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GAS–LIQUID CHROMATOGRAPHY OF TRIMETHYLSILYLATED DISACCHARIDE OXIMES

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SUMMARY

The relative retention times of trimethylsilyl derivatives of ten disaccharide oximes on SE-52 and OV-17 liquid phases are reported. Maltose, lactose, gentiobiose, turanose and lactulose gave a single main peak, while the others gave two or three separated peaks which probably resulted from isomeric forms of the sugar oximes.

INTRODUCTION

Gas–liquid chromatography (GLC) has become an important method for the analysis of carbohydrates since Sweeley *et al.*[1] described a simple and rapid procedure for preparing trimethylsilyl (TMS) derivatives of sugars and related substances. The application of this technique to TMS ethers of disaccharides was summarized by Haverkamp *et al.*[2]. A number of procedures[3–8] have been described for the trimethylsilylation of carbohydrates, including several variations of the reagents of Sweeley *et al.*[1] that require completely dry samples. Further improvements to accommodate moderate amounts of water and to avoid the precipitation of presumably ammonium chloride were described by Brobst and Lott[9]. Gas chromatographic separations of disaccharides have also been performed on O-acetyl[10], O-methyl[11] and O-trifluoroacetyl[12] derivatives. However, the separation of disaccharides from each other is generally difficult, as most disaccharides give multiple peaks in gas chromatograms owing to the presence of tautomeric forms of reducing sugars[1]. This tautomerization has also been found to complicate the chromatograms of carbohydrate mixtures, overlapping peaks being obtained[2]. In order to prevent the formation of multiple peaks, the analysis of disaccharides has been performed successfully by utilizing their reduction products[13,14]. On the other hand, the possibility of reducing the multiplicity of peaks by converting the sugars into oximes before forming the TMS ethers was investigated by Sweeley *et al.*[1] and Mason and Slover[15]. The latter found that lactose gives a single peak, although glucose and galactose give two well-separated peaks and maltose gives two distinct partially separated peaks. In this work, we investigated the separation of ten disaccharides, including two ketodisaccharides, as the sugar oxime TMS ethers.

MATERIALS AND METHODS

Materials and reagents

Melibiose was purchased from Difco Labs. (Detroit, Mich., U.S.A.). Kojibiose, nigerose and neolactose were gifts. Lactulose was prepared from lactose by one of the authors. All other carbohydrates were of guaranteed grade and were purchased from the following commercial sources: trehalose, maltose, cellobiose, lactose and gentiobiose from Wako (Osaka, Japan), turanose from Tokyo Kasei Kogyo (Tokyo, Japan) and sucrose from Nakarai (Kyoto, Japan).

Pyridine stored over potassium hydroxide pellets was used in the trimethylsilylation reaction. Hydroxylamine hydrochloride (guaranteed reagent), hexamethyldisilazane (HMDS, extra pure reagent) and trifluoroacetic acid (TFA, guaranteed reagent) were products of Wako.

Apparatus

A gas chromatograph, Model JGC-20KFP (Japan Electron Optics Laboratory Co.) equipped with a hydrogen flame-ionization detector and coiled stainless-steel columns (2 m × 3 mm I.D.) was used. The packing materials were 1.5% silicone SE-52 on 60–80-mesh Chromosorb W AW DMCS (Gas Chro Kogyo Co., Tokyo, Japan) and 1.5% silicone OV-17 on 80–100-mesh Shimalite W (Wako); the injection and detector port temperatures were 270° and the column oven was maintained at 215°. Nitrogen was used as the carrier gas at a flow-rate of 40 ml/min.

Oximation of disaccharides[15]

Sugars were anomerized in water for 48 h at room temperature, and subsequently evaporated to dryness at 45°. To *ca.* 5 mg of a solid disaccharide, 1 ml of a solution of hydroxylamine hydrochloride in pyridine (25 mg/ml) was added and the mixture heated at 70–80° for 30 min. Pyridine was then removed by evaporating the mixture to dryness at 45°; the last traces of water were removed as an azeotrope by adding a drop of benzene and again evaporating to dryness[7].

Preparation of TMS derivatives[9]

The sample was dissolved in 0.25 ml of anhydrous pyridine and trimethylsilylated with 0.225 ml of HMDS plus 0.025 ml of TFA. The tube was capped, shaken vigorously for 30 sec and allowed to stand for 30 min with occasional shaking. No precipitate was formed during this derivatization of anomerized solid sugars. With sugar oximes, however, the solution became cloudy on addition of HMDS owing to the precipitation of presumably unreacted hydroxylamine hydrochloride. No attempt was made to remove this fine precipitate, which did not interfere in the subsequent gas chromatography. A volume of 1–2 μl of the resulting reaction mixture was injected into the gas chromatograph.

