Food Analysis

Fourth Edition

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S. Suzanne Nielsen

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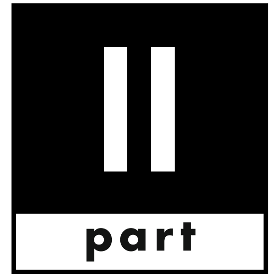

Compositional Analysis of Foods

Moisture and Total Solids Analysis

Robert L. Bradley, Jr.
Department of Food Science, University of Wisconsin, Madison, WI 53706, USA
rbradley@wisc.edu

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6.1 INTRODUCTION

Moisture assays can be one of the most important analyses performed on a food product and yet one of the most difficult from which to obtain accurate and precise data. This chapter describes various methods for moisture analysis – their principles, procedures, applications, cautions, advantages, and disadvantages. Water activity measurement also is described, since it parallels the measurement of total moisture as an important stability and quality factor. With an understanding of techniques described, one can apply appropriate moisture analyses to a wide variety of food products.

6.1.1 Importance of Moisture Assay

One of the most fundamental and important analytical procedures that can be performed on a food product is an assay for the amount of moisture (1–3). The dry matter that remains after moisture removal is commonly referred to as **total solids**. This analytical value is of great economic importance to a food manufacturer because water is an inexpensive filler. The following listing gives some examples in which moisture content is important to the food processor.

1. Moisture is a quality factor in the preservation of some products and affects stability in
   (a) Dehydrated vegetables and fruits
   (b) Dried milks
   (c) Powdered eggs
   (d) Dehydrated potatoes
   (e) Spices and herbs
2. Moisture is used as a quality factor for
   (a) Jams and jellies to prevent sugar crystallization
   (b) Sugar syrups
   (c) Prepared cereals – conventional, 4–8%; puffed, 7–8%
3. Reduced moisture is used for convenience in packaging or shipping of
   (a) Concentrated milks
   (b) Liquid cane sugar (67% solids) and liquid corn sweetener (80% solids)
   (c) Dehydrated products (these are difficult to package if too high in moisture)
   (d) Concentrated fruit juices
4. Moisture (or solids) content is often specified in compositional standards (i.e., Standards of Identity)
   (a) Cheddar cheese must be $\leq$39% moisture.
   (b) Enriched flour must be $\leq$15% moisture.
   (c) Pineapple juice must have soluble solids of $\geq$10.5°Brix (conditions specified).
   (d) Glucose syrup must have $\geq$70% total solids.
   (e) The percentage of added water in processed meats is commonly specified.
5. Computations of the nutritional value of foods require that you know the moisture content.
6. Moisture data are used to express results of other analytical determinations on a uniform basis [i.e., dry weight basis (dwb), rather than wet weight basis (wwb)].

6.1.2 Moisture Content of Foods

The moisture content of foods varies greatly as shown in Table 6-1 (4). Water is a major constituent of most food products. The approximate, expected moisture content of a food can affect the choice of the method of measurement. It can also guide the analyst in determining the practical level of accuracy required when measuring moisture content, relative to other food constituents.

6.1.3 Forms of Water in Foods

The ease of water removal from foods depends on how it exists in the food product. The three states of water in food products are:

1. **Free water:** This water retains its physical properties and thus acts as the dispersing agent for colloids and the solvent for salts.
2. **Adsorbed water:** This water is held tightly or is occluded in cell walls or protoplasm and is held tightly to proteins.
3. **Water of hydration:** This water is bound chemically, for example, lactose monohydrate; also some salts such as $Na_2SO_4 \cdot 10H_2O$.

Depending on the form of the water present in a food, the method used for determining moisture may measure more or less of the moisture present. This is the reason for official methods with stated procedures (5–7). However, several official methods may exist for a particular product. For example, the AOAC International methods for cheese include: Method 926.08, vacuum oven; 948.12, forced draft oven; 977.11, microwave oven; 969.19, distillation (5). Usually, the first method listed by AOAC International is preferred over others in any section.

6.1.4 Sample Collection and Handling

General procedures for sampling, sample handling and storage, and sample preparation are given in Chap. 5. These procedures are perhaps the greatest potential source of error in any analysis. Precautions must be taken to minimize inadvertent **moisture losses or gains** that occur during these steps.

6-1 table Moisture Content of Selected Foods

Food Item	*Approximate Percent Moisture (Wet Weight Basis)*
Cereals, bread, and pasta	
Wheat flour, whole-grain	10.3
White bread, enriched (wheat flour)	13.4
Corn flakes cereal	3.5
Crackers saltines	4.0
Macaroni, dry, enriched	9.9
Dairy products	
Milk, reduced fat, fluid, 2%	89.3
Yogurt, plain, low fat	85.1
Cottage cheese, low fat or 2% milk fat	80.7
Cheddar cheese	36.8
Ice cream, vanilla	61.0
Fats and oils	
Margarine, regular, hard, corn, hydrogenated	15.7
Butter, with salt	15.9
Oil-soybean, salad, or cooking	0
Fruits and vegetables	
Watermelon, raw	91.5
Oranges, raw, California navels	86.3
Apples, raw, with skin	85.6
Grapes, American type, raw	81.3
Raisins	15.3
Cucumbers, with peel, raw	95.2
Potatoes, microwaved, cooked in skin, flesh and skin	72.4
Snap beans, green, raw	90.3
Meat, poultry, and fish	
Beef, ground, raw, 95% lean	73.3
Chicken, broilers and fryers, light meat, meat and skin, raw	68.6
Finfish, flatfish (flounder and sole species), raw	79.1
Egg, whole, raw, fresh	75.8
Nuts	
Walnuts, black, dried	4.6
Peanuts, all types, dry roasted with salt	1.6
Peanut butter, smooth style, with salt	1.8
Sweeteners	
Sugar, granulated	0
Sugar, brown	1.3
Honey, strained or extracted	17.1

From US Department of Agriculture, Agricultural Research Service (2009) USDA National Nutrient Database for Standard Reference. Release 22. Nutrient Data Laboratory Home Page, http://www.ars.usda.gov/ba/bhnrc/ndl

Obviously, any exposure of a sample to the open atmosphere should be as short as possible. Any heating of a sample by friction during grinding should be minimized. Headspace in the sample storage container should be minimal because moisture is lost from the sample to equilibrate the container environment against the sample. It is critical to control temperature fluctuations since moisture will migrate in a sample to the colder part. To control this potential error, remove the entire sample from the container, reblend quickly, and then remove a test portion (8,9).

To illustrate the need for optimum efficiency and speed in weighing samples for analysis, Bradley and Vanderwarn (10) showed, using shredded Cheddar cheese (2–3 g in a 5.5-cm aluminum foil pan), that moisture loss within an analytical balance was a straight line function. The rate of loss was related to the relative humidity. At 50% relative humidity, it required only 5 s to lose 0.01% moisture. This time doubled at 70% humidity or 0.01% moisture loss in 10 s. While one might expect a curvilinear loss, the moisture loss was actually linear over a 5-min study interval. These data demonstrate the necessity of absolute control during collection of samples through weighing, before drying.

6.2 OVEN DRYING METHODS

In **oven drying methods**, the sample is heated under specified conditions, and the loss of weight is used to calculate the moisture content of the sample. The amount of moisture determined is highly dependent on the type of oven used, conditions within the oven, and the time and temperature of drying. Various oven methods are approved by AOAC International for determining the amount of moisture in many food products. The methods are simple, and many ovens allow for simultaneous analysis of large numbers of samples. The time required may be from a few minutes to over 24 h.

6.2.1 General Information

6.2.1.1 Removal of Moisture

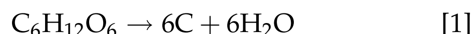
Any oven method used to evaporate moisture has as its foundation the fact that the boiling point of water is 100°C; however, this considers only pure water at sea level. Free water is the easiest of the three forms of water to remove. However, if 1 molecular weight (1 mol) of a solute is dissolved in 1.0 L of water, the boiling point would be raised by 0.512°C. This boiling point elevation continues throughout the moisture removal process as more and more concentration occurs.

Moisture removal is sometimes best achieved in a two-stage process. Liquid products (e.g., juices, milk) are commonly predried over a **steam bath** before drying in an oven. Products such as bread and field-dried grain are often air dried, then ground and oven dried, with the moisture content calculated from moisture

loss at both air and oven drying steps. Particle size, particle size distribution, sample sizes, and surface area during drying influence the rate and efficiency of moisture removal.

6.2.1.2 Decomposition of Other Food Constituents

Moisture loss from a sample during analysis is a function of time and temperature. Decomposition enters the picture when time is extended too much or temperature is too high. Thus, most methods for food moisture analysis involve a compromise between time and a particular temperature at which limited decomposition might be a factor. One major problem exists in that the physical process must separate all the moisture without decomposing any of the constituents that could release water. For example, carbohydrates decompose at 100°C according to the following reaction:

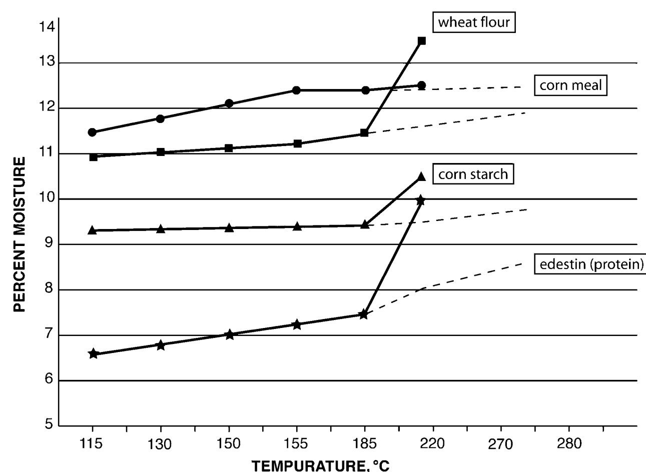


$$C_6H_{12}O_6 \rightarrow 6C + 6H_2O \quad [1]$$

The moisture generated in carbohydrate decomposition is not the moisture that we want to measure. Certain other chemical reactions (e.g., sucrose hydrolysis) can result in utilization of moisture, which would reduce the moisture for measurement. A less serious problem, but one that would be a consistent error, is the loss of **volatile constituents**, such as acetic, propionic, and butyric acids; and alcohols, esters, and aldehydes among flavor compounds. While weight changes in oven drying methods are assumed to be due to moisture loss, weight gains also can occur due to oxidation of unsaturated fatty acids and certain other compounds.

Nelson and Hulett (11) determined that moisture was retained in biological products to at least 365°C, which is coincidentally the critical temperature for water. Their data indicate that among the decomposition products at elevated temperatures were CO, CO_2, CH_4, and H_2O. These were not given off at any one particular temperature but at all temperatures and at different rates at the respective temperature in question.

By plotting moisture liberated against temperature, curves were obtained that show the amount of moisture liberated at each temperature (Fig. 6-1). Distinct breaks were shown that indicated the temperature at which decomposition became measurable. None of these curves showed any break before 184°C. Generally, proteins decompose at temperatures somewhat lower than required for starches and celluloses. Extrapolation of the flat portion of each curve to 250°C gave a true moisture content based on the assumption that there was no adsorbed water present at the temperature in question.



6-1 figure Moisture content of several foods held at various temperatures in an oven. The hyphenated line extrapolates data to 250°F, the true moisture content. [Reprinted with permission from (11) Nelson OA and Hulett GA. 1920. The moisture content of cereals. *J. Industrial Eng. Chem.* 12:40–45. Copyright 1920, American Chemical Society.]

6.2.1.3 Temperature Control

Drying methods utilize specified drying temperatures and times, which must be carefully controlled. Moreover, there may be considerable variability of temperature, depending on the type of oven used for moisture analysis. One should determine the extent of variation within an oven before relying on data collected from its use.

Consider the temperature variation in three types of ovens: **convection** (**atmospheric**), **forced draft**, and **vacuum**. The greatest temperature variation exists in a convection oven. This is because hot air slowly circulates without the aid of a fan. Air movement is obstructed further by pans placed in the oven. When the oven door is closed, the rate of temperature recovery is generally slow. This is dependent also upon the load placed in the oven and upon the ambient temperature. A 10°C temperature differential across a convection oven is not unusual. This must be considered in view of anticipated analytical accuracy and precision. A convection oven should not be used when precise and accurate measurements are needed.

Forced draft ovens have the least temperature differential across the interior of all ovens, usually not greater than 1°C. Air is circulated by a fan that forces air movement throughout the oven cavity. Forced draft ovens with air distribution manifolds appear to have

added benefit where air movement is horizontal across shelving. Thus, no matter whether the oven shelves are filled completely with moisture pans or only half filled, the result would be the same for a particular sample. This has been demonstrated using a Lab-Line oven (Melrose Park, IL) in which three stacking configurations for the pans were used (10). In one configuration, the oven shelves were filled with as many pans holding 2–3 g of Cheddar cheese as the forced draft oven could hold. In the two others, one-half of the full load of pans with cheese was used with the pans (1) in orderly vertical rows with the width of one pan between rows, or (2) staggered such that pans on every other shelf were in vertical alignment. The results after drying showed no difference in the mean value or the standard deviation.

Two features of some **vacuum ovens** contribute to a wider temperature spread across the oven. One feature is a glass panel in the door. Although from an educational point of view, it may be fascinating to observe some samples in the drying mode; the glass is a heat sink. The second feature is the way by which air is bled into the oven. If the air inlet and discharge are on opposite sides, conduct of air is virtually straight across the oven. Some newer models have air inlet and discharge manifolds mounted top and bottom. Air movement in this style of vacuum oven is upward from the front and then backward to the discharge in a broad sweep. The effect is to minimize cold spots as well as to exhaust moisture in the interior air.

6.2.1.4 Types of Pans for Oven Drying Methods

Pans used for moisture determinations are varied in shape and may or may not have a cover. The AOAC International (5) moisture pan is about 5.5 cm in diameter with an insert cover. Other pans have covers that slip over the outside edge of the pan. These pans, while reusable, are expensive, in terms of labor costs to clean appropriately to allow reuse.

Pan covers are necessary to control loss of sample by spattering during the heating process. If the cover is metal, it must be slipped to one side during drying to allow for moisture evaporation. However, this slipping of the cover also creates an area where spattering will result in product loss. Examine the interior of most moisture ovens and you will detect odor and deposits of burned-on residue, which, although undetected at the time of occurrence, produce erroneous results and large standard deviations (10).

Consider the use of **disposable pans** whenever possible; then purchase **glass fiber discs** for covers. At 5.5 cm in diameter, these covers fit perfectly inside disposable aluminum foil pans and prevent spattering while allowing the surface to breathe. Paper filter discs foul with fat and thus do not breathe effectively. Drying studies done on cheese using various pans and covers have shown that fat does spatter from pans with slipped covers, and fiberglass is the most satisfactory cover.

6.2.1.5 Handling and Preparation of Pans

The preparation and handling of pans before use requires consideration. Use only **tongs** to handle any pan. Even fingerprints have weight. All pans must be oven treated to prepare them for use. This is a factor of major importance unless disproved by the technologist doing moisture determinations with a particular type of pan. Disposable aluminum pans must be vacuum oven dried for 3 h before use. At 3 and 15 h in either a vacuum or forced draft oven at 100°C, pans varied in their weight within the error of the balance or 0.0001 g (10). Store dried moisture pans in a functioning **desiccator**. The glass fiber covers should be dried for 1 h before use.

6.2.1.6 Control of Surface Crust Formation (Sand Pan Technique)

Some food materials tend to form a semipermeable crust or lump together during drying, which will contribute to erratic and erroneous results. To control this problem, analysts use the **sand pan technique**. Clean, dry sand and a short glass stirring rod are preweighed into a moisture pan. Subsequently, after weighing in a sample, the sand and sample are admixed with the stirring rod left in the pan. The remainder of the procedure follows a standardized method if available; otherwise the sample is dried to constant weight. The purpose of the sand is twofold: to prevent **surface crust** from forming and to disperse the sample so evaporation of moisture is less impeded. The amount of sand used is a function of sample size. Consider 20–30 g sand/3 g sample to obtain desired distribution in the pan. Similar to the procedure, applications, and advantages of using sand, other heat-stable inert materials such as diatomaceous earth can be used in moisture determinations, especially for sticky fruits.

The inert matrices such as sand and **diatomaceous earth** function to disperse the food constituents and minimize the retention of moisture in the food products. However, the analyst must ascertain that the inert matrix used does not give erroneous results for the assay because of decomposition or entrapped moisture loss. Test the sand or other inert matrix for weight loss before using in any method. Add approximately 25 g of sand into a moisture pan and heat at 100°C for 2 h and weigh to 0.1 mg. Add 5 ml of water and mix

with the matrix using a glass rod. Heat dish, matrix, cover, and glass rod for at least 4 h at 100°C, reweigh. The difference between weighing must be less than 0.5 mg for any suitable matrix (12).

6.2.1.7 Calculations

Moisture and total solids contents of foods can be calculated as follows using oven drying procedures:

$$\%\text{Moisture (wt/wt)} = \frac{\text{wt } H_2O \text{ in sample}}{\text{wt of wet sample}} \times 100 \quad [2]$$

$$\%\text{Moisture (wt/wt)} = \frac{\text{wt of wet sample} - \text{wt of dry sample}}{\text{wt of wet sample}} \times 100 \quad [3]$$

$$\%\text{Total solids (wt/wt)} = \frac{\text{wt of dry sample}}{\text{wt of wet sample}} \times 100 \quad [4]$$

6.2.2 Forced Draft Oven

When using a forced draft oven, the sample is rapidly weighed into a predried moisture pan covered and placed in the oven for an arbitrarily selected time if no standardized method exists. Drying time periods for this method are 0.75–24 h (Table 6-2), depending on the food sample and its pretreatment; some liquid samples are dried initially on a steam bath at 100°C to minimize spattering. In these cases, drying times are shortened to 0.75–3 h. A forced draft oven is used with or without a steam table predrying treatment to determine the solids content of fluid milks (AOAC Method 990.19, 990.20).

An alternative to selecting a time period for drying is to weigh and reweigh the dried sample and pan until two successive weighings taken 30 min apart agree within a specified limit, for example, 0.1–0.2 mg for a 5-g sample. The user of this second method must be aware of sample transformation, such as browning which suggests moisture loss of the wrong form. Lipid oxidation and a resulting sample weight gain can occur at high temperatures in a forced draft oven. Samples high in carbohydrates should not be dried in a forced draft oven but rather in a vacuum oven at a temperature no higher than 70°C.

6.2.3 Vacuum Oven

By drying under reduced pressure (25–100 mm Hg), one is able to obtain a more complete removal of water and volatiles without decomposition within a 3–6-h drying time. Vacuum ovens need a dry air purge in addition to temperature and vacuum controls to operate within method definition. In older methods, a vacuum flask is used, partially filled with concentrated sulfuric acid as the desiccant. One or two air bubbles per second are passed through the acid. Recent changes now stipulate an air trap that is filled with calcium sulfate containing an indicator to show moisture saturation. Between the trap and the vacuum oven is an appropriately sized rotameter to measure air flow (100–120 ml/min) into the oven.

The following are important points in the use of a vacuum drying oven:

table 6-2 Forced Draft Oven Temperature and Times for Selected Foods

Product	*Dry on Steam Bath*	*Oven Temperature (°C ± 2)*	*Time in Oven (h)*
Buttermilk, liquid	X[a]	100	3
Cheese, natural type only		100	16.5 ± 0.5
Chocolate and cocoa		100	3
Cottage cheese		100	3
Cream, liquid and frozen	X	100	3
Egg albumin, liquid	X	130	0.75
Egg albumin, dried	X	100	0.75
Ice cream and frozen desserts	X	100	3.5
Milk	X	100	3
Whole, low fat, and skim		100	3
Condensed skim		100	3
Nuts: almonds, peanuts, walnuts		130	3

From (6) p. 492, with permission. Copyright 2004 by the American Public Health Association, Washington, DC.

[a] X = samples must be partially dried on steam bath before being placed in oven.

1. **Temperature** used depends on the product, such as 70°C for fruits and other high-sugar products. Even with reduced temperature, there can be some decomposition.
2. If the product to be assayed has a high concentration of **volatiles**, you should consider the use of a correction factor to compensate for the loss.
3. Analysts should remember that in a **vacuum**, heat is not conducted well. Thus pans must be placed directly on the metal shelves to conduct heat.
4. **Evaporation** is an endothermic process; thus, a pronounced cooling is observed. Because of the cooling effect of evaporation, when several samples are placed in an oven of this type, you will note that the temperature will drop. Do not attempt to compensate for the cooling effect by increasing the temperature, otherwise samples during the last stages of drying will be overheated.
5. The **drying time** is a function of the total moisture present, nature of the food, surface area per unit weight of sample, whether sand is used as a dispersant, and the relative concentration of sugars and other substances capable of retaining moisture or decomposing. The drying interval is determined experimentally to give reproducible results.

6.2.4 Microwave Analyzer

Determination of moisture in food products has traditionally been done using a standard oven, which, though accurate, can take many hours to dry a sample. Other methods have been developed over the years including infrared and various types of instruments that utilize halogen lamps or ceramic heating elements. They were often used for "spot checking" because of their speed, but they lacked the accuracy of the standard oven method. The introduction of microwave moisture/solids analyzers in the late 1970s gave laboratories the accuracy they needed and the speed they wanted. **Microwave moisture analysis**, often called **microwave drying**, was the first precise and rapid technique that allowed some segments of the food industry to make in-process adjustment of the moisture content in food products before final packaging. For example, processed cheese could be analyzed and the composition adjusted before the blend was dumped from the cooker. The ability to adjust the composition of a product in-process helps food manufacturers reduce production costs, meet regulatory requirements, and ensure product consistency. Such control could effectively pay for the microwave analyzer within a few months.

A particular microwave moisture/solids analyzer (CEM Corporation, Matthews, NC), or equivalent, is specified in the AOAC International procedures for total solids analysis of processed tomato products (AOAC Method 985.26) and moisture analysis of meat and poultry products (AOAC Method 985.14).

The general procedure for use of a microwave moisture/solids analyzer has been to set the microprocessor controller to a percentage of full power to control the microwave output. Power settings are dependent upon the type of sample and the recommendations of the manufacturer of the microwave moisture analyzer. Next, the internal balance is tared with two sample pads on the balance. As rapidly as possible, a sample is placed between the two pads, then pads are centered on the pedestal, and weighed against the tare weight. Time for the drying operation is set by the operator and "start" is activated. The microprocessor controls the drying procedure, with percentage moisture indicated in the controller window. Some newer models of microwave moisture analyzers have a temperature control feature to precisely control the drying process, removing the need to guess appropriate time and power settings for specific applications. These new models also have a smaller cavity that allows the microwave energy to be focused directly on the sample.

There are some considerations when using a microwave analyzer for moisture determination: (1) the sample must be of a uniform, appropriate size to provide for complete drying under the conditions specified; (2) the sample must be centrally located and evenly distributed, so some portions are not burned and other areas are underprocessed; and (3) the amount of time used to place an appropriate sample weight between the pads must be minimized to prevent moisture loss or gain before weight determination. Sample pads also should be considered. There are several different types, including fiberglass and quartz fiber pads. For optimum results, the pads should not absorb microwave energy, as this can cause the sample to burn, nor should they fray easily, as this causes them to lose weight and can affect the analysis. In addition, they should absorb liquids well.

Another style of microwave oven that includes a vacuum system is used in some food plants. This vacuum microwave oven will accommodate one sample in triplicate or three different samples at one time. In 10 min, the results are reported to be similar to 5 h in a vacuum oven at 100°C. The vacuum microwave oven is not nearly as widely used as conventional microwave analyzers, but can be beneficial in some applications.

Microwave drying provides a fast, accurate method to analyze many foods for moisture content. The method is sufficiently accurate for routine assay.

The distinct advantage of rapid analysis far outweighs its limitation of testing only single samples (13).

6.2.5 Infrared Drying

Infrared drying involves penetration of heat into the sample being dried, as compared with heat conductivity and convection with conventional ovens. Such heat penetration to evaporate moisture from the sample can significantly shorten the required drying time to 10–25 min. The infrared lamp used to supply heat to the sample results in a filament temperature of 2000–2500 K (degrees Kelvin). Factors that must be controlled include distance of the infrared source from the dried material and thickness of the sample. The analyst must be careful that the sample does not burn or case harden while drying. Infrared drying ovens may be equipped with forced ventilation to remove moisture air and an analytical balance to read moisture content directly. No infrared drying moisture analysis techniques are approved by AOAC International currently. However, because of the speed of analysis, this technique is suited for qualitative in-process use.

6.2.6 Rapid Moisture Analyzer Technology

Many rapid moisture/solids analyzers are available to the food industry. In addition to those based on infrared and microwave drying as described previously, compact instruments that depend on high heat are available, such as analyzers that detect moisture levels from 50 ppm to 100% using sample weights of 150 mg to 40 g (e.g., Computrac®, Arizona Instrument LLC, Chandler, AZ). Using a digital balance, the test sample is placed on an aluminum pan or filter paper and the heat control program (with a heating range of 25–275°C) elevates the test sample to a constant temperature. As the moisture is driven from the sample, the instrument automatically weighs and calculates the percentage moisture or solids. This technology is utilized to cover a wide range of applications within the food industry and offers quick and accurate results within minutes. These analyzers are utilized for both production and laboratory use with results comparable to reference methods.

6.3 DISTILLATION PROCEDURES

6.3.1 Overview

Distillation techniques involve codistilling the moisture in a food sample with a high boiling point solvent that is immiscible in water, collecting the mixture that distills off, and then measuring the volume of water. Two distillation procedures are in use today: **direct** and **reflux distillations**, with a variety of solvents. For example, in direct distillation with immiscible solvents of higher boiling point than water, the sample is heated in mineral oil or liquid with a flash point well above the boiling point for water. Other immiscible liquids with boiling point only slightly above water can be used (e.g., toluene, xylene, and benzene). However, reflux distillation with the immiscible solvent toluene is the most widely used method.

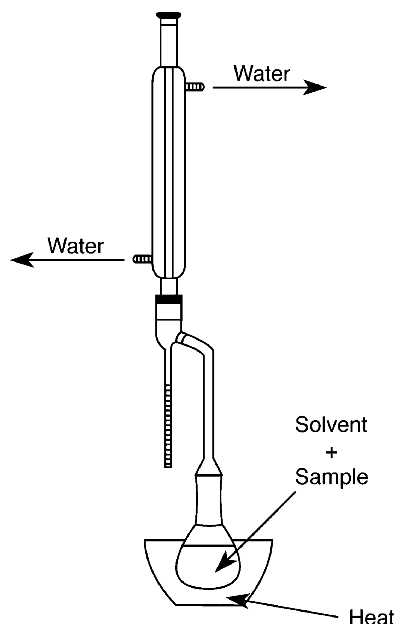
Distillation techniques were originally developed as rapid methods for quality control work, but they are not adaptable to routine testing. The distillation method is an AOAC-approved technique for moisture analysis of spices (AOAC Method 986.21), cheese (AOAC Method 969.19), and animal feeds (AOAC Method 925.04). It also can give good accuracy and precision for nuts, oils, soaps, and waxes.

Distillation methods cause less thermal decomposition of some foods than oven drying at high temperatures. Adverse chemical reactions are not eliminated but can be minimized by using a solvent with a lower boiling point. This, however, will increase distillation times. Water is measured directly in the distillation procedure (rather than by weight loss), but reading the volume of water in a receiving tube may be less accurate than using a weight measurement.

6.3.2 Reflux Distillation with Immiscible Solvent

Reflux distillation uses either a solvent less dense than water (e.g., toluene, with a boiling point of 110.6°C; or xylene, with a boiling range of 137–140°C) or a solvent more dense than water (e.g., tetrachlorethylene, with a boiling point of 121°C). The advantage of using this last solvent is that material to be dried floats; therefore it will not char or burn. In addition, there is no fire hazard with this solvent.

A **Bidwell–Sterling moisture trap** (Fig. 6-2) is commonly used as part of the apparatus for reflux distillation with a solvent less dense than water. The distillation procedure using such a trap is described in Fig. 6-3, with emphasis placed on dislodging adhering water drops, thereby minimizing error. When the toluene in the distillation just starts to boil, the analyst will observe a hazy cloud rising in the distillation flask. This is a vaporous emulsion of water in toluene. Condensation occurs as the vapors rise, heating the vessel, the Bidwell–Sterling trap, and the bottom of the condenser. It is also hazy at the cold surface of the condenser, where water droplets are visible. The emulsion inverts and becomes toluene dispersed in water. This turbidity clears very slowly on cooling.



6-2 figure Apparatus for reflux distillation of moisture from a food. Key to this setup is the Bidwell–Sterling moisture trap. This style can be used only where the solvent is less dense than water.

Three potential sources of error with distillation should be eliminated if observed:

1. Formation of emulsions that will not break. Usually this can be controlled by allowing the apparatus to cool after distillation is completed and before reading the amount of moisture in the trap.
2. Clinging of water droplets to dirty apparatus. Clean glassware is essential, but water seems to cling even with the best cleaning effort. A burette brush, with the handle end flattened so it will pass down the condenser, is needed to dislodge moisture droplets.
3. Decomposition of the sample with production of water. This is principally due to carbohydrate decomposition to generate water ($C_6H_{12}O_6 \rightarrow 6H_2O + 6C$). If this is a measurable problem, discontinue method use and find an alternative procedure.

REFLUX DISTILLATION

Place sample in distillation flask and cover completely with solvent.

⇓

Fill the receiving tube (e.g., Bidwell-Sterling Trap) with solvent, by pouring it through the top of the condenser.

⇓

Bring to a boil and distill slowly at first then at increased rate.

⇓

After the distillation has proceeded for approximately 1 hr, use an adapted buret brush to dislodge moisture droplets from the condenser and top part of the Bidwell-Sterling trap.

⇓

Slide the brush up the condenser to a point above the vapor condensing area.

⇓

Rinse the brush and wire with a small amount of toluene to dislodge adhering water drops.

⇓

If water has adhered to the walls of the calibrated tube, invert the brush and use the straight wire to dislodge this water so it collects in the bottom of the tube.

⇓

Return the wire to a point above the condensation point, and rinse with another small amount of toluene.

⇓

After no more water has distilled from the sample, repeat the brush and wire routine to dislodge adhering water droplets.

⇓

Rinse the brush and wire with toluene before removing from the condenser.

⇓

Allow the apparatus to cool to ambient temperatures before measuring the volume of water in the trap.

⇓

Volume of water x 2 (for a 50 g sample) = % moisture

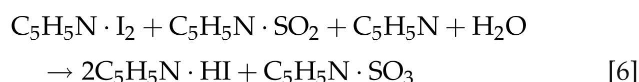
6-3 figure Procedures for reflux distillation with toluene using a Bidwell–Sterling trap. Steps to dislodge adhering moisture drops are given.

6.4 CHEMICAL METHOD: KARL FISCHER TITRATION

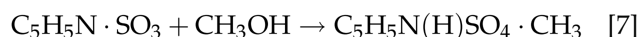
The **Karl Fischer titration** is particularly adaptable to food products that show erratic results when heated or submitted to a vacuum. This is the method of choice for determination of water in many low-moisture foods such as dried fruits and vegetables (AOAC Method 967.19 E-G), candies, chocolate (AOAC Method 977.10), roasted coffee, oils and fats (AOAC Method 984.20), or any low-moisture food high in sugar or protein. The method is quite rapid, is accurate, and uses no heat. This method is based on the fundamental reaction described by Bunsen in 1853 (14) involving the reduction of iodine by SO_2 in the presence of water:

$$2H_2O + SO_2 + I_2 \rightarrow C_5H_2SO_4 + 2HI \quad [5]$$

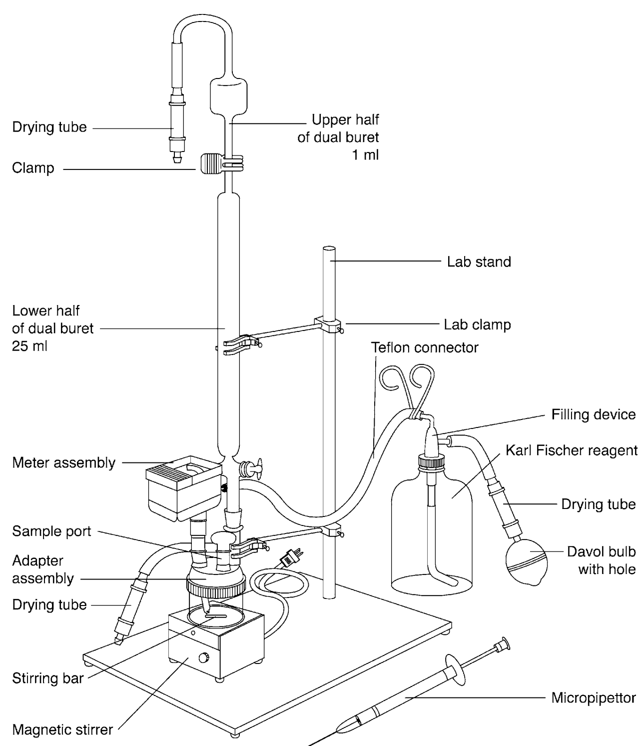
This was modified to include methanol and pyridine in a four-component system to dissolve the iodine and SO_2:



$$C_5H_5N \cdot I_2 + C_5H_5N \cdot SO_2 + C_5H_5N + H_2O \rightarrow 2C_5H_5N \cdot HI + C_5H_5N \cdot SO_3 \quad [6]$$



$$C_5H_5N \cdot SO_3 + CH_3OH \rightarrow C_5H_5N(H)SO_4 \cdot CH_3 \quad [7]$$



6-4 figure Manual Karl Fischer titration unit. (Courtesy of Lab Industries, Inc., Berkeley, CA.)

6-5 figure Automated Karl Fischer volumetric titration unit. (Courtesy of Mettler-Toledo, Columbus, OH.)

These reactions show that for each mole of water, 1 mol of iodine, 1 mol of SO_2, 3 mol of pyridine, and 1 mol of methanol are used. For general work, a methanolic solution is used that contains these components in the ratio of 1 iodine:3 SO_2:10 pyridine, and at a concentration so that 3.5 mg of water = 1 ml of reagent. A procedure for standardizing this reagent is given below.

In a **volumetric titration** procedure (Fig. 6-4 is manual titration unit; Fig. 6-5 is example of automated titration unit), iodine and SO_2 in the appropriate form are added to the sample in a closed chamber protected from atmospheric moisture. The excess of I_2 that cannot react with the water can be determined **visually**. The endpoint color is dark red-brown. Some instrumental systems are improved by the inclusion of a potentiometer (i.e., **conductometric method**) to electronically determine the endpoint, which increases the sensitivity and accuracy. The volumetric titration can be done manually (Fig. 6-4) or with an automated unit (Fig. 6-5 is one example instrument). The automated volumetric titration units (used for 100 ppm water to very high concentrations) use a pump for mechanical addition of titrant and use the conductometric method for endpoint determination (i.e., detection of excess iodine is by applying a current and measuring the potential).

The volumetric titration procedure described above is appropriate for samples with a moisture content greater than ~0.03%. A second type of titration, referred to as **coulometric titration**, is ideal for products with very low levels of moisture, from 0.03% down to parts per million (ppm) levels. In this method, iodine is electrolytically generated ($2I \rightarrow I_2 + 2e^-$) to titrate the moisture. The amount of iodine required to titrate the moisture is determined by the current needed to generate the iodine. Just like for volumetric titration, automated coulometric titration units are available commercially.

In a Karl Fischer volumetric titration, the **Karl Fischer reagent** (KFR) is added directly as the titrant if the moisture in the sample is accessible. However, if moisture in a solid sample is inaccessible to the reagent, the moisture is extracted from the food with an appropriate solvent (e.g., methanol). (Particle size affects efficiency of extraction directly.) Then the methanol extract is titrated with KFR.

The obnoxious odor of pyridine makes it an undesirable reagent. Therefore, researchers have experimented with other amines capable of dissolving iodine and sulfur dioxide. Some aliphatic amines and several other heterocyclic compounds were found suitable. On the basis of these new amines, **one-component reagents** (solvent and titrant components

together) and **two-component reagents** (solvent and titrant components separate) have been prepared. The one-component reagent may be more convenient to use, but the two-component reagent has greater storage stability.

Before the amount of water found in a food sample can be determined, a **KFR water (moisture) equivalence** (KFReq) must be determined. The KFReq value represents the equivalent amount of moisture that reacts with 1 ml of KFR. Standardization must be checked before each use because the KFReq will change with time.

The KFReq can be established with **pure water**, a **water-in-methanol standard**, or **sodium tartrate dihydrate**. Pure water is a difficult standard to use because of inaccuracy in measuring the small amounts required. The water-in-methanol standard is premixed by the manufacturer and generally contains 1 mg of water/ml of solution. This standard can change over prolonged storage periods by absorbing atmospheric moisture. Sodium tartrate dihydrate ($Na_2C_4H_4O_6{\cdot}2H_2O$) is a primary standard for determining KFReq. This compound is very stable, contains 15.66% water under all conditions expected in the laboratory, and is the material of choice to use.

The KFReq is calculated as follows using sodium tartrate dihydrate:

$$\text{KFReq}\,(\text{mg}\,H_2O/\text{ml}) = \frac{36\,\text{g}\,H_2O/\text{mol}\,Na_2C_4H_4O_6 \cdot 2H_2O \times S \times 1000}{230.08\,\text{g/mol} \times A} \quad [8]$$

where:

KFReq = Karl Fischer reagent moisture equivalence
S = weight of sodium tartrate dihydrate (g)
A = ml of KFR required for titration of sodium tartrate dehydrate

Once the KFReq is known, the moisture content of the sample is determined as follows:

$$\%H_2O = \frac{\text{KFReq} \times \text{Ks}}{S} \times 100 \quad [9]$$

where:

KFReq = Karl Fischer reagent water (moisture) equivalence
Ks = ml of KFR used to titrate sample
S = weight of sample (mg)

The major difficulties and sources of error in the Karl Fischer titration methods are as follows:

1. **Incomplete moisture extraction**. For this reason, fineness of grind (i.e., particle size) is important in preparation of cereal grains and some foods.
2. **Atmospheric moisture**. External air must not be allowed to infiltrate the reaction chamber.
3. **Moisture adhering** to walls of unit. All glassware and utensils must be carefully dried.
4. **Interferences** from certain food constituents. **Ascorbic acid** is oxidized by KFR to dehydroascorbic acid to overestimate moisture content; **carbonyl compounds** react with methanol to form acetals and release water to overestimate moisture content (this reaction also may result in fading endpoints); **unsaturated fatty acids** will react with iodine, so moisture content will be overestimated.

6.5 PHYSICAL METHODS

6.5.1 Dielectric Method

The electrical properties of water are used in the **dielectric method** to determine the moisture content of certain foods, by measuring the change in **capacitance** or **resistance to an electric current** passed through a sample. These instruments require calibration against samples of known moisture content as determined by standard methods. Sample density or weight/volume relationships and sample temperature are important factors to control in making reliable and repeatable measurements by dielectric methods. These techniques can be very useful for process control measurement applications, where continuous measurement is required. These methods are limited to food systems that contain no more than 30–35% moisture.

The moisture determination in dielectric-type meters is based on the fact that the dielectric constant of water (80.37 at 20°C) is higher than that of most solvents. The **dielectric constant** is measured as an index of capacitance. As an example, the dielectric method is used widely for cereal grains. Its use is based on the fact that water has a dielectric constant of 80.37, whereas starches and proteins found in cereals have dielectric constants of 10. By determining this properly on samples in standard metal condensers, dial readings may be obtained and the percentage of moisture determined from a previously constructed standard curve for a particular cereal grain.

6.5.2 Hydrometry

Hydrometry is the science of measuring **specific gravity** or **density**, which can be done using several different principles and instruments. While hydrometry is considered archaic in some analytical circles, it is still widely used and, with proper technique, is highly

accurate. Specific gravity measurements with various types of **hydrometers** or with a **pycnometer** are commonly used for routine testing of moisture (or solids) content of numerous food products. These include beverages, salt brines, and sugar solutions. Specific gravity measurements are best applied to the analysis of solutions consisting of only one solute in a medium of water.

6.5.2.1 Hydrometer

A second approach to measuring specific gravity is based on **Archimedes' principle**, which states that a solid suspended in a liquid will be buoyed by a force equal to the weight of the liquid displaced. The weight per unit volume of a liquid is determined by measuring the volume displaced by an object of standard weight. A hydrometer is a standard weight on the end of a spindle, and it displaces a weight of liquid equal to its own weight (Fig. 6-6). For example, in a liquid of low density, the hydrometer will sink to a greater depth, whereas in a liquid of high density, the hydrometer will not sink as far. Hydrometers are available in narrow and wide ranges of specific gravity. The spindle of the hydrometer is calibrated to read specific gravity directly at 15.5 or 20°C. A **hydrometer** is not as accurate as a pycnometer, but the speed with which you can do an analysis is a decisive factor. The accuracy of specific gravity measurements can be improved by using a hydrometer calibrated in the desired range of specific gravities.

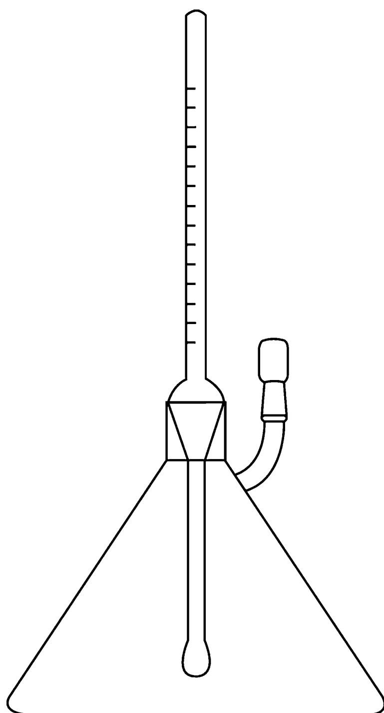
The rudimentary but surprisingly accurate hydrometer comes equipped with various modifications depending on the fluid to be measured:

1. The Quevenne and New York Board of Health **lactometer** is used to determine the density of milk. The Quevenne lactometer reads from 15 to 40 lactometer units and corresponds to 1.015 to 1.040 specific gravity. For every degree above 60°F, 0.1 lactometer unit is added to the reading, and 0.1 lactometer unit is subtracted for every degree below 60°F.
2. The **Baumé hydrometer** was used originally to determine the density of salt solutions (originally 10% salt), but it has come into much wider use. From the value obtained in the Baumé scale, you can convert to specific gravity of liquids heavier than water. For example, it is used to determine the specific gravity of milk being condensed in a vacuum pan.
3. The **Brix hydrometer** is a type of **saccharometer** used for sugar solutions such as fruit juices and syrups and one usually reads directly the percentage of sucrose at 20°C. **Balling saccharometers** are graduated to indicate percentage of sugar by weight at 60°F. The terms **Brix** and **Balling** are interpreted as the weight percentage of pure sucrose.
4. **Alcoholometers** are used to estimate the alcohol content of beverages. Such hydrometers are calibrated in 0.1 or 0.2° proof to determine the percentage of alcohol in distilled liquors (AOAC Method 957.03).
5. The **Twaddell hydrometer** is only for liquids heavier than water.

6-6 figure Hydrometers. (Courtesy of Cole-Parmer Instrument Company, Vernon Hills, IL.)

6.5.2.2 Pycnometer

One approach to measuring specific gravity is a comparison of the weights of equal volumes of a liquid and water in standardized glassware, a **pycnometer** (Fig. 6-7). This will yield density of the liquid compared to water. In some texts and reference books, 20/20 is given after the specific gravity number. This indicates that the temperature of both fluids was 20°C when the weights were measured. Using a clean, dry pycnometer at 20°C, the analyst weighs it empty, fills it to the full point with distilled water at 20°C, inserts the thermometer to seal the fill opening, and then touches off the last drops of water and puts on the cap for the overflow tube. The pycnometer is wiped dry in case of



6-7 figure Pycnometer.

any spillage from filling and is reweighed. The density of the sample is calculated as follows:

$$\frac{\text{weight of sample-filled pycnometer} - \text{weight of empty pycnometer}}{\text{weight of water-filled pycnometer} - \text{weight of empty pycnometer}} = \text{density of sample} \quad [10]$$

This method is used for determining alcohol content in alcoholic beverages (e.g., distilled liquor, AOAC Method 930.17), solids in sugar syrups (AOAC Method 932.14B), and solids in milk (AOAC Method 925.22).

6.5.3 Refractometry

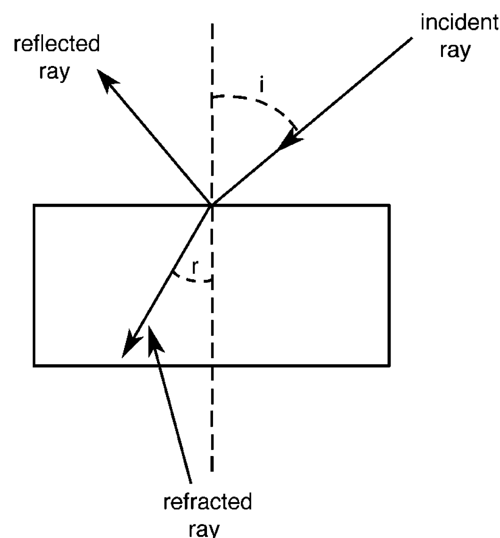
Moisture in liquid sugar products and condensed milks can be determined using a Baumé hydrometer (solids), a Brix hydrometer (sugar content), gravimetric means, or a **refractometer**. If it is performed correctly and no crystalline solids are evident, the refractometer procedure is rapid and surprisingly accurate (AOAC Method 9.32.14C, for solids in syrups). The refractometer has been valuable in determining the soluble solids in fruits and fruit products (AOAC Method 932.12; 976.20; 983.17).

The **refractive index** (RI) of an oil, syrup, or other liquid is a dimensionless constant that can be used to describe the nature of the food. While some refractometers are designed only to provide results as refractive indices, others, particularly hand-held, quick-to-use units, are equipped with scales calibrated to read the percentage of solids, percentage of sugars, and the like, depending on the products for which they are intended. Tables are provided with the instruments to convert values and adjust for temperature differences. Refractometers are used not just on the laboratory bench or as hand-held units. Refractometers can be installed in a liquid processing line to monitor the °Brix of products such as carbonated soft drinks, dissolved solids in orange juice, and the percentage of solids in milk (15).

When a beam of light is passed from one medium to another and the density of the two differs, then the beam of light is bent or refracted. Bending of the light beam is a function of the media and the sines of the angles of incidence and refraction at any given temperature and pressure and is thus a constant (Fig. 6-8). The (RI) (η) is a ratio of the sines of the angles:

$$\eta = \frac{\text{sine incident ray angle}}{\text{sine refracted ray angle}} \quad [11]$$

All chemical compounds have an index of refraction. Therefore, this measurement can be used for the qualitative identification of an unknown compound by comparing its RI with literature values. RI varies with **concentration** of the compound, **temperature**, and **wavelength of light**. Instruments are designed to give a reading by passing a light beam of a specific wavelength through a glass prism into a liquid, the sample. Bench-top or hand-held units use **Amici prisms** to obtain the **D line of the sodium spectrum** or



6-8 figure Reflection and refraction concepts of refractometry.

589 nm from white light. Whenever refractive indices of standard fluids are given, these are prefaced with $\eta_{\mathrm{D}}^{20} = a$ value from 1.3000 to 1.7000. The Greek letter η is the symbol for RI; the 20 refers to temperature in °C; and D is the wavelength of the light beam, the D line of the sodium spectrum.

Bench-top instruments are more accurate compared with hand-held units mainly because of temperature control (Fig. 6-9). These former units have provisions for water circulation through the head where the prism and sample meet. **Abbe refractometers** are the most popular for laboratory use. Care must be taken when cleaning the prism surface following use. Wipe the contact surface clean with lens paper and rinse with distilled water and then ethanol. Close the prism chamber and cover the instrument with a bag when not in use to protect the delicate prism surface from dust or other debris that might lead to scratches and inaccuracy.

The fact that the RI of a solution increases with concentration has been exploited in the analysis of total soluble solids of carbohydrate-based foods such as sugar syrups, fruit products, and tomato products. Because of this use, these refractometers are calibrated in °Brix (g of sucrose/100 g of sample), which is equivalent to percentage sucrose on a wt/wt basis. Refractive index measurements are used widely to approximate sugar concentration in foods, even though values are accurate only for pure sucrose solutions.

6.5.4 Infrared Analysis

Infrared spectroscopy (see Chap. 23) has attained a primary position in monitoring the composition of food products before, during, and following processing (16). It has a wide range of food applications and has proven successful in the laboratory, at-line, and on-line. Infrared spectroscopy measures the absorption of radiation (near- or mid-infrared) by molecules in foods. Different frequencies of infrared radiation are absorbed by different functional groups characteristic of the molecules in food. Similar to the use of ultraviolet (UV) or visible (Vis) light in UV–Vis spectroscopy, a sample is irradiated with a wavelength of infrared light specific for the constituent to be measured. The concentration of that constituent is determined by measuring the energy that is reflected or transmitted by the sample, which is inversely proportional to the energy absorbed. Infrared spectrometers must be calibrated for each analyte to be measured and the analyte must be uniformly distributed in the sample.

For water, near-infrared (NIR) bands (1400–1450; 1920–1950 nm) are characteristic of the –OH stretch

6-9 figure Rhino Brix hand-held refractometer, R^2 mini digital hand-held refractometer, and Mark III Abbe refractometer. (Courtesy of Reichert Analytical Instrument, Depew, NY.)

of the water molecule and can be used to determine the moisture content of a food. NIR has been applied to moisture analysis of a wide variety of food commodities.

The use of mid-infrared milk analyzers to determine fat, protein, lactose, and total solids in milk (AOAC Method 972.16) is covered in Chap. 23. The midrange spectroscopic method does not yield moisture or solids results except by computer calculation because these instruments do not monitor at wavelengths where water absorbs. The instrument must be calibrated using a minimum of eight milk samples that were previously analyzed for fat (F), protein (P), lactose (L), and total solids (TS) by standard methods. Then, a mean difference value, a, is calculated for all samples used in calibration:

$$a = \Sigma(\mathrm{TS} - F - P - L)/n \qquad [12]$$

where:

a = solids not measurable by the F, P, and L methods
n = number of samples
F = fat percentage
P = protein percentage
L = lactose percentage
TS = total solids percentage

Total solids then can be determined from any infrared milk analyzer results by using the formula

$$\mathrm{TS} = a + F + P + L \qquad [13]$$

The a value is thus a standard value mathematically derived. Newer instruments have the algorithm in their computer software to ascertain this value automatically. Moreover, Fourier transform infrared spectroscopy (FTIR) is the latest development that allows greater flexibility in infrared assays.

