Understanding Carbohydrate Analysis

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Understanding Carbohydrate Analysis

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2.1 Introduction

Carbohydrates are one of the most important ingredients in foods and raw materials. They may occur naturally or be added to food products to provide nutrients and, in most cases, to improve the texture and overall quality of a food product.

Naturally occurring polysaccharides in foods are an intrinsic or innate part of the raw material. For example, starch is the most abundant naturally occurring carbohydrate in food products, followed by pectin, hemicellulose and cell wall materials.

Many polysaccharides are also added to food systems as stabilizers and dietary fiber. For example, locust bean and guar gum are used to stabilize emulsions and prohibit ice crystal growth in ice cream. Many other polysaccharide gums have been widely used in bakery and dairy products to improve the texture and organoleptic properties of baked goods and as gelling agents for making desserts (see Chapter 6 for details). In addition, polysaccharides may be produced as a by-product of bacteria, such as in yogurt. Recent applications of nonstarch polysaccharides in breakfast cereals and snack food products have increased due to their perceived physiological effects and health benefits. This group of components are called dietary fiber. For example, galactomannans and cereal β-glucans can reduce serum cholesterol and attenuate blood glucose levels whereas psyllium and flaxseed gum have been used as laxatives.

Regardless if they were added or present in food indigenously, it is important to know what kind and how much carbohydrates are present in a food system. This chapter describes current methods used for the determination of total carbohydrates and uronic acids, as well as for analyzing oligosaccharides and dietary fiber in food products.

2.2 Total Sugar Analysis

As a group, carbohydrates are quite heterogeneous, differing in primary structure (ring size and shape), degree of polymerization (mono vs. oligo vs. polysaccharides), macromolecular characteristics (linear structure vs. branched compact structure), linkage (i.e., α or β glycosidic linkage, linkage position), and charge (see Chapter 1 for more detailed coverage of carbohydrate structure). The physical and chemical differences give rise to disparate properties, including solubilities, reactivities, and susceptibility to digestive enzymes.

From an analytical perspective, the simplest situation is where there is only one type of carbohydrate present in a sample with minimal interfering compounds — measuring glucose oligomers in corn syrup, for example. In this case the disparate reactivities of sugars based on structure (e.g., ketose or hexose forms), charge, and type (e.g., glucose vs. arabinose) do not present a problem. It is the case most often though, especially with raw materials (e.g., seeds, cereal grains) and food products (ice cream, baked goods), that various types of carbohydrates are present in a sample with other compounds including lipid solubles, proteins, and minerals. The heterogeneity of this group of compounds can make analyzing the total carbohydrate content of a sample quite complex.

2.2.1 Sample Preparation

Before analyzing for any class of carbohydrate, whether it is monosaccharides or insoluble cellulosic material, the sample must be prepared so as to remove substances that can interfere with analysis. For samples that are already essentially sugar solutions (juice, honey) very little sample preparation is required. For other samples, such as oil seeds, cereals or whole foods, fats, proteins, pigments, vitamins, minerals, and various other compounds should be removed prior to analysis. There are several detailed procedures outlined in the literature for the removal of these substances prior to the analysis of simple sugars or polysaccharides.[1,2] It should be noted that the extent of sample preparation required is also dependent on the analytical technique and/or equipment being used. Generally, samples are dried and ground first, followed by a defatting step. Drying can be done under a vacuum, at atmospheric pressure, or for samples that are sensitive to heat,

in a freeze dryer. Samples, once ground to a specified mesh size, are defatted using a nonpolar solvent such as hexane or chloroform. Low molecular weight carbohydrates can then be extracted using hot 80% ethanol. The ethanol extract will contain mineral salts, pigments, and organic acids as well as low molecular weight sugars and proteins, while the residue will mainly contain proteins and high molecular weight carbohydrates including cellulose, pectin, starch, and any food gums (hydrocolloids) that may be present. Protein is generally removed from samples using a protease such as papain. Water soluble polysaccharides can be extracted using water and separated from insoluble material by centrifugation or filtration. Depending on the compound of interest in the sample, an enzymatic treatment with α-amylase and/or amyloglucosidase can be used to rid the sample of starch. In this case starch is hydrolyzed to glucose, which can be separated from high molecular weight polysaccharides by dialysis or by collecting the high molecular weight material as a precipitate after making the solution to 80% ethanol. Glucose is soluble in 80% ethanol while polysaccharide material is not.

The chemical methods applicable to neutral sugars, the phenol–sulfuric acid assay and the anthrone assay, are classic methods with a long history of use, although the phenol–sulfuric acid assay is probably the more popular of the two. A chemical method for the analysis of uronic acids is also outlined. Enzymatic methods, while widely used and readily available, are generally specific for only one or more sugars in a sample. Chemical methods such as the phenol–sulfuric acid assay can be utilized to provide an approximate value, but because different sugars in solution react differently with the assay reagents, a measurement of the total sugar would be an estimate based on the reactivity of the sugar used to construct the calibration curve (usually glucose). If an approximate or estimated value is all that is required then this method is sufficient. When the exact amount of each sugar present is required, highly specific enzymatic methods are more appropriate. This is frequently done by instrumental analysis as described in Section 2.3.

2.2.2 Phenol–Sulfuric Acid Assay

2.2.2.1 Reaction Theory

In the presence of strong acids and heat, carbohydrates undergo a series of reactions that leads to the formation of furan derivatives such as furanaldehyde and hydroxymethyl furaldehyde.[1,3] The initial reaction, a dehydration reaction (Figure 2.1) is followed by the formation of furan derivatives, which then condense with themselves or phenolic compounds to produce dark colored complexes. Figure 2.2 displays some furan derivatives and the carbohydrates from which they originate. The developed complex absorbs UV-VI light, and the absorbance is proportional to the sugar concentration in a linear fashion. An absorbance maximum is observed at 490 nm for hexoses and 480 nm for pentoses and uronic acids as measured by a UV-VI spectrophotometer (Figure 2.3).[4]

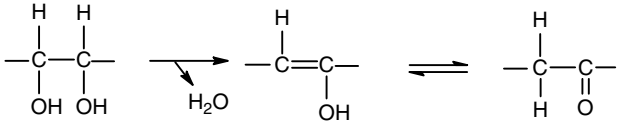

FIGURE 2.1
Dehydration reaction.

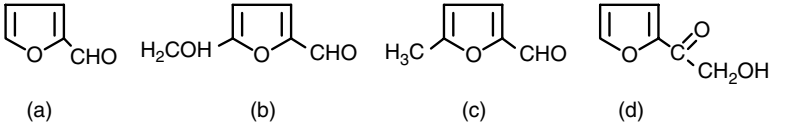

FIGURE 2.2
Furan derivatives from (a) pentoses and hexuronic acids, (b) hexoses, (c) 6-deoxyhexoses, and (d) keto-hexoses, respectively.

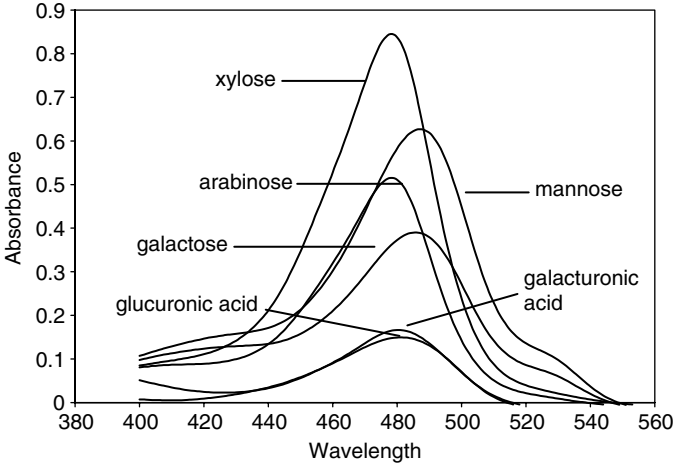

FIGURE 2.3
Phenol–sulfuric acid assay absorbance maxima for hexoses and pentoses.

2.2.2.2 Operating Procedure

Phenol, in a 5 or 80% solution is added to a glass test tube containing a clear sample solution. Concentrated sulfuric acid is added in a rapid stream directly to the surface of the liquid in the test tube. The mixture is thoroughly combined using a vortex mixter and then permitted to stand a sufficient time to allow for color development. The solution absorbance is read at 490 or 480 nm using a spectrophotometer, depending on the type of sugar present. Mixing and standing time should be kept the same for all samples to assure reproducible results.[4]

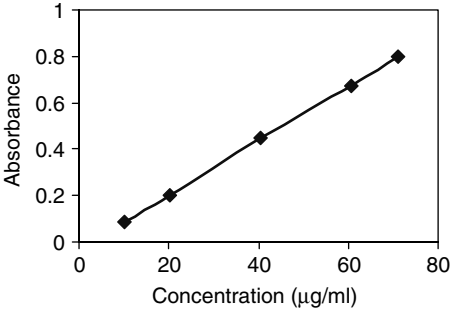

FIGURE 2.4
Phenol–sulfuric acid assay calibration curve.

2.2.2.3 Quantification

A calibration curve is constructed using the sugar being assayed. A stock 1 mg/ml aqueous sugar standard solution is used to prepare 5 or 6 standards ranging from 10 to 100 μg/ml. Each standard is subjected to the reaction procedure outlined above, transferred to a cuvette, and its absorbance read at 480 or 490 nm. The absorbance of the blank should be subtracted from the absorbance of the standards manually, or the blank should be used to zero the spectrophotometer. A graph of absorbance vs. concentration is constructed and the amount of analyte is derived from the calibration curve (Figure 2.4).

