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SEPARATION OF SACCHARIDES AND THEIR ANOMERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Optimization of the separation of monosaccharides, oligosaccharides and their anomers was studied. Silica gel with chemically bonded 3-aminopropyl groups, particle size 10 μm, served as the stationary phase. The mobile phase for the separation of oligosaccharides was water–acetonitrile (50:50). In order to separate anomers, aminopropyl-silica gel in the sulphate form and water–acetonitrile (20:80) as the mobile phase were used. The temperature range 20–30° was the most suitable for the separation of mono- and oligosaccharides, and 0° for anomers.

INTRODUCTION

Paper and thin-layer chromatography (TLC) have lost their former significance in the analysis of sugars, mainly owing to their poor separation efficiencies and long analysis times. Ion-exchange chromatography on strong anion- and cation-exchange resins is widely used, but the time of analysis is often several hours. The same applies to gel permeation chromatography, which is sometimes used for separating oligosaccharides.

The separation of sugars by high-performance liquid chromatography (HPLC) is based on silica gels with chemically bonded aminopropyl groups[1–5], and the method can easily be modified for the analysis of mono-, di- and oligosaccharides. For separating sugars by HPLC some other bonded phases[6,7] can also be applied, but aminopropyl-silica gels are used most often.

Anomers of furanose and pyranose forms of various sugars can also be separated by chromatography. For gas chromatography, the formation of derivatives that cannot mutarotate or otherwise change[8,9] is crucial. The reasons why sugar anomers are not separated by paper chromatography were discussed by Jäger *et al.*[10]. TLC was used successfully to separate anomers at a decreased temperature[11]. Ramnās and Samuelson[12] separated anomers of some mono- and disaccharides at −10° on a strong anion exchanger in the sulphate form by elution with 75% aqueous ethanol. The time required for the total separation of glucose anomers was 6 h. As shown by Goulding[13], anomers can also be separated on a strong cation exchanger in the calcium or strontium form. The separation of glucose into the anomers takes only 18 min but the efficiency is low.

We have attempted to optimize the separation of sugars on aminopropyl-silica gel and to determine the factors responsible for the changes in the separation process. The influences of the mobile phase composition, temperature and specific surface area of the sorbents were studied. The separation of anomers is affected by binding of sulphate counter ion on the 3-aminopropyl groups. Thanks to the great rapidity or by decreasing the temperature, the mutarotation is suppressed and the separation is highly efficient.

EXPERIMENTAL

The liquid chromatograph was constructed from available parts. A pressure reservoir for the liquid of our own design, with a volume of 500 ml, was used as a pump. The mobile phase was forced from the pressure cylinder equipped with a reduction valve by nitrogen at a maximal pressure of 2.5 MPa. Stainless-steel tubes (100 × 4 mm I.D.) served as columns. Injection of samples was performed with a 5-μl Hamilton syringe through a silicone-rubber septum. An RIDK 101 differential refractometer (Laboratory Instruments, Prague, Czechoslovakia) served as a detector. A Varian CDS 111 integrator was used for quantitative evaluation.

Irregular graded Silasorb silica gel (Lachema, Brno, Czechoslovakia) with a particle size of 10 μm and original specific surface areas of 260 and 600 m^2/g was used for packing the column. Dried silica gel was dispersed in toluene, 3-aminopropyltriethoxysilane was added with stirring and the mixture was refluxed for 1 h, filtered and washed[3]. According to the results obtained by elemental analysis, samples with an original surface area of 600 m^2/g contained 7% of carbon and those with a surface area of 260 m^2/g contained 3% of carbon. The viscosity method was applied to column packing. A weighed amount of the sorbent was stirred in 25 ml of cyclohexanol with 5% of methanol and the suspension thus obtained was treated ultrasonically for 2 min. The suspension, with a concentration of 4–6% (w/v), was poured into a packing device to which a pump was connected, capable of feeding ethanol under a pressure of 45 MPa. The packing of one column took 15–20 min.

The mobile phase was a mixture of acetonitrile plus 15–50% of water. Prior to being filled into the pump, the mobile phase was degasified ultrasonically for 5 min. The used mobile phase was regenerated by distilling the azeotrope with a boiling point of 76.5° and a composition of 16% of water and 84% of acetonitrile under normal pressure. New mobile phase was prepared by adding the calculated amount of water.

Operating conditions

The use of 100 × 4 mm I.D. columns, sorbent with a particle size of 10 μm and a mobile phase with a high content of acetonitrile makes it possible to operate with relatively low pressures (less than 2.5 MPa) at satisfactory flow-rates (1–2 ml/min) and thus also at reasonable linear velocities of 10–20 cm/min.

In order to separate anomers, the aminopropyl-silica gel had to be converted into the sulphate form. By means of a syringe, 0.1 *N* sulphuric acid was forced through until saturation was reached. The increase in acidity was checked at the column outlet with an acid–base indicator. Sulphuric acid could be removed from the column by washing with several millilitres of 1 *N* ammonia solution.

The preparation of samples involved adjustment of the viscosity so that they

could be injected with a high-pressure syringe. The samples were conserved with methanol or isopropanol.

In order to investigate the concentrations of anomers, 10% solutions of sugars in water were prepared. All of the samples were stored in a refrigerator.

RESULTS AND DISCUSSION

Effect of mobile phase composition on the separation of sugars

Of the variable factors investigated, the content of water in the mobile phase has a significant effect on the capacity factors of sugars. Fig. 1 shows the capacity factors for various components of the test mixture for various concentrations of water in the mobile phase. The capacity factors increase considerably with decreasing content of water. The column efficiency for various components of the test mixture increases slightly with increasing water content in the mobile phase.

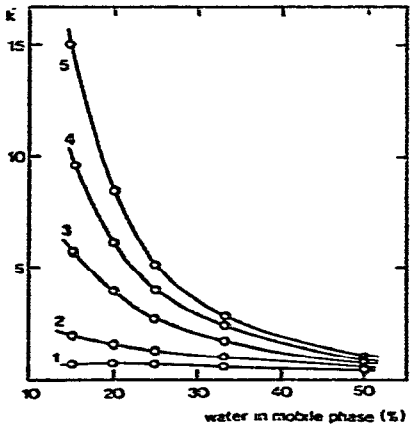


Fig. 1. Dependence of capacity factors on composition of mobile phase. Aminopropyl-silica gel with an original specific surface area of 600 m^2/g. Operating temperature 20°. 1 = Ethylene glycol; 2 = glycerol; 3 = xylose; 4 = fructose; 5 = glucose.

Effect of temperature on the separation of sugars

Temperatures in the range 20–30° are the most suitable for the separation of sugars on aminopropyl-silica gel. Increasing the operation temperature to 40° leads to tailing and at 50° the appearance of the chromatograms is changed entirely. With increasing temperature the retention decreases to a certain extent. The column efficiency increases slightly when the operating temperature is increased from 20° to 30°.

Column efficiency

The efficiencies of both columns were determined. The results obtained for the column used for the separation of anomers before conversion into the sulphate form are listed in Table I and for those on which glucose and malto-oligosaccharides were separated in Table II. The poor efficiency for glucose was caused by partial separation of the anomers. When complete separation occurs on aminopropyl-silica gel in the sulphate form, the efficiency for single anomers doubles.

TABLE I

EFFICIENCY OF SEPARATION ON A COLUMN PACKED WITH AMINOPROPYL-SILICA GEL WITH AN ORIGINAL SPECIFIC SURFACE AREA OF 600 m²/g

Mobile phase, water–acetonitrile (20:80); flow-rate, 1.6 ml/min; pressure, 2.0 MPa; operating temperature, 20°. k' = capacity factor; N = number of theoretical plates; h = reduced plate height.

Compound	k'	N	h
Ethylene glycol	0.8	1050	9.5
Glycerol	1.7	1000	10.0
Xylose	3.9	900	11.0
Fructose	6.1	1050	9.5
Glucose	8.4	750	13.3

TABLE II

EFFICIENCY OF SEPARATION ON A COLUMN PACKED WITH AMINOPROPYL-SILICA GEL WITH AN ORIGINAL SPECIFIC SURFACE AREA OF 260 m²/g

Conditions as in Fig. 2.

Compound	k'	N	h
Glucose	0.7	890	11.2
Maltose	1.2	820	12.2
Maltotriose	1.6	740	13.5
Maltotetraose	2.2	720	13.9

Separation of oligosaccharides

The column packed with the sorbent with an original specific surface area of 260 m²/g was used for the separation of oligosaccharides. The separation of malto-

