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IMPROVED GAS–LIQUID CHROMATOGRAPHIC SEPARATION OF METHYLATED ACETYLATED ALDITOLS

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SUMMARY

A 0.3% OV-275–0.4% XF-1150 gas chromatography column has been developed which, together with gas chromatography–mass spectrometry facilities, replaces the previous necessity of using at least two different columns to separate methylated alditol acetates.

INTRODUCTION

Analysis of the structure of primary cell walls relies largely at present on the process of methylation to identify and quantitate the glycosidic linkages. Determinations of fully acetylated sugars give the total quantity of sugars in the cell wall, whereas methylation analysis allows the determination of the glycosidic linkages. In recent years, advances in gas chromatography–mass spectrometry (GC–MS) of partially methylated sugars have helped in such structural analyses[1–4]. It has become apparent that the polysaccharide structure within the primary cell wall is very complex[5–8], thus placing great demands on any method of determination of this structure.

Previously, at least two of four GC columns, *i.e.*, surface-coated open-tubular (SCOT) OV-225, OV-225, ECNSS-M and polyethylene glycol adipate–polyethylene glycol succinate (PEGA–PEGS), have been used to give a complete analysis of partially methylated alditol acetates obtained from cell wall samples[9]. It is also difficult to separate completely the fully acetylated deoxysugars rhamnose and fucose. The aim of this work has been to develop a GC column that will replace the need to use several columns to characterize a single sample. This paper gives an account of the success obtained with a 0.3% OV-275–0.4% XF-1150 stationary phase coated on to Gas-Chrom Q (100–120 mesh) and used in conjunction with a GC–MS system. The time saved using such a column is considerable compared with a multiple-column system.

EXPERIMENTAL

Gas–liquid chromatography (GLC)

All GLC separations were made on a Pye 104 Series gas chromatograph fitted with dual hydrogen-flame ionization detectors.

Materials

Gas-Chrom Q (100–120 mesh) was obtained from Jones Chromatography, (Machen, Great Britain), silicone XF-1150 from Field (Richmond, Great Britain) and OV-275 from Pierce (Rockford, Ill., U.S.A.).

Standard references consisting of partially methylated alditol acetates of sugars likely to be found in plant cell-wall hydrolysates were obtained by methylation and acetylation of 5-mg amounts of xylan (CHR grade), araban (pure arabinan), mannan (from yeast) and galactan (from larch), all obtained from Koch-Light (Colnbrook, Great Britain) and starch (from potato), from BDH (Poole, Great Britain). All other chemicals used in acetylation and methylation were obtained from BDH. Alditol acetate standards were prepared from the appropriate sugars.

Preparation of alditol acetates

Alditol acetate standards for GLC analysis of aldoses[10] and for aldoses and uronic acids[11] were prepared from 5-mg samples of the respective sugars. *myo*-Inositol was used as an internal standard (IS) in order to obtain relative retention times.

Preparation of partially methylated alditol acetates (methylation analysis)

Carbohydrate samples were methylated according to a modification[12] of a standard method[13], as employed in recent cell-wall investigations[1]. The process involved permethylation of the polysaccharides followed by hydrolysis, reduction and acetylation.

Preparation and use of the OV-275–XF-1150 GLC column

The stationary phases OV-275 (30 mg) and XF-1150 (40 mg) were suspended in dry glass-distilled acetone (50 ml). Dry Gas-Chrom Q (10 g, 100–120 mesh) was then added and gently stirred. The acetone was removed by rotary evaporation using a vacuum-assisted evaporator. This material was then packed into a GC column (1.5 m $\times$ 4 mm I.D.) using vibration and a nitrogen pressure of 5 p.s.i. The column was baked at 180° for 18 h with a nitrogen flow-rate of 50 ml/min.

Several conditions were tested in order to obtain the most efficient separation. The optimum conditions were: column temperature 120°, held for 5 min and then raised at 1°/min to 180°; carrier gas flow-rate, 50 ml/min (nitrogen). The normal amount injected was 1.0–10.0 μl of 100-μl samples of the standards or cell-wall materials.

Identification of alditol acetates and partially methylated alditol acetates by GC–MS

Standard samples of fully acetylated alditol acetates gave mass spectra which were in agreement with those already presented[14]. The partially methylated alditol acetates gave mass spectra which were analysed as previously described[15]. All stan-

TABLE I

RELATIVE RETENTION TIMES OF ALDITOL ACETATE DERIVATIVES ON THE OV-275–XF-1150 COLUMN

5-mg amounts of the standard sugars were acetylated, and the amount injected was 1 μl from 2.0-ml samples. GC conditions: temperature, 120° held for 5 min, then raised at 1°/min to 180°; carrier gas (nitrogen) flow-rate, 50 ml/min; recorder range, 10 mV; chart speed, 120 mm/h. Internal standard, *myo*-inositