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REVIEW

RECENT DEVELOPMENTS IN THE CHROMATOGRAPHIC ANALYSIS OF CARBOHYDRATES

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1. INTRODUCTION

The analysis of mixtures of carbohydrates is important in many diverse fields such as biology, biochemistry, medicine, pharmacy and various industries, especially that concerned with the preparation and stabilization of food, in addition to the crucial role played in synthetic and structural carbohydrate chemistry *per se*. Many problems arise from the necessity to distinguish between small molecules solely on the basis of differences in stereochemistry or mode of linkage, or homologous oligosaccharides differing in chain length by a single sugar unit. Chromatographic methods have long been recognized as a vital part of such analyses. The necessity to isolate each sugar or methylated derivative by laborious, time-consuming procedures such as

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adsorption chromatography on charcoal–Celite or partition chromatography on cellulose columns (for a review, see ref. 1) was obviated, for analytical if not for preparitive purposes, by the development 25 years ago of gas–liquid chromatographic (GLC) methods for analysis of sugars after conversion to suitably volatile derivatives, such as alditol acetates[2,3], trimethylsilyl ethers[4] or, for methylated sugars, methyl glycosides[5,6]. However, the full potential of GLC analysis of carbohydrates has been realized only since the advent of capillary columns[7] and coupled GLC–mass spectrometry (GLC–MS)[8]. The first applications of high-performance liquid chromatography (HPLC) to the analysis of sugars were reported in 1975 (bonded amine phases[9,10] and microparticulate cation-exchange resins in various ionic forms[11]) and in 1979 the power of reversed-phase HPLC to resolve homologous series of oligosaccharides as their peracetylated derivatives was amply demonstrated[12]. In this case also the real value of such methods has become apparent only in more recent years, with the development of highly efficient packings based on silica of very small particle diameter (3–5 μm, compared with the 10 μm that was standard for the earlier packings) and high performance ion-exchange resins (particle diameter 25 μm or less). A survey of the literature of the past 10 years shows that HPLC methods of carbohydrate analysis have proliferated far more rapidly than any other, even GLC. In addition to the growth of the modes of chromatography just mentioned, which were designed for HPLC, much effort has been expended on the adaptation to HPLC of other classical methods such as ion-exchange[13] and gel-permeation[14] (now steric-exclusion) chromatography and the possibilities of adaptation of the newer technique of affinity chromatography on immobilized lectins[15] are currently being explored.

Planar chromatography of carbohydrates has not developed to the same extent in recent years as have the column methods. In the case of paper chromatography (PC), which has been applied to the qualitative analysis of mixtures of sugars for over 40 years[16,17] and remains an invaluable technique, the method was so well established that there was little room for improvement, except perhaps with regard to the sensitivity and specificity of detection reagents[18]. Thin-layer chromatography (TLC), which is now generally preferred to PC, owing to its greater speed and sensitivity, was also an established technique in carbohydrate chemistry by 1976 (by which date some very comprehensive reviews had been published[19,20]), and subsequent improvements have stemmed largely from the use of high-performance TLC plates[21,22] and, more recently, bonded-phase plates[23].

The aim of this review is to summarize and put into perspective the main developments in the chromatographic analysis of carbohydrates during the past decade. These include not only vast improvements in GLC and HPLC techniques, owing to the application of novel methods of derivatization and more efficient or selective columns, but also the use of new techniques, such as supercritical fluid chromatography and ion chromatography with pulsed amperometric detection. As many of these innovations were introduced in papers published in the *Journal of Chromatography*, it seems appropriate to consider their resultant impact upon carbohydrate chemistry and biochemistry in this, the 500th volume of the journal.

2. GAS–LIQUID CHROMATOGRAPHY

2.1. Developments in methods existing before 1980

GLC analysis of mixtures of sugars or methylated sugars as their alditol acetate derivatives has remained the most widely used method. This is due to the simplicity of the resulting chromatograms, as the production of multiple peaks by each sugar, owing to the presence of α- and β-anomers and pyranoside and furanoside rings, is eliminated by the opening of the sugar rings by borohydride reduction. The wealth of mass spectral data accumulated also adds to the value of these derivatives. Recent improvements include changes in the standard method of derivatization with a view to greater speed and quantitative recovery of the products.

Blakeney *et al.*[24] recommend reduction of monosaccharides, dissolved in 1 *M* ammonia, with sodium borohydride in anhydrous dimethyl sulphoxide, rather than water, increase in the rate of reduction by use of a high concentration of sodium borohydride (20 mg/ml) and elevated temperature (40°C), and the addition of 1-methylimidazole, instead of pyridine or sodium acetate, to serve as a catalyst in acetylation with acetic anhydride. This results in complete reduction to alditols within 90 min and acetylation in 10 min at room temperature, the necessity to remove the borate formed on decomposition of excess borohydride by acidification with acetic acid being eliminated by the use of the new conditions for acetylation. In addition to the advantage of speed, selective losses of volatile alditols, resulting from the repeated evaporation with methanol required to remove borate, are obviated in this method. For derivatization of methylated sugars, the same workers[25] advocate reduction of the dry sample with 0.5 *M* sodium borohydride in 2 *M* ammonia at 60°C for 1 h (reduction in dimethyl sulphoxide being incomplete in this instance). To permit acetylation without removal of borate, acid- rather than base-catalysed acetylation is necessary, perchloric acid being used as the catalyst, with acetic anhydride–ethyl acetate, and 1-methylimidazole is then added to catalyse the conversion of excess acetic anhydride to acetic acid. This entire process is very rapid, acetylation requiring only 5 min at room temperature and the reaction with 1-methylimidazole 5 min after cooling on ice. Each of these derivatization procedures[24,25] can be carried out in a single vessel and losses are thereby minimized.

Further significant improvements in the GLC analysis of alditol acetates that have been achieved by the Australian group cited above have resulted from their use of very efficient capillary columns in GLC, a fused-silica column with the highly polar phase OV-275 bonded (BP-75) or a Silar 10C glass capillary column giving excellent resolution of both peracetylated alditols[24,26,27] and partially methylated alditol acetates[25,28]. GLC of the latter on a wall-coated SP-2100 fused-silica capillary column has also been reported by this group[29], some differences in order of elution from that observed with the other, more polar phases providing useful evidence to aid in the identification of partially methylated alditol acetates analysed on both types of column (see Table 1). This multiple-column approach has been recommended also by Lomax and co-workers[30,31], who used GLC on three different capillary columns differing in polarity, *viz.*, a glass[30] or fused-silica[31] column wall-coated with SP-1000, a fused-silica column wall-coated with CP-Sil88, and another with OV-1 bonded (DB-1). Using a database of retention coefficients obtained for a wide range of partially methylated alditol acetates on each of the three phases, these workers pro-

TABLE 1

RELATIVE RETENTION TIMES OF SOME PARTIALLY METHYLATED ALDITOL ACETATES ON PHASES OF DIFFERENT POLARITY

Parent O-methyl sugar	*Relative retention time*[a]	
	BP-75[b]	*SP-2100*[c]
Rhamnose:		
3-O-Methyl	1.22	1.12
2,3-Di-O-methyl	0.96	0.91
2,4-Di-O-methyl	0.96	0.93
3,4-Di-O-methyl	0.94	0.91
2,3,4-Tri-O-methyl	0.67	0.69
Fucose:		
3-O-Methyl	1.26	1.15
4-O-Methyl	1.25	1.15
2,3-Di-O-methyl	1.04	0.93
2,4-Di-O-methyl	1.01	0.96
2,3,4-Tri-O-methyl	0.78	0.75
Arabinose:		
2-O-Methyl	1.33	1.09
4-O-Methyl	1.36	1.10
2,3-Di-O-methyl	1.09	0.88
2,5-Di-O-methyl	1.01	0.81
3,5-Di-O-methyl	0.94	0.80
2,3,4-Tri-O-methyl	0.81	0.68
2,3,5-Tri-O-methyl	0.70	0.60
Xylose:		
3-O-Methyl	1.43	1.12
4-O-Methyl	1.44	1.11
2,3-Di-O-methyl	1.18	0.89
2,4-Di-O-methyl	1.12	0.87
2,3,4-Tri-O-methyl	0.86	0.66
Mannose:		
6-O-Methyl	1.56	1.60
2,3-Di-O-methyl	1.56	1.56
2,6-Di-O-methyl	1.44	1.43
4,6-Di-O-methyl	1.42	1.46
2,3,6-Tri-O-methyl	1.27	1.26
2,4,6-Tri-O-methyl	1.23	1.28
3,4,6-Tri-O-methyl	1.22	1.24
2,3,4,6-Tetra-O-methyl	0.95	1.01
Galactose:		
2-O-Methyl	1.78	1.78
6-O-Methyl	1.62	1.63
2,3-Di-O-methyl	1.66	1.61
2,4-Di-O-methyl	1.68	1.68
3,4-Di-O-methyl	1.72	1.67
2,3,4-Tri-O-methyl	1.44	1.42
2,3,6-Tri-O-methyl	1.33	1.26
2,4,6-Tri-O-methyl	1.28	1.30
2,3,4,6-Tetra-O-methyl	1.06	1.02
Glucose:		
2-O-Methyl	1.78	1.76
3-O-Methyl	1.83	1.82
4-O-Methyl	1.90	1.85

TABLE I (*continued*)

Parent O-methyl sugar	*Relative retention time*[a]	
	BP-75[b]	*SP-2100*[c]
6-O-Methyl	1.46	1.62
2,3-Di-O-methyl	1.64	1.58
2,4-Di-O-methyl	1.60	1.60
2,6-Di-O-methyl	1.51	1.45
2,3,4-Tri-O-methyl	1.34	1.33
2,3,6-Tri-O-methyl	1.36	1.28
2,4,6-Tri-O-methyl	1.25	1.24
3,4,6-Tri-O-methyl	1.26	1.23
2,3,4,6-Tetra-O-methyl	1.00	1.00

[a] Relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol; these values were calculated from published retention times relative to *myo*-inositol hexaacetate[28,29].

