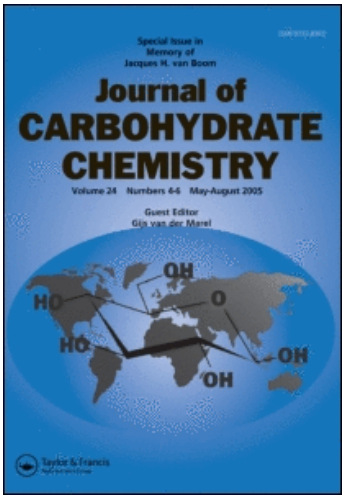

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PREPARATION, SEPARATION AND IDENTIFICATION OF PARTIALLY METHYLATED ALDITOL ACETATES FOR USE AS STANDARDS IN METHYLATION ANALYSIS

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ABSTRACT

A procedure is described for a simple preparation that produces mixtures of a majority of the possible O-methylated alditol acetate derivatives of neutral monosaccharides. The derivatives can be used as standards to determine both the linkage positions and the precise identities of sugar constituents in cell wall polysaccharides.

INTRODUCTION

Methylation analysis is one of the methods used to elucidate the structures of complex carbohydrates isolated from biological materials.[1] The methylated monosaccharides obtained on methylation and hydrolysis of the polysaccharides are usually analyzed by gas chromatography - mass spectrometry of the corresponding partially O-methylated alditol acetates.

The methylated alditol acetates can be identified either by comparison with standards using gas chromatography or directly using gas chromatography - mass spectrometry. However, mass spectrometry alone, without reference to known retention times on

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a gas chromatograph, cannot unequivocally identify the starting monosaccharides. Standard mixtures of methylated alditol acetates have been prepared from oligo- and polysaccharides,[2] methyl glycosides[3] and alditols.[4] This paper describes a relatively simple, rapid procedure for preparing standard mixtures of the methylated alditol acetates from the monosaccharides. Using this procedure, we succeeded in preparing partially methylated derivatives corresponding to every form which has been found in the structural components of plant cell walls.

RESULTS AND DISCUSSION

The method for the permethylation of complex carbohydrates described by Hakomori[5] was modified for this procedure, in which methylated alditol acetates were prepared from monosaccharides. Potassium dimsylate was used as the base to improve ease of reagent preparation and product purity.[6,7] Various volumes of 3.0 N potassium dimsylate were added in different experiments to make the reaction solution 0.02, 0.05, 0.075, 0.1 or 0.4 N in base. A base strength of 0.075 N produced a mixture containing all of the methylated derivatives. Higher base strengths produced mixtures containing predominantly the fully methylated products and little or none of the mono- and dimethylated derivatives. Conversely, lower base strengths produced mixtures devoid of the higher methylated derivatives. Length of sonication time did not appear to affect the methylated product distribution; therefore, a 1-h sonication treatment was used. The reaction mixture was treated with a ten-fold excess of methyl iodide to react with the sugar alkoxide and quench the reaction.

The reaction mixtures were partially purified on a C_{18} reverse-phase column, and the methylated sugars were eluted with a water-acetonitrile step gradient. The C_{18} column is used to remove the DMSO solvent and some of the other impurities produced by the reaction from the sample. The individual fractions were tested with anthrone for the presence of carbohydrates, but it was found that of the seven neutral sugars studied, only D-glucose and D-

mannose gave a test with anthrone sufficiently sensitive at the levels used to be suitable for monitoring eluted fractions. Therefore, the D-glucose sample was put through a C_{18} column first and the anthrone-positive fractions identified for D-glucose were also collected for the other methylated sugars. The more polar, less-methylated species were eluted first with water or 10% acetonitrile, whereas the more-methylated ones required higher concentrations of acetonitrile for elution. While this work was in progress, comparable separations with C_{18} Sep-Pak cartridges were reported by others.[8,9] For L-rhamnose, L-fucose, L-arabinose, D-mannose and D-galactose, the initial effluent and the first water wash were discarded and the subsequent water and acetonitrile fractions which contained the complete range of methylated sugars were combined. The methylated derivatives of D-xylose were contained in the water eluates only including the first 1-mL wash and showed evidence of a number of extraneous peaks probably from reaction products of the methylating reagents. For D-glucose, the complete range of methylated derivatives was found in the acetonitrile eluates.

After the C_{18} column purification, the methylated sugar samples were treated with 2.0 N aqueous trifluoroacetic acid to cleave the methyl glycosides. The methylated aldoses are reduced to the corresponding alditols with sodium borodeuteride. Use of the borodeuteride reagent labels the C-1 carbon and introduces asymmetry into the molecule, thus aiding in mass spectral identification.[10] Excess reductant is decomposed with acetic acid and the resultant borate is converted to its volatile trimethyl ester and removed by evaporation. The partially methylated alditols are converted to the corresponding acetates by reaction with acetic anhydride; and the O-methylated alditol acetates thus formed are analyzed by GC-MS.

Gas Chromatography/Electron Ionization Mass Spectrometry (GC/EIMS). The retention times for the C-1 linked terminal (T-) sugars, which elute first from the GC column, were obtained by

separately injecting each of the fully methylated sugars. Retention times for the unmethylated alditol acetates were obtained by injecting a mixed standard for which the order of elution was known.[11] In this way the earliest (terminal) and latest (unmethylated) eluting peaks were identified. All other partially methylated compounds eluted between these two. Retention times relative to myo-inositol hexaacetate (RRT) were calculated for each peak. The relative retention times were constant to within 3 RRT units when the myo-inositol hexaacetate peak eluted at 22.5 ± 0.5 min. As peaks were identified by RRT and confirmed by mass spectrometry, Table 1 was compiled listing the RRT's of many of the twenty-four possible unique derivatives of the hexoses (pyranose and furanose forms) and twelve unique derivatives of the pentoses and 6-deoxyhexoses (pyranose and furanose forms). The nomenclature used for the derivatives is found in a footnote to the table. It should be noted that there is some overlap of RRT's for different methylated species, so positive identification in an unknown sample will be difficult without mass spectrometry.

It may be seen in Table 1 that all the pyranose forms were found for all seven neutral sugars. In addition, all the furanose forms were found for L-arabinose and D-xylose and all but one of the furanose forms were found for L-fucose. None of the furanose forms were found for L-rhamnose; however, even the pyranose forms for this sugar were present in low concentrations since most of the starting material did not react. The low level of reactivity, as shown by the decreased peak area, probably indicated that methylation was inhibited by the presence of a significant quantity of α-L-rhamnose monohydrate. D-galactose gave rise to a greater weight percent of partially methylated alditol acetates derived from the furanose form than the other hexoses. This fact is consistent with a previous study[12] on equilibrium mixtures of hexoses in an organic solvent. Typical gas chromatograms are shown in Figures 1 through 3.

TABLE 1
Relative Retention Times (Myo-inositol = 1000) of Methylated Alditol Acetates of Neutral Sugar Standards

Glycosidic Linkage[a]	Rha	Fuc	Ara	Xyl	Man	Gal	Glu
terminal-(p)	435	465	431	432	595	618	592
terminal-(f)	ND[b]	446	389	393	598	601	597
2-(p)	540	567	532	532	704	729	730
2-(f)	ND	538	490	489	ND	719	698
3-(p)	552	567	539	530	720	729	704
3-(f)	ND	538	490	496	673	698	682
4-(p) = 5-(f)	540	550	532	540	709	709	718
6-(p)	[c]	-	-	-	748	786	745
6-(f)	-	-	-	-	762	776	774
2,3-(p)	639	659	632	640	802	817	813
2,3-(f)	ND	ND	571	567	ND	ND	774
2,4-(p) = 2,5-(f)	650	656	645	644	822	821	816
2,6-(p)	-	-	-	-	868	901	866
2,6-(f)	-	-	-	-	ND	ND	ND
3,4-(p) = 3,5-(f)	619	622	626	640	790	794	797
3,6-(p)	-	-	-	-	883	901	866
3,6-(f)	-	-	-	-	ND	826	ND
4,6-(p) = 5,6-(f)	-	-	-	-	850	869	857
3,4,6-(p) = 3,5,6-(f)	-	-	-	-	930	947	936
2,4,6-(p) = 2,5,6-(f)	-	-	-	-	970	989	977
2,3,6-(p)	-	-	-	-	970	989	977
2,3,6-(f)	-	-	-	-	ND	ND	ND
2,3,4-(p) = 2,3,5(f)	698	705	707	731	865	878	877
2,3,4,6-(p) = 2,3,5,6-(f)	-	-	-	-	1012	1033	1020

a. The numbers indicate the position of carbons bearing acetoxy groups. In addition, for the pyranose ring form (p) carbon numbers 1 and 5 have acetoxy groups and for the furanose ring form (f) carbon numbers 1 and 4 have acetoxy groups. All carbons which do not bear acetoxy groups are attached to methoxy groups. In all the sugar residues carbon number 1 is spectroscopically labelled by its attachment to a single deuterium atom. b. Derivative which was not found. c. Derivatives which are not applicable to pentoses and 6-deoxyhexoses.

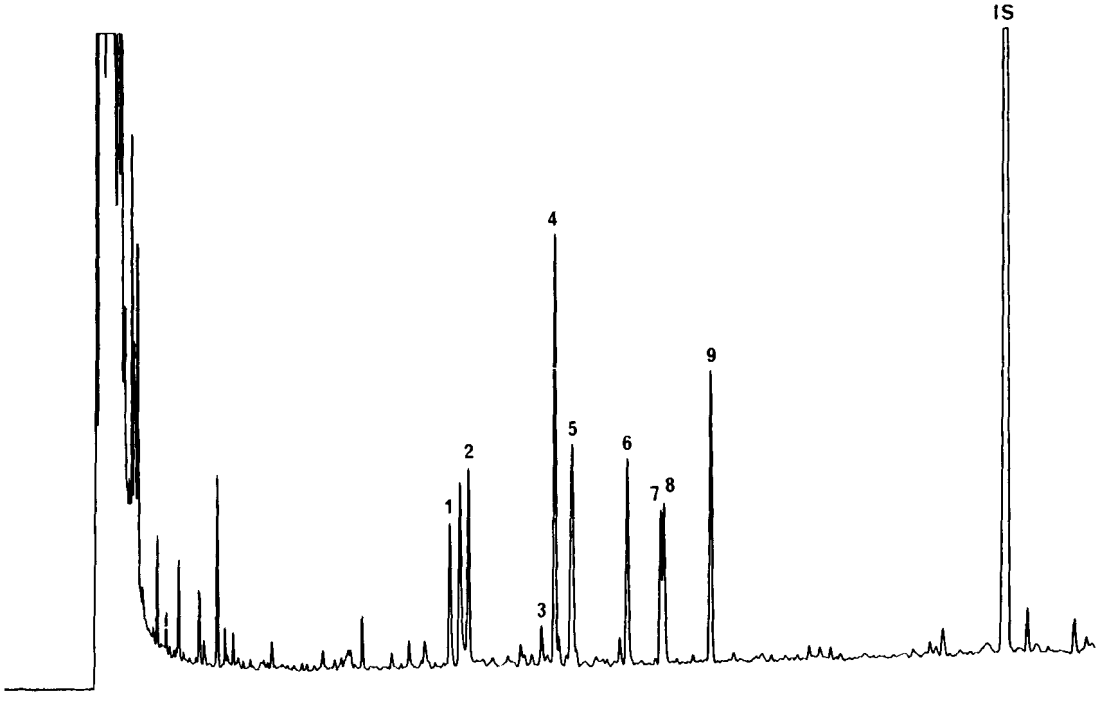

FIG. 1. Capillary gas chromatogram of the partially methylated alditol acetates of L-fucose. Peak: 1 = T-Fuc(f); 2 = T-Fuc(p); 3 = 2-Fuc(f) + 3-Fuc(f); 4 = 4-Fuc(p); 5 = 2-Fuc(p) + 3-Fuc(p); 6 = 3,4-Fuc(p); 7 = 2,4-Fuc(p); 8 = 2,3-Fuc(p); 9 = 2,3,4-Fuc(p); and IS = Myo-inositol hexaacetate.