The time between oximation of sugars and injection into the gas chromatograph was kept to the minimum (within 24 h at the most), as the sugar oxime TMS ethers were extremely unstable[15].

Thin-layer chromatography of disaccharide oxime TMS ethers

The thin-layer plates were coated with a 0.25-mm layer of Kieselgel G nach

Stahl (E. Merck, Darmstadt, G.F.R.) and dried at 120° for 2 h[16]. Before use, the plates were activated at 120° for 15 min, spotted while hot and immediately developed[17]. In order to minimize the appearance of spots caused by partial degradation, the time between sample spotting and development was kept to within 15 min. *n*-Hexane–benzene–ethyl acetate (5:4:1) was used as the developing solvent and the front was allowed to migrate 15.0 cm. Sulphuric acid (50%) was used as the spray reagent.

RESULTS AND DISCUSSION

The retention times (R_T) and peak area ratios of the oxime TMS derivatives and TMS derivatives of disaccharides on the two stationary phases are presented in Table I. The disaccharides comprised two non-reducing sugars that do not form oximes and ten reducing sugars, consisting of eight aldodisaccharides and two ketodisaccharides. In general, the retention times of sugar oxime TMS ethers were higher than those of corresponding sugar TMS ethers, with the following exceptions: The retention time of lactose oxime TMS ether was between that of α- and β-lactose TMS ethers on OV-17. The relationship between the retention times of cellobiose oxime TMS ether and α- and β-cellobiose TMS ethers on both SE-52 and OV-17 was similar to that of lactose mentioned above. The retention time of gentiobiose oxime TMS ether was lower than that of the corresponding TMS ether on OV-17.

Although a single peak was obtained from turanose oxime TMS ether alone, almost all oxime TMS ethers of reducing disaccharides gave a main peak associated with sub- or trace peak(s). Representative gas chromatograms of some aldodisaccharide oxime TMS ethers are shown in Fig. 1. Lactose and maltose gave a single main peak on both SE-52 and OV-17. In this connection, however, Mason and Slover[15] reported that lactose gives a single peak but maltose two distinct partially separated peaks. Gentiobiose gave a single main peak on OV-17 and two distinct major and sub-peaks on SE-52.

In order to account for the assignment of peaks encountered with the oxime TMS ethers, cellobiose and melibiose oxime in aqueous equilibrium solutions allowed to stand at different temperatures were examined for changes of the peak ratio. These oximes in aqueous solution were maintained overnight at temperatures of 0, 20 and 40°, trimethylsilylated by the Brobst and Lott method[9], which accomodates moderate amounts of water, and immediately injected into the gas chromatograph. The peak area ratio of R_T 1.24 or 1.09 to R_T 1.35 or 1.16 on SE-52 or OV-17, respectively, for cellobiose oxime TMS ether with low-temperature treatment was smaller than that with high-temperature treatment. The peak area ratio of the oxime TMS ether with low-temperature treatment slowly changed to give that with high-temperature treatment. With melibiose oxime TMS ether, the peak area ratios of R_T 2.20 or 1.95 to R_T 2.46 or 2.12 on SE-52 or OV-17 were similar with both high- and low-temperature treatments.

Two isomeric forms of glucose oxime are known; one, a cyclic modification, is known to be the only form of a crystalline oxime[18], whereas an equilibrium with an acyclic modification probably exists in solution[19]. Sweeley *et al.*[1] concluded that both a cyclic and an acyclic modification of glucose oxime cause single symmetrical peaks with different retention times on the gas chromatogram. However, two isomeric

TABLE I

R_T VALUES AND PEAK AREA RATIOS OF THE OXIME TMS ETHERS AND TMS ETHERS OF DISACCHARIDES

TMS-trehalose was used as an internal standard (R_T = 1.00); the retention times of this compound were 14.50 and 17.05 min on SE-52 and OV-17, respectively. Figures in parentheses represent peak area ratio (main peak = 10).

Carbohydrate	*Oxime TMS ethers*		*TMS ethers*	
	1.5% SE-52	*1.5% OV-17*	*1.5% SE-52*	*1.5% OV-17*
α,α-Trehalose	—	—	1.00	1.00
Kojibiose	1.36 (t)*	1.17 (t)	1.02 (10)	1.03 (10)
	1.56 (10)	1.36 (10)	1.28 (9)	1.36 (9)
	1.75 (2)	1.50 (2)		
Nigerose	0.51 (t)	0.54 (t)	0.94 (10)	0.95 (10)
	0.62 (t)	0.66 (t)	1.01 (8)	1.07 (8)
	1.21 (1)	1.05 (1)		
	1.48 (10)	1.23 (10)		
	1.57 (2)	1.35 (2)		
Maltose	1.11 (t)	1.01 (t)	0.83 (8)	0.87 (8)
	1.21 (t)	1.14 (t)	0.97 (10)	0.98 (10)
	1.45	1.26		
Cellobiose	0.51 (t)	0.55 (t)	0.86 (7)	0.91 (7)
	0.59 (t)	0.62 (t)	1.27 (10)	1.30 (10)
	1.03 (t)	0.82 (t)		
	1.24 (10)	1.09 (10)		
	1.35 (3)	1.16 (3)		
Lactose	0.97 (t)	0.52 (t)	0.73 (7)	0.80 (7)
	1.16	0.97	1.12 (10)	1.15 (10)
Neolactose	0.57 (t)	0.52 (t)	0.60 (5)	0.57 (5)
	0.91 (t)	0.82 (t)	0.68 (10)	0.62 (10)
	1.22 (10)	0.99 (10)		
	1.35 (3)	1.08 (3)		
Gentiobiose	0.81 (t)	0.85 (t)	1.89	2.13
	0.93 (t)	1.58 (t)		
	1.53 (t)	1.82		
	2.01 (10)	2.36 (t)		
	2.16 (5)			
Melibiose	0.86 (t)	0.62 (t)	1.53 (8)	1.65 (8)
	0.96 (t)	0.79 (t)	1.69 (10)	1.81 (10)
	1.82 (t)	1.73 (t)		
	2.20 (10)	1.95 (10)		
	2.46 (3)	2.12 (3)		
Sucrose	—	—	0.73	0.71
Turanose	1.47	1.23	0.94	0.92
Lactulose	0.27 (t)	0.30 (t)	0.70	0.62 (3)
	0.36 (t)	0.40 (t)		0.67 (10)
	0.52 (t)	0.56 (t)		
	0.72 (t)	0.66 (t)		
	1.15	0.94		

* t = trace.

forms (α- and β-) of a cyclic modification, and *syn*- and *anti*-forms of an acyclic modification, could be possible theoretically[19]. Newstead and Gray[20] found that after formation of the TMS ethers less than 4% of anomerization (α- to β-) occurred in 16 h. It was further reported that cyclic oxime TMS ethers in pyridine solution

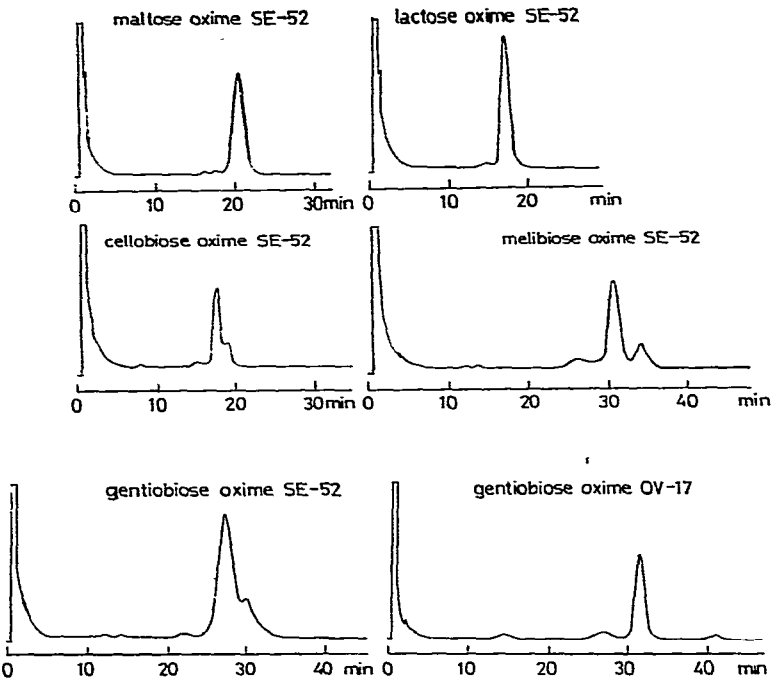

Fig. 1. Gas chromatograms of TMS derivatives. Column, 1.5% SE-52 or 1.5% OV-17 (2 m × 3 mm I.D.); column temperature, 215°; injection temperature, 270°; detector temperature, 270°; carrier gas (nitrogen) flow-rate, 40 ml/min.