6.5.5 Freezing Point

When water is added to a food product, many of the physical constants are altered. Some properties of solutions depend on the number of solute particles as ions or molecules present. These properties are vapor pressure, freezing point, boiling point, and osmotic pressure. Measurement of any of these properties can be used to determine the concentration of solutes in a solution. However, the most commonly practiced assay for milk is the change of the freezing point value. It has economic importance with regard to both raw and pasteurized milk. The **freezing point** of milk is its most constant physical property. The secretory process of the mammary gland is such that the osmotic pressure is kept in equilibrium with blood and milk. Thus, with any decrease in the synthesis of lactose, there is a compensating increase in the concentrations of Na^+ and Cl^-. While termed a physical constant, the freezing point varies within narrow limits, and the vast majority of samples from individual cows fall between −0.503°C and −0.541°C (−0.525 and −0.565°H, temperature in °H or **Hortvet**, the surname of the inventor of the first freezing point apparatus). The average is very close to −0.517°C (−0.540°H). Herd or bulk milk will exhibit a narrower range unless the supply was watered intentionally or accidentally or if the milk is from an area where severe drought has existed. All values today are given in °C by agreement. The following is used to convert °H to °C, or °C to °H (5, 6):

$$^\circ\mathrm{C} = 0.9623^\circ\mathrm{H} - 0.0024 \qquad [14]$$

$$^\circ\mathrm{H} = 1.03916^\circ\mathrm{C} + 0.0025 \qquad [15]$$

The principal utility of freezing point is to measure for **added water**. However, the freezing point of milk can be altered by mastitis infection in cows and souring of milk. In special cases, nutrition and environment of the cow, stage of lactation, and processing operations for the milk can affect the freezing point. If the solute remains constant in weight and composition, the change of the freezing point varies inversely with the amount of solvent present. Therefore, we can calculate the percent H_2O added:

$$\%\mathrm{H_2O\ added} = \frac{0.517 - T}{0.517} \times 100 \qquad [16]$$

where:

0.517 = freezing point in °C of all milk entering a plant
T = freezing point in °C of a sample

The AOAC Method 961.07 for water added to milk uses a **cryoscope** to test for freezing points, and assumes a freezing point for normal milk of −0.527°C (−0.550°H). The Food and Drug Administration will reject all milk with freezing points above −0.503°C (−0.525°H). Since the difference between the freezing points of milk and water is slight and since the freezing point can be used to calculate the amount of water added, it is essential that the method be as precise as possible. The thermister used can sense temperature change to 0.001°C (0.001°H). The general technique is to supercool the solution and then induce crystallization by a vibrating reed. The temperature will rise rapidly to the freezing point or eutectic temperature as the water freezes. In the case of pure water, the temperature remains constant until all the water is frozen. In the case of milk, the temperature is read when there is no further temperature rise.

6-10 figure A model 4D3 advanced instruments cryoscope for freezing point determination in milk. (Courtesy of Advanced Instruments, Inc., Norwood, MA.)

Instrumentation available is manufactured by Advanced Instruments (Fig. 6-10). Time required for the automated instruments is 1–2 min per sample using a prechilled sample.

6.6 WATER ACTIVITY

Water content alone is not a reliable indicator of food stability, since foods with the same water content differ in their perishability (17). This is at least partly due to differences in the way that water associates with other constituents in a food. Water tightly associated with other food constituents is less available for microbial growth and chemical reactions to cause decomposition. **Water activity** (a_w) is a better indication of food perishability than is water content. Water activity is defined as follows:

$$a_w = \frac{P}{P_o} \quad [17]$$

$$a_w = \frac{\text{ERH}}{100} \quad [18]$$

where:

a_w = water activity
P = partial pressure of water above the sample
P_o = vapor pressure of pure water at the same temperature (specified)
ERH = equilibrium relative humidity surrounding the product

There are various techniques to measure a_w. A commonly used approach relies on measuring the amount of moisture in the equilibrated headspace above a sample of the food product, which correlates directly with sample a_w. A sample for such analysis is placed in a small closed chamber at constant temperature, and a relative humidity sensor is used to measure the ERH of the sample atmosphere after equilibration. A simple and accurate variation of this approach is the chilled mirror technique in which the water vapor in the headspace condenses on the surface of a mirror that is cooled in a controlled manner. The dew point is determined by the temperature at which condensation takes place, and this determines the relative humidity in the headspace. Two other general approaches to measuring a_w are (1) using the sample freezing point depression and moisture content to calculate a_w, and (2) equilibrating a sample in a chamber held at constant relative humidity (by means of a saturated salt solution) and then using the water content of the sample to calculate a_w (17).

6.7 COMPARISON OF METHODS

6.7.1 Principles

Oven drying methods involve the removal of moisture from the sample and then a weight determination of the solids remaining to calculate the moisture content. Nonwater volatiles can be lost during drying, but their loss is generally a negligible percentage of the amount of water lost. Distillation procedures also involve a separation of the moisture from the solids, and the moisture is quantitated directly by volume. Karl Fischer titration is based on chemical reactions of the moisture present, reflected as the amount of titrant used.

The dielectric method is based on electrical properties of water. Hydrometric methods are based on the relationship between specific gravity and moisture content. The refractive index method is based on how water in a sample affects the refraction of light. Near-infrared analysis of water in foods is based on measuring the absorption at wavelengths characteristic of the molecular vibration in water. Freezing point is a physical property of milk that is changed by a change in solute concentration.

6.7.2 Nature of Sample

While most foods will tolerate oven drying at high temperatures, some foods contain volatiles that are lost at such temperatures. Some foods have constituents that undergo chemical reactions at high temperatures to generate or utilize moisture or other

compounds, to affect the calculated moisture content. Vacuum oven drying at reduced temperatures may overcome such problems for some foods. However, a distillation technique is necessary for some food to minimize volatilization and decomposition. For foods very low in moisture or high in fats and sugars, Karl Fischer titration is often the method of choice. The use of a pycnometer, hydrometer, and refractometer requires liquid samples, ideally with limited constituents.

6.7.3 Intended Purposes

Moisture analysis data may be needed quickly for quality control purposes, in which high accuracy may not be necessary. Of the oven drying methods, microwave drying, infrared drying, and the moisture analyzer technique are fastest. Some forced draft oven procedures require less than 1 h drying, but most forced draft oven and vacuum oven procedures require a much longer time. The electrical, hydrometric, and refractive index methods are very rapid but often require correlation to less empirical methods. Oven drying procedures are official methods for a variety of food products. Reflux distillation is an AOAC method for chocolate, dried vegetables, dried milk, and oils and fats. Such official methods are used for regulatory and nutrition labeling purposes.

6.8 SUMMARY

The moisture content of foods is important to food processors and consumers for a variety of reasons. While moisture determination may seem simplistic, it is often one of the most difficult assays in obtaining accurate and precise results. The free water present in food is generally more easily quantitated as compared to the adsorbed moisture and the water of hydration. Some moisture analysis methods involve a separation of moisture in the sample from the solids and then quantitation by weight or volume. Other methods do not involve such a separation but instead are based on some physical or chemical property of the water in the sample. A major difficulty with many methods is attempting to remove or otherwise quantitate all water present. This often is complicated by decomposition or interference by other food constituents. For each moisture analysis method, there are factors that must be controlled or precautions that must be taken to ensure accurate and precise results. Careful sample collection and handling procedures are extremely important and cannot be overemphasized. The choice of moisture analysis method is often determined by the expected moisture content, nature of other food constituents (e.g., highly volatile, heat sensitive), equipment available, speed necessary, accuracy and precision required, and intended purpose (e.g., regulatory or in-plant quality control).

6.9 STUDY QUESTIONS

1. Identify five factors that one would need to consider when choosing a moisture analysis method for a specific food product.
2. Why is standardized methodology needed for moisture determinations?
3. What are the potential advantages of using a vacuum oven rather than a forced draft oven for moisture content determination?
4. In each case specified below, would you likely overestimate or underestimate the moisture content of a food product being tested? Explain your answer.
   (a) Forced draft oven:
   - Particle size too large
   - High concentration of volatile flavor compounds present
   - Lipid oxidation
   - Sample very hygroscopic
   - Alteration of carbohydrates (e.g., Maillard browning)
   - Sucrose hydrolysis
   - Surface crust formation
   - Splattering
   - Desiccator with dried sample not sealed properly

   (b) Toluene distillation:
   - Emulsion between water in sample and solvent not broken
   - Water clinging to condenser

   (c) Karl Fischer:
   - Very humid day when weighing original samples
   - Glassware not dry
   - Sample ground coarsely
   - Food high in vitamin C
   - Food high in unsaturated fatty acids
5. The procedure for an analysis for moisture in a liquid food product requires the addition of 1–2 ml of deionized water to the weighed sample in the moisture pan. Why should you add water to an analysis in which moisture is being determined?
6. A new instrument based on infrared principles has been received in your laboratory to be used in moisture analysis. Briefly describe the way you would ascertain if the new instrument would meet your satisfaction and company standards.
7. A technician you supervise is to determine the moisture content of a food product by the Karl Fischer method. Your technician wants to know what is this "Karl Fischer reagent water equivalence" that is used in the equation to calculate percentage of moisture in the sample, why it is necessary, and how it is determined. Give the technician your answer.

8. To explain and contrast the principles (not procedures) in determining the moisture content of food products by the following method, complete the table below. (Assume that sample selection and handling has been done appropriately.)

	What is actually measured?	*How is water removed/ reacted/ identified?*	*What assumptions are made in trusting the data obtained (or precautions taken to ensure accurate data)?*
Microwave oven			
NIR			
Karl Fischer			
Toluene distillation			

9. You are fortunate to have available in your laboratory the equipment for doing moisture analysis by essentially all methods – both official and rapid quality control methods. For each of the food products listed below (with the purpose specified as rapid quality control or official), indicate (a) the name of the method you would use, (b) the principle (not procedure) for the method, (c) a justification for use of that method (as compared to using a hot air drying oven), and (d) two cautions in use of the method to ensure accurate results.
   (a) Ice cream mix (liquid) – quality control
   (b) Milk chocolate – official
   (c) Spices – official
   (d) Syrup for canned peaches – quality control
   (e) Oat flour – quality control
10. You are a manufacturer of processed cheese. The maximum allowed moisture content for your product is 40%. Your current product has a mean moisture content of 38%, with a standard deviation of 0.7. It would be possible to increase your mean moisture content to 39.5% if you could reduce your standard deviation to 0.25. This would result in a saving of $3.4 million per year. You can accomplish this by rapidly analyzing the moisture content of the cheese blend prior to the cooking step of manufacture. The cheese blend is prepared in a batch process, and you have 10 min to adjust the moisture content of each batch.
    (a) Describe the rapid moisture analysis method you would use. Include your rationale for selecting the method.
    (b) How would you ensure the accuracy and precision of this method (you need to be sure your standard deviation is below 0.25)?
11. You work in a milk drying plant. As part of the production process, you need to rapidly analyze the moisture content of condensed milk.
    (a) What rapid secondary method would you use, and what primary method would you use to calibrate the secondary method? Additionally, how would you ensure the accuracy and precision of your secondary method?
    (b) Your results with the secondary method are consistently high (about 1%), based on the secondary method you chose. What are some potential problems and how would you correct them?
12. During a 12-h period, 1000 blocks (40 lbs each) from ten different vats (100 blocks per vat) of Cheddar cheese were produced. It was later realized that the cooking temperature was too low during cheesemaking. You are concerned that this might increase the moisture content of the cheese above the legal requirement. Describe the sampling plan and method of analysis you would use to determine the moisture content of the cheese. You want the results within 48 h so you can determine what to do with the cheese.

6.10 PRACTICE PROBLEMS

1. As an analyst, you are given a sample of condensed soup to analyze to determine if it is reduced to the correct concentration. By gravimetric means, you find that the concentration is 26.54% solids. The company standard reads 28.63%. If the starting volume were 1000 gallons at 8.67% solids and the weight is 8.5 pounds per gallon, how much more water must be removed?
2. Your laboratory just received several sample containers of peas to analyze for moisture content. There is a visible condensate on the inside of the container. What is your procedure to obtain a result?
3. You have the following gravimetric results: weight of dried pan and glass disc is 1.0376 g, weight of pan and liquid sample is 4.6274 g, and weight of the pan and dried sample is 1.7321 g. What was the moisture content of the sample and what is the percent solids?

Answers

1. The weight of the soup initially is superfluous information. By condensing the soup to 26.54% solids from 8.67% solids, the volume is reduced to 326.7 gal $[(8.67\%/26.54\%) \times 1000\,\text{gal}]$. You need to reduce the volume further to obtain 28.63% solids $[(8.67\%/28.63\%) \times 1000\,\text{gal}]$ or 302.8 gal. The difference in the gallons obtained is 23.9 gal $(326.7\,\text{gal} - 302.8\,\text{gal})$, or the volume of water that must be removed from the partially condensed soup to comply with company standards.
2. This problem focuses on a real issue in the food processing industry – when do you analyze a sample and when don't you? It would appear that the peas have lost moisture that should be within the vegetable for correct results. You will need to grind the peas in a food mill or blender. If the peas are in a Mason jar or one that fits a blender head, no transfer is needed. Blend the peas to a creamy texture. If a container transfer was made, then

put the blended peas back into the original container. Mix with the residual moisture to a uniform blend. Collect a sample for moisture analysis. You should note on the report form containing the results of the analysis that the pea samples had free moisture on container walls when they arrived.

3. Note Equations [2]–[4]. To use any of the equations, you must subtract the weight of the dried pan and glass disc. Then you obtain 3.5898 g of original sample and 0.6945 g when dried. By subtracting these results, you have removed water (2.8953 g). Then $(0.6945\,g/3.5898\,g) \times 100 = 19.35\%$ solids and $(2.8953\,g/3.5898\,g) \times 100 = 80.65\%$ water.

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Ash Analysis

Maurice R. Marshall
Department of Food Science and Human Nutrition, University of Florida, Gainesville, FL 32611-0370, USA
martym@ufl.edu

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7.1 INTRODUCTION

Ash refers to the inorganic residue remaining after either ignition or complete oxidation of organic matter in a foodstuff. A basic knowledge of the characteristics of various ashing procedures and types of equipment is essential to ensure reliable results. Two major types of ashing are used: dry ashing, primarily for proximate composition and for some types of specific mineral analyses; wet ashing (oxidation), as a preparation for the analysis of certain minerals. Microwave systems now are available for both dry and wet ashing, to speed the processes. Most dry samples (i.e., whole grain, cereals, dried vegetables) need no preparation, while fresh vegetables need to be dried prior to ashing. High-fat products such as meats may need to be dried and fat extracted before ashing. The ash content of foods can be expressed on either a wet weight (as is) or on a dry weight basis. For general and food-specific information on measuring ash content, see references (1–11).

7.1.1 Definitions

Dry ashing refers to the use of a muffle furnace capable of maintaining temperatures of 500–600°C. Water and volatiles are vaporized, and organic substances are burned in the presence of oxygen in air to CO_2 and oxides of N_2. Most minerals are converted to oxides, sulfates, phosphates, chlorides, and silicates. Elements such as Fe, Se, Pb, and Hg may partially volatilize with this procedure, so other methods must be used if ashing is a preliminary step for specific elemental analysis.

Wet ashing is a procedure for oxidizing organic substances by using acids and oxidizing agents or their combinations. Minerals are solubilized without volatilization. Wet ashing often is preferable to dry ashing as a preparation for specific elemental analysis. Wet ashing often uses a combination of acids and requires a special perchloric acid hood if that acid is used.

7.1.2 Importance of Ash in Food Analysis

Ash content represents the total mineral content in foods. Determining the ash content may be important for several reasons. It is a part of proximate analysis for nutritional evaluation. Ashing is the first step in preparing a food sample for specific elemental analysis. Because certain foods are high in particular minerals, ash content becomes important. One can usually expect a constant elemental content from the ash of animal products, but that from plant sources is variable.

7.1.3 Ash Contents in Foods

The average ash content for various food groups is given in Table 7-1. The ash content of most fresh foods rarely is greater than 5%. Pure oils and fats generally contain little or no ash; products such as cured bacon may contain 6% ash, and dried beef may be as high as 11.6% (wet weight basis).

Fats, oils, and shortenings vary from 0.0 to 4.1% ash, while dairy products vary from 0.5 to 5.1%. Fruits, fruit juice, and melons contain 0.2–0.6% ash, while dried fruits are higher (2.4–3.5%). Flours and meals vary from 0.3 to 1.4% ash. Pure starch contains 0.3% and wheat germ 4.3% ash. It would be expected that

7-1 table **Ash Content of Selected Foods**

Food Item	*Percent Ash (Wet Weight Basis)*
Cereals, bread, and pasta	
Rice, brown, long-grain, raw	1.5
Corn meal, whole-grain, yellow	1.1
Hominy, canned, white	0.9
White rice, long-grain, regular, raw, enriched	0.6
Wheat flour, whole-grain	1.6
Macaroni, dry, enriched	0.9
Rye bread	2.5
Dairy products	
Milk, reduced fat, fluid, 2%	0.7
Evaporated milk, canned, with added vitamin A	1.6
Butter, with salt	2.1
Cream, fluid, half-and-half	0.7
Margarine, hard, regular, soybean	2.0
Yogurt, plain, low fat	1.1
Fruits and vegetables	
Apples, raw, with skin	0.2
Bananas, raw	0.8
Cherries, sweet, raw	0.5
Raisins	1.9
Potatoes, raw, skin	1.6
Tomatoes, red, ripe, raw	0.5
Meat, poultry, and fish	
Eggs, whole, raw, fresh	0.9
Fish fillet, battered or breaded, and fried	2.5
Pork, fresh, leg (ham), whole, raw	0.9
Hamburger, regular, single patty, plain	1.9
Chicken, broilers or fryers, breast meat only, raw	1.0
Beef, chuck, arm pot roast, raw	1.1

From US Department of Agriculture, Agricultural Research Service (2009) USDA National Nutrient Database for Standard Reference. Release 22. Nutrient Data Laboratory Home Page: http://www.ars.usda.gov/ba/bhnrc/ndl

grain and grain products with bran would tend to be higher in ash content than such products without bran. Nuts and nut products contain 0.8–3.4% ash, while meat, poultry, and seafoods contain 0.7–1.3% ash.

7.2 METHODS

Principles, materials, instrumentation, general procedures, and applications are described below for various ash determination methods. Refer to methods cited for detailed instructions of the procedures.

7.2.1 Sample Preparation

It cannot be overemphasized that the small sample used for ash, or other determinations, needs to be very carefully chosen so that it represents the original materials. A 2–10-g sample generally is used for ash determination. For that purpose, milling, grinding, and the like probably will not alter the ash content much; however, if this ash is a preparatory step for specific mineral analyses, contamination by microelements is of potential concern. Remember, most grinders and mincers are of steel construction. Repeated use of glassware can be a source of contaminants as well. The water source used in dilutions also may contain contaminants of some microelements. Distilled-deionized water always should be used.

7.2.1.1 Plant Materials

Plant materials are generally dried by routine methods prior to grinding. The temperature of drying is of little consequence for ashing. However, the sample may be used for multiple determinations – protein, fiber, and so on – which require consideration of temperature for drying. Fresh stem and leaf tissue probably should be dried in two stages (i.e., first at a lower temperature of 55°C, then a higher temperature) especially to prevent artifact lignin. Plant material with 15% or less moisture may be ashed without prior drying.

7.2.1.2 Fat and Sugar Products

Animal products, syrups, and spices require treatments prior to ashing because of high fat, moisture (spattering, swelling), or high sugar content (foaming) that may result in loss of sample. Meats, sugars, and syrups need to be evaporated to dryness on a steam bath or with an infrared (IR) lamp. One or two drops of olive oil (which contains no ash) are added to allow steam to escape as a crust is formed on the product.

Smoking and burning may occur upon ashing for some products (e.g., cheese, seafood, spices). Allow this smoking and burning to finish slowly by keeping the muffle door open prior to the normal procedure. A sample may be ashed after drying and fat extraction. In most cases, mineral loss is minimal during drying and fat extraction. Under no circumstances should fat-extracted samples be heated until all the ether has been evaporated.

7.2.2 Dry Ashing

7.2.2.1 Principles and Instrumentation

Dry ashing is incineration at high temperature (525°C or higher). Incineration is accomplished with a muffle furnace. Several models of muffle furnaces are available, ranging from large-capacity units requiring either 208 or 240 V supplies to small benchtop units utilizing 110-V outlets.

Crucible selection becomes critical in ashing because the type depends upon the specific use. **Quartz crucibles** are resistant to acids and halogens, but not alkali, at high temperatures. **Vycor® brand crucibles** are stable to 900°C, but **Pyrex® Gooch crucibles** are limited to 500°C. Ashing at a lower temperature of 500–525°C may result in slightly higher ash values because of less decomposition of carbonates and loss of volatile salts. **Porcelain crucibles** resemble quartz crucibles in their properties, but will crack with rapid temperature changes. Porcelain crucibles are relatively inexpensive and usually the crucible of choice. **Steel crucibles** are resistant to both acids and alkalies and are inexpensive, but they are composed of chromium and nickel, which are possible sources of contamination. **Platinum crucibles** are very inert and are probably the best crucibles, but they are currently far too expensive for routine use for large numbers of samples. **Quartz fiber crucibles** are disposable, unbreakable, and can withstand temperatures up to 1000°C. They are porous, allowing air to circulate around the sample and speed combustion. This reduces ashing times significantly and makes them ideal for solids and viscous liquids. Quartz fiber also cools in seconds, virtually eliminating the risk of burns.

All crucibles should be marked for identification. Marks on crucibles with a felt-tip marking pen will disappear during ashing in a muffle furnace. Laboratory inks scribed with a steel pin are available commercially. Crucibles also may be etched with a diamond point and marked with a 0.5 *M* solution of $FeCl_3$, in 20% HCl. An iron nail dissolved in concentrated HC1 forms brown goo that is a satisfactory marker. The crucibles should be fired and cleaned prior to use.

The *advantages* of conventional dry ashing are that it is a safe method, it requires no added reagents or blank subtraction, and little attention is needed once ignition begins. Usually a large number of crucibles

can be handled at once, and the resultant ash can be used additionally in other analyses for most individual elements, acid-insoluble ash, and water-soluble and insoluble ash. The *disadvantages* are the length of time required (12–18 h or overnight) and expensive equipment. There will be a loss of the volatile elements and interactions between mineral components and crucibles. Volatile elements at risk of being lost include As, B, Cd, Cr, Cu, Fe, Pb, Hg, Ni, P, V, and Zn.

7.2.2.2 Procedures

AOAC International has several dry ashing procedures (e.g., AOAC Methods 900.02 A or B, 920.117, 923.03) for certain individual foodstuffs. The general procedure includes the following steps:

1. Weigh a 5–10-g sample into a tared crucible. Predry if the sample is very moist.
2. Place crucibles in a cool muffle furnace. Use tongs, gloves, and protective eyewear if the muffle furnace is warm.
3. Ignite 12–18 h (or overnight) at about 550°C.
4. Turn off muffle furnace and wait to open it until the temperature has dropped to at least 250°C, preferably lower. Open door carefully to avoid losing ash that may be fluffy.
5. Using safety tongs, quickly transfer crucibles to a desiccator with a porcelain plate and desiccant. Cover crucibles, close desiccator, and allow crucibles to cool prior to weighing.

Note. Warm crucibles will heat air within the desiccator. With hot samples, a cover may bump to allow air to escape. A vacuum may form on cooling. At the end of the cooling period, the desiccator cover should be removed gradually by sliding to one side to prevent a sudden inrush of air. Covers with a ground glass sleeve or fitted for a rubber stopper allow for slow release of a vacuum.

The ash content is calculated as follows:

$$\%\text{ ash (dry basis)} = \frac{\text{wt after ashing} - \text{tare wt of crucible}}{\text{original sample wt} \times \text{dry matter coefficient}} \times 100 \qquad [1]$$

where:

$$\text{dry matter coefficient} = \%\text{ solids}/100$$

For example, if corn meal is 87% dry matter, the dry matter coefficient would be 0.87. If ash is calculated on an as-received or wet weight basis (includes moisture), delete the dry matter coefficient from the denominator. If moisture was determined in the same crucible prior to ashing, the denominator becomes (dry sample wt - tared crucible wt).

7.2.2.3 Special Applications

Some of the AOAC procedures recommend steps in addition to those listed previously. If carbon is still present following the initial incineration, several drops of water or nitric acid should be added; then the sample should be re-ashed. If the carbon persists, such as with high-sugar samples, follow this procedure:

1. Suspend the ash in water.
2. Filter through ashless filter paper because this residue tends to form a glaze.
3. Dry the filtrate.
4. Place paper and dried filtrate in muffle furnace and re-ash.

Other suggestions that may be helpful and accelerate incineration:

1. High-fat samples should be extracted either by using the crude fat determination procedure or by burning off prior to closing the muffle furnace. Pork fat, for example, can form a combustible mixture inside the furnace and burn with the admission of oxygen if the door is opened.
2. Glycerin, alcohol, and hydrogen will accelerate ashing.
3. Samples such as jellies will spatter and can be mixed with cotton wool.
4. Salt-rich foods may require a separate ashing of water-insoluble components and salt-rich water extract. Use a crucible cover to prevent spattering.
5. An alcoholic solution of magnesium acetate can be added to accelerate ashing of cereals. An appropriate blank determination is necessary.

7.2.3 Wet Ashing

7.2.3.1 Principle, Materials, and Applications

Wet ashing is sometimes called **wet oxidation** or **wet digestion**. Its primary use is preparation for specific mineral analysis and metallic poisons. Often, analytical testing laboratories use only wet ashing in preparing samples for certain mineral analyses (e.g., Fe, Cu, Zn, P), because losses would occur by volatilization during dry ashing.

There are several *advantages* to using the wet ashing procedure. Minerals will usually stay in solution, and there is little or no loss from volatilization because of the lower temperature. The oxidation time is short and requires a hood, hot plate, and long tongs, plus safety equipment.

The *disadvantages* of wet ashing are that it takes virtually constant operator attention, corrosive reagents are necessary, and only small numbers of samples can

be handled at any one time. If the wet digestion utilizes perchloric acid, all work needs to be carried out in an expensive special fume hood called a **perchloric acid hood**.

Unfortunately, a single acid used in wet ashing does not give complete and rapid oxidation of organic material, so a mixture of acids often is used. Combinations of the following acid solutions are used most often: (1) **nitric acid**, (2) **sulfuric acid-hydrogen peroxide**, and (3) **perchloric acid**. Different combinations are recommended for different types of samples. The nitric–perchloric combination is generally faster than the sulfuric–nitric procedure. While wet digestion with perchloric acid is an AOAC procedure (e.g., AOAC Method 975.03), many analytical laboratories avoid if possible the use of perchloric acid in wet ashing and instead use a combination of nitric acid with either sulfuric acid, hydrogen peroxide, or hydrochloric acid.

Wet oxidation with perchloric acid is *extremely* dangerous since the perchloric acid has a tendency to explode. The perchloric acid hood that must be used has wash-down capabilities and does not contain plastic or glycerol-base caulking compounds. Precautions for use of perchloric acid are found in the AOAC methods under “Safe Handling of Special Chemical Hazards.” Cautions must be taken when fatty foods are wet ashed using perchloric acid. While perchloric acid does not interfere with atomic absorption spectroscopy, it does interfere in the traditional colorimetric assay for iron by reacting with iron in the sample to form ferrous perchlorate, which forms an insoluble complex with the *o*-phenanthrolene in the procedure.

7.2.3.2 Procedures

The following is a wet ash procedure using concentrated nitric and sulfuric acids (*to be performed in a fume hood*) (John Budin, Silliker Laboratories, Chicago, IL, personal communication):

1. Accurately weigh a dried, ground 1-g sample in a 125-ml Erlenmeyer flask (previously acid washed and dried).
2. Prepare a blank of 3 ml of H_2SO_4 and 5 ml of HNO_3, to be treated like the samples. (Blank is to be run with every set of samples.)
3. Add 3 ml of H_2SO_4 followed by 5 ml of HNO_3 to the sample in the flask.
4. Heat the sample on a hot plate at ca. 200°C (boiling). Brown-yellow fumes will be observed.
5. Once the brown-yellow fumes cease and white fumes from decomposing H_2SO_4 are observed, the sample will become darker. Remove the flask from the hot plate. Do not allow the flask to cool to room temperature.
6. *Slowly* add 3–5 ml of HNO_3.
7. Put the flask back on the hot plate and allow the HNO_3 to boil off. Proceed to the next step when all the HNO_3 is removed and the color is clear to straw yellow. If the solution is still dark in color, add another 3–5 ml of HNO_3 and boil. Repeat the process until the solution is clear to straw yellow.
8. While on the hot plate, reduce the volume appropriately to allow for ease of final transfer. Allow the sample to cool to room temperature, then quantitatively transfer the sample to an appropriately sized volumetric flask.
9. Dilute the sample to volume with ultrapure water, and mix well. Dilute further, as appropriate, for the specific type of mineral being analyzed.

The following procedure for a modified dry–wet ash sample destruction may be used. It is listed under “Minerals in Infant Formula, Enteral Products, and Pet Foods” (AOAC Method 985.35).

1. Evaporate moist samples (25–50 ml) in an appropriate dish at 100°C overnight or in a microwave drying oven until dry.
2. Heat on a hot plate until smoking ceases.
3. Ash in a 525°C furnace for 3–8 h.
4. Remove dish from furnace and allow to cool. Ash should be grayish white to white and free from carbon.
5. Cool and wet with deionized distilled water plus 0.5–3.0 ml of HNO_3.
6. Dry on a hot plate or steam bath and then return to a 525°C furnace for 1–2 h.
7. Repeat steps 5 and 6 if carbon persists. (*Caution*: Some K may be lost with repeated ashing.)
8. Dissolve the ash in 5 ml of 1 *M* HNO_3 by warming on a hot plate for 2–3 min to aid solution. Transfer to an appropriate size volumetric flask (i.e., 50 ml), then repeat with two additional portions of 1 *M* HNO_3.

7.2.4 Microwave Ashing

Both **wet ashing** and **dry ashing** can be done using microwave instrumentation, rather than the conventional dry ashing in a muffle furnace and wet ashing in a flask or beaker on a hot plate. The CEM Corporation (Matthews, NC) has developed a series of instruments for dry and wet ashing, as well as other laboratory systems for microwave-assisted chemistry. While the ashing procedures by conventional means can take many hours, the use of microwave instrumentation

can reduce sample preparation time to minutes, allowing laboratories to increase their sample throughput significantly. This advantage has led to widespread use of microwave ashing, especially for wet ashing, both within analytical laboratories and quality control laboratories within food companies.

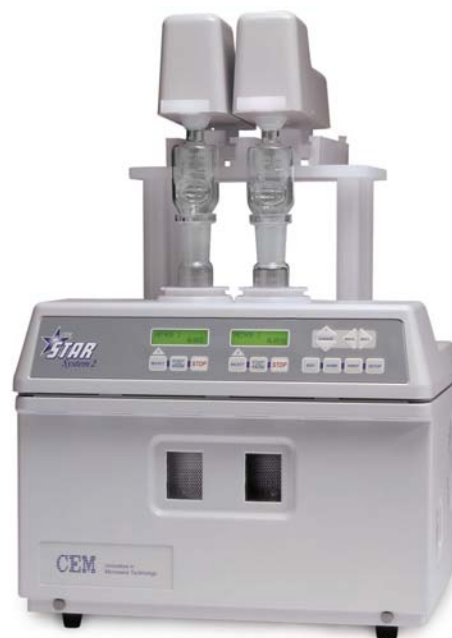
7.2.4.1 Microwave Wet Ashing

Microwave wet ashing (acid digestion) may be performed safely in either an open- or closed-vessel microwave system. Choice of the system depends on the amount of sample and the temperatures required for digesting. Because of the ability of the closed vessels to contain higher pressures (some vessels can handle up to 1500 psi), acids may be heated past their boiling points. This ensures a more complete dissolution of hard-to-digest substances. It also allows the chemist to use nitric acid with samples that might normally require a harsher acid, such as sulfuric or perchloric. In closed vessels specifically designed for high-temperatures/high-pressure reactions, nitric acid can reach a temperature of 240°C. Thus, **nitric acid** is often the acid of choice, though hydrochloric, hydrofluoric, and sulfuric acids also are used, depending on the sample and the subsequent analysis being performed. **Closed-vessel microwave digestion systems** (Fig. 7-1) can process up to 40 samples at a time, with vessel liners available in Teflon®, TFM™ Fluoropolymer, and quartz. These systems allow the input of time, temperature, and pressure parameters in a step-by-step format (ramping). In addition, some instruments enable the user to adjust the power and offer "change-on-the-fly" software, which allows the method to be changed while the reaction is running.

Typically, in a closed-vessel microwave system, sample is placed in vessels with the appropriate amount of acid. The vessels are sealed and set on a carousel where the temperature and pressure sensors are connected to a control vessel. The carousel then is placed in the microwave cavity, and the sensors are connected to the instrument. Time, temperature, pressure, and power parameters are chosen and the unit is started. Digestions normally take less than 30 min. Because of the pressure generated by raising the temperature of a reaction, the vessels must be allowed to cool before being opened. The ability to process multiple samples simultaneously provides the chemist with greater throughput than traditional methods. (Note that some closed-vessel microwave digestion systems may also be used for acid concentration, solvent extraction, protein hydrolysis, and synthesis with the proper accessories.)

Open-vessel digestion systems (Fig. 7-2) are used often for larger sample sizes (up to 10 g) and for samples that generate substantial amounts of gas as they are digested. Open-vessel systems can process up to six samples, each according to its own parameters in a sequential or simultaneous format. Teflon®, quartz, or Pyrex® vessels are used, and condensers are added for refluxing. Acid (reagent) is automatically added according to the programmed parameters. Sulfuric and nitric acids are used most often with open-vessel systems, as they process reactions under atmospheric conditions; however, hydrochloric and hydrofluoric acids, as well as hydrogen peroxide, can be used.

7-1 figure Microwave closed-vessel digestion system. (Courtesy of CEM Corporation, Matthews, NC.)



7-2 figure Microwave open-vessel system. (Courtesy of CEM Corporation, Matthews, NC.)

These instruments do not require the use of a fume hood, because a vapor containment system contains and neutralizes harmful fumes.

Generally, in an open-vessel microwave system, the sample is placed in a vessel and the vessel is set in a slot in the microwave system. Time, temperature, and reagent addition parameters are then chosen. The unit is started, the acid is added, and the vapor containment system neutralizes the fumes from the reaction. Samples are typically processed much faster and more reproducibly than on a conventional hot plate. (Note that some open-vessel systems may be used for evaporation and acid concentration as well.)

7.2.4.2 Microwave Dry Ashing

Compared with conventional dry ashing in a muffle furnace that often takes many hours, **microwave muffle furnaces** (Fig. 7-3) can ash samples in minutes, decreasing analysis time by as much as 97%. Microwave muffle furnaces can reach temperatures of up to 1200°C. These systems may be programmed with various methods and to automatically warm up and cool down. In addition, they are equipped with exhaust systems that circulate the air in the cavity to help decrease ashing times. Some also have scrubber systems to neutralize any fumes. Any crucible that may be used in a conventional muffle furnace may be used in a microwave furnace, including those made of porcelain, platinum, quartz, and quartz fiber. Quartz fiber crucibles cool in seconds and are not breakable. Some systems can process up to 15 (25 ml) crucibles at a time.

Typically, in microwave dry ashing, a desiccated crucible is weighed and then sample is added and it is weighed again. The crucible then is placed in the microwave furnace, and the time and temperature parameters are set. A step-by-step (ramping) format may be used when programming the method. The system is started and the program is run to completion. The crucible then is carefully removed with tongs and reweighed. The sample then may be further analyzed, if necessary. Some tests call for acid to be added to a dry ashed sample, which is then digested for further analysis.

7-3 figure Microwave muffle furnace. (Courtesy of CEM Corporation, Matthews, NC.)

A comparative study (9) showed that dry ashing various plants for 40 min using a microwave system (CEM Corporation, Matthews NC) was similar to the 4-h time in a conventional muffle furnace. Twenty minutes was shown to be adequate for the plant material used except for Cu determinations, which needed 40 min to obtain similar results. Other comparative examples include dried egg yolks, which can be ashed in 20 min in a microwave system, but require 4 h in a conventional muffle furnace. It takes 16 h to ash lactose in a conventional muffle furnace, but only 35 min in a microwave furnace. Though microwave furnaces may not hold as many samples as a conventional furnace, their speed actually allows significantly more samples to be processed in the same amount of time. Also, microwave furnaces do not require fume hood space.

7.2.5 Other Ash Measurements

The following are several special ash measurements and their applications:

1. **Soluble and insoluble ash** (e.g., AOAC Method 900.02) – Applied to fruits.
2. **Ash insoluble in acid** – A measure of the surface contamination of fruits and vegetables and wheat and rice coatings; contaminants are generally silicates and remain insoluble in acid, except HBr.
3. **Alkalinity of ash** (e.g., AOAC Method 900.02, 940.26) – Ash of fruits and vegetable is alkaline; ash of meats and some cereals is acid.
4. **Sulfated ash** (AOAC Method 900.02, 950.77) – Applied to sugars, syrups, and color additives.

7.3 COMPARISON OF METHODS

Ash determination by dry ashing requires expensive equipment, especially if many samples are analyzed. The muffle furnace may have to be placed in a heat room along with drying ovens and it requires a 220-V outlet. It is important to make sure that large furnaces of that type are equipped with a double-pole, single-throw switch. Heating coils are generally exposed, and care must be taken when taking samples in and out with metal tongs. Desktop furnaces (110 V) are available for fewer samples. Wet ashing requires a hood (a special hood if perchloric acid is used), corrosive

reagents, and constant operator attention. While wet oxidation causes little volatilization, dry ashing will result in the loss of volatile elements. The type of further elemental analyses will dictate the equipment. Some micro- and most volatile elements will require special equipment and procedures. Refer to Chaps. 12 and 24 for specific preparation procedures for elemental analyses. Both dry and wet ashing can be done using microwave systems that utilize relatively expensive instrumentation, but they greatly reduce the time for ashing and do not require use of a fume hood.

7.4 SUMMARY

The two major types of ashing, dry ashing and wet oxidation (ashing), can be done by conventional means or using microwave systems. The procedure of choice depends upon the use of ash following its determination, and limitations based on cost, time, and sample numbers. Conventional dry ashing is based upon incineration at high temperatures in a muffle furnace. Except for certain elements, the residue may be used for further specific mineral analyses. Wet ashing (oxidation) often is used as a preparation for specific elemental analysis by simultaneously dissolving minerals and oxidizing all organic material. Wet ashing conserves volatile element, but requires more operator time than dry ashing and is limited to a smaller number of samples. Dry and wet ashing using microwave technology reduces the time for analyses and requires little additional equipment (special fume hood) or space (heat room).

7.5 STUDY QUESTIONS

1. Identify four potential sources of error in the preparation of samples for ash analysis and describe a way to overcome each.
2. You are determining the total ash content of a product using the conventional dry ashing method. Your boss asks you to switch to a conventional wet ashing method because he/she has heard it takes less time than dry ashing.
   (a) Do you agree or disagree with your boss concerning the time issue, and why?
   (b) Not considering the time issues, why might you want to continue using dry ashing, *and* why might you change to wet ashing?
3. Your lab technician was to determine the ash content of buttermilk by conventional dry ashing. The technician weighed 5 g of buttermilk into one weighed platinum crucible, immediately put the crucible into the muffle furnace using a pair of all stainless steel tongs, and ashed the sample for 48 h at 800°C. The crucible was removed from the muffle furnace and set on a rack in the open until it was cool enough to reweigh. Itemize the instructions you should have given your technician before beginning, so there would not have been the mistakes made as described above.
4. How would you recommend to your technician to overcome the following problems that could arise in conventional dry ashing of various foods?
   (a) You seem to be getting volatilization of phosphorus, when you want to later determine the phosphorus content.
   (b) You are getting incomplete combustion of a product high in sugar after a typical dry ashing procedure (i.e., the ash is dark colored, not white or pale gray).
   (c) The typical procedure takes too long for your purpose. You need to speed up the procedure, but you do not want to use the standard wet ashing procedure.
   (d) You have reason to believe the compound you want to measure after dry ashing may be reacting with the porcelain crucibles being used.
   (e) You want to determine the iron content of some foods but cannot seem to get the iron solubilized after the dry ashing procedure.
5. Identify an advantage and disadvantage of using microwave wet digesters or microwave muffle furnaces compared with conventional units.

7.6 PRACTICE PROBLEMS

1. A grain was found to contain 11.5% moisture. A 5.2146-g sample was placed into a crucible (28.5053 g tare). The ashed crucible weighed 28.5939 g. Calculate the percentage ash on (a) an as-received (wet weight) basis and (b) a dry matter basis.
2. A vegetable (23.5000 g) was found to have 0.0940-g acid-insoluble ash. What is the percentage of acid-insoluble ash?
3. You wish to have at least 100 mg of ash from a cereal grain. Assuming 2.5% ash on average, how many grams of the grain should be weighed for ashing?
4. You wish to have a coefficient of variation (CV) below 5% with your ash analyses. The following ash data are obtained: 2.15%, 2.12%, 2.07%. Are these data acceptable, and what is the CV?
5. The following data were obtained on a sample of hamburger: sample wt, 2.034 g; wt after drying, 1.0781 g; wt after ether extraction, 0.4679 g; and wt of ash, 0.0233 g. What is the percentage ash on (a) a wet weight basis and (b) a fat-free basis?

Answers

1. (a) 1.70%, (b) 1.92%
   Calculate ash from sample:

Crucible + ash:	28.5939 g
Tared crucible:	28.5053 g
Ash:	0.0886 g

(a) Calculate for ash on a wet weight basis (a):

$$\frac{0.0886\text{ g ash}}{5.2146\text{ g sample}} \times 100\% = 1.70\%\text{ or }1.7\%$$

(b) Calculate for ash on a dry weight basis (b):

$$0.0886\text{ g ash} \div \left[5.2146\text{ g sample} \times \left(\frac{100\% - 11.5\%}{100\%}\text{ dry matter coeff}\right)\right] \times 100\% = 1.92\%$$

or

$$5.214\text{ g sample} \times \frac{11.5\text{ g water}}{100\text{ g sample}} = 0.5997\text{ g water}$$

$$5.214\text{ g sample} - 0.5997\text{ g water} = 4.6149\text{ g sample dry wt}$$

$$\frac{0.0886\text{ g ash}}{4.6149\text{ g dry wt sample}} \times 100\% = 1.92\%$$

2. 0.4%
   Calculate % insoluble ash:

$$\frac{0.0940\text{ g acid insoluble ash}}{23.5\text{ g sample}} \times 100\% = 0.4\%$$

3. 4 g
   100 mg = 0.1 g ash
   2.5% = 2.5 g ash/100 g sample

$$\frac{2.5\text{ g ash}}{100\text{ g sample}} = \frac{0.1\text{ g ash}}{x}$$

$$2.5x = 10$$

$$x = 4\text{ g sample}$$

4. Yes, 1.9%
   Calculate the mean:

$$\frac{2.15 + 2.12\% + 2.07\%}{3} = 2.11\%$$

Calculation of mean and standard deviation was done using Excel:

1. 2.15%
2. 2.12%
3. 2.07%

Average = 2.11%
Std. deviation = 0.0404

$$\text{Coefficient of variation(CV)} = \frac{\text{SD}}{x} \times 100\%$$

$$\text{CV} = \frac{0.0404}{2.11} \times 100\% = 1.91\%$$

It is within the 5% level for CV? YES.

5. (a) 1.1%, (b) 1.64%

Sample wet wt: 2.034 g
Sample dry wt: 1.0781 g
Wt after extraction: 0.4679 g
Wt of ash: 0.0233 g

(a) Calculate for wet weight basis:

$$\frac{0.0233\text{ g ash}}{2.034\text{ g sample}} \times 100\% = 1.15\%$$

(b) Calculate for fat-free basis:

$$2.034\text{ g wet sample} - 1.0781\text{ g solids} = 0.9559\text{ g water this is 47\% moisture})$$

$$1.0781\text{ g solids dry wt} - 0.4679\text{ g solids after extraction} = 0.6102\text{ g fat}$$

$$2.034\text{ g wet sample} - 0.6102\text{ g fat} = 1.4238\text{ g wet sample wt without fat}$$

$$\frac{0.0233\text{ g ash}}{(1.4238\text{ g wet sample wt without fat})} \times 100\% = 1.64\%\text{ ash, fat-free basis}$$

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Fat Analysis

David B. Min

Department of Food Science and Technology, The Ohio State University, Columbus, OH 43210, USA
min2@osu.edu

and

Wayne C. Ellefson

Nutritional Chemistry and Food Safety, Covance Laboratories, 3301 Kinsman Boulevard, Madison, WI 53714, USA
Wayne.Ellefson@covance.com

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8.1 INTRODUCTION

8.1.1 Definitions

Lipids, proteins, and carbohydrates constitute the principal structural components of foods. Lipids are a group of substances that, in general, are soluble in ether, chloroform, or other organic solvents but are sparingly soluble in water. However, there exists no clear scientific definition of a lipid, primarily due to the water solubility of certain molecules that fall within one of the variable categories of food lipids (1). Some lipids, such as triacylglycerols, are very hydrophobic. Other lipids, such as di- and monoacylglycerols, have both hydrophobic and hydrophilic moieties in their molecules and are soluble in relatively polar solvents (2). Short-chain fatty acids such as C1–C4 are completely miscible in water and insoluble in nonpolar solvents (1). The most widely accepted definition is based on solubility as previously stated. While most macromolecules are characterized by common structural features, the designation of "lipid" being defined by solubility characteristics is unique to lipids (2). Lipids comprise a broad group of substances that have some common properties and compositional similarities (3). Triacylglycerols are fats and oils that represent the most prevalent category of the group of compounds known as lipids. The terms lipids, fats, and oils are often used interchangeably. The term "lipid" commonly refers to the broad, total collection of food molecules that meet the definition previously stated. Fats generally refer to those lipids that are solid at room temperature and oils generally refer to those lipids that are liquid at room temperature. While there may not be an exact scientific definition, the US Food and Drug Administration (FDA) has established a regulatory definition for nutrition labeling purposes. The FDA has defined total fat as the sum of fatty acids from C4 to C24, calculated as triglycerides. This definition provides a clear path for resolution of any nutrition labeling disputes.

8.1.2 General Classification

The general classification of lipids that follows is useful to differentiate lipids in foods (3).

8.1.2.1 Simple Lipids

Ester of fatty acids with alcohol:

- **Fats:** Esters of fatty acids with glycerol – triacylglycerols
- **Waxes:** Esters of fatty acids with long-chain alcohols other than glycerols (e.g., myricyl palmitate, cetyl palmitate, vitamin A esters, and vitamin D esters)

8.1.2.2 Compound Lipids

Compounds containing groups in addition to an ester of a fatty acid with an alcohol:

- **Phospholipids:** Glycerol esters of fatty acids, phosphoric acids, and other groups containing nitrogen (e.g., phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, and phosphatidyl inositol)
- **Cerebrosides:** Compounds containing fatty acids, a carbohydrate, and a nitrogen moiety (e.g., galactocerebroside and glucocerebroside)
- **Sphingolipids:** Compounds containing fatty acids, a nitrogen moiety, and phosphoryl group (e.g., sphingomyelins)

8.1.2.3 Derived Lipids

Derived lipids are substances derived from neutral lipids or compound lipids. They have the general properties of lipids – examples are fatty acids, long-chain alcohols, sterols, fat-soluble vitamins, and hydrocarbons.

8.1.3 Content of Lipids in Foods

Foods may contain any or all types of the lipid compounds previously mentioned. The lipid content in bovine milk (Table 8-1) illustrates the complexity and variability of lipids in a food system, having lipids that differ in polarity and concentrations.

Foods contain many types of lipids, but those which tend to be of greatest importance are the triacylglycerols and the phospholipids. **Liquid triacylglycerols** at room temperature are referred to as **oils**,

8-1 table **Lipids of Bovine Milk**

Kinds of Lipids	*Percent of Total Lipids*
Triacylglycerols	97–99
Diacylglycerols	0.28–0.59
Monoacylglycerols	0.016–0.038
Phospholipids	0.2–1.0
Sterols	0.25–0.40
Squalene	Trace
Free fatty acids	0.10–0.44
Waxes	Trace
Vitamin A	(7–8.5 μg/g)
Carotenoids	(8–10 μg/g)
Vitamin D	Trace
Vitamin E	(2–5 μg/g)
Vitamin K	Trace

Adapted from (4) with permission of S. Patton and (5) *Principles of Dairy Chemistry.* Jenness R. and Patton S. Copyright ©1959, John Wiley & Sons, Inc with permission.

such as soybean oil and olive oil, and are generally of plant origin. **Solid triacylglycerols** at room temperature are termed as **fats**. Lard and tallow are examples of fats, which are generally from animals. The term *fat* is applicable to all triacylglycerols whether they are normally solid or liquid at ambient temperatures. Table 8-2 shows the wide range of lipid content in different foods.

8.1.4 Importance of Analysis

An accurate and precise quantitative and qualitative analysis of lipids in foods is important for accurate nutritional labeling, determination of whether the food meets the standard of identity, and to ensure that the product meets manufacturing specifications. Inaccuracies in analysis may prove costly for manufacturers and could result in a product of undesirable quality and functionality.

8.2 GENERAL CONSIDERATIONS

By definition, lipids are soluble in organic solvents and insoluble in water. Therefore, water insolubility is the essential analytical property used as the basis for the separation of lipids from proteins, water, and carbohydrates in foods. Glycolipids are soluble in alcohols and have a low solubility in hexane. In contrast, triacylglycerols are soluble in hexane and petroleum ether, which are nonpolar solvents. The wide range of relative hydrophobicity of different lipids makes the selection of a single universal solvent impossible for lipid extraction of foods. Some lipids in foods are components of complex lipoproteins and liposaccharides; therefore, successful extraction requires that bonds between lipids and proteins or carbohydrates be broken so that the lipids can be freed and solubilized in the extracting organic solvents.

8.3 SOLVENT EXTRACTION METHODS

The total lipid content of a food is commonly determined by organic solvent extraction methods or by alkaline or acid hydrolysis followed by Mojonnier extraction. For multicomponent food products, acid hydrolysis is often the method of choice. Both acid hydrolysis and alkaline hydrolysis methods can be performed using Mojonnier extraction equipment. The use of acid hydrolysis eliminates some of the matrix effects that may be exhibited by simple solvent extraction methods. The accuracy of direct solvent extraction methods (i.e., without prior acid or alkaline hydrolysis) greatly depends on the solubility of the lipids in the solvent used and the ability to separate the lipids from complexes with other macromolecules. The lipid content of a food determined by extraction with one solvent may be quite different from the

8-2 table **Fat Content of Selected Foods**

Food Item	*Percent Fat (Wet Weight Basis)*
Cereals, bread, and pasta	
Rice, white, long-grain, regular, raw, enriched	0.7
Sorghum	3.3
Wheat, soft white	2.0
Rye	2.5
Wheat germ, crude	9.7
Rye bread	3.3
Cracked-wheat bread	3.9
Macaroni, dry, enriched	1.5
Dairy products	
Milk, reduced fat, fluid, 2%	2.0
Skim milk, fluid	0.2
Cheddar cheese	33.1
Yogurt, plain, whole milk	3.2
Fats and oils	
Lard, shortening, oils	100.0
Butter, with salt	81.1
Margarine, regular, hard, soybean	80.5
Salad dressing	
Italian, commercial, regular	28.3
Thousand Island, commercial, regular	35.1
French, commercial, regular	44.8
Mayonnaise, soybean oil, with salt	79.4
Fruits and vegetables	
Apples, raw, with skin	0.2
Oranges, raw, all commercial varieties	0.1
Blackberries, raw	0.5
Avocados, raw, all commercial varieties	14.7
Asparagus, raw	0.1
Lima beans, immature seeds, raw	0.9
Sweet corn, yellow, raw	1.2
Legumes	
Soybeans, mature seeds, raw	19.9
Black beans, mature seed, raw	1.4
Meat, poultry, and fish	
Beef, flank, separable lean and fat	5.0
Chicken, broilers or fryers, breast meat only	1.2
Bacon, pork, cured, raw	45.0
Pork, fresh, loin, whole, raw	12.6
Finfish, halibut, Atlantic and Pacific, raw	2.3
Finfish, cod, Atlantic, raw	0.7
Nuts	
Coconut meat, raw	33.5
Almonds, dried, unblanched, dry roasted	52.8
Walnuts, black, dried	56.6
Egg, whole, raw, fresh	10.0

From US Department of Agriculture, Agricultural Research Service (2009) USDA National Nutrient Database for Standard Reference. Release 22. Nutrient Data Laboratory Home Page, http://www.ars.usda.gov/ba/bhnrc/ndl

content determined with another solvent of different polarity. In addition to solvent extraction methods, there are nonsolvent wet extraction methods and several instrumental methods that utilize the physical and chemical properties of lipids in foods for fat content determination. For nutrition labeling purposes, total fat is most commonly determined by gas chromatography (GC) analysis.

