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SOME APPLICATIONS OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO OLIGOSACCHARIDE SEPARATIONS

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SUMMARY

Oligosaccharide separations on reversed-phase high-performance liquid chromatographic columns have been examined using a range of aqueous solvents. Addition of anionic, cationic and non-ionic surfactants, tetramethyl urea and organic solvents to the mobile phase cause faster elution of oligosaccharides, and allow the separation of the larger oligomers in an acceptable time. Addition of neutral, inorganic salts increases the retention factors considerably, and allows good resolution of some compounds poorly resolved in water alone.

The mechanism operating in the separations approximates to that invoked in the solvophobic theory of reversed-phase chromatography. There is some evidence also of hydrogen bond effects. The improvements described should prove useful in the isolation and analysis of neutral oligosaccharides in general, and in structural analyses of polysaccharides in particular.

INTRODUCTION

Neutral oligosaccharides may be separated by a variety of methods [1]. Gel permeation chromatography (GPC) separates mainly on the basis of molecular size [1, 2] and sometimes on the basis of chemical structure [3, 4]. The latter observations imply some solute—matrix interactions. Some separations are excellent up to a degree of polymerization (D.P.) of 40 or more [2], but retention times of up to 20 h are involved.

High-performance liquid chromatography (HPLC) of oligosaccharides on anion-exchange resins is not often reported. Cation-exchange resins, in a number of ionic forms, e.g. calcium [5, 6] or silver [7], using water as the sole eluent have proved useful.

Polyfunctional amines added to the solvent (usually acetonitrile—water) allow the use of silica columns [8, 9]. Of the chemically bonded silica based HPLC phases, aminopropyl is the most widely used [10—12], though octadecyl-bonded silica is becoming increasingly popular [13—15]. It was with a

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view to extending the usefulness of the latter type of column that the present work was undertaken. It was observed [13] that oligosaccharides greater than about D.P. 9 were eluted as broad peaks having relatively long retention times. Large oligosaccharides were retained on the column, eventually causing deterioration of its performance and an increase in back pressure. A means of eluting higher oligomers was thus sought. Observations by ourselves confirm those of Vrátný et al. [14] for both steel and glass columns, that increased temperature reduces the retention times of oligosaccharides markedly. It is not possible, however, to heat Dextropak columns in the Radial Compression Module used in the present work. In other cases we found that resolution between closely related oligosaccharides was poor and this initiated the work on added neutral salts.

EXPERIMENTAL

The HPLC system consisted of the following Waters Assoc. equipment: U6K injector, M6000 pump, R401 refractive index detector, and Radial Compression Module RCM-100. Columns: (a) Waters Dextropak Cartridge 10 × 0.8 cm (10 μm particle size, spherical silica, 125 Å pore size, 10% loading of octadecyl silane bonded phase). (b) LiChrosorb RP-8 steel (E. Merck) 25 × 0.4 cm (10 μm particle size, LiChrosorb SI-60 porous silica; octylsilane bonded phase). Sodium dodecyl sulphate (SDS) and cetyl trimethylammonium bromide (CTAB) were Eastman products; Triton X-100 (TX) was purchased from Rohm & Haas and tetramethylurea (TMU) from Fluka. Water used as HPLC solvent was purified in a Milli-Q system (Millipore).

Coating of the column was carried out by pumping a solution of detergent (0.1%, w/v, in water) through the column at a flow-rate of 1 ml/min. The standard compound used to determine the extent of column modification was isomaltotetraose (IM_4) approx. 10 mg/ml in water.

IM_4 (10 μl) was injected periodically during the coating process. The coating was stopped at a particular IM_4 retention time by changing the solvent to water. Detergent on the column was removed completely by flushing with methanol (200 ml) when it was desired to return the column to its unmodified state. The mass of TX adsorbed was found by rotary evaporation of the methanol flushings, followed by drying of the detergent in vacuo at room temperature over phosphorus pentoxide and paraffin wax, and weighing.

RESULTS AND DISCUSSION

The standard compound used to determine the extent of column modification was IM_4. On the unmodified column used for the anionic and cationic detergents, IM_4 had a retention time of 4.9 min at a flow-rate of 2 ml/min.

Effect of cetyl trimethylammonium bromide

An 0.1% solution of CTAB was pumped through the column at a flow-rate of 2 ml/min. The extent of the modification was followed by injection of IM_4 solution periodically. The decrease in the retention time of IM_4 was linear with respect to solvent volume. As the coating of the column proceeded, the back-

pressure of the column rose from 33 bars to almost 133 bars (the limit for the Radial Compression Module). The experiment was stopped at this point (IM_4 retention time = 2.6 min). The solvent was changed to water, and the retention time of IM_4 was again checked periodically. The water removed CTAB from the column, the retention time increased continually, so the CTAB treatment was considered unsuitable for practical purposes.

Effect of sodium dodecyl sulphate

The effect of an 0.1% solution of SDS on the retention time of IM_4 was similar to that of CTAB. The rate of pressure increase was less than for CTAB, and IM_4 could be made to elute at the void of the column. The solvent was changed to water, and again detergent was removed, leading to increasing retention times for IM_4. In summary, the ionic detergents yielded columns whose elution characteristics changed continuously, and were thus unsuitable for most practical purposes. A coating material which would not be washed off with the solvent was sought.

Effect of Triton X-100

The non-ionic detergent Triton X-100 is a mixture of *p-tert.*-octylphenoxypolyethoxyethanol oligomers, with an average ethylene oxide (EO) number of approx. 9.5 [16]. Triton was used as a column modifier in the hope that the neutral detergent molecules might not be as easily removed by water as the anionic and cationic groups previously used. This appeared to be the case: TX (0.1% solution) could be coated onto a column to achieve for IM_4 any desired retention time from 4.9 min to the column void (1.3 min). When the eluting solvent was changed from TX solution to water, the retention time of IM_4 at that stage of modification remained constant, i.e. TX which was coating the C_{18} on the column was not removed. Up to 1 l of water was passed through the modified column without significantly changing the retention time of IM_4. Several experiments confirmed this. While the column was being coated with 0.1% TX, none of the detergent emerged in the eluent, as shown by freeze-drying of the collected water.

The change in IM_4 retention time brought about by TX solutions was linear with time (Fig. 1). This plot was used in subsequent coating experiments to

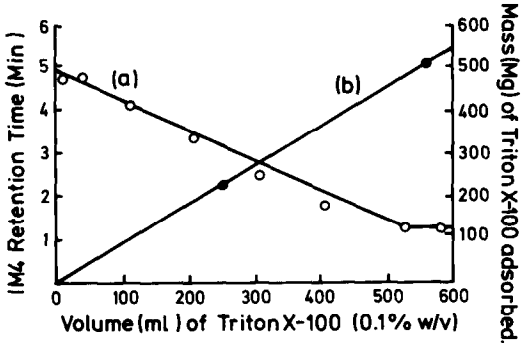

Fig. 1. (a) Isomaltotetraose (IM_4) retention time as a function of solvent volume of Triton X-100 (0.1%) passed through the column. (b) Mass of Triton X-100 adsorbed as a function of solvent volume passed through the column.

determine the volume of TX solution needed to achieve a chosen IM_4 retention time.

Experiments to determine the quantity of TX coating the C_{18} column showed that when IM_4 emerged at the column void volume, 500 ± 10 mg had been taken up. This occurred after the passage of 540 ml of 0.1% TX solution.

It was possible to absorb more TX than 500 mg. The back-pressure increased rapidly above this level so no limiting value was determined.

The coating process with TX was completely reversible. The retention time of IM_4 returned to the original value when the TX was flushed out using

TABLE I

RETENTION TIMES FOR MALTO-OLIGOSACCHARIDES ON TRITON X-100 COATED DEXTROPAK COLUMN

Extent of coating is shown by retention time of IM_4. Flow-rate: 2.0 ml/min; solvent: water.

