Journal of Chromatography, 321 (1985) 393–399
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,413

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

ZIVKO L. NIKOLOV, MICHAEL M. MEAGHER and PETER J. REILLY*
Department of Chemical Engineering, Iowa State University, Ames, IA 50011 (U.S.A.)
(Received November 19th, 1984)

SUMMARY

Thirteen different trisaccharides (3-O-β-cellobiosyl-D-glucose, cellotriose, 3-O-α-isomaltosyl-D-glucose, isomaltotriose, isopanose, 1-kestose, 4-O-β-laminaribiosyl-D-glucose, laminaritriose, maltotriose, melezitose, panose, raffinose, and xylotriose) were subjected to high-performance liquid chromatography on prepacked amino-bonded silica columns, using acetonitrile–water eluents. As with mono- and disaccharides, replacement of glucose by xylose or fructose caused shorter retention times, while its replacement by galactose led to delayed elution. Retention times of trisaccharides containing only glucose were shortest when only (1 → 3) bonds were present, and increased with increasing numbers of (1 → 4) and especially (1 → 6) bonds. When a trisaccharide contained two different types of bonds, retention times were roughly halfway between those of the two trisaccharides having only one type.

INTRODUCTION

Many laboratory and industrial processes produce oligosaccharides of the same chain length but of different composition or structure. These mixtures can often be analyzed by paper, thin-layer, and low-pressure liquid chromatography, though these usually require many hours and quantitative results are often insufficiently precise. Because gas chromatography (GC) and high-performance liquid chromatography (HPLC) are quicker and usually yield trustworthy quantitative data, they would be preferable to other analytical methods if separations between different oligosaccharides of the same chain length were sufficient.

While there have been a number of investigations of disaccharide separations by GC and HPLC (including two from this laboratory using fused-silica capillary columns coated with SE-54 silicone oil[1] and packed amino-bonded silica columns[2], respectively), little work has been conducted on the separation of large numbers of trisaccharides. This is a significantly more difficult problem, as derivatized trisaccharides are less volatile than the corresponding disaccharides, while structural differences of naturally occurring trisaccharides are generally less pronounced and therefore could be expected to lead to less complete separation than among disaccharides.

0021-9673/85/$03.30 © 1985 Elsevier Science Publishers B.V.

Two groups have dealt with the HPLC analysis of trisaccharides most completely, both using packed silica amine-modified columns. Boersma *et al.*[3] separated four nitrogen-containing trisaccharides with a decreasing linear gradient of aqueous acetonitrile, while Baust *et al.*[4] chromatographed melezitose, gentianose, raffinose, maltotriose, and isomaltotriose with an acetonitrile–water–tetraethylenepentamine (75:25:0.02) mixture, probably separating the first and last from the other three.

To investigate further their analysis, we have subjected thirteen different trisaccharides to HPLC using amino-bonded silica columns and acetonitrile–water eluents, following closely our previous work with disaccharides[2]. Nine of the trisaccharides contained only glucose, one only xylose, two glucose and fructose, and the last glucose, fructose, and galactose.

EXPERIMENTAL

Chromatographic apparatus and procedures

The chromatographic apparatus, as well as DuPont Zorbax-NH_2 and Supelcosil LC-NH_2 aminopropyl-bonded silica columns, Bio-Rad Bio-Sil NH_2 precolumn, solvents, separation conditions (23 ± 1°C, 1 ml/min acetonitrile–water eluent), and methods to determine capacity factors (k'), retention times (t_R), column dead times (t_0), and resolutions (R_s), were described earlier[2]. The refractometer output was processed with a Cyborg (Newton, MA, U.S.A.) ISAAC Model 42A chromatography workstation and an Apple (Cupertino, CA, U.S.A.) IIe microcomputer.

