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ANALYSIS OF SYNTHETIC MIXTURES OF PARTIALLY METHYLATED ALDITOL ACETATES BY CAPILLARY GAS CHROMATOGRAPHY, GAS CHROMATOGRAPHY–ELECTRON IMPACT MASS SPECTROMETRY AND GAS CHROMATOGRAPHY–CHEMICAL IONIZATION MASS SPECTROMETRY

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SUMMARY

The identification of naturally methylated neutral monosaccharides in acid hydrolysates as their alditol acetates requires appropriate standards. The availability of such standards also facilitates the analysis of complex mixtures of partially methylated alditol acetates (PMAAs) which appear upon methylation analysis of polysaccharides.

For this purpose the alditols of eight common monosaccharides have been partially methylated using the Haworth methylation. The resulting mixtures of partially methylated alditols have been acetylated and analysed by capillary gas chromatography, gas chromatography–electron impact mass spectrometry and gas chromatography–chemical ionization mass spectrometry.

Identification of the obtained PMAAs is further elaborated by reduction of the aldoses with sodium borodeuteride and the use of partially methylated aldoses or disaccharides.

INTRODUCTION

Until recently very few naturally occurring methylated sugars had been reported. Over the past few years they have been more frequently encountered as building blocks of, for example, polysaccharides of bacteria[1,2], cyanobacteria[3] and coccoliths[4] and of lipopolysaccharides of bacteria[5] and cyanobacteria[6,7].

In the geochemical literature partially methylated monosaccharides have only been reported to occur in soils[8] and in peat samples[9]. During the course of our geochemical research on the occurrence and composition of carbohydrates in recent sediments we demonstrated the presence of various methylated monosaccharides in acid hydrolysates[10]. The monosaccharides obtained were analysed as their alditol acetates by capillary gas–liquid chromatography[11].

In order to identify the observed methylated sugars we systematically analysed

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the mono-, di-, tri-, and tetra-O-methyl ethers of the common pentitols, hexitols and 6-deoxyhexitols. For this purpose we synthesized mixtures of partially methylated alditol acetates (PMAAs) starting from each alditol. These standard mixtures were analysed by capillary gas chromatography (GC), capillary gas chromatography–electron-impact mass spectrometry (GC–EI-MS) and capillary gas chromatography–chemical-ionization mass spectrometry (GC–CI-MS). This paper presents the identification of methylated monosaccharides in complex mixtures and gives details of the procedure used.

EXPERIMENTAL

Some of the alditols used in this study were commercially available [ribitol, L-arabitol, xylitol, D-mannitol, D-galactitol and D-glucitol (= sorbitol)]. The others (L-rhamnitol and L-fucitol) were prepared by reduction of the corresponding aldoses.

Reduction of the aldoses

The reduction of the aldoses was carried out by addition of sodium borohydride or sodium borodeuteride to an ammoniacal solution of the mono- or disaccharide. After at least 3 h the excess of borohydride was decomposed by addition of glacial acetic acid. In order to remove the boric acid methanol was added and the mixture was evaporated to dryness. The residue was suspended in dry methanol and again evaporated to dryness. This procedure was repeated twice.

Synthesis of PMAA mixtures (Haworth methylation)

The alditol, reduced monosaccharide or reduced disaccharide (50–100 mg) was dissolved in 0.5 ml 4 *M* sodium hydroxide and freshly distilled dimethyl sulphate (100 µl) was added. After 1 h at 70°C the reaction mixture was cooled, acidified with glacial acetic acid and evaporated to dryness *in vacuo*. The dry residue was dissolved in 1 ml acetic anhydride and 100 mg sodium acetate were added. The mixture was heated in a closed vial for 2 h at 100°C and subsequently evaporated *in vacuo*. The dry residue was suspended in 2 ml dichloromethane washed several times with water and dried over anhydrous sodium sulphate. Aliquots of 1 µl of the resulting dichloromethane solution were injected into the gas chromatograph.

