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Note

Capillary gas chromatography of partially methylated alditol acetates on a high-polarity bonded-phase vitreous-silica column

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Methylation analysis is commonly used to establish the glycosyl linkage positions between monosaccharides in polysaccharides and glycoproteins[1–6]. The resulting partially methylated alditol acetates are separated by gas chromatography (GC) and can be identified by mass spectrometry (MS) and by their retention times relative to an internal standard. Capillary columns coated with phases ranging in polarity from the low polarity SE-30[5] to the high polarity OV-275[7] have been used for the separation of partially methylated alditol acetates. Geyer *et al.*[8] chromatographed thirty partially methylated alditol acetates on glass capillary columns coated with four different phases (OV-101, SE-30, Dexsil 410, Silar 9CP) and found the best resolution was obtained using the most polar of these phases, Silar 9CP.

Vitreous-silica capillary columns have distinct advantages over glass capillary columns due to their greater flexibility and mechanical strength. This is particularly important for GC–MS where the flexibility of the vitreous-silica column enables direct interfacing of the column with the ion source of the mass spectrometer. However, high-polarity phases are unstable when coated onto the non-polar surface of vitreous-silica columns. This can be overcome by cross-linking within the phase to give a bonded or immobilized phase[9,10]. In addition, bonded phases have lower bleed characteristics and contaminants can be removed by washing the column with solvents without affecting the resolution of the column[9,10]. In this paper, we report the retention times of a range of partially methylated alditol acetates, on a high-polarity wall-coated open-tubular (WCOT) column, BP-75, produced by bonding the highly polar phase, OV-275, on vitreous-silica. The partially methylated alditol acetates include those that commonly result from the methylation analysis of plant polysaccharides[11]. Columns with these specifications have been found to give good separation of alditol acetates and some partially methylated alditol acetates[12].

EXPERIMENTAL

Materials

Sugars were obtained from the following sources: rhamnose, methyl-α-D-mannopyranoside, galactose and *myo*-inositol from Sigma (St. Louis, MO, U.S.A.); methyl-β-D-glucopyranoside, methyl-β-D-galactopyranoside and methyl-α-D-xylopyrano-

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side from Pfanstiehl Labs. (Waukegan, IL, U.S.A.); and arabinose from BDH (Poole, U.K.). Methyl-α-L-fucopyranoside was synthesized from α-L-fucose (Sigma) by the method of Springer and Williamson[13]. Potassium hydride (approx. 20% in oil), methyl iodide (Puriss, inhibited with silver) and 1-methylimidazole (stored over molecular sieve type 4A) were obtained from Fluka (Buchs, Switzerland), 2,2-dimethoxypropane from Aldrich (Milwaukee, WI, U.S.A.) and sodium borodeuteride (98 atom% ^2H) from Sigma. *Myo*-inositol hexaacetate was prepared as described by Henry *et al.*[14]. All other reagents were of the highest purity commercially available.

Preparation of partially methylated alditol acetate standards

Sugars were undermethylated by a modification of the procedure of Harris *et al.*[15] by using a limiting amount of potassium methylsulphinyl carbanion for alkoxide formation. Samples (approx. 5 mg) were undermethylated in a single step methylation using 15 μl of potassium methylsulphinyl carbanion and 150 μl of methyl iodide. Where partially methylated alditol acetates derived from both the pyranose and furanose forms of a monosaccharide were required the monosaccharide was used as the starting compound.

Partially methylated sugars were reduced with sodium borodeuteride (rather than sodium borohydride) to introduce asymmetry where derivatives would otherwise have a symmetrical substitution pattern (*e.g.* 2,3- and 3,4-di-O-methyl pentitols). This allows unequivocal identification of the derivatives from their fragmentation patterns by electron impact ionisation MS[1]. *Myo*-inositol hexaacetate was used as the internal standard.

Capillary gas chromatography–mass spectrometry

Partially methylated alditol acetates were separated and identified by capillary GC–MS using a fully automated Finnigan MAT 1020B GC–MS instrument (Sunnyvale, CA, U.S.A.).