Trisaccharides

Isomaltotriose [O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→6)-D-glucose], maltotriose [O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucose], and melezitose [O-α-D-glucopyranosyl-(1→3)-O-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside) were purchased from Sigma (St. Louis, MO, U.S.A.). Cellotriose [O-β-D-glucopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→4)-D-glucose] was supplied by V-Labs (Covington, LA, U.S.A.), panose [O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→4)-D-glucose] by BDH (Poole, U.K.), and raffinose [O-α-D-galactopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside] by Pfanstiehl (Waukegan, IL, U.S.A.). Xylotriose [O-β-D-xylopyranosyl-(1→4)-O-β-D-xylopyranosyl-(1→4)-D-xylose] was prepared in our laboratory by partial acid hydrolysis of xylan followed by charcoal-Celite and Aminex AG1-X4 strong-base column chromatography. The following compounds were gifts: 3-O-β-cellobiosyl-D-glucose [O-β-D-glucopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→3)-D-glucose], isopanose [O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→6)-D-glucose], and 4-O-β-laminaribiosyl-D-glucose [O-β-D-glucopyranosyl-(1→3)-O-β-D-glucopyranosyl-(1→4)-D-glucose] from Dr. Bent Stig Enevoldsen of the Carlsberg Research Laboratory; 3-O-α-isomaltosyl-D-glucose [O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→3)-D-glucose] from Professor John F. Robyt of Iowa State University; 1-kestose [O-β-D-fructofuranosyl-(2→1)-O-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside] from Dr. Robert M. Sequeira of Amstar; and laminaritriose [O-β-D-glucopyranosyl-(1→3)-O-β-D-glucopyranosyl-(1→3)-D-glucose] from Dr. Elwyn T. Reese of the U.S. Army Natick Research and Development Laboratories.

RESULTS AND DISCUSSION

Capacity factors and resolutions for trisaccharides are shown on Table I. As with disaccharides[2], highest resolution occurred with the Supelcosil LC-NH_2 column at higher acetonitrile concentrations than with the Zorbax-NH_2 column. Again, improved resolution accompanied higher retention times and increasing acetonitrile contents, at least up to 80% with the former column and 75% with the latter.

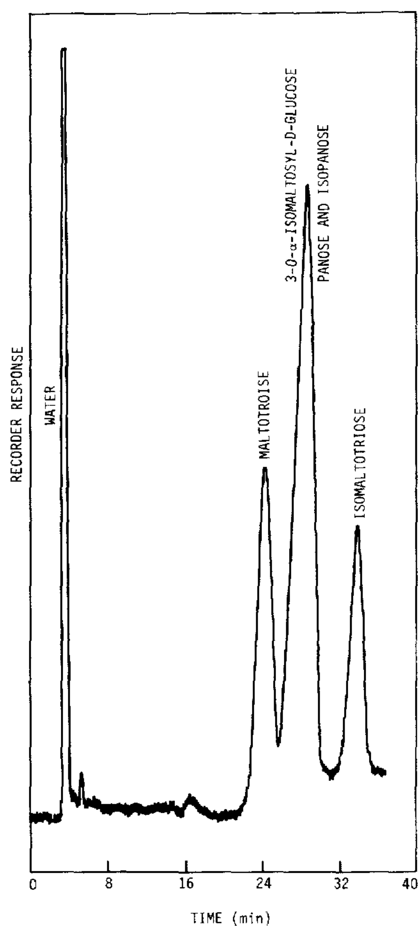
There is a greater range of capacity factors among trisaccharides than among disaccharides under roughly the same conditions, with the ratio between isomaltotriose and laminaritriose averaging 1.8 while that between isomaltose and laminaribiose[2] is *ca.* 1.4. However, because the α-linked compounds studied here have capacity factors mainly at the upper end of the range, with those for β-linked compounds at lower values, separations of each seem little better than those of the disaccharides (Figs. 1 and 2).

When the trisaccharide was composed solely of glucosyl units, retention times increased in the order of bonding (1→3), (1→4), (1→6), following the pattern of the

