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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF DISACCHARIDES ON AMINE-BONDED SILICA COLUMNS

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SUMMARY

Twenty different disaccharides (cellobiose, gentiobiose, isomaltose, kojibiose, lactose, lactulose, laminaribiose, leucrose, maltose, maltulose, melibiose, nigerose, palatinose, sophorose, sucrose, α,α-trehalose, α,β-trehalose, β,β-trehalose, turanose, and xylobiose) were subjected to high-performance liquid chromatography on prepacked amine-bonded silica columns, using acetonitrile–water eluents. Glucopyranosyl-glucoses had increasing retention times in the order of linkage (1→3), (1→4), (1→2) and (1→1), and (1→6). Replacement of one of the glucosyl residues by galactose led to longer retention times, while substitution by a fructosyl residue yielded shorter ones. Forced assumption of the furanose ring form by the fructosyl residue, as in sucrose and palatinose, gave greatly reduced retentions.

INTRODUCTION

The need for rapid quantitative analysis of carbohydrate mixtures has led to use of gas chromatography (GC) and high-performance liquid chromatography (HPLC) in place of paper and thin-layer chromatography and low-pressure liquid chromatographic columns. GC of derivatized carbohydrates is an accurate and sensitive method, but sample preparation and derivatization are time-consuming.

Non-derivatized carbohydrates have been analyzed by HPLC chiefly with columns containing one of three materials: a strong-acid ion exchanger on a polystyrene–divinylbenzene matrix with Ca^{2+} or Ag^{+} counter-ions[1,2], an octadecyl moiety chemically attached to silica[3], and an amino moiety linked chemically or physically to silica. Ion exchangers can separate some monosaccharides from each other, but are more suited for separations of carbohydrates of different numbers of monosaccharide residues. Octadecyl columns are better at separating trisaccharides and longer chains. Amino columns, on the other hand, have the ability to separate carbohydrates of either the same or different molecular weight.

Since their first use for carbohydrate analysis by Linden and Lawhead[4] and by Palmer[5], amino columns have been employed for the separation of many sugars[6]. However, much of the published research has dealt primarily with monosaccharides or sugar alcohols; work on oligosaccharides has been limited mainly to lactose, su-

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crose, raffinose, and members of the maltooligosaccharide series. Only a few papers have deviated from this pattern in their treatment of the analysis of underivatized oligosaccharides with an amino column[4,7]. As yet there has been no investigation where a sufficiently large number of oligosaccharides of the same chain length have been separated to discern fully the influence of type of linkage, ring structure, and sugar residue on the retention.

For that reason, we studied in this work the separation of twenty disaccharides, using two commercially prepacked aminopropyl-bonded silica columns with various compositions of acetonitrile–water eluent. These disaccharides contain all possible glycosidic bonds linking a glucopyranosyl unit to another glycosyl residue; in addition, a number of disaccharides containing fructosyl, galactosyl and xylosyl residues were tested.

EXPERIMENTAL

Apparatus

The chromatographic apparatus consisted of a Waters (Milford, MA, U.S.A.) Model 6000A solvent delivery system, a Model U6K injector, and a Model R401 refractive index detector. The detector output was recorded on an Omniscribe (Houston Instruments, Austin, TX, U.S.A.) recorder and Varian (Palo Alto, CA, U.S.A.) CDS111C integrator. The two aminopropyl-bonded silica columns employed in this work were a DuPont (Wilmington, DE, U.S.A.) Zorbax-NH_2 (column T 2535) and a Supelco (Bellefonte, PA, U.S.A.) Supelcosil LC-NH_2 (column 91,164), both 250 × 4.6 mm I.D. Some of the column packing properties are summarized in Table I. Each column had *ca.* 7000 plates measured with sucrose at a flow-rate of 1 ml/min of 75% aqueous acetonitrile. A Bio-Rad (Richmond, CA, U.S.A.) Bio-Sil NH_2 precolumn, 40 × 4.6 mm I.D., was used with both columns.

Materials

Solvent mixtures were prepared with Fisher (Pittsburgh, PA, U.S.A.) HPLC grade acetonitrile and distilled water purified with the Barnstead (Boston, MA, U.S.A.) Nanopure II System.

Seventeen disaccharides (β-cellobiose, β-gentiobiose, isomaltose, α-kojibiose, α-lactose, lactulose, laminaribiose, maltose, maltulose, α-melibiose, nigerose, palatinose, α-sophorose, sucrose, α,α-trehalose, turanose, and xylobiose) were obtained as

TABLE I

CHARACTERISTIC COLUMN PACKING PROPERTIES*

Property	*Zorbax-NH_2*	*Supelcosil LC-NH_2*
Particle shape	Spherical	Spherical
Average particle size (μm)	7	5
Specific surface area (m^2/g)	300–350	160–180
Pore diameter of parent silica (nm)	6–7	9–13
Aminopropyl surface concentration (μmol/m^2)	*ca.* 3.6	*ca.* 2.8

* Information obtained from the suppliers.

described previously[8]. Leucrose was donated by Professor John F. Robyt of Iowa State University, α,β-trehalose by Dr. Fred W. Parrish of the Southern Regional Research Center, and β,β-trehalose by Dr. Riaz Khan of Tate & Lyle.

Chromatographic conditions and measurements

All separations were carried out at 23 ± 1°C with a flow-rate of 1.0 ml/min. Acetonitrile–water mixtures, prepared daily, were filtered and degassed before use. Samples were 1% disaccharide in water, and 10-μl portions were usually injected.

Capacity factor values [$k' = (t_R - t_0)/t_0$] were calculated from retention time (t_R) and column dead time values (t_0). Values of t_0 were determined from the retention time of the water peak. Resolutions between adjacent peaks were calculated with the equation $R_s = 2(t_{R2} - t_{R1})/(w_1 + w_2)$, where w_1 and w_2 are baseline peak widths.

RESULTS AND DISCUSSION

Capacity factor and resolution values of disaccharides analyzed on Supelcosil LC-NH_2 and Zorbax-NH_2 columns are presented in Table II. Disaccharides were

TABLE II

CAPACITY FACTOR (k') AND RESOLUTION (R_s) VALUES FOR DISACCHARIDES CHROMATOGRAPHED ON SUPELCOSIL LC-NH_2 AND ZORBAX-NH_2 COLUMNS WITH AQUEOUS ACETONITRILE

Disaccharide	*Supelcosil LC-NH_2*				*Zorbax-NH_2*			
	Acetonitrile (%)				*Acetonitrile (%)*			
	75		*80*		*72*		*77*	
	k'	R_s	k'	R_s	k'	R_s	k'	R_s
Xylobiose [O-β-D-xylopyranosyl-(1→4)-D-xylose]	1.00		1.36		1.80		2.32	
Sucrose [O-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside]	1.41	0.9	2.22	1.4	2.41	1.2	3.49	1.3
Turanose [O-α-D-glucopyranosyl-(1→3)-D-fructose]	1.53	0.1	2.47	0.2	2.65	0.1	3.80	0.4
Palatinose [O-α-D-glucopyranosyl-(1→6)-D-fructose]	1.54	0.7	2.50	0.9	2.67	0.6	3.89	0.8
Maltulose [O-α-D-glucopyranosyl-(1→4)-D-fructose]	1.63	0.4	2.65	0.6	2.81	0.1	4.09	0.4
Leucrose [O-α-D-glucopyranosyl-(1→5)-D-fructose]	1.68		2.76		2.83		4.20	
Laminaribiose [O-β-D-glucopyranosyl-(1→3)-D-glucose]	1.46	0.9	2.41	1.3	2.61	1.0	3.77	1.5
Nigerose [O-α-D-glucopyranosyl-(1→3)-D-glucose]	1.66	0.1	2.77	0.0	2.93	0.1	4.31	0.2
Cellobiose [O-β-D-glucopyranosyl-(1→4)-D-glucose]	1.68	0.0	2.77	0.1	2.96	0.1	4.36	0.1
Maltose [O-α-D-glucopyranosyl-(1→4)-D-glucose]	1.68	0.2	2.80	0.3	2.98	0.3	4.38	0.4
Sophorose [O-β-D-glucopyranosyl-(1→2)-D-glucose]	1.72	0.2	2.92	0.3	3.05	0.4	4.50	0.5
α,β-Trehalose [O-α-D-glucopyranosyl-(1→1)-β-D-glucopyranoside]	1.76	0.4	2.98	0.6	3.12	0.3	4.61	0.9
Kojibiose [O-α-D-glucopyranosyl-(1→2)-D-glucose]	1.83	0.2	3.14	0.3	3.19	0.1	4.85	0.2
α,α-Trehalose [O-α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside]	1.86	0.6	3.22	1.1	3.21	0.7	4.91	1.1
β,β-Trehalose [O-β-D-glucopyranosyl-(1→1)-β-D-glucopyranoside]	1.95	0.3	3.42	0.4	3.34	0.7	5.16	0.8
Isomaltose [O-α-D-glucopyranosyl-(1→6)-D-glucose]	2.00	0.3	3.51	0.4	3.50	0.5	5.39	0.7
Gentiobiose [O-β-D-glucopyranosyl-(1→6)-D-glucose]	2.07		3.64		3.65		5.65	
Lactulose [O-β-D-galactopyranosyl-(1→4)-D-fructose]	1.82		3.03		3.09		4.63	
Lactose [O-β-D-galactopyranosyl-(1→4)-D-glucose]	1.96	1.6	3.31	2.3	3.48	1.3	5.22	2.4
Melibiose [O-α-D-galactopyranosyl-(1→6)-D-glucose]	2.25		3.94		3.87		6.02	

