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# Note

## The detection and quantification of apiose by capillary gas chromatography of its alditol acetates*

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The branched-chain sugar, apiose [3-*C*-(hydroxymethyl)-D-*glycero*-aldotetrose] occurs widely in plants, in low molecular weight glycosides[1], and as a component of cell-wall polysaccharides[1–5].

Problems have been encountered in the analysis of apiose as its alditol acetate derivatives. For example, when pyridine was used as the acetylation catalyst[6], the alditol gave two peaks by g.l.c.[7]. Similar results were obtained when sodium acetate was used as the catalyst[2], and underacetylation was suggested[8]. We have reported[9] the application to apiose of the method of Blakeney *et al.*[10], which uses reduction with sodium borohydride in methyl sulphoxide and acetylation catalysed by *N*-methylimidazole. The two peaks detected were assigned to apiitol tetra- and penta-acetate and reflected the slow acetylation[11,12] of the tertiary hydroxyl group at C-3. Complete acetylation requires elevated temperatures and extended times (90 min)[9]. Kindel and Cheng[13] obtained similar results, but reported that substantial proportions of 3-*C*-(acetoxymethyl)-1,2,4-tri-*O*-acetyl-3-*O*-(methylthiomethyl)-D-*glycero*-tetritol were formed as a side product.

We now present additional evidence that application of the method of Blakeney *et al.*[10] to apiose gives apiitol 1,2,3,4,4′-penta-acetate and 1,2,4,4′-tetra-acetate, and propose a method for quantification based on the latter derivative.

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*Gas chromatography.* — G.l.c. of the products obtained by application of the method of Blakeney *et al.*[10] to apiose gave two peaks of unequal size. With columns coated with cyanoalkyl silicone phases, namely, BP 225, BPX70, and SP 2340 (used for g.l.c.–e.i.-m.s.), the smaller peak (apiitol penta-acetate) eluted before the larger one (apiitol tetra-acetate), but, on a methyl silicone phase (CP Sil 5) (used for g.l.c.–c.i.-m.s.), the order was reversed. Only partial separation of apiitol penta-acetate from xylitol penta-acetate was achieved with the cyanoalkyl silicone phases (Fig. 1). However, apiitol tetra-acetate was well separated from the alditol acetates derived from the monosaccharides found in acid hydrolysates of plant cell-wall polysaccharides, and from allitol hexa-acetate used as an internal standard[14]. With BP 225, BPX70, and SP 2340, the order of elution of the acetates of rhamnitol, fucitol, arabinitol, xylitol, mannitol, galactitol, and glucitol was the same as that reported for Silar 10C[10] and BP75[15]. Apiitol tetra-acetate eluted before allitol hexa-acetate on BP225, before mannitol hexa-acetate on BPX70 (Fig. 1), and before galactitol hexa-acetate on SP2340. We now use BPX70 routinely for the analysis of mixtures of alditol acetates. No peak was observed that could be attributed to 3-*C*-(acetoxymethyl)-1,2,4-tri-*O*-acetyl-3-*O*-(methylthiomethyl)-D-*glycero*-tetritol when methyl sulfoxide was used as the solvent for sodium borohydride, and acetylation was carried out for 90 min at 40° as described by Blakeney *et al.*[10] or at 80°.

The c.i.- and e.i.-mass spectra of apiitol penta- and tetra-acetates were similar to those reported[2,5,13].