The relative retention time data are used in conjunction with the mass spectral data to complete the identification of the partially methylated alditol acetates. Mass spectrometry cannot usually distinguish between isomers. Thus, similarly substituted derivatives of D-mannose, D-galactose and D-glucose have identical mass spectra, as do similarly substituted derivatives of L-rhamnose and L-fucose, and similarly substituted derivatives of L-arabinose and D-xylose. By examination of the mass spectra alone it is generally possible to determine only the degree and location of acetylation and whether the starting sugar was a hexose, 6-deoxy-hexose, or pentose, but not the precise starting sugar.

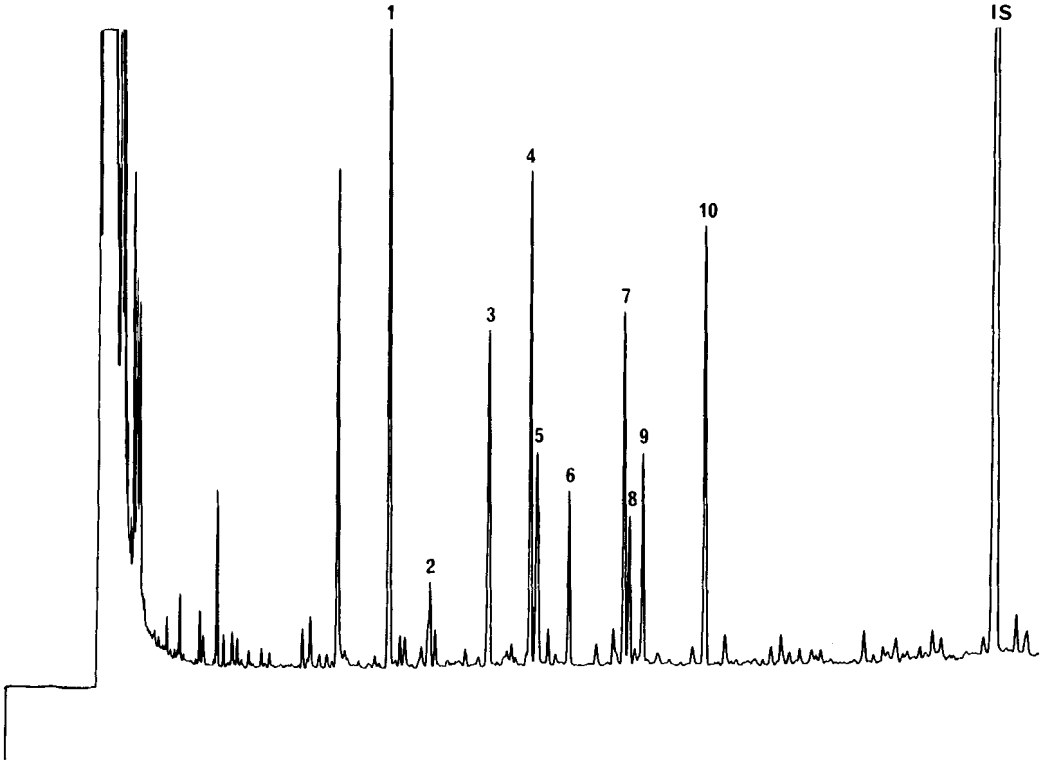

FIG. 2. Capillary gas chromatogram of the partially methylated alditol acetates of L-arabinose. Peak: 1 = T-Ara(f); 2 = T-Ara(p); 3 = 2-Ara(f) + 3-Ara(f); 4 = 5-Ara(p) + 2-Ara(p); 5 = 3-Ara(p); 6 = 2,3-Ara(f); 7 = 3,5-Ara(f); 8 = 2,3-Ara(p); 9 = 2,5-Ara(f); 10 = 2,3,5-Ara(f); and IS = Myo-inositol hexaacetate.