Methylation of the aldoses was carried out by the method of Hirst and Percival[12]. The aldose (100 mg) was dissolved in 300 µl water, then 140 µl dimethyl sulphate and 450 µl 8.8 *M* sodium hydroxide were added in small portions at 0°C. A second portion of 140 µl dimethyl sulphate was added and the temperature was raised to 35°C. Subsequently 450 µl 8.8 *M* sodium hydroxide was added and the mixture was stirred for 3 h. The resulting methyl glycosides were hydrolysed by adding sulphuric acid to an end concentration of 0.5 *M*. The mixture was kept in a closed vial at 100°C for at least 10 h. The pH was raised to 9 by adding concentrated ammonia solution and the resulting mixture was reduced and acetylated as described above.

Gas–liquid chromatography

Gas–liquid chromatography of the alditol acetates (AAs) and PMAAs on a glass capillary coated with OV-275 (25 m × 0.25 mm I.D., Chrompack, Middelburg, The Netherlands) was carried out as described earlier[11]. The temperature was programmed from 165 to 215°C at 2°C/min and finally kept isothermal at 215°C.

As an example of the separation which can be obtained on this stationary phase, Fig. 1 shows the separation of a standard mixture of 24 AA, of which 22 are clearly separated. The relative retention times of the components shown in Fig. 1 are listed in Table I.

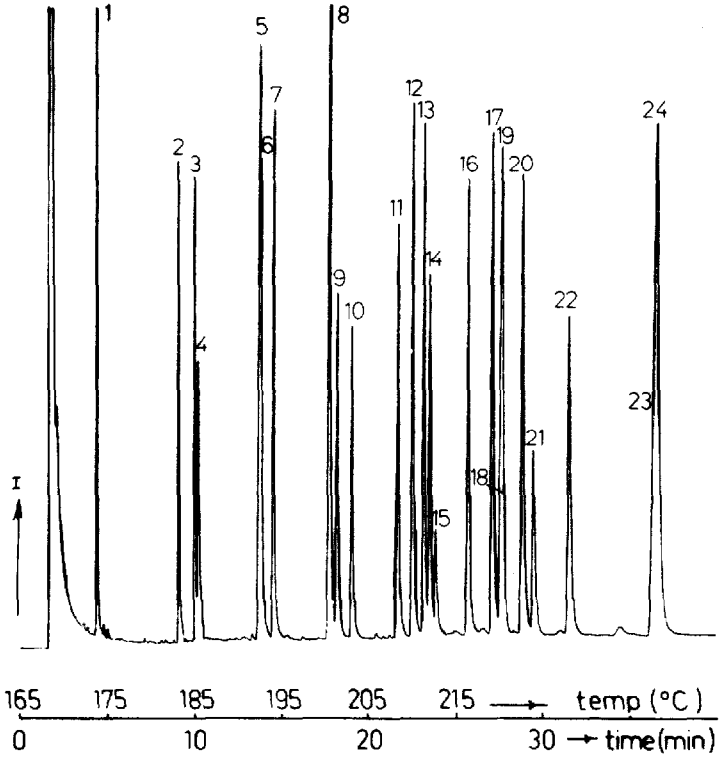

Fig. 1. Gas chromatogram of a standard mixture of 24 alditol acetates. Identifications are given in Table I.

Mass spectrometry

GC–MS was carried out on a Varian 3700 gas chromatograph connected to a Varian/MAT-44 mass spectrometer. In the EI mode the mass spectrometer was operated at 70 eV and a source temperature of 200°C. For CI isobutane was used as reagent gas at 200 eV, keeping the source temperature at 150°C and the pressure of the ionization chamber at 0.5 Torr.

RESULTS AND DISCUSSION

Partial methylation of alditols results in mixtures of methyl ethers. Depending on the symmetry of the starting alditol different numbers of derivatives are expected theoretically. For each alditol the number of positional isomers within each group of O-methyl ethers (mono-, di-, tri-O-methyl ethers, etc.) are given in Table II. Since enantiomers are not separated on the non-chiral OV-275 stationary phase they have not been considered.

From Table II it is clear that glucitol yields the most complex mixture of

TABLE I

RELATIVE RETENTION TIMES OF STANDARD ALDITOL ACETATES SHOWN IN FIG. 1

The retention time of xylitol pentaacetate is taken as standard.

