10 chapter

# Carbohydrate Analysis

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S.S. Nielsen, *Food Analysis*, Food Science Texts Series, DOI 10.1007/978-1-4419-1478-1_10,

## 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<br>D-Glucose |
| Lactose | In milk and products derived from milk | D-Galactose<br>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<br>D-Fructose<br>D-Galactose |
| Stachyose | Small amounts in beans | D-Glucose<br>D-Fructose<br>D-Galactose |
| **Polysaccharides** | | |
| Starch[b] | Widespread in cereal grains and tubers. Added to processed foods. | D-Glucose |
| **Food gums/hydrocolloids**[c] | | |
| Algins<br>Carboxymethylcelluloses<br>Carrageenans<br>Curdlan<br>Gellan<br>Guar gum<br>Gum arabic<br>Hydroxypropylmethyl-celluloses<br>Inulin<br>Konjac glucomannan<br>Locust bean gum<br>Methylcelluloses<br>Pectins<br>Xanthan | Added as ingredients | [d] |
| **Cell-wall polysaccharides**[c] | | |
| Pectin (native)<br>Cellulose<br>Hemicelluloses<br>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]

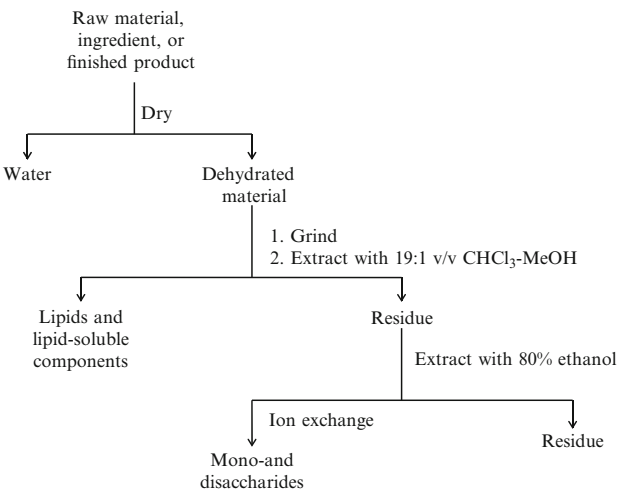
| *Food* | *Approximate Percent Carbohydrate (Wet Weight Basis)* |
|---|---|
| Cereals, bread, and pasta | |
| Corn flakes | 80.4 |
| Macaroni, dry, enriched | 74.7 |
| Bread, white, commercially prepared | 50.6 |
| Dairy products | |
| Ice cream, chocolate | 28.2 |
| Yogurt, plain, low fat (12 g protein/8 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.

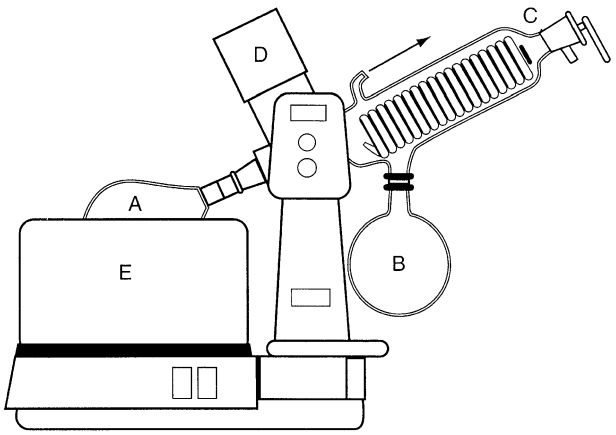
The 80% ethanol extract will contain components other than carbohydrates, in particular ash, pigments, organic acids, and perhaps free amino acids and low-molecular-weight peptides. Because the mono- and oligosaccharides are neutral and the contaminants are charged, the contaminants can be removed by **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

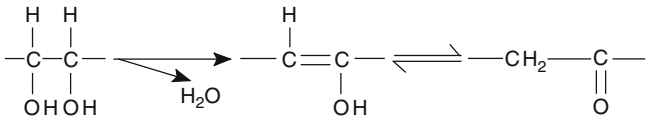
#### *10.3.2.1 Principle and Characteristics*

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.

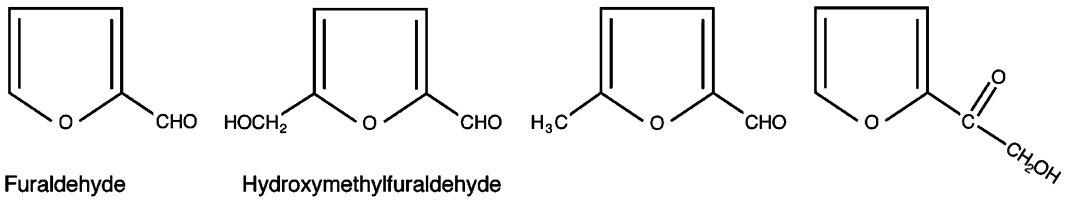
#### *10.3.2.2 Outline of Procedure*

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*

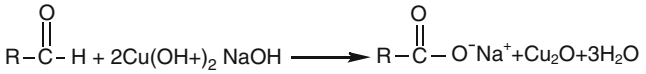
*10.3.3.1.1 Principle* Oxidation is a loss of electrons; reduction is a gain of electrons. Reducing sugars are those sugars that have an aldehydo group (aldoses) that can give up electrons (i.e., act as a reducing agent)




**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.




[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

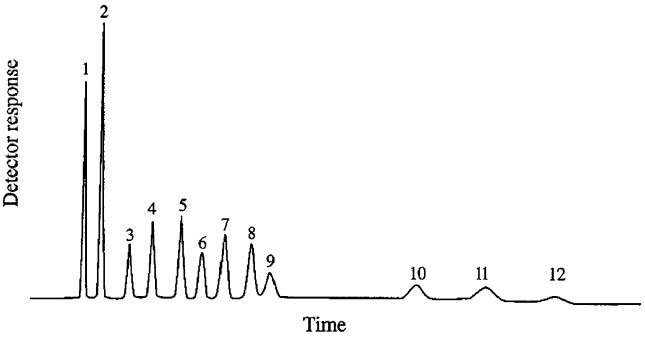
### *10.3.4.1 High-performance Liquid Chromatography*

**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 p$K_a$ 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]




**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.

and of providing separation of homologous series of oligosaccharides into their components (27,28).

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.

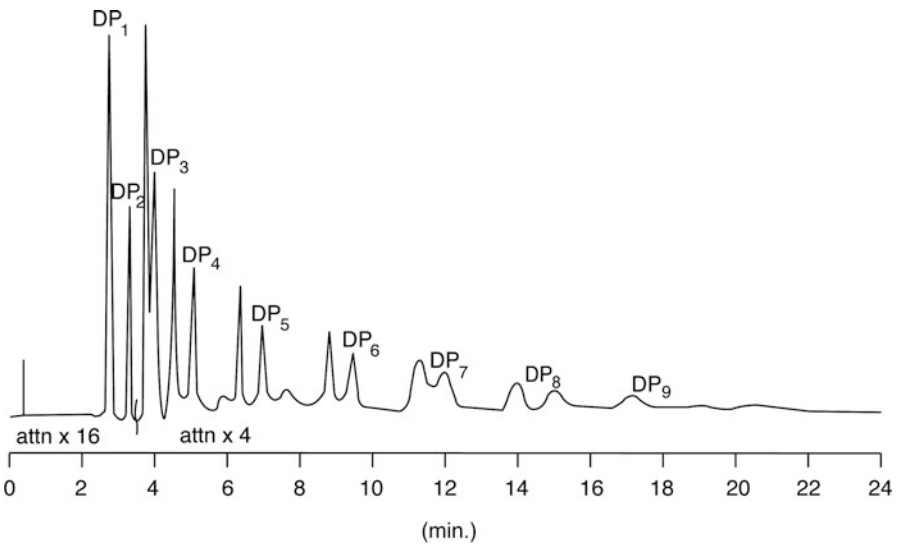
4. **Reversed-phase chromatography**. In reversed-phase chromatography, the stationary phase is hydrophobic, and the mobile phase is largely water. The hydrophobic stationary phase is made by reacting silica gel with a reagent that adds alkyl chains, such as an 18-carbon-atom alkyl chain (a C18 column) 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.

