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SEPARATION OF METHYLATED ALDITOL ACETATES BY GLASS CAPILLARY GAS CHROMATOGRAPHY AND THEIR IDENTIFICATION BY COMPUTER

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SUMMARY

A gas chromatographic procedure has been developed for the separation and identification of more than 50 partially methylated alditol acetates during a single run. Separation has been achieved using an on-column injection technique with a glass-capillary column wall-coated with SP-1000. Retention coefficients, based on two widely separated compounds as standards, are calculated using a program written for a computing integrator. These coefficients are stable under the chromatographic conditions employed, and enable accurate identification of methylated alditol acetates found during methylation analysis of polysaccharides.

INTRODUCTION

A technique of major importance in structural investigations of polysaccharides and oligosaccharides is that of methylation analysis in which the mixture of methylated sugars obtained after hydrolysis of a methylated polysaccharide is derivatised and separated by gas chromatography (GC). To eliminate complications arising from anomeric forms, the derivatives most commonly employed are the alditol acetates. Such derivatives have the additional advantage that they give readily identifiable fragments when examined by mass spectrometry, enabling their structure to be determined.

GC separation of alditol acetates was first achieved with the phase ECNSS-M[1]. Use of this phase was extended to the separation of partially methylated alditol acetates[2], and for several years it was considered uniquely suitable because of the separations achieved. However, its low thermal stability and the consequent limited column life led to a search for alternatives, especially for GC–mass spectrometry studies[3–6]. The introduction of glass capillary columns resulted in more efficient separations of partially methylated alditol acetates, and various attempts have been made to achieve separation of all the methylated sugars commonly found in natural polysaccharides[7–9]. More recently, the availability of fused-silica, wall-coated glass capillary columns, which offer less active surfaces and significant mechanical advantages, has led to further improvements in chromatographic separations, though published studies to date have dealt mainly with non-methylated sugars[10,11].

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Polysaccharides from plant cell walls contain a considerable number of sugars, the most common being glucose, galactose, mannose, xylose, arabinose, rhamnose and fucose. In the course of studies on changes in plant polysaccharide structures arising from various degradative processes, it became necessary to examine products containing mixtures of polysaccharides and oligosaccharides using methylation analysis. Since each sugar can theoretically produce many different methylated derivatives, it was necessary to develop a chromatographic procedure which would separate, if possible in a single run, the methylated sugars liable to be found in such polysaccharides.

Test mixtures containing a range of methylated derivatives of each sugar have been prepared by partial methylation of the corresponding glycosides. After derivatisation and separation, the identity of each component of the mixture was established by mass spectrometry. Where possible the test mixtures were supplemented by methylated sugars which had been synthesised or which originated from polysaccharides of established structure.

For separation of such complex mixtures in a single chromatographic run, only wall-coated glass capillary columns were capable of giving the necessary resolution. Of the stationary phases tested, SP-1000 was the most effective, enabling more than 50 methylated derivatives to be separated under suitable chromatographic conditions. It would be desirable if identification could be based on GC evidence without routine recourse to mass spectrometry.

A major problem in GC is the reproducibility of relative retentions. Numerous attempts have been made to overcome this problem. Kováts[12] introduced a system, using the homologous series of *n*-alkanes as standards, which has been widely used and works well for non-polar solutes on non-polar phases. Various efforts have been made to extend the usefulness of Kováts indices to more polar solutes and phases[13–15]. All these methods utilise more than one standard, usually members of a homologous series. None is particularly applicable to partially methylated sugars, especially when dealing with highly complex natural mixtures. Lindberg[16], working with methylated sugars, makes reference to the use of two standards to improve reproducibility of retention data though he quotes their results relative to a single standard.

It can be confusing to refer to retention data obtained by interpolation using two standards as relative retentions. We have therefore used the term *retention coefficient* for data which we obtained using two widely separated internal standards. These values for a particular component are much more stable than relative retention values based on a single standard. They are less dependent on temperature and other chromatographic conditions. We have obtained retention coefficients for more than 50 commonly encountered partially methylated sugars on SP-1000 and, using a programmable computing integrator, have been able to identify automatically large numbers of unknown peaks in methylated biological samples. The method described here is applicable to data obtained from any capillary column which gives adequate resolution and sufficiently symmetrical peaks for accurate computer identification.