Peak*		Retention time (min)			
		IM_4 = 4.9**	IM_4 = 4.1	IM_4 = 3.7	IM_4 = 3.0
3	i	3.0	2.9	2.6	1.9
	ii				
4	i	3.5	3.5	3.2	2.2
	ii	3.9	3.8		
5	i	4.8	4.5	4.0	2.6
	ii	5.4	4.9		
6	i	6.7	5.9	5.1	3.0
	ii	7.2	6.2		
7	i	8.6	7.1	6.0	3.8
	ii	9.1			
8	i	10.1	8.0	7.0	4.5
	ii	10.9	8.3		
9	i	12.4	9.2	8.0	5.2
	ii	14.1	10.2	8.7	
10	i	16.4	11.4	9.6	6.2
	ii	19.2	12.9	10.8	
11	i	22.6	14.6	12.2	7.6
	ii	26.5	16.6	13.6	
12	i	31.1	18.9	15.7	9.9
	ii	35.5	21.0	17.5	
13	i	41.8	23.9	19.9	12.2
	ii	47.0	26.3	22.1	
14	i	—	29.8	25.1	15.9
	ii		32.6	28.4	
15	i	—	37.2	32.0	19.4
	ii		41.5	35.5	

*Peak numbers refer to degree of polymerisation (D.P.) of the oligosaccharide. i and ii refer to the β- and α-anomers. Where only one retention time is shown, anomers were not resolved.

**Unmodified column.

methanol. The column to date has been used for several absorption/desorption cycles. (Another column whose performance in oligosaccharide separations had deteriorated slightly was actually improved after one adsorption/desorption cycle with TX, i.e. the retention time of IM_4 increased.)

Results for experiments involving the separation of oligosaccharides of the maltose series are summarized in Table I. On the unmodified column, the α and β anomers of all oligosaccharides above D.P. 3 are resolved (Fig. 2). [The data in Table I (IM_4 = 4.9 min) were not derived from Fig. 2, which was run under conditions such that a compact chromatogram resulted.] As the amount of TX was increased, the lower-molecular-weight oligosaccharides began to elute as single peaks. Finally, when IM_4 emerged at 3.0 min, all oligosaccharides eluted as single peaks (Fig. 3). The peaks are still well resolved and quantitative and qualitative analyses are possible. On the untreated column, peaks of the higher oligosaccharides are broad, resolved into anomers, and have inconveniently long retention times. The modified columns may also be used for preparative purposes. As the Triton remains on the column, freeze-drying of collected samples yields clean products. About 5 mg carbohydrate can be injected at one time.

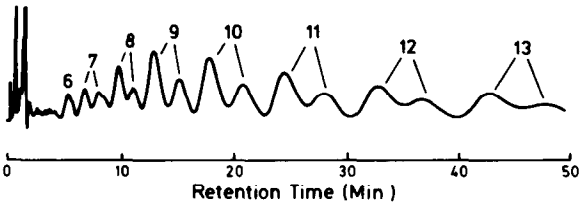

Fig. 2. Elution profile of large malto-oligosaccharides on an unmodified Dextropak column. Numbers indicate degree of polymerization (D.P.). Solvent: water; flow-rate: 2.0 ml/min.

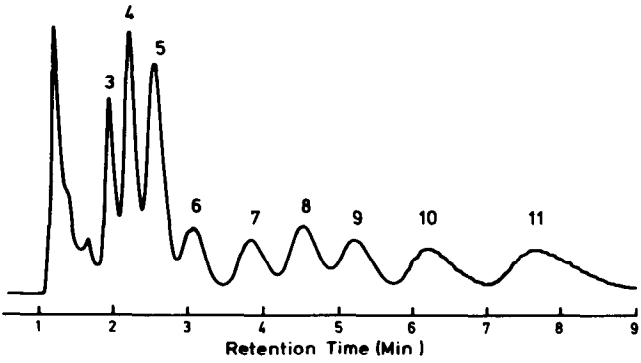

Fig. 3. Elution profile of malto-oligosaccharides on a Triton X-100 modified Dextropak column (IM_4 at 3.0 min). Numbers indicate degree of polymerization (D.P.). Solvent: water; flow-rate: 2.0 ml/min.

Similar comments may be made about the isomaltose series (Table II). Even more dramatic reductions in retention times are obtained, and resolution remains excellent (Fig. 4). Table II also shows that pumping 840 ml of water through the column does not remove the Triton X-100. In fact, a slight reduction in retention times occurs. This could be due to build up of organic compounds from the solvent.

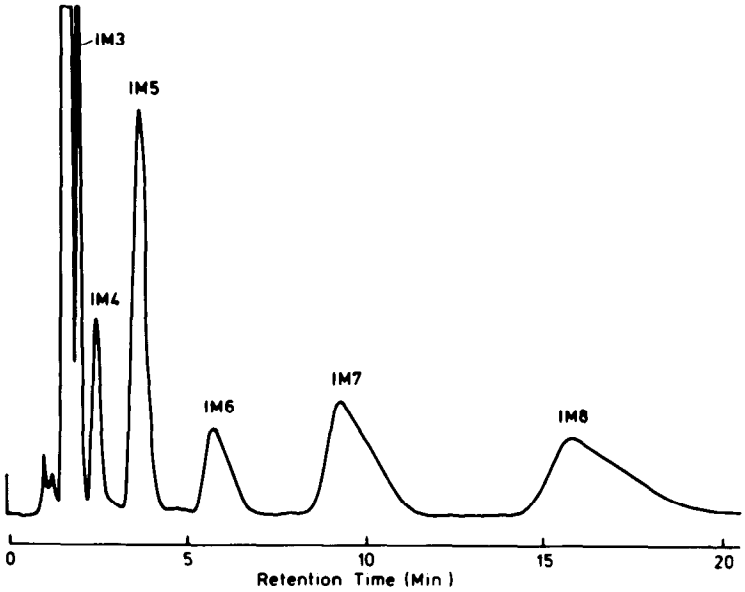

Fig. 4. Elution profile of isomalto-oligosaccharides on a Triton X-100 modified Dextropak column (IM_4 at 2.7 min). Numbers indicate degree of polymerization (D.P.). Solvent: water; flow-rate: 2.0 ml/min.

TABLE II

RETENTION TIMES FOR ISOMALTO-OLIGOSACCHARIDES ON TRITON X-100 TREATED DEXTROPAK COLUMN

Peak numbers refer to degree of polymerization (D.P.). Flow-rate: 2.0 ml/min; solvent: water.

Treatment	Retention time (min)				
	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8
1. Untreated column	5.1	9.1	17.5	29.5	66.0
2. 132 ml of 0.1% Triton X-100	3.5	5.45	9.2	15.8	28.3
3. 220 ml of 0.1% Triton X-100	2.9	4.5	7.4	12.3	21.6
4. 260 ml of 0.1% Triton X-100	2.7	3.9	6.0	9.6	16.1
5. 260 ml of 0.1% Triton X-100 + 840 ml water	2.5	3.6	5.4	8.4	14.0

The Triton modification should also be useful in reducing the retention times of other oligosaccharide series, as the column is capable of separating oligosaccharides on a structural, as well as a size, basis [1, 13]. In the case of columns used in the Radial Compression Module, increased temperatures cannot be used to decrease retention times, which is possible for steel columns [14].