2.2.2.4 Applicability

The phenol–sulfuric method is widely used to determine the total concentration of carbohydrates in a sample. It can be used on lipid-free extracts from cereals, seeds, and plants, provided the sample is in solution, and is appropriate for both reducing and nonreducing sugars. It is advantageous in that the reagents are low cost and readily available, the required equipment is minimal, and the assay is simple. Additionally, it can be used to quantify monosaccharides, oligosaccharides, and polysaccharides. Absorption curves are characteristic for different sugars; therefore, this method provides the most accurate results when applied to samples containing only one type of carbohydrate.

This assay has been used to quantify total sugars in a sample containing more than one type of carbohydrate. In this case, glucose is often used to construct the calibration curve and the results are then approximate only and should be stated as glucose equivalents.

2.2.3 Anthrone–Sulfuric Acid Assay

2.2.3.1 Reaction Theory

Similar to the phenol–sulfuric acid assay, the anthrone method is based on the condensation of furaldehyde derivatives, generated by carbohydrates in

the presence of a strong acid, with a reagent, in this case anthrone (9,10-dihydro-9-ozoanthracene), to produce colored compounds.[5] The reaction of carbohydrates in a strongly acidic environment with anthrone results in a blue-green color and the absorbance is read at 625 nm.

2.2.3.2 Operating Procedure

A cooled mixture of 2% anthrone in concentrated sulfuric acid is mixed with an aliquot of a clear sample solution containing the sugar being assayed. After incubation in a temperature-controlled environment for sufficient time to allow color development, the solution is poured into an appropriate spectrophotometric cuvette and the absorbance measured at 625nm.[5,6]

2.2.3.3 Quantification

Similar to the phenol–sulfuric acid assay, the anthrone reaction is nonstoichemetric and therefore requires the construction of a standard curve for quantitative purposes.

2.2.3.4 Applicability

The anthrone–sulfuric method is most applicable to solutions containing one type of hexose because even sugars with similar structures result in different rates and quantities of color development. Other sugars, such as pentoses and hexuronic acids, will also react to produce colored compounds that absorb at the same wavelength, but this only becomes a problem if they are present in a solution above a certain level.[5] This assay can also be used for quantitative analysis of oligo- and polysaccharides provided only one type is present in solution.

The anthrone method has been modified for use with a micro-plate, thus permitting the analysis of many samples within a short period of time and reducing the quantity of reagent needed.[7]

2.2.4 Analysis of Uronic Acids

Colorimetric methods for determining uronic acids are similar to the phenol–sulfuric acid and the anthrone assay in that they are based on the reaction of a reagent with carbohydrate derivatives formed in concentrated acid. The carbazole assay reported by Dishe[8] essentially involves mixing a sample containing uronic acid with concentrated sulfuric acid, heating it at 100°C, cooling it and then reacting it with 0.1% carbazole in ethanol. After sufficient time for color development, absorbance is read at 535 nm. Modifications to this assay include alterations to the timing of steps and reagent concentrations.[9] The carbazole assay, while simple, rapid, and sensitive suffers interferences from hexoses and pentoses. The replacement of carbazole with *m*-hydroxydiphenyl[10] increased the specificity and sensitivity of the assay.

2.2.4.1 Reaction Theory — m-Hydroxydiphenyl Method

While all carbohydrates react in concentrated acid to form colored compounds, uronic acids react with *m*-hydroxydiphenyl in a strongly acidic environment to form pink colored complexes. Absorbance measurements read at 520 nm increase linearly with uronic acid concentration from 0 to 100 μg/ml.

2.2.4.2 Operating Procedure

A sample solution is thoroughly mixed with sulfuric acid containing tetraborate in a test tube and placed in a boiling water bath for 5 minutes. After rapid cooling in an ice water bath, *m*-hydroxydiphenyl is added to each sample test tube and vortexed to ensure adequate mixing. The absorbance for each sample is read after allowing the color to develop for 20 minutes. A sample blank (containing a sample solvent) should be prepared at the same time as the samples.

2.2.4.3 Quantification

A solution of an appropriate uronic acid standard (i.e., galacturonic acid) is used to prepare several dilutions and these are used to prepare a standard curve. A graph of concentration vs. absorbance is constructed and the sample absorbance reading plotted along the curve to obtain concentration values. Standard curves typically range from 10 to 100 μg/ml.

2.2.4.4 Applicability

The *m*-hydroxydiphenyl assay can tolerate the presence of nonuronic acid sugars, up to ~200 μg/ml has been reported,[11] but higher concentrations of neutral sugars may artificially increase absorbance readings. Additionally, the presence of protein in a sample may interfere with the absorbances.[12] This method is appropriate for the quantification of pectic material in fruits and vegetables[13] and has been adapted for use with a micro-plate.[12]

2.3 Monosaccharide Analysis

The most frequently used methods for determining the concentration of monosaccharides in a sample are probably gas chromatography (GC) and high performance liquid chromatography (HPLC). These methods can also be suitable for qualitative determinations provided appropriate detection systems and/or standards are used. Unlike enzymatic methods, which tend to be specific for one type of monosaccharide only, chromatographic techniques provide qualitative and quantitative information about one or several monosaccharides in a sample.

Sample preparation is simplest when starting with a relatively pure, dry sample. For an HPLC analysis, the sample needs only to be finely ground, solubilized, diluted if required, and filtered prior to injection onto the column. In some cases, especially when analyzing complex food products, sample preparation becomes somewhat more complex and one or more clean up steps may be required. Lipid soluble compounds are generally removed via extraction with a suitable solvent, such as ether or hexane. Protein can be removed from samples enzymatically using a suitable protease (i.e., papain). The presence of inorganic salts can negatively affect column life and these can be removed using pre-packed preparatory columns that employ a mixed anion/cation exchange resin.

When the sample starting material is a polysaccharide and a quantitative and/or qualitative analysis of its constituent monosacharides is required, the sample must be depolymerized. This is most commonly accomplished using acid hydrolysis.

2.3.1 Acid Hydrolysis

In the presence of a strong acid and heat, the glycosidic bond between monosaccharide residues in a polysaccharide is cleaved. During this reaction, one molecule of water is consumed for every glycosidic linkage cleaved. During an acid hydrolysis released monosaccharides are susceptible to degradation in the presence of hot concentrated acid. However, not all glycosidic linkages are cleaved at the same rate and the hydrolysis time must be sufficient to hydrolyze all linkages in the sample. These two needs must be balanced; the need for hydrolysis of sufficient strength and length to permit complete hydrolysis, but not so long so as to lead to sample degradation.

Sulfuric acid and trifluoracetic acid (TFA) are commonly used for hydrolysis. It has been reported that sulfuric acid is superior to TFA for the hydrolysis of fibrous substrates such as wheat bran, straw, apples, and microcrystalline cellulose.[14] However, sulfuric acid can be difficult to remove post-hydrolysis and its presence can interfere with some analyses. TFA is volatile and can be easily removed prior to an HPLC analysis.

A hydrolysis procedure appropriate for neutral polysaccharide gums using TFA requires heating ~10 mg of polysaccharide material in 1 ml of 1M TFA at 121°C for 1 hour.[2] After cooling to room temperature, the TFA can be removed under a stream of nitrogen. A hydrolysis procedure using sulfuric acid appropriate for water soluble dietary fiber material in foods has been outlined. It requires mixing the sample material with 1M sulfuric acid and heating at 100°C for 2.5 hours.[6] Another recommended procedure for neutral polysaccharides involves mixing 2 to 5 mg of accurately weighed dry sample with 0.1 to 0.25 ml of 2M HCL and heating at 100°C for 2 to 5 hours.[15] While many hydrolysis procedures exist in the literature, when working with a new or unknown polysaccharide, it is best to check that the hydrolysis protocol is not resulting in excessive decomposition and is also cleaving bonds quantitatively. This is done by subjecting the sample to a chosen

TABLE 2.1

Acid Hydrolysis Methods

Substrate (s)	Acid Used for Hydrolysis	Hydrolysis Conditions	Reference
Xylan, wheat bran	H_2SO_4	72% H_2SO_4 1 hr at 30°C 2.5% H_2SO_4 1 hr at 125°C	Garleb et al., 1989
Wheat flour	HCL	2M HCL, 100°C for 90 min	Houben et al., 1997
Celluose	H_2SO_4	72% H_2SO_4 1 hr at 30°C, 3% H_2SO_4, 100°C 4.5 hr	Adams, 1965 Saeman et al., 1963
Neutral polysaccharides	HCL	2M HCL, 2-5 hr	Pazur, 1986

hydrolysis protocol and monitoring the quantity of each sugar present in the solution at timed intervals. As hydrolysis proceeds, the quantity of each sugar should increase until all of that sugar present has been released from the polysaccharide. Degradation of released sugars will be evident as a decrease in monosaccharide concentration as hydrolysis time increases. Table 2.1 summarizes various hydrolysis procedures found in the literature. The following two sections outline the chromatographic techniques most frequently used for the analysis of monosaccharides.

Samples containing acidic sugar residues such as pectins and certain fungal polysaccharides can be difficult to hydrolyze quantitatively using traditional methods that employ TFA or sulfuric acid. The difficulty arises from the disparate susceptibilities to hydrolysis of neutral and acidic sugar residues as well as different linkage types. In cases where quantitative hydrolysis cannot be achieved, qualitative information can be obtained using an appropriate chromatographic technique post–acid hydrolysis (see next sections), and the uronic acid content determined using the spectrophotometric method outlined in Section 2.2.4.