Many of the methods cited in this chapter are official methods of AOAC International. Refer to these methods and other original references cited for detailed instructions of procedures. There are many methods available for the determination of lipid content. This chapter will focus on some of the primary methods in common use.

8.3.1 Sample Preparation

The validity of the fat analysis of a food depends on proper sampling and preservation of the sample before the analysis (see also Chap. 5). An ideal sample should be as close as possible in all of its intrinsic properties to the material from which it is taken. However, a sample is considered satisfactory if the properties under investigation correspond to those of the bulk material within the limits of the test (7).

The sample preparation for lipid analysis depends on the type of food and the type and nature of lipids in the food (8). The extraction method for lipids in liquid milk is generally different from that for lipids in solid soybeans. To analyze the lipids in foods effectively, knowledge of the structure, the chemistry, and the occurrence of the principal lipid classes and their constituents is necessary. Therefore, there is no single standard method for the extraction of all kinds of lipids in different foods. For the best results, sample preparation should be carried out under an inert atmosphere of nitrogen at low temperature to minimize chemical reactions such as lipid oxidation.

Several preparatory steps are common in lipid analysis. These act to aid in extraction by removal of water, reduction of particle size, or separation of the lipid from bound proteins and/or carbohydrates.

8.3.1.1 Predrying Sample

Lipids cannot be effectively extracted with ethyl ether from moist food because the solvent cannot easily penetrate the moist food tissues due to the hydrophobicity of the solvents used or the hydroscopic nature of the solvents. The ether, which is hygroscopic, becomes saturated with water and inefficient for lipid extraction. Drying the sample at elevated temperatures is undesirable because some lipids become bound to proteins and carbohydrates, and bound lipids are not easily extracted with organic solvents. Vacuum oven drying at low temperature or lyophilization increases the surface area of the sample for better lipid extraction. Predrying makes the sample easier to grind for better extraction, breaks fat–water emulsions to make fats dissolve easily in the organic solvent, and helps to free fat from the tissues of foods (7).

8.3.1.2 Particle Size Reduction

The extraction efficiency of lipids from dried foods depends on particle size; therefore, adequate grinding is very important. The classical method of determining fat in oilseeds involves the extraction of the ground seeds with selected solvent after repeated grinding at low temperature to minimize lipid oxidation. For better extraction, the sample and solvent are mixed in a high-speed comminuting device such as a blender. It can be difficult to extract lipids from whole soybeans because of the limited porosity of the soybean hull and its sensitivity to dehydrating agents. The lipid extraction from soybeans is easily accomplished if the beans are broken mechanically by grinding. Extraction of fat from finished products can be a challenge, based on the ingredients (e.g., energy bars with nuts, caramel, protein, granola, soybean oil). Such products may best be ground after freezing with liquid nitrogen.

8.3.1.3 Acid Hydrolysis

A significant portion of the lipids in foods such as dairy, bread, flour, and animal products is bound to proteins and carbohydrates, and direct extraction with nonpolar solvents is inefficient. Such foods must be prepared for lipid extraction by acid hydrolysis. This includes a significant percentage of finished food products. Table 8-3 shows the inaccuracy that can occur if samples are not prepared by acid hydrolysis. Acid hydrolysis can break both covalently and ionically bound lipids into easily extractable lipid forms. The sample can be predigested by refluxing for 1 h

8-3 table **Effects of Acid Digestion on Fat Extraction from Foods**

	Percent Fat	
	Acid Hydrolysis	*No Acid Hydrolysis*
Dried egg	42.39	36.74
Yeast	6.35	3.74
Flour	1.73	1.20
Noodles	3.77–4.84	2.1–3.91
Semolina	1.86–1.93	1.1–1.37

Adapted from (6), p. 154, with permission.

with 3 N hydrochloric acid. Ethanol and solid hexametaphosphate may be added to facilitate separation of lipids from other components before food lipids are extracted with solvents (6, 7). For example, the acid hydrolysis of two eggs requires 10 ml of HCl and heating in a water bath at 65°C for 15–25 min or until the solution is clear (6).

8.3.2 Solvent Selection

Ideal solvents for fat extraction should have a high solvent power for lipids and low or no solvent power for proteins, amino acids, and carbohydrates. They should evaporate readily and leave no residue, have a relatively low boiling point, and be nonflammable and nontoxic in both liquid and vapor states. The ideal solvent should penetrate sample particles readily, be in single component form to avoid fractionation, and be inexpensive and nonhygroscopic (6,7). It is difficult to find an ideal fat solvent to meet all of these requirements. Ethyl ether and petroleum ether are the most commonly used solvents, but pentane and hexane are used to extract oil from soybeans.

Ethyl ether has a boiling point of 34.6°C and is a better solvent for fat than petroleum ether. It is generally expensive compared to other solvents, has a greater danger of explosion and fire hazards, is hygroscopic, and forms peroxides (6). **Petroleum ether** is the low boiling point fraction of petroleum and is composed mainly of pentane and hexane. It has a boiling point of 35–38°C and is more hydrophobic than ethyl ether. It is selective for more hydrophobic lipids, cheaper, less hygroscopic, and less flammable than ethyl ether. The detailed properties of petroleum ether for fat extraction are described in AOAC Method 945.16 (8).

A combination of two or three solvents is frequently used. The solvents should be purified and peroxide free and the proper solvent-to-solute ratio must be used to obtain the best extraction of lipids from foods (7).

8.3.3 Continuous Solvent Extraction Method: Goldfish Method

8.3.3.1 Principle and Characteristics

For continuous solvent extraction, solvent from a boiling flask continuously flows over the sample held in a ceramic thimble. Fat content is measured by weight loss of the sample or by weight of the fat removed.

The continuous methods give faster and more efficient extraction than semicontinuous extraction methods. However, they may cause channeling which results in incomplete extraction. The Goldfish (as well as the Wiley and Underwriters) tests are examples of continuous lipid extraction methods (6,7).

8.3.3.2 Procedure (See Fig. 8-1)

1. Weigh predried porous ceramic extraction thimble. Place vacuum oven dried sample in thimble and weigh again. (Sample could instead be combined with sand in thimble and then dried.)
2. Weigh predried extraction beaker.
3. Place ceramic extraction thimble into glass holding tube and then up into condenser of apparatus.
4. Place anhydrous ethyl ether (or petroleum ether) in extraction beaker and put beaker on heater of apparatus.
5. Extract for 4 h.
6. Lower the heater and let sample cool.
7. Remove the extraction beaker and let air dry overnight, then at 100°C for 30 min. Cool beaker in desiccator and weigh.

8.3.3.3 Calculations

$$\text{Weight of fat in sample} = (\text{beaker} + \text{fat}) - \text{beaker} \quad [1]$$

$$\%\ \text{Fat on dry weight basis} = (\text{g of fat in sample/g of dried sample}) \times 100 \quad [2]$$

8-1 figure Goldfish fat extractor. (Courtesy of Labconco Corp., Kansas City, MO.) (http://www.labconco.com/_scripts/EditItem.asp?ItemID=487)

8.3.4 Semicontinuous Solvent Extraction Method: Soxhlet Method

The Soxhlet method (AOAC Method 920.39C for Cereal Fat; AOAC Method 960.39 for Meat Fat) (8) is an example of the semicontinuous extraction method and is described below.

8.3.4.1 *Principle and Characteristics*

For semicontinuous solvent extraction, the solvent builds up in the extraction chamber for 5–10 min and completely surrounds the sample and then siphons back to the boiling flask. Fat content is measured by weight loss of the sample or by weight of the fat removed.

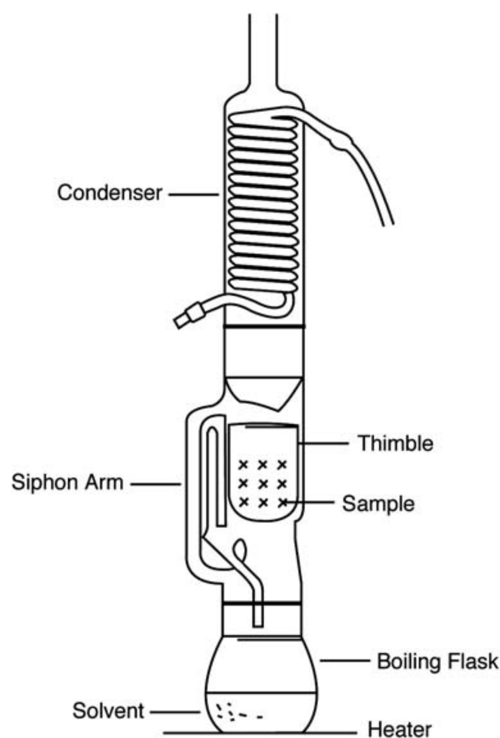
This method provides a soaking effect of the sample and does not cause channeling. However, this method requires more time than the continuous method. Instrumentation for a more rapid and automated version of the Soxhlet method is available (e.g., Soxtec™, FOSS in North America, Eden Prairie, MN) and is used for some quality control applications.

8.3.4.2 *Preparation of Sample*

If the sample contains more than 10% H_2O, dry the sample to constant weight at 95–100°C under pressure $\leq$ 100 mm Hg for about 5 h (AOAC Method 934.01).

8.3.4.3 *Procedure* (See Fig. 8-2)

1. Weigh, to the nearest mg, about 2 g of predried sample into a predried extraction thimble, with porosity permitting a rapid flow of ethyl ether. Cover sample in thimble with glass wool.
2. Weigh predried boiling flask.
3. Put anhydrous ether in boiling flask. *Note*: The anhydrous ether is prepared by washing commercial ethyl ether with two or three portions of H_2O, adding NaOH or KOH, and letting stand until most of H_2O is absorbed from the ether. Add small pieces of metallic Na and let hydrogen evolution cease (AOAC Method 920.39B). Petroleum ether may be used instead of anhydrous ether (AOAC Method 960.39).
4. Assemble boiling flask, Soxhlet flask, and condenser.
5. Extract in a Soxhlet extractor at a rate of five or six drops per second by condensation for about 4 h, or for 16 h at a rate of two or three drops per second by heating solvent in boiling flask.
6. Dry boiling flask with extracted fat in an air oven at 100°C for 30 min, cool in desiccator, and weigh.



8-2 figure Soxhlet extraction apparatus.

8.3.4.4 *Calculation*

% Fat on dry weight basis

$$= (\text{g of fat in sample}/\text{g of dried sample}) \times 100 \quad [3]$$

8.3.5 Discontinuous Solvent Extraction Methods

8.3.5.1 *Mojonnier Method*

8.3.5.1.1 Principle and Characteristics Fat is extracted with a mixture of ethyl ether and petroleum ether in a Mojonnier flask, and the extracted fat is dried to a constant weight and expressed as percent fat by weight.

The Mojonnier test is an example of the discontinuous solvent extraction method and does not require removal of moisture from the sample. It can be applied to both liquid and solid samples. If petroleum ether is used to purify the extracted fat, this method is very similar to the **Roese-Gottlieb Method** (AOAC Method 905.02) in both principle and practice. The Mojonnier flasks (Fig. 8-3) are used not only for the Mojonnier and Roese-Gottlieb methods, but also to do the hydrolysis (acid, alkaline, or combination) prior to fat extraction and GC analysis to determine fat content and fatty acid profile (Sect. 8.3.6). (Note that sometimes the terms Mojonnier, Roese Gottlieb, and alkaline hydrolysis are used interchangeably.)

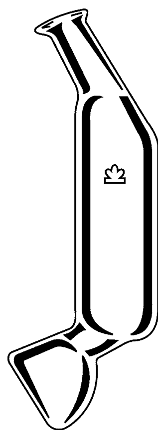
The Mojonnier method was developed for and is applied primarily to dairy foods (procedure as described below for milk fat), but is applicable to other foods. Specifically, methods for fat in flour (AOAC Method 922.06) and fat in pet food (AOAC Method 954.02) both involve an acid hydrolysis with HCl, followed by extraction with a combination of ethyl ether and petroleum ether as described in AOAC Method 989.05 below for milk fat.

8.3.5.1.2 Procedure: Milk Fat Method (AOAC Method 989.05)

1. **Preparation of Sample**. Bring the sample to about 20°C; mix to prepare a homogeneous sample by pouring back and forth between clean beakers. Promptly weigh or measure the test portion. If lumps of cream do not disperse, warm the sample in a water bath to about 38°C and keep mixing until it is homogeneous, using a "rubber policeman" if necessary to reincorporate the cream adhering to the container or stopper. When it can be done without interfering with dispersal of the fat, cool warmed samples to about 20°C before transferring the test portion.
2. **Procedure**
   - (a) Weigh, to the nearest 0.1 mg, 10 g of milk into a Mojonnier fat extraction flask (Fig. 8-3).
   - (b) Add 1.5 ml of NH_4OH and shake vigorously. Add 2 ml if the sample is sour. NH_4OH neutralizes the acidic sample and dissolves protein.
   - (c) Add 10 ml of 95% ethanol and shake for 90 s. The alcohol prevents possible gel formation.
   - (d) Add 25 ml of ethyl ether and shake for 90 s. The ether dissolves the lipid.
   - (e) Cool if necessary, and add 25 ml of petroleum ether and shake for 90 s. The petroleum ether removes moisture from the ethyl ether extract and dissolves more nonpolar lipid.
   - (f) Centrifuge for 30 s at 600 rpm.
   - (g) Decant ether solution from the Mojonnier flask into the previously weighed Mojonnier fat dish.
   - (h) Perform second and third extractions in the same manner as for the first extraction described previously (ethanol, ethyl ether, petroleum ether, centrifugation, decant).
   - (i) Evaporate the solvent in the dish on the electric hot plate at ≤100°C in a hood.
   - (j) Dry the dish and fat to a constant weight in a forced air oven at 100°C ± 1°C.
   - (k) Cool the dish to room temperature and weigh.
3. **Calculations**

$$\begin{aligned}\%\ \text{Fat} = 100 \times \{&[(\text{wt dish} + \text{fat}) - (\text{wt dish})]\\ &-(\text{avg wt blank residue})\}/\text{wt sample}\end{aligned} \quad [4]$$

A pair of reagent blanks must be prepared every day. For reagent blank determination, use 10 ml of distilled water instead of milk sample. The reagent blank should be <0.002 g. Duplicate analyses should be <0.03% fat.



8-3 figure Mojonnier fat extraction flask. (Courtesy of Kontes Glass Co., Vineland, NJ.)

8.3.5.2 Chloroform–Methanol Procedure

8.3.5.2.1 Principle and Characteristics The combination of chloroform and methanol has been used commonly to extract lipids. The "Folch extraction" (9) applied to small samples, and the "Bligh and Dyer extraction" (10) applied to large samples of high moisture content, both utilize this combination of solvents to recover lipids from foods. These methods have been reviewed and procedures modified by Christie (11) and others. The Bligh and Dyer procedure (10) is a modification of the Folch extraction (9), designed for more efficient solvent usage for low-fat samples. The Christie modification (11) of these former methods replaced water with 0.88% potassium chloride aqueous solution to create two phases.

In both the modified Folch extraction and Bligh and Dyer procedure, food samples are mixed/homogenized in a chloroform–methanol solution, and the homogenized mixture is filtered into a collection tube. A 0.88% potassium chloride aqueous solution is added to the chloroform–methanol mixture

containing the extracted fats. This causes the solution to break into two phases: the aqueous phase (top) and the chloroform phase containing the lipid (bottom). The phases are further separated in a separatory funnel or by centrifugation. After evaporation of the chloroform, the fat can be quantitated by weight.

The various methanol–chloroform extraction procedures are rapid, well suited to low-fat samples, and can be used to generate lipid samples for subsequent fatty acid compositional analysis. The procedure has been more applied to basic commodities, rather than to finished product samples. For consistent results, the procedures must be followed carefully, including the ratio of chloroform and methanol. A cautionary note is that chloroform and methanol are highly toxic, so the extraction procedure must be done in well-ventilated areas.

8.3.5.2.2 Procedure (Modified Folch Extraction) (9, 11)

1. Weigh 1 g accurately and homogenize 1 min in 10 ml of methanol.
2. Add 20 ml of chloroform and homogenize for 2 min.
3. Filter on a Buchner funnel and reextract cake and filter paper with 20 ml of chloroform plus 10 ml methanol.
4. Filter and combine two filtrates. Measure volume. Calculate 25% of the total volume and add this amount of 0.88% KCl. Let separate in a separatory funnel.
5. Remove the lower lipid-containing layer and measure the volume. Calculate 25% of the volume and add this volume of water to wash the sample.
6. Remove water from the sample by adding sodium sulfate. Filter to separate sample from hydrated sodium sulfate. Use chloroform to wash the sodium sulfate on the filter paper.
7. Place the chloroform–lipid solution in a weighed round bottom flask and remove chloroform from the lipid using a rotary evaporator with water temperature below 50°C. (If the extract is cloudy, add 20 ml of acetone and 20 ml of chloroform and reevaporate.) Weigh the flask to determine the % lipid in the sample.

8.3.6 Total Fat by GC for Nutrition Labeling (AOAC Method 996.06)

8.3.6.1 Principle

AOAC International (8) gives an excellent description of the principle of AOAC Method 996.06: "Fat and fatty acids are extracted from food by hydrolytic methods (acidic hydrolysis for most products, alkaline hydrolysis for dairy products, and combination for cheese). Pyrogallic acid is added to minimize oxidative degradation of fatty acids during analysis. Triglyceride, triundecanoin ($C_{11:0}$), is added as internal standard. Fat is extracted into ether, then methylated to *fatty acid methyl esters* (*FAMEs*) using BF_3 (boron trifluoride) in methanol. FAMEs are quantitatively measured by capillary gas chromatography (GC) against $C_{11:0}$ internal standard. Total fat is calculated as sum of individual fatty acids expressed as triglyceride equivalents. Saturated and monounsaturated fats are calculated as sum of respective fatty acids. Monounsaturated fat includes only *cis* form." Trans fat can be quantified utilizing this method in conjunction with identification criteria established by the American Association of Oil Chemists (AOCS Method Ce 1h-05) (12) and Golay et al. (13).

8.3.6.2 Sample Preparation Procedures

Samples are methylated by either a standard or alternative method (Fig. 8-4) to form the FAMEs prior to GC analysis. In both methods, the sample is combined with a specified amount of the **internal standard** triglyceride, triundecanoin.

Most foods and 100% fat products can be subjected to the standard sample preparation procedure. Before using the standard method, unless the sample is 100% fat, it is subjected to the appropriate fat extraction procedure (including, but not limited to, acid hydrolysis, alkaline hydrolysis, and Soxhlet extraction). Before any samples are subjected to acid or alkaline hydrolysis, 1.0 ml of triundecanoin internal standard solution is added to the sample. In the case of a sample extracted by the Soxhlet method, or if the sample is 100% fat, 1.0 ml of the internal standard is added directly to the flask used as the sample is methylated. Extracted samples can be stored refrigerated (5 ± 3°C), with hexane added, for up to 2 weeks if methylation will not be done immediately. (The hexane is dried off on a steam bath with a gentle stream of nitrogen before the sample is weighed and methylation occurs.)

The alternative sample preparation method is efficient for infant formulas and other similar matrices containing short chain fatty acids (e.g., butyric acid) and long chain fatty acids (e.g., eicosapentaenoic or docosahexaenoic acids) and for products containing microencapsulated fatty acids. This alternative procedure provides for more thorough recovery of short chain fatty acids, such as butyric acid, and eliminates the preliminary fat extraction step, thereby saving time.

Standard Sample Preparation Procedure:

1. For oil samples (100% lipid) or samples that were extracted by the Soxhlet method, accurately weigh a portion of the lipid (usually 0.15–0.2 g) into a 125-ml, or 50-ml, round bottom flask, containing the internal standard. For low lipid amounts, the lipid may be transferred into the flask using hexane. The weight of lipid is the difference in the weight of the empty flask and the weight of the flask after the hexane is evaporated on a steam both under a gentle stream of nitrogen.
2. Add 4–5 ml of 0.5 *N* sodium hydroxide (NaOH) in methanol (MeOH), then a few boiling chips.
3. Attach a water-cooled condenser and reflux on a boiling steam bath for ∼15 min.
4. Add 5 ml of boron trifluoride (BF3): methanol (MeOH) (14%, w/v) through the top of the condenser.
5. Reflux for ∼5 min.
6. Add an appropriate amount of heptane, usually 10 ml.
7. Reflux for ∼1 min.
8. Raise the round bottom flask still attached to the condenser above the steam bath and let it cool for ∼15 min. Disconnect the condensers.
9. Add 2–3 ml of saturated sodium chloride (NaCl) solution to the flask and stopper the flask.
10. Let stand until it reaches room temperature (∼15 min).
11. Add saturated NaCl solution so that the heptane layer will float in the neck of the flask.
12. Place a small scoop of sodium sulfate in a vial.
13. Transfer the heptane layer containing the methyl esters into the vial and place the screw cap on the vial. (Sample is stable for 2 wk)
14. Inject an appropriate amount of the heptane layer into the GC system.

Alternative Sample Preparation Procedure:

Note: Samples with butryric and caproic acids (typically found in matrices containing dairy products) are treated differently after Step 8 than those without. Step 9 provides direction concerning this.

1. For a final volume of 5 ml (usually samples with less than 10% fat, or known to have low concentrations of the fatty acids of interest), add 1.0 ml of the tridecanoic tritridecanoin internal standard to a 50-ml round-bottom flask. If a final volume of 10 ml is used (usually samples known to contain more than 10% fat), add 2.0 ml of the tritridecanoic tritridecanoin internal standard. Evaporate the solvent on a steambath under a gentle stream of nitrogen until dry. Let the flask cool to room temperature.
2. Accurately weigh an appropriate amount of sample (depending on the amount of lipid in the sample) into the round-bottom flask.
3. Add approximately 6 ml of the 0.5 *N* NaOH in MeOH solution and a magnetic stir bar to the round-bottom flask.
4. Attach the flask to a condenser and reflux the solution for 1–2 hr on a heating stirplate.
5. Dry moist samples or powder samples under a gentle nitrogen stream without heat to avoid bumping. Liquid samples may be dried on a steam bath with a gentle nitrogen stream.
6. Place the flask in an oven set to maintain 100°C to dry completely for (usually 20–30 min) (cover samples that splatter in oven). Cool to room temperature. At this point, the samples may be covered and left at room temperature overnight (6–12 hr).
7. Add approximately 5 ml MeOH and a few boiling chips to the round-bottom flask. Connect the flask to condensers over a steambath.
8. Bring the samples to a boil and add approximately 5 ml of BF_3 in MeOH (14% w/v) through the condenser.
9. For samples not containing butyric and caproic acids skip to Step 11; samples containing butyric and caproic acid continue to Step 10.
10. Methylation of fatty acids including butyric and caproic acids.
    - 10.1. Continue to reflux the sample for ∼1 min.
    - 10.2. Raise the sample above the steam bath and add an appropriate amount of methylene chloride [either 5.0 or 10.0 ml depending on the amount (1 or 2 ml, respectively) of tridecanoic tritridecanoin internal standard added in Step 1].
    - 10.3. Place the flask in a cup containing ice chips to quickly cool the sample, while still leaving it attached to the condenser and let sample cool.
    - 10.4. Remove flask from condenser and add 5–20 ml saturated NaCl solution. Stopper and shake.
    - 10.5. Transfer contents of flask to a 50-ml glass centrifuge tube and centrifuge to achieve separation of the methylene chloride.
    - 10.6. Continue as specified in Steps 11–14 of the Standard Sample Preparation section, replacing heptane with methylene chloride. The methylene chloride layer will be the lower layer.
11. Methylation of fatty acids excluding butyric and caproic acids.
    - 11.1. Continue to reflux the sample for ∼2–5 min.
    - 11.2. Add an appropriate amount of heptane through the condenser [either 5 or 10 ml, depending on the amount (1 or 2 ml, respectively) of tridecanoic tritridecnoin internal standard added in Step 1]. Continue to reflux the solution for ∼1 min.
    - 11.3. Continue as specified in Steps 9–14 of the Standard Sample Preparation section.

8-4 figure Standard and alternative procedures to methylate samples prior to gas chromatographic analysis for fatty acid composition.

8.3.6.3 Chromatographic Conditions

The following are two sets of conditions that might be used, but conditions may be modified as needed to optimize separation. Electronic pressure control of gases may be used.

Column	Any 0.25 mm ID × 100 m long, 0.20 μm film thickness 100% biscyanopropanol (nonbonded) capillary column such as SP-2560, HP-88, CP-Sil 88, etc.
Column temperature	170°C, hold 11.10 min; increase 7.9°C/min to 200°C, hold 1.27 min; increase 7.9°C/min to 210°C, hold 16 min
Injection port temperature	250°C
Detector temperature	300°C
Carrier gas (hydrogen) flow	1.2 ml/min (26 cm/s)
Split ratio	100:1
Hydrogen flow	30 ml/min
Air flow	300 ml/min
Makeup gas flow	30 ml/min

OR

Column	Any 0.25 mm ID × 100 m long, 0.20 μm film thickness 100% cyanopropanol capillary column such as SP-2560, HP-88, CP-Sil 88, etc.
Column temperature	170°C, hold 5.0 min; increase 2.0°C/min to 190°C, hold 5.0 min; increase 10.0°C/min to 210°C, hold 5.0 min; increase 10.0°C/min to 230°C, hold 9 min

8.3.6.4 Calculations

Calculations can be made by peak height or by integrated area (Fig. 8-5; Table 8-4). Fatty acids may be calculated as triglycerides, methyl esters, ethyl esters, or acids. Use a current validated computer software (e.g., Waters Empower Chromatography Manager, Waters Corporation, Milford, MA) to perform calculations, entering data for sample weight (mg) and final volume (ml).

$$\text{\% Fatty acid on a lipid basis} = \text{mg/mg of fatty acid} \times 100 \qquad [5]$$

or

$$\text{\% Fatty acids on a sample basis} = \text{mg/mg of fatty acid} \times \text{\% lipid} \qquad [6]$$

Alternative calculations can be made using peak areas obtained from current validated computer software. Calculate the response factor (RF) for each standard (by dividing the peak area by the internal standard area) and the standard factor (F) (by dividing the response factor by the internal standard concentration, mg/ml). Measure the peak area of each respective fatty acid and the internal standard, then calculate the sample response factor (SRF) by dividing the sample fatty acid peak area by the internal standard peak area.

$$\text{\% Fatty acid} = \frac{\text{SRF}}{F} \times \frac{\text{final sample volume (ml)}}{\text{sample weight (mg)}} \times 100 \qquad [7]$$

where:

SRF = sample response factor
F = standard factor

8.4 NONSOLVENT WET EXTRACTION METHODS

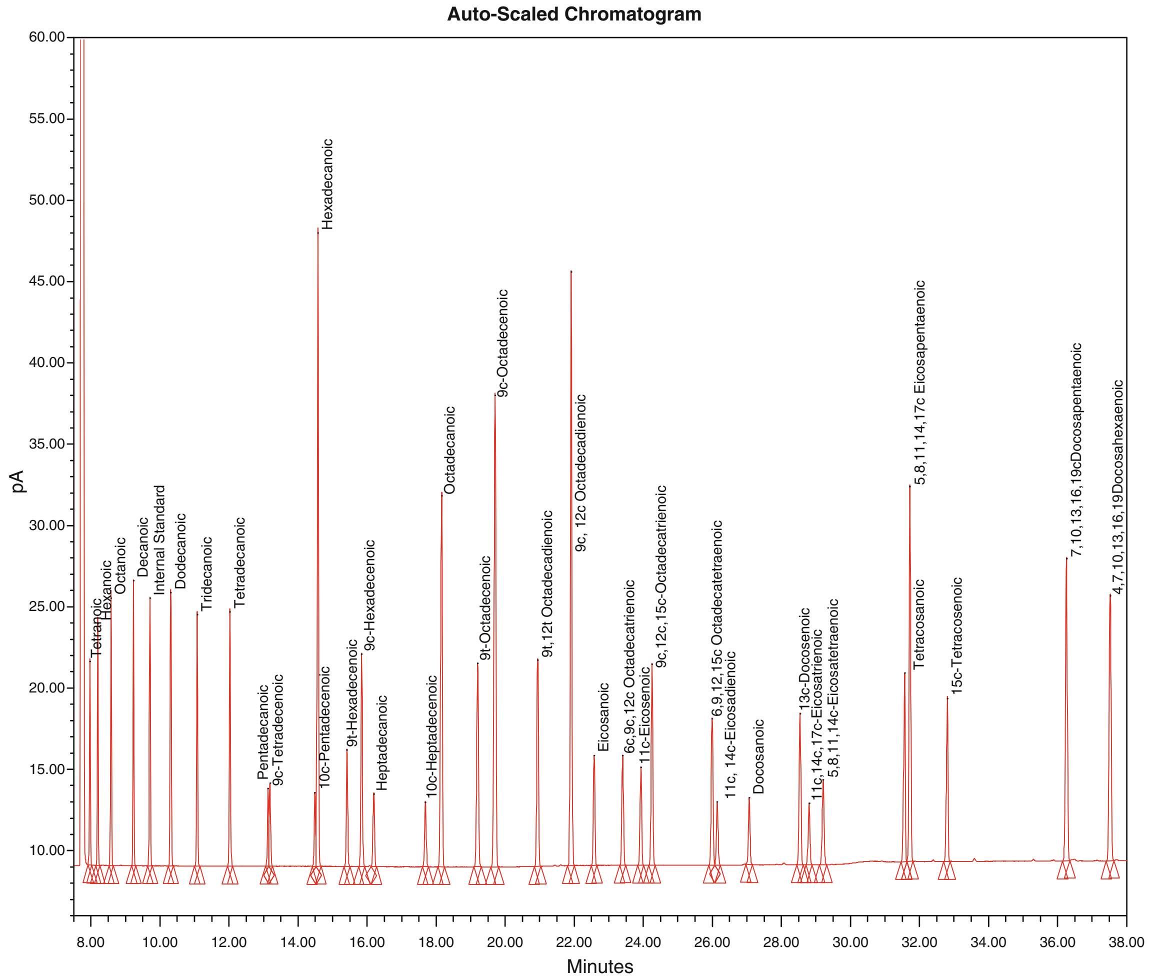
8.4.1 Babcock Method for Milk Fat (AOAC Method 989.04 and 989.10)

8.4.1.1 Principle

In the Babcock method, H_2SO_4 is added to a known amount of milk in the Babcock bottle. The sulfuric acid digests protein, generates heat, and releases the fat. Centrifugation and hot water addition isolate fat for quantification in the graduated portion of the test bottle. The fat is measured volumetrically, but the result is expressed as percent fat by weight.

8.4.1.2 Procedure

1. Accurately pipette the milk sample (17.6 ml) into a Babcock test bottle (Fig. 8-6).
2. Add reagent grade (1.82 specific gravity) sulfuric acid (17.5 ml) to the bottle, allowing the acid to flow gently down the neck of the bottle as it is being slowly rotated. The acid digests proteins to liberate the fat.
3. Centrifuge the mixture for 5 min and liquid fat will rise into the calibrated bottle neck. The centrifuge must be kept at 55–60°C during centrifugation.
4. Add hot water to bring liquid fat up into the graduated neck of the Babcock bottle.
5. The direct percentage of fat by weight is read to the nearest 0.05% from the graduation mark of the bottle.



8-5 figure Example of chromatogram from gas chromatography analysis.

8-4 table

Fatty Acid Conversion Table

	Molecular Weight				Conversion Factors[b]		
Fatty Acid[a]	*Acid*	*Methyl Ester*	*Triglyceride*	*1/3 Triglyceride*	*Triglyceride/ Methyl Ester*	*Acid/Methyl Ester*	*Acid/ Triglyceride*
4:0 Butyric Tetranoic	88.11	102.14	302.38	100.79	0.9868	0.8627	0.8742
5:0 Valeric Pentanoic	102.40	116.43	345.25	115.08	0.9885	0.8795	0.8898
6:0 Caproic Hexanoic	116.16	130.19	386.53	128.84	0.9897	0.8923	0.9016
7:0 Heptanoic	130.19	144.22	428.62	142.87	0.9907	0.9027	0.9112
8:0 Caprylic Octanoic	144.21	158.24	470.69	156.90	0.9915	0.9114	0.9192
10:0 Capric Decanoic	172.27	186.30	554.85	184.95	0.9928	0.9247	0.9314
12:0 Lauric Dodecanoic	200.35	214.38	639.02	213.01	0.9937	0.9346	0.9405
13:0 Tridecanoic	214.35	228.38	681.10	227.03	0.9941	0.9386	0.9441

(continued)

8-4 table **Fatty Acid Conversion Table (continued)**

Fatty Acid[a]	Molecular Weight				Conversion Factors[b]		
	Acid	Methyl Ester	Triglyceride	1/3 Triglyceride	Triglyceride/ Methyl Ester	Acid/Methyl Ester	Acid/ Triglyceride
14:0 Myristic Tetradecanoic	228.38	242.41	723.18	241.06	0.9945	0.9421	0.9474
14:1 Myristoleic 9-Tetradecenoic	226.38	240.41	717.18	239.06	0.9944	0.9417	0.9469
15:0 Pentadecanoic	242.41	256.44	765.26	255.09	0.9948	0.9453	0.9503
15:1 Pentadecenoic 10-Pentadecenoic	240.40	254.43	759.26	253.09	0.9947	0.9449	0.9499
16:0 Palmitic Hexadecanoic	256.43	270.46	807.34	269.11	0.9950	0.9481	0.9529
16:1 Palmitoleic 9-Hexadecenoic	254.43	268.46	801.46	267.11	0.9950	0.9477	0.9525
17:0 Heptadecanoic	270.48	284.51	849.42	283.14	0.9953	0.9507	0.9552
17:1 Heptadecenoic 10-Heptadecenoic	268.48	282.51	843.42	281.14	0.9952	0.9503	0.9549
18:0 Stearic Octadecanoic	284.48	298.51	891.50	297.17	0.9955	0.9530	0.9573
18:1 Oleic 9-Octadecenoic	282.48	296.51	885.50	295.17	0.9955	0.9527	0.9570
18:2 Linoleic 9-12 Octadecadienoic	280.48	294.51	879.50	293.17	0.9954	0.9524	0.9567
18:3 Gamma Linolenic 6-9-12 Octadecatrienoic	278.48	292.51	873.50	291.17	0.9954	0.9520	0.9564
18:3 Linolenic 9-12-15 Octadecatrienoic	278.48	292.51	873.50	291.17	0.9954	0.9520	0.9564
18:4 Octadecatetraenoic 6-9-12-15 Octadecatetraenoic	276.48	290.51	867.50	289.17	0.9954	0.9517	0.9561
20:0 Arachidic Eicosanoic	312.54	326.57	975.66	325.22	0.9959	0.9570	0.9610
20:1 Eicosenoic 11-Eicosenoic	310.54	324.57	969.66	323.22	0.9959	0.9568	0.9608
20:2 Eicosadienoic 11-14 Eicosadienoic	308.53	322.56	963.66	321.22	0.9958	0.9565	0.9605
20:3 Eicosatrienoic 11-14-17 Eicosatrienoic	306.53	320.56	957.66	319.22	0.9958	0.9562	0.9603
20:4 Arachidonic 5-8-11-14 Eicosatetraenoic	304.52	318.55	951.66	317.22	0.9958	0.9560	0.9600
20:5 Eicosapentaenoic 5-8-11-14-17 Eicosapenatenoic	302.52	316.55	945.66	315.22	0.9958	0.9557	0.9598
22:0 Behenic Docosanoic	340.59	354.62	1,059.82	353.27	0.9962	0.9604	0.9641
22:1 Erucic 13-Docosenoic	338.59	352.63	1,053.82	351.27	0.9962	0.9602	0.9639
22:5 Docosapentaenoic 7-10-13-1619 Docosapentaenoic	330.50	344.53	1,029.55	343.18	0.9961	0.9593	0.9630
22:6 Docosahexaenoic 4-7-10-13-16-19 Docosahexaenoic	328.57	342.60	1,023.82	341.27	0.9961	0.9591	0.9628
24:0 Lignoceric Tetracosanoic	368.64	382.67	1,143.98	381.33	0.9965	0.9633	0.9667
24:1 Nervonic 15-Tetracosenoic	366.63	380.66	1,137.98	379.33	0.9965	0.9632	0.9666

[a]Top number indicates carbon chain length and degree of unsaturation. Bottom number(s) indicate position of double bond(s) on the carbon chain.

[b]Conversion factors: To convert methyl ester to triglyceride: multiply by T/M ratio; To convert methyl ester to acid: multiply by A/M ratio; To convert triglyceride to acid: multiply by A/T ratio; To convert tridecanoic to methyl ester: multiply by 1.065 or divide by A/M ratio.

8-6 figure Babcock milk test bottles for milk (**a**), cream (**b**), and cheese (Paley bottle) (**c**) testing. (Courtesy of Kimble Glass Co., Vineland, NJ.)

8.4.1.3 Applications

The Babcock method, which is a common official method for the determination of fat in milk, takes about 45 min and duplicate tests should agree within 0.1%. The Babcock method does not determine the phospholipids in the milk products. It is not applicable to products containing chocolate or added sugar without modification because of charring of chocolate and sugars by sulfuric acid. A modified Babcock method is used to determine essential oil in flavor extracts (AOAC Method 932.11) and fat in seafood (AOAC Method 964.12).

8.4.2 Gerber Method for Milk Fat

8.4.2.1 Principle

The principle of the Gerber method is similar to that of the Babcock method, but it uses sulfuric acid and amyl alcohol. The sulfuric acid digests proteins and carbohydrates, releases fat, and maintains the fat in a liquid state by generating heat.

8.4.2.2 Procedure

1. Transfer 10 ml of H_2SO_4 at 15–21°C into a Gerber milk bottle.
2. Accurately measure milk sample (11 ml) into the Gerber bottle, using a Gerber pipette.
3. Add 1 ml of isoamyl alcohol to the bottle.
4. Tighten the stopper and mix by shaking the bottle.
5. Centrifuge the bottle for 4 min.
6. Place the bottle in a water bath at 60–63°C for 5 min and then read the fat content from the graduations on the bottle neck.

8.4.2.3 Applications

The Gerber method is comparable to the Babcock method but is simpler and faster and has wider application to a variety of dairy products (14). The isoamyl alcohol generally prevents the charring of sugar found with the regular Babcock method. This test is more popular in Europe than in America.

8.5 INSTRUMENTAL METHODS

Instrumental methods offer numerous attractive features compared with the previously described extraction methods. In general, they are rapid, nondestructive, and require minimal sample preparation and chemical consumption. However, the equipment can be expensive and measurements often require the establishment of calibration curves specific to various compositions. Despite these drawbacks, several of the following instrumental methods are very widely used in quality control as well as research and product development applications. The following section describes several of these instrumental methods.

8.5.1 Infrared Method

The infrared (IR) method is based on absorption of IR energy by fat at a wavelength of 5.73 μm. The more the energy absorption at 5.73 μm, the higher is the fat content of the sample (15). Mid-IR spectroscopy is used in Infrared Milk Analyzers to determine milk fat content (AOAC Method 972.16). Near-infrared (NIR) spectroscopy has been used to measure the fat content of commodities such as meats, cereals, and oilseeds in the laboratory and is being adapted for on-line measurement. See Chap. 23 for a discussion of IR spectroscopy.

8.5.2 Specific Gravity (Foss-Let Method)

Fat content by the Foss-Let method (Foss North America, Eden Prairie, MN) is determined as a function of the specific gravity of a sample solvent extract. A sample of known weight is extracted for 1.5–2 min in a vibration-reaction chamber with perchloroethylene. The extract is filtered, and using a thermostatically controlled device with digital readout, its specific

gravity is determined. The reading can then be converted to oil or fat percentage using a conversion chart.

8.5.3 Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) can be used to measure lipids in food materials in a nondestructive way. It is one of the most popular methods for use in determining lipid melting curves to measure solid fat content (see Chap. 14), and with more affordable instruments is becoming more popular for measuring total fat content. Total fat content can be measured using low-resolution pulsed NMR. The principles and applications of NMR are described in Chap. 25. NMR analysis is a very rapid and accurate method, and while the principles of NMR are relatively complex, the use of NMR can be quite simple, especially due to the high degree of automation and computer control.

8.6 COMPARISON OF METHODS

Soxhlet extraction or its modified method is a common crude fat determination method in many food commodities. However, this method requires a dried sample for the hydroscopic ethyl ether extraction. If the samples are moist or liquid foods, the Mojonnier method is generally applicable to determination of the fat content. Acid hydrolysis or alkaline hydrolysis is widely used on many finished food products. The instrumental methods such as IR and NMR are very simple, reproducible, and fast, but are available only for fat determination for specific foods. The application of instrumental methods for fat determination generally requires a standard curve between the signal of the instrument analysis and the fat content obtained by a standard solvent extraction method. However, a rapid instrumental method could be used as a quality control method for fat determination of a specific food.

Major uses of the Goldfish, Soxhlet, and Mojonnier (or Roese-Gottlieb) methods include the following: (1) extract fat prior to GC analysis, (2) quality control of formulated products, (3) determine fat content during product development, (4) verify when fat content is <0.5 g per serving (so nutrient content claim can be made), and (5) defat samples prior to fiber analysis. Compared with GC analysis of fat content by AOAC Method 996.06, these three methods are faster and cheaper, but give a higher fat content (which must be recognized when using these methods for product development).

8.7 SUMMARY

Lipids are generally defined by their solubility characteristics rather than by some common structural feature. Lipids in foods can be classified as simple, compound, or derived lipids. The lipid content of foods varies widely, but quantitation is important because of regulatory requirements, nutritive value, and functional properties. To analyze food for the fat content accurately and precisely, it is essential to have a comprehensive knowledge of the general compositions of the lipids in the foods, the physical and chemical properties of the lipids as well as the foods, and the principles of fat determination. There is no single standard method for the determination of fats in different foods. The validity of any fat analysis depends on proper sampling and preservation of the sample prior to analysis. Predrying of the sample, particle size reduction, and acid hydrolysis prior to analysis also may be necessary. The total lipid content of foods is commonly determined by organic solvent extraction methods, which can be classified as continuous (e.g., Goldfish), semicontinuous (e.g., Soxhlet), discontinuous (e.g., Mojonnier, Folch), or by GC analysis for nutrition labeling. Nonsolvent wet extraction methods, such as the Babcock or Gerber, are commonly used for certain types of food products. Instrumental methods, such as NMR, infrared, and Foss-Let, are also available for fat determination of specific foods. These methods are rapid and so may be useful for quality control but generally require correlation to a standard solvent extraction method.

8.8 STUDY QUESTIONS

1. What are some important considerations when selecting solvents to be used in continuous and noncontinuous solvent extraction methods?
2. To extract the fat from a food sample, you have the choice of using ethyl ether or petroleum ether as the solvent, and you can use either a Soxhlet or a Goldfish apparatus. What combination of solvent and extraction would you choose? Give all the reasons for your choice.
3. Itemize the procedures that may be required to prepare a food sample for accurate fat determination by a solvent extraction method (e.g., Soxhlet method). Explain why each of these procedures may be necessary.
4. You performed fat analysis on a new superenergy shake (high carbohydrate and protein) using standard Soxhlet extraction. The value obtained for fat content was much lower than that expected. What could have caused the measured fat content to be low and how would you modify the standard procedure to correct the problem?

5. What is the purpose of the following chemicals used in the Mojonnier method?
   (a) Ammonium hydroxide
   (b) Ethanol
   (c) Ethyl ether
   (d) Petroleum ether
6. What is a key application of the GC method and what does it specifically quantify?
7. What is the purpose of the following procedures used in Babcock method?
   (a) Sulfuric acid addition
   (b) Centrifugation and addition of hot water
8. Which of the following methods are volumetric and which are gravimetric determinations of lipid content: Babcock, Soxhlet, Mojonnier, Gerber?
9. Explain and contrast the principles (not procedures) involved in determining the fat content of a food product by the following methods. Indicate for each method the type of sample and application that would be appropriate for analysis.
   (a) Soxhlet
   (b) Babcock
   (c) Mojonnier
   (d) GC analysis

8.9 PRACTICE PROBLEMS

1. To determine the fat content of a semimoist food by the Soxhlet method, the food was first vacuum oven dried. The moisture content of the product was 25%. The fat in the dried food was determined by the Soxhlet method. The fat content of the dried food was 13.5%. Calculate the fat content of the original semimoist product.
2. The fat content of 10 g of commercial ice cream was determined by the Mojonnier method. The weights of extracted fat after the second extraction and the third extraction were 1.21 g and 1.24 g, respectively. How much of fat, as a percentage of the total, was extracted during the third extraction?

Answers

1. If the sample weight of a semimoist food is 10 g and the moisture content is 25%, the dried weight of the original food is 7.5 g ($10\,g \times 75\% = 7.5\,g$). If the fat content of the dried food is 13.5%, the 7.5 g of dried sample has 1.0125 g fat (7.5 g dried food $\times$13.5% fat = 1.0125 g fat). The 10 g of semimoist food contains the same amount of fat, i.e., 1.0125 g. Therefore, the fat content of the semimoist food is 10.125% (1.0125 g fat/10 g semimoist food).
2. $[(1.24\,g–1.21\,g)/10\,g] \times 100 = 0.3\%$

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Protein Analysis

Sam K. C. Chang

Department of Cereal and Food Sciences, North Dakota State University,
Fargo, ND 58105, USA
kow.chang@ndsu.edu

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9.1 INTRODUCTION

9.1.1 Classification and General Considerations

Proteins are an abundant component in all cells, and almost all except storage proteins are important for biological functions and cell structure. Food proteins are very complex. Many have been purified and characterized. Proteins vary in molecular mass, ranging from approximately 5000 to more than a million Daltons. They are composed of elements including hydrogen, carbon, nitrogen, oxygen, and sulfur. Twenty α-amino acids are the building blocks of proteins; the amino acid residues in a protein are linked by peptide bonds. Nitrogen is the most distinguishing element present in proteins. However, nitrogen content in various food proteins ranges from 13.4 to 19.1% (1) due to the variation in the specific amino acid composition of proteins. Generally, proteins rich in basic amino acids contain more nitrogen.

Proteins can be classified by their composition, structure, biological function, or solubility properties. For example, simple proteins contain only amino acids upon hydrolysis, but conjugated proteins also contain non-amino-acid components.

Proteins have unique conformations that could be altered by denaturants such as heat, acid, alkali, 8 *M* urea, 6 *M* guanidine-HCl, organic solvents, and detergents. The solubility as well as functional properties of proteins could be altered by denaturants.

The analysis of proteins is complicated by the fact that some food components possess similar physicochemical properties. Nonprotein nitrogen could come from free amino acids, small peptides, nucleic acids, phospholipids, amino sugars, porphyrin, and some vitamins, alkaloids, uric acid, urea, and ammonium ions. Therefore, the total organic nitrogen in foods would represent nitrogen primarily from proteins and to a lesser extent from all organic nitrogen-containing nonprotein substances. Depending upon methodology, other major food components, including lipids and carbohydrates, may interfere physically with analysis of food proteins.

Numerous methods have been developed to measure protein content. The basic principles of these methods include the determinations of nitrogen, peptide bonds, aromatic amino acids, dye-binding capacity, ultraviolet absorptivity of proteins, and light scattering properties. In addition to factors such as sensitivity, accuracy, precision, speed, and cost of analysis, what is actually being measured must be considered in the selection of an appropriate method for a particular application.

9.1.2 Importance of Analysis

Protein analysis is important for:

1. **Nutrition labeling**
2. **Pricing:** The cost of certain commodities is based on the protein content as measured by nitrogen content (e.g., cereal grains; milk for making certain dairy products, e.g., cheese).
3. **Functional property investigation:** Proteins in various types of food have unique food functional properties: for example, gliadin and glutenins in wheat flour for breadmaking, casein in milk for coagulation into cheese products, and egg albumen for foaming (see Chap. 15).
4. **Biological activity determination:** Some proteins, including enzymes or enzyme inhibitors, are relevant to food science and nutrition: for instance, the proteolytic enzymes in the tenderization of meats, pectinases in the ripening of fruits, and trypsin inhibitors in legume seeds are proteins. To compare between samples, enzymes activity often is expressed in terms of specific activity, meaning units of enzyme activity per mg of protein.

Protein analysis is required when you want to know:

1. Total protein content
2. Content of a particular protein in a mixture
3. Protein content during isolation and purification of a protein
4. Nonprotein nitrogen
5. Amino acid composition (see Chap. 15)
6. Nutritive value of a protein (see Chap. 15)

9.1.3 Content in Foods

Protein content in food varies widely. Foods of animal origin and legumes are excellent sources of proteins. The protein contents of selected food items are listed in Table 9-1.

9.2 METHODS

Principles, general procedures, and applications are described below for various protein determination methods. Refer to the referenced methods for detailed instructions of the procedures. The Kjeldahl, Dumas (N combustion), and infrared spectroscopy methods cited are from the *Official Methods of Analysis* of AOAC International (3) and are used commonly in nutrition labeling and quality control. The other methods

9-1 table **Protein Content of Selected Foods (2)**

Food Item	Percent Protein (Wet Weight Basis)
Cereals and pasta	
Rice, brown, long-grain raw	7.9
Rice, white, long-grain, regular, raw, enriched	7.1
Wheat flour, whole-grain	13.7
Corn flour, whole-grain, yellow	6.9
Spaghetti, dry, enriched	13.0
Cornstarch	0.3
Dairy products	
Milk, reduced fat, fluid, 2%	3.2
Milk, nonfat, dry, regular, with added vitamin A	36.2
Cheese, cheddar	24.9
Yogurt, plain, low fat	5.3
Fruits and vegetables	
Apple, raw, with skin	0.3
Asparagus, raw	2.2
Strawberries, raw	0.7
Lettuce, iceberg, raw	0.9
Potato, whole, flesh and skin	2.0
Legumes	
Soybeans, mature seeds, raw	36.5
Beans, kidney, all types, mature seeds, raw	23.6
Tofu, raw, firm	15.8
Tofu, raw, regular	8.1
Meats, poultry, fish	
Beef, chuck, arm pot roast	21.4
Beef, cured, dried beef	31.1
Chicken, broilers or fryers, breast meat only, raw	23.1
Ham, sliced, regular	16.6
Egg, raw, whole, fresh	12.6
Finfish, cod, Pacific, raw	17.9
Finfish, tuna, white, canned in oil, drained solids	26.5

From US Department of Agriculture, Agricultural Research Service (2009). USDA National Nutrient Database for Standard Reference. Release 22. Nutrient Data Laboratory Home Page, http://www.ars.usda.gov/ba/bhnrc/ndl

described are used commonly in research laboratories working on proteins. Many of the methods covered in this chapter are described in somewhat more detail in recent books on food proteins (4–6).

9.2.1 Kjeldahl Method

9.2.1.1 Principle

In the Kjeldahl procedure, proteins and other organic food components in a sample are digested with sulfuric acid in the presence of catalysts. The **total organic nitrogen** is converted to ammonium sulfate. The digest is neutralized with alkali and distilled into a boric acid solution. The borate anions formed are titrated with standardized acid, which is converted to nitrogen in the sample. The result of the analysis represents the crude protein content of the food since nitrogen also comes from nonprotein components (note that the Kjeldahl method also measures nitrogen in any ammonia and ammonium sulfate).

9.2.1.2 Historical Background

9.2.1.2.1 Original Method In 1883, Johann Kjeldahl developed the basic process of today's Kjeldahl method to analyze organic nitrogen. General steps in the original method include the following:

1. **Digestion** with sulfuric acid, with the addition of powdered potassium permanganate to complete oxidation and conversion of nitrogen to ammonium sulfate.
2. **Neutralization** of the diluted digest, followed by **distillation** into a known volume of standard acid, which contains potassium iodide and iodate.
3. **Titration** of the liberated iodine with standard sodium thiosulfate.

