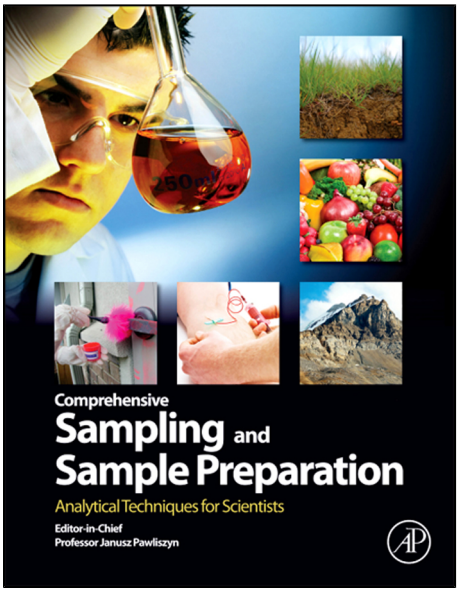

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4.11 Sample Preparation for the Determination of Carbohydrates in Food and Beverages

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4.11.1 Introduction

Carbohydrates are one of the major classes of food components along with lipids and proteins. Carbohydrates appear as natural components in almost every food, but they can also be added to increase sweetness, to improve other properties (increase viscosity, improve texture, etc.), or even as inexpensive additives. They can occur as free saccharides (monosaccharides, oligosaccharides, or polysaccharides) but can also be linked to proteins or lipids, forming glycoproteins or glycolipids.

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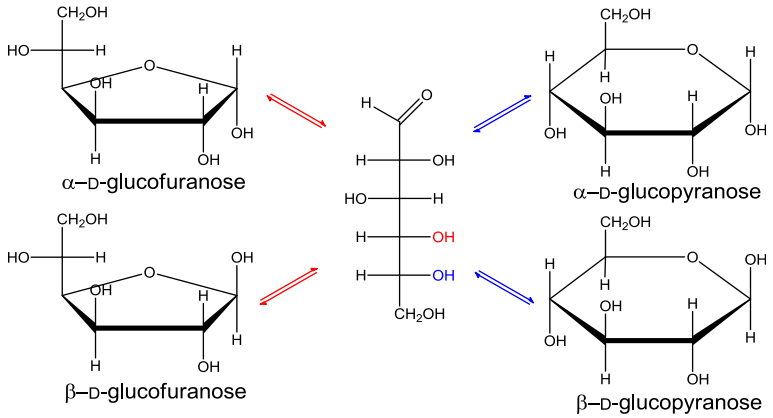

Figure 1 Tautomeric forms of D-glucose.

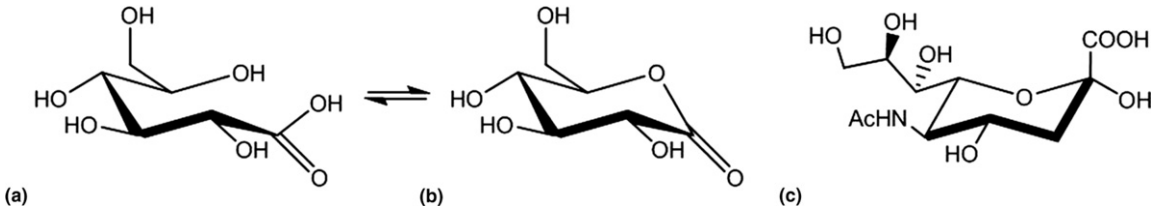

Figure 2 Chemical structure of (a) D-gluconic acid, (b) D-glucono-δ-lactone, and (c) *N*-acetylneuraminic acid.

This chapter follows the International Union of Pure and Applied Chemistry (IUPAC) recommendations for nomenclature of carbohydrates. Nevertheless, some traditional terms commonly used in food analysis have also been considered. The generic term 'sugar' is applicable to soluble carbohydrates of low molecular weight.

Carbohydrates have some common features: they are all polar molecules with a high number of free hydroxyl groups (linear as polyalcohols and cyclic as inositols), but they usually have at least one other functional group.

Monosaccharides are the basic constituents of all superior carbohydrates. Monosaccharides are polyhydroxyaldehydes and polyhydroxyketones, with 3–8 carbon atoms. Those that contain four or more carbons can exist in an open-chain form or a heterocyclic ring form, where the carbonyl group (aldehyde or ketone) has reacted reversibly with an intramolecular hydroxyl to form a hemiacetal. These heterocyclic forms consist of five (furanoses) or six (pyranoses) atoms (Figure 1).

Other functional groups commonly found in food sugars are carboxylic acids (aldonic, uronic, aldaric) able to form lactones (reversibly), amines or amides (amino sugars), and both carboxyl and amine groups (sialic acids) (Figure 2).

A carbonyl group of a monosaccharide can react with a hydroxyl group of another monosaccharide, forming an acetal group to give a disaccharide. The free carbonyl group of the second monosaccharide can in turn react with another monosaccharide to give a trisaccharide, and so on. The term 'oligosaccharide' is used for polymers with a degree of polymerization (DP) up to 20 units; the term 'polysaccharide' is usually reserved for polymers with DP > 20, and is frequently used in a more general way to refer to both oligosaccharides and polysaccharides. Polysaccharides can appear as linear or branched chains (Figure 3).

4.11.1.1 Properties of Carbohydrates

Some properties of carbohydrates should be taken into account in selecting the most adequate preparative strategies for their further analysis.

4.11.1.1.1 Molecular Weight

As previously mentioned, carbohydrates can be classified according to their polymerization degree as monosaccharides, disaccharides, trisaccharides, and polysaccharides; their molecular weight ranging from 120 g mol^{-1} corresponding to tetraoses ($C_4H_8O_3$) to 10^8 g mol^{-1} corresponding to amylopectin (one of the naturally occurring polysaccharides with the highest molecular weight).

4.11.1.1.2 Isomerism

The number and type of isomers that carbohydrates are capable of displaying is higher than that of any other class of compounds. As an example, inositol has eight stereoisomers that are not mirror images of each other (diastereoisomers) (Figure 4).

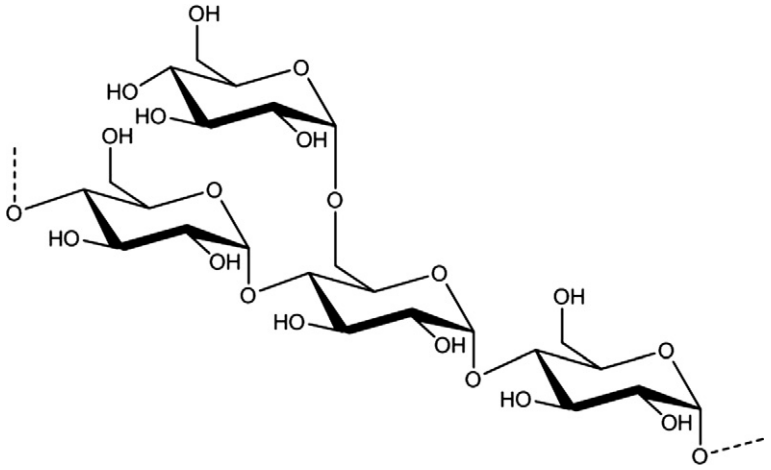

Figure 3 Branched sequence of a polysaccharide.

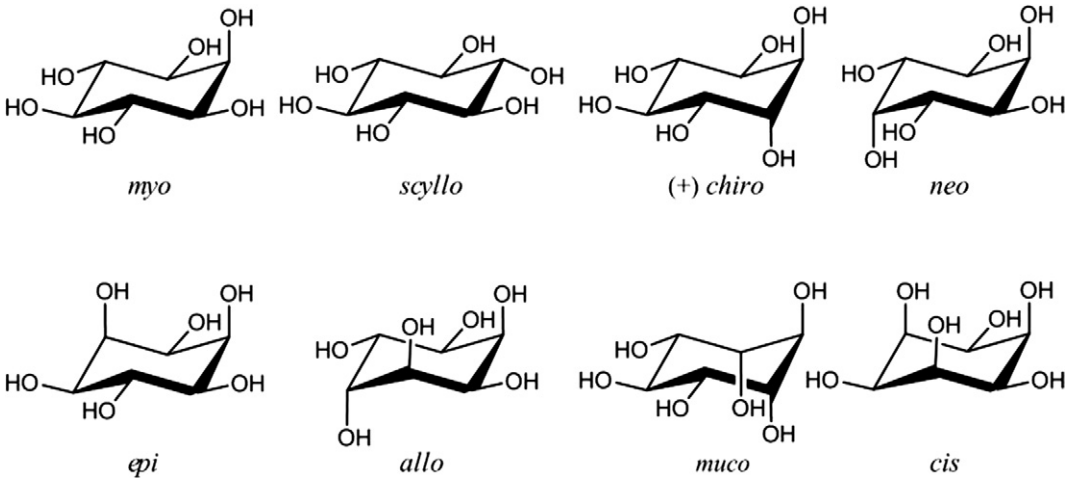

Figure 4 Diastereoisomers of inositol.

The number of possible isomers is notably high when sugars are polymerized; whereas two identical amino acids can be combined to give a unique dipeptide, two identical monosaccharides can give up to 13 different disaccharides.

Chirality is another characteristic property of carbohydrates. Although most food sugars belong to D-series (glucose, fructose, sucrose, cellulose), others are L-isomers, such as arabinose, sorbose, and rhamnose.

4.11.1.1.3 Solubility

The high number of hydroxyl groups in the molecule allows carbohydrates to be soluble in water, where they form complex equilibrium mixtures. Lower saccharides are also soluble in polar classic solvents, their solubility decreasing as molecular weight increases; many polysaccharides are totally insoluble.

Solubility increases with temperature and varies depending on the cosolutes present. Recently, ionic liquids have been shown to be satisfactory solvents for carbohydrates, extending their capability up to compounds traditionally considered as insoluble, such as cellulose (see Section 4.11.3.2.1).

It is noteworthy that the solubility of the different anomeric forms of a given saccharide is different; although interconversion in solution means that they finally attain the equilibrium, which is different for every solvent.

4.11.1.1.4 Rotary Power

The specific rotation $[\alpha]_D$ is defined using the sodium D-line at 20–25 °C.[1] Rotary power is very characteristic of every sugar in solution, and is not related to its molecular weight (Table 1). It depends on concentration, temperature, and solvent. When

Table 1 Specific rotation of different sugars in equilibrium, measured with $[\alpha]_D$ at 20–25 °C

Sugar (in equilibrium)	*Specific rotation*
L-Arabinose	+105
D-Fructose	−92
D-Glucose	+52.7
D-Rhamnose	−7.0
Sucrose	+66.5
Maltose	+130
Maltulose	+64
Gentiobiose	+10
Maltotriose	+160
Melezitose	+88.2
Panose	+154
Maltopentaose	+178
γ-Cyclodextrin	+180

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a crystalline sugar is dissolved, the rotary power of the solution slowly changes until the equilibrium is reached (mutarotation). Each tautomeric form has their own rotary power, and the equilibrium value reflects the individual values of all present forms.

4.11.1.1.5 Spectroscopic Properties

Carbohydrates do not have fluorescence properties and only absorb in ultraviolet (UV) at very short wavelengths.

4.11.1.1.6 Chemical Reactivity

Although some carbohydrates are hygroscopic, those appearing in food are rather stable in solid state, at neutral pH and room temperature. Polyalcohols are noticeably more stable than saccharides. Thermal stability of the latter is low: when heating, they easily undergo dehydration and further degradation such as caramelization or pyrolysis. Saccharides can easily react with acids, bases, amino groups, and different reagents. Some of these reactions are as follows.

- *Reactions in acid medium*: glycosides, oligosaccharides, and polysaccharides may be hydrolyzed to monosaccharides, which in turn may be dehydrated and degraded to low molecular weight compounds such as furanic compounds (furfural, hydroxymethylfurfural (HMF)) and to different colored products.
- *Reactions in basic medium*: reducing sugars undergo isomerization reactions through enediol intermediates (Lobry De Bruyn–Van Ekenstein reaction); a high number of by-products can be produced.
- *Maillard reaction*: carbonyl group of reducing sugars can condensate with free amino groups (from proteins or amino acids), giving rise to multiple reactions that take place in different foods and generate a high number of by-products. This reaction takes place at intermediate temperature and water activity.
- *Oxidation*: free carbonyl groups are able to reduce alkaline solutions of metal (mainly copper) salts to the free metal or to the oxide. Carbohydrates can also be oxidized by reagents such as chloramine T, picric acid, or 3,5-dinitrosalycilic acid (DNS). These reactions have been the basis of a high number of classic analytical methods.
- *Reduction*: the carbonyl group of an aldose or ketose can be reduced with different reagents (e.g., sodium borohydride) to give a linear polyalcohol (alditol) with formula $HOCH_2(CHOH)nCH_2OH$.
- *Derivatization*: the active hydrogen of hydroxyl, carboxyl, or amine groups can be replaced by nonpolar groups; the carbonyl group can be substituted by a fluorophore. The obtained derivatives are relevant for chromatographic or electrophoretic analysis.
- *Fermentation*: many sugars in aqueous solution are easily fermented by different microorganisms. Special care is therefore required for preservation of aqueous standard solutions; they should be stored at low temperature (or even frozen), and addition of preservatives such as dichromate or sodium azide is sometimes recommended.

4.11.1.2 Sample Preparation for Carbohydrate Analysis

Sampling is the first step within sample preparation procedures for carbohydrate analysis. Their determination in foods and beverages also requires different sample pretreatments according to the complexity of the sample or the analytical procedure to be used. Thus, whereas some foodstuffs require a simple procedure such as dilution followed by filtration/dialysis, others necessitate of different stages of sample preparation including, among others, sample cleanup, fractionation, derivatization, etc., prior to their analysis. Figure 5 shows a diagram of the basic steps necessary in the analysis of food carbohydrates. Although cleanup is usually considered as a previous step to further treatments, some basic procedures are applied sometimes as cleanup and sometimes as the final step. A brief discussion on the fundamentals of the most common of these steps can be found below, and their application to foods and beverages has been set out in Section 4.11.4.

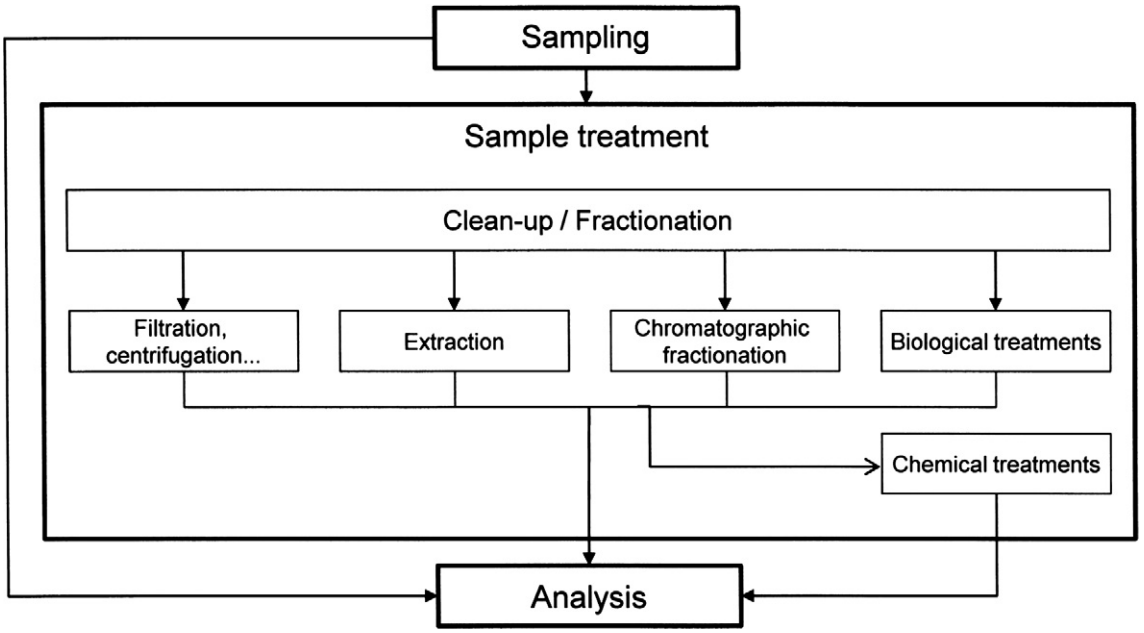

Figure 5 Diagram showing the most important steps of sample preparation for carbohydrate analysis.

4.11.2 Sampling

This step should be carried out under controlled conditions, depending on the type of food to be analyzed. Carbohydrates available as crystalline solids, and those appearing as liquid solutions are the easiest foods to be sampled and stored at room temperature in adequate containers. As an exception, the high viscosity and the frequent presence of crystals make the sampling of honey much more difficult. Complex foods such as milk, vegetables, or precooked food require specific protocols to avoid undesirable reactions. Therefore, sampling is only mentioned in Section 4.11.4 of this chapter in those cases where a specific treatment is necessary.

4.11.3 Sample Treatments

4.11.3.1 Filtration, Reverse Osmosis, and Dialysis

4.11.3.1.1 Filtration

Filtration is the process of removing particulate matter from a sample by forcing it through a porous media. Filtration processes have been widely used to purify, concentrate, and fractionate carbohydrates. It is well known that filtration is an important stage in sample preparation, particularly prior to high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) analysis, as this procedure discards insoluble material to avoid blocking the column. Nearly all filtration methods work with the size-exclusion mechanism, which allows small molecules to pass through freely but stops the flow of large molecules. The molecular size cutoff value is thus a key factor in characterizing a filter. The term microfiltration usually refers to conventional media filtration (paper, glass fiber, nylon, etc.) with pore size in the 0.1–0.5-μm range, while ultrafiltration (UF) and nanofiltration (NF) are usually carried out with membrane filters (polysulfone and cellulose acetate) of approximately 0.01 and 0.001 μm, respectively.

UF and NF are commonly used for removal of soluble components of the matrix; desalting and separation of interfering compounds such as proteins being some of their main applications in carbohydrate purification and concentration. Since macromolecules are often identified by their molecular weight, the value of the molecular weight cutoff (MWCO), which is the molecular mass of the smallest compound retained to an extent larger than 90%, is the parameter of choice for selecting UF and NF membranes. Disposable centrifugal ultrafiltration devices are also commonly used for purifying and concentrating carbohydrate solutions in foods and beverages. In these devices, where concentration and purification occur simultaneously, sample losses due to material transfer are eliminated (Figure 6).

In carbohydrate chemistry, the use of filtration procedures also constitutes an important tool for the fractionation of carbohydrate mixtures according to their DP and substitution. Although most applications deal with the use of UF and NF membranes with different MWCO and of different materials for separation of oligosaccharides from polysaccharides, filtration processes have also been used to obtain oligosaccharide fractions free from monosaccharides.[2]

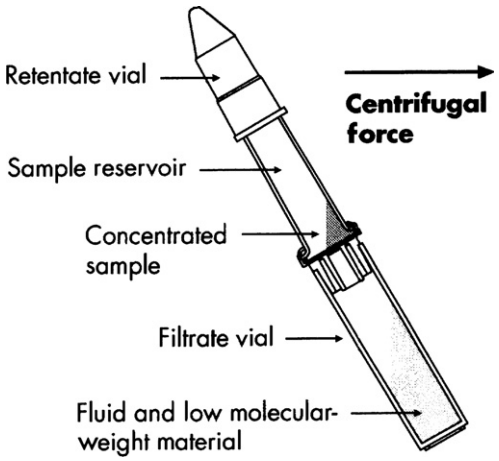

Figure 6 Amicon Centricon centrifugal filter device. *Reprinted with permission from Millipore Corporation, Danvers, MA (USA).*

4.11.3.1.2 Reverse Osmosis

Reverse osmosis is another membrane-based procedure commonly used for concentration of carbohydrates. Basically it is the inverse process to osmosis, that is, pressure in excess of the osmotic pressure forces the water to move through a semipermeable membrane (polyethylene terephthalate, polysulfone, polyamide, etc.) from the more concentrated solution of carbohydrate to the weaker (water). Reverse osmosis filters have a pore size of around 0.0005 μm.

4.11.3.1.3 Dialysis

Dialysis is another common laboratory technique for the purification, concentration, or fractionation of carbohydrates. Dialysis works on the principles of the diffusion of solutes and ultrafiltration of fluids across a semipermeable membrane or dialysis bag, which contains the carbohydrate solution. This sealed dialysis bag is placed in a container of a different solution (generally pure water). Molecules small enough to pass through the tubing (often water, salts, and other small molecules) tend to move into or out of the dialysis bag, in the direction of decreasing concentration. Larger molecules (e.g., polysaccharides) that have dimensions significantly greater than the pore diameter are retained inside the dialysis bag. This procedure can be used in reverse mode; i.e., placing pure water in the dialysis bag and immersing it in a complex, diluted sample; sugars are concentrated into the bag and they are obtained as a clear solution, which then can be lyophilized prior to analysis.[3]

4.11.3.2 Extraction

Extraction is one of the most basic sample preparation methods, which has been applied to carbohydrates under two traditional modes: liquid–liquid and liquid–solid. Modern solvent extraction methods such as supercritical fluid extraction or pressurized liquid extraction (PLE) are at present extensively applied to nonpolar analytes, but the number of applications to carbohydrates is low.

Extraction is frequently used for purification of carbohydrates, but as the molecular weight range of food carbohydrates is very broad, it is also often used as a fractionation or enrichment step.

Before extraction, the sample has to be adequately conditioned. Liquid samples have to be homogenized and solid samples have to be ground to improve their contact with the solvent. Moisture must be controlled in order to keep the calculated solvent polarity. In certain cases, when water is not compatible with the chosen solvent system, water must be removed by evaporation or lyophilization.

4.11.3.2.1 Liquid Extraction

A number of standard sample preparation methods are based on liquid extraction, which are discussed in detail in Section 4.11.4.

Liquid extraction is based on solubility differences among analytes and matrix. In general, carbohydrate solubility increases with temperature and decreases when molecular weight increases. It is necessary to take into account the low thermal stability of carbohydrates to make extractions at moderate temperature (usually less than 65 °C). As an exception, polyalcohols are rather stable and can be extracted at 100 °C.

As previously mentioned in Section 4.11.1.1.3, carbohydrates are soluble in three types of solvents:

- *Water and alcohols of low molecular weight*. Although water is the best solvent for carbohydrates, high molecular weight saccharides can be totally insoluble. When comparing linear and branched alcohols, solubility in alcohols decreases as the number of carbon atoms of the solvent increases; thus, methanol > ethanol > propanol… or isopropanol > *tert*-butanol; solubility is also higher in branched alcohols than in linear ones[4] (**Table 2**). Mixtures of ethanol and water are commonly used in the extraction of soluble

Table 2 Experimental equilibrium concentrations of glucose in different alcohols at 40 °C

Solvent	*Glucose (g l^{-1})*
tert-Pentyl alcohol	1.08
tert-Butyl alcohol	1.24
1-Butanol	0.59
Ethanol	3.81
Methanol	28.76
Ethanol + 10% water	21.23

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sugars in foods. As shown in Figure 7, the addition of small amounts of water usually provokes a high increase in solubility, allowing the possibility of tuning the extraction power of the system.[5]

- *A small group of polar organic solvents such as pyridine, 1,4-dioxane, dimethyl sulfoxide, dimethylformamide, etc*. These polar solvents dissolve low molecular weight sugars, but they have high boiling points and are difficult to eliminate; they are less used than alcohols for carbohydrate extraction. A special case is their use when chemical reactions are to be carried out. For example, an extraction with pyridine allows the subsequent silylation of sugars in the obtained solution (see Section 4.11.3.4.2). Extraction with solvents such as dimethyl sulfoxide (DMSO) is an adequate treatment to afford the direct obtainment of nuclear magnetic resonance spectra. Native starch from edible plants is extracted with DMSO and precipitated with ethanol before its stable carbon isotopic analysis.[6]
- *Ionic liquids* (ILs). These are a new class of solvents with unique properties: they entirely consist of ions, they are liquid at room temperature, they have almost no vapor pressure, and they are thermally stable, recyclable, and nonflammable. ILs are also commonly considered 'greener' than other organic solvents, since only small quantities of them pass to the environment. The solubility power of ILs can be modulated to dissolve a high number of carbohydrates, even those traditionally considered insoluble, as cellulose. ILs can also be mixed with traditional solvents either for tuning their

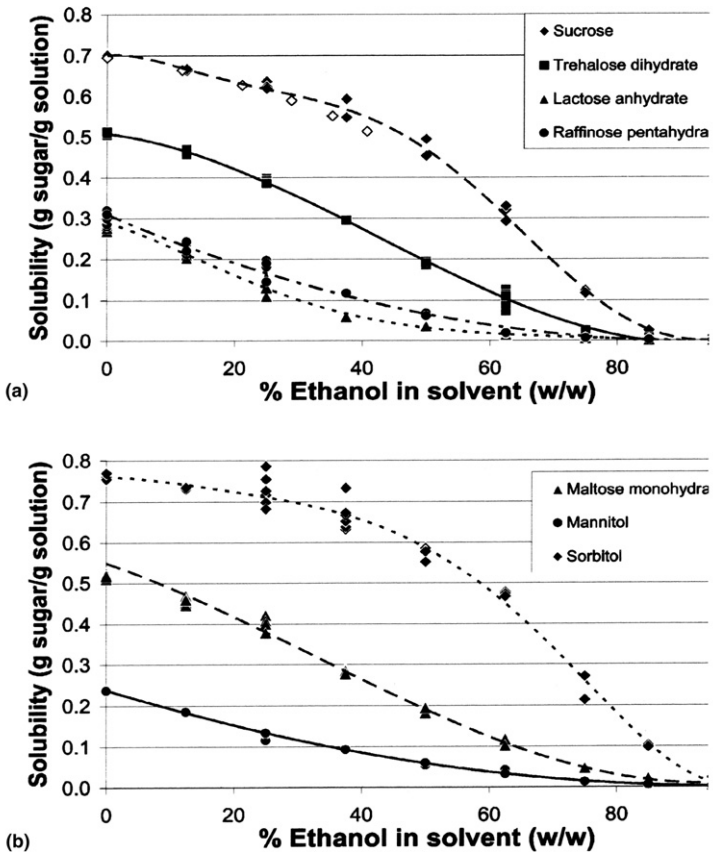

Figure 7 Solubility of (a) sucrose, trehalose, lactose, and raffinose, and (b) maltose, mannitol, and sorbitol, in different ethanol/water mixtures. *Reprinted with permission from J. Chem. Eng. Data 52, 1839. Copyright 2007 American Chemical Society.*

Table 3 Some ionic liquids used as solvents of carbohydrates

Cations						Anions		
				R = C_6H_{13}	[(di-h)$_2$DMG]	 [ACES]	Cl^-	[Cl]
				$CH_3OCH_2CH_2$	[$(C_3O)_4$DMG]		Br^-	[Br]
R=H,	R' = C_2H_5	[EMIM]		R = C_8H_{17}	[Aliquat®]		BF_4^-	[BF_4]
	C_4H_9	[BMIM]					PF_6^-	[PF_6]
	C_8H_{17}	[OMIM]					NCS^-	[SCN]
	$C_{10}H_{21}$	[C_{10}MIM]					$(NC)_2N^-$	[DCA]
	CH_3OCH_2	[MOMMIM]		R = C_6H_{13}	[$P_{6.6.6.14}$]	 [SAC]	$CF_3SO_3^-$	[TfO]
	$CH_3OCH_2CH_2$	[MOEMIM]		R'= $C_{14}H_{29}$			$(CF_3SO_3)_2N^-$	[Tf_2N]
	$CH_3CH_2OCH_2CH_2$	[EOEMIM]					$CH_3SO_3^-$	[MS]
	$CH_3OCH_2CH_2OCH_2CH_2$	[MOEOEMIM]		R = C_4H_9	[Bt_{14}]		CH_3COO^-	[ACET]
R=CH_3,	R' = C_4H_9	[BM_2IM]		$CH_2C_6H_5$	[Bt_{1Bn}]			

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properties, or to precipitate the substances previously dissolved in them. Despite the high number of reports about ILs in carbohydrate synthesis, their application for analytical extraction is still scarce. Table 3 presents the structures of several ILs reported for dissolution of carbohydrates.[7] A review on this subject has recently been published, including a large volume of data.[8]

4.11.3.2.2 Reactive Extraction

Alternative methods have been devised to extract sugars from aqueous solutions. Reactive extraction permits the removal of hydrophilic compounds from water solutions using a reversible reaction that improves solubility of analytes in organic solvents. The reagents used (called carriers) for the transport of carbohydrates from an aqueous medium into an immiscible organic solvent work by forming reversible covalent unions or inclusion complexes. Amines have been used for the extraction of sugar acids; boronic acid carriers (such as phenylboronic acids) have been used for the transport of mono- and disaccharides. Cyclodextrines have been used as carriers for small saccharides, which can easily enter the chiral cavity of these molecules.[9] Ionic compounds such as trioctyl methylammonium chloride have been used along with phenylboronic acid for the extraction of acid sugars such as *N*-acetylneuraminic acid.[10]Although this technique seems to be attractive in chemical or industrial processes, at present it is not applied in food analysis.

In some cases polysaccharides such as hemicelluloses are extracted with alkaline solutions. Partial hydrolysis and perhaps isomerization can be produced during extraction; therefore, operating conditions should be optimized.[11]

4.11.3.2.3 Ultrasound-Assisted Extraction

Ultrasound has been widely used for extracting polysaccharides from vegetable material. Acoustic waves are of purely mechanical nature; thus, ultrasound has mechanical effects that facilitate mass transfer, disrupt cells, and improve penetration of solvent. The mechanism of ultrasonic extraction involves two processes of physical activity: the diffusion from the solid toward the bulk of the liquid phase, which is a slow step, and the dissolution of the extractive substances near the solid surface, which is faster. As a counterpart, cavitation energy can create 'hot spots' and local pressure increments[12] (Figure 8).

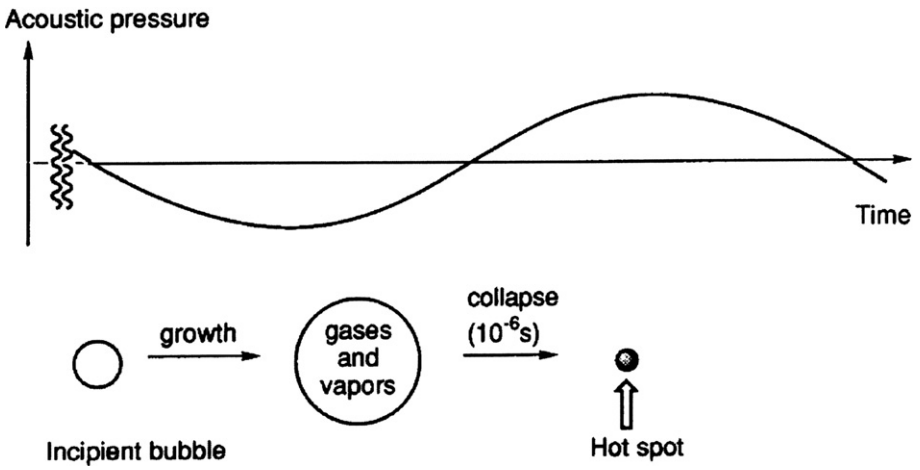

Figure 8 Cavitation in ultrasonic extraction. *Reprinted with permission from Elsevier (Carbohydr. Res. 2001, 332, 117).*

Although small molecules are usually not affected by ultrasonic treatment, polysaccharide molecules can be degraded. Under the sound-waves effect, carbohydrates become more sensitive to chemical attacks: ultrasound-mediated hydrolysis can be carried out at room temperature without collateral reactions occurring, e.g., in alkaline hydrolysis.[12] Ultrasonic extraction is an interesting analytical tool when a hydrolysis step is necessary before carbohydrate analysis.[13] It has been observed that recovery of polysaccharides increases when the extraction time increases, but a longer extraction time decreases yield, probably by degradation of the extractives. Ultrasonics has been successfully tried out in industrial food processes such as the extraction of polysaccharides from buckwheat hull, sugar cane bagasse, and apple pomace. Although these processes have been carried out without apparent changes in the properties of the extractives, it is usual to find some cleavage of links.[14]

Ultrasound equipment can be classified into two main classes: the low-frequency domain reaching 100 kHz and the high-frequency domain above 100 kHz. Ultrasonic cleaners are probably the simplest systems used in analytical laboratories for ultrasound extraction. This equipment supplies low energy (1–2 W cm^{-2}) at frequencies of 30–50 kHz; since energy distribution is very irregular through the tank, the position of extraction vessel is important. It is recommended to work with baths controlling time and power. Other used systems are immersion probes (as cell disruptors). These probes are immersed in the extraction vessel and the ultrasound waves easily attain the whole sample; temperature can be controlled by introducing the extraction vessel into a thermostatic bath. High-frequency generators are less common.

Important parameters are the type of the vessel and its geometric configuration, the bulk temperature, the frequency and, particularly, the acoustic energy.

4.11.3.2.4 Microwave-Assisted Extraction

Microwave-assisted extraction (MAE) is a technique based on the use of the microwave energy to help the transfer of the solutes from the matrix into the solvent. As consequence, extraction yield is increased, whereas time and solvent consumption are decreased.

Similarly to ultrasound extraction, this technique has been mainly used for the extraction of polysaccharides. Different studies have been conducted to optimize MAE conditions to avoid possible degradations of the polysaccharides.

MAE can also be assisted by the application of external pressure to the extraction vessels via nitrogen. As an example, moderate viscosity pectins[15] and alkaline-soluble polysaccharides[11] have been extracted and isolated from fresh sugar beet pulp by MAE (1200 W of microwave power at a frequency of 2450 MHz) under pressure in short times (3 min) and medium temperatures (60 °C), rather than hours and 80 °C as is required by conventional heating.

4.11.3.2.5 Supercritical Fluid Extraction

This extraction technique has been scarcely used for carbohydrates, although it is capable of giving good results in certain difficult separations. Carbon dioxide (probably the most used supercritical fluid) is nontoxic, nonflammable, can act at low temperatures, and is relatively cheap; unfortunately, the solubility of carbohydrates in the supercritical phase of this fluid is low. Solubility can be increased by the addition of polar cosolvents, usually low alcohols and/or water. Modification of the fluid along with changes in pressure and temperature allows solubility modulation in order to recover the searched analyte. For example, extraction of ketoses (tagatose and lactulose) from mixtures with their isomeric aldoses (galactose and lactose, respectively) was achieved with high yields[16]; prebiotic galactooligosaccharides can be purified from a commercial sample using CO_2 with ethanol/water as cosolvent.[17] In both cases, purity was confirmed by gas chromatography (GC) analysis. An ethanol/water mixture at 150 bar and 80 °C completely removed monosaccharides from a galactooligosaccharide mixture (Table 4).

4.11.3.2.6 PLE

PLE is also called accelerated solvent extraction or pressurized fluid extraction. However, when water is used as extractant, some terms such as subcritical water extraction, superheated water extraction, or hot liquid water extraction are frequently used. PLE is based on conventional heating at elevated temperatures and pressures up to 200 bar. The high temperature increases solubility of the analytes, and also improves their desorption from the matrix, whereas high pressure is maintained to keep the solvent in a liquid state.[18] This technique offers several advantages over classic methods, such as the shorter extraction time and the lower solvent consumption; most commercial equipment is also automated. Although PLE is in widespread use for the extraction of nonpolar compounds, a number of applications for polar analytes, mainly in plant materials, is also available.

Table 4 Supercritical fluid two-step extraction of a galactooligosaccharide mixture using CO_2 with ethanol 95% as cosolvent

		Extraction yield (%)			*Extract composition (%)*		
Step	*P/T/F/MF*	*Monosac*	*Disac*	*Trisac*	*Monosac*	*Disac*	*Trisac*
1	150/80/1.2/0.6	103.4	17.0	0.0	68.0	32.0	0.0
2	100/100/0.4	13.8	65.1	5.7	6.6	89.2	4.2

Disac, disaccharides; F, CO_2 flow (ml min^{-1}); MF, modifier flow (ml min^{-1}); Monosac, monosaccharides; P, pressure (bar); T, temperature (°C); Trisac, trisaccharides.
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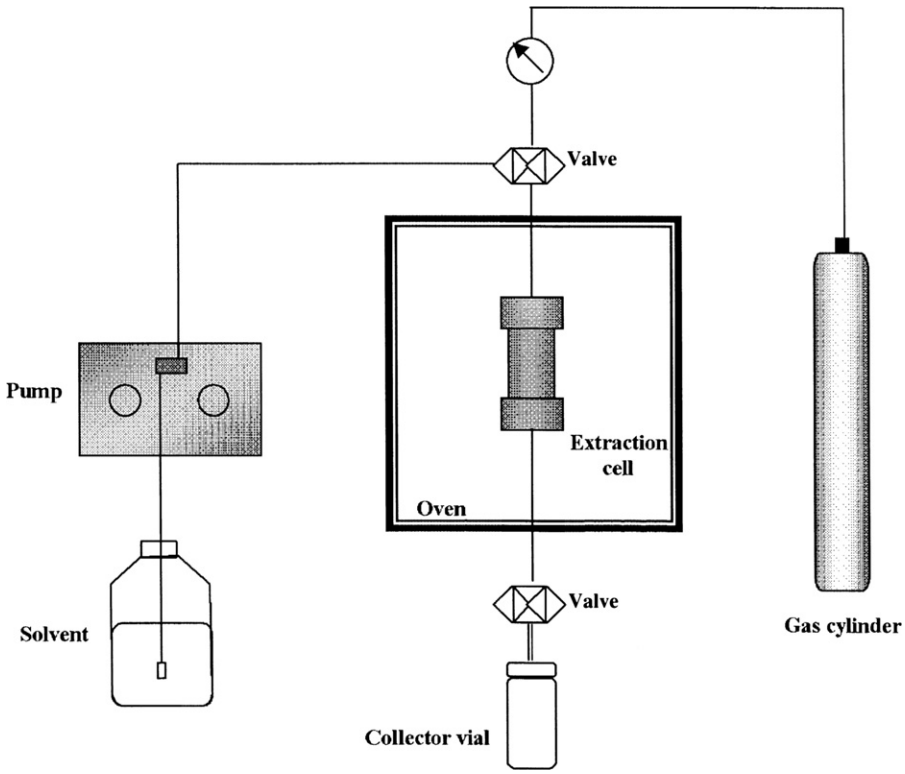


Figure 9 Scheme of equipment used for PLE extraction of honey carbohydrates.

PLE has been recently applied for the first time to the fractionation of carbohydrates.[19] Lactulose was separated from lactose with 70:30 (v/v) ethanol/water at 40 °C and 1500 psi using a commercial apparatus (ASE-200; Dionex, Sunnyvale, CA, USA); yield was assessed by GC. PLE has also been applied for obtaining honey fractions enriched in di- and trisaccharides for further GC analysis.[20] A scheme of the homemade extractor[21] used is shown in Figure 9. Optimum results were obtained at 10 MPa and 40 °C using two consecutive PLE cycles: first, 1:99 (v/v) ethanol/water for 5 min and second, 50:50 (v/v) ethanol/water for 10 min. Di- and trisaccharide fractions were enriched after PLE treatment, accounting for 73 and 8% of total carbohydrates, respectively. PLE has also been applied to the extraction of sugars and polyalcohols from woods used in winemaking.[22]

4.11.3.2.7 Solid-Phase Extraction

Solid-phase extraction (SPE) is one of the most common techniques used for sample cleanup. Samples are percolated through a solid sorbent (stationary phase), previously conditioned. Either the analytes of interest or the undesired impurities in the sample are retained by the sorbent according to their physical or chemical properties, and further eluted in a small volume of an appropriate solvent (mobile phase).

This technique has several advantages such as easy automation, high analyte recovery and reproducibility, possible hyphenation with chromatographic techniques, and commercial availability of several stationary phases.

Different formats have also emerged: SPE can be carried out not only in columns or cartridges but also in disks, tips, or 96-well plate designs. Moreover, online methods with analytical techniques (mainly HPLC) are currently used.

Solvents used for carbohydrate purification in SPE are those already indicated for liquid extraction, mainly water and alcohols, in which these compounds are soluble. Stationary phases can be grouped as follows.

- Reverse phase

Reverse-phase (RP) cartridges are commonly used for the purification of carbohydrates. They contain a stationary phase constituted by silica that has been modified by the covalent incorporation of nonpolar ligands. The retention mechanism is based on the interaction of the hydrophobic moiety of the analyte and the nonpolar ligand.[23] Therefore, the most polar compounds elute first whereas those with lower polarity are more retained.

Octyl (C_8) and octadecyl (C_{18}) silica phases are the most common RP cartridges used for carbohydrate cleanup. These sorbents show high affinity for hydrophobic compounds but little affinity for hydrophilic solutes such as oligosaccharides.[24] It has been observed that glucose and maltose are not retained on either C_8 or C_{18} sorbents, whereas maltoheptaose is not retained in C_8 cartridges.[25] Moreover, C_{18} cartridges are useful for the fractionation of (1–4)-α-glucans depending on their DP. The separation of salts from low molecular weight carbohydrates cannot be carried out using these cartridges.

Other cleanup procedures are based on the use of hydrophobic resins such as styrenic-based polymeric bead types.[26] These have been assayed for the removal of polymeric contaminants from low-level oligosaccharides, and have proved to be superior to C_{18} packing material in the elimination of interferences.

- Hydrophilic interaction liquid chromatography (HILIC)

Hydrophilic interaction liquid chromatography cartridges have also been used for carbohydrate cleanup. This chromatography belongs to the normal-phase liquid chromatographic mode where the aqueous mobile phase has been replaced by an aqueous/organic mixture (typically acetonitrile in water or a volatile buffer). The hydrophilic groups of the stationary phase attract water molecules from the mobile phase to form water-enriched layers. The chromatographic mechanism is therefore mainly based on partitioning equilibrium between both mobile and stationary phases facilitated by the aqueous layers. However, electrostatic interactions or hydrogen bonds can also take place in these separations (Figure 10).

- Ion exchange

Regarding ion-exchange SPE, different phases for weak cation exchange such as cyanopropyl-bonded silica or weak anion exchange such as aminopropyl-bonded silica are used. Both anion and cation-exchange resins are used for desalting oligosaccharide mixtures; special care should be taken to avoid the loss of charged sugars (acid or amine sugars) during their purification.

- Graphitized charcoal

In recent years, graphitized charcoal cartridges have become widely used for carbohydrate separation and purification. Their mechanism is based on the adsorption of polar compounds such as carbohydrates, and interaction is enhanced with increasing molecular size. This is a disadvantage for the purification of neutral monosaccharides, which elute in the water wash together with salts or other impurities. Nevertheless, neutral and acidic oligosaccharides are clearly separated from the undesired compounds using acetonitrile as organic modifier or acetonitrile with trifluoroacetic acid, respectively.

4.11.3.3 Chromatographic Fractionation

Previous to their analysis, the procurement of simple fractions constituted by carbohydrates with different configuration of hydroxylic groups or molecular weight distributions is sometimes required. Chromatographic techniques, usually set up in open columns with stationary phases based on ion exchange, adsorption, or size-exclusion mechanisms, are commonly used for this purpose.

Simulated moving bed chromatography has also been used for the fractionation of carbohydrates, mainly for industrial applications.[27] This technique is based on the use of columns with different stationary phases connected in series and with a continuous counter-current movement of mobile phases.

Except for size exclusion, chromatographic mechanisms have been commented on in Section 4.11.3.2.7, related to sample cleanup. Only those aspects concerning sample fractionation are remarked on here.

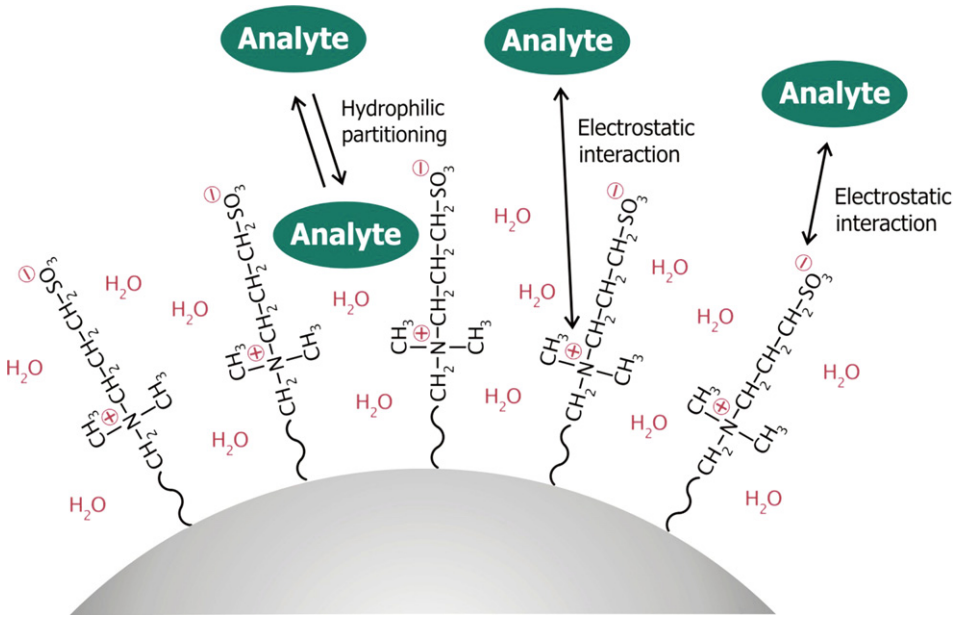


Figure 10 Schematic representation of the retention processes on a sulfoalkylbetaine zwitterionic stationary phase. The mechanism of retention is based on the hydrophilic partitioning of the analytes into the water-enriched stationary phase, and weak electrostatic interactions with either the positive or negative charge of the functional group. *Reprinted with permission from SeQuant, Umea, Sweden.*

- Ion exchange

A mixture of different effects such as ligand exchange, exclusion mechanism, complex formation, ion–dipole interactions, or hydrogen bonds are involved in the retention mechanism for the fractionation of carbohydrates using cation-exchange resins (loaded with Ca^{2+}, Na^{+}, or K^{+}).[28] The retention is also related to the number of equatorial-axial oriented sugar OH groups for complexation with the cation. Resins loaded with K^{+} are suitable for the separation of glucose from oligosaccharides, whereas Ca^{2+} are the best choice for the separation of fructose from oligosaccharides. Chloride-form strong base anion-exchange resins previously conditioned with low concentrations of sodium hydroxide have been shown to be useful for the separation of inositols from sugars and sugar alcohols.[29]

- Adsorption

Columns of activated charcoal (Celite™) have been traditionally used for the separation of carbohydrates of different molecular weight. The use of different ethanol concentrations allows the selective extraction of the carbohydrates previously adsorbed onto the charcoal column: from 1% (v/v) ethanol/water for monosaccharides to 50% (v/v) for oligosaccharides. Batch systems have also been used for the separation of oligosaccharides from mono- and disaccharides of honey previous to their analysis.[30]

- Size exclusion

Size-exclusion chromatography (SEC) is another operation mode widely applied in its classic form with open columns for fractionation of carbohydrates. The stationary phase is constituted by cross-linked polysaccharides or polyacrylamide. These packing materials are available in a range of pore sizes. Therefore, the retention time of the analytes in the column depends on the ratio of their molecular dimensions to the diameter of the pore of the stationary phase. Those compounds with a molecular weight higher than that retained in the pores elute in the void volume. Water is the most common mobile phase used.

