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Note

High-performance liquid chromatography of oligosaccharides in water on a reversed phase doped with primary amino groups

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The separation of oligosaccharides contained in hydrolysates from natural polysaccharides is of interest in a number of branches of the food industry, from both the analytical and the preparative standpoints. A more or less standard high-performance liquid chromatographic (HPLC) technique[1] consists in the use of aminopropyl-bonded silica with acetonitrile–water as the mobile phase and refractometric detection. Recently, silica reversed phases have been described[2,3], specially developed for the separation of oligosaccharides in water. A common shortcoming of these sorbents at room temperature and with pure water as the mobile phase is the splitting of peaks of the individual oligosaccharides because their α- and β-anomers are separated on the columns, which complicates the chromatograms. The splitting may be removed by reducing oligosaccharides to the corresponding alditols[2] or by increasing the temperature[3], and by basic catalysis[2]. An increase in temperature decreases the resolution to such an extent that the baseline separation of peaks becomes very difficult to achieve. Basic catalysis either failed completely[2] or led[4] to incomplete suppression of double peaks, a decrease in k' values and a loss of baseline separation; if amines are employed to increase the pH, the possible losses of aldehydic sugars due to the formation of glycosyl amines are also of concern[5,6].

It is known that under the usual conditions the peak splitting caused by anomerization does not occur in the separation of sugars on aminopropyl-bonded silica. As has been shown by Verhaar and Kuster[7], an explanation is that amino groups bound on silica gel increase the rate of anomerization sufficiently.

In this study we have found that doping of the surface of the silica-based reversed phase with chemically bound primary amino groups in chromatography carried out in pure water and at room temperature has a sufficient catalytic effect on the rate of anomerization of oligosaccharides that no peak splitting takes place, and the resulting special sorbent gives a sufficient separation efficiency.

EXPERIMENTAL

Materials

Separon SIX RPS reversed phase (average particle size, $d_p = 5$ μm), optimized for the separation of oligosaccharides in pure water, was produced by Laboratory Instrument Works (Prague, Czechoslovakia). The reversed phase doped with primary

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amino groups (introduced commercially by Laboratory Instrument Works as Separon SIX RPDA) was prepared[8] by reacting Separon SIX silica (Laboratory Instrument Works) (specific surface area 450 m^2/g, d_p = 5 and 10 μm), activated by boiling with dilute HCl (1:1), with octadecyltrichlorosilane (Aldrich, Milwaukee, WI, U.S.A.) in anhydrous toluene using pyridine as catalyst[9] to a bound carbon content of at least 18%, followed by reaction of the product with γ-aminopropyltriethoxysilane (Fluka, Buchs, Switzerland) in dry toluene[10]. Starch syrup was a commercial product. The content of primary amino groups[11] was determined by a common procedure, *viz.*, titration with 0.1 *N* perchloric acid in anhydrous acetic acid using crystal violet as the indicator.

Apparatus and columns

The liquid chromatograph consisted of a VCM 300 membrane pump (Development Workshop, Czechoslovak Academy of Sciences, Prague, Czechoslovakia), an LCI 20 stop-flow injection device (Laboratory Instrument Works), an R 401 differential refractometer (Waters Assoc., Milford, MA, U.S.A.) and a Servogor 2S recorder (Goerz Electro, Vienna, Austria). CGC high-pressure glass columns (150

