GEORGE F.M. BALL

VITAMINS IN FOODS

Analysis, Bioavailability, and Stability

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Dedication

This work is dedicated to my wife and dearest friend, Kazuko (Kako).

About the Author

George Ball has accumulated many years of commercial and research laboratory experience in pharmaceutical analysis, clinical analysis, biochemical analysis, and food analysis. He has contributed to original research publications relating to biochemistry (platelet function) and endocrinology (control of ovulation) and is the author of several books and book chapters on vitamins. He received the B.Sc. honors degree in agricultural sciences from the University of Nottingham, UK in 1975.

Preface

Optimal vitamin status is a prerequisite for good health, and government-approved food fortification strategies are deemed necessary to ensure adequate intake of certain vitamins. Knowledge about vitamin bioavailability from food is essential for the estimation of dietary requirements. Equally important is knowledge of a vitamin's stability toward post-harvest handling of food, processing, storage, and preparation for consumption. To acquire this knowledge, one must learn about vitamin chemistry and how the vitamin is absorbed and metabolized. Successful research into vitamin bioavailability and stability is entirely dependent on the development and validation of suitable analytical methods. Vitamin bioavailability from food is subject to many variables imposed by food constituents and the preparation of food. Great progress has been made over the past decade, largely attributable to innovative analytical methodology, but there are many inconsistencies and the continuation of a multipronged research effort from independent laboratories must be encouraged to achieve solid and vital data.

I would like to acknowledge the expertise and diligence of Lynn Saliba at the British Library.

George F. M. Ball

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Part I

Properties of Vitamins

1

Nutritional Aspects of Vitamins

1.1 Definition and Classification of Vitamins

Vitamins are a group of organic compounds that are, in very small amounts, essential for the normal functioning of the human body. They have widely varying chemical and physiological functions and are broadly distributed in natural food sources. Thirteen vitamins are recognized in human nutrition and these may be conveniently classified into two groups according to their solubility. The fat-soluble vitamins are represented by vitamins A, D, E, and K; also included are the 50 or so carotenoids that possess varying degrees of vitamin A activity. The water-soluble vitamins comprise vitamin C and the members of the vitamin B group, namely thiamin (vitamin B_1), riboflavin (vitamin B_2), niacin, vitamin B_6, pantothenic acid, folate, and vitamin B_{12}. This simple classification reflects to some extent the bioavailability of the vitamins, as the solubility affects their mode of intestinal absorption and their uptake by tissues. The solubility properties also relate to the distribution of vitamins in the various food groups, and have a direct bearing on the analytical methods employed.

For many of the vitamins, biological activity is attributed to a number of structurally related compounds known as vitamers. The vitamers pertaining to a particular vitamin display, in most cases, similar qualitative biological properties to one another, but, because of subtle differences in their chemical structures, exhibit varying degrees of potency. Provitamins are vitamin precursors, that is, naturally occurring substances which are not vitamins themselves, but which can be converted into vitamins by normal body metabolism.

Most of the vitamins are absolutely essential in the human diet because the body tissues cannot synthesize them. Two notable exceptions are vitamin D and niacin. Cutaneous synthesis of vitamin D depends on adequate exposure of the skin to sunlight, and the synthesis of niacin depends on a sufficient intake of its amino acid precursor, tryptophan, bound within protein. Plants have the ability to synthesize vitamins, except for vitamin B_{12}, and serve as primary sources of these dietary essentials.

1.2 Nutritional Vitamin Deficiency

Several B-group vitamins serve as coenzymes for enzymes that function in the catabolism of foodstuffs to produce energy for the organism. A typical coenzyme consists of a protein (apoenzyme) to which the vitamin is attached. The vitamin portion of the coenzyme is usually responsible for the attachment of the enzyme to the substrate. If the specific vitamin is not available to form the coenzyme, the sequence of chemical changes in the metabolic process cannot proceed and the product whose change is blocked accumulates in the tissues: alternatively, metabolism is diverted to another pathway.

For some B-group vitamins, clinical deficiency results in a biochemical defect, which is manifested as a disease with characteristic symptoms. Other vitamins have less dramatic deficiency symptoms in humans, but their deficiency in certain animal species may give rise to distinctive signs. Some human individuals can benefit from vitamin supplements, indicating that they may have been subclinically vitamin deficient to begin with.

The causes of nutritional vitamin deficiency are any one or combination of the following: inadequate ingestion, poor absorption, inadequate utilization, increased requirement, increased excretion, and increased catabolism. The capacity of the body to store vitamins is another factor: humans can store thiamin for only about 2 weeks, whereas vitamin B_{12} can be stored in the liver for several years.

1.3 Vitamin Requirements

Metabolic processes must respond to the immediate needs of the body and therefore vitamin requirements are subject to continuous variation between certain limits. The Food and Nutrition Board of the Institute of Medicine in the U.S. defines a requirement as the lowest continuing intake level of a nutrient that, for a specific indicator of adequacy, will maintain a defined level of nutriture in an individual. A recommended dietary allowance (RDA) of a nutrient is the average daily dietary intake level that is sufficient to meet the requirement of nearly all (97–98%) apparently healthy individuals in a particular life stage and gender group. The RDA is derived from an estimated average requirement (EAR), which is an estimate of the intake at which the risk of inadequacy to an individual is 50%. RDAs have been published for vitamins A, D, E, and K, thiamin, riboflavin, niacin, vitamin B_6, folate, vitamin B_{12}, and vitamin C. In the case of pantothenic acid and biotin, there is insufficient evidence to calculate an EAR, and a reference adequate intake (AI) is

provided instead of an RDA. The AI is a value based on experimentally derived intake levels or approximations of observed mean nutrient intakes by a group (or groups) of apparently healthy people [1].

1.4 Vitamin Enhancement of Foods

The terms restoration, fortification, enrichment, standardization, and nutrification have been used to describe various ways of enhancing the vitamin content of foods [2].

Restoration involves the replacement, in full or in part, of vitamin losses incurred during processing. The addition of vitamins A and D to skimmed milk powder, and of vitamin D to evaporated milk, are examples of nonlegislative vitamin restoration in the U.K. Other examples are the replacement of B-group vitamins in flour to compensate for the losses incurred in the milling of cereals to low extraction rates, and the addition of vitamin C to instant potato.

Fortification refers to the addition of vitamins to foods that are suitable carriers for a particular vitamin, but which do not necessarily contain that vitamin naturally. It is especially carried out to fulfill the role of a food in the diet. Thus margarine is fortified with vitamin A in many countries to replace the vitamin A that is lost from the diet when margarine is substituted for butter. Vitamin D is added to margarine at higher levels than found in butter as a public health measure, as the extra is considered necessary for the population as a whole. In the U.S., fortification of cereals and grains with folic acid began in 1996 and, since January 1998, all cereal grain products are fortified with 140 μg of folic acid/100 g. In the U.K., fortification of foods with folic acid is still voluntary.

Enrichment refers to the addition of vitamins above the initial natural levels to make a product more marketable. Standardization refers to additions designed to compensate for natural fluctuations in vitamin content. For instance, milk and butter are subject to seasonal variations in their vitamin A and D contents, and hence these vitamins are added to some dairy products to maintain constant levels. Nutrification means the addition of vitamins to formulated or fabricated foods marketed as meal replacers.

Vitamins are also added to perform specific processing functions. For instance, β-carotene (the principal source of dietary vitamin A) is added to products such as pasta, margarine, cakes, and processed cheeses to impart color. Vitamin E and vitamin C (in the form of ascorbyl palmitate) can be used as antioxidants to stabilize pure oils and fats. Ascorbic acid is used for a variety of purposes in food processing, such as a reducing agent involved in the formation of the cured meat color in the curing of bacon and ham.

1.5 Stability of Vitamins

New food product development aims to retain as much of the naturally occurring vitamin content as possible, to protect added vitamins, and to minimize the appearance of undesirable breakdown products. Factors that play a role in the degradation of vitamins during processing and storage include temperature, air or oxygen, light, moisture content, water activity, pH, degradative enzymes, and metal trace elements, particularly iron and copper. Ryley et al. [3] discussed the use of kinetic data and mathematical models for predicting vitamin retention in food processing.

1.5.1 Water Activity and Lipid Oxidation

The water activity of a food is one of the primary factors determining the rate of food deterioration by various biochemical reactions. Raw foods with a high content of active water, such as leafy vegetables and meat, deteriorate in only a few days, whereas dry seeds, containing only structural water, can be stored for years under proper conditions.

Water activity measures the availability of the water present in the system: it is more closely related to the physical and chemical properties of food than is total moisture content. Within the heterogeneous food matrix, the reactivity of each constituent is influenced by its affinity for surrounding water molecules and the competing influences of neighboring hydrophilic or hydrophobic chemical groups. Changes in the environment, such as heat, light, pressure, pH, additives, and modification of particle size, may alter the molecular state of water, and thereby influence constituent reactivities and functional properties.

The total water-binding energies of constituent chemical groups are reflected in the equilibrium water vapor pressure or absolute humidity of the food material. At constant temperature, the vapor pressure may be expressed as equilibrium relative humidity (ERH) or the related analogous water activity. Water activity (A_w) can be defined as the ratio of the equilibrium vapor pressure exerted by the food material (p) to the vapor pressure of pure water (p_0) at the same temperature:

$$A_w = \frac{p}{p_0} = \frac{EHR}{100} \tag{1.1}$$

Small changes in temperature, or other factors which influence A_w, may induce profound changes in the quality and stability of a food product. Therefore, A_w measurements provide a simple and convenient

means of evaluating water-binding activity. The nonlinear, generally sigmoidal relationship between A_w and total moisture content, expressed as a moisture sorption isotherm, is a fundamental characteristic of a food product [4].

A low water activity ($A_w < 0.6$) does not permit microbial or enzymatic activity. It is generally accepted that growth of bacteria and most yeasts virtually ceases at $A_w < 0.9$, while molds do not grow at $A_w < 0.7$ [5].

The presence of water has a profound effect on lipid oxidation η as discussed by Labuza [6]. This has relevance to vitamin A and carotenoids, which undergo coupled oxidation in the presence of lipids. The kinetics of lipid oxidation are best studied in model systems using a swellable solid matrix, since the variables can be controlled. At $A_w = 0$ (as in a dehydrated food), lipid oxidation is rapid, but the rate of oxidation decreases as the water activity increases up to $A_w = 0.5$, where a leveling-off occurs. The protective effect of water within this range of water activity may be due to two mechanisms working together. First, hydrogen bonding takes place between water and lipid hydroperoxides produced during the propagation stage of lipid peroxidation. This bonding prevents the metal-catalyzed decomposition of the hydroperoxides into peroxyl and alkoxyl radicals that intensify propagation of the chain reaction. Second, hydration of metal catalysts makes them less effective through changes in their coordination sphere.

In the intermediate moisture range of $A_w = 0.55–0.85$, the solvent and mobilization properties of water become dominant, and lipid oxidation increases with increasing water activity. The catalysts present are more easily mobilized and swelling of the solid matrix exposes new catalytic sites. This detrimental effect of water has been confirmed in actual intermediate moisture foods [7].

As might be expected from the effect of water on lipid oxidation, the loss of all-*trans*-retinol suspended on microcrystalline cellulose in the dark, in air, increased with increasing water activity in the range of intermediate-moisture foods ($A_w = 0.4–0.75$) [8]. In contrast, increasing the water activity from 0 to 0.73 decreased the rate of β-carotene degradation in the dark [9]. A protective effect of water against oxidation of carotenoids in the intermediate-moisture region was also observed by Ramakrishnan and Francis [10]. This difference in behavior between retinol and β-carotene can possibly be attributed to the poorer solubility of β-carotene in water.

1.5.2 First-Order Kinetics

The analytical approach in calculating and predicting food quality deterioration involves a kinetic/mathematical model. The model may

include equations of mass and energy balance, thermodynamics, transport and chemical properties, and coefficients. These equations have been simplified for specific conditions. Saguy and Karel [11] produced a status report on the modeling of quality deterioration during food processing and storage.

Most reactions involving the deterioration of a nutrient fit a first-order mathematical expression:

$$-\frac{dC}{dt} = kC \tag{1.2}$$

where C is the nutrient concentration at a given reaction time (t), and k is the degradation reaction rate constant. When Equation (1.1) is integrated, and log (C/C_0) versus t is plotted, the slope of the straight line obtained defines the rate constant (k), where C_0 is the initial concentration of nutrient.

As an example, consider the effect of microwave processing on water-soluble vitamins, as carried out by Okmen and Bayindirli [12]. Vitamin solutions were heated at constant temperatures of 60, 70, 80 and 90°C in the microwave oven. Aliquots (1 ml) were taken from the heat-treated samples after 20, 40, 60 and 80 min intervals and analyzed for vitamin concentration. The first-order reaction rate constants at the four different temperatures were calculated from the slopes of the log (C/C_0) versus t plots shown in Figure 1.1.

Other reaction orders besides first are possible. A zero-order reaction occurs if the loss of nutrient is so small in the time period studied that the value of C does not change significantly. Therefore, the right-hand side of Equation (1.2) is a constant [13]. Ascorbic acid oxidation in liquid infant formula under limited presence of dissolved oxygen takes place by a second-order reaction [14].

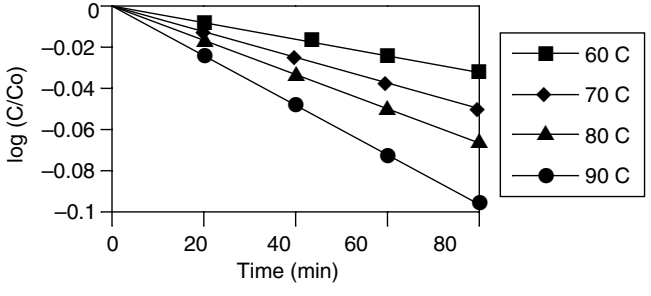

FIGURE 1.1
A typical graph of log (C/C_0) vs. time for vitamin C. (From Okmen, Z.A. and Bayindirli, A.L., *Int. J. Food Prop.*, 2 (3), 255, 1999. Copyright 1999. With permission.)

1.5.3 Effects of Food Processing on Vitamin Retention

Commercial food processing preserves food quality and extends shelf life by destruction of food-spoilage microorganisms and certain endogenous enzymes, which could otherwise promote spoilage and/or reduce nutritive value. Since all processed foods have to be stored until they are consumed, proper food packaging is essential to maintain preservation. Time and temperature during processing and storage are closely controlled in good manufacturing practice. Distribution controls, however, are less stringent, except for very perishable products.

The main factors contributing to vitamin losses are oxidation (air exposure), heat (temperature and time), catalytic effect of metals, pH, action of enzymes, moisture, irradiation (light or ionizing radiation), and various combinations of these factors. Some vitamins are sensitive to processing and storage, while others are more or less stable. The water-soluble vitamins are susceptible to leaching losses during commercial washing and blanching, and domestic cooking. Vitamin C is very susceptible to chemical oxidation during processing, storage, and cooking. Thiamin is heat-sensitive in neutral and alkaline foods, and is unstable in air. Riboflavin is notoriously susceptible to decomposition by light. Niacin and vitamin B_6 are stable under a variety of processing conditions. Vitamins A and E are destroyed under conditions that accelerate the oxidation of unsaturated fats, such as access of air, heat, light, trace metal ions, and storage time. Vitamin K is stable to heat, but extremely sensitive to both fluorescent light and sunlight. Vitamin D is little affected by processing and storage.

Some losses of certain vitamins during food processing are inevitable. However, one should consider the relative importance of the loss of a specific vitamin from a particular commodity. For example, vitamin C loss from milk during pasteurization and refrigerated storage is relatively unimportant, as milk is an insignificant source of this vitamin in the daily diet compared to other foods, such as citrus fruits and juices. Another point to consider is that natural variations in the vitamin content of a raw food material may affect the content of that vitamin in the final product more than the processing itself.

Retention studies of vitamins to assess the effects of food processing on the nutritive value of foods are of great importance to food technologists and consumers. In this section, the broad effects of various processing techniques on vitamin retention are discussed. The effects of processing on specific vitamins are discussed in the relevant chapters.

1.5.3.1 Dehydration

Removal of the biologically active water from foods through dehydration stops the growth of microorganisms, whilst also reducing the rate of

enzyme activity and chemical reactions. Rancidity of the lipid constituents of dehydrated foods is reduced if the protective structural water is left intact. Fruits, vegetables, juices, meats, fish, milk, and eggs are among the foodstuffs commonly subjected to dehydration processes.

Appropriate drying techniques are used in the processing of dehydrated foods. The sun-drying of fruit, fish, meat, and grain is still of importance in certain parts of the world. In the tunnel-drying of fruits and vegetables, the produce is spread onto trays or a conveyer and passed into a high-velocity air stream in the temperature range of 60–93°C. Spray-drying is a highly efficient process for drying milk, eggs, and coffee. In spray-drying, liquids are dispersed in fine droplets and sprayed into an upward-flowing stream of hot air. Materials that can be made into a paste, such as mashed potatoes and tomato puree, can be dried by spreading thinly on steam-heated revolving drums. As the product is in direct contact with the hot drums, vitamin losses would be expected to be greater than those resulting from tunnel driers and spray driers. In commercial freeze-drying, the frozen food is placed into a chamber, which is evacuated and then heated. Because of the low pressure, ice does not melt, and the water vapor passes directly from a solid to a vapor phase (sublimation). The freeze-drying process is applied mainly to meats and results in the least change in the physical characteristics of the product. The result is a dry, porous product, though there will always be some small amount of residual water. Compression of freeze-dried foods gives improved stability in storage by reducing the surface area exposed to oxygen and moisture.

Except for sun-drying, the actual process of dehydration does not cause major losses of vitamins. Retention of ascorbic acid is better in rapid drying at high temperatures than in slower drying at lower temperatures. Drying methods that expose the food to air result in losses of vitamin A, β-carotene, and vitamin C due to oxidation. Freeze-drying, which is carried out in the absence of oxygen, does not cause loss of vitamin C.

1.5.3.2 Blanching

The blanching of vegetables and fruits entails subjecting the fresh produce to temperatures in the range of 75–95°C for 1–10 min prior to canning, freezing, or dehydration. Blanching serves several purposes: as a cleaning process; to reduce the volume of bulky vegetables by wilting; to expel air from the plant tissues, thereby reducing the potential for oxidative changes; and to inactivate endogenous enzymes that would otherwise cause quality deterioration. Blanching with hot water is still the most common system, despite the significant loss of water-soluble vitamins through leaching. This is because of the relatively low capital and

running costs compared with more efficient procedures, such as steam or microwave blanching.

The immediate scalding of plant tissues is desirable to minimize the oxidation of ascorbic acid by ascorbic acid oxidase. If the inactivating temperature of about 85°C is not immediately reached, the breakdown of cell structure will allow contact between active enzyme and substrate. Thus inefficient blanching will incur some loss of vitamin C by oxidation, as well as by leaching [15]. Unlike thiamin, ascorbic acid is essentially stable to the heating conditions during blanching. When peas were blanched at 82–88°C for 3 min, losses of thiamin, niacin, vitamin B_6, and vitamin C were 4, 20, 18, and 17%, respectively [16].

1.5.3.3 Canning

The heat treatment of canned foods will ideally sterilize the food, that is, destroy all viable microorganisms present. Some microorganisms and their spores are extremely resistant to heat, and very stringent heat treatments would be required to destroy them. Unfortunately, such treatments would promote unacceptable organoleptic changes and nutrient losses, therefore the temperatures applied in commercial practice aim to achieve maximum microbial destruction commensurate with acceptable organoleptic and nutritional value. The rate of destruction of heat-resistant bacteria is increased approximately tenfold with a 10°C increase in temperature [17]. Canned foods are considered to be "commercially sterile" because the conditions within the can are such that any remaining microorganisms or spores will not grow during storage [18].

Heat sterilization causes destructive losses of the thermolabile vitamins. The extent of the losses depends on the time/temperature conditions and the rate of heat transfer into the product. In the case of canned cured pork luncheon meat, the retentions of thiamin and pantothenic acid were greater after processing at a high retort temperature for a short time than after processing to the same sterilizing value at lower retort temperatures for longer periods [17]. Heat transfer is slow in canned solids such as meat, and excessive heat must be applied to the container to ensure sterility in the center. Canned beans (a semisolid) retain approximately 55% of the thiamin content, while canned tomatoes (a more liquid product) retain a larger percentage [19]. Vitamin C losses are increased by the inclusion of oxygen during canning. Low-temperature storage improves retention of vitamins in canned foods.

1.5.3.4 Pasteurization and Ultra-High-Temperature Processing

In the pasteurization process, the foodstuff is subjected to a temperature high enough to destroy all pathogenic microorganisms present. Unlike

sterilization procedures, some nonpathogenic microorganisms survive pasteurization. It is fundamental that the higher the temperature and the shorter the processing time, the greater is the nutrient retention. The accepted methods of milk pasteurization in the U.S. include the batch or holding method of heating the milk for 30 min at 145°F (62.8°C) and the high temperature–short time (HTST) method of heating at 161°F (71.7°C) for 15 sec [20]. Orange juice is traditionally pasteurized by heat treatment at 95°C for 15 sec, or at 90°C for 1 min [21]. In aseptic packaging, fluid products such as milk and fruit juices are pasteurized by HTST treatment, rapidly cooled, then placed into sterile containers and sealed. The heat treatment is applied to a thin layer of the milk or juice in a heat exchanger, or by direct steam injection. These packages are likely to have a higher content of residual and headspace oxygen than "in-container" pasteurized products, which are exhausted at blanch temperatures and vacuum-seamed prior to heat treatment [22]. The pasteurization of milk has little or no effect on the water-soluble vitamins, apart from a 20–25% loss of the vitamin C content [23]. The pasteurization of fruit juices inactivates endogenous enzymes that would otherwise promote the oxidation of ascorbic acid.

Ultra-high-temperature (UHT)-milk generally refers to milk that has been heated to at least 130°C for not less than 1 sec and then aseptically packaged. The process may be described as either UHT-sterilization or UHT-pasteurization, depending on the temperature and holding time. In general, UHT-milk processing involves temperatures of 130–150°C and holding times of 2–8 sec. Heating and cooling methods employed in UHT processing involve direct heating with saturated steam under pressure followed by expansion cooling, or indirect heating and cooling through a heat-conducting barrier. Equipment for indirect heat processing may sometimes include vacuum flash vessels to remove gas and vapor [24]. UHT-milk can be stored unrefrigerated for several months.

1.5.3.5 Microwave Heating

Microwaves are electromagnetic waves of radiant energy, which easily penetrate materials containing dielectric molecules such as water. The rapidly oscillating electromagnetic field forces the molecules to undergo rapid reorientation. By this process, electromagnetic energy is converted into thermal energy. In conventional cooking, heat is applied to the surface of the food and then conducted to the inner parts with uneven distribution. Microwaves generate heat throughout the bulk of the food, resulting in a comparatively uniform and rapid temperature rise throughout the product.

Microwave processing offers precise control of heating, making it useful for several commercial food processing operations [25,26]. Some examples

are as follows: (i) in the microwave tempering of frozen meat, frozen blocks of meat are brought to a temperature of -4 to -2°C. The partially thawed meat can then be used to make hamburger patties or other products. (ii) In the processing of pasta products, the pasta is first dried by conventional drying with controlled humidity. Microwave drying is then used to redistribute water from the moist inner parts to the surface. A third conventional drying step achieves the final moisture content. (iii) For the precooking of meat, fish, and chicken products, microwave cooking is often combined with conventional cooking methods. This results in less overcooking of surface parts and lower losses of moisture. (iv) Microwave-vacuum-drying is used for production of fruit juice concentrates. (v) Microwave heating within a conveyerized tunnel is used for pasteurizing pasta products, bread, and prepared meals.

Microwave heating has the potential for a greater retention of heat-labile vitamins compared with other more conventional methods because the heating time is shortened. Ascorbic acid content is higher in vegetables cooked by microwave heating than by conventional methods [27]. The cooking of pork and chicken in microwave ovens led to greater retentions of vitamins B_1, B_2, and B_6 than in conventional electric ovens [28,29]. Vitamin B_1 retentions in conventionally roasted and microwaved samples ranged between 48–67% and 86–94%, respectively. Comparative retentions of vitamin B_6 were 22–48% and 60–87% [29].

1.5.3.6 Hydrothermal Processes (Flaking, Puffing, and Extrusion)

Subjecting whole or almost whole cereal grains to the simultaneous effect of moisture and heat creates a fine and voluminous structure, making these products suitable for breakfast cereals and snacks. Flaking entails steam treatment followed by passing through rollers, drying, and toasting. For puffing, the grains are treated with water, then enclosed in a pressure chamber (puffing gun) and heated to 267–294°C for 3–5 sec to increase the vapor pressure. Sudden opening of the gun releases the pressure, and the expansion of water vapor and other gases results in a 5–15 times increase in volume of the cereals. In the extrusion process, food ingredients are conveyed through a screw within a tightly fitting stationary barrel. The food is subjected to a combination of heat sources, including frictional heat, direct steam injection, and heat transfer from steam or water in jackets surrounding the extruder barrel. At the discharge of the extruder, the high-temperature, pressurized, cooked dough mass is forced through a small restrictive opening called a die. The sudden change from high pressure and high temperature to ambient conditions on emergence through the die leads to product expansion.

Barna et al. [30] measured the changes in the content of four water-soluble vitamins (thiamin, riboflavin, niacin, and vitamin B_6) during

flaking, puffing, and extrusion of cereals. During the flaking of rye, oats, wheat, and barley, the retention of thiamin ranged between 65 and 89%. Retentions of riboflavin, niacin, and vitamin B_6 were mostly around 100%, except for wheat which retained only 78% of its niacin content. The puffing of wheat and rice had a dramatic effect on thiamin content, with only 1–6% of this vitamin being retained. Thus high-temperature treatment for only a few seconds can destroy thiamin almost totally. The retention ranges of riboflavin and vitamin B_6 were 65–80 and 60–78%, respectively; niacin retention was nearly 100%. The extrusion of a raw mixture consisting mainly of maize grits resulted in thiamin, niacin, and vitamin B_6 average retentions of 70, 89 and 77%, respectively. There was no loss of riboflavin.

1.5.3.7 Freezing

Low temperature inhibits microbial growth and slows down the rates of chemical and enzymatic reactions. The commercial freezing process consists of prefreezing treatments, freezing, frozen storage, and thawing. Vitamin losses occur mainly in the prefreezing treatments (e.g., blanching, washing, peeling, trimming, and grinding). The actual freezing step does not destroy vitamins. Using proper packaging and processing conditions, losses of most vitamins (but not folate) during the freezing process are negligible. Proper freezing conditions are important to vitamin retention. Foods must be frozen quickly and solidly, and maintained at a constant low temperature ($-18^{\circ}C$ or lower), otherwise the product will exhibit a greater than normal drip loss when thawed and, hence, greater loss of water-soluble vitamins. Gradual oxidation of vitamins A, C, and E can take place during frozen storage if the food is exposed to air through ineffective packaging.

Freezing damages plant cell structure by expansion of the intracellular fluid. In theory, slow freezing causes more damage to cell structure than rapid freezing at lower temperatures, because larger ice crystals are formed. Disruption of cell membranes by freezing brings endogenous folate conjugase in contact with polyglutamyl folate, converting it to monoglutamyl folate, which is more readily leached from plant tissues during subsequent blanching.

1.5.3.8 Irradiation

The commercial irradiation of foods by exposure to ionizing radiation is carried out to inhibit the growth of spoilage microorganisms and prolong product shelf-life. Mild heat treatment prior to low-dose irradiation enhances the antimicrobial effects of irradiation [31]. Ionizing radiation passes through the packaging material as it travels to the food inside the container. A number of polymeric films and multilayered

flexible materials have been approved by the U.S. Food and Drug Administration. Ionizing radiation has sufficient energy to remove an electron from water, thereby creating highly active free radical species. These free radicals can react with the DNA of microorganisms, causing death. The applied irradiation dose is measured in kiloGrays (kGy), where 1 Gy = 1 J/kg. For the sterilization of meats, doses of 30 kGy and higher are required. The major emphasis on food irradiation is on pasteurization doses of 1–10 kGy.

Two forms of ionizing radiation are used in the commercial irradiation of foods: (i) gamma rays from a radioactive source, ^{60}cobalt or ^{137}cesium and (ii) high-energy electrons generated by machines such as a linear accelerator. The high penetrating power of gamma radiation facilitates its use in the treatment of bulk food items in containers. Electron beams have a limited penetration depth, up to a maximum of about 8 cm in food. Typical applications include the treatment of grain on a conveyer, low-density foods such as ground spices, and prepared meals.

Meat, vegetables, and fruits are suitable for irradiation. Because irradiation initiates the autoxidation of fats and the subsequent development of off-flavors, oils, dairy products, and eggs are unsuitable. The vitamins receive some protection from other food constituents, including interacting vitamins. Irradiation of pork at doses greater than 0.57 kGy resulted in considerable destruction of thiamin [32]. The rigorous exclusion of oxygen did not prevent the loss of thiamin on irradiation [33]. There was no loss of riboflavin on irradiation of pork under anaerobic conditions [33]. Irradiated pork retained much more thiamin than canned meats sterilized by heat [34]. Thiamin destruction in meat irradiated in the frozen state was markedly less than when irradiated under ambient conditions [35]. When wheat was irradiated with a ^{60}cobalt source, the thiamin content was not reduced, even after 24 months of storage [35]. In irradiated fruits, retention of vitamin C ranged from 72 to 100%. β-Carotene and vitamin K were stable to the irradiation of vegetables [35].

During storage of irradiated rolled oats, the peroxides formed during fat autoxidation destroyed vitamin E [35]. Vitamin A is also sensitive to irradiation, but as the main sources of this vitamin are dairy products and eggs, this has little nutritional relevance. Vacuum packing and low temperature protect vitamins A and E during irradiation and subsequent storage. Vitamin D is unaffected by irradiation.

1.5.3.9 High Hydrostatic Pressure Treatment

Pressure can inhibit cell growth by interfering with DNA replication and can also cause cell death through damage to the cell membrane. Many other cellular processes are affected by pressure and its effects on volume [36,37]. The application of pressures between 300 and 600 MPa

(1 MPa = 10 bar = 145.038 psi) can inactivate yeasts, molds, and bacteria in the vegetative state. Most spores from yeasts and molds are easily inactivated by pressures around 400 MPa. However, pressures higher than 1000 MPa are needed to kill bacterial spores. Pressures of 50–300 MPa can often stimulate bacterial spores to germinate, after which the vegetative cells can be killed by relatively mild pressure or heat treatments. However, in most cases, a small fraction of the spore population can survive this treatment [37]. The use of multiple high-pressure pulses with an end temperature above 105°C under pressure produces sterility (destruction of all bacterial spores) with minimal impact on flavor, texture, and color of foods [38].

High hydrostatic pressure (HHP) treatment of foods at ambient temperature, or combined with moderate heat treatment (below 70°C), is a promising alternative to pasteurization, as there is less of an effect on food quality [39]. A range of pressure-treated products has been on the Japanese market for several years, including fruit preparations, fruit juices, rice cakes, and raw squid [37]. The applied pressure acts instantaneously and uniformly throughout the food, irrespective of product size and shape. Only noncovalent bonds (i.e. hydrogen, ionic, and hydrophobic bonds) are disrupted, thus the food's quality attributes (e.g., color, texture, and flavor) are unimpaired, and in some cases even improved. HHP eliminates or reduces the spoilage microflora commonly found in citrus juices [40–43]. HHP also reduces the activity of the enzyme pectinmethylesterase in citrus juice, thereby maintaining good cloud stability [40,43,44]. In the technique of cold isostatic pressing, foods other than liquids are placed into sterilized plastic containers, and the sealed containers are placed into a hydrostatic pressure unit. Liquids, such as fruit juices, are pumped to the bottom of the pressure chamber, and pressure is applied by operating an hydraulically-driven piston [45]. Due to adiabatic heating, an initial temperature rise is to be expected on pressurizing (about 3°C per 100 MPa) [46].

High-pressure treatment of egg white and yolk from 588 to 784 MPa at 25°C for 30 min resulted in almost total retention of thiamin, riboflavin, folate, retinol, and tocopherol [47]. Similarly, there was no significant effect on vitamin C concentrations in a strawberry sauce or egg yolk [48]. High-pressure treatment of milk (400 MPa at 25°C for 30 min) did not produce significant loss of thiamin or vitamin B_6 [49]. Orange juice was fully stabilized for commercial purposes at a pressure not lower than 350 MPa during 1 min, with no significant effect on vitamin C content [42].

1.5.3.10 Curing and Smoking

Curing consists of the addition of salt, sodium nitrate, sodium nitrite, and other chemicals to meat, both as a preservation technique and to promote

or improve the organoleptic properties of the food. Smoking has a similar function. These procedures have no major effects on vitamin retention, apart from a 15–20% loss of thiamin as a result of smoking [50].

1.5.4 Milling

Milling of grains such as wheat, corn (maize), rice, oats, barley, and rye is a process used to produce white flour, rice, starch, dextrose, and corn syrups. Milling has detrimental effects on the vitamin content of the resultant flour. The bran, germ, and scutellum portion of the grain endosperm contain significant concentrations of vitamins, which are lost as a result of separation of these components during the milling process. The amount of loss depends on the duration of the extraction process. In the United States, milling results in an approximate 40–60% loss of vitamins. Grain products are therefore required to be enriched with thiamin, riboflavin, and niacin (also iron) to replace these losses. Calcium and vitamin D addition is optional, and is frequently done.

1.5.5 Effects of Food Storage on Vitamin Retention

The residual oxygen in canned products is used primarily to oxidize vitamin C, but no further oxidation takes place once the residual oxygen has been used up. The vitamins in dehydrated foods are stable, given proper packaging to avoid moisture gain and oxygen access. Vitamins A and C in puffed products become rapidly oxidized during storage due to the extensive exposure to oxygen. The main factors affecting vitamin loss in frozen foods are the oxygen permeability and light transmission characteristics of the packaging material. Subfreezing temperatures have an impact on vitamin retention in stored frozen foods. During frozen storage of vegetables at temperatures of -20 to -25°C, a 10% loss of vitamin C might be expected over a period of 1 yr. At temperatures of -25 to -30°C and below, vitamin C losses are practically zero. However, at temperatures around -10°C, vitamin C losses can be 80–90% after storage for 1 yr [15].

1.5.6 Effects of Domestic Cooking on Vitamin Retention

Overall, the major loss of vitamin from a food usually occurs during the final preparation step before consumption. This may be either home cooking or foodservice operations. Thus the actual vitamin content of a particular table-ready food is frequently about the same, regardless of the type of processing, or lack of processing, that the food has undergone. Significant losses of vitamins can occur in each of the various stages of

food preparation, including (i) preliminary trimming, washing, soaking, slicing, or chopping; (ii) the cooking process; (iii) holding of the cooked food on a steam table, in a warming oven or insulated transport equipment, or under an infrared lamp; (iv) storage of prepared food in refrigerator or freezer; and (v) reheating of stored food. The greatest losses during home cooking are due to the leaching of water-soluble vitamins into the cooking water.

De Ritter et al. [51] measured eight vitamins in various frozen convenience dinners and pot pies (14 products of 10 different types) before and after normal oven heating, in accordance with packaging directions. Losses of vitamin A (preformed vitamin A + carotene) averaged only 3%, with a range of 0–55%. Vitamin E losses averaged 13% (range 0–33%). Among the water-soluble vitamins, riboflavin and niacin showed no significant losses. Average losses and ranges for others were: thiamin, 30% (0–85%); vitamin B_6, 7% (0–25%); vitamin B_{12}, 4% (0–50%); and ascorbic acid in ten products having significant levels, 77% (49–91%).

Attention to several procedures in the home preparation of foods allows vitamin losses to be minimized. Proper procedures include: (i) refraining from excessive trimming and over-chopping; (ii) cooking in covered pans (to shorten cooking time); (iii) cooking with a minimum amount of water (just enough to prevent scorching); (iv) cooking vegetables until only tender; (v) refraining from overcooking; and (vi) using cooking water for soups. Steaming or pressure cooking and stir-frying result in less vitamin loss than boiling or typical pan frying.

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2

Intestinal Absorption and Bioavailability of Vitamins: Introduction

2.1 General Principles of Solute Translocation

Mammalian epithelia are enveloped by a plasma membrane composed of a phospholipid bilayer interspersed frequently with cholesterol molecules. Integral transmembrane proteins span the lipid bilayer in a weaving fashion and account for most membrane-associated receptors and transporters and certain enzymes. Tight junctions prevent the passage of water and molecular solutes between adjacent epithelial cells.

The plasma membrane constitutes a selective barrier to the transcellular movement of molecules and ions between the extracellular and intracellular fluid compartments. Fat-soluble substances, water, and small uncharged polar solutes can simply diffuse through the membrane, but ions and water-soluble molecules having five or more carbon atoms cannot do so. Most biologically important water-soluble substances (e.g., glucose, amino acids, water-soluble vitamins, and certain inorganic ions) are translocated across the plasma membrane by means of protein transporters, which exert their effect through a change in their three-dimensional shape. Specific transporters are responsible for the translocation of a specific molecule or a group of closely related molecules. Specificity is imparted by the tertiary and quaternary structures of the transporter molecule — only if a solute's spatial configuration fits into the protein, will the solute be transferred across the membrane. Transporters fall into two main classes: carriers and ion channels. Ion pumps are a type of carrier protein, which is also an enzyme.

At physiological concentrations, the translocation of several water-soluble vitamins (thiamin, riboflavin, pantothenic acid, biotin, and vitamin C) across cell membranes is mediated by carrier proteins. The term "transport" implies a carrier-mediated translocation. The interaction of a transportable substrate with its carrier is characterized by saturation at high substrate concentration, stereospecificity, and competition with structural analogs. These properties are shared by the interaction of a substrate and an enzyme, and therefore, the terms V_{max} and K_m can be

used to describe the kinetics of transport. The maximum rate of transport (V_{max}) is the point at which all of the available binding sites on the carrier are occupied by substrate — a further increase in the substrate concentration has no effect on the transport rate. V_{max} values are expressed in picomoles of substrate per milligram protein during a specified period of minutes. Each carrier protein has a characteristic binding constant (K_m) for its substrate. K_m is defined as the concentration of substrate (expressed in units of molarity, m*M*) at which half of the available carrier sites are occupied and is determined experimentally as $V_{max}/2$. K_m describes the affinity of the carrier for its substrate in a reciprocal manner and is independent of the amount of carrier. The lower the value of K_m, the greater the affinity of the carrier for its substrate and the greater the transport rate.

The downhill movement of a substance from a region of higher concentration to one of lower concentration is a passive process driven by the concentration gradient. There are two types of passive movement: facilitated diffusion, which is carrier mediated, and simple diffusion, which is not. The uphill movement of a substance is referred to as active transport, either primary or secondary, and requires the expenditure of metabolic energy. Primary active transport is driven *directly* by metabolic energy and is carried out exclusively by ion pumps, such as the calcium pumps, the sodium pump, and the proton pumps. Ion pumps are ATPases, which utilize the energy released by the hydrolysis of ATP. Secondary active transport is *indirectly* linked to metabolic energy through a coupling of the solute to the pump-driven movement of an inorganic ion (usually Na^+).

At many places in the body, substances must be translocated all the way through an epithelium, instead of simply through the plasma membrane. Movement of this type occurs, for example, through the epithelia of the intestine and renal tubules. The vectorial nature of such movement is made possible by the polarity of the cell surface, whereby distinct sets of surface components (carriers, ion channels, and ion pumps) are localized to separate plasma membrane domains. Transepithelial movement may involve concentrative active transport through the apical membrane domain, and facilitated diffusion for the downhill exit through the basolateral membrane domain.

2.2 Intestinal Absorption

2.2.1 The Villus

The functional absorptive unit of the small intestine is the villus, a finger-like projection of the mucosa. Contained within the lamina propria core of each

villus is a capillary network with a supplying arteriole and draining venule. A blind-ending lymphatic vessel (lacteal) in the center of each villus drains into a plexus of collecting vesicles in the submucosa. Each villus is covered by an epithelium composed of a single layer of columnar absorptive cells (enterocytes) interspersed occasionally with mucus-secreting goblet cells. The enterocyte constitutes the only anatomical barrier of physiological significance controlling the absorption of nutrients. The apical membrane of the enterocyte (i.e., the membrane facing the intestinal lumen) is covered with microvilli, which are minute projections of the plasma membrane. Because of its brush-like appearance under the microcope, the apical membrane is also known as the brush-border membrane.

2.2.2 The Luminal Environment

Bulk contents of the intestinal lumen are mixed by segmentation and peristalsis, and water and solutes are brought to the surface of the mucosa by convection. However, the luminal environment immediately adjacent to the brush-border membrane is stationary and unaffected by gut motility. The lack of convective mixing in this region creates a series of thin layers, each progressively more stirred, extending from the surface of the enterocyte to the bulk phase of the lumen. These thin layers constitute the so-called "unstirred layer," whose effective thickness has been calculated to be 35 μm [1].

Solute movement within an unstirred layer takes place by diffusion, which is slow compared with the convective movement in the bulk luminal phase. The pH at the luminal surface is approximately two units lower than that of the bulk phase and varies less than ± 0.5 units, despite large pH variations in the intestinal chyme. It has been suggested that the formation of the low-pH microclimate is due to the presence of mucin, which covers the entire surface of the epithelium [2,3]. Mucopolysaccharides possess a wide range of ionizable groups and hence mucin is an ampholyte. If the luminal chyme is of low pH, the ampholyte is positively charged, and so it repels additional hydrogen ions entering the microclimate. If, on the other hand, the chyme is alkaline, the ampholyte becomes negatively charged, and retains hydrogen ions within the microclimate. In this manner, the mucin layer functions as a restrictive barrier for hydrogen ions diffusing in and out of the microclimate.

2.2.3 Adaptive Regulation of Intestinal Nutrient Transport

2.2.3.1 Nonspecific Anatomical Adaptations to Changing Metabolic Requirements and Food Deprivation

Increases in metabolic requirements, such as arise during pregnancy, lactation, growth, exercise, and cold stress, are met by an increased

absorption of all available nutrients, mediated at least in part by an induced increase in food intake. The increased absorption is due to an increase in mucosal mass per unit length of intestine and a consequent increase in absorptive surface area. Not only is there an increase in the total number of cells, but the villi become taller.

The mammalian intestine adapts to prolonged food deprivation by dramatically slowing the rate of epithelial cell production in the crypts in order to conserve proteins and biosynthetic energy. This effect on mitosis and enterocyte renewal leads to markedly shortened villi. Because cell migration along the crypt-villus unit is also slowed, more cells lining the villi are functionally mature. Therefore, food deprivation, by reducing mucosal mass and increasing the ratio of transporting to nontransporting cells, effectively increases solute transport per unit mass of intestine.

2.2.3.2 Dietary Regulation of Intestinal Nutrient Carriers

It is well established that certain intestinal nutrient carriers (e.g., those transporting glucose and amino acids) are adaptively regulated by their substrates. In response to a signal for regulation of transport, the number of carriers at both the apical and basolateral membranes of enterocytes is increased or decreased as appropriate. According to Karasov's adaptive modulation hypothesis [4], a carrier should be repressed when its biosynthetic and maintenance costs exceed the benefits it provides. The benefits can be provision of either metabolizable calories or an "essential" nutrient, that is, a nutrient which cannot be synthesized by the body and must be obtained from the diet. Glucose carriers are up-regulated when the dietary supply of glucose is adequate or high because glucose provides valuable calories. The down-regulation of glucose carriers during a deficiency of glucose can be explained by the biosynthetic and maintenance costs outweighing the benefits of transporting this "nonessential" nutrient.

One might expect carriers for water-soluble vitamins to be down-regulated by their substrates and up-regulated in deficiency of the vitamins. The rationale in this case is that carriers for these essential nutrients are most needed at low dietary substrate levels; at high levels, the required amount of the vitamin could be extracted from the lumen by fewer carriers, or even cross the enterocyte by simple diffusion. As vitamins do not provide metabolizable energy, there is nothing to gain from the cost of synthesizing and maintaining carriers when the vitamin supply is adequate or in excess.

The prediction of suppressed transport of vitamins at high dietary intakes has proved to be true for ascorbic acid, biotin, and thiamin, but not for pantothenic acid, for which carrier activity is independent of dietary levels [5]. It appears that intestinal carriers are regulated only if

they make the dominant contribution to uptake, as is the case for the three regulated vitamins. It can also be reasoned that carriers for ascorbic acid, biotin, and thiamin would need to be regulated, because nutritional deficiencies of these vitamins can and do occur. In contrast, there is no need to regulate pantothenic acid carriers, because this vitamin is found naturally in almost all foods, and cases of deficiency are very rare.

2.2.4 Digestion, Absorption, and Transport of Dietary Fat

Absorption of the fat-soluble vitamins takes place mainly in the proximal jejunum and depends on the proper functioning of the digestion and absorption of dietary fat. The stomach is the major site for emulsification of fat. The coarse lipid emulsion, on entering the duodenum, is emulsified into smaller globules by the detergent action of bile. Pancreatic lipase hydrolyses triglycerides at the 1 and 3 positions, yielding 2-monoglycerides and free fatty acids. During their detergent action, bile salts exist as individual molecules. Above a critical concentration of bile salts, the bile constituents (bile salts, phospholipids, and cholesterol) form aggregates called micelles, in which the polar ends of the molecules are orientated toward the surface and the nonpolar portion forms the interior. The 2-monoglycerides and free fatty acids are sufficiently polar to combine with the micelles to form mixed micelles. These are stable water-soluble structures, which can dissolve fat-soluble vitamins and other hydrophobic compounds in their oily interior.

Mixed micelles do not cross the brush-border membrane of enterocytes as intact structures: the products of lipolysis must dissociate from these structures before they can be absorbed. Shiau and Levine [6] showed that a low-pH microclimate, representing the unstirred layer lining the luminal surface of the jejunum, facilitates micellar dissociation. Presumably, the fatty acid components of the mixed micelles become protonated when the mixed micelles enter the unstirred layer. This protonation reduces fatty acid solubility in the mixed micelles, allowing release of the fatty acids together with other lipid constituents. Individual lipids, including fat-soluble vitamins, can then be passively absorbed across the brush-border membrane. The bile salts are left behind to be actively reabsorbed in the distal ileum, whence they return to the liver to be recycled via the gall-bladder.

After the lipolytic micellar products enter the enterocytes, a cytosolic fatty acid-binding protein (FABP) facilitates intracellular transport of fatty acids by directing them from the cell membrane to the smooth endoplasmic reticulum, where triglyceride synthesis takes place. The triglycerides are packaged into chylomicrons, together with free and esterified cholesterol, phospholipids, apolipoproteins, fat-soluble vitamins, and

carotenoids. After further processing, the chylomicrons are discharged from the enterocyte by exocytosis across the basolateral membrane and enter the central lacteal of the villus. From there, they pass into the larger lymphatic channels draining the intestine, into the thoracic duct, and ultimately into the systemic circulation.

Medium-chain triglycerides, which contain fatty acids with a chain length of 6–12 carbon atoms, are not found in appreciable amounts in the normal diet. However, they deserve mention because they are included in specialized diets for patients who have fat malabsorption. Medium-chain triglycerides are absorbed in a more efficient manner to that described above for the longer-chain triglycerides. Being water-soluble, they can be absorbed directly as intact triglycerides. Once inside the enterocyte, they are hydrolyzed to medium-chain fatty acids by specific cellular lipases. Medium-chain fatty acids do not bind to FABP, are not reesterified to triglycerides, and are not packaged in chylomicrons. After leaving the enterocyte, medium-chain fatty acids enter the portal vein where they are bound to albumin and transported to the liver [7].

The chylomicrons are carried by the blood to all the tissues. Associated with the endothelium of blood capillaries in most tissues is the enzyme lipoprotein lipase, which attacks circulating chylomicrons and converts them into much smaller triglyceride-depleted particles known as chylomicron remnants. These particles contain apolipoprotein E (apoE) acquired from other circulating lipoproteins. The released free fatty acids and diglycerides can then be absorbed by the tissue cells.

The liver has the capacity to rapidly remove chylomicron remnants from the circulation, the apoE on the remnants serving as the ligand for receptors present on the surface of hepatocytes. The fates of individual fat-soluble vitamins after liver uptake of chylomicron remnants are discussed in their respective chapters (3–6).

2.2.5 Transport of Glucose and Fructose: A Model for the Absorption of Some Water-Soluble Vitamins

Glucose and fructose transport have been well studied [8], and the experimental techniques and postulated mechanisms help toward understanding the absorption of water-soluble vitamins.

Figure 2.1 shows how physiological amounts of glucose and fructose are absorbed by the small intestine. Luminal glucose crosses the epithelial brush border and accumulates in the enterocyte by means of secondary active transport. Transport is mediated by a sodium–glucose cotransporting carrier (SGLT1), which binds the substrates at a stoichiometric ratio of

two sodium ions to one glucose molecule. The immediate driving force for the sodium-coupled entry of glucose is the electrochemical gradient for sodium. This has two components: an electrical potential difference of about 40 mV across the brush-border membrane (cell interior negative) and a sodium concentration gradient. Both the electrical and chemical

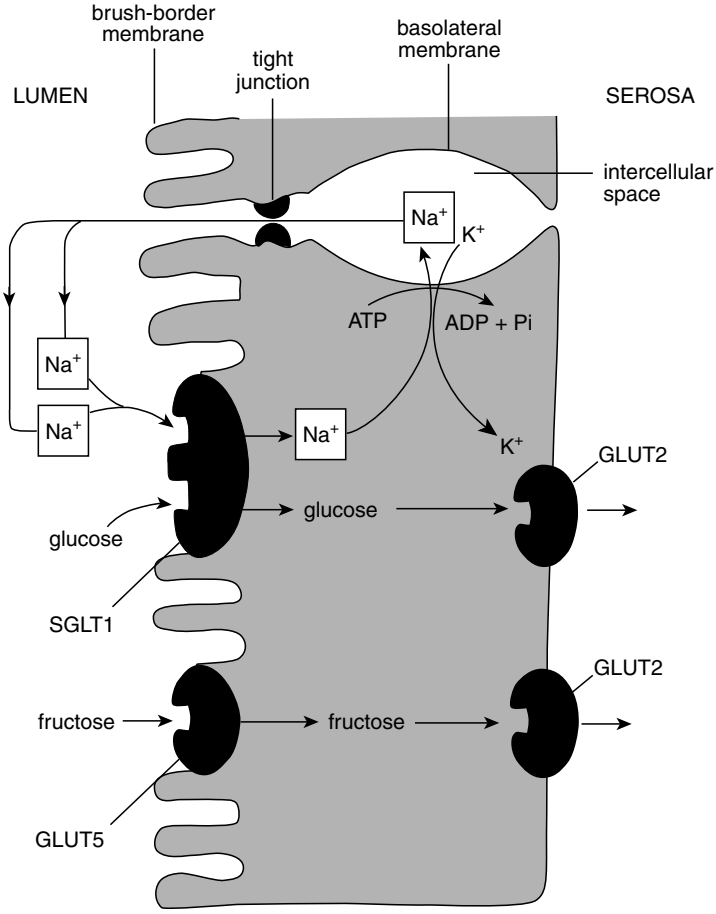

FIGURE 2.1
The carrier-mediated transport of D-glucose and D-fructose across the apical membrane and basolateral membrane of an enterocyte. Na^+ extruded into the intercellular space by the basolateral Na^+–K^+-ATPase (sodium pump) is able to equilibrate with Na^+ on the luminal side of the enterocyte by permeation through the tight junction. ATP, adenosine triphosphate; ADP, adenosine diphosphate; Pi, inorganic phosphate. (From Ball, G.F.M., *Vitamins. Their Role in the Human Body*, Blackwell Publishing Limited, Oxford, 2004, p. 12. With permission.)

components are established by the constant extrusion of sodium out of the enterocyte by the action of the basolateral sodium pump. Fructose crosses the brush border by facilitated diffusion mediated by the glucose transporter GLUT5. Exit of both glucose and fructose from the enterocyte to the serosa takes place by facilitated diffusion at the basolateral membrane and is mediated by GLUT2.

2.2.6 Effects of Dietary Fiber on Absorption of Nutrients

Dietary fiber consists of plant material that cannot be digested by the endogenous secretions of the human digestive tract. From an analytical standpoint, dietary fiber can be divided into insoluble fibers and soluble fibers. The insoluble fibers include cellulose, lignin, and many hemicelluloses; the soluble fibers include pectins, some hemicelluloses, gums, and mucilages. The various gums and mucilages are widely used in the food and pharmaceutical industries as emulsifiers, thickeners, and stabilizers. The nature and physical properties of the main fiber components are summarized in Table 2.1 [9].

Vahouny and Cassidy [10] discussed potential mechanisms by which dietary fiber can modify nutrient absorption. Intestinal absorption of nutrients can be influenced by modifying the rates at which food enters or leaves the stomach. Bulky, high fiber foods may require longer periods for ingestion, and therefore modify rates of gastric filling. Viscous fiber components slow stomach emptying. The delayed release of gastric emptying and modified intestinal pH might alter the regulation of pancreatic and biliary secretions. Insoluble fibers accelerate small intestinal transit, allowing less time for nutrient absorption; in contrast, viscous fibers slow transit. Many fiber components can alter the activities of pancreatic enzymes by affecting viscosity and pH, and by adsorption. Dietary fiber impairs lipid absorption by interfering with micelle formation. Evidence is the *in vitro* binding of bile salts and other micellar components by lignin and guar gum, and the increase in fecal bile salts in response to ingestion of dietary fiber. Viscous fibers can influence nutrient absorption by interfering with bulk phase diffusion of nutrients in the intestinal lumen. The mucin layer covering the mucosal surface has been suggested to be an important diffusion barrier to absorption. Reported changes in mucin content or turnover in response to various fiber types is a possible mechanism by which dietary fiber alters the transport characteristics of nutrients at the mucosal surface. Prolonged feeding of diets supplemented with cellulose or pectin significantly increased villus height and thickness, thereby increasing the absorptive surface area. The dietary supplements also improved nutrient uptake by the small intestine *in vitro*.

TABLE 2.1

Dietary Fiber Components

Fiber	Chemistry	Solubility in Water	Natural Source	Physical Properties
Cellulose	Linear polymer of glucose with beta 1–4 linkages	Insoluble	Main structural component of plant cell wall	Binding of water
Lignin	Highly complex nonpolysaccharide polymer derived from phenolics	Insoluble	Structural component of woody plants	Binding of bile salts and other organic material
Hemicelluloses	Heterogeneous group of polysaccharides which contain a variety of different sugars in the polymeric backbone and side chains	Many insoluble, some soluble	Matrix of plant cell wall	Binding of water and cations
Pectins	Polymer composed primarily of galacturonic acid and rhamnose with a variable degree of methyl esterification	Soluble, capable of forming gels with sugar and acid	Matrix of plant cell wall, ripe fruits	Formation of gels, binding of bile salts and other organic material
Gums	Complex group of highly branched polysaccharides (e.g., gum acacia)	Soluble to give very viscous colloidal solutions	Extruded at site of injury to plants	Similar to pectins
Mucilages	Polysaccharides resembling hemicelluloses (e.g., guar gum)	Soluble to give slimy, colloidal solutions	Mixed with starch in endosperm	Binding of water, formation of gels, binding of bile salts and other organic material

Source: From Anderson, J.W. and Chen, W.-J.L., *Am. J. Clin. Nutr.*, 32, 346, 1979. With permission.

2.3 Bioavailability

2.3.1 General Concepts

The term "bioavailability," as applied to food-borne vitamins in human nutrition, refers to the proportion of the quantity of vitamin ingested that undergoes intestinal absorption and utilization by the body. Utilization encompasses transport of the absorbed vitamin to the tissues, cellular uptake, and ultimate fate of the vitamin. The latter can be conversion to a form that can fulfill some biochemical or physiological function, conversion to a nonfunctional form for subsequent excretion, and storage. The major component of bioavailability and the rate-limiting factor is absorption. Many ways of determining vitamin bioavailability have been reported, most of which give an estimate of relative rather than absolute bioavailability. Relative bioavailability is commonly expressed as a percentage of the response obtained with a reference material of high bioavailability. Bioavailability is an operational term defined by the method used to determine it. Different values will be obtained within a given study if different endpoints are used.

Intestinal absorption, and therefore bioavailability, of a vitamin depends on the chemical form and physical state in which the vitamin exists within the food matrix. These properties may be influenced by the effects of food processing and cooking, particularly in the case of provitamin A carotenoids, niacin, vitamin B_6, and folate. The food matrix enhances vitamin absorption by stimulating the secretion of digestive enzymes and bile salts. Bile salts inhibit gastric emptying and proximal intestinal transit, resulting in an increased residence time at the absorption sites. Thus, absorption of a riboflavin supplement taken with a meal was about 60%, as compared to 15% on an empty stomach [11].

In foods derived from animal and plant tissues, the B-group vitamins occur as their coenzyme derivatives, usually associated with their protein apoenzyme. In addition, niacin in cereals and vitamin B_6 in certain fruits and vegetables occur largely as bound storage forms. In milk and eggs, which are derived from animal secretions, the B-group vitamins occur, at least to some extent, in the underivatized form, a proportion of which may be associated with specific binding proteins. Vitamins that exist as chemically bound complexes with some other material in the food matrix exhibit lower efficiencies of digestion and absorption compared with the free (unbound) vitamin ingested, for example, in tablet form.

Certain dietary components can retard or enhance a vitamin's absorption, therefore the composition of the diet is an important factor in bioavailability. For example, the presence of adequate amounts of

dietary fat is essential for the absorption of fat-soluble vitamins. Fibrous plant material can interfere with the physiological mechanisms of absorption, as is evident by the poor bioavailability of β-carotene in a raw carrot compared with that in a cooked carrot [12]. The binding of bile salts to various wood fiber sources and isolated fiber components has been demonstrated [13]. Furthermore, certain types of dietary fiber may either interfere with the formation of mixed micelles in the intestinal lumen or effectively alter the normal diffusion and accessibility of micellar lipids to the absorptive surface of the intestinal mucosa. Such events could compromise the absorption of lipids, including the fat-soluble vitamins. The absorption of folate is impaired by ethanol in cases of chronic alcoholism.

2.3.2 Methods for Estimating Vitamin Bioavailability in Human Subjects

There are two main experimental approaches for estimating vitamin bioavailability in human subjects: (i) determining the extent of vitamin absorption by measuring the concentration of vitamin in the plasma, the chylomicron fraction of plasma, or urine and (ii) comparing the mass of vitamin consumed with the mass excreted in the feces. A difficulty arises in the plasma sampling method when newly ingested vitamin mixes with endogenous circulating vitamin, but this can be overcome by the use of stable isotopes as tracers. A difficulty also arises in the oral–fecal balance method because colonic flora can utilize unabsorbed vitamin and also synthesize new vitamin. Surgical bypassing of the colon using pigs or human ileostomy subjects overcomes this problem to a large extent.

The application of stable isotope techniques has given much needed impetus to the study of vitamin bioavailability, particularly the provitamin A carotenoids and folate.

2.3.2.1 Plasma Response

This method involves measuring the increase in plasma vitamin concentration over baseline level at several time intervals after ingestion of the test meal, and plotting these values against time. The procedure is repeated after oral dosing with a reference vitamin standard. The area under the curve (AUC) obtained for the test meal, expressed as a percentage of the AUC for the reference dose, gives the relative bioavailability of the vitamin in the meal. The post-absorption positive AUC (AUC+) might be followed by a negative AUC (AUC−), depending on the degree of diurnal fluctuation (Figure 2.2). In practice, AUR+ is calculated. Only in the case of fortified foodstuffs or foods with naturally very

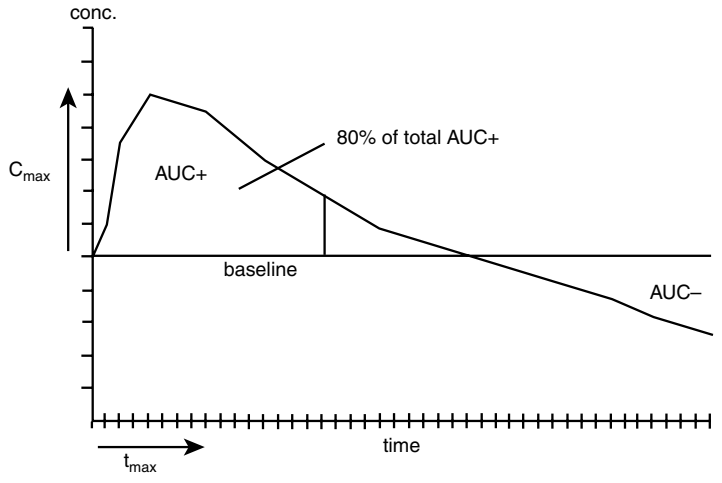

FIGURE 2.2
Parameters of bioavailability. AUC+, post-absorption area under curve; AUC−, negative area under curve; C_{max}, maximal concentration; t_{max}, time for maximal concentration to be reached. (From Pietrzik, K., Hages, M., and Remer, T., *J. Micronutr. Anal.*, 7, 207–222, 1990. With permission.)

high vitamin contents can a significant increase in vitamin blood level be expected and AUC to be measurable [14]. The validity of the AUC depends on the *in vivo* handling of the reference dose and test dose being equivalent [15].

2.3.2.2 Urinary Excretion

Urinary excretion can be used to measure relative bioavailability because it is proportional to the plasma concentration if urinary clearance is constant. Subjects are preloaded with synthetic vitamin in order to saturate the tissues and ensure that the additionally absorbed vitamin will be excreted.

2.3.2.3 Oral-Fecal Balance Studies and the Determination of Prececal Digestibility

In a classical balance study, human subjects are fed a diet containing a known amount of the test vitamin, and the difference between what is ingested and what is recovered in the feces is considered to be apparent absorption. Absolute absorption is not measured because not all of the vitamin in the feces will originate from the ingested food: some will

originate from sloughed mucosal cells. There is no information about the utilization of the vitamin. The natural presence of microflora in the colon creates a possible bias in the balance method. Unabsorbed vitamin, on reaching the colon, can become metabolized by the intestinal flora, leading to overestimation of bioavailability. The gut microflora can also synthesize B-vitamins and vitamin K, leading to underestimation of bioavailability.

An effective and practical way of circumventing the problem of intestinal microflora is to surgically bypass the colon, causing the digesta to move straight from the ileum to the rectum. This allows the *in vivo* determination of prececal digestibility, and the calculation of absolute absorption. The domestic pig is chosen as the animal model, because the digestive physiology of this species resembles that of the human. The surgical technique is an end-to-end ileo-rectal anastomosis [16,17]. The technique has been used successfully for determining the prececal digestibility of thiamin [18], niacin [19], vitamin B_6 [20], and pantothenic acid [19]. Human patients with ileostomies fulfill a similar function to the pig model in allowing the assessment of absolute absorption, and have been used to determine the bioavailability of β-carotene [21] and folate [22] from food. The body conserves folate and vitamin B_{12} through their excretion in bile and subsequent reabsorption by the small intestine. This enterohepatic circulation complicates interpretation of the results for these vitamins.

2.3.2.4 Use of Stable Isotopes

Isotopes of a particular atom contain a different number of neutrons and can be either stable or radioactive. Unlike radioactive isotopes, stable isotopes emit no radiation and can therefore be used safely as tracers in human studies of nutrient metabolism. Stable isotopic methods using deuterium (^2H) and ^{13}C are being used to estimate body stores of vitamin A, and to study the bioavailability and bioefficacy of dietary carotenoids [23]. They are also being used to assess folate bioavailability [24,25]. The use of stable isotopes allows differentiation between isotopically labeled vitamin from the dose and unlabeled endogenous vitamin from body stores. The labeled vitamin or its metabolites can be specifically determined in blood, urine, and feces, allowing the detailed study of absorption, metabolism, and excretion. Detection methods for stable isotopes are less sensitive than those for radioisotopes, thus the dose administered needs to be relatively high to reach measurable levels. Owing to complicated methodology and expensive instrumentation, stable isotopic procedures are confined to specialized laboratories.

Isotopic labeling of the vitamin can be performed intrinsically or extrinsically. Intrinsic labeling involves biological incorporation of isotope into

the tissues of the plant or animal food source during growth and development, so that the labeled vitamin is in the same matrix as the food consumed. For example, kale was labeled intrinsically with ^{13}C by growing plants continuously in an atmosphere containing $^{13}CO_2$, starting approximately 8 days after sowing [26]. Broccoli was labeled intrinsically with deuterium by adding deuterium oxide (heavy water) to the nutrient solution of hydroponically grown plants [27]. Extrinsic labeling refers to the chemical incorporation of isotope into the vitamin molecule of interest. Multiple labeling is desirable to increase the molecular mass and improve the sensitivity of detection by mass spectrometry. The labeled vitamin is mixed with the food just before consumption. Interpretation depends on the assumption that the labeled vitamin behaves in a manner similar to the naturally occurring vitamin in the diet.

Mass spectrometry is an analytical technique that measures the masses of individual molecules and atoms. During the initial conversion of analyte molecules into gas-phase ionic species (ionization), the excess energy transferred to the molecules leads to fragmentation. A mass analyzer separates these molecular ions and their charged fragments according to their m/z (mass/charge) ratio. The ion current due to these mass-separated ions is detected by a suitable detector and displayed in the form of a mass spectrum. The mass spectrum is a plot of m/z values of all ions that reach the detector versus their abundance. For quantitative analysis, the ions are usually detected by selected-ion monitoring, in which selected m/z values are exclusively monitored [28].

Mass spectrometers designed for the analysis of organic molecules include gas chromatography–mass spectrometry (GC–MS), high-performance liquid chromatography–mass spectrometry (LC–MS), tandem mass spectrometry (MS–MS), and LC–MS–MS instruments. LC–MS methods have the advantage over GC–MS methods in that they do not require such labor-intensive sample preparation. Tandem mass spectrometry refers to the coupling of two mass spectrometers (MS-1 and MS-2) in series. MS-1 mass-selects a specified ion, which undergoes fragmentation in the intermediate region, and MS-2 mass-analyzes the ionic fragments. Molecular specificity is guaranteed because the product ions are derived exclusively from the preselected precursor.

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3

Vitamin A: Retinoids and the Provitamin A Carotenoids

3.1 Background

Vitamin A-active compounds are represented by retinoids (designated as vitamin A) and their carotenoid precursors (provitamin A carotenoids). The retinoids comprise retinol, retinaldehyde, and retinoic acid, together with their naturally occurring and synthetic analogs. Generally speaking, dietary vitamin A is obtained from animal-derived foods, while plant foods provide carotenoid precursors.

Retinol derived from ingested provitamin A carotenoids, along with that ingested as such, is stored in the liver and secreted into the bloodstream when needed. The circulating retinol is taken up by target cells and converted in part to retinoic acid, which functions as a ligand to a nuclear retinoid receptor. The liganded receptor interacts with specific enhancer sites on the DNA and, in collaboration with many other regulatory proteins, induces the synthesis of proteins through the direct control of gene transcription. This type of action establishes vitamin A (in the form of the retinoic acid metabolite) as a hormone, similar to the steroid hormones and thyroid hormone. Vision is a nonhormonal, biochemical process involving a different vitamin A metabolite, 11-*cis*-retinaldehyde.

Vitamin A is an essential dietary factor for normal embryogenesis, cell growth and differentiation, reproduction, maintenance of the immune system, and vision. Malnourished children in famine-stricken countries are at risk of clinical vitamin A deficiency, which manifests as keratinization of the conjunctiva, and later of the cornea, causing permanent blindness. There is also increased infant mortality from infectious diseases due to impaired immunocompetence.

Excessive dietary intakes of vitamin A produce symptoms of acute and chronic toxicity, including teratogenicity in developing fetuses. Normally, toxicity results from the indiscriminate use of pharmaceutical supplements, and not from the consumption of usual diets. The only naturally occurring products that contain sufficient vitamin A to induce toxicity are the livers of animals at the top of long food chains, such as large marine fish and carnivores.

Carotenoids are widely believed to protect human health. In particular, some epidemiological studies have correlated the intake of carotenoid-rich fruits and vegetables with protection from some forms of cancer, cardiovascular disease, and age-related macular degeneration. This action is not restricted to provitamins and, therefore, may be attributable to the carotenoids' antioxidant properties rather than to their vitamin A activity.

3.2 Chemical Structure, Biopotency, and Physicochemical Properties

3.2.1 Structure and Biopotency

3.2.1.1 Retinol

The structures of retinoids found in foods and fish-liver oils are shown in Figure 3.1. The parent vitamin A compound, retinol, has the empirical formula $C_{20}H_{30}O$ and a molecular weight (MW) of 286.4. The molecule comprises a β-ionone (cyclohexenyl) ring attached at the carbon-6 (C-6) position to a polyene side chain whose four double bonds give rise to *cis–trans* (geometric) isomerism. Theory predicts the existence of 16 possible isomers of retinol, but most of these exhibit steric hindrance, and some are too labile to exist [1]. The predominant isomer, all-*trans*-retinol,

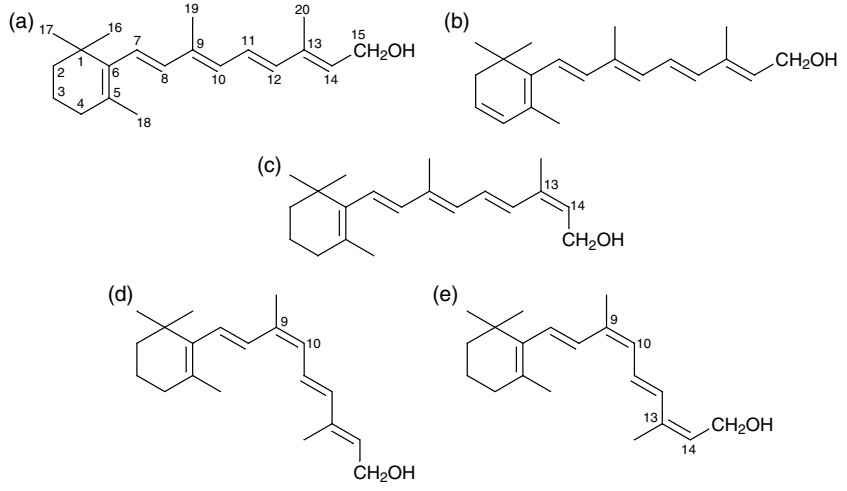

FIGURE 3.1
Structures of retinoids found in foods and fish-liver oils. (a) All-*trans*-retinol (vitamin A_1); (b) all-*trans*-3-dehydroretinol (vitamin A_2); (c) 13-*cis*-retinol; (d) 9-*cis*-retinol; (e) 9,13-di-*cis*-retinol.

TABLE 3.1

Biopotency of Isomers of Vitamin A Esters Relative to the All-*trans* Forms (set at 100%) as Determined in Rat Vaginal Smear Assays

Isomers	Retinyl Acetate	Retinyl Palmitate
All-*trans*	100	100
13-*cis*	76	73
9-*cis*	19	19
9,13-di-*cis*	16	21

Source: From Weiser, H. and Somorjai, G., *Int. J. Vitam. Nutr. Res.*, 62, 201, 1992. With permission.

possesses maximal (100%) vitamin A activity and is frequently accompanied in natural foodstuffs by smaller amounts of the less potent 13-*cis*-retinol. Lower potency 9-*cis*-retinol and 9,13-di-*cis*-retinol occur in small amounts in fish-liver oils. 3-Dehydroretinol (vitamin A_2) represents the major form of vitamin A in the liver and flesh of freshwater fish.

Synthetic retinyl acetate ($C_{22}H_{32}O_2$, MW = 328.5) and retinyl palmitate ($C_{36}H_{60}O_2$, MW = 524) are used commercially to supplement the vitamin A content of foodstuffs.

The biopotencies of isomers of vitamin A esters determined by means of rat vaginal smear assays are presented in Table 3.1 [2]. Retinaldehyde possesses about 90% of the biological activity of all-*trans*-retinol and 3-dehydroretinol is about 40% as active [3].

3.2.1.2 Provitamin A Carotenoids

Carotenoids can be considered chemically as derivatives of lycopene — a $C_{40}H_{56}$ polyene composed of eight isoprenoid units (Figure 3.2). Using the abbreviation ip for the isoprenoid unit, the carotenoids can be represented as ip-ip-ip-ip-pi-pi-pi-pi, that is, the arrangement of the units is reversed at the center of the molecule. Derivatives are formed by a variety of reactions that include cyclization, hydrogenation, dehydrogenation, and insertion of oxygen. Hydrocarbon carotenoids are known as carotenes, and the oxygenated derivatives are termed xanthophylls. The oxygen functions of xanthophylls are most commonly hydroxy, keto, epoxy, methoxy, and carboxy groups. Some acyclic carotenoids occur widely, for example, lycopene, but monocyclic and bicyclic compounds are more common. Most carotenoids of plant tissues contain 40 carbon atoms, but shortened molecules known as apocarotenoids can arise as a result of partial oxidative cleavage.

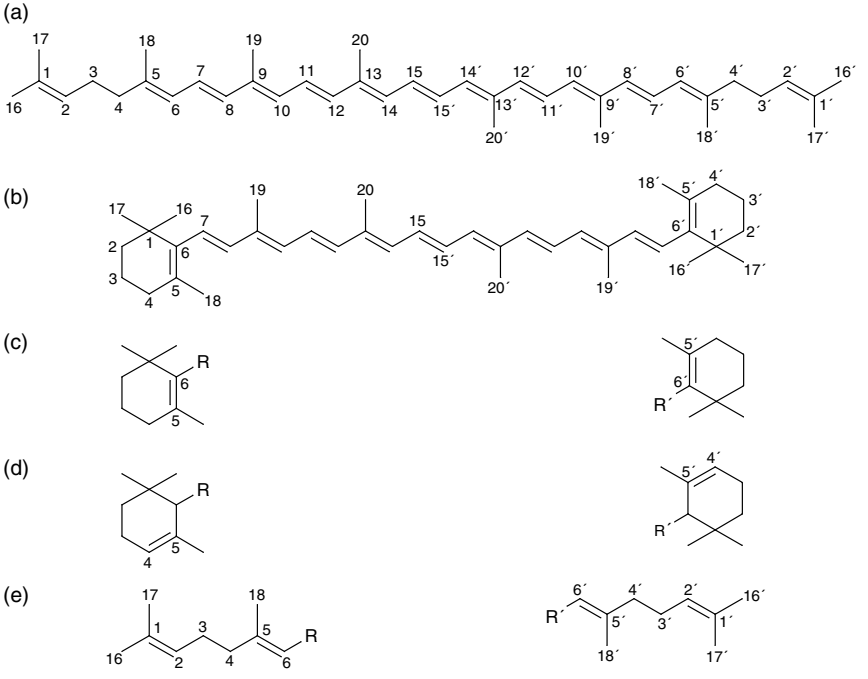

FIGURE 3.2
Structures of lycopene, β-carotene, and three of the six carotenoid end group designations. (a) Lycopene; (b) β-carotene; (c) β (beta); (d) ε (epsilon); (e) ψ (psi).

A semisystematic nomenclature for carotenoids has been devised to convey structural information [4]. According to this scheme, the carotenoid molecule is considered as two halves, and the nature of the end group of each half is specified. Each carotenoid is considered to be a derivative of a parent carotene, indicated by two Greek letters describing the end groups. The nomenclature recognizes six end groups: β (beta), ε (epsilon), κ (kappa), ϕ (phi), χ (chi), and ψ (psi). Three of the more common end groups (β, ε, and ψ) are shown in Figure 3.2. Changes in hydrogenation level and the presence of substituent groups are indicated by the use of conventional prefixes and suffixes. The numbering system for carotenoids is shown in the structure of β-carotene (Figure 3.2).

Many of the naturally occurring carotenoids are chiral, bearing one to five asymmetric carbon atoms. In most cases, a given carotenoid occurs in only one chiral form. The absolute configuration at a chiral center is designated by use of the *R,S* convention. Unless stated otherwise, all double bonds have the *trans* configuration. *Cis–trans* isomerism is indicated by citing the double bond or bonds with a *cis* configuration.

The *Z, E* terminology for geometric isomerism is seldom used in vitamin A and carotenoid nomenclature. The semisystematic names of some common carotenoids of plant foods are given in Table 3.2 [5].

From a nutritional viewpoint, the carotenoids are classified as provitamins and inactive carotenoids. To have vitamin A activity, the carotenoid molecule must incorporate a molecule of retinol, that is an unsubstituted β-ionone ring with an 11-carbon polyene chain. β-Carotene ($C_{40}H_{56}$, MW = 536.9), the most ubiquitous provitamin A carotenoid, is composed of two molecules of retinol joined tail to tail; thus the compound possesses maximal (100%) vitamin A activity. The structures of all other provitamin A carotenoids incorporate one molecule of retinol and hence theoretically contribute 50% of the biological activity of β-carotene. Among the 600 or so carotenoids that exist in nature, only about 50 possess vitamin A activity in varying degrees of potency.

In nature, carotenoids exist primarily in the all-*trans* configuration, but small amounts of 9-*cis*, 13-*cis*, and 15-*cis* isomers of β-carotene have been found in fresh and processed fruits and vegetables [6,7,7a,8]. With asymmetrical carotenoids, such as α-carotene and β-cryptoxanthin, the number of theoretically possible *cis* isomers is approximately twofold greater than with symmetrical carotenoids, such as β-carotene. In fruits, hydroxycarotenoids (carotenols) exist mainly as mono or bis esters of saturated long-chain fatty acids, such as lauric (C12), myristic (C14), and palmitic (C16) acids [9,10].

Synthetic β-carotene, β-apo-8′-carotenaldehyde (apocarotenal), the ethyl ester of β-apo-8′-carotenoic acid (apocarotenoic ester), and the nonprovitamin carotenoid canthaxanthin are permitted food color additives [11].

13-*cis*-β-Carotene and 9-*cis*-β-carotene exhibit, respectively, 53 and 38% of the provitamin A activity of all-*trans*-β-carotene in a rat growth assay [12].

3.2.2 Physicochemical Properties

3.2.2.1 Appearance and Solubility

Retinol and retinyl acetate are yellow crystalline powders; retinyl palmitate is a pale yellow oil or crystalline mass. β-Carotene is a reddish-brown to deep violet crystalline powder; β-apo-8′-carotenaldehyde is a deep violet crystalline powder; β-apo-8′-carotenoic acid ethyl ester is a rust-red crystalline powder.

Retinol and its esters are insoluble in water; soluble in alcohol; and readily soluble in diethyl ether, petroleum ether, chloroform, acetone, and fats and oils. β-Carotene is insoluble in water; very sparingly soluble in alcohol, fats and oils; sparingly soluble in ether and acetone; and slightly soluble in chloroform.

TABLE 3.2

Chemical Nomenclature and Provitamin A Activity of some Common Carotenoids of Plant Foods

Trivial Name	Semisystematic Name	Type	Vitamin A Activity (%)[a]
Lycopene	ψ,ψ-Carotene	Acyclic carotene	Inactive
ζ-Carotene	7,8,7′,8′-Tetrahydro-ψ,ψ-carotene	Acyclic carotene	Inactive
γ-Carotene	β,ψ-Carotene	Monocyclic carotene	42–50
β-Zeacarotene	7′,8′-Dihydro-β,ψ-carotene	Monocyclic carotene	20–40
β-Carotene	β,β-Carotene	Bicyclic carotene	100
α-Carotene	(6′*R*)-β,ε-Carotene	Bicyclic carotene	50–54
β-Cryptoxanthin	(3*R*)-β,β-Caroten-3-ol	Bicyclic, monohydroxy-carotenoid	50–60
Zeinoxanthin	(3*R*,6′*R*)-β,ε-Caroten-3-ol	Bicyclic, monohydroxy-carotenoid	Inactive
Zeaxanthin	(3*R*,3′*R*)-β,β-Carotene-3,3′-diol	Bicyclic, dihydroxycarotenoid	Inactive
Lutein	(3*R*,3′*R*, 6′*R*)-β,ε-Carotene-3,3′-diol	Bicyclic, dihydroxycarotenoid	Inactive
β-Carotene-5,6-epoxide	5,6-Epoxy-5,6-dihydro-β,β-carotene	Bicyclic, monoepoxy-carotenoid	21
β-Carotene-5,8-epoxide (Mutatochrome)	5,8-Epoxy-5,8-dihydro-β,β-carotene	Bicyclic, monoepoxy-carotenoid	50
β-Cryptoxanthin-5, 6-epoxide	5,6-Epoxy-5,6-dihydro-β,β-carotene-3-ol	Bicyclic, monoepoxy-, monohydroxycarotenoid	Active (not quoted)
Neoxanthin	(3*S*,5*R*,6*R*,3′*S*,5′*R*,6′*S*)-5′,6′-Epoxy-6, 7-didehydro-5,6,5′,6′-tetrahydro-β, β-carotene-3,5,3′-triol	Bicyclic, monoepoxy-, trihydroxycarotenoid	Inactive
Violaxanthin	(3*S*,5*R*, 6*S*,3′*S*, 5′*R*,6′*S*)-5,6,5′, 6′-Diepoxy-5,6,5′,6′-tetrahydro-β, β-carotene-3,3′-diol	Bicyclic, diepoxy-, dihydroxycarotenoid	Inactive

[a]Activity of all-*trans* forms relative to the activity of β-carotene.

Source: From Bauernfeind, J.C., *J. Agric. Food Chem.*, 20, 456, 1972. With permission.

3.2.2.2 Stability in Nonaqueous Solution

3.2.2.2.1 Retinoids

Retinol is readily oxidized by atmospheric oxygen, resulting in an almost complete loss of biological activity. The 5,6-epoxide and 5,8-furanoxide are among the oxidation products. Retinyl esters are somewhat more stable towards oxidation than retinol. Retinol is extremely sensitive towards acids, which can cause rearrangement of the double bonds and dehydration. Solutions of all-*trans*-retinol or retinyl palmitate in hexane undergo slow isomerization to the lower potency *cis* isomers when exposed to white light. The photoisomerization rate is greatly increased in the presence of chlorinated solvents, but under gold fluorescent light (wavelengths greater than 500 nm) no significant isomerization occurs within 23 h [13]. Retinyl palmitate is stable in chlorinated solvents when it is stored in the dark [14]. Irradiation also rearranges double bonds to form inactive retro structures, which are responsible for much of the yellow color of decomposing vitamin A [15].

3.2.2.2.2 Carotenoids

The carotenoids are stable within their natural plant cell environment, but once isolated they are prone to molecular rearrangement, *trans* to *cis* isomerization, and degradation by heat, light, oxygen, trace amounts of acids, and active surfaces such as silica. Xanthophylls are particularly susceptible to these agents and are also destroyed in alkaline environments. Chlorophyll compounds naturally present in extracts of green leafy vegetables have the ability to sensitize the photoisomerization of carotenoids, giving rise to appreciable amounts of *cis* isomers during even a brief exposure to white light [16]. Solutions of β-carotene undergo slow isomerization, giving rise to 9-*cis* and 13-*cis* isomers, even when stored in the dark. In general, isomerization is higher in nonpolar solvents than in polar solvents [17]. Chlorinated solvents are often contaminated with trace amounts of hydrochloric acid, which can promote stereoisomerization.

3.3 Vitamin A in Foods

3.3.1 Occurrence

All natural sources of vitamin A are derived ultimately from provitamin A carotenoids, which are synthesized for metabolic purposes exclusively by higher plants and photosynthetic microorganisms. Meat and milk contain vitamin A as a consequence of the animal converting ingested provitamin A carotenoids to retinol. Carotenoids present in milk

products, egg yolk, shellfish, and crustacea result from the deposition of unmetabolized dietary carotenoids in the animal's tissues. In green, photosynthetic plant tissue, carotenoids are localized with the chlorophylls in the thylakoid membranes of chloroplasts, bound noncovalently to proteins in pigment—protein complexes. In nonphotosynthetic tissues (e.g., in fruits, carrots, and sweet potatoes), carotenoids are primarily found in chromoplasts, either within lipid droplets or associated with proteins, depending on the chromoplast type [18]. Carotenoids also occur as very fine dispersions in aqueous systems, such as orange juice.

Foods are supplemented with vitamin A in the form of standardized preparations of synthetic esters of retinol, nowadays chiefly retinyl palmitate. The preparations are available commercially as either dilutions in high-quality vegetable oils containing added vitamin E as an antioxidant or as dry, stabilized beadlets, in which the vitamin A is dispersed in a solid matrix of gelatin and sucrose or gum acacia and sucrose. The oily preparations are used to supplement fat-based foods such as margarines. The dry preparations are used in dried food products such as milk powder, infant formulas, and dietetic foods. β-Carotene, in the form of microcrystals suspended in vegetable oil, is used to impart color to fat-based foods such as margarines, butter, and cheese. Dried emulsions of carotenoids can be rehydrated and used to color and nutrify a variety of water-based foods [19].

3.3.1.1 Vitamin A

The distribution of vitamin A in some common foods is given in Table 3.3 [20]. The liver of meat animals is a rich source of vitamin A, for this organ is the body's main storage site of the vitamin. Whole milk, butter, cheese, and eggs are important dietary sources. Margarine is fortified with vitamin A to make it nutritionally equivalent to butter. Fortification of skim milk, partially skimmed milk, and nonfat dry milk with vitamin A is mandatory in the U.S. and Canada. The edible portions of fatty fish (e.g., herring, mackerel, pilchards, sardines, and tuna) contain moderate amounts of vitamin A, but white fish, apart from the haddock, contain only trace amounts. In most of the foods that contain vitamin A, the retinol forms esters with long-chain fatty acids, particularly palmitic acid. An exception is egg yolk, in which unesterified retinol represents the major retinoid, accompanied by retinaldehyde and retinyl esters [21]. *Cis* isomers of vitamin A occur in foods to varying extents, with fish-liver oils and eggs containing as much as 35 and 20%, respectively, of their total retinol in this form [3].

In margarines, the various naturally occurring vitamin A isomers and provitamins that may have been present in the original crude oils are removed during the refining process [22]. Thus the only vitamin A that

TABLE 3.3

Vitamin A Content of Various Foods

Food	Micrograms of Retinol per 100 g Edible Portion
Cow's milk, pasteurized, whole	30
semi-skimmed	19
skimmed	1
Butter	958
Cheese, cheddar	364
Egg, chicken, whole, raw	190
Beef, trimmed lean, raw, average	Tr
Lamb, trimmed lean, raw, average	6
Pork, trimmed lean, raw, average	Tr
Chicken meat, raw, average	11
Liver, lamb, fried	19,700
Cod, raw, fillets	2
Herring, raw	44
grilled	34
Pilchards, canned in tomato sauce	7
Sardines, canned in brine, drained	6
canned in oil, drained	7
canned in tomato sauce	9
Tuna, canned in brine, drained	N
canned in oil, drained	N

Note: Tr, trace; N, the vitamin is present in significant quantities but there is no reliable information on the amount.
Source: From Food Standards Agency, *McCance and Widdowson's The Composition of Foods*, 6th summary ed., Royal Society of Chemistry, Cambridge, 2002. With permission.

is present in the final margarine is the retinyl ester and β-carotene combination that is added during production [23].

3.3.1.2 Provitamin A Carotenoids

Carrots, sweet potatoes, and green leafy vegetables are major contributors of provitamin A in the American diet [24]. Carotenoid concentrations of fruits and vegetables are affected by factors such as: (1) cultivar/variety; (2) part of the plant consumed; (3) uneven distribution of the carotenoids in a given food sample; (4) stage of maturity; (5) climate/geographic site of production; (6) harvesting and postharvest handling; and (7) processing and storage [25]. Compared to vegetables, fruits contain a greater variety of carotenoids in varying concentrations. Citrus fruits are the most complex fruits in terms of the number of carotenoids found. In ripening fruits, the decrease in chlorophylls is frequently accompanied by an increase in the concentration of carotenoids and an increase in the

ratio of carotenes to xanthophylls. Red palm oil is an important food source of vitamin A in South America, Southeast Asia, and some countries of Africa. This oil is extracted from the fleshy mesocarp of the palm nut; the oil extracted from the palm kernel is without value as a source of vitamin A [26]. Cereals (apart from yellow maize) are negligible sources of provitamin A.

The quantitative distribution of the major provitamin isomers in a selection of fresh and processed fruits and vegetables is presented in Table 3.4. Fresh broccoli, collards, and spinach have 29, 25, and 22%, respectively, of their total β-carotene content in the form of *cis* isomers. This high proportion of *cis* isomers in fresh green vegetables can be attributed to the ability of chlorophylls to sensitize photoisomerization of carotenoids [16]. The presence of *cis* isomers in fresh orange juice, peaches, and tomatoes may be due to the same effect of chlorophylls before these fruits ripen. In most vegetables and fruits, β-carotene constitutes more than 85% of the total provitamin A activity. Notable exceptions are carrots and oranges, which contain both β-carotene and α-carotene [27]. β-Cryptoxanthin is a major provitamin in orange juice [28] and in some varieties of sweet corn [29]. In many fruits and vegetables, the concentrations of provitamins are low relative to the concentrations of inactive carotenoids. For example, lutein is the most abundant carotenoid in green leafy vegetables [30], lycopene predominates in tomatoes [31], and capsanthin is the major pigment in red peppers [32]. Other inactive carotenoids found in fruits and vegetables include ζ(zeta)-carotene, zeinoxanthin, zeaxanthin, neoxanthin, and violaxanthin.

3.3.2 Stability

3.3.2.1 Introduction

In foods, the indigenous retinyl esters are dissolved in the lipid matrix, in which they are protected from the oxidizing action of atmospheric oxygen by vitamin E and other antioxidants that might be present. The carotenoids locked in plant tissues are also protected from oxidation. On depletion of the antioxidants, the retinyl esters become vulnerable to direct oxidation and subject to attack by free radicals produced during lipid oxidation. Thus factors that accelerate lipid oxidation, such as exposure to air, heat, traces of certain metals (notably copper and, to a lesser extent, iron), and storage time, will also result in the destruction of the vitamin A compounds.

The many studies of the effects of processing and domestic cooking on the levels of carotenoids in foods have produced conflicting results, owing to differences in the experimental approach [33]. One of the factors that may lead to considerable variation in analytical data on raw and

TABLE 3.4

Quantitative Distribution of β-Carotene, α-Carotene, and β-Cryptoxanthin Isomers in Fresh and Processed Fruits and Vegetables[a,b]

	β-Carotene						α-Carotene						β-Cryptoxanthin				
Extract	**All-*trans***	**9-*cis***	**13-*cis***	**15-*cis***	**other *cis*[c]**	**Total**	**All-*trans***	**9-*cis***	**13-*cis***	**13′-*cis***	**other *cis*[d]**	**Total**	**All-*trans***	**13/13′-*cis***	**15-*cis***	**Total**	**Total[e]**
Broccoli																	
Fresh	29.2	5.0	3.3	1.9	2.0	41.4											41.4
Boiled	36.5	6.9	4.2	2.2	2.2	52.0											52.0
Carrot																	
Fresh	534.4					534.4	372.7					372.7					907.1
Canned	420.4	32.7	90.5	30.4		574.0	290.9	6.1	91.0	55.9	37.1	481.0					1055.0
Collard																	
Fresh	205.5	33.4	15.6	8.5	10.9	273.9											273.9
Canned	229.5	128.5	18.7	9.2	23.8	409.7											409.7
Orange juice																	
Fresh	2.2	Tr[f]	0.4	Tr		2.6	1.9	Tr	0.2	0.1		2.2	2.5	0.2	Tr	2.7	7.5
Pasteurized	1.5	Tr	0.3	Tr		1.8	1.3	Tr	0.1	0.1		1.5	1.3	0.2	Tr	1.5	4.8
Peach																	
Fresh	2.2	0.3	0.5	Tr		3.0							0.3	0.1	0.1	0.5	3.5
Canned	0.9	0.2	0.4	Tr		1.5							0.2	0.1	Tr	0.3	1.8

(*Table continued*)

TABLE 3.4 *Continued*

	β-Carotene						α-Carotene						β-Cryptoxanthin				
Extract	**All-*trans***	**9-*cis***	**13-*cis***	**15-*cis***	**other *cis*[c]**	**Total**	**All-*trans***	**9-*cis***	**13-*cis***	**13′-*cis***	**other *cis*[d]**	**Total**	**All-*trans***	**13/13′-*cis***	**15-*cis***	**Total**	**Total[e]**
Spinach																	
Fresh	311.9	38.6	24.5	Tr	22.5	397.5											397.5
Canned	309.8	96.9	28.6	14.9	22.9	473.1											473.1
Sweet potato																	
Fresh	256.5					256.5											256.5
Canned	191.0	25.3	76.6	19.4		312.3											312.3
Tomato																	
Fresh	71.0	4.8	5.8			81.6											81.6
Canned	49.1	5.5	12.0	4.8		71.4											71.4
Juice	40.0	4.5	10.1	4.8		59.4											59.4

[a]Data based on an average of two lots, except for orange juice, which had only one lot. Each lot was analyzed by HPLC in duplicate.
[b]Concentrations are in micrograms per gram of dry weight tissue.
[c]Totals the concentration of one unidentified cis isomer of β-carotene.
[d]Totals the concentration of two unidentified cis isomers of α-carotene.
[e]Total provitamin A carotenoid concentration includes all isomers of β-carotene, α-carotene, and β-cryptoxanthin.
[f]Tr, trace.
Source: Reprinted with permission from Lessin, W.J., Catigani, G.L., and Schwartz, S.J., *J. Agric. Food Chem.*, 45, 3728, © 1997 American Chemical Society.

cooked vegetables is the incomplete extraction of protein-bound carotenoids from the raw samples. Other factors are unaccounted loss of water and leaching of soluble solids. The most reliable data are provided by well-designed protocols that employ high-performance liquid chromatography (HPLC) to determine individual carotenoids.

3.3.2.2 Vitamin A in Milk

Low-fat fluid milks are fortified commercially by adding small quantities of a carrier oil containing an emulsifier and retinyl palmitate [34]. More than 99% of the native vitamin A in milk occurs as retinyl esters of saturated and unsaturated fatty acids; the remaining fraction occurs as free retinol. The distribution of the predominant retinyl esters in pasteurized bovine milk, expressed as percent retinol equivalents, is as follows: palmitate (16:0), 36.7%; oleate (18:1), 20.3%; linolenate (18:3), 8.5%; stearate (18:0), 8.4%; linoleate (18:2), 7.3%; pentadecanoate (15:0), 6.0% [35].

Vitamin A stability in milk depends on the method of processing, the fat content, whether or not the product is fortified with vitamin A, the packaging, and the holding conditions prior to consumption. Dissolved oxygen and oxygen in the container's headspace adversely affect the stability of vitamin A [36]. During heat processing and storage of milk, the vitamin A is potentially subject to *trans*–*cis* isomerization as well as oxidation. *Cis* isomers of vitamin A exhibit reduced biopotency relative to the all-*trans* form and, therefore, the analytical method employed for determining vitamin A must be capable of distinguishing between *cis* and all-*trans* forms. This can be achieved by means of normal-phase HPLC. Saponification simplifies the analysis by hydrolyzing the various esters to retinol. The loss of ester information is of no practical consequence as it has been demonstrated that all the retinyl ester species have similar stability in milk fat [35].

Panfili et al. [37] determined the degree of isomerization of vitamin A in various dairy products marketed in Italy. In unprocessed "raw" milk, there was no conversion of all-*trans* retinol to *cis* forms. Pasteurized milk treated for 15 s at temperatures ranging from 72 to 76°C had an average 13-*cis*:all-*trans* ratio of 6.4%. Ratios for ultra-high temperature (UHT) and sterilized milks were 15.7 and 33.5%, respectively, consistent with increased isomerization at the higher processing temperatures. The heating of pasteurized whole milk in a household microwave oven does not cause loss of vitamin A [38].

UHT milk and powdered milk can be stored in a safe edible state for long periods at ambient temperature, provided that the packaging is impervious to moisture, air, and light. It is important to recognize that "ambient temperature" can be higher than 20–25°C in some parts of the world and that the rate of biochemical reactions depends largely on the

storage temperature. During storage of UHT milk, the main cause of eventual spoilage is proteolysis and gelation [39]. Milk powder is frequently consumed in developing countries after long storage periods at tropical temperatures. The keeping quality of milk powder is dependent on its low water activity ($A_w < 0.6$), which does not permit microbial or enzymic activity [40].

Woollard and Edmiston [41] monitored the stability of vitamin A in fortified whole and skim milk powder stored at ambient temperature in heat-sealed, laminated aluminum foil sachets. Six-monthly checks were made over a 2-year period. Losses of vitamin A in six whole milk powder samples after 6 months ranged from 24 to 50%; loss in another sample was 2%. After 24 months, losses in the whole milk powder samples were not much greater (24–66%). In contrast, the two samples of skim milk powder tested lost 93% and 95% vitamin A after 6 months, and 100% after 18 months.

Woollard and Fairweather [42] studied the storage stability of vitamin A in 2% low-fat UHT milk sampled at the time of manufacture. The UHT treatment was an indirect heating process (138°C for 4 s) and the milk was packaged aseptically in 250-ml Tetrapak cartons formed from polyethylene-coated paper. The use of normal-phase HPLC on unsaponified samples allowed natural (palmitate) and supplemental (acetate) vitamin A esters to be determined independently. Losses of both natural and added vitamin A occurred with time. In two trials at ambient temperature, total vitamin A decayed progressively up to 15 weeks of storage, when the losses were 33 and 45%. The respective losses at the product's 28-week expiry date were 35 and 47%. At simulated tropical temperature (35°C), the total losses of vitamin A in two trials increased to 53 and 61% at 28 weeks.

Woollard and Indyk [43] studied the effects of processing and storage on vitamin A isomerization in UHT milk and milk powders. They first studied retinyl ester isomerization in vitamin A-fortified 2% low-fat UHT milk stored at 20°C for up to 8 months. The UHT treatment was indirect and the milk was packaged in 250-ml Tetrapak cartons. *Cis* isomers were undetectable in raw milk, but appeared after UHT treatment. Both supplementary and native all-*trans* esters showed degradation with time, but the *cis* isomers maintained their concentrations until the eventual onset of milk spoilage. The next experiment was to compare the effects of direct and indirect UHT treatment on two identical skim milk samples fortified with retinyl acetate. After 3 days of storage, survival of all-*trans* retinyl acetate in the directly heated sample was markedly higher than in the indirectly heated sample. However, the *cis* isomer concentrations (mainly 13-*cis* retinyl acetate) differed little between the two milks. Fortified whole milk powders sealed in aluminum foil sachets exhibited little difference in *cis*:*trans* ratios (mean ratio 0.20, $n = 6$) when

analyzed as soon as possible after production, except for one sample (ratio 0.42) where an abnormally poor quality vitamin premix was used. The *cis*:*trans* ratio gradually increased during storage of the milk powder for up to 3 years. Storing canned fortified whole milk powders at 37°C and 45°C for 3 months caused significant temperature-related increases in all-*trans* vitamin A degradation and concomitant increases in *cis* isomer concentration. At 27°C, there were no significant changes from initial concentrations.

Vidal-Valverde et al. [44] studied the effects of storage temperature and time on the retinol content of four commercial unfortified whole UHT milk samples, two directly heated and two indirectly heated. Analysis involved saponification of samples and reversed-phase HPLC. Significant losses of retinol were observed after 1 month of storage at 30°C, generally increasing with storage time. Increasing the storage temperature from 30 to 40°C had a variable effect, depending on the particular milk. Frozen storage (−20°C) for up to 60 days had no effect on the retinol content, but after 4–8 months there was a significant ($P < 0.05$) decrease of 17–18%.

Albalá-Hurtado et al. [45] assessed the extent of vitamin A loss in three liquid milk-based infant formulas stored for 1 year at 20, 30, and 37°C. Two samples (brands A and B) were "follow-on" milks for infants aged from 4–6 months to 12 months, and the other a "junior milk" (brand B) for infants after the first year of life. The expiry date of these products was 6 months. Vitamin A remained stable in the brand A follow-on milks stored for 12 months at 20 and 30°C, but there were significant ($P < 0.01$) losses during storage at 37°C, such that 34% was lost by the end of this period. In the brand B follow-on and junior milks there were significant ($P < 0.05$) losses of vitamin A at all three storage temperatures, the greatest losses being at 37°C (57–69%). In a comparison of liquid and powdered milk-based infant formulas claimed to have the same final composition, contents of vitamin A throughout storage at 20 and 30°C only showed a slight decrease, which was not statistically significant. At 37°C, losses after 12 months of storage were practically identical in powdered (36%) and in liquid (34%) samples. However, the decrease occurred earlier in powdered samples than in liquid samples [46].

The presence of fat in milk protects vitamin A from degradation, as demonstrated by Woollard and Edmiston [41] in stored fortified milk powders. Lau et al. [47] prepared milk samples with 0.15, 3, 6, and 10% fat and found that the rate of degradation of added retinyl palmitate in beadlet form increased with decreasing fat content. Final vitamin A concentrations at the end of 3 weeks of storage were higher in milk with high fat and closely corresponded to the native vitamin A concentrations present in the 3-, 6-, and 10%-fat milks. Natural vitamin A, being localized in the fat globules, would be protected from oxygen. On the other hand,

the added retinyl palmitate, being dispersed in the serum phase, could be preferentially oxidized due to greater contact with oxygen. Cox et al. [48] reported that the added vitamin A (retinyl palmitate dissolved in glyceryl mono-oleate) is more stable in dry whole milk than in nonfat dry milk. Whereas the added vitamin A is likely to be dissolved in the fat phase of dry whole milk, the vitamin added to nonfat dry milk would be subject to more surface exposure on nonfat material.

Some studies of UHT milk have shown an association between vitamin A degradation and residual oxygen in the sample [36,49], pointing to oxidation as one of the mechanisms involved. In addition to dissolved oxygen, the antioxidant NDGA effectively retarded vitamin A degradation in fluid milks and air-packed dry milks [48]. UHT milk that has been processed by direct heating has a very low oxygen content, whereas the indirect process, when employed without a deaerator, gives a product that may be near saturated in oxygen [50]. Vitamin A has been shown to be more stable in directly heated than in indirectly heated UHT milk [43], which is consistent with oxidation being partly responsible for loss of the vitamin. Le Maguer and Jackson [36] observed that rapid degradation of vitamin A in UHT milk only occurred after a delayed period (12–16 weeks) and suggested that the initial lag phase may be due to the effective scavenging of residual oxygen by free sulfhydryl groups made available as a result of the heat treatment upon whey proteins, chiefly β-lactoglobulin [51]. In support of this suggestion, vitamin A destruction was less in dried milks which had received a preheat treatment at 82.2°C for 30 min when compared with a preheat treatment at 62.8°C for 30 min [48].

A distinct hay-like off-flavor is frequently detectable in vitamin A-fortified pasteurized low-fat milk that has been stored in a refrigerator for several days. The hay-like flavor is definitely associated with the presence of added vitamin A in the milk, because it does not occur in non-fortified milk [52]. In a study of fortified nonfat dry milk [53], the intensity of the hay-like flavor became weaker as the level of milk fat increased, thus the milk fat masks the off-flavor. The incidence of hay-like flavor increased as the vitamin A levels decreased during storage, suggesting that the off-flavor was a chemical product(s) of vitamin A decomposition. The addition of ascorbic acid prevented the degradation of vitamin A in skim milk stored at 4°C, indicating that oxidation of vitamin A was the likely cause of hay-like flavor development. Two major oxidation products of retinyl palmitate, namely β-ionone and dihydroactinidiolide, have a mild hay-like odor in the pure state and may be responsible for the flavor defect [54]. The best fortification methods for preventing vitamin A degradation and hay-like flavor production in nonfat dry milk were dry-blending with vitamin A beadlets or addition of beadlets at the agglomeration chamber during instantization [53].

Milk in a transparent container is inevitably exposed to light during processing, handling and storage. Exposure of milk to light causes a partial destruction of vitamin A, which increases as the fat content decreases (Table 3.5) [55]. The addition of 3% nonfat milk solids to skim milk protects the retinyl palmitate added to the milk [56]. Retinyl palmitate added to homogenized whole milk is more susceptible to photodestruction than indigenous retinyl esters [57]. Aqueous-based vitamin A concentrate provided more stability to fluorescent light in 2% low-fat milk than oil-based concentrate, while the reverse occurred in skim milk [58]. The type of carrier oil influences the stability of added vitamin A toward light; butter oil and coconut oil were much more protective than corn oil or peanut oil [59]. No correlation was found between peroxide value of the carrier oil and vitamin A loss [60], as would be expected if degradation was a consequence of lipid peroxidation. Photodegradation of added vitamin A was not prevented by the addition of NDGA, although this antioxidant effectively retarded vitamin A degradation in all air-packed milk samples tested [48]. These latter two observations imply that the destructive effect of light on vitamin A in milk is not due to autoxidation, at least not entirely. When skim milk fortified with retinyl palmitate is exposed to fluorescent light, the concentration of all-*trans* retinyl palmitate decreases along with the concentration of the minor 13-*cis* isomer; this is accompanied by the appearance of 9-*cis* retinyl palmitate [61,62]. Jung et al. [62] reported that addition of ascorbic acid to the fortified skim milk inhibited the loss of all-*trans* and 13-*cis* retinyl palmitate, but increased the formation of the 9-*cis* isomer. It appeared that exposing the milk to light induced isomerization of all-*trans* retinyl palmitate to the 9-*cis* form as well as oxidation of the isomers. Ascorbic acid decreased oxidation of retinyl palmitate isomers, but did not prevent isomerization of all-*trans* to 9-*cis.* The photodegradation of riboflavin in milk may be important in the

TABLE 3.5

Effect of Fat Content on Loss of Added Retinyl Palmitate in Milk Exposed to Fluorescent Light

	Percentage Loss of Vitamin A	
Type of Milk/Fat Content (%)	**2 h Exposure**	**4 h Exposure**
Whole (3.4%)	18	37
Low-fat (2%)	26	44
Low-fat (1%)	28	49
Skim	31	57

Source: From Senyk, G.F. and Shipe, W.F., *Dairy Field*, 164 (3), 81, 1981.

loss of vitamin A because the decrease in all-*trans* retinyl palmitate was more pronounced when 3 μg/ml riboflavin was added to the skim milk. Addition of ascorbic acid reduced this loss. Photodegradation of riboflavin in milk generates superoxide anion radical and singlet oxygen, two highly reactive oxygen species [63]. The results suggest that these reactive species are, to some extent, involved in the light-induced destruction of all-*trans* retinyl palmitate. Ascorbic acid is an effective scavenger of all aggressive reactive oxygen species [64] and conjecturally, by performing this role when added to milk, it reduces the light-induced loss of vitamin A to a considerable extent. Fortified skimmed milk in glass or plastic containers showed similar losses of all-*trans* and 13-*cis* retinyl palmitate when exposed to 3 days of continuous illumination. No loss of either isomer occurred in paperboard cartons [65].

3.3.2.3 Supplemental Vitamin A in Corn Flakes and Rice

Kim et al. [66] studied the degradation of retinyl palmitate added to corn flakes either by itself or as part of a vitamin mixture (A, B_1, B_6, B_{12}, C, and D) during storage of the flakes at ambient temperature (average 23°C) and at elevated temperature (45°C). Individual 400-g samples were stored in the original sealed plastic liner and cardboard box packaging system, protected from light. The initial isomer composition of the retinyl palmitate was 5% 13-*cis*, <1% 9-*cis*, and the remainder all-*trans*. The distribution of these isomers remained nearly constant throughout storage, irrespective of sample type and storage conditions. After 6–8 weeks of storage, about 90% of the retinyl palmitate was lost in all samples, except those fortified with the vitamin mixture and kept at ambient temperature. In the latter samples, about 30–40% of retinyl palmitate was lost after the same period. Thus the presence of other vitamins reduced the loss of retinyl palmitate from corn flakes stored at ambient temperature, but not at elevated temperature.

Rice is an ideal candidate for vitamin A fortification because vitamin A deficiency is prevalent in certain countries where rice is a staple food. A preparation called Ultra Rice® (Bon Dente International, Inc., Lynden, Washington) has been developed that overcomes the problem of loss of the fortificant during the typical washing and cooking methods. Ultra Rice is made from broken rice grains, which typically comprise 20–30% of the harvest. The grains are milled into rice flour, combined with a binder and retinyl palmitate, and reformed into rice grains with similar appearance and texture to whole rice grains. This preparation is then used as a premix for blending with virgin, unfortified rice at a ratio of 1:100 to 1:200 to yield a final product that can provide 100% of the daily vitamin A requirement for a child eating a typical daily quantity of rice. Lee et al. [67] mixed Ultra Rice with a long grain rice at the ratio of

1:100 and studied the stability of retinyl palmitate in the mixture during cooking and storage for 6 months. After cooking, 75–87% of the retinyl palmitate was retained, depending upon the cooking methods. A significant ($P < 0.05$) difference in retinyl palmitate stability between samples stored at 23 and 35°C was observed, but no difference in the stability was found between 55 and 80% relative humidity at the two temperatures. When stored at 23°C for 6 months, 85% of the retinyl palmitate was retained compared with about 50% retention at 35°C. The product half-life (time required to reach 50% loss of retinyl palmitate) at different storage conditions is given in Table 3.6. The results show that storage under tropical conditions results in extensive losses of vitamin A.

3.3.2.4 Provitamin A Carotenoids

Being highly unsaturated, carotenoids are prone to *trans–cis* isomerization and oxidation, resulting in loss of color and provitamin A activity. The major cause of carotenoid destruction during processing and storage of foods is enzymatic or nonenzymatic oxidation. The enzyme lipoxygenase in plant tissues catalyzes lipid peroxidation, giving rise to hydroperoxides. The hydroperoxides decompose to form peroxyl and alkoxyl radicals which attack carotenoids. The cutting of fruits and vegetables into small pieces or maceration increases exposure to oxygen and brings the carotenoids and enzymes together. Exclusion of oxygen, by vacuum- or hot-filling, impermeable packaging, or inert atmosphere, diminishes carotenoid decomposition during storage.

Moderate heat treatments such as blanching and cooking denature carotenoid binding proteins, thereby releasing the carotenoids so that they can be more readily extracted. Blanching of fruits and vegetables before processing inactivates lipoxygenase and other enzymes (e.g., peroxidase) that are involved in carotenoid destruction. Steam blanching

TABLE 3.6

Half-Life of Retinyl Palmitate in Rice Fortified with Ultra Rice® Under Different Storage Conditions

Storage Condition	Half-Life (days)
0°C, under nitrogen	1240
23°C, 55% RH	548
23°C, 80% RH	554
35°C, 55% RH	169
35°C, 80% RH	159

Note: RH, relative humidity.
Source: From Lee, J., Hamer, M.L., and Eitenmiller, R.R., *J. Food Sci.*, 65, 915, 2000. With permission.

increased the β-carotene concentration of broccoli and carrots relative to raw concentrations and only slightly decreased the β-carotene concentration of green beans [68]. Overcooking can cause loss of provitamin A activity due to the action of heat and oxygen to form *cis* isomers and epoxy-carotenoids. Optimal retention of carotene is obtained by steaming vegetables or cooking with minimal water until the vegetables are cooked but still crisp [69]. Khachik et al. [70] examined the effect of various cooking methods on the carotenoid concentration of green vegetables and tomatoes determined by HPLC. Samples were taken from several sources and the carotenoid content in the cooked vegetables and tomatoes was compared to that in raw samples of equal weight taken from the same starting material. Care was taken to ensure the complete extraction of the protein-bound carotenoids from the raw samples. The cooking conditions and concentrations of α- and β-carotene in broccoli, spinach, green beans, and tomatoes are shown in Table 3.7. The moderate heat treatments of steaming, microwaving, 9-min boiling, and stewing did not significantly affect the concentrations of α- and β-carotene; surprisingly, neither did the more severe treatment of boiling green beans for 1 h.

TABLE 3.7

Effect of Cooking on Carotene Content of Green Vegetables and Tomatoes

		mg Carotene per/100 g of Edible Food (Raw) (mean ± S.D.)	
Vegetable	**Cooking Method**	**α-Carotene**	**β-Carotene**
Broccoli[a]	Raw	nd	2.33 ± 0.10
	Steaming (5 min)	nd	2.76 ± 0.20
	Microwaving (5 min)	nd	2.45 ± 0.07
Spinach[a]	Raw	nd	8.90 ± 0.70
	Steaming (3 min)	nd	9.86 ± 0.47
	Microwaving (1.5 min)	nd	9.10 ± 0.71
Green beans[b]	Raw	0.08 ± 0.01	0.47 ± 0.05
	Boiling (9 min)	0.08 ± 0.01	0.54 ± 0.11
	Microwaving (4 min)	0.09 ± 0.02	0.53 ± 0.09
Green beans[c]	Raw	0.09 ± 0.01	0.51 ± 0.04
	Boiling (1 h)	0.10 ± 0.02	0.57 ± 0.03
Tomatoes[b]	Raw	nd	0.28 ± 0.02
	Stewing (8 min)	nd	0.30 ± 0.01

Note: nd, not detected.
[a]Mean values are for three batches.
[b]Mean values are for four batches.
[c]Mean values are for two batches.
Source: From Khachik, F., Goli, M.B., Beecher, G.R., Holden, J., Lusby, W.R., Tenorio, M.D., and Barrera, M.R., *J. Agric. Food Chem.*, 40, 390, 1992. With permission.

Direct sun exposure of leafy vegetables, as often practiced in the tropics, results in considerable destruction of total carotene, but retention is improved by minimizing light exposure through shade provision [71]. Dehydrated plant tissues undergo oxidation of carotenoids due to the increased surface area exposed to oxygen. The loss of water further reduces carotenoid stability through the relationship between water activity and lipid oxidation (Section 1.5.1). Carotenoid content in dehydrated carrots, broccoli, and spinach significantly decreased, regardless of the drying method [72]. High hydrostatic pressure treatment of orange juice combined with moderate heat treatment led to an increase in the extraction of provitamin A carotenoids [73]. This was attributed to the denaturation of the carotenoid-binding protein induced by pressure.

Lessin et al. [8] reported the quantitative distribution of provitamin A isomers in fresh and processed fruits and vegetables using a polymeric C_{30} stationary phase for HPLC (Table 3.4). Analysis of fresh and processed produce was performed on the same lot. On a quantitative basis, processing produced 50, 26, 22, 19, and 16% increases in total provitamin A carotenoids in collards, broccoli, sweet potatoes, spinach, and carrots, respectively, relative to the provitamin content in the fresh samples. Since little or no degradation of carotene reportedly occurs during thermal processing [70,74], these increases are most likely a result of increased extraction efficiency due to disruption of carotoprotein complexes, inactivation of carotene oxidizing enzymes, and loss of soluble solids into the liquid canning medium. For the majority of the samples analyzed, the provitamin all-*trans* isomer was lower in the processed samples as compared to the fresh samples on a percent basis. This is because thermal processing causes the conversion of *trans* isomers to *cis* isomers. Solubilization of carotenes is a prerequisite for the formation of *cis* isomers. Severe heat treatment causes solubilization of carotenes by cellular lipids released after thermal breakdown of cell structures [75]. Canning of sweet potatoes caused the largest increase in total *cis* isomers (39%), followed by processing of carrots (33%), tomato juice (20%), collards (19%), tomatoes (18%), spinach (13%), peaches (10%) and orange juice (3%). The predominant *cis* isomer of provitamins in processed red, yellow, and orange fruits and vegetables was 13-*cis*, whereas in green vegetables the predominant isomer of β-carotene (the only provitamin detected) was the 9-*cis* isomer. The boiling of broccoli for 7 min did not cause significant change in the isomer distribution. Chen and Chen [76] reported significant isomerization of α- and β-carotene after boiling carrots for 30 min. The *trans* to *cis* isomerization of thermally processed fruits and vegetables results in some loss of provitamin A activity because of the lower potency of the *cis* isomers.

The freezing of fruits and vegetables preserves the carotenoids, but increasing the lag time after thawing progressively decreases

the carotenoid content [72]. This is due partly to resumption of lipoxygenase activity. The β-carotene content of green beans and broccoli did not change significantly during the retail-marketing period or during frozen storage at −20°C for 16 weeks [77].

3.3.3 Vitamin A Equivalency

In 2001, the U.S. Institute of Medicine [78] changed the interconversion of vitamin A and carotenoids from a system based on retinol equivalents (μg RE) to one based on retinol activity equivalents (μg RAE). The retinol activity equivalency ratio for β-carotene from food is set at 12:1 and 24:1 for other provitamin A carotenoids. In other words, 1 μg of retinol is nutritionally equivalent to 12 μg β-carotene or 24 μg of other provitamin A carotenoids. The 12:1 ratio for dietary β-carotene is derived from the following experimental observations: (1) 6 μg of β-carotene from a mixed diet is nutritionally equivalent to 1 μg of β-carotene in oil; and (2) 2 μg of β-carotene in oil yields 1 μg of retinol. The 24:1 ratio for other provitamin A carotenoids is based on the observation that the vitamin A activity of β-cryptoxanthin and α-carotene is approximately half of that for β-carotene. The RE and RAE systems are compared in Table 3.8. Food composition data tables should report food content in amounts of each carotenoid whenever possible.

Based on total liver vitamin A stores in Mongolian gerbils, the retinol equivalency for 9-*cis*-β-carotene was 38%, and that for 13-*cis*-β-carotene was 62% that of all-*trans*-β-carotene [79].

The new retinol activity equivalency ratio of 12:1 for β-carotene in a mixed diet was based on data from healthy people in developed countries. Field studies conducted in Indonesia and Vietnam indicate a lower ratio of 21:1. This implies that populations in developing countries

TABLE 3.8

Comparison of Equivalency Factors for Interconversion of Vitamin A and Provitamin A Carotenoids

Retinol Equivalent (RE)	Retinol Activity Equivalent (RAE)
1 retinol equivalent (μg RE) =1 μg of all-*trans*-retinol =2 μg of supplemental all-*trans*-β-carotene =6 μg of dietary all-*trans*-β-carotene =12 μg of other dietary provitamin A carotenoids	1 retinol activity equivalent (μg RAE) =1 μg of all-*trans*-retinol =2 μg of supplemental all-*trans*-β-carotene =12 μg of dietary all-*trans*-β-carotene =24 μg of other dietary provitamin A carotenoids

Source: From Institute of Medicine, Vitamin A, in *Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc*, National Academy Press, Washington, D.C., 2001, pp. 82. With permission.

are unable to meet their vitamin A requirements from existing dietary sources [80].

3.3.4 Applicability of Analytical Techniques

Since the mid-1970s till the present day, the method of choice for determining vitamin A and the provitamin A carotenoids in food has been HPLC. In the 1995 edition of *Official Methods of Analysis of AOAC International*, HPLC methods have been introduced for the first time for the determination of vitamin A in milk [81] and milk-based infant formulas [82].

Of the vitamin A commonly found in foods, only all-*trans*-retinol and smaller amounts of 13-*cis*-retinol, both in esterified form, are usually present in significant quantities. For the analysis of vitamin A-fortified foods, HPLC can be applied to determine either the total retinol content or the added retinyl ester (acetate or palmitate), depending on the extraction technique employed.

The vitamin A activity of plant foods is usually based on the HPLC determination of the three most ubiquitous provitamins, namely α- and β-carotene, and β-cryptoxanthin. It is necessary to separate the provitamins from other carotenoids and to quantify them individually. An obvious prerequisite to accurate quantitation is the conclusive identification of the provitamins.

To assess the effects of processing on the nutritional value of a plant food with respect to vitamin A activity, the various isomeric forms of provitamin A carotenoids present in both the fresh and processed states must be accurately measured. In such investigations it must be demonstrated that the analytical procedure does not itself cause *trans*–*cis* isomerization of carotenoids.

The HPLC methodology for carotenoids depends on their known distribution in plant tissues, which can be classified into three main groups: (1) those in which the vitamin A value is due almost exclusively to β-carotene (e.g., green leafy vegetables, peas, broccoli, sweet potatoes, tomatoes, water-melon, mango); (2) those in which primarily α- and β-carotene account for the vitamin A value (e.g., carrots, some varieties of squash); and (3) those in which β-cryptoxanthin and β-carotene are the major contributors (e.g., cashew, apple, peach, persimmon, loquat) [83].

3.4 Intestinal Absorption, Metabolism, and Transport

The following discussion of absorption, metabolism, and transport is taken largely from a more detailed account by Ball published in 2004 [84].

3.4.1 Absorption

The absorption of β-carotene and retinol and principal metabolic events within the enterocyte are shown diagrammatically in Figure 3.3 [85]. Absorption of vitamin A and carotenoids depends on the proper functioning of the digestion and absorption of dietary fat, which are described in Section 2.2.4. Ingested retinyl esters and provitamin A carotenoids are liberated from their association with membranes and lipoproteins by the action of pepsin in the stomach and of proteolytic enzymes in the small intestine. In the stomach, the free carotenoids and retinyl esters congregate in fatty globules, which then pass into the duodenum. Extensive hydrolysis of retinyl esters takes place within the duodenum, catalyzed mainly by a nonspecific pancreatic hydrolase that can act on a wide variety of esters. Retinyl ester hydrolysis is completed by brush-border hydrolases. Esterified xanthophylls are hydrolyzed by esterases. In the presence of bile salts, the fatty globules are broken up into smaller globules, which renders them more easily digestible by a variety of pancreatic lipases. Above a critical concentration of bile salts,

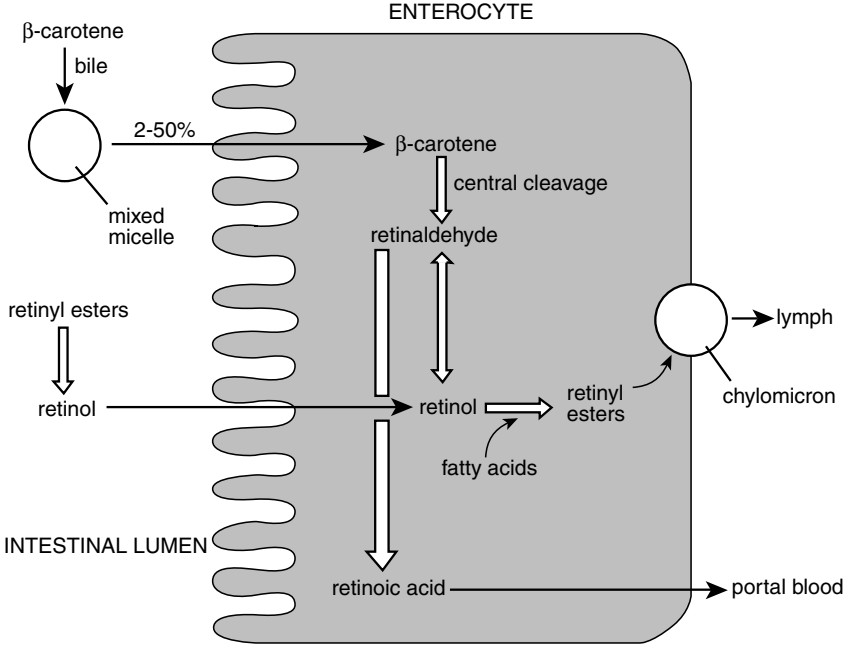

FIGURE 3.3
Intestinal absorption and metabolism of β-carotene and retinyl esters. (Reproduced from Erdman, J.W., Jr., Bierer, T.L., and Gugger, E.T., *Ann. N.Y. Acad. Sci.*, 691, 76, 1993. With permission.)

the bile constituents form micelles. The lipolysis products, together with retinol and carotenoids, combine with the micelles to form mixed micelles.

The retinol and carotenoids contained within the mixed micelles cross the unstirred layer of the intestinal lumen and are released as a result of micelle dissociation in the brush-border region. Physiological concentrations of retinol derived from natural food sources are absorbed by facilitated diffusion, mediated by a carrier; at higher concentrations, a process of simple diffusion takes over. The carrier-mediated absorption of retinol shows specificity toward all-*trans*-retinol; uptake of 9-*cis*- and 13-*cis*-retinol and retinaldehyde takes place by simple diffusion [86]. *In vitro* studies in Caco-2 cells have suggested that carotenoid uptake by enterocytes is a facilitated process [87]; however, no transporter proteins have as yet been identified for carotenoids.

Human ingestion of an algal preparation consisting of a 50:50 mixture of all-*trans*- and 9-*cis*-β-carotene produced only a modest increase in the serum concentration of the 9-*cis* isomer with respect to the increase in the all-*trans* isomer [88,89]. In contrast, ingestion of 13-*cis*-β-carotene from natural palm oil resulted in significant elevation in its plasma concentration [90]. Stahl et al. [91] reported the preferential accumulation of all-*trans*-β-carotene in chylomicrons compared with the 9-*cis* isomer, suggesting an efficient isomer-selective mechanism for intestinal uptake of β-carotene. In stable isotope studies, isomerization of 9-*cis* to all-*trans*-β-carotene has been demonstrated in human intestinal mucosa at physiologic doses, and shown to occur before its secretion into the bloodstream [92]. Whereas 13-*cis*-β-carotene has no known specific function, the 9-*cis* isomer can be converted to both all-*trans*- and 9-*cis*-retinoic acid [93,94], which are hormones involved in the regulation of gene expression [95,96]. The isomer-selective ability of the intestinal mucosa may be important in limiting the potential supply of 9-*cis* retinoids to tissues.

3.4.2 Metabolic Events Within the Enterocyte

3.4.2.1 Esterification of Retinol

Within enterocytes, retinol becomes bound in a 1:1 molar ratio to cellular retinol-binding protein type II (CRBP-II), which is present exclusively and abundantly in these cells. The protein-bound retinol is esterified with saturated long-chain fatty acids, preferentially palmitic acid (C16:0). The esterification uses a different pool of fatty acids, and hence different enzymes, than are used for the synthesis of triglycerides.

3.4.2.2 Conversion of Provitamin Carotenoids to Retinoids

A scheme of the intestinal metabolism of β-carotene is shown in Figure 3.4 [97]. Both symmetric and asymmetric oxidative cleavage of provitamin

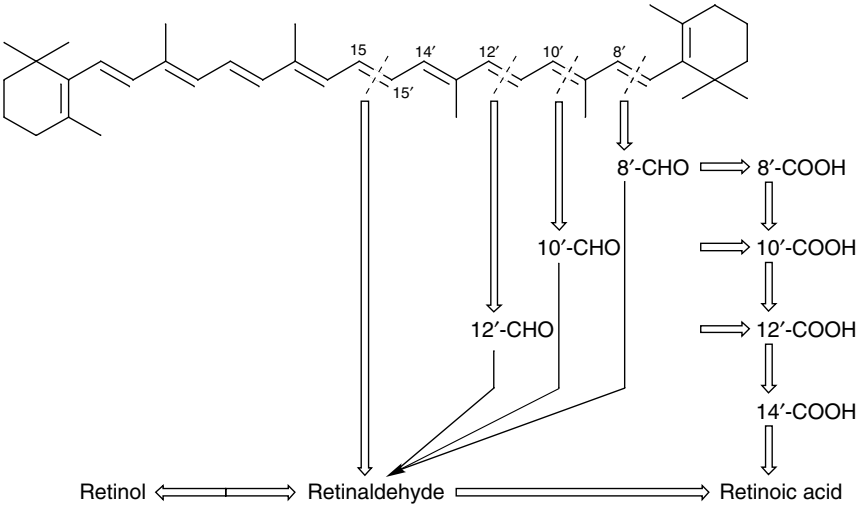

FIGURE 3.4
Intestinal metabolism of β-carotene. The enzyme β-carotenoid-15,15′-dioxygenase forms retinaldehyde directly. Cleavage at other double bonds forms β-apocarotenals (e.g., 8′-CHO), which can be shortened to retinaldehyde. β-Apocarotenals may be oxidized to β-apocarotenoic acids (e.g., 8′-COOH), which can form retinoic acid. Retinol is esterified, incorporated in chylomicrons together with some intact β-carotene, and secreted into lymph. Retinoic acid enters portal blood accompanied by other polar metabolites. (Reprinted from Sharma, R.V., Mathur, S.N., Dmitrovskii, A.A., Das, R.C., and Ganguly, J., *Biochim. Biophys. Acta*, 486, 183, 1977, with permission from Elsevier.)

carotenoids take place within the enterocyte [98]. Symmetric cleavage at the 15,15′ double bond of β-carotene yields two molecules of retinaldehyde; other provitamin carotenoids yield one molecule of retinaldehyde. The enzyme responsible for the central cleavage of β-carotene, β-carotene-15,15′-dioxygenase (EC 1.13.11.21), has been cloned from chicken and mouse tissues and found to be expressed not only in duodenal villi, but also in liver and in tubular structures of lung and kidney [99]. Redmond et al. [100] confirmed that the dioxygenase is a cytosolic enzyme and showed that it is also highly expressed in the testis. Leuenberger et al. [101] provided evidence that the central cleavage of β-carotene actually takes place by a monooxygenase-catalyzed procedure rather than a dioxygenase-catalyzed one.

In the excentric cleavage of β-carotene described by Glover [102], one molecule of β-carotene ultimately yields one molecule of retinaldehyde. The initial reaction is cleavage of the terminal 7′,8′ double bond to produce β-apo-8′-carotenal (Figure 3.5). The stepwise degradation of this compound to retinoic acid is postulated to take place by a β-oxidative-type enzyme system [103]. All of the β-apocarotenals formed from β-carotene can

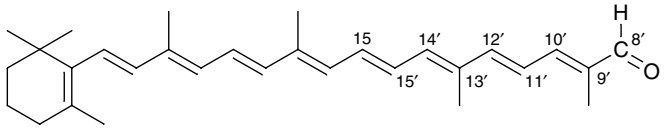

FIGURE 3.5
Structure of β-apo-8′-carotenal.

be shortened to retinaldehyde. Kiefer et al. [104] cloned a mammalian enzyme that specifically cleaves the 9′,10′ double bond of β-carotene.

Most of the retinaldehyde formed from carotenoids becomes bound to CRBP-II and reversibly reduced to retinol by retinaldehyde reductase [105]. The resulting retinol–CRBP-II complex is then used as a substrate for esterification.

Bioconversion of provitamins to retinoids is regulated both up and down according to vitamin A status. The activity of the dioxygenase was shown to be higher in rats [106] and in hamsters [107] fed a vitamin A-deficient diet compared with normally fed controls. This up-regulation was confirmed in rats by van Vliet et al. [108], who also found that a high intake of either retinyl ester or β-carotene down-regulated (decreased) cleavage activity. Using a stable isotope-dilution procedure to indicate total body stores of vitamin A, Ribaya-Mercado et al. [109] showed that bioconversion of plant carotenoids to vitamin A in Filipino school-aged children increases when vitamin A status is low.

In the dioxygenase assay, the 9000 g supernatant (S-9, cell fraction of intestinal mucosa with the highest cleavage activity) is incubated with the carotenoid of interest and the retinaldehyde yield is measured. Using this *in vitro* measurement of cleavage activity, retinaldehyde formed from α-carotene and β-cryptoxanthin was 29 and 55%, respectively, of the amount formed from β-carotene. Addition of 9 μg of lutein to an incubation with 3 μg β-carotene reduced retinaldehyde formation, while lycopene had no effect [110]. Many green vegetables (e.g., broccoli, kale, spinach, watercress, Brussels sprouts, green beans, and peas) contain more lutein than β-carotene [30,111,112] and so a predominant vegetable diet might lower the provitamin A activity of β-carotene *in vivo*. This could at least partly account for the vitamin A deficiency found in some developing countries, in spite of adequate intake of β-carotene.

Dioxygenase activity is also affected by dietary protein and fat. The reduction in dioxygenase activity in response to protein insufficiency [113] may account for the reduced conversion of β-carotene to vitamin A in the intestinal wall of rats fed low-protein diets [114]. During et al. [115] reported that feeding rats a diet rich in polyunsaturated fatty acids (PUFA) (basal diet with 15% soybean oil) enhanced the activity of β-carotene-15,15′-dioxygenase and also the level of CRBP-II in the

intestine. This dual enhancement was not observed for a diet rich in saturated fatty acids (basal diet with 15% hydrogenated soybean oil). The data suggested that the carotene-cleaving enzyme and retinol-binding protein are regulated by a common mechanism involving PUFA. CRBP-II is involved in the conversion of retinaldehyde to retinol, therefore the simultaneous increases of the enzyme activity and protein level would enhance the total ability of intestine to convert β-carotene to vitamin A. In support of this hypothesis, the feeding of Mongolian gerbils with a diet providing high amounts of PUFA resulted in higher liver vitamin A and lower liver β-carotene levels [116].

3.4.3 Liver Uptake of Chylomicron Remnants and Storage of Vitamin A

The combined retinyl esters, accompanied by varying small amounts of unchanged carotenoids, are secreted from the enterocytes into the bloodstream via the lymphatic system as components of chylomicrons [117]. The plasma concentrations of chylomicrons typically peak at 4–5 h after a meal [118]. The circulating chylomicrons undergo lipolysis and the resultant chylomicron remnants are taken up by the liver and to a lesser extent by extrahepatic tissues. Within the liver, the chylomicron remnant retinyl esters are hydrolyzed and most of the retinol is transferred from hepatocytes to stellate cells, where it is esterified and stored.

The amount of vitamin A stored in the liver influences retinol utilization by extrahepatic tissues, and therefore hepatic liver reserves are a true indication of vitamin A status. Green et al. [119] determined the retinol utilization rate in rats provided with different intakes of vitamin A, such that the rats had low, marginal, or high liver vitamin A reserves. Vitamin A-depleted rats exhibited a lower utilization rate, which was positively correlated with the size of the plasma retinol pool; that is, the lower the plasma retinol concentration, the lower the vitamin A utilization rate. The increased rate of utilization observed in rats of higher vitamin A status was reflected in an increased rate of retinol catabolism. It appeared that some minimal utilization rate is maintained as long as dietary supply and/or liver stores of vitamin A can maintain normal plasma retinol concentrations. The decreased utilization rate in depleted states could be a way of conserving vitamin A for its most critical functions, whereas in vitamin A sufficiency increased catabolism prevents excessive accumulation of retinol. Accelerated catabolism as a function of increase in liver vitamin A stores was also reported in rats fed excessive amounts of vitamin A [120].

3.4.4 Plasma Transport of Retinol and Carotenoids

Upon demand, the retinyl esters in the liver are hydrolyzed and the retinol is released into the bloodstream bound to retinol-binding protein

(RBP), which is synthesized in the liver. In the plasma, retinol–RBP combines with a protein called transthyretin, and the resultant complex delivers retinol to vitamin A-requiring cells. Plasma concentrations of vitamin A are maintained relatively constant through a homeostatically controlled interchange of the vitamin among plasma, liver, and extrahepatic tissues.

Remnant carotenoids do not accumulate in liver cells: they are released into the circulation as components of very low density lipoproteins (VLDL), which are subsequently delipidated to low density lipoproteins (LDL). In the fasted state, LDLs are the main carriers of nonpolar carotenoids. Carotenoids released from lipoproteins, especially LDL, are taken up by many tissues, particularly adipose tissue, where they accumulate [121].

Rock et al. [122] examined the plasma carotenoid response to a diet of natural foods that was very low (<4 mg/day) in carotenoids. Plasma carotenoid concentrations were measured on study days 2–3, 14–15, 35–36 and 63–64. A significant ($P < 0.005$) 46% decline in mean plasma β-carotene occurred between days 2–3 and 14–15, but did not occur between days 14–15 and 35–36. Plasma levels on days 63–64 averaged 23% of mean initial levels. The initial rapid plasma response to a low-carotenoid diet suggests that measurement of plasma carotenoid levels is useful only in the assessment of short-term intake. The slower rate of decline after 2 weeks suggests that, during dietary carotenoid deprivation, carotenoids are released from tissues back into plasma. Regular consumption of carotenoid-containing foods seems to be necessary to maintain plasma levels of these compounds.

3.4.5 Tissue Uptake and Metabolism of Retinol

The RBP component of the circulating retinol complex is recognized by a receptor on the surface of target cells and, after negotiating the lipid layer of the plasma membrane, the retinol interacts with a specific cellular binding protein. Cytoplasmic retinol is subject to a variety of metabolic fates, including oxidation to retinoic acid. Cellular binding proteins play major roles in controlling retinol metabolism and may regulate the movement of retinoic acid to the nucleus, where it acts as a hormone to influence gene expression.

3.5 Bioavailability

3.5.1 Introduction

The published literature on carotenoid bioavailability is extensive, but comprehensive reviews are available [98,123,124]. To simplify matters,

this discussion focuses on the major provitamin A carotenoid, β-carotene. Bioavailability is an ambiguous term and therefore it is important to state the method when assigning a bioavailability value to a food or supplement. Bioavailability of carotenoids is frequently assessed by absorption efficiency, which is defined as the percentage of ingested carotenoid that is secreted into the general circulation and therefore made available for tissue uptake [124]. Two related terms have been introduced into the carotenoid literature, bioaccessibility and bioefficacy. Bioaccessibility is defined as the fraction of carotenoid transferred during digestion from the food matrix to mixed micelles and thus made accessible for absorption [125]. Bioefficacy defines the percentage efficiency with which ingested β-carotene is absorbed and converted to retinol in the body [126]. Because 1 μmol β-carotene theoretically could form 2 μmol retinol, 100% bioefficacy would mean that 1 μmol dietary β-carotene (0.537 μg) is 100% absorbed and converted 100% to retinol, yielding 2 μmol retinol (0.572 μg). Thus, the amount of β-carotene required to form 1 μg retinol would be $0.537/0.572 = 0.94$ μg.

When meals containing natural amounts of vitamin A and provitamin A carotenoids are consumed, vitamin A is absorbed with an efficiency of 70–90% compared with 20–50% for the provitamins. The absorption efficiency of vitamin A remains high (60–80%) as the amount ingested increases beyond physiological levels, whereas that of the provitamins falls dramatically to less than 10% [127], indicative of a saturable process. When a large dose (40 mg) of β-carotene (in the form of capsules containing palm oil carotenes) was administered with a standardized, low-carotenoid meal to men and women, only 3.5% of the dose appeared in the chylomicron-rich fraction of plasma [128]. Tyssandier et al. [129] reported a very low (<7%) recovery of vegetable-borne carotenoids in the micellar phase of the duodenum (bioaccessibility), which could account for their poor bioavailability.

3.5.2 *In vivo* Methods of Assessing β-Carotene Bioavailability

3.5.2.1 Use of Radioisotopes in Cannulated Patients

In two studies designed to investigate the intestinal handling of β-carotene in humans [130,131], single doses within the range of 45 μg to 39 mg of either ^{14}C- or ^{3}H-labeled β-carotene in a fat-containing liquid formula were administered to four male hospitalized patients with cannulated thoracic ducts. Radioactivity was measured in four fractions of the lymph; namely, β-carotene, retinyl ester, retinaldehyde, and retinol fractions. Recoveries of radioactivity in the lymph of three patients were 9, 15, and 17%. Of the recovered radioactivity in these patients, 68, 73, and 88% was found in the retinyl ester fraction, and 1.7, 11.3, and 27.9% was

found in the β-carotene fraction. In the fourth patient, 52% of the administered radioactivity was recovered in the lymph, the majority (90%) of this occurring in the β-carotene fraction. These studies clearly showed that, apart from the fourth patient, humans are moderate absorbers of intact β-carotene, and bioconvert β-carotene primarily to retinyl esters.

3.5.2.2 Animal Models

The use of radioisotopes in healthy human subjects cannot be justified because of the risks involved. Animal models offer the advantages of allowing use of radiolabeled compounds and accessing tissues. Rats absorb β-carotene in a different manner to humans and therefore, are not appropriate models for studying carotenoid absorption and bioavailability. When rats are fed radioactive β-carotene, the β-carotene is converted almost entirely to retinyl esters at the level of the enterocyte, and there is no labeled β-carotene in the lymph [132]. This means that, unlike in humans, accumulation of β-carotene in rat tissues does not occur and neither does it occur in the tissues of mice, hamsters, guinea pigs, rabbits, chickens, pigs, or sheep. In the search for a more suitable animal model, the ferret and gerbil have been found to resemble the human in their ability to absorb and accumulate intact carotenoids [133]. Gerbils are the more suitable because they are small (an adult male weighs 80 g) and easily maintained in large numbers. In addition, gerbils convert dietary β-carotene to vitamin A with efficiency similar to that currently estimated for humans. Ferrets are large enough (an adult male weighs 1200 g) to permit surgical cannulation of the lymphatics and the portal vein, allowing direct measurement of carotenoid absorption. Preruminant calves also resemble humans in their ability to absorb β-carotene intact [133]. Their utility lies in the availability of multiple blood and liver biopsy samples.

3.5.2.3 Serum, Plasma, or Chylomicron Responses not Involving Isotopic Tracers

Plasma retinol levels are homeostatically maintained within a narrow range (except in cases of vitamin A deficiency or excess) and so do not reflect dietary intake of either vitamin A or provitamin A carotenoids in adequately nourished subjects. This explains why serum vitamin A levels were unchanged when human subjects were fed carrot juice [134]. Plasma or serum carotenoid responses (concentration versus time curves) do reflect dietary intake of carotenoids. The relative bioavailability of β-carotene in a particular food or meal has been determined as the serum β-carotene response relative to that after simultaneous ingestion of pure β-carotene and a low-carotenoid meal.

Average serum β-carotene concentrations in women increased 4.1- and 4.0-fold in response to single oral doses of 60 and 210 mg β-carotene, respectively. Maximum serum β-carotene levels were reached 24 h post-dosing and, after 8 days, serum levels remained ca. twofold higher than baseline levels [135]. Increases in serum β-carotene concentrations varied from 3.5- to 11-fold (mean: 7.4-fold) among women after a 28-day period of supplementation with 30 mg purified β-carotene [136]. These increases conformed to first-order kinetics with a half-time of 5.5 days.

An increase in dietary intake of fruits and vegetables is associated with an increase in plasma carotenoid concentrations [137–141]. The plasma response is very variable in magnitude and duration, and some people consistently exhibit a low response [142]. Johnson and Russell [143] measured β-carotene concentrations in plasma and various lipoproteins in healthy males for ten days after a single oral dose (120 mg) of β-carotene in capsule form. Seven of the eleven subjects were nonresponders, showing little or no increase in plasma β-carotene and only a small response in chylomicrons. Of interest with the four responders was that surges of chylomicron β-carotene occurred every few days following the single dose, suggesting delayed release of β-carotene from enterocytes or re-uptake of β-carotene following sloughing-off of enterocytes.

Density gradient ultracentrifugation isolates a triglyceride-rich lipoprotein (TRL) fraction of plasma, the $d < 1.006$ fraction, which contains mainly intestinally derived chylomicrons and their remnants together with some VLDL of hepatic origin. Measurement of postprandial changes in the concentration of retinyl esters in the TRL fraction provides information as to the mass of vitamin A derived from a test meal containing provitamin A carotenoids. The value of this approach stems from the fact that chylomicrons and their remnants are the exclusive means by which newly absorbed β-carotene and derived retinyl esters are transported from the intestine to the liver. In the postprandial chylomicron (PPC) response model, using human subjects, data from multiple blood samples drawn over periods ranging from 8 to 16 h after a test meal are used to construct concentration versus time plots, from which area-under-the-curve (AUC) values are computed. Such values are presumed to be proportional to the amount of retinyl ester produced by the intestinal mucosa [144].

Response curves in whole human plasma or serum do not measure accurately the extent of absorption of carotenoids from a test meal because they are unable to distinguish newly absorbed carotenoids from endogenous carotenoids circulating within LDL. Response curves in TRL fractions specifically measure newly absorbed carotenoids, as well as accounting for intestinal conversion of provitamin A carotenoids to retinyl esters. Another advantage of TRL response curves is the

allowance for smaller doses that results from lower baseline levels. van Vliet et al. [145] reported the following observations supporting the presence of mainly intestinally derived lipoproteins in the TRL fraction: (1) the TRL fraction from fasting plasma contained no β-carotene; (2) control subjects showed no response or only a very low response; and (3) the increase of plasma β-carotene seen up to 16 h after the first meal (probably due to liver-derived lipoproteins) was not seen in the TRL fraction. Hu et al. [146] subfractionated TRLs into particles of varying size and composition in an effort to better distinguish particles of exogenous and endogenous origin.

3.5.2.4 Methods Involving Stable Isotopes

Parker and colleagues [147] adapted the PPC model by administering 6.0 μmol deuterium-labeled (d_4) retinyl acetate in oil solution simultaneously with the test meal. The labeled compound constituted an extrinsic reference with which to measure the mass of unlabeled retinyl ester (d_0-RE) derived from intestinal cleavage of absorbed provitamin A carotenoids and the mass of intact carotenoid (as β-carotene) absorbed. The extrinsic reference also controlled for variation in *in vivo* chylomicron kinetics and for variation in retinyl ester recovery during the preparation and analysis of the TRL fraction. The retinyl ester-derived retinol (d_0-retinol plus d_4-retinol) in the TRL fraction of plasma was collected from HPLC and derivatized to a trimethylsilyl (TMS) ether. The molar ratio of d_0-retinol-TMS and d_4-retinol-TMS, determined by GC–MS, was multiplied by the concentration of total TRL retinyl ester, as determined by HPLC, to give the concentration of d_0- and d_4-retinyl ester in the TRL fraction at each sampling time point. Baseline-corrected response curves for d_0-RE, d_4-RE and β-carotene were constructed and AUC values were calculated. Figure 3.6 illustrates typical PPC response curves in a subject given a test meal of raw carrot with 20 g fat. An absorption efficiency of 80% for the 6.0 μmol d_4-retinyl acetate reference dose was assumed, that is, the AUC for d_4-retinyl ester was set equivalent to 4.8 μmol retinyl ester. The mass of d_0-retinyl ester derived from the provitamin A carotenoids in the test meal was calculated by dividing the AUC of d_0-retinyl ester by that of d_4-retinyl ester and multiplying this quotient by 4.8. The same approach was used to estimate the mass of β-carotene absorbed intact. Test meals comprised raw carrot or raw spinach in amounts that contained 6 mg β-carotene, and 20 g of added fat. The amount of vitamin A reaching the bloodstream after consumption of the meals ranged from 0.14 to 0.53 mg (mean: 0.30 ± 0.13 mg). This amount was more consistent for the carrot meal (mean: 0.28 ± 0.01 mg). Thus, in the presence of ample fat, the amount of β-carotene in raw carrot or raw spinach required to form 1 μg retinol was 20 μg (0.3 mg

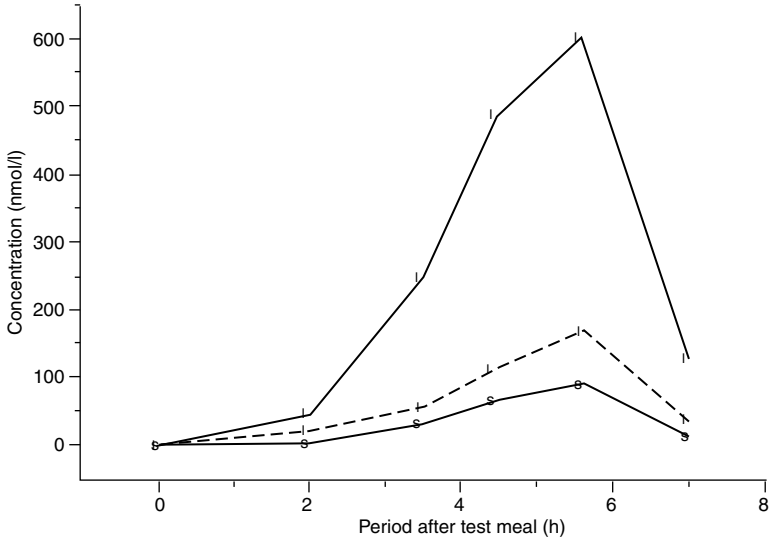

FIGURE 3.6
Postprandial chylomicron response curves in a subject given a test meal containing 5.25 mg β-carotene as raw carrot and 20 g fat plus 2 mg deuterium-labeled (d_4) retinyl ester. Labeled retinyl ester (•—•); unlabeled retinyl ester (• – – •); and β-carotene (▲—▲). Adapted from Ref. 124. (Reprinted in modified from Parker, R.S., Swanson, J.E., You, C.-S., Edwards, A.J., and Huang, T., *Proc. Nutr. Soc.*, 58, 155, 1999. With permission.)

retinol from 6 mg β-carotene); this corresponds to a bioefficacy of 4.7%. The absorption efficiency (absolute bioavailability) of β-carotene from these vegetable meals ranged from 3 to 16% (mean 7%), assuming central cleavage of β-carotene.

van Lieshout et al. [126] used a double-tracer technique for quantifying the bioefficacy and bioavailability of β-carotene dissolved in oil. Every day for 10 weeks, 35 Indonesian school children consumed two capsules, each containing 80 μg of [$^{13}C_{10}$]β-carotene and 80 μg of [$^{13}C_{10}$]retinyl palmitate dissolved in sunflower oil with added vitamin E as an antioxidant. The ingestion of these labeled compounds enabled [$^{13}C_{10}$]retinol to be distinguished from circulating unlabeled retinol and from [$^{13}C_5$]retinol formed in the body from the [$^{13}C_{10}$]β-carotene administered. The capsules were consumed after a low-retinol, low-carotenoid meal Monday through Friday, and after a chocolate wafer on the weekend. The foods provided contained some fat to promote the absorption of the retinol and β-carotene from the capsules. Three blood samples were drawn per child over a period of 10 weeks. HPLC coupled with atmospheric pressure chemical ionization liquid chromatography–mass spectrometry

(APCI LC–MS) was used to measure the isotopic enrichment in serum of retinol with [$^{13}C_5$]retinol and [$^{13}C_{10}$]retinol and of β-carotene with [$^{13}C_{10}$]β-carotene. On the basis of data from the plateau isotopic enrichment from days 21 to 70, it was calculated, using the CarRet PIE model, that the amount of β-carotene in oil required to form 1 μg retinol was 2.4 μg (95% CI: 2.1, 2.7). The labeled β-carotene fed in this study had a *cis–trans* ratio of 3:1. In a second study with 77 Indonesian children [148], 90% of the labeled β-carotene was in the all-*trans* configuration. In this case, 2.7 μg (95% CI: 2.5, 2.8) β-carotene in oil was required to form 1 μg retinol. If the data from the two studies are pooled, then 2.6 μg (median value) of β-carotene in oil is required to form 1 μg of retinol and the bioefficacy of β-carotene in oil is 36%. To calculate the bioavailability of β-carotene using the CarRet PIE model requires an assumption about the stoichiometry of cleavage of β-carotene into retinol. Using the most efficient stoichiometry possible, that is, 1 mol β-carotene yields 2 mol retinol, the median bioavailability of β-carotene in oil is 86%.

A double-tracer technique was also used to measure the intrinsic variability in the bioefficacy of β-carotene in healthy, well-nourished women [149] and men [150] living in the controlled environment of a metabolic research unit. The volunteers were given capsules containing hexadeuterated retinyl acetate and β-carotene (both in crystalline form) with a meal providing 16 g fat. Only 6 of 11 women volunteers had measurable plasma [$^2H_6$]β-carotene and [$^2H_3$]retinol concentrations (responders). The mean absorption of β-carotene in these six women was $6.1 \pm 0.02\%$, and the mean conversion ratio was 1.47 ± 0.49 mol retinol to 1 mol β-carotene (i.e., 1.27 μg crystalline β-carotene was required to form 1 μg retinol). The remaining five women were low responders with $\leq 0.01\%$ absorption and a mean conversion ratio of 0.014 ± 0.004 mol retinol to 1 mol β-carotene (i.e., 134 μg crystalline β-carotene was required to form 1 μg retinol). The mean conversion ratio in all 11 women was 0.81 ± 0.34 mol retinol to 1 mol β-carotene (i.e., 2.3 μg β-carotene was required to form 1 μg retinol) [149]. Only 6 of 11 men volunteers had measurable plasma [$^2H_6$]β-carotene and [$^2H_3$]retinol concentrations (responders). The mean absorption of β-carotene in these six men was $4.097 \pm 1.208\%$, and the mean conversion ratio was 0.0540 ± 0.0128 mol retinol to 1 mol β-carotene (i.e., 34.6 μg β-carotene was required to form 1 μg β-carotene). The ratio ranged from 0.0162 to 0.0919 within the responder group (i.e., 115 to 20.4 μg β-carotene was required to form 1 μg retinol). Absorption of β-carotene in the remaining five men was too low to be measured. The mean conversion ratio in all 11 men was 0.0296 ± 0.0108 mol retinol to 1 mol β-carotene (i.e., 63 μg β-carotene was required to form 1 μg retinol) [150].

3.5.3 *In vitro* Methods of Assessing β-Carotene Bioaccessibility and Bioavailability

3.5.3.1 In vitro Digestion Methods to Assess β-Carotene Bioaccessibility

Garrett et al. [151] developed an *in vitro* digestion method to determine the degree of micellarization of carotenoids as an indicator of carotenoid bioaccessibility from meals. The homogenized meal was cooled on ice prior to acidification (pH 2) with 1 *M* hydrochloric acid and the addition of pespsin/hydrochloric acid solution. The homogenate was transferred to a clean amber bottle and incubated at 37°C in a shaking water bath. Next, the pH of the partially digested meal was raised to 5.3 with sodium bicarbonate solution, followed by the addition of a mixture of pancreatin and bile extract dissolved in 0.1 *M* sodium bicarbonate. The pH was increased to 7.5 with 1 *M* sodium hydroxide and 10 ml aliquots of the incompletely digested meal were incubated at 37°C for 2 h in sealed, argon-filled bottles. This completed the intestinal phase of the *in vitro* digestion process. The aqueous fraction of the digestate that contained micelles was isolated by ultracentrifugation and filtered (cellulose acetate, 0.22 μm pore size) to remove microcrystalline, nonmicellarized carotenoids that were not pelleted during centrifugation. Aliquots of filtrates were stored at −20°C under a blanket of argon until extraction and analysis by HPLC. To validate that the carotenoids transferred to the aqueous fraction during the *in vitro* digestion were actually accessible for absorption, the uptake of the micellarized carotenoids by cultures of differentiated Caco-2 human intestinal cells was examined. It was observed that the cells accumulated carotenoids in proportion to both the carotenoid content of the medium and the duration of exposure.

Garrett et al. [152] used the *in vitro* digestion method described above to determine carotenoid bioaccessibility from a stir-fried meal consisting of fresh carrots, fresh spinach, and tomato paste as sources of α- and β-carotene, lutein, and lycopene, respectively. The percentages of α- and β-carotene transferred from the meal to the micellar fraction were 14.7 ± 0.3% and 16.0 ± 0.4%, respectively. Values for lutein and lycopene were 29.0 ± 0.6% and 3.2 ± 0.1%, respectively. All four carotenoids were accumulated by cultures of differentiated Caco-2 human intestinal cells in a linear manner throughout a 6-h incubation period.

Hedŕen et al. [153] developed an *in vitro* digestion method that, in contrast to the method of Garrett et al. [151], estimates the maximum amount of carotenoids released, not necessarily micellarized, from the food matrix that is accessible for absorption. To start with, an internal standard (20 μg β-apo-8-carotenal dissolved in 100 μl hexane) was added to a screw-capped conical flask and the solvent was evaporated with a flush of nitrogen. About 0.5 g freeze-dried vegetable sample was accurately weighed into the flask and suspended in 10 ml distilled water containing

1% (w/v) ascorbic acid. Nitrogen was blown into the flask before it was screw-capped and left for 30 min to absorb moisture. The digestion procedure commenced with the addition of pepsin/hydrochloric acid solution containing physiological amounts of calcium, magnesium, sodium, potassium, and phosphate. If necessary, the pH was adjusted to 2.0 with 2 *M* hydrochloric acid. Nitrogen was blown into the flask before it was screw-capped and incubated at 37°C in a water bath with orbital shaking for 1 h. The pH was adjusted to ca. 5 with 2 *M* sodium hydroxide before adding 3 ml of a mixture of pancreatin and bile salt extract dissolved in 0.1 *M* sodium bicarbonate and stabilized with 1% (w/v) α-tocopherol. The pH was increased to 7.5 and, after flushing with nitrogen, the mixture was incubated again for 30 min. Finally, the sample was centrifuged and the supernatant was taken for extraction and analysis by HPLC.

Hedŕen et al. [153] used their *in vitro* digestion method to assess the impact of cooking, particle size, and presence of cooking oil on the bioaccessibility of α- and β-carotene in carrots. Carrots were peeled and cut into pieces (ca. 10 × 10 × 40 mm) and half the samples were boiled in water. The samples were then rapidly frozen at −40°C for 24 h before they were freeze-dried. Prior to digestion, the freeze-dried carrot samples were either finely ground to pass a 150 μm aperture sieve ("pulped samples") or cut into pieces similar to chewed items in size. The samples, with or without added canola (rapeseed) oil, were exposed to the digestion procedure and the carotenes released from the carrot matrix were extracted and quantified by HPLC. The results are given in Table 3.9. In samples without added cooking oil, raw carrot pieces released 3% of the total β-carotene after digestion, and cooked carrot pieces released 6%. When the carrots were pulped, the β-carotene

TABLE 3.9

Percentage *In Vitro* Accessible β-Carotene in Freeze-Dried Carrots[a,b]

	Raw		Cooked	
Oil Addition (%)[c]	**Pieces**	**Pulped**	**Pieces**	**Pulped**
0	3	21[a]	6	27[a]
20	4	28[b]	8	38[b]
40	4	31[b]	8	42[b]
60	4	34[b]	8	45[b]

[a]The total amount of β-carotene was 1347 ± 52 μg/g dry matter in the cooked carrots.
[b]Values in the same column not sharing the same superscript were significantly different ($P < 0.05$). The ANOVA analysis was done on absolute values (μg/g dry matter).
[c]In percentage of dry weight of carrots.
Source: From Hedŕen, E., Diaz, V., and Svanberg, U., *Eur. J. Clin. Nutr.*, 56, 425, 2002. With permission.

release increased to 21% and 27% in the raw and cooked samples, respectively. Addition of 20% oil per gram of dry matter resulted in a significant ($P < 0.05$) increase in the amount of β-carotene released from the pulped samples. Additional oil (up to 60%) slightly increased β-carotene release from the pulped samples. Adding oil to the carrot piece samples had no significant effect on the amount of released β-carotene, although there was a tendency for a slight increase. The trends for α-carotene were similar to those for β-carotene, except that the absolute values were about 60% lower. Overall, pulping is more important than cooking for carotene bioaccessibility in carrots, and addition of oil is more effective when carrot is pulped rather than in pieces.

3.5.3.2 In vitro Studies of β-Carotene Absorption Using Caco-2 Cells

At the confluent stage, human-derived Caco-2 cells differentiate spontaneously to become enterocyte-like absorptive cells, having well defined brush-border and basolateral membranes and intercellular junctional complexes. Under normal cell culture conditions, Caco-2 cells are unable to form chylomicrons, but when cultured as monolayers on membranes under defined conditions, and supplemented with oleic acid and taurocholate, are able to do so [154]. During et al. [87] employed this cell culture system to investigate fundamental aspects of carotenoid absorption at physiological concentrations (1 μM). Two components of absorption were studied separately, namely cellular uptake and secretion in chylomicrons. β-Carotene and other carotenoids were presented to Caco-2 cells in aqueous solution with Tween 40 micelles. Both cellular uptake and secretion of β-carotene were shown to be curvilinear, time-dependent, saturable, and concentration-dependent processes. Saturation occurred at β-carotene concentrations equivalent to a daily intake of 100 mg — well above the normal daily intake of 5 mg. From total β-carotene secreted, 80% was associated with chylomicrons. Under linear concentration conditions, 11% of all-*trans*-β-carotene was absorbed (i.e., passed through Caco-2 cell monolayers) in contrast to only 2–3% of 9-*cis*- and 13-*cis*-β-carotene. This discrimination between β-carotene isomers occurred at the levels of cellular uptake and incorporation into chylomicrons. The preferential uptake of the all-*trans* isomer was not observed in endothelial cells or monocyte-macrophages, demonstrating cellular specificity. A differential absorption was found among different carotenoids tested, with all-*trans*-β-carotene (11%) >α-carotene (10%) >lutein (7%) >lycopene (2.5%). Cellular uptake for these four carotenoids was similar, however, ranging from 15 to 18%.

During et al. [87] also studied potential interactions between β-carotene and either α-carotene, lutein or lycopene during their absorption by Caco-2 cells. Lycopene reduced the extent of β-carotene absorption by

1.4-fold ($P < 0.05$) and 4.9-fold ($P < 0.001$), respectively, for β-carotene/lycopene ratios of 1:1 and 1:5, compared with β-carotene alone. Similar and significant reductions in the cellular uptake of β-carotene were also observed at these ratios of β-carotene and lycopene. α-Carotene and lutein did not show any effect on cellular uptake or secretion of β-carotene. In a second experiment, β-carotene significantly reduced the extent of α-carotene secretion by 1.4- and 2.2-fold, respectively, for α-carotene/β-carotene ratios of 1:1 and 1:5 ($P < 0.05$). The cellular uptake of α-carotene was also decreased with increasing β-carotene concentration, but not significantly. β-Carotene reduced both cellular uptake and secretion of lycopene by 1.7- and 2.3-fold, respectively, for a lycopene/β-carotene ratio of 1:5, but not significantly. Finally, β-carotene did not interfere with the intestinal absorption of lutein.

The interactions between β-carotene/α-carotene and β-carotene/lycopene suggest that these carotenes could follow similar pathways for their cellular uptake and/or incorporation into chylomicrons. The kinetics of CaCo cell uptake and secretion of β-carotene, the inhibition of the intestinal absorption of one carotenoid by another, and the cellular specificity of isomer discrimination all suggest that carotenoid uptake by enterocytes is a facilitated process mediated by a specific transport protein(s).

Lutein was reported to inhibit β-carotene absorption *in vivo*, according to data obtained from PPC response curves [155,156], whereas the Caco-2 cell culture system showed no interaction between these two carotenoids [87]. Interpretation of *in vivo* data is confounded by metabolic events such as variations in plasma clearance rates. The *in vivo* inhibition could be attributable at least partly to the inhibitory effect of lutein on the β-carotene cleavage enzyme observed *in vitro* [110].

3.5.4 Host-Related Factors Affecting the Bioavailability of β-Carotene

The absorption efficiency of carotenoids naturally present in foods is influenced by host-related factors such as the presence of intestinal parasites and atrophic gastritis, a condition common in elderly people in which little or no acid is secreted by the stomach. With regard to the latter, Tang et al. [157] reported that raising the gastric pH of human subjects to >4.5 by administering omeprazole (a drug which suppresses gastric acid secretion) reduced the serum response curve after a β-carotene dose to 50% compared with the response at a normal gastric pH of 1.0–1.5.

3.5.5 Dietary Factors Affecting the Bioavailability of β-Carotene

3.5.5.1 Location of Carotenoids in the Plant Source

In a study of Indonesian school children [158], the increase in serum β-carotene in relation to the amount of β-carotene ingested was 5–6

times higher for a fruit meal (orange fruits) than for a vegetable meal (dark-green leafy vegetables and carrots). This difference in bioavailability may be attributable to differences in the intracellular location of carotenoids. In photosynthetic plant tissue, carotenoids are localized as protein complexes in the inner membranes of chloroplasts. In nonphotosynthetic tissues, carotenoids are primarily found in chromoplasts. There are four main types of chromoplasts, and one or more different types may be found in the same plant tissue [18]. Globulous chromoplasts, the most common type, contain carotenoid in lipid droplets with only traces of associated protein. They are found in orange and yellow fruits, pumpkins, and sweet potatoes. Carotenoids in these substructures can be readily released during digestion, hence the higher serum β-carotene response observed for orange fruits compared with vegetables. Tubulous chromoplasts contain tubules consisting of a liquid crystal core stabilized by phospholipids and a major 30-kD protein. Membranous chromoplasts contain lipid and carotenoid-rich concentric membrane structures, which are associated with a variety of proteins. Crystalline chromoplasts occur in the carrot root and contain crystalline carotenes. Each crystal is surrounded by a membrane to form a carotene body.

In preruminant calves fed a carotenoid-free milk replacement, the serum β-carotene response to dosing with crystalline β-carotene in oil was only 4% of the response to dosing with an equal amount of β-carotene in the form of water-soluble, antioxidant-stabilized beadlets [159]. The serum β-carotene reponse to beadlets was also greater than the response to powdered β-carotene in women [160]. These observations suggest that crystals of β-carotene are not easily broken up and transferred to mixed micelles during the digestion process, whereas β-carotene dispersed in an emulsified solution is readily incorporated into mixed micelles. This comparison of crystalline and water-soluble forms of β-carotene is relevant to the poor bioavailability of β-carotene in carrots.

3.5.5.2 Food Matrix

The ability of the digestion process to release food-borne β-carotene for absorption depends largely on the nature of the plant food matrix at the point of consumption. The effect of the food matrix on β-carotene bioavailability is evident from studies in children showing that the plasma β-carotene response is greater from synthetic β-carotene supplements than from cooked carrots [161,162]. The higher plasma β-carotene response to drinks supplemented with β-carotene as a water dispersible powder compared to drinks supplemented with carrot juice [163] further demonstrated the matrix effect. Food processing and cooking improve carotenoid bioavailability by reducing the particle size, disrupting the plant cells, and breaking carotenoid–protein bonds.

van het Hof et al. [164] examined the plasma β-carotene response in healthy subjects to chronic ingestion of three types of vegetables representing leaves (spinach), flowers (broccoli), and seeds (green peas). Following consumption of meals supplemented with 300 g of broccoli or green peas, the plasma β-carotene response (per milligram of β-carotene supplied) was 74 and 96%, respectively, of the response induced by 1 mg synthetic β-carotene (microcrystalline suspension in oil). Consumption of the spinach-supplemented meal did not affect plasma levels of β-carotene, even though the β-carotene content of spinach was tenfold that of broccoli and green peas. If the carotenoids of broccoli or green peas are present in chromoplasts, as they are in fruits, this may explain the higher β-carotene response compared with spinach.

Zhou et al. [165] evaluated the effect of the carrot matrix on the relative bioavailability of α- and β-carotene. Ferrets were fed a carotenoid-free diet supplemented with one of the following: commercial β-carotene beadlets, isolated carrot chromoplasts, or carrot juice. The carrot chromoplasts mainly contained carotene bodies and the inner and outer membranes of the chromoplasts, while the carrot juice contained not only carrot chromoplasts, but also the rest of the food matrix, including carrot fiber. The effect of the natural structure of carotenoids in carrots, namely crystalline carotenoids surrounded by membranes, was ascertained by comparing the bioavailability of carotenes in animals receiving the chromoplasts to those receiving the β-carotene beadlets. The difference in bioavailabilities between animals receiving the carrot juice and those receiving the chromoplasts determined the effect of other components of the carrot matrix. The results of the study showed that the chromoplasts contributed significantly to the decreased relative bioavailability of β-carotene from carrots. It was not possible to separate the effects of the carotenes being in crystalline form from the effects of the surrounding chromoplast membranes.

Huang et al. [166] determined the relative bioavailability of vegetables typically consumed in a Taiwanese diet by examining the serum β-carotene response to a single ingestion of 12 mg β-carotene from the vegetables compared with that from β-carotene beadlets. A cross-over design was employed so that each healthy young male subject served as his own control in calculating the bioavailability data. The relative bioavailabilities of β-carotene from stir-fried shredded carrot (33%), stir-fried water convolvulus leaves (26%) and deep-fried sweet potato ball (37%) did not differ significantly from one another. Simultaneous ingestion of oriental radish (a root vegetable free of β-carotene) reduced the serum response to beadlets to two thirds, demonstrating interference of β-carotene absorption by the vegetable matrix. In women, the relative bioavailabilities obtained from chronic ingestion of β-carotene (12 mg) from raw carrots and carrot juice were 26 and 45%, respectively [167].

In men, the relative bioavailability of 29 mg β-carotene from cooked carrots was 21% after single ingestion [137] and 18% after chronic ingestion [168]. In a comparison of whole leaf, minced, and enzymatically liquefied spinach, relative bioavailabilities of β-carotene were 5.1, 6.4, and 9.5%, respectively [169]. The relative bioavailability of β-carotene from cooked mixed vegetables was 14% [170]. The consumption of vegetable juice elicited a three-times greater plasma α-carotene response in women as compared to raw or cooked vegetables, but no significant difference in the β-carotene response [171]. In women, the plasma β-carotene response to chronic ingestion of thermally processed and puréed carrots and spinach was three times that elicited by the raw vegetables. This enhancement was not negated by the heat-induced partial conversion of all-*trans*-β-carotene to *cis* forms [172].

Livny et al. [173] compared the bioavailability of β-carotene from two single carrot meals, one composed of ground raw carrot and the other of cooked puréed carrot. Test meals, each containing 15 mg all-*trans*–carotene and 40 g added fat, were given to ileostomy volunteers and complete ileal effluent samples were collected over a 24-h period. Mass-balance calculations indicated that 65.1 $\pm$ 7.4% of the β-carotene ingested was absorbed from the cooked puréed carrot meals as compared to 41.4 $\pm$ 7.4% from the raw ground carrot meals.

Edwards et al. [174] used the adapted PPC model [145] to compare the absolute and relative bioavailabilities of vitamin A and β-carotene derived from a single source of carrots prepared by three ways — commercially puréed, boiled-mashed, or raw-chopped (in a household food processor). Each test meal consisted of the carrot preparation, supplying 18.6 mg β-carotene, plus a blended drink containing the d_4-labeled retinyl acetate and 5 g safflower oil. The amounts of β-carotene absorbed intact from raw-chopped, puréed, and boiled-mashed carrots were 0.38 $\pm$ 0.28, 0.44 $\pm$ 0.15, and 0.16 $\pm$ 0.11 mg, respectively. The corresponding vitamin A (retinol) yields were 0.43 $\pm$ 0.27, 0.53 $\pm$ 0.2, and 0.44 $\pm$ 0.17 mg, respectively. Both cooking and comminution improve the bioavailability of β-carotene of vegetables [175] and therefore it was expected that the carotene and vitamin A masses derived from the raw-chopped carrot would be significantly lower than those of the two cooked preparations. However, this turned out not to be the case. The authors speculated that longer gastrointestinal residence time may have compensated for the lack of heat treatment and relatively large particle size of the raw-chopped carrot.

3.5.5.3 Dietary Protein

There are many published reports of an association between vitamin A deficiency and protein malnutrition [176]. In Guatemala,

Arroyave et al. [177] found low serum vitamin A levels in some patients with severe protein malnutrition. When these patients were given adequate dietary protein without a source of vitamin A, their serum vitamin A levels rose, provided they had sufficient liver reserves of the vitamin. This observation, supported by experiments with rats [178], suggests that adequate dietary protein is necessary for the mobilization of vitamin A from the liver into the bloodstream. Ingested proteins tend to stimulate absorption of vitamin A by serving as surface-active agents, both within the intestinal lumen and at the surface of enterocytes [179]. In rats, the enzymatic conversion of β-carotene to vitamin A in the intestinal wall is dependent on the intake of protein [113].

3.5.5.4 Dietary Fat and Energy

Absorption of the hydrophobic carotenes is facilitated by their solubilization within micelles in the intestinal lumen. Because adequate amounts of dietary fat are essential for micelle formation, dietary fat is a major determinant of carotene absorption. Essentially no β-carotene is absorbed in the absence of dietary fat.

The effect of additional fat on provitamin A carotenoid bioavailability depends on the amount of provitamin and fat in the normal diet. A number of studies have been conducted in situations where the level of dietary fat is very low. In a Ruandan village area in Central Africa, the addition of 18 g per day of olive oil to a carotenoid-sufficient, but low-fat, diet increased the absorption of vegetable carotenoids from 5 to 25% in boys showing clear signs of vitamin A deficiency (Bitot spots) [180]. In a study of Indian children, Jayarajan et al. [181] added 0, 5, or 10 g of fat (groundnut oil) to rice meals supplemented with 40 g of cooked spinach (ca. 1.2 mg of β-carotene). The results (Table 3.10) show that the greatest improvement in serum retinol occurred in children with poor vitamin A status ($\leq$20 μg/100 ml), who were fed 10 g of fat together with the spinach. The addition of 5 g of fat was no more effective than the provision of spinach without fat in the vitamin A-deficient children. No improvement in serum retinol occurred in children with better vitamin A status ($>$20 μg/100 ml), who were fed spinach without fat. Among these children, the addition of 5 g of fat per meal was sufficient to improve serum retinol. Thus, initial vitamin A status is an important factor in determining the serum retinol response to green leafy vegetables and the effect of dietary fat. In a study of village children in West Sumatra, Indonesia [182], supplementing a basic low-fat (ca. 3 g fat), low-β-carotene diet with β-carotene-rich food sources (mainly from red sweet potatoes) significantly increased serum retinol concentrations. Adding 15 g of fat to the basic diet and antihelminthic treatment (deworming) was nearly as effective in raising serum retinol as was the addition of β-carotene

TABLE 3.10

Effects of Adding Fat to a Diet Supplemented with Spinach on Serum Vitamin A Levels in Children

			Serum Vitamin A (μg/100 ml)		
Group	**Initial Serum Vitamin A (μg/100 ml)**	**No. of Children**	**Before Fat Additon**	**After Fat additon**	**Difference Before/After Fat Addition**
Without added fat	≤20	12	13.1 ± 1.18	22.7 ± 1.43[a]	9.6
	>20	14	26.7 ± 1.30	25.6 ± 1.61	−1.1
With 5 g added fat	≤20	14	16.1 ± 0.97	25.4 ± 2.05[a]	9.3
	>20	8	26.7 ± 1.29	33.9 ± 2.37[a]	7.2
With 10 g added fat	≤20	8	12.4 ± 1.38	25.7 ± 1.58[a]	13.3
	>20	14	25.4 ± 0.87	30.6 ± 1.17[a]	5.2

[a] $P < 0.001$. Compared to values before addition of fat.

Note: Values are mean ± S.E.

Source: From Jayarajan, P., Reddy, V., and Mohanran, M., *Ind. J. Med. Res.*, 71, 53, 1980. With permission.

sources. The addition of fat to the β-carotene-supplemented diet further improved serum retinol, but only if intestinal helminth infection was low. In a study of village children in northern Ghana [183], supplementation of a basic low-vitamin A, low-carotene diet with green leafy vegetables significantly increased serum retinol concentrations. The leaves were first pounded in wooden mortars, using wooden pestles, followed by high-speed homogenization in a blender. In a village setting, milling on flat-surfaced stone or grinding in earthenware would replace the use of a blender. The addition of fat to the carotene-supplemented diet further improved vitamin A status. Antihelminthic treatment enhanced the utilization of vegetable carotenes in children who were fed additional fat. Among children who were not dewormed, the effect of additional fat on plant β-carotene utilization was minimal.

Addition of 20% cooking oil per gram of dry matter carrot resulted in a 30% increase of bioaccessible β-carotene in pulped raw carrot, but had no significant effect on raw carrot pieces [153]. This suggested an interaction of added fat with the integrity of the plant matrix.

When sun-dried green leafy vegetables from Tanzania were cooked without oil, 8–29% of the β-carotene content became bioaccessible (i.e., available for absorption) after *in vitro* digestion [184]. The effects of cooking with sunflower oil or red palm oil were also determined. Red palm oil is not commonly consumed, but is known to be a good source of α- and β-carotene; sunflower oil is a poor source of provitamin A carotenoids. The amount of bioaccessible β-carotene increased three to eight times when the leaves were cooked with sunflower oil. Cooking with

red palm oil instead of sunflower oil resulted in about twice as much bioaccessible β-carotene, owing to its high β-carotene content. Overall, 39–94% of the β-carotene became available for absorption after cooking the leaves with sunflower oil or red palm oil. The results showed that by eating vegetable relish cooked with oil daily, it should be possible to provide the recommended intake level of vitamin A.

The situation of very-low-fat diets does not apply to Western diets, in which an average hot meal can contain as much as 40 g fat. In a study of well-nourished Dutch adults [185], subjects consumed a low-fat, low-carotenoid evening meal with a low-fat (3 g) or high-fat (36 g) spread enriched with 8 mg of α- plus β-carotene. No difference in plasma α- and β-carotene concentration was found in the subjects. Based on these results, a threshold of 3–5 g fat per meal is taken as a guideline to promote optimal absorption of β-carotene.

Brown et al. [125] compared carotenoid absorption from a test salad of mixed fresh vegetables consumed with fat-free, reduced-fat, or full-fat salad dressing containing 0, 6, or 28 g canola (rapeseed) oil, respectively. The total carotenoid content of the test salad was 31 mg, of which 12 mg was β-carotene. Newly absorbed carotenoids were isolated in the chylomicron fraction of plasma and α-carotene, β-carotene, and lycopene were determined using high-sensitivity HPLC with coulometric array (electrochemical) detection. When the salads were ingested with fat-free salad dressing, the absorption of carotenoids was negligible. When the salads were ingested with reduced-fat salad dressing, the added fat promoted absorption of α-carotene, β-carotene, and lycopene. Similiarly, there was substantially more absorption of these carotenes when salads were consumed with full-fat than with reduced-fat salad dressing. These results show that salads consumed with fat-free salad dressings, in the absence of other sources of fat, provide practically no available β-carotene. The threshold for optimal absorption of β-carotene from the salads exceeded 6 g added fat. Therefore, the guideline threshold of 3–5 g fat, based on the absorption of added β-carotene [185], should be considered a minimal amount that may not be sufficient for optimal bioaccessibility of carotenoids within the intact structures of plants.

Carotenoid absorption in humans has been assessed by the metabolic balance technique after cleansing the entire gastrointestinal tract of ingesta. Subjects given a 15-mg dose of β-carotene (in beadlet form) excreted 83% of the dose in their feces, indicating 17% availability for absorption. In the presence of a meal, containing 20 or 40% fat, the amount of ingested β-carotene available for absorption increased to 29–51% [186].

Whereas a single 51-mg dose of β-carotene given in the absence of dietary fat did not measurably change baseline serum β-carotene, the same dose given with 200 g fat increased serum β-carotene 2.5-fold [187].

Subjects receiving 45-mg doses of β-carotene daily with a high-fat diet for five days achieved high plasma β-carotene concentrations that could be sustained with intermittent (4–5 days) doses of β-carotene, without a need for continuous high-fat levels in the diet [188]. This may reflect a rapid saturation of storage tissues, facilitating the maintenance of high levels of plasma β-carotene.

Henderson et al. [189] demonstrated that the serum response to doses of β-carotene in humans is affected by meal timing. Variable dose–response experiments were designed to determine the pattern of the serum concentration versus time curve, the effect of β-carotene dose, and the relationship of serum response to meal-ingested lipids (as measured by the triglyceride level) to serum β-carotene response. A fasting venous blood sample was drawn from each subject to establish serum baseline levels. Subjects were then given a gelatin capsule containing 0, 15 30, 60, 90, 120, or 180 mg pure β-carotene to consume with a liquid formula diet containing 18 g fat. Blood samples were taken hourly for 8 h and at 24 h. Some subjects consumed a second meal (the liquid diet) at mid-day, ca. 4 h after the start of the study. Five other subjects omitted the second meal and fasted until the evening meal, ca. 9 h after the start of the study. All subjects were asked to omit carotenoid-containing foods from their evening meal. One of the control subjects took 15 mg β-carotene with water instead of the liquid diet, but consumed no other food or liquid diet until 16 h after the start of the study. In this subject, blood was drawn hourly for 24 h following the β-carotene dose. Results from the subjects receiving β-carotene and the two liquid meals showed the onset of a rise in serum β-carotene at 2–3 h, peak levels at 5 h, and above-baseline levels at 24 h, independent of dose. The β-carotene response curve ($AUC_{8\,h}$) increased linearly with β-carotene dose. The rise in serum triglyceride occurred at 1 h, well before the onset of the rise in β-carotene. The triglyceride level rose further following the second meal. The AUC/dose positively correlated with the peak serum triglyceride level, suggesting that individuals with a greater triglyceride response to meal lipids may be better absorbers of β-carotene. For the subjects who omitted the mid-day meal, the mean time of the β-carotene peak was 7.1 ± 0.6 h compared to 5.1 ± 0.2 h for those subjects who consumed this meal. This delay was highly significant ($P < 0.0004$). However, β-carotene levels in the former subjects rose in response to the carotenoid-free evening meal, and remained elevated at 24 h after the β-carotene dose. The further rise in serum β-carotene following subsequent β-carotene-free meals may be due to additional triglyceride becoming available to allow packaging of previously ingested β-carotene into chylomicrons within the enterocyte. The interval between the ingestion of β-carotene and the next meal cannot be too long, otherwise the β-carotene will be cleared by the time the triglyceride enters the

enterocyte. Indeed, no increase in serum β-carotene was observed in the control subject who took 15 mg β-carotene without food, and fasted for 16 h.

Shiau et al. [186] investigated the effect of total energy intake, dietary fat, and the age of the subject on the serum response curve of β-carotene. Male subjects were recruited from two different age groups: 18–30 years and 65–80 years. The subjects were given 15 mg β-carotene (water-soluble beadlets) or placebo with a preselected meal of energy content 2092 or 4184 kJ, and fat content 20 or 40%. The results showed a significant increase in serum peak β-carotene concentration within 8 h and at 24 h postdosing in young subjects receiving 40% as compared to 20% fat in their meal. Different energy contents of meals had no significant effect on any parameter of the serum response curve in young subjects. In elderly subjects, the percent fat in the meal had only a small effect on the serum β-carotene peak concentration. However, the lower energy meal resulted in a significantly higher peak concentration of serum β-carotene at 8 h as compared to the higher energy meal. Statistical analysis revealed a significant interaction between age and dietary energy intake on the change of serum β-carotene levels within 8 and at 24 h ($P < 0.05$). This indicated that higher energy intake significantly decreased the apparent serum response to β-carotene in elderly as compared to young men. However, the interaction between dietary fat intake, β-carotene level, and age was not statistically significant.

Borel et al. [190] reported that β-carotene absorption, as measured by the postprandial appearance of β-carotene in chylomicrons, was markedly diminished when human subjects ingested β-carotene with a meal containing medium-chain triglycerides (MCT) rather than one containing long-chain triglycerides (LCT). In addition, a significant increase in postprandial chylomicron triglycerides was observed after the LCT meal, but not after the MCT meal. These observations can be explained by the different ways in which MCT and LCT are absorbed (Section 2.2.4). LCT contributes ultimately to the formation of chylomicrons, which are essential vehicles for β-carotene release from the enterocytes and transport in the blood. In contrast, only a small proportion of medium-chain fatty acids produced from absorbed MCT is incorporated into chylomicrons, the majority leaving the enterocyte via the portal circulation. MCT did not affect the rate of intestinal conversion of β-carotene into vitamin A.

Hollander and Ruble [191], using the perfused rat intestine, showed that addition of linoleic acid (18:2) resulted in lower rates of absorption of β-carotene than did addition of oleic acid (18:1). They hypothesized that fatty acid-binding protein (FABP), which is necessary for the intracellular transport of long-chain fatty acids [192], may also function in the intracellular transport of β-carotene. Long-chain PUFAs have a greater binding affinity for FABP than do more saturated fatty acids

[193] and therefore, may more effectively compete with β-carotene for FABP-mediated intracellular transport, resulting in lower β-carotene absorption. In a human study, Hu et al. [146] compared the effects of a formulated meal containing 60 g fat in the form of either sunflower oil (high in PUFA) or beef tallow (high in monounsaturated and saturated fatty acids) on the intestinal absorption of supplemental β-carotene (25 mg), as measured by the postprandial appearance of β-carotene in the TRL fraction of plasma. The estimated average absorption efficiencies of β-carotene from the sunflower oil-rich meal and beef tallow-rich meal were 10.9 and 17.3%, respectively. Thus, in accordance with the earlier hypothesis, β-carotene ingested with a meal high in PUFA is absorbed more efficiently than when ingested with a meal containing more saturated fatty acids.

3.5.5.5 Dietary Fiber

The lower bioavailability of carotenoids from food than from synthetic carotenoid supplements can probably be at least partly explained by the inhibitory effects of soluble dietary fiber on the absorption of lipids (Section 2.2.6). Human subjects who had consumed a high-fiber diet containing fruits and vegetables excreted more vitamin A activity in feces than subjects who had consumed a low-fiber diet containing comparable amounts of carotene in the form of synthetic α- and β-carotene [194]. The concentration of vitamin A in the serum of fiber-fed rats was significantly lower than that of rats on a fiber-free diet [195]. The enrichment of chick diets with various dietary fiber components depressed β-carotene utilization by the chick, as measured by percent of consumed β-carotene stored in the liver as vitamin A relative to the 0% fiber control. With pectin, there was an inverse relationship between the extent of methyl esterification of the pectin and β-carotene utilization [196]. Deming et al. [116] reported that addition of citrus pectin (7 g/100 g) to a formulated fiber-free diet for Mongolian gerbils resulted in lower hepatic vitamin A stores. Moreover, hepatic β-carotene was higher in pectin-fed animals compared with all other groups, suggesting less conversion of β-carotene to vitamin A after consumption of pectin. In contrast, addition of oat gum to the fiber-free diet did not affect hepatic vitamin A stores. In fact, the higher vitamin A levels and lower β-carotene levels observed in the livers of oat-gum-fed gerbils compared with pectin-fed gerbils suggested that this purified β-glucan fraction from oats had no adverse effect on uptake and/or conversion of β-carotene to vitamin A. In a similar report [197], addition of pectin (7 g/100 g) to a rat diet containing 24 μg synthetic β-carotene significantly reduced the concentration of plasma β-carotene, and the liver contents of retinol, β-carotene, and retinyl palmitate.

In a study conducted in seven young women [198], the increase in plasma β-carotene concentration in response to a single 25-mg dose of purified β-carotene added to a standard meal with negligible fiber content was reduced by more than 50% at 30, 48, and 192 h after the meal, when 12 g citric pectin was added to the meal. In a similar study of six women [199], enrichment of a standard meal with pectin, guar, or alginate (all water-soluble dietary fiber components) significantly ($P < 0.05$) decreased the plasma response ($AUC_{24\ h}$) to supplemented β-carotene by 42, 43, and 33%, respectively. When the results of one atypical subject were excluded from the statistical analysis, the inhibitory effects of cellulose and wheat bran (water-insoluble types) also reached significance. Castenmiller et al. [169] found that the serum β-carotene response to liquefied spinach was not altered when sugar beet fiber was added to compensate for the fiber that was broken down. It seemed, therefore, that once the cell wall structure is broken down, addition of dietary fiber in amounts originally present in the food has no effect on bioavailability of carotenoids.

Erdman et al. [69] opined that very high levels of dietary fiber (especially pectin) may cause some reduction of carotenoid utilization in humans, but typical dietary levels of fiber in the U.S. should not significantly affect carotenoid utilization. Carrots, which contribute the highest amount of dietary β-carotene in U.S. diets [24], contain 3.2% dietary fiber (wet weight basis), 44% of which is the soluble fraction containing pectin [165].

3.5.5.6 Plant Sterols

The commercial practice of adding plant sterol esters or stanol esters to margarine and other suitable vehicles is a public health measure to lower plasma concentrations of cholesterol, and thus reduce the incidence of cardiovascular disease. Plant sterols and stanols (saturated sterols) and their esters reduce the intestinal absorption of dietary and biliary cholesterol by competing with cholesterol for solubilization within mixed micelles. This results in a lowering of blood total and LDL cholesterol, but not of the beneficial high density lipoprotein (HDL) cholesterol. Sterols and stanols are potentially atherogenic, like cholesterol, but atherosclerosis does not take place. This is because sterols and especially stanols are very poorly absorbed, and the small amounts that are absorbed are actively excreted in bile [200]. In addition to lowering cholesterol, consumption of sterol- and stanol-enriched margarines lower serum α- and β-carotene levels [201,202].

A well-designed clinical trial in normocholesterolemic men was carried out to investigate whether daily consumption of 2.2 g plant sterols, either as free or ester form (at the same equivalent dose), would affect the

bioavailability of β-carotene, as determined by the postprandial chylomicron response with deuterium-labeled β-carotene [203]. The 1-week duration of plant sterol administration ensured a 60% reduction in cholesterol absorption. Bioavailability of β-carotene was reduced by ca. 50% overall, the reduction being significantly less with plant free sterols than with esters. The chronic consumption of plant sterols would be expected to induce a progressive decrease in plasma carotenoid concentration, but concentrations can be maintained by increasing the intake of dietary carotenoids [204].

3.5.6 Conclusions

The absorption efficiency of dietary β-carotene is low to moderate and highly variable between individiduals. The food matrix is an important determinant of carotenoid bioavailability. Processing of vegetables by mechanical homogenization or heat treatment greatly improves the bioavailability of carotenoids by releasing them from the plant tissue. The consumption of vegetables with added fat also improves carotenoid bioavailability by aiding their absorption.

The use of stable isotopes as tracers in bioavailability studies allows discernment between dosed and endogenous carotenoids, and also allows assessment of carotenoid bioefficacy. The very small doses of labeled carotenoid do not perturb endogenous pool sizes. Bioavailability of carotenoids from different matrices can be compared simultaneously using different isotopomers of the same carotenoid. *In vitro* methods allow the screening of carotenoid bioavailability from foods and meals.

3.6 β-Carotene Supplementation

3.6.1 Effect of Vegetable Consumption on Vitamin A Status in Populations at Risk of Vitamin A Deficiency

In industrialized countries, much of the dietary vitamin A is provided by natural and supplemental preformed vitamin A, which is well absorbed and available all the year round. In less industrialized parts of the world, the people have to rely on the provitamin A carotenoid content of vegetables and fruits as their source of vitamin A. The same is true for vegans wherever they live. Besides having poor bioavailability in terms of vitamin A contribution, the plant sources of provitamin A carotenoids are seasonal and subject to crop failure, resulting in fluctuations in intake and the risk of vitamin A deficiency. In some populations,

carotenoid absorption is impaired by factors such as low-fat intake, protein deficiency, and infestation with intestinal parasites.

Seasonal fluctuations in the availability of provitamin A-rich vegetables and fruits occurs, for example, in the Shandong province of northern China. In the summer there is an abundance of dark-green and yellow vegetables, and also fruits, but in the fall and winter these rich sources of provitamin A carotenoids are not readily available, and children, in particular, become susceptible to vitamin A deficiency.

Tang et al. [205] conducted a 10-week vegetable intervention study among Chinese children in the Shandong province to determine whether green and yellow vegetables could sustain or improve vitamin A status (total-body stores of vitamin A) during the fall and winter seasons. One month before the beginning of the intervention, each child was given 400 mg of albendazole to rid them of intestinal parasites. The children, aged 5 to 6.5 years, were divided into two groups and ate their meals at school five days per week starting in the fall. One group (group A) was fed mainly dark-green and yellow vegetables and the other group (group B) was fed mainly the customary light-colored vegetables. A stable isotope technique was used to measure total-body vitamin A stores before and after the vegetable intervention. Serum retinol and carotenoid concentrations were measured by HPLC. The initial concentrations of serum carotenoids were similar in both groups. After the intervention, the serum concentration of most carotenoids increased significantly in group A, and these higher concentrations were sustained for as long as 23 days after the intervention period ended. In the group B children, the serum carotenoid levels decreased. The serum concentration of retinol was sustained in group A, but significantly ($P < 0.01$) decreased in group B. The total-body vitamin A stores of group A did not change, whereas those of group B tended to decrease ($P < 0.06$). The results showed that dietary dark-green and yellow vegetables can provide adequate vitamin A nutrition to Chinese children, and protect them from becoming vitamin A deficient at times when the provitamin A food source is limited.

Haskell et al. [206] assessed the effect of daily consumption of Indian spinach and sweet potatoes on total-body vitamin A stores in Bangladeshi men. One week before beginning the study procedures, subjects were given 800 mg of albendezole to rid them of intestinal parasites. Twice daily low-vitamin A meals were supplemented with either: (1) low-vitamin A vegetables (0 μg RE/day); (2) sweet potato (cooked and puréed); (3) Indian spinach (steamed, puréed, and then sautéed in oil); (4) low-vitamin A vegetables and a capsule containing retinyl palmitate in corn oil; or (5) low-vitamin A vegetables and a capsule containing β-carotene in corn oil. Supplements 1–5 provided 375 μg RE/meal, and supplements 1–3 included a corn oil capsule. Total-body vitamin A

stores were determined by a stable isotopic technique [207] before and after 60 days of supplementation. Relative to the low-vitamin A control group, the estimated mean changes in vitamin A stores were 0.029 mmol for sweet potato (not significant, $P = 0.21$), 0.041 mmol for Indian spinach (significant, $P = 0.033$), 0.065 mmol for retinyl palmitate ($P < 0.001$) and 0.062 mmol for β-carotene ($P < 0.002$). Plasma β-carotene concentrations increased by 300 and 385% in response to supplementation with sweet potato and Indian spinach, respectively. The results provided further evidence that daily consumption of cooked, puréed green leafy vegetables or sweet potatoes has a positive effect on vitamin A status in populations at risk of vitamin A deficiency.

3.6.2 Effects of β-Carotene Supplementation on Breastmilk Carotenoids

Carotenoids are present in human milk at 5–10% of their concentrations in serum [208]. Changes in breastmilk β-carotene concentrations after supplementation can be predicted from incremental changes in serum β-carotene concentration, using a simple equation derived from linear-regression analysis [135].

In a study in well-nourished, lactating American women, Canfield et al. [136] measured changes in concentrations of milk and serum β-carotene over a 28-day supplementation trial with 30 mg purified β-carotene, and for 4 weeks thereafter. Increases in milk β-carotene concentrations averaged 6.4-fold higher than baseline concentrations, and the pattern of changes in concentrations of milk β-carotene paralleled that in serum. Concentrations of retinol in serum and milk were similar and unaffected by β-carotene supplementation. The data showed that short-term supplementation of healthy, lactating mothers with purified β-carotene at ca. fivefold the average daily dietary intake substantially increases milk and serum β-carotene concentrations, while not interfering with concentrations of other carotenoids, retinol, or α-tocopherol in milk or serum.

Sub-optimal vitamin A status is prevalent in lactating women in developing countries [209] and, therefore, the question arises as to whether increasing the dietary intake of provitamin A carotenoids (either as plant foods or β-carotene supplements) could satisfy the infant's need for retinol. Canfield et al. [210] investigated the effects of β-carotene supplementation in Honduran mothers whose initial milk β-carotene concentrations were ca. 20% those of the American mothers in the previous study [136]. Three capsules, each containing 30 mg β-carotene in water-miscible beadlet form, were administered on three consecutive days to the lactating women. The capsules were administered with a meal that provided 8 g of fat. Supplementation elevated maternal serum and milk β-carotene

concentrations nine- and seven-fold, respectively. Maternal serum and milk retinol concentrations were unchanged. In contrast, serum retinol concentrations in the infants significantly ($P < 0.001$) increased, but β-carotene concentrations remained unchanged. This report was the first to demonstrate that maternal supplementation with β-carotene can provide retinol for the suckling infant.

In Honduras, red palm oil is a locally available rich natural source of provitamin A, and has the potential to solve the problem of vitamin A deficiency if introduced into the diet. Canfield et al. [211] investigated the effects of red palm oil supplementation in Honduran mothers and their suckling infants over 10 days. On days 1, 3, 5, 7, and 9, the lactating women were given a 90-mg supplement of β-carotene, either as red palm oil mixed with black beans or as capsules containing powdered β-carotene. A third group of women received placebo capsules. Supplements and placebo were given with a meal containing ca. 8 g of fat. Maternal serum α-carotene and β-carotene concentrations increased significantly more ($P < 0.001$) for the palm oil supplement group compared with the β-carotene supplement and placebo groups. Serum α-carotene, but not β-carotene, concentrations were significantly increased ($P < 0.05$) in serum of infants in the palm oil group compared with the other groups. Maternal serum retinol concentrations were not significantly changed by any of the treatments. Serum retinol of infants increased significantly ($P < 0.05$) relative to initial concentrations in response to maternal supplementation with red palm oil; however, this increase was not significant when compared to concentrations in the placebo group. Increases in milk β-carotene concentrations were greater for the palm oil group (2.5-fold, $P < 0.001$) than for the β-carotene supplement group (1.6-fold, $P < 0.006$) relative to placebo. Similarly, increases in milk α-carotene concentrations in the palm oil group (3.2-fold) were greater than those in the β-carotene group (1.6-fold). When expressed relative to milk lipid, changes in milk retinol concentrations were not significantly different among the treatment groups. No biological explanation could be offered for the large increase in milk α-carotene following supplementation with β-carotene capsules. Serum α-carotene was not increased in mothers supplemented with β-carotene. In summary, dietary supplementation of low-vitamin A-consuming nursing mothers with red palm oil significantly increases β-carotene levels in maternal serum and milk, and also retinol levels in the serum of the suckling infant [160].

Lietz et al. [212] also reported that supplementing pregnant women with red palm oil increased α- and β-carotene concentrations significantly in plasma and breastmilk, but did not increase retinol levels. In addition, consumption of red palm oil retarded the decline in breastmilk retinol concentration (as seen in the control group) during the progression of lactation, thereby conserving retinol for the benefit of the suckling infant.

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4

Vitamin D

4.1 Background

Vitamin D is represented by cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2), which are structurally similar secosteroids derived from the UV irradiation of provitamin D sterols. Exposure of the skin to sunlight converts 7-dehydrocholesterol to vitamin D_3, which, on reaching the blood capillaries of the dermis, is conveyed to the liver on a specific plasma transport protein. Vitamin D_2 is produced in plants, fungi, and yeasts by the solar irradiation of ergosterol. On irradiation, the provitamins are initially converted to previtamin D, which undergoes thermal transformation to vitamin D. In parts of the world where there is ample sunlight throughout the year, the human vitamin D requirement is met entirely by biogenesis in the skin; otherwise, populations depend on dietary sources of the vitamin.

Vitamin D itself is biologically inactive and must be metabolized to 1α,25-dihydroxyvitamin D [1α,25$(OH)_2$D], which acts as a hormone in controlling calcium homeostasis and regulating the growth of various cell types. In the liver, the vitamin D of both cutaneous and dietary origin is hydroxylated to 25-hydroxyvitamin D [25(OH)D], which is released without delay into the bloodstream. On reaching the kidneys, this circulating metabolite undergoes the second hydroxylation to 1α,25$(OH)_2$D. This hormone stimulates the intestine to absorb calcium and phosphate, and acts with parathyroid hormone to mobilize calcium, accompanied by phosphate, from the bone fluid compartment into the bloodstream. 1α,25-Dihydroxyvitamin D is also involved in the formation of osteoclasts – giant cells that are solely responsible for the resorption of bone matrix. Resorption is an essential process for the development, growth, maintenance, and repair of bone. Another metabolite, 24*R*,25-dihydroxyvitamin D [24*R*,25$(OH)_2$D], is the initial product in the catabolism of 1α,25$(OH)_2$D and plays a crucial role in bone formation and repair of bone fractures.

The hormonal effects of 1α,25$(OH)_2$D are mediated via a specific intracellular receptor, which belongs to the superfamily of steroid receptors. After binding to 1α,25$(OH)_2$D, the receptor–ligand complex binds to vitamin D responsive elements on the DNA and, depending on the

target cell, it regulates the expression of a number of genes involved in calcium homeostasis or in the control of cell proliferation and differentiation.

Vitamin D deficiency in children causes rickets, in which the bones do not develop properly and become deformed through inadequate deposition of calcium and phosphorus. The equivalent disease in adults is called osteomalacia, in which the newly deposited bone matrix fails to mineralize adequately, causing the bones to become brittle. Toxicity can result from chronic oversupplementation of vitamin D, but not from unlimited exposure to sunshine or the consumption of usual diets. The physiological basis for toxicity is bone demineralization, causing increased serum calcium levels. The excess calcium is deposited in the kidneys, causing hypertension, cardiac insufficiency, and renal failure.

4.2 Chemical Structure, Biopotency, and Physicochemical Properties

4.2.1 Structure and Biopotency

Irradiation of the parent steroid causes breakage of the B ring at the 9,10-carbon bond, resulting in the conjugated triene system of double bonds. The numbering system of the carbon atoms of the vitamin D molecule is identical to that of the parent steroid (Figure 4.1). Vitamin D_2 ($C_{28}H_{44}O$, MW = 396.6) and vitamin D_3 ($C_{27}H_{44}O$, MW = 384.2) differ structurally only in the C-17 side chain, which in vitamin D_2 has a double bond and an additional methyl group (Figure 4.1). Both compounds occur naturally with 5,6 double bond in the *cis* configuration.

The biological potencies of vitamins D_2 and D_3 in humans are essentially equal. The circulating metabolite 25(OH)D_3, which is found in significant quantities in animal-derived foods, induces all the responses of vitamin D_3, but its biological activity is five-times higher based on its ability to enhance intestinal calcium absorption [1]. Animal tissues contain a small proportion of vitamin D esterified with both saturated and unsaturated fatty acids. All the esters have biological activity equivalent on a molar basis to that shown by cholecalciferol [2].

4.2.2 Physicochemical Properties

4.2.2.1 Appearance and Solubility

Vitamins D_2 and D_3 are white to yellowish crystalline powders, which are insoluble in water; soluble in 95% ethanol, acetone, fats, and oils; and readily soluble in chloroform and ether.

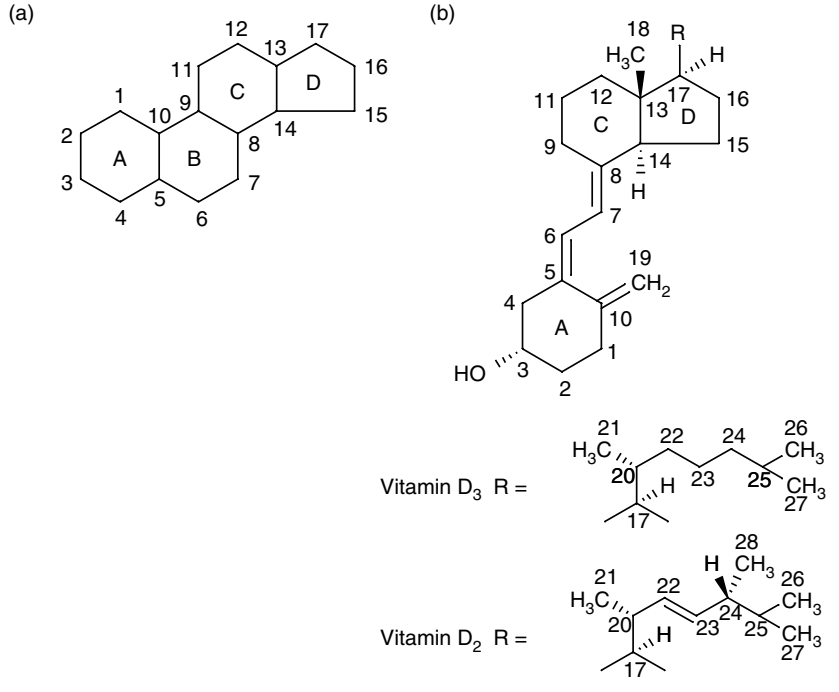

FIGURE 4.1
Structural relationship of (a) the parent steroid nucleus to (b) vitamin D.

4.2.2.2 Stability in Nonaqueous Solution

In solution, but not in the solid state, vitamins D_2 and D_3 exhibit reversible thermal isomerization to their corresponding previtamins, forming an equilibrium mixture. Equations and calculations have been published to determine the ratio of previtamin D to vitamin D as a function of temperature and reaction time [3]. The previtamin D/vitamin D ratios and the equilibration times attained at different temperatures are given in Table 4.1 [4]. When equilibrated at 20°C, the ratio of previtamin D to vitamin D is 7:93. The isomerization rates of vitamins D_2 and D_3 are virtually equal [5] and are not affected by solvent, light, or catalysis [4].

Like vitamin A, the stability of vitamin D in fats and oils corresponds to the stability of the fat itself. Vitamin D, however, is more stable than vitamin A under comparable conditions. Once freed from the protection of the food matrix, vitamin D is susceptible to decomposition by oxygen and light. Conditions which promote destruction of vitamin D include exposure of thin films to air (especially with heat), and dispersion of an alcoholic solution of the vitamin into an aqueous phase in the presence of dissolved oxygen [6]. The vitamin is stable toward alkali, but

TABLE 4.1

The Previtamin D and vitamin D Equilibrium

Temperature (°C)	%Previtamin D	%Vitamin D	Equilibrium Time[a]
−20	2	98	16 yr
10	4	96	350 days
20	7	93	30 days
30	9	91	10 days
40	11	89	3.5 days
50	13	87	1.3 days
60	16	84	0.5 days
80	22	78	0.1 days
100	28	72	30 min
120	35	65	7 min

[a]The time necessary to reach equilibrium, starting with pure vitamin D or pure previtamin D.
Source: From Mulder, F.J., de Vries, E.J., and Borsje, B., *J. Assoc. Off. Anal. Chem.*, 54, 1168, 1971. With permission.

under conditions of even mild acidity the molecule isomerizes to form the 5.6-*trans* and isotachysterol isomers (Figure 4.2), neither of which possesses any significant antirachitic activity [7].

4.3 Vitamin D in Foods

4.3.1 Occurrence

As with vitamin A, fish-liver oils are exceedingly rich in vitamin D. However, natural foods contain only very small amounts and significant sources are confined to a limited number of animal products. Fatty fish, such as herring, sardines, pilchards, and tuna, are rich natural food sources; smaller amounts are found in mammalian liver, eggs, and dairy products (Table 4.2) [8]. The concentration of vitamin D_3 in milk shows a seasonal variation, which is related to the amount of sunlight available for vitamin D biogenesis in the cow. Milk also contains vitamin D_2, but in smaller concentrations than vitamin D_3. The D_2 vitamer is derived by UV irradiation of ergosterol in sun-dried green forage (hay); ergosterol cannot be converted by the animal into vitamin D_2.

The vitamin D activity in animal products is contributed by both vitamin D and its immediate metabolite, 25(OH)D. Typical values of this metabolite (micrograms per 100 g) are bovine muscle, 0.2–0.3; bovine liver, 0.3–0.5; bovine kidney, 0.5–1.0 [9]; and egg yolk, 1.0 [10]. Supplementation of chicken feed with vitamin D increases the content

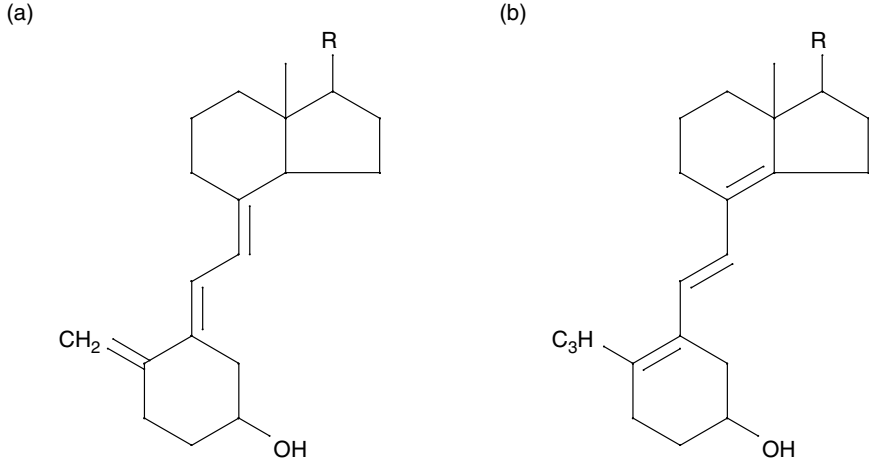

FIGURE 4.2
Structures of (a) 5,6-*trans* vitamin D and (b) isotachysterol.

of both vitamin D_3 and 25(OH)D_3 in egg yolk [11]. 25-Hydroxyvitamin D is of nutritional significance: in milk, for example, it accounts for 75% of the total vitamin D activity as estimated by the calcium transport assay [12].

In the United States and Canada, fluid milk is supplemented by law with vitamin D to a level of 400 international units (IU) per quart (10 μg/0.95 l) to meet the recommended dietary allowance (RDA) of 10 μg. In the United Kingdom, the Margarine Regulations [13] state that "every ounce of margarine shall contain not less than 80 IU and not more than 100 IU of vitamin D," equating to limits of between 7 and 9 μg/100 g. Other foodstuffs commonly enriched with vitamin D include skimmed milk powder, evaporated milk, milk-based beverages, breakfast cereals, dietetic products of all kinds, infant formulas, and soup powders. Vitamin D is either added as an oily solution or in combination with a vitamin A formulation [14].

Reports of water-soluble vitamin D sulfate present in large quantities in milk [15,16] have not been confirmed in later studies [17]. The water-soluble vitamin D activity in milk can now be explained by the presence of protein-bound 25(OH)D [18]. In any event, synthetic vitamin D sulfate has been shown to possess negligible biological activity [19].

The RDA of vitamin D for humans exposed to inadequate sunlight is 10 μg. It has been estimated that to maintain adequate plasma 25-hydroxyvitamin D levels without any input from skin irradiation

TABLE 4.2

Vitamin D Content of Various Foods

Food	Micrograms of Vitamin D per 100 g Edible Portion
Cow milk, whole, pasteurized	Tr
Butter	0.9
Cheese, cheddar	0.3
Egg, chicken, whole, raw	1.8
Beef, trimmed lean, raw, average	0.5[a]
Lamb, trimmed lean, raw, average	0.4[a]
Pork, trimmed lean, raw, average	0.5[a]
Chicken meat, raw, average	0.1[a]
Liver, lamb, fried	0.9[a]
Cod, raw, fillets	Tr
Haddock, raw, fillets	Tr
Herring, raw	19.0 (range 7–31)
grilled	16.1
Pilchards, canned in tomato sauce	14.0
Sardines, canned in brine, drained	4.6
Sardines, canned in oil, drained	5.0
Sardines, canned in tomato sauce	8.0
Tuna, canned in brine, drained	3.6
Tuna canned in oil, drained	3.0

Note: Tr, trace.

[a]The total vitamin D activity for meat, meat products and poultry is taken as the sum of vitamin D_3 and five times 25-hydroxyvitamin D_3.

Source: From Food Standards Agency, McCane and Widdowon's *The Composition of Foods*, 6th summary ed., Royal Society of Chemistry, Cambridge, 2002. With permission.

would require ingestion of at least 12.5 μg of vitamin D per day in the form of dietary supplements [20]. Except for eggs and fatty fish, a serving of food containing only natural sources of the vitamin D would probably supply less than 1 μg [21].

4.3.2 Stability

Vitamin D is destroyed in oxidizing fats; otherwise, food processing, cooking, and storage of foods do not generally affect the vitamin's activity. The vitamin will withstand smoking of fish, pasteurization and sterilization of milk, and spray drying of eggs [22].

Indyk et al. [23] evaluated the stability of supplemental vitamin D_3 in spray-dried milk. Measured losses through the pasteurization, high-pressure evaporation, and drying processes were demonstrated to be statistically insignificant ($P > 0.05$). Milk should not be exposed to

light during processing and storage because the vitamin D present can be oxidized to an inactive 5,6-epoxide by riboflavin-photosensitized singlet oxygen [24].

4.3.3 Expression of Dietary Values

One IU of vitamin D is the activity obtained from 0.025 μg of crystalline cholecalciferol in a bioassay. Despite a recommendation by an expert committee in 1970 that the IU be abandoned, the vitamin D content of foods is often expressed in such units. There is as yet no consensus on which conversion factor should be used for expressing the content of 25(OH)D in IU. For calculation of the "total" vitamin D activity present in foods, a factor of 5 [1 IU = 0.005 μg 25(OH)D] has been used [18].

4.3.4 Applicability of Analytical Techniques

The estimation of the very low concentrations of indigenous vitamin D in foodstuffs is difficult owing to the need to remove interfering subtances, such as cholesterol, vitamin A, and vitamin E, which are invariably present in gross excess (Table 4.3) [25]. Most of the published HPLC methods for determining vitamin D in foods are concerned with estimating the vitamin D content in supplemented products, such as milk in various forms, infant formulas, and margarine. In supplemented foods, the amount of naturally occurring vitamin D (if any) is usually considered to be negligible, and it is deemed necessary to determine only the vitamin D that is added. Even so, supplementation levels are very low (e.g., 7.5–12.5 μg/100 g in milk powder) [26] and the determination of vitamin D is by no means a simple task.

A bioassay will account for the activity of previtamin D as well as vitamin D and its various active metabolites. A valid estimate of the

TABLE 4.3

Ratios of Vitamins A and E and Cholesterol to Vitamin D in Some Foodstuffs

	Ratio Relative to Vitamin D on a Weight Basis			
Foodstuff	**D**	**A**	**E**	**Cholesterol**
Whole milk	1	1,500	5,000	600,000
Ox liver	1	6,000	1,400	300,000
Whole egg	1	75	500	80,000

Source: From Osborne, D.R. and Voogt, P., *The Analysis of Nutrients in Foods*, Academic Press, London, 1978. With permission.

vitamin D value of a food should therefore represent "potential vitamin D," that is, the sum of the vitamin D and previtamin D contents.

When determining naturally occurring vitamin D in animal products for nutritional evaluation purposes, 25(OH)D_3 should be included, because this metabolite contributes significantly to the total biological activity, particularly in milk. 25-Hydroxyvitamin D_3 is present in dairy products, eggs, and meat tissues in sufficient concentration to permit its determination by HPLC, using an absorbance detector. In bovine milk, the concentration of this metabolite is less than 1 ng/ml [27]; hence it is usually determined by a competitive protein-binding assay after fractionation of the extracted sample by HPLC [28].

4.4 Intestinal Absorption, Transport, and Metabolism

The following discussion of absorption, transport, and metabolism is taken from more a detailed account in a book by Ball [29] published in 2004.

The vitamin D activity in the human diet is contributed mainly by free vitamin D, with 25(OH)D making a quantitatively small but nutritionally significant contribution. Ingested vitamin D is solubilized within mixed micelles in the duodenum and passively absorbed in the jejunum along with other lipids. Esters of vitamin D, if present, are hydrolyzed during solubilization [18]. A cytosolic vitamin D-binding protein (cDBP), with a preference for binding 25(OH)D, has been isolated from rat enterocytes [30,31] and may play a role in the absorption of 25(OH)D. Vitamin D is incorporated into chylomicrons within the enterocytes and the released chylomicrons convey the vitamin in the mesenteric lymph to the systemic circulation. During the journey in the lymph, an appreciable amount of the vitamin D in the chylomicrons is transferred to the serum vitamin D-binding protein (sDBP) [32]. This protein has a higher affinity for 25(OH)D than for vitamin D [33] and is present in large excess of the normal blood contents of its ligands [34]. After lipolysis of the chylomicrons, the vitamin D remaining on the chylomicron remnants, and also the vitamin D bound to sDBP, is initially taken up by the liver [35].

Absorption of 25-hydroxyvitamin D is faster than that of vitamin D in normal humans and in patients with fat malabsorption syndromes [36]. Dueland et al. [37] compared the distribution of [^{14}C]vitamin D_3 and 25-[^{3}H]hydroxyvitamin D_3 in mesenteric lymph after simultaneous instillation of these compounds in the duodenum of rats. They showed that vitamin D_3 is mainly carried with chylomicrons, whereas 25(OH)D_3 is carried mainly by proteins. The protein responsible for carrying the bulk of 25(OH)D and part of the vitamin D is very likely sDBP. The results suggested that the portal circulation plays only a minor role in

the total absorption of vitamin D and 25(OH)D. The association of $25(OH)D_3$ with sDBP rather than with chylomicrons implies that absorption of $25(OH)D_3$ is far less dependent on bile salts. Thus $25(OH)D_3$ is an important pharmacological agent in cases of bile salt deficiency.

Three key enzymes are involved in the conversion of vitamin D to active hormonal forms: vitamin D-25-hydroxylase in the liver converts vitamin D to 25(OH)D; a multicatalytic 1α-hydroxylase in the kidney converts 25(OH)D to 1α,25$(OH)_2$D; and 24*R*-hydroxylase, another renal multicatalytic enzyme, converts 25(OH)D to 24*R*,25$(OH)_2$D. The mammalian liver does not store vitamin D to any significant effect, and any vitamin D which is not metabolized in the liver is stored in the adipose tissue and skeletal muscle. The liver metabolite, 25(OH)D, is the major circulating form of vitamin D.

In contrast to the rapid hepatic uptake of dietary vitamin D and inactivation of surplus amounts, the supply of cutaneous vitamin D to the liver is gradual, allowing continuous prolonged production of 25(OH)D. Thus, the plasma concentration of this metabolite is maintained, even when exposure of the skin to sunlight occurs only intermittently. The hydroxylating enzyme, vitamin D-25-hydroxylase, is only loosely regulated, and circulating concentrations of 25(OH)D continue to rise in response to ingestion of pharmacological doses of vitamin D. Levels can rise to more than 400 ng/ml, leading to vitamin D toxicity. In contrast, extensive UV irradiation of the skin does not cause hypervitaminosis D, and raises 25(OH)D levels in plasma to no more than 80 ng/ml [20].

Almost all the 25(OH)D produced in the liver is released without delay into the bloodstream, where it becomes tightly bound to sDBP. The normal concentration range of plasma 25(OH)D is 10–40 ng/ml. The blood constitutes the largest single body pool of 25(OH)D, since extrahepatic tissues take up only small amounts. During vitamin D deprivation, the 25(OH)D blood pool is maintained through the prolonged release of vitamin D from its skin reservoir and from its muscle and adipose tissue storage sites. Plasma concentrations of the highly potent 1α,25$(OH)_2$D are kept within the range of 25 to 70 pg/ml by reciprocal changes in the rates of synthesis and degradation at the cellular level.

4.5 Bioavailability

In the only known study of vitamin D bioavailability from natural sources [18], the average relative bioavailability of vitamin D_2 from meat sources was estimated to be ca. 60% as compared with a vitamin supplement. Vitamin D given with milk has been reported to be 3–10 times more potent than that given with oil, the stimulatory factor being attributable

to the lactalbumin fraction [38]. It is not known whether enhanced absorption or some other factor is responsible for the greater apparent biological activity of vitamin D given in milk.

The serum 25(OH)D concentration reflects vitamin D stores in humans, and its measurement by protein-binding assay is an index of nutritional status [39]. In the rat, chronic ethanol ingestion promotes the biliary loss of 25(OH)D and this loss may be a contributing factor in the impaired vitamin D status in alcoholics [40]. Human subjects receiving a high-fiber diet exhibited a reduced plasma half-life of $25(OH)D_3$, indicating a more rapid elimination of the metabolite from the body [41]. Smoking seems to depress the serum levels of 25(OH)D, 1α,$25(OH)_2D$, and parathyroid hormone [42]. This may partly account for the decreased bone mass and increased fracture risk seen among smokers later in life [43].

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5

Vitamin E

5.1 Background

Vitamin E is represented by eight vitamers of varying biological potency: four tocopherols and four tocotrienols. The vitamin functions as a biological antioxidant by protecting the vital phospholipids in cellular and subcellular membranes from peroxidative degeneration.

A deficiency of vitamin E in animals results in a variety of pathological conditions that affect the muscular, cardiovascular, reproductive, and central nervous systems, as well as the liver, kidney, and red blood cells. The diversity of these disorders is attributable to secondary effects of the widespread damage caused to the membranes of muscle and nerve cells by lipid peroxidation. There is a marked difference between animal species in their susceptibility to different deficiency disorders. A complex biochemical inter-relationship exists between vitamin E and the trace element selenium. Unsaturated fat, sulfur-containing amino acids, and synthetic fat-soluble antioxidants are also implicated in some disorders. It is well documented that a diet rich in polyunsaturated fat, but which does not contain a correspondingly high amount of vitamin E, induces deficiency signs in animals.

Aside from instances of fat malabsorption or genetic abnormalities of lipid metabolism, clinical vitamin E deficiency is rare in adult humans and no recognizable deficiency syndrome has been demonstrated. This is due to the occurrence of the vitamin in a wide variety of foods, its widespread storage distribution throughout the body tissues, and the consequent extended period required for depletion. However, various symptoms have been reported in preterm infants; these include hemolytic anemia, oedema, colic, and failure to thrive.

Vitamin E, being fat-soluble, accumulates in the body, especially in the liver and pancreas. Unlike vitamins A and D, however, vitamin E is essentially nontoxic.

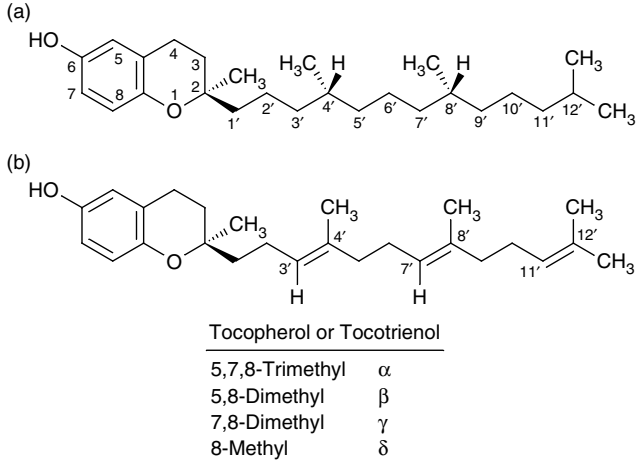
A possible role for vitamin E as a preventative factor for cardiovascular disease, cancer, Alzheimer's disease, and other disease states involving oxidative stress is under intensive investigation.

5.2 Chemical Structure, Biopotency, and Physicochemical Properties

5.2.1 Structure

Tocopherols are methyl-substituted derivatives of tocol, which comprises a chroman-6-ol ring attached at C-2 to a saturated isoprenoid side chain. Tocotrienols are analogous structures whose side chains contain three *trans* double bonds. In nature, there are four tocopherols and four corresponding tocotrienols; these are designated as alpha- (α), beta- (β), gamma- (γ) and delta- (δ) according to the number and position of the methyl substituents in the chromanol ring (Figure 5.1). The β- and γ-forms are positional isomers. Tocopherol molecules contain three chiral centers at C-2, C-4′, and C-8′, making possible eight stereoisomers. Tocotrienols possess only the chiral center at C-2.

The *RS* system of nomenclature for some α-tocopherols is given in Table 5.1. The most biologically active vitamer is the naturally occurring *RRR*-α-tocopherol ($C_{29}H_{50}O_2$, MW = 430.7). Totally synthetic α-tocopherol is produced by the condensation of trimethylhydroquinone with synthetic phytol or isophytol. This method of synthesis results in all-racemic 2*RS*,4′ *RS*,8′ *RS*-α-tocopherol (*all-rac*-α-tocopherol), which is a mixture of all eight possible diastereoisomers in virtually equal proportions. The four enantiomeric pairs are *RRR*/*SSS*, *RRS*/*SSR*, *RSS*/*SRR*, and *RSR*/*SRS*.



Tocopherol or Tocotrienol	
5,7,8-Trimethyl	α
5,8-Dimethyl	β
7,8-Dimethyl	γ
8-Methyl	δ

FIGURE 5.1
Stereochemical structures of tocol and tocotrienol: (a) *RRR*-tocol and (b) 2*R*,3′-*trans*, 7′-*trans*-tocotrienol.

TABLE 5.1

Nomenclature for Some α-Tocopherols

Configuration	Designated Name	Trivial Name	Description
2*R*,4′ *R*,8′ *R*	*RRR*-α-Tocopherol	*d*-α-Tocopherol	The only isomer of α-tocopherol found in nature
2*S*,4′ *R*,8′ *R*	2-*epi*-α-Tocopherol	*I*-α-Tocopherol	C-2 epimer of *RRR* form
2*RS*,4′ *RS*,8′ *RS* (mixture of four enantiomeric pairs)	*all-rac*-α-Tocopherol	*dl*-α-Tocopherol	Totally synthetic (produced from synthetic phytol or isophytol)

The principal commercially available forms of vitamin E used in the food, feed, and pharmaceutical industries are the acetate esters of *RRR*-α-tocopherol and *all-rac*-α-tocopherol. In commercial circles, *all-rac*-α-tocopheryl acetate ($C_{31}H_{52}O_3$, MW = 472.8) is commonly referred to by the trivial name of *dl*-α-tocopheryl acetate. *RRR*-α-tocopheryl acetate is obtained by extraction from vegetable oils. Since it is not isolated without chemical processing, it cannot legally be called natural, but it can be described as derived from natural sources. Another commercial preparation, not commonly used, is the hydrogen succinate of *RRR*-α-tocopherol.

The term "vitamin E" is the generic descriptor for all tocol and tocotrienol derivatives that exhibit qualitatively the biological activity of α-tocopherol. The term "tocopherol" refers to the methyl-substituted derivatives of tocol and is not synonymous with the term vitamin E. The tocopherols and tocotrienols may be referred to collectively as tocochromanols. In food and clinical analysis, commonly used methods do not distinguish the stereoisomers of vitamin E and therefore the tocochromanols are referred to without their stereochemical designation.

5.2.2 Biopotency

Only the naturally occurring *RRR*-α-tocopherol and the 2*R*-stereoisomeric forms of α-tocopherol (*RRR*-, *RRS*-, *RSS*-, and *RSR*-α-tocopherol) obtained from synthetic *all-rac*-α-tocopherol ester are maintained in plasma and delivered to tissues. This is because of the discriminating ability of the hepatic α-tocopherol transfer protein (Section 5.4.3) and the fact that the vitamin E vitamers are not interconvertible in the human body. Therefore, when establishing recommended intakes, the definition of vitamin E is limited to the 2*R*-stereoisomeric forms of α-tocopherol [1]. On the basis of this definition, *all-rac*-α-tocopherol has one-half the activity of

RRR-α-tocopherol. This 2:1 activity ratio for natural and synthetic vitamin E has been demonstrated in human studies [2] and is more relevant to human needs than the officially accepted 1.36:1.00 ratio that is based on the rat resorption–gestation assay [3].

Deuterium-labeling of *RRR*-α-tocopherol and its acetate and succinate esters in healthy humans showed that these compounds are absorbed to an equal extent overall, although the initial rate or absorption is higher from the acetate ester than from the succinate ester [4]. These compounds can therefore be accorded equal potency on a molar basis.

5.2.3 Physicochemical Properties

5.2.3.1 Appearance and Solubility

In the pure state, tocopherols and tocotrienols are pale yellow, nearly odorless, clear viscous oils which darken on exposure to air. α-Tocopheryl acetate is of similar appearance. The hydrogen succinate ester is a white granular powder.

Nonesterified tocopherols and tocotrienols are insoluble in water and readily soluble in ethanol, other organic solvents (including acetone, chloroform, and ether), and in vegetable oils. The vitamin E acetates are less readily soluble in ethanol than the unesterified vitamers.

5.2.3.2 Stability in Nonaqueous Solution

Tocopherols and tocotrienols are destroyed fairly rapidly by sunlight and artificial light containing wavelengths in the UV region. The vitamers are slowly oxidized by atmospheric oxygen to form mainly biologically inactive quinones; the oxidation is accelerated by light, heat, alkalinity, and certain trace metals. The presence of ascorbic acid completely prevents the catalytic effect of iron(III) and copper(II) on vitamin E oxidation by maintaining these metals in their lower oxidation states [5]. The tocotrienols, by virtue of their unsaturated side chains, are more susceptible to destruction than the tocopherols. The vitamers can withstand heating in acid or alkaline solution provided that oxygen and UV radiation are excluded. Because α-tocopheryl acetate lacks the reactive hydroxyl group, air and light have practically no destructive effect.

5.2.3.3 In Vitro Antioxidant Activity

α-Tocopherol (unesterified) is frequently used as an antioxidant to stabilize animal fats, which have a much lower vitamin E content than vegetable oils. In the absence of an antioxidant, unsaturated fats undergo autoxidation to produce hydroperoxides. These break down further to give a variety of volatile compounds such as aldehydes and ketones, which produce the disagreeable odors and flavors of rancidity.

The order of *in vitro* antioxidant activities of the tocopherols conforms to their oxidation potentials and parallels their biological activities, that is, $\alpha > \beta > \gamma > \delta$. This order of antioxidant activity has been confirmed in detailed studies, although there was no significant difference between the β and γ positional isomers [6].

5.3 Vitamin E in Foods

5.3.1 Occurrence

The important plant sources of vitamin E are the cereal grains and those nuts, beans, and seeds that are also rich in high-potency oils. The vegetable oils extracted from these plant sources are the richest dietary sources of vitamin E. Cereal grain products, fish, meat, eggs, dairy products, and green leafy vegetables also provide significant amounts. Major sources of vitamin E in the United States include margarine, mayonnaise and salad dressings, fortified breakfast cereals, vegetable shortenings and cooking oils, peanut butter, eggs, potato crisps, whole milk, tomato products, and apples [7].

Vegetable oils are highly unsaturated and contain a correspondingly high concentration of vitamin E to maintain the oxidative stability of their constituent polyunsaturated fatty acids (PUFAs). The distribution of tocopherols and tocotrienols in different plant oils varies greatly, as shown in Table 5.2 [8]. In some vegetable oils, notabley soybean oil, γ-tocopherol is the major vitamer present and in palm oil γ-tocotrienol predominates. Thus measurement of total tocopherols does not accurately

TABLE 5.2

Distribution of Tocopherols and Tocotrienols in Selected Vegetable Oils

Oil	α-T[a]	β-T[a]	γ-T[a]	δ-T[a]	α-T3[a]	β-T3[a]	γ-T3[a]	δ-T3[a]
Corn (maize)	25.69	0.95	75.23	3.25	1.50	—	2.03	—
Olive	11.91	—	1.34	—	—	—	—	—
Palm	6.05	—	tr	—	5.70	0.82	11.34	3.33
Peanut	8.86	0.38	3.50	0.85	—	—	—	—
Rapeseed	18.88	—	48.59	1.20	—	—	—	—
Safflower	44.92	1.20	2.56	0.65	—	—	—	—
Soyabean	9.53	1.31	69.86	23.87	—	—	—	—
Sunflower	62.20	2.26	2.67	—	—	—	—	—

Note: —, Not detected; tr, trace.

[a]Mean values (6–10 determinations) of each oil purchased from 3 to 5 different manufacturers in mg/100/g.

Source: Syväoja, E.-L. et al., *J. Am. Oil Chem. Soc.*, 63, 328, 1986. With permission.

represent the vitamin E biological activity of vegetable oils or food products containing them.

Table 5.3 shows the vitamin E content of a selection of Finnish foods determined using high-performance liquid chromatography (HPLC)

TABLE 5.3

Distribution of Tocopherols and Tocotrienols in Selected Finnish Foods

Item	α-T[a]	β-T[a]	γ-T[a]	δ-T[a]	α-T3[a]	β-T3[a]	γ-T3[a]	δ-T3[a]	Ref.
Wheat flour									[9]
1.2–1.4% ash[b]	1.6	0.8	—	—	0.3	1.7	—	nd	
ca. 0.7% ash[c]	0.4	0.2	—	—	0.2	1.5	—	nd	
ca. 0.5% ash[d]	0.2	0.1	—	—	0.1	1.4	—	nd	
Wheat bran	1.6	0.8	—	—	1.5	5.6	—	nd	[9]
Wheat germ	22.1	8.6	—	<0.1	0.3	1.0	—	nd	[9]
Peanut	10.89	0.27	8.39	0.17	—	nd	—	nd	[10]
Broccoli	0.68	tr	0.14	—	—	nd	—	nd	[10]
Lettuce	0.63	tr	0.34	—	—	nd	—	nd	[10]
Spinach	1.22	—	—	—	—	nd	—	nd	[10]
Tomato	0.66	tr	0.20	tr	—	nd	—	nd	[10]
Sweet pepper	2.16	0.11	0.02	tr	—	nd	—	nd	[10]
Orange	0.36	tr	tr	—	—	nd	—	nd	[10]
Banana	0.21	tr	tr	—	—	nd	—	nd	[10]
Peach (flesh only)	0.96	tr	0.05	—	—	nd	—	nd	[10]
Raspberry	0.88	0.15	1.47	1.19	—	nd	—	nd	[10]
Blackcurrant	2.23	tr	0.83	tr	—	nd	—	nd	[10]
Milk, raw									[11]
summer	0.11	—	—	nd	tr	nd	nd	nd	
winter	0.06	—	—	nd	tr	nd	nd	nd	
Butter									[11]
summer	2.00	—	—	nd	0.07	nd	nd	nd	
winter	1.01	—	—	nd	0.11	nd	nd	nd	
Egg, whole[e]	1.96	0.04	0.08	nd	0.25	nd	nd	nd	[11]
Beef, raw									[12]
spring	0.34	—	0.01	nd	0.05	—	nd	nd	
autumn	0.60	tr	0.01	nd	0.04	tr	nd	nd	
Liver, cow									[12]
spring	0.25	—	0.01	nd	0.01	tr	nd	nd	
autumn	1.37	—	0.01	nd	tr	—	nd	nd	
Pork, raw, shoulder	0.47	—	0.01	nd	0.05	tr	nd	nd	[12]
Chicken, raw	0.70	tr	0.06	nd	0.03	tr	nd	nd	[12]
Cod, raw	1.05	—	—	nd	nd	nd	nd	nd	[13]
Salmon, raw	2.02	—	0.02	nd	nd	nd	nd	nd	[13]

Note: —, Not detected; nd, not determined; tr, trace.
[a]Pooled samples in mg/100 g.
[b]Milled mainly from the aleurone tissue.
[c]Extraction rate ca. 78%.
[d]Extraction rate ca. 74%.
[e]Eggs from hens fed with vitamin supplements containing α-tocopheryl acetate.

with fluorometric detection. Among the cereal grains, wheat, maize, barley, rye, rice, and oats are important plant sources of vitamin E. The vitamin E content of cereal grains is influenced by plant genetics and is adversely affected by too much rain and humidity during harvest [14]. The germ fraction of the cereal grains contains a far higher proportion of tocopherols, and therefore a greater vitamin E activity, than the endosperm and other nongerm fractions in which most of the tocotrienol content of the grain in found [15,16]. Thus flour, which is derived from endosperm, has a low vitamin E activity compared with milling fractions containing germ and aleurone tissue. Wheat germ is the richest source of vitamin E among the various milling products.

Most of the common nontropical vegetables and fruits contain less than 1 mg α-tocopherol equivalents/100 g fresh weight, α-tocopherol being the predominant vitamer present. Green leafy vegetables are included among the richer vegetable sources of vitamin E. The mature dark green outer leaves of brassicae, which are usually discarded, contain more vitamin E than the lighter green leaves which are consumed. The almost colorless heart of white cabbage and the florets of cauliflower contain practically no vitamin E, the determined tocopherol values for these vegetables being attributable to the green parts included [17]. Paradoxically, yellow senescent leaves that have lost their chlorophyll contain much more α-tocopherol than fresh leaves [18]. Presumably α-tocopherol, which resides in the chloroplasts, protects chlorophyll from destruction by the action of oxygen produced by photosynthesis, and is used up during high photosynthetic activity. In apples and pears, the concentration of vitamin E is greater in the skin than in the flesh. Green cooking apples contain more tocopherol than red or yellow types [17].

The concentration of vitamin E in animal tissues depends on the amount of vitamin in the animal's diet. In raw muscle, fat, and organs from mammals and birds, most of the vitamin E is in the form of α-tocopherol. The α-tocopherol content of mammalian muscle is generally less than 1 mg/100 g. There is a marked seasonal variation in the α-tocopherol content of beef and mutton, the values being about twice as high in the autumn as in the spring. Cow liver shows a much greater seasonal variation. The feeding of grass or fresh silage during the summer and dry forage and concentrates during the winter explains the higher autumn values observed in ruminants. During the same season, the tocopherol concentration in different meat cuts of a given animal species increases with increasing fat content. The α-tocopherol content of cow's milk is higher in summer than in winter owing to the changes in the animal's diet. In eggs, all the vitamin E is in the yolk; the concentration varies greatly depending on the level of supplemental α-tocopheryl acetate (if any) contained in the chicken feed. Contrasting values of 0.46 and 1.10 [19], 0.70 [20], and 1.96 [11] mg α-tocopherol/100 g whole egg have been reported.

In general, fish is a better source of vitamin E than meat. Tuna and salmon canned in water contained, respectively, 0.53 [21] and 0.7 [22] mg α-tocopherol/100 g and sardines canned in tomato sauce contained 3.9 mg/100 g [22].

Vitamin E is sometimes added to whole milk powder and breakfast cereals to supplement dietary requirements. The vitamin E requirement increases with an increased intake of PUFA and hence several types of high-quality dietetic margarines are enriched with vitamin E. The acetate ester of α-tocopherol, rather than the free alcohol, is used as a food supplement on account of its greater stability.

5.3.2 Stability

The effects of processing on vitamin E retention have been reviewed in Ref. [23]. During processing, the food is exposed to the destructive influences of oxygen, light, heat, and metal ions. Therefore, refined and processed foods are variable and usually less predictable sources of vitamin E than whole fresh foods. Frozen vegetables retain much of their vitamin E content, but losses in the canning of beans, peas, and sweetcorn can be as high as 70–90% [24]. Frozen foods which have been fried in vegetable oil suffer a great loss of vitamin E during freezer storage. This loss is presumably due to destruction by hydroperoxides, which are more stable at low temperatures than at high temperatures, and hence accumulate [25]. Vitamin E is not destroyed during the normal cooking of meat and vegetables. Little loss of the vitamin occurs during deep-fat frying in fresh vegetable oil, but shallow-pan frying is destructive.

The thermal stabilities of the vitamin E vitamers in food vary with the heating time, heating method, and food composition. The order of vitamer stability in rice bran heated in a microwave for 12 min is δ-T > β-T > γ-T = δ-T3 > α-T3 > γ-T3 > α-T [26]. In frying oils, stabilities depend on the plant source of the oil. The stabilities are: α-T > δ-T > β-T > γ-T (soybean oil), α-T > γ-T > δ-T > γ-T3 (corn oil), and α-T > δ-T3 > α-T3 > γ-T3 (palm oil) [27].

In the production of white wheat flour from whole-grain wheat, the vitamin E content is reduced by about 50% due to the removal of bran and germ [28]. This reduction of vitamin E content is not usually compensated for by fortification. Storage of wholewheat flour at 20°C for 1 yr resulted in the following losses of constituent tocochromanols: α-T, 44%; α-T3, 41%; β-T, 23%; β-T3, 22%. Corresponding losses for stored white flour were: α-T, 42%; α-T3, 42%; β-T, 27%; β-T3, 29% [29].

In the making of French bread by the Chorleywood process, doughmaking was the only stage which resulted in major loss of

vitamin E (30–40%). Preparation of sour dough in the making of wheat/rye bread decreased the vitamin E content of whole rye flour by about 60% [29].

Among various industrial processes for the manufacture of cereal products such as breakfast cereals, drum drying and extrusion cooking of wheat flour result in a 90% loss of vitamin E [28]. Vitamin E destruction during these two processes is due partly to the lipid degradation caused by endogenous enzymes. Polyunsaturated fatty acids originating from the lipase-catalyzed hydrolysis of cereal lipids are readily peroxidized by lipoxygenase, and peroxidizing lipids cause loss of vitamin E due to the vitamin's antioxidant action. Peroxidation begins immediately after water is added to the cereal, as in the first stage of drum drying, and is enhanced in the presence of copper and iron. Nonenzymatic oxidation of vitamin E may also take place when the process temperature has passed the point at which the enzymes are heat-inactivated (about 60°C). Håkansson and Jägerstad [30] reported that the steam flaking of whole-grain wheat inactivated lipoxygenase with no loss of vitamin E. After drum drying, about 50% of the vitamin E of the steam-flaked flour was retained compared with 10–15% in the untreated flour. Microwave treatment was another effective way of inactivating enzymes and improving vitamin E retention.

Shin et al. [31] studied the effect of variations in extrusion temperature (110, 120, 130, and 140°C) and holding time (0, 3, and 6 min) on the concentration of individual of vitamin E vitamers in rice bran. Rice bran oil contains a relatively high proportion of tocotrienols compared with other vegetable oils. Increasing the extrusion temperature from 110 to 140°C with 0-min holding time resulted in a 5–10% loss of total vitamin E. At an extrusion temperature of 110°C, increasing the holding time from 0 to 6 min resulted in a 3–6% loss of total vitamin E. Storage losses of the raw and extruded rice bran were also determined. Raw rice bran lost 44% of total vitamin E after 35 days of storage at ambient temperature, the least stable vitamers being α-tocopherol and α-tocotrienol (ca. 57% loss). After 1 yr of storing raw bran, 73% of the total vitamin E was lost, the least stable vitamers being γ-tocotrienol (78% loss) and α-tocopherol (75% loss). Rice bran extruded at 110°C with 0-min holding time lost 21 and 46% of its initial total vitamin E after 7 and 105 days of storage, respectively, with no difference in degradation rates among vitamers. Lipoxygenase activity probably accounted for the destruction of vitamin E in raw bran. Extrusion temperature, exposure to trace metals, and damage to the grains would account for the vitamin E losses in extruded bran.

Vitamin E is the most radiation-sensitive of the fat-soluble vitamins. The γ-irradiation of rice bran at doses of 5, 10, and 15 kGy caused the following losses: 46, 62, and 74%, respectively, of total tocopherols and 51, 69, and

85%, respectively, of total tocotrienols. The order of loss of individual vitamers in bran irradiated at 5 kGy was α-T3 > α-T > β-T = γ-T3 > δ-T > γ-T > δ-T3 [32]. Irradiation decreases α-tocopherol in meats [33], but as meats are a poor source of vitamin E, this is of little nutritional significance.

Wyatt et al. [34] measured the cooking losses of vitamin E (determined as α- and γ-tocopherols on a dry weight basis) from selected foods commonly consumed in the Mexican diet. Among grains, oats lost 22% of vitamin E, while rice, corn, and wheat lost 42–55%. Among legumes, garbanzo beans (chick peas) and black and pinto beans gave low (9–17%) losses compared with bayo and faba beans, lentils and split peas (38–59%). Legumes giving low cooking losses contained predominantly γ-tocopherol, which is more resistant to cooking temperatures than α-tocopherol. Corn (maize) tortillas are low in vitamin E content due to destruction of the vitamin when the corn is steeped in lime water prior to dough-making.

5.3.3 Expression of Dietary Values

Vitamin E activity is commonly expressed as milligrams of α-tocopherol equivalents using data from rat fetal resorption assays to calculate the equivalencies [3]. However, in 2000, the Institute of Medicine [1] redefined vitamin E in human physiology as solely the 2*R*-stereoisomers of α-tocopherol (Section 5.2.2). In the light of the new findings in humans, it is necessary to reevaluate the relative biological potencies of the vitamin E vitamers. Therefore, it is best to measure and report the actual concentrations of each vitamer in food.

5.3.4 Applicability of Analytical Techniques

HPLC is ideally suited for the measurement of the individual tocopherols and tocotrienols. For the analysis of those animal products known to contain predominantly α-tocopherol, only this vitamer need be determined. In vitamin E-fortified foods, it is usually sufficient to determine either the added α-tocopheryl acetate or the total α-tocopherol (natural plus added vitamin).

5.4 Intestinal Absorption and Transport

The following discussion of absorption and transport is taken from a more detailed account in a book by Ball [35] published in 2004.

5.4.1 Absorption

Before absorption, any ingested supplemental α-tocopheryl acetate or succinate is hydrolyzed in the lumen of the small intestine by pancreatic esterase, which requires bile salts as cofactors. The free α-tocopherol thus formed, together with vitamin E of natural origin, is solubilized within mixed micelles for passage across the unstirred water layer of the intestinal lumen. The micelles dissociate and the liberated vitamin E is absorbed by simple diffusion. Studies in thoracic duct-cannulated rats have shown no significance in the rate of absorption of α- and γ-tocopherol; even a 50-fold excess of α-tocopherol did not affect the absorption of γ-tocopherol [36]. Within the enterocyte, vitamin E is incorporated into chylomicrons and the chylomicrons are secreted into the bloodstream via the lymphatic route. The appearance of vitamin E in chylomicrons is delayed by 0.5 h compared with that of triglycerides, suggesting that the transport of α-tocopherol in the enterocyte is less efficient than that of lipids [37].

5.4.2 Plasma Transport and Distribution

On entering the bloodstream, the chylomicrons are attacked by lipoprotein lipase located on the capillary walls of most tissues. Hydrolysis of the core triglycerides results in the formation of chylomicron remnants. During chylomicron lipolysis and erosion of the triglyceride core, the excess surface components, including some vitamin E, are transferred to circulating high density lipoproteins (HDLs) [38]. HDLs can readily exchange surface components with other types of lipoprotein [39], this interchange being accelerated by the phospholipid transfer protein [40]. Thus the various tocochromanols that were consumed are distributed to all of the circulating lipoproteins.

5.4.3 Preferential Secretion of 2*R*-α-Tocopherol Stereoisomers by the Liver

The chylomicron remnants, containing the major proportion of absorbed vitamin E, are taken up by the liver parenchymal cells by receptor-mediated endocytosis. The liver then secretes into the plasma the newly absorbed vitamin E within very low density lipoproteins (VLDLs). α-Tocopherol is secreted in preference to γ-tocopherol, the latter being preferentially excreted in the bile [41]. This discrimination explains why α-tocopherol is the predominant circulating form of vitamin E, despite γ-tocopherol being the predominant dietary form. The liver further discriminates between stereoisomers of α-tocopherol, preferentially

secreting *RRR*-α-tocopherol and probably the other 2*R*-α-tocopherol stereoisomers [42]. This discriminatory ability has been attributed to a hepatic tocopherol-binding protein known as the α-tocopherol transfer protein that is present exclusively in hepatocytes [43] and has been identified in human liver [44]. The protein seems to function by stimulating the secretion of cytosolic α-tocopherol into the sinusoidal space, where it becomes associated with nascent VLDL [45].

Catabolism of VLDL in plasma results in the enrichment with 2*R*-α-tocopherol stereoisomers of other circulating lipoproteins and hence, eventually, the tissues [46,47]. The α-tocopherol transfer protein salvages α-tocopherol that is returned to the liver and promotes its resecretion in VLDL [48]. Thus α-tocopherol delivered to the liver is recycled rather than excreted in the bile. The ability of the transfer protein to select 2*R*-stereoisomers of α-tocopherol ensures that the most effective form of vitamin E reaches the tissues. Ultimately, all four 2*R* stereoisomers will be preferentially retained and all four 2*S* stereoisomers will be preferentially eliminated from the body. This means that natural vitamin E (*RRR*-α-tocopherol) should, in theory, have twice the potency of synthetic vitamin E (*all-rac*-α-tocopherol). This 2:1 ratio has been demonstrated in humans using stable isotopes [2].

5.4.4 Storage

More than 90% of the human body pool of α-tocopherol accumulates in fat droplets within adipose tissue, but the vitamin is not readily mobilized from this tissue [49].

5.5 Bioavailability

5.5.1 Efficiency of Vitamin E Absorption

The few reported studies in normal human subjects have shown that the absorption of vitamin E is incomplete. Estimates of the absorption of physiological oral doses of tritiated *all-rac*-α-tocopherol based on the measurement of unabsorbed fecal radioactivity gave efficiencies ranging from 51 to 86% (mean 72%) [50] and 55 to 79% (mean 69%) [51]. Pharmacological doses of vitamin E are absorbed with progressively less efficiency as the dose is increased until, eventually, a limit is reached. Even if doses exceed the daily requirement 100-fold, the plasma concentrations do not increase beyond 2–4-fold [47]. There is no compensatory increase in absorption in the presence of vitamin E deficiency [52].

Traber et al. [53] investigated in healthy human subjects the mechanisms by which plasma α-tocopherol concentrations are limited in response to supplemental vitamin E at doses up to 150 mg of deuterated *RRR*-α-tocopherol acetate. Incorporation of labeled *RRR*-α-tocopherol into chylomicrons and subsequent secretion into plasma increased linearly with increasing dosage. However, plasma total α-tocopherol did not similarly increase. This indicated that the newly absorbed vitamin E replaces the α-tocopherol in circulating lipoproteins. This phenomenon can be explained by the role of the hepatic α-tocopherol transfer protein in maintaining homeostasis of plasma *RRR*-α-tocopherol concentration [54]. The limitation on plasma vitamin E concentration presumably occurs because the ability of the liver to secrete *RRR*-α-tocopherol in VLDL is limited by the restricted number of transfer protein molecules present. At supplemental dose levels, the system becomes saturated. Biliary excretion prevents accumulation of vitamin E in the liver.

5.5.2 Effects of Polyunsaturated Fats on Vitamin E Absorption

It has been clearly demonstrated that a diet rich in PUFA depresses the absorption of vitamin E [55]. Various mechanisms to explain this phenomenon have been proposed [56]. For example, Hollander [57] suggested that PUFAs enhance the solubility of vitamin E in micelles, thereby shifting the partitioning of the vitamin between mixed micelles and the mucosal brush-border membrane in favor of the micelles, which would impair intestinal uptake.

5.5.3 Effects of Dietary Fiber on Vitamin E Absorption

The feeding of pectin to rats at levels comprising 6 and 8% of the total diet for 8 weeks reduced the bioavailability of vitamin E based on decreases in plasma and liver tocopherol as well as on increased erythrocyte hemolysis [58]. The results were consistent with the working hypothesis that pectin binds bile acids and makes them unavailable for micelle formation, thus decreasing the absorption of vitamin E. A previous report [59] showed that pectin does not bind to α-tocopherol *in vitro*. It is unlikely that a high-fiber human diet would ever contain more than 3% pectin, therefore a typical human diet would not be expected to adversely affect vitamin E status.

A diet containing a high level of wheat bran also influences vitamin E bioavailability in rats (the fiber content of wheat bran is ca. 50%). Feeding a diet containing 20% wheat bran for 36 days resulted in a 28% decrease in plasma tocopherol concentration compared with a diet containing 5% wheat bran [60]. A similar decrease was observed between

35 and 42 days in a subsequent study [61], but after 56 days on the 20% wheat bran diet the plasma tocopherol concentrations had returned to normal. The authors concluded that the high-fiber diet induced structural and functional changes of the intestinal tract, which led to a temporary reduction of vitamin E bioavailability. The reversal of this effect after 56 days was presumably due to the ability of the intestinal tract to adapt to such changes. Kahlon et al. [62] reported that coarse wheat bran significantly lowered ($P < 0.05$) the liver α-tocopherol in rats as compared to fine bran or high-cellulose fiber diets at the same dietary fiber level. This apparent decrease in α-tocopherol bioavailability would not have been caused by adsorption of vitamin E by bran. If adsorption was the mechanism responsible, the fine bran, with nearly twice the surface area of coarse bran, would have had the greater effect on lowering liver α-tocopherol. The effect of coarse bran could have been due to morphological and physiological alterations of the intestinal mucosa, which impaired absorption of vitamin E.

5.5.4 Effect of Plant Sterols on Vitamin E Bioavailability

In a well-designed clinical trial in normocholesterolemic men, dietary supplementation with plant sterols for 1 week lowered the absorption of cholesterol by 60%; however, it but also reduced the bioavailability of α-tocopherol by ca. 20%. The effect on vitamin E was greater with sterol esters than with free sterols at the same equivalent doses [63].

5.6 Vitamin E Requirements

Is has been established in animals and in humans that an increase in the intake of unsaturated fat accelerates the depletion and increases the requirement for vitamin E [64]. This is because dietary PUFAs are concentrated in cellular and subcellular membranes, where they have the capacity to sequester corresponding amounts of vitamin E to maintain their oxidative stability. High PUFA intakes should be accompanied by correspondingly high intakes of vitamin E. In general, the total vitamin E content of animal and vegetable oils parallels their PUFA content [65].

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6

Vitamin K

6.1 Background

Vitamin K is obtained from plant foods in the form of phylloquinone and from bacterial sources as a series of menaquinones with side chains of different length. The vitamin is essential for the activation of specific proteins involved in blood clotting (e.g., prothrombin) and bone mineralization (e.g., osteocalcin) through its role as a cofactor for γ-glutamylcarboxylase. This enzyme catalyzes a unique posttranslational conversion of selected glutamate residues in the proteins to γ-carboxyglutamate residues, allowing the proteins to bind calcium and thus become activated.

In adult humans, a prolonged blood-clotting time is the predominant, if not sole, clinical sign of vitamin K deficiency. Aortic calcification is also associated with a reduced vitamin K status. Deficiency is more likely to arise from secondary causes such as malabsorption syndromes or biliary obstruction than from a dietary inadequacy of vitamin K. Breast-fed newborn infants, especially if premature, are susceptible to an hemorrhagic syndrome because (i) the placenta imposes a barrier to the transport of vitamin K to the fetus, (ii) there is inadequate colonization of intestinal microflora during the first few days of life, and (iii) human milk provides inadequate vitamin K. Newborn infants in many countries now routinely receive intramuscular or (less effectively) oral doses of vitamin K as a prophylactic measure against the syndrome.

There is evidence for a role of phylloquinone as a protective dietary factor against hip fracture in the elderly. It is assumed that the beneficial effects of phylloquinone on bone are mediated through the carboxylation of γ-carboxyglutamic acid residues in vitamin K-dependent proteins in bone, including osteocalcin, matrix γ-carboxyglutamic acid protein, and protein S. A Japanese group demonstrated that menaquinone-4, but not phylloquinone, inhibits bone resorption by targeting osteoclasts to undergo programmed cell death. Menaquinone-4 may have different effects on bone metabolism than phylloquinone by virtue of its geranylgeranyl side chain. A number of Japanese studies have claimed beneficial results using synthetic menaquinone-4 (menatetranone) in the treatment of osteoporosis.

6.2 Chemical Structure, Biopotency, and Physicochemical Properties

6.2.1 Structure and Biopotency

Vitamin K is a generic term used for all compounds possessing cofactor activity for γ-glutamylcarboxylase. Two forms of vitamin K exist in nature. Phylloquinone (vitamin K_1) is a specific vitamer synthesized by green plants in the chloroplasts. Menaquinones (vitamin K_2) are a series of structural analogs, which are synthesized by bacteria. The chemical stuctures of these compounds are shown in Figure 6.1.

Phylloquinone ($C_{31}H_{46}O_{21}$, MW = 450.7) comprises a methyl-substituted naphthoquinone ring structure attached at C-3 to a phytyl side chain containing 20 carbon atoms, and may be designated $K_{1(20)}$ to distinguish it from synthetic structural analogs such as $K_{1(25)}$. Naturally occurring phylloquinone has the C-2′ double bond in the *trans* configuration. Synthetic phylloquinone usually contains both the *trans* and *cis* isomers, but only the *trans* isomer is essentially responsible for the vitamin's biological activity. The predominant members of the menaquinone family have polyisoprene side chains comprised of 6–10 unsaturated isoprene units and are designated menaquinone-6 (MK-6) to menaquinone-10 (MK-10) accordingly. Practically nothing is known about the relative biopotency of the menaquinones.

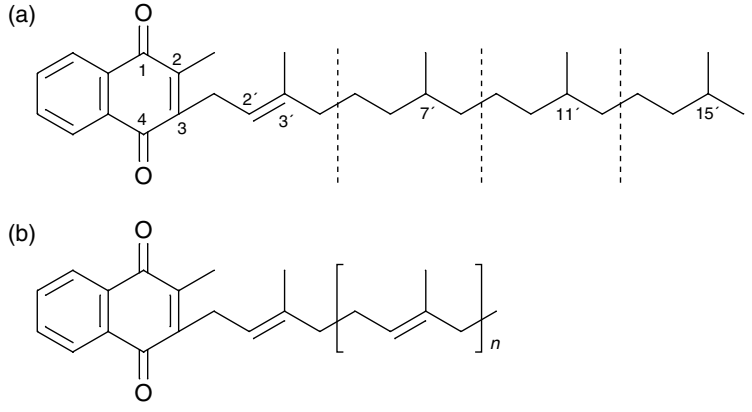

FIGURE 6.1
Structures of the vitamin K-active compounds: (a) phylloquinone (vitamin K_1) and (b) menaquinones (vitamin K_2). The vertical dotted lines in (a) delineate the four isoprene units.

6.2.2 Physicochemical Properties

6.2.2.1 Appearance and Solubility

Phylloquinone is a golden-yellow viscous oil; the various menaquinones can be obtained in crystalline form; menadione is a yellow crystalline powder.

Phylloquinone is insoluble in water; sparingly soluble in ethanol; and readily soluble in acetone, diethyl ether, chloroform, hexane, fats, and oils.

6.2.2.2 Stability in Nonaqueous Solution

Vitamin K compounds are decomposed by UV radiation, alkali, strong acids, and reducing agents, but are reasonably stable to oxidizing conditions and heat.

6.3 Vitamin K in Foods

6.3.1 Occurrence

Supplementation of foods with vitamin K is uncommon, except for infant formula feeds to which synthetic preparations of phylloquinone are invariably added.

Booth et al. [1] tabulated the phylloquinone content of 261 foods using high-performance liquid chromatography (HPLC) as the method of analysis [2]. Representative data from this table are presented in Table 6.1. The foods selected for analysis were those from the Food and Drug Administration's (FDA) Total Diet Study, which lists core foods in the American food supply on the basis of data from the U.S. Department of Agriculture's 1987–1988 Nationwide Food Consumption Survey [3]. Bolton-Smith et al. [4] compiled a U.K. database for the phylloquinone content of foods.

The highest concentrations of vitamin K (in the form of phylloquinone) are found in green leafy vegetables, with values for cabbage, broccoli, Brussels sprouts, spinach, and collards ranging between 98 and 440 μg of phylloquinone per 100 g of vegetable. Such vegetables are the top contributors to vitamin K intake in the American diet [5]. Phylloquinone contents of green leafy vegetables (but not cabbage) increase during plant maturation. Cabbage contains 3–6 times more phylloquinone in the outer leaves compared with the inner leaves. The phylloquinone content of a given vegetable differs according to the geographical growth location, suggesting that climate, soil, and growing conditions may be influencing factors [6]. Other types of vegetable (roots, bulbs, and tubers), cereal grains and their milled products, fruits and fruit

TABLE 6.1

Phylloquinone Content of Selected Foods

Item	Micrograms Phylloquinone per 100 g Mean (SD)	Average Serving size (g)	Micrograms Phylloquinone per serving
Whole milk, fluid	0.3 (0.02)	244	0.7
Cheddar cheese	2.1 (0.2)	28	0.6
Eggs, boiled	0.3 (0.05)	50	0.2
Beef steak, loin, pan-cooked	1.8 (0.2)	85	1.5
Pork chop, pan-cooked	3.1 (0.07)	85	2.6
Pork sausage, pan-cooked	3.4 (1.4)	56	1.9
Liver, beef, fried	2.7 (0.1)	85	2.3
Chicken breast, roasted	<0.01 (<0.01)	85	<0.01
Haddock, pan-cooked	5.2 (0.1)	85	4.4
Tuna, canned in oil, drained	24 (1.2)	56	14
Kidney beans, dry, boiled	8.4 (0.9)	88	7.4
Pinto beans, dry, boiled	3.7 (0.20)	86	3.2
Peanut butter, smooth	0.3 (0.02)	32	0.1
White bread	1.9 (0.1)	50	1.0
Whole wheat bread	3.4 (0.05)	57	1.9
Orange, raw	<0.01 (<0.01)	154	<0.01
Apple, red, raw	1.8 (0.09)	154	2.8
Banana, raw	0.2 (0.02)	126	0.3
White potato			
baked with skin	1.1 (0.1)	140	1.5
boiled without skin	0.3 (0.02)	136	0.4
French fries, fast food	4.4 (0.2)	68	3.0
Spinach, fresh or frozen, boiled	360 (70)	90	324
Broccoli, fresh or frozen, boiled	113 (2.5)	78	88
Carrot, fresh, boiled	15 (0.5)	78	12
Tomato sauce, plain, bottled	2.9 (0.4)	61	1.8
Green beans, fresh or frozen, boiled	16 (6.6)	62	9.7
Brussels sprouts, fresh or frozen, boiled	289 (55)	78	225
Celery, raw	32 (4.0)	55	17
Iceberg lettuce, raw	31 (8.6)	89	28
Mayonnaise, regular, bottled	41 (1.2)	14	5.8
French salad dressing	51 (5.7)	29	15
Margarine, stick, regular	33 (4.3)	14	4.6

Source: From Booth, S.L., Sadowski, J.A., and Pennington, J.A.T., *J. Agric. Food Chem.*, 43, 1574, 1995. With permission.

juices are poor sources of vitamin K. Animal products (meat, fish, milk products, and eggs) contain low concentrations of phylloquinone, but appreciable amounts of menaquinones are present in liver. The *cis* isomer of phylloquinone contributes up to about 15% of total phylloquinone in certain foods [7].

Some vegetable oils, including canola (rapeseed), soybean, and olive oils, are rich sources of phylloquinone, whereas peanut oil and corn (maize) oil are not (Table 6.2) [8]. Soybean oil is the most commonly consumed vegetable oil in the American diet. The addition of phylloquinone-rich vegetable oils in the processing and cooking of foods that are otherwise poor sources of vitamin K makes them potentially important dietary sources of the vitamin. This is particularly evident, for example, when chicken, fish, eggs, and potatoes are fried in certain vegetable oils.

Those margarines, mayonnaises and regular-calorie salad dressings that are derived from phylloquinone-rich vegetable oils are second to green leafy vegetables in their phylloquinone content. The addition of these fats and oils to mixed dishes and desserts has an important impact on the amount of vitamin K in the American diet [5].

Livers of ruminant species contain significant concentrations (10–20 μg/100 g) of some menaquinones [9], while cheese contains significant quantities of MK-8 (5–10 μg/100 g) and MK-9 (10–20 μg/100 g) [10].

6.3.2 Stability

Stability studies on the phylloquinone content of vegetable oils demonstrated that the vitamin is significantly affected by heat [8]. When subjected to temperatures of 185–190°C for 40 min, 7% of the original phylloquinone was lost during 20 min of heating and 11% during 40 min of heating. Phylloquinone is sensitive to both fluorescent light and sunlight. After only 2 days of exposure, fluorescent light decreased the phylloquinone content of rapeseed and safflower oils by 46 and 59%, respectively, and daylight by 87 and 94%, respectively. Amber

TABLE 6.2

Phylloquinone Content in Various Vegetable Oils

Type	Number of Brands Analyzed	Phylloquinone (μg/100 g of oil)[a]
Peanut	3	0.65 ± 0.27
Corn (maize)	2	2.91 ± 1.28
Sunflower	2	9.03 ± 0.17
Safflower	2	9.13 ± 2.64
Olive	6	55.5 ± 6.3
Canola (rapeseed)	4	141 ± 17.0
Soybean	5	193 ± 28

[a]Average value (mean ± SEM). Each brand was analyzed in triplicate.
Source: From Ferland, G. and Sadowski, J.A., *J. Agric. Food Chem.*, 40, 1869, 1992. With permission.

glass bottles were effective in protecting the phylloquinone in oils from the destructive effects of light.

Richardson et al. [11] compared the relative vitamin K activity of raw vegetables which had been preserved by freezing, heat processing (canning), and γ-irradiation. The frozen foods were stored at −20°C, while the heat processed and irradiated foods were stored at ambient temperature (24–27°C). Vitamin K activity of the foods was determined by prothrombin times of chick plasma. The results indicated that there was no appreciable loss of vitamin K activity in the foods preserved by any of the methods examined or when stored for 15 months.

Langenberg et al. [12] used an HPLC method to demonstrate that neither cooking nor γ-irradiation of vegetables lowered the phylloquinone content. The phylloquinone content of commercially available vegetable products in cans or glass containers, or dried or deep-frozen, did not differ significantly from the content of fresh vegetables.

6.3.2.1 Effects of Hydrogenation

Hydrogenation is a common process used by the food industry to increase the oxidative stability of polyunsaturated oils and to convert liquid oils into semi-solid fats, thereby increasing their commercial applications. During the hydrogenation of vegetable oils, a percentage of the phylloquinone present is converted to 2′,3′-dihydrophylloquinone, owing to saturation of the 2′,3′ double bond of the phytyl side chain [13]. Dihydrophylloquinone has been identified in human plasma following its ingestion in the form of margarine [14], indicating that it is absorbed. Of the 261 foods analyzed in the Food and Drug Administration's Total Diet Study, 36 foods contain dihydrophylloquinone in concentrations greater than 1.0 μg/100 g of food [15]. All these 36 foods contain hydrogenated fats or oils, with the highest concentrations of dihydrophylloquinone being found in assorted cookies and stick margarine. However, when expressed in micrograms per average serving size, the richest sources of the hydrogenated vitamin are commercial apple pies (27 μg) followed by fast-food french-fries (25 μg). In a metabolic study with human participants, dihydrophylloquinone was not as well absorbed as phylloquinone and was less effective in carboxylating glutamic acid residues in the hepatic vitamin K-dependent protein, prothrombin. In contrast to phylloquinone, dihydrophylloquinone had no measurable effect on biochemical markers of bone turnover [16].

6.3.3 Applicability of Analytical Techniques

Reliable methods of determining vitamin K in foods only became available in the 1980s with the development of specific HPLC techniques.

Among the various vitamin K vitamers, only phylloquinone is accounted for in routine food analysis. Milk-based and soy protein-based infant formulas for the full-term infant are supplemented with synthetic preparations of phylloquinone, which invariably contain about 10% of the biologically inactive *cis* isomer [17]. The analytical method for infant formulas must therefore exclude *cis*-phylloquinone in the measurement. HPLC techniques using gradient elution are capable of separating the individual menaquinones from one another and from phylloquinone.

6.4 Intestinal Absorption and Transport

The following discussion of absorption and transport is taken from a more detailed account in a book by Ball [18] published in 2004.

6.4.1 Absorption and Transport of Dietary Vitamin K

Phylloquinone, the major form of vitamin K in the diet, is absorbed in the jejunum, and less efficiently in the ileum, in a process that is dependent on the normal flow of bile and pancreatic juice [19]. Both long- and short-chain menaquinones are readily absorbed by rats after oral ingestion [20] and therefore dietary menaquinones are likely to be incorporated into mixed micelles through the action of bile salts and absorbed along with phylloquinone.

Absorbed phylloquinone enters the systemic circulation chemically unchanged via the lymphatic route in association with chylomicrons. Following lipolysis of the chylomicrons in the bloodstream, the chylomicron remnants containing the phylloquinone are taken up the liver, where the vitamin can be utilized for the synthesis of clotting factors. Bone marrow may also take up chylomicron remnants. Three hours after a meal, triglyceride-rich lipoproteins account for 91% of plasma phylloquinone. The proportion of plasma phylloquinone carried by low density lipoproteins and high density lipoproteins during the postprandial phase increases progressively from 3 and 4% at 3 h to 14 and 11% at 12 h, respectively [21].

6.4.2 Bacterially Synthesized Menaquinones as a Possible Endogenous Source of Vitamin K

The large intestine of healthy adult humans contains a microflora of bacteria, many species of which synthesize menaquinones ranging mainly from MK-6 to MK-12. Conly and Stein [22] reported quantitative

and qualitative measurements of phylloquinone and menaquinones at different sites within the human intestinal tract. Overall, the long-chain menaquinones (MK-9, -10, and -11) predominated. Menaquinones were found mostly in the distal colon (ten fecal samples) and totalled 19.85 ± 0.36 μg/g dry weight. Menaquinones in two samples of terminal ileal contents taken during appendectomy totaled 8.85 μg/g dry weight. Little menaquinone was found in samples of proximal jejunal contents collected by means of a nasojejunal tube (total, 0.03 μg/g dry weight).

The menaquinones incorporated into membranes of viable bacteria are not available for absorption. However, Conly and Stein [23] described *in vitro* experiments showing that significant amounts of biologically active menaquinones can be secreted or liberated from bacteria. Being strongly lipophilic, the bacterially synthesized menaquinones require the presence of bile salts and the formation of mixed micelles for absorption to take place. Because bile salts are reabsorbed in the distal ileum and the amounts remaining are degraded by colonic bacteria, there is no opportunity for absorption of menaquinones to take place in the colon. Indeed, colonic absorption of MK-9 in rats is extremely poor [20,24]. However, bearing in mind the appreciable amounts of menaquinones found in the terminal ileum of two subjects [22], and considering the possibility that contents from the cecum (where large amounts of bacteria reside) may backwash past the ileocecal valve into the ileum, one can envisage some degree of bile salt-mediated absorption taking place in this region. In addition, Hollander et al. [25] demonstrated ileal absorption of MK-9 in the conscious rat and showed that the absorption rate increased with increasing bile salt concentration.

Direct evidence to support absorption of menaquinones from the distal human intestinal tract, where intestinal microflora are most prevalent, is lacking. Indirect evidence that enteric menaquinones are absorbed is the fact that 90% of liver stores of vitamin K is in the form of menaquinones [26], despite phylloquinone predominating in the diet. Moreover, the various menaquinones found in liver are remarkably consistent with the menaquinone profile of human intestinal content. However, it has not been possible to prove that the hepatic menaquinones do not originate from the diet. Studies performed on human volunteers placed on a vitamin K-deficient diet have consistently failed to demonstrate any significant changes in prothrombin times. However, bleeding episodes associated with a prolonged prothrombin time have been reported in vitamin K-deprived volunteers receiving broad-spectrum antibiotics [27]. The data from the latter study did not support the hypothesis discussed by Lipsky [28] that *N*-methylthiotetrazole-containing antibiotics suppress vitamin K-dependent clotting factor biosynthesis. Collectively,

these data imply that enteric menaquinones are absorbed and utilized to some extent.

Conly et al. [29] demonstrated that menaquinones can be absorbed directly from the human ileum and be functionally active. Their study consisted of an experimental phase followed by a control phase, using the same four volunteers. The volunteers were started on a vitamin K-deficient diet and then given adjusted doses of warfarin to maintain a stable elevated prothrombin time. A 1.5-mg dose of mixed menaquinones (MK-4–MK-9) extracted from harvested *Staphylococcus aureus* was then placed directly into the ileum by means of a nasoileal tube after an overnight fast. Within 24 h of menaquinone administration, the prothrombin time decreased significantly and the factor VII level increased significantly, indicating that the menaquinones had been absorbed and utilized. The results of this study provide an explanation as to why starvation or a complete lack of dietary intake of vitamin K alone cannot induce a clinically manifest vitamin K deficiency.

In conclusion, a report by Ferland et al. [30] that subclinical vitamin K deficiency can be induced in healthy adults by dietary deprivation of the vitamin suggests that absorption of bacterially synthesized menaquinones may not be sufficient to sustain adequate vitamin K status.

6.5 Bioavailability

The efficiency of vitamin K absorption varies widely, depending on the source of the vitamin and the amount of fat in the meal. Pure phylloquinone is absorbed with an efficiency of 80% [19]. The phylloquinone present in cooked spinach was only 4% as bioavailable as that from a commercial detergent suspension of phylloquinone. Adding butter to the spinach increased this to 13% [31]. The absorption of phylloquinone was six times higher after the ingestion of a 500-μg phylloquinone tablet than after the ingestion of 150 g of raw spinach containing 495 μg phylloquinone [32]. The phylloquinone from a phylloquinone-fortified oil was absorbed better than that from an equivalent amount from cooked broccoli, regardless of adjustment to triglyceride concentrations [33]. The tight binding of phylloquinone to the thylakoid membranes of chloroplasts explains the poor bioavailability of the vitamin in green plants. The free phylloquinone in vegetable oils, margarines, and dairy products is well absorbed, owing to the stimulating effect of fat.

As mentioned in Section 6.3.2, the bioavailability of dihydrophylloquinone (produced by the commercial hydrogenation of vegetable oils) is lower than that of phylloquinone.

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7

Thiamin (Vitamin B_1)

7.1 Background

Thiamin, as thiamin diphosphate (also known as thiamin pyrophosphate), serves as a coenzyme for enzymes involved in carbohydrate and amino acid metabolism. There is, therefore, an increased requirement for thiamin in situations where metabolism is heightened, for example, during high muscular activity, pregnancy, and lactation, and also during protracted fever and hyperthyroidism.

The classic disease resulting from a gross deficiency of thiamin in humans is beriberi, which manifests as a complicated picture of neurological and cardiovascular disorders. Beriberi is prevalent in Far Eastern populations where unfortified polished rice is the staple diet. In chronic alcoholics, thiamin deficiency resulting from poor nutrition can lead to Wernicke–Korsakoff's encephalopathy, which is characterized by mental confusion and deterioration of nerve function. In addition, decreased liver function in alcoholics impairs the utilization of thiamin.

Thiamin is practically nontoxic when administered orally owing to its limited absorption and rapid excretion of excess amounts.

7.2 Chemical Structure, Biopotency, and Physicochemical Properties

7.2.1 Structure and Potency

The thiamin molecule (Figure 7.1) comprises substituted pyrimidine and thiazole moieties linked by a methylene bridge. It is a quaternary amine and exists as a monovalent or divalent cation, depending on the pH of the solution. Three phosphorylated forms of thiamin occur in nature. In living tissues, the predominant form is the coenzyme, thiamin diphosphate (TDP) (Figure 7.1). Small amounts of the monophosphate (TMP) and triphosphate (TTP) esters also occur in animal tissues. TMP and TTP have no known coenzyme function, but the latter has a role (as yet

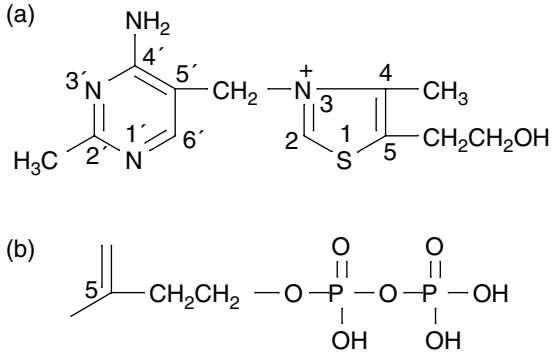

FIGURE 7.1
Structures of (a) thiamin and (b) TDP.

not understood) in nerve transmission. Thiamin is the preferred generic term for this vitamin, formerly designated as vitamin B_1.

For food supplementation purposes, thiamin is chiefly used in the form of its chloride hydrochloride double salt (known commercially as thiamin hydrochloride) $C_{12}H_{17}N_4OSCl \cdot HCl$, MW = 337.3. The hydrochloride salt is hygroscopic and usually exists as the hemihydrate containing the equivalent of about 4% water. Another commercial form of the vitamin, thiamin mononitrate, is practically nonhygroscopic and is especially recommended for the enrichment of flour mixes.

7.2.2 Physicochemical Properties

7.2.2.1 Appearance and Solubility

Thiamin hydrochloride is a colorless, crystalline substance with a yeast-like odor and a salty nut-like taste. Its mp is about 207°C, with decomposition. It is readily soluble in water (100 g/100 ml), sparingly soluble in 95% ethanol (1 g/100 ml) and absolute ethanol (0.3 g/100 ml), and insoluble in fat solvents. The pH of a 1% solution of thiamin hydrochloride in water is 3.13 and that of a 0.1% solution is 3.58.

7.2.2.2 Stability in Aqueous Solution

Thiamin itself is stable at acid pH, but as the pH of the medium is increased it becomes increasingly susceptible to deactivation by heat and chemical reaction. Mild oxidation produces thiamin disulfide without loss of thiamin activity; more vigorous oxidation with alkaline potassium hexacyanoferrate(III) [potassium ferricyanide, $K_3Fe(CN)_6$] produces the biologically inactive thiochrome (Figure 7.2). Thiamin phosphate esters are oxidized to thiochrome compounds in a similar

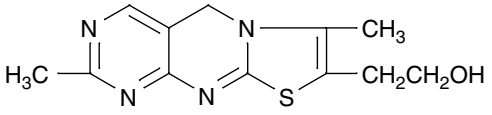

FIGURE 7.2
Structure of thiochrome.

manner without splitting of the phosphate bonds. The thiamin molecule is cleaved by sulfite ions, even at room temperature, yielding 2-methyl-5-sulfomethylpyrimidine and the thiazole moiety [1]. This reaction is instantaneous at pH 6 and is complete within hours at pH 5, but at pH 3, thiamin may be kept in solution with sulfite for several months without serious loss of potency [2]. Solutions of thiamin are degraded by UV irradiation [3].

7.3 Thiamin in Foods

7.3.1 Occurrence

All plant and animal tissues contain thiamin, hence it is present in all natural unprocessed foods. The thiamin content of a selection of foods is presented in Table 7.1 [4]. Rich sources of thiamin include yeast and yeast extract, wheat bran, oatmeal, whole-grain cereals, pulses, nuts, lean pork, heart, kidney, and liver. Beef, lamb, chicken, eggs, vegetables, and fruits contain intermediate amounts, while milk contains a relatively low amount.

In cereal grains, the thiamin is unevenly distributed, being relatively low in the starchy endosperm and high in the germ. The milling of cereals removes most of the thiamin, so white flour, ready-to-eat breakfast cereals and, in certain countries, polished rice are enriched by addition of the vitamin. Other frequently enriched foodstuffs are breads, macaroni, spaghetti, and milk modifiers (chocolate, malt, etc.).

In most animal tissues, over 90% of the thiamin present is phosphorylated, with TDP predominating. Exceptions are pig skeletal muscle and chicken white skeletal muscle, in which TTP comprises 70–80% of the total thiamin present [5]. Bovine milk contains mainly free, nonphosphorylated thiamin [6]. The natural thiamin content of most cereals and cereal products, including white flour made from wheat, is present almost entirely in the form of nonphosphorylated thiamin; exceptions are wheat germ and bran, which contain TDP [7,8]. There is evidence that the absence of phosphorylated thiamin in plant material is caused by phosphatase activity when the material is extracted for assay [9].

TABLE 7.1

Thiamin Content of Various Foods

Food	mg Thiamin/100 g Edible Portion
Cow milk, whole, pasteurized	0.03
Egg, chicken, whole, raw	0.09
Wheat flour, wholemeal	0.47[a]
Wheat flour, white, plain	0.31[a]
Rice, brown, raw	0.59
Beef, trimmed lean, raw, average	0.10
Lamb, trimmed lean, raw, average	0.09
Pork, trimmed lean, raw, average	0.98
Chicken meat, raw	0.14
Liver, lamb, fried	0.38
Cod, raw, fillets	0.04
Potato, main crop, old, average, raw	0.21
Chick peas, dried, raw	0.39
Soya beans, dried, raw	0.61
Red kidney beans, dried, raw	0.65
Peas, raw	0.74
Broccoli, raw	0.10
Apples, eating, average, raw	0.03
Orange juice, unsweetened	0.08
Peanuts, plain	1.14
Yeast extract	4.10

[a]This level is for fortified flour.
Source: From Food Standards Agency, *McCance and Widdowson's The Composition of Foods*, 6th summary ed., Royal Society of Chemistry, Cambridge, 2002. With permission.

White bread contains TDP due to the phosphorylation of thiamin added for enrichment during the yeast fermentation of the dough as well as the contribution from the yeast itself [10].

7.3.2 Stability

A range of conditions, such as pH, temperature, and moisture content, promote loss of thiamin in foods. Thiamin is the most heat-labile of the B vitamins. The thermal destruction of thiamin during the processing and subsequent storage of foods has been thoroughly discussed by Farrer [11]. Thiamin loss follows a first-order reaction, and temperature dependence of the rate constants is similar among different food and model systems, facilitating the prediction of thiamin retention in processed or stored foods that have received known heat treatments [12]. Proteins are known to protect thiamin [3] and thiamin in a food matrix is more resistant to thermal breakdown than is the pure vitamin in aqueous or buffered solutions [13]. Frozen foods and dehydrated foods generally incur little loss of thiamin during storage.

An alkaline environment during processing or cooking will promote loss of thiamin; for example, the use of baking powder in cake making can destroy over 50% of the original thiamin content of the flour [14]. In the baking of bread with yeasts, the medium is slightly acidic and the loss is reduced to 15–25%, mostly in the crust [14]. Sulfites are used commercially to preserve the color of various food products, such as orange juice, dehydrated fruits and vegetables, and canned green beans and peas. As an oxygen scavenger, the sulfite ion protects ascorbic acid and β-carotene from oxidation, but as a nucleophile, it is destructive to thiamin. Because sulfites destroy thiamin, their use is not permitted in meats or in foods recognized as important sources of the vitamin. The effects of chemical interactions on thiamin stability in complex food systems have been reviewed by Clydesdale et al. [15].

In a detailed study of the commercial canning of vegetables [16], the retention of thiamin during the blanching of asparagus, green beans, lima beans, and peas ranged from 64 to 100% and during the sterilization process, from 58 to 79%. The overall retention for nine products studied, based on the total contents of the can, ranged from 31 to 89% with an average of 57%. The liquid portion of canned vegetables contained between 27 and 45% of the total thiamin in the can. The thiamin content of canned vegetables decreased upon storage. Greenwood et al. [17] studied the effects of processing time and temperature on the retention of thiamin in canned pork luncheon meat. The rate of thiamin destruction was doubled with a 10°C increase in temperature in contrast to a tenfold increase in the rate of destruction of heat-resistant bacteria. At a constant temperature, the degree of thiamin destruction was a function of time. Retention of thiamin in the canned meat (average of whole 2.5-pound can) was 52%.

The kinetics of thiamin loss has been determined in extrusion cooking of wheat flour [18] and corn grits [19]. The rate constant of thiamin destruction (k) was most dependent on temperature of the mix in the barrel, water content of the food mix, and speed of screw rotation. Analysis of the data showed that shear stress had a direct influence on the destruction of the thiamin molecule. Barrel temperature had the greatest effect on thiamin loss, and at 200°C, the vitamin was almost completely destroyed. Thiamin destruction decreased markedly with increased water content of the mix. The water acted as a plasticizer on the starch-based material, thereby reducing the shear stress. Increasing the screw speed accelerated thiamin destruction by increasing the shear stress in the cooking zone. Beetner et al. [20] reported a thiamin loss of 10–81% during extrusion cooking of corn grits with 13–16% moisture content. The puffing of wheat and rice results in only 1–6% retention of the initial thiamin content [21].

Low-dose gamma irradiation of vacuum-packaged, ground fresh pork resulted in a dose-dependent, first-order rate of thiamin destruction [22].

Thiamin losses at irradiation doses of 0.57, 1.91, 3.76, 5.52, and 7.25 kGy were 7.7, 23.5, 38.1, 49.8, and 57.6%, respectively, of the nonirradiated sample. Storage and cooking had little additional effect on the thiamin content of irradiated pork. The rate of loss of thiamin in irradiated beef was higher than that in lamb, pork, and turkey leg, but not turkey breast, with losses of 16%/kGy in beef and 8%/kGy in lamb [23]. The rigorous exclusion of oxygen did not prevent the loss of thiamin resulting from the irradiation of pork [24].

In the processing of milk, the following losses of thiamin have been reported: pasteurization, 9–20%; sterilization, 30–35%; spray-drying, 10%; roller drying, 15%; and condensing (canning), 40% [25].

7.3.3 Applicability of Analytical Techniques

The major form of thiamin in meat products is protein-bound TDP. In cereals and cereal products, including white flour made from wheat, free thiamin predominates, but wheat germ, bran, and white bread contain TDP. For the analysis of these products, it is customary to determine the combined vitamin B_1 activity of the various forms present. Thiamin can be determined microbiologically, or by fluorometry after conversion to thiochrome, or by gas chromatography. The principal technique is HPLC using either absorbance detection of thiamin itself or fluorescence detection of thiochrome. The use of a common extraction procedure allows both thiamin and riboflavin to be assayed by HPLC either simultaneously or successively.

One international unit (IU) of vitamin B_1 is the activity obtained from 3 μg of crystalline thiamin hydrochloride in a bioassay. The IU has become obsolete and analytical results are nowadays expressed in terms of weight (mg) of thiamin hydrochloride.

7.4 Intestinal Absorption

Humans cannot synthesize thiamin and so must obtain this vitamin from the diet. Thiamin is synthesized by the microflora that normally inhabit the large intestine, but this source of the vitamin does not appear to be available to the human host [26]. The following discussion is taken largely from a book by Ball [27] published in 2004.

Dietary sources of phosphorylated thiamin, mainly TDP, are completely hydrolyzed to free thiamin in the intestinal lumen by different phosphatases, including alkaline phosphatase. Absorption of thiamin then takes place in the duodenum and proximal jejunum by a dual mechanism that is predominantly saturable at low, physiological concentrations of the vitamin

(<2.5 μmol/l) and largely nonsaturable (diffusive) at higher concentrations [28]. A significant amount of the thiamin that is taken up by the enterocyte is phosphorylated, mainly to TDP, thereby trapping the transported thiamin inside the absorptive cell [29]. Intracellular TDP is dephosphorylated by microsomal phosphatases to free thiamin before exit [30].

Said et al. [31] examined the uptake of radioactive [^{3}H]thiamin by a cell culture system comprising confluent monolayers of the human-derived Caco-2 intestinal epithelial cells. Thiamin uptake was found to be:

(1) Dependent on temperature and metabolic energy
(2) pH-sensitive
(3) Na^+-independent
(4) Saturable as a function of concentration
(5) Competitively inhibited by structural analogs of thiamin.

The saturability and competitive inhibition indicate a carrier-mediated transport process. Inhibition of the Ca^{2+}/calmodulin second messenger system resulted in a significant inhibition of thiamin uptake, implicating this system in the regulation of thiamin absorption. The effect of one such inhibitor, trifluoperazine, was to decrease V_{max} (maximal transport rate) but not the K_m (half-saturation concentration) of the thiamin uptake process. This indicates that the inhibitory effect of trifluoperazine is mediated via down-regulation of thiamin carriers at the brush-border membrane, with no effect on carrier affinity.

The use of purified intestinal membrane vesicles permits transport mechanisms in brush-border and basolateral membranes to be studied separately, with no interference from intracellular metabolism. Laforenza et al. [32] reported the existence of a thiamin/H^+ antiporter mechanism for thiamin entry into brush-border membrane vesicles from rat small intestine. In the absence of H^+ gradient, changes in transmembrane electrical potential did not affect thiamin uptake. Studies using basolateral membrane vesicles showed that exit of thiamin from the enterocyte took place by primary active transport mediated by the basolateral membrane's Na^+–K^+-ATPase [33].

7.5 Bioavailability

7.5.1 Bioavailability of Thiamin in Foods

Relatively little quantitative information is available regarding the bioavailability of thiamin. A review by Gregory [34] concluded that the

thiamin in most foods tested is either highly or totally available for absorption and utilization by humans.

Roth-Maier et al. [35] determined the prececal digestibility of thiamin naturally present in a variety of foods and feedstuffs using pigs subjected to an end-to-end ileo-rectal anastomosis. The foods and feedstuffs selected were boiled eggs, bananas, white cabbage, corn, milk powder, stewed fish, barley, boiled soybeans, boiled rice, wheat bran, brewer's yeast, rye, and soybean meal. In addition, thiamin-free supplementary diets were individually formulated and combined with the test food to provide adequate amounts of nutrients, minerals, and vitamins. The prececal digestibility of thiamin in all tested foods and feedstuffs was found to be relatively high, ranging from 73% in stewed fish to 94% in boiled soybeans, boiled rice, and barley. When compared to the animal products, the plant products showed on average a nearly equal prececal digestibility of thiamin (87.3 versus 83.5%).

7.5.2 Antithiamin Factors

Antithiamin factors refer to naturally occurring compounds that act on thiamin in such a way that the products formed no longer possess the biological activity of the vitamin *in vivo*. They may be divided into two categories: thermolabile enzymes (thiaminases) and thermostable plant polyphenols.

7.5.2.1 Thiaminases

Thiaminase I (EC 2.5.1.2) has been found in shellfish, the viscera of freshwater fish, ferns, and in some bacteria (e.g., *Bacillus thiaminolyticus*). It cleaves the thiamin molecule by an exchange reaction with an organic base or a sulfhydryl compound via a nucleophilic displacement on the methylene group of the pyrimidine moiety. Thiaminase II (EC 3.5.99.2), which has been found only in microorganisms, is a hydrolytic enzyme that hydrolyzes the methylene-thiazole-*N* bond [36]. Thiaminases are usually inaccessible to thiamin in living cells, but become accessible when tissues are disrupted at pH 4–8 [37]. The enzymes can act during food storage and preparation, and during passage through the gastrointestinal tract [38].

Thiaminase activity of raw fermented fish in the diet of rural Thais was estimated to contain 4.5 units per gram. The amount consumed daily was found to be about 50 g of the fish, which is equivalent to 225 units of the enzyme [39]. Habitual intakes of raw freshwater fish (with or without fermentation) and raw shellfish are risk factors for the development of thiamin deficiency. Since thiaminases are inactivated by heat, the cooking of such foods destroys the enzymes completely.

7.5.2.2 Polyphenols

High antithiamin activity attributable to thermostable substances has been found in tea, coffee, rice bran, betel nuts, ferns, and a variety of fruits and vegetables, including blueberries, blackcurrents, red chicory, beetroot, red cabbage, and Brussels sprouts [40]. Taungbodhitham [41] analyzed 14 types of vegetable commonly consumed by people in southern Thailand and found antithiamin factors in 10 types. Samples with a high antithiamin activity tended to have a low thiamin content. The consumption of vegetables containing antithiamin activity has been demonstrated as a factor leading to poor thiamin status among populations in Thailand.

The thermostable antithiamin activity in plants is associated with polyphenols such as hydroxylated derivatives of cinnamic acid, flavonoids, and tannins. The chemical structures of some of these compounds are shown in Figure 7.3. In plant tissues, caffeic acid occurs mainly in

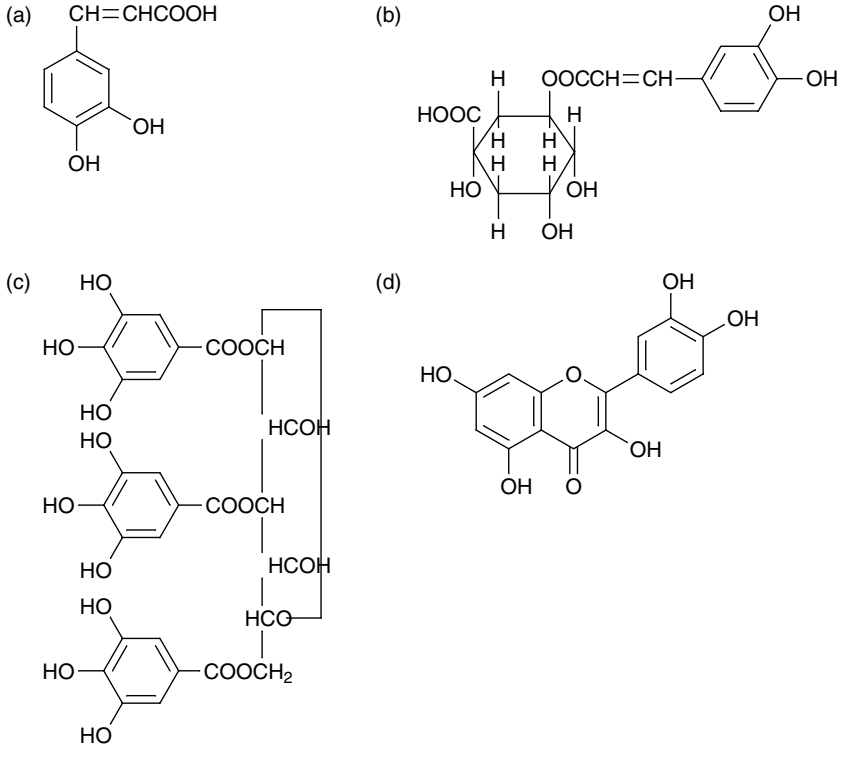

FIGURE 7.3
Polyphenolic compounds possessing antithiamin activity. (a) Caffeic acid (3,4-dihydroxycinnamic acid), (b) chlorogenic acid, (c) corilagin (a tannin), and (d) quercetin (a flavone).

conjugated forms such as chlorogenic acid, and is released from such forms by hydrolysis. Roasted coffee has a high content of chlorogenic acid and contains smaller quantities of caffeic acid and other diphenols. In tests using pure solutions of plant polyphenols, two cinnamic acid derivatives (caffeic acid and chlorogenic acid) showed a greater degradation of thiamin than did two flavonoids (quercetin and dihydroquercetin), although they were less effective as antioxidants [42]. The major product of thiamin degradation was found to be thiamin disulfide.

Tannic acid or tannin is a generic term to describe a complex and nonuniform group of polyhydroxyphenols, which occur in fruits and vegetables commonly consumed by people in various parts of the world [43]. Tannins may be divided into two groups: (i) derivatives of flavonols — so-called condensed tannins, and (ii) hydrolyzable tannins, which are esters of a sugar, usually glucose, with one or more trihydroxybenzenecarboxylic acids. Tannins comprise 7–14% of tea leaf dry weight, which gives tea the astringent taste. Betel nuts are also good sources of tannins.

In all polyphenolic antithiamin factors, the positions of the hydroxyl groups are important for activity. Compounds with *ortho*-hydroxyl groups have the highest activity, followed by *para*-hydroxylated compounds with medium activity, and *meta*-hydroxylated compounds with no activity [40]. Panijpan and Ratanaubolchai [44] proposed a broad scheme to explain the mechanism for thiamin–polyphenol interaction. In Figure 7.4, only the thiazole moiety and changes thereon are depicted. At a sufficiently high pH (>6.5), the polyphenol ionizes and the OH^- groups attack the C-2 to open the ring and yield a sulfhydryl derivative (ThSH) which exists in equilibrium with thiamin (Th). In the presence of oxygen, *ortho*- and *para*-polyphenolic compounds undergo oxidation and polymerization to yield active quinones and less active polymerized products. The quinones interact with the sulfhydryl compound to give thiamin disulfide (ThSSTh) as the major reaction product. In the absence of the polyphenol compound, the oxidation of the sulfhydryl compound to thiamin disulfide is slow. Thiamin disulfide shows the full activity of the vitamin itself because it is easily reduced to thiamin

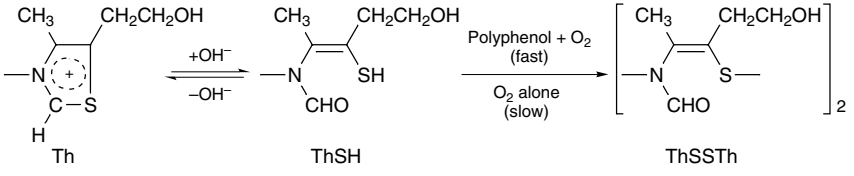

FIGURE 7.4
A scheme showing the effect of OH^- on the opening of the thiazole ring of thiamin (Th) to the sulfhydryl (ThSH) form leading to formation of thiamin disulfide (ThSSTh).

in vivo by reducing agents such as cysteine, ascorbic acid, and glutathione [36]. However, in the absence of reducing agents, thiamin disulfide is not the ultimate major product of thiamin degradation by polyphenolic compounds. Further hydrolysis of the disulfide and subsequent oxidation yield inactive degradation products.

Ascorbic acid was found to prevent thiamin modification by tannic acid *in vitro* [43,45]. Under the conditions used, ascorbic acid appeared not only to protect the remaining thiamin from further modification by tannic acid, but also to be capable of partially reversing the reaction. Cysteine and reduced glutathione were found to be less effective than ascorbic acid.

In rural parts of Thailand, the chewing of fermented tea leaves and betel nuts as stimulants is common practice. Vimokesant et al. [39] reported on studies of the effects of tea drinking and chewing of fermented tea leaves on the thiamin status of Thai subjects. Thiamin status was measured as the effect of TDP stimulation of blood transketolase activity — a high effect indicates thiamin deficiency. After the drinking of tea or chewing of fermented tea leaves, the TDP effect was significantly increased and thereafter decreased to normal in response to thiamin supplementation. Abstention from chewing betel nuts reduced a high TDP effect to a normal level, which was again increased significantly when the subjects resumed their chewing habits. A high TDP effect could be prevented by a delay in tea-leaf chewing after meals or consuming foods high in ascorbic acid along with the meals. The consumption of coffee, either caffeinated or decaffeinated, also produced an antithiamin effect in human subjects, as shown by a decreased urinary excretion of thiamin [40].

Definite effects of antithiamin factors on thiamin status have not been demonstrated in rat feeding trials [40]. However, experiments using rat everted intestinal sacs showed that thiamin passage through the intestinal wall was diminished in the presence of caffeic acid [46]. These investigators suggested that, in the case of a limited supply of thiamin, deficiency could arise from a disturbance in the saturable transport process.

7.5.3 Effects of Alcohol

Acute and chronic effects of alcohol on thiamin absorption in rats have been summarized by Hoyumpa [47]. The acute effect of single doses of ethanol in rats is to significantly reduce the absorption of thiamin by inhibiting in a reversible manner the low concentration, saturable component of the dual transport process. More specifically, ethanol appears to allow the initial uptake of thiamin across the apical membrane of the enterocyte, but impairs the subsequent movement of the vitamin across the

basolateral membrane into the serosa. The fall in the rate of cellular thiamin exit is associated with inhibition of the basolateral Na^+-K^+-ATPase activity. In contrast, chronic ethanol feeding for 6–8 weeks fails to alter thiamin absorption or Na^+-K^+-ATPase activity in rats. These data suggest that inhibition of thiamin absorption is dependent more on the concentration of ethanol bathing the basolateral membrane of the enterocyte than on the duration of exposure. Thiamin malabsorption may, therefore, be intermittent, depending on the ethanol concentration.

Studies in rats may not be completely relevant to humans. Jejunal perfusion studies in chronic alcoholic patients demonstrated that neither alcoholism nor acute exposure to alcohol (5% concentration of ethanol) significantly affected thiamin absorption [48].

7.5.4 Effects of Dietary Fiber

Omaye et al. [49] demonstrated certain *in vitro* interactions of thiamin with selected dietary fiber constituents, which could influence vitamin bioavailability in long-term high-fiber regimens. The interactions were consistent with binding of thiamin to the fiber matrix or trapping by interstitial water. Ranhotra et al. [50] reported that the bioavailability of thiamin present in whole wheat bread was slightly higher than that in thiamin-restored white bread, as determined by erythrocyte transketolase (ETK) activity and liver thiamin content in the rat. Thus, the higher fiber content of whole-wheat bread did not impair the bioavailability of the thiamin present. Yu and Kies [51] determined the bioavailability of thiamin to humans from wet- and dry-milled maize brans which were coarsely or finely ground. Using a double cross-over design, the nine subjects were fed laboratory-controlled diets containing unsupplemented bread or bread supplemented with each type of bran to supply 20 g fiber per day. Urinary excretion values were not significantly affected by the experimental treatment.

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8

Flavins: Riboflavin, FMN, and FAD (Vitamin B_2)

8.1 Background

Riboflavin exerts its biological function through two flavin coenzymes, inappropriately named flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). The coenzymes participate in oxidation–reduction reactions in numerous metabolic pathways exemplified by the oxidation of reduced nicotinamide adenine dinucleotide (NADH), the oxidation of succinate to fumarate in the tricarboxylic acid cycle, and the oxidation of saturated fatty acyl coenzyme A to α,β-unsaturated fatty acyl coenzyme A in the β-oxidation of fatty acids. In all of these reactions, the reduced flavin ($FADH_2$ or $FMNH_2$) is reoxidized by the cytochrome electron-transport chain. In addition, flavin coenzymes are necessary for specific reactions in the interconversion of vitamin B_6 vitamers and folate vitamers, and in the conversion of kynurenine to 3-hydroxykynurenine in the synthesis of nicotinamide adenine dinucleotide (NAD) from tryptophan.

No pathologically severe symptoms attributed to vitamin B_2 deficiency have been observed in humans. Volunteers fed vitamin B_2 antagonists or diets lacking the vitamin exhibited lesions of the muco-cutaneous surfaces and intense photophobia.

The low solubility of riboflavin and the limited capacity of intestinal absorption probably account for the lack of toxicity.

8.2 Chemical Structure, Biopotency, and Physicochemical Properties

8.2.1 Structure and Potency

The principal vitamin B_2-active flavins found in nature are riboflavin, riboflavin-5′-phosphate (FMN) and riboflavin-5′-adenosyldiphosphate

(FAD), which have equal vitamin activity in human nutrition. The structures of these compounds are depicted in Figure 8.1. The parent riboflavin molecule ($C_{17}H_{20}N_4O_6$, MW = 376.4) comprises a substituted isoalloxazine moiety with a ribitol side chain. In biological tissues, FAD and, to a lesser extent, FMN occur almost entirely as coenzymes for a large variety of flavin enzymes (flavoproteins). The molecular weights of FMN and FAD are 514.4 and 865.6, respectively. In most flavoproteins, the flavins are bound tightly but noncovalently to the apoenzyme. In mammalian tissues, less than 10% of the FAD is covalently attached to specific amino acid residues of four important apoenzymes. These are found within succinate and sarcosine dehydrogenases, monoamine oxidase, and L-gluconolactone oxidase in which FAD is peptide linked to an *N*-histidyl or *S*-cysteinyl residue via the 8-methyl group [1].

The term riboflavin can be confusing as it may be used in two different contexts. It is either synonymous with vitamin B_2 (a generic descriptor for all biologically active flavins) or it refers specifically to the parent riboflavin molecule. In this chapter, the names riboflavin, FMN, and FAD will be used as specific terms, and flavins or vitamin B_2 will be used as generic terms.

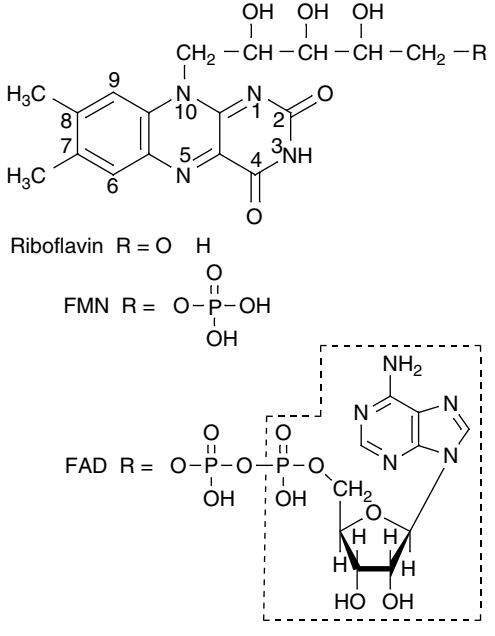

FIGURE 8.1
Structures of riboflavin, FMN, and FAD.

8.2.2 Physicochemical Properties

8.2.2.1 Appearance and Solubility

Riboflavin crystallizes in the form of yellow to orange-yellow needles with an mp of about 280°C. The compound is odorless and has an unpleasant bitter taste. In neutral aqueous solution, riboflavin exhibits a strong yellow-green fluorescence. Riboflavin is only sparingly soluble in water (10–13 mg/100 ml at 25–27.5°C; 19 mg/100 ml at 40°C; 230 mg/100 ml at 100°C) and even less soluble in absolute ethanol (4.5 mg/100 ml at 27°C). Riboflavin is soluble in dilute hydrochloric acid and in dilute alkali (but unstable in alkali). There is no solubility in acetone, diethyl ether, or chloroform. A saturated aqueous solution of riboflavin has a pH of 5.5–7.2. The solubility of riboflavin in water is increased in the presence of aromatic compounds such as nicotinamide, and practical use of this property is made in pharmaceutical preparations. The sodium salt of FMN is very soluble in water, and FAD is freely soluble in water. The pK_a values of riboflavin, FMN, and FAD are 10.2, 1.3, and 6.5, respectively.

8.2.2.2 Stability in Aqueous Solution

Crystalline riboflavin is stable in dry form. In solution, riboflavin is destroyed by exposure to both UV radiation and visible light, the rate of destruction increasing with an increase in temperature and pH. Sattar et al. [2] reported that the wavelength range of 350–520 nm was generally damaging to riboflavin in aqueous solution, with the 415–455 nm range being particularly destructive. The principal photodegradation product under neutral and acidic conditions is lumichrome (7,8-dimethylalloxazine), while under alkaline conditions the major product is lumiflavin (7,8-trimethylisoalloxazine) (Figure 8.2). Neither of these degradation product exhibits vitamin B_2 activity.

If protected from light, aqueous solutions of riboflavin in the pH range between 2 and 5 are heat-stable up to 120°C; above pH 7, the isoalloxazine ring is rapidly destroyed at elevated temperatures. Optimal stability is

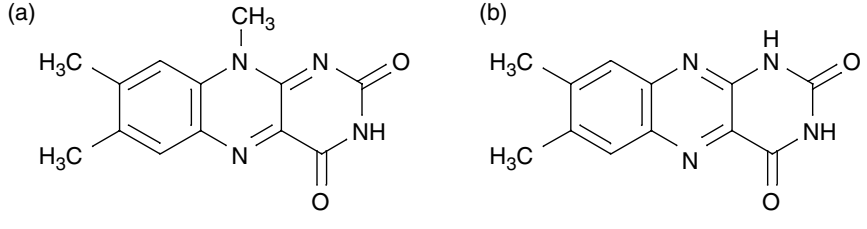

FIGURE 8.2
Photodegradation products from riboflavin: (a) lumiflavin and (b) lumichrome.

obtained at pH 3.5–4.0. Riboflavin is stable toward oxygen and many oxidizing agents in the absence of light.

8.3 Vitamin B_2 in Foods

8.3.1 Occurrence

Living cells require FMN and FAD as the prosthetic groups of a variety of enzymes, and hence the flavins are found, at least in small amounts, in all natural unprocessed foods. The vitamin B_2 content of a selection of foods is presented in Table 8.1 [3]. Yeast extract is exceptionally rich in vitamin B_2, and liver and kidney are also rich sources. Wheat bran, eggs, meat, milk, and cheese are important sources in diets containing these foods.

TABLE 8.1

Vitamin B_2 Content of Various Foods

Food	mg Riboflavin/ 100 g Edible Portion
Cow milk, whole, pasteurized	0.23
Cheese, cheddar, average	0.39
Egg, chicken, whole, raw	0.47
Wheat flour, wholemeal	0.09
Wheat flour, white, plain	0.03
Rice, brown, raw	0.07
Beef, trimmed lean, raw, average	0.21
Lamb, trimmed lean, raw, average	0.20
Pork, trimmed lean, raw, average	0.24
Chicken, meat, raw	0.18
Liver, lamb, fried	5.65
Cod, raw, fillets	0.05
Potato, main crop, old, average, raw	0.02
Chick peas, dried, raw	0.24
Soya beans, dried, raw	0.27
Red kidney beans, dried, raw	0.19
Peas, raw	0.02
Broccoli, raw	0.06
Apples, eating, average, raw	0.02
Orange juice, unsweetened	0.02
Peanuts, plain	0.10
Yeast extract	11.9

Source: From Food Standards Agency, *McCance and Widdowson's The Composition of Foods*, 6th summary ed., Royal Society of Chemistry, Cambridge, 2002. With permission.

Cereal grains contain relatively low concentrations of flavins, but are important sources in those parts of the world where cereals constitute the staple diet. The milling of cereals results in considerable loss (up to 60%) of vitamin B_2, so white flour is enriched by addition of the vitamin. The enrichment of bread and ready-to-eat breakfast cereals contributes significantly to the dietary supply of vitamin B_2. Polished rice is not usually enriched, because the vitamin's yellow color would make the rice visually unacceptable to the major rice-consuming populations. However, most of the flavin content of the whole brown rice is retained if the rice is steamed prior to milling. This process drives the flavins in the germ and aleurone layers into the endosperm [4].

Free riboflavin is naturally present in foods along with protein-bound FMN and FAD. Bovine milk contains mainly free riboflavin, with a minor contribution from FMN and FAD [5]. In whole milk, 14% of the flavins is bound noncovalently to specific proteins [6]. Egg white and egg yolk contain specialized riboflavin-binding proteins, which are required for storage of free riboflavin in the egg for use by the developing embryo [7].

8.3.2 Stability

Riboflavin is generally stable during the heat processing and normal cooking of foods if light is excluded. The alkaline conditions in which riboflavin is unstable are rarely encountered in foodstuffs. Riboflavin degradation in milk can occur slowly in the dark during storage in the refrigerator [8]. Mũnoz et al. [9] reported riboflavin losses of 16–23% from UHT-processed milk in opened polyethylene cartons after 6 days of refrigerated (8°C) storage. Milk exposed to light undergoes major loss of riboflavin due to photodegradation in the wavelength range of 400–550 nm [10]. As much as 85% of the vitamin B_2 content of milk in glass bottles may be destroyed after only 2 h exposure to bright sunlight [11]. Photodegradation of riboflavin in milk proceeds by a single-phase, first order mechanism, and increases with an increase in storage temperature and light intensity [12]. Riboflavin acts as a sensitizer for destruction of ascorbic acid by light, and its removal from milk will prevent the light-induced oxidation of ascorbic acid from taking place [13]. Photodegradation of riboflavin in milk generates singlet oxygen, which can indirectly react with polyunsaturated fatty acids to give hydroperoxides [14]. The hydroperoxides decompose to a wide range of volatile compounds responsible for "off flavors".

Woodcock et al. [15] studied the effect of light on the rate of riboflavin degradation in single layers of enriched elbow macaroni. Riboflavin photodegradation was rapid at first, with 50% of the initial riboflavin being

lost within 12 h. Riboflavin loss then proceeded much more slowly, with a further 10% loss after 35 days of exposure. The slower second phase was probably due to lack of light penetration into the interior of the macaroni. An increase in temperature increased the rate of riboflavin photodegradation, but there was no significant difference in the rate of vitamin loss using light intensities of 100, 200, or 300 footcandles (ft-c). Further studies [16] showed that lower light intensities (25, 50, and 100 ft-c) resulted in two-phased degradation with no significant differences between their effects. An extremely dim light (0 ft-c) provided sufficient energy for riboflavin to degrade at a slow but constant rate during the 2 weeks of exposure. There was no loss of riboflavin in macaroni completely protected from light. Exposing macaroni to a light of 75 ft-c at room temperature and a broad range of relative humidities revealed a general trend of increased photolability with increasing water activity. However, most macaroni is within the moisture range of maximum riboflavin stability. The conversion of riboflavin to lumichrome was incomplete during light exposure and therefore lumichrome cannot be used as an exact measure of riboflavin loss.

In another study [17], storage of enriched elbow macaroni under controlled lighting conditions for 3 months resulted in an 80% loss of the original riboflavin content. The initial riboflavin content did not greatly influence the percent vitamin retention with light exposure. The leaching of riboflavin with cooking was studied in enriched spaghetti that was either not previously exposed to light or previously exposed to light of 150 ft-c for 2 days. Cooking and handling procedures were performed in a darkened room. The percent retention of riboflavin was relatively constant (ca. 58%) in the previously unexposed samples and the riboflavin found in the cooking water accounted for most of the vitamin not retained in the spaghetti. Total recovery from the sum of the riboflavin in the cooked spaghetti and in the cooking water was ca. 95%. There was no significant difference in percent cooking retention between the exposed and unexposed samples. Thus, the heating of spaghetti did not degrade its riboflavin content: cooking losses in the absence of light were entirely due to leaching, which was also independent of the initial riboflavin content. Cooking enriched pasta in the normal way and then freeze-drying prior to analysis of riboflavin results in total riboflavin recovery of only about 60% [18,19]. Photodegradation of the vitamin during cooking and freeze drying is probably responsible for this low recovery.

With regard to the packaging materials commonly used for pasta, paperboard cartons with or without transparent windows completely protected riboflavin from photodegradation. Pasta packaged in transparent bags allowed approximately one third of the riboflavin to photodegrade in 1 week of light exposure [20].

8.3.3 Applicability of Analytical Techniques

Vitamin B_2 occurs naturally in foods as free riboflavin and as protein-bound FAD and FMN. It is usually required to determine the total vitamin B_2 content, which is defined in the nutritional context as the sum of FAD, FMN, and riboflavin. Current techniques for determining total vitamin B_2 are microbiological assay, fluorometry, and HPLC using fluorometric detection. HPLC can also be used to determine the individual flavins.

No international unit of vitamin B_2 activity has been defined, and analytical results are expressed in terms of weight (mg) of chemically pure riboflavin.

8.4 Intestinal Absorption

The following discussion of absorption is taken from a more detailed account in a book by Ball [21] published in 2004.

8.4.1 Absorption of Dietary Vitamin B_2

The FMN and FAD present in the ingested food are released from noncovalent binding to flavoproteins as a consequence of acidification in the stomach and gastric and intestinal proteolysis. Riboflavin is similarly released from its association with binding proteins [7].

The flavin coenzymes are hydrolyzed in the upper small intestine to free riboflavin, which is then absorbed. Hydrolysis of both FMN and FAD is effected by alkaline phosphatase (E.C. 3.1.3.1), which has a broad specificity and is located on the brush-border membrane of the enterocyte [22]. Two additional brush-border enzymes, FMN phosphatase and FAD pyrophosphatase, participate in the degradation of the flavin coenzymes [23]. The considerably smaller amounts of covalently bound flavins are released as 8α-(peptidyl) riboflavins, which are absorbed along with free riboflavin [24].

In vitro studies using rat everted jejunal sacs have shown that absorption of riboflavin takes place by a saturable, energy-dependent process at physiologically relevant concentrations and by simple diffusion at higher concentrations [25–27]. This dual process of absorption has been confirmed under *in vivo* conditions [28]. Casirola et al. [29] showed that the ribitol side chain and the NH group at position 3 of the isoalloxazine moiety are essential for riboflavin binding to specific sites on the brush-border membrane of rat small intestine.

The absorption of riboflavin is efficient along the length of the small intestine (i.e., jejunum and ileum) in the guinea pig [30] and the rabbit [31]. Absorption is enhanced by the presence of bile salts in the intestinal lumen [32]. The importance of bile salts is also indicated by impaired absorption of riboflavin in cases of biliary obstruction in children [33].

Transport of riboflavin across brush-border and basolateral membrane vesicles prepared from rabbit small intestine was found to be independent of sodium and electroneutral in nature [31,34]. Using human-derived Caco-2 intestinal epithelial cells, Said and Ma [35] confirmed the involvement of a carrier-mediated process in the initial phase of riboflavin uptake (a 3-min incubation time). Riboflavin uptake was Na^+- and pH-independent and the initial phase occurred without metabolic alteration of the transported riboflavin. Inhibitors of anion transport did not produce inhibition of riboflavin uptake by Caco-2 cells, thus riboflavin does not appear to act as an anion with regard to its intestinal transport.

Some of the absorbed riboflavin is phosphorylated to FMN within the cytosol of the enterocyte by flavokinase (ATP:riboflavin 5′-phosphotransferase, EC 2.7.1.26) and most of the FMN is further converted to FAD by FAD synthetase (ATP:FMN adenylyltransferase, EC 2.7.7.2). Both these metabolic steps require ATP, that is, they are energy-dependent.

To summarize, the small intestine is well adapted to completely extracting the small amounts of riboflavin that are largely bound within the ingested flavin coenzymes. The coenzymes are dephosphorylated in the lumen and the liberated riboflavin is extracted very efficiently by a high-affinity, carrier-mediated transport system, which is distributed along the entire length of the small intestine. The uptake mechanism is Na^+-independent and electroneutral in nature. After uptake, some of the riboflavin is metabolically trapped within the enterocyte as FMN. The energy used in riboflavin absorption is not required for membrane uptake, but rather for riboflavin metabolism within the enterocyte. Thus, intracellular metabolism is probably the driving force behind the internalization of riboflavin. The vitamin is dephosphorylated to permit exit of riboflavin across the basolateral membrane; this also takes place by a carrier-mediated, Na^+-independent and electroneutral mechanism.

8.4.2 Absorption of Bacterially Synthesized Vitamin B_2 in the Large Intestine

The normal microflora of the large intestine synthesize considerable amounts of vitamin B_2, a significant portion of which exists as free riboflavin. The amount of vitamin B_2 synthesized depends on the diet, being significantly higher following consumption of a vegetable-based diet compared with a meat-based diet [36]. In a study with human subjects, Sorrell et al. [37] showed that riboflavin instilled directly into the lumen

of the mid-transverse colon was absorbed, as judged by an increase in plasma riboflavin concentrations. Colonic absorption of FMN sodium has also been demonstrated in the rat [38].

Said et al. [39] demonstrated the existence of a high-affinity, carrier-mediated transport system in the large intestine using cultured human colonic epithelial cells (NCM460 cells). Saturable uptake of riboflavin by these cells was energy-dependent and Na^+-independent. An adaptive up- and down-regulation of riboflavin uptake took place when NCM460 cells were grown in a riboflavin-deficient or over-supplemented medium, respectively. These findings are similar to those in the small intestine and suggest that the same mechanism may be operating in the large intestine to absorb the bacterially synthesized riboflavin.

8.5 Bioavailability

Bates [40] concluded that vitamin B_2 bioavailability has been little studied and is poorly characterized. Roe et al. [41] examined the influence of various dietary fiber sources on riboflavin absorption as measured by urinary excretion of the vitamin in healthy male subjects. Coarse and fine wheat bran, cellulose, and cabbage were incorporated as single fiber sources into the diet on an isofibrous basis (12 g fiber/day). Compared with a control group receiving a low-fiber diet, each fiber source increased urinary excretion when 15 mg FMN was ingested with the breakfast meal. It was concluded that the dietary fiber sources enhanced riboflavin absorption, probably by slowing the passage of chyme in the intestine and thereby increasing the duration of vitamin exposure at the absorption sites.

Chronic alcoholism is associated with a high prevalence of vitamin B_2 deficiency. In rats, ethanol markedly diminished the bioavailability of FAD and, to a lesser extent, riboflavin. Ethanol impaired intraluminal hydrolysis of FAD, and also markedly inhibited activities of FMN phosphatase and FAD pyrophosphatase *in vitro* [42]. These findings suggest that ethanol inhibits the enzymes necessary to release riboflavin from FMN and FAD, thereby diminishing the amount of vitamin available for absorption.

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9

Niacin

9.1 Background

Niacin is the generic descriptor for two vitamers, nicotinic acid and nicotinamide, which have equal bioactivity in the free form. American usage of the term "niacin" to denote specifically nicotinic acid, and "niacinamide" to denote the amide is not used in this text. In living tissues nicotinamide is the reactive moiety of the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). The coenzymes function as proton and electron carriers in a wide variety of oxidation–reduction reactions. Examples are reactions concerned with the release of energy from carbohydrates, fatty acids, and amino acids, and with the synthesis of amino acids, fatty acids, and pentose sugars.

Niacin can be synthesized *in vivo* from the essential amino acid L-tryptophan. An average intake of protein will probably provide more than enough tryptophan to meet the body's requirement for niacin without the need for any preformed niacin in the diet. Cases of primary niacin deficiency are rare (at least in industrialized countries), but secondary deficiencies may arise from gastrointestinal disorders or alcoholism.

A deficiency in niacin results in pellagra, which historically reached epidemic proportions in populations subsisting chiefly on maize. Early neurological symptoms of this disease include tremor, irritability, anxiety, and depression, with delerium and dementia sometimes occurring in severe and chronic cases. The response to nicotinamide therapy is rapid and dramatic: untreated pellagra results eventually in death.

Nicotinic acid administered orally at doses as low as 100 mg/day causes peripheral vasodilation, with the appearance of skin flushing. In high doses, nicotinic acid competes with uric acid for excretion, leading to an increase in the incidence of gouty arthritis. Of greatest concern is possible liver damage. Nicotinamide does not cause vasodilation, but is otherwise two to three times as toxic as the acid.

9.2 Chemical Structure, Biopotency, and Physicochemical Properties

9.2.1 Structure and Potency

Nicotinic acid and nicotinamide are systematically named as pyridine 3-carboxylic acid ($C_6H_5O_2N$, MW = 123.1) and pyridine 3-carboxamide ($C_6H_6ON_2$, MW = 122.1), respectively (Figure 9.1). The nicotinamide nucleotide coenzymes are composed of a nucleotide (adenonine monophosphate, AMP) and a pseudonucleotide containing the nicotinamide moiety

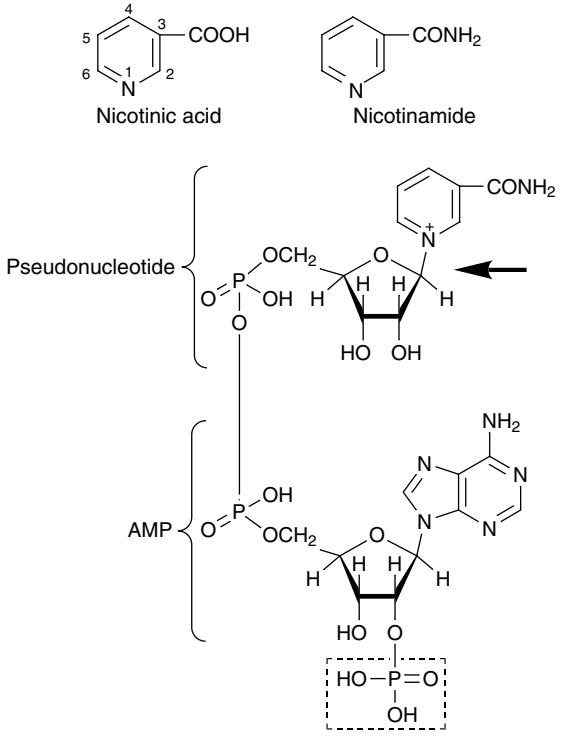

FIGURE 9.1
Structures of niacin compounds and the nicotinamide nucleotides. The nitrogen in the pyridine ring of nicotinamide is positively charged when it is a component of NAD(P). Arrow shows the β-*N*-glycosidic linkage separating the ADP-ribose and nicotinamide moieties.

(Figure 9.1). The oxidized forms of the coenzymes carry a positive charge (NAD^+ and $NADP^+$); reduced forms carrying two electrons and one proton (and associated with an additional proton) are represented as NADH and NADPH. Although the coenzymes are only loosely associated with the apoenzyme during catalysis, most cellular NAD and NADP are stored in the cytoplasm bound to protein.

Nicotinic acid and nicotinamide possess equal vitamin activity, the free acid being converted to the amide in the body.

9.2.2 Physicochemical Properties

9.2.2.1 Appearance, Solubility, and Other Properties

Nicotinic acid crystallizes into colorless needles (mp 235°C), which are odorless and have a tart taste. The crystals of nicotinamide (mp 129°C) are similar, but have a bitter taste. Nicotinic acid is sparingly soluble in water at 25°C (1.67 g/100 ml) and ethanol (0.73 g/100 ml) and insoluble in acetone and diethyl ether. It is, however, freely soluble in boiling water. The pH of a saturated aqueous solution is 3 and the pK_a is 4.8 (25°C). The sodium salt of nicotinic acid is readily soluble in water (71 g/100 ml) and yields a solution of about pH 7. Nicotinamide is readily soluble in water (100 g/100 ml) and in ethanol (67 g/100 ml); there is solubility in acetone and very slight solubility in diethyl ether and chloroform. An aqueous solution of nicotinamide yields a pH of 6 and the pK_a is 3.3 (20°C). Both nicotinic acid and nicotinamide have the properties of bases and form quaternary ammonium salts when in acid solution. Nicotinic acid, being amphoteric, forms carboxylic acid salts when in a basic solution, but nicotinamide possesses no acidic properties.

9.2.2.2 Stability in Aqueous Solution

Both nicotinic acid and nicotinamide are unaffected by atmospheric oxygen, light, and heat in the dry state and in neutral aqueous solution. Nicotinamide is hydrolyzed quantitatively to nicotinic acid when autoclaved in a medium of 1 *N* mineral acid or in alkaline solution; nicotinic acid is resistant to these conditions. The oxidized coenzymes (NAD^+ and $NADP^+$) are labile to alkali [1], whereas the reduced coenzymes (NADH and NADPH) are labile to acid [2].

9.3 Niacin in Foods

9.3.1 Occurrence

Because of the contribution of tryptophan, foods containing balanced protein are important contributors to total niacin equivalent intake.

The niacin, tryptophan, and total niacin equivalent contents of a selection of foods are presented in Table 9.1.

Lean red meat, poultry, and liver contain high levels of both niacin and tryptophan and, together with legumes, are important sources of the vitamin. In meat, niacin is largely in the form of nicotinamide formed by postmortem hydrolysis of NAD. Peanut butter is an excellent source of niacin. Cheese and eggs are relatively poor sources of preformed niacin, but these high-protein foods have a high niacin equivalent. Fruits and vegetables provide useful amounts, depending on the dietary intake. Trigonelline (*N*-methyl nicotinic acid), an abundant constituent of green coffee beans, is demethylated to nicotinic acid during the roasting process. The amount of nicotinic acid formed is 80–150 mg/kg in medium-roasted coffee and up to 500 mg/kg in a dark

TABLE 9.1

Niacin Content of Various Foods (mg/100 g Edible Portion)

Food	Niacin	Niacin Equivalent From Tryptophan[a]	Total Niacin Equivalents
Cows milk, whole, pasteurized	0.2	0.6	0.8
Cheese, cheddar, average	0.1	6.8	6.9
Egg, chicken, whole, raw	0.1	3.7	3.8
Wheat flour, wholemeal	5.7[b]	2.5	8.2
Wheat flour, white, plain	1.7[b]	1.9	3.6
Rice, brown, raw	5.3	1.5	6.8
Beef, trimmed lean, raw, average	5.0	4.7	9.7
Lamb, trimmed lean, raw, average	5.4	3.9	9.3
Pork, trimmed lean, raw, average	6.9	4.5	11.4
Chicken meat, raw	7.8	4.3	12.1
Liver, lamb, fried	19.9	4.9	24.8
Cod, raw, fillets	2.4	3.4	5.8
Potato, main crop, old, average, raw	0.6	0.5	1.1
Chick peas, dried, raw	1.9	2.9	4.8
Soya beans, dried, raw	2.2	5.7	7.9
Red kidney beans, dried, raw	2.1	3.5	5.6
Peas, raw	2.5	1.1	3.6
Broccoli, raw	0.9	0.8	1.7
Apples, eating, average, raw	0.1	0.1	0.2
Bananas	0.7	0.2	0.9
Orange juice, unsweetened	0.2	0.1	0.3
Peanuts, plain	13.8	5.5	19.3
Yeast extract	64.0	9.0	73.0
Coffee, instant	24.8	2.9	27.7

[a]Niacin equivalent = tryptophan mg/60.
[b]This level is for fortified flour.
Source: From Food Standards Agency, *McCance and Widdowson's The Composition of Foods*, 6th summary ed., Royal Society of Chemistry, Cambridge, 2002. With permission.

roast [4]. Fortification or enrichment of foods with niacin uses free nicotinamide because of its high solubility in water.

In mature cereal grains, most of the niacin is present as bound nicotinic acid and is concentrated in the aleurone and germ layers (Figure 9.2). Milling to produce white flour removes most of the vitamin with the bran. In the U.K. it is compulsory by law to add niacin to white flour (mostly 70% extraction rate) at 16 mg/kg. All flour other than wholemeal (100% extraction) must be enriched.

As discussed in Section 9.5.1, some plant-derived foods contain niacin in chemically bound forms that result in their bioavailabilities being low. In mature cereal grains, for example, as much as 70% of the niacin may be biologically unavailable after conventional cooking. Most food composition tables give total niacin, and are compiled from the results of analyses in which nicotinic acid is liberated from unavailable bound forms by hydrolysis with acid or alkali. Therefore, tabulated niacin contents for many plant foods, particularly mature cereals, overestimate their value in providing biologically available niacin.

9.3.2 Stability

Apart from leaching losses, niacin is stable during processing, storage, and cooking of foods. Niacin is present in uncooked foods mainly as NAD and NADP, but these nucleotides may undergo some degree of hydrolysis during cooking to yield nicotinamide. The use of baking powder liberates much of the bound vitamin from cereal flours during baking. The steaming of rice prior to milling does not significantly

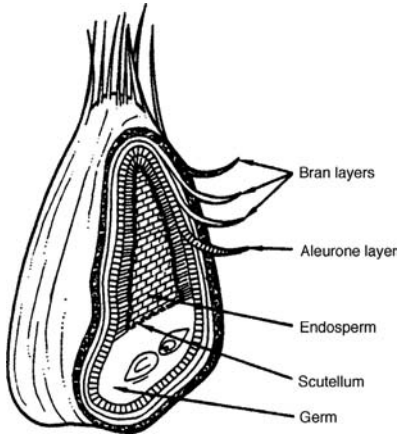

FIGURE 9.2
Longitudinal section of a wheat grain.

increase the niacin content of polished rice as, unlike other B-group vitamins, the nicotinic acid in its bound form does not diffuse into the endosperm [5].

9.3.3 Applicability of Analytical Techniques

The niacin in mature cereal grains exists largely as chemically bound forms of nicotinic acid that are nutritionally unavailable, whereas in pulses and other noncereal plant foods it is present mainly as free nicotinic acid. In meat, the niacin content is mainly in the form of nicotinamide, and this is the form used in the fortification of processed foods.

It has been customary in the past when employing *in vitro* analytical methods to measure the total niacin (i.e., free plus bound) content of the food sample; however, this provides a gross overestimate of the biologically available niacin of several staple cereal-based foods. Several nutritionists have suggested that the measurement of free niacin in foods would provide an accurate estimate of the content of available niacin but, in view of the apparent partial utilization of bound nicotinic acid present in mature cereal grains, this may underestimate the niacin value in cereal products. Nevertheless, the measurement of free niacin would seem to be more appropriate for such products as a means of ensuring an adequate level of enrichment. Both total and free niacin can be measured microbiologically, colorimetrically, or by HPLC, using an appropriate extraction procedure.

To account for the contribution to niacin activity by dietary tryptophan, the term "niacin equivalent" was introduced. In humans, approximately 60 mg of L-tryptophan yield 1 mg of niacin, so 1 niacin equivalent is equal to either 1 mg of available niacin or 60 mg of tryptophan. To calculate the niacin equivalent of a diet, one therefore adds one-sixtieth of the weight of tryptophan present to the weight of nicotinamide. Many factors, such as hormonal status, can affect the equivalence of tryptophan and dietary niacin. Horwitt et al. [6] predicted that 89 mg of tryptophan to 1 mg of niacin would be the ratio to cover 97.5% of the population.

The standard microbiological assay procedure for determining total niacin does not account for tryptophan, because the mild acid digestion used to extract the niacin does not hydrolyze the proteins, and the lactic acid bacterium employed as the assay organism cannot utilize peptide-bound tryptophan. Apart from using an animal bioassay, or possibly a protozoan assay, an estimate of the niacin equivalent content would necessitate the determination of L-tryptophan in a separate assay.

No international unit of niacin activity has been defined, and analytical results are expressed in weight units (mg) of pure nicotinamide. The niacin activity of food samples can be assayed by a growth test in rats or chicks.

9.4 Intestinal Absorption

The following discussion on absorption is taken largely from a book by Ball [7] published in 2004.

Much of the available niacin in the diet will be in the form of nicotinamide nucleotides (NAD and NADP), with meat and milk containing free nicotinamide. It has been assumed that the digestion process gives rise to nicotinamide after ingestion of these coenzymes, since nicotinamide appears to be the primary circulating vitamer and it is not significantly hydrolyzed to nicotinic acid in the intestine of the rat. Biosynthesis of niacin by gut flora may occur, but whether or not it is absorbed by the host has not been established.

Studies using various rat tissue preparations [8] have led to a possible pathway of NAD (or NADP) degradation to nicotinamide in the intestine. The initial step is an attack at the phosphodiester bond of NAD to form nicotinamide mononucleotide and AMP. The enzyme responsible, NAD pyrophosphatase (EC 3.6.1.22), is present in intestinal secretions and to a much lesser extent in pancreatic juice. Nicotinamide mononucleotide is rapidly hydrolyzed to nicotinamide riboside and inorganic phosphate, followed by reaction of the riboside with inorganic phosphate to form nicotinamide and ribose-1-phosphate. The latter two reactions require the presence of intestinal cells, indicating that the enzymes are membrane-bound or intracellular.

Schuette and Rose [9] reported that entry of [^{14}C]nicotinamide into isolated enterocytes was rapid and neither energy-dependent nor saturable at the physiological concentrations employed (11.7 μM). Upon entry into the cells, the nicotinamide was immediately metabolized to NAD. Data obtained from this and other reports [10,11] have led to the general conclusion that cellular uptake of nicotinamide and nicotinic acid occurs primarily by simple diffusion. This is followed by the rapid conversion of the vitamers to NAD within the cytosol. The NAD and intermediate metabolites are not freely permeable to the cell membrane, resulting in the intracellular trapping of nicotinamide and nicotinic acid. This metabolic trapping creates a concentration gradient across the brush-border membrane, which provides the impetus for passive but rapid uptake of the two vitamers.

9.5 Bioavailability

9.5.1 Niacin

In mature cereal grains (those examined included maize, wheat, rice, barley, and sorghum), 85–90% of the total niacin content exists in

chemically bound forms of nicotinic acid, the remainder being present as free nicotinic acid [12]. The majority of this bound nicotinic acid is biologically unavailable after conventional cooking [13]. At one time there were two views concerning the nature of the bound nicotinic acid in cereal grains. The nicotinic acid was contained either within a polysaccharide, referred to as niacytin [14], or within a polypeptide, which was called niacinogen [15]. Investigations by Mason et al. [16] revealed that the bound nicotinic acid of wheat bran is not present as a single substance, but is incorporated in a number of macromolecules that are both polysaccharide and glycopeptide in character. Mason and Kodicek [17] subjected preparations of bound nicotinic acid of wheat bran to partial acid hydrolysis and identified a subunit as nicotinoyl glucose (Figure 9.3). The nicotinoyl ester bond linking the nicotinic acid and glucose moieties most probably exists in the polysaccharide and glycopeptide fractions isolated from wheat bran, and is so far the only established linkage in bound nicotinic acid.

Nicotinoyl glucose itself is readily utilized, so why should this compound be unavailable when present in plant tissues? Mason and Kodicek [17] suggested that its incorporation within indigestible celluloses and hemicelluloses prevents access of the gastrointestinal esterases to the nicotinoyl ester bonds. Alternatively, esterase activity may be poor: the methyl ester of nicotinic acid was only 15% as effective as the free acid in supporting the growth of rats [13].

Pellagra, the disease caused by a deficiency of both niacin and tryptophan, has commonly been found in population groups having maize as their staple food. The generally accepted explanation for this association is the unavailability of niacin in maize, coupled with a very low proportion of tryptophan in zein (the major protein in maize). Mexican and Central American peasants, and also Hopi Indians in Arizona, rely upon maize as a food staple, and yet do not experience pellagra. The explanation for this paradox lies in the way in which these people prepare the maize for bread-making. In the traditional preparation of

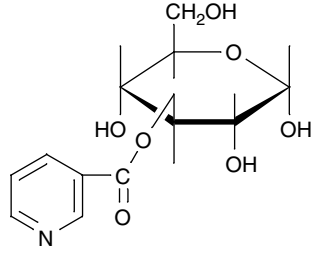

FIGURE 9.3
Structure of β-3-*O*-nicotinoyl-D-glucose.

Mexican tortillas, the maize is soaked at alkaline pH in lime-water before baking and this process releases the nicotinic acid from its bound forms. In the making of piki bread, the Hopi Indians use wood ash, which is alkaline and also results in the liberation of nicotinic acid. The availability of nicotinic acid in tortillas baked from maize treated with lime-water has been demonstrated in pigs [18].

Wall et al. [19] established that niacin exists in different forms during the development of the maize grain, the changes being consistent with the biological needs of the seed. In the early milky and dough stages (21–28 days after pollination), the predominant niacin-containing compounds are the pyridine nucleotides NAD and NADP. These coenzymes are required by the plant to supply energy for producing polysaccharides, proteins, and fats for deposition and storage within the seed. The oxidized forms of these coenzymes (NAD^+ and $NADPH^+$) are biologically available as a source of niacin, and release nicotinamide on heating. The reduced coenzymes (NADH and NADPH), which may also be present in small amounts, are unavailable, because they are acid-labile and break down in the gastric juice. As the grain matures, some free nicotinamide and nicotinic acid are present and, finally, nutritionally unavailable, carbohydrate-bound nicotinic acid and trigonelline are formed for storage. Small amounts of pyridine nucleotides are retained in the germ and aleurone layer to facilitate germination.

Carpenter et al. [20] confirmed in rat growth assays the high available niacin value of maize harvested at the milky stage, whether it was of a sweet or starchy type, and the much lower value of each type at maturity. The higher level of tryptophan in the maize protein at the milky stage also contributes to its overall niacin equivalent value. Therefore, the niacin of sweet corn (corn-on-the-cob) and also immature starchy corn has a high bioavailability both raw, when it is present mostly in NAD, and cooked (at neutral pH), when NAD has largely broken down to yield free nicotinamide.

Carter and Carpenter [21] compared the available niacin values of foods determined by the weight gain in young rats with the contents of free and total niacin determined by chemical analysis (Table 9.2). The bioavailability of niacin in each food is calculated by dividing the rat assay value by the total niacin content (both in units of mg/kg) and multiplying by 100. The niacin in liver and beans was present largely, if not entirely, as free niacin and was fully bioavailable. In mature cereals (maize, sorghum, rice, and wheat), only a small proportion of free niacin, or none, was detected and bioavailabilities ranged from 26 to 37%. The complete bioavailability of niacin in tortilla demonstrated the effect of alkali in liberating free nicotinic acid from its bound forms in maize. Potato and peanut flour contained little or no free niacin and exhibited bioavailabilities of 52 and 46%, respectively. The coffee extract had about half its niacin

TABLE 9.2

Bioavailability of Niacin in Cereal and Noncereal Foods

	Total Niacin (Chemical) mg/kg	Free Niacin[a] (Chemical) mg/kg	Available Niacin (Rat Assay) mg/kg	Bioavailability[b] %
Cereal foods				
Maize, boiled	18.8	1.1	7 ± 1.8	37
Tortilla	12.6	11.7	14 ± 1.8	111
Sweet corn, raw	51.3	Tr	40 ± 6.4	78
Sweet corn, steamed	56.4	45	48 ± 7.2	85
Sorghum, boiled	45.5	1.6	15 ± 3.8	33
Rice, boiled	70.7	17.3	29 ± 6.6	26[c]
Whole wheat, raw	51.4	ND	16 ± 3.0	31
Whole wheat, boiled	57.3	ND	18 ± 5.4	31
Concentrate from wheat bran	2830	Tr	480 ± 240	17
Non-cereal foods				
Potato, baked	50.7	12	32 ± 6.9	52[c]
Liver, baked, beef	306	297	314 ± 30	103
Beans, autoclaved	26.2	22	31 ± 3.1	118
Peanut flour	255	Tr	117 ± 20	46
Coffee extract	597	315	420 ± 67	70

Note: ND, none detected; Tr, trace.

[a]Free nicotinic acid + nicotinamide.

[b]Bioavailability = (available niacin ÷ total niacin) × 100%.

[c]Estimate of the bioavailability of the (total − free niacin) assuming that the free niacin is fully available to the rat.

Source: From Carter, E.G.A. and Carpenter, K.J., *J. Nutr.*, 112, 2091, 1982.

in free form and the bioavailability was 70%. High bioavailabilities were obtained for both raw and steamed sweet corn, owing to their niacin being mainly in the form of NAD and free nicotinamide, respectively.

Carter and Carpenter [22] measured the urinary excretion of two major niacin metabolites in a human study of niacin bioavailability from wheat bran. Two preparations were compared: (i) a portion of wheat bran concentrate cooked in steam (bound niacin, BN) and (ii) another portion of the concentrate made alkaline with calcium oxide and then cooked (alkali-treated bound niacin [ABN]). About 90% of a 24-mg dose of synthetic nicotinic acid was recovered in the urine. The corresponding values for BN and ABN averaged 24 and 62%, respectively, and were significantly different ($P < 0.05$).

Roth-Maier et al. [23] determined the prececal digestibility of endogenous niacin in five different foods using pigs subjected to an end-to-end ileo-rectal anastomosis. The foods selected were wheat, coarse wholemeal bread, steamed potatoes, boiled pork, and boiled beef. These test foods

were mixed with basal ingredients to provide edible meals, and to supply a minimum of niacin. All diets were fortified with minerals and fat-soluble vitamins, and the wheat, bread and potato meals were additionally enriched with soybean oil and amino acids. The digestibilities of niacin from the wheat, potato, and meat meals ranged between 59 and 69% and were not statistically different from one another. The digestibility of niacin from the coarse wholemeal bread diet was 40%, significantly lower ($P < 0.05$) than values for the other commodities.

9.5.2 Tryptophan

Estimates of tryptophan bioavailability in selected foods using a rat growth assay were 90–100% for most products tested [24]. One notable exception was pinto beans, whose low value of 59% could be explained by a low protein digestibility. Kies [25] reported that the protein contained in the bran fraction of wheat is not available to the human, presumably because of interference of absorption by dietary fiber.

The utilization of tryptophan as a precursor of NAD depends on an adequate dietary supply of other essential amino acids. If the supply falls, less tryptophan is utilized in protein synthesis and more becomes available for conversion to NAD. A high dietary intake of leucine inhibits the synthesis of NAD from tryptophan, without affecting the utilization of niacin obtained directly from the diet. Leucine is found in high concentration in sorghum. In parts of India, where sorghum is the staple food, leucine may be pellagragenic at times of food shortage when intakes of both tryptophan and niacin are very low [26].

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10

Vitamin B_6

10.1 Background

Pyridoxal phosphate (PLP), the physiologically active B_6 vitamer, serves as a coenzyme for over 100 different enzymes involved in almost all aspects of cellular biochemistry and metabolism. Vitamin B_6 also plays an important role in the development and maintenance of a competent immune system. Because of this versatility, vitamin B_6 is crucial for normal growth, development, and homeostasis.

In amino acid metabolism, vitamin B_6 is required in a variety of enzymes concerned with the interconversion of amino acids, the synthesis of nonessential amino acids, and the metabolism of amino acids in excess of the amounts required for protein synthesis. Aminotransferases facilitate the transfer of amino groups between amino acids and keto acids, and thus represent an important link among amino acid, carbohydrate, and fat metabolism. Many neurotransmitters are formed by the PLP-dependent decarboxylation of amino acids. PLP is the coenzyme for glycogen phosphorylase, a key enzyme in the utilization of liver and muscle glycogen reserves. In lipid metabolism, PLP is the coenzyme for phosphatidylserine decarboxylation. PLP is also involved in the synthesis of sphingosine, and a deficiency of vitamin B_6 leads to impaired development of brain lipids and incomplete myelination of nerve fibers in the central nervous system. Cellular levels of thymine, one of the four constituent DNA bases, depend on PLP through its involvement as a coenzyme in folate metabolism.

Vitamin B_6 is widely distributed in foods, and any diet so poor as to be insufficient in this vitamin would most likely lack adequate amounts of other B-group vitamins. For this reason, a primary clinical deficiency of B_6 in the adult human is rarely encountered. The administration of the antagonist deoxypyridoxine to adult volunteers receiving diets low in vitamin B_6 resulted in lesions of the skin and mouth that resembled those of riboflavin and niacin deficiency. These symptoms responded to vitamin B_6 therapy, but did not respond to thiamin, riboflavin, or niacin.

Chronic overdosing with vitamin B_6 has been reported to cause peripheral neuropathy in women. Acute toxicity of the vitamin, however, is low.

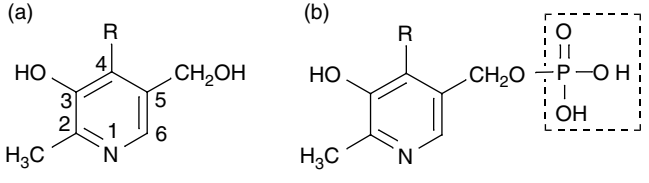
10.2 Chemical Structure, Biopotency, and Physicochemical Properties

10.2.1 Structure and Potency

Vitamin B_6 is the generic descriptor for all 3-hydroxy-2-methylpyridine derivatives that exhibit qualitatively in rats the biological activity of pyridoxine. Six B_6 vitamers are known, namely pyridoxine or pyridoxol (PN), pyridoxal (PL), and pyridoxamine (PM), which possess, respectively, alcohol, aldehyde, and amine groups in the 4-position; their respective 5′-phosphate esters are designated as PNP, PLP, and PMP (Figure 10.1). Pyridoxine is systematically named as 3-hydroxy-4,5-bis(hydroxymethyl)-2-methylpyridine and is available commercially as its hydrochloride salt, PN·HCl ($C_8H_{11}O_3N$·HCl, MW = 205.6). PN·HCl is the only form of vitamin B_6 used in the fortification of foods. The six B_6 vitamers are considered to have approximately equivalent biopotency on the basis of their ultimate conversion to coenzymes.

In its role as a coenzyme, PLP is bound tightly to the apoenzyme by a Schiff base (aldimine) linkage formed through condensation of the 4-carbonyl group with the ε-amino group of specific lysine residues. The resultant Schiff base compound may be subject to nucleophilic attack by a neighboring amino, sulfhydryl, or imidazole group to form a substituted aldamine (Figure 10.2) [1]. All these forms of vitamin B_6 are reversibly bound and easily dissociated from the apoenzymes.

An ubiquitous bound form of PN that occurs in plant tissues is 5′-*O*-(β-D-glucopyranosyl)-pyridoxine (Figure 10.3), abbreviated to PN-glucoside [2]. Two minor derivatives, in which an organic acid is esterified to the C-6 position of the glucose moiety of PN-glucoside, have been



R	Vitamers
CH_2OH	PN, PNP
CHO	PL, PLP
CH_2NH_2	PM, PMP

FIGURE 10.1
Structures of vitamin B_6 compounds showing (a) nonphosphorylated and (b) phosphorylated forms.

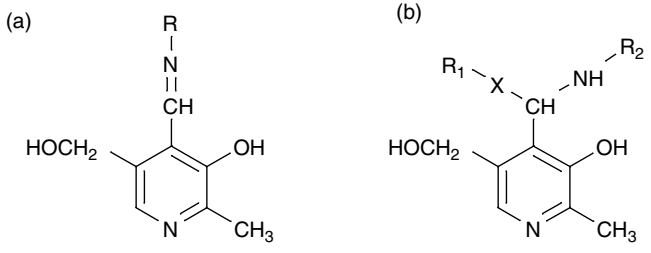


FIGURE 10.2
Potential interactions involving pyridoxal and amino groups of food proteins. (a) Schiff base, and (b) substituted aldamine (X = amino, sulfhydryl, or imidazole). Analogous reactions would occur with PLP and proteins.

identified in legume seedlings [2]. A more complex derivative of PN-glucoside containing cellobiose and 5-hydroxydioxindole-3-acetic acid moieties has been identified as a major form of vitamin B_6 in rice bran and legumes [3].

10.2.2 Physicochemical Properties

10.2.2.1 Appearance and Solubility

PN·HCl is a white, odorless, crystalline powder with a salty taste and an mp of 204–206°C (with decomposition). It is readily soluble in water (1 g/5 ml), sparingly soluble in ethanol (1 g/100 ml), and very slightly soluble in diethyl ether and chloroform. The pH of a 5% aqueous solution is 2.3–3.5; pK_a values are 5.0 and 9.0 (25°C). The free base is readily soluble in water and slightly soluble in acetone, chloroform, and diethyl ether.

In aqueous solutions, the B_6 vitamers exist in a variety of equilibrium forms, depending upon the pH [4]. The structures of some of these forms are shown in Figure 10.4. PN exists as the cation in acidic solutions, as the anion in alkaline solutions, and primarily as the electrically neutral

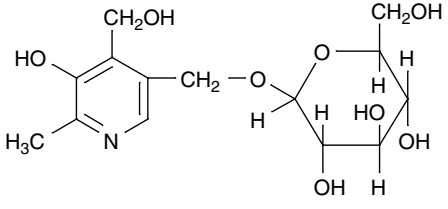


FIGURE 10.3
Structure of 5′-*O*-(β-D-glucopyranosyl)-pyridoxine.

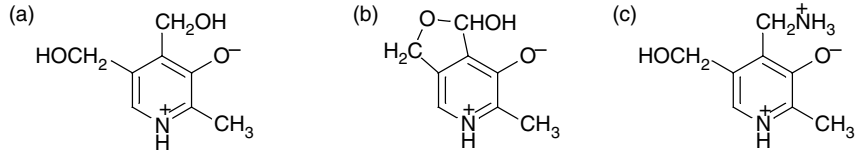


FIGURE 10.4
Ionic forms of vitamin B_6 that exist in aqueous solution at neutral pH. (a) Pyridoxine (dipolar ion), (b) pyridoxal hemiacetal (dipolar ion), and (c) pyridoxamine (tripolar ion).

dipolar ion at neutral pH. PM also exists as the cation in acidic solutions and the anion in alkaline solutions, but at neutral pH the predominant form is the tripolar ion. The situation is more complicated with PL owing to the possibility of hemiacetal formation or hydration. At neutral pH, the predominant form of PL is the hemiacetal dipolar ion.

10.2.2.2 Stability in Aqueous Solution

Saidi and Warthesen [5] observed that no significant degradation of PN·HCl took place when aqueous solutions protected from light were held at 40 and 60°C for up to 140 days at pH levels ranging from 4 to 7. Under the same conditions, PM·2HCl showed a trend of increasing loss with increasing pH, while PL·HCl showed a marked loss at pH 5, but only a moderate loss above and below that pH value. Ang [6] showed that PN·HCl was the most stable and PM·2HCl the least stable of the three vitamers after exposure of aqueous solutions to normal laboratory light at different pH values. Low-actinic amber glassware or gold fluorescent lighting protected solutions of PLP from photodegradation, but low-ultraviolet "white" fluorescent lamps failed to do so [7]. The principal photodegradation product of PLP is 4-pyridoxic acid 5′-phosphate [8].

Shephard and Labadarios [9] investigated the degradation of vitamin B_6 standard solutions after experiencing difficulties in the reproducibility of B_6 vitamer standard determinations in an HPLC method. Of the crystalline vitamer standards used (PN·HCl, PM·2HCl, PL·HCl, PLP, and PMP·HCl), all but PLP were stable when stored individually in the dark. Solutions of PLP in water were stable when stored frozen (−20°C) at a concentration of 1 mg/ml (pH 3.3). However, storage at room temperature in the dark for 24 h of a laboratory working solution (1 μg/ml) either in sodium acetate buffer (pH 5.5) or in distilled water (pH 6.1) resulted in a 20 or 95% loss of PLP, respectively, due to hydrolysis to PL. When prepared in 0.01 *M* HCl, PLP was stable for at least 2 days at room temperature. It was shown that Schiff base formation

and transamination reactions can occur between the vitamers themselves at room temperature or below. To prevent these reactions, vitamer solutions must be stored separately and compound standards must be prepared before use in a fairly acid medium (pH 3.0).

10.3 Vitamin B_6 in Foods

10.3.1 Occurrence

Vitamin B_6 is present in all natural unprocessed foods, with yeast extract, wheat bran, and liver containing particularly high concentrations. Other important sources include whole-grain cereals, nuts, pulses, lean meat, fish, kidney, potatoes, and other vegetables. In cereal grains, over 90% of the vitamin B_6 is found in the bran and germ [10], and 75–90% of the B_6 content of the whole grain is lost in the milling of wheat to low-extraction flour [11]. Thus, white bread is considerably lower in vitamin B_6 content than is whole wheat bread. Milk, eggs, and fruits contain relatively low concentrations of the vitamin. Table 10.1 [12] gives the vitamin B_6 content of selected foods.

In raw animal and fish tissue, the major form of vitamin B_6 is PLP, which is reversibly bound to proteins as Schiff bases and substituted aldamines. PN and PNP are virtually absent in animal tissues, one exception being liver tissue, in which they are detectable at very low levels. Using a nonhydrolytic extraction procedure and HPLC, the vitamin B_6 content of whole pasteurized homogenized milk was found to comprise the following vitamers: PL (53%), PLP (23%), PMP (12%), and PM (12%); PN was not detected [13]. Siegel et al. [14] estimated the free and total vitamin B_6 content of milk by assaying aliquots before and after acid hydrolysis; the difference indicated the amount of bound vitamin, which was found to be 14% of the total. PNP does not occur to any measurable extent in natural products.

Roth-Maier et al. [15] reported that the vitamin B_6 in all tested foods of plant origin occurs in the form of PN and PM, except for corn (maize) where more than 50% of the vitamin B_6 content occurred as PL. A proportion of the PN in plant tissues may be present as PN-glucoside and/or more complex derivatives of PN-glucoside. No generalizations can be made as to one group of foods consistently having a high PN-glucoside content. Typical sources of PN-glucoside (expressed as a percentage of the total vitamin B_6 present) are bananas (5.5%), raw broccoli (35.1%), raw green beans (58.5%), raw carrots (70.1%), and orange juice (69.1%) [16].

TABLE 10.1

Vitamin B_6 Content of Various Foods

Food	mg Vitamin B_6/100 g Edible Portion
Cow milk, whole, pasteurized	0.06
Cheese, cheddar, average	0.15
Egg, chicken, whole, raw	0.12
Wheat flour, wholemeal	0.50
Wheat flour, white, plain	0.15
Rice, brown, raw	N
Beef, trimmed lean, raw, average	0.53
Lamb, trimmed lean, raw, average	0.30
Pork, trimmed lean, raw, average	0.54
Chicken meat, raw	0.38
Liver, lamb, fried	0.53
Cod, raw, fillets	0.18
Potato, main crop, old, average, raw	0.44
Chick peas, dried, raw	0.53
Soybeans, dried, raw	0.38
Lentils, green and brown, whole, dried, raw	0.93
Red kidney beans, dried, raw	0.40
Peas, raw	0.12
Broccoli, raw	0.14
Cauliflower, raw	0.28
Bananas	0.29
Orange juice, unsweetened	0.07
Peanuts, plain	0.59
Yeast extract	1.60

Note: N, the vitamin is present in significant quantities but there is no reliable information on the amount.
Source: From Food standards Agency, McCance and Widdowson's. The composition of foods, 6th summary ed., Royal Society of Chemistry, Cambridge, 2002. With permission.

10.3.2 Stability

In general, vitamin B_6 is unstable during prolonged heat treatment, and not sensitive to oxidation by air. The stability of vitamin B_6 toward food processing and storage depends to some extent on the B_6 vitamer content of the food, because PN is considerably more stable to heat than PL or PM [17]. This means that plant foods (containing mostly PN) are likely to be rather more stable than foods of animal origin (containing mostly PL or PM). Interconversions between the aldehyde (PL and PLP) and amine (PM and PMP) B_6 vitamers via reversible interaction with proteins or carbonyl compounds occur during the processing or storage of meat and dairy products [18]. The kinetics of vitamin B_6 degradation have been discussed by Gregory [19].

Raab et al. [20] reported that water-blanching caused a loss of 19–24% of vitamin B_6 from lima beans, while steam-blanching caused a loss of 13–17%. Ekanayake and Nelson [21] measured total vitamin B_6 (acid-hydrolyzed) and available vitamin B_6 (enzyme-treated) in a study of thermally processed limas beans. About 20% of the PN content was lost during the blanching of the beans, but the subsequent heat sterilization did not affect the total vitamin B_6 content. There was, however, a 50% drop in available vitamin B_6. The data suggested that the PL and PLP of the heat-processed beans were bound in some way, and this prevented the vitamers from being released by digestion. A 55% loss of vitamin B_6 activity in canned lima beans relative to frozen lima beans was also reported by Richardson et al. [22] using a rat growth assay. Sterilization and subsequent 5-year storage did not affect the vitamin B_6 content of canned military rations [23]. Thermal processing had little effect on the concentration of PN-glucoside in alfalfa sprouts, demonstrating the stability of the glycosidic bond as well as the stability of the PN moiety of the conjugate [24]. There is good stability of vitamin B_6 in wheat flour during bread making [25].

Most of the research into the effects of food processing upon vitamin B_6 bioavailability has been directed to the heat-sterilization of canned evaporated milk by retort processing. The heat-sterilization of milk and unfortified infant formula resulted in losses of 36–67% of naturally occurring vitamin B_6 using *Saccharomyces cerevisiae* as the assay organism, the losses appearing progressively during the first 10 days after processing [26]. The reduction of vitamin activity was due mainly to the loss of the predominant B_6 vitamer, PL. In the same study, PM and PL added to milk were degraded to roughly the same extent as natural vitamin B_6, whereas added PN·HCl was not appreciably destroyed by autoclaving. Gregory and Hiner [17] reported that PN·HCl is stable during retort processing, whereas PM·2HCl and PL·HCl are 2.5- to 3.5-fold less stable. Vitamin B_6 losses are not as great when processing spray-dried milk products and condensed milk as when heat sterilization is employed [26]. Ford et al. [27] reported no loss of vitamin B_6 during the ultra-high temperature (UHT) processing of milk, but up to 50% of the vitamin was lost during 90 days' storage. The UHT processing (heating at 110–112°C for 15–20 min) was unlikely to be responsible for these storage losses since similar losses during storage were found with raw milk held at −30°C. Conventional pasteurization of milk has no effect on the vitamin B_6 content [28].

Losses of indigenous vitamin B_6 in foods during storage have been observed [22,29–31]. The decrease in vitamin B_6 activity on storage of beef liver, boned chicken, cabbage, and green beans was not observed in lima beans and sweet potatoes using a rat growth assay [22]. Using selective extraction techniques and HPLC, Addo and Augustin [32]

showed that PLP and PM in stored potatoes remained unchanged, whereas the PN-glucoside increased more than fourfold, and PN was reduced by half. These observations indicated a possible synthesis of vitamin B_6 during storage. The storage of various foods at −18°C for 5 months resulted in a 19–60% decrease in total vitamin B_6. The loss was significantly greater in foods of animal origin (an average of 55%) than in plant-derived foods [33]. Unlike the loss in indigenous vitamin B_6 during storage, the stability of PN added to fortify various products is high. Bunting [34] reported the retention of 90–100% of the PN added to corn meal and macaroni following storage at 38°C and 50% relative humidity for 1 yr. There is good stability of vitamin B_6 in fortified cereal products after storage [35–37]. However, only 18–44% of the vitamin B_6 in a commercial rice-based PN-fortified breakfast cereal was found to be biologically available using a rat bioassay [38].

In a report on the influence of cooking [39], stewing reduced the content of total vitamin B_6 by 56% and braising by 58% in beef and by 58 and 45%, respectively, in pork. Part of the loss was due to release of meat juice. With meat juice included, the percentage of vitamin retained was 80 and 63% in beef, and 68 and 62% in pork. Losses of the total vitamin B_6 in vegetables after boiling in water were between 16 and 61%; losses were lower after steaming (between 10 and 24%) due to less leaching.

The research effort has led to the general conclusion that the thermal processing of milk products and other animal-derived foods promotes the chemical reduction of protein-bound Schiff base forms of PL and PLP to peptide-linked ε-pyridoxyllysine (Figure 10.5) or its 5′-phosphate derivative. This reaction has been demonstrated to take place in a model food system during thermal processing [40], in evaporated milk, chicken liver, and muscle that were autoclaved to simulate retort processing [18] and in a dehydrated model food system that was stored at 37°C and 0.6 water activity (A_w) for 128 days [41].

Protein-bound ε-pyridoxyllysine can be phosphorylated by pyridoxal kinase, the enzyme that phosphorylates the free B_6 vitamers after they

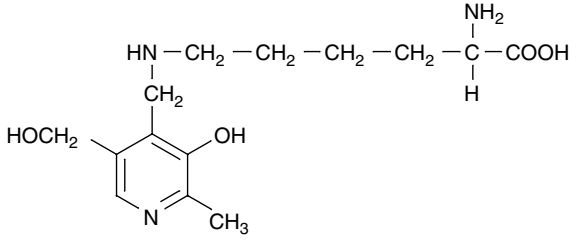


FIGURE 10.5
Structure of ε-pyridoxyllysine.

have been absorbed into the enterocytes [42]. The 5′-phosphorylated ε-pyridoxyllysine can then be oxidized to PLP by pyridoxamine (pyridoxine) 5′-phosphate oxidase, the enzyme responsible for converting PMP and PNP to PLP [42]. These two enzyme reactions provide a metabolic basis for the observed 50% vitamin B_6 activity of ε-pyridoxyllysine relative to the molar potency of PN [43]. When fed to rats at low levels in vitamin B_6-deficient diets, ε-pyridoxyllysine exhibited antivitamin B_6 activity, which could be counteracted by dietary supplementation with PN [43]. This antivitamin B_6 activity may be at least partly attributable to the competitive inhibition of pyridoxal kinase. This antagonistic effect of ε-pyridoxyllysine, when present in vitamin B_6-deficient diets, may have been responsible for the severe deficiency developed in infants fed unfortified, heat-sterilized, canned infant formulas [44].

10.3.3 Applicability of Analytical Techniques

In foods of animal origin, PL, PLP, PM, and PMP are found as a result of interconversion of aldehyde and amine forms during processing and storage. Plant-derived foods contain mostly PN, a significant proportion of which may be present as PN-glucoside. PNP does not occur to any significant extent in natural products. PN·HCl is used in the fortification of foods. The PLP that is bound to the apoenzyme by a Schiff base in animal tissues is presumed to be totally bioavailable. The PN-glucoside and other conjugated forms found in plant-derived foods appear to be largely unavailable to humans.

The total available vitamin B_6 activity of a food or diet containing all forms of the vitamin is measured most conclusively by an animal assay. By feeding the material directly, both the free and bound forms can exert their combined effect. The free vitamers are equally active mole for mole when fed to animals as separate supplements in solution. However, when mixed in the ration, PM and PL are less active than PN [45]. Both the rat and the chick are sensitive to influences of diet composition, especially with respect to fermentable carbohydrates, which provide the means for vitamin B_6 production by microflora in the large intestine. The utilization of microbially produced vitamin B_6 by coprophagy or direct intestinal absorption can bias quantitative bioassays.

Total vitamin B_6 activity is usually estimated microbiologically using a turbidimetric yeast assay; the radiometric microbiological assay is a more recent innovation. With the aid of HPLC, it is possible to measure simultaneously all of the B_6 vitamers likely to be present in a food extract, together with the inactive metabolite 4-pyridoxic acid. For routine food analysis, it is customary to hydrolyze the phosphorylated forms and to determine PN, PL, and PM, each of which represents the sum of the

phosphorylated and nonphosphorylated forms. This approach is valid because, in humans, all six vitamers exhibit equal biological activity on a molar basis. The chromatographic separation of three compounds, rather than six, is obviously less demanding of the HPLC system, and the data obtained can be compared with data obtained by microbiological assay, in which dephosphorylation is obligatory. Traditional methods used to assay vitamin B_6 in food products may overestimate the bioavailability of the vitamin due to the use of extraction techniques that completely liberate PN from glycosylated forms. The use of selective extraction procedures allows the various bound forms of vitamin B_6 to be determined by HPLC. Gregory [2] has critically reviewed methods for determination of vitamin B_6 in foods and other biological materials.

No international unit of vitamin B_6 activity has been defined, and analytical results are expressed in weight units (mg) of pure PN·HCl. All six vitamers are considered to have approximately equivalent biological activity in humans as a result of their enzymatic conversion to the major coenzyme form, PLP.

10.4 Intestinal Absorption

The following discussion of absorption is taken from a more detailed account in a book by Ball [46] published in 2004.

Humans cannot synthesize vitamin B_6 and thus must obtain the vitamin from exogenous sources via intestinal absorption. The intestine is exposed to vitamin B_6 from two sources: (1) the diet and (2) the bacterially synthesized vitamin B_6 in the large intestine. Whether or not the latter source of vitamin B_6 is available to the host tissues (apart from the colonic epithelial cells) in nutritionally significant amounts is unknown.

Vitamin B_6 is present in foods mainly as PN, PLP, and PMP vitamers. In many fruits and vegetables, 30% or more of the total vitamin B_6 is present as PN-glucoside. The binding of PLP to protein through aldimine (Schiff base) and substituted aldamine linkages is reversibly dependent on pH, the vitamin–protein complexes being readily dissociated under normal gastric acid (low pH) conditions. The release of PLP from its association with protein is an important step in the subsequent absorption of vitamin B_6, as binding to protein inhibits the next step, hydrolysis of PLP by alkaline phosphatase [47]. It would appear, therefore, that the widespread practice of raising the postprandial gastric and upper small intestinal pH by the use of pharmaceutical antacids may impair vitamin B_6 absorption.

Physiological amounts of PLP and PMP are largely hydrolyzed by alkaline phosphatase in the intestinal lumen before absorption of free

PL and PM [48,49]. When present in the lumen at nonphysiological levels that saturate the hydrolytic enzymes, substantial amounts of PLP and PMP are absorbed intact, but at a slower rate than their nonphosphorylated forms.

The absorption of PN, PL, and PM takes place mainly in the jejunum and is a dynamic process involving several inter-related events. The vitamers cross the brush-border membrane by simple diffusion. In humans, PM is absorbed more slowly or metabolized differently, or both, than either PL or PN [50]. Within the enterocyte, PN, PL, and PM are converted to their corresponding phosphates by the catalytic action of cytoplasmic pyridoxal kinase, and transaminases interconvert PLP and PMP. The conversion of a particular vitamer to other forms by intracellular metabolism creates a concentration gradient across the brush border for that vitamer, thus enhancing its uptake by diffusion [51]. The phosphorylated vitamers formed in the cell are largely dephosphorylated by nonspecific phosphatases, thus permitting easy diffusion of B_6 compounds across the basolateral membrane. The major form of vitamin B_6 released to the portal circulation is the nonphosphorylated form of the vitamer predominant in the intestinal lumen.

Part of the PN-glucoside in plant-derived foods is hydrolyzed to PN and glucose by brush-border and cytosolic intestinal β-glucosidases; the PN is conveyed to the liver where it is converted to biologically active PLP. Another part of dietary PN-glucoside is absorbed intact by simple diffusion, but not metabolized by the liver. A small part is unabsorbed and eliminated with the feces.

10.5 Bioavailability

10.5.1 Bioavailability of Vitamin B_6 in Foods

Gregory [52] discussed factors affecting the bioavailability of vitamin B_6 in foods and presented a critical assessment of the methodology. Inherent problems with rat bioassays make them unreliable for the determination of vitamin B_6 bioavailability of foods and attention has turned to the use of protocols with human subjects. The principal indices of B_6 bioavailability are urinary excretion of 4-pyridoxic acid and total vitamin B_6, and plasma PLP concentration [53].

The bioavailability of vitamin B_6 in foods is highly variable, owing largely to the presence of poorly utilized PN-glucoside in plant tissues. As expected, vitamin B_6 generally has a lower availability from plant-derived foods than from animal tissues [54]. In humans, the vitamin B_6 from whole wheat bread and peanut butter was 75 and 63%, respectively,

as available as that from tuna [55]. The vitamin in soybeans was 6–7% less available than that in beef [56]. Kies et al. [57] obtained experimental data indicating that vitamin B_6 provided by the bran fractions of wheat, rice, and corn (maize) is unavailable to humans. On the basis of plasma PLP levels in male human subjects, the availability of the vitamin in an average American diet ranged from 61 to 81%, with a mean of 71% [58].

Roth-Maier et al. [15] determined the prececal digestibility of endogenous vitamin B_6 in selected foods and feedstuffs using pigs subjected to an end-to-end ileo-rectal anastomosis. The foods selected were boiled eggs, bananas, white cabbage, corn, milk powder, stewed fish, barley, boiled soybeans, boiled brown rice, wheat bran, brewer's yeast, rye, and soybean meal. In addition, vitamin B_6-free supplementary diets were individually formulated and combined with the test food to provide adequate amounts of nutrients, minerals, and vitamins. The results are presented in Table 10.2. Prececal digestibility for PN ranged between 14% from corn and 98% from white cabbage and boiled

TABLE 10.2

Prececal Digestibility (%) of the Vitamin B_6 Vitamers (Pyridoxine, Pyridoxal, and Pyridoxamine) from Different Food Sources

Food Sources	Number of Observations (n)	Prececal Digestibility (%)		
		Pyridoxine (PN)	Pyridoxal (PL)	Pyridoxamine (PM)
Eggs, boiled	3	*	82 ± 3^{a}	27 ± 6^{d}
White cabbage	3	98 ± 1^{a}	69 ± 2^{b}	76 ± 2^{b}
Bananas	3	95 ± 1^{a}	*	91 ± 1^{a}
Corn (maize)	2	14 ± 8^{b}	79 ± 5^{a}	64 ± 4^{c}
Fish, stewed (Alaska pollack)	6	22 ± 31^{d}	89 ± 1^{a}	85 ± 2^{a}
Milk powder	6	85 ± 17^{a}	87 ± 5^{a}	75 ± 5^{a}
Brown rice, boiled	6	43 ± 13^{c}	*	*
Soybeans, boiled	6	98 ± 2^{a}	46 ± 8^{b}	32 ± 16^{b}
Barley	6	91 ± 3^{a}	*	36 ± 14^{b}
Wheat bran	6	69 ± 2^{b}	*	26 ± 21^{b}
Brewer's yeast, dried	3	78 ± 8	*	80 ± 5^{a}
Rye	3	71 ± 2	41 ± 21^{b}	29 ± 21^{b}
Soybean meal	3	85 ± 1	82 ± 11^{a}	57 ± 10^{a}

Note: Data represent mean $\pm$ SD. Significantly different means are denoted with different superscripts.

*Intake of the vitamin B_6 vitamers below the detection limit.

Source: From Roth-Maier, D.A., Kettler, S.I., and Kirchgessner, M., *Int. J. Food Sci. Nutr.*, 53, 171, 2002. With permission.

soybeans; for PL between 41% from rye and 89% from stewed fish; and for PM between 27% from boiled eggs and 91% from bananas.

10.5.2 Effects of Alcohol

The excessive consumption of alcohol meets most of the human energy needs and decreases food intake by as much as 50%. Alcoholic beverages that replace food are practically devoid of vitamin B_6 and the alcoholic is therefore likely to be consuming a diet that is deficient in the vitamin.

Chronic, excessive alcohol ingestion can interfere with the normal processes of vitamin B_6 metabolism, thus leading to an increased requirement for the vitamin [59]. The conversion of intravenously administered PN to PLP in the plasma is impaired in alcoholic patients, suggesting that alcohol or its oxidation products may interfere directly with the metabolism of vitamin B_6. There is *in vitro* evidence that acetaldehyde facilitates the dissociation of PLP from its binding with protein, thereby making the PLP available for hydrolysis by membrane-bound alkaline phosphatase [60]. Thus the generation of acetaldehyde associated with alcoholism accelerates the degradation of PLP, lowering plasma concentrations and also body stores. Ethanol may also stimulate the urinary excretion of nonphosphorylated B_6 vitamers [60].

Absorption of vitamin B_6 from food is significantly impaired in alcoholic patients with liver disease, although such patients are able to absorb synthetic vitamin B_6 normally. Liver disease may also impair the ability of the liver to synthesize PLP.

10.5.3 Effects of Dietary Fiber

Experimental studies using rats or chicks have shown that dietary fiber in purified or semi-purified form does not appear to be a major factor influencing the bioavailability of vitamin B_6 in animals. A wide variety of dietary fiber material (polysaccharides, lignin, and wheat bran) did not bind or entrap B_6 vitamers *in vitro* [61] and in a rat jejunal perfusion study, cellulose, pectin, and lignin did not adversely affect vitamin B_6 absorption [62]. Cellulose, pectin, and bran had little or no effect on the availability of vitamin B_6 in a chick bioassay [63] and neither cellulose nor the indigestible component in wheat bran impaired the bioavailability in rats [64].

Little is known about the possible inhibitory effect of dietary fiber on the bioavailability of vitamin B_6 in humans. The availability of vitamin B_6 from whole wheat bread was only 5–10% less than that from white bread supplemented with vitamin B_6 [65], and the addition of wheat bran (15 g/day) to human diets resulted in only a minor decrease

(maximum of 17%) in availability of the vitamin [66]. Supplementation of human diets with pectin (15 g supplement/day) had no effect on vitamin B_6 utilization [67]. Although the pectin supplement stimulated the synthesis of vitamin B_6 by the intestinal microflora, the newly synthesized vitamin was apparently not absorbed by the host. An inhibitory effect of a component(s) of orange juice upon vitamin B_6 absorption has been shown using perfused segments of human jejunum [68]. Absorption of the vitamin from orange juice was only about 50% of that from synthetic solutions. Further investigations [69] revealed that vitamin B_6 in orange juice was bound to a small dialyzable molecule, which was heat stable and nonprotein in nature.

Since vegetarian diets are relatively high in fiber, one might predict that such a diet may lead to a reduced bioavailability of vitamin B_6. However, two independent studies have shown no significant difference in vitamin B_6 status between vegetarian and nonvegetarian women [70,71]. Consumption of plant-derived foods would most likely exert a positive effect on vitamin B_6 intake and status because of their higher vitamin B_6 to protein ratio.

10.5.4 Glycosylated Forms of Vitamin B_6

A major fraction of the total vitamin B_6 content of many plant-derived foods consists of glycosylated forms of PN, most frequently PN-glucoside. This compound has been found in a variety of vegetables, fruits, and grain products, including carrots, broccoli, green beans, bananas, orange juice, wheat bran, and rice bran [16,72]. PN-glucoside accounted for 5–70% of the total vitamin B_6 present in selected vegetables and fruits [16] and represented 10–15% of the total vitamin B_6 in typical American mixed diets [73]. The glucoside was not detected in animal-derived foods, including meats, human milk, and cow's milk [16].

In a rat bioassay, the bioavailability of PN-glucoside was 10–34% relative to that of free PN [74]. Vitamin B_6 status has no influence on the bioavailability of PN-glucoside in the rat [75]. The relative bioavailability of PN-glucoside differed among rodent species: 69% in mice, 70% in hamsters, and 92% in guinea pigs [76]. In human subjects, using stable isotopic methods, the relative bioavailability of PN-glucoside was ca. 50% [77]. The potential effect of the incomplete bioavailability of PN-glucoside was demonstrated in a study of women in which chronic intake of a diet high in the glucoside led to significant decreases in indicators of vitamin B_6 status [52]. However, vegetarian women did not demonstrate any significant difference in vitamin B_6 status compared with nonvegetarian women [70,71], suggesting that, in general, there may be little nutritional significance to the human consumption of glycosylated vitamin B_6.

Bills et al. [78] investigated the relationship between the PN-glucoside content of foods and vitamin B_6 bioavailability to ascertain if the PN-glucoside content could be used as an *in vitro* index of vitamin B_6 bioavailability. HPLC measurements of urinary 4-pyridoxic acid excretion were used to assess vitamin B_6 bioavailability in human subjects. A strong inverse correlation between percent PN-glucoside and bioavailability was found in six of the ten foods examined (walnuts, bananas, tomato juice, spinach, orange juice, and carrots) but not in others (wheat bran, shredded wheat, broccoli, and cauliflower). These data indicate that the PN-glucoside content of foods is not a reliable index of vitamin B_6 bioavailability as previously supposed by Kabir et al. [79].

PN-glucoside is adequately absorbed by the rat intestine by simple diffusion [80]. The extent of absorption is approximately half that of PN, but its hydrolysis to PN and glucose is limited, and most of the compound is excreted unchanged in the urine [24]. Thus, the limiting factor in the utilization of PN-glucoside is its enzymatic hydrolysis and not its absorption. The *in vivo* utilization of PN-glucoside is much greater when the compound is administered orally to human subjects than when injected intravenously, implicating the intestine as a major site of β-glucosidase activity [73].

The nonphosphorylated B_6 vitamers released from the enterocytes after absorption of dietary vitamin B_6 are conveyed in the portal blood and taken up by the liver. The liver plays a central role in vitamin B_6 metabolism. Hepatic vitamin B_6 metabolism involves phosphorylation of B_6 vitamers catalyzed by pyridoxal kinase, oxidation of PNP and PMP to PLP by pyridoxamine (pyridoxine) 5′-phosphate oxidase, and interconversion of PLP and PMP through transamination reactions. The principal forms of vitamin B_6 in liver are PLP and PMP. PNP is usually present in only trace quantities because of its rapid oxidation to PLP. Vitamin B_6 vitamers (mainly PL and PLP) derived from the partial hydrolysis and metabolism of ingested PN-glucoside serve as a limited source of the vitamin secreted in the milk of lactating rats; PN-glucoside itself is not secreted in milk. Thus the vitamin B_6 present in rat milk is expected to be highly available to the pup [81].

Nakano et al. [77] reported that PN-glucoside partially inhibits the utilization of co-ingested PN in humans, although the effect was less pronounced than that previously seen in rats [82–84]. The inhibitory effect may be due to the competitive inhibition of hepatic uptake of PN by PN-glucoside, as demonstrated in isolated rat hepatocytes [85]. PN-glucoside does not inhibit pyridoxal kinase and pyridoxamine (pyridoxine) 5′-phosphate oxidase [85], the two key enzymes in the metabolic utilization of vitamin B_6. Nakano et al. [77] concluded that the inhibitory effect of PN-glucoside on PN utilization probably has little nutritional significance when typical U.S. diets are consumed.

The uptake of PN-glucoside by isolated rat hepatocytes is only about 20% that of equimolar PN [85]. Zhang et al. [85] postulated that, on entry into the hepatocyte, PN-glucoside undergoes hydrolysis by a broad-specificity β-glucosidase. However, it was later demonstrated unequivocally that rat liver does not hydrolyze PN-glucoside [84,86]. Evidently, the broad-specificity β-glucosidase in the liver does not act upon PN-glucoside, and the glucoside is rapidly cleared from the cell.

Enzyme activity capable of hydrolyzing PN-glucoside was found in the cytosolic fractions of rat small intestinal mucosa and kidney [84]. The ability of kidney to hydrolyze PN-glucoside may contribute to the post-absorptive release of PN for entry into vitamin B_6 metabolism. Contrary to previous results [84], intestinal hydrolytic activity toward PN-glucoside did not increase in vitamin B_6-deficient rats. Enzyme activity was, however, affected by the basal nutrient composition of the diets, regardless of dietary PN concentration [87].

McMahon et al. [88] identified and purified a novel cytosolic β-glucosidase, designated pyridoxine-5′-β-D-glucosidase hydrolase, from porcine intestinal mucosa. This enzyme, not broad-specificity β-glucosidase, was responsible for the hydrolysis of PN-glucoside immediately after absorption into the cytosolic compartment of the enterocyte. Partial characterization of the hydrolase revealed its ability to hydrolyze lactose and cellobiose (but not sucrose) in addition to PN-glucoside.

PN-glucoside hydrolase activity has also been found in the brush-border membrane fraction of rat small intestinal mucosa, accounting for 50–60% of hydrolytic activity in the mucosa [87]. The only known mammalian brush-border β-glucosidase is lactase-phlorizin hydrolase (LPH). This enzyme is primarily responsible for the hydrolysis of dietary lactose, but it can also hydrolyze PN-glucoside [89].

The intestinal absorption and subsequent fate of dietary PN-glucoside can be summarized as follows. A proportion of the ingested PN-glucoside is hydrolyzed at the brush-border membrane by LPH and absorbed as free PN and glucose. Another proportion is absorbed intact; of this, part is hydrolyzed to free PN by pyridoxine-5′-β-D-glucosidase hydrolase in the cytosol of enterocytes and part is excreted in the urine unchanged. A third proportion of the ingested PN-glucoside is neither absorbed nor hydrolyzed, and is eliminated in the feces.

Since lactose, the preferred substrate for hydrolysis by LPH, is a competitive inhibitor of PN-glucoside hydrolysis, the question is raised as to whether the utilization of dietary PN-glucoside would be reduced by co-ingested lactose. *In vitro* specific activities toward PN-glucoside and lactose were greater in brush border than in cytosol [90]. Brush-border hydrolytic activity toward PN-glucoside and lactose declined in concert with the decline in brush-border LPH activity that occurs after weaning in rats [90].

Cheng and Trumbo [91] reported that the utilization of PN-glucoside in pregnant rats was nearly equivalent to that of PN. This is in contrast to the situation in male rats, where PN-glucoside is poorly utilized relative to PN [75]. The increased utilization of PN-glucoside during pregnancy may be due to up-regulation of PN-glucoside-hydrolyzing enzymes in response to hormonal changes, ensuring a supply of highly available vitamin B_6 to the fetus. This finding in rats warrants investigation of PN-glucoside bioavailability in pregnant women.

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11

Pantothenic Acid

11.1 Background

The biological activity of pantothenic acid is attributable to its incorporation into the molecular structures of coenzyme A and acyl carrier protein. As a component of coenzyme A, pantothenic acid is essential for numerous reactions involved in the release of energy from carbohydrates, fats, and amino acids. In carbohydrate metabolism, the formation of acetyl-coenzyme A is necessary for introducing acyl groups into the tricarboxylic acid cycle. Other roles of acetyl-coenzyme A include its requirement for the acetylation of amino sugars, which are constituents of various mucopolysaccharides of connective tissues, and also for the acetylation of choline to form the neurotransmitter acetylcholine. Succinyl-coenzyme A is a precursor for porphyrin and hence for hemoglobin and cytochromes. Acyl carrier protein plays a major role in the biosynthesis of fatty acids.

Because of the widespread distribution of pantothenic acid in foods, a dietary deficiency of the vitamin is virtually impossible, apart from circumstances of severe malnutrition. When volunteers were fed a metabolic antagonist, omega-methylpantothenic acid, along with a diet low in pantothenic acid, the most persistent of the many symptoms were fatigue, headache, and the sensation of weakness. Reported neurological manifestations include numbness and "burning feet" syndrome.

Pantothenic acid has very low toxicity; even with oral amounts as high as 10–20 g of the calcium salt, the only reported problem was occasional diarrhea.

11.2 Chemical Structure, Biopotency, and Physicochemical Properties

11.2.1 Structure and Potency

Structures of pantothenic acid and compounds containing a pantothenate moiety are shown in Figure 11.1. Pantothenic acid ($C_9H_{17}O_5N$,

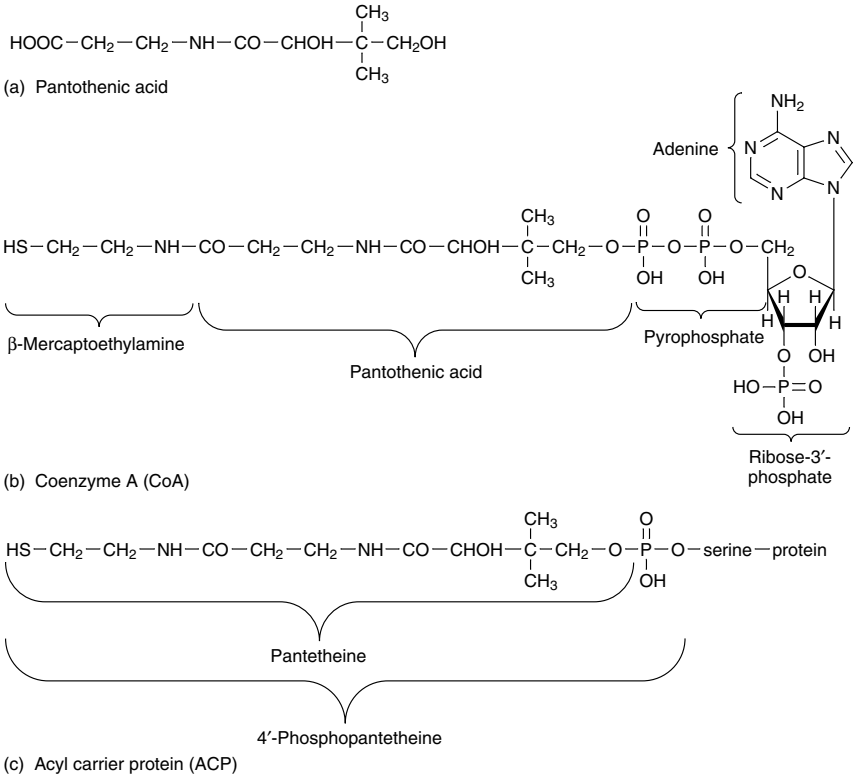

FIGURE 11.1
Structures of (a) pantothenic acid, (b) coenzyme A, and (c) acyl carrier protein.

MW = 219.2) comprises a derivative of butyric acid (pantoic acid) joined by a peptide linkage to the amino acid β-alanine. The molecule is optically active but only the D(+)-enantiomorph occurs in nature. Synthetic pantothenic acid is a racemic mixture and, because only the D isomer is biologically active, this fact must be considered if the DL mixture is to be used therapeutically. Pantothenic acid is a pale yellow oil which is extremely hygroscopic, and so is unsuitable for commercial application. For human food supplements, calcium D-pantothenate [$(C_9H_{16}O_5N)_2Ca$, MW = 476.5] is used.

11.2.2 Physicochemical Properties

11.2.2.1 Appearance and Solubility

Calcium pantothenate is a colorless, odorless, bitter-tasting, moderately hygroscopic, microcrystalline powder which decomposes at ca. 200°C.

The pH of an aqueous 5% (w/v) solution of calcium pantothenate is 7.2–8.0; the pK_a value is 4.4 (dissociation of the carbonyl group). Pantothenic acid is readily soluble in water and ethyl acetate and slightly soluble in diethyl ether; the calcium salt is more soluble in water (40 g/100 ml), only slightly soluble in ethyl acetate, and insoluble in diethyl ether.

11.2.2.2 Stability in Aqueous Solution

The stability of pantothenic acid and its calcium salt in aqueous solution is highly dependent on the pH. In contrast to other B-vitamins, pantothenic acid becomes more stable as the pH of the solution increases. Solutions of calcium pantothenate are most stable between pH 5 and 7 but, even so, are not stable to autoclaving, and therefore sterilization by ultrafiltration is necessary. Below and above these pH values, solutions of calcium pantothenate are thermolabile. Alkaline hydrolysis yields pantoic acid and β-alanine, whereas acid hydrolysis yields the gamma-lactone of pantoic acid [1]. Pantothenic acid is unaffected by atmospheric oxygen and light.

11.3 Pantothenic Acid in Foods

11.3.1 Occurrence

Pantothenic acid is widely distributed in foods of animal and plant origin. The vitamin is particularly abundant in liver, kidney, yeast, egg yolk, and broccoli, which contain more than 50 μg pantothenate/g dry weight. Exceptionally high levels are found in royal jelly (511 μg/g) and in ovaries of tuna and cod (2.32 mg/g) [2].

Pantothenic acid exists in foodstuffs in its free form, as well as bound in coenzyme A and acyl carrier protein. Pakin et al. [3] used selective enzymes and HPLC analysis to determine free and bound pantothenic acid in various foods (Table 11.1). Free pantothenic acid was found in all foods analyzed. Coenzyme A was the major form of the vitamin in yeast, pig liver, and peas, but this compound was not significantly present in avocado, carrots, French beans, or salmon. Several foods of both animal and plant origin contained significant amounts of bound pantothenic acid other than coenzyme A.

11.3.2 Stability

Pantothenic acid has good stability in most foods during processing, but is susceptible to leaching in the blanching of vegetables and home cooking. The vitamin content was higher in peas cooked in steam as

TABLE 11.1

Contents of Free and Bound Pantothenic Acid in Various Foods

Food	Concentration (μg/g)[a]		
	Free PA	PA Free and Bound in Coenzyme A	Total PA
Avocado	8.2 (0.2)[b]	8.4 (0.2)[b,c]	8.9 (0.3)[c]
Carrot	3.45 (0.05)[b]	3.6 (0.2)[b,c]	3.9 (0.3)[c]
French beans	1.8 (0.2)[b]	2.0 (0.2)[b]	2.5 (0.2)
Lentils	10.6 (0.4)	14.7 (0.4)	17.7 (0.3)
Peas	2.0 (0.2)	4.4 (0.2)	5.0 (0.2)
Spinach	0.80 (0.08)	1.10 (0.09)[c]	1.13 (0.03)[c]
Chicken meat	10.2 (0.2)	13.0 (0.1)	15.1 (0.8)
Pig liver	30.7 (0.1)	63 (2)	73 (2)
Salmon	11.2 (0.05)[b]	11.9 (0.4)[b]	15.3 (0.7)
Chicken egg	17.8 (0.2)	22.3 (0.5)[c]	23.0 (0.5)[c]
Powdered milk (fortified)	46.3 (0.7)	54 (2)	57.1 (0.9)
Yeast	24.1 (0.7)	73 (3)	80 (3)

Note: PA, pantothenic acid.

[a]Average of three determinations (standard deviation in parentheses).

[b,c]Not significantly different ($P > 0.05$) when the means of two samples are compared.

Source: From Pakin, C., Bergaetzlé, M., Hubscher, V., Aoudé-Werner, D., and Hasselmann, C., *J. Chromatogr. A*, 1035, 87, 2004. With permission from Elsevier.

compared with boiling [4]. Water blanching incurred more loss of pantothenic acid than steam blanching for spinach and broccoli. Free pantothenic acid was lost to greater extent than total pantothenic acid during water blanching of both vegetables [5]. Hoppner and Lampi [6] investigated the effect of pre-soaking procedures and cooking times on the retention of pantothenic acid (and also biotin) in 25 different dried legumes. Before cooking for 20, 90, or 150 min, pooled samples of each legume were subjected to either a short soak or a long soak. For the short soak, one volume of the legume in three volumes of water was brought to boil and boiled for 2 min, covered, and left to soak for 1 h while cooling to room temperature. For the long soak, one volume of the legume in three volumes of water was left to soak overnight (16 h) at room temperature. Legumes in the 20-min cooking category were also prepared without pre-soak. After cooking, samples were weighed, freeze-dried, finely ground, and analyzed for pantothenic acid content by microbiological assay. Legumes cooked for 20 min without pre-soaking retained more pantothenic acid than those subjected to pre-soaking. When pre-soaked, most of the legumes in the 20- and 90-min cooking group retained more pantothenic acid after the long soak than after the short soak with boiling.

This was true in only half the legumes in the 150-min cooking group. As shown in Table 11.2, average retentions for pantothenic acid in the long-soaked legumes were higher ($P < 0.05$) than those in the short-soaked legumes for the 20- and 90-min cooking times. There was no significant difference ($P > 0.05$) between the two pre-soaking treatments for legumes cooked for 150 min. The more severe short soak with boiling prior to cooking for 20 or 90 min probably liberated bound pantothenic acid, thereby increasing leaching of free vitamin into the soak water.

Retention of pantothenic acid in oven-roasted beef loin averaged 89%, with an average recovery in the drip of 19%. By comparison, average retention in oven-braised beef was 56%, with 44% recovered in the drip. Thus approximately twice as much pantothenic acid was transferred to the drip by braising as by roasting [7]. There is no appreciable loss of pantothenic acid during frozen storage of meat [8].

In an evaluation of data compiled by Orr [9], Schroeder [10] reported that the canning and freezing of foods incurs large losses of pantothenic acid. In canned foods of animal origin, losses ranged from 20 to 35%, and in canned vegetable foods from 46 to 78%. Freezing losses in animal foods ranged from 21 to 70%, and in vegetable foods from 37 to 57%. Grains lost 37–74% of the original pantothenic acid content during conversion to various cereal products, and meats lost 50–75% during conversion to comminuted products. Later studies [11] have also indicated large losses of pantothenic acid in many highly processed foods, including products made from refined grains, fruit products, and fat- or cereal-extended meats and fish. When stored at room temperature ($22 \pm 2^{\circ}C$), canned Dutch Army meals (consisting of meat, vegetables, pulses, and potatoes) lost approximately 50% of their pantothenic acid

TABLE 11.2

Effect of Pre-Soak Methods on the Mean Pantothenic Acid Retention in Dried Legumes After Cooking[a]

Cooking Time (min)	No Pre-Soak (%)	Short-Soak (%)	Long-Soak (%)	Statistical Difference
20	75.8 ± 15.0	—	—	
20		33.0 ± 6.6	43.8 ± 9.0	*
90		40.2 ± 7.7	58.1 ± 13.9	*
150		50.8 ± 14.6	55.1 ± 18.4	ns

Note: *Significant ($P < 0.05$); ns, not significant ($P > 0.05$).

[a]Values were derived from analysis of 25 different legumes.

Source: From Hoppner, K. and Lampi, B., Pantothenic acid and biotin retention in cooked legumes, *J. Food Sci.*, 58, 1084, 1993. With permission.

in 5 years of storage [12]. Losses of pantothenic acid during milk pasteurization, sterilization, and drying are usually less than 10% [13].

11.3.3 Applicability of Analytical Techniques

Pantothenic acid is routinely determined by microbiological assay. Gas chromatographic determination of the pantoyl lactone formed from pantothenic acid by acid hydrolysis has been applied to foodstuffs. HPLC methods have also been reported. Biospecific methods of analysis include the radioimmunoassay and the enzyme-linked immunosorbent assay (ELISA).

No international unit of pantothenic activity has been defined. Analytical results are generally expressed in weight units (mg) of pantothenic acid, using calcium pantothenate as the standard material: 1 mg of pantothenic acid is equivalent to 1.087 mg of calcium pantothenate.

11.4 Intestinal Absorption

The following discussion of absorption is taken from a more detailed account in a book by Ball [14] published in 2004.

Humans and other mammals cannot synthesize pantothenic acid and therefore they rely on dietary sources of the vitamin. Pantothenic acid is synthesized by the normal microflora in the large intestine, but the quantitative contribution of this endogenous vitamin to the host tissues is unknown.

11.4.1 Digestion and Absorption of Dietary Pantothenic Acid

Ingested coenzyme A is hydrolyzed in the intestinal lumen to pantetheine by the nonspecific action of pyrophosphatases and phosphatase. Pantetheine is then split into pantothenic acid and β-mercaptoethylamine by the action of pantotheinase secreted from the intestinal mucosa into the lumen [15]. Within the alkaline medium of the intestinal chyme, the vitamin exists primarily as the pantothenate anion.

Absorption of the free pantothenic acid takes place mainly in the jejunum. A so-called sodium-dependent multivitamin transporter (SMVT), which mediates placental and intestinal uptake of pantothenate, biotin, certain biotin analogs, and the essential metabolite lipoate, has been cloned from rat [16] and human [17] placenta and from rabbit intestine [18]. The functional characteristics of the cloned transporter are

similar to those observed in native intestinal membranes regarding substrate specificity, kinetics, and inhibitor profiles [19]. The transporter appears to interact primarily, though not exclusively, with the long side-chain of the substrate containing the carboxylate group, which is present in pantothenate, biotin, and lipoate. Messenger RNA transcripts of SMTV were shown to be present in all the tissues that were tested (intestine, liver, kidney, heart, lung, skeletal muscle, brain, and placenta), suggesting that this carrier protein may be involved in the uptake of pantothenate, biotin, and lipoate by all cell types [16]. The stoichiometry of the Na^+: vitamin cotransport appears to be 2:1, indicating an electrogenic transport process. Thus, both the Na^+ concentration gradient and the potential difference across the brush-border membrane drive the transport process.

Unlike other water-soluble vitamins which are absorbed by specific carrier-mediated mechanisms (ascorbic acid, biotin, and thiamin), the absorption of pantothenic acid is not adaptively regulated by its level of dietary intake [20]. Clear-cut pantothenic acid deficiency symptoms in humans are rarely found in practice and the vitamin is nontoxic at high doses. These factors could explain why a regulated absorption mechanism has not evolved for pantothenic acid.

11.4.2 Absorption of Bacterially Synthesized Pantothenic Acid in the Large Intestine

The normal microflora of the large intestine synthesizes pantothenic acid, but it is not known how much, if any, of this endogenous vitamin is available to the host tissues. In human subjects, absorption of pantothenic acid takes place equally well whether the vitamin is given orally or instilled directly into the lumen of the mid-transverse colon [21]. Said et al. [22] showed that human colonic NCM460 cells took up pantothenic acid by a Na^+-dependent, carrier-mediated process that was shared by biotin.

11.5 Bioavailability

Little information is at hand regarding the nutritional availability to the human of pantothenic acid in food commodities. Based on the urinary excretion of pantothenic acid, the availability for male human subjects ingesting the "average American diet" ranged from 40 to 61% with a mean of 50% [23]. Roth-Maier et al. [24] determined the prececal digestibility of endogenous pantothenic acid in five different foods using pigs subjected to an end-to-end ileo-rectal anastomosis. The foods

selected were wheat, coarse wholemeal bread, steamed potatoes, boiled pork, and boiled beef. These test foods were mixed with basal ingredients to provide edible meals, and to supply a minimum of pantothenic acid. All diets were fortified with minerals and fat-soluble vitamins, and the wheat, bread, and potato meals were additionally enriched with soybean oil and amino acids. The digestibilities of pantothenic acid from the wheat, potato, and meat meals ranged between 65 and 81% and were not statistically different from one another. The digestibility of pantothenic acid from the coarse wholemeal bread diet reached a level of only 28%.

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12

Biotin

12.1 Background

In mammals, biotin functions as a covalently bound prosthetic group in four carboxylase enzymes that play key roles in gluconeogenesis, fatty acid biosynthesis, amino acid metabolism, and odd-chain fatty acid catabolism.

The human requirements for biotin are normally met through the combined dietary supply and endogenous microbial synthesis in the gut. A primary biotin deficiency state is therefore extremely rare, especially when a well-balanced diet prevails. Deficiency states have been induced in adult volunteers by feeding low-biotin diets containing a high proportion of raw egg white. An initial scaly dermatitis was followed by nonspecific symptoms that included extreme lassitude, anorexia, and muscular pains. All these symptoms responded to injections of 150–300 μg of biotin per day. Seborrhoeic dermatitis of the scalp and a more generalized dermatitis known as Leiner's disease have been reported in breast-fed infants when the mother is malnourished. These symptoms are relieved when biotin is administered to the mother.

Biotin toxicity in humans has not been reported and is presumably low.

12.2 Chemical Structure, Biopotency, and Physicochemical Properties

12.2.1 Structure and Potency

The biotin molecule ($C_{10}H_{16}O_3N_2S$, MW = 244.3) is a fusion of an imidazolidone ring with a tetrahydrothiophene ring bearing a pentanoic acid side chain. The molecule contains three asymmetric carbon atoms, and hence eight stereoisomers are possible. Of these, only the dextrorotatory (+) *d*-biotin occurs in nature and possesses vitamin activity. Biotin synthesized industrially by the Hoffmann-La Roche process is also in the *d*-form [1]. The stereochemical structure of *d*-biotin (Figure 12.1) reveals

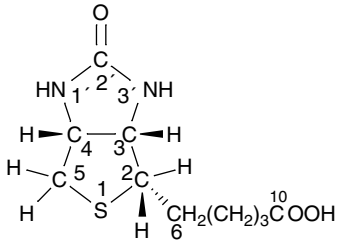

FIGURE 12.1
Structure of *d*-biotin

that the two rings are fused in the *cis* configuration and the aliphatic side chain is *cis* with respect to the imidazolidone ring.

In animal and plant tissues, only a small proportion of the biotin occurs in the free state. The majority is covalently bound to the protein structure (apoenzyme) of biotin-dependent enzymes via an amide bond between the carboxyl group of biotin and the ε-amino group of a lysine residue [2]. Proteolysis of the enzyme liberates a natural water-soluble fragment called biocytin (ε-*N*-biotinyl-L-lysine) (Figure 12.2), which is biologically active.

12.2.2 Physicochemical Properties

12.2.2.1 Appearance and Solubility

Synthetic *d*-biotin crystallizes as fine colorless needles which melt at 232°C with decomposition. In its free acid form, biotin is only very sparingly soluble in water at 25°C (20 mg/100 ml) and in 95% ethanol (80 mg/100 ml) but is more soluble in hot water. It is soluble in dilute alkali, sparingly soluble in dilute acid, and practically insoluble in fat solvents, including chloroform, diethyl ether, and petroleum ether.

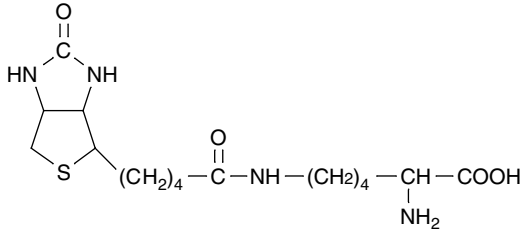

FIGURE 12.2
Structure of biocytin

The pH of a 0.01% aqueous solution of biotin is 4.5; the pK_a value is 4.51. Biotin salts are significantly more water-soluble than the acid form of biotin.

12.2.2.2 Stability in Aqueous Solution

Dry crystalline biotin is stable to heat and atmospheric oxygen but is gradually destroyed by UV radiation. Aqueous solutions within the pH range 4–9 are stable up to 100°C, but solutions that are more acid or alkaline are unstable to heating. The sulfur atom can be readily oxidized with various reagents to yield a mixture of isomeric *d*- and *l*-biotin sulfoxides; stronger oxidants form biotin sulfone. These oxidation products are essentially inactive for humans, although the *d*-sulfoxide can be metabolically slowly reduced to biotin [3]. Bound forms of biotin may be freed by strong acid hydrolysis at elevated temperature or with a proteolytic enzyme [4]. Aqueous solutions of biotin are very susceptible to mold growth.

12.3 Biotin in Foods

12.3.1 Occurrence

Biotin is present in all natural foodstuffs, but the content of even the richest sources is very low when compared with the content of most other water-soluble vitamins. Table 12.1 [5] gives typical values of some dietary sources of biotin. Liver, eggs, soy beans, and peanuts are particularly rich sources of the vitamin. Other good sources include yeast, wheat bran, oatmeal, and some vegetables. Muscle meats, fish, dairy products, and cereals contain smaller amounts, but are important contributors to the dietary intake. Biotin is not commonly used in fortified foods, apart from infant formulas. Most of the biotin content of animal products, nuts, cereals, and yeast is in a protein-bound form. A higher percentage of free water-extractable biotin occurs in vegetables, green plants, fruit, milk, and rice bran [4].

12.3.2 Stability

In vegetables, in which a significant proportion of the biotin present is not bound, leaching of the vitamin may occur during the washing and blanching steps of commercial processing. Hoppner and Lampi [6] investigated the effect of pre-soaking procedures and cooking times on the retention of biotin (and also pantothenic acid) in 25 different dried legumes. Before cooking for 20, 90, or 150 min, pooled samples of each legume were

TABLE 12.1

Biotin Content of Various Foods

Food	Micro grams of Biotin per 100 g Edible Portion
Cow milk, whole, pasteurized	2.5
Cheese, cheddar, average	4.4
Egg, chicken, whole, raw	20
Wheat bran	45
Oatmeal, quick cook, raw	21
Wheat flour, wholemeal	7
Wheat flour, white, plain	1
Rice, brown, raw	N
Beef, trimmed lean, raw, average	1
Lamb, trimmed lean, raw, average	2
Pork, trimmed lean, raw, average	2
Chicken, meat, raw	2
Liver, lamb, fried	33
Kidney, lamb, fried	73
Cod, raw, fillets	1
Herring, grilled	7
Potato, main crop, old, average, raw	0.3
Chick peas, dried, raw	N
Soybeans, dried, raw	65
Red kidney beans, dried, raw	N
Peas, raw	0.5
Cauliflower, raw	1.5
Carrots, old, raw	0.6
Mushroom, common, raw	12.0
Apples, eating, average, raw	1.2
Bananas	2.6
Orange juice, unsweetened	1
Peanuts, plain	72
Yeast extract	N
Coffee, instant	67

Note: N, The vitamin is present in significant quantities but there is no reliable information on the amount.
Source: From Food Standards Agency, 6th summary ed., Royal Society of Chemistry, Cambridge, 2002. With permission.

subjected to either a short soak or a long soak. For the short soak, one volume of the legume in three volumes of water was brought to boil and boiled for 2 min, covered, and left to soak for 1 h while cooling to room temperature. For the long soak, one volume of the legume in three volumes of water was left to soak overnight (16 h) at room temperature. Legumes in the 20-min cooking category were also prepared without pre-soak. After cooking, samples were weighed, freeze-dried,

TABLE 12.2

Effect of Pre-Soak Methods on the Mean Biotin Retention in Dried Legumes After Cooking[a]

Cooking Time (min)	No Pre-Soak (%)	Short Soak (%)	Long Soak (%)	Statistical Difference
20	94.6 ± 5.4	—	—	
20		86.6 ± 11.06	90.0 ± 8.2	ns
90		98.9 ± 11.1	92.3 ± 12.0	ns
150		90.3 ± 14.4	85.1 ± 14.4	ns

Note: ns, not significant ($P > .05$).
[a] Values were derived from analysis of 25 different legumes.
Source: From Hoppner, K. and Lampi, B., *J. Food Sci.*, 58, 1084, 1993. With permission.

finely ground, and analyzed for biotin content by microbiological assay. The mean values presented in Table 12.2 [6] showed that there was no significant difference between the two pre-soak methods. Mean retention time for biotin in legumes cooked for 20 min with no pre-soak was 95%, while means for both pre-soak methods for the 20, 90, and 150 min cooking times were 88, 95, and 88%, respectively. Thus biotin retention in cooked legumes is much less affected by leaching and heat destruction than is pantothenic acid (Section 11.3.2.). The high degree of biotin retention can be explained by the existence of protein-bound biotin in plant tissues, as shown by the rigorous extraction procedures (acid hydrolysis or enzymatic digestion) required before the vitamin can be analyzed.

The canning of carrots, maize, mushrooms, green peas, spinach, and tomatoes resulted in biotin losses of 40–78%, in contrast to asparagus with 0% loss [7]. In canned infant foods, a ca. 15% reduction in biotin content after a 6-month storage period has been observed [8]. Evaporated milk and milk powder incur losses of 10–15% during processing [9].

12.3.3 Applicability of Analytical Techniques

Biotin occurs covalently bound to proteins as well as in the free state. In addition to the conventional microbiological assay for determining biotin, biospecific methods have been developed using radiometric protein-binding assays (radioassays), nonisotopic protein-binding assays, and a biosensor-based immunoassay. An HPLC method involving post column derivatization has been reported for the determination of biotin and biocytin in a variety of foods. No international unit of biotin activity has been defined and analytical results are expressed in weight units (μg) of pure *d*-biotin.

12.4 Intestinal Absorption

The following discussion of absorption is taken from a more detailed account in a book by Ball [10] published in 2004.

Humans and other mammals cannot synthesize biotin and thus must obtain this vitamin from food. The liver extracts the majority of the newly absorbed biotin from the portal blood and is the major site of biotin utilization and metabolism. Biotin is synthesized by the microflora that normally inhabit the large intestine. Presumably, this endogenous vitamin is not available to the host tissues in nutritionally significant amounts because an inherited impairment of biotin recycling results in symptoms of biotin deficiency.

12.4.1 Digestion and Absorption of Dietary Biotin

Biotin in foods exists as the free vitamin and as protein-bound forms in variable proportions. Because the amide bond linking biotin and lysine is not hydrolyzed by gastrointestinal proteases, proteolytic digestion of protein-bound biotin releases not biotin, but biocytin and biotin-containing short peptides (biotinyl peptides). Biotinidase, which is present in pancreatic juice and in intestinal mucosa, is capable of hydrolyzing biocytin to yield free biotin. The release of biotin might occur during the luminal phase of proteolysis by the action of pancreatic biotinidase or in the intestinal mucosa by the action of mucosal biotinidase. Biocytin may also be absorbed intact and be acted upon by biotinidase present in plasma. Biotinyl peptides can also be absorbed directly.

Avidin, the glycoprotein in egg white, uniquely binds biotin, both in the free form and as the prosthetic group of enzymes. The complex is stable over a wide pH range and is not dissociated in the gastrointestinal tract. When eggs are cooked, the avidin is denatured and the biotin is liberated.

Transport of biotin across the brush-border membrane of the enterocyte takes place by a carrier-mediated process at physiological concentrations and by simple diffusion at higher concentrations [11–13]. Transport occurs without metabolic alteration to the biotin molecule. In the human intestine, the carrier-mediated system is sodium- and energy-dependent, and is predominant in the jejunum [14]. The system resembles that for D-glucose in that a sodium gradient maintained by the basolateral sodium pump provides the energy required for the uphill movement of biotin against its concentration gradient. Biotin at physiological pH exists mostly in the anionic form. The sodium-dependent multivitamin transporter (SMVT), which handles pantothenate, biotin, and lipoate,

mediates an electrogenic process with an assumed Na^+:biotin$^-$ stoichiometry of 2:1 [15].

The rate of biotin transport was shown to increase with a decrease in pH of the incubation medium from 8 to 5.5 [16]. This effect presumably occurs through an increase in the transport of biotin by simple diffusion and is due to an increase in the percentage of the easily diffusible unionized form of the vitamin under acidic conditions.

The exit of biotin from the enterocyte, that is, transport across the basolateral membrane, is a carrier-mediated process which has a higher affinity for the substrate than the transport system at the brush-border membrane. The basolateral transport process is independent of sodium, electrogenic in nature, and not capable of accumulating biotin against a concentration gradient [17].

There are conflicting data regarding the intestinal transport of biocytin. Dakshinamurti et al. [12] concluded that the transport is carrier-mediated and more efficient than the uptake of free biotin. Said et al. [18], on the other hand, found that the transport of biocytin occurs by a nonmediated process that is independent of sodium, pH, energy, and temperature, and is significantly less efficient than that of free biotin. The mechanism for the transport of biotinyl peptides is unknown; it might involve a specific biotin transporter or a nonspecific pathway for peptide absorption [19].

Transport in both the jejunum and ileum of the rat is appropriately increased and suppressed by biotin deficiency and biotin excess, respectively, due to changes in the number of functional carriers [20].

12.4.2 Absorption of Bacterially Synthesized Biotin in the Large Intestine

The normal microflora of the large intestine (mainly in the cecum) can synthesize large amounts of biotin, much of which exists as the unbound molecule. This is inferred by the fecal content of biotin exceeding dietary intake three to sixfold. Balance studies in humans have shown that urinary content often exceeds dietary intake, suggesting that enterically synthesized biotin can be absorbed and utilized to some extent by the host. In a study with human subjects [21], when biotin was instilled directly into the lumen of the mid-transverse colon, the concentration of plasma biotin increased, although not as much as when the same dose of vitamin was given orally. Bowman and Rosenberg [22] examined biotin absorption using the rat intestinal loop, an *in vivo* preparation in which the intestinal blood supply and lymphatics remain intact. Their data showed that the large intestine of the rat is capable of absorbing nutritionally significant amounts of biotin.

Said et al. [23] studied the mechanism and regulation of biotin transport in the large intestine using the human colonic epithelial cell line NCM460

as an *in vitro* model system. Uptake of biotin by these cells involved a Na^+-dependent, carrier-mediated transport system that was competitively inhibited by pantothenic acid and appeared to be under the regulation of an intracellular protein kinase C-mediated pathway. These findings are similar to those in the small intestine and suggest that a common mechanism mediated by the SMVT may be involved in the transport of biotin and pantothenic acid in the small and large intestinal epithelia.

12.5 Bioavailability

Growth assays have shown that biotin in wheat is largely unavailable to the chick, in contrast to biotin in maize (corn) which is almost completely available [24,25]. This has been confirmed using the ileal digestibility of biotin as an index of availability [26]. In growing pigs, corrected ileal digestibilities of biotin in soybean meal, canola meal, barley, maize, and wheat were 55.4, 3.9, 4.8, 4.0, and 21.6%, respectively; the digestibility of supplemental biotin was 93.5% [27]. Another study [28] found that apparent ileal digestibilities of biotin in wheat, sorghum, barley, and soybean meal were −3, −123, 18, and 12% in pigs, and −10, −73, 5, and 28% for the same grains in chickens. The negative digestibility of biotin from the sorghum diet for both chickens and pigs suggests that some components of sorghum are having an influence on biotin digestibility, perhaps by forming unabsorbable complexes with proteins. The validity of extrapolating values determined with chickens to pigs is questionable in view of marked differences in the processes of digestion in the two species.

Zemplini and Mock [29] quantitated the bioavailability of biotin at pharmacologic doses in healthy adults. The doses were administered orally or intravenously, and the urinary excretion of biotin and biotin metabolites was measured using a chemically specific HPLC/avidin-binding assay. The urinary recovery of the oral doses was compared with the intravenous dose as a standard for complete bioavailability. Urinary recovery of biotin plus metabolites was similar (ca. 50%) following the two largest oral doses and the one intravenous dose, suggesting 100% bioavailability of the two largest oral doses. These findings suggest that free biotin is absorbed nearly completely even when pharmacologic doses of biotin are administered. The bioavailability of biotin in the human diet awaits investigation. Most, if not all, of the biotin content of human milk is in the free form and thus is likely to be completely bioavailable to the infant [30].

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13

Folate

13.1 Background

Biologically active folate is not folic acid itself but its reduced metabolite tetrahydrofolate (THF). THF acts as a carrier of one-carbon units in biochemical reactions that lead to the synthesis of the purines (adenine and guanine) and one of the pyrimidines (thymine), which are the base constituents of DNA. In another important reaction, 5-methyl-THF donates its methyl group to homocysteine to form methionine.

A deficiency in folate leads to a lack of adequate DNA replication and consequent impaired cell division, especially in the hemopoietic tissue of the bone marrow and the epithelial cells of the gastrointestinal tract. In the bone marrow, the erythroblasts fail to divide properly and become enlarged, and the circulating red blood cells are macrocytic and fewer in number than normal. This condition, megaloblastic anemia, is particularly common in pregnancy, during which there is an increased metabolic demand for folate. The effect of folate deficiency on cell renewal of the intestinal mucosa causes gastrointestinal disturbances and also has adverse consequences on overall nutritional status. Division of cells with unrepaired or misrepaired DNA damage through folate deficiency leads to mutations. If these relate to critical genes, such as proto-oncogenes or tumor suppressor genes, cancer may result. Elevated plasma homocysteine, a consequence of marginal folate deficiency, is a risk factor for occlusive vascular disease. There is an association between the incidence of neural tube defects (e.g., spinal bifida) and maternal folate deficiency, possibly attributable to hyperhomocysteinemia. Polymorphisms in genes encoding key enzymes in folate and homocysteine metabolism play a major role in occlusive vascular disease and neural tube defects.

Folate is generally considered to have a low acute and chronic toxicity for humans. However, in the event of vitamin B_{12} deficiency, there is a danger of irreversible neurological damage if folic acid supplements are taken without including vitamin B_{12}. This is because the obvious symptoms of anemia will be alleviated by the folate treatment, but the concomitant nerve degeneration caused by lack of vitamin B_{12} will be

undetected. For this reason, prophylactic vitamin supplementation always includes vitamin B_{12} with folic acid.

13.2 Chemical Structure, Biopotency, and Physicochemical Properties

13.2.1 Structure and Potency

The term "folate" is used as the generic descriptor for all derivatives of pteroic acid that exhibit vitamin activity in humans. The chemical structures of folate vitamers are shown in Figure 13.1. The parent compound, folic acid ($C_{19}H_{19}N_7O_6$, MW = 441.4), comprises a bicyclic 2-amino-4-hydroxypteridine (pterin) moiety joined by a methylene bridge to *p*-aminobenzoic acid, which in turn is coupled via an α-peptide bond to a single molecule of L-glutamic acid.

The term "folic acid" refers specifically to pteroylmonoglutamic acid which, with reference to the pteroic acid and glutamate moieties, can be abbreviated to PteGlu. "Folate" is a nonspecific term referring to any folate compound with vitamin activity. "Folacin" is a nonapproved term synonymous with "folate."

Folic acid is not a natural physiological form of the vitamin. In nature, the pteridine ring is reduced to give either the 7,8-dihydrofolate (DHF) or 5,6,7,8-tetrahydrofolate (THF). These reduced forms can be substituted with a covalently bonded one-carbon adduct attached to nitrogen positions 5 or 10 or bridged across both positions. The following substituted forms of THF are important intermediates in folate metabolism: 10-formyl-, 5-methyl-, 5-formimino-, 5,10-methylene-, and 5,10-methenyl-THF. 5-Formyl-THF is also known as folinic acid and leucovorin.

An important structural feature of the THFs is the stereochemical orientation at the C-6 asymmetric carbon of the pteridine ring. Of the two 6*S* and 6*R* stereoisomers, only the 6*S* is biologically active and occurs in nature. Methods of chemical synthesis of tetrahydrofolates, whether by catalytic hydrogenation or chemical reduction, yield a racemic product (i.e., a mixture of both stereoisomers).

All folate compounds exist predominantly as polyglutamates containing typically from five to seven glutamate residues in γ-peptide linkage. The γ-peptide bond is almost unique in nature, its only other known occurrence being in peptides synthesized by two *Bacillus* species [1]. Folate conjugates are abbreviated to $PteGlu_n$ derivatives, where *n* is the number of glutamate residues; for example, $5\text{-}CH_3\text{-}H_4PteGlu_3$ refers to triglutamyl-5-methyl-tetrahydrofolic acid. Assuming that the polyglutamyl side chain extends to no more than seven residues, the theoretical number of folates approaches 150 [2].

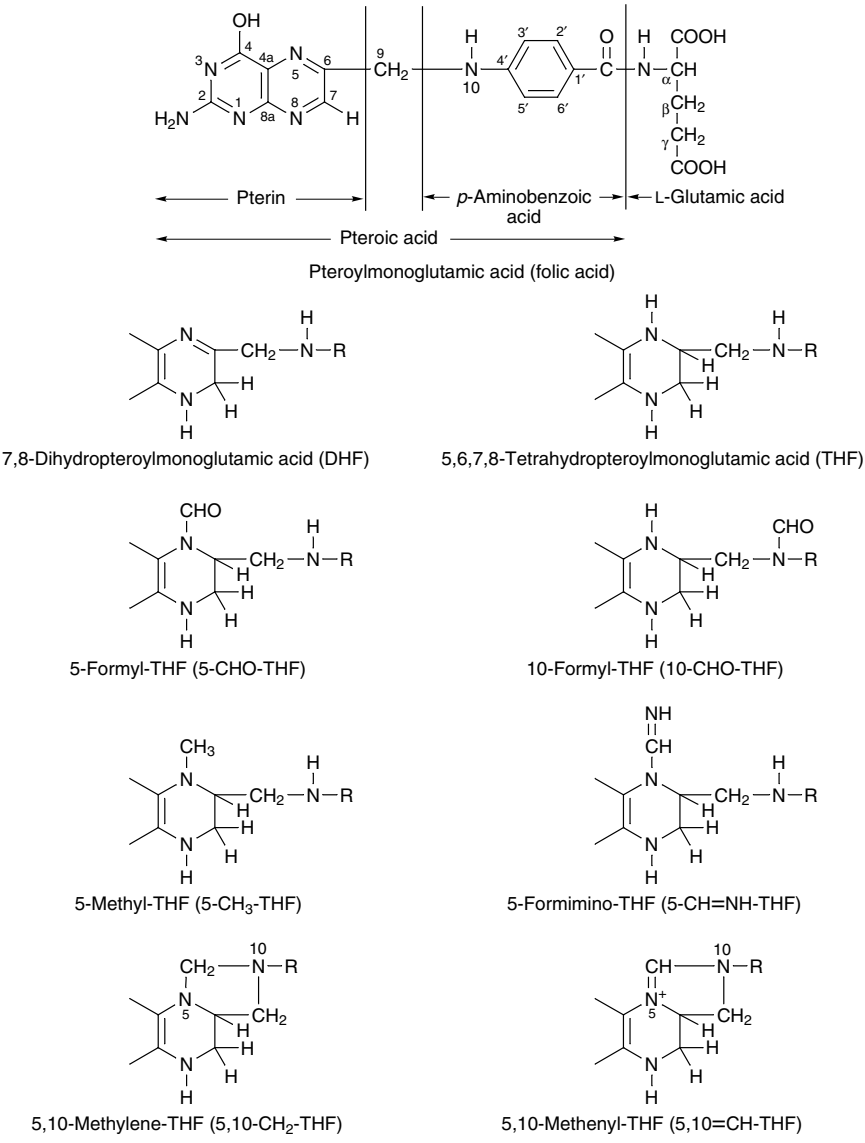

FIGURE 13.1
Structures of folate compounds.

13.2.2 Physicochemical Properties

13.2.2.1 Appearance, Solubility, and Ionic Characteristics

Folic acid is synthesized commercially for use in food fortification as either the free acid or the disodium salt. Synthetic folic acid is an

orange–yellow, microcrystalline, almost odorless, tasteless powder. It has no well-defined mp but darkens at 250°C, followed by charring. Commercially available folic acid contains on average 8.0–8.5% of water of hydration, which can be removed at 140°C under vacuum to give a hygroscopic product [3]. The free acid is practically insoluble in cold water and sparingly soluble in boiling water (20 mg/100 ml). There is slight solubility in methanol, appreciably less solubility in ethanol, and no solubility in acetone, diethyl ether, chloroform, or benzene. Folic acid dissolves in warm dilute hydrochloric acid, but with degradation that increases with increasing temperature and acid strength. It is soluble and stable in dilute alkaline solution; aqueous solutions prepared with sodium bicarbonate have a pH between 6.5 and 6.8. The disodium salt is soluble in water (1.5 g/100 ml at 0°C).

Folates are ionogenic and amphoteric molecules. Ionogenic groups of particular significance in the range of pH values relevant to foods and biological systems are the N-5 positions of THF ($pK_a = 4.8$) and the glutamate carboxyl groups ($\gamma\ pK_a = 4.8$; $\alpha\ pK_a = 3.5$). Polyglutamyl folates, because of the free α-carboxyl groups situated on each glutamate residue, exhibit greater ionic character than the monoglutamyl forms when dissociated under neutral to alkaline conditions [4].

13.2.2.2 Stability in Aqueous Solution

The folate vitamers differ widely with respect to their susceptibility to oxidative degradation, their thermal stability, and the pH dependence of their stability. The length of the glutamyl side chain has little or no influence on the stability properties of the folate compounds [5]. The most stable of the various folates at ambient and elevated temperatures is the parent compound, folic acid. In aqueous solution, folic acid is stable at 100°C for 10 h in a pH range 5.0–12.0 when protected from light, but becomes increasingly unstable as the pH decreases below 5.0 [6]. Alkaline hydrolysis under aerobic conditions promotes oxidative cleavage of the folic acid molecule to yield *p*-aminobenzoylglutamic acid (PABG) and pterin-6-carboxylic acid, whereas acid hydrolysis under aerobic conditions yields 6-methylpterin [7]. Polyglutamyl derivatives of folic acid can be hydrolyzed by alkali in the absence of air to yield folic and glutamic acids [7]. Folate activity is gradually destroyed by exposure to sunlight, especially in the presence of riboflavin, to yield PABG and pterin-6-carboxaldehyde [7].

The stability of folic acid (and indeed most folates) is greatly enhanced in the presence of an antioxidant such as ascorbic acid. The one known exception is 5-methyl-DHF, which is converted by antioxidants to 5-methyl-THF [8].

The unsubstituted reduced structure THF is extremely susceptible to oxidative cleavage. DHF is included with the PABG and pterin breakdown products at pH 10, but not at pH 4 or 7 [9]. Trace metals, particularly iron(III) and copper(II) ions, catalyze the oxidation of THF [10].

The presence of substituent groups in the N-5 position greatly increases the oxidative stability of the reduced folates relative to that of THF. 5-Methyl-THF, an important dietary folate in its polyglutamate form, exhibits a half-life of 21 min at 100°C in aqueous solution compared with 2 min for THF [11]. Temperature rather than light is the predominant factor influencing the stability of 5-methyl-THF. The rate of 5-methyl-THF loss varies dramatically with pH. At pH 9, in the absence of antioxidant, it is very unstable at 25°C, whilst at pH 7.3 and 3.5 the stability is much greater, with the latter two pH values producing very similar rates of loss. In the presence of an antioxidant (dithiothreitol) at 25°C, 5-methyl-THF is relatively stable at pH 7.3 and 9.0, but at pH 3.5 the antioxidant has little or no protective effect [12].

Oxidation of 5-methyl-THF under mild conditions at or near neutral pH yields 5-methyl-5,6-DHF [13]. The latter compound is rapidly reduced back to 5-methyl-THF by ascorbate and mercaptoethanol, which are commonly used as antioxidants in folate analysis, and therefore the specific presence of 5-methyl-5,6-DHF would not be detected in most chromatographic methods for determining folates. In strongly acidic media, 5-methyl-5,6-DHF undergoes C-9—N-10 bond cleavage [14,15], whereas in mildly acidic media it undergoes rearrangement of the pteridine ring system [6]. In both cases, there is a consequent loss of folate activity [6].

10-Formyl-THF is readily oxidized by air to 10-formylfolic acid [14,15] with no loss of biological activity [16]. The stability of 10-formylfolic acid is comparable to that of folic acid [6]. Under anaerobic conditions, 10-formyl-THF undergoes isomerization to 5-formyl-THF at neutral pH after prolonged standing and especially at elevated temperature [17].

5-Formyl-THF exhibits equal thermal stability to folic acid at neutral pH, but under acidic conditions, and especially at high temperatures, it loses a molecule of water to form 5,10-methenyl-THF [7]. The latter compound is stable to atmospheric oxidation in acid solution, but is hydrolyzed to 10-formyl-THF in neutral and slightly alkaline solutions [18].

The milk protein casein, iron(II), and ascorbate, which are capable of lowering the concentration of dissolved oxygen, have all been shown to increase the thermal stability of folic acid and 5-methyl-THF [19]. Other reducing agents that occur in foods, such as thiols and cysteine, would also retard folate oxidation.

Lucock et al. [20] reported that at pH 6.4, 5-methyl-THF is profoundly unstable in the presence of 0.1 *M* $ZnCl_2$. Instability to a lesser degree was also observed in the presence of other metal cations, the order of the effect

being $Zn^{2+} > Ca^{2+} \simeq K^{+} > Mg^{2+} \simeq Na^{+}$. This effect was negated in the presence of reduced glutathione, suggesting that the loss is due to oxidative degradation. The oxidative process seems to depend on the ionic state of 5-methyl-THF. At pH 6.4, 5-methyl-THF exists in its anionic form, which renders it more labile in the presence of metal cations through the formation of a complex. Since the stability of 5-methyl-THF increased at pH 3.5 in the presence of the same cations, the protonated free acid is probably less available for complex formation and consequently is more stable.

Sulfurous acid and nitrite, two chemicals that are used in food processing, cause a loss of folate activity in aqueous systems. Reactions between folates and sulfurous acid promote C-9—N-10 bond cleavage [5]. Nitrite ions react with folic acid to yield exclusively 10-nitrosofolic acid and with 5-formyl-THF to yield the 10-nitroso derivative; interaction with THF and 5-methyl-THF yields PABG and several pterin products [21].

13.3 Folate in Foods

13.3.1 Occurrence

Polyglutamyl folate is an essential biochemical constituent of living cells, and most foods contribute some folate to the diet. In the United States, dried beans, eggs, greens, orange juice, sweet corn, peas, and peanut butter are good sources of folate that are inexpensive and available all the year round. The folate content of a selection of vegetables and fruits is shown in Table 13.1 [22]. Meat, fish, and poultry are poor or moderate sources relative to plant products. Liver, all types of fortified breakfast cereals, cooked dried beans, asparagus, spinach, broccoli, and avocado provide the highest amount of folate per average serving; however, several of these foods do not rank highly in terms of actual dietary folate intake in the United States because of their low rate of consumption. According to data from a 1976–1980 national health and nutrition survey [23], orange juice is the major source, contributing nearly 10% to dietary folate intake. Rychlik [24] revised the folate content of foods determined by stable isotope dilution assays.

Since January 1, 1998, it has been mandatory in the United States for food manufacturers to add folic acid to certain specified grain products, including enriched flour used in breadmaking, corn meals, rice, noodles, and macaroni, at a level of 140 μg/100 g. These foods were chosen for folate fortification because they are staple products for most

TABLE 13.1

Folate Composition of Selected Vegetables and Fruits

	Folate Concentration (μg Per 100 g Fresh Weight)			
Product	**5-Methyl-THF**	**THF**	**5-Formyl-THF**	**Total (as Folic Acid)**
Vegetables				
Potato, raw	21	3	nd	23
Carrot, raw	16	1	msk	16
Broccoli	98	18	nd	114
Brussel sprouts	88	9	msk	94
Cauliflower	80	9	msk	85
Swede	50	2	nd	49
Tomato	11	1	nd	11
Onion	13	1	nd	13
Sweet pepper, red	50	5	3	55
Lettuce	44	9	nd	51
Peas, green frozen	51	10	nd	59
Fruits				
Banana	12	1	nd	13
Orange	27	<1	msk	27
Orange juice	16	<1	nd	15

Note: nd, not detected; msk, HPLC peak masked by impurities.
Source: From Vahteristo, L., Lehikoinen, K., Ollilainen, V., and Varo, P., *Food Chem.*, 59, 589, 1997. With permission.

of the U.S. population, and because they are suitable vehicles for this purpose [25]. This public health measure was designed to reduce the incidence of infantile neural tube defects.

Approximately 75% of the folate in mixed American diets is present in the form of polyglutamates [26]. The predominant folate vitamers in animal tissues are polyglutamyl forms of THF, 5-methyl-THF, and 10-formyl-THF [27]. 5-Formyl-THF is a minor vitamer in most animal tissues, but thermal processing can promote its formation through isomerization of 10-formyl-THF [4]. THF is the main vitamer in several fish species [28], as well as in pork and beef liver [29]. Plant tissues contain mainly polyglutamyl 5-methyl-THF, accounting for up to 90% of folate activity [27]. Folic acid is present in liver, but in most other foods it does not occur naturally to a significant extent. It is, however, often found in small quantities as an oxidation product of THF in foods stored under conditions that permit exposure to oxygen [30]. 5-Methyl-5,6-DHF and, to a lesser extent, 10-formylfolic acid may be present in processed foods as oxidation products of 5-methyl-THF and 10-formyl-THF, respectively.

The folates generally exist in nature bound to proteins [2] and they are also bound to storage polysaccharides (various types of starch and glycogen) in foods [31].

13.3.2 Stability

Folate, being water-soluble, is lost during the water blanching of vegetables due to both thermal degradation and leaching into the blanch effluent. The loss of folate by leaching increases with the amount of blanch water used. In the water blanching of spinach, folate loss with a spinach-to-water ratio of 1 : 1.6 was 33% [32] compared with a loss of 83% with a spinach-to-water ratio of 1 : 7 [33]. Steam blanching and microwave blanching incur less loss of folate owing to the absence of leaching by water. Folate loss in green beans was 10% after steam blanching compared with 21% after water blanching [34]. Water blanching of spinach caused a 33% loss of folate compared with a 14% loss with microwave blanching [32]. Malin [35], studying effects of blanching, freezing, and storage on Brussels sprouts, found that the folate was stable during all processing stages. This finding reflects minimal leaching due to the small exposed surface area of Brussels sprouts as compared, for example, to spinach.

Melse-Boonstra et al. [34] subjected vegetables to various processing treatments and, using HPLC, determined total folate (after conjugation with rat plasma conjugase), monoglutamyl folate (no enzyme treatment), and polyglutamyl folate (difference between total folate and monoglutamyl folate). The vegetables selected were leeks (rings, 5 mm), cauliflower (florets, 2–4 cm), and green beans (pieces, 5 cm). The treatments applied are summarized in Table 13.2 and the results are shown in Table 13.3. Freezing and thawing or high-pressure treatment, both followed by blanching, resulted in total folate losses of more than 55% and an increase in the proportion of monoglutamyl folate (in leeks, 100 and 65% of total folate, respectively). In contrast, freezing and thawing or high-pressure treatment, both preceded by blanching, resulted in no more loss of total folate than blanching alone ($<$38%) and only a slight increase in the proportion of monoglutamyl folate (in leeks, 10 and 9% of total folate, respectively). Evidently, freezing and thawing or high-pressure treatment disrupted plant cell structure, establishing contact between endogenous conjugase and polyglutamyl folate, thereby stimulating conversion of polyglutamate to monoglutamate. Subsequent blanching caused leaching of the monoglutamyl folate from the damaged plant tissues. Conversely, blanching before these treatments inactivated endogenous conjugase, preventing the conversion of polyglutamyl folate to the monoglutamate and reducing the net loss of folate.

TABLE 13.2

Description of the Processing Treatments for Leeks, Cauliflower, and Green Beans

Treatment		Description
A	Raw	No treatment
B	Storage	Storage for 24 h in a refrigerator at 4°C
C	Blanching	Blanching in an industrial blanching kettle (10 l of water/200 g of fresh vegetable weight) for 5 min (leeks), 8 min (cauliflower), or 6 min (green beans)
D	Steaming	Steaming in a steaming sieve of 200 g of vegetable above 1 l of boiling water for 5 min (leeks), 7 min (cauliflower), or 6 min (green beans)
E	High-pressure treatment	High-pressure treatment at 200 MPa for 5 min; pressure established by compression of water surrounding the vegetables
F	Freezing, thawing, blanching	Freezing at −18°C for 16 h, followed by thawing during storage (treatment B) and then blanching (treatment C)
G	High-pressure treatment, blanching	High-pressure treatment (treatment E) followed by storage (treatment B) and then blanching (treatment C)
H	Blanching, freezing, thawing	Blanching (treatment C) followed by freezing at −18°C for 16 h and thawing during storage (treatment B)
I	Blanching, high-pressure treatment	Blanching (treatment C) followed by high-pressure treatment (treatment E) and storage (treatment B)

Source: From Melse-Boonstra, A., Verhoef, P., Konings, E.J.M., van Dusseldorp, M., Matser, A., Hollman, P.C.H., Meyboom, S., Kok, F.J., West, C.E., *J. Agric. Food Chem.*, 50, 3473, 2002. American Chemical Society. With permission.

The addition of ascorbic acid to canned vegetables before retorting provides an additional stability to the folate content during subsequent storage, but little protective effect takes place during the heat process itself [10]. Lin et al. [36] reported a 30% loss of folate in canned garbanzo beans (chick peas) regardless of whether or not 0.2% ascorbic acid was added to the curing brine. Folate analysis was performed on the homogenized contents of the whole can, that is, beans plus brine. No further loss of folate took place when the process time at 118°C was extended from 30 to 53 min. The pH of canned garbanzo beans is between 5.8 and 6.2, a range at which folate is quite stable toward heat.

Lane et al. [37] investigated the effect of freeze-drying on the folate content of precooked vegetable dishes prepared for astronauts. Decreases in folate content (29–49% retention) were observed with some dishes (asparagus, cauliflower with cheese, and green beans with broccoli) probably due to oxidative reactions that result from the increase in

TABLE 13.3

Folate Content (Total, Monoglutamate, and Polyglutamate) of Vegetables after Various Processing Treatments[a]

	Total			Monoglutamate		Polyglutamate[c]
Treatment[b]	**Dry Matter (%)**	**μg/100 g of Dry Wt.**	**% Loss**	**μg/100 g of Dry wt.**	**% of Total**	**μg/100 g of Dry wt.**
Leeks						
Raw (A)	8.6 ± 0.1	580 ± 56	0	187 ± 38	33 ± 9	392 ± 81
Storage (B)	8.6	491	15	260	53	231
Blanching (C)	6.5	417	28	23	6	394
Steaming (D)	8.7	431	26	49	11	382
High-pressure treatment (E)	6.5	236	81	174	74	62
Freezing, thawing, blanching (F)	5.6	85	85	85	100	0
High-pressure treatment, blanching (G)	5.3	86	85	56	65	30
Blanching, freezing, thawing (H)	6.5	418	28	40	10	378
Blanching, high-pressure treatment (I)	5.6	359	38	31	9	328
Cauliflower						
Raw (A)	7.7 ± 0.3	696 ± 111	0	62 ± 11	9 ± 1	634 ± 105
Storage (B)	7.7	519	25	23	4	496
Blanching (C)	6.9	626	10	16	3	610
Steaming (D)	7.7	640	8	10	2	630
High-pressure treatment (E)	6.8	394	43	48	12	346
Freezing, thawing, blanching (F)	6.7	246	65	62	25	184
High-pressure treatment, blanching (G)	6.3	311	55	27	9	284
Blanching, freezing, thawing (H)	6.7	587	16	18	3	569
Blanching, high-pressure treatment (I)	6.1	576	17	23	4	553

Green beans						
Raw (A)	7.4 ± 0.2	526 ± 65	0	170 ± 7	33 ± 5	355 ± 71
Storage (B)	7.4	560	−7	231	41	329
Blanching (C)	7.9	414	21	27	7	387
Steaming (D)	7.8	471	10	10	2	461
High-pressure treatment (E)	7.4	277	47	226	82	51
Freezing, thawing, blanching (F)	8.2	108	79	83	77	25
High-pressure treatment, blanching (G)	7.4	102	81	73	72	29
Blanching, freezing, thawing (H)	8.5	340	35	16	5	32
Blanching, high-pressure treatment (I)	8.1	397	24	15	4	382

[a]Values for raw vegetables expressed as mean ± SD ($n = 5$). All other data are based on single measurements.
[b]See Table 13.2 for a description of treatments.
[c]Polyglutamate content was calculated as the total folate content (after deconjugation) minus the monoglutamyl folate content (before conjugation).
Source: From Melse-Boonstra, A., Verhoef, P., Konings, E.J.M., van Dusseldorp, M., Matser, A., Hollman, P.C.H., Meyboom, S., Kok, F.J., and West, C.E., *J. Agric. Food Chem.*, 50, 3473, 2002. American Chemical Society. With permission.

internal surface area. Apparent increases in folate content were observed in a combination of corn, broccoli and pasta (129% retention), and creamed spinach (160% retention). One possible reason for this is that the freeze-drying process destroys conjugase inhibitors, thereby increasing the amount of free folate that is utilized by the assay organism, *Lactobacillus rhamnosus*.

Müller and Diehl [38] used HPLC to determine the effect of ionizing radiation on folate vitamers in foods. Irradiation with doses of 2.5, 5.0, and 10 kGy affected THF, 5-methyl-THF, and 5-formyl-THF about equally, therefore the results obtained on these vitamers were pooled. A radiation dose of 2.5 kGy, the highest dose practically feasible for fresh vegetables, caused ca. 10% loss of total folate in spinach, green cabbage, and Brussels sprouts. At 10 kGy, the highest dose legally permitted for food irradiation in many countries, losses of total folate ranged from 0 to ca. 30% in dehydrated vegetables, baker's yeast, beef liver, and Camembert cheese. The results indicated some radiation-induced conversion of polyglutamyl folates to the more bioavailable monoglutamates.

High-pressure processing at moderate temperature (high hydrostatic pressure, HHP) is currently being used for the pasteurization of various foods. Nguyen et al. [39] studied the stability of folic acid and 5-methyl-THF in phosphate buffer (0.2 *M*, pH 7) toward combined thermal and high-pressure treatments. Folic acid was highly stable, tolerating 600 MPa and 60°C for 7 h without degradation. In contrast, 5-methyl-THF was degraded by a synergistic effect of pressure and temperature at temperatures above 40°C. Indrawati et al. [40] studied the effects of HHP treatment on the stability of endogenous 5-methyl-THF in orange juice, kiwi puree, carrot juice, and asparagus. Recognizing that folate loss is mainly caused by leaching, these researchers employed a closed system in their investigation. 5-Methyl-THF in orange juice and kiwi puree was relatively stable at different pressure–temperature combinations up to 500 MPa/60°C for 40 min, but in carrot juice and asparagus there was significant degradation of the vitamer. The addition of ascorbic acid increased the barostability of 5-methyl-THF in carrot juice and to a lesser extent in asparagus. The authors concluded that endogenous ascorbic acid in foods plays an important role in increasing folate barostability and suggested that ascorbic acid addition could be considered as a strategy in preventing folate degradation during processing. Butz et al. [41] also found that excess ascorbate strongly protected folates against pressure and heat in model orange juices. Treatment of fresh orange juice at 600 MPa/80°C for 5 min did not cause severe loss of folates, owing to the stabilizing action of intrinsic substances other than ascorbate.

Leichter et al. [42] examined the effect of cooking (vigorous boiling for 10 min) on the folate content of vegetables that are good sources of the vitamin. Folate was determined in both the raw and cooked vegetables, as well as the water in which they were cooked. With the exception of spinach, the amount of folate retained in the cooked vegetable and the amount in the cooking water was in excess of 100% of the amount found in the raw vegetable. Thus, the loss of folate during the cooking of vegetables is caused mostly by leaching into the surrounding cooking water and not by actual destruction of the vitamin. In the case of broccoli, cabbage, cauliflower, and spinach, the cooking water contained higher quantities of folate than the cooked vegetables. Brussels sprouts and asparagus were exceptions, presumably because of their lower surface-to-weight ratios. There was no loss of folate (101% retention) in microwave-cooked spinach [43].

Petersen [44] compared the influence of steaming and sous-vide processing (heating the product when it is vacuum packed) on folate retention in broccoli florets. Percent folate retentions were as follows: steaming (100°C) for 20 min, 74%; steaming for 40 min, 59%; sous-vide processing for 40 min at 100°C and 97% vacuum, 89%.

Williams et al. [45] compared two foodservice systems for their effect on retention of 5-methyl-THF in vegetables: (1) cook hot-hold and (2) cook and chill, where food is cooked, chilled, and held up to 5 days before reheating. Retention in reheated chilled food was less than in food held hot 30 min (74 versus 81%) and not different from that in food held hot 2 h (74 versus 68%).

The soaking of dried forms of chick peas and peas for 16 h caused folate losses of 17 and 21%, respectively, through leaching. Subsequent boiling of the soaked legumes increased the leaching losses to 28 and 32% for chick peas and peas, respectively, whereas pressure-cooking increased the leaching losses to 20 and 27%. The retention of folate in the legumes after soaking and boiling was 53 and 45% for chick peas and peas, respectively. Corresponding retentions after soaking and pressure-cooking were 62 and 50% [46].

Oven-baking experiments on fish and chicken confirmed a lower stability for constituent THF compared with 5-methyl-THF [47], suggesting that animal-derived foods containing a high proportion of THF are especially prone to folate losses during processing and cooking. Folate vitamers (THF, 5-methyl-THF, and 5-formyl-THF) were well retained in the processing of liver sausage [47]. Frozen storage at -18°C for up to 6 months did not affect the 5-methyl-THF content of raw liver or strawberries [47].

Folate loss in UHT-processed milk amounted to 12.5% [48]; the loss in pasteurized milk is <10% [49]. The presence of ascorbic acid in milk protects the folate from oxidation by the dissolved oxygen.

The folic acid added to flour and cereal-grain products in accordance with the mandatory U.S. fortification policy shows good stability toward processing and storage [50]. The baking of bread caused a ca. 20% loss of added folic acid and a similar loss of native folate [51].

13.3.3 Applicability of Analytical Techniques

Folate compounds constitute a large family of pteroylpolyglutamates which differ in the reduction state of the pteridine nucleus, the nature of the single carbon substituent at the N-5 and/or N-10 positions, and the number of glutamate residues. The parent compound, folic acid, is relatively stable and is used in food fortification. Although up to 150 different folate structures are theoretically possible, fewer than 50 principal folates probably exist in most animal and plant tissues [4]. Naturally occurring folates exist in protein-bound form, the predominant vitamers in most foods being polyglutamyl forms of THF, 5-methyl-THF, and 10-formyl-THF [30]. THF is highly susceptible to oxidation, but the presence of substituent groups in the N-5 position greatly increases the oxidative stability.

The multiplicity and diversity of natural folates, their instability, and their existence at low concentration in biological tissues pose formidable obstacles to the separation, identification, and quantification of these compounds. The study of folates is further complicated by their association with enzymes capable of modifying or degrading them during sample preparation. Attempts to determine intact pteroylpolyglutamates in biological tissues have involved fractionation of tissue extracts by column chromatography and comparison of elution profiles with those of authentic folate standards. The effluent fractions are then further characterized and quantified by differential microbiological assay, both before and after treatment with conjugase [52].

In food analysis, the determination of the chain length of folate polyglutamates is not required, and the folates are determined as their monoglutamyl forms after enzymatic deconjugation. For several decades, a microbiological assay has been the standard method for quantification of food folates. The radiolabeled protein-binding assay, enzyme-labeled protein-binding assay, and biosensor-based immunoassay represent biospecific techniques. HPLC with UV, fluorescence, or electrochemical detection permits the separation and determination of the major folate vitamers that occur in foods. The coupling of HPLC with mass spectrometry and the use of stable isotopes as internal standards is currently the most accurate analytical technique.

No international unit for folate activity has been defined, and analytical results are expressed in weight units (μg or mg) of pure

crystalline folic acid. The various monoglutamyl folates, being totally interconvertible in mammalian metabolism, are considered to have equal biological activity in humans on a molar basis.

13.4 Absorption, Transport, and Metabolism

Much of the following discussion of absorption, transport, and metabolism is taken from a book by Ball [53] published in 2004.

13.4.1 Deconjugation of Polyglutamyl Folate

Folylpolyglutamates, being large and strongly electronegative molecules, are not transportable into cells and, before they can be absorbed, they must be hydrolyzed to monoglutamate forms. None of the known proteases in saliva, gastric juice, or pancreatic secretions are capable of splitting the γ-peptide bonds in the polyglutamyl side chain. Polyglutamyl folate can, however, be hydrolyzed by folate conjugase, which is a trivial name for pteroylpolyglutamate hydrolase (EC 3.4.12.10, also known as folylpoly-γ-glutamyl carboxypeptidase). As much as 50–75% of dietary polyglutamyl folate can be absorbed after deconjugation to monoglutamyl folate [54]. The presence of conjugase activity in many raw foods of both plant and animal origin results in a high proportion of the dietary folate being already monoglutamyl when presented to the intestinal mucosa [4].

Two folate conjugases have been found in human jejunal tissue fractions: one, a brush-border exopeptidase, has a pH optimum of 6.7–7.0 and is activated by Zn^{2+}; the other, an intracellular endopeptidase of mainly lysosomal origin, has a pH optimum of 4.5 and no defined metal requirement. The brush-border conjugase splits off terminal glutamate residues one at a time and is thought to be the principal enzyme in the hydrolysis of polyglutamyl folate. Brush-border conjugase from the jejunum of the human and pig possess similar enzymatic properties [55] and thus the porcine enzyme can be used to study folate bioavailability in humans. Interestingly, the human and the pig are the only species in which intestinal brush-border conjugase activity has been demonstrated. The intracellular conjugase may play no role in the digestion of dietary folate, being, instead, concerned with folate metabolism within the enterocyte [56].

Significant conjugase activity has also been reported in the pancreatic juice of pigs and humans [57]. Bhandari et al. [58] found the porcine pancreatic enzyme to be Zn^{2+}-dependent, with maximum activity at pH 4.0–4.5. Feeding stimulated secretion of pancreatic juice, including

conjugase activity. Chandler et al. [59] calculated that conjugase activity in porcine pancreatic juice was minor relative to the activity of the jejunal brush-border conjugase.

13.4.2 Absorption of Dietary Folate

In the human, the entire small intestine is capable of absorbing monoglutamyl folate. Absorption is somewhat greater in the proximal than in the distal jejunum which, in turn, is much greater than in the ileum. Folate transport across the brush-border membrane of the enterocyte proceeds by two parallel processes [60]. At physiological concentrations, ($<5\ \mu m$) of luminal folate, transport occurs primarily by a saturable process, which is competitively inhibited by structural analogs of folic acid, unaffected by transmembrane electrical potential, and Na^+-independent [61]. At higher concentrations, transport occurs by a nonsaturable process with characteristics of simple diffusion. This process may occur in part through a conductance pathway that involves anionic folate and a cation (perhaps Na^+) whose membrane permeation properties affect the rate of folate transport [62]. The saturable component is discussed in the following with no further mention of unsaturable transport.

Transport of folate is mediated by the reduced folate carrier, which is an organic anion exchange protein present in the plasma membrane of a wide variety of cells [63]. Transport is markedly influenced by changes in pH [64]. Folate exists primarily as an anion at the pH of the luminal contents. *In vitro* studies using everted rat jejunal rings showed that absorption was maximal at pH 6.3 and fell off sharply between pH 6.3 and 7.6 [65]. In studies using brush-border membrane vesicles [61], folate uptake increased as the pH of the incubation buffer was decreased from 7.4 to 5.5. This increase in folate uptake appeared to be partly mediated through $folate^-/OH^-$ exchange and/or $folate^-/H^+$ cotransport mechanisms, driven by the proton gradient across the membrane and partly through a direct effect of acidic pH on the carrier. Inhibition of folate transport by the anion transport inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) suggested the involvement of the $folate^-/OH^-$ exchange mechanism. Data reported by Mason et al. [66] suggest that the effect of pH on the carrier is attributable to an increased affinity of the carrier for its folate substrate. The physiological relevance of the pH dependency may be related to the existence of the acidic microclimate at the luminal surface of the jejunum. This so-called "unstirred layer" has a pH that is ca. 2 units lower than the bulk luminal pH and therefore provides the necessary extracellular acidic conditions for folate uptake.

During intestinal transport, some of the folate is converted within the enterocyte to 5-methyl-THF in a pH-dependent manner [67]. This conversion is extensive at pH 6.0 and negligible at pH 7.5, presumably because dihydrofolate reductase, the rate-limiting enzyme in the reduction and methylation process, has an acidic pH optimum. The percent conversion is reduced by increasing the concentration of folate in the mucosal medium, thus indicating saturation of the process. Since at higher concentrations most transported folate remains unmodified, intestinal conversion of absorbed folic acid is not obligatory for transport into the circulation.

The mechanism of folate exit from the enterocyte into the lamina propria of the villus is also carrier-mediated and sensitive to the effect of anion exchange inhibition. In addition, the exit mechanism is electroneutral and Na^+-independent, and has a higher affinity for the substrate than has the system at the brush-border membrane [68].

13.4.3 Influence of Folate-Binding Protein on the Absorption of Folate from Milk

Human milk and the milk of other mammals contain soluble proteins that avidly and specifically bind the folate present in the milk. These proteins are referred to as folate-binding proteins (FBPs). Cow's milk FBP exists as an aggregating system at pH values above 6.0 in which a polymer composed of at least 16 monomers is reversibly formed [69,70]. Concentration and pH effects on the equilibrium constant of this reversible system explain the variations in the molecular weight of FBP reported in the literature. FBP has both physicochemical and immunological species-specificity among mammals [71]. The binding of folate to FBP in milk is tight, but not covalent [72], and the molar stoichiometry is 1 : 1 [73]. FBP is present in excessive amounts, so that the milk has the capacity to bind added folate [74]. The folate in bovine milk occurs predominantly as 5-methyl-THF, of which about 60% is the monoglutamate [75]. In unprocessed and pasteurized milk, the folate is bound to FBP, but in UHT-processed milk and yogurt the folate exists in the free (unbound) form [76].

Little is known about the manner of absorption of bound milk folate. Tani et al. [77] showed that, *in vitro*, folic acid was entirely dissociated from FBP at pH 4.5 and below, but at pH 7.4, dissociation was negligible. The dissociation was completely reversible and pepsin did not impair the binding activity of the protein. These observations indicate that, *in vitro*, the bovine milk FBP is not inactivated under the acidic conditions in the stomach.

Tani et al. [77] studied the influence of FBP from bovine milk on the absorption of folic acid in the gastrointestinal tract of the rat. When

bound ^{14}C-labeled folic acid was administered intragastrically to rats by stomach tube, a considerable amount of folic acid was released from FBP in the stomach and it recombined with FBP in the jejunum. It appears that, when the ingested material is transferred from the stomach to the duodenum, it is not yet fully neutralized, allowing a small part of the unbound folic acid to be absorbed before being recombined with FBP as the material is neutralized in the jejunum. Compared with free folic acid, bound folic acid was more gradually absorbed in the small intestine, but finally the total amount of bound folic acid absorbed was almost the same as that of free folic acid. Free folic acid was rapidly absorbed in the jejunum, whereas bound folic acid was only slightly absorbed in this region. In the ileum, however, the absorption rates of the two forms of folic acid were almost equal. These results suggest that folic acid bound to FBP is absorbed by a manner different from that of free folic acid in the adult rat. A slower rate of folate absorption was also demonstrated in adult rats by Said et al. [78], using human milk FBP and 5-methyl-THF as the substrate.

Milk has evolved for the nutritional support of the suckling animal and absorption studies in neonates might shed more light on the role of FBP in milk than studies in adults. Mason and Selhub [79] demonstrated that, with respect to preferential jejunal uptake and sulfasalazine inhibition, free folic acid absorption in the neonatal rat is similar to that in the adult. However, the characteristics of FBP-bound folic acid absorption are very different from those of free folic acid. The uptake of folate bound to FBP from rat milk occurs preferentially in the ileum of the suckling rat as opposed to the jejunum. The absorption rate was slower than that for free folic acid and sulfasalazine was ineffective. In the suckling animal, a variety of pancreatic and intestinal absorptive functions are not fully developed, a situation which could allow the FBP to reach the ileum in an active form. This situation was demonstrated by Salter and Mowlem [80], who showed that a proportion of goat's milk FBP administered orally to neonatal goats survived along the length of the small intestine. Protease inhibitors inherent to colostrum may assist the passage of bound folate into the small intestine [81]. Additional evidence for the role of FBP in the absorption of milk folate by neonates was provided by Salter and Blakeborough [82], who reported that the addition of goat's milk FBP enhanced the transport of 5-methyl-THF in brush-border membrane vesicles isolated from the small intestine of neonatal goats.

Tani and Iwai [83] reported that the incorporation of radiolabeled folic acid into folate-requiring intestinal bacteria was considerably diminished when it was bound to FBP. Also, the presence of goat's colostrom considerably reduced the uptake of folic acid by folate-requiring

bacteria [74]. These observations suggest that milk FBP prevents the uptake of bound folate by intestinal bacteria, thereby indirectly promoting folate absorption by the infant.

Mason and Selhub [79] postulated that, in neonates, the absorption of bound folate occurs by endocytosis (or similar process) of the intact folate–protein complex. Subsequent proteolytic digestion inside the enterocyte would then release the bound folate for export into the portal circulation. Suckling animals are known to absorb proteins by a process that decreases markedly at the time of weaning and is more active in the ileum than in the jejunum [84].

13.4.4 Adaptive Regulation of Folate Absorption

Said et al. [85] induced folate deficiency in rats by feeding a folate-deficient diet that contained an antibiotic to decrease the bacterial synthesis of folate in the intestine. Using everted intestinal sacs and brush-border membrane vesicles, they showed that folate deprivation causes a specific up-regulation in the transport of physiological concentrations of folic acid across the brush-border membrane of both the small and large intestine. The effect in the small intestine took place not only in the jejunum, but also in the ileum, a region that does not usually absorb folate. The up-regulation was mediated through an increase in the number and activity of functional reduced folate carriers (increased V_{max}) with no significant effect on the affinity of the transport system (unchanged K_m). The up-regulation was associated with a marked increase in the levels of carrier mRNA and protein, suggesting a possible involvement of transcriptional regulatory mechanisms. In addition to the up-regulation of transepithelial folate transport, folate deficiency was associated with a 10-fold increase in the activity of brush-border membrane conjugase. The intestine is therefore able to maximize its ability to extract the limited amount of folate ingested during periods of deprivation.

13.4.5 Salvage of Dietary 5-Methyl-5,6-DHF

As much as 50% of the total folate in processed foods may be in the form of 5-methyl-5,6-DHF as a result of the facile oxidation of 5-methyl-THF [86]. The physiological significance of the presence of 5-methyl-5,6-DHF in the human diet has been studied by Lucock et al. [87]. In the mildly acidic postprandrial gastric environment, 5-methyl-5,6-DHF is rapidly degraded, whereas 5-methyl-THF is relatively stable. Fortunately, when gastric histology is normal, endogenous ascorbic acid present in gastric juice can salvage labile 5-methyl-5,6-DHF by reducing it back to

5-methyl-THF. This appears to be a true physiological process as 5-methyl-5,6-DHF exhibits nearly complete folate activity when given orally to the rat and chick [16], presumably via nonenzymatic reduction *in vivo*.

In patients with severely impaired gastric function, the stomach pH will be high and endogenous gastric ascorbate levels will be low. Under these conditions, 5-methyl-5,6-DHF is stable within the stomach, but it will not be reduced to 5-methyl-THF. 5-Methyl-5,6-DHF, although rapidly absorbed, persists in the bloodstream without being metabolized and does not therefore appear to enter the folate metabolic pool [86]. It follows that such patients are likely to have a reduced bioavailability of dietary folate if 5-methyl-5,6-DHF is a major constituent. There will be no problem with supplemental folic acid, which is stable within a broad pH range.

13.4.6 Absorption of Bacterially Synthesized Folate in the Large Intestine

A significant proportion of the bacterially synthesized folate in the large intestine exists in the form of monoglutamyl folate. Rong et al. [88] have shown that a portion of this folate is absorbed by the rat and is incorporated into the various tissues. In studies using a human-derived colonic epithelial cell line NCM460 [89] and purified apical membrane vesicles isolated from the colon of human organ donors [90], clear evidence was presented to show the existence of a specific, pH-dependent, carrier-mediated system for folate uptake. The uptake system was found to be similar in most respects to that of the small intestine, including intracellular regulation by protein tyrosine kinase and cyclic adenosine 3′,5′-monophosphate (cyclic AMP). Energy dependency was also demonstrated using different metabolic inhibitors. The K_m for the brush-border transport system of the human colon was ca. 8 μM compared with 1.9 μM for the small intestinal system, indicating a relatively low affinity of the colonic carrier for the folate substrate. Conversely, the maximal velocity (V_{max}) of the colonic system was three to four times higher than that of the small intestinal system, indicating a greater capacity. The mechanism for folate exit across the basolateral membrane of the human colon was similar to that for uptake [91].

A role for the colonic transport system in human folate nutrition was suggested by a report of a significant association between serum folate levels and consumption of dietary fiber [92]. The authors of this report suggested that an increase in fiber intake may promote increased folate biosynthesis by the large intestinal microflora and subsequent absorption of some of this folate.

13.4.7 Plasma Transport and Intracellular Metabolism

Following the ingestion of foods containing natural amounts of folate, the vitamin circulates in the plasma as reduced monoglutamates, primarily 5-methyl-THF, in concentrations ranging from 10 to 35 n*M*. High oral doses of folic acid bypass the normal folate absorption mechanism, resulting in both folic acid and 5-methyl-THF appearing in the bloodstream [93]. Folate in plasma seems to be distributed in three fractions: free folate and that loosely bound to plasma proteins such as albumin exist in similar proportions; less than 5% is bound to high-affinity binders [94].

Approximately 10–20% of the reduced monoglutamyl folate in the portal blood is taken up by the liver during the first pass through this organ. The remainder is taken up by the extrahepatic tissues and demethylated by the action of vitamin B_{12}-dependent methionine synthetase [95]. The resultant THF can now have glutamate residues (usually five to eight) added to it. The resultant polyglutamated THF is the functional coenzyme, accepting and transferring one-carbon units in intermediary metabolism.

13.4.8 Folate Homeostasis

The majority of 5-methyl-THF arriving at the liver from the intestine and taken up is not demethylated and converted to polyglutamate; instead, it is quickly released for distribution to extrahepatic tissues. The initial route for this distribution is the enterohepatic circulation, whereby the folate is discharged into the bile and subsequently reabsorbed by the small intestine before re-entering the systemic circulation. Accompanying 5-methyl-THF in the bile are larger amounts of nonmethylated tetrahydrofolates, which represent folates salvaged from dying cells such as senescent erythrocytes and hepatocytes [84]. Any folic acid that might have been absorbed and released into the portal circulation without modification is exclusively taken up by the liver and either converted into one-carbon derivatives of THF prior to rapid release into bile or polyglutamated and incorporated into the hepatic folate pool [95]. Hepatic reduction and derivatization of folic acid provides another source of nonmethylated tetrahydrofolates present in bile [96].

The recycling of folates via the enterohepatic pathway may account for as much as 50% of the folate that ultimately reaches the extrahepatic tissues. Disruption of the enterohepatic cycle by bile drainage results in a fall of the serum folate levels to 30–40% of normal within 6 h — a much more dramatic drop than that seen with a folate-deficient diet. Eventually, the serum folate level stabilizes, despite continuing losses in the bile. This suggests a net flux of folate into the plasma compartment from tissue

pools. Release of stored folate from cells of any tissue requires hydrolysis of the polyglutamates to monoglutamates by intracellular conjugase.

The maintenance of a normal level of plasma folate depends on regular increments of exogenous folate from the diet. The enterohepatic circulation of folate evens out the intermittent intake of dietary folate. The liver plays a major role in maintaining folate homeostasis because of its capacity to store about 50% of the total body folate, its relatively rapid folate turnover, and the large folate flux through the enterohepatic circulation [95]. In situations of dietary folate deficiency, the liver does not respond by releasing its folate stores. Rather, the nonproliferating, less metabolically active tissues mobilize their folate stores and return monoglutamyl folate to the liver. This folate is released by the liver via the enterohepatic cycle and distributed to the tissues that most need it — in particular, those with actively proliferating cells. Preferential uptake of folate by certain tissues (e.g., placenta and choroid plexus) is made possible by the presence of folate receptors on their cellular surfaces. The kidney plays its part in conserving body folate by actively reabsorbing folate from the glomerular filtrate. In addition, a pathway exists that is capable of salvaging folate released from senescent erythrocytes.

13.5 Bioavailability

13.5.1 Introduction

Recent reviews of folate bioavailability are in Ref. [97–99]. The bioavailability of folate in a wide variety of foods is incomplete and highly variable. It can be affected by dietary constituents or physiological conditions influencing: (1) the rate or extent of intestinal deconjugation of polyglutamyl folates and (2) intestinal absorption. Bioavailability can be a major determinant of nutritional status when folate intakes are in the marginal range.

13.5.2 Methods for Assessing Folate Bioavailability

13.5.2.1 Plasma Response

Comparison of the plasma or serum folate response (area under curve, AUC) from food with that from a reference dose of folic acid in water is regarded as a valid indicator of food folate bioavailability [100,101]. The fasting of subjects during the test period is not appropriate because fasting results in increased plasma folate concentrations due to a

disturbance of the enterohepatic recirculation of folate [102,103]. Subjects must have adequate folate status because folate is transported more rapidly from plasma to the tissues if tissue levels are low.

In a procedure reported by Prinz-Langenohl et al. [100], healthy volunteers received: A, the test meals; B, 0.4 mg folic acid in water (reference dose); and C, folate-free control meal. The test meals were consumed at 0800 h after an overnight fast. At regular intervals during the test days, the subjects received an energetically adequate but folate-free formula diet as a means of preventing increasing blood-folate levels induced by fasting. A fasting blood sample was drawn directly before consumption of the test meals, followed by seven postprandial samples (1, 2, 3, 4, 6, 8, and 10 h). Plasma folate was determined and the AUC calculated after subtracting predose plasma folate concentrations from the test dose concentrations. Konings et al. [101] found that taking only two samples in the first 2 h after food consumption is insufficient. The maximum serum folate concentration was reached 45 min after the consumption of test meals, thus frequent sampling about this time is of importance to obtain an accurate AUC. The use of pharmaceutical folic acid given orally as a reference dose might result in overestimation of relative folate absorption from food samples, due to destruction of the folic acid dose during gut passage. Witthöft et al. [104] used (6*S*)-5-methyl-THF, given by intramuscular injection, to ensure similar handling of the reference dose to the food folate by the body.

13.5.2.2 Stable-Isotopic Methods

Several stable isotopic methods for assessing folate bioavailability are based on urinary excretion ratios using single-dose, dual-label protocols. Initially, two deuterium-labeled forms of folic acid were available: benzene-ring labeled 3′,5′-bideuterofolic acid ([$^2H_2$]folic acid) and glutamate-labeled tetradeuterofolic acid ([$^2H_4$]folic acid) in which the four deuterium atoms are located on the β and γ carbons of the glutamate side chain [105]. These two labeled forms of folic acid exhibit equivalent *in vivo* handling through absorption, transport, metabolism, and excretion processes when administered orally [106]. Later, the method for synthesizing [$^2H_4$]folic acid was modified to synthesize [$^{13}C_5$]folic acid, in which the 5 carbon positions of the glutamate side chain are labeled with the ^{13}C stable isotope [105]. The $^{13}C_5$ and $^2H_2$ forms of folic acid exhibit similar absorption and *in vivo* handling when administered orally [107]. More recently, [$^{13}C_6$]folic acid, with the 6 carbon positions of the benzene ring labeled, has been synthesized [108].

In later single-dose, dual-label protocols, a ^{13}C-labeled folate is taken orally and a ^2H-labeled folate is injected intravenously. Pfeiffer et al. [109] used such an approach to evaluate the relative bioavailability of

folic acid in fortified cereal-grain products without prior loading of subjects with nonlabeled folate. In separate trials, subjects received a single oral dose of [$^{13}C_5$]folic acid in one of the fortified products or in water (control) concurrently with an intravenous injection of [$^{2}H_2$]folic acid. For each trial, complete urine collections were made during the 24 h before administration of labeled folate, and during the 36-h postdose period. The predose urine collection allowed the assessment of any isotopic carryover and basal folate excretion. Urine volumes were recorded and the total folate content of each urine collection was measured by HPLC after affinity chromatographic purification and concentration. Urinary folates were prepared for gas chromatographic–mass spectral analysis by affinity chromatography isolation, followed by chemical cleavage of the C-9—N-10 bond. The *p*-aminobenzoylglutamate (*p*ABG) fragment was isolated by HPLC and was then converted to a lactam derivative that retained the isotopic labeling. Mass spectral data were acquired in the selected ion monitoring mode at charge-to-mass ratios (m/z) of 426, 428, and 431, corresponding to the molecular anions of the nonlabeled (^{1}H), $^{2}H_2$, and $^{13}C_5$ folate isotopomers, respectively. The molar ratios of $^{2}H_2$- and $^{13}C_5$-labeled folic acid to [^{1}H]folic acid were determined on the basis of working response curves relating the ratio of observed peak areas to actual molar ratios of standards of derivatized *p*ABG. The molar ratios were then corrected for the natural abundance of stable isotopes. Total urinary excretion of $^{2}H_2$- and $^{13}C_5$-labeled folates was calculated from the daily urine volume, the total folate concentration, and the molar ratios of labeled to nonlabeled folic acid. Excretion data were normalized by calculating molar urinary excretion ratios, as follows:

$$\text{Urinary excretion ratio} = \frac{\text{Percentage of dose of } [^{13}C_5]\text{folic acid in urine}}{\text{Percentage of dose of } [^{2}H_2]\text{folic acid in urine}} \tag{13.1}$$

The urinary excretion ratio (UER) is the primary indicator of absorption of oral doses of [$^{13}C_5$]folic acid. Expressing the UER of a fortified food sample as a percentage of the UER of the control gives the relative bioavailability of folic acid in that sample.

Rogers et al. [107] applied dual-label stable isotope methodology to the short-term plasma folate response and found that the response to intravenous [$^{2}H_2$]folic acid was ca. 15 times greater than to oral [$^{13}C_5$]folic acid, when adjusted for the difference in dose. This poor response to a relatively large oral dose of folic acid limits the usefulness of plasma kinetics in assessing the relative bioavailability of nutritionally relevant oral doses of labeled folate.

Wright et al. [110] reported important problems in interpreting folate bioavailability studies based on short-term plasma folate responses. Human plasma 5-methyl-THF response profiles (AUC) were obtained over a period of 8 h following oral administration of single physiological doses of [$^{13}C_6$]folic acid or (6*S*-) [$^{13}C_6$]-5-formyl-THF. Folate-binding affinity columns were used to isolate extracted plasma folate and, following HPLC analysis of total plasma 5-methyl-THF concentrations, a newly developed LC–MS analytical method [111] was used to determine the proportions of ^{13}C-labeled and unlabeled 5-methyl-THF.

An average 60 and 40% of the total plasma 5-methyl-THF response to an oral dose of labeled folic acid and labeled 5-formyl-THF, respectively, was unlabeled and must have originated from displacement of tissue folates. This makes interpretation of absorption studies using unlabeled folates problematic because the portion of plasma response due to oral versus endogenous folate cannot be reliably determined. Wright et al. [110] found no relationship between this unlabeled folate response and folate status. Furthermore, there was no apparent simple relationship between oral and endogenous response. The labeled plasma 5-methyl-THF AUC response to folic acid was smaller than that to 5-formyl-THF. Wright et al. [110] speculated that, unlike naturally occurring reduced folates, physiological doses of folic acid are not metabolized within the enterocytes to any great extent and enter the hepatic portal vein largely unmodified. There is extensive first-pass uptake of the folic acid by the liver, and the subsequent plasma 5-methyl-THF response is entirely a function of enterohepatic recirculation.

The data reported by Wright et al. [110] reveal that the relative bioavailability of food folate cannot be reliably estimated by comparing the labeled plasma response with that from an equivalent reference dose of folic acid. The use of isotopically labeled (6*S*)-5-methyl-THF (now commercially available) as the reference dose would overcome the problem of different rates and patterns of plasma response between folic acid and reduced folates. This adaptation would also be appropriate to bioavailability methods based on urinary excretion, using isotopically labeled (6*S*)-5-methyl-THF as the intravenous dose, rather than isotopically labeled folic acid. Finglas et al. [112] discussed the inherent advantages of LC–MS–MS over GC–MS in stable isotope methodology and ventured that development of compartmental modeling techniques could lead to measurement of absolute absorption of folates and *in vivo* kinetics.

13.5.2.3 Use of Ileostomy Subjects

Ileostomy subjects provide the opportunity to study absolute folate absorption on the principle that absorption of dietary folate takes place

exclusively in the small intestine. Folate excretion into the ileostomal effluent reflects nonabsorbed folate. In a procedure reported by Konings et al. [101], ileostomy bags are emptied immediately after the test meal and every 2 h thereafter during the following 9 h. The bags are emptied into a container stored in dry ice (−79°C) to minimize the influence of bacteria on the effluent. Witthöft et al. [104] recommended a 10–12 h collection period of individual ileostomy samples. Ileal folate excretion from bile was accounted for by introducing one study day without folate dosing.

Absolute absorption of a food folate is calculated from the difference between folate intake and folate content of ileostomal effluents.

$$\text{Absolute absorption (\%)} = \frac{\begin{array}{c}\text{Intake} + \text{Individual background level} \\ - \text{Excretion in ileostomy bag}\end{array}}{\text{Intake}} \times 100\%$$

Actually, "apparent" absolute absorption is calculated because no correction is made for physiological losses.

13.5.3 Inherent Bioavailability of Monoglutamyl and Polyglutamyl Folates

Gregory [57] has reviewed the inherent bioavailability of various folates in human subjects. Absorption of monoglutamyl folic acid ($PteGlu_1$) is essentially complete when administered orally under fasting conditions. There appears to be little nutritionally significant difference in the *in vivo* absorption and utilization among the various monoglutamyl folate vitamers (THF, 5-formyl-THF, 10-formyl-THF, and 5-methyl-THF) under normal dietary conditions. The bioavailability of polyglutamyl folate, as evaluated by the urinary excretion of radiolabeled folates in fasting subjects, ranges from approximately 60 to 80% relative to $PteGlu_1$. Gregory et al. [113] reported a bioavailability of ca. 50% for $PteGlu_6$ relative to $PteGlu_1$ using stable-isotope methodology based on urinary excretion ratios.

Melse-Boonstra et al. [114] conducted a long-term study in 180 healthy Dutch men and women to quantify the bioavailability of low doses of polyglutamyl relative to monoglutamyl folic acid. The volunteers, aged 50–70 years, ingested capsules containing 323 nmol heptaglutamyl folic acid/day, 262 nmol monoglutamyl folic acid/day, or placebo in a randomized parallel trial. Folate intake was restricted to the habitual diet and to the supplements supplied during the study. Because food fortification with folic acid is not allowed in the Netherlands, additional sources of folic acid could be ruled out. Serum and erythrocyte folate concentrations were measured after an overnight fast at baseline and after 12 weeks of

intervention. Mean serum and erythrocyte folate concentrations increased less in the polyglutamyl folic acid group [6.1 (95% confidence interval 5.3–7.0) and 155 (122–188) nmol/l, respectively] than in the monoglutamyl folic acid group [11.8 (10.3–13.3) and 282 (246–318) nmol/l, respectively]. Differences remained statistically significant ($P < 0.05$) after correction for the difference in the amount of folic acid administered. The relative bioavailability of polyglutamyl folic acid was 64% (95% confidence interval 52–75%) on the basis of serum folate and was 68% (51–84%) on the basis of erythrocyte folate concentrations, giving an overall value of 66%. In the Netherlands, about two-thirds of dietary folate is provided by polyglutamates and one-third is provided by monoglutamates [115]. The bioavailability of folate from a normal mixed diet would then be calculated as $[(2/3) \times 0.66 + (1/3)] \times 100\% = 77\%$, compared with a diet in which all folate would be present in the monoglutamate form.

13.5.4 Bioavailability of Naturally Occurring Folate in Fruits and Vegetables

Certain fruits and vegetables exhibit incomplete folate bioavailability as reviewed by Gregory [57]. Brouwer et al. [116] performed a dietary controlled trial with 66 healthy subjects to show that increased intake of dietary folate from vegetables and citrus fruit improved folate status and decreased total plasma homocysteine concentration. The relative bioavailability of folate from vegetables and citrus fruits compared with folic acid tablets was 78% using plasma folate concentration as the endpoint. In a 92-day controlled metabolic study in women, the relative bioavailability of folate from a mixed diet was less than 50% based on the plasma folate response [25].

A possible explanation for the incomplete bioavailability of polyglutamyl folate is that the intestinal deconjugation process is rate-limiting. Reisenauer and Halsted [117] calculated that human intestinal brush-border conjugase is present in sufficient quantity as to not limit folate absorption. However, changes in the pH of the luminal contents and the presence of dietary conjugase inhibitors and folate binders could adversely affect the rate of deconjugation and absorption of folate. These factors may account for the variation in folate bioavailability in individual foods.

As previously mentioned, plant tissues contain conjugase which attacks folate polyglutamates after mechanical disruption of the plant cell structure [118]. The bioavailability of folate in vegetables may be affected by the extent of polyglutamyl folate deconjugation by endogenous conjugase. Thus raw cabbage, which had undergone enzymatic deconjugation

during diet preparation and storage, was found to be completely biologically available by a chick bioassay [119]. In the same study, ca. 60% of the folate in cooked cabbage, which corresponded to the polyglutamate fraction, was not biologically available. The heat treatment would have inactivated the endogenous conjugase in the cooked cabbage, leaving the majority of the folate content in the polyglutamyl form.

Using 11 ileostomy subjects, Konings et al. [101] reported an absolute absorption of spinach folate between 73 and 91%. Chopped spinach was immediately heated in a microwave oven to give a mono- : polyglutamate ratio of 40 : 60 or it was stored for 24 h in a refrigerator at 4°C and then microwaved to give a 100 : 0 ratio. There was no difference in folate absorption between spinach subjected to these two treatments.

Folate bioavailability can be influenced by the way in which foods are processed. Consumption of spinach, in which the matrix was disrupted or destroyed by mincing or liquefying with enzymes prior to cooking, resulted in a higher plasma folate response than the consumption of whole-leaf spinach [120]. In this study, all spinach was blanched beforehand to destroy conjugases and other enzymes. Melse-Boonstra et al. [34] subjected vegetables (leeks, cauliflower, and green beans) to various processing treatments with the aim of increasing the enzymatic conversion of folate polyglutamate to the monoglutamate form by the endogenous conjugase and thereby increasing the vegetables' bioavailability. Both freezing/thawing and high-pressure treatments increased the proportion of monoglutamyl folate 2–3-fold. These treatments incurred a loss of total folate (>55%) due to leaching into the processing water, especially of the monoglutamyl form, but processing in a closed system would prevent this loss.

13.5.5 Bioavailability of Folate in Milk

The physiological function of the milk FBP is uncertain. It may ensure the folate content of milk by sequestering folate from the blood plasma into the mammary gland [121] and it might facilitate the absorption of folate from milk in the suckling animal, as previously discussed. It is also very effective in stabilizing the very labile THF and moderately labile 5-methyl-THF *in vitro* [122].

Smith et al. [123] monitored the folate status of infants by measuring serum and erythrocyte folate concentrations from birth to 1 yr. When solid foods were introduced after 3 months of life, blood concentrations of folate were significantly correlated with the total amount of folate ingested from milk (either human milk or bovine milk-based formula) but not with the total amount of folate in the diet. This strong correlation between folate status and milk folate intake suggests that folate present

in the milk component of the diet (whether from human milk, bovine milk, or bovine milk-based formula) is more available to the infant than is folate ingested from the remainder of the diet.

All folate in pasteurized cow's milk is protein-bound, despite a 20% loss of FBP during processing [76]. UHT processing reduces the FBP concentration by 97%, with accompanying loss of folate-binding capacity [76]. Thus UHT milk does not afford the suckling infant the potential benefits of protein-bound folate, although the infant is able to absorb unbound folate. Wigertz and Jägerstad [124] found no significant difference in the relative bioavailability of folate between processed milk (pasteurized, UHT-treated, or fermented milk) and raw milk in a rat bioassay, suggesting that the loss of protein-binding capacity does not affect the bioavailability of milk folate in the adult rat.

The use of a computer-controlled *in vitro* dynamic gastrointestinal model (TIM) permits the direct quantification of FBP before and after passage through a simulated gastrointestinal tract. The model comprises four connected compartments that represent the stomach, duodenum, jejunum, and ileum. It is strictly controlled for variables such as pH curves, enzyme activities, peristaltic movements, and transit times. Because TIM has no intestinal receptor systems, it cannot answer the question of whether or not folate absorption actually takes place. The TIM model has been used to study the bioaccessibility of folic acid and 5-methyl-THF in fortified milk and yogurt [125,126]. Bioaccessible folate describes the amount of folate released from the food matrix that is available for absorption in the small intestine. The TIM and food samples were analyzed by HPLC after cleanup and concentration by FBP affinity columns. FBP was quantified by an enzyme-linked immunosorbent assay (ELISA).

Verwei et al. [125] investigated folate-fortified pasteurized or UHT-processed milk with or without additional FBP. Folate bioaccessibility from folic acid-fortified milk without additional FBP was 58–61%. This was lower ($P < 0.05$) than that of milk fortified with 5-methyl-THF (71%). Addition of FBP reduced ($P < 0.05$) folate bioaccessibility from folic acid-fortified milk (44–51%) but not from 5-methyl-THF-fortified milk (72%). Approximately 15% of the FBP from folic-acid-fortified milk passed through the TIM system intact, in contrast to only 0–1% from pasteurized milk fortified with 5-methyl-THF. These results suggest that folic acid remains partly bound to FBP during passage through the small intestine, and this reduces the bioaccessibility of folic acid from milk.

The bioaccessibility for both folic acid and 5-methyl-THF from yogurt without FBP was 82% [126]. No difference in folate bioaccessibility was found between folate-fortified yogurt and folate-fortified pasteurized milk. However, the addition of FBP to yogurt lowered folate bioaccessibility more than the addition of FBP to pasteurized milk. This was

accompanied by a 2–16-fold higher ileal recovery of intact FBP from yogurt compared with pasteurized milk.

Verwei et al. [127] demonstrated that FBP in a whey suspension has a higher affinity for folic acid than for 5-methyl-THF at neutral pH under static experimental conditions. Incubation at pH 3 had no effect on the extent of binding of folic acid and 5-methyl-THF to FBP after adjustment to pH 7. This simulates the *in vivo* situation in which dissociation of folate from FBP takes place at low pH in the stomach and reassociation takes place at neutral pH in the duodenum. The stability and binding characteristics of FBP for folic acid and 5-methyl-THF were then investigated during passage through the TIM system. There was no change in the extent of folic acid binding to FBP during gastric passage, but the FBP-bound 5-methyl-THF fraction gradually decreased from 79 to 5%. From both folic acid—FBP and 5-methyl-THF—FBP mixtures, 70% of the initial amount of FBP was retained after gastric passage. This study revealed that FBP is partly stable during gastric passage, but exhibits different binding characteristics for folic acid and 5-methyl-THF in the duodenal lumen. This difference in extent of binding to FBP for the two folate compounds can influence the bioavailability from milk products, as mentioned in the experiments with milk [125] and yogurt [126].

13.5.6 Effects of Soluble Food Components on Folate Bioavailability

Bhandari and Gregory [128] extracted various foods into a pH 7.0 Tris–HCl buffer with the objective of determining the *in vitro* effect of soluble food components on the activity of intestinal brush-border conjugase. Brush-border membrane vesicles from human and pig jejunum were used as a source of conjugase activity to mimic the natural intestinal environment of the enzyme. As shown in Table 13.4, extracts from beans and peas caused a moderate (26–36%) inhibition of porcine conjugase activity, but more dramatic inhibition was caused by extracts from tomato (46%) and orange juice (80%). The observed inhibition was not accompanied by binding of enzyme to the substrate, and pH was not a factor because all the food extracts were neutralized before testing. Although citrate significantly inhibited the enzyme activity, it was not the causative factor of inhibition for tomato or orange juice. Inhibition was specific for the conjugase; two other brush-border enzymes, alkaline phosphatase and sucrase, were not affected by the foods tested. No inhibitory effect was shown by dietary FBP, soluble anionic polysaccharides, sulfated compounds, phytohemagglutinins, or trypsin inhibitors at nutritionally relevant concentrations.

Wei et al. [129] characterized further the *in vitro* inhibition of porcine conjugase by soluble fractions of selected foods (orange juice, tomatoes,

TABLE 13.4

In Vitro Effect of Extracts from Different Food Substances on the Conjugase Activity of Human and Porcine Jejunal Brush-Border Membrane Vesicles

	Relative Activity (% of Control)	
Food Substance	**Porcine**	**Human**
Red kidney beans	64.5[a]	84.1[a]
Pinto beans	64.9[a]	66.8[a]
Green lima beans	64.4[a]	64.8[a]
Cut green beans	74.1[a]	80.7[a]
Black-eyed peas	64.7[a]	71.3[a]
Yellow-corn meal	102.5	100
Wheat bran	91.9	85.8[a]
Tomato	54.1[a]	54.0[a]
Banana	74.8[a]	74.8[a]
Cauliflower	85.8	84.3[a]
Spinach	78.9[a]	86.1[a]
Orange juice	20.0[a]	26.6[a]
Whole egg	88.5	94.7
Evaporated milk	86.3	nd
Cabbage	87.9	nd
Lettuce	93.8	nd
Whole-wheat flour	99.7	nd
Medium rye flour	100.2	nd

Note: Control, the enzyme assayed in the absence of any food extract; nd, not determined.
[a]Significantly different from control, $P < 0.01$.
Source: From Bhandari, S.D. and Gregory, J.F., III, *Am. J. Clin. Nutr.*, 51, 87, 1990. American Society for Clinical Nutrition. With permission.

and lima beans) and by organic anions. Organic acids tested were competitive inhibitors with respect to the polyglutamyl folate substrate, with the following K_i values (mmol/l): citrate, 6.42; malate, 10.1; phytate, 6.48; ascorbate, 19.6. Neutralized orange juice strongly inhibited conjugase activity, neutralized tomato juice homogenate caused weaker inhibition, and lima bean homogenate inhibited much more weakly. Fractionation of food extracts indicated that the inhibitors were anions of low molecular mass (<6–8 kDa). Chromatographic separation of food extracts indicated that citrate was the major inhibitor, with less inhibition by malate and phytate. Although ascorbic acid is a relatively weak inhibitor, the taking of large doses of supplemental ascorbic acid at meal times could possibly reduce the extent of folate absorption.

The nutritional significance of the above *in vitro* findings was investigated *in vivo* using human subjects and dual-label, stable isotope methodology [130]. When $[^2H_4]PteGlu_1$ and $[^2H_2]PteGlu_6$ blended in a single portion of tomatoes or lima beans were consumed, the

polyglutamyl folate exhibited 100% relative bioavailability based on urinary excretion ratios. In contrast, the polyglutamate exhibited 66% relative bioavailability when consumed in a single serving of orange juice. Citrate did not appear to be solely responsible for the inhibitory effect of orange juice because a dose of citrate buffer of equivalent volume, concentration and pH had no significant effect. These findings indicate that the effect of orange juice components on folate absorption is specific for polyglutamyl folate and thus occurs at the level of folate deconjugation. Rhode et al. [131] measured serum folate concentrations in women taking orange juice or folic acid tablets during 9 weeks of a folate-restricted diet. Concentrations were lower in the orange juice group than in the folic acid group, but the difference did not reach statistical significance. The less than complete bioavailability of folate in orange juice is compensated by the high content of folate and its stability afforded by the high concentration of the antioxidant ascorbic acid.

An unidentified heat-activated factor associated with the skin of legumes was reported to inhibit the activity of hog kidney conjugase *in vitro* [132]. The conjugases of human plasma, chicken pancreas, and rat liver were similarly inhibited [133]. Inhibition of jejunal brush-border conjugase was not examined in either of these studies because the existence of this particular enzyme was not known. Wei and Gregory [129] found no evidence of such an inhibitor in their studies with jejunal conjugase. In a human study, the bioavailability of $PteGlu_7$ added to a formulated diet was not affected by the presence of beans in the diet, and was not significantly different from the bioavailability of added $PteGlu_1$ [134].

13.5.7 Effects of Dietary Fiber on Folate Bioavailability

The possible influence of dietary fiber on folate bioavailability is of interest, because many of the food products reported to have low folate bioavailability are also high in dietary fiber.

The bioavailability of monoglutamyl folate does not appear to be inhibited by high-fiber foods. Neither spinach nor bran cereal impaired the absorption of folic acid in humans [135], and high-fiber Iranian breads were also without effect [136]. Ristow et al. [137] investigated the ability of purified dietary fiber components to sequester folic acid *in vitro* using equilibrium dialysis under neutral isotonic conditions. The recovery of essentially 100% of the folic acid from each material (cellulose, pectin, lignin, sodium alginate, and wheat bran) indicated the absence of any binding or entrapment. *In vivo* effects were evaluated by a chick bioassay with graded levels of folic acid in semipurified diets containing the fiber material at 3% (w/w). There were no differences between any of

the materials with respect to the rise in plasma and liver folate as a function of dietary folic acid. The results of this study suggest that the fiber component of human diets would have a negligible effect on the bioavailability of folic acid employed in food fortification. A similar conclusion can be drawn from data produced by Keagy and Oace [138], who showed that cellulose, xylan, pectin, or wheat bran had no detectable effect on the utilization of folic acid added to rat diets.

In contrast to the lack of effect of dietary fiber on the bioavailability of monoglutamyl folate, certain forms of fiber (e.g., wheat bran) may lessen the bioavailability of polyglutamyl folate. The serum folate response of human subjects to $PteGlu_7$ when added to bran cereal was significantly less than the response to $PteGlu_1$ added to bran cereal [135]. In another human study [134], the addition of 30 g of wheat bran to a formulated meal delayed slightly the absorption of added $PteGlu_7$ relative to added $PteGlu_1$. The authors presented possible factors that may explain the lower bioavailability of polyglutamyl folate compared with monoglutamyl folate when consumed with wheat bran. The six additional alpha carboxyl groups in the $PteGlu_7$ molecule increase the potential for anionic interactions. Wheat bran has cation exchange properties and can decrease intragastric concentrations of hydrogen ions and pepsin. Thus it has the potential for direct interaction with folate compounds, may interfere with folate binding to other diet components, may alter the pH of the medium, and may alter the rate and extent of digestion of other diet components. In addition, wheat bran reaches the colon faster than other sources of fiber [139].

13.5.8 Bioavailability of Folate in Fortified Foods

Colman et al. [140] compared the efficiency of absorption of folic acid from fortified whole wheat bread, rice, maize meal (porridge), and an aqueous solution of folic acid in human subjects. Each helping of the food contained 1 mg of added folic acid, the same amount as in the aqueous dose. The absorption profile for each subject was determined as the summated increases in serum folate 1 and 2 h after administration of the test materials. The absorption efficiencies of the rice and maize were similar (ca. 55%), relative to the response observed with aqueous folic acid, while the absorption efficiencies of the bread was lower (ca. 30%). The folic acid was found to resist destruction by the conventional cooking conditions used to prepare the test foods. The long-term rate of change of erythrocyte folate during daily administration of fortified maize meal [141] or fortified bread [142] corroborated these findings.

The results of Colman and associates suggested that interactions of the added folic acid with food components, possibly during cooking, caused

impaired absorption. To investigate this possibility, Ristow et al. [119] prepared lactose–casein liquid model food systems fortified with folic acid or 5-methyl-THF and subjected them to retort processing (121°C for 20 min). Microbiological and HPLC analysis indicated that folic acid was very stable during thermal processing, whereas 5-methyl-THF was ca. 75% degraded. A chick bioassay showed that the bioavailability of the remaining folate was complete for both fortified model food systems, indicating that no complexes were formed during processing that inhibited folate utilization.

Finglas et al. [143] used an oral/intravenous, dual-label, stable-isotope protocol to determine potential matrix effects on the bioavailability of fortificant folic acid from cereal-based foods. The relative 48-h urinary excretion ratio for white bread and bran flakes, when compared with that for folic acid capsules, was 0.71 and 0.37, respectively, indicating that some cereal-based vehicles may inhibit absorption of fortificant.

13.5.9 Effects of Alcohol on Folate Status

Except for certain beers, little or no folate is present in alcoholic beverages. Thus chronic alcoholics who substitute ethanol for other sources of calories typically deprive themselves of dietary folate and are potential cases of megaloblastic anemia. The well-nourished alcoholic rarely manifests this condition.

Halsted [144] has reviewed the etiologies of folate deficiency in alcoholism. Aside from the poor diet of alcoholics, chronic alcoholism impairs both conjugase activity and jejunal uptake of folate, decreases liver folate levels, and increases urinary folate excretion. Destruction of the folate molecule as a consequence of ethanol metabolism may also contribute to the vitamin deficiency.

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14

Vitamin B_{12} (Cobalamins)

14.1 Background

There are two vitamin B_{12} coenzymes with known metabolic activity in humans, namely methylcobalamin and adenosylcobalamin.

Vitamin B_{12} deficiency has a drastic effect on folate metabolism because methylcobalamin is a coenzyme for methionine synthetase, the enzyme that catalyzes the methylation of homocysteine to methionine using 5-methyl-tetrahydrofolate (5-methyl-THF) as the methyl donor. The inability to synthesize methionine from homocysteine in the absence of vitamin B_{12} means that THF cannot be regenerated from the demethylation of 5-methyl-THF. The folate thus becomes trapped in the form of 5-methyl-THF because the formation of this derivative by reduction of 5,10-methylene-THF is thermodynamically irreversible. This situation could lead to the inability to form the other THF derivatives that are necessary for purine and pyrimidine synthesis. The consequent lack of DNA synthesis causes many hemopoietic cells to die in the bone marrow. In this event, a megaloblastic anemia that is clinically indistinguishable from that induced by folate deficiency results. When this type of anemia is caused by deficiency of vitamin B_{12}, it is called pernicious anemia, because it is accompanied by a neuropathy which is unrelated to folate deficiency. The neuropathy is caused by the inability to produce the lipid component of myelin, which results in a generalized demyelinization of nerve tissue. Neuropathy begins in the peripheral nerves, affecting first the feet and fingers, and then progressing to the spinal cord and brain.

The body is extremely efficient at conserving vitamin B_{12}. Unlike the other water-soluble vitamins, vitamin B_{12} is stored in the liver. Vitamin B_{12} deficiency is rarely, if ever, caused by a lack of dietary B_{12}; rather it is attributable to various disorders of absorption and transport. Absorption of vitamin B_{12} is facilitated by intrinsic factor, a protein secreted by the parietal cells of the stomach lining. Elderly people are prone to atrophic gastritis, a condition in which the gastric oxyntic mucosa

atrophies to such an extent that virtually no intrinsic factor (or hydrochloric acid) is secreted. In patients with diverticula, strictures, and fistulas of the small intestine, stagnant regions of the lumen may become contaminated with colonic bacteria. The bacteria can take up much of the dietary vitamin B_{12} passing by, whether it be the free vitamin or vitamin bound to intrinsic factor. A common inherited disorder is an autoimmune reaction with formation of antibodies against intrinsic factor. Vitamin B_{12} is nontoxic when taken orally.

14.2 Chemical Structure, Biopotency, and Physicochemical Properties

14.2.1 Structure and Potency

In accordance with the literature on nutrition and pharmacology, the term vitamin B_{12} is used in this text as the generic descriptor for all cobalamins that exhibit antipernicious anemia activity. Individual cobalamins will be referred to by their specific names (e.g., cyanocobalamin).

The cobalamin molecule is a six-coordination cobalt complex containing a corrin ring system substituted with numerous methyl, acetamide, and propionamide radicals (Figure 14.1). Methylene bridges link the pyrrole rings A to B, B to C, and C to D but not A to D, which are linked directly. The cobalt atom, which may assume an oxidation state of (I), (II), or (III), is linked by four equatorial coordinate bonds to the four pyrrole nitrogens, and by an axial coordinate bond to a 5,6-dimethylbenzimidazole (DMB) moiety, which extends in α-glycosidic linkage to ribose-3-phosphate. The phosphate group is linked to the D ring of the corrin structure via a substituted propionamide chain. The pseudonucleotide (DMB base plus sugar phosphate) is oriented perpendicularly to the corrin structure. The remaining axial coordinate bond at the X position links the cobalt atom to a cyano (—CN) group in the case of cyanocobalamin ($C_{63}H_{88}O_{14}N_{14}PCo$, MW = 1355.4) or, depending on the chemical environment, to some other group (e.g., —OH in hydroxocobalamin and —HSO_3 in sulfitocobalamin).

There are two vitamin B_{12} coenzymes with known metabolic activity in humans, namely, methylcobalamin and 5′-deoxyadenosylcobalamin (frequently abbreviated to adenosylcobalamin and also known as coenzyme B_{12}). The methyl or adenosyl ligands of the coenzymes occupy the X position in the corrin structure. The coenzymes are bound intracellularly to their protein apoenzymes through a covalent peptide link, or in milk and plasma to specific transport proteins.

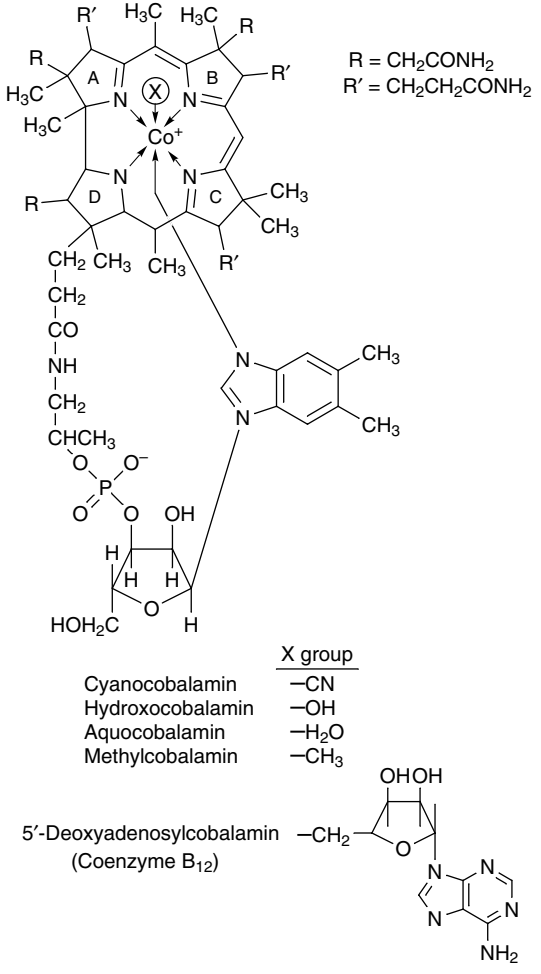

FIGURE 14.1
Structures of vitamin B_{12} compounds.

14.2.2 Physicochemical Properties

14.2.2.1 Appearance and Solubility

Cyanocobalamin is an artificial product used in pharmaceutical preparations because of its stability. It is a tasteless, dark red, crystalline hygroscopic powder, which can take up appreciably more than the 12% of moisture permitted by the British Pharmacopoeia. The anhydrous material can be obtained by drying under reduced pressure at 105°C. Cyanocobalamin is soluble in water (1.25 g/100 ml at 25°C) and in lower alcohols, phenol, and other hydroxylated solvents like ethylene diol; it is

insoluble in acetone, chloroform, ether, and most other organic solvents. Aqueous solutions of cyanocobalamin are of neutral pH.

14.2.2.2 Stability in Aqueous Solution

Cyanocob(III)alamin is the most stable of the vitamin B_{12}-active cobalamins and is the one mostly used in pharmaceutical preparations and food supplementation. Aqueous solutions of cyanocobalamin are stable in air at room temperature if protected from light. The pH region of optimal stability is 4.5–5 and solutions of pH 4–7 can be autoclaved at 120°C for 20 min with negligible loss of vitamin activity. The addition of ammonium sulfate increases the stability of cyanocobalamin in aqueous solution. Heating with dilute acid deactivates the vitamin owing to hydrolysis of the amide substituents or further degradation of the molecule. Mild alkaline hydrolysis at 100°C promotes cyclization of the acetamide side chain at C-7 to form a biologically inactive γ-lactam or, in the presence of an oxidizing agent, a γ-lactone [1]. The vitamin B_{12} activity in aqueous solutions is destroyed in the presence of strong oxidizing agents and high concentrations of reducing agents, such as ascorbic acid, sulfite, and iron(II) salts. On exposure to light, the cyano group dissociates from cyanocobalamin and hydroxocob(III)alamin is formed. In neutral and acid solution, hydroxocobalamin exists in the form of aquocobalamin [2]. This photolytic reaction does not cause a loss of activity. Adenosylcobalamin and methylcobalamin are reduced cob(I)alamin derivatives, which are easily oxidized by light to the hydroxo compound [3].

14.3 Vitamin B_{12} in Foods

14.3.1 Occurrence

Naturally occurring vitamin B_{12} originates solely from synthesis by bacteria and other microorganisms growing in soil or water, in sewage, and in the rumen and intestinal tract of animals. Any traces of the vitamin that may be detected in plants are due to microbial contamination from the soil or manure or, in the case of certain legumes, to bacterial synthesis in the root nodules.

Vitamin B_{12} is ubiquitous in foods of animal origin and is derived from the animal's ingestion of cobalamin-containing animal tissue or microbiologically contaminated plant material, in addition to vitamin absorbed from the animal's own digestive tract. The vitamin B_{12} contents of some foods in which the vitamin is found are listed in Table 14.1 [4]. Liver is the outstanding dietary source of the vitamin, followed by kidney and heart. Muscle meats, fish, eggs, cheese, and milk are other important food

TABLE 14.1

Vitamin B_{12} Content of Various Foods

Food	μg Vitamin B_{12} per 100 g Edible Portion
Cow milk, whole, pasteurized	0.9
Cheese, cheddar, average	2.4
Egg, chicken, whole, raw	2.5
Beef, trimmed lean, raw, average	2
Lamb, trimmed lean, raw, average	2
Pork, trimmed lean, raw, average	1
Chicken meat, raw	Tr
Liver, lamb, fried	83
Kidney, lamb, fried	54
Cod, raw, fillets	1
Herring, grilled	15
Pilchards, canned in tomato sauce	13
Salmon, grilled	5

Note: Tr, trace.
Source: From Food Standards Agency, *McCance and Widdowson's The Composition of Foods*, 6th summary ed., Royal Society of Chemistry, Cambridge, 2002. With permission.

sources. Vitamin B_{12} activity has been reported in yeast, but this has since been attributed to the presence of noncobalamin corrinoids or vitamin B_{12} originating from the enriching medium [5]. Spirulina, a type of seaweed, is claimed to be a source of vitamin B_{12}, but in fact is practically devoid of the vitamin. The so-called vitamin B_{12} in spirulina is actually inactive analogs, two of which have been shown to block vitamin B_{12} metabolism in human cell cultures [5]. About 5–30% of the reported vitamin B_{12} in foods may be microbiologically active noncobalamin corrinoids rather than true B_{12} [6].

Vitamin B_{12} in foods exists in several forms. Meat and fish contain mostly adenosyl- and hydroxocobalamins; these compounds, accompanied by methylcobalamin, also occur in dairy products, with hydroxocobalamin predominating in milk. Sulfitocobalamin is found in canned meat and fish. Cyanocobalamin was not detected, apart from small amounts in egg white, cheese, and boiled haddock [7]. In bovine milk, naturally occurring vitamin B_{12} is bound to proteins, with a high proportion being present in whey proteins [8].

14.3.2 Stability

The cobalamins present in food are generally resistant to thermal processing and cooking in a nonalkaline medium. There was no distinctive destruction of vitamin B_{12} during sterilization and storage of canned meals containing beef [9]. A 27–33% loss of vitamin B_{12}, expressed per

unit of nitrogen, occurred during the cooking of beef due to the loss of moisture and fat; the vitamin content of raw and cooked beef was, however, similar on a moisture basis [10]. Thus there is little loss of the vitamin in the cooking of meat provided that the meat juices are utilized. Microwave heating of raw beef and pork and pasteurized cow's milk for 6 min resulted in an appreciable loss (ca. 30–40%) of vitamin B_{12} [11]. Degradation products of hydroxovitamin B_{12} formed in foods by microwave heating had no vitamin activity and were nontoxic [12].

In the heat treatment of milk, the following losses of vitamin B_{12} have been reported: boiling for 2–5 min, 30%; pasteurization for 2–3 sec, 7%; sterilization in the bottle at 120°C for 13 min, 77%; rapid sterilization at 143°C for 3–4 sec with superheated steam, 10%; evaporation, 70–90%; and spray-drying, 20–35% [13]. The light-sensitive coenzyme forms of vitamin B_{12} are largely converted to hydroxocobalamin in light-exposed milk, but with no loss of vitamin activity [7].

Arkbåge et al. [14] used a validated radio protein-binding assay to evaluate the retention of vitamin B_{12} at key stages during the manufacture of six different fermented dairy products and at subsequent ripening and storage until "use-by" date. Two different heat treatments of milk (76°C for 16 sec and 96°C for 5 min) caused no loss of vitamin B_{12}. Milk after heat treatment was therefore chosen as starting point for calculating the retention of vitamin B_{12}, and set to 100%. The addition of starter cultures did not affect vitamin B_{12} concentrations in any of the fermented dairy products tested. For the fermented milks, fermentation of heat-treated milk resulted in vitamin B_{12} losses of 15% for Filmjölk and 25% for yoghurt. Storage of an unopened package of product at 4°C for 14 days, until "use-by date," reduced the vitamin B_{12} concentrations further by 26 and 33% for Filmjölk and yoghurt, respectively. Taken together, Filmjölk and yoghurt contained 40–60% of the vitamin B_{12} originally present in the milk, when consumed at use-by date. During the manufacture of cottage cheese, about 82% of the original vitamin B_{12} was removed with the whey fraction, whereas 16% of the vitamin was retained in the curd. A vitamin B_{12} addition corresponding to 6% of the starting milk content was obtained in the final product by mixing the curd with dressing (made from skimmed milk, cream, and salt). The level of vitamin B_{12} at packaging remained unaltered during storage of an unopened package for 10 days, until use-by date. Thus, in total, the manufacture and storage of cottage cheese retained 16% of the original vitamin B_{12} content. During hard cheese production, 44–52% of the original vitamin B_{12} was removed with the whey. In total, the hard cheeses that ripened for up to 32 weeks retained about 60–70% of the original vitamin B_{12} content. The manufacture of blue cheese incurred the removal of 38% of the original vitamin B_{12} with the whey. The added mold did not contain any detectable vitamin B_{12}. After a ripening period of 5–6 weeks, 45%

of the original vitamin B_{12} was still present in the blue cheese. The additional 8 weeks of storage until use-by date did not seem to alter the vitamin B_{12} content.

14.3.3 Applicability of Analytical Techniques

Vitamin B_{12} occurs intracellularly in the living tissues of animals in the form of the two coenzymes, adenosylcobalamin and methylcobalamin, which are covalently bound to their protein apoenzymes. In milk, these coenzymes are bound noncovalently to specific transport proteins. Hydroxocobalamin is present in animal-derived foods, especially in milk, as a result of the photooxidation of the coenzyme forms. Cyanocobalamin is a synthetic stable form of the vitamin and is used in fortification. The potential vitamin B_{12} activity of a food sample is represented by the total cobalamin content, regardless of the ligand attached. The determination of total vitamin B_{12} may be performed by microbiological assay or by radioassay. Supplemental vitamin B_{12} (cyanocobalamin) may be determined by a nonisotopic protein-binding assay. A biosensor-based protein-binding assay has been developed for determining supplemental and endogenous vitamin B_{12}.

No international unit for vitamin B_{12} activity has been defined, and the assay results are expressed in milligrams, micrograms, or nanograms of pure crystalline cyanocobalamin. The measurement of biological activity in preparations containing vitamin B_{12} relies on microbiological assays, there being no animal bioassay.

14.4 Absorption and Conservation

Much of the following discussion of absorption and conservation is taken from a book by Ball [15] published in 2004.

Humans appear to be entirely dependent on a dietary intake of vitamin B_{12}. Although microbial synthesis of the vitamin occurs in the human colon, it is apparently not absorbed [16]. Strict vegetarians may obtain limited amounts of vitamin B_{12} through ingestion of the vitamin-containing root nodules of certain legumes and from plant material contaminated with microorganisms.

The absorption and transport of the vitamin B_{12} naturally present in foods takes place by specialized mechanisms that accumulate the vitamin and deliver it to cells that require it. The high efficiency of these mechanisms enables 50–90% of the minute amount of B_{12} present in a typical omnivorous diet (ca. 10 μg) to be absorbed and delivered to cells. The specificity is such that all natural forms of vitamin B_{12} are

absorbed and transported in the same way; structurally similar but biologically inactive analogs, which are metabolically useless and possibly harmful, bypass the transport mechanisms and are eliminated from the body.

14.4.1 Digestion and Absorption of Dietary Vitamin B_{12}

Ingested protein-bound cobalamins are released by the combined action of hydrochloric acid and pepsin in the stomach. Gastric juice also contains two functionally distinct cobalamin-binding proteins: (1) haptocorrin (there are actually several haptocorrins, which are also known as R binders or cobalophilin) and (2) intrinsic factor. Haptocorrin binds a wide variety of cobalamin analogs in addition to vitamin B_{12}, whereas intrinsic factor binds B_{12} vitamers with high specificity and equal affinity. The 5,6-dimethylbenzimidazole moiety is essential for recognition by intrinsic factor [17]. The haptocorrin originates in saliva, while intrinsic factor is synthesized and secreted directly into gastric juice by the parietal cells of the stomach. At the acid pH of the stomach, cobalamins have a greater affinity for haptocorrin than for intrinsic factor. Therefore cobalamins leave the stomach and enter the duodenum bound to haptocorrin and accompanied by free intrinsic factor. In the mildly alkaline environment of the jejunum, pancreatic proteases, particularly trypsin, partially degrade both free haptocorrin and haptocorrin complexed with cobalamins. Intrinsic factor, which is resistant to proteolysis by pancreatic enzymes, then binds avidly to the released B_{12} vitamers.

The intrinsic factor–B_{12} complex is carried down to the ileum where it binds avidly to specific receptors on the brush border of the ileal enterocyte. The presence of calcium ions and a pH above 5.5 are necessary to induce the appropriate configuration of the receptor for binding [18]. The intrinsic factor–B_{12} complex is absorbed intact [19], but the precise mechanism of absorption and subsequent events within the enterocyte have yet to be elucidated. It is possible that ileal absorption of the intrinsic factor–B_{12} complex is accomplished by receptor-mediated endocytosis [20], but clathrin-coated pits or vesicles have not been found. The B_{12} is subsequently released at an intracellular site, possibly in either lysosomes or prelysosomal vesicles, both of which are acidic [21]. The intrinsic factor appears to be degraded by proteolysis after releasing its bound B_{12}, there being no apparent recycling of intrinsic factor to the brush-border membrane.

The intrinsic factor-mediated system is capable of handling between 1.5 and 3.0 μg of vitamin B_{12}. The limited capacity of the ileum to absorb B_{12} can be explained by the limited number of receptor sites, there being only about one receptor per microvillus. Saturation of the system at one meal

does not preclude absorption of normal amounts of the vitamin some hours later. The entire absorptive process, from ingestion of the vitamin to its appearance in the portal vein, takes 8–12 h.

Absorption can also occur by simple diffusion across the entire small intestine. This process probably accounts for the absorption of only 1–3% of the vitamin consumed in ordinary diets, but can provide a physiologically significant source of the vitamin when it is administered as free cobalamin in pharmacological doses of 30 μg or more.

14.4.2 Conservation of Vitamin B_{12}

The body is extremely efficient at conserving vitamin B_{12}. Unlike the other water-soluble vitamins, vitamin B_{12} is stored in the liver, primarily in the form of adenosylcobalamin. In an adult man, the total body store of vitamin B_{12} is estimated to be 2000–5000 μg, of which about 80% is in the liver. The remainder of the stored vitamin is located in muscle, skin, and blood plasma. Only 2–5 μg of vitamin B_{12} are lost daily through metabolic turnover, regardless of the amount stored in the body.

Vitamin B_{12} excreted in the bile is reabsorbed in the ileum along with dietary sources of the vitamin. This enteroheptic cycle allows the excretion of unwanted nonvitamin cobalamin analogs, which constitute about 60% of the corrinoids secreted in bile, and returns vitamin B_{12} relatively free of analogs.

The binding of vitamin B_{12} with specific plasma proteins (transcobalamins I and II) prevents the vitamin molecule from being excreted in the urine as it passes through the kidney. Only if the circulating B_{12} exceeds the vitamin-binding capacity of the blood is the excess excreted; this typically occurs only after injection of cobalamin. The binding with plasma proteins, negligible urinary loss, and an efficient enterohepatic circulation, together with the slow rate of turnover, explains why strict vegetarians normally take 20 yr or more to develop signs of deficiency. People with absorptive malfunction develop deficiency signs within 2–3 yr.

14.5 Bioavailability

14.5.1 Efficiency of Absorption

The percentage of ingested vitamin B_{12} that is absorbed decreases as the actual amount in the diet increases. At intakes of 0.5 μg or less, ca. 70% of the available vitamin B_{12} is absorbed. At an intake of 5.0 μg, a mean of 28% is absorbed (range, 2–50%) while at a 10-μg intake the mean

absorption is 16% (range, 0–34%) [22]. When 100 μg or more of crystalline vitamin B_{12} is taken, the absorption efficiency drops to 1%, and the excess vitamin is excreted in the urine.

The efficiency of vitamin B_{12} absorption from a variety of foods has been determined in human subjects using extrinsically labeled vitamin B_{12} and whole body counting or stool counting techniques. The mean percentage absorption of the extrinsic vitamin B_{12} label was as follows: lean mutton, 65% [23]; chicken, 60% [24]; fish, 39% [25]; eggs, 24–36% [26]; milk, 65% [27]; and fortified bread, 55% [27]. In all these foods, with the exception of eggs, vitamin B_{12} was absorbed as efficiently as a comparable amount of crystalline cyanocobalamin administered orally in aqueous solution. The relatively poor absorption of vitamin B_{12} in eggs was attributed to the presence of distinct vitamin B_{12}-binding proteins in egg white and egg yolk [28].

14.5.2 Bioavailability Studies

14.5.2.1 Effects of Dietary Fiber

Vitamin B_{12} depletion occurs more rapidly in the presence than in the absence of intestinal microorganisms, presumably due to competition for available B_{12} between the gut flora and the host. Cullen and Oace [29] investigated the possibility that dietary fiber, by stimulating the growth of intestinal bacteria, might increase the rate of vitamin B_{12} utilization by the rat. The results showed that cellulose or pectin added to purified vitamin B_{12}-deficient diets increased the fecal excretion of radioactive B_{12} that was injected after several weeks of depletion. Thus, both cellulose and pectin may have bound or adsorbed biliary B_{12} and carried it past the ileal absorptive sites. In addition, pectin, which is hydrolyzed to an appreciable extent by intestinal microorganisms, might have served as a substrate for the growth of vitamin B_{12}-requiring bacteria.

There was no significant difference in the urinary excretion of vitamin B_{12} in human subjects receiving controlled diets supplemented with or without wheat bran [30].

14.5.2.2 Effects of Alcohol

Although alcohol ingestion has been shown to decrease vitamin B_{12} absorption in volunteers after several weeks of intake, alcoholics do not commonly suffer from vitamin B_{12} deficiency, probably because of the large body stores of the vitamin and the reserve capacity for absorption [31].

14.5.2.3 Effects of Smoking

Several constituents of cigarette smoke react with vitamin B_{12} and convert it to biologically inactive forms. For example, cyanides and hydrogen sulfide have a high affinity for the vitamin's cobalt atom and form cyanocobalamin and sulfocobalamin, respectively. Nitrous oxide inactivates methylcobalamin through oxidation of the cobalt atom [32]. Cigarette smokers, but not nonsmokers, showed a high urinary thiocyanate excretion, which was associated with increased vitamin B_{12} excretion and a relatively low serum B_{12} concentration [33]. Because urinary thiocyanate excretion is an index of cyanide detoxication, it appears possible that a high plasma cyanide concentration caused through smoking disturbs the equilibrium between plasma and urinary vitamin B_{12}. Piyathilake et al. [32] reported that vitamin B_{12} concentrations in buccal mucosal cells of smokers were significantly lower than in cells of nonsmokers. Salivary vitamin B_{12} was higher in smokers, possibly because of leakage of cyanocobalamin from the smoke-exposed buccal mucosal cells.

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15

Vitamin C

15.1 Background

Humans, in common with other primates, guinea pigs, and fruit-eating bats, rely on their diet to provide vitamin C in the form of ascorbic acid. The functions of ascorbic acid are based primarily on its properties as a reversible biological antioxidant.

Hydroxylase enzymes that require iron or copper ions as cofactors also require the specific presence of ascorbic acid as an electron donor to maintain these ions in the reduced form. In the synthesis of collagen, the initial polypeptide undergoes post-translational modifications to produce the crosslinked triple helix. These modifications entail the hydroxylation of proline and lysine residues by enzymes that require ferrous ions. Other iron-containing hydroxylases are required for the post-translational modification of osteocalcin (a protein involved in bone mineralization) and protein C (one of the blood-clotting factors). Iron-dependent hydroxylations also feature in the synthesis of carnitine from protein-incorporated lysine. Many peptide hormones and hormone-releasing factors are synthesized as precursor molecules that, after a series of copper-dependent modifications, are converted to their active forms. A copper-dependent enzyme also catalyzes the hydroxylation of dopamine to noradrenaline.

Ascorbic acid, in collaboration with α-tocopherol and β-carotene, plays an important role in the defense against cellular damage by oxidants. In this role, ascorbic acid scavenges many types of free radical and also regenerates the reduced form of α-tocopherol.

A deficiency of vitamin C results in scurvy, the primary symptoms of which are hemorrhages in the gums, skin, bones and joints, and the failure of wound healing. These symptoms are accompanied by listlessness, malaise, and other behavioral effects.

Ascorbic acid is generally regarded as being nontoxic, although excessive daily amounts can cause an increased production of oxalic acid in some individuals, leading to an increased risk of kidney stone formation.

15.2 Chemical Structure, Biopotency, and Physicochemical Properties

15.2.1 Structure and Potency

The term vitamin C is used as the generic descriptor for all compounds exhibiting qualitatively the biological activity of ascorbic acid. The principal natural compound with vitamin C activity is L-ascorbic acid ($C_6H_8O_6$, MW = 176.1). There are two enantiomeric pairs, namely L- and D-ascorbic acid, and L- and D-isoascorbic acid (Figure 15.1). D-Ascorbic acid and L-isoascorbic acid are devoid of vitamin C activity and do not occur in nature. D-Isoascorbic acid (also known as erythorbic acid) is also not found in natural products, apart from its occurrence in certain microorganisms. It is, however, a byproduct of biosynthetic vitamin C, produced from glucose by a combined chemical and microbial procedure, and has been detected in beer after addition of commercial vitamin C [1]. Erythorbic acid possesses similar reductive properties to L-ascorbic acid, but exhibits only 5% of the antiscorbutic activity of L-ascorbic acid in guinea pigs [2,3].

Ascorbic acid is used extensively in food technology as a stabilizer for the processing of beverages, wines, and meat products. Ascorbyl palmitate is a stable, fat-soluble form of the vitamin which does not occur in

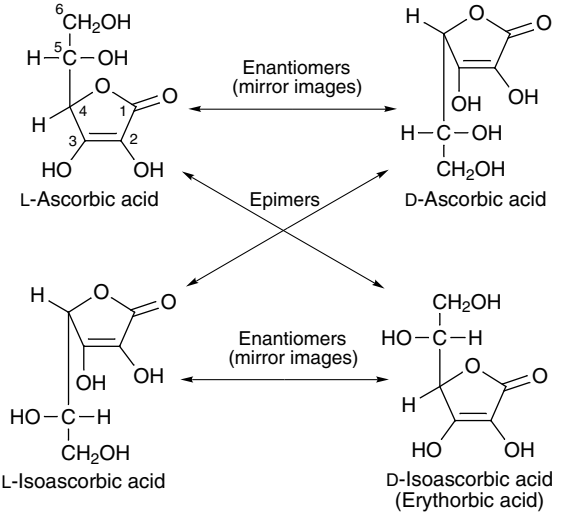

FIGURE 15.1
Stereoiosomers of 2-hexenono-1,4-lactone.

nature. It exhibits the full antiscorbutic activity of ascorbic acid on a molar basis: that is, 1 g of ascorbyl palmitate is equivalent to the potency of 0.425 g of ascorbic acid. Erythorbic acid is cheaper to manufacture on a commercial scale than L-ascorbic acid. In some countries, it is legal to substitute erythorbate for ascorbate when, for technological reasons, the antioxidant or reducing properties and not the vitamin C activity of the additive is required. In the United Kingdom and certain other countries, erythorbate is not permitted as an antioxidant, and it is also prohibited for use with raw and unprocessed meats.

L-Ascorbic acid is easily and reversibly oxidized to dehydroascorbic acid, forming the ascorbyl radical anion (also known as semidehydroascorbate) as an intermediate (Figure 15.2). Dehydroascorbic acid possesses full vitamin C activity because it is readily reduced to ascorbic acid in the animal body. Dehydroascorbic acid is not an acid in the chemical sense, as it lacks the dissociable protons that ascorbic acid has at the carbon 2 and 3 positions.

15.2.2 Physicochemical Properties

15.2.2.1 Solubility and Other Properties

Ascorbic acid is an almost odorless white or very pale yellow crystalline powder with a pleasant sharp taste and an mp of about 190°C (with decomposition). Pure dry crystalline ascorbic acid and sodium ascorbate are stable on exposure to air and daylight at normal room temperature for long periods of time. Commercial vitamin C tablets possess virtually their original potency even after 8-yr storage at 25°C [4]. Ascorbic acid is readily soluble in water (33 g/100 ml at 25°C), less soluble in 95% ethanol (3.3 g/100 ml), absolute ethanol (2 g/100 ml), acetic acid (0.2 g/100 ml), and acetonitrile (0.05 g/100 ml) and insoluble in fat solvents [5]. A 5% aqueous solution of ascorbic acid has a pH of 2.2–2.5, the acidic nature being due to the facile ionization of the hydroxyl group on C-3 ($pK_1 = 4.17$); the hydroxyl group on C-2 is much more

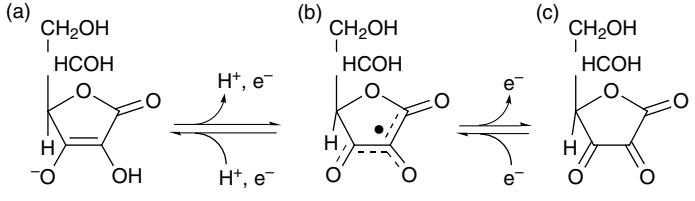

FIGURE 15.2
Oxidation of ascorbate. (a) L-Ascorbate (AH^-), (b) ascorbyl radical anion ($A^{-\bullet}$), and (c) dehydroascorbic acid. Note the delocalized unpaired electron in the ascorbyl radical anion.

resistant to ionization ($pK_2 = 11.79$) [6]. Sodium ascorbate is freely soluble in water (62 g/100 ml at 25°C; 78 g/100 ml at 75°C) and practically insoluble in ethanol, diethyl ether, and chloroform; the pH of an aqueous solution is 5.6–7.0.

Ascorbyl palmitate is practically insoluble at 25°C in water (0.2 g/100 ml), soluble in ethanol (20 g/100 ml), and slightly soluble in diethyl ether. The solubility in vegetable oils at room temperature is very low (30 mg/100 ml) but increases sharply with increasing temperature.

The carbonyl enediol group of ascorbic acid confers strong reducing properties to the molecule, as indicated by its ability to reduce Fehling's or Tollen's solution at room temperature. The redox potential of the first stage at pH 5.0 is $\varepsilon_0^1 = +0.127$ V.

15.2.2.2 Stability in Aqueous Solution

Oxidation of ascorbic acid follows first-order kinetics in the pH range 3–7 in aqueous model systems containing traces of copper. Stability is higher in the pH range 3.0–4.5 than in the range 5.0–7.0 [7]. At alkaline pH, ascorbic acid is unstable. At pH 1, the ionization of ascorbic acid is suppressed, and the fully protonated molecule is relatively slowly attacked by oxygen. Consequently, the rate of oxidation of ascorbic acid accelerates as the pH is increased from 1.5 to 3.5.

Dehydroascorbic acid in solution at neutral or alkaline pH undergoes a nonreversible oxidation to form the biologically inactive, straight-chained compound, 2,3-diketogulonic acid. The half-life for this breakdown is 6 min at 37°C [8], 2 min at 70°C, and less than 1 min at 100°C [9]. Dehydroascorbic acid is, however, stable for several days at 4°C at pH 2.5–5.5 [5].

15.3 Vitamin C in Foods

15.3.1 Occurrence

The ascorbic acid and dehydroascorbic acid contents of some vegetables and fruits are listed in Table 15.1 [10]. The values shown are typical of the observed concentrations found in these samples, but they can vary greatly (Table 15.2) [11] and should not be taken as absolute. Genetic variation, maturity, climate, sunlight, method of harvesting, and storage all can affect the levels of vitamin C.

Fresh fruits (especially blackcurrants and citrus fruits) and green vegetables constitute rich sources of vitamin C. Potatoes contain moderate amounts but, because of their high consumption, represent the most

TABLE 15.1

Vitamin C Content of Some Vegetables and Fruits

	Concentration (mg/100 g)[a,b]		
Food	**AA**	**DHAA**	**Total**
Vegetables			
Broccoli			
Fresh, raw	89.0 ± 2.0	7.7 ± 0.6	97 ± 2
Boiled	37.0 ± 1.0	2.6 ± 0.6	40 ± 1
Microwaved	111.0 ± 2.0	4.7 ± 0.6	116 ± 2
Cabbage			
Fresh, raw	42.3 ± 3.4	—	42 ± 3
Boiled	24.4 ± 1.6	—	24 ± 2
Cauliflower, fresh, raw	54.0 ± 1.0	8.7 ± 0.6	63 ± 1
Spinach			
Fresh, raw	52.4 ± 2.5	—	52 ± 3
Boiled	19.6 ± 1.0	—	20 ± 1
Microwaved (4 min)	48.3 ± 3.7	5.8 ± 0.6	54 ± 4
Peppers			
Red, fresh	151.0 ± 3.0	4.0 ± 1.0	155 ± 4
Green, fresh	129.0 ± 1.0	5.0 ± 0.0	134 ± 1
Potatoes (without skin)			
Raw	8.0 ± 0.0	3.0 ± 0.0	11 ± 0
Boiled	7.0 ± 1.0	1.3 ± 0.6	9 ± 1
Tomatoes, fresh	10.6 ± 0.6	3.0 ± 0.0	14 ± 1
Fruits			
Bananas	15.3 ± 2.5	3.3 ± 0.6	19 ± 3
Grapefruit, fresh	21.3 ± 0.6	2.3 ± 0.6	24 ± 1
Oranges			
Florida	54.7 ± 2.5	8.3 ± 1.2	63 ± 3
California navel	75 ± 4.5	8.2 ± 1.6	83 ± 5

[a]Values reported are mean ± standard deviation based on three measurements. When no values are listed, the concentration was <1 mg/100 g sample.
[b]Foods analyzed by HPLC together with robotic extraction procedures.
Source: From Vanderslice, J.T., Higgs, D.J., Hayes, J.M., and Block, G., *J. Food Comp. Anal.*, 3, 105, 1990. With permission.

important source of the vitamin in the British diet. Liver (containing 10–40 mg/100 g), kidney, and heart are good sources, but muscle meats and cereal grains do not contain the vitamin. Human milk provides enough ascorbic acid to prevent scurvy in breast-fed infants, but preparations of cow's milk are a poor source owing to oxidative losses incurred during processing. Cabbage and other brassica contain a bound form of ascorbic acid known as ascorbigen, which exhibits 15–20% bioavailability in guinea pigs [12].

TABLE 15.2

Range of Vitamin C Values in Some Vegetables and Fruits

Sample	Total Vitamin C Content (AA + DHAA) (mg/100 g)
Broccoli, raw	97–163
Cabbage, raw	42–83
Spinach, fresh	25–70
Potatoes, Idaho	11–13
Tomatoes	14–19
Bananas	12–19
Grapefruit, red	21–31
Oranges	
Florida	53–63
California navel	52–78

Source: From Vanderslice, J.T. and Higgs, D.J., *Am. J. Clin. Nutr.*, 54, 1323S, 1991. With permission.

15.3.2 Stability

Ascorbic acid is very susceptible to chemical and enzymatic oxidation during the processing, storage, and cooking of food. The catalyzed oxidative pathway of ascorbic acid degradation is the most important reaction pathway for loss of vitamin C in foods. In the presence of molecular oxygen and trace amounts of transition metals [particularly copper(II) and iron(III)], a metal–oxygen–ascorbate complex is formed. This complex has a resonance form of a diradical that rapidly decomposes to give the ascorbate radical anion, the original metal ion, and hydrogen peroxide. The radical anion then rapidly reacts with oxygen to give dehydroascorbic acid [13].

In the absence of free oxygen, an anaerobic pathway of ascorbic acid degradation leads to the formation of diketogulonic acid [13]. The rate of degradation is maximum at pH 3–4 and therefore this pathway could be responsible for the anaerobic loss of vitamin C in canned grapefruit and orange juices, which have a pH of ca. 3.5. Degradation of ascorbic acid beyond diketogulonic acid is closely tied to nonenzymatic browning in some food products [13].

The enzyme mainly responsible for enzymatic degradation of ascorbic acid in plant tissues after harvesting is ascorbate oxidase (EC 1.10.3.3), which catalyzes the oxidation of ascorbic acid to dehydroascorbic acid. This enzyme exhibits maximum activity at 40°C and is almost completely inactivated at 65°C [14]. Hence rapid heating, such as the blanching of fruit and vegetables or the pasteurization of fruit juices, prevents

the action of this enzyme during post-process storage. The ascorbic acid in intact plant tissue is protected from ascorbate oxidase and other enzymes by cellular compartmentation. However, when tissues are disrupted after bruising, wilting, rotting, or during advanced stages of senescence, the enzymes gain access to the vitamin and begin to destroy it.

Enzymatic destruction of ascorbic acid in plant tissues begins as soon as a crop is harvested. Maintaining cool conditions during transport and storage can markedly reduce vitamin C loss in some vegetables. For example, newly harvested peas retained 70% of their ascorbic acid content after 14 days' storage at 4°C compared with 20% retention at ambient temperature (20°C). Corresponding retentions for broccoli were 90 and 30% [15].

Vegetables grown for commercial freezing are processed as soon as possible after harvest. Some loss of vitamin C takes place during blanching, but little further loss occurs during deep frozen storage. Blanching losses are greatest in green leafy vegetables with large surface areas. Thus, steam blanching of broccoli decreased ascorbic acid by about 30%, whereas losses in green beans were only slight [16]. In a study of frozen stored green beans [17], no significant oxidation of ascorbic acid occurred during the blanching and freezing steps. During frozen storage, there was a progressive conversion of ascorbic acid to dehydroascorbic acid, which was almost complete after 20 days of storage at −7°C. The dehydroascorbic acid was very stable at freezer temperatures, the average loss after 250 days of frozen storage being only 8%. Thus vitamin C, in the form of dehydroascorbic acid, is well retained during the frozen storage of green beans.

Chemical oxidation of ascorbic acid is lowered during processing by carrying out vacuum deaeration and inert gas treatment where feasible. The headspace in containers should be minimized and hermetically sealed systems used. Ascorbic acid is very stable in canned or bottled foods after the oxygen in the headspace has been used up, provided the food is not subjected to high-temperature storage or exposed to light. In contrast to glass containers, plastic bottles and cardboard cartons are permeable to oxygen, so a lowered vitamin C retention is to be expected. Bronze, brass, copper, and iron equipment should be avoided, while sequestering agents such as ethylenediaminetetraacetic acid (EDTA), polyphosphates, and citrates prevent the catalytic action of traces of copper and iron. The sulfites and metabisulfites, which are added to juices or beverages as a source of SO_2, exert a stabilizing effect on ascorbic acid in addition to their role as antimicrobial agents. The addition of food-grade antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate also protect the vitamin.

The loss of ascorbic acid from orange juice correlates with the amount of oxygen initially present in the headspace and that dissolved in the juice,

and with temperature and storage time [18–20]. In the presence of dissolved oxygen, ascorbic acid decomposition is predominantly aerobic, but when the dissolved oxygen is depleted decomposition continues by an anaerobic pathway that is mainly influenced by temperature [18]. The loss of ascorbic acid also correlates with an increase in the browning of the juice [20]. Light has little or no effect upon ascorbic acid levels in orange juice [19]. The vitamin loss is rapid in the early stage of storage, coincident with the consumption of dissolved oxygen, and then becomes gradual. The packaging of orange juice in an experimental oxygen-scavenging film reduced ascorbic acid loss in the first 3 days at 25°C compared with the loss from juice packaged in a film with no oxygen scavenger [20]. This demonstrated that the oxygen scavenger can remove oxygen from the juice before it has the opportunity to react with the ascorbic acid. Furthermore, the residual oxygen-scavenging capacity in the experimental film provided an ongoing barrier to oxygen permeation.

The storage of broccoli spears in elevated (20%) carbon dioxide atmosphere suppresses tissue respiration and ethylene production rates, whilst also delaying loss of chlorophyll and ascorbic acid [21]. The packaging of broccoli spears in polymeric film modified the atmosphere by elevating carbon dioxide to 8% and lowering oxygen to 10%. While ascorbic acid retention in packaged broccoli dropped about 15% from initial values in the first 48 h, losses were minimal during the following 48 h. In contrast, the degradation of ascorbic acid in nonpackaged samples showed a steady decline over time and decreased 31% by 96 h [22].

Ascorbic acid can leach away from fruits and vegetables during processing or cooking. This is of little importance with canned, bottled, or stewed fruits where the juice is eaten with the tissue, but may represent a serious loss with vegetables, where the liquor is drained away before serving. If vegetables are steamed or pressure-cooked instead of boiled, the leaching effect is greatly reduced, but a greater loss of ascorbic acid is to be expected from oxidation. Cold water washing or steeping does not normally leach out a significant amount of the vitamin in whole undamaged fruits and vegetables. In jam making, when the fruit is boiled with sugar, ascorbic acid is remarkably stable [23].

Vitamin C in freshly secreted cow's milk is predominantly in the form of ascorbic acid, but this is rapidly oxidized by the dissolved oxygen content [24]. The photochemical destruction of riboflavin accelerates the oxidation of ascorbic acid in milk through a sensitizing effect [25,26]. Losses of vitamin C content during high-temperature-short time (HTST) and ultrahigh temperature (UHT) treatment of milk average 20% [27].

Graham and Stevenson [28] studied the effect of ionizing radiation on vitamin C content of strawberries and potatoes using ^{60}cobalt as the

source of gamma rays. Their HPLC method of analysis permitted the separate measurement of total vitamin C, ascorbic acid, and dehydroascorbic acid. Fresh strawberries have a relatively short shelf-life, mainly because of fungal spoilage. Irradiation at doses of 2–3 kGy combined with refrigeration has been shown to extend the shelf-life of strawberries. Low doses (0.075–0.15 kGy) of γ-radiation are very effective in inhibiting the sprouting of potatoes during post-harvest storage.

Strawberries were assayed immediately after irradiation at doses of 1, 2, or 3 kGy and then after storage for 5 and 10 days at 6°C. Irradiation had the effect of increasing the dehydroascorbic acid content in accordance with dose, whilst decreasing the concentrations of total vitamin C and ascorbic acid. During the storage of irradiated strawberries, the total vitamin C and ascorbic acid levels increased, whereas dehydroascorbic acid levels decreased. Overall, irradiation of the strawberries caused some loss of total vitamin C, which increased during storage (Table 15.3).

Raw, boiled, and microwaved potatoes were assayed immediately after irradiation at 0.15 kGy and then after storage for 1, 2, and 5 months at 12°C. The potatoes were irradiated 1 month after harvesting to allow them to recover from the effects of post-harvest stress. Losses of total vitamin C in the raw potatoes immediately after irradiation were about 8%; corresponding losses in the cooked potatoes were about 20%. There was no significant difference in the dehydroascorbic acid content between irradiated and nonirradiated samples. After 2 and 3 months' storage, irradiated potatoes contained less vitamin C than nonirradiated potatoes. However, after 5 months' storage, both irradiated and nonirradiated potatoes had comparable vitamin C levels. The vitamin C content in both irradiated and nonirradiated cooked potatoes showed similar changes on storage to those of raw potatoes. Similar findings for potatoes were reported by Shirsat and Thomas [29]. The loss of total vitamin C in nonirradiated potatoes after 3 months in storage (15°C) was 26–45%, depending on the cultivar. Additional losses of 6.5–13% were

TABLE 15.3

Percent Loss of Vitamin C in Four Varieties of Strawberry Following a 3-kGy Dose of Gamma Radiation

Storage (days)	Variety 1	Variety 2	Variety 3	Variety 4	Mean
0	12.8	12.2	6.4	6.3	9.4
5	14.1	3.8	7.5	20.2	11.4
10	22.7	14.0	13.6	19.5	17.5

Source: From Graham, W.D. and Stevenson, M.H., *J. Sci. Food Agric.*, 75, 371, 1997. With permission.

recorded in the irradiated samples stored for 3 months. From the fourth month onwards, the vitamin C levels in the irradiated samples began to increase. In conclusion, irradiation keeps the potatoes in good marketable condition during proper storage conditions for at least 5 months, with no additional loss of vitamin C after this period.

Two studies were carried out to evaluate the possibility of using high hydrostatic pressure (HHP) treatment as an alternative to the blanching of vegetables. Potato cubes retained 90% of their ascorbic acid content after treatment at 400 MPa and 5°C for 15 min, but only 35% when the temperature was increased to 50°C [30]. Green peas retained 82% ascorbic acid after treatment at 900 MPa and 43°C for 5 min [31]. Other studies have evaluated HHP treatment as a possible alternative to the thermal pasteurization of freshly squeezed fruit juices. Ascorbic acid in orange juice and tomato juice was shown to be unstable at a combination of relatively high pressure (850 MPa) and temperature (60–85°C). At the same pressure level and lower temperature (50°C), no degradation of ascorbic acid occurred within 1 h [32]. High-pressure treatment of chilled orange juice (500 and 800 MPa for 5 min) and storage up to 21 days at 4°C caused no significant difference in vitamin C [33]. Similar treatment of orange juice (800 MPa at 25°C for 1 min) greatly extended the shelf-life, with less than 20% loss of ascorbic acid after 3 months' storage at 4°C or after 2 months at 15°C [34]. Pressure-processing of guava puree (600 MPa and 25°C for 15 min) and storage at 4°C for 40 days did not alter initial content of ascorbic acid [35].

Sánchez-Morino et al. [36] tested three processes that combined high-pressure treatment with heat treatment for their effect on vitamin C retention in orange juice: T0, fresh juice (without treatment); T1, 100 MPa/60°C/5 min; T2, 350 MPa/30°C/2.5 min; T3, 400 MPa/40°C/1 min. Fresh and treated samples were kept refrigerated (4°C) and assayed at intervals over 10 days. T1 and T3 juices showed a small (<10%) loss of vitamin C just after processing, whereas T2 juices showed no loss of the vitamin. There was no further degradation of vitamin C during the 10-day storage period. Therefore, the intermediate pressure/low-temperature treatment best preserved the vitamin C content.

A major factor contributing to the variability in vitamin C content of potatoes is the storage time. A sharp decrease in vitamin content was observed during the first 4 months of storage at 7°C and 95% relative humidity, followed by either a complete leveling out or a less pronounced decrease [37].

Wang et al. [38] reported on the losses of added ascorbic acid during the pilot-scale processing and storage of potato flakes, and during the reconstitution and holding of the mashed potatoes prior to serving. Cumulative losses were: 56% after addition of ascorbic acid to the cooked mashed potatoes followed by drum-drying; 82% storing the flakes 4.3 months

at 25°C; and 96% reconstituting mashed potatoes and holding them 30 min on a steam table. The total cumulative loss of ascorbic acid was still severe (82%) when the dried potato flakes were stored for 1 month at 25°C and the reconstituted mashed potatoes were held on the steam table for 15 min. The initial 56% loss occurred during the cooking and mashing steps, with little loss during drum-drying. The entrapped air, high moisture content and high temperature of the mashed potatoes explain the high losses of ascorbic acid. There was no loss of ascorbic acid during reconstitution of the mashed potatoes. Adding ascorbic acid at a level of 251 ppm to the freshly mashed potatoes gave a final level of 10 ppm ascorbic acid in the reconstituted mashed potatoes at the point of consumption. In contrast, addition of magnesium L-ascorbate 2-monophosphate or sodium L-ascorbate 2-polyphosphate at about 250 ppm ascorbic acid equivalents produced mashed potato with ascorbic acid equivalents of 201 or 171 ppm, respectively (20 or 30% overall losses). These two compounds are more stable toward oxygen than ascorbic acid.

Williams et al. [39] compared two foodservice systems for their effect on retention of ascorbic acid in vegetables: (1) cook/hot-hold and (2) cook/chill, where food is cooked, chilled and held up to 5 days before reheating. The cook/chill system retained less vitamin C than food held hot for 30 min (50 versus 65%), but more than food held hot for 2 h.

The amount of water used in domestic cooking and, to a lesser extent, the cooking time affect vitamin C losses more than the source of energy or the type of cooking [14]. If short cooking times and small amounts of water are used, more vitamin C will be retained in any cooking method. Theoretically, stir-frying should provide maximum vitamin C retention. When the same ratio of water to vegetable (1 : 4) was used in the microwaving and boiling of frozen peas, ascorbic acid retentions were similar (70%), but lower than when no water was used in the microwave oven (>96%). In most frozen vegetables, sufficient ice clings to the product to provide adequate moisture for cooking in the microwave oven. When microwaving fresh vegetables, it is advisable to add a minimum amount of water to prevent scorching. When boiling vegetables, boiling water should be added to the vegetables and boiling maintained in order to rapidly inactivate enzymes that would otherwise destroy the vitamin C.

15.3.3 Applicability of Analytical Techniques

In food analysis, a method for determining vitamin C should ideally account for both ascorbic acid and its reversible oxidation product, dehydroascorbic acid, to give a total value for vitamin C. In addition, the ability

to distinguish ascorbic acid from its epimer D-isoascorbic acid (erythorbic acid) is useful in the analysis of processed foods.

The classic titrimetric method using 2,6-dichlorophenolindophenol accounts for ascorbic acid, but not dehydroascorbic acid. Nonchromatographic methods for determining total vitamin C include colorimetry and fluorometry, in which ascorbic acid is oxidized to dehydroascorbic acid and then reacted with a chemical reagent to form a colored or fluorescent compound. Total vitamin C can be determined by HPLC using absorbance or electrochemical detection after reduction of dehydroascorbic acid to ascorbic acid, or using fluorescence detection after oxidation of ascorbic acid and derivatization of the dehydroascorbic acid formed. Capillary electrophoresis offers an alternative to HPLC and eliminates the need for organic mobile phases and expensive chromatography columns. Flow-injection analysis coupled with immobilized enzyme and using electrochemical detection confers high specificity and provides a rapid automated procedure using relatively simple apparatus. Results obtained by chemical analysis are usually expressed in milligrams of pure L-ascorbic acid.

15.4 Intestinal Absorption

Much of the following discussion of absorption is taken from a book by Ball [40] published in 2004.

15.4.1 General Principles

Approximately 80–90% of the vitamin C content of a given foodstuff exits in the reduced form, ascorbic acid; the remainder is in the oxidized form, dehydroascorbic acid. Ascorbic acid and dehydroascorbic acid are absorbed by separate transport mechanisms in animal species that depend upon dietary vitamin C (Figure 15.3). Inside the absorptive cell (enterocyte) of the intestinal epithelium, dehydroascorbic acid is enzymatically reduced and the accumulated ascorbic acid is transported across the basolateral membrane to the bloodstream. In addition to uptake at the brush-border membrane, dehydroascorbic acid from the bloodstream can be taken up at the basolateral membrane, reduced within the cell, and returned to the circulation in the form of the useful and nontoxic ascorbic acid. The serosal uptake of dehydroascorbic acid from the bloodstream and intracellular reduction to ascorbic acid take place in animal species which do not have a dietary vitamin C requirement as well as those species that do. The ability of the enterocyte to

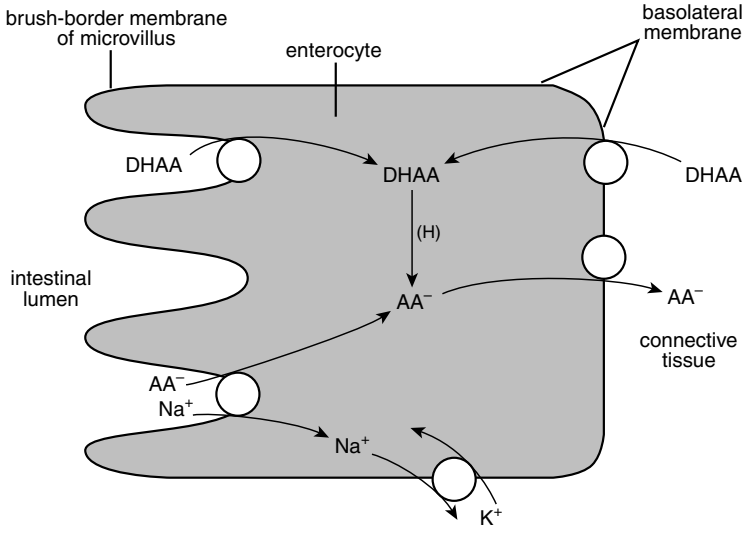

FIGURE 15.3
Model of intestinal transport of the L-ascorbate anion (AH^-) and uncharged dehydroascorbic acid (A) in vitamin C-dependent animals. Thick arrowed lines indicate directional pathways; [H] signifies enzymatic reduction. (From Ball, G.F.M., *Vitamins. Their Role in the Human Body*, Blackwell Publishing Limited, Oxford, 2004, p. 393. With permission.)

absorb dehydroascorbic acid efficiently is important because, apart from the indigenous dehydroascorbic acid content of the diet, additional oxidation of ascorbic acid occurs in the gastrointestinal tract as the vitamin functions to maintain other nutrients such as iron in the reduced state. The intestinal uptake and reduction of dehydroascorbic acid explains why this compound, orally administered, maintains plasma concentrations of ascorbic acid and prevents scurvy. The overall system of intestinal transport and metabolism is designed to maximize the conservation of vitamin C and also to maintain the vitamin in its nontoxic reduced state, whether it is derived from the diet or from the circulation.

15.4.2 Transport Mechanisms

15.4.2.1 Ascorbic Acid

Absorption of physiological intakes of ascorbic acid by guinea pigs takes place mainly in the ileum and occurs as a result of specific carrier-mediated mechanisms in the brush-border and basolateral membranes of enterocytes [41–44]. Ascorbic acid is 99.9% ionized within the pH range of intestinal chyme, and therefore it is the ascorbate anion

(specifically L-ascorbate$^-$) that is transported across the brush-border membrane. The absorption mechanism is sodium-coupled, secondary active transport. Experiments using brush-border membrane vesicles from guinea pig small intestine have shown that ascorbate transport is unaffected by changes in the membrane potential [44]. The transport system is therefore electrically neutral, indicating a 1 : 1 cotransport of ascorbate$^-$ and Na^+ by the same carrier. The immediate energy for the sodium-coupled transport of ascorbate is provided by the inward sodium concentration gradient, which in turn is created and maintained by the sodium pump at the basolateral membrane. Phloridzin, a well-known fully-competitive inhibitor of D-glucose transport across the small intestinal brush-border membrane, does not inhibit L-ascorbate uptake, demonstrating that D-glucose and L-ascorbate do not share the same carrier. Uptake of L-ascorbate is, however, competitively inhibited by D-isoascorbic acid, making the latter a potential antivitamin C.

Ascorbate leaves the enterocyte by sodium-independent, facilitated diffusion at the basolateral membrane [43]. Although the high intracellular concentration of ascorbate and the negative membrane potential are energetically favorable toward the exit of ascorbate, a carrier protein is required to facilitate transport of the hydrophobic anion across the lipid bilayer.

15.4.2.2 Dehydroascorbic Acid

In the guinea pig intestine, intraluminal dehydroascorbic acid is transported across the brush-border membrane by facilitated diffusion, driven by the steep concentration gradient maintained by its intracellular reduction to ascorbate. Dehydroascorbic acid is also taken up from the blood across the basolateral membrane by facilitated diffusion [45]. Dehydroascorbic acid, lacking the dissociable hydrogens at carbon atoms 2 and 3, does not ionize and is therefore unable to be co-transported with sodium. It can, however, be transported by the glucose transporter GLUT1 with an affinity similar to or lower than that for glucose [46].

15.4.3 Efficiency of Ascorbate Absorption in Humans

The usual dietary intake of vitamin C ranges from 30 to 180 mg/day and over this range the efficiency of absorption is 70–90% [47]. Brush-border uptake by the sodium-coupled, secondary active transport mechanism reaches its maximum rate at a relatively low luminal concentration. Beyond physiological intakes, absorption becomes progressively less efficient, falling from 75% of a single 1-g dose to 16% of a single 12-g dose (Table 15.4) [48,49]. This fall-off in efficiency occurs because absorption of high luminal concentrations of vitamin C takes place mainly by simple diffusion, and this passive movement proceeds at a very low

TABLE 15.4

Absorption of Large, Single, Oral Doses of Ascorbic Acid in Humans

	Absorption Efficiency (%)	
Dose Ingested (g)	**Experiment 1[a]**	**Experiment 2[b]**
1	75	—
1.5	—	50
2	44	—
3	39	36
4	28	—
5	20	—
6	—	26
12	—	16

Source: [a]From Hornig, D., Vuilleumier, J.-P., and Hartmann, D., *Int. J. Vitam. Nutr. Res.*, 50, 309, 1980. [b]Kübler, W. and Gehler, J., *Int. J. Vitam. Nutr. Res.*, 40, 442, 1970 (in German). With permission.

rate. In addition, when large amounts of vitamin C are ingested, appreciable amounts are broken down in the intestinal lumen to compounds that are degraded to carbon dioxide, which is eliminated by exhalation [50].

The ingestion of eight 0.125-g doses of ascorbate spaced throughout the day produced a 72% increase in absorption compared with a single 1-g dose [51]. The absorption efficiency of a single dose can be improved if the ascorbate is ingested in the form of a sustained-release capsule [52]. The ingestion of 1 g of ascorbate immediately after a fatty meal produced a 69% increase in absorption compared with the same dose given on an empty stomach [51]. The divided dose effect is consistent with a saturable absorption mechanism, whereas the after-meal effect indicates a slowing of gastric emptying.

15.5 Bioavailability

15.5.1 Bioavailability of Vitamin C in Foods

Nelson et al. [53] compared the absorption of naturally occurring ascorbic acid from orange juice with synthetic ascorbic acid from a chemically defined nutrient solution, using an intestinal perfusion technique in human subjects. No significant difference was found in the uptake of the vitamin from the two sources.

In a human study involving 68 adult male nonsmokers, Mangels et al. [54] examined the relative bioavailability of ascorbic acid from several sources. Subjects underwent two 8-week ascorbic acid depletion–repletion cycles. In repletion, subjects were randomized to receive 108 mg of ascorbic acid per day as a tablet taken with or without an iron tablet (63 mg of ferrous fumarate to release 20 mg of elemental iron), as orange segments or juice, or as raw or cooked broccoli. The experiment was designed with a crossover within each major treatment group (e.g., cooked to raw broccoli) for the second repletion. The response to the tested source of ascorbic acid was quantified as the rate of change of plasma ascorbate concentration over the first 3 weeks of each repletion period. The relative bioavailability of ascorbic acid from the various sources was determined by comparing these responses. Statistical analysis of the data showed no significant overall differences in ascorbic acid bioavailability among the three main sources of the vitamin (i.e., tablets, orange, and broccoli). In addition, the bioavailability of ascorbic acid in the tablet alone did not differ from that in the tablet plus iron, and there was no difference in bioavailability between orange segments and juice. The bioavailability of ascorbic acid from raw broccoli was about 20% lower than from cooked broccoli. The lack of a significant effect of the iron supplement on the bioavailability of synthetic ascorbic acid suggests that the presence of iron has no influence on ascorbate absorption or its stability in the intestinal lumen prior to absorption. This is rather surprising, as ascorbic acid is known to enhance the absorption of nonheme iron when the two nutrients are ingested together [55]. In summary, the observation that ascorbic acid bioavailability from the fruit and vegetable sources examined was not significantly different from that from synthetic ascorbic acid indicates that the bioavailability of vitamin C in a typical American diet is high.

Bates et al. [56] used stable isotope labeled-ascorbate to explore the possible influence of ferrous iron and red grape juice on the kinetics of ascorbate absorption in human subjects. The effects of red grape juice were studied because this drink is rich in polyphenols and polyphenols in fruits or fruit extracts have been reported to protect ascorbate against oxidative destruction [57,58]. In agreement with the findings of Mangels et al. [54], ferrous iron had no significant effect on the extent of degradation of the [^{13}C]ascorbate dose to $^{13}CO_2$ or on ascorbate absorption kinetics. The red grape juice exerted a transient inhibitory effect on ascorbate absorption, reaching significance ca. 20 min after dosing.

15.5.2 Effects of Dietary Fiber

In vitro studies using 1-^{14}C-ascorbic acid have shown that ascorbic acid interacts with wheat bran, possibly due to adsorption or entrapment by water held in the fiber matrix [59].

15.5.3 Effects of Alcohol

In a study of the acute effects of alcohol on plasma ascorbic acid in healthy subjects, Fazio et al. [60] observed the increase in plasma ascorbate concentrations over fasting levels after the ingestion of 2.0 g of ascorbic acid and breakfast. When 35 g of ethanol was ingested with ascorbic acid and breakfast, plasma concentrations were significantly lower for at least 24 h. This effect was probably due to an impairment in absorption of ascorbic acid by ethanol. These findings indicated that ethanol may reduce the availability of ascorbic acid from food and predispose to deficiency of the vitamin. Whether ethanol increases the excretion, catabolism, or utilization of ascorbic acid is not known [61].

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Part II

Analysis of Vitamins

16

Analytical Considerations

16.1 Bioassays

Vitamins are physiologically active substances and a true assessment of the nutritional value of a food commodity or diet with respect to a particular vitamin can only be obtained by determining the vitamin's biological activity. The only direct means of determining biological activity is a bioassay based on a biological function. Such assays were the means by which the vitamins were originally discovered and they are still in use today to measure the biopotency of novel vitamin analogs that may have potential therapeutic benefit. A discussion of bioassay methodology is beyond the scope of this book, since such assays are in the province of specialized laboratories.

The result from a bioassay accounts for the combined *in vivo* response to all vitamin-active compounds and vitamin precursors present in the test sample. The response may be either the overall growth response or a specific response, such as calcification of bone induced by vitamin D. Bioassays based on biological function depend on a series of sequential physiological events: intestinal absorption, plasma transport, tissue uptake, metabolism, and biological function at the target cell.

Bioassays may be either curative or prophylactic. In a typical bioassay of the curative type, rats or chicks are fed a formulated basal diet, which provides all their nutritional requirements except for the vitamin under assay. When a decline in body weight or the appearance of a specific vitamin deficiency disorder is observed, the animals are divided into treatment groups and fed with either the test food sample or a standard preparation of the vitamin incorporated into the basal diet. The extent to which the growth retardation or deficiency disorder has been cured is then estimated, and from this estimate the vitamin content of the test sample can be calculated. In prophylactic assays, the animals receive the test sample and standard vitamin preparation at the beginning of the assay, and their ability to prevent the deficiency is compared. In both types of assay, control animals receive only the basal diet throughout.

Bioassays based on biological function can be of long duration and expensive. Consequently, alternative bioassays have been developed, which are simpler to perform, more rapid, and less expensive. Among the latter, assays based on elevated plasma vitamin levels take into account the efficiency of intestinal absorption and plasma transport. Liver-storage assays go one step further because they depend on tissue uptake of the vitamin. However, neither of these bioassays accounts for biological function at the cellular level and, therefore, they may not reflect the true physiological vitamin activity of the test sample.

16.2 *In Vitro* Analytical Techniques

Nowadays, vitamin determinations in foods are routinely performed using microbiological assays or physicochemical methods of analysis. These, along with biospecific assays, can be described collectively as *in vitro* techniques. Microbiological assays, like animal bioassays, measure the combined response of the active substances and take into account utilization at the cellular level. Physicochemical assays permit the quantification of the principal substances that are responsible for the biological activity, and can achieve a high degree of precision. The great drawback of *in vitro* analytical techniques is that they do not account for the complexities of mammalian digestion and absorption and thus do not provide a reliable estimate of vitamin bioavailability. This is particularly the case for vitamins which are chemically bound with other constituents of the food matrix.

Since the mid-1970s up to the present, the method of choice for determining the fat-soluble vitamins in foods has been high-performance liquid chromatography (HPLC). This is due to the technique's ability to separate the vitamins without the need for chemical derivatization, the nondestructive operation, and the detection selectivity. HPLC can be used in the preparative mode to purify sample extracts, as well as in the quantitative mode. Microbiological assays, developed in the early 1940s, remain the official (AOAC) methods for determining vitamin B_6, pantothenic acid, folate, and vitamin B_{12}.

16.3 Analytical Approach

The selection of a suitable analytical method depends on what needs to be measured to provide the required information to fulfil the objective. For example, the chemist in the food manufacturing industry must

conform with the nutritional labeling regulations and achieve a consistent product without necessarily being concerned with bioavailability. The main concern is how much of the vitamin that was added in fortification is actually present in the finished product. In practice, this requirement is comparatively simple, as the vitamin in its parent form will be added as a stabilized preparation, which can be readily extracted from an analytical sample in a measurable quantity. The fortified product should be reasonably consistent for every batch, and therefore, no unforeseen problems associated with a changing food matrix should be encountered once a suitable analytical method has been established. The quality control manager will need a quick and reliable result on a regular basis, with minimum cost per analysis; therefore, the method should be simple and robust, automated as far as possible, and provide good repeatability (i.e., within-laboratory precision).

The investigator who needs to determine the nutritional value of a food commodity or diet is faced with a more demanding analytical challenge. Most of the vitamins occur naturally in trace amounts and in a variety of forms (vitamers), and some are chemically bound to proteins or carbohydrates in the food matrix. Moreover, the matrix will vary enormously in composition if different diets are being studied. For nutritional purposes, the accuracy of the method (i.e., nearness to truth) is more important than the precision.

16.4 Preparation of Sample Extracts for Analysis

Before a food sample can be analyzed, consideration must be given to obtaining a representative sample [1,2]. In fortified foods, there is a tendency for the beadlets containing the vitamin to separate during mixing and handling, and then to agglomerate if exposed to moisture. The amount of sample material to be taken for analysis depends on the inherent homogeneity of the material or the homogeneity achieved after comminution.

Most of the vitamins are photosensitive and, therefore, vitamin solutions must be protected from light throughout the analysis. This can be achieved by artificial lighting provided by gold fluorescent tubes that exclude radiation wavelengths of less than 500 nm, and by the use of low-actinic amber glassware. Several of the water-soluble vitamins are rapidly destroyed by alkali, and glassware should be acid-washed before rinsing to prevent inactivation of vitamins by surface films of alkaline detergents.

The general analytical procedure for microbiological and physicochemical assays can be broken down into five main stages: sampling, extraction,

cleanup, measurement, and calculation of results. Other factors to be considered are maintenance of sample integrity, storage of the sample pending analysis, and preparation of the sample for analysis. In analysis for carotenoids, some plants must be blanched to inactivate lipoxidase enzymes that otherwise would accelerate destruction of carotenes during storage and mixing.

16.4.1 Extraction

Often, the extraction step in a method is designed to liberate vitamin that is chemically bound, thus providing an assay value that represents the total amount of vitamin present. For some vitamins, especially niacin, this approach can lead to a gross overestimation of the amount of biologically available vitamin in the food. Some methods employ more selective extraction procedures, which simulate (as far as possible) mammalian digestion, in attempts to estimate the biologically available vitamin content.

16.4.2 Cleanup

The extracts prepared by treatment of the test material may require some form of cleanup before the vitamins are measured. The requirement for cleanup depends upon the ratio of analyte to interfering substances, and also upon the sensitivity and selectivity of the analytical technique employed. Colorimetric, titrimetric, and spectrophotometric techniques have rather poor specificity and require extensive sample cleanup. Direct fluorometric techniques are rather more specific, while chromatographic techniques, particularly HPLC, have relatively high specificity. Microbiological assays are subject to interference from many substances, although these can usually be diluted out owing to the high sensitivity of such assays. Nonchromatographic cleanup techniques include deproteinization, sterol precipitation, solvent extraction, and dialysis. Chromatographic techniques include open-column chromatography, solid-phase extraction, and high-pressure gel permeation chromatography.

16.5 Method Evaluation

16.5.1 Measurement Value and Uncertainty

All scientific measurements have an uncertainty associated with the measured value. This uncertainty represents the total error of the analytical method, comprising systematic errors and random errors. Systematic

errors are nonrandom and are due to bias. Causes are exemplified by defects in the methodology, instrument malfunction, improper calibration, and the presence of interfering compounds. These errors are usually constant for a given method and it is sometimes possible to reduce or eliminate them. Random errors are unpredictable and cannot be eliminated. Typical causes are sample inhomogeneity, incomplete extraction, and the inherent variability of the measurement system. Random errors follow the normal distribution curve and hence can be expressed in units of standard deviation. Precision is an estimate of the variability of a method and deals only with random errors. Accuracy is the nearness of an individual measured value, or the arithmetic mean of a set of values, to the true, expected, or accepted value. Statistically, accuracy represents the total error of the analytical method. A measurement is accurate when the determined value is both precise and free of systematic error.

Precision is a statistically defined term, stated as a standard deviation of the mean, and can be ascertained by multiple analysis of an homogenous sample. The relative standard deviation (RSD) and coefficient of variation (CV) are synonymous terms for standard deviation $\times$ 100 divided by the mean. The RSD allows the analyst to tell quickly if the measured value falls within the acceptable precision of the method, independent of the size of the value. The precision depends on the concentration range of the analyte. Under optimal conditions the standard deviation should increase linearly with the concentration, while the RSD should remain more or less constant. When the RSD begins to increase rapidly with lower concentration or amount, it determines the limit of reliable measurement.

With regard to accuracy, it is impossible to determine with absolute certainty the true value of a nutrient in a natural food product. Accuracy can be assessed by comparing the results with those obtained using a method that is different in principle to the method normally employed. If two independent methods give numerically similar results, it is more probable that the measured values are near to the true value than that both methods have the same systematic error by chance. If a certified standard reference material is available, whose compositional properties simulate those of the samples of interest, its "correct" measurement implies correct measurement of the samples. The sample itself can serve as the matrix for recovery studies to ensure that at least the added analyte is recovered in a satisfactory amount. Ideally, recovery experiments should be carried out using a zero reference material (i.e., a manufactured product matrix lacking the vitamin analyte), since such materials can be spiked with analyte at levels below those of the finished product. Recovery data provide useful information regarding the completeness and precision of the extraction process, but the recovery

of exogenously added vitamin does not necessarily reflect the extraction efficiency of the indigenous vitamin.

The method of standard additions is an important technique for evaluating systematic error caused by proportional matrix bias [3]. To perform this method, three or four identical aliquots of the sample extract are spiked with increasing known amounts of the pure analyte (aliquots from a standard solution), and each solution is analyzed. The analytical response (e.g., HPLC peak height) is plotted as a function of the added amount of analyte. The graph obtained represents the standard curve of the analyte in the presence of matrix, offset by the amount of endogenous analyte in the sample. The linearity and slope of this curve, when compared to an equivalent standard curve in matrix-free solution, gives information about the presence or absence of matrix effects. The method of standard additions also allows for correction for proportional error if such error is consistent over the entire calibration range (i.e., the curve is linear).

16.5.2 Quality Assurance

Quality assurance is the broad management concept of maintaining the ability of a laboratory to furnish reliable information. A quality assurance program encompasses the entire laboratory operation from sampling to application of the analytical result. The analyst is usually confronted with the problem of having too few data with which to apply statistical analysis to a set of results, and hence other techniques for verifying how a method behaves in actual practice must be used. A laboratory that routinely performs a particular determination should periodically analyze a working standard (house standard) along with routine samples, and monitor such analyses using a statistical control chart. The working standard consists of a large amount of a homogeneous product such as nonfat dry milk or flour, which is dispensed into small sealed bottles and stored under conditions that inhibit deterioration. In the absence of a suitable working standard, previously analyzed samples, on which several analyses have been performed, can be used instead.

16.5.3 Food Reference Materials

Food reference materials with certified contents of vitamins are needed to validate the analytical methods that are used to ensure compliance with nutrition labeling laws.

In 1989, a project funded by the European Commission's Community Bureau of Reference (BCR) (in 1993 renamed as the Standard, Measurement and Testing programme) was initiated. The main objective was to improve the reliability and accuracy of methods for the determination

of vitamins in food. The project included research into methodology (extraction and cleanup, end-method of determination, and calibration), inter-laboratory comparison of the methods, and the preparation of homogeneous food reference materials having good stability properties. Six reference materials were selected to cover the range of food matrices in which vitamins are commonly determined, namely, Brussels sprouts, mixed vegetables, wholemeal flour, milk powder, pig's liver, and margarine. With the exception of margarine, which is a canned product, these materials can be prepared in the form of dry powders. The vitamin contents of the materials have been certified on the basis of acceptable statistical agreement of results produced by collaborating laboratories using technically valid analytical procedures. Certified reference materials (CRMs) available in Europe are listed in Table 16.1. The preparation and certification of the six listed CRMs are described in two reports [4,5].

The National Institute of Standards and Technology (NIST) in the United States is in the process of introducing standard reference materials (SRMs) for use in determining the nutritional contents of foods, and developing or adapting reliable methods with which to analyze these

TABLE 16.1

European Certified Reference Materials (CRMs) with Certified Vitamin and Provitamin Constituents

Material	Certified Constituents	Designation
Wholemeal flour[a]	Vitamin B_1, vitamin B_6, and folate	CRM 121
Margarine	Vitamin D_3 and α-tocopherol	CRM 122
Milk powder[b]	Vitamin D_3, α-tocopherol, vitamin B_1, B_2, B_6, B_{12}, niacin, folate, and vitamin C	CRM 421
Lyophilized Brussels sprouts powder	Niacin and vitamin C	CRM 431
Lyophilized mixed vegetables[c]	Vitamin B_1, B_6, folate, *trans*-α-carotene, total α-carotene, *trans*-β-carotene, and total β-carotene	CRM 485
Lyophilized pig's liver	Vitamin B_1, B_2, B_6, B_{12}, and folate	CRM 487

[a] Commercially obtained wheat flour.
[b] Spray-dried and vitamin enriched powder produced from cow's milk.
[c] Sweet corn, carrot, and canned tomatoes.
Source: From Finglas, P.M., van den Berg, H., and de Froidmont-Görtz, I., The certification of the mass fractions of vitamins in three reference materials: margarine (CRM 122), milk powder (CRM 421), and lyophilized Brussels sprouts powder (CRM 431), Report EUR 18039 EN, Office for Official Publications of the European Communities, Luxembourg, 1997 and Finglas, P.M., Scott, K.J., Witthöft, C.M., van den Berg, H., and de Froidmont-Görtz, I., The certification of the mass fractions of vitamins in four reference materials: wholemeal flour (CRM 121), milk powder (CRM 421), lyophilized mixed vegetables (CRM 485), and lyophilized pigs liver (CRM 487), Report EUR 18320 EN, Office for Official Publications of the European Communities, Luxembourg, 1998. With permission.

TABLE 16.2

Standard Reference Materials (SRMs) with Certified Vitamin and Provitamin Constituents

Material	Certified Constituents	Certificate Issue Date	Designation
Coconut oil (fortified)	DL-α-Tocopheryl acetate	28 February 2003	SRM 1563
Infant formula	α-Tocopherol, vitamin C, vitamin B_2, vitamin B_6, and niacin	14 January 2004	SRM 1846
Baby food composite	all-*trans*-Retinol, α-tocopherol, γ-tocopherol, δ-tocopherol, total α-carotene, and total β-carotene	26 July 2002	SRM 2383
Slurried spinach	Total β-carotene	9 December 2003	SRM 2385

materials. Certified values for a given SRM are the equally weighted means of the measurements made by the NIST laboratory and the grand-mean of measurements made by collaborating laboratories. The expanded uncertainties are expressed at the 95% level of confidence and include within- and between-laboratory uncertainties. SRMs that have certified values for vitamins and provitamins are listed in Table 16.2. Reference values are noncertified values that are the best estimate of the true values based on available data.

16.5.4 Method Validation

Method validation is the process used to confirm that the analytical procedure is acceptable for its intended purpose. A valid method is necessary, but not sufficient, for the production of reliable data; for instance, a degree of skill is required on the part of the analyst. Data obtained by a valid method used in a well-defined quality assurance program should allow the assignment of limits of uncertainty that can be used to judge the reliability of the data.

First, the scope of the method and its validation criteria should be defined. These include analytes, matrices, type of equipment, quantitation limits, precision, and accuracy. The parameters for method validation are:

- *Specificity*: A specific method is one in which an analyte measurement is not subject to interference from other sample components.
- *Linearity*: Linearity is achieved when results are directly or, after mathematical transformation, linearly proportional to analyte concentration within a given range.

- *Precision*: Precision is achieved when results of multiple analyses of an homogenous sample fall within the acceptance criteria for the method's precision. The relative standard deviation can be subdivided into three categories: repeatability, intermediate precision, and reproducibility. Repeatability refers to the within-laboratory precision and is determined from a set of replicate results obtained simultaneously or in quick succession by one operator using the same equipment throughout over a relatively short time span. Intermediate precision is the long-term variability of the measurement process and is determined by comparing the results of a method run within one laboratory over a number of weeks. A method's intermediate precision may reflect discrepancies in results obtained by different analysts using different standards, reagents, HPLC columns, and so on. Reproducibility refers to the between-laboratory precision and is determined from results obtained by a number of analysts in different laboratories. The 95% confidence intervals of the repeatability and reproducibility for the expected assay concentration are useful information of the precision.
- *Accuracy*: This is assessed by recovery experiments.
- *Range*: The range of a method is the interval between the upper and lower analyte concentrations (inclusive) over which acceptable linearity, precision, and accuracy can be obtained.
- *Detection limit*: The detection limit of a method is the lowest concentration of analyte in a sample that can be detected (but not necessarily quantified) and is typically three times the noise level of the measurement system.
- *Quantification limit*: This is the lowest concentration of analyte that can be precisely measured.
- *Robustness*: The robustness of a method is its ability to remain unaffected by small changes in operational parameters, such as mobile-phase composition and flow-rate, column temperature, and injection volume in chromatographic systems.

The reproducibility of a candidate method is determined in an inter-laboratory collaborative study using identical samples provided by the peer laboratory. The ideal sample is a certified food reference material. Such a study will reveal any bias in the method. A method is considered to have been successfully validated when all participants in the study can obtain results comparable to those of the peer laboratory. Statistical equivalence can be used to compare results; alternatively, the peer laboratory stipulates a range of values as a criterion of acceptability. When the

candidate method has been validated and standardized by an organization such as AOAC International (AOAC) in the United States. or the International Organization for Standardization (ISO), it becomes a standard test method.

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17

Extraction Techniques for the Water-Soluble Vitamins

In vitro analytical techniques require prior extraction of the vitamins from the food matrix in order to facilitate their measurement. The appropriate method of extraction depends upon the following criteria: the analytical information required, the nature of the food matrix, the form in which the vitamin occurs naturally or is added (different bound forms of vitamins are often found in meat, plant, and dairy products), the nature and relative amounts of potentially interfering substances, the stability of the vitamin towards heat and extremes of pH, and the selectivity and specificity of the analytical method to be used. Extraction procedures for water-soluble vitamins include hydrolysis of the sample with a mineral acid [hydrochloric acid (HCl) or sulfuric acid (H_2SO_4)], alkaline hydrolysis with calcium hydroxide, deproteinization with trichloroacetic acid or similarly acting agent, and digestion with an appropriate enzyme.

17.1 Vitamin B_1

The extraction procedure generally used for the determination of total vitamin B_1 by fluorometry, GC, HPLC, and microbiological assay involves hot mineral acid digestion to release the thiamin and thiamin phosphate esters from their association with proteins, followed by enzymatic hydrolysis of the phosphate esters to complete the liberation of thiamin. Food samples of animal origin can be autoclaved at 121°C for 30 min with 0.1 *N* HCl, as the phosphorylated forms of thiamin present in such samples are not degraded under these conditions. For the majority of cereals and cereal products, which contain mostly nonphosphorylated thiamin, it is necessary to lower the autoclaving temperature to 108°C in order to avoid vitamin loss.

A commercial diastatic enzyme preparation of fungal origin (e.g., Takadiastase, Claradiastase, or Mylase) is suitable for the hydrolysis step, as such preparations contain phosphatase activity in addition to

α-amylase and other enzymes [1]. The enzyme treatment can be omitted for the analysis of those grain products that do not contain phosphorylated thiamin. For proteinaceous samples such as meat, the proteolytic enzyme papain is sometimes added to the diastase in order to dissolve the proteins that have been denatured during the previous acid digestion.

Instead of using an enzyme hydrolysis procedure for thiamin extraction prior to HPLC, rice flour samples can be refluxed with a mixture of hydrochloric acid and methanol (0.1 *N* HCl–40% aqueous methanol) for 30 min at 60°C [2]. For the analysis of milk, the extraction procedure simply entails precipitation of the protein by acidification at room temperature, and filtration. This nonhydrolytic extraction procedure has the advantage of leaving the biologically inactive thiamin monophosphate intact, so this compound can be excluded from the measurement.

17.2 Vitamin B_2

When carrying out physicochemical or microbiological assays for vitamin B_2, it is necessary to release the flavins from their intimate association with proteins and to completely convert the FAD to FMN. Both of these requirements are readily accomplished (for noncovalently bound flavins) by autoclaving food samples at 121°C for 30 min with dilute mineral acid (usually 0.1 *N* HCl) at a pH of <3. During acid digestion some of the FMN is hydrolyzed to riboflavin, and a small fraction of the FMN is converted to the isomeric 2′-, 3′-, and 4′-phosphates [3]. The complete conversion of FMN to riboflavin can only be achieved by subsequent enzymatic hydrolysis, for which a standardized diastatic enzyme preparation such as Takadiastase or Claradiastase is used. Watada and Tran [4] reported that Mylase was as effective as Takadiastase, the latter being unobtainable at that time. These are relatively inexpensive and crude preparations that contain varying degrees of phosphatase activity. In practice, the complete enzymatic conversion of FMN to riboflavin may not always be achieved, the degree of hydrolysis depending on the source and batch-to-batch phosphatase activity of the enzyme and on the incubation conditions.

For the analysis of milk, eggs, and dairy products, it is common practice to determine the riboflavin specifically, on the assumption that free or loosely bound riboflavin is the predominant naturally occurring flavin present. In this case, the extraction procedure simply entails precipitation of the protein by acidification and filtration, omitting the acid and enzyme digestion steps. Rashid and Potts [5] removed the protein from milk and milk products by filtration after treatment with acidified lead acetate solution.

Acid and enzymatic hydrolysis carried out successively are incapable of liberating the covalently bound FAD of certain enzymes, and hence this source of FAD will not be measured. This is perhaps fortuitous when the nutritional value of the food sample is under assessment, as there is evidence that covalently bound FAD is largely unavailable to the host.

17.3 Niacin

In order to assess the nutritional value of a foodstuff with respect to its niacin content, it is necessary to determine the niacin that is biologically available. As discussed in Section 9.5.1, the majority of the niacin in mature cereal grains exists in chemically bound forms of nicotinic acid that are not biologically available. Therefore, measurement of total niacin (i.e., free plus bound) provides a gross overestimate of the biologically available niacin of several staple cereal-based foods.

The terms "total" and "free" (bioavailable) niacin are defined by the extraction methods employed in the analysis. Total niacin generally refers to the niacin that is extractable by autoclaving the sample with alkali or 1 *N* mineral acid; free niacin is frequently defined as the niacin extractable by heating or autoclaving with 0.1 *N* mineral acid.

In the AOAC colorimetric method for determining total niacin [6], noncereal foods are extracted by autoclaving for 30 min at 121°C in the presence of 1 *N* (0.5 *M*) H_2SO_4. This same procedure is used in the AOAC microbiological method for determining niacin in milk-based infant formulas [7]. The acid treatment liberates nicotinamide from its coenzyme forms and simultaneously hydrolyzes it to nicotinic acid; it does not, however, completely liberate the bound nicotinic acid from cereal products. A procedure that has been used for extracting total niacin from cereal products is autoclaving at 121°C for 1 h in the presence of 0.22 *M* calcium hydroxide [8,9]. This alkali treatment readily liberates the nicotinic acid from its chemically bound forms; it also converts nicotinamide to nicotinic acid, but with a yield lower than 80%. Sodium hydroxide, although more effective at hydrolyzing nicotinamide, is not used because it induces gelation of the cereal sample. If the microbiological assay with *Lactobacillus plantarum* or the AOAC colorimetric assay are to be used, complete conversion of nicotinamide to nicotinic acid is not necessary, as these procedures account for both vitamers.

Autoclaving meat samples with 1 *N* HCl in the presence of urea resulted in a significant increase in the niacin content when compared with extraction using 1 *N* acid alone [10]. This suggests the release of

niacin from nonester conjugates by the acid–urea combination, possibly from amide-linked forms.

Windahl et al. [11] found that autoclaving food samples at 121°C for 30 min in the presence of 1 *N* H_2SO_4 did not completely hydrolyze nicotinamide to nicotinic acid. These authors ensured complete hydrolysis by autoclaving samples in the presence of 1.6 *N* (0.8 *M*) H_2SO_4 for 2 h at 121°C. They also performed alkaline extraction by autoclaving samples in the presence of saturated calcium hydroxide for 2 h at 121°C. Acid and alkali extractions gave similar levels of niacin in foods as determined by capillary electrophoresis and HPLC. In meat samples, acid extraction resulted in slightly higher niacin values compared with alkali extraction. Conversely, in cereal samples, alkali extraction yielded slightly higher values compared with acid extraction.

Among the many published HPLC methods for determining niacin in foods, several have used extraction procedures designed to yield a value for bioavailable niacin. Lahély et al. [12] added 0.1 *N* HCl to ground food samples and heated the suspensions in a water-bath at 100°C for 1 h. A portion of the diluted and filtered digest was then autoclaved at 120°C in a medium of 0.8 *N* NaOH for 1 h to ensure complete conversion of nicotinamide to nicotinic acid. Thus, ultimately, only nicotinic acid needed to be measured chromatographically. The application of this method to beef liver and yeast gave comparable niacin values to those obtained when simulating gastric digestion conditions (0.1 *N* HCl hydrolysis at 37°C for 3 h, followed by an alkaline treatment). However, when the method was applied to cereal products, the alkaline treatment induced the formation of impurities, which interfered with the chromatography. Rose-Sallin et al. [13] found that a one-step acid hydrolysis (0.1 *N* HCl, 1 h, 100°C water-bath) yielded similar concentrations of niacin to those following two-step acid-alkaline or acid-enzymatic hydrolysis in a range of fortified foods, including cereal products. The one-step procedure also yielded slightly better recoveries for niacin compared to the two-step methods. Rose-Sallin et al. [13] adopted the one-step extraction and calculated bioavailable niacin from the nicotinic acid and nicotinamide peaks in the chromatogram. Vidal-Valverde and Reche [14] found that treatment of acid hydrolysates with Takadiastase was absolutely necessary in the case of legume samples, because the high starch content made the hydrolysate extremely viscous.

Ndaw et al. [15] replaced the usual 0.1 *N* acid extraction by enzymatic hydrolysis, using a NADase that hydrolyses only the bound forms of niacin clearly bioavailable (i.e., NAD and NADP). This enzymatic hydrolysis (incubation at 37°C for 18 h) did not induce any subsequent conversion of nicotinamide into nicotinic acid. The one-step enzymatic treatment was always sufficient, even when the foodstuff contained large quantities of starch (rice, wheat flour) or proteins (wheat germ,

TABLE 17.1

Influence of the Extraction Protocol on the Niacin Concentration in Various Foodstuffs as Determined by HPLC with Fluorometric Detection

Food	Extraction Protocol[a]	Nicotinic Acid (μg/g)	Nicotinamide (μg/g)	Niacin (μg/g of Nicotinic Acid Equivalents)
Peas	NADase	0.29 (0.01)	11.0 (0.1)	11.3 (0.1)
	0.1 *N* HCl	1.22 (0.08)	10.2 (0.4)	11.4 (0.4)
Spinach	NADase	0	0.72 (0.06)	0.72 (0.06)
	0.1 *N* HCl	0	0.69 (0.04)	0.69 (0.04)
French beans	NADase	0.19 (0.01)	2.8 (0.2)	3.0 (0.2)
	0.1 *N* HCl	0.37 (0.06)	2.6 (0.2)	3.0 (0.2)
Sweet corn	NADase	3.6 (0.2)	13.8 (1.0)	17.4 (1.0)
	0.1 *N* HCl	4.3 (0.4)	12.7 (0.9)	17.0 (1.0)
Rice	NADase	10.3 (0.2)	0	10.3 (0.2)
	0.1 *N* HCl	10.0 (0.5)	0	10.0 (0.5)
Wheat flour	NADase	3.4 (0.1)	1.7 (0.1)	5.2 (0.1)
	0.1 *N* HCl	5.7 (0.4)	1.9 (0.1)	7.6 (0.4)
Wheat germ	NADase	10.8 (0.2)	0	10.8 (0.2)
	0.1 *N* HCl	13.8 (0.5)	0	13.8 (0.5)
Peanuts	NADase	26.5 (0.9)	3.7 (0.3)	30.2 (1.0)
	0.1 *N* HCl	93.4 (0.7)	1.9 (0.3)	95.8 (0.8)
Yeast	NADase	17 (1)	182 (5)	199 (5)
	0.1 *N* HCl	22.0 (0.3)	174 (5)	196 (5)
Beef fillet	NADase	3.8 (0.2)	53 (1)	57 (1)
	0.1 *N* HCl	3.6 (0.7)	50 (2)	54 (2)
Pork escalope	NADase	0	64 (2)	64 (2)
	0.1 *N* HCl	0.2 (0.1)	57 (1)	58 (1)

Note: Concentrations are averages of three determinations (standard deviations in parentheses).

[a](1) NADase (pH 4.5, 18 h, 37°C); (2) 0.1 *N* HCl (water-bath at 100°C during 1 h).

Source: From Ndaw, S. et al. *Food Chem*, 78, 129–134, 2002. With permission from Elsevier.

peanuts, beef fillet). Table 17.1 compares the niacin contents of various foods extracted either by NADase treatment or acid hydrolysis (0.1 *N* HCl, 1 h, 100°C water-bath). Acid hydrolysis led to significantly higher niacin contents in the analysis of wheat flour, wheat germ, and peanuts, attributable to the release of nicotinic acid from bound forms that are probably nonbioavailable. On analysis of peas, French beans, and yeast (foods in which nicotinamide is by far the major vitamer), nicotinic acid contents were slightly higher after acid hydrolysis than they were after enzymatic hydrolysis. This increase most probably resulted from a partial conversion of the nicotinamide to nicotinic acid. When the acid hydrolysis was applied to standard solutions of NAD (1.35 μ*M*) and NADP (1.17 μ*M*), about 10% of the nicotinamide liberated was converted to nicotinic acid.

For the determination of added nicotinic acid as a color fixative in fresh meat (illegal in Japan), meat samples have been extracted by boiling with 96% ethanol [16] and blending with water [17–19], acetonitrile [20], methanol [21], or methanol after addition of a small amount of phosphoric acid [22].

17.4 Vitamin B_6

Because animal and plant tissues differ greatly with respect to the forms of vitamin B_6 contained in them, there is no single set of conditions that can quantitatively extract vitamin B_6 from both plant and animal products. In the AOAC microbiological method [23] for determining total vitamin B_6 in food extracts, animal-derived foods are autoclaved with 0.055 *N* HCl for 5 h at 121°C. This treatment hydrolyzes phosphorylated forms of vitamin B_6, whilst also liberating PL from its Schiff base and substituted aldamine bound forms. Plant-derived foods are autoclaved with 0.44 *N* HCl for 2 h at 121°C, the stronger acid environment being necessary to liberate PN from its glycosylated form. Autoclaving whole-wheat samples with 0.055 *N* HCl, instead of 0.44 *N* HCl, yielded a similar PL value, but lower values of PN and PM [24]. Conversely, autoclaving meat products with 0.44 *N* HCl, instead of 0.055 *N* HCl, gave approximately the same PN and PL values, but only about half of the PM [25]. The superiority of the lower concentration of acid used for animal products does not result from destruction of vitamin B_6 by the stronger acid; rather, it is due to the incomplete liberation of the vitamin by the more concentrated acid [26]. The optimum release occurs between pH 1.5 and 2.0, with a maximum at pH 1.7–1.8 [27]. To satisfy these strict pH criteria, one must always ensure that the acid is added in amounts that exceed the buffering capacity of the sample. Another factor to consider is that PMP is more resistant to acid hydrolysis than is PLP. Autoclaving for 3 h at 125°C in 0.055 *N* HCl was required for complete hydrolysis of PMP, while PLP was completely hydrolyzed in 30 min under the same conditions [28].

The possibility of interaction of PL or PLP with amino acids during the AOAC extraction procedure for animal foods has been investigated [29]. No loss of activity for *Saccharomyces cerevisiae* was observed when PL or PLP was autoclaved in the presence of a relatively high concentration of glutamic acid, which indicated that transamination does not occur under these conditions.

PN-glucoside exhibits around 60% bioavailability relative to PN in humans [30]. Since the AOAC extraction procedure for plant foods hydrolyzes glycosylated forms of PN, analyses based on this procedure

would overestimate the biologically available vitamin B_6 in foods that contain significant quantities of β-glucoside conjugates.

The AOAC acid hydrolysis procedures have no effect upon the peptide-bound ε-pyridoxyllysine and its 5′-phosphate derivative, which are formed during the heat-sterilization of evaporated milk and other animal-derived canned foods (Section 10.3.2). These conjugates, which possess anti-vitamin B_6 activity under certain conditions, exhibit 75–80% stability when subjected to 6 *N* HCl at 105°C for 48 h [31].

Bognår and Ollilainen [32] investigated the use of hydrochloric acid and trichloroacetic acid alone, and in combination with several commercial enzyme preparations, as extractants for the determination of total vitamin B_6 in food by HPLC. Three reference materials were tested: CRM 121 (wholemeal flour), CRM 485 (lyophilized mixed vegetables) and CRM 487 (lyophilized pig liver). Also included in the investigation were broccoli, Brussels sprouts, kidney beans, spelt (a kind of wheat), potatoes, sunflower seeds, pork meat, cod, and milk. The highest values of total vitamin B_6 were achieved by autoclaving samples at 120°C for 30 min in 0.1 *N* HCl, followed by incubation with acid phosphatase and β-glucosidase at 37°C for 18 h after adjustment to pH 4.8. Enzymatic hydrolysis of food by Takadiastase, degraded PL distinctly and also produced a compound that interfered with the PN peak during gradient elution. The content of glycosylated PN could be determined by analyzing the acid hydrolysate before and after the double enzyme treatment. The difference in PN content before and after enzyme treatment gives an estimate of glycosylated PN.

The simultaneous separation of all six B_6 vitamers, plus pyridoxic acid, can be achieved using HPLC. Treatment of samples with deproteinizing agents such as metaphosphoric, perchloric, trichloroacetic, or sulfosalicylic acid at ambient temperature readily hydrolyzes Schiff bases, whilst preserving the phosphorylated vitamers. These acids also preserve PN-glucoside, and hence their use provides better estimates of available vitamin B_6 than the use of mineral acids. The high efficiency of extraction using these acidic reagents is partly due to the conversion of the pyridine bases to quaternary ammonium salts, thereby increasing their solubility in water. Their use as extracting agents also prevents enzymatic interconversion of B_6 vitamers during homogenization of samples. In such procedures it is usually necessary to remove excess reagent, which might otherwise interfere with the analytical chromatography. Trichloroacetic acid can be removed by extraction with diethyl ether; perchloric acid by reaction with 6 *M* potassium hydroxide and precipitation as insoluble potassium perchlorate; and sulfosalicylic acid by chromatography on an anion exchange column [33]. An extraction procedure using 5% sulfosalicylic acid has been successfully applied to such complex foods as pork, dry milk, and cereals [34]. Recoveries of B_6 vitamers added to

samples were 95–105% for all vitamers except for PNP, where the recovery was 85%. Other workers [35,36] have found perchloric acid to be a better extracting agent of the B_6 vitamers for animal tissues than sulfosalicylic acid.

17.5 Pantothenic Acid

Before pantothenic acid can be determined by methods other than an animal bioassay, it is necessary to liberate the vitamin from its bound forms, chiefly coenzyme A. Neither acid nor alkaline hydrolysis can be used, as the pantothenic acid is degraded by such treatments. The only practicable alternative is enzymatic hydrolysis, and this was successfully accomplished through the simultaneous action of intestinal phosphatase and an avian liver enzyme [37]. This double enzyme combination liberates practically all of the pantothenic acid from coenzyme A, but it does not release the vitamin from acyl carrier protein [38]. The phosphatase splits the coenzyme A molecule between the phosphate-containing moiety and pantethiene, while the liver enzyme breaks the link in pantethiene between the pantothenic acid and β-mercaptoethylamine moieties. The double enzyme combination is used in the AOAC microbiological method for determining pantothenic acid in milk-based infant formula [39].

17.6 Biotin

Bound forms of biotin, including biocytin, cannot be utilized by *L. plantarum*, the organism usually employed in microbiological biotin assays, and strong mineral acid hydrolysis at elevated temperature is required to liberate biotin completely from natural materials [40]. Animal tissues require more stringent hydrolysis conditions than do plant tissues, because the latter contain a higher proportion of free water-extractable biotin [41]. Experimental studies with meat and meat products [42] and feedstuffs of animal origin [43] showed that maximum liberation of biotin in animal-derived products is obtained by autoclaving with 6 *N* H_2SO_4 for 2 h at 121°C. This procedure promotes losses of biotin in plant materials, which are extracted more efficiently by autoclaving with 4 *N* H_2SO_4 for 1 h at 121°C [41] or with 2 *N* H_2SO_4 for 2 h at 121°C [43]. Because of the differences in extractability between animal and plant tissues, a single acid extraction procedure to cover all food commodities must be a compromise, and no such procedure has

been universally adopted. Representative methods for extracting foods of any type entail autoclaving with 2 *N* H_2SO_4 at 121°C for 2 h [41,44] or 3 *N* H_2SO_4 at 121°C for 1 h [45] or 30 min [46].

Hydrolysis with 6 *N* H_2SO_4 destroys the synthetic sodium salt of biotin added to feed premixes. A suggested procedure for extracting feed premixes with biotin potencies up to 1 g/lb entailed the addition of 50 ml of 0.1 *N* NaOH and 250 ml of water to 5 g of sample, shaking vigorously, and then standing for 30 min at room temperature with occasional swirling [47].

Sulfuric acid, rather than hydrochloric acid, is invariably used for sample hydrolysis, as the biotin content of dilute (30 ng/ml) solutions is almost completely destroyed by autoclaving with 2 *N* HCl [48]. Evidence from differential microbiological assay points to the oxidation of biotin to a mixture of its sulfoxide and sulfone derivatives, possibly caused by trace impurities (e.g., chlorine) in the acid. This loss of vitamin activity does not necessarily occur when autoclaving actual food samples, as many natural products are capable of preventing this oxidation [49]. Finglas et al. [50] reported no loss of biotin from liver using 3 *N* HCl.

It is evident from the foregoing that sulfuric acid hydrolysis is an unreliable way of extracting biotin from food. The results depend on both the concentration of acid and the duration of autoclaving. This makes the microbiological assay of biotin problematic, since acid hydrolysis is used to convert biocytin to biotin. A proposed HPLC method [51] solves the problems associated with acids by eliminating acid extraction. Instead, food samples are digested with papain for 18 h, a treatment that releases biotin from its association with proteins, but leaves biocytin intact. There is no degradation of biotin during the digestion at 37°C. Biotin and biocytin are measured separately after postcolumn conversion to fluorescent derivatives. The addition of Takadiastase is necessary for starchy foods such as cereals and yeast.

17.7 Folate

The AOAC microbiological method for determining folic acid in infant formula [52] employs a single-enzyme digestion with folate conjugase (pteroylpoly-γ-glutamyl hydrolase; EC 3.4.22.12). The chicken pancreas conjugase specified in the method converts folylpolyglutamates to diglutamates, which can be utilized by the assay organism, *L. rhamnosus.* HPLC methods for determining folate require deconjugation of folylpolyglutamates to monoglutamates, and therefore chicken pancreas conjugase is unsuitable. Conjugases from hog kidney and human or rat plasma do yield folylmonoglutamates and can be used in HPLC and other

nonmicrobiological methods. Chicken pancreas conjugase is most active at neutral pH, in contrast to hog kidney conjugase and plasma (human or rat) conjugase whose pH optimum is 4.5 [53]. The various conjugases are not commercially available in purified form and enzyme solutions have to be prepared in the laboratory from their crude sources, such as lyophilized human or rat plasma and hog kidney acetone powder.

In 1990, DeSouza and Eitenmiller [54] reported that increased folate levels could be obtained in microbiological and radioassays by including protease (EC 3.4.24.31) and α-amylase (3.2.1.1) with the conjugase treatment. Martin et al. [55] then published a tri-enzyme digestion procedure using chicken pancreas conjugase, α-amylase, and protease in the microbiological determination of total folate in foods. This was followed by reports from other laboratories advocating tri-enzyme treatment as a means of extracting the maximum possible amount of folate from foods as diverse as cereal-grain products [56], American fast foods [57], dairy products [58], foods commonly consumed in Korea [59], and complete food composites [60]. Folate values in 8 of 16 fortified bakery products, and 4 of 13 fortified products in the rice, macaroni, and noodle category were significantly higher following the additional protease and α-amylase treatments [61].

In order to achieve maximum extraction of bound folate from the food matrix, food samples suspended in buffered aqueous medium are first autoclaved to break up particles, gelatinize starch, and denature folate-binding proteins and enzymes that may catalyze folate degradation or interconversion. The inclusion of an antioxidant is essential in preventing the destruction of labile folates during heat treatment. The most effective reducing conditions are provided by the presence of both ascorbic acid and mercaptoethanol, with the air displaced by nitrogen. The autoclaved samples are digested with protease to liberate the folate bound to proteins, then heated to inactivate the protease. Digestion with α-amylase then follows to liberate the folate bound to starch. Prolonged digestion with conjugase completes the tri-enzyme treatment. A variety of foods cause detectable inhibition of conjugase activity [62], but this problem can be partly overcome by extracting at near neutral pH using a large excess of conjugase [63].

In 2000, the American Association of Cereal Chemists (AACC) [64] published a microbiological assay using tri-enzyme extraction for the determination of total folate in cereal products. The extraction procedure is as follows: Weigh an amount of ground sample equal to 0.25–1.0 g dry solids and containing about 1 μg folic acid into a 125-ml conical flask. Add 20 ml 0.1 *M* phosphate buffer (pH 7.8) containing 1% ascorbic acid, mix thoroughly, then add enough water to bring the total volume to 50 ml. Add 0.1–1.0 ml octanol (antifoaming agent), cover flasks with 50-ml

beakers, and autoclave for 15 min at 121–123°C. Cool and add a further 10 ml of the pH 7.8 buffer. Add 1 ml protease solution, cover the flask, and incubate for 3 h at 37°C. Autoclave for 3 min at 100°C, then cool. Add 1 ml α-amylase solution and incubate for 2 h at 37°C. Add 4 ml chicken pancreas conjugase solution and incubate for 16 h (or overnight) at 37°C. Inactivate the enzymes by autoclaving for 3 min, then cool. Adjust to pH 4.5, dilute to 100 ml with water, and filter approximately 20 ml through 2V filter paper. Dilute an aliquot of the clear filtrate with 0.1 *M* phosphate buffer (pH 6.7 $\pm$ 0.1) to a final volume such that the folate concentration is about 0.2 ng/ml, and assay microbiologically using *L. rhamnosus.*

Rader et al. [61] tested the efficiency of the tri-enzyme extraction using chicken pancreas conjugase at four different pHs (pH 4.3, 6.0, 6.8, and 7.8) for the microbiological assay of four cereal-grain products and found no significant differences among folate values. A pH 7.8 buffer is used in the AACC method, but this pH is not optimal for the tri-enzyme extraction of all food types. Tamura et al. [60], for example, found that complex food composites were extracted more efficiently at pH 4.1 than at pH 6.3 or 7.85. The pH optima and incubation times for protease and α-amylase can vary, depending on the substrates present in the foods [65], and this creates a dilemma in deciding which conditions should be used for the tri-enzyme treatment.

17.8 Vitamin B_{12}

Procedures for extracting vitamin B_{12} generally have the dual purpose of liberating protein-bound cobalamins and converting the labile naturally occurring forms to a single, stable form — cyanocobalamin or sulfitocobalamin. Conversion to the sulfitocobalamin by reaction with metabisulfite avoids the use of lethally toxic cyanide solutions required to form cyanocobalamin.

The extraction procedure employed in the AOAC microbiological method for determining vitamin B_{12} activity in vitamin preparations [66] is also applicable to foods, having been found satisfactory by interlaboratory collaborative analysis of a crude liver paste, condensed fish solubles, and a crude vitamin B_{12} fermentation product [67]. The procedure entails homogenizing the sample with 0.1 *M* phosphate–citrate buffer at pH 4.5 containing freshly prepared sodium metabisulfite ($Na_2S_2O_5$), and then autoclaving the mixture for 10 min at 121°C. The heat treatment denatures the proteins, inactivates the enzymes, and accelerates the conversion of liberated cobalamins to sulfitocobalamin.

In the AOAC method for determining vitamin B_{12} in milk-based infant formula [68], protein is removed by filtration after adjustment of the autoclaved extract to the point of maximum precipitation (ca. pH 4.5).

Methods, in which the sample is heated on a boiling water-bath, rather than autoclaved, may not completely extract all of the bound vitamin [69].

17.9 Vitamin C

An effective means of extracting vitamin C from foods is homogenization with a solution of 3% (w/v) metaphosphoric acid dissolved in 8% glacial acetic acid [70]. This extracting solution denatures and precipitates proteins (thereby inactivating all enzymes) and provides a medium below pH 4, which favors the stability of ascorbic acid and dehydroacorbic acid. Furthermore, metaphosphoric acid prevents catalysis of the oxidation of ascorbic acid by copper(II) or iron(III) ions [71]. Addition of ethanol or acetone to the metaphosphoric extract precipitates solubilized starch [72]. Dilute (5 or 6%) metaphosphoric acid forms precipitates when mixed with certain ion-pairing reagents [73], which warrants caution in its use for ion-pair chromatography. It is recommended to deoxygenate extracting solutions by bubbling an inert gas (e.g., oxygen-free nitrogen) through the solution before use.

Krall and Andersen [74] extracted fruits and vegetables with an aqueous solution of 1% (w/v) metaphosphoric acid and 0.5% (w/v) oxalic acid adjusted to pH 2. Homogenization of high-starch food samples with aqueous 2% metaphosphoric acid and 1% oxalic acid mixed 1 : 1 with ethanol resulted in precipitation of starch. Use of these extractants was compatible with the reversed-phase ion-pair HPLC system.

Bognár and Daood [75] compared two solvent systems, A and B, for their effect on the stability of vitamin C derivatives in standard solutions and spiked extracts of fruits and vegetables. Solvent A was the classic solution of 3% metaphosphoric acid in 8% acetic acid; solvent B was solvent A mixed with acetonitrile (1 : 2). In solvent A, dehydroascorbic acid was unstable in standard solution: only 64 and 40% of the initial concentration was retained after 8 and 24 h of ambient storage, respectively. In contrast, the corresponding retentions in solvent B were 100 and 98%. In fruit and vegetable extracts prepared in solvent A, 36–54% of the spiked quantity of dehydroascorbic acid was lost after 12 h of storage time. These decreases in dehydroascorbic acid were accompanied by remarkable increases in ascorbic acid concentration (29–53%), most probably due to the presence of reducing agents in the extracts of fruits and vegetables. There was little or no loss of dehydroascorbic acid added to

food extracts prepared in solvent B. After a 24-h storage, ascorbic acid was highly stable in all of the standard solutions and food extracts tested. Taking these findings into account, Bognár and Daood [75] added acetonitrile to the standard solutions or to food extracts immediately after preparation or extraction with 3% metaphosphoric acid in 8% acetic acid. This modification of the extraction procedure resulted in complete recoveries of dehydroascorbic acid and total vitamin C.

In an interlaboratory study [76], fruit juices and processed foods were extracted by diluting or blending with water, then immediately adding dithiothreitol to reduce dehydroascorbic acid to ascorbic acid, thereby stabilizing the vitamin C. Proteinaceous samples were treated with 5% trichloroacetic acid to precipitate the proteins.

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18

Microbiological Methods for the Determination of the B-Group Vitamins

18.1 Introduction

All the B-group vitamins can be assayed microbiologically. The assay organisms are particular strains obtained from various culture collections such as the American Type Culture Collection (ATCC). The current and previous names of the microorganisms used in this chapter are listed in Table 18.1. With the exception of vitamin B_6, official (AOAC) microbiological methods for determining B-vitamins in foods (Table 18.2) employ lactic acid bacteria, and measure either the lactic acid produced after a 72-h incubation period (titrimetric method) or the growth of the organism (turbidimetric method). The latter is nowadays generally preferred, as it is simpler and requires shorter incubation times. Methods based on the measurement of metabolic carbon dioxide have also been developed.

18.2 General Principles

18.2.1 Turbidimetric Methods

Turbidimetric methods are based on the absolute requirement of certain microorganisms for the vitamin in question; that is, the organisms can multiply only when the vitamin is present in the surrounding medium. Conventional procedures, such as those adopted by the AOAC, use test tubes, but 48-well or 96-well microtiter plates can also be used. Aliquots of a standard solution of the vitamin to be determined, or aliquots of the sample extract containing the vitamin, are added to an initially translucent basal nutrient medium, complete in all respects except for the vitamin in question. Following inoculation with the assay organism, the organism multiplies in proportion to the concentration of limiting vitamin in the standard or sample solution, and the extent of the growth is ascertained by measuring the turbidity produced. Over a defined concentration range, the measured response will be directly proportional to the amount of limiting vitamin present and, within this

TABLE 18.1

Assay Organisms Used for Determining B-Group Vitamins

Current Name	Previous Name(s)	ATCC No.
Weissella viridescens	*Lactobacillus viridescens*	12706
Lactobacillus rhamnosus	*Lactobacillus casei*	7469
Lactobacillus fermentum		9338
Lactobacillus plantarum	*Lactobacillus arabinosus*	8014
Lactobacillus delbrueckii subsp. *lactis* (strain 326)	*Lactobacillus leichmannii*	4797
Lactobacillus delbrueckii subsp. *lactis* (strain 313)	*Lactobacillus leichmannii*	7830
Enterococcus hirae	*Streptococcus lactis*	8043
Enterococcus faecalis		10100
Leuconostoc mesenteroides subsp. *mesenteroides*		9135
Saccharomyces cerevisiae	*Saccharomyces carlsbergensis* *Saccharomyces uvarum*	9080
Kloeckera apiculata	*Kloeckera brevis*	9774
Poterioochromonas malhamensis	*Ochromonas malhamensis*	11532

Source: http://www.atcc.org/SearchCatalogs/longview.cfm.

range, the sample solution and standard vitamin solution can be compared. The response is highly dependent upon whether or not bound forms of the vitamin are released during the extraction stage of the assay.

Cultures of assay organisms are despatched in lyophilized (freeze-dried) form sealed under vacuum in glass ampoules. Media can be obtained in a dehydrated form from Difco Laboratories, Michigan, U.S. They are reconstituted for use simply by suspending the required weighed amount in distilled water, heating to effect solution, and autoclaving to sterilize them. The use of dehydrated media allows a fresh batch of medium to be made up in the amount required with the minimum of time and effort.

Lactic acid bacteria are ideally suited as assay organisms for determining all but one of the B-vitamins turbidimetrically, the one exception being vitamin B_6. The nutritional requirements of lactic acid bacteria are specific and complex, they grow readily in synthetic and semisynthetic media, and they are nonpathogenic. They are not prone to mutation and have maintained their characteristics unimpaired after many years of subculture in the laboratory. The rod forms (genus, *Lactobacillus*) are microaerophilic and the coccus forms (genus, *Enterococcus, Leuconostoc*) are facultative aerobes [1]. This ability to grow well in limited amounts of air means that test tubes of liquid medium can conveniently be used for assay purposes.

Lactic acid bacteria do not respond to all forms of vitamin B_6, and certain species of yeasts are used instead for the determination of this

TABLE 18.2

Official (AOAC[a]) Microbiological Methods of Analysis of B-Group Vitamins

Vitamin	AOAC No.	Assay Organism (ATCC No.)	Applicability
Vitamin B_2	940.33	*L. rhamnosus* (7469)	All food products
Niacin	944.13	*L. plantarum* (8014)	All food products; cereal products would require alkaline hydrolysis to completely extract bound forms of nicotinic acid
Niacin	985.34	*L. plantarum* (8014)	Milk-based infant formula
Vitamin B_6	961.15	*S. cerevisiae* (9080)	All food products
Vitamin B_6	985.32	*S. cerevisiae* (9080)	Milk-based infant formula
Pantothenic acid (free form only)	945.74	*L. plantarum* (8014)	Vitamin preparations; double enzyme treatment would be required to liberate pantothenic acid from coenzyme A in food samples
Pantothenic acid	992.07	*L. plantarum* (8014)	Milk-based infant formula
Folate (free form only)	944.12	*E. hirae* (8043)	Vitamin preparations; enzymatic deconjugation would be required to determine total folate
Folate (total)	992.05	*L. rhamnosus* (7469)	Milk-based infant formula
Vitamin B_{12}	952.20	*L. delbrueckii* subsp. *lactis* (7830)	All food products
Vitamin B_{12}	986.23	*L. delbrueckii* subsp. *lactis* (7830)	Milk-based infant formula

[a]Official Methods of Analysis of AOAC International, 17th ed., revision 2 (2003).

vitamin. Because yeasts grow aerobically, such assays have the inconvenience of requiring constant and uniform shaking during incubation.

Protozoa have more highly developed ingestive and digestive systems than do bacteria and yeasts, and therefore exhibit a more mammalian-like response to the various naturally occurring forms of the vitamins. However, the test growth period for protozoa is longer than that for lactic acid bacteria (3–5 days versus 24–48 h), conditions of growth are more demanding, and growth response is more difficult to measure.

18.2.2 Methods Based on the Measurement of Metabolic Carbon Dioxide

The radiometric microbiological assay (RMA) is based upon the measurement of radioactive $^{14}CO_2$ generated from the metabolism of a ^{14}C-labeled

substrate by the test organism in the presence of the specific vitamin to be analyzed. This technique has been applied to the determination of vitamin B_1, niacin, pantothenic acid, vitamin B_6, and biotin in foods using the yeast *Kloeckera apiculata* as the test organism and L-[1-^{14}C]valine as the radiolabeled substrate [2]. Folate was determined from the metabolism of [1-^{14}C]gluconate by *Lactobacillus rhamnosus* [3] and vitamin B_{12} from the metabolism of L-[guanido-^{14}C]arginine by *Lactobacillus delbrueckii* subsp. *lactis* [4]. In these applications, the radioactivity is measured automatically by means of a commercially available gas flow system incorporating an ionization chamber [2]. The RMA combines the biological specificity of measuring a vitamin-dependent microbiological metabolic reaction with the sensitivity and accuracy of radioactive decay measurement. Extraction methods suitable for the analysis of vitamin B_1, niacin, pantothenic acid, and biotin with the RMA have been discussed by Guilarte [5]. Sample preparation is simplified because colored, turbid, or precipitated debris do not interfere with the $^{14}CO_2$ output or detection; furthermore, the scrupulous cleaning of glassware, so important for conventional tube assays, is unnecessary.

A nonradiometric technique for measuring metabolic CO_2 employs an infrared CO_2 analyzer, which measures automatically the infrared radiation absorbed by the CO_2 band at 4.2 μm [6].

18.3 Conventional Turbidimetric Method Using Test Tubes

18.3.1 Summary

In the standard procedure, the appropriate assay medium, free of the vitamin to be determined, is prepared at twice its final concentration (i.e., double strength). Multiple aliquots of a standard solution of the pure vitamin and of suitably prepared extracts of the test food are added to a series of uniform test tubes in amounts suitable to produce gradations in growth between no growth and maximum growth. The contents of all tubes are diluted with water to the same volume, and an equal volume of the translucent assay medium is added. The tubes are then covered and sterilized by autoclaving. After cooling to a uniform temperature, the tubes are aseptically inoculated with an actively growing culture of the test organism. The tubes are then incubated for 22–24 h at a constant temperature near the optimum for the test organism until growth has reached the maximum permitted by the limiting vitamin present. The growth response to standard and test extract is determined by measuring the turbidity produced in the tubes. The data obtained from the standards are used to construct a standard curve from which the vitamin concentrations of the various sample aliquots are derived.

The use of multiple aliquots allows a validity check to be carried out: the vitamin concentration found should be directly proportional to the volume of aliquot taken. The amount of vitamin present in the original sample is then calculated at the different test levels, and the results are averaged to obtain the final result.

18.3.2 Laboratory Facilities and Cleaning of Glassware

Microbiological manipulations should be performed well away from the sample preparation area, preferably in a separate room of structural simplicity to facilitate cleaning. An essential requirement is a readily available supply of fresh glass-distilled water; deionized water is not suitable for the highly sensitive folate and vitamin B_{12} assays [7]. All glassware and other items should be kept exclusively for microbiological assays and segregated. A meticulous washing-up routine is essential to ensure that the glassware is scrupulously clean before use. A cleaning procedure used for folate and B_{12} assays involves the following steps:

1. Boiling in a water bath containing a special type of detergent for 3 h
2. Machine-washing and rinsing with deionized water
3. Soaking in dilute acid
4. Rinsing with deionized water
5. Baking in an oven at 100°C overnight [8]

The use of disposable glassware eliminates this meticulous washing, but items that are not disposable require special attention [9].

Essential equipment includes:

1. An autoclave large enough to admit all racks of tubes for a given assay and capable of accurate adjustment to a constant pressure of 15 lb/in.^{2}. A steam-injected autoclave is preferred to an electrically heated autoclave, because it allows steaming at 100°C and has a more rapid overall operation sequence from heating up to cooling down.
2. Incubators of the forced draught or circulating water bath type, capable of maintaining a constant accurate temperature (± 0.5°C) in the range of 27–37°C. A shaking incubator or water bath is required for assays with yeasts; assays with protozoa require incubation with both shaking and illumination.
3. A nephelometer equipped to accept the assay tubes for direct measurement of turbidity.

18.3.3 Media

Three types of media are used:

1. Maintenance media to preserve the viability and sensitivity of the assay organism. Such media contain agar and all the nutritional factors essential for the organism's normal growth and metabolism. A buffer salt is included to prevent the pH from rapidly dropping to levels which would inhibit growth, although growth will proceed until a pH of at least 4 is reached. The medium is formulated to give an initial pH (typically 6.8) that is somewhat higher than the optimum pH of 5.5–6.5 for most lactic acid bacteria [10]. This is to allow for the production of acid that occurs when the medium is sterilized by autoclaving.
2. Inoculum media (broths) to condition the test culture for immediate use. Difco supply dehydrated inoculum media, whose formulations are the same as those of the corresponding maintenance media, except that the agar component is omitted. The vitamin to be assayed is present in an amount that barely supports the growth of the assay organism. This limits the accumulation of the vitamin in the bacterial cell and thus maintains the sensitivity of the assay.
3. Assay (basal) media to permit quantitation of the vitamin under test. The assay medium contains all the nutritional factors necessary for the normal growth and metabolism of the assay organism, except the vitamin to be determined. It is formulated from highly purified natural products, synthetic vitamins, and other reagent-grade compounds. An assay medium used for lactic acid bacteria must contain a fermentable carbohydrate, a variable assortment of essential amino acids, various vitamins and mineral salts, certain purine and pyrimidine bases, and an appropriate buffer system. Glucose is universally used as a source of carbon and energy; the amino acids are provided in the form of acid-hydrolyzed casein plus tryptophan or a mixture of the specific amino acids; and the buffer salt is usually sodium acetate, which also has a stimulatory effect on growth.

18.3.4 General Assay Procedure

The standard assay procedure using lactic acid bacteria can be broken down into a number of steps:

1. Maintenance of stock cultures
2. Preparation of the inoculum culture
3. Preparation of the assay medium
4. Extraction of the vitamin from the test material

5. Setting up the assay
6. Quantification

A scheme of steps 1 and 2 is depicted in Figure 18.1.

18.3.4.1 Maintenance of Stock Cultures

The assay organism purchased in lyophilized culture is regenerated by incubation for 24 h at 37°C in sterile inoculum broth. The cells are then concentrated by centrifugation and transferred by stab to tubes containing agar-based maintenance medium. These tubes are the original stock cultures. Stock cultures of the microaerophilic lactobacilli can be maintained over a period of several years by employing a regular schedule of subculturing into maintenance medium. At regular weekly or monthly intervals (depending on the medium employed), three fresh stab cultures are prepared from a refrigerated stock culture. One of these cultures is reserved as the new stock culture until the next time for transfer, when it will be used to prepare three more agar stabs. The other two cultures are used to prepare inocula for the assay.

The usual procedure for subculturing lactobacilli is to distribute 10-ml quantities of molten maintenance medium into lipless 16–20-mm diameter test tubes and autoclave the plugged tubes for 20 min at 15 lb pressure (121°C). After cooling, stab inoculations are made into the solidified agar. The tubes are incubated under the appropriate conditions for each vitamin that produce distinctly visible growth along the line of the stab. Since the growth rate of an organism is a function of temperature, the incubation must be precisely controlled to within ± 0.5°C of the selected temperature. The stab cultures thus prepared are stored in the refrigerator under aseptic conditions.

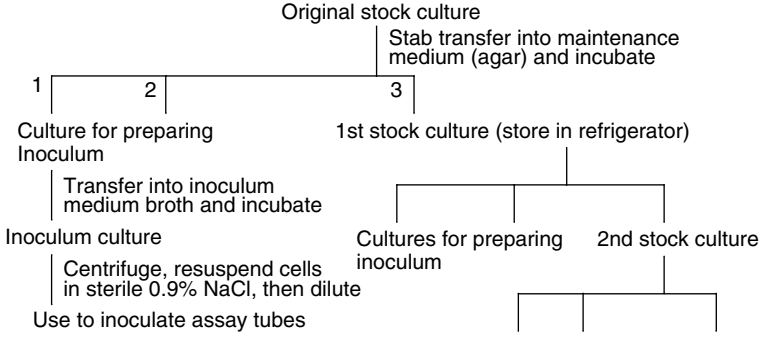

FIGURE 18.1
Preparation of stock cultures and inocula (standard assay procedure).

For the determination of vitamin B_6, Bell [11] maintained the yeast *Saccharomyces cerevisiae* under aerobic conditions on an agar slope using 10 ml of sterilized maintenance medium in a 1-oz McCartney bottle. The yeast was subcultured weekly by taking a loopful of yeast from the current culture onto a fresh agar slope, incubating at 32°C overnight, and storing in a refrigerator.

18.3.4.2 Preparation of the Inoculum Culture

The inoculum is prepared 1 day before the assay day by making a transfer from the stab culture into a tube containing sterile inoculum medium. Following an overnight incubation at 37°C, the cells of the resultant culture are washed three times with sterile saline (0.9% NaCl solution) to reduce carry-over of vitamin into the assay tubes during the subsequent inoculation. This washing operation is performed by centrifuging the tube contents, discarding the supernant, and resuspending the cells in 10 ml of saline. After the third wash, the cells are resuspended in sterile assay medium and incubated at 37°C until used. The final inoculum is prepared by diluting the vitamin-depleted cell suspension with assay medium.

Bell [11] found that procedures using centrifugal washing gave an inoculum in the lag phase of growth, thus necessitating unduly long assay incubation periods. Problems of airborne bacterial contamination were also encountered. Bell prepared inocula for the determination of vitamin B_2, niacin, pantothenic acid, biotin, folate, and vitamin B_{12} using Bacto-Micro Inoculum Broth (Difco Code 0320) as the inoculum medium. The common procedure for all these determinations was to subculture the assay organism from the most recent agar stab into 5 ml of sterile inoculum broth and incubate overnight at 37°C. The following morning, one drop of this subculture was added to 5 ml of single-strength basal medium (basal medium diluted with an equal volume of water) containing a controlled amount of the vitamin being assayed. These amounts were 25 ng nicotinic acid/ml, 20 ng pantothenic acid/ml, 0.05 ng biotin/ml, 2 ng riboflavin/ml, 1.0 ng folic acid/ml, and 0.04 ng cyanocobalamin/ml. Both the inoculum broth and the basal medium were held at 37°C during transfer of the organism. After a further 6-h incubation at 37°C, two drops of the resultant culture were transferred to 10 ml of single-strength basal medium (without added vitamin). This final suspension was used as the inoculum and contained cells in the exponential (acceleration) growth phase.

Bell [11] prepared the inoculum for the determination of vitamin B_1 in a similar manner as described earlier, except that the inoculum medium was Bacto-APT Broth (Difco Code 0655) and the incubation temperature was 30°C. To prepare the inoculum for the determination of vitamin B_6,

a loop of *S. cerevisiae* from the agar slope was added to 5 ml of single-strength medium containing a glass bead, and this subculture was incubated for 20 h at 27°C with constant shaking. One milliliter of the suspension obtained was pipetted into a tube containing 5 ml of sterile single-strength basal medium and mixed to give the inoculum culture.

Variations of the inoculum culture can be eliminated by the use of glycerol-cryoprotected lactobacilli. This entails preparing a large volume of inoculated basal medium, adding glycerol, and dispensing aliquots of the sterilized mixture into vials for storage at −70°C. The bacteria stored in this manner remain viable for several months. A thawed vial is then used as the inoculum for each assay. This technique has the practical advantages over serial agar-stab culturing in diminishing the need for microbiological expertise and saving time. The washing of cells by centrifugation with saline is unnecessary, thus saving further time and effort. Standard curves made from a single batch of cryoprotected inoculum are superimposable. Because blank values are lower, a heavier inoculum can be used, which results in a greater growth rate and hence a reduction in incubation time. Thus, assays using cryoprotected inocula are more sensitive and reproducible, besides being cheaper and quicker [12].

Horne [13] prepared glycerol-cryoprotected *L. rhamnosus* as inoculum for a microtiter plate assay of folate as follows. Dissolve 9.4 g of dehydrated basal medium (Bacto-Folic Acid Casei Medium; Difco Code No. 0822) and 50 mg of ascorbic acid in 200 ml of distilled water. To this single-strength basal medium add 120 ng of (6*RS*)-5-formyl-THF, calcium salt pentahydrate, and then sterilize by filtration (0.22-μm pore size). Suspend the lyophilized *L. rhamnosus*, in its shipping vial, in 1 ml of the prepared medium. Transfer 0.25 ml of this suspension to the remaining 199 ml of medium and incubate at 35–37°C for about 18 h. Cool in an ice bath and add a equal volume of cold, sterile glycerol (80%, v/v in water). Store 4-ml aliquots in sterile tubes at about −70°C.

18.3.4.3 Preparation of the Assay (Basal) Medium

In traditional assay procedures, the assay media are prepared at double strength, either from commercial dehydrated formulations (if available) or from individual ingredients.

In addition to factors essential for bacterial growth, other substances which stimulate growth must be considered. Ideally, the medium should contain sufficient amount of all stimulatory substances so that the effects of these nutrients added with the hydrolyzed food extract being assayed will be eliminated. In practice, the ideal is seldom achieved and the adequacy of a basal medium depends upon the experimental conditions [10]. A basal medium which, when supplied with the missing

vitamin, contains all of the nutrients *essential* for growth, but is lacking in one or more substances that markedly stimulate growth, may give satisfactory assays if the period of incubation is long enough to eliminate the effects of the nonessential growth stimulants. Such a medium will also be satisfactory for assaying samples which are rich in the vitamin to be determined, as the high dilution of sample will result in a negligible addition of stimulatory substances. Conversely, such a medium will tend to give erroneous results if used with a short incubation period, and with samples of low vitamin potency.

Fatty acids are notorious growth stimulants for a number of lactic acid bacteria [14,15], but it is not customary to add these acids to the basal medium; rather they are removed from the food sample after the extraction step.

18.3.4.4 Extraction of the Vitamin from the Test Material

The vitamins are extracted from the food matrix in a form that can be utilized by the particular assay organism being used. This generally involves autoclaving the food sample in the presence of acid or, for acid-labile vitamins, digesting the sample with suitable enzymes. After precipitating the proteins at their isoelectric point (ca. pH 4), the pH of the extract is adjusted to that of the basal medium (typically pH 6.8). This step is necessary to ensure that the pH of the medium is not altered by the addition of different amounts of the extract. The extract is then diluted to bring the concentration of the vitamin to be assayed within the range of the standard curve. Hopefully, the dilution factor will be sufficiently high to dilute out any interfering substances that would cause drift and invalidate the assay. The minimum dilutions of foods necessary to avoid the inhibitory effects of food preservatives and neutralization salts have been calculated [16]. Finally, the extracts are filtered to remove the precipitated protein and lipoidal material, and to obtain a clear solution for assay.

18.3.4.5 Setting Up the Assay

At all stages during the analytical procedure, the solutions must be protected from daylight. Aliquots of the working standard vitamin solution are added in increasing volumes up to 5 ml to a duplicate series of tubes for the construction of a standard curve; duplicate blanks containing no vitamin are included. Similar volumes of the neutralized test extracts are added to a single series of tubes. A range of concentration levels of each test extract is assayed in the expectation that at least three will fall on the standard curve. Fresh glass-distilled water is added to all tubes to bring the volume in each tube to 5.0 ml, after which 5.0 ml

of double-strength basal medium is added so that the total volume is 10 ml. The addition of basal medium to the extract and water gives better mixing of the two solutions than adding the extract and water to the medium. The entire rack of filled tubes is covered with a sheet of aluminum foil and sterilized in an autoclave. The tubes are removed immediately from the autoclave when atmospheric pressure is reached, and the tubes are cooled to below the subsequent incubation temperature. It is imperative that all tubes are cooled to the same temperature, because turbidity is a measure of the rate of growth rather than the extent of growth, and small differences in the temperature between tubes at the start of the incubation influence the growth rate.

All tubes are inoculated with one drop of freshly prepared inoculum culture. The inclusion of a control run, which is not inoculated but otherwise treated identically, is advisable to check the sterility of the basal medium. For turbidimetric assays using lactic acid bacteria or yeasts, the tubes are incubated for 22–24 h at a constant temperature near the optimum for these organisms. Suitable temperatures are 37°C for *L. rhamnosus, L. plantarum*, and *L. delbrueckii* subsp. *lactis*; 30°C for *Weissella viridescens*; and 28°C (with constant shaking) for *S. cerevisiae* and *K. apiculata.*

The setting-up procedure described by Bell [11] involves less manipulations than the conventional procedure, owing to the volumes of standard or sample assay solutions being reduced from milliliter aliquots to microliter aliquots ranging from 0 to 250 μl. The addition of such small volumes to 10 ml of assay medium has two advantages. First, no significant volume change occurs so that single-strength medium can be used. Second, the need for adjustment of the pH value of samples extracted with acids is greatly reduced. For example, no pH adjustment is required when acids less than 0.1 *N* are used, and for strong acid hydrolysates the solution need only be adjusted to pH 1–2.

18.3.4.6 Quantification

At the end of the incubation period, the cells are uniformly suspended by shaking the tubes, and time is allowed for the air bubbles to disperse before measurement. The turbidities of all tubes are measured in a nephelometer using a neutral filter, colorimeter with a filter in the region of 640 nm, or spectrophotometer at 540–660-nm wavelength. The turbidity may be expressed as an absorption (extinction), as a transmittance (in % T), as a difference $100 - T$ (in %) or simply as a galvanometer reading. The arithmetic means of the replicates are calculated, and the means for the standard solutions are plotted on semilogarithmic graph paper with the turbidity values as ordinates (linear scale) and concentrations in ng/ml as abscissae (logarithmic scale). The calibration curve

is drawn through these points. The vitamin concentrations of the sample tubes are read off from the calibration curve and the concentration values for the original samples are calculated from simple dilution factors. Concentration values for a given sample calculated from at least three dilutions should not differ by more than $\pm 10\%$ from their common mean [17].

The reliability of a determination can be assessed by testing for the presence or absence of drift. This simply entails plotting the mean turbidity values on the calibration curve for the sample dilutions against the corresponding dilution factors. A check curve that is roughly parallel to the calibration curve signifies the absence of drift. Drift is manifested by a check curve that deviates widely from the calibration curve, either increasing or decreasing with concentration of the sample. The occurrence of drift in assay solutions is evidence for the presence of interfering materials in the test solution presented for assay. If a drift has been established, the determination is invalid and the assay must be repeated. In this event, measures must be taken to remove the interfering substances by improving the efficiency of the extraction procedure. If these measures fail, the assay conditions must be changed or a different assay organism employed [17].

18.3.5 Partial Automation of the Assay Procedure

Haggett et al. [18] described an inter-connected modular system which provided automated dispensing of sample, buffer, and assay medium into the tubes; automatic measurement of growth response by turbidimetry after incubation; and computer software to control the equipment and to process the analytical data. The automation process facilitated detection of errors and halved the operator time per assay.

18.4 Turbidimetric Method Using Microtiter Plates

A microbiological assay for folate adapted for the use of disposable 96-well microtiter plates and an automatic plate reader was introduced by Newman and Tsai [19]. The organism used in this assay was a folate-depleted culture of *L. rhamnosus* prepared by serial agar-stab culturing and centrifugal washing. Horne and Patterson [20] simplified this method by using the glycerol-cryoprotected *L. rhamnosus*, which requires only dilution before use as the inoculum. An inherent problem of overestimated results in the two perimeter rows of clear-wall 96-well plates was overcome by the use of plates with opaque, black walls [13].

The use of microtiter plates allows automated determination and computer analysis of data. The saving in time is substantial owing to the speed of multiwell plate readers, which can measure the light transmission in 96 wells in less than 2 min. Other advantages over the standard tube assay are minimized reagent costs and a 10-fold increase in sensitivity.

Molloy and Scott [21] provided details of a microtiter plate method for determining folate in blood, which can be applied to suitably prepared food extracts. For each diluted sample extract in duplicate (replicates a and b), duplicate aliquots of 100 and 50 μl are transferred to four separate wells of a 96-well microtiter plate. The working folate standard is dispensed into wells of a separate microtiter plate at volumes of 0 (blanks), 5, 10, 15, 20, 25, 30, 40, 60, 80, and 100 μl. Eight replicates of each volume from 5 to 100 μl and 16 blank replicates are dispensed. Volumes of all wells are made up to 100 μl with 0.5% (w/v) sodium ascorbate and to each well is added 200 μl of inoculum (a cryopreserved, chloramphenicol-resistant strain of *L. rhamnosus* in assay medium). Each plate is covered with a plastic plate sealer and a roller is used to ensure that all wells are sealed and air bubbles removed. Plates are incubated at 37°C for about 42 h. After this time, each plate is inverted and mixed to produce an even cell suspension and the plate sealer is removed. The optical densities at 590 nm are measured in a microtiter plate reader linked to a computer. A commercial enzyme-linked immunosorbent assay (ELISA) program is used to collect data, draw a standard curve, and calculate the concentration of folate in each well. Appropriate dilution factors are used to calculate folate concentrations in the sample extracts. For sample replicate a, the concentration to be reported is calculated by averaging the final results (ng/ml) from the duplicated 100-μl and 50-μl aliquots. The 50-μl average is then checked against the 100-μl average and the result is accepted if there is no more than a 10% difference between the two. The mean of these two averages is then taken as the result for replicate a. This procedure is repeated for replicate b. Finally, the results for replicates a and b are checked against each other. If there is agreement within $\pm 5\%$ of the mean, the result for that sample is accepted.

18.5 Assays of Individual B-Group Vitamins

18.5.1 Vitamin B_1

Two *Lactobacilli* species have been widely used as assay organisms for the determination of vitamin B_1, namely, *L. fermentum* and *W. viridescens.* Of the two, the latter is generally preferred as it is less susceptible to

inhibitory or stimulatory substances [22]. *W. viridescens* requires the intact thiamin molecule for growth. Thiamin pyrophosphate is approximately 60% as active as thiamin when equimolar concentrations are compared [23]. The conventional tube assay using *W. viridescens* has been compared with manual and semiautomated fluorometric methods in terms of its ability to recover added thiamin hydrochloride from commercially processed foods whose natural vitamin B_1 content was destroyed with thiaminase [24]. The microbiological method gave the best results for eight products tested, with a recovery of $99.9 \pm 1.03\%$. In a microtiter plate assay for determining vitamin B_1 in biological fluids and foods, Bui [25] used *L. fermentum* maintained as stab cultures.

To ensure the complete utilization of total vitamin B_1 by *W. viridescens*, the extraction procedure involves both acid and enzymatic hydrolysis as a means of liberating B_1 vitamers from all bound forms. The enzyme treatment can be omitted for the analysis of grain products and milk, and for the determination of the added thiamin hydrochloride in fortified foods.

18.5.2 Vitamin B_2

The organism traditionally used for determining vitamin B_2 is *L. rhamnosus.* Lactic acid bacteria cannot utilize flavin adenine dinucleotide (FAD), and the growth response of *L. rhamnosus*, measured turbidimetrically, differs significantly between riboflavin and flavin mononucleotide (FMN) [26]. As most of the vitamin B_2 activity in foods is present in the form of FMN after acid extraction, it would be more accurate to use FMN as the standard in the microbiological assay instead of riboflavin.

L. rhamnosus is stimulated by starch [27], and either stimulated or inhibited by long-chain free fatty acids (e.g., palmitic, stearic, oleic, and linoleic acids) and other lipids, including lecithin [28–30]. Kornberg et al. [31] proposed the use of *Enterococcus faecalis* which, with a sensitivity to 0.1 ng riboflavin/ml [32], is 50 times more sensitive than *L. rhamnosus.* Lipids also stimulate the growth of *E. faecalis*, but its higher sensitivity to riboflavin allows samples to be prepared for assay at a higher dilution, resulting in negligible amounts of interfering lipids and other extraneous matter in the assay tubes. Barton-Wright [33] used a basal medium that was more synthetic in composition than the original medium used by Kornberg et al. [31]. Comparative assay values using *E. faecalis* and *L. rhamnosus* (Table 18.3) show excellent agreement for many of the foods tested. The higher value of 0.33 μg/g for white flour using *L. rhamnosus* is apparently due to the stimulatory effect of lipids, as a prior ether extraction lowered this value to 0.20 μg/g, making it comparable to the value of 0.21 μg/g using *E. faecalis.* The ether extraction produced no lowering of the *E. faecalis* assay value. On the basis of these comparisons, *E. faecalis* is preferred to *L. rhamnosus* as an assay organism for determining vitamin B_2 [33].

TABLE 18.3

Comparative Assay Values for Vitamin B_2 Using *E. faecalis* and *L. rhamnosus*

	Riboflavin (μg/g)	
Material	***E. faecalis***	***L. rhamnosus***
Yeast	45	45
Navy beans	2.6	2.5
Green pea soup (dehydrated)	1.00	1.01
Pea puree	0.90	1.05
Sweet potato	0.95	0.94
Tomato juice	0.32	0.35
Carrots	1.68	1.83
Carrot puree	0.16	0.22
White potatoes	0.36	0.52
White flour (nonenriched)	0.21	0.33

Source: From Kornberg, H.A., Langdon, R.S., and Cheldelin, V.H., *Anal. Chem.*, 20, 81, 1948. With permission from American Chemical Society.

The extraction procedure for the *L. rhamnosus* assay necessitates autoclaving the food sample with 0.1 *N* HCl at 121°C for 30 min. During acid hydrolysis, FAD, which cannot be utilized by lactic acid bacteria, is completely degraded to FMN and riboflavin, and some of the FMN is also degraded to riboflavin. The complete conversion of FMN to riboflavin is not necessary, because FMN, riboflavin, and the various isomeric riboflavin monophosphates are all nutrients for the growth of *L. rhamnosus.* The acid digestion eliminates the troublesome starch, but it liberates interfering free fatty acids.

The general extraction procedure used in *L. rhamnosus* assays for analyzing foods of very low fat content (such foods include many cereals) involves acid hydrolysis, followed by precipitation of the denatured proteins at pH 4.5. The precipitated proteins, together with the small amount of lipoidal material and any nonhydrolyzed starch, are removed by simply filtering through paper. For samples of negligible fat content, the filtration step results in an assay free from drift. Omission of the filtration step produced a pronounced drift in vitamin B_2 values for whole wheat flour, which was typical of cereal products in general [29].

High-fat foods, such as wheat germ, maize, oats, soya beans, meat, cheese, and mixed diets, require a preliminary extraction with petroleum ether (bp 40–60°C) before acid hydrolysis to remove neutral fats. This involves extracting the dried, finely ground sample with petroleum ether for 16–18 h in a Soxhlet apparatus. The defatted material is autoclaved, adjusted to pH 4.5, diluted and filtered. A 50-ml aliquot of the filtrate is shaken with two or three 30-ml portions of diethyl ether in a separating funnel. The combined ether extracts are washed two or three times

with water, and the washings are added to the bulked aqueous layers. Finally, the pH of the extract is adjusted to 6.8, and the extract is filtered, if necessary, and diluted to 100 ml (or other suitable volume) for direct assay [32].

Milk should be separated by centrifugation and the serum shaken in a separating funnel with diethyl ether. Petroleum ether is not a suitable fat solvent with milk, as the mixture tends to form an emulsion [34].

The published extraction procedure using *E. faecalis* [31] entailed the addition of 20 ml of water and 3 ml of 1 *N* H_2SO_4 to 1 g of the dry material to be assayed, and autoclaving for 30 min. The pH was adjusted to 4.5–5.0, and the extract diluted to contain ca. 0.001–0.002 μg riboflavin/ml.

18.5.3 Niacin

Microbiological techniques currently employed for determining total niacin are based on the assay method developed by Snell and Wright [35] using *L. plantarum. L. plantarum* responds equally well on a molar basis to nicotinic acid, nicotinamide, nicotinuric acid (an inactive metabolite), and nicotinamide adenine dinucleotide (NAD) without preliminary hydrolysis [35]; hence this organism cannot be used to differentiate between nicotinic acid and nicotinamide. The growth of *L. plantarum* on a nicotinic acid basal medium is not affected by free fatty acids or phospholipids, apart from an inhibitory effect of linoleic acid at a relatively high concentration [36], so there are no problems of growth stimulation or inhibition due to lipids. Nevertheless, Barton-Wright [32] recommended that high-fat samples be Soxhlet-extracted with light petroleum ether for 16–18 h before acid or alkaline hydrolysis to prevent the formation of oily emulsions which may hinder complete extraction.

Sølve et al. [37] described an automated microtiter plate assay for the determination of niacin and introduced digital image processing as a measurement of turbidity.

18.5.3.1 Determination of Total Niacin

The extraction procedure recommended by the Association of Vitamin Chemists, Inc. [38] for the microbiological determination of total niacin for all foodstuffs, including cereals and cereal products, entails autoclaving the sample at 121°C for 30 min in the presence of 1 *N* H_2SO_4. This treatment liberates nicotinamide from its coenzyme forms and simultaneously hydrolyzes it to nicotinic acid. The treatment does not completely liberate the bound nicotinic acid from cereal products; alkaline hydrolysis is necessary to do this. However, extraction of cereal products with 1 *N* H_2SO_4 yielded similar nicotinic acid values obtained microbiologically as did extraction with 1 *N* NaOH [39], and

microbiological values obtained on wheat bran, corn bran, and rice bran were uniformly higher than chemical values when 1 *N* H_2SO_4 was used for extraction [40]. These findings support the experimental evidence presented by Krehl and Strong [41] that *L. plantarum* is able to utilize the bound nicotinic acid in cereals to a considerable extent. Although alkaline hydrolysis has been used to ensure the complete liberation of bound nicotinic acid in microbiological assays [42], the use of H_2SO_4 is preferred for practical reasons because alkaline extraction of fatty samples produces extracts that are difficult to clarify.

18.5.3.2 Determination of Bound Nicotinic Acid

In mature cereal grains, most of the niacin present exists in the form of bound nicotinic acid, which is biologically unavailable to humans unless pretreated with alkali. The general approach to estimating the bound nicotinic acid content of a cereal sample is to treat the sample with weak (0.1 *N*) acid, which extracts the free niacin but does not liberate nicotinic acid from the bound form. The resultant extract therefore contains both free and bound nicotinic acid, but provides a value only for the free form when analyzed microbiologically using a selective organism. A portion of the acid extract is subsequently treated with 1 *N* NaOH to liberate bound nicotinic acid, and the resultant solution is assayed for total niacin. The difference between the results obtained for total and free niacin is a measure of bound nicotinic acid. This approach depends on the assay organism being unresponsive to bound nicotinic acid; thus the usual organism, *L. plantarum*, is unsuitable. The two methods described below utilize organisms that fulfill this requirement.

In a method described by Clegg [43], food samples weighing 2–4 g are extracted with 50 ml of 0.1 *N* HCl on a boiling water bath for 30 min. After cooling, the pH of the solution is adjusted to 3.5 with 0.1 *N* NaOH with constant stirring to avoid localized alkalinity. The extract is made up to 100 ml, filtered, and an aliquot of the filtrate is washed with an equal volume of chloroform. An aliquot of the fat-free extract is heated with 1 *N* NaOH in a boiling water bath for 30 min to liberate the bound niacin. The alkali-treated solution and an untreated aliquot of the fat-free extract at pH 3.5 are adjusted to pH 6.5 and diluted to contain ca. 30 ng of total or free niacin/ml, respectively. To avoid the liberation of bound nicotinic acid during the subsequent autoclaving of medium plus sample, aliquots of the alkali-untreated solution are added aseptically to previously autoclaved assay tubes containing the medium. The alkali-treated solution is dispensed into assay tubes before autoclaving, in accordance with standard procedure. The difference between the values for total and free niacin, using *L. rhamnosus* as the assay organism, represents the bound niacin content of the sample.

A similar procedure for determining the bound nicotinic acid in natural materials was reported by Ghosh et al. [44] using *Leuconostoc mesenteroides* subsp. *mesenteroides* as the assay organism. In this procedure, the homogenized sample was extracted with 0.1 *N* HCl in a boiling water bath for 45 min, and the extract was cooled and centrifuged. The residue was treated twice with 0.1 *N* HCl in the cold and then centrifuged. The combined centrifugate was used for the assay of free nicotinic acid, bound nicotinic, and nicotinamide. Free nicotinic acid was determined by adjusting an aliquot of the acid extract to pH 6.2–6.4 and centrifuging off the precipitated material. The centrifugate was adjusted to pH 3.5 then sterilized by steaming at atmospheric pressure for 15 min. It was found that a portion of the bound nicotinic acid was hydrolyzed when the materials were sterilized at 10 lb/in.^{2} for 15 min at pH 6.2–6.4, but when the sterilization was effected at pH 3.5–4 by steaming, only traces of nicotinic acid were released from the bound form. Bound nicotinic acid, together with free nicotinic acid, was determined by treating a second aliquot of the extract with 0.5 *N* NaOH for 10 min at room temperature, and assaying the nicotinic acid content after removal of the material precipitated at pH 6.2–6.4. Nicotinamide, together with free and bound nicotinic acid, was estimated by hydrolyzing the bound nicotinic acid with 0.5 *N* NaOH, then hydrolyzing the amide with 1 *N* HCl at 15 lb/in.^{2} for 1 h. The total nicotinic acid was assayed after removal of the material precipitated at pH 6.2–6.4. The difference in *Leuc. mesenteroides* assay results between the nicotinic acid content of the extract before and after alkaline hydrolysis represented the bound nicotinic acid content of the sample. The difference between the total nicotinic acid content and the nicotinic acid content obtained after alkaline hydrolysis represented the nicotinamide content of the sample.

18.5.3.3 Determination of Added Nicotinic Acid

Nicotinic acid added to fresh meat can be determined directly using *Leuc. mesenteroides.* Gorin and Schütz [45] suspended the minced meat sample in 0.2 *M* acetate buffer (pH 4.8) and shook the suspension for 10 min at room temperature. Nicotinic acid was extracted while the buffer flocculated the meat proteins. This mild extraction process does not convert nicotinamide to nicotinic acid.

18.5.4 Vitamin B_6

The ideal assay organism for the microbiological determination of total vitamin B_6 should exhibit an equal growth response to pyridoxine (PN),

pyridoxal (PL), and pyridoxamine (PM), as these vitamers have equal biological activities in humans [46]. Lactic acid bacteria have no utility, because they do not respond to PN [47]. The yeast *S. cerevisiae* responds vigorously to all three free bases and is the most widely used assay organism for determining vitamin B_6. Acid hydrolysis of food samples is necessary for the determination of total vitamin B_6 because *S. cerevisiae* utilizes only the unbound nonphosphorylated forms of the vitamin [48]. The growth response of *S. cerevisiae* to PL relative to that to PN is practically equal, or somewhat less, but the response to PM is markedly less than that to PN and is dose-dependent within the working concentration range of 2–10 ng molar equivalents of PN per tube [49,50]. This unequal response leads to an underestimation of the total vitamin B_6 content if the sample extract contains predominantly PM (e.g., a processed meat product), but is of little concern in plant-derived foods or in foods that are substantially fortified with PN. Morris et al. [48] pointed out that cultures of *S. cerevisiae* obtained from different sources, but reputed to be derived from the same parent strain, possess different nutritional requirements, particularly in their response to thiamin and vitamin B_6. It is therefore important to study the growth requirements of any strain of *S. cerevisiae* before it is used as an assay organism.

The problem of differential response in the *S. cerevisiae* assay can be overcome by separating PN, PL, and PM chromatographically and assaying each vitamer individually. Toepfer and Polansky [51] reported the results of a collaborative study using such a technique, which was adopted as Final Action by the AOAC in 1975 [52]. In this method, acid-hydrolyzed food samples are adjusted to pH 4.5 with 6 *N* KOH to precipitate the denatured proteins, then diluted with water and filtered. An aliquot of the filtrate is applied to an open column packed with Dowex 50W-X8 cation exchange resin, and fractions containing PL, PN, and PM are obtained by elution with a sequence of boiling buffer solutions. Each vitamer is then assayed using its own standard curve. For a number of animal products, total vitamin B_6 values, obtained by adding the results of the individual vitamers, were statistically higher than nonchromatographed values calculated using a PN standard curve [53]. An additional benefit of the chromatographic technique is the removal from high-starch plant products of acid-treated glucose compounds which would otherwise stimulate yeast growth [54]. Data obtained by the chromatographic procedure compared well with data obtained by rat growth assay for total vitamin B_6 in a few selected food samples [55]. For routine purposes, the chromatographic step is omitted, and aliquots of the filtrate are diluted according to the expected vitamin B_6 content before addition to the assay tubes. A more efficient and reproducible fractionation of B_6 vitamers can be obtained using

high-performance liquid chromatography (HPLC), as performed by Gregory et al. [56].

Guilarte [57] used a modified version of the AOAC procedure [52] in which 200 ml of 0.5 *N* HCl or 0.05 *N* HCl, for plant and animal products, respectively, was added to 1–2 g of the dry product, and the mixtures were autoclaved for 19 min at 121°C. The cooled extracts were adjusted to pH 4.5 using 6 *N* NaOH, diluted to 250 ml with water, centrifuged or filtered, and finally diluted to the appropriate concentration for turbidimetric assay.

Guilarte et al. [49] compared *S. cerevisiae* with another yeast, *K. apiculata*, for their ability to utilize PN, PL, and PM in the concentration range needed for the measurement of vitamin B_6 in biological materials. The results showed that, unlike *S. cerevisiae*, *K. apiculata* responded equally to all three vitamers at a concentration range of 2–10 ng molar equivalents of PN per tube. A practically equal response of *K. apiculata* to PN, PL, and PM was previously reported by Barton-Wright [58] and Daoud et al. [59]. Guilarte et al. [49] proposed that *K. apiculata* should be used instead of *S. cerevisiae* as the standard turbidimetric microbiological assay organism for vitamin B_6 in biological materials. However, this proposal has not found acceptance among certain other research groups. Gregory [50] conducted a study under conditions comparable to those employed by Guilarte et al. [49], and found that *K. apiculata* exhibited an even lower relative response to PM than that obtained with *S. cerevisiae.* A similar disparity in the response to PM with *K. apiculata* was reported by Polansky [60]. These conflicting data [61,62] suggest that subtle environmental factors or culturing variables affect the specificity of *K. apiculata.* This as yet unresolved discrepancy between results from different laboratories illustrates the importance of checking the growth response to PN, PL, and PM of any assay organism before proceeding to routine determinations.

The growth response of *S. cerevisiae* and *K. apiculata* is influenced by KCl and NaCl formed as a result of pH adjustment of acid-hydrolyzed food samples with 6 *N* KOH or NaOH [57]. This potential source of interference can be reduced or eliminated by treating the standard in a similar fashion as the food sample to be assayed.

Barton-Wright [58] encountered the occasional problem of excessive growth in the blanks using *K. apiculata*, which could not be reduced using the PN-depletion technique of Gare [63]. The problem was overcome by maintaining the organism in liquid stock culture containing PN.HCl instead of on the conventional malt agar slope. The basal medium was modified in several respects, notably by substituting a 10% charcoal-treated malt extract solution for vitamin-free casein hydrolysate, which often proved difficult to free completely from vitamin B_6.

18.5.5 Pantothenic Acid

The usual assay organism for the microbiological determination of pantothenic acid in foods is *L. plantarum.* The basal medium has the same composition as that used for the determination of niacin, except that pantothenic acid instead of nicotinic acid is the limiting factor. Fatty acids are stimulatory with suboptimal amounts of pantothenic acid [64], so a preliminary ether extraction step may be necessary.

Both intestinal phosphatase and avian liver enzyme preparations required to liberate bound pantothenic acid are available commercially as powdered extracts. Liver enzyme preparations contain a relatively high amount of coenzyme A, which is converted to pantothenate during the incubation period, thus creating an unacceptably high blank value. Pigeon or chicken acetone-dried liver powder obtained from Sigma Chemicals can be purified quite simply by treatment with Dowex 1-X4 anion exchange resin [11,65,66]. Purification of the enzyme reduces the blank value to a very low level without appreciable loss of coenzyme A-splitting activity. Intestinal phosphatase preparations contain negligible amounts of coenzyme A and do not require purification.

Toepfer et al. [65] used the phosphatase-liver enzyme treatment in a standardized microbiological assay and obtained pantothenic acid values for whole egg powder, kale, peanuts, pig liver, and brewer's yeast that did not differ significantly from values obtained on the same samples using a rat bioassay. The microbiological results for carrots, however, were significantly lower than the bioassay results.

A collaborative study [67] showed that using Dowex-treated pigeon liver enzyme or a commercially purified hog kidney enzyme in combination with intestinal phosphatase produced similar results for the analysis of alfalfa leaf meal, whole egg powder, and dried brewer's yeast. However, there was considerable variation among the data reported by collaborators. A loss of activity of these enzyme preparations showed a need for establishing the activity of the enzymes before they could be relied upon for use in an official assay procedure. Measurement of enzyme activity requires a stable standard of bound pantothenic acid, but such a standard has yet to be found. Coenzyme A did not prove satisfactory because minute quantities of enzymes were sufficient to release most of the bound pantothenate [68].

In the procedure employed by Bell [11], the test material is homogenized with 10 ml of 0.2 *M* tris-(hydroxymethyl)-methylamine buffer (pH 8.0) at 70°C, and then autoclaved for 15 min at 121°C. After mixing and cooling to room temperature, 1.0 ml of 2% (w/v) alkaline phosphatase (Sigma) in "Tris" buffer and 0.5 ml of Dowex-treated pigeon liver enzyme solution are added, and the mixture is incubated at 37°C overnight. On the following day, the extracts are steamed in an autoclave for 5 min to

destroy any remaining enzyme activity, cooled to room temperature, and diluted to a suitable volume with Tris buffer. The extracts are centrifuged at 15,000 × *g* for 20 min (an additional step [69]), and then filtered through Whatman No. 42 (or, if nonfatty, through Whatman No. 541) filter paper into polythene bottles for storage at −20°C pending analysis. For conventional assay procedures using double-strength media, the filtrates are adjusted to pH 6.8 with NaOH solution and diluted to place them in the range of the standard curve. A reagent blank is taken through the same procedure. Walsh et al. [70] omitted the slow and troublesome filtration step and used dialysis to isolate the liberated pantothenate from the large amount of protein in the food digest.

18.5.6 Biotin

The assay organism *L. plantarum* shows a high specificity toward free *d*-biotin and does not respond to biocytin or other bound forms. Biotin-*d*-sulfoxide elicits an equal growth response to that of *d*-biotin, whereas biotin sulfone is inhibitory [71]. However, these oxidation products are not likely to be present in food samples in sufficient concentration to cause significant interference. *L. plantarum* is stimulated by nonesterified unsaturated fatty acids and other lipids when biotin is present in suboptimal amounts [72–74]. The high sensitivity of the biotin assay (0.1–1.0 ng per assay tube) [75] necessitates a high sample dilution, and this will reduce this growth stimulation. The high sample dilution has the added advantage of lowering the initially high concentration of salt produced on neutralization of the acid extracts. The basal medium used for nicotinic acid and pantothenic acid can be used for biotin assays with the exclusion of the relevant vitamin (in this case biotin) from the formulation. The use of the same organism (*L. plantarum*) for assaying the three vitamins also eliminates the need for preparing separate stock cultures.

It has been recommended to remove lipid material by adjusting the pH of the acid hydrolysate to 4.5 and filtering through paper [76]. (Note: Guilarte [77] reported a possible loss of biotin during filtration.) This procedure might not be adequate for samples with a high fat content (e.g., wheat germ, meat, and eggs), which should be defatted by a preliminary Soxhlet extraction with light petroleum [32].

18.5.7 Folate

For routine food analysis purposes, total folate is measured using cryoprotected *L. rhamnosus* after treatment of sample extracts with conjugase. The Folic Acid Casei Medium supplied by Difco has a pH of 6.7 ± 0.1. The validity of the assay depends on all active monoglutamyl folates having identical equimolar growth-support activities for *L. rhamnosus.* Whether

or not this is the case is a subject of controversy. While some investigators [78,79] reported that various monoglutamates gave essentially equivalent responses, others [6,80–83] have demonstrated different responses.

Phillips and Wright [83] reported a reduced response to 5-methyl-THF relative to folic acid under conventional assay conditions, resulting in an underestimation of total folate in food samples. However, they obtained approximately equivalent responses by adjusting the initial pH of the assay medium to 6.2. Rader et al. [84] investigated the response of *L. rhamnosus* to different calibrants at media pHs of 6.2 and 6.7 $\pm$ 0.1. An enriched corn meal, an enriched rice product, and unfortified shredded whole-wheat cereal were assayed at the two pHs using folic acid to construct the standard curve. Growth was slightly better at pH 6.2 than at pH 6.7 $\pm$ 0.1 in both the standard and sample tubes. Similar results for the folate content of the three products were obtained at both pHs (Table 18.4). Analysis of the unfortified shredded whole-wheat cereal at pH 6.2 and pH 6.7 $\pm$ 0.1 against folic acid and 5-methyl-THF as the calibrants gave comparable results. Rader et al. [84] concluded from their studies that an assay pH of 6.7 $\pm$ 0.1 is satisfactory for the analysis of cereal-grain products. Increased sensitivity can be achieved when the assay is performed at pH 6.2 and this modification may be useful for samples of low folate content.

18.5.8 Vitamin B_{12}

Most current procedures for the microbiological determination of vitamin B_{12} activity in foods use *L. delbrueckii* subsp. *lactis.* Either the 326 or the 313 strain may be used, although the latter requires a shorter time to reach nearly maximum growth (20 h versus 48 h) [85]. The growth response of the 313 strain to cyanocobalamin is similar to its growth response to

TABLE 18.4

Effect of pH of Microbiological Assay With *L. rhamnosus* on Determination of Folate in Three Cereal-Grain Products

	Folate (μg/100 g)	
Product	**pH 6.2**	**pH 6.7 $\pm$ 0.1**
Enriched stone-ground yellow corn meal	282.0 $\pm$ 17.7	277.2 $\pm$ 4.0
Enriched rice, medium grain	192.4 $\pm$ 14.6	200.2 $\pm$ 5.6
Shredded whole-wheat cereal (unfortified)	45.1 $\pm$ 3.0	42.7 $\pm$ 2.4

Note: Values are means $\pm$ SD of two or three determinations. The calibrant was folic acid.
Source: From Rader, J.I., Weaver, C.M., and Angyal, G., *Food Chem.*, Vol. 62, pp. 451–465, 1998. With permission from Elsevier.

hydroxocobalamin, sulfitocobalamin, dicyanocobalamin, and nitritocobalamin, but lower than that to adenosylcobalamin and higher than that to methylcobalamin. Accurate measurement of vitamin B_{12} in foods can be made using *L. delbrueckii* subsp. *lactis* 313 strain and cyanocobalamin as the calibration standard if the sample extracts are exposed to light before analysis. Complete conversion of adenosylcobalamin and methylcobalamin to hydroxocobalamin takes place in 20 min after exposure to white light from a 15 W bulb at a distance of 20 cm [86].

L. delbrueckii does not respond specifically to vitamin B_{12}, as biologically inactive analogs, in which the 5,6-dimethylbenzimidazole moiety is substituted by a purine base or a purine derivative, can replace the vitamin as a growth factor [87]. Such analogs are found mainly in natural material that has undergone microbial fermentation, and they do not occur to any significant extent in foods. Examples of potentially interfering analogs include the so-called pseudovitamin B_{12} and Factor A, in which the base substituents are adenine and 2-methyladenine, respectively. DNA, deoxyribonucleotides, and deoxyribonucleosides can also replace vitamin B_{12} for the growth of *L. delbrueckii* [88]. The nucleotides and nucleosides are less active than vitamin B_{12} by a factor of 10^4, while DNA is less active by a factor of 10^6 [89]. The activity attributable to deoxyribonucleosides can be determined by *L. delbrueckii* assay after the cobalamins have been destroyed by heating to 100°C at pH 11 for 30 min [85]. Deoxyribonucleosides do not occur in usual foods at levels likely to constitute a significant interference [90], and they only substitute for B_{12} at concentrations above 1 μg/ml of assay solution [91]. Any such interference can therefore be eliminated by simply diluting to an inactive concentration.

Ford [92] developed an assay using the protozoan *Poterioochromonas malhamensis*, which is as sensitive as the *L. delbrueckii* assay, but is more specific in that it responds only slightly, if at all, to clinically inactive cobalamins. A disadvantage of the *P. malhamensis* assay is the long incubation period of 72 h, during which the culture must be shaken continuously in the dark. A comparative study between the *P. malhamensis* and *L. delbrueckii* methods applied to a cross-section of 27 different foods did not reveal any problems of interference with the latter method. On the contrary, the *P. malhamensis* results were statistically higher at the 5% level of significance than the *L. delbrueckii* results, suggesting the presence in certain foods of unknown noncobalamin substances which stimulate the growth of the protozoan [93]. Ford's method was recommended for the determination of vitamin B_{12} in animal feedstuffs [94], although Shrimpton [95] reported that *P. malhamensis* was no better than *L. delbrueckii* in estimating the vitamin B_{12} activity of feeding stuffs for chicks.

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19

Physicochemical Analytical Techniques (Excluding HPLC)

Physicochemical methods for determining vitamins in foods began with colorimetric methods, which required open-column chromatography or thin-layer chromatography to isolate the vitamins from interfering substances. In the 1960s, gas chromatography (GC) became the dominant technique for determining vitamins D and E. The columns used at that time were of the packed type and lacked the sophistication of modern capillary columns. Cleanup of sample extracts by open-column or thin-layer chromatography was still necessary, as was derivatization to increase the vitamins' thermal stability and volatility. The subsequent development of fused-silica open tubular capillary columns has revived the interest in GC. The introduction of high-performance liquid chromatography (HPLC) in the early 1970s led to its becoming the technique of choice for the determination of fat-soluble vitamins and carotenoids. Other modern separation techniques of potential application are supercritical fluid chromatography, capillary electrophoresis, and continuous-flow analysis.

19.1 AOAC Titrimetric Method for Vitamin C

The AOAC method for determining vitamin C in vitamin preparations and juices [1] is based upon the reduction of the dye 2,6-dichlorophenolindophenol (DCPIP) with ascorbic acid in an acid solution (Figure 19.1). Dehydroascorbic acid does not participate in the redox reaction, so the method does not yield the total vitamin C activity of a sample if this compound is present in significant quantities. In its oxidized form, DCPIP is purplish-blue in neutral or alkaline solution, and pink in acid solution; the reduced leuco compound is colorless. The procedure entails titrating a standardized solution of the dye into an acid extract of the sample. The pink end-point signals the presence of excess unreduced dye. The titration should be performed rapidly (within 1–2 min) in the pH range of 3–4, taking the first definite end-point. In the absence of interfering substances, the capacity of the extract to reduce the dye is directly proportional to the ascorbic acid content.

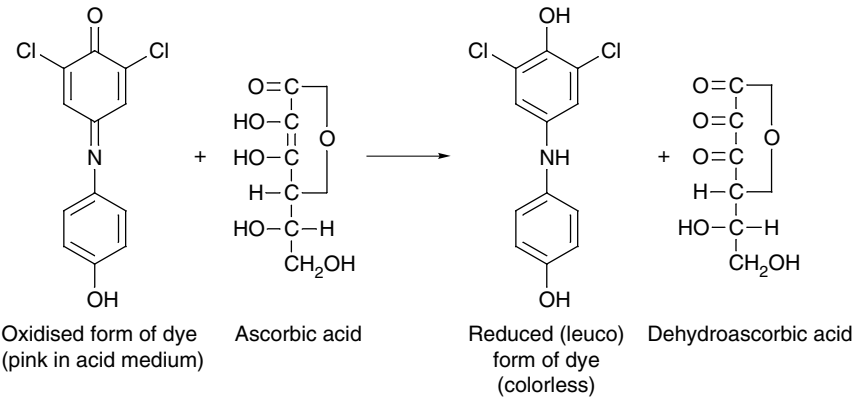

FIGURE 19.1
Reduction of DCPIP dye with ascorbic acid in an acid medium.

Unless suitable measures are taken, substances (other than ascorbic acid) that have reduction potentials lower than that of the DCPIP indicator, will react with the dye and give a falsely high result for the vitamin C content of the sample. Substances known to interfere in the assay include sulfhydryl compounds (e.g., glutathione and cysteine), phenols, sulfites, metal ions (copper(I), iron(II) and tin(II), and reductones such as reductic acid (Figure 19.2). Sulfites are a common cause of difficulty because of their use as food preservatives. Provided that the titration is performed rapidly, sulfhydryl and phenolic compounds should not cause interference, as the reduction of DCPIP by these compounds is relatively slow [2]. Metal ions are not normally present in sufficient concentration to cause a significant interference. However, iron(II) and tin(II) ions can be leached from nonlacquered cans containing fruit drinks and juices and, in combination with the traces of naturally occurring oxalic acid, can produce a measurable interference [3]. Reductones are only likely to be found in processed foods after prolonged boiling or in canned foods after standing at elevated temperatures [2].

The DCPIP titrimetric method gives results that generally agree with the biological estimation of vitamin C in raw and canned fruit and

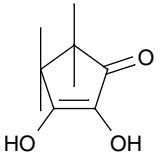

FIGURE 19.2
Reductic acid.

vegetables and their juices, which usually contain negligible amounts of dehydroascorbic acid. Analytical results for fresh fruits and vegetables showed good general agreement between the ascorbic acid values obtained by the DCPIP titrimetric method and by HPLC [4]. The DCPIP and HPLC methods gave comparable results for fresh and three-week stored broccoli, cauliflower, green beans, turnips, and three-week stored Brussels sprouts and spinach. Ascorbic acid contents, when measured by the HPLC method, were higher for fresh Brussels sprouts and spinach, presumably because of interfering compounds that disappeared during storage [5].

The DCPIP titration method may be performed potentiometrically instead of visually as a means of overcoming the end-point difficulty encountered with colored solutions. Spaeth et al. [6] described a procedure in which manual potentiometric measurements were made with a pH meter using standard calomel and platinum electrodes to establish the titration curve (Figure 19.3). An automatic titrator was set to 35 mV above the baseline potential, and the standard and test solutions were titrated to ± 10 mV of this arbitrary end-point.

Verma et al. [7] revitalized the DCPIP titration method by employing preliminary solid-phase extraction (SPE) to remove coloring matter and

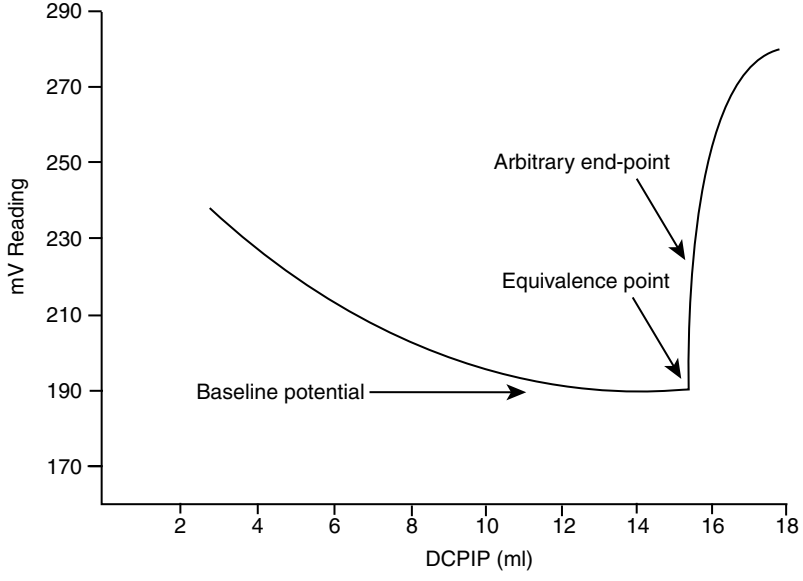

FIGURE 19.3
Titration curve of ascorbic acid with DCPIP. (Reprinted from Spaeth, E.E., Baptist, V.H., and Roberts, M., *Anal. Chem.*, 34, 1342, 1962. With permission.)

interfering substances from samples. Their procedure allowed the determination of total vitamin C as well as ascorbic acid. Solid-phase extraction cartridges (2.8 ml) containing 500 mg C_{18}-bonded silica were preconditioned by passing 1–2 column volumes of methanol and then 1–2 column volumes of water through the sorbent. The sorbent was then impregnated with 2,2′-bipyridyl and 2,9-dimethyl-1,10-phenanthroline, which form complexes with iron(II) and copper(II) ions, respectively, and with *N*-ethylmaleimide, which reacts rapidly with both sulfite and sulfhydryl compounds. Impregnation was carried out by passing 2 ml of a solution containing these reagents through the sorbent under mild positive pressure. A 2-ml aliquot of sample solution was passed though the column with the application of gentle suction and the effluent was collected in a titration flask. The column was then washed with 1–2 ml water and the effluent collected in the same flask. The coloring matter, metal complexes and sulfur adducts were retained on the column. The combined effluents containing the ascorbic acid were mixed with 1 ml anhydrous acetic acid, and the solution was titrated with DCPIP to the first appearance of a pink color. The endpoint was very sharp.

To determine total vitamin C, a second 2-ml aliquot of the sample solution was passed through an SPE cartridge and washed with 1–2 ml water. The combined effluents were mixed with 2 ml phosphate buffer (pH 6.8) and 2 ml 0.1% cysteine hydrochloride, and the solution was allowed to stand for 15 min to reduce dehydroascorbic acid. The solution was then passed through an SPE cartridge previously preconditioned and impregnated with *N*-ethylmaleimide. Titration of the effluent with DCPIP gave a result for total vitamin C. The dehydroascorbic acid content could be obtained by subtracting the ascorbic acid result from the total vitamin C result.

The DCPIP titration method preceded by SPE was applied successfully to highly colored fruit and vegetable juices, such as blackcurrant, black grape, and beetroot, and also to cola-type soft drinks. The efficacy of the SPE to remove interfering materials was shown by comparing ascorbic acid recoveries using the titration procedure with or without SPE. Recoveries from tomato, lime, watermelon, and mausambi were higher by 32, 115, 58, and 88%, respectively, when SPE was omitted.

19.2 Direct Spectrophotometric Determination of Vitamin C

The application of direct spectrophotometry to the determination of water-soluble vitamins in food extracts is subject to spectral interference from many substances. The extent of the interference depends upon the intensity of the absorbance of the vitamin relative to the absorbances of

accompanying substances at the selected wavelengths. Direct spectrophotometry has not found widespread routine application in the determination of the water-soluble vitamins in food, owing to the rigorous sample preparation that would be required to obtain a sufficiently pure solution for assay. Furthermore, for certain vitamins, fluorometric assay offers superior sensitivity and selectivity.

Direct spectrophotometry has been applied to the determination of ascorbic acid in soft drinks, fruit juices, and cordials after correction for background absorption in the UV region [8]. Background correction was made by measuring the absorbance of the sample solution before and after the catalytic oxidation of ascorbic acid with copper(II) sulfate, and then calculating the concentration of ascorbic acid from the difference. The sample blank was prepared by adding copper(II) sulfate to an aliquot of diluted sample and heating at 50°C for 15 min. The heating step was necessary to overcome the inhibitory effect of citrate upon the copper-catalyzed oxidation. To correct for the absorption due to Cu(II), ethylenediaminetetraacetic acid (EDTA) was added after the oxidation. Samples and standard solutions were prepared to contain the same concentration of the Cu(II)–EDTA complex, which does not catalyze the oxidation of ascorbic acid at room temperature. The absorption due to the Cu(II)–EDTA complex constituted part of the reagent blank against which the ascorbic acid standard solutions were read. Absorbance measurements were made at 267 nm and at pH 6. The calibration graph was linear within the range of 0–20 μg ascorbic acid/ml. The precision was 0.1–0.5% for ascorbic acid in the concentration range of 5–13 μg/ml.

19.3 Colorimetric Methods for Niacin and Vitamin C

19.3.1 Determination of Niacin by the König Reaction (AOAC Method)

The AOAC 1990 colorimetric method for the determination of niacin in foods and feeds [9] is based on the König reaction, in which pyridine derivatives are reacted with cyanogen bromide and an aromatic amine, sulfanilic acid. The pyridine ring is opened up, and the intermediate product is coupled with the amine to form a yellow dye, whose absorbance can be measured photometrically.

The AOAC method employs two different procedures: one for noncereal foods and feeds, and the other for cereal products. Noncereal foods and feed are extracted by autoclaving with 1 *N* H_2SO_4 for 30 min at 104 kPa pressure in order to liberate nicotinamide from its coenzyme forms and hydrolyze it to nicotinic acid. The reaction with cyanogen bromide and sulfanilic acid is carried out at room temperature, and the

resulting color is measured at 450 nm. Cereal products are autoclaved with calcium hydroxide solution for 2 h at 104 kPa pressure to liberate the nicotinic acid from its chemically bound forms. The reaction with cyanogen bromide and sulfanilic acid is carried out in the cold under somewhat different conditions, and the color is measured at 470 nm.

19.3.2 Colorimetric Methods for Vitamin C

Many colorimetric methods for determining vitamin C have been published. A well known example is the method of Roe and Kuether [10], which involves the oxidation of ascorbic acid to dehydroascorbic acid and subsequent reaction with 2,4-dinitrophenylhydrazine (DNPH) to form the osazone of diketogulonic acid. Treatment with 85% sulfuric acid yields a stable brownish-red color with an absorption maximum of 500–550 nm. The absorbance of this color is measured photometrically at 540 nm, and is proportional to the quantity of ascorbic acid (plus dehydroascorbic acid) present in the solution before oxidation. The assay procedure comprises four main steps: extraction, oxidation, condensation reaction, and color formation [2,11]. The method is not applicable to food containing erythorbic acid, because this epimer participates in the reaction. Unlike with the DCPIP titrimetric method, metal reducing ions do not interfere, but sugars such as glucose, fructose and glucuronic acid react with DNPH to form yellow osazones. Although the absorption maxima of these sugars lie toward shorter wavelengths, they nevertheless absorb sufficient light at 540 nm to constitute serious interferences in samples containing high levels of sugars or sugar degradation products [12]. Pigments do not interfere because they are removed by adsorption on the active carbon used in the oxidation step. Pelletier and Brassard [13] proposed manual and automated discrete sample analytical methods for total vitamin C in foods based on the Roe and Kuether method. The interference from high concentrations of sugars was rendered negligible by incubating at 15°C for 17 h after addition of DNPH, and by measuring the absorbance 75 min after the addition of sulfuric acid.

A recent colorimetric method for determining total vitamin C in fruit juices is based on the oxidation of ascorbic acid to dehydroascorbic acid by iron(III), followed by reaction between the iron(II) thus produced and the reagent 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol (Br-PADAP) to form a brown complex which is stabilized with EDTA [14]. The analytical procedure is as follows. Transfer a portion of the sample solution containing 1.0–60.0 μg of ascorbic acid to a 25-ml volumetric flask. Add 1.0 ml of iron(III) (ferric sulfate) solution, 4.0 ml of Br-PADAP solution and 2.5 ml of acetate buffer (pH 4.75). Mix, and after 5 min add 1.0 ml

of 0.1% EDTA. Dilute to volume with demineralized water and measure the absorbance after 5 min at 560 nm against an appropriate blank.

The above method can be used in the presence of the following substances (at ten times the concentration of ascorbic acid): citric acid, oxalic acid, calcium phosphate, sodium chloride, sodium citrate, benzoic acid, thiamin hydrochloride, pyridoxine hydrochloride, calcium pantothenate, vitamin B_{12}, starch, tartaric acid, ribose, leucine, alanine, methionine, cysteine, arginine, sucrose, fructose, and glucose.

19.4 Fluorometric Methods for Thiamin, Riboflavin, Vitamin B_6, and Vitamin C

19.4.1 Thiamin (AOAC Method)

The AOAC (1990) fluorometric method for determining thiamin in foods [15], grain products [16], bread [17] and milk-based infant formula [18] is based on the conversion of thiamin to its fluorescent oxidation product, thiochrome, by reaction with alkaline potassium hexacyanoferrate(III) (potassium ferricyanide, $K_3Fe(CN)_6$). In the procedure described for foods containing thiamin pyrophosphate [15], the food sample and a standard solution of thiamin hydrochloride are taken through the following steps: acid digestion, enzymatic hydrolysis, purification by open-column chromatography, oxidation of thiamin to thiochrome, extraction of the thiochrome into isobutanol, and measurement of the fluoresence. Thiamin monophosphate is insoluble in isobutanol, so it will not be measured in this assay. Alyabis and Simpson [19] modified the AOAC method by using a reversed-phase C_{18} 50-μm packing material for the open-column chromatography in place of the Bio-Rex 70 cation exchange resin.

For the analysis of grain products such as wheat flour, macaroni, and noodle products, which do not contain significant amounts of phosphorylated or protein-bound thiamin, the enzymatic hydrolysis and chromatographic purification steps have been omitted [16]. The enzymatic hydrolysis step, but not the chromatography, is essential for bread and wheat germ, which both contain phosphorylated thiamin [17].

19.4.2 Riboflavin (AOAC Method)

The native fluorescence exhibited by riboflavin enables this vitamin to be assayed fluorometrically without the need for chemical derivatization. The approach taken for the determination of vitamin B_2 using direct fluorometry is dictated by the relative fluoresence intensities of the

three major flavins. Flavin mononucleotide (FMN) and riboflavin exhibit equal fluorescence intensity on a molar basis, whereas the fluorescence of flavin adenine dinucleotide (FAD) is much less intense. It is therefore necessary to completely convert the FAD to FMN and riboflavin by autoclaving the food sample at 121°C for 30 min with 0.1 *N* HCl. The AOAC has adopted a fluorometric method for the determination of vitamin B_2 in foods, including ready-to-feed milk-based infant formulas. The general procedure [20] involves the following steps: acid digestion, precipitation of proteinaceous material, oxidation, and measurement of the fluorescence.

19.4.3 Vitamin B_6

The first published methods for the fluorometric determination of vitamin B_6 in foods [21–23] involved acid hydrolysis of the food samples, chromatographic purification, chemical conversion of the eluted vitamers to 4-pyridoxic acid, and acid treatment of this intermediate to form the lactone derivative. Different pretreatment procedures were necessary for selectively determining each of the vitamers. PN was eluted from an activated Decalso ion exchange column and oxidized with potassium permanganate to 4-pyridoxic acid. PM was converted to PN by deamination with nitrous acid before Decalso chromatography, and calculated by subtracting the PN value of the unconverted fraction from that of the converted fraction. PL was eluted from an Amberlite IR-112 ion exchange column and oxidized with ammoniacal silver to 4-pyridoxic acid.

The procedure described by Fujita et al. [21] for determining PN was adapted by Hennessy et al. [24] to the analysis of white flour enriched by the addition of PN.HCl, as well as bread made from this flour. Modifications included an additional enzymatic (Mylase) digestion step after acid hydrolysis. A simplified modification of the Hennessy method was applied to PN.HCl-enriched foods in general [25]. Ion exchange purification of the extract was not always adequate, and in these cases the alternative use of thin-layer chromatography was suggested.

The Strohecker and Henning [25] method was modified by Šebečić and Vedrina-Dragojević [26] for the determination of total vitamin B_6 in foods. Soya bean samples, which have a complex composition and are notoriously difficult to analyze, were chosen to test the applicability of the suggested procedure. Sample extraction involved the following steps: autoclaving in the presence of sulfuric acid, buffering to pH 4.5, digestion with Claradiastase, dilution, and filtration. PM was converted to PN by boiling the filtrate with sulfuric acid/nitrous acid solution and the resultant solution was neutralized and filtered. As PL is an intermediate product in the oxidation of PN to 4-pyridoxic acid by permanganate,

a separate procedure of PL oxidation was not carried out. The filtrate was applied to an open column containing Permutit-T ion exchange resin and the column bed was washed with distilled water to remove unwanted material. Both PN and PL were eluted in one step with warm sulfuric acid and the eluate was diluted with acid. The PN was oxidized to 4-pyridoxic acid by the addition of ice-cold potassium permanganate solution, and surplus permanganate was removed by the dropwise addition of 3% hydrogen peroxide. Lactonization was accomplished by the addition of hydrochloric acid and boiling for 12 min. After cooling, EDTA was added and the solution was diluted with ammonia solution and filtered. The fluorescence intensity of the 4-pyridoxic acid lactone produced was measured at 350 nm (excitation) and 430 nm (emission). Total vitamin B_6 was calculated on the basis of the difference in fluorescence of the sample and fluorescence of the sample with added known amount of B_6 vitamers (method of standard additions). To prepare a sample blank, a duplicate sample was taken through the procedure up to the oxidation of PN with permanganate, and the 4-pyridoxic acid thus formed was destroyed by incubating for 12 min in boiling water (without HCl). EDTA was then added to the cooled solution, and the solution was diluted with ammonia solution and filtered.

19.4.4 Vitamin C (AOAC Method)

A fluorometric method for determining microgram quantities of total vitamin C in pharmaceutical preparations, beverages, and special dietary foods has been described [27]. The method involves the oxidation of ascorbic acid to dehydroascorbic acid with active charcoal, followed by the reaction of dehydroascorbic acid with 1,2-phenylenediamine dihydrochloride (OPDA) to form the fluorescent quinoxaline derivative 3-(1,2-dihydroxyethyl)furol[3,4-*b*]quinoxaline-1-one (DFQ) (Figure 19.4). The blank reveals any fluorescence due to interfering substances, and is prepared by complexing the oxidized vitamin with boric acid to prevent the formation of the quinoxaline derivative.

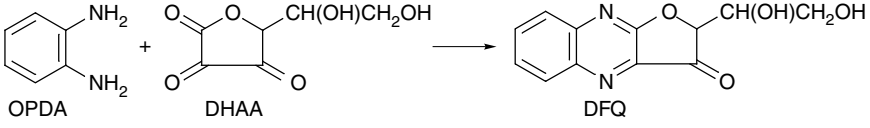

FIGURE 19.4
Reaction between dehydroascorbic acid and *o*-phenylenediamine to form the quinoxaline derivative (DFQ).

The fluorometric method for determining vitamin C in vitamin preparations was adopted as Final Action by the AOAC in 1968 [28]. The method shows a high degree of specificity. Deutsch and Weeks [27] ascertained that a substance will only interfere in the assay if all of the following conditions are satisfied: (i) the substance must have α-diketo groups, which react with OPDA under the assay conditions; (ii) the excitation and emission wavelengths of the quinoxaline derivative must be within the regions prescribed for the assay; (iii) it must contain adjacent *cis* hydroxyl groups, which react with the boric acid solution to form a complex. Additionally, the substance must be present in the sample assay solution in sufficient quantity to have an effect. Of a large number of possibly interfering substances tested [27,29], no individual compound was found which satisfied all of the above criteria. The procedure was therefore judged to be suitable for samples containing large amounts of reducing substances. An additional advantage is the method's ability to cope with highly colored materials.

The fluorometric method has been reported to have successful application to a wide range of foodstuffs, including liver, milk, fresh and canned fruit, raw and cooked vegetables, and potato powder [30]. However, Wills et al. [4] found that total vitamin C values for green leafy vegetables were higher when measured by the fluorometric assay than when measured by HPLC with UV detection, suggesting a pigment-related interference in the fluorescence measurement. Also, Augustin et al. [31] obtained unrealistically high vitamin C values with the fluorometric method in the analysis of processed potato products.

19.5 Enzymatic Methods for Nicotinic Acid and Ascorbic Acid

19.5.1 Nicotinic Acid

Hamano et al. [32] proposed an enzymatic method for the determination of nicotinic acid in meat products based on the stoichiometric consumption of oxygen that accompanies the hydroxylation of nicotinic acid. The conversion of nicotinic acid to 6-hydroxynicotinic acid is catalyzed by nicotinic acid hydroxylase in the presence of oxygen as a hydrogen acceptor. Samples of meat (5 g) were homogenized with 30 ml deionized water, the pH was adjusted to 7.2, and the volume was made up to 50 ml with water. The suspension was filtered through a Millipore filter and 100 μl of the filtrate was introduced into the reaction cell of a glucose analyzer containing 800 μl phosphate buffer (pH 7.2). After a 60 sec incubation at 30°C with stirring, 20 μl enzyme solution was injected to

start the reaction, and the rate of oxygen consumption was monitored by means of an oxygen electrode. Quantitative measurements were made from derivative signals, which corresponded to the maximum rate of oxygen depletion and were related to the amount of nicotinic acid present. None of the following compounds interfered with the determination when tested at their expected concentrations in commercial meat products: nicotinamide, L-tryptophan, L-phenylalanine, ascorbic acid, sorbic acid, and nitrite. Satisfactory recoveries were achieved for three spiked meat products, analyzed in triplicate. The limit of detection for a 5-g sample was 5 μg/g. There was close agreement ($r = 0.960$) between the results obtained by the enzymatic method and the HPLC method of Takatsuki et al. [33]. The run time per sample was 3 min compared to about 30 min with the HPLC method.

19.5.2 Ascorbic Acid

Tsumura et al. [34] developed a method of ascorbic acid determination which directly measures the change in absorbance of ascorbic acid during oxidation by guaiacol peroxidase. In contrast to ascorbate peroxidase, which is unstable and commercially unavailable in purified form, guaiacol peroxidase, extracted from horseradish, is stable and available at reasonable cost. In the procedure described, food samples were extracted with 2% metaphosphoric acid, then centrifuged and filtered. Into a quartz spectrophotometer cuvette were mixed an aliquot of the sample filtrate or ascorbic acid standard solution, *M*/30 phosphate buffer (pH 7.0) and guaiacol peroxidase in phosphate buffer containing 1.81 m*M* EDTA and 0.13 m*M* 2-mercaptoethanol. The initial absorbance at 265 nm was recorded with a spectrophotometer and the reaction was initiated by adding 50 m*M* hydrogen peroxide. Temperature was controlled at 37°C by circulating water around the cuvette. The decrease in absorbance at 265 nm due to oxidation of ascorbic acid to dehydroascorbic acid was recorded until absorbance reached the final value. The difference between initial and final absorbance corresponding to ascorbic acid was then calculated. A calibration graph was constructed in the ascorbic acid concentration range of 0.2–1.0 mg/100 ml using pH 7.0 phosphate buffer and 0.05 mg/ml peroxidase at 37°C. The plot of absorbance at 265 nm against ascorbic acid concentration gave a straight line passing through the origin. No interference was seen for any of 30 different food samples tested, including vegetables, fruits, ham, liver, and jam. Erythorbic acid also acts as a substrate for guaiacol peroxidase and, if present in a processed food, will interfere with the assay. The method was applicable to colored and sugar-rich samples and was more precise than officially adopted chemical methods.

19.6 Continuous-Flow Analysis

Continuous-flow analysis has been applied to the determination of certain vitamins using either segmented-flow or continuous-flow methods.

19.6.1 Segmented-Flow Methods

The Technicon AutoAnalyzer II modular system, which was introduced commercially in 1957 for use in clinical analysis, has been adapted to automate the chemical stages of the AOAC fluorometric methods for determining thiamin, riboflavin, and vitamin C, and the AOAC colorimetric method for determining niacin in foods [35,36]. Within this system, the chemical reactions take place in a continuously flowing stream segmented by air bubbles to limit sample dispersion and to promote mixing of sample with diluent or reagent by so-called bolus flow. The mixing is enhanced by arrangement of the tubing as a horizontally orientated helical coil. For some applications, a dialyser is incorporated into the system. A peristaltic proportioning pump moves sample (or standard) and reagents through the system to the photometric or fluorometric detector. The concentration of reaction product corresponding to a state of chemical equilibrium is recorded as a flat-topped profile, the height of which can be compared with that of a standard to give the analyte concentration [37]. Segmented-flow methods are usually operated at sampling rates of 30–60 per hour.

Extraction of the vitamins from the food matrix must be performed manually, and so the modified official methods can only be truly described as semi-automated procedures. Comparison of results obtained by semi-automated and corresponding manual procedures show overall good agreement. As well as reducing the labor requirement, the automation significantly improves the precision of reactions in which timing and reagent volumes are critical.

19.6.2 Flow-Injection Analysis

Flow-injection analysis is a more recent innovation, in which there is no air segmentation and it is not necessary for a state of chemical equilibrium to be reached. The sample is introduced into the carrier stream as a discrete plug, and the presence of a sample–carrier interface allows diffusion-controlled dispersion of the sample as it is swept through narrow-bore tubing to create a concentration gradient. The flow-through detector monitors the change in concentration of the reaction product,

which is displayed as a well-defined peak. Quantification of analyte concentration can be achieved by comparing the peak height of the sample with that of a standard. In practice, the area of the peak is measured by integration, since the peak width will not vary significantly. Flow-injection systems permit faster sampling rates and consume less reagent than comparable segmented-flow methods. The technique depends on precise sample injection, reproducible timing, and controlled sample dispersion. These features preclude the requirement for reaching the state of chemical equilibrium, since the residence time of the sample in the analytical system is constant. Optimization of a flow-injection system for a given application is achieved by empirical selection of manifold design (tubing bore size and length), sample and reagent volume, and low rate [38,39]. Osborne and Tyson [40] have reviewed the principles, instrumentation and techniques of flow-injection analysis, and considered the scope of its actual and potential applications in food and beverage analysis.

19.6.3 Applications to Food Analysis

19.6.3.1 Fat-Soluble Vitamins

Segmented-flow analysis has been used to automate fluorometric methods for determining vitamin A in milk, including the saponification step [41], and α-tocopherol in the unsaponifiable fraction of foods and feeds [42].

19.6.3.2 Thiamin

Segmented-flow analysis has been used to automate the oxidation reaction and subsequent steps of the AOAC fluorometric methods at a sampling rate of 30 per hour [43–46]. Partial separation of thiamin from extraneous substances was achieved by inline dialysis [35], and interferences from the remaining impurities were corrected by measuring the blank of the sample solution. Pelletier and Madére [47] showed that acid digestion carried out before enzymatic hydrolysis can result in low yields of thiamin from fish and certain nonprocessed meat such as beefsteak. Losses of thiamin incurred during the separation of insoluble matter from certain fruits was prevented by addition of ethylene glycol monoethyl ether [47]. Kirk [43] observed that the chromatographic purification step, normally used for sample cleanup, was not required for most samples. When high blank values interfered with the assay results, interference was eliminated by extracting the sample with water-saturated isobutanol before automated analysis. Only sample extracts containing chocolate were found to require the use of column

chromatography [44]. Pelletier and Madére [46] included the chromatographic purification step in the extraction procedure, although they observed that with certain foods it appeared to be unnecessary. Soliman [48] used neither column chromatography nor isobutanol washing to clean up sample extracts. Instead, extracts were analyzed before and after addition of benzenesulfonyl chloride, which inhibited thiochrome formation and provided a more representative blank based on the fluorescence of all the reactants except thiochrome.

19.6.3.3 Riboflavin

The AOAC fluorometric method has been semi-automated using segmented-flow analysis to perform inline dialysis and permanganate oxidative cleanup after manual acid digestion of food samples [49]. Excess permanganate was reduced with sodium bisulfite, and metaphosphoric acid was added as a manganese-sequestering agent to prevent the precipitation and build-up of manganese dioxide in the reagent lines. A collaborative study [50] led to the adoption of the method by the AOAC as Final Action in 1982 [51].

Russell and Vanderslice [52] applied flow-injection analysis to the standard AOAC fluorometric method [20] using sodium dithionite ($Na_2S_2O_4$) dissolved in 0.4% sodium acetate as the blank determination, as specified in the AOAC semi-automated method [51].

19.6.3.4 Thiamin and Riboflavin Simultaneously

Dunbar and Stevenson [53] reported the simultaneous determination of thiamin and riboflavin in infant formulas using segmented-flow analysis and a common extraction procedure involving both acid and enzymatic hydrolysis.

19.6.3.5 Niacin

The AOAC colorimetric method has been semi-automated using segmented-flow analysis with inline dialysis for the determination of nicotinic acid (representing total niacin) released from food samples by autoclaving with calcium hydroxide solution [54]. A reference flow cell was employed to eliminate blank color interference. The semi-automated procedure was shown to compare favorably with a microbiological assay for 63 different food products ($r = 0.9937$). The method was adopted as Final Action by the AOAC in 1976 for the determination of niacin in cereal products [55,56] and in 1982 for the determination of niacin in foods and feeds [57,58]. The inline generation of cyanogen chloride using the Technicon AutoAnalyzer II system has been investigated [59] and shown to represent a definite improvement in the safe handling of the reagents.

19.6.3.6 Vitamin C

Segmented-flow analysis has been utilized for modifying the AOAC fluorometric procedure for total vitamin C using DCPIP [60] or *N*-bromosuccinimide [48] as the oxidizing agent instead of activated charcoal. Roy et al. [61] reported that cocoa and chocolate, known to contain significant amounts of reductic acids, reductones, and alkaloids, required prior filtration through charcoal to remove interfering fluorescent compounds. On the basis of these observations, Egberg et al. [62] proposed a semi-automated method involving simultaneous oxidation and cleanup with charcoal prior to automated analysis. Dunmire et al. [63] compared the methods of Roy et al. [61] and Egberg et al. [62] and concluded that the latter method, with its potential for sample cleanup, was more appropriate for the majority of food commodities analyzed. Following a collaborative study [64], the AOAC in 1985 adopted the semi-automated microfluoromeric procedure as Final Action for the determination of total vitamin C in foods [65].

Lázaro et al. [66] proposed a flow-injection method for the simultaneous determination of ascorbate and sulfite in soft drinks based on the reaction with chloramine-T. The sample, dissolved in an acidic medium, is injected into the chloramine-T stream after merging with a starch-iodide stream. Iodide forms hydrogen iodide in an acidic medium, which subsequently reacts with chloramine-T to liberate iodine. The iodine oxidizes ascorbate and sulfite and, as soon as these reducing analytes have disappeared, the remaining iodine binds to starch to form the starch–iodine complex, whose absorbance is monitored at 581 nm. Maximum absorbance is obtained with blank sample because no iodide is consumed when the reducing analytes are absent. Thus the difference in absorbance between the blank and sample solutions is related to the concentration of analyte. The absorbance due to sulfite alone is determined by simultaneously injecting a sample previously mixed with NaOH to destroy the ascorbate. The ascorbic acid content can then be calculated by subtracting the result of the NaOH-treated sample from the untreated sample.

Ensafi et al. [67] developed a flow-injection kinetic spectrophotometric method based on the inhibitory effect of ascorbic acid on the oxidation of pyrogallol red by potassium iodate in acidic medium. Another flow-injection method was based on the oxidation of ascorbic acid by thallium(III) in the presence of potassium chloride and measurement of the fluorescence produced by thallium(I) [68]. The violet fluorescence was attributed to the $TlCl_3^{2-}$ anion complex. Both methods were applied to the analysis of fruit juices. Vanderslice and Higgs [69] combined a flow-injection system with a robotic extraction procedure to automate the determination of total vitamin C in fruits, juices, and vegetables from

after sample weighing to final quantification. The procedure was based on the AOAC microfluorometric method [28], except that mercury(II) chloride was used as the oxidizing agent.

Greenway and Ongomo [70] devised a flow-injection system for determining ascorbic acid in fruit and vegetable juices using a three-electrode amperometric detector and an inline column containing ascorbate oxidase (EC 1.10.3.3) immobilized on activated Sepharose 4B. On passage through the immobilized enzyme, a fraction of the ascorbic acid was converted into the electrochemically inactive dehydroascorbic acid, and the decrease in signal compared with that obtained using a blank (no enzyme) column was measured. The signal from the blank column represented total oxidizable material, while the signal from the enzyme column represented total oxidizable material less ascorbic acid. The difference between the two signals therefore gave the ascorbic acid content, with the enzyme reaction providing the selectivity. Glucose, oxalic acid, citric acid, and tartaric acid did not seriously interfere, even in great excess, while interference from copper was eliminated by addition of EDTA. The immobilized enzyme was used continuously each day for a period of 3 weeks without significant loss in activity.

In a flow-injection method for determining total vitamin C, Daily et al. [71] extracted food samples with 15 m*M* phosphate buffer (pH 5.0) containing 1 m*M* dithiothreitol, which reduced dehydroascorbic acid present in the sample to ascorbic acid whilst also stabilizing the ascorbic acid. Initially, an aliquot of each sample was taken and divided into two. The first aliquot was passed through an enzyme packed-bed (ascorbate oxidase immobilized on aminopropyl controlled-pore glass beads) that had been previously heat-denatured. An amperometric signal, proportional to the amount of ascorbic acid plus other electro-oxidizable species (interferents) present in the sample, was produced at the electrochemical detector. The second aliquot was then passed through a similar bed containing active enzyme, where ascorbic acid was selectively removed giving a second signal attributable only to the interfering species. The difference between the two signals generated at the detector electrode could be related to the concentration of ascorbic acid, representing total vitamin C. With the automated instrumentation, the method provided high sample throughput (15 samples per hour).

Marques et al. [72] immobilized ascorbate oxidase on alkylamine glass beads and used this biosensor in a flow injection system equipped with an oxygen electrode to measure the fall in oxygen concentration that accompanied ascorbic acid oxidation. The oxygen consumed by the enzyme reaction is proportional to the ascorbic acid content of the sample. This system was applied successfully to the determination of ascorbic acid in soft drinks and fruit juices. The immobilized enzyme retained its initial activity for 2 months with more than 600 assays.

19.7 Gas Chromatography

19.7.1 Principle

Gas chromatography (GC) is a technique for separating volatile substances by passing a stream of inert gas (mobile phase) over a film of nonvolatile organic liquid (stationary phase) contained within a heated column. The sample extract is introduced at the column inlet, and the volatile components are retained as a result of selective molecular interactions between the components and the stationary phase. Separation is effected as a consequence of the numerous sorption–desorption cycles that take place as the volatile sample material passes through the column. Detection of the separated components is carried out by continuous monitoring of the gaseous column effluent.

19.7.2 Column Technology

GC may be carried out using either packed-bed (packed) columns or capillary columns. In packed columns the stationary phase is coated onto microparticles of a porous support material (diatomaceous earth) contained in a glass column. The surfaces of untreated diatomaceous earth are covered with silanol (≡Si—OH) groups, which function as active sites in promoting severe peak tailing of polar compounds. The diatomaceous earth is therefore deactivated before coating with stationary phases of low or intermediate polarity by treatment with dimethyldichlorosilane (DMCS) or similar silylating reagent. Typical packed column dimensions are 2–4 mm internal diameter (ID) and 1.5 m up to 3 or 4 m length.

In modern capillary columns, the stationary phase is chemically bonded to the inner surface of a fused-silica capillary, leaving a lumen extending throughout the length of the column. Separation of sample components is governed solely by the rate of mass transfer in the stationary phase, and the absence of a support leads to an improved inertness. The standard capillary columns are of 0.25 or 0.32 mm ID and 10 m up to 50 m or even 100 m in length, with a film thickness of up to 1 μm (typically 0.25 μm). The column is coated on the outside with a protective layer of polyimide resin to impart mechanical strength and flexibility.

Capillary columns provide superior separation efficiencies compared with packed columns, and the sharper peaks facilitate a more accurate integration, as well as a greatly improved detectability. Another feature of capillary columns is a low stationary phase bleed, which is an advantage in temperature-programmed separations. On the other hand, packed

columns offer the advantages of high sample capacity, sample ruggedness, ease of operation, and low cost.

Capillary columns require special injection systems to cope with the limited sample capacity. Since 1983, wide-bore, thick-film capillary columns have become available which bridge the gap between capillary and packed column GC. The combination of increased ID and increased film thickness permits a significant increase in the sample capacity, allowing the use of a simple on-column injector (as used for packed columns) with its inherent ease of operation and quantitative assay. The column ID is most commonly 0.53 mm, and cross-linked nonpolar polysiloxane phases can be coated in thicknesses of 1–8 μm in 1 μm increments. The immobilized stationary phase is nonextractable, which allows the column to be flushed with pure solvents to remove contaminants, nonvolatiles and pyrolysis products. A 10-m length wide-bore capillary column approximates the sample capacity and separation efficiency of a 1.8×2 mm ID packed column with a 3–5% phase loading at the same operating conditions [73]. However, when the carrier gas flow rate is optimized for the capillary tubing, the wide-bore column produces far superior separations, with an increased speed of analysis. The low pressure drop across the column offers a very practical way to increase the efficiency several-fold, simply by increasing the column length.

19.7.3 Detectors

The detector monitors the gaseous column effluent and measures compositional variations attributable to eluted components. The output signal is produced in response to the mass or concentration of the eluted compound passing through the detector. The linear range refers to the range of solute concentrations over which the detector response is linear, from the minimum detectable level (two or three times the noise level) to the upper concentration, which produces a departure from linearity of about 5%.

Three types of GC detector employed for vitamin determinations are ionization detectors: they are the flame ionization detector, the nitrogen–phosphorus detector, and the electron capture detector. Interfacing a gas chromatograph with a mass spectrometer (GC–MS) provides information on the molecular weight and structure of the compound, in addition to its quantitative detection.

19.7.4 Derivatization Techniques

A prerequisite for a sample to be analyzed by GC is that the sample components are sufficiently volatile without decomposing under the

conditions of the separation. The required volatility and stability can be achieved either by making a suitable degradation product or preparing a chemical derivative by reaction with a suitable reagent. The most widely used derivatization technique is silylation, whereby a trimethylsilyl (TMS) group is introduced into a wide variety of organic compounds containing —OH, —COOH, —SH, —NH_2, and =NH groups. The replacement of active hydrogen by the silyl group reduces the polarity of the compound and decreases the possibility of hydrogen bonding, with the resultant increase in volatility. Furthermore, stability is enhanced by reduction in the number of reactive sites. The trifluoroacetate (TFA) derivative offers the advantage of compatibility with the highly sensitive electron capture detector [74].

19.7.5 Quantification

In vitamin assays by GC, quantification is usually performed by internal standardization, which compensates for analyte losses incurred during the sample work-up. A known amount of a suitable compound (internal standard) is added to the sample at the earliest possible point in the extraction stage. The quantification is based upon the comparison of the ratio of the internal standard peak size to analyte peak size in the sample with that ratio in a standard solution containing known amounts of the analyte and internal standard. Any loss of analyte will be accompanied by the loss of an equivalent amount of internal standard. As the calculation involves ratios of peak sizes, and not the absolute peak size, the injection volumes need not be precise.

19.7.6 Applications to Food Analysis

19.7.6.1 Vitamin E

During the 1960s, packed-column GC was widely applied to the determination of vitamin E in foods. Saponification of the sample was necessary, followed in most cases by further purification to remove interfering sterols. Capillary columns facilitate the separation of the TMS ethers of all eight vitamin E vitamers, and enable the tocopherols, tocotrienols, and major plant sterols in margarines [75] and vegetable oils [76] to be determined simultaneously.

A major problem encountered in the GC analysis of animal-derived foods is cholesterol, which accompanies vitamin E in the unsaponifiable fraction. Even using capillary columns, the TMS derivatives of cholesterol and α-tocopherol are poorly resolved from one another. However, this separation problem can be overcome by forming the heptafluorobutyryl esters [77].

19.7.6.2 Thiamin

Attempts to chromatograph TMS and other derivatives of thiamin have been unsuccessful because the derivatives are not sufficiently volatile at normal GC operating temperatures, whereas they pyrolyze above 250°C [78]. The ability of sulfite treatment to quantitatively split the thiamin molecule into substituted pyrimidine and thiazole moieties has been utilized in GC methods for determining thiamin in food samples [79–82]. The thiazole compound possesses the necessary attributes of being soluble in organic solvents and sufficiently volatile to permit its direct determination by GC. The general analytical procedure entails acid and enzyme hydrolysis of the sample, cleavage of thiamin by bisulfite, extraction of the thiazole compound with chloroform, and GC analysis. The flame ionization detector provides adequate sensitivity for the analysis of food samples containing relatively high concentrations of thiamin; the nitrogen–phosphorus detector is about 1000 times more sensitive and is more selective.

19.7.6.3 Niacin

No GC applications have been reported for determining naturally occurring niacin in foods. Nicotinamide, added to meats and meat products to stabilize the red color (this practice is not permitted in the U.K. and in certain other countries), has been determined as 3-cyanopyridine after dehydration by heptafluorobutyric anhydride [83]. Although nicotinamide can be chromatographed as such, 3-cyanopyridine was found to be over six times more sensitive towards GC.

Lin et al. [84] determined nicotinamide in vitamin drinks consumed in Taiwan without the need for sample pretreatment. Samples of drinks (1 ml) were mixed with 0.1 ml of internal standard solution (0.2% w/v 1,9-nonanediol in methanol) and 0.1 μl of the resultant solution was injected directly onto a wide-bore capillary column (30 m × 0.53 mm ID) coated with a mid-polar stationary phase (CP-SIL 8CB). The liner of the injector was packed with glass wool as a means of preventing nonvolatile compounds from reaching the column. The liner could be used for more than 100 injections before cleaning and replacement of the glass wool. The detection limit for nicotinamide was 2–5 μg/ml. Nicotinic acid was not detected due to its low volatility. Chromatograms of the nicotinamide in a tonic drink and amino acid drink are shown in Figure 19.5.

19.7.6.4 Vitamin B_6

TMS derivatives are not ideal for the GC analysis of vitamin B_6-fortified foods, as pyridoxine-TMS generally yields two peaks whose relative

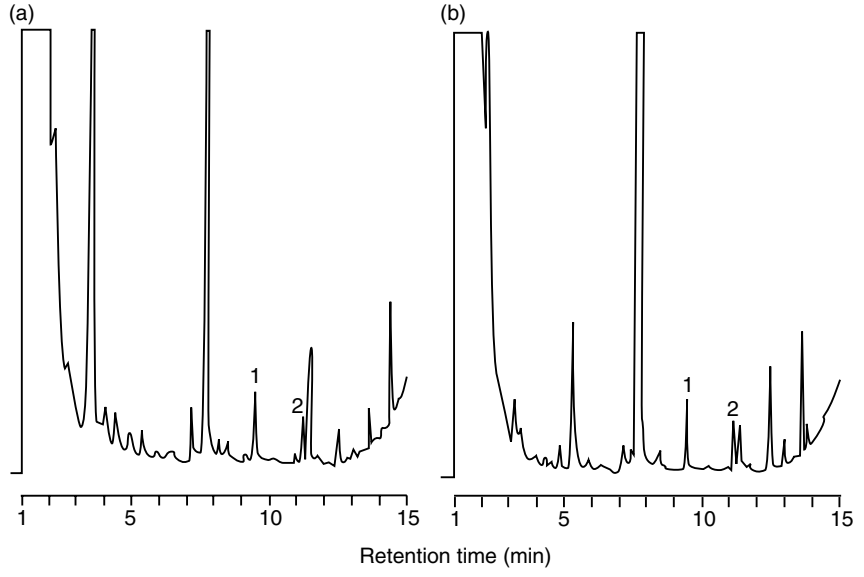

FIGURE 19.5
Gas chromatograms of the nicotinamide in (a) tonic drink, and (b) amino acid drink after direct injection of spiked sample. Peaks: (1) 1,9-nonanediol (internal standard); (2) nicotinamide. Fused-silica capillary column, 30 m × 0.53 mm ID; stationary phase, CP-SIL 8CB; film thickness 1.5 μm; column temperature, 110°C (1.5 min) → 8°C/min → 190°C (1 min) → 40°C/min → 290°C; FID. (From Lin, H.-J., Chen, C.-W., Hwang, B.-S., and Choong, Y.-M., *J. Food Drug Anal.*, 8, 113, 2000. With permission.)

areas are dependent on the silylation reaction conditions [85]. Of the numerous other derivatives that have been investigated [78], the TFA offers advantages of greater volatility, as well as compatibility with the electron capture detector. A suitable internal standard for chromatographing the TFA derivatives of vitamin B_6 vitamers is desoxypyridoxine [86]. The derivatizing agent employed is *N*-methyl-bis-trifluoroacetamide (MBTFA), which reacts with primary and secondary amines, and hydroxyl and thiol groups under mildly acidic conditions. Lim et al. [87] used this reagent to determine the TFA derivatives of pyridoxine, pyridoxamine, and pyridoxal in hydrolyzed extracts of enriched white bread, nonfat dry milk, and peas. After reaction of the extracts with MBTFA, absolute ethanol was added to convert pyridoxal-TFA to its hemiacetal in order to distinguish it from pyridoxine-TFA.

19.7.6.5 Pantothenic Acid

Salts of pantothenic acid have been analyzed by GC after conversion to volatile acetate, TFA or TMS derivatives [88,89], but such methods are

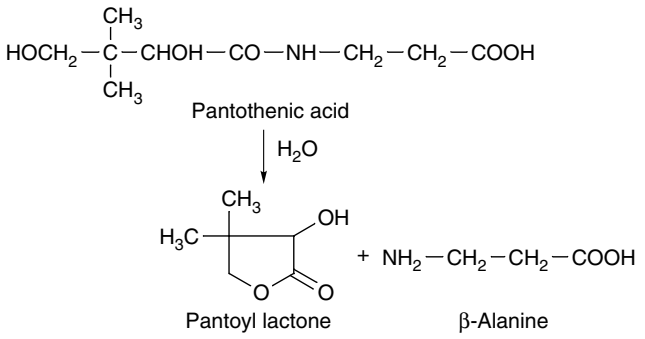

FIGURE 19.6
Hydrolysis of pantothenic acid.

only applicable to relatively pure samples such as pharmaceutical preparations. An alternative approach, which does not involve derivatization, is to chromatograph the pantoyl lactone formed from pantothenic acid by acid hydrolysis [90] (Figure 19.6). This approach is applicable to foodstuffs because the hydrolysis reaction liberates the lactone from the free and bound pantothenic acid in the food matrix with a recovery greater than 95% [78]. Davídek et al. [91] applied the technique to the analysis of fresh beef liver, spray-dried egg yolk, soybean flour, wholegrain wheat flour, and dried bakers' yeast. On comparison of the GC results with results obtained by the currently accepted microbiological method, no significant difference was found at the 95% probability level ($P > 0.05$), and the correlation coefficient was $r = 0.975$.