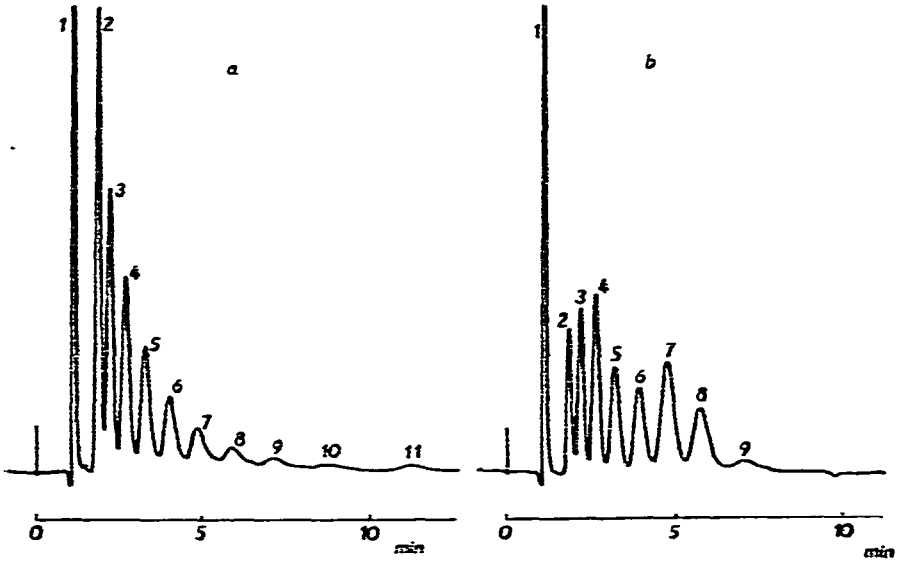


Fig. 2. Separation of malto-oligosaccharides of (a) starch syrup and (b) maltose syrup. Mobile phase, water–acetonitrile (40:60); flow-rate, 0.9 ml/min; pressure, 1.5 MPa; temperature, 20°. 1 = Water, isopropanol; 2 = glucose; 3 = maltose; 4 = maltotriose; 5 = maltotetraose; 6–11 = higher malto-oligosaccharides.

oligosaccharides from glucose to maltodecaose can be performed within 4 min using water–acetonitrile (50:50) as the mobile phase. A better separation is obtained with water–acetonitrile (40:60) within 12 min (Fig. 2a). A different distribution of malto-oligosaccharides is found in maltose syrup obtained by enzymatic hydrolysis of starch (Fig. 2b).

Separation of anomers

After converting aminopropyl-silica gel into the sulphate form, anomers can be separated chromatographically. It is interesting that the arithmetic mean of the capacity factors (k') of both anomers of one sugar is approximately the same as k' for the given sugar on free aminopropyl-silica gel. With the use of perchlorate as a counter ion no separation of anomers occurs and the capacity factors decrease significantly, *e.g.*, by 80% for glucose. Anomers are also separated on aminopropyl-silica gel in the phosphate form but the separation is less sharp than with the sulphate form.

Table III summarizes the capacity factors, separation factors at 0° and 20° and the mean relative contents of various anomers obtained by integration of peak areas. The anomer contents in aqueous solutions in the equilibrium state were taken from the paper by Pigman and Isbell[14]. Individual peaks obtained for aldoses could then be ascribed to alpha- and beta-anomers. The interpretation of chromatograms obtained by separating ketoses is difficult, owing to the lack of data and the poor separation of individual peaks.

By decreasing the operating temperature from 20° to 0° a substantial improvement in the separation of all of the sugars except glucose was obtained. As an example, Fig. 3a and b shows the separation of anomers of lyxose at these tempera-

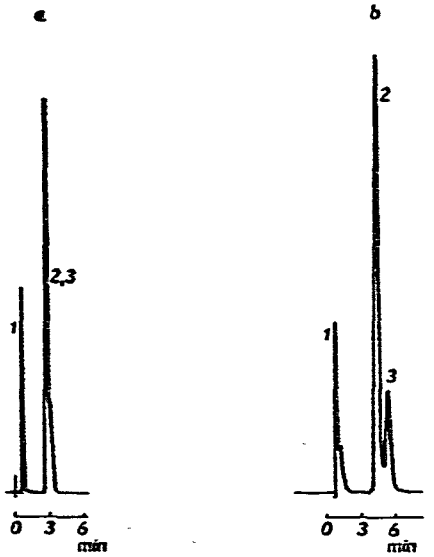


Fig. 3. Separation of lyxose anomers at (a) 20° and (b) 0°. Mobile phase, water–acetonitrile (20:80); flow-rate, 1.2 ml/min at 0° and 1.6 ml/min at 20°; pressure, 2.0 MPa. 1 = Water; 2 = α-D-lyxose; 3 = β-D-lyxose.

TABLE III

RETENTION DATA FOR SACCHARIDES AND COMPOSITION OF THE EQUILIBRIUM AQUEOUS SOLUTIONS

k_α, capacity factors of α-anomers; k_β, capacity factors of β-anomers; α, separation factor.

