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CHROMATOGRAPHY OF SUGARS ON DEAE-SPHERON*

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SUMMARY

Chromatography of borate complexes of sugars was carried out on diethylaminoethyl derivatives of glycol methacrylate macroporous gel, Spheron™ in borate form. The dependence of the separation of sugars on the exchanging capacity of the ion exchanger was tested. Sugar mixtures were best separated on an ion exchanger of maximum capacity 2.2 mequiv./g. The effect of the molarity of boric acid as well as the effects of temperature, flow-rate through the column, and boric acid gradient on the quality of the separation were investigated, and expressed by resolution and efficiency values (number of theoretical plates) and also by elution volume. For chromatography of the investigated mixture of sugars the following conditions were selected: buffer concentration 0.1 *M*, pH 8.5, temperature 50°, eluent flow, through a 500 × 6 mm I.D. column, 50 ml/h.

The separation of a mixture of sugars on DEAE-Spheron under these conditions was compared with the separation on Aminex A-15. The separation efficiency for individual sugars was also compared on the basis of the number of theoretical plates achieved, and the values of un-reduced heights of the theoretical plates.

INTRODUCTION

Automatic analysis of sugars is an important aid in the investigation of macromolecules of biological origin (glycoproteins, glycopeptides) and in the analysis of hydrolysates of wood, pulp and paper. Separation is effected by gas–liquid, liquid–solid, liquid–liquid and gas-partition chromatography (GLC, LSC, LLC, GPC)[1–5]. The method of liquid chromatography (LC) on ion exchangers has two basic approaches: partition chromatography of sugars, in which the sugars are distributed

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between the ion exchanger and an aqueous solution of ethanol[6], or the separation of borate complexes of sugars (Boeseken[7]) on an anion exchanger in borate form. The latter method was introduced by Khym and Zill[8–10] and developed by Kesler[11] as rapid quantitative chromatography with further improvements and uses being published by a number of investigators[12–15].

The papers on ion-exchange chromatography of borate complexes of sugars published so far most often use ion exchangers with strongly basic groups of the quaternary ammonium base type. In this paper an ion exchanger based on a microporous homogeneous matrix with strongly-basic groups (Aminex A-15) is compared with another ion exchanger based on a macroporous structure carrying more weakly-basic diethylaminoethyl groups (DEAE-Spheron). The Spheron ion-exchanging materials were already tested for the separation of biopolymers[16] and technical enzymes[17]. As shown in this investigation the DEAE-Spheron can be used under the conditions mentioned as a packing for columns in automatic analyzers used for the separation of sugars.

EXPERIMENTAL

Materials

Weak to medium-basic ion exchangers of the DEAE-Spheron type were prepared in our laboratory according to procedures published elsewhere[16]. The starting Spheron P-300, exclusion limit 500,000 Daltons for polydextran, diameter of the most abundant pores 250 Å and particle size 20–40 μm, was a product of Lachema (Brno, Czechoslovakia). The sugars, concentrated sulphuric acid and boric acid (all analytical grades) were from the same firm. Orcinol monohydrate (a.g.) was from Loba-Chemie (Wien-Fischamend, Austria). The strongly-basic Aminex A-15, X-8, granulation 17.5 $\pm$ 2 μm, capacity 3.2 mequiv./g (1.4 mequiv./ml) supplied by the producer of the analyzer, was from Bio-Rad Labs. (Richmond, Calif., U.S.A.).

Methods

Preparation of column. Before use DEAE-Spheron was regenerated with 2 *M* NaCl, 2 *M* HCl, 2 *M* NaOH and washed to neutrality. The 300 × 6 mm I.D. or 500 × 6 mm I.D. column was packed with a suspension of the equilibrated ion exchanger in the elution buffer at 25 atm, using the slurry technique. After isocratic elution the column packing was not regenerated. Between individual experiments the column was equilibrated with approx. 800 ml of buffer at 50° and a 50 ml/h throughflow. Before the comparative experiments, the DEAE-Spheron column was washed with 800 ml of a 0.355 *M* borate buffer pH 8.5, at a 50 ml/h flow-rate, then equilibrated as above. The Aminex A-15 column was washed with 800 ml of a 0.61 *M* borate buffer, pH 8.5, at the same flow-rate and also equilibrated with the elution buffer.

Buffers, sugar solutions, detection reagent. For chromatography on Aminex A-15, borate buffer of pH 8.5 was employed (10.5 g of H_3BO_3 and 42.0 g of $Na_2B_4O_7 \cdot 10H_2O$ per l). For chromatography on DEAE-Spheron, borate buffers were also used, the given molarity of which always refers to boric acid.

The mixture of sugars was prepared from stock solutions (10^{-2} *M*) in 5% isopropyl alcohol by dilution with water. Sugar mixtures (0.2 ml) containing about 30 μg of pentoses and 60 μg of hexoses were used for the injection into the column. The

orcinol reagent for detection consisted of 1 g of orcinol in 1 l of 85% H_2SO_4 stored in a brown glass bottle at 4°, for a maximum of three weeks.

Sugar analyzer. The chromatography of sugars was carried out on a modular analyzer 71000 A produced in the Developmental Laboratories of the Czechoslovak Academy of Sciences. The variant used for sugar analysis had the following parameters: a piston pump operating through a filtration insert, a 200 μl injection loop for a 500 × 6 mm I.D. column at 50°, buffer flow-rate 50 ml/h. An aliquot of the eluate, withdrawn with a peristaltic pump for colorimetry at 0.42 ml/h, was mixed with the orcinol reagent introduced at 1.92 ml/h and stirred with bubbles of air introduced at 0.42 ml/h. The absorbance of the resulting solutions was followed using a flow-through photocell at 420 nm. The degree of peak spreading in the detection system was determined by introducing 50 μl of a solution of saccharides into the stream of effluent from the column, with an injection loop inserted after the latter.

The evaluation of the results was carried out on a computer, HP 9820, using known programmes[18].

RESULTS

Dependence of the separation of sugars on the exchanging capacity of ion exchanger

A series of DEAE-Spherons of 0.2, 0.4, 0.6, 0.8, 1.2, 1.6, 2.0 and 2.2 mequiv./g capacity was prepared. Chromatography of a mixture of five sugars (trehalose, rhamnose, lyxose, ribose and arabinose) in borate buffers was tested on these derivatives

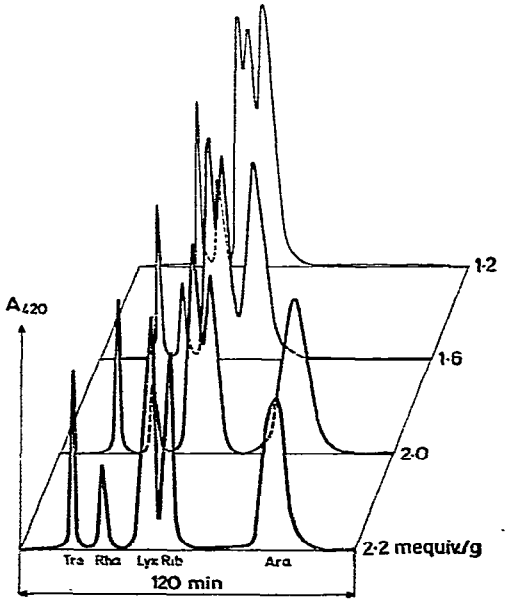

Fig. 1. Comparison of chromatographic separation of five sugars in 0.06 *M* borate buffer of pH 8.5 on DEAE-Spheron of different capacities. Column 300 × 6.0 mm I.D., flow-rate 50 ml/h. Vertical axis: record of absorbance at 420 nm for the detection using orcinol–sulphuric acid method, horizontal axis: elution time in min. The ion-exchange capacities of individual packings are given at corresponding zero lines. Other parameters are given in the Experimental part and in the legend to Fig. 2.

