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ACCELERATED REVERSED-PHASE CHROMATOGRAPHY OF CARBOHYDRATE OLIGOMERS

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SUMMARY

Two different ways of accelerating analyses of glucose oligomers by reversed-phase chromatography and of improving their sensitivity are presented. A specially developed glass cartridge packed with a C_{18} bonded silica of the Separon type was used for high speed separations of maltodextrins up to a degree of polymerization of 8 at ambient temperature in 5 min using water as eluent. The effect of temperature upon the separation of carbohydrates on a conventional reversed-phase column in terms of heats of transfer, capacity factors, selectivity and column efficiency was also considered. The capacity factors decrease markedly with increasing column temperature owing to relatively high values of the heat of transfer. The non-linear increase in the heat of transfer with the degree of polymerization leads also to some reduction in the column selectivity at increased temperatures. An increase in temperature accelerates the rate of interconversion between the α- and β-anomers of saccharides, thus eliminating unwanted double peaks. The combined effects of elevated temperature result in a considerable acceleration in the separation of maltose (10 min for up to a degree of polymerization of 10) and cellobiose oligomers (10 min for a degree of polymerization of 6 at 70–80°C), which means a 2–20 fold acceleration of their analyses with a substantial gain in sensitivity.

INTRODUCTION

Separation of saccharides according to their degree of polymerization (DP) in enzymatic and acid hydrolysates of natural materials is important in biochemistry, agriculture and the food industry. For these purposes, the following techniques have been investigated: partition chromatography on cellulose[1] or ion-exchange resins[2,3],

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separation of saccharides in the form of borate complexes on anion exchangers[4,5], gel filtration on cation exchangers[6,7] or polyacrylamide gels[8,9] and chromatography on silica with chemically bonded amino phases[10,11]. Cheetham *et al.*[12] pointed out the time consuming character of the majority of these procedures together with problems arising in connection with the limited solubility of higher oligomers in mobile phases and suggested a simple procedure for the separation of oligosaccharides using a special Dextropak cartridge packed with reversed-phase bonded silica optimized for the given purpose.

In spite of this, however, even this procedure suffers from drawbacks. With respect to the practical application of the method in the food industry and the like, the separation of α- and β-anomers (which complicates the chromatogram) is of no use. Attempts at a substantial shift of the equilibrium between the anomers by using chemical agents have proved unsuccessful, with the exception of reduction. The sensitivity of the procedure is too low for the determination of glucose oligomers of the maltose series with DPs above 6, while for the determination of cellodextrins it is too time-consuming (*ca.* 30–40 min for DP 4).

We tried to eliminate these disadvantages by employing two procedures: first, by using specially prepared high-pressure glass cartridges (CGC System) packed with a covalently bonded silica reversed phase, and secondly, by using the effect of elevated temperature on the separation of saccharides on a stainless steel column of standard design packed with C_{18} silica.

While the aim of the former method was to accelerate the analyses and raise the sensitivity by increasing the efficiency of the column, the latter procedure allowed us to expect (in addition to the effects just mentioned) also a pronounced acceleration in the establishment of equilibrium between the α- and β-anomers of the saccharides undergoing separation, which might result in the elimination of double peaks. Furthermore, one might expect an increase in the linear capacity of the column[13,14] observed in the separation of other compounds on columns with chemically bonded reversed phases.

The dependence of the capacity factor on temperature is given by

$$\ln k' = \frac{\Delta H}{RT} - \frac{\Delta S^0}{R} + \ln \frac{A}{V_M}$$

where ΔH is a change in enthalpy related to the transport of solute between the phases, ΔS^0 is a corresponding change in standard entropy and A is the total surface of the sorbent in a column with a dead volume of V_M. Neither the ΔH values for the separation of saccharides on reversed-phase columns nor the respective experimental dependences of k' on temperature have so far been reported in the literature.