[b] Fused-silica column, 25 m × 0.22 mm I.D., with OV-275 (highly polar) as bonded phase; carrier gas, He; flow-rate, 0.78 ml/min; temperature programme, 150 to 250°C at 4°C/min, isothermal at 250°C for 10 min.

[c] Fused-silica column, 15 m × 0.24 mm I.D., wall-coated with SP-2100 (low polarity), film thickness 0.25 μm; carrier gas, He; flow-rate, 0.78 ml/min; temperature programme, 120 to 200°C at 2°C/min.

grammed a computer to process the large amounts of data produced on GLC of complex samples on these columns, all of the results being merged to assist in identification in difficult cases[31].

Confirmation of identification from retention times was obtained from GLC–MS in all the studies cited above, the BP-75 column or that wall-coated with SP-2100 being interfaced with the ion source of the mass spectrometer used by the Australian group[25,28,29], and the SP-1000 glass capillary column being employed in GLC–MS by the Lomax group[30]. A large database for GLC–MS of partially methylated alditol acetates has resulted not only from the well known, pioneering work of the Lindberg group in Stockholm[32], but also from an extremely comprehensive study undertaken by Klok *et al.*[33], who systematically synthesized all possible methyl ethers of the common pentitols, hexitols and 6-deoxyhexitols by partial methylation of each alditol, using the Haworth method under carefully controlled conditions. The acetylated products were examined by GLC on a glass capillary column coated with OV-275 and by GLC–MS in which this column was coupled to a mass spectrometer operating in both electron impact (EI) and chemical ionization (CI) modes, isobutane being used as reagent gas in the latter, which was used to discriminate between groups of derivatives differing in degree of methyl substitution. The paper cited, which contains a complete list of retention times of the numerous partially methylated alditol acetates synthesized and m/z values for the M + 1, M + 1 − 32 and M + 1 − 60 ions predominating in their CI mass spectra, with some useful suggestions for using deuterium labelling introduced by sodium borodeuteride reduction of aldoses to distinguish between pairs of components with similar EI mass spectra, must be regarded as a classical contribution to the field.

Another important advance has resulted from the work of the Albersheim group, who scaled down the methylation analysis procedure to permit complete anal-

ysis of less than 10 μg of complex carbohydrates[34]. Multiple selected-ion monitoring (MSIM) is used in capillary GLC–MS of the deuterium-labelled partially methylated alditol acetates obtained by sodium borodeuteride reduction, followed by acetylation, of the hydrolysate from the per-O-methylated carbohydrate, usually pre-reduced with sodium borodeuteride to form the oligosaccharide– or polysaccharide-alditol. If uronic acid residues are present, the carboxylate groups in the methyl-esterified product of methylation can be reduced with sodium borodeuteride in 95% ethanol–oxolane (27:73) to the corresponding 6,6-dideuteriohexosyl residues[34,35]; reduction with lithium aluminium deuteride[36] is also widely used. Diagnostic fragment ions for EI-MS of the deuterium-labelled, partially methylated alditol acetates derived from hexosyl and 6,6-dideuteriohexosyl residues in all possible glycosidic linkages have been listed by Waeghe *et al.*[34], who reported methylation analysis of samples as small as 5 μg by GLC–MS using these ions in MSIM, which is about seven times more sensitive than standard GC-MS.

The MSIM technique has also proved useful in GLC–MS of hydrolysates from oligosaccharides derived from glycoproteins. For example, 2-acetamido-2-deoxy-D-galactose (GalNAc) residues occurring in the oligosaccharide chain can be distinguished from 2-acetamido-2-deoxygalactitol (produced at the end of the chain by alkaline borohydride hydrolysis of the linkage through GalNAc to Ser or Thr) by sodium borodeuteride reduction and acetylation of the hydrolysate, followed by GLC–MS. MSIM, using the pairs *m/z* 84–85, 102–103 and 144–145, differentiates between the GalNAc originally in the chain, which is deuterium-labelled, and that already present as GalNAc-ol, which is not[37]. In analysis of polysaccharides from bacterial cell walls[38,39] by GLC–MS of the derived alditol acetates, sensitivity of detection of the late-eluting components 2-amino-2-deoxy-D-glucose and muramic acid is enhanced by monitoring the ions of *m/z* 318 and 403; these components are thus distinguished from one another and from amino acid constituents of the cell wall (derivatized by the butyl heptafluorobutyl procedure).

The very long retention times of alditol acetates derived from aminodeoxy- and acetamidodeoxyhexoses[40–42] must be regarded as a disadvantage of this GLC method. The problem can be overcome by nitrous acid deamination of the amino sugars[27,43], which yields 2,5-anhydromannose and -talose from GlcN and GalN, respectively, and D-glucose from ManN, or selective methylation of the amino groups by reaction of the aminodeoxyalditols with formaldehyde and sodium cyanoborohydride prior to acetylation, which gives alditol acetates eluting between the pentitol and the hexitol acetates on GLC on an EGSS-X packed column[44].

Notwithstanding the great success of the alditol acetate method in qualitative analyses of mixtures of sugars and methylated sugars, a *caveat* must be expressed with regard to quantification of the results. In analyses of sugars as peracetylated alditols the response factors, used to correct the peak areas for differences in the response of the flame-ionization detector to alditol acetates derived from different sugars, are far from reproducible, especially when a capillary column is used, and should therefore be re-determined at intervals during a series of analyses. It is essential to include an internal standard (*myo*-inositol hexaacetate is often used) in both the samples to be analysed and the standard mixture used in calibration. There is also some doubt about the precision and accuracy of quantitative methylation analysis by the alditol acetate method. The molar response factors of partially methylated alditol acetates

are sometimes assumed to be equal, but this has been refuted by Sweet *et al.*[45], and the "effective carbon response factors' calculated by these workers are widely used to correct peak areas.

Better reproducibility has been reported for methylation analyses performed with the methylated sugars present as their methyl glycosides, with GLC on a packed column containing ethylene glycol succinate polyester[6]. This method offers the further advantage of direct determination of uronic acid[46] and neuraminic acid[47] residues as the methyl glycoside methyl esters and, although the complication of multiple peaks arises, the pattern of these peaks can aid identification of methylated sugars and may thus be turned to advantage if only a few components are present.

Another disadvantage of the alditol acetate method is the formation of the same alditol by two different sugars in some cases, *e.g.*, arabinose and lyxose, 2- and 4-O-methyl-D-xylose (although this can be obviated by deuterium labelling). For this reason, an alternative method of eliminating the anomeric centres in sugars by conversion to aldononitrile acetates, in which such symmetry is impossible, is preferred by some workers for GLC analysis of both sugars[48,49] and methylated sugars [50–54]. Seymour and co-workers have published detailed information on the mass spectrometry of peracetylated aldononitriles[49], both EI and CI (with ammonia and methane as reagent gases) being used, and EI mass spectral data for GLC–MS of acetylated aldononitriles from methyl ethers of D-mannose[50], D-glucose[51,52] and D-galactose[53] are available. The method has been applied to methylation analyses of dextrans[51,54], which were facilitated by the superior resolution of capillary GLC[54]; again, the use of two capillary columns differing in polarity (SP-2100 and Carbowax 20M) gave the best results. Capillary GLC (on a fused-silica column, coated with OV-1) also gave improved resolution of the peracetylated aldononitriles from neutral and aminodeoxy sugars[55]. A high column temperature (260°C) was necessary to avoid delayed elution of the latter and, as in analysis by the alditol acetate method, deamination of these sugars prior to derivatization is recommended[56].

The aldononitrile acetate method has the advantage that derivatization is relatively rapid, the processes of oxime formation by treatment with hydroxylamine hydrochloride and simultaneous dehydration to nitrile and acetylation by acetic anhydride requiring only 20 min each at 60°C when pyridine is used as solvent and catalyst[48,49]. The use of 1-methylimidazole instead of pyridine decreases the time of each step to 5 min[57], and the reaction is not affected by the presence of water or mineral acid[58].

The validity of the quantitative analysis of mixtures of sugars as peracetylated aldononitriles has been called into question by Furneaux[59], who has identified by-products, N-hydroxy-D-glycosylamine hexaacetates, mainly in the β-furanose form, in appreciable proportion (33–37%) in addition to the nitriles when D-glucose and D-galactose are derivatized by the traditional method, with pyridine as solvent and catalyst. With regard to analysis of methylated sugars, however, Slodki *et al.*[54] have made the point that glycofuranosylamine formation should be blocked in methylated sugars other than those derived from residues linked 1→4 in the original polysaccharide; only a trace (3–5%) of by-product was detected among the aldononitrile acetates derived from permethylated dextrans.

Analysis of oligosaccharides by GLC–MS of their permethylated, reduced derivatives[60–62] has remained an important technique, especially in the examination of

oligosaccharides obtained in degradative structural studies of the carbohydrate moieties of glycoproteins[63], including sialo-oligosaccharides[64]. With packed columns containing Dexsil 300, a carborane with high thermal stability (up to 500°C), GLC–MS data have been obtained for oligosaccharides to tetrasaccharide level (temperature programmed up to 320°C)[64]. Sialo-oligosaccharides were examined only after N-deacetylation and deamination (GLC on an SE-30 packed column at 265°C)[64]; detailed MS data are available for these trisaccharides, which were derived from sialyllactose and -lactosamine with sialic acid linked 2→3 or 2→6 to D-galactose. By N-trifluoroacetylation of 2-acetamido-2-deoxyhexose residues by trifluoroacetolysis under carefully controlled conditions, the volatility of oligosaccharides containing such residues is increased: Nilsson and Zopf[65] have obtained GLC–MS data for oligosaccharides containing up to seven sugar residues by this means, using a fused-silica capillary column wall-coated with methylsilicone (*e.g.*, OV-101), with temperature programming to 350°C.

