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GAS-CHROMATOGRAPHIC ANALYSIS OF SUGARS AND RELATED HYDROXY ACIDS AS ACYCLIC OXIME AND ESTER TRIMETHYLSILYL DERIVATIVES*

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ABSTRACT

A g.l.c. method which makes possible the simultaneous analysis of the common types of acidic and neutral sugars is described. Particular advantages are achieved with hydroxy acids, ketoses, and uronic acids. Acids and lactones are first converted into their sodium salts. Free or potential aldehydo and keto groups are transformed into oximes or *O*-methyloximes. Fully substituted acyclic ester and oxime trimethylsilyl derivatives are prepared with bis(trimethylsilyl)trifluoroacetamide and chlorotrimethylsilane. *Syn* and *anti* isomers of the oximes are formed, but a stationary phase can often be chosen on which the two peaks coincide. The silicone stationary phases OV-1, OV-17, QF-1, and XE-60 were used. Retention data are given for aldonic and deoxyaldonic acids, aldoses, ketoses, and uronic acids. Relationships between structure and retention are discussed. The derivatives are suited to structural analysis by gas chromatography–mass spectrometry.

INTRODUCTION

A major problem in the application of g.l.c. to sugars is that many compounds give rise to multiple peaks. These are due to the structurally different species obtained on preparation of common types of derivatives. The most fruitful approach to diminishing the number of peaks from each sugar has been to prepare acyclic derivatives. Reduction to alditols, followed by acetylation and g.l.c., has developed into a standard technique for aldoses[1]. This technique, in combination with characterization by mass spectrometry (m.s.), is also advantageous for methylated aldoses from methylation analysis[2]. Alternatively, aldoses can be transformed into acyclic aldononitrile acetates, permitting facile separation[3] by g.l.c. as well as structure determination[4] by m.s.

In recent years, trimethylsilyl (TMS) derivatives have found increased use in g.l.c., largely because they are appropriate for a wide range of functional groups. The usefulness of TMS ether derivatives for carbohydrates was first demonstrated by Sweeley *et al.*[5]. The preparation and analytical advantages of TMS ester derivatives

*Editor's footnote: R. A. Laine and C. C. Sweeley, *Carbohyd. Res.*, 27 (1973) 199, have recently described the use of g.l.c.–m.s. for the analysis of *O*-methyloxime TMS derivatives of aldoses and ketoses.

were described by Horii *et al.*[6] for acids of the Krebs cycle. Keto groups were protected by the preparation of oxime derivatives. In a study of sugar-related hydroxy acids[7], it was later shown that TMS ester derivatives can be prepared from salts of the acids. TMS amine derivatives have been used for many classes of compound, including sugar amines[8].

By combining the advantages of acyclic derivatives for sugars with those of TMS derivatives for different functional groups, the present investigation extends the range of compounds that can be analyzed simultaneously. The potential of this approach is illustrated in Fig. 1, by its application to some compounds of different structural types.

ALDONIC AND DEOXYALDONIC ACIDS

Acids with hydroxyl groups at C-4 and C-5 exist in solution as acyclic molecules and as 1,4- and 1,5-lactones and may form corresponding derivatives, *e.g.*, TMS derivatives[9]. Transformation of each acid into a single derivative is desirable for g.l.c. analysis. A previously used method[10,11] involves treatment with a strong acid to promote the formation of aldono-1,4-lactones, the TMS derivatives[9–12] of which are amenable to g.l.c. However, the lactonization procedure exposes sensitive compounds to rather severe conditions and it is difficult to obtain complete conversion of some 1,5-lactones, *e.g.*, glucono-1,5-lactone[12]. Furthermore, the method is not suited to dicarboxylic and non-lactone-forming acids.

These drawbacks are avoided with the present technique, which is based on the preparation of TMS esters from carboxylate groups[7], and which was first used for saccharinic acids obtained from hydrocellulose[13]. Similar methods, which make use of TMS esters, have been applied to aldonic[14,15] and aldaric[15,16] acids.

Ester trimethylsilyl derivatives. The preparation of acyclic TMS derivatives from lactone-forming acids requires initial saponification of the lactones. This was achieved by keeping aqueous solutions of the samples at a constant, alkaline pH by automatic addition of sodium hydroxide. Saponification was usually complete after 4 h at pH 8.5 and room temperature, but lower saponification rates were observed for 2- and 3-deoxyaldonolactones and tetronolactones. The 2-deoxyaldonolactones were particularly stable and required 4 h at pH 10. Similar conditions were used for some dicarboxylic acids[16]. Several non-lactone-forming acids, *e.g.*, glycolic, lactic, and hydracrylic acids, formed intermolecular esters on storage, some of which were difficult to saponify. This complication did not arise when evaporation to dryness before neutralization was avoided.

