20 chapter

Vitamin Analysis

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20.1 INTRODUCTION

20.1.1 Definition and Importance

Vitamins are defined as relatively low-molecular-weight compounds which humans, and for that matter, any living organism that depends on organic matter as a source of nutrients, require in small quantities for normal metabolism. With few exceptions, humans cannot synthesize most vitamins and therefore need to obtain them from food and supplements. Insufficient levels of vitamins result in deficiency diseases [e.g., scurvy and pellagra, which are due to the lack of ascorbic acid (vitamin C) and niacin, respectively].

20.1.2 Importance of Analysis

Vitamin analysis of food and other biological samples has played a critical role in determining animal and human nutritional requirements. Furthermore, accurate food composition information is required to determine dietary intakes to assess diet adequacy and improve human nutrition worldwide. From the consumer and industry points of view, reliable assay methods are required to ensure accuracy of food labeling. This chapter provides an overview of techniques for analysis of the vitamin content of food.

20.1.3 Vitamin Units

When vitamins are expressed in units of mg or μg per tablet or food serving, it is very easy to grasp how much is present. Vitamins also can be expressed as **international units** (IU), **United States Pharmacopeia** (USP) **units**, and **% Daily Value** (DV). The IU is a unit of measurement for the amount of a substance, based on measured biological activity or effect. For details about IU and USP units of various vitamins, see the Vitamin Analysis chapter in the fourth edition of this textbook. For details about % DV for vitamins, see Chap. 3 in this textbook. When analysis of a foodstuff or dietary supplement is required for its content of vitamins, as might be the case for labeling and quality control purposes, being able to report the findings on different bases becomes important.

20.1.4 Extraction Methods

With the exception of some biological feeding studies, vitamin assays in most instances involve the extraction of a vitamin from its biological matrix prior to analysis. This generally includes one or several of the following treatments: **heat**, **acid**, **alkali**, **solvents**, and **enzymes**.

In general, extraction procedures are specific for each vitamin and designed to stabilize the vitamin. Some procedures are applicable to the combined extraction of more than one vitamin, for example, for thiamin and riboflavin as well as some of the fat-soluble vitamins [1, 2, 12]. Typical extraction procedures are as follows:

- *Ascorbic acid*: Cold extraction with *meta*phosphoric acid/acetic acid.
- *Vitamin B1 and B2*: Boiling or autoclaving in acid plus enzyme treatment.
- *Niacin*: Autoclaving in acid (noncereal products) or alkali (cereal products).
- *Folate*: Enzyme extraction with α-amylase, protease, and γ-glutamyl hydrolase (conjugase).
- *Vitamins A, E, or D*: Organic solvent extraction, saponification, and re-extraction with organic solvents. For unstable vitamins such as these, antioxidants are routinely added to inhibit oxidation.

For fat-soluble vitamins, the initial extraction with a hydrophobic organic solvent removes all fat-soluble compounds from the food, including all of the triacylglycerols. The **saponification** step that follows (generally either overnight at room temperature or by refluxing at 70 °C, using an antioxidant that protects the sample from oxidation) renders liberated fatty acids from the triacylglycerols insoluble in an organic solvent (because they now exist as soap, typically as a potassium salt), but the fat-soluble vitamins remain soluble. These vitamins are then re-extracted with a hydrophobic organic solvent and concentrated as needed.

20.1.5 Overview of Methods

Vitamin assays can be classified as follows:

1. **Bioassays** involving humans and animals
2. **Microbiological assays** making use of protozoan organisms, bacteria, and yeast
3. **Chemical assays** that include spectrophotometric, fluorometric, chromatographic, enzymatic, immunological, and radiometric methods

In terms of ease of performance, but not necessarily with regard to accuracy and precision, the three systems follow the reverse order. It is for this reason that bioassays, on a routine basis at least, are very limited in their use to those instances in which no satisfactory alternative method is available.