For GC, a 25 m × 0.22 mm I.D., BP-75, vitreous-silica wall-coated open tubular (WCOT) column (SGE, Melbourne, Australia) was used. The column was interfaced with the ion source via a separator oven which was maintained at 250°C. The injection port was held at 240°C and the oven was programmed from 150°C to 250°C at 4°C/min and held at the final temperature for 10 min. Samples, in dichloromethane, were introduced via a Finnigan MAT split/splitless injector using the split mode. Helium (ultra-high purity, C.I.G., Melbourne, Australia) at a flow-rate of 0.78 ml/min was used as the carrier gas.

For MS, electron impact ionisation, at an ionisation potential of 70 eV, was used throughout. Compounds eluting from the gas chromatograph were detected in the mass spectrometer using the total ion current by scanning from *m/e* 100 to *m/e* 350 in 0.3 sec.

Peaks were identified by comparison of their mass spectra with standard spectra (kindly made available by Drs. P. Albersheim and A. Darvill, University of Colorado, CO, U.S.A.). Where no reference spectra were available the unknown spectra were interpreted from established fragmentation rules for partially methylated alditol acetates[1,16].

RESULTS AND DISCUSSION

The retention times, relative to *myo*-inositol hexaacetate, of a range of partially methylated alditol acetates separated on a BP-75, vitreous-silica WCOT column are shown in Table I. Reproducibility between different injections was within ±4%. As would be expected from a bonded-phase column, the column bleed during chromatography was minimal[9,10], thus providing excellent conditions for detection and identification of low abundance derivatives as well as efficient data acquisition by MS. Partially methylated alditol acetates with retention times differing by more than 2% were clearly resolved. Where one of two closely eluting (less than 2% retention time difference) derivatives was dominant, the minor component was often masked, and only careful examination of the mass spectrum revealed that the apparently single peak consisted of two derivatives.

TABLE I

RETENTION TIMES OF PARTIALLY METHYLATED ALDITOL ACETATES ON A BP-75 CAPILLARY COLUMN

*Position of O-methyl group**	*Retention time***						
	Parent monosaccharide						
	Ara	*Xyl*	*Fuc*	*Rha*	*Gal*	*Glc*	*Man*
2-	0.647	0.701***,§§§	0.569	0.554	0.868	0.869	0.843
3-	0.665	0.697§§§	0.614	0.595	0.924§	0.892	0.894***
4-	0.660	0.701***,§§§	0.610	0.577	0.924§	0.925	0.894***
5-	0.569	–	–	–	–	–	–
6-	–	–	–	–	0.787	0.810	0.758§§§
2,3-	0.531	0.576§	0.506	0.470***	0.809	0.797***	0.761§§§
2,4-	0.538	0.547	0.491	0.470***	0.820	0.781	0.788§
2,5-	0.489	–	–	–	–	–	–
2,6-	–	–	–	–	0.720§§§	0.735	0.699
3,4-	0.541	0.576§	0.518	0.457	0.837	0.797***	0.788§
3,5-	0.459	–	–	–	–	–	–
3,6-	–	–	–	–	0.756	0.753	0.741
4,6-	–	–	–	–	0.724§§§	0.743	0.694
2,3,4-	0.393	0.417	0.379	0.326	0.703	0.654	0.639
2,3,5-	0.341	–	–	0.338	–	–	–
2,3,6-	–	–	–	–	0.647§§	0.664	0.617
2,4,6-	–	–	–	–	0.624	0.607	0.601
2,5,6-	–	–	–	–	–	–	–
3,4,6-	–	–	–	–	0.643§§	0.614	0.595
2,3,4,6-	–	–	–	–	0.514	0.487	0.465
2,3,5,6-	–	–	–	–	0.495	–	–
none	0.727	0.804	0.631	0.611	0.939	0.961	0.912

* 2-0-Methyl arabinitol = 1,3,4,5-tetra-O-acetyl-2-O-methyl arabinitol; etc.

** Retention times relative to *myo*-inositol hexaacetate = 1.000.

*** and § Co-eluting derivatives from the same parent monosaccharide.

§§ and §§§ Incompletely resolved derivatives from the same parent monosaccharide.