slowly change to give a mixture containing probably an acyclic oxime[1]. Thus, examination of cellobiose and melibiose oxime TMS ethers indicates that the two separated peaks of cellobiose oxime result from two isomeric forms of a cyclic and an acyclic modification, whereas those of melibiose are due to two isomeric forms (α- and β-) of a cyclic modification or *syn*- and *anti*-forms of an acyclic modification. In disaccharides, only the oximes of cellobiose[21], lactose[22] and melibiose[23] could be obtained in crystalline form; however, in none of these instances has it been reported whether the oximes have a cyclic or an acyclic modification.

The suggestions that multiple peaks for each oxime TMS ether are due to the presence of such isomers are also supported by the fact that several spots were detected from some aldodisaccharide oxime TMS ethers by the following thin-layer chromatographic procedure. Thin-layer chromatography with *n*-hexane–benzene–ethyl acetate (5:4:1) separated the oxime TMS ethers of lactose, maltose, cellobiose, melibiose and gentiobiose into several sub-fractions (Fig. 2). All of the compounds remained at low R_F values with benzene. However, all of the corresponding sugar TMS ethers moved with the solvent front with *n*-hexane–benzene–ethyl acetate (5:4:1) and separated into isomers with benzene, as reported by Kärkkäinen *et al.*[16]. Consequently, the spots obtained from the oxime TMS ethers are not due to the corresponding sugar TMS ethers remaining in the reaction products as impurities, but arise directly from parental oxime TMS ethers.

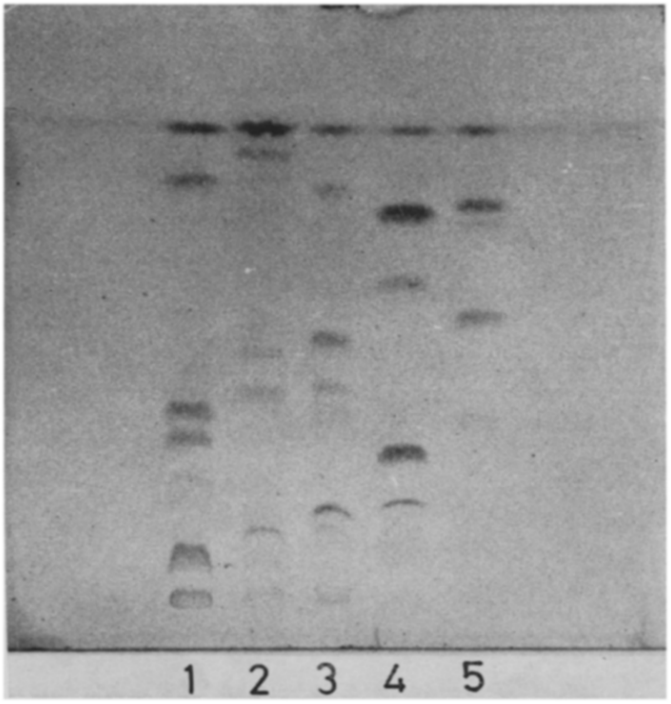

Fig. 2. Thin-layer chromatogram of sugar oxime TMS ethers. Solvent system, *n*-hexane–benzene–ethyl acetate (5:4:1); spray reagent, 50% sulphuric acid. Carbohydrates: 1 = lactose; 2 = maltose; 3 = cellobiose; 4 = melibiose; 5 = gentiobiose.

CONCLUSIONS

In this study, in order to prevent multiplicity of peaks, GLC analysis of reducing disaccharides using their oxime TMS ethers has, in general, been unsuccessful. Sugar oxime TMS ethers of lactose, maltose, gentiobiose, turanose and lactulose, however, which give a single main peak, were found to have a possibile application in their determinations.

Gas chromatograms of sugar oxime TMS ethers commonly gave two or three distinct peaks, probably resulting from isomeric forms of the corresponding sugar oximes. Although single peaks were not generally obtained for TMS oximes of a number of disaccharides, the analysis of disaccharides as oxime TMS ethers may be feasible when a complicated mixture of sugars has not been separated as the TMS ethers or those of the corresponding reduction products.

Further, there is little doubt that the gas chromatographic analysis of sugar oxime TMS ethers will be a convenient method for the study of interconversions of isomeric forms of sugar oximes.

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