The efficiency of this technique for the separation of oligosaccharides with different DPs is very high. For example, Hernandez et al.[31] obtained pure fractions of mono-, di-, tri-, tetra-, and pentagalactooligosaccharides by this technique, whereas hexa-, hepta-, and octasaccharides were obtained with a minor degree of purity.

4.11.3.4 Biological Treatments

Biological treatments have scarcely been used for purification of carbohydrate samples. However, there are some applications in the literature that are worth mentioning here. These procedures are based on the use of yeast to mainly remove low molecular weight carbohydrates for further analysis of oligosaccharides. *Saccharomyces cerevisiae* has been shown to selectively remove certain mono-, di-, and trisaccharides,[32] and has been used to decrease glucose and fructose concentrations in honey that interfere in the analysis of minor oligosaccharides.[33] **Figure 11** shows the GC profile of trimethylsilyl (TMS) oximes of honey carbohydrates before and after yeast treatment. As can be observed, monosaccharides (fraction I) have almost disappeared with this treatment.

Immobilized cells of the bacterium *Zymomonas mobilis* have also been used to remove low molecular weight carbohydrates from food-grade oligosaccharide mixtures.[34]

4.11.3.5 Chemical Treatments

Chemical treatments are often required in cases such as the extraction of insoluble polysaccharides or derivatization prior to chromatographic or electrophoretic analysis.

4.11.3.5.1 Hydrolysis and Methanolysis

The analysis of larger oligo- and polysaccharides by several chromatographic techniques (planar chromatography or GC) requires an initial chemolysis step to release soluble saccharides. For this purpose acidic hydrolysis and methanolysis are used; in some cases microwave irradiation or sonication (as shown in Section 4.11.3.2.3) are used as an aid to shorten the reaction times. Moreover, enzymatic hydrolysis can be also carried out, although chemical hydrolyses are more common for analytical purposes.

Depending on the strength of hydrolysis conditions – temperature, reaction time, and acid concentration – polysaccharides undergo degradations, producing oligosaccharides, disaccharides, and monosaccharides. The most commonly used hydrolytic agents are trifluoroacetic acid (TFA), sulfuric acid, and hydrochloric acid in water. However, in some cases it is necessary to achieve a trade-off between incomplete cleavage of the glycosidic and glucuronosyl linkages under relatively mild conditions, and decomposition of the liberated monosaccharides.

The major disadvantage of sulfuric acid is its inability to completely hydrolyze certain linkages in polyglucuronic acid and the difficulty of removing the acid after hydrolysis.

Hydrochloric acid can cause more degradation than sulfuric acid, with losses of up to 40%

Due to its effectiveness at hydrolyzing without causing extensive destruction of sugars and its easy elimination by evaporation after reaction, TFA is commonly used for analysis of most carbohydrates. On the other hand, it is reported to be insufficient for the complete hydrolysis of β(1–4) linked cellulose polymers or mucins.

Methanolysis is an alternative to hydrolysis, and is preferred for analysis of hemicellulose and pectins because it is less destructive to liberated glycosides. Hydrochloric or sulfuric acid in anhydrous methanol used for this purpose releases both neutral

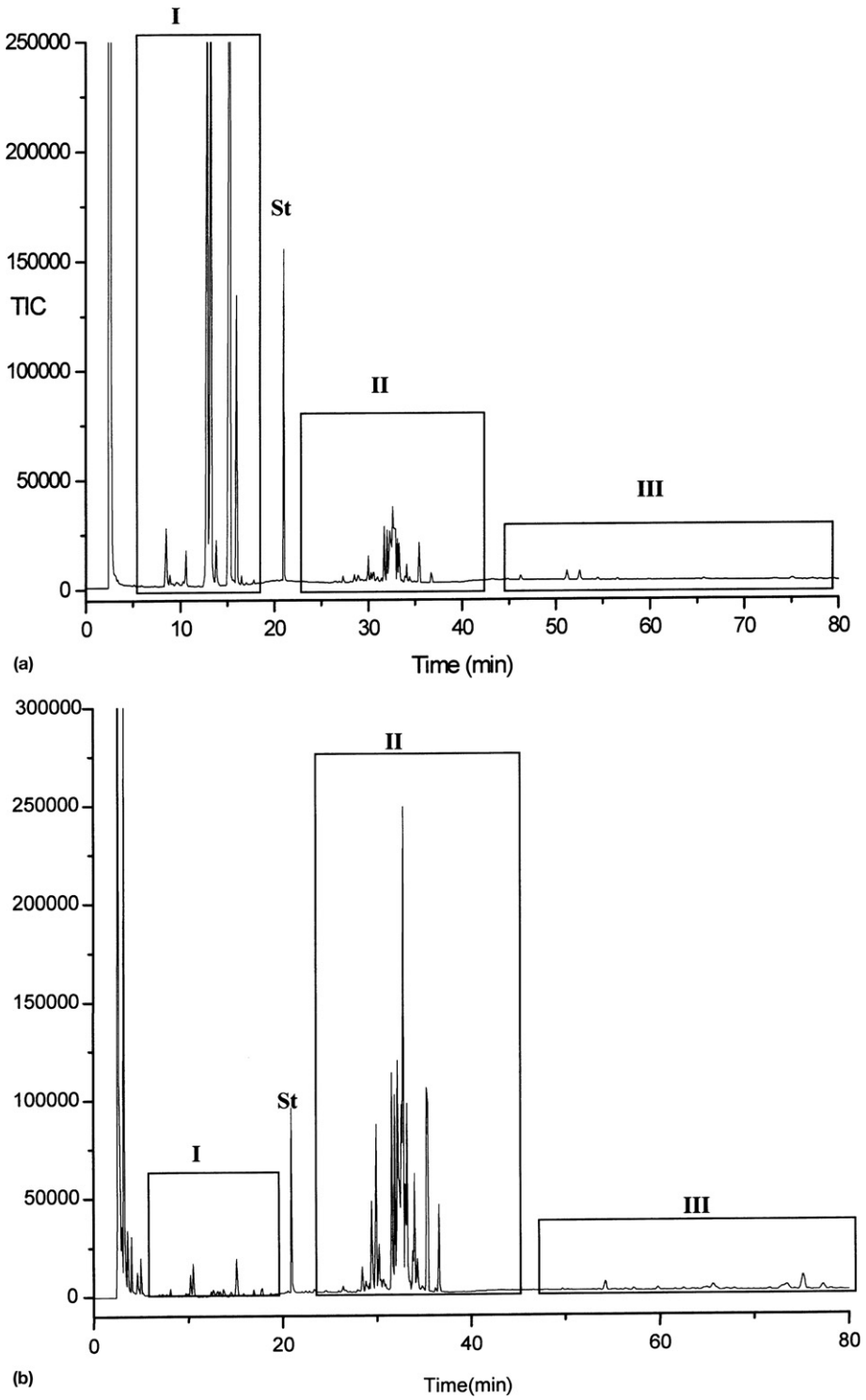


Figure 11 Gas chromatography profile of trimethylsilyl oximes of honey carbohydrates before (a) and after (b) yeast treatment. I, monosaccharides; II, disaccharides; III, trisaccharides. St: Internal standard. *Reprinted with permission from* J. Agric. Food Chem. 55, *7265. Copyright 2007 American Chemical Society.*

and acid sugar units from hemicelluloses and pectic substances and converts them into the corresponding methyl glycosides, and carboxyl groups of uronic acids are esterified with methyl groups.

In some cases the combination of methanolysis with acid hydrolysis gives superior results compared with separate performance of these methods.

4.11.3.5.2 Derivatization

The most common use of derivatization is focused on the enhancement of detection sensitivity and the treatment of polar compounds to improve thermal stability, increase volatility, and change chromatographic properties. General requirements for derivatization include, among others, quantitative yield with minimum side reactions, chemical stability of derivatives, specificity of derivatization reactions, and the use of reactions with only one or two steps. In addition to the cost of reactives, the selection of the most adequate derivatization procedure will also take into account the compatibility of derivatives with the analytical technique to be used.

The most common derivatization methods of carbohydrates for their chromatographic and electrophoretic separation, as well as for their structural characterization by mass spectrometry (MS), are provided below.

- Derivatives for GC

Derivatization of carbohydrates is a necessary step prior to GC analysis, as the presence of multiple polar –OH groups is responsible for their low volatility. Replacement of active hydrogen in this group with nonpolar groups, usually by alkylation, acylation, or silylation, increases the volatility, making GC analysis of carbohydrates possible. An important aspect regarding the chromatographic analysis of sugars concerns the fact that monosaccharides in solution are present as several tautomeric forms (see **Figure 1**).

Methyl ethers

Methyl ethers were the first volatile derivatives of carbohydrates used for GC. Methyl iodide, in the presence of silver oxide, is usually used as a methyl group donor; a two-step reaction with methylsulfinyl carbanion and methyl iodide can also be used. The time-consuming preparation and the lack of good resolution in GC, however, militate against their general use. Methylation is the first step used for structural investigation, which is further discussed below.

Silyl derivatives

One of the most common derivatization methods is silylation, where the introduction of several TMS groups into the molecule is performed to form ethers (**Figure 12**). Compared with their parent compounds, silyl derivatives are more volatile, less polar, and more thermally stable. Despite the high number of possible derivatives per carbohydrate, the TMS ethers have become the derivatives of choice for analysis of most carbohydrates. Various reagents used for trimethylsilylation are listed in **Table 5**.[35]

The *t*-butyldimethylsilyl group, much more stable to hydrolysis in comparison with TMS, is commonly used as an –OH protecting group for the acid group, since TMS esters are more labile groups than TMS ethers and can be easily decomposed. The use of other silyl derivatives such as dimethylsilyl, dimethylethylsilyl, dimethylpropylsilyl, chloromethyldimethylsilyl, or bromoethyldimethylsilyl (the latter two to improve response when electron capture detectors are used) has also been reported.

The drawback of silyl derivatives is that in the case of reducing sugars, up to five previously mentioned tautomeric silylated forms appear; nonreducing sugars and sugar alcohols give single peaks. Formation of multiple chromatographic peaks makes difficult the quantitation of complex mixtures, but in counterpart can be an aid for identification of simple mixtures.

A solution for this problem is to convert the carbonyl group of reducing sugars into oximes prior to silylation (**Figure 13**). This process reduces the number of chromatographic peaks to only two: *anti*-(*E*)- and *syn*-(*Z*)-isomers, one of which usually preponderates (in aldoses). Nonreducing sugars provide a single silylation product, as they do not undergo the oximation process.

Acetyl derivatives

Similarly to TMS ethers, carbohydrate acetates (**Figure 14**) may give rise to multiple peaks. Their volatility is low in comparison with TMS, but their stability is high, thus they are used for analysis of simple mixtures of low molecular weight compounds.

To decrease the number of chromatographic peaks, the reduction of carbonyl groups is performed: monosaccharides are transformed into the corresponding alditols, followed by acetylation, resulting in formation of very stable alditol acetates (**Figure 15**). In this way the anomeric center is eliminated, and there is only one chromatographic peak for each aldose. However, when a ketose is reduced, the formation of two isomeric alditols takes place. For example, reduction of fructose gives glucitol and mannitol; moreover, the same alditol can be formed from different sugars (e.g., mannitol is formed from the reduction of both mannose and fructose), which is the main disadvantage of these derivatives.

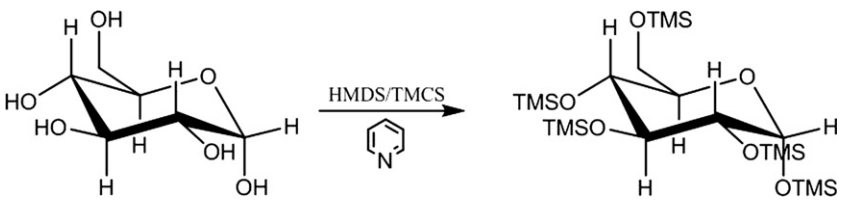
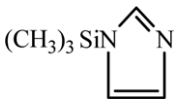


Figure 12 Formation of trimethylsilyl ethers (TMS: $-Si(CH_3)_3$). HMDS, hexamethyldisilazane; TMCS, trimethylchlorosilane.

Table 5 Common trimethylsilylating reagents listed in order of increasing silyl donor strength

Reagent	*Structure*	*Abbreviation*
Hexamethyldisilazane	$(CH_3)_3Si\text{-}N(H)\text{-}Si(CH_3)$	HMDS
Trimethylchlorosilane (base catalyzed)	$(CH_3)_3Si\text{-}Cl$	TMCS
N-Methyl-*N*-trimethylsilylacetamide	$CH_3\text{-}C(=O)\text{-}N(CH_3)\text{-}Si(CH_3)_3$	MSA
N-Trimethylsilyldiethylamine	$(CH_3)_3Si\text{-}N(C_2H_5)_2$	TMSDEA
N-Trimethylsilyldimethylamine	$(CH_3)_3Si\text{-}N(CH_3)_2$	TMSDMA
N-Methyl-*N*-trimethylsilyltrifluoroacetamide	$CF_3\text{-}C(=O)\text{-}N(CH_3)\text{-}Si(CH_3)_3$	MSTFA
N,O-Bis(trimethylsilyl)acetamide	$CH_3\text{-}C(=N\text{-}Si(CH_3)_3)\text{-}O\text{-}Si(CH_3)_3$	BSA
N,O-Bis(trimethylsilyl)trifluoroacetamide	$CF_3\text{-}C(=N\text{-}Si(CH_3)_3)\text{-}O\text{-}Si(CH_3)_3$	BSTFA
N-Trimethylsilylimidazole		TMSI

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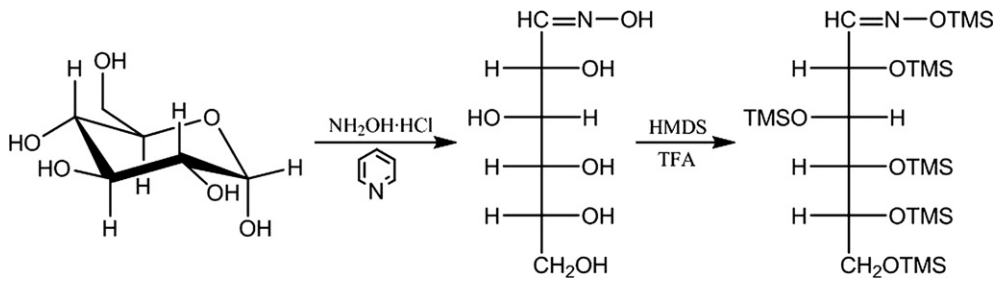


Figure 13 Two-step oximation–silylation reaction (TMS: $-Si(CH_3)_3$).