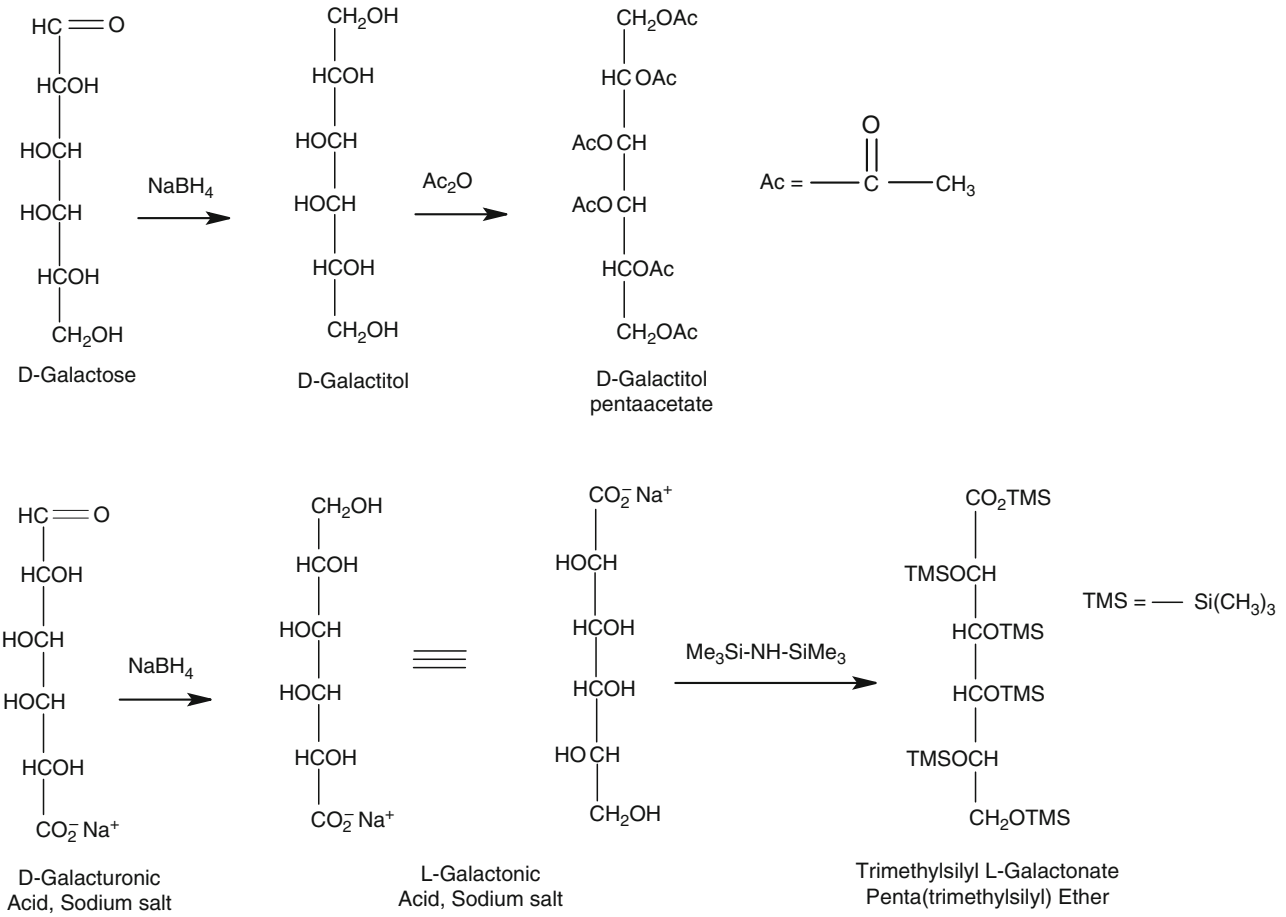
#### *10.3.4.2.1 Neutral Sugars: Outline of Procedure (38)*