Similarly, data on the effect of temperature on the column selectivity in such separations are also missing in the literature. Selectivity has been observed to decrease slightly with temperature in the separations of chemically different compounds on columns with a reversed phase. This dependence may be represented by

$$\frac{\alpha_{T_1}}{\alpha_{T_0}} = e^{-\frac{T_1 - T_0}{T_1 T_0} \cdot \frac{A(\Delta H)}{R}}$$

EXPERIMENTAL

Equipment for liquid chromatography manufactured by Laboratorní Přístroje (Prague, Czechoslovakia) was used in the experiments. The instrument consisted of a high-pressure HPP 4001 linear pump, an LCI-02 septum injection valve and an LCI-20 stop–flow valve with an RIDK 101 refractometric detector. The following types of columns were used: a standard Type 6023 stainless steel column (250 × 6 mm I.D.), packed with spherical Separon six silica with a covalently bonded octadecyl stationary phase (mean particle diameter, $\bar{d}_p$ = 10 μm) and a special glass column (high-pressure CGC glass cartridge) optimized for the separation of oligosaccharides. These cartridges (150 × 3.2 mm I.D.) were packed with spherical Separon 6 silica with an optimized covalently bonded C_{18} phase ($\bar{d}_p$ = 5 μm).

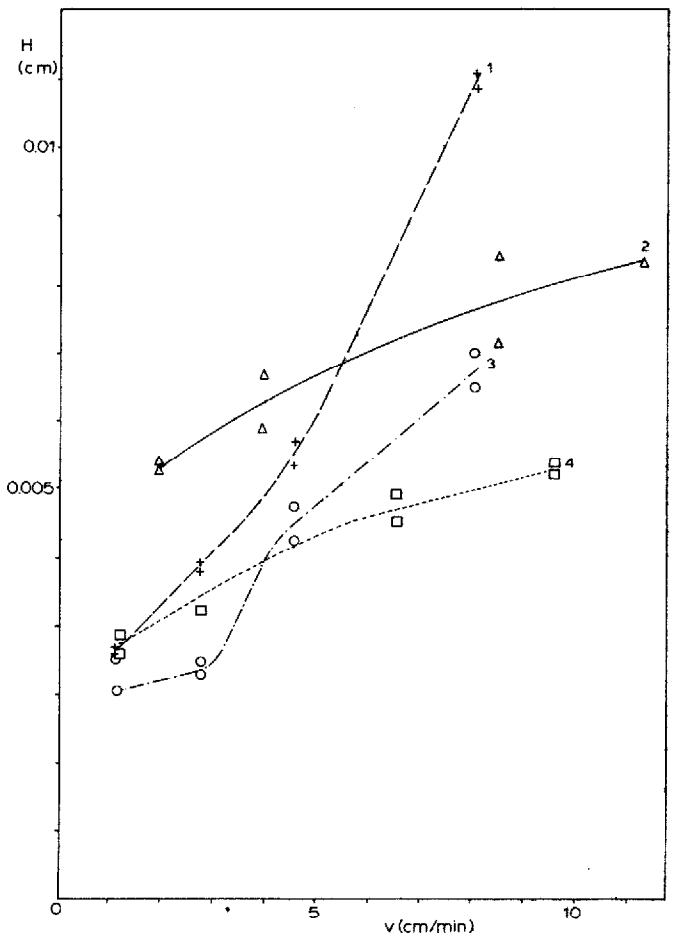

Fig. 1. Dependence of the height equivalent to a theoretical plate on linear flow-rates of eluent for various experimental conditions. Curves: 1 = maltose, metallic column, particle size 10 μm, 60°C; 2 = glucose, glass cartridge, particle size 5 μm, 24°C; 3 = glucose, metallic column, particle size 10 μm, 60°C; 4 = glucose, glass cartridge, particle size 5 μm, 60°C. Mobile phase: distilled water. Sorbent: silica C_{18}.

The preparation of standard cellodextrins has been reported elsewhere[5]. A commercial sample of starch syrup served as a source of maltodextrins. Maltose, cellobiose and glucose were supplied by Labora, maltotriose and maltotetraose were products of Koch-Light (Colnbrook, Great Britain).

The chromatographic columns were stored filled with methanol. Prior to use they were conditioned by washing with methanol, distilled water, 0.2 *M* boric acid and distilled water (25 column volumes each). The same procedure was employed for the regeneration of contaminated columns.