EXPERIMENTAL

Gas chromatography

Separations were carried out with a Carlo-Erba Fractovap 4160 gas chromato-

graph fitted with a flame ionization detector and a non-vaporizing septumless on-column injector of the Grob type[17] with adjustable temperature profile. The column oven was cooled to 60°C during injection and then raised rapidly to 206°C and maintained at this temperature. The detector temperature was 250°C and the carrier gas (helium) pressure 60 Pa. Glass capillary columns (20 m × 0.3 mm) wall-coated with SP-1000 were obtained from Erba Science (UK), Swindon, Great Britain.

Recording and calculation of chromatographic data was carried out with a Spectra-Physics SP-4100 computing integrator. BASIC programmes were written enabling retention coefficients to be calculated based on two standards, and peaks to be identified and named.

Mass spectrometry

Mass spectra were obtained from a VG Micromass 16 mass spectrometer connected to a VG 2025 data collection and processing system. The ionization potential was 70 eV and the source temperature 200°C.

Standards and test mixtures

Mixtures of partially methylated derivatives of each sugar were obtained by partial Purdie methylation[18,19] of the corresponding methyl glycosides prepared as previously described[20]. Periods of methylation were 120 min for rhamnose, 90 min for the hexoses and 35 min for xylose and arabinose. After derivatisation and separation by GC, the compounds were identified by mass spectrometry. Other mixtures were obtained by methylation analysis of commercial samples of xylan, arabinan and galactan.

Authentic samples of 2, 3, and 6-O-methylgalactose were kindly donated by Dr. R. Begbie. Samples of 2- and 3-O-methylglucose, 3,4-di-O-methylglucose, 2,4- and 2,6-di-O-methylgalactose and 2,3,6-tri -O-methylglucose were from the collection of the late Dr. D. J. Bell. A sample of 2,3-di-O-methylglucose was prepared from glycogen, and 2- and 3-O-methyl-, 2,4- and 3,6-di-O-methyl-, and 2,4,6- and 3,4,6-tri-O-methylmannose were obtained from original ovomucoid glycopeptides and glycopeptides derived from them. Quebrachitol (monomethyl-L-inositol) was obtained from Calbiochem, Bishops Stortford, Great Britain.

Methylation analysis of polysaccharides

Samples of soluble carbohydrate polymers from the rumen fluid of sheep, and of milled barley straw were methylated by the method of Stellner *et al.*[21], hydrolysed by a formolysis/hydrolysis procedure[16], and the liberated methylated sugars converted to the corresponding alditol acetates. The mixtures were subjected to GC using the procedure described above.

RESULTS AND DISCUSSION

As the first step in the development of a standard separation procedure we examined the efficiency of glass capillary columns coated with a number of stationary phases most of which had been used previously, mainly in packed columns, for separating methylated alditol acetates. These included OV-330, OV-225[3], OV-17[22], OV-1[23] and SP-1000[7]. Of these phases, SP-1000 gave the most satisfactory separa-

tions of our mixtures, with no tailing or asymmetry of the peaks. Better separation of the 2- and 3-O-methylxylitol derivatives was obtained with OV-1 (resolution ≈ 0.6), but this phase was not quite as efficient for the separation of some of the other compounds, and gave less symmetrical peaks with non-linear isotherms, making peak identification less accurate. It has, however, proved to be the most satisfactory phase for the separation and determination of methylated aminosugar derivatives[24].

The first step towards the identification of peaks obtained using packed columns is the relation of the peak retention time to that of a standard. Such relative retention values are, however, affected by column temperature and by the composition and condition of the column packings, especially for packings containing mixed stationary phases. A comparison of relative retentions obtained by different laboratories will often show considerable variation. When capillary columns are used, the problem of obtaining reproducible retention times and relative retentions is greatly increased because of difficulties in coating and because many different methods of preparing and deactivating the columns are employed.

The method of transferring samples which has been shown to give the most reproducible quantitative results is that of on-column injection[24,25]. In this procedure, the chromatographic run is started with the column oven cool or cold, and after injection the temperature is rapidly raised to the operating value[17]. Under such conditions, however, there is no accurate zero time which is implicity required for the ratio method of identification described above.