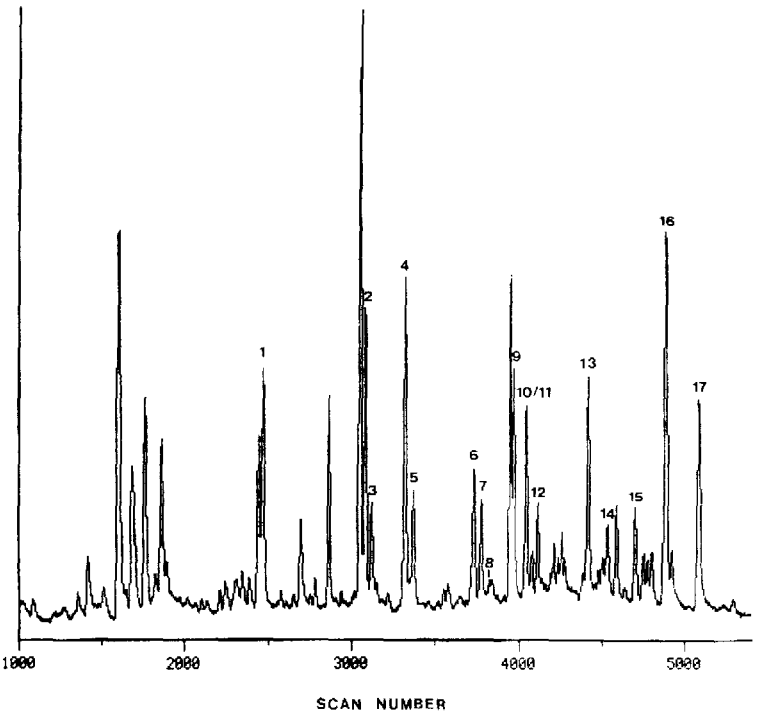

Fig. 1. Reconstructed ion chromatogram (RIC-total ion trace) of partially methylated glucitol acetates, obtained from partial methylation of methyl-β-D-glucopyranoside, separated by capillary GC on a BP-75 column. 1 = 2,3,4,6-OCH_3; 2 = 2,4,6-OCH_3; 3 = 3,4,6-OCH_3; 4 = 2,3,4-OCH_3; 5 = 2,3,6-OCH_3; 6 = 2,6-OCH_3; 7 = 4,6-OCH_3; 8 = 3,6-OCH_3; 9 = 2,4-OCH_3; 10 = 2,3-OCH_3; 11 = 3,4-OCH_3; 12 = 6-OCH_3; 13 = 2-OCH_3; 14 = 3-OCH_3; 15 = 4-OCH_3; 16 = glucitol hexaacetate; 17 = *myo*-inositol hexaacetate (internal standard).

As an example, Fig. 1 shows the separation of the partially methylated glucitol acetates obtained by the partial methylation of methyl-β-D-glucopyranoside. In addition to demonstrating the excellent resolving power of capillary chromatography, it suggests caution is necessary in the assignment of peaks as partially methylated alditol acetates based solely on their retention times. The unmarked peaks in this reconstructed ion chromatogram (RIC) are contaminants, for example, plasticisers, introduced during the derivatization procedure[17]. Thus, both mass spectra and retention times relative to an internal standard are required for unequivocal identification of partially methylated alditol acetates[3].

With one exception, the order of elution of the partially methylated alditol acetates from each parent monosaccharide is the same on BP-75 as that reported by Klok *et al.*[7], who used a glass capillary column coated with OV-275. The exception is the reversed order of elution of 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl mannitol and 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl mannitol. This result is surprising as BP-75 is cross-linked OV-275. We have no explanation for this observation.

The high-polarity bonded phase, BP-75, is an excellent phase for the separation of partially methylated alditol acetates. However, it is advisable to chromatograph partially methylated alditol acetates on two separate columns coated with stationary

phases of different polarities, *e.g.* BP-75 and SE-30, since the order of elution can be different[8]. This will provide those without access to MS facilities with a greater degree of certainty in the assignment of peak identity based solely on retention times. Such an approach has been used for the study of partially methylated alditol acetates derived from plant cell wall polysaccharides[18,19].

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