9.2.1.2.2 Improvements Several important modifications have improved the original Kjeldahl process:

1. Metallic catalysts such as mercury, copper, and selenium are added to sulfuric acid for complete digestion. Mercury has been found to be the most satisfactory. Selenium dioxide and copper sulfate in the ratio of 3:1 have been reported to be effective for digestion. Copper and titanium dioxide also have been used as a mixed catalyst for digestion (AOAC Method 988.05) (3). The use of titanium dioxide and copper poses less **safety concern** than mercury in the postanalysis disposal of the waste.
2. Potassium sulfate is used to increase the boiling point of the sulfuric acid to accelerate digestion.
3. Sulfide or sodium thiosulfate is added to the diluted digest to help release nitrogen from mercury, which tends to bind ammonium.
4. The ammonia is distilled directly into a boric acid solution, followed by titration with standard acid.
5. Colorimetry Nesslerization, or ion chromatography to measure ammonia, is used to determine nitrogen content after digestion.

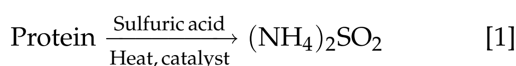
An excellent book to review the Kjeldahl method for total organic nitrogen was written by Bradstreet (7). The basic AOAC Kjeldahl procedure is Method 955.04.

Semiautomation, automation, and modification for microgram nitrogen determination (micro Kjeldahl method) have been established by AOAC in Methods 976.06, 976.05, and 960.52, respectively.

9.2.1.3 General Procedures and Reactions

9.2.1.3.1 Sample Preparation Solid foods are ground to pass a 20-mesh screen. Samples for analysis should be homogeneous. No other special preparations are required.

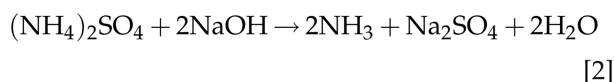
9.2.1.3.2 Digestion Place sample (accurately weighed) in a Kjeldahl flask. Add acid and catalyst; digest until clear to get complete breakdown of all organic matter. Nonvolatile ammonium sulfate is formed from the reaction of nitrogen and sulfuric acid.



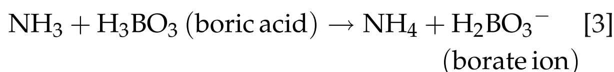
$$\text{Protein} \xrightarrow[\text{Heat, catalyst}]{\text{Sulfuric acid}} (NH_4)_2SO_2 \qquad [1]$$

During digestion, protein nitrogen is liberated to form ammonium ions; sulfuric acid oxidizes organic matter and combines with ammonium formed; carbon and hydrogen elements are converted to carbon dioxide and water.

9.2.1.3.3 Neutralization and Distillation The digest is diluted with water. Alkali-containing sodium thiosulfate is added to neutralize the sulfuric acid. The ammonia formed is distilled into a boric acid solution containing the indicators methylene blue and methyl red (AOAC Method 991.20).



$$(NH_4)_2SO_4 + 2NaOH \rightarrow 2NH_3 + Na_2SO_4 + 2H_2O \qquad [2]$$



$$NH_3 + H_3BO_3 \text{ (boric acid)} \rightarrow NH_4 + H_2BO_3^- \text{ (borate ion)} \qquad [3]$$

9.2.1.3.4 Titration Borate anion (proportional to the amount of nitrogen) is titrated with standardized HCl.

$$H_2BO_3^- + H^+ \rightarrow H_3BO_3 \qquad [4]$$

9.2.1.3.5 Calculations

$$\text{Moles of HCl} = \text{moles of } NH_3 = \text{moles of N in the sample} \qquad [5]$$

A reagent blank should be run to subtract reagent nitrogen from the sample nitrogen.

$$\%\,N = N\,HCl \times \frac{\text{Corrected acid volume}}{\text{g of sample}} \times \frac{14\,\text{g N}}{\text{mol}} \times 100 \qquad [6]$$

Table 9-2 Nitrogen to Protein Conversion Factors for Various Foods

	Percent N in Protein	*Factor*
Egg or meat	16.0	6.25
Milk	15.7	6.38
Wheat	18.76	5.33
Corn	17.70	5.65
Oat	18.66	5.36
Soybean	18.12	5.52
Rice	19.34	5.17

Data from (1, 8).

where:

NHCl = normality of HCl, in mol/1000 ml

Corrected acid vol. = (ml std. acid for sample) – (ml std. acid for blank)

14 = atomic weight of nitrogen

A factor is used to convert percent N to percent crude protein. Most proteins contain 16% N, so the conversion factor is 6.25 (100/16 = 6.25).

$$\%\,N/0.16 = \%\,\text{protein} \qquad [7]$$

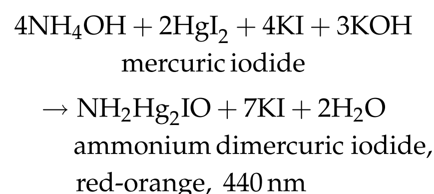
or

$$\%\,N \times 6.25 = \%\,\text{protein}$$

Conversion factors for various foods are given in Table 9-2.

9.2.1.3.6 Alternate Procedures In place of distillation and titration with acid, ammonia or nitrogen can be quantitated by:

1. Nesslerization



$$4NH_4OH + 2HgI_2 \text{ (mercuric iodide)} + 4KI + 3KOH \rightarrow NH_2Hg_2IO + 7KI + 2H_2O \text{ (ammonium dimercuric iodide, red-orange, 440 nm)} \qquad [8]$$

   This method is rapid and sensitive, but the ammonium dimercuric iodide is colloidal and color is not stable.
2. NH_3 + phenol + hypochloride $\xrightarrow[OH^-]{}$ indophenol (blue, 630 nm) [9]
3. pH measurement after distillation into known volume of boric acid
4. Direct measurement of ammonia, using ion chromatographic method

9.2.1.4 Applications

Advantages:

1. Applicable to all types of foods
2. Inexpensive (if not using an automated system)
3. Accurate; an official method for crude protein content
4. Has been modified (micro Kjeldahl method) to measure microgram quantities of proteins

Disadvantages:

1. Measures total organic nitrogen, not just protein nitrogen
2. Time consuming (at least 2 h to complete)
3. Poorer precision than the biuret method
4. Corrosive reagent

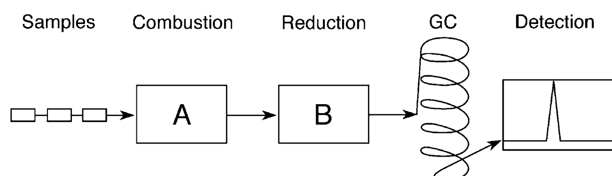
9.2.2 Dumas (Nitrogen Combustion) Method

9.2.2.1 Principle

The combustion method was introduced in 1831 by Jean-Baptiste Dumas. It has been modified and automated to improve accuracy since that time. Samples are combusted at high temperatures (700–1000°C) with a flow of pure oxygen. All carbon in the sample is converted to carbon dioxide during the flash combustion. Nitrogen-containing components produced include N_2 and nitrogen oxides. The nitrogen oxides are reduced to nitrogen in a copper reduction column at a high temperature (600°C). The total nitrogen (including inorganic fraction, i.e., including nitrate and nitrite) released is carried by pure helium and quantitated by **gas chromatography** using a **thermal conductivity detector** (TCD) (9). Ultra-high purity acetanilide and EDTA (ethylenediamine tetraacetate) may be used as the standards for the calibration of the nitrogen analyzer. The nitrogen determined is converted to protein content in the sample using a protein conversion factor.

9.2.2.2 Procedure

Samples (approximately 100–500 mg) are weighed into a tin capsule and introduced to a combustion reactor in automated equipment. The nitrogen released is measured by a built-in gas chromatograph. Figure 9-1 shows the flow diagram of the components of a Dumas nitrogen analyzer.



9-1 figure General components of a Dumas nitrogen analyzer. *A*, the incinerator; *B*, copper reduction unit for converting nitrogen oxides to nitrogen; and *GC*, gas chromatography column.

9.2.2.3 Applications

The combustion method is an alternative to the Kjeldahl method (10) and is suitable for all types of foods. AOAC Method 992.15 and Method 992.23 are for meat and cereal grains, respectively.

Advantages:

1. Requires no hazardous chemicals.
2. Can be accomplished in 3 min.
3. Recent automated instruments can analyze up to 150 samples without attention.

Disadvantages:

1. Expensive equipment is required.
2. Measures total organic nitrogen, not just protein nitrogen.

9.2.3 Infrared Spectroscopy

9.2.3.1 Principle

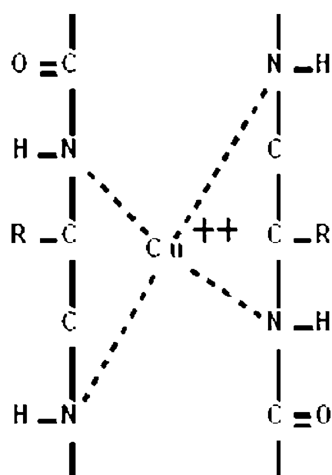
Infrared spectroscopy measures the **absorption of radiation** (near- or mid-infrared regions) by molecules in food or other substances. Different functional groups in a food absorb different frequencies of radiation. For proteins and peptides, various **mid-infrared** bands (6.47 μm) and **near-infrared** (NIR) bands (e.g., 3300–3500 nm; 2080–2220 nm; 1560–1670 nm) characteristic of the **peptide bond** can be used to estimate the protein content of a food. By irradiating a sample with a wavelength of infrared light specific for the constituent to be measured, it is possible to predict the concentration of that constituent by measuring the energy that is reflected or transmitted by the sample (which is inversely proportional to the energy absorbed) (11).

9.2.3.2 Procedure

See Chap. 23 for a detailed description of instrumentation, sample handling, and calibration and quantitation methodology.

9.2.3.3 Applications

Mid-infrared spectroscopy is used in Infrared Milk Analyzers to determine milk protein content, while near-infrared spectroscopy is applicable to a wide range of food products (e.g., grains; cereal, meat, and dairy products) (3,12,13) (AOAC Method 997.06). Instruments are expensive and they must be calibrated properly. However, samples can be analyzed rapidly (30 s to 2 min) by analysts with minimal training.



9-2 figure Reaction of peptide bonds with cupric ions.

9.2.4 Biuret Method

9.2.4.1 Principle

A violet-purplish color is produced when **cupric ions** are complexed with **peptide bonds** (substances containing at least two peptide bonds, i.e., biuret, large peptides, and all proteins) under **alkaline conditions** (Fig. 9-2). The absorbance of the color produced is read at 540 nm. The color intensity (absorbance) is proportional to the protein content of the sample (14).

9.2.4.2 Procedure

1. A 5-ml biuret reagent is mixed with a 1-ml portion of protein solution (1–10 mg protein/ml). The reagent includes copper sulfate, NaOH, and potassium sodium tartrate, which is used to stabilize the cupric ion in the alkaline solution.
2. After the reaction mix is allowed to stand at room temperature for 15 or 30 min, the absorbance is read at 540 nm against a reagent blank.
3. Filtration or centrifugation before reading absorbance is required if the reaction mixture is not clear.
4. A standard curve of concentration versus absorbance is constructed using **bovine serum albumin** (BSA).

9.2.4.3 Applications

The biuret method has been used to determine proteins in cereal (15, 16), meat (17), soybean proteins (18), and as a qualitative test for animal feed [AOAC Method 935.11 (refers to Methods 22.012–22.013, AOAC, 10th edn, 1965)] (19). The biuret method also can be used to measure the protein content of isolated proteins.

Advantages:

1. Less expensive than the Kjeldahl method; rapid (can be completed in less than 30 min); simplest method for analysis of proteins.
2. Color deviations are encountered less frequently than with Lowry, ultraviolet (UV) absorption, or turbidimetric methods (described below).
3. Very few substances other than proteins in foods interfere with the biuret reaction.
4. Does not detect nitrogen from nonpeptide or nonprotein sources.

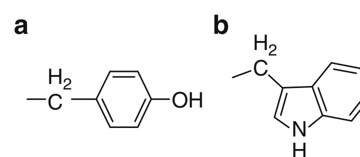
Disadvantages:

1. Not very sensitive as compared to the Lowry method; requires at least 2–4 mg protein for assay.
2. Absorbance could be contributed from bile pigments if present.
3. High concentration of ammonium salts interfere with the reaction.
4. Color varies with different proteins; gelatin gives a pinkish-purple color.
5. Opalescence could occur in the final solution if high levels of lipid or carbohydrate are present.
6. Not an absolute method: color must be standardized against known protein (e.g., BSA) or against the Kjeldahl nitrogen method.

9.2.5 Lowry Method

9.2.5.1 Principle

The Lowry method (20, 21) combines the **biuret reaction** with the reduction of the **Folin–Ciocalteau phenol reagent** (phosphomolybdic-phosphotungstic acid) by **tyrosine** and **tryptophan** residues in the proteins (Fig. 9-3). The bluish color developed is read at 750 nm (high sensitivity for low protein concentration) or 500 nm (low sensitivity for high protein concentration). The original procedure has been modified by Miller (22) and Hartree (23) to improve the linearity of the color response to protein concentration.



9-3 figure Side chains of amino acids tyrosine (**a**) and tryptophan (**b**).

9.2.5.2 Procedure

The following procedure is based on the modified procedure of Hartree (23):

1. Proteins to be analyzed are diluted to an appropriate range (20–100 μg).
2. K Na Tartrate-Na_2CO_3 solution is added after cooling and incubated at room temperature for 10 min.
3. $CuSO_4$-K Na Tartrate-NaOH solution is added after cooling and incubated at room temperature for 10 min.
4. Freshly prepared Folin reagent is added and then the reaction mixture is mixed and incubated at 50°C for 10 min.
5. Absorbance is read at 650 nm.
6. A standard curve of BSA is carefully constructed for estimating protein concentration of the unknown.

9.2.5.3 Applications

Because of its simplicity and sensitivity, the Lowry method has been widely used in protein biochemistry. However, it has not been widely used to determine proteins in food systems without first extracting the proteins from the food mixture.

Advantages:

1. Very sensitive
   (a) 50–100 times more sensitive than biuret method
   (b) 10–20 times more sensitive than 280-nm UV absorption method (described below)
   (c) Similar sensitivity as Nesslerization; however, more convenient than Nesslerization
2. Less affected by turbidity of the sample.
3. More specific than most other methods.
4. Relatively simple; can be done in 1–1.5 h.

Disadvantages:

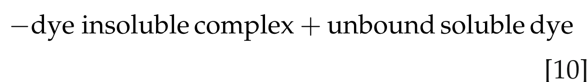
For the following reasons, the Lowry procedure requires careful standardization for particular applications:

1. Color varies with different proteins to a greater extent than the biuret method.
2. Color is not strictly proportional to protein concentration.
3. The reaction is interfered with to varying degrees by sucrose, lipids, phosphate buffers, monosaccharides, and hexoamines.
4. High concentrations of reducing sugars, ammonium sulfate, and sulfhydryl compounds interfere with the reaction.

9.2.6 Dye-Binding Methods

9.2.6.1 Anionic Dye-Binding Method

9.2.6.1.1 Principle The protein-containing sample is mixed with a known excess amount of **anionic dye** in a buffered solution. Proteins bind the dye to form an insoluble complex. The unbound soluble dye is measured after equilibration of the reaction and the removal of insoluble complex by centrifugation or filtration.



$$\text{Protein} + \text{excess dye} \rightarrow \text{Protein} \\ -\text{dye insoluble complex} + \text{unbound soluble dye} \quad [10]$$

The anionic sulfonic acid dye, including acid orange 12, orange G, and Amido Black 10B, binds cationic groups of the **basic amino acid residues** (imidazole of histidine, guanidine of arginine, and ϵ-amino group of lysine) and the **free amino terminal group** of the protein (24). The amount of the unbound dye is inversely related to the protein content of the sample (24).

9.2.6.1.2 Procedure

1. The sample is finely ground (60 mesh or smaller sizes) and added to an excess dye solution with known concentration.
2. The content is vigorously shaken to equilibrate the dye binding reactions and filtered or centrifuged to remove insoluble substances.
3. Absorbance of the unbound dye solution in the filtrate or supernatant is measured and dye concentration is estimated from a dye standard curve.
4. A straight calibration curve can be obtained by plotting the unbound dye concentration against total nitrogen (as determined by Kjeldhal method) of a given food covering a wide range of protein content.
5. Protein content of the unknown sample of the same food type can be estimated from the calibration curve or from a regression equation calculated by the least squares method.

9.2.6.1.3 Applications Anionic dye binding has been used to estimate proteins in milk (25, 26), wheat flour (27), soy products (18), and meats (17). The AOAC approved methods include two dye-binding methods [Method 967.12 using Acid Orange 12 and Method 975.17 using Amido Black (10B) for analyzing proteins in milk]. AACC Method 46–14.02 uses Acid Orange 12 binding for measuring proteins in wheat flour and soy samples (28). An automated Sprint Rapid Protein

Analyzer has been developed by the CEM Company (Matthews, NC) based on the anionic dye-binding method. This automated method requires calibration for each type of food protein determined using other official methods.

Advantages:

1. Rapid (15 min or less), inexpensive, and relatively accurate for analyzing protein content in food commodities.
2. May be used to estimate the changes in available lysine content of cereal products during processing since the dye does not bind altered, unavailable lysine. Since lysine is the limiting amino acid in cereal products, the available lysine content represents protein nutritive value of the cereal products (29).
3. No corrosive reagents.
4. Does not measure nonprotein nitrogen.
5. More precise than the Kjeldahl method.

Disadvantages:

1. Not sensitive; milligram quantities of protein are required.
2. Proteins differ in basic amino acid content and so differ in dye-binding capacity. Therefore, a calibration curve for a given food commodity is required.
3. Not suitable for hydrolyzed proteins due to binding to N-terminal amino acids.
4. Some nonprotein components bind dye (i.e., starch) or protein (calcium or phosphate) and cause errors in final results. The problem with calcium and heavy metal ions can be eliminated using properly buffered reagent that contains oxalic acid.

9.2.6.2 Bradford Dye-Binding Method

9.2.6.2.1 Principle When **Coomassie Brilliant Blue G-250** binds to protein, the **dye changes color** from reddish to bluish, and the absorption maximum of the dye is shifted from 465 to 595 nm. The change in the absorbance at 595 nm is proportional to the protein concentration of the sample (30). Like other dye-binding methods, the Bradford relies on the **amphoteric nature of proteins**. When the protein-containing solution is acidified to a pH less than the isoelectric point of the protein(s) of interest, the dye added binds electrostatically. Binding efficiency is enhanced by hydrophobic interaction of the dye molecule with the polypeptide backbone adjoining positively charged residues in the protein (4). In the case of the Bradford method, the dye bound to protein has a change in absorbance spectrum relative to the unbound dye.

9.2.6.2.2 Procedure

1. Coomassie Brilliant Blue G-250 is dissolved in 95% ethanol and acidified with 85% phosphoric acid.
2. Samples containing proteins (1–100 μg/ml) and standard BSA solutions are mixed with the Bradford reagent.
3. Absorbance at 595 nm is read against a reagent blank.
4. Protein concentration in the sample is estimated from the BSA standard curve.

9.2.6.2.3 Applications The Bradford method has been used successfully to determine protein content in worts and beer products (31) and in potato tubers (32). This procedure has been improved to measure microgram quantities of proteins (33). Due to its rapidity, sensitivity, and fewer interferences than the Lowry method, the Bradford method has been used widely for the analysis of low concentrations of proteins and enzymes in their purification and characterizations.

Advantages:

1. Rapid; reaction can be completed in 2 min
2. Reproducible
3. Sensitive; several fold more sensitive than the Lowry method
4. No interference from ammonium sulfate, polyphenols, carbohydrates such as sucrose, or cations such as K^+, Na^+, and Mg^{+2}
5. Measures protein or peptides with molecular mass approximately equal to or greater than 4000 Da

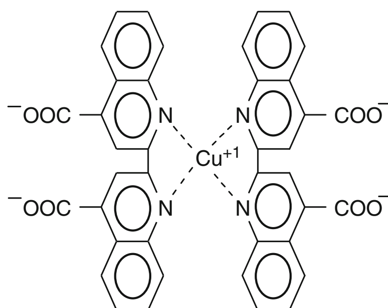
Disadvantages:

1. Interfered with by both nonionic and ionic detergents, such as Triton X-100 and sodium dodecyl sulfate. However, errors due to small amounts (0.1%) of these detergents can be corrected using proper controls.
2. The protein–dye complex can bind to quartz cuvettes. The analyst must use glass or plastic cuvettes.
3. Color varies with different types of proteins. The standard protein must be selected carefully.

9.2.7 Bicinchoninic Acid Method

9.2.7.1 Principle

Proteins and peptides (as short as dipeptides) reduce **cupric ions** to **cuprous ions** under **alkaline conditions** (34), which is similar in principle to that of the biuret reaction. The cuprous ion then reacts with the apple-greenish **bicinchoninic acid** (BCA) **reagent** to



9-4 figure Protein reaction with cupric ions under alkaline conditions to form cuprous ions, which react with bicinchoninic acid (BCA) to form purple color, measured at 562 nm. (Figure Courtesy of Pierce Biotechnology Technical Library, Thermo Fisher Scientific, Inc., Rockford, IL.)

form a purplish complex (one cuprous ion is chelated by two BCA molecules) (Fig. 9-4). The color measured at 562 nm is near linearly proportional to protein concentration over a wide range of concentration from micrograms up to 2 mg/ml. Peptide bonds and four amino acids (cysteine, cystine, tryptophan, and tyrosine) contribute to the color formation with BCA.

9.2.7.2 Procedure

1. Mix (one step) the protein solution with the BCA reagent, which contains BCA sodium salt, sodium carbonate, NaOH, and copper sulfate, pH 11.25.
2. Incubate at 37°C for 30 min, or room temperature for 2 h, or 60°C for 30 min. The selection of the temperature depends upon sensitivity desired. A higher temperature gives a greater color response.
3. Read the solution at 562 nm against a reagent blank.
4. Construct a standard curve using BSA.

9.2.7.3 Applications

The BCA method has been used in protein isolation and purification. The suitability of this procedure for measuring protein in complex food systems has not been reported.

Advantages:

1. Sensitivity is comparable to that of the Lowry method; sensitivity of the micro-BCA method (0.5–10 μg) is better than that of the Lowry method.
2. One-step mixing is easier than in the Lowry method.
3. The reagent is more stable than for the Lowry reagent.
4. Nonionic detergent and buffer salts do not interfere with the reaction.
5. Medium concentrations of denaturing reagents (4 *M* guanidine-HCl or 3 *M* urea) do not interfere.

Disadvantages:

1. Color is not stable with time. The analyst needs to carefully control the time for reading absorbance.
2. Any compound capable of reducing Cu^{+2} to Cu^{+} will lead to color formation.
3. Reducing sugars interfere to a greater extent than in the Lowry method. High concentrations of ammonium sulfate also interfere.
4. Color variations among proteins are similar to those in the Lowry method.

9.2.8 Ultraviolet 280 nm Absorption Method

9.2.8.1 Principle

Proteins show strong absorption in the region at **ultraviolet** (UV) **280 nm**, primarily due to **tryptophan** and **tyrosine** residues in the proteins. Because the content of tryptophan and tyrosine in proteins from each food source is fairly constant, the absorbance at 280 nm could be used to estimate the concentration of proteins, using **Beer's law**. Since each protein has a unique aromatic amino acid composition, the extinction coefficient (E_{280}) or molar absorptivity (E_m) must be determined for individual proteins for protein content estimation.

9.2.8.2 Procedure

1. Proteins are solubilized in buffer or alkali.
2. Absorbance of protein solution is read at 280 nm against a reagent blank.
3. Protein concentration is calculated according to the equation

$$A = abc \quad [11]$$

where:

A = absorbance
a = absorptivity
b = cell or cuvette path length
c = concentration

9.2.8.3 Applications

The UV 280-nm method has been used to determine the protein contents of milk (35) and meat products (36). It has not been used widely in food systems. This technique is better applied in a purified protein system or to proteins that have been extracted in alkali or denaturing agents such as 8 *M* urea. Although peptide

bonds in proteins absorb more strongly at 190–220 nm than at 280 nm, the low UV region is more difficult to measure.

Advantages:

1. Rapid and relatively sensitive; At 280 nm, 100 µg or more protein is required; several times more sensitive than the biuret method.
2. No interference from ammonium sulfate and other buffer salts.
3. Nondestructive; samples can be used for other analyses after protein determination; used very widely in postcolumn detection of proteins.

Disadvantages:

1. Nucleic acids also absorb at 280 nm. The absorption 280 nm/260 nm ratios for pure protein and nucleic acids are 1.75 and 0.5, respectively. One can correct the absorption of nucleic acids at 280 nm if the ratio of the absorption of 280 nm/260 nm is known. Nucleic acids also can be corrected using a method based on the absorption difference between 235 and 280 nm (37).
2. Aromatic amino acid contents in the proteins from various food sources differ considerably.
3. The solution must be clear and colorless. Turbidity due to particulates in the solution will increase absorbance falsely.
4. A relatively pure system is required to use this method.

9.3 COMPARISON OF METHODS

- **Sample preparation:** The Kjeldahl, Dumas, and infrared spectroscopy methods require little preparation. Sample particle size of 20 mesh or smaller generally is satisfactory for these methods. Some of the newer NIR instruments can make measurements directly on whole grains and other coarsely granulated products without grinding or other sample preparation. Other methods described in this chapter require fine particles for extraction of proteins from the complex food systems.
- **Principle:** The Dumas and Kjeldahl methods measure directly the nitrogen content of foods. However, the Kjeldahl method measures only organic nitrogen plus ammonia, while Dumas measures total nitrogen, including the inorganic fraction. (Therefore, Dumas gives a higher value for products that contain nitrates/nitrites.) Other methods of analysis measure the various properties of proteins. For instance, the biuret method measures peptide bonds, and the Lowry method measures a combination of peptide bonds and the amino acids tryptophan and tyrosine. Infrared spectroscopy is an indirect method to estimate protein content, based on the energy absorbed when a sample is subjected to a wavelength of infrared radiation specific for the peptide bond.
- **Sensitivity:** Kjeldahl, Dumas, and biuret methods are less sensitive than Lowry, Bradford, BCA, or UV methods.
- **Speed:** After the instrument has been properly calibrated, infrared spectroscopy is likely the most rapid of the methods discussed. In most other methods involving spectrophotometric (colorimetric) measurements, one must separate proteins from the interfering insoluble materials before mixing with the color reagents or must remove the insoluble materials from the colored protein–reagent complex after mixing. However, the speed of determination in the colorimetric methods and in the Dumas method is faster than with the Kjeldahl method.
- **Applications:** Although both Kjeldahl and Dumas methods can be used to measure N content in all types of foods, in recent years the Dumas method has largely replaced the Kjeldahl method for nutrition labeling (since Dumas method is faster, has a lower detection limit, and is safer). However, the Kjeldahl method is the preferred method for high-fat samples/products since fat may cause an instrument fire during the incineration procedure in the Dumas method. Also, the Kjeldahl method is specified to correct for protein content in an official method to measure the fiber content of foods (see Chap. 10, Sect. 10.5). Melamine, a toxic nitrogen adulterant, is included in the total nitrogen content if measured by the Kjeldahl or Dumas methods.

9.4 SPECIAL CONSIDERATIONS

1. To select a particular method for a specific application, sensitivity, accuracy, and reproducibility as well as physicochemical properties of food materials must be considered. The data should be interpreted carefully to reflect what actually is being measured.
2. Food processing methods, such as heating, may reduce the extractability of proteins for analysis and cause an underestimation of the protein content measured by methods involving an extraction step (9).
3. Except for the Dumas and Kjeldahl methods, and the UV method for purified proteins, all

methods require the use of a standard or reference protein or a calibration with the Kjeldahl method. In the methods using a standard protein, proteins in the samples are assumed to have similar composition and behavior compared with the standard protein. The selection of an appropriate standard for a specific type of food is important.

4. **Nonprotein nitrogen** is present in practically all foods. To determine **protein nitrogen**, the samples usually are extracted under alkaline conditions then precipitated with trichloroacetic acid or sulfosalicylic acid. The concentration of the acid used affects the precipitation yield. Therefore, nonprotein nitrogen content may vary with the type and concentration of the reagent used. Heating could be used to aid protein precipitation by acid, alcohol, or other organic solvents. In addition to acid precipitation methods used for nonprotein nitrogen determination, less empirical methods such as dialysis and ultrafiltration and column chromatography could be used to separate proteins from small nonprotein substances.
5. In the determination of the nutritive value of food proteins, including **protein digestibility** and **protein efficiency ratio** (PER), the Kjeldahl method with a 6.25 conversion factor usually is used to determine crude protein content. The PER could be underestimated if a substantial amount of nonprotein nitrogen is present in foods. A food sample with a higher nonprotein nitrogen content (particularly if the nonprotein nitrogen does not have many amino acids or small peptides) may have a lower PER than a food sample containing similar protein structure/composition and yet with a lower amount of nonprotein nitrogen.

9.5 SUMMARY

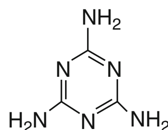
Methods based on the unique characteristics of proteins and amino acids have been described to determine the protein content of foods. The Kjeldahl and Dumas methods measure nitrogen. Infrared spectroscopy is based on absorption of a wavelength of infrared radiation specific for the peptide bond. Copper–peptide bond interactions contribute to the analysis by the biuret and Lowry methods. Amino acids are involved in the Lowry, dye-binding, and UV 280 nm methods. The BCA method utilizes the reducing power of proteins in an alkaline solution. The various methods differ in their speed and sensitivity.

In addition to the commonly used methods discussed, there are other methods available for protein quantification. Because of the complex nature of various food systems, problems may be encountered to different degrees in protein analysis by available methods. Rapid methods may be suitable for quality control purposes, while a sensitive method is required for work with a minute amount of protein. Indirect colorimetric methods usually require the use of a carefully selected protein standard or a calibration with an official method (e.g., Kjeldahl).

9.6 STUDY QUESTIONS

1. What factors should one consider when choosing a method for protein determination?
2. The Kjeldahl method of protein analysis consists of three major steps. List these steps in the order they are done and describe in words what occurs in each step. Make it clear why milliliters of HCl can be used as an indirect measure of the protein content of a sample.
3. Why is the conversion factor from Kjeldahl nitrogen to protein different for various foods, and how is the factor of 6.25 obtained?
4. How can Nesslerization or the procedure that uses phenol and hypochlorite be used as part of the Kjeldahl procedure, and why might they be best for the analysis?
5. Differentiate and explain the chemical basis of the following techniques that can be used to quantitate proteins in quality control/research:
   (a) Kjeldahl method
   (b) Dumas method (N combustion)
   (c) Infrared spectroscopy
   (d) Biuret method
   (e) Lowry method
   (f) Bradford method
   (g) Bicinchoninic acid method
   (h) Absorbance at 280 nm
   (i) Absorbance at 220 nm
6. Differentiate the principles of protein determination by dye binding with an anionic dye such as Amido Black vs. with the Bradford method, which uses the dye Coomassie Blue G-250.
7. With the anionic dye-binding method, would a sample with a higher protein content have a higher or a lower absorbance reading than a sample with a low protein content? Explain your answer.
8. For each of the situations described below, identify a protein assay method most appropriate for use, and indicate the chemical basis of the method (i.e., what does it really measure?)
   (a) Nutrition labeling
   (b) Intact protein eluting from a chromatography column; qualitative or semiquantitative method
   (c) Intact protein eluting from a chromatography column; colorimetric, quantitative method
   (d) Rapid, quality control method for protein content of cereal grains
9. The FDA found melamine (see structure below) in pet food linked to deaths of pets in the United States. The

FDA also found evidence of melamine in wheat gluten imported from China used as one of the ingredients in the production of the pet food. Melamine is a nitrogen-rich chemical used to make plastic and sometimes used as a fertilizer.



(a) Knowing that each ingredient is tested and analyzed when imported, explain how melamine in wheat gluten could have escaped detection.
(b) How can the adulteration of wheat gluten be detected (not necessarily detecting melamine specifically), using a combination of protein analysis methods? Explain your answer.

9.7 PRACTICE PROBLEMS

1. A dehydrated precooked pinto bean was analyzed for crude protein content in duplicate using the Kjeldahl method. The following data were recorded:
   - Moisture content = 8.00%
   - Wt of Sample 1 = 1.015 g
   - Wt of Sample 2 = 1.025 g
   - Normality of HCl used for titration = 0.1142 *N*
   - HCl used for Sample 1 = 22.0 ml
   - HCl used for Sample 2 = 22.5 ml
   - HCl used for reagent blank = 0.2 ml

   Calculate crude protein content on both wet and dry weight basis of the pinto bean, assuming pinto bean protein contains 17.5% nitrogen.
2. A 20 ml protein fraction recovered from a column chromatography was analyzed for protein using the BCA method. The following data were the means of a duplicate analysis using BSA as a standard:

BSA (mg/ml)	*Mean Absorbance at 562 nm*
0.2	0.25
0.4	0.53
0.6	0.74
0.8	0.95
1.0	1.15

The average absorbance of a 1-ml sample was 0.44. Calculate protein concentration (mg/ml) and total protein quantity of this column fraction.

Answers

1. Protein content = 19.75% on a wet weight basis; 21.47% on a dry weight basis.

   Calculations:

$$\%\,\mathrm{N} = N\,\mathrm{HCl} \times \frac{\text{Corrected acid volume}}{\text{g of sample}} \times \frac{14\,\mathrm{g\,N}}{\mathrm{mol}} \times 100 \qquad [6]$$

where:

NHCl = normality of HCl, in mol/1000 ml
Corrected acid vol. = (ml std. acid for sample) – (ml std. acid for blank)
14 = atomic weight of nitrogen

Corrected acid volume for Sample 1
= 22.0 ml − 0.2 ml = 21.8 ml
Corrected acid volume for Sample 2
= 22.5 ml − 0.2 ml = 22.3 ml

%N for Sample 1

$$= \frac{0.1142\,\mathrm{mol}}{1000\,\mathrm{ml}} \times \frac{21.8\,\mathrm{ml}}{1.015\,\mathrm{g}} \times \frac{14\,\mathrm{g\,N}}{\mathrm{mol}} \times 100\% = 3.433\%$$

%N for Sample 2

$$= \frac{0.1142\,\mathrm{mol}}{1000\,\mathrm{ml}} \times \frac{22.3\,\mathrm{ml}}{1.025\,\mathrm{g}} \times \frac{14\,\mathrm{g\,N}}{\mathrm{mol}} \times 100\% = 3.478\%$$

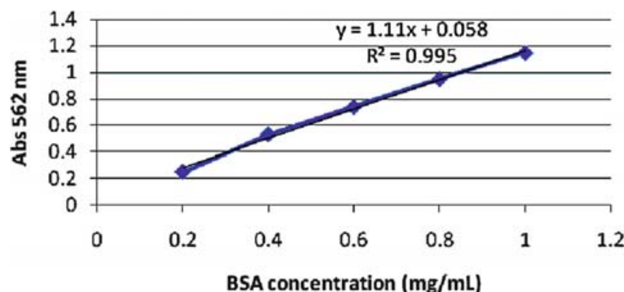
Protein conversion factor = 100%/17.5% N = 5.71
Crude protein content for Sample 1
= 3.433% × 5.71 = 19.6%
Crude protein content for Sample 2
= 3.478% × 5.71 = 19.9%

The average for the duplicate data = (19.6% + 19.9%)/2 = 19.75% = ∼19.8% wet weight basis.

To calculate protein content on a dry weight basis: Sample contain 8% moisture, therefore, the sample contains 92% dry solids, or 0.92 g out of 1-g sample. Therefore, protein on a dry weight basis can be calculated as follows = 19.75%/0.92 g dry solids = 21.47% = ∼21.5% dry weight basis.

2. Protein content = 0.68 mg/ml. Total protein quantity = 6.96 mg

   Calculations:
   Plot absorbance (y-axis, absorbance at 562 nm) vs. BSA protein concentration (x-axis, mg/ml)) using the data above. Determine the equation of the line ($y = 1.11x + 0.058$), then use this equation and the given absorbance ($y = 0.44$) to calculate the concentration ($x = 0.344$ mg/ml). Since 1 ml of sample gives a concentration of 0.344 mg/ml and we have a total of 20 ml collected from column chromatography, we will have a total of (0.344 mg/ml × 20 ml) = 6.88 mg protein in this collected column fraction.



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10

chapter

Carbohydrate Analysis

James N. BeMiller
Department of Food Science, Purdue University,
West Lafayette, IN 47907–2009, USA
bemiller@purdue.edu

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10.1 INTRODUCTION

Carbohydrates are important in foods as a major source of energy, to impart crucial textural properties, and as dietary fiber which influences physiological processes. Digestible carbohydrates, which are converted into monosaccharides, which are absorbed, provide metabolic energy. Worldwide, carbohydrates account for more than 70% of the caloric value of the human diet. It is recommended that all persons should limit calories from fat (the other significant source) to not more than 30% and that most of the carbohydrate calories should come from starch. Nondigestible polysaccharides (all those other than starch) comprise the major portion of dietary fiber (Sect. 10.5). Carbohydrates also contribute other attributes, including bulk, body, viscosity, stability to emulsions and foams, water-holding capacity, freeze-thaw stability, browning, flavors, aromas, and a range of desirable textures (from crispness to smooth, soft gels). They also provide satiety. Basic carbohydrate structures, chemistry, and terminology can be found in references (1,2).

Major occurrences of major carbohydrates in foods are presented in Table 10-1. Ingested carbohydrates are almost exclusively of plant origin, with milk lactose being the major exception. Of the **monosaccharides** (sometimes called **simple sugars**), only D-glucose and D-fructose are found in other than minor amounts. Monosaccharides are the only carbohydrates that can be absorbed from the small intestine. Higher saccharides (**oligo**- and **polysaccharides**) must first be digested (i.e., hydrolyzed to monosaccharides) before absorption and utilization can occur. (Note: There is no official definition of an oligosaccharide. Most sources consider an oligosaccharide to be a carbohydrate composed of from 2 to 10 sugar (saccharide) units. A polysaccharide usually contains from 30 to at least 60,000 monosaccharide units.) Humans can digest only sucrose, lactose, maltooligosaccharides/maltodextrins, and starch. All are digested with enzymes found in the small intestine.

At least 90% of the carbohydrate in nature is in the form of polysaccharides. As stated above, starch polymers are the only polysaccharides that humans can digest and use as a source of calories and carbon. All other polysaccharides are nondigestible. **Nondigestible polysaccharides** can be divided into **soluble** and **insoluble** classes. Along with lignin and other nondigestible, nonabsorbed substances, they make up **dietary fiber** (Sect. 10.5). As dietary fiber, they regulate normal bowel function, reduce the postprandial hyperglycemic response, and may lower serum cholesterol, among other effects. However, nondigestible polysaccharides most often are added to processed foods because of the functional properties they impart, rather than for a physiological effect. Nondigestible oligosaccharides serve as prebiotics and are, therefore, increasingly used as ingredients in functional foods and neutraceuticals. The foods in which dietary fiber components can be used, and particularly the amounts that can be incorporated, are limited because addition above a certain level usually changes the characteristics of the food product. Indeed, as already stated, they are used often as ingredients because of their ability to impart important functional properties at a low level of usage.

Carbohydrate analysis is important from several perspectives. Qualitative and quantitative analysis is used to determine compositions of foods, beverages, and their ingredients. **Qualitative analysis** ensures that ingredient labels present accurate compositional information. **Quantitative analysis** ensures that added components are listed in the proper order on ingredient labels. Quantitative analysis also ensures that amounts of specific components of consumer interest, for example, **β-glucan**, are proper and that caloric content can be calculated. Both qualitative and quantitative analysis can be used to authenticate (i.e., to detect adulteration of) food ingredients and products.

In this chapter, the most commonly used methods of carbohydrate determination are presented. [A thorough description of the analytical chemistry of carbohydrates was published in 1998 (3).] However, methods often must be made specific to a particular food product because of the nature of the product and the presence of other constituents. Approved methods are referenced, but method approval has not kept pace with methods development; so where better methods are available, they are also presented. Methods that have been in long-time use, although not giving as much or as precise information as newer methods, nevertheless may be useful for quality assurance and product standardization in some cases.

In general, evolution of analytical methods for carbohydrates has followed the succession: qualitative color tests, adaptation of the color test for reducing sugars based on reduction of Cu(II) to Cu(I) (Fehling test) to quantitation of reducing sugars, qualitative paper chromatography, quantitative paper chromatography, gas chromatography (GC) of derivatized sugars, qualitative and quantitative thin-layer chromatography, enzymic methods, and high-performance liquid chromatography (HPLC). Multiple official methods for the analysis of mono- and disaccharides in foods are currently approved by AOAC International (4, 5); some are outdated, but still used. Methods continue to be developed and refined. Methods employing nuclear magnetic resonance, near-infrared (NIR) spectrometry (Sect. 10.6.2 and Chap. 23), antibodies (Immunoassays; Chap. 17),

10-1 table Occurrences of Some Major Carbohydrates in Foods

Carbohydrate	*Source*	*Constituent(s)*
Monosaccharides[a]		
D-Glucose (Dextrose)	Naturally occurring in honey, fruits, and fruit juices. Added as a component of corn (glucose) syrups and high-fructose syrups. Produced during processing by hydrolysis (inversion) of sucrose.	
D-Fructose	Naturally occurring in honey, fruits, and fruit juices. Added as a component of high-fructose syrups. Produced during processing by hydrolysis (inversion) of sucrose.	
Sugar alcohol[a]		
Sorbitol (D-Glucitol)	Added to food products, primarily as a humectant	
Disaccharides[a]		
Sucrose	Widely distributed in fruit and vegetable tissues and juices in varying amounts. Added to food and beverage products	D-Fructose D-Glucose
Lactose	In milk and products derived from milk	D-Galactose D-Glucose
Maltose	In malt. In varying amounts in various corn (glucose) syrups and maltodextrins	D-Glucose
Higher oligosaccharides[a]		
Maltooligosaccharides	Maltodextrins. In varying amounts in various glucose (corn) syrups	D-Glucose
Raffinose	Small amounts in beans	D-Glucose D-Fructose D-Galactose
Stachyose	Small amounts in beans	D-Glucose D-Fructose D-Galactose
Polysaccharides		
Starch[b]	Widespread in cereal grains and tubers. Added to processed foods.	D-Glucose
Food gums/hydrocolloids[c]		
Algins Carboxymethylcelluloses Carrageenans Curdlan Gellan Guar gum Gum arabic Hydroxypropylmethyl-celluloses Inulin Konjac glucomannan Locust bean gum Methylcelluloses Pectins Xanthan	Added as ingredients	[d]
Cell-wall polysaccharides[c]		
Pectin (native) Cellulose Hemicelluloses Beta-glucan	Naturally occurring	

[a]For analysis, see Sect. 10.3.4.
[b]For analysis, see Sect. 10.4.1.1.
[c]For analysis, see Sect. 10.4.2.
[d]For compositions, characteristics, and applications, see reference (2) and Table 10-2.

fluorescence spectrometry (Chap. 22), capillary electrophoresis (Sect. 10.3.4.6), and mass spectrometry (Sect. 10.3.4.4) have been published, but are not yet in general use for carbohydrate analysis.

It should be noted that, according to the nutrition labeling regulations of the US Food and Drug Administration, the "**total carbohydrate**" content of a food (Table 10-2), which is declared in relation to a serving, which is defined as the amount of food customarily consumed per eating occasion by persons 4-years of age or older [(6), paragraph (b)(1)], must be calculated by subtraction of the sums of the weights of crude protein, total fat, moisture, and ash in a serving from the total weight of the food in a serving [(6), paragraph (c)(6)] (i.e., carbohydrate is determined by difference). The grams of dietary fiber (Sect. 10.5) in a serving also must be stated on the label [(6), paragraph (c)(6)(i)]. The content of "**other carbohydrate**" (formerly called "**complex carbohydrate**") is obtained by calculating the difference between the amount of "total carbohydrate" and the sum of the amounts of dietary fiber and sugars (Table 10-1). For labeling purposes, **sugars** are defined as the sum of all free monosaccharides (viz., D-glucose and -fructose) and disaccharides [viz., sucrose, lactose, and maltose (if a maltodextrin or glucose/corn syrup has been added)] [(6), paragraph (c)(6)(ii)] (Table 10-1). Other carbohydrates are likely to be **sugar alcohols** (alditols, polyhydroxy alcohols, polyols), such as sorbitol and xylitol, the specific declaring of which is also voluntary [(6), paragraph (c)(6)(iii)].

10-2 table **Total Carbohydrate Contents of Selected Foods[a]**

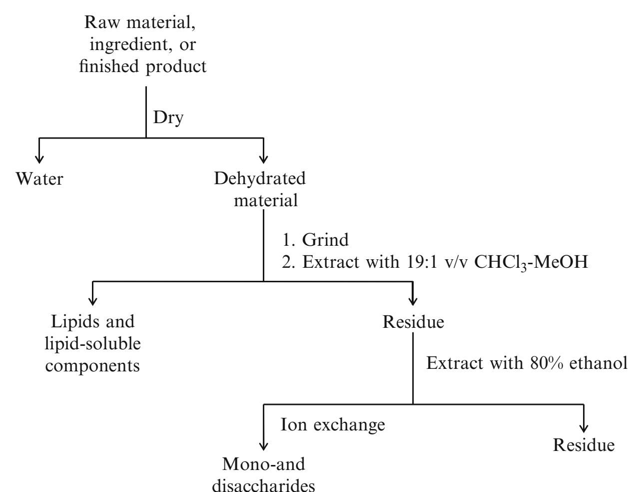
Food	*Approximate Percent Carbohydrate (Wet Weight Basis)*
Cereals, bread, and pasta	
Corn flakes	80.4
Macaroni, dry, enriched	74.7
Bread, white, commercially prepared	50.6
Dairy products	
Ice cream, chocolate	28.2
Yogurt, plain, low fat (12 g protein/8 oz)	7.0
Milk, reduced fat, fluid, 2%	4.7
Milk, chocolate, commercial, whole	10.3
Fruits and vegetables	
Apple sauce, canned, sweetened, with salt	19.9
Grapes, raw	17.2
Apples, raw, with skin	13.8
Potatoes, raw, with skin	12.4
Orange juice, raw	10.4
Carrots, raw	9.6
Broccoli, raw	6.6
Tomato, tomato juice, canned, with salt	4.2
Meat, poultry, and fish	
Fish fillets, battered or breaded, fried	17.0
Bologna, beef	4.0
Chicken, broilers or fryers, breast meat	0
Other	
Honey	82.4
Salad dressing, Italian, fat free	11.0
Salad dressing, Italian, regular	10.4
Carbonated beverage, cola, contains caffeine	9.6
Cream of mushroom soup, from condensed and canned	6.7
Light beer	1.6

[a]In part from US Department of Agriculture, Agricultural Research Service (2009). USDA National Nutrient Database for Standard Reference. Release 22. Nutrient Data Laboratory Home Page, http://www.ars.usda.gov/ba/bhnrc/ndl

10.2 SAMPLE PREPARATION

Sample preparation is related to the specific raw material, ingredient, or food product being analyzed and the specific carbohydrate being determined, because carbohydrates have such a wide range of solubilities. However, some generalities can be presented (Fig. 10-1).

For most foods, the first step is drying, which also can be used to determine moisture content. For other than beverages, drying is done by placing a weighed amount of material in a vacuum oven and drying to constant weight at 55°C and 1 mm Hg pressure. Then, the material is ground to a fine powder, and lipids are extracted using 19:1 vol/vol chloroform–methanol



10-1 figure Flow diagram for sample preparation and extraction of mono- and disaccharides.

in a Soxhlet extractor (Chap. 8). (Note: Chloroform–methanol forms an azeotrope boiling at 54°C with a mole ratio of 0.642:0.358 or a vol/vol ratio of 3.5:1 in the vapor.) Prior extraction of lipids makes extraction of carbohydrates easier and more complete.

However, other sample preparation schemes may be required. For example, the AOAC International method (3) for presweetened, ready-to-eat breakfast cereals calls for removal of fats by extraction with petroleum ether (hexane) rather than the method described above and extraction of sugars with 50% ethanol (AOAC Method 982.14), rather than the method described below.

10.3 MONO- AND OLIGOSACCHARIDES

10.3.1 Extraction

Food raw materials and products and some ingredients are complex, heterogeneous, biological materials. Thus, it is quite likely that they may contain substances that interfere with measurement of the mono- and oligosaccharides present, especially if a spectrophotometric method is used. Interference may arise either from compounds that absorb light of the same wavelength used for the carbohydrate analysis or from insoluble, colloidal material that scatters light, since light scattering will be measured as absorbance. Also, the aldehydo or keto group of the sugar can react with other components, especially amino groups of proteins, a reaction (the **nonenzymatic browning** or **Maillard reaction**) that simultaneously produces color and destroys the sugar. Even if chromatographic methods, such as HPLC (Sect. 10.3.4.1), are used for analysis, the mono- and oligosaccharides must usually be separated from other components of the food before chromatography. Thus, for determination of any mono- (glucose, fructose), di- (sucrose, lactose, maltose), tri- (raffinose), tetra- (stachyose), or other oligo- (maltodextrins) saccharides present, the dried, lipid-free sample is extracted with **hot 80% ethanol** (final concentration) in the presence of precipitated calcium carbonate to neutralize any acidity (AOAC Method 922.02, 925.05). Higher oligosaccharides from added malto- or fructooligosaccharides also may be extracted. Carbohydrates are soluble in polar solvents. However, much of the composition of a food (other than water) is in the form of polymers, and almost all polysaccharides and proteins are insoluble in hot 80% ethanol. Thus, this extraction is rather specific. Extraction is done by a batch process. Refluxing for 1 h, cooling, and filtering is standard practice. (A Soxhlet apparatus cannot be used because aqueous ethanol undergoes azeotropic distillation as 95% ethanol.) Extraction should be done at least twice to check for and ensure completeness of extraction. If the foodstuff or food product is particularly acidic, for example a low-pH fruit, neutralization before extraction may be necessary to prevent hydrolysis of sucrose, which is particularly acid labile; thus, precipitated calcium carbonate is routinely added.

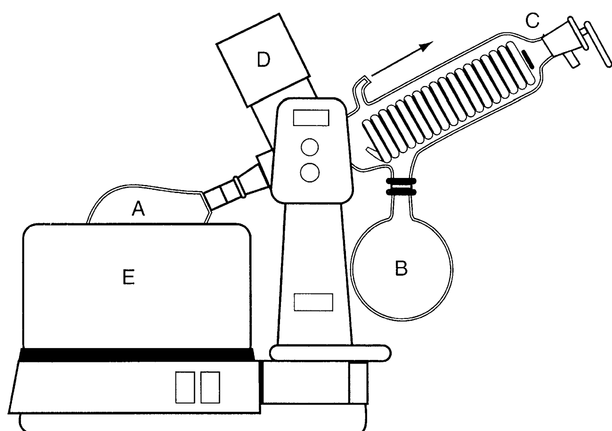
The 80% ethanol extract will contain components other than carbohydrates, in particular ash, pigments, organic acids, and perhaps free amino acids and low-molecular-weight peptides. Because the mono- and oligosaccharides are neutral and the contaminants are charged, the contaminants can be removed by **ion-exchange** techniques (Chap. 27). Because reducing sugars can be adsorbed onto and be isomerized by strong anion-exchange resins in the hydroxide form, a weak anion-exchange resin in the carbonate (CO_3^{2-}) or hydrogencarbonate (HCO_3^-) form is used. [**Reducing sugars** are those mono- and oligosaccharides that contain a free carbonyl (aldehydo or keto) group and, therefore, can act as reducing agents; see Sect. 10.3.3.] Because sucrose and sucrose-related oligosaccharides are very susceptible to acid-catalyzed hydrolysis, the anion-exchange resin should be used before the cation-exchange resin. However, because the anion-exchange resin is in a carbonate or hydrogencarbonate form, the cation-exchange resin (in H^+ form) cannot be used in a column because of CO_2 generation. Mixed-bed columns are not recommended for the same reason. AOAC Method 931.02C reads basically as follows for cleanup of ethanol extracts: Place a 50-ml aliquot of the ethanol extract in a 250-ml Erlenmeyer flask. Add 3 g of anion-exchange resin (hydroxide form) and 2 g of cation-exchange resin (acid form). Let it stand for 2 h with occasional swirling.