The selection criteria for a particular assay depend on a number of factors, including accuracy and precision, but also economic factors and the sample load to be handled. Applicability of certain methods for a particular matrix also must be considered. It is important to bear in mind that many official methods presented by regulatory agencies are limited in their applicability to certain matrices, such as vitamin concentrates, milk, or cereals, and thus cannot be applied to other matrices without some procedural modifications, if at all.

Because of the sensitivity of certain vitamins to adverse conditions such as light, oxygen, pH, and heat, proper precautions need to be taken to prevent any deterioration throughout the analytical process, regardless of the type of assay employed. Such precautionary steps need to be followed with the test material in bioassays throughout the feeding period. They are required with microbiological and chemical methods during extraction as well as during the analytical procedure.

Just as with any type of analysis, proper sampling and subsampling as well as the preparation of a homogeneous sample are critical aspects of vitamin analysis. General guidelines regarding this matter are provided in Chap. 5 of this text.

The principles and procedures for select vitamin analysis methods are described in this chapter. Calculations for select vitamins are described with the Practice Problems. Many of the methods cited are official methods of AOAC International [2], the British Standards Institution [3–10], or the US Pharmacopeial Convention [11]. Refer to these methods and other original references cited for detailed instructions on procedures. A summary of commonly used regulatory and other methods is provided in Table 20.1. The sections below on bioassay, microbiological, and chemical methods are not comprehensive, but rather just give examples of each type of analysis.

20.1 table Commonly used regulatory methods for vitamin analysis

Vitamin	*Method designation*	*Application*	*Approach*
Fat-soluble vitamins			
Vitamin A (and precursors)			
Retinol	AOAC[a] 992.04	Vitamin A in milk and milk-based infant formula	HPLC[b] 340 nm
Retinol	AOAC 2001.13	Vitamin A in foods	HPLC 328 or 313 nm
All-*trans*-retinol	AOAC 2011.07	Vitamin A in infant formula and adult nutritionals	UHPLC[c] 326 nm
All-*trans*-retinol 13-*cis*-retinol	EN 1283-1 [3]	All foods	HPLC 325 nm or Fluorescence[d] E_x λ = 325 nm E_m λ = 475 nm
β-Carotene	AOAC 2005.07	β-Carotene in supplements and raw materials	HPLC 445 or 444 nm
β-Carotene	EN 1283-2 [3]	All foods	HPLC 450 nm
Vitamin D			
Cholecalciferol Ergocalciferol	AOAC 936.14	Vitamin D in foods	Bioassay
Cholecalciferol Ergocalciferol	AOAC 995.05	Vitamin D in infant formula and enteral products	HPLC 265 nm
Cholecalciferol Ergocalciferol	AOAC 2002.05	Vitamin D in selected foods	HPLC 265 nm
Cholecalciferol Ergocalciferol	AOAC 2011.11	Vitamin D in infant formula and adult/pediatric nutritional formula	UHPLC-MS/MS[e]
Cholecalciferol Ergocalciferol	AOAC 2012.11	Simultaneous determination of vitamins D_2 and D_3 in infant formula and adult/pediatric nutritional formula	ESI[f] LC-MS/MS
Cholecalciferol Ergocalciferol	EN 1282172 [5]	Vitamin D in foods	HPLC 265 nm
Vitamin E			
All-racemic α-tocopherol	AOAC 2012.10	Simultaneous determination of vitamins E and A in infant formula and adult nutritionals	NP-HPLC[g] Fluorescence E_x λ = 280 nm E_m λ = 310 nm
α-tocopherol	AOAC 2012.09	Vitamins A and E in infant formula and adult/pediatric nutritional formula	HPLC Fluorescence E_x λ = 295 nm E_m λ = 330 nm

(continued)

20.1 table (continued)