Peak number	*Identity*	*Rel. retention time*
1	Glycerol	184
2	2,3,4,6-Tetra-O-methylglucitol	392
3	Erythritol	431
4	Digitoxitol	440
5	2-Deoxyribitol	589
6	Rhamnitol	596
7	Fucitol	625
8	6-Deoxyglucitol	764
9	Ribitol	784
10	Arabitol	820
11	1,4-Anhydromannitol	936
12	1,5-Anhydromannitol	971
13	Xylitol	1000
14	2-Deoxyglucitol	1016
15	2-Deoxygalactitol	1033
16	Allitol	1110
17	Mannitol	1170
18	3-O-Methylglucitol	1188
19	Altritol	1192
20	Galactitol	1249
21	4-O-Methylglucitol	1277
22	Glucitol	1366
23	Iditol	1584
24	*myo*-Inositol	1589

PMAAs. As an example of the application of this procedure, the identification of the PMAAs in this mixture will be elaborated in more detail. Fig. 2 shows the gas chromatogram of this mixture. Peak numbers correspond with those in Table III.

Identification of the individual components is based on the GC–EI-MS and GC–CI-MS (isobutane) data. The simplicity of the CI-fragmentation patterns, in which the masses $M + 1 - 60$, $M + 1 - 32$ and $M + 1$ dominate[13,14], offers the

TABLE II

NUMBER OF POSITIONAL ISOMERS OF METHYLATED ALDITOLS

Alditol	*Number of methyl substituents*						
	0	*1*	*2*	*3*	*4*	*5*	*6*
Rhamnitol	1	5	10	10	5	1	–
Fucitol	1	5	10	10	5	1	–
Ribitol	1	3	6	6	3	1	–
Arabitol	1	5	10	10	5	1	–
Xylitol	1	3	6	6	3	1	–
Mannitol	1	3	9	10	9	3	1
Galactitol	1	3	9	10	9	3	1
Glucitol	1	6	15	20	15	6	1

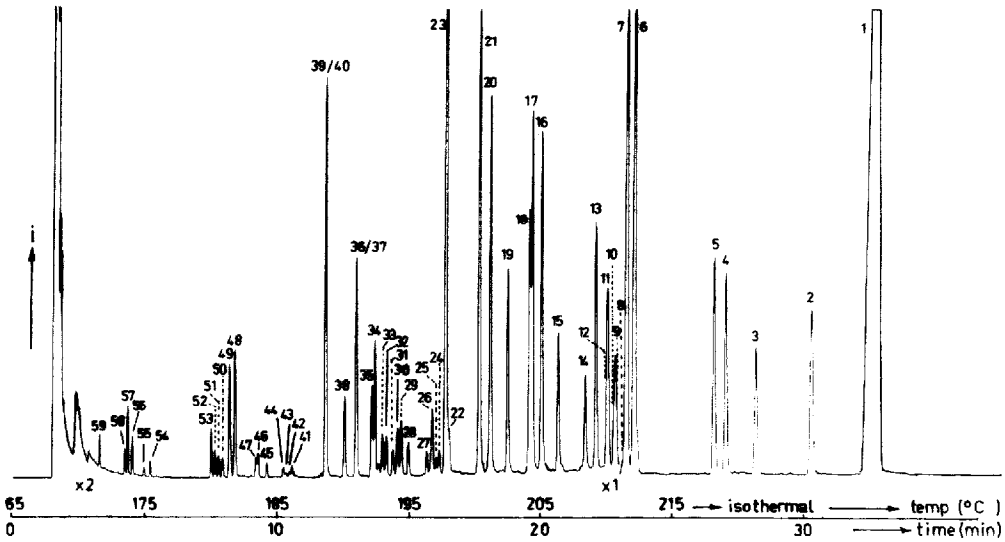

Fig. 2. Gas chromatogram of the glucitol-derived PMAA mixture. Identifications are given in Table III.

