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The Application of HPLC to the Determination of Low Molecular Weight Sugars and Polyhydric Alcohols in Foods: A Review

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ABSTRACT

HPLC methods for the determination of individual low molecular weight sugars (mono- to tetrasaccharides) and related polyhydric alcohols in foods are surveyed. Methods of sample preparation, detection and separation modes are discussed. Specific analytical procedures are tabulated according to sample type, sample preparation and HPLC conditions.

INTRODUCTION

Sugars or syrups may be added to processed foods for a variety of technological purposes (Pirisino, 1984). Acid or enzymatic hydrolysis of corn starch yields glucose and short chain oligomers of glucose with varying degrees of polymerisation, such as maltose (Dp2) and maltotriose (Dp3). The resultant corn syrup is frequently used instead of sucrose and invert sugar as a sweetener in food processing. Enzymatic conversion of the glucose in corn syrups to fructose produces a much sweeter high-fructose corn syrup. Lactose is used in foods for infants and convalescents; it also provides the basis for lactic acid fermentation in yoghurts and other foods. Enzymatic conversion of lactose from whey to glucose and galactose yields a sweet syrup which can substitute for corn syrup in many food applications. Lactose-reduced milks are targeted for the lactose-intolerant person who, otherwise, would avoid milk and other dairy products (Pirisino, 1983).

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Of the polyhydric alcohols, mannitol acts as a stabiliser, thickener and anticaking agent, whilst sorbitol retards the solidification of sugars and acts as a sequestrant in soft drinks and wines. These polyols, and also xylitol, are not absorbed by the intestine and hence are used as sugar substitutes in dietetic foods, confectionery products and chewing gums.

Glucose and fructose occur naturally in the free state in fruits and other plant tissues and in honey. Free glucose is also found in animal fluids (blood, lymph and cerebrospinal fluid). The pentose monosaccharides, arabinose, xylose and ribose, and the hexoses, mannose and galactose, rarely occur free in nature, except as breakdown products during fermentation. Of the disaccharides, maltose is formed only during the synthesis or degradation of starch; lactose occurs solely in mammary tissue, whilst sucrose is ubiquitous in root crops and many fruits. The trisaccharide, raffinose, and the tetrasaccharide, stachyose, occur in legume seeds and are predominant sugars in soybeans. Sorbitol occurs in many berries (but not grapes) as well as in apples, pears, plums and cherries, whilst mannitol is produced in vegetable fermentation products such as sauerkraut.

This review deals with the determination of individual low molecular weight sugars, which are arbitrarily defined as mono- to tetrasaccharides (Folkes & Crane, 1988). The principal sugars of interest in food products are fructose, glucose, galactose, maltose, sucrose, lactose, raffinose and stachyose. Also included are the chief polyhydric alcohols; xylitol, sorbitol and mannitol.

An unfortunate drawback in the application of HPLC is the absence of a strong chromophore in the carbohydrate molecule, since this rules out absorbance detection of the underivatised sugar at wavelengths above 200 nm. Underivatised sugars can be determined using a variety of chromatographic modes in conjunction with refractive index detection. With anion exchange chromatography, electrochemical detection can be exploited. Different chromatographic modes used with absorbance or fluorescence detection can be utilised if the carbohydrates are derivatised.

SAMPLE PREPARATION

The methods of sample preparation discussed here relate to the determination of underivatised sugars.

Sample preparation involves extraction of the sugars from the food matrix and clean-up of the extracts, where necessary, to provide a solution that can be analysed by HPLC. The treatment of the sample is dictated by its chemical nature and by the mode of HPLC employed.

Water is obviously an excellent sugar extractant, but is non-selective in

that polysaccharides and proteins are soluble to different extents, in addition to organic acids, salts and other polar material.

A commonly-used sugar extraction solvent is 80% aqueous ethanol, which provides a good compromise between efficiency and selectivity. This solvent is particularly suitable when applied to dairy products as it precipitates the milk proteins. In fatty foods, aqueous ethanol or methanol will extract a proportion of the lipid along with the sugars. Further extraction of the aqueous alcoholic extract with chloroform or petroleum spirit will leave a relatively lipid-free aqueous extract of the sugars. Another technique is to freeze-dry food samples, and then defat with hexane before extracting with 80% methanol. Chocolate can be defatted by shaking with petroleum spirit and centrifuging; free sugars are then recovered from the defatted residue by mixing with water.

In the analysis of dairy products, the milk proteins and fats may be removed by treatment of the sample with 80% aqueous ethanol (Warthesen & Kramer, 1979; Richmond *et al.*, 1982, 1987), trichloroacetic acid (Euber & Brunner, 1979), dilute perchloric acid followed by acetonitrile (West & Llorente, 1981) or acetate buffer (pH 4·6) and acetonitrile (Brons & Olieman, 1983). Kwak and Jeon (1988) reported that propan-2-ol was more effective than ethanol in precipitating milk proteins, and it also incurred a lower dilution of the sample. In the analysis of defatted soy flour, a 1:1 v/v ratio of acetonitrile–soy extract produced protein precipitation in excess of 98% without reducing the sugar concentrations (Black & Glover, 1980). The use of Carrez solutions (potassium ferrocyanide and zinc acetate) (Macrae, 1985), neutral lead acetate (Wight & van Niekerk, 1983) and a solution containing zinc acetate, phosphotungstic acid and acetic acid (Van Riel & Olieman, 1986) as deproteinising agents have also found application. Kennedy *et al.* (1985) deproteinised aqueous extracts of defatted soybean meal by centrifuging at 1000 × *g* through a 25 000 molecular weight cut-off filter.

The acids present in acidic foods, such as citric acid in fruit juices, can be precipitated by treatment with calcium carbonate (Davis & Hartford, 1979).

Sample clean-up can be effected chromatographically by means of open minicolumns or syringe-operated cartridges. Reversed-phase (C18) cartridges retain molecules with hydrophobic properties such as lipids, pigments and proteins. Jeon *et al.* (1984) employed a Sep-Pak C18 (Waters Chromatography Division, Millipore Corp., Milford, MA, USA) cartridge to remove residual proteins afer heating whey and whey permeates to precipitate the bulk of the proteins.

HPLC guard columns protect the analytical column from damage caused by particulate matter, irreversibly bound material and aggressive reagents in samples or solvents. In addition, they provide a convenient method for in-

line sample clean-up by removing contaminants which could foul the analytical column and reducing or eliminating interferences caused by anions, cations, organics, salts, insolubles and particulates (Bio-Rad Laboratories, 1988).

Guard columns may be packed with a pellicular material having a similar stationary phase to that of the analytical column. In this case, strongly retained sample components will be prevented from entering the analytical column and causing loss of efficiency. Alternatively, guard columns may be packed with a different type of chromatographic material than the analytical column and hence will selectively retain unwanted compounds. Thus, a combined anion and cation exchange guard column would deionise a sample, whilst a reversed-phase C18 guard column would retain lipids.

Sample extracts should be passed through a 0·22–0·45 μm clarification filter before injection into the liquid chromatograph.

HPLC SYSTEMS

Separation techniques

The most common types of HPLC column packings in commercial use for separating low molecular weight sugars in foods are metal-loaded cation exchangers based on a resin or a silica substrate, and amino-bonded silica. Plain silica operated with an amine modifier in the mobile phase provides similar separations to amino-bonded silica. Anion exchange resins are gaining in popularity and can be used with electrochemical detection. Adsorption chromatography on unmodified silica and reversed-phase chromatography on octadecylsilane(ODS)-bonded silica only provide a limited separation of underivatised sugars, but can be utilised for sugars subjected to precolumn derivatisation. Phases that merit further attention include polyol-bonded silica, cyclodextrin-bonded silica and copper-modified silica.

Cation exchangers

Resin-based cation exchangers usually comprise microparticulate (9–11 μm diameter) spheres of a sulphonated polystyrene–divinylbenzene (PS–DVB) copolymer lattice with 6% or 8% crosslinking. The resin is loaded with a metal counter-ion, usually calcium (Ca^{2+}), lead (Pb^{2+}) or silver (Ag^{+}), which remains ionically bound to the polymer under the appropriate elution conditions. Columns packed with such resins are designed to be operated with distilled water as the mobile phase, but up to 30% vol. of acetonitrile

can be added to the eluent as an organic modifier. The addition of 0·001M triethylamine (TEA) to the water eluent improves the chromatography of sugars on Ca^{2+}-form resins, and allows a column temperature below 80°C to be used. The TEA raises the pH of the eluent to 10·8, which increases the rate of mutarotation of reducing sugars and hence prevents the separation of the α and β anomers. The TEA will gradually displace the calcium from the resin, thus periodic column regeneration is required (Verhaar & Kuster, 1981*a*).

Owing to the exclusion properties of the resin, elution takes place in order of decreasing molecular weight, i.e. higher oligosaccharides elute first, followed by disaccharides, monosaccharides and polyhydric alcohols. Individual monosaccharides and polyhydric alcohols are well separated from one another, but the separation of individual disaccharides and higher oligosaccharides is limited.

Columns with a particular counterion tend to be dedicated to a particular application, depending upon which carbohydrates need to be separated. This is because there is little or no opportunity to alter chromatographic selectivity by adjusting the composition of the mobile phase. The specificity of each column type is attributable to the different counterions forming characteristic complexes with the carbohydrates; the retentive properties depend on the stability of the complex; the elution order within a class of carbohydrates is governed by the stereochemistry of the polyol molecule (Goulding, 1975). The use of metal-loaded cation exchangers for the separation of polar non-ionic compounds is frequently referred to as ion moderated partition (IMP) chromatography (Jupille *et al.*, 1981).

Columns packed with sulphonated PS–DVB resins require operation at high temperatures (usually in the range 80–90°C) for optimum efficiency. The elevated temperature increases the rate of mass transfer between the mobile phase and the stationary phase, thereby reducing band spreading and improving peak resolution. The resultant decrease in viscosity of the mobile phase reduces the column back pressure and thus allows a higher flow-rate to be used. The elevated temperature also accelerates mutarotation and ensures that only one peak will be obtained for each reducing sugar; at room temperature the α and β anomers are separated. According to Charles (1981), the equilibrium mixture gives a single peak by about 55°C.

Sulphonated PS–DVB columns are typically 30 cm in length by 6·5 mm (e.g. Sugar-PAK I, Waters Chromatography Division) or 7·8 mm (e.g. Aminex HPX range, Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK) internal diameter. The compressibility of the resins depends upon the percentage of crosslinking. The maximum back pressure that can be applied to a 30 cm × 7·8 mm 8% crosslinked Ca^{2+}- or Pb^{2+}-form column is 1500 lb in^{-2} (103 bar), which imposes a maximum flow-rate

of 1·2 ml min^{-1} (Bio-Rad Laboratories, 1987). In practice, a flow-rate of 0·5–0·6 ml min^{-1} for a standard column is the highest that can be obtained (Verzele *et al.*, 1987).

It is essential to remove Na^+ and K^+ ions from the injected sample when using IMP chromatography, otherwise these ions would exchange with the counterion. Na^+ and K^+ ions can be removed by means of a strong acid cation exchanger in the Ca^{2+} or H^+ form. The presence of salt in the injected sample complicates the chromatogram, since the Cl^- ion elutes with the higher saccharides on Ca^{2+}-form PS–DVB resins using water as the mobile phase. The Cl^- ion can be removed by using an anion exchanger converted to the OH^- form.

It has been frequently stated that only very small amounts of anion exchange resins should be used in clean-up procedures, as the sugars themselves can be retained on these resins. However, the degree of sugar retention depends upon the type of anion exchanger employed. A resin with tertiary amine functional groups, such as AG-3 (Bio-Rad Laboratories Ltd), is preferable to a resin with quaternary amine functional groups, such as A-25 (Bio-Rad Laboratories Ltd), because the former causes less retention of sugars and also less on-column hydrolysis of sucrose. A weak anion exchanger in the carbonate form is more suitable as it incurs still less sugar retention.

The interfering Na^+, K^+ and Cl^- ions can be conveniently removed by employing a de-ashing guard cartridge system comprising a Ca^{2+}-form strong cation exchanger and a CO_3^{2-}-form weak anion exchanger.

Anion exchange columns will also remove weak organic acids such as the citrate and lactate ions. The removal of acids from the injected sample is necessary, otherwise the H^+ ions will replace the calcium counterion and cause resin-bed shrinkage. The resultant protonated resin, in combination with the high elution temperature (80–90°C), will catalyse on-column hydrolysis of sucrose. Calcium leaching can be avoided by the addition of a calcium salt to the mobile phase, but interactions between Ca^{2+} and COO^- groups result in increased retention of the nonvolatile acids and their co-elution with sugars (Spanos & Wrolstad, 1987). Woollard (1983) added the calcium–sodium salt of ethylenediaminetetraacetic acid (EDTA) to the mobile phase as a metal sequestrant to preserve column life.

Table 1 lists applications of cation exchange columns to the analysis of canned soft drinks, commercial fruit juices and concentrates, wines, yoghurts, skimmed milk and infant formulae.

The separation of typical oligosaccharides, monosaccharides and polyhydric alcohols on a Ca^{2+}-form column eluted with water at 90°C is shown in Fig. 1. Mannitol, xylitol and sorbitol can be separated using a mobile phase of water:acetonitrile (75:25 v/v), the latter two being

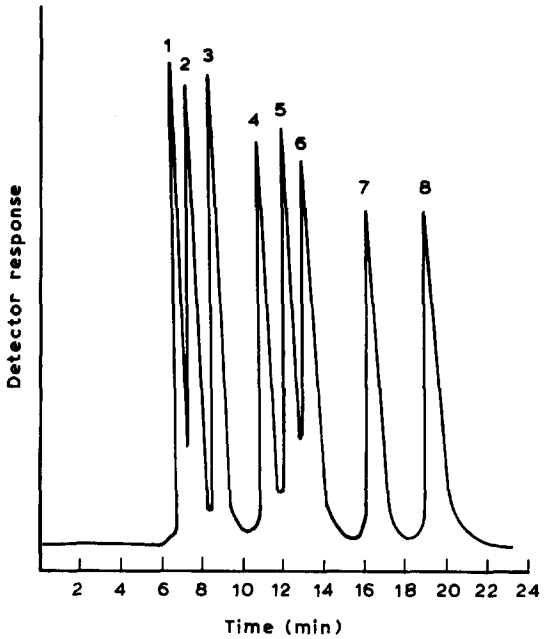

Fig. 1. HPLC of carbohydrate standards on a 30 cm × 6·5 mm i.d. Sugar-PAK I Ca^{2+}-form column eluted with water (90°C) at 0·4 ml min^{-1}. Detection: RI. Peaks: 1, stachyose; 2, raffinose; 3, sucrose; 4, glucose; 5, galactose; 6, fructose; 7, mannitol; 8, sorbitol. Reprinted from Vidal-Valverde *et al.*, 1985*b*, by courtesy of Marcel Dekker, Inc., NY, USA.

inseparable using water as the eluent (Vidal-Valverde *et al.*, 1984*a*). Ca^{2+}-form resins have been employed to determine lactose, glucose and galactose in various dairy products (Richmond *et al.*, 1982, 1987; Pirisino, 1983; Woollard, 1983; Jeon *et al.*, 1984). The presence of sucrose in the dairy product causes a problem as it is poorly resolved from lactose using a Ca^{2+}-form resin (Vidal-Valverde *et al.*, 1984*b*) (Fig. 2). For this application, a Pb^{2+}-form column would be more suitable as it gives a good separation of sucrose, lactose, glucose, galactose and fructose (Fig. 3).

Scott and Hatina (1988) used a Pb^{2+}-form column to analyse infant formulae and found small amounts of the non-absorbed lactose isomer, lactulose, in liquid milk-based preparations. A chromatogram of a standard mixture of sucrose, lactose, glucose, lactulose and fructose is shown in Fig. 4(a). A typical chromatogram of a formula for premature infants is shown in Fig. 4(b). Clearer evidence for the presence of maltose and higher glucose polymers was obtained by treating the sample with the enzyme amyloglucosidase. This treatment virtually completely converted the polymers to glucose, as shown by the enhanced glucose peak in Fig. 4(c).

An Ag^{+}-form 6% cross-linked resin column operated at 85°C has been applied to the determination of maltose, lactose, glucose and galactose in

TABLE 1
Applications of HPLC in Sugar Analysis

Sugars[a]	*Sample*	*Extraction*	*Clean-up*	*Guard column*	*Analytical column*	*Mobile phase*[b]	*Detector*	*Reference*
Sucrose, lactose, glucose, lactulose, fructose	Infant formulae	Freeze-dry liquid samples; defat powdered formula with hexane; centrifuge; extract with 80% methanol at 65°C; centrifuge; repeat extraction	Evaporate pooled extracts to dryness; redissolve in H_2O; Sep-Pak C18; 0·22 μm membrane filter	Cation exchanger and anion exchanger in series	Aminex HPX-87P (85°C)	H_2O	RI	Scott & Hatina (1988)
Lactose, glucose, galactose	Lactose-reduced milk	Aqueous acetonitrile	Centrifuge; 0·45 μm membrane filter	None	Aminex HPX-87C (80°C)	H_2O	RI	Pirisino (1983)
Lactose, glucose, galactose	Dairy products	80% ethanol	Centrifuge; evaporate to dryness under vacuum; dissolve residue in H_2O; filter; Sep-Pak C18	Aminex A-25 Anion/OH^-	Aminex HPX-87C (80°C)	H_2O	RI	Richmond *et al.* (1982)
Lactose, glucose, galactose	Yoghurt	80% ethanol	Centrifuge; concentrate by rotary evaporation; dilute with H_2O; filter (paper then membrane)	Aminex A-25 Anion/OH^-	Aminex HPX-87C (80°C)	H_2O	RI	Richmond *et al.* (1987)
Lactose, glucose, galactose	Hydrolysed whey products	Syrups: add mannitol as internal standard; dilute with water Powders: add mannitol as internal standard; H_2O (70°C)	Heat at 60°C for 1 h to flocculate residual protein; filter Deproteinize with 5% TCA; dilute with H_2O; centrifuge; membrane filter	None	Sugar-PAK I (85°C)	H_2O + 2 mg each of calcium acetate and $Ca(Na)_2EDTA$ per litre	RI	Woollard (1983)
Lactose, glucose, galactose	Hydrolysed whey and whey permeate	Adjust pH of acid whey to 5·2 with 10% KOH; heat to complete protein aggregation; cool	Filter; dilute with water; Sep- Pak C18	LiChrosorb AX-GU anion exchange resin	μ-Spherogel Carbohydrate (Altex) (80°C)	H_2O	RI	Jeon *et al.* (1984)
Lactose	Milk (skimmed)	H_2O	Acetate buffer, pH 4·60/acetonitrile to precipitate fats and proteins; stand 15 min; filter	Equivalent amounts of cation (AG 50W-X4, H^+) and anion (AG 3-X4A, OH^-) exchange resins (45:55 wt ratio of dry cation to dry anion exchange material)	Aminex HPX-87C (85°C)	H_2O	RI	Brons & Olieman (1983)