RESULTS AND DISCUSSION

Use of CGC glass cartridges

Glass cartridges are characterized by having plate heights which are less dependent on the linear flow-rate of the eluent (*cf.* Fig. 1) than conventional stainless steel columns, and seem therefore to be better suited for rapid separations. Using a glass cartridge, oligosaccharides can be resolved at room temperature up to DP 8 in less than 5 min. The use of a glass cartridge (3.2 × 150 mm, packed with silica C_{18}, $\bar{d}_p$ = 5 μm) gives an increase in sensitivity of approximately four to five times and a decrease in sample consumption compared with a 6.0 × 250 mm standard stainless

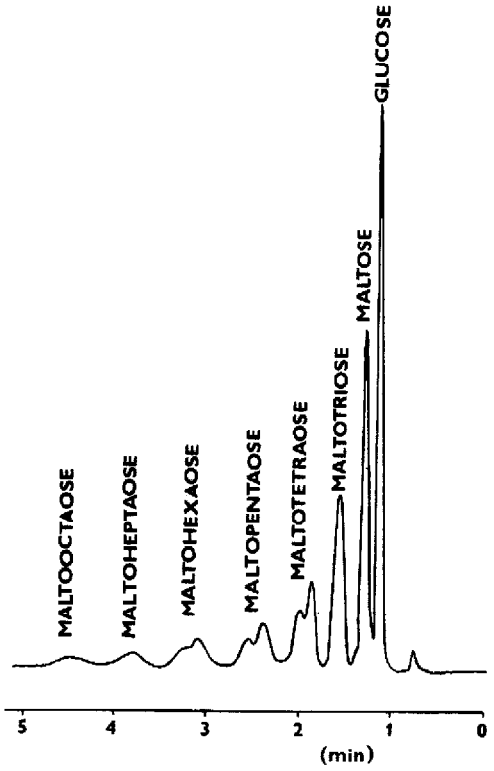

Fig. 2. Separation of maltodextrins by reversed-phase liquid chromatography. Glass cartridge, 150 × 3.2 mm. Sorbent: silica C_{18} 5 μm. Eluent: water. Flow-rate: 54.1 ml/h. Column temperature: 24°C. Pressure drop: 15 MPa. Sample introduction: stop-flow valve. Detector: refractometer.

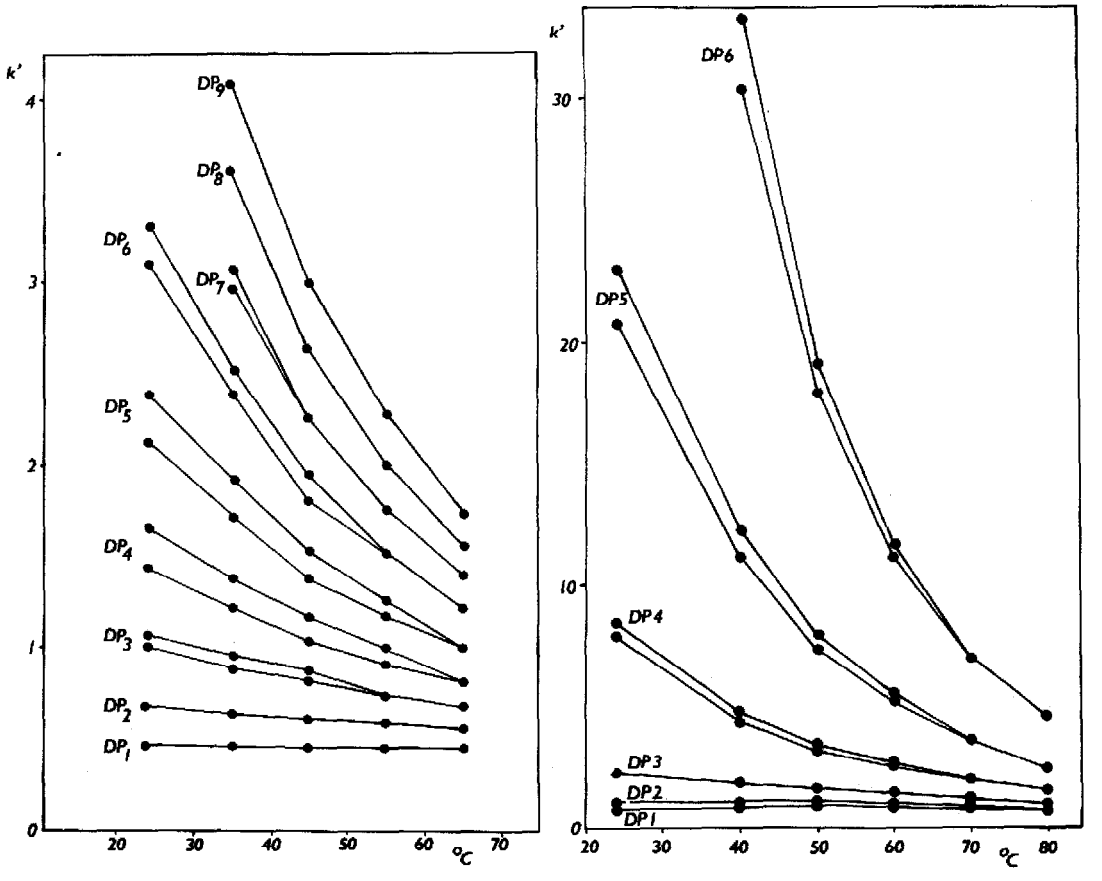

Fig. 3. Dependence of capacity factors of maltodextrins on temperature. Metallic column 250 × 6.0 mm, sorbent silica C_{18}, 10 μm, elution with water. Plots: DP_1 = glucose, DP_2 = maltose, DP_3 = maltotriose, DP_4 = maltotetraose, DP_5 = maltopentaose, DP_6 = maltohexaose, DP_7 = maltoheptaose, DP_8 = maltooctaose, DP_9 = maltononaose. Double curves for DP_{3-7} represent partial resolution between α and β anomers.

Fig. 4. Dependence of capacity factors of cellodextrins on temperature. Chromatographic conditions as for Fig. 3. Plots: DP 1 = glucose, DP 2 = cellobiose, DP 3 = cellotriose, DP 4 = cellotetraose, DP 5 = cellopentaose, DP 6 = cellohexaose. Double curves for DP 4–6 represent partial resolution between α- and β-anomers.

steel column packed with the same sorbent ($\bar{d}_p$ = 10 μm) and eluted with the same linear flow-rate of water. The time needed for the separation is approximately four times shorter than that reported in the literature[12]; however, the separation of saccharides of various degrees of polymerization remains complete. The distinction between α- and β-anomers is somewhat reduced (*cf.* Fig. 2).

Effect of increasing the column temperature

The effect of increased column temperature was checked mainly on stainless steel columns (250 × 6 mm) packed with silica C_{18}, particle size 10 μm.

The dependence of the saccharide retention on temperature was investigated

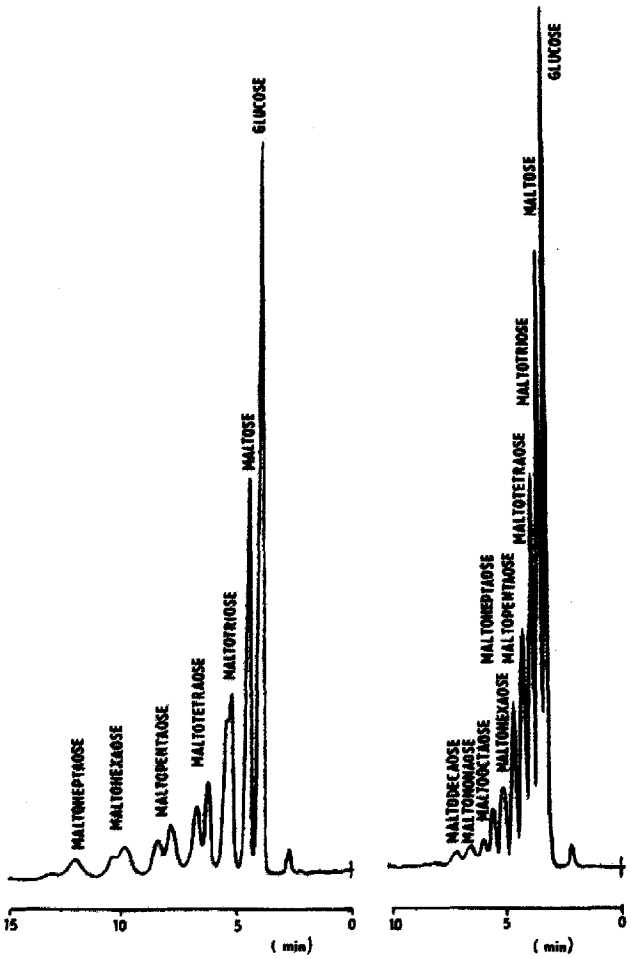

Fig. 5. Separation of maltodextrins at various temperatures. Metallic column, 250 × 6 mm. Sorbent: silica C_{18}, 10 μm. Sample injection: septum valve. Left: separation at 24°C; flow-rate of mobile phase (water), 47.4 ml/h; pressure, 1.6 MPa. Right: separation at 60°C; flow-rate of mobile phase (water), 57.6 ml/h; pressure, 1.0 MPa. Detector: refractive index.