best separated by the Supelcosil LC-NH_2 column using between 75% and 80% aqueous acetonitrile, with 80% acetonitrile being optimal. Similarly, the best composition range for the separation of disaccharides by the Zorbax-NH_2 column was between 72% and 77%, with the optimal resolution values being obtained at 77% acetonitrile in water. In general, increasing the percentage of acetonitrile in the mobile phase led to better resolution, but at the cost of extended elution times. However, increasing the acetonitrile concentration above the upper limit of the optimal eluent composition range for the specific column caused significant increase in peak width and peak tailing, resulting in negligible improvement of resolution.

Resolution values of disaccharides given in Table II indicate no appreciable difference in separation efficiencies between Supelco LC-NH_2 and Zorbax-NH_2 columns. The higher aminopropyl concentration of the Zorbax packing (Table I) resulted in higher k' values and a better resolution of disaccharides than those obtained on the Supelcosil LC-NH_2 column at the same eluent composition. On the other hand, almost comparable resolution values in much lower retention times could be obtained with the Supelcosil LC-NH_2 column by using higher acetonitrile concentrations.

Although capacity factors were a function of the mobile phase composition and the column used, elution order was not appreciably affected by either. Elution order of disaccharides with the same glycosidic bond was governed by the identity of the monosaccharide units that constituted the disaccharide molecule. When a glucosyl residue made up one part of the molecule, retention time varied with the other constituent in the order fructose < glucose < galactose, as observed with the individual monosaccharides[5,9]. Accordingly, elution order was turanose < nigerose, maltulose < maltose, cellobiose < lactose, and palatinose < isomaltose < melibiose.

The order of elution among the glucopyranosyl-glucoses is striking: first the (1→3)-linked disaccharides, then those bonded (1→4), then a mixture of those linked (1→2) and (1→1), and finally the (1→6)-bonded compounds, the only ones linked through a primary hydroxyl group. These findings hold for the only two galactopyranosyl-glucoses tested, with lactose eluting before melibiose. However, extrapolation to the glucopyranosyl-fructoses fails because of the forced assumption of the furanosyl ring form by the fructosyl residues in sucrose and palatinose, which leads to low retention times in amino columns[10]. This causes earlier-than-expected elution of these two compounds within the glucopyranosyl-fructose group, whose other three members have fructose residues partially (turanose and maltulose) or almost completely (leucrose) in the pyranosyl form[11]. To the extent that the disaccharides tested coincide with those of Baust *et al.*[7] (ten cases), elution order agrees with the exception of cellobiose.

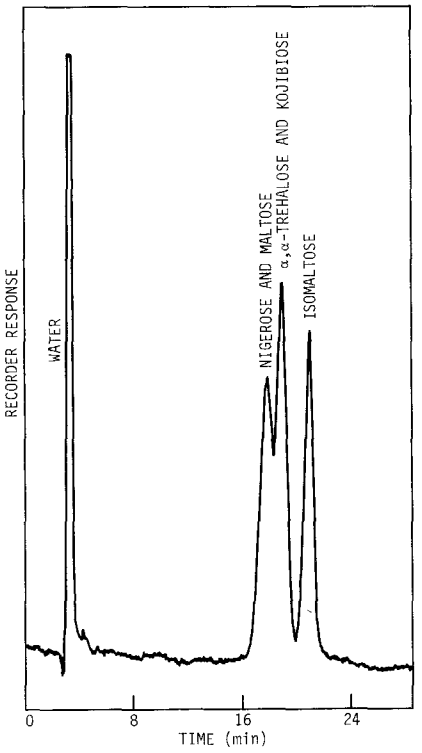
Whether separation of disaccharides on amine-modified silica columns is caused by partitioning between the water-enriched stationary phase layer and the mobile phase[12,13] or by adsorption via hydrogen bonding between the hydroxyl groups of the carbohydrate molecule and the amino groups of the stationary phase[14,15], the delayed elution of disaccharides linked through primary hydroxyl groups appears to be caused by the more easy rotation of these compounds about their glycosidic bonds and their consequent lower free energy.

For disaccharides possessing (1→2), (1→3), and (1→4) links, those with β-bonds eluted before those with α-bonds, as would be expected from the results of

earlier work with alkyl and aryl disaccharide derivatives[10]. This pattern is not upheld by (1→1)- and (1→6)-linked monosaccharides, however. This variation in the order of elution leads to more complete separation of β-linked glucopyranosylglucoses from each other than of α-linked ones (Figs. 1 and 2).