This problem can be overcome by using as standards two compounds whose peaks are widely separated in the chromatogram. The two standards are assigned retention coefficients (ρ) usually based on relative retention values either previously determined or taken from the literature. Assuming a linear relationship between retention times and retention coefficients, it is then possible to derive retention coefficients for any compound by interpolation or extrapolation from the standards. The equation used is

$$\rho_x = \frac{t_{Rx}(\rho_2 - \rho_1) + \rho_1 t_{R2} - \rho_2 t_{R1}}{t_{R2} - t_{R1}}$$

where ρ_x = retention coefficient of compound x
t_{Rx} = retention time of compound x
ρ_1 = retention coefficient of first standard
ρ_2 = retention coefficient of second standard
t_{R1} = retention time of first standard
t_{R2} = retention time of second standard

The equation is derived from that for a straight line ($y = mx + c$). The retention times can be measured from any convenient starting-point, as it is only the difference between the retention times of the two standards which is important in the calculation. The retention coefficients so calculated need no correction for the gas hold-up volume of the column. In practice the retention coefficients do vary slightly with temperature, though this is only noticeable with a large temperature change. They are also affected to a slight extent by the column being used, according to its source and method of manufacture. For greatest accuracy it is therefore preferable

that new values for retention coefficients should be determined for new columns before general use.

With our equipment and procedures, retention times varied between runs within ± 0.3% and tended to decrease in the course of a day, probably due to small changes in carrier gas pressure. This did not affect retention coefficients, but because of this variability it is necessary to use internal standards in every sample. The standard peaks are located by a computer program which compares the retention time of each peak with the retention time of the standard found in the previous run, and selects the largest peak within a set "window" around this retention time. The size of window used —a percentage of the retention time— is double that used for the identification of peaks and is usually 2%. In difficult situations this procedure can be bypassed and appropriate values for the standard peaks inserted manually. The program uses the found internal standards to calculate the retention coefficients of each peak. Over a period of months the calculated values were stable within about 0.15% relative standard deviation.

Controlled, incomplete methylation of sugars can be used to produce a range of partially methylated compounds, though usually not all those theoretically possible. After separation by GC and identification of the individual sugars by mass

TABLE I

RETENTION COEFFICIENTS (ρ)* OF PARTIALLY METHYLATED ALDITOL ACETATES

Location of methyl group	*Rha*	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*
Unmethylated	1.892	2.081	2.623	3.023	6.630	7.419	6.430
2	1.403	1.462	1.815	1.988	4.793	5.176	5.023
3	1.547		1.936	1.988	5.488	6.185	5.399
4	1.470			**	***	***	
6	—		—	—	3.529	4.096	
2,3	0.945	1.027	1.125	1.208	3.218	3.620	3.417
2,4	1.000	1.076	1.205	1.154	3.691		3.417
2,5			0.977				
2,6	—	—	—	—	2.603	2.736	2.707
3,4	0.888		1.171	‡	3.276		
3,5			0.935		‡‡		
3,6	—	—	—	—	2.907	3.037	2.893
4,6	—	—	—	—	2.603		2.893
2,3,4	0.540	0.649	0.671	0.686	1.807	2.379	1.931
2,3,5			0.564				
2,3,6	—	—	—	—	1.765	1.842	1.889
2,4,6	—	—	—	—	1.803	1.912	1.680
3,4,6	—	—	—	—	1.621		
2,3,4,6	—	—	—	—	1.000	1.143	1.000

* Standard 1 was the 2,3,4,6-tetra-O-methyl-D-glucitol derivative (ρ = 1.000) and Standard 2 was the monomethyl-L-inositol (quebrachitol) derivative (ρ = 4.300).

** This compound yields an alditol acetate identical to that obtained from 2-O-methylxylose. By using sodium borodeuteride for reduction the two compounds can be distinguished by mass spectrometry.

*** Yields identical product to corresponding 3-O-methylhexose (see footnote **).

‡ Yields identical product to corresponding 2,3-di-O-methylxylose (see footnote **).