Rychlik [91a] developed a stable isotope dilution assay using GC–MS to determine total and free pantothenic acid in rice, skimmed milk powder, and apple juice. The internal standard, calcium [^{15}N,$^{13}C_3$]-(*R*) pantothenate, was synthesized by coupling labeled β-alanine to pantolactone. TMS derivatives of pantothenate were prepared by evaporating the sample extracts to dryness and heating the residues with pyridine and *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA). The method exhibited excellent recovery, repeatability, and a detection limit that was low enough to measure the vitamin even in poor food sources. Results obtained by the method confirmed the literature data of apple juice and milk powder, whereas lower contents were found in unpolished rice.

19.8 Supercritical Fluid Chromatography

19.8.1 Principle

If a gas is compressed, it will at some pressure liquefy. However, if the gas is held above its critical temperature, while being simultaneously

compressed to a pressure exceeding its critical pressure, it will not liquefy, but instead will become what is called a supercritical fluid. The physical properties of supercritical fluids are somewhere between liquids and gases. For supercritical carbon dioxide, the critical temperature is 31°C and the critical pressure is 73 atm. [92]. The solvent polarity of supercritical carbon dioxide is low and similar to that of hexane.

Supercritical fluid chromatography (SFC) combines some of the best properties of GC and HPLC, thus creating a complementary separation technique. SFC has greater resolving power than HPLC because the high diffusivities of supercritical fluids relative to liquids improves the analyte mass transfer between the mobile and stationary phase. The resolving power of SFC is not as good as that of capillary GC. However, whereas the carrier gas in GC has no effect upon selectivity, the mobile phase in SFC has the solvating power of a liquid, so the different solubilities of individual sample compounds provide selectivity. Another advantage of SFC over GC is the low operating temperature when supercritical CO_2 is used, which permits the analysis of thermolabile compounds. In summary, the supercritical fluid viscosity and diffusion properties allow fast efficient analytical separations to be performed (as in the case of GC) but the solvating ability provides selectivity (as in HPLC).

19.8.2 Instrumentation

A typical SFC system consists of three basic components: a high-pressure pump that delivers the mobile phase under supercritical pressures; a precision oven equipped with injector, column, and detector; and a microprocessor-based control system. The standard detector is the flame ionization detector (FID) — the universal detector most commonly used in GC — but selective GC detectors can also be installed. One of the major advantages of SFC over HPLC is that, interfacing with mass spectrometry is much more straightforward. The density of the supercritical fluid, and hence its solvating power, can be changed by programmed pressure ramping, enabling complex mixture to be separated at moderate temperatures.

SFC is often preceded by supercritical fluid extraction (SFE), which is discussed in Section 20.2.6. Ibáñez et al. [93] developed a system of inline SFE–SFC coupling using packed capillary columns. A schematic diagram of the system is shown in Figure 19.7. The extraction cell and the coupling interface device are placed inside the oven of the chromatograph equipped with a FID. One syringe pump only serves to deliver the mobile phase (SF-grade CO_2) either through the extraction cell in the SFE step or through the column in the SFC analysis. Switching of the CO_2 flow is performed with a six-way Rheodyne model 7000 valve positioned outside the oven. Depressurization of the SFE extract in the

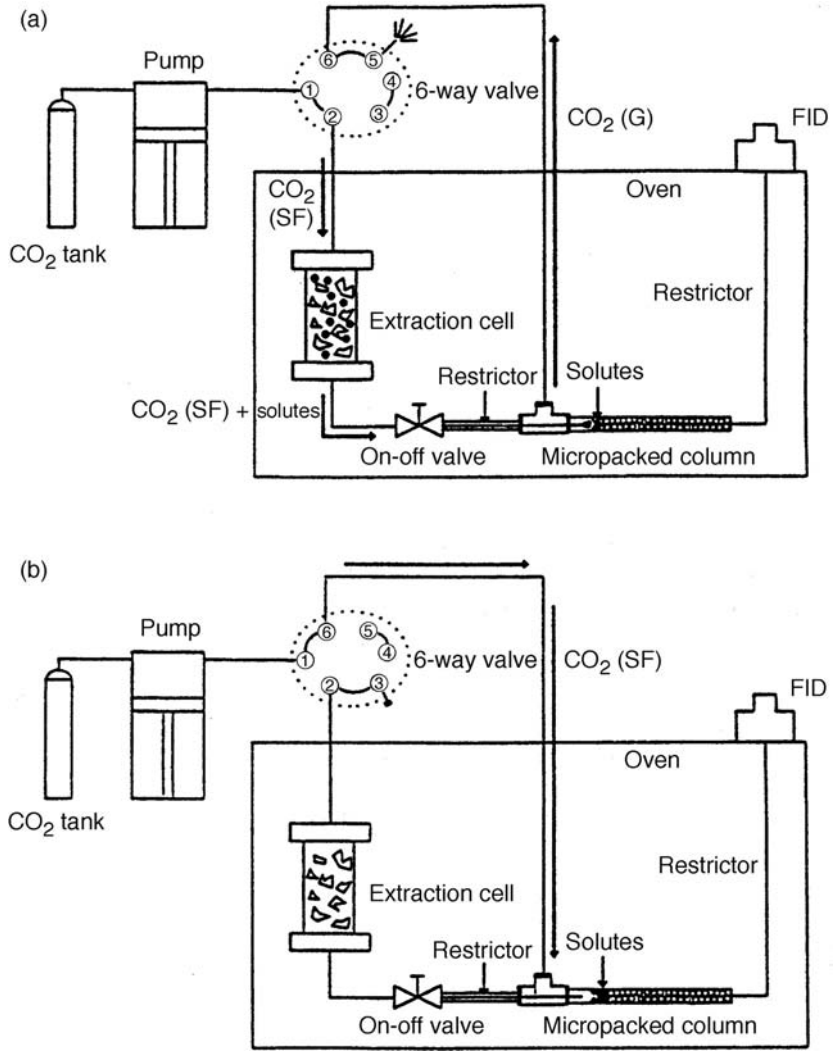

FIGURE 19.7
Schematic diagram of on-line SFE–SFC coupling using a packed capillary column: (a) extraction mode; (b) chromatography mode. In SFE mode, the CO_2 delivered by the pump goes through the extraction cell containing the sample. CO_2 gas is vented through port 5 of the valve. Once extraction is complete, the on–off valve is closed and the pressure and temperature conditions for the analysis are established. After reaching the initial conditions, the 6-way valve is switched to position 6 and the supercritical carbon dioxide operating as the mobile phase flows through the column for the SFC separation. The solutes, retained in the head of the column during the extraction step, are carried into the column and detected by the FID. (From Ibáñez, E., Herraiz, M., and Reglero, G., *J. High Res. Chromatogr.*, 18, 507, 1995. With permission.)

capillary column is achieved by means of a fused silica integral restrictor. The expansion of the supercritical carbon dioxide inside the column reduces the temperature in this zone and enables cryofocusing of the extracted compounds in the stationary phase.

19.8.3 Columns

SFC can be performed using open tubular capillary GC columns, packed HPLC columns of 4.6 mm ID, or SFC-dedicated packed capillary columns. Column efficiency in SFC is adversely affected by the pressure drop per unit column length. A large pressure drop causes significant differences in fluid densities along the column and hence substantial variations of solute retention, since mobile phase solvating power is directly proportional to density [94]. Open tubular capillary columns are constructed of highly deactivated fused silica with internal diameters of 100 μm or less, and are coated internally with an immobilized polysiloxane-based stationary phase. Being highly permeable, these columns exhibit relatively small pressure drops per unit length, allowing long columns of high overall efficiency to be used for the separation of complex mixtures. Open tubular capillary columns suffer from a low sample capacity, making them unsatisfactory for trace compound determinations. Packed 4.6-mm ID columns containing particles of 3–10 μm diameter have a greater sample capacity than open tubular capillary columns, and hence improved sensitivity. Because of a large pressure drop per unit length, only a short column (typically 250 mm length) can be used. This limits the resolution power but allows fast analysis times. Capillary columns packed with large (100–125 μm) particles give higher permeabilities and enhanced efficiencies compared to 4.6-mm ID packed columns, and have greater sample capacity than open tubular capillary columns.

HPLC columns containing silica particles with a bonded octadecyl (C_{18}) hydrocarbon as the stationary phase have been used in packed column SFC. Even after extensive chemical deactivation, remaining silanol groups on the silica surface cause serious peak tailing or even permanent adsorption of polar compounds when neat supercritical carbon dioxide is used as the mobile phase. Polar mobile phase modifiers are therefore used to deactivate the active sites by competitive adsorption.

Ibáñez et al. [95] tested packed capillary columns constructed of 180 μm ID fused silica for their ability to separate lycopene and β-carotene using neat supercritical carbon dioxide. The column packings were silica particles (10 μm) deactivated by means of a siloxane reagent, which, at high temperature, forms O—Si—CH_3 bonds. Silica particles were also coated with typical GC silicone stationary phases, namely SE-54 and

OV-17. Separation of the two carotenes was achieved using all three columns, but β-carotene showed bad peak tailing with the SE-54 column. The deactivated silica column gave the best separation, with no peak tailing.

19.8.4 Applications to Food Analysis

Most of the vitamin-related applications of SFC are dedicated to the determination of carotenes. Lesellier et al. [96] tested 22 different commercial HPLC columns packed with C_{18}-bonded stationary phases for their ability to separate *cis*/*trans* α- and β-carotenes using supercritical carbon dioxide and organic modifier as the mobile phase. The carotenes were detected by absorbance measurement at 450 nm. The use of a polyfunctional stationary phase was found to be preferable for this separation, although one monofunctional phase (Ultrabase UB 225) was also successful. The separations were obtained at a subcritical temperature of 22°C, at which the mobile phase is in the liquid state. However, since the chromatographically relevant physical properties (including compressibility) do not change abruptly at the critical temperature, it is usual to retain the term "SFC". A chromatogram of a carrot extract on a polyfunctional Spheri-5 ODS-5A column is shown in Figure 19.8. Total elution time was 16 min, whereas the separation required 45 min when the same column was used in nonaqueous reversed-phase HPLC [97].

19.9 Capillary Electrophoresis

19.9.1 Principle

A capillary electrophoresis system consists basically of a buffer-filled length of capillary tubing suspended between two reservoirs filled with buffer. The capillaries are made of fused silica that has been coated with an external layer of polyimide to make them flexible and less susceptible to fracture. They vary in length and typically have internal diameters of 50 or 75 μm. Samples are introduced at one end of the capillary and, under the influence of an applied electric field, migrate towards the other end at different rates according to their electrophoretic mobility. An inline detector monitors the UV absorbance of the eluted solutes and produces an electropherogram which is analogous to a chromatogram [98]. A photodiode array detector provides spectral information for each peak in the electropherogram, enabling peak purity and identification as well as quantitative measurement. The window for detection is made by removing a small section (0.5–1.0 cm) of the polyimide coating at the

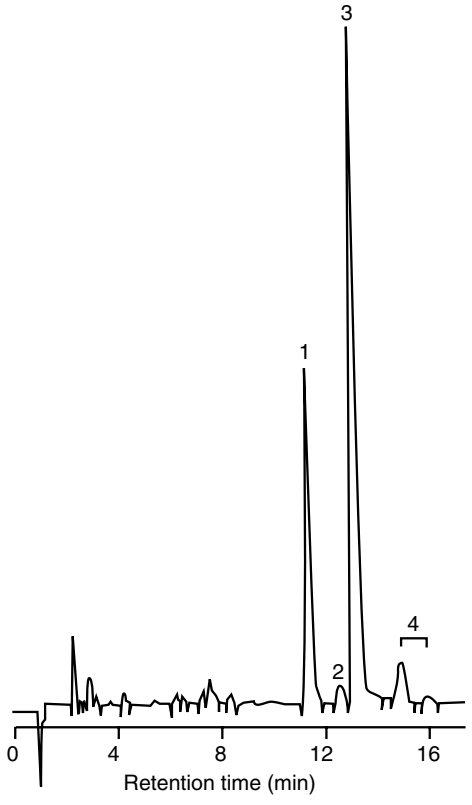

FIGURE 19.8
SFC of the carotenes present in a carrot extract. Peaks: (1) all-*trans*-α-carotene; (2) *cis* isomers of α-carotene; (3) all-*trans*-β-carotene; and (4) *cis*-isomers of β-carotene. The early-eluting peaks are probably oxygen-containing degradation products. Column: Spheri-5 ODS-5A (250 × 4.6 mm ID). Mobile phase: SFC-grade carbon dioxide/methanol (85 : 15, v/v). Temperature, 22°C; pressure 15 MPa; flow-rate, 3 ml/min; detection wavelength, 450 nm. (Reprinted from Lesellier, E., Marty, C., Berset, C., and Tchapla, A., *J. High Res. Chromatogr.*, 12, 447, 1989. With permission.)

distal end of the capillary. This optical window is mounted in an assembly where the UV light beam is focused radially by a spherical sapphire lens into the center of the capillary [99]. It is important to maintain the system at a constant temperature, because the flow of the buffer solution itself (electroosmotic flow) and the mobility of sample ions are dependent on the viscosity of the solution.

Two modes of capillary electrophoresis have found application in vitamin analysis. Capillary zone electrophoresis (CZE) is limited to the separation of charged solutes according to their mobility in free solution. Micellar electrokinetic capillary chromatography (MECC) facilitates the

separation of neutral solutes as well as charged solutes according to their partition coefficients between solution and micelle.

19.9.2 Capillary Zone Electrophoresis

The separation mechanisms that take place in CZE depend upon whether the internal surface of the fused silica column is coated or uncoated. When the capillary is uncoated, solute migration is dominated by the process of electroosmosis, which causes the flow of the buffer solution itself. Electroosmosis occurs because the uncoated silica surface, under normal aqueous conditions with small binary electrolytes, has an excess of anionic charge resulting from ionization of the silanol groups. Counterions to these anions are in the stagnant double layer adjacent to the capillary walls. This cationic nature extends into the diffuse layer, which is mobile. When an electric field is applied, the cationic counterions in the diffuse layer, accompanied by their spheres of hydration, migrate towards the cathode, causing a concomitant flow of solvent through the capillary [100]. Electroosmotic flow is much stronger than the electrophoretic mobility of an ion, therefore all solutes are carried towards the cathode, regardless of charge. Cations with the highest charge/mass ratio will be eluted first, followed by cations with reduced ratios. Neutral solutes move with the speed of the electroosmotic flow and follow the cations. Finally, the anions elute, those with the highest charge/mass ratio appearing last. An increase in pH causes more ionization of silanol groups and hence an increased electroosmotic flow rate. Other variables that affect flow rate are viscosity, ionic strength, the voltage, and the dielectric constant of the buffer [101].

When a coated fused-silica capillary is used, the electroosmotic flow is strongly reduced and the neutral solutes exhibit a negligible mobility under an electric field. In this case, the separation is only affected by the differences in solute charge [102].

Jegle [103] reported the separation of (in order of elution) thiamin, nicotinamide, pyridoxine, D(+) pantothenate, L-ascorbic acid, folic acid, orotic acid, and nicotinic acid within about 15 min, using an uncoated capillary of 48.5 cm total length $\times$ 50 μm ID, a citrate buffer of pH 7, and UV detection at 215 nm. Biotin could be determined in a pharmaceutical preparation, but not in natural samples, with the detector set at 200 nm [104].

19.9.3 Micellar Electrokinetic Capillary Chromatography

When an ionic surfactant is added to the buffer solution above a certain critical concentration, the surfactant monomers form spherical aggregates called micelles. The monomers are arranged with the charged groups

facing the surface of the micelle and the hydrophobic tails forming the interior. MECC is most commonly performed with anionic surfactants, especially sodium dodecyl sulfate (sodium lauryl sulfate), which forms micelles with a large net negative charge, giving them a high electrophoretic mobility towards the anode. However, as the electroosmotic flow of the aqueous phase is predominant, the micelles are transported towards the cathode, but at a slower rate than the bulk aqueous phase. The separation is based on the partitioning of the neutral solute molecules between the aqueous mobile phase and the hydrophobic interior of the slower moving micelle [100]. This separation mechanism is much the same as observed in traditional liquid–liquid partition chromatography, with the micellar phase functioning as a "pseudo-stationary" phase. MECC will separate ionic solutes as well as neutral solutes, the retention mechanisms being a combination of charge/mass ratios, hydrophobicity and charge interactions at the surface of the micelles [101]. The choices of anionic and cationic surfactants further increase the diversity of MECC.

Fujiwara et al. [105] reported the separation of thiamin, riboflavin phosphate, nicotinamide, nicotinic acid, pyridoxine, cyanocobalamin and L-ascorbic acid within 22 min by MECC using 0.05 *M* sodium dodecyl sulfate as the surfactant at a buffered pH of 9.0. Similar conditions were used in another study [106] to separate thiamin, riboflavin, riboflavin phosphate, nicotinic acid, nicotinamide, vitamin B_6 vitamers (pyridoxine, pyridoxal, pyridoxamine, pyridoxal phosphate, and pyridoxamine phosphate) and cyanocobalamin (vitamin B_{12}). The analysis of B_6 vitamers has been reported using MECC and laser-excited fluorescence [107] and electrochemical [108] detection.

19.9.4 Operational Aspects

The food matrix has profound effects on the quality of separation. For example, proteins can bind to the silica internal surface of the capillary and cause changes in solute migration. It may therefore be necessary to purify sample extracts before introduction onto the capillary. Solid-phase extraction (SPE) is a particularly useful purification technique, because it isolates the analyte from interfering material.

Quantitative precision depends largely on the reproducibility of sample introduction. This is most commonly performed using pressure-differential introduction. In this technique, the sampling end of the capillary is immersed in the sample solution contained in a vial. Application of positive or negative pressure causes a few nanoliters of sample solution to be either pushed or drawn into the capillary. The volume introduced is determined by the time and level of the pressure difference. An alternative technique is electrokinetic introduction where the electroosmotic

flow is allowed to transport a sample aliquot into the capillary. Solutes with high mobilities will be overrepresented in the introduced sample plug, but this is not a problem if there is only one analyte. If there is more than one analyte, internal standards can be used to compensate for the bias. Sample introduction times can range from 1 to 20 sec, whichever technique is used, but short (1–3 sec) times are not recommended if high precision is desired.

Simplicity, ease of operation, and robustness of the capillaries are key features of capillary electrophoresis. The electrophoretic peaks are sharper and better resolved than those obtained with HPLC. Despite these features, many food analysts are deterred from using capillary electrophoresis, because the short optical pathlength and extremely low sample introduction volumes (a few nanoliters) results in high concentration limits of detection compared with HPLC.

There are two techniques which the analyst can use to improve sensitivity. The first is SPE, which achieves trace enrichment of the sample. If the eluting solvent used in SPE is not compatible with the capillary electrophoresis buffer, the eluate can be evaporated to dryness and the residue dissolved in a small volume of the appropriate buffer. The second technique is to concentrate a large volume of the purified sample extract onto the capillary by sample stacking. This entails dissolving the final sample extract in a buffer whose concentration is about ten times less than that of the separation buffer, and introducing a long plug of this extract into the capillary. Because the buffer in the sample plug has a low concentration of ions, the resistivity in the sample plug region will be higher than the resistivity of the rest of the capillary. Consequently, a high electric field is set up in this region, causing ionized solute molecules to migrate very rapidly until they reach the interface between the sample buffer and the higher ionic strength separation buffer. Once the ions pass this interface, they immediately encounter a lower electric field and slow down. A narrow zone of concentrated solute ions is thus formed, resulting in enhanced detector signals for the separated sample components. Reducing the concentration of sample buffer to below the optimum will generate laminar flow, thus causing peak broadening [109].

Choi and Jo [110] compared three sample introduction methods to examine their effect on sample stacking using CZE with a 100 m*M* borate separation buffer. The methods were: (A) pressure injection for 5 sec of an ascorbic acid sample dissolved in 100 m*M* borate buffer; (B) pressure injection of a water plug for 3 sec in front of the pressure-injected sample dissolved in 5 m*M* borate buffer; and (C) the pressure injection of a water plug for 18 sec in front of the electrokinetic injection of sample for 18 sec at 20 kV in a 5 m*M* borate buffer. The peak area and height of the ascorbic acid peak obtained with (C) were about four times higher than those obtained with (A).

Altria et al. [111] discussed considerations necessary for the selection and preparation of separation buffers. Because the injection volume is related to the solvent viscosity, the same solvent must be used for both samples and standards. It is good practice to regenerate the capillary before and after the daily runs by sequential flushing with water for 3 min, 0.1 *M* NaOH for 3 min, and water for 5 min. The capillary should also be washed for 3 min with the separation buffer before each run.

19.9.5 Applications to Food Analysis

Capillary electrophoresis is routinely used in pharmaceutical laboratories for quantitative assay and purity testing. The availability of chiral selectors such as cyclodextrins makes the technique an important tool for separating enantiomers (mirror-image isomers). Chiral resolution of racemic (DL) pantothenic acid was performed using 2-hydroxypropyl-β-cyclodextrin [112]. With regard to food analysis, the technique has the potential to determine any water-soluble vitamin that can be determined by HPLC. The necessary criteria are that the vitamin is present at sufficient concentration in a food sample and that it possesses the necessary chromophore or fluorophore to permit its detection. Endogenous vitamins that meet these criteria are thiamin, riboflavin, niacin, vitamin B_6, folate, and vitamin C. Table 19.1 summarizes procedures for determining thiamin, flavins, niacin, and vitamin C in foods.

19.9.5.1 Thiamin

For the determination of thiamin in meat by MECC, 5-g samples were extracted by autoclaving in a medium of 0.3 *N* HCl at 121°C for 30 min, followed by enzymatic hydrolysis with Takadiastase. The extracts were treated with 90% ethanol to precipitate the proteins and then purified using a weak cation-exchange column [113]. Milk samples were treated in a similar manner, except that the protein precipitation was carried out with 90% ethanol followed by 1.2% trichloroacetic acid [114]. Electropherograms for thiamin in purified extracts of meat and milk are shown in Figure 19.9.

19.9.5.2 Riboflavin, FMN, and FAD

Cataldi et al. [115] determined riboflavin, FMN, and FAD in common foods by CZE with laser-induced fluorescence detection. Samples were shaken with methanol/dichloromethane (9:10), 0.1 *M* ammonium acetate at pH 6 was added, and the suspensions were shaken again. After centrifugation, the supernatants were diluted to volume with

TABLE 19.1

Capillary Electrophoresis Methods for Determining Water-Soluble Vitamins in Foods

Food	Sample Preparation	Capillary	Separation Buffer	Voltage (kV)	Compounds Separated	Detection	Ref.
Thiamin *Micellar electrokinetic capillary chromatography*							
Meat	*Extraction*: autoclave 5 g sample with 0.3 *N* HCl at 121°C for 30 min, cool. Adjust pH to 4.0–4.5, incubate at 45°C with Takadiastase for 3 h, filter, dilute to volume with water. *Cleanup*: add 90% ethanol to precipitate the proteins, hold in an ice bath for 30 min, centrifuge. Adjust pH of supernatant to 5.0–5.2, pass through Amberlite CG-50 weak cation-exchange column. Wash column with water, elute thiamin with 0.15 *N* HCl. Evaporate eluate to dryness under vacuum, dissolve residue in 2 ml water, filter (0.22 μm)	Fused-silica 70 cm effective length × 75 μm ID, $T = 50$°C	0.1 *M* sodium tetraborate, 0.05 *M* NaH_2PO_4 (pH 7.6), 0.05 *M* sodium dodecyl sulfate, 10% 2-propanol	15	Thiamin	UV 254 nm	[113]

Milk	*Extraction*: autoclave 10 g of milk powder or 100 ml of fluid milk with 0.3 *N* HCl (30 ml) at 121°C for 20 min, cool. Adjust pH to 5.0–5.2, incubate with Takadiastase at 45°C for 3 h, filter, dilute to volume with water. *Cleanup*: add 90% ethanol and then TCA to precipitate the proteins, centrifuge. Continue as in preceding entry from "Adjust pH of supernatant. . ."	As in preceding entry	As in preceding entry	15	Thiamin	UV 254 nm	[114]
Flavins (vitamin B_2) *Capillary zone electrophoresis*							
Vegetables, wheat flours, tomatoes, baker's yeasts	Shake sample with methanol/dichloromethane, 9 : 10. Add 0.1 *M* ammonium acetate, pH 6. Shake, centrifuge, dilute supernatant to volume with water, filter (0.22 μm)	Fused-silica, uncoated 84 cm effective length × 75 μm ID, $T = 15$°C	0.03 *M* phosphate buffer, pH 9.8	30	Riboflavin, FAD, FMN	Laser-induced fluorescence: ex 442 nm em > 515 nm	[115]

(*Table continued*)

TABLE 19.1 *Continued*

Food	Sample Preparation	Capillary	Separation Buffer	Voltage (kV)	Compounds Separated	Detection	Ref.
Niacin							
Raw and cooked meat and fish, cereal products, vegetables, fruits	*Extraction of meat and fish*: autoclave with 0.8 *M* H_2SO_4 at 121°C for 2 h. *Extraction of cereals, vegetables and fruits*: autoclave with saturated $Ca(OH)_2$ at 121°C for 2 h. After acid or alkali extraction, cool, dilute to volume with water, mix, centrifuge. Adjust an aliquot of the supernatant to pH 7, dilute to volume with water, centrifuge. *Cleanup*: solid-phase extraction using coupled C_{18} and strong cation exchange cartridges. Evaporate eluate to dryness, dissolve residue in water containing saccharin as internal standard	Fused-silica, uncoated 56 cm effective length × 50 μm ID, $T = 28$°C	0.02 *M* KH_2PO_4/ 0.02 *M* Na_2HPO_4, 1 : 1 (pH 7), 7.5% acetonitrile	25	Nicotinic acid (representing total niacin), saccharin (internal standard)	UV 254 nm	[116]
Micellar electrokinetic capillary chromatography							

Legumes (lentils, faba beans)	*Extraction of available niacin*: autoclave ground sample with dilute HCl at 121°C for 15 min, cool. Adjust pH to 4.0–4.5, incubate with Takadiastase at 48°C for 3 h. Cool, filter, dilute to volume with water. *Extraction of total niacin*: to 1.5 g ground sample add 80 ml water, 10 ml 5% $Ca(OH)_2$ suspension, and 1 drop *n*-octanol, to prevent foaming. Autoclave at 8.27 kPa of pressure for 2 h, cool, centrifuge, adjust pH of supernatant to 5.0–5.2, dilute to volume with water. *Cleanup*: pass aliquot of extract through Dowex 1-X8 anion-exchanger. Wash column with water, elute nicotinic acid with 0.15 *N* HCl. Repeatedly evaporate under vacuum and dissolve residue in water to completely remove residual HCl. Dissolve the final residue in 1 ml water and filter (0.22 μm)	Fused-silica 70 cm effective length × 75 μm ID, $T = 25$ or 30°C	0.02 *M* sodium tetraborate (pH 9.2), 0.015 *M* sodium dodecyl sulfate, 20% 2-propanol	30	Nicotinic acid (representing free or total niacin, depending on method of extraction)	UV 254 nm	[117]
Vitamin C *Capillary zone electrophoresis*							
Lemon and orange juices	Filter (0.2 μm), dilute with 0.1 *M* phosphate buffer, pH 5.0	Fused-silica, coated 20 cm × 25 μm ID	0.1 *M* phosphate, buffer, pH 5.0	8	AA	UV 265 nm	[118]

(*Table continued*)

TABLE 19.1 *Continued*

Food	Sample Preparation	Capillary	Separation Buffer	Voltage (kV)	Compounds Separated	Detection	Ref.
Fruit beverages	Add EA as internal standard, dilute with HPO_3 (100 g/l), vortex-mix, filter (0.45 μm) by centrifugation	Fused-silica, uncoated 30 cm effective length × 75 μm ID	0.1 *M* tricine buffer, pH 8.8	11	AA, EA (internal standard)	UV 254 nm	[119]
Orange juice	*For AA*: add 12.5% TCA, centrifuge, filter. *For total vitamin C*: adjust pH of filtrate to 7, add 0.8% homocysteine (allow 15 min reaction time), filter	Fused-silica, coated 40 cm × 100 μm ID	0.02 *M* phosphate buffer, pH 7.0	6	AA (representing total vitamin C after treatment of sample extract with homocysteine)	UV 254 nm	[102]
Citrus juices, fruit beverages	Add cysteine to water-diluted samples, filter (0.2 μm)	Fused-silica, uncoated 51 cm effective length × 50 μm ID, $T = 25°C$	20 m*M* phosphate buffer, pH 8.0	−30	AA	UV 266 nm (diode array)	[104]
Plant tissues	Pulverize in liquid nitrogen. Extract twice with 3% HPO_3/1 mM EDTA, centrifuge. Pass 2 ml of extract through C_{18} solid-phase extraction cartridge. Keep only the last 500 μl for analysis	Fused-silica, uncoated 50 cm effective length × 75 μm ID, $T = 25°C$	0.2 *M* borate buffer, pH 9	25	AA, EA	UV 260 nm	[120]
Candy, chocolate, biscuit, balanced nutrition food, vegetables, fruits, juices	Blend with 5% HPO_3 and L-cysteine, centrifuge, filter (0.45 μm)	Fused-silica, uncoated 27 cm total length × 57 μm ID, $T = 25°C$	0.1 *M* borate buffer, pH 8.0	15	AA	UV 245 and 265 nm	[110]

Vegetables	Homogenize chopped vegetables with 2% thiourea/ 10 m*M* HCl, add 10 m*M* HCl, allow to stand for 15 min, add water, centrifuge, filter (0.45 μm)	Fused-silica, uncoated 50 cm effective length × 50 μm ID, $T = 35°C$	0.02 *M* sodium tetraborate, pH 9.2	20	AA	UV 270 nm	[121]
Citrus juice	Dilute with water containing 1 g/l EDTA and 0.2% dithiothreitol, filter through cellulose acetate filter, add ferulic acid as internal standard	Fused-silica, uncoated 70 cm × 50 μm ID, $T = 23°C$	0.35 *M* sodium borate buffer (pH 9.3), 5% acetonitrile	21	AA, (representing total vitamin C), ferulic acid (internal standard)	UV 270 nm	[122]
Micellar electrokinetic capillary chromatography							
Fruits, vegetables	Blend with 3% HPO_3, filter, add 0.2% dithiothreitol containing EA (internal standard). Pass through C_{18} solid-phase extraction cartridge, discard the first 5 ml, filter remaining eluate (0.8 μm)	Fused-silica. *For fruits*: 40 cm effective length × 75 μm ID. *For vegetables*: 50 cm effective length × 75 μm ID, $T = 28°C$	0.05 *M* sodium deoxycholate (surfactant), 0.01 *M* sodium borate, 0.01 *M* KH_2PO_4, (pH 8.6)	25	AA (representing total vitamin C), EA (internal standard)	UV 254 nm	[123]
Beers, wines, fruit juices	Degas beers, add 0.2% dithiothreitol containing internal standard (EA for fruit juices; nicotinic acid for beers and wines), filter (0.45 μm)	Fused-silica 75 cm effective length × 75 μm ID, $T = 28°C$	As in preceding entry	25	AA (representing total vitamin C), EA or nicotinic acid (internal standards)	UV 254 nm	[124]

Note: AA, ascorbic acid; EA, erythorbic acid; HPO_3, metaphosphoric acid; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid.

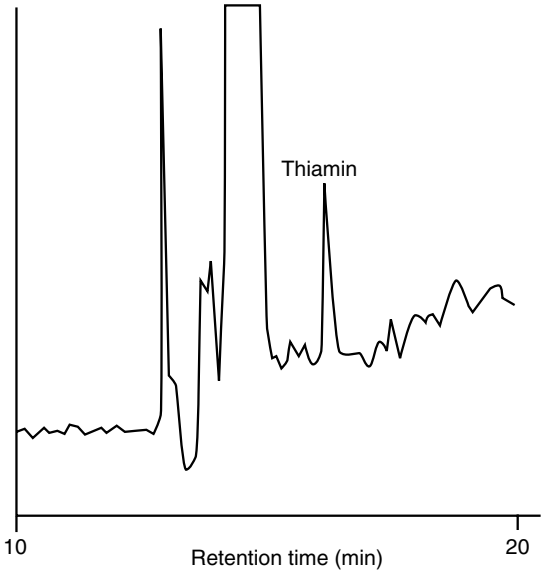

FIGURE 19.9
Electropherogram (MECC) of thiamin present in a meat extract. Capillary, 70 cm effective length × 75 μm ID. Separation buffer, 0.1 *M* sodium tetraborate, 0.05 *M* NaH_2PO_4 (pH 7.6), 0.05 *M* sodium dodecyl sulfate, 10% 2-propanol. Temperature, 50°C; voltage, 15 kV; detection wavelength, 254 nm. (From Vidal-Valverde, C. and Diaz-Pollán, C., *Eur. Food Res. Technol.*, 209, 355, 1999. With permission.)

water and filtered. Electrophoresis was performed using a 0.03 *M* phosphate buffer at pH 9.8. At this pH, all three flavins are ionized and the elution order of riboflavin, FAD, and FMN reflects the different charge/mass ratios. The fluorescence intensities of FAD and FMN were, respectively, only 36% and 44% of the intensity of riboflavin, therefore separate calibration curves were required. An electropherogram of flavins in Baker's yeast is shown in Figure 19.10.

19.9.5.3 Niacin

In a method for determining total niacin in selected foods, Windahl et al. [116] extracted raw and cooked meat and fish by autoclaving in the presence of 0.8 *M* sulfuric acid; cereal products, vegetables, and fruits were extracted by autoclaving with saturated aqueous calcium hydroxide. Neutralized extracts were centrifuged and a 20-ml aliquot of the supernatant was loaded onto a C_{18} SPE cartridge placed on top of a strong cation exchange (SCX) cartridge. Water was passed through the SPE assembly, the C_{18} cartridge was discarded, and the SCX cartridge was washed with 5 ml of methanol. Nicotinic acid was eluted from the SCX

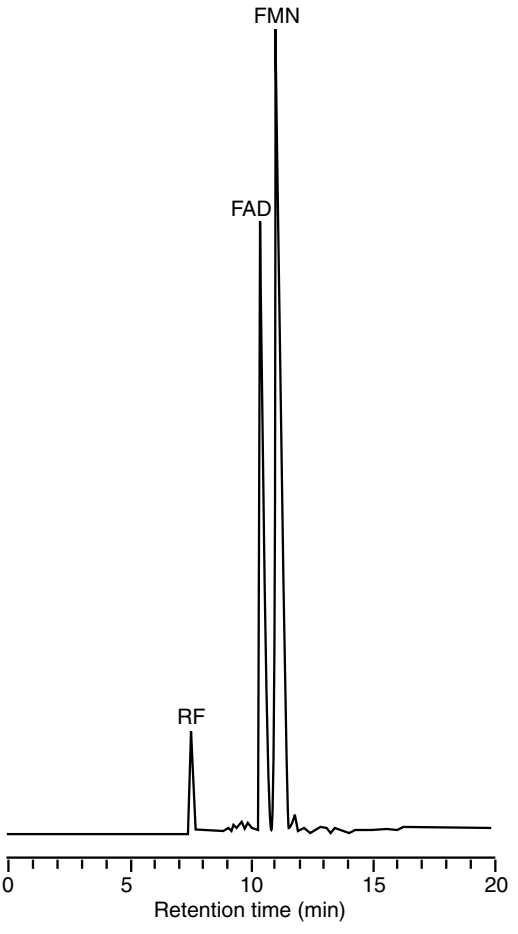

FIGURE 19.10
Electropherogram (CZE) of flavins present in an extract of baker's yeast. Capillary (uncoated), 84 cm effective length × 75 μm ID. Separation buffer, 0.03 *M* phosphate buffer (pH 9.8). Temperature, 15°C; voltage, 30 kV; detection, laser-induced fluorescence (excitation/emission wavelengths of 442/>515 nm). (Reprinted from Cataldi, T.R.I., Nardiello, D., Carrara, V., Cirello, R., and De Benedetto, G.E., *Food Chem.*, 82, 309, 2003. With permission.)

cartridge with 5 ml of freshly prepared 2% concentrated ammonium hydroxide in methanol. The eluate was evaporated to dryness under a stream of nitrogen at room temperature and the residue was dissolved in 1 ml of water containing 40 μg/ml of saccharin (internal standard). This final solution was analyzed by CZE. An electropherogram of nicotinic acid extracted from cooked chicken is shown in Figure 19.11. The SPE step achieved a tenfold concentration of the sample extract and overcame the problem of low sensitivity due to the small injection volume.

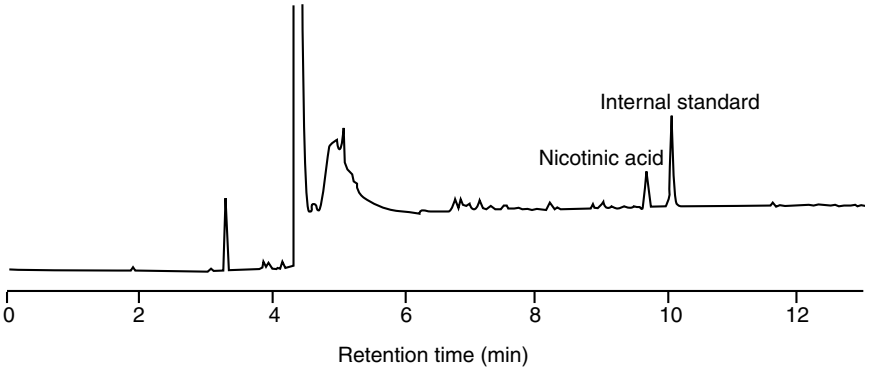

FIGURE 19.11
Electropherogram (CZE) of nicotinic acid (representing total niacin) in an extract of cooked chicken. Capillary (uncoated), 56 cm effective length × 50 μm ID. Separation buffer, 0.02 *M* KH_2PO_4/0.02 *M* Na_2HPO_4, 1:1 (pH 7), 7.5% acetonitrile. Temperature, 28°C; voltage, 25 kV; detection wavelength, 254 nm. (Reprinted from Windahl, K.L., Trenerry, V.C., and Ward, CM., *Food Chem.*, 65, 263, 1998. With permission.)

19.9.5.4 Vitamin C

Both CZE and MECC have been applied to the determination of ascorbic acid in foods and erythorbic acid (D-isoascorbic acid) can be used as an internal standard. The optimal conditions for the epimeric separation of ascorbic acid and erythorbic acid by CZE in a model system were as follows: separation buffer, 0.2 *M* borate buffer, pH 9.0; applied voltage, 25 kV, with an uncoated fused-silica capillary of 57 cm effective length × 75 μm ID maintained at 25°C ± 0.1°C [125].

Dehydroascorbic acid cannot be directly determined electrophoretically because its molar absorptivity is very weak. However, total vitamin C can be determined by first reducing the dehydroacorbic acid to ascorbic acid by treatment with homocysteine [102] or dithiothreitol [122,123].

Thompson and Trennery [123] reported a method for determining total vitamin C in fruits and vegetables by MECC. The vitamin was extracted from the foods by blending with 3% metaphosphoric acid, and the resultant slurry was filtered through a Whatmann No. 1 filter paper. A 5-ml aliquot of the filtrate was diluted to 10 ml with aqueous 0.2% dithiothreitol containing erythorbic acid as internal standard. The resultant solution was passed through a C_{18} SPE cartridge, which had been previously activated with methanol and water. The first 5 ml of effluent were discarded and the remaining effluent was syringe-filtered through a 0.8 μm cellulose acetate disc before introduction into the capillary. The method proved to be faster and more cost effective than

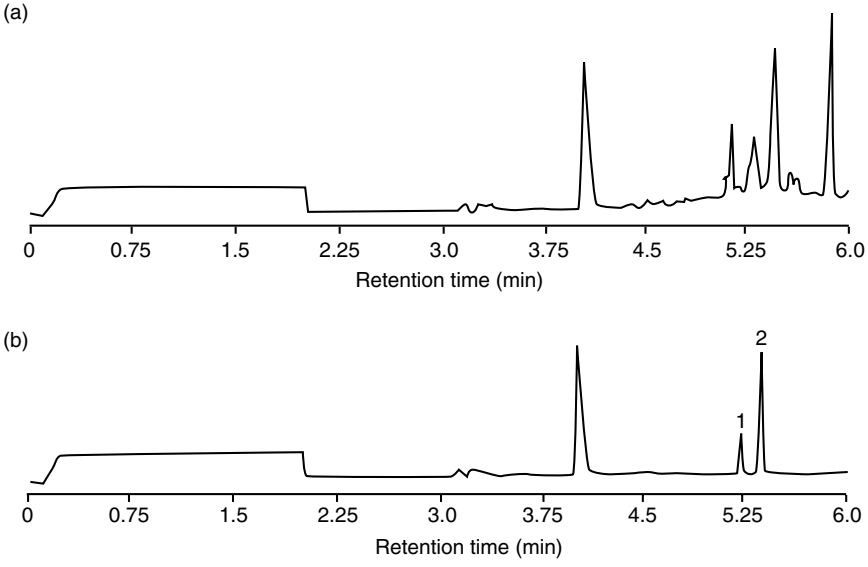

FIGURE 19.12
Electropherograms (MECC) of ascorbic acid from extract of blueberry (a) before and (b) after cleanup by C_{18} SPE. Peaks: (1) ascorbic acid; (2) erythorbic acid (internal standard). Capillary, 40 cm effective length × 75 μm ID. Separation buffer, 0.05 *M* sodium deoxycholate; 0.01 *M* sodium borate; 0.01 *M* KH_2PO_4 (pH 8.6). Temperature, 28°C; voltage, 25 kV; detection wavelength, 254 nm. (Reprinted from Thompson, C.O. and Trennery, V.C., *Food. Chem.*, 53, 43, 1995. With permission.)

the authors' current HPLC method. Separations of ascorbic acid and erythorbic acid and the effect of SPE are shown in Figure 19.12.

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20

Determination of the Fat-Soluble Vitamins by HPLC

20.1 Nature of the Sample

The lipid fraction of foods containing the fat-soluble vitamins is composed mainly of triglycerides, with much smaller amounts of sterols, carotenoids, phospholipids, and minor lipoidal constituents. All of these substances exhibit solubility properties similar to those of the fat-soluble vitamins, and therefore they constitute a potential source of interference. A proportion of the indigenous fat-soluble vitamin content of a food is bound up with a lipoprotein complex, and hence the fat—protein bonds must be broken to release the vitamin. The protective gelatine coating used in certain proprietary vitamin premixes will need to be dissolved before commencing the analysis of supplemented foods.

20.2 Extraction Procedures

It is essential for a successful assay that the vitamins be quantitatively extracted from the food matrix in a form that can be accurately measured by the particular high-performance liquid chromatography (HPLC) technique to be used. An effective extraction procedure serves to homogenize and concentrate the sample, isolate the vitamin analyte from its association with protein, eliminate as far as possible known interfering substances, and destroy any indigenous enzyme activity. Methods of extracting the fat-soluble vitamin from food matrices include alkaline hydrolysis, enzymatic hydrolysis, alcoholysis, direct solvent extraction, and supercritical fluid extraction of the total lipid component.

20.2.1 Alkaline Hydrolysis (Saponification)

Alkaline hydrolysis (saponification) effectively removes the preponderance of triglycerides from fatty food samples and is a practical way of extracting a relatively large amount of material. The hydrolysis reaction

affects ester linkages, releasing the fatty acids from glycerides and phospholipids, and also from esterified sterols and carotenol esters. The reaction also liberates indigenous vitamins from any combined form in which they may exist (e.g., lipoprotein complex) and breaks down chlorophylls into small, water-soluble fractions. In addition, it dissolves any gelatine that might have been present in the vitamin premix added to supplemented foods. Saponification can be used in assays for vitamins A, D, and E, but it is not expedient for vitamin K vitamers, which are rapidly decomposed in alkaline media.

Prepared samples of many types of food can be saponified directly. High-starch samples, such as breakfast cereals, may be digested with the enzyme Takadiastase before saponification to avoid the formation of lumps [1].

Saponification is conventionally carried out by refluxing the suitably prepared sample with a mixture of ethanol and 50% (w/v) aqueous potassium hydroxide (KOH) solution in the presence of pyrogallol or ascorbic acid as an antioxidant for 30 min. The amount of ethanolic KOH required for an efficient saponification is calculated on the basis that 3 moles of KOH are needed for each mole of fat (taken to be triglyceride) [2]. A slow stream of nitrogen is introduced into the saponification flask via a side-arm at the start and end of the process. A nitrogen flow is not necessary during the actual refluxing because a blanket of alcohol vapor prevents aerial oxidation during boiling. Rapid cooling after-saponification is important. The liberation of the unstable retinol and tocopherols from their relatively stable esters demands protective measures against light and oxygen during saponification and throughout the subsequent analytical procedure.

The sterols, carotenoids, fat-soluble vitamins, and so forth, which constitute the unsaponifiable fraction, are extractable from the saponification digest by liquid–liquid extraction using a water-immiscible organic solvent, after adding water to the digest to facilitate the separation of the aqueous and organic phases. Multiple extractions are necessary to ensure a quantitative transference of the vitamin analyte in accordance with partition theory. The combined solvent extracts are washed free of alkali with successive portions of water until the washings give no color on addition of phenolphthalein. The solvent extract is dried over anhydrous sodium sulfate and concentrated to ca. 1 ml on a rotary evaporator. The extract is quantitatively transferred to a glass tube and evaporated to dryness using a gentle stream of nitrogen. The residue is redissolved in a small volume of a suitable solvent for chromatographic analysis or further purification.

Vitamins A, D, and E, being slightly polar compounds, are extracted more efficiently from the saponification digest using a slightly polar solvent, such as petroleum ether/diethyl ether (1 + 1) than with a

nonpolar hydrocarbon solvent, such as petroleum ether or hexane. The washing of diethyl ether-containing extracts to remove the alkali is troublesome, owing to the solubility of soaps (potassium salts of fatty acids) in this solvent and the formation of stable emulsions when soaps, water, and hydrophobic solvents are shaken in the absence of ethanol. Therefore the washing step must be performed using a gentle swirling motion of the separatory funnel. The use of hexane is advantageous in that soaps are not extracted and the hexane extracts are nearly neutral. However, large amounts of soaps confer hydrophobic properties to the saponification digest, therefore, when hexane is used, the minimum number of extractions needed to achieve a quantitative recovery of the vitamins is affected by the amount of fat present in the original sample. It is also important, when using hexane or other hydrocarbon solvent, to maintain the optimum proportion of water and ethanol in the extraction system. For the efficient extraction of retinols [3] and tocopherols [4] using hexane, the ethanol strength must be below 40%.

Instead of refluxing for 30 min, saponification of homogeneous liquid samples can be scaled down and performed rapidly in a microwave oven. In a method for determining vitamins A and E in beverages [5], 1 ml of 50% aqueous KOH and 5 ml of an ethanolic solution of ascorbic acid are added to a 2-ml sample in a reaction tube, and the mixture is microwaved for 2 min. After saponification, the tube is removed from the microwave oven and rapidly cooled to room temperature. Acetic acid (1 ml), saturated sodium chloride solution (10 ml), and cyclohexane (20 ml) containing 500 mg/l butylated hydroxytoluene (BHT, antioxidant) are added, and the mixture is mechanically shaken for 10 min. The tube is then centrifuged and the supernatant organic layer is analyzed by HPLC. The addition of the salt solution and the choice of cyclohexane as an extraction solvent allow the extraction procedure to be performed in a single step. Neutralization of the digest helps to prevent the formation of stable emulsions.

20.2.1.1 Vitamin A

Retinol is stable in alkaline solution and has been reported to survive at least 1 week while steeping in ethanolic KOH containing pyrogallol [6].

Zahar and Smith [7] developed a rapid saponification method for the extraction of vitamin A from milk and other fluid dairy products, which avoids the need for multiple extractions and washings using separating funnels. Into a series of 50-ml stoppered centrifuge tubes is placed 2 ml of sample, 5 ml of absolute ethanol containing 1% (w/v) pyrogallol, and 2 ml of 50% (w/v) aqueous KOH. The tubes are stoppered, agitated carefully, and placed in a water bath at 80°C for 20 min with periodic agitation. After saponification, the tubes are cooled with

running water and then placed in an ice-water bath before adding 20 ml of diethyl ether/petroleum ether (1 + 1) containing 0.01% (w/v) BHT. The tubes are again stoppered and vortex-mixed vigorously for 1 min, allowed to stand for 2 min, and again vortexed for 1 min. To each tube is added 15 ml of ice-cold water, and the tubes are inverted at least ten times. After centrifugation, 10 ml of the upper organic layer is accurately pipetted into a tube, and the solvent is evaporated to dryness in a stream of nitrogen or under vacuum at 40°C using a rotary evaporator. The residue is dissolved in 1.0 ml of methanol (for milk samples) to provide a final solution for HPLC.

20.2.1.2 Carotenoids

Saponification causes a significant loss of xanthophylls, even when carried out under relatively mild conditions (ambient temperature for 3 h) [8]. In addition, several different saponification procedures have been shown to promote the formation of *cis* isomers of β-carotene [9]. Since saponification prolongs the analysis and is error-prone, it should only be carried out when needed, as in high-fat samples or those containing carotenol esters.

Kimura et al. [9] recommended a procedure in which the carotenoids are dissolved in petroleum ether, an equal volume of 10% methanolic KOH is added, and the mixture is left standing overnight (ca. 16 h) in the dark at room temperature. This treatment caused no loss or isomerization of β-carotene, while completely hydrolyzing β-cryptoxanthin ester. Losses of xanthophylls could be reduced to insignificant levels by using an atmosphere of nitrogen or antioxidant.

To reduce the time and costs of the saponification process, Granado et al. [10] proposed a "shortcut" protocol in which a 0.5-ml sample is placed into a disposable test tube followed by 0.5 ml ethanol containing 0.1 *M* pyrogallol and 0.5 ml of 40% KOH. The tube is nitrogen-flushed and the contents are vortex-mixed for 3 min to effect saponification. To the tube are added 1 ml water and 2 ml hexane/dichloromethane (5:1). The tube contents are vortex-mixed for 30 sec and then centrifuged. The organic phase is evaporated and the residue is dissolved in HPLC mobile phase. Compared to the standard protocol, the shortcut can save up to 90% of time and costs without noticeable loss of accuracy or precision.

20.2.1.3 Vitamin D

Saponification is obligatory for the determination of vitamin D in fatty foods because of the need to remove the vast excess of triglycerides present. Hot saponification results in the thermal isomerization of

vitamin D to previtamin D, and the consequent need to determine the potential vitamin D content. Thompson et al. [11] reported that saponification of milk at 83°C in the presence of pyrogallol results in a 10–20% loss of added vitamin D due to thermal isomerization. Several workers have avoided the problem of thermal isomerization by employing cold saponification (i.e., prolonged alkaline digestion at room temperature). Whatever the saponification temperature, it is necessary to perform the reaction in an inert atmosphere. Indyk and Woollard [12] avoided vitamin D losses of 10–20% by flushing the saponification vessel with oxygen-free nitrogen and then sealing the vessel before cold saponification.

A mixture of petroleum ether/diethyl ether (1 + 1) is suitable for extracting vitamin D from the unsaponifiable material and allows vitamins A and D to be coextracted. For the determination of vitamin D alone in fortified milks, margarine, and infant formulas, Thompson et al. [3] extracted the unsaponifiable matter three times with hexane in the presence of a 6:4 ratio of water to ethanol. The combined hexane layers were then washed with 55% aqueous ethanol, after the initial 5% aqueous KOH and water washes, to remove material, including 25-hydroxyvitamin D, that was more polar than vitamin D. This extraction process was based on partition studies that showed that insignificant amounts of vitamin D were extracted from hexane by aqueous ethanol when the ratio of ethanol to water was less than 6:4.

20.2.1.4 Vitamin E

Saponification at 70°C under nitrogen in the presence of pyrogallol for 45 min gave quantitative recoveries (96.2–105.4%) of all eight tocochromanols from a barley sample spiked with a standard mixture. Tocochromanol concentrations following hot and cold saponification of barley samples and analysis by HPLC were not significantly different, but standard deviations were higher when cold saponification was employed [13].

Saponification of meat is essential to release the α-tocopherol, which is incorporated into the cell membranes. Pfalzgraf et al. [14] reported a rapid saponification method using a single vessel for the extraction of α-tocopherol in pork tissues. Samples of homogenized tissue are weighed into amber 50-ml laboratory bottles, followed by the addition of ascorbic acid and methanolic KOH. The bottles are flushed with nitrogen, sealed, and heated at 80°C for 40 min, with occasional shaking. To the cooled digest is added 20 ml water/ethanol (4:1 for muscle or 1:1 for adipose tissue) and 10 ml hexane/toluene containing 0.01% BHT. The mixture is vigorously shaken for 10 min and centrifuged. A 20-μl aliquot of the upper layer is injected onto the HPLC column.

Indyk [15] extracted cholesterol, phytosterols, and tocopherols from dairy and nondairy foods using the following procedure. Into a series of 200 × 24-mm test tubes is placed 0.5 g of solid food or milk powder, 5.0 g of fluid milk, or 0.1–0.2 g of oil or fat. Ethanol (10.0 ml) is added to each sample, and the mixture is agitated to avoid agglomeration. Ethanolic KOH solution (2.0 ml of 50%, w/v) is added immediately, and the loosely stoppered tubes are incubated for 8 min at 70°C with periodic agitation. After cooling, 20.0 ml of hexane/diisopropyl ether (3 + 1) is added. The tubes are then stoppered securely and shaken mechanically for 5 min. Water (30 ml) is added and the tubes are re-stoppered, inverted ten times, and centrifuged (180 × *g*) for 10 min. The upper organic phase is retained for analysis by HPLC with no need for cleanup.

Saponification results in the hydrolysis of α-tocopheryl acetate (and other esters) to α-tocopherol. This can create a problem if the food sample under analysis is supplemented with *all-rac*-α-tocopheryl acetate, because the hydrolysis product, *all-rac*-α-tocopherol, has only 74% of the biological activity of naturally occurring *RRR*-α-tocopherol, and these two forms cannot be separated by the analytical HPLC techniques usually employed. If the food sample originally contained both naturally occurring *RRR*-α-tocopherol and supplemental *all-rac*-α-tocopheryl acetate, it is impossible to calculate a true potency value from the single total α-tocopherol peak in the HPLC chromatogram. This problem does not arise if the supplement used is *RRR*-α-tocopheryl acetate.

20.2.2 Alcoholysis

The lipid content of a food sample can be removed by converting the parent glycerides into their methyl esters by reaction with methanolic KOH solution under conditions that favor alcoholysis rather than saponification [16]. Alcoholysis depends upon the KOH and methanol reacting to form potassium methoxide, which, in turn, converts the glycerides into glyceride methyl esters and soaps. The reaction is completed within 2 min at ambient temperature; hence alcoholysis is a very rapid process compared with saponification. Alcoholysis is also a milder process than saponification and does not hydrolyze vitamin A esters; consequently, there is less potential for destruction of vitamin A. Alcoholysis has been used in the HPLC determination of vitamin A in fortified nonfat milk and vitamin D_3 in fortified whole milk [17].

20.2.3 Enzymatic Hydrolysis

Enzymatic hydrolysis is a nondestructive alternative to saponification for removing triglycerides in vitamin K determinations. For the simultaneous

determination of vitamins A, D, E, and K in milk- and soy-based infant formulas, and dairy products fortified with these vitamins [18], an amount of sample containing ca. 3.5–4.0 g of fat was digested for 1 h with lipase at 37°C and at pH 7.7. This treatment effectively hydrolyzed the glycerides, but only partially converted retinyl palmitate and α-tocopheryl acetate to their alcohol forms; vitamin D and phylloquinone were unaffected. The hydrolysate was made alkaline to precipitate the fatty acids as soaps and then diluted with ethanol and extracted with pentane. A final water wash yielded an organic phase containing primarily the fat-soluble vitamins and cholesterol.

Woollard et al. [19] found that removal of lipids from foods by lipase digestion, followed by single extraction into hexane, yielded a vitamin K fraction that was free from co-eluting contaminants when analyzed for vitamin K by HPLC. In their procedure, the following amounts of foods are weighed into 100-ml Schott bottles: milk powders, infant formula powders, and hard cheeses (1 g), retorted baby foods (5–10 g, depending on estimated fat content), yogurt (2.5 g), fluid milk, soymilk, health beverages (10 ml), vegetable oils and other high-fat foods (0.25 g), and meats and raw vegetables (minced, 5–10 g). Lipase (1.0–1.5 g, ca. 1000 U/mg from *Candida rugosa*) and 20 ml of 0.2 *M* phosphate buffer (pH 7.9–8.0) are added, and the suspensions are incubated at 37°C for 2 h with frequent shaking. Additional buffer is added to the digest, if necessary, to maintain the optimal pH range of 7.6–8.2. An alternative source of lipase (from porcine pancreas) is used for some hard cheeses. Addition of the proteolytic enzyme papain (ca. 200 mg, >30,000 USP U/mg from *Carica papaya*) aids the digestion of meat and animal-derived products. After incubation, the digests are cooled to ambient temperature. Ethanol (10 ml) and solid potassium carbonate (1.0 g) are added and the bottle contents are mixed by inversion. Hexane (30 ml) is then added and the bottles are shaken mechanically for 30 min. After phase separation, an aliquot of the hexane layer is evaporated under nitrogen, and the residue is redissolved in methanol for analysis by HPLC.

20.2.4 Direct Solvent Extraction

The fat-soluble vitamins can be extracted from the food matrix without chemical change using a solvent system that is capable of effectively penetrating the tissues and breaking lipoprotein bonds. A total lipid extraction is required for the simultaneous determination of vitamers or vitamins with a wide range of polarities and, for this purpose, a mixture of chloroform and methanol (2 + 1) is highly efficient [20]. The Röse-Gottlieb method is particularly suitable for extracting the total fat from milk

products and infant formulas. It entails treatment of the reconstituted milk samples with ammonia solution and alcohol in the cold and extraction with a diethyl ether/petroleum ether mixture. The alcohol precipitates the protein, which dissolves in the ammonia, allowing the fat to be extracted with the mixed ethers. The method is suitable for the extraction of vitamins A and D, but not for extracting vitamins E and K, which are labile under alkaline conditions.

A new technology called accelerated solvent extraction (ASE) has been developed and marketed as the ASE 200® (Dionex Corp., Sunnyvale, CA). Solid or semisolid samples are loaded onto the ASE system and the solvent is pumped into an extraction cell, which is then pressurized and heated for several minutes. After the extraction, the solvent containing the analyte is collected. Extraction under pressure allows solvents to be heated while maintaining their liquid state. The increased temperature allows extractions to be completed in a fraction of the time required for traditional extractions performed at room temperature or with warm solvent.

Some methods of selectively extracting the lipid fraction from various foods prior to the determination of the fat-soluble vitamins by HPLC are discussed below.

20.2.4.1 Vitamin A and Carotene

In fortified fluid milks, in which the vitamin A ester (palmitate or acetate) in the form of an oily premix is thoroughly dispersed in the bulk product, the total vitamin A content can be extracted directly with hexane. The hexane solution, after removal of the polar material, is then injected into the liquid chromatograph. Thompson et al. [21] developed a method in which sufficient absolute ethanol (5.0 ml) is added to a 2-ml sample of milk in a centrifuge tube so that the milk constituents are suspended in 71% aqueous ethanol; this solvent denatures the proteins and fractures the fat globules. The lipid fraction is then partitioned into hexane, and water is added to induce the aqueous and organic phases to separate. After centrifugation, the upper phase is a hexane solution of the milk lipids containing the vitamin A, and the lower phase is aqueous ethanol in which is dissolved salts, denatured proteins, and polar lipids. The interface contains a mixture of upper and lower phases plus insoluble protein. This extraction technique can, with slight modification, also be applied to the determination of vitamin A and carotene in margarine. It is not recommended for milk powders, because the added vitamin A may be contained within a gelatine matrix, and a quantitative extraction may not be achieved.

20.2.4.2 Carotenoids

When green leafy vegetables are undergoing analysis, the carotenoids are prone to photoisomerization by the sensitizing action of coextracted chlorophylls.

For the determination of carotenoids in fruits and nonleafy vegetables, which contain a large percentage of water, direct solvent extraction using a suitable water-miscible organic solvent is appropriate. Tetrahydrofuran has been found suitable, because it readily solubilizes carotenoids without causing isomerization, and it prevents the formation of emulsions by denaturing the associated proteins [8]. However, tetrahydrofuran is known to promote peroxide formation, so it must be stabilized with an antioxidant such as BHT. The extraction may be carried out in the presence of anhydrous sodium sulfate as a drying agent. The addition of magnesium carbonate to the extraction system serves to neutralize traces of organic acids that can cause destruction and structural transformation of carotenoids.

In an extraction procedure described by Khachik and Beecher [22], homogenized vegetables are blended with anhydrous sodium sulfate (200% of the weight of the test portion of vegetable), magnesium carbonate (10% of the weight of the test portion), and tetrahydrofuran. The extract is filtered under vacuum, and the solid materials are re-extracted with tetrahydrofuran until the resulting filtrate is colorless. Most of the solvent is removed on a rotary evaporator at 30°C, and the concentrated filtrate is partitioned between petroleum ether and water to remove the majority of contaminating nonterpenoid lipids. The water layer is washed with petroleum ether several times, and the resulting organic layers are combined, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue is taken up in a small volume of HPLC solvent for analysis.

Taungbodhitham et al. [23] evaluated an extraction method for the analysis of carotenoids in tomato juice, carrot, and spinach in which 2–5 g samples are extracted twice with 35 ml of ethanol/hexane mixture (4:3).

20.2.4.3 Vitamin D

In a simplified method for screening vitamin D levels in fortified skimmed milk, the milk sample was mixed with water, ethanol, and ammonium hydroxide and then extracted four times with diethyl ether/hexane. The dried residue obtained from the combined organic phase could be analyzed by HPLC without the need for purification [24].

20.2.4.4 Vitamin E

For the determination of vitamin E in seed oils by HPLC, the oils can simply be dissolved in hexane and analyzed directly. Solid-food samples demand a more rigorous method of solvent extraction. In a modified Röse-Gottlieb method to extract vitamin E from infant formulas [25], dipotassium oxalate solution (35%, w/v) was substituted for ammonia to avoid alkalizing the medium, and methyl *tert*-butyl ether was substituted for diethyl ether because of its stability against the formation of peroxides.

Sánchez-Pérez et al. [26] performed continuous extraction of vitamin E from vegetable oils using a silicone nonporous membrane coupled online with the liquid chromatographic system. The donor solution is obtained by dissolving the oil sample in a nonionic surfactant (Triton X-114) in the presence of methanol and hexane. As this solution passes along one side of the membrane, the acceptor solution (acetonitrile) is stopped and it extracts the vitamin E that has previously diffused across the membrane. After a preset period of enrichment, the acceptor solution is moved on via a diluter, and a given volume is introduced into the injection loop. Quantitation of α-, γ-, and δ-tocopherols is performed by reversed-phase HPLC using coulometric detection in the redox mode. A washing step is performed between each successive determination. A similar technique was used to extract vitamin E from seeds and nuts [27]. In this case, the donor solution was Triton X-114 in the presence of methanol and acetonitrile.

Katsanidis and Addis [28] tested several solvents for their ability to quantitatively extract vitamin E from muscle tissue. Methanol was unsuitable as it extracts and denatures proteins in muscle, causing foaming, and making volume reduction by rotary evaporation impossible. Extraction with methanol/chloroform (2:1) resulted in poor recovery (ca. 60%). The following adopted procedure gave ca. 96% recoveries for all tocopherols and tocotrienols; recovery of cholesterol was 94%. Place 2 g of muscle tissue (meat) into a 100-ml plastic tube, add 8 ml of absolute ethanol, and homogenize for 30 sec. Add 10 ml of distilled water and homogenize for 15 sec. Add 8 ml of hexane and homogenize for 15 sec. Cap the tubes and centrifuge at 1500 rpm for 10 min. Collect the upper (hexane) layer for analysis.

20.2.4.5 Vitamin K

For the analysis of infant formulas, Ayi and Burgher [29] modified the Röse-Gottlieb procedure by replacing the ammonia/ethanol treatment by acidified ethanol. Shearer [30] extracted phylloquinone from vegetables, fruits, cereals, meats, and fish by grinding in a mortar with fine quartz granules before extracting with acetone. After the addition of water and hexane to the acetone extract, the phylloquinone partitioned

entirely in the upper hexane phase, leaving polar impurities in the acetone/water phase.

Chase and Thompson [31] employed the ASE 200 accelerated solvent extraction system in conjunction with matrix solid-phase dispersion for the determination of phylloquinone in medical foods. In their procedure, the reconstituted sample is blended into C_{18}–isopropyl palmitate using a mortar and pestle, and the blended material is accurately transferred to an ASE extraction cell that contains a cellulose filter at the bottom. The cell is tapped gently on a hard surface to pack the material into the cell. The top cellulose filter is inserted and the material is compressed into the cell using the filter insertion tool. The void created is filled with cell fill material and the extraction cell cap is screwed into place. The extraction cell is placed into the ASE 200 cell rack with the C_{18}–matrix blend side of the cell oriented downward. The phylloquinone is extracted with ethyl acetate at 50°C and 1500 psi pressure with a programmed 5-min heatup time and a 5-min static extraction time. The eluate is collected in a 50-ml Turbo Vap vessel and evaporated at 45°C in a turboevaporator. The residue is dissolved in hexane, ready for analysis by HPLC.

20.2.5 Matrix Solid-Phase Dispersion

Matrix solid-phase dispersion (MSPD) was originally developed for isolating drug residues from tissues [32], but it can also be applied to foods of animal origin [33], and processed infant formulas [34]. By blending tissues with a lipophilic solid-phase packing material, one obtains a semi-dry material which can be placed in a column. Drugs can then be eluted from the column using selective solvents. Chase et al. [35] extracted retinyl acetate from soy-based infant formula using the following procedure. Into a glass mortar is placed 2 g of prewashed C_{18} packing material (bulk octadecylsilane-coated silica microparticles, 40 μm, end-capped, 18% carbon load). Isopropyl palmitate (100 μl) is added and gently blended onto the C_{18} phase with a glass pestle. An accurately weighed 5-g portion of reconstituted formula is added and the mortar contents are blended, using the pestle, to obtain a fluffy, slightly sticky powder. The blended material is quantitatively transferred to a purpose-made (Varian) 15-ml syringe barrel-column with a frit at the bottom. Another frit is placed on top of the powdery mix and the column contents are tightly compressed with a 10 cm^3 syringe plunger. The column is eluted with 7 ml of hexane containing 0.5% 2-propanol, followed by 7 ml of dichloromethane, collecting all 14 ml into a 50-ml Turbo Vap vessel. The combined eluates are evaporated at 45°C in a turbo-evaporator under 5 psi of nitrogen to near dryness. The residue is diluted to 1.0 ml with hexane ready for analysis by HPLC.

20.2.6 Supercritical Fluid Extraction

Supercritical fluid carbon dioxide is an excellent extraction medium for nonpolar compounds and is beginning to replace the use of organic solvents in analytical methods for determining fat-soluble vitamins in foods [36,37]. Analytical supercritical fluid extraction (SFE) using supercritical fluid carbon dioxide is a welcome technology in view of the environmental and health problems associated with the use of solvents, especially chlorinated ones.

20.2.6.1 Principle

Supercritical carbon dioxide is produced by holding gaseous CO_2 above its critical temperature (31°C) while simultaneously compressing it to a pressure exceeding its critical pressure (73 atm) [38]. Supercritical fluids have densities and solvating power similar to liquid organic solvents, but, like gases, have extremely high diffusivities and very low viscosities. These unique properties make supercritical fluids particularly suitable for extracting compounds from solid or semisolid food samples. Their low viscosity and absence of surface tension allow them to penetrate a matrix very rapidly, and their solvating power enables them to dissolve the solutes. The high diffusion coefficients of solutes in the supercritical fluid media permit rapid mass transfer out of the matrix. The solvating power of a supercritical fluid is directly related to its density, with density a function of pressure. Therefore, stepping up the pressure will increase the solvating power, and this provides the means by which the extraction can be optimized. Organic modifiers may be added to an extraction process for two reasons: (1) to increase the polarity of the supercritical carbon dioxide in order to improve the solubility of the analytes and (2) to facilitate desorption of analytes from the sample matrix.

20.2.6.2 Instrumentation

In a typical instrumental configuration, a high-pressure pump is used to deliver the supercritical fluid at a constant controllable pressure to the extraction vessel, which is placed in an oven or heating block to maintain the vessel at a temperature above the critical temperature of the supercritical fluid. During the extraction, the soluble compounds are partitioned from the bulk sample matrix into the supercritical fluid, then swept through a flow restrictor into a collection device that is at ambient pressure. The depressurized supercritical fluid (now a gas in the case of carbon dioxide) is vented, and the extracted compounds are retained. There are basically two different ways of collecting the analytes and the coextractives: into a solvent or onto a solid-phase trap. For collection into a solvent, it is important that the organic modifier is

compatible with the collection solvent to avoid formation of two-phase systems [36].

SFE may be performed in three ways. In the dynamic mode, the supercritical fluid is continuously flowing through the extraction vessel and the analyte is collected continuously. In the static mode, the extraction vessel is pressurized and the sample is extracted with no outflow of the supercritical fluid. After a set period of time, a valve is opened to allow the soluble compounds to be swept into the collection device. In the recirculating mode, the same supercritical fluid is pumped through the sample repeatedly then, after some time, it is pumped to the collection device. Of the three approaches, the dynamic method seems preferable because the supercritical fluid is constantly renewed during the extraction [39].

SFE can be employed either as an offline method, in which a "stand-alone" extraction instrument is used to collect the sample extract for subsequent analysis, or an online method, in which the extraction instrument is coupled directly to an analytical chromatographic instrument. Offline SFE is inherently simpler to perform and allows the extract to be analyzed repeatedly if required. Online SFE is usually coupled either with capillary gas chromatography or supercritical fluid chromatography (SFC); reports of online coupling with HPLC are rare. SFE–SFC offers prospects for the extraction and determination of the fat-soluble vitamins in food. The SFE–SFC coupling has been achieved by flowing the extract through a cold injector loop, by cryogenically trapping the extract on an adsorbent column for subsequent backflushing onto the SFC column, or by quantitatively transferring the extract directly onto the chromatographic column. The elimination of sample handling between extraction and chromatographic analysis reduces the possibility for loss and degradation of the analyte, thereby improving the precision and accuracy of the determination. There is also the potential to achieve maximum sensitivity by direct transfer of the extract onto the chromatographic column. A major drawback of online SFE is the danger of introducing bulk amounts of interfering co-extractants into the chromatographic system, which could exceed its analytical capacity and possibly ruin the SFC column.

20.2.6.3 Applications

Phylloquinone has been extracted from powdered infant formula using supercritical carbon dioxide at 8000 psi and 60°C for 15 min [40]. The extracted material was readily recovered by depressurization of the carbon dioxide across an adsorbent trap and then washed from the trap with a small volume of dichloromethane/acetone (1 + 1) to give a sample suitable for direct HPLC analysis. Trial experiments gave

recoveries of 92% of phylloquinone from a Chromosorb W matrix. A similar technique was applied to the extraction of retinyl palmitate from cereal products [41]. Berg et al. [2] showed that SFE is a suitable alternative to conventional solvent extraction for determination of vitamins A and E in meat and milk.

Marsili and Callahan [42] compared a supercritical carbon dioxide extraction procedure with an ethanol/pentane solvent extraction procedure for the HPLC determination of α- and β-carotene in vegetables. A combination of static and dynamic modes of extraction with ethanol modifier at 338 atm and 40°C was necessary to achieve optimum recovery with the SFE procedure. The extracted material was recovered by depressurization of the carbon dioxide across a solid-phase trap and rinsed from the trap into a 2-ml vial with HPLC-grade hexane. β-Carotene results obtained using the SFE procedure averaged 23% higher than results using the solvent extraction process.

Fratianni et al. [13] compared traditional methods and an SFE method for the extraction of tocochromanols from pearled barley. The experimental plan is summarized in Figure 20.1. The Folch extraction method [20] involves extraction with chloroform/methanol (2:1) and washing of

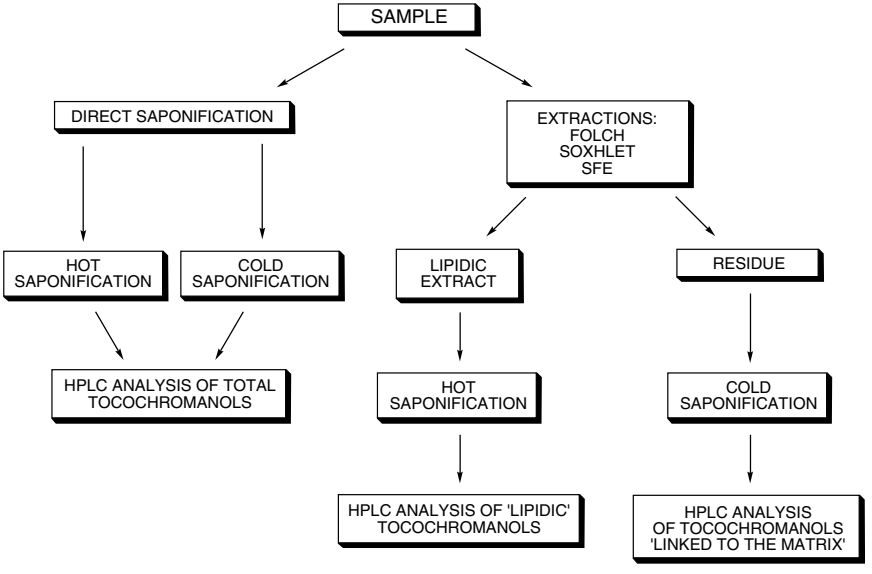

FIGURE 20.1
Experimental plan used for the extraction of tocochromanols from the barley sample. (From Fratianni, A., Caboni, M.F., Irano, M., and Panfili, G., *Eur. Food Res. Technol.*, 215, 353, Figure 1, © 2002 Springer-Verlag. With permission.)

the extract with salt solution, and the Soxhlet method involves continuous extraction with petroleum ether in a Soxhlet apparatus. In preliminary experiments, the barley sample was successively extracted with supercritical carbon dioxide at increasing pressure values of 200, 350, and 450 bar at a constant temperature of 40°C. As shown by HPLC analysis of the extracts, most of the tocochromanol (about 96% of total recovery) was extracted during the first step at 200 bar; ca. 4% was extracted at 350 bar, and a negligible amount was extracted at 450 bar. It was therefore decided to collect only the first two fractions at 200 and 350 bar for data analysis. A single extraction step was tested by using 350 bar as experimental pressure and by extending the dynamic extraction time, using the same conditions as used in the multistep SFE extraction. The lipid yield was lower than that obtained by the multistep SFE, confirming that several extraction steps, rather than a single step, are necessary to quantitatively recover lipids.

Table 20.1 shows the tocochromanols recovered using the SFE, the Soxhlet, and the Folch extraction procedures. The results are expressed as a percentage of those obtained using hot saponification — the method that gives the highest tocochromanol recoveries. Recoveries with the Soxhlet and the Folch procedures were higher than recoveries with SFE. The ratio of tocopherols to tocotrienols was almost the same in all the methods tested (0.3), proving that all extraction procedures, including saponification, showed the same selectivity toward the different tocochromanols. This comparison of extraction methods clearly shows that neither SFE nor the traditional methods are able to give complete recoveries of cereal tocochromanols with the normal-phase HPLC procedure used.

Table 20.2 shows the percentage of tocochromanol recoveries after saponification of the lipidic extracts and of the residues of SFE, Soxhlet,

TABLE 20.1

Tocochromanol Content of the Barley Sample using Different Extraction Conditions Expressed as a Percentage of the Results Obtained using Hot Saponification

Tocochromanols	Multistep SFE[a]	SFE 350 bar	Soxhlet	Folch
T	68	68	82	82
T3	75	71	81	86
T + T3	73	70	81	84
T/T3	0.3	0.3	0.3	0.3

T, sum of tocopherols; T3, sum of tocotrienols.

[a]The reported results are the sum of those separately obtained from 200 and 350 bar.

Source: Fratianni, A., Caboni, M.F., Irano, M., and Panfili, G., *Eur. Food Res. Technol.*, 215, 353, 2002. With permission.

TABLE 20.2

Percentage of Tocochromanol Recoveries after Saponification of the Lipidic Extracts and Residues of SFE, Soxhlet, and Folch Extractions

	Multistep SFE[a]			SFE 350 bar			Soxhlet			Folch		
Tocochromanols	Extracts (A)	Residue (B)	A + B	Extracts (C)	Residue (D)	C + D	Extracts (E)	Residue (F)	E + F	Extracts (G)	Residue (H)	G + H
T	82	9	91	79	9	88	88	6	94	94	3	97
T3	84	4	88	82	4	86	93	3	96	95	1	96
T + T3	83	4	87	82	5	87	92	4	96	94	1	95
T/T3	0.3	0.6	0.3	0.3	0.5	0.3	0.3	0.4	0.3	0.3	0.7	0.3

Capital letters refer to the percentages of the different compounds. T, sum of tocopherols; T3, sum of tocotrienols.
[a]The reported results are the sum of those separately obtained from 200 and 350 bar.
Source: Fratianni, A., Caboni, M.F., Irano, M., and Panfili, G., *Eur. Food Res. Technol.*, 215, 353, 2002. With permission.

and Folch extractions. In the lipidic extracts, an increase in the tocochromanol content was evident (compare with the data in Table 20.1) and attributed to the hydrolysis of tocochromanol esters. Tocochromanol amounts of 4–5% (SFE, Soxhlet) and 1% (Folch) were extracted from the residues after saponification. These amounts represent tocochromanols tightly bound to the cereal matrix. The tocopherol to tocotrienol ratios of the residues obtained from all extraction methods are higher than the consistent ratio (0.3) of the corresponding lipidic extracts, revealing a prevalence of tocopherols over tocotrienols. Table 20.2 shows that the Folch method has the highest extractive capacity, having higher recoveries in the lipidic extracts and lower amounts in the residue after saponification. In summary, SFE extracts free and esterified tocochromanols, but is unable to extract tocochromanols tightly bound to cereal matrix components.

Comparisons of analytical SFE with traditional methods for extracting fat-soluble vitamins (A, D, E, and β-carotene) in foods have also been reported by Perretti et al. [43]. The results obtained reveal limitations with the SFE method employed, and further studies are in progress to improve the efficiency of extraction.

20.3 Cleanup Procedures

The unsaponifiable fraction of whole milk constitutes 0.3–0.45% by weight of the total fat and is composed largely of cholesterol [44]. In vegetable oil types of margarine, the larger part of the unsaponifiable matter is composed of phytosterols, which are predominantly β-sitosterol, stigmasterol, and campesterol; only trace amounts of cholesterol are generally present [45]. Cleanup of the unsaponifiable matter is obligatory in HPLC methods for vitamin D in order to remove the excessive amounts of sterols and other interfering substances, including carotenoids and vitamin E vitamers. Sterols exhibit similar chromatographic properties to vitamin D and, if not removed, would alter the vitamin's retention time. Although sterols exhibit only very weak absorbance at the HPLC detection wavelength used for vitamin D, a vast excess will cause a detector response sufficient to constitute an interference.

Cleanup or fractionation procedures that have been used in the more recent fat-soluble vitamin assays include sterol precipitation, open-column chromatography, solid-phase extraction, and high-pressure gel permeation chromatography. HPLC has been used on a semipreparative scale in vitamins D and K assays to obtain purified fractions of sample extracts.

20.3.1 Precipitation of Sterols

The bulk of the sterols can be removed from the dried unsaponifiable matter by treatment of this material with ice-cold methanol/water (90:10) at $-20^{\circ}C$ [46] or with methanol [47], followed by removal of the precipitated sterols by membrane filtration. Alternatively, the dried unsaponifiable matter can be dissolved in digitonin solution, diluted with methanol, and stored at $-20^{\circ}C$ overnight to precipitate the sterols [44].

20.3.2 Open-Column Chromatography

The more recent applications of open-column chromatography in fat-soluble vitamin assays utilize liquid–solid (adsorption) chromatography using gravity-flow glass columns dry-packed with magnesia, alumina, or silica gel. Such columns enable separations directly comparable with those obtained by thin-layer chromatography to be carried out rapidly on a preparative scale.

20.3.2.1 Magnesia

A glass column packed with 3–4 g of a mixture of magnesia and diatomaceous earth (1 + 1, w/w) was employed to remove interfering pigments from the unsaponifiable fraction of vegetables or fruits prior to the determination of carotenes and monohydroxycarotenoids by HPLC [48]. The unsaponifiable residue was dissolved in hexane and applied in a total volume of 5 ml to the column. The carotenes and monohydroxycarotenoids were eluted with hexane/acetone (90 + 10 or 85 + 15) leaving residual chlorophylls and dihydroxy- and polyoxycarotenoids on the column. A magnesia column will also retain lycopene, which is a potential interference in tomato extracts [49].

20.3.2.2 Alumina

Alumina must be supplied in the neutral condition, that is, pretreated with acid to reduce its basic behavior [50] and partially deactivated to provide the necessary chromatographic resolution. The practical working range for alumina is 2–10% water-deactivated, that is, where the adsorbent is fully activated by driving out the water at $600^{\circ}C$ and then deactivated by shaking with 2–10% of its weight of water. Alumina-column chromatography has found application as a cleanup step in vitamin D assays, with the chief aim of removing cholesterol, phytosterols, and carotenes; vitamins A and E will also be removed, if present.

For the determination of vitamin D in fortified milk [51], the unsaponifiable residue was dissolved in 5 ml of hexane, and 0.1 or 0.2 ml of a tracer solution (chlorophyll-*a*) and 1 g of dry 8% water-deactivated alumina were added. The solvent was evaporated off, and the dried alumina containing the sample was poured on top of a prepared column packed with 15 g of alumina. Elution of the column was effected with chloroform using the visible chlorophyll-*a* band to locate the purified vitamin D fraction.

20.3.2.3 Silica Gel

The weak adsorption of phylloquinone on silica gel [52] (Table 20.3) provides the basis for silica purification of lipid extracts of milk and infant formulas in vitamin K assays. Haroon et al. [53] washed the hydrocarbons from a silica gel column with petroleum ether, after which the phylloquinone fraction was eluted with petroleum ether containing 3% diethyl ether; lipids that were more polar than phylloquinone were retained on the column.

20.3.3 Solid-Phase Extraction

20.3.3.1 General Considerations

Solid-phase extraction, a refinement of open-column chromatography, uses disposable prepacked cartridges to facilitate rapid cleanup of sample extracts prior to analysis by HPLC [54]. The full range of silica-based polar and nonpolar stationary phases encountered in HPLC column packings is commercially available, but only silica and C_{18}-bonded silica have so far found widespread application in fat-soluble vitamin assays. The average silica particle size is typically 40 μm (BondElut and Bakerbond) or 60 μm (SepPak) and allows easy elution

TABLE 20.3

Relative Retentions of Fat-Soluble Vitamins on Silica Gel

Weakly adsorbed	Anhydroretinol, retinyl esters, β-carotene, phylloquinone
↑	α-Tocopherol
	Retinoic acid and its isomers, 13-*cis*-retinaldehyde
	All-*trans*-retinaldehyde
	Vitamin D_2
↓	13-*cis*-Retinol, 9,13-di-*cis*-retinol
Strongly adsorbed	All-*trans*-retinol, 9-*cis*-retinol

Source: DeLuca, H.F., Zile, M.H., and Neville, P.F., in *Lipid Chromatographic Analysis*, Marinetti, G.V., Ed., Marcel Dekker, New York, 1969, p. 345. With permission.

under low pressure. The small mean pore diameter, which is typically 60 Å (1 Å $= 10^{-10}$ m), excludes proteins of molecular weight higher than 15,000–20,000, and the large surface area (typically 500–600 m^2/g) confers a high sample loading capacity. The successive conditioning, loading, washing, and elution of solid-phase extraction cartridges are carried out by a step change in solvent strength using the smallest possible volumes of solvent. The cartridges may be operated under positive pressure using a hand-held syringe or under negative pressure using a vacuum manifold. The latter technique is preferable because multiple samples can be processed simultaneously and the solvent flow rate can be precisely controlled with the aid of a vacuum gauge.

Purification of the sample extract can be effected in two ways, after first conditioning the sorbent with an appropriate solvent to solvate the functional groups of the stationary phase. In the sample cleanup mode, a stationary phase is selected that has a very high affinity for the analyte and little or no affinity for the matrix; therefore the sorbent retains the analyte, and unwanted material passes through. After loading the sample, the cartridge is washed with an appropriate solvent to remove further unwanted material, and the analyte is finally eluted with the minimum volume of a solvent that is just strong enough to displace it from the sorbent. This technique provides the opportunity for trace enrichment, in which a large volume of dilute sample is passed through the cartridge, and the enriched sample can be displaced with a small volume of solvent.

In the matrix removal mode, the sample extract is simply passed through the cartridge. Unwanted material will be retained, while the analyte will pass through the sorbent. This strategy is usually chosen when the analyte is present in high concentration.

20.3.3.2 Application in Vitamin D Determinations

Solid-phase extraction in the sample cleanup mode is proving to be an effective means for purifying the unsaponifiable fraction of food samples in HPLC methods for determining vitamin D. In one technique, the unsaponifiable residue is dissolved in a nonpolar solvent; the resultant solution is loaded onto a cartridge containing silica, a highly polar sorbent. The hydroxyl group on the vitamin D molecule bestows sufficient polarity to cause retention onto the silica surface. Nonpolar material in the sample solution has a greater affinity for the solvent and hence is discarded. The silica bed is then washed with a solvent that is sufficiently polar to remove further interfering material without displacing the vitamin D. The vitamin D is then eluted with a slightly more polar solvent, thus achieving isolation of the vitamin from its less polar coextractants. Bui [1] used this approach to remove the bulk of the sterols

from high-fat vitamin D-fortified milk products and diet foods prior to analysis by HPLC. After loading the unsaponifiable extract onto the cartridge, the silica bed was washed with hexane/ethyl acetate (85 + 15). The vitamin D was then eluted with hexane/ethyl acetate (80 + 20). The recovery of vitamin D_3 following solid-phase extraction was 98%.

Solid-phase extraction effectively separates vitamin D from its more polar 25-hydroxy metabolite. In the analysis of egg yolk [55], the unsaponifiable residue was dissolved in 10 ml hexane and loaded onto a preconditioned Mega Bond Elut silica cartridge. The sample was fractionated using the following elution sequence: 20 ml hexane (discard), 50 ml 0.5% 2-propanol in hexane (discard), 35 ml 0.5% 2-propanol in hexane (collect: vitamins D_2 and D_3), 50 ml 0.5% 2-propanol in hexane (discard), 40 ml 6% 2-propanol in hexane (collect: 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3).

In an HPLC method for determining vitamin D in fully vitaminized infant formulas, Indyk and Woollard [56] loaded the sample unsaponifiable extract onto a silica cartridge and washed the sorbent with 60 ml (or 65–75 ml) of hexane/chloroform (21.5 + 78.5) to remove the carotenoids and vitamin E. The carotenoids appeared in the first 10 ml of eluate; α-tocopherol appeared in the first 30 ml of eluate, and γ-tocopherol and some of the tocotrienols appeared in the following 30 ml. The vitamin D was then eluted with 10 ml of methanol, along with the retinols, sterols, xanthophylls, δ-tocopherol, and other unidentifiable polar excipients.

For the determination of vitamin D in fortified skimmed milk powder, Reynolds and Judd [57] dissolved the unsaponifiable residue in 2 ml of ethanol, added 1 ml of water, and applied this solution to a C_{18} reversed-phase cartridge. The cartridge was flushed with 15 ml of methanol/THF/water (1 + 1 + 2) to remove material that was more polar than vitamin D. The vitamin D was eluted with 5 ml of methanol, leaving the nonpolar material retained on the sorbent.

20.4 HPLC Systems

20.4.1 Principle

In HPLC, a small volume (typically 10–100 μl) of the suitably prepared sample extract is applied to a column packed with a microparticulate material, whose surfaces constitute the stationary phase, and the sample components are eluted under high pressure with a liquid mobile phase. Detection of the separated components is achieved by continuously monitoring the UV absorption of the column effluent.

Fluorometric and electrochemical detection provide improved selectivity and sensitivity for certain vitamins. HPLC allows hundreds of individual separations to be carried out on a given column with high speed, efficiency, and reproducibility.

20.4.2 Explanations of Chromatographic Terms

The goal of all chromatographic separations is the resolution of peaks within the shortest analysis time. Factors controlling resolution are the retention factor, k (formerly called the capacity factor, k') and the separation factor, α (formerly called selectivity). The number of theoretical plates (N) in a column is a measure of column efficiency, relating band broadening and retention volume.

20.4.2.1 Retention

The retention factor, k, is a measure of a solute's retention on a chromatographic column, corrected for the void volume. It is defined as the ratio of the quantity of solute in the stationary phase to the quantity in the mobile phase at equilibrium. The term k is dimensionless and can be calculated from measurements obtained from the chromatogram (Figure 20.2).

$$k = \frac{t_r - t_0}{t_0} \tag{20.1}$$

where t_0 is the void volume, and refers to the time required for an unretained solute to reach the detector from the point of injection; t_r is the retention time of the retained solute. Retention may be measured in units of time or chart distance, given a constant mobile phase flow rate.

A permeating but nonsorbed solute has a k value of zero; the k value increases by 1 for each column volume needed to elute the solute. A k value of 8–10 means that the solute takes a long time to elute. For rapid analysis a low k value is desired, whereas for complex separations a high k is needed. The compromise is a k value of 2–6.

20.4.2.2 Separation

The separation factor, α, is a measure of how well two adjacent solute peaks (peaks 1 and 2) are separated. It is defined as the relative retention of the two solutes by the stationary phase:

$$\alpha = \frac{k_2}{k_1} \tag{20.2}$$

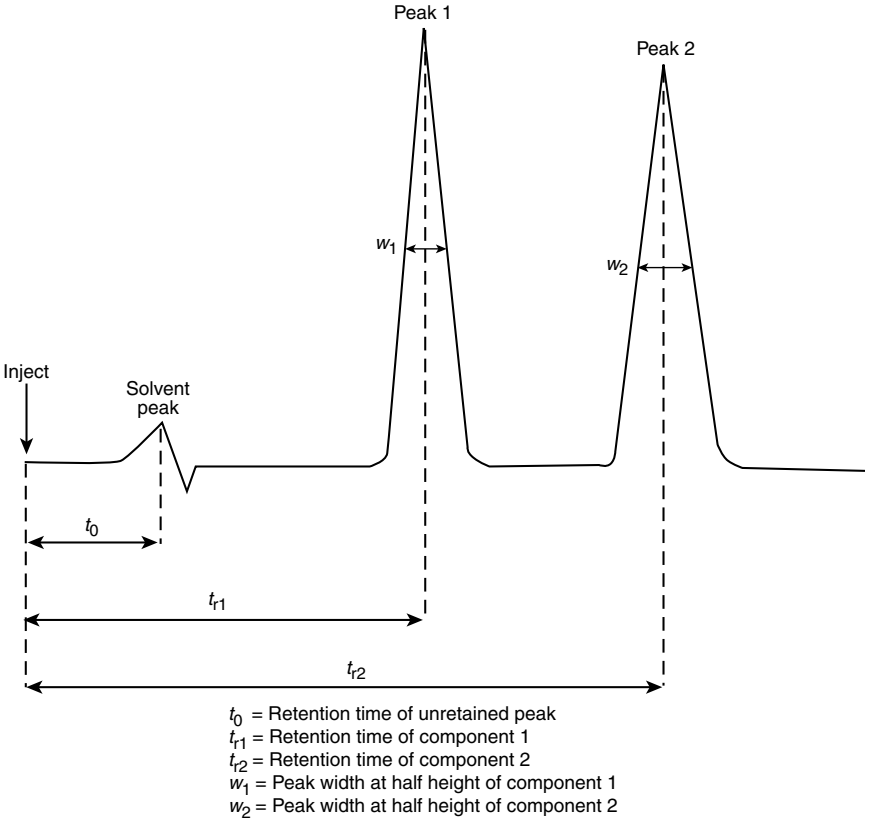

FIGURE 20.2
Model of an HPLC chromatogram.

The higher the value of α, the greater is the separation between two solutes; if α is 1.0 the separation is zero.

20.4.2.3 Resolution

The actual separation of two peaks is not adequately described by α alone, as it does not contain any information about peak widths. The resolution, R_s, between two adjacent peaks is calculated as

$$R_s = 1.18\left(\frac{t_{r2} - t_{r1}}{w_1 + w_2}\right) \tag{20.3}$$

where w_1 and w_2 are widths at half-height for peaks 1 and 2, respectively. When two peaks are baseline resolved, $R_s \geq 1.5$.

20.4.2.4 Efficiency

The efficiency of a column is expressed as the number of theoretical plates, N:

$$N = 5.54\left(\frac{t_r}{w}\right)^2 \tag{20.4}$$

where w is the peak width at half-height.

20.4.3 The Column

The majority of published HPLC techniques used in fat-soluble vitamin assays have utilized 5- or 10-μm particles of porous silica or derivatized silica packed into stainless steel tubes of typical length of 250 mm and standard internal diameter (ID) of 4.6 mm. Radially compressed cartridge-type columns (Waters Chromatography Division) manufactured from heavy-wall polyethylene of dimensions 10 cm × 8 mm have also found application. The insertion of a short guard column between the injector and the analytical column protects the latter against the loss of efficiency caused by strongly retained sample components and from pump or valve wear particles. The column-packing material is held in the column by fine-porosity frits of stainless steel or some other material. Membrane filtration of all test extracts is important for the removal of particulate material or macromolecules that might otherwise enter the guard or analytical column. Rabel [58] discussed the care and maintenance of HPLC columns.

Narrow-bore columns of between 1.0 and 2.5 mm ID are available for use in specially designed liquid chromatographs having an extremely low extracolumn dispersion. For a concentration-sensitive detector such as the absorbance detector, the signal is proportional to the instantaneous concentration of the analytes in the flow cell. Peaks elute from narrow-bore columns in much smaller volumes compared to those from standard-bore columns. Consequently, because of the higher analyte concentrations in the flow cell, the use of narrow-bore columns enhances detector sensitivity. The minimum detectable mass is directly proportional to the square of the column radius [59]; therefore, in theory, a 2.1-mm-ID column will provide a mass sensitivity about five times greater than that of a 4.6-mm-ID column of the same length.

The enhanced detectability obtained using a 2.0-mm-ID column with respect to a 4.0-mm-ID column of the same length (250 mm) is illustrated in Figure 20.3, which compares the HPLC-UV chromatograms of a standard solution of fat-soluble vitamins. The narrow-bore column was used with a 2-mm^3 volume flow cell and the standard-bore column

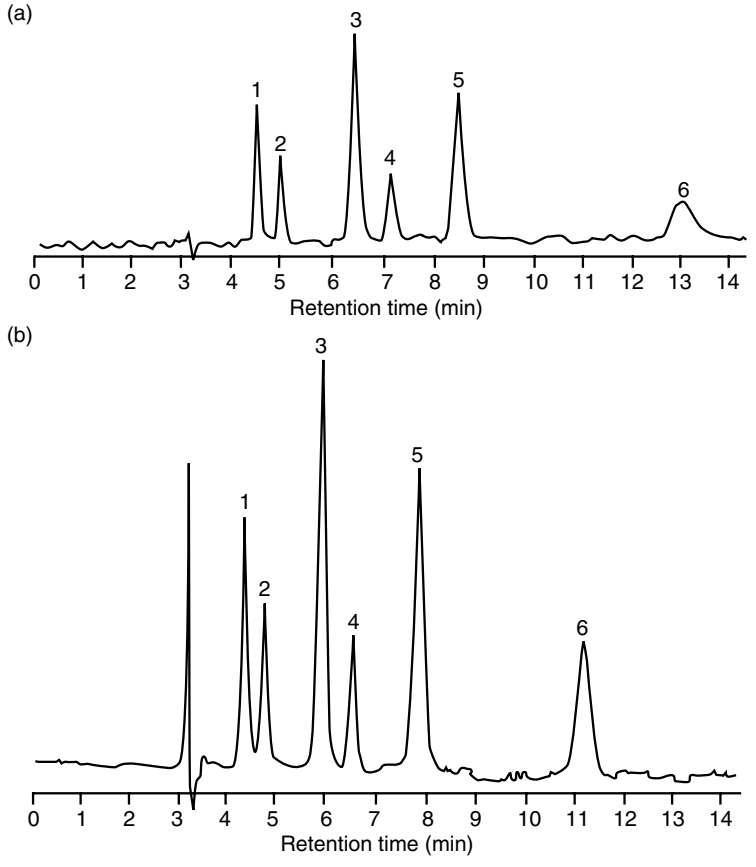

FIGURE 20.3
Comparison of a 4.0-mm-ID column and a 2.0-mm-ID column for the separation of fat-soluble vitamins in a standard solution. Reversed-phase, Nucleosil-120-5 C8 (octyl); mobile phase, methanol and water (92:8); programmable UV detector for optimal wavelength selection. Column dimensions: (a) 250 × 4.0 mm ID; (b) 250 × 2.0 mm ID. Peaks: (1) retinol; (2) retinyl acetate; (3) vitamin D_3; (4) α-tocopherol; (5) α-tocopheryl acetate; (6) retinyl palmitate. (From Andreoli, R., Careri, M., Manini, P., Mori, G., and Musci, M., *Chromatographia*, 44, 605, 1997. With permission.)

with an 8-mm^3 cell. The flow rate of the narrow-bore system was adjusted to give the same linear velocity as the standard-bore system. Limits of detection using the narrow-bore system were between 1.5 (α-tocopherol) and 90 (retinol) times lower than those obtained using the standard-bore system (Table 20.4). For a component eluting with a k value of 1, the two columns had comparable efficiency as determined by calculation of the number of theoretical plates (N). However, in the case of a late-eluting peak (the retinyl palmitate peak), the narrow-bore column

TABLE 20.4

Absolute Detection Limits (DLs) of Fat-Soluble Vitamins using Standard-Bore (4.0-mm ID) and Narrow-Bore (2.0-mm ID) Columns and UV Detection

	DL (ng)[a]	
Vitamin	**4.0 mm ID**	**2.0 mm ID**
Retinol	0.090	0.001
Retinyl acetate	0.100	0.003
Retinyl palmitate	0.320	0.030
D_3	0.800	0.040
α-Tocopherol	0.900	0.600
α-Tocopheryl acetate	2.500	0.700

Detection limits based on a signal-to-noise ratio of 3:1.
Source: Andreoli, R., Careri, M., Manini, P., Mori, G., and Musci, M., *Chromatographia*, 44, 605, 1997. With permission.

proved to be more efficient ($N = 8215$) than the standard-bore column ($N = 3850$) [60].

Food analysts have been somewhat reluctant to convert to narrow-bore HPLC. However, several vitamin methods utilize 3.2-mm-ID columns, which in a properly designed system give a twofold increase in sensitivity over 4.6-mm-ID columns.

20.4.4 Chromatographic Modes

The chromatographic mode selected for analytical separations depends on the extraction and cleanup procedures employed and the vitamers required to be measured.

20.4.4.1 Normal-Phase Chromatography

In the normal-phase mode, a polar (hydrophilic) stationary phase is used in conjunction with a nonpolar mobile phase. Separation is based on the relative polarity of the solutes and their affinity for the stationary phase. Relatively nonpolar solutes prefer the mobile phase and elute first; more polar solutes prefer the stationary phase and elute later.

20.4.4.1.1 Adsorption Chromatography

This is liquid–solid chromatography in which the surface of microparticulate silica or other adsorbent constitutes the polar stationary phase.

The adsorption sites on the surface of silica are silanol (≡Si—OH) groups, which are present both as isolated groups and hydrogen-bonded to one another. Separations result from the interactions of polar functional groups in the solute molecule with the adsorption sites on the silica surface. The strength of these polar interactions is responsible for the selectivity of the separation. Solute retention is very sensitive to changes in temperature; therefore column thermostatting is recommended, especially when peak height measurements are used in quantitative assays.

Adsorption chromatography is generally suitable for the separation of nonionic molecules that are soluble in organic solvents: such compounds are of moderate polarity, and contain at least one polar functional group. The unique ability of adsorbents to differentiate solutes based on differences in their polar functional groups enables compounds to be separated into classes or groups of similar chemical type. Adsorption chromatography also provides a powerful means of separating *cis* and *trans* isomers of unsaturated compounds, the separation mechanism being attributed to a steric fitting of solute molecules with the discrete adsorption sites. This is illustrated in the isocratic separation of six geometric isomers of vitamin A obtained by photolysis of all-*trans*-retinol [61].

The silica particles are characterized by their shape (irregular or spherical), size and size distribution, and pore structure (mean pore diameter, specific surface area, and specific pore volume). Chromatographic silicas can be arbitrarily classified into two types, A and B. Type A silicas generally are more acidic and less purified, and, when used in normal-phase chromatography with more polar or basic solutes, are more likely to exhibit tailing, misshapen peaks. Type B silicas are more highly purified and less acidic; they generally give superior results and better reproducibility than type A silicas [62].

The nonpolar mobile phase is typically hexane containing a small percentage (usually $<5\%$ by volume) of a polar organic solvent to act as moderator. The moderator is preferentially adsorbed from the mobile phase by the hydrogen-bonded silanol groups on the silica surface, and effectively deactivates these strong adsorption sites. The isolated silanol groups that remain are those responsible for the adsorptive properties of the deactivated silica. Various organic moderators have been used, including 2-propanol (isopropanol), diethyl ether, diisopropyl ether, methyl *tert*-butyl ether, ethyl acetate, and 1,4-dioxane. The use of alcohols is accompanied by difficulties in achieving accurate mobile phase proportions, as relatively very small volumes of these highly polar solvents are needed. Compared to other ethers, methyl *tert*-butyl ether is less prone to peroxide formation.

Water, being a very polar solvent, is a very strong moderator. In practice, all systems in adsorption chromatography are moderated systems,

because, unless specifically dried, all organic solvents contain an inherent amount of water. The lower the polarity of the mobile phase, the bigger is the influence of small changes in water concentration. With hexane or heptane, a change in water concentration of a few parts per million is sufficient to greatly affect sample retention. It is difficult to standardize the water content of a mobile phase, but a possible alternative is to prepare an isohydric mobile phase, that is, a mobile phase which corresponds to the same hydration level as the adsorbent. Isohydric mobile phases avoid the long equilibrium times usually required with silica columns when changing the eluent, as the eluent is in equilibrium with the adsorbent with respect to water. The mobile phase can be made isohydric with respect to silica by maintaining the water saturation of the mobile phase at about 50% by volume. Mobile phases with approximately 50% water saturation can be prepared by mixing equal volumes of dry mobile phase with completely water-saturated mobile phase. In the case of a highly nonpolar solvent (e.g., hexane or heptane), in which the solubility of water is very low, it is difficult to achieve a complete saturation by simply shaking or stirring the solvent with water. Engelhardt [63] described a moisture control system as a means of conditioning the mobile phase and column together.

Gradient elution in adsorption chromatography is performed by increasing the solvent strength of the mobile phase exponentially during the separation. In practice, it is difficult to ensure that equilibrium between the silica adsorbent and the changing mobile phase is occurring sufficiently rapidly. The problem is due to the susceptibility of the silica to water, regardless of whether or not isohydric solvents are used. If both solvents A and B are isohydric at the start of the gradient program, intermediate compositions of A and B are usually nonisohydric. This leads to the possible uptake of water by the adsorbent, resulting in nonreproducible separations and long regeneration times. Because of this problem, gradient elution is best avoided in adsorption chromatography for routine applications.

Silica columns can tolerate relatively heavy loads of triglyceride and other nonpolar material. Such material is not strongly adsorbed and can easily be washed from the column with 25% diethyl ether in hexane after a series of analyses [21]. Procedures for determining vitamins A and E have been devised in which the total lipid fraction of the food sample is extracted with a nonaqueous solvent, and any polar material that might be present is removed. An aliquot of the nonpolar lipid extract containing these vitamins is then injected into the liquid chromatograph without further purification. Direct injection of the lipid extract is possible because the lipoidal material is dissolved in a nonpolar solvent that is compatible with the predominantly nonpolar mobile phase. Procedures based on this technique are rapid and simple, because there is no need to saponify the sample.

The silica surface, even when deactivated, can tenaciously hold very polar compounds present as contaminants in the injected sample or as impurities in the mobile phase. This leads to an eventual loss of chromatographic efficiency, as strongly adsorbed material accumulates with continued use. It then becomes necessary to regenerate the column by washing off the adsorbed material and then to re-equilibrate the column. This process may be carried out by pumping a sequence of solvents of increasing polarity through the column and then reversing the sequence [64].

20.4.4.1.2 Polar Bonded-Phase Chromatography

Bonded-phase column packings for use in normal-phase chromatography are available in which the stationary phase is a polar functional group chemically bonded onto the silica surface. Polar-bonded phases, unlike unmodified silica, are not sensitive to traces of water, and, because they respond rapidly to changes in solvent polarity, can be used in gradient systems. Moderately polar nitrile-bonded phases containing propylcyano [—$(CH_2)_3$—CN] as the functional group generally show less retention when substituted for silica, but similar selectivity. Amino-bonded phases containing propylamino [—$(CH_2)_3$—NH_2] functional groups are of high polarity, and the basic nature of the functional groups imparts a quite different selectivity when compared with the acidic surface of silica. Ando et al. [65] have studied the retention behavior of fat-soluble vitamins using propylcyano- and propylamino-bonded phases and mobile phases containing ethyl acetate, tetrahydrofuran, or 2-propanol as the polar components in hexane. A mobile phase of 2-propanol in hexane generally gave the best peak shape. A unique type of polar-bonded phase is Partisil PAC (Whatman). This moderately polar silica-based material contains both secondary amine and cyano functional groups in a ratio of 2:1, and is nonendcapped.

A diol-bonded phase has been used as an alternative to silica for the separation of vitamin E vitamers. The Supelcosil LC-Diol bonded phase is prepared by reacting 5-μm spherical silica particles containing surface ≡Si—OH groups with 3-glycidoxypropyltrimethoxysilane to furnish an epoxysilica (Figure 20.4). The functional group of this phase should therefore be considered as an "epoxide" rather than as a "diol." The epoxy column reportedly gives more reproducible and constant separations than silica columns, owing to its stability to polar and non-polar solvents, its incapability to form any strong hydrogen-bonded structures with proton-donor analytes, and its moderate polarity [66].

Among the newer generation of packing materials, PVA-Sil (Yamamura Chemicals) is prepared by bonding a polymer coating of vinyl alcohol to silica. Because the polymer completely covers both the external and

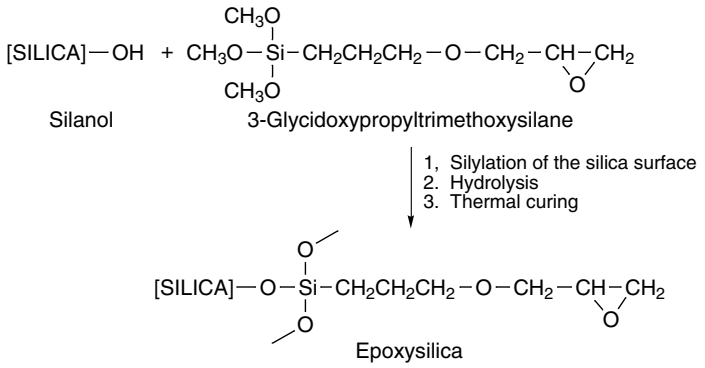

FIGURE 20.4
Preparation of the Supelcosil LC-Diol bonded phase. (From Kramer, J.K.G., Blais, L., Fouchard, R.C., Melnyk, R.A., and Kallury, K.M.R., *Lipids*, 32, 323, © 1997 by AOCS Press. With permission.)

internal surfaces of the silica support, the silica is protected against aggressive, high-pH (up to 9.5) mobile phases.

20.4.4.2 Reversed-Phase Chromatography

In this mode, a nonpolar stationary phase is used in conjunction with a polar mobile phase. Solute elution is the reverse of that observed in the normal mode, that is, polar solutes are eluted before nonpolar solutes.

The majority of reversed-phase HPLC applications utilize an octadecylsilane (ODS, C_{18}) stationary phase manufactured by reaction of an organosilane reagent with silanol groups on the silica substrate to form stable siloxane-bond linkages (Si—O—Si). The concentration of bonded ligands on the high surface area of the porous substrate is designated by the term surface coverage or ligand density, expressed in units of micromoles per square-meter. In comparison, carbon loading is a measure of the percentage of carbon on a bonded stationary phase, and does not take into account the surface area of the substrate. Phase coverage is a general term usually taken to mean carbon loading. Owing to steric hindrance during the bonding process, not all sites on the silica surface react. Accessible residual silanols trapped by steric hindrance can be endcapped by carrying out a secondary silanization reaction using a small monofunctional silane, such as trimethylchlorosilane. Fully endcapped stationary phases are almost completely hydrophobic and exhibit true reversed-phase properties. Nonendcapped phases contain a percentage of accessible silanol groups that impart some secondary normal-phase properties.

Depending on the reaction conditions and the type of silane reagent used, both monomeric (monolayer) and polymeric stationary phases can be prepared [67] (Figure 20.5). Monomeric phases result from the reaction of monofunctional organosilanes (e.g., dimethyl-*n*-octadecylchlorosilane) with silanols at the silica surface. Owing to steric limitations,

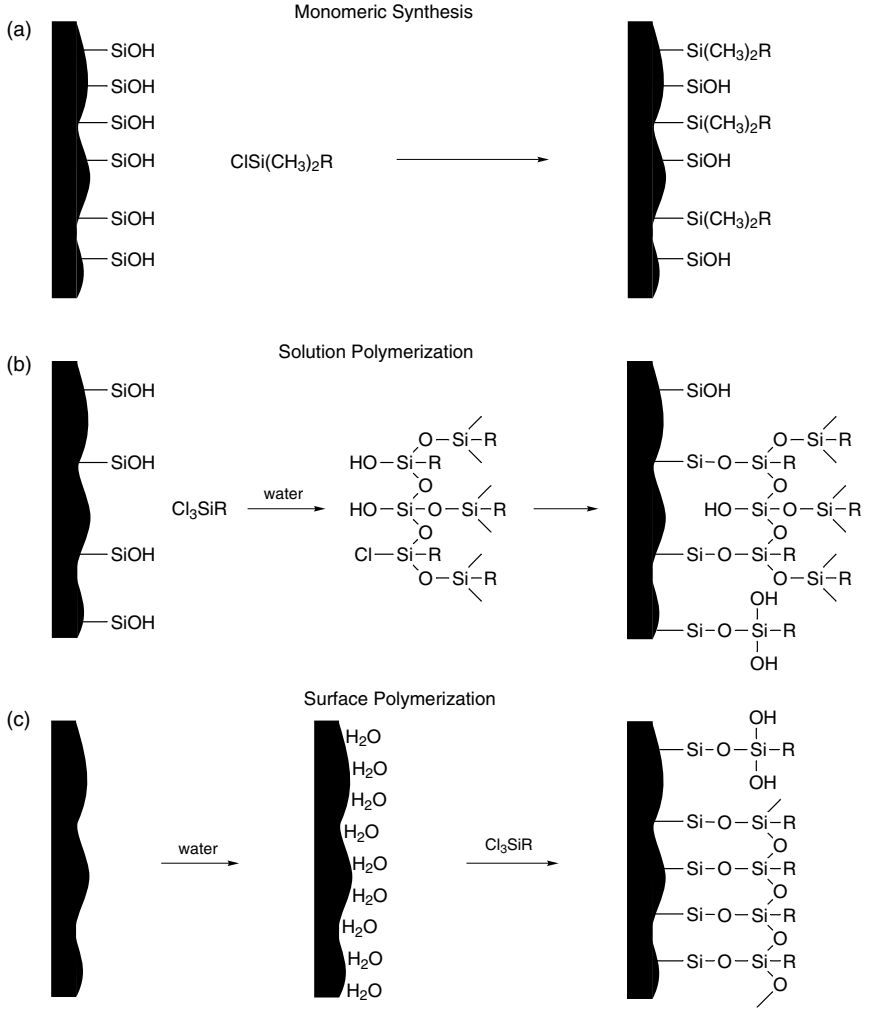

FIGURE 20.5
Synthesis schemes for surface modification of silica. (Reprinted with permission from Sander, LC., Pursch, M., and Wise, S.A., *Anal. Chem.*, 71, 4821, © 1999 American Chemical Society.)

only about half of the available silanol groups are covalently modified. Higher bonding densities can be achieved with polymeric synthesis. Polymeric phases are manufactured by intentionally introducing a measured quantity of water into a synthesis involving di- or trifunctional silanes. There are two ways of synthesizing polymeric phases: solution polymerization and surface polymerization. In solution polymerization, water is added to the reaction slurry, resulting in deposition and linkage of the polymerized silane to the silica surface. In surface polymerization, water is adsorbed onto dry silica before introduction of the silane. Only siloxane bonds are involved in polymerization, and both linear addition and crosslinking reactions are possible [68]. (*Note*: The term polymeric phase should not be confused with polymer-based substrate phases, which have a polymer substrate instead of a silica substrate.)

The physical structure of the stationary phase depends on the compatibility of the solvent with the bonded *n*-alkyl chains. Compatible nonpolar solvents tend to promote extension of the chains, allowing full penetration by the solvent. Conversely, fairly polar solvents tend to promote collapse of the chains on each other, allowing negligible solvent penetration.

Two contrasting theories of retention in reversed-phase liquid chromatography are the solvophobic theory proposed in 1976 by Horváth et al. [69] and revived in 1998 [70], and a partitioning model [71]. According to the solvophobic theory, the very high cohesive density of the mobile phase, arising from the three-dimensional hydrogen-bonding network, causes the less polar solutes to be literally "squeezed" out of the mobile phase, enabling them to bind with the hydrocarbon ligands of the stationary phase. The selectivity of the separation results, therefore, almost entirely from specific interactions of the solute with the mobile phase. Many features of retention are well predicted by ordinary bulk-phase partitioning, which is strongly dependent upon the surface density of the grafted chains [71]. Other workers have found that retention on monomeric [72] and polymeric [73] alkyl-bonded silica phases with octyl chains or longer are dominated by a partitioning-like mechanism in which solutes accumulate on the mobile phase–bonded-phase boundary region rather than distributing uniformly in the bonded phase.

The solvophobic theory and partitioning models can be used to explain the retention behavior of solutes with different polarity and hydrocarbonaceous surface area. They are unable, however, to account for differences in retention among geometric isomers or for selectivity differences observed among C_{18} column packings from different manufacturers. Wise and Sander [74] proposed the "slot model" to explain these differences. In this model, the spaces between the alkyl chains are viewed as slots into which solute molecules penetrate. Planar (flat) molecules can

slot in more readily than nonplanar molecules, and long narrow molecules more readily than cuboid molecules. The stationary phase is therefore shape selective. Wise and Sander [74] observed that polymeric phases exhibit a greater selectivity for molecular shape than monomeric phases, and that shape selectivity increases with increasing phase coverage for polymeric phases. Shape selectivity also increases with increasing alkyl chain length for both monomeric and polymeric phases [67]. This is evident from the superior resolution of β-carotene *cis* and *trans* isomers obtained using a C_{30} (triacontyl) stationary phase compared to a C_{18} phase [75]. In addition to being more shape selective, C_{30} columns have a higher recovery rate than C_{18} columns. In HPLC studies of β-carotene extracts from algae, only 66% of the injected β-carotene could be detected after passage through a polymeric C_{18} column, whereas the recovery with a C_{30} column of the same dimensions (250 × 4.6 mm ID) was 97%. On the downside, C_{30} columns are less efficient in terms of number of theoretical plates and therefore peaks with larger half-widths are to be expected [75].

Shape discrimination with C_{30} phases is increased by reducing the temperature from ambient to subambient. This is because at a lower temperature the number of bends and kinks (gauche defects) in the alkyl chains decreases and the population of straight (all-*trans*) chains increases. Chains with all-*trans* configurations are more rigid than ones with gauche defects. In the more solid-like state, the chains are more or less frozen into a disordered state on the surface. Molecules such as carotenoids will find it difficult to penetrate into the stationary phase, unless there are open cavities ("slots") into which they can move by diffusion. In contrast, increasing the temperature above ambient allows the solutes to penetrate the more fluid environment by means of a partitioning process, and shape discrimination will be lost [76]. The loss of chromatographic resolution with increasing temperature correlates with the increasing number of gauche defects, showing that shape selectivity depends on a more ordered arrangement of alkyl chains, that is, chains with predominantly all-*trans* configurations.

In reversed-phase chromatography, the main component of the organic/aqueous mobile phase is frequently methanol or acetonitrile. Increasing the proportion of water causes an increased retention of the more hydrophobic solutes relative to the more polar solutes. The surface tension of the mobile phase plays a major role in governing solute retention, so an increase in temperature, by reducing viscosity, increases column efficiency and shortens retention times. However, as explained earlier, heating the column impairs shape selectivity. Methanol and acetonitrile are weak solvents that have only a minor influence on the ordered arrangement of alkyl chains of the stationary phase. In contrast,

chlorinated solvents and methyl *tert*-butyl ether cause a disordering of the alkyl chains and an impairment of shape selectivity [77].

Several methods for determining fat-soluble vitamins employ nonaqueous reversed-phase (NARP) chromatography as a means of increasing their solubility. A typical NARP mobile phase consists of a polar basis (usually acetonitrile), a solvent of lower polarity (e.g., dichloromethane) to act as a solubilizer, and to control retention by adjusting the solvent strength, and, occasionally, a small amount of a third solvent with hydrogen bonding capacity (e.g., methanol) to optimize selectivity. To compensate for the increased affinity of the lipophylic compounds for the mobile phase, a highly retentive stationary phase, such as Zorbax ODS (20% carbon loading), is required.

The removal of triglycerides from the sample before injection is essential when using reversed-phase chromatography. Triglycerides are insoluble in water and only sparingly soluble in methanol and acetonitrile. If injected, they may not be completely eluted from the column, and the retained material would impair chromatographic efficiency, peak shape, and reproducibility. In the absence of nonpolar material in the final sample extract, reversed-phase chromatography exhibits improved reproducibility of solute retention times compared with normal-phase systems. This is largely because retention in reversed-phase chromatography is little affected by small variations in the mobile-phase composition, and, unlike adsorption chromatography, no significant effect is seen from slight changes in water content. Re-equilibration of reversed-phase columns is easier and quicker compared with silica columns, owing to the weaker interactive forces between the solute and the nonpolar stationary phase. Elution with several column volumes of methanol is usually sufficient to restore the column to its original condition.

20.4.4.3 Two-Dimensional HPLC

For determining the trace amounts of naturally occurring vitamins D and K in foods, it is often impossible to achieve an adequate separation for quantitation using a single column. This is because prior chromatographic cleanup techniques fail to remove lipoidal substances that are of similar polarity to the vitamin in question, and these substances interfere in the analytical HPLC. It has therefore been found necessary in such cases to perform the HPLC in two systems. A true two-dimensional combination involves two distinct chromatographic modes (e.g., normal phase and reversed phase) and can be expected to provide better selectivity than the use of two columns operated in the same mode [78]. The first system (semipreparative HPLC) is designed to isolate and collect a fraction of the sample extract that contains the

vitamin analyte and the internal standard. Ideally, the analyte and internal standard should be unresolved from each other so that they can be collected by reference to a single peak in the chromatogram. If the analyte/internal standard peak is masked by coeluting peaks, the obvious method of collecting the fraction is to refer to the retention time of an analyte standard. However, this method may not be reliable if the chromatographic mode is normal phase, because retention times may vary from run to run. To overcome this problem, the chromatogram can be compared with one obtained by spiking a small amount of the sample extract with the pure analyte. This enables the fraction to be collected by observing the analyte peak in relation to UV-absorbing contaminants in the sample extract.

The fraction of column effluent containing the analyte and internal standard can be either collected manually for subsequent reinjection onto the second (analytical) column (offline operation) or diverted directly onto the second column via a high-pressure switching valve (online operation). For manual collection, a drop counter-fraction collecting system rather than a volume collection system has been recommended [79]. The fraction is collected in a small tapered tube, and the solvent is carefully evaporated off under a stream of nitrogen. The residue is then dissolved in a small volume of a suitable solvent for the analytical separation. Because the sample is reconstituted in offline operation, the potential problem of mobile-phase incompatibility between the two HPLC systems is avoided, and hence any semipreparative and analytical combination can be used.

Online operation has the potential for being completely automated, because the switching valve can be actuated by time-programmable events from a microprocessor-based chromatograph. Limitations are that the two mobile phases must be miscible with each other, and the mobile phase from the first column must be of weaker elution strength than that used for the second column. The latter criterion facilitates the concentration of solute onto the head of the second column, this being essential to maintain the effect of the first separation. If a stronger solvent is injected onto the second column, band spreading will occur, because the injected solvent will preferentially move the solutes down the column until the concentration of this solvent is diluted sufficiently that solutes begin to be retained [80].

20.4.5 Detection Systems

20.4.5.1 Introduction

Three types of inline HPLC detector have been routinely employed in methods for determining vitamins in foods: photometric, fluorometric,

and electrochemical detectors. Each of these detectors provides a continuous electrical output that is a function of the concentration of solute in the column effluent passing through the flow cell. The photodiode array detector permits simultaneous absorbance detection at several wavelengths and continuous memorizing of spectra during the evolution of a peak. This detector has proved invaluable for the assessment of peak purity and aids peak identification in carotenoid analysis. Peak identification based on chromatographic retention and spectral or electrochemical properties is equivocal. Mass spectrometry (MS) is a powerful tool for analyte identification, but coupling MS to HPLC is not as straightforward as coupling it to gas chromatography.

20.4.5.2 Absorbance Detection

Radiation absorption monitoring of the column effluent at an appropriate wavelength provides the most versatile means of detection for the fat-soluble vitamins. Vitamins A, D, E, and K exhibit characteristic absorption spectra in the UV region, whereas the carotenoid pigments absorb light in the visible region.

Absorbance measurement in HPLC is generally most frequently performed using a continuously variable-wavelength spectrophotometer, which permits any wavelength to be selected throughout the UV–visible range of the spectrum (i.e., 190–900 nm). This type of detector allows operation at the wavelength of maximum absorption (λ_{max}) of the analyte or at a wavelength that provides optimum selectivity. Fixed-wavelength photometers are suitable provided that the analyte displays sufficient absorbance to permit its measurement at the operational wavelength (most commonly 254 nm). The photodiode array detector can monitor absorbance at several wavelengths simultaneously and can record the complete absorption spectrum of a chromatographic peak in less than 1 sec. Peak purity, that is, the presence or absence of a coeluting compound, can be assessed by recording spectra at the upstroke, apex, and downstroke of the peak. The three spectra, after normalization, should be superimposable if the peak is attributable to a single compound.

The selectivity of absorbance measurement for a given vitamin is dependent upon the wavelength employed in the measurement and the strength of absorbance of the vitamin relative to the absorbance of accompanying substances. Obviously, it is desirable to isolate the vitamin analyte from interfering substances on the HPLC column. If this cannot be achieved, it is sometimes possible to select a detection wavelength that reveals the vitamin peak in the chromatogram but not the accompanying substances.

The relationship between the molar absorbance coefficient (ε) and the specific absorbance coefficient ($A^{1\%}_{1\,cm}$) is

$$\varepsilon = A^{1\%}_{1\,cm} \times \frac{\text{Molecular weight}}{10} \tag{20.5}$$

The analytical techniques employed in the determination of fat-soluble vitamins in foods always involve extraction of the sample or unsaponifiable fraction of the sample into an organic solvent; therefore accompanying substances in the final extract will be lipoidal or lipophylic in nature. Most lipids found in foods do not have chromophores that absorb strongly in the UV region above 220 nm. Exceptions are free or esterified conjugated fatty acids that occur in at least trace amounts in fats or oils that have undergone autoxidation or bleaching. Conjugated fatty acids exhibit very strong UV absorption, with λ_{max} of 230–235 nm for dienes and 260–280 nm for trienes [81]. Glycerides and sterols exhibit weak, but measurable, UV absorbance within the spectral range of vitamins D and K; hence they constitute potential sources of interference in the determination of these vitamins. For example, the λ_{max} for triolein (265 nm in hexane; $A^{1\%}_{1\,cm} = 0.74$) and for cholesterol (266 nm in chloroform; $A^{1\%}_{1\,cm} = 0.68$) coincide with the λ_{max} (265 nm) for vitamin D [81]. The absorption spectra of vitamins A and E lie beyond those for glycerides and sterols. Other potential sources of spectral interference include vitamers of low biological potency, vitamin oxidation and decomposition products, and added antioxidants.

20.4.5.3 Fluorescence Detection

Retinol and its esters and unesterified tocopherols and tocotrienols possess strong native fluorescence, but neither vitamin D nor vitamin K fluoresce. The carotenoids commonly associated with foods do not fluoresce to any significant extent, except notably phytofluene, which is found in considerable amounts in tomatoes [82] and in smaller amounts in carrots [83] and which fluoresces six times more intensely than retinyl acetate [84].

Fluorescence detection is more selective than absorbance detection because two wavelengths are required in the measurement, and the structural features necessary for a molecule to fluoresce are more limited. Most lipids, including glycerides and sterols, do not fluoresce. Maximum sensitivity is obtained by selecting the wavelengths corresponding to the intensity maxima in the excitation and emission spectra. At other wavelengths the sensitivity, although reduced, may still be adequate for measurement purposes. The fluorescence intensity of a compound is highly dependent on the composition of the mobile phase.

Coeluting compounds that absorb radiation at either the excitation or emission maximum of the fluorescent compound of interest can partially or even completely quench the fluorescence by absorbing the excitation or emission energy [85].

When using normal-phase HPLC with fluorescence detection, the presence of dissolved oxygen in the predominantly hexane mobile phase quenches the fluorescence of vitamins A and E, causing a progressive reduction in peak size. Quenching refers to the loss of energy from excited molecules before they can emit radiation. The energy is transferred directly to other molecules, especially molecules of dissolved oxygen. Oxygen is 7–8 times more soluble in organic solvents than in aqueous solvents, which explains the propensity of normal-phase systems to fluorescence quenching. The problem of quenching by dissolved oxygen can be overcome by thoroughly "degassing" the mobile phase with helium before use, and maintaining a blanket of helium above the mobile-phase reservoir with a low continuous flow [86]. Excessive degassing causes preferential evaporation of the modifier, 2-propanol, in the mobile phase, and consequent increase in retention time. This is due to the formation of an azeotrope between 2-propanol and hexane. Without azeotrope formation, hexane would be evaporated in preference to 2-propanol, causing a decrease in retention time. The preferential evaporation of 2-propanol from the mobile phase can be prevented by presaturating the helium with the azeotrope. This is achieved by passing the helium through a flask containing hexane/2-propanol (78:22) before it reaches the mobile phase [86].

20.4.5.4 Electrochemical Detection

All the fat-soluble vitamins, including provitamin carotenoids, exhibit some form of electrochemical activity. Both amperometry and coulometry have been applied to electrochemical detection. In amperometric detectors, only a small proportion (usually $<20\%$) of the electroactive solute is reduced or oxidized at the surface of a glassy carbon or similar nonporous electrode; in coulometric detectors, the solute is completely reduced or oxidized within the pores of a graphite electrode. The operation of an electrochemical detector requires a semiaqueous or alcoholic mobile phase to support the electrolyte needed to conduct a current. This restricts its use to reverse-phase HPLC (but not NARP) unless the electrolyte is added post-column. Electrochemical detection is incompatible with NARP chromatography, because the mobile phase is insufficiently polar to dissolve the electrolyte. A stringent requirement for electrochemical detection is that the solvent delivery system should be virtually pulse-free.

Amperometric detection in the oxidative mode produced on-column detection limits of 0.07, 4.3, and 0.19 ng for retinol, vitamin D_3, and α-tocopherol, respectively [87]. A limitation of amperometric detection in vitamin E assays is that it cannot measure α-tocopheryl acetate, owing to the absence of the oxidizable hydroxyl group.

20.4.5.5 Mass Spectrometry

van Breemen [88] has discussed various ways of interfacing HPLC with MS and their suitability for carotenoid analysis. Particle beam MS detection has been studied as a means of identifying vitamins A, D, and E in various foods [89] and determining vitamin K_1 in vegetables [90]. Fat-soluble vitamin molecules cannot be ionized by standard electrospray ionization because they lack a site for protonation or deprotonation. However, the formation of Ag^+–carotenoid and Ag^+–tocopherol adducts by the addition of silver ions greatly enhances the ionization of carotenoids and tocopherols, and renders these compounds amenable to electrospray ionization MS [91]. Atmospheric pressure chemical ionization (APCI), like electrospray ionization, combines nebulization and ionization of the column effluent at atmospheric pressure. All carotenoids can form protonated molecules and molecular ions during positive-ion APCI and deproteinated molecules and molecular ions during negative-ion APCI. HPLC coupled with positive-ion APCI-MS has been used for the simultaneous quantification of vitamins A, D_3, and E in fortified infant formulas [92].

20.5 Applications of HPLC

20.5.1 Vitamin A

Methods for determining vitamin A, and sometimes β-carotene as well, are summarized in Table 20.5. Federal legislation in the U.S. does not differentiate between *trans* retinyl esters and the less active *cis* isomers, predominantly 13-*cis.* Therefore, individual isomer identification is not a legal requirement of the analytical process.

20.5.1.1 Detection

Retinol and its esters exhibit similar UV absorption spectra within a broad wavelength range and have practically equal molar absorptivities when dissolved in a given solvent. The absorption spectrum of

TABLE 20.5

HPLC Methods Used for the Determination of Vitamin A and Carotene in Food

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Normal-phase chromatography						
Margarine	Dissolve sample in heptane containing 500 mg BHT and 200 mg α-tocopherol per liter. Pass emulsion through glass column containing sodium sulfate and sodium chloride	LiChrosorb Si-60 5 μm 250 × 4.6 mm	Heptane/ diisopropyl ether, 95:5	Retinyl palmitate or retinyl acetate	UV 325 nm	[93]
Fortified fluid milk (whole, semi-skimmed, skimmed)	Mix 2 ml milk and 5 ml absolute EtOH in a centrifuge tube, let stand 5 min. Vortex-mix with 5 ml hexane, let stand 2 min. Repeat mixing and standing procedure twice. Add 3 ml water, mix by inversion, centrifuge	LiChrosorb Si-60 5 μm 250 × 3.2 mm	Hexane/diethyl ether, 98:2	All-*trans*-retinyl palmitate	UV 325 nm	[21]
Fortified fluid milk	As in preceding entry, but initial 5 ml ethanol contains retinyl acetate (internal standard)	Silica column 5 μm 250 × 4.6 mm	0.15% 2-PrOH in hexane	Retinyl palmitate retinyl acetate (internal standard)	UV 325 nm	[94]

Margarine	Dissolve sample in hexane, shake with 60% EtOH, centrifuge.	LiChrosorb Si-60 5 μm 250 × 3.2 mm	Hexane/diethyl ether, 98:2	Carotene, all-*trans*-retinyl palmitate	Vis 453 nm (carotene) UV 325 nm (retinyl palmitate)	[21]
Milk-based infant formula	Saponify (hot), extract with hexane/CH_2Cl_2, 3 + 1. Wash organic layer three times with water/absolute ethanol, 3 + 2. Evaporate a 20-ml portion under nitrogen, dissolve residue in HPLC mobile phase	Silica–CN 5 μm 150 × 4.6 mm	0.25% 2-PrOH in hexane	13-*cis*- and all-*trans*-retinol	UV 325 nm	[95]
Milk, infant formula	Saponify (ambient), extract with hexane/diethyl ether, 85:15. Evaporate a portion of clear solvent under nitrogen, dissolve residue in heptane	Apex silica 3 μm 150 × 4.5 mm	1–5% 2-PrOH in heptane	13-*cis*- and all-*trans*-retinol	UV 340 nm (filter)	[96]
Cheese	Saponify (ambient), extract with hexane. Concentrate by rotary evaporation	LiChrosorb Si-60 5 μm 250 × 4 mm	Hexane/methyl ethyl ketone, 90:10	Carotene, all-*trans*-retinol	Vis 450 nm (carotene) UV 340 nm (retinol)	[97]
Milk	Dilute 400 mg milk with water/MeOH/EtOH (55:9:36). Saponify in a culture tube, extract with heptane/isopropyl ether (3:1), centrifuge. Repeat extraction	Perkin-Elmer HS-5-Silica 125 × 4 mm	6% 2-PrOH in heptane	Retinol	Fluorescence: ex 344 nm em 472 nm	[98]

(*Table continued*)

TABLE 20.5 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Soy-based infant formula	Extract sample by matrix solid-phase dispersion. Elute retinyl acetate from C_{18} column with 7 ml hexane containing 0.5% 2-PrOH followed by 7 ml CH_2Cl_2. Evaporate pooled eluates under nitrogen, dissolve residue in hexane	LiChrosorb Si-60 5 μm 250 × 4.6 mm	0.28% 2-PrOH in hexane	Retinyl acetate	Fluorescence: ex 325 nm em 470 nm	[35]
Various foods	Saponify (ambient), dilute digest with water and absolute EtOH to yield a volumetric ratio of water to EtOH of 1:1. Pipet a 20-ml aliquot onto a Kieselguhr cartridge, elute with petroleum ether, evaporate, dissolve residue in isooctane	Spherisorb SW silica gel 3 μm 100 × 2 mm (narrow-bore)	0.3% 1-octanol in hexane	13-*cis*- and all-*trans*-retinol	UV 325 nm	[99]
Milk, milk energy drink, multivitamin fruit juice, orange juice	Saponify 2-ml sample in a microwave system for 2 min, cool. Add acetic acid, saturated sodium chloride solution, and cyclohexane containing 0.05% BHT, shake, centrifuge	LiChrosorb Si-60 5 μm 125 × 4 mm	Hexane/2-PrOH, 98:2	13-*cis*- and all-*trans*-retinol	UV 325 nm	[5]

Reversed-phase chromatography

All food categories	Saponify (hot), extract with diethyl ether/petroleum ether (1:1), wash pooled extracts to neutral pH. Evaporate under vacuum, dissolve residue in MeOH	μBondapak C_{18} 10 μm 300 × 3.9 mm	MeOH/H_2O, 90:10	Retinol	UV 325 nm	[100]
Unfortified fluid dairy products	Saponify (hot) 2-ml sample in a centrifuge tube, extract with diethyl ether/petroleum ether (1:1) containing 0.01% BHT, centrifuge. Evaporate under nitrogen, dissolve residue in MeOH	Nova-PAK C_{18} 150 × 3.9 mm	MeOH/H_2O, 95:5	Retinol	UV 325 nm	[7]
Milk powder, flour	Saponify (hot), extract with diethyl ether/petroleum ether (1:1), wash pooled extracts with water, filter through cellulose to clarify. Evaporate under vacuum, dissolve residue in MeOH	Zorbax ODS 5 μm 150 × 4.6 mm	MeCN/H_2O, 80:20	Retinol	UV 325 nm	[101]
Fortified fluid milk (whole, skimmed), infant formula, margarine	Saponify (hot) 1-ml sample of milk or formula or 50 mg margarine in a centrifuge tube, extract with hexane. Evaporate, dissolve residue in MeOH	LiChrosorb RP-18 10 μm 250 × 3.2 mm	MeOH/H_2O, 90:10 (retinol) MeOH/H_2O, 99:1 (β-carotene)	Retinol and β-carotene (separate chromato-grams)	UV 325 nm (retinol) Vis 453 nm (β-carotene)	[102]

(*Table continued*)

TABLE 20.5 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Selected foods of animal origin and processed foods	Saponify (hot), extract with hexane, wash pooled extracts to neutral pH, dry with sodium sulfate, evaporate, dilute with hexane	μBondapak C_{18} 10 μm 300 × 3.9 mm	MeCN/MeOH/ ethyl acetate, 88:10:2	Retinol, α- and β-carotenes	UV 313 nm (retinol) Vis 436 nm (carotenes)	[103]
Breakfast cereals, margarine, butter	Saponify (hot), precipitate soaps with acetic acid in MeCN, filter, dilute with water	Vydac 201 TP C_{18} 10 μm 250 × 3.2 mm	MeCN/H_2O, 65:35	13-*cis*- and all-*trans*-retinol	UV 328 nm	[104]
All food categories	Saponify (hot), neutralize KOH with glacial acetic acid. Transfer the cooled solution to a 100-ml volumetric flask using THF/95% EtOH (1 + 1), dilute to volume. Refrigerate overnight to precipitate fatty acid salts, centrifuge	C_{18} 10 μm 250 × 4.6 mm	MeOH/H_2O, 86:14	Retinol (all-*trans* separated from *cis* isomers)	UV 328 nm	[105]
Fortified cereal products	Supercritical fluid extraction	Altex C_8 (octyl) 5 μm 150 × 3.9 mm	MeCN/2-PrOH/ aqueous 25-mM sodium perchlorate, 45:45:10	Retinyl palmitate	Amperometric (oxidative mode) glassy carbon electrode, +1.2 V vs. saturated calomel electrode	[41]

BHT, butylated hydroxytoluene; MeOH, methanol; EtOH, ethanol; 2-PrOH, 2-propanol; MeCN, acetonitrile; CH_2Cl_2, dichloromethane (methylene chloride); THF, tetrahydrofuran.

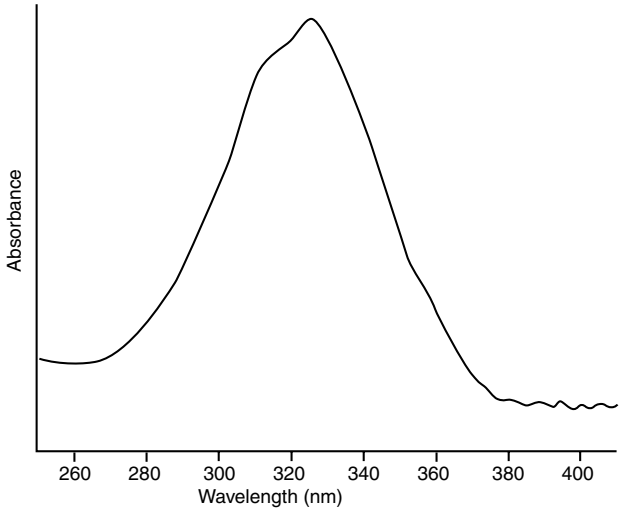

FIGURE 20.6
UV absorption spectrum of all-*trans*-retinol recorded by photodiode array detector during elution of a sample extract through a silica HPLC column using a mobile phase of 1–5% 2-propanol in heptane. The absorption maximum occurs at 326 nm. (Reprinted from Thompson, J.N. and Duval, S., *J. Micronutr. Anal.*, 6, 147–159, © 1989, with permission from Elsevier.)

all-*trans*-retinol is shown in Figure 20.6. The ε value of crystalline all-*trans*-retinol in 2-propanol at the λ_{max} of 325 nm is 52,300 [106], which corresponds to an $A_{1\,cm}^{1\%}$ of ca. 1830. The on-column minimum detectable quantity of vitamin A using UV absorption is ca. 2 ng [107].

The fluorescence excitation spectra of retinol and its esters correspond to their absorption spectra, with wavelength maxima in the 324–328 nm region; emission takes place between 470 and 490 nm (λ_{max} 470 nm) [108]. The excitation and emission spectra of retinyl acetate are shown in Figure 20.7. The use of fluorescence, rather than UV detection, has the advantage that β-carotene does not interfere with vitamin A determination, even if there is coelution. Otherwise, fluorescence detection offers no real advantages over absorbance detection, and the linear response range is more limited. Moreover, the fluorescence response of 13-*cis*-retinol is less than that of all-*trans*-retinol, the relative fluorescence depending on the solvent [104,109].

20.5.1.2 Quantification

Several different quantification procedures for vitamin A have been described in the literature, some using retinol directly as a standard and some using retinyl acetate, which is converted to retinol by saponification.

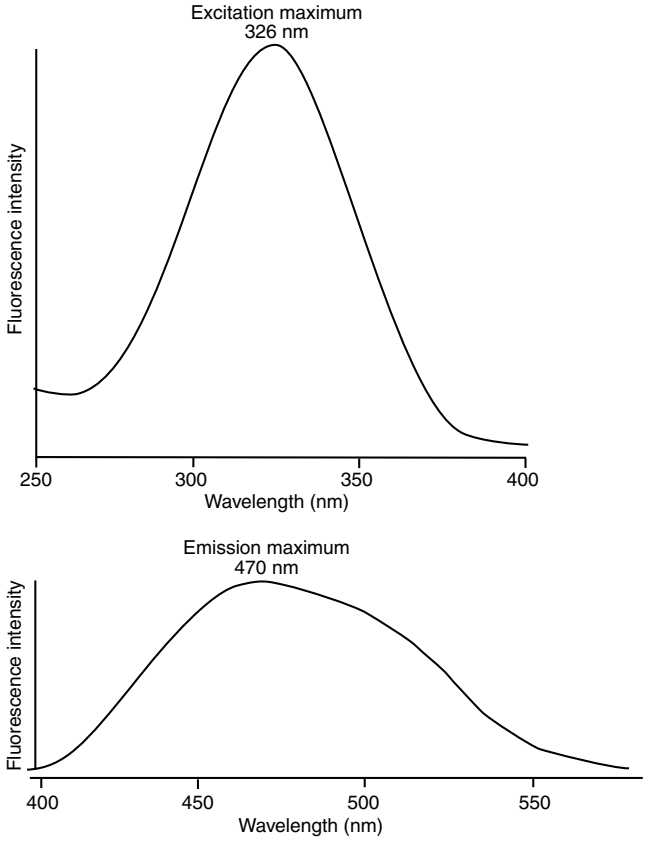

FIGURE 20.7
Fluorescence excitation and emission spectra of retinyl acetate obtained by stop-flow scanning after passage through a silica HPLC column eluted with hexane containing 0.03% 2-propanol.

The latter approach is generally preferred, because crystalline all-*trans*-retinyl acetate is commercially available in high purity and is free from *cis* isomers. Commercial sources of retinol are oily preparations and are at best only about 70% pure. There are two ways of preparing a retinol standard from retinyl acetate.

1. A relatively large amount (typically 25 mg) of retinyl acetate is saponified and extracted, and the residue is dissolved in 2-propanol to give a stock solution of retinol, which can be stored for 2–3 months in a refrigerator. This stock solution is diluted with 2-propanol to give a suitable working standard

solution, whose concentration is determined spectrophotometrically ($A^{1\%}_{1\,cm} = 1830$ at λ_{max} of 325 nm) immediately before use as an external standard in the HPLC procedure.

2. An accurately prepared standard solution of retinyl acetate (i.e., a solution of known concentration) is taken through the saponification and extraction procedure along with each batch of samples, and the resultant retinol solution is used as an external standard without spectrophotometric standardization. This technique, which is recommended by COST 91 [100], compensates for losses of vitamin A incurred during the saponification and subsequent manipulations (i.e., the calculated vitamin A value is recovery-corrected).

20.5.1.3 Normal-Phase Separations

Adsorption chromatography on underivatized silica is capable of separating the four isomers of retinol most likely to occur in nature, namely all-*trans*, 13-*cis*, 9-*cis*, and 9,13-di-*cis.* An example of such a separation is shown in Figure 20.8, which depicts a chromatogram of the above four isomers, plus 11-*cis*- and 11,13-di-*cis*-retinol, obtained by photolysis of all-*trans*-retinol. The separation of 13-*cis*- and all-*trans*-retinol in a food extract is shown in Figure 20.9. Adsorption chromatography is also capable of separating the retinol and dehydroretinol isomers found in fish liver (Figure 20.10).

Adsorption chromatography has been applied to the determination of added retinyl palmitate in margarine and milk [21]. The overall procedure entails extracting the total lipid fraction of the sample with hexane, removing the polar material, and then injecting an aliquot of the hexane extract directly into the chromatograph. The vitamin A is maintained in its relatively stable ester form throughout the assay and is protected by the lipids to the point of chromatographic separation. A major practical advantage is that there is no need to evaporate extracts to dryness. The method of Thompson et al. [21], with the addition of retinyl acetate as an internal standard, has been studied collaboratively [94] and adopted, first action 2002, by AOAC International [112] as a method for determining retinyl palmitate in fortified fluid milk. Under normal-phase conditions, the various indigenous long-chain esters of retinol (e.g., stearate, oleate, and linoleate) coelute with retinyl palmitate. A chromatogram of a milk sample extract is shown in Figure 20.11.

Saponification of the sample simplifies the analysis by converting the vitamin A esters to retinol. The unsaponifiable material is extracted with hexane, or a predominantly hexane solvent mixture, which is compatible with the nonpolar mobile phase [97,104,113]. In vitamin A-fortified foods there is no need to concentrate the unsaponifiable extract — an aliquot can be injected directly into the chromatograph [104].

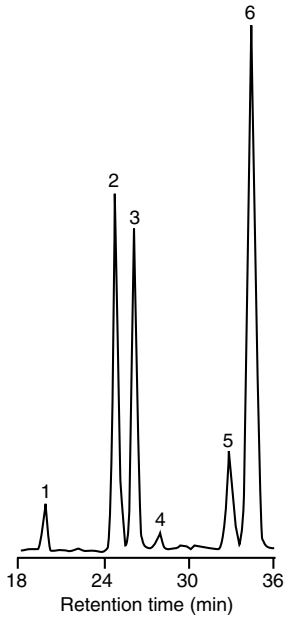

FIGURE 20.8
Normal-phase HPLC of retinol isomers in ethanol obtained by photolysis of all-*trans*-retinol. Column, LiChrosorb Si-60; column temperature, 45°C; mobile phase, hexane containing 0.4% 2-propanol; UV detection, 326 nm. Peaks: (1) 11,13-di-*cis*-retinol; (2) 13-*cis*-retinol; (3) 11-*cis*-retinol; (4) 9,13-di-*cis*-retinol; (5) 9-*cis*-retinol; (6) all-*trans*-retinol. (Reprinted from Stancher, B. and Zonta, F., High-performance liquid chromatography of the unsaponifiable from samples of marine and freshwater fish: fractionation and identification of retinol (vitamin A_1) and dehydroretinol (vitamin A_2) isomers, *J. Chromatogr.*, 287, 353–364, © 1984, with permission from Elsevier.)

20.5.1.4 Reversed-Phase Separations

The removal of triglycerides from the food sample by saponification provides the opportunity to utilize reversed-phase chromatography. The unsaponifiable matter is conventionally extracted into a solvent [e.g., diethyl ether/petroleum ether (50:50) or hexane] that is incompatible with a semiaqueous mobile phase. It then becomes necessary to evaporate the unsaponifiable extract to dryness and to dissolve the residue in a small volume of methanol (if methanol is the organic component of the mobile phase). The AOAC method for determining retinol in all food categories [105] avoids the time-consuming extraction of the unsaponifiable matter and the evaporation step. Samples are saponified and then neutralized with glacial acetic acid. After mixing and cooling, the solution is transferred to a 100-ml volumetric flask using tetrahydrofuran/95% ethanol (1 + 1) and diluted to volume with the same solvent mixture.

FIGURE 20.9
Normal-phase HPLC of retinol isomers extracted from a liver-rice casserole. Column, LiChrosorb Si-60; mobile phase, 3% 2-propanol in hexane; UV detection, 325 nm. Peaks: (1) 13-*cis*-retinol; (2) all-*trans*-retinol. (Reprinted from Heinonen, M., Ollilainen, V., Linkola, E., Varo, P., and Koivistoinen, P., *J. Food Comp. Anal.*, 1, 221–230, © 1988, with permission from Elsevier.)

The solutions are refrigerated overnight to precipitate fatty acid salts formed during saponification. Aliquots of the clear solution can then be injected onto a C_{18} column, using a mobile phase of methanol/water (86:14) for the subsequent analysis.

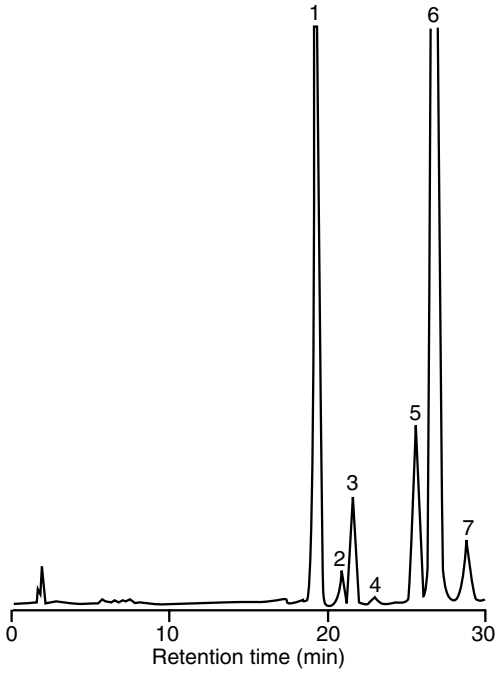

FIGURE 20.10
Normal-phase HPLC of isomers of retinol and dehydroretinol obtained from the unsaponifiable matter of cod liver oil. Operating parameters as in Figure 20.8, but different mobile-phase flow-rate. Peaks: (1) 13-*cis*-retinol; (2) 13-*cis*-dehydroretinol; (3) 9,13-di-*cis*-retinol; (4) 9,13-di-*cis*-dehydroretinol (tentative); (5) 9-*cis*-retinol; (6) all-*trans*-retinol; (7) all-*trans*-dehydroretinol. (Reprinted from Stancher, B. and Zonta, F., High-performance liquid chromatography of the unsaponifiable from samples of marine and freshwater fish: fractionation and identification of retinol (vitamin A_1) and dehydroretinol (vitamin A_2) isomers, *J. Chromatogr.*, 287, 353–364, © 1984, with permission from Elsevier.)

Schneiderman et al. [41] extracted retinyl palmitate from commercial breakfast cereals using supercritical CO_2 and determined the vitamin by means of reversed-phase HPLC and electrochemical detection. Chromatograms of an unfortified wheat sample and a fortified bran-based cereal product are shown in Figure 20.12.

Reversed-phase chromatography using semiaqueous mobile phases can separate all-*trans*-retinol from 13-*cis*-retinol, albeit rather poorly (Figure 20.13). Further separation of the minor *cis* isomers is not achieved, but this is of little practical concern in routine food analysis. In practice, it is convenient to adjust the elution conditions to obtain a single peak encompassing all the geometric isomers, ignoring the reduced biopotency of the *cis* isomers. Alternatively, the peak areas of the all-*trans* and 13-*cis*

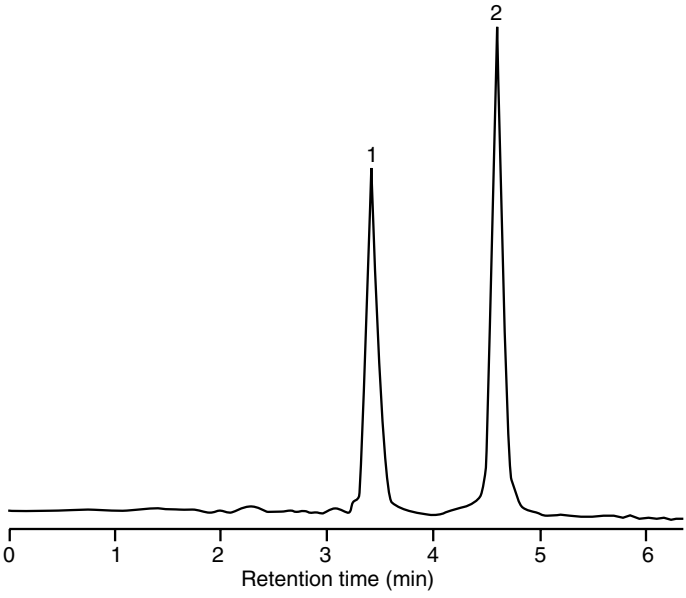

FIGURE 20.11
Normal-phase chromatography and UV detection of an extract of vitamin A-fortified milk. Operating parameters as in Table 20.5 [94]. Peaks: (1) retinyl palmitate; (2) retinyl acetate (internal standard). (Reprinted from the Hite, D.A., *J. AOAC Int.*, 86, 375–385, Copyright 2003 by AOAC International.)

isomers can be measured separately and then multiplied by their respective relative potencies of 100 and 75%, and added.

20.5.2 Provitamin A Carotenoids

20.5.2.1 Sources of Variation in the Methodology

Interlaboratory studies of HPLC procedures using a candidate reference material have shown that the effect of the chromatographic system and standardization of the carotenoid solutions are probably not major variables. The preparation of sample extracts for injection onto the HPLC column may account for about half of the total variance [114].

20.5.2.2 Detection

Most carotenoids absorb radiation in the visible region of the spectrum, the chromophore being the extensive conjugated double-bond system of the molecule. The absorption spectra of most carotenoids exhibit three maxima, whose positions are influenced by the solvent in which the

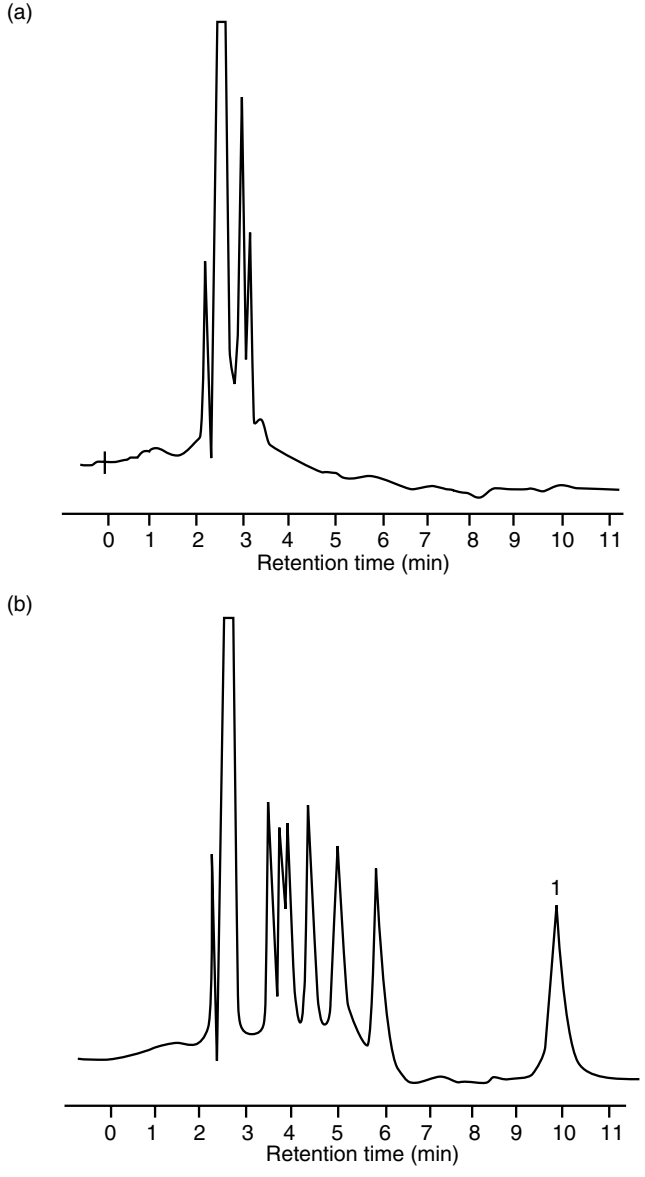

FIGURE 20.12
Reversed-phase HPLC and amperometric detection of supercritical fluid extracts of (a) an unfortified wheat sample and (b) a vitamin A-fortified bran-based ready-to-eat breakfast cereal. Operating parameters as in Table 20.5 [41]. Peak: (1) retinyl palmitate. (Reprinted from Schneiderman, M.A., Sharma, A.K., and Locke, D.C., *J. Chromatogr. A*, 765, 215–220, © 1997, with permission from Elsevier.)

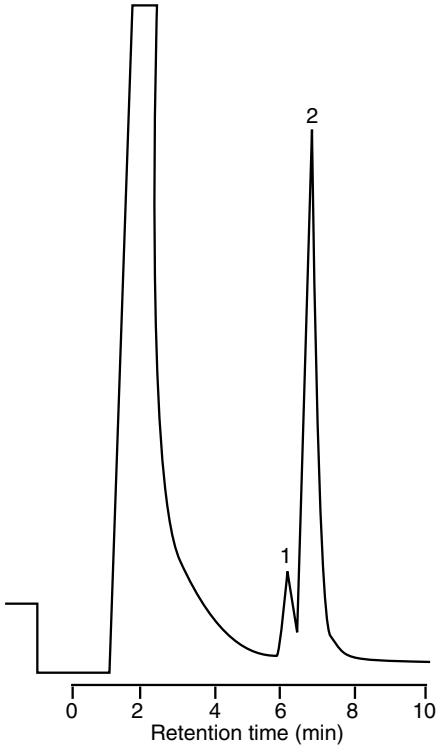

FIGURE 20.13
Reversed-phase HPLC and UV detection of vitamin A extracted from a fortified breakfast cereal. Operating parameters as in Table 20.5 [104]. Peaks: (1) 13-*cis*-retinol; (2) all-*trans*-retinol. (Reprinted with permission from Egberg, D.C., Heroff, J.C., and Potter, R.H., *J. Agric. Food Chem.*, 25, 1127, © 1999 American Chemical Society.)

spectrum is obtained. Spectra of carotenes (hydrocarbons) are usually determined in petroleum ether or hexane, and those of xanthophylls in ethanol. The λ_{max} is mainly a function of the length of the conjugated double-bond system. A cyclic carotenoid in which the conjugation extends into the ring (β-ring) will normally have its λ_{max} at shorter wavelengths than an acyclic pigment with the same number of conjugated double bonds. Rings having nonconjugated double bonds (e.g., ε-ring) do not contribute to the chromophore. A hydroxy substitution, among others, has little effect on λ_{max}, therefore β-carotene, β-cryptoxanthin, and zeaxanthin have virtually identical spectra, with λ_{max} at 428, 450, and 476–478 nm in ethanol. The spectrum of all-*trans*-β-carotene in petroleum ether is shown in Figure 20.14.

Formation of a *cis* isomer from an all-*trans* carotenoid results in a shift of the spectral bands to shorter wavelengths (hypsochromic shift) together

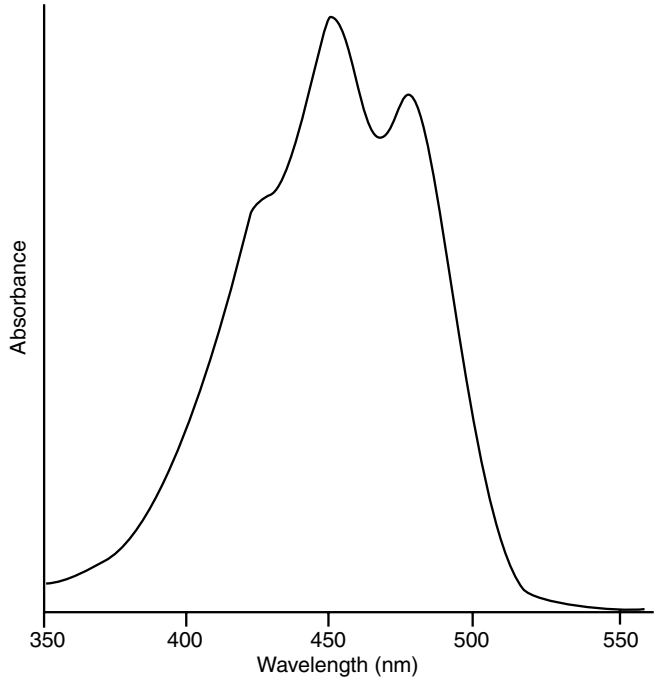

FIGURE 20.14
Absorption spectrum of β-carotene in petroleum ether. The absorption maximum occurs at 453 nm.

with a loss of fine structure and a hypochromic effect on absorbance. The hypsochromic shift of mono-*cis* isomers and di-*cis* isomers is about 2–5 and 10 nm, respectively [115]. These changes are accompanied by the appearance of a chromatographic "*cis* peak" in the UV region of the spectrum, generally between 320 and 380 nm.

Absorption maxima and $A_{1\,\mathrm{cm}}^{1\%}$ values for a large number of carotenoids have been compiled by De Ritter and Purcell [116]. The $A_{1\,\mathrm{cm}}^{1\%}$ value for β-carotene dissolved in hexane or petroleum ether is 2592 at λ_{max} 453 nm.

20.5.2.3 Potential Problems with the Chromatography

It is important to correctly identify the provitamin A peaks of interest in the chromatogram. A tentative identification can be made by a combination of retention time and spectral characteristics, using a photodiode array detector. Identification is aided by comparison with authentic carotenoid standards in more than one chromatographic mode.

An aggravating problem is the possibility of *trans–cis* isomerization occurring during analysis. This can be investigated by subjecting a

solution containing pure all-*trans*-β-carotene to the extraction process and obtaining an HPLC chromatogram of the resultant solution. The absence of *cis* peaks indicates that the extraction process does not promote isomerization.

Chromatographic artifacts can be produced if the injection solvent is not compatible with the mobile phase. Khachik et al. [117] employed four mobile phases (eluents A, B, C, D; Table 20.6) and various injection solvents to study factors that produced artifacts in the reversed-phase separation of carotenoids. Using an injection volume of 20 μl, it was found that the injection solvents could be divided into two groups. The first group, comprising acetonitrile, acetone, methanol, hexane, and the four mobile phase solvent mixtures, produced a single symmetrical peak for β-carotene and other carotenoids. The second group, comprising dichloromethane, chloroform, tetrahydrofuran, benzene, and toluene, produced artifacts such as peak splitting, peak distortion, and multiple peak formation. From solubility data (Table 20.6) and chromatographic profiles, it appears that artifacts arose in cases where there was a dramatic difference between the relative solubilities of β-carotene in the injection solvent and in the mobile phase. Hence artifacts were produced when tetrahydrofuran and dichloromethane were used in injection solvents with eluents A and B. Conversely, the solubility of β-carotene in hexane and acetone is similar to those of eluents A and B and therefore no

TABLE 20.6

Chromatographic Conditions for the Separation of Carotenoids and Solubility Data

Eluent or Injection Solvent	% Composition	Solubility of β-Carotene (mg/100 ml)
Eluent A	MeOH/MeCN/CH_2Cl_2 (25:55:20)	22
Eluent B	MeOH/MeCN/CH_2Cl_2/hexane (25:55:10:10)	22
Eluent C	MeCN/THF (78:22)	No data
Eluent D	MeOH/MeCN/CH_2Cl_2/hexane (gradient)	No data
THF		930
CH_2Cl_2		325
Hexane		39
Acetone		21

Note: MeOH, methanol; MeCN, acetonitrile; CH_2Cl_2, dichloromethane (methylene chloride); THF, tetrahydrofuran.

Source: Khachik, F., Beecher, G.R., Vanderslice, J.T., and Furrow, G., *Anal. Chem.*, 60, 807, 1988. With permission.

artifacts were observed. The injection of smaller (5 and 10 μl) volumes of sample eliminated the artifacts produced using injection solvents of the second group.

Owing to reported losses of carotenoids by rapid decomposition through oxidation in the presence of a stainless steel column frit [118], it has been recommended to purchase HPLC columns fitted with inert metal-free frits [119,120].

20.5.2.4 Normal-Phase Separations

Where silica columns have been utilized to determine vitamin A in margarine [21] and cheese [97], the sole carotenoid present, β-carotene, which is eluted near the solvent front, can be determined at the same time. Apart from such applications, silica is an unsuitable stationary phase for carotenoid analysis, because the carotenes are very weakly adsorbed and the xanthophylls are highly retained.

Microparticulate alumina specially prepared for HPLC is available commercially and facilitates the isocratic separation of all-*trans*-β-carotene from its lower potency *cis* isomers. Using an alumina column and a mobile phase of isooctane containing 0.5% stabilized tetrahydrofuran [121], the *cis* isomers of β-carotene are eluted before the all-*trans* isomer to form a single composite peak in the chromatogram. α-Carotene coelutes with the *cis* isomers of β-carotene and therefore cannot be accurately quantified. γ-Carotene, β-cryptoxanthin, and canthaxanthin are not eluted.

Calcium hydroxide is an excellent adsorbent for resolving carotenoid *cis*–*trans* isomers; compared with alumina, it is less retentive and less sensitive to temperature and moisture content of the mobile phase. However, calcium hydroxide columns are not commercially available, and separations using them can be difficult to reproduce, owing to extreme sensitivities to mobile phase composition and temperature. Nine *cis*-β-carotenes were characterized from a chromatogram of 18 peaks after isocratic elution of a mixture obtained by thermal isomerization and photoisomerization of β-carotene [122]. Chandler and Schwartz [123], using a calcium hydroxide column and a mobile phase of hexane containing 0.3% acetone, separated six carotene isomers from canned carrots. These were, in order of elution, two *cis*-α-carotenes, all-*trans*-α-carotene, 13-*cis*-β-carotene, all-*trans*-β-carotene, and 9-*cis*-β-carotene. A mobile phase of hexane modified with 2% *p*-methylanisole separated all-*trans*-β-carotene and its 9-, 13-, and 15-*cis* isomers in an iodine-isomerized mixture [124].

Of the polar bonded-phase packings that have been investigated, the interactions between carotenes and nitrile stationary phases are very weak; thus the limitations described for silica apply [125].

Amino-bonded phases eluted with isooctane containing 0.5% stabilized tetrahydrofuran separate α- and β-carotene (unresolved) from γ-carotene; canthaxanthin and β-cryptoxanthin are not eluted [121]. The *cis* isomers of β-carotene are separated from the all-*trans* isomer; thus amino columns offer an alternative option to alumina columns for determining all-*trans*-β-carotene without its *cis* isomers interfering.

20.5.2.5 Reversed-Phase Separations

Reversed-phase chromatography is generally preferred to normal-phase chromatography for the determination of provitamin carotenoids in food, because the carotenes are retained and the separation of α- and β-carotene is easily achieved. Owing to the weak hydrophobic forces on which the separation depends, there is little risk of on-column degradation of carotenoids. The xanthophylls are eluted well before the carotenes, the latter requiring strong mobile phases containing little or no water to displace them.

The more recent published methods (Table 20.7) employ NARP chromatography, which overcomes the problem of poor solubility of carotenes in semiaqueous mobile phases. Recoveries of carotenoids are better with methanol-based mobile phases than with acetonitrile-based ones [140]. Dichloromethane, tetrahydrofuran, and methyl *tert*-butyl ether appear to be the best modifiers for optimizing selectivities as well as reducing analysis times [141].

20.5.2.5.1 C_{18}-Bonded Phases

Cis–trans isomers of carotenes cannot normally be separated on a monomeric ODS phase. However, the separation of all-*trans*-β-carotene from its principal 9-*cis* and 13-*cis* isomers has been achieved using silica-based polymeric ODS bonded-phase column packings, such as Vydac TP [48,121,142–145] and Spheri-5 ODS [127,146,147]. Vydac TP and Spheri-5 ODS column packings have very different particle characteristics and surface coverages, as shown in Table 20.8. It appears that the polymeric surface configuration, which is common to Vydac TP and Spher-5 ODS, is responsible for the separation of the *cis–trans* isomers of β-carotene, endcapping being unimportant.

Using the polymeric Vydac 201 TP C_{18} column, Chen and Chen [133] obtained the optimum separation of *cis* isomers of α- and β-carotene using a mobile phase of methanol/dichloromethane (99:1). Figure 20.15 shows the separation of all-*trans*-β-carotene, four β-carotene *cis* isomers (9-*cis*, 13-*cis*, 15-*cis*, and 13,15-di-*cis*), all-*trans*-α-carotene, 9-*cis*- plus 13-*cis*-α-carotene (unresolved), and 15-*cis*-α-carotene. Chromatograms of carotene isomers from fresh and cooked carrots are shown in Figure 20.16.

TABLE 20.7

HPLC Methods Used for the Determination of Provitamin A Carotenoids in Food

	Quantitative HPLC					
Food	**Sample preparation**	**Column**	**Mobile phase**	**Compounds separated**	**Detection**	**Ref.**
Normal-phase chromatography						
Medical foods	Extract sample by matrix solid-phase dispersion. Elute β-carotene from C_{18} column with 7 ml hexane containing 0.5% 2-PrOH, followed by 7 ml CH_2Cl_2/ethyl acetate/hexane (3 + 3 + 4) containing 0.5% 2-PrOH. Evaporate pooled eluates under nitrogen, dissolve residue in hexane	LiChrosorb Si-60 5 μm 250 × 4.6 mm	0.125% 2-PrOH in hexane	β-Carotene	Vis 436 nm	[126]
Reversed-phase chromatography						
Raw and cooked vegetables	Extract sample with THF containing 0.01% BHT in a blender. Add echinenone (internal standard). Stir under nitrogen with solid sodium sulfate and solid magnesium carbonate. Filter under suction, re-extract filter cake with THF until filtrate is colorless. Evaporate pooled filtrates under vacuum, dissolve residue in THF	Spheri-5 ODS 5 μm 220 × 4.6 mm	MeCN/CH_2Cl_2/MeOH, 70:20:10	α-Carotene, all-*trans*-β-carotene (separated from *cis*-β-carotenes), echinenone (internal standard)	Vis 450 nm	[127]

Leafy vegetables, sweet potatoes, mangoes	Extract homogenized material with solid sodium sulfate, solid calcium carbonate, and THF containing 0.01% BHT using a rod mixer. Filter through paper, re-extract filter cake with THF until filtrate is colorless. Evaporate under vacuum, dilute to volume with THF/MeOH, 1 + 1	Vydac 218 TP54 C_{18} 5 μm (metal frits replaced with PAT (Peek alloyed with Teflon) 250 × 4.6 mm	MeOH/THF, 98:2	All-*trans*-α-carotene, all-*trans*-β-carotene, *cis*-β-carotene	Vis 450 nm	[128]
Raw and cooked vegetables, salads, fruits	Extract freeze-dried samples with MeOH, filter under suction, re-extract filter cake with THF until filtrate is colorless. Evaporate under vacuum, partition concentrated extract between petroleum ether and water, dry organic layer with sodium sulfate, evaporate, dissolve residue in HPLC mobile phase	Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm	MeOH/THF, 95:5	Lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, lycopene	Vis 445 nm	[129]
Olive oil	Saponify (ambient), extract with diethyl ether, wash pooled extracts twice with phosphate buffer (pH 7.4) and once with water, dry with sodium sulfate. Evaporate under vacuum, dissolve residue in 1,2-dichloroethane	Supelcosil LC18 5 μm 150 × 4.6 mm	MeCN/2-PrOH/1,2-dichloroethane, 92.5:5.0:2.5	α- and β-Carotenes	Vis 458 nm	[130]

(*Table continued*)

TABLE 20.7 *Continued*

	Quantitative HPLC					
Food	**Sample preparation**	**Column**	**Mobile phase**	**Compounds separated**	**Detection**	**Ref.**
Vegetables	Saponify (hot), extract with diisopropyl ether, wash pooled extracts with 5% KOH solution and then with water to neutral pH. Evaporate under vacuum, dissolve residue in HPLC mobile phase	Hypersil-ODS 3 μm 250 × 4.6 mm	MeCN/MeOH/$CHCl_3$/H_2O, 250 +200 + 90 + 11	β-Carotene	Vis 445 nm	[131]
Malaysian vegetables	Saponify (hot), extract with hexane, wash pooled extracts to neutral pH, evaporate under nitrogen, dissolve residue in HPLC mobile phase	μBondapak C_{18} 10 μm 300 × 3.9 mm	MeCN/MeOH/ethyl acetate, 88:10:2	β-Cryptoxanthin, γ-, α-, β-carotenes	Vis 436 nm	[132]
Vegetables, fruits	Saponify (hot), extract with hexane, wash pooled extracts with water. Evaporate under vacuum, dissolve residue in hexane. Load extract onto magnesia column, elute carotenes and cryptoxanthins with hexane/acetone (90 + 10). Evaporate, dissolve residue in $CHCl_3$, add Sudan I as internal standard, dilute to volume with HPLC mobile phase	Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm	MeOH/$CHCl_3$, 94:6	Sudan I, zeinoxanthin, β-cryptoxanthin, α-carotene, all-*trans*-β-carotene (separated from *cis*-β-carotenes)	Vis 475 nm	[48]

Carrots	Shake sample with hexane/acetone/absolute EtOH/toluene (10:7:6:7) + 40% methanolic KOH. Leave for 16 h in the dark for saponification, extract with hexane, evaporate, dissolve residue in hexane	Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm	MeOH/CH_2Cl_2, 99:1	α- and β-Carotenes (separated from their 15-*cis* isomers and from 13-*cis*-β-carotene)	Vis 450 nm	[133]
Dark green vegetables	Blanch leaves in boiling water. Homogenize with water containing 0.5% ascorbic acid. Extract an aliquot of the resultant mixture with acetone/petroleum ether (3:2) containing 0.5% BHT by shaking for 10 min. Re-extract until colorless (three extractions). Add ethanolic KOH to pooled extracts, stand for 15 min (saponification). Wash saponified extract with 10% sodium chloride solution, then with water to remove acetone, dry with sodium sulfate. Evaporate under vacuum, dissolve residue in MeOH/CH_2Cl_2 (9:1) containing 0.5% BHT	Vydac 201 TP C_{18} 5 μm 250 × 4.6 mm	MeOH/CH_2Cl_2/H_2O, 80:15.2:4.8	All-*trans*-, 9-*cis*-, 13-*cis*-β-carotene, all-*trans*-α-carotene	Vis 450 nm	[134]

(*Table continued*)

TABLE 20.7 *Continued*

	Quantitative HPLC					
Food	**Sample preparation**	**Column**	**Mobile phase**	**Compounds separated**	**Detection**	**Ref.**
Milk	Mix 3 ml milk with 6 ml 2-PrOH. Extract twice with hexane, centrifuge. Wash pooled extracts with 0.47 *M* sodium sulfate, evaporate under nitrogen. Determine the lipid content of the extract by weight. Evaporate a portion of the hexane extract, dissolve residue in 1 ml 95% EtOH containing 12.5 mg/ml pyrogallol. Add 1 ml 60% KOH, saponify at 30°C, extract three times with hexane, centrifuge. Evaporate, dissolve residue in hexane/CH_2Cl_2, 95:5	Chromsep-ChromSpher PAH, 5 μm 100 × 3 mm (two columns connected in series)	MeCN/MeOH/CH_2Cl_2, 80:14:6	β-Carotene	Vis 450 nm	[135]
Chinese vegetables	Extract sample with hexane/acetone/EtOH/toluene (10:7:6:7) + solid magnesium carbonate + internal standard (β-apo 8′-carotenal) in a blender, filter under vacuum. Saponify (ambient), extract with hexane, evaporate, dissolve residue in ethyl acetate	Ultramex C-18 5 μm 250 × 4.6 mm	MeCN/MeOH/ethyl acetate, 75:15:10	β-Apo 8′-carotenal (interal standard), β-cryptoxanthin, α- and β-carotenes	Vis 450 nm	[136]

Vegetables, fruits	Homogenize sample with THF/MeOH (1:1) + solid magnesium carbonate + internal standard (β-apo-8′-carotenal or, for green vegetables, echinenone), filter. Partition into petroleum ether containing 0.1% BHT. Evaporate under vacuum, dissolve residue in CH_2Cl_2. *Saponification*: (applicable to fruits and peppers). Saponify (ambient), extract with petroleum ether containing 0.1% BHT, wash pooled extracts to neutral pH. Evaporate under vacuum, dissolve residue in CH_2Cl_2	Vydac 201 TP54 C_{18} 5 μm (metal frits replaced with Teflon frits) 250 × 4.6 nm	MeCN/MeOH/CH_2Cl_2 (75:20 15) containing 0.1% BHT and 0.05% triethylamine (the MeOH contains 0.05 *M* ammonium acetate)	β-Cryptoxanthin, α-carotene, all *trans*-β-carotene (separated from *cis*-β-carotenes), internal standard	Vis 450 nm	[137]
Vegetables, fruits	Homogenize freeze-dried sample with THF/MeOH (1:1) + solid magnesium carbonate + internal standard (β-apo-8′-carotenoate) at 0°C, centrifuge. *Saponification*: (if β-cryptoxanthin needs to be determined). Saponify (ambient), extract with petroleum ether, wash pooled extracts to neutral pH, evaporate, dissolve residue in MeOH/THF, 75:25	Vydac 201 TP C_{18} 5 μm (Hastalloy frits replaced with PAT (Peek alloyed with Teflon frits) 250 × 4.6 mm	MeOH/THF, 95:5	Internal standard, all-*trans*-α-carotene, all-*trans*-β-carotene, (separated from their *cis* isomers)	Vis 450 nm	[138]

(*Table continued*)

TABLE 20.7 *Continued*

	Quantitative HPLC					
Food	**Sample preparation**	**Column**	**Mobile phase**	**Compounds separated**	**Detection**	**Ref.**
Vegetables	Supercritical fluid extraction	Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm	MeOH/MeCN/ CH_2Cl_2/hexane, 65:27:4:4	α- and β-Carotenes	Vis 450 nm	[42]
Fresh and processed fruits and vegetables	Homogenize pureed tissue with water, solid calcium carbonate, and Celite then extract with MeOH. Filter through paper under vacuum, re-extract filter cake with acetone/hexane (1:1) until colorless. Wash hexane layer with water, dry with sodium sulfate. *Separate extraction procedure for orange juice:* vortex samples with MeOH, centrifuge, filter. Extract pellets with acetone/ hexane (1:1) until colorless. Wash hexane layer with water, dry with sodium sulfate.	Polymeric C_{30} 5 μm 250 × 4.6 mm	MeOH/MTBE, 89:11	All-*trans*-α-carotene, all-*trans*-β-carotene (separated from their *cis* isomers)	Vis 410 nm	[139]

	Saponification: applicable to hexane extracts (prepared as stated above) of broccoli, collards, orange juice, peaches, spinach, vegetable soup. Saponify (ambient), wash hexane layer to neutral pH. *Solid-phase extraction:* applicable to extracts of carrot, orange juice, peach, tomato, vegetable soup. Load hexane extracts onto alumina *N* solid-phase extraction cartridge, wash with hexane, elute α- and β-carotenes with hexane/acetone (96.5:3.5), elute β-cryptoxanthin with acetone. Evaporate collected fractions, dissolve residues in hexane

BHT, butylated hydroxytoluene; MeOH, methanol; EtOH, ethanol; 2-PrOH, 2-propanol; MeCN, acetonitrile; CH_2Cl_2, dichloromethane (methylene chloride); $CHCl_3$; chloroform; THF, tetrahydrofuran; MTBE, methyl *tert*-butyl ether.

TABLE 20.8

Properties of Polymeric ODS Bonded-Phase Column Packings

	Vydac 218 TP[a]	**Vydac 201 TP[a]**	**Spheri-5-ODS[b]**
Surface configuration	Polymeric	Polymeric	Polymeric
Endcapped	Yes	No	Yes
Percentage carbon loading (w/w)	9	9	14
Mean pore diameter (Å)	300	300	80
Specific surface area (m^2/g)	80	80	200

[a]Separations Group.
[b]Brownlee Laboratories.

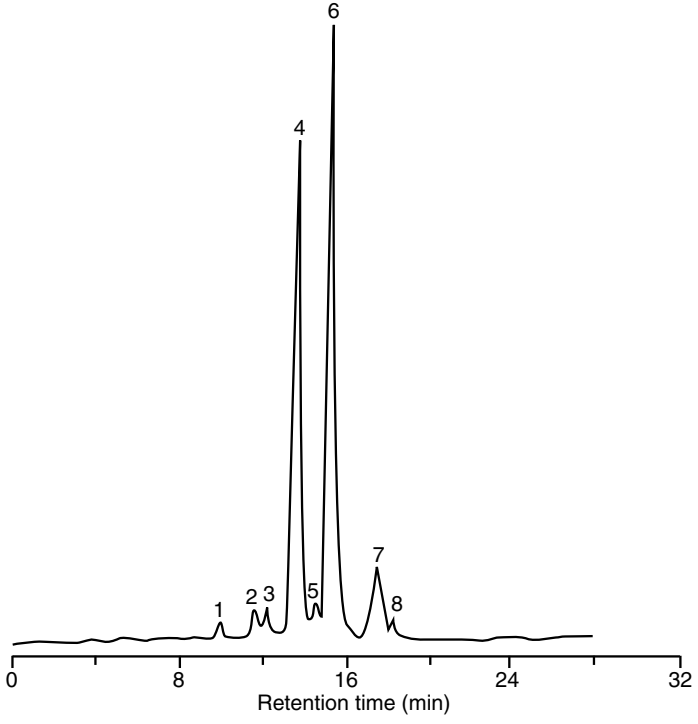

FIGURE 20.15
Reversed-phase HPLC of isomers of α-carotene and β-carotene. Operating parameters as in Table 20.7 [133]. Peaks: (1) 15-*cis*-α-carotene; (2) 13,15-di-*cis*-β-carotene; (3) 15-*cis*-β-carotene; (4) all-*trans*-α-carotene; (5) 9-*cis*-α-carotene plus 13-*cis*-α-carotene; (6) all-*trans*-β-carotene; (7) 13-*cis*-β-carotene; (8) 9-*cis*-β-carotene. (From Chen, T.M. and Chen, B.H., *Chromatographia*, 39, 346, 1994. With permission.)

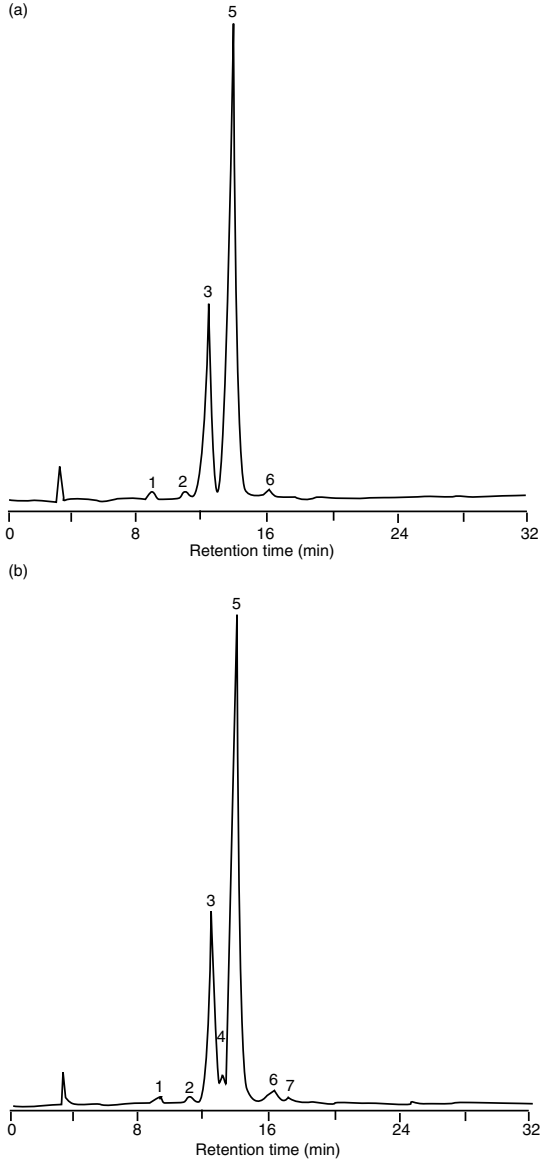

FIGURE 20.16
Reversed-phase HPLC of carotenes extracted from (a) fresh carrots and (b) cooked carrots. Operating parameters as in Table 20.7 [133]. Peaks: (1) 15-*cis*-α-carotene; (2) 15-*cis*-β-carotene; (3) all-*trans*-α-carotene; (4) 9-*cis*-α-carotene plus 13-*cis*-α-carotene; (5) all-*trans*-β-carotene; (6) 13-*cis*-β-carotene; (7) 9-*cis*-β-carotene. (From Chen, T.M. and Chen, B.H., *Chromatographia*, 39, 346, 1994. With permission.)

After cooking for 30 min, two new peaks appeared, attributable to 9-*cis*-β-carotene and 9-*cis*- and 13-*cis*-α-carotene (unresolved).

Nyambaka and Ryley [134] used a Vydac 201 TP column and a mobile phase of methanol/dichloromethane/water (80:15.2:4.8) to separate carotene isomers in dark green vegetables. Typical chromatograms from sample extracts are shown in Figure 20.17. Three isomers of β-carotene (all-*trans*, 9-*cis*, and 13-*cis*) were resolved in all the extracts studied, except the cowpea leaves which also contained all-*trans*-α-carotene.

20.5.2.5.2 C_{30}-Bonded Phases

Sander et al. [148] obtained a high degree of selectivity toward the *cis–trans* isomers of β-carotene using a 5-μm polymeric C_{30}-bonded phase prepared without endcapping. (The 250 × 4.6-mm-ID column used in this study is available commercially.) Further studies [149,150] confirmed that the C_{30} phase was superior to C_{18} phases commonly employed for carotenoid analysis. Among reversed-phase materials, the C_{30} phase is uniquely capable of resolving geometric isomers of asymmetric carotenoids. For example, the predominant geometric isomers of α-carotene were separated in the following order of elution: 13-*cis*, 13′-*cis*, all-*trans*, 9-*cis*, and 9′-*cis* [150]. Chromatograms of carotenoids extracted from raw and thermally processed carrots are shown in Figure 20.18. The 9′-*cis* isomer of α-carotene coelutes with all-*trans*-β-carotene under the chromatographic conditions employed. (*Note*: The thermal treatment used in the processing was more intensive than that used commercially.)

Retention characteristics and elution order of carotenoid *cis–trans* isomers with C_{30}-bonded phases are strikingly similar to those obtained with normal-phase systems using calcium hydroxide columns [149]. Different carotenoids exhibit varying retention behavior in response to temperature changes for C_{30} and C_{34} polymeric stationary phases as compared to a C_{18} polymeric phase [141]. These behaviors are believed to be related to conformational changes in the longer stationary phases with temperature. The "slot model" proposed for the retention of planar and nonplanar polycyclic aromatic hydrocarbons [74] could account for the excellent shape selectivity of the C_{30} polymeric phase toward carotenoid *cis–trans* isomers and the preferential retention of lycopene. Lycopene, being acyclic, is relatively planar compared to β-carotene and therefore fits more easily into slots within the rigid polymeric stationary phase. It is hypothesized that C_{18} polymeric phases possess insufficient thickness to permit complete penetration of carotenoid molecules. Only part of the molecule interacts with the bonded phase, and poorer isomer separation results.

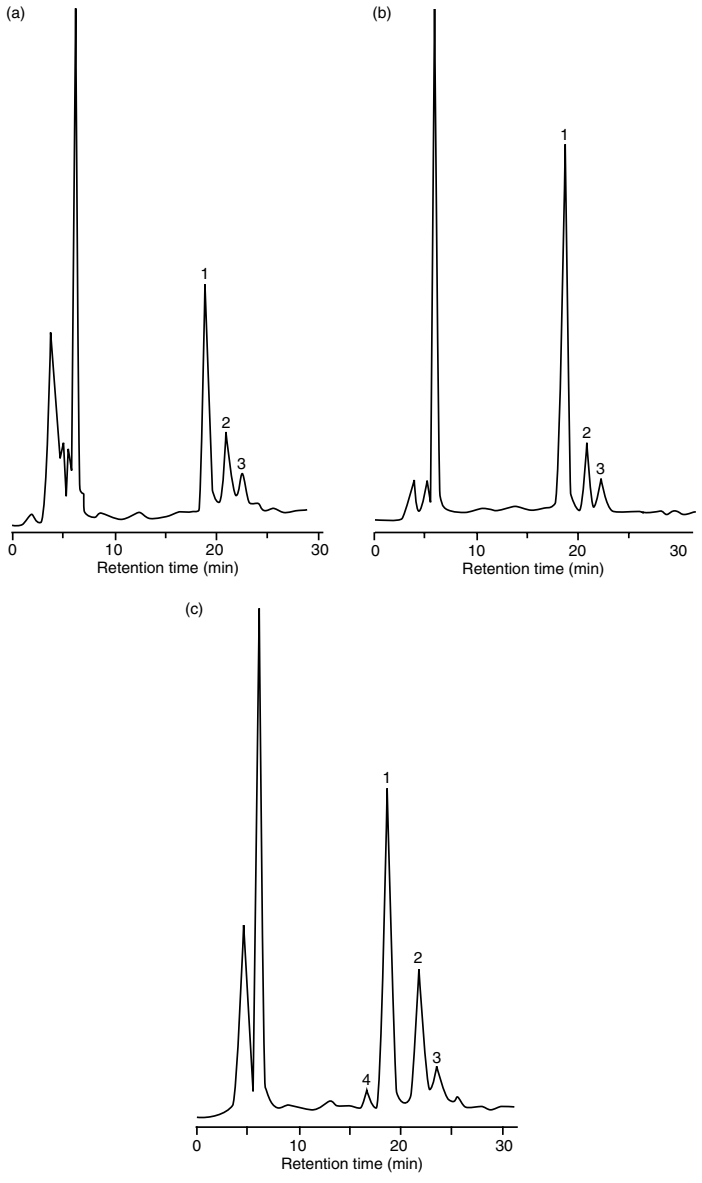

FIGURE 20.17
Reversed-phase HPLC of carotenes extracted from dark green vegetables: (a) spring cabbage, (b) spinach, and (c) cowpea leaves. Operating parameters as in Table 20.7 [134]. Peaks: (1) all-*trans*-β-carotene; (2) 9-*cis*-β-carotene; (3) 13-*cis*-β-carotene; (4) all-*trans*-α-carotene. (Reprinted from Nyambaka, H. and Ryley, J., *Food Chem.*, 55, 63–72, © 1996, with permission from Elsevier.)

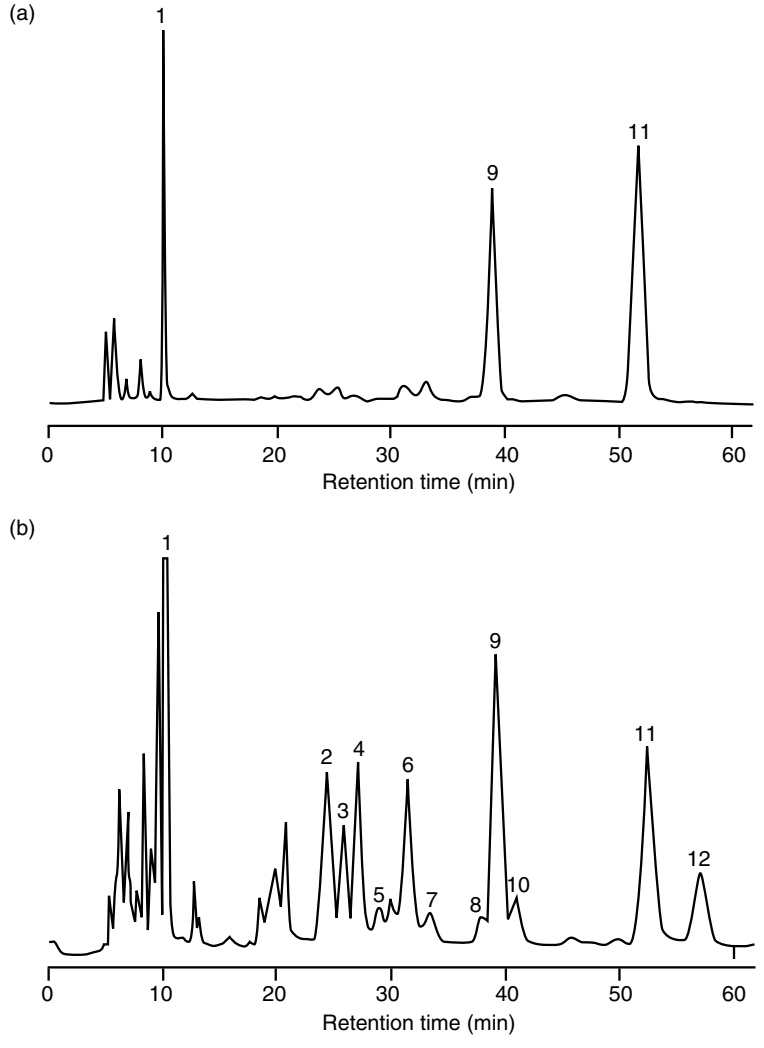

FIGURE 20.18
Reversed-phase HPLC of carotenoids extracted from (a) raw and (b) thermally processed carrots. Column, 5-μm polymeric C_{30}; mobile phase, methanol and methyl *tert*-butyl ether (89:11); Vis detection, 453 nm. Tentative peak identifications: (1) all-*trans*-lutein; (2) 13-*cis*-α-carotene; (3) a *cis*-α-carotene isomer; (4) 13′-*cis*-α-carotene; (5) 15-*cis*-β-carotene; (6) 13-*cis*-β-carotene; (7, 8) *cis*-β-carotene isomers; (9) all-*trans*-α-carotene; (10) 9-*cis*-α-carotene; (11) all-*trans*-β-carotene; (12) 9-*cis*-β-carotene. (Reprinted with permission from Emenhiser, C., Sander, L.C., and Schwartz, S.J., *J. Agric. Food Chem.*, 44, 3887, © 1996 American Chemical Society, With permission.)

20.5.3 Vitamin D

Selected methods for determining vitamin D are presented in Table 20.9.

20.5.3.1 Detection

Vitamins D_2 and D_3 exhibit identical UV absorption spectra, with λ_{max} at 265 nm (Figure 20.19) and an ε value of 18,000 in ethanol or hexane. The ε value is less than that predicted for a conjugated *cis*-triene structure because the degree of conjugation is reduced by the C-19 methylene group being above the plane of the other two double bonds. Reported on-column detection limits range from 1 to 10 ng [1,167,168].

20.5.3.2 Quantification

The most effective means of removing the vast excess of triglycerides from samples of full-fat foods is saponification. Hot saponification promotes thermal isomerization of vitamin D, with the formation of previtamin D. During subsequent chromatographic cleanup, either the previtamin D is separated and discarded, or it is retained in the final sample extract. In the latter event, the previtamin D peak in the chromatogram is far removed from the vitamin D peak when either normal-phase or reversed-phase chromatography is used [169]. Unfortunately, the smaller previtamin D peak cannot usually be measured because of interference from coeluting contaminants. Thus, in practice, the potential vitamin D content cannot be obtained by measuring the amounts of previtamin D and vitamin D and adding them together.

The reversibility of the isomerization reaction is very slow, and therefore the percentage of the previtamin will remain virtually unchanged during the subsequent stages of the analytical procedure. This equilibrium allows the potential vitamin D to be calculated from measurements of the vitamin D peak alone, and the same principle applies to the hydroxylated metabolites of vitamin D [170]. In an HPLC method that involved saponification at 80°C for 45 min [171], a vitamin D_3 external standard subjected to the entire analytical procedure gave 80% vitamin D_3 on analysis. Therefore, results for the samples were multiplied by a factor of 1.25 to allow for the formation of previtamin D and give a result for the potential vitamin D content of the sample.

Another way of determining the potential vitamin D is to saponify a standard vitamin D solution in parallel to the sample and use the resultant solution as an external standard in the quantification [79]. Alternatively, if vitamins D_2 and D_3 can be separated during quantitative HPLC, one of these vitamers can be used as an internal standard to quantify the other vitamer. Because the isomerization rates of vitamins D_2 and D_3 are virtually equal, the previtamin D to vitamin D ratio will be the

TABLE 20.9

HPLC Methods Used for the Determination of Vitamin D in Food

Food	Sample Preparation	Semipreparative HPLC	Quantitative HPLC	Compounds Separated	Ref.
Normal-phase chromatography					
Infant formula	Saponify (ambient), extract with hexane, wash pooled extracts to neutral pH, dry with sodium sulfate. Concentrate extract to ca. 5 ml on rotary evaporator, transfer to tube, evaporate under nitrogen, dissolve residue in MeOH. Convert vitamin D to isotachysterol with acidified butanol. Add saturated sodium carbonate solution to destroy excess reagent. Extract isotachysterol with $CHCl_3$, dry all extracts with sodium sulfate, evaporate extract, dissolve residue in MeOH	Supelcosil LC-18-DB 5 μm 250 × 4.6 mm MeCN/MeOH, 90:10. UV 301 nm Collect isotachysterol fraction, evaporate under nitrogen, dissolve residue in hexane	Spherisorb silica 5 μm 250 × 2 mm. Hexane/ethyl acetate/MeOH, 97:2.5:0.5. UV 301 nm	Isotachysterols D_2 or D_3 (same retention time)	[152]
Reversed-phase chromatography					
Milk (unfortified)	Add vitamin D_2 to sample as internal standard. Saponify (ambient), extract with hexane/diethyl ether (90:10), wash pooled extracts to neutral pH. Evaporate under vacuum, dissolve residue in hexane. Load extract onto silica solid-phase extraction cartridge, wash with 3 ml hexane/ethyl acetate (90:10), elute vitamin D with 5 ml hexane/ethyl acetate, 80:20. Evaporate eluate under nitrogen to ca. 100 μl	Radial-PAK cartridge containing Resolve silica 5 μm. Hexane/2-PrOH, 99:1. UV 265 nm. Collect vitamin D fraction, evaporate under nitrogen, dissolve residue in MeOH	Radial-PAK cartridge containing Resolve C_{18} 5 μm, column temperature 30°C MeOH/THF/H_2O, 93:2:5. UV 265 nm	Vitamins D_2 and D_3	[153]

Fortified milk	Saponify (ambient), extract with petroleum ether/diethyl ether (90:10), wash pooled extracts to neutral pH. Concentrate to 2–3 ml on rotary evaporator, evaporate under nitrogen, dissolve residue in hexane. Load extract onto silica solid-phase extraction cartridge, elute vitamin D with 5 ml hexane/chloroform, 21.5:78.5. Evaporate eluate under nitrogen, dissolve residue in MeCN	Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm. MeCN/MeOH, 90:10 UV 254 nm	Vitamins D_2 and D_3	[154]
Pasteurized process cheese (fortified)	As in preceding entry, except saponify at 70°C	Discovery C_{18} 5 μm 150 × 4.6 mm MeOH/MeCN, 70:30 UV 254 nm	Vitamin D_3	[155]
Infant formula	Add vitamin D_2 to sample as internal standard. Saponify (hot), extract with hexane, wash extract to neutral pH using 10% acetic acid solution. Evaporate under vacuum, dissolve residue in CH_2Cl_2/2-PrOH, 99.8 + 0.2. Load extract onto silica solid-phase extraction column, wash with 2 ml CH_2Cl_2/2-PrOH (99.9 + 0.2), elute vitamin D with 7 ml CH_2Cl_2/2-PrOH, 99.8 + 0.2. Evaporate eluate under nitrogen, dissolve residue in MeCN	Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm, column temperature 27°C MeCN/MeOH, 91:9 UV 265 nm	Vitamins D_2 and D_3	[156]

(*Table continued*)

TABLE 20.9 *Continued*

Food	Sample Preparation	Semipreparative HPLC	Quantitative HPLC	Compounds Separated	Ref.
Fortified milk	Add vitamin D_2 to sample as internal standard. Saponify (hot), extract with hexane, wash extract to neutral pH. Evaporate under vacuum, dissolve residue in hexane. Load extract onto Florisil solid-phase extraction cartridge, wash with 5 ml hexane, elute vitamin D with 5 ml 2-PrOH. Evaporate eluate under nitrogen, dissolve residue in MeCN		Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm. MeCN/MeOH, 97+3 UV 265 nm	Vitamins D_2 and D_3	[157]
Margarine, fats, and oils	Add vitamin D_2 to sample as internal standard. Saponify (hot), extract with diethyl ether, wash extract to neutral pH. Evaporate under vacuum, dissolve residue in hexane/2-PrOH/THF, 99:0.5:0.5	LiChrosorb Si-60 7 μm 250 × 4.6 mm Hexane/2-PrOH/THF, 98:1:1. UV 264 nm. Collect vitamin D fraction, evaporate under vacuum, dissolve residue in MeCN/$CHCl_3$/MeOH, 94:4:2	ChromSphere C_{18} 8 μm 100 × 3 mm. MeCN/$CHCl_3$/MeOH, 91:6:3 UV 264 nm	Vitamins D_2 and D_3	[158]

Fortified milk, infant formula, gruel, margarine, cooking oil, fish oil	Add vitamin D_2 to sample as internal standard. Saponify (hot), extract with heptane, wash pooled extracts to neutral pH. Evaporate under vacuum, dissolve residue in cyclohexane/heptane, 1 + 1	Nucleosil 50–5 5 μm 250 × 4.6 mm, column temperature 35°C 0.5% 2-PrOH and 2% MTBE in cyclohexane/heptane, 1+1. UV 265 nm. Collect vitamin D fraction, evaporate under vacuum, dissolve residue in MeCN/MeOH, 80 + 20	Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm. MeCN/MeOH, 80 + 20 UV 265 nm	Vitamins D_2 and D_3	[159]
Margarine, vegetable oils, fortified milk	Add vitamin D_2 to sample as internal standard. Saponify (hot), extract with hexane, wash pooled extracts to neutral pH. Evaporate under vacuum, dissolve residue in isooctane/$CHCl_3$/THF, 88:10:2	Polygosil 60 5 μm 300 × 8 mm, column temperature 30°C Isooctane/$CHCl_3$/THF/isobutanol, 94:3:2:1. UV 254 nm. Collect vitamin D fraction, evaporate under nitrogen, dissolve residue in MeOH	Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm, column temperature 30°C MeCN/MeOH/$CHCl_3$, 82:12:6 UV 265 nm	Vitamins D_2 and D_3	[160]

(*Table continued*)

TABLE 20.9 *Continued*

Food	Sample Preparation	Semipreparative HPLC	Quantitative HPLC	Compounds Separated	Ref.
Infant formula	Add vitamin D_2 to sample as internal standard. Saponify (hot), extract with petroleum ether/diethyl ether (90:10), wash pooled extracts to neutral pH, dry by passing through phase-separating filter. Evaporate under vacuum, dissolve residue in isooctane	Polygosil 60 5 μm 250 × 8 mm Isooctane/ isobutanol, 99:1 UV 265 nm Collect vitamin D fraction, evaporate under vacuum, dissolve residue in MeOH containing 50 mg/l BHT	Hypersil ODS 5 μm 250 × 4.6 mm (two columns connected in series) 100% MeOH UV 265 nm	Vitamins D_2 and D_3	[161]
Fish products	Add vitamin D_2 to sample as internal standard. Saponify (hot), extract with hexane/ethyl acetate (8 + 2), wash pooled extracts with 5% sodium chloride. Evaporate under vacuum, transfer extract to tube with hexane, evaporate under nitrogen, dissolve residue in hexane	μPorasil 10 μm 300 × 3.9 mm Hexane/THF/2-PrOH, 98:1:1. UV 265 nm. Collect vitamin D fraction, evaporate under nitrogen, dissolve residue in $MeOH/H_2O$, 93:7	Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm. $MeOH/H_2O$, 93:7. UV 265:nm	Vitamin D_3 and D_2 (internal standard)	[162]

Emulsified nutritional supplements	Dissolve 10-g sample in 0.2 *M* KH_2PO_4 containing 1 m*M* EDTA. Load solution onto C_{18} solid-phase extraction cartridge, wash with 10 ml water, followed by 5 ml 10% aqueous MeOH, elute vitamin D with 10 ml MeOH. Evaporate under vacuum, dissolve residue in MeOH	Hitachigel 3011–0 5 μm 100 × 4.6 mm precolumn. Wash column with MeOH. Flush adsorbed material onto the analytical column with MeCN/MeOH, 75:25 at 40°C by column switching	Inertsil ODS-2 5 μm 150 × 4.6 mm UV 265 nm	Vitamin D_2	[163]
Nutritionally complete liquid-formula diet	Saponify (hot), extract with diethyl ether, wash pooled extracts to neutral pH. Add vitamin D_2 as internal standard. Evaporate under vacuum, dissolve residue in hexane, evaporate under nitrogen, redissolve in hexane	Nucleosil 50–5 5 μm 250 × 4.6 mm 0.05% 2-PrOH in hexane. UV 265 nm. Collect vitamin D fraction, evaporate under nitrogen, dissolve residue in MeOH	Hitachi Gel 3056 reversed- phase column 5 μm 250 × 4.6 mm. MeCN/MeOH/50% perchloric acid (970 + 30 + 1.2) containing 0.057 *M* sodium perchlorate Dual-cell electrochemical detector (redox mode): +0.65 V (oxidation) −0.20 V (reduction)	Vitamins D_2 and D_3	[164]

(*Table continued*)

TABLE 20.9 *Continued*

Food	Sample Preparation	Semipreparative HPLC	Quantitative HPLC	Compounds Separated	Ref.
Egg yolk	Add vitamin D_2 and 25(OH)D_2 to homogenized sample as internal standards. Saponify (ambient), extract with diethyl ether/petroleum ether (1:1), wash pooled extracts to neutral pH, dry with sodium sulfate, filter. Evaporate under vacuum, dissolve residue in hexane. Load extract onto silica solid-phase extraction cartridge, wash with 20 ml hexane followed by 50 ml 0.5% 2-PrOH in hexane. Elute vitamin D with 35 ml 0.5% 2-PrOH in hexane, wash with 50 ml 0.5% 2-PrOH in hexane, elute 25(OH)D with 40 ml 6% 2-PrOH in hexane. Evaporate the vitamin D and 25(OH)D fractions under vacuum, dissolve residues in hexane	*Vitamin D fraction*: μPorasil silica 10 μm 300 × 3.9 mm Hexane/THF/2-PrOH, 98:1:1. UV 264 nm. Collect vitamin D fraction, evaporate under nitrogen, dissolve residue in MeOH; *25(OH)D fraction*: μPorasil silica 10 μm 300 × 3.9 mm Hexane/2-PrOH, 97:3. UV 264 nm Collect 25(OH)D fraction, evaporate under nitrogen, dissolve residue in MeOH	*Vitamin D_3*: Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm. MeOH/H_2O, 94:6. UV 264 nm *25(OH)D_3*: Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm. MeOH/H_2O, 83:17. UV 264 nm	Vitamins D_2 and D_3 25(OH)D_2 and 25(OH)D_3	[55,165]

Raw meat and liver; milk and milk products	Add vitamin D_2 and 25(OH)D_2 to homogenized sample as internal standards. Saponify and extract samples as in preceding entry	μPorasil silica 10 μm 300 × 3.9 mm Gradient elution with hexane/2-PrOH. UV 265 nm. Collect vitamin D fraction and 25(OH)D fraction, evaporate under nitrogen, dissolve residues in MeOH/H_2O (93:7) and MeOH/H_2O (87:13), respectively.	*Vitamin D_3*: Zorbax ODS + Vydac 201 TP54 C_{18} (connected in series). MeOH/H_2O, 96:4. UV 265 nm *25(OH)D_3*: Spherisorb S5NH_2 5-μm 250 × 4.6 mm + μ Porasil 10 μm (connected in series) Hexane/2-PrOH, 97:3. UV 265 nm	Vitamins D_2 and D_3 25(OH)D_2 and 25(OH)D_3	[166]

TABLE 20.9 *Continued*

Food	Sample Preparation	Semipreparative HPLC	Quantitative HPLC	Compounds Separated	Ref.
		Vitamin D fraction: Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm MeOH/H_2O, 93:7 UV 265 nm Collect vitamin D fraction, evaporate under nitrogen, dissolve residue in MeOH/H_2O, 96:4. *25(OH)D fraction*: Vydac 201 TP54 C_{18} 5 μm. MeOH/H_2O, 83:17. UV 265 nm. Collect 25(OH)D fraction, evaporate under nitrogen, dissolve residue in hexane or 0.5% 2-PrOH in hexane			

BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; MeOH, methanol; 2-PrOH, 2-propanol; MeCN; acetonitrile; CH_2Cl_2, dichloromethane (methylene chloride); $CHCl_3$, chloroform; THF, tetrahydrofuran; MTBE, methyl *tert*-butyl ether.

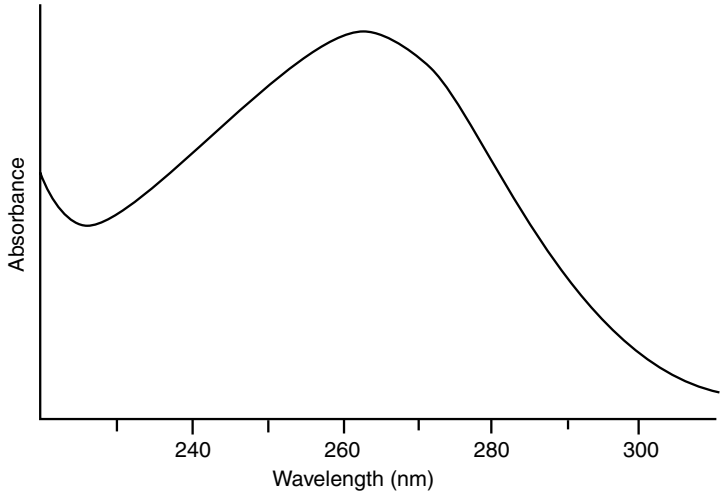

FIGURE 20.19
UV absorption spectrum of vitamin D_2 in ethanol. The absorption maximum occurs at 265 nm.

same for both vitamers at any given temperature. Therefore, the quantification will compensate for the formation of previtamin D. When using vitamin D_2 as an internal standard to quantify vitamin D_3 (or vice versa), it is necessary to analyze a separate portion of the test sample in parallel to confirm the absence of endogenous D_2.

In several methods (Table 20.9), the problem of thermal isomerization is avoided by saponifying overnight at ambient temperature.

In a method for determining added vitamin D_3 in pasteurized process cheese, Upreti et al. [155] were unable to use vitamin D_2 as an internal standard because of an interfering peak in the test extract. They prepared a standard curve by adding 1 ml of a series of vitamin D_3 standard solutions to 5 g of specially prepared unfortified cheese (spiked samples) and analyzing these samples in parallel with the fortified test samples. The areas of the vitamin D_3 peaks in the spiked samples were adjusted by deducting the area in the unfortified cheese without added vitamin D_3, and plotted against the amounts of vitamin D_3 actually added. This external standard technique has the merit of compensating for potential matrix interferences, whereby the presence of other compounds in the test extract may affect the retention and/or the peak size of the analyte.

20.5.3.3 Cleanup Procedures

The removal of sterols, vitamin E vitamers, carotenoids, and other interfering material from the unsaponifiable fraction of food samples has

been achieved using one or more of the following techniques: coprecipitation of sterols with digitonin [44], precipitation of sterols from a methanolic solution [46,47], adsorption chromatography on open columns of alumina [3,44,51], thin-layer chromatography on silica plates [46], and solid-phase extraction on silica [1,56] and reversed-phase [57] cartridges.

Semipreparative HPLC has been employed to obtain a vitamin D-rich fraction of the unsaponifiable matter for subsequent quantitative HPLC. Combinations of chromatographic modes used for offline semipreparative and quantitative analysis have included polar bonded-phase/adsorption [171,172] and reversed-phase/adsorption [173]. An online two-dimensional HPLC technique using two polar bonded-phase columns has also been described [174]. The ideal combination is adsorption/reversed-phase chromatography, because vitamins D_2 and D_3 coelute during the semipreparative stage, allowing a narrow retention window to be collected for analysis using vitamin D_2 as an internal standard. By this means, Johnsson et al. [160] obtained a vitamin D_3 detection limit of 0.1 μg/kg for milk and milk products.

20.5.3.4 Normal-Phase Separations

Normal-phase HPLC, using either silica or polar-bonded stationary phases, separates, isocratically, vitamin D_2 or D_3 from their respective previtamins and inactive isomers [169] (Figure 20.20). Vitamin D ($D_2 + D_3$), 25-hydroxyvitamin D_2, and 25-hydroxyvitamin D_3 can be separated from one other and from other hydroxylated metabolites [175], but vitamins D_2 and D_3 cannot be separated from each another. The inability to resolve vitamins D_2 and D_3 precludes the use of one D vitamer as an internal standard for the other.

In a novel method for determining vitamin D in infant formulas [152], previtamin D and vitamin D were converted to a common derivative, isotachysterol, with acidified butanol following saponification of the sample and extraction with hexane. This derivatization step circumvented the isomerization problem previously discussed. Derivatized extracts were purified by semipreparative reversed-phase HPLC and the collected fraction containing isotachysterol was analyzed by adsorption HPLC. The isotachysterol was detected at its λ_{max} of 301 nm, which provided greater sensitivity and selectivity compared with the detection wavelength of 265 nm usually used for vitamin D. Chromatograms of milk-based liquid infant formula on the reversed-phase and silica columns are shown in Figure 20.21 and Figure 20.22, respectively.

20.5.3.5 Reversed-Phase Separations

Reversed-phase chromatography also separates, isocratically, vitamin D_2 or D_3 from their respective previtamins and inactive isomers [169]

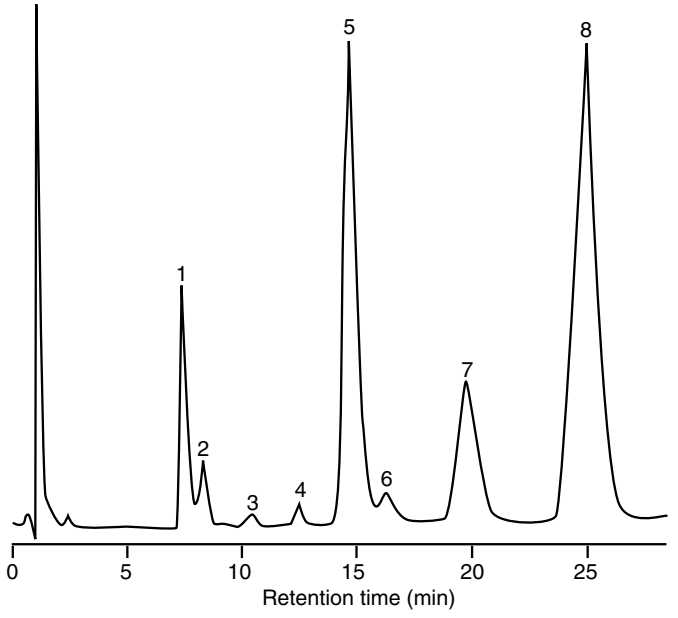

FIGURE 20.20
Normal-phase HPLC and UV detection of vitamin D_3 and its isomers. Column, LiChrosorb Si-60; mobile phase, 0.3% amyl alcohol in hexane; UV detection, 254 nm. Peaks: (1) previtamin D_3; (2) *trans*-vitamin D_3; (3) lumisterol$_3$; (4) isotachysterol$_3$; (5) vitamin D_3; (6) tachysterol$_3$; (7) 4,6-cholestadienol; (8) 7-dehydrocholesterol. (Reprinted from de Vries, E.J., Zeeman, J., Esser, R.J.E., Borsje, B., and Mulder, F.J., *J. AOAC Int.*, 62, 129–135, Copyright 1979 by AOAC International.)

(Figure 20.23) but, unlike normal-phase chromatography, it can separate vitamin D_2 from D_3 using nonendcapped stationary phases [156]. The 25-hydroxylated metabolites of vitamins D_2 and D_3 can be separated from one another using a Vydac 201 TP column [55]. The separation of vitamin D_2 from vitamin D_3, and 25-hydroxyvitamin D_2 from 25-hydroxyvitamin D_3, allows the D_2 form of the vitamin or its hydroxylated metabolite to be used as an internal standard for quantifying the corresponding D_3 form.

Renken and Warthesen [154] developed a method for determining the added vitamin D_3 in both skim and whole milk. The overall procedure involved overnight saponification at room temperature, cleanup by silica solid-phase extraction, and nonaqueous, reversed-phase HPLC. Figure 20.24 compares chromatograms of skim milk extracts with and without solid-phase cleanup. In skim milk, the main purpose of the cleanup step was to remove later eluting material which could interfere with subsequent analyses or necessitate an increased running time.

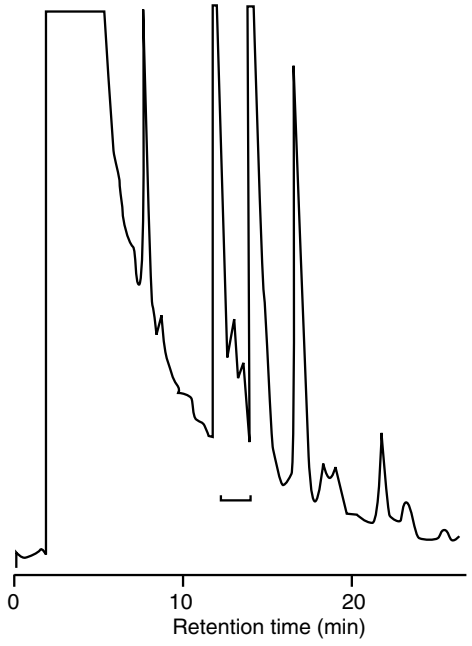

FIGURE 20.21
Semipreparative reversed-phase HPLC and UV detection of derivatized vitamin D (isotachysterol$_3$) in the unsaponifiable matter from a milk-based liquid infant formula. Operating parameters as in Table 20.9 [152]. The bracket shows the fraction collected. (Reprinted from Agarwal, V. K., *J. AOAC Int.*, 72, 1007–1009, Copyright 1989 by AOAC International. With permission.)

Figure 20.25 compares chromatograms of whole milk extracts with and without solid-phase cleanup. In this case, the cleanup step removed some of the material that eluted before vitamin D_3 and eliminated a peak adjacent to vitamin D_3.

Kurmann and Indyk [153] developed a method for determining endogenous levels of vitamin D_3 in bovine milk to study the influence of season. Samples with added internal standard (vitamin D_2) were saponified overnight at ambient temperature, extracted with hexane/diethyl ether (90:10), and purified by silica solid-phase extraction. Eluates were further purified by normal-phase semipreparative HPLC before analysis by reversed-phase HPLC. A typical reversed-phase chromatogram of a sample extract is shown in Figure 20.26.

In a method for determining vitamin D_3 in milk- and soy-based infant formulas [161], samples and internal standard (vitamin D_2) were saponified and extracted, purified by normal-phase semipreparative

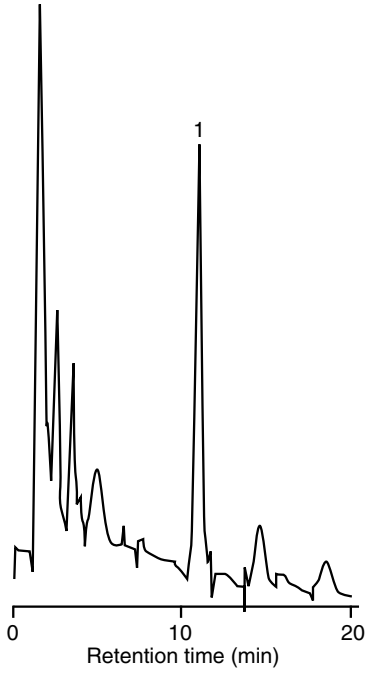

FIGURE 20.22
Quantitative normal-phase HPLC and UV detection of the isotachysterol$_3$ fraction collected by semipreparative HPLC (Figure 20.21). Operating parameters as in Table 20.9 [152]. Peak: (1) isotachysterol$_3$. (Reprinted from Agarwal, V.K., *J. AOAC Int.*, 72, 1007–1009, Copyright 1989 by AOAC International. With permission.)

HPLC and analyzed by reversed-phase HPLC. Chromatograms of the semipreparative and quantitative HPLC are shown in Figure 20.27 and Figure 20.28, respectively.

Sliva et al. [156] devised a method for determining vitamin D which involved saponification, extraction with hexane, solid-phase extraction cleanup, concentration, and reversed-phase quantitation. The method is applicable to milk-, soy-, and protein hydrolysate-based infant formulas and enteral nutritional products, both liquid and powder. Figure 20.29 is a chromatogram of a vitamin D_3 standard and vitamin D_2 internal standard that was taken through the sample preparation process. Chromatograms of a milk-based infant formula made specifically without vitamin D_3 fortification, and the same formulation, but with vitamin D_3 added, are shown in Figure 20.30. The method has been studied collaboratively [176] and adopted, first action 1995, by AOAC International [177].

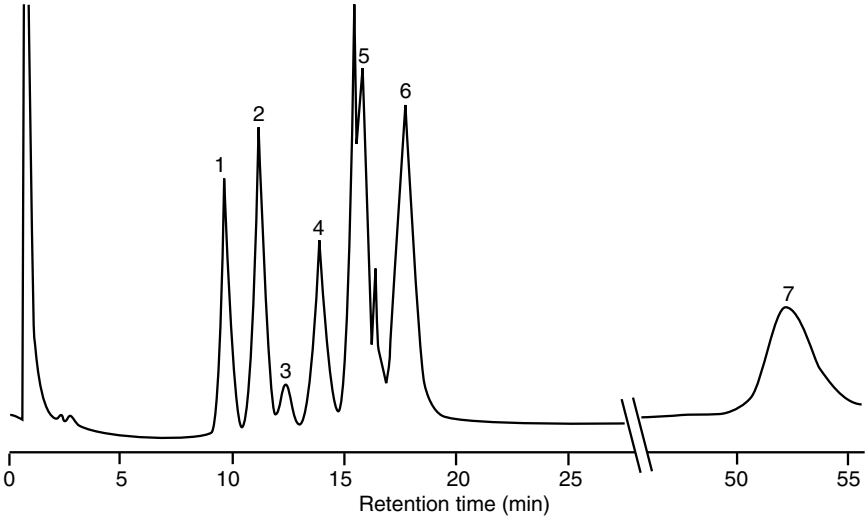

FIGURE 20.23
Reversed-phase HPLC of vitamin D_3 and its isomers. Column, LiChrosorb RP-18; mobile phase, acetonitrile, propionitrile, and water (79:15:6); UV detection, 254 nm. Peaks: (1) *trans*-vitamin D_3; (2) isotachysterol$_3$; (3) previtamin D_3; (4) tachysterol$_3$; (5) vitamin D_3; (6) lumisterol$_3$; (7) 7-dehydrocholesterol. (Reprinted from de Vries, E.J., Zeeman, J., Esser, R.J.E., Borsje, B., and Mulder, F.J., *J. AOAC Int.*, 62, 129–135, Copyright 1979 by AOAC International. With permission.)

A collaborative study presented by a Nordic Committee on Food Analysis for the determination of vitamin D_3 in selected foods [159] has been adopted, first action 2002, by AOAC International [178]. The method is based on the addition of vitamin D_2 as internal standard, followed by saponification and extraction with heptane. The fraction that contains vitamin D_2 and D_3 is isolated by semipreparative normal-phase HPLC and the analysis is completed by reversed-phase HPLC. Chromatograms from the semipreparative and quantitative columns are shown in Figure 2.31 and Figure 20.32, respectively.

Mattila et al. [166] described a procedure using two steps of semi-preparative HPLC for determining vitamin D_3 and 25-hydroxyvitamin D_3 in raw meat and liver. Samples were saponified in the presence of vitamin D_2 and 25-hydroxyvitamin D_2 as internal standards, and the extracted unsaponifiable matter was subjected to normal-phase semipreparative HPLC to obtain a fraction containing 25-hydroxyvitamin D_2 + 25-hydroxyvitamin D_3 and a fraction containing vitamin D_2 + vitamin D_3. The collected fractions were evaporated and purified by reversed-phase HPLC using Vydac 201 TP54 C_{18} columns. Fractions were again collected, after which vitamin D_3 was quantified by tandem-column

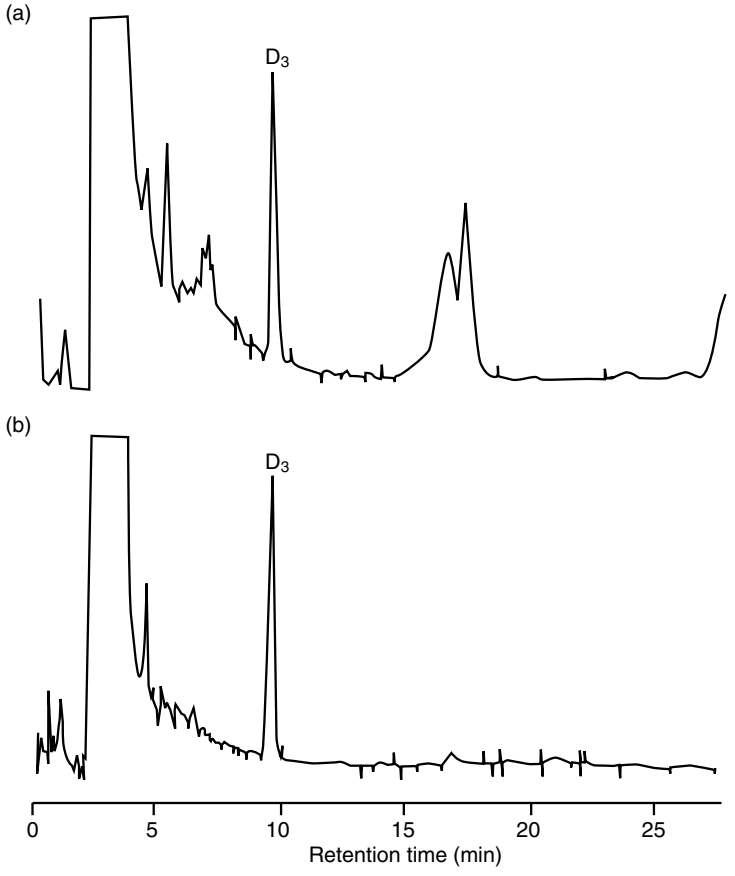

FIGURE 20.24
Reversed-phase HPLC and UV detection of vitamin D_3-fortified skim milk extracts (a) without and (b) with silica solid-phase cleanup. Operating parameters as in Table 20.9 [154]. (From Renken, S.A. and Warthesen, J.J., *J. Food Sci.*, 58, 552, 1993. With permission.)

reversed-phase HPLC and 25-hydroxyvitamin D_3 by tandem-column normal-phase HPLC. Analytical chromatograms of a purified extract of chicken are shown in Figure 20.33. Mattila et al. [179] summarized HPLC procedures developed in Finland for determining vitamin D in foods.

20.5.4 Vitamin E

Methods for estimating vitamin E values in foods are summarized in Table 20.10.

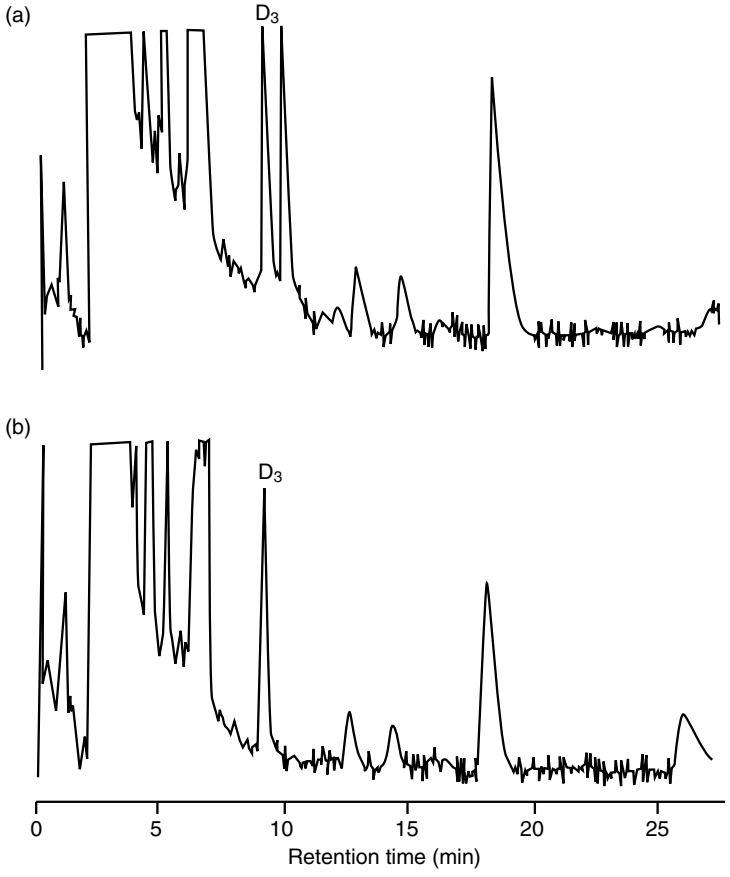

FIGURE 20.25
Reversed-phase HPLC and UV detection of vitamin D_3-fortified whole milk extracts (a) without and (b) with silica solid-phase cleanup. Operating parameters as in Table 20.9 [154]. (From Renken, S.A. and Warthesen, J.J., *J. Food Sci.*, 58, 552, 1993. With permission.)

20.5.4.1 Detection

It is fortuitous that indigenous concentrations of vitamin E in the principal food sources are on the order of mg rather than μg/100 g, for the tocopherols and tocotrienols exhibit relatively low intensities of UV absorption. Individual vitamers are characterized by a slightly different absorption maximum within the wavelength range of 292–298 nm in ethanol. Published $A^{1\%}_{1\,cm}$ values for the tocopherols (T) and tocotrienols (T3) at their λ_{max} in ethanol are: α-T, 71 at 294 nm; β-T, 86.4 at 297 nm; γ-T, 92.8 at 298 nm; δ-T, 91.2 at 298 nm; α-T3, 91 at 292.5 nm; β-T3, 87 at 296 nm [205]. The different absorption characteristics among the vitamers

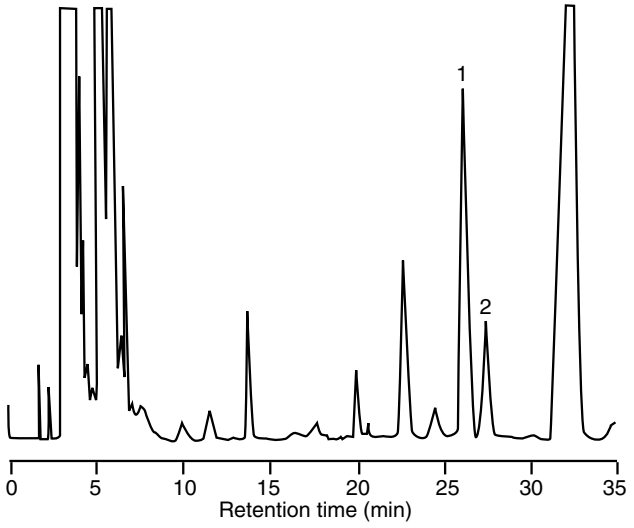

FIGURE 20.26
Reversed-phase HPLC and UV detection of vitamin D extracted from unfortified bovine milk. Operating parameters as in Table 20.9 [153]. Peaks: (1) vitamin D_2 (internal standard); (2) vitamin D_3. (Reprinted from Kurmann, A. and Indyk, H., *Food Chem.*, 50, 75–81, © 1994, with permission from Elsevier.)

necessitate the running of individual standards for accurate quantitation of each vitamer. The absorption intensity of α-tocopheryl acetate is lower still with an $A_{1\,cm}^{1\%}$ value of only 40–44 at the λ_{max} of 285.5 nm [206]. Reported minimum detectable quantities of α-, β-, γ-, and δ-tocopherols at 295 nm are, respectively, 50, 70, 90, and 130 ng [207]. Figure 20.34 and Figure 20.35 show the absorption spectra of α-tocopherol and α-tocopheryl acetate, respectively, in ethanol.

Unesterified α-tocopherol displays fluorescence excitation and emission spectra with wavelength maxima at 295 and 330 nm, respectively. The fluorescence maxima of the other tocopherols are at longer wavelengths, in accordance with their absorbance spectra. The fluorescence activity of α-tocopheryl acetate is very weak, and the excitation and emission maxima of 285 and 310 nm (Figure 20.36) are even closer together than those for α-tocopherol. Only those spectrofluorometers equipped with a high-energy (150-W) xenon lamp and narrow-band excitation and emission monochromators are capable of stimulating and measuring the weak fluorescence [25].

Thompson and Hatina [186] showed that the sensitivity of a fluorescence detector toward unesterified vitamin E compounds under normal-phase conditions was at least 10 times greater than that of a

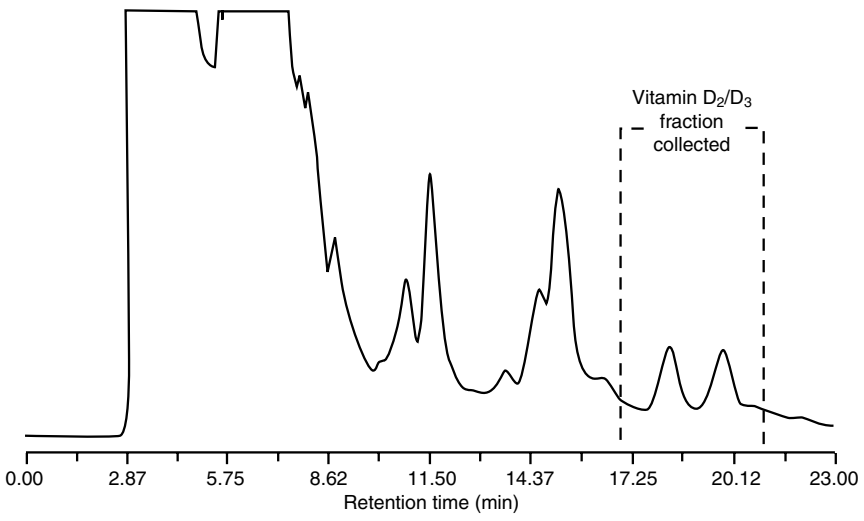

FIGURE 20.27
Semipreparative normal-phase HPLC and UV detection of vitamin D in the unsaponifiable matter from a vitamin D_3-fortified infant formula. Operating parameters as in Table 20.9 [161]. (Reprinted from Konings, E.J.M., *Neth. Milk Dairy J.*, 48, 31–39, © 1994, with permission from Elsevier.)

variable-wavelength absorbance detector. The relative fluorescence responses of the tocopherols at 290 nm (excitation) and 330 nm (emission), as measured by HPLC peak area, were α-T, 100; β-T, 129; γ-T, 110; and δ-T, 122. The fluorescence responses of the corresponding tocotrienols were very similar to those of the tocopherols, and therefore tocotrienol standards were not needed for calibration purposes. The fluorescence detector also allows the simultaneous monitoring of ubiquinone derivatives, for example, ubiquinone-10 has been detected in tomato [208].

The fluorescent intensities of the E vitamers are highly dependent on the solvent. Polar solvents such as diethyl ether and alcohols provide greater intensities compared with hexane. The inclusion of an ether or an alcohol in the hexane mobile phase increases the sensitivity of vitamin E detection measurably in normal-phase HPLC. The fluorescence is negligible when the compounds are dissolved in chlorinated hydrocarbons [209]. Hewavitharana [210] stressed the importance of adequately degassing the mobile phase when fluorescence detection is used in normal-phase chromatography.

The fluorescence detector response to α-tocopherol can be increased by up to 20-fold in some instruments by using short-wavelength

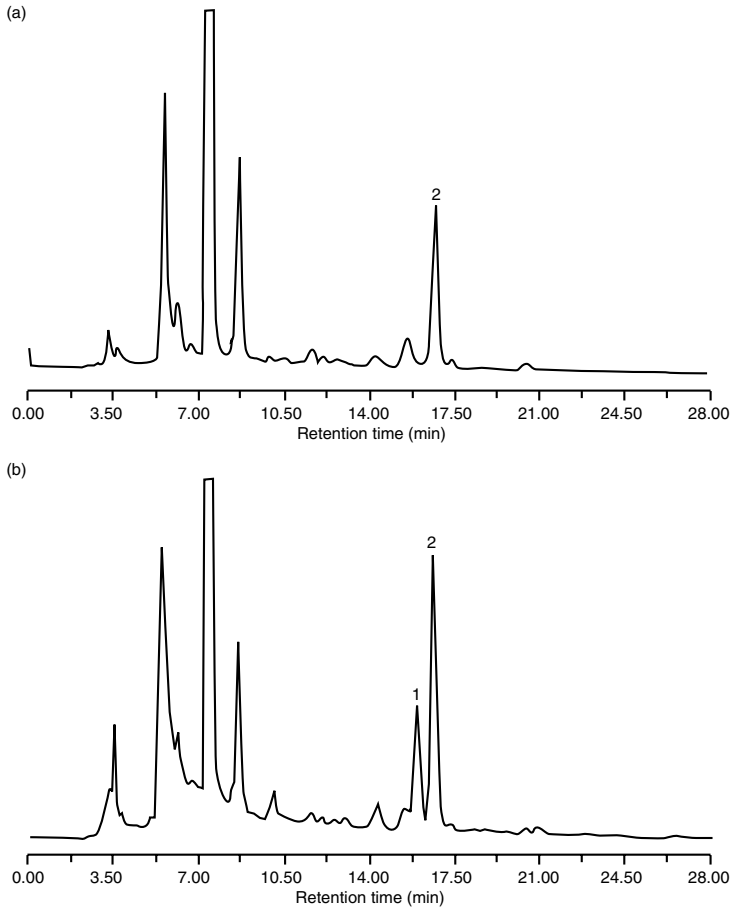

FIGURE 20.28
Quantitative reversed-phase HPLC and UV detection of the vitamin D fraction collected by semipreparative HPLC (Figure 20.27). (a) Sample without vitamin D_2 added as internal standard. (b) Sample spiked with vitamins D_2 and D_3. Operating parameters as in Table 20.9 [161]. Peaks: (1) vitamin D_2 (internal standard); (2) vitamin D_3. (Reprinted from Konings, E.J.M., *Neth. Milk Dairy J.*, 48, 31–39, © 1994, with permission from Elsevier.)

excitation at 205 nm [211]. A disadvantage of short-wavelength excitation is a marked loss of selectivity and an aggravation of quenching effects [212]; hence the longer wavelength (295 nm) is usually employed in food analysis applications.

Delgado-Zamarreño's group [26,27] employed coulometric detection in the redox mode to measure tocopherols separated by reversed-phase HPLC. The detector was equipped with a dual-electrode cell in which

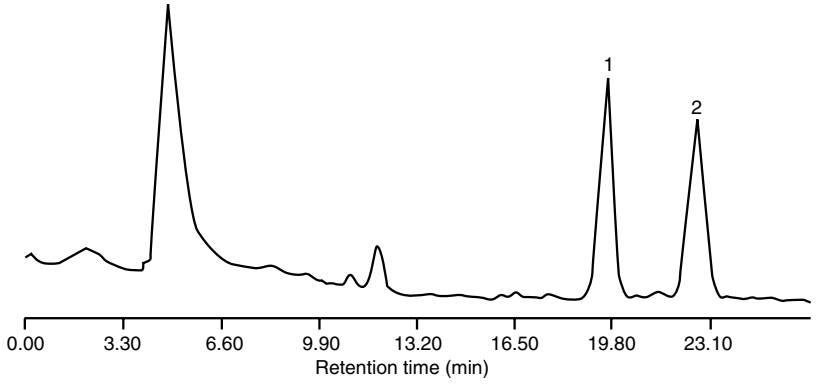

FIGURE 20.29
Reversed-phase HPLC and UV detection of a vitamin D_3 standard and vitamin D_2 internal standard that was taken through the sample preparation process. Operating parameters as in Table 20.9 [156]. Peaks: (1) vitamin D_2 (internal standard); (2) vitamin D_3. (Reprinted from Sliva, M.G., Green, A.E., Sanders, J.K., Euber, J.R., and Saucerman, J.R., *J. AOAC Int.*, 75, 566–571, Copyright 1992 by AOAC International. With permission.)

vitamin E is first reduced upstream at the generator electrode and then reoxidized downstream at the detector electrode. The chromatographic system also incorporated a "guard cell" to treat the mobile phase before it entered the column. The guard cell comprised a porous graphite working electrode, a reference electrode, and a counterelectrode, the latter two made of stainless steel. A potential of +1.0 V was applied to the guard cell as a means of oxidizing possible impurities entering the mobile phase, hence reducing background noise.

20.5.4.2 Quantification

External standard calibration is generally used. The quality of an α-tocopherol standard can easily be checked by making a solution in hexane and measuring the UV absorbance at minimum (A_{min}) and maximum (A_{max}) wavelengths of 255 and 292 nm, respectively. If the quotient A_{min}/A_{max} exceeds 0.18, the standard contains less than 90% α-tocopherol [213].

If the food sample contains both supplemental *all-rac*-α-tocopheryl acetate and naturally occurring *RRR*-α-tocopherol, it will not be possible (using conventional HPLC techniques) to calculate a true vitamin E potency value if the sample is saponified or otherwise hydrolyzed (see Section 20.2.1.4).

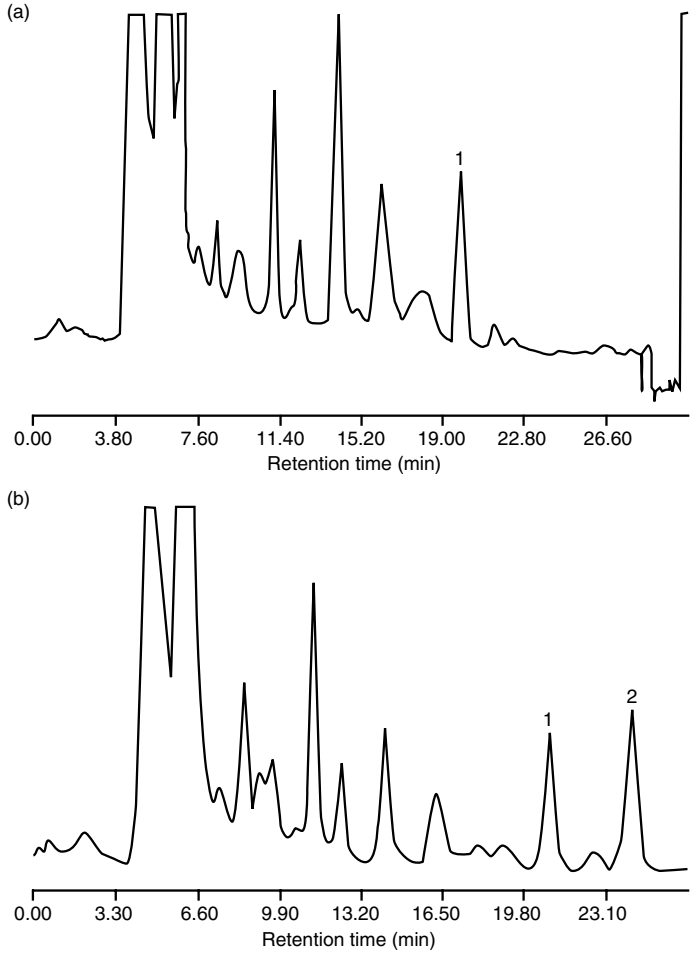

FIGURE 20.30
Reversed-phase HPLC and UV detection of an extract of (a) unfortified and (b) vitamin D_3-fortified milk-based infant formula. Operating parameters as in Table 20.9 [156]. Peaks: (1) vitamin D_2 (internal standard); (2) vitamin D_3. (Reprinted from Sliva, M.G. and Sanders, J.K., *J. AOAC Int.*, 75, 566–571, Copyright 1992 by AOAC International. With permission.)

20.5.4.3 Normal-Phase Separations

Normal-phase HPLC is capable of separating, isocratically, all of the eight unesterified tocopherols and tocotrienols that occur in nature, and this chromatographic mode has been utilized in determining the distribution of E vitamers in a wide variety of fats, oils, and foodstuffs.

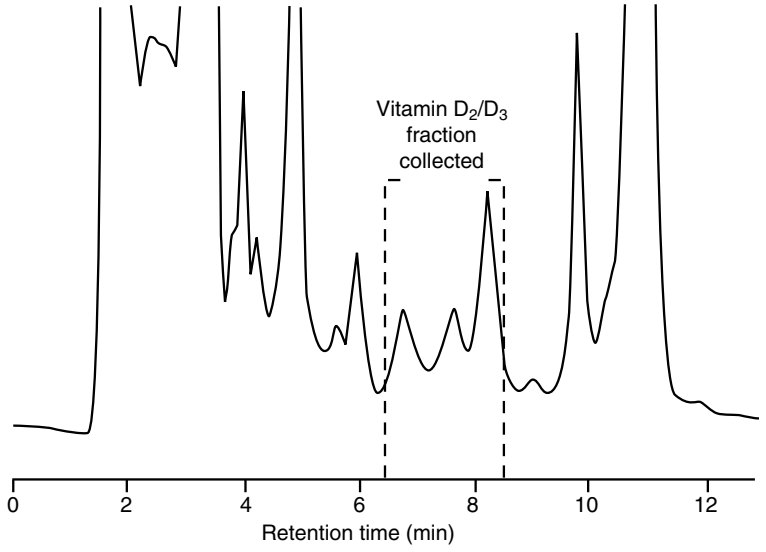

FIGURE 20.31
Semipreparative normal-phase HPLC and UV detection of vitamin D in the unsaponifiable matter from margarine. Operating parameters as in Table 20.9 [159]. (Reprinted from Staffas, A. and Nyman, A., *J. AOAC Int.*, 86, 400–406, Copyright 2003 by AOAC International. With permission.)

The E vitamers are separated by adsorption chromatography according to the number of methyl groups on their chromanol rings. Increasing the number of methyl groups makes the molecule more hydrophobic and therefore less retentive. In addition, the differently positioned methyl groups impart different steric effects on the phenolic group, influencing its interaction with the silanol groups of the silica. Furthermore, the unsaturation in the side chain of tocotrienols makes these molecules slightly more polar than the corresponding tocopherols. The elution sequence using normal-phase chromatography is α-T, α-T3, β-T, γ-T, β-T3, γ-T3, δ-T, and δ-T3. Supplemental α-tocopheryl acetate is eluted before α-T, and the acetate and palmitate esters of vitamin A elute separately before α-tocopheryl acetate.

Hewavitharana [210] reported that injections of tocopherol and tocotrienol standards or sample extracts containing E vitamers onto a silica column at the beginning of each HPLC session produced only very small chromatographic peaks or even no peaks at all. It was only after several successive injections of standards or samples containing vitamin E that normal peak sizes were obtained. This phenomenon was attributed to the presence of extra active sites that irreversibly retain tocopherols

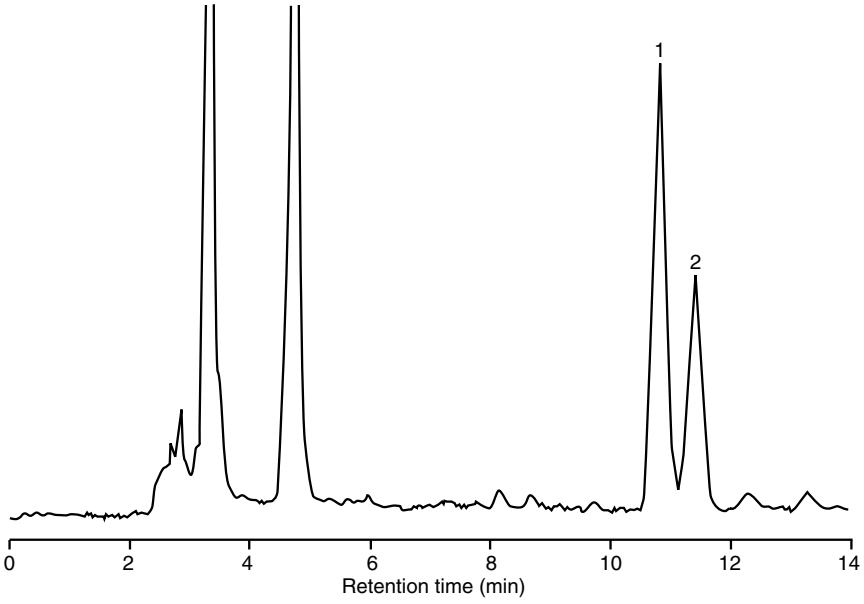

FIGURE 20.32
Quantitative reversed-phase HPLC and UV detection of the vitamin D fraction collected by semipreparative HPLC (Figure 20.31). Operating parameters as in Table 20.9 [159]. Peaks: (1) vitamin D_2 (internal standard); (2) vitamin D_3. (Reprinted from Staffas, A. and Nyman, A., *J. AOAC Int.*, 86, 400–406, Copyright 2003 by AOAC International. With permission.)

and tocotrienols by hydrophobic interaction. Normal-phase chromatography involving silanol groups is restored when these extra active sites are blocked by tocopherols and tocotrienols. Hewavitharana [210] suggested preconditioning the silica column after a period of non-use, such as a weekend break, by adding 0.02 mg/l of α-tocopherol to the initial mobile phase and injecting two 200-μl aliquots of 2000 mg/l of α-tocopherol. This pretreatment blocks the extra active sites and allows subsequent chromatography to take place by normal-phase mechanisms. A commercial silica column regenerating solution, comprising a mixture of acetic acid, 2,2-dimethoxypropane (DMP), and dichloromethane made the irreversible retention worse, necessitating injection of additional aliquots of α-tocopherol to block the extra active sites. DMP reacts with water to form 2 moles of methanol and 1 mole of acetone in the presence of an acid catalyst [214]. The removal of water in this manner would promote the conversion of some of the silanols (Si—OH) to siloxanes (Si—O—Si), making the latter likely candidates for the hydrophobic interactions. The column preconditioning step was included in a method for determining vitamin E in chicken meat [191].

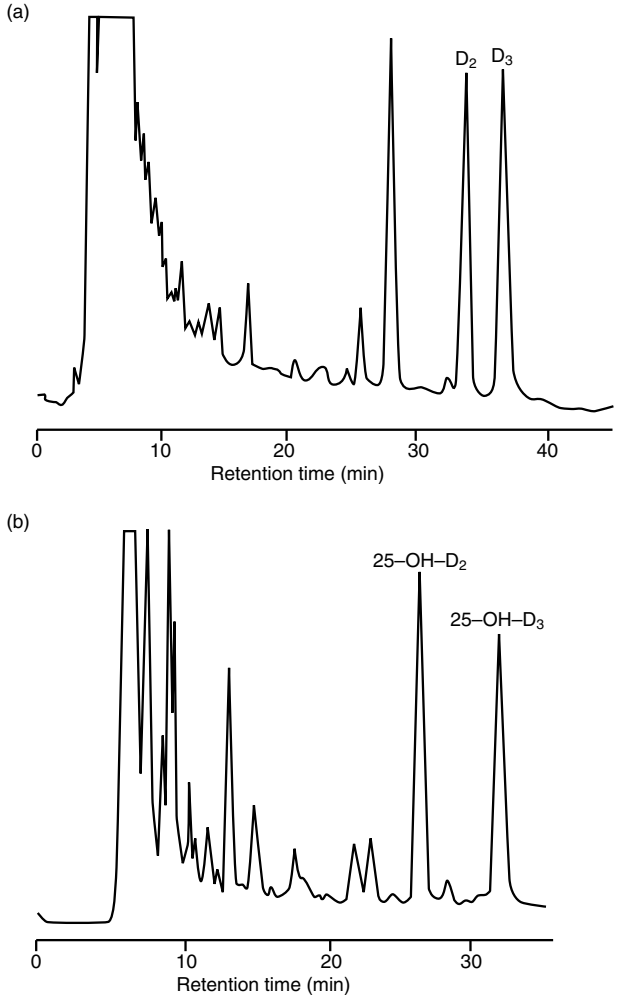

FIGURE 20.33
Quantitative HPLC and UV detection of the vitamin D fraction from a saponified chicken sample collected by semipreparative HPLC. (a) Reversed-phase: vitamins D_2 (internal standard) and D_3. (b) Normal-phase: 25-hydroxyvitamin D_2 (internal standard) and 25-hydroxyvitamin D_3. Operating parameters as in Table 20.9 [166]. (Reprinted with permission from Mattila, P.H., Piironen, V.I., Uusi-Rauva, E.J., and Koivistoinen, P.E., *J. Agric. Food Chem.*, 43, 2394, © 1995 American Chemical Society. With permission.)

Kamal-Eldin et al. [215] compared several commercial normal-phase HPLC columns packed with underivatized or derivatized type B silica for their ability to separate the eight E vitamers. The best separations were obtained on three silica columns and two amino-bonded-silica

TABLE 20.10

HPLC Methods Used for the Determination of Vitamin E in Food

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Vitamers Separated**	**Detection**	**Ref.**
Normal-phase chromatography						
Seed oils	Filter oil through sodium sulfate. Dissolve in hexane	LiChrosorb Si-60 5 μm 250 × 4 mm	0.3% 2-PrOH in hexane	α-, β-, γ-, δ-T α-, β-, γ-T3	Fluorescence: ex 290 nm em 330 nm	[180]
Seed oils, margarine, butter	Dissolve sample in hexane	LiChrosorb Si-60 5 μm 250 × 4 mm (column temperature 45°C for separation of γ-T and β-T3)	Diisopropyl ether gradient of 8–17% in hexane	α-, β-, γ-, δ-T α-, β-, γ-, δ-T3	Fluorescence: ex 290 nm em 325 nm	[181]
Wheat flour, wheat germ	Hexane extraction in Soxhlet apparatus	LiChrosorb Si-60 5 μm 250 × 4 mm	Hexane/ diisopropyl ether, 97:3	α-, β-, γ-, δ-T α-, β-T3	Fluorescence: ex 290 nm em 330 nm	[182]
Margarine, vegetable oil spreads	Dissolve 5 g margarine or spread in hexane containing 0.1% BHT, add three drops of Tween 80 and 3 g anhydrous magnesium sulfate, let stand for ≥2 h. Filter through fritted glass, dilute to volume with hexane–BHT	LiChrosorb Si-60 5 μm 250 × 4.6 mm	0.9% 2-PrOH in hexane	α-, γ-, δ-T	Fluorescence: ex 290 nm em 330 nm	[183]

(*Table continued*)

TABLE 20.10 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Vitamers Separated**	**Detection**	**Ref.**
Infant formula	Disperse sample in nonaqueous solvent mixture (DMSO/DMF/$CHCl_3$, 2 + 2 + 1) containing 0.1% (w/v) ascorbic acid. Partition total lipid fraction into hexane, centrifuge	Radial-PAK cartridge containing. Resolve silica 5 μm	0.08% 2-PrOH in hexane	α-Tocopheryl acetate	UV 280 nm	[184]
Infant formula	Mix 1 ml reconstituted formula with 5-ml $CHCl_3$/MeOH (2:1), add 1 ml water, mix by inversion, centrifuge. Evaporate $CHCl_3$ phase under nitrogen, dissolve residue in hexane	Nova-Pak silica 150 × 3.9 mm	Hexane/ethyl acetate, 98:2	α-Tocopheryl acetate, α-, γ-, δ-T	Fluorescence: ex 295 nm em 330 nm	[185]
Cereals, flour foods (unfortified)	Homogenize sample with boiling 2-PrOH, add acetone, filter, extract residue with acetone. Partition into hexane, wash pooled hexane extracts with water, evaporate under vacuum, dissolve residue in hexane	LiChrosorb Si-60 5 μm 250 × 3.2 mm	0.2% 2-PrOH or 5% diethyl ether in dry hexane/water-saturated hexane, 1 + 1	α-, β-, γ-, δ-T α-, β-, γ-T3	Fluorescence: ex 290 nm em 330 nm	[186]
Infant formula	Saponify (hot), extract with diethyl ether, wash pooled extracts to neutral pH. Evaporate under vacuum, dissolve residue in hexane	As in preceding entry	As in preceding entry	Total α-T, β-, γ-, δ-T α-, β-, γ-T3	Fluorescence: ex 290 nm em 330 nm	[186]

Infant formula	Saponify (hot), extract with hexane, wash pooled extracts to neutral pH. Evaporate under vacuum, dissolve residue in HPLC mobile phase	LiChrosphere Si-60 5 μm 120 × 4.6 mm	1% 2-PrOH and 0.5% EtOH in hexane	Total α-T, β-, γ-, δ-T	Fluorescence: ex 292 nm em 320 nm	[187]
Milk-based infant formula	Saponify (hot) in a tube with shaking, cool, extract once with hexane/CH_2Cl_2 (3 + 1), wash hexane layer three times with water/absolute EtOH (3 + 2). Evaporate portion under nitrogen, dissolve residue in HPLC mobile phase	Silica 5 μm 250 × 4.6 mm	0.08% 2-PrOH in hexane	α-Tocopherol	UV 280 nm	[188]
Various foodstuffs, dairy products, infant formula	Saponify (hot) 0.5-g sample in a tube with periodic agitation, cool, shake with petroleum ether/diisopropyl ether (3 + 1), add water, mix, centrifuge	Radial-PAK cartridge containing Resolve silica 5 μm	1% 2-PrOH in hexane	Total α-T, β-, γ-, δ-T	Fluorescence: ex 295 nm em 330 nm	[189]
Pork (muscle, adipose tissue)	Saponify (hot), extract with 20 ml water/EtOH (4:1 for muscle or 1:1 for adipose tissue) + 10 ml hexane/toluene (1:1) containing 0.01% BHT, centrifuge	LiChrosorb Si-60 5 μm 250 × 4 mm	Hexane/ethyl acetate, 95:5	α-T	Fluorescence: ex 295 nm em 330 nm	[14]
Beef (muscle)	Saponify (hot) 1-g sample in a tube, extract with isooctane	Resolve silica 5 μm 150 × 3.9 mm	Isooctane/THF, 96:4	α-T	Fluorescence: ex 296 nm em 325 nm	[190]
Chicken meat	Add α-tocopheryl acetate solution (internal standard) and 4 ml absolute ethanol to 1 g minced meat. Homogenize, add 5 ml water, homogenize, add 4 ml hexane containing 200 mg/ml BHT, homogenize, centrifuge	LiChrosphere Si-100 5 μm 250 × 4.6 mm	4% 1,4-dioxane, 0.04% acetic acid and 0.02 mg/l α-tocopherol in hexane	BHT, α-tocopheryl acetate (internal standard), α-T, γ-T	Fluorescence: ex 295 nm em 330 nm	[191]

(*Table continued*)

TABLE 20.10 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Vitamers Separated**	**Detection**	**Ref.**
Meat and meat products	Saponify (ambient), extract with hexane. Wash pooled hexane extracts with water, evaporate, dissolve residue in hexane	LiChrosorb Si-60 5 μm 250 × 4 mm	Hexane/ diisopropyl ether, 93:7	α, β-, γ-T α-, β-T3	Fluorescence: ex 292 nm em 324 nm	[192]
Fish and fish products	As in preceding entry	As in preceding entry	As in preceding entry	α, β-, γ-T	As in preceding entry	[193]
Infant formula	As in preceding entry	As in preceding entry	As in preceding entry	Total α-T, β-, γ-T α-, β-T3	As in preceding entry	[194]
Cereal products	As in preceding entry	As in preceding entry	As in preceding entry	α-, β-, γ-, δ-T α-, β-, γ-T3	As in preceding entry	[195]
Cereals	Saponify (hot), extract with hexane/ ethyl acetate (9:1), evaporate pooled extracts, dissolve residue in hexane containing 1% 2-PrOH	Kromasil Phenomenex Si 250 × 4.6 mm	Hexane/ethyl acetate/acetic acid, 97.3:1.8:0.9	α-, β-, γ-, δ-T α-, β-, γ-, δ-T3	Fluorescence: ex 290 nm em 330 nm	[196]
Peanut, almond, spinach, wheat bran	Saponify (ambient), extract with hexane, wash twice with saturated sodium chloride solution and once with water. Evaporate, dissolve residue in hexane	Chromcart CC Nucleosil 50-5 250 × 4 mm	Isooctane/1,4-dioxane, 97:3	α-, β-, γ-, δ-T α-, β-, γ-, δ-T3	Fluorescence: ex 295 nm em 330 nm	[197]
Milk, milk energy drink, multivitamin fruit juice, orange juice	Saponify 2-ml sample in a microwave system for 2 min, cool. Add acetic acid, saturated sodium chloride solution, and cyclohexane containing 0.05% BHT, shake, centrifuge	LiChrosorb Si-60 5 μm 125 × 4 mm	Hexane/dioxane, 97:3	α-Tocopherol	UV 292 nm	[5]

Breakfast cereals, infant formula	*Cereals*: add water and MeOH to ground sample, shake, then sonicate. Extract with MTBE/petroleum ether (10 + 14), centrifuge. Extract twice more. Evaporate pooled extracts under vacuum, dissolve residue in hexane, centrifuge. *Formulas*: reconstitute sample with water, add 35% (w/v) dipotassium oxalate followed by EtOH, extract twice with MTBE/petroleum ether, 25 + 35. Evaporate pooled extracts under vacuum, dissolve residue in hexane, centrifuge	LiChrosphere 100 Diol 5 μm 250 × 4 mm	Two-step gradient composed of hexane and an increasing concentration of MTBE. 0–4 min hexane, 4–5 min up to hexane/MTBE (97:3), 5–41 min isocratic, 41–42 min up to hexane/MTBE (95:5), 42–50 min isocratic	α-Tocopheryl acetate α-, β-, γ-, δ-T α-, β-, γ-, δ-T3 plasto-chromanol-8	Fluorescence: ex 295 nm em 330 nm	[25]
Margarine, infant formula broccoli	Saponify (hot), extract with hexane/ethyl acetate (90 + 10) containing 20 mg BHT per liter, wash pooled extracts to neutral pH, dry by filtration through phase-separating filter. Evaporate under vacuum, dissolve residue in hexane	LiChrosorb Diol 5 μm 250 × 4.6 mm	Hexane/MTBE, 94 + 6	α-, β-, γ-, δ-T α-, β-, γ-, δ-T3 plastochro-manol-8	Fluorescence: ex 295 nm em 330 nm	[198]
Reversed-phase chromatography						
Vegetable oils	Dilute oil in hexane (1:10). Take 200 μl, add 600 μl MeOH and 200 μl of internal standard solution (300 μg/ml α-tocopheryl acetate in EtOH), mix, centrifuge	Tracer Extrasil ODS-2 150 × 4.4 mm Column temperature 45°C	MeOH/H_2O, 96:4	α-, (β + γ)-, δ-T, α-tocopheryl acetate (internal standard)	UV 292 nm	[199]

(*Table continued*)

TABLE 20.10 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Vitamers Separated**	**Detection**	**Ref.**
Vegetable oils	Dilute oil with THF, further dilute with MeOH	Spherisorb ODS 5 μm 250 × 4.6 mm	0.05 *M* aqueous sodium perchlorate/ MeOH, 10 + 90	α-, (β + γ)-, δ-T α-, (β + γ)-, δ-T3	Amperometric +0.600 V	[200]
Secds, nuts	Solubilize peeled and ground samples in Triton X-114 in the presence of MeOH and MeCN (donor solution). Pump through extraction membrane system coupled on-line with the HPLC system. Extract vitamin E into MeCN (acceptor solution). PMC (2,2,5,7,8-pentamethyl-6-chromanol) added as internal standard	OD-224 RP-18 5 μm 220 × 4.6 mm	2.5 m*M* acetate buffer in MeOH/ H_2O, 97:3	α-, (β + γ)-, δ-T PMC (internal standard)	Dual-electrode coulometric detection (redox mode), porous graphite electrodes, −1.0 V (generator electrode) +0.5 V (detector electrode)	[27]
Vegetable oils, biscuits	Extract oil from biscuits with petroleum ether/diethyl ether (1 +1) using a Soxhlet apparatus. Dissolve vegetable oil or oil extracted from biscuits in hexane. Pass a 1-ml aliquot through silica solid-phase extraction cartridge, elute vitamin E with 2 ml MeOH, add 0.3 ml CH_2Cl_2	μBondapak C_{18} 10 μm 300 × 3.9 mm	MeOH/H_2O, 95 + 5	α-, (β + γ)-, δ-T α-, (β + γ)-, δ-T	Fluorescence: ex 296 nm em 330 nm	[201]

Emulsified nutritional supplements	Dissolve sample in 5% sodium sulfate containing 1 m*M* EDTA. Load 1 ml onto C_{18} solid-phase extraction cartridge, wash with water, and then sequentially with 5, 25, and 50% aqueous MeCN, elute vitamin E with 100% MeCN	Inertsil ODS-2 5 μm 150 × 4.6 mm	MeOH/EtOH, 50:50	α-Tocopheryl acetate	Fluorescence: ex 295 nm em 325 nm	[202]
All food categories	Saponify (ambient), extract with hexane, wash pooled extracts twice with saturated sodium chloride solution, followed by a water wash, filter through sodium sulfate. Evaporate under vacuum, dissolve residue in HPLC mobile phase	Zorbax ODS 5 μm 250 × 4.6 mm	MeCN/CH_2Cl_2 containing 0.001% triethylamine/ MeOH, 700 + 300 + 50	α-, (β + γ)-, δ-T	Fluorescence: ex 290 nm em 330 nm	[203]
Seed oils	Dissolve sample in HPLC mobile phase	Taxsil PFPS 3 μm (pentafluorophenyl-silica) 250 × 4.6 mm	MeOH/H_2O, 9:1	α-, β-, γ-, δ-T α-, β-, γ-, δ-T3	Fluorescence: ex 298 nm em 345 nm	[204]

BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; MeOH, methanol; EtOH, ethanol; 2-PrOH, 2-propanol; MeCN, acetonitrile; CH_2Cl_2, dichloromethane (methylene chloride); $CHCl_3$, chloroform; THF, tetrahydrofuran; DMF, dimethylformamide; DMSO, dimethylsulfoxide; MTBE, methyl *tert*-butyl ether.

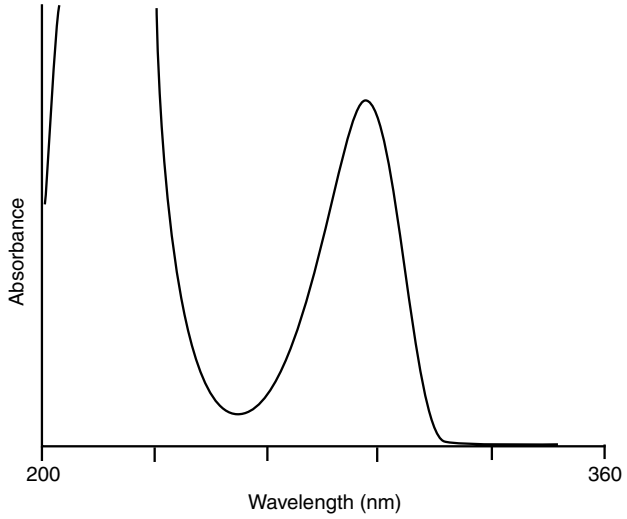

FIGURE 20.34
UV absorption spectrum of *all-rac*-α-tocopherol in ethanol. The absorption maximum occurs at 294 nm.

columns using 4 or 5% 1,4-dioxane in hexane as the mobile phase, as well as on a diol-bonded silica column eluted with 4% methyl *tert*-butyl ether in hexane. Figure 20.37 shows the separation of a balanced mixture of tocopherols and tocotrienols on a silica column using a mobile phase of hexane/1,4-dioxane, 96:4.

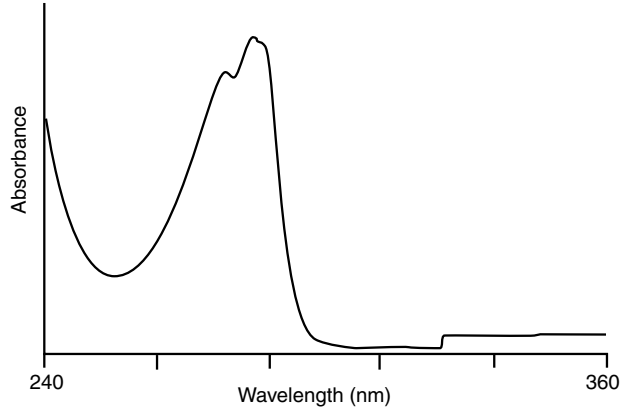

FIGURE 20.35
UV absorption spectrum of *all-rac*-α-tocopheryl acetate in ethanol. The absorption maximum occurs at 285.5 nm.

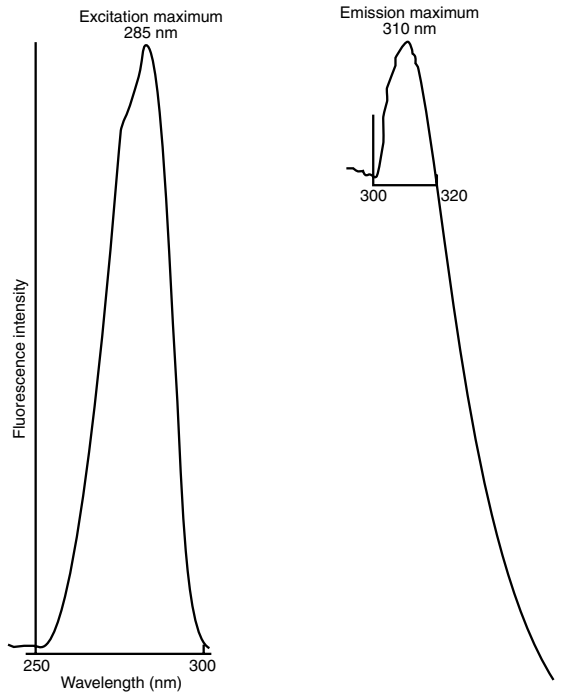

FIGURE 20.36
Fluorescence excitation and emission spectra of *all-rac*-α-tocopheryl acetate obtained by stop-flow-scanning after passage through a silica HPLC column eluted with hexane containing 0.03% 2-propanol.

The distribution of naturally occurring E vitamers in oils and fats may be determined by simply dissolving the sample in hexane (typically 0.5 g in 50 ml) and injecting an aliquot of the solution, without concentration, onto a silica column [181]. Complex food matrices require a more rigorous initial extraction before partitioning of the total lipid into hexane. Fluorescence detection is usually obligatory when the total lipid fraction is analyzed, because absorbance detection reveals peaks of lipid origin that interfere with the peaks of the E vitamers. If the sample is saponified, absorbance detection can be utilized, as hexane extracts of the unsaponifiable matter are usually free from interfering lipoidal material. For the analysis of saponified vitamin E-supplemented foods, fluorescence detection provides a much higher sensitivity, because the liberated α-tocopherol fluoresces more strongly than α-tocopheryl acetate.

For the determination of supplemental vitamin E in infant formulas, Woollard and Blott [184] employed a radially compressed Radial-PAK

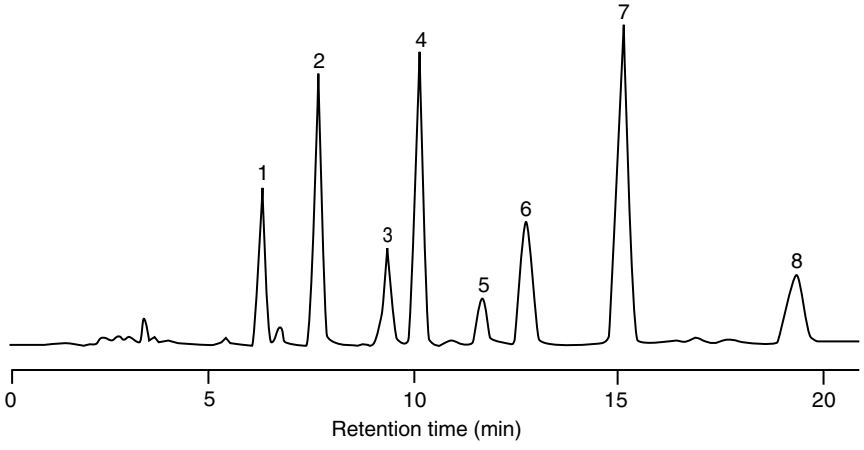

FIGURE 20.37
Normal-phase (adsorption) HPLC of a standard mixture of tocopherols and tocotrienols in hexane. Column, 4-μm Genesis silica (Jones), 250 × 4.6-mm-ID; mobile phase, hexane and 1,4-dioxane (96:4); fluorescence detection, excitation 294 nm, emission 326 nm. Peaks: (1) α-T; (2) α-T3; (3) β-T; (4) γ-T; (5) β-T3; (6) γ-T3; (7) δ-T; (8) δ-T3. (Reprinted from Kamal-Eldin, A., Görgen, S., Pettersson, J., and Lampi, A.-M., *J. Chromatogr. A*, 881, 217–227, © 2000, with permission from Elsevier.)

cartridge. This enabled lipid material to be rapidly cleared by stepping up the mobile-phase flow rate from 2 to 10 ml/min after elution of the α-tocopheryl acetate. Fluorescence detection, using a filter-type fluorometer, allowed the indigenous α-tocopherol to be conveniently estimated, while UV absorbance detection was used to quantify the α-tocopheryl acetate. Supplemental retinyl acetate could be assayed simultaneously with either added or indigenous vitamin E using the appropriate detection mode. With the aid of a dual-monochromator spectrofluorometer, α-tocopheryl acetate and α-tocopherol could be determined simultaneously with wavelengths of 280 nm (excitation) and 335 nm (emission), but the increased selectivity eliminated detection of the vitamin A esters [216].

Konings et al. [198] reported the isocratic separation of the eight unesterified E vitamers using a diol stationary phase (Figure 20.38). Some interferences were encountered from tocomoneols, tocodienols, plastochromanol-8, and the synthetic antioxidant, butylated hydroxyanisole (BHA) (Figure 20.39). Butylated hydroxytoluene (BHT) eluted immediately after injection. A chromatogram of tocopherols and tocotrienols in an extract of powdered milk-based infant formula is shown in Figure 20.40.

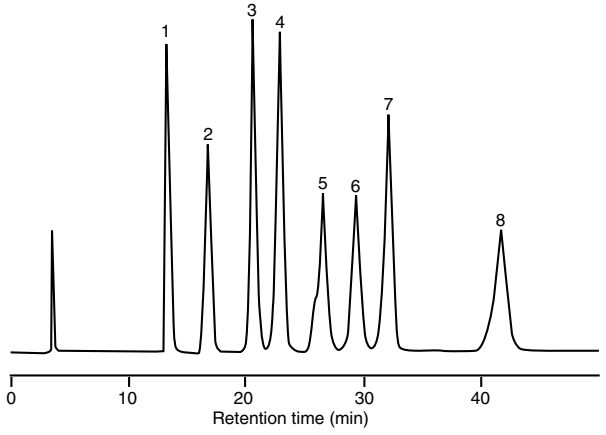

FIGURE 20.38
Normal-phase (polar bonded-phase) HPLC and fluorescence detection of a standard mixture of tocopherols and tocotrienols in hexane. Operating parameters as in Table 20.10 [198]. Peaks: (1) α-T; (2) α-T3; (3) β-T; (4) γ-T; (5) β-T3; (6) γ-T3; (7) δ-T; (8) δ-T3. (Reprinted from Konings, E.J.M., Roomans, H.H.S., and Beljaars, P.R., *J. AOAC Int.*, 79, 902–906, Copyright 1996 by AOAC International. With permission.)

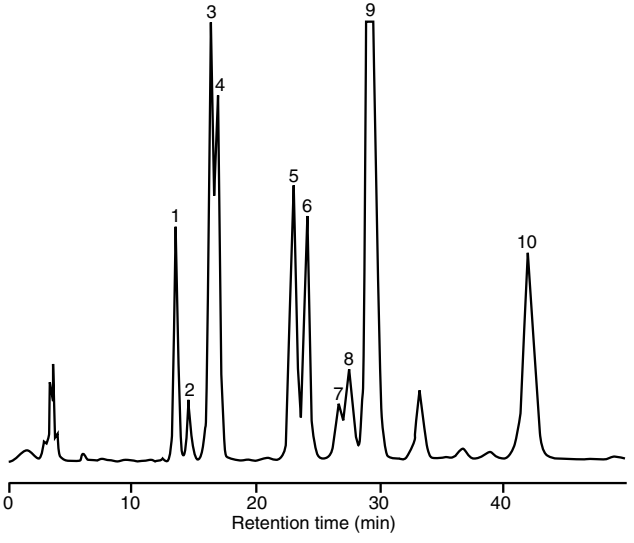

FIGURE 20.39
Normal-phase (polar bonded-phase) HPLC and fluorescence detection of vitamin E extracted from a mixture of palm oil, linseed oil, BHA, and BHT in hexane. Operating parameters as in Table 20.10 [198]. Peaks: (1) α-T; (2) α-tocomoneol; (3) α-tocodienol; (4) α-T3; (5) γ-T; (6) BHA; (7) β-T3; (8) plastochromanol-8; (9) γ-T3; (10) δ-T3. BHT elutes immediately after injection. (Reprinted from Konings, E.J.M., Roomans, H.H.S., and Beljaars, P.R., *J. AOAC Int.*, 79, 902–906, Copyright 1996 by AOAC International. With permission.)

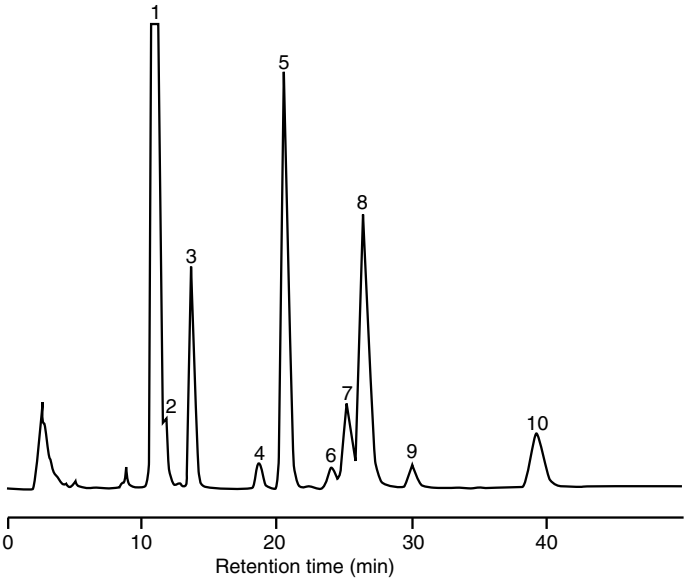

FIGURE 20.40
Normal-phase (polar bonded-phase) HPLC and fluorescence detection of vitamin E extracted from powdered milk-based infant formula. Operating parameters as in Table 20.10 [198]. Peaks: (1) α-T; (2) α-tocomoneol; (3) α-T3; (4) β-T; (5) γ-T; (6) β-T3; (7) plastochromanol-8; (8) γ-T3; (9) δ-T; (10) δ-T3. (Reprinted from Konings, E.J.M., Roomans, H.H.S., and Beljaars, P.R., *J. AOAC Int.*, 79, 902–906, Copyright 1996 by AOAC International. With permission.)

20.5.4.4 Reversed-Phase Separations

In reversed-phase HPLC, the E vitamers are eluted in the order δ-T3, γ-T3, and β-T3 (unresolved), α-T3, δ-T, γ-T, and β-T (unresolved), and α-T (Figure 20.41). This elution profile contrasts with that obtained using normal-phase chromatography, in which α-T is eluted first and δ-T is eluted last. Tocopheryl acetate elutes immediately in front of α-tocopherol, with baseline separation. The positional β and γ isomers of tocopherols and tocotrienols cannot be separated using C_{18} reversed-phase columns of standard dimensions, even if gradient elution is used. The isomeric tocopherols can, however, be resolved, but not baseline separated, using a polymeric C_{30} phase [77].

Abidi [204] reported the complete separation of the eight naturally occurring tocochromanols within 35 min using a pentafluorophenylsilica polar reversed-phase column and a methanol/water mobile phase (Figure 20.42). The ability of this system to isocratically separate the β–γ isomers of tocopherol and tocotrienol is an improvement on hydrocarbon-bonded reversed phases and avoids the pitfalls encountered with normal-phase chromatography.

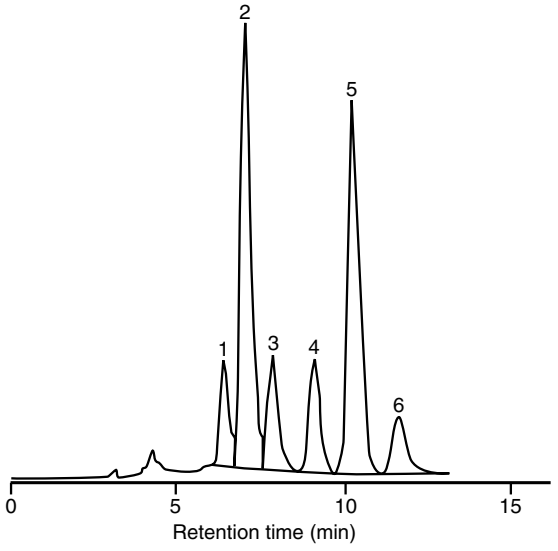

FIGURE 20.41
Reversed-phase HPLC and fluorescence detection of a standard mixture of tocopherols and tocotrienols on a C_{18} column. Operating parameters as in Table 20.10 [201]. Peaks: (1) δ-T3; (2) β + γ-T3 (unresolved); (3) α-T3; (4) δ-T; (5) β + γ-T (unresolved); (6) α-T. (Reprinted from Bonvehi, J.S., Coll, F.V., and Rius, I.A., *J. AOAC Int.*, 83, 627–634, Copyright 2000 by AOAC International. With permission.)

Reversed-phase HPLC with fluorescence detection is the preferred system for the routine determination of total α-tocopherol in vitamin E-supplemented foods after saponification. The use of NARP chromatography with a predominantly hexane mobile phase allows aliquots of hexane extracts of the unsaponifiable matter to be injected directly onto the column, thus avoiding the evaporation step necessary when a semiaqueous mobile phase is used [217].

Indyk [15] described a method for the simultaneous determination of cholesterol, phytosterols, and tocopherols in dairy and nondairy foods. Analysis of the unsaponifiable matter was accomplished using a C_{18} Rad-PAK cartridge and an isocratic mobile phase of either hexane/2-propanol (99.9 + 0.1, v/v) or methanol (100%). Sterols were monitored by short-wave UV spectrophotometry, and tocopherols by fluorometry.

20.5.5 Vitamin K

Selected methods for determining vitamin K in a variety of foods are summarized in Table 20.11.

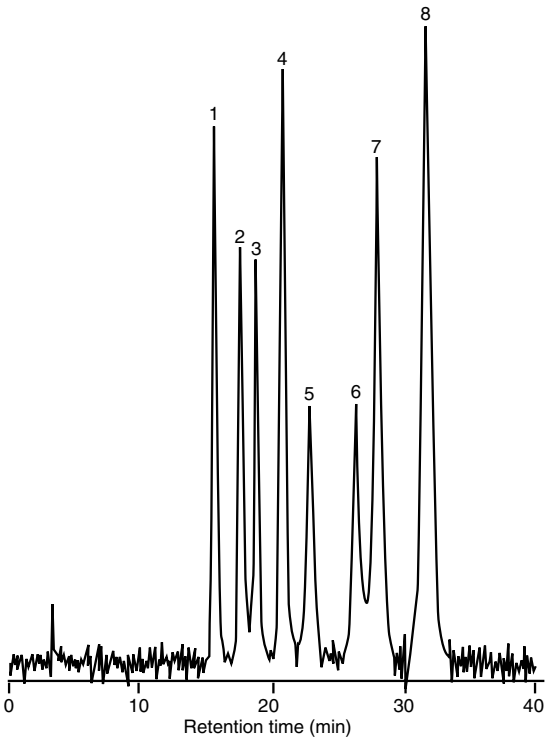

FIGURE 20.42
Reversed-phase HPLC and fluorescence detection of a standard mixture of tocopherols and tocotrienols on a pentafluorophenylsilica column. Operating parameters as in Table 20.10 [204]. Peaks: (1) δ-T3; (2) β-T3; (3) γ-T3; (4) α-T3; (5) δ-T; (6) β-T; (7) γ-T; (8) α-T. (From Abidi, S.L., *J. Am. Oil Chem. Soc.*, 80, 327, © 2003 AOCS Press. With permission.)

20.5.5.1 Detection

Phylloquinone and the menaquinones all possess the same chromophore and exhibit identical UV absorption spectra, which contain five maxima (Figure 20.43). The ε value at the λ_{max} of 248 nm is 18,900 [238]. The $A_{1\,cm}^{1\%}$ for phylloquinone in hexane at 248 nm is 419 [239]. Hwang [218] reported an on-column detection limit of 0.3 ng for both *cis* and *trans* isomers of phylloquinone using photometric detection at 254 nm. Photometric determination of phylloquinone has sufficient sensitivity for the analysis of green leafy vegetables and vitamin K-supplemented infant formulas, but it lacks the required sensitivity for the analysis of foods that contain smaller amounts of the vitamin.

The first combined HPLC–electrochemical measurements of vitamin K used the reductive mode, but this technique suffered from interference

TABLE 20.11

HPLC Methods Used for the Determination of Vitamin K in Food

Food	Sample Preparation	Semipreparative HPLC	Quantitative HPLC	Compounds Separated	Ref.
Normal-phase chromatography					
Infant formula	Mix reconstituted sample with concentrated NH_4OH/MeOH, extract twice with CH_2Cl_2/isooctane, 2 + 1. Evaporate pooled extracts under vacuum, further dry by co-evaporation with acetone, dissolve residue in isooctane containing 0.01% 2PrOH. Load extract onto silica open column, rinse column with portions of same solvent, totaling 10 ml, elute vitamin K with isooctane/CH_2Cl_2/2-PrOH, 85 + 15 + 0.02. Evaporate to near dryness under vacuum, dissolve residue in isooctane		Apex Silica 5 μm 250 × 4.6 mm Isooctane/CH_2Cl_2/2-PrOH, 70 + 30 + 0.02 UV 254 nm	*Cis*- and *trans*-phylloquinone	[218]
Reversed-phase chromatography					
Infant formula	Supercritical fluid extraction		μBondapak C_{18} 10 μm 150 × 3.9 mm. MeCN/CH_2Cl_2/aqueous 0.025 *M* sodium perchlorate, 90:5:5 Amperometric detection (reductive mode), silver electrode, −1.1 V vs. saturated calomel reference electrode	Phylloquinone	[40]

(*Table continued*)

TABLE 20.11 *Continued*

Food	Sample Preparation	Semipreparative HPLC	Quantitative HPLC	Compounds Separated	Ref.
Milk, milk powder	Digest milk sample with lipase over 90 min at 37°C, treat with alcoholic NaOH (15 s contact time). Extract hydrolysate twice with hexane. Evaporate an aliquot under vacuum, dissolve residue in hexane. Load hexane extract onto silica solid-phase extraction cartridge, wash with 4 ml hexane, elute vitamin K with 4 ml hexane/diethyl ether (96 + 4)		OD-224 RP18 5 μm 220 × 4.6 mm (Brownlee Labs) MeOH/H_2O (99 + 1) containing 2.5 m*M* acetate buffer Dual-amperometric detection (redox mode) glassy carbon dual electrode at −1.1 V and +0.7 V vs. Ag/AgCl reference electrode	Phylloquinone	[219]
All food categories	*Vegetables, fruits, cereals, meat and fish*: extract sample and internal standard with acetone, filter. Partition the phylloquinone into hexane. *Fats, oils, and dairy products*: digest sample and internal standard with lipase, extract with hexane. The internal standard is phylloquinone 2,3-epoxide (unlabeled for UV detection and tritium labeled for coulometric detection). Load hexane extracts from either extraction technique onto silica solid-phase extraction cartridge, wash with 10 ml hexane, elute vitamin K with 10 ml 3% diethyl ether in hexane, evaporate under nitrogen, dissolve residue in semipreparative HPLC mobile phase	A. Partisil silica 5 μm 250 × 4.6 mm. 50% water-saturated CH_2Cl_2/hexane, 15:85. UV 254 nm B. Spherisorb nitrile 5 μm 250 × 4.6 mm 50% water-saturated CH_2Cl_2/hexane, 3:97. UV 254-nm. Collect vitamin K fraction, evaporate, dissolve residue in quantitative HPLC mobile phase	A. Hypersil ODS 250 × 4.6 mm. CH_2Cl_2/MeOH, 15:85. UV 270 nm B. Spherisorb C_8 (octyl) 5 μm 250 × 4.6 mm MeOH/50 mM acetate buffer, pH 3.0, 97:3 containing 0.1 m*M* EDTA. Dual-electrode coulometric detection (redox mode), porous graphite electrodes, −1.5 V (generator electrode) +0.05 V (detector electrode)	Phylloquinone, phylloquinone 2,3-epoxide (internal standard)	[220,221]

Oils, margarines, butter	Add menaquinone-4 (internal standard) to sample. Shake with hexane, dilute to volume with hexane, let stand 30 min, concentrate an aliquot by evaporation	μPorasil 10 μm 300 × 3.9 mm. 1% diethyl ether in hexane. UV 248 nm Collect vitamin K fraction, evaporate, dissolve residue in quantitative HPLC mobile phase	Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm. MeOH/ 0.05 *M* sodium acetate buffer (pH 3). Dual-electrode coulometric detection (redox mode), porous graphite electrodes, −1.1 V (generator electrode) 0 V (detector electrode)	Phylloquinone, menaquinone-4 (internal standard)	[222]
Vegetables, fruits, berries	Add menaquinone-4 (internal standard) to homogenized sample. Add 2-PrOH, digest in boiling water bath, cool, add 2-PrOH, homogenize. Add hexane, homogenize, repeat, add water, shake vigorously, centrifuge. Evaporate aliquot of hexane layer, dissolve residue in hexane	As in preceding entry	As in preceding entry	Phylloquinone, menaquinone-4 (internal standard)	[223]
Cereal products	As in preceding entry	As in preceding entry	As in preceding entry	Phylloquinone, menaquinone-4 (internal standard)	[224]
Soybean oil	Add $K_{1(25)}$ (internal standard) to oil sample. Shake with 0.9% sodium chloride + EtOH and partition vitamin K into hexane. Centrifuge, evaporate under nitrogen, dissolve residue in hexane. Load onto silica solid-phase extraction cartridge, wash with 10 ml hexane, elute vitamin K with 10 ml hexane/diisopropylether (90:10). Evaporate, dissolve in 1 ml hexane, re-evaporate, dissolve residue in HPLC mobile phase		Beckman XL C_8 (octyl) 3 μm 70 × 4.7 mm cartridge. MeCN/EtOH (95:5) containing 5 mM sodium perchlorate Postcolumn coulometric reduction. Fluorescence: ex 320 nm em 430 nm	Phylloquinone, $K_{1(25)}$ (internal standard)	[225]

(*Table continued*)

TABLE 20.11 *Continued*

Food	Sample Preparation	Semipreparative HPLC	Quantitative HPLC	Compounds Separated	Ref.
Spinach	Mix 10 g boiled spinach with 1 ml acetone and grind with sand. Add 100 ml acetone, shake for 30 min. Add 100 ml water, extract three times with hexane. Evaporate pooled hexane extracts under nitrogen, dissolve residue in 1 ml hexane		Econosphere C_{18}. MeOH/2-PrOH/H_2O, 88.5:10:1.5 containing 0.045% tetramethylammonium octahydrotriborate. Postcolumn electrochemical reduction. Fluorescence: ex 246 nm em 430 nm	Phylloquinone	[226]
Emulsified nutritional supplements	Dissolve 10-g sample in 5% sodium sulfate containing 1 mM EDTA. Load onto C_{18} solid-phase extraction cartridge, wash with 10 ml water and then 10% aqueous EtOH, elute vitamin K with 10 ml EtOH		Inertsil ODS-2 5 μm 150 × 4.6 mm Column temperature 40°C MeOH/EtOH, 50:50 Postcolumn chemical reduction with platinum oxide catalyst. Fluorescence: ex 320 nm em 430 nm	Phylloquinone	[227]

Canola (rape-seed) oil	Digest oil sample + internal standard (menaquinone-4) with lipase in pH 7.7 buffer at 37°C. Shake hydrolysate with 5 ml 10 M NaOH and 100 ml 95% EtOH. Extract with hexane, wash pooled hexane extracts, dry with sodium sulfate, evaporate, dissolve residue in hexane. Load onto silica solid-phase extraction cartridge, wash with hexane, elute vitamin K with hexane/diethyl ether (96:4), concentrate under nitrogen, dilute to volume with hexane	PartiSphere C_{18} 5 μm 150 × 4.6 mm. 850 ml MeOH + 150 ml MeCN combined with 5 ml of a MeOH/MeCN (85:15) solution containing 2 *M* zinc chloride, 1 *M* sodium acetate and 1 *M* acetic acid. Postcolumn chemical reduction with zinc powder column. Fluorescence: ex 254 nm em 400 nm (filter)	Phylloquinone, menaquinone-4 (internal standard)	[228]
Medical foods	Mix 2 ml reconstituted or diluted sample with 2 ml enzyme mixture consisting of lipase and α-amylase in 0.05 *M* phosphate buffer, pH 7.7. Incubate for 2 h at 37°C. Cool, add 1% sodium bicarbonate/2-PrOH (1 + 1), vortex-mix. Load digest onto C_{18} solid-phase extraction cartridge, wash sequentially with 10 ml 1% sodium bicarbonate/2-PrOH (1 + 1), 10 ml water, 10 ml MeCN/MeOH/water (250 + 200 + 50). Dry cartridge by applying full vacuum. Elute vitamin K with two 4-ml portions of CH_2Cl_2/MeOH (8 + 2), evaporate, dissolve residue in HPLC mobile phase	Zorbax C_{18} 5 μm 250 × 4.6 mm. 900 ml MeOH + 100 ml CH_2Cl_2 combined with 10 ml of a methanolic solution containing 2 *M* zinc chloride, 1 *M* sodium acetate and 1 *M* acetic acid. Postcolumn chemical reduction with zinc powder column. HPLC column and zinc powder column placed in column oven set to 60°C. Fluorescence: ex 248 nm em 400 nm	Phylloquinone	[229]

(*Table continued*)

TABLE 20.11 *Continued*

Food	Sample Preparation	Semipreparative HPLC	Quantitative HPLC	Compounds Separated	Ref.
Fish (edible part)	Homogenize fish homogenate with MeOH, add CH_2Cl_2, stir for 2 h, add further CH_2Cl_2, stir for 5 min. Add water and 1–2 drops of 1 *M* magnesium chloride, stir, filter, leave overnight to obtain complete separation of organic and aqueous phases. Evaporate the lower CH_2Cl_2 layer under vacuum, dissolve residue in hexane. Load aliquot of hexane extract onto silica solid-phase extraction cartridge, wash with 8 ml hexane, elute vitamin K with hexane/diethyl ether (96:4), evaporate under nitrogen, dissolve residue in hexane	LiChrosorb Si-60 5 μm 250 × 4 mm 2% MTBE in hexane. UV 248 nm Collect vitamin K fraction, evaporate under nitrogen, dissolve residue in MeOH/CH_2Cl_2 (90:10)	Hypersil ODS 5 μm 250 × 4 mm. Gradient elution. Eluents: (A) MeOH/CH_2Cl_2, 90:10. (B) MeOH/CH_2Cl_2, 60:40. To each liter of A and B is added 5 ml of a methanolic solution containing 2 *M* zinc chloride, 1 *M* sodium acetate and 1 *M* acetic acid. 0–6 min 0% B; 6–11 min up to 100% B; 11–18 min 100% B isocratic; 18–20 min down to 0% B. Postcolumn chemical reduction with zinc powder column. Fluorescence: ex 240 nm em 430 nm	Phylloquinone, MK-4, MK-5, MK-6, MK-8, MK-10	[230]

All food categories	Add $K_{1(25)}$ (internal standard) to homogenized, ground samples. Add 2-PrOH/hexane (3 + 2) followed by water, disperse by sonication, then vortex-mix, and centrifuge. Evaporate hexane layer under vacuum, dissolve residue in hexane. Load onto silica solid-phase extraction cartridge, wash with 8 ml hexane, elute vitamin K with 8 ml hexane/diethyl ether, 97:3. Evaporate, dissolve residue in 2-PrOH for further purification or in HPLC mobile phase *Lipid-rich samples*: load 2-PrOH extract onto C_{18} solid-phase extraction cartridge, wash with 6 ml MeOH/H_2O (95:5), followed by 6 ml MeCN, elute vitamin K with 10 ml MeOH/CH_2Cl_2, 80:20. Evaporate, dissolve residue in HPLC mobile phase	Hypersil ODS 3 μm 150 × 4.6 mm. 900 ml MeOH + 100 ml CH_2Cl_2 combined with 5 ml of a methanolic solution containing 2 *M* zinc chloride, 1 *M* sodium acetate and 1 *M* acetic acid. Postcolumn chemical reduction with zinc powder column. Fluorescence: ex 244 nm em 418 nm	Phylloquinone, 2′,3′dihydro-phylloquinone, $K_{1(25)}$ (internal standard)	[231]

(*Table continued*)

TABLE 20.11 *Continued*

Food	Sample Preparation	Semipreparative HPLC	Quantitative HPLC	Compounds Separated	Ref.
Egg, whole milk, yoghurt, cheese (Emmentaler), oatmeal, carrot, potato, broccoli, cauliflower, edible oils	Blend samples with appropriate solvent* and added dihydrophylloquinone (internal standard), centrifuge. Evaporate sample extracts (except edible oils), dissolve residue in hexane; add equal volume of MeOH/H_2O (9:1), mix, centrifuge. Evaporate hexane layer, dissolve residue in HPLC mobile phase.* *Cheese, oatmeal, broccoli, cauliflower*: CH_2Cl_2/MeOH, 2:1. *Egg, milk, yoghurt, carrot, potato*: 2-PrOH/hexane, 3:1. *Edible oils*: 100% hexane		Hypersil ODS 5 μm 250 × 4.6 mm. 900 ml MeOH + 100 ml CH_2Cl_2 combined with 5 ml of a methanolic solution containing 2 *M* zinc chloride, 1 *M* sodium acetate and 1 *M* acetic acid. Column temperature 40°C. Postcolumn chemical reduction with zinc powder column. Fluorescence: ex 243 nm em 430 nm	Phylloquinone, dihydrophyl-loquinone (internal standard)	[232]
Baby food products	Blend sample with CH_2Cl_2/MeOH (2:1), filter through sodium sulfate, dilute to volume with MeOH. Take 1-ml aliquot, add dihydrophylloquinone (internal standard), evaporate, dissolve residue in hexane. Add MeOH/H_2O (9:1), mix, centrifuge. Evaporate hexane layer, dissolve residue in HPLC mobile phase		As in preceding entry	Phylloquinone dihydrophyl-loquinone (internal standard)	[233]

Foods of animal origin	*Meat and fish products*: Add $K_{1(25)}$ (internal standard) to 3 g homogenized sample. Add 2-PrOH, digest in boiling water bath, cool, add 2-PrOH, homogenize. Add hexane, homogenize, repeat, add water, shake vigorously, centrifuge. Evaporate aliquot of hexane layer, dissolve residue in hexane *Dairy products*: mix 3-g sample with internal standard, 10 ml water and 10 ml 37% HCl, digest in a boiling water bath. Cool, shake with diethyl ether, and then with petroleum ether. Separate the two phases, repeat the ether extraction twice. Evaporate pooled extracts under vacuum *Samples with high fat content and all cheeses*: extract with 2-PrOH/hexane or acid hydrolysis as above followed by lipase digestion. Add EtOH to digest, mix, extract twice with hexane, evaporate, dissolve residue in hexane	μPorasil 10 μm 300 × 3.9 mm. 1% diethyl ether in hexane. UV 248 nm Collect vitamin K fraction, evaporate, dissolve residue in quantitative HPLC mobile phase	Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm. MeOH/EtOH (83:17) containing 10 m*M* zinc chloride, 5 m*M* sodium acetate and 5 m*M* acetic acid. Postcolumn chemical reduction with zinc powder column. Fluorescence: ex 238 nm em 425 nm	Phylloquinone, $K_{1(25)}$ (internal standard), MK-4, MK-6, MK-7, MK-8, MK-9, MK-10	[234]

(*Table continued*)

TABLE 20.11 *Continued*

Food	Sample Preparation	Semipreparative HPLC	Quantitative HPLC	Compounds Separated	Ref.
Milk-based infant formula	Extract sample by matrix solid-phase dispersion. Elute vitamin K from C_{18} column with 9 ml 0.5% 2-PrOH in hexane followed by 9 ml ethyl acetate. Evaporate pooled eluates under nitrogen, dissolve residue in hexane		Alltech C_8 (octyl) 3 μm 150 × 4.6 mm. 900 ml MeOH + 100 ml hexane combined with 5 ml of a methanolic solution containing 2 *M* zinc chloride, 1 *M* sodium acetate and 1 *M* acetic acid. Postcolumn chemical reduction with zinc powder column. Fluorescence: ex 248 nm em 418 nm	Phylloquinone	[235]
Soy-based infant formula	As in preceding entry		As in preceding entry		[236]
Medical foods	Accelerated solvent extraction with ethyl acetate in conjunction with matrix solid-phase dispersion		As in preceding entry except that column is Supelco C_{18} 5 μm 250 × 4.6 mm	Phylloquinone	[31]

Milk, infant formula	Digest sample with lipase, cool, add EtOH + solid potassium carbonate, mix, add hexane, shake for 7 min, centrifuge. Evaporate hexane layer under nitrogen, dissolve residue in MeOH	Radial-PAK cartridge containing Resolve C_{18} 5 μm. 900 ml MeOH + 100 ml CH_2Cl_2 combined with 5 ml of a methanolic solution containing 2 *M* zinc chloride, 1 *M* sodium acetate and 1 *M* acetic acid. Postcolumn chemical reduction with zinc powder column. Fluorescence: ex 243 nm em 430 mn	MK-4, MK-5, phylloquinone, 2′,3′-dihydrophylloquinone, MK-6	[237]
All food categories	Digest sample with lipase, cool, add EtOH + solid potassium carbonate, mix, add hexane, shake vigorously for 30 min, centrifuge. Evaporate aliquot of hexane layer under nitrogen, dissolve residue in MeOH or (for high-fat samples) MeOH/2-PrOH, 1 +1	YMC polymeric C_{30} 5 (or 3) μm 250 × 4.6 mm MeOH/CH_2Cl_2 (92:8) containing 10 m*M* zinc chloride, 5 m*M* sodium acetate and 5 m*M* acetic acid. Postcolumn chemical reduction with zinc powder column. Fluorescence: ex 243 nm em 430 nm	*Cis*- and *trans*-phylloquinone	[19]

EDTA, ethylenediaminetetraacetic acid; MeOH, methanol; EtOH, ethanol; 2-PrOH, 2-propanol; MeCN, acetonitrile; CH_2Cl_2, dichloromethane (methylene chloride); MTBE, methyl *tert*-butyl ether.

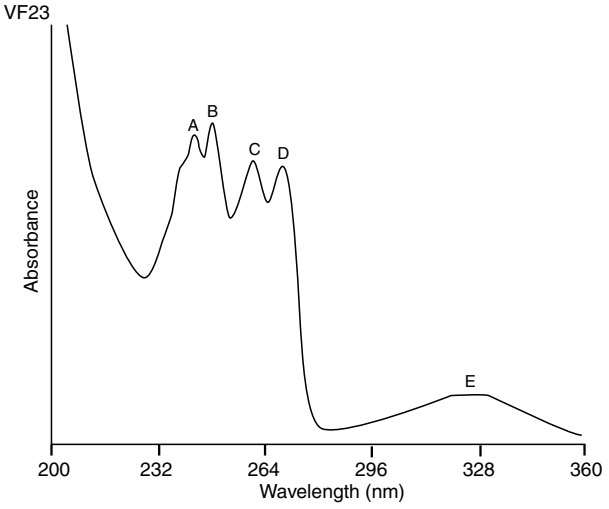

FIGURE 20.43
UV absorption spectrum of phylloquinone in hexane. The absorption maximum occurs at 248 nm (peak B).

from the reduction of oxygen. A redox method was later developed that eliminated this interference, and provided a 10-fold increase in sensitivity over photometric detection and an improved selectivity. The coulometric detector employed in the redox mode is equipped with a dual-electrode cell in which phylloquinone is first reduced upstream at the generator electrode and the hydroquinone is reoxidized downstream at the detector electrode.

Phylloquinone can be detected fluorometrically after electrochemical [225] or chemical [240] postcolumn reduction to its hydroquinone (quinol) form. Chemical reduction is commonly carried out using a reactor column packed with zinc metal powder. When operating the zinc reducer column, 5 ml of a reductive ionic solution containing 2.0 *M* zinc chloride, 1.0 *M* sodium acetate, and 1.0 *M* acetic acid per liter of methanol is added to 1 liter of mobile phase. Indyk [241] reported an HPLC method in which a commercial fluorescence detector facilitates photochemical reduction of phylloquinone and instantaneous detection of the hydroquinone during normal passage of the column effluent through the flow cell.

20.5.5.2 Normal-Phase Separations

Adsorption chromatography using a silica column facilitates the separation of the inactive *cis* isomer of phylloquinone from the active

trans isomer. Hwang [218] applied adsorption HPLC to the determination of phylloquinone in infant formulas using photometric detection at 254 nm. Both the *cis* and *trans* isomers could be measured in standards and in liquid formulas, but matrix interferences prevented measurement of the *cis* isomer in powdered formulas. Figure 20.44 shows chromatograms of a vitamin K standard, and purified extracts of milk-based and soy protein-based infant formula powders.

20.5.5.3 Reversed-Phase Separations

Reversed-phase chromatography using C_{18} stationary phases can separate phylloquinone from closely related structures, but cannot separate *cis*- and *trans*-phylloquinone. However, this separation can be achieved using a polymeric C_{30} phase [242]. Postcolumn reduction of vitamin K coupled with fluorescence detection permits the use of highly efficient NARP chromatography, which is incompatible with electrochemical detection. NARP-HPLC facilitates the separation of the homologous series of menaquinones, separated from phylloquinone (Figure 20.45). The long-chain menaquinones are so lipophilic that they are insoluble in pure methanol, hence NARP-HPLC is well suited for determining these compounds.

Schneiderman et al. [40] developed a rapid, single-step extraction procedure using supercritical fluid carbon dioxide to isolate phylloquinone from powdered infant formulas. The extracted vitamin is trapped on a short tube packed with silica and is eluted with 30 ml of dichloromethane/acetone (1 + 1). The eluate is evaporated to dryness and the residue is dissolved in 1 ml of the HPLC mobile phase. Phylloquinone is determined by reversed-phase HPLC using amperometric detection in the reductive mode. A typical chromatogram of an extract of milk-based infant formula is shown in Figure 20.46. The single peak corresponds to about 45 ng phylloquinone and originated from a 1-g sample. For comparison, 10 g of a soy protein-based formula spiked with 10 μg phylloquinone per g sample was extracted by the same procedure, and the extract was analyzed by reversed-phase HPLC using UV detection at 248 nm. The chromatogram (Figure 20.47) shows that the supercritical carbon dioxide extracts early-eluting material as well as vitamin K, but this material is not electroactive under the conditions used. The peak in Figure 20.47 corresponds to approximately 5 μg phylloquinone.

In a method developed by Shearer [221] for determining phylloquinone, food sample extracts are purified by silica solid-phase extraction and semipreparative normal-phase HPLC, and then analyzed by reversed-phase HPLC. Either of two analytical systems are employed, one using UV absorbance detection and the other electrochemical

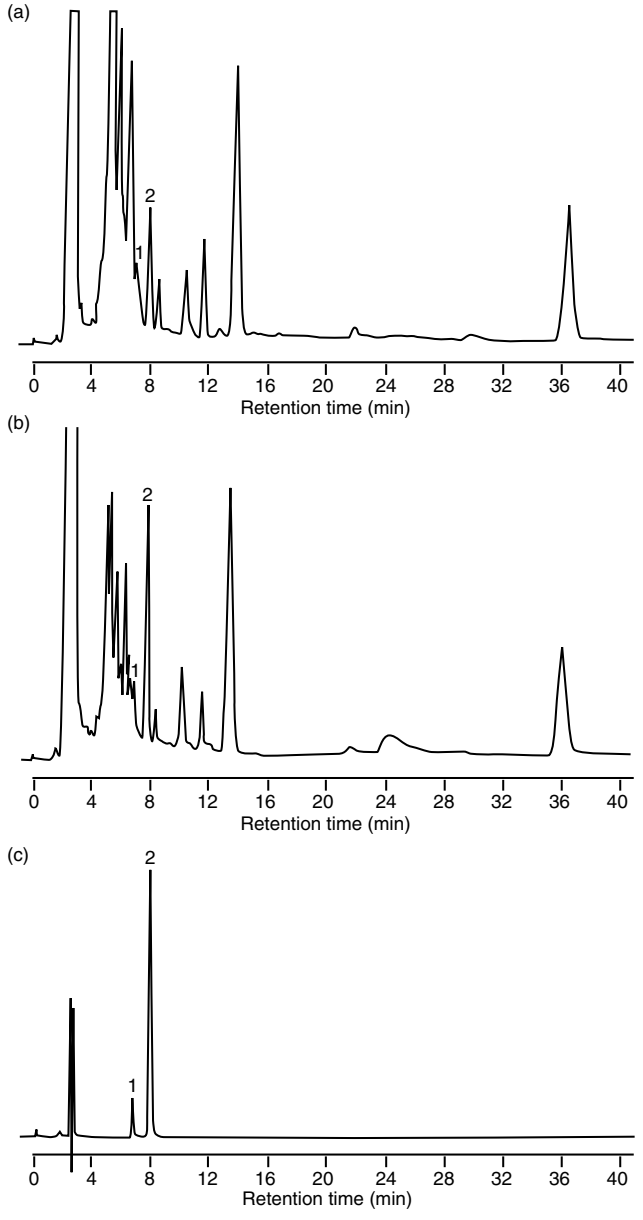

FIGURE 20.44
Normal-phase HPLC and UV detection of phylloquinone *cis* and *trans* isomers. (a) Phylloquinone standard. (b) Extract of milk-based and (c) extract of soy-protein-based infant formula powders. Operating parameters as in Table 20.11 [218]. Peaks: (1) *cis*-phylloquinone; (2) *trans*-phylloquinone. (Reprinted from Hwang, S.-M., *J. AOAC Int.*, 68, 684–689, Copyright 1985 by AOAC International.)

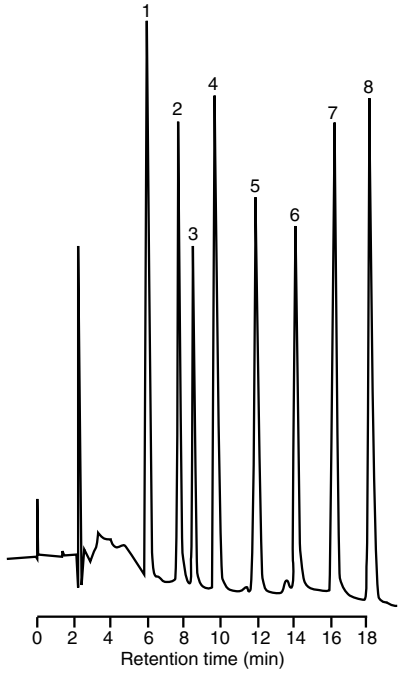

FIGURE 20.45
Separation of vitamin K vitamers by nonaqueous reversed-phase HPLC. Column, Zorbax ODS; mobile phase, linear gradient in which the concentration of dichloromethane in methanol was increased from an initial concentration of 20% to a final concentration of 50% at a rate of 5%/min; UV detection, 270 nm. Peaks: (1) MK-4; (2) MK-5; (3) phylloquinone; (4)–(8) MK-6 to MK-10. (Reprinted with permission from M.J. Shearer.)

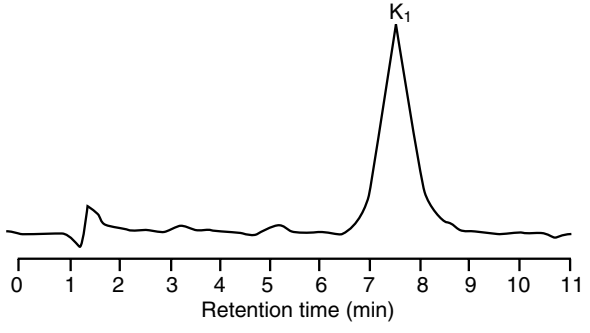

FIGURE 20.46
Reversed-phase HPLC and amperometric detection of phylloquinone (K_1) extracted from a milk-based infant formula with supercritical carbon dioxide. Operating parameters as in Table 20.11 [40]. (Reprinted from Schneiderman, M.A., Sharma, A.K., Mahanama, K.R.R., and Locke, D.C., *J. AOAC Int.*, 71, 815–817, Copyright 1988 by AOAC International. With permission.)

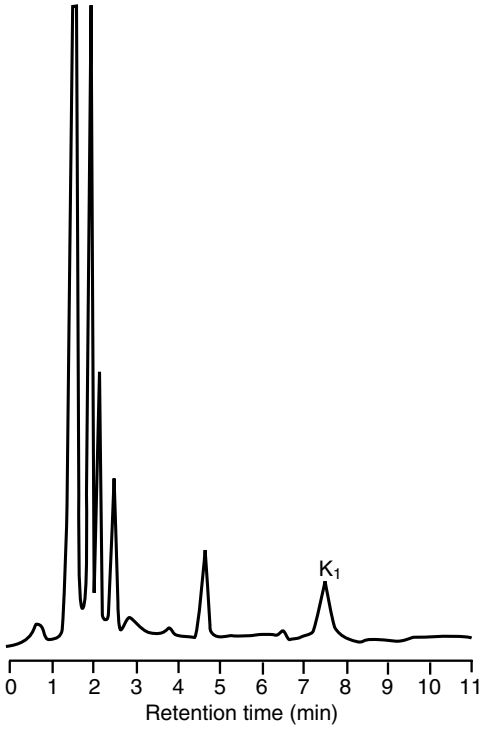

FIGURE 20.47
Reversed-phase HPLC and UV detection of added phylloquinone (K_1) extracted from a soy-protein-based infant formula with supercritical carbon dioxide. Column, Whatman C_{18}; mobile phase, acetonitrile, dichloromethane, and water (90 + 5 + 5); UV detection, 248 nm. (Reprinted from Schneiderman, M.A., Sharma, A.K., Mahanama, K.R.R., and Locke, D.C., *J. AOAC Int.*, 71, 815–817, Copyright 1988 by AOAC International. With permission.)

detection. The retention window for collecting the fraction containing the phylloquinone and internal standards excludes *cis*-phylloquinone. With UV detection, a nonaqueous mobile phase is used, and the phylloquinone is quantified by the method of peak height ratios using phylloquinone 2,3-epoxide as the internal standard. With electrochemical detection, a semiaqueous mobile phase is used in conjunction with a less retentive octyl-bonded (C_8) stationary phase. The addition of 0.1 m*M* EDTA to the semiaqueous mobile phase prevents the reduction of metal ions at the generator electrode. Phylloquinone 2,3-epoxide is electrochemically inactive; therefore quantification is accomplished by the technique of radioisotopic dilution using tritiated phylloquinone 2,3-epoxide as the internal standard. A chromatogram showing the analytical separation of the phylloquinone fraction from

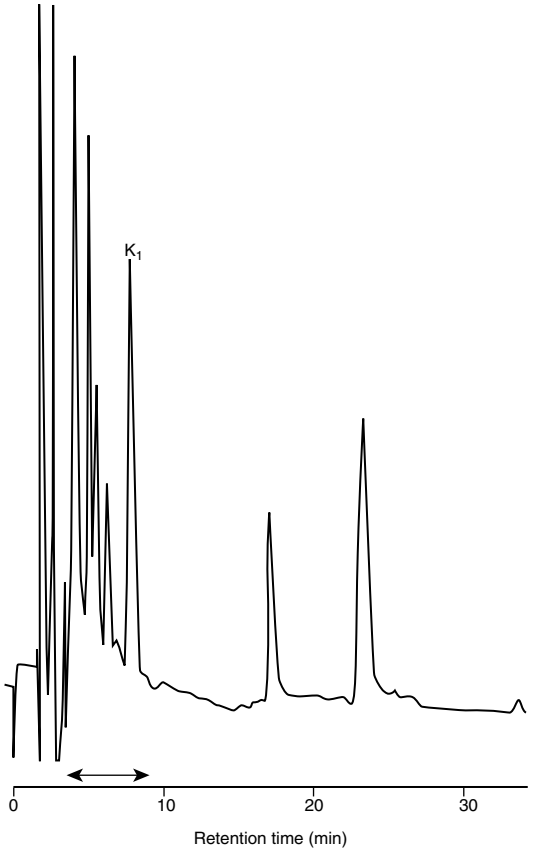

FIGURE 20.48
Quantitative reversed-phase HPLC of the phylloquinone fraction collected by semi-preparative HPLC from an extract of brown rice. Column, Spherisorb C_8 (octyl); mobile phase, methanol and 50 m*M* acetate buffer pH 3.0 (97:3) containing 0.1 m*M* EDTA; dual-electrode coulometric detection (redox mode), porous graphite electrodes, −1.5 V (generator electrode), +0.05 V (detector electrode). The arrows signify the fraction containing tritiated phylloquinone 2,3-epoxide (internal standard) and phylloquinone (analyte) that is collected for quantitation by radioisotopic dilution. (Reprinted with permission from M.J. Shearer.)

a sample of brown rice isolated by semipreparative HPLC is shown in Figure 20.48.

For the determination of phylloquinone in canola (rapeseed) oils, Gao and Ackman [228] removed the triglycerides by enzymatic hydrolysis, and then purified the hydrolysate by silica solid-phase extraction. Analysis was performed by NARP-HPLC with fluorescence detection after postcolumn chemical reduction. Menaquinone-4 was used as an

internal standard. Solid-phase extraction was found to be necessary to remove late-eluting nonpolar material that, although nonfluorescent, would have accumulated in the C_{18} column and impaired chromatographic performance. Solid-phase extraction also removed interfering fluorescent compounds at or near the position of MK-4 (Figure 20.49) and made internal standardization possible.

For the determination of phylloquinone in various food types, Booth and Sadowsi [231] performed lipid extraction followed by silica solid-phase extraction. Lipid-rich samples, such as oils, required further purification using C_{18} solid-phase extraction to remove the interfering lipid. Analysis was performed by NARP-HPLC, and the fluorescent hydroquinone reduction products of phylloquinone and related compounds were produced by postcolumn chemical reduction. The synthetic analog, $K_1(_{25})$, was used as the internal standard. The analytical separation of phylloquinone, 2′,3′-dihydrophylloquinone, and $K_1(_{25})$ in an extract of partially dehydrogenated soybean oil is shown in Figure 20.50.

Koivu-Tikkanen et al. [234] extracted meat and fish products with 2-propanol/hexane and dairy products with an acid hydrolysis method. In addition, the fats of cheese and rainbow trout were removed by lipase treatment. Sample extracts were purified by normal-phase HPLC and the fraction containing the K vitamers was analyzed by NARP-HPLC using postcolumn chemical reduction and fluorescence detection. Figure 20.51 shows an analytical chromatogram of K vitamers in a purified extract of bovine liver.

A method developed by Indyk and Woollard [237] for the determination of phylloquinone, menaquinones, and dihydrophylloquinone in milk and infant formulas has been subjected to interlaboratory collaborative study [244] and adopted, first action 1999, by AOAC International [245]. The analytical protocol involves lipase digestion, solvent extraction, and NARP chromatography using a C_{18} column, postcolumn reduction, and fluorescence detection. Woollard et al. [19] extended the enzymatic digestion procedure (Section 20.2.3) and employed a C_{30} column to determine *cis*- and *trans*-phylloquinone as well as menaquinones and dihydrophylloquinone. Figure 20.52 compares the chromatography of vitamin K standards on a C_{30} column and a C_{18} column. A similar comparison of a purified sample of canola oil is shown in Figure 20.53.

20.5.6 Simultaneous Determination of Two or Three Vitamins

Few HPLC separations of two or more fat-soluble vitamins in foods have been reported, mainly because of the presence of many interfering

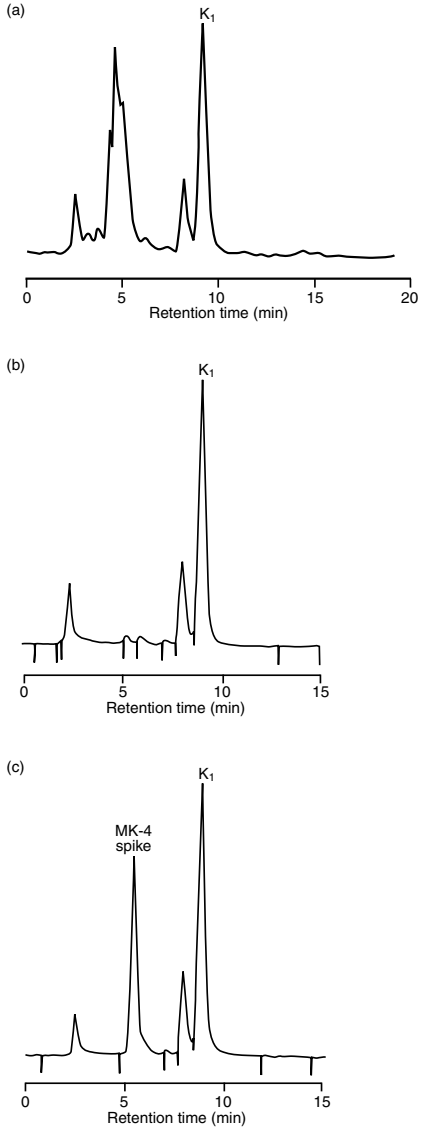

FIGURE 20.49
Reversed-phase HPLC and fluorescence detection of vitamin K in canola oil. (a) Oil subjected to enzymatic hydrolysis and hexane extraction without subsequent solid-phase extraction. (b) Oil subjected to enzymatic hydrolysis, hexane extraction, and solid-phase extraction. (c) As for (b) but spiked with MK-4. Operating parameters as in Table 20.11 [228]. The identity of the peak preceding the phylloquinone (K_1) peak is unknown. (Reprinted from Gao, Z.H. and Ackman, R.G., *Food Res. Int.*, 28, 61–69, © 1995, with permission from Elsevier.)

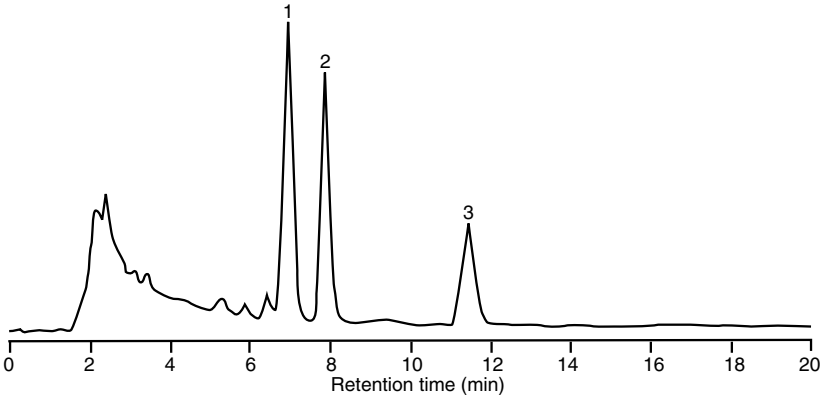

FIGURE 20.50
Reversed-phase HPLC and fluorescence detection of vitamin K extracted from partially hydrogenated soybean oil. Operating parameters as in Table 20.11 [231]. Peaks: (1) phylloquinone; (2) 2′,3′-dihydrophylloquinone: (3) $K_{1(25)}$ (internal standard). (Reprinted from Booth, S.L. and Sadowski, J.A., *Meth. Enzymol.*, 282, 446–456, © 1997, with permission from Elsevier.)

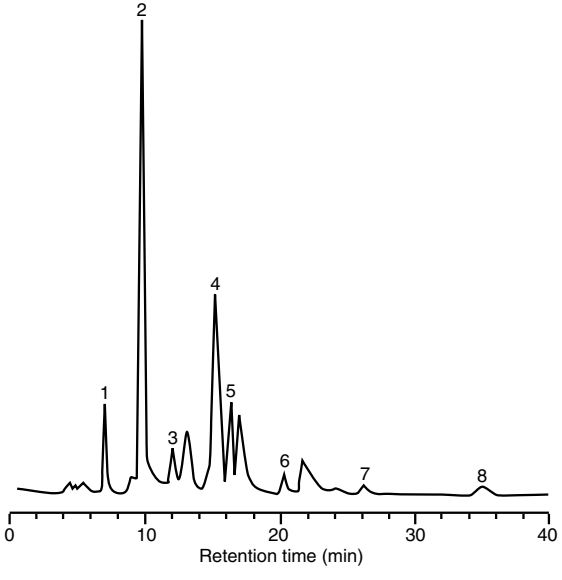

FIGURE 20.51
Reversed-phase HPLC and fluorescence detection of the vitamin K fraction from an extract of bovine liver collected by semipreparative HPLC. Operating parameters as in Table 20.11 [234]. Peaks: (1) MK-4; (2) phylloquinone; (3) MK-6; (4) $K_{1(25)}$ (internal standard); (5)–(8) MK-7 to MK-10. (Reprinted with permission from Koivu-Tikkanen, T.J., Ollilainen, V., and Piironen, V.I., *J. Agric. Food Chem.*, 48, 6325, © 2000 American Chemical Society. With permission.)

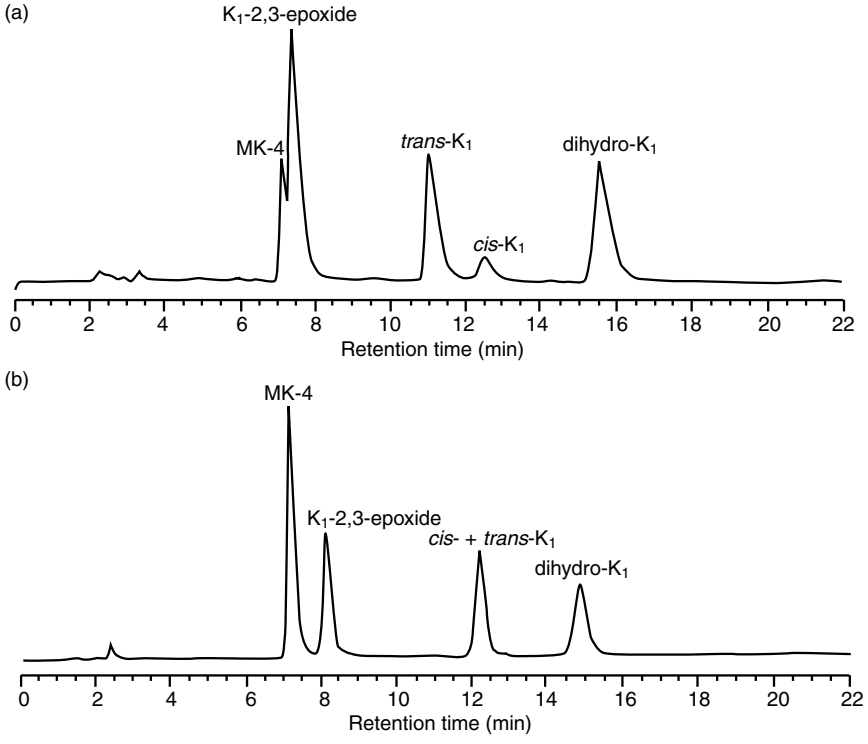

FIGURE 20.52
Reversed-phase HPLC and fluorescence detection of vitamin K standards on (a) YMC polymeric C_{30} column and (b) a C_{18} column (unspecified). Operating parameters as in Table 20.11 [19]. (Reprinted from Woollard, D.C., Indyk, H.E., Fong, B.Y., and Cook, K.K., *J. AOAC Int.*, 85, 682–691, Copyright 2002 by AOAC International. With permission.)

substances in the test extract. Examples of simultaneous determinations are shown in Table 20.12.

20.5.6.1 Normal-Phase Separations

Normal-phase HPLC with gradient elution has been employed for the simultaneous determination of tocopherols, β-carotene, and retinol and its geometric isomers in Italian cheeses [256]. Absorbance monitoring of β-carotene and fluorescence monitoring of the A and E vitamers are accomplished by wavelength-programmable detectors connected in series. Chromatograms of a saponified standard vitamin solution and a saponified cheese sample are shown in Figure 20.54. A similar HPLC system was utilized for the simultaneous assay of total vitamins A

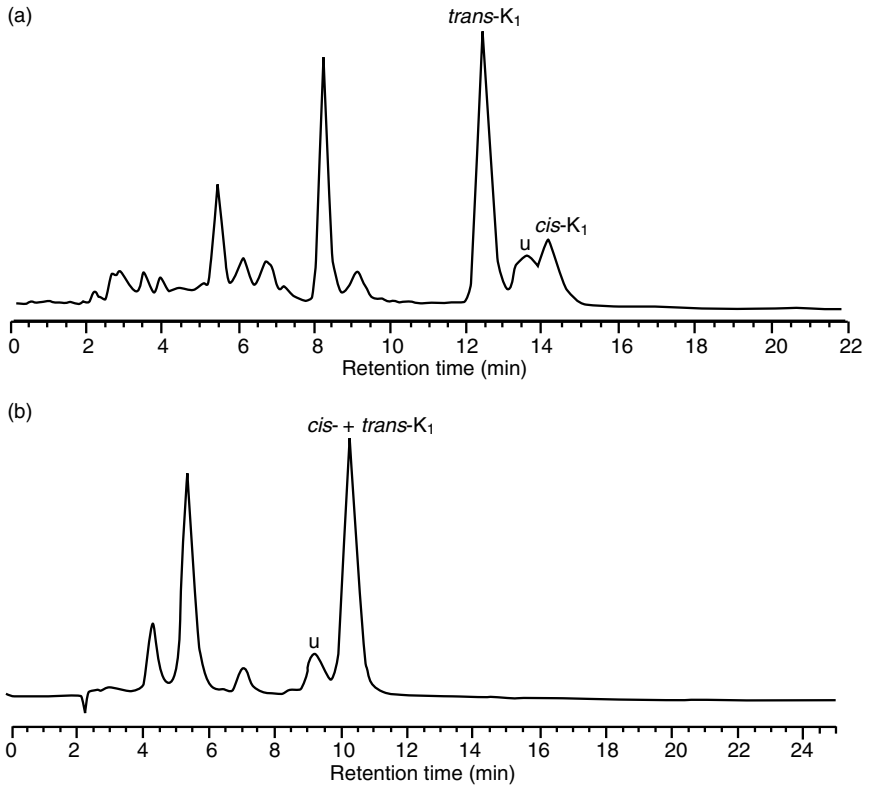

FIGURE 20.53
Reversed-phase HPLC and fluorescence detection of vitamin K extracted from canola oil on (a) YMC polymeric C_{30} column and (b) a C_{18} column (unspecified). Operating parameters as in Figure 20.52. Unidentified component is labeled as "u". (Reprinted from Woollard, D.C., Indyk, H.E., Fong, B.Y., and Cook, K.K., *J. AOAC Int.*, 85, 682–691, Copyright 2002 by AOAC International. With permission.)

and E activity in fortified, plant-based foods [250]. The vitamins, including encapsulated retinyl palmitate, were extracted in 2-propanol and hexane without saponification. A chromatogram of a margarine extract is shown in Figure 20.55. Isocratic normal-phase chromatography separated geometric isomers of retinyl acetate and retinyl palmitate, α-tocopheryl acetate, and α-tocopherol in dairy products [258]. A programmable fluorescence detector monitored the A and E vitamers, while β-carotene was detected photometrically at 450 nm. The fluorescence trace obtained for a fat extract of infant formula powder

TABLE 20.12

HPLC Methods Used for the Simultaneous Determination of Two or More Fat-Soluble Vitamins in Food

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Vitamins Separated**	**Detection**	**Ref.**
Normal-phase chromatography						
Breakfast cereals fortified with vitamins A and E	Reflux sample with a solvent mixture of $CHCl_3$, EtOH, and H_2O at 50°Cl. Filter $CHCl_3$ extract through sodium sulfate, evaporate under vacuum, dissolve residue in HPLC mobile phase	μPorasil 10 μm 300 × 4 mm	Hexane/$CHCl_3$ containing 1% EtOH, 85:15	Retinyl palmitate, α-tocopheryl acetate	UV 280 nm	[246]
Milk-based infant formula	Mix reconstituted sample sequentially with hot 2-PrOH, solid magnesium sulfate, hexane/ethyl acetate (85:15), BHT in hexane (360 μg/ml). Homogenize, filter through sintered glass under vacuum, wash $MgSO_4$ cake with hexane/ethyl acetate. Repeat extraction procedure. Evaporate pooled filtrate under vacuum, dissolve residue in hexane, filter (0.45 μm). Evaporate filtrate plus washings to <5 ml using a turbo-evaporator, dissolve residue in hexane	LiChrosorb Si-60 5 μm 250 × 4.6 mm	0.5% 2-PrOH in hexane flow rates: (1) 1.0 ml/min (2) 0.5 ml/min	(1) α-Tocopheryl acetate, tocopherols (2) Retinyl palmitate	Fluorescence: (1) ex 285 nm em 310 nm (2) ex 325 nm em 470 nm	[247]

(*Table continued*)

TABLE 20.12 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Vitamins Separated**	**Detection**	**Ref.**
Soy-based infant formula	As in preceding entry	As in preceding entry	(1) 0.5% 2-PrOH in hexane (2) 0.125% 2-PrOH in hexane	(1) α-Tocopheryl acetate (2) Retinyl palmitate	As in preceding entry	[248]
Medical foods	As in preceding entry	As in preceding entry	As in preceding entry	As in preceding entry	As in preceding entry	[249]
Fortified breakfast cereal, fortified peanut butter, margarine	*Cereal and peanut butter*: add hot water to sample, sonicate. Add 2-PrOH, solid magnesium sulfate, and hexane containing 0.003% BHT. Homogenize, filter through sintered glass under vacuum, wash $MgSO_4$ cake with hexane/BHT. Repeat extraction procedure. Dilute combined filtrates to volume (100 ml) with hexane. Evaporate a 2-ml aliquot under nitrogen, dissolve residue in HPLC mobile phase *Margarine*: add hexane/ethyl acetate (90:10) containing 0.01% BHT to sample, sonicate, add solid $MgSO_4$, mix, let stand for 2 h, filter. Evaporate a 2-ml aliquot under nitrogen, dissolve residue in HPLC mobile phase	LiChrosorb Si-60 5 μm 250 × 4.6 mm	0.5% 2-PrOH in hexane with a gradient flow rate from 0.9 to 1.3 ml/min	β-Carotene retinyl palmitate, α-tocopheryl acetate, tocopherols	Vis: β-carotene, 450 nm. Fluorescence: retinyl palmitate, ex 325 nm, em 470 nm; α-tocopheryl acetate, ex 285 nm, em 310 nm; tocopherols, ex 290 nm, em 330 nm	[250]

Reduced fat mayonnaise	As in preceding entry except evaporate a 4-ml aliquot to dryness	As in preceding entry	0.27% 2-PrOH in hexane with a gradient flow rate from 0.9 to 1.5 ml/min	β-Carotene, α-tocopheryl acetate, tocopherols	Vis: β-carotene, 450 nm Fluorescence: α-tocopheryl acetate and, tocopherols as in preceding entry	[251]
Milk-based infant formula	Extract sample by matrix solid-phase dispersion. Elute vitamins from C_{18} column with 7 ml hexane containing 0.5% 2-ProH followed by 7 ml CH_2Cl_2. Evaporate pooled eluates under nitrogen, dissolve residue in hexane	LiChrosorb Si-60 5 μm 250 × 4.6 mm	(1) 0.5% 2-PrOH in hexane (2) 0.125% 2-PrOH in hexane	(1) α-Tocopheryl acetate, tocopherols (2) Retinyl palmitate	Fluorescence: (1) ex 285 nm em 310 nm (2) ex 325 nm em 470 nm	[252]
Soy-based infant formula	As in preceding entry	As in preceding entry	As in preceding entry	As in preceding entry	As in preceding entry	[253]
Medical foods	As in preceding entry, except first elution is followed by 7 ml CH_2Cl_2/ethyl acetate/hexane containing 0.5% 2-PrOH (3 + 3 + 4).	As in preceding entry	As in preceding entry	As in preceding entry	As in preceding entry	[254]

(*Table continued*)

TABLE 20.12 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Vitamins Separated**	**Detection**	**Ref.**
Butter, whole milk powder, infant formula	Saponify (hot), extract with diethyl ether, wash pooled extracts to neutral pH. Evaporate under vacuum, dissolve residue in hexane	LiChrosorb Si-60 5 μm 250 × 4 mm	Hexane containing 8% 1,4-dioxan	α-Tocopherol, all-*trans*-retinol	UV and fluorescence detectors connected in series UV (retinol) 325 nm. Fluorescence (tocopherol) ex 293 nm em 326 nm	[255]
Italian cheeses	Saponify (hot) a 0.5-g sample in a tube, cool to 0°C, add 1% sodium chloride, extract twice with hexane/ethyl acetate, 9 + 1. Evaporate pooled extracts, dissolve residue in HPLC mobile phase	Ultrasphere Si 5 μm 250 × 4.6 mm	(A) 1% 2-PrOH in hexane and (B) hexane in a multi-linear gradient elution	Total carotenes, α-, β-, γ-, δ- tocopherols, 13-*cis*- and all-*trans*-retinol	Programmable UV/Vis and fluorescence detectors connected in series. Vis (carotenes) 450 nm. Fluorescence (tocopherols): ex 280 nm em 325 nm (retinols): ex 325 nm em 475 nm	[256]

Milk	Saponify (hot), a 1-g sample in a tube, cool to 0°C, add water, extract twice with hexane/ethyl acetate, 8 + 2. Evaporate pooled extracts under nitrogen, dissolve residue in hexane	μPorasil 10 μm 300 × 3.9 mm, column temperature 30°C	Diisopropyl ether/ hexane step gradient elution	Tocopherols, β-carotene, all-*trans*-retinol	UV/Vis 450 nm (β-carotene) 325 nm (retinol). Fluorescence (tocopherols) ex 292 nm em 325 nm	[257]
Dairy foods	*Butter, anhydrous milk fats*: dissolve in hexane, centrifuge. *Milk, milk powder, cream, infant formula*: reconstitute milk powder or infant formula. To a 5-ml sample, add 1 ml conc. (25%) ammonia and 5 ml EtOH, mix. Add internal standard solution (retinyl acetate or α-tocopheryl acetate + 2 mg/l BHA). Shake vigorously with diethyl ether containing 40 mg/l BHT, add hexane, mix by inversion. After phase separation, wash organic layer with 0.35 *M* calcium chloride. Evaporate under vacuum, dissolve residue in hexane	Econosphere silica 3 μm 150 × 4.6 mm	3% Diisopropyl ether and 0.04% acetic acid in hexane. *For samples containing both retinyl acetate and α-tocopheryl acetate*: 1.2% diisopropyl ether and 0.016% acetic acid in hexane	β-Carotene; 13-*cis*-, 9-*cis*-, and all- *trans*-retinyl palmitate; 13-*cis*-, 9-*cis*-, and all- *trans*-retinyl acetate; α- tocopheryl acetate, α- tocopherol	Programmable UV/Vis and fluorescence detectors connected in series. Vis (β-carotene) 450 nm. Fluorescence (vitamin A): ex 330 nm em 470 nm (vitamin E): ex 295 nm em 330 nm	[258]

(*Table continued*)

TABLE 20.12 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Vitamins Separated**	**Detection**	**Ref.**
Breakfast cereals, infant formula, margarine	*Cereals*: add water and MeOH to ground sample, shake, then sonicate. Extract with MTBE/petroleum ether (10 + 14), centrifuge. Extract twice more. Evaporate pooled extracts under vacuum, dissolve residue in hexane, centrifuge	Nucleosil 100-5 NO_2 5 μm 250 × 4 mm	Three-step gradient composed of hexane and an increasing concentration of MTBE. 0–3.5 min hexane, 3.5–4.5 min up to hexane/MTBE	β-Carotene, retinol and its esters (*cis* and *trans* forms), α-tocopheryl acetate, tocopherols, tocotrienols	UV/Vis: β-carotene, 451 nm: retinols, 325 nm. Fluorescence: retinyl esters, ex 325 nm, em 475 nm; α-tocopheryl acetate, tocopherols, tocotrienols, ex 295 nm, em 330 nm	[259]
	Formulas: reconstitute sample with water, add 35% (w/v) dipotassium oxalate followed by EtOH, extract twice with MTBE/petroleum ether, 25 + 35. Evaporate pooled extracts under vacuum, dissolve residue in hexane, centrifuge. *Margarine*: melt in a water bath, centrifuge, dissolve separated fat in hexane	(98 + 2), 4.5–14 min isocratic, 14–15 min up to hexane/MTBE (92 + 8), 15–38 min isocratic, 38–39 min up to hexane/MTBE (85 + 15), 39–60 min isocratic				

Fortified infant formulas, infant cereals	Add vitamin D_2 and 5,7-dimethyltocol (internal standards) to warm homogeneous slurry. For starch-containing products, incubate with Takadiastase for 30 min at 45°C. Saponify (hot). Load a 20-μl aliquot of the hydrolysate onto a Chromabond XTR solid-phase extraction cartridge, allow to absorb for 15 min, elute vitamins with 100 ml hexane containing 5 mg BHT. Evaporate eluate under nitrogen, dissolve residue in HPLC mobile phase	Nucleosil 100–5 250 × 4.6 mm	Hexane/dioxane/ 2-PrOH, 96.7:3:0.3	All-*trans*-retinol, vitamin D_3, α- tocopherol, internal standards	APCI-MS	[92]
Reversed-phase chromatography						
Infant formulas	Dissolve 1-g sample in 4 ml absolute EtOH, vortex-mix for 2 min, add 400 μl hexane, mix for 1 min, centrifuge	Spherisorb ODS-2 5 μm 250 × 4.6 mm column temperature 50°C	100% MeOH	All-*trans*-retinol, retinyl acetate, retinyl palmitate, α-, β/γ-, δ-tocopherol, α-tocopheryl acetate	UV (diode array detector) 325 nm (vitamin A) 292 nm (vitamin E)	[260]
Infant formulas	Saponify (ambient), extract with hexane, wash pooled extracts to neutral pH, filter through sodium sulfate. Evaporate under vacuum, dissolve residue in MeOH	Spherisorb ODS-2 5 μm 250 × 4.6 mm	MeOH/MeCN/H_2O, 95:1:4	Retinol, α-tocopherol	UV 323 nm (retinol) 292 nm (α-tocopherol)	[261]

(*Table continued*)

TABLE 20.12 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Vitamins Separated**	**Detection**	**Ref.**
Ready-to-eat meals, milk, milk products	Saponify (hot), extract with diethyl ether, wash pooled extracts to neutral pH, dry with sodium sulfate. Evaporate under vacuum, dissolve residue in absolute EtOH containing retinyl acetate (internal standard)	Spherisorb ODS-2 5 μm 250 × 4.6 mm	MeOH/water, 96:4	Retinol, α-tocopherol, retinyl acetate (internal standard)	UV 325 nm (retinol), 294 nm (α-tocopherol)	[262]
Milk-based infant formula	Fully automated robotic system (sample handling, saponification, extraction, injection). Saponify (hot), extract with hexane/CH_2Cl_2 (75 + 25), wash extract three times with water/EtOH (60 + 40). Evaporate an aliquot of the organic layer under nitrogen, dissolve residue in MeOH	Ultrabase C_{18} 5 μm 250 × 4.6 mm	MeOH/2-PrOH, 90 + 10	Retinol, α-tocopherol	UV 235 nm (retinol) 292 nm (α-tocopherol)	[263]
Milk powder	Extract with supercritical CO_2 containing 5% MeOH. Evaporate under nitrogen, saponify the residue at (40°C), extract with petroleum ether, wash twice with water. Evaporate an aliquot of the organic layer under argon, dissolve residue in EtOH	LiChrospher RP-18 5 μm 250 × 4 mm	MeOH/H_2O, 96:4	Retinol. α-tocopherol	UV 325 nm (retinol) 295 nm (α-tocopherol)	[264]

Milk powder, infant formula, liver paste, minced pork, minced beef	Online supercritical fluid extraction/enzymatic hydrolysis using immobilized lipase. Evaporate extracts under nitrogen, dissolve residue in EtOH	LiChrospher RP-18 5 μm 250 × 4 mm	MeOH/H_2O, 98:2	Retinol, retinyl palmitate, α-tocopheryl acetate, α-, β/γ-, δ-tocopherols	UV 325 nm (retinol, retinyl palmitate) 284 nm (α-tocopheryl acetate). Fluorescence: ex 294 nm em 330 nm (tocopherols)	[265]
Vitamin supplemented (ATBC) drinks	Extract with acetone/hexane (1:1), remove emulsion by adding 10% sodium chloride, allow to separate. Wash hexane layer with sodium chloride solution to remove acetone, dry with sodium sulfate. Evaporate under vacuum, dissolve residue in 2-PrOH	Polymeric C_{30} (YMC) 5 μm 250 × 4.6 mm	Gradient elution; eluents: (A) MeOH/MTBE/H_2O, 81:15:4 (B) MTBE/MeOH/H_2O, 90:6:4. Linear gradient from 100% A to 56% B within 50 min	α-, β-, γ-, δ-tocopherol, α-tocopheryl acetate, all-*trans*-α-carotene, all-*trans*-β-carotene	UV at spectral maxima of analytes	[266]
Olive oil	Saponify (hot) a 400-mg sample in a tube, cool, add sodium chloride (25 g/l), extract three times with hexane/ethyl acetate, 85:15. Evaporate pooled extracts, dissolve residue in MeOH	Tracer Extrasil ODS-2 5 μm 150 × 4 mm, column temperature 45°C	Gradient elution; eluents: (A) MeOH; (B) water; (C) butanol. Isocratic with A/B/C, 92:3:5 for 3 min, linear gradient to A/C, 92:8 in 1 min and hold for 5 min. Return to initial conditions	α-Tocopherol, β-carotene	UV 292 nm (α-tocopherol), Vis 450 nm (β-carotene)	[267]

(*Table continued*)

TABLE 20.12 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Vitamins Separated**	**Detection**	**Ref.**
Milk, milk powder	Saponify (hot), extract with hexane, evaporate, dissolve residue in MeOH	Spheri-5 RP-18 5 μm 220 × 4.6 mm	MeOH/H_2O (99:1) containing aqueous 0.1 *M* lithium perchlorate	Retinol, vitamin D_3, α-tocopherol	Amperometric (oxidative mode) glassy carbon electrode, + 1.05 V vs. silver–silver chloride reference electrode	[87]
Milk, butter	Sonicate samples with EtOH to precipitate proteins. Extract twice with hexane, wash pooled extracts with MeOH/water (9 + 1), filter (0.45 μm). Evaporate under nitrogen, dissolve residue in MeOH	Extrasil ODS2 3 μm 150 × 2.1 mm (narrow-bore)	Gradient elution; eluents: (A) MeOH/H_2O, 99 + 1; (B) MeOH/THF, 70 + 30. 0–20 min 0% B, 20–22 min up to 85% B, 22–29 min 85% B isocratic, 29–33 min back to 0% B	Retinol, retinyl acetate, vitamin D_2, vitamin D_3, α-tocopherol, ergosterol, 7-dehydrocholesterol, α-tocopheryl acetate, vitamin K_1, retinyl palmitate	Programmed UV at optimal wavelengths for vitamins	[268]

BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; MeOH, methanol; EtOH, ethanol; 2-PrOH, 2-propanol; $CHCl_3$, chloroform; THF, tetrahydrofuran; MTBE, methyl *tert*-butyl ether.

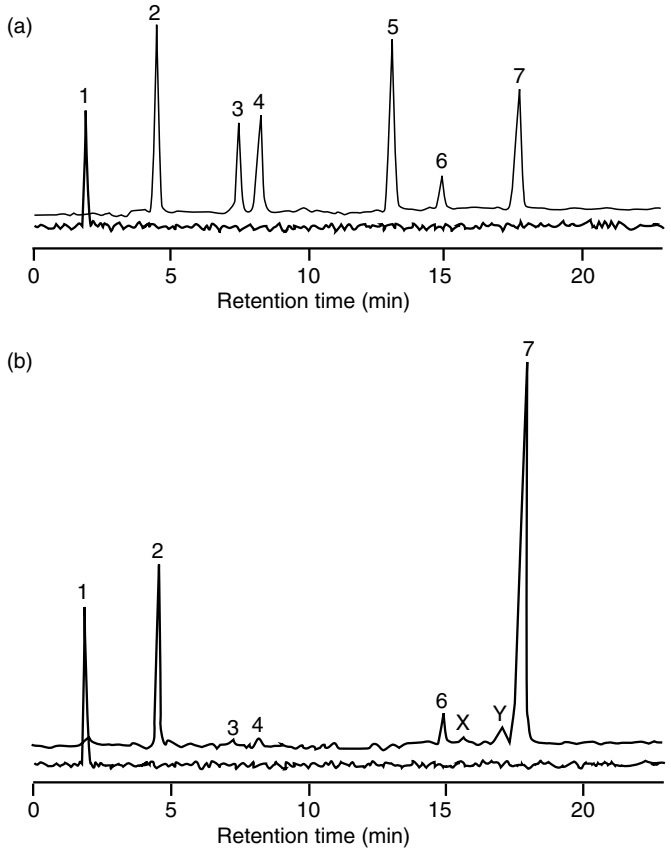

FIGURE 20.54
Normal-phase HPLC of (a) standard vitamin solution after saponification and (b) a saponified cheese sample. Column, Ultrasphere Si; mobile phase, (A) 1% 2-propanol in hexane and (B) hexane in a multilinear gradient. Lower traces, visible absorbance; upper traces, fluorescence. Peaks: (1) β-carotene; (2) α-tocopherol; (3) β-tocopherol; (4) γ-tocopherol; (5) δ-tocopherol; (6) 13-*cis*-retinol; (7) all-*trans*-retinol. Other retinol isomers tentatively identified: (X) 9,11-di-*cis* + 9,13-di-*cis*; (Y) 9-*cis*. (From Panfili, G., Manzi, P., and Pizzoferrato, L., *Analyst*, 119, 1161, 1994. Reproduced by permission of the Royal Society of Chemistry.)

fortified with both retinyl acetate and α-tocopheryl acetate is shown in Figure 20.56.