under comparable conditions. Fig. 1 illustrates the results achieved on the last four anion exchangers mentioned, with capacities from 1.2 to 2.2 mequiv./g. The results of chromatography on anion exchangers of lower capacity are not shown. Elution volumes of individual sugars decrease almost linearly with decreasing ion exchanger capacity, until all the sugars are eluted as a single peak in the proximity of the hold-up volume of the column on an anion-exchanger of 0.2 mequiv./g capacity. The observed linear dependence of the elution volume on the column capacity is in agreement with the equation describing the chromatographic behaviour of glycols in borate buffers on a strongly-basic anion exchanger[19]. From Fig. 1 it is evident that the best separation was achieved on the anion exchanger with the highest capacity (2.2 mequiv./g). Further experiments were carried out on an anion exchanger with a 2.0 mequiv./g capacity on a column increased from 300 mm to 500 mm.

Effect of concentration of borate ions

The chromatography of individual sugars and their mixtures was carried out in borate buffers with varying concentrations of borate, at pH 8.5. The dependence of the elution volumes and resolution of some sugars on the borate concentration in the buffer is shown in Fig. 2. The dependence of the elution volumes on the molarity of the borate has a distinct maximum for the majority of sugars at 0.025 *M* concentration. Decreasing the borate concentration below this limit causes a steep decrease in

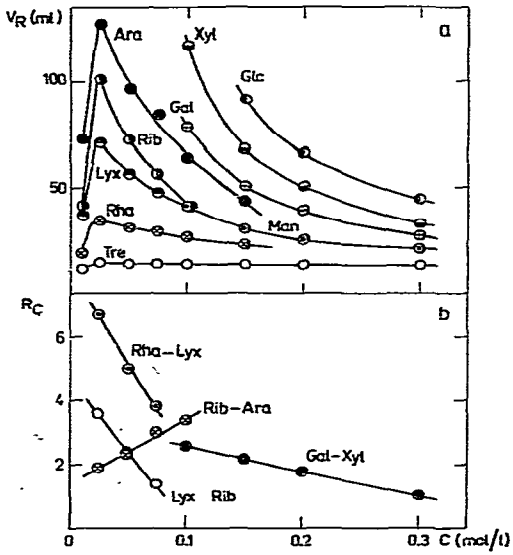

Fig. 2. Dependence of retention volume V_R (ml) of individual sugars (a), and dependence of the resolution R_C (b) on the concentration of borate in the elution buffer in chromatography on DEAE-Spheron. The retention volume is corrected with respect to the volume of the detection system. The concentration of borate buffers is given in moles/l, pH was always 8.5. The capacity of DEAE-Spheron was 2.0 mequiv./g, particle size 20–40 μm, column dimension 500 × 6 mm I.D., temperature 50°, flow-rate through the column 50 ml/h, flow through the detector 1.93 ml/h of orcinol–sulphuric acid plus 0.42 ml/h of effluent from the column. The amount of individual pentoses was about 30 μg, of hexoses about 60 μg, in 200 μl of water. Symbols of sugars: Ara = D-arabinose, Gal = D-galactose, Glc = D-glucose, Lyx = D-lyxose, Man = D-mannose, Rib = D-ribose, Rha = L-rhamnose, Tre = trehalose, Xyl = D-xylose. The resolution is corrected for the peak spreading in the detection system.

the retention of sugars on the column, while increasing the borate concentration above 0.025 *M* also leads to a decrease in the retention volumes of individual sugars. Below 0.15 *M* concentration the values of the elution volumes and the distribution constants for individual sugars are affected by two equilibria: the formation of borate complexes of sugars, and the equilibrium between the borate form of the anion exchanger and its other forms. In both equilibria a decrease in concentration of borate reduces the concentrations of the components responsible for the retention of the sugar on the column. At a borate concentration below 0.025 *M* this effect prevails over the displacing effect of borate ions. For molarities of borate higher than 0.15 *M* the decrease of elution volumes already begins to follow a linear relationship with the distribution constant and a reciprocal relationship with the concentration of borate ions in the elution medium. These relations were also derived from chromatography of glycols in borate buffers on a strongly-basic, fully dissociated, anion exchanger[19].

Fig. 2b shows the dependence of the resolution R_c for four pairs of neighbouring sugars on the borate concentration in the eluent. While for column efficiency (calculated as the number of theoretical plates) no distinct change with borate concentration was found, the resolution for individual pairs is strongly dependent on the molarity of borate. For the majority of pairs examined, the resolution decreases with increasing molarity of borate. An exception is the pair ribose–arabinose where an opposite trend was observed. For further experiments the concentrations 0.1 *M* and 0.15 *M* were selected.

Effect of pH

The effect of the pH change on chromatography of sugars in 0.1 *M* borate buffers is illustrated in Fig. 3. Within the 8.0–9.0 interval a decrease of pH of the

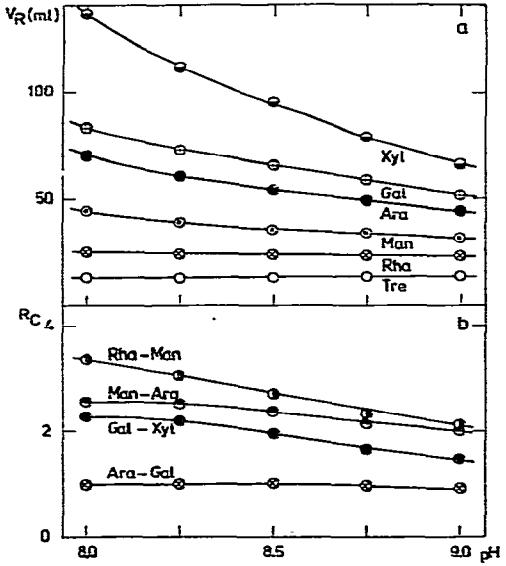

Fig. 3. Dependence of retention volumes V_R of sugars (a) and the resolution R_C (b) on pH value of borate buffers (0.1 *M*) in chromatography of sugars on DEAE-Spheron. The symbols for sugars and the conditions of chromatography are the same as in Fig. 2.

elution buffer increases the elution volumes of sugars (Fig. 3a) and also their resolution (Fig. 3b). With the exception of the slowest among the tested sugars (xylose) the increase of elution volume with pH decrease, by one unit, is not striking. At a lower pH, the time necessary for elution increases but a decrease in concentration of hydroxyl ions overlaps this unfavourable effect, because the possibility of destruction of sugars is decreased. Column efficiency, calculated as the number of theoretical plates of the column, was not affected by pH change. A mild but clear increase in efficiency with increased pH was observed for xylose only. For further experiments pH 8.5 was chosen.

Effect of temperature

In the 30–60° range the effect of temperature was observed on the elution volumes of sugars, the resolution and the column efficiency. A decrease in retention of sugars on the column with increasing temperature was noticed, while the resolution was only slightly affected by temperature (Fig. 4a,b). A decrease in retention is more pronounced when the temperature is increased from 30° to 40°, while a further increase in temperature only slightly decreases the retention volumes of the investigated sugars (Fig. 4a). An increase in column efficiency, *i.e.* in the number of theoretical plates of the column, n_c, was observed when the temperature was increased, due to an increase in the diffusion rate of the solute (Fig. 4b). In view of the thermal stability of sugars, resolving power and column efficiency, the 50° temperature was selected for further experiments.

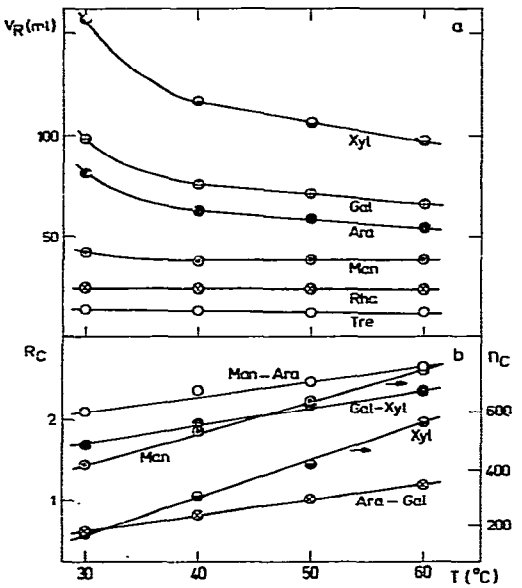

Fig. 4. Dependence of elution volume V_R (a), column efficiency, expressed by the number of theoretical plates of the column, n_c, and resolution R_C (b), on temperature in chromatography of sugars with borate buffers on DEAE-Spheron. Concentration of the borate buffer 0.1 *M*, pH 8.50, other conditions equal to those in Fig. 2.

Effect of flow-rate

In Fig. 5 the elution volumes of 6 sugars are plotted against the flow-rate. Chromatography on DEAE-Spheron was carried out at pH 8.5, a 0.1 *M* concentration of borate buffer, and 50° at a flow-rate of the elution medium varying from 106 ml/h cm² to 354 ml/h cm². With increasing flow-rate the elution volume also increased, almost linearly. In the case of the most rapid sugars (trehalose and rhamnose) the relative increase in elution volumes is considerable. This is probably due to the fact that the dynamic equilibrium in the moving chromatographic zone is affected by some of the numerous reactions of the borate anion taking place in the column, as for example the formation of a complex with a sugar, or the binding of borate or the borate complex on the anion-exchanger. Some of the reactions can be so slow that they can affect the retention of sugars at different flow-rates to a variable extent. This assumption is also confirmed by the behaviour of sugars on a DEAE-Spheron column during elution with linear concentration gradients of borate ions.

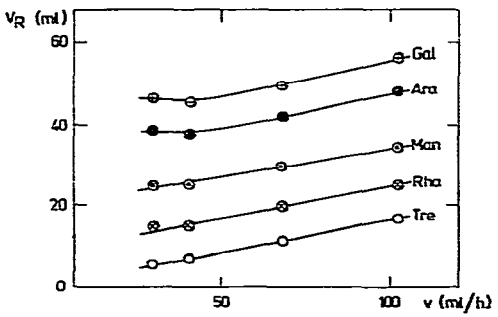

Fig. 5. Dependence of retention volumes of sugars V_R in chromatography on DEAE-Spheron, on flow-rate, *v*. 0.1 *M* borate buffer, other conditions equal to those in Fig. 2.

When the flow-rate was increased a decrease in column efficiency could be observed. This decrease is contributed to not only by the spreading of the chromatographic zone in the column but also by greater peak spreading in the detection system at a higher flow-rate. The curve of the dependence of the elution volumes of the sugars on the flow-rate (Fig. 5) are almost parallel from the 145 ml/h cm² rate; a decrease in resolution, observed at higher flow-rates, is thus caused mainly by peak spreading.

Effect of gradient

The effect of linear concentration gradients of borate ions on the elution of sugars from a DEAE-Spheron column, and the effect of the combination of these gradients, was also investigated. In Fig. 2 the dependence of the elution volumes of sugars on the molarity of borate was plotted, and for molarities higher than 0.025 it was found that, with increasing molarity, the elution volume decreases. With linear concentration gradients formed by two buffers from this region, and with a column stabilized in the weaker, starting buffer, the sugars were always eluted with shorter elution volumes than would correspond to isocratic elution with a stronger buffer. The difference was dependent on the steepness of the gradient. Gradients from 0.025 *M* up to 0.1 *M* concentration of borate, and gradients from 0.05 *M* concentration to

0.075 *M*, 0.1 *M* and 0.3 *M* were tested. The retention of sugars in gradient elutions with the above mentioned gradients increased when the column was equilibrated with a buffer of a higher borate concentration than that in the starting buffer before elution. For the mentioned gradients, from 0.05 to 0.15 *M* and from 0.05 to 0.2 *M*, the stronger terminal buffer of the gradient was always used for the equilibration of the column before chromatography. Fig. 6 shows the separation of 8 sugars achieved in 150 min by this method. The application of various gradients during the separation of sugars on DEAE-Spheron may lead to an improved separation, but the use of a gradient can hardly counterbalance the other advantages of isocratic elution. In an isocratic operation it is easier to maintain constant chromatographic conditions and, mainly, it does not require a re-equilibration of the packing after each chromatography.