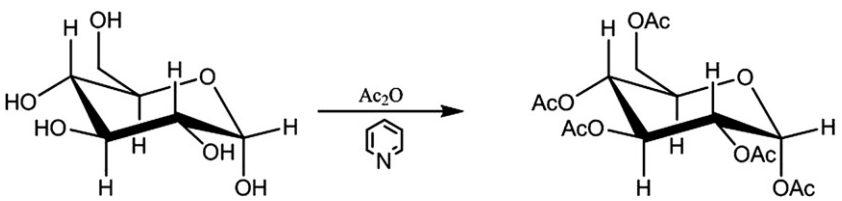


Figure 14 Formation of acetates (Ac: CH_3CO-).

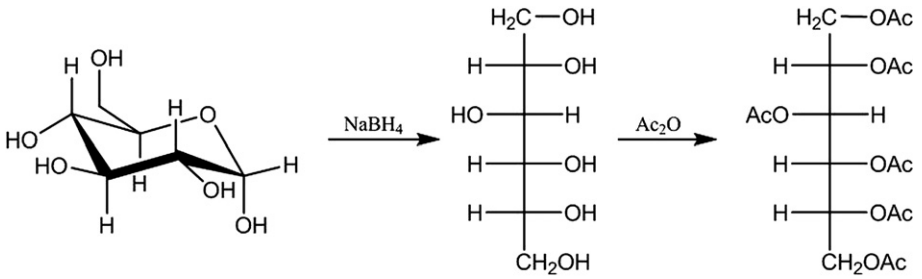


Figure 15 Formation of alditol acetates (Ac: CH_3CO–).

Trifluoroacetyl derivatives

The advantage of trifluoroacetates (Figure 16) is that they are more volatile than either acetates or TMS ethers, which allows the use of lower analysis temperatures and permits application to saccharides of high molecular weight. Moreover, their molecular size is also smaller, which allows their use in the GC separation of enantiomers, since they interact well with the chiral cavity of cyclodextrins. Many researchers, however, have encountered difficulty in achieving satisfactory quantitation of sugars as trifluoroacetyl derivatives.

Aldononitrile acetates

Aldononitrile acetates (Figure 17), also called Wohl derivatives, are derivatives that form a single peak for each aldose, as a result of the elimination of the anomeric center by the nitrile group. Unfortunately, the formation of nonvolatile products for ketoses discards them for GC analyses of most real samples where aldoses and ketoses exist simultaneously.

Diethyldithioacetal derivatives

Diethyldithioacetals (Figure 18), also forming just one chromatographic peak, are reported to be good derivatives for the analysis of polysaccharides such as gums, because of the possibility to derivatize uronic acids (which fails with some derivatives) as well as aldoses. In this case, mercaptalation removes the anomeric center, which can be followed either by trimethylsilylation or trifluoroacetylation, and yields stable products for chromatography.

Chiral derivatives

Of special interest are chiral derivatives that permit the separation of enantiomers by GC. The resolution of racemic mixtures is achieved by conversion of the enantiomers into diastereoisomers by a chiral reagent, usually an optically active alcohol such as 2-butanol or 2-octanol, and subsequent separation in a nonchiral phase. This group of derivatives includes acyclic dithioacetals (acetylated or trimethylsilylated), TMS ethers or acetates of the diastereoisomeric α-methylbenzylaminoalditols, and trifluoroacetylated (−)-methyl- or (−)-bornyloximes. Nowadays, alternative methods of separation of enantiomers are based on the use of chiral stationary phases.

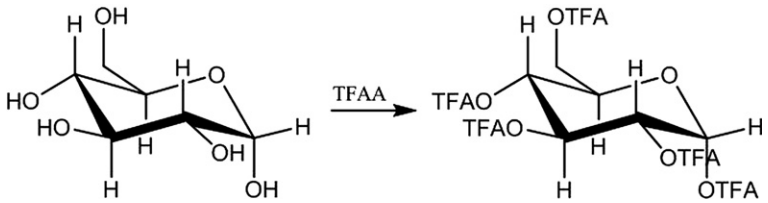


Figure 16 Formation of trifluoroacetates (TFA: CF_3CO–). TFAA, trifluoroacetic anhydride.

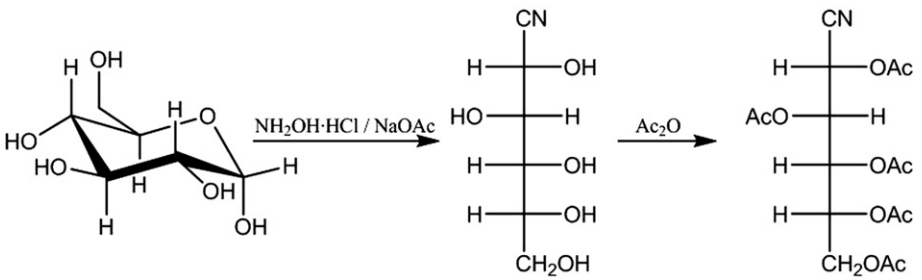


Figure 17 Formation of aldononitrile acetates (Ac: CH_3CO–).

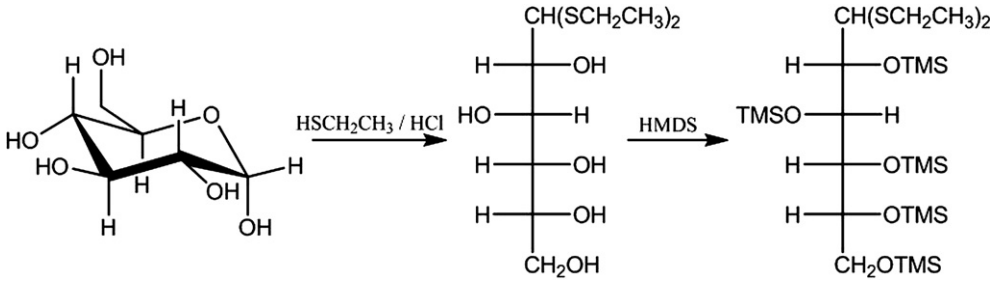


Figure 18 Formation of diethyldithioacetal trimethylsilylated derivatives (TMS: $-Si(CH_3)_3$).

Partially methylated alditol acetates

The Hakomori procedure[36] and its further modifications are the classic methods for structural analysis of polysaccharides. These procedures are composed of four stages. The first two consist of methylation of free hydroxyl groups of the macromolecule followed by the cleavage of glycosidic linkages. Partially methylated alditol acetates are formed by subsequent reduction of newly formed hydroxyls followed by acylation, which finally reveals the position of the original glycosidic linkages (**Figure 19**). Other procedures based on the obtainment of partially ethylated alditol acetates or partially methylated aldononitrile acetates are also used.

- Derivatives for HPLC and CE

Derivatization in HPLC generally involves the use of UV-absorbing or fluorescent molecules for better detection sensitivity. As most carbohydrates are hydrophilic and neutral, derivatives are also prepared to change carbohydrate properties (hydrophobicity, charge, etc.) and, therefore, to improve their resolution by either HPLC or CE. Although derivatization may occur prior to, during, or after the separation technique, this chapter focuses on precolumn derivatization protocols as part of sample pretreatment in carbohydrate analysis.[37,38]

Carbonyl derivatization

The carbonyl group of reducing sugars is the main target of derivatization reactions, and several schemes have been developed based on carbonyl derivatization.

Reductive amination

The labeling reaction starts with the nucleophilic addition of the amino group of the labeling reagent to the carbon of carbonyl groups of reducing sugars, yielding a Schiff base (imine derivative) (**Figure 20**). Under the mild acid conditions necessary to promote sugar ring opening, the acid-labile Schiff base formed is reduced with sodium cyanoborohydride to a stable secondary amine. A large number of reagents has been described for sugar derivatization by reductive amination, and a summary of the most widely used is presented in **Table 6**.

Despite its relatively high yield and absence of side products, difficulties have been described in the application of this procedure to derivatization of sialic acid-containing oligosaccharides (particularly labile at the acid conditions used for labeling), and to derivatization of ketoses, since only a few derivatizing agents react with them, and in some cases not quantitatively.

Other factors to be considered in reductive amination are the purity of the labeling agent and the presence of salts that may cause a decrease in derivatization yield. These derivatives are stable under various structure–elucidation conditions, including methylation analysis, acid or alkaline hydrolysis, and so forth.

Formation of imines (Schiff bases)

Derivatization can also be carried out in the absence of reducing agent, the product of the reaction being an imine or glycosylamine rather than a secondary amine. As formation of these compounds is a reversible reaction, the derivatives are somewhat unstable. However, this characteristic could be advantageous if the derivatives are sufficiently stable during chromatography, because the parent oligosaccharides often need to be regenerated after separation for further characterization (convertible derivatization).

7-Nitro-2,1,3-benzoxadiazole derivatives

Reducing sugars are converted to *N*-methylglycosylamines in the presence of methylamine and dimetlylamine–borane complex. The resultant products are labeled with 7-nitro-2,1,3-benzoxadiazole-4-fluoride (NBD-F) (**Figure 21**). Advantages of this rapid and quantitative one-pot reaction include that the derivatives show strong absorbance at 490 nm and their fluorimetric properties match with the Ar-ion laser line of laser-induced fluorescence (LIF) detectors. It is also a rapid and quantitative reaction and, under the mild conditions of derivatization, sialylated oligosaccharides can be analyzed. These derivatives are less specific to sugars than those obtained by reductive amination, as the reagents may also react with the amino groups of nonsugar materials concomitantly present in the sample.

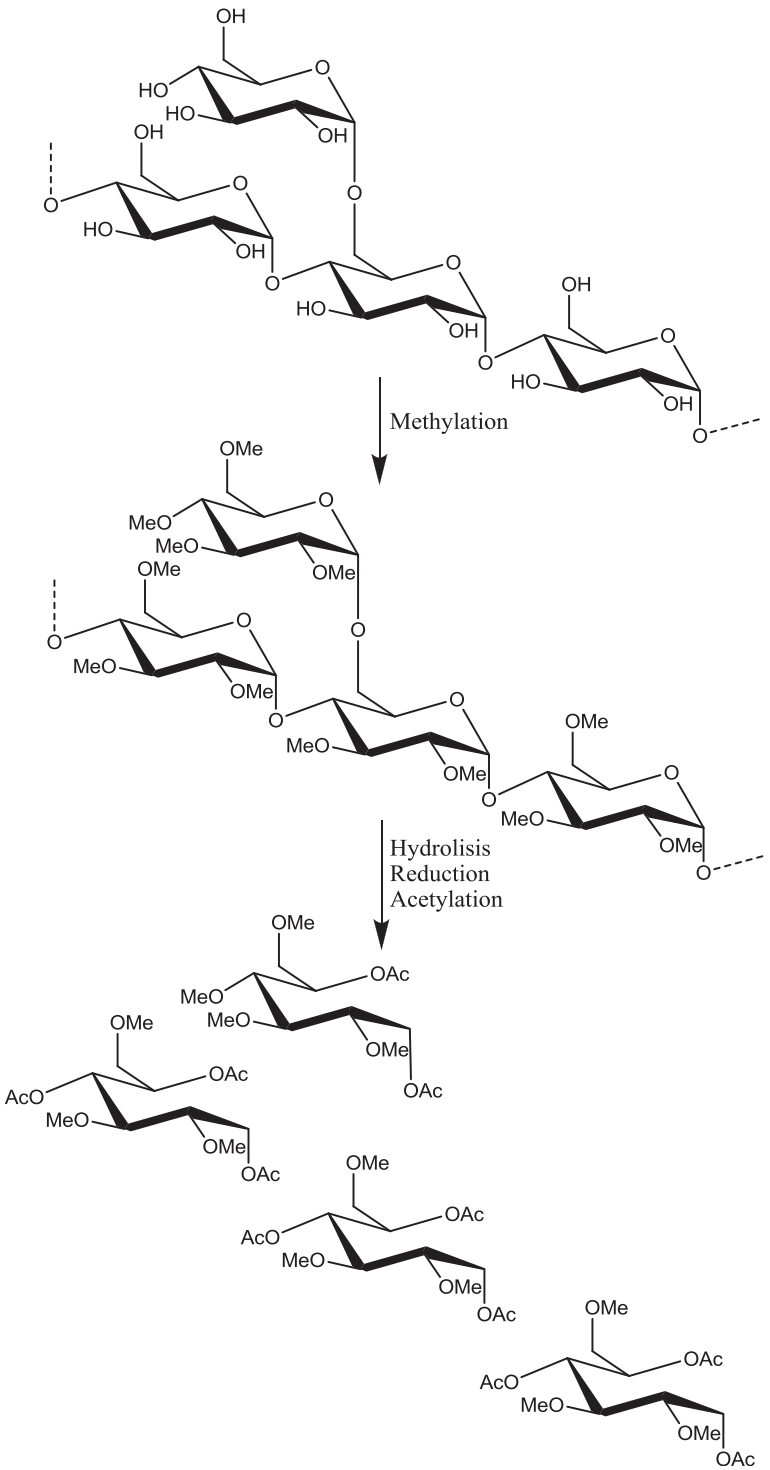


Figure 19 Formation of partially methylated alditol acetates from a polysaccharide.

Pyrazolone derivatives

1-Phenyl-3-methyl-2-pyrazolin-5-one (PMP) and its more reactive methoxy analog 1-(*p*-methoxy)-phenyl-3-methyl-5-pyrazolone (PMPMP) have been used to derivatize reducing sugars (both aldoses and ketoses) in the presence of carbodiimide by a condensation reaction under mild conditions, which does not cause desialylation or desulfation (Figure 22). This method, which yields UV-absorbing derivatives, has been used for the derivatization of carbohydrates from simple monosaccharides to sialylated *N*-glycans. The bis-PMP/PMPMP derivatives behave like weak anions in aqueous basic solutions and can be detected

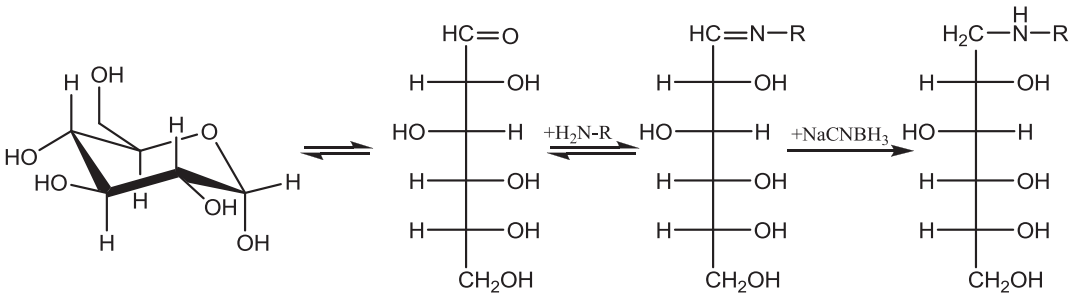
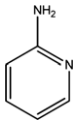
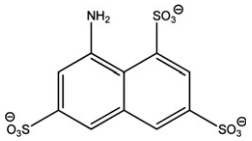
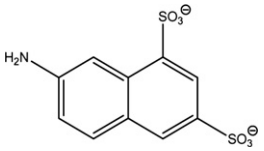
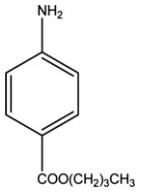
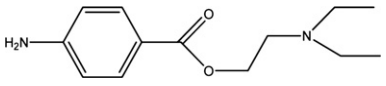


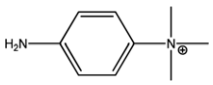
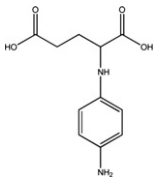
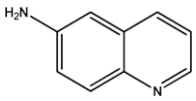
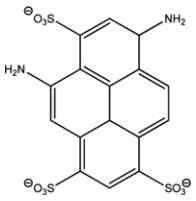
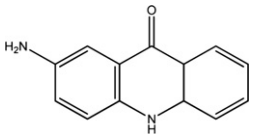
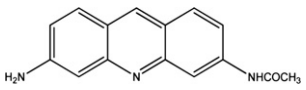
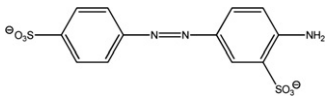
Figure 20 Reductive amination of carbohydrates.

Table 6 Reagents for reductive amination of carbohydrates

Reagent	*Structure*	*Abbreviation*
2-Aminopyridine		2-AP
8-Aminonaphthalene-1,3,6-trisulfonic acid		ANTS
7-Aminonaphthalene-1,3-disulfonic acid		ANDS
4-Aminobenzoic butyl ester		4-ABBE
4-Aminobenzoic 2-(diethylamino)ethyl ester		ABDEAE

(*Continued*)

Table 6 Reagents for reductive amination of carbohydrates—cont'd

Reagent	*Structure*	*Abbreviation*
4-Trymethylaminoaniline		
N-(4-Aminobenzoyl)-L-glutamic acid		ABG
6-Aminoquinoline		
1-Aminopyrene-3,6,8-trisulfonate		APTS
2-Aminoacridone		2-AMAC
3-Acetamido-6-aminoacridine		AA-Ac
4-Amino-1,1′-azobenzene-3,4′-disulfonic acid		

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electrochemically. In addition, PMP labeling of sialylated oligosaccharides enables very clean and informative electrospray ionization (ESI) mass spectra with high sensitivity.

Hydrazone derivatives

The carbonyl group of an aldehyde or ketone reacts with the amine group of a fluorescent or UV-absorbing hydrazine (e.g., dansylhydrazine, *N*,*N*-diphenylhydrazine, 2,4-dinitrophenylhydrazine, Girard's T reagent), forming a hydrazone product (**Figure 23**). A drawback of this derivatization procedure is the formation of side products or various isomers.

Tritium labeling

Less frequently used is the reduction with radioactive compounds. When reducing groups of oligosaccharides are reduced with $NaBT_4$ in an alkaline solution, radiolabeled alditols are obtained. These labeled carbohydrates can be monitored with a radiochemical detector.

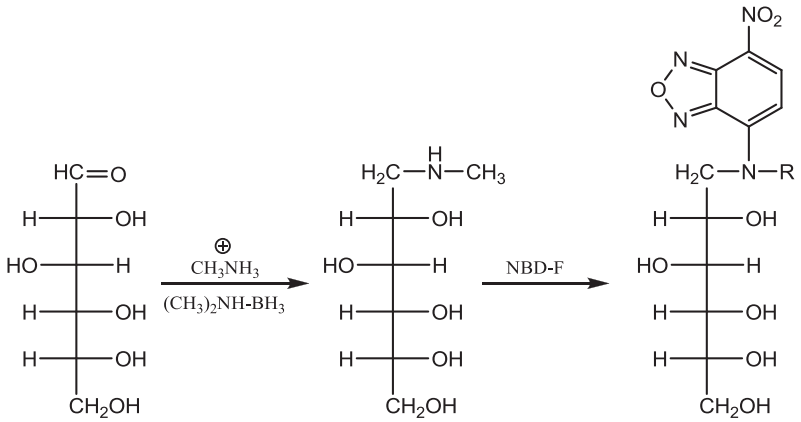

Figure 21 Derivatization of D-glucose with 7-nitro-2,1,3-benzoxadiazole (NBD)-4-fluoride.

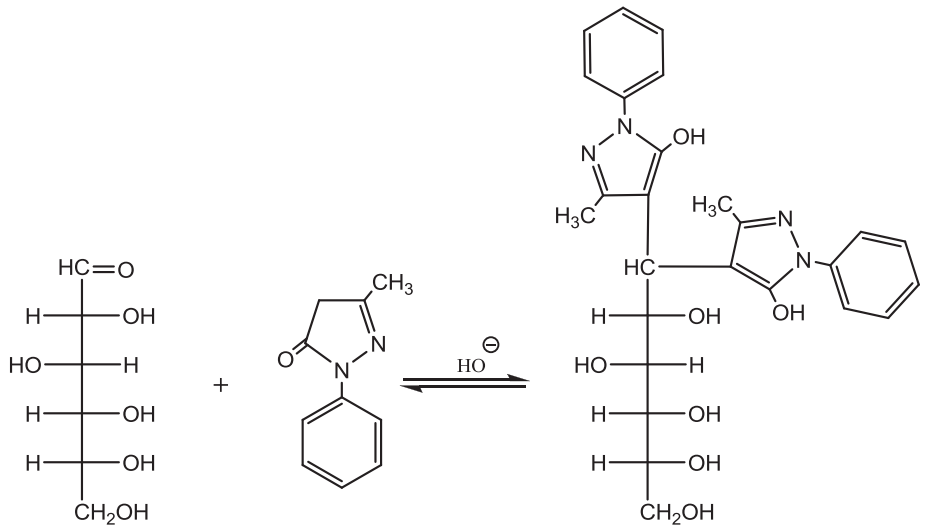

Figure 22 Derivatization reaction with 1-phenyl-3-methyl-2-pyrazolin-5-one (PMP).

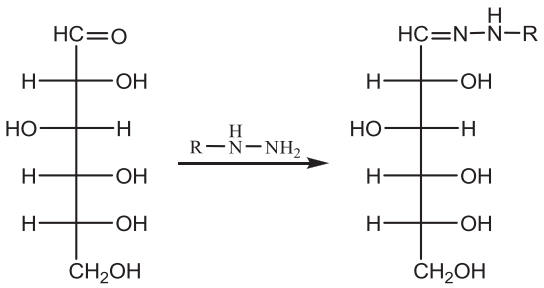

Figure 23 Hydrazone derivatives.

Derivatization of hydroxyl, amino, and carboxyl groups

The hydroxyl, amino, and carboxyl groups of carbohydrates are also points of substitution where chromophores can be introduced.

Per-O-benzoylation has been extensively and successfully used for derivatization at hydroxyl groups and sensitive detection of carbohydrates. In general terms, to the dry carbohydrate sample, the benzoylation mixture (benzoyl chloride and *p*-dimethylaminopyridine in pyridine) is added, and the mixture is subsequently heated. The reaction is terminated by the addition of water and vigorous shaking. Other labeling agents used to substitute all the hydroxyl groups in mono-, di-, and trisaccharides include benzoic anhydride and *p*-nitrobenzoyl chloride, among others.

Amino sugars react readily and quantitatively with 3-(4-carboxybenzoyl-)-2-quinoline carboxyaldehyde in the presence of potassium cyanide, while neutral sugars are converted to 1-amino-1-deoxyalditols by reductive amination. The isoindole derivatives have excitation maxima near the blue line of the He/Cd laser and the secondary line of the argon laser. This procedure has the advantage that it is not necessary to remove the excess of reagent, since it does not fluoresce. Aminated carbohydrates can also be labeled with 5-carboxytetramethylrhodamine succinimidyl ester.[39] However, the succinimidyl ester is not stable in basic buffers, yielding two hydrolysis products.

Amino groups of amino sugars are derivatized with *o*-phthalaldehyde and 2-mercaptoethanol, derivatives being detected by fluorescence. Thus, picomole amounts of amino sugars can be conveniently and reliably analyzed in the presence of amino acids. *p*-Toluenesulfonylchloride or dansyl-Cl have also been used for derivatization of hexosamines, the derivatives being detected by UV and fluorescence, respectively.

The *α-ketocarboxylic acid* group of the sialic acid reacts with 1,2-diamino-4,5-methyl-enedioxybenzene (DMB) to form quinoxaline derivatives with strong fluorescence. Under the acidic conditions of reaction, hydrolysis or migration of acetyl groups from one to another hydroxyl group is prevented, this method being appropriated for the analysis of *O*-acetylated derivatives. In liquid chromatography–ESI–MS, derivatization with DMB allows the definition of the type and position of the various *O*-acetyl substituents.

Acid sugars can react with aminated derivatizing labels such as 7-aminonaphthalene-1,3-disulfonic acid (**Table 6**) via a scheme different from reductive amination. The amine groups react with the carboxyl group in the presence of carbodiimide to form a peptide bond. The mild reaction conditions allow the application of this derivatization to labeling of acid monosaccharides, sialooligosaccharides, etc., for their sensitive detection by UV absorbance or LIF. The reaction yield is also very high.

- Derivatives for structural characterization by MS

Native carbohydrates are difficult to analyze by any MS procedure: they are not ionized very efficiently by the soft ionization methods such as ESI, matrix-assisted laser desorption/ionization, etc., since they are polar, thermally labile, and relatively nonvolatile.[40] Derivatization may offer several advantages such as increased response and less complicated spectra. Most derivatives used for GC analysis (methyl and TMS ethers, TMS oximes, TFA derivatives, etc.) have proved to be excellent for MS characterization of saccharides and glycosides. Formation of partially methylated alditol acetates has been used to enhance MS characterization of oligosaccharides.

Reducing carbohydrates can also be derivatized with a number of compounds containing amino groups (e.g., reductive amination with 1-aminopyrene-3,6,8-trisulfonate) in order to make easier their MS characterization. The glycosylamine approach has also been described as providing more information on linkage and anomeric configuration than reductive amination.

4.11.4 Basic Sample Types

Carbohydrates occur in foodstuffs in different physical states and also in very different matrices. One can find pure crystalline products such as sucrose, or amorphous solids such as starch, and simple solutions such as syrups or more complex liquid matrices such as honey or milk. In most cases, carbohydrates occur along with fat and proteins in very complex solid matrices such as vegetables, fruits, meat, and fish. The increasing complexity of the matrix makes the analysis more laborious and complicated, raising the number of required sample pretreatment steps required to suppress interferences.

It is also worth noting that official/standard methods of analysis include a number of classic procedures, mainly colorimetric and polarimetric measurements, which require many various previous sample treatments. However, modern techniques that mainly rely on chromatography often allow the use of simpler treatments.

In this section, both sampling process and sample treatment of a wide range of foods are considered. However, sampling of most of these products (mainly aqueous solutions and some emulsions) only requires a homogenization step. Therefore, sampling is only detailed for those samples that need specific protocols. Moreover, it is worth noting that general treatments such as derivatization procedures required for GC, HPLC, or CE analyses are common for most edible products, and have been generally discussed in Section 4.11.3.5.2. Consequently, the specific treatments are not emphasized in this section, and only some examples are considered.

4.11.4.1 Aqueous Solutions

In this group one can distinguish different foods and beverages such as nonalcoholic drinks (soft drinks and juices), alcoholic drinks (beer, wine, and spirits), coffee, tea, cocoa and surrogates, syrups, and honey.

4.11.4.1.1 Nonalcoholic Drinks

- Soft drinks

The main carbohydrates of soft drinks are sucrose, glucose, and fructose. No sample preparation other than filtration, dilution, or derivatization is necessary for the analysis of carbohydrates from noncarbonated beverages. For carbonated drinks, degassing by sonication or by purging with an inert gas is required prior to analysis.

- Juices

The major components of fruit juices are water, carbohydrates, acids, nitrogen compounds, polyphenols, minerals, and vitamins. Although fructose, glucose, and sucrose are the main carbohydrates in most fruit juices, pectin or starch are also present.

Before analysis, suspended particles of fruit juices can be removed by filtering, whereas soluble carbohydrates remain in the juice. Juices are commonly diluted at a fixed soluble solid content of 10° Brix, centrifuged and filtered through cellulose acetate membranes. For example, dilution procedures using 70% methanol followed by filtration through Whatman no. 40 filter paper have been carried out. Extracts are evaporated under vacuum and derivatized for their GC analyses.[41]

Official methods include a clarification treatment of fruit juices with lead acetate and alcohol, followed by the removal of the lead precipitated obtained. Supernatants are washed with different percentages of alcohol and used for further analyses.[42]

4.11.4.1.2 Alcoholic Drinks

- Beer

Beer is a fermented beverage made from starch-rich cereals (usually barley), hops, yeast, and water. In general, it is constituted by 3–4% of carbohydrates, mainly dextrins of DP > 4 ($\sim$70% of total carbohydrates).[43] Glucose and fructose are the main monosaccharides ($\sim$10% of total carbohydrates) but traces of ribose, arabinose, and xylose have also been detected. Maltose and sucrose are the most abundant disaccharides, whereas maltotriose is the main trisaccharide. Only minor amounts of isomaltose, kojibiose, nigerose, maltulose, isomaltose, panose, and isopanose have been observed. Polysaccharides such as arabinoxylans, β-glucans, and dextrins ($\sim$5% of total carbohydrates) are also present as unfermented substances.

Most analytical methods only require a previous degasification of the sample in an ultrasonic bath and an appropriate dilution. Official methods also comprise the removal of the suspended material by filtering through dry filter paper, whereas alcohol can be evaporated in a water bath at temperatures not higher than 80 °C.[42] Samples are submitted to hydrolysis with HCl to determine the dextrin content.

- Wine

Sugars determine the classification of a wine regarding its quality, and contribute to the taste of wines conferring sweetness, which varies from a background note in dry wines to a clear taste in sweet wines. Free carbohydrates in wine are mainly constituted by monosaccharides (glucose, galactose, fructose, mannose, arabinose, rhamnose, ribose, xylose), sugar alcohols (erythritol, threitol, ribitol, arabitol, *myo*-inositol, *chiro*-inositol, *scyllo*-inositol), sugar acids (gluconic and galacturonic acids), and disaccharides (α,α-trehalose, cellobiose, sophorose, laminaribiose, gentiobiose).[44] Oak-aged wines can be also constituted by carbohydrates coming from barrels.[45]

Polysaccharides constitute one of the main groups of macromolecules in wine, and contribute to increasing its viscosity and stability. The main polysaccharides coming from berries' cell walls (pectic polysaccharides) are rhamnogalacturonans II and arabinogalactan proteins, whereas those from yeast cell walls are mainly mannoproteins and mannans.

Official methods for sample preparation to determine the carbohydrate content in wine are based on the evaporation of alcohol and clarification with lead acetate.[42] Lead is then removed with dry potassium oxalate. Although sample cleanup for monosaccharide analysis has been carried out using C_{18} SPE cartridges, other methods require only the ethanol evaporation. The preliminary separation of the neutral from the acidic compounds has also been carried out before their analysis using anion-exchange cartridges.

The analysis of high molecular weight carbohydrates also requires the evaporation of the samples under reduced pressure to eliminate ethanol and to concentrate total solids. The material is then dialyzed to remove the small molecules. Other methods suggest the previous precipitation of total colloids using high concentrations of ethanol (80–95%) and acidifying with HCl (0.5%). Samples are kept at low temperature (4 °C) to help the precipitation and are centrifuged to remove the supernatant. A further separation of oligosaccharides from polysaccharides present in the pellets can be carried out either by ion-exchange or SEC columns. In other cases, UF membranes (5 kDa) have been used for retaining the polysaccharides.

- Liquors

The term spirit refers to a distilled beverage of a fermented sugar-containing mash for which no sugar has been added and contains at least 20% alcohol. Distilled beverages that are bottled with added sugar and added flavorings are liquors. Therefore, carbohydrates in spirits are only present at trace level, whereas higher amounts can be found in liquors.

Liquors may arise from alcohol-containing liquids (wine, beer, fruit wines, etc.), sugar-containing sources (sugar cane, beet, molasses, etc.), and starch or inulin-containing materials.[1]

Similarly to wines, oak-aged liquors can also contain some carbohydrates such as monosaccharides, including anhydrosugars from the toasting process of the barrel or chips.