‡‡ Yields identical product to corresponding 2,4-di-O-methylmannose (see footnote **).

spectrometry, the mixtures can be used as a source of reference compounds for the assembly of retention coefficients for a particular column. We have also assembled additional data from authentic derivatives obtained by synthesis or from methylation analysis of polysaccharides of known structure. Using these sources, it has been possible to produce a table containing retention coefficients for more than 60 partially methylated sugars, including most of those commonly encountered during methylation analysis of polysaccharides from plant and other sources (Table I). As with almost all other types of column, it has not proved possible to separate the 2,3,4,6 tetra-O-methyl-glucitol and mannitol derivatives. These cannot be distinguished by

TABLE II

METHYLATION ANALYSIS REPORT FOR COMPUTER PROGRAM APPLIED TO DATA FROM FIG. 1

Peak number	*Area (%)*	*Retention coefficient (ρ)**	*Identification***		
			1st	*2nd*	*3rd*
1	4.3	0.540	*2,3,4-Rha****		
2	1.0	0.649	*2,3,4-Fuc*		
3	0.4	0.673	2,3,4-Ara		
4	0.6	0.681	2,3,4-Xyl		
5	3.0	0.885	*3,4-Rha*		
6	2.1	0.940	3,5-Ara	*2,3-Rha*	
7	16.5	1.000	*2,3,4,6-Glc* ‡	*2,3,4,6-Man* ‡	*2,4-Rha*
8	3.3	1.028	*2,3-Fuc*		
9	4.3	1.075	*2,4-Fuc*		
10	0.5	1.125	2,3-Ara		
11	5.0	1.139	*2,3,4,6-Gal*		
12	2.2	1.200	*2,4-Ara*	*2,3-Xyl*	
13	0.8	1.397	*2-Rha*		
14	3.4	1.460	2-Fuc	*4-Rha*	
15	0.5	1.542	*3-Rha*		
16	2.4	1.625	*3,4,6-Man*		
17	4.8	1.677	*2,4,6-Glc*		
18	1.7	1.750	*2,3,6-Man*		
19	1.9	1.804	*2,4,6-Man*	2-Ara	
20	3.1	1.829	*2,3,6-Gal*	2-Ara	
21	14.2	1.891	Rha	*2,3,6-Glc*	
22	5.2	1.931	*2,3,4-Glc*	3-Ara	
23	1.0	2.322			
24	2.0	2.359	*2,3,4-Gal*		
25	0.6	2.586	*4,6-Man*	2,6-Man	
26	3.4	2.709	*2,6-Glc* ‡	*2,6-Gal* ‡	
27	0.5	2.830			
28	1.3	3.393	*2,3-Glc*	*2,4-Glc*	
29	0.8	4.074	6-Gal		
30		4.300	Quebrachitol (standard)		

* Based on the standards 2,3,4,6-Glc (ρ = 1.000) and quebrachitol (ρ = 4.300).
** Identifications confirmed by mass spectrometry are shown in italics.
*** 2,3,4-Rha = 1,5-di-O-acetyl-2,3,4-tri-O-methylrhamnitol etc.
‡ These pairs of compounds cannot be distinguished by mass spectrometry.

TABLE III

METHYLATION ANALYSIS REPORT FOR COMPUTER PROGRAM APPLIED TO DATA FROM FIG. 2

Peak number	*Area (%)*	*Retention coefficient (ρ)**	*Identification***		
			1st	*2nd*	*3rd*
1	2.6	0.569	*2,3,5-Ara****		
2	0.1	0.597			
3	0.1	0.667	2,3,4-Ara		
4	0.2	0.688	*2,3,4-Xyl*		
5	0.5	0.754			
6	1.1	0.851			
7	0.1	0.887	3,4-Rha		
8	0.3	0.937	3,5-Rha	2,3-Rha	
9	0.5	0.976	*2,5-Ara*		
10	1.4	1.000	*2,3,4,6-Glc*	*2,3,4,6-Man*	2,4-Rha
11	0.3	1.030	2,3-Fuc		
12	0.1	1.075	2,4-Fuc		
13	0.5	1.125	2,3-Ara		
14	1.1	1.143	*2,3,4,6-Gal*	2,4-Xyl	
15	23.8	1.218	*2,3-Xyl*		
16	0.2	1.536	3-Rha		
17	1.0	1.677	2,4,6-Glc		
18	0.3	1.756	2,3,6-Man		
19[§]	50.4	1.929	3-Ara	2,4,6-Gal	
20	6.8	1.984	*2-Xyl*	*3-Xyl*	
21	1.3	2.687	*2,6-Glc*		
22[§§]	0.9	2.863			
23	0.4	3.000	Xyl		
24	2.3	3.397	*2,3-Glc*	2,4-Glc	
25	0.9	3.844			
26		4.300	Quebrachitol (standard)		

* Based on the standards 2,3,4,6-Glc (ρ = 1.000) and quebrachitol (ρ = 4.300).