After vacuum-drying over phosphorus pentaoxide, many sodium salts, in contrast to neutral sugars, still retained approximately their own weight of water. This had to be allowed for during preparation of TMS derivatives, but evaporation of dichloromethane from the hydrated sodium salts effectively removed the water.

The sodium salts are poorly soluble in pyridine and could not be converted easily into TMS derivatives. The use of the reagents of Sweeley *et al.*[5], hexamethyl-

disilazane (HMDS) together with chlorotrimethylsilane (TMCS), required an elevated temperature[15] or a better solvent[14]. In pyridine, bis(trimethylsilyl)acetamide (BSA), or the more-volatile bis(trimethylsilyl)trifluoroacetamide[17] (BSTFA), was found to be more satisfactory. Addition of TMCS further promoted the reaction. With pyridine+BSTFA+TMCS (8:2:1), quantitative conversion into ether–ester TMS derivatives was normally observed after 2 h at room temperature with mechanical shaking or ultrasonic treatment. The amount of BSTFA was chosen to give an excess of reagent, as calculated for the transfer of one TMS group from BSTFA.

When BSTFA+TMCS were used, evaporation after completed reaction removed the reagents together with pyridine. In this way, contamination problems with flame-ionisation detectors in g.l.c. and ion sources in m.s. were avoided. At the same time, tailing could be diminished by the use of another solvent for g.l.c. On the other hand, the removal of the reagents made the TMS ethers, and especially the TMS esters, more sensitive to decomposition. Deterioration due to water and other reactive compounds in the solvent was prevented by the addition of BSTFA to the solvent. Hydrolysis from air moisture was avoided by keeping the solutions in sealed glass capillaries. Derivatives protected by these measures were shown by g.l.c. to be unchanged after storage for several years.

Sodium salts were used routinely for the preparation of TMS esters because of their convenience, but it was shown that other salts (*e.g.*, Ca and Ba) can also be used.

Effect of the stationary phase. The structure of the TMS derivatives is similar to the polysiloxane structure of the silicone stationary phases, and the best chromatographic results were obtained with this type of phase. In contrast, several polar stationary phases, including some organosilicones, gave small, specific retention-volumes and tailing peaks as might be expected with non-polar solutes.

Relative retention times for four of the most useful silicones are given in Table I for aldonic and deoxyaldonic acids. Corresponding data for aldaric and deoxyaldaric acids were given previously[16]. When different temperatures were used for a column, relative retention times closer to unity were normally obtained at higher temperatures.

As with the hydroxy dicarboxylic acids[16], the compounds were eluted in almost the same order on all stationary phases. This order also applies to data for aldonic acids[15] on other stationary phases, and to earlier scattered data on aldonic[7,9,14] and aldaric[7,15] acids.

For structurally related compounds, the retention increases in the order alditol<aldonic acid<aldaric acid, and the differences are larger for XE-60, QF-1, and OV-17 phases than for OV-1. This is explained by contributions to the retention from polar interactions with the ester functional groups. These are smaller for the essentially non-polar methyl silicone OV-1 than for the phenyl silicone OV-17, the trifluoropropyl silicone QF-1, and the cyanoethyl silicone XE-60.

Theoretically, the OV-17, QF-1, and XE-60 phases could give different retentions for enantiomers because of a chiral structure. However, within experimental errors, identical, relative retentions were obtained for D- and L-galactonic acids and for the oxime TMS derivatives of D- and L-arabinose on both OV-17 and

TABLE I

G.L.C. DATA FOR ALDONIC AND DEOXYALDONIC ACIDS AS TRIMETHYLSILYL DERIVATIVES: RELATIVE RETENTIONS[a]