Sampling pretreatment of liquors prior to their analysis resembles that previously mentioned for wines.

4.11.4.1.3 Coffee, Tea, Cocoa, and Surrogates

- Coffee

Carbohydrates of green coffee are mainly water insoluble and consist of polymers of mannan, arabinogalactan, and cellulose. Regarding low molecular weight carbohydrates, sucrose is the most abundant while only small quantities of free reducing sugars are present.

Green coffee beans cannot be directly consumed, and are submitted to roasting processes that can be carried out with and without sugar addition (torrefacto and conventional, respectively). During roasting, polysaccharides can be degraded, giving rise to soluble sugars. Nevertheless, roasted coffees are still composed of around 30–40% of polysaccharides (mainly galactomannans and arabinogalactans). Sucrose is also partially hydrolyzed to glucose and fructose. Mannose, glucose, fructose, and galactose are the main free monosaccharides in coffee extracts; small amounts of rhamnose, fucose, arabinose, xylose, and polyalcohols (mannitol, *myo*-inositol, and bornesitol) have also been reported.

Ground samples and instant coffees are dissolved in hot water and filtered. Filtrates are usually deproteinated and defatted by stirring with methanol and standing for at least 1 h. The clear methanolic solutions obtained are used for the analysis of soluble carbohydrates by HPLC. These extracts can be evaporated and derivatized for GC analysis.[46] Purification of the extracts with SPE C_{18} cartridges has been carried out prior to high-performance anion exchange chromatography–pulsed amperometric detection (HPAEC-PAD) analysis.[47]

Soxhlet has been also used to extract polysaccharides from coffee beans. For this purpose, lipids are firstly removed using petroleum ether or hexane. The ground and defatted coffee is later extracted with hot water (80–90 °C), filtered, concentrated, dialyzed (using a 10 kDa dialysis membrane), and freeze-dried. The galactomannan-rich fraction can be obtained by precipitation in 50% ethanol and purification by anion exchange chromatography.[48]

To carry out an exhaustive characterization of polysaccharides, their isolation is a required step, different extraction procedures being necessary to achieve a high purity, as shown in **Figure 24**.[49]

- Coffee surrogates

Coffee surrogates are the parts of roasted plants such as barley, rye, chicory, sugar beet, and figs, which are treated with hot water to provide a coffee-like brew. The carbohydrate content in these beverages is 74.7% for malt, 68.4% for chicory, 70.2% for fig, and 73.0% for acorn coffee[1]: most of them contain both mono- and oligosaccharides, and sample treatment is similar to that previously indicated for coffee.

- Tea

Glucose, fructose, sucrose, arabinose, and ribose are present in tea leaves and extracted in tea brew. Water-soluble polysaccharides (hemicelluloses or pectic substances) are also present. Previous to the analysis of polysaccharides by HPAEC-PAD, samples are treated with ethanol (70% v/v) at 4 °C for precipitation, followed by enzymatic treatments and charcoal filtration.[50]

- Cocoa

Contrary to coffee or tea, cocoa is consumed as a suspension instead of as a water extract. After different treatments of cocoa beans including fermentation, roasting, grinding, partial defatting, and addition of ingredients (sucrose, milk powder, etc.), either cocoa powder or chocolate is produced.[1]

Among other constituents, cocoa beans are composed of mono- and oligosaccharides such as stachyose, raffinose, and sucrose (around 1.5% of total constituents) and polysaccharides such as starch, pentosans, and cellulose (around 16% of total constituents). Similarly to green coffee beans, changes in these carbohydrates occur during roasting.

Prior to their analysis, skins are removed from beans and the latter are milled for their extractions. Samples are treated with methanol/chloroform (1:1, v/v) and the supernatant is dried for its further reextraction in water.

Separation of mono- and oligosaccharides from soluble polysaccharides in the water extract is carried out by dialysis (3.5 kDa MWCO). Sugars are then fractionated from other interfering substances such as amino acids and acidic components through ion-exchange columns (cationic and anionic), and analyzed by HPLC.[51]

Starch and pectic polysaccharides present in the insoluble water residue are the major components of cocoa bean polysaccharides. For the analysis of cell wall polysaccharides, a previous purification of the extracts by consecutive washing with different solvents (i.e., methanol/chloroform followed by phenol/acetic acid/water followed by DMSO) is required.[51]

4.11.4.1.4 Honeys and Syrups

- Honey

Honey is the most complex mixture of carbohydrates found in nature. It is mainly composed of monosaccharides, glucose, and fructose (around 70–80% of honey), although lower amounts of 16 disaccharides and 12 trisaccharides (around 10% of honey) have also been detected. The presence of oligosaccharides of higher molecular weight (up to DP 14) at trace levels has also been described.

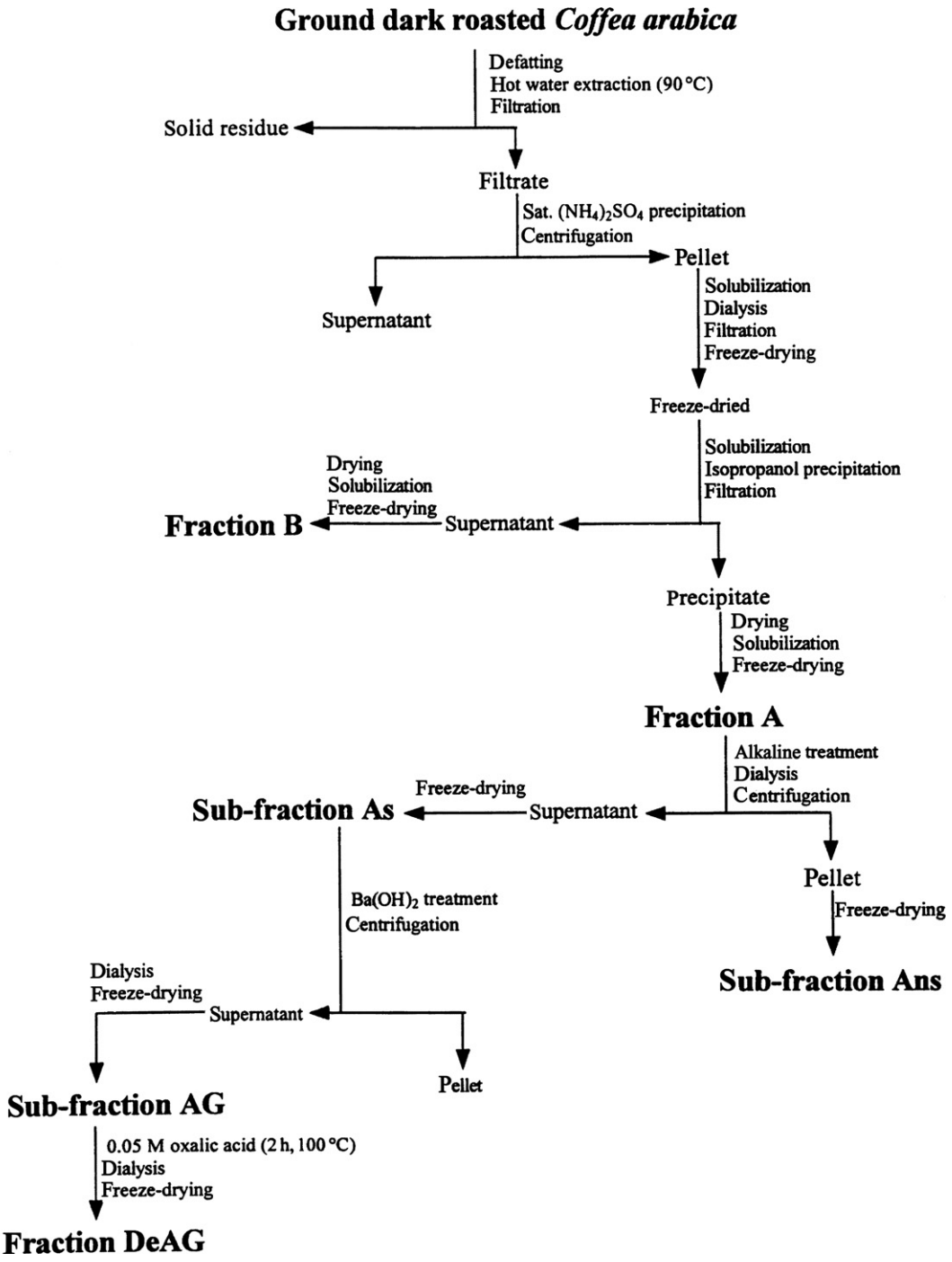

Figure 24 Extraction and isolation scheme of polysaccharides from ground dark roasted coffee. Fraction B is unknown; Fraction AG is composed of arabinogalactan; fraction Ans is composed of β(1–4) mannan substituted with galactose and mannose. *Reprinted with permission from Elsevier (*Carbohydr. Polym. ***1999***, 40, *73).*

Sampling

As previously mentioned, the first step for carbohydrate analysis is to have a homogeneous sample. Honey is a supersaturated solution of carbohydrates and can crystallize depending on storage conditions (time and temperature). In such a case, honey should be submitted to mild heating (temperature lower than 65 °C) to create homogeneity.

Sample treatment

Most studies on honey have focused on the analysis of mono- and disaccharides, which only require the dilution and filtration or derivatization of the sample prior to their HPLC or GC analysis. However, the determination of high molecular weight

oligosaccharides (from DP 3 to 14) needs the removal of the most abundant carbohydrates. Different processes based on the use of activated charcoal in batch, columns, yeast treatments, or graphitized charcoal cartridges have been carried out for this purpose.

The use of PLE combined with an in-cell packed adsorbent bed of activated charcoal has also been used to remove monosaccharides from honey and to analyze its minor constituents (**Figure 11**).[20]

- Syrups

Molasses, caramels, sugar syrups from corn, sugar cane, and sugar beet, inverted by acids and enzymes, as well as corn syrups (CS) or high-fructose corn syrups (HFCS) obtained by isomerization from CS, are found under 'syrups.' Most of these nutritive sweeteners are composed of low molecular weight carbohydrates, but oligosaccharides can also be found in CS or HFCS.

Similar treatments to those applied in honey for the removal of oligosaccharides have been carried out for the analysis of carbohydrates in syrups. The use of clarifying agents such as lead acetate or other lead salts are also used to neutralize the organic acids present in cane syrups.[42] Salts, coagulated albumin, and some fats, waxes, and gums form a soft precipitate that traps finely suspended matter and a part of the colloids. Filtration produces a clear juice of about neutral pH which contains the sugars.[52] However, one of the main problems of these reagents is their high toxicity. Different reagents such as mixtures with activated bentonite are being introduced to the clarification process to replace lead salts.[52]

4.11.4.2 Aqueous Emulsions

Foods constituted by aqueous emulsions can be split into two main groups: dairy products and eggs. Some products can be found in solid state (dry milk, dry egg) or concentrated (condensed milk). After the determination of moisture, the first step prior to carbohydrate analysis consists of the addition of water (reconstitution). Sauces are also considered under this heading.

4.11.4.2.1 Milk and Dairy Products

This group includes milk (liquid, concentrated, condensed, dried), fermented milks (yoghourts and others), cream and ice creams, butter, whey, and cheese as main products. Their analysis has been recently reviewed.[53]

Fresh liquid milk is constituted by an aqueous solution of salts, carbohydrates, proteins, amino acids, vitamins, and others. It also contains casein micelles that form a fine dispersion and a coarse emulsion of fat globules surrounded by a membrane. The globules can separate after storage, forming a layer (cream) on the skim milk. Cream is similar to milk but it contains a very high proportion of fat globules (18–60%). Butter is an emulsion of milk fat that contains a relatively high percentage of water (about 18%). Ice creams are frozen masses containing fat, milk solids (or not), and sugars. Cheese is a fraction of milk containing mainly proteins, fat, and water; the sugar content is very low.

Free carbohydrates in dairy products are usually dissolved in the aqueous phase, and a small part is conjugated as glycolipids and glycoproteins. The main sugar of milk is lactose (4-*O*-β-D-galactopyranosyl-glucose); its content ranges from 4.4 to 5.2% in cow's milk. Other free carbohydrates that occur at lower concentrations in fresh milk include monosaccharides (glucose, galactose, *myo*-inositol, amino sugars, sugar phosphates), neutral and acid oligosaccharides, and nucleotide sugars. Thermal treatments such as sterilization form tagatose, 2-deoxypentulose, lactulose (4-*O*-β-D-galactopyranosyl-D-fructose), epilactose (4-*O*-β-D-galactopyranosyl-D-mannose), and saccharinic acids; the fate of oligosaccharides during thermal treatment has been less studied. Although most sugars are expelled in the whey, cheese can contain minor amounts of lactose and galactose; lactulose can also be found in processed cheese. Whey contains a high amount of lactose; it can also have monosaccharides formed by hydrolysis and sugar acids by fermentation, depending on the storage conditions or cheese manufacturing process.

Lactose in milk can be determined by many different methods: gravimetric, polarimetric, enzymatic, spectrophotometric, flow injection analysis, and chromatographic.

- Sampling

Dairy products may be found in different forms, and the first step for their carbohydrate analysis is to have a liquid, homogeneous sample.

Raw liquid without treatment, as appears when it is collected from farms, is a microbiologically unstable product that needs to be kept refrigerated; cream separation can also occur. Although this fact does not interfere in its qualitative analysis, since carbohydrates are dissolved in the aqueous phase a homogeneous sample is required for its quantitative determination. To avoid microbial spoilage, it is sometimes recommended to add some preservative (potassium dichromate, sodium azide, etc.) to the sample with the aim of keeping it unaltered until analysis. This is also valid for raw whey.

Ultrahigh-temperature and sterilized milks are usually homogenized prior to their commercialization. Homogenization is a mechanical process consisting of the division of the fat globules into smaller-sized ones until cream separation does not occur. Homogenized milks are easy to sample because cream does not separate and the amount of microorganisms is very low.

Cream is treated as liquid milk, but special attention must be paid to its homogenization, since this product has a high viscosity due to its fat content. Cream should be thoroughly mixed and warmed at 30–35 °C, if it is very thick.

Condensed milk should be mixed and reconstituted with water.

For ice cream, blocks are cut from the frozen product; they are allowed to soften at room temperature and mixed, avoiding fat separation.

For dried milk and dried whey the powder products are hygroscopic but rather stable. Once sampled, the product is reconstituted with water; homogenization is carried out at about 30–40 °C with occasional stirring, and treated as liquid milk.

Cheese can exist as grated, as a cream, or in loaves or portions. Small loaves of cheese can be cut, taking narrow wedges from the edge to the center; if the cheese loaf is too big, a cheese trier can be used to obtain small plugs, perpendicular to the surface and extending either entirely or halfway through. Cheese pieces are then grated or blended (depending on hardness) and emulsified with water.

- Sample treatment

With some exceptions such as automatic analyzers based on Fourier transform infrared technology, which do not require sample preparation, dairy products usually need some pretreatment before analysis.

Caseins and caseinates are rather insoluble in water. They require a first step of dissolution of a test portion; the International Dairy Federation (IDF)[54] recommends hot water for caseinates, hot water with the addition of sodium hydrogen carbonate for acid caseins, and hot water with the addition of pentasodium triphosphate in the case of rennet casein.

The second step is to obtain a clear solution, where proteins and fat have been suppressed. The clear solution can be submitted to diverse analytical methods, including reductimetric, colorimetric, and polarimetric; chromatography (HPLC and GC) is preferred when sugar mixtures have to be determined. Clarification is carried out by the addition of different reagents, which cause the precipitation of proteins and fat. The most common reagents are as follows.