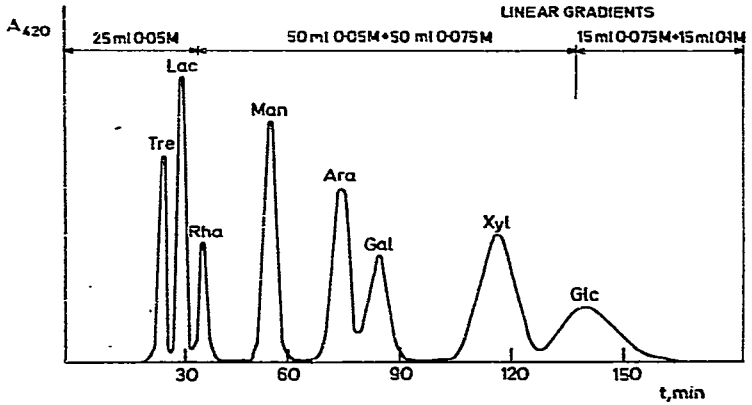

Fig. 6. Chromatography of a mixture of eight sugars on DEAE-Spheron column with concentration gradients of borate buffers. The column was equilibrated with a buffer of 0.1 *M* concentration of borate, pH 8.5. The first stage of elution without a gradient (25 ml) with a 0.05 *M* buffer was followed by two linear gradients, from 0.05 *M* to 0.075 *M*, and from 0.075 *M* to 0.1 *M* of borate. Other parameters were identical with those given for Fig. 2.

A mixture of 7 sugars (trehalose, rhamnose, mannose, arabinose, galactose, xylose and glucose) was separated under isocratic conditions with a 0.15 *M* borate buffer at pH 8.5, 50° and a flow-rate of 50 ml/h. As shown in Fig. 7, separation was achieved in 135 min when a column equilibrated with the elution buffer was used, which makes immediate chromatography of a next sample possible.

Weak to medium-basic DEAE-derivative enables elution of borate complexes of sugars with acceptable elution volumes at a lower ionic strength. From this point of view a weak anion exchanger is more suitable than a strongly-basic derivative that requires a substantially higher ionic strength, or gradients, because it binds borate complexes much more strongly.

Comparison of the separation efficiency of sugars on DEAE-Spheron and Aminex A-15

Both ion exchangers were compared in a separation of equal amounts of the same sugars under chromatographic conditions shown in Table I, and the elution volumes of the slowest sugar (xylose) were practically the same (Fig. 8). The values of

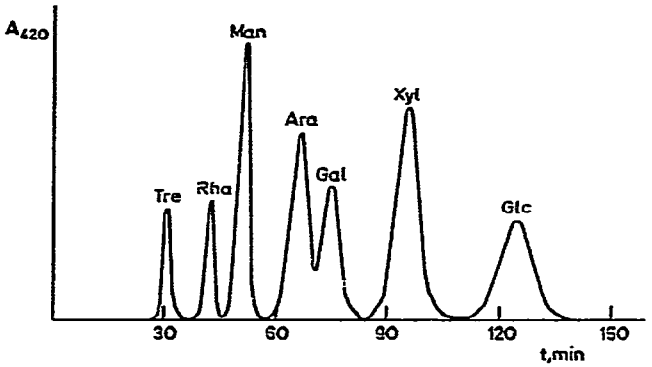

Fig. 7. Chromatography of a mixture of seven sugars using isocratic elution with a 0.15 *M* borate buffer, pH 8.5, on a DEAE-Spheron column. Other parameters are the same as those in Fig. 2.

