CHROM. 6115

COMMENTS ON GAS–LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY OF METHYLATED ALDITOL ACETATES

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(First received December 22nd, 1971; revised manuscript received April 24th, 1972)

SUMMARY

Butanediol succinate is shown to be a useful column for the gas–liquid chromatographic separation of methylated alditol acetates. These compounds are readily identified by mass spectrometry even when the two instruments are physically separate. Substances not separated by gas–liquid chromatography may be collected from the effluent resolved by thin-layer chromatography, and the components identified by gas chromatography–mass spectrometry.

INTRODUCTION

The determination of polysaccharide structures by methylation analysis has been greatly simplified in recent years by three major improvements in experimental technique. These are the HAKOMORI method of methylation[1], the experimental details of which have been well described by SANDFORD AND CONRAD[2], the separation of methylated sugars or their derivatives by gas–liquid chromatography (GLC)[3], and the determination of the position of methoxyl groups by mass spectrometry (MS). Some aspects of these methods have been reviewed recently[4]. The HAKOMORI method of methylation has been used by many investigators as has GLC for the separation of methylated compounds. By contrast, the identification of methylated sugars and derivatives by MS has not been widely adopted.

PETERSSON AND SAMUELSON[5,6] have studied the fragmentation of the trimethylsilyl derivatives of methylated sugars while LINDBERG and his associates have examined methylated alditol acetates[7]. In view of the simpler gas chromatogram obtained from alditols the latter method may be considered the more generally useful and is the only method considered here. A further advantage of acetates is that they yield fragments of lower mass and are thus preferable for low-resolution mass spectrometers. It should, however, be noted that GLC of trimethylsilyl derivatives of methylated sugars is one of the useful alternatives where the alditol acetates, *e.g.*, 2- and 3-O-methyl-D-xylose[8], are inseparable.

The identification of methylated sugars by MS of the derived alditol acetates has been widely used by LINDBERG and his associates, primarily in studies on the O-antigen specific polysaccharides of *Salmonella* (see ref. 9 and earlier papers) and by a few other Swedish workers in studies on the xylans of eucalypt and birch[10] and of red cotton wood[11]. Outside of the group originating this method of analysis, the identification of methylated sugars by MS has been adopted by a few other workers, amongst whom may be cited ASPINALL AND COTTRELL[12] in studies on soybeans, and WHYTE AND ENGLAR[13,14] working on red algae.

In the original publication[7] and in all the cases cited above, except the work mentioned in refs. 13 and 14, a gas–liquid chromatograph linked to a mass spectrometer was used. While such a combined unit has many advantages it is not mandatory. The main purpose of this paper is to emphasize this fact. We have studied polysaccharides of several different types, and have analyzed the methylated sugars therefrom by means of an isolated mass spectrometer using samples which were collected in glass melting point capillary tubes from the exit port of the gas chromatograph. The handling of such samples is facilitated by a collector, described elsewhere[15], but this is not essential. A secondary purpose of this paper is to comment on the interpretation of the mass spectra, and on suitable columns for GLC.

MATERIALS AND METHODS

An F & M Model 720 dual-column gas–liquid chromatograph was used, with stainless-steel columns (6 ft. × 0.25 in.) containing 5% butanediol succinate (BDS) on Diatoport S (60–80 mesh), and programmed at 3°/min from 140° to 205°. Helium flow-rate was 88 ml/min.

Mass spectra were obtained with an AEI MS 9 apparatus operated at 70 eV, and with a Nuclide 1290 G spectrometer.

Thin-layer chromatography (TLC) was carried out on silica gel with butanone–water.

Methylated alditol acetates were demethylated with boron trichloride[16], and the product was acetylated by heating for 1 h at 100° in a sealed tube with acetic anhydride–pyridine (1:1).

Methylated polysaccharides have been obtained from corn leaf and corn stalk xylan[17], lemon gum[18,19], sapote gum[20], glucomannans of coniferous woods[21], and *Klebsiella* Type 21 capsular polysaccharide[22].

RESULTS AND DISCUSSION

Lemon gum has been shown by STODDARD AND JONES[23] to be a branch-on-branch type of polysaccharide containing L-arabinose, D-galactose, and 4-O-methyl-D-glucuronic acid. These authors separated by GLC the methylated sugars from the fully methylated gum, in the form of methyl glycosides. This gum was, therefore, a convenient source of methylated sugars whose separation as alditol acetates and identification by MS could be studied. It is also the first gum to be examined in this manner.

The gas–liquid chromatogram reproduced in Fig. 1 shows the peaks obtained when the methylated alditol acetates from the neutral sugars in the hydrolyzate

of methylated lemon gum were analyzed. Fig. 2 shows the thin-layer chromatogram of each of the ten fractions obtained by GLC. It is clear that certain peaks represent mixtures. While this may be anticipated from the shape of peaks 5, 6 and 7, it is more surprising in the case of peaks 3 and 9, which give the impression of being homogeneous. Thus one advantage of having the gas chromatograph divorced from the mass spectrometer is that it permits a check by another technique, *e.g.*, TLC, on the homogeneity of each component separated by GLC. In the present instance TLC permitted the separation of each of the pairs of components represented by peaks 6 and 7. The pure compounds thus obtained were re-injected individually onto the GLC column and analyzed by MS. Peak 6 was thus shown to represent 2,3-di-O-methyl-L-arabinitol acetate (R_F 0.47) and 2,3,4,6-tetra-O-methyl-D-galactitol acetate (R_F 0.32) while peak 7 was composed of 2,3,4-tri-O-methyl-D-glucitol acetate (R_F 0.43) and a mixture of 2,3,6- and 2,4,6-tri-O-methyl-D-galactitol acetates (R_F 0.35).

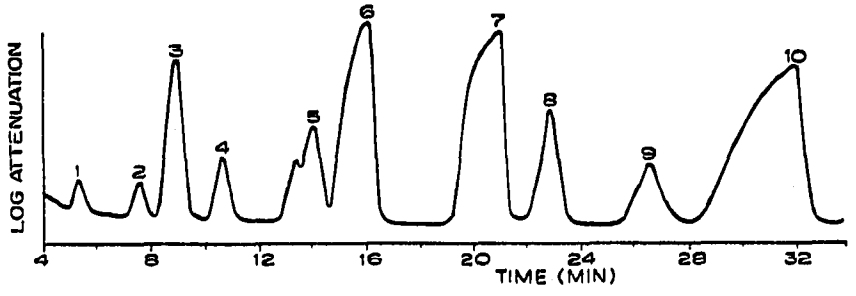

Fig. 1. Gas–liquid chromatogram of methylated alditol acetates from neutral sugar portion of methylated lemon gum hydrolyzate.

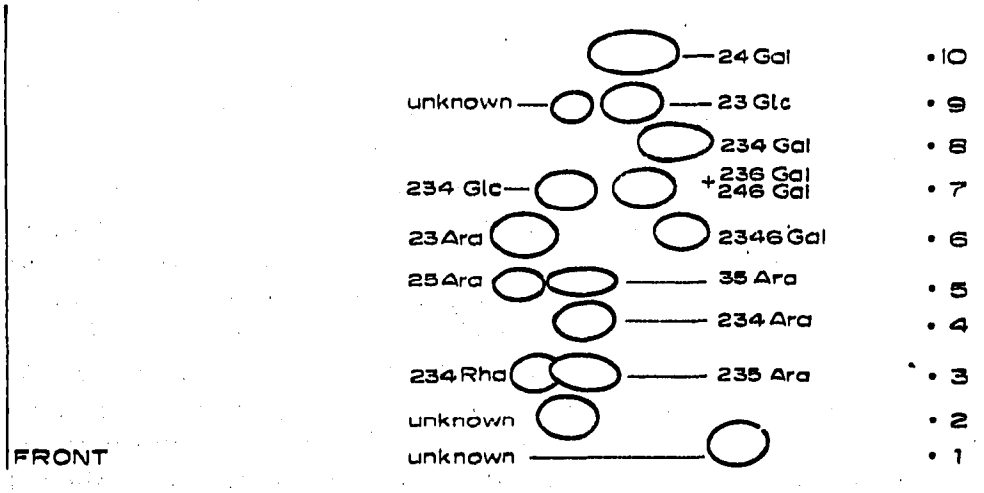

Fig. 2. Thin-layer chromatogram of the ten fractions shown in Fig. 1.