TABLE III

IDENTIFICATION OF THE GLUCITOL-DERIVED PMAAs SHOWN IN FIG. 2

Peak number	*Position(s) of the O-methyl groups*	*Peak number*	*Position(s) of the O-methyl groups*
1	None	25	2,3,5
2	4	26	1,4,5
3	3	27	2,3,4
4	5	28	1,3,5
5	2	29	1,2,5
6	6	30	2,5,6
7	1	31	3,5,6
8	3,5	32	3,4,6
9	3,4	33	1,3,4
10	2,3	34	1,4,6
11	2,5	35	2,4,6
12	4,5	36	1,2,3
13	1,4	37	4,5,6
14	2,4	38	1,3,6
15	3,6	39	1,2,6
16	4,6	40	1,5,6
17	2,6	41–45	Tetra
18	1,5	46	2,3,4,6
19	1,3	47	1,3,4,5
20	5,6	48–51	Tetra
21	1,2	52	1,3,4,6
22	2,3,6	53	Tetra
23	1,6	54–58	Penta
24	3,4,5	59	Hexa*

* Tentative identification.

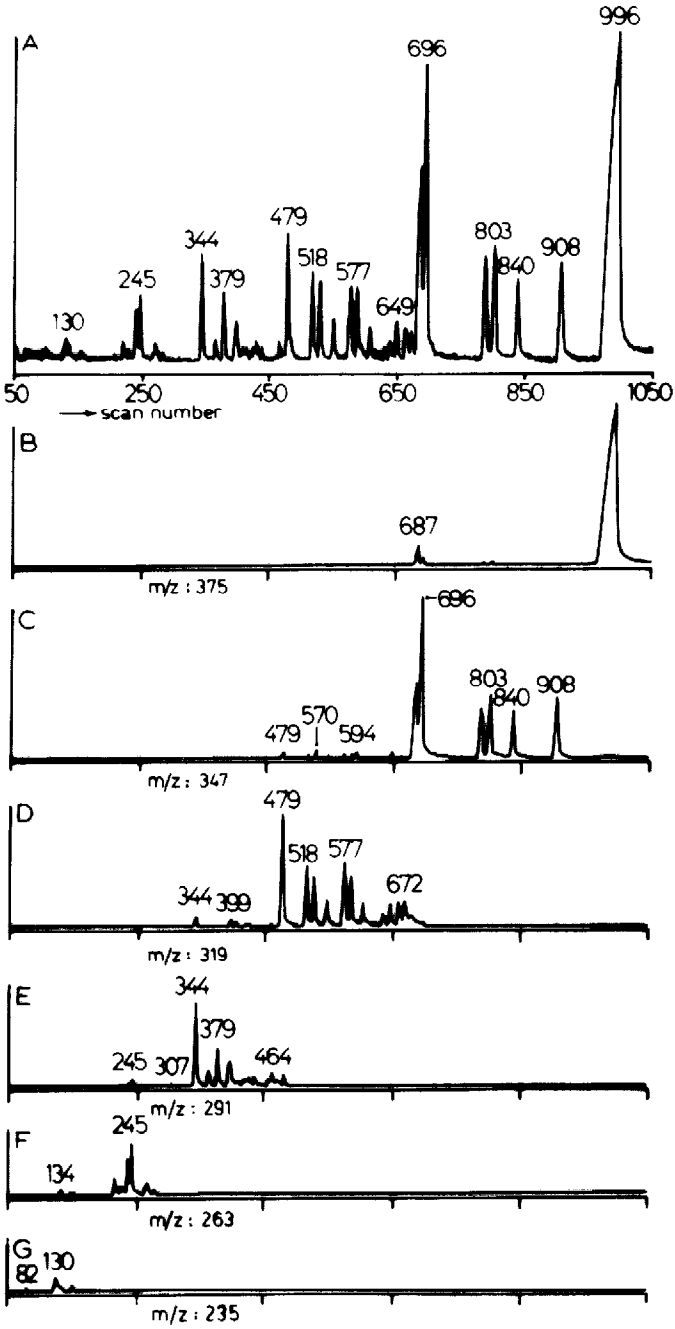

Fig. 3. Total ion current of the gas chromatogram of the glucitol-derived PMAAs (A) and mass chromatograms of $M + 1 - 60$ ion of hexitol hexaacetate and $M + 1 - 32$ ion of mono-O-methylhexitol pentaacetates (B); $M + 1 - 60$ ion of mono-O-methylhexitol pentaacetates and $M + 1 - 32$ ion of di-O-methylhexitol tetraacetates (C); $M + 1 - 60$ ion of di-O-methylhexitol tetraacetates and $M + 1 - 32$ ion of tri-O-methylhexitol triacetates (D); $M + 1 - 60$ ion of tri-O-methylhexitol triacetates and $M + 1 - 32$ ion of tetra-O-methylhexitol diacetates (E); $M + 1 - 60$ ion of tetra-O-methylhexitol diacetates and $M + 1 - 32$ ion of penta-O-methylhexitol acetates (F); and $M + 1 - 60$ ion of penta-O-methylhexitol acetates and $M + 1 - 32$ ion of hexa-O-methylhexitol (G).