Heudi et al. [92] developed a novel method for the simultaneous determination of vitamins A, D_3, and E in fortified infant formulas using isocratic normal-phase HPLC and positive-ion atmospheric pressure chemical ionization mass spectrometry (APCI-MS). Samples

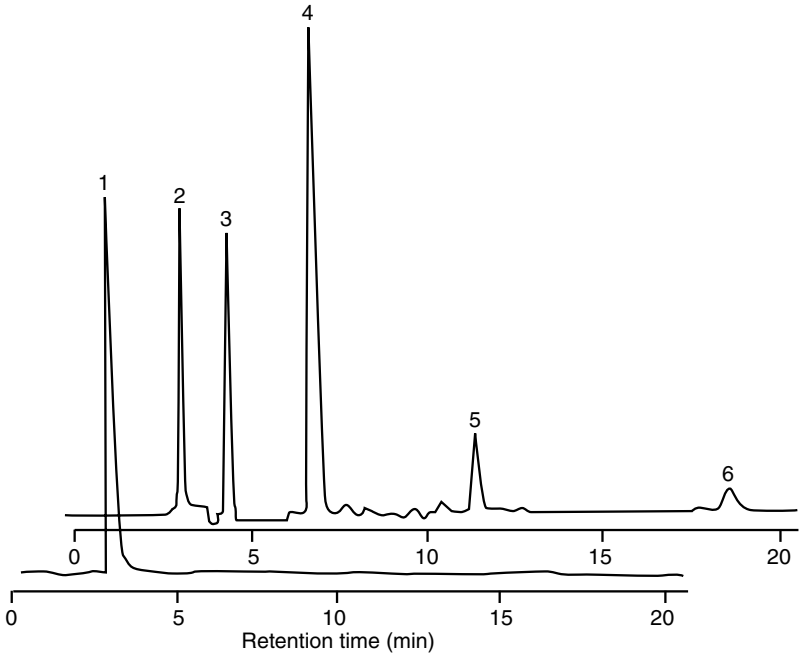

FIGURE 20.55
Normal-phase HPLC of vitamins extracted from margarine. Operating parameters as in Table 20.12 [250]. Lower trace, visible absorbance; upper trace, fluorescence. Peaks: (1) β-carotene; (2) retinyl palmitate; (3) α-tocopheryl acetate; (4) α-tocopherol; (5) γ-tocopherol; (6) δ-tocopherol. (Reprinted with permission from Ye, L., Landen, W.O., and Eitenmiller, R.R., *J. Agric. Food Chem.*, 48, 4003, © 2000 American Chemical Society.)

were saponified and the vitamins in the hydrolysate were isolated using solid-phase extraction. Quantification of vitamins D_3 and E were performed using vitamin D_2 and 5,7-dimethyltocol as internal standards, respectively; no internal standard was used for vitamin A. Detection of the vitamins was made in the selected ion monitoring mode.

20.5.6.2 Reversed-Phase Separations

The sequential separation of retinyl acetate, α-tocopheryl acetate, phylloquinone, vitamin D_3, and retinyl palmitate can be achieved isocratically on a standard C_{18} column eluted with 10% dichloromethane in acetonitrile (Figure 20.57). This method can be used to determine the pure or encapsulated fat-soluble vitamins in vitamin premixes after extraction with hexane. Blanco et al. [268], using a narrow-bore column packed with 3-μm C_{18}-bonded silica particles, separated retinol, retinyl acetate,

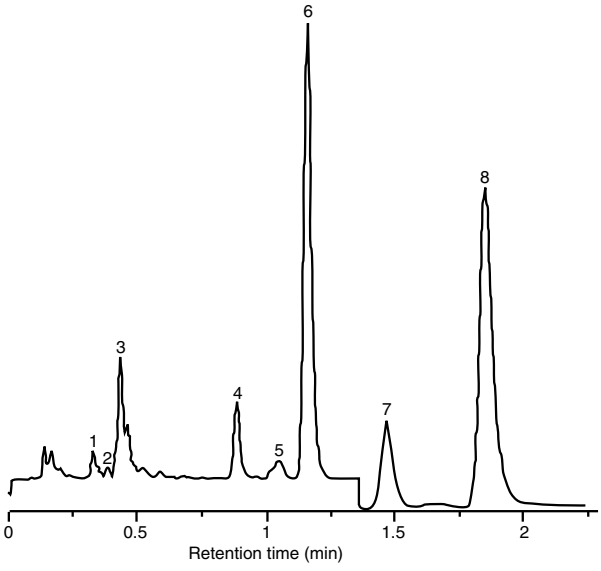

FIGURE 20.56
Normal-phase HPLC and fluorescence detection of an extract of milk-based infant formula fortified with both retinyl acetate and α-tocopheryl acetate. Operating parameters as in Table 20.12 [258]. Gains of 1000 and 100 were used with the fluorescence detector for vitamins A and E, respectively. Peaks: (1) 13-*cis*-retinyl palmitate; (2) 9-*cis*-retinyl palmitate; (3) all-*trans*-retinyl palmitate; (4) 13-*cis*-retinyl acetate; (5) 9-*cis*-retinyl acetate; (6) all-*trans*-retinyl acetate; (7) α-tocopheryl acetate; (8) α-tocopherol. (Reprinted from Hewavitharana, A.K., van Brakel, A.S., and Harnett, M., *Int. Dairy J.*, 6, 613–624, © 1996, with permission from Elsevier.)

vitamins D_2 and D_3, α-tocopherol, ergosterol, 7-dehydrocholesterol, α-tocopheryl acetate, vitamin K_1, and retinyl palmitate in a 33-min gradient run (Figure 20.58). Milk and butter samples were deproteinized and extracted with hexane. Evaporation of the combined hexane extracts and dissolution into a small volume of methanol achieved the necessary concentration of sample to facilitate peak measurement using a programmable UV–Vis detector with a 3-μl flow cell.

Gámiz-Gracia et al. [263] developed a fully automated method for the determination of vitamins A and E in milk-based infant formulas in which a robotic station performed sample handling, saponification, extraction, and injection into a liquid chromatograph. Reversed-phase chromatography and diode array detection completed the analysis.

Delgado Zamarreño et al. [87] proposed a rapid method for the simultaneous determination of vitamins A, D_3, and E in saponified milk and milk powders using reversed-phase HPLC and amperometric

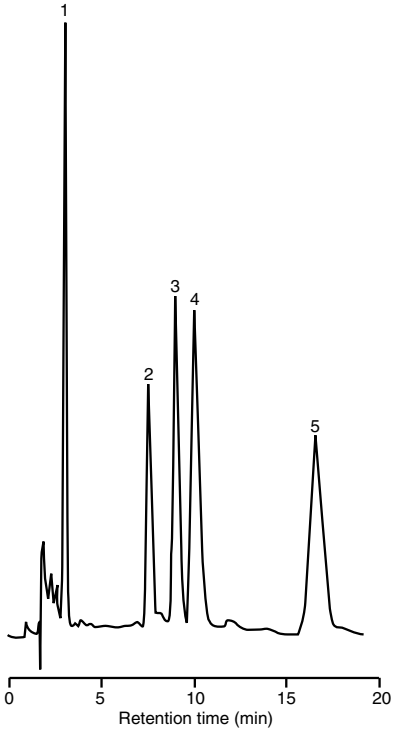

FIGURE 20.57
Reversed-phase separation of fat-soluble vitamins. Column, LiChrosorb RP-18; mobile phase, acetonitrile and dichloromethane (90:10); UV detection, 248 nm. Peaks: (1) retinyl acetate; (2) α-tocopheryl acetate; (3) phylloquinone; (4) vitamin D_3; (5) retinyl palmitate. (From Stary, E., Cruz, A.M.C., Donomai, C.A., Monfardini, J.L., and Vargas, J.T.F., *J. High Resolut. Chromatogr.*, 12, 421, 1989. With permission.)

detection. The high sensitivity of amperometry allows the determination of vitamin D_3 in unenriched fluid milk; this determination is not possible using UV detection without a preconcentration step. A typical chromatogram showing the indigenous vitamins in fluid bovine milk is depicted in Figure 20.59. A directly coupled sample treatment–HPLC system was later developed for the online automatic determination of the same vitamins in liquid and powdered milk [270] and butter and margarine [271]. Alkaline hydrolysis of the samples was performed using two confluent channels through which the sample solution and hydrolysis reagent flowed for a given period of time. A third channel merged with the other two to neutralize the solution before it arrived at a C_{18} solid-phase extraction cartridge. The latter, inserted into a loop with a six-port injection valve, retained the fat-soluble vitamins. The vitamins

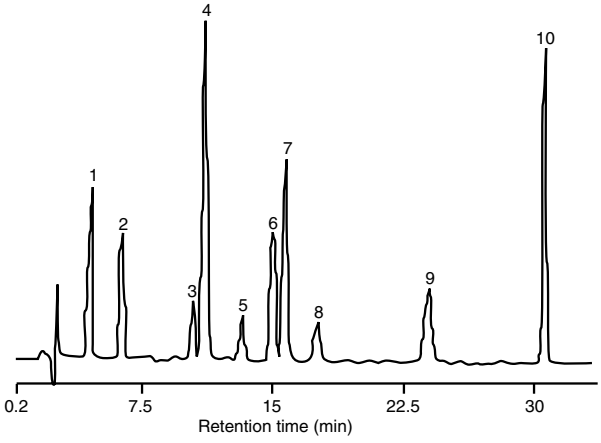

FIGURE 20.58
Reversed-phase separation of fat-soluble vitamins using a narrow-bore column and gradient elution. Programmed UV detection at optimal wavelengths. Operating parameters as in Table 20.12 [268]. Peaks: (1) retinol; (2) retinyl acetate; (3) vitamin D_2; (4) vitamin D_3; (5) α-tocopherol; (6) ergosterol; (7) 7-dehydrocholesterol; (8) α-tocopheryl acetate; (9) phylloquinone; (10) retinyl palmitate. (From Blanco, D., Fernández, M.P., and Gutiérrez, M.D., *Analyst*, 125, 427, 2000. Reproduced by permission of the Royal Society of Chemistry. With permission.)

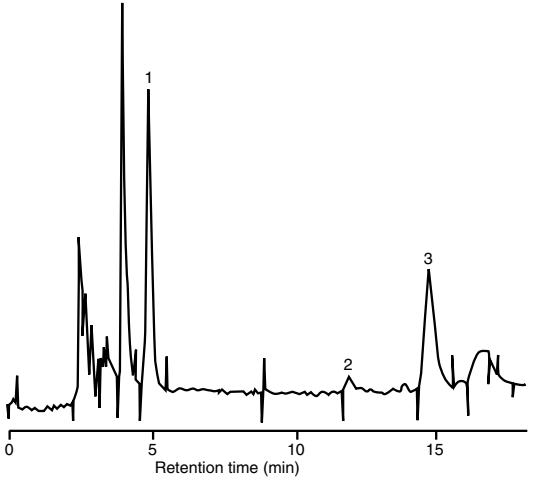

FIGURE 20.59
Reversed-phase HPLC and amperometric detection of vitamins A, D_3, and E in the unsaponifiable fraction of milk. Operating parameters as in Table 20.12 [87]. Peaks (1) retinol; (2) vitamin D_3; (3) α-tocopherol. (Reprinted from Delgado Zamarreño, M.M., Sánchez Pérez, A., Gómez Pérez, C., and Hernández Méndez, J., *J. Chromatogr.*, 623, 69–74, © 1992, with permission from Elsevier.)

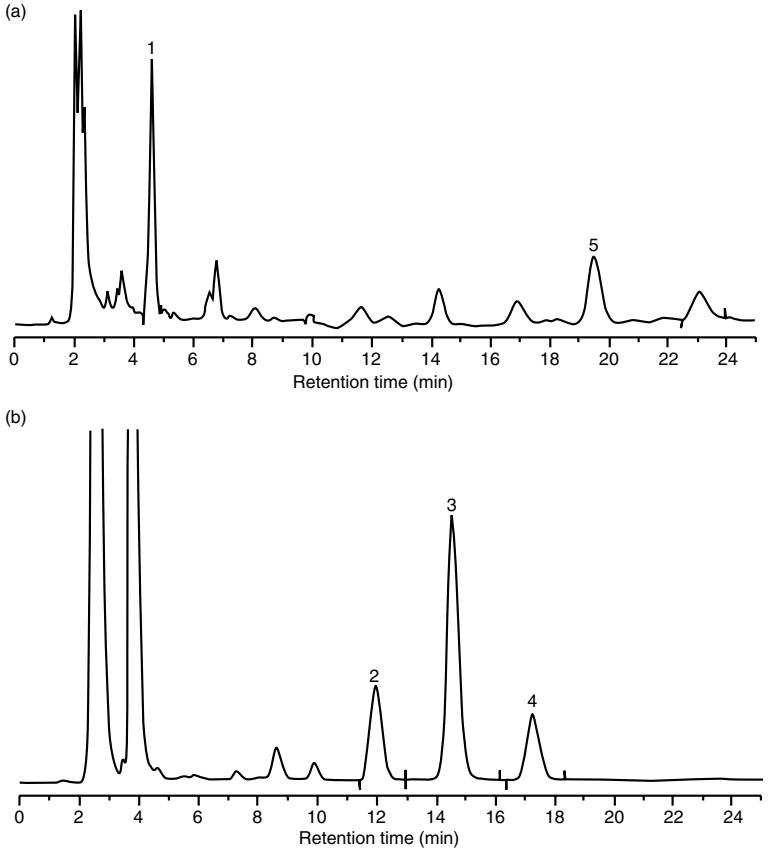

FIGURE 20.60
Reversed-phase HPLC of an infant formula sample after supercritical fluid extraction or enzymatic hydrolysis using immobilized lipase. Column, LiChrospher RP-18; mobile phase, methanol and water (98:2). Serially connected detectors: (a) Programmable UV, 325 nm for retinol and 284 nm for α-tocopheryl acetate; (b) fluorescence, 294 nm excitation or 330 nm emission for tocopherols. Peaks: (1) retinol; (2) δ-tocopherol; (3) β + γ-tocopherol; (4) α-tocopherol; (5) α-tocopheryl acetate. (Reprinted with permission from Turner, C., King, J.W., and Mathiasson, L., *J. Agric. Food Chem.*, 49, 553, © 2001 American Chemical Society.)

were eluted with a stream of methanol, and a 100-μl loopful of eluate was automatically injected into the HPLC column. The problem of solubilizing butter and margarine was overcome by dissolving these samples in an aqueous micellar medium using the surfactant Triton X-100 before introduction into the online system.

In an automated method for determining vitamins A and E in food [265], lipase-catalyzed hydrolysis of glycerides and retinyl esters

was performed during supercritical fluid extraction by immobilizing the enzyme in the SFE extraction cell. Collected extracts were analyzed directly by reversed-phase HPLC using UV detection for retinol, retinyl acetate and α-tocopheryl acetate, and fluorescence detection for tocopherols (Figure 20.60).

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21

Determination of the Water-Soluble Vitamins by HPLC

21.1 HPLC Systems

21.1.1 The Column

HPLC columns used for the analysis of water-soluble vitamins are of the same type as those used in fat-soluble vitamin assays (see Chapter 20, Section 20.4.3).

21.1.2 Chromatographic Modes

The choice of chromatographic mode for quantitative analysis depends on the extraction and cleanup procedures employed and the vitamins required to be measured. Chromatographic modes used in water-soluble vitamin assays include normal- and reversed-phase chromatography (see Section 20.4.4), ion exchange chromatography, ion exclusion chromatography, and reversed-phase ion-pair (ion interaction) chromatography.

21.1.2.1 Ion Exchange Chromatography

An ion exchange material comprises a porous support bearing fixed ionogenic groups, which, when ionized, function as the ion exchange sites. Depending on their function, ion exchange materials are either anion exchangers or cation exchangers, bearing positively charged and negatively charged functional groups, respectively. The positive charges of anion exchangers result from the protonation of basic groups, while the negative charges of cation exchangers are produced by the protolysis of acidic groups (Table 21.1). The functional groups are located mainly within the extensive pore structure of the matrix. To preserve electrical neutrality, each fixed ion is paired with an exchangeable counterion of

TABLE 21.1

Characterization of Ion Exchangers

Type	Functional Group	Usable pH Range
Strong cation exchanger (SCX)	Sulfonic acid (SO_3^-)	>1
Strong anion exchanger (SAX)	Quaternary amine (NR_3^+)	<11
Weak cation exchanger (WCX)	Carboxylic acid (COO^-)	>6
Weak anion exchanger (WAX)	Primary amine (NH_3^+)	<8

opposite charge. The type of counterion specifies the "form" of the ion exchanger; for example, a strong anion exchanger is usually supplied in the chloride form, that is, the counterion is Cl^-.

In ion exchange chromatography, the separation of sample ions depends on the selectivity at the numerous sorption–desorption cycles that take place as the sample material passes through the column. Ions having a strong affinity for the functional groups will be retained on the column, whereas ions that interact only weakly will be easily displaced by competing ions and eluted early.

Ion exchangers are further classified as strong or weak according to the ionization properties of the basic or acidic functional groups (Table 21.1). The degree of ionization depends on the pK_a of the functional group and on the pH of the mobile phase, and is directly proportional to the ion exchange capacity. The capacity is maximal when all of the functional groups are ionized. The maximum exchange capacity for strong anion and cation exchangers is maintained over a wide pH range, whereas for weak exchangers the usable pH range is limited (Table 21.1).

Most classical ion exchange resins are polystyrene-divinylbenzene (PS-DVB) copolymers to which the ionogenic functional groups are attached. Such resins exhibit a relatively slow diffusion of solutes within the deep pores containing stagnant mobile phase, and this leads to major band broadening. For this reason, such resins were often operated at elevated temperatures to speed mass transfer through a decrease in mobile phase viscosity. One way of minimizing the diffusion path and improving the efficiency of the separation is to use pellicular particles, which have a nonporous, impervious solid core surrounded by a thin coating of active stationary phase. Pellicular packings have been superseded by totally porous microparticulate silica-based packings. Silica-based packings are stable at temperatures up to 80°C, but strongly acidic (pH < 2) or mildly basic (pH > 7.5) conditions destroy the silicon structure, leading to a drastic increase in column resistance and loss of efficiency. This problem has prompted investigation into new supports for a second generation of microparticulate column packings.

The chief mobile phase parameters that control sample retention and separation selectivity are ionic strength and pH. The role of the buffer component is to maintain the pH at the selected value and to provide the desired solvent strength in terms of the appropriate type of counterion at the right concentration. The ionic strength can be regarded as a measure of the number of counterions present. The sample ions and mobile phase counterions of the same charge compete for the ion exchange sites, and hence an increase in ionic strength will proportionately decrease solute retention and vice versa. In other words, the solvent strength increases with increasing ionic strength, accompanied by a minimal change in solute selectivity. The ionic strength of the mobile phase can be increased by either increasing the molarity of the buffer solution while holding the pH constant, or adding a nonbuffer salt such as sodium nitrate when it is undesirable to increase the buffer concentration. The primary effect of pH is to control the ionization of weak organic acids and bases in the sample. Increasing the pH leads to an increased ionization of weak acids and decreased ionization of weak bases, and vice versa for a decrease in pH. An increase in ionization in each case leads to increased solute retention.

Water-miscible organic solvents such as acetonitrile, 2-propanol, and ethanol are frequently added as modifiers to the aqueous mobile phase as a means of lowering the viscosity and improving mass transfer kinetics. Typical amounts of added solvent range between 3 and 10% by volume. The effect of the organic modifier on the ion exchange equilibria is relatively minor, and any significant changes that result from such additions are mainly attributed to hydrophobic mechanisms. In weak anion exchange chromatography, an appreciable proportion of an organic acid solute will exist in the nonionized form, and thus behave differently to the ionized form (anion). The resultant peak tailing caused by the mixed-mode chromatography can be eliminated by use of an organic modifier, which also decreases the retention time. In general, using a modifier can dramatically improve a separation, although the effect is unpredictable and has to be determined empirically. It is obviously important to ascertain beforehand that the column packing material is compatible with the proposed organic solvent.

21.1.2.2 Ion Exclusion Chromatography

In this technique, an ion exchange resin is employed for separating ionic molecules from nonionic or weakly ionic molecules. Ions having the same charge as the functional groups of the support (i.e., co-ions) are repelled by the electrical potential across the exchanger–solution interface (Donnan potential) and excluded from the aqueous phase within

the pore volume of the resin beads. Nonionic or weakly ionic molecules are not excluded and, provided they are small enough, may freely diffuse into the matrix, where they can partition between the aqueous phase within the resin beads and the aqueous phase between the resin beads. Therefore, ionized sample solutes pass quickly through the column, whereas nonionic or weakly ionic solutes pass through more slowly. The retention mechanisms of the nonionic solutes include polar attraction between the solute and the resin functional groups (i.e., adsorption), van der Waal's forces between the solute and the hydrocarbon portion of the resin (primarily the benzene rings), and size exclusion. The overall separation is accomplished without any exchange of ions, so the column does not require regeneration after use.

Ion exclusion chromatography using a strong cation exchange resin has been successfully applied to the separation of organic acids, including ascorbic acid. The technique here is to suppress the ionization of the weak organic acid by adding sulfuric acid to the water mobile phase so that the highly ionized sulfate ion is excluded and quickly eluted, while the undissociated organic acid enters the resin pore structure and is retained. The mobile phase pH should be lower than the pK_a of the organic acid to ensure that the acid is undissociated. The volume of aqueous phase within the resin bead must be sufficient to allow partition of the nonionic solutes to take place and, to achieve optimum separation, must be greater than the sample volume. For this reason, PS-DVB types of resin, which are capable of swelling, are used in preference to silica-based exchangers.

21.1.2.3 Reversed-Phase Chromatography

Ionic compounds cannot be analyzed as such by reversed-phase HPLC, since they elute near the void volumes. Ion suppression is a reversed-phase chromatographic technique in which the ionic equilibrium of the sample is controlled by adjusting the pH of the mobile phase to obtain retention and separation of the components according to their pK_a values [1]. By buffering of the mobile phase at 1–2 units below the pK_a value for a weak acid, and a corresponding amount above the pK_b value for a weak base, the ionization is suppressed and the undissociated compound, having a greater affinity for the stationary phase, is retained. Thus, weak acids and weak bases can be retained in the pH regions 2–5 and 7–8, respectively.

A potential problem with silica-based reversed-phase column packings is that the siloxane bond linking the alkyl ligand to the silica support is prone to hydrolysis at low pH, resulting in a progressive loss of bonded

phase. Although longer-chain ligands such as C_{18} are relatively stable at pH 3 and below, short-chain bonded phases, including small endcapping groups, are especially susceptible. The problem of loss of column performance due to hydrolysis can be largely overcome by the use of 'shielded' stationary phases, which are sterically protected from attack by hydrolyzing protons. One such material is Zorbax SB-C18, which has large, bulky diisobutyl groups on the silane silicon atom and is nonendcapped. Outstanding long-term ruggedness under highly aggressive low-pH conditions (pH 2) has been demonstrated using Zorbax SB-C18 [2]. Another approach is to use a totally polymeric column packing such as PLRP-S, a PS-DVB copolymer. Such materials are not attacked by extremes of pH, but they exhibit appreciably lower separation efficiencies than reversed-phase silica-based packings for small molecules such as vitamins [3].

21.1.2.4 Reversed-Phase Ion-Pair Chromatography

Reversed-phase ion-pair chromatography (also known as ion interaction chromatography) employs the same types of column packing and water/organic mobile phases as those used in conventional reversed-phase HPLC. The pH of the mobile phase is adjusted to encourage ionization of the ionogenic solutes, and retention is controlled by adding to the mobile phase an amphiphilic ion-pairing agent bearing an opposite charge to that of the analyte. The ion-pairing agent should be univalent, aprotic, and soluble in the mobile phase. It should ideally give a low UV-absorbing background, although for special applications a reagent with a strong chromophore can be used to enhance the response of an absorbance detector. The retention behavior of nonionic solutes is not affected by the presence of the ion-pairing agent, so both ionized and nonionized solutes may be resolved in the same chromatographic run. Use of ion-pair chromatography is advantageous for determining water-soluble vitamins because many polar interferences elute in the dead volume, and hydrophobic compounds would be in low concentration in the aqueous extract of the sample.

For the determination of anionic solutes such as ascorbic acid, a variety of organic amines have been used as ion pairing agents, representing primary, secondary, tertiary, and quaternary amines. One of the more popular of these is tetrabutylammonium (Bu_4N^+) phosphate, which is commercially available as a prepared 5 m*M* solution in pH 7.5 buffer (PIC A reagent, Waters Associates). This aprotic quaternary amine interacts with strong and weak acids, and the buffering to pH 7.5 suppresses weak base ions.

For the determination of cationic solutes such as thiamin (a protonated amine), a range of alkyl sulfonates having the formula $CH_3(CH_2)_nSO_3^-$

($n = 4–7$) predominates. Selection of the appropriate reagent is based on solute retention time, which increases with an increase in the length of the alkyl chain. Prepared 5 m*M* solutions of the sodium salts in pH 3.5 buffer are available from Waters Associates; namely, pentane sulfonic acid (PIC B5), hexane sulfonic acid (PIC B6), heptane sulfonic acid (PIC B7), and octane sulfonic acid (PIC B8). These reagents interact with strong and weak bases, and the buffering to pH 3.5 suppresses weak acid ions.

Most ion-pair chromatographic applications reported for water-soluble vitamin assays up to the present day have utilized 5- or 10-μm silica-based C_{18} bonded-phase packings. Monomeric phases yield better-shaped peaks than do polymeric phases, and high carbon loadings ensure good retention properties [4]. PS-DVB copolymers developed for HPLC have also been utilized for ion-pair chromatography [5].

The practice of ion-pair chromatography has been discussed by Gloor and Johnson [6]. Retention and selectivity are optimized mainly by altering the concentration of the ion-pairing agent and the pH of the mobile phase. Ionic strength is not a variable for controlling retention and it should be kept as low as possible, commensurate with satisfactory retention characteristics and reproducibility.

Variation of the concentration of ion-pairing agent in the mobile phase provides a simple means of controlling solvent strength. An increase in the concentration causes an increase in solute retention but, beyond a certain limit, a further increase in concentration causes a decrease in retention. A possible explanation for this reversal effect is that the increased amount of adsorbed surfactant lowers the interfacial tension between the modified stationary phase and the surrounding aqueous medium to a point at which solute retention is decreased [7]. This nonionic theory also accounts for the observed decrease in retention of neutral solutes with increasing concentration of ion-pairing agent.

Alterations in the pH of the mobile phase will have a pronounced effect on separation selectivity for weak acids and weak bases because of the effect of pH on solute ionization. Maximal retention is obtained where the solute and ion-pairing agent are completely ionized. The reagents, being strong acids or salts of strong bases, remain completely dissociated over a wide pH range, so that the pH can be adjusted to an optimal value for the separation. Weak acid solutes ($pK_a > 2$) are usually separated at a pH of 6–7.4, and weak bases at pH 2–5, using a buffer to hold the pH constant. Buffer salts should have poor ion association properties, but good solubilities in the mobile phase. An excessive concentration of buffer salt, or the addition of neutral salt to the mobile phase, results in the surplus ions of such salts competing successfully with analyte ions for association with the adsorbed ion-pairing agent,

thus causing a decrease in retention. Solute pK_a values are affected by a change in temperature, so significant changes in selectivity can occur with relatively small changes in column temperature. To ensure reproducible separations, it is thus good practice to maintain a constant column temperature with the aid of a column heating oven. Ion-pair chromatography is usually carried out at a few degrees above ambient, although operation at 50–60°C will improve peak resolution (with a slight decrease in retention) by reducing the viscosity of the mobile phase.

Increasing the proportion of organic modifier increases the solvent strength, resulting in an overall lowering of solute retention. The concentration of organic modifier affects the surface potential (and hence solute retention) by influencing the sorption of the ion-pairing agent onto the stationary phase [8].

The general strategy for separating complex mixtures of nonionic and ionic solutes is firstly to adjust the percentage of organic modifier (usually methanol) to obtain optimum retention and separation of nonionic solutes. One then adds a suitable ion-pairing reagent in the appropriate buffer to the previously established mobile phase to separate the ionic compounds isocratically. Gradient elution programs usually involve a decrease in the concentration of ion-pairing agent with time as a means of decreasing solute retention.

Ion-pairing agents may irreversibly adsorb onto the stationary phase, thereby changing the phase chemistry and reducing the apparent pore volume. Columns used for ion-pair chromatography should therefore be reserved exclusively for this purpose.

21.1.3 Derivatization

It is sometimes necessary to make a chemical derivative of an analyte in order to facilitate the use of a more suitable means of detection and/or a more suitable chromatographic mode. Either pre- or post-column derivatization may be employed, depending on whether one wishes to chromatograph the derivatized analyte or the underivatized analyte. In precolumn derivatization, the reaction is carried out before the sample is analyzed by HPLC, so it is the derivatized compounds that are actually chromatographed. In postcolumn derivatization, the test solution is injected into the chromatograph, and the separated compounds in the column effluent are reacted with the derivatizing agent in a heated reaction coil located between a mixing tee and the detector [9].

A postcolumn derivatization system requires a second pump to introduce the derivatizing agent but, once set up, the system provides an

automatic and standardized means of preparing the derivatives. There will inevitably be some degree of peak broadening due to the increased distance between the HPLC column and the detector. Another disadvantage is that there is no opportunity to remove or separate excess reagent or impurities within the reagent that might impair the sensitivity of detection. Precolumn derivatization requires manual manipulations, and hence more skill and nonstandardized reaction conditions, unless rigorously controlled. Advantages are the opportunity to clean up the reaction mixture before injection, and the operation of a simpler and more efficient chromatographic system.

21.2 Applications of HPLC

In this section, applications are arbitrarily divided into single vitamin analyses and multiple vitamin analyses. The requirement to determine the naturally occurring vitamin of a foodstuff allows little scope for determining more than one vitamin at a time. This is because of difficulties of quantitatively extracting the vitamins from their various bound forms, the need to measure low indigenous concentrations in the presence of a complex matrix, and the requirement to determine several vitamers of some vitamins.

21.2.1 Thiamin

21.2.1.1 Detection

The absorption spectrum of thiamin hydrochloride is pH-dependent, as shown in Figure 21.1. At pH 2.9 a single maximum at 246 nm occurs; the ε value at this wavelength is 11,305. At pH 5.5 two maxima occur at 234 and 264 nm, which correspond to the substituted pyrimidine and thiazole moieties, respectively.

Thiamin itself does not fluoresce, but the vitamin and its phosphate esters can be reacted with alkaline potassium hexacyanoferrate(III) [potassium ferricyanide, $K_3Fe(CN)_6$] to form the corresponding thiochrome compound (Figure 7.2), which displays a strong blue fluorescence. The fluorescence excitation and emission spectra of thiochrome possess wavelength maxima at 375 and 432–435 nm, respectively (Figure 21.2). Equimolar amounts of the thiochrome derivatives of thiamin, TMP, TDP, and TTP produce different fluorescence intensities [10].

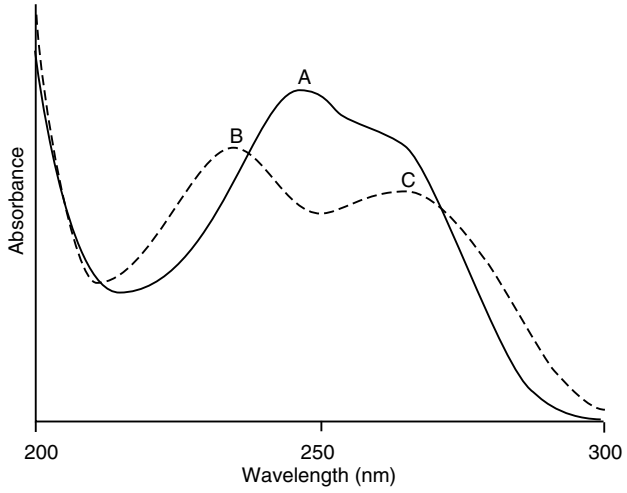

FIGURE 21.1
UV absorption spectra of thiamin hydrochloride in 0.1 *M* phosphate buffer at pH 2.9 (solid line) and 5.5 (broken line) (λ_{max} of peak A = 246 nm; B = 234 nm; C = 264 nm).

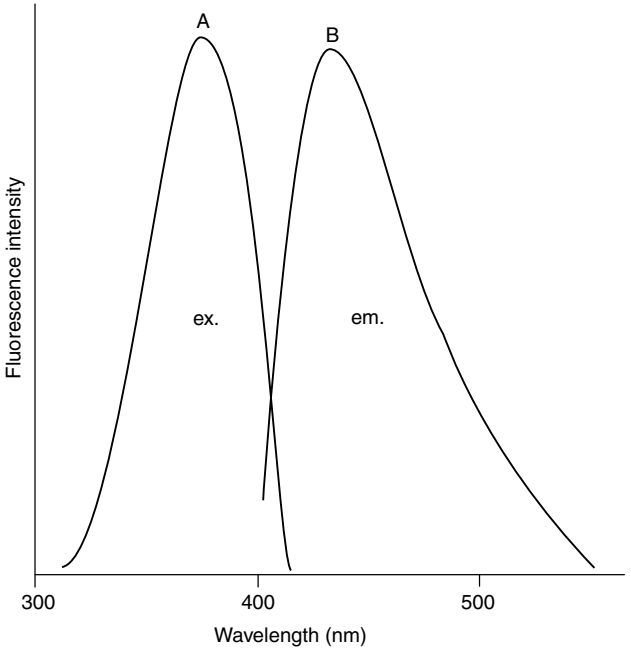

FIGURE 21.2
Fluorescence excitation and emission spectra of thiochrome (λ_{max} of peak A = 375 nm; B = 432–435 nm).

21.2.1.2 Methodology

HPLC methods used for determining thiamin *per se* are summarized in Table 21.2. Methodology has been well discussed in a review by Lynch and Young [15].

When determining the total thiamin content of a food commodity, the test material is extracted by autoclaving with dilute mineral acid (usually 0.1 *N* hydrochloric acid) followed by enzymatic hydrolysis, in order to convert protein-bound and phosphorylated forms of the vitamin to free thiamin. Although thiamin exhibits a rather low molar absorptivity ($\varepsilon = 11{,}305$ at λ_{max} 246 nm), absorbance detection has adequate sensitivity for fortified foods [16,17] and also for foods that are relatively rich in the vitamin, such as legumes and pork muscle [14]. For other food commodities, absorbance detection is inadequate, and it is necessary to employ the more sensitive fluorescence detection after oxidation of the thiamin to thiochrome by pre- or postcolumn reaction with alkaline hexacyanoferrate(III).

Precolumn derivatization allows the relatively nonpolar thiochrome to be determined using conventional reversed-phase chromatography, with its attendant ease of operation and long-term stability. Some workers [18–20] added orthophosphoric acid 45 sec after treatment with alkaline hexacyanoferrate(III) to minimize formation of thiamin disulfide, a pH-dependent side reaction of the thiamin to thiochrome oxidation. Cleanup of the reaction mixture prior to HPLC has been effected using C_{18} solid-phase extraction cartridges [18,19,21]. An alternative approach is to selectively extract the thiochrome into isobutanol, and then to inject an aliquot of the organic solution onto an HPLC column of underivatized silica eluted with chloroform/methanol (80:20) [11]. An on-column fluorescence detection limit of 0.05 ng thiamin was reported using this approach [22].

If the derivatization is carried out postcolumn, it is actually thiamin that is being chromatographed, and this compound in the ionized state is not retained under simple reversed-phase conditions. However, reversed-phase columns can be utilized for thiamin assay by means of ion-pair chromatography using hexane (or heptane) sulfonic acid as the ion-pairing reagent, either after postcolumn derivatization of thiamin and fluorescence detection, or without derivatization, using UV detection. Reversed-phase columns can also be used with ion suppression [23,24].

Postcolumn derivatization is not only more reproducible and convenient than precolumn derivatization, but the alkaline pH of the effluent is more conducive to the fluorometric detection of thiochrome. This is because the fluorescence intensity of thiochrome is pH-dependent and reaches a steady state at pH above 8 [10].

TABLE 21.2

HPLC Methods Used for the Determination of Thiamin in Food

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Normal-phase chromatography						
Liver, semi-synthetic animal diet	*Acid and enzymatic hydrolysis*: digest homogenized sample with 0.1 *N* HCl at 95–100°C for 1 h. Cool, adjust pH to 4.5. Incubate with Takadiastase and papain at 45–50°C for 3 h. Cool, dilute to volume with 0.1 *M* HCl, centrifuge, filter *Derivatization*: oxidize thiamin to thiochrome with alkaline $K_3Fe(CN)_6$, partition into isobutanol, centrifuge	LiChrosorb Si-60 5 μm 250 × 4 mm	$CHCl_3$/MeOH, 80:20	Thiochrome	Fluorescence ex 375 nm em 430 nm	[11]
Reversed-phase chromatography						
Cooked sausages	*Acid and enzymatic hydrolysis*: autoclave ground sample with 0.1 *N* HCl at 120°C for 20 min. Cool, adjust pH to 4.0–4.5. Incubate with Claradiastase at 50°C for 3 h. Precipitate proteins by heating at 90°C with 50% (w/v) TCA, dilute to volume with water, filter	Spherisorb C_8 (octyl) 5 μm 250 × 4 mm Column temperature 35°C	5 m*M* phosphate buffer, pH 7.0/MeCN, 70:30	Thiochrome	Fluorescence ex 360 nm em 430 nm	[12]

(*Table continued*)

TABLE 21.2 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
	Derivatization: oxidize thiamin to thiochrome with alkaline $K_3Fe(CN)_6$ *Cleanup and concentration*: pass oxidized extract through a C_{18} solid-phase extraction cartridge, wash cartridge with 5 m*M* phosphate buffer, pH 7.0/MeOH (95:5). Elute thiamin with 3 ml MeOH, dilute to 5 ml with MeOH					
Reversed-phase ion-pair chromatography						
Infant formula, milk, low-fat yoghurt, eggs, salad dressing	Stir sample with water. Precipitate proteins by pH adjustment to 1.7–2.0 and then to 4.0–4.2. Dilute to volume with water, filter	μBondapak C_{18} 10 μm 300 × 3.9 mm Column temperature 50°C	Water/MeOH (80:20) containing 0.15% sodium hexane sulfonate, 0.75% acetic acid and 0.1% EDTA	Thiamin	UV 248 nm	[13]

Legumes, pork muscle, full cream milk powder	*Acid and enzymatic hydrolysis*: autoclave ground sample with dilute HCl at 121°C for 15 min. Cool, adjust pH to 4.0–4.5. Incubate with Takadiastase at 48°C for 3 h. Cool, dilute to volume with water, filter *Cleanup and concentration*: readjust pH of extract to 5.0–5.5 and keep at 0°C. Pass aliquot of extract through an Amberlite CG-50 ion exchange column, wash column bed with water, elute thiamin with 0.15 *M* HCl. Evaporate eluate to dryness, dissolve residue in water, adjust pH to 5.5–6.0. Pass aliquot of solution through a C_{18} solid-phase extraction cartridge, wash cartridge with aqueous 5 m*M* sodium hexane sulfonate, elute thiamin with methanolic 5 m*M* sodium hexane sulfonate	μBondapak C_{18} 10 μm 300 × 3.9 mm Column temperature 30°C (legumes) or 50°C (pork and milk products)	Water/MeOH (69:31) containing 5 m*M* sodium hexane sulfonate/5 m*M* sodium heptane sulfonate (75 + 25) and 0.5% acetic acid	Thiamin	UV 254 nm	[14]

Note: $K_3Fe(CN)_6$, potassium hexacyanoferrate(III); MeOH, methanol; MeCN, acetonitrile; $CHCl_3$, chloroform; trichloroacetic acid, TCA; EDTA, ethylenediaminetetraacetic acid.

21.2.2 Vitamin B_2

21.2.2.1 Detection

Riboflavin in aqueous solution exhibits a UV–visible spectrum containing four major bands centered around 223, 266, 373, and 445 nm (Figure 21.3). The positions of the maxima and their absorbance coefficients vary somewhat according to the nature of the solvent. The 373-nm band is the most affected by solvents, generally shifting to shorter wavelengths with decreasing solvent polarity. The position of the visible 445-nm band is not greatly affected. The spectra of riboflavin and FMN are practically identical to one another under similar conditions, but the spectrum of FAD is slightly different. All three flavins lose their absorbance in the visible region when they are reduced to their colorless 1,5-dihydro forms, known as leuco bases. Molar absorptivity (ε) values of flavins dissolved in 0.1 *M* phosphate buffer, pH 7.0, are given in Table 21.3.

Riboflavin, FMN, and FAD in aqueous solution exhibit an intense yellowish-green fluorescence with an emission maximum at around 530 nm when excited at 440–500 nm. The fluorescence spectra of

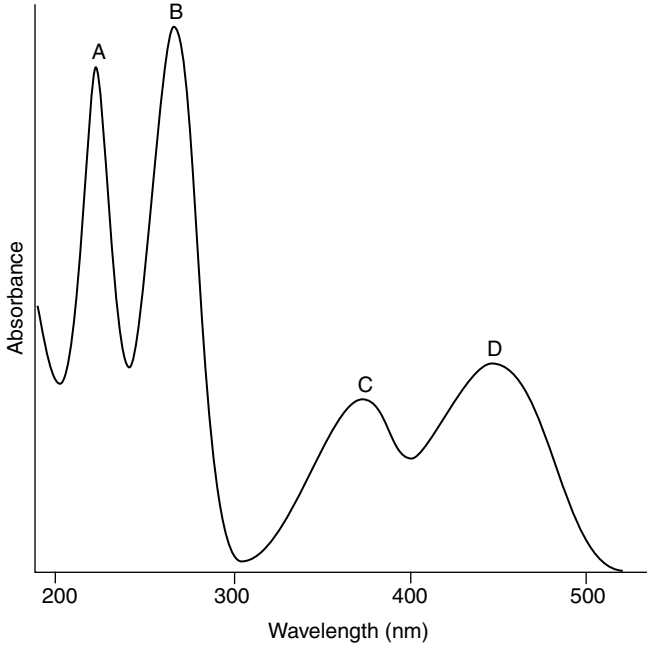

FIGURE 21.3
UV-visible absorption spectrum of riboflavin dissolved in water (pH 7.4) (λ_{max} of peak A = 223 nm; B = 266 nm; C = 373 nm; D = 444 nm).

TABLE 21.3

The Molar Absorptivity (ε) Values of Flavins Dissolved in 0.1 *M* Phosphate Buffer, pH 7.0

	Wavelength (nm)		
Flavins	**260**	**375**	**450**
Riboflavin	27,700	10,600	12,200
FMN	27,100	10,400	12,200
FAD	37,000	9,300	11,300

Source: Yagi, K., Chemical determination of flavins, in *Methods of Biochemical Analysis*, Glick, D., Ed., Vol. 10, John Wiley & Sons, New york, 1962, p. 319. With Permission.

riboflavin are shown in Figure 21.4. The fluorescence of flavins is a characteristic of uncharged neutral forms of isoalloxazines; anionic and cationic forms do not fluoresce. Riboflavin shows a maximum and equal fluorescence in the pH range between 3.5 and 7.5. The same is true for FMN, but FAD displays maximal intensity at pH 2.7–3.1. In equimolar neutral

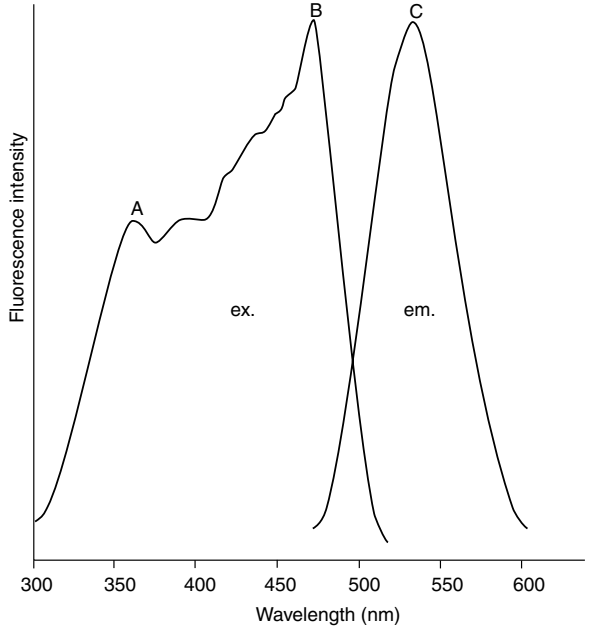

FIGURE 21.4

Fluorescence excitation and emission spectra of riboflavin dissolved in water (pH 7.4) (λ_{max} of peak A = 360 nm; B = 465 nm; C = 521 nm).

aqueous solutions, riboflavin and FMN exhibit practically the same fluorescence intensity, whereas the intensity of FAD is only about 15% of that of riboflavin. The fluorescence property of riboflavin depends on the 3-imino group being free. Because protein binding takes place via the 3-imino group, protein-bound forms of the vitamin do not fluoresce. It is therefore essential to release flavins from proteins if the quantification is to rely on fluorescence detection.

21.2.2.2 Methodology

HPLC methods for determining riboflavin *per se* are summarized in Table 21.4.

A standard extraction technique suitable for HPLC, and applicable to any kind of foodstuff, is to convert the bound flavins to free riboflavin by autoclaving the sample with dilute mineral acid, followed by enzymatic digestion at the optimum pH. In some applications, the hydrolyzed extract is heated with 50% (w/v) trichloroacetic acid to deactivate and precipitate the previously added enzyme. The total vitamin B_2 content (excluding covalently bound flavins) is then calculated, based on measurement of the riboflavin peak. The chromatography is capable of separating FMN and riboflavin, so a valid estimate of the total vitamin B_2 using this technique depends on the conversion of FMN to riboflavin being complete. If the hydrolysis is incomplete, as indicated by the presence of an FMN peak in the sample chromatogram, an alternative enzyme preparation and/or a longer incubation period can be tried. If these steps fail to achieve a complete hydrolysis, the FMN peak must be measured in addition to the riboflavin peak. Summation of FMN and riboflavin peak heights provides a total vitamin B_2 content without significant error, provided that the FMN peak height is $<25\%$ of the sum of the FMN and riboflavin peak heights [37]. An unavoidable source of error is the formation during acid digestion of biologically active isomeric riboflavin monophosphates, which are chromatographically separated from FMN.

The above extraction technique has been used for the determination of total vitamin B_2 in a wide range of foods, and the results compared with a standard microbiological assay using *Lactobacillus rhamnosus* [34]. The importance of the enzyme treatment in converting FMN to riboflavin is illustrated in Figure 21.5. High correlations were obtained between the two assay techniques for vitamin-enriched samples such as baby foods, gruels, porridges, breakfast cereals, and flour ($r = 0.9996$) and for milk products ($r = 0.9999$). This work was reported from Sweden, where FMN, rather than riboflavin, is generally used for enrichment. In contrast, the riboflavin values found with the HPLC method in nonenriched flours and flours with a high rate of extraction were 25–50% lower than those

TABLE 21.4

HPLC Methods Used for the Determination of Riboflavin and Other Flavins in Food

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Reversed-phase chromatography						
Italian cheeses	Homogenize sample with water/MeOH, 2:1. Add acetic acid, mix, centrifuge. Resuspend the pellet three times in water/MeOH/acetic acid, 65:25:10. Dilute pooled supernatants to volume with the same solvent, centrifuge	LiChrosorb RP-18 5 μm 250 × 4 mm	Water/MeCN, 80:20	Riboflavin (free)	Visible 446 nm	[26]
Milk, yoghurt, cheese	Milk: pass sample through C_{18} solid-phase extraction cartridge, wash column bed twice with water, elute riboflavin with 20 m*M* acetate buffer (pH 4.0)/MeOH, 1 + 1 Yoghurt and cheese: blend sample with acetate buffer, filter, then solid-phase extraction as for milk	Bio-Sil ODS-5S 250 × 4 mm	Water containing 0.1% acetic acid/ MeOH, 65:35	Riboflavin (free)	UV 270 nm	[27]

(*Table continued*)

TABLE 21.4 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Pasta (enriched)	*Acid hydrolysis*: autoclave ground sample with 0.1 *N* HCl at 121°C for 30 min, cool, centrifuge. Resuspend the pellet twice in 0.1 *N* HCl. Dilute pooled supernatants to volume	μBondapak C_{18} 10 μm 300 × 3.9	(1) Water/MeOH/acetic acid, 56:43:1 (2) Water/MeOH/acetic acid, 50/49/1	(1) Riboflavin (2) Lumichrome	Fluorescence (1) ex 450 nm em 510 nm (filters) (2) ex 300–350 nm em 479 nm (filters)	[28]
Wines, beers, fruit juices	Filter (0.22 μm), inject directly or after dilution with water	Hypersil C_{18} 5 μm 200 × 2.1 mm	Gradient of solvent (A) 0.05 *M* phosphate buffer, pH 3.0 and (B) MeCN	FAD, FMN, riboflavin	Fluorescence ex 265 nm em 525 nm with a 500 nm cut-off filter	[29]
Dairy products (milk, whole milk powder, cheese)	Homogenize sample with 6% formic acid containing 2 *M* urea, centrifuge. Mix a 2-ml aliquot with sorboflavin (internal standard) *Cleanup and concentration*: pass solution through C_{18} solid-phase extraction column, wash column bed with 10% formic acid, elute the flavins with 10% formic acid/MeOH, 4:1	LC-18 3 μm (Supelco) 75 × 4.6 mm	14% MeCN in 0.1 *M* KH_2PO_4 (final pH 2.9)	FAD, FMN, sorboflavin (internal standard), riboflavin	Fluorescence ex 450 nm em 530 nm	[30]

Bovine milk	Boil 1.0 g milk to inactivate pyrophosphatase. Digest with buffered pronase (pH 6.8) for 1 h at 45°C to simultaneously release the flavins bound to milk proteins and deproteinize. Cool, adjust volume with phosphate buffer, adjust pH to 5.5. Centrifuge, filter	Capcell Pak C_{18} 5 μm 250 × 4.6 mm Column temperature 40°C	90% MeOH in water (Solvent A) and 0.01 *M* phosphate buffer, pH 5.5 (Solvent B). Linear gradient from 35% of A and 65% of B to 95% of A and 5% of B over 8 and 5 min	FAD, FMN, riboflavin	Fluorescence ex 462 nm em 520 nm	[31]
Raw and cooked meats, dairy products, eggs, cereal products	Homogenize sample with $MeOH/CH_2Cl_2$ (9 + 10) and 7-ethyl- 8-methyl-riboflavin (internal standard). Add 0.1 *M* citrate-phosphate buffer, pH 5.5 containing 0.1% sodium azide, rehomogenize, filter	2 PLRP-S 5 μm (Polymer Laboratories) columns in series 250 × 4.6 mm Column temperature 40°C	MeCN/0.1% sodium azide in 0.01 *M* citrate-phosphate buffer (pH 5.5) in multistep gradient elution program	FAD, FMN, riboflavin, 7-Et-8-Me-RF (internal standard)	Fluorescence ex 450 nm em 522 nm	[32]
Cereals, milk (whole, semi skimmed, skimmed, evaporated)	Online system consisting of microwave extraction, dialysis and trace enrichment with a C_{18} mini-column	Spherisorb ODS-2 5 μm 250 × 4.6 mm	*Initial*: 94% of 0.1 *M* sodium acetate buffer (pH 4.8) and 6% of water/MeCN/MeOH (50 + 40 + 10) *Linear gradient elution*: proportion of water/MeCN/MeOH mixture	FMN, riboflavin	Fluorescence ex 450 nm em 520 nm (cut-off filter 400 nm)	[33]

(*Table continued*)

TABLE 21.4 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
			increased from 6% to 100% over 30 min			
Baby foods, breakfast cereals, enriched flours, dairy products, health foods	*Acid and enzymatic hydrolysis*: autoclave homogenized sample with 0.1 *N* HCl at 121°C for 30 min. Cool, adjust pH to 4.5. Incubate with acid phosphatase at 45°C overnight. Precipitate proteins by heating at 100°C with 50% (w/v) TCA for 5 min. Adjust pH to 4.5, dilute to volume with water, filter	Hypersil-ODS 5 μm 250 × 4.6 mm	Water/MeOH (3 + 2) adjusted to pH 4.5 with acetic acid	Riboflavin (representing total vitamin B_2)	Fluorescence ex 440 nm em 520 nm	[34]
Dairy products, meat products, fish, fruit, vegetables, flour, baked products, beer, coffee	*Acid and enzymatic hydrolysis*: autoclave homogenized sample with 0.1 *N* H_2SO_4 at 121°C for 20 min. Cool, adjust pH to 4.5. Incubate with Claradiastase at 45°C overnight. Cool, dilute to volume, filter *Cleanup and concentration*: pass aliquot of filtrate through C_{18} solid-phase	Spherisorb ODS-2 5 μm 250 × 4.6 mm	Water/MeOH, 65:35	Riboflavin (representing total vitamin B_2)	Fluorescence ex 445 nm em 525 nm	[35]

	extraction cartridge, wash cartridge with water, elute the riboflavin with 40–70% MeOH					
Milk- and soy-based infant formula, beer, fruit juices, juice with milk, honey, milk powder CRM 421 and pig's liver CRM 487	Solid samples and CRMs: homogenize with MeCN, add 10 m*M* phosphate buffer (pH 5), homogenize, centrifuge, dilute to volume Liquid samples: filter (0.45 μm nylon)	Discovery RP-AmideC_{16} (hexadecyl) 5 μm 150 × 4.6 mm	10 m*M* phosphate buffer, pH 5/ MeCN, 90:10	FAD, FMN, riboflavin	Fluorescence; two channels at wavelengths of 270/516 and 452/ 516 nm (ex/em)	[36]
Reversed-phase ion-pair chromatography						
Flour, bread, raw beef, corned beef, chicken liver, mushrooms, milk and milk products, cereals	*Acid hydrolysis*: defat high-fat samples with hexane. Autoclave homogenized sample with 0.1 *N* HCl at 121°C for 30 min. Adjust pH to 6.0, dilute to volume, filter	LiChrosorb RP-8 (octyl) 10 μm 250 × 4.0 mm	Water/MeOH, 60:40 containing 5 m*M* sodium hexane sulfonate	FMN, riboflavin (representing total vitamin B_2)	Fluorescence ex 440 nm (filter), em 565 nm (filter)	[37]
Fresh fruit and vegetables	*Acid and enzymatic hydrolysis*: digest ground dry sample with 0.1 *N* HCl at 99°C for	Ultrasphere-ODS 5 μm 250 × 4.6 mm	MeOH/water (40:60) containing 5 mM sodium	Riboflavin (representing total vitamin B_2)	Fluorescence ex 450 nm, em 530 nm	[38]

(*Table continued*)

TABLE 21.4 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
	30 min, cool. Incubate with buffered Mylase at 38°C overnight. Precipitate proteins by heating at 60°C with 50% (w/v) TCA for 5 min. Adjust pH to 4.0, dilute to volume with water, filter		heptane sulfonate; adjusted to pH 4.5 with H_3PO_4			
Cooked sausages	*Acid and enzymatic hydrolysis*: autoclave ground sample with 0.1 *N* HCl at 120°C for 20 min. Cool, adjust pH to 4.0–4.5; incubate with Claradiastase at 50°C for 3 h. Precipitate proteins by heating at 90°C with 50% (w/v) TCA, dilute to volume with water, filter	Spherisorb ODS-2 5 μm 250 × 4 mm Column temperature 35°C	5 m*M* heptanesulfonic acid adjusted to pH 2.7 with phosphoric acid/MeCN, 75:25	Riboflavin (representing total vitamin B_2)	Fluorescence ex 227 nm em 520 nm	[39]
Chick peas, green beans, full cream milk powder	*Acid and enzymatic hydrolysis*: autoclave ground sample with dilute HCl at 121°C for 15 min. Cool, adjust pH to 4.0–4.5. Incubate with	μBondapak C_{18} 10 μm 300 × 3.9 mm	(1) Water/MeOH/acetic acid (67:32:1) containing 5 m*M* sodium hexane sulfonate	Riboflavin (representing total vitamin B_2)	UV 254 nm	[40]

	Takadiastase at 48°C for 3 h. Cool, dilute to volume with water, filter *Cleanup and concentration*: readjust pH of extract to 4.0–4.5. Pass through Florisil column, wash column bed with water, elute riboflavin with triethylamine/MeOH/water, 7:30:13. Evaporate eluate to dryness, dissolve residue in MeOH/water (same proportion as mobile phase). Pass aliquot of solution through C_{18} solid-phase extraction cartridge, wash cartridge with aqueous 5 m*M* sodium hexane sulfonate, elute riboflavin with methanolic 5 m*M* sodium hexane sulfonate	(2) Water/MeOH/acetic acid (68.5:31:0.5) containing 5 m*M* sodium heptane sulfonate/5 m*M* sodium hexane sulfonate, 25:75

Note: TCA, trichloroacetic acid; MeOH, methanol; MeCN, acetonitrile.

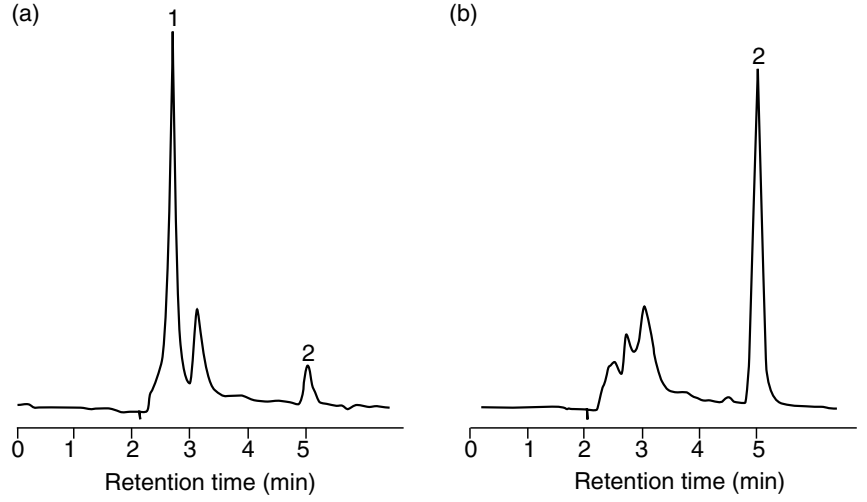

FIGURE 21.5
Reversed-phase HPLC with fluorescence detection of the flavins extracted from a typical foodstuff by treatment with (a) 0.1 *N* hydrochloric acid and (b) acid and enzyme. Operating parameters as in Table 21.4 [34]. Peaks: (1) FMN; (2) riboflavin. (From Johnsson, H. and Branzell, C., *Int. J. Vitam. Nutr. Res.*, 57, 53, 1987. With permission.)

found with the microbiological assay. A possible reason for this disparity was the loss of fluorescence by the inner filter effect. This refers to the absorption of incident or emitted radiation by substances in the complex food matrix which coeluted with riboflavin. Further purification of the filtered extract using C_{18} solid-phase extraction failed to increase the fluorescence signal, and unchanged results were obtained. Vidal-Valverde and Reche [40] reported that C_{18} solid-phase extraction was inadequate for removing all interfering substances present in hydrolyzed food extracts. These authors employed a cleanup procedure involving adsorption chromatography on a Florisil (fuller's earth) column, followed by C_{18} solid-phase extraction, which removed the major interfering substances present in legumes, and made absorbance detection at 254 nm sufficiently sensitive for these products (detection limit: 0.4 ng per injection).

An alternative technique for determining total vitamin B_2 is to measure the FMN and riboflavin liberated during autoclaving with dilute mineral acid, after precipitation of proteinaceous material by pH adjustment to about 4.5 (as in the AOAC fluorometric procedure). The enzymatic hydrolysis step is omitted. For acid extracts containing significant

amounts of FMN, that is, where the FMN peak height is >25% of the sum of the FMN and riboflavin peak heights (such as were obtained from raw beef, corned beef, fresh and cooked liver, and canned mushrooms), FMN and riboflavin were calculated separately, using their corresponding response factors (ratio of concentration of standard-to-peak height), and the results were summed to obtain total vitamin B_2. For smaller amounts of FMN, the total vitamin B_2 could be obtained by summation of FMN and riboflavin peak heights without significant error [37]. The necessity of applying a correction factor to compensate for the lack of purity of commercial FMN preparations has been stressed by Russell and Vanderslice [32]. It is important to understand that the quantity of FMN found does not represent the original FMN content of the food sample, as the FMN peak originates largely from hydrolyzed FAD.

For the analysis of milk, eggs, and dairy products, in which free or loosely bound riboflavin is considered to be the predominant naturally occurring flavin present, riboflavin can be determined specifically without the need for acid and enzyme hydrolysis. A common extraction procedure for milk entails precipitation of the protein to below pH 4.5 (the isoelectric point of most proteins) and filtration. This procedure has been employed using acetic acid [41,42], trichloroacetic acid [43,44], and acidified lead acetate solution [45] as the deproteinizing agent.

Most published riboflavin HPLC assays utilize C_{18} stationary phases, either with aqueous/organic mobile phases in the reversed-phase mode or using ion-pairing agents (hexane or heptane sulfonic acid). Fluorescence monitoring is the preferred means of detection in most cases. The limits of fluorescence detection at 450/522 nm (excitation/emission) for the biologically active flavins at a signal-to-noise ratio of 3:1 were 0.55 pmol or 0.21 ng riboflavin; 1.96 pmol or 0.89 ng FMN; 14.9 pmol or 11.15 ng FAD [32]. The sensitivity can be increased even further to achieve a detection limit of 0.02 ng per injection by irradiating the riboflavin at high pH to form lumiflavin [22], but this technique has not been widely adopted.

The use of a 254-nm fixed-wavelength absorbance detector provided a minimum on-column detection limit of 0.4 ng riboflavin [40], which was adequate for the analysis of legumes and full cream milk powder. Absorbance monitoring at 270 nm was also sufficiently sensitive for the analysis of milk and milk products after cleanup and concentration using C_{18} solid-phase extraction [27] and has the advantage in simultaneously detecting riboflavin decomposition products [42]. Stancher and Zonta [26] utilized visible absorbance detection at 446 nm to avoid detection interference in the analysis of Italian cheeses.

The on-column detection limit of 2.5 ng (cf. 0.1 ng by fluorescence) was more than adequate for determining the relatively high concentration of riboflavin (at least 100 μg/100 g) present in the cheese commodities analyzed.

For samples with low riboflavin content (<10 μg/100 g), Lumley and Wiggins [46] employed a trace-enrichment technique based on the extremely long retention time of riboflavin on a reversed-phase column when pure water was used as a mobile phase. Concentration was achieved by loading successive 100-μl volumes of sample extract onto the guard column. The riboflavin, which concentrated at the column head, could then be eluted as a tight band by changing the eluent from water to the methanol/water mobile phase. Jaumann and Engelhardt [47] described an online enrichment technique for separation of riboflavin in food using a column-switching device.

Greenway and Kometa [33] developed an online sample preparation method for the determination of riboflavin and FMN in milk and cereal samples by reversed-phase HPLC. The online system consisted of microwave extraction followed by dialysis and trace enrichment with a C_{18} mini-column. Sample preparation was minimal, with milk samples being directly introduced into the system and cereal samples only needing to be ground prior to analysis. During the microwave extraction, the FAD was converted into FMN, and 15% of the FMN was converted into riboflavin. The full analysis time on the ground samples was about 20 min. Results were found to be in agreement with those obtained using the AOAC fluorometric method and a previously reported HPLC method.

Russell and Vanderslice [32] employed a nondegradative two-step extraction procedure for the simultaneous quantification of riboflavin, FMN and FAD in a variety of foods. An internal standard, 7-ethyl-8-methyl-riboflavin, was added at the start of the extraction procedure, and separation of the flavins and internal standard was accomplished with a polymer-based column packing and a multistep gradient elution program (Figure 21.6). Appropriate correction factors were applied to account for the impurities in the commercial FMN and FAD standards. Russell et al. [48] employed a robotic system to automate the extraction, duplicating the manual steps as closely as possible. The robotic method was faster and generally produced slightly higher results than the manual process. Higher molar concentrations of FMN and FAD relative to riboflavin indicated that less degradation or interconversion of the individual vitamers was taking place. The advantage of operating the robotic system in complete darkess could be responsible for this protective effect. Bilic and Sieber [30] extracted riboflavin, FMN and FAD from dairy products by homogenization with 6% formic acid containing 2 *M* urea in the presence of sorboflavin added as an internal standard. Sorboflavin contains a glucityl side chain instead of a ribityl chain on the isoalloxazine ring.

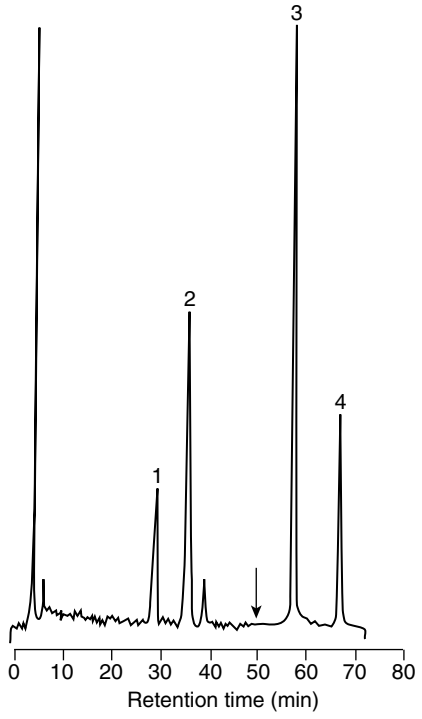

FIGURE 21.6
Reversed-phase HPLC with fluorescence detection of the flavins extracted from hamburger. Operating parameters as in Table 21.4 [32]. Arrow indicates change of detector attenuation. Peaks: (1) FAD; (2) FMN; (3) riboflavin; (4) 7-ethyl-8-methyl-riboflavin (internal standard). (Reprinted from Russell, L.F. and Vanderslice, J.T., *Food Chem.*, 43, 151–162, 1992. With permission from Elsevier.)

Viñas et al. [36] used a C_{16} stationary phase with embedded amide groups and trimethylsilyl endcapping for the separation of riboflavin, FMN, and FAD by isocratic elution. A two-channel fluorescence detector allowed two excitation wavelengths to be selected: 270 nm was optimal for maximum fluorescence and 425 nm overcame interferences from the matrix. Fluorescence spectra were continuously measured during passage of the solute through the flow cell. The standard additions method was used to investigate the possibility of interference by the matrix. Slopes of the standard additions calibration graphs for the food samples were similar to those of aqueous standards, confirming that the matrix did not interfere. The procedure was applied to the determination of B_2 vitamers in milk- and soy-based infant formulas, beer, fruit juices, and honey of different types. On-column detection limits were

0.03, 0.05, and 0.24 ng for riboflavin, FMN, and FAD, respectively. The method was validated using two certified reference materials, and results within the certified range were obtained.

21.2.3 Niacin

21.2.3.1 Detection

Nicotinic acid and its amide exhibit similar absorption spectra in the UV region. The absorptivity is strongly affected by pH, being higher in an acidic than in an alkaline solution, but the λ_{max} remains almost unchanged at 261 nm (Figure 21.7). The $A^{1\%}_{1\,cm}$ value for nicotinamide in 0.1 *N* sulfuric acid at 261 nm is 478. The presence of electrolytes also has a marked effect on the absorbances of the solutions [49].

Nicotinic acid and nicotinamide do not fluoresce, but fluorescent derivatives can be formed by treatment with a mixture of cyanogen bromide and *p*-aminoacetophenone [50]. Ultraviolet irradiation of niacin solutions in the presence of hydrogen peroxide and copper(II) ions also induces fluorescence [51].

21.2.3.2 Methodology

Selected HPLC methods for determining niacin are summarized in Table 21.5.

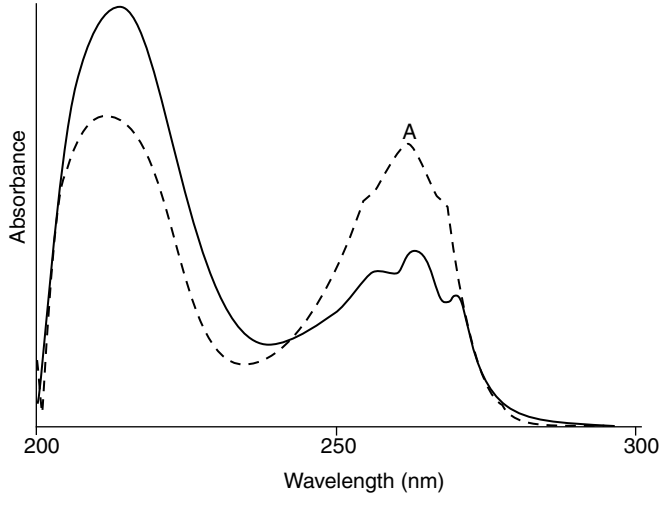

FIGURE 21.7
UV absorption spectra of nicotinic acid in 0.1 *M* phosphate buffer at pH 7.0 (solid line) and 2.0 (broken line) (λ_{max} of peak A = 261 nm).

TABLE 21.5

HPLC Methods Used for the Determination of Niacin in Food

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds separated**	**Detection**	**Ref.**
Anion exchange chromatography						
Milk-based and soy-based infant formula	*Acid hydrolysis*: autoclave at 121°C for 45 min in a medium of ca. 2.5 *N* H_2SO_4, allow to cool. Precipitate proteins by pH adjustment to 6.5 then immediately to pH $\leq$ 1.0. Filter through paper, dilute to volume with water *Cleanup:* apply 3.0-ml aliquot of filtrate to pretreated ArSCX solid-phase extraction cartridge. Flush column bed with water, elute nicotinic acid with 2 × 6.0 ml 0.25 *M* acetate buffer, pH 5.6. Bring to final volume with 0.5 *M* acetate buffer	PRP-X100 250 × 4.1 mm	0.1 *M* acetate buffer, pH 4.0	Nicotinic acid (representing total niacin)	UV 260 nm	[52]

(*Table continued*)

TABLE 21.5 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds separated**	**Detection**	**Ref.**
Strong cation exchange chromatography						
Fresh meat (to test for added nicotinic acid)	Homogenize sample in water then boil, cool, and filter	Partisil SCX 10 μm 250 × 4.6 mm	0.05 *M* phosphate buffer, pH 3.0	Nicotinic acid, nicotinamide (free forms) ascorbic acid, sorbic acid	UV 260 nm	[53]
Reversed-phase ion-pair chromatography						
Instant coffee	Dissolve sample in hot water, cool, dilute to volume, filter. Pass through C_{18} solid-phase extraction cartridge	Spherisorb ODS-2 150 × 5 mm	MeOH/water (8:92) containing 5 m*M* tetrabutyl-ammonium hydroxide (final pH adjusted to 7.0)	Nicotinic acid (free)	UV 254 nm	[54]
Beef, pork, tuna (to test for added nicotinic acid)	Add H_3PO_4 to homogenized sample, extract with MeOH, filter	Shim-Pack FLC ODS 50 × 4.6 mm Column temperature 50°C	1 m*M* sodium dodecyl sulfate and 0.02 *M* H_3PO_4/MeOH, 7:3 (pH 2.4)	Nicotinic acid, nicotinamide (free forms)	UV 261 nm	[55]

Fresh meat (to test for added nicotinic acid)	Extract homogenized sample with MeOH *Cleanup*: alumina N solid-phase extraction cartridge, eluting nicotinamide with MeOH and nicotinamide with 0.1 *M* $NaHCO_3$	Lichrosorb RP-18	MeOH/acetate buffer (pH 5), 2:10 containing 0.1 *M* sodium acetate and 10 m*M* tetrabutyl ammonium hydroxide	Nicotinic acid, nicotinamide (free forms in separate chromatograms)	UV 261 nm	[56]
Fresh beef and pork, fresh fish, fish products (to test for added nicotinic acid)	Homogenize sample with water, dilute to volume with water, centrifuge, filter through paper. Precipitate proteins by successive addition of saturated zinc sulfate solution and 1 *N* NaOH. Dilute to volume with water, let stand for 30 min, filter through paper, refilter (0.45 μm)	Radial-PAK μBondapak C_{18} 10 μm 100 × 8 mm	(1) 5 m*M* tetrabutyl-ammonium phosphate acid (PIC-A) in MeOH/water, 1 + 9 (2) 10 m*M* heptane sulfonic acid (PIC-B7) in water	(1) Nicotinic acid, (2) Nicotinamide (free forms)	UV 263 nm	[57]
Cooked sausages	As in preceding entry	Spherisorb ODS-2 5 μm 250 × 4 mm Column temperature 35°C	5 m*M* heptane sulfonic acid adjusted to pH 3.3 with phos phoric acid/MeCN, 75:25	(1) Nicotinic acid, (2) Nicotinamide (free forms)	UV 261 nm	[58]

(*Table continued*)

TABLE 21.5 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds separated**	**Detection**	**Ref.**
Beef, semolina, cottage cheese	*Alkaline hydrolysis*: blend sample with water and $Ca(OH)_2$ mixture. Autoclave at 121°C for 15 min. Cool to 0°C, filter, adjust pH to 6.5–7.5 with oxalic acid, filter *Cleanup and concentration*: pass 10 ml of filtrate through C_{18} solid-phase extraction cartridge. Discard first 6 ml of eluate and collect next 3.5 ml. Add one drop 85% H_3PO_4, mix	C_{18} LC-18-DB 5 μm (Supelco) 150 × 4.6 mm	23% MeCN, 0.10% H_3PO_4, 0.10% sodium dodecyl sulfate in water (final pH 2.8)	Nicotinic acid (representing total niacin)	UV 254 nm	[59]
Legumes, lyophilized pork muscle	*Acid hydrolysis*: autoclave ground sample with dilute HCl at 121°C for 15 min. Cool, adjust pH to 4.0–4.5. Incubate with Takadiastase at 48°C for 3 h. Cool, filter, dilute to volume with water	μBondapak C_{18} 10 μm 300 × 3.9 mm	0.01 *M* sodium acetate buffer, pH 4.66/ MeOH (9:1) containing	Nicotinic acid (representing bioavailable niacin)	UV 254 nm	[60]

	Cleanup and concentration: readjust pH of extract to 4.7 ± 0.02. Pass an aliquot through a Dowex 1-X8 (Bio-Rad) anion exchange column. Wash column bed with water, elute nicotinic acid with 0.15 *M* HCl. Evaporate eluate to dryness. Dissolve residue in MeOH/0.1 *M* acetate buffer of the required pH to obtain a final pH of 4.7–4.9		0.005 *M* tetrabutyl-ammonium bromide (final pH 4.72)			
Reversed-phase chromatography						
Cereal products, mushrooms	*Acid hydrolysis*: autoclave sample with 0.2 *N* (0.1 *M*) H_2SO_4 at 1 bar for 1 h, cool. Incubate with buffered Clarase at 45°C for 3 h. Cool, dilute to volume, filter *Alkaline hydrolysis*: heat sample with $Ca(OH)_2$ suspension at 95–100°C for 30 min. Autoclave at 1 bar for 30 min. Cool, dilute to volume. Refrigerate overnight, centrifuge	Nicotinic acid fraction transferred from reversed-phase column (Nucleosil C_{18} 5 μm) to anion exchange column (Nucleosil SB 5 μm) using automatic column switching. Both columns 150 × 4.6 mm	A. 0.57% acetic acid adjusted to pH 3.0 with NaOH to elute the nicotinic acid from the C_{18} column and to place it on to the anion exchange column. B. Mobile phase A/MeOH, 5:95 to flush the C_{18} column	Nicotinic acid (representing bioavailable niacin after acid hydrolysis and total niacin after alkaline hydrolysis)	UV 254 nm	[61]

(*Table continued*)

TABLE 21.5 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds separated**	**Detection**	**Ref.**
			C. 2.28% acetic acid adjusted to pH 3.0 with NaOH to elute the nicotinic acid from the ion exchange column			
Beef liver, fruit juice, brewer's yeast, tomato, biscuits, green peas, peanuts, crystallized fruit, beer, pork, veal	*Acid and alkaline hydrolysis*: add 30 ml 0.1 *N* HCl to 1–5 g ground sample, heat suspension in a water bath at 100°C for 1 h. Cool, dilute to volume with water, filter. Autoclave at 120°C in a medium of 0.8 N NaOH for 1 h. Cool adjust pH to 4.5, dilute to volume with water, filter through paper then through cellulose nitrate filter (0.45 μm)	Lichrospher 100 RP 18 5 μm 250 × 5 mm	0.07 *M* KH_2PO_4, 0.075 *M* hydrogen peroxide, 5 μ*M* copper (II) sulfate	Nicotinic acid (representing bioavailable niacin)	Fluorescence ex 322 nm em 380 nm, after postcolumn derivatization	[62]

Frozen vegetables (peas, spinach, French beans), fresh meat (beef, pork), sweet corn, yeast, wheat flour, wheat germs, rice, peanuts	*Enzymatic hydrolysis*: add 50 m*M* sodium acetate (pH 4.5) and NADase solution to ground sample, incubate at 37°C for 18 h. Dilute to volume with water	Uptisphere C_{18} HDO (Interchim) 5 μm 150 × 4.6 mm	As in preceding entry	Nicotinic acid and nicotinamide (representing bioavailable niacin)	As in preceding entry	[63]
Fortified foods (infant formula, cereal products, chocolate drink, clinical nutrition products)	*Acid hydrolysis*: add 70 ml 0.1 *N* HCl to 5 g (dry weight) or liquid sample, heat suspension in a water-bath at 100°C for 1 h with magnetic stirring. Cool, adjust pH to 4.5–4.6. Dilute to volume with water, filter	Inertsil ODS 3 5 μm 250 × 4.6 mm	As in preceding entry	As in preceding entry	As in preceding entry	[64]

Note: MeOH, methanol; MeCN, acetonitrile.

In determining the naturally occurring niacin in foodstuffs, it must be decided whether to estimate total niacin or free (*available*) niacin, or both. The terms "total" and "free" niacin are defined by the extraction methods employed, as discussed in Section 17.3. Ingested NAD and NADP are readily converted to nicotinamide in the body, and so the extraction must be capable of at least hydrolyzing these compounds to nicotinamide. Enzymatic hydrolysis using an NADase yields lower niacin values than does mildly acidic extraction (boiling 0.1 *N* hydrochloric acid for 1 h) for certain foods that contain large amounts of bound nicotinic acid [63]. This suggests that the acid conditions hydrolyze some of the bound nicotinic acid, and that the enzyme treatment yields a more reliable estimate of bioavailable niacin.

The free nicotinamide and nicotinic acid naturally present in fresh meat, as well as that possibly added, can be extracted quantitatively with water or methanol and determined by HPLC with reasonable precision. Strong cation exchange chromatography permits the separation of the two vitamers, together with ascorbic acid and sorbic acid, in aqueous extracts of meat [53]. Other investigators have used reversed-phase ion-pair chromatography for determining the niacin vitamers. Takatsuki et al. [57] found it necessary to use two different mobile phases for determining each vitamer in deproteinized aqueous extracts of meat. The detection limit for a 10-g sample was 1 mg/100 g for nicotinic acid and nicotinamide. Values greater than 20 mg/100 g indicated the illegal addition of either vitamer in Japan. Tsunoda et al. [55] reported the simultaneous determination of the two vitamers in methanolic extracts of meat and fish using sodium dodecylsulfate as the ion-pairing agent. Oishi et al. [56] obtained nicotinic acid and nicotinamide fractions from methanolic extracts of fresh meat by selective elution from an alumina solid-phase extraction cartridge, and chromatographed each fraction separately.

Tyler and Genzale [59] reported a simple, yet efficient, means of purifying alkaline digests of three major food representatives (beef, semolina, and cottage cheese). After autoclaving with calcium hydroxide solution, digests were cooled in an ice bath, filtered, and then adjusted to pH 6.5–7.0 with oxalic acid to precipitate the excess calcium. A 10-ml aliquot of the filtered suspension was loaded onto a C_{18} solid-phase extraction cartridge, the first 6 ml of effluent was discarded, and the remaining effluent was collected and acidified with phosphoric acid. Analysis of the purified extracts was performed by reversed-phase ion-pair chromatography. A feature of this method is the unusually sharp nicotinic acid peak (Figure 21.8), which resulted from the injection of the analyte in a large volume (200 μl) of dilute phosphoric acid, and which led to a detection limit of ca. 0.5 mg/100 g. The high efficiency of the single cleanup step is attributable to the amphoteric nature of nicotinic

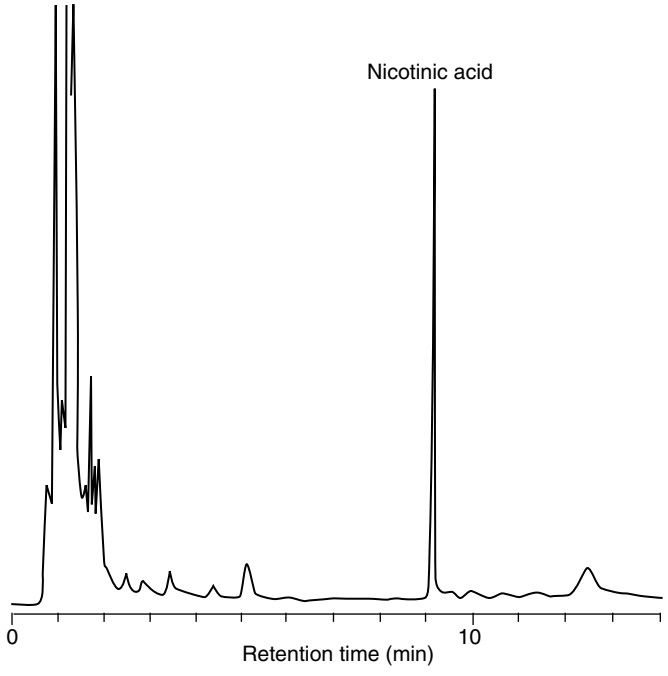

FIGURE 21.8
Reversed-phase ion-pair HPLC with UV detection of nicotinic acid extracted from semolina after cleanup by Sep-Pak C_{18} solid-phase extraction and acidification of Sep-Pak effluent. Operating parameters as in Table 21.5 [59]. (Reprinted from Tyler, T.A. and Genzale, J.A., *J. AOAC Int.*, 73, 467–469, Copyright 1990 by AOAC International. With permission.)

acid. At pH 7, nicotinic acid is not retained on the C_{18} cartridge, but a large number of pigmented compounds are retained. Conversely, nicotinic acid is strongly retained on the C_{18} analytical column at acidic pH in the presence of an ion-pairing agent, whereas many polar interferences elute in the dead volume. Tyler and Genzale [59] compared HPLC results with results obtained from a microbiological assay. Agreement between the two methods was obtained for the analysis of beef, semolina and cottage cheese, but certain food samples (e.g., instant coffee) gave higher niacin values using HPLC.

van Niekerk et al. [61] did not include a separate cleanup step in their assay procedure for the analysis of cereal products and mushrooms subjected to acid or alkaline hydrolysis. They employed, instead, inline two-dimensional HPLC, whereby the nicotinic acid fraction was transferred from a reversed-phase column to an anion exchange column. The HPLC method gave lower values for the acid extracts than for the alkaline extracts (except for the standard), whereas results obtained

microbiologically on both acid and alkaline extracts showed no difference. This suggests that part of the nicotinic acid in the acid extract was present in a bound form that was available to the assay organism (*Lactobacillus plantarum*).

For the estimation of free (*available*) niacin in legumes and meat [60], samples (1–10 g, containing 10–100 μg of niacin) were mixed with hydrochloric acid (30 ml of 0.1 N + 1 ml of 6 N HCl) and autoclaved at 121°C for 15 min. The acid digest was then adjusted to pH 4.0–4.5 and incubated with Takadiastase to hydrolyze the starch present in the legumes. The extracts were purified by strong anion exchange chromatography, and then analyzed by reversed-phase ion-pair HPLC. Each different food required a trial-and-error procedure to ascertain the column temperature required for optimum separation.

LaCroix and Wolf [52] presented the following method for the determination of total niacin in milk- and soy-based infant formula. Samples are autoclaved at 121°C for 45 min in a medium of ca. 2.5 N sulfuric acid to free endogenous niacin from protein and to convert nicotinamide to nicotinic acid. The digest is adjusted to pH 6.5 and then immediately to pH <1.0 to precipitate the proteins. The clarified solution is filtered through paper and the filtrate is diluted to volume with water. Sample cleanup is achieved by passing an aliquot of the filtrate through a strong cation exchange solid-phase extraction cartridge. The column bed is flushed with water and the nicotinic acid is eluted with two 6-ml portions of 0.25 M acetate buffer (pH 5.6). HPLC is performed using an anion exchange polystyrene–divinylbenzene column packing and UV detection at 260 nm. Results obtained from the analysis of SRM 1846 Infant Formula were consistent with the certified niacin value. The method has achieved AOAC recognition as Peer-Verified Method (PVM) 1:2000 [65].

Most of the published HPLC methods to date have used UV absorbance detection of nicotinamide and nicotinic acid at 261 nm (λ_{max}) or 254 nm. Because absorbance detection is not very selective, sample extracts have had to be purified to remove interfering UV-absorbing material. Kral [66] used pulsed amperometric detection for the analysis of fruit juices subjected to acid hydrolysis, but, despite the improved selectivity, it was still necessary to employ open-column cation exchange chromatography as a cleanup step. In 1991, Mawatari et al. [51] described an ingenious way of converting nicotinamide and nicotinic acid to fluorescent derivatives postcolumn, thereby increasing the selectivity and sensitivity of detection. The derivatives are formed instantaneously by UV irradiation of the column effluent in the presence of hydrogen peroxide and copper(II) ions, and the fluorescence is monitored by an inline detector. A postcolumn pump is not required as the reagents are components of the

mobile phase. The derivatization method, originally applied to the analysis of human serum, has been applied to food samples subjected to acid-alkaline hydrolysis [62], acid hydrolysis [64], and enzymatic (NADase) hydrolysis [63]. Typical chromatograms obtained after acid hydrolysis and enzymatic hydrolysis are shown in Figure 21.9 and Figure 21.10, respectively. The increased selectivity of fluorescence detection over UV absorbance detection eliminated the need to purify the hydrolysates. The detection limit for a 5-g food sample was 20 μg/100 g [62] compared to 500 μg/100 g by HPLC -UV [61]. Rose-Sallin et al. [64] checked the accuracy of their HPLC-fluorimetric method by analyzing two standard reference materials, SRM 1846 Infant Formula and a fortified cereal, VMA 195. Results were in good accordance with the certified values.

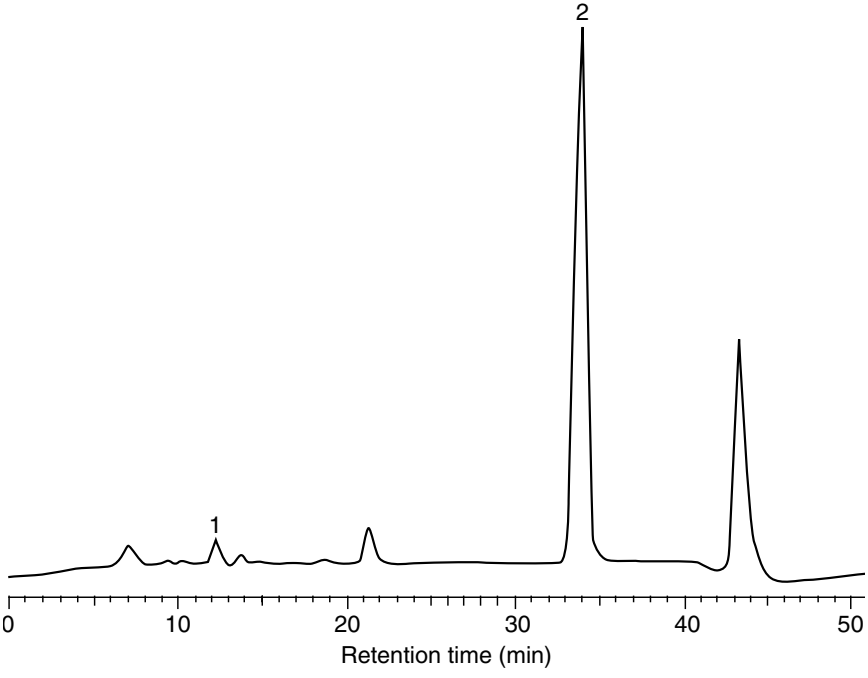

FIGURE 21.9
Reversed-phase HPLC with fluorescence detection of nicotinic acid and nicotinamide after acid hydrolysis of a cereal-based reference material. Operating parameters as in Table 21.5 [64]. Peaks: (1) nicotinic acid; (2) nicotinamide. (Reprinted from Rose-Sallin, C. et al., *Food Chem.*, 73, 473–480, 2001. With permission from Elsevier.)

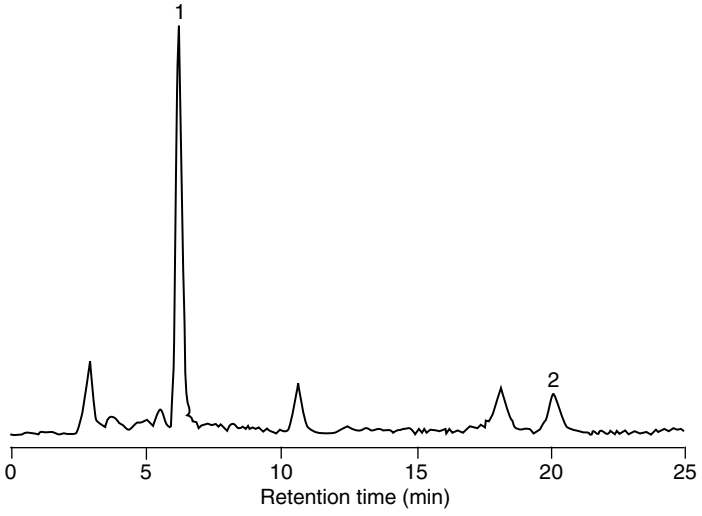

FIGURE 21.10
Reversed-phase HPLC with fluorescence detection of nicotinic acid and nicotinamide after enzymatic hydrolysis of a peanut sample. Operating parameters as in Table 21.5 [63]. Peaks: (1) nicotinic acid; (2) nicotinamide. (Reprinted from Ndaw, S., *Food Chem.*, 78, 129–134, 2002. With permission from Elsevier.)

21.2.4 Vitamin B_6

21.2.4.1 General Considerations

HPLC has the ability to isolate pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), and their 5′-phosphate esters (PNP, PLP, and PMP), and also glycosylated pyridoxine (PN-glucoside). The proportion of PN-glucoside in a food sample is a determinant of the bioavailability of vitamin B_6 in that sample. The analyst can obtain values for total or bioavailable vitamin B_6 by using the appropriate sample pretreatment. One approach is to hydrolyze the phosphate esters and PN-glucoside in one step, and measure the PN, PL, and PM that together represent total vitamin B_6. This approach is unable to determine bioavailable vitamin B_6 for plant foods that contain significant amounts of β-glucoside conjugates. However, by using selective enzymes (acid phosphatase and β-glucosidase), differential hydrolysis of the phosphate esters and PN-glucoside can be achieved. Taking the difference between PN data obtained with both enzymes (total PN) and with acid phosphatase only (free and phosphorylated PN) gives the content of PN-glucoside. Another approach is to preserve the phosphorylated and glycosylated forms and measure all six vitamers, together with PN-glucoside.

21.2.4.2 Detection

The absorption spectrum of PN·HCl is strongly dependent on the pH of the solution (Figure 21.11). At pH 1.8, there is a single maximum at 290 nm; this diminishes in intensity at pH 4.5 and a new maximum appears at 324 nm. At pH 7.0, the 324-nm maximum increases in intensity, the 290 nm maximum disappears, and a new maximum appears at 253 nm. The spectra of PL·HCl at corresponding pH values are similar to those of PN·HCl, the only obvious difference being at pH 7.0, where the maximum occurs at 316 nm. The $A_{1\ cm}^{1\%}$ values of acidified solutions (pH ca. 2) of PN·HCl and PL·HCl are, respectively, ca. 430 at λ_{max} 290.5 and 440 at λ_{max} 286.5.

PN, PL, and PM and their 5′-phosphorylated derivatives exhibit native fluorescence, which is utilized in HPLC methods for detection purposes. The intensity of fluorescence among the B_6 vitamers is pH dependent. The fluorescence spectra of PN·HCl in 0.1 *M* phosphate buffer, pH 7.0, are shown in Figure 21.12. Equivalent molar fluorescence of PN-glucoside and PN has been demonstrated [67], and therefore the glucoside may be quantified using a PN standard. HPLC methods currently used for determining vitamin B_6 in foods employ acidic mobile phases.

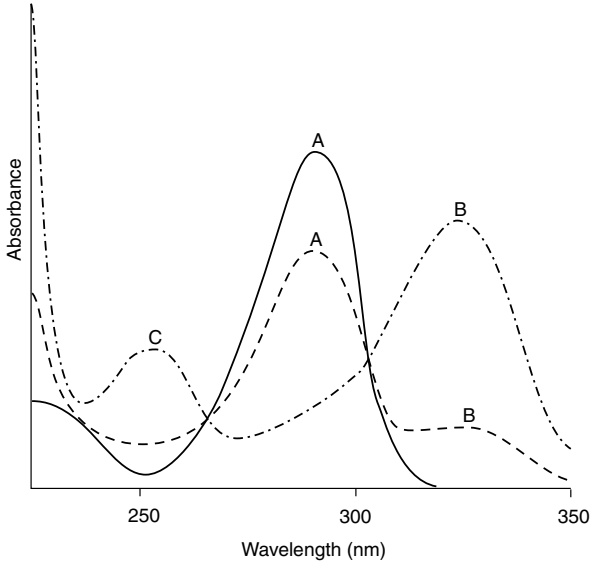

FIGURE 21.11
UV absorption spectra of pyridoxine hydrochloride dissolved in 0.1 *M* phosphate buffer at pH 1.8 (solid line), 4.5 (broken line) and 7.0 (discontinuous line) (λ_{max} of peak A = 290 nm; B = 324 nm; C = 253 nm.)

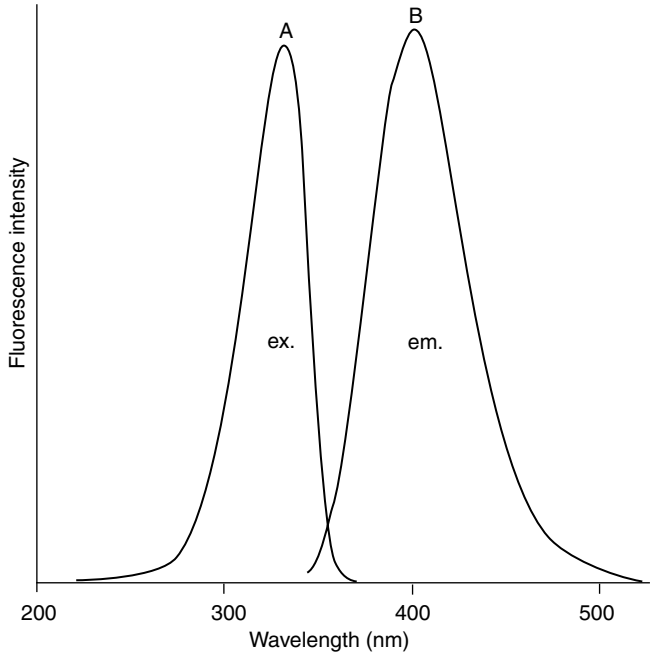

FIGURE 21.12
Fluorescence excitation and emission spectra of pyridoxine hydrochloride dissolved in 0.1 *M* phosphate buffer at pH 7.0 (λ_{max} of peak A = 327 nm; B = 393 nm).

At low pH, the fluorescence intensity of PLP is weak relative to that of the other five B_6 vitamers. However, this problem can be overcome by the post-column addition of sodium bisulfite (1 mg/ml) in 1.0 *M* phosphate buffer, pH 7.5 [68]. Neutralization of the column effluent permits detection of all B_6 vitamers at the same wavelength, and formation of the hydroxysulfonate adduct enhances the fluorescence of PLP specifically. Methods in which the phosphorylated B_6 vitamers are not hydrolyzed during extraction employ either the buffered sodium bisulfite [69–73] or bisulfite in distilled water [74]. In some methods which do hydrolyze the phosphorylated B_6 vitamers, the column effluent is neutralized by the addition of dipotassium hydrogen phosphate (pH 7) in order to change the selectivity of fluorescence detection. This step has been taken to avoid fluorescence interference from impurities present in wheat and rice products [75] and legumes [76].

21.2.4.3 Methodology

Selected methods for determining vitamin B_6 in food are presented in Table 21.6.

TABLE 21.6

HPLC Methods Used for the Determination of Vitamin B_6 Compounds in Food

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Reversed-phase chromatography						
Fortified breakfast cereals	Sonicate sample with 0.5 *M* potassium acetate (pH 4.5), centrifuge. Precipitate proteins by heating at 50°C with 33.3% (w/v) TCA, centrifuge	μBondapak C_{18} 10 μm 300 × 3.9 mm	0.033 *M* KH_2PO_4 buffer, pH 2.2	PM, PL, PN (only PN present at any significant level in breakfast cereals)	Fluorescence; ex 295 nm, em 405 nm (filters)	[77]
Pork meat, pork meat products	*Acid and enzymatic hydrolysis*: shake ground sample with 0.1 *N* HCl and 4-dPN (internal standard). Heat in a water-bath at 100°C for 30 min. Cool, adjust pH to 4.0–4.5. Incubate with Takadiastase at 45°C for 3 h with shaking. Cool, precipitate proteins by heating with 50% (w/v) TCA at 100°C for 5 min. Cool, dilute to volume with water, filter	Spherisorb ODS 5 μm 250 × 4 mm Column temperature 30°C	0.01 *M* H_2SO_4	PM, PL, PN (representing total vitamin B_6), 4-dPN (internal standard)	Fluorescence; ex 290 nm, em 395 nm	[78]

(*Table continued*)

TABLE 21.6 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Legumes	*Deproteination and enzymatic hydrolysis*: homogenize with 5% (w/v) TCA for 30 min, filter, dilute to volume with water. Adjust an aliquot of the filtrate to pH 4.8, incubate with acid phosphatase at 37°C for 5 h. Stop reaction by addition of 20% TCA. Cool, filter (0.22 μm) *Enzymatic hydrolysis of glycosylated PN*: adjust an aliquot of the initial filtrate to pH 5.0, incubate with β-glucosidase at 37°C for 5 h. Stop reaction by addition of 20% TCA, filter (0.22 μm)	Spherisorb ODS-2 10 μm 300 × 3.9 mm	0.033 *M* potassium phosphate buffer, pH 2.2/MeOH, 98:2	PM, PL, PN (representing total vitamin B_6)	Fluorescence; ex 328 nm, em 390 nm, after post-column addition of 0.3 M K_2HPO_4 (pH 7 ± 0.5)	[76]
Dried pig liver, vegetable mix, whole wheat flour	*Acid and enzymatic hydrolysis*: autoclave sample with 0.1 *N* HCl at 120°C for 30 min. Adjust to pH 4.8, incubate with acid phosphatase and β-glucosidase at 37°C for 18 h with stirring. Cool, adjust to pH 3.0, filter	Nucleosil 120 C_{18} 5 μm 250× 4 mm Column temperature 30°C	0.04 *M* H_2SO_4 and 0.005 M TCA (pH 1.0)	PM, PL, PN (representing total vitamin B_6)	Fluorescence; ex 333 nm, em 375 nm, after post-column addition of 0.13 M $K_2HPO_4 \cdot 3H_2O$ (pH 7)	[79]

Baker's yeast extract, egg yolk, milk	Vortex-mix sample with 1 *M* perchloric acid and isopyridoxal (internal standard), centrifuge. Adjust pH to 3–4 with KOH, refrigerate for a few hours, centrifuge, filter (0.45 μm)	Phenosphere ODS2 5 μm 250 × 4.6 mm	0.15 *M* NaH_2PO_4 adjusted to pH 2.5 with perchloric acid	PMP, PM, PLP, PNP, PL, iso-PL, PN, 4-PA	Fluorescence; ex 290 nm, em 389 nm after post-column reaction with sodium bisulfite in distilled water	[74]
Ground beef, lima beans, whole wheat flour, nonfat dry milk	Partially defat sample by cold extraction with $CHCl_3$/petroleum ether, 1:2. Air dry, store overnight at −40°C. Slurry in water, acidify to pH 2.0. Digest with pepsin at 37°C for 3 h, adjust pH to 8.0. Digest with pancreatin at 37°C for 12 h. Add TCA followed by MeOH, mix, centrifuge. Resuspend pellet in HPLC mobile phase, centrifuge. Pass combined supernatants through C_{18} solid-phase extraction cartridge	μBondapak C_{18} 10 μm 300 × 3.9 mm	0.075 *M* KH_2PO_4 containing monochloroacetic acid at 1.5 g/l and acidified to pH 2.75 with H_3PO_4	PMP, PM, PLP, PL, PN (representing biologically available vitamin B_6)	Fluorescence; ex 310 nm, em 390 nm, after post-column addition of sodium bisulfite in pH 7.5 phosphate buffer	[69]

(*Table continued*)

TABLE 21.6 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Reversed-phase ion-pair chromatography						
All food types	*Deproteination and enzymatic hydrolysis*: homogenize sample with 5% (w/v) TCA and 4-dPN (internal standard). Dilute with 5% TCA, shake vigorously for 30 min, centrifuge, filter. Dilute aliquot of filtrate with 4 *M* acetate buffer, pH 6.0. Incubate with Takadiastase at 45°C for 3 h. Cool, add 16.7% (w/v) TCA, centrifuge, filter	Hypersil ODS 3 μm 125 × 4.6 mm	3% MeOH and 1.25 m*M* 1-octane-sulfonic acid (PIC-B8) in 0.1 *M* KH_2PO_4 adjusted to pH 2.15 with H_3PO_4	PL, PN, PM (representing total vitamin B_6), 4-dPN (internal standard)	Fluorescence; ex 333 nm em 375 nm after post-column addition of 1 *M* $K_2HPO_4 \cdot 3H_2O$	[75]
Yeast, wheat germ, breakfast cereal (unfortified), muesli	*Dephosphorylation and conversion of PM to PL*: to ground sample add 0.05 *M* acetate buffer (pH 4.5), 1 *M* glyoxylic acid (adjusted to pH 4.5), ferrous sulfate and acid phosphatase. Incubate with continuous shaking at 37°C, dilute to volume with water, filter *Reduction of PL to PN*: add aliquot of filtrate to a solution containing 0.2 *M* NaOH and 0.1 *M* sodium borohydride, shake, filter	LiChrosphere 60 RP Select B (C_8) 5 μm 250 × 5 mm	0.05 *M* KH_2PO_4/MeCN, 96:4 containing 0.5 m*M* sodium heptane sulfonate; total solution adjusted to pH 2.50 with H_3PO_4	PN (representing total vitamin B_6)	Fluorescence; ex 290 nm em 395 nm	[80]

Pork liver (raw), milk	To minced liver sample or to milk add 4-dPN as internal standard. Homogenize with ice-cold 0.1–0.5 *M* perchloric acid, centrifuge, adjust pH to 4	LiChrosphere RP-18 5 μm 125 × 4 mm	Gradient elution program. Solvent A: MeOH; solvent B: 0.03 *M* KH_2PO_4 buffer (pH 2.7) containing 4 m*M* octanesulfonic acid. Linear gradient: 90% B to 60% B to 90% B	PLP, 4-PA, PMP, PL, PN, 4-dPN (internal standard), PM	Fluorescence; ex 330 nm em 400 nm, after post-column reaction with sodium bisulfite in pH 7.5 phosphate buffer	[72]
Potatoes	Mix sample with 5% SSA plus 4-dPN as internal standard, centrifuge *Cleanup*: SSA removed by passage through a 40 × 8 mm 100–120 mesh AG1-X8 anion exchange column (Bio-Rad)	Radial-PAK C_{18} 4 μm 100 × 8 mm	Binary step gradient. Solvent A: water containing 0.033 *M* H_3PO_4, 4 m*M* octane- and heptanesulfonic acid (pH 2.2)/2-PrOH, 97.5:2.5; solvent B: water containing 0.33 *M* H_3PO_4 (pH 2.2)/2-PrOH, 82.5:17.5. Equilibrate column with B for 1 h. Switch to A 10 min before injection, then to B 3 min after injection	PLP, PMP, PN-glucoside, PL, PN, 4-dPN (internal standard), PM	Fluorescence; ex 338 nm em 425 nm, after post-column reaction with sodium bisulfite in pH 7.5 phosphate buffer	[71]

(*Table continued*)

TABLE 21.6 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Wheat	Add 4-dPN as internal standard to ground sample, homogenize. Precipitate proteins by addition of 5% meta phosphoric acid, centrifuge	Ultremex C_{18} 3 μm 150 × 4.6 mm	Gradient elution program. Solvent A: 0.033 *M* H_3PO_4 + 8 m*M* 1-octane sulfonic acid adjusted to pH 2.2 with 6 *M* KOH; solvent B: 0.033 *M* H_3PO_4/MeCN, 90:10 adjusted to pH 2.2. Linear gradient from 100% A to 100% B during 10 min after injection, hold at 100% B for 15 min, then return to 100% A in 4.5 min	PLP, 4-PA, PMP, PN-glucoside, PL, PN, 4-dPN (internal standard), PM	Fluorescence; ex 311 nm em 360 nm, after post-column reaction with sodium bisulfite in pH 7.5 phosphate buffer	[73]

Note: MeOH, methanol; 2-PrOH, 2-propanol; MeCN, acetonitrile; $CHCl_3$, chloroform; TCA, trichloroacetic acid; SSA, sulfosalicylic acid; 4-dPN, 4′-deoxypyridoxine.

Argoudelis [74] demonstrated the separation of a standard mixture of B_6 vitamers plus isopyridoxal (internal standard) and 4-pyridoxic acids using reversed-phase chromatography with ion suppression (Figure 21.13). It can be seen that PLP is the only B_6 vitamer whose fluorescence intensity is enhanced by postcolumn reaction with sodium bisulfite. A chromatogram of an extract from milk (2% fat) is shown in Figure 21.14.

Ekanayake and Nelson [69] estimated the biologically available vitamin B_6 content of foods by subjecting partially defatted samples to a two-stage enzymatic digestion (pepsin and pancreatin), followed by treatment with trichloroacetic acid and then methanol to terminate the hydrolysis reaction and precipitate the proteins. The hydrolysates were centrifuged at 28,000 × *g*, and the supernatant fraction was transferred to a hypodermic

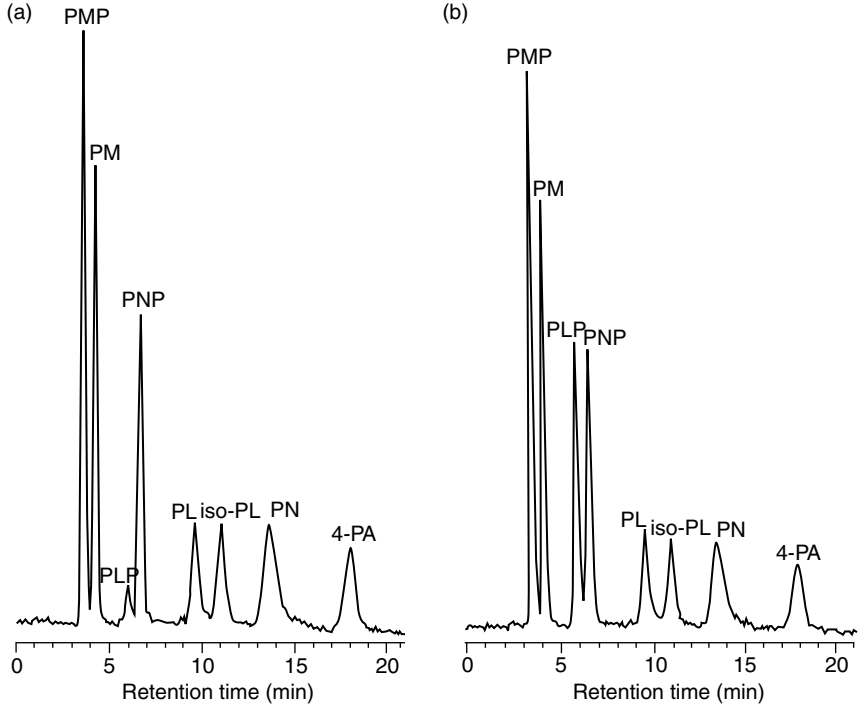

FIGURE 21.13
Reversed-phase HPLC with fluorescence detection of standards of B_6 vitamers (A) without and (B) with post-column reaction with sodium bisulfite. Operating parameters as in Table 21.6 [74]. (Reprinted from *J. Chromatogr. A*, 790, 83–91, 1997. With permission from Elsevier.)

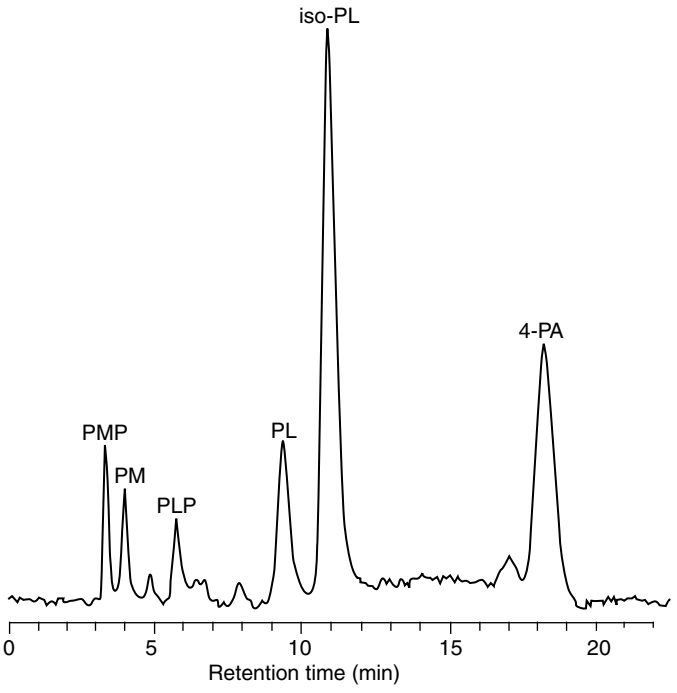

FIGURE 21.14
Reversed-phase HPLC with fluorescence detection of B_6 vitamers in an extract of milk. Operating parameters as in Table 21.6 [74]. (Reprinted from Argoudelis, C.J., *J. Chromatogr. A*, 790, 83–91, 1997. With permission from Elsevier.)

syringe. The pellet was resuspended in HPLC mobile phase and centrifuged. The supernatant was collected in the same syringe, and the pooled fractions were passed through a C_{18} solid-phase extraction cartridge. The purified extracts were analyzed by reversed-phase HPLC with ion suppression, using postcolumn reaction with buffered (pH 7.5) sodium bisulfite. To calculate the available vitamin B_6, nonphosphorylated vitamer equivalents were calculated from the phosphorylated forms by using the molarity conversion factors of 0.6764 for PLP to PL and 0.6777 for PMP to PM. Transamination of PLP to PMP and PL to PM occurred on digestion and was taken into account when calculating the recoveries. Total vitamin B_6 contents of the food samples were determined after acid hydrolysis and HPLC analysis. The validity of the *in vitro* enzymatic digestion procedure as a true measure of available vitamin B_6 was evaluated by comparing the ratio of available to total vitamin B_6, obtained

by the methods described, with the same ratio obtained by rat bioassay and yeast growth assay taken from published data. When the 95% confidence intervals for each of the ratios for a given food were compared, they were found to be comparable for ground beef, nonfat dry milk, and whole wheat flour, but not for lima beans. The latter discrepancy may have been due to varietal differences in lima beans.

For the determination of total vitamin B_6 in legumes, Sierra and Vidal-Valverde [76] deproteinized samples by homogenization with 5% (w/v) trichloroacetic acid followed sequentially by incubation with acid phosphatase and β-glucosidase. Quantitation of PM, PL, and PN was performed by reversed-phase chromatography with ion suppression, fluorometric detection, and postcolumn addition of dipotassium hydrogen phosphate solution (Figure 21.15). The content of glycosylated

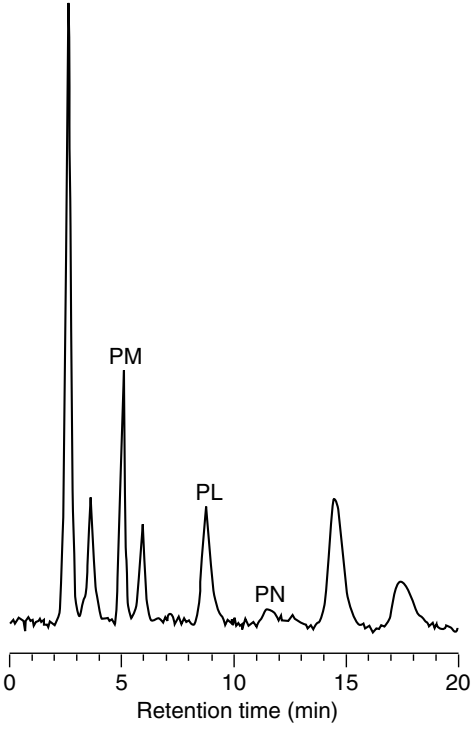

FIGURE 21.15
Reversed-phase HPLC with fluorescence detection of B_6 vitamers in hydrolyzed extract from haricot beans. Operating parameters as in Table 21.6 [76]. (From Sierra, I. and Vidal-Valverde, C. *J. Liq. Chromatogr. Rel. Technol.*, 20 (6), 957, Copyright 1997. With permission of Taylor & Francis. Inc., http://www.taylorandfrancis.com.)

pyridoxine could be obtained by calculating the difference between PN data obtained with and without β-glucosidase treatment.

Addo and Augustin [71] demonstrated the separation by ion-pair chromatography of B_6 vitamers and PN-glucoside extracted from potatoes (Figure 21.16). Sampson et al. [73] reported the separation of PN, PN-glucoside, PL, PLP, PM, and PMP, together with 4-pyridoxic acid and 4′-deoxypyridoxine as internal standard, by means of ion-pair HPLC with a single binary gradient elution program. The separation method was applied to the determination of free, phosphorylated, and glycosylated vitamin B_6 in deproteinized wheat samples. The major form found was PN-glucoside, accompanied by PN. Small amounts of PMP, PLP, and 4-pyridoxic acid were found in two out of three wheat cultivars analyzed. van Schoonhoven et al. [75] extracted samples of foods and feeds with 5% trichloroacetic acid, and dephosphorylated B_6 vitamers by incubation with Takadiastase. PL, PN, PM, and 4′-deoxypyridoxine (internal standard) were separated isocratically within 35 min by means of reversed-phase ion-pair HPLC. An approximately 40% higher total vitamin B_6 content was found with the HPLC method than with the classical microbiological method using *Saccharomyces cerevisiae*. It was demonstrated that the Takadiastase used for dephosphorylation contained β-glucosidase activity, so the HPLC result represented total vitamin B_6 from free, phosphorylated and glycosylated forms.

In a method proposed by Reitzer-Bergaentzlé et al. [80] for the determination of free and phosphorylated vitamin B_6 in yeast, wheat germ, breakfast cereal (with bran), and muesli, the sample preparation entailed dephosphorylation of B_6 vitamers with acid phosphatase and simultaneous conversion of PM to PL by treatment with glyoxylic acid in the presence of catalytic ferrous ions. After filtration, the PL was reduced to PN by treatment with sodium borohydride in alkaline medium, and the PN (originating from phosphorylated and free vitamers) was determined using reversed-phase ion-pair chromatography and fluorescence detection. The retention time of PN was 7 min under the selected conditions and the detection limit was 0.02 μg/g. The chromatograms depicted in Figure 21.17 show the effects of the glyoxylic acid and sodium borohydride treatments. In most of the foods studied, interfering substances prevented satisfactory separation and quantification of PN when dephosphorylation was performed with hydrochloric acid hydrolysis. On the other hand, phosphatase hydrolysis led to a very good resolution of the PN peak in yeast, breakfast cereal, and muesli. In wheat germ, however, the separation of PN was not entirely satisfactory, but could be markedly improved by increasing the concentration of ion-pairing agent from 0.5 to 0.7 m*M*. The proposed method has been subjected to a collaborative study [81] in which 12 participating laboratories analyzed eight samples

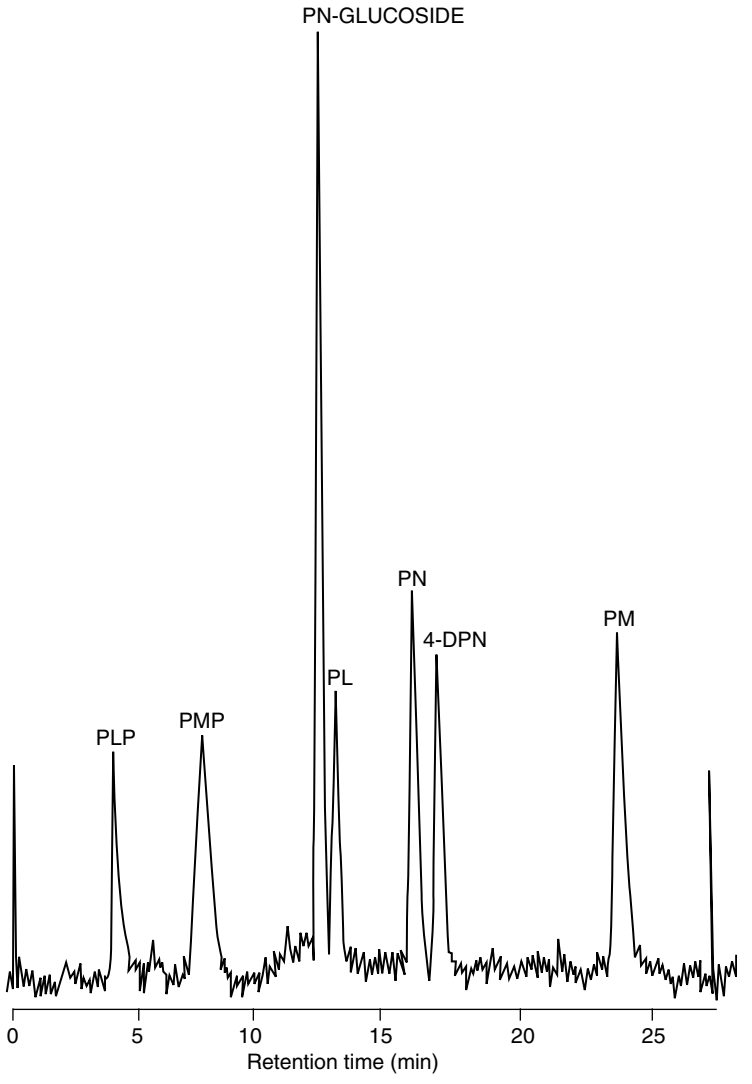

FIGURE 21.16
Reversed-phase ion-pair HPLC with fluorescence detection of B_6 vitamers extracted from potato. Operating parameters as in Table 21.6 [71]. (From Addo, C. and Augustin, J., *J. Food Sci.*, 53, 749, 1988. With permission.)

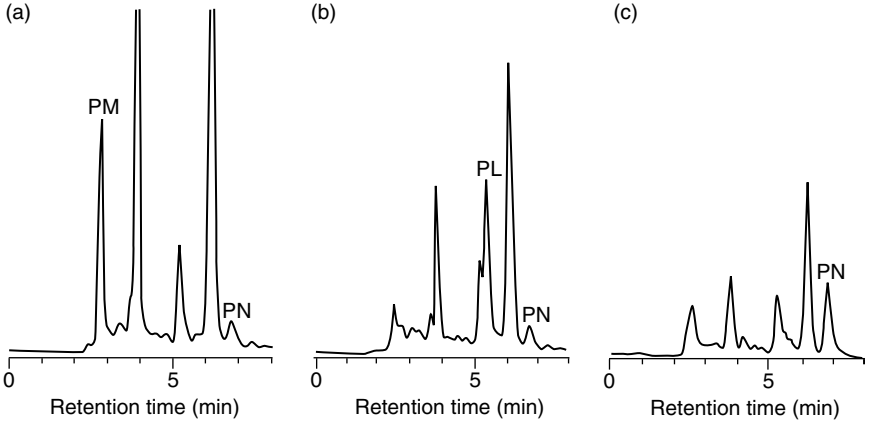

FIGURE 21.17
Reversed-phase ion-pair HPLC with fluorescence detection of B_6 vitamers in yeast (a) with deletion of the glyoxylic acid and sodium borohydride treatments, (b) with deletion of the sodium borohydride treatment, and (c) without deletion. Operating parameters as in Table 21.6 [80]. (Reprinted from Reitzer-Bergaentzlé, M., Marchioni, E., and Hasselmann, C., *Food Chem.*, 48, 321–324, 1993. With permission from Elsevier.)

containing various amounts of vitamin B_6 (from 0.6 to 32.8 μg/g of PN). Repeatability RSDs ranged from 3 to 18%, depending on the vitamin B_6 concentration. Reproducibility RSDs were around 30% for samples containing lower concentrations of vitamin B_6 (<3 μg/g of PN) and 12–13% when concentration exceeded 5 μg/g of PN, except for a sample of yeast for which the RSD was 26%. The method of Reitzer-Bergaentzlé et al. [80] has been chosen as the official French method for vitamin B_6 determination in foods for nutritional purposes. As the acid phosphatase treatment does not hydrolyze glycosylated PN, the method yields results that assuredly represent bioavailable vitamin B_6. The method has also been evaluated for the analysis of vitamin B_6 in soy-based infant formula [82]. Assay of SRM 1846 Infant Formula (milk-based) gave results within the certified range.

21.2.5 Pantothenic Acid

21.2.5.1 Detection

The pantothenic acid molecule does not contain a characteristic chromophore, and hence it exhibits only very weak absorbance at 204 nm, owing to the presence of carbonyl groups. Detection at wavelengths below

220 nm is subject to interference from the many organic compounds present in a typical food sample extract prepared for analysis. The problem of weak and nonspecific absorbance, coupled with the low concentrations of pantothenic acid in foods, has thwarted attempts to apply HPLC–UV to the determination of pantothenic acid in foodstuffs, although the technique has been successfully applied to pharmaceutical products.

21.2.5.2 Applications

HPLC methods for determining pantothenic acid in foods and infant formulas are presented in Table 21.7.

Woollard et al. [83] developed an HPLC–UV method for determining the free endogenous D-pantothenic acid in milk and supplemental calcium pantothenate in infant formulas. The chromatographic mode was reversed-phase with ion suppression. Sample preparation simply entailed addition of 3% (v/v) acetic acid to the milk or reconstituted infant formula, vortex-mixing and standing for 20 min, followed by centrifugation and membrane filtration. This treatment resulted in a protein- and fat-free extract that could be directly injected (10-μl aliquot). The problems of poor detection specificity in the low-ultraviolet region of the spectrum were overcome by the use of a photodiode array detector that provided multiwavelength detection (selected wavelengths were 200, 205, and 240 nm) and inline spectral analysis. The HPLC system incorporated an inline mobile phase degasser — an important feature, as dissolved oxygen constitutes a source of interference at low ultraviolet wavelengths.

Among several reversed-phase columns investigated by Woollard et al. [83], the column selected for routine use was of dimensions 250 × 4.6 mm ID and packed with Luna (Phenomenex) 5 μm C_8 (octyl) of 100 Å pore size, 400 m^2/g surface area, and 13.5% carbon load. The Luna material is based on low-acidity silica, exhaustive end-capping, and shielded bonded phase ligand. The mobile phase was phosphate buffer (0.1 *M*, pH 2.25)/acetonitrile (97:3, v/v) delivered initially at 1.4 ml/min and increased to 1.8 ml/min at 18 min. Following completion of the sample schedule, the column was purged sequentially with acetonitrile/water (50:50), water (100%), acetonitrile/water (50:50), and finally acetonitrile (100%) for column storage between runs. The retention time of the pantothenic acid was around 15 min, but the next sample was not injected until a major unknown peak with a retention time of about 35 min was removed (Figure 21.18). Results obtained by HPLC correlated with those obtained by microbiological assay utilizing *L. plantarum* ($r = 0.971$), and there was no significant difference ($P > 0.05$) between the two sets of results.

TABLE 21.7

HPLC Method Used for the Determination of Pantothenic Acid in Food

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Reversed-phase chromatography						
Milk, milk-based infant formula supplemented with calcium pantothenate	Add 3% (v/v) acetic acid to milk or reconstituted formula, vortex-mix, stand for 20 min. Dilute to volume, centrifuge, filter (0.45 μm)	Luna C_8 (octyl) 5 μm 250 × 4.6 mm	0.1 *M* Phosphate buffer, pH 2.25/ MeCN, 97:3	Pantothenic acid	UV 200, 205, 240 nm (diode array detector)	[83]
Fortified food products: breakfast cereals, milk- and soy-based infant formulas	Dissolve homogenized sample in warm water to make a slurry. Add water to a weighed portion under agitation plus hopantenic acid (HOPA) as internal standard). Autoclave at 103°C for 20 min, cool, dilute to known volume, shake, filter (0.22 μm)	YMC C_{18} 3 μm 150 × 3.0 mm	Multi-step binary gradient. Solvent A: water containing 0.025% TFA (pH 2.6); solvent B: MeCN	Pantothenic acid, HOPA (internal standard)	Mass spectrometry: electrospray ionization in positive ion mode with single ion monitoring at mass m/z 220.	[84]

Whole egg, pork liver, mushrooms, corn, hazel nuts, cocoa	Freeze-dry liver, eggs and mushrooms. Stir powdered samples for 1 h at 20°C in 0.02 *M* sodium acetate, pH 5.6 containing calcium [^{15}N,$^{13}C_3$]-(*R*)-pantothenate (internal standard). Autoclave filtered extract at 120°C for 15 min, cool. Add pantetheinase, alkaline phosphatase, $NaHCO_3$ and aqueous solution of calcium [^{15}N,$^{13}C_3$]-(*R*)-pantothenate. Adjust to pH 8, incubate for 8 h at 37°C. Adjust to pH 5.6	Aqua C-18 5 μm (Phenomenex) 250 × 4.6 mm	Gradient elution. Solvent A: 0.1% aqueous formic acid; solvent B: MeCN	Pantothenic acid, calcium [^{15}N,$^{13}C_3$]- (*R*)-pantothenate (internal standard)	Mass spectrometry: electrospray ionization in positive ion mode using selected ion monitoring; spray voltage, 5 kV; capillary temperature, 200°C	[85]
Yeast, pig liver, chicken meat, salmon,	*Extraction of free pantothenic acid*: shake ground sample with 200 m*M* Tris buffer (pH 8), dilute with water, centrifuge, filter *Extraction of total (free and bound) pantothenic acid:* incubate ground sample	Lichrospher 100 RP 18 5 μm 250 × 5 mm	Gradient of MeOH and 33 m*M* phosphate buffer (pH 2.5). Proportion of MeOH increased linearly from 0 to 10% during 25 min, and held at 10% for 8 min	Free or total pantothenic acid	Fluorescence; ex 345 nm, em 455 nm, after postcolumn addition of reagent (see text for composition)	[86]

(*Table continued*)

TABLE 21.7 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
whole egg, powdered milk (supplemented with pantothenic acid), frozen vegetables (spinach, peas, French beans), lentils, avocado	with buffered (pH 4.5) pepsin at 50°C for 3 h. Adjust to pH 8, add 200 m*M* Tris buffer (pH 8), alkaline phosphatase, and pantetheinase. Incubate at 20°C for 18 h, dilute to volume with water, centrifuge, filter (0.45 μm) *Cleanup of extracts*: successive passages through strong anion exchange and strong cation exchange solid-phase extraction cartridges					

Note: MeCN, acetonitrile; TFA, trifluoroacetic acid.

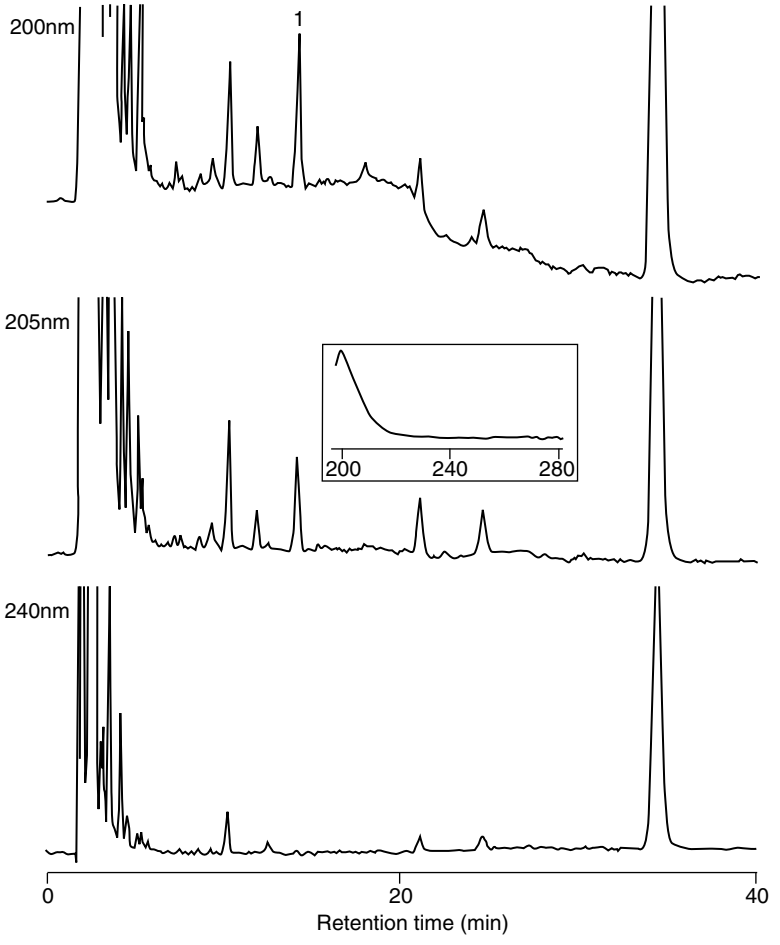

FIGURE 21.18
Reversed-phase HPLC of pantothenic acid in an extract of a typical infant formula supplemented with calcium pantothenate. Multiwavelength UV detection (200, 205, 240 nm). The inset illustrates a UV spectral scan of pantothenic acid. Operating parameters as in Table 21.7 [83]. Flow, 1.4 ml/min (increased to 1.8 ml/min at 18 min); injection volume, 10 μl. Peak (1) pantothenic acid. (Reprinted from Woollard, D.C., Indyk, H.E., and Christiansen, S.K., *Food Chem.*, 69, 201–208, 2000. With permission from Elsevier.)

Mittermayr et al. [84] developed a method for determining supplemental calcium pantothenate in breakfast cereals and infant formulas using reversed-phase HPLC–mass spectrometry with electrospray ionization. Hopantenic acid was added as internal standard to the slurried sample before autoclaving. The binary gradient elution program using

0.025% aqueous trifluoroacetic acid (pH 2.6) and acetonitrile did not produce ion suppression. Chromatograms of a hypoallergenic infant formula using UV or MS detection are shown in Figure 21.19. The limit of detection of the method was 800 pg on-column and the limit of quantification was 0.25 μg/ml, which is equivalent to 0.28 mg/100 g in a dry food product.

Rychlik [85] developed a stable isotope dilution assay based on HPLC–tandem mass spectrometry for the determination of naturally occurring free and total pantothenic acid in a variety of foods. The quantification limit was 0.024 mg/100 g in starch-containing foods, which was sufficient as cereals contain more than 0.2 mg/100 g.

Pakin et al. (86) determined free and total (free and bound) pantothenic acid in foods by reversed-phase HPLC with fluorometric detection. This

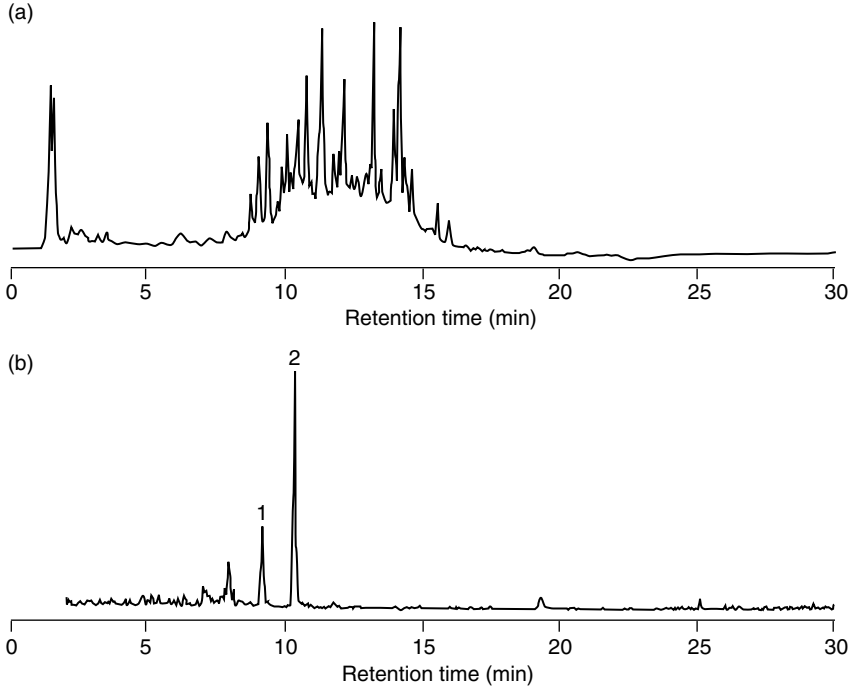

FIGURE 21.19
Gradient reversed-phase HPLC of pantothenic acid in an extract of a hypoallergenic infant formula with (a) UV detection and (b) mass spectrometric detection. Operating parameters as in Table 21.7 [84]. Peaks: (1) pantothenic acid; (2) hopantenic acid (internal standard). (Reprinted from Mittermayr, R., et al., *J. Chromatogr. A*, 1032, 1–6, 2004. With permission from Elsevier.)

entailed postcolumn chemistry involving alkaline hydrolysis of pantothenic acid to β-alanine and reaction of this amino acid with ortho-phthaldialdehyde in the presence of 2-mercaptoethanol to form fluorescent 1-alkylthio-2-alkylisoindole. Total pantothenic acid was obtained by treatment of samples with pepsin then pantetheinase and alkaline phosphatase. Purification of sample extracts was achieved by successive passages through strong anion exchange and strong cation exchange solid-phase extraction cartridges. A chromatogram of an extract from chicken egg is shown in Figure 21.20. The detection limit was 0.65 μg/g, making the method applicable to the determination of pantothenic acid in any foodstuff.

21.2.6 Biotin

21.2.6.1 Detection

The low concentration of biotin in most foods and the absence of a strong chromophore in its molecule have precluded any sensitive detection of this vitamin by UV photometry. The sensitivity of mass spectrometry is also inadequate [87]. HPLC methods employing precolumn fluorescence derivatization of the carboxylic acid group have been proposed for the determination of biotin in pharmaceutical preparations, but these systems are not suitable for food analysis owing to interference from fluorescent contaminants. Avidin, the egg-white protein, can be labeled with a fluorescent marker (fluorescein 5-isothiocyanate, FITC) and the complex

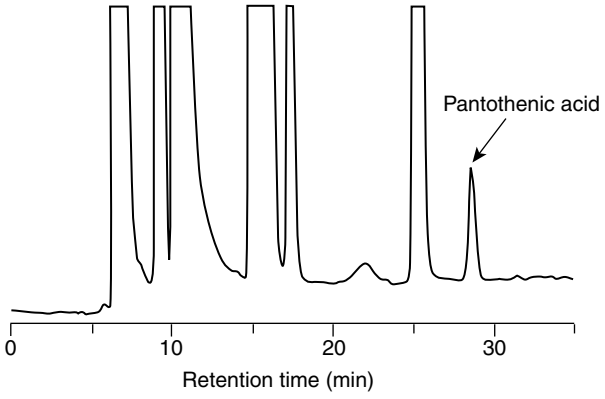

FIGURE 21.20
Reversed-phase HPLC with fluorescence detection of pantothenic acid in a purified extract of whole chicken egg. Operating parameters as in Table 21.7 [86]. (Reprinted from Pakin, C., et al., *J. Chromatogr. A*, 1035, 87–95, 2004. With permission from Elsevier.)

used as a postcolumn derivatizing agent. The fluorescence of the labeled protein is enhanced on binding of its specific ligands, biotin and biocytin [88].

21.2.6.2 Application

Lahély et al. [89] developed a highly specific and sensitive HPLC method for determining biotin in any food (summarized in Table 21.8). Biotin and biocytin are separated by reversed-phase chromatography and then derivatized with the avidin–FITC reagent prior to fluorescence detection. By increasing the injection volume to 200 μl, it was possible to determine biotin in foods at concentrations as low as 0.004 μg/g, without any problems of interference. Chromatographic separations are shown in Figure 21.21. Enzymatic hydrolysis of samples using papain and Takadiastase allowed a good estimation of bioavailable biotin.

21.2.7 Folate

21.2.7.1 General Considerations

Except for a few cases, such as liver and yeast extract, levels of naturally occurring folate in foods are extremely low (<100 ng/g). The folate vitamers most likely to be naturally present in a food matrix at measurable concentration are polyglutamyl forms of THF, 5-methyl-THF, 5-formyl-THF, and 10-formyl-THF. 10-Formylfolic acid may be present in processed foods as an oxidation product of 10-formyl-THF. Folic acid is present in significant amounts only in fortified foods. In HPLC methods for food folate, polyglutamyl folates are converted enzymatically to monoglutamyl forms, and 10-formyl-THF is converted by heat treatment to 5-formyl-THF. This leaves THF, 5-methyl-THF, 5-formyl-THF, and folic acid as the folates of analytical concern. Of the naturally occurring vitamers, 5-methyl-THF is by far the most abundant, as measured by HPLC with fluorometric detection.

The reduced folates are susceptible to oxidation by air, and protection of samples and standards from oxidation is an inherent and essential part of the analytical procedure. Oxygen can be removed from folate solutions by ultrasonication and flushing with pure nitrogen gas. Sodium ascorbate is an effective antioxidant when present at a concentration of 10 m*M* [90]. Other commonly used antioxidants are dithiothreitol (also known as dithioerythritol) and 2-mercaptoethanol. In using stabilizing agents, the subsequent HPLC method has to be optimized to achieve sufficient separation of these substances from folates and their degradation products.

TABLE 21.8

HPLC Method Used for the Determination of Biotin in Food

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Reversed-phase chromatography						
Cereals, fruit juice, infant formula, beef liver, tomato, peanuts, brewer's yeast, yogurt, carrot, pork, chicken, raw egg	To ground sample add reduced glutathione solution, 1% EDTA, citrate buffer (pH 5.7) and papain. For high-starch sample add Takadiastase. Incubate at 37°C with shaking overnight, cool, dilute to volume with water. Filter through paper then through membrane (0.45 μm)	Lichrospher 100 RP 18 5 μm 250 × 5 mm	0.1 M Phosphate buffer, pH 6/ MeOH, 81:19	Biotin, biocytin	Fluorescence; ex 490 nm em 520 nm, after postcolumn addition of avidin– FITC reagent	[89]

Note: EDTA, ethylenediaminetetraacetic acid; MeOH, methanol; FITC, fluorescein 5-isothiocyanate.

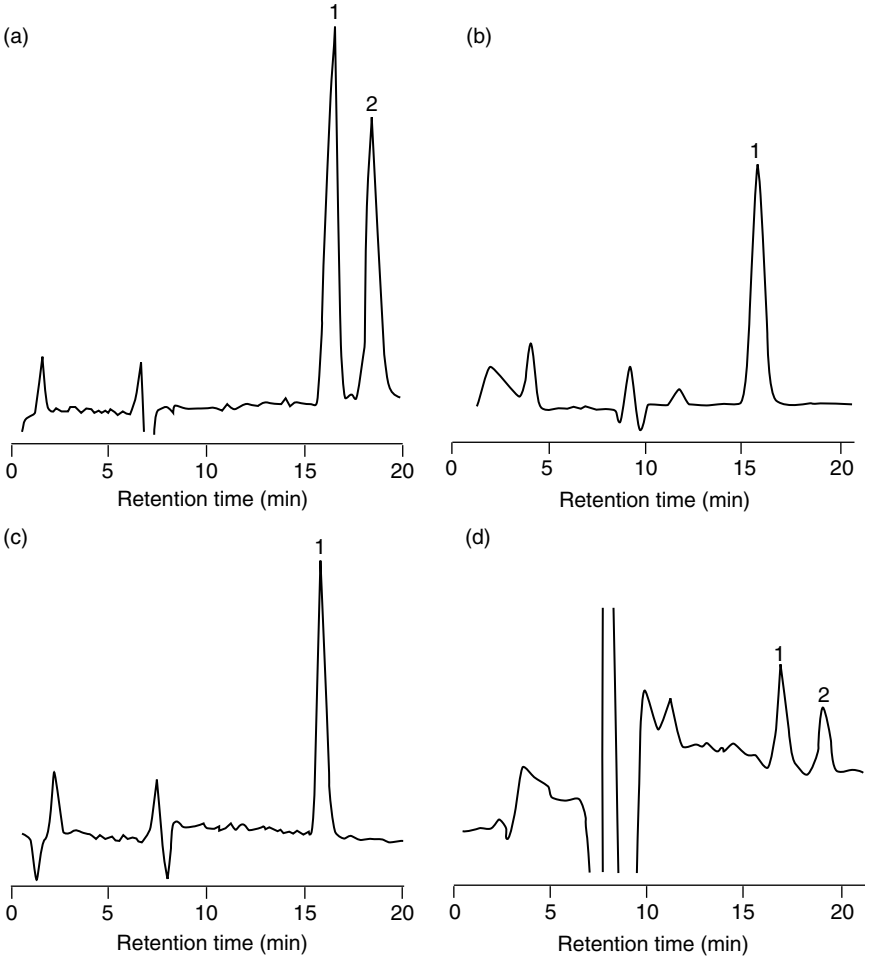

FIGURE 21.21
Reversed-phase HPLC with fluorescence detection of biotin and biocytin in (a) a standard mixture, (b) cereal, (c) beef liver, and (d) tomato. Operating parameters as in Table 21.8 [89]. Peaks: (1) biotin; (2) biocytin. (Reprinted from Lahély, S., *Food Chem.*, 65, 253–258, 1999. With permission from Elsevier.)

21.2.7.2 Cleanup Procedures

Food extracts require some form of cleanup to remove material that interferes with the chromatographic determination of folates. Solid-phase extraction using silica-based strong anion-exchange (SAX) materials with quaternary amine functional groups is a well-tested technique.

In a cleanup procedure reported by Vahteristo et al. [91], 3-ml (bed volume) SAX cartridges are conditioned by application of 10 ml of 0.01 *M* phosphate buffer (pH 7.0) containing 0.1% 2-mercaptoethanol. Sample extracts (3–5 ml) are diluted with water (6–10 ml) and 15 μl of 2-mercaptoethanol is added. The extracts are then syringe-filtered through nylon (0.8 μm) before loading onto the conditioned cartridge. Folates are retained as the solution is passed slowly (<1 ml/min) through the column bed. The cartridge is washed twice with 1.5 ml of conditioning buffer, and the folates are eluted with 2.5 ml of 0.1 *M* sodium acetate (pH 4.5) containing 10% (w/v) sodium chloride and 1% (w/v) ascorbic acid.

The use of SAX solid-phase extraction cartridges to purify food extracts commonly provides high recovery for different folate vitamers, but preconcentration of sample extracts is usually not possible due to the limited capacity of the sorbent and the need to use large buffer volumes to elute folates quantitatively. Furthermore, the use of SAX cartridges failed to remove fluorescent compounds that interfered with the measurement of 5-formyl-THF during HPLC analysis of foods [92]. Nilsson et al. [93] evaluated the use of phenyl and cyclohexyl sorbents for the purification of dietary folate extracts by reversed-phase solid-phase extraction. The ionization of folates must be suppressed for these compounds to be retained on nonpolar, reversed-phase sorbents and this was achieved by the use of 0.03 *M* H_3PO_4 as a conditioning and washing buffer. The elution of folates was carried out with 0.1 *M* sodium acetate containing 12% (v/v) acetonitrile, 1% (w/v) ascorbic acid, and 0.1% (v/v) 2-mercaptoethanol (pH 4.5). Under these conditions, phenyl and cyclohexyl sorbents with trimethyl endcapping of silanol groups showed strong retention of THF, 5-methyl-THF, and 5-formyl-THF. The losses of folates were negligible (<4%) during application and washing steps. Preconcentration of sample extracts was possible because extract volumes up to 5 ml could be loaded onto both phenyl and cyclohexyl cartridges without any noticable losses in 5-methyl-THF. For complex matrices, the combined use of SAX and phenyl cartridges minimized matrix interference for all food samples tested. Purification on either SAX or phenyl cartridges failed to remove chromatographic peaks in the vicinity of 5-formyl-THF.

Sample extracts can be effectively purified and concentrated by affinity chromatography using immobilized folate-binding protein (FBP). Affinity columns contain 2 ml of a gel matrix prepared by the chemical coupling of bovine milk FBP with sepharose gel. Ready-to-use affinity columns are not commercially available, but columns can be prepared in the laboratory from commercially available sepharose gel and FBP [94]. The procedure for purifying sample extracts [95,96] is as follows.

Apply the centrifuged or filtered sample extract to an affinity column that has been previously equilibrated with 10 ml of 0.1 *M* phosphate buffer, pH 7.0. Wash the column with 5 ml of 25 m*M* phosphate buffer (pH 7.0) containing 1.0 *M* sodium chloride, followed by 5 ml of 25 m*M* phosphate buffer (pH 7.0). Apply 2 ml of elution solution (water containing 20 m*M* trifluoroacetic acid and 10 m*M* dithiothreitol) corresponding to the void volume, and discard the effluent. Elute the folates with 4.95 ml of the elution solution, collecting the eluate in a 5-ml volumetric flask containing 30 μl of 1 *M* piperazine, 5 μl 2-mercaptoethanol, and 50 μl of a 20% (w/v) solution of ascorbic acid (0.2% final concentration). Fill the flask to the exact volume. After elution, wash the column with 1.5 ml of elution solution and then with 10 ml of equilibrating buffer. Store the columns at 4°C in equilibrating buffer containing 0.2% sodium azide.

Kariluoto et al. [96] found that an affinity column could be used more than 20 times over a period of 6 months, by which time the capacity of the column was reduced by 16%. 5-Formyl-THF is known to have a lower affinity for the immobilized FBP than the other reduced folates. The recovery of 5-formyl-THF was over 90%, provided that the total folate load did not exceed 25% of the column capacity [95,96]. Affinity columns exhibit higher affinities toward the natural (6*S*) folate vitamers than to racemic (6*R*,*S*) vitamers. The binding affinities of racemic THF, 5-methyl-THF, and 5-formyl-THF relative to those of the natural vitamers were 97, 84, and 74%, respectively [97]. The neutralization of the acidic eluate with piperazine is important in preventing the conversion of 5-formy-THF to 5,10-methenyl-THF at low pH.

21.2.7.3 Detection

Folates exhibit UV absorption (Table 21.9) and produce spectra whose characteristics are affected by pH. Absorption spectra of folic acid and THF are shown in Figure 21.22. The spectrum of folic acid in phosphate buffer at pH 7.1 exhibits two maxima at wavelengths above 220 nm, namely at 282 and 347 nm. At pH 13, three maxima above 220 nm are observed, namely at 255, 283, and 364 nm. THF in phosphate buffer at pH 7.1 shows a single maximum at 286 nm. UV absorption detection has sufficient sensitivity to detect folic acid in fortified foods, but it lacks the sensitivity to detect naturally occurring folates such as 5-methyl-THF.

The naturally occurring folates and several pterin derivatives exhibit native fluorescence whose intensity is markedly dependent on pH [99]. Gounelle et al. [100] obtained the highest fluorescence at pH 3.0 with THF, at pH 2.5 with 5-methyl-THF, and at pH 2.0 with 5-formyl-THF.

TABLE 21.9

Absorption Spectral Properties of Folates

Compound	pH[a]	λ_{max} (nm)	Molar extinction coefficient (ε)
Folic acid	7	282, 350	27,000, 7,000
	13	254, 282, 363	25,000, 23,800, 8,700
THF	7	297	27,000
10-Formyl-THF	7	288	18,200
5-Formyl-THF	7	287	31,500
	13	282	32,600
5-Methyl-THF	7	290	32,000
	13	290	31,600
5-Formimino-THF	7	285	35,400
5,10-Methenyl-THF	7	352	25,000
5,10-Methylene-THF	7.2	294	32,000

[a]pH 7, usually phosphate buffer; pH 13, 0.1 *M* NaOH.
Source: From Temple, C., Jr. and Montgomery, J.A., in *Folates and Pterins*, Vol. 1, *Chemistry and Biochemistry of Folates*, Blakley, R.L.P and Benkovic, S.J., Eds., John Wiley & sons, New York, 1984, p. 61. With permission.

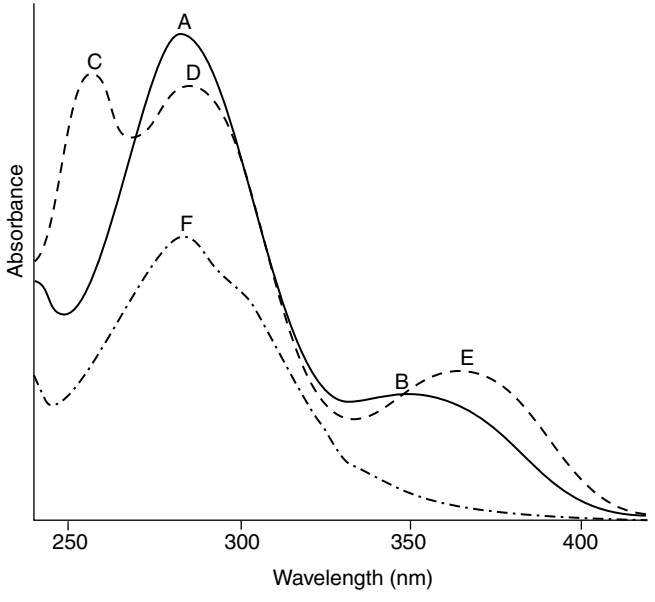

FIGURE 21.22
UV absorbance spectra of folic acid and tetrahydrofolic acid (THF). For folic acid in 0.1 *M* phosphate buffer at pH 7.1 (solid line), λ_{max} of peak A = 282 nm; B = 347 nm. For folic acid in 0.1 *M* NaOH, pH 13 (broken line), λ_{max} of peak C = 255 nm; D = 283 nm; E = 364 nm. For THF in 0.1 *M* phosphate buffer at pH 7.1 (discontinuous line), λ_{max} of peak F = 286 nm.

Fluorescent intensity remained constant from pH 3.0 up to at least pH 8.0 for 5-formyl-THF, and decreased to zero at pH 7.0 for 5-methyl-THF and THF. The native fluorescence of 5-methyl-THF was about 10 times stronger than that of 5-formyl-THF and twice as strong as that of THF. The fluorescence of reduced folates in an acidic medium provides a sensitive and specific means of HPLC detection. The following wavelength maxima (excitation/emission) were obtained from stopped-flow fluorescence spectra during chromatography with an acidic (pH 2.3) mobile phase: THF (288/355 nm), 5-methyl-THF (288/353 nm), and 5-formyl-THF (292/356 nm) [101]. Folic acid fluoresces at neutral, acid, and alkaline pH (optimum, pH 9.0), but not at pH 6.0. However, the intensity is weak compared with that of reduced folates and is not sufficient to allow quantitation of folic acid in fortified foods.

Postcolumn oxidative cleavage of folic acid to a highly fluorescent pterin fragment enables the parent vitamin to be measured fluorometrically with greatly improved sensitivity and specificity compared with UV detection. 5-Methyl-THF is not converted to a fluorescent product and the fluorescence of the oxidation products of THF and 5-formyl-THF is negligible compared with their intact forms [102]. It is therefore not possible to derivatize all the folates and measure the products with a single fluorescence detector. The system employed by Gregory et al. [101] consisted of the following modules installed inline after the column: (1) a fluorescence detector to monitor the native fluorescence of the reduced folates in the acidic mobile phase, (2) a pump-driven derivatization system, and (3) a second fluorescence detector to measure the fluorescence of the folic acid cleavage product. Hahn et al. [102] tested a number of oxidizing agents, including calcium hypochlorite, a reagent first used by Day and Gregory [103]; the best results were obtained with potassium peroxodisulfite ($K_2S_2O_8$). Doherty and Beecher [104] converted folic acid postcolumn to a fluorescent product by UV-induced photolysis. Gauch et al. [105] performed precolumn derivatization of folic acid to a fluorescent product using FMOC-Cl (chloroformic acid-9-fluorenylmethyl ester).

Electrochemical detection in the oxidative mode is equally as sensitive as fluorescence detection for 5-methyl-THF [106], but has not been extensively applied to food analysis. Holt et al. [107] reported that the high concentrations of reducing agents (e.g., ethanol ascorbate and 2-mercaptoethanol) included during the extraction steps created significant interferences in the oxidative electrochemical detection of folates.

The coupling of a mass spectrometer to the HPLC system (LS–MS) allows specific identification and provides greatly improved selectivity over the more conventional detectors. The use of an internal standard is obligatory to compensate for variations in ionization yields due to

matrix interferences. The best internal standards, if available, are stable isotope-labeled analyte standards, because they exactly correct for analyte losses during extraction, cleanup, and derivatization. Assays which use isotopomers of the analytes as internal standards are known as stable isotope dilution assays. Deuterated standards have been synthesized for the main folate vitamers expected to occur in foods, namely $^{2}H_{4}$-labeled THF, 5-methyl-TF, 5-formyl-THF 10-formylfolic acid, and folic acid [108].

Freisleben et al. [97] developed a stable isotope dilution assay using LC–tandem MS for determining endogenous folate in foods. The liquid chromatograph was coupled to an ion-trap mass spectrometer fitted with an electrospray ionization source, which was operated in the positive ion mode using selected reaction monitoring. Identification and quantitation of solutes in the column effluent is based on the mass spectral analysis of the unique fragmentation pattern obtained using collision-induced dissociation of the molecular anion formed in the electrospray. Evidence was presented that, compared to single-stage MS, tandem MS (MS–MS) results in a markedly increased selectivity and therefore requires less elaborate sample cleanup.

21.2.7.4 Methodology

Selected HPLC methods for determining folic acid and the principal naturally occurring folates in foods are summarized in Table 21.10.

Current HPLC systems for separating folates use either reversed-phase chromatography with ion suppression or reversed-phase ion-pair chromatography. The former generally require gradient elution to analyze food folates (Figure 21.23), whereas the latter can separate folates and related compounds isocratically (Figure 21.24). Ion suppression uses an acidic mobile phase, so that the reduced folate analytes are directly fluorescent in the column effluent. The ion-pair technique requires that the analyte be ionized and, for folates, this necessitate a neutral or basic pH. This negates the use of direct fluorometric detection of 5-methyl-THF and THF. Fluorescence detection is possible, however, if the column effluent is acidified. Mobile phases containing phosphate buffers or ion-pairing reagents are not compatible with LC–MS.

A method for determining the most abundant folates in unfortified and fortified cereal-grain products involved tri-enzyme extraction (rat plasma conjugase, α-amylase, and protease), affinity chromatography, and reversed-phase HPLC with UV diode array detection [95]. The trienzyme treatment produced extracts that were clearer and less viscous than extracts obtained with conjugase alone. The extracts passed through the affinity columns more quickly and left less starchy residues on the top of the column. The affinity chromatography concentrated the extracts

TABLE 21.10

HPLC Methods Used for the Determination of Folate Compounds in Food

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Reversed-phase chromatography						
Infant formulas and liquid medical nutritional diets fortified with folic acid	Dilute sample with water. Sterilize by autoclaving at 100°C for 5 min. Incubate with buffered (pH 8.4) papain + bacterial protease enzyme solution at 40°C for 1 h with continuous shaking, filter	Automated inline column system using column switching *Preliminary separation*: Bio Series SAX 89 × 6.2 mm Column temperature 25°C *Analytical separation*: Zorbax RX C8 (octyl) 250 × 4.6 mm Column temperature 25°C	Solvent A: 0.02 *M* sodium acetate + 0.02 *M* Na_2SO_4, pH 5.3; solvent B: solvent A/MeCN, 64:36. *Preliminary separation*: use Solvent A. *Analytical separation*: MeCN gradient	Folic acid	UV 345 nm	[109]
Milk	Place milk sample in centrifuge tube, add sodium ascorbate to yield 1%. Place tube in a boiling water bath for 15 min, cool in ice. Add human plasma conjugase containing MCE,	LiChrospher 100 RP-18 5 μm 250 × 4 mm Column temperature 27°C	8% MeCN in acetic acid, pH 2.3	THF, 5-CH_3-THF, 5-CHO-THF	Fluorescence ex 310 nm em 352 nm	[110]

	followed by extraction buffer (0.1 *M* phosphate buffer, pH 6.0, containing sodium ascorbate). Incubate at 37°C for 1 h, centrifuge. Cleanup with SAX cartridges					
Beetroots (raw and processed)	*Extraction and deconjugation*: homogenize in 0.1 *M* phosphate buffer (pH 6.0) containing 2% (w/v) sodium ascorbate and 0.1% MCE. Flush tubes with nitrogen, cap, heat in boiling water bath for 10 min. Cool in ice, adjust pH to 4.9. Incubate with hog kidney conjugase at 37°C for 3 h. Heat in boiling water bath for 5 min, cool in ice, centrifuge *Cleanup*: SAX solid-phase extraction	Zorbax SB C_8 (octyl) 5 μm 150 × 4.6 mm	Gradient elution: 6% MeCN in 30 m*M* phosphate buffer (pH 2.3) isocratic for 5 min, then increase MeCN linearly to 25% over 20 min	THF, 5-CH_3-THF, 5-CHO-THF	Fluorescence ex 290 nm em 360 nm	[111]
Various	*Extraction and deconjugation*: homogenize samples with 75 m*M* phosphate buffer (pH 6.0) containing 52 m*M* ascorbic acid and 0.1% MCE. Microwave then heat on a boiling water bath for 10 min. Cool extracts in ice centrifuge, adjust pH to 4.9 with acetic acid. Incubate with	Hypersil ODS 3 μm 150 × 4.6 mm. Column temperature 30°C	Gradient elution with MeCN and 30 m*M* phosphate buffer, pH 2.2. 10% MeCN isocratic for 4 min, increase MeCN linearly to 24% within 8 min,	THF, 5-CH_3-THF, 5-CHO-THF, folic acid (main folates present in foods). 10-CHO-folic acid (oxidation product of	Programmed fluorescence ex 290 nm em 356 nm for THF, 5-CH_3-THF and 5-CHO-THF ex 360 nm em 460 nm for	[91]

(*Table continued*)

TABLE 21.10 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
	hog kidney and chicken pancreas conjugases for 2 h at 37°C. Heat on boiling water bath for 5 min, cool in ice, filter. *Cleanup:* SAX solid-phase extraction		isocratic for 3 min, decrease MeCN back to 10%	10-CHO-THF) could also be determined	10-CHO-folic acid UV 290 nm for folic acid. Detectors connected in series	
Liver (pig, beef, chicken), kidney (beef)	As in preceding entry, except that deconjugation is performed using hog kidney conjugase only	As in preceding entry	As in preceding entry	As in preceding entry and also 5,10-methenyl-THF	As in preceding entry ex 360 nm em 460 nm for 10-CHO-folic acid and 5, 10-methenyl-THF	[112]
Vegetables, fruits, berries	As in preceding entry. Sample extracts rich in starch are incubated with α-amylase before deconjugation, omitting prior centrifugation	As in preceding entry	As in preceding entry	As in preceding entry	As in preceding entry	[92]

Fish, meat, meat products, egg, dairy products	As in entry for various foods	As in preceding entry	As in preceding entry, with slight modification		As in preceding entry but not 5,10-methenyl-THF	[113]
Cereal-grain food products (unfortified and fortified)	*Extraction and deconjugation*: mix with 50 m*M* Hepes/50 m*M* Ches buffer (pH 7.85) containing 2% sodium ascorbate and 10 m*M* MCE, heat on boiling water bath for 10 min, cool in ice, homogenize. Incubate homogenate with rat plasma conjugase and α-amylase for 4 h at 37°C followed by protease for 1 h at 37°C. Heat on boiling water bath for 5 min, cool in ice, centrifuge. Resuspend residue in extraction buffer, recentrifuge *Cleanup and concentration*: affinity chromatography	Ultremex C_{18} 5 μm. 250 × 4.6 mm	Gradient elution: 5% MeCN in 33 m*M* phosphate buffer (pH 2.3) isocratic for 8 min, then increase MeCN linearly to 17.5% over 25 min	5-CH_3-THF, 10-CHO-DHF, 10-CHO-folic acid, 5-CHO-THF, folic acid	UV diode array 280 nm	[95]

(*Table continued*)

TABLE 21.10 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Italian reference diet, chickpeas, beans, salami Milano, Parma ham	*Extraction and deconjugation*: mix with boiling Hepes/Ches buffer, pH 7.85 (diet, chickpeas, beans) or 0.1 *M* phosphate buffer, pH 6.0 (salami, ham). Heat in boiling water bath for 10 min, cool, homogenize, dilute to known volume with extraction buffer, adjust pH to 4.9; incubate with hog kidney conjugase at 37°C for 3 h. For diet, chickpeas and beans, add α-amylase to the conjugase. Adjust pH to 7.0, incubate with protease at 37°C for 1 h. Heat in boiling water bath for 10 min, centrifuge. Resuspend pellet in 0.1 *M* phosphate buffer, recentrifuge *Cleanup and concentration*: affinity chromatography	Hypersil ODS 3 μm 150 × 4.6 mm	Gradient elution: 5% MeCN in 30 m*M* phosphate buffer (pH 2.2) isocratic for 9 min, then increase MeCN linearly to 17% over 30 min	THF, 5-CH_3-THF, 10-CHO-DHF, 10-CHO-folic acid, 5-CHO-THF, folic acid	Programmed fluorescence; ex 290 nm em 356 nm for THF, 5-CH_3-THF, 5-CHO-THF ex 360 nm, em 460 nm for 10-CHO-folic acid UV 290 nm for 10-CHO-DHF, folic acid. Detectors connected in series	[114]

Pig liver, milk powder (enriched with folic acid), mixed vegetables, wholemeal flour	*Extraction and deconjugation*: homogenize in Ches/Hepes buffer (pH 7.85), heat on boiling water bath for 10 min, re-homogenize, cool in ice, adjust to pH 7. Incubate a portion with rat plasma conjugase, α-amylase and aminopeptidase for 4 h at 37°C, heat on boiling water bath for 5 min, cool in ice, centrifuge *Cleanup and concentration*: affinity chromatography	Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm	Gradient elution: 5% MeCN in 33 m*M* phosphate buffer (pH 2.1) isocratic for 3 min, then increase MeCN linearly to 10% over 10 min. Change proportion of MeCN to 5% between 10 and 12 min, to 10% after 12.5 min, and to 5% between 13 and 14 min	THF, 5-CH_3-THF, 5-CHO-THF, folic (main folates present in foods). 10-CHO-folic acid (oxidation product of 10-CHO-THF) could also be determined	Programmed fluorescence; ex 280 nm, em 359 nm for THF and 5-CH_3-THF ex 360 nm, em 460 nm for 10-CHO-folic acid. UV 280 nm for folic acid and 5-CHO-THF. Detectors connected in series	[94]
Frozen peas, frozen spinach, powdered milk, yeast, wheat flour, egg yolk, apples, fresh beef liver and fillet, liquid fruit juice supplemented with folic acid	*Extraction and deconjugation*: mix with phosphate buffer (pH 7), heat on boiling water bath for 10 min (omitted for wheat flour samples), cool, dilute with buffer, centrifuge. Precipitate milk powder proteins by pH adjustment to 4.5, restore to pH 7 after centrifugation. Incubate a portion with chicken pancreas conjugase for 1 h at 37°C. *Conversion of folates to 5-CH_3-THF*: react with sodium borohydride (see main text) *Cleanup and concentration*: affinity chromatography	LiChrospher 100 RP 18 5 μm 250 × 5 mm	Gradient elution: increase MeCN in 50 m*M* phosphate buffer (pH 4.6) from 0 to 10% in 15 min, then to 20% in 5 min and maintain at 20% for 5 min	5-CH_3-THF	Fluorescence; ex 295 nm em 356 nm	[115]

(*Table continued*)

TABLE 21.10 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
CRMs: wholemeal flour, dried milk, mixed vegetables, infant formula *Foods*: orange juice, refined flour, frozen spinach, nonfat dried milk	*Extraction and deconjugation*: mix with extraction buffer [0.1 *M* phosphate (pH 6.0) containing 10 m*M* ascorbic acid, 10 m*M* MCE and 10 mg/l sodium azide]. Incubate with α-amylase at 37°C for 1 h then with protease at 37°C for 3 h. Heat in boiling water bath for 15 min, cool. Incubate with rat plasma conjugase at 37°C overnight. Repeat heating and cooling, centrifuge. *Cleanup:* load the extract onto a SAX/PS-DVB solid-phase extraction column. Wash column with extraction buffer, elute the folates with 6 ml 0.1 *M* phosphate buffer (pH 6.0) containing 1 *M* sodium chloride and 250 ml/l MeCN. Bubble nitrogen through eluate to remove MeCN	Adsorbosphere C18 HS 3 μm 150 × 4.6 mm	Gradient elution: 1% MeCN in 30 m*M* phosphate buffer (pH 2.2) with 10 mg/l sodium azide isocratic for 5 min, then increase MeCN linearly to 20% over 20 min and to 40% over next 5 min	5-CH_3-THF, folic acid	Programmed fluorescence ex 290 nm em 355 nm, gain 10 for 5-CH_3-THF ex 230 nm em 440 nm, gain 11 for postcolumn photolytic product of folic acid	[104]

Fortified breakfast cereals, powdered infant formula (SRM 1846)	Suspend crushed sample in 0.03 m*M* dibasic potassium phosphate containing 0.1% each trifluoroacetic acid and MCE. Add $^{13}C_5$-labeled folic acid (internal standard), homogenize. Heat in boiling water bath for 1 h. Mix an aliquot with ethanol, stand overnight at 4°C, centrifuge. Cleanup with C_{18} solid-phase extraction cartridges	Luna C-18 5 μm (Phenomenex). 150 × 4.6 mm	Gradient of solvent (A) aqueous formic acid (0.1%) and (B) MeCN/H_2O/MeOH, 26:60:14 + 0.1% formic acid	Folic acid	Mass spectrometry: electrospray ionization in negative ion mode using selected ion monitoring. Spray voltage 4.5 kV; capillary temperature, 200°C	[116]
Citrus juices	Add juice to 0.1 *M* dibasic potassium phosphate containing 10 m*M* each ascorbic acid and MCE with 10 mg/l sodium azide. Add $^{13}C_5$-labeled 5-CH_3-THF and folic acid (internal standards), adjust to pH 6.0 with 5% KOH. Add rat plasma conjugase, homogenize, incubate at 37°C for 16 h, centrifuge at 4°C, adjust to pH 3.0 with trifluoro-acetic acid. Cleanup with phenyl solid-phase extraction cartridges	Luna C-18 5 μm (Phenomenex). 150 × 4.6 mm	Gradient of solvent (A) aqueous formic acid (0.1%) and (B) MeCN/H_2O/MeOH, 26:60:14 + 0.1% formic acid	5-CH_3-THF, folic acid	Mass spectrometry: electrospray ionization set to positive ion mode for 5-CH_3-THF and negative ion mode for folic acid using selected ion monitoring. Spray voltage, 4.5 kV; capillary temperature, 200°C	[117]

(*Table continued*)

TABLE 21.10 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Spinach, wheat bread, beef	*Extraction and deconjugation*: freeze with liquid nitrogen, blend with 50 m*M* Hepes/ 50 m*M* Ches buffer (pH 7.85) containing 2% sodium ascorbate and 0.2 *M* MCE. Add $^2H_4$-labeled THF, 5-CH_3-THF, 5-CHO-THF, 10-formylfolic acid, folic acid (internal standards), purge with argon, heat in boiling water bath for 10 min, cool in ice. Incubate with protease at 37°C for 6 h, heat at 100°C for 10 min, cool in ice; incubate with rat plasma conjugase at 37°C overnight, heat at 100°C, cool in ice, centrifuge, filter (0.4 μm) *Cleanup*: strong anion-exchange solid-phase extraction for spinach extract. Affinity chromatography for wheat bread and beef extracts	Aqua C-18 5 μm (Phenomenex). 250 × 4.6 mm	Gradient elution with variable mixtures of aqueous formic acid (0.1%) and MeCN. 7% MeCN isocratic for 9 min, 13 min linear gradient to 13% and a further 4 min linear gradient to 25% MeCN. Increase MeCN to 100% over 4 min then back to 7% for 5 min to equilibrate	THF, 5-CH_3-THF, 10-CHO-folic acid, 5-CHO-THF, folic acid	Mass spectrometry: positive electrospray mode using selected-reaction monitoring. Spray voltage, 5.5 kV; capillary temperature, 200°C; capillary voltage, 24.3 V	[97]

Reversed-phase ion-pair chromatography						
Orange juice, grapefruit juice	*Deconjugation*: centrifuge diluted samples, adjust to pH 5.0. Incubate with hog kidney conjugase at 37°C for 1 h, cool in ice, centrifuge at 2°C *Cleanup*: direct injection of deconjugated sample onto a Nova-PAK C_{18} 4 μm pre-column (75 × 3.9 mm) followed by automatic backflush of the analyte to the analytical column	Zorbax ODS. 250 × 4.6 mm	A. 10% MeOH in phosphate-acetate buffer (pH 5.0) containing 5 m*M* tetrabutyl-ammonium phosphate for pre-column elution B. Similar to A but containing 30% MeOH for backflushing and analysis	5-CH_3-THF	Amperometric: glassy carbon electrode, +0.2 V vs. Ag/AgCl	[118]
Breakfast cereals and beverages enriched with folic acid	Digest homogenized sample with Claradiastase and trypsin for 3 h at 40°C, centrifuge, filter *Cleanup*: DEAE- Sephadex weak anion-exchange column chromatography followed by C_{18} solid-phase extraction. Analyze directly using System 1 or proceed as follows for System 2. Adjust pH of purified sample extract to 4.0–4.5 and derivatize by addition of FMOC-CI	Systems 1 and 2 Nucleosil C_{18} 5 μm	System 1: 0.5% tetrabutyl-ammonium hydrogen sulfate, pH 8.5/MeCN, 85 + 15. System 2: 0.5% tetrabutyl-ammonium hydrogen sulfate/MeOH, 70 + 30	Folic Acid	System 1: UV 300 and 340 nm. System 2: Fluorescence ex 360 nm em 450 nm	[105]

(*Table continued*)

TABLE 21.10 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Fortified cereal products	*Extraction:* stir for 1 h with 0.1 *M* phosphate buffer (pH 8–9) containing 0.05% (w/v) ascorbate, adjust to pH 6.9. Incubate with α-amylase at 65°C for 1 h, heat to 90°C, cool, centrifuge *Cleanup*: dilute test extract with the pH 8–9 phosphate buffer, load the extract onto a preconditioned SAX solid-phase extraction cartridge. Wash cartridge with diluted buffer, elute folic acid with 4 ml of 0.1 *M* sodium acetate (pH 4.5) containing 5% Na_2HPO_4 and 0.05% ascorbic acid	Microsorb-MV C_{18} 3 μm. 100 × 4.6 mm	25% MeOH in aqueous phosphate buffer (pH 6.8) containing 5 m*M* tetrabutyl-ammonium dihydrogen phosphate	Folic acid	UV 280 nm	[119]

Flour, dough, bread	*Extraction and deconjugation*: stir for 1 h in 0.1 *M* phosphate buffer (pH 8–9), adjust to pH 6.6. Incubate with α-amylase at 65°C for 1 h, cool. Incubate with rat plasma conjugase at 37°C for 3 h. Heat in boiling water bath for 5 min, centrifuge *Cleanup* (*for endogenous folates*): as in preceding entry but with 0.1% MCE included in eluting solution	Microsorb-MV C_{18} 3 μm. 150 × 4.6 mm	24% MeOH in aqueous phosphate buffer (pH 6.8) containing 5 m*M* tetrabutyl-ammonium dihydrogen phosphate	THF, 5-CHO-THF, 10-CHO-folic acid, 5-CH_3-THF, folic acid	Post-column adjustment of the mobile phase to pH 2.7–3.0. Programmed fluorescence: ex 290 nm em 350 nm for THF, 5-CH_3-THF and 5-CHO-THF. ex 290 nm em 450 nm for 10-CHO-folic acid. UV 280 nm for folic acid. Detectors connected in series	[120]

Note: MCE, 2-mercaptoethanol; MeOH, methanol; MeCN, acetonitrile; FMOC-CI, chloroformic acid-9-fluorenylmethyl ester.

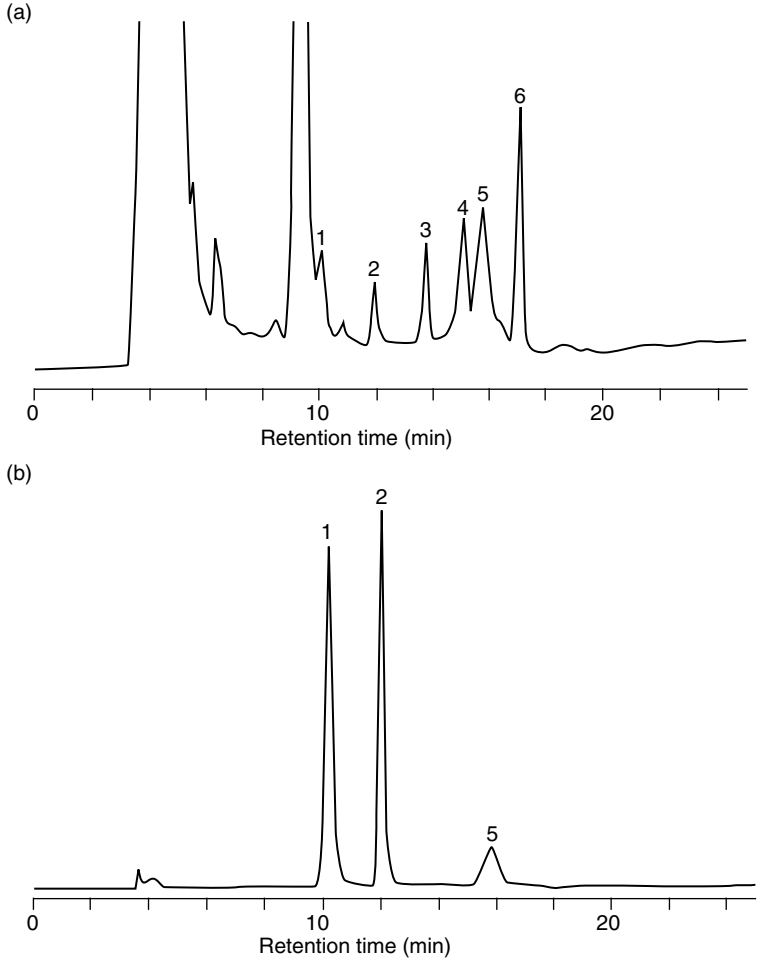

FIGURE 21.23
Gradient reversed-phase HPLC of folates in a standard mixture. Operating parameters as in Table 21.8 [94]. Detection: (a) UV at 280 nm and (b) fluorescence (ex = 280 nm, em = 359 nm). Peaks: (1) THF; (2) 5-methyl-THF; (3) 10-formyl-DHF; (4) 10-formylfolic acid; (5) 5-formyl-THF; (6) folic acid. (Reprinted from Konings, E.J.M. *J. AOAC Int.*, 82, 119–127, Copyright 1999 by AOAC International. With permission.)

10-fold or more, so that even the small amounts of endogenous folates in the cereal-grain products could be quantified with sufficient sensitivity using UV detection and an injection volume of 1 ml. Calibration plots were prepared for 5-methyl-THF, 5-formyl-THF, 10-formylfolic acid, and folic acid. Chromatograms of folates in a standard mixture and in unfortified and fortified white bread are shown in Figure 21.25.

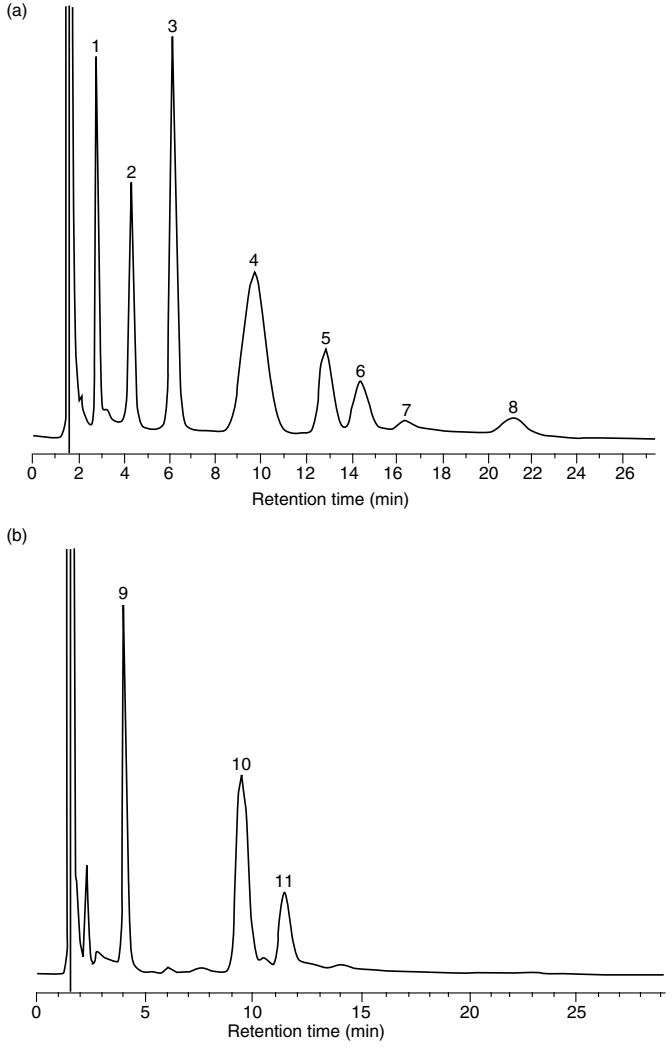

FIGURE 21.24
Isocratic reversed-phase ion-pair HPLC of 11 equimolar folylmonoglutamates and related compounds (100 pmol on column for each molecule). Column (75 × 3.9 mm ID), 4 μm Nova-Pak phenyl; mobile phase, 15% methanol in 50 m*M* phosphate buffer (pH 3.5); flow rate, 0.4 ml/min; UV diode array detection, 280 nm. The main chromatogram (a) shows baseline resolution of (1) *p*-aminobenzoylglutamate; (2) 5-methyl-DHF; (3) 5-methyl-THF; (4) 5-formyl-THF; (5) DHF; (6) pteroylmonoglutamic acid (folic acid); (7) aminopterin; (8) 5,10-methylene-THF. Chromatogram (b) shows folates which coelute with the folates in (A): (9) THF; (10) pteroyltriglutamic acid; (11) 5,10-methenyl-THF. (Reprinted from Lucock, M.D., et al., *Food Chem.*, 53, 329–338, 1995. With permission from Elsevier.)

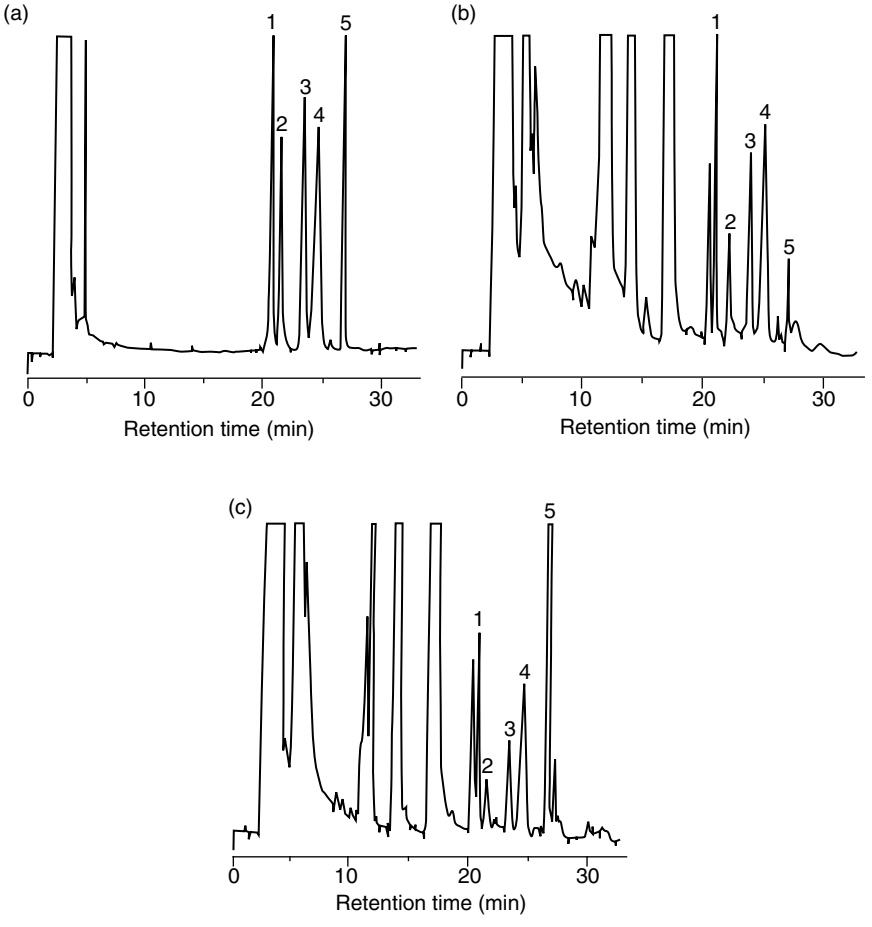

FIGURE 21.25
Gradient reversed-phase HPLC with UV diode array detection of folates in (a) a standard mixture and extracts of (b) unfortified and (c) fortified white bread. Operating parameters as in Table 21.10 [95]. Peaks: (1) 5-methyl-THF; (2) 10-formyl-DHF; (3) 10-formylfolic acid; (4) 5-formyl-THF; (5) folic acid. (Reprinted from Pfeiffer, C.M., Rogers, L.M., and Gregory, J.F. III, *J. Agric. Food Chem.*, 45, 407, 1997, American Chemical Society. With permission)

Ndaw et al. [115] reported a method for determining total folate in foodstuffs in which the folates were deconjugated and chemically converted to 5-methyl-THF before purification by affinity chromatography, and quantitation by reversed-phase HPLC with fluorescence detection. For the conversion, 5 ml of 10 m*M* phosphate buffer (pH 7.4) containing 40% sodium ascorbate, 15 ml of 66 m*M* Tris buffer (pH 7.8), 1 ml of 2-octanol, and 10 ml of sodium borohydride solution (120 g/l) were added sequentially to the deconjugated sample extract. After shaking, the extract was left to

stand for 10 min, then adjusted to pH 7.4 with 5 *M* acetic acid. An 80-μl volume of 37% formaldehyde was added and, after mixing, 10 ml of sodium borohydride solution was cautiously added. The pH was then adjusted to below 1 with concentrated hydrochloric acid. The extract was left to stand for 10 min, then the pH was adjusted to 5 with 5 *M* sodium hydroxide. A 10-ml volume of sodium borohydride solution was once again added cautiously and the extract was left to stand for 20 min. The volume of the extract was finally made up to 100 ml with Tris buffer before filtration through a cellulose acetate filter (0.45 μm). Chromatograms showing the 5-methyl-THF peak in extracts of fruit juice and beef liver are shown in Figure 21.26.

Doherty and Beecher [104] described a robust procedure for determining 5-methyl-THF and folic acid in foods as a means of distinguishing between naturally occurring folate and added synthetic folic acid. Following a tri-enzyme treatment (rat plasma conjugase, α-amylase, and protease), samples were purified by solid-phase extraction using a large-capacity 20-ml column containing a polystyrene–divinylbenzene-based strong anion-exchange resin. An inline photochemical reactor comprising a 10-W short-wavelength mercury lamp installed between the diode array and fluorescence detectors converted folic acid to a fluorescent product. The reversed-phase HPLC system was controlled so that the appropriate segment of the column effluent was subjected to photolysis and the photolytic product of folic acid could be detected at the appropriate fluorescence wavelengths. The authors defined a level of positive identification as the lowest concentration at which the UV spectrum of the analytical peak is indistinguishable from that of the synthetic standard. For both folic acid and 5-methyl-THF, this level was somewhere between 10 and 20 μg/100 g in foods, depending on the matrix. A chromatogram showing 5-methyl-THF and folic acid peaks in an extract of BCR 421 Nonfat Dried Milk is shown in Figure 21.27.

Pawlosky et al. [121] reported procedures for the determination of 5-methyl-THF and folic acid in foods using the HPLC/fluorescence method of Doherty and Beecher [104] that was validated using a stable isotope LC–MS method with electrospray ionization [117]. The mean values for the concentration of the analytes given in Table 21.11 represent independent determinations of the two analytes from 10 food products (five certified reference materials and five frozen foods). The relative standard deviations (RSD) observed in the determination of the concentrations of 5-methyl-THF in the frozen broccoli, oranges, spinach and strawberries and in the BCR 421 (nonfat dried milk) and BCR 485 (mixed vegetables) using either the HPLC or the LC–MS assay were on the order of 10% or lower. This indicates an acceptable precision of the methods. A good agreement between results from the two methods suggest that both methods are accurate. For BCR 485 and BCR 421 the

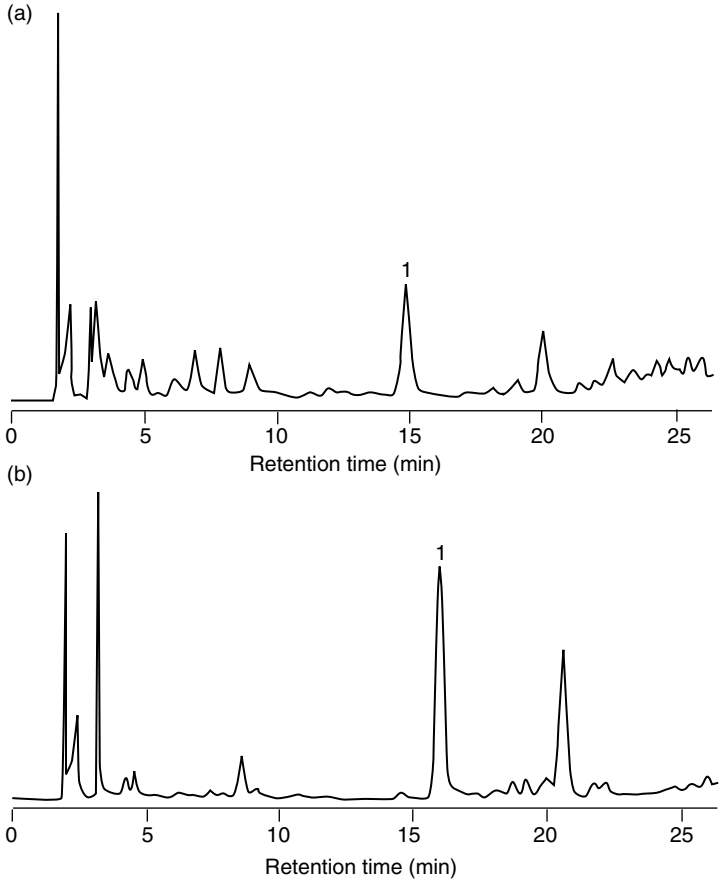

FIGURE 21.26
Gradient reversed-phase HPLC with fluorescence detection of extracts of (A) fruit juice and (B) beef liver after chemical conversion of folates to 5-methyl-THF. Operating parameters as in Table 21.10 [115]. Peak: (1) 5-methyl-THF. (Reprinted from Ndaw, S., *J. Chromatogr. A*, 928, 77–90, 2001. With permission from Elsevier.)

differences in the values of 5-methyl-THF were 1 and 5%, respectively. Corresponding differences for frozen broccoli and frozen strawberries were 17 and 12%, respectively. Greater variations were observed in the determination of 5-methyl-THF in BCR 121 (whole meal flour), BCR 487 (pig liver), SRM 1846 (infant formula), and frozen potatoes. The two methods yielded similar RSDs (ca. 25%) for the analysis of whole meal flour, suggesting that the low concentration of the analyte and an inherent substrate variability contributed to the lower precision. The formation of an emulsion during the extraction phase of the analysis may have

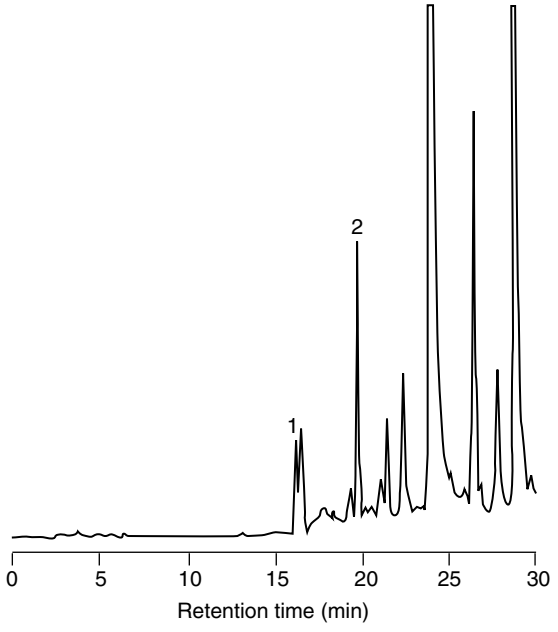

FIGURE 21.27
Gradient reversed-phase HPLC with programmed fluorescence detection of folates in an extract of BCR 421 Non-Fat Dried Milk. Operating parameters as in Table 21.10 [104]. Peaks: (1) 5-methyl-THF; (2) folic acid (detected as post-column photolytic product). (Reprinted with permission from Doherty, R.F. and Beecher, G.R., *J. Agric. Food Chem.*, 51, 354, 2003, American Chemical Society. With permission.)

contributed to the highly variable 5-methyl-THF values in pig liver (47 and 20% RSD). Only three certified reference materials (nonfat dried milk, infant formula, and pig liver) contained measurable quantities of folic acid that could be detected by both assays. For nonfat dried milk and pig liver, the differences in the values of folic acid between methods were 3 and 12%, respectively. Although the RSDs associated with the determination of folic acid in infant formula were similar in both assays (8 and 9%), the difference in the values between methods was significant (19%).

Freisleben et al. [97] also performed a direct method comparison between stable isotope LC–MS and reversed-phase HPLC with fluorometric detection. After deconjugation, spinach extracts were subjected to solid-phase extraction cleanup using strong anion-exchange cartridges, and wheat bread and beef extracts were purified by affinity chromatography. The LC–MS chromatograms were almost devoid of background interference and revealed the presence of 5-formyl-THF in the spinach sample (Figure 21.28). In the HPLC chromatogram, 5-formyl-THF was

TABLE 21.11

Concentrations of 5-Methyl-THF and Folic Acid from Five Certified Reference Materials and Five Frozen Foods as Determined by HPLC Analysis with Fluorescence Detection and Stable-Isotope LC–MS Analysis

	5-Methyl-THF						Folic Acid					
	HPLC			LC–MS			HPLC			LC–MS		
	μg/100 g ($n = 3$)	SD	RSD (%)	μg/100 g ($n = 3$)	SD	RSD (%)	μg/100 g ($n = 3$)	SD	RSD (%)	μg/100 g ($n = 3$)	SD	RSD (%)
Whole meal flour (BCR 121)	6.45	1.77	27	3.82	0.95	25	ND	—	—	ND	—	—
Nonfat dried milk (BCR 421)	14.87	1.08	7	15.74	0.7	5	66.95	2.61	4	64.85	7.21	11
Mixed vegetables (BCR 485)	195.82	6.61	3	197.7	6.65	5	ND	—	—	0.2	0.07	34
Pig liver (BCR 487)	168.93	79.38	47	410.54	81.28	20	80.89	9.9	12	70.81	12.1	17
Infant formula [SRM 1846 (NIST)]	3.11	0.24	8	4.79	0.88	18	88.02	7.08	8	109.44	9.98	9
Frozen broccoli	27.6	0.57	2	34.45	1.81	5	ND	—	—	ND	—	—
Frozen oranges	20.46	1.79	9	30.32	3.28	11	ND	—	—	ND	—	—
Frozen potatoes	9.76	0.71	7	5.25	0.85	16	ND	—	—	ND	—	—
Frozen spinach	79.58	8.86	11	88.69	6.65	8	ND	—	—	0.58	0.03	5
Frozen strawberries	31.98	3.34	10	27.87	0.87	3	ND	—	—	ND	—	—

Note: ND, not detected.

Source: Pawlosky, R.J., Flanagan, V.P., and Doherty, R.F., *J. Agric. Food Chem.*, 51, 3726, 2003. With permission.

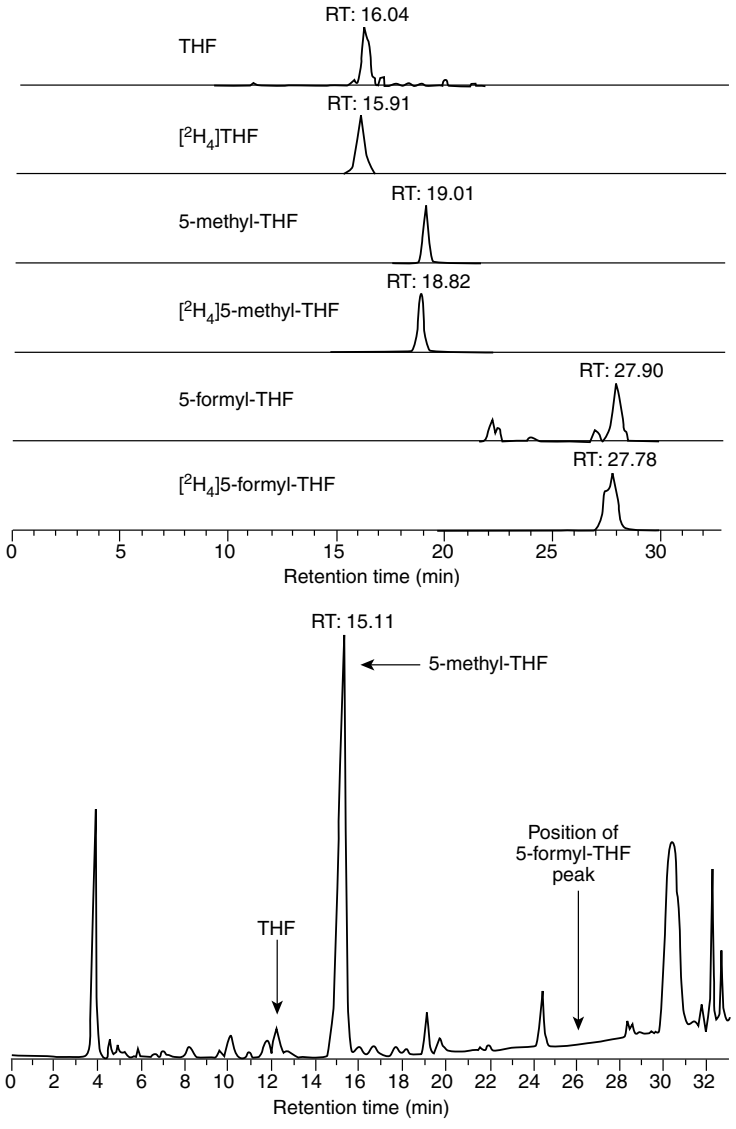

FIGURE 21.28
Gradient reversed-phase HPLC of folate vitamers in a spinach extract. Top: chromatograms of native vitamers and deuterated internal standards detected by mass spectrometry. Operating parameters as in Table 21.10 [97]. Bottom: chromatogram of vitamers detected by fluorescence. Note the absence of 5-formyl-THF peak in bottom chromatogram. (Reprinted from Freisleben, A., Schieberle, P., and Rychlik, M., *Anal. Biochem.*, 315, 247–255, 2003. With permission from Elsevier.)

not detectable at all (Figure 21.28). As is evident from Table 21.12, the amount of folate in spinach is significantly underestimated by HPLC. By applying LC–MS, a 67% higher folate content was acquired from the identical aliquot, even though the HPLC values were recovery corrected. The main reason for the differing total folate content is the severe difference in the value for 5-methyl-THF (77.2 μg/100 g) determined by HPLC compared to the LC–MS value (137 μg/100 g). The quantitative results of wheat bread (Table 21.13) show a significantly different distribution of folate vitamers. Beef extracts contained only low interference in the HPLC chromatogram, and the very low total folate value (0.7 μg/100 g) was quite close to the LC–MS value (1.2 μg/100 g). These comparisons demonstrate the superior correction for losses by using isotopically labeled standards, and suggest that the HPLC/fluorescence assay produces reliable results only if the matrix is not too demanding.

Osseyi et al. [120] used a reversed-phase ion-pair HPLC procedure to determine the stability and distribution of added folic acid and some endogenous folates during breadmaking. Samples of flour and freeze-dried dough and bread were treated with α-amylase and rat plasma conjugase, then purified by strong anion-exchange solid-phase extraction. HPLC analysis was performed isocratically with postcolumn acidification of the mobile phase. Detection modes were UV (280 nm) for folic acid and fluorescence (excitation: 290 nm, emission: 350 nm) for THF, 5-methyl-THF, and 5-formyl-THF. The fluorescence of 10-formylfolic acid was measured at 290 nm excitation and 450 nm emission. A chromatogram of added folic acid in bread is shown in Figure 21.29. Chromatograms of endogenous folates in bread are shown in Figure 21.30. For

TABLE 21.12

Folate Content[a] in Spinach Measured by Stable Isotope LC–MS and HPLC/Fluorescence in μg/100 g Fresh Weight

	LC–MS	HPLC
5-Methyl-THF	137.0 (3.0)	77.2 (18.5)[b]
5-Formyl-THF	12.0 (7.1)	ND
THF	15.5 (5.6)	21.5 (11.5)[c]
Sum (as folic acid)	159.2	95.5

Source: From Freisleben, A., Schieberle, P., and Rychlik, M., *Anal. Biochem.*, 315, 247, 2003. With permission.

Note: ND, not detected.

[a]Inter-assay coefficient of variation ($n = 3$) is given in parentheses.

[b]Corrected for the recovery value of 43.8 ± 2.1%.

[c]Corrected for the recovery value of 56.3 ± 3.0%.

TABLE 21.13

Folate Content[a] in Wheat Bread Measured by Stable Isotope LC–MS and HPLC/Fluorescence in μg/100 g Fresh Weight

	LC–MS	HPLC
5-Methyl-THF	3.3 (18.8)	5.5 (2.5)[b]
5-Formyl-THF	8.7 (13.3)	ND
THF	6.4 (1.4)	1.0 (22.0)[c]
10-Formylfolic acid	2.3 (11.1)	10.6 (2.4)[d]
Folic acid	NQ	ND
Sum (as folic acid)	19.8	16.2

Source: From Freisleben, A., Schieberle, P., and Rychlik, M., *Anal. Biochem.*, 315, 247, 2003. With permission.
Note: ND, not detected; NQ, below quantifiable levels.
[a]Inter-assay coefficient of variation ($n = 3$) is given in parentheses.
[b]Corrected for the recovery value of 84.2 ± 3.3%.
[c]Corrected for the recovery value of 65.4 ± 2.4%.

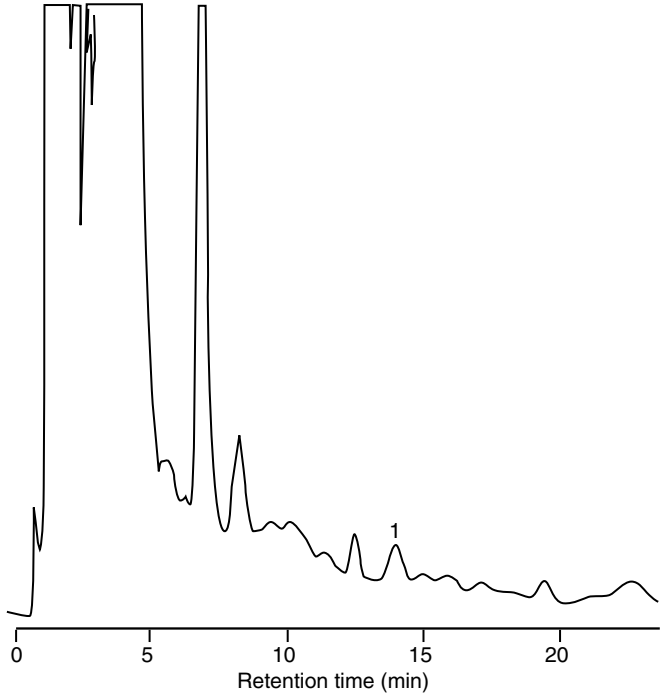

FIGURE 21.29
Isocratic reversed-phase ion-pair HPLC with UV detection of added folic acid in an extract of bread. Operating parameters as in Table 21.10 [120]. Peak 1, folic acid. (From Osseyi, E.S., Wehling, R.L., and Albrecht, J.A., *Cereal Chem.*, 78, 375, 2001. With permission.)

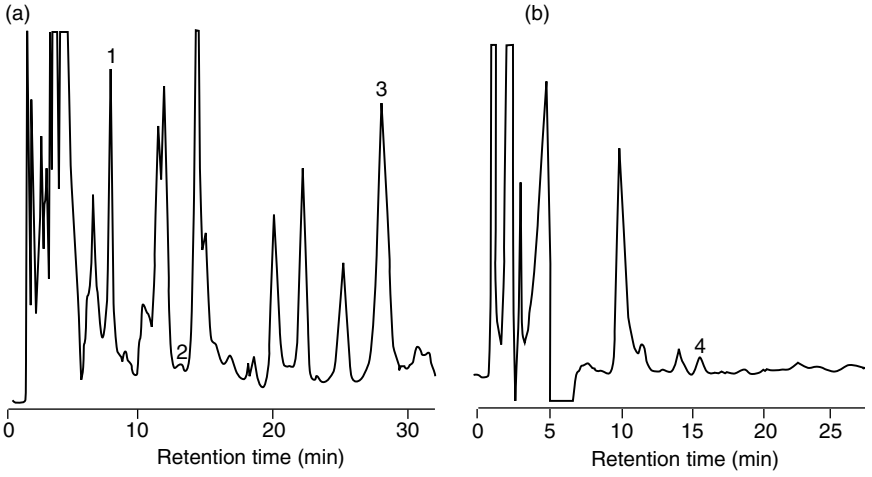

FIGURE 21.30
Isocratic reversed-phase ion-pair HPLC with fluorescence detection of endogenous folates in an extract of bread. (a) Excitation/emission wavelengths, 290/350 nm; (b) excitation/emission wavelengths, 290/450 nm. Operating parameters as in Table 21.10 [120]. Peaks: (1) THF; (2) 5-formyl-THF; (3) 5-methyl-THF; (4) 10-formylfolic acid. (From Osseyi, E.S., Wehling, R.L., and Albrecht, J.A., *Cereal Chem.*, 78, 375, 2001. With permission.)

the determination of added folic acid in fortified cereal products [119], samples were treated with α-amylase and purified using strong anion-exchange cartridges. Quantitation was achieved by UV absorption at 280 nm. The solid-phase extraction was effective in eliminating some substances that eluted in the vicinity of folic acid, and often resulted in baseline resolution of folic acid. Chromatograms of extracts of unfortified and fortified cornflakes (Figure 21.31) show that folic acid can be resolved from other sample components.

21.2.8 Vitamin B_{12}

HPLC lacks the sensitivity to measure the extremely small concentrations of naturally occurring vitamin B_{12} in foods and almost all data have been obtained using microbiological assays. Choi et al. [122] determined cyanocobalamin in fortified foods using a column switching technique that incorporated three different types of reversed-phase column for pre-separation, focusing and analysis (Table 21.14). The limit of quantification ($5 \times$ limit of detection) with an injection volume of 400 μl was 1 μg/100 g, therefore the method could be applicable for foods fortified with cyanocobalamin above this level. Comparison of

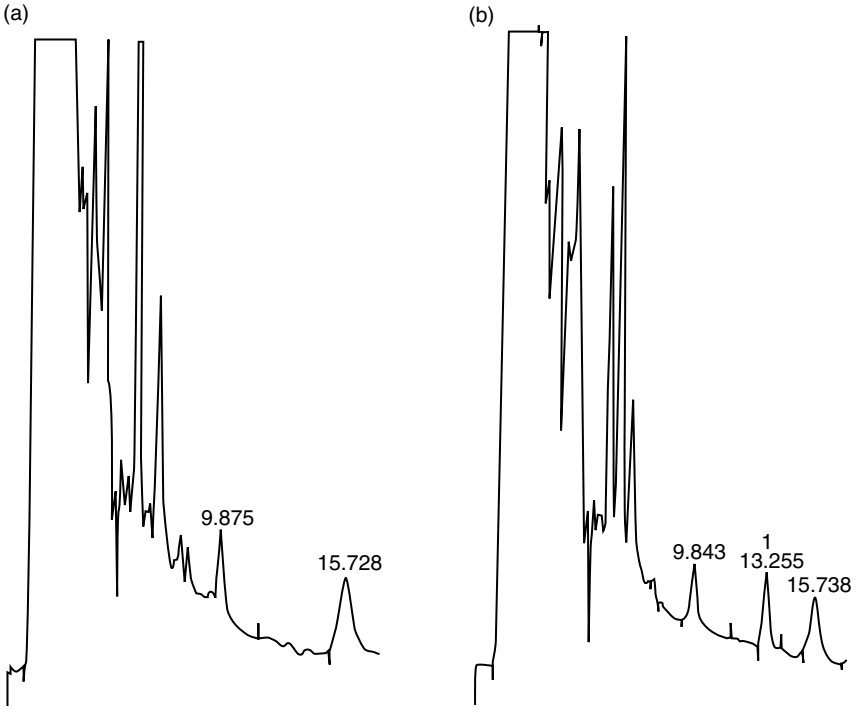

FIGURE 21.31
Isocratic reversed-phase ion-pair HPLC with UV detection of an extract of (a) unfortified cornflake and (b) cornflakes fortified with folic acid. Operating parameters as in Table 21.10 [119]. Peak 1, folic acid. (Reprinted from Osseyi, E.S., Wehling, R.L., and Albrecht, J.A., *J. Chromatogr. A*, 826, 235–240, 1998. With permission from Elsevier.)

results obtained by HPLC and microbiological assay showed no significant difference ($P < 0.01$) with $r = 0.9781$. The HPLC method could be used in monitoring and quality control of infant formulas and nutritional supplements.

21.2.9 Vitamin C

21.2.9.1 Detection

Ascorbic acid displays an absorption spectrum which is attributable to its enediol structure. The λ_{max} depends on the ionic state of the molecule and is therefore influenced by the pH of the medium. For the nondissociated form (pH 2, with dilute hydrochloric acid), the $A_{1\,cm}^{1\%}$ at λ_{max} 245 nm is 695; for the monodissociated form (pH 6.4, with phosphate

TABLE 21.14

HPLC Method Used for the Determination of Vitamin B_{12} in Food

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Reversed-phase chromatography						
Infant formula, nutritional supplement (all fortified)	Suspend in 5 m*M* KH_2PO_4, sonicate for 10 min, dilute with KH_2PO_4, centrifuge. Collect middle layer, centrifuge. Transfer middle layer to test tube, add chloroform to remove lipids, centrifuge. Collect aqueous layer, centrifuge, filter (0.45 μm)	Pre-separation column: Capcellpak MF C_8 (octyl) 5 μm 150 × 4.6 mm. Focusing column: Capcellpak MG C_{18} 5 μm 35 × 2.0 mm Analytical column: Capcellpak UG C_{18} 5 μm 250 × 1.5 mm. Column temperature 40°C	Solvent A: 5 m*M* KH_2PO_4/MeOH, 80:20. Solvent B: 5 m*M* KH_2PO_4	Cyanocobalamin	Visible 550 nm	[122]

Note: MeOH, methanol.

buffer) the $A_{1\ cm}^{1\%}$ at λ_{max} 265 nm is 940 [123]. Dehydroascorbic acid has a relatively very weak absorptivity with λ_{max} at 223 nm [124]. Spectra of ascorbic acid and dehydroascorbic acid are shown in Figure 21.32. The spectra of erythorbic acid at pH 2.0 and 6.0 are virtually identical to those of ascorbic acid.

Ascorbic acid can be detected directly by either absorbance or electrochemical monitoring, and fluorescence detection can be utilized after chemical derivatization to a fluorescent compound. A photodiode array detector combines absorbance detection with the ability to assess peak purity. The detection of dehydroascorbic acid, however, poses a problem as the molar absorptivity of this compound is relatively very weak and it is electrochemically inactive.

Some HPLC methods for analyzing fruits, fruit juices and vegetables have employed dual absorbance detection, ascorbic acid being measured at 254 nm and dehydroascorbic acid at 210 nm [125,126] or 214 nm [127]. This dual detector technique is simple to perform and does not involve the analytes in any chemical reactions. However, the accuracy of

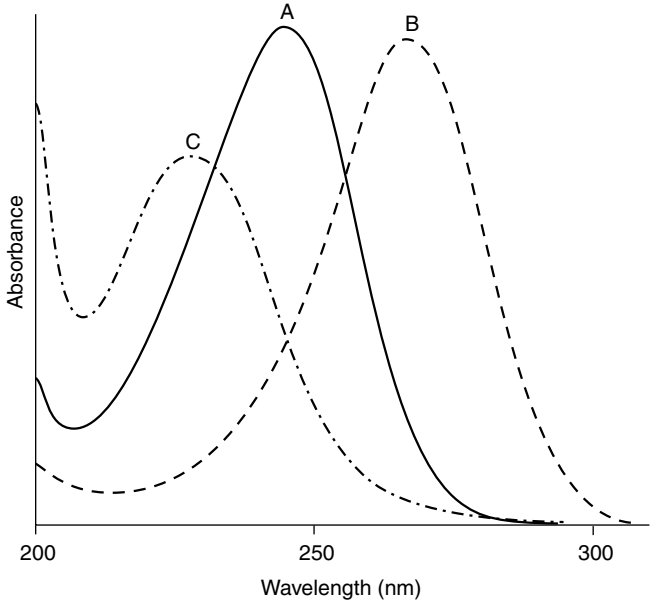

FIGURE 21.32
UV absorption spectra of L-ascorbic acid (16 μg/ml) in 0.1 *M* phosphate buffer at pH 2.0 (solid line) and pH 6.0 (broken line). L-Dehydroascorbic acid (1 mg/ml) in water at pH 3.1 (discontinuous line). For L-ascorbic acid, λ_{max} of peak A = 244 nm; B = 265 nm. For L-dehydroascorbic acid, λ_{max} of peak C = 223 nm.

dehydroascorbic acid measurement is expected to be poor at such low detection wavelengths.

A more popular approach is to reduce the dehydroascorbic acid in the sample extract to ascorbic acid by precolumn reaction with either homocysteine, dithiothreitol or L-cysteine, and then to determine the ascorbic acid (representing total vitamin C) by HPLC using absorbance detection. The dehydroascorbic acid value can be calculated by subtracting the ascorbic acid value (obtained without chemical reduction) from the total vitamin C value, but the error will be appreciable if the concentration of dehydroascorbic acid is very low relative to that of ascorbic acid. Sufficient time must be allowed for the reduction reaction to proceed at ambient temperature. Bates [128] reported that homocysteine in a pH 4.0 medium does not reduce dehydroascorbic acid to ascorbic acid, but conversion is virtually 100% at pH 7.5.

Ziegler et al. [129] determined dehydroascorbic acid directly, in addition to ascorbic acid, by separating the two compounds using reversed-phase HPLC with ion suppression, and reducing dehydroascorbic acid to ascorbic acid with dithiothreitol in a postcolumn inline reaction system. This technique enabled the dehydroascorbic acid to be measured photometrically (267 nm) at the same sensitivity as ascorbic acid with a detection limit of 1.4 ng per 10 μl injection volume (signal-to-noise ratio of 2:1).

Doner and Hicks [130] used a refractive index monitor to detect dehydroascorbic acid, dehydroerythorbic acid, diketogulonic acid and diketogluconic acid, which were all transparent at the absorbance wavelength (268 nm) employed for detecting ascorbic acid and erythorbic acid. Refractive index detection is nonselective and only moderately sensitive, and hence it has no utility in the accurate measurement of the small amounts of dehydroascorbic acid and other oxidation products that might be present in foods.

Hydrodynamic voltammograms for ascorbic acid show that the peak current reaches a plateau at +0.8 V using either a platinum or glassy carbon electrode [131]. An oxidative potential of +0.8 V is relatively high, and the many compounds with lower or very close potentials that are present in biological materials will also be detected. Thus, the specificity of the assay relies largely on the chromatographic separation. In practice, a potential of +0.6 V is frequently chosen, which, though not as sensitive as +0.8 V, results in improved selectivity. The minimum detection limit of an amperometric detector towards ascorbic acid, using an oxidation potential of +0.6 V, was reported to be 0.3 ng on-column (based on a signal-to-noise ratio of 3:1) and was an order of magnitude lower than the limit obtained with a photometric detector used for comparison [132]. The higher sensitivity

gives electrochemical detection the advantage over absorbance detection for the analysis of foods that contain relatively small amounts of vitamin C.

Amperometry with a conventional single applied potential is prone to errors resulting from the loss of detector sensitivity that occurs as the working electrode becomes contaminated. These errors can be minimized by injecting standard solution alternately with sample injections [133]. Operating a standard amperometric cell connected to a pulsed amperometric detection system enabled cleaning cycles to be continually applied, and maintained the integrity of the working electrode [134].

Dehydroascorbic acid, being electrochemically inactive, must be reduced to ascorbic acid if it is to be taken into account using electrochemical detection. These circumstances also apply to erythorbic acid and its oxidation product, dehydroerythorbic acid. The use of dithiothreitol in a postcolumn reaction system was unsuccessful with electrochemical detection, owing to a high background signal and electrode poisoning that resulted from the excess dithiothreitol [129]. Karp et al. [132] solved this problem by reacting the excess dithiothreitol with *N*-ethylmaleimide, thereby permitting the electrochemical detection of both ascorbic acid and dehydroascorbic acid with a detection limit of 0.3 ng per 20 μl injection volume. Karp et al. [135] later employed a solid-state postcolumn reactor packed with sulfhydryl cellulose, which eliminated the cumbersome equipment needed to introduce reagents into the effluent stream and completely avoided the problem of electrode poisoning.

Another approach is to oxidize the ascorbic acid in the sample extract to dehydroascorbic acid and then to react the latter compound with *o*-phenylenediamine dihydrochloride (OPDA) to form the fluorescent quinoxaline derivative 3-(1,2-dihydroxyethyl)furo[3,4-*b*]quinoxaline-1-one (DFQ). HPLC analysis of the resulting solution, with fluorescence detection of the DFQ, gives the total vitamin C value. Omitting the oxidation step allows dehydroascorbic acid to be determined independently as DFQ. Zapata and Dufour [136] used absorbance detection for the simultaneous determination of ascorbic acid and DFQ after precolumn derivatization. The detector wavelength was initially set at 348 nm and, after elution of DFQ, it was manually changed to 261 nm for ascorbic acid and erythorbic acid (internal standard) detection. Speek et al. [137] used ascorbate oxidase to oxidize the ascorbic acid in buffered extracts of citrus juices and vegetables. Stability studies showed that the quinoxaline derivatives deteriorated rapidly under daylight exposure and in the dark at 22°C, whereas they were fairly stable in the dark at +4 or −20°C for at least 12 h. Kacem et al. [138] employed tandem absorbance and fluorometric detection for the simultaneous determination of ascorbic acid and

dehydroascorbic acid, respectively, after postcolumn derivatization of the latter compound to DFQ.

Most HPLC systems can separate ascorbic acid and erythorbic acid, together with their respective oxidation products. This provides the opportunity for postcolumn inline chemistry to form the quinoxaline derivatives. There are two ways in which this can be done. First, oxidation of ascorbic acid and erythorbic acid in the column effluent, followed by derivatization with OPDA and fluorescent detection of all four peaks [139,140]. Second, derivatization without prior oxidation. In this case, ascorbic acid and erythorbic acid are detected by UV monitoring and the quinoxaline derivatives of dehydroascorbic acid and dehydroerythorbic acid are detected fluorometrically [141].

Bognár and Daood [140] used a short column of activated charcoal for postcolumn oxidation and optimized the derivatization conditions. The use of acetic and metaphosphoric acid solutions to prepare the OPDA reagent resulted in much higher detector responses and reagent stability than those obtained when water was the solvent for the fluorogenic reagent. The pH of the mixed streams was close to the optimum value (ca. 3) recommended by Zapata and Dufour [136] for the condensation of dehydroascorbic acid and OPDA to form DFQ. The optimum concentration of OPDA was found to be 0.4% and the maximum response was approached at a reaction temperature of 60°C. The detection limit was 0.1 mg of each analyte per 100 g of food.

21.2.9.2 Methodology

Selected HPLC methods for determining vitamin C in foods are summarized in Table 21.15. The chromatographic modes employed are weak anion exchange, ion exclusion, reversed-phase with and without ion suppression, and reversed-phase ion-pair chromatography. Techniques that separate ascorbic acid from erythorbic acid (D-isoascorbic acid) provide the opportunity to use erythorbic acid as an internal standard for the quantification of ascorbic acid, provided it is shown that erythorbic acid is absent in the food sample extract presented for analysis. Erythorbic acid does not occur naturally in foods, as demonstrated by Bognár and Daood [140].

Aminopropyl-bonded stationary phases operated in the WAX mode have the potential to separate, isocratically, not only ascorbic acid from its oxidation products, dehydroascorbic acid and diketogulonic acid, but, simultaneously, erythorbic acid from its analogous oxidation products, dehydroerythorbic acid and diketogluconic acid. Since there is no significant difference in pK_a values of the ionizable protons between ascorbic acid and erythorbic acid, one must assume that factors other than degree of ionization affect interactions with the stationary phase.

TABLE 21.15

HPLC Methods Used for the Determination of Vitamin C in Food

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Weak anion exchange chromatography						
Cooked sausages	Stir ground sample with 5% HPO_3 and 0.1 mg/ml EDTA, dilute with water, centrifuge, filter	Spherisorb NH_2 5 μm 250 × 4 mm Column temperature 35°C	MeCN/0.02 *M* KH_2PO_4 buffer, pH 3.6 (60:40)	AA	UV 248 nm	[142]
Fruits, vegetables	Homogenize with 3% citric acid, dilute to volume, filter. Pass through C_{18} solid-phase extraction cartridge	μBondapak-NH_2 10 μm 300 × 3.9 mm	MeCN/0.01 *M* $NH_4H_2PO_4$ buffer, pH 4.3 (70:30)	AA, DHAA	Dual UV; 214 nm (DHAA), 254 nm (AA)	[127]
Tropical root crops	Homogenize with 5% HPO_3, filter	μBondapak-NH_2 10 μm in Z-module cartridge	MeCN/0.005 *M* KH_2PO_4 buffer, pH 4.6 (70:30)	AA, DHAA	Dual UV; 210 nm (DHAA), 254 nm (AA)	[126]
Nonfat dry milk	Dissolve sample in water containing 1 mg/ml dithiothreitol, stir, add 12% HPO_3, stir, add 2 ml MeCN, stir, centrifuge	μBondapak-NH_2 10 μm 300 × 7.8 mm Column temperature 21°C	MeCN/0.005 *M* KH_2PO_4 (70 + 30) + 0.125 ml/l of mercaptoethanol	AA (representing total vitamin C)	UV 268 nm	[143]

(*Table continued*)

TABLE 21.15 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Frozen apples, potato products, concentrated fruit and vegetable juices, natural and artificially flavored drink mixes, Hi-C drinks, cured meat products	*For AA and EA*: suspend blended sample in water (or EtOH for potato products), filter. Pass through C_{18} solid-phase extraction cartridge *For total vitamin C and isovitamin C*: treat filtrate with dithiothreitol. Pass through C_{18} solid-phase extraction cartridge	LiChrosorb-NH_2 250 × 4.6 mm	MeCN/0.05 *M* KH_2PO_4 buffer, pH 5.95 (75:25)	AA and EA (representing total vitamin C and isovitamin C, respectively, if sample extract is treated with dithiothreitol	UV 268 nm	[144]
Fresh apple and potato	*For AA*: blend with 2.5% HPO_3 and mobile phase, filter through Celite, refilter. Pass through C_{18} solid-phase extraction cartridge *For total vitamin C*: treat second filtrate with dithiothreitol after adjusting to pH 6. Pass through C_{18} solid-phase extraction cartridge	Dynamax-60A-NH_2 8 μm (Rainin) 250 × 4.6 mm	MeCN/0.05 *M* KH_2PO_4, 75:25	AA (representing total vitamin C if sample extract is reacted with, dithiothreitol), ascorbic acid-2-phosphate	UV 254 nm	[145]

Orange juice	*For AA*: dilute juice with extraction solution (1% citric acid containing 0.05% EDTA·2Na in 50% MeOH), filter *For total vitamin C*: add 0.8% DL-homocysteine, adjust pH to 7 with 45% K_2HPO_4. Dilute with extraction solution, filter	Zorbax-NH_2 5 μm 250 × 4.6 mm	0.25% KH_2PO_4 buffer (pH 3.5)/MeOH, 60:40	AA (representing total vitamin C if sample extract is reacted with DL-homocysteine)	UV 244 nm	[146]
Fresh fruit and vegetables, fruit drinks	Homogenize with 3% HPO_3, filter. Pass through C_{18} solid-phase extraction cartridge	NH_2-phase (Alltech)	MeCN/0.05 *M* KH_2PO_4 buffer, pH 5.9 (75:25)	AA, DHAA	AA: UV 254 nm DHAA: fluorescence (filters) after postcolumn reaction with OPDA to form DFQ; detectors connected in series	[138]
Fruits, vegetables, milk, liver, sausage	Homogenize with extracting solution (8% acetic acid and 3% HPO_3 in water). Add 2 vol of MeCN per 1 vol of extract, filter	Grom-Sil-NH_2 5 μm 250 × 4.6 mm	MeCN/0.05 *M* $NH_4H_2PO_4$ buffer, pH 5 (70:30)	AA, DHAA	Fluorescence; ex 350 nm, em 420 nm. Postcolumn derivatization (oxidation of AA to DHAA using short column of activated charcoal followed by reaction with OPDA to form DFQ)	[140]

(*Table continued*)

TABLE 21.15 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Ion-exclusion chromatography						
Citrus fruit juices, fruits, vegetables	Dilute or extract sample with 0.05% EDTA·2Na in 0.1 *M* H_2SO_4, filter or centrifuge	Aminex HPX-87 SCX sulfonated PS-DVB resin 300 × 7.8 mm	4.5 m*M* H_2SO_4	AA	UV 245 nm	[147]
Potatoes	Homogenize with 95% EtOH/water containing 0.2% conc. H_2SO_4 (60:40), filter	Aminex HPX-87 SCX sulfonated PS-DVB resin, 9 μm 300 × 7.8 mm	9 m*M* H_2SO_4	AA + oxalic, citric, malic and fumaric acids	UV 260 nm (AA) UV 210 nm (other organic acids)	[148]
Fruits	Blend with 95% ethanol, vacuum-filter through paper, wash residue twice with 80% ethanol. Evaporate ethanolic extract to dryness at 50°C in the dark, dissolve residue in 1 ml 0.1 *M* H_2SO_4 containing 0.05% EDTA. Load onto C_{18} solid-phase extraction cartridge and elute with the same solution	Ion-300 10 μm 300 × 7.8 mm	4.25 m*M* H_2SO_4	AA, citric acid, malic acid	UV 245 nm for AA only UV 195 nm for AA, citric acid and malic acid	[149]

Strawberries (fresh), potatoes (raw and cooked)	Blend samples with 62.5 m*M* HPO_3, centrifuge, filter, dilute with 62.5 mM HPO_3 *For AA*: analyze extract directly *For total vitamin C:* add 30 m*M* DL-homocysteine to extract, adjust pH to 6.8–7.0 with 2.6 *M* K_2HPO_4. After 30 min, stop reduction by addition of 6.25 *M* HPO_3	Aminex HPX-87H sulfonated PS-DVB resin. 300 × 7.8 mm	4.5 m*M* H_2SO_4	AA (representing total vitamin C if sample extract is reacted with DL-homocysteine)	UV 245 nm	[150]
Milk (fluid and powdered)	Powdered milk: dilute with 1% HPO_3, centrifuge. Fluid milk: centrifuge	SCX sulfonated PS-DVB resin, H^+-form (Bio-Rad) 9 μm 100 × 7.8 mm	1 m*M* H_2SO_4	AA	Amperometric: glassy carbon electrode, + 0.70 vs. Ag/AgCl	[151]
Fresh fruits, fruit juices, dry mixed fruits, potatoes	Homogenize solid samples or dilute liquid samples with 0.005 *M* H_2SO_4 (pH 2) + 0.01 *M* mannitol, centrifuge, filter (0.45 μm) or (for some fresh fruits) pass through C_{18} solid-phase extraction cartridge	Polypore H 10 μm (Bioanalytical Systems). 100 × 4.6 mm	10 m*M* H_2SO_4 + 10 m*M* mannitol	AA, free sulfite	Amperometric: dual-electrode; free sulfite: Pt, + 0.70 V. AA: glassy carbon, + 0.80 V vs. Ag/AgCl	[131]
Beer	Degas, filter, dilute with 20 m*M* $Na_2HPO_4^-$ 10 m*M* D-mannitol buffer, pH 9	HPICE-AS1	10 m*M* H_2SO_4 containing 4% MeCN	AA, free sulfite	Pulsed amperometric: Pt electrode, + 0.70 V vs. Ag/AgCl	[134]

(*Table continued*)

TABLE 21.15 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Fruits, fruit drinks, vegetables	Dilute or homogenize with 0.02 *M* H_2SO_4, centrifuge, filter (0.45 μm). Mix with 0.05 *M* phosphate buffer (pH 7) and 0.01 *M* dithiothreitol	SCX sulfonated PS-DVB resin (Wescan) 100 × 4.6 mm	20 m*M* H_2SO_4	AA (representing total vitamin C)	Amperometric: Pt electrode, + 0.60 V or +0.80 V vs. Ag/AgCl	[152]
Reversed-phase chromatography						
Honey	Dissolve in 0.25% HPO_3, filter	Shimadzu C-18-ODS 5 μm 250 × 4.6 mm	MeOH/H_2O (15:85) adjusted to pH 2.5 with HPO_3	AA	UV 254 nm	[153]
Fruits, vegetables	Extract with 6% HPO_3 containing 1 μ*M* EDTA and 0.1 μ*M* diethylthiocarbamate	μBondapak C_{18} 10 μm in a 100 × 8 mm radial compression module (Waters)	1.5% $NH_4H_2PO_4$ buffer, pH 3	AA	UV 254 nm	[154]
Fruits, vegetables, juices	Homogenize with 0.2 *M* phosphate buffer (pH 2.0), extract with 3% HPO_3, centrifuge	PLRP-S 100 Å, 5 μm 250 × 4.6 mm	1.8% THF and 0.3% HPO_3 in water	AA	UV 244 nm	[155]
Citrus juices	Mix with 5% HPO_3, centrifuge. Pass through C_{18} solid-phase extraction cartridge, add quinic acid as internal standard	Zorbax ODS 250 × 4.6 mm	2% KH_2PO_4 buffer, pH 2.4	AA, quinic acid (internal standard)	UV 245 nm	[156]

Fresh citrus fruit juices	Dilute with mobile phase, filter	PLRP-S 100 Å, 5 μm (Polymer Laboratories) 250 × 4.6 mm	0.2 *M* NaH_2PO_4 buffer, pH 2.14	AA, EA	UV 244 nm (AA). UV 220 nm (AA, EA)	[157,158]
Fruits, vegetables, commercial orange juices	Blend sample with 0.05 *N* H_3PO_4, centrifuge, dilute. Pass through C_{18} solid-phase extraction cartridge, filter (0.45 μm)	Spheri-5 RP-18 5 μm 110 × 4.6 mm and two Polypore H organic acid columns (110 × 4.6 and 220 × 4.6 mm) connected in series	2% KH_2PO_4 buffer, pH 2.3	AA (separated from DHAA and oxalic, malic, succinic and citric acids)	UV 260 nm	[159]
Fresh fruits	Shake homogenized sample with extracting solution (8% acetic acid and 3% HPO_3 in water), dilute with the same solution, filter	Spherisorb ODS-2 5 μm. 250 × 4.6 mm	Water adjusted to pH 2.2 with H_2SO_4	AA (separated from quinic, malic and citric acids)	254 nm for AA. 214 nm for other organic acids	[160]
Green beans	Stir homogenized sample with 4.5% HPO_3 for 15 min, filter, dilute with 4.5% HPO_3, filter (0.22 μm)	Spherisorb ODS-2 5 μm. 250 × 1.6 mm	Water adjusted to pH 2.2 with H_2SO_4	AA (separated from oxalic, malic, citric, succinic and fumaric acids)	245 nm for AA. 215 nm for other organic acids	[161]
Fruit juices, fruit drinks	React 1 ml of sample with 1 ml 50 m*M* dithiothreitol, centrifuge through 0.22 μm filter	J'sphere ODS-H80 4 μm 250 × 4.6 mm	2% acetic acid in water (ca. pH 2.5)	AA (representing total vitamin C)	UV 243 nm (photodiode array detector)	[162]

(*Table continued*)

TABLE 21.15 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Fruit juices, selected processed foods	Juices: dilute with water, add dithiothreitol Fruits and processed foods: blend with water, add dithiothreitol and (if sample is proteinaceous) 5% TCA; filter or centrifuge	C_{18} (unspecified) 250 × 4 mm	0.5% KH_2PO_4 buffer (pH 2.5) containing 0.1% dithiothreitol	AA (representing total vitamin C)	UV 254 nm	[163]
Fruits, vegetables	*For AA:* homogenize with water *For total vitamin C*: homogenize with water containing dithiothreitol	C_{18} 5 μm (unspecified) 250 × 4 mm	0.2 *M* KH_2PO_4 buffer (pH 2.4)	AA (representing total vitamin C if sample extract is reacted with dithiothreitol)	UV 210 and 254 nm (photodiode array detector)	[164]
Rose hips	Extract with 1% HPO_3. Pass through C_{18} solid-phase extraction cartridge	LiChrosorb RP-18 5 μm 250 × 4 mm	0.25% HPO_3	AA, DHAA	UV 267 nm for both peaks after post-column reduction of DHAA to AA with dithiothreitol at 50°C	[129]
Orange juice	Centrifuge, mix 1:1 with 6% HPO_3, filter	Brownlee RP-18 5 μm 220 × 4.6 mm (or 100 × 4.6 mm)	2% $NH_4H_2PO_4$, pH 2.8	AA	Amperometric: glassy carbon electrode, +0.6 V vs. Ag/AgCl	[165]

Fruit juices	Dilute juice with water, filter *For AA*: add α-methyl-L-DOPA (internal standard) and 2% HPO_3. *For total vitamin C*: add internal standard and L-cysteine diluted in 0.01 *M* phosphate buffer, pH 6.8 (allow 15 min reaction time)	Inertsil ODS-2 5 μm 150 × 4.6 mm	0.1 *M* KH_2PO_4 buffer (pH 3) containing 1 m*M* EDTA·2Na	AA (representing total vitamin C if sample extract is reacted with L-cysteine), α-methyl-L-DOPA (internal standard)	Amperometric: +0.3 V vs. Ag/AgCl	[166]
Processed meats, skimmed and condensed milk, cherry cheesecake, chocolate diet product, fruit preservative	Homogenize in cold 17% HPO_3, centrifuge, filter *For AA and EA*: dilute with phosphate buffer (pH 9.8) to give a final pH of 7.1. After 30 min at 25°C, further dilute with cold 0.85% HPO_3. Dilute an aliquot with mobile phase buffer *For total vitamin C and isovitamin C*: dilute with 1% homocysteine in phosphate buffer (pH 9.8) to give a final pH of 7.1. Then as for AA and EA	PLRP-S 100 Å, 5 μm 250 × 4.6 mm. Two columns connected in series	0.02 *M* $NaH_2PO_4·H_2O$ containing 0.17% HPO_3 (final pH 2.2)	AA, EA (representing total vitamin C and isovitamin C, respectively, if sample extract is reacted with homocysteine)	Amperometric: glassy carbon electrode, +0.7 V vs. Ag/AgCl	[167]

(*Table continued*)

TABLE 21.15 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Citrus juices, vegetables	*For total vitamin C and total isovitamin C:* blend with 0.3 *M* TCA, dilute to volume, filter. Add 4.5 *M* acetate buffer (pH 6.2), incubate at 37°C for 5 min with ascorbate oxidase. Add 0.1% OPDA; react at 37°C for 30 min. *For DHAA and DHEA*: as above, but omit enzymatic oxidation	Hypersil-ODS 3 μm 125 × 4.6 mm	0.08 *M* KH_2PO_4/MeOH, 80:20 (final pH 7.8)	Quinoxaline derivatives of DHAA and DHEA (representing total vitamin C and isovitamin C, respectively, if sample extract is reacted with ascorbate oxidase)	Fluorescence ex 365 nm (filter) em 418 nm (filter)	[137]
Infant formulas, potato crisps, breakfast cereals (fortified), canned vegetables (sweetcorn, green beans, potatoes), cranberry juice, cranapple juice	Mix homogenized or blended sample with extraction solution (3% HPO_3 in 8% acetic acid), centrifuge. Re-extract, centrifuge *Conversion to DHAA*: shake with acid-washed Norit (carbon), filter. Clarify by mixing with sodium acetate solution and MeOH, filter or centrifuge *Derivatization*: add OPDA solution, dilute with HPLC mobile phase (allow 1 h reaction time)	μBondapak C_{18} 10 μm 300 × 3.9 mm	MeOH/H_2O, 55 + 45	DFQ, representing total vitamin C	Fluorescence ex 350 nm em 430 nm	[168]

Solid and liquid foods	Add extraction solution (30 g HPO_3, 0.5 g EDTA·2Na and 80 ml glacial acetic acid/l plus EA (internal standard), blend *For nonfat and low starch samples*: centrifuge, filter *For low-starch samples with fat*: add hexane, vortex-mix, centrifuge, filter the aqueous layer *For high-starch samples*: add *n*-butanol, vortex-mix, centrifuge, filter the aqueous layer	Two PLRP-S 100 Å, 5 μm columns in series. 150 × 4.6 mm, 250 × 4.6 mm	0.2 *M* NaH_2PO_4 adjusted to pH 2.14 with H_3PO_4	DHEA, DHAA, AA, EA	Fluorescence ex 350 nm em 430 nm Postcolumn derivatization (oxidation of AA to DHAA and EA to DHEA using $CuCl_2$ followed by reaction with OPDA to form the quinoxaline derivatives)	[139]
Reversed-phase ion-pair chromatography						
Wine and beer	Membrane-filter (0.2 μm)	Nucleosil 120 C_{18} 7 μm. 250 × 4 mm	0.5% aqueous MeOH containing 0.05 *M* acetate buffer (pH 5.4) and 5 m*M* *n*-octylamine	AA, EA	UV 266 nm	[169]
Processed meat	Homogenize with 0.5% HPO_3, centrifuge. Dilute with 0.5% HPO_3, add MeOH to give a 4% final concentration	Hypersil ODS 5 μm, 250 × 4 mm	H_2O/MeOH/ acetate buffer/ 1,5-dimethyl-hexylamine (945 + 40 + 15 + 1.5)	AA, EA	UV 254 nm	[170]

(*Table continued*)

TABLE 21.15 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Fresh tomatoes	Homogenize with 6% HPO_3, mix aliquot of slurry with MeOH, add 0.05% pentanophenone (internal standard), centrifuge, dilute with MeOH containing 0.0015 *M* pyrogallol	Vydac 201-HS C_{18} 10 μm 250 × 4.6 mm	MeOH/H_2O/MeCN (60 + 40 + 1) containing 0.5 m*M* tridecyl ammonium formate, adjusted to pH 4.25	AA, pentanophenone (internal standard)	UV 247 nm	[171]
Fresh fruits and vegetables	Mix macerated sample with 2% HPO_3, shake, filter	Spherisorb ODS-2 10 μm 250 × 4.6 mm	0.01 *M* KH_2PO_4/ 20% tetrabutyl-ammonium hydroxide/ MeOH (970 + 1 + 30) adjusted to pH 2.75 using 85% H_3PO_4	AA (separated from succinic, citric, fumaric, and gallic acids)	UV 244 nm	[172]

Fruits, fruit juice products, vegetables	Dilute or extract with 6% HPO_3/EtOH (10:90), filter (paper), centrifuge, filter (0.45 μm membrane). Pass through C_{18} solid-phase extraction cartridge	Spherisorb ODS 10 μm 250 × 4 mm	(1) 0.1 *M* phosphate buffer (pH 4.2) containing 5 m*M* tetrabutyl-ammonium hydroxide (2) 0.2 *M* phosphate buffer (pH 4.2) containing 10 m*M* hexadecyl-trimethyl-ammonium bromide/MeOH (1 + 1)	AA	UV 254 nm	[173]
Fresh fruits	Homogenize with 0.4% HPO_3, dilute to volume, filter (0.2 μm). Pass through C_{18} solid-phase extraction cartridge (if colored)	μBondapak C_{18} 10 μm 300 × 3.9 mm	0.1 *M* KH_2PO_4/MeOH (9:1) containing 5 m*M* cetyltrimethyl-ammonium bromide	AA	UV 265 nm	[174]
Beer	Ultrasonic degassing	μBondapak C_{18} 10 μm 300 × 3.9 mm	Citrate buffer (pH 4.4) containing 0.05 m*M* EDTA and 1 m*M* *N*-methyldode-cylamine	AA	Amperometric: glassy carbon electrode, + 0.60 V vs. Ag/AgCl	[175]

(*Table continued*)

TABLE 21.15 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Beer	Ultrasonic degassing	Zorbax ODS 250 × 4.6 mm	0.1 *M* acetate buffer (pH 5.5) containing 200 mg/l EDTA·2Na and 1 m*M* octylamine	AA or EA	Amperometric: glassy carbon electrode, +0.60 V vs. Ag/AgCl	[176]
Orange juice, milk	Dilute with cold 0.05 *M* perchloric acid, centrifuge	Ultrasphere ODS 5 μm 250 × 4.6 mm	0.04 *M* acetate buffer containing 1 m*M* decylamine and 15% MeOH	AA, EA	Amperometric: carbon paste electrode, +0.7 V vs. Ag/AgCl	[177]
Fruits, infant foods, milk	Fruits, infant foods: extract with 3% HPO_3–8% acetic acid; centrifuge, dilute to volume with cold 0.05 *M* perchloric acid. Milk: dilute to volume with cold 0.05 *M* perchloric acid, centrifuge	LiChrosorb RP-18 150 × 4.6 mm	0.08 *M* acetate buffer containing 1 m*M* tridecylamine and 15% MeOH (final pH 4.5)	AA	Amperometric: carbon paste electrode, +0.7 V vs. Ag/AgCl	[178]
Bread	Grind freeze-dried bread, extract with 3% HPO_3, centrifuge, dilute to volume with cold 0.05 *M* perchloric acid	Alltech C-18 5 μm 250 × 4.6 mm Column temperature 25°C	0.08 *M* acetate buffer (pH 4.2) containing 0.1 m*M* EDTA· 2Na and 1 m*M* octyltriethyl-ammonium phosphate	AA	Amperometric: glassy carbon electrode, +0.72 V vs. Ag/AgCl	[179]

Cured meats	Grind with 5% HPO_3 containing 0.1 mg/ml EDTA·2Na, centrifuge, dilute with mobile phase	Ultrasphere ODS 250 × 4.6 mm. Column temperature 30°C	0.04 *M* acetate buffer containing 5 m*M* tetrabutyl-ammonium phosphate and 0.2 mg/ml EDTA·2Na (final pH 5.25)	AA, EA	Amperometric: glassy carbon electrode, +0.6 V vs. Ag/AgCl	[180]
Fresh oranges, canned orange juice, orange soft drink, beer, fresh kiwi fruit, fresh tomato	Dilute homogenized sample with MeOH/H_2O (5:95), add EA (internal standard), centrifuge. Adjust pH to between 2.20 and 2.45 if necessary, bring final volume to 10 ml. Pass through C_{18} solid-phase extraction cartridge. Discard first 5 ml of eluate and collect the next 3 ml. Add OPDA solution, filter (0.22 μm). Allow 37 min reaction time before HPLC analysis	μBondapak C_{18} 10 μm 300 × 3.9 mm	MeOH/H_2O (5:95) containing 5 m*M* hexadecyl-trimethyl-ammonium bromide (cetrimide) and 0.05 *M* KH_2PO_4 (final pH 4.59)	DFQ (representing DHAA), AA, EA (internal standard)	UV 348 nm for DFQ and 261 nm for AA and EA	[136]

(*Table continued*)

TABLE 21.15 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Fruits, vegetables	Place sample and extracting solution (1% HPO_3 and 0.5% oxalic acid adjusted to pH 2) into blender, bubble carbon dioxide through the suspension, blend for 5 min. Dilute with extraction solution, centrifuge, filter (0.45 μm)	Jupiter C_{18} 5 μm 250 × 4.6 mm	66 m*M* phosphate–20 m*M* acetate buffer containing 2.3 m*M* dodecyltrimethylammonium chloride and 2.5 m*M* Na_2EDTA (final pH 4.50)	DHEA, DHAA, AA, EA	AA and EA: UV 247 nm DHAA and DHEA: Fluorescence ex 350 nm em 430 nm; after post-column reaction with OPDA to form the quinoxaline derivatives; detectors connected in series	[141]

AA, ascorbic acid: DHAA, dehydroascorbic acid; EA, erythorbic acid (isoascorbic acid); DHEA, dehydroerythorbic acid; HPO_3, metaphosphoric acid; EDTA, ethylenediaminetetraacetic acid; TCA, trichloroacetic acid; MeOH, methanol; EtOH, ethanol; MeCN, acetonitrile; OPDA, *o*-phenylenediamine: DFQ, quinoxaline derivative.

One possible mechanism is hydrogen bonding between hydroxyl protons in the compounds with the neutral amino group in the stationary phase [130].

In order to obtain a quantitative recovery of dehydroascorbic acid using WAX chromatography and absorbance detection, it is essential to neutralize sample extracts to pH 6 if metaphosphoric acid is used as the extractant. Recovery of dehydroascorbic acid was only 14% in non-neutralized extracts (pH 2.9) and 45% at pH 4 [145]. Wimalasiri and Wills [127] also experienced interference from metaphosphoric acid in the measurement of dehydroascorbic acid at 214 nm and used, instead, citric acid as the extractant for fruit and vegetables.

In WAX chromatographic methods for the determination of total vitamin C and/or total isovitamin C after precolumn reduction of the oxidized forms, the reducing agent dithiothreitol elutes near the solvent front, and hence does not interfere in the determination [143]. An alternative reducing agent, homocysteine, produced a strongly absorbing doublet peak in front of the ascorbic acid peak, and so would mask the erythorbic acid peak. Tuan et al. [144] reported that phenylalanine could be used as an internal standard for determining total vitamin C in fruit and vegetable samples, but could not be used for determining total isovitamin C in meat products, owing to the presence of interfering peaks.

The column life of aminopropyl-bonded phases is somewhat limited compared to that of ODS-bonded reversed phases, owing to the loss of the amine function when it reacts irreversibly with the carbonyl groups of reducing sugars or other compounds to form Schiff bases. Bui-Nguyên [181] reported that the resolution of ascorbic acid and erythorbic acid decreased after 50–100 injections using an aminopropyl-bonded column. The resolution was not significantly improved by increasing the percentage of methanol in the mobile phase (decreasing the polarity), but increasing the column temperature from 35 to 60°C restored the separation.

Kim and Kim [182] employed ion exclusion chromatography with amperometric detection to determine ascorbic acid in foods and beverages. The method was rapid (total analysis time: 5 min), sensitive ($<$0.1 mg/100 g food sample detectable), and selective. A useful feature of this technique was its ability to determine simultaneously the sulfite present in fruit juices and instant mashed potato. The results obtained for these commodities using the AOAC titrimetric method were higher than those obtained chromatographically, due to the interference by free sulfite. The method was extended to include the determination of dehydroascorbic acid after its reduction to ascorbic acid with dithiothreitol [152]. Dithiothreitol is electrically active, but its

peak appears well after the ascorbic acid peak in the chromatogram, so it does not hinder the quantitative determination. A disadavantage of ion exclusion chromatography is that ascorbic acid and erythorbic acid are inseparable.

Vanderslice and Higgs [139] used two PLRP-S copolymer reversed-phase columns connected in series and a pH 2.14 phosphate buffer mobile phase to separate dehydroerythorbic acid, dehydroascorbic acid, ascorbic acid, and erythorbic acid. Postcolumn inline chemistry involving oxidation and reaction with OPDA enabled total vitamin C and total isovitamin C in solid and liquid foods to be determined using fluorometric detection. The PLRP-S column packing was compatible with metaphosphoric acid present in the injected samples and in the mobile phase, providing a very stable system. Brause et al. [163] incorporated dithiothreitol into the mobile phase to maintain vitamin C in the reduced form.

Reversed-phase chromatography at around neutral pH (i.e., without ion suppression) can be utilized if DFQ is chromatographed after precolumn oxidation of ascorbic acid and derivatization [137,168].

In reversed-phase ion-pair chromatography, buffering of the mobile phase to pH 4.2 (the pK_{a1} of ascorbic acid) results in a sharp, well defined ascorbic acid peak [173]. The tetrabutylammonium salts that are generally popular as ion-pairing agents cause a relatively poor retention of ascorbic acid, but they have been used without an organic modifier to determine ascorbic acid and erythorbic acid in meat products [180]. Carnevale [183] reported that tetrabutylammonium salts are unsuitable for the analysis of citrus juices, because the relatively high concentration of ionizable material (mostly citric acid) exceeds their ion pairing capacity, resulting in loss of resolution, distortion of peak shapes, and changes in elution volume.

Most published ion-pair chromatographic methods for determining vitamin C and related compounds use relatively hydrophobic ion pairing agents, representing a variety of primary, secondary, or tertiary amines. Some mobile phases include methanol as an organic modifier. Kall and Andersen [141] used dodecyltrimethylammonium chloride in a method for separating ascorbic acid and erythorbic acid together with their corresponding dehydro forms. Ascorbic acid and erythorbic acid were detected by UV absorbance (Figure 21.33) and the dehydro forms were detected fluorometrically after postcolumn derivatization (Figure 21.34). Some ion pairing agents, namely *n*-octylamine, *n*-decylamine, hexadecyltrimethylammonium bromide [180], and tridecylammonium formate [154,184] reportedly form precipitates (presumably phosphate complexes) when mixed with dilute (5 or 6%) metaphosphoric acid used in the extraction procedure. This precipitation causes the

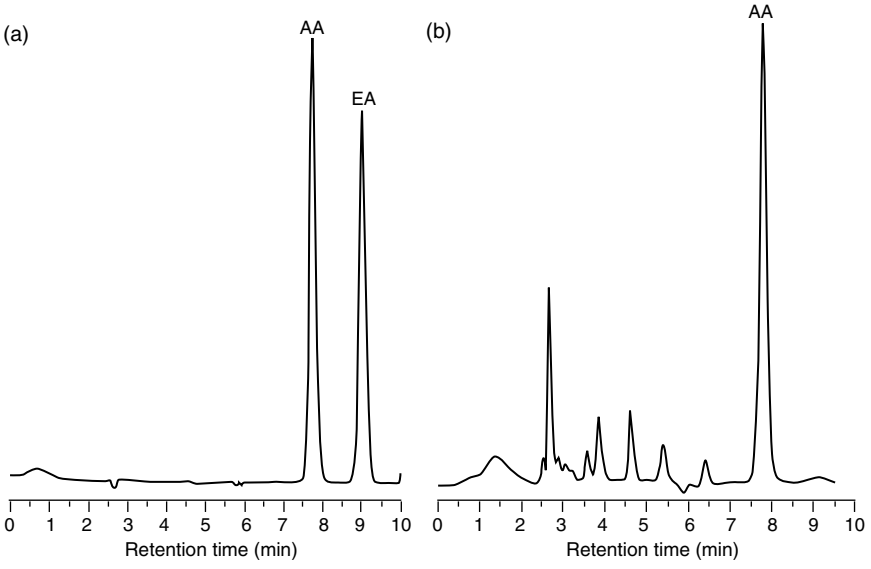

FIGURE 21.33
Reversed-phase ion-pair HPLC with UV detection of ascorbic acid (AA) and erythorbic acid (EA) in (a) standard solution and (b) extract of frozen green beans. Operating parameters as in Table 21.14 [141]. (Reprinted from Kall, M.A. and Andersen, C., *J. Chromatogr. B*, 730, 101–111, 1999. With permission from Elsevier.)

problem of pressure build-up within the HPLC system, which is not prevented by the addition of methanol to the mobile phase.

21.2.10 Multiple Vitamin Analyses

Methods discussed here are those that chromatograph two or more vitamins from the same sample extract, either simultaneously or successively. Selected methods are summarized in Table 21.16. Naturally occurring thiamin and riboflavin can be coextracted and determined together, but analyses of other combinations of vitamins are usually restricted to synthetic vitamins added to enrich certain foods.

21.2.10.1 Thiamin and Riboflavin

Autoclaving food samples with dilute mineral acid, followed by enzymatic hydrolysis, liberate both thiamin and riboflavin, enabling these vitamins to be determined simultaneously or successively from one

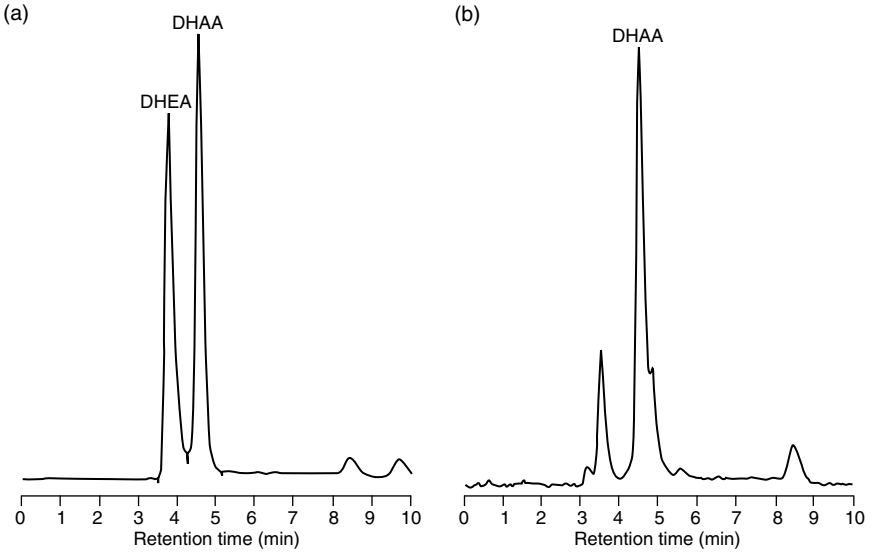

FIGURE 21.34
Reversed-phase ion-pair HPLC with fluorescence detection of dehydroascorbic acid (DHAA) and dehydroerythorbic acid (DHEA) as their quinoxaline derivatives after post-column reaction with OPDA. (a) Standard solution, (b) extract of frozen green beans. Operating parameters as in Table 21.14 [141]. (Reprinted from Kall, M.A. and Andersen, C., *J. Chromatogr. B*, 730, 101–111, 1999. With permission from Elsevier.)

sample hydrolysate. The diastatic enzymes used to dephosphorylate vitamins B_1 and B_2 also have protease activity. Ndaw et al. [197] showed that the acid hydrolysis step was superfluous for the determination of these vitamins if the protease activity of the selected diastase was sufficiently high.

For the analysis of fortified foods, such as breakfast cereals, thiamin and riboflavin can be determined simultaneously, and without the need for derivatization, by reversed-phase ion-pair chromatography and photometric detection at 254 nm [16]. Most applications, however, use fluorescence detection as a means of obtaining adequate sensitivity for thiamin after conversion to its fluorescent oxidation product, thiochrome, by reaction with alkaline potassium hexacyanoferrate(III) [$K_3Fe(CN)_6$]. The chromatographic mode employed depends on whether the derivatization is carried out precolumn or postcolumn. Precolumn derivatization provides the opportunity for detecting thiochrome and riboflavin simultaneously using simple reversed-phase chromatography. This technique has been carried out using two

TABLE 21.16

HPLC Methods for Determining Multiple Water-Soluble Vitamins in Foods

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Thiamin and riboflavin						
Reversed-phase chromatography						
Raw and cooked potatoes	Reflux sample with 0.1 *N* HCl for 30 min, cool to below 50°C. Incubate with buffered (pH 4.5) Takadiastase at 45–50°C for 2 h. Cool, dilute to volume with water, filter *Derivatization*: oxidize thiamin to thiochrome with alkaline $K_3Fe(CN)_6$	μBondapak C_{18} 10 μm 300 × 3.9 mm	Water/MeOH (70:30)	Thiochrome Riboflavin (separate chromatograms)	Fluorescence *Thiochrome*: ex 365 nm; em 435 nm, *Riboflavin*: ex 450 nm; em 510 nm	[185]
Dietetic foods	Digest sample with 0.1 *N* HCl at 95–100°C for 30 min, cool. Adjust pH to 4.5, incubate with β-amylase and Takadiastase at 37°C overnight. Dilute to volume with water, filter (0.2 μm). *For riboflavin*: analyze filtrate directly *For thiamin*: oxidize to thiochrome by treating aliquot of filtrate with alkaline $K_3Fe(CN)_6$. Pass solution through C_{18}	μBondapak C_{18} 10 μm 300 × 3.9 mm	50 m*M* acetate buffer (pH 4.5)/MeOH (40:60)	Thiochrome Riboflavin (separate chromatograms)	Fluorescence *Thiochrome*: ex 366 nm; em 435 nm; *Riboflavin*: ex 422 nm, em 522 nm	[21]

(*Table continued*)

TABLE 21.16 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
	solid-phase extraction cartridge, wash cartridge with 50 m*M* sodium acetate, elute thiochrome with MeOH/H_2O (60:40)					
Soy products	Hydrate dry samples heat at 90°C for 30 min: Adjust pH to 2, autoclave at 20 psi for 15 min. Adjust pH of cooled extract to 4.5, centrifuge, filter *For riboflavin*: analyze filtrate directly *For thiamin*: oxidize to thiochrome by treating aliquot of filtrate with alkaline $K_3Fe(CN)_6$ and neutralizing with conc. H_3PO_4 after 45 sec	Ultrasphere C_{18} 5 μm, 150 × 4.6 mm	10 m*M* acetate buffer (pH 5.5)/ MeCN (87:13)	Thiochrome Riboflavin (separate chromato-grams)	Fluorescence *Thiochrome*: filters *Riboflavin*: ex 436 nm, em 535 nm (filters)	[20]
All food types	Autoclave sample with 0.1 *N* HCl at 121°C for 30 min, cool, adjust pH to 4.5. Incubate with β-amylase and Takadiastase at 37°C overnight. Add 50% TCA to precipitate soluble proteins, dilute to volume with water, filter *For riboflavin*: analyze filtrate directly *For thiamin*: oxidize to thiochrome by treating aliquot of filtrate with alkaline $K_3Fe(CN)_6$ and then neutralizing with conc. H_3PO_4.	Novapak C_{18} 4 μm 150 × 3.9 mm Column temperature 30°C	50 m*M* phosphate buffer (pH 7.0)/ MeOH (70:30)	Thiochrome Riboflavin (separate chromato-grams)	Fluorescence *Thiochrome*: ex 366 nm; em 435 nm *Riboflavin*: ex 445 nm, em 522 nm	[186]

	Cleanup and concentration: pass the oxidized extract through C_{18} solid-phase extraction cartridge, wash cartridge with 50 m*M* phosphate buffer (pH 7.0), elute thiochrome with MeOH/phosphate buffer (80:20)					
All food types	Autoclave sample with 0.1 *N* HCl at 121°C for 15 min, cool, adjust pH to 4.0–4.5. Incubate with Claradiastase at 50°C for 3 h. Precipitate proteins by heating at 90°C with 50% (w/v) TCA for 15 min, cool, adjust pH to 3.5, dilute with water, filter through paper *Derivatization*: oxidize thiamin to thiochrome by treating filtrate with alkaline $K_3Fe(CN)_6$, neutralize with conc. H_3PO_4. *Cleanup and concentration*: pass the oxidized extract through C_{18} solid-phase extraction cartridge, wash cartridge sequentially with 5 m*M* phosphate buffer (pH 7.0) and 5 m*M* phosphate buffer/MeOH (95:5). Elute vitamins with 50% aqueous MeOH, dilute to volume	Radial-PAK C_{18} 10 μm 100 × 8 mm Column temperature 30°C	5 m*M* phosphate buffer (pH 7.0)/ MeOH (65:35)	Thiochrome Riboflavin (separate chromato-grams)	Fluorescence *Thiochrome*: ex 360 nm, em 425 nm, *Riboflavin*: ex 440 nm, em 520 nm	[187]

(*Table continued*)

TABLE 21.16 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Cereals	Autoclave sample with 0.1 *N* HCl at 121°C for 30 min, cool, adjust pH to 4.5, dilute with water, filter through paper *Derivatization*: oxidize thiamin to thiochrome with alkaline $K_3Fe(CN)_6$ *Cleanup and concentration*: pass the oxidized extract through C_{18} solid-phase extraction cartridge, wash cartridge with 5 m*M* ammonium acetate (pH 5.0), elute vitamins with MeOH/5 m*M* ammonium acetate (60:40)	μBondapak C_{18} 10 μm 300 × 3.9 mm	5 m*M* acetate buffer (pH 5.0)/MeOH (72:28)	Thiochrome and riboflavin (in same chromatogram)	Programmed fluorescence *Thiochrome*: ex 370 nm, em 435 nm *Riboflavin*: ex 370 nm, em 520 nm	[188]
Peas, beans, liver, skim milk, whole and enriched wheat flour	Autoclave sample with dilute HCl at 121°C for 15 min, cool, adjust pH to 4.0–4.5. Incubate with Takadiastase at 48°C for 3 h. Precipitate proteins by heating at 95–100°C with 50% (w/v) TCA for 15 min, cool, adjust pH to 3.5, dilute to volume with water, filter	Radial-PAK C_8 (octyl) 10 μm 100 × 8 mm	10 m*M* phosphate buffer (pH 7.0)/MeOH (63:37)	Thiochrome and riboflavin (in same chromatogram)	Dual fluorescence *Thiochrome and riboflavin*: ex 360 nm, em 415 nm (filters). *Riboflavin alone*: ex 450 nm, em 530 nm (filters)	[18]

	Derivatization: oxidize thiamin to thiochrome with alkaline $K_3Fe(CN)_6$, neutralize with conc. H_3PO_4 *Cleanup and concentration*: pass the oxidized extract through C_{18} solid-phase extraction cartridge, wash cartridge sequentially with 10 m*M* phosphate buffer (pH 7.0) and 10 m*M* phosphate buffer/MeOH (95:5). Elute vitamins with 50% aqueous MeOH, dilute to volume					
Cereal products	Autoclave ground samples with 0.1 *N* HCl at 121°C for 30 min, cool, adjust pH to 4.0–4.5. Incubate with Takadiastase at 50°C for 3 h, cool, dilute to volume with water *Derivatization and cleanup/concentration*: as in preceding entry	Radial-PAK C_{18} 10 μm 100 × 8 mm	5 m*M* phosphate buffer (pH 7.0)/MeOH (65:35)	Thiochrome and riboflavin (in same chromatogram)	Fluorescence (filters)	[19]
Reversed-phase ion-pair chromatography						
Cereal products, fortified breakfast, cereals	Autoclave sample with 0.1 *N* HCl at 121°C for 30 min, cool, centrifuge	μBondapak C_{18}10 μm 300 × 3.9 mm	10 m*M* phosphate buffer (pH 7.0)/MeCN (12.5:87.5) containing 5 m*M* sodium heptane sulfonate	Thiamin and riboflavin (in same chromatogram)	UV 254 nm	[16]

(*Table continued*)

TABLE 21.16 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Cereal products, fresh meat, meat products, fresh fruit and vegetables, eggs, milk, yoghurt	Digest sample with 0.1 *N* HCl at 95–100°C for 30 min, cool, dilute to volume, adjust pH of aliquot to 4.0–4.5. Incubate with Clarase at 45–50°C for 3 h, cool, filter. *Cleanup and concentration*: pass filtrate through C_{18} solid-phase extraction cartridge, wash cartridge with aqueous 5 m*M* sodium hexane sulfonate, elute vitamins with methanolic 5 m*M* sodium hexane sulfonate	Radial-PAK C_{18} 10 μm 100 × 8 mm	MeOH/water (40:60) containing 5 m*M* hexane sulfonic acid	Thiamin and riboflavin (in same chromatogram)	Dual fluorescence *Thiochrome*: 1st detector, ex 360 nm, em 425 nm (filters) after post-column oxidation of thiamin with alkaline $K_3Fe(CN)_6$ *Riboflavin*: 2nd detector, ex 360 nm em 500 nm (filters)	[189,190]
Enriched cereal-based products	Digest sample with 0.1 *N* H_2SO_4 at 95–100°C for 10 min, cool. Incubate with buffered Mylase at 56°C for 1 h, cool, dilute to volume, filter	μBondapak C_{18} 10 μm 300 × 3.9 mm	MeOH/water (36:64) containing 5 m*M* hexane sulfonic acid and 1% acetic acid	Thiamin and riboflavin (in same chromatogram)	Dual fluorescence *Thiochrome*: 1st detector (filters) after post-column derivatization of thiamin with	[191]

					alkaline $K_3Fe(CN)_6$ *Riboflavin*: 2nd detector (filters)	
Riboflavin and pyridoxine						
Reversed-phase ion-pair chromatography						
Infant formula (fortified)	Extract sample with water. Precipitate proteins by pH adjustment to 1.7 and then to 4.6. Dilute to volume with water, filter	Spherisorb ODS-1 5 μm 150 × 4.6 mm	40 mM triethyl ammonium phosphate buffer (pH 3.0) containing 7.5 m*M* sodium octane sulfonate, 10% MeOH and 5% MeCN	Pyridoxal, pyridoxamine, riboflavin	Fluorescence; ex 285 nm, em 546 nm (filter)	[192]
Nicotinamide and pyridoxine						
Reversed-phase ion-pair chromatography						
Fortified foods (e.g., milk products, powdered meals)	Digest sample with 2 *N* H_2SO_4 at 95–100°C for 30 min, cool, dilute to volume with water, filter	Partisil ODS 10 μm or Ultrasphere ODS 5 μm	2.5 *M* sodium acetate (80 ml), acetic acid (50 ml), and water (25 ml) containing 1.1 g sodium heptane sulfonate; mixture diluted to 1 l with water	Nicotinamide and pyridoxine	*Nicotinamide*: UV 260 nm *Pyridoxine*: Fluorescence; ex 296 nm, em 396 nm Detectors connected in series	[193]

(*Table continued*)

TABLE 21.16 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Three or more vitamins						
Reversed-phase ion-pair chromatography						
Fortified cereal products	Digest ground sample with 0.1 *N* H_2SO_4 at 95–100°C for 30 min, cool. Incubate with buffered Clarase at 55°C for 1 h, centrifuge dilute to volume	μBondapak C_{18} 10 μm 250 × 4.6 mm and 5 × 4.6 mm short column. Sample cleanup achieved by column switching	MeOH/water (30:70) containing 1% acetic acid and 5 m*M* sodium hexane sulfonate	Pyridoxine, riboflavin, thiamin	Dual fluorescence *Pyridoxine and riboflavin*: 1st detector, ex 288 nm, em 418 nm (cut-off filter). *Thiochrome*: 2nd detector, ex 360 nm em 460 nm (cut-off filter) after post-column oxidation of thiamin with alkaline $K_3Fe(CN)_6$	[194]

Infant formulas, medical foods (fortified)	Precipitate proteins by adding perchloric acid, stirring for 0.5 h, then adjusting pH to 3.2. Dilute with mobile phase, refrigerate overnight, filter through paper, refilter through nylon (0.45 μm)	Nova-PAK C_{18} 300 × 3.9 mm	Water/MeCN (90.5:9.5) containing sodium hexane sulfonate and NH_4OH, adjusted to pH 3.6 with H_3PO_4	Pyridoxine, riboflavin, thiamin	Programmed fluorescence. A specially designed flow system allows the same detector to be used for two injection sequences 1st sequence: *Pyridoxine*: ex 295 nm, em 395 nm *Riboflavin*: ex 440 nm em 565 nm 2nd sequence: *Thiochrome:* ex 360 nm em 435 nm after post-column oxidation of thiamin with alkaline $K_3Fe(CN)_6$	[195]

(*Table continued*)

TABLE 21.16 *Continued*

		Quantitative HPLC				
Food	**Sample Preparation**	**Column**	**Mobile Phase**	**Compounds Separated**	**Detection**	**Ref.**
Supplemented infant formulas	Shake liquid or reconstituted sample with 0.6 *M* TCA for 15 min to precipitate proteins, filter (0.45 μm)	Luna Prodigy ODS (3) 5 μm 150 × 4.6 mm	Solvent (A): Water/MeOH (75:25) containing 1% formic acid and 0.1% (w/v) sodium dioctylsulfo-succinate adjusted to pH 8 with 50% KOH Solvent (B): Water/MeOH (45:55) containing 1% formic acid and 0.1% (w/v) sodium dioctylsulfo-succinate adjusted to pH 4.4 with 50% KOH	(A) FMN, riboflavin, nicotinamide, pyridoxal, pyridoxine. (B) Thiamin	UV 258 nm for nicotinamide. Programmed fluorescence for riboflavin and FMN (ex 450 nm, em 510 nm), pyridoxal and pyridoxine (ex 290 nm, em 390 nm). UV 254 nm for thiamin. Detectors connected in series	[196]

Supplemented infant formulas and baby foods	Homogenize ultrasonically, heat in water bath at 90°C for 30 min, allow to cool, adjust pH to 4 using 1 *M* sodium acetate. Incubate with Takadiastase at 50°C for 2 h, add 50% (w/v) TCA, re-heat at 90°C for 10 min. Cool, adjust pH to 6 with 10 *M* KOH, dilute to volume with mobile phase buffer, centrifuge, filter (0.45 μm)	Discovery RP-AmideC_{16} (hexadecyl) 5 μm 150 × 4.6 mm	Gradient elution. Isocratic with 10 m*M* phosphate buffer (pH 6) for 13 min, then linear gradient to MeCN–buffer (6:94) during 1 min and hold for 6 min. Second linear gradient to MeCN–buffer (12:88) during 1 min and hold for 10 min. Return to initial conditions in 1 min and hold for 15 min	Nicotinic acid, pyridoxal, pyridoxine, thiamin, nicotinamide, inosine, folic acid, cyanoco–balamin, riboflavin	UV/Vis (photodiode array detector)	[203]

Note: TCA, trichloroacetic acid; MeOH, methanol; MeCN, acetonitrile.

fluorescence detectors, each equipped with suitable excitation and emission filters to obtain adequate responses for each compound [18,19], or using a wavelength-programmable spectrofluorometer [188]. It is usually preferred to chromatograph thiochrome and riboflavin successively so that the optimum detection parameters can be selected [20,21,185–187]. Chromatograms of thiochrome and riboflavin extracted from a pork sample are shown in Figure 21.35. Although the separation of thiochrome from riboflavin might seem to be unnecessary if they are to be determined successively, it is in fact essential. If these compounds coeluted, the radiation emitted by thiochrome could be reabsorbed by riboflavin by the inner filter effect, causing a diminished fluorescent signal. With postcolumn derivatization, thiamin and riboflavin can be chromatographed simultaneously using reversed-phase ion-pair chromatography. This technique, used with two fluorescence detectors, has been applied to the analysis of a wide range of foods [189–191].

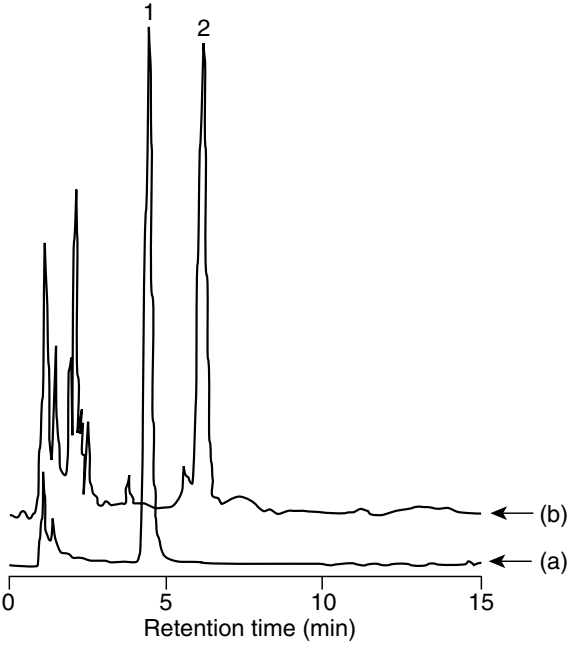

FIGURE 21.35
Successive reversed-phase HPLC with fluorescence detection of (a) thiochrome (representing thiamin after pre-column derivatization) and (b) riboflavin extracted from pork. Operating parameters as in Table 21.15 [186]. Peaks: (1) thiochrome; (2) riboflavin. (Reprinted from Ollilainen, V., et al., *J. Food Comp. Anal.*, 6, 152–165, 1993. With permission from Elsevier.)

The method of Hasselmann et al. [21] has been subjected to a collaborative study [198]. Thiamin and riboflavin were co-extracted by enzyme hydrolysis using a mixture of Takadiastase and β-amylase. Minor modifications to the method were filtration of the solution obtained after the incubation step through a fine filter paper and a slight change of the mobile phase composition [from 40:60 (v/v) to 30:70 (v/v) methanol and 0.05 *M* sodium acetate without adjustment to pH 4.5]. With all nine foodstuffs studied, the recovery rate was always greater than 89%, except with chocolate powder, for which it was reduced to ca 50% for thiamin and 75% for riboflavin. The proposed method, despite this drawback, was confirmed as the official French method for thiamin and riboflavin determination in foodstuffs for nutritional purposes.

21.2.10.2 Riboflavin and Pyridoxine

Ayi et al. [192] developed a comparatively simple method for the simultaneous determination of supplemental riboflavin and pyridoxine in fortified milk- and soy-based infant formula products. The method took advantage of the natural fluorescence of these vitamins and was performed using a single fluorescence detector equipped with carefully selected filters. The ion-pair mode of reversed-phase chromatography was capable of isolating pyridoxine from the other B_6 vitamers and from 4-pyridoxic acid. Similarly, riboflavin was well separated from its potential degradation products, lumiflavin and lumichrome.

21.2.10.3 Nicotinamide and Pyridoxine

Rees [193] used reversed-phase ion-pair chromatography, and absorbance and fluorescence detectors connected in series, for the simultaneous determination of nicotinamide and pyridoxine, respectively, in fortified food products. The extraction process (heating the sample with 2 *N* H_2SO_4 for 30 min at 100°C) caused a 6% conversion of nicotinamide to nicotinic acid, but this was catered for by subjecting the nicotinamide standard to the same treatment as the sample extract. A Partisil ODS column containing residual free silanol groups was used for routine analyses and gave retention times of 8.0 and 5.8 min for nicotinamide and pyridoxine, respectively. If a new product was being analyzed, or it was known that the pyridoxine peak was subject to interference from other peaks, the analysis was repeated using a fully endcapped Ultrasphere ODS column. On this column, the order of elution was reversed, nicotinamide and pyridoxine having retention times of 4.2 and 8.6 min, respectively. The detection limit for nicotinamide was 0.1 mg/100 g, and that for pyridoxine was 0.01 mg/100 g. Pyridoxine is usually present in fortified food products at levels 10 times lower than those of nicotinamide,

and hence the higher sensitivity of detection for pyridoxine was fortuitous. HPLC results for milk powder, high-protein meal powder, and diet meal powder were in good agreement with results obtained by microbiological assay.

21.2.10.4 Three or More Vitamins

Reversed-phase ion-pair chromatography eliminates the differences in ionization between the various water-soluble vitamins, and permits their isocratic separation. The technique is routinely applied to the analysis of pharmaceutical multivitamin preparations [199] and has also been applied to the determination of B vitamins in supplemented foods and infant formulas. Triethylamine is used as a mobile phase additive to reduce the peak tailing of the strongly basic thiamin [200].

Albalá-Hurtado et al. [201] reported the simultaneous determination of (in order of elution) nicotinamide, pyridoxal, pyridoxine, pyridoxamine, folic acid, riboflavin cyanocobalamin, and thiamin in supplemented infant milk formulas using ion-pair chromatography and programmed UV detection. The first seven analytes eluted within 25 min, but thiamin took 50 min to elute. Agostini and Godoy [202] used gradient elution to separate nicotinic acid, nicotinamide, pyridoxine, thiamin, and riboflavin within 25 min. Woollard and Indyk [196] separated FMN, riboflavin, nicotinamide, pyridoxal, and pyridoxine simultaneously (Figure 21.36), and thiamin successively (Figure 21.37), from deproteinized extracts of supplemented infant formulas. The ion-pairing agent used for these separations was (uniquely for food analysis) dioctylsulfosuccinate, which, by virtue of its highly lipophilic double-branched alkyl functionality, conferred enhanced retention.

Wehling and Wetzel [194] accomplished the simultaneous determination of thiamin, riboflavin, and pyridoxine in fortified cereal products after extraction of the samples by heating with 0.1 *N* H_2SO_4, followed by Clarase treatment to break down the starch. Reversed-phase ion-pair chromatography with isocratic elution separated the three vitamins within 20 min, but more strongly retained components took up to 50 min to elute. The 50-min chromatographic analysis time was halved by the use of online sample cleanup, utilizing a short (5 cm) C_{18} column in addition to the C_{18} analytical (25 cm) column, and a column switching arrangement. Aliquots of the test extract were injected onto the short column, with the effluent being routed directly to waste. Solvent flow through the analytical column and detector was maintained independently by a second pump. At 0.60 min after injection, the fraction of effluent containing the vitamins was diverted onto the analytical column, and at 3.5 min the valve was switched back to its original position. During the remaining time, as the vitamins were undergoing analysis, the more

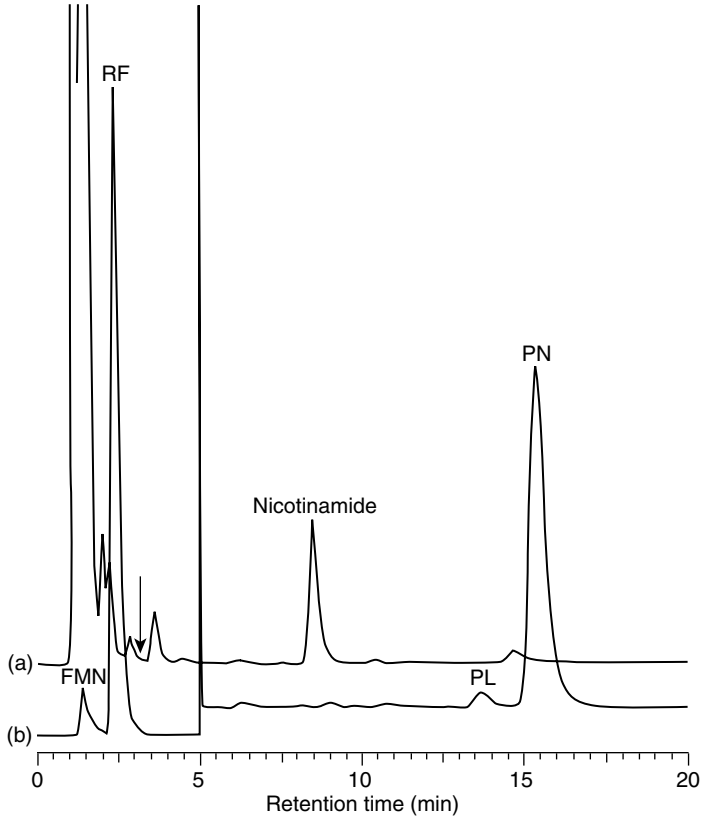

FIGURE 21.36
Simultaneous reversed-phase ion-pair HPLC of supplemental vitamin B_2, niacin, and vitamin B_6 in milk-based infant formula. (a) UV at 258 nm for nicotinamide; (b) programmed fluorescence for FMN, riboflavin (RF), pyridoxal (PL) and pyridoxine (PN). Arrow indicates where nicotinic acid would elute. Operating parameters as in Table 21.15 [196]. (Reprinted from Woollard. D.C. and Indyk, H.E. *J. AOAC Int.*, 85, 945–951, Copyright 2002 by AOAC International. With Permission)

strongly retained components were eluted from the short column directly to waste. Fluorescence detection was used to measure the native fluorescence of pyridoxine and riboflavin. Thiamin, in the form of thiochrome, was detected by a second fluorescence monitor after postcolumn derivatization, the strong alkaline conditions suppressing the fluorescence of pyridoxine and riboflavin. The concentration of potassium hexacyanoferrate(III) added for postcolumn thiochrome formation was decreased significantly from levels used in other procedures in order to minimize the quenching effect of excess reagent during detection. Detection

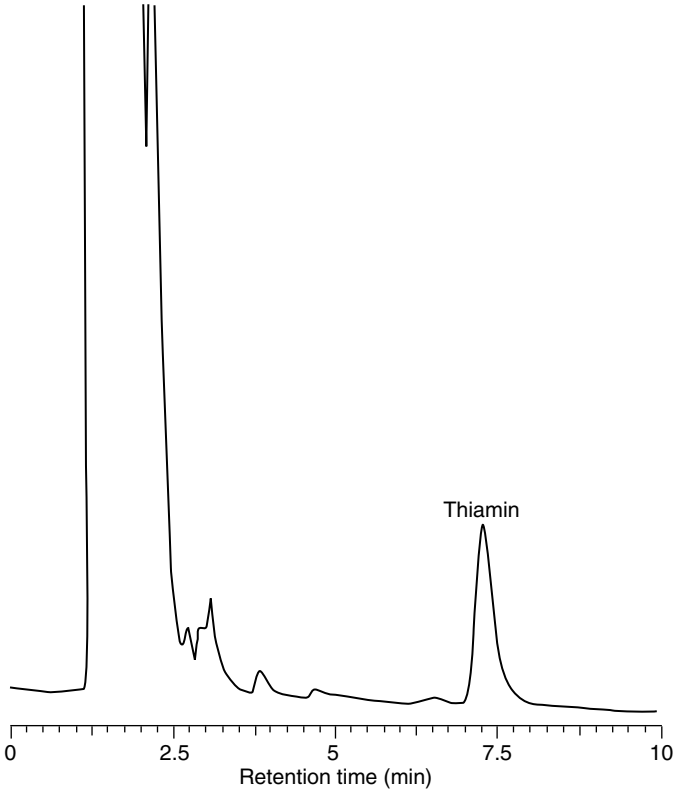

FIGURE 21.37
Reversed-phase ion-pair HPLC with UV detection of supplemental thiamin in milk-based infant formula. Operating parameters as in Table 21.15 [196]. (Reprinted from Woollard, D.C. and Indyk, H.E., *J. AOAC Int.*, 85, 945–951, Copyright 2002 by AOAC International. With permission.)

limits were 2 μg/g for pyridoxine and 1 μg/g for both thiamin and riboflavin.

Viñas et al. [203] reported the reversed-phase separation of thiamin, riboflavin, nicotinamide, nicotinic acid, pyridoxine, pyridoxal, folic acid, and cyanocobalamin within 30 min using gradient elution with acetonitrile/phosphate buffer (pH 6) as mobile phase. Under these conditions, the vitamins were not ionized and therefore an ion-pairing reagent was not necessary. The stationary phase was RP-Amide C_{16} (Supelco) which contains embedded amide groups and is endcapped with trimethylsilyl. A photodiode array detector permitted simultaneous absorbance detection at several wavelengths and continuous memorizing of spectra during the evolution of a peak. Chromatograms of the B vitamins and inosine at different wavelengths are shown in Figure 21.38.

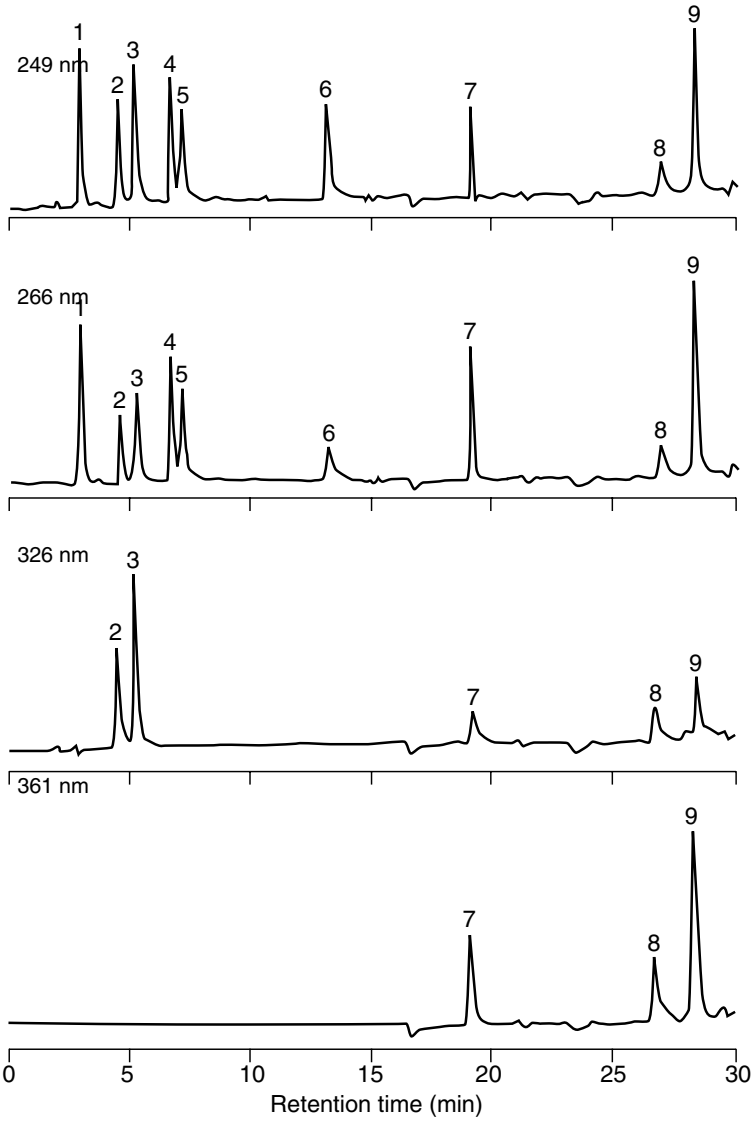

FIGURE 21.38
Reversed-phase HPLC on an amide stationary phase with photodiode array detection of B vitamins and inosine in standard solution. Operating parameters as in Table 21.16 [203]. Peaks: (1) nicotinic acid; (2) pyridoxal; (3) pyridoxine; (4) thiamin; (5) nicotinamide; (6) inosine; (7) folic acid; (8) cyanocobalamin; (9) riboflavin. (Reprinted from Viñas, P., et al., *J. Chromatogr. A*, 1007, 77–84, 2003. With permission from Elsevier.)

Advantages of the amide-based stationary phase over reversed-phase ion-pair chromatography were sharper peaks and a longer column life. Viñas et al. [203] applied their HPLC technique to the determination of supplemental vitamins in infant formulas, cereals and fruit products. Validation was performed using two certified reference materials, milk powder (CRM 421) and pig's liver (CRM 487).

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22

Biospecific Methods for Some of the B-Group Vitamins

22.1 Introduction

Biospecific methods of analysis for selected vitamins of the B group can be broadly classified as immunoassays and protein-binding assays [1]. Immunoassays are based on the specific interaction of an antibody with its antigen, and are represented by the radioimmunoassay (RIA) and the enzyme-linked immunosorbent assay (ELISA). Protein-binding assays utilize naturally occurring vitamin-binding proteins with either radiolabels (as in the radiolabeled protein-binding assay, RPBA) or enzyme labels (as in the enzyme-labeled protein-binding assay, EPBA). A more recent innovation is the optical biosensor-based immunoassay/protein-binding assay. Biospecific assays can be performed on complex biological matrices, so they require minimal sample cleanup. The analytical stages can be automated using equipment that is commercially available, but the methods can only be described as semiautomated, as it is necessary to liberate the vitamins from their bound forms using manual extraction procedures.

22.2 Immunoassays

22.2.1 The Immunological Reaction

If a test animal such as a rabbit is given repeated small injections of an immunogenic antigen, antibodies against the antigen are produced by lymphoid tissues and circulate in the rabbit's bloodstream. When the serum of the rabbit (referred to now as antiserum) is added *in vitro* to a solution containing the antigen, the antigen binds to specific sites on the surface of the antibody as it did *in vivo*. Proteins of molecular weight >5000 Da can usually be both antigens and immunogens. Smaller compounds, though antigenic, must be coupled to a large protein carrier such

as albumin to be immunogenic. When coupled, the small compound is called a hapten, and the carrier–hapten complex is called a conjugate. Usually, the immunogen is mixed with an adjuvant, which, when injected, serves to both enhance and prolong the immune response [2].

The following terms are encountered in immunoassays:

- *Antibody* A binding protein (immunoglobulin) which is synthesized by the immune system of an animal in response to the injection of an immunogenic antigen.
- *Antigen* A substance capable of binding to a specific antibody.
- *Immunogen* A substance that, when injected into a suitable animal, elicits an immune response.
- *Antiserum* The serum of the test animal containing polyclonal antibodies.
- *Polyclonal antibodies* These are antibodies which are present in the antiserum of an immunized animal and which are derived from several clones of lymphocyte. They are reactive for several antigenic sites.
- *Monoclonal antibodies* These are antibodies derived from a single clone of lymphocytes produced in cell culture by hybridoma cells, which are formed by the fusion of lymphocytes with myeloma cells (cancerous lymphocytes) from an immunized animal donor. The antibody molecules, being chemically identical, exhibit identical binding properties.
- *Cross-reactivity* This is the ability of substances, other than the antigen, to bind to the antibody, and the ability of substances, other than the antibody, to bind the antigen. Cross-reactants may be substances that carry on their surface a molecular configuration similar to the antigenic determinants on the antigen being measured.
- *Antigenic determinant* This is the structural feature of an antigen which defines the recognition pattern of an antibody.
- *Affinity* This is the energy with which the combining sites of an antibody bind its specific antigen. It is analogous to the association constant (K_A) in physical chemistry and has the dimensions of moles per liter.
- *Avidity* There are several populations of antibodies with different affinities in a polyclonal antiserum, the mean affinity being referred to as its avidity. The high-affinity antibodies dictate the sensitivity of an immunoassay.

The production of monoclonal antibodies [3] is more expensive, labor-intensive, and time-consuming than the production of polyclonal

antiserum. However, the provision of potentially unlimited amounts of a homogeneous reagent is a major advantage in the development of commercial assay kits. The higher specificity compared with polyclonal antibodies is another advantage. On the demerit side, monoclonal antibodies rarely exhibit such a high affinity for the antigen as do polyclonal antibodies, and this can result in a less sensitive assay. The affinity is less important for the sensitivity of an excess reagent assay than it is for a competitive assay.

22.2.2 Radioimmunoassay

22.2.2.1 Principle

The RIA is based on the competition for a fixed, but limited, number of antibody-binding sites by antigen (the vitamin analyte) and a trace amount of radiolabeled antigen added to the sample extract. Thus, the presence of larger amounts of unlabeled analyte results in less radioactivity being bound to the antibody. The free and antibody-bound fractions are separated by adsorption or precipitation, followed by centrifugation, and the radioactivity in the supernatant or precipitate is measured. A comparison of the ratio of the bound to free labeled analyte with that obtained from a series of standards permits quantification of unknown samples.

22.2.2.2 Determination of Pantothenic Acid

Walsh et al. [4] compared an RIA method with the microbiological (*Lactobacillus plantarum*) method for the determination of pantothenic acid in 75 processed and cooked foods. The results of the individual foods analyzed have been reported [5]. The pantothenic acid was released from aqueous sample homogenates by autoclaving at 121°C for 10 min, followed by incubation with phosphatase–liver enzyme, and the protein was removed by dialysis. Antibody was prepared by injecting rabbits with a pantothenic acid–bovine serum albumin (PA–BSA) conjugate, and the resulting antiserum was diluted 100-fold with a solution of rabbit albumin. Each assay tube contained 0.5 ml of the diluted antiserum, 0.50 ml of standard solution or sample extract, and 50 μl of [^{3}H]sodium D-pantothenate. After incubation at room temperature for 15 min, neutral saturated ammonium sulfate was added to achieve a 50% saturation, and the suspension was centrifuged. The precipitate was washed with 0.5 ml of 50%-saturated ammonium sulfate and recentrifuged. The washed precipitate, containing antibody-bound pantothenic acid, was dissolved in 0.5 ml of tissue solubilizer and transferred quantitatively to vials containing 12 ml of scintillation fluid. The radioactivity

results in counts per minute (cpm) were read on a 5–150 ng (per 0.5 ml) standard curve. An enzyme blank value was subtracted from each sample value.

Although the results from the RIA and microbiological assay were highly correlated ($r = 0.94$), the microbiological assay produced a higher average result for the food types meats, fruits and vegetables, and breads and cereals. It was postulated that either bacterial enzymes in the assay organism promote further breakdown of bound pantothenic acid, or nonenzymatic breakdown occurs during the long microbiological incubation period.

22.2.3 Enzyme-Linked Immunosorbent Assay

22.2.3.1 Principle

An ELISA is an enzyme-linked immunoassay in which one of the reactants is immobilized by physical adsorption onto the surface of a solid phase. In its simplest form, as used in food analysis applications, the solid phase is provided by the plastic surface of a 96-well microtiter plate. The ELISA can be performed manually, with the aid of push-button dispensers, or it can be totally automated, complete with computer for calculation of standard curves, statistical analysis of data, and data storage.

There are many variants of the ELISA, but in a discussion of basic principles, they fall into two main types, namely competitive and noncompetitive (reagent excess) immunoassays.

In the direct competitive ELISA, the analyte vitamin molecules and added enzyme–vitamin conjugate compete for a limited number of binding sites on the immobilized antibody. The proportion of added enzyme–vitamin conjugate present in either the free or bound phases after equilibrium has been reached is dependent upon the amount of analyte initially present. The phases are separated by emptying the well contents and washing the plate. The amount of bound enzyme is then determined by addition of substrate and spectrophotometric measurement of the colored product. A variation of this format is the indirect competitive ELISA, in which the analyte and immobilized analyte compete for a limited number of binding sites on the enzyme-labeled antibody. The characteristic feature of competitive ELISAs is that the higher the optical density, the lower is the amount of analyte present.

The generally preferred ELISA format for vitamin assays in food analysis is a two-site noncompetitive assay used in the indirect mode. This format employs two antibodies: a primary antivitamin antibody raised against a hapten–protein conjugate, and an enzyme-labeled, species-specific second antibody, which binds specifically to the primary antibody. The scheme for performing such an ELISA is depicted in

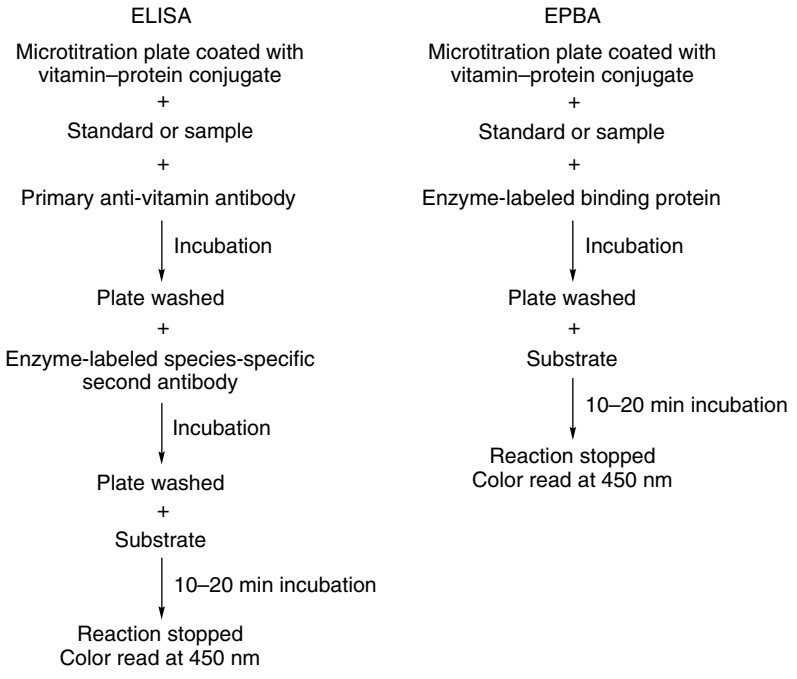

FIGURE 22.1
Comparison of methodologies for the two-site noncompetitive ELISA (indirect mode) and the EPBA (indirect mode). (Taken from Lee, H.A., Mills, E.N.C., Finglas, P.M., and Morgan, M.R.A., *J. Micronutr. Anal.*, 7, 261–270, 1990. With permission.)

Figure 22.1 [6]. A protein conjugate of the vitamin is immobilized to the well surface of the microtitration plate, the attached protein being different to that used for the immunogen. The protein adsorbs passively and strongly to the plastic and, once coated, plates can usually be stored for several months. To perform the assay, the sample or standard is added to the well, followed by a limited amount of primary antibody. After incubation, the antibody becomes distributed between immobilized vitamin and free vitamin according to the amount of analyte initially present. After phase separation, achieved by well emptying and washing, the second antibody is added in excess, and the plate is incubated for a second time. Excess unbound material is removed and substrate is added. Optical densities are measured after a suitable time, and unknown samples are quantified by reference to the behavior of vitamin standards.

In contrast to the competitive ELISA, the noncompetitive assay uses an excess of antibody, so that the optical densities increase with increasing amount of analyte. Although the competitive assay produces its greatest

signal (optical density) for low concentrations of analyte, the noncompetitive assay gives lower detection limits. It is also more specific, since the two antibodies recognize separate antigenic determinants on the analyte. Two other advantages of the noncompetitive assay are that the affinity of the primary antibody is less important than in the competitive assay, because excess antibody is used, and the accuracy of pipetting the primary antibody is less critical, as it is no longer a limiting factor [7]. Enzyme-labeled second antibodies are widely available commercially, active against different species, and labeled with a variety of enzymes. In summary, noncompetitive assays are more reliable and more rugged than competitive assays, with the added advantages of improved sensitivity and specificity.

22.2.3.2 Determination of Pantothenic Acid

Morris et al. [8] developed an indirect two-site noncompetitive ELISA by raising polyclonal antibodies in rabbits against pantothenic acid. The PA–BSA immunogen was prepared by reacting the primary alcohol group of the pantothenic acid molecule with bromoacetyl bromide to form bromoacetyl pantothenate, which, in turn, was reacted with denatured reduced BSA. The immunogen was purified by extensive dialysis and column chromatography on Sephadex G-25. The protein conjugate for plate coating was pantothenic acid–keyhole limpet hemocyanin (PA–KLH) produced by the bromoacetyl procedure, as for the immunogen. Anti-rabbit immunoglobulin–alkaline phosphatase conjugate was used as the enzyme-labeled second antibody. The ELISA system was highly specific for pantothenic acid, and did not recognize coenzyme A, pantothenol, or pantetheine. The lower limit of detection was 0.5 ng pantothenic acid per well.

The validation and application of the ELISA system for the analysis of six foods representing major sources of pantothenic acid in the U.K. diet was reported by Finglas et al. [9]. Sample preparation entailed autoclaving at 121°C for 15 min, homogenization, and overnight incubation with phosphatase—pigeon liver enzyme. The following day, the sample hydrolysates were autoclaved at 121°C for 5 min to destroy any remaining enzyme activity. The ELISA values obtained for the six foods compared favorably ($r = 0.999$) with values obtained by the microbiological method of Bell [10] using *L. plantarum.*

Gonthier et al. [11] improved the sensitivity of the ELISA by using an immunogen composed of pantothenic acid coupled to thyroglobulin by a 6-carbon atom linker (adipoyl dichloride). In contrast, the bromoacetyl linker used in Finglas' pantothenic acid–BSA system 1 immunogen contains two carbon atoms.

22.2.3.3 Determination of Vitamin B_6

The ideal biospecific assay for vitamin B_6 is one which exhibits a broad specificity and provides a value for the total B_6 content, but no such assay has yet been reported. Alcock et al. [12] raised polyclonal antibodies in rabbits using a purified PL–BSA conjugate as the immunogen, but the antisera all showed a preference for PM. A corresponding preference for PMP was reported for antisera raised against PLP–BSA using polyclonal [13] and monoclonal [14] immunization techniques. The general preference displayed for the amine forms probably reflects the fact that the protein linkage to the 4-carbonyl groups is an ε-amino group of a lysine residue, and hence the conjugate is an amine derivative most resembling PM or PMP.

Alcock et al. [12] set up an indirect two-site noncompetitive ELISA procedure using a PM-specific antiserum preparation and anti-rabbit immunoglobulin horseradish peroxidase conjugate as the enzyme-labeled second antibody. The microtiter plates were coated with a PL–KLH conjugate. The assay limit of detection was 7 pg of PM per well. Food samples were autoclaved with 0.2 *N* H_2SO_4 at 121°C for 20 min, cooled, homogenized, adjusted to pH 4.5, centrifuged, and filtered.

PM measurement alone by ELISA is of little practical use in food analysis as, after acid hydrolysis, PL and PN frequently predominate in animal- and plant-derived foods, respectively. One of the antiserum preparations, which exhibited 80% cross-reactivity with PN with a detection limit of 100 pg/well, would be useful for the determination of added PN in fortified foods. There are two possible approaches toward developing an ELISA for determining total vitamin B_6. Experiments with different conjugation procedures might produce an antibody that could recognize vitamin B_6 irrespective of the functional group at the C-4 position, although such an antibody would probably also recognize the nonactive metabolite, 4-pyridoxic acid. Alternatively, antisera or monoclonal antibodies specific for each nonphosphorylated B_6 vitamer could be produced, and mixed to allow the determination of total vitamin B_6 after acid hydrolysis, or used separately to determine the individual vitamers.

22.3 Protein-Binding Assays

22.3.1 Radiolabeled Protein-Binding Assays

22.3.1.1 Principle

RPBAs, also known as radioassays, have been applied to the determination of biotin, folate, and vitamin B_{12} in biological materials. The assays are based on the radioisotope-dilution principle, whereby the unknown

quantity of the vitamin in the test material, after first being liberated from bound materials, is used to dilute the radioactivity of an added measured quantity of tracer (radioactively labeled vitamin). The analysis usually involves an initial heating step to denature indigenous binding proteins. The assay procedure is carried out as follows.

Into a centrifuge tube are placed measured volumes of a suitable buffer solution, the test extract or unlabeled vitamin standard, and the tracer. The labeled vitamin is available commercially in powdered form or in solution, and can be standardized against the unlabeled vitamin standard by the method of Lau et al. [15]. The standardization technique allows the actual quantity of labeled vitamin to be calculated for any percentage change in the binding capacity of the protein for the labeled vitamin. A soluble natural vitamin-binding protein is then added in a predetermined quantity that has a maximal capacity to bind only some of the labeled vitamin present. Typical binding capacities are 80–90% for biotin assays [16], 50–60% for folate assays [17], and 60–80% for vitamin B_{12} assays [15]. The binding protein has a high affinity and specificity for the vitamin in question, but it does not discriminate between labeled and unlabeled vitamin.

The tubes are stored at ambient temperature in the dark for a prescribed period. During this time, unlabeled and labeled vitamin will compete stoichiometrically for the limited number of binding sites on the protein molecule. The amount of labeled vitamin that is subsequently bound is inversely related to the amount of indigenous vitamin present. Activated charcoal coated with hemoglobin, albumin, or dextran is added and the tube contents are mixed thoroughly. The charcoal coating acts as a molecular sieve, allowing the unbound vitamin to pass through and be adsorbed onto the charcoal, but excluding the protein-bound vitamin. The unbound vitamin is separated from bound vitamin by centrifugation.

The specific radioactivity in the supernatant fluid (bound fraction) or in the pellet (unbound fraction) is measured in counts per minute (cpm) in a liquid scintillation counter for β-emitters such as ^{3}H or ^{14}C isotopes or in a gamma counter for γ-emitters such as ^{125}I, ^{75}Se, or ^{57}Co isotopes. Included in the assay procedure is a control tube, which contains only the tracer and coated charcoal (plus buffer solution to make up the volume). The control represents the amount of radioactivity that is not bound to the charcoal and is due to radioactive degradation products of the tracer. The cpm for the control is subtracted from those for the unknown and the standards to obtain net counts.

Quantification is achieved by assaying a range of unlabeled vitamin standards of known concentration. Let B represent the amount of bound tracer (net cpm) corresponding to each concentration of standard and B_M represent the amount of bound tracer (net cpm) corresponding to a zero amount of standard (i.e., the maximal binding capacity of the

fixed amount of protein). A linear calibration curve is obtained on logit–log paper (1×3 cycle log–log paper) by plotting the percentage of tracer bound at each concentration of standard ($B/B_M \times 100$) as the logit function (ordinate) versus the log concentration (abscissa) of standard in nanograms per milliliter. Alternatively, the reciprocal of the percentage of tracer bound versus concentration can be plotted as a straight line on nonlogarithmic graph paper [18]. The concentration of vitamin in the assay solution can be obtained from the standard curve by interpolation of the percentage of tracer binding found or by calculation from the regression equation of the standard curve [19].

22.3.1.2 Determination of Biotin

RPBA techniques for determining biotin are based on the high affinity of the glycoprotein avidin for the functional ureido group of the biotin molecule. Early methods used [^{14}C]biotin, which has a specific radioactivity of 45 mCi/mmol, but a higher sensitivity can be obtained using [^{3}H]biotin of specific activity 2.5 Ci/mmol [16]. In a procedure described by Hood [20], samples of pelleted poultry feeds and wheat were autoclaved with 2 *N* H_2SO_4 for 1 h at 121°C, and then neutralized with 20% NaOH. The filtered extracts were incubated with [^{14}C]biotin and the avidin–biotin complex was precipitated with 2% zinc sulfate solution. The method was reported to be capable of measuring biotin levels down to 5 μg/kg of biological material and was more than adequate for analyzing wheat and poultry feeds, which contained 68–341 μg/kg. Results obtained by RPBA and microbiological (*L. plantarum*) assay were similar for poultry feeds, but the RPBA values for two wheat samples were approximately 20 and 55% higher than the microbiological assay values.

Bitsch et al. [21,22] released protein-bound biotin from food samples by means of papain digestion rather than acid hydrolysis. [^{3}H]Biotin was used as the tracer, and nonbound biotin was removed by adsorption on dextran-coated charcoal. Values for biotin concentration obtained by this method for meat, offal, cereal products, milk, and vegetables generally agreed with data from food composition tables, but RPBA values for cabbage and bananas were higher than literature values.

22.3.1.3 Determination of Folate

Waxman et al. [17] developed an RPBA for measuring folate levels in blood serum using a folate-binding protein (FBP) isolated from milk. Subsequently, many variations of this technique have been applied to the measurement of folate levels in serum, plasma, or red blood cells, and several radioassay kits are commercially available for such analyses.

Sources of FBP used in radioassays have included nonfat dry milk, skim milk, and whey protein concentrate [23], as well as crystalline bovine β-lactoglobulin [24]. At the physiological pH range of 7.3–7.6, milk FBP shows a greater affinity for binding folic acid than it does for 5-methyl-THF, whereas at pH above 9.4, its affinity is greater for 5-methyl-THF. At pH 9.3, FBP exhibits a similar binding capacity for these two folates [25]. The presence of at least one glutamate residue is required for binding to take place, as shown by the nonbinding of pteroic acid [26]. Pterin-6-carboxylic acid and *p*-aminobenzoylglutamic acid exhibit little or no affinity for FBP, indicating that these folate degradation products would not significantly interfere with the accuracy of the assay [27].

Radioactive folic acid, labeled with tritium in the 3′-, 5′-, 7-, and 9-positions, is commercially available in high specific activity (43 Ci/mmol) [28]. The use of γ-emitters such as ^{125}I- and ^{75}Se-labeled folic acid simplifies the assay procedures by eliminating liquid scintillation counting [24].

In contrast to blood plasma or serum, in which monoglutamyl 5-methyl-THF is virtually the sole folate present [29], the naturally occurring folates in foodstuffs comprise a variety of polyglutamyl forms. In applying the pH 9.3 RPBA to foods, the extraction procedure should avoid or minimize the thermal conversion of 10-formyl-THF to 5-formyl-THF, because FBP does not exhibit significant affinity for 5-formyl-THF [27]. An initial heating step is, however, essential when analyzing milk and other dairy products in order to denature indigenous FBP. Deconjugation of folates to monoglutamyl forms is obligatory, because of the dependency of the binding affinity on polyglutamyl chain length. Shane et al. [29] reported that the molar response of different folate compounds in RPBA procedures varied considerably, depending on the stereochemical form, the reduction state of the pteridine nucleus, the nature of the one-carbon substituent, and the number of glutamate residues. This observation appears to rule out the application of RPBAs for accurately determining the various naturally occurring folates in foods.

Several studies have been conducted in which the results of the pH 9.3 RPBA have been compared with those of the *Lactobacillus rhamnosus* (*casei*) assay for the determination of total folate in foods [27,30–33]. Strålsjö et al. [34] optimized and validated a commercial RBPA kit for reliable folate quantification in berries and milk. The optimized procedure, using 5-methyl-THF as external calibrant, could only be recommended in foods containing mainly this vitamer. The affinity of FBP for THF was much stronger than for 5-methyl-THF and there was almost no affinity for 5-formyl-THF. Analysis of two European certified reference materials (CRM 421, 485) gave results that were within the range of results from previously reported HPLC methods.

22.3.1.4 Determination of Vitamin B_{12}

Published RPBA techniques for determining vitamin B_{12} in foods [35–42] are based on the original method of Lau et al. [15], which was developed for measurement of serum B_{12}. Food extracts prepared for radioassay contain 20–1000 pg vitamin B_{12}/ml, which is well within the 50–2000 pg/ml range of commercially available assay kits. The RPBA utilizes [^{57}Co]cyanocobalamin as the tracer, and hog intrinsic factor as the binding protein. Cyanocobalamin has a binding affinity for hog intrinsic factor equal to that of methylcobalamin, dicyanocobalamin, and nitrotocobalamin, but not to that of hydroxocobalamin, sulfitocobalamin, and adenosylcobalamin. For an accurate assay, it is therefore necessary to extract foods in the presence of excess cyanide, in order to convert the latter three cobalamins to dicyanocobalamin [43]. Ellenbogen [44] stressed the importance of using purified intrinsic factor to avoid the binding of inactive noncobalamin corrinoids to extraneous proteins.

The extraction techniques employed for the determination of serum vitamin B_{12} are not sufficiently rigorous to liberate the more tightly bound cobalamins present in many foods [45], and it is necessary in food analysis to implement a more rigorous extraction step prior to the RPBA. Beck [36] developed an extraction procedure that was compatible with RPBA techniques for the determination of cyanocobalamin in seafoods. Homogenized tissue was mixed with sodium nitrite and sodium cyanide, adjusted to pH 4.0 with hydrochloric acid, and boiled for 1 h. The extract was cooled and the coagulated protein was removed by suction filtration. The filtrates from oily fish were extracted with petroleum ether. The extracts were purified and concentrated by extracting the cyanocobalamin into benzyl alcohol, followed by reextraction into water after the addition of chloroform. Richardson et al. [35] extracted the cobalamins from various foods using pH 4.6 acetate buffer containing sodium cyanide and heating in a boiling water bath for 30 min. Results obtained by RPBA were found to be somewhat lower than comparative results obtained by microbiological assay using *Lactobacillus delbrueckii.* The differences between the two sets of results were postulated to be caused by the extraction not releasing all of the vitamin B_{12} in a form capable of binding to intrinsic factor, although it was usable by the assay microorganism. On the other hand, Österdahl et al. [39], using a similar extraction procedure to that of Richardson et al. [35], obtained a very high correlation ($r = 0.987$) between the results from the RPBA method and the microbiological method for the determination of vitamin B_{12} in gruel. Gruel is an infant food based mainly on dry milk and cereals, and its vitamin B_{12} content is mainly derived from the dried milk component. Some gruel products are also fortified with vitamin B_{12}, so these free and bound forms are apparently readily extractable by the method employed.

Casey et al. [37] mixed ground food samples with 1.3% (w/v) anhydrous sodium phosphate dibasic (Na_2HPO_4), 1.2% (w/v) citric acid, and 1.0% sodium metabisulfite ($Na_2S_2O_5$), and then autoclaved the mixture for 10 min at 121°C according to the extraction procedure described in the AOAC [46] microbiological method for determining vitamin B_{12} in vitamin preparations. The problems of incomplete extraction reported by Richardson et al. [35] were not encountered, probably because of the more rigorous heat treatment employed, and the results compared quite favorably with results obtained by the AOAC microbiological method. The purification step involving partitioning into benzyl alcohol [36] was not necessary, as the increased specificity of the highly purified intrinsic factor effectively eliminated interference by other biochemical compounds.

Andersson et al. [40] established, by statistical analysis of experimental data, the optimal extraction conditions (cyanide concentration, buffer concentration, pH, method of heating, and heating time) for the determination of total vitamin B_{12} in milk by RPBA. The optimized procedure entailed mixing 2 ml of milk with 5 ml of sodium acetate buffer (0.4 *M*, pH 4.5) and sodium cyanide (2000 ppm), and autoclaving at 121°C for 25 min. When the optimized extraction technique was used in the *L. delbrueckii* assay, there was no significant difference compared with the RPBA technique. However, the RPBA had a better reproducibility than the microbiological assay. It was shown that autoclaving gave a significantly higher yield than a boiling water bath.

Arkbåge et al. [42] extracted pasteurized milk and fermented dairy products by mixing samples with extraction buffer containing 0.08 m*M* sodium cyanide and then autoclaving at 121°C for 25 min. Autoclaved milk samples were cooled and then centrifuged. The pellet was resuspended in extraction buffer and recentrifuged. The combined supernatants were made to a known volume with extraction buffer ready for analysis. Autoclaved hard cheese and blue cheese samples were cooled, the pH was adjusted to 7, and pancreatin was added. The samples were incubated at 37°C for 3.5 h during constant shaking and then heated on a boiling water bath for 5 min to inactivate the enzyme. An enzyme blank was always prepared to correct for addition of vitamin B_{12}. After cooling, the pH was adjusted to 4.5 with glacial acetic acid. The samples were then centrifuged and further treated as for the milk samples.

In one of two commercial assay kits evaluated by Richardson et al. [35], the binding agent is supplied in the form of a Sephadex (dextran)–intrinsic factor complex, which simplifies the analysis and was found to function more satisfactorily with food extracts than the separate use of intrinsic factor and albumin-coated charcoal. The results obtained by RPBA for the determination of vitamin B_{12} in food were compared with those obtained by the *L. delbrueckii* (ATTC No. 7830) microbiological

assay using the same sample extracts [41]. The agreement between the two methods was very good in eight out of the ten foods analyzed. In the case of pork, the RPBA gave the higher figure, whereas the opposite was observed for yoghurt. The RPBA is presumably more specific for vitamin B_{12}, as intrinsic factor binds with a very narrow range of corrinoids. Arkbåge et al. [42] validated a commercial assay kit and showed that, with modification, it was both precise and accurate for fermented dairy products.

22.3.2 Enzyme-Labeled Protein-Binding Assays

22.3.2.1 General Procedure

An EPBA has been developed for food analysis applications using the 96-well microtitration plate as the solid phase. Individual methods have been reported for the determination of biotin, folate, and vitamin B_{12} using avidin, FBP, and R-protein as the respective vitamin-specific binding proteins. The principle of the assay is based on the competition between immobilized vitamin and free vitamin (analyte) in the assay solution for a limited number of binding sites on the enzyme-linked vitamin-binding protein. The amount of protein bound to the well surface is inversely proportional to the concentration of free vitamin in the assay solution and is determined, after plate washing, by measuring the enzyme activity. The scheme for performing such an assay is compared with an ELISA format in Figure 22.1.

22.3.2.2 Determination of Biotin

In an EPBA, for the determination of biotin in fresh lamb's liver [47], biotin–KLH conjugate was used to form the immobilized phase. The binding protein–enzyme conjugate was avidin–horseradish peroxidase, which is commercially available, and the substrate was 2,2′-azino-bis-(3-ethylbenzthiazoline sulfonic acid) (ABTS). The extraction of liver samples entailed autoclaving with $6\,N\,H_2SO_4$ at 121°C for 30 min, followed by neutralization to pH 7.0, filtration, and dilution with phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20. The value obtained for biotin in the pooled liver sample was 37.0 μg per 100 g fresh weight, which compared favorably with the microbiological assay value of 41 μg per 100 g fresh weight of lamb's liver quoted by Paul and Southgate [48]. From a biotin standard curve, the detection limit was calculated to be 10 pg/well.

22.3.2.3 Determination of Folate

An EPBA developed for folate determination in foods [49] has been applied to the determination of folate in raw and cooked vegetables

[50]. The immobilized phase was folic acid–KLH, the enzyme–protein conjugate was peroxidase–FBP, and the substrate was ABTS. On the basis of the interpretation of cross-reactivity data, the assay has no utility for the simultaneous determination of folic acid, 5-formyl-THF, and 5-methyl-THF in foods. However, the assay, using a folic acid standard, would be applicable for determining added folic acid in fortified food, with cross-reactions of less than 10% for the two THF derivatives and a detection limit of 6 pg folic acid per well. The requirement for analyzing nonfortified foods is to determine 5-formyl-THF and 5-methyl-THF, since these two compounds represent the major naturally occurring folates in food samples subjected to prolonged heat treatment during extraction [51]. Using the more stable 5-formyl-THF as the standard, the cross-reaction with 5-methyl-THF was 87%, which implies that the method would be suitable for estimating naturally occurring folate at detection limits of 34 and 36 pg per well for the two respective THF derivatives. Experiments using 5-formyl-THF–KLH-coated plates were unsuccessful in obtaining similar responses for the three folate compounds.

22.3.2.4 Determination of Vitamin B_{12}

In an EPBA, for the determination of cyanocobalamin in fortified breakfast cereals [52], the immobilized phase was cyanocobalamin–KLH, the enzyme–protein conjugate was peroxidase–R-protein, and the substrate was 3,3′,5,5′-tetramethylbenzidine. The immobilized phase was prepared by the bromoacetyl bromide coupling procedure using the primary alcohol group on the ribose 3-phosphate moiety. This method of synthesis gave an improved assay sensitivity compared to methods in which the conjugate was synthesized using the carboxylamide groups on the corrin ring structure [53,54]. The binding-protein–enzyme conjugate was prepared by reacting a dialysate of periodate-activated horseradish peroxidase with R-protein in pH 9.2 buffer. Sodium borohydride solution was added to the reaction mixture, and the mixture was stirred for 1 h before overnight dialysis against phosphate-buffered saline (pH 7.4) at 4°C. The conjugate was purified on a Superose 6 column and stored in glycerol/water (1:1, v/v) at −20°C. R-protein was used in preference to intrinsic factor because of its lower cost. R-protein binds all corrinoids (cobalamins and nonactive analogs), whereas intrinsic factor binds only cobalamins. However, for application to fortified foods, where a single vitamin B_{12} form (cyanocobalamin) predominates, the lack of absolute specificity is of no practical significance.

The extraction step in the sample preparation was designed to be compatible with the maintenance of protein-binding activity. Finely ground 10-g samples of breakfast cereals were shaken with 40 ml of buffered

(pH 7.0) methanol/water (1:1, v/v) containing sodium nitrate, and then centrifuged. The pellet was twice extracted with 25 ml and then 15 ml of extraction buffer, and the combined supernatants were made up to 100 ml with buffer. The lack of matrix interference was demonstrated by comparing standard curves prepared in extracts of nonfortified cereals and in assay buffer, and observing correlation coefficients of 0.971 and 0.972 for two different cereals. The assay exhibited a detection limit of 9 pg of cyanocobalamin standard per well, which was not sufficiently sensitive to measure the levels of naturally occurring vitamin B_{12} in foods.

22.4 Biomolecular Interaction Analysis

22.4.1 Principle

Biomolecular interaction analysis (BIA) is a biospecific technique based on biosensor technology. A biosensor is an instrument that combines a biological recognition mechanism with a transducer, which generates a measurable signal in response to changes in the concentration of a given biomolecule at the sensor. The BiacoreQuant® biosensor system (Biacore AB, Uppsala, Sweden) is a fully automated continuous-flow system, which exploits the phenomenon of surface plasmon resonance (SPR) to detect and measure biomolecular interactions [55]. The essential components are the sensor chip where the biomolecular interactions take place, the liquid handling flow system with an autosampler, precision pumps and an integrated μ-fluidic cartridge (IFC), and an optical detection unit. The continuous-flow technology with microfluidics allows rapid switching between sample and buffer at the sensor surface. The principal advantages of BIA compared with other biospecific techniques include real-time measurement, freedom from enzyme or radioisotope requirement, and enhanced precision.

The sensor chip consists of three layers: glass, a thin gold film, and a dextran matrix to which the analyte is covalently immobilized. The autosampler facilitates the transference of samples and reagents to mixing-positions in the microtiter plate or to the IFC injection port. Two syringe pumps, one for buffer flow and other for the autosampler functions, deliver a smooth pulse-free flow through the system. The IFC controls delivery of solutions to the sensor surface. By pressing the IFC against the sensor chip, the flow cells for detection are formed.

The optical detection unit is responsible for generation and detection of the SPR signal. A surface plasmon is a charged density wave that occurs at an interface between a thin metal film and another medium. Surface

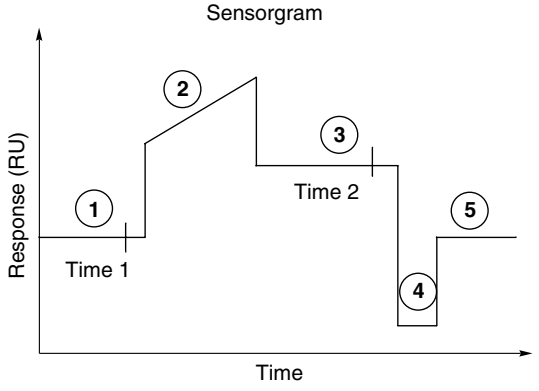

FIGURE 22.2
Plot of the SPR signal (expressed in resonance units, RU) against time in a sensorgram. Phases during a typical analytical cycle: (1) baseline, (2) binding of free analyte to specific protein or antibody, (3) response plateau, (4) regeneration of sensor surface, and (5) back to baseline.

plasmon waves become excited whenever energy is incident upon the thin film. SPR occurs when the energy from incident light of a particular frequency and angle of incidence is absorbed by the surface plasmon wave, resulting in a drop in intensity of the reflected light at a specific angle of reflection. This angle (the resonance angle) is very sensitive to the refractive index of the solution close to the sensor chip surface. Changes in the refractive index (e.g., after biomolecular interactions) will change the resonance angle and can be measured as a change in the SPR signal (expressed in resonance units, RU). The SPR signal is plotted against time in a sensorgram (Figure 22.2).

22.4.2 Biosensor-Based Immunoassay for Supplemental Biotin and Folate

In a fully validated BIA method for determining supplemental biotin and folic acid in infant formulas and milk powders [56], sample preparation for biotin analysis simply involved dissolution in water, sonication, centrifugation, and syringe filtration (0.22 μm). For folate analysis, the sonicated solution was heated at 100°C for 15 min to liberate folate from the milk FBP by protein denaturation. The BiacoreQuant system was configured as an immunoassay using monoclonal antibodies raised against analyte-conjugate. An excess of antibody was added to standard or sample extract and allowed to reach equilibrium binding with free analyte. When injected, noncomplexed antibodies were measured by

TABLE 22.1

Antibody Specificity

Antibiotin Antibody		Antifolic Acid Antibody	
Substance	**Cross-Reactivity (%)**	**Substance**	**Cross-Reactivity (%)**
Biotin	100	Folic acid	100
Biocytin	10	5-Methyl-THF	100[a]
Biotinyl-4-amidobenzoic acid	37	DHF	17
Lumichrome	38	THF	8
Riboflavin	0	5-Formyl-THF	0

[a] In the presence of ascorbate (1%, w/v).

Source: Indyk, H.E., Evans, E.A., Caselunghe, M.C.B., Persson, B.S., Finglas, P.M., Woollard, D.C., and Filonzi, E.L., *J. AOAC Int.*, 83, 1141, 2000. With permission.

the biosensor system when they bound to the analyte immobilized on the sensor chip. At the end of each analytical cycle, the sensor surface was prepared for a new sample by injection of a regeneration solution that dissociated the analyte–antibody complex on the surface. Antibody specificity for target analytes and cross-reactivities against related vitamers and potential interferences were evaluated and are summarized in Table 22.1. Dose–response sigmoid calibration curves established quantitation ranges for biotin and folic acid of 2–70 ng/ml.

22.4.3 Biosensor-Based Protein-Binding Assay for Supplemental and Endogenous Vitamin B_{12}

A fully validated BIA method has been reported for determining supplemental vitamin B_{12} in infant formulas and endogenous vitamin B_{12} in milk, beef, and liver [57]. Sample preparation involved vortex mixing with extraction buffer, standing for 30 min to allow conversion of all B_{12} vitamers to cyanocobalamin, autoclaving at 121°C for 25 min, cooling to ambient temperature, and syringe filtration (0.22 μm). The BiacoreQuant system was configured as a protein-binding assay using nonintrinsic R-protein. An excess of R-protein was added to standard or sample extract and allowed to reach equilibrium binding with free analyte. When injected, noncomplexed R-protein molecules were measured by the biosensor system when they bound to the analyte immobilized on the sensor chip. Regeneration solution was injected at the end of each analytical cycle. Dose–response calibration curves established quantitation ranges for cyanocobalamin of 0.08–2.40 ng/ml.

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23

Summarized Appraisal of Analytical Techniques

The purpose of this chapter is to make some inferences from the analytical methodology described in previous chapters and to illustrate the application of current analytical techniques.

23.1 Microbiological Assays

The turbidimetric microbiological assay is useful for estimating the total vitamin activity of a food or diet because it can be applied to all of the B-group vitamins and it is sensitive enough to measure the levels of naturally occurring vitamin. The assay organisms are selected on the basis of their specific requirement for vitamin forms that are biologically active in humans. Microbiological assays using lactic acid bacteria or yeasts require the chemical or enzymatic liberation of bound vitamin. The equipment, bacterial cultures, and ready-made media are commercially available and the assay procedure follows a standardized protocol. Once everything is set up, batches of samples can be analyzed simultaneously for most of the B vitamins. The use of microtiter plates allows automated determination and computer analysis of data. The results obtained from a microbiological assay provide an estimate of the total biological activity of a particular vitamin in the sample extract presented for analysis. Whether the results reflect bioavailable vitamin depends on the extraction procedure employed.

The standard turbidimetric method for determining vitamin B_6 using the yeast *Saccharomyces cerevisiae* underestimates the total vitamin B_6 content if the sample contains predominantly pyridoxamine (PM) (e.g., a processed meat product), as the microbial growth response to this vitamer is markedly less than that to pyridoxal (PL) or pyridoxine (PN). This unequal response is of little concern in plant-derived foods or foods that are fortified with PN · HCl. *Kloeckera apiculata* has been proposed as the assay organism in the standard turbidimetric and semiautomated radiometric–microbiological assay on the basis of an equivalent

growth response to all three free vitamers. However, this proposal has not found acceptance in certain other laboratories, in which *K. apiculata* was found to exhibit an even lower relative response to PM than that obtained with *S. cerevisiae.*

The microbiological assay using *Lactobacillus plantarum* is the standard method for determining biotin in foods, but the nutritional significance of the results obtained is largely dependent on the extraction procedure employed. Maximum liberation of bound biotin from animal tissues requires autoclaving the sample with 6 *N* H_2SO_4 for 2 h at 121°C, but these conditions promote losses of biotin in plant tissues, and a somewhat milder acid hydrolysis must be used for plant foods and food composites. Bitsch et al. [1] reported that treatment of samples with liquid nitrogen, followed by digestion with papain, was capable of quantitatively releasing bound biotin (including biotin from biocytin) from both plant and animal tissues.

Total folate is determined using *Lactobacillus rhamnosus* (*casei*) after enzymatic deconjugation to monoglutamyl forms. The basic premise using this approach is that all active monoglutamyl folates have identical equimolar growth-support activities for *L. rhamnosus* under the conditions of the assay. This assumption is, however, a subject of controversy. Careful control of pH and buffering capacity of the medium appear to be essential prerequisites. The overall picture reveals that the microbiological assay for folate is fragile, rather than robust. A comparison of HPLC and microbiological results using the same extract of each food for both assays showed significant differences [2,3]. For oat flakes, cabbage, orange juice, and particularly whole-wheat flour, the *L. rhamnosus* assay yielded higher folate values, suggesting the influence of nonfolate compounds on the bacterial growth response.

23.2 High-Performance Liquid Chromatography

23.2.1 Introduction

HPLC is the current method of choice for determining the fat-soluble vitamins and can distinguish between added and indigenous vitamin. The ability to quantify individual vitamers is important if the vitamers have different potencies. The ability to separate carotenoids provides the opportunity to identify these compounds using sophisticated detectors. Increases in lower potency *cis* isomers of carotenoids are an early indication of heat damage in thermally processed foods.

The high repeatability of HPLC makes it ideal for determining the added thiamin, riboflavin, nicotinamide, pyridoxine, pantothenic acid, and folic

acid in fortified foods. The determination of naturally occurring B vitamins by HPLC is made more complicated by the existence of several vitamers (especially in the case of vitamin B_6 and folate) and the need for more complicated extraction procedures to deal with the various bound forms. Pantothenic acid and biotin, which lack a strong chromophore, can be determined at naturally occurring levels after conversion to a fluorescent derivative. HPLC lacks the required sensitivity to accurately measure the extremely low indigenous levels of vitamin B_{12} in foods. HPLC is a popular technique for vitamin C analysis, despite the poor optical absorptivity of dehydroascorbic acid and its lack of electrochemical activity. It cannot be guaranteed that HPLC will account for all of the biologically active forms of a vitamin that would be measured by microbiological assay and, in this respect, the inherent specificity of HPLC may lead to an underestimation of total vitamin activity. Thiamin results in food products analyzed by the microbiological assay were found to be 15–39% higher than results obtained by HPLC [4].

23.2.2 Fat-Soluble Vitamins

23.2.2.1 Vitamin A

The AOAC method for determining retinyl palmitate in fortified fluid milk [5] is based on the method of Thompson et al. [6], with the addition of retinyl acetate as internal standard. A 2-ml milk sample is treated with ethanol to denature the proteins and fracture the fat globules. The total lipid fraction is extracted into hexane, and the proteins are removed by centrifugation. An aliquot of the hexane extract is injected onto a silica column for normal-phase chromatography with UV detection.

The AOAC method for determining naturally occurring retinol in all food categories [7] involves saponification and neutralization with glacial acetic acid. The solution is then diluted with tetrahydrofuran/95% ethanol (1 + 1) and refrigerated overnight to precipitate the fatty acid salts formed during saponification. After centrifugation, analysis is performed by reversed-phase HPLC with UV detection.

23.2.2.2 Carotenoids

Most published methods for carotenoid analysis employ nonaqueous reversed-phase chromatography with photodiode array detection. Silica-based polymeric C_{18}-bonded-phase column packings achieve the separation of all-*trans*-β-carotene from its principal 9-*cis* and 13-*cis* isomers [8]. The introduction of a polymeric C_{30}-bonded phase has advanced carotenoid analysis, as it is capable of resolving geometric isomers of asymmetric carotenoids such as α-carotene as well as those

of β-carotene [9]. The polymeric C_{30}-bonded phase has been used successfully to analyze provitamin A carotenoids in foods [10,11].

23.2.2.3 Vitamin D

The AOAC method for determining vitamin D_3 in infant formulas and enteral nutritional products [12] is based on the method of Sliva et al. [13] and involves saponification, solid-phase cleanup and concentration, and quantitative reversed-phase HPLC. The AOAC method for determining vitamin D_3 in selected foods [14] involves saponification, semipreparative normal-phase HPLC, and quantitative reversed-phase HPLC. In both AOAC methods, vitamin D_2 is used as an internal standard. Vitamin D_3 and 25-hydroxyvitamin D_3 have been determined in raw meat and liver using two steps of semipreparative HLPC [15].

23.2.2.4 Vitamin E

All of the eight unesterified tocopherols and tocotrienols can be separated isocratically by normal-phase HPLC and detected fluorometrically, making this technique ideal for the analysis of vegetable oils and fats [16]. Normal-phase HPLC with UV detection is the current AOAC method for determining supplemental vitamin E in milk-based infant formula [17].

Reversed-phase C_{18} columns of standard dimensions are unable to resolve the positional β and γ isomers of tocol and tocotrienol, even if gradient elution is used. Isocratic separation of all E vitamers can, however, be achieved using a pentafluorophenylsilica polar reversed-phase column and a methanol/water mobile phase [18].

23.2.2.5 Vitamin K

The AOAC method for determining vitamin K in milk and infant formulas [19] was developed by Indyk and Woollard [20]. The analytical protocol involves lipase digestion, solvent extraction, and nonaqueous reversed-phase HPLC using a C_{18} column, postcolumn reduction, and fluorescence detection. Woollard et al. [21] extended the enzymatic digestion procedure and employed a C_{30} column to determine *cis*- and *trans*-phylloquinone as well as menaquinones and dihydrophylloquinone. A C_{30} column was also used to determine *trans*-phylloquinone and dihydrophylloquinone in margarines and margarine-like products [22]. For the determination of phylloquinine and menaquinones in foods of animal origin, semipreparative normal-phase HPLC was used to isolate a vitamin K fraction, which was then analyzed by reversed-phase HPLC using $K_{1(25)}$ as an internal standard [23].

23.2.3 Water-Soluble Vitamins

23.2.3.1 Thiamin and Flavins

HPLC methods are well suited for determining thiamin and riboflavin in fortified foods and foods containing appreciable amounts of these vitamins. The use of a common extraction procedure allows these vitamins to be chromatographed either simultaneously or successively with a high degree of precision. Recovery data have indicated that riboflavin remains stable during the precolumn oxidation of thiamin to thiochrome [24]. Postcolumn chemistry eliminates the problem of reducing sugars (produced during acid hydrolysis) competing with thiamin for the oxidizing agent (ferricyanide) [25]. The HPLC procedure proposed by Reyes and Subryan [26] incorporates a number of desirable features designed to provide reliable results with high sensitivity and to prolong the life of the analytical column. Simultaneous determination of thiamin and riboflavin is achieved through simple reversed-phase chromatography at neutral pH using a single fluorescence detector. Cleanup and concentration of the ferricyanide-treated sample extract is effected by solid-phase extraction, which provides a purified and neutralized solution for injection. Thiamin values for raw and processed foods were the same (within experimental error) as those obtained by the 1984 AOAC fluorometric method [27], which uses Bio-Rex 70 in the purification step. The HPLC was further validated by the good agreement obtained between HPLC values for both thiamin and riboflavin with certified values on three dry AACC (American Association of Cereal Chemists) check samples.

Abdel-Kader [28] analyzed various enriched and nonenriched foods for thiamin using the AOAC manual fluorometric method and an HPLC method involving postcolumn derivatization and fluorometric detection of thiochrome. There was no statistical difference between the values obtained by the two procedures.

Several investigators have compared riboflavin values obtained by HPLC with those obtained by the AOAC manual fluorometric method. Good or reasonable agreement has been reported for the determination of riboflavin in rice and rice products [29]; milk, eggs, and dairy products [30,31]; infant formula products [32], blanched soya beans [33]; and various raw and processed foods [24,34–36]. Dramatically higher riboflavin values using the AOAC method were reported for soy products due to interference from fluorescent impurities that were not removed by oxidation with permanganate [37]. Evidence for this statement was that treatment of the samples with permanganate before HPLC analysis failed to eliminate all the nonvitamin peaks from the chromatogram. Higher riboflavin values have also been reported for fortified cereal products using a semiautomated modification of the AOAC method [25].

Favorable comparisons between HPLC and microbiological assay (*L. rhamnosus*) for riboflavin determinations have been reported for the analysis of infant formula products [32], blanched soya beans [33], and a wide range of foods [35,38–40]. For nonenriched flours and flours with a high rate of extraction, the HPLC values were as much as 25–50% lower than those found with microbiological assay [39]. This discrepancy was considered to be caused by an underestimation of vitamin B_2 activity by the HPLC procedure, possibly due to the chromatographic separation and nonmeasurement of biologically active isomeric riboflavin monophosphates.

23.2.3.2 Niacin

An HPLC method for determining niacin with solid-phase cleanup and UV detection produced results for SRM 1846 milk-based infant formula that were within uncertainty ranges of the certified value [41]. Lahély et al. [42] revitalized HPLC methodology for niacin by postcolumn conversion of nicotinamide and nicotinic acid to fluorescent derivatives by UV irradiation, thereby increasing the selectivity and sensitivity of detection. Cleanup of acid hydrolyzates was not necessary. The quantification limit was estimated at approximately 0.2 μg/g for a 5-g test sample of food. Rose-Sallin et al. [43] tested this method on a range of fortified products and obtained results that were in good accordance with certified values of both SRM 1846 and VMA 195 (a fortified cereal).

23.2.3.3 Vitamin B_6

Total vitamin B_6 in food is determined by HPLC after liberation of all bound forms by autoclaving samples at 120°C for 30 min in 0.1 *N* HCl followed by β-glucosidase/acid phosphatase hydrolysis [44]. The results obtained would overestimate bioavailable vitamin B_6 in plant-derived food samples, which contain significant amounts of pyridoxine-β-glucoside (PN-glucoside). However, the content of PN-glucoside could be estimated indirectly by analyzing the sample with and without the double-enzyme treatment. The difference in PN content between the two sets of results gives an estimate of PN-glucoside. The validated method of Reitzer-Bergaentzlé et al. [45] employs an acid phosphatase treatment that does not hydrolyze glycosylated PN and therefore yields results that represent bioavailable vitamin B_6. The sample preparation includes chemical conversion of PM to PL and subsequent reduction of PL to PN, thus only one peak (orginating from phosphorylated and free vitamers) need be measured in the chromatogram.

23.2.3.4 Pantothenic Acid

Woollard et al. [46] developed an HPLC method for determining the free endogenous D-pantothenic acid in milk and supplemental calcium pantothenate in infant formulas. The problem of poor spectral specificity in the low UV was tackled by combining photodiode array detection and online spectral analysis. A comparison of results with microbiological assay data showed good agreement.

23.2.3.5 Biotin

The low concentrations (5–50 ng/g) of biotin in most foods and the absence of a strong chromophore in the biotin molecule have precluded direct sensitive detection of this vitamin by HPLC. However, postcolumn derivatization with avidin–fluorescein 5-isothiocyanate (FITC) reagent enables biotin to be determined in natural foods by HPLC–fluorescence with a detection limit of 5 ng/g [47].

23.2.3.6 Folate

Doherty and Beecher [48] developed a robust procedure for distinguishing between naturally occurring 5-methyl-THF and added folic acid in foods. Folate deconjugation was accomplished by a tri-enzyme treatment and a large-capacity polystyrene–divinylbenzene-based solid-phase extraction column provided sample cleanup with quantitative recovery of analytes. Folic acid was converted to a fluorescent product by photolysis of the appropriate segment of the column effluent and detected by programmed fluorescence. The earlier-eluting 5-methyl-THF is naturally fluorescent. Results for foods were in close agreement with those from micobiological assay of total folate. Ndaw et al. [49] determined total folate in foods by chemically converting folates to 5-methyl-THF before sample purification by affinity chromatography.

23.2.3.7 Vitamin C

Many HPLC methods for determining vitamin C in foods have been reported, using a variety of separation techniques and detection systems. HPLC is accurate and sensitive for the determination of L-ascorbic acid, but it is less than ideal for determining total vitamin C. The poor optical absorptivity of dehydroascorbic acid and its lack of electrochemical activity necessitate the chemical reduction of this compound to ascorbic acid if absorbance or electrochemical detection is to be used. Fluorescence detection requires the chemical oxidation of ascorbic acid to dehydroascorbic acid and subsequent derivatization. If this derivatization is performed postcolumn, and ascorbic acid and dehydroascorbic acid are separated, the oxidation step can be omitted.

A simple and convenient protocol is to reduce the dehydroascorbic acid in the sample extract with dithiothreitiol and measure the ascorbic acid (representing total vitamin C) by reversed-phase HPLC and UV detection, using a photodiode array detector [50–52]. A low-pH mobile phase is necessary to achieve ion suppression, but a "shielded" stationary phase will be sterically protected from attack by hydrolyzing protons. Reversed-phase HPLC separates ascorbic acid from other organic acids and from erythorbic acid. This methodology has been subjected to an interlaboratory study and found to be suitable for the routine determination of total vitamin C in fruit juices and selected foods at 5–60 mg/100 g [52].

23.3 Supercritical Fluid Chromatography

Supercritical fluid chromatography (SFC) offers the possibility of a completely automated analysis of fat-soluble vitamins as it can be coupled directly to a supercritical fluid extraction (SFE) module. The exclusion of light, moderate temperature, and an oxygen-free environment backed by the solvating power of supercritical carbon dioxide are conducive to the analysis of fat-soluble vitamins and carotenoids. The elimination of organic solvents, normally consumed in large volumes, is beneficial with regard to pollution, inhalation, fire risk, and cost. Published applications include the separation of *cis–trans* isomers of β-carotene [53] and α-, β-, γ-, and δ-tocopherols using both packed [54] and capillary [55] columns.

23.4 Capillary Electrophoresis

Capillary electrophoresis has been used successfully in various application fields such as biochemistry, biotechnology, pharmaceutical analysis, and clinical chemistry. Relatively little impact has been made in food analysis. Advantages of capillary electrophoresis over HPLC are superior resolution of sample components, shorter run times, more robust columns, and cheaper operating costs. Disadvantages are a lower sensitivity attributable to the extremely low (nanoliter) injection volumes and small volume of the detector cell (a small section of the capillary column).

Micellar electrokinetic capillary chromatography (MECC) has been applied to the determination of thiamin in meat [56] and milk [57] and found to compare favorably with HPLC. Sample cleanup using a

cation-exchange column allowed concentration of the purified sample extract.

Cataldi et al. [58] established the optimal conditions to quantify riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) in common food samples by capillary zone electrophoresis (CZE) with laser-induced fluorescence detection. The sensitivity of the detector is two or three orders of magnitude higher than a conventional fluorescence detector. This allows dilution of the sample extract, thereby reducing matrix effects and the interference of other native fluorescent compounds. Samples were extracted with a solvent mixture and centrifuged, with no requirement for additional cleanup.

In a CZE method applied to the determination of niacin in fortified and natural foods [59–61], sample cleanup by solid-phase extraction concentrated the sample and provided the opportunity to redissolve the purified extract in a solvent that was compatible with the running buffer. Analysis of a standard reference cereal sample (VMA 195) gave an average concentration of niacin that compared well with the certified value [59]. Niacin values obtained for the various cereals, meats, and other foods compared favorably with results obtained by HPLC [59,61]. Analysis of concentrated yeast spreads gave results that were in good agreement with results obtained by the AOAC colorimetric procedure [60].

Capillary electrophoresis has been applied to the determination of vitamin C in fruits and vegetables [62] and citrus juice [63] using MECC and CZE modes, respectively. In both applications, results compared well with those obtained by HPLC. Choi and Jo [64] used the technique of sample stacking to concentrate sample extracts.

23.5 Flow-Injection Analysis

Flow-injection analysis has been used to automate several AOAC colorimetric and fluorometric methods for water-soluble vitamins, with a resultant improvement in precision. The use of immobilized ascorbate oxidase in flow-injection systems with amperometic detection [65,66] offers the opportunity for a precise, accurate, and automated vitamin C analysis using relatively simple apparatus.

23.6 Biospecific Methods

Enzyme-linked immunosorbent assays (ELISAs), enzyme-labeled protein-binding assays (EPBAs), and biomolecular interaction analysis

(BIA) use microtitration plates, thereby allowing the batchwise analysis of large numbers of sample extracts, without the problems of handling and disposal of radioactive material encountered with radio-labeled protein-binding assays (RPBAs) and radioimmunoassays. The high technology is built into the reagents, so the assays are simple to perform using automated procedures. It is still necessary, however, to chemically or enzymatically extract the vitamins from the food matrix, as these biospecific techniques depend upon the vitamins being in their free forms.

A biospecific assay suitable for determining total vitamin B_6 has not as yet been reported. Antisera raised against PL respond mainly to PM, although one antiserum exhibited 80% cross-reactivity with PN and could therefore be used in an ELISA for the determination of added PN in fortified foods [67].

An ELISA developed by Finglas et al. [68] can substitute for the microbiological assay in the determination of total pantothenic acid in foods, as shown by the high correlation for the analysis of foods using the same extract. The detection limit of the ELISA was at least tenfold lower than that of the microbiological assay.

Individual EPBAs have been reported for the determination of biotin, folate, and vitamin B_{12} using avidin, milk folate-binding protein, and R-protein as the respective vitamin-specific binding proteins. The biotin EPBA [69] has broad specificity and will include analogs of this vitamin, making it comparable to the microbiological assay. The folate EPBA [70] shows similar responses for 5-methyl-THF and 5-formyl-THF, but a different response for folic acid. Therefore, it is not possible to assay 5-methyl-THF, 5-formyl-THF, and folic acid simultaneously. However, the folate content of nonfortified foods (containing mainly 5-methyl-THF and 5-formyl-THF) and the added folic acid in fortified foods can be determined in separate assays, using 5-formyl-THF and folic acid as the respective calibrants. Folate results from the EPBA and the *L. rhamnosus* assay of Phillips and Wright [71] have been compared using regression analysis [72]. The data gave a linear relationship over the range 0–400 μg/100 g with a correlation coefficient, *r*, of 0.939, which signified good agreement. The folate values obtained using both assays were significantly higher than values reported in food composition tables, which were obtained using the *L. rhamnosus* method of Bell [73]. In an interlaboratory study [74], the analysis of a candidate reference material (lyophilized Brussels sprouts) by EPBA yielded results that were highly variable, indicating that further work is necessary to standardize the methodology. The vitamin B_{12} EPBA [75] could be applied to fortified foods, but lacks the sensitivity to measure the levels of naturally occurring vitamin B_{12} in foods.

The commercial availability of BIA assay kits for specific B vitamins and automated operation qualify BIA as a practical alternative to established microbiological techniques. The BiacoreQuant® system (Biacore AB, Uppsala, Sweden) has been applied to the determination of supplemental biotin and folic acid in infant formulas and milk powders using specific antibodies raised against these analytes [76]. The BIA for both vitamins was evaluated with reference to standard microbiological assays. Both methods demonstrated statistical equivalence for biotin, confirming an absence of significant difference ($P = 0.45$) with r of 0.9963. Comparative data were also obtained for folate ($P = 0.06$) with r of 0.9917. The BIA results were in agreement with assigned biotin and folate values for the standard reference material SRM 1846.

The BiacoreQuant system in the protein-binding configuration has been applied to the determination of supplemental vitamin B_{12} in infant formulas and endogenous vitamin B_{12} in milk, beef, and liver [77]. A range of samples was tested by BIA, microbiological assay, and RPBA. BIA yielded data statistically equivalent to the standard microbiological assay ($P = 0.32$ and $r = 0.9922$). RPBA estimations were generally low ($P < 0.05$) with an overall bias of ca. 8% relative to the microbiological assay. BIA results for three certified food reference materials were generally consistent with the assigned values based exclusively on microbiological assay.

23.7 Evaluation of Vitamin Bioavailability From Food Analysis Data

23.7.1 Fat-Soluble Vitamins

The fat-soluble vitamins are most commonly determined by HPLC. The various extraction techniques employed (saponification, enzymatic hydrolysis, solvent extraction, supercritical fluid extraction) are designed to extract the total vitamin present, a proportion of which exists in intimate association with membranes and lipoproteins. The analytical results obtained are accurate for vitamin content of the sample, but they provide no information about bioavailability.

23.7.2 Water-Soluble Vitamins

23.7.2.1 Thiamin

Endogenous thiamin is extracted by high-temperature acid digestion of the food sample followed by enzymatic hydrolysis. This two-step

procedure converts protein-bound and phosphorylated forms of thiamin to free thiamin. Thiamin is considered to be totally bioavailable in a wide range of food products, and therefore a chemical assay which measures total thiamin can substitute for a biological assay as a measure of bioavailable thiamin. This was illustrated in a study which compared a semiautomated fluorometric method with a rat bioassay for the determination of thiamin in green beans [78]. Calculation of bioavailable thiamin using the various rat assay dose–response curves indicated a mean value of 7.30 ± 2.46 μg/g, as thiamin hydrochloride, which compared favorably with the mean chemically determined value of 7.46 ± 0.14 μg/g.

23.7.2.2 Vitamin B_2

In the human digestive system, the FMN and FAD present in the ingested food are released from noncovalent binding to proteins as a consequence of acidification and proteolysis. The free coenzymes are then hydrolyzed to riboflavin, which is absorbed by active transport. In food analysis, endogenous riboflavin is extracted in a similar manner to thiamin. The acid/enzymatic hydrolysis converts coenzyme forms of vitamin B_2 (but not covalently bound FAD) to free riboflavin. The covalently bound FAD is largely unavailable as a source of vitamin B_2 in the rat [79] and therefore its nonextraction is fortuitous when bioavailable vitamin B_2 is being assessed. Because the extraction conditions liberate riboflavin in the manner of the human digestive system, the results obtained provide a good estimation of bioavailable vitamin B_2.

23.7.2.3 Niacin

In mature cereal grains, much of the niacin exists as chemically bound, largely unabsorbable nicotinic acid, which renders the cereals poor sources of dietary niacin, unless pretreated with alkali. In the analysis of cereal products, extraction techniques that liberate bound nicotinic acid will yield niacin values that overestimate the bioavailability of the product. The measurement of "free" (0.1 *N* acid-extractable) niacin in foods by HPLC is a convenient way of assessing bioavailable niacin. Values obtained probably underestimate the true bioavailability because bound nicotinic acid is partially used after ingestion [80]. Rose-Sallin et al. [43] reported that, using a common extraction procedure (0.1 *N* HCl, 100°C, 1 h), niacin values obtained by the microbiological assay with *L. plantarum* were higher than those obtained by HPLC. The most significant differences were observed for cereal-based foods. Thus, the *L. plantarum* assay is not completely specific for the bioavailable forms of niacin and HPLC yields a more accurate value.

23.7.2.4 Vitamin B_6

The vitamin B_6 content of many plant-derived foods is only partly bioavailable, owing to the presence of PN-glucoside, which is only partly utilized by the human. PN-glucoside is thus a determinant of vitamin B_6 bioavailability. The rat bioassay has been judged to be unsuitable for use in studies of vitamin B_6 bioavailability, even with the control of coprophagy [81]. Treatment of partially defatted food samples with pepsin and pancreatin is an attempt to simulate the human digestive process so that subsequent HPLC analysis represents bioavailable vitamin B_6 [82]. A more informative approach is to separate and quantify PN-glucoside, along with other B_6 vitamers, after an extraction procedure that preserves the integrity of the phosphorylated and glycosylated forms of vitamin B_6 [83–85].

23.7.2.5 Pantothenic Acid

In foods, pantothenic acid occurs mainly as coenzyme A, except in milk where free pantothenic acid predominates. The double-enzyme extraction technique that has become standard practice mimics enzyme actions in the human digestive system and therefore the results obtained are likely to be a good approximation of bioavailable pantothenic acid.

23.7.2.6 Biotin

The bioavailability of biotin in foods has not been studied directly in human subjects. The majority of naturally occurring biotin is covalently bound to the protein portion of biotin-dependent enzymes. Extraction of food by autoclaving in the presence of sulfuric acid is unreliable owing to the different conditions required for plant and animal tissues. In contrast, enzymatic hydrolysis with papain leads to a good approximation of bioavailable biotin [47].

23.7.2.7 Folate

For typical mixed diets, the bioavailability of naturally occurring folate is incomplete. Factors that affect folate bioavailability include the nature of the food matrix, the relative content of monoglutamyl and polyglutamyl forms, and the presence of naturally occurring inhibitors of intestinal folate deconjugation [86]. In food analysis, the tri-enzyme combination of conjugase, protease, and α-amylase extracts more folate from the sample than any other known method [87]. The tri-enzyme extraction technique can be used for microbiological or HPLC methods of analysis. HPLC provides information on individual folate vitamers present in food, whereas the microbiological assay gives a value for total folate.

Whichever analytical method is employed, it is unclear how well the results reflect bioavailable folate.

23.7.2.8 Vitamin B_{12}

Vitamin B_{12} is determined by microbiological assay following an extraction procedure that involves protein denaturation and conversion of B_{12} vitamers to a single stable form, sulfitocobalamin. The values obtained for meat and fish represent bioavailable B_{12} because the vitamin in these foods is absorbed by humans as efficiently as a comparable amount of crystalline cyanocobalamin dissolved in water. This is not the case with eggs, whose vitamin B_{12} content is poorly absorbed, owing to the presence of binding proteins in the white and yolk.

23.7.2.9 Vitamin C

Extraction techniques currently employed achieve high recoveries of ascorbic acid and dehydroascorbic acid from food matrices. The efficiency of vitamin C absorption of foods in typical western diets is also high (ca. 90%). Thus analytical methods that account for both ascorbic acid and dehydroascorbic acid give results that represent bioavailable vitamin C.

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Index

Vitamins in Foods: Analysis, Bioavailability, and Stability presents the latest information on vitamin analysis, bioavailability, and stability in foods. The contents of the book are divided into two sections to facilitate comprehension and use. The first section discusses vitamins' specific attributes and absorption capabilities. It then focuses on the major vitamin groups and their related compounds. The second section presents analytical considerations and techniques and information pertaining to both water- and fat-soluble vitamins.

Topics of particular interest include the problems associated with quantitatively extracting vitamins from the food matrix; assay techniques including immunoassays, protein binding, microbiological, and biosensor assays; the high-performance liquid chromatography (HPLC) methodology illustrated in tables with step-by-step details of sample preparation; representative separations (chromatograms) taken from original research papers reproduced together with ultraviolet and fluorescence spectra of vitamins; and appraisal of the various analytical approaches employed.

Comprehensive and complete, **Vitamins in Foods: Analysis, Bioavailability, and Stability** is a resource for those who need the latest information about vitamins, their chemical stability, analysis, and the way the body assimilates them.

Features

- Presents information on vitamin chemistry, absorption, and metabolism
- Discusses vitamin stability during the post-harvest handling, processing, storage, and preparation of food
- Explores the development of suitable analytical methods for research into vitamin bioavailability
- Examines the relationship of vitamin bioavailability to variables imposed by food constituents and food preparation

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