Maltose, lactose, glucose, galactose	Dairy products	H_2O	Precipitate proteins with solution containing zinc acetate, phosphotungstic acid and acetic acid; filter	Mixed bed of cation (AG 50W-X4, H^+) and anion (AG3-X4A, OH^-) exchange resins (10:15, meq) (corresponding to a 35:65 wt ratio) of dry cation to dry anion exchange material	Aminex HPX-65A (85°C)	H_2O	RI	Van Riel & Olieman (1986)
Sucrose, lactose, glucose, galactose	Yoghurt	Boil with 80% ethanol	Filter; evaporate to dryness under vacuum; dissolve residue in H_2O; extract with diethyl ether; reduce to dryness; dissolve residue in H_2O; Sep-Pak C18; 0·45 μm membrane filter; precolumn filter	None	Sugar-PAK I (85 or 90°C)	H_2O	RI	Vidal-Valverde *et al.* (1984*b*)
Sucrose, glucose, fructose, sorbitol	Apple juice		AG1-X8, OH^- anion exchange resin column	None	Aminex HPX-87C (86°C)	H_2O	RI	Mattick & Moyer (1983)
Sucrose, glucose, fructose, sorbitol	Soft drinks, fruit juices	Boil fruit juices with 80% ethanol; extract with 80% ethanol; evaporate to dryness under vacuum; dissolve residue in H_2O; extract with diethyl ether; evaporate to dryness; dissolve residue in H_2O	Sep-Pak C18; 0·45 μm membrane filter; precolumn filter	None	Sugar-PAK I (85°C)	H_2O	RI	Vidal-Valverde *et al.* (1985*a*)
Sucrose, glucose, fructose, sorbitol	Wines, blackberry juice concentrates	H_2O; boil off the ethanol from wines	AG1-X2 anion exchange resin column	None	Aminex HPX-87C (87°C)	H_2O	RI	Wrolstad *et al.* (1982)
Sucrose, glucose, fructose	Sweet potatoes (raw)	Boil with 80% ethanol; filter	0·45 μm membrane filter	Dual cation (Aminex HPX-85, H^+) and anion (Aminex A-25, OH^-) system	Aminex HPX-87C (75°C)	H_2O	RI	Picha (1985)

(*continued*)

TABLE 1—*contd.*

Sugars[a]	*Sample*	*Extraction*	*Clean-up*	*Guard column*	*Analytical column*	*Mobile phase*[b]	*Detector*	*Reference*
Sucrose, glucose, fructose	Raspberry juice	Mix 5 ml juice with mannitol solution as internal standard in HPLC mobile phase	Sep-Pak C18; apply eluate to Bio-Rex 5, Cl^- anion exchange minicolumn; filter eluate through 0·45 μm membrane	Carbo C (Bio-Rad)	Aminex HPX-87C (85°C)	H_2O + 200 mg calcium nitrate per litre	RI	Spanos & Wrolstad (1987)
Sucrose, glucose, fructose, mannitol	Vegetable fermentations	Blend without added liquid then filter or centrifuge	0·45 μm membrane filter	Dual cation (Aminex HPX-85, H^+) and anion (Aminex A-25, OH^-) system	Aminex HPX-87P (68°C)	H_2O	RI	McFeeters *et al.* (1984)
Sucrose, glucose, fructose	Cabbage	Heat at 80–90°C with 80% ethanol + sorbitol as internal standard; blend; reheat; dilute with H_2O; filter	0·45 μm membrane filter	None	Sugar-PAK I (77°C)	H_2O + 50 mg calcium disodium EDTA per litre	RI	Hughes & Lindsay (1985)
Residual sugars, mannitol	Sauerkraut	Neutralize with $CaCO_3$; extract with 80% ethanol + sorbitol as for cabbage	Sep-Pak alumina; 0·45 μm membrane filter					
Fructose, glucose, sucrose	Fruit juice	H_2O	Sep-Pak C18; AG 50W-X8, H^+ cation exchange resin column; AGMP-1, Cl^- anion exchange resin column; concentrate under vacuum; membrane filter	None	Brownlee amine column	Acetonitrile: H_2O (85:15)	UV 190 nm	Shaw & Wilson (1983)
Sucrose, glucose, fructose	Soft drinks		Vacuum degas; neutralize with $CaCO_3$; filter; evaporate an aliquot to dryness; dissolve residue in water and dilute with acetonitrile, 0·45 μm membrane filter	None	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (75:25)	RI	Vidal-Valverde *et al.* (1981)
Fructose, glucose, sucrose, maltose	Honey	H_2O	0·45 μm membrane filter	None	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (83:17)	RI	Thean & Funderburk (1977)
Fructose, glucose, sucrose, maltose	Honey, apple juice	H_2O	0·45 μm membrane filter	None	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (65:35)	RI	Conrad & Palmer (1976)
Sucrose, lactose	Cocoa mix, milk	H_2O	Precipitate proteins and fats in acetonitrile; 0·45 μm membrane filter	None	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (80:20)	RI	Conrad & Palmer (1976)

Fructose, glucose, sucrose, maltose, lactose	Wide range: cereals, cream, beverages, etc.	H_2O	0·45 μm membrane filter	None	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (80:20)	RI	Iverson & Bueno (1981)
Fructose, glucose, sucrose, maltose, lactose	Breakfast cereals and other foods	H_2O (80–90°C)	0·45 μm membrane filter	None	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (85:15)	RI	De Vries *et al.* (1979)
Fructose, glucose, sucrose, lactose	Confectionery, redcurrant jelly	H_2O (add ribose as internal standard)	0·45 μm membrane filter	None	Amino-bonded (unspecified)	Acetonitrile: H_2O (90:10)	RI	Hunt *et al.* (1977)
Fructose, glucose, sucrose, maltose, lactose	Confectionery	Petroleum spirit; extract defatted residue with H_2O	Centrifuge (48 000 × g)	Corasil I	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (80:20)	RI	Timbie & Keeney (1977)
Fructose, glucose, sucrose, maltose, lactose	Milk chocolate	Petroleum spirit; centrifuge, extract defatted residue with H_2O (85–90°C)	Centrifuge (2000 rpm); 0·45 μm membrane filter	None	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (80:20)	RI	Hurst & Martin (1977)
Lactose	Milk products	H_2O (add xylose as internal standard)	Deproteinise and defat with TCA; hold at 40°C for 30 min; filter; 0·8 μm membrane filter	None	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (80:20)	RI	Euber & Brunner (1979)
Lactose	Milk	Propan-2-ol to precipitate proteins	Centrifuge; filter; Sep-Pak C18	Amino Spheri-5 polar phase AS-GU cartridge	Amino Spheri-5 (Brownlee Labs)	Acetonitrile: H_2O (75:25)	RI	Kwak & Jeon (1988)
Fructose, glucose, sucrose, maltose	Liquorice extracts	H_2O (85–90°C)	0·45 μm membrane filter; Sep-Pak C18	Corasil AX	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (83:17)	RI	Vora & Tuorto (1984)
Fructose, glucose, sucrose, maltose, lactose	Milk, ice cream	80% ethanol	Centrifuge; 0·2 μm membrane filter	Corasil CX/ Corasil AX	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (75:25 or 82:18)	RI	Warthesen & Kramer (1979)

(continued)

TABLE 1—*contd.*

Sugars[a]	*Sample*	*Extraction*	*Clean-up*	*Guard column*	*Analytical column*	*Mobile phase*[b]	*Detector*	*Reference*
Fructose, glucose, sucrose, maltose, lactose	Wide range: beverages, processed fruits, dairy products, cakes, snacks	Dairy products, cakes, snacks: H_2O (70–80°C) Processed fruit: methanol:H_2O: acetic acid (79:20:1)	0·45 μm membrane filter (beverages)	Bondapak C18/Porasil B	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (80:20)	RI	Hurst *et al.* (1979)
Fructose, glucose, sucrose, maltose, lactose	Diet composite reference material	Defat freeze-dried samples with hexane, then extract with either 80% methanol or H_2O	Evaporate methanol extracts to dryness under N_2; redissolve in H_2O; mix aliquot of aqueous extract with xylose solution as internal standard; evaporate to one-half volume under N_2 at 60°C; mix with acetonitrile; allow precipitation (30 min standing); centrifuge;	None	Radial-PAK μ-Bondapak NH_2 cartridge	Acetonitrile: H_2O (75:25)	RI	Li *et al.* (1985)
Sucrose, raffinose, stachyose	Defatted soya flour	H_2O	Sep-Pak C18 and 0·45 μm membrane filter Centrifuge; add 5 ml acetonitrile to 5 ml supernatant to precipitate proteins; filter	μ-Bondapak precolumn	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (70:30)	RI	Black & Glover (1980)
Sucrose, raffinose, stachyose	Defatted soya flour	Shake with $CHCl_3$:methanol (1:1) then with H_2O; centrifuge; concentrate aqueous layer to 5 ml		Co-Pell-PAC	Partisil 10 PAC	Acetonitrile: phosphate buffer, 2·5 mM, pH 5	RI	Havel *et al.* (1977)
Sucrose, raffinose, stachyose	Leguminous seeds	Boiling H_2O; blend in H_2O (60°C)	Centrifuge; precipitate proteins with acetonitrile (5°C); 0·45 μm membrane filter	None	LiChrosorb NH_2	Acetonitrile: H_2O (65:35)	RI	Knudsen (1986)
Fructose, glucose, sucrose, maltose, lactose, melibiose, raffinose, stachyose	Wide range: infant foods, cereals, chocolate products, cakes, preserves, soya protein products	50% ethanol (or H_2O for samples containing >50% protein); heat for 1 h at 50°C in sonic bath	Wash high (>8%) fat samples with $CHCl_3$; adjust pH of high protein samples to 4·5 with acetic acid to precipitate protein; centrifuge; pass supernatant through minicolumn packed with 1/8 in Corasil AX and 3/4 in Porasil B/C18	None	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (75:25)	RI	Dunmire & Otto (1979)