The aqueous alcohol of the ethanol extract is removed under reduced pressure using a **rotary evaporator** (Fig. 10-2) and a temperature of 45–50°C. The residue is dissolved in a known, measured amount of water. Filtration should not be required, but should be used if necessary. Some methods employ a final passage through a hydrophobic column such as a Sep-Pak C18 cartridge (Waters Associates, Milford, MA) as a final cleanup step to remove any residual lipids, proteins, and/or pigments, but this should not be necessary if the lipids and lipid-soluble components were properly removed prior to extraction. (Extracts may contain minor carbohydrates, such as cyclitols and naturally occurring or added sugar alcohols. These are not considered in Sects. 10.3.2 or 10.3.3.)

10.3.2 Total Carbohydrate: Phenol-Sulfuric Acid Method

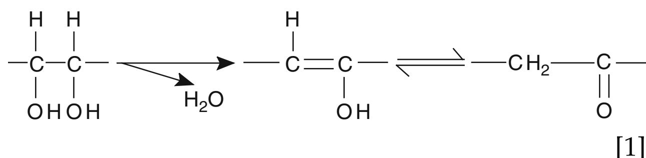
10.3.2.1 Principle and Characteristics

Carbohydrates are destroyed by strong acids and/or high temperatures. Under these conditions, a series of



10-2 figure Diagram of a rotary evaporator. The solution to be concentrated is placed in the round-bottom Flask A in a water bath (E) at a controlled temperature. The system is evacuated by means of a water aspirator or pump; connecting tubing is attached at the arrow. Flask (A) turns (generally slowly). Evaporation is relatively rapid from a thin film on the inside walls of flask (A) produced by its rotation because of the reduced pressure, the large surface area, and the elevated temperature. C is a condenser. D is the motor. Condensate collects in flask B. The stopcock at the top of the condenser is for releasing the vacuum.

complex reactions takes place, beginning with a simple dehydration reaction as shown in Equation [1].



[1]

Continued heating in the presence of acid produces various furan derivatives (Fig. 10-3). These products then condense with themselves and other products to produce brown and black substances. They will also condense with various phenolic compounds, such as phenol, resorcinol, orcinol, α-naphthol, and napthoresorcinol, and with various aromatic amines, such as aniline and *o*-toluidine, to produce colored compounds that are useful for carbohydrate analysis (3, 6).

The most often used condensation is with phenol itself (3, 7–10) (AOAC Method 44.1.30). This method is simple, rapid, sensitive, accurate, specific for carbohydrates, and widely applied. The reagents are inexpensive, readily available, and stable. Virtually all classes of sugars, including sugar derivatives and oligo- and polysaccharides, can be determined with the phenol-sulfuric acid method. (Oligo- and polysaccharides react because they undergo hydrolysis in the presence of the hot, strong acid, releasing monosaccharides.) A stable color is produced, and results are reproducible. Under proper conditions, the phenol-sulfuric method is accurate to ±2%.

Neither this method nor those for measuring reducing sugar content (Sect. 10.3.3) involves stoichiometric reactions. The extent of reaction is, in part, a function of the structure of the sugar. Therefore, a standard curve must be used. Ideally, the standard curve will be prepared using mixtures of the same sugars present in the same ratio as they are found in the unknown. If this is not possible, for example, if a pure preparation of the sugar being measured is not available, or if more than one sugar is present either as free sugars in unknown proportions or as constituent units of oligo- or polysaccharides or mixtures of them, D-glucose is used to prepare the standard curve. In these cases, accuracy is determined by conformity of the standard curve made with D-glucose to the curve that would be produced from the exact mixture of carbohydrates being determined. In any analysis, the concentrations used to construct the standard curve must span the sample concentrations and beyond (i.e., all sample concentrations must fall within the limits of the standard concentrations), and both must be within the limits reported for sensitivity of the method. If any concentrations are greater than the upper limit of the sensitivity range, dilutions should be used.

The phenol-sulfuric acid procedure is often used as a qualitative test for the presence of carbohydrate. Neither sorbitol nor any other alditol (polyol, polyhydroxyalchol) gives a positive test.

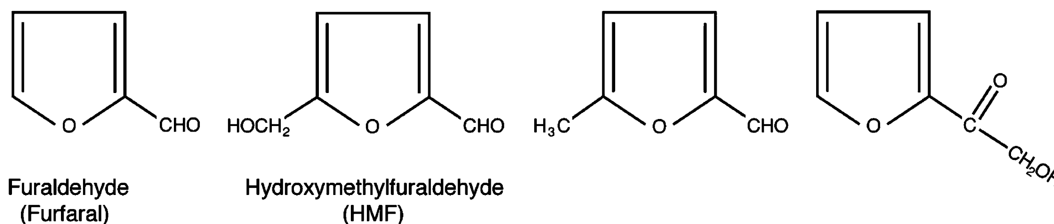
10.3.2.2 Outline of Procedure

1. A clear, aqueous solution of carbohydrate(s) is transferred using a pipette into a small tube. A blank of water also is prepared.
2. An aqueous solution of phenol is added, and the contents are mixed.
3. Concentrated sulfuric acid is added rapidly to the tube so that the stream produces good mixing. The tube is agitated. (Adding the sulfuric acid to the water produces considerable heat.) A yellow-orange color results.
4. Absorbance is measured at 490 nm.
5. The average absorbance of the blanks is subtracted, and the amount of sugar is determined by reference to a standard curve.

10.3.3 Total Reducing Sugar

10.3.3.1 Somogyi–Nelson Method

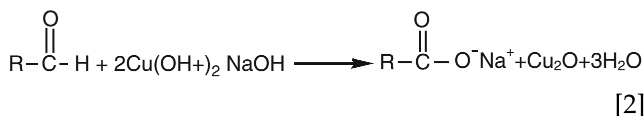
10.3.3.1.1 Principle Oxidation is a loss of electrons; reduction is a gain of electrons. Reducing sugars are those sugars that have an aldehydo group (aldoses) that can give up electrons (i.e., act as a reducing agent)



10-3 figure Furan products that could arise from, in order, pentoses and hexuronic acids, hexoses, 6-deoxyhexoses, and ketohexoses (1).

to an oxidizing agent, which is reduced by receiving the electrons. Oxidation of the aldehydo group produces a carboxylic acid group. Under alkaline conditions, ketoses behave as weak reducing sugars because they will partially isomerize to aldoses.

The most often used method to determine amounts of reducing sugars is the Somogyi–Nelson method (7, 11–14), also at times referred to as the Nelson–Somogyi method. This and other reducing sugar methods (Sect. 10.3.3.2) can be used in combination with enzymic methods (Sect. 10.3.4.3) for determination of oligo- and polysaccharides. In enzymic methods, specific hydrolases are used to convert the oligo- or polysaccharide into its constituent monosaccharide or repeating oligosaccharide units, which are measured using a reducing sugar method.



$$R-\overset{O}{\overset{\|}{C}}-H + 2Cu(OH+)_2\ NaOH \longrightarrow R-\overset{O}{\overset{\|}{C}}-O^-Na^+ + Cu_2O + 3H_2O \quad [2]$$

The Somogyi–Nelson method is based on the **reduction of Cu(II) ions to Cu(I) ions by reducing sugars**. The Cu(I) ions then reduce an arsenomolybdate complex, prepared by reacting ammonium molybdate [$(NH_4)_6Mo_7O_{24}$] and sodium arsenate (Na_2HAsO_7) in sulfuric acid. Reduction of the arsenomolybdate complex produces an intense, stable blue color that is measured spectrophotometrically. This reaction is not stoichiometric and must be used with a standard curve of the sugar(s) being determined or D-glucose.

10.3.3.1.2 Outline of Procedure

1. A solution of copper(II) sulfate and an alkaline buffer are added by pipettes to a solution of reducing sugars(s) and a water blank.
2. The resulting solution is heated in a boiling water bath.
3. A reagent prepared by mixing solutions of acidic ammonium molybdate and sodium arsenate is added.
4. After mixing, dilution, and remixing, absorbance is measured at 520 nm.
5. After subtracting the absorbance of the reagent blank, the A_{250} is converted into glucose equivalents using a standard plot of micrograms of glucose vs. absorbance.

10.3.3.2 Other Methods (3)

The **dinitrosalicylic acid method** (15) will measure reducing sugars naturally occurring in foods or released by enzymes, but is not much used. In this reaction, 3,5-dinitrosalicylate is reduced to the reddish monoamine derivative.

There are other methods that, like the Somogyi–Nelson method, are based on the **reduction of Cu(II) ions in alkaline solution to Cu(I) ions** that precipitate as the brick-red oxide Cu_2O. Tartrate or citrate ions are added to keep the Cu(II) ions in solution under the alkaline conditions. The **Munson–Walker method** (AOAC Method 906.03) has various forms. The precipitate of cuprous oxide can be determined **gravimetrically** (AOAC Method 31.039), by **titration** with sodium thiosulfate (AOAC Method 31.040), by titration with potassium permanganate (AOAC Method 31.042), by titration in the presence of methylene blue (the **Lane–Eynon method**; AOAC Method 923.09, 920.183b), and **electrolytically** (AOAC Method 31.044). These methods also must be used with standard curves because each reducing sugar reacts differently. Because assay conditions affect the outcome, they generally also must be done by trained, experienced analysts so that they always are done in exactly the same way. They are still used where specified.

A keto group cannot be oxidized to a carboxylic acid group, and thus ketoses are not reducing sugars. However, under the alkaline conditions employed, ketoses are isomerized to aldoses (1) and, therefore, are measured as reducing sugars. The response is less with ketoses, so a standard curve made with D-fructose as one of the sugars in the mixture of sugars should be used if it is present.

Methods that both identify individual carbohydrates present and determine their amounts are preferred over general reducing sugar methods and are described next.

10.3.4 Specific Analysis of Mono- and Oligosaccharides

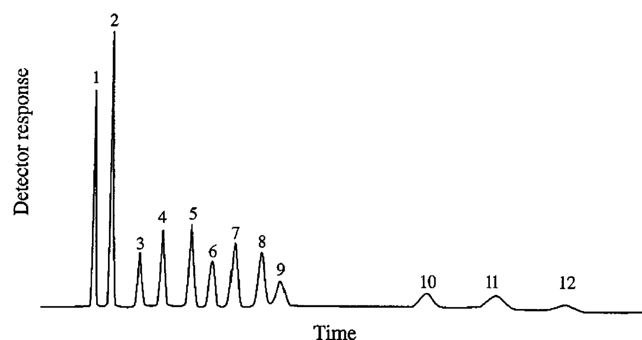
10.3.4.1 High-performance Liquid Chromatography

HPLC (Chap. 28) is the method of choice for analysis of mono- and oligosaccharides and can be used for analysis of polysaccharides after hydrolysis (Sect. 10.4.2). HPLC gives both qualitative analysis (identification of the carbohydrate) and, with peak integration, quantitative analysis. HPLC analysis is rapid, can tolerate a wide range of sample concentrations, and provides a high degree of precision and accuracy. HPLC requires no prior derivatization of carbohydrates, unlike GC of sugars (Sect.10.3.4.2), but does require micron-filter filtration prior to injection. Complex mixtures of mono- and oligosaccharides can be analyzed. The basic principles and important parameters of HPLC (the stationary phase, the mobile phase, and the detector) are presented and discussed in Chap. 28. Some details related to carbohydrate analysis are discussed here. Use of HPLC to determine soluble food and other carbohydrates has been reviewed many times. Selected reviews can be found in references (16–21). Specific details of methods of analyses of specific food ingredients or products should be obtained from the literature. The USDA Food Safety and Inspection Service recommends the use of HPLC for determination of sugars and sugar alcohols in meat and poultry products (22). Results of interlaboratory collaborative studies done to validate the reproducibility of HPLC methods are available (20, 23, 24).

10.3.4.1.1 Stationary Phases Stationary phases are presented in probable order of use for carbohydrate analysis.

1. **Anion-exchange chromatography** (*AE-HPLC*). Carbohydrates have pK_a values in the pH range 12–14 and are, therefore, very weak acids. In a solution of high pH, some carbohydrate hydroxyl groups are ionized, allowing sugars to be separated on columns of anion-exchange resins. Special column packings have been developed for this purpose. The general elution sequence is sugar alcohols (alditols), monosaccharides, disaccharides, and higher oligosaccharides.

AE-HPLC is most often used in conjunction with electrochemical detection (see Chap. 27 and Section "Detectors") (18–21, 23–27). AE-HPLC has been used to examine the complex oligosaccharide patterns of many food components and products. The method has the advantage of being applicable to baseline separation within each class of carbohydrates [see Fig. 10-4 for separation of some monosaccharides, disaccharides, alditols (sugar alcohols), and raffinose] and of providing separation of homologous series of oligosaccharides into their components (27, 28).



10-4 figure High-performance liquid chromatogram of some common monosaccharides, disaccharides, alditols, and the trisaccharide raffinose at equal wt/vol concentrations separated by anion-exchange chromatography and detected by pulsed amperometric detection (see Sect. 10.3.4.1.2). Peak *1*, glycerol; *2*, erythritol; *3*, L-rhammose; *4*, D-glucitol (sorbitol); *5*, mannitol; *6*, L-arabinose; *7*, D-glucose; *8*, D-galactose; *9*, lactose; *10*, sucrose; *11*, raffinose; *12*, maltose.

2. **Normal-phase chromatography** (29). Normal-phase chromatography is a widely used HPLC method for carbohydrate analysis. In normal-phase chromatography, the stationary phase is polar and elution is accomplished by employing a mobile phase of increasing polarity. Silica gel that has been derivatized with one or more of several reagents to incorporate amino groups is often used. These so-called amine-bonded stationary phases that are generally used with acetonitrile–water (50–85% acetonitrile) as the eluent are effective in carbohydrate separations. The elution order is monosaccharides and sugar alcohols, disaccharides, and higher oligosaccharides. Amine-bonded silica gel columns have been used successfully to analyze the low-molecular-weight carbohydrate content of foods (17).

A severe disadvantage of amine-bonded silica gel is the tendency for reducing sugars to react with the amino groups of the stationary phase, which results in a deterioration of column performance over time and loss of some of the carbohydrate being measured. This situation can be partially alleviated through the use of amine-modified silica gel columns. To prepare amine-modified silica gel columns, small amounts of modifiers, which are soluble amine compounds, are added to the mobile phase to modify the packing in situ. The modifier must have at least two amino groups, for one is needed to adsorb to the silica gel and the other must be free to interact with the carbohydrate. Because the modifier is in the eluent, the column is continuously regenerated.

3. **Cation-exchange chromatography**. Microparticulate spheres of sulfonated resin are used for cation-exchange stationary phases. The resin is loaded with one of a variety of metal counter ions, depending on the type of separation desired. Usually Ca^{2+}, Pb^{2+}, or Ag^{+} is used as the counter ion. The mobile phase used with these columns is water plus varying amounts (typically <40%) of an organic solvent such as acetonitrile and/or methanol. These columns normally are operated at elevated temperatures (>80°C) to increase column efficiency by increasing the mass transfer rate between the stationary and mobile phases which effects peak narrowing and improved resolution (30).

Carbohydrate elution from cation-exchange resins takes place in the order of decreasing molecular weight. Oligosaccharides with a degree of polymerization (DP) greater than 3 elute first, followed by trisaccharides, disaccharides, monosaccharides, and alditols. There is some resolution of disaccharides, but the real strength of this stationary phase is in the separation of individual monosaccharides.

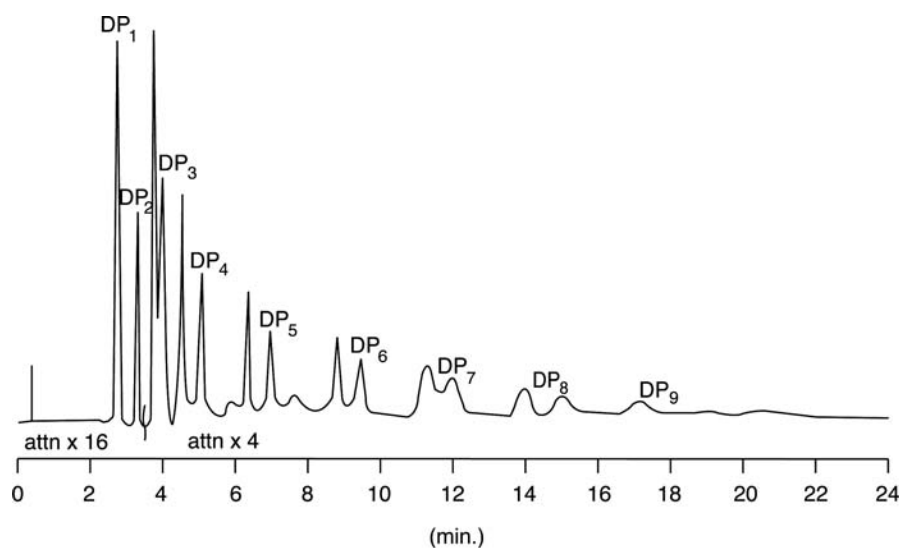
4. **Reversed-phase chromatography**. In reversed-phase chromatography, the stationary phase is hydrophobic, and the mobile phase is largely water. The hydrophobic stationary phase is made by reacting silica gel with a reagent that adds alkyl chains, such as an 18-carbon-atom alkyl chain (a C18 column) or a phenyl group (a phenyl column). Reversed-phase chromatography has been used for separation of mono-, di-, and trisaccharides by groups (31, 32) (Fig. 10-5).

A major disadvantage of this stationary phase is the short retention times of monosaccharides, which result in elution as a single unresolved peak. The addition of salts (such as sodium chloride) can increase retention on the stationary phase and the utility of this method for monosaccharide analysis. Reversed-phase chromatography is complicated by peak doubling and/or peak broadening due to the presence of anomers. This problem can be alleviated by the addition of an amine to the mobile phase to accelerate mutarotation (anomerization), but separation may be negatively affected by the shorter retention times that usually result.

A wide variety of stationary phases is available, including phases not included in one of the four groups given above, and new improved phases continue to be developed. Both normal- and reversed-phase columns have long lives, have good stability over a wide range of solvent compositions and pH values (from pH 2 to pH 10), are suitable for the separation of a range of carbohydrates, and are of relatively low cost. All silica-based stationary phases share the disadvantage that silica dissolves to a small extent in water-rich eluents.

10.3.4.1.2 Detectors Detectors and their limitations and detector limits have been reviewed (19).

1. **Refractive index detection**. **The refractive index** (*RI*) detector is commonly employed for carbohydrate analysis (33) (Sect. 10.6.4). RI measurements are linear over a wide range of carbohydrate concentrations and can be universally applied to all carbohydrates, but the RI detector has its drawbacks. RI is a bulk physical property that is sensitive to changes



10-5 figure High-performance, reversed-phase liquid chromatogram of maltodextrins (DP 1–9). [From (32), used with permission.]

in flow, pressure, and temperature; but with modern HPLC equipment and a temperature-controlled detector, problems arising from these changes can be minimized. The most significant limiting factor with RI detection is that gradient elution cannot be used. The other is that, since an RI detector measures mass, it is not sensitive to low concentrations.

2. **Electrochemical detection**. The triple-pulsed electrochemical detector, called a **pulsed-amperometric detector** (PAD), which relies on oxidation of carbohydrate hydroxyl and aldehydo groups, is universally used with AE-HPLC (18–21, 23–27, 34). It requires a high pH. Gradient and graded elutions can be used with the PAD. The solvents employed are simple and inexpensive (sodium hydroxide solution, with or without sodium acetate). (Water may be used, but when it is, postcolumn addition of a sodium hydroxide solution is required.) The detector is suitable for both reducing and nonreducing carbohydrates. Limits are approximately 1.5 ng for monosaccharides and 5 ng for di-, tri-, and tetrasaccharides.

3. **Postcolumn derivatization** (35). The purpose of pre- and postcolumn derivatization is to increase detection sensitivity by addition of a substituent whose concentration can be measured using an ultraviolet (UV) or fluorescence detector. However, with the development of the PAD detector, neither pre- nor postcolumn derivatization is much used. Postcolumn derivatization involves addition of reagents that will provide compounds whose concentration can be measured using absorbance (visible) or fluorescence detection. It is straightforward; requires only one or two additional pumps, a mixing coil, and a thermostatted bath; and provides greater sensitivity than does an RI detector.

4. **Precolumn derivatization** (35). Precolumn derivatization reactions must be stoichiometric. Oligosaccharides derivatized with aromatic groups are often separated with higher resolution in normal-phase HPLC.

10.3.4.2 Gas Chromatography

GC (**gas-liquid chromatography**, GLC), like HPLC, provides both qualitative and quantitative analysis of carbohydrates. For GC, sugars must be converted into volatile derivatives. The most commonly used derivatives are the alditol peracetates (and aldonic acid pertrimethylsilyl ethers from uronic acids) (36–39). These derivatives are prepared as illustrated in Fig. 10-6 for D-galactose and D-galacturonic acid. Conversion of sugars into peracetylated aldononitrile (aldoses) and peracetylated ketooxime (ketoses) derivatives for GC has also been done (40), although this procedure is not used nearly as much as the preparation of peracetylated aldoses and aldonic acids. A **flame ionization detector** is the detector of choice for peracetylated carbohydrate derivatives.

The most serious problem with GC for carbohydrate analysis is that two preparation steps are involved: reduction of aldehyde groups to primary alcohol groups and conversion of the reduced sugar into a volatile peracetate ester or pertrimethylsilyl ether derivative. Of course, for the analysis to be successful, each of these steps must be 100% complete (i.e., stoichiometric). The basic principles and important parameters of GC (the stationary phase, temperature programming, and detection) are presented and discussed in Chap. 29.

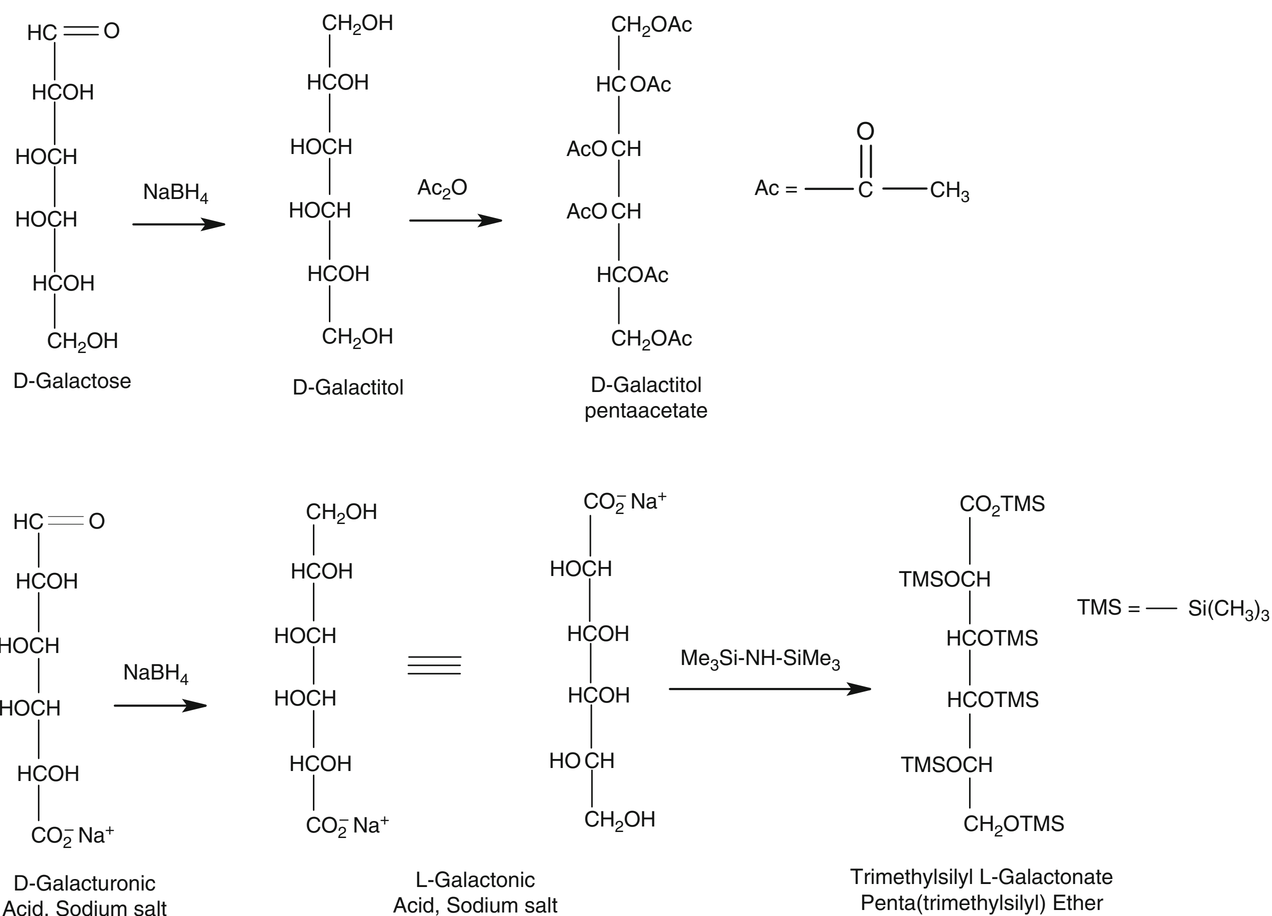
10.3.4.2.1 Neutral Sugars: Outline of Procedure (38)

1. **Reduction to alditols**. Neutral sugars from the 80% ethanol extract (Sect. 10.3.1) or from hydrolysis of a polysaccharide (see Sects. 10.4.2.2 and “Overview”) are reduced with an excess of sodium or potassium borohydride dissolved in dilute ammonium hydroxide solution. After reaction at 40°C, glacial acetic acid is added dropwise until no more hydrogen is evolved. This treatment destroys excess borohydride. The acidified solution is evaporated to dryness. Borate ions may be removed as methyl borate by successive additions and evaporation of methanol, but this step is not necessary.

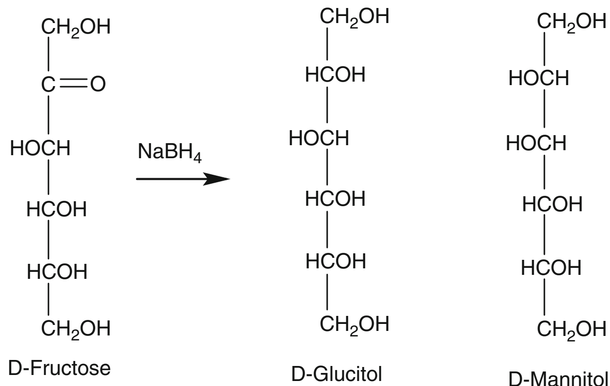
A potential problem is that, if fructose is present, either as a naturally occurring sugar, from the hydrolysis of inulin, or as an additive [from high fructose syrup (HFS), invert sugar, or honey], it will be reduced to a mixture of D-glucitol (sorbitol) and D-mannitol (Fig. 10-7).

2. **Acetylation of alditols**. Acetic anhydride and 1-methylimidazole (as a catalyst) are added. After 10 min at room temperature, water and dichloromethane are added. The dichloromethane layer is washed with water and evaporated to dryness. The residue of alditol peracetates is dissolved in a polar organic solvent (usually acetone) for chromatography.

3. **GC of alditol peracetates** (38, 39). Alditol acetates may be chromatographed isothermally and identified by their retention times relative to that of inositol hexaacetate, inositol being added as an internal standard prior to acetylation. It is wise to run standards of the additol peracetates of the sugars being determined with inositol hexaacetate as an internal standard.



10-6 figure Modification of D-galactose and D-galacturonic acid in preparation for gas chromatography.



10-7 figure Reduction of D-fructose to a mixture of alditols.

10.3.4.2.2 Hydrolyzates of Polysaccharides Containing Uronic Acids: Outline of Procedure (41) A method different from that used for neutral sugars (Sect. 10.3.4.2.1) is required when uronic acids are present.

1. **Reduction**. As with hydrolyzates containing only neutral sugars, the hydrolyzate is evaporated to dryness. The residue is dissolved in sodium carbonate solution and treated with an excess of sodium borohydride. Excess borohydride is decomposed by addition of glacial acetic acid; borate may be removed by addition and evaporation of methanol (Sect. 10.3.4.2.1). This procedure reduces uronic acids to aldonic acids and aldoses to alditols (Fig. 6).

2. **Preparation and chromatography of trimethylsilyl (TMS) derivatives**. The aldonic acids are converted into per-TMS ethers rather than per-acetate esters (Fig. 10-6). Trimethylsilyation of free aldonic acids gives derivatives of lactones (predominately the 1,4-lactone), while trimethylsilyation of the sodium salt produces the ester. Several procedures and packaged reagents have been developed for this etherification. The reaction mixture is injected directly into the chromatograph. Temperature programming is required. Components are identified by their retention times.

10.3.4.3 Enzymic Methods

10.3.4.3.1 Overview The method of choice for the determination of starch employs a combination of enzymes in sequential **enzyme-catalyzed reactions** and is specific for starch, as long as purified enzyme preparations are used (Sect. 10.4.1.1).

Other enzymic methods for the determination of carbohydrates have been developed (Table 10-3) [see also Equation (3) and Chap. 16]. They are often, but not always, specific for the substance being measured. Kits for several enzymic methods have been developed and marketed. The kits contain specific enzymes, other required reagents, buffer salts, and detailed instructions that must be followed because enzyme concentration, substrate concentration, concentration of other required reagents, pH, and temperature all affect reaction rates and results. A good description of a method will point out any interferences and other limitations.

Limits of detection by methods involving enzyme- or coupled enzyme-catalyzed reactions are generally low. In addition, enzymic methods are usually quite specific for a specific carbohydrate, although not always 100% specific. However, it is not often that determination of a single component is desired, the notable exception being the determination of starch (Sect. 10.4.1.1). Other exceptions are the identification and quantitative determination of β-glucan and inulin. Thus, chromatographic methods (Sects. 10.3.4.1 and 10.3.4.2) that give values for each of the sugars present are preferred.

10.3.4.3.2 Sample Preparation It sometimes is recommended that the **Carrez treatment** (7), which breaks emulsions, precipitates proteins, and absorbs some colors, be applied to food products prior to determination of carbohydrates by enzymic methods. The Carrez treatment involves addition of a solution of potassium hexacyanoferrate ($K_4[Fe(CN)_6]$, potassium ferrocyanide), followed by addition of a solution of zinc sulfate ($ZnSO_4$), followed by addition of a solution of sodium hydroxide. The suspension is filtered, and the clear filtrate is used directly in enzyme-catalyzed assays.

10.3.4.3.3 Enzymic Determination of D*-Glucose* The enzyme **glucose oxidase** oxidizes D-glucose quantitatively to D-glucono-1,5-lactone (glucono-delta-lactone), the other product being hydrogen peroxide (Fig. 10-8). To measure the amount of D-glucose present, **peroxidase** is added along with a colorless compound that can be oxidized to a colored compound. In a second enzyme-catalyzed reaction, the leuco dye is oxidized to a colored compound which is measured spectrophotometrically. Various dyes are used in commercial kits. The method using this combination of two enzymes and an oxidizable colorless compound is known as the **GOPOD (glucose oxidase-peroxidase) method**.

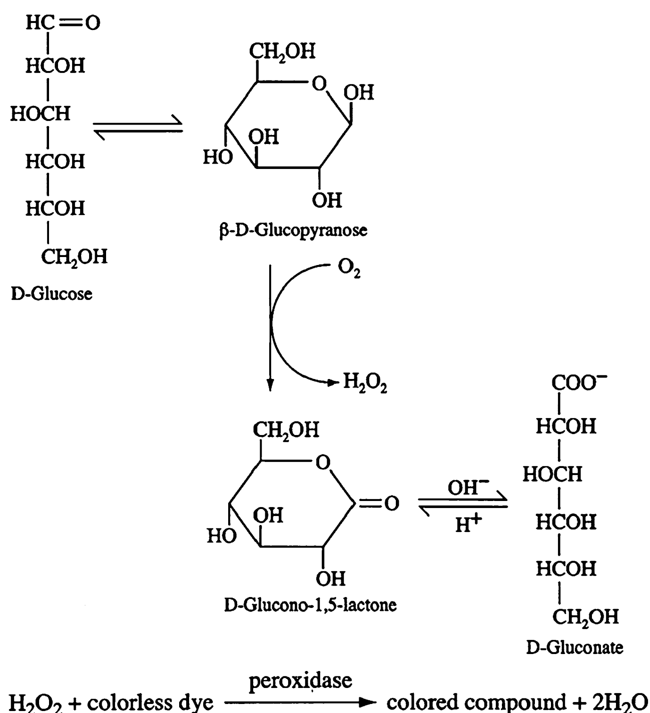
Table 10-3 Selected Enzymic Methods of Carbohydrate Analysis

Carbohydrate	*Reference*	*Kit Form*[a]
Monosaccharides		
Pentoses		
L-Arabinose	(42, 43)	
D-Xylose	(42, 43)	
Hexoses		
D-Fructose	(42, 43)	x
D-Galactose	(42, 43)	x
D-Galacturonic acid	(42)	
D-Glucose		
Using glucose oxidase	(43), Sect. 10.3.4.3.3	x
Using glucose dehydrogenase	(42, 43)	
Using glucokinase (hexokinase)	(42, 43)	x
D-Mannose	(42, 43)	
Monosaccharide derivatives		
D-Gluconate/D-glucono-δ-lactone	(42, 43)	x
D-Glucitol/sorbitol	(42, 43)	x
D-Mannitol	(42, 43)	
Xylitol	(42, 43)	x
Oligosaccharides		
Lactose	(42, 43)	x
Maltose	(42, 43)	x
Sucrose	(42, 43)	x
Raffinose, stachyose, verbascose	(42, 43)	x
Polysaccharides		
Amylose, amylopectin (contents and ratio)		x
Cellulose	(42, 43)	
Galactomannans (guar and locust bean gums)	(42)	
β-Glucan (mixed-linkage)	(42)	x
Glycogen	(42, 43)	
Hemicellulose	(42, 43)	
Inulin	(42, 43)	x
Pectin/poly(D-galacturonic acid)	(42, 43)	
Starch	Sect. 10.4.1.1 (42, 43)	x

[a] Available in kit form from companies such as R-Biopharm, Megazyme, and Sigma-Aldrich.

10.3.4.4 Mass Spectrometry

There are many different variations of mass spectrometry (MS) (Chap. 26). With carbohydrates most of the techniques are used for structural analysis; MS has



10-8 figure Coupled enzyme-catalyzed reactions for the determination of D-glucose.

been used for analysis of carbohydrates, but not in a routine manner (44). Particularly useful is the **matrix-assisted laser desorption time-of-flight** (MALDI-TOF) technique for analysis of a homologous series of oligosaccharides (Fig. 10-9). A comparison was made between anion-exchange HPLC (Sect. 10.3.4.1) (the most used carbohydrate analysis technique today), capillary electrophoresis (Sect. 10.3.4.6), and MALDI-TOF mass spectrometry for the analysis of maltooligosaccharides, with the conclusion that the latter technique gave the best results (28).

10.3.4.5 Thin-layer Chromatography

Thin-layer chromatography has been used for identification and quantitation of the sugars present in the molasses from sugar beet and cane processing (45). It is particularly useful for rapid screening of several samples simultaneously.

10.3.4.6 Capillary Electrophoresis (46, 47)

Capillary zone electrophoresis (Chap. 15) has also been used to separate and measure carbohydrates, but because carbohydrates lack chromophores, precolumn derivatization and detection with a UV or fluorescence detector is required (35). Generally, this method provides no advantage over HPLC methods for carbohydrate analysis.

10.4 POLYSACCHARIDES

10.4.1 Starch

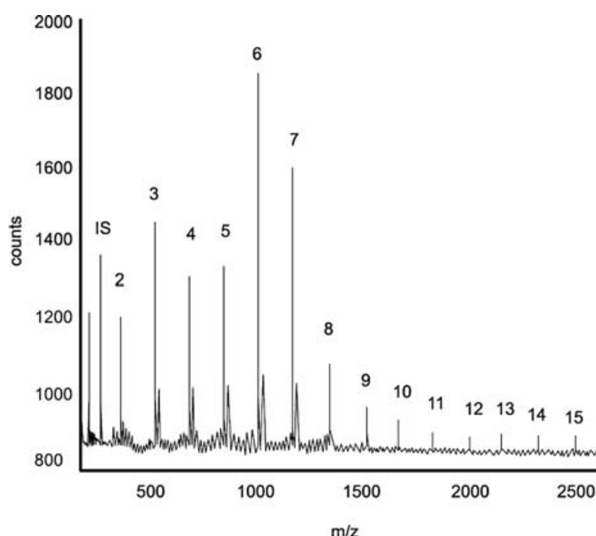
Starch is second only to water as the most abundant component of food. Starch is found in all parts of plants (leaves, stems, roots, tubers, seeds). A variety of commercial starches are available worldwide as food additives. These include corn (maize), waxy maize, high-amylose corn (amylomaize), potato, wheat, rice, tapioca (cassava), arrowroot, and sago starches. In addition, starch is the main component of wheat, rye, barley, oat, rice, corn, mung bean, and pea flours and certain roots and tubers such as potatoes, sweet potatoes, and yams.

10.4.1.1 Total Starch

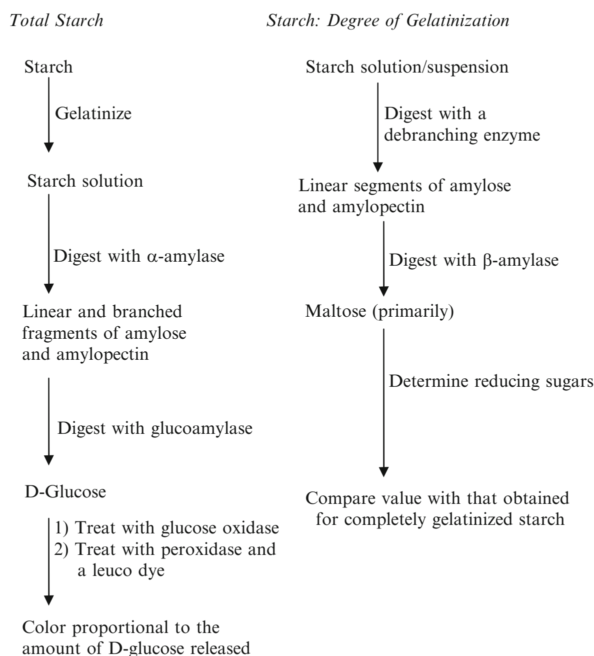
10.4.1.1.1 Principle The only reliable method for determination of total starch is based on complete conversion of the starch into D-glucose by purified enzymes specific for starch and determination of the D-glucose released by an enzyme specific for it (Fig. 10-8) (see also Chap. 16).

10.4.1.1.2 Potential Problems Starch-hydrolyzing enzymes (amylases) must be purified to eliminate any other enzymic activity that would release D-glucose (e.g., cellulases, invertase or sucrase, β-glucanase) and catalase, which would destroy the hydrogen peroxide on which the enzymic determination of D-glucose depends (Sect. 10.3.4.3.3). The former contamination would give false high values and the latter, false low values. Even with purified enzymes, problems can be encountered with this method. It may not be quantitative for high-amylose or another starch at least partially resistant to enzyme-catalyzed hydrolysis. **Resistant starch** (RS), by definition, is composed of starch and starch-degradation products that escape digestion in the small intestine (48). There are generally considered to be four starch sources that are resistant to digestion or so slowly digested that they pass through the small intestine:

1. Starch that is physically inaccessible to amylases because it is trapped within a food matrix (RS1),
2. Starch that resists enzyme-catalyzed hydrolysis because of the nature of the starch granule (uncooked starch) (RS2),
3. Retrograded starch (i.e., starch polymers that have recrystallized after gelatinization of the granules, e.g., cooled cooked potatoes contain resistant starch) (Sect. 10.4.1.3) (RS3), and
4. Starch that has been modified structurally in such a way as to make it less susceptible to digestion (RS4).



10-9 figure MALDI-TOF mass spectrum of maltooligosaccharides produced by hydrolysis of starch. *Numbers* indicate DP. *IS*, internal standard. [From (28), used with permission, Copyright Springer-Verlag, 1998.]



10-10 figure Flow diagrams for determination of total starch (Sect. 10.4.1.1) and determination of the degree of starch gelatinization (Sect. 10.4.1.2).

RS is at best only partially converted into D-glucose by the method described below to measure starch; rather most of it is usually included in the analysis for dietary fiber (Sect. 10.5.4.1).

One method of starch analysis purports to overcome at least the first three of these problems (49). In it, the starch is dispersed in dimethyl sulfoxide (DMSO) and then is converted quantitatively to D-glucose by treatment with a thermostable α-amylase to effect depolymerization and solubilization of the starch (Fig. 10-10). Glucoamylase (amyloglucosidase) effects quantitative conversion of the fragments produced by the action of α-amylase into D-glucose. D-glucose is determined with a glucose oxidase-peroxidase (GOPOD) reagent (Sect. 10.3.4.3.3) (AOAC Method 969.39; AACC Method 76-13). This reagent contains a colorless (leuco) dye that is oxidized to a colored compound by the hydrogen peroxide (produced by the glucose oxidase-catalyzed oxidation of glucose, Fig. 10-8) in a reaction catalyzed by peroxidase. The method determines total starch. It does not reveal the botanical source of the starch or whether it is native starch or modified food starch. The botanical source of the starch may be determined microscopically (Sect. 10.6.1) if the material being analyzed has not been cooked. Some information about modification also may be determined with a microscope.

10.4.1.1.3 Outline of Procedure

1. A sample of finely milled material is placed in a glass test tube and wetted with 80% vol/vol ethanol. DMSO is added to the ethanol-wetted sample, and the contents of the tube are mixed vigorously. The tube is then heated in a boiling water bath.
2. A buffered solution of a thermostable α-amylase is added. Tube contents are vortex mixed, and the tube is returned to the boiling water bath.
3. After 5 min, the tube is brought to 50°C. Sodium acetate buffer, pH 4.5, and glucoamylase (amyloglucosidase) solution is added, and the contents are mixed. The tube then is incubated at 50°C.
4. The tube contents are transferred quantitatively to a volumetric flask using distilled water to

wash the tube and to adjust the contents to volume.

5. After thorough mixing of the flask, aliquots are removed, treated with GOPOD reagent, and incubated at 50°C. Absorbance of the test sample and a reagent blank is measured at the wavelength required by the GOPOD reagent being used.

Glucose and a starch low in protein and lipid content (such as potato starch) are used as standards after determination of their moisture contents. Addition of DMSO can be omitted, and diluted thermostable α-amylase solution can be added directly to the ethanol-wetted sample if it is known from experience that no starch resistant to the α-amylase under the conditions used is present in the samples being analyzed.

10.4.1.2 Degree of Gelatinization of Starch

When starch granules are heated in water to a temperature specific for the starch being cooked, they swell, lose their crystallinity and birefringence, and become much more susceptible to enzyme-catalyzed hydrolysis. Heating starch in water produces phenomena that result from two processes: **gelatinization** and **pasting**, often together referred to simply as gelatinization, which are very important in determining the texture and digestibility of foods containing starch.

Several methods have been developed that make use of the fact that certain enzymes act much more rapidly on cooked starch than they do on native starch. A particularly sensitive method employs a combination of pullulanase and β-amylase, neither of which is able to act on uncooked starch granules (50). With gelatinized or pasted starch, the enzyme **pullulanase** debranches amylopectin and any branched amylose molecules, giving a mixture of linear segments of various sizes. (Another debranching enzyme, **isoamylase**, may also be used.) ***β*-Amylase** then acts on the linear chains, releasing the disaccharide maltose, starting at the nonreducing ends (Fig. 10-10) and a small amount of maltotriose (from chains containing an odd number of glucosyl units). The **degree of gelatinization** is determined by measuring the amount of reducing sugar formed (Sect. 10.3.3).

10.4.1.3 Degree of Retrogradation of Starch

Upon storage of a product containing cooked starch, the two starch polymers, **amylose** and **amylopectin**, associate with themselves and with each other, forming polycrystalline arrays. This process of reordering is called **retrogradation**. (Retrogradation is a contributing factor to the staling of bread and other bakery products, for example.) Retrograded starch, like native starch, is acted on very slowly by the combination of pullulanase plus β-amylase. Therefore, the basic method described in Sect. 10.4.1.2 can be used to determine retrogradation. The decrease in reducing power (from maltose released by action of the enzyme combination) after storage is a measure of the amount of retrograded starch at the time of analysis and/or the degree of retrogradation.

10.4.2 Nonstarch Polysaccharides (Hydrocolloids/Food Gums)

10.4.2.1 Overview

A starch (or starches) may be used as ingredients in a food product, either as isolated starch or as a component of a flour, or may occur naturally in a fruit or vegetable tissue. Other polysaccharides are almost always added as ingredients, although there are exceptions. These added polysaccharides, along with the protein gelatin, comprise the group of ingredients known as **food gums** or **hydrocolloids**. Their use is widespread and extensive. They are used in everything from processed meat products to chocolate products, from ice cream to salad dressings.

Analytical methods are required for these polysaccharides to enable both suppliers and food processors to determine the purity of a gum product, to ensure that label declarations of processors are correct, and to monitor that hydrocolloids have not been added to standardized products in which they are not allowed. It also may be desirable to determine such things as the ***β*-glucan** content of oat or barley flour or a breakfast cereal for a label claim or the **arabinoxylan** content of wheat flour to set processing parameters. Another processor may want to determine other polysaccharides not declared on the ingredient label, such as those introduced by microorganisms during fermentation in making yogurt and yogurt-based products.

Food gum analysis is problematic because polysaccharides present a variety of chemical structures, solubilities, and molecular weights. Plant polysaccharides do not have uniform, repeating-unit structures; rather the structure of a specific polysaccharide such as *κ*-carrageenan varies from molecule to molecule. In addition, the average structure can vary with the source and the conditions under which the plant is grown. Some polysaccharides are neutral; some are anionic. Some are linear; some are branched. Some of the branched polysaccharides are still effectively linear; some are bushlike. Some contain ether, ester, and/or cyclic acetal groups in addition to sugar units, either naturally or as a result of chemical modification. Some are soluble only in hot water; some are soluble only in room temperature or colder water; some are soluble in both hot and cold water, and some

require aqueous solutions of acids, bases, or metal ion-chelating compounds to dissolve them. And all polysaccharide preparations are composed of a mixture of molecules with a range of molecular weights. All this structural diversity complicates qualitative analysis of food gums when their nature is unknown or when more than one is present, and structural heterogeneity complicates quantitative analysis of a specific gum.

Current methods depend on extraction of the gum(s), followed by fractionation of the extract. Fractionation invariably results in some loss of material. Most often, an isolated gum is identified by identifying and quantitating its constituent sugars after acid-catalyzed hydrolysis. However, sugars are released from polysaccharides by hydrolysis at different rates and are destroyed by hot acids at different rates, so the exact monosaccharide composition of a polysaccharide may be difficult to determine. Problems associated with the determination of gums in foods and various procedures that have been used for their measurement have been reviewed (51,52).

Qualitative identification tests, specifications, and analytical methods for many food-approved gums/hydrocolloids, including modified starches, have been established for the United States (53) and Europe (54). None of the qualitative methods is conclusive. AOAC International has established methods for analysis of some specific food products. But not all gums approved for food use are included; not all methods that determine total gums can be used if starch is present; and not all methods can be used to determine all gums. Hydrocolloid/gum suppliers and food processors usually have their own specifications of purity and properties.

10.4.2.2 Hydrocolloid/Food Gum Content Determination

Several schemes, some published, some unpublished, have been developed for analysis of food products for food gums. Most are targeted to a specific group of food products, as it is difficult, perhaps impossible, to develop a universal scheme. A general scheme that is reported to work successfully (41) is presented here. Figure 10-11 presents the scheme for isolation and purification of nonstarch, water-soluble polysaccharides. Letters in the parentheses below refer to the same letters in Fig. 10-11. Many of the steps in the method utilize principles previously described.

(a) It is usually difficult to extract polysaccharides quantitatively when fats, oils, waxes, and proteins are present. Therefore, lipid-soluble substances are removed first. Before this can be effected, the sample must be dried. Freeze drying is recommended. If the dried material contains lumps, it must be ground to a fine powder. A known weight of dry sample is placed in a Soxhlet apparatus, and the lipid-soluble substances are removed with 19:1 vol/vol chloroform–methanol (see note in Sect. 10.2). (*n*-Hexane has also been used.) Solvent is removed from the sample by air drying in a hood, then by placing the sample in a desiccator, which is then evacuated.

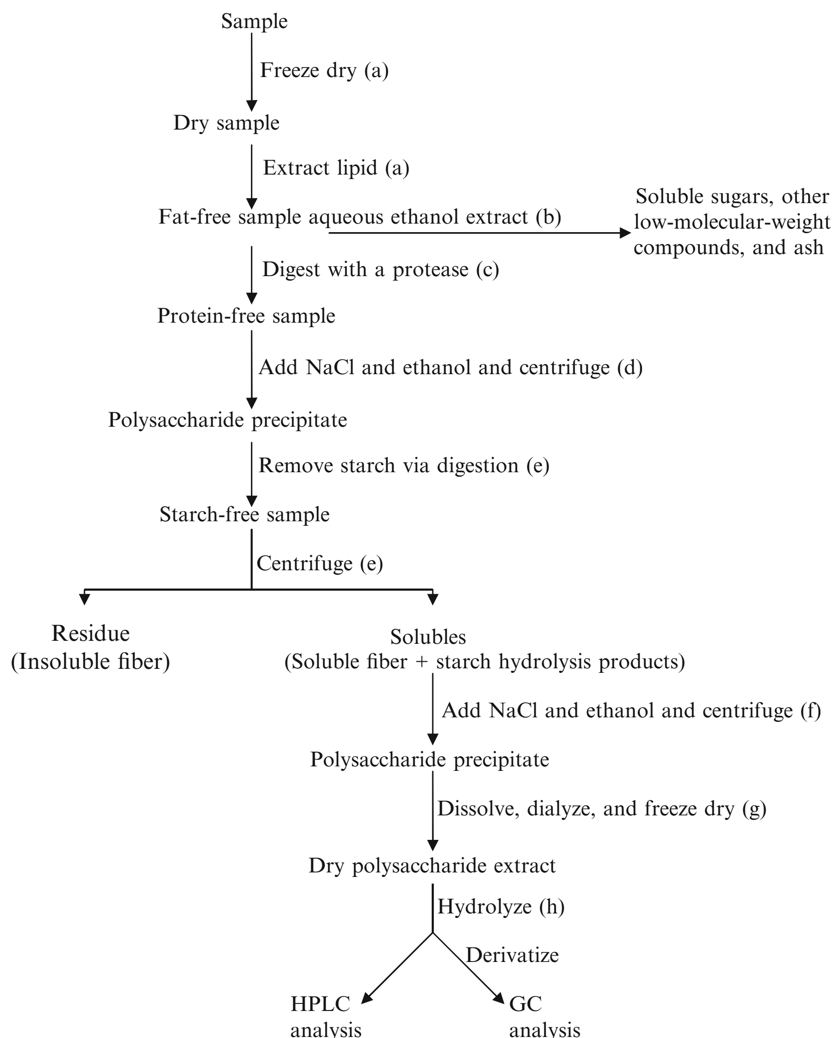
(b) Although not in the published scheme, soluble sugars, other low-molecular-weight compounds, and ash can be removed at this point using hot 80% ethanol as described in Sect. 10.3.1. (Hot 80% methanol has also been used.)

