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CAPILLARY COLUMN GAS CHROMATOGRAPHY OF TRIFLUOROACETYL TRISACCHARIDES

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SUMMARY

Relative retention times are reported for thirteen trifluoroacetyl 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) subjected to gas chromatography on a fused-silica capillary column coated with DB-5. Unlike trimethylsilylated derivatives, trifluoroacetylated trisaccharides containing (1→6) links are eluted quickly, while those composed of pentoses are strongly retarded by the column. Two peaks were obtained for each of the reducing compounds and one for each nonreducing compound. Virtually complete separation of the mixture was possible by programming between 180 and 200°C.

INTRODUCTION

This article is one of a series devoted to the gas chromatography (GC) and high-performance liquid chromatography (HPLC) of oligosaccharides. The first in the series dealt with the capillary column GC of trimethylsilyl (TMS) disaccharides[1]. Subsequent papers covered the HPLC of disaccharides[2] and trisaccharides[3] on amine-bonded silica columns. Because HPLC is so far incapable of separating complex trisaccharide mixtures, a successful GC analytical technique would be very desirable for research and industrial purposes.

There have been occasional reports of derivatized trisaccharides being separated from each other by GC. Trimethylsilylation yields compounds of only moderate volatility[4,5], and temperatures necessary for analysis approach the limits of most columns. Bhatti *et al.*[4] employed 300°C to separate four TMS trisaccharides, and Nurok and Reardon[5], analyzing the TMS derivatives of the three kestoses and melezitose (all of which contain fructose and therefore would be expected to be quite volatile), required 260°C. In our hands trimethylsilylation and capillary GC along the lines of our earlier disaccharide work[1] was unsuccessful. Although non-reducing trisaccharides yielded symmetrical narrow peaks, peaks of reducing trisaccharides were broad, and in no case was complete separation of anomers achieved. Column temperatures necessary for minimal separations between different compounds ex-

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ceeded 300°C, close to the maximum allowed for the bonded-phase DB-5 column being used, and little operational flexibility remained.

In the most complete work dealing with the GC of derivatized trisaccharides, Kärkkainen separated 21 permethylated methylglycosides[6] and permethylated alditols[7] at 260 or 265°C. However, he furnished only relative retention times and an indication of peak size, and it is unclear how complete a separation he obtained.

To obtain trisaccharide derivatives of greater volatility than those previously studied, we resorted to trifluoroacetylation. Because the high efficiency of capillary columns gives promise of separating complex mixtures, even when multiple peaks arise from each reducing sugar, we did not reduce mixtures before derivatization, as has often been done. There also seemed to be little reason to produce the oximes or glycosides, which also usually give multiple peaks.

Free sugars have been subjected to GC after trifluoroacetylation on a number of occasions[8–17]. However, the technique has been employed only rarely[8,14,15,18] with oligosaccharides of length sufficient to use the high volatility of the resulting derivatives to advantage. Trifluoroacetylation also has disadvantages, in that hydrolysis occurs easily, and therefore initial reaction and subsequent storage must take place under dry conditions[19,20]. Degradation can also occur during chromatography, especially on catalytic surfaces[13,20–22].

To obtain more reproducible trifluoroacetylation, Sullivan and Schewe[14] used N-methyl-bis(trifluoroacetamide) (MBTFA) for derivatization in place of trifluoroacetic anhydride. It has since been employed by others[18,22] and was used in this project.

Therefore, to investigate the separation of trifluoroacetyl (TFA) trisaccharides, we have subjected thirteen to capillary GC. Nine of the trisaccharides contained only glucose, one only xylose, two glucose and fructose, and the last glucose, fructose, and galactose.

EXPERIMENTAL

Trisaccharides

The following trisaccharides were used in this work: 3-O-β-cellobiosyl-D-glucose [O-β-D-glucopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→3)-D-glucose], cellotriose [O-β-D-glucopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→4)-D-glucose], 3-O-α-isomaltosyl-D-glucose [O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→3)-D-glucose], isomaltotriose [O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→6)-D-glucose], isopanose [O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→6)-D-glucose], 1-kestose [O-β-D-fructofuranosyl-(2→1)-O-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside], 4-O-β-laminaribiosyl-D-glucose [O-β-D-glucopyranosyl-(1→3)-O-β-D-glucopyranosyl-(1→4)-D-glucose], laminaritriose [O-β-D-glucopyranosyl-(1→3)-O-β-D-glucopyranosyl-(1→3)-D-glucose], maltotriose [O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucose], melezitose [O-α-D-glucopyranosyl-(1→3)-O-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside], panose [O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranoyl-(1→4)-D-glucose], raffinose [O-α-D-galactopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside], and xylotriose [O-β-D-xylopyranosyl-(1→4)-O-β-D-xylopyranosyl-(1→4)-D-xylose]. Their sources have been described previously[3].

Reagents

MBTFA and pyridine (silylation grade) were purchased from Pierce (Rockford, IL, U.S.A.).

Derivatization

About 2 mg of each trisaccharide were dissolved in 0.5 ml pyridine and left for 36 h at 40°C to achieve mutarotation equilibrium. The mixtures were derivatized by the method of Sullivan and Schewe[14] with 0.5 ml each of MBTFA added. The resulting solutions were maintained at 65°C for 1 h to complete the reaction.

Gas chromatography

Derivatized samples were analyzed with a Hewlett-Packard (Palo Alto, CA, U.S.A.) 5890A gas chromatograph coupled to a Hewlett-Packard 3492A integrator. The column was a 30 m × 0.26 mm I.D. fused-silica capillary manufactured by J & W Scientific (Rancho Cordova, CA, U.S.A.) coated with a 0.1-μm film of DB-5 liquid phase.

The column oven was held at 180°C for 5 min, then increased at 5°C/min to 200°C and held there for the duration of the run. Injector and flame ionization detector temperatures were 220°C and 240°C, respectively. Helium carrier gas flow-rate was 0.204 m/sec measured by methane, and the splitting ratio was 1:100.

RESULTS AND DISCUSSION

When trifluoroacetylated and subjected to capillary GC, the three non-reducing trisaccharides raffinose, 1-kestose, and melezitose each yielded one peak, while the ten reducing trisaccharides yielded two peaks each, the larger always being eluted first. Separation of the 23 peaks of the mixture was almost complete (Fig. 1), with only two instances of overlap: the second TFA 4-O-β-laminaribiosyl-D-glucose peak

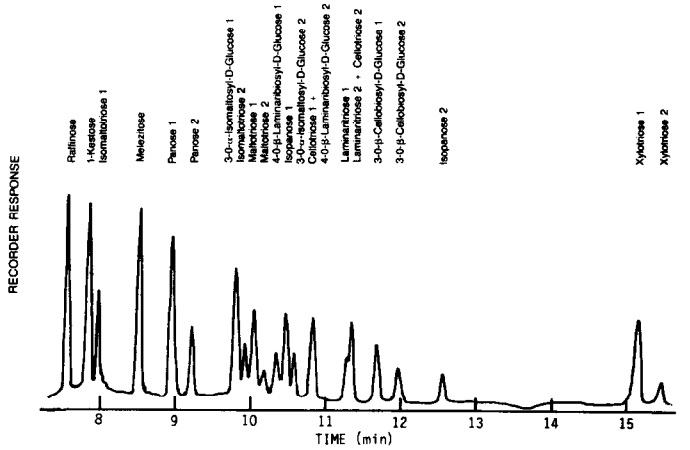

Fig. 1. Gas chromatogram of thirteen TFA trisaccharides conducted on a 30 m × 0.26 mm I.D. fused-silica capillary column coated with DB-5. Temperature programme: 180°C for 5 min, followed by an increase of 5°C/min to 200°C.

and the first TFA cellotriose peak, and the two TFA laminaribiose peaks and the second TFA cellotriose peak.

The relative retention times, resolutions (R_s), and baseline peak widths (w_i) of the 23 peaks are presented in Table I. Relative retention times (t_R) were based on TFA raffinose; resolutions between neighboring peaks were defined as the difference in their retention times divided by the average baseline peak width:

$$R_s = \frac{t_2 - t_1}{(w_1 + w_2)/2}$$

Several observations can be made about the order of elution of these thirteen TFA trisaccharides. First, the three non-reducing trisaccharides, the only compounds studied here possessing fructosyl residues, as a group have very low retention times. Presence of fructosyl groups also led to early elution of most TMS disaccharides[1]. Second, although (1→6) bonds in TMS disaccharides caused late elution, with TFA trisaccharides the same bond generally was associated with low retention times. Third, although the two TMS xylobiose peaks had very low retention times[1], in this study the two TFA xylotriose peaks were by far the last to be eluted. Finally, with

TABLE I

RELATIVE RETENTION TIMES AND RESOLUTIONS OF TFA TRISACCHARIDES

Trisaccharide	*Peak*	*Peak width (min)*	*Relative retention time**	*Resolution (R_s)*
Raffinose		0.104	1.0000	2.84
1-Kestose		0.125	1.0426 ± 0.0008	0.88
Isomaltotriose	1	0.103	1.0557 ± 0.0008	5.08
Melezitose		0.123	1.1310 ± 0.0005	3.51
Panose	1	0.096	1.1814 ± 0.0009	2.63
Panose	2	0.096	1.2145 ± 0.0013	5.04
3-O-α-Isomaltosyl-D-glucose	1	0.148	1.2949 ± 0.0002	0.61
Isomaltotriose	2	0.129	1.3059 ± 0.0008	1.03
Maltotriose	1	0.162	1.3255 ± 0.0013	0.96
Maltotriose	2	0.103	1.3422 ± 0.0019	0.82
4-O-β-Laminaribiosyl-D-glucose	1	0.129	1.3546 ± 0.0005	0.78
Isopanose	1	0.205	1.3717 ± 0.0007	0.96
3-O-α-Isomaltosyl-D-glucose	2	0.124	1.3924 ± 0.0006	1.51
4-O-β-Laminaribiosyl-D-glucose	2	0.120	1.4166 ± 0.0007	0.36
Cellotriose	1	0.133	1.4226 ± 0.0010	2.98
Laminaritriose	1	0.140	1.4760 ± 0.0010	0.67
Laminaritriose	2	0.107	1.4869 ± 0.0005	0.04
Cellotriose	2	0.122	1.4875 ± 0.0015	3.25
3-O-β-Cellobiosyl-D-glucose	1	0.109	1.5367 ± 0.0015	2.52
3-O-β-Cellobiosyl-D-glucose	2	0.089	1.5695 ± 0.0016	5.61
Isopanose	2	0.111	1.6433 ± 0.0010	14.57
Xylotriose	1	0.250	1.9879 ± 0.0012	1.36
Xylotriose	2	0.172	2.0255 ± 0.0007	

* TFA raffinose was used as an internal standard (retention time = 7.63 min) and relative retention times were normalized to it. Standard deviations are based on four determinations.

the exception of one TFA isopanose and one TFA 4-O-β-laminaribiosyl-D-glucose peak, α-linked TFA glucotrisaccharides had lower retention times than β-linked ones.

It is particularly noteworthy that TFA trisaccharides containing the same bonds but in reversed order, such as panose and isopanose as well as 3-O-β-cellobiosyl-D-glucose and 4-O-β-laminaribiosyl-D-glucose, are easily separated. This suggests that capillary GC of longer TFA oligosaccharides, which is easily possible because of the extreme volatility of these derivatives, will distinguish the location of branch points in compounds having the same backbones.

Relative amounts and resolutions of the two peaks of each reducing trisaccharide are shown in Table II. Those compounds having their anomeric residues linked (1→6) to the rest of the molecule (TFA isopanose and TFA isomaltotriose) had the greatest peak separations. The two peaks of TFA laminaritriose could not be sufficiently separated to estimate their relative amounts.

Little variation existed in relative amounts of the two peaks of most reducing trisaccharides from one injection of a trifluoroacetylated preparation to the next,

TABLE II

PROPORTIONS AND RESOLUTIONS OF TFA TRISACCHARIDE PEAKS IN PYRIDINE

Trisaccharide	*Peak*	*Proportion (%)*	*R_s*
Isomaltotriose	1	56.7 ± 3.4*	16.48
	2	43.3	
Panose	1	61.4 ± 1.9	2.63
	2	38.6	
3-O-α-Isomaltosyl-D-glucose	1	69.3 ± 1.2	5.48
	2	30.7	
Maltotriose	1	73.1 ± 4.1	0.96
	2	26.9	
4-O-β-Laminaribiosyl-D-glucose	1	62.6 ± 5.6	3.80
	2	37.4	
Isopanose	1	77.7 ± 3.4	13.12
	2	22.3	
Cellotriose	1	61.8 ± 1.4	3.88
	2	38.2	
Laminaritriose	1	–	0.67
	2	–	
3-O-β-Cellobiosyl-D-glucose	1	76.4 ± 1.0	2.52
	2	23.6	
Xylotriose	1	76.7 ± 1.3	1.36
	2	23.3	

* Standard deviation based on four determinations.

giving some assurance that the relative stabilities of the two forms were the same over several hours of storage. It should not be assumed that the two forms are necessarily derived from the two pyranosyl anomers and, furthermore, that the relative amounts will yield the anomeric ratios of each trisaccharide at mutarotation equilibrium before derivatization. Previous work has shown that ratios of peaks from reducing sugars subjected to trifluoroacetylation are rather variable, especially with changes in reaction solvent[9] and catalyst[12]. In addition, a very preliminary study of ours using proton nuclear magnetic resonance has indicated that the major chromatographic peaks of a number of TFA reducing sugars corresponded to α-anomers, in direct contradiction to their known mutarotation equilibria. Further elucidation of this point requires a much more extensive study, currently in progress.

In conclusion, it may be stated that capillary GC at moderate temperatures can successfully separate an extensive mixture of TFA trisaccharides not previously subjected to reduction or other types of derivatization. Of thirteen compounds tested, only three, laminaritriose, cellotriose, and 4-O-β-laminariobiosyl-D-glucose, have peaks that overlap.

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