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.

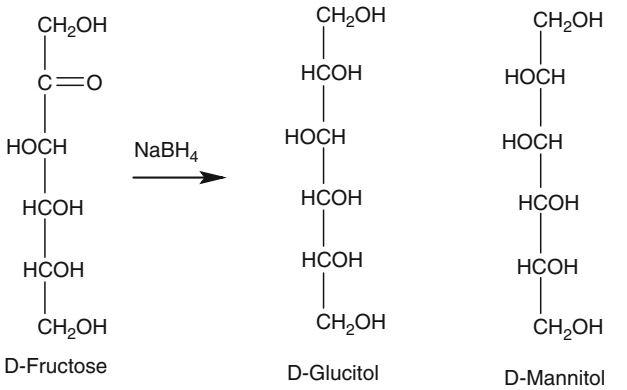
A potential problem is that, if fructose is present, either as a naturally occurring sugar, from the hydrolysis of inulin, or as an additive [from high fructose syrup (HFS), invert sugar, or honey], it will be reduced to a mixture of D-glucitol (sorbitol) and D-mannitol (Fig. 10-7).

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 peracetate 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**.

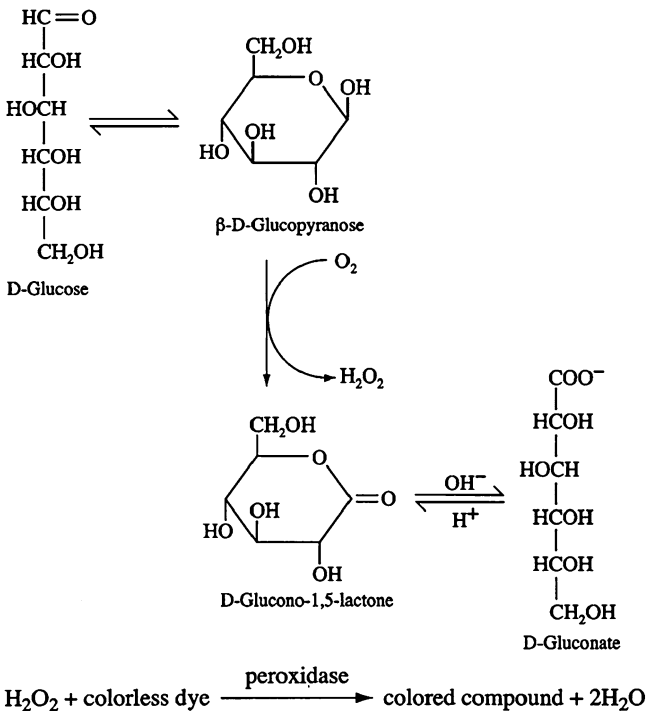
**10-3 table** **Selected Enzymic Methods of Carbohydrate Analysis**

| *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, pre-column 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