2.3.2 Gas-Liquid Chromatography (GC)

Gas-liquid chromatography is a technique whereby components in a mixture are separated based on their degree of affinity for or interaction with a liquid stationary phase. In the case of GC, the sample components are dissolved in a gas phase and moved through a very small bore column, the interior of which is coated with the stationary phase. These separations occur at high pressure and high temperatures. Components in the sample mixture with a high affinity for the stationary phase will stay in the column longer and elute later than those with less affinity for the stationary phase. The degree of affinity for or interaction with the stationary phase that a molecule has is governed by its structure, properties, and the chemistry of the stationary phase being used.

The prerequisite of a GC separation in the sample must be volatile. Given that monosaccharides are not volatile, they must be derivatized prior to analysis.

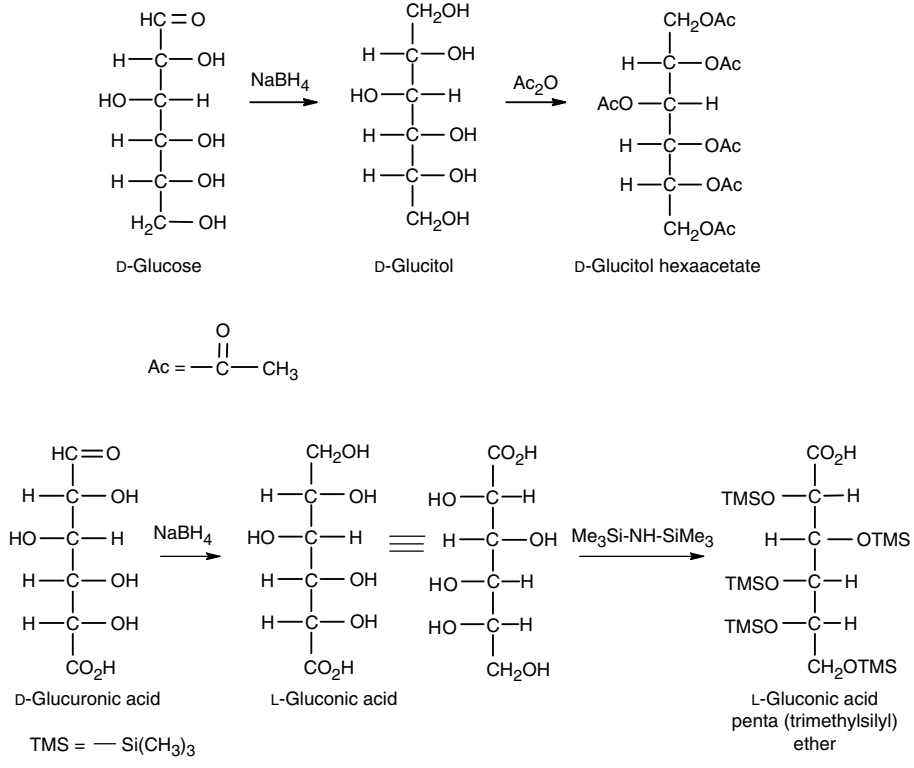

FIGURE 2.5
Alditol acetates and TMS derivatives from neutral and acidic sugars, respectively.

2.3.2.1 Derivatization

Neutral monosacharides are most often derivatized into alditol acetates prior to GC analysis. The age of this derivatization technique and the frequency with which it is used is evident from the number of methods for this procedure available in the literature.[6,16,17] The essential elements of this derivitization procedure are the reduction of neutral sugars to alditols and their subsequent acetylation. The resulting alditol acetates are then dissolved in a suitable solvent and injected onto a GC column. Acidic sugars are treated differently to yield trimethylsilyl (TMS) derivatives. The derivitization process is shown in Figure 2.5 for both neutral (alditol acetates) and acidic (TMS derivatives) sugars.

2.3.2.1.1 Neutral Sugars

The starting material must be a dry sample of one or more monosaccharides. Polysaccharide material must first be hydrolyzed and the acid removed prior to analysis. Trifluoracetic acid works well for this purpose because it is volatile and can be easily removed by rotary evaporation.

The dry sample containing a small amount (~10mg) of accurately weighed monosacharide material and inositol hexa-acetate (internal standard) is mixed with a solution of sodium borohydride in ammonium hydroxide to convert monosaccharides into alditols. Acetic acid is added to acidify the sample and destroy excess sodium borohydride after the reaction has reached completion. The mixture is dried (rotary evaporation or under a stream of nitrogen) and methanol is added and removed by drying with a nitrogen stream several times. Treatment with methanol removes borate ions as volatile methyl borate. When the final portion of methanol has been removed and the sample is dry, the mixture of alditols is acetylated by adding acetic anhydride and heating it at 121°C for a few hours. A few drops of water are added to the reaction vial to destroy any residual acetic anhydride and the entire mixture is brought to dryness.[2] The resulting alditol acetates are solubilized in methylene chloride in preparation for GC analysis.

Various conditions for separating alditol acetates exist in the literature; the one most appropriate is dependant on the column used. There are many columns on the market that are appropriate for the separation of alditol acetates including SPB-1701 (30 m × 0.25 mm ID), which separates alditols isothermally at 220°C using helium as the carrier gas, and SP-2380 (30 m × 0.25 m ID), which separates the derivatized sugars isothermally at 275°C in helium (Supelco, Bellefonte, PA). A DB-210 column (30 m × 0.25 ID) has also been used to separate alditol acetates isothermally at 220°C using nitrogen as a carrier gas.[18]

2.3.2.1.2 Acidic Sugars

As with neutral polysaccharides, polysaccharides containing uronic acids must be hydrolyzed and dried prior to analysis. It is very difficult to obtain a quantitative yield of acidic monosaccharides using acid hydrolysis, and when this is required, it is advisable to use a spectrophotometric method such as is outlined in Section 2.2.4.

A sample containing a small amount (~10 mg) of accurately weighed carbohydrate material is dissolved in sodium carbonate and then treated with sodium borohydride. Acetic acid is added to destroy excess borohydride and borate ions are removed with methanol as described for neutral monosaccharides. The resultant mixture of aldonic acids and aldoses (from neutral sugars if present) is made into TMS derivatives by treating the dry residue with a mixture containing pyridine, hexamethyldisilazane, and trifluoracetic acid.[2] An internal standard, such as docosane, should be used for quantification.

2.3.2.2 Quantification

A flame ionization detector (FID) is the most commonly used detector. With an FID, quantification requires using an internal standard and the formulation of response factors (RF). Response factors are used to correct for disparate GC response to monosaccharides and losses arising from hydrolysis and derivatization. They are obtained for each monosacharide by subjecting a

mixture of standard monosaccharides corresponding to the monosaccharides present in the sample to the same hydrolytic and derivatization conditions and using the following equation:

$$RF = (A_S \times W_M)/(A_M \times W_S)$$

where A_S = peak area of internal standard, W_M = weight in mg of individual monosaccharide standard, A_M = peak area of monosaccharide standard, and W_S = weight in mg of internal standard.

Once response factors have been determined for each monosacharide present, they are used to determine the percent content of each monosaccharide residue, %M, in the sample according to the following equation:

$$\%M = (RF \times A_M \times W_S \times F \times 100)/(A_S \times S)$$

where RF = the response factor for each monosaccharide, A_M = peak area for monosacharide in sample, W_S = weight in mg of internal standard in sample solution, F is a factor for converting monosaccharides to polysaccharide residues (use 0.88 for pentoses and 0.90 for hexoses), A_S = peak area of internal standard in sample solution, and S is the dry weight in mg of starting sample material.

2.3.2.3 Advantages/Disadvantages

GC analysis of carbohydrates is advantageous because it is an established technique and much of the method optimization has been done. It also requires small sample sizes and is very sensitive, a vital advantage when only a small amount of sample is available. The disadvantages of this technique originate chiefly from the preparatory steps. If either the reduction or the acetylation steps do not proceed to completion, the quantity of derivatised sugars will be underestimated. In short, there is ample opportunity for sample loss. Additionally, preparation may appear prohibitively laborious, especially when compared to HPLC techniques (see next section).

2.3.3 High Performance Liquid Chromatography (HPLC)

Similar to GC, HPLC is a separation technique whereby compounds in a mixture are separated on a stationary phase. In this case, the mobile phase (eluant) containing the sample and the stationary phase are both liquids. Unlike GC, HPLC separation is a function of the compatibility (or differing compatibility) of sample components for the eluant and stationary phase. A HPLC separation can be manipulated by changing the concentration and/or makeup of the eluant. For example, a compound that is very compatible with the stationary phase (and therefore one that stays on the column longer and elutes later) can be forced off the column faster by changing the solvent makeup such that sample components favor it vs. the stationary phase.

Under the broad category of HPLC there are several sub-types characterized by the type of stationary and mobile phases employed and therefore the chemistry of the separation. In normal phase chromatography the stationary phase is hydrophilic and the mobile phase varies in its hydrophilic/hydrophobic nature depending on the sample components being separated. In reverse phase chromatography, the stationary phase is hydrophobic. High performance anion exchange chromatography (HPAEC), an increasingly popular choice for the separation of carbohydrates, is characterized by an anionic stationary phase and a mobile phase with a high pH. At high pH (10 to 14), carbohydrate hydroxyl groups ionize and their separation is based on their differing affinity for the oppositely charged stationary phase and the mobile phase.