TABLE I

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

Trisaccharide	*Supelcosil LC-NH_2*				*Zorbax-NH_2*			
	Acetonitrile (%)				*Acetonitrile (%)*			
	75		*80*		*69*		*75*	
	k'	R_s	k'	R_s	k'	R_s	k'	R_s
Xylotriose [β-Xylp-(1→4)-β-Xylp-(1→4)-Xyl]	1.64		2.62		1.68		2.93	
1-Kestose [β-Fruf-(2→1)-β-Fruf-(2→1)-α-Glcp]	–		–		3.04		5.84	
Melezitose [α-Glcp-(1→3)-β-Fruf-(2→1)-α-Glcp]	2.45		4.51		3.07		5.87	
Raffinose [α-Galp-(1→6)-α-Glcp-(1→2)-β-Fruf]	3.00		5.68		3.61		7.22	
Laminaritriose [β-Glcp-(1→3)-β-Glcp-(1→3)-Glc]	2.21		3.99		2.92		5.66	
		–		–		1.1		1.8
3-O-β-Cellobiosyl-D-glucose [β-Glcp-(1→4)-β-Glcp-(1→3)-Glc]	–		–		3.29		6.61	
		–		–		0.0		0.1
4-O-β-Laminaribiosyl-D-glucose [β-Glcp-(1→3)-β-Glcp-(1→4)-Glc]	–		–		3.31		6.67	
		–		–		1.1		1.3
Maltotriose [α-Glcp-(1→4)-α-Glcp-(1→4)-Glc]	2.84		5.28		3.66		7.31	
		0.2		0.5		0.0		0.2
Cellotriose [β-Glcp-(1→4)-β-Glcp-(1→4)-Glc]	2.88		5.53		3.67		7.42	
		1.0		1.4		0.9		1.4
3-O-α-Isomaltosyl-D-glucose [α-Glcp-(1→6)-α-Glcp-(1→3)-Glc]	3.14		6.22		3.96		8.15	
		0.4		0.4		0.4		0.7
Panose [α-Glcp-(1→6)-α-Glcp-(1→4)-Glc]	3.26		6.45		4.11		8.52	
		0.3		0.5		0.1		0.3
Isopanose [α-Glcp-(1→4)-α-Glcp-(1→6)-Glc]	3.36		6.77		4.15		8.69	
		1.9		2.3		1.6		2.7
Isomaltotriose [α-Glcp-(1→6)-α-Glcp-(1→6)-Glc]	3.93		8.23		4.70		10.22	



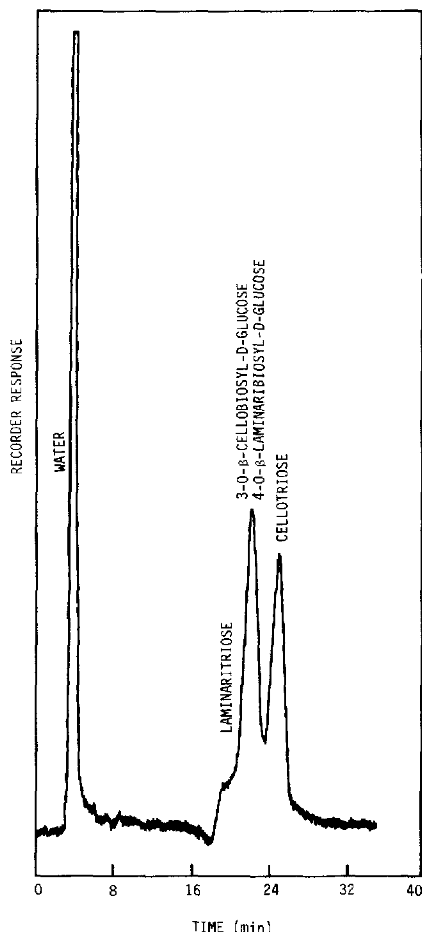


Fig. 1. Elution pattern of α-linked trisaccharides from Supelcosil LC-NH_2 column 91399. Mobile phase, acetonitrile–water (75:25); flow-rate, 1.0 ml/min.

Fig. 2. Elution pattern of β-linked trisaccharides. Column and conditions as in Fig. 1.

disaccharides. Accordingly, laminaritriose eluted before maltotriose and cellotriose, which eluted from the column before isomaltotriose. When two different bonds were present in a trisaccharide, capacity factors were roughly halfway between those of the two trisaccharides that contained only one type. In this work, 3-O-β-cellobiosyl-D-glucose and 4-O-β-laminaribiosyl-D-glucose, which contained both β-(1→3) and β-(1→4) bonds, had capacity factors that were almost the average of laminaritriose, with two β-(1→3) links, and cellotriose, with two β-(1→4) bonds. Likewise, panose and isopanose eluted roughly halfway between maltotriose and isomaltotriose. The bond closer to the reducing end had slightly more influence on the capacity factor, as 4-O-β-laminaribiosyl-D-glucose exited after 3-O-β-cellobiosyl-D-glucose, and isopanose after panose.