Mechanism of the detergent effect

The decrease in retention times by added detergents is caused by the coating of the detergent on the C_{18} phase. As a result, the stationary phase—solute

interactions are reduced. Possible reasons for the reduced interactions include (a) reduction of the surface tension between stationary phase and the mobile phase, which affects the transfer of solute from the mobile phase to the stationary phase, and vice versa. This behaviour is characteristic of systems in which solvophobic (hydrophobic here) interactions determine the separation [17]. (b) Reduction in the size of the hydrocarbonaceous area of the stationary phase by adsorption of the detergent. This also assumes the separation mechanism to be largely hydrophobic in nature. Use of smaller alkyl chains, e.g. C_8, in reversed-phase chromatography results in shorter retention times than for C_{18} columns [18]. This effect also applies to the separation of oligosaccharides in water. The maltose oligosaccharides of D.P. 3—7 were poorly resolved on a C_8 steel column, but those of the isomaltose series were almost baseline-resolved and emerged at much earlier retention times than those on an unmodified, or even a significantly modified, column (Fig. 5 cf. Fig. 4). The Dextropak columns have a high loading of C_{18} chains (10%). Other C_{18} columns with lower loadings do not retain oligosaccharides to the same extent.

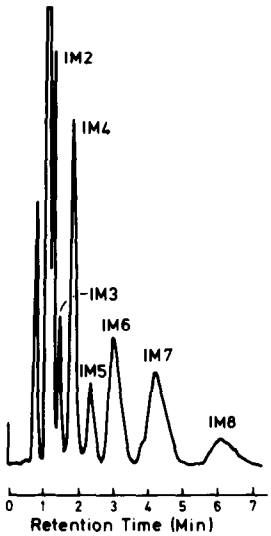

Fig. 5. Elution profile of isomalto-oligosaccharides on an unmodified steel 25 cm × 0.4 cm, octylsilane (C_8) column. Solvent: water; flow-rate: 2.0 ml/min.

Effect of mixed organic—aqueous solvents

An obvious way to reduce the retention times in reversed-phase separations is to use mixed water—organic solvents. A range of organic solvents viz. methanol, ethanol, acetonitrile, tetrahydrofuran and dimethyl formamide (DMF) were tested in amounts as low as 0.05%. Organic solvents at a concentration greater than 0.1% result in poor resolution, and up to this level they do not reduce retention times sufficiently to be useful. The results of using 0.2% methanol in water are typical (Fig. 6). The oligosaccharides elute more rapidly than in water, but are not well resolved. Use of solvents having DMF concentrations above 4% changed the separation mechanism completely. The presence of DMF reduced the solute—C_{18} and solute—silica interactions so

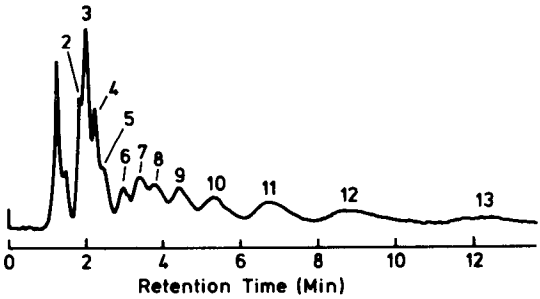

Fig. 6. Elution profile of malto-oligosaccharides on an unmodified Dextropak column. Numbers refer to degree of polymerization (D.P.). Solvent: 0.2% methanol—water; flow-rate: 2.0 ml/min.

completely that a gel permeation mode was observed, i.e. the larger oligosaccharides eluted first. Resolution in the gel permeation mode was poor, so the change in mechanism could not be put to practical use.

Effect of tetramethylurea

Tetramethylurea is reported to disrupt hydrogen bonds. It has been used in polysaccharide chemistry to improve one-step methylations by the Hakomori method, presumably by reducing hydrogen bonding between chains [19]. It was used here in an attempt to show whether hydrogen bonding to polar surface groups on the silica support was involved in the separation mechanism.