TABLE I

CONDITIONS OF CHROMATOGRAPHY OF SUGARS ON AMINEX A-15 AND DEAE-SPHERON

	Column packing	
	Aminex A-15	*DEAE-Spheron*
Capacity for small ions (mequiv./g)	3.2	2.0
Particle diameter (μm)	17.5 ± 2	20–40
Length of ion exchanger bed (mm)	508*	475**
Inner diameter of the column (mm)	6.0	6.0
Column temperature (°C)	50.1 ± 0.1	50.1 ± 0.1
Effluent flow-rate (ml/h)	49.0–49.5	49.7–50.3
Column resistence (atm)	5.8–8.0	1.5–2.5
pH of eluent	8.80	8.50
Concentration of eluent (gramatom B/l)	0.355	0.100

* At the end of a series of analyses at operating pressure (original length 517 mm).
** At the end of a series of analyses at operating pressure (original length 489 mm).

the elution volumes, height equivalent to a theoretical plate values, the numbers of plates in the column, and the variation coefficients for the two latter quantities are given in Table II.

Comparative chromatography could not be carried out in a buffer of equal concentration owing to very different elution volumes of individual sugars on DEAE-Spheron and Aminex A-15. The sequence of the sugars in the effluent from both columns was the same (Fig. 8). Sugars with lower distribution coefficients (especially trehalose and rhamnose) exhibit relatively greater retention on Aminex A-15 (with respect to the slowest xylose) than on DEAE-Spheron, their peaks are substantially broader, and therefore the column efficiency is in these cases lower on Aminex A-15 (Table II). An exceptionally low efficiency was found for trehalose. The effect of separation on Aminex A-15 increases with increasing elution volume. The determination of the column-efficiency for individual sugars was carried out repeatedly (Table II) and it could be proved with a 95% level of probability that the efficiency of the separation of sugars on Aminex A-15 depends on the retention volume of the sugars

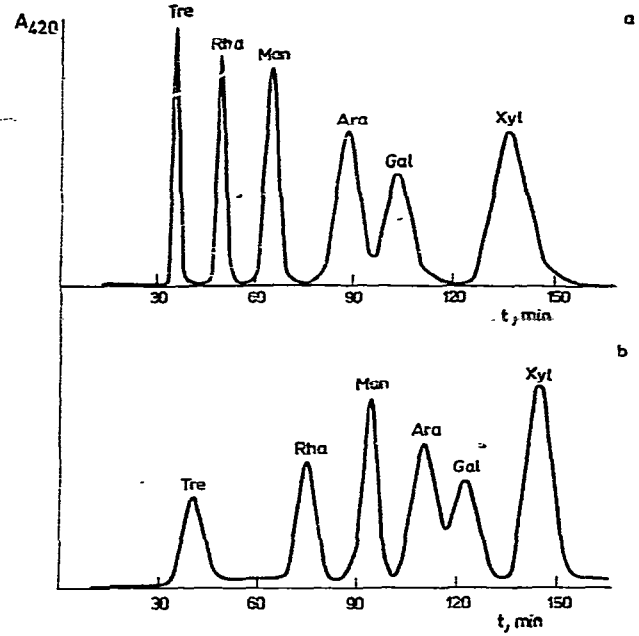

Fig. 8. Comparison of chromatography of a mixture of six sugars on columns with (a) medium-basic macroporous ion exchanger DEAE-Spheron, and (b) microporous strongly-basic Aminex A-15. The parameters for both are summarized in Table I. The column with DEAE-Spheron used for this determination was packed under different conditions from the columns used in preceding experiments. For the packing, the slurry technique was again used, flow-rate during the packing was 50 ml/h.

TABLE II

COMPARISON OF THE EFFICIENCY OF AMINEX A-15 AND DEAE-SPHERON FOR THE SEPARATION OF SUGARS BY ION-EXCHANGE CHROMATOGRAPHY WITH BORATE BUFFERS

Conditions of chromatographic comparison are given in Table I.
V_R retention volume, n_c number of plates of the column corrected for the peak spreading in the detection system, v relative standard deviation (variation coefficient), H_c height equivalent to a theoretical plate corrected for the peak spreading in the detection system, h_r reduced height of the theoretical plate.