2.3.3.1 High Performance Anion Exchange Chromatography (HPAEC)

A sample containing monosaccharides, either as its natural state or after a hydrolysis step, must be separated chromatographically so that each monosaccharide can be identified and quantified. Over the past few years, high performance anion exchange chromatography has proven invaluable in the analysis of carbohydrate material. This type of chromatography is based on the fact that carbohydrates in a strongly alkaline environment will ionize, thereby rendering them amenable to separation on an ion exchange column. HPAEC columns used for carbohydrates are coated with an anion exchange resin. For example, the Dionex (Sunnyvale, CA) PA1 column, optimized for the separation of mono-, di-, oligo-, and low molecular weight polysaccharides, is composed of 10 μm nonporous beads covered in a quaternary amine anion exchange material. HPAEC systems typically use sodium hydroxide as the eluant to separate mono- and disaccharides, while eluants for larger molecules often include sodium acetate to increase ionic strength. The detector of choice for HPAEC is the pulsed amperometric detector (PAD). In general, amperometry measures the change in current resulting from the oxidation or reduction of a compound at an electrode. In PAD, it is the change in current resulting from carbohydrate oxidation at a gold or platinum electrode that is measured. The advantage of PAD is not only its low detection limits, reportedly in the picomole range,[19] but also its suitability for gradient elution, which provides an analyst with more flexibility when optimizing separation conditions.

Figure 2.6 presents the HPAEC separation profile of arabinose, rhamnose, glucose, galactose, mannose, and xylose. These six standards were separated using a sodium hydroxide gradient. Excellent baseline separation is also achieved for mixtures of mono- and oligosacchrides (Figure 2.7), sugar alcohols,[20,21] and uronic acids.[22] HPAEC-PAD permits the separation and quantification of both neutral and acidic monosaccharides in one analytical run. A sodium hydroxide/sodium acetate gradient and PA1 column (Dionex, Sunnyvale Ca.) has been used to separate and identify neutral sugars (rhamnose, arabinose, galactose, glucose, mannose) and uronic acids (galacturonic and glucuronic acid) from citrus pectin and fungal polysaccharides after

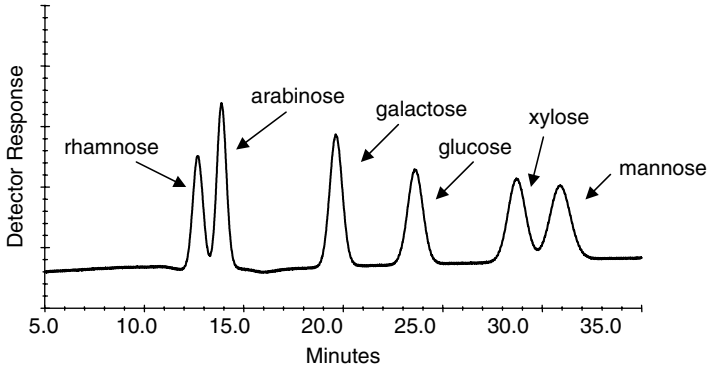

FIGURE 2.6
HPAEC-PAD chromatogram showing separation of rhamnose, arabinose, glucose, galactose, xylose, and mannose.

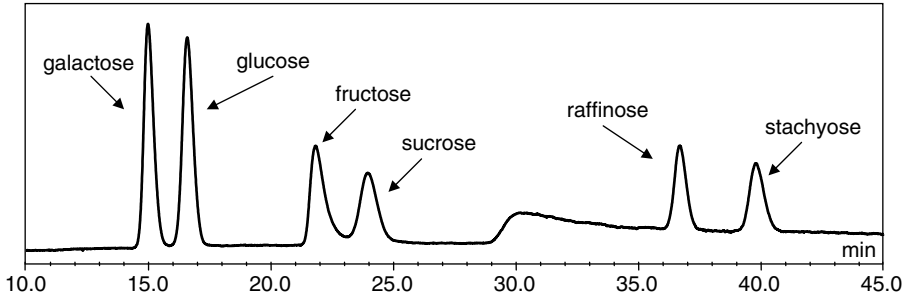

FIGURE 2.7
HPAEC-PAD chromatogram showing separation of glucose, galactose, sucrose, fructose, raffinose, and stachyose.

hydrolysis with TFA, sulfuric acid, and methanolysis combined with TFA.[22] Furthermore, a sodium hydroxide/sodium acetate gradient can also be used to separate neutral and acidic monosaccharides as well as oligogalacturonic acids from strawberries after treatment with pectolytic enzymes.[23] For many samples, preparation entails extraction of the desired components with water, centrifugation to remove precipitated matter, and filtering.[20,21] The final filtering step prior to injection onto the HPLC column is usually through at 0.45 μm filter.

2.3.3.2 Quantification

Quantifying the amount of each individual mono-, di-, oligo-, or polysaccharide from an HPAEC chromatogram is quite simple provided 4 to 5 dilutions of appropriate standards are run with a set of samples. The software associated with the HPLC system will integrate peaks, provide peak areas/heights, and give concentration values provided standards with known concentrations have been run. It is important to run standards corresponding

to sample constituents because PAD response varies with the analyte. Detector response decreases with the increasing degree of polymerization (DP),[19] therefore, the relative percentage of each component in a chromatogram (and therefore the sample) cannot be determined by peak areas alone, especially when the sample contains mono-, di-, oligo-, and polysaccharides. Sugar concentration values obtained for acid hydrolysates need to be further adjusted to account for the molecule of water that was added to each residue upon hydrolysis. For hexoses this requires a conversion factor of 0.90 and for pentoses a factor of 0.88 is used.

2.3.3.3 Advantages/Disadvantages

The advantage of HPAEC for the analysis of carbohydrates is that samples do not require derivatization and the analysis itself is usually quite fast. In the past, disadvantages of HPLC originated with detection systems, which for the most part were not very sensitive. Refractive index detectors have traditionally been the detector of choice, because more sensitive detectors, for example UV or fluorescence detectors, are not appropriate for analyzing carbohydrates since carbohydrates do not contain moieties that respond to these detection systems. HPAEC-PAD has overcome this disadvantage, enabling the separation and quantification of monosaccharides with low detection limits. In addition, using sodium hydroxide as eluant is inexpensive and relatively safe. Because carbohydrates are typically soluble in the mobile phase, derivatization is unnecessary and sample preparation is usually limited to the removal of interfering substances such as lipids and proteins. The PAD is also appropriate for use with gradient elution.

2.3.4 Enzymatic Analysis

Enzymatic methods for determining sugar content rely on the ability of an enzyme to catalyze a specific reaction and employ a suitable method for monitoring the progression of the reaction or the concentration of reaction product. Enzymatic methods are highly specific, usually rapid and sensitive to low sugar concentrations.

There are enzymatic methods for most common sugars, and for some such as glucose several different enzymes can be used, and these assays are available in kit form. Enzymatic methods for the quantitative analysis of glucose, fructose, sucrose, lactose, and galactose are presented below. Enzymatic methods have been developed for the quantification of various polysaccharides, including β-glucan[24] (American Association of Cereal Chemists [AACC] method 32-23; Association of Official Analytical Chemists [AOAC] method 995.16), starch[25] (AACC method 76-13; AACC method 76-11), and the galactomannans locust bean gum and guar gum.[26,27] These generally require enzymatic hydrolysis of the polymer followed by enzymatic determination of the released monosaccharides using one of the methods below.

2.3.4.1 Analysis of Glucose

2.3.4.1.1 Reaction Theory

One of the earliest and most widely used enzymes for the quantitative determination of glucose is glucose oxidase. This highly specific enzyme, which can be obtained from *Penicillium notatum* and *Aspergillis niger,* catalyzes the oxidation (2 hydrogen atoms removed) of β-D-glucopyranose to form D-glucono-1,5-lactone, which is a short lived species that hydrolyzes to yield D-gluconic acid.[28] The reaction of glucose with glucose oxidase also yields H_2O_2 in a ratio of 1:1.

Early methods for detecting the quantity of glucose in a sample after treatment with glucose oxidase relied on the volumetric titration of gluconic acid formed.[28] Today, colorimetric methods based on the use of glucose oxidase are more common. In these reactions peroxidase is used in combination with a chromogen to yield a colored complex in the presence of H_2O_2. Glucose is oxidized to yield gluconic acid and peroxide in the presence of glucose oxidase (EC.1.1.34) and peroxidase catalyzes the oxidation of a chromogen (i.e., *o*-dianisidine, Figure 2.8) in the presence of H_2O_2, thereby enabling quantitative measurement spectrophotometrically when an appropriate standard curve has been established. Detailed methods for this assay

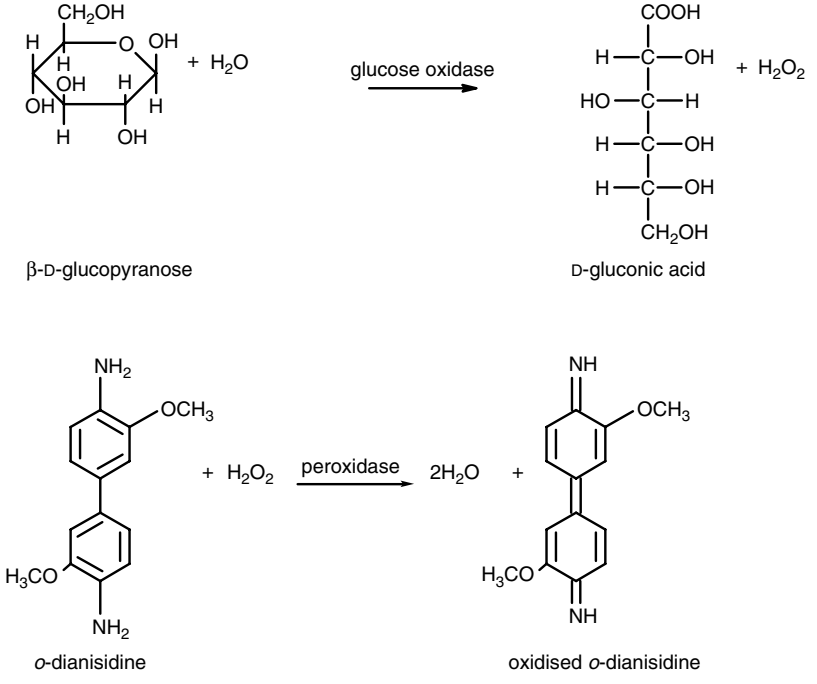

FIGURE 2.8
Glucose oxidase assay reaction.

are available elsewhere[6] (AACC method 80-10) and complete kits containing the method and reagents are available commercially.

Hexokinase is another enzyme frequently used in the quantitative determination of glucose. Glucose reacts with hexokinase in the presence of adenosine triphosphate (ATP) to form glucose-6-phosphate and adenosine diphosphate (ADP). Glucose-6-phosphate reacts with glucose-6-phosphate dehydrogenase in the presence of nicotinamide-adenine dinucleotide (NAD) to produce 6-phosphogluconate and NADH.[6] NADH concentration is measured spectrophotometrically at 340 nm, or the reaction is modified so it is amenable to colorimetric determination.

$$\text{1. D-Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{Glucose-6-phosphate} + \text{ADP}$$

$$\text{2. Glucose-6-phosphate} + \text{NAD} \xrightarrow[\text{dehydrogenase}]{\text{Glucose-6-phosphate}} \text{Gluconate-6-phosphate} + \text{NADH}$$

2.3.4.1.2 Operating Procedure — Glucose Oxidase

Aliquots of sample solution are mixed with a buffered solution containing glucose oxidase, peroxidase, and the chosen chromogen and incubated under temperature controlled conditions for a specified period of time (time and temperature dependant on a chosen analytical method). After sufficient time for color development, the absorbance is read at an appropriate wavelength. For example, when using AACC method 80-10, which uses *o*-dianisodine hydrochloride as chromogen in 0.1 M acetate buffer at pH 5.5, sample solutions are incubated at 30°C for 5 min and absorbance read at 525 mn against a reagent blank.

2.3.4.1.3 Quantification

A calibration curve is constructed by plotting absorbance vs. concentration for 5 separate glucose dilutions. Quantification is achieved using the calibration curve.

2.3.4.1.4 Applicability

Glucose oxidase is a highly specific enzyme. Therefore this method is applicable for use with samples that contain other sugars.

2.3.4.2 Analysis of Galactose and Lactose

D-Galactose has been assayed enzymatically using galactose dehydrogenase[6] and galactose oxidase.[29] In the presence of oxygen, galactose is oxidized to D-galacto-hexodialdo-1,5-pyranose by galactose oxidase (EC 1.1.3.9). This reaction generates hydrogen peroxide, which can be measured colorimetrically in the presence of hydrogen donors. Alternately, D-galactose is oxidized to galactonic acid by NAD when β-galactose dehydrogenase (EC 3.2.1.23) is

present, and the resultant formation of NADH can be monitored spectrophotometrically. Lactose can also be detected as galactose after treatment with β-galactosidase, an enzyme that catalyzes the hydrolysis of lactose to D-glucose and D-galactose. Both galactose and lactose may be determined in a sample by correcting the lactose content of a sample obtained post–β-galactosidase digestion by subtracting the free galactose determined in the absence of the enzyme. Enzymatic methods for determining lactose and galactose are available in the literature[6,30] and in kit form (Megazyme, Ireland). The procedure outlined below is adapted from Mustranta and Ostman.[30]

2.3.4.2.1 Reaction Theory

Galactose is oxidized to galactonic acid by NAD in the presence of galactose dehydrogenase. NADH generated by this reaction is present in solution at a concentration proportional to the galactose content.

$$1.\ \text{D-Lactose} + H_2O \xrightarrow{\beta\text{-Glactosidase}} \text{D-Glucose} + \text{D-Galactose}$$

$$2.\ \text{D-Galactose} + NAD^+ \xrightarrow[\text{Dehydrogenase}]{\text{Galactose}} \text{Galactonic acid} + NADH + H^+$$

2.3.4.2.2 Operating Procedure

Samples containing galactose exclusively are mixed with a buffer containing NAD, the initial absorbance is read at 340 nm and then measured again after incubation with galactose dehydrogenase. Lactose containing samples must first be treated with β-galactosidase to convert lactose to glucose and galactose and then incubated with galactose dehydrogenase. Both galactose and lactose can be determined in one assay using these two enzymes. Blanks (each containing all required enzymes and buffer but no sample) need to be prepared at the same time as the samples.

2.3.4.2.3 Quantification

The quantity of NADH formed is stoichiometric with the amount of galactose present in the sample. The absorption differences for the samples and blanks must be determined first by subtracting initial absorbance values (pre–galactose dehydrogenase addition) from the final absorbance values (post–galactose dehydrogenase addition).

2.3.4.2.4 Applicability

This assay is applicable for determining galactose concentration in solutions that are free of fat and protein. Samples to be analyzed should be clear and less than 0.5 g/L total galactose and lactose. Samples containing protein can be treated with perchloric acid[6] or Carrez reagent,[1,3] which precipitates proteins and absorbs some colored compounds.

The enzymatic assay for lactose and galactose can be used for many different food products, including meat, dairy, and bakery products, provided sample treatment includes steps to extract sugars and remove interfering substances. Galactose dehydrogenase also oxidises L-arabinose, therefore, the presence of arabinose will interfere with the analysis.

2.3.4.3 Analysis of Fructose, Glucose, and Sucrose

2.3.4.3.1 Reaction Theory

Fructose can also be quantitatively assayed using hexokinase. Glucose and fructose can be assayed together using hexokinase, glucose-6-phosphate dehydrogenase, and phosphoglucose isomerase (PGI) to catalyze specific reactions. In the presence of ATP and hexokinase, glucose and fructose are phosphorylated to glucose-6-phosphate and fructose-6-phosphate (F-6-P) respectively. Adding NAD and glucose-6-phosphate dehydrogenase oxidises G-6-P to gluconate-6-phosphate and results in the formation of NADH which can be measured at 340 nm. Adding phospho-glucose isomerase changes F-6-P into G-6-P, which is then oxidized to gluconate-6-phosphate in the presence of NAD and leads to the formation of NADH. Sucrose may also be assayed using hexokinase or glucose oxidase (Section 2.3.4.1.) by first treating it with invertase (EC.3.2.1.26) to release glucose and fructose.

$$\text{1. D-Fructose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{Fructose-6-phosphate} + \text{ADP}$$

$$\text{2. Fructose-6-phosphate} \xrightarrow[\text{Isomerase}]{\text{Phospho-Glucose}} \text{Glucose-6-phosphate}$$

$$\text{3. Glucose-6-phosphate} + \text{NAD} \xrightarrow[\text{Dehydrogenase}]{\text{Glucose-6-Phosphate}} \text{Gluconate-6-phosphate} + \text{NADH}$$

2.3.4.3.2 Operating Procedure

Buffer, NAD, ATP, and sample solution containing glucose and fructose are combined in a test tube. A mixture of hexokinase and glucose-6-phosphate dehydrogenase is added and after sufficient time for the reaction to proceed, the solution absorbance is read at 340 nm. The absorbance is read again after adding phospho-glucose isomerase. A sample blank should be subjected to the same procedure as the samples and used to zero the spectrophotometer. A detailed procedure to determine both glucose and fructose is available in the literature[6] and in kit form (Megazyme, Ireland).

2.3.4.3.3 Quantification

Glucose is quantified from absorbance values obtained after adding hexokinase and glucose-6-phosphate dehydrogenase. To quantify fructose in the

same sample, absorbance values read after adding phospho-glucose isomerase are corrected for initial glucose absorbance values. The quantity of NADH produced is stoichiometric with the quantity of glucose and fructose.

2.3.4.3.4 Applicability

This method is appropriate for the determination of glucose and fructose in many different varieties of food stuffs including jam, honey, and ice cream, as long as they have been treated to remove interfering substances such as lipids and proteins. Samples should be clear, relatively colorless, and free from precipitated material. Solutions that are turbid or contain interfering matter can be filtered or treated with Carrez reagents for clarification.

2.3.4.4 Analysis of (1→3) (1→4)-β-D-Glucans

2.3.4.4.1 Reaction Theory

(1→3) (1→4)-β-D-glucans are cell wall polysaccharides found in greatest abundance in cereal grains such as oats and barley, while smaller quantities are also found in wheat and rye grains. β-Glucans from cereal grains have been extensively researched over the past several decades based on their postulated health benefits, including attenuating blood glucose levels[31] and lowering serum cholesterol levels.[32,33] The analysis of β-glucan requires enzymatic hydrolysis using (1→3)(1→4)-β-D-glucan-4-glucanohydrolase (E.C. 3.2.1.73) (lichenase) to yield oligosacchrides and subsequent hydrolysis with β-glucosidase (E.C.3.2.21.). Released glucose is assayed using glucose oxidase as outlined in Section 2.3.4.1. This assay is available in kit form from Megazyme (Ireland).

$$1.\ (1\rightarrow 3)\ (1\rightarrow 4)\text{-}\beta\text{-D-Glucan} \xrightarrow{\text{Lichenase}} 1\rightarrow 3 \text{ linked cellodextrins}$$

$$2.\ (1\rightarrow 3) \text{ linked cellodextrins} \xrightarrow{\beta\text{-glucosidase}} \text{Glucose}$$

2.3.4.4.2 Operating Procedure

An accurately weighed amount of dry sample (usually flour or milling fraction) is mixed with phosphate buffer (pH 6.5), boiled briefly, and mixed thoroughly. The mixture is incubated with high purity lichenase at 50°C, combined with sodium acetate buffer (pH 4.0) and centrifuged. A portion of the supernatant is treated with glucosidase and another portion with acetate buffer to serve as a blank. After incubation at 40°C with glucosidase the samples are diluted and glucose determined enzymatically using the glucose oxidase-peroxidase method (Section 2.3.4.1). Standards containing 50 and 100 μg/ml glucose, reagent blanks (containing acetate buffer and glucose oxidase-peroxidase reagent) and flour controls with known β-glucan values are prepared and run with each set of samples.

2.3.4.4.3 Quantification

Absorbance values from glucose standards are used to evaluate percent glucose in the sample. When calculating the percent glucose it is important to include a conversion factor of 0.9 to account for the difference in molecular weight of free glucose vs. glucose in polysaccharides.

2.3.4.4.4 Applicability

This assay is appropriate for use with dry flours and milling fractions of cereal grains such as rye, oats, barley, wheat, and unsweetened cereals provided all of the above have been milled to pass a 0.5 mm mesh. This assay may also be used for samples containing simple sugars by extracting the sample with 50% ethanol to remove these sugars (which can artificially increase measured glucose values) prior to lichenase treatment. Assay steps have been modified and optimized for different applications including flours and milling fractions from cereal grains[24] (AACC method 32-22 and 32-23) and β-glucan in malt, wort, and beer.[34]

2.3.4.5 Analysis of Galactomannans

Galactomannans are polysaccharides consisting of a β-1→4 linked mannosyl backbone substituted to varying degrees at the C-6 position by an α-galactose unit. The level of substitution varies depending on galactomannan source; for example, galactomannan from guar has a galactose:mannose (G:M) ratio of approximately 1:2 while the ratio for locust bean gum (carob gum) is 1:4.[35] An enzymatic method for quantitatively determining galactomannans has been developed that is based upon the known G:M ratios of common galactomannans.[26,27]

2.3.4.5.1 Reaction Theory

The concentration of galactomannan in a sample is determined from the quantity of galactose released by α-galactosidase after β-mannanase digestion. Galactose is quantified spectrophotometrically after treatment with NAD and galactose dehydrogenase.

$$\text{1. Galactomannan} \xrightarrow{\beta\text{-mannanase}} \text{Oligosaccharide mixture}$$

$$\text{2. Oligosaccharide mixture} \xrightarrow{\alpha\text{-galactosidase}} \text{Galactose + manno-oligosaccharides}$$

2.3.4.5.2 Operating Procedure

Dry flour samples from guar or locust bean gum are extracted with 80% ethanol to remove simple sugars and oligosaccharides which may contain galactose. The galactomannan present is solubilized by boiling the flour in a buffer followed by incubation at 40°C with β-mannanase. Sample mixtures are centrifuged to separate insoluble material and portions of the supernatant are incubated with α-galactosidase to release galactose and another portion

is treated with an acetate buffer (blank). Galactose is quantitatively determined in the sample after treatment with NAD and galactose dehydrogenase.

2.3.4.5.3 Quantification

Blank absorbance values are subtracted from sample absorbances and the resulting values used to quantitatively determine galactose by accounting for sample dilutions, the conversion of free galactose to anhydro-galactose, and a factor to relate free galactose to galactomannan content (×0.90). The average percentage of galactose in guar and locust bean gum is 38 and 22%, respectively.[36]

2.3.4.5.4 Applicability

This method can be used for analysing seed flours provided the galactose:mannose ratio of the galactomannan is known. It can also be used when low molecular weight sugars are present in the sample provided they are removed via ethanol extraction prior to analysis. The presence of 1→6 linked α-D-galactose units in some oligosaccharides, such as raffinose and stachyose, are also susceptible to α-galactosidase; these oligosaccharides will artificially increase the gum content if they are not removed before enzyme treatment.

2.4 Oligosaccharide Analysis

Strictly speaking, oligosaccharides are carbohydrates composed of between two and ten monosaccharide residues glycosidically linked.[37] Practically speaking, saccharides with a DP greater than 10 are often referred to as oliogsaccharides. For example, inulin is often referred to as a nondigestible oligosaccharide, despite the fact that it is a polydisperse mixture with a DP ranging from 2 to 65.[38] Raffinose and stachyose, tri- and tetrasaccharides respectively composed of galactose, glucose, and fructose are found in beans. Other common oligosaccharides in foods are dextrins or starch hydrolysates. Starch hydrolysates are characterized based on their dextrose equivalent (DE), or the relative reducing power of all sugars in the mixture. Hydrolysates with a DE of 20 or below are referred to as maltodextrins and are mainly used for adding bulk to food products as well as their viscosity-modifying and film-forming properties. Oligosaccharides are also found in beer, the large majority being starch hydrolysates that result from the enzymatic activity of amylases during malting.[39]

Analysis of oligosaccharides released by partial acid hydrolysis or enzymatic attack of polysaccharide material is a technique often used to provide valuable information about molecular structure. For example, the (1→4) linkage attached to a (1→3) linked glucose unit in mixed linked (1→3)

(1→4)-β-D-glucan is preferentially cleaved by (1→3) (1→4)-β-D-glucan-4-glucanohydrolase (lichenase). The oligosaccharides released from a lichenase digestion of β-glucan are primarily 3-O-β-cellobiosyl and 3-O-β-cellotriosyl-D-glucose; the relative proportion of which is indicative of the dominant polysaccharide structure. The analysis of these oligosaccharides is thus an important step in fully understanding the parent polysaccharide. Details on structural analysis of polysaccharides are given in Chapter 3.

Regardless of whether or not oligoaccharides are present in a sample as an inherent component of its makeup (e.g., beans) or owing to some hydrolytic treatment resulting from direct sample manipulation (i.e., acid or enzymatic hydrolysis), there is a necessity to identify and quantify them. Methods for analyzing oligosaccharides are similar to methods for analyzing monosaccharides. For example, the extraction of oligosaccharides from food products is accomplished as for monosaccharides, with hot 80% ethanol. They can be hydrolysed with acid or enzymes to their constituent monosaccharides and the hydrolysate subjected to analysis by chromatographic, chemical or enzymatic methods. In addition, size exclusion techniques such as high performance size exclusion chromatography or gel permeation chromatography can be used to separate oligosaccharide mixtures by size.

2.4.1 Monosaccharide Composition

The monosaccharide composition of oligosaccharides can be determined by hydrolysis (acid or enzymatic) followed by a suitable method for identifying the released monosaccharides. Released monosaccharides may be identified using chromatographic techniques (HPLC, GC) provided suitable standards are used for comparing the retention times (see Section 2.3 for details on monosaccharide analysis).

2.4.2 Size Exclusion Chromatography (SEC)

Size exclusion chromatography is a chromatographic technique whereby molecules in a sample are separated based on their size. Molecules in an eluant stream (usually a buffer) are directed into a column filled with a gel packing of clearly defined pore size. The smaller molecules in a sample get held up in the pores, therefore spending more time in the column and eluting later than larger molecules, which essentially pass through the spaces between the pores and elute first. Separation is influenced by the size of the pores in the column packing. Effluant from the column is monitored using one or more detectors. Refractive index detectors are often used alone or in combination with light scattering and/or viscosmetric detectors.

Size exclusion chromatography has been used as a method for separating and characterizing mixtures of oligosaccharides based on size. Dextrins, or starch hydrolysates, can be rather complex mixtures of oligosaccharides with the size and proportion of linear and branched oligomers varying depending

on the starting material (rice starch, corn starch, etc.) and hydrolysis method (enzymes, acid) even when the DE is the same. Dextrins of DE ranging from 4 to 25 were analyzed on a SEC system equipped with multi-angle light-scattering detector, enabling the acquisition of information with respect to molecular weight distribution.[40] In combination with high performance anion exchange chromatography (HPAEC), which provided information about the relative occurrence of individual oligosaccharides, SEC coupled with a molecular weight sensitive detector allowed a more complete dextrin sample profile than the DE value alone (which only reflects the number of reducing ends).

Conventional or low pressure size exclusion chromatography predates SEC. Low pressure systems employ large capacity columns (i.e., 1.6×70 cm and larger) singly or several in series that operate at a relatively low pressure compared to SEC and separate larger sample volumes. Therefore, they are often used for preparative or semi-preparative purposes, to clean samples (remove salts or other small molecular weight material from a sample) or isolate a specific fraction of interest in a sample prior to further analysis. Eluent concentration is often monitored with a detector (i.e., RI detector) so a chromatogram of sample distribution is obtained. Alternately, a eluent containing separated material is collected in tubes and the tube contents analyzed using another analytical technique, such as the phenol–sulfuric acid assay. By plotting the tube number vs. the carbohydrate concentration, a curve representing sample distribution can be obtained. For example, large and small molecular weight starch fractions in native and acid-modified starches from cereals, pulses, and tubers were separated on a Sepharose CL 4B gel (Pharmacia Fine Chemicals, Sweden) at 30ml/h with water as eluent.[41] Oligosaccharides arising from enzymatic degradation of carrageenan have also been separated successfully on a low pressure SEC system. Using Superdex™ 30 (Pharmacia Fine Chemicals, Sweden) preparatory grade gel (a dextran-agarose composite), good separation and resolution of carrageenan oligosaccharides in large quantities with run times ranging from 16 hr[42] to several hours[43] depending on the sample type and the analytical conditions used. The structure of isolated oligosaccharides can then be determined using methods outlined in Chapter 3. Other examples of the use of gel-permeation chromatography for the purpose of purifying and/or separating oligosaccharides generated by the hydrolysis of polysaccharides abound, including galactoglucomannan oligosaccharides from kiwi fruit,[44] xyloglucan oligosaccharides generated by the action of cellulase,[45] and oligosaccharides from olive xylogucan.[46]

Using gel-permeation chromatography is rooted in the fact that relatively large sample sizes may be applied to the column and therefore separated fractions can be collected in relatively large amounts. Additionally, it has been reported that conventional SEC separates some classes of oligosaccharides, such as a homologous series of maltodextrins, with better resolution than HPSEC.[47] One of the disadvantages of low pressure SEC systems is the time required for a single run, often requiring many hours or even days.