(c) Protein is removed by enzyme-catalyzed hydrolysis. The cited procedure (41) uses papain as the protease. Bacterial alkaline proteases are recommended by some because carbohydrases have acidic pH optima. However, one must always be aware of the fact that commercial enzyme preparations, especially those from bacteria or fungi, almost always have carbohydrase activities in addition to proteolytic activity. In this procedure, proteins are denatured for easier digestion by dispersion of the sample in sodium acetate buffer, pH 6.5, containing sodium chloride and heating the mixture. Papain [activated by dispersing it in sodium acetate buffer, pH 6.5, containing cysteine and ethylenediaminetetraacetic acid (EDTA)] is added to the sample, and the mixture is incubated.

(d) Any solubilized polysaccharides are precipitated by addition of sodium chloride to the cooled dispersion, followed by the addition of four volumes of absolute ethanol (to give an ethanol concentration of 75%). The mixture is centrifuged.

(e) The pellet is suspended in acetate buffer, usually pH 4.5. To this suspension is added a freshly prepared solution of glucoamylase (amyloglucosidase) in the same buffer. This suspension is then incubated. Just as in the analysis of starch, highly purified enzyme must be used to minimize hydrolytic breakdown of other polysaccharides (Sect. 10.4.1.1.2). This step may be omitted if it is known that no starch is present. Centrifugation after removal of starch isolates and removes insoluble fiber (cellulose, some hemicelluloses, lignin) (Sect. 10.5).

The presence of starch can be tested for by adding a solution of iodine and potassium iodide and observing the color. A color change to blue or brownish-red indicates the presence of starch. A microscope may be used to look for stained intact or swollen granules or granule



10-11 figure Flow diagram for isolation and analysis of polysaccharides.

fragments (Sect. 10.6.1). However, unless a definite blue color appears, the test may be inconclusive. A better check is to analyze the ethanol-soluble fraction from step (f) for the presence of glucose (Sect. 10.3.4). If no glucose is found, the starch digestion part of step (e) may be omitted in future analyses of the same product.

(f) Solubilized polysaccharides are reprecipitated by addition of sodium chloride to the cooled dispersion, followed by the addition of four volumes of absolute ethanol (to give an ethanol concentration of 75%). The mixture is centrifuged. The precipitate (pellet) of water-soluble polysaccharides (often added hydrocolloids/food gums) is soluble dietary fiber (Sect. 10.5).

(g) The pellet is suspended in deionized water, transferred to dialysis tubing, and dialyzed against frequent changes of sodium azide solution (used to prevent microbial growth). Finally, dialysis against deionized water is done to remove the sodium azide. The retentate is recovered from the dialysis tubing and freeze dried.

(h) Polysaccharide identification relies on hydrolysis to constituent monosaccharides and identification of these sugars (Sect. 10.3.4). For hydrolysis, polysaccharide material is added to a Teflon-lined, screw-capped vial. Trifluoroacetic acid solution is added (usually 2 *M*), and the vial is tightly capped and heated (usually for 2 h at 120°C). After cooling, the contents are evaporated to dryness in a hood with a stream of air or nitrogen. Then, sugars are determined by HPLC (Sect. 10.3.4.1) or GC (Sect. 10.3.4.2). If GC is used, inositol is added as an internal standard. Qualitative and quantitative analysis of the polysaccharides present

can be determined by sugar analysis. For example, guaran, the polysaccharide component of guar gum, yields D-mannose and D-galactose in an approximate molar ratio of 1.00:0.56.

The described acid-catalyzed hydrolysis procedure does not release uronic acids quantitatively. The presence of **uronic acids** can be indicated by either the modified **carbazole assay** (55, 56), the ***m*-hydroxydiphenyl assay** (11, 57, 58), or the **3,5-dimethylphenol assay** (58). All three methods are based on the same principle as the phenol-sulfuric acid assay (Sect. 10.3.2) (i.e., condensation of dehydration products with a phenolic compound to produce colored compounds that can be measured quantitatively by means of spectrophotometry).

10.4.2.3 Pectin

10.4.2.3.1 Nature of Pectin Even though pectin is a very important food polysaccharide, no official methods for its determination have been established. What few methods have been published basically involve its precipitation (by addition of ethanol) from jams, jellies, etc. in which it is the only polysaccharide present.

Even the definition of pectin is somewhat ambiguous. What may be called "**pectin**" in a native fruit or vegetable is a complex mixture of polysaccharides whose structures depend on the source, including the stage of development (degree of ripeness) of the particular fruit or vegetable. Generally, much of this native material can be described as a main chain of α-D-galactopyranosyluronic acid units (some of which are in the methyl ester form) interrupted by L-rhamnopyranosyl units (1, 2). Many of the rhamnosyl units have arabinan, galactan, or arabinogalactan chains attached to them. Other sugars, such as D-apiose, also are present. In the manufacture of commercial pectin, much of the neutral sugar part is removed. Commercial pectin is, therefore, primarily poly(α-D-galacturonic acid methyl ester) with various degrees of esterification and sometimes amidation.

Enzyme action during development/ripening or during processing can partially deesterify and/or depolymerize native pectin. These enzyme-catalyzed reactions are important determinants of the stability of fruit juices, tomato sauce, tomato paste, apple butter, etc. in which some of the texture/body is supplied by pectin and its interaction with calcium ions. It is probable that the fact that pectin is not a single substance has precluded development of methods for its determination (see also Sect. 10.5.2.1.3).

10.4.2.3.2 Pectin Content Determination The constant in pectins is **D-galacturonic acid** as the principal component (often at least 80%). However, glycosidic linkages of uronic acids are difficult to hydrolyze without decomposition, so methods involving acid-catalyzed hydrolysis to release D-galacturonic acid and chromatography are generally not applicable.

One method employed for pectin uses saponification in sodium hydroxide solution, followed by acidification, and addition of Ca^{2+} to precipitate the pectin. **Calcium pectate** is collected, washed, dried, and measured gravimetrically. Precipitation with the quaternary ammonium salt cetylpyridinium bromide has been used successfully because there is a much lower critical electrolyte concentration for its salt formation with pectin than with other acidic polysaccharides (60), and because pectin and other acidic polysaccharides are not likely to be found together. For a review of methods for determination of pectin, see references (61, 62).

Because of the dominance of D-galacturonic acid in its structure, pectins are often determined using the **carbazole** or ***m*-hydroxydiphenyl methods** (Sect. 10.4.2.2). Isolation of crude pectin usually precedes analysis.

10.4.2.3.3 Degree of Esterification The **degree of esterification** (DE) is a most important parameter in both natural products and added pectin. DE may be measured directly by titration before and after saponification. First, the isolated pectin (Sect. 10.4.2.2) is washed with acidified alcohol to convert carboxylate groups into free carboxylic acid groups and then washed free of excess acid. Then, a dispersion of the pectinic acid in water is titrated with dilute base, such as standardized sodium hydroxide solution, to determine the percentage of nonesterified carboxyl ester groups. Excess base is added to saponify the methyl ester groups. Back-titration with standardized acid to determine excess base following saponification gives the DE. Also, DE can be determined by measuring methanol released by saponification via GC (63) and by nuclear magnetic resonance (NMR) (see Chap. 25) (64, 65).

10.5 DIETARY FIBER

10.5.1 Introduction

Although there is an ongoing discussion about what constitutes dietary fiber within both domestic and international organizations (66), **dietary fiber** is essentially the sum of the nondigestible components of a foodstuff or food product. Most, but not all, dietary fiber is plant cell-wall material (cellulose, hemicelluloses, lignin) and thus is composed primarily of

polysaccharide molecules (see Sect. 10.5.1.2 for definitions of dietary fiber). Because only the amylose and amylopectin molecules in cooked starch are digestible (Sect. 10.4.1.2), all other polysaccharides are also components of dietary fiber. Some are components of insoluble fiber; some make up soluble fiber. **Insoluble dietary fiber** components are cellulose, microcrystalline cellulose added as a food ingredient, lignin, hemicelluloses entrapped in a lignocellulosic matrix, and resistant starch (Sect. 10.4.1.1.2). Other polysaccharides, including many, but not all, hemicelluloses not entrapped in a lignocellulosic matrix, much of the native pectin, and the majority of hydrocolloids/food gums (Sect. 10.4.2), are classified as **soluble dietary fiber**. Often, their determination is important in terms of making food label claims and is described in Sect. 10.4.2. Determination of the β-glucan content of products made with oat or barley flours is an example. (Nondigestible protein is not considered to be a significant contributor to dietary fiber.)

Since the scheme presented in Fig. 10-11 is designed to separate nonstarch, water-soluble polysaccharides from other components for quantitative and/or qualitative analysis, the pellet from the centrifugation step (e) is insoluble fiber, and those components precipitated from the supernatant with alcohol [step (f)] constitute soluble fiber; but specific fiber determination methods have been established and are presented in Sect. 10.5.4.3.

Measurement of insoluble fiber is important not only in its own right, but also for calculating the caloric content of a food. According to nutrition labeling regulations, one method allowed to calculate calories involves subtracting the amount of insoluble dietary fiber from the value for total carbohydrate, before calculating the calories based on protein, fat, and carbohydrate content (approximately 4, 9, and 4 Calories per gram, respectively) (Chap. 3). This method ignores the fact that soluble fiber, like insoluble fiber, is also essentially noncaloric. [Fiber components can contribute calories via absorption of products of fermentation (mostly short-chain fatty acids) from the colon].

10.5.1.1 Importance of Dietary Fiber

In 1962, it was postulated that the prevalence of heart disease and certain cancers in Western societies was related to inadequate consumption of dietary fiber (67). Much research has been done since then to test the fiber hypothesis. While the research has not always produced consistent results, it is clear that adequate consumption of dietary fiber is important for optimum health.

Adequate consumption of dietary fiber from a variety of foods will help protect against colon cancer and also help to keep blood lipids within the normal range, thereby reducing the risk of obesity, hypertension, and cardiovascular disease in general. Certain types of fiber can slow D-glucose absorption and reduce insulin secretion, which is of great importance for diabetics and probably contributes to the well-being of nondiabetics as well. Fiber helps prevent constipation and diverticular disease. However, dietary fiber is not a magic potion that will correct or prevent all diseases. Rather, dietary fiber is an essential component of a well-balanced diet that will help minimize some common health problems. References (68–73) provide an extensive compilation of articles related to the physiological action of dietary fiber.

The Dietary Reference Intake (DRI) value for dietary fiber to promote optimal health has been set at 25 g per 2000 kcal per day. However, dietary fiber includes a variety of materials that in turn produce a variety of physiological actions (68–73). For example, the pentosan fraction of dietary fiber seems to be most beneficial in preventing colon cancer and reducing cardiovascular disease. Pectin and the hydrocolloids are most beneficial in slowing glucose absorption and in lowering insulin secretion. A mixture of hemicellulose and cellulose will help prevent diverticulosis and constipation.

Recognition of the importance of dietary fiber and of the fact that certain physiologic effects can be related to specific fiber components has led to the emergence of a number of methodologies for determining dietary fiber.

10.5.1.2 Definition

Because labeling of food products for dietary fiber content is required, an official analytical method(s) for its determination is required. The first step in adopting a method must be agreement on what constitutes dietary fiber. Then, there must be a method that measures what is included in the definition. A definition and a method related to it are also needed: (a) to determine the dietary fiber content of any new ingredient such as new resistant or slowly digesting starch products, and (b) to ensure that scientific studies of the physiological effects of dietary fiber are based on the same measure of dietary fiber content.

Following extensive international consultation, the American Association of Cereal Chemists (now AACC International) adopted the following definition in 2001 (74–78). "**Dietary fiber** is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small

intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fiber promotes beneficial physiological effects, such as laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation."

No polysaccharide other than starch is digested in the human small intestine; so all polysaccharides other than nonresistant starch are included in this definition of fiber. Of the oligosaccharides, only sucrose, lactose, and those derived from starch (maltooligosaccharides/maltodextrins) are digested. The term **analogous carbohydrates** is defined as those carbohydrate-based food ingredients that are nondigestible and nonabsorbable, but are not natural plant components. Wax (suberin and cutin) is included within associated substances. The definition also includes some of the health benefits known to be associated with ingestion of dietary fiber.

Since adoption of the definition, modified versions have been adopted by both governmental and nongovernmental organizations around the world. However, there is yet no consensus of national and international organizations as to a definition (66). One reason that formulating a definition acceptable to all is so difficult is that dietary fiber materials from different sources are often different mixtures of nondigestible and nonabsorbable carbohydrates and other substances with different effects on human physiology. However, there is general agreement that dietary fiber consists of oligo- and polysaccharides, lignin, and other substances not digested by digestive enzymes in the human stomach or small intestine.

10.5.2 Major Components of Dietary Fiber

The major components of natural dietary fiber are **cellulose**, **hemicelluloses**, **lignin**, and **other nonstarch plant polysaccharides** such as pectin. In a food product, added hydrocolloids/food gums, resistant starch, and certain oligosaccharides such as those derived from inulin are included because they are also nondigestible and provide certain of the physiological benefits of dietary fiber. An example is polydextrose, which is often, but not always, used in product formulations specifically because it is considered to be soluble dietary fiber.

10.5.2.1 Cell-Wall Polysaccharides of Land Plants

10.5.2.1.1 Cellulose Cellulose is a linear polymer of β-D-glucopyranosyl units (1). Some molecules may contain 10,000 or more glucosyl units. Hydrogen bonding between parallel polymers forms strong microfibrils. Cellulose microfibrils provide the strength and rigidity required in primary and secondary plant cell walls.

10.5.2.1.2 Hemicelluloses Hemicelluloses are a heterogeneous group of polysaccharides, the only similarity between them being their association with cellulose in plant cell walls (1). Units of D-xylose, D-mannose, and D-galactose frequently form the main-chain structures of hemicelluloses; units of L-arabinose, D-galactose, and uronic acids are often present as branch units or in side chains. Hemicelluloses may be soluble or insoluble in water. Molecular sizes and degrees of branching vary widely.

10.5.2.1.3 Pectins What food scientists generally call pectin (Sect. 10.4.2.3) is (like the hemicelluloses) a family of polysaccharides, although in this case there is structural similarity. The main feature of all commercial pectins is a linear chain of 1,4-linked α-D-galactopyranosyluronic acid units. Interspersed segments of neutral sugar units may be branched, sometimes with other polysaccharides. The carboxylic acid groups of the D-galacturonic acid units are often in the methyl ester form. When present primarily in a calcium and/or magnesium salt form, they are generally water insoluble and extractable only with dilute solutions of acid, chelators such as EDTA, or ammonium oxalate. These molecules are present in the middle lamella of plant tissues.

10.5.2.2 Hydrocolloids/Food Gums as Dietary Fiber

As mentioned in Sect. 10.5.1, all polysaccharides other than those in cooked starch are nondigestible and, therefore, classified as dietary fiber. Therefore, those polysaccharides classified as food gums or hydrocolloids (Sect. 10.4.2) fall within the definition of dietary fiber. Those obtained from marine algae (alginates and carrageenans) and certain of those from higher land plants (cellulose, the hemicelluloses, and the pectic polysaccharides) are cell-wall or middle lamella structural components. Others are either nonstructural plant polysaccharides (guar gum, locust bean gum, and inulin) or bacterial polysaccharides (xanthan and gellan), but neither do we humans have small intestinal enzymes that can digest them.

10.5.2.3 Resistant Starch

See Sect. 10.4.1.1.2. Resistant starch content in a food or food ingredient can be determined using AOAC Method 2002.02 (AACC Method 32-40.01).

10.5.2.4 Lignin

Lignin is a noncarbohydrate, three-dimensional, water-insoluble polymer and a major component of the cell walls of higher land plants (79). Lignin may be covalently linked to hemicellulose.

10.5.3 General Considerations

Fiber components or subfractions of them are usually not distinct entities, rather their compositions are methodology dependent. Although considerable progress has been made in relating fiber composition to physiological effects, much remains to be learned, and improving the nutritional value of foods by adding fiber or modifying resistant starch content remains a challenge for the food scientist.

10.5.4 Methods

10.5.4.1 Overview

Dietary fiber is often determined **gravimetrically**. In such a procedure, digestible carbohydrates, lipids, and proteins are selectively solubilized by chemicals or removed by enzyme-catalyzed hydrolysis. Then, nonsolubilized and/or nondigested materials are collected by filtration, and the fiber residue is recovered, dried, and weighed.

The food component that may be most problematic in fiber analysis is **starch**. In any method for determination of dietary fiber, it is essential that all digestible starch be removed, for incomplete removal of digestible starch increases the residue weight and inflates the estimate of fiber. [Resistant starch (Sect. 10.4.1.1.2) is a component of dietary fiber.]

Alpha-amylase, debranching enzymes, and glucoamylase (amyloglucosidase) are enzymes used in starch analysis (76). **α-Amylase** catalyzes hydrolysis of unbranched segments of 1,4-linked α-D-glucopyranosyl units forming primarily maltooligosaccharides composed of 3–6 units. **Debranching enzymes** (both pullulanase and isoamylase are used) catalyze hydrolysis of the 1,6 linkages that constitute the branch points and thereby produce short linear molecules. **Glucoamylase** (amyloglucosidase) starts at the nonreducing ends of starch chains and releases D-glucose, one unit at a time; it will catalyze hydrolysis of both 1, 4 and 1, 6 α-D-glucosyl linkages.

All fiber methods include a heating step (95–100°C for 35 min) to **gelatinize starch granules** and make them susceptible to hydrolysis. Resistant starch molecules (Sect. 10.4.1.1.2) remain unhydrolyzed and, therefore, are usually measured as dietary fiber, but not all nondigestible products made from starch may be determined as dietary fiber by the approved methods.

Nondigestible oligosaccharides such as those derived from inulin and certain specially prepared maltodextrins also are problematic in an analytical sense since they are in the soluble portion that is not precipitated with ethanol.

It is essential either that all digestible materials be removed from the sample so that only nondigestible polysaccharides remain or that the nondigestible residue be corrected for remaining digestible contaminants. **Lipids** are removed easily from the sample with organic solvents (Sect. 10.5.4.2) and generally do not pose analytical problems for the fiber analyst. **Protein** and **minerals** that are not removed from the sample during the solubilization steps should be corrected for by Kjeldahl nitrogen analysis (Chap. 9) and by ashing (Chap. 7) portions of the fiber residue.

Because labeling of dietary fiber content is required, because dietary fiber is a complex heterogeneous material containing several substances with different solubilities and other properties, and because of its physiological importance, methods for fiber determination continue to be researched and refined (76,77).

10.5.4.2 Sample Preparation

Measures of fiber are most consistent when the samples are low in fat (less than 10% lipid), dry, and finely ground. If necessary, the sample is ground to pass through a 0.3–0.5-mm mesh screen. If the sample contains more than 10% lipid, the lipid is removed by extraction with 25 parts (vol/wt) of petroleum ether or hexane in an ultrasonic water bath. The mixture is then centrifuged and the organic solvent decanted. This extraction is repeated. The sample is air dried to remove the organic solvent. It may then be dried overnight in a vacuum oven at 70°C if a measure of lipid and moisture content is required. Loss of weight due to fat and moisture removal is recorded, and the necessary correction is made in the calculation of the percentage dietary fiber value determined in the analysis.

If samples contain large amounts of soluble sugars (mono-, di-, and trisaccharides), they should be extracted three times with 80% aqueous ethanol in an ultrasonic water bath at room temperature for 15 min. The supernatant liquid is discarded and the residue is dried at 40°C.

Nonsolid samples with less than 10% fiber are best analyzed after freeze drying. Nonsolid samples with greater than 10% fiber can be analyzed without drying if the sample is homogeneous and low in fat and if particle size is sufficiently small to allow efficient removal of digestible carbohydrate and protein.

10.5.4.3 Methods

10.5.4.3.1 Overview A variety of methods have been developed and used at different times for different products. *AOAC International Official Methods of Analysis* in reference (5) and *AACC International Approved Methods* in reference (80) are listed in Table 10-4. It is obvious from the list that methods are generally specific for the type of fiber or the fiber component desired to be measured. For example, when inulin (a fructan) or its breakdown products (fructooligosaccharides, FOS) are added to food products, not all of the inulin and perhaps none of the FOS are precipitated by addition of four volumes of alcohol (because of their low molecular weights) and measured as soluble dietary fiber, although both inulin and FOS undergo fermentation in the colon and are, therefore, components of dietary fiber. As a result special methods have been designed for them. The same is true of polydextrose and resistant maltodextrins. In other cases, determination of a specific component of dietary fiber, such as β-glucan and resistant starch, may be desired. The most widely used general method for total, soluble, and insoluble dietary fiber (AOAC Method 991.43, AACC Method 32-07.01) is outlined below. Table 10-5 gives the fiber content of select foods analyzed by this method.

10.5.4.3.2 AOAC Method 991.43 (AACC Method 32-07.01) This method determines soluble, insoluble, and total dietary fiber in cereal products, fruits and vegetables, processed foods, and processed food ingredients.

1. **Principle**. Starch and protein are removed from a sample by treating the sample sequentially with a thermostable α-amylase, a protease, and glucoamylase

table 10-4 Official Methods of Analysis for Dietary Fiber

AOAC Method No. (5)	*AACC Method No. (80)*	*Description of Method and Measured Substance*
994.13	32-25.01	Total dietary fiber determined as neutral sugar and uronic acid monomer units and Klason lignin by a gas chromatographic–spectrophotometric–gravimetric method
993.21		Nonenzymic-gravimetric method for total dietary fiber applicable to determination of >10% TDF in foods and food products with <2% starch
985.29	32-05.01	Enzymic-gravimetric method for total dietary fiber in cereal grains and cereal grain-based products
	32-06.01	A rapid gravimetric method for total dietary fiber
991.42		Enzymic-gravimetric method for insoluble dietary fiber in vegetables, fruits, and cereal grains
993.19		Enzymic-gravimetric method for soluble dietary fiber
991.43	32-07.01	Enzymic-gravimetric method for total, soluble, and insoluble dietary fiber in grain and cereal products, processed foods, fruits, and vegetables
2002.02	32-40.01	Enzymic method for RS2 and RS3 in products and plant materials
	32-21.01	Enzymic-gravimetric method for insoluble and soluble dietary fiber in oats and oat products
	32-32.01	Enzymic-spectrophotometric method for total fructan (inulin and fructooligosaccharides) in foods
993.03		Enzymic-spectrophotometric method for fructan (inulin) in foods (not applicable to fructooligosaccharides)
997.08	32-31.01	Anion-exchange chromatographic method for fructan in foods and food products applicable to the determination of added inulin in processed foods
2000.11	32-28.01	Anion-exchange chromatographic method for polydextrose in foods
	32-22.01	Enzymic method for β-glucan in oat fractions and unsweetened oat cereals
	32-23.01	Rapid enzymic procedure for β-glucan content of barley and oats
2001.03	32-41.01	Enzymic-gravimetric and liquid chromatographic method for dietary fiber containing added resistant maltodextrin
2001.02	32-33.01	Anion-exchange chromatographic method for *trans*-galactooligosaccharides (TGOS) applicable to added TGOS in selected food products

table 10-5 Total, Soluble, and Insoluble Dietary Fiber in Foods as Determined by AOAC Method 991.43[a]

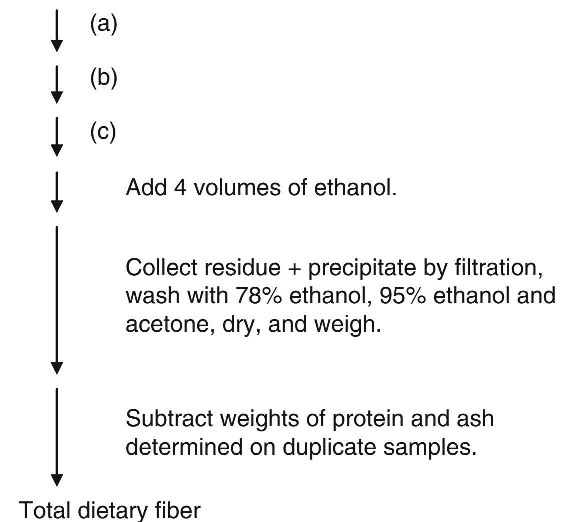
Food	*Soluble[b]*	*Insoluble[b]*	*Total[b]*
Barley	5.02	7.05	12.14
High-fiber cereal	2.78	30.52	33.30
Oat bran	7.17	9.73	16.90
Soy bran	6.90	60.53	67.56
Apricots	0.53	0.59	1.12
Prunes	5.07	4.17	9.37
Raisins	0.73	2.37	3.03
Carrots	1.10	2.81	3.92
Green beans	1.02	2.01	3.03
Parsley	0.64	2.37	3.01

[a]Adapted from *Official Methods of Analysis*, 18th edn. Copyright 2005 by AOAC International.
[b]Grams of fiber per 100 g of food on a fresh weight basis.

(amyloglucosidase). The insoluble residue is recovered and washed (**insoluble dietary fiber**). Ethanol is added to the soluble portion to precipitate soluble polysaccharides (**soluble dietary fiber**). To obtain **total dietary fiber** (TDF), the alcohol is added after digestion with the glucoamylase, and the soluble and insoluble dietary fiber fractions are collected together, dried, weighed, and ashed.

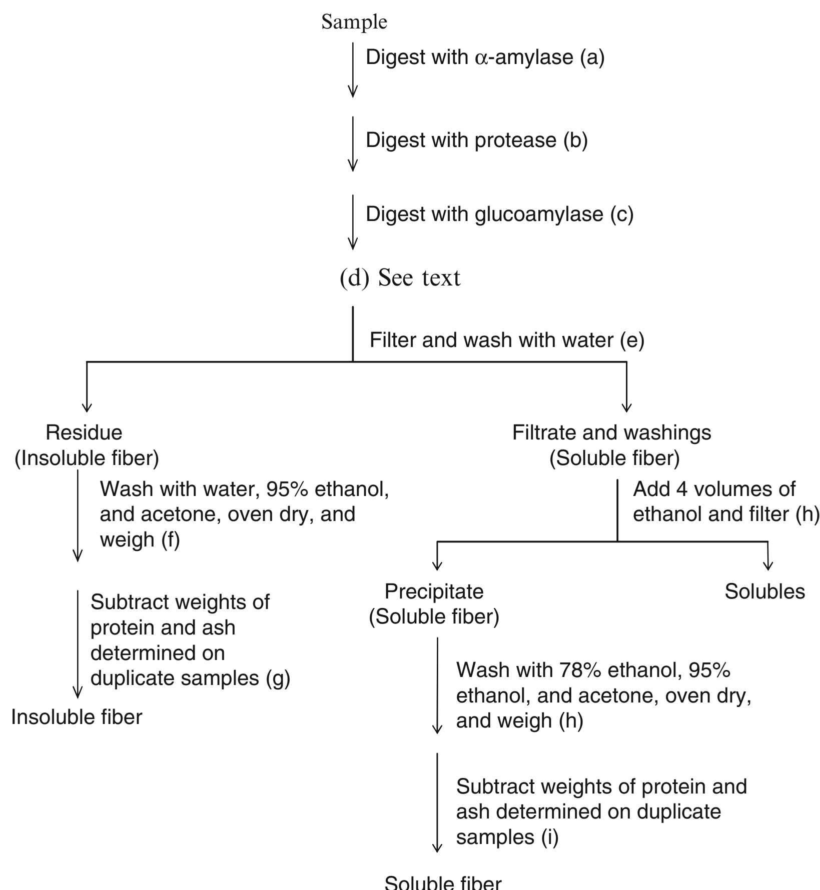
2. **Outline of procedure**. A flow diagram outlining the general procedure for the method is given in Fig. 10-12. Letters in the parentheses refer to the same letters in Fig. 10-12. If necessary, lipids are removed by extraction (Sect. 10.5.4.2).

(a) To samples devoid of significant lipid solvent-soluble substances is added 2-(*N*-morpholino)ethanesulfonic acid-tris(hydroxymethyl)aminomethane (MES-TRIS) buffer (0.05 *M* each, pH 8.2) and a thermostable α-amylase. The mixture is heated 35 min at 95–100°C to gelatinize any starch so that the α-amylase can break it down.
(b) After cooling to 60°C, a protease is added, and the mixture is incubated at 60°C for 35 min to break down the protein.
(c) The pH is adjusted to 4.1–4.8, glucoamylase is added, and the mixture is incubated at 60°C for 30 min to complete the digestion of any starch.
(d) To determine TDF, four volumes of 95% ethanol are added. The residue plus precipitate is collected by filtration, washed with 78% ethanol, 95% ethanol, and acetone in that order, dried, and weighed (see below). Protein and ash are determined on duplicate samples and the weight is corrected for them. Alternatively, TDF can be calculated as the sum of the insoluble and soluble dietary fiber determined in the remainder of the procedure.



(e) The mixture obtained after step (c) is filtered through a crucible containing fritted glass disk and preashed Celite (a siliceous filter aid).
(f) The residue is washed with water, 95% ethanol, and acetone in that order, dried, and weighed.
(g) The dried residue is analyzed for protein using the Kjeldahl method (Chap. 9). A duplicate residue is analyzed for ash (Chap. 7). The weights of protein and ash are subtracted from the residue weight obtained in step (f) to determine insoluble dietary fiber.
(h) To determine soluble dietary fiber, to the filtrate and washings from steps (e) and (f) at 60°C are added four volumes of 95% ethanol (to give an ethanol concentration of 76%). The precipitate is collected by filtration through a crucible containing a fritted glass disk and preashed Celite. The residue is washed with 78% ethanol, 95% ethanol, and acetone. The crucible is dried at 103°C and weighed.
(i) Protein and ash are determined as in step (f) and the weights of protein and ash are subtracted from the residue weight obtained in step (h) to determine soluble dietary fiber. Total dietary fiber may be determined as described in (d) or obtained by adding the values for insoluble (g) and soluble (i) dietary fiber.

Duplicate reagent blanks must be run through the entire procedure for each type of fiber determination. Table 10-6 shows a sample and blank sheet



10-12 figure Flow diagram of AOAC Method 991.43 (AACC International Method 32-07.01) for determining soluble, insoluble, and total dietary fiber.

used to calculate fiber percentages. Using the equations shown, percent dietary fiber is expressed on a dry weight basis if the sample weights are for dried samples. If it is believed that resistant starch is present, it can be determined separately using AOAC Method 2002.02 (AACC Method 32-40.01).

10.6 PHYSICAL METHODS

10.6.1 Microscopy

Microscopy can be a valuable tool in food analysis. Various kinds of microscopy [light, fluorescence, confocal scanning laser (CSLM), Fourier transform infrared (FTIR), scanning electron (SEM), and transmission electron (TEM) microscopies] have been used to study the organization of food products and the stability of emulsions and foams and to identify extraneous matter and its amount (Chap. 19). Microscopy is particularly useful in examinations of starchy foods. Granule size, shape, and form, the birefringence endpoint temperature determined using a polarizing microscope with a hot stage, and, in some cases, iodine-staining characteristics can be used to identify the starch source (81). In cooked starch products, the extent of retrogradation (82) and the effects of storage on microstructure have been evaluated by iodine staining and light microscopy (83–89). The degree that starch has been damaged mechanically during dry milling (90), the extent of digestion by enzymes, and whether the starch-based product has been overcooked, undercooked, or correctly cooked also can be determined microscopically. Quantitative microscopy has been employed for analysis of the nonstarch polysaccharides of cereal grains (91).

10.6.2 Mass and NIR Transmittance Spectrometry

Mass and NIR transmittance spectrometry have been used to determine sugar content (92). NIR spectrometry is described in Chap. 23. Mass spectrometry is mentioned in Sect. 10.3.4.4.

10-6 table **Dietary Fiber Data Sheet**[a]

	Sample				*Blank*			
	Insoluble Fiber		*Soluble Fiber*		*Insoluble Fiber*		*Soluble Fiber*	
Sample wt (mg)	m_1	m_2						
Crucible + Celite wt (mg)								
Crucible + Celite + residue wt (mg)								
Residue wt (mg)	R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2
Protein (mg) P								
Crucible + Celite + ash wt (mg)								
Ash wt (mg) A								
Blank wt (mg) B[b]								
Fiber (%)[c]								

[a]Adapted with permission from J AOAC Int (1988), 71:1019. Copyright 1988 by AOAC International.

[b]$\text{Blank(mg)} = \frac{R_1 + R_2}{2} - P - A$

[c]$\text{Fiber(\%)} = \frac{\frac{R_1+R_2}{2} - P - A - B}{\frac{m_1+m_2}{2}} \times 100$

10.6.3 Specific Gravity

Specific gravity is defined as the ratio of the density of a substance to the density of a reference substance (usually water), both at a specified temperature. The concentration of a carbohydrate solution can be determined by measuring the specific gravity of the solution, then referring to appropriate specific gravity tables (11).

Measurement of specific gravity as a means of determining sugar concentration is accurate only for pure sucrose or other solutions of a single pure substance (AOAC Method 932.14), but it can be, and is, used for obtaining approximate values for liquid products (Chap. 6). Two basic means of determining specific gravity are used. By far the most common is use of a hydrometer calibrated either in °**Brix**, which corresponds to sucrose concentrations by weight, or in **Baumé Modulus** (Bé). The values obtained are converted into concentrations by use of tables constructed for the substance in the pure solution, e.g., sucrose or glucose syrups.

10.6.4 Refractive Index

When electromagnetic radiation passes from one medium to another, it changes direction (i.e., is bent or refracted). The ratio of the sine of the angle of incidence to the sine of the angle of refraction is termed the **refractive index** (RI). The RI varies with the nature of the compound, the temperature, the wavelength of light, and the concentration of the compound. By holding the first three variables constant, the concentration of the compound can be determined by measuring the RI. Thus, measurement of refractive index is another way to determine total solids in solution (Chap. 6). Like determination of specific gravity, use of RI to determine concentrations is accurate only for pure sucrose or other solutions of a single pure substance, and also like the determination of specific gravity, it is used for obtaining approximate sugar concentrations in liquid products (11). In this case, the solution must be clear. Refractometers that read directly in sucrose units are available.

10.7 SUMMARY

For determination of low-molecular-weight carbohydrates, older colorimetric methods for total carbohydrate, various reducing sugar methods, and physical measurements have largely been replaced by chromatographic methods. The older chemical methods suffer from the fact that they are not stoichiometric and, therefore, require standard curves. This makes them particularly problematic when a mixture of sugars is being determined. Physical measurements are not specific for carbohydrates. Chromatographic methods (HPLC and GC) separate mixtures into the component sugars, identify each component by retention time, and provide a measurement of the mass of each component. Enzymic methods are specific and sensitive, but seldom, except in the case of starch, is determination of only a single component desired. HPLC is widely used for identification and measurement of mono- and oligosaccharides.

Polysaccharides are important components of many food products. Yet there is no universal procedure for their analysis. Generally, isolation must precede measurement. Isolation introduces errors because

no extraction or separation technique is stochiometric. Identification and measurement are done by hydrolysis to constituent monosaccharides and their determination. An exception is starch, which can be digested to glucose using specific enzymes (amylases), followed by measurement of the glucose released.

Insoluble dietary fiber, soluble dietary fiber, and total dietary fiber are each composed primarily of nonstarch polysaccharides. The method for the determination of starch is based on its complete conversion to, and determination of, D-glucose. Methods for the determination of total dietary fiber and its components rely on removal of the digestible starch in the same way and often on removal of digestible protein with a protease, leaving nondigestible components.

10.8 STUDY QUESTIONS

1. Give three reasons why carbohydrate analysis is important.
2. "Proximate composition" refers to analysis for moisture, ash, fat, protein, and carbohydrate. Identify which of these components of "proximate composition" are actually required on a nutrition label. Also, explain why it is important to measure the nonrequired components quantitatively if one is developing a nutrition label.
3. Distinguish chemically between monosaccharides, oligosaccharides, and polysaccharides, and explain how solubility characteristics can be used in an extraction procedure to separate monosaccharides and oligosaccharides from polysaccharides.
4. Discuss why mono- and oligosaccharides are extracted with 80% ethanol rather than with water. What is the principle involved?
5. Define reducing sugar. Classify each of the following as a reducing or nonreducing carbohydrate: D-glucose, D-fructose (Conditions must be described. Why?), sorbitol, sucrose, maltose, raffinose, maltotriose, cellulose, amylopectin, κ-carrageenan.
6. Briefly describe a method that could be used for each of the following:
   (a) To prevent hydrolysis of sucrose when sugars are extracted from fruits via a hot alcohol extraction
   (b) To remove proteins from solution for an enzymic analysis
   (c) To measure total carbohydrate
   (d) To measure total reducing sugars
   (e) To measure the sucrose concentration in a pure sucrose solution by a physical method
   (f) To measure glucose enzymically
   (g) To measure simultaneously the concentrations of individual free sugars
7. What are the principles behind total carbohydrate determination using the phenol-sulfuric acid method? Give an example of another assay procedure based on the same principle.
8. What is the principle behind determination of total reducing sugars using the Somogyi–Nelson and similar methods?
9. The Munson–Walker, Lane–Eynon, and Somogyi–Nelson methods can be used to measure reducing sugars. Explain the similarities and differences among these methods with regard to the principles involved and the procedures used.
10. Describe the principle behind AE-HPLC of carbohydrates.
11. Describe the general procedure for preparation of sugars for GC. What is required for this method to be successful?
12. What difference is there between the preparation of an extract of reducing sugars for GC and the preparation of polysaccharide hydrolyzates containing uronic acids for GC? What two differences are there in the final derivatives?
13. Why has HPLC largely replaced GC for analysis of carbohydrates?
14. Compare and contrast RI and PAD detectors.
15. What is the advantage of an enzymic method? What is the limitation (potential problem)?
16. Describe the principles behind the enzymic determination of starch. What are the advantages of this method? What are potential problems?
17. Describe the principle behind each step in Fig. 10-11. What is the reason for each step?
18. Describe the principles behind separation and analysis of water-soluble gums and starch.
19. Describe two methods for determination of pectin.
20. Describe the principles behind and the limitations of determining sugar (sucrose) concentrations by (a) specific gravity determination and (b) RI measurement.
21. Define dietary fiber.
22. List the major constituents of dietary fiber.
23. Explain how measurement of dietary fiber relates to calculating the caloric content of a food product.
24. Explain the purpose(s) of each of the steps in the AOAC Method 994.13 for total dietary fiber listed below as applied to determination of the dietary fiber content of a high-fiber snack food.
    (a) Heating sample and treating with α-amylase
    (b) Treating sample with glucoamylase
    (c) Treating sample with protease
    (d) Adding four volumes of 95% ethanol to sample after treatment with glucoamylase and protease
    (e) After drying and weighing the filtered and washed residue, heating one duplicate final product to 525°C in a muffle furnace and analyzing the other duplicate sample for protein.
25. What is the physiological definition and the chemical nature of resistant starch? What types of foods have relatively high levels of resistant starch?

10.9 PRACTICE PROBLEMS

1. The following data were obtained when an extruded breakfast cereal was analyzed for total fiber by AOAC Method 991.43 (AACC Method 32-07).

Sample wt (mg)	1002.8
Residue wt (mg)	151.9
Protein wt (mg)	13.1
Ash wt (mg)	21.1
Blank wt (mg)	6.1
Resistant starch (mg)	35.9

What is percent total fiber (a) without and (b) with correction for resistant starch, determined to the appropriate number of significant figures?

2. The following tabular data were obtained when a high-fiber cookie was analyzed for fiber content by AOAC Method 991.43 (AACC Method 32-07).

	Sample			
	Insoluble		*Soluble*	
Sample wt (mg)	1002.1	1005.3		
Crucible + Celite wt (mg)	31,637.2	32,173.9	32,377.5	33,216.4
Crucible + Celite + residue wt (mg)	31,723.5	32,271.2	32,421.6	33,255.3
Protein (mg)	6.5		3.9	
Crucible + Celite + ash wt (mg)		32,195.2		33,231.0

	Blank			
	Insoluble		*Soluble*	
Crucible + Celite wt (mg)	31,563.6	32,198.7	33,019.6	31,981.2
Crucible + Celite + residue wt (mg)	31,578.2	32,213.2	33,033.4	33,995.6
Protein (mg)	3.2		3.3	
Crucible + Celite + ash wt (mg)		32,206.8		31,989.1

What is the (a) insoluble, (b) soluble, and (c) total fiber content of the cookie determined to the appropriate number of significant figures?

Answers

1. Number of significant figures = 2 (6.1 mg)

(a) $$\frac{151.9 - 13.1 - 21.1 - 6.0}{1002.8} \times 100 = 11\%$$

(b) $$\frac{151.9 - 13.1 - 21.1 - 6.1 - 35.9}{1002.8} \times 100 = 7.5\%$$

2. (a) 6.1%, (b) 2.0%, (c) 8.1%
(Calculations are done a little differently than those at the bottom of Table 10-6.)

a. *Insoluble dietary fiber*

Number of significant figures = 2 (6.5 mg, 3.2 mg)

$$\text{Blank residue} = 31,578.2\text{ mg} - 31,563.6\text{ mg} = 14.6\text{ mg}$$
$$32,231.2\text{ mg} - 32,198.7\text{ mg} = 14.5\text{ mg}$$
$$\text{Average} = 14.6\text{ mg}$$

$$\text{Blank ash} = 32,206.8\text{ mg} - 32,198.7\text{ mg} = 8.1\text{ mg}$$

First sample residue:

$$= 31,723.5\text{ mg} - 31,637.2\text{ mg} = 86.3\text{ mg}$$

$$\text{Ash} = 32,195.2\text{ mg} - 32,173.9\text{ mg} = 21.3\text{ mg}$$

86.3 mg (residue weight)
− 14.6 mg (blank)
− 3.3 mg (protein, 6.5 − 3.2 [blank])
− 13.2 mg (ash, 21.3 − 8.1 [blank])
= 55.2 mg

$$(55.2\text{ mg} \div 1,002.1[\text{sample wt.}]) \times 100 = 5.5\%$$

Second sample residue :

$$= 32,271.2\text{ mg} - 32,173.9\text{ mg} = 97.3\text{ mg}$$

$$97.3 - 14.5 - 3.3 - 13.2 = 66.3\text{ mg}$$

$$(66.3\text{ mg} \div 1005.3\text{ mg}\,[\text{sample wt.}]) \times 100 = 6.6\%$$

Average of 5.5% and 6.6% = 6.1%

b. *Soluble dietary fiber*

Number of significant figures = 2 (3.9 mg, 3.3 mg)

$$\text{Blank residue} = 33,033.4\text{ mg} - 33,019.6\text{ mg} = 13.8\text{ mg}$$
$$33,995.6\text{ mg} - 31,981.2\text{ mg} = 14.4\text{ mg}$$
$$\text{Average} = 14.1\text{ mg}$$

$$\text{Blank ash} = 31,989.1\text{ mg} - 31,981.2\text{ mg} = 7.9\text{ mg}$$

First sample residue:

$$= 32,421.6\text{ mg} - 32,377.5\text{ mg} = 44.1\text{ mg}$$

$$\text{Ash} = 33,231.0\text{ mg} - 33,216.4\text{ mg} = 14.6\text{ mg}$$

44.1 mg (residue weight)
− 14.1 mg (blank)
− 0.6 mg (protein, 3.9 − 3.3 [blank])
− 6.7 mg (ash, 14.6 − 7.9 [blank])
= 22.7 mg

$$(22.7\text{ mg} \div 1,002.1[\text{sample wt.}]) \times 100 = 2.3\%$$

Second sample residue:

$$= 33,255.3\text{ mg} - 33,216.4\text{ mg} = 38.9\text{ mg}$$

$$38.9 - 14.1 - 0.6 - 6.7 = 17.5\text{ mg}$$

$$(17.5\text{ mg} \div 1005.3\,[\text{sample wt.}]) \times 100 = 1.7\%$$

Average of 2.3% and 1.7% = 2.0%

c. *Total dietary fiber* (TDF)

$$\begin{aligned} \text{TDF} &= 6.1\%\ (\text{insolublefiber}) \\ &\quad + 2.0\%\ (\text{solublefiber}) = 8.1\% \end{aligned}$$

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Vitamin Analysis

Ronald B. Pegg*, W.O. Landen, Jr., and Ronald R. Eitenmiller
Department of Food Science and Technology, The University of Georgia, 100 Cedar Street, Athens, GA 30602–7610, USA
rpegg@uga.edu
woland@uga.edu
eiten@uga.edu

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11.1 INTRODUCTION

11.1.1 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 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].

11.1.2 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 and some of the problems associated with these techniques. Please note that the sections below on bioassay, microbiological, and chemical methods are not comprehensive, but rather just give examples of each type of analysis.

11.1.3 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 can also be expressed as **international units** (IU), **United States Pharmacopeia** (USP) **units**, and % **Daily Value** (DV). To many, these definitions are unclear. 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.

The IU is a unit of measurement for the amount of a substance, based on measured biological activity or effect. It is used for vitamins, hormones, vaccines, and similar biologically active substances. The precise definition of 1 IU differs from substance to substance, but has been established by international agreement for each substance. There is no equivalence among different substances; that is, 1 IU or USP unit of vitamin E does not contain the same number of micrograms as 1 IU or USP unit of vitamin A. Although IUs are still employed in food fortification and for nutrition labeling in the US (e.g., dietary supplements), many regulators feel that their use should be abandoned.

Concerning vitamin E, the USP discontinued the use of the IU in the US after 1980 and replaced it with USP units derived from the same biological activity values as the IU. Thus, 1 USP unit is defined as the activity of 1 mg of all-*rac*-α-tocopheryl acetate on the basis of biological activity measured by the rat fetal resorption assay. This equals the activity of 0.67 mg of *RRR*-α-tocopherol or 0.74 mg of *RRR*-α-tocopheryl acetate. Biological activities relative to *RRR*-α-tocopherol have been a convenient way to compare the different forms of vitamin E on the basis of IU or USP units, and were used to calculate milligram α-tocopherol equivalent (mg α-TE) values for reporting vitamin E contents. As vitamin E is available in different forms, conversion factors have been established (1) (Table 11-1).

Some other IU definitions for vitamins include the following:

- 1 IU of vitamin A is the biological equivalent of 0.3 μg retinol, 0.6 μg β-carotene, and 1.2 μg of other provitamin A active carotenoids (e.g., α-carotene and β-cryptoxanthin). One retinol equivalent (RE) is defined as 1 μg of all-*trans*-retinol. Varying dietary sources of vitamin A have different potencies. For calculation of RE values in foods, 100% efficiency of absorption of all-*trans*-retinol is assumed; however, incomplete absorption and conversion of β-carotene as well as other provitamin A active carotenoids must be taken into account. The conversion factors of 1 RE equals 6 μg and 12 μg for β-carotene and other provitamin A active carotenoids, respectively, are applied. A more recent international standard of measure of vitamin A established by the Institute of Medicine of the National Academies is to report μg retinol activity equivalents (RAE). For example, 2 μg of β-carotene in oil provided as a supplement can be converted by the body to 1 μg of retinol giving it an RAE ratio of 2:1, whereas 12 μg of all-*trans*-β-carotene from foods are required to provide the body with 1 μg of retinol giving dietary β-carotene an RAE ratio of 12:1. Other provitamin A carotenoids in foods are less easily absorbed than β-carotene resulting in RAE ratios of 24:1. So in food, unlike a dietary supplement, there is no direct comparison between an IU and μg RE or RAE. As a guide to convert IUs of vitamin A to μg RE, multiply the number of IUs by 0.1 if the food is of plant origin and by 0.2 if it is of animal origin. The result will be the approximate number of μg RE in the food.

11-1 table **Conversion Factors to Calculate α-Tocopherol from International Units or United States Pharmacopeia Units to Meet Dietary Reference Intakes for Vitamin E**

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

USP, United States Pharmacopeia; *IU*, international unit; α*T*, α-tocopherol.
From: Reference (1), used with permission of Taylor & Francis Group, CRC Press, Boca Raton, FL.

- 1 IU of vitamin C is the biological equivalent of 50 μg L-ascorbic acid.
- 1 IU of vitamin D is the biological equivalent of 0.025 μg cholecalciferol/ergocalciferol.

The % Daily Value (DV) is a newer dietary reference value designed to help consumers to use label information to plan a healthy overall diet (see also Chap. 3). The DVs are reference numbers based on Recommended Dietary Allowances (RDAs) established by the Food and Nutrition Board of the Institute of Medicine. On food labels, the numbers tell you the % DV that one serving of this food provided as a percentage of established standards. In fact, DVs actually comprise two sets of reference values for nutrients: Daily Reference Values, or DRVs, and Reference Daily Intakes, or RDIs. The % DV is based on a 2000-Calorie diet for adults older than 18.

11.2 METHODS

11.2.1 Overview

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. **Physicochemical 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 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.

On account 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 physicochemical 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 book.

The principles, critical points, procedures, and calculations for various vitamin analysis methods are described in this chapter. Many of the methods cited are official methods of AOAC International (2), the European Committee for Standardization (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 methods is provided in Table 11-2. The sections below on bioassay, microbiological, and chemical methods are not comprehensive, but rather just give examples of each type of analysis.

11-2 table **Commonly Used Regulatory Methods for Vitamin Analysis**

Vitamin	*Method Designation*	*Application*	*Approach*
Fat-Soluble Vitamins			
Vitamin A (and precursors)			
Retinol	AOAC Method 992.04 (2)	Vitamin A in milk-based infant formula	LC[a] 340 nm
Retinol	AOAC Method 2001.13 (2)	Vitamin A in foods	LC 328 or 313 nm
all-*trans*-retinol 13-*cis*-retinol	EN 1283-1 (3)	All foods	LC 325 nm or Fluorometric[b] E_x $\lambda = 325$ nm E_m $\lambda = 475$ nm
β-Carotene	AOAC Method 2005.07 (2)	β-Carotene in supplements and raw materials	LC 445 or 444 nm
β-Carotene	EN 1283–2 (3)	All foods	LC 450 nm
Vitamin D			
Cholecalciferol Ergocalciferol	AOAC Method 936.14 (3)	Vitamin D in foods	Bioassay
Cholecalciferol Ergocalciferol	AOAC Method 995.05 (3)	Vitamin D in infant formula and enteral products	LC 265 nm
Cholecalciferol Ergocalciferol	EN 1282172 (5)	Vitamin D in foods	LC 265 nm
Vitamin E			
R, *R*, *R* – tocopherols	EN 12822 (6)	Vitamin E in foods	LC Fluorescence E_x $\lambda = 295$ nm E_m $\lambda = 330$ nm
Vitamin K			
Phylloquinone	AOAC Method 999.15 (2)	Vitamin K in milk and infant formulas	LC postcolumn reduction Fluorescence E_x $\lambda = 243$ nm E_m $\lambda = 430$ nm
Phylloquinone	EN 14148 (7)	Vitamin K in foods	LC postcolumn reduction Fluorescence E_x $\lambda = 243$ nm E_m $\lambda = 430$ nm
Water-Soluble Vitamins			
Ascorbic acid (Vitamin C)			
Ascorbic acid	AOAC Method 967.21 (2)	Vitamin C in juices and vitamin preparations	2,6-Dichloroindophenol titration
Ascorbic acid	AOAC Method 967.22 (2)	Vitamin C in vitamin preparations	Fluorescence E_x $\lambda = 350$ nm E_m $\lambda = 430$ nm
Ascorbic acid	EN 14130 (8)	Vitamin C in foods	LC 265 nm
Thiamin (Vitamin B_1)			
Thiamin Thiamin·HCl	AOAC Method 942.23 (2)	Thiamin in foods	Thiochrome Fluorescence E_x $\lambda = 365$ nm E_m $\lambda = 435$ nm
Thiamin	EN 14122 (9)	Thiamin in foods	LC Thiochrome Fluorescence E_x $\lambda = 366$ nm E_m $\lambda = 420$ nm

(continued)

11-2 table **Commonly Used Regulatory Methods for Vitamin Analysis**

Vitamin	*Method Designation*	*Application*	*Approach*
Riboflavin (Vitamin B_2)			
Riboflavin	AOAC Method 970.65 (2)	Riboflavin in foods and vitamin preparations	Fluorescence E_x $\lambda = 440$ nm E_m $\lambda = 565$ nm
Riboflavin	EN 14152 (10)	Riboflavin in foods	LC Fluorescence E_x $\lambda = 468$ nm E_m $\lambda = 520$ nm
Niacin			
Nicotinic acid Nicotinamide	AOAC Method 944.13 (2)	Niacin and niacinamide in vitamin preparations	Microbiological
Vitamin B_6			
Pyridoxine Pyridoxal Pyridoxamine	AOAC Method 2004.07 (1,2)	Total Vitamin B_6 in infant formula	LC Fluorescence E_x $\lambda = 290$ nm E_m $\lambda = 395$ nm
Folic Acid, Folate			
Total folates	AOAC Method 2004.05 (2)	Total folates in cereals and cereal products – Trienzyme procedure	Microbiological
Vitamin B_{12}			
Cyanocobalamin	AOAC Method 986.23 (2)	Cobalamin (Vitamin B_{12}) in milk-based infant formula	Microbiological
Biotin			
Biotin	USP29/NF24, Dietary supplements official monograph (11)	Biotin in dietary supplements	LC 200 nm or Microbiological
Pantothenic acid			
Ca pantothenate	AOAC Method 992.07 (2)	Pantothenic acid in milk-based infant formula	Microbiological

[a] LC, liquid chromatography (high-performance liquid chromatography).
[b] Fluorometric test, giving excitation (E_x) and emission (E_m) wavelengths.

