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Review

Recent progress in carbohydrate separation by high-performance liquid chromatography based on hydrophilic interaction

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Abstract

This article surveys recent developments in the separation and analysis of carbohydrates by high-performance liquid chromatography, in adsorption or partition modes, on polar sorbents with less polar eluents, a technique that is now termed hydrophilic interaction chromatography. A variety of chromatographic methods are included under this generic heading, the most important being adsorption chromatography on silica and partition chromatography on silica-based sorbents bearing bonded polar phases. Examples are given of the applications of these stationary phases, as well as the newer polymer-based polar sorbents, in high-performance liquid chromatography of carbohydrates and their derivatives.

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1. Introduction

The mode of chromatography in which a polar stationary phase is used, with a less polar mobile phase, has long been known as "normal-phase" partition chromatography, as opposed to reversed-phase chromatography which was introduced at a later date. However, as applications of reversed-phase chromatography have proliferated rapidly since the advent of C_{18}-silica stationary phases, the term "normal" no longer seems appropriate when applied to chromatography driven by polar forces, and for this reason the name "hydrophilic interaction chromatography", with the acronym HILIC, was proposed in 1990 by Alpert [1]. This name, which is rapidly gaining currency, is used in the present review as a generic term encompassing adsorption and partition chromatography on silica, either as such or modified by the presence of bonded polar phases (or of a polyfunctional amine in the mobile phase), and partition on macroporous polymers carrying bonded polar phases, which are now being manufactured specifically for this type of chromatography.

Recent developments in chromatographic systems based on HILIC, and their applications in the carbohydrate field, are surveyed in this review. Emphasis has been placed on progress in this area during the last five years; for an overview of earlier developments in the application of this and other HPLC methods to carbohydrate analysis the reader is referred to the author's 1990 review [2] and to comprehensive articles by Hicks [3] and Verzele et al. [4]. More recently Herbreteau [5] has published a critical review of HPLC methodologies used for the analysis of sugar mixtures.

2. Chromatographic systems used in HILIC

2.1. Stationary phases

The stationary phases used in HILIC are by definition polar, and include silica gel (which is particularly suitable for chromatography of polar substances, as its surface is covered with hydrated hydroxyl groups), sorbents in which polar phases are chemically bonded to silica gel, and polymers bearing polar functional groups.

2.1.1. Silica and silica-based sorbents

The silica gel used in HPLC today is the microparticulate, macroporous type, the use of the older pellicular packings being confined mainly to guard columns or extraction columns. The properties of silica gel preparations manufactured specifically for chromatography have been described in detail by Poole and Poole in a recently published book [6]. The introduction of silica packings having average particle diameters of 5 or 3 μm has resulted in greatly increased chromatographic efficiency. Improvements have also come from the increasing use of silica in the form of spherical particles, which form more homogeneous beads than do the irregularly shaped particles produced by some manufacturers.

Although partition chromatography of sugars on unmodified silica has been reported [7,8], this type of chromatography is usually performed on stationary phases in which the silica is modified by chemical bonding of suitable functional groups. Since 1975 bonded aminopropyl silica packings have been widely used in HILIC of sugars and other carbohydrates. However, these sorbents are not ideal for this purpose, as the formation of glycosylamines by interaction of the amino groups on the stationary phase with reducing sugars results in loss of sugars from the analyte and deactivation of the column. There has, therefore, been much research directed at the development and evaluation of alternative bonded-phase packings for HILIC of carbohydrates; these include silica-based sorbents carrying bonded amide, cyano, diol and polyol phases. In 1990 Alpert [1] demonstrated the use of a novel stationary phase, of the type described as polymer-coated or polymer-encapsulated [6], which was prepared by incorporation of ethanolamine into a coating of polysuccinimide, covalently bonded to silica. Since 1989, when their high selectivity for a diversity of carbohydrates was shown by Armstrong and Jin [9], packings in which cyclodextrins are bonded to 5-μm spheri-

cal beads of silica gel have found increasing application in the chromatographic analysis of carbohydrates.

Some examples of silica and silica-based packings that have proved effective in HILIC of carbohydrates are given in Table 1.

2.1.2. Polymer-based sorbents

Silica has a limited solubility (about 100 mg/l) [10] in aqueous solvents of pH 2–7 at room temperature, but solubility increases rapidly at high pH (above 8), owing to the formation of the soluble silicate anion. Therefore, silica-based packings are not suitable for HPLC at pH greater than 8, or under strongly acidic conditions (pH below 2). With mixed aqueous–organic eluents the solubility of silica depends upon the proportion of water and the pH and concentration of any buffer present [6]. Solubility increases with increasing proportion of water, and also at elevated temperatures. For HPLC under conditions that preclude the use of silica-based packings owing to dissolution of the silica, more stable polymeric packings have been introduced. Such polymers are supplied in microparticulate bead form, having an average particle diameter of 5–10 μm; they must be rigid enough to withstand high pressures and flow-rates without deformation and should not shrink or swell with changes of solvent. Macroporous polymers carrying polar functional groups that have been used in HILIC of carbohydrates include highly cross-linked sulphonated polystyrene cation-exchange resins and vinyl polymers. Recently polymers normally used in size exclusion chromatography (SEC) were shown [11] to separate maltodextrins by partition chromatography if the concentration of organic solvent (acetonitrile) in the eluent was raised to above 50% (see Section 3.4).

Table 1
Examples of silica and silica-based packings for HILIC[a]

Packing	Bonded phase	Particle diameter (μm)	Supplier
Silica gel			
Hypersil		3 or 5	Shandon
LiChrospher Si		5 or 10	Merck
Partisil		5 or 10	Whatman
Spherisorb-S		3 or 5	Phase Separations
Zorbax Sil		5	Du Pont
Silica-based			
ERC-NH-1171	Amino	3	Erma (Tokyo)
Hypersil APS	Amino	3 or 5	Shandon
LiChrospher NH_2	Amino	5 or 10	Merck
LiChrospher DIOL	Diol	5 or 10	Merck
Partisil PAC	Amino-cyano	5 or 10	Whatman
Polyol-RSIL	Polyol	5 or 10	Alltech
Supelcosil LC-NH_2	Amino	5	Supelco
Supelcosil LC-DIOL	Diol	5	Supelco
TSK gel Amide-80	Amide	5	Toyo Soda (Tokyo)
Newer packings			
Cyclobond			Advanced
I	β-Cyclodextrin	5	Separation
III	α-Cyclodextrin	5	Technologies
PolyHydroxyethyl A	Hydroxyethyl on polymer coating	5	PolyLC

[a] Inclusion of these products in the table does not imply endorsement by the author or publishers; equivalent packings from other suppliers may be equally effective.