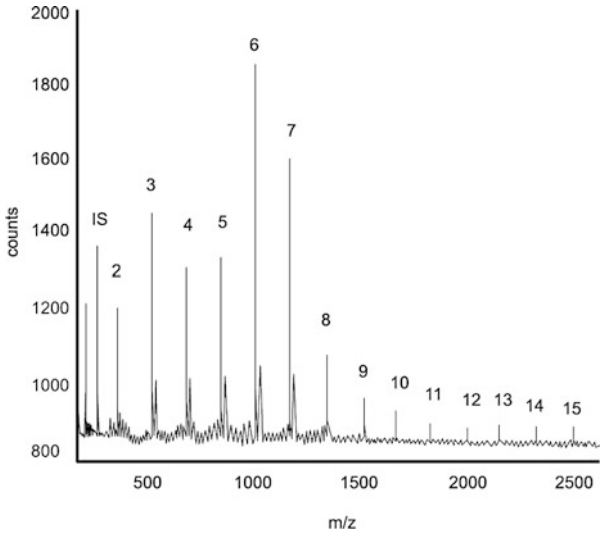
Starch is second only to water as the most abundant component of food. Starch is found in all parts of plants (leaves, stems, roots, tubers, seeds). A variety of commercial starches are available worldwide as food additives. These include corn (maize), waxy maize, high-amylose corn (amylomaize), potato, 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*

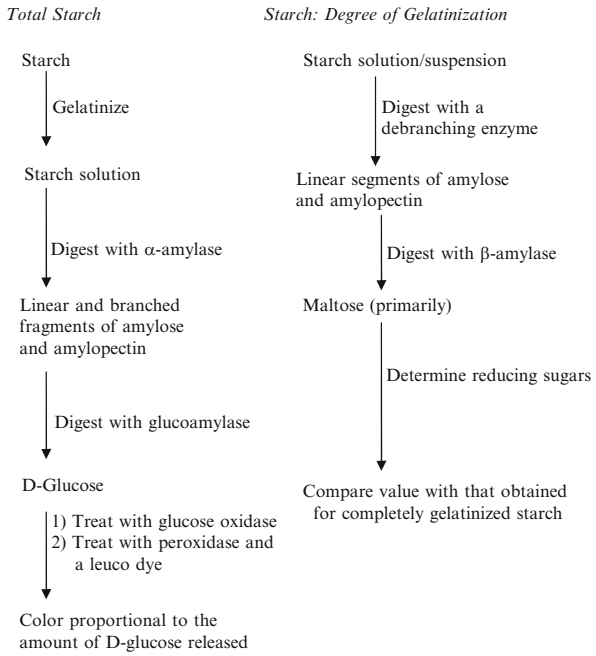
*10.4.1.1.1 Principle* The only reliable method for determination of total starch is based on complete conversion of the starch into D-glucose by purified enzymes specific for starch and determination of the D-glucose released by an enzyme specific for it (Fig. 10-8) (see also Chap. 16).

*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.