Vitamin	*Method designation*	*Application*	*Approach*
R,R,R – tocopherols	EN 12822 [6]	Vitamin E in foods	HPLC Fluorescence E_x λ = 295 nm E_m λ = 330 nm
Vitamin K			
Phylloquinone	AOAC 999.15	Vitamin K in milk and infant formulas	HPLC postcolumn reduction, Fluorescence E_x λ = 243 nm E_m λ = 430 nm
Phytonadione (K_1)	AOAC 2015.09	*Trans*-vitamin K1 in infant, pediatric, and adult nutritionals	NP-HPLC postcolumn reduction, Fluorescence E_x λ = 245 nm E_m λ = 440 nm
Phylloquinone	EN 14148 [7]	Vitamin K in foods	HPLC postcolumn reduction, Fluorescence E_x λ = 243 nm E_m λ = 430 nm
Water-soluble vitamins			
Ascorbic acid (vitamin C)			
Ascorbic acid	AOAC 967.21	Vitamin C in juices and vitamin preparations	2,6-dichloroindophenol titration
Ascorbic acid	AOAC 967.22	Vitamin C in vitamin preparations	Fluorescence E_x λ = 350 nm E_m λ = 430 nm
Ascorbic acid	AOAC 2012.21	Vitamin C in infant formula and adult/pediatric nutritional formula	HPLC 254 nm
Ascorbic acid	AOAC 2012.22	Vitamin C in infant formula and adult/pediatric nutritional formula	UHPLC 254 nm
Thiamine (vitamin B_1)			
Thiamine	AOAC 942.23	Thiamine in foods	Thiochrome Fluorescence E_x λ = 365 nm E_m λ = 435 nm
Thiamine	AOAC 2015.14	Total vitamins B_1, B_2, and B_6 in infant formula and related nutritionals	Enzymatic digestion and UHPLC-MS/MS
Thiamine	EN 14122 [9]	Thiamine in foods	HPLC Thiochrome Fluorescence E_x λ = 366 nm E_m λ = 420 nm
Riboflavin (Vitamin B_2)			
Riboflavin	AOAC 970.65	Riboflavin in foods and vitamin preparations	Fluorescence Ex λ = 440 nm E_m λ = 565 nm
Riboflavin	AOAC 2015.14	Total vitamins B_1, B_2, and B_6 in infant formula and related nutritionals	Enzymatic digestion and UHPLC-MS/MS

(continued)

20.1 table (continued)

Vitamin	*Method designation*	*Application*	*Approach*
Riboflavin	EN 14152 [10]	Riboflavin in foods	HPLC Fluorescence E_x λ = 468 nm E_m λ = 520 nm
Niacin			
Nicotinic acid Nicotinamide	AOAC 944.13	Niacin and niacinamide in vitamin preparations	Microbiological
Nicotinic acid Nicotinamide	AOAC 985.34	Niacin and niacinamide in ready-to-feed milk-based infant formula	Microbiological
Vitamin B_6			
Pyridoxine Pyridoxal Pyridoxamine	AOAC 2004.07	Total vitamin B_6 in infant formula	HPLC Fluorescence E_x λ = 468 nm E_m λ = 520 nm
Pyridoxine Pyridoxal Pyridoxamine	AOAC 2015.14	Total vitamins B_1, B_2, and B_6 in infant formula and related nutritionals	Enzymatic digestion and UHPLC-MS/MS
Folic acid, folate			
Total folates	AOAC 2004.05	Total folates in cereals and cereal products – trienzyme procedure	Microbiological
Total folates	AOAC 2011.06	Total folates in infant formula and adult nutritionals	Trienzyme extraction and HPLC-MS/MS
Folic acid 5-methyl tetrahydrofolic acid	AOAC 2013.13	Folate in infant formula and adult/pediatric nutritional formula	Trienzyme extraction and UHPLC-MS/MS
Vitamin B_{12}			
Cyanocobalamin	AOAC 986.23	Cobalamin (vitamin B_{12}) in milk-based infant formula	Microbiological
Cyanocobalamin	AOAC 2011.10	Vitamin B_{12} in infant and pediatric formulas and adult nutritionals	HPLC 550 nm
Cyanocobalamin	AOAC 2014.02	Vitamin B_{12} in infant and pediatric formulas and adult nutritionals	UHPLC 361 nm
Biotin			
Biotin	USP29/NF24, dietary supplements official monograph [11]	Biotin in dietary supplements	HPLC 200 nm or microbiological
Pantothenic acid			
Calcium pantothenate	AOAC 992.07	Pantothenic acid in milk-based infant formula	Microbiological
Calcium pantothenate	AOAC 2012.16	Pantothenic acid (vitamin B_5) in infant formula and adult/pediatric nutritional formula	UHPLC-MS/MS