2.2. Mobile phases

The eluting strength of a solvent in HPLC depends upon both its own polarity and that of the sorbent. The common solvents have been arranged in eluotropic series according to polarity index by some authors, while others have adopted as a criterion the solvent strength parameter (ϵ°), which is determined by the capacity factor for a given solute on a specified sorbent of known activity with that solvent as mobile phase. Originally solvent strength parameters were measured with alumina as the sorbent; however, scales of ϵ° values measured on silica are now available [6] for use in modern HPLC. In Table 2 some of the solvents used in HILIC of carbohydrates and their derivatives are listed according to these ϵ° values, which clearly do not always parallel polarity index [12].

For adsorption chromatography of carbohydrate derivatives on silica solvents of low polarity and solvent strength should preponderate in the mobile phase. A common choice is *n*-hexane, with ethyl acetate as a polar modifier [13]. For partition chromatography of carbohydrates on any of the stationary phases used in HILIC, water is generally present in the mobile phase, in proportions of 10–20% (v/v) for chromatography of analytes of low molar mass, 25–50% if the analytes are oligosaccharides of higher molar mass. The organic solvent is usually acetonitrile, but the use of acetone–ethyl acetate–water (5:3:2) in HPLC of sugars on an aminopropyl silica stationary phase has been reported [4] to produce better separation of the disaccharides maltose and lactose than did aqueous acetonitrile. Recently Herbreteau et al. have strongly recommended the use of dichloromethane–methanol solvent systems in HILIC of sugars and alditols on unmodified silica [8] and on silica carrying bonded diol phases [14].

Table 2
Eluotropic series of solvents used in HILIC of carbohydrates

Solvent	Solvent strength parameter[a] [6]	Polarity index [12]
n-Hexane	0.01	0.1
Benzene	0.25	2.7
Chloroform	0.26	4.1
Dichloromethane	0.30	3.1
Ethyl acetate	0.48	4.4
Acetonitrile	0.52	5.8
Acetone	0.53	5.1
Methanol	0.70	5.1
Water	–	10.2

[a] Measured on silica; reference solvent is *n*-pentane ($\epsilon^\circ = 0$).

2.3. Detection systems

The sensitivity of the widely used refractive index detector has been much improved in recent years, the detection limit for sugars having been decreased to below 25 ng [3]. However, these detectors have the major disadvantage that their sensitivity to changes in solvent composition prevents their use with gradient elution. For this reason the evaporative light-scattering detector, which has been strongly recommended by several authors [5,8,14–16], is finding increasing application in HILIC of carbohydrates. The detector is compatible with the solvent systems used in HILIC, detects sugars and alditols with higher sensitivity than does the refractive index detector and with greater stability of baseline, and is not dependent on temperature [16]. Its major advantage is that it can be used when gradient elution is employed to extend the upper limit of resolution of oligosaccharides to a higher degree of polymerisation (dp), or effect difficult separations of sugars and polyols [8], so that this detector is considerably more versatile than the refractive index detector in the analysis of carbohydrates.

Precolumn derivatization to introduce chromophoric or fluorescent groups into carbohydrate molecules is now much used to increase sensitivity of detection in HILIC on silica. Such methods, which are reviewed elsewhere in this issue, include benzoylation, 4-nitrobenzoylation and reaction with the fluorogenic reagent 5-dimethylaminonaphthalene-1-sulphonylhydrazine (dansylhydrazine) [13]. The dansyl derivatives of sugars are detectable at levels in the 3–20 pmol range. More recently Ikemoto et al. [17] have reported detection limits as low as 0.1 pmol

when sugars were derivatized by reduction to alditols followed by reaction with the fluorogenic reagent 2-naphthylimidazole. The resulting alditol pernaphthoates (which included those derived from glucosamine, galactosamine and quinovose, as well as the common monosaccharides) were analysed at sub-picomolar levels by HPLC on a silica column, with fluorimetric detection (excitation wavelength 234 nm, emission 374 nm). UV detection (234 nm) was also sensitive, but detection limits were an order of magnitude higher than those given by fluorimetry.

Postcolumn derivatization with 2-cyanoacetamide, which permits sensitive detection by fluorimetry or UV photometry [18], or electrochemically [19], is compatible with the aqueous acetonitrile eluents commonly used in HILIC of sugars, as is the method involving reactions of reducing sugars with ethylenediamine in a borate buffer at pH 9.0, which also gives products that are both fluorescent and electrochemically oxidisable [20]. Detection methods of this type are reviewed by Honda elsewhere in this issue.

3. Applications of HILIC to carbohydrates

3.1. Adsorption and partition chromatography on silica

HPLC on silica was initially much used in the separation and analysis of derivatized carbohydrates, especially benzoates and dansylhydrazones [13,21], but the method has now been largely superseded by reversed-phase chromatography (see the review by El Rassi in this issue). However, adsorption chromatography on silica packings remains an option favoured by some authors, a notable example being its recently reported use in HILIC of sugars as the pernaphthoates of the derived alditols [17], already mentioned in Section 2.3. Excellent resolution of these derivatives, from an artificial mixture of the nine monosaccharides most commonly encountered in bioconjugates, was obtained by chromatography on a column (250 × 4.6 mm I.D.) packed with 5-μm silica, by isocratic elution for 15 min (at a flow-rate of 1 ml/min) with dichloromethane containing 2-propanol (0.021%, v/v) and acetonitrile (0.6%), followed by a gradient of acetonitrile (0.6% to 3.2% in 15 min) in the dichloromethane-2-propanol mixture. Separation of the derivatives from glucosamine and galactosamine required isocratic elution with the final solvent mixture for a further 10 min.

Silica columns have been widely used in the separation and analysis of glycolipids, usually as their perbenzoylated derivatives [13]. Gottfries et al. [22] described a strategy for the isolation and purification of individual mono- and disialogangliosides that included a step in which the peracetylated derivatives of the gangliosides were separated by HPLC on a column packed with 5-μm silica, with chloroform–methanol (17:3) as the mobile phase. The original compounds were then regenerated by de-O-acetylation in methanolic sodium hydroxide. After further purification by anion-exchange chromatography the individual gangliosides were isolated, without derivatization, by HPLC on the silica column, the monosialogangliosides being eluted with a gradient of chloroform–methanol–water from 65:25:3 to 60:35:8 in 50 min, the disialogangliosides with a separate gradient of 60:30:4.5 to 60:35:8 in 60 min. The advantage of obtaining a separation of the pure compounds without derivatization is that the products are amenable to structural characterization by methods such as fast atom bombardment mass spectrometry (FAB-MS) and enzyme-linked immunosorbent assay (ELISA). The use of sensitive detectors such as the evaporative light-scattering detector in HPLC of glycolipids [23] obviates the necessity for precolumn derivatization. The approach of using HPLC without derivatization, followed by characterization of the products by FAB-MS, in structural studies of gangliosides and glycosphingolipids is rapidly gaining favour, and analyses of nanogram samples by a micro method based on this technique have been reported [24].