Glucose, sucrose, maltose, lactose	Sausage products	52% ethanol	Filter; defat with petroleum spirit; Sep-Pak C18; evaporate to dryness under vacuum; dissolve residue in H_2O; transfer to two ion exchange minicolumns connected in series, (i) AG 50W-X8, H^+ cation exchanger, (ii) AG 3-X4A, OH^- anion exchanger; evaporate to dryness; dissolve residue in water; 0·45 μm membrane filter	None	Bio-Sil Amino 5S (Bio-Rad)	Acetonitrile: H_2O (78:22)	RI	Sher Ali (1988)
Fructose, glucose, sucrose	Potatoes	50% methanol	Centrifuge; Sep-Pak C18	Porasil B/C18	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (75:25)	RI	Wilson *et al.* (1981)
Fructose, glucose, sorbitol, sucrose, maltose	Fruits	80% ethanol; concentrate by rotary evaporation; filter	Sep-Pak C18; 0·45 μm membrane filter	None	Dual column Partisil PAC and μ-Bondapak Carbohydrate	Acetonitrile: H_2O:ethanol (80:15:5)	RI	Richmond *et al.* (1981)
Fructose, glucose, sucrose, maltose	Vegetables	H_2O	Deionisation (Biodeminrolit); Sep-Pak C18	Porasil B/C18	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (75:25)	RI	Martin-Villa *et al.* (1982)
Fructose, glucose, sucrose, maltose	Sweet potatoes (baked)	Boil with 80% ethanol; filter	0·45 μm membrane filter	Bio-Sil NH_2	Bio-Sil Amino 5S	Acetonitrile: H_2O (70:30)	RI	Picha (1985)
Fructose, glucose, sucrose, maltose	Presweetened cereals	50% ethanol	Centrifuge; 0·45 μm membrane filter; Sep-Pak C18	None	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (80:20)	RI	Zygmunt (1982)
Fructose, glucose, sucrose, maltose	Pizza	Hot 85% methanol; concentrate by evaporation	Membrane filtration	None	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (80:20) + 2·5 mM tetrabutyl-ammonium phosphate	RI	Wills *et al.* (1982)
Fructose, glucose	Tomato juice	Boil with 80% ethanol; filter; concentrate by rotary evaporation; filter	Sep-Pak C18; 0·45 μm membrane filter	Co-Pell PAC	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (80:20)	RI	Gancedo & Luh (1986)

(continued)

TABLE 1—*contd.*

Sugars[a]	*Sample*	*Extraction*	*Clean-up*	*Guard column*	*Analytical column*	*Mobile phase*[b]	*Detector*	*Reference*
Fructose, glucose, sucrose, maltose, lactose	Wide range: infant foods, cakes, snacks, etc.	80% ethanol	Defat with $CHCl_3$; filter; centrifuge	None	μ-Bondapak Carbohydrate	Acetonitrile: H_2O (82:18)	RI	Smith *et al.* (1986)
Sucrose	Breakfast cereal (sugar coated)	H_2O; centrifuge; 70% ethanol	Centrifuge; Sep-Pak C18	Corasil AX	Radial-PAK B silica cartridge	Acetonitrile: H_2O (81:19) + 0·02% TEPA	RI	Lee *et al.* (1983)
Fructose, glucose, sucrose, maltose, maltotriose	Brewery wort and beers	Add rhamnose as internal standard	0·45 μm membrane filter	None	Radial-PAK silica cartridge	Acetonitrile: H_2O (70:30) + 0·01% TEPA	RI	Buckee & Long (1982)
Fructose, glucose, sucrose, maltose, lactose	Wide range: unspecified	H_2O (80–90°C) with added $CaCO_3$	Clarify with neutral lead acetate; add AG 501-X8, CO_3^{2-} anion exchange resin; dilute with ethanol; 0·6 μm membrane filter	None	LiChrosorb Si-60	Acetonitrile: H_2O (75:25) or (80:20) + 0·01% amine modifier	VIS 550 nm after post-column reduction of tetrazolium blue	Wight & van Niekerk (1983)
Lactose	Milk	Dilute perchloric acid to remove fat and protein	Centrifuge; add acetonitrile; 0·45 μm membrane filter	HC-Pellosil	Micropak Si-10 (Varian)	Acetonitrile: H_2O (80:20)	RI	West & Llorente (1981)
Sucrose, raffinose, stachyose	Soybean meal (defatted)	H_2O	Centrifuge with a 25 000 molecular weight cut-off filter to remove protein	None	Dextro-PAK Radial-PAK C_{18} cartridge (Waters)	H_2O	RI	Kennedy *et al.* (1985)
Sucrose, raffinose, stachyose	Soybeans	Defat with petroleum spirit; extract with H_2O (80–90°C)	Clarify with saturated neutral lead acetate; deionize with mixed bed resin AG 501-X8; membrane filter	LiChrosorb RP-18	LiChrosorb RP-18	H_2O	RI	Wight & Datel (1986)

[a] In elution order from HPLC column.
[b] Proportions by volume.
EDTA, ethylenediaminetetraacetic acid; TCA, trichloroacetic acid; TEPA, tetraethylenepentamine.

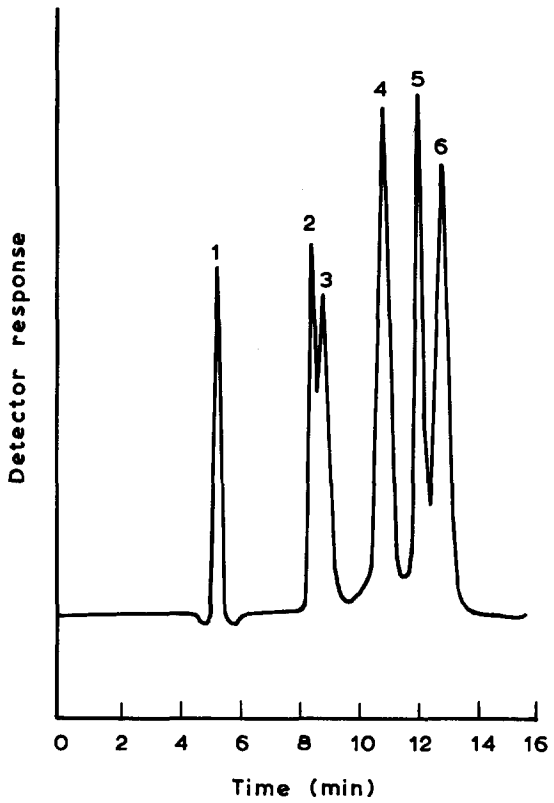

Fig. 2. HPLC of sugar standards. Conditions as in Fig. 1. Peaks: 1, $CaCl_2$; 2, sucrose; 3, lactose; 4, glucose; 5, galactose; 6, fructose. Reprinted from Vidal-Valverde *et al.*, 1984*b*, by courtesy of American Dairy Science Association, IL, USA.

dairy products (Van Riel & Olieman, 1986) (Fig. 5). This technique cannot be applied in the presence of sucrose, which would undergo acid-catalysed hydrolysis, resulting in multiple or broad peaks. The hydrolysis is due to the presence of residual H^+-form sulphonic groups in the resin, which is prepared commercially at less than 100% silver ion loading to obtain optimum efficiency. Anomeric separation of the reducing sugars does not occur with Ag^+-form resins; hence the peaks are narrower than those obtained used Ca^{2+}- or Pb^{2+}-form resins and the columns can be operated at lower temperatures. However, operating Ag^+-form columns at room temperature as a means of avoiding acid-catalysed hydrolysis of sucrose is of little benefit, since the resolution between lactose–glucose and galactose–fructose is lost.

Ca^{2+}-form resins have been employed to determine sucrose, glucose and fructose in sweet raw potatoes (Picha, 1985) and raspberry juice (Spanos &

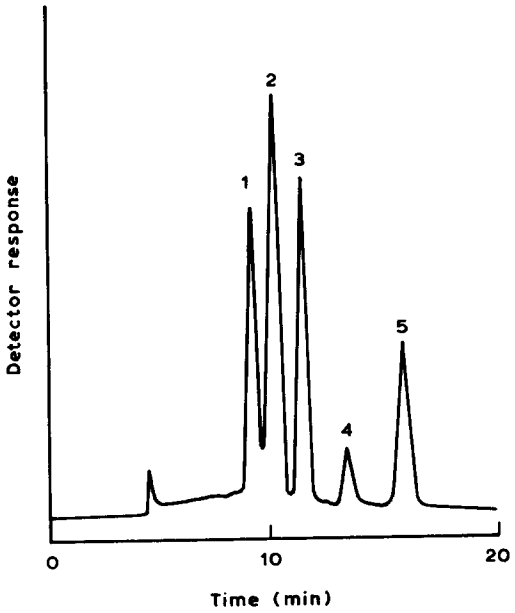

Fig. 3. HPLC of sugars extracted from strawberry yoghurt on a 30 cm × 7·8 mm i.d. Aminex HPX-87P Pb^{2+}-form column eluted with water (85°C) at 0·6 ml min^{-1}. Detection: RI. Peaks: 1, sucrose; 2, lactose; 3, glucose; 4, galactose; 5, fructose. Reprinted from Bio-Rad Laboratories, 1988, by courtesy of Bio-Rad Laboratories Ltd, Hemel Hempstead, UK.

Wrolstad, 1987) and these sugars plus sorbitol in fruit juices, soft drinks and wines (Wrolstad *et al.*, 1982; Mattick & Moyer, 1983; Vidal-Valverde *et al.*, 1985*a*).

IMP chromatography has found commercial application in monitoring the fermentation of vegetables by measuring the rate of sugar depletion and the formation of mannitol among the fermentation products. For instance, Hughes & Lindsay (1985) employed a Ca^{2+}-form column to separate sucrose, glucose, fructose and ethanol in unfermented cabbage and sodium chloride, glucose, mannitol and ethanol in sauerkraut. Sorbitol was used as an internal standard in both separations. The presence of sodium chloride, which is added to vegetables to direct the course of fermentation, did not interfere with the analysis of the fermented product as it eluted before the sugars of interest (Fig. 6). A Ca^{2+}-form column was more convenient than a Pb^{2+}-form column since, using the latter, sodium chloride elutes as a broad peak with a retention volume similar to that of the sugars (McFeeters *et al.*, 1984).