The first step in identifying an unknown alditol acetate is to submit its mass spectrum to a search of a library which had been built previously using the alditol acetate standards prepared earlier. If there is a corresponding entry in the library, a partial identification is made, giving the degree and location of acetylation, and the type of sugar. For example, a peak in the GC separated mixture might be classified as a 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylhexitol. The relative retention time (relative to myo-inositol hexaacetate) is used to determine the probable sugar, for example, that the hexitol is a D-glucose derivative.

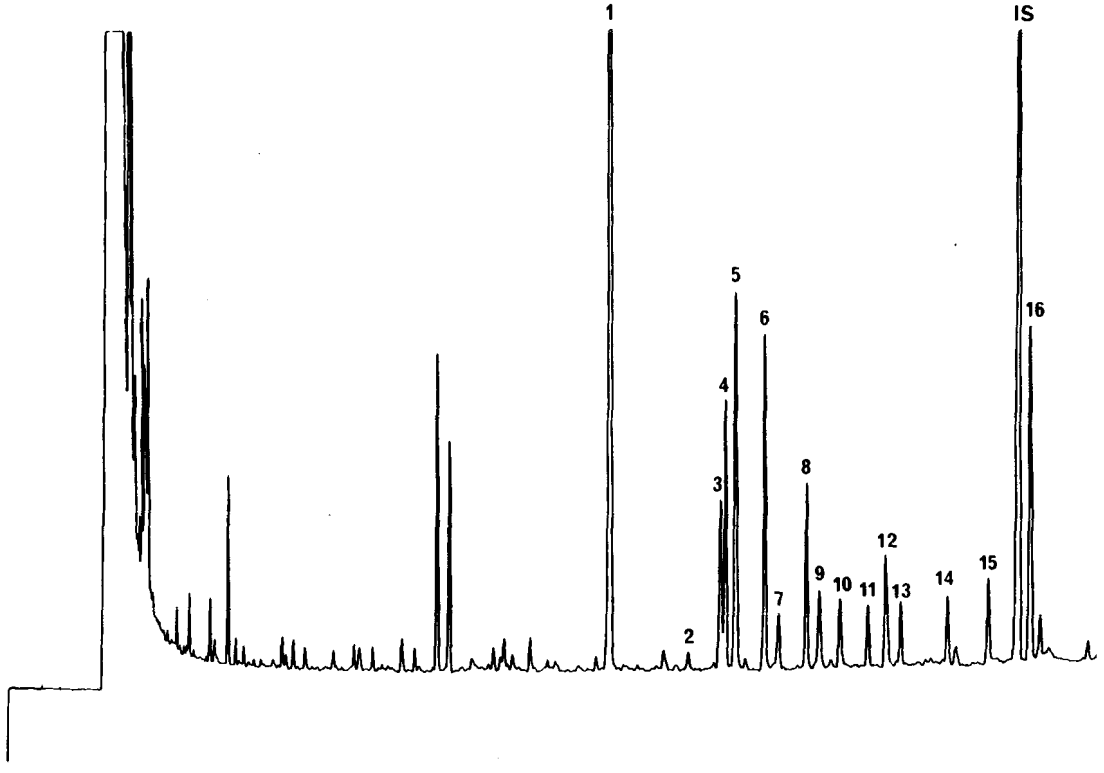

FIG. 3. Capillary gas chromatogram of the partially methylated alditol acetates of D-mannose. Peak: 1 = T-man(p) + T-Man(f); 2 = 3-Man(f); 3 = 2-Man(p); 4 = 4-Man(p); 5 = 3-Man(p); 6 = 6-Man(p); 7 = 6-Man(f); 8 = 3,4-Man(p); 9 = 2,3-Man(p); 10 = 2,4-Man(p); 11 = 4,6-Man(p); 12 = 2,6-Man(p) + 2,3,4-Man(p); 13 = 3,6-Man(p); 14 = 3,4,6-Man(p); 15 = 2,4,6-Man(p); 16 = 2,3,4,6-Man(p); and IS = Myo-inositol hexaacetate

In those cases where no library match is found, an attempt is made to deduce the structure from the mass spectral fragmentation pattern by applying the rules described by Bjorndal et. al.[10] If a structure can be assigned with reasonable certainty, that mass spectrum may be entered into the library, and the compound's relative retention time recorded.