11.2.2 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. In some instances, 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, 13). Typical extraction procedures are as follows:

- *Ascorbic acid*: Cold extraction with metaphosphoric acid/acetic acid.
- *Vitamin B_1 and B_2*: 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.

Analysis of fat-soluble vitamins may require **saponification**, generally either overnight at room temperature or by refluxing at 70°C. In the latter case, an air-cooled reflux vessel as depicted in Fig. 11-1 provides excellent control of conditions conducive to oxidation.

11.2.3 Bioassay Methods

Outside of vitamin bioavailability studies, bioassays at the present are used only for the analysis of **vitamins B_{12}** and **D**. For the latter, it is the reference standard method of analysis of food materials (AOAC Method

11-1 figure Reflux vessel useful for saponification.

936.14), known as the **line test** (Fig. 11-2), based on bone calcification. Because the determination of vitamin D involves deficiency studies as well as sacrificing the test organisms, it is limited to animals rather than humans as test organisms.

11.2.4 Microbiological Assays

11.2.4.1 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.

11.2.4.2 Principle

The growth of microorganisms is proportional to their requirement for a specific vitamin. 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™) (12301 Parkway Drive, Rockville, MD 20852).

11.2.4.3 Niacin

The procedural sequence for the microbiological analysis of niacin is outlined in Fig. 11-3 (AOAC Method 944.13, 45.2.04) (2, 14). *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.

In general, growth is measured by turbidity. If lactobacilli are employed as the test organism, acidimetric measurements can be used as well. The latter may be necessary if a clear sample extract cannot be obtained prior to inoculation, and incubation (which is a prerequisite for turbidimetry) cannot be obtained. In making a choice between the two methods of measurement, one needs to bear in mind that a prolonged incubation period of 72 h is required for acidimetry.

11.2.4.4 Folate

Folate is the general term including folic acid (pteroylglutamate, PteGln) and poly-γ-glutamyl conjugates with the biological activity of folic acid. Folates present a diverse array of compounds that vary by oxidation state of the pteridine ring structure, one-carbon moieties carried by the specific folate, and the number of conjugated glutamate residues on the folate. Folates are labile to oxidation, light, thermal losses, and leaching when foods are processed. Because of the presence of multiple forms in food products and its instability, folate presents a rather difficult analytical problem. To account for differences in biological availability of synthetic folic acid used for food fortification and food folate, the Institute of Medicine Panel on Folate, Other B Vitamins and Choline established the dietary folate equivalent (DFE) value (5). Based on research showing that folic acid is 85% bioavailable whereas food folate

VITAMIN D BIOASSAY PROCEDURE

Sample Preparation

AOAC International provides specific instructions for preparation of various matrices for the bioassay. In some cases, saponification is used.

Depletion Period

Rats are suitable for depletion at age ≤30 days with body weight of ≥44 g but ≤60 g. A rachitogenic diet is fed for 18–25 days.

Assay Period

The assay period is the interval of life of the rat between the last day of the depletion period and the eighth or eleventh day thereafter. Feeding protocols are specified. During the assay, depleted rats are fed known and unknown amounts of vitamin D from standards and samples, respectively.

Potency of Sample

Vitamin D in the sample is determined by the line test from staining of the proximal end of the tibia or distal end of the radius or ulna.

11-2 figure The bioassay of vitamin D by the line test, AOAC Method 936.14, 45.3.01 (2).

NIACIN MICROBIOLOGICAL ASSAY PROCEDURE

Test Sample Preparation

Weigh out a sufficient amount of sample to contain ≤5.0 mg niacin/ml, add volume of 1 *N* H_2SO_4 equal in ml to ≥10X dry weight of test portion in g, macerate, autoclave 30 min at 121–123°C, and cool. If dissolved protein is not present, adjust mixture to pH 6.8 with NaOH solution, dilute with deionized H_2O to volume (*ca*. 0.1–0.4 μg niacin/ml), mix, and filter.

Assay Tube Preparation

In at least duplicate use 0.0, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 ml test sample filtrate and make up the difference to 5.0 ml with deionized H_2O, then add 5.0 ml of Difco™ Niacin Assay Medium to each tube, autoclave 10 min at 121–123°C, and cool.

Niacin Standard Preparation

Prepare assay tubes in at least duplicate using 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 ml standard working solution (0.1–0.4 μg niacin/ml), make up difference to 5.0 ml with deionized H_2O, then add 5.0 ml of Difco™ Niacin Assay Medium and treat identically as the sample tubes.

Inoculation and Incubation

Prepare inoculum using *Lactobacillus plantarum* ATCC™ 8014 in Difco™ Lactobacilli Broth AOAC. Add one drop of inoculum to each tube, cover tubes, and then incubate at 37°C for 16–24 hr; that is, until maximum turbidity is obtained as demonstrated by lack of significant change during a 2-hr additional incubation period in tubes containing the highest concentration of niacin.

Determination

Measure %T at any specific wavelength between 540 and 660 nm. Set transmittance to 100% with the inoculated blank sample. Prepare a standard concentration-response curve by plotting %T readings for each level of standard solution used against amount of reference standard contained in respective tubes. Determine the amount of niacin for each level of the test solution by interpolation from the standard curve.

11-3 figure The microbiological assay of niacin, AOAC Method 944.13, 45.2.04 (2).

is only 50%, it can be stated that folic acid in fortified products is 85/50 or 1.7 times more bioavailable than food folate. Therefore, the μg of DFEs provided equals the μg of food folate plus (1.7 × μg folic acid). Calculation of the μg DFE for any food requires quantitation of folic acid as a separate entity from food folate. Currently, quite sophisticated liquid chromatography methods are necessary for accurate quantitation of folic acid and the multiple forms of folates in foods. A collaborated microbiological procedure based on the trienzyme extraction quantifies only total folate and cannot differentiate between added folic acid and food folate. The microbiological assay for total food folate with *Lactobacillus casei* (spp. *rhamnosus*) ATCC™ 7469 and trienzyme digestion follows AOAC International (2, 15).

11.2.4.4.1 Principle Folate in the sample is extracted with a buffer at 100°C (boiling water bath). The extract is then digested with α-amylase and protease (i.e., to free macromolecularly bound folates) and conjugase (i.e., to cleave poly-γ-glutamyl folates to $PteGln_3$ or lower.) Growth response of the assay microorganism is measured by percent transmittance. Transmittance depends on folate concentration.

11.2.4.4.2 Critical Points Care must be exercised to protect labile folates from oxidation and photochemical degradation. Reducing agents including ascorbic acid, β-mercaptoethanol, and dithiothreitol are effective in preventing oxidation. Strict adherence to microbiological assay techniques is necessary to assay folate with accuracy and precision.

11.2.4.4.3 Procedure Analysis of food folate by *Lactobacillus casei* (spp. *rhamnosus*) ATCC™ 7469 and a trienzyme extraction procedure (Fig. 11-4) is provided by AOAC International (2). The analytical protocol has also been easily adapted using 96-well microtiter plates and a reader (16).

11.2.4.4.4 Calculations Results are calculated manually or from the regression line of the standard curve responses using 4th degree polynomial plots and a computer program written to conform to the AOAC microbiological analysis protocol. Software provided for microplate readers is suitable for calculating results from analyses using 96-well microplates. Results are reported as micrograms of vitamin per 100 g or per serving.

FOLATE MICROBIOLOGICAL ASSAY PROCEDURE

Sample Preparation

To 1.2–2.0 g of sample, add 50 ml of specified buffer, homogenize, and proceed to digestion step. (Note: High fat samples should be extracted with hexane, and all samples should be protected from light and air.)

Trienzyme Digestion

Boil samples for 5 min and cool to room temperature. Digest each sample with specified α-amylase, protease, and conjugase. Deactivate enzymes by boiling for 5 min. Cool tubes, filter, and dilute an appropriate aliquot to a final concentration of *ca.* 0.15 ng/ml.

Preparation of Standard Curve and Blank Tubes

Construct an 8-point standard curve using a working standard solution of folate. Add 5 ml of *Lactobacillus casei* (spp. *rhamnosus*) ATCC™ 7469 assay medium to each tube. Prepare an uninoculated blank and an inoculated blank to zero the spectrophotometer, and an enzyme blank to determine the contribution of the enzymes to microbial growth.

Assay

Folic acid is assayed by the growth of *Lactobacillus casei* (spp. *rhamnosus*) ATCC™ 7469 according to AOAC International (2). Prepared tubes of samples, standard curve, inoculated and uninoculated blanks, and enzyme blank are autoclaved at 121–123°C for 5 min and then inoculated with one drop of the prepared inoculum per tube. After tubes have been incubated at 37°C for 20–24 hr, the growth response is measured by percent transmittance at $\lambda = 550$ nm.

11-4 figure Analysis of folate in cereals and cereal products or other foods using *Lactobacillus casei* (spp. *rhamnosus*) ATCC™ 7469 and a trienzyme extraction procedure (2).

11.2.5 Chemical Methods

11.2.5.1 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 using low actinic glassware, nitrogen, and/or vacuum, as well as avoiding excessively high temperatures. The addition of an antioxidant at the onset of the procedure is highly recommended. **High-performance liquid chromatographic** (HPLC) methods are considered the only acceptable methods to provide accurate food measurements of vitamin A activity.

Details follow for 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):

11.2.5.1.1 Principle 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.

11.2.5.1.2 Critical Points All work must be performed in subdued artificial light. Care must be taken to avoid oxidation of the retinol throughout the entire procedure. Solvent evaporation should be completed under nitrogen, and hexadecane is added to prevent destruction during and after solvent evaporation.

11.2.5.1.3 Procedure Figure 11-5 outlines the procedural steps of the assay. Pyrogallol is added prior to saponification as an antioxidant.

11.2.5.1.4 Calculations

$$\text{all-}trans\text{-retinol(ng/ml milk or diluted formula)} = (A_t/A_{st}) \times W_t \times C_t \times \text{DF} \quad [1]$$

where:

A_t = peak area, all-*trans*-retinol in test sample
A_{st} = peak area, all-*trans*-retinol in standard
W_t = weight, mg, oil solution used to prepare working standard solution
C_t = concentration, ng/ml, all-*trans*-retinol in oil solution
DF = dilution factor = $1/50 \times 25/15 \times 100/3 \times 1/2 \times 1/40 = 5/360$

VITAMIN A HPLC ANALYSIS PROCEDURE

Test Sample Saponification

Transfer 40 ml of ready-to-use formula or fluid milk to a 100-ml digestion flask containing a stirring bar. For saponification, add 10 ml of ethanolic pyrogallol solution (*i.e.*, 2% (w/v) pyrogallol in 95% ethanol) and 40-ml ethanolic KOH (*i.e.*, 10% (w/v) KOH in 90% ethanol). Wrap the flask in aluminum foil and stir at room temperature for 18 hr, or at 70°C using the reflux vessel as depicted in Fig. 11-1. Dilute to volume with ethanolic pyrogallol solution.

Extraction of Digest

Pipet 3 ml of digestate into a 15-ml centrifuge tube and add 2 ml of deionized H_2O. Extract Vitamin A with 7-ml of hexane:diethyl ether (85:15, v/v). Repeat extraction 2X with 7-ml portions of extractant. After extractions, transfer the organic layer to a 25-ml volumetric flask. Add 1 ml of hexadecane solution (*i.e.*, 1-ml hexadecane in 100-ml hexane) and dilute to volume with hexane. Pipette 15 ml of diluted extract into a test tube and evaporate under nitrogen. Dissolve the residue in 0.5 ml of heptane.

Chromatography Parameters

Column	4.6 mm x 150 mm packed with 3-µm silica (Apex 3-µm silica)
Mobile Phase	Isocratic elution; heptane containing 2-propanol (1-5%, v/v)
Injection Volume	100 µl
Detection	UV, 340 nm
Flow Rate	1-2 ml/min

Inject 100-µl standard working solutions (see AOAC Method 992.04, 50.1.02 for details) into the HPLC. Inject 100-µl test extract. Measure peak areas for all-*trans*-retinol and 13-*cis*-retinol.
Note: The exact mobile phase composition and flow rate are determined by system suitability test to give retention times of 4.5 and 5.5 min for 13-*cis*-retinol and all-*trans*-retinol, respectively.

11-5 figure The HPLC analysis of vitamin A in milk and milk-based infant formula, AOAC Method 992.04, 50.1.02 (2).

$$13\text{-}cis\text{-retinol(ng/ml milk or diluted formula)} = (A_c/A_{sc}) \times W_c \times C_c \times DF \quad [2]$$

where:

A_c = peak area, 13-*cis*-retinol in test sample
A_{sc} = peak area, 13-*cis*-retinol in standard
W_c = weight, mg, oil solution used to prepare working standard solution
C_c = concentration, ng/ml, 13-*cis*-retinol in oil solution
DF = dilution factor = $1/50 \times 25/15 \times 100/3 \times 1/2 \times 1/40 = 5/360$

11.2.5.2 Vitamin E (Tocopherols and Tocotrienols)

11.2.5.2.1 Vitamin E Compounds 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-). All homologs in nature are (R, R, R)-isomers. Recently, the Institute of Medicine Panel on Dietary Antioxidants and Related Compounds recommended that human requirements for vitamin E include only the 2R-stereoisomeric forms of α-tocopherol for establishment of recommended intakes (17). For the past two decades human requirements have been stated in terms of α-tocopherol equivalents.

Details follow of vitamin E analysis in food products using HPLC (18):

11.2.5.2.2 Principle

1. General food products. The sample is saponified under reflux (see Fig. 11-1), extracted with hexane, and injected onto a normal phase HPLC column connected to a fluorescence detector, $E_x\ \lambda = 290$ nm, $E_m\ \lambda = 330$ nm (E_x, excitation; E_m, emission; see Chap. 22, Sect. 22.3).
2. Margarine and vegetable oil spreads. The sample is dissolved in hexane, anhydrous $MgSO_4$ is added to remove water, and the filtered extracts are assayed by HPLC.
3. Oils. Oil is dissolved in hexane and injected directly onto the HPLC column.

11.2.5.2.3 Critical Points Vitamin E is subject to oxidation. Therefore, saponification is completed under reflux, in the presence of the antioxidant, pyrogallol, with the reaction vessel protected from light.

11.2.5.2.4 Procedure The vitamin E assay is detailed in Fig. 11-6 and an example chromatogram is depicted in Fig. 11-7.

VITAMIN E HPLC ANALYSIS PROCEDURE

Sample Preparation

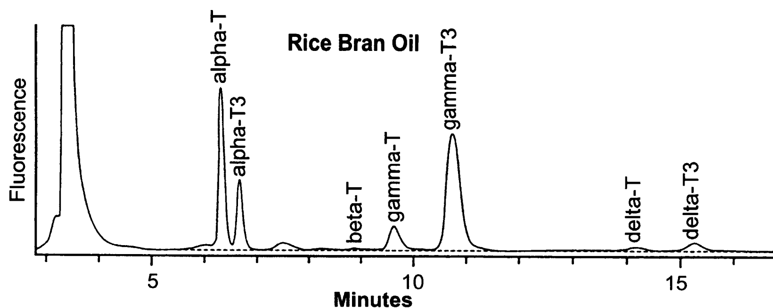
a. ***General food products***: Add 10 ml of 6% (w/v) pyrogallol in 95% ethanol to sample, mix, and flush with N_2. Heat at 70°C for 10 min with sonication. Add 2 ml of 60% (w/v) KOH solution, mix, and flush with N_2. Digest for 30 min at 70°C. Sonicate 5 min. Cool to room temperature, and add sodium chloride and deionized H_2O. Extract 3X with hexane (containing 0.1% BHT). Combine hexane extracts. Add 0.5 g of anhydrous $MgSO_4$ and mix. Filter through a Millipore filtration apparatus (0.45 μm). Dilute to volume with hexane. Inject sample into HPLC.
b. ***Margarine and vegetable oil spreads***: Add 40 ml of hexane (containing 0.1% BHT) to a 10-g sample and mix. Add 3 g of anhydrous $MgSO_4$, mix, let stand ≥2 hr. Filter and dilute combined filtrate to volume with hexane (0.1% BHT). Inject sample into HPLC.

Chromatography Parameters

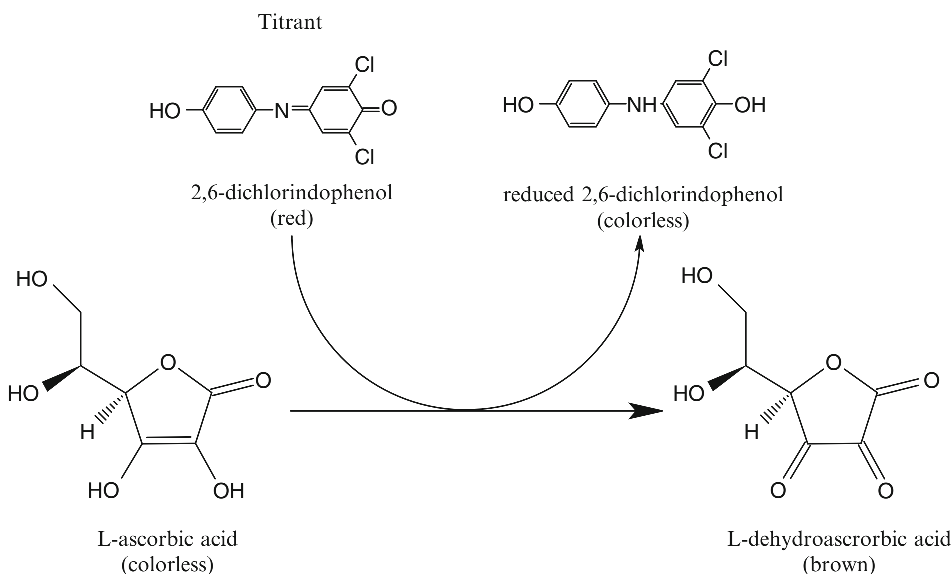
Column	Hibar® LiChrosorb Si 60 (4 mm × 250 mm, 5-μm particle size) and LiChromCART® 4-4 guard column packed with LiChrospher® Si 60 (5 μm)
Mobile Phase	Isocratic, 0.85% (v/v) 2-propanol in hexane
Injection Volume	20 μl
Flow	1 ml/min
Detector	Fluorescence, $E_x\lambda = 290$ nm, $E_m\lambda = 330$ nm

(Note: Determine recovery for each food product.)

11-6 figure Analysis of vitamin E in food products using HPLC. [Adapted from (18).] Refer to (18) for details on applications.



11-7 figure Chromatogram of rice bran oil showing tocopherols and tocotrienols.



11-8 figure Chemical reaction between L-ascorbic acid and the indicator dye, 2,6-dichloroindophenol.

11.2.5.2.5 Calculation Vitamin E is quantitated by external standards from peak area by linear regression.

11.2.5.3 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.

11.2.5.3.1 2,6-Dichloroindophenol Titrimetric Method (AOAC Method 967.21, 45.1.14) (2, 9)

1. **Principle**. L-ascorbic acid is oxidized to L-dehydroascorbic acid by the oxidation–reduction indicator dye, 2,6-dichloroindophenol. At the endpoint, excess unreduced dye appears rose-pink in acid solution (see Fig. 11-8).
2. **Procedure**. Figure 11-9 outlines the protocol followed for this method. In the presence of significant amounts of ferrous Fe, cuprous Cu, and stannous Sn ions in the biological matrix to be analyzed, it is advisable to include a **chelating agent** such as ethylenediaminetetraacetic acid (EDTA) with the extraction to avoid overestimation of the ascorbic acid content.

   The light but distinct rose-pink endpoint should last more than 5 s to be valid. With colored samples such as red beets or heavily

VITAMIN C ASSAY PROCEDURE 2,6-DICHLOROINDOPHENOL TITRATION

Sample Preparation

Weigh and extract by homogenizing test sample in metaphosphoric 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 2,6-dichloroindophenol sodium salt and 42 mg of $NaHCO_3$ to 200 ml with deionized H_2O) to a light but distinctive rose pink endpoint lasting $\geq$5 sec.

11-9 figure Analysis of vitamin C by the 2,6-dichloroindophenol titration, AOAC Method 967.21, 45.1.14 (2). [Adapted from (19), pp. 334–336.]

browned products, the endpoint is impossible to detect by human eyes. 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.

3. **Calculations**.

$$\text{mg of ascorbic acid/g or ml of sample} = (X - B) \times (F/E) \times (V/Y) \quad [3]$$

where:

X = average ml for test solution titration
B = average ml for test blank titration
F = mg ascorbic acid equivalents to 1.0-ml indophenol standard solution
E = sample weight (g) or volume (ml)
V = volume of initial test solution
Y = volume of test solution titrated

Note. The (V/Y) term represents the dilution factor employed.

11.2.5.3.2 Microfluorometric Method (AOAC Method 967.22, 45.1.15) (2, 19)

1. **Principle**. This method measures both ascorbic acid and dehydroascorbic acid. Ascorbic acid, following oxidation to dehydroascorbic acid, is reacted with **o-phenylenediamine** to produce a **fluorescent quinoxaline compound**.
2. **Procedure**. The procedural sequences for this method are outlined in Fig. 11-10. To compensate for the presence of interfering extraneous material, blanks need to be run using boric acid prior to the addition of the *o*-phenylenediamine solution.
3. **Calculations**.

$$\text{mg of ascorbic acid/g or ml} = [(X - D)/(C - B)] \times S \times (\text{DF}/E) \quad [4]$$

where:

X and C = average fluorescence of sample and standard, respectively
D and B = average fluorescence of sample blank and standard blank, respectively
S = concentration of standard in mg/ml
DF = dilution factor
E = sample weight, g, or sample volume, ml

11.2.5.4 Thiamin (Vitamin B$_1$) in Foods, Thiochrome Fluorometric Procedure (AOAC Method 942.23) (2)

11.2.5.4.1 Principle Following extraction, enzymatic hydrolysis of thiamin's phosphate esters and chromatographic cleanup (i.e., purification), this method is based on the fluorescence measurement of the oxidized form of thiamin, **thiochrome**, in the test solution compared to that from an oxidized thiamin standard solution.

11.2.5.4.2 Critical Points Thiochrome is light sensitive. Therefore, the analytical steps following the oxidation must be performed under subdued light. Thiamin is sensitive to heat, especially at alkaline

VITAMIN C MICROFLUOROMETRIC ASSAY PROCEDURE

Sample Preparation

Prepare sample extract as outlined in Fig. 11-8. To 100 ml each of the ascorbic acid standard and test sample solutions add 2 g of acid-washed Norit® Neutral, shake vigorously, and filter, discarding the first few ml.

Ascorbic Acid Standard and Test Sample Blanks

Transfer 5 ml of each filtrate to separate 100-ml volumetric flasks containing 5 ml of H_3BO_3-NaOAc solution. Let stand 15 min, swirling occasionally. Designate as standard or test sample blank. Development of the fluorescent quinoxaline is prevented by the formation of a H_3BO_3-dehydroascorbic acid complex prior to the addition of the *o*-phenylenediamine reagent. At the appropriate time, dilute blank solutions to volume with deionized H_2O. Transfer 2 ml of these solutions to each of three fluorescence tubes.

Ascorbic Acid Standard and Test Samples

Transfer 5 ml of each standard and test sample filtrate to separate 100-ml volumetric flasks containing 5 ml of 50% (w/v) NaOAc trihydrate and *ca*. 75 ml of H_2O, swirl contents, and then dilute to volume with deionized H_2O. Transfer 2 ml of these solutions to each of three fluorescence tubes.

Formation of Quinoxaline

To all sample and blank tubes, add 5 ml of 0.02% (w/v) aqueous *o*-phenylenediamine reagent, swirl contents using a Vortex mixer, and allow to stand at room temperature with protection from light for 35 min.

Determination

Measure fluorescence of sample and blank tubes at $E_x\ \lambda = 350$ nm, $E_m\ \lambda = 430$ nm.

11-10 figure Analysis of vitamin C by the microfluorometric method, AOAC Method 967.22, 45.1.15 (2). [Adapted from (20), pp. 338–341.]

pH. The analytical steps beginning with the oxidation of thiamin through to the fluorescence measurement (Fig. 11-11) must be carried out rapidly and precisely according to the instructions.

11.2.5.4.3 Procedure Figure 11-11 outlines the procedural sequence of the thiamin analysis. The enzymatic treatment and subsequent chromatographic cleanup may not be necessary with certain matrices, such as vitamin concentrates that contain nonphosphorylated thiamin and no significant amounts of substances that could interfere with the determination.

11.2.5.4.4 Calculations

$$\mu\text{g of thiamin in 5 ml of test solution} = [(I - b/S - d)] \quad [5]$$

where:

I and b = fluorescence of extract from oxidized test sample and sample blank, respectively

S and d = fluorescence of extract from oxidized standard and standard blank, respectively

$$\mu\text{g of thiamin/g} = [(I - b/S - d)] \times C/A \times 25/V_p \times V_o/\text{WT} \quad [6]$$

where:

I and b = fluorescence of extract from oxidized test sample and sample blank, respectively

S and d = fluorescence of extract from oxidized standard and standard blank, respectively

C = concentration of thiamin · HCl standard, µg/ml

A = aliquot taken, ml

25 = final volume of column eluate, ml

V_p = volume passed through the chromatographic column, ml

V_o = dilution volume of original sample, ml

WT = sample weight, g

THIAMIN ANALYSIS BY THIOCHROME PROCEDURE

Sample Preparation

Weigh out sample containing thiamin, add volume of 0.1 *M* HCl equal in ml to ≥10X dry weight of test portion in g, mix, autoclave for 30 min at 121–123°C, then cool. Dilute with 0.1 *M* HCl to measured volume containing *ca.* 0.2–5 μg thiamin/ml.

Enzyme Hydrolysis

Take aliquot containing *ca*. 10–25 μg thiamin, dilute to *ca*. 65 ml with 0.1 *M* HCl and adjust pH to 4.0–4.5 with *ca*. 5-ml 2 *M* CH_3COONa. Add 5 ml of enzyme solution, mix, incubate for 3 hr at 45–50°C. Cool, adjust to *ca*. pH 3.5, dilute to 100 ml with deionized H_2O, and filter.

Sample Extract Cleanup

Apply an aliquot of the test sample extract containing *ca*. 5 μg thiamin to a specified ion-exchange resin column, and wash column with 3X 5-ml portions of almost boiling water. Then elute thiamin from the resin with 5X 4.0 to 4.5-ml portions of almost boiling acid-KCl solution. Collect the eluate in a 25-ml volumetric flask and dilute to volume with acid-KCl solution. Treat standards identically.

Oxidation of Thiamin to Thiochrome

To a test tube, add 1.5 g of NaCl and 5 ml of the thiamin · HCl standard solution (1 μg/ml). Add 3 ml of oxidizing reagent [*i.e.*, basic $K_3Fe(CN)_6$], swirl contents, then add 13 ml of isobutanol, shake vigorously, and centrifuge. Repeat steps for the standard blank but instead replace the oxidizing reagent with 3 ml of 15% (w/v) NaOH. Decant the isobutanol extracts (*i.e.*, the standard and blank) into fluorescence reading tubes, and measure at E_x $\lambda = 365$ nm and E_m $\lambda = 435$ nm. Treat the test solution identically, and record the fluorescence intensity of the test sample and blank.

Analysis of thiamin (vitamin B_1) by the thiochrome fluorometric procedure, AOAC Method 942.2.3, 45.1.05 (2). Refer to (2) for more details on procedure.

11.2.5.5 Riboflavin (Vitamin B_2) in Foods and Vitamin Preparations, Fluorometric Method (AOAC Method 970.65, 45.1.08) (2)

11.2.5.5.1 Principle Following extraction, cleanup, and compensation for the presence of interfering substances, riboflavin is determined fluorometrically.

11.2.5.5.2 Critical Points Due to the extreme sensitivity of the vitamin to UV radiation, all operations need to be conducted under subdued light. The analyst also needs to be aware that exact adherence to the permanganate oxidation process is essential for reliable results.

11.2.5.5.3 Procedure An outline of the procedural protocol for this analysis is shown in Fig. 11-12. In spite of the fact that riboflavin is classified as a water-soluble vitamin, it does not readily dissolve in water. When preparing the standard solution, the analyst must pay special attention and ensure that the riboflavin is completely dissolved.

11.2.5.5.4 Calculations

$$\text{mg of riboflavin/ml final test solution} = [(B - C)/(X - B)] \times 0.10 \times 0.001 \quad [7]$$

where:

B and C = fluorescence of test sample containing water and sodium dithionite, respectively
X = fluorescence of test sample containing riboflavin standard

Note. Value of $[(B - C)/(X - B)]$ must be ≥0.66 and ≤1.5)

$$\text{mg of riboflavin/g of sample} = [(B - C)/(X - B)]\ x\ (\text{CS}/V)\ x\ (\text{DF}/\text{WT}) \quad [8]$$

where:

B and C = fluorescence of sample containing water and sodium hydrosulfite, respectively
X = fluorescence of sample containing riboflavin standard
CS = concentration of standard expressed as mg/ml

RIBOFLAVIN ASSAY PROCEDURE BY FLUORESCENCE

Sample Preparation

Weigh out homogenized sample, add volume of 0.1 *M* HCl equal in ml to $\geq$10X dry weight of test portion in g; the resulting solution must contain $\leq$0.1 mg riboflavin/ml. Mix contents, autoclave for 30 min at 121–123°C and then cool. Precipitate interfering substances by adjusting pH to 6.0–6.5 with dilute NaOH immediately followed by a pH readjustment to 4.5 with dilute HCl. Dilute with deionized H_2O to *ca*. 0.1 µg of riboflavin/ml, and filter.

Oxidation of Interfering Materials

Oxidize as follows: Transfer 10 ml of test filtrate to each of four tubes. To two of these tubes, add 1.0 ml of deionized H_2O, and to the remaining ones add 1.0 ml of a standard solution (*i.e.*, 1 µg/ml of riboflavin). Then to each tube, one at a time, add 1.0 ml of glacial HOAc followed by 0.5 ml of 4% (w/v) $KMnO_4$. Allow the mixture to stand for 2 min, and then add 0.5 ml of 3% (v/v) H_2O_2. Shake vigorously until excess O_2 is expelled.

Measurement of Fluorescence

Measure fluorescence at E_x $\lambda = 440$ nm and E_m $\lambda = 565$ nm. First read test samples containing 1 ml of added standard riboflavin solution, and then samples containing 1 ml of deionized H_2O. Add, with mixing, 20 mg of $Na_2S_2O_4$ to two of the tubes, and measure the minimum fluorescence within 5 sec.

11-12 figure Analysis of riboflavin (vitamin B_2) by fluorescence, AOAC Method 970.65, 45.1.08 (2).

V = volume of sample for fluorescence measurement, ml
DF = dilution factor
WT = weight of sample, g

11.3 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?

Bioassays are extremely time consuming. Their employment is generally limited to those instances in which no suitable alternate method is available, or for cases in which bioavailability of the analyte is desired, especially if other methods have not been demonstrated to provide this information. Bioassays have the advantage that they sometimes do not require the preparation of an extract, thus eliminating the potential of undesirable changes of the analyte during the extract preparation. On the other hand, in the case of deficiency development requirements prior to analysis, bioassays are limited to animals rather than humans.

Both microbiological and physicochemical methods require vitamin extraction (i.e., solubilization prior to analysis). In general, the results obtained through these methods represent the total content of a particular vitamin in a certain biological matrix, such as food, and not necessarily its bioavailability to humans.

The applicability of microbiological assays is limited to water-soluble vitamins, and most commonly applied to 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 physicochemical assays.

Because of their relative simplicity, accuracy, and precision, the physicochemical methods, in particular the chromatographic methods using HPLC, are preferred. For example, standard HPLC is commonly employed as an official method of analysis for vitamins A, E, and D, and as a quality control method for vitamin C. While HPLC 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 multianalyte 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.

Over the past decade, liquid chromatography in combination with mass spectrometry (MS) (see Chap. 26) has added a new dimension to vitamin analysis. In general, LC–MS methods are now 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 are rapidly becoming 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), and LC–MS/MS for folate (vs. the microbiological method). The reader is referred to reference (12) for applications of LC–MS to specific vitamins.

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 European Committee for Standardization (3–10), the US Pharmacopeial Convention (11), or the AACC International (21). Again, one must realize that these methods are limited to certain biological matrices.

11.4 SUMMARY

The three most used types of methods for the analysis of vitamins – bioassays and microbiological and physicochemical assays – have been outlined in this chapter. They 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.

11.5 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 two vitamins must be listed on the standard nutritional label?
4. The standard by which all chemical methods to measure vitamin D content are compared is a bioassay method. Describe this bioassay method.
5. Explain why it is possible to use microorganisms to quantitate a particular vitamin in a food product, and describe such a procedure.
6. Niacin and folate both can be quantitated by microbiological methods. What extra procedures and precautions are necessary in the folate assay compared to the niacin assay, and why?
7. 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.
8. 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.
9. What are the advantages and disadvantages of using HPLC for vitamin analysis?
10. Vitamin contents can be presented as units of mg or µg, as International Units (IU), or as % DV. Discuss the differences between these approaches for reporting the result.

11.6 PRACTICE PROBLEMS

1. A 3.21-g tuna sample (packed in water and drained before sampling) was analyzed for its niacin content. The sample was digested in 50 ml of 1 *N* H_2SO_4. After dissolved protein was removed by precipitation according to the AOAC method, a 20-ml aliquot was diluted to 100 ml, and then a 25-ml aliquot of the intermediate solution was taken and diluted to 250 ml. The concentration of niacin in the working solution was determined to be 0.168 µg/ml. (a) How much niacin is present in the tuna sample, and (b) how closely does this value compare with

that provided in the USDA Nutrient Database for Standard Reference (i.e., fish, tuna, light, canned in water, drained solids)?

2. Vitamin C in a nutraceutical formulation was assayed using the 2,6-dichloroindophenol titrimetric method. Determine the concentration of vitamin C (mg/g) in the nutraceutical based on the data given below from the assay.
   - Sample weight. 101.7 g, diluted to 500 ml with HPO_3/HOAc solution and filtered
   - Volume of sample filtrate titrated: 25 ml
   - Volume of dye used for the test solution titration: 9.2 ml
   - Volume of dye used for the test blank titration: 0.1 ml
   - mg ascorbic acid equivalents to 1.0 ml of indophenol standard solution: 0.175 mg/ml
3. Thiamin in a pet food sample was analyzed using the AOAC fluorometric method. Based on the assay conditions described below, determine the concentration of thiamin (μg/g) in the original pet food sample.
   - Sample weight. 2.0050 g
   - Dilutions. Diluted sample to 100 ml, applied 25 ml onto the Bio-Rex 70 ion-exchange column, then diluted the eluate to 25 ml and used 5 ml for fluorometry
   - Concentration of thiamin·HCl standard working solution: 0.1 μg/ml
   - Fluorometry reading ratio: 0.850
4. Riboflavin in raw almonds was analyzed using the AOAC fluorometric method. Based on the assay conditions described below, (a) determine the concentration of riboflavin (mg/g) in the almonds and (b) how closely this value compares with that provided in the USDA Nutrient Database for Standard Reference.
   - Sample weight: 1.0050 g
   - Dilutions: to 50 ml; used 10 ml for fluorometry
   - Fluorometry readings: $B_{60}/X_{85}/C_{10}$
   - Concentration or riboflavin standard solution: 1 μg/ml
5. 1.7 g of a braised, loin pork chop was analyzed for thiamin. The sample was digested with 20 ml of 0.1 *M* HCl and then diluted to 50 ml. A 40-ml aliquot of the digest was treated with the enzyme preparation and eventually diluted to 100 ml. A 45-ml aliquot of the enzyme-treated filtrate was purified using a Bio-Rex 70 ion-exchange column. The analyte from the 45-ml aliquot was applied to the column and recovered with five 4.0-ml portions of hot acid-KCl solution. The portions were pooled in a 25-ml volumetric flask and diluted to mark. A 5-ml aliquot as well as appropriate blanks and standard (concentration = 1 μg/ml) were converted to thiochrome and measured spectrofluorometrically. The following results were found:
   - Fluorescent intensity of the oxidized test sample and blank were 62.8 and 7.3, respectively
   - Fluorescent intensity of the thiamin·HCl standard and blank were 60.4 and 5.2, respectively

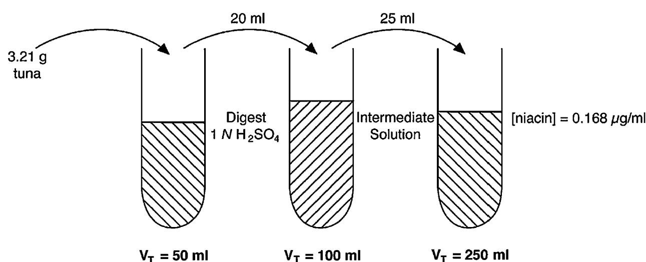
   Determine (a) how many μg of thiamin are in 5 ml of the test solution; (b) how many μg of thiamin/g braised loin pork chop; (c) how the answer from (b) compares with that reported in the USDA Nutrient Database for Standard Reference.
6. A graduate student is analyzing the antioxidant activity of phenolic compounds in apple juice samples and purchases some apple cider from a Farmer's market. The student worries that the product might have been fortified with ascorbic acid, and if so, will mess up his antioxidant assay. So, a vitamin C analysis is conducted by the 2,6-dichloroindophenol titrimetric method. 120 ml of the apple cider was mixed with 120 ml of the HPO_3/HOAc solution. In triplicate, a 10-ml aliquot of the resultant solution was taken and titrated against the indophenol standard solution. The following results were found:
   - An average of 13.3 and 0.1 ml of the indophenol standard was consumed during titration of the test sample and blank, respectively
   - From preliminary work it was determined that 0.1518 mg of ascorbic acid was equivalent to 1.0 ml of indophenol standard solution

   Determine (a) how many mg of ascorbic acid/ml of apple cider; (b) how do these results compare to tinned or bottled fresh apple juice according to the USDA Nutrient Database for Standard Reference; (c) has the apple cider been fortified with vitamin C; and (d) can this apple cider sample be used for the intended antioxidant activity study?
7. In the 2,6-dichloroindophenol titrimetric method, the indophenol reagent is prepared by dissolving 50.0 mg of 2,6-dichloroindophenol sodium salt plus some sodium bicarbonate in deionized water to 200 ml. The ascorbic acid standard solution is prepared by dissolving 50.0 mg of USP ascorbic acid reference standard in 50 ml of deionized water. If a 4.5-ml aliquot of the ascorbic acid reference standard is treated with 5.0 ml of the HPO_3/HOAc solution and then titrated against the indophenol reagent, how many milliliters should be consumed? Note: Remember the stoichiometry for the reaction.
   - FW of 2,6-dichloroindophenol sodium salt is 290.08 g/mol
   - FW of ascorbic acid is 176.12 g/mol
8. A new infant formula was developed for delivery to a third world country. Unfortunately, the dried infant formula was stored outdoors for 3 months in the sunlight before use and there is fear that the vitamin A has degraded. The reported vitamin A content (expressed as all-*trans* retinol equivalents) in the formula at the point of shipping was 3.0 μg/g dry formula. The following test was performed: 140 g of dry formula (i.e., after 3 months of storage outdoors in the sunlight) were dissolved in water and made up to 1 L. The AOAC method was followed with the following observations:
   - Peak area for the all-*trans*-retinol in the standard was 934
   - Weight of oil solution used to prepare the working retinol standard was 53 mg
   - Concentration of all-*trans*-retinol in oil standard solution was 1996 ng/ml
   - Peak area for the test solution after outdoor storage was 89

Determine (a) the concentration (ng/ml) of all-*trans*-retinol in the rehydrated infant formula; (b) by what percentage has the vitamin A content, expressed as all-*trans*-retinol, in the dried infant formula degraded?

Answers

1. (a) 420 μg in the 3.2-g test portion; (b) there are 13.08 mg niacin/100 g tuna in the test sample. The USDA Nutrient Database for Standard Reference lists a value of 13.280 ± 0.711 mg niacin/100 g tuna; so, the values compare well.



Calculations:

(a) Niacin present in tuna sample is ...

$$0.168\,\frac{\mu g}{ml} \times \frac{250\,ml}{25\,ml} \times \frac{100\,ml}{20\,ml} \times 50\,ml$$

$$= 420\,\mu g = 0.420\,mg\,niacin$$

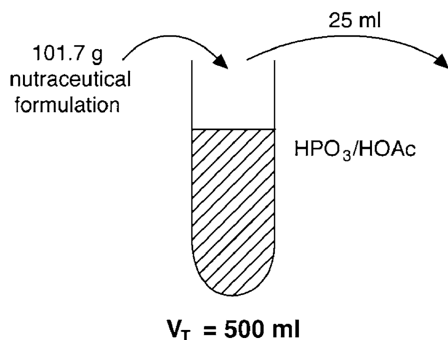
So,

$$\frac{0.420\,mg\,niacin}{3.21\,g}$$

$$= 0.1308\,mg/g\,tuna\ or\ 13.08\,mg\,niacin/100\,g\,tuna$$

(b) The USDA Nutrient Database for Standard Reference lists a niacin content of 13.28 mg/100 g tuna for “fish, tuna, light, canned in water, drained solids.” Thus, the 13.08 mg niacin/100 g tuna value is very close to the 13.28 mg value reported in the database.

2. 0.313 mg ascorbic acid/g nutraceutical



Calculations:

$F = 0.175$ mg AA eq./ml indophenol standard
$X = 9.2$ ml of dye for test solution titration
$B = 0.1$ ml of dye for test blank titration

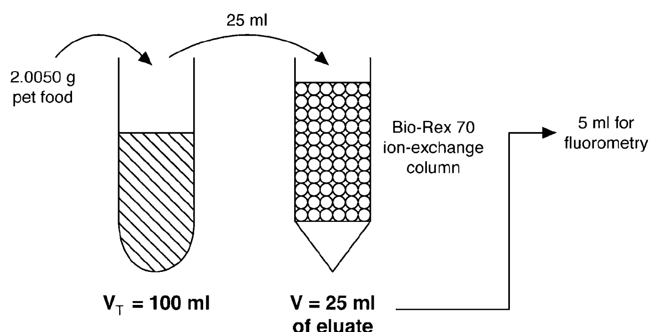
mg ascorbic acid/g sample

$$= (X - B) \times (F/E) \times (V/Y)$$

$$= \frac{(9.2\,ml - 0.1\,ml) \times 0.175\,mg/ml}{101.7\,g} \times \frac{500\,ml}{25\,ml}$$

= 0.313 mg of ascorbic acid/g nutraceutical formulation

3. 0.8479 μg thiamin/g pet food



Calculations:

Concentration of thiamin·HCl standard = 0.1 μg/ml

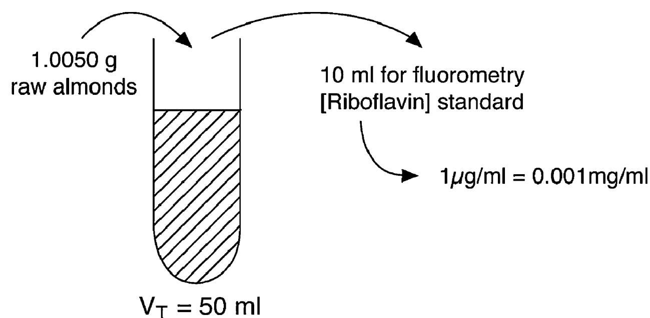
$$\text{Fluorometry reading ratio} = 0.850\left[\frac{(I-b)}{(S-d)}\right]$$

$$\mu g\ thiamin/g = \left[\frac{(I-b)}{(S-d)}\right] \times \frac{C}{A} \times \frac{25}{V_p} \times \frac{V_o}{WT}$$

$$= 0.850 \times \frac{0.1\,\mu g/ml}{5\,ml} \times \frac{25}{25\,ml} \times \frac{100\,ml}{2.0050\,g}$$

= 0.8479 μg thiamin/g pet food

4. (a) 9.95×10^{-3} mg riboflavin/g raw almonds; (b) there is 0.995 mg riboflavin/100 g raw almonds in the test sample. The USDA Nutrient Database for Standard Reference lists a value of 1.014 ± 0.025 mg riboflavin/100 g raw almonds; so, the values compare well.

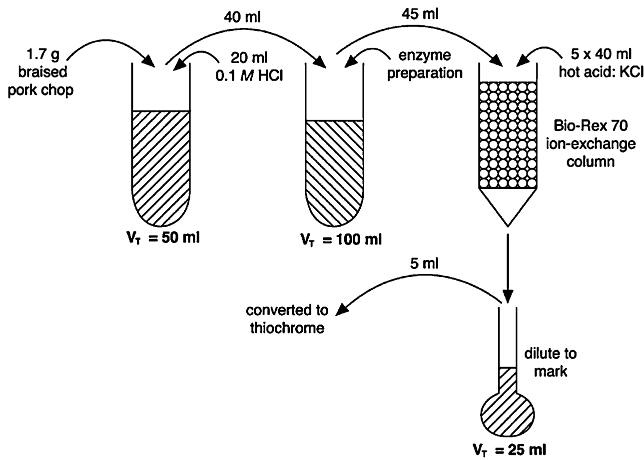


Calculations:
(a) mg of riboflavin/g raw almonds

$$= \left[\frac{(B-C)}{(X-B)}\right] \times \frac{CS}{V} \times \frac{DF}{WT}$$
$$= \left[\frac{(60-10)}{(85-60)}\right] \times \frac{0.001\,mg/ml}{10\,ml} \times \frac{50\,ml}{1.0050\,g}$$
$$= 9.95 \times 10^{-3}\ \text{mg of riboflavin/g raw almonds}$$

(b) The USDA Nutrient Database for Standard Reference lists a riboflavin content of 1.014 ± 0.025 mg/100 g for "nuts, almonds." Thus, the 9.95×10^{-3} mg of riboflavin/g raw almonds is very close to the value reported in the database.

5. (a) 1.005 µg in 5 ml; (b) 8.214 µg/g; (c) very close, as each 100 g of braised, pork loin chop contains 0.822 mg of thiamin.



Calculations:
(a) µg of thiamin in 5 ml of test solution

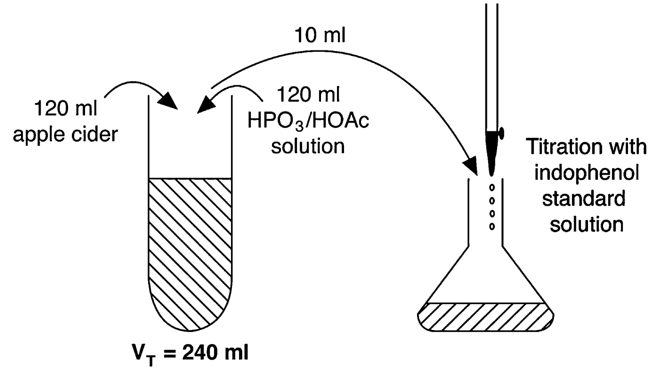
$$= \left[\frac{(I-b)}{(S-d)}\right] = \frac{(62.8-7.3)}{(60.4-5.2)} = \frac{55.5}{55.2}$$
$$= 1.005\ \mu g\ \text{thiamin/5 ml}$$

(b) µg of thiamin/g braised pork chops

$$= \left[\frac{(I-b)}{(S-d)}\right] \times \frac{C}{A} \times \frac{25}{V_p} \times \frac{V_o}{WT}$$
$$= \left[\frac{(62.8-7.3)}{(60.4-5.2)}\right] \times \frac{1\,\mu g/ml}{5\,ml} \times \frac{25\,ml}{45\,ml}$$
$$\times 50\,ml \times \frac{100\,ml}{40\,ml} / 1.7\,g$$
$$= 8.214\ \mu g/g\ \text{braised pork chop}$$

(c) The USDA Nutrient Database for Standard Reference lists a thiamin content of 0.822 mg/100 g for "pork, fresh loin, center loin (chops), bone-in, separable lean only, cooked, braised." Thus, the 8.214 µg thiamin/g braised pork chops is very close to the value reported in the database.

6. (a) 0.401 mg ascorbic acid/ml; (b) tinned or bottled juice contains 38.5 mg/100 g juice, so the results are similar; (c) Yes. The cider has been fortified with vitamin C; and (d) No. The high content of vitamin C will likely sacrifice itself as the antioxidant in the antioxidant activity assay before any endogenous phenolic compounds in the cider do so.



Calculations:
(a) mg of ascorbic acid (AA)/ml apple cider

$$= (X-B) \times \frac{F}{E} \times \frac{V}{Y}$$
$$= (13.3\,ml - 0.1\,ml)$$
$$\times \frac{0.1518\ \text{mg AA eq./1.0 ml indophenol std. solution}}{120\,ml}$$
$$\times \frac{240\,ml}{10\,ml}$$
$$= 0.401\ \text{mgAA/ml apple cider}$$

(b) According to the USDA Nutrient Database for Standard Reference, "apple juice, canned or bottled, unsweetened, with added ascorbic acid" contains 38.5 mg ascorbic acid/100 g apple juice; so, the results are similar.
(c) Yes. The cider has been fortified with vitamin C.
(d) No. The high content of vitamin C will likely sacrifice itself as the antioxidant in the antioxidant assay before any endogenous phenolic compounds in the cider do so.