Metal-loaded cation exchangers based on a silica substrate give similar elution profiles to resin-based exchangers when water is used as the mobile phase, but the separations can be obtained at room temperature. Higher

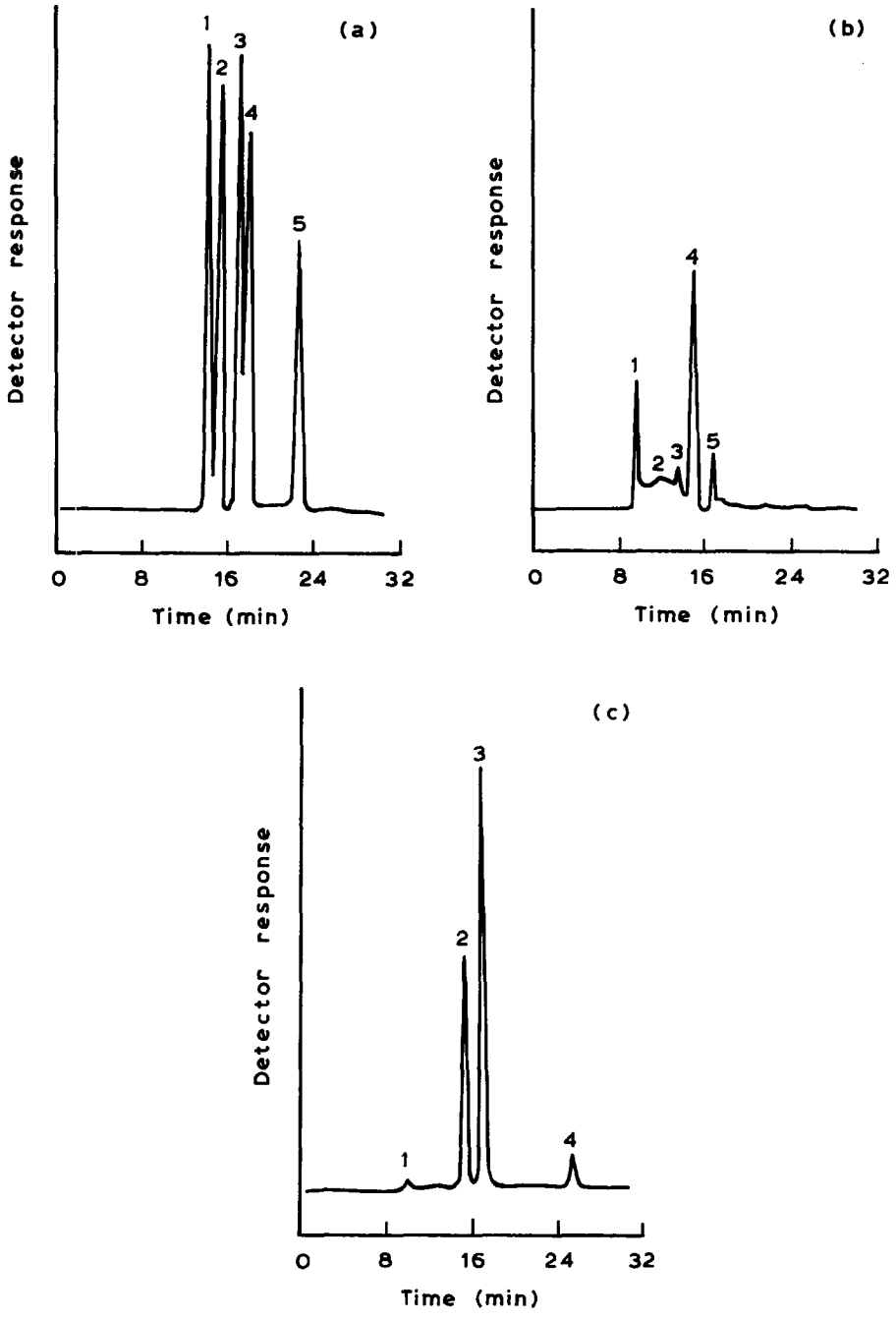

Fig. 4. HPLC of sugars on a 30 cm × 7·8 mm i.d. Aminex HPX-87P Pb^{2+}-form column eluted with water (85°C) at 0·5 ml min^{-1}. Detection: RI. (a) Sugar standards, peaks: 1, sucrose; 2, lactose; 3, glucose; 4, lactulose; 5, fructose. (b) extract from infant formula, peaks: 1, unknown; 2, oligosaccharides; 3, maltose; 4, lactose; 5, glucose. (c) The same extract as in (b) but treated with amyloglucosidase, peaks: 1, unknown; 2, lactose; 3, glucose; 4, glycerol (from the filter). Reprinted from Scott & Hatina, 1988, © Institute of Food Technologists.

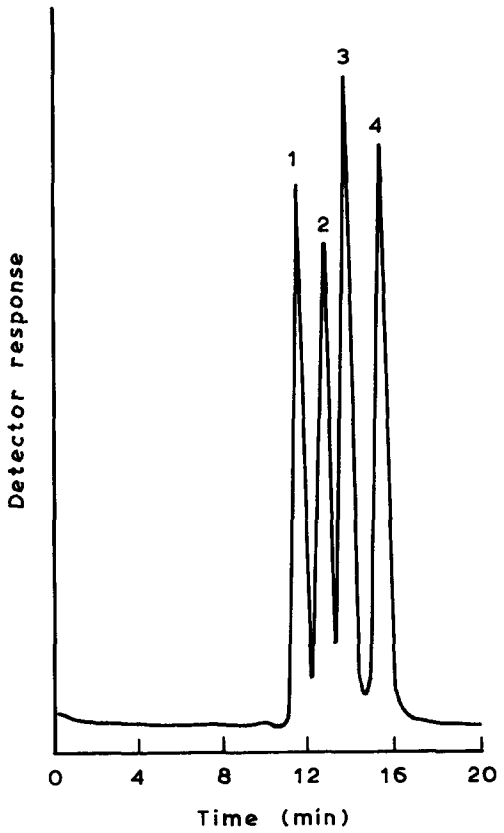

Fig. 5. HPLC of sugar standards on a 30 cm × 7·8 mm i.d. Aminex HPX-65A Ag^+-form column eluted with water (85°C) at 0·6 ml min^{-1}. Detection: RI. Peaks: 1, maltose; 2, lactose; 3, glucose; 4, galactose. Reprinted from Van Riel & Olieman, 1986.

flow-rates, and hence faster analysis times, can be obtained with silica-based packings because the silica substrate is more pressure resistant compared with PS–DVB resins.

Chromatographic efficiency on the silica packings improves considerably by using a mobile phase of acetonitrile : water (75 : 25 v/v), containing 0·075% TEA to accelerate mutarotation. This system elutes the monosaccharides before the higher saccharides and facilitates the separation of glucose, fructose, maltose, lactose and maltotriose (Fig. 7). Thus the difficult pairs for other HPLC systems are well separated; namely, the lactose–maltose and the glucose–fructose pairs, but now sucrose and fructose coelute. The addition of methanol to the acetonitrile/water mobile phase shortens the retention time of the common sugars (except for fructose) and the separation can be adjusted in this way (Verzele *et al.*, 1987). The gradual dissolution of the silica substrate in the alkaline eluent can be prevented by installing a precolumn in

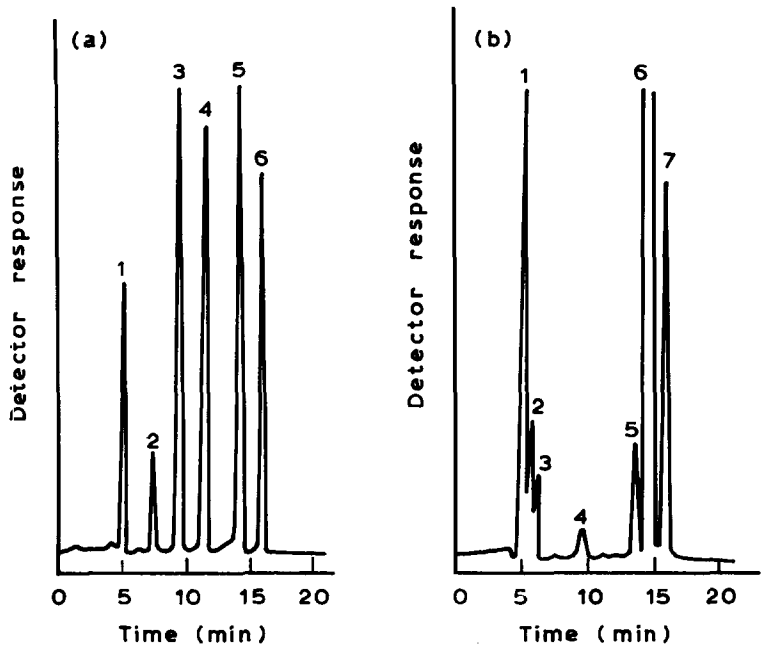

Fig. 6. HPLC of carbohydrates on a 30 cm × 6·5 mm i.d. Sugar-PAK I Ca^{2+}-form column eluted with water (77°C) containing 50 mg calcium disodium EDTA l^{-1} at 0·5 ml min^{-1}. Detection: RI. (a) Extract from cabbage, peaks: 1, unknown; 2, sucrose; 3, glucose; 4, fructose; 5, ethanol; 6, sorbitol (internal standard). (b) Extract from sauerkraut, peaks: 1, NaCl; 2 and 3, unknown; 4, glucose; 5, mannitol; 6, ethanol; 7, sorbitol (internal standard). Reprinted from Hughes & Lindsay, 1985, © Institute of Food Technologists.

front of the injection valve, packed with silica of similar quality. The precolumn saturates the mobile phase with silica and thus prevents it from dissolving the silica substrate of the analytical column.

Anion exchange resins

Carbohydrates are weak acids with dissociation constants (pK_a values) in the range 12–14. In a strongly alkaline medium, such as 10–200 mM NaOH, they are partially or completely ionised owing to the dissociation of the hydroxyl groups. The anions can be retained and separated on a strongly basic hydroxide-form anion exchange resin eluted with an aqueous solution of NaOH. The general elution sequence is polyhydric alcohols, mono-, di- and higher oligosaccharides, but retention order can be controlled by changing the composition of the mobile phase and/or by varying the column temperature. Non-ionic and basic molecules elute in the void volume and do not interfere.