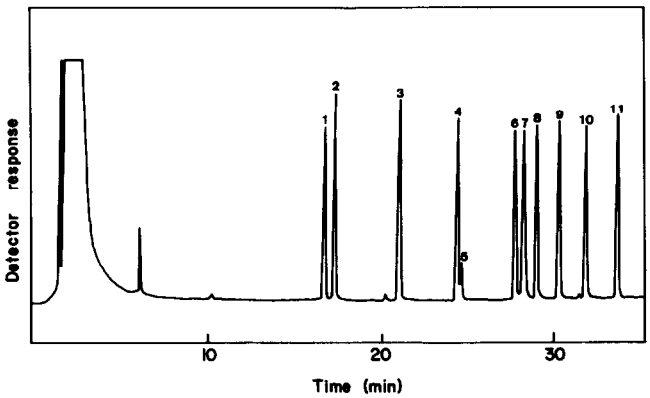


Fig. 1. Gas chromatogram of alditol acetates separated on a 25-m BPX70 column (reduction and acetylation were carried out by the procedure of Blakeney *et al.*[10]): 1, rhamnitol; 2, fucitol; 3, arabinitol; 4, xylitol; 5, apiitol penta-acetate; 6, allitol; 7, apiitol tetra-acetate; 8, mannitol; 9, galactitol; 10, glucitol; 11, *myo*-inositol.

*$^{13}C$-N.m.r. spectroscopy.* — The $^{13}C$ chemical shift data for apiitol tetra- and penta-acetate are summarised in Table I. The assignments were made with the help of INEPT[16] and data for apiose[17]. The differences confirm that HO-3 in the tetra-acetate is not acetylated. Thus, in the tetra-acetate, the signal for C-3 was shifted upfield (8.5

TABLE I

$^{13}C$ Chemical shift data[a] for solutions of apiitol penta-acetate and tetra-acetate in $Me_2SO$-$d_6$

| | *Penta-acetate* | *Tetra-acetate* |
|---|---|---|
| Carbonyls | 170.01, 169.64, 169.57, 169.39, 169.35 | 170.66, 170.47, 170.11 |
| Quaternary C-3 | 80.68 | 72.31 |
| Methine C-2[b] | 69.71 | 71.52 |
| Methylenes[c] C-1, C-4, C-4′ | 61.85, 61.25, 61.16 | 64.5, 63.88, 62.29 |
| Methyls[d] | 21.26, 20.38, 20.33 | 20.83 |

[a] Referenced to the central peak of the multiplet for the solvent, which was set at 39.5 p.p.m. relative to the signal for $Me_4Si$. [b] $^1J_{C,H}$ 152 and 147 Hz. [c] $^1J_{C,H}$ 150, 152, 152, 112, 116, and 150 Hz. [d] $^1J_{C,H}$ 130 Hz.

p.p.m.) and that of C-2 was shifted downfield (1.8 p.p.m.) compared to the corresponding signals for the penta-acetate.

*Proposed method for the quantification of apiose.* — The procedure of Blakeney *et al.*[10] (acetylation at 40° for 90 min) gave 84% of apiitol tetra-acetate and 16% of the penta-acetate (based on peak areas). Acetylation at 0° for 1 min yielded 98% of the tetra-acetate, and at 80° for 90 min yielded 95% of the penta-acetate. When reduction was conducted in a freshly prepared solution of sodium borohydride in diglyme followed by acetylation at 80° for 90 min, >99% conversion into the penta-acetate was achieved.

In order to avoid the formation of 3-*C*-(acetoxymethyl)-1,2,4-tri-*O*-acetyl-3-*O*-(methylthiomethyl)-D-*glycero*-tetritol when the method of Blakeney *et al.*[10] was used under conditions that gave complete conversion of apiitol, Kindel and Cheng[13] replaced the methyl sulfoxide with *N*,*N*-dimethylformamide as the solvent in the reduction step and acetylated for 4 h at 85°, thereby obtaining >99% of the penta-acetate. However, acetylation of apiitol in methyl sulfoxide in the presence of 1-methylimidazole under the conditions described by Blakeney *et al.*[10] did not give the reported[13] derivative. The acetylation of sulphoxides in the Pummerer reaction is well documented[18], but side products are not formed when the reaction is conducted below 80°.