	OV-1 160°	*OV-17* 160°	*QF-1* 120°	*XE-60* 120°
Aldonic acids				
Glyceric	0 052	0.092	0 086	0.087
Erythronic	0.151	0.218	0.212	0.214
Threonic	0.156	0.251	0.237	0.256
Ribonic	0.438	0.569	0.565	0.567
Arabinonic	0.463	0.646	0.632	0.661
Xylonic	0.428	0.617	0.570	0.621
Lyxonic	0.457	0.599	0.621	0.641
Allonic	1.115	1.276	1.410	1.380
Altronic	1.247	1.531	1.568	1.637
Gluconic	1.286	1.569	1.639	1.713
Mannonic	1.113	1.254	1.385	1.433
Gulonic	1.087	1.256	1.309	1 332
Idonic	1.368	1.743	1.812	1.930
Galactonic	1.256	1.561	1.570	1.671
Talonic	1.235	1.507	1.656	1.656
Deoxyaldonic acids (unbranched)				
2-Deoxytetronic	0.084	0.146	0.145	0.149
3-Deoxytetronic	0.078	0.136	0.129	0.135
4-Deoxyerythronic	0.058	0.094	0 091	0.086
4-Deoxythreonic	0.061	0.099	0.092	0.094
2-Deoxy-*erythro*-pentonic	0.252	0.379	0.395	0 409
2-Deoxy-*threo*-pentonic	0.251	0.373	0.392	0.402
3-Deoxy-*erythro*-pentonic	0 236	0.380	0.375	0.404
3-Deoxy-*threo*-pentonic	0 252	0.419	0.418	0.455
2-Deoxy-*arabino*-hexonic	0.659	0.912	1.007	1.031
2-Deoxy-*lyxo*-hexonic	0.673	0.935	1.035	1.080
3-Deoxy-*ribo*-hexonic	0.673	0.946	1.007	1.107
3-Deoxy-*arabino*-hexonic	0.676	0.971	1.004	1.080
3-Deoxy-*xylo*-hexonic	0.659	0.995	1.026	1.116
3-Deoxy-*lyxo*-hexonic	0.644	0.894	0.973	1.070
6-Deoxymannonic	0.528	0.629	0.687	0.685
6-Deoxygalactonic	0.615	0.809	0.801	0.805
Deoxyaldonic acids (branched)				
2-*C*-Methylglyceric	0.054	0.082	0.072	0.068
3-Deoxy-2-*C*-(hydroxymethyl)tetronic	0.224	0.326	0.319	0.331
2-*C*-Methylribonic	0.564	0.689	0.742	0.706
3-Deoxy-2-*C*-hydroxymethyl-*erythro*-pentonic (α-isosaccharinic)	0.707	0.942	1.003	1.114
3-Deoxy-2-*C*-hydroxymethyl-*threo*-pentonic (β-isosaccharinic)	0.670	0.896	0.933	1.028

[a]Adjusted retention times relative to those of the glucitol derivative (11.4 min for OV-1; 5.6 min for OV-17; 15.2 min for QF-1; 11.8 min for XE-60). The last decimal figure was difficult to reproduce, especially over large time-intervals and with different columns.

QF-1. These results indicate that differences for enantiomers are negligible, and the enantiomeric prefixes are therefore omitted throughout this paper.

Effect of compound structure. From the data in Table I, it is seen that all acids are eluted in the order of an increasing number of TMS groups. The same is true for polyhydric alcohols[18], hydroxy dicarboxylic acids[16], and most other types of sugar TMS derivative. With few exceptions, aldonic acids are eluted before deoxyaldonic acids having the same number of TMS groups. These observations are consistent with a predominant influence of non-polar interactions, the strength of which is known to depend mainly on the size of the molecule.

The 2- and 3-deoxyaldonic acids are retained more strongly than the isomeric 2-*C*-methylaldonic and ω-deoxyaldonic acids. Presumably, the ester functional group in the latter derivatives is better protected from interactions with the stationary phase by surrounding, non-polar groups.

The relative retention times for the diastereomeric aldonic acids differ much more than those of the corresponding alditols, but less than those of the corresponding aldaric acids. This indicates that the availability of the ester groups for polar interactions with the stationary phase is the principal factor that determines the retention order for the diastereomeric acids.

Dreiding models demonstrate that the ester groups in derivatives with OTMS groups at C-2 and C-3 are best protected when these groups are antiparallel. The *erythro* and *threo* configurations correspond to antiparallel and gauche conformations, respectively, in the favoured, zigzag conformation. Accordingly, erythronic and 4-deoxyerythronic acids are eluted before the corresponding *threo* isomers. For pentonic and hexonic acids, deviations from the planar, zigzag conformation influence the order of retention. Thus, parallel 1,3-interactions in the planar, zigzag conformers promote rotation between C-2 and C-3 for talonic acid and between C-3 and C-4 for allonic acid. As a result, the antiparallel conformation at C-2 and C-3 should be favoured for allonic, mannonic, and gulonic acids, and these compounds are eluted much faster than the five remaining hexonic acids. The retention order of diastereomeric aldaric acids is explained by similar considerations[16].

Analytical applications. From Table I, it is seen that all non-isomeric acids can be conveniently separated by using one of the four stationary phases. The same is true for most of the constitutionally isomeric deoxyaldonic acids, but several 2- and 3-deoxyaldonic acids exhibit similar, relative retention times. Many of the diastereomeric acids can also be well separated, and the relative retention times of almost all diastereomers differ enough to permit qualitative identification.

For acids which are not separated as acyclic TMS esters, complementary analysis of their 1,4-lactones as TMS derivatives may be advantageous. The lactones appear before the TMS esters on non-polar stationary phases (*e.g.*, OV-1 and SE-30), as expected from the lower molecular weight of the lactone derivatives. On QF-1, XE-60, and other more-polar stationary phases, the TMS esters appear first.