It must be recognized that a mass spectrum alone is able to show clearly the presence of a mixture and may also permit a quantitative analysis of the composition[7]. This was demonstrated in the present context where peak 3 was shown by analysis of the mass spectrum to be a mixture of 2,3,5-tri-O-methyl-L-arabinitol and 2,3,4-tri-O-methyl-L-rhamnitol acetates. The heterogeneity of a GLC fraction

may also be ascertained by comparing the mass spectra obtained from the first and last portions of the peak.

Using these techniques it was possible to identify the peaks in Fig. 1 as shown in Table I. In a similar manner the methylated alditol acetates obtained in studies on corn hemicelluloses[17], coniferous glucomannans[21], sapote gum[20], and *Klebsiella* Type 21 capsular polysaccharide[22] have all been identified by MS following separation by GLC.

TABLE I

METHYLATION ANALYSIS OF LEMON GUM

Alditol acetate	*GLC peak (No.)*	*GLC peak area*[a] (%)
Unknown	1	0.51
Unknown	2	0.70
2,3,5-Me_3-Ara	3S[b]	7.02
2,3,4-Me_3-Rha	3F	
2,3,4-Me_3-Ara	4	1.40
2,5-Me_2-Ara	5F	1.83
3,5-Me_2-Ara	5S	3.62
2,3-Me_2-Ara	6F	21.7
2,3,4,6-Me_4-Gal	6S	
2,3,4-Me_3-Glc	7F	28.5
2,3,6-Me_3-Gal	7S	
2,4,6-Me_3-Gal	7S	
2,3,4-Me_3-Gal	8	3.96
2,3-Me_2-Glc	9S	3.16
Unknown	9F	
2,4-Me_2-Gal	10	27.6

[a] As computed by Infotronics digital integrator Model CRS-100.
[b] F, faster; S, slower on TLC (see Fig. 2).

The most reliable method for identifying an unknown by MS is by comparison with the spectrum of a standard, run under identical conditions. Failing this, use may be made of the excellent data provided by BJÖRNDAL *et al.*[7]. These authors gave a table of the *m/e* values for the principal fragments whose peaks had intensities greater than 10% of the base peak. The relative intensity of different peaks, however, is known to change with experimental parameters concerning the instrument used and the method of introduction of the sample. The discounting of weak peaks (admittedly arbitrary[7]) is in most cases justified but occasionally an ion with a high *m/e* value, yet of low relative intensity, may be particularly useful for diagnostic purposes, *e.g.*, *m/e* 333 (M—73) from 2-O-methylglucitol acetate. In our experience good agreement may be obtained with the published data[7] and in view of the high reproducibility of the fragmentation patterns it is surprising that this method of identification has not been more widely adopted.

MS analysis of methylated alditol acetates permits determination of the position of the methoxyl groups but not of the stereochemistry since diastereoisomers give the same fragmentation patterns. LINDBERG and colleagues[7] have recommended the use of two standard substances, with short and long retention times, respectively, when analyzing methylated sugars as methylated alditol acetates. In this way

accurate values for relative retention times can be obtained and these, together with MS fragmentation patterns, enable many components in a mixture to be identified. A more positive method of distinguishing between diastereoisomers is to collect samples of the GLC effluent and to demethylate using boron trichloride[10]. The resulting alditol may then be acetylated, purified by GLC, collected, and in the case of the common hexitols, identified by melting point. This method has been successfully used to distinguish between D-glucose and D-mannose derivatives in wood glucomannans[21] and between D-mannose and D-galactose compounds in *Klebsiella* Type 21 capsular polysaccharides[22]. Likewise, the uronic acid occurring in this capsular polysaccharide has been positively confirmed as D-glucuronic acid by transformation to D-glucitol hexaacetate which was separated by GLC and identified by melting point[22].

The GLC separation of alditol acetates was not readily achieved until the introduction of ECNSS-M as a liquid phase[24]. Almost all authors who have employed this material have used it at a concentration of 3 % on Gas-Chrom Q. An important paper by SHAW AND MOSS[25] in this regard seems to have been largely overlooked. These authors studied the related liquid phases EGSS-X, EGSS-Y, and EGSP-Z with particular reference to the separation of difficult pairs of acetates such as those of rhamnitol and fucitol or glucitol and galactitol. They found that the efficiency of the separation was very dependent on the nature of the solid support. For glucitol and galactitol acetates they obtained the best resolution on the most active support, non-acid washed Chromosorb W, rather than on this material treated with dimethyl chlorosilane or on Gas-Chrom Q[25]. In extending these observations to methylated alditol acetates we have confirmed that in certain cases resolution is improved by using 3 % ECNSS-M on Chromosorb W although the actual retention time is somewhat longer. Using this system, for example, 2,3,6-tri-O-methyl-D-mannitol acetate (24.8 min) and 2,3,6-tri-O-methyl-D-glucitol acetate (26.4 min) are clearly resolved[21]. There is, however, a much greater separation between these two compounds when they are chromatographed on ECNSS-M as the acetates of the free sugars[26]. This method, the use of sugar acetates, is an excellent one for the resolution of 2,3,4,6-tetra-O-methyl-D-glucose, -D-galactose, and -D-mannose, which are otherwise hard to separate[26].

A second mystique which has become firmly established in the literature is that methylated alditol acetates are only separable on ECNSS-M although BROWN AND LINDBERG[27] have used polyphenyl ether as an alternative. The liquid phase ECNSS-M has a low maximum operating temperature and often columns packed with this material have a short life. Columns packed with BDS are also highly effective in separating methylated alditol acetates and in some cases give better resolution than ECNSS-M. Thus, the chromatogram shown in Fig. 1 represents a separation on a BDS column which gave much better resolution of peaks 5 and 6 than ECNSS-M. Furthermore, 2,4,6- and 3,4,6-tri-O-methyl-D-mannitol acetates are not separated on an ECNSS-M column but are well resolved on 5 % BDS[22]. Other examples are to be found in the procedure for determining uronic acids after reduction as neutral sugars[28,29]. A common uronic acid is 4-O-methyl-D-glucuronic acid but the acetates of galactitol and 4-O-methylglucitol are not resolved on ECNSS-M whereas the separation is readily achieved on 5 % BDS[29]. Columns of BDS have a higher maximum operating temperature than ECNSS-M, which facili-

ates the separations of compounds of low volatility and, more importantly, means that such columns have a longer serviceable life. LÖNNGREN AND PILOTTI[30] have also commented unfavorably on the thermal stability of ECNSS-M as a liquid phase and have proposed OV-225 in its place. ALBERSHEIM and colleagues[31] recommended a three-component liquid phase as superior to ECNSS-M for the separation of alditol acetates. This same packing also gives excellent results with methylated alditol acetates[14]. It is thus clear that several liquid phases are suitable for the chromatography of this class of compound.

CONCLUSIONS

MS of methylated alditol acetates is confirmed as a reliable method of identification. Excellent results may be obtained even when the gas–liquid chromatograph and the mass spectrometer are physically separate.

Separation of the GLC unit permits the collection of the effluent and verification of the homogeneity of each peak by TLC (or other methods). BDS and other liquid phases are shown to be excellent alternatives to ECNSS-M for the separation of methylated alditol acetates.

The identification of the parent alditol may be readily achieved by demethylation and identification of the alditol peracetate.

Many of these manipulations are facilitated by an apparatus designed to handle small amounts of material in melting point capillaries.

ACKNOWLEDGEMENTS

The financial support of the National Research Council of Canada and of the University of British Columbia is gratefully acknowledged. We thank Mr. G. D. GUNN and Mr. J. LOVE for the mass spectra.

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