for the maltodextrin (Fig. 3) and cellodextrin series (Fig. 4). Figs. 3–6 show that the capacity factors decrease non-linearly with increasing temperature. The steepness of the curves is larger for the cellodextrin series and increases with the degree of polymerization. The Figures also show that with increasing temperature the peaks due to the α- and β-anomers of the saccharides coincide. This may be explained, on the one hand, by a decrease in the selectivity of the column (*cf.* below) and, on the other, by an accelerated establishment of equilibrium between the α- and β-forms of the saccharides. Hence, by raising the column temperature, it is possible to achieve simultaneously an important acceleration of separation, a decrease in the column resistance against the flow and elimination of double peaks.

In the column under investigation, the doublets due to the α- and β-anomers for maltodextrins were eliminated at 60°C (*cf.* Fig. 3) while those for cellodextrins

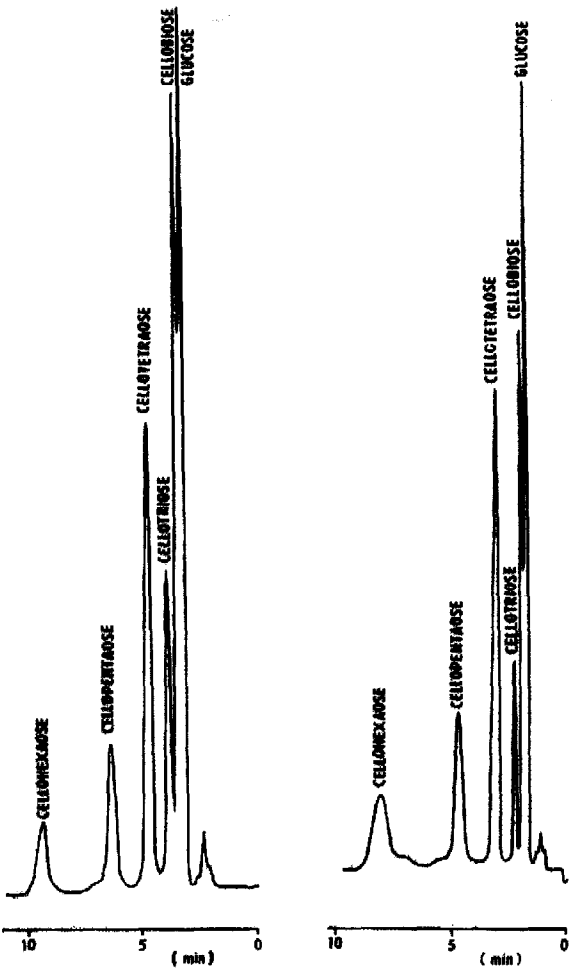

Fig. 6. Separation of cellodextrins at elevated temperature. Left: metallic column, 250 × 6 mm; sorbent, silica C_{18}, 10 μm; column temperature, 80°C; flow-rate, 57.6 ml/h; pressure, 0.8 MPa; septum injection. Right: glass cartridge, 150 × 3.2 mm; sorbent, silica C_{18}, 5 μm; column temperature, 70°C; flow-rate, 31.0 ml/h; pressure, 4.0 MPa; stop-flow injection. Mobile phase: water. Detector: refractive index.

disappeared at 80°C (*cf.* Fig. 4). While glucose can be readily distinguished from maltose at 60°C, the separation of cellobiose from glucose at 80°C and at large concentration differences may be insufficient. A somewhat better distinction between these saccharides was obtained on a glass cartridge at 70°C (*cf.* Fig. 6).

The slopes of the Van 't Hoff lines (Figs. 7 and 8) (in the maltose series there was very good linearity over the range DP 1–10, and in the cellobiose series over the range DP 3–6 observed) were used in calculating the enthalpy changes connected with the mass transfer between the phases given in Table I. In constructing the Van 't Hoff curves, the average retention values of the α- and β-anomers of saccharides were used, so that the ΔH values reflect only the effect of the chain length and bond type of the monomer units and not the effect of the orientation of the semiacetal hydroxy group.