Until recently, attempts to separate underivatized sugars and alditols by partition chromatography on unmodified silica gel had achieved only

limited success. Iwata et al. [25] reported on HPLC of a mixture of rhamnose, xylose, arabinose, mannose and glucose on a column packed with microparticulate silica, with ethyl formate–methanol–water (12:3:1) as the mobile phase; the sugars were separated but resolution of the two pentoses was poor. Separation of the disaccharides sucrose, maltose and lactose on the silica column, with ethyl acetate replacing ethyl formate in the eluent, was better and if the proportion of water was increased (ethyl acetate–methanol–water, 7:3:2), good resolution of D-glucose and its α-(1→4)-linked oligomers, dp 2–6, was obtained. Nikolov and Reilly [7], who determined the HPLC capacity factors of several sugars of different molecular structure on both unmodified silica (particle size 5 μm) and the same silica carrying a bonded amino phase, with acetonitrile–water mobile phases of water content ranging from 10% to 40%, concluded that the selectivity of the former stationary phase was lower than that of the latter with a mobile phase having the same water content. This was related to differences in the preferential sorption of water by the two stationary phases, which affect the partition coefficients for the polar sugars.

A recent study by Herbreteau et al. [8] has demonstrated that higher selectivities for sugars are shown by silica if eluents consisting of dichloromethane–methanol (4:1), which contain only a trace (0.2%) of water, are used, thus overcoming the difficulties arising from variation in the degree of hydration of the silica with eluents of different water contents. Under these conditions capacity factors of various unmodified silica packings for sugars and polyols were found to be similar to or higher than those shown by an aminopropyl silica packing in acetonitrile–water (4:1). Good resolution of mono- and disaccharides, with reproducible retention times, was achieved on a column of 5-μm silica eluted isocratically with dichloromethane–methanol–water (80:19.8:0.2). The use of gradient elution permitted complete separation of fructose, glucose, sucrose and raffinose on this column. After isocratic elution with dichloromethane–methanol–water (80:19.8:0.2) for 5 min, the proportions in the solvent mixture were changed to 45:54.8:0.2 in 2 min, and these proportions were maintained during isocratic elution for a further 7 min. The polyols erythritol, ribitol, arabinitol, mannitol and *myo*-inositol were also resolved on the silica column by gradient elution (dichloromethane–methanol–water, 75:24.8:0.2 for 4 min, then changing to 60:39.8:0.2 in 11 min). This high selectivity, coupled with the sensitivity afforded by the evaporative light-scattering detector, makes the use of unmodified silica sorbents under the conditions recommended by Herbreteau et al. [8] an attractive alternative to the conventional procedure using the less stable aminopropyl silica stationary phases for HILIC of sugars and polyols.

3.2. Use of amine-modified silica

Another alternative to the use of aminopropyl silica stationary phases for chromatography of sugars is the in situ modification of silica by addition of a trace (0.01–0.02%, v/v) of a polyfunctional amine to the eluent, and about 0.1% to the solvent used to regenerate the column. This procedure is favoured by many as a relatively inexpensive HILIC system, the columns having a longer life than aminopropyl silica packings because of the continuous regeneration of the amine adsorbed on the surface of the silica. Retention data for a variety of mono- and oligosaccharides (to dp 4) on HPLC with systems of this type, usually with tetraethylenepentamine (TEPA) as the amine modifier in aqueous acetonitrile solvent systems, have been collated [13], as have data for HPLC of higher oligosaccharides (to dp above 20) by this method. In the latter case the diamine 1,4-diaminobutane (putrescin) has proved to be a better modifier than TEPA, giving superior resolution of the higher members of homologous series of oligosaccharides. Praznik et al. [26] recommended the addition of not only 1,4-diaminobutane (0.02%, v/v) but also a small proportion (0.2%) of polyethylene glycol (average molar mass 35 000) to the solvent system; the role of the polyethylene glycol was to decrease the interaction of the amino groups with the hydroxyl groups of the saccharides. Further improvement in res-

olution was effected by operation of the column at 35°C. Under these conditions resolution of the (1→2)-linked D-fructo-oligosaccharide series to dp 30 was achieved within 40 min (at a flow-rate of 1 ml/min) on a standard column (250 × 4 mm I.D.) packed with 5-μm silica. The eluent was acetonitrile–water (1:1), containing the additives mentioned. More recently piperazine has been recommended as an amine modifier for HILIC of both mono- and oligosaccharides on silica [4,27]. Verzele et al. [4] found this amine and TEPA to be equally effective in separation of sugars but expressed a preference for piperazine as, being a solid, it is more easily added in the trace amounts required.

The use of amine-modified silica in HILIC of carbohydrates has some disadvantages, which include variation in retention times, due to fluctuations in the loading of the silica with amine, and unsteady base lines arising from variable delivery of amine from the column into the mobile phase. The claim that these columns are more durable than aminopropyl silica columns has been disputed [3], since amine-modified silica shares with other silica-based packings the tendency to dissolve appreciably in water-rich eluents, especially in the presence of base. Nevertheless, because of their simplicity, relatively low cost and high capacity for carbohydrate analytes, such systems remain a useful option for laboratories concerned only with simple separations of sugars, such as that of fructose, glucose, sucrose and maltose, which is often required in the food industry.

3.3. Partition chromatography on silica-based bonded polar phases

3.3.1. Aminopropyl silica sorbents

The bonded aminopropyl silica sorbents that were the first of this type to be used in HPLC of carbohydrates are still used extensively for this purpose, despite the disadvantage of glycosylamine formation. The optimal conditions for separations of sugars by this method, which were established during the 1970s, remain the generally accepted conditions, namely, the use of an aqueous acetonitrile mobile phase containing 80–85% (v/v) of acetonitrile for separations of mono- and disaccharides, 65–75% for oligosaccharides to dp 5. A detailed study of HPLC of mono-, di- and trisaccharides under these conditions enabled Nikolov and Reilly [7] to relate observed capacity factors to structural features such as the number of hydroxyl groups in the sugar molecule and their orientation, their position in the ring and the conformation of the molecule, all of which affect hydration and the affinity of the sugar for the polar sorbent. Systematic determination of the capacity factors of a chromatographic system of this type for twenty disaccharides [28] and thirteen trisaccharides [29] revealed correlations with the ring forms of the constituent sugar units and the positions of glycosidic linkage. These studies allowed the prediction of separations of sugars by HPLC, from a consideration of their molecular features.