The method described for the analysis of hydroxy acids has been widely applied in this department (*e.g.*, refs. 19 and 20) in studies of the degradation of

polysaccharides. Isolated samples of acids were normally analyzed on QF-1 only, but sometimes also on OV-1 or OV-17. Acids of the disaccharide type were usually analyzed on OV-1 at 250°. The volatile derivatives of lactic, glycolic, and hydracrylic acids were separated in that order on QF-1 at 60°. When pyridine solutions containing such derivatives were evaporated, care had to be taken to avoid losses. No losses in the preparation of derivatives or in g.l.c. were observed with any acids. Rough, quantitative results were obtained from peak-area ratios. More-detailed determinations can be based on calibration curves[15] or relative molar responses[14].

A major advantage of the technique is its applicability to such different types of acids as aldonic and saccharinic, mono- and di-carboxylic (Fig. 1A), and lactone-forming and non-lactone-forming. Its versatility makes it suited to acids of unknown structure, and the relationships between retention and structure are useful in structural analysis.

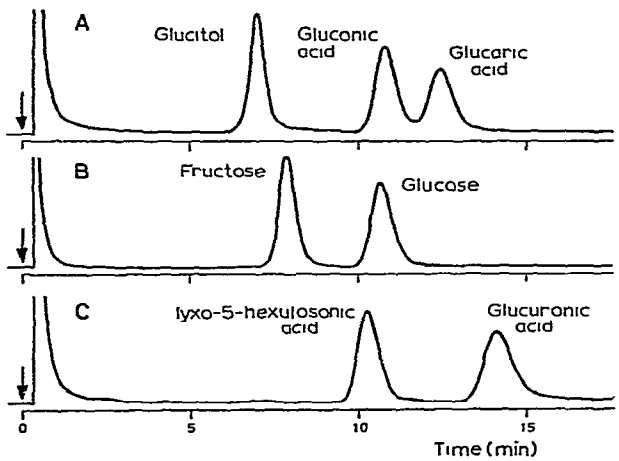


Fig. 1. Gas chromatographic separations (OV-17; 160°) of glucose-related compounds as acyclic derivatives: A, ether–ester trimethylsilyl derivatives; B, ether–oxime trimethylsilyl derivatives; C, ether–ester–oxime trimethylsilyl derivatives.

Mass-spectrometric identification. Conclusive identifications of the sugar derivatives were made by using g.l.c.–m.s. QF-1 columns did not give rise to any troublesome background from column bleeding below 180°, and OV-1 and OV-17 are even more thermally stable.

The use of mass spectra for structural analysis was dealt with in papers on aldonic and deoxyaldonic acids[21], hydroxy-dicarboxylic acids[22], and aldonolactones[23]. Non-isomeric derivatives and constitutional isomers give rise to spectra which are characteristic of the structure. The spectra of diastereomers are similar, but differences in relative intensities can often be used for identification. The molecular weight is usually obtained from M (molecular ion) or M−15 peaks. The spectra of the acyclic derivatives are easily correlated with the structure. Abundant ions from carbon-chain cleavages reveal structural features such as the position of deoxy groups.

Odd-electron[24], as well as even-electron[22], rearrangement ions are also useful for structural conclusions. For unknown acids, the structure can often be partly or completely deduced from the mass spectrum.

ALDOSES AND KETOSES

The present study was started with the aim of developing a method for neutral sugars suitable for combination with that for acidic sugars described in the previous section. Results with oxime[6] and *O*-methyloxime[25] TMS derivatives of keto acids suggested that these types of derivatives might be suitable. Sweeley *et al.*[5] prepared oxime TMS derivatives of aldohexoses, but relied mainly on other types of TMS derivatives for g.l.c. TMS derivatives of oximes prepared in aqueous solution have been used for analysis of glycolaldehyde and glyceraldehyde from Smith degradations[26]. Recently, g.l.c. analysis of fructose and a number of aldoses as oxime TMS derivatives was successfully applied to sugars in foods[27].

Trimethylsilyl derivatives of oximes. TMS derivatives were prepared as with the sugar acids, but with allowance for BSTFA consumption from the oximation reagents. The oximes were converted into *O*-trimethylsilyloximes, whereas the *O*-methyloxime functional group was unaffected. The silylation was normally completed within a few minutes at room temperature. The reaction was less critical than with the sugar acids, permitting HMDS+TMCS to be used[27] as an alternative to BSTFA+TMCS.

All oxime TMS derivatives studied by m.s. were acyclic, and no evidence for the formation of cyclic oxime derivatives was found. Both *syn* and *anti* isomers of the oximes were formed. Similar amounts of the two isomers were obtained for ketoses and 2-deoxyaldoses, whereas one major and one minor (10–30%) isomer were observed for aldoses. It seems likely that steric reasons explain these facts and that the major component is the *syn* isomer having the two bulky groups on opposite sides of the double bond. Only one isomer was observed with dihydroxyacetone, in accordance with its symmetry properties.