Carrez, which consists of two aqueous solutions added consecutively (solution I: $K_4[Fe(CN)_6]\cdot 3H_2O$ 3.60 g/100 ml; solution II: $ZnSO_4\cdot 7H_2O$ 4.20 g/100 ml), is a widely used precipitating agent for determination of lactose in milk and milk products by polarimetric, reductimetric, and HPLC methods.

Biggs/Szijarto solution: obtained by dissolving zinc acetate hydrate (25 g), phosphotungstic acid hydrate (12.5 g), and glacial acetic acid (20 ml) in water (up to 200 ml). It is recommended by the International Organization for Standardization/IDF for HPLC analysis of lactose in milk and milk products.

Aqueous solution of phosphotungstic acid (5% for milk and 20% for processed cheese).

Trichloroacetic acid 50%: for detection of gums (polysaccharides) in cheese and ice cream.

Mercuric nitrate is used for clarifying condensed milk before polarimetric sucrose analysis, but this reagent is acid and hydrolysis starts when it is added; moreover, some investigators point out that precipitation of proteins is incomplete and they recommend the addition of phosphotungstic acid.

Methanol 60% has been proposed as precipitant previous to chromatographic analyses of milks, cheeses, and infant formulas.[53] It is easy to use and provides a clear solution adequate for chromatographic or electrophoretic analysis of major carbohydrates. Free fatty acids can appear as minor interfering peaks in the analysis; thus, an intermediate washing with an apolar solvent such as pentane is recommended when the original product contains fat (whole milk or cheese) and minor carbohydrates have to be analyzed.

Ultracentrifugation can separate fat (at the top of centrifuge tube) and proteins (at the bottom) from the serum, which remains as a clear solution in the middle. This method can be applied to water-dispersed cheese. In any event, some cleanup might be necessary.

Most of these methods form a precipitate constituted by proteins and fat, giving a clear serum adequate for carbohydrate analysis.

After precipitation, filtration or centrifugation allows a clear solution. The ulterior analysis may require an additional step such as:

addition of a buffer
addition of acetonitrile
SPE with a C_{18} cartridge to remove hydrophobic compounds
Evaporation or freeze-drying previous to derivatization

Analysis of milk oligosaccharides by matrix-assisted laser desorption/ionization time-of-flight MS has been carried out without clarification, probably because of the matrix effect that suppresses interferences.

4.11.4.2.2 Eggs and Egg Products

Eggs are constituted by several structures: shell, different membranes, an aqueous solution (white), and a fat-in-water emulsion (yolk). Carbohydrates are minor constituents of egg (about 1% of total). Free carbohydrates include *myo*-inositol and monosaccharides, mainly constituted by glucose, along with minor amounts of mannose, galactose, arabinose, xylose, ribose, and deoxyribose. Carbohydrates also occur in the form of *N*-linked and *O*-linked oligosaccharides conjugated to glycoproteins and consisting of galactose, mannose, *N*-acetylglucosamine, galactosamine, and uronic and sialic acids. Eggs also contain glycolipids with units of glucose, galactose, hexosamine, and sialic acids.[1]

In dried egg, the carbohydrate content has usually been reduced (by fermentation or enzymatic treatments) to 0.1% in order to avoid a Maillard reaction.

- Sampling

The Association of Official Analytical Chemists (AOAC)[42] specifies sampling details for liquid, dried, and frozen eggs. Egg samples are especially unstable, and should be conserved frozen and tightly closed without air in adequate containers.

- Sample treatment

As carbohydrates are minor components of eggs, they are not usually analyzed. Some attention has been paid to complex carbohydrates, especially to sialic acids. Previous hydrolysis with adequate enzymes (pronase for shell membrane, papain for other structures), trichloroacetic acid addition, and dialysis (to remove any interfering substances from the papain digest) are required before analysis. Extraction of glycans from shell requires decalcification prior to papain digestion. The sample is pulverized and decalcified by incubating in 25% acetic acid at room temperature for 24 h; the incubation mixture is dialyzed in water.

Analysis of soluble sugars requires two successive clarifications: the first with $Ca_2CO_3 + NaCl +$ ethanol, and the second with powdered phosphotungstic acid. It is also necessary to add dry powders of KCl to the filtrate to precipitate any excess phosphotungstic acid; this may require a third filtration.

4.11.4.2.3 Sauces and Dressings

These denominations encompass a high diversity of products industrially or homemade elaborated. Although some are true solutions, most sauces and salad dressings are emulsions of fat and water and, for this reason, all of them are included here. In certain products, a high viscosity is reached by the addition of polysaccharides, as starch, flour, gums, etc. Soluble carbohydrates are often present to impart more or less intense sweetness. Official methods[42] recommend the following treatment of these samples after homogenization of the sample.

Defatting can be carried out by successive extractions with light hydrocarbon solvents as pentane or heptane (hexane is more toxic) until the solvent does not show residue when some drops are allowed to evaporate. The excess of solvent in the sample is removed with a stream of air. Mayonnaise and French dressing can be clarified without previous defatting, since the emulsion is relatively stable. Alginates require a special deffating procedure using acetone and ethanol.

Clarification can be performed with different reagents. Some examples are described below:

Starch content in mustard is analyzed after addition of $CaCl_2$ and boiling
Gums in mayonnaise: fat and proteins are precipitated with 50% trichloroacetic acid (TCA)
Sugars in dressings: after defatting, sample is clarified with 5% H_3PO_4
Gums in dressings: clarification with $MgCO_3 + CaCl_2$ is recommended
Alginates in dressings: precipitation is carried out with dioxane. The use of H_3PO_4 or TCA requires neutralization with alkali to avoid further hydrolysis.

Filtration or centrifugation is the final step in obtaining a clear serum adequate for analysis.

4.11.4.3 Complex Matrices

4.11.4.3.1 Vegetables

The predominant sugars in fresh vegetables are glucose and fructose (0.3–4%) as well as sucrose (0.1–12%); oligosaccharides and minor carbohydrates occur in smaller amounts. Depending on the vegetable considered, starch or inulin occur as storage carbohydrates. Other polysaccharides in vegetables are cellulose, hemicelluloses, and pectins. It is also worth noting that the sugar content of vegetables is greatly affected by factors such as the variety, time of harvest and sowing, and processing and storage conditions.

- Sampling

Depending on the type of vegetable to be analyzed (fresh, dehydrated, canned, or frozen), sampling consists of different stages. For fresh vegetables, sampling includes either washing with tap water, rinsing with deionized water, draining, removal of outer and/or damaged parts of the vegetable, or peeling. Samples are then cut and mixed thoroughly prior to extraction. Drained canned vegetables are ground in a mortar or food chopper, thoroughly mixed, and stored in a glass-stoppered container. If comminuted products are to be analyzed, unopened containers are shaken to incorporate any sediment. Dehydrated samples are usually ground, sieved, and stored in tightly sealed containers prior to analysis. Frozen samples are submitted to air thawing, or direct or indirect water thawing under mild temperature conditions ($\leq$30 °C); the thawing procedure will depend on the characteristics of the vegetable and should be selected to avoid altering or degrading product characteristics.[42]

- Sample pretreatment

Sample pretreatment prior to either GC or HPLC analysis of low molecular weight carbohydrates in vegetables generally includes extraction with 70–80% alcoholic solution (methanol or ethanol), centrifugation, evaporation of the extract under vacuum and, if necessary, conversion to appropriate derivatives.[55] As part of the sample pretreatment, defatting with ethyl ether or hexane is also necessary for the analysis of certain samples.[56]

A simple method of isolation and purification of α-galactosides, also called the raffinose family oligosaccharides (RFOs), has been applied to different legumes (lentil, pea seeds). The method includes imbibitions of seeds in distilled water, extraction with 50% ethanol overnight, precipitation of RFOs in ethanol, purification on diatomaceous earth and charcoal, and cation-exchange chromatography. This method allows one to obtain high-purity RFO preparations with yields of α-galactosides higher than 4%.[57]

With the aim of obtaining an enriched fraction of oligosaccharides (e.g., fructooligosaccharides (FOS) from dehydrated onion and garlic), samples are homogenized in 70% ethanol and boiled under reflux in a water bath. The pellets and supernatants are

centrifuged and decanted, and the extraction–centrifugation procedure is repeated twice to achieve a complete extraction of FOS. All supernatants are mixed and vacuum evaporated to dryness to be finally redissolved in water. After filtration, samples are directly analyzed by HPAEC-PAD.[58] Another procedure for analysis of oligosaccharides includes extraction with different binary mixtures to remove low molecular weight compounds, extraction with water, and precipitation of extracted polysaccharides with ethanol, dialysis, and freeze-drying. Protocols for chemical treatment (hydrolysis, methylation, and derivatization) previously mentioned in Section 4.11.3.5 are usually used for determination of monosaccharide composition and elucidation of the linkage type of oligosaccharides.

4.11.4.3.2 Cereals and Cereal-Based Products

Two of the major constituents of most cereals (wheat, rye, corn, barley, oats, rice, and millet) are starch (40–70% weight) and other carbohydrates (5–23% weight). In oats, barley, and rye the content of starch is slightly lower, being replaced by other nonstarchy polysaccharides (hemicelluloses, pentosans, cellulose, β-glucans, and glucofructans).

Cereal starches consist of about 25% amylase and 75% amylopectin. The building constituents of pentosan polysaccharides are D-xylose, L-arabinose, and glucose. β-Glucans are linear polysaccharides with D-glucopyranose units joined by β-1,3 and β-1,4 linkages. Wheat flour contains 1% water-soluble, nonreducing oligosaccharides (glucofructans), consisting of D-glucose and D-fructose. Cellulose is a minor constituent of the carbohydrate fraction, and mono-, di-, and trisaccharides, as well as other low molecular weight degradation products of starch, occur in cereals at relatively low concentration.

- Sampling

Sampling of most cereals and cereal-based products includes air-drying (if necessary), grinding, homogenizing, and sieving. Samples should be kept in tightly sealed containers to prevent moisture changes.

- Sample pretreatment

Official methods for determination of starch in cereals are based on enzymatic procedures. For instance, in the glucoamylase method[42] samples extracted with boiling water and gelatinized in an autoclave are hydrolyzed with glucoamylase solution. After filtration, samples are mixed with an enzyme-buffer–chromogen mixture, and determination is carried out by measurement of absorbance.

Total dietary fiber in flour and bread is determined by an enzymatic–gravimetric method.[42] After extraction of fats, previously dried and milled samples are gelatinized with a heat-stable α-amylase, and then enzymatically digested with protease and amyloglucosidase to remove protein and starch. The residue obtained after precipitation with ethanol is filtered, dried, and weighed. Total dietary fiber is calculated as the difference between the residue weight and the joint protein and ash weight.

During manufacture of cereal-based foods, starch and nonreducing sugars such as sucrose might be hydrolyzed to form reducing sugars. Several methods have been developed for their determination. Extraction with 80% ethanol followed by derivatization into TMS oximes has been used for GC-MS analysis of fructose, glucose, lactose, lactulose, maltose, and maltulose in cookies, crackers, and breakfast cereals,[59] and in flours commonly used in the cereal products industry.[60] Alternatively, clarification of sample with Carrez I and II solutions, extraction with hot water, centrifugation, and filtration are the only sample pretreatments prior to HPLC analysis of sugars in cereal-based products.[61] Fat extraction with petroleum ether prior to carbohydrate extraction is also a requirement before direct HPLC–refractive index (RI) analysis of glucose, fructose, sucrose, and maltose in presweetened cereals.[42]

In flour, reducing and nonreducing sugars are measured by a titrimetric method that requires pretreatment of alcohol extracts with sodium tungstate and alkaline ferricyanide.[42] Similarly, sample pretreatment for analysis of lactose in bread[42] includes Soxhlet extraction with an alcohol/water mixture, treatment with yeast, and precipitation of proteins to obtain a clear filtrate.

4.11.4.3.3 Meat and Fish

- Meat

Meat, when referring to muscle freed from adhering fat, contains on average 0.05–0.2% of carbohydrates. In muscle, the content of glycogen, the polysaccharide that is the principal storage form of glucose in animals, varies greatly from 0.2 to 1.0% on a fresh tissue weight basis, and is influenced by the age and conditions of the animal prior to slaughter. Sugars are only 0.1–0.15% of the weight of fresh muscle, of which 0.1% is shared by glucose-6-phosphate and other phosphorylated sugars. The free sugars present are glucose (0.009–0.09%), fructose, and ribose.[1] By contrast, meat products may contain added polysaccharides or sugars.

Sampling

Sampling of meat and meat products generally includes separation from any bone, grinding with a food chopper, and mixing. Ground samples should be placed in containers with airtight and watertight covers and, if analysis is not going to be carried out promptly, samples should be chilled to inhibit decomposition. Alternatively, samples can be dried either in vacuo or by evaporation on a steam bath for nonimmediate analysis. Extraction of fat with petroleum ether is also commonly required.[42] For preparation of meat extracts and similar products, only mixing thoroughly (for liquid and semiliquid samples) or grinding in a mortar (for samples in the form of cubes) is required.

Sample pretreatment

As sugars are present in meat as minor components, their individual analysis is not very common. However, flour or other cereal fillers are widely used in the formulation of many meat products, and these contribute minor amounts of sugars but significant amounts of starch. In meat, free sugars can be determined after extraction with aqueous alcohol, and the starch can be measured after acid or enzymatic hydrolysis of the meat extract.

Sample pretreatment for determination of starch in meat includes clarification with Carrez I and II, hydrolysis with HCl, and treatment with phosphotungstic acid solution prior to determination of reducing sugars by titration. Digestion with 8% KOH solution followed by dilution with alcohol is used for analysis of starch from starchy flour and spices in meat (chopped sausage, etc.), the percentage of flour present in meat being calculated from the volume of sediment obtained. Determination of inuline, an additive in meat products, has also been carried out after hot-water extraction followed by enzymatic hydrolysis and determination of the released fructose by HPLC-RI.[62]

The addition of milk powder to some meat products provides lactose for these foods, and colorimetric and enzymatic procedures have been described in official methods[42] for measuring lactose in mixtures of this type. For instance, qualitative tests for detection of nonfat dry milk in absence of maltose are based on the change in color after addition of 5% $MeNH_2 \cdot HCl$ solution, boiling, and addition of 20% NaOH to hot-water solutions of meat. Alternatively, lactose can be quantitatively determined (either in the absence or presence of maltose) by the Benedict solution method, after treatment with 20% phosphotungstic acid solution for precipitation of proteins and incubation with yeasts.

Several qualitative tests based on colorimetric reactions with iodine solution, Benedict reactive, etc., or gelling have been developed for the detection of agar or other hydrolyzable gum in meat, consommé, or broth.[42] General sample pretreatments including extraction, centrifugation, precipitation, and acid hydrolysis are required for these tests.

- Fish

Fish flesh contains small concentrations of sugars, which are usually ignored in food analysis. Cereal fillers are used in the formulation of many fish products, and the carbohydrates in these can be measured by the procedures outlined for similar meat products. In general, samples of fish and other marine products are usually obtained after discarding bones and other inedible parts, and blending until homogeneous or grinding through a meat chopper. For fish packed in salt or brine, draining and rinsing off adhering salt crystals is also done. As previously discussed for meat sampling, determinations from fish should be carried out as soon as practicable.

See also: Fundamentals of Supercritical Fluid Extraction; Principles and Practice of Solid-Phase Extraction; Microwave Extraction; Membrane Extraction: General Overview and Basic Techniques; Ionic Liquids; Analytical Derivatization Techniques; Headspace Sampling in Flavor and Fragrance Field; Theory of Extraction

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