In general, alditols are not well resolved on aminopropyl silica stationary phases. However, in recent work by Vicente et al. [30] on HPLC analysis of the mixtures of sugars and polyols in extracts of lichens and in sugar cane juice some resolution of ribitol and arabinitol, both well separated from ribose, was achieved on such a sorbent, with 80% acetonitrile as the mobile phase. Galactitol, clearly distinguished from ribitol and arabinitol, was identified by this method as a component in an acid hydrolysate of sugar cane juice.

The addition of a phosphate buffer, pH 5.9–6.0, to the mobile phase has been shown [31] to eliminate losses of reducing sugars due to glycosylamine formation in HPLC on a stationary phase carrying a bonded amino group. The presence of the buffer is essential in HPLC analysis of acidic carbohydrates such as ascorbic acid, dehydroascorbic acid and ascorbic acid-2-phosphate, which have been successfully analysed [32] in extracts of apple and potato tissue, treated with the 2-phosphate to prevent browning. The mobile phase used was a 3:1 (v/v) mixture of acetonitrile and 0.05 *M* potassium dihydrogen phosphate. Even hexuronic acids, which are irreversibly adsorbed on an amine column when acetonitrile–water eluents are used, can be analysed by this method if the

mobile phase contains a phosphate buffer high enough in concentration and proportion to prevent acid-base interaction [33]. With a mobile phase consisting of acetonitrile–0.015 *M* sodium dihydrogen phosphate (7:3, v/v), pH 7.0, glucuronic acid and galacturonic acid are eluted from an aminopropyl silica column as symmetrical peaks, not much later than the neutral monosaccharides and sucrose. It has been suggested that the role of the dihydrogen phosphate anion may be not only to adjust the pH of the mobile phase but also to form a complex with the amino groups on the stationary phase. Ion exchange must clearly play a role in the chromatographic separation, in addition to partition chromatography, when acidic compounds are subjected to HPLC on amine columns.

The analysis of mixtures of oligosaccharides having dp above 3 is important in many fields, especially nutrition. A recent example of the use of HILIC on an aminopropyl silica column in this way is the study by Muzquiz et al. [34] of the effect of germination on the levels of oligosaccharides of the raffinose family in lupin seeds, which are considered a potentially important source of protein for animal and human consumption. The presence of these oligosaccharides in high quantities is undesirable as the absence of α-1,6-galactosidase from intestinal mucosa makes their digestion impossible, the oligosaccharides containing $(1\rightarrow6)$-linked α-D-galactopyranosyl residues bound to the D-glucose moiety of sucrose. HPLC analysis of seed extracts, on a 5-μm aminopropyl silica column eluted with 65% acetonitrile, showed the presence of stachyose in high proportion, as well as the pentasaccharide verbascose, raffinose and sucrose. However, analysis of extracts from lupin seeds that had been allowed to germinate for periods ranging from 24 to 120 h, demonstrated a significant reduction in the levels of raffinose and the higher oligosaccharides as germination progressed.

Analysis of maltodextrins is of great importance in the food industry and much research has been devoted to extending the upper limit of the dp range within which these α-$(1\rightarrow4)$-linked D-glucose oligomers can be resolved by HPLC. The detailed study by Koizumi et al. [35] on optimising the conditions for the separation of the D-gluco-oligosaccharides of various homologous series by HPLC on an aminopropyl silica packing based on silica having an average particle diameter of 3 μm led to a separation of the malto-oligosaccharide series to dp 30 by isocratic elution with 57% acetonitrile. More recently, superior resolution of the malto-oligosaccharides in the same range, with baseline separation of the oligomers to dp 17 or higher, has been reported by Niemann et al. [36], who analysed the oligosaccharides in the form of their 4-nitrophenyl glycosides, as produced by phosphorolytic synthesis. This made possible sensitive detection by photometry at 300 nm. With this detection system gradient elution could be used, and excellent resolution was achieved with an aminopropyl silica sorbent (3-μm silica) eluted with a linear gradient (75 to 25% in 60 min) of acetonitrile in water. Better separations were achieved by this method than by the use of reversed-phase chromatography with a water in methanol gradient.

The range of dp over which resolution of the oligosaccharides in a homologous series by HPLC is possible varies with the nature of the monomer and the glycosidic linkage. The linkage type affects the degree of compactness of the molecule as well as the interaction of hydroxyl groups with the polar phase in HILIC. Among the D-gluco-oligosaccharides the β-$(1\rightarrow2)$-linked series obtained on partial acid hydrolysis of cyclosophoraose were resolved by Koizumi et al. [35] to dp 35 on a 3-μm aminopropyl silica packing by isocratic elution with 58% acetonitrile, whereas those that were $(1\rightarrow6)$-linked (α- or β-) were resolvable to dp 26 with 55–56% acetonitrile. The β-$(1\rightarrow3)$-linked series obtained by partial acetolysis of curdlan was resolved only to dp 18 (with 60% acetonitrile), but this lower limit was imposed mainly by restricted solubility. The even less soluble β-$(1\rightarrow4)$-linked cellodextrin series, produced by acetolysis of cellulose, could be separated only up to dp 10.

The use of a 3-μm aminopropyl silica stationary phase, eluted with 57–58% acetonitrile, has also proved effective in HPLC of cyclic D-gluco-oligosaccharides. The cyclosophoraoses isolated from cell cultures of *Agrobacterium* and

Rhizobium strains have been resolved over the dp range 17–40 [37], and cyclodextrins, together with series carrying branches of α-D-glucosyl residues, (1→4)-linked, have been analysed by this HPLC method [38–40], although better resolution has been obtained with different bonded phases (see Section 3.3.2).