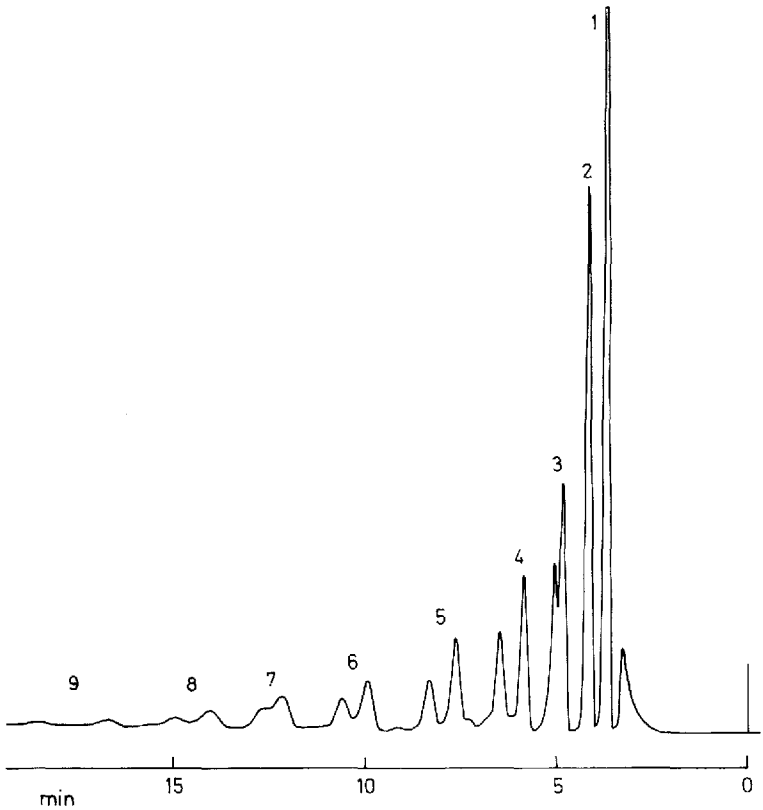

Fig. 1. Separation of starch hydrolysate on a CGC column packed with Separon SIX RPS (d_p = 5 μm). Sample: 1.5 μl of a 10% solution of hydrolysate. 1 = Glucose; 2 = maltose; 3 = maltotriose; 4 = maltotetraose; ...; 9 = maltononaose. Distilled water as the mobile phase, flow-rate 0.26 ml/min. R 401 refractometer, attenuation × 8. Room temperature.

× 3 mm I.D.) (Laboratory Instrument Works) were slurry-packed at 50 MPa in ethyl acetate. In each chromatographic run a saturation column (100 × 6 mm I.D., stainless steel, packed with Separon SIX NH_2, particle size 10 μm) was placed between the pump and the injection device.

RESULTS AND DISCUSSION

Fig. 1 shows an example of the separation of starch hydrolysate on a CGC column packed with Separon SIX RPS using pure water at room temperature. The separation of the individual oligosaccharides and their anomers is almost identical with that achieved by Cheetham *et al.*[2] using a Waters Assoc. Dextropak column, which suggests that both packings are very similar.

Fig. 2 shows a chromatogram of the same starch hydrolysate on the reversed phase doped with 0.25 mmol/g of primary amino groups; peak splitting disappeared completely. It can be seen by comparing Figs. 1 and 2 that in addition to the effect on the rate of anomerization, primary amino groups reduce the k' values of the individual oligosaccharides. However, the resolution of the individual oligosaccharides still remains more than satisfactory, and the analysis proceeds faster. According to Vrátný *et al.*[3], an increase in temperature when Separon SIX RPS is used does

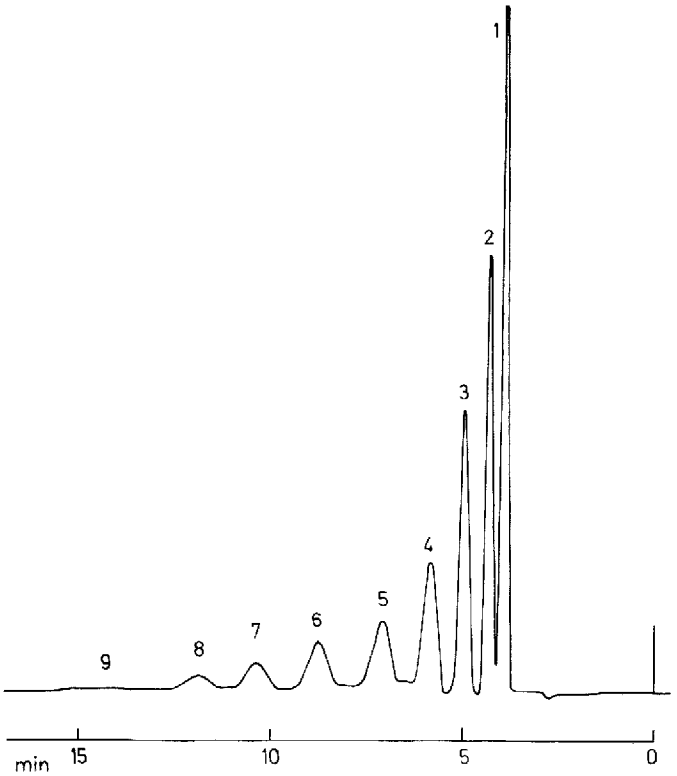

Fig. 2. Separation of starch hydrolysate on a CGC column packed with the reversed phase (d_p = 10 μm) doped with 0.25 mmol/g of primary amino groups. Flow-rate of mobile phase, 0.23 ml/min. Other conditions as in Fig. 1.

not provide such a favourable result. The finding that doping of the surface of the reverse phase reduces the capacity factors of oligosaccharides is probably due to the fact that primary amino groups reduce the hydrophobicity of the surface, which seems to play a decisive role in separation[2] and is in agreement with the results obtained by Verhaar *et al.*[4].