** Identifications confirmed by mass spectrometry are shown in italics.

*** 2,3,5-Ara = 1,4-di-O-acetyl-2,3,5-tri-O-methylarabinitol etc.

[§] Peak 19 has been identified as 2,3,6-Glc, but, due to deliberate overloading, was not identified correctly in this run.

[§§] This peak was identified as a 3,6-hexose by mass spectrometry.

electron impact mass spectrometry, but the few other compounds which co-chromatograph can readily be distinguished by such means. A recent paper describes the separation of these components using a Silar-10C column, though the separation of some other derivatives was inferior to that on our SP-1000 column[9]. Our program uses the table of retention coefficients established for the column to identify each peak in a chromatogram. The identification window is again set as a percentage of the retention coefficient (usually 1%) and possible identifications are given in the printed output ranked in order of closeness of fit (see Tables II and III)*.

* Copies of the BASIC computer program for use with the Spectra-Physics SP-4100 computing integrator may be obtained from the authors.

Application of the procedure is illustrated by two examples showing separations obtained during methylation analysis and the identifications obtained using the computer program. The first example (Fig. 1) shows the methylated sugars obtained from the soluble oligosaccharide complexes found in the rumen liquor of sheep fed on dried grass. Table II shows the report for this run, together with the results of GC–mass spectrometric analysis which confirms almost all the identifications made by GC alone, and enables positive assignment of most of the peaks with multiple identification. Fig. 2 and Table III show the chromatogram and report for a methylated sample of milled barley straw. In this case the column has been heavily loaded to

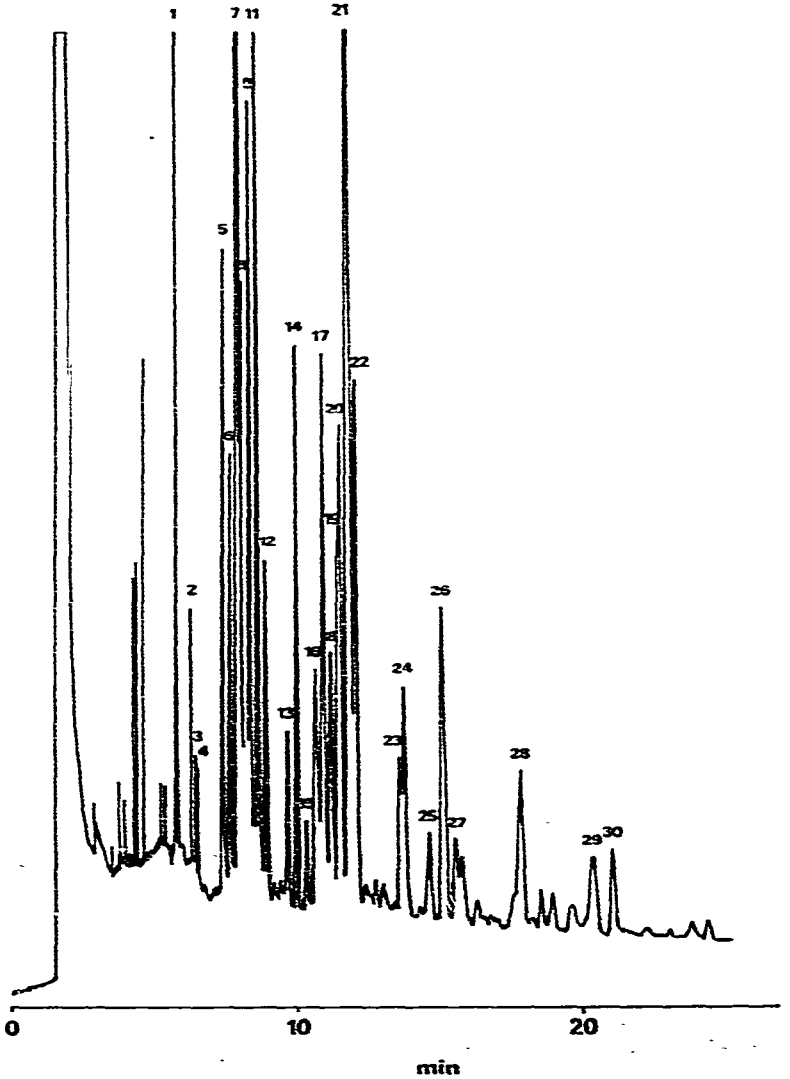

Fig. 1. Gas chromatogram showing separation of the partially methylated alditol acetates from the soluble oligosaccharide fraction from sheep rumen liquor. For details of conditions see text. Peak numbers are given in Table II.