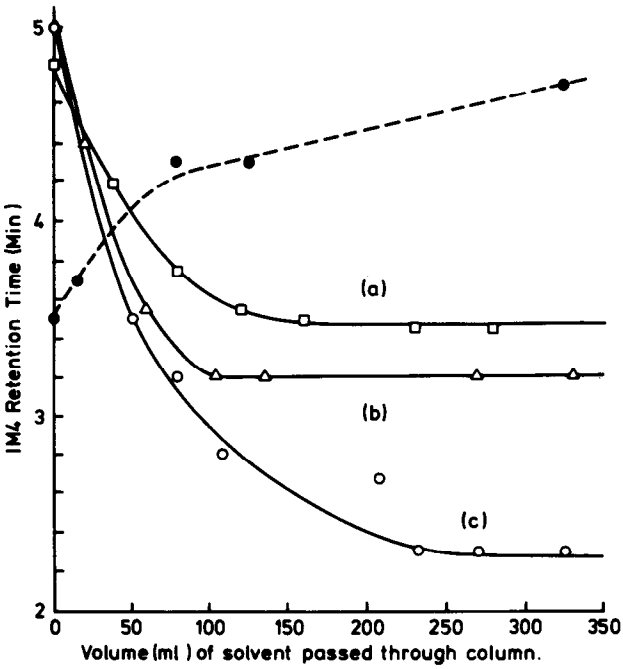

Fig. 7. Effect of tetramethyl urea (TMU) concentration on the retention time of isomaltotetraose (IM_4). (a) 0.025% TMU (□); (b) 0.05% TMU (△); (c) 0.1% (v/v) TMU in water (○); removal of 0.025% TMU with water (●). Column: Dextropak; flow-rate: 2.0 ml/min.

Results using the retention time of IM_4 as an index of column modification are shown in Fig. 7. A distinct difference between the effect of TMU and Triton is immediately evident. For the TMU, the retention time of IM_4 reaches a constant value, the magnitude of which depends on the TMU concentration in the solvent. This suggests that a dynamic equilibrium is being established, rather than a mere coating of the C_{18} as appears to be the case with detergents. A simple decrease in surface tension should have an immediate effect, as with methanol etc. The effect of TMU is reduced retention times, thus decreased hydrophobicity of the stationary phase is almost certainly involved. Involvement of hydrogen bonding in the separation mechanism has not been proven*. If it is involved, description of such hydrogen bonding by the presence of TMU would reduce retention times also, and be consistent with the above results.

TABLE III

RETENTION TIMES FOR MALTO- AND ISOMALTO-OLIGOSACCHARIDES ON A DEXTROPAK COLUMN USING 0.025% TETRAMETHYL UREA IN WATER

Flow-rate: 2.0 ml/min.

D.P.	Retention time (min)	
	Maltose series	Isomaltose series
2		2.1
3	2.8	2.6
4	3.1	3.5
5	3.7	5.3
6	4.6	8.3
7	5.4	14.3
8	6.3	—
9	7.3	—
10	8.8	—
11	10.8	—
12	14.0	—
13	18.8	—

Table III summarizes results for 0.025% TMU obtained with the maltose and isomaltose oligosaccharides. The maltose series shows single somewhat tailing peaks at this level, but well resolved. The isomaltose series in 0.025% TMU yields a chromatogram similar to that using the Triton coating in Fig. 4. The TMU-treated columns are thus useful. The major difference is that the requisite concentration of TMU must be maintained in the solvent at all times, as it is washed from the column by neat water (Fig. 7). This is a disadvantage for preparative work, but the low level of TMU present may be removed by passing the sample of oligosaccharide through a column of Sephadex G-25.

To further check for the possible involvement of hydrogen bonding, 0.1 *M* urea was used as solvent. The results (Table IV) show a large decrease in capacity factor. However, use of an ionized hydrogen bond disrupting agent, guanidine hydrochloride, up to 0.3 *M* concentration, had much less of an effect

*Editor's remark: The data presented here support the concept of tetramethyl urea as a hydrophobic bond splitting agent rather than being involved in the breakage of hydrogen bridges.