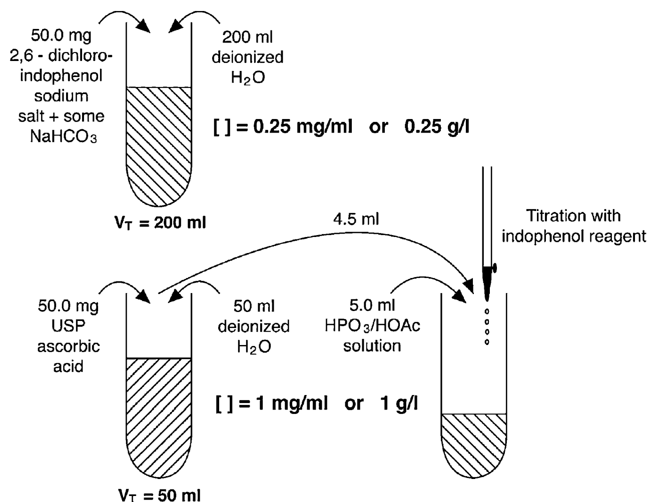
7. 29.6 ml
Calculations:
Formula Weight: 2,6-dichloroindophenol sodium salt = 290.08 g/mol
Formula Weight: ascorbic acid = 176.12 g/mol

$$\text{moles (mol)} = \frac{\text{mass (g)}}{\text{formula weight (g/mol)}}$$
$$\text{mol} = \frac{0.25\,g}{290.08\,g/mol}$$

So, concentration of 2,6-dichloroindophenol sodium salt solution is $= 8.618 \times 10^{-4}$ mol/L

$$\text{mol} = \frac{g}{g/mol};\ \text{mol} = \frac{1\,g}{176.12\,g/mol}$$

So, concentration of ascorbic acid stock solution $= 5.678 \times 10^{-3}$ mol/L



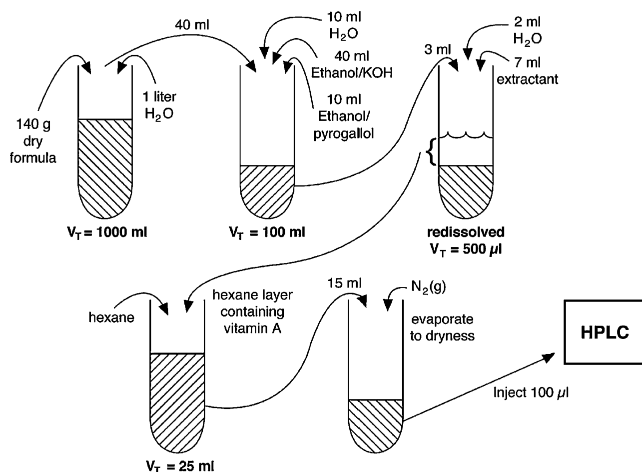
In the reaction vessel, there is:

$$5.678 \times 10^{-3}\,\text{mmol/ml} \times 4.5\,\text{ml}$$
$$= 2.555 \times 10^{-2}\,\text{mmol ascorbic acid}$$

The reaction stoichiometry is 1:1. See Fig. 11-8 for the chemical reaction. So, 2.555×10^{-2} mmol of 2,6-dichloroindophenol reagent need to be consumed.

$$8.618 \times 10^{-4}\text{mmol/ml} \times X\,\text{ml} = 2.555 \times 10^{-2}\text{mmol}$$
$$x = 29.6\,\text{ml}$$

8. (a) 140 ng all-*trans*-retinol/ml; and (b) 66.7%, or 2/3.



Calculations:

(a) all-*trans*-retinol (ng/ml rehydrated formula)

$$= \frac{A_t}{As_t} \times W_t \times C_t \times DF$$
$$= \frac{89}{934} \times 53\,\text{mg} \times 1996\,\text{ng/ml} \times \frac{5}{360}$$
$$= 140\,\text{ng all-}trans\text{-retinol/ml rehydrated milk}$$

(b) Originally there was 3 µg all-*trans* retinol/g dry formula

So, in 140 g of formula → 420 µg all-*trans*-retinol → in 1 L → 420 ng/ml

$$420\,\text{ng/ml} \times x = 140\,\text{ng/ml}$$
$$X = 0.333 \text{ or } {}^{1}/_{3}$$

Therefore, the vitamin A content, expressed as all-*trans*-retinol, had degraded by 66.7% or 2/3.

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12

chapter

Traditional Methods for Mineral Analysis

*Robert E. Ward** and *Charles E. Carpenter*

Department of Nutrition and Food Sciences, Utah State University,
Logan, UT 84322-8700, USA
robert.ward@usu.edu
chuck.carpenter@usu.edu

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12.1 INTRODUCTION

This chapter describes traditional methods for analysis of minerals involving titrimetric and colorimetric procedures, and the use of ion selective electrodes. Other traditional methods of mineral analysis include gravimetric titration (i.e., insoluble forms of minerals are precipitated, rinse, dried, and weighed) and redox reactions (i.e., mineral is part of an oxidation–reduction reaction, and product is quantitated). However, these latter two methods will not be covered because they currently are used little in the food industry. The traditional methods that will be described have maintained widespread usage in the food industry despite the development of more modern instrumentation such as atomic absorption spectroscopy and inductively coupled plasma-atomic emission spectroscopy (Chap. 24). Traditional methods generally require chemicals and equipment that are routinely available in an analytical laboratory and are within the experience of most laboratory technicians. Additionally, traditional methods often form the basis for rapid analysis kits (e.g., Quantab® for salt determination) that are increasingly in demand. Procedures for analysis of minerals of major nutritional or food processing concern are used for illustrative purposes. For additional examples of traditional methods refer to references (1–6). Slight modifications of these traditional methods are often needed for specific foodstuffs to minimize interferences or to be in the range of analytical performance. For analytical requirements for specific foods see the *Official Methods of Analysis* of AOAC International (5) and related official methods (6).

12.1.1 Importance of Minerals in the Diet

Calcium, phosphorus, sodium, potassium, magnesium, chlorine, and sulfur make up the dietary macro minerals, those minerals required at more than 100 mg/day by the adult (7–9). An additional ten minerals are required in milli- or microgram quantities per day and are referred to as **trace minerals**. These include iron, iodine, zinc, copper, chromium, manganese, molybdenum, fluoride, selenium, and silica. There is also a group of minerals called **ultra trace minerals**, including vanadium, tin, nickel, arsenic, and boron, that are being investigated for possible biological function, but that currently do not have clearly defined biochemical roles. Some mineral elements have been documented to be **toxic** to the body and should, therefore, be avoided in the diet. These include lead, mercury, cadmium, and aluminum. Essential minerals such as fluoride and selenium also are known to be harmful if consumed in excessive quantities, even though they do have beneficial biochemical functions at proper dietary levels.

The Nutrition Labeling and Education Act of 1990 (NLEA) mandated labeling of **sodium**, **iron**, and **calcium** contents largely because of their important roles in controlling hypertension, preventing anemia, and impeding the development of osteoporosis, respectively (see Fig. 3-1, Chap. 3). The content of these minerals in several foods is shown in Table 12-1. The content of other minerals may be included on the label at the producer's option, although this becomes mandatory if the mineral is the subject of a nutrient claim on the label. Implementation of the NLEA has led to an increased need for more rapid and accurate analysis of minerals and other food components.

12.1.2 Minerals in Food Processing

Minerals are of **nutritional** and **functional** importance, and for that reason their levels need to be known and/or controlled. Some minerals are contained at high levels in natural foodstuffs. For example, milk is a good source of calcium, containing about 300 mg of calcium per 8-ounce cup. However, direct acid cottage cheese is very low in calcium because of the action of the acid causing the calcium bound to the casein to be freed and consequently lost in the whey fraction. Similarly, a large portion of the phosphorus, zinc, manganese, chromium, and copper found in a grain kernel is lost when the bran layer is removed in processing. The enrichment law for flour requires that iron be replaced in white flour to the level at which it occurred naturally in the wheat kernel before removal of the bran.

Fortification of some foods has allowed addition of minerals above levels ever expected naturally. Prepared breakfast cereals often are fortified with minerals such as calcium, iron, and zinc, formerly thought to be limited in the diet. Fortification of salt with iodine has almost eliminated goiter in the USA. In other cases, minerals may be added for functionality. Salt is added for flavor, to modify ionic strength that effects solubilization of protein and other food components, and as a preservative. This increases significantly the sodium content of products such as processed meats, pickles, and processed cheese. Phosphorus may be added as phosphates to increase water-holding capacity. Calcium may be added to promote gelation of proteins and gums.

Water is an integral part of food processing, and **water quality** is a major factor to be considered in the food processing industry. Water is used for washing, rinsing, blanching, cooling, and as an ingredient in formulations. Microbiological safety of water used in food processing is very important. Also important, but generally not appreciated by the consuming public, is the mineral content of water used in food processing.

12-1 table Mineral Content of Selected Foods

Food Item	mg/g (Wet Weight Basis)		
	Calcium	Iron	Sodium
Cereals, bread, and pasta			
Rice, brown, long-grain, raw	23	2	7
Corn flakes, plain	3	19	950
White rice, long-grain, regular, real, enriched	28	4	5
Wheat flour, whole-grain	34	4	5
Wheat flour, white, all-purpose, unenriched	15	1	2
Macaroni, dry, enriched	21	3	6
Rye bread	73	3	660
Dairy products			
Milk, whole, fluid, 3.3% fat	110	<1	40
Evaporated milk, whole	260	<1	110
Butter, with salt	24	<1	580
Cream, fluid, half and half	110	<1	41
Cheese, cottage, low fat, 2% milk fat	91	<1	330
Yogurt, plain, low fat	200	<1	77
Fruits and vegetables			
Apples, raw, with skin	6	<1	1
Bananas, raw	5	<1	1
Cherries, sweet, raw	13	<1	<1
Raisins, seedless	50	2	11
Potatoes, raw, skin	30	3	10
Tomatoes, red, ripe, raw	10	<1	5
Meats, poultry, and fish			
Eggs, whole, raw, fresh	53	2	40
Fish fillet, battered or breaded, and fried	18	2	530
Pork, fresh, leg (ham), whole, raw	5	<1	47
Bologna, chicken, pork, beef	92	1	1,100
Chicken, broilers or fryers, breast meat only, raw	11	<1	65
Beef, chuck, arm pot roast, raw	16	2	74

From US Department of Agriculture, Agricultural Research Service (2009) USDA National Nutrient Database for Standard Reference. Release 22. Nutrient Data Laboratory Home Page, http://www.ars.usda.gov/ba/bhnrc/ndl

Waters that contain excessive minerals can result in clouding of beverages. Textural properties of fruits and vegetables can be influenced by the "**hardness**" or "**softness**" of the water used during processing.

12.2 BASIC CONSIDERATIONS

12.2.1 Nature of Analyses

Mineral analysis is a valuable model for understanding the basic structure of analysis procedures to separate and measure. Separation of minerals from the food matrix is often specific, such as **complexometric titrations** (Sect. 12.3.1) or **precipitation titrations** (Sect. 12.3.2). In these cases of specific separation, nonspecific measurements such as volume of titrant are made and are later converted to mass of mineral based on fundamental stoichiometric relationships. In other cases, separation of mineral involves nonspecific procedures such as **ashing** or **acid extraction**. These nonspecific separations require that a specific measurement be made as provided by **colorimetry** (Sect. 12.3.3), **ion-selective electrodes** (ISE) (Sect. 12.3.4), **atomic absorption spectroscopy**, or **inductively coupled plasma-atomic emission spectroscopy** (Chap. 24).

Because determination of mass of mineral is the final objective of analysis, measures other than mass are considered to be surrogate, or stand-in, measures. **Surrogate measures** are converted into mass of mineral via fundamental stoichiometric and physiochemical relationships or by empirical relationships. Empirical relationships are those associations that need to be established by experimentation because they do not follow any well-established physiochemical relationship. An example of a surrogate measurement is the absorbance of a chromogen–mineral complex (Sect. 12.3.3). It may be possible to convert absorbance into mass of mineral using the fundamental relationships defined by the molar absorptivity and stoichiometry of the chromogen–mineral complex. However, it is more commonly required that the absorbance: concentration relationship be empirically developed using a series of standards (i.e., a standard curve).

12.2.2 Sample Preparation

Some sample preparation is generally required for traditional methods of mineral analysis to ensure a well-mixed and representative sample and to make the sample ready for the procedure to follow. A major concern in mineral analysis is **contamination** during sample preparation. **Comminution** (e.g., grinding or chopping) and mixing using metallic instruments can add significant mineral to samples and, whenever possible, should be performed using nonmetallic instruments or instruments not composed of the sample mineral. For example, it is standard practice in our laboratories to use an aluminum grinder for comminution of meat samples undergoing iron analysis. **Glassware** used in sample preparation and analysis should be scrupulously cleaned using acid washes and triple rinsed in the purest water. The latter may necessitate installation of an **ultrapure water system** in the laboratory to further purify the general supply of distilled water.

Solvents, including water, can contain significant quantities of minerals. Therefore, all procedures involving mineral analysis require the use of the

purest reagents available. In some cases, the cost of ultrapure reagents may be prohibitive. When this is the case, the alternative is to always work with a reagent blank. A **reagent blank** is a sample of reagents used in the sample analysis, quantitatively the same as that used in the sample but without any of the material being analyzed. This reagent blank, representing the sum of the mineral contamination in the reagents, is then subtracted from the sample values to more accurately quantify the mineral.

A method such as near-infrared spectroscopy (Chap. 23) allows for mineral estimation without destruction of the carbon matrix of carbohydrates, fats, protein, and vitamins that make up foods. However, traditional methods generally require that the minerals be freed from this organic matrix in some manner. Chapter 7 describes the various methods used to ash foods in preparation for determination of specific mineral components of the food. In water samples, minerals may be determined without further preparation.

12.2.3 Interferences

Factors such as **pH**, **sample matrix**, **temperature**, and other **analytical conditions** and **reagents** can interfere with the ability of an analytical method to quantify a mineral. Often there are specific interfering substances that must be removed or suppressed for accurate analysis. Two of the more common approaches are to isolate the sample mineral, or remove interfering minerals, using selective precipitations or ion exchange resins. Water may need to be boiled to remove carbonates that interfere with several traditional methods of mineral analysis.

If other interferences are suspected, it is a common practice to develop the standard curve using sample mineral dissolved in a background matrix containing interfering elements known to be in the food sample. For example, if a food sample is to be analyzed for calcium content, a **background matrix solution** of the known levels of sodium, potassium, magnesium, and phosphorus should be used to prepare the calcium standards for developing the standard curve. In this manner, the standard curve more closely represents the analysis response to the sample mineral when analyzing a food sample. Alternatively, the standard curve can be developed using a series of sample mineral spikes added to the food sample. A **spike** is a small volume of a concentrated standard that is added to the sample. The volume is small enough so as to not appreciably change the overall composition of the sample, except for the mineral of interest. Thus, measurements of both the standards and the sample are made in the presence of the same background. If the spikes are added before implementation of the analysis protocol, possible effects of incomplete extractions, sample mineral degradation, and other losses are integrated into the standard curve.

12.3 METHODS

12.3.1 EDTA Complexometric Titration

12.3.1.1 Principles

The hexadentate ligand **ethylenediaminetetraacetate** (EDTA) forms stable 1:1 complexes with numerous mineral ions. This gives complexometric titration using EDTA broad application in mineral analysis. Stability of mineral–EDTA complexes generally increases with valence of the ion, although there is significant variation among ions of similar valence due to their coordination chemistry. The complexation equilibrium is strongly pH dependent. With decreasing pH the chelating sites of EDTA become protonated, thereby decreasing its effective concentration. Endpoints are detected using mineral chelators that have coordination constants lower than EDTA (i.e., less affinity for mineral ions) and that produce different colors in each of their complexed and free states. **Calmagite** and **Eriochrome Black T** (EBT) are such indicators that change from blue to pink when they complex with calcium or magnesium. The endpoint of a complexometric EDTA titration using either Calmagite or EBT as the indicator is detected as the color changes from pink to blue.

The pH affects a complexometric EDTA titration in several ways and must be controlled for best performance. The pH must be 10 or more for calcium or magnesium to form stable complexes with EDTA. Also, the sharpness of the endpoint increases with increasing pH. However, magnesium and calcium precipitate as their hydroxides at pH 12, and titration pH should probably be no more than 11 to ensure their solubility. Considering all factors, EDTA complexometric titration of calcium and magnesium is specified at pH 10 ± 0.1 using an ammonia buffer (10).

12.3.1.2 Procedure: Hardness of Water Using EDTA Titration

Water hardness is determined by EDTA complexometric titration of the total of calcium and magnesium, in the presence of Calmagite, and expressed as the equivalents of calcium carbonate (mg/L) (*Standard Methods for the Examination of Water and Wastewater*, Method 2340, Hardness) (10) (Fig. 12-1). The calcium–Calmagite complex is not stable, and calcium alone cannot be titrated using the Calmagite indicator. However, Calmagite becomes an effective indicator for

WATER HARDNESS-EDTA TITRATION

Titration of Water Sample

Dilute 25 ml sample (or such volume to require <15 ml titrant) to 50 ml in a flask.

⇓

Bring pH to 10±0.1 by adding 1–2 ml buffer solution (NH_4 in NH_4OH, combined with Na_2EDTA and $MgSO_4$ or $MgCl_2$) and 1–2 drops Calmagite indicator solution.

⇓

Titrate with a standard solution of ca. 0.01 *M* EDTA to a blue endpoint.

Standardization of EDTA

Weigh 1.000 mg $CaCO_3$ into a 500-ml Erlenmeyer flask and add HCl (1 : 1 dilution with water) until dissolved. Add 200 ml H_2O and boil a few minutes to expel CO_2. Let cool.

⇓

Add a few drops of methyl red indicator and adjust to intermediate orange color with 3 *N* NH_4OH or HCl (1 : 1) as required. Transfer to 1 L flask and dilute to volume.

⇓

Titrate calcium standard solution with EDTA solution, to Calmagite endpoint.

⇓

Determine $CaCO_3$ equivalents as mg $CaCO_3$/ml EDTA solution.

Calculations

Hardness (EDTA) as mg $CaCO_3$/L = (mg $CaCO_3$/ml EDTA x ml EDTA)/L sample

12-1 figure Procedure for determination of water hardness by EDTA titration. *Standard Methods for the Examination of Water and Wastewater*, Method 2340, Hardness. [Adapted from (10).]

calcium titration if we include in the buffer solution a small amount of neutral magnesium salt and enough EDTA to bind all magnesium. Upon mixing sample into the buffer solution, calcium in the sample replaces the magnesium bound to EDTA. The free magnesium binds to Calmagite, and the pink magnesium–Calmagite complex persists until all calcium in the sample has been titrated with EDTA. The first excess of EDTA removes magnesium from Calmagite and produces a blue endpoint.

12.3.1.3 Applications

The major application of EDTA complexometric titration is testing calcium plus magnesium as an indicator of water hardness (10). However, EDTA complexometric titration is suitable for determining calcium in the ash of fruits and vegetables (AOAC Method 968.31) (5) and other foods that have calcium without appreciable magnesium or phosphorus. The water hardness application of the EDTA complexometric titration is made easy using test strips impregnated with Calmagite and EDTA (e.g., **AquaChek**, Environmental Test Systems, Inc., a HACH Company, Elkhart, IN). The strips are dipped into the water to test for total hardness caused by calcium and magnesium. The calcium displaces the magnesium bound to EDTA, and the released magnesium binds to Calmagite, causing the test strip to change color.

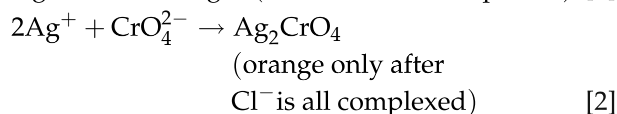
12.3.2 Precipitation Titration

12.3.2.1 Principles

When at least one product of a titration reaction is an insoluble precipitate, it is referred to as **precipitation titrimetry**. Few of the many gravimetric methods, however, can be adapted to yield accurate volumetric methods. Some of the major factors blocking the adaptation are long times necessary for complete precipitation, failure of the reaction to yield a single product of definite composition, and lack of an endpoint indicator for the reaction.

Nonetheless, precipitation titration has resulted in at least two methods that are used widely in the food industry today. The **Mohr method** for chloride determination is a direct or **forward titration** method, based on the formation of an orange-colored solid, silver chromate, after silver from silver nitrate has complexed with all the available chloride.

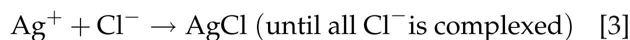
$$Ag^{+} + Cl^{-} \rightarrow AgCl \text{ (until all } Cl^{-} \text{ is complexed)} \quad [1]$$



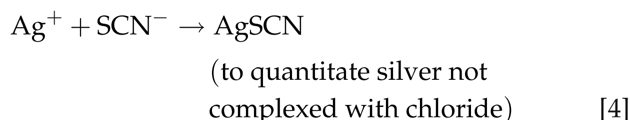
$$2Ag^{+} + CrO_4^{2-} \rightarrow Ag_2CrO_4 \text{ (orange only after } Cl^{-} \text{ is all complexed)} \quad [2]$$

The **Volhard method** is an indirect or **back-titration** method in which an excess of a standard solution of silver nitrate is added to a chloride-containing sample solution. The excess silver is then back-titrated using

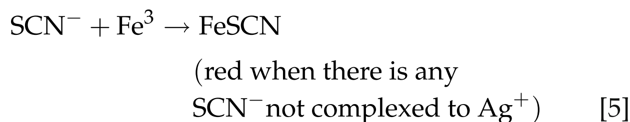
a standardized solution of potassium or ammonium thiocyanate with ferric ion as an indicator. The amount of silver that is precipitated with chloride in the sample solution is calculated by subtracting the excess silver from the original silver content.



$$Ag^+ + Cl^- \rightarrow AgCl \text{ (until all } Cl^- \text{is complexed)} \quad [3]$$



$$Ag^+ + SCN^- \rightarrow AgSCN \text{ (to quantitate silver not complexed with chloride)} \quad [4]$$



$$SCN^- + Fe^3 \rightarrow FeSCN \text{ (red when there is any } SCN^- \text{not complexed to } Ag^+) \quad [5]$$

12.3.2.2 Procedures

12.3.2.2.1 Mohr Titration of Salt in Butter (AOAC Method 960.29) Salt in foods may be estimated by titrating the chloride ion with silver (Fig. 12-2). The orange endpoint in this reaction occurs only when all chloride ion is complexed, resulting in an excess of silver to form the colored silver chromate. The endpoint of this reaction is therefore at the first hint of an orange color. When preparing reagents for this assay, use boiled water to avoid interferences from carbonates in the water.

12.3.2.2.2 Volhard Titration of Chloride in Plant Material (AOAC Method 915.01) In the Volhard method (Fig. 12-3), water must be boiled to minimize errors due to interfering carbonates, because the solubility product of silver carbonate is much less than the solubility product of silver chloride. Once chloride is determined by titration, the chloride weight is multiplied by 1.648 to obtain salt weight, if salt content is desired.

12.3.2.3 Applications

Precipitation titration methods are well suited for any foods that may be high in chlorides. Because of added salt in processed cheeses and meats, these products should certainly be considered for using this method to detect chloride; then salt content is estimated by calculation. Precipitation titrations are easily automated, thus ensuring that these traditional methods will see continued use in the analytical food laboratory. For example, the automatic titration system commonly used to rapidly measure the salt content of potato

SALT — MOHR TITRATION

Titration of Butter Sample

Weigh about 5 g of butter into 250-ml Erlenmeyer flask and add 100 ml of boiling H_2O.

⇓

Let stand 5–10 min with occasional swirling.

⇓

Add 2 ml of a 5% solution of K_2CrO_4 in d H_2O.

⇓

Titrate with 0.1 *N* $AgNO_3$ standardized as below until an orange-brown color persists for 30 sec.

Standardization of 0.1 *N* $AgNO_3$

Accurately weigh 300 mg of recrystallized dried KCl and transfer to a 250-ml Erlenmeyer flask with 40 ml of water.

⇓

Add 1 ml of K_2CrO_4 solution and titrate with $AgNO_3$ solution until first perceptible pale red-brown appears.

⇓

From the titration volume subtract the milliliters of the $AgNO_3$ solution required to produce the endpoint color in 75 ml of water containing 1 ml of K_2CaO_4.

⇓

From the next volume of $AgNO_3$ calculate normality of the $AgNO_3$ as:

$$\text{Normality } AgNO_3 = \frac{\text{mg KCl}}{\text{ml } AgNO_3 \times 74.555 \text{ g KCl/mole}}$$

Calculating Salt in Butter

$$\text{Percent salt} = \frac{\text{ml } 0.1\,N\ AgNO_3 \times 0.585}{\text{g of sample}}$$

[0.585 = (58.5 g NaCl/mol)/100]

12-2 figure Procedure of Mohr titration of salt in butter. AOAC Method 960.29 [Adapted from (5)].

SALT — VOLHARD TITRATION

Titration of Sample

Moisten 5 g of sample in crucible with 20 ml of 5% Na_2CO_3 in water.

⇓

Evaporate to dryness.

⇓

Char on a hot plate under a hood until smoking stops.

⇓

Combust at 500°C for 24 hr.

⇓

Dissolve residue in 10 ml of 5 *N* HNO_3.

⇓

Dilute to 25 ml with d H_2O.

⇓

Titrate with standardized $AgNO_3$ solution (from the Mohr method) until white AgCl stops precipitating and then add a slight excess.

⇓

Stir well, filter through a retentive filter paper, and wash AgCl thoroughly.

⇓

Add 5 ml of a saturated solution of $FeNH_4(SO_4)2 \bullet 12H_2O$ to the combined titrate and washings.

⇓

Add 3 ml of 12 *N* HNO_3 and titrate excess silver with 0.1 *N* potassium thiocyanate.

Standardization of Potassium Thiocyanate Standard Solution

Determine working titer of the 0.1 *N* potassium thiocyanate standard solution by accurately measuring 40–50 ml of the standard $AgNO_3$ and adding it to 2 ml of $FeNH_4(SO_4)2 \cdot 12H_2O$ indicator solution and 5 ml of 9 *N* HNO_3.

⇓

Titrate with thiocyanate solution until solution appears pale rose after vigorous shaking.

Calculating Cl Concentration

Net volume of the $AgNO_3$ = Total volume $AgNO_3$ added – Volume titrated with thiocyanate

1 ml of 0.1 *M* $AgNO_3$ = 3.506 mg chloride

12-3 figure Procedure for Volhard titration of chloride in plant material. AOAC Method 915.01. [Adapted from (5).]

chips is simply doing a Mohr titration. Also, the **Quantab® chloride titration** used in AOAC Method 971.19 is an adaptation of the principles involved in the Mohr titration method. This test strip adaptation allows for very rapid quantitation of salt in food products and is accurate to ±10% over a range of 0.3–10% NaCl in food products.

12.3.3 Colorimetric Methods

12.3.3.1 Principles

Chromogens are chemicals that, upon reaction with the compound of interest, form a colored product. Chromogens are available that selectively react with a wide variety of minerals. Each chromogen reacts with its corresponding mineral to produce a soluble colored product that can be quantified by absorption of light at a specified wavelength. The relationship between concentration and absorbance is given by **Beer's law** as detailed in Chap. 22. Generally, concentration of mineral in a sample is determined from a standard curve developed during the analysis, although in some cases it is possible to directly calculate concentration based on molar absorptivity of the chromogen–mineral complex.

Samples generally must be ashed or treated in some other manner to isolate and/or release the minerals from organic complexes that would otherwise inhibit their reactivity with the chromogen. The mineral of interest must be solubilized from a dry ash and subsequently handled in a manner that prevents its precipitation. The soluble mineral may need to be treated (e.g., reduced or oxidized) to ensure that all mineral is in a form that reacts with the chromogen (2). Ideally, the chromogen reacts rapidly to produce a stable product. This is not always the case in practice, and time constraints may be established for color development and reading of absorbance. As with all mineral analysis of food, special efforts must be put in place to avoid contamination during sampling and analysis.

12.3.3.2 Procedures: Colorimetric Determination of Iron in Meat

The total iron content of foods can be quantified spectrophotometrically as shown in Fig. 12-4. In this method, the absorption of light at 562 nm is converted to iron concentration in the sample via a regression equation generated from a standard curve developed during the analysis using a standard solution. In meat systems, this method has been coupled with a method specific for heme iron to determine the ratio of heme iron to total iron, which is important nutritionally as the former is more bioavailable (11). Another interesting aspect of this method of interest to the food scientist is the fact that the ferrozine reagent only reacts with ferrous iron, and not ferric. The addition of ascorbic acid in the second to last step is necessary to convert all ionic iron to the detectable ferrous form. Repeating the procedure with and without ascorbic acid allows determination of total and ferrous ionic iron, respectively. Ferric iron is calculated by difference.

12.3.3.3 Applications

Colorimetry is used for the detection and quantification of a wide variety of minerals in food, and it is often a viable alternative to atomic absorption spectroscopy and other mineral detection methods. Colorimetric methods generally are very specific and usually can be performed in the presence of other minerals, thereby avoiding extensive separation to isolate the mineral of interest. They are particularly robust and often immune to matrix effects that can limit the usefulness of other methods for mineral analysis. With minimal effort and expense, many colorimetric methods will perform with precision and accuracy similar to that obtained by experienced personnel using atomic absorption spectroscopy (11).

12.3.4 Ion-Selective Electrodes

12.3.4.1 Principles

Many electrodes have been developed for the selective measurement of various cations and anions, such as bromide, calcium, chloride, fluoride, potassium, sodium, and sulfide (12, 13). The pH electrode described in Chap. 13 is a specific example of an **ISE**. For any ISE, an **ion-selective sensor** is placed such that it acts as a “bridging electrode” between two reference electrodes carefully designed to produce a constant and reproducible **potential**. The sensor can take on many forms (e.g., glass, single crystal, precipitate based, solvent polymer), although each provides an ion-selective electronic coupling that allows a potential to develop across the sensor. The exact mechanism(s) of charge transport across the sensor is not completely understood, but it is brought about by ion-selective species incorporated within the sensor itself and has been described by analogy to the response of billiard balls to an impact. If the ion-selective species within the sensor are imagined to be a row of billiard balls, it can be envisioned how the impact of sample ions on one sensor surface is translocated to the other surface. In this manner, the potential within the

IRON DETERMINATION OF MEAT—COLORIMETRIC ASSAY

Preparation of Standards

Prepare solutions of 10, 8, 6, 4, 2 μg iron/ml from a stock solution of 10 μg iron/ml.
Make dilutions using 0.1 *N* HCl.

Analysis of Sample

Place ~5 g sample into crucible and accurately weigh.

⇓

Heat on hot plate until well charred and sample has stopped smoking.

⇓

Ash in furnace at ca 550°C until ash is white.

⇓

Dissolve ash in small amount 1 *N* HCl and dilute to 50 ml volume with 0.1 *N* HCl

⇓

Transfer 0.500 ml of diluted sample and standards into 10 ml test tubes.

⇓

Add 1.250 ml ascorbic acid (0.02% in 0.2 *N* HCl, made fresh daily). Vortex and let set 10 min.

⇓

Add 2.000 ml 30% ammonium acetate. Vortex. (pH needs to be >3 for color development)

⇓

Measure absorbance at 562 nm. Determine iron concentration in sample digest (μg iron/ml) from standard curve.

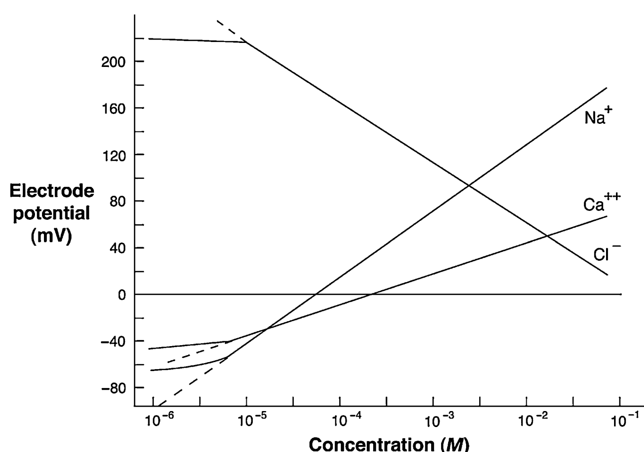
12-4 figure Procedure for determination of iron in meat by colorimetry. [Adapted from (11).]

sensor remains constant, while potentials develop at the sensor surfaces according to the **Nernst equation** (Sect. 13.3.2.2), dependent on sample **ion activity** in the solutions contacting each surface.

Typically, the inside surface of the ion-selective sensor is in contact with the **negative reference electrode** (anode) via a filling solution having a constant concentration of sample ion, while the outside surface of the ion-selective sensor is in contact with the **positive reference electrode** (cathode) via sample solutions having varying concentration of sample ion. Because sample ion activity of the internal solution is fixed, the potential varies across the sensor depending solely on the activity of sample ion in the sample solution. As described by the Nernst equation, the potential of the outside sensor surface increases by $0.059/n$V (where n is the number of electrons involved in the half reaction of the sample ion) for each tenfold increase in activity of a mineral cation. Conversely, the potential decreases by $0.059/n$V for each tenfold increase in activity of mineral anion. These changes have a direct effect on the overall ISE potential because the outside sensor surface is orientated toward the positive reference electrode. **Ion concentration** is generally substituted for ion activity, which is a reasonable approximation at low concentrations and controlled ionic strength environments. Indeed, this is observed within limitations set by electrode and instrumental capabilities (Fig. 12-5).

12.3.4.2 General Methodology

For ISE analysis, one simply attaches an ISE for the sample ion to a **pH meter** set on the **mV scale** and follows instructions for determination. However, the performance of an ISE must be considered when first



12-5 figure Examples of ion-selective electrode calibration curves for ions important in foods. (Courtesy of Van London pHoenix Co., Houston, TX.)

selecting an electrode and later when designing sampling and analysis protocols. Detailed information regarding the performance of specific ISEs is available from vendor catalogs. Typical ISEs likely to be employed for analysis of foods operate in the range of $1–10^{-6}$ M, although the electrode response may be distinctly nonlinear at the lower concentrations.

Electrode performance is affected by the presence of **interfering ions**, often with the strongest interference from those ions having size and charge similar to the ion of interest. Relative response of ISE to interfering ions may be expressed as selectivity coefficients or as concentration of interfering ion that leads to 10% error. If the selectivity coefficient relative to an interfering ion is 1000 (i.e., the ISE is 1000-fold more responsive to the sample ion than the interfering ion), 10% or greater error can be expected when measuring μM levels of the sample ion with interfering ion present at mM levels. Most ISEs operate over a broad pH range, although pH may need to be controlled for best performance. Minimum response times for ISEs fall in the range of 20 s to 1 min.

Despite inherent limitations of electrode design and construction, the analyst can adjust the sample and control measurement conditions to minimize many practical problems that otherwise limit the specificity and precision of ISEs. Because the ISE responds to ionic activity, it is important that the **activity coefficient** be kept constant in samples and calibration standards. The activity coefficient (γ) is used to relate ion activity (A) to ion concentration (C) ($A = \gamma C$). Activity coefficient is a function of ionic strength, so **ionic strength adjustment** (ISA) **buffers** are used to adjust the samples and standards to the same ionic strength. These ISA buffers are commercially available. The use of ISA buffers also adjusts the pH, which may be necessary if H^+ or OH^- activities affect the ion-specific sensor or if they interact with the analyte. In the case of metals having insoluble hydroxides, it is necessary to work at a pH that precludes their precipitation. Depending on the selectivity of the ISE, it may be necessary to remove interfering ions from the sample by selective precipitation or complexation.

In view of temperature effects on standard potentials and slopes of electrodes (see Nernst Equation [8] in Chap. 13), it is important to keep the electrode and solutions at a constant temperature. This may involve working in a room that is thermostatically controlled to 25 °C (one of the internationally accepted temperatures for electrochemical measurements), and allowing sufficient time for all samples and standards to equilibrate to this temperature. Solutions should be gently stirred during the measurement to attain rapid equilibrium and to minimize concentration gradients at the sensor surface. Finally, it is important to allow sufficient time for the electrode to stabilize

before taking a reading. ISEs may not completely stabilize within a practical timeframe, so a decision needs to be made of when to take the reading. The reading may be taken when the rate of change has fallen below some predetermined value or at a fixed time after the electrode was placed in solution. A problem with the latter is that many ISEs respond more rapidly, as samples are changed, to an increase in concentration of sample ion as compared to a decrease in concentration of sample ion.

12.3.4.3 Electrode Calibration and Determination of Concentration

In using an ISE, ion concentration can be determined using either a calibration curve, standard addition, or endpoint titration. It is common practice to develop a **calibration curve** when working with an ISE because it allows a large number of samples to be measured rapidly. The electrode potential (volts) is developed in a series of solutions of known concentration and plotted on **semilog paper** against the standard concentrations. Examples of calibration curves for various ions are given in Fig. 12-5. Upon analysis of a test sample, the observed electrode potential is used to determine ion concentration by referring to the calibration curve. Note the nonlinear region of the curve at the lowest concentrations. Total ionic strength and the concentration of interfering ions are especially important factors limiting selective detection of low levels of ions.

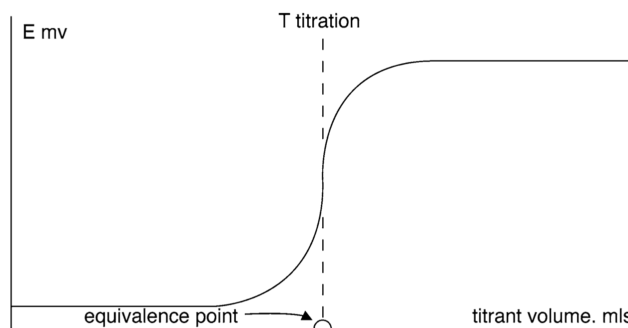
The **standard addition method** is of great value when only a few samples are to be measured and time does not permit the development of a calibration curve. This method also eliminates complex and unknown background effects that cannot be replicated when developing a calibration curve using standards. The ISE is immersed in the sample and the resulting voltage is recorded (E_{sample}). An aliquot, or **spike**, containing a known amount of the measured species is added to the sample, and a second measurement of electrode potential is determined (E_{spike}). Concentration of active species in the original sample is determined from the absolute difference in the voltage readings ($\Delta E = |E_{\text{spike}} - E_{\text{sample}}|$) according to the following relationship algebraically derived from the Nernst equations.

$$C_O = \frac{C_\Delta}{(10^{\Delta E/S} - 1)} \quad [6]$$

where:

C_O = original concentration of sample ion (mol/L)

C_Δ = change in sample ion concentration when spike was added (mol/L)



12-6 figure A typical T-type titration. [From (2), used with permission.]

E = difference in potential between the two readings (V)

S = 0.059/number of electrons in the 1/2 reaction of the sample ion

Finally, ISEs can be used to detect the **endpoints of titrations** using species that form a precipitate or strong complex with the sample ion. If an ISE is selected that detects titrant species, a T-type titration curve results from the large increase in titrant activity detected at the equivalence point (see Fig. 12-6 for a cation titrant). If an ISE is selected that detects the sample ion, an S-type titration curve results from the removal of sample ion activity at the equivalence point. In either case, sample concentration is calculated from titrant volume to reach the equivalence point and the stoichiometric relationship between titrant species and sample ion.

12.3.4.4 Applications

Some examples of applications of ISEs are salt and nitrate in processed meats, salt content of butter and cheese, calcium in milk, sodium in low-sodium ice cream, carbon dioxide in soft drinks, potassium and sodium levels in wine, and nitrate in canned vegetables. An ISE method applicable to foods containing $<$100 mg sodium/100 g is an official method of AOAC International (Method 976.25). This method employs a sodium combination ISE, pH meter, magnetic stirrer, and a semilog graph paper for plotting a standard curve. Obviously, there are many other applications, but the above serve to demonstrate the versatility of this valuable measuring tool.

A major *advantage* of ISEs is their ability to measure many anions and cations directly. Such measurements are relatively simple compared to most other analytical techniques, particularly because a pH meter may be used as the voltmeter. Analyses are independent of sample volume when making direct

measurements, while turbidity, color, and viscosity are all of no concern.

A major *disadvantage* in the use of ISEs is their inability to measure below 2–3 ppm, although there are some electrodes that are sensitive down to 1 part per billion. At low levels of measurement (below 10^{-4} M), the electrode response time is slow. Finally, some electrodes have had a high rate of premature failure or a short operating life and possible excessive noise characteristics.

12.4 COMPARISON OF METHODS

For labeling, processing, and even practical nutrition, we are concerned only with a few minerals, which generally can be analyzed by traditional methods. The traditional methods available for mineral analysis are varied, and only a very limited number of examples have been given in this chapter. Choice of methods for mineral analysis must be made considering method performance regarding accuracy, sensitivity, detection limit, specificity, and interferences. Information on method performance is available from the collaborative studies referenced with official methods (Chap. 1). Other factors to be considered include cost per analysis completed, equipment availability, and analytical time compared to analytical volume.

Generally, for a small laboratory with skilled analytical personnel, the traditional methods can be carried out rapidly, with accuracy, and at minimal costs. If a large number of samples of a specific element are to be run, there is certainly a time factor in favor of using atomic absorption spectroscopy or emission spectroscopy, depending on the mineral being analyzed. The graphite furnace on the atomic absorption spectrophotometer is capable of sensitivity in the parts per billion range. This is beyond the limits of the traditional methods. However, for most minerals of practical concern in the food industry, this degree of sensitivity is not required.

Modern instrumentation has made it possible to quantify an entire spectrum of minerals in one process, some into the parts per billion range. Instrumentation capable of such analysis is expensive and beyond the financial resources of many quality assurance laboratories. Large numbers of samples to be analyzed may justify the automation of some routine analyses and perhaps the expense of some of the modern pieces of equipment. However, the requirements for only occasional samples to be analyzed for a specific mineral will not justify the initial costs of much instrumentation. This leaves the options of sending samples out to certified laboratories for analysis or utilizing one of the more traditional methods for analysis.

12.5 SUMMARY

The mineral content of water and foodstuffs is important because of their nutritional value, toxicological potential, and interactive effects with processing and texture of some foods. Traditional methods for mineral analysis include titrimetric and colorimetric procedures. The basic principles of these methods are described in this chapter, along with discussion of ISE methodology that has general application for mineral analysis.

Procedures are described in this chapter that illustrate use of these traditional methods to quantify minerals of concern in the food industry. These procedures generally require chemicals and equipment routinely available in an analytical laboratory and do not require expensive instrumentation. These methods may be suited to a small laboratory with skilled analytical personnel and a limited number of samples to be analyzed. The traditional procedures will often perform similarly to procedures requiring more instrumentation and may be more robust in actual practice.

Foods are typically ashed prior to traditional analyses because the methods generally require that the minerals be freed from the organic matrix of the foods. Sample preparation and analysis must include steps necessary to prevent contamination or loss of volatile elements and must deal with a variety of potential interferences. Various approaches are described to account for these possible errors including use of reagent blanks, addition of spikes, and development of standard curves using appropriate mineral matrix background.

Traditional methods for mineral analysis are often automated or adapted to test kits for rapid analysis. Tests for water hardness and the Quantab® for salt determination are examples currently being used. The basic principles involved in traditional methods will continue to be utilized to develop inexpensive rapid methods for screening mineral content of foods and beverages. Familiarity with the traditional principles will allow the food analyst to obtain the best possible performance with the kits and adapt to problems that may be encountered.

12.6 STUDY QUESTIONS

1. What is the major concern in sample preparation for specific mineral analysis? How can this concern be addressed?
2. If the ammonia buffer is pH 11.5 rather than pH 10 in the EDTA complexometric titration to determine the hardness of water, would you expect to overestimate or underestimate the hardness? Explain your answer.

3. This chapter includes descriptions of the EDTA complexometric titration method and ISE methodology for quantifying calcium. Differentiate these techniques with regard to the principles involved, and discuss primary advantages and disadvantages of these two techniques.
4. The Mohr and Volhard titration methods often are used to determine the NaCl content of foods. Compare and contrast these two methods, as you explain the principles involved.
5. In a back-titration procedure, would overshooting the endpoint in the titration cause an over- or underestimation of the compound being quantified? Explain your answer.
6. Describe how and why to employ standards in background matrix, spikes, and reagent blanks.
7. Explain the principles of using an ISE to measure the concentration of a particular inorganic element in food. List the factors to control, consider, or eliminate for an accurate measure of concentration by the ISE method.
8. You have decided to purchase an ISE to monitor the sodium content of foods produced by your plant. List the advantages this would have over the Mohr/Volhard titration method. List the problems and disadvantages of ISE that you should anticipate.
9. What factors should be considered in selecting a specific method for mineral analysis for a food product?

12.7 PRACTICE PROBLEMS

1. If a given sample of food yields 0.750 g of silver chloride in a gravimetric analysis, what weight of chloride is present?
2. A 10-g food sample was dried, then ashed, and analyzed for salt (NaCl) content by the Mohr titration method $(AgNO_3 + Cl \rightarrow AgCl)$. The weight of the dried sample was 2 g, and the ashed sample weight was 0.5 g. The entire ashed sample was titrated using a standardized $AgNO_3$ solution. It took 6.5 ml of the $AgNO_3$ solution to reach the endpoint, as indicated by the red color of Ag_2CO_4 when K_2CrO_4 was used as an indicator. The $AgNO_3$ solution was standardized using 300 mg of dried KCl as described in Fig. 12-2. The corrected volume of $AgNO_3$ solution used in the titration was 40.9 ml. Calculate the salt (NaCl) content of the original food sample as percent NaCl (wt/wt).
3. A 25-g food sample was dried, then ashed, and finally analyzed for salt (NaCl) content by the Volhard titration method. The weight of the dried sample was 5 g, and the ashed sample weighed 1 g. Then 30 ml of 0.1 *N* $AgNO_3$ was added to the ashed sample, the resultant precipitate was filtered out, and a small amount of ferric ammonium sulfate was added to the filtrate. The filtrate was then titrated with 3 ml of 0.1 *N* KSCN to a red endpoint.
   (a) What was the moisture content of the sample, expressed as percent H_2O (wt/wt)?
   (b) What was the ash content of the sample, expressed as percent ash (wt/wt) on a dry weight basis?
   (c) What was the salt content of the original sample in terms of percent (wt/wt) NaCl? (molecular weight Na = 23; molecular weight Cl = 35.5)
4. Compound X in a food sample was quantified by a colorimetric assay. Use the following information and Beer's law to calculate the content of Compound X in the food sample, in terms of mg Compound X/100 g sample:
   (a) A 4-g sample was ashed.
   (b) Ashed sample was dissolved with 1 ml of acid and the volume brought to 250 ml.
   (c) A 0.75-ml aliquot was used in a reaction in which the total volume of the sample to be read in the spectrophotometer was 50 ml.
   (d) Absorbance at 595 nm for the sample was 0.543.
   (e) The absorptivity constant for the reaction (i.e., extinction coefficient) was known to be 1574 L/M cm.
   (f) Inside diameter of cuvette for spectrophotometer was 1 cm.
5. Colorimetric analysis
   (a) You are using a colorimetric method to determine the concentration of Compound A in your liquid food sample. This method allows a sample volume of 5 ml. This volume must be held constant but can comprise diluted standard solution and water. For this standard curve, you need standards that contain 0, 0.25, 0.50, 0.75, and 1.0 mg of Compound A. Your stock standard solution contains 5 g/L of Compound A.

   Devise a dilution scheme(s) for preparing the samples for this standard curve that could be followed by a lab technician. Be specific. In preparing the dilution scheme, use no volumes less than 0.5 ml.
   (b) You obtain the following absorbance values for your standard curve:

Sample (mg)	*Absorbance (500 nm)*
0.00	0.00
0.25	0.20
0.50	0.40
0.75	0.60
1.00	0.80

   Construct a standard curve and determine the equation of the line.
   (c) A 5-ml sample is diluted to 500 ml, and 3 ml of this solution is analyzed as per the standard samples; the absorbance of 0.50 units at 500 nm. Use the equation of the line calculated in part (b) and information about the dilutions to calculate what the concentration is of Compound A in your original sample in terms of g/L.
6. What is the original concentration of copper in a 100-ml sample that shows a potential change of 6 mV after the addition of 1 ml of 0.1 *M* $Cu(NO_3)_2$?

Answers

1.

$$\frac{x \text{ g Cl}}{0.750 \text{ g AgCl}} = \frac{35.45 \text{ g/mol}}{143.3 \text{ g/mol}}$$

$$x = 0.186 \text{ g Cl}$$

2.

$$N\text{AgNO}_3 = \frac{0.300\,\text{g KCl}}{\text{ml AgNO}_3 \times 74.555\,\text{g KCl/mol}}$$

$$0.0984\,N = \frac{0.300\,\text{g}}{40.9\,\text{ml} \times 74.555}$$

Percent salt

$$= \left(\frac{0.0065\,\text{L} \times 0.0984\,N\,\text{AgNO}_3 \times 58.5\,\text{g/mol}}{10\,\text{g}}\right) \times 100$$

Percent salt = 0.37%

3.

(a) $$\frac{25\,\text{g wet sample} - 5\,\text{g dry sample}}{25\,\text{g wet sample}} \times 100 = 80\%$$

(b) $$\frac{1\,\text{g ash}}{5\,\text{g dry sample}} \times 100 = 20\%$$

(c) mol Ag added = mol Cl^- in sample +mol SCN^- added

$$\text{mol Ag} = (0.1\,\text{mol/L}) \times (0.03\,\text{L}) = 0.003\,\text{mol}$$

$$\text{mol SCN}^- = (0.1\,\text{mol/L}) \times (0.003\,\text{L}) - 0.0003\,\text{mol}$$

$$0.003\,\text{mol Ag} = \text{mol Cl}^- + 0.0003\,\text{mol SCN}^-$$

$$0.0027\,\text{mol} = \text{mol Cl}^-$$

$$(0.0027\,\text{mol Cl}^-) \times \frac{58.5\,\text{g NaCl}}{\text{mol}} = 0.1580\,\text{g NaCl}$$

$$\frac{0.1580\,\text{g NaCl}}{25\,\text{g wet sample}} = \frac{0.00632\,\text{g NaCl}}{\text{g wet sample}} \times 100$$

$$= 0.63\%\,\text{NaCl (w/w)}$$

4.

$$A = abc$$

$$0.543 = (1574\,\text{L g}^{-1}\,\text{cm}^{-1})(1\,\text{cm})\,c$$

$$c = 3.4498 \times 10^{-4}\,\text{g/L}$$

$$c = 3.4498 \times 10^{-4}\,\text{mg/ml}$$

$$\frac{3.4498 \times 10^{-4}\,\text{mg}}{\text{ml}} \times 50\,\text{ml} = 1.725 \times 10^{-2}\,\text{mg}$$

$$\frac{1.725 \times 10^{-2}\,\text{mg}}{0.75\,\text{ml}} \times \frac{250\,\text{ml}}{4\,\text{g}} = 1.437\,\text{mg/g}$$

$$= 143.7\,\text{mg/100 g}$$

5.

(a) Lowest dilution volume for 1 ml of stock:

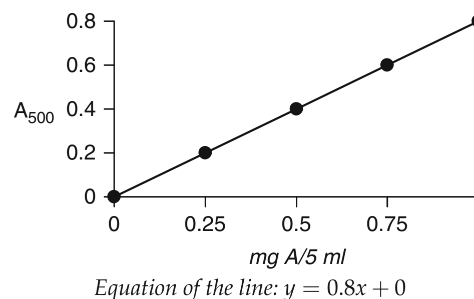
$$\frac{0.25\,\text{mg}}{0.5\,\text{ml}} = \frac{1\,\text{ml}}{x\,\text{ml}} \times \frac{5\,\text{mg}}{\text{ml}}$$

$$x = 10\,\text{ml}$$

Therefore, use a volumetric pipette to add 1 ml of stock to a 10-ml volumetric flask. Bring to volume with ddH_20 to give a diluted stock solution of 0.25 mg/0.5 ml. Use this to make up standards according to the following table.

mg A/5 ml	*ml Diluted stock solution*	*ml H_2O*
0	0	5.0
0.25	0.5	4.5
0.50	1.0	4.0
0.75	1.5	3.5
1.0	2.0	3.0

(b)



Equation of the line: $y = 0.8x + 0$

(c)

$$A_{500} = 0.50 = y$$

$$0.50 = 0.8x + 0$$

$$x = 0.625$$

$$\frac{0.625\,\text{mg}}{5\,\text{ml}} \times \frac{5\,\text{ml}}{3\,\text{ml}} \times \frac{500\,\text{ml}}{5\,\text{ml}} = 20.8\,\text{mg/ml}$$

$$= 20.8\,\text{g/L}$$

6.

$$C_O = \frac{0.001\,\text{L} \times \frac{0.001\,\text{moles}}{\text{L}} \times \frac{1}{0.100\,\text{L}}}{10^{0.006/0.0285} - 1} = 1.6\,\text{m}M$$

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