Increasing the concentration of NaOH in the eluent has a two-fold effect

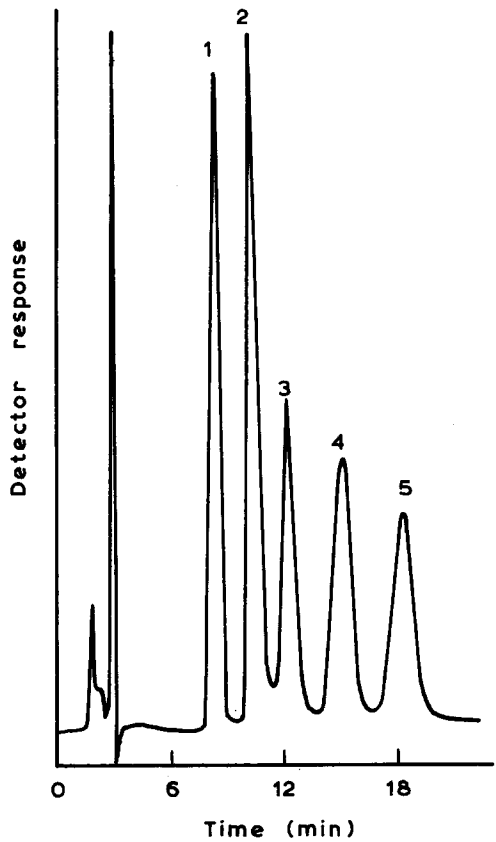

Fig. 7. HPLC of sugar standards on a 25 cm × 4·6 mm i.d. 5 μm RSiL-CAT Ca^{2+}-form column (Alltech Associates) eluted with acetonitrile:water (75:25 v/v) containing 0·075% triethylamine at ambient temperature. Detection: RI. Peaks: 1, glucose; 2, fructose; 3, maltose; 4, lactose; 5, maltotriose. Reprinted from Verzele *et al.*, 1987, by courtesy of Friedr. Vieweg & Sohn, Verlagsgesellschaft MBH, Wiesbaden, FRG.

on retention. The hydroxyl ions displace the carbohydrate anions from the resin sites, thereby reducing retention; they also increase the degree of dissociation, thereby increasing retention. The two opposing effects tend to cancel each other until quantitative dissociation is reached and the carbohydrate is eluted earlier. The pH at which this point occurs is dependent on the pK_a of the carbohydrate. Sodium acetate can be added to increase the ionic strength without affecting the pH and is used to elute the more strongly retained oligosaccharides (Dionex Corp., 1987).

Gradient elution is a useful means (detection method permitting) of improving the resolution for early eluting carbohydrates, whilst also eluting the more strongly retained ones. The gradient elution of mono- and

disaccharides is accomplished by means of a pH gradient, whereby the eluent concentration of NaOH is increased during the run. For oligosaccharides, an ionic strength gradient is used; this is accomplished by holding the NaOH concentration constant and increasing the acetate concentration.

An advantage of anion exchange over IMP is that the high operating pH accelerates mutarotation; thus the α and β anomers of the reducing sugars are eluted as a single peak, without the need to heat the column. However, column temperature can be used as a variable to control retention. Retention decreases for all solutes as the column temperature is increased; the observed magnitude of this effect is in the descending order of oligosaccharides, disaccharides, polyhydric alcohols and monosaccharides. Anion exchange columns are usually operated at temperatures between 20°C and 45°C (Rocklin & Pohl, 1983).

The Dionex Corporation (Sunnyvale, CA, USA) market a liquid chromatography system in which a strongly basic anion exchange column in the OH^- form is connected to a pulsed amperometric detector (see 'Detection systems'). This system provides a selective method of determining carbohydrates and can be used in a gradient mode. The Carbopac PA2 (also known as HPIC-AS6A, Dionex (UK) Ltd, Camberley, Surrey, UK) column packing consists of 5 μm diameter non-porous PS–DVB beads with sub-micron particles of a quaternary amine type anion exchange material electrostatically bonded to the surface. This arrangement facilitates a high exchange capacity per bead in combination with a short diffusion path. The column packing is resistant to extremes of both acid and alkaline conditions.

A potential problem is the effect of the alkaline environment upon the carbohydrate analytes. Reducing sugars are known to undergo epimerisation and aldose–ketose conversion when exposed to dilute alkali for several hours, i.e. the Lowbry de Bruyn–van Ekenstein transformation. Olechno *et al.* (1987) left glucose in the most alkaline eluent used with the Dionex system (150 mM NaOH) for four days at room temperature and found no evidence of the presence of mannose or fructose. Rocklin and Pohl (1983) observed tailing and secondary peaks for glucose and mannose when the column temperature exceeded the recommended maximum of 45°C. It is concluded that the chromatography is usually completed before any transformation products have time to be formed.

The isocratic elution of xylitol, sorbitol, rhamnose, arabinose, glucose, fructose, lactose, sucrose, raffinose, stachyose and maltose using a Carbopac PA1 (Dionex (UK) Ltd) column eluted with 150 mM NaOH at 36°C is shown in Fig. 8. Glucose, galactose, mannose and xylose are only poorly resolved, but these sugars can be separated isocratically at low NaOH concentration with post-column addition of NaOH.

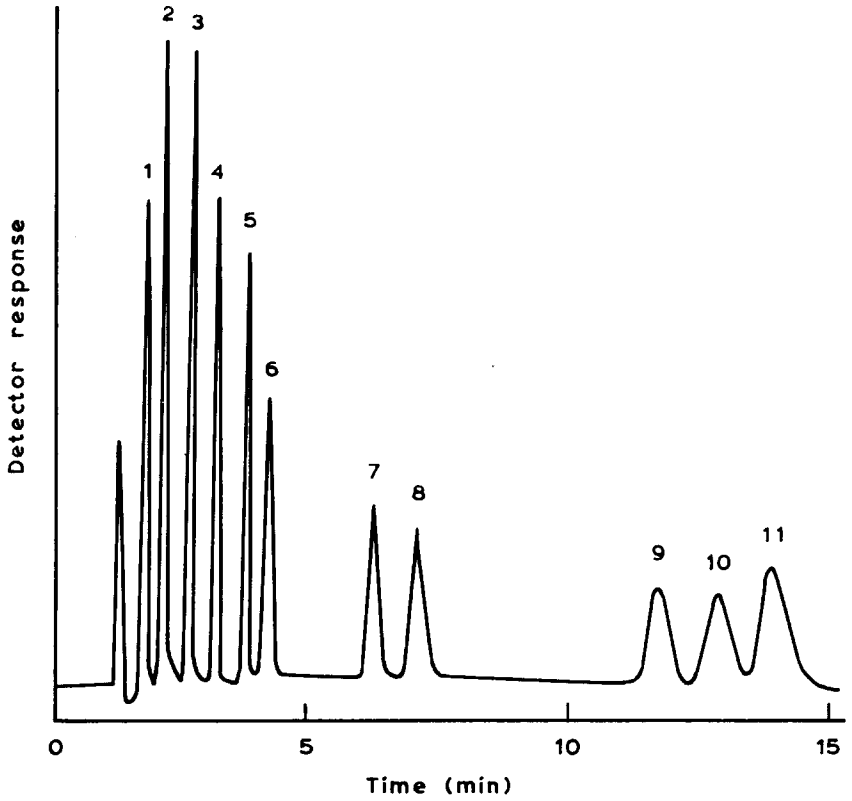

Fig. 8. HPLC of sugar standards on a 25 cm × 4 mm i.d. 10 μm Dionex Carbopac PA1 anion exchange column eluted with 150 mM NaOH (36°C) at 1 ml min^{-1}. Detection: pulsed amperometric. Peaks: 1, xylitol; 2, sorbitol; 3, rhamnose; 4, arabinose; 5, glucose; 6, fructose; 7, lactose; 8, sucrose; 9, raffinose; 10, stachyose; 11, maltose. The late elution of maltose is an effect of the raised column temperature. Reprinted from Dionex Corp. (1987), by courtesy Dionex (UK) Ltd.

Sample preparation should include removal of proteins and other hydrophobic material on C18-bonded silica and membrane filtration. Amino acids, which are retained by anion exchange resins and detected electrochemically, can be removed by passage through a guard column packed with a mixed anion/cation exchange resin.

Amino-bonded silica

The elution order of carbohydrates using an aminopropylsilane-bonded silica column packing and an acetonitrile/water mobile phase is monosaccharides and polyhydric alcohols, then disaccharides, and finally the higher oligosaccharides. Amino-bonded columns facilitate the separation of fructose, glucose, sucrose, maltose, lactose, raffinose and stachyose. Xylitol

coelutes with fructose, whilst sorbitol and mannitol coelute with glucose (Vidal-Valverde *et al.*, 1985*b*). Comparative separations of sugar standards on three commercial amino-bonded columns from different suppliers are depicted in Fig. 9. A single amino-bonded column of standard dimensions cannot usually separate glucose from galactose. Two amino columns connected in series can achieve this separation, but lactose elution is unacceptably prolonged (Yang *et al.*, 1981). Tandem columns have also been used to separate sorbitol from glucose in the analysis of apples, pears, plums and cherries (Richmond *et al.*, 1981).

The separation mechanism has been attributed either to partition of the sugars between the water-enriched stationary phase and the mobile phase (Verhaar & Kuster, 1982) or adsorption via hydrogen bonding between the sugar hydroxyl groups and the amino functional groups (D'Amboise *et al.*, 1980). Whatever the mechanism may be, it is normal-phase chromatography as an increased water content of the mobile phase reduces carbohydrate retention, and elution order follows carbohydrate polarity (Abbott, 1980). The acetonitrile:water ratio in the mobile phase lies typically between 80:20 and 75:25 v/v. Increasing the water content of the mobile phase decreases the retention of the sugars.

Amino-bonded silica columns can be operated at ambient temperature and, for the usual columns of 3·9 mm or 4·6 mm internal diameter, at a flow-rate of $2\,ml\,min^{-1}$. Anomeric separation of the reducing sugars is not normally a problem, as the bonded amino group catalyses the mutarotation reaction (Verhaar & Kuster, 1982).