[a]AOAC method [2]
[b]HPLC, high-performance liquid chromatography (in some methods simply called liquid chromatography)
[c]UHPLC, ultra-HPLC
[d]Fluoremetric test giving excitation (E_x) and emission (E_m) wavelengths
[e]MS/MS, tandem mass spectrometry
[f]ESI, eletrospray ionization
[g]NP, normal phase

20.2 BIOASSAY METHODS

Outside of vitamin bioavailability studies, bioassays at the present are used only for the analysis of **vitamins B_{12}** and **D**, and even for them, the bioassays have very limited use. For vitamin D, the bioassay reference standard method (AOAC Method 936.14) (specified for milk, vitamin preparations, and feed concentrates) is known as the

line test, which is based on bone calcification. Rats are initially fed a diet that depletes rats of vitamin D and then groups of the rats are fed a diet with known (for standard curve) or unknown (sample) amounts of vitamin D. The rats are then sacrificed, and the sections of specific bones are stained to show the extent of bone calcification.

20.3 MICROBIOLOGICAL ASSAYS

20.3.1 Principle

The growth of microorganisms is proportional to their requirement for a specific vitamin, if all other nutritional needs of the microorganisms are met. Thus, in microbiological assays the growth of a certain microorganism in an extract of a vitamin-containing sample is compared against the growth of this microorganism in the presence of known quantities of that vitamin. Bacteria, yeast, or protozoans are used as test organisms. **Growth** can be measured in terms of **turbidity**, **acid production**, **gravimetry**, or by **respiration**. With bacteria and yeast, turbidimetry is the most commonly employed system. If turbidity measurements are involved, clear sample and standard extracts vs. turbid ones are essential. With regard to incubation time, turbidity measurement is also a less time-consuming method. The microorganisms are specified by ATCC™ numbers and are available from the *American Type Culture Collection* (ATCC™) (10801 University Blvd., Manassas, VA 20110).

20.3.2 Applications

Microbiological assays are limited to the analysis of water-soluble vitamins. The methods are very sensitive and specific for each vitamin. The methods are somewhat time consuming, and strict adherence to the analytical protocol is critical for accurate results. All microbiological assays can use microtiter plates (96-well) in place of test tubes. Microplate usage results in significant savings in media and glassware, as well as labor.

20.3.3 Niacin

The microbiological analysis of niacin and nicotinamide, as an example of such an assay, is briefly described here (AOAC Method 944.13, 45.2.04) [2, 13]. *Lactobacillus plantarum* ATCC™ 8014 is the test organism. A stock culture needs to be prepared and maintained by inoculating the freeze-dried culture on Bacto *Lactobacilli* agar followed by incubation at 37 °C for 24 h prior to sample and standard inoculation. A second transfer may be advisable in the case of poor growth of the inoculum culture. The final inoculum is added to tubes of niacin assay medium, that contain added known amounts of a USP niacin reference standard (for standard curve) and unknown amounts of niacin (food sample extract). The tubes are incubated at 37 °C for 16–24 h. The percent transmittance at a specific wavelength is measured to determine microbial growth as indicated by turbidity. Using *Lactobacilli sp*. as the test organism, acidimetric measurements could be used instead of turbidity, but the required incubation time would be 72 h.