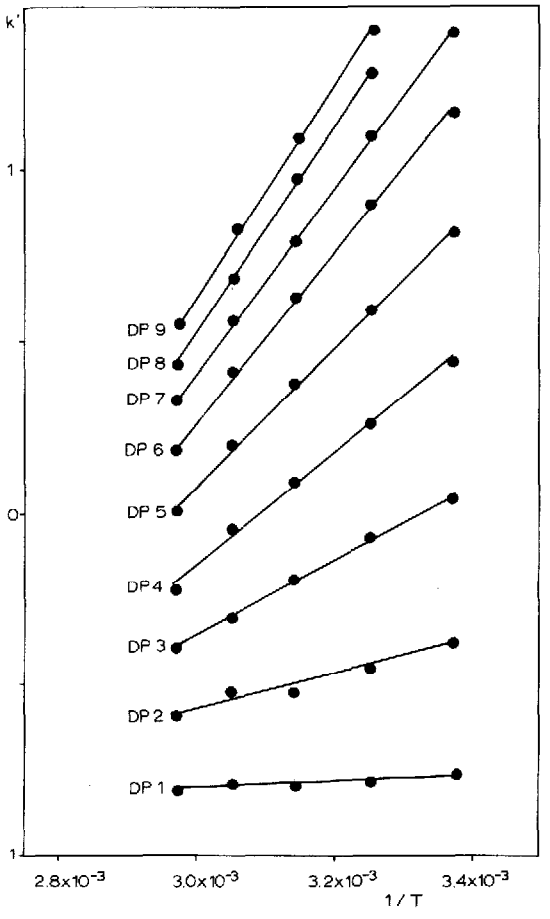

Fig. 7. Van 't Hoff lines for maltodextrin series. Chromatographic conditions and designation of saccharides as for Fig. 3.

The tabulated values are found to be partly in the range cited in the literature[13–15] for interaction of other compounds with non-polar chemically bonded phases, and partly (particularly for the cellodextrin series) outside this range. On the other hand, the enthalpy values given in Table I are greater by one order of magnitude than those given by Heyraud and Rinaudo[16] for adsorption interactions on polyacrylamide gels. Of course, on these materials the main separation mechanism occurring is gel filtration. The inverse ratio of ΔH between the maltose and cellobiose series for a given degree of polymerization (*cf.* Table I and data in ref. 16) may be explained by the inverse polarity of the stationary phase. The increase in ΔH with increasing molecular size which can be seen in Fig. 9 is in accordance with other observations[13–15], even though the dependence ought to be linear.

The comparatively high ΔH values (particularly for the cellodextrin series) explain why the effect of temperature on the saccharide retention in the reversed-phase column is so pronounced compared with gel chromatography. The fact that the

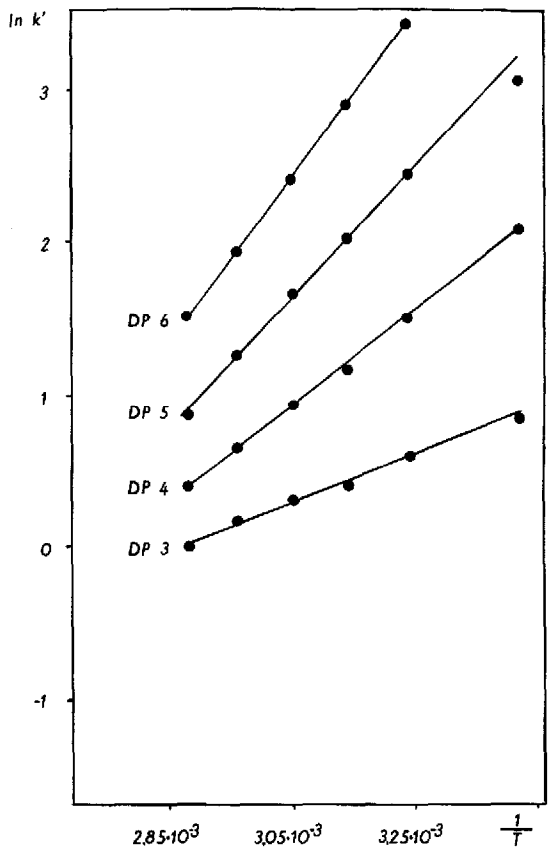

Fig. 8. Van 't Hoff lines for cellodextrin series. Chromatographic conditions and designation of saccharides as for Fig. 4. Values for DP 1 and DP 2 are not plotted owing to inaccurate reading of k' values.