O-Trifluoroacetylation has also been used to increase the volatility of carbohydrate derivatives to be analysed by GLC; by 1979 this method of derivatization, which has the advantage of making possible the use of the sensitive electron-capture detector[66–68], had been applied to monosaccharides[66,69,70], oligosaccharides (to DP4)[69], methyl glycosides produced on methanolysis of glycoproteins and gangliosides[67,68,70], alditols[67,68,70] and the mono-O-methyl ethers of glucitol[71] and rhamnitol[72]. Use of capillary columns with the electron-capture detector allows the detection of sugars at the picogram level[66], and analyses of glycoprotein samples as small as 0.1 μg by trifluoroacetylation of the methanolysate[67,68]. Even with the ordinary flame-ionization detector, analyses of bacterial lipopolysaccharide samples as small as 0.1 mg have been achieved by the latter method[73], with GLC on a fused-silica capillary column coated with SE-30. The multiple peaks obtained are not considered a disadvantage in such analyses as they serve as a "fingerprint" for each sugar present, aiding identification. However, for analyses of mixtures containing many sugars, reduction to alditols is preferable; good resolution of trifluoroacetylated alditols derived from all of the common sugars has been reported (fused-silica capillary column with cyanopropyl bonded phase, 150°C)[74]. An advantage of the volatility of these derivatives is that aminodeoxy sugars can be analysed as their trifluoroacetylated alditols, without prior deamination, with retention times of less than 10 min on a phase of low polarity (*e.g.*, OV-101 at 120°C)[75]. GLC of oligosaccharides up to the hexasaccharide level is possible by this method if a thermally stable column packing is used (Dexsil 410 in a packed column, programmed to 310°C)[76]. In all instances derivatization with N-methylbis(trifluoroacetamide) is recommended[69,76], since this reagent does not affect the GLC column as does trifluoroacetic anhydride.

Because of the speed and relative simplicity of the derivatization process, trimethylsilyation[4] has been widely used in GLC analysis of diverse carbohydrates. Monosaccharides (including aminodeoxy sugars, neuraminic acids and uronic acids), oligosaccharides to DP4, glycosides (especially methyl glycosides produced on methanolysis of complex carbohydrates), partially methylated sugars, methyl glycosides and alditols have been analysed by this method, which is effective also in the analysis of cyclitols (but not alditols), reduced oligosaccharides, aldonic and aldaric acids and their lactones, and oximes. The extensive application of this technique during the period 1963–79 has been reviewed[77,78]. The more recent literature dealing with this

well established method has been concerned mainly with improvements in resolution resulting from the replacement of packed columns by capillary columns. Trimethylsilylated pentitols and hexitols remain poorly resolved, even on a 25-m fused-silica capillary column[79], but an interesting consequence of the capillary GLC–MS of human urine by this method (30-m glass capillary, OV-101) was the identification of many uncommon deoxyalditols, including 4-deoxyerythritol and -threitol, 5-deoxyxylitol and -arabinitol and 6-deoxyallitol and -gulitol[80,81]. The greatly enhanced sensitivity of capillary GC (25-m fused-silica capillary CP-Si15) allows the determination of subnanomolar amounts of carbohydrates in the trimethylsilylated methanolysates of glycoproteins[82]. This method is also recommended for the simultaneous determination of neutral sugars and uronic acids in small samples; *e.g.* gums of industrial importance, such as gums arabic, karaya and tragacanth, and xanthans, have been analysed very rapidly and efficiently as the trimethylsilylated methyl glycosides on a 30-m fused-silica bonded-phase (DB-5) capillary column[83]. Here again the pattern of multiple peaks given by each sugar is characteristic and facilitates identification.

GLC traces of sugars as trimethylsilyated oximes or O-methyloximes are simpler, each sugar giving rise to only two peaks (not always resolved) corresponding to geometric isomers. These derivatives give distinctive mass spectra[84,85], and the method is applicable to aldoses and ketoses, from trioses and tetroses[86] to heptoses[84], and to disaccharides[87,88]. Excellent resolution of C_3- and C_4-aldoses and -ketoses as their trimethylsilylated oximes has been obtained with a 60-m SP-2100 glass capillary column[89]. A 50-m fused-silica column coated with the same phase has been used in GLC-MS analyses of a variety of sugars (pentose, hexose, pentulose, hexulose and heptulose, deoxyhexose, gluconic and glucuronic acid and acetamidodeoxyhexoses) as the trimethylsilylated O-methyloxime derivatives[90], for which *m/z* values of diagnostic ions used in selected-ion monitoring are listed.

2.2. Newer methods of derivatization

In view of the advantages of trimethylsilylated oximes as volatile derivatives for the GLC and GLC–MS of sugars, oximes have been a focus of interest during the past decade, and the GLC behaviour and mass spectrometry of both acetylated and trifluoroacetylated oximes have been studied. As an extension of their earlier investigations of GLC–MS of aldononitrile acetates[49], Seymour *et al.*[91] published GLC retention times (on a packed OV-17 column) and very comprehensive MS data (both EI and ammonia CI modes) for peracetylated oximes derived from ketoses, pentulose to heptulose. Using a capillary column (fused silica, 50 m, OV-1), Guerrant and Moss[55] obtained an excellent resolution of 27 carbohydrates as acetylated O-methyloximes; in addition to pentoses, hexoses, hexuloses, heptuloses and deoxy sugars, the method was succesfully applied to aminodeoxy sugars, 3-deoxy-D-*manno*-2-octulosonic acid (KDO), N-acetylneuraminic (sialic) acid and muramic acid, all of which eluted within 35 min under the conditions used (temperature programmed from 175 to 260°C). This permitted the analysis of the carbohydrates in whole cell walls of bacteria. Neeser and Schweizer[92] obtained similar results for neutral and amino sugars as acetylated O-methyloximes on a fused-silica Carbowax 20M capillary column (25 m), and resolution of these derivatives and acetylated aminodeoxy- and acetamidodeoxyalditols likely to be present in hydrolysates of carbohydrate chains released from glycoproteins by treatment with alkaline borohydride made possible the complete analysis of such hydrolysates in one GLC run[93].

In a series of papers published in 1982, Schweer and co-workers reported GLC retention times, on a 50-m glass capillary column wall-coated with OV-225, of trifluoroacetylated O-methyl- and O-butyloximes derived from pentoses[94,95], hexoses[96], tetroses and tetruloses[95] and pentuloses[97], with some data for the O-2-methyl-2-propyloxime derivatives of pentoses[94] and hexoses[96]. CI (isobutane) mass spectral data for the trifluoroacetates of these O-alkyloximes were listed for the pentoses and hexoses[94,96], which were examined by GLC–MS with selected ion monitoring. The O-butyloxime derivatives were used in analyses, by GLC[97] and GLC–MS[98], of the products of oxidation of pentitols and hexitols with bromine (2- and 3-pentuloses, 2- and 3-hexuloses and 2,5-hexodiuloses) and the complex mixture of sugars (trioses to hexoses, including 2-pentuloses and 3-hexuloses) formed by autocatalytic condensation of formaldehyde[99]. With selected ion monitoring, excellent resolution of the various oximes, and of the geometric isomers of each oxime, was obtained.

The trifluoroacetylated oximes have the advantage of rapid preparation at low temperatures and of requiring low column temperatures for GLC. However, they decompose on metal surfaces and therefore require all-glass equipment[95,97], which is the probable reason for their limited use.

A method for the simultaneous analysis of aldonic acids and alditol acetates, the former being converted to N-alkylaldonamide acetates giving single peaks on GLC, well separated from the latter, has been proposed by Lehrfeld[100–102]. After lactonization, the aldonic acids are treated with a 1-alkylamine in pyridine to form the N-alkylaldonamide, and the product is acetylated. Of several 1-alkylamines tested[102], 1-propylamine was selected by Lehrfeld for this derivatization, but recently 1-hexylamine has been successfully used by others[103]. The method can be applied to alduronic acids[103,104] if they are first converted to aldonic acids by sodium borohydride reduction of the alduronates, the uronic acids being treated with sodium carbonate at 30°C for 45–60 min to destroy any alduronolactone, which would form alditol on borohydride reduction. This step is crucial to the success of the analysis of alduronic acids as N-alkylaldonamide acetates. The inverted aldonic acids are formed, *e.g.*, L-gulonic acid from D-glucuronic acid. Lehrfeld[104] obtained excellent resolution of peracetylated N-propylaldonamides derived from uronic acids, and of alditol acetates, on a wide-bore SP-2330 capillary column in only 20 min (temperature programmed from 200 to 235°C).

A derivatization method that gives a single peak for each sugar is that proposed by Honda *et al.*[105], in which the sugars are first mercaptalated by reaction with ethanethiol in the presence of trifluoroacetic acid and the resulting diethyl dithioacetals are trimethylsilylated. This entire procedure is complete in less than 1 h and GLC of the derivatives on a capillary column (50-m SCOT column, SF-96, 225°C) permits sugars to be determined at the micromole level, including uronic acids and aminodeoxy sugars, although the retention times of the latter are long, necessitating deamination prior to derivatization[106]. Pentoses are well separated from hexoses but the resolution of some isomers within each group is poor, *e.g.*, the ribose and xylose derivatives are not resolved and those from glucose and mannose separate very little. The method was applied succesfully to the analysis of hydrolysates from gum arabic and gum tragacanth, which do not contain these pairs; the simultaneous analysis of neutral sugars and uronic acids is possible by this method[105]. The trimethylsilylated diethyl dithioacetals derived from partially methylated sugars give simple, character-

istic mass spectra, which permit the identification of individual methylated sugars in GLC–MS[107], but the resolution is such that only simple mixtures of methylated sugars can be analysed, and therefore the technique has never gained general acceptance.

In a novel derivatization method developed by Das Neves *et al.*[108], sugar O-methyloximes are reduced with sodium cyanoborohydride to the corresponding deoxy(methoxyamino)alditols. These are resolved by capillary GLC after acetylation or trimethylsilylation, each aldose giving a single peak but each ketose two peaks due to diastereoisomers. Both acetylated and trimethylsilylated derivatives have highly characteristic mass spectra. The resolution of isomeric sugars is better with the acetates, but greatly improved resolution has been reported[109] after permethylation of the deoxy(methoxyamino)alditols, and the permythylated deoxy(methylmethoxyamino) alditol glycosides derived from disaccharides[110] are also amenable to capillary GLC. Mass spectral data for these derivatives are available: the presence of intense diagnostic ions makes possibly highly sensitive, specific selected ion monitoring. An alternative detector is the nitrogen–phosphorus-selective detector. The good resolution and the possibility of sensitive detection suggest that this new method may have great potential for the GLC of complex mixtures of sugars.

Of special interest are chiral derivatives that permit the resolution of enantiomers by GLC. After the success of two groups in distinguishing between enantiomeric sugars by capillary GLC of derivatives of the glycosides formed with (+)-2-octanol[111] or (−)-2-butanol[112], the latter method being applied not only to neutral sugars but also to uronic acids and 2-acetamido-2-deoxy sugars[113], several different chiral derivatives giving simpler chromatograms, with only one or two peaks for each enantiomer, have been employed. Little [114] used (+)-1-phenylethanethiol to convert pairs of sugar enantiomers into diastereoisomeric, acyclic dithioacetals, which could be separated as acetylated or trimethylsilylated derivatives by capillary GLC. Oshima *et al.*[115] achieved resolution of enantiomers by capillary GLC of the trimethylsilyl ethers or acetates of the diastereoisomeric α-methylbenzylaminoalditols formed on reaction of the sugar with L-(−)-α-methylbenzylamine in the presence of sodium cyanoborohydride. Hara *et al.*[116] have recently reported the GLC separation of aldose enantiomers as trimethylsilyl ethers of the methyl 2-(polyhydroxyalkyl) thiazolidine-4-(*R*)-carboxylates obtained by reaction of the sugars with L-cysteine methyl ester. Some retention data for these chiral derivatives, all of which give a single peak for each enantiomer, are given in Table 2. Resolution of enantiomers of the common pentoses, hexoses and deoxyhexoses has also been achieved by capillary GLC of trifluoroacetylated (−)-menthyl- or (−)-bornyloximes[117,118], but the chromatograms are more complicated owing to the presence of two isomers of each oxime.

2.3. Chiral phases

An alternative approach to the resolution of sugar enantiomers by GLC is the use of chiral stationary phases. König and co-workers, the leaders in this field, have published separation factors for enantiomeric pairs of sugars, methyl glycosides and chiral alditols, obtained by capillary GLC of the trifluoroacetylated derivatives at various temperatures from 80 to 140°C or, for glycosides[119,120], with temperature programming over the range 100–145°C or 120–180°C, on two types of stationary

TABLE 2

RESOLUTION OF SUGAR ENANTIOMERS BY GLC OF CHIRAL DERIVATIVES

Sugar	*Enantiomer*	*Relative retention time*[a]			
		(+)-PED, Ac[b]	*(+)-PED, TMS*	*(−)-MBAA, TMS*[c]	*MPTC, TMS*[d]
Arabinose	D	0.56	0.46	0.52	0.67
	L	0.60	0.49	0.53	0.61
Xylose	D	–	–	0.53	0.62
	L	–	–	0.54	0.67
Lyxose	D	0.57	0.47	0.54	0.65
	L	0.61	0.50	0.55	0.63
Ribose	D	–	–	0.55	0.63
	L	–	–	0.56	0.70
Rhamnose	D	0.54	0.62	0.57	0.71
	L	0.57	0.65	0.58	0.68
Fucose	D	0.59	0.67	0.59	0.73
	L	0.57	0.63	0.61	0.81
Mannose	D	0.94	0.98	0.95	1.01
	L	1.00	0.98	0.93	0.98
Glucose	D	1.00	1.00	1.00	1.00
	L	0.92	0.92	0.98	1.07
Galactose	D	1.03	0.98	0.96	1.09
	L	0.97	0.93	1.00	1.22

[a] Relative to D-glucose derivative; calculated from published data.

[b] Bis[(+)-1-phenylethyl] dithioacetal[114]; Ac = acetylated; TMS = trimethylsilylated; both derivatives run on a fused-silica column, 30 m × 0.25 m I.D., coated with SE-54; carrier gas, H_2; flow-rate, 1.5 ml/min; splitting ratio, 1:100; column temperature, 280°C.

[c] 1-(L-(−)-α-methylbenzylamino)-1-deoxyalditol[115], on a fused-silica column, 25 m × 0.2 mm I.D., wall-coated with Carbowax 20; carrier gas, He; flow-rate, 0.7 ml/min; splitting ratio, 1:100; column temperature, 158°C.

[d] Methyl 2-(polyhydroxyalkyl)thiazolidine-4(*R*)-carboxylate[116], on a glass column, 50 m × 0.3 mm I.D., OV-17; carrier gas, He; flow-rate, 0.8 ml/min; splitting ratio, 1:75; column temperature, 200°C.

phase. The first was prepared by saponification of the cyanoethyl groups in the methyl(cyanoethyl)silicone phase XE-60 with alkali and coupling the carboxylic groups thus produced to L-valine-(*S*)-α-phenylethylamide by reaction with dicyclohexylcarbodiimide in chloroform solution[119,120]. The second, currently under investigation, is per-O-pentyl-α-cyclodextrin [hexakis(2,3,6-tri-O-pentyl)cyclomaltohexaose], prepared by reaction of α-cyclodextrin with 1-bromopentane and sodium hydroxide in dimethyl sulphoxide, followed by further pentylation in the presence of sodium hydride in tetrahydrofuran[121]. The pentylated cyclodextrin has been found to exhibit a high degree of enantioselectivity towards trifluoroacetylated aldoses, methyl glycosides, alditols, 1,4- and 1,5-anhydroalditols and polyhydroxy acid methyl esters[122,123],with baseline resolution of enantiomers within 5–10 min. It affords a thermally stable phase for capillary GLC, with no deterioration in column performance after operation at temperatures up to 200°C. As trifluoroacetylated carbohydrate enantiomers are resolved, but not the non-polar permethylated or trimethylsilylated derivatives, specific dipole–dipole interactions are believed to be responsible, rather than inclusion phenoma involving the cyclodextrin matrix[123].

3. SUPERCRITICAL FLUID CHROMATOGRAPHY

In supercritical fluid chromatography (SFC), the mobile phase is a highly compressed gas just above its critical temperature and pressure, so that its viscosity and density are intermediate between those of gas and liquid, thus combining the mobility of the gaseous phase with the solvating power of liquid. This new technique is rapidly gaining acceptance as a separation method for thermally labile and non-volatile materials[124,125]. Elution can be selectively controlled not only by temperature programming as in GLC but also by pressure programming, which varies the density of the mobile phase, very sensitive to pressure in the supercritical region, where the gas laws no longer apply. Carbon dioxide is widely used as the mobile phase in SFC because of its low critical temperature (32°C), chemical inertness and minimal response in most detection systems. As carbon dioxide is non-polar, polar solutes such as carbohydrates require derivatization to enhance miscibility, and to date there have been very few applications of SFC in the carbohydrate field. However, the pioneering work of Chester and Innis[126] and of Reinhold and co-workers[127,128] in applying capillary SFC to the separation of suitably derivatized oligosaccharides and glycoconjugates has demonstrated the potential of the technique, and its use is expected to increase, particularly in combination with CI mass spectrometry, with which it has now been interfaced[127].

The homologous series of α-(1→4)-linked D-glucose oligomers from corn syrup has been examined by SFC as both trimethylsilylated[126,127] and permethylated[127,128] derivatives. Resolution of the trimethylsilylated oligomers to degree of polymerization (DP) 18 has been obtained on fused-silica columns (10 m × 50 μm I.D.) with bonded methylpolysiloxane phases (DB-1 or DB-5) at 90°C, the pressure of the carbon dioxide mobile phase being programmed from 115 to 400 atm over 75 min, while the permethylated series is resolved to DP 15 at 120°C under similar conditions of pressure[128]. Detection is flame ionization detection or, with the column coupled to the chemical ionization chamber (ammonia reagent gas) of a mass spectrometer[127], total ion current, with selected ion monitoring [$(M + NH_4)^+$] of individual oligomers allowing detection down to *ca.* 2 pmol. The α- and β-anomers of the trimethylsilylated maltodextrins are resolved[126,127], and application of SFC, under similar conditions, to glycosphingolipids[128] gives multiple peaks due to alkane heterogeneity. The high resolution and sensitivity possible with SFC, at relatively low temperatures, should prove valuable in studies of glycolipids and glycoproteins.

4. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

The ever-increasing application of HPLC methods to carbohydrate analysis since 1980 is reflected in the number of reviews[129–134] devoted entirely or largely to this topic that have appeared during the period 1981–88. The literature is now so extensive that comprehensive coverage is beyond the scope of the present review; the reader is referred to those cited above, especially the excellent chapter by Hicks[134] and the critical comparison of various HPLC systems given by Verzele *et al.*[133]. The discussion that follows focuses on some of the most striking improvements in carbohydrate analysis that have resulted from the phenomenal development of HPLC during recent years.

4.1. HPLC resolution of higher oligosaccharides on silica-based packings

4.1.1. Normal-phase partition chromatography. Before 1979, the upper limit of resolution for normal-phase HPLC of linear D-gluco-oligosaccharides on silica-based packings with the usual aminopropyl bonded phase was *ca.* 6, although resolution of malto-oligosaccharides to DP 10 had been reported by Rabel *et al.*[135] using Partisil-10 PAC (Whatman), which also contains bonded cyanopropyl groups. These early packings were based on silica of average particle diameter 10 μm. Eluents were acetonitrile–water mixtures containing 65–75% of acetonitrile; Rabel *et al.*[135] adjusted the pH to 5.0 by use of an acetate buffer as the aqueous component. Isocratic elution was essential, owing to the use of the differential refractometer as a detector. The replacement of this by spectrophotometric detection following post-column reaction of the oligosaccharides with tetrazolium blue allowed gradient elution and, using this method, Noël *et al.*[136] were able to distinguish (but with poor resolution) oligomers having DP 2-20 in hydrolysed starch and xylan, which were eluted from Chromosorb-NH_2 (Johns-Manville) within 30 min with a reverse linear gradient (70–62.5%) of acetonitrile in water. Similar fractionation of the fructo-oligosaccharides from hydrolysed inulin was achieved[137] with an acetonitrile gradient of 66–57%.

Using a column of MicroPak AX-5 (Varian), in which the amine phase is bonded to 5-μm silica, and detection by scintillation counting after precolumn labelling of the oligosaccharide with $NaB[^3H]_4$ reduction, Mellis and Baenziger[138] obtained excellent resolution (to the baseline for DP 1–13) of the α-(1→6)-linked D-gluco-oligosaccharides in a dextran hydrolysate. Oligomers to DP 18 were eluted within 1 h with a reverse linear gradient (65–35%) of acetonitrile in water. Complex hetero-oligosaccharides derived from glycoproteins by hydrazinolysis or endoglycosidase digestion were also resolved under these conditions; these included bi-, tri- and tetra-antennary oligosaccharides containing 9–14 sugar residues and high-mannose oligosaccharides with 5–7 D-mannosyl residues[138].

The development of packings based on 3-μm silica has now permitted resolution of linear D-gluco-oligosaccharides to DP 30–35, with isocratic elution allowing the use of refractive index (RI) detection. Outstanding results have been reported by Koizumi *et al.*[139], using a column (200 × 6 mm I.D.) designated ERC-NH-1171 (Erma Optical Works, Tokyo, Japan): malto-oligosaccharides were resolved to DP 30 within 35 min, and the β-(1→2)-linked D-gluco-oligosaccharides obtained on hydrolysis of cyclosophoraose to DP 35 within 45 min, with 57–58% acetonitrile as eluent. D-Gluco-oligosaccharides linkd α- or β-(1→6), from hydrolysed dextran or luteose, respectively, were resolved to DP 26 within 40 min with 55–56% acetonitrile. Upper limits of DP 18 and 10 for the fractionation of β-(1→3)-linked D-gluco-oligosaccharides from hydrolysed curdlan and β-(1→4)-linked cello-oligosaccharides from acetolysis of cellulose, respectively, were due to the restricted solubility of these oligosaccharides in the eluent rather than the inability of the column to resolve higher members of these series.

The Koizumi group[140] have recently applied two novel column packings to the HPLC of malto-oligosaccharides: both YMC-Pack PA-03 (Yamamura Chemical, Kyoto, Japan) and TSK gel Amide-80 (Toyo Soda, Tokyo, Japan) resolved these oligosaccharides to DP 25 in 40 min, with eluents containing 50% and 53% acetonitrile, respectively. The former packing has a polyamine resin bonded to silica gel and the latter carbamoyl instead of amine groups in the bonded phase. These pack-

ings and ERC-NH-1171 were also successfully applied[140] to the HPLC analysis of branched cyclodextrins containing up to thirteen D-glucosyl residues, the best resolution being obtained on YMC-Pack PA-03 with 55% acetonitrile. However, the TSK gel Amide-80 column, which is stable at temperatures up to 80°C, gave improved resolution with higher acetonitrile concentrations (up to 62%) at higher temperatures (50–70°C). The ERC-NH-1171 column resolved cyclosophoraoses [the cyclic β-(1→2)-linked D-gluco-oligosaccharides produced by *Rhizobium* and *Agrobacterium* strains] of DP 17–40 within 50 min by isocratic elution with 57% acetonitrile[141].

Good resolution of complex oligosaccharides, containing up to twelve sugar residues, derived from glycoproteins has been achieved by normal-phase partition chromatography on amino phases bonded to 5-μm silica[142,143], with eluents consisting of acetonitrile (50–80%) and water or phosphate buffer (pH 5.2–5.6). UV detection (at 195–202 nm) permits the use of a buffer gradient in acetonitrile[142]. Similar HPLC methods have been applied to the higher oligosaccharides (lacto-N-hexaose and mono- and difucosylated derivatives) from human milk[144] and to brain gangliosides[145,146]. When acetonitrile is a major component of the eluent, as in the examples cited above, the normal-phase partition mechanism is operative; amino-phase packings can also act as weak anion exchangers in the HPLC of acidic oligosaccharides (see later).

A disadvantage of the use of packings with bonded amino phases in the HPLC of carbohydrates is the tendency for glycosylamine formation between reducing sugars and the amino groups on the stationary phase, which causes a deterioration in column performance on prolonged use[133,134]. An alternative method of performing normal-phase partition chromatography is the use of pure silica gel modified *in situ* by equilibration with a mobile phase containing a polyfunctional amine that is adsorbed, the same amine being added to the chromatographic eluent so that the column is continuously regenerated[147,148]. White *et al.*[149], after testing various di- and polyamine modifiers, achieved satisfactory resolution of malto-oligosaccharides to DP 20 on a column of 5-μm silica equilibrated by passage of 50% aqueous acetonitrile (30 column volumes) containing 0.1% of 1,4-diaminobutane; the eluent contained acetonitrile and water in the same proportion but only 0.01% of the amine modifier. Fructan oligomers of DP 2–30 were resolved by Praznik *et al.*[150] using a similar amine-modified silica column heated to 35°C and with 0.2% of polyethylene glycol 35 000 added to both equilibrating and eluting solvent systems to decrease the interaction of the amino groups of 1,4-diaminobutane with the oligosaccharides.

Amine-modified silica columns have several advantages over those containing amine-bonded silica: exceptionally long life, high stability over a wide range of pH and solvent composition, high capacity for carbohydrate solutes and relatively low cost[129,147–151]. Disadvantages are the difficulty of using UV detection with such systems, owing to the presence of amine in the eluent[129], variability in retention times due to non-reproducibility of the amine loading of the silica, and fluctuating baselines due to variable amine delivery[133]. All silica-based packings share the disadvantage that the silica dissolves to a small extent in water-rich eluents, and amine-modified silica is no exception[134].

4.1.2. Use of octadecylsilica packings. Packings in which a long alkyl chain (usually C_{18}) is bonded to silica are designed for reversed-phase chromatography, in which mode oligosaccharides are resolved only after derivatization. The resolution of

acetylated malto-oligosaccharides to DP 35 on gradient elution (10–70% of acetonitrile in water) from a pellicular C_{18}-silica packing at 65°C that was reported in 1979 by Wells and Lester[12] has never been developed further, probably because of the advent of normal-phase packings capable of such resolution without derivatization of the oligosaccharides. The same applies to the separation of the high-mannose oligosaccharides isolated from the side-chains of ovalbumin that was achieved by the same group[152] using a similar chromatographic system, after $NaB[^3H]_4$ reduction and peracetylation of the oligosaccharides. However, one application of reversed-phase HPLC of derivatized oligosaccharides that has been outstandingly successful and remains an invaluable technique in sequencing of glycosyl residues in complex carbohydrates is its use in fractionating the mixtures of partially methylated, partially ethylated, reduced oligosaccharides obtained in the structural analysis of such carbohydrates using the methodology developed by Albersheim and co-workers[36, 153–155]. The alkylated, reduced oligosaccharides, containing up to six sugar residues and varying in degree of branching, were fractionated by this group on microparticulate C_{18}-silica (Zorbax ODS, DuPont) with aqueous acetonitrile eluents. Interfacing the column with the chemical ionization chamber of a mass spectrometer, the eluent serving as the CI reactant gas[154,155], permitted direct MS detection and partial identification (by their M+1 ions) of the individual alkylated oligosaccharide–alditols, which were then isolated and further examined by GLC coupled with EI-MS. This procedure makes possible the sequencing of complex carbohydrates with samples on the milligram scale.

Octadecylsilica packings have found application in the HPLC of underivatized carbohydrates, especially oligosaccharides, with water or aqueous solutions as eluents; in this instance the mechanism governing separation is not reversed-phase partition but is probably a form of hydrophobic chromatography, involving interaction of the van der Waals type between the oligosaccharides and the bonded C_{18} chains. Early attempts to exploit this effect in the HPLC of malto-oligosaccharides[156,157] were not very successful, owing to the separation of anomers under these conditions, which resulted in double peaks or, at DP 6–8, very broad peaks owing to incomplete resolution of the anomeric forms. The flattened peaks became undetectable at DP above 8. Subsequently several groups have investigated possible means of obtaining sharper, well resolved peaks, so that the method could be applied to oligosaccharides of higher DP. Vrátny *et al.*[158] separated malto-oligosaccharides to DP 10 at 60°C, at which temperature all doublets were eliminated owing to accelerated equilibration between α- and β-anomers, but this was at the expense of resolution of the lower members of the series. Verhaar *et al.*[159] investigated the effects of eluents of low or high pH, which should accelerate mutarotation; the use of 5 m*M* sulphuric acid (pH 2.0) had no effect, whereas elution with 1 m*M* triethylamine in water (pH 10.0) improved the separation of malto-oligosaccharides to DP 6, owing to the elimination of doublets, but those of higher DP were not affected and the improvement was not sufficient to justify risking possible alkali-catalysed degradation of the silica support. The best results were reported by Cheetham and Teng[160] using a Dextropak C_{18} cartridge (Waters Assoc.) which had been coated with a non-ionic detergent, Triton X-100, a solution (0.1%) of this detergent being pumped through the column for about 5 h before use; this gave good resolution of malto-oligosaccharides of DP 3–15, and those of the α-(1→6)-linked isomaltoseries of DP 3–8, eluting as single

peaks. Similar improvement through modification of hydrophobic interaction was achieved[160] by addition of tetramethylurea (0.025%) to the eluent, which gave single, sharp peaks, well resolved, for oligomers of DP 3–13 in the maltoseries and 2–7 in the isomaltoseries.

This form of chromatography is successfully applied to complex oligosaccharides only if no reducing sugars, and therefore no anomeric centres, are present. Dua and co-workers, using a packing based on 5-μm silica (Alltech 605 RP) and water as eluent, applied the method to a series of glycoprotein-derived oligosaccharides, containing 2–7 sugar residues, with the GalNAc residue at the reducing terminal reduced to GalNAc-ol by alkaline borohydride treatment[143], and high-mannose glycopeptides containing 7–9 sugar residues, with the terminal GlcNAc still linked to asparagine, were resolved[161] on elution with 1 m*M* phosphate buffer (pH 6.0). Cyclic oligosaccharides, which contain no reducing sugars, are well resolved on columns packed with octadecylsilica of particle diameter 5 or 3 μm, cyclosophoraoses of DP 17–33 and branched cyclodextrins being separated by Koizumi and co-workers[141,162] using eluents containing 5–7% of methanol in water. Octadecylsilica is now considered superior to packings giving normal-phase partition[140] for the resolution of branched cyclodextrins, although the reverse holds for oligosaccharide–alditols from glycoproteins[143].

An interesting recent development is the use by the Koizumi group of a new column packing in which the C_{18} chains are bonded not to silica but to a vinyl alcohol copolymer gel (5-μm particles). This column (Asahipak ODP-50; Asahi Kasei, Tokyo, Japan) was less successful than the silica-based packings in the HPLC of branched cyclodextrins[162], but greatly superior in the chromatography of linear D-gluco-oligosaccharides[163], resolving those of the maltoseries to DP 23, (1→6)-linked oligomers (both isomalto and gentioseries) to DP 11 and the β-(1→2)-linked sophoroseries to DP 12. Solubility considerations imposed an upper limit of DP 6 on resolution of the β-(1→4)-linked cello-oligosaccharides, and of 5 and 4, respectively, on that of α- and β-(1→3)-linked oligomers. Elution with water resulted in the separation of anomers but this could be precluded by elution with aqueous sodium hydroxide at pH 11. The stability of this packing at high pH is a major advantage over the silica-based packings, and it seems likely that future work on this form of HPLC will focus on such polymer-based columns.

Yet another mode of chromatography in which octadecylsilica packings have found application is ion-pair chromatography. As an alternative to the use of ion-exchange, acidic oligosaccharides have been fractionated on C_{18}-silica columns with eluents, usually phosphate buffers (pH 6.5–7.5) mixed with methanol or acetonitrile (10–30%), containing an ion-pairing reagent. The tetrabutylammonium cation has been found to be particularly effective for this purpose, conferring sufficient hydrophobic character on the complex formed with the anionic oligosaccharide to permit resolution on a reversed-phase column. Thus Voragen *et al.*[164] resolved the normal and unsaturated oligogalacturonic acids produced by degradation of pectic acid with *endo*-polygalacturonase or *endo*-pectic acid lyase, respectively, on a column of LiChrosorb RP-18 (Merck) at 40°C within 15 min, with eluents composed of 0.05 *M* phosphate buffer (pH 7.0), methanol (10% for normal, 30% for unsaturated acids) and 25 m*M* tetrabutylammonium bromide. The normal oligogalacturonic acids were resolved only to DP 4, but the unsaturated acids could be resolved to DP 7, as were

the β-(1→4)-linked oligomers of D-mannuronic acid with a 4,5-unsaturated residue at the non-reducing end, produced by lyase degradation of alginate[165]; these were separated at ambient temperature on a μBondapak C_{18} cartridge (Waters Assoc.) eluted with a 0.1 *M* phosphate buffer (pH 6.5) containing acetonitrile (10%) and tetrabutylammonium hydroxide (10 m*M*).

Thus, octadecylsilica packings are very versatile. Other advantages are their stability at neutral pH, under which conditions they do not dissolve in aqueous mobile phase to the same extent as other silica-based packings[134], and the ease with which they can be regenerated, adsorbed impurities being removed by washing with methanol[133,134]. A disadvantage in addition to the tendency to resolve anomers, which has been discussed, is the low capacity, which limits the use of such columns in preparative chromatography[134].

4.1.3. Silica-based anion exchangers. Packings with strong-base anion-exchange groups (quaternary ammonium) bonded to 5- or 10-μm silica have been used in the anion-exchange HPLC of acidic oligosaccharides, *e.g.*, the oligogalacturonic acids also separated by ion-pair chromatography on C_{18}-silica (see above). Voragen *et al.*[164] resolved the normal oligogalacturonic acids and those bearing a 4,5-unsaturated residue to DP 4 and 6, respectively, within 30 min on such packings (Nucleosil 10 SB or Zorbax SAX) with a 0.3 *M* acetate buffer, pH 5.4, as eluent; use of a more concentrated buffer (0.4 *M*) resolved the unsaturated oligogalacturonic acids to DP 8 within 15 min. As has been mentioned, aminopropylsilica packings used in normal-phase partition chromatography can also act as weak-base anion exchangers. These give poorer separations than the strong-base type, *e.g.*, the same unsaturated oligogalacturonic acids were resolved only to DP 5 in 15 min on LiChrosorb 10-NH_2[164], but are more stable; strong-base anion exchangers bonded to silica have very limited lifetimes[134].

Other examples of the use of silica-based anion exchangers in the HPLC of acidic oligosaccharides include resolution of the oligosaccharides (di-, tetra, hexa-, octa- and decasaccharide) produced on digestion of chondroitin 4- and 6-sulphates with hyaluronidase; this was achieved, after a preliminary fractionation by SEC, on a Partisil-10 SAX column (Whatman; strong base), by gradient elution for 35 min with phosphate buffer (0.25→0.55 *M*)[166]. Nebinger *et al.*[167] have reported the complete separation of all the saturated oligosaccharides, even- and odd-numbered, derived from hyaluronic acid; the members of this series, containing 2–8 sugar residues, were resolved within 30 min on Ultrasil-NH_2 (Beckman) eluted with 0.1 *M* phosphate buffer (pH 4.75). Micropak AX-10 (Varian), with gradient elution (25→500 m*M* KH_2PO_4, pH 4.0), resolved glycoprotein-derived neutral oligosaccharides and those bearing 1, 2, 3 and 4 sialic acid residues within 45 min[168]; these oligosaccharides contained 6–18 sugar residues. Phosphorylated oligosaccharides were also fractionated by this means.

4.2. HPLC of higher oligosaccharides on microparticulate ion-exchange resins

Developing in parallel with the silica-based ion exchangers have been high-performance resins of particle size 25 μm or less, which allow HPLC in the ion-exchange mode. These resins, which are far more stable than silica-based packings, have proved valuable in applications such as the analysis of sialylated oligosaccharides from sugar chains of glycoproteins, milk or urine of patients with sialidosis. For

example, Tsuji *et al.*[169], using a custom-made resin and elution at 55°C with concave gradients of sodium chloride in water, were able to separate not only oligosaccharides and oligosaccharide–alditols differing in chain length and sialic acid content, but also isomeric oligosaccharides differing in sequence and glycosidic linkage. However, this applied only to oligosaccharides containing up to four sugar residues; larger biantennary and triantennary oligosaccharides containing eleven and thirteen sugar residues, with sialic acid terminating one branch in each instance, were poorly resolved. Mono Q (Pharmacia), the strong-base anion-exchange resin (particle diameter 10 μm) developed for medium-pressure liquid chromatography, gives a better resolution of sialylated oligosaccharides containing up to fourteen sugar residues, mono-, di- and trisialylated. Using this resin, Van Pelt *et al.*[170] reported the analysis, on the microgram scale with sensitive UV detection, of such oligosaccharides from sialidosis urine and preparative fractionation of those produced by hydrazinolysis of human serotransferrin. With a prepacked, short column (50 × 5 mm I.D.) of Mono Q, analysis using gradient elution with aqueous sodium chloride is rapid (10 min or less).

Microparticulate cation-exchange resins have been widely applied to "ion-moderated partitioning" of carbohydrates where the mechanism can involve ion or size exclusion, hydrophobic adsorption or ligand exchange, depending on the counter ion associated with the resin. Such columns are run at elevated temperatures to preclude the formation of broad peaks or doublets due to resolution of anomers[11] and to accelerate the slow, diffusion-controlled partitioning process[134]. Unless the resin is used in the H^+ form, water is the eluent in this mode of chromatography, which is a major advantage. Oligosaccharides are eluted in order of decreasing molecular weight, the size-exclusion mechanism being predominant[171]. The use of 4% cross-linked resin in the Ca^{2+} form to resolve cello-oligosaccharides to DP 7 was reported by Ladisch *et al.*[172] in 1978 and the resolution of malto-oligosaccharides to DP 8 in 1980 by Fitt *et al.*[171]. Using Aminex HPX-42C (Bio-Rad Labs.) at 85°C, Schmidt *et al.*[173] were able to resolve each of these oligosaccharide series, as well as the β-(1→4)-linked D-xylo-oligosaccharides from xylan (to DP 9), together with some monosaccharides (glucose, xylose, rhamnose and arabinose), within 1 h. Cyclodextrins are resolved[174,175] on a similar resin at 90°C, eluting in the order $\alpha < \gamma < \beta$, at retention times later than those of the corresponding linear D-gluco-oligosaccharides; this and the unexpected early elution of α-cyclodextrin (cyclomaltohexaose) indicate that specific interactions with the polystyrenesulphonate matrix of the resin are involved. Brunt[175] performed this separation and that of the malto-oligosaccharides with Ca-EDTA (50 ppm) in the eluent, to protect the Ca^{2+} resin from contamination by traces of metal ions leached from the stainless-steel column. Resin in the Ag^+ form is more stable, and use of 4% cross-linked resin in this form allows the resolution of oligosaccharides to higher DP; cello-oligosaccharides from hydrothermally degraded poplar wood were resolved to DP 8 by Bonn *et al.*[176] and malto-oligosaccharides can be resolved to DP 11 on Aminex HPX-42A (Bio-Rad Labs.) at 85°C[134,177].

Recently, the feasibility of using 2% cross-linked resin in the H^+ form in the HPLC of oligosaccharides has been demonstrated both by Derler *et al.*[178] and by Hicks and Hotchkiss[179]. Provided that flow-rates, and hence back-pressures, are kept low (not more than 0.35 ml/min at 60°C and 0.5 ml/min at 85°C), this softer resin can be used in HPLC, with a dilute acid as eluent. On this resin (designated HPX-22H by Bio-Rad Labs.), eluted with 5 m*M* sulphuric acid at 76–88°C, malto-oligosaccharides

can be resolved to DP 12–14 and cello-oligosaccharides to DP 9 in 40–60 min[178,179]. The H^+ form resin has the advantage over those in the Ca^{2+} and Ag^+ forms that it can be used in the analysis of ionic oligosaccharides[179]; oligogalacturonic acids have been resolved to DP 10 and chito-oligosaccharides to DP 8 at 85°C, the latter being poorly resolved, possibly owing to partial de-*N*-acetylation. Lower column temperatures are obviously advisable for the chromatography of acid-labile oligosaccharides on this column, which could be applied in the direct analysis, without work-up procedures, of the products of the acid hydrolysis of polysaccharides.

4.3. Ion chromatography

Most carbohydrates have pK_a values in the range 12–13, and therefore will become anionic at pH $\geqslant$ 13. With eluents of this pH, such as 0.1–0.2 *M* sodium hydroxide solution, carbohydrates may be analysed by the relatively new technique of ion chromatography. Special column packings, which are pellicular, having a thin layer of strongly basic OH^- form anion exchanger coated on to 5- or 10-μm latex beads (Dionex), have been developed for the rapid chromatography of anionic solutes, and the application of such a packing to the chromatography of alditols, sugars and oligosaccharides was demonstrated in 1983 by Rocklin and Pohl[180]. Use of the triple-pulsed amperometric detector permits the very sensitive detection of carbohydrates in the ion chromatographic system. The carbohydrates are oxidized at the surface of a gold electrode, the voltage on the electrode is then reversed to convert the resulting gold oxide layer back to native gold and the potential is cycled back to the analytical voltage.

With the Dionex HPIC-AS6 column and a pulsed amperometric detector, sugars, including aminodeoxyhexoses, can be separated within 15–20 min on elution with 0.15 *M* sodium hydroxide solution at 1 ml/min[180,181]. Simple oligosaccharides, such as sucrose, raffinose and stachyose, are also resolved under these conditions. The addition of acetate decreases the capacity factors of all solutes, and this is used to extend the application of the method to the chromatography of higher oligosaccharides and the resolution of isomeric disaccharides. Initially, Rocklin and Pohl[180] used an eluent in which both sodium hydroxide and sodium acetate were 0.2 *M* to resolve the malto-oligosaccharides to DP 10 on HPIC-AS6. The potential of gradient elution was demonstrated by the excellent resolution of inositol, sorbitol, ten monosaccharides (including four deoxy sugars, arabinose and xylose, galactose, glucose, mannose and fructose) and six disaccharides (melibiose, isomaltose, gentiobiose, cellobiose, maltose and turanose) on HPIC-AS6A (5-μm particles) with a gradient in which the proportion of a solution containing 50 m*M* sodium hydroxide and 1.5 m*M* acetic acid to pure water was increased from 7% (15 min) to 100% (over 10 min)[181,182]. In this instance, with low concentrations of sodium hydroxide in the eluent, post-column addition of 0.3 *M* sodium hydroxide was used to raise the pH to the optimum range for the detector. This is unnecessary if the pH of the eluent is kept constant, with only the concentration of acetate changing during gradient elution. In this way, with the HPIC-AS6 column and a linear gradient, from 0 to 600 m*M* sodium acetate in 100 m*M* sodium hydroxide over 30 min, followed by isocratic elution at the final concentration for 5 min, resolution of the malto-oligosaccharide series to DP 43 has been achieved[181], the sensitivity of the detector being increased 3-fold for the oligomers of DP 25–35, and 30-fold to detect those of DP above 35. Hence ion chromatography is

TABLE 3

RESOLUTION OF MALTO-OLIGOSACCHARIDES BY VARIOUS HPLC SYSTEMS

DP	*Relative retention time*[a]				
	Silica-based systems		*Resin-based systems*		
	Amino phase[b]	*Reversed phase*[c]	*HPX-42A*[d]	*HPX-22H*[e]	*HPIC-AS6*[f]
1	–	–	1.33	1.23	–
2	0.91	–	1.13	1.11	0.51
3	1.00	1.00	1.00	1.00	1.00
4	1.09	1.16	0.87	0.89	1.34
5	1.18	1.37	0.77	0.82	1.65
6	1.26	1.58	0.71	0.76	1.95
7	1.33	2.00	0.64	0.70	2.25
8	1.39	2.37	0.60	0.65	2.52
9	1.45	2.74	0.56	0.60	2.79
10	1.53	3.26	0.53	0.56	2.99
11	1.62	4.00	0.51	0.53	3.19
12	1.72	5.21	–	0.50	3.39
13	1.82	6.42	–	0.48	3.58
14	1.95	8.37	–	0.46	3.70
15	2.09	10.2	–	–	3.79
20	3.00	–	–	–	4.29
25	4.18	–	–	–	4.73
30	5.90	–	–	–	5.06
35	–	–	–	–	5.29
40	–	–	–	–	5.47
43	–	–	–	–	5.57

[a] Relative to retention time for maltotriose: 5.5 min[b]; 1.9 min[c]; 16.8 min[d]; 30.0 min[e]; 6.3 min[f].

[b] ERC-NH-1171 (Erma), 3-μm particles; column, 200 × 6 mm I.D.; eluent, acetonitrile–water (57:43); flow-rate, 1 ml/min; ambient temperature[139].

[c] Dextropak C_{18} (Waters Assoc.), 10-μm particles, coated with Triton X-100, in plastic cartridge, 100 × 8 mm I.D., used under radial compression; eluent, water; flow-rate, 2 ml/min; ambient temperature[160].

[d] Column, 300 × 7.8 mm I.D.; eluent, water, flow-rate, 0.4 ml/min; column temperature, 85°C[177].

[e] Column, 300 × 10 mm I.D.; eluent 5 m*M* sulphuric acid; flow-rate, 0.5 ml/min; column temperature, 85°C[179].

[f] Column, 250 × 4 mm I.D.; linear gradient, 0 to 600 m*M* sodium acetate in 100 m*M* sodium hydroxide over 30 min, isocratic for 5 min; flow-rate, 1 ml/min; ambient temperature[181].

clearly the most powerful method for the HPLC of higher oligosaccharides (see Table 3).

Recently, Koizumi *et al.*[183] have applied ion chromatography on HPIC-AS6 with pulsed amperometric detection to branched cyclodextrins containing up to twelve D-glucosyl residues and to cyclosophoraoses of DP 17–25; isocratic elution with 0.15 *M* sodium hydroxide containing sodium acetate (0.20 and 0.14 *M* for cyclodextrins and cyclosophoraoses, respectively) was used. With acetate gradients, this versatile technique has given excellent resolutions of many of the neutral, sialylated and phosphorylated oligosaccharides derived from glycoproteins[184,185].

4.4. High-performance steric-exclusion and affinity chromatography

Many of the new column packings developed for steric-exclusion chromatography (SEC) in HPLC systems have been tested with characterized dextran fractions to determine their fractionation ranges for polysaccharides. These packings include microparticulate, chemically cross-linked agarose (Superose; Pharmacia)[186], hydrophilic, cross-linked, semi-rigid polymers, such as the TSK-gel PW series (Toyo Soda)[187,188], and porous silica deactivated by chemically bonded polyether or hydroxylic phases[189], such as μBondagel (Waters Assoc.)[190] or the TSK-gel SW series (Toyo Soda)[191]. They have also been applied in the rapid determination of average molecular weights of a variety of other polysaccharides, *e.g.*, amylose, amylopectin and pullulan[192–194] (for which the validity of the 'universal calibration' relating elution volume to the logarithm of the size-related parameter $[\eta] \cdot \bar{M}_w$, where $[\eta]$ is the intrinsic viscosity, has been demonstrated on TSK-PW gels[195]), inulins[196], guar[197] and glycosaminoglycuronans[198–200]. In general, the speed of analysis using these HPLC packings is at the expense of the resolution possible at the slower flow-rates of the gels used in conventional, low-pressure SEC, so that distinct peaks corresponding to various components of polymolecular polysaccharides are seldom seen. Molecular-weight distributions can, however, be determined directly during SEC by monitoring the effluent carbohydrate by low-angle laser-light scattering[192]. Because of the tendency, already mentioned, of silica-based packings to dissolve in aqueous eluents, the polymer-based SEC packings are now generally preferred.

With the objective of high-speed fractionation or purification of glycoproteins and glycopeptides, some of the carbohydrate-binding lectins (notably concanavalin A, also wheat germ agglutinin and the phytohaemagglutinin from red kidney beans, *Phaseolus vulgaris*[15]) that have proved useful in affinity chromatography when coupled to agarose have been bonded to microparticulate silica to form packings for high-performance affinity chromatography[201–204]. With such packings affinity chromatography is complete within 20 min, but peaks are broad, so that the resolution of mixtures is poor. A detailed thermodynamic and kinetic study of affinity chromatography on silica-based concanavalin A[205–207] has shown that the main factor responsible for this is slow dissociation of the carbohydrate solute from the immobilized lectin, dissociation constants for glycosides[205] being *ca.* ten times smaller than those in solution and those for glycoproteins even smaller[206]. For this reason, the feasibility of the use of weakly binding ligands, such as monoclonal antibodies[208], coupled to silica for more efficient high-performance affinity chromatography of carbohydrates is currently under investigation.

4.5. Novel packings and derivatization methods for HPLC of sugars

Although this section has focused mainly on the application of HPLC to higher oligosaccharides, no review of this topic would be complete without mention of certain novel column packings which give improved resolution of sugars, overcoming some of the disadvantages of the older packings. Following the testing[209] of diol-modified silica (LiChrosorb DIOL; Merck) as an alternative to aminopropylsilica, without the complication of glycosylamine formation, the application of polyol-modified silica to the HPLC of sugars has been investigated[133,210]. Good resolution of mono-, di- and trisaccharides and also of sugars within each group, *e.g.*, mannose–galactose, maltose–lactose and maltotriose–raffinose, was obtained, but addition of

amine (0.1% of triethylamine) to the acetonitrile–water eluents (68–75% acetonitrile) was necessary to prevent peak broadening due to resolution of anomers, and this must be detrimental to the stability of the silica-based packing. Far more promising results have been achieved in a recent evaluation of new packings in which a cyclodextrin (α- or β-) is bonded to 5-μm silica[211]; with eluents consisting of mixtures of water with acetonitrile or acetone only (80–90% organic component, or gradient), highly efficient and selective separations of a diversity of sugars (including monosaccharides from triose to heptose, di-, tri and tetrasaccharides), of alditols and even of the cyclodextrins have been obtained at ambient temperature. The analysis times are generally comparable to those on octadecylsilica columns and shorter than those on amino and ion-exchange stationary phases, and the new packings are far more stable than other silica-based packings. It seems likely that these cyclodextrin–silica columns will soon supersede the older packings for the HPLC analysis of sugars.

As it is now generally accepted that accuracy and precision of quantitative HPLC[134] are comparable to or better than those obtainable by the various GLC methods, detectors more sensitive than the commonly used refractive index and UV types are being sought for the quantitation of HPLC analyses of carbohydrates at sub-microgram levels. In addition to instrumental methods for direct detection, such as the pulsed amperometric detector, and several post-column derivatization methods[134], precolumn derivatization to introduce fluorescent centres into carbohydrate molecules, and so permit sensitive detection by fluorimetry, is being increasingly applied in HPLC on reversed-phase or unmodified silica columns. Chief among these derivatives are the dansylhydrazones, produced by reaction of neutral aldoses with 5-dimethylaminonaphthalene-1-sulphonylhydrazine in the presence of trichloroacetic acid, which have been used in the analysis of sugars by HPLC on both reversed-phase[212–214] and silica[215] columns; the use of fluorimetric detection (excitation wavelength 360 nm, emission >470 nm) permits the analysis of dansyl-sugars at levels of 5–15 pmol[213]. Quantitative analysis of picomole amounts of neutral and amino sugars in glycoconjugates by reversed-phase HPLC with fluorimetric detection of the 2-pyridylamino derivatives has been reported by Takemote *et al.*[216]. More recently, reversed-phase HPLC of neutral aldoses as the dabsylhydrazones, produced on reaction with 4′-N,N,dimethylamino-4-azobenzene-1-sulphonylhydrazine in the presence of acetic or trichloroacetic acid, has made possible the analysis of the constituent neutral sugars in 5-μg samples of glycoproteins[217] and of individual sugars at levels down to 10 pmol[218]. In this instance the derivatives are not fluorescent, but strongly chromophoric (absorbance maximum 460 nm, shifting to 495 nm at low pH). This detection method has also been applied to the TLC of sugars[218].

5. THIN-LAYER CHROMATOGRAPHY

5.1. Sensitive detection methods

The dabsylhydrazone derivatives just mentioned appear as bright yellow spots on TLC (silica gel plates), turning bright pink when the developed plates are briefly exposed to hydrogen chloride vapour. Visual detection of the strongly chromophoric spots is possible down to 0.1 nmol of the sugar dabsylhydrazone[218]. Fluorimetric detection also greatly enhances sensitivity in the TLC of sugars. Honda *et al.*[219] listed detection limits for the detection of sugar spots with a malonamide spray (1% solu-

tion in 1 *M* carbonate buffer, pH 9.2) followed by heating at 120°C for 20 min and irradiation by a mercury lamp. Most aldoses, ketoses, uronic acids, amino sugars and even reducing disaccharides can be detected at levels as low as 0.25 nmol, only aldonic acids, alditols, glycosides and non-reducing oligosaccharides being insensitive to this reagent. Fluorogenic reagents used in the TLC assay of amino sugars include dansyl chloride [the sugars being derivatized before TLC and the developed plates sprayed with triethanolamine–isopropanol (1:4) before *in situ* scanning][220] and fluorescamine, which reacts with primary amines at pH 9 to give highly fluorescent pyrrolines[221]. The former method allows the detection of GlcN and GalN down to 0.3 nmol, the latter to 50 pmol. A procedure for the quantitative TLC of sugars, sugar acids and lactones, and polyols, separation on HPTLC plates being followed by treatment of the plates with lead tetraacetate in glacial acetic acid and then the fluorogenic reagent 2,7-dichlorofluorescein, which permits detection of nanogram amounts, has been described[222,223].

5.2. Use of HPTLC and bonded-phase plates

Good separations of sugars and alditols, often improved by the presence of boric acid in the eluent, have been achieved by use of HPTLC plates with a variety of solvent systems[222]. The limits of resolution of malto-oligosaccharides by HPTLC, initially DP 10–12, were extended by Koizumi *et al.*[139] to DP 26, using macroporous Si 50 000 HPTLC plates (Merck), developed four times with 1-butanol–pyridine–water (6:5:4); β-(1→2)-linked D-gluco-oligosaccharides of the sophoroseries were resolved to DP 30 under the same conditions. Doner[224] has reported a good resolution of cello- and xylo-oligosaccharides (to DP 8 and 10, respectively) on these plates after three-fold irrigation with 1-butanol–pyridine–water (8:5:4), and of oligogalacturonic acids to DP 9 on Whatman HPTLC plates (type HP-K, 60 Å pore diameter) with a single development with ethanol–25 m*M* acetic acid (21:29) at 35°C[225]. Greatly improved resolution of gangliosides was achieved by Ando *et al.*[226] using silica gel 60 HPTLC plates (Merck) with the solvent system acetonitrile–2-propanol–50 m*M* aqueous potassium chloride (10:67:23). Koizumi *et al.*[227] resolved branched cyclodextrins on Si 50 000 HPTLC plates, using two developments with 1-butanol–pyridine–water systems (6:3:2, then 6:4:3), and also NH_2-bonded HPTLC plates, developed five times with acetonitrile–water (3:2).

This successful use of amino-bonded TLC plates was not paralleled in the TLC of monosaccharides[23], aldotetroses, pentoses and some hexoses and deoxy sugars remaining at the origin on development of aminopropyl-bonded silica plates in 70% acetonitrile. This can be overcome by impregnation of the amino-bonded plates with NaH_2PO_4 to minimize covalent interactions of sugars with the amino groups. With such plates Doner and Biller[228] resolved a diversity of monosaccharides, including pentoses, hexoses, heptuloses, deoxy sugars and acetamidodeoxyhexose, in addition to acetals, methyl glycosides, isomeric disaccharides and oligosaccharides of DP 2–7, separations being achieved in less than 30 min. These bonded-phase TLC plates are a useful complement to HPLC systems and as such represent an important advance in the planar chromatography of carbohydrates.

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7. SUMMARY

The impact on carbohydrate chemistry and biochemistry of the many significant developments in chromatographic analysis during the past decade is discussed and assessed. New techniques, such as supercritical fluid chromatography and ion chromatography with pulsed amperometric detection, have been applied with advantage, and the use of highly efficient columns has greatly improved resolution in high-performance liquid chromatography, especially of longer or more complex oligosaccharides. Novel methods of derivatization for gas–liquid, high-performance and thin-layer chromatography have enhanced both resolution and sensitivity; some of these derivatization methods make possible the resolution of enantiomers, which has been achieved also by the development of chiral stationary phases for gas–liquid chromatography. The results stemming from these new approaches to the chromatography of carbohydrates are compared with a view to a critical evaluation of the contribution of each new method to solving problems in carbohydrate analysis.

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