The main advantage of the doped reversed phase is that the rate of the analysis is increased, baseline separation remains and peak splitting in the chromatography using pure water at room temperature is removed, which is particularly important in the preparative HPLC of oligosaccharides. It should be borne in mind, moreover, that malto-oligosaccharides possess a free aldehyde group, which may[5,6] form glycosyl amines with the primary amino group. As the hydrolytic stability of glycosyl amines rapidly decreases[5,6] at pH values below 9 (the pH of a suspension of aminopropyl-bonded silica is about 9.5, whereas the doped reversed phase is approximately neutral), it may be inferred that this reaction is much less operative in this instance than with aminopropyl-bonded silica and, consequently, losses of glucose are also negligible. We did not observe any differences when we compared the chromatograms of glucose at markedly different flow-rates (similarly to the procedure employed earlier[6]).

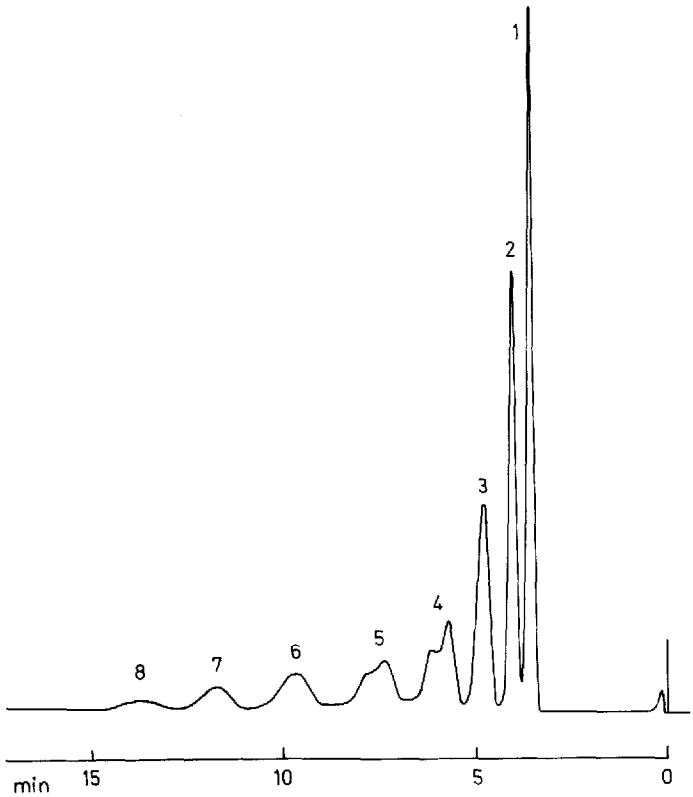

Fig. 3. Separation of starch hydrolysate on a CGC column packed with the reversed phase (d_p = 10 μm) doped with 0.16 mmol/g of primary amino groups. Flow-rate of mobile phase, 0.23 ml/min. Other conditions as in Fig. 1.

TABLE I

COMPARISON OF THE EFFICIENCIES OF COLUMNS PACKED WITH THE REVERSED PHASE CONTAINING VARIOUS QUANTITIES OF PRIMARY AMINO GROUPS AT VARIOUS FLOW-RATES OF THE MOBILE PHASE

NH_2 content (mmol/g)	*Flow-rate (ml/min)*	*Number of theoretical plates**					
		2	*3*	*4*	*5*	*6*	*7*
0.16	0.23	2440	875	–**	–**	690	–
0.16	0.11	2920	1340	–**	–**	1020	–
0.25	0.23	3150	2560	1110	915	1000	1210
0.25	0.11	3290	3190	1260	1290	1750	1800

* 2 = Maltose; 3 = maltotriose; ...; 7 = maltoheptaose.
** Double peak.

Verhaar and Kuster[12] demonstrated the connection between column efficiency and the rate of anomerization of sugars. From this viewpoint, it is of interest to compare chromatographic efficiencies under conditions where peak splitting has been suppressed completely and in part only (Fig. 3; amount of dopant 0.16 mmol/g) at different flow-rates, as summarized in Table I. The efficiencies calculated for the particular peaks increase when the flow-rate decreases and when the amount of dopant is increased. Hence, it may be inferred that even under conditions such that the rate of anomerization is increased to such an extent that no peak splitting takes place, it still affects the column efficiencies calculated from the particular peaks. It should be noted that under the conditions of slow anomerization in Fig. 1 at a flow-rate of 0.11 ml/min we obtain for maltose $N = 3960$ and for anomeric peaks of maltotetraose $N = 5120$ and 4640. The effect of anomerization on the efficiency is also confirmed by the steady increase in column efficiency at flow-rates below 0.1 ml/min; this does not appear, *e.g.*, for D-mannitol, which within the limits of experimental error and with a column having a content of amino groups of 0.25 mmol/g in the flow-rate range 0.06–0.25 ml/min gives a constant average value of $N = 3990$. This means, of course, that in the characterization of columns intended for the separation of sugars the efficiency should never be determined using sugars capable of anomerization.

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