The capacity factors shown by hetero-oligosaccharides on HPLC are much influenced by the nature and position of the sugar units present, as well as their dp. From the earliest studies of the behaviour of oligosaccharides derived from glycoproteins, and those occurring in human milk, on HILIC on aminopropylsilica columns it was concluded [41,42] that structural features that affect retention of oligosaccharides having the same dp include the presence of residues of fucose or 2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine), which decrease retention, especially when the latter is at the reducing end, or (1→6)-linked residues, whether in a branch or the main chain, which increase retention. More recently, in studies of the separation of reduced oligosaccharides obtained on alkaline borohydride degradation of glycopeptides from mucins [43], the effects of molecular structure on retention in HILIC on aminopropyl silica columns have been rationalised in terms of the accessibility of hydroxyl groups for hydrophilic interaction with the stationary phase. Separation of glycoprotein-derived oligosaccharides by HPLC in various modes is reviewed elsewhere in this issue.

Brain gangliosides, including highly sialylated members of the series, are well separated by HILIC on aminopropyl silica columns [44,45]. Gradient elution with increasing concentrations and proportions of phosphate buffers (pH 5.5–5.6) is employed. The proportions of phosphate required for elution increase with increasing sialylation in the ganglioside molecule, which indicates that ion exchange plays an important role in the mechanism of this separation.

3.3.2. Polyamine, amide and amino-cyano phases

In the search for an alternative to aminopropyl silica as a stationary phase for HILIC of carbohydrates Koizumi et al. [39] tested a sorbent consisting of a polyamine resin bonded to silica gel, and an amide bonded phase, with carbamoyl groups bonded to the silica. The former (YMC-Pack PA-03; Yamamura, Kyoto, Japan) retained the malto-oligosaccharides longer than did the 3-μm aminopropyl silica column (ERC-NH-1171; Erma, Tokyo, Japan) with which it was compared, and it was necessary to increase the water content of the aqueous acetonitrile eluent to 50% to allow the separation of the larger oligomers in an acceptable time. However, the upper limit of resolution for these oligosaccharides on the polyamine column was 25 and the resolution of the lower members of the series was inferior to that obtained with the ERC-NH-1171 column. The resolving power of the amide column (TSK gel Amide-80; Toyo Soda, Tokyo, Japan) for the maltodextrins was better than that of the polyamine column, but the upper limit (with 53% acetonitrile as the eluent) remained at dp 25 (cf. 30 for the ERC-NH-1171 column). In the same study the three columns were also tested with respect to their selectivity for branched cyclodextrins and in this case it was the polyamine column, eluted with 55% acetonitrile, that gave the best resolution of isomeric members of the series. However, the resolution of branched cyclodextrins on the amide column was greatly enhanced if the acetonitrile content of the mobile phase was increased to 60–62% and the temperature was raised (to 70°C). An important advantage of the amide column is that it is stable at temperatures up to 80°C, whereas the amino and polyamine stationary phases are stable only below 50°C.

Because of its lower stability and inferior resolving power the polyamine column has found little application in HPLC of carbohydrates. However, it does give baseline resolution of fructose, glucose, sucrose and maltose, which has recently been exploited in a method for monitoring the nutrient status of fermentation broths used in the production of antibiotics [46]. After removal of interfering substances by solid-phase extraction, samples from the broths could be rapidly analysed for these sugars by HPLC on the polyamine column, eluted with 75% acetonitrile.

The amide column has been utilised in an

improved method for HPLC analysis of the unsaturated disaccharides obtained on enzymatic degradation of glycosaminoglycans [47], in which nine different disaccharides were resolved on this column, eluted with a mobile phase consisting of acetonitrile, methanol and 0.5 *M* ammonium formate buffer, pH 4.8 (70:5:25). The stationary phase is also finding increasing application in HPLC analysis of glycoprotein-derived oligosaccharides, derivatized by reductive amination with ethyl 4-aminobenzoate [48] for UV detection or the fluorogenic 2-aminopyridine [49]. The use of this method as one of the two complementary HPLC techniques involved in mapping of glycoprotein-derived oligosaccharides [49–53] is reviewed elsewhere in this issue. Mapping by two-dimensional HPLC, on amide and reversed-phase columns, has also been applied to the oligosaccharides released from glycosphingolipids by digestion with endoglycoceramidase; like the glycoprotein oligosaccharides, these can be mapped as either the ethyl 4-aminobenzoate [54] or the 2-aminopyridyl derivatives [55].

Partisil PAC (Whatman), the amino-cyano phase that was one of the first to be introduced as a more stable alternative to aminopropyl silica for HPLC of carbohydrates, has now been largely superseded by the amide stationary phase discussed above. However, this sorbent shares with the amide column the advantage of stability at elevated temperatures, and Partisil-5 PAC, based on 5-μm silica, has recently been effectively utilised in achieving sharp resolution of α-, β and γ-cyclodextrins, glucose and the linear malto-oligosaccharides to dp 7, the column, eluted with 73% acetonitrile, being maintained at 45°C, which prevented peak broadening due to anomeric separation [56]. This HPLC method affords a rapid, reliable method of monitoring the purity of commercial cyclodextrin preparations. The same sorbent has also proved useful in analysis of all the unsaturated disaccharides derived from glycosaminoglycans by chondroitinase digestion [57]. The mobile phase used in this HPLC separation consisted of a 48:14:38 mixture of acetonitrile, methanol and 0.5 *M* Tris-HCl buffer, pH 8.0, to which boric acid (0.1 *M*) and sulphuric acid (23.4 m*M*) were added. The presence of borate was essential to prevent peak broadening and the addition of sulphuric acid optimised the differences in retention among the sulphated oligosaccharides, which were eluted in order of increasing sulphation, indicating that not only partition but also ion exchange contributed to the separation.

3.3.3. Hydroxylic phases

Modification of silica gel by the introduction of chemically bonded diol groups gives a sorbent that functions like aminopropyl silica in HPLC of sugars but is more stable, as glycosylamine formation is eliminated. In an early investigation of the use of diol-modified silica as a stationary phase for HPLC analysis of the sugars present in dairy products, Brons and Olieman [58] found it necessary to operate the column at 40°C and to add a small proportion (0.1%) of the base diisopropylethylamine to the mobile phase, 85% acetonitrile, in order to eliminate peak broadening due to anomeric separation. The presence of base in the mobile phase is, however, not desirable as it promotes the dissolution of the silica support. Lafosse et al. [59] employed gradient elution (85–50% acetonitrile in 20 min) in separating fructose, glucose, sucrose, lactose and raffinose on a column of diol-modified silica (LiChrospher DIOL); baseline resolution of the sugars was achieved, with sharp peaks showing no sign of splitting, as anomeric resolution was diminished in the presence of high proportions of water in the mobile phase. The evaporative light-scattering detector used by these authors [8,14,59] was compatible with the use of gradient elution.