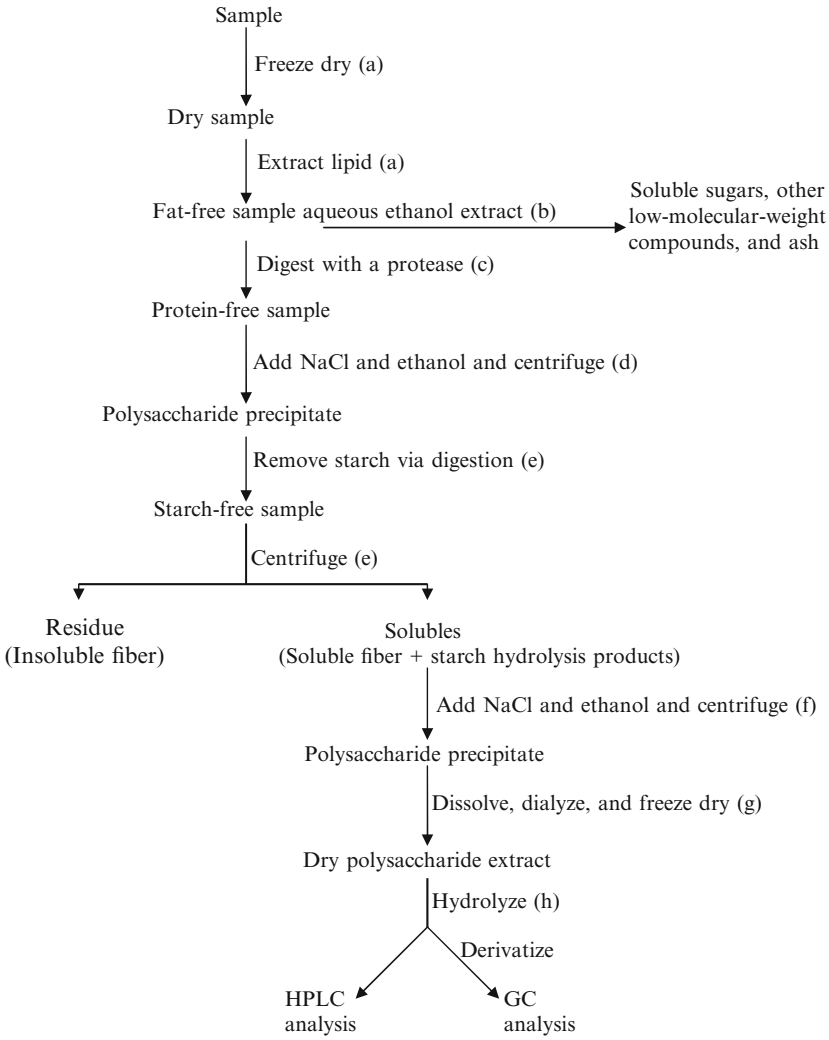
(b) Although not in the published scheme, soluble sugars, other low-molecular-weight compounds, and ash can be removed at this point using hot 80% ethanol as described in Sect. 10.3.1. (Hot 80% methanol has also been used.)

(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

**10-4 table** **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 |

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

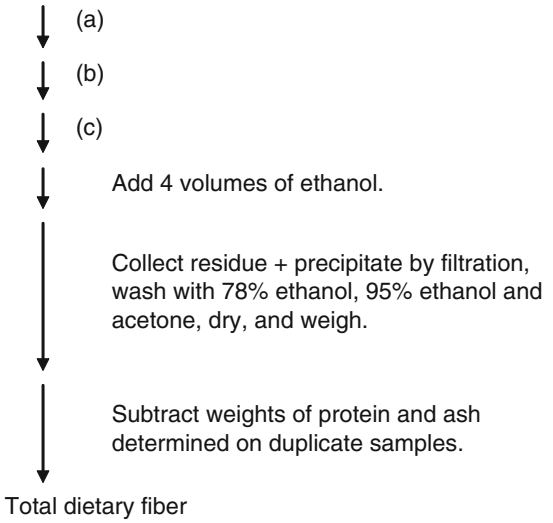
| *Food* | *Soluble*[b] | *Insoluble*[b] | *Total*[b] |
|---|---|---|---|
| Barley | 5.02 | 7.05 | 12.14 |
| High-fiber cereal | 2.78 | 30.52 | 33.30 |
| Oat bran | 7.17 | 9.73 | 16.90 |
| Soy bran | 6.90 | 60.53 | 67.56 |
| Apricots | 0.53 | 0.59 | 1.12 |
| Prunes | 5.07 | 4.17 | 9.37 |
| Raisins | 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.