2.4.3 High Performance Anion Exchange Chromatography (HPAEC)

Using HPAEC for carbohydrate analysis is highlighted again when the separation of oligosaccharides is considered. The combination of column packing, eluent composition, and sensitive electrochemical detection enables the baseline resolution of a homologous series of oligosaccharides such as dextrins (starch hydrolysates) up to DP 40. Unlike monosaccharide analysis, where the mobile phase is typically sodium hydroxide alone, mobile phases for oligosaccharides contain sodium acetate as well to increase ionic strength and to ensure adequate pushing off of the oligosaccharides from the column. The Dionex Carbopac PA1 column has been used to separate dextrins up to DP 30[40] using a mobile phase combination of 80% A and 20%B at time zero with a linear gradient to 90% B and 10% A (where A = 100 mM NaOH and B = 100 mM NaOH containing 600 mM NaOAC). In the case of dextrins, sample preparation consists only of drying the sample, diluting an appropriate amount with water, and filtering (0.45 um filter) prior to injection. HPAEC has also been used as a preparative technique to separate the oligosaccharides in beer.[39] Again, using a NaOH/NaOAC buffer, an effluant was collected from the column in 20 second fractions and subjected to further analysis. Oligosaccharides (composed of glucose, galactose, and xylose) released by the action of cellulase on seed xyloglucan were separated on a CarboPak PA100 column (Dionex, Sunnyvale, CA) using gradient elution of sodium hydroxide and sodium acetate buffers.[45] Oligosaccharides such as isomaltose, kojibiose, gentiobiose, nigerose, and maltose from different varieties of honey have also been separated using HPAEC-PAD.[48]

Not limited to the separation of neutral oligosaccharides, oligogalacturonic acids from strawberry juice have been separated on a HPAEC system using a sodium hydroxide gradient and a CarboPac PA-100 column.[23] Oligogalacturonic acids resulting from pectin enzymatic depolymerisation were separated on a Mono-Q anion exchange column (Pharmacia, Upsala, Sweden) using gradient elution (Na_2SO_4 in phosphate buffer) and detected on a photodiode array detector. The anion exchange resin permitted good resolution of oligomers up to Dp 13.[49]

The main disadvantage of using HPAEC-PAD for analysing oligosaccharides is the absence of adequate standards for many oligosaccharide types (i.e., oligosaccharides from β-glucan, malto-oligosaccharides over DP 7), which prevents their quantification. In addition, pulsed amperometric detector response is not equivalent for all sample types and, in fact, detector response decreases with the increase of DP;[19] therefore purified standards are required in order to facilitate accurate quantification.

2.4.4 Enzymatic Analysis

Enzymatic hydrolysis of oliogsaccharides coupled with chromatographic separation and identification of released oligosaccharides is commonly used to quantify oligosaccharides in both simple and complex food systems. This

method has successfully been applied to the analysis of fructo-oligosaccharides and inulin (AOAC 997.08). Inulin and oligofructose are fructans, (2→1) linked β-D-fructofuranosyl units with or without a terminal glucose,[50] found naturally in chicory, Jeruselem artichoke, and onions. Both inulin and oligofructose are polydisperse mixtures with DP values ranging from 2 to 60 and 2 to 10, respectively.[51] These fructans have received much attention in recent years based on their postulated positive nutritional benefits, including improved laxation and the stimulation of beneficial gut microflora, and their potential uses as functional food additives.[50] Because of inulins' larger DP it is less soluble than oligofructose and able to form micro-crystals in solution that impart a fat-like mouthfeel, enabling it to function as a fat mimetic. Oligofructose is more soluble than inulin and sweet tasting, making it appropriate for use as a humectant in baked goods and a binder in granola bars with improved nutritional functionality vs. corn syrup.

Both inulin and oligofructose in a food product may be quantified by subjecting a hot water extract to an enzymatic treatment that permits fructan determination by difference (sugar content before and after treatment) (AOAC 997.08).[51,52] Free fructose and sucrose are determined in the original sample, free glucose and glucose from starch are determined after amyloglucosidase treatment, and total glucose and total fructose are determined after fructozym hydrolysis. Glucose and fructose from fructans are then determined by difference. HPAEC-PAD is ideally suited for the analysis of these enzyme hydrolysates because it enables baseline resolution of all sugars of interest and facilitates straightforward quantification.

Similarly, a method has been developed for the extraction, detection, and quantification of inulin in meat products. Inulin is extracted from meat products[53] in hot aqueous solvent, treated with inulinase, and the released fructose quantified by HPLC with RI detection. The quantity of inulin and oligofructose is determined by subtracting the quantitiy of free fructose (determined from a blank run without enzyme) from the total fructose in a sample after enzyme treatment.

Alternately, fructans can be determined as glucose and fructose after enzyme hydrolysis using chemical/spectrophotometric methods[54] (AACC method 32-32). This method uses *p*-hydroxybenzoic acid hydrazide (PAHBAH) to measure fructans as reducing sugars after treatment with a fructanase. As with other methods, fructans are extracted in hot water and the extract is treated with a succession of enzymes to cleave sucrose into glucose and fructose and starch into glucose. The released monosaccharides are reduced to sugar alcohols to avoid interfering in the fructan assay. Fructanase is added to release fructose and glucose from the fructans and the quantity of reducing sugars present in the solution is then determined using the *p*-hydroxybenzoic acid hydrazide method where color generated by the reaction is measured at 410 nm. Advantages of this method vs. chromatographic methods include its relatively fast speed and that expensive chromatographic equipment is not required. The disadvantage of this method is that fructan content will be underestimated if fructans have been extensively depolymerized because the

reducing ends will be reduced with other sugars prior to fructanase addition and therefore omitted from quantification when the PAHBAH is added.

2.5 Dietary Fiber Analysis

2.5.1 Definition of Dietary Fiber

The past few decades have seen much discussion and debate over the definition and measurement of dietary fiber. The definition, as it existed in the early 1970s, was a response to the observed physiological behavior of plant cell wall material in the human digestive system and the postulated benefits of this behavior.[55] This definition held that dietary fiber was plant cell wall material that was impervious to the action of digestive enzymes. This definition was later expanded to include all indigestible polysaccharides such as gums, mucilages, modified celluloses, oligosaccharides, and pectins.[56] The expanded definition arose because these additional substances behaved physiologically in a similar manner to compounds included in the original definition; they were edible and not digested and absorbed in the small intestine. Clearly the definition of dietary fiber is intricately linked with the physiological behavior of food compounds, but scientific methodology has tended to overshadow physiological function as the basis for definition.[57] The challenge over the past few years has been to arrive at a widely accepted (by nutrition professionals, regulators, and scientists) definition that reflects the physiological function of dietary fiber and to pursue methodologies that facilitate its quantitative analysis. The official AACC definition of dietary fiber[57] is as follows;

> 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 fibers promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation.

Other definitions have been put forth that differentiate between intact, intrinsic fiber in plants termed dietary fiber (i.e., that found in fruits or cereal grains) and which have been isolated or synthesized and added to a product, termed functional or added fiber.[58] Still others have argued that only plant cell wall material should be considered dietary fiber,[59,60] excluding such components as resistant starch and other noncell wall nonstarch polysaccharides. Despite the above, it is generally accepted that what constitutes dietary fiber are those compounds outlined in the AACC definition above.

Explicit in the definition above is the expectation of beneficial physiological consequences based on the ingestion of dietary fiber. Dietary fiber intake has been associated with reducing blood cholesterol levels, reducing the risk of coronary heart disease, attenuating postprandial blood glucose response, and improving laxation. Because dietary fiber is a term representing a fairly heterogeneous group of compounds, it is not expected that every type of dietary fiber will play a role in each of these beneficial physiological consequences. For example, insoluble fiber such a cellulose is associated with increasing fecal bulk and improving laxation,[61,62] while viscous dietary fiber, also termed soluble fiber, which includes β-glucan, psyllium gum, and pectin, is associated with lowering cholesterol[32,33,63,64] and moderating postprandial blood glucose levels.[31,65–67] There are several postulated mechanisms for the effects of dietary fiber. Soluble fiber is believed to bind cholesterol and bile acids, increasing their excretion.[68] Alternately, it has been suggested that short chain fatty acids produced by bacterial fermentation of fiber may beneficially affect lipid metabolism.[68,69] With respect to plasma glucose levels, it is thought that viscosity development in the gut caused by the presence of soluble fiber slows the movement of glucose into the blood stream, flattening the blood glucose peak that usually occurs after a meal. Included in the group termed viscous dietary fiber is β-glucan, psyllium gum, and guar gum. Despite this, the distinction between soluble and insoluble fiber in terms of physiological function is not clear, and in fact, there is support for the abandonment of the terms soluble and insoluble altogether.[58] For example, inulin and oligofructose are soluble dietary fibers that have a demonstrated ability to increase fecal weight[70] despite the fact they are soluble fibers. Additionally, many fiber sources contain both soluble and insoluble fiber and therefore provide the benefits associated with both types of fiber.