TABLE IV

RETENTION TIMES FOR MALTO-OLIGOSACCHARIDES CHROMATOGRAPHED ON A DEXTROPAK COLUMN WITH WATER, UREA AND GUANIDINE HYDROCHLORIDE SOLUTION AS SOLVENTS

Flow-rate: 2.0 ml/min; peak numbers refer to D.P.

Peak	Retention time (min)			
	Water*	0.1 *M* Urea	0.1 *M* Guanidine·HCl	0.3 *M* Guanidine·HCl
3	4.2	1.9	2.1	2.05
4	5.2	2.25	2.6	2.5
5	6.7	2.8	3.45	3.4
6	8.8	3.5	4.55	4.45
7	10.9	4.2	5.6	5.4

*In water, for D.P. 4 and above, anomers were resolved. Only the first of these has been included. A single peak was observed for each D.P. in the other solvents.

(Table IV). The latter result suggests operation of two competing effects on the capacity factor: an increase due to the ionic strength (hence surface tension) effect, and a decrease due to the disruption of hydrogen bonding.

Effect of added salts

The foregoing column treatments were designed to reduce the retention times of larger oligosaccharides. There is also a requirement for the better resolution of smaller oligosaccharides. During studies on the branching of dextrans [20], it was found that IM_4 was not resolved from a branched oligosaccharide of the same D.P., 3^3-α-D-glucopyranosylisomaltotriose (B_4).

As the work involving TX and TMU treatment was consistent with the solvophobic theory of reversed-phase chromatography, it was decided to utilize

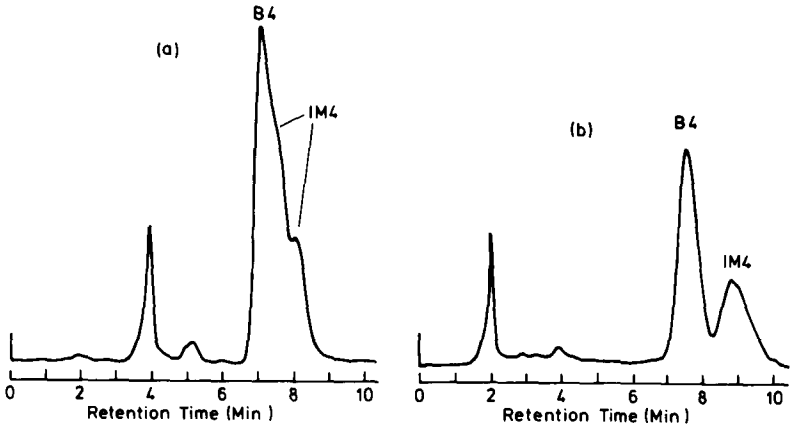

Fig. 8. Effect of the addition of ammonium sulphate on the HPLC separation of isomaltotetraose (IM_4) and 3^3-α-D-glucosylisomaltotriose (B_4). (a) Flow-rate: 1 ml/min; solvent: water. (b) Flow-rate: 2 ml/min; solvent: 1 *M* ammonium sulphate solution. Column: Dextropak.