In addition to capacity factors, peak widths of disaccharides vary markedly (Table III). So long as mutarotation rates are high, anomeric forms of the same reducing sugar will elute as one peak with a capacity factor equal to the sum of the weighted capacity factors of the individual forms[16]. However, peak width will increase with increasing differences in retention times between the anomeric forms, with decreasing interconversion rates between them and, of course, with increasing overall peak retention times. One would therefore expect sharper peaks as a function of k' for those disaccharides that are completely or almost completely in one form and for those with rapidly mutarotating anomeric fructosyl residues. Among the first group, this is certainly true for the non-reducing sugars α,α-, α,β-, and β,β-trehalose, more so than for sucrose. Among the second group, lactulose has somewhat narrow peak widths, considering its retention time, while peak widths for turanose, palatinose, maltulose, and leucrose are within the normal range.

To determine the influence of differences in anomeric retention times on peak



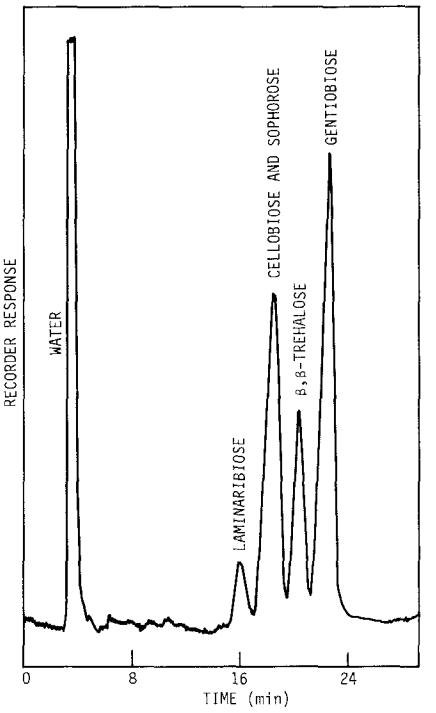


Fig. 1. Elution pattern of α-linked disaccharides from a Zorbax-NH_2 column. Mobile phase, acetonitrile–water (77:23); flow-rate, 1.0 ml/min.

Fig. 2. Elution pattern of β-linked disaccharides from a Zorbax-NH_2 column. Conditions as in Fig. 1.

TABLE III

PEAK WIDTHS (w_i/t_0) FOR DISACCHARIDES CHROMATOGRAPHED ON SUPELCOSIL LC-NH_2 AND ZORBAX-NH_2 COLUMNS WITH AQUEOUS ACETONITRILE

Disaccharide	*Supelcosil LC-NH_2*		*Zorbax-NH_2*	
	Acetonitrile (%)		*Acetonitrile (%)*	
	75	*80*	*72*	*77*
Xylobiose	0.11	0.15	0.18	0.23
Sucrose	0.11	0.15	0.18	0.23
Laminaribiose	0.19	0.25	0.32	0.34
Turanose	0.16	0.20	0.22	0.24
Palatinose	0.13	0.16	0.22	0.24
Maltulose	0.14	0.18	0.21	0.24
Leucrose	0.14	0.18	0.21	0.24
Nigerose	0.24	0.30	0.32	0.37
Cellobiose	0.16	0.23	0.24	0.28
Maltose	0.19	0.25	0.26	0.32
Sophorose	0.16	0.22	0.18	0.26
α,β-Trehalose	0.16	0.22	0.16	0.22
Lactulose	0.16	0.20	0.21	0.24
Kojibiose	0.17	0.28	0.26	0.29
α,α-Trehalose	0.16	0.20	0.17	0.22
Lactose	0.17	0.25	0.27	0.29
β,β-Trehalose	0.16	0.22	0.18	0.24
Isomaltose	0.21	0.29	0.28	0.35
Gentiobiose	0.22	0.30	0.32	0.37
Melibiose	0.20	0.30	0.34	0.38

widths, the Zorbax-NH_2 column was treated with 0.1 *M* hydrochloric acid, similar to the sulfuric acid treatment by Kahle and Tesařík of an amino column to decrease the mutarotation rate[17]. Use of 1 ml/min of an aqueous eluent containing 72% (v/v) acetonitrile of 23°C caused anomeric peak separation of all reducing disaccharides except sophorose and those containing fructose. Widest separation occurred with xylobiose, nigerose, laminaribiose, and melibiose, where differences between k' values of the two anomeric peaks exceeded 10% of their average value. The first three disaccharides yielded the broadest peaks on untreated Zorbax-NH_2 and Supelcosil LC-NH_2 columns, when k' is taken into account.

In conclusion, use of amine-bonded silica columns to separate disaccharides by HPLC is feasible if the mixture is not too complex. Separation of highly complex mixtures is best accomplished by capillary GC following trimethylsilylation of the disaccharides, since the number of theoretical plates that can be feasibly obtained is much greater and the range of retention times is somewhat larger[8].

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