Given the nutritional importance of dietary fiber, there must be an adequate standardized method for determining the dietary fiber content in food products. This is important from a regulatory perspective, ensuring nutrition labels are reporting accurate data and that all those compounds behaving as a dietary fiber (are eaten, impervious to digestion in the small intestine, promote laxation, and/or attenuation of blood cholesterol and/or blood glucose levels) are captured and counted as dietary fiber in the analysis. For several reasons this has proven a less than simple task. Compounds that behave similarly physiologically do not necessarily have analogous solubility properties. For example, in what is probably the most used and widely recognized dietary fiber method (AOAC 985.29) there is an ethanol precipitation step (1 part sample solution, 4 parts ethanol) intended to separate those compounds considered digestible (glucose from starch hydrolysis, amino acids from protein hydrolysis, simple sugars) and therefore excluded from what is considered dietary fiber, and the indigestible residue. There are compounds, such as fructans which are partially soluble in 80% ethanol[71] and as such would be excluded from the analysis despite the fact research has suggested they are able to increase fecal bulk[72] and reduce serum lipid

levels.[73,74] Polydextrose (a highly branched glucose polymer) and galacto-oligosaccharides have also historically been excluded from dietary fiber measurements based on their solubility in 80% ethanol. Realization of this fact necessitated the development of methods for quantifying fructo-oligosaccharides, inulin, polydextrose, and galacto-oligosaccharides.

2.5.2 Dietary Fiber Analysis

2.5.2.1 Uppsala Method

There are two fundamentally different approaches to analyzing dietary fiber. The Uppsala method[75] (AOAC 994.13; AACC 32-25) requires measuring the neutral sugars, uronic acids (pectic material), and Klason lignin (noncarbohydrate dietary fiber including native lignin, tannins, and proteinatous material) and summing these components to obtain a dietary fiber value (Figure 2.9). The sample is first subjected to an amylase and amyloglucosidase digestion to remove starch. Starch hydrolysates and low molecular weight sugars are separated from soluble fiber using an 80% ethanol precipitation, leaving a residue containing both soluble and insoluble fiber. Neutral sugars are determined after derivitisation as their alditol acetates by GC, uronic acids are assayed colorimetrically, and Klason lignin is determined gravimetrically.

2.5.2.2 Enzymatic/Gravimetric Methods

The second method is a measure by difference where dietary fiber residue is isolated, dried, weighed, and then this weight is adjusted for nondietary fiber material (i.e., protein and ash). Enzymatic-gravimetric methods such as official AOAC method 985-29 involve subjecting a sample to a succession of enzymes (α-amylase, amyloglucosidase, protease) to remove digestible material, an ethanol precipitation step to isolate nonstarch polysaccharide, and finally an ash and protein determination (Figure 2.10). The quantity of protein and ash is subtracted from the dry residue weight to obtain a total dietary fiber value. This method has been modified to permit the determination of total soluble and insoluble dietary fiber (AOAC 991.43).

With appropriate methods in place for the measurement of the majority of dietary fiber constituents, recent efforts have focused on establishing official methods for classes of compounds that behave physiologically like dietary fiber, but are soluble in 80% ethanol and hence excluded from commonly used official methods for measuring dietary fiber. These compounds include fructans (AOAC official method 997.08 or AOAC method 999.03), which are found in onions, leeks, chicory, and Jeruselum artichoke. Polydextrose, also soluble in 78% ethanol owing to its extensively branched structure, can be analyzed using AOAC method 2000.11. Fructans include both inulin and oligofructose, which rather than being single molecular species, are polydisperse mixtures of fructose polymers. Oligofructose has a DP range from

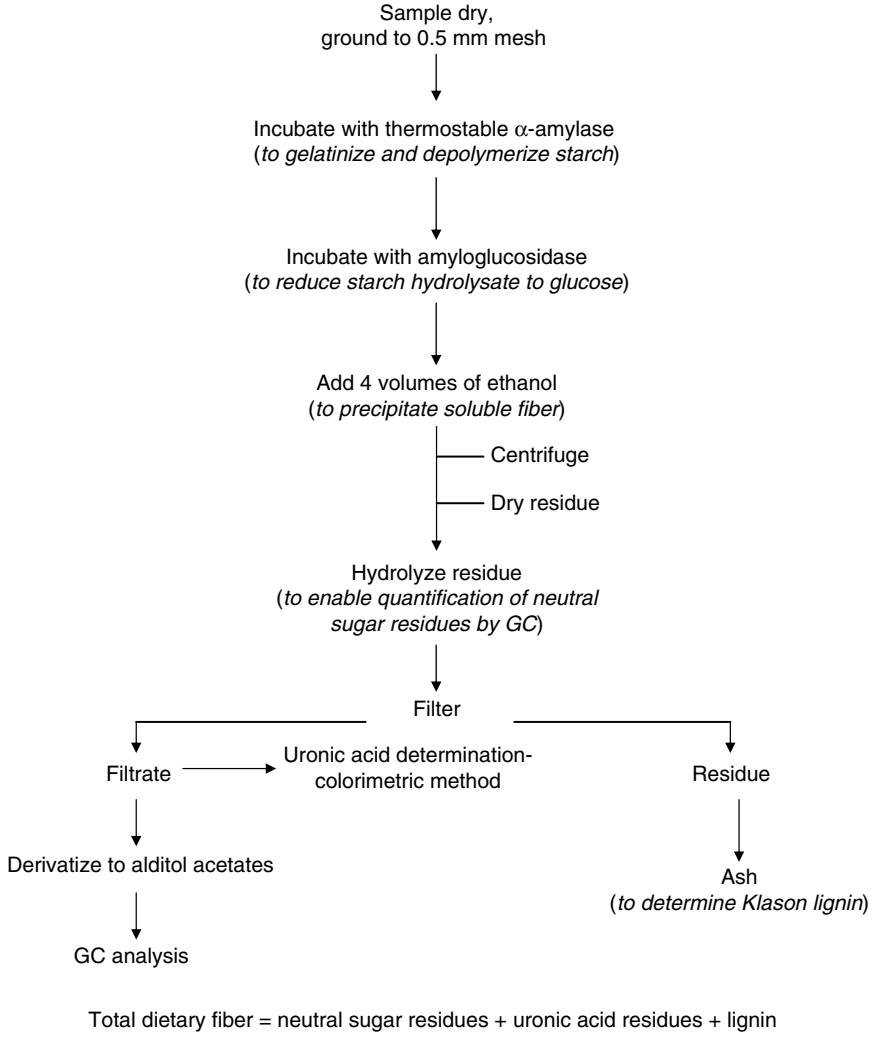

FIGURE 2.9
Total dietary fiber procedure — Uppsala method. (Adapted from AACC method 32–25.)

~2 to 10, while inulin typically ranges from ~2 to 60, but there is variation in this range depending on the inulin source. Because the DP of these compounds varies, their solubility in 80% ethanol varies, and therefore their inclusion in total dietary fiber (TDF) measurements is not complete.[76] In order to avoid overestimating the contribution of fructans by virtue of counting them twice, the inclusion of an inulinase in the TDF procedure will completely exclude fructans from being counted as a dietary fiber. Inulin and oligofructose can then be determined in a separate analytical procedure and their quantity as dietary fiber added to the TDF determined using

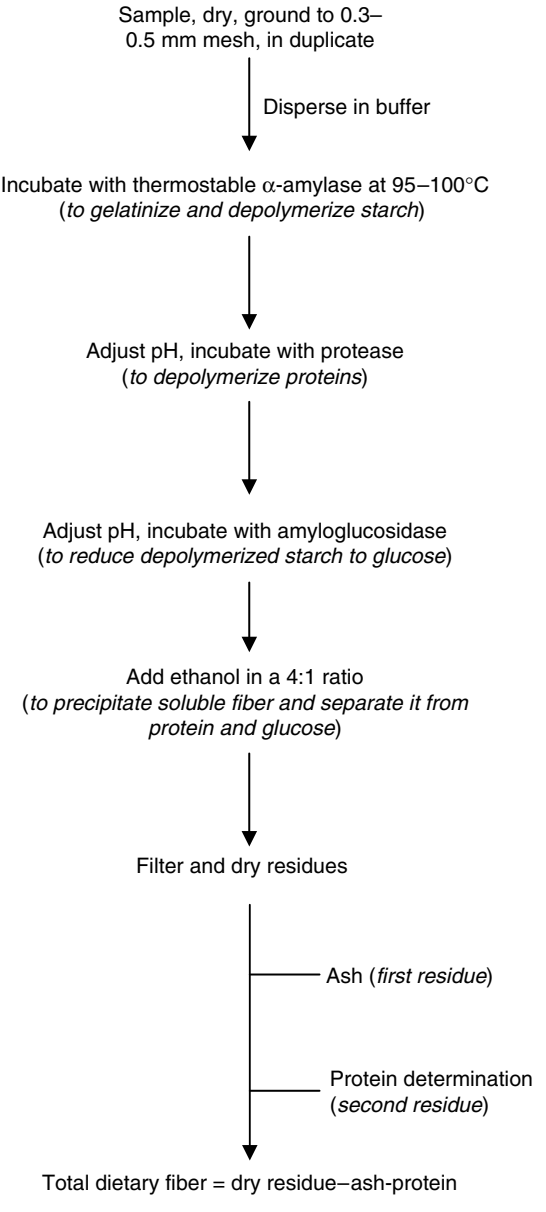

FIGURE 2.10
Total dietary fiber procedure — enzymatic/gravimetric method. (Adapted from AACC method 32–05.)

conventional procedures.[77] Fructans are determined in food products as their constituent monosaccharides after extraction in aqueous solvent and enzymatic hydrolysis. Released monosaccharides are determined using spectrophotometric or HPLC methods (see Section 2.4 for details).

2.5.3 Summary of Dietary Fiber Analysis

Given the nutritional implications of dietary fiber intake, its quantitative determination in food products is very important, but the measurement of dietary fiber has been hampered by controversies surrounding its very definition and the fact that dietary fiber encompasses a class of compounds with disparate properties. There are now several methods in place that permit the measurement of TDF and additional methods to specifically measure those compounds (i.e., polydextrose and fructans) excluded from conventional dietary fiber methods.

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