Amino-bonded columns have a rather short lifetime expectancy owing to the gradual stripping off of the aminopropyl ligand bound by the silyl ether. This is due to the hydrolysis of the siloxane bond in an alkaline environment. The siloxane bonds within the structure of the silica substrate undergo a similar hydrolysis, resulting in the gradual dissolution of the silica particle, but this can be avoided by using a silica precolumn (Verhaar & Kuster, 1981*b*). Under acidic conditions, the siloxane bond remains stable and will not release the ligand. However, at low pH the retention is diminished and anomeric separation starts to appear. In practice, the pH of the mobile phase must be kept as low as possible while still being compatible with separation performance; pH 7 seems to be the best compromise (Verzele *et al.*, 1987).

A crosslinked aminopropyl phase, such as Carbohydrate I (Alltech Applied Science Ltd, Carnforth, Lancashire, UK) is more resistant to hydrolysis than the usual non-crosslinked phase and therefore has a longer life expectancy. By anchoring the primary ligand to a number of neighbouring ligands, the hydrolysis reaction does not necessarily result in the loss of the aminopropyl functional group. Instead, the free primary ligand is held in place by its neighbours so that the amino stationary phase remains relatively intact. However, crosslinking shortens the retention, thus

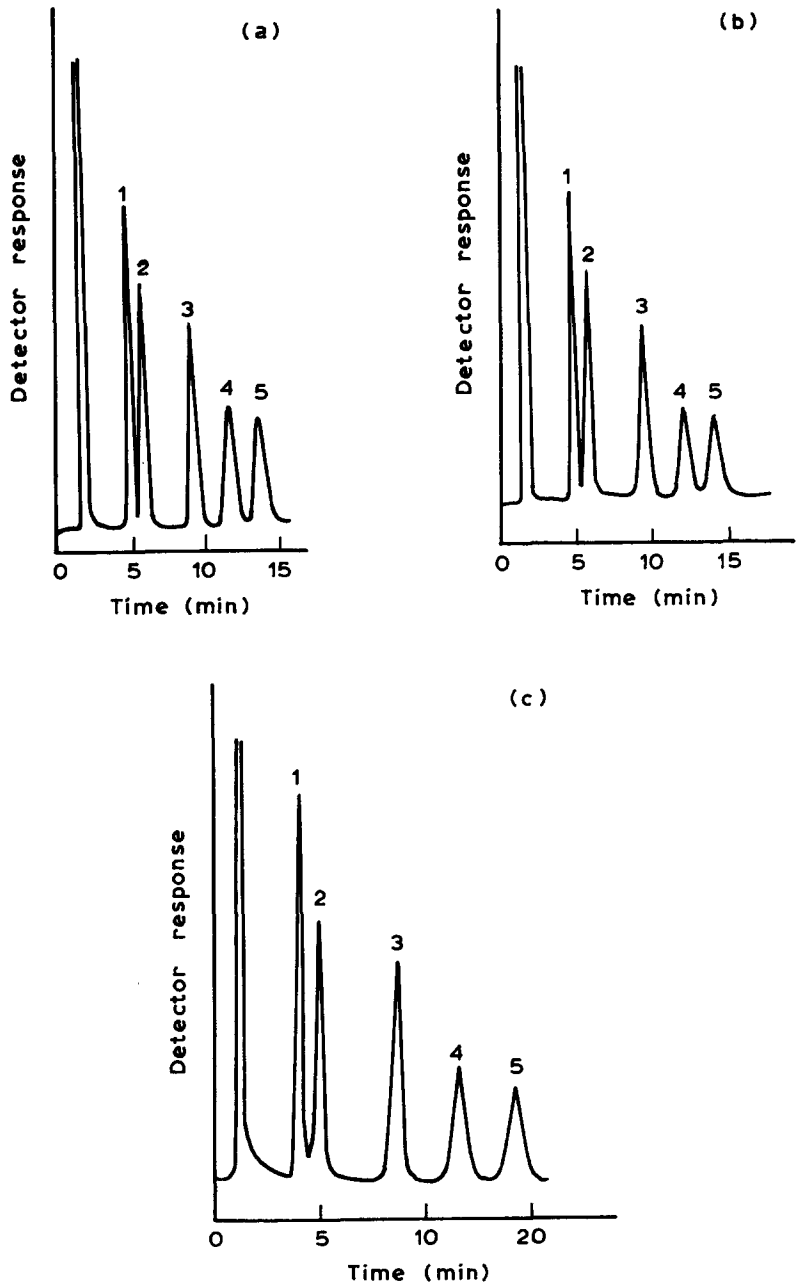

Fig. 9. HPLC of sugar standards on various amino-bonded silica columns eluted with acetonitrile:water (80:20 v/v) at 2 ml min^{-1}. Column temperature: ambient. Detection: RI. Peaks: 1, fructose; 2, glucose; 3, sucrose; 4, maltose; 5, lactose. (a) Waters μ-Bondapak/Carbohydrate column, (b) Merck LiChrosorb NH_2 column, (c) Dupont Zorbax NH_2 column.
Reprinted from Pirisino, 1984, by courtesy of Marcel Dekker, Inc., NY, USA.

the acetonitrile:water ratio of the mobile phase must be increased to 85:15 v/v in order to obtain a similar elution profile to a non-crosslinked phase. The high acetonitrile content of the eluent could cause solubility problems for some carbohydrates.

A reduced column lifetime may also be attributed to the loss of the amine function when it reacts irreversibly with the carbonyl groups of the reducing sugars or other compounds to form Schiff bases. A deteriorating column is characterised by a decreased retention and loss of resolution, especially between fructose and glucose. Although decreased retention can be compensated for by decreasing the water content of the acetonitrile/water mobile phase by 1–2%, the resolution will finally become unacceptable (Pirisino, 1984). The gradual loss of the amino functional groups from the stationary phase can be overcome by using columns packed with plain silica and adding an amine modifier to the mobile phase so that the amino phase is generated continuously (see 'Plain silica').

The deterioration in column performance caused by Schiff base formation is accompanied by a loss of reducing sugars on amino-bonded silica columns. This was demonstrated by Brons and Olieman (1983) who noted the increased loss of galactose and lactose with increasing column temperature, but no loss of the non-reducing sugar, sucrose. The most reactive sugars in the Schiff base reaction are the aldopentoses (e.g. ribose) and galactose. Secondary amines are less reactive towards carbonyl groups than primary amines. Thus Partisil PAC (Whatman Ltd, Maidstone, Kent, UK) columns, which contain cyano and secondary amine groups in a 2:1 ratio in the stationary phase, could be more amenable to sugar chromatography than amino-bonded columns containing a primary amine function (Abbott, 1980).

The presence of salt in the injected sample extract interferes with the HPLC analysis due to the chloride ion eluting closely after glucose. Wills *et al.* (1982) eliminated the interference by adding the ion-pairing reagent tetrabutylammonium phosphate (PIC A, Waters Chromatography Division) to the acetonitrile/water mobile phase in order to increase the retention of the sugars, whilst reducing the retention of chloride.

Applications of amino-bonded columns to the analysis of honey, preserves, beverages, presweetened breakfast cereals, dairy products, chocolate, ice cream, confectionery, cakes, snacks, infant foods, fruits, vegetables, sausage products and soy products are listed in Table 1.

Diol- and polyol-bonded silica

Diol and polyol phases eluted with acetonitrile/water mixtures exhibit similar elution profiles to amino phases, but without the disadvantage of

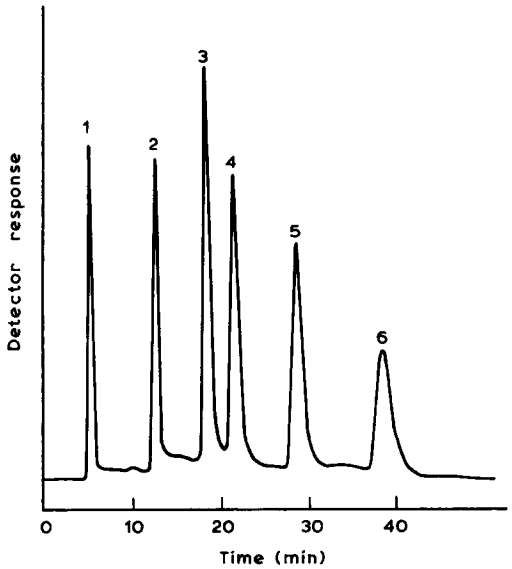

Fig. 10. HPLC of sugar standards on a 25 cm × 4·6 mm i.d. 10 μm RSiL-Polyol column eluted with acetonitrile:water (75:25 v/v) containing 0·075% triethylamine at ambient temperature. Detection: RI. Peaks: 1, solvent; 2, rhamnose; 3, fructose; 4, glucose; 5, sucrose; 6, lactose. Reprinted from Verzele *et al.*, 1987, by courtesy of Friedr. Vieweg & Sohn, Verlagsgesellschaft MBH, Wiesbaden, FRG.

Schiff base formation. With diol phases, the ratio of acetonitrile:water has to be rather high (85:15 v/v) which could lead to solubility problems. With a polyol phase the acetonitrile content is not so high and varying the amount alters the retention according to the needs of the analysis. Figure 10 shows the separation of rhamnose, fructose, glucose, sucrose and lactose on a RSiL-Polyol (Alltech Applied Science Ltd) column eluted with acetonitrile:water (75:25 v/v) containing 0·075% triethylamine to accelerate mutarotation. With a silica precolumn for eluent saturation, the column stability is reported to be very good for a long period of time (Verzele *et al.*, 1987).