During the course of the work reported herein, an attempt was made to identify as many of all the methylated alditol acetates as possible, originating from all seven sugars, in both the furanose

and pyranose forms. We succeeded in preparing partially methylated derivatives corresponding to every form which has been found in the structural components of plant cell walls. These derivatives were identified by electron ionization mass spectrometry and characterized on the basis of relative retention time. These methylated alditol acetate mixtures can be used as standards to calibrate other GC columns, probes to optimize GC conditions for those regions where peak overlap occurs to improve resolution or to identify unknown compounds by coinjection on a gas chromatograph.

EXPERIMENTAL

A. General. Chemicals were purchased from the following sources and used without further purification: L-rhamnose, L-fucose, L-arabinose, D-xylose, D-glucose, D-mannose, D-galactose and myo-inositol (Sigmal Chemical Company, St. Louis, MO); potassium hydride (35% dispersion in mineral oil) and methyl iodide (Aldrich Chemical Company, Milwaukee, WI); and trifluoroacetic acid (Fisher Scientific Company, Fair Lawn, NJ).

Dimethyl sulfoxide (Aldrich Chemical Co.) was dried by vacuum distillation from calcium hydride and stored over molecular sieves 4A. 4-Heptanone (Aldrich Chemical Co.) was distilled at 144 °C and stored at 5 °C.

It was essential before starting the methylation that the sample, glassware and reagents be thoroughly dry and remain so at all stages of the methylation.

B. Methylation. The monosaccharides were methylated by a modification of the Hakomori[5] procedure as adapted by Phillips and Fraser.[7] A stock solution of 3 N potassium dimsylate was prepared from potassium hydride and dry dimethyl sulfoxide, standardized by titration and stored at 0 °C. The monosaccharide sample (1 mg) was weighed into a glass test tube having a Teflon®-lined screwcap and dried in vacuo at 60 °C over phosphorus pentoxide for 24 hours. For all preparations, dry dimethyl sulfoxide (0.5 mL) and a dry Teflon® stirring-bar were added, and the mixture was stirred until

the sample had completely dissolved. A measured volume of the stock 3 N potassium dimsylate was added to make a solution that was 0.075 N in base. The reaction mixture was sonicated for one h, and then a 10-fold stoichiometric excess of methyl iodide was added. The mixture was stirred magnetically for one hour, and the excess of methyl iodide was removed under a gentle flow of nitrogen on an analytical evaporator using a water-bath maintained at 40°C.

C. C_{18} Column Cleanup. A disposable C_{18} extraction column (1 mL capacity, J. T. Baker Chemical Co.) first was conditioned by passing through it two 1-mL portions of acetonitrile, followed by four 1-mL portions of deionized water using a gentle stream of nitrogen to force the liquid through.

The sample was diluted 1:1 with deionized water (~ 0.6 mL), the solution applied to the head of the column with a Pasteur pipet and the solute adsorbed on the column by using N_2 pressure. A 1-mL wash of deionized water was passed through the column, added to the first effluent, and discarded. The methylated derivatives were eluted from the column with two additional 1-mL portions of deionized water, two 1-mL portions of 10% aqueous CH_3CN, and two 1-mL portions of 50% aqueous CH_3CN, and the eluates collected in clean 13 x 100 mm test tubes. The appropriate eluates were combined and evaporated to dryness under N_2 on an analytical evaporator in a water bath maintained at <40 ^{o}C.

D. Hydrolysis, Reduction and Acetylation. The samples were hydrolyzed in 2.0 N trifluoroacetic acid containing myo-inositol as an internal standard, the sugars reduced to the alditols with sodium borodeuteride and the products acetylated with acetic anhydride as described by Ryan.[11] The methylated alditol acetates were partitioned into chloroform using two successive 1-mL portions of chloroform, and the extracts were carefully transferred to a clean, dry test tube and evaporated to dryness under N_2.