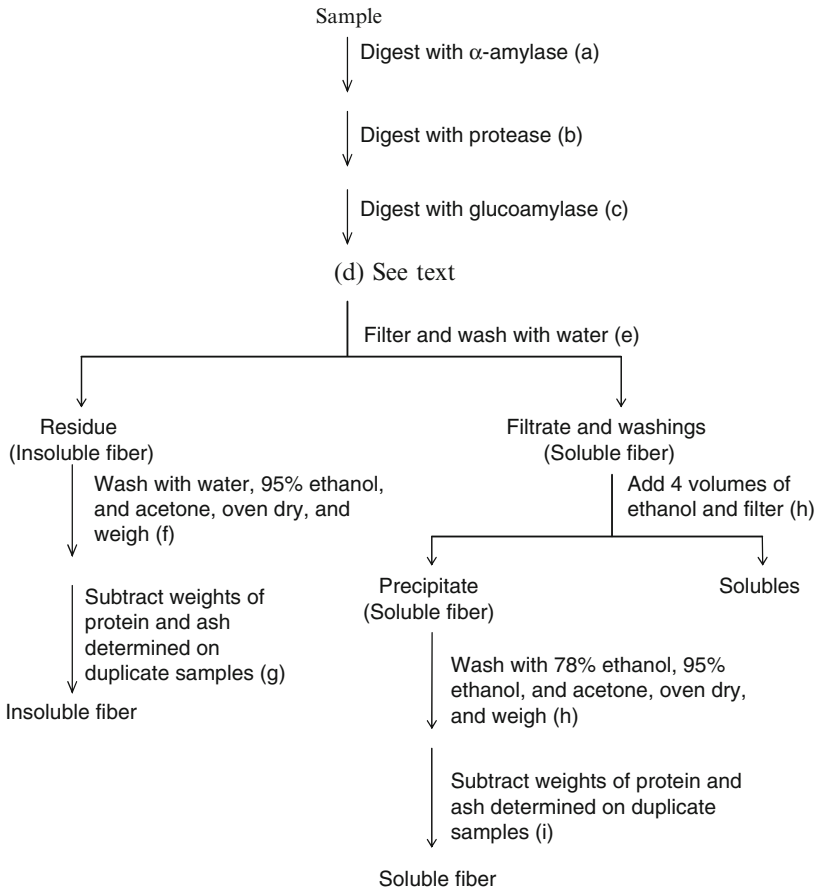
2. **Outline of procedure**. A flow diagram outlining the general procedure for the method is given in Fig. 10-12. Letters in the parentheses refer to the same letters in Fig. 10-12. If necessary, lipids are removed by extraction (Sect. 10.5.4.2).

(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)

Blank residue = 31,578.2 mg − 31,563.6 mg = 14.6 mg
32,231.2 mg − 32,198.7 mg = 14.5 mg
Average = 14.6 mg

Blank ash = 32,206.8 mg − 32,198.7 mg = 8.1 mg

First sample residue:
= 31,723.5 mg − 31,637.2 mg = 86.3 mg
Ash = 32,195.2 mg − 32,173.9 mg = 21.3 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 mg ÷ 1,002.1[sample wt.]) × 100 = 5.5%

Second sample residue :
= 32,271.2 mg − 32,173.9 mg = 97.3 mg
97.3 − 14.5 − 3.3 − 13.2 = 66.3 mg
(66.3 mg ÷ 1005.3 mg [sample wt.]) × 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)

Blank residue = 33,033.4 mg − 33,019.6 mg = 13.8 mg
33,995.6 mg − 31,981.2 mg = 14.4 mg
Average = 14.1 mg

Blank ash = 31,989.1 mg − 31,981.2 mg = 7.9 mg

First sample residue:
= 32,421.6 mg − 32,377.5 mg = 44.1 mg
Ash = 33,231.0 mg − 33,216.4 mg = 14.6 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 mg ÷ 1,002.1[sample wt.]) × 100 = 2.3%

Second sample residue:
= 33,255.3 mg − 33,216.4 mg = 38.9 mg
38.9 − 14.1 − 0.6 − 6.7 = 17.5 mg
(17.5 mg ÷ 1005.3 [sample wt.]) × 100 = 1.7%
Average of 2.3% and 1.7% = 2.0%

c. *Total dietary fiber* (TDF)

$$\text{TDF} = 6.1\%\ (\text{insolublefiber}) + 2.0\%\ (\text{solublefiber}) = 8.1\%$$

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