To investigate further the effect of varying saccharide compositions and lin-

kages on capacity factors, advantage was taken of the fact that the standard free energy difference between a component i in mobile and stationary phases is related to the logarithm of its capacity factor:

$$\ln k'_i = \ln (V_s/V_m) - \Delta G_i^0/RT \quad (1)$$

where V_s/V_m is the ratio of the effective volume of the stationary phase to that of the mobile phase. Free energy differences of two components may be compared[5,6]:

$$\ln (k'_i/k'_p) = (\Delta G_p^0 - \Delta G_i^0)/RT = \sum_{j=1}^{m} \tau_{ji} \quad (2)$$

where τ_{ji} is a substituent parameter arising from the addition of a substituent j at the reducing end of a parent compound p, and m is the total number of substitutions[5]. There are numerous indications that τ_{ji} values for a given substituent in a given position do not vary with different parent compounds, but are additive with different substituents in the same or different locations, simplifying τ_{ji} to τ_j[5]. A relationship like eqn. 2 has been used at least once with an amino column eluted with aqueous

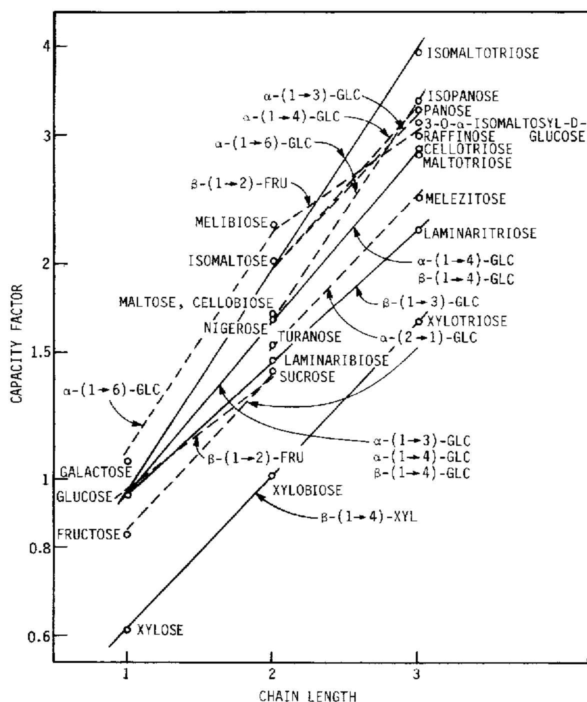


Fig. 3. Capacity factors of mono-, di-, and trisaccharides obtained with a Supelcosil LC-NH_2 column using 75% aqueous acetonitrile at 1.0 ml/min. Solid lines: calculated by linear regression for members of a homologous series; dashed lines: calculated by averaging at least two slopes obtained from formation of the same bond.

acetonitrile[7]. As expected, values of τ_j did not vary with change of parent molecule when the effect on capacity factors of substituting a 6-hydroxyl group for a hydrogen atom in a number of monosaccharides was studied.

Accordingly, values of capacity factors as a function of chain length are plotted on Fig. 3. Included are four monosaccharide parent compounds, nine disaccharides derived from them by anomeric hydroxyl substitution by a xylosyl, fructosyl, or glucosyl residue, and ten trisaccharides derived from the disaccharides by further reducing-end addition. These values were obtained using the Supelcosil LC-NH_2 column with 75% aqueous acetonitrile eluent, though similar plots occurred at 80% acetonitrile with the Supelcosil LC-NH_2 column and at 72% and 75% acetonitrile with the Zorbax-NH_2 column. Most capacity factors for disaccharides were taken from earlier work[2].

Values of τ_j were calculated from slopes between parent monosaccharides and daughter disaccharides and between parent disaccharides and daughter trisaccharides for each of the four cases. When a minimum of two values for the same substituent were available, the means were calculated and are presented on Table II.

Inspection of Fig. 3 demonstrates that agreement between experimentally obtained capacity factors (shown as discrete points) and those calculated from mean values of τ_j (shown as lines between sugars of different chain length) is quite acceptable. Xylotriose has a low capacity factor, mainly because xylose has a low value of k'_p. Raffinose has a lower capacity factor than other trisaccharides having glucosyl residues linked by α-(1→6) bonds, because it contains a β-(1→2)-fructosyl bond, which has a very low τ_j. An increase of acetonitrile concentration causes a sharp increase in τ_j.

Peak widths normalized to column dead time (w_i/t_0) are shown on Table III. As with disaccharides[2], there is a tendency for the non-reducing sugars, especially

TABLE II

MEAN SUBSTITUENT PARAMETERS (τ_j) FOR VARIOUS SUBSTITUENTS CHROMATOGRAPHED ON SUPELCOSIL LC-NH_2 AND ZORBAX-NH_2 COLUMNS WITH AQUEOUS ACETONITRILE

Substituent	*No. of slopes*	*Supelcosil LC-NH_2* Acetonitrile (%)		*Zorbax-NH_2* Acetonitrile (%)	
		75	*80*	*72*	*75*
β-(1→4)-Xylosyl	2	0.51	0.66	—	—
β-(1→2)-Fructosyl (from α-glucosyl)	2	0.35	0.47	0.32	0.38
α-(2→1)-Glucosyl (from β-fructosyl)	2	0.50	0.69	0.48	0.59*
α-(1→3)-Glucosyl	2	0.51	0.68	0.52	0.61
β-(1→3)-Glucosyl	2	0.43	0.58	0.44	0.53*
α-(1→4)-Glucosyl	3	0.53	0.68	0.55	0.63
β-(1→4)-Glucosyl	2	0.56	0.74	0.56	0.66*
α-(1→6)-Glucosyl	4	0.72	0.96	0.70	0.82

* Three slopes.

TABLE III

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

Trisaccharide	*Supelcosil LC-NH_2*		*Zorbax-NH_2*	
	Acetonitrile (%)		*Acetonitrile (%)*	
	75	*80*	*69*	*75*
Xylotriose	0.17	0.20	0.19	0.22
Laminaritriose	0.25	0.43	0.26	0.46
1-Kestose	–	–	0.21	0.40
Melezitose	0.19	0.30	0.24	0.39
3-O-β-Cellobiosyl-D-glucose	–	–	0.39	0.60
4-O-β-Laminaribiosyl-D-glucose	–	–	0.28	0.51
Raffinose	0.24	0.32	0.28	0.39
Maltotriose	0.26	0.48	0.34	0.50
Cellotriose	0.26	0.45	0.30	0.50
3-O-α-Isomaltosyl-D-glucose	0.27	0.55	0.34	0.56
Panose	0.26	0.50	0.34	0.50
Isopanose	0.32	0.58	0.35	0.57
Isomaltotriose	0.33	0.68	0.36	0.60

raffinose, to have disproportionately low peak widths, while those with β-(1→3) bonds (especially when they are at the reducing end, as with laminaritriose and 3-O-β-cellobiosyl-D-glucose) have abnormally high values. The latter again seems to be caused by large differences in retention times of anomers; suppression of mutarotation by use of an amine-bonded column treated with hydrochloric acid causes wide anomer peak separation in these two compounds when they are chromatographed.

Again, as already found with a selection of disaccharides[2], use of amine-bonded silica columns can give adequate separations of trisaccharides if the mixture is not too complex. The effect of composition and bond type on capacity factors and peak widths, first noted with disaccharides, has been confirmed using trisaccharides.

ACKNOWLEDGEMENTS

This project was supported by National Science Foundation Grant CPE-8311521 and by the Engineering Research Institute, Iowa State University.

REFERENCES

1 Z. L. Nikolov and P. J. Reilly, *J. Chromatogr.*, 254 (1983) 157.
2 Z. L. Nikolov, M. M. Meagher and P. J. Reilly, *J. Chromatogr.*, 319 (1985) 51.
3 A. Boersma, G. Lamblin, P. Degand and P. Roussel, *Carbohydr. Res.*, 94 (1981) C7.
4 J. G. Baust, R. E. Lee, Jr., R. R. Rojas, D. L. Hendrix, D. Friday and H. James, *J. Chromatogr.*, 261 (1983) 65.
5 B. K. Chen and C. Horváth, *J. Chromatogr.*, 171 (1979) 15.
6 C. M. Riley, E. Tomlinson and T. M. Jeffries, *J. Chromatogr.*, 185 (1979) 197.
7 K.-I. Fukuhara, A. Nakamura and M. Tsuboi, *Chem. Pharm. Bull.*, 30 (1982) 1121.