Cyclodextrin-bonded silica

Cyclodextrin-bonded silica columns operated with acetonitrile/water mobile phases provide a similar elution sequence to amino- and diol- or polyol-bonded phases; i.e. monosaccharides elute first, followed by di-, tri- and tetrasaccharides. Sugar retention is probably due to adsorption via hydrogen bonding and dipolar interactions and generally increases with an increased acetonitrile content of the mobile phase. Raising the percentage of acetonitrile tends to enhance anomeric separation and *vice versa*. α-

Cyclodextrin columns exhibit a greater retentive power than β-cyclodextrin columns and both appear to be more efficient and selective than cation exchange and amino columns. Cyclodextrin columns exhibit good stability and reproducibility. Armstrong and Jin (1989) reported that a β-cyclodextrin column, approximately one year old and that had received over 3000 injections, was nearly as selective and efficient as a new column.

Plain silica

Unmodified silica column packings operated with acetonitrile/water mobile phases have limited application for the determination of underivatised low molecular weight sugars, because the monosaccharides are eluted rapidly and cannot be adequately resolved from one another. One useful application, however, is the determination of lactose in milk (West & Llorente, 1981).

An alternative and cheaper option to the amino-bonded column is to use a column of plain silica and to generate the amino stationary phase *in situ* by adding a suitable amine modifier to the acetonitrile/water mobile phase. Such dynamically coated amino columns provide similar elution profiles to amino-bonded columns but, according to Verzele *et al.* (1987), sugar retention is stronger, and this allows more water to be used in the mobile phase. Of the various modifiers that have been suggested, piperazine and tetraethylenepentamine (TEPA) are equally effective for the separation of fructose–glucose–sucrose (Verzele *et al.*, 1987). However, TEPA contains two primary amino groups per molecule and its use leads to considerable losses of reducing sugars, presumably due to Schiff base formation (Brons & Olieman, 1983). Piperazine would be the better choice on account of its more rigid structure and absence of a primary amino group. The use of a silica precolumn is recommended to prevent the dissolution of silica in the analytical column caused by the high eluent pH.

Elution profiles that are also similar to those provided by amino-bonded columns can be obtained using a stationary phase of copper-modified silica. Such phases have been prepared in the laboratory by shaking silica with copper–ammonia solution, then rinsing, drying and packing into a column. The presence of sugars in the mobile phase modifies the structure of the cuprammonium complex and shifts the absorption spectrum towards a longer wavelength. Leonard *et al.* (1984) reported such a system using a mobile phase of acetonitrile : water (75 : 25 v/v) containing copper (Cu^{2+}) and ammonia with UV detection at 254 nm. This system seems promising in view of the absence of Schiff base formation and improved detection over refractive index.

Reversed-phases

Reversed-phase chromatography is of limited practical use for determining underivatised low molecular weight sugars because such sugars are only weakly retained on the hydrophobic stationary phase. Certain oligosaccharides can be separated on C_{18} bonded phases using distilled water as the mobile phase, but the monosaccharides are eluted rapidly as a single unresolved peak. Retention can be increased slightly by using a shorter alkyl chain than octadecyl (Verhaar *et al.*, 1984) or by using a C_{18} stationary phase with a low (12%) carbon loading as opposed to a 17–18% loading (Verzele *et al.*, 1987). The addition of a salt to the water eluent also increases retention and improves the selectivity of separation (Wight & Datel, 1986).

Another problem with reversed-phase systems is that anomeric separation of the reducing oligosaccharides can complicate the chromatogram. Lactose readily exhibits peak doubling, while maltose can give peak broadening. Again, the mutarotation process can be accelerated by adding an amine, such as triethylamine, to the water eluent, but this reduces the overall sugar retention (Verzele *et al.*, 1987).

Reversed-phase chromatography has found application in the analysis of soybeans and soybean products in which the major oligosaccharides are sucrose, raffinose and stachyose (Kennedy *et al.*, 1985; Wight & Datel, 1986). Other applications include the determination of invert sugar, sucrose and raffinose in juices and syrups (Palla, 1981) and invert sugar, maltose, sucrose and maltotriose in brewery wort (Verzele *et al.*, 1987). The advantages of reversed-phase packings over amino-modified packings for these analyses are a greater chemical inertness and a longer column life.

Detection systems

Sugars exhibit an absorption maximum between 187 and 188 nm (Binder, 1980) due to the carbonyl groups, but almost all possible contaminants also absorb light at this low wavelength. Absorbance monitoring at below 200 nm therefore demands extensive purification of the injected sample and the use of high purity eluents that have no intrinsic absorbance in this range. Shaw and Wilson (1983) used an amino-bonded column eluted with acetonitrile:water (85:15 v/v) and absorbance monitoring at 190 nm to determine fructose, glucose and sucrose extracted from fruit-juice. Sample clean-up involved cation exchange followed by anion exchange chromatography. The method was sensitive to on-column loadings of 1·2 μg of fructose or 9 μg of glucose or sucrose.

The most commonly used HPLC detector in carbohydrate analysis is the refractive index (RI) detector. Because RI is a bulk physical property, the RI

of the column effluent will respond to the addition of any solute or change in solvent composition. Thus, potentially interfering solutes must either be removed from the sample before injection onto the analytical column or be separated in the chromatogram from the sugars of interest. Gradient elution is not feasible with RI detection, but flow programming has been tried. RI detectors also respond to temperature fluctuations and hence must be thermally insulated to provide stable baselines. In cases where the HPLC column is heated (as in IMP chromatography), extra thermal ballasting is necessary to ensure that the temperature of the eluent in the detector is held constant.

The RI of acetonitrile is higher than that of water ($n_D^{20} = 1{\cdot}3441$ cf. 1·330); hence the detection limits are lower when the eluent is distilled water (as in IMP chromatography) compared with acetonitrile/water mixtures. Modern refractometers, designed to high optical and thermal specifications, can detect on-column loadings of 100 ng of an individual analyte (Scott & Hatina, 1988). With such a detector, concentrations of individual sugars down to 0·1% in the original sample can be reliably determined.

In the mass detector the solvent is removed by evaporation prior to the determination of the non-volatile solutes by light scattering. Thus gradient elution is possible with this instrument (Charlesworth, 1978).

Electrochemical detection, in combination with anion exchange chromatography, enables carbohydrates to be detected by measuring the current caused by their oxidation. Only molecules containing oxidisable functional groups are detected; these include hydroxyl, amine and sulphide groups. In the Dionex system, a pulsed amperometric detector with a gold working electrode is employed to overcome the problem of reaction products accumulating on the electrode surface. A repeating sequence of three applied potentials first oxidises the carbohydrates, then cleans the electrode of the oxidation products by applying a large positive and then a large negative potential. This sequence is repeated at a rate of three times per second. Using a 50 μl injection, the on-column detection limits are approximately 1·5 ng for polyhydric alcohols and monosaccharides and 5 ng for di-, tri- and tetrasaccharides (Rocklin & Pohl, 1983). Non-reducing carbohydrates are detected with the same apparent sensitivity as reducing sugars.

The optimum pH for the electrochemical detection of carbohydrates is 13, although lower pHs can be used. Electrochemical detectors are sensitive to changes in pH below pH 13; therefore, during a pH gradient, in which the eluent concentration of NaOH is < 100 mM, NaOH up to 0·3M must be added post-column to maintain a stable baseline. Little or no baseline drift occurs during ionic strength gradients at constant pH.

Absorbance and fluorescence detection can be employed if the sugars are derivatised using precolumn or postcolumn techniques. The potential

benefits are an increased sensitivity and selectivity compared with refractive index detection and the ability to utilise plain silica or reversed-phase HPLC columns, which have only limited use in separating underivatised sugars. In addition, difficult sugar mixtures can often be separated by means of gradient elution. Depending upon the derivatisation reaction, there are certain disadvantages. Isomeric derivatives may be formed that, if separated, can complicate the chromatogram. If the reaction involves the carbonyl group of the reducing sugar molecule, non-reducing sugars can only be determined indirectly after hydrolysis.

Derivatisation techniques

Precolumn derivatisation of sugars to compounds that contain a strong UV-absorbing chromaphore facilitates absorbance monitoring in the region 230–280 nm. A number of derivatives have been proposed which possess hydrophobic properties allowing normal-phase chromatography on plain silica. These include acetates, benzoates, *p*-nitrobenzoates, *p*-bromobenzoates, and phenyldimethylsilyl derivatives (Honda, 1984). Sugars with free anomeric centres (i.e. reducing sugars) yield multiple peaks corresponding to the α- and β-forms of pyranose and furanose structures and this constitutes a hindrance in routine food analysis applications. Anomeric separation of the reducing sugars can be avoided by forming an acyclic oxime derivative at the carbonyl group but, using an amino-bonded HPLC column, the *syn* and *anti* isomers of fructose and glucose derivatives are separated (Lawson & Russell, 1980).

Fluorescence detection of reducing sugars using dansyl hydrazine as a precolumn reagent has been reported using plain silica (Takeda *et al.*, 1982) and reversed-phase (Mopper & Johnson, 1983) HPLC column packings. Non-reducing sugars do not react with dansyl hydrazine and therefore cannot be detected by this method.

Post-column derivatisation can be carried out automatically using a post-column reactor and reagent supply plumbed in between the column and detector. The reducing sugars in foods were determined by the post-column reduction of tetrazolium blue using an amino-modified silica column and absorbance detection at 530 nm; sucrose was determined indirectly as glucose after hydrolysis by β-fructosidase (Wight & van Niekerk, 1983). Woollard (1983) used post-column reaction with *p*-hydroxybenzoyl hydrazide and absorbance detection at 436 nm to determine glucose, galactose and residual lactose in hydrolysed whey products after separation on a Ca^{2+}-form cation exchange resin column. Vrátný *et al.* (1985) developed a post-column reaction system to determine both reducing and non-reducing sugars

simultaneously. The sugars are first separated on a metal-loaded cation exchange column and then passed through a solid phase catalytic reactor to hydrolyse the non-reducing sugars into their reducing subunits. The sugars are then derivatised with *p*-aminobenzoyl hydrazide and measured photometrically at 410 nm. The post-column reaction of sugars (including sucrose) with cuprammonium reagent and UV detection has been reported on silica columns (Grimble *et al.*, 1983).

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