Therefore, we have used the milder, rapid acetylation conditions and media in the method of Blakeney *et al.*[10] and calculated the yield of apiose on the basis of the area of the peak of apiitol tetra-acetate. For this approach to be valid, the ratio of the penta- and tetra-acetates must be constant under given conditions of acetylation. When nine separate samples of 1,2:3,5-di-*O*-isopropylidene-α-D-apiose were hydrolysed with 0.25M sulphuric acid and the apiose released was reduced and acetylated by the method of Blakeney *et al.*[10], the proportions of the penta- and tetra-acetates based on peak areas were 16.4 ±0.94% and 83.6 ±2.0%, respectively. When the penta- and tetra-acetates were extracted into dichloromethane containing *myo*-inositol hexa-acetate (150 μg/

mL), four consecutive extracts (1 mL each) contained 83.8, 16.3, 0, and 0%, respectively, of the penta-acetate recovered, and 44.4, 34.3,14.8 and 6.6%, respectively, of the tetra-acetate recovered. The recovery of the penta-acetate was similar to that found by Kindel and Cheng[13], and for 12 other alditol acetates by Blakeney *et al.*[10].

The recovery of alditol acetates after reduction and acetylation of the products in the hydrolysates of cell-wall preparations can be made difficult due to the formation of emulsions. The separation of the phases can be improved by increasing the volume of the dichloromethane phase, drying with sodium sulphate, back-extraction of the dichloromethane phase with water, or by centrifugation. Cell-wall preparations contaminated with lipids are a major contributor to the phase-separation problems, which can be minimised by extraction with a hydrophobic solvent prior to hydrolysis.

The method proposed for the quantification of apiose avoids the long acetylation step (4 h at 85°) suggested by Kindel and Cheng[13], and possible confusion between the penta-acetates of apiitol and xylitol which may be resolved only partially on cyanoalkyl silicone phases. The resolution of these polar columns deteriorates in routine use, probably due to air oxidation. Methods based on the complete acetylation of apiitol may not detect apiose in mixtures of monosaccharides from hydrolysates of low molecular weight glycosides or cell-wall polysaccharides.

Apiose is a relatively rare sugar and, in the analysis of cell-wall monosaccharides as their alditol acetates, its presence will be indicated by a peak for apiitol tetra-acetate. It is recommended that standards containing 1,2:3,5-di-*O*-isopropylidene-α-D-apiose and *myo*-inositol be processed by hydrolysis, reduction, and acetylation, then chromatographed in conjunction with the samples containing apiose derived from cell walls.

## EXPERIMENTAL

1,2:3,5-Di-*O*-isopropylidene-α-D-apiose was obtained from Sigma. Diglyme (bis-2-methoxyethyl ether) (Sigma) was distilled under reduced pressure[19] immediately prior to use. Methyl sulphoxide was stored over molecular sieve 4A. *myo*-Inositol hexa-acetate was prepared as described[20]. All other reagents were of analytical grade.

*Preparation of apiitol acetates.* — Apiose was obtained by hydrolysing 1,2:3,5-di-*O*-isopropylidene-α-D-apiose (0.5–2 mg) by (*a*) 2M trifluoroacetic acid (0.5 mL) under argon at 121° for 1 h[21] (the acid was removed in a stream of $N_2$ at room temperature) and (*b*) 0.25M $H_2SO_4$ at 95° for 1.5 h (the $H_2SO_4$ was neutralised with $BaCO_3$ to pH 7, and the hydrolysate was concentrated under reduced pressure at 40°). The residue was dissolved in M ammonium hydroxide (0.1 mL) and the apiose was reduced by the method of Blakeney *et al.*[10] or by modifications of this method in which (*a*) the temperature and time of the acetylation were altered and (*b*) reduction was achieved using a freshly prepared M solution of sodium borohydride in freshly redistilled diglyme[19] (0.25 mL) at various temperatures and for various times, and the products were analysed by g.l.c. and g.l.c.–m.s.