More recently, better separations of sugars and polyols on this stationary phase have been obtained by replacing the aqueous acetonitrile eluent by a dichloromethane–methanol solvent system, containing 75–84% of dichloromethane [14]. Higher selectivity was achieved, and anomeric resolution caused peak broadening only in the case of galactose when the proportion of dichloromethane was 84%. This was again eliminated by the use of gradient elution (83 to 80% dichloromethane in 5 min, then 80 to 70% in 3 min, followed by isocratic elution with the

70% mixture for 10 min), which gave sharp resolution of a mixture of sugars ranging from 2-deoxyribose to raffinose. With a flow-rate of 1 ml/min this separation was complete within 20 min. Some sugars have lower solubility in the less polar dichloromethane–methanol solvent system, but use of the sensitive evaporative light-scattering detector permits detection of sugars at nanogram levels.

Verzele and co-workers [4, 60] tested the performance of a stationary phase in which polyol is bonded to silica (Polyol-RSiL; Alltech) in HILIC of sugars and found it effective in resolving mixtures of mono-, di- and trisaccharides with aqueous acetonitrile mobile phases of acetonitrile content of 68–70%. However, it was necessary to add the base triethylamine (0.1%) to the eluent to prevent peak broadening. For this reason these stationary phases have not found wide application in HPLC of sugars, and are likely to be completely superseded by diol-modified silica used under the conditions described above.

3.3.4. Other polar phases

The polymer-coated stationary phase, poly (2-hydroxyethyl aspartamide)-silica, known as Poly-Hydroxyethyl A (PolyLC, Columbia, MD, USA), that has been developed especially for HILIC, has been shown by Alpert [1] to be capable of resolving reducing sugars. However, these are eluted in doublets due to anomeric splitting, which complicates the chromatogram. As in the case of diol- and polyol-modified silica, this problem can be overcome by addition of a small amount of amine to the aqueous acetonitrile mobile phase, to accelerate mutarotation of the anomers. The polymer-coated silica is less liable to degradation under basic conditions than is the bare silica surface.

Application of PolyHydroxyethyl A to the separation of oligosaccharides was demonstrated by Alpert [1] in a series of chromatograms showing the elution of malto-oligosaccharides, as their 3-hydroxy-2-nitropridinyl β-glycosides, from a column (200 × 4.6 mm I.D.) packed with this sorbent, with aqueous acetonitrile eluents containing different proportions of acetonitrile (55–80%). Resolution of the oligomeric glycosides was optimal with 70% acetonitrile at a flow-rate of 2 ml/min, under which conditions the series was resolved over the dp range 2–13.

The cyclodextrin-bonded silica stationary phases, Cyclobond I and III (Advanced Separation Technologies, Whippany, NJ, USA), which carry β- and α-cyclodextrin, respectively, have been evaluated as sorbents for HPLC of carbohydrates and related molecules by Armstrong and Jin [9]. Lists of capacity factors for HPLC of a wide variety of compounds, including monosaccharides from triose to heptose, deoxy sugars, alditols, di-, tri- and tetrasaccharides and cyclodextrins, on columns (250 × 4.6 mm I.D.) containing these novel packings, with mobile phases consisting of aqueous acetonitrile (80–85%) or acetone (85–90%), indicate the feasibility of many separations not easily achieved by other HPLC methods. Gradient elution further increases the resolving power of the phases: for example, a noteworthy separation of 2-deoxyribose, the pentoses ribose and xylose, the hexoses glucose and talose, the ketose sorbose, the polyol sorbitol (D-glucitol), the disaccharides sucrose, turanose, maltose, lactose and melibiose, the trisaccharides melezitose and maltotriose, the tetrasaccharide stachyose, and α-, β- and γ-cyclodextrins has been achieved [9] by HPLC on Cyclobond I with an acetonitrile gradient (92% for 6 min, 92 to 70% in 30 min, then isocratic at 70% for 20 min) at a flow-rate of 1 ml/min. Good resolution of a mixture of polyols, including glycerol, erythritol, ribitol, arabinitol, sorbitol, galactitol, *myo*-inositol and maltitol, was obtained with the same column, eluted isocratically with acetonitrile–methanol–water (19:1:1).

These studies yielded evidence that the mechanism governing separations of carbohydrates on the cyclodextrin-bonded silica stationary phases is HILIC, involving the hydrophilic exterior of cyclodextrins, rather than the formation of inclusion complexes with the cyclodextrin cavity, which is hydrophobic. The hydrophilic interaction is suggested by a correlation of retention on these columns with the number of available hydroxyl groups per molecule: a deoxy sugar is

eluted before the parent sugar, which in turn is eluted before the related alditol. Furthermore, retention increases with increasing concentration of the organic (less polar) component of the eluent, as expected in HILIC. The hydrophilic interaction of polar solutes with the polar solvated hydroxyl groups around the rim of the cyclodextrins is further supported by the results of a recent study of cyclodextrin-bonded silicas by electron paramagnetic resonance (EPR) with spin probes of varying polarity [61].

In addition to their high selectivity for polar analytes, including carbohydrates, the cyclodextrin-bonded silica sorbents have the further advantage of high efficiency, calculated plate counts for the columns indicating that the α-cyclodextrin phase is more efficient than the β-cyclodextrin phase [9]. The sharp peaks obtained may be attributable to suppression of anomeric resolution under the conditions used (although separation of anomers may be achieved by operation at low temperatures [62]). A further reason for the high efficiency may be steric hindrance by the bulky cyclodextrins, which prevents the polar solute molecules from coming into contact with reactive groups on the silica surface. The kinetics of exchange of polar solutes between stationary and mobile phases may thus be accelerated.

Further applications for cyclodextrin-bonded silica stationary phases in the chromatographic analysis of carbohydrates have emerged from recent studies by Simms et al., who have demonstrated the use of the β-cyclodextrin-bonded phase, Cyclobond I, in the separation of neutral oligosaccharides of various homologous series [63] and of lactobionic acid and its lactone from the parent compound, lactose [64]. As in HPLC on aminopropyl silica sorbents (see Section 3.3.1), the dp range over which resolution of oligosaccharides is possible depends upon both monosaccharide composition and glycosidic linkage [63]. For example, the D-gluco-oligosaccharides of the malto- and isomalto-series can be resolved to dp 25 and 15, respectively, by HPLC on the standard Cyclobond I column with 65% acetonitrile in water as the mobile phase, whereas separation of the β-(1→4)-linked D-xylo-oligosaccharides from xylan, which have shorter retention times, requires a higher concentration of acetonitrile (80%) for resolution of the oligomers having dp 2–8. The inulin-derived fructo-oligosaccharides can be separated to dp 13 with 70% acetonitrile as the mobile phase.