20.4 CHEMICAL METHODS

20.4.1 High-Performance Liquid Chromatography (HPLC)

20.4.1.1 *Overview*

Because of their relative simplicity, accuracy, and precision, the chemical methods, in particular the chromatographic methods using HPLC/UHPLC, are preferred (see Chap. 13). Numerous vitamins are now commonly measured by HPLC (e.g., A, D, E, K, C, various B vitamins), many as official methods and some unofficial. Liquid chromatography in combination with mass spectrometry (MS) (see Chap. 11) has added a new dimension to vitamin analysis. In general, LC-MS or electrospray ionization (ESI) LC-MS/MS methods are available for each fat- and water-soluble vitamin. Detection by MS leads to increased sensitivity as well as unequivocal identification and characterization of the vitamin. The LC-MS assays have become a mainstay of accurate, cost-effective vitamin analyses. For example, LC-MS is commonly employed for verification of vitamin D content of products with difficult matrices (i.e., comparing results to those with standard LC analysis, e.g., AOAC Method 2012.11, Simultaneous Determination of Vitamins D_2 and D_3 in Infant Formula and Adult/Pediatric Nutritional Formula) and LC-MS/MS for folate (AOAC Method 2013.13, Folate in Infant Formula and Adult/Pediatric Nutritional Formula by a UHPLC-MS/MS assay vs. the microbiological method).

Standard HPLC is commonly employed as an official method of analysis for vitamins A (e.g., AOAC Method 992.04, 50.1.02), E (e.g., AOAC Method 992.03, 50.1.04), and D (e.g., AOAC Method 2002.05, 45.1.22A) and as a quality control method for vitamin C. While HPLC/UHPLC involves a high capital outlay, it is applicable to most vitamins and lends itself in some instances to simultaneous analysis of several vitamins and/or vitamers (i.e., isomers of vitamins). Implementation of multi-analyte procedures for the analysis of water-soluble vitamins can result in assay efficiency with savings in time and materials. To be useful, a simultaneous assay must not lead to loss of sensitivity, accuracy, and precision when compared to single-analyte methods. In general terms, multi-analyte methods for water-soluble vitamin assay of high-concentration products including pharmaceuticals, supplements, and vitamin premixes are quite easily developed. Though the applicability of HPLC has been demonstrated to a wide variety of biological matrices with no or only minor modifications in some cases, one must always bear in mind that all chromatographic

techniques, including HPLC, are separation and not identification methods. Therefore, during adaptation of an existing HPLC method to a new matrix, establishing evidence of peak identity and purity is an essential step of the method adaptation or development.

20.4.1.2 *Vitamin A*

Vitamin A is sensitive to ultraviolet (UV) light, air (and any prooxidants, for that matter), high temperatures, and moisture. Therefore, steps must be taken to avoid any adverse changes in this vitamin due to such effects. Steps include: (1) using low actinic glassware, nitrogen, and/or vacuum, (2) avoiding excessively high temperatures, (3) working in subdued artificial light, and (4) adding pyrogallol as an antioxidant prior to saponification.

HPLC methods are considered the only acceptable methods to provide accurate food measurements of vitamin A activity. For example, in the HPLC method of vitamin A (i.e., retinol isomers) in milk and milk-based infant formula (AOAC Method 992.04, 50.1.02) [2], the test sample is saponified with ethanolic KOH, vitamin A (retinol) is extracted into organic solvent, and then concentrated. Vitamin A isomers – all-*trans*-retinol and 13-*cis*-retinol – levels are determined by HPLC on a silica column (i.e., normal phase). Vitamin A also can be analyzed using reversed-phase HPLC columns.

20.4.1.3 *Vitamin D*

Vitamin D is typically analyzed by HPLC with a UV-Vis detector (some version of AOAC Method 2002.05) but by HPLC-MS for verification of analyte presence, as needed. Protection against oxidation is done as described for vitamin A above. For the HPLC-UV-Vis analysis, an internal standard (vitamin D_2) is added to the sample that is subjected to basic hydrolysis then saponified in ethanolic KOH. This sample is extracted with heptane, and the heptane organic phase is evaporated to dryness. The reconstituted sample is subjected to a semi-preparative normal-phase HPLC column, from which the fractions are collected, concentrated, and diluted in acetonitrile-methanol. These samples are subjected to a reversed-phase HPLC column with UV detection to quantitate the D_3. A separate sample is tested in parallel to confirm the absence of endogenous D_2.

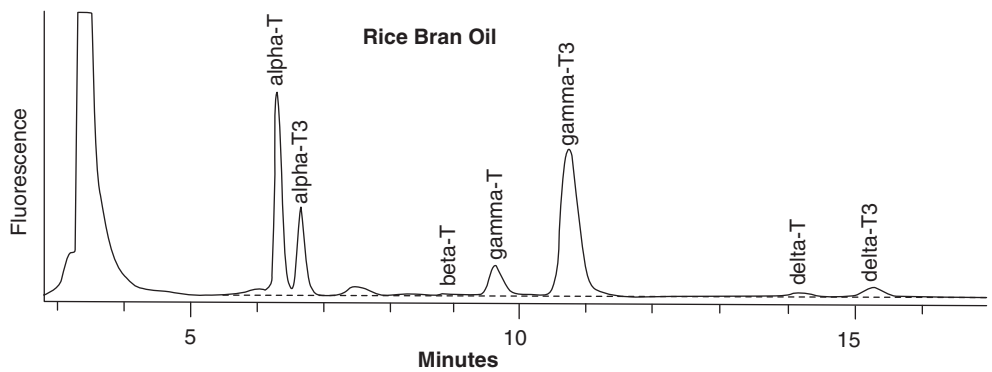
20.4.1.4 *Vitamin E (Tocopherols and Tocotrienols)*

Vitamin E is present in foods as eight different compounds: all are 6-hydroxychromans. The vitamin E family is comprised of α-, β-, γ-, and δ-tocopherol, characterized by a saturated side chain of three isoprenoid units and the corresponding unsaturated tocotrienols (α-, β-, γ-, and δ-). Like vitamins A and D, vitamin E must be protected from oxidation during sample preparation and is commonly analyzed by HPLC. Typically a normal or reversed-phase HPLC column is connected to a fluorescence detector: E_x $\lambda = 290$ nm and E_m $\lambda = 330$ nm (E_x, excitation; E_m, emission) (for fluorescence spectroscopy, see Chap. 7, Sect. 7.3) [14]. An example chromatogram is depicted in Fig. 20.1. Vitamin E is quantitated by external standards from peak area by linear regression.

20.4.2 Other Chemical Methods

20.4.2.1 *Vitamin C*

The vitamin (**L-ascorbic acid** and **L-dehydroascorbic acid**) is very susceptible to oxidative deterioration, which is enhanced by high pH and the presence of ferric and cupric ions. For these reasons, the entire analytical procedure needs to be performed at low pH and, if necessary, in the presence of a chelating agent. Mild oxidation of ascorbic acid results in the formation of dehydroascorbic acid, which is also biologically active and is reconvertible to ascorbic acid by treatment with **reducing agents** such as β-mercaptoethanol and dithiothreitol. Two AOAC official methods for vitamin C are described below, but vitamin C also can be analyzed in infant formula and adult/pediatric nutritional formula by HPLC with UV detection (AOAC Method 2012.21) and UHPLC with UV detection (AOAC Method 2012.22).