TABLE I

ENTHALPY CHANGES CONNECTED WITH MASS TRANSFER BETWEEN PHASES IN THE SEPARATION OF SACCHARIDES BY CHROMATOGRAPHY WITH CHEMICALLY BONDED REVERSED PHASE

Saccharide	$-\Delta H$ *(kcal)*
Glucose	0.20
Maltose	1.05
Maltotriose	2.23
Maltotetraose	3.30
Maltopentaose	4.07
Maltohexaose	5.02
Maltoheptaose	5.41
Maltooctaose	6.05
Maltononaose	6.23
Cellotriose	3.17
Cellotetraose	6.18
Cellopentaose	8.46
Cellohexaose	10.7

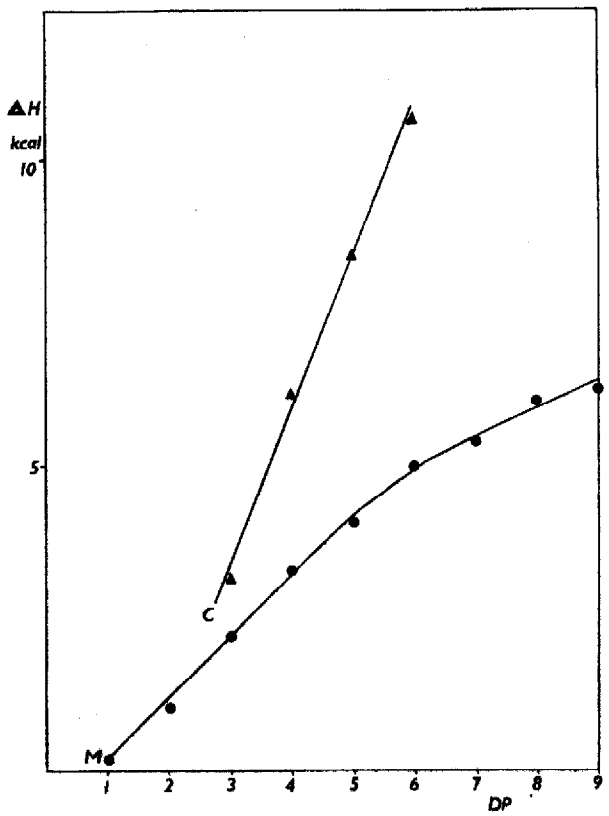

Fig. 9. Relationship between the enthalpy change in the transfer between phases and the degree of polymerization for two series of glucose oligomers. C = cellodextrins, M = maltodextrins.

differences between the ΔH values of the individual members of the maltodextrin and cellodextrin series are not zero causes the column selectivity to fall along with decreasing temperature (*cf.* the experimental dependence for the cellodextrin series in Fig. 10). For these reasons an increase in the column temperature has a negative effect on resolution in the reversed-phase chromatography of saccharides, while in the gel chromatography of saccharides an increase in the column temperature leads to the elimination of adsorption effects, raises the efficiency of the column, and hence has a positive effect of resolution.

In practice, however, in the reversed-phase chromatography of saccharides, the negative effect of increased temperature on the selectivity and capacity term in the expression for resolution is partly compensated for by an increased column efficiency (*cf.* Fig. 5) and by the removal of the effect of formation of the α- and β-anomers of the saccharides. For this reason, it is possible by, for example, merely raising the column from room temperature to 60°C, to accelerate the separation of maltodextrin by more than 50% while retaining sufficient resolution.

When applying the method separation of saccharides at elevated temperature to other types of column packings with reversed phase, the following procedure can be recommended: first, a temperature should be found at which the double peaks of