Saccharide	*Operating temperature 0°*			*Operating temperature 20°*			*Contents of O-pyranose in equilibrium aq. solution (%)* *		*Configuration of hydroxyl groups on C-1 and C-2 for predominant anomer*[14]	*Sequence of predominant pyranose anomer peak on HPLC separation*
	k_α	k_β	α	k_α	k_β	α	*1*	*2*		
D-Ribose	3.0**		—	2.7**		—	—	—	β c-c	—
L-Arabinose	8.5	6.5	1.30	5.6	4.1	1.36	61	73.5	α c-c	2
D-Xylose	4.7	5.9	1.24	3.4	4.4	1.28	36	34.8	β c-c	2
D-Lyxose	5.0	6.4	1.28	3.5**		—	79	76.0	α a-a	1
D-Glucose	10.2	11.3	1.11	7.6	8.8	1.16	36***	36.2	β e-e	2
D-Mannose	8.0	12.6	1.58	6.0	9.5	1.58	77	68.8	α a-a	1
D-Galactose	12.0	14.3	1.19	9.1	11.7	1.28	32	29.6	β e-e	2
L-Rhamnose	3.5	5.5	1.57	2.1	3.3	1.59	66	73.1	α a-a	1
L-Fucose	4.6	5.9	1.28	4.0	3.0	1.33	30	—	—	—
D-Fructose	7.0	9.6	1.37	6.1**		—	—	—	—	—
L-Sorbose	7.7**		—	5.7**		—	—	—	—	—
D-Tagarose	5.6	8.3	1.48	5.3**		—	—	—	—	—
Maltose	—	—	—	20.6	24.1	1.17	41***	36.8	β e-e	2
Lactose	—	—	—	25.9	29.2	1.13	38***	36.0	β e-e	2

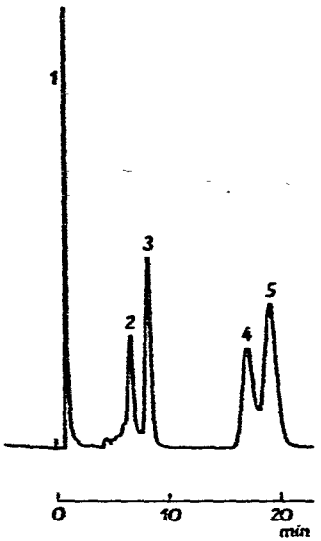
* 1, Determined by HPLC at an operating temperature of 0°; 2, rotation measurement[14].

** Anomers not separated.

*** Operating temperature 20°.

tures. Sorbose and ribose only gave one peak each, owing to the substantial predominance of the alpha-anomer in aqueous solution with sorbose, and to the rapid mutarotation of ribose.

For the aldoses under study, except talose, maltose and lactose, the interpretation of the chromatograms is unambiguous. The separation of galactose and lactose anomers is obvious from Fig. 4. Talose, which in aqueous solution exists in the furanose form to the extent of almost 50%[15], gives a complicated chromatogram. The identification would require a better resolution of individual peaks.



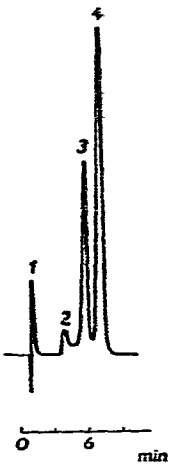


Fig. 4. Separation of galactose and lactose anomers. Conditions as in Fig. 3; operating temperature, 20°. 1 = Water; 2 = α-D-galactose; 3 = β-D-galactose; 4 = α-lactose; 5 = β-lactose.

Fig. 5. Separation of arabinose anomers. Conditions as in Fig. 3; operating temperature, 0°. 1 = Water; 2 = arabinofuranoses; 3 = β-L-arabinose; 4 = α-L-arabinose.

Furanose forms of arabinose (Fig. 5) seem to be separated, similarly to those of galactose. The first small peak has an area corresponding to a furanose in aqueous solution[15]. It is not an impurity, as the separation on aminopropyl-silica gel without sulphate as counter ion leads to only one peak with either of the sugars.

The retention sequence of anomers of various aldoses and L-rhamnose is in agreement with the suggestion by Jäger *et al.*[10] that a larger number of equatorial hydroxyl groups in the molecule of a sugar increases the retention. As can be seen from Table III, such anomers, in which the hydroxyl group on C-1 is in the equatorial position always have higher k' values.

CONCLUSION

Optimal operation conditions were determined for the separation of oligosaccharides and anomers of sugars on aminopropyl-silica gel with water–acetonitrile as the mobile phase. The retention of sugars increases with increasing content of

water in the mobile phase. The optimal operation temperature for the separation of sugars is 20–30° and for that of anomers 0°.

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