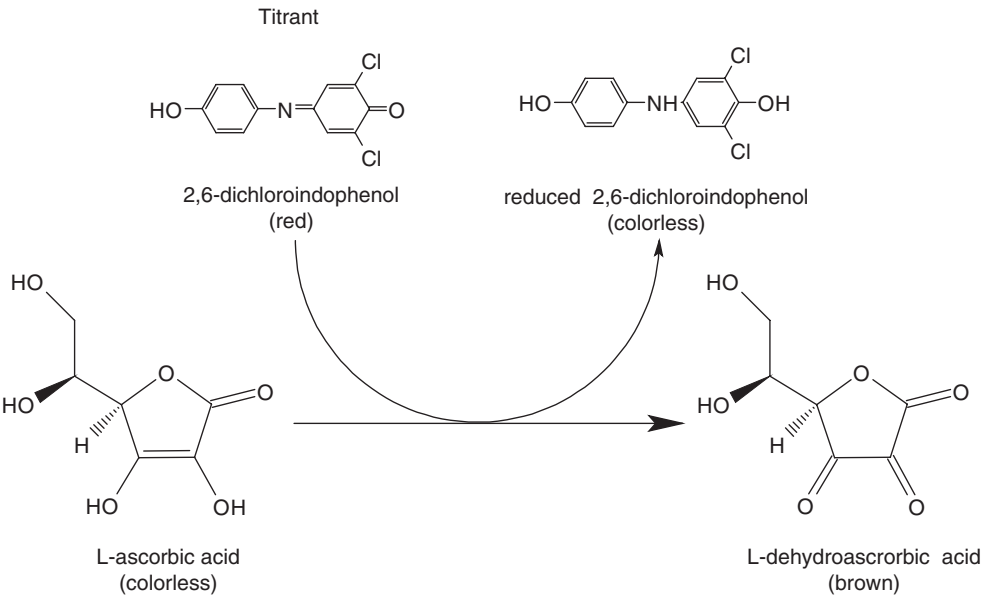
20.1 figure Chromatogram of rice bran oil showing tocopherols and tocotrienols

20.4.2.1.1 2,6-Dichloroindophenol (DCIP) Titrimetric Method

This method is specified as an AOAC official method (AOAC Method 967.21, 45.1.14) [2] for vitamin preparation and juices (i.e., fruits), but it is sometimes used as a secondary method for other foods, because it is a more rapid method than the microfluorometric method (described in Sec. 20.4.2.1.2) applicable to other foods. In the DCIP method, L-ascorbic acid is oxidized to L-dehydroascorbic acid by the oxidation-reduction indicator dye, DCIP. At the endpoint, excess unreduced dye appears rose pink in acid solution (see Figs. 20.2 and 20.3). With colored samples such as red beets or heavily browned products, the rose-pink endpoint is impossible to detect by the human eye. In such cases it, therefore, needs to be determined by observing the change of transmittance using a spectrophotometer with the wavelength set at 545 nm.

20.4.2.1.2 Microfluorometric Method

The vitamin C AOAC microfluorometric (AOAC Method 967.22, 45.1.15) assay is specified for vitamin preparations, but a semiautomated fluorometric AOAC method (AOAC Method 984.26, 45.1.16) is specified as applicable to all food products in the absence of erythorbate [2, 15]. The microfluorometric method mea-



20.2 figure Chemical reaction between L-ascorbic acid and the indicator dye, 2,6-dichloroindophenol

VITAMIN C ASSAY PROCEDURE
2,6-DICHLOROINDOPHENOL (DCIP) TITRATION

Sample Preparation

Weigh and extract by homogenizing test sample in *meta*phosphoric acid-acetic acid solution (*i.e.*, 15 g of HPO_3 and 40 ml of HOAc in 500 ml of deionized H_2O). Filter (and/or centrifuge) sample extract, and dilute appropriately to a final concentration of 10-100 mg of ascorbic acid/100 ml.

Standard Preparation

Weigh 50 mg of USP L-ascorbic acid reference standard and dilute to 50 ml with HPO_3-HOAc extracting solution.

Titration

Titrate three replicates each of the standard (*i.e.*, to determine the concentration of the indophenol solution as mg ascorbic acid equivalents to 1.0 ml of reagent), test sample, and blank with the indophenol reagent (*i.e.*, prepared by dissolving 50 mg of DCIP sodium salt and 42 mg of $NaHCO_3$ to 200 ml with deionized H_2O) to a light but distinctive rose pink endpoint lasting ≥ 5 sec.

20.3 figure Analysis of vitamin C by the 2,6-dichloroindophenol titration, AOAC Method 967.21, 45.1.14 [2] (Adapted from Pelletier [15])

sures both ascorbic acid and dehydroascorbic acid. All ascorbate forms are oxidized to dehydroascorbic acid (using a boric acid-sodium acetate solution), and then the dehydroascorbic acid is reacted with ***o*-phenylenediamine** to produce a **fluorescent quinoxaline compound**. The amount of fluorescence in the sample (compared to a standard and corrected with blanks) is used to quantitate the amount of vitamin C.