Larger amounts of apiitol acetates were prepared as follows. A solution of 1,2:3,5-di-*O*-isopropylidene-α-D-apiose (400 mg) in 2M trifluoroacetic acid (400 mL)

under argon was kept at 121° for 1 h and then the acid was evaporated. To a solution of the residue in 2.38M ammonium hydroxide (96 mL) was added aq. 2% sodium borohydride (200 mL), and the mixture was kept at 90° for 90 min. 17.4M Acetic acid (80 mL) was added, followed by AG 501-X8 ($HCO_3^-$) resin (20–50 mesh) (1 kg, Bio-Rad Laboratories). When the evolution of carbon dioxide ceased, the resin was collected and washed with water, and the combined filtrate and washings were concentrated. A solution of the white crystalline residue in methanol (300 mL) and 17.4M acetic acid (0.5 mL) was concentrated and the procedure was repeated until a clear syrup of apiitol remained, which was then washed through a bed of activated charcoal. Colorimetric assay of this product for reducing groups[22] showed that the reduction of apiose was complete, and p.c. using 6:4:3 1-butanol–pyridine–water on pre-washed Whatman 3MM paper gave a single white spot with anisidine–metaperiodate reagent[23]. Apiitol penta-acetate was prepared by adding a solution of apiitol (50 mg) in water (100 mL) to a mixture of 13M ammonium hydroxide (80 mL), 1-methylimidazole (160 mL), and acetic anhydride (1.6 L) at 80°. The mixture was kept at 80° for 90 min, then diluted with water (8 L), cooled to room temperature, and extracted with dichloromethane (1 L). The extract was washed with water (500 mL) and dried ($Na_2SO_4$), and the solvent evaporated in a stream of $N_2$ at 40° to leave a slightly yellow syrup. Apiitol tetra-acetate was prepared as described above, except that the acetylation was performed at room temperature for 10 min.

*G.l.c.* — Alditol acetates were separated and quantified using a Hewlett–Packard 5890A gas chromatograph fitted with a flame-ionisation detector. High-purity hydrogen or helium (CIG Australia) was used as the carrier gas. The columns used, obtained from SGE Pty (Melbourne), were (*a*) a 25 m × 0.22 mm i.d. (film thickness, 0.1 μm) BP-225, wall-coated, open tubular (WCOT) vitreous silica column and (*b*) a 25 m × 0.33 mm i.d. (film thickness, 0.25 μm) BPX70 WCOT fused-silica column. BPX70 is a thermally stable, fully cross-linked, polar stationary phase equivalent to 70% of cyanopropyl siloxane. Alditol acetates were applied to column (*a*) with a split injector (1:40), and to column (*b*) using a dedicated, cool, on-column capillary inlet. Column (*a*) was used at 210°; column (*b*) was used at 38° for 30 s following injection, then heated to 170° at 50°/min and to 230° at 2°/min, and kept at 230° for 5 min. With each column, the detector was maintained at 250°.

*G.l.c.–c.i.-m.s.* — Mass spectra were recorded on a Kratos MS25RF mass spectrometer fitted with a 25 m × 0.32 mm i.d. Chrompak CP Sil 5 WCOT fused-silica capillary column. Chromatography was carried out at 200° using helium as the carrier gas at 0.55 mL/min. Compounds eluted from the column were detected using the total ion current by scanning in the range *m/z* 40–400 at 0.6 s/decade. The ionisation gas was ammonia.

*G.l.c.–e.i.-m.s.* — Mass spectra were recorded with a Finnigan MAT 1020B spectrometer at 70 eV. A 30 m × 0.25 mm i.d. SP 2340 WCOT vitreous silica column (Supelco Inc.) was used. The temperature programme was 170° to 240° at 5°/min and then 240° for 10 min. Helium at 0.78 mL/min was used as the carrier gas. Compounds were detected using the total ion current by scanning in the range *m/z* 100–350 in 0.5 s.

*$^{13}$C-N.m.r. spectroscopy.* — Spectra were recorded on solutions of apiitol tetra- and penta-acetate in $Me_2SO$-$d_6$ in 5-mm tubes at room temperature with a Bruker AM400 F.t. spectrometer operating at 100 MHz. The solvent was used for the internal lock and as the reference (39.5 p.p.m.).

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