Retention data. Relative retention times for oxime TMS derivatives of aldoses, ketoses, and deoxyaldoses are given in Table II. The distribution coefficients of the *syn* and *anti* forms are similar, and frequently only one peak was recorded for both species. For aldoses, the relative positions of the two isomers depend on the configuration, the molecular weight, and the stationary phase. The minor isomer appears earlier for the smaller molecules and earlier on OV-17 than on QF-1.

The relative retention times on OV-1 and the more-polar QF-1 are similar and demonstrate that the oxime derivatives behave essentially like non-polar solutes. The stronger retention on OV-17 suggests that interactions between the π-electron systems of solute and stationary liquid are important.

As expected, the oxime derivatives are eluted primarily according to the molecular weight. Most aldoses are retained more strongly than the isomeric ketoses, and the 2-deoxyaldoses more strongly than the 6-deoxyaldoses, in accordance with the different accessibilities of the oxime functional groups for interactions with the

stationary phase. The differences between diastereomers are smaller than with the TMS esters.

Similar behaviour was observed for the *O*-methyloxime derivatives, but the retention times were considerably shorter.

TABLE II

G.L.C. DATA FOR NEUTRAL MONOSACCHARIDES AS ACYCLIC OXIME AND *O*-METHYLOXIME TRIMETHYLSILYL DERIVATIVES: RELATIVE RETENTIONS[a]

	OV-1 160°		*OV-17* 160°		*QF-1* 120°	
Oximes, aldoses[b]						
Glyceraldehyde	0.06[c]		0.09	0.08	0.06	
Erythrose	0.16[c]		0 23	0.20	0.15	
Ribose	0.48		0.60[c]		0.46[d]	
Arabinose	0.43		0.53		0.40[d]	
Xylose	0.42		0.55		0.40	
Glucose	1.34		1.55		1.32	1.50
Mannose	1.30[d]		1.45		1.31	1.51
Galactose	1.27	1.41	1.43	1.57	1.26	1.54
Oximes, ketoses						
Triulose (dihydroxyacetone)	0.05		0.09		0.05	
Tetrulose	0.14[e]		0 20		0.13	
erythro-Pentulose (ribulose)	0.41		0.50		0.37[e]	
threo-Pentulose (xylulose)	0.40		0.51		0.36	
arabino-Hexulose (fructose)	1.07		1.13		1.03	
xylo-Hexulose (sorbose)	1.07		1.19		1.02	
Oximes, deoxyaldoses						
2-Deoxy-*erythro*-pentose (2-deoxyribose)	0 28		0.41	0 44	0.31	
2-Deoxy-*arabino*-hexose (2-deoxyglucose)	0.78		1.01	1.08	0.89	
2-Deoxy-*lyxo*-hexose (2-deoxygalactose)	0.78[e]		1.02	1.10	0.90	
6-Deoxymannose (rhamnose)	0.58		0.68		0.58[d]	
6-Deoxygalactose (fucose)	0.58[d]		0.69		0.57	0.65
O-Methyloximes[b]						
Xylose	0.25		0.39		0.25	
Glucose	0.80[d]		1.04[d]		0.84	1.07
Mannose	0.77		0.96		0.83	1.02
arabino-Hexulose (fructose)	0.73		0.94	1.02	0.72	

[a]Adjusted retention times relative to those of the glucitol derivative (13.2 min for OV-1; 6.6 min for OV-17; 14.4 min for QF-1). [b]Major isomer in main columns. [c]Shoulder before main peak. [d]Shoulder after main peak. [e]Two unresolved peaks of similar size.

Analytical applications. Coincident peaks from the *syn* and *anti* isomers and large differences in retention times are advantageous in most analytical applications. From Table II, it is seen that glucose, fructose, and mannose are better separated as

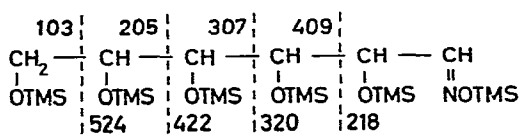
oxime than as *O*-methyloxime derivatives, particularly on OV-17. All three stationary phases in Table II were useful for various applications, whereas tailing peaks were obtained with XE-60.

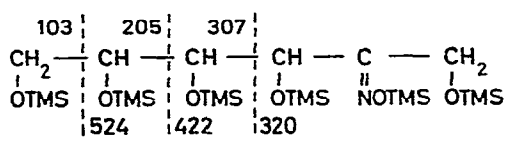
The oxime TMS derivatives are particularly suitable for the analysis of samples containing sugars of different structural types. In this study, the application to ketoses was emphasized since these sugars are usually difficult to analyze by g.l.c. Direct silylation was recently shown to give several products, including enediol derivatives, for many types of ketoses[28]. Reduction, followed by acetylation, produces two alditol acetates from each ketose and these may interfere with the analysis of aldoses. By the present method, ketoses are conveniently analyzed in mixtures with aldoses (Fig. 1B). For compounds of low molecular weight, lower column-temperatures were preferred. Glyceraldehyde and dihydroxyacetone, which form intermolecular hemiacetals in solution, were completely converted into the monomeric oxime TMS derivatives.

Suitable conditions for the separation of any neutral sugar from any hydroxy acid can usually be found because of the strong retention of the oxime TMS derivatives on OV-17 and because of other phase-dependent differences. The *O*-methyloxime derivatives can also be used to achieve such separations.

The oxime TMS derivatives are suitable for quantitative determinations[27], and the present procedure was applied to samples containing 0.1 mg or more of each sugar. In qualitative studies, analysis with and without oximation was useful to demonstrate the presence or absence of aldehydo or keto groups in the components of a sample.

Mass-spectrometric identification. The fragmentation of the *O*-methyloxime TMS derivative of glucose was studied by Laine and Sweeley[29]. It was shown that prominent and structurally significant ions were formed by carbon-chain cleavages. In the present study, the fully TMS-substituted oximes were used for analysis by g.l.c.–m.s. The fragmentation is analogous to that of the *O*-methyloxime derivatives, but ions containing the oxime functional group are 58 mass units larger.





Scheme 1. Mass-spectrometric, chain-cleavage fragmentations of oxime trimethylsilyl derivatives of hexoses and hexuloses.

The peaks from chain-cleavage ions were the most useful for structural determinations of various types of sugars. These cleavages and the masses of the ions are indicated in Scheme 1 for hexoses and hexuloses. α-Cleavages at the oxime functional group do not occur easily, and the derivatives are rather stable towards fragmentation. As a result, the molecular weight is usually obtained easily from M (odd mass number) or M−15 (even mass number) peaks. Of the remaining chain cleavages, β-cleavage with charge retention on the non-nitrogen-containing fragment is particularly favoured and useful for structural conclusions. The spectra of diastereomers and of *syn* and *anti* isomers are similar.

URONIC ACIDS

Preparation of TMS derivatives of uronic acids may produce as many as six different species for lactone-forming hexuronic acids. Although many papers have described g.l.c. of samples containing one or two uronic acids by this method, it is clearly desirable to diminish the number of components for g.l.c. One approach is the reduction to aldonic acids[10], followed by g.l.c. of the silylated aldono-1,4-lactones[9–12] or of the silylated, acyclic aldonic acids as described here. The alternative elimination of the uronolactone derivatives by initial saponification leaves the four monocyclic pyranose and furanose derivatives. The present study shows that saponification can be combined with the preparation of oximes to give a further, simplified g.l.c. procedure.

Preparation of derivatives. The uronic acids were converted into their sodium salts and dried in the same way as for other hydroxy acids. Glucuronic, mannuronic, guluronic, and iduronic acids, which form furanurono-6,3-lactones, required 4 h at pH 8.5 for complete saponification, whereas shorter times could be used for other hexuronic acids and for penturonic and hexulosonic acids, which normally do not form lactones.

The oximation process was slower with sodium salts than with neutral sugars, and prolonged times were sometimes used to ensure complete reaction. Silylation was effected in the pyridine solutions of the oximes. Direct oximation of glucofuranurono-6,3-lactone was shown by m.s. to produce the monocyclic *syn* and *anti* oxime derivatives of the lactone. Incomplete saponification was therefore detected by g.l.c. peaks from such lactone TMS derivatives, whereas incomplete formation of oximes was revealed by peaks mainly from pyranose TMS derivatives of the uronic acids.

5-Hexulosonic acids appear as reactive, isomerization products from uronic acids. The mild conditions of the procedure for the preparation of derivatives permitted its application to these acids as well.

G.l.c. Retention data are given in Table III for some hexuronic acids and for the 5-hexulosonic acids corresponding to sorbose and fructose.

The retention values reflect the combined acid and sugar character of the structure. The relative amounts of the *syn* and *anti* isomers and the retention differences between them are similar to those of the corresponding sugars, with the

TABLE III

G.L.C. DATA FOR URONIC ACIDS AS ACYCLIC OXIME TRIMETHYLSILYL DERIVATIVES: RELATIVE RETENTIONS[a]

	OV-1 160°		*OV-17* 160°		*QF-1* 160°	
Uronic acids[b]						
Glucuronic	1.47		2.04		1.57	1.74
Mannuronic	1.47	1.65	1.96		1.57	1.84
Guluronic	1.62	1.90	2.30	2.53	1.73	2.20
Galacturonic	1.56	1.74	2.20	2.41	1.60	1.91
Hexulosonic acids						
xylo-5-Hexulosonic	1.42		2.00		1.55	
lyxo-5-Hexulosonic	1.20		1 46		1.31	

[a]Adjusted retention times relative to those of the glucitol derivative (13.2 min for OV-1; 6.6 min for OV-17; 4 3 min for QF-1). [b]Major isomer in main columns.

major isomer appearing first. The TMS ester group explains the higher relative retention on QF-1 than on OV-1, and the TMS oxime group the still higher relative retention on OV-17. Acids having the *erythro* configuration at the two carbon atoms adjacent to the ester group are eluted before diastereomers having the *threo* configuration. This effect is large, particularly with the 5-hexulosonic acids, and is explained in the same way as with other 2,3-dihydroxy acids.

As indicated by the tabulated data, the OV-17 phase is better suited to the analysis of most uronic acids than OV-1 and QF-1. In Fig. 1C, the separation of glucuronic acid and its isomerization product, *lyxo*-5-hexulosonic acid, is illustrated. From Tables I–III, it is seen that uronic acids can be easily separated from other hydroxy acids and from neutral sugars.

Mass spectrometry. G.l.c.–m.s. was used for structural analysis of the derivatives. The mass spectra of the derivatives of glucuronic acid (the major oxime isomer) and *lyxo*-5-hexulosonic acid are shown in Fig. 2. The base peak at *m/e* 73 is due to the trimethylsiliconium ion, and the peak at *m/e* 147 to the nonspecific pentamethyldisiloxonium ion. The M−15 ions are formed by the well-known loss of a silicon-linked methyl group, and the M−15−90 ions by the further elimination of trimethylsilanol. For the aldoxime, a characteristic M−15−117 ion is obtained by the additional loss of the elements of HCN.

The most-striking differences between the two types of spectra are explained by the characteristic β-cleavage fragmentation. For the hexuronic acids, the abundant *m/e* 333 ion is formed from the β-cleavage ion of mass 423 by elimination of trimethylsilanol. With the 5-hexulosonic acids, β-cleavage produces the *m/e* 321 ion and, through the further loss of CO_2, the *m/e* 277 ion, as demonstrated by a metastable peak. The formation and further fragmentation of chain-cleavage ions from TMS esters were discussed in more detail for aldaric acids[22].

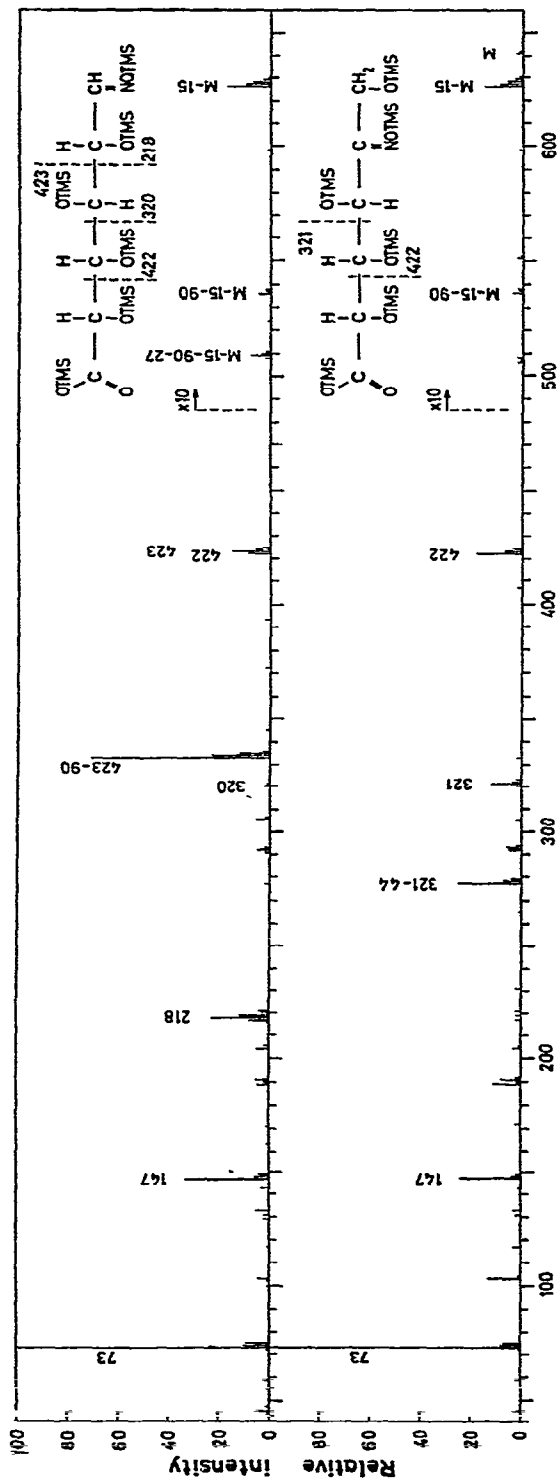


Fig. 2. Mass spectra at 70 eV of acyclic oxime trimethylsilyl derivatives of glucuronic acid and *lyxo*-5-hexulosonic acid.

Reproducible differences in relative intensities were found to exist between *syn* and *anti* isomers and to a smaller extent between diastereomers. Thus, the *m/e* 422 ion was more abundant for the major than for the minor oxime isomers of the hexuronic acids.

The interpretation of the spectra of the hexuronic acids was supported by the appropriate shifts of 58 mass units in the spectrum of 4-*O*-methylglucuronic acid for the ions comprising the OMe group. To obtain further evidence, the d_9-TMS derivative was prepared[22] for galacturonic acid. Mass shifts in accordance with all ion structures proposed were obtained.

Fig. 2 illustrates that even the most complicated structures studied can be easily correlated with the mass spectra. Clearly, the acyclic oxime and ester TMS derivatives are suitable for structural analysis by g.l.c.–m.s.

EXPERIMENTAL

The reference compounds were either commercial samples, or were prepared in previously published work from this department.

The procedure described below was applied to all types of compounds studied in this paper.

Conversion of acids and lactones into sodium salts. — The sample (0.1–10 mg) was dissolved in water (2–5 ml), and 0.05M NaOH was added until pH 8.5 was reached. Unless lactones were known to be absent, the solution was stirred magnetically for 4 h at pH 8.5 and room temperature. Constant pH was maintained by automatic titration with 0.05M NaOH (Titrator Type TTTl, Radiometer, in combination with Dosimat E412, Metrohm). If the sample was not known to be alkali-stable, the pH was adjusted to 7 before evaporation, by the addition of 0.01M HCl. Water was removed at 35° in a vial (5–10 ml) connected to a rotary evaporator. The sample was further dried by two successive evaporations of dichloromethane (1–2 ml) at 35°. Alternatively, the residue was kept at least one day in vacuum over P_2O_5. After the sample had been dried, 1–2 ml of NaOH-dried pyridine was added.

Preparation of oximes. — To a solution or suspension of the sample in pyridine was added approximately the same amount of hydroxylamine hydrochloride. The solution was kept in an ultrasonic water-bath (Varian Aerograph Model 220, 50 kHz) at 30° for 2 h, or shaken mechanically for at least 2 h at room temperature. *O*-Methyloximes were prepared analogously by using $CH_3ONH_2 \cdot HCl$ (Applied Science Lab.).

Preparation of trimethylsilyl derivatives. — Required amounts of reagents were calculated from the weights of dry sample (*a* mg), $HONH_2 \cdot HCl$ or $CH_3ONH_2 \cdot HCl$ (*b* mg), and remaining water (*c* mg). To the solution or suspension in pyridine were added BSTFA ($10a+10b+25c+100$ µl) followed by half the volume of TMCS. The reagents (Pierce or Macherey-Nagel) in capped vials were transferred with a 1-ml syringe. The reaction was completed by vibrating the reaction vials for at least 2 h at room temperature, permitting subsequent, direct g.l.c. Normally, however, pyridine

and excess reagents were removed at 30° (rotary evaporator) and the sample was dissolved in anhydrous ethyl ether (0.5–1 ml) containing 0.5% of BSTFA. For storage, the solution was transferred by means of a 1-ml syringe to glass capillaries which were sealed in a gas flame without cooling.

G.l.c. data. — A Perkin–Elmer Model 900 instrument with flame-ionization detectors was used. Columns [0.2 cm (i.d.) × 2 m] of stainless steel were packed with 0.5% OV-1 (methyl silicone) on 100/120 mesh Chromosorb G, 0.5% OV-17 (50% phenyl, methyl silicone) on 100/120 mesh Chromosorb G, 3% DC QF-1 (50% trifluoropropyl, methyl silicone) on 100/120 mesh Gas Chrom Q, or 1% GE XE-60 (25% cyanoethyl, methyl silicone) on 100/120 mesh Gas Chrom Q. Injector and manifold temperatures were ~50° above the column temperature. The carrier gas was purified nitrogen; 50 p.s.i. inlet pressure and 30–35 ml/min flow-rate at room temperature and pressure. Samples (0.1–1 μl) were injected with Hamilton Model 7101 (1.0-μl) or Model 701 (10-μl) syringes.

Adjusted retention times were measured from the ethyl ether solvent-front. Relative retention times were determined from runs of the reference compound before and after the sample studied.

Mass spectrometry. — Reference spectra were recorded on a LKB 9000 gas chromatograph–mass spectrometer. Operation data[21–24]: molecular separator 210°, ion source 270°, accelerating voltage 3.5 kV, trap current 60 μA, exit slit 0.05 mm, collector slit 0.10 mm.

Routine identifications were made on an AEI Organic MS 20 mass spectrometer connected *via* a Biemann separator to a Varian Model 1200 gas chromatograph.

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