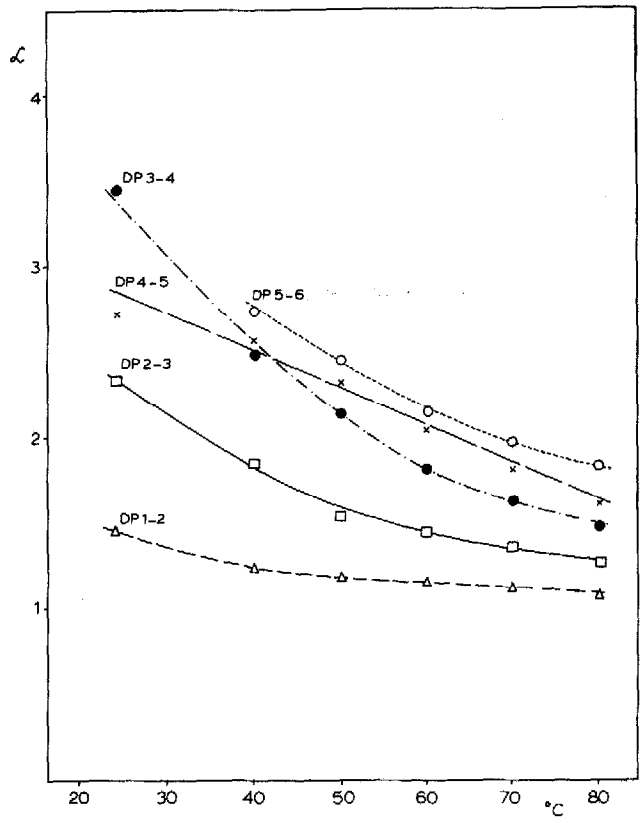

Fig. 10. Dependence of column selectivity for cellodextrins on column temperature. Chromatographic conditions as for Fig. 4.

the α- and β-anomers disappear. After that, resolution and the rate of the separation may be optimized by changing the flow-rate of the mobile phase.

It is obvious that by separation at elevated temperatures the two basic disadvantages of the method of Cheetham *et al.*[12] can be overcome. Thus, in this way, the double peaks which these authors tried in vain to remove by shifting the equilibrium between the anomers are eliminated; this facilitates both the qualitative as well as the quantitative evaluation of the chromatograms. Another contribution is the increased sensitivity, which becomes most pronounced for compounds having very high degrees of polymerization for which the sensitivity is normally the lowest. At the same time, the speed of separation increases two-fold [for maltodextrins (Fig. 5) separated at 60°C] or even by a factor of 20 [for cellodextrins (Fig. 6)] compared with at room temperature, at which temperature maltodextrins with DPs greater than 7 (low sensitivity) and cellodextrins with DPs greater than 4 (low sensitivity, time of determination greater than 1 h) can be determined only with difficulty.

Using a glass cartridge it was not possible at an equivalent linear flow-rate of the mobile phase, to achieve a comparable resolution of maltodextrins at elevated temperatures to that shown in Fig. 5. The cause can be seen in the increased role of

the extra-column contributions in the spreading of the very narrow peaks eluted from this miniaturized and very efficient column at elevated temperature. Hence, the application of this cartridge to the separation of oligosaccharides at elevated temperatures is most probably limited by the performance of the equipment used.

Other factors affecting the separation of saccharides

The initial experiments were carried out in columns which before use were washed with distilled water only. After several dozens of analyses the separation of the saccharides became poorer. However, it was found that the properties of the sorbent in the columns may be renewed by gradual washing with methanol, water, boric acid and water as described in the Experimental section. The role of boric acid in the regeneration remains obscure: it is not clear whether this compound contributes to the washing-out of some impurities from the sample or from the sorbent or whether it may be partly adsorbed on the column, affecting the interactions between the sugars and the sorbent. In glass cartridges the effect of washing with boric acid was comparatively weak, but these columns are less expensive than the traditional metal ones and it may be more reasonable to replace them with new ones rather than attempt to regenerate them.

The use of 0.05–0.2 *M* boric acid as the eluent did not have any effect on the retention of the saccharides in the chromatographic system discussed. The use of 4% methanol–water as the mobile phase strongly impaired the resolution of the saccharides by reducing the capacity factors. When 10% methanol–water was used, all the saccharides were eluted in a single diffuse peak.

CONCLUSIONS

With water as the mobile phase, the separation of oligosaccharides by reversed-phase chromatography could be accelerated in two ways. By optimizing the column for separation under standard conditions (room temperature), the separation could be markedly accelerated along with a simultaneous rise in sensitivity. The undesired resolution between the α- and β-anomers of the saccharides partly remained.

The other way became interesting when we studied the effect of the column temperature on separation. It was found that the effect of temperature on resolution is opposite to that observed in gel chromatography. The ΔH values indicate pronounced and strongly temperature-dependent interactions between the saccharides and the octadecyl stationary phase, especially with oligosaccharides of the cellodextrin series. The selectivity of the column decreases with increasing temperature on account of the ΔH differences between the individual members of the oligosaccharide series. In spite of this, however, very rapid separations of oligosaccharides may be achieved at elevated column temperatures while the effects of the formation of α- and β-anomers are completely suppressed.

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