The rapid and complete separation of lactose, lactobionic acid and lactobionolactone by HPLC on Cyclobond I, with a 70:30 mixture of acetonitrile and a sodium phosphate buffer (50 m*M*, pH 5.0) as the mobile phase, that was reported in 1994 [64] gives this system an important application as a means of monitoring the chemical or enzymatic conversion of lactose into its pharmaceutically valuable aldonic acid derivative. It is of interest to note that the behaviour of the cyclodextrin-bonded phase in this separation parallels that of an aminopropyl silica phase, which acts in this case as a weak-base anion exchanger as well as by a HILIC mechanism. The presence of the buffer in the mobile phase is therefore necessary to prevent irreversible adsorption of the acid (see Section 3.3.1).

The cyclodextrin-bonded silicas are very stable and retention times have been found to be reproducible, even after a year of frequent operation of the columns. In general, analysis times on these columns, under conditions of maximum efficiency, are comparable with those for reversed-phase chromatography of carbohydrates, and less than those on aminopropyl silica and ion-exchange phases.

3.4. Partition chromatography on polymer-based sorbents

Polymeric sorbents that are rigid enough for use in HPLC include highly cross-linked polystyrenesulphonate ion-exchange resins, which function in mixed organic–aqueous media as supports for partition chromatography. In this case the separation mechanism is governed by the hydrophilicity of the resin groups and their counter-ions, not by ion exchange. An example of a resin that has proved useful in HILIC of carbohydrates is Shodex RSPak DC-613 (Showa Denko, Tokyo, Japan), which has a very high degree of cross-linking (55% divinylbenzene)

and is available as spherical beads of average particle diameter 6 or 10 μm. This resin, used in the H^+ form, has been applied in a useful HPLC method for the simultaneous analysis of all the monosaccharide constituents of glycoproteins, including hexosamines and sialic acids; the eluent was 92% acetonitrile and the column temperature 30°C [65]. With detection by the sensitive method of postcolumn derivatization with 2-cyanoacetamide (see Section 2.3) this technique permits analysis at nanomolar levels. The same resin, in the sodium or calcium forms, is capable of resolving anomers of sugars when the column is operated at 4°C, with 80% acetonitrile as the mobile phase [66]. The sodium form, with a mobile phase consisting of acetonitrile, methanol and 0.8 *M* ammonium formate, pH 4.5 (13:3:4) and a column temperature of 70°C, has been successfully applied to the analysis of the mixtures of unsaturated disaccharides produced by lyase digestion of glycosaminoglycans [67]. Recently, the use of the resin, in the H^+ form, in the separation of monomers and the lower oligomers obtained on hydrolysis of polymers of sialic acids and of deaminoneuraminic acid has been reported [68]. With a column temperature of 35°C, elution was carried out isocratically with mixtures of acetonitrile (67–75%) and a sodium phosphate buffer (0.02–0.025 *M*), pH 7.4. Separations of the monomers, dimers, trimers and tetramers of the two sialic acids, N-acetyl- and N-glycolylneuraminic acid, and deaminoneuraminic acid (2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid, KDN), of oligomers of KDN, according to the presence or absence of 9-O-acetyl groups, and of the homologous oligosaccharides, to dp 5–7, derived from each of the three polymers, have been achieved by this HPLC method.

In a recent study of the separation of maltodextrins in aqueous acetonitrile by HPLC on three stationary phases used in aqueous size exclusion chromatography, viz. the silica-based phases TSK G2000 SW (Toyo Soda, Tokyo, Japan) and Protein-Pak 60 (Waters, Milford, MA, USA) and the polymeric sorbent G-Oligo-PW (Toso Haas, Philadelphia, PA, USA), Feste and Khan [11] showed that the chromatographic mechanism varied with the acetonitrile content of the mobile phase for all three phases. With eluents containing 0–40% of acetonitrile, the homologous oligosaccharides were separated by SEC, being eluted in order of decreasing dp. However, if the acetonitrile content was 50% or higher, the order of elution was reversed and retention increased as the polarity of the mobile phase decreased. With an eluent containing 65% of acetonitrile, a correlation between retention and dp was found for HPLC of these oligosaccharides on the three different phases. It is evident from these observations that HILIC is the mechanism of separation on such columns with mobile phases of acetonitrile content above 50%. It is of interest to note that this study marks the application to HPLC of the principles originally utilised by Havlicek and Samuelson [69,70], who reported similar reversals in elution order as the proportion of ethanol in the mobile phase was varied in studies of the separation of oligosaccharides of various homologous series on ion-exchange resins in aqueous ethanol.

Polymers manufactured specifically for HPLC include the macroporous cross-linked vinylpyridium polymers prepared by Sugii et al. [71], which have proved effective in HPLC of sugars in aqueous acetonitrile [72,73]. These polymers, used in the phosphate or sulphate forms, are stable at elevated temperatures. For HPLC of sugars the column temperature is usually 70°C and the polymer (average particle diameter 10–15 μm) in the phosphate form; under these conditions elution with 80% acetonitrile gives baseline resolution of the common monosaccharides, and of disaccharides such as maltose and lactose.

Stationary phases in which a polymer replaces silica as the support for the conventional amino-bonded phase have been introduced for use in HILIC. Akiyama [74] has reported excellent resolution of a number of mono- and disaccharides, including N-acetylglucosamine and its β-(1→4)-linked dimer, chitobiose, on a column (250 × 4.5 mm I.D.) packed with an amine-bonded vinyl alcohol copolymer, Asahipak NH2P-50 (Asahi Kasei, Tokyo, Japan). The sugars were derivatized by reductive amination

with ethyl 4-aminobenzoate prior to chromatography. HPLC of the derivatives on the polymer (average particle diameter 5 μm), with 85% acetonitrile as the mobile phase gave resolution of fucose, xylose, N-acetylglucosamine, mannose and glucose, and of maltose, cellobiose and isomaltose that was superior to that obtained on chromatography of the free sugars under the same conditions. Hydrophobic interaction between the aromatic ring in the derivatized sugars and the polymer matrix probably contributes to the total interaction, in addition to the hydrophilic interaction between the polar groups of solute and stationary phase, resulting in the higher selectivity. The relatively early elution of N-acetylglucosamine in this HPLC method is a feature that should facilitate analysis of glycoprotein hydrolysates. The lower members (to dp 5 or 6) of the malto-, cello- and isomalto-oligosaccharide series, similarly derivatized, are also well resolved on this column, with 70% acetonitrile as the mobile phase, and the upper limit of resolution can be extended (to dp 13 for the isomalto-oligosaccharides) by increasing the water content of the mobile phase to 35%. Some resolution of the chito-oligosaccharides (to dp 4) was achieved by Akiyama [74] with this HPLC system, but these oligosaccharides had shorter retention times than their counterparts in the cellodextrin series, which have the same linkage type. The presence of the N-acetyl groups at the 2-position in the chito-oligosaccharides clearly decreases hydrophilic interaction, as in the case of HPLC of glycoprotein-derived oligosaccharides on silica-based polar sorbents (see Section 3.3.1).