possibility of discriminating between the mono-, di-, tri-, tetra- and penta-O-methyl derivatives by mass chromatography. Fig. 3 shows the total ion current and the appropriate mass chromatograms of the D-glucitol PMAA mixture.

In Table IV the *m/z* values of the expected fragments are compiled for the PMAAs derived from pentitols, hexitols and deoxy-hexitols. The $M + 1 - 60$ fragment is the ion with the highest intensity in the CI mass spectra of most PMAAs[13,14]. Discrimination by mass chromatography of, *e.g.*, mono-O-methylhexitol pentaacetates only on account of their $M + 1 - 60$ ion is prevented by the $M + 1 - 32$ ion of the di-O-methyl hexitol tetraacetates interfering (both *m/z* 347, Table III). Nevertheless mass chromatography of the $M + 1 - 60$ and $M + 1 - 32$ ions clearly distinguishes between groups of derivatives with different degrees of methyl substitution (mono-, di-, tri-O-methyl derivatives, etc.), as shown in Fig. 3.

TABLE IV

m/z VALUES OF THE VARIOUS IONS RESULTING FROM CHEMICAL IONIZATION OF PMAAs DERIVED FROM PENTITOLS, HEXITOLS AND DEOXYHEXITOLS

Parent	*Fragment*	*Number of methyl substituents*						
		0	*1*	*2*	*3*	*4*	*5*	*6*
Deoxyhexitol	$M + 1$	377	349	321	293	265	237	
	$M + 1 - 32$	–	317	289	261	233	205	
	$M + 1 - 60$	317	289	261	233	205	–	
Pentitol	$M + 1$	363	335	307	279	251	223	
	$M + 1 - 32$	–	303	275	247	219	191	
	$M + 1 - 60$	303	275	247	219	191	–	
Hexitol	$M + 1$	435	407	379	351	323	295	267
	$M + 1 - 32$	–	375	347	319	291	263	235
	$M + 1 - 60$	375	347	319	291	263	235	–

The identitity of a number of the components in the D-glucitol PMAA mixture can be established directly by comparison of the EI mass spectra with data published by Jansson *et al.*[15]. However in many cases pairs of components are encountered in the chromatogram which give very similar mass spectra. In this way the mono-O-methylglucitol pentaacetates consist of three pairs of derivatives (1- and 6-, 2- and 5-, and 3- and 4-O-methylglucitol pentaacetates). Final identification of these components is achieved by comparison of the gas chromatograms of the D-glucitol- and D-glucose-derived PMAAs and/or by comparison of the EI mass spectra of the D-glucitol- and D-glucitol(1-^{2}H)-derived PMAAs.

D-Glucose, because of its pyranose structure, yields PMAAs in which the 1- and 5-O-methyl derivatives are absent. Thus comparison of the gas chromatograms of D-glucose- and D-glucitol-derived PMAAs allows one to distinguish between the 1- and 6-, and 2- and 5-, but not between the 3- and 4-O-methylglucitol pentaacetates. The latter problem can be solved by interpretation of the EI mass spectra of the D-glucitol(1-^{2}H) PMAAs. On the basis of the known EI fragmentation[16], predictions

TABLE V

RELATIVE RETENTION TIMES OF PARTIALLY METHYLATED ALDITOL ACETATES ON AN OV-275 GLASS CAPILLARY COLUMN

The retention time of xylitol pentaacetate was taken as standard. Temperature programme: 165°C then 2°C/min to 215°C (isothermal). The observed deviation was in the order of 1%. Derivatives marked – were not clearly distinguishable among the PMAAs

Position of O-methyl group	*Rha*	*Fuc*	*Rib*	*Ara*	*Xyl*	*Man*	*Gal*	*Glu*
None	596	625	784	820	1000	1170	1249	1366
1	370	386	518	534	666	865	918	983
2	511	541	640	685	777	1035	1098	1123
3	589	623	623	714	768	1166	1247	1188
4	554	611	640	707	777	1166	1247	1277
5	445	505	518	550	666	1035	1098	1142
6						865	918	995
1,2	274	278	357	379	443	671	715	743
1,3	296	300	335	368	430	746	798	789
1,4	337	347	397	425	492	857	875	931
1,5	276	304	333	339	411	758	799	824
1,6						586	631	689
2,3	398	459	424	493	545	883	989	961
2,4	390	425	408	498	492*	922	994	914
2,5	–	–	397	433	492	893	974	951
2,6						758	799	829
3,4	383	475	424	509	545	951	1049	963
3,5	–	–	335	384	430	922	994	980
3,6						857	875	871
4,5	–	–	357	401	443	883	989	949
4,6						746	798	844
5,6						671	715	761
1,2,3	–	–	195	232	256	498	579	543
1,2,4	–	–	210	252	254	565	602	–
1,2,5	–	–	216	232	266	560	629	616
1,2,6						446	469	494
1,3,4	–	–	218	250	277	590	661	585
1,3,5	–	–	182	194	231	577	612	625
1,3,6						493	501	524
1,4,5	–	–	216	227	266	623	678	667
1,4,6						493	501	573
1,5,6						446	469	494
2,3,4	234	297	216	305	314	657	776	654
2,3,5	–	279	218	255	277	690	756	673
2,3,6						623	678	694
2,4,5	–	–	210	258	254	690	756	–
2,4,6						577	612	568
2,5,6						560	629	610
3,4,5	–	–	195	239	256	657	776	679
3,4,6						590	661	593
3,5,6						565	602	602
4,5,6						498	579	543
1,3,4,5						387	447	388
1,3,4,6						315	361	317
2,3,4,6						387	447	392
2,3,5,6						396	432	–

* The 2,4-di-O-methylxylitol derivative probably co-elutes with the 1,4- and 2,5-di-O-methylxylitol derivatives.

can be made on which fragment will carry the deuterium label. In addition this method confirms the identifications based on the D-glucose-derived PMAAs.

Within the group of di-O-methylglucitol derivatives, all the components can be identified as described above, except for the 1,2-, 5,6- and 1,6-di-O-methylglucitol tetraacetates for which no spectra are available in the literature. The presence in this group of two components having identical EI mass spectra implies that this pair represents the 1,2- and 5,6-di-O-methylglucitol derivatives. Consequently the third component must be 1,6-di-O-methylglucitol tetraacetate. The EI fragmentation patterns of the D-glucitol(1-^{2}H)-derived PMAAs allow distinction between 1,2- and 5,6-di-O-methylglucitol tetraacetate and confirm the identity of 1,6-di-O-methylglucitol tetraacetate.

Components with three or more O-methyl groups per molecule can be identified similarly. In some cases additional information was obtained from a study of the PMAAs derived from sodium-borohydride-reduced maltose.

Although many PMAAs having a high degree of methyl substitution were synthesized from the alditols used in this study, some are not considered, partly because their identification was too tentative and partly because they are less relevant both for our purposes and for methylation studies on polysaccharides.

The PMAAs derived from all the other alditols are characterized as described for the glucitol PMAAs. Table V is a compilation of the relative retention times of all the identified PMAAs.

CONCLUSIONS

The synthesis of PMAAs outlined above affords mixtures of all the theoretically possible derivatives of each alditol which can be well separated using a glass capillary column coated with OV-275.

With the help of these standard mixtures the preliminary identification of the methylated monosaccharides encountered in acid hydrolysates of recent marine sediments has been possible[10].

In addition the data obtained after analysis of these synthesized mixtures will be of importance for structural analyses of polysaccharides, because it is possible to synthesize all the possible PMAAs that might appear upon methylation analysis. Thus it overcomes the problem of the non-availability of individual reference compounds for GC and GC–MS studies.

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Copies of the chromatograms of the various PMAA mixtures and of the EI mass spectra of the components identified are available from the authors on request.

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