Saccharide	*Ion exchanger*	V_R *(ml)*	n_c	v *(%)*	t *(test)*	H_c *(mm)*	h_r
Trehalose	Aminex	16.0	57.3	5.2	+	8.87	552
	Spheron	11.5	829.5	20.4		0.573	21.2
L-Rhamnose	Aminex	43.8	435.6	2.7	+	1.17	68.8
	Spheron	22.8	727.4	3.7		0.653	24.2
D-Mannose	Aminex	58.5	658.2	3.2	—	0.772	45.4
	Spheron	35.7	732.5	4.5		0.648	24.0
D-Arabinose	Aminex	does not separate sufficiently from galactose					
	Spheron	54.6	457.6	5.3		1.04	38.5
D-Galactose	Aminex	80.8	742.7	1.8	+	0.684	40.23
	Spheron	66.8	551.3	6.1		0.862	31.9
D-Xylose	Aminex	97.8	1170	5.4	+	0.434	25.5
	Spheron	94.1	611.9	2.9		0.776	28.7

tested. When DEAE-Spheron was used the effect of this factor on efficiency was not demonstrable even though differences in column efficiency have been found for some sugars. The peak spreading in the detector during the elution of trehalose from DEAE-Spheron participates up to 46% in the resulting peak width, which affects the error of measurement significantly. Therefore the values for trehalose and DEAE-Spheron in Table II are given with less reliability and were not plotted in graphs (Figs. 2b–4b). When the efficiency of the packings and the rates of flow are expressed in reduced quantities (Table II), *i.e.* in values which refer to the particle size of the packing, the higher efficiency of DEAE-Spheron in comparison with Aminex is quite evident for more rapid sugars. For a computation of the reduced quantities both values are usually used, *i.e.* both the mean particle size and the size of the largest particles of a given distribution. It was shown that the use of the second method is more correct[20]. As both chromatographic materials were fractionated in different ways, we used the average size of the particles (Aminex A-15 17 μm, DEAE-Spheron 27 μm) in the calculation of the reduced height of the theoretical plate h_r.

DISCUSSION

The Spheron type ion-exchange materials have already been used for high-performance liquid chromatography of biopolymers[16] and technical enzymes[17]. This paper demonstrates that they are also suitable for chromatography of low-molecular-weight substances, such as sugars. For the chromatography of sugars in the form of borate complexes the medium-basic diethylaminoalkyl derivatives are also suitable. The basicity of the diethylaminoalkyl group is sufficient to achieve suitable values of distribution constants in the chromatography of the complex at a lower molarity of borate. The diethylaminoethyl group had certain advantages over the strongly-basic quaternary ammonium group, comprising its higher chemical stability, especially in alkaline medium where these ion exchangers are not degraded and do not lose part of their capacity after a period of use. Sugars in borate complexes are more weakly bound on DEAE-Spheron, which enables a lower concentration of borates and an isocratic elution operation. The macroporous ion exchanger permits the use of higher through-flows and pressures owing to its mechanical strength, and the macroporous structure probably also partly decreases the mass-transport restrictions in beads, and this can increase the separation efficiency.

From the results of the comparison of DEAE-Spheron and Aminex A-15 it is evident that the use of DEAE-Spheron permits a speeding-up of the analysis by a decrease of distribution constants without a decrease of efficiency, which is impossible on Aminex A-15 because of the low efficiency of this ion exchanger at low values of distribution constants. For some dissaccharides (saccharose, cellobiose, maltose and lactose) we observed that they are chromatographed on Aminex A-15 with the same low efficiency as trehalose, while the efficiency on DEAE-Spheron was the same as for other saccharides mentioned.

CONCLUSIONS

Sugars can be separated chromatographically in the form of borate complexes not only on ion exchangers with quaternary ammonium groups, but also on more

weakly-basic ion exchangers. It was demonstrated that both the weak to medium-basic groups of DEAE-Spheron and its macroporous structure, may be of some advantage for chromatography of borate complexes of sugars, and therefore it can be used as a packing for automatic analyzers of sugars.

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