allow identification of those components present in small amounts. This has resulted in an overload of the flame ionization detector amplifier and an incorrect retention time for the largest peak which from other runs is known to be 2,3,6-tri-O-methylglucitol. In both examples the standards used for calculation of retention coefficients were the 2,3,4,6-tetra-O-methylglucitol and mono-methyl-L-inositol (quebrachitol) derivatives. The identification window was 1%, though in fact most of the peaks were identified even with a window of 0.5%.

While simpler mixtures of methylated sugars may be resolved satisfactorily using other columns and less complex identification techniques, we consider that the chromatographic procedure described, allied to a programed computing integrator,

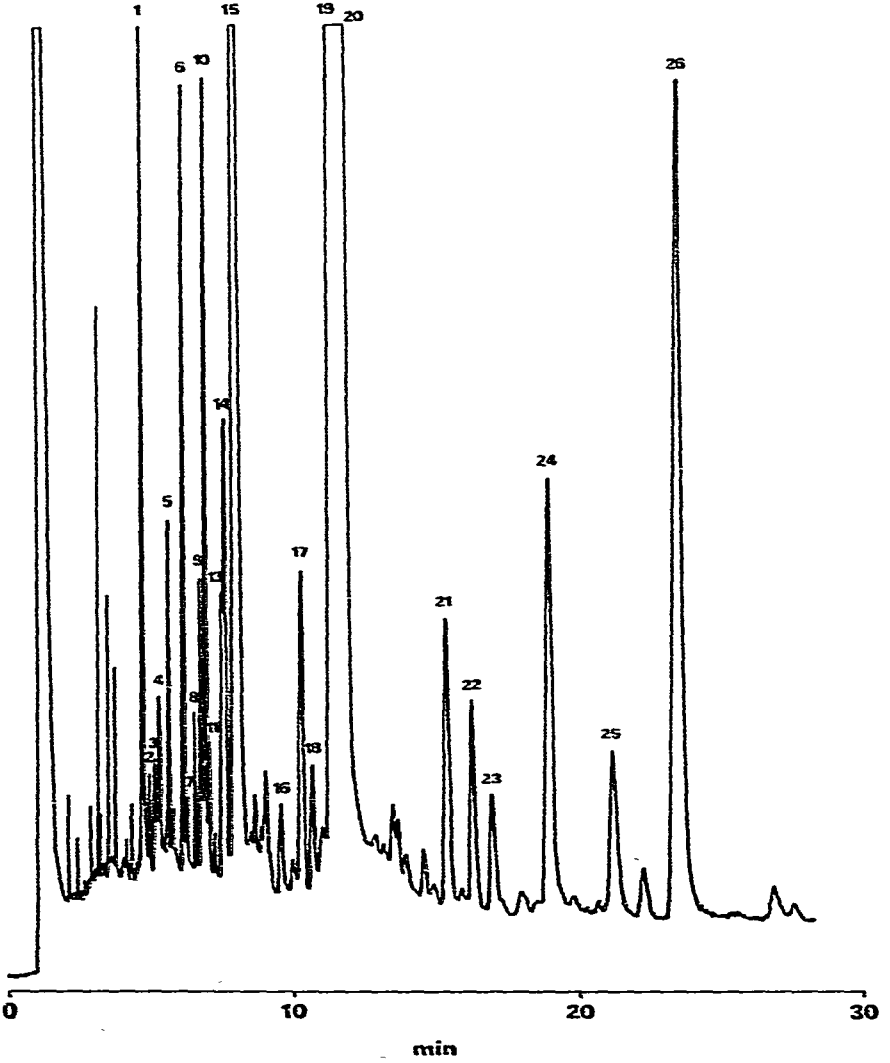

Fig. 2. Gas chromatogram showing separation of the partially methylated alditol acetates from barley straw. For details of conditions see text. Peak numbers are given in Table III.

provides a satisfactory means of obtaining with a single column the identification and approximate quantitation of most of the methylated sugars liable to be encountered in the course of applying methylation analysis to carbohydrate polymers from plant and other sources.

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