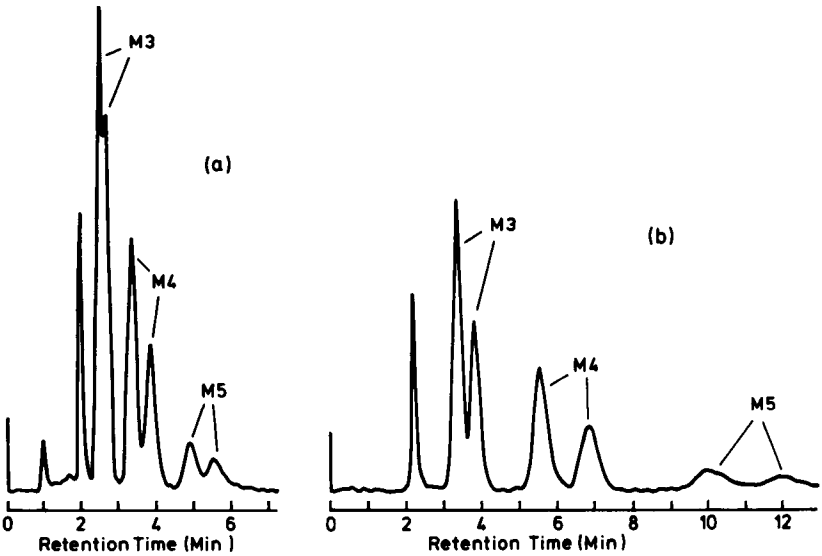

Fig. 9. Effect of the addition of ammonium sulphate on the HPLC separation of D-malto-oligosaccharides. (a) Solvent: water. (b) Solvent: 1 *M* ammonium sulphate solution. Column: Dextropak; flow-rate: 2 ml/min. Peak numbers refer to degree of polymerization (D.P.). Note resolution into α- and β-anomers.

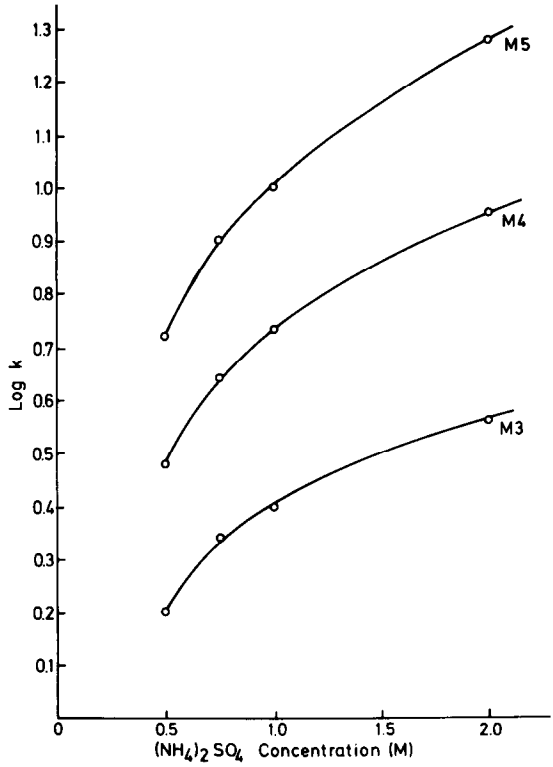

Fig. 10. The dependence of the retention factor, k, for malto-oligosaccharides M_3, M_4 and M_5 on the concentration of ammonium sulphate in the solvent. Data are for one anomer only. Column: Dextropak; flow-rate: 2 ml/min.

another prediction of that theory in order to increase the retention times of, and perhaps enhance the resolution between, IM_4 and B_4. Solvophobic theory predicts, inter alia, an increase in the retention factor of neutral solutes with an increase in ionic strength [17], the effect being due to an increase in surface tension of the solvent. The effect on the retention and resolution of IM_4 and B_4 (Fig. 8) when 1 *M* ammonium sulphate solution is used as solvent instead of water, is quite dramatic. The effect on the maltose series of oligosaccharides (Fig. 9) and other oligosaccharide separations indicate that the effect is general, and should prove extremely useful in oligosaccharide separations.

The solvophobic theory predicts that for a neutral solute, the logarithm of its capacity factor, k, should increase linearly with salt concentration. This seems to be almost so for ammonium sulphate and sodium sulphate up to 1 *M* concentration, but a curved plot is more closely followed over the range 0.5–2 *M*. Fig. 10 illustrates this point for the maltose series in ammonium sulphate solution. Similar curved plots are obtained with the isomaltose series and with sodium sulphate solution as solvent. The reason for the departure from linearity is not known. The increase surface tension of ammonium sulphate solutions is linear with concentration up to 4 *M* [21]. Perhaps as suggested above, there are hydrogen bonding or other contributions to the retention mechanism which are affected by ionic strength.

ACKNOWLEDGEMENTS

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