A hydroxylated polymeric sorbent, Glyco-Pak N, was used by Bendiak et al. [75] in separating mixtures of neutral oligosaccharides derived from glycoproteins by hydrazinolysis, re-N-acetylation and mild acid hydrolysis. By stepwise elution with aqueous acetonitrile containing increasing proportions of acetonitrile (from 65 to 77%) oligosaccharides containing from 11 sugar residues down to 2, including some having isomeric structures, could be completely separated. The method has been used to advantage in the isolation of these oligosaccharides for subsequent characterization by NMR spectroscopy, which is greatly facilitated by the absence of contaminants from the highly stable sorbent [75,76]. The reproducibility of retention times and the elimination of contamination due to column breakdown are important advantages of the use of polymeric, as opposed to silica-based, stationary phases in applications of this type.

4. Conclusion: summary of main trends

The most important trend in recent advances in HILIC of carbohydrates is evident from Table 3, in which the performance of several of the HPLC systems discussed in this review is compared with respect to their capacity factors for sugars and polyols. The long domination of the aminopropyl silica stationary phase in this application is clearly being challenged by several of the newer phases, which show selectivity for these compounds that is equal to or greater than that of the amino phase. Chief among these are diol-bonded and cyclodextrin-bonded silica, which have the advantage of much greater stability and, in the latter case, higher efficiency than aminopropyl silica. Even unmodified silica can sometimes be used to advantage in separations of sugars if the dichloromethane–methanol mobile phase recommended by Herbreteau et al. [8] is employed. This system is likely to supersede amine-modified silica (see Section 3.2) as the method of choice for those seeking a simple HPLC system for separations of sugars. The dichloromethane–methanol mobile phase has also proved effective in HPLC of sugars and polyols on diol-modified silica; thus, the role of aqueous acetonitrile as the mobile phase for HILIC of these compounds is now being challenged.

Aminopropyl silica of small average particle diameter (3 μm) remains the best stationary phase for HILIC of series of homologous oligosaccharides, as it is capable of resolving some series of oligosaccharides to dp 30 or more (see Section 3.3.1). However, the newer amide-bonded silica phase (see Section 3.3.2) is a strong contender in this application, as its resolv-

Table 3
Retention data for HILIC of sugars and polyols

Compound	Capacity factor (k')[a]					
	P1	P2	P3	P4	P5	P6
Fucose					0.97	0.25
Rhamnose	1.8	1.35	1.78		0.73	0.26
Ribose	1.6	1.12	1.35		0.78	
Xylose	2.2	1.53	2.22		0.96	0.41
Arabinose	2.2	1.82	2.04		1.05	0.46
Mannose	2.9	2.76	3.30		1.34	0.70
Galactose	3.7[b]	3.53	3.46[b]		1.65	0.90
Glucose	4.0	3.29	3.78		1.55	0.82
Fructose	2.8	2.35	1.62		1.25	
Sorbose	3.0	2.29	2.08		1.29	
N-Acetylglucosamine						0.54
Chitobiose						1.69
Sucrose				2.08	2.71	
Maltose				2.52	3.33	2.24
Cellobiose				2.48	3.30	2.61
Isomaltose						2.24
Gentiobiose					4.12	3.11
Trehalose				4.40		
Lactose				2.44	3.84	2.39
Melibiose				2.92	4.51	2.80
Raffinose				3.92	7.19	
Polyols						
Erythritol	2.5	1.18	1.17		0.94	
Xylitol	4.8	3.00	1.48	1.00	1.33	
Mannitol	5.7	3.12	2.42	1.52	1.76	
Sorbitol				1.32	1.75	
myo-Inositol				3.00	3.10	

[a] P1 = Zorbax Sil, 5 μm (250 × 4.6 mm I.D.); dichloromethane–methanol–water (80:19.8:0.2), 1.5 ml/min [8]. P2 = aminopropyl silica, 10 μm (Waters Carbohydrate Analysis Column, 300 × 3.9 mm I.D.); acetonitrile–water (80:20), 1 ml/min [14]. P3 = LiChrospher 100 DIOL, 5 μm (250 × 4 mm I.D.); dichloromethane–methanol (84:16), 1 ml/min [14]. P4 = LiChrospher 100 DIOL, 5 μm (250 × 4 mm I.D.); dichloromethane–methanol (75:25), 1 ml/min [14]. P5 = Cyclobond I, β-cyclodextrin bonded to silica, 5 μm (250 × 4.6 mm I.D.); acetonitrile–water (85:15), 1.5 ml/min [9]. P6 = amine-bonded vinyl alcohol copolymer, 5 μm (Asahipak NH2P-50, 250 × 4.6 mm I.D.); acetonitrile–water (85:15), 0.5 ml/min; sugars derivatized by reductive amination with ethyl 4-aminobenzoate [74].
[b] Some anomeric resolution.

ing power is only slightly inferior to that of the aminopropyl silica phase and it is more stable, even at elevated temperatures. This stationary phase is now widely used in HPLC of oligosaccharides derived from glycoconjugates.

Polymeric sorbents are finding increasing application in HILIC of carbohydrates. These are, in general, more stable than silica-based sorbents, which is clearly advantageous in preparative chromatography of oligosaccharides for subsequent characterization by spectroscopic methods (see Section 3.4). A stationary phase consisting of a vinyl alcohol copolymer carrying amino groups is another that has proved effective in HILIC of sugars (see Table 3). Precolumn derivatization of the sugars by reductive amination with ethyl 4-aminobenzoate greatly enhances selectivity on this column [74], as well as increasing the sensitivity of detection by the introduction of a chromophoric group. This HPLC

method, which is also applicable to the resolution of homologous oligosaccharides if mobile phases of higher water content are used, is potentially one of the most useful of the novel techniques recently developed for HILIC of carbohydrates.

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