20.4.2.2 *Thiamine (Vitamin B_1) Thiochrome Fluorometric Method*

While thiamine can be quantitated by HPLC, it is still commonly analyzed by the longtime official thiochrome fluorometric procedure (AOAC Method 942.23) [2]. Following sample extraction with dilute acid, enzymatic hydrolysis of thiamine's phosphate esters, and chromatographic cleanup (i.e., purification), thiamine is oxidized to **thiochrome**, which is fluorescent. This method is based on the fluorescence measurement of thiochrome in the test solution compared to that from an oxidized thiamine standard solution.

20.4.2.3 *Riboflavin (Vitamin B_2) Fluorometric Method*

Like other B vitamins, riboflavin can be analyzed by HPLC, but its natural fluorescence allows for measurement based on this characteristic. Following sample extraction, cleanup, and compensation for the presence of interfering substances, riboflavin is determined fluorometrically, compared to a riboflavin standard (AOAC Method 970.65, 45.1.08) [2].

20.5 COMPARISON OF METHODS

Each type of method has its advantages and disadvantages. In selecting a certain method of analysis for a particular vitamin or vitamins, a number of factors need to be considered, some of which are listed below:

1. Method accuracy and precision
2. The need for bioavailability information
3. Time and instrumentation requirements
4. Personnel requirements
5. The type of biological matrix to be analyzed
6. The number of samples to be analyzed
7. Regulatory requirements – Must official AOAC International methods be used?

At present, the applicability of microbiological assays is limited to water-soluble vitamins (most commonly niacin, B_{12}, and pantothenic acid). Though somewhat time consuming, they generally can be used for the analysis of a relatively wide array of biological matrices without major modifications. Furthermore, less sample preparation is often required compared to chemical assays; yet, with more and more official methods being developed for HPLC and UHPLC, the employment of these microbiological assays is expected to decrease with time.

When selecting a system for analysis, at least initially, it is wise to consider the use of official methods that have been tested through interlaboratory studies and that are published by such organizations as AOAC International [2], the British Standards Institution [3–10], the US Pharmacopeial Convention [11], or the AACC International [16]. Again, one must realize that these methods are limited to certain biological matrices.

20.6 SUMMARY

Three types of methods for the analysis of vitamins – bioassays and microbiological and chemical assays – have been outlined in this chapter, with emphasis on the chemical methods. The methods are, in general, applicable to the analysis of more than one vitamin and several food matrices. However, the analytical procedures must be properly tailored to the analyte in question and the biological matrix to be analyzed; issues concerning sample preparation, extraction, and quantitative measurements are also involved. It is essential to validate any new application appropriately by assessing its accuracy and precision. Method validation is especially important with chromatographic methods such as HPLC, because these methods basically accent separations rather than identification of compounds. For this reason, it is essential to ensure not only identity of these compounds but also, just as important, their purity.

20.7 STUDY QUESTIONS

1. What factors should be considered in selecting the assay for a particular vitamin?
2. To be quantitated by most methods, vitamins must be extracted from foods. What treatments are commonly used to extract the vitamins? For one fat-soluble vitamin and one water-soluble vitamin, give an appropriate extraction procedure.
3. What vitamin must be listed on the US standard nutrition label as of 2018 (see Chap. 3, Sect. 3.2.1.1), and what would be an official method for its analysis?
4. Explain why it is possible to use microorganisms to quantitate a particular vitamin in a food product, and describe such a procedure.
5. There are two commonly used AOAC methods to measure the vitamin C content of foods. Identify these two methods; then compare and

contrast them with regard to the principles involved.
6. Would the vitamin C content as determined by the 2,6-dichloroindophenol method be underestimated or overestimated in the case of heat processed juice samples? Explain your answer.
7. What are the advantages and disadvantages of using HPLC for vitamin analysis?

20.8 PRACTICE PROBLEMS

Please refer to the fourth edition of this Food Analysis textbook for practice problems.

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