E. Gas Chromatography. A Hewlett-Packard Model 5880 A gas chromatograph equipped with a flame ionization detector was used. The methylated alditol acetates were separated on a fused silica

capillary column (30 m x 0.259 mm ID) coated with a 0.25 μm film of DB-1 phase (J&W Scientific, Inc., Rancho Cordova, CA). The carrier gas was helium with a linear velocity of 20 cm/sec at a column head pressure of 96.5 kPa (14 psig). The injection port and detector temperatures were respectively 240 °C and 300 °C. The oven-temperature program was 2 min at an initial temperature of 150 °C, and then increased to 225 °C at 3 °C/min. Injections of the methylated alditol acetates were made from a 4-heptanone solution using the split mode (split ratio 120:1).

The retention times for each peak of interest were calculated relative to myo-inositol hexaacetate which elutes at 22.5 min.

F. Gas Chromatography/Mass Spectrometry. Electron ionization mass spectra were recorded for the methylated alditol acetates following GC separation, the column used being identical to that just described. The column was installed in a Varian 3700 GC in such a manner that the column itself extended into the source of the mass spectrometer. Details of the heated GC/MS interface have been reported by Magin, et al.[13] Splitless injections of the methylated alditol acetate were made from a 4-heptanone solution directly onto the head of the GC column (at 150 °C) through an unheated on-column injector (J&W Scientific). The oven temperature for the column was programmed at the same rate as for the GC, i.e., held for 2 min at 150 °C, then heated at 3 °C/min to a final temperature of 225 °C and held there until the last peaks had been eluted. The carrier gas was helium at 35 cm/sec (at 150 °C). The GC/MS interface was held at 220 °C.

Electron ionization mass spectra of the methylated alditol acetates were recorded with a Finnegan (Varian) MAT 112-S mass spectrometer. The source temperature was 210-220 °C, the ionizing voltage was 80 eV, and the accelerating voltage was 820 V. Spectra from m/z 35 to 500 were obtained at the rate of 1 sec/decade, with a 0.3 sec interscan time. All data were collected by and examined with an SS-200 MS data system. A library generated from various standards prepared during a period of a year was used to aid in the tentative identification of the methylated alditol acetates.

Identifications were made by comparing mass spectra with fragmentations expected from the structures and by comparison to published mass spectral data.[2]

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REFERENCES

1. H. Rauvala, J. Finne, T. Krusius, J. Karkkanen, and J. Jarnefelt, Adv. Carbohydr. Chem. Biochem., 38, 389-416 (1981).

2. P. E. Jansson, L. Kenne, H. Liedgren, B. Lindberg, and J. Lonngren, Chem. Commun. (Stockholm Univ.), No. 8, (1976).

3. J. A. Lomax and J. Conchie, J. Chromatogr., 236, 385-394 (1982).

4. J. Klok, H. C. Cox, J. W. DeLeeum, and P. A. Schenck, J. Chromatogr., 253, 53-64 (1982).

5. S. I. Hakomori, J. Biochem. (Tokyo), 55, 205-208 (1964).

6. A. G. Darvill, M. McNeil, and P. Albersheim, Plant Physiol., 62, 418-422 (1978).

7. L. R. Phillips and B. A. Fraser, Carbohydr. Res., 90, 149-152 (1981).

8. A. J. Mort, S. Parker and M. S. Kuo, Anal. Biochem. 133, 380-384 (1983).

9. T. J. Waeghe, A. G. Darvill, M. McNeil, and P. Albersheim, Carbohydr. Res., 123, 281-304 (1983).

10. H. Bjorndal, C. G. Hellerquist, B. Lindberg, and S. Svensson, Angew. Chem. Internat. Edit., 9, 610-619 (1970).

11. W. S. Ryan, Jr., Beitr. Tabakforsch, 12, 105-111 (1984).

12. R. J. Ferrier and P. M. Collins, "Monosaccharide Chemistry," William Clowes and Sons Limited, London, 1972, p. 52.

13. D. F. Magin, W. N. Einolf, G. B. Wilkes and S. Marrs, "Modification of Carter Cook Varian MAT GC/MS Interface for Use with Fused Silica Columns," in "The Mass Spec Handbook of Service," (Vol. 1); J. J. Manura, Ed.; Scientific Instrument Services, Inc., Pennington, NJ, 1983, p. 189-196.