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ANALYSIS OF UNDERIVATISED D-GLUCO-OLIGOSACCHARIDES (d.p. 2–20) BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

The behaviour of oligosaccharides in high-pressure liquid chromatography (h.p.l.c.) has been investigated by using silica columns dynamically modified with different di- or poly-amines. Oligosaccharides having d.p. of up to 20 (from partially hydrolysed starch) are well-resolved, without derivatisation, in under 40 min by using a simple isocratic system. The results agreed well with those obtained by automated gel-permeation chromatography.

INTRODUCTION

Polymers of D-glucose, such as those present in corn syrups, are usually separated by low-pressure ion-exchange[1] or by permeation chromatography (g.p.c.) using soft gels, such as cross-linked dextran (*e.g.*, Sephadex G-series)[2] or polyacrylamide (*e.g.*, Bio-Gel P-series)[3]. The best separations are achieved by using fully automated g.p.c. analysers[4,5] which separate oligosaccharides up to d.p. 15 (G1–G15), but require analysis times of between 8 and 22 h.

The use of small-bore columns and small particle-sizes permits oligosaccharides up to d.p. 11 to be resolved in 4.5 h[6] on Bio-Gel P2 and up to d.p. 15 in 8 h[7] on Bio-Gel P4, but analysis times cannot be reduced further because of the compressibility of these packings at high operating pressures. Silica of small particle size has been used for g.p.c. of dextrans of high molecular weight[8] or oligosaccharides[9] having d.p. up to 6, but intermediate fractionations have not been achieved. In the absence of suitable rigid permeation-supports, alternative chromatographic methods for such analyses have been sought.

Oligosaccharides up to d.p. 7 can be separated on ion-exchange resins[10–12] of small particle size in 30 min by elution with water or aqueous ethanol, but apparently the columns are unstable. Oligosaccharides with d.p. higher than 7 cannot be separated, due to possible precipitation at high concentrations of ethanol.

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Separations by adsorption on chemically bonded phases have been achieved by using μBondpak carbohydrate[13] (up to d.p. 5), aminopropyl-bonded phase[14] (up to d.p. 7), and Partisil-10PAC[15] (up to d.p. 11). Refractometers can only be used satisfactorily in conjunction with isocratic elution and higher oligomers cannot be analysed under these conditions. Amino-bonded phases, however, tend to deteriorate with prolonged use[16]. In an alternative approach, Wells and Lester[17] used an octadecyl-bonded phase to fractionate mixtures of peracetylated oligosaccharides. Gradient elution was required to separate oligosaccharides having d.p. >11, but, with a convex gradient, those up to d.p. 30 could be separated in 75 min. Pure silica[18] has been used for adsorption chromatography of carbohydrates, but is not generally applicable because of the low solubility of oligosaccharides in the aqueous acetonitrile mixtures required.

We have found that silica columns, dynamically modified with polyfunctional amines, provide a rapid, inexpensive method of separating not only simple sugars[16,19] but also oligosaccharides up to d.p. 20.

EXPERIMENTAL

Apparatus. — The system used consisted of a Hewlett–Packard 1010B chromatograph with a Waters R401 refractometer for detection and a Watanabe Servocorder. Stainless-steel columns (100 × 5 mm i.d. and 200 × 8 mm i.d.) were packed with a slurry of irregular silica (~5 μm diameter, H.S. Chromatography Packings) in 50% (v/v) glycerol in methanol. A pre-column (50 × 5 mm i.d.) containing LiChroprep Si60 (15–25 μm diameter, Merck) was fitted between the pump and septum injector.

Modifiers. — 1,3-Diaminopropane, 1,4-diaminobutane, 1,3-diaminobenzene, 1,5-diaminopentane, 1,6-diaminohexane, 1,8-diaminooctane, tetraethylenepentamine, and 3-aminopropiononitrile (as fumarate) were commercial materials. Polyamine modifier was obtained from H.S. Chromatography Packings, triethylenetetramine and di-(4-aminodiphenyl)methane were gifts from Ciba–Geigy, and pentaethylenehexamine was a gift from Mr. B. B. Wheals.

General methods. — The eluant was prepared daily by mixing appropriate volumes of acetonitrile and water. Percentages recorded are for the water content. The solution was filtered, 0.01% (v/v; w/v for solid samples which would not melt readily) of modifier was added, and the mixture was degassed by sonication. Columns were conditioned immediately before use with 300 ml of aqueous acetonitrile containing 0.1% of the appropriate modifier. Modifiers were removed from the silica by washing with 0.5% aqueous phosphoric acid (~30 ml) and water (until acid free, ~100 ml). Removal of an amine was checked by confirming that a sample was not resolved when the aqueous acetonitrile contained no modifier. The silica was then equilibrated, as described above, with a different modifier.

Starches were hydrolysed by chemical and/or enzymic methods, to provide various mixtures of oligosaccharides. All samples were analysed by automated g.p.c.[4,5] on Bio-Gel P2. One malto-saccharide sample[20] was a gift from Dr. M. Ohnishi.

TABLE I

EFFECT OF MODIFIER ON RETENTION AND RESOLUTION OF OLIGOSACCHARIDES ON DYNAMICALLY MODIFIED SILICA[a]

Modifier	*k′ Values for oligosaccharides*[b]								*Resolution between oligosaccharides*						
	G1	*G2*	*G3*	*G4*	*G5*	*G6*	*G7*	*G8*	*G1→G2*	*G2→G3*	*G3→G4*	*G4→G5*	*G5→G6*	*G6→G7*	*G7→G8*
1,3-Diaminopropane	1.0	1.4	2.0	2.6	3.5	4.6	5.7	7.2	1.7	1.6	1.6	1.7	1.5	1.3	1.3
1,4-Diaminobutane	1.0	1.5	2.0	2.8	3.8	5.0	6.3	7.9	1.6	1.8	1.9	1.9	1.6	1.4	1.7
1,5-Diaminpentane	0.9	1.3	1.8	2.4	3.2	4.1	5.1	6.4	1.5	1.6	1.6	1.6	1.3	1.3	1.1
1,6-Diaminohexane	0.8	1.1	1.5	2.0	2.6	3.2	4.0	4.8							
1,8-Diamino-octane	0.6	0.7	0.9	1.1	1.3	1.6	1.9	2.2							
Triethylenetetramine	0.8	1.2	1.7	2.2	3.0	3.8	4.7	5.9	1.4	1.5	1.6	1.5	1.4	1.2	1.4
Tetraethylenepentamine	0.9	1.2	1.7	2.3	3.1	4.0	5.0	6.2	1.4	1.7	1.9	1.7	1.5	1.5	1.6
Pentaethylenehexamine	0.9	1 2	1.7	2.4	3.1	4.0	5.0	6.2	1.5	1.8	1.9	1.7	1.5	1.5	1.5
Polyamine modifier	0.9	1.2	1.7	2.3	3.0	3.8	4.7	5.8	1.6	1.9	2.0	1.8	1.7	1.5	1.5
3-Aminopropiononitrile	0.3	0.4	0.4	0.5	0.5	0.6	0.6	—							
1,3-Diaminobenzene									No resolution						
Diaminodiphenylmethane									No resolution						

[a]See text for details of chromatography. [b]G1 = D-glucose, G2 = maltose, G3 = D-glucotrisaccharide, *etc.*

TABLE II

EFFECT OF FLOW RATE ON THE RESOLUTION OF OLIGOSACCHARIDES BY DYNAMICALLY MODIFIED SILICA[a]

Oligosaccharides[b]	*Flow rate (ml/min)*			
	0.2	*0.5*	*1.0*	*1.5*
	Resolution			
G1→G2	1.6	1.7	1.6	1.3
G2→G3	2.3	2.1	1.9	1.5
G3→G4	2.4	2.1	2.0	1.6
G4→G5	2.1	1.8	1.8	1.5
G5→G6	1.9	1.8	1.7	1.3
G6→G7	1.6	1.7	1.5	1.3
G7→G8	1.5	1.8	1.5	1.2
Efficiency (N)	2200	1700	1400	1000

[a]See text for details of chromatography. [b]For key, see Table I.

TABLE III

EFFECT OF WATER CONTENT OF ELUANT ON THE RETENTION OF OLIGOSACCHARIDES BY DYNAMICALLY MODIFIED SILICA[a]

Oligosaccharide[b]	*Water content (% v/v) of eluant*			
	40	*45*	*50*	*55*
	k' Value			
G1	0.93	0.82	0.60	0.45
G2	1.33	1.10	0.76	0.53
G3	1.90	1.41	0.95	0.61
G4	2.57	1.82	1.15	0.71
G5	3.47	2.31	1.38	0.79
G6	4.40	2.85	1.60	0.89
G7	5.60	3.41	1.85	0.98
G8	7.07	4.13	2.10	—
Flow rate	2.0	1.5	1.5	1.0
Efficiency (N)	2800	3600	5700	6500

[a]See text for details of chromatography. [b]For key, see Table I.

Effect of different modifiers. — Using the 100-mm column and 45% aqueous acetonitrile, the effect of various modifiers on the elution pattern of one sample of hydrolysed starch was studied. Phase-capacity ratios (k') are given in Table I. Where possible, the resolution between successive peaks (R_s) was calculated by using peak widths at half height for successive peaks and the relationship

$$R_s = \sqrt{5.54}/2 \times (t'' - t')/(w''_{\frac{1}{2}} + w'_{\frac{1}{2}}),$$

where t′ and t″ are the elution times and $w'_{\frac{1}{2}}$ and $w''_{\frac{1}{2}}$ the widths at half height, respectively, for successive peaks.

Effect of flow rate. — The effect of different flow-rates on the resolution was studied by using the 100-mm column and 45% aqueous acetonitrile containing 0.01% of polyamine modifier (Table II). The column efficiency (number of theoretical plates, N) was measured with respect to D-glucose. Flow rates of 2.0 ml.min^{-1} and above gave inadequate resolution.

Effect of eluant composition. — The effect of varying the water content of the aqueous acetonitrile containing 0.01% of 1,4-diaminobutane was studied by using the 200-mm column. For lower concentrations of water, the flow rate was increased to avoid excessive times of analysis. Water contents below 40% were not employed because of insolubility of the oligosaccharides, and concentrations above 55% gave no resolution (Table III). The concentration of 1,4-diaminobutane (between 0.005 and 0.02%) in 40% aqueous acetonitrile had no significant effect on the retention.

Analytical separations. — Partial hydrolysates of starch were analysed by using the 200-mm column and 50% aqueous acetonitrile containing 0.01% of either 1,4-

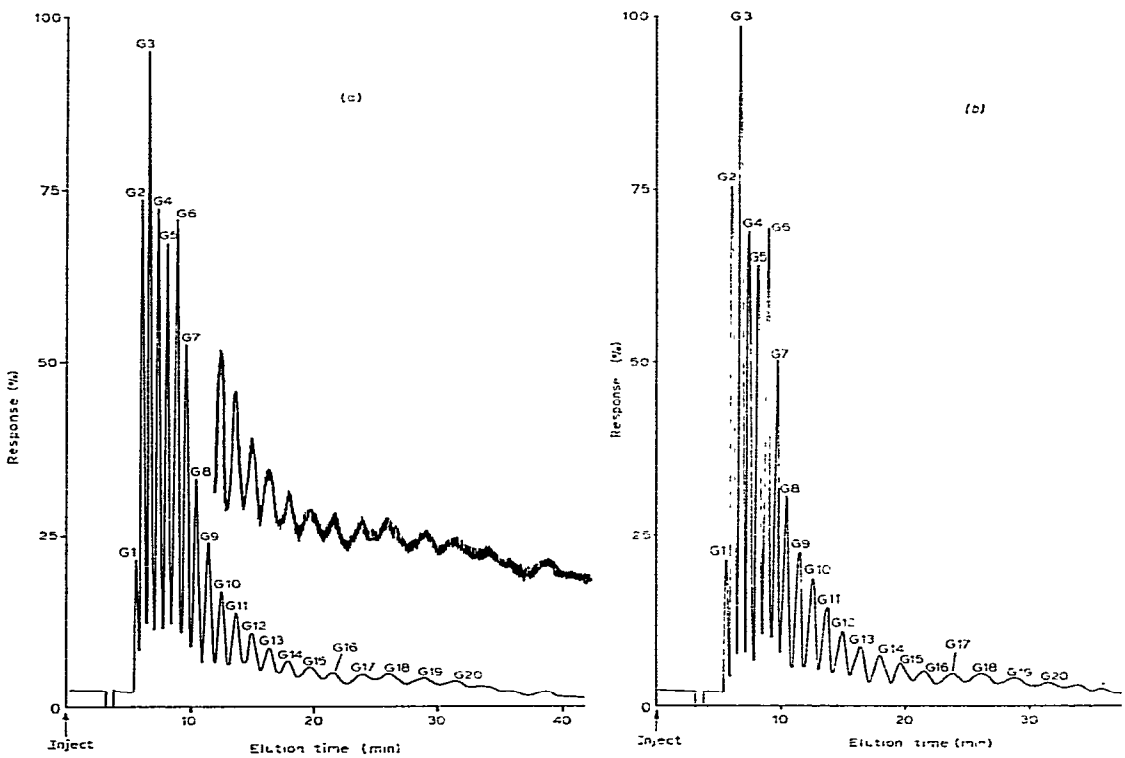

Fig. 1. Fractionation of D-gluco-oligosaccharides present in a sample of hydrolysed starch. Chromatographic conditions: column (200 mm × 8 mm i.d.) eluted at 2.0 ml/min with 50% aqueous acetonitrile containing 0.01% of (*a*) polyamine modifier or (*b*) 1,4-diaminobutane; the upper trace in (*a*) shows the signal-to-noise ratio at twice the normal sensitivity.

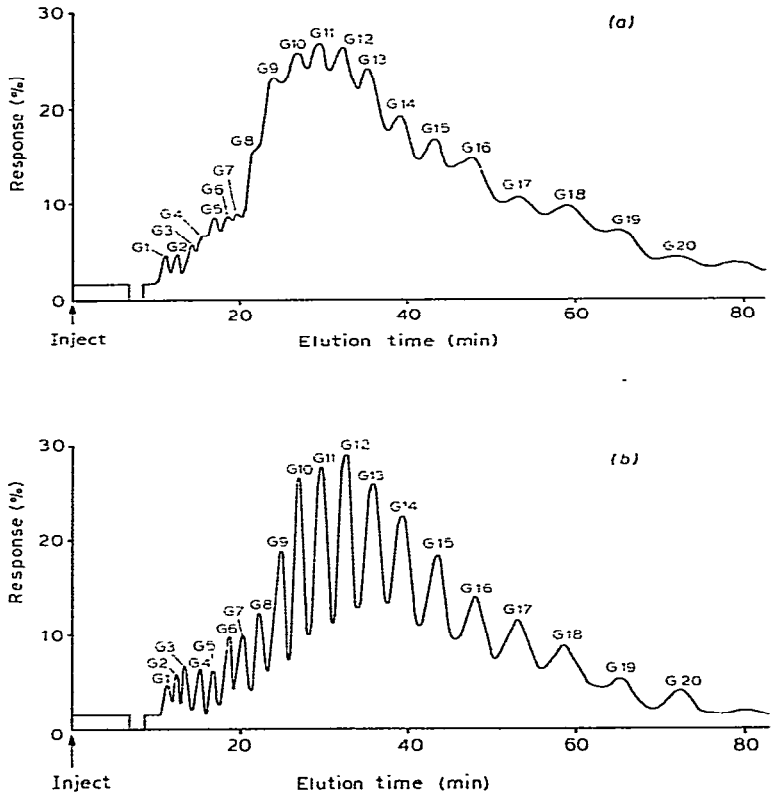

Fig. 2. Fractionation of D-gluco-oligosaccharides present in the maltosaccharide[20] having average d.p. 17. Chromatographic conditions: column (200 mm × 8 mm i.d.) eluted at 1.0 ml/min with 50% aqueous acetonitrile containing 0.01% of (*a*) polyamine modifier or (*b*) 1,4-diaminobutane.

diaminobutane or polyamine modifier. Injections were usually of 25 μl of a solution (up to 100 mg/ml) of the hydrolysate in water. Percentage compositions were calculated by expressing the area under each peak in terms of the total area, using an interpolated baseline (Figs. 1 and 2).

DISCUSSION

In agreement with previous work[16,19], the use of various di- and poly-amines to modify silica dynamically provided a useful system for separation of sugars. The retention data given in Tables I and II show that, at higher concentrations of water than used by Wheals and White[19], there was little difference between the various polyamines used in this study. Fig. 1 shows the separation obtained by using polyamine modifier and 1,4-diaminobutane. The improved separation obtained with the diamine can be seen, notably in the range G1–G8 where troughs between the peaks are deeper. This effect is more marked with higher oligomers. Using 1,4-diamino-

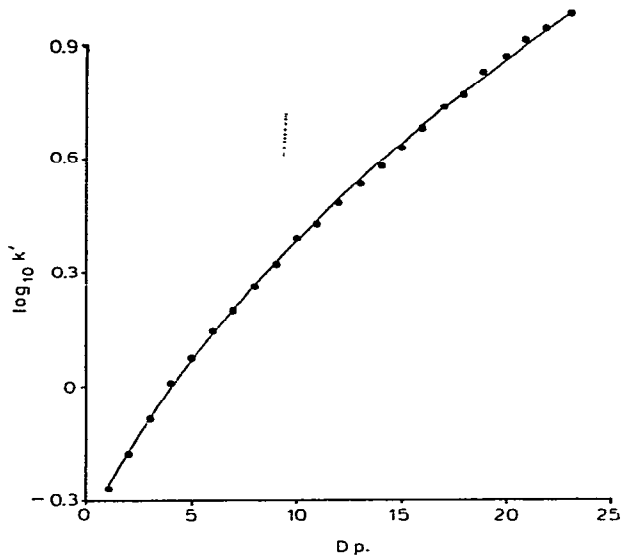

Fig. 3. Relationship between $\log_{10}$ k′ and d.p. of D-gluco-oligosaccharides. Chromatographic conditions as in Fig. 1*a*.

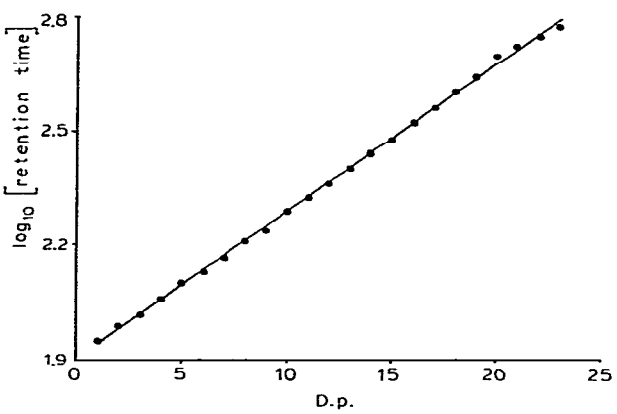

Fig. 4. Relationship between $\log_{10}$ [retention time] and d.p. of D-gluco-oligosaccharides. Chromatographic conditions as in Fig. 1*a*.

butane (Fig. 2), good separation up to G20 was achieved with a sample of maltosaccharide[20] (average d.p. 17) chosen because of its high content of oligomers above G8.

The effect of these modifiers is complex, as can be seen from plots of $\log_{10}$ k′ against d.p. (Fig. 3). A straight-line relationship might be expected, but the higher oligomers were eluted more rapidly than would be predicted. This may be because the dynamically modified packing has some permeation properties analogous to amino-bonded phases[8], or because interaction between the amine present in the mobile phase and oligosaccharides (analogous to the interaction between poly-

saccharides and polyaromatic amines[21]) reduces the interaction with the packing. The overall effect is to permit analysis of higher oligomers without loss of resolution of the lower oligomers. The relationship between elution volumes (or retention time) and d.p. was logarithmic (Fig. 4) and provides a method of identifying specific components in an incomplete series.

Modifiers can be removed from the system with orthophosphoric acid and the system regenerated without significant loss of performance. Whilst the acid was passing through the column, the pH of the eluate rose from neutrality to 10 before dropping to <7. This effect seems to be associated with displacement of bound amine from the silica. The pre-column is unable to protect the analytical column from these pH changes. Repeated treatment with acid causes an increase in column back-pressure (ultimately column blockage), a drop of >50% in efficiency, and decreased column life, although phase-capacity ratios are unaffected. For routine use, it is not necessary to remove the modifier, and storage of the column overnight in eluant had no adverse effects. Columns stored for longer periods were washed with water (50 ml) and methanol (50 ml), and then stored in methanol. Re-equilibration at high concentrations of modifier, as described above, was necessary before re-use.

Resolution of the oligosaccharides can be increased by lowering the flow rate (Table II). Flow rates of 1–2 ml/min with the 200-mm column provide a satisfactory compromise between time of analysis and resolution. Lowering the water content of the eluant increased the resolution (Table III). However, the proportion of water was maintained as high as possible in order to avoid the risk of precipitation of higher oligomers. Variation in modifier content did not affect the separation. Variation in

TABLE IV

COMPARISON OF RESULTS OBTAINED BY H.P.L.C. AND AUTOMATED G.P.C. FOR G1–G12 IN SAMPLES OF HYDROLYSED STARCHES

Oligosaccharide[a]	*Composition (% total)*			
	Sample 1		*Sample 2*	
	H.p.l.c.	*G.p.c.*	*H.p.l.c.*	*G.p.c.*
G1	3.0	2.6	10.5	8.9
G2	11.7	10.5	10.5	9.7
G3	15.4	14.3	10.0	9.7
G4	9.6	9.8	10.2	9.5
G5	9.4	9.8	9.0	9.3
G6	22.0	23.0	8.5	8.9
G7	16.1	16.3	7.6	9.1
G8	5.4	4.9	6.4	8.2
G9	2.7	2.9	6.7	8.0
G10	1.6	2.1	7.5	7.2
G11	1.2	2.0	7.6	6.2
G12	0.8	1.8	5.4	5.6

[a]For key, see Table I.

retention times due to changes in flow rate or eluant composition can be overcome by use of an internal standard, for example, erythritol (k' 0.5; D-glucose, k' 0.7; resolution 1.5).

Using the system described above, it is possible to detect 10 μg of D-glucose. This result is comparable with the detection limit of 40 nequiv. of acetylated hexose (~8 μg of D-glucose) using u.v. detection[16]. However, the system described here has the advantage of faster analysis times, requires no derivatisation (pre- or post-column), and uses simpler equipment with isocratic elution. Moreover, the column is cheaper and probably more stable than amino-bonded phases[19], and its higher efficiency allows the use of lower proportions of acetonitrile, thereby decreasing the risk of precipitation of higher oligosaccharides.

The present method is capable of resolving more peaks than automated g.p.c., but in a more restricted way (polysaccharides of high molecular weight are not detected). Consequently, direct comparison of results is not possible; over the range G1–G12, for which information is available for both methods, comparison of the results shows good agreement (Table IV).

We consider that this procedure could be the basis of an automated system similar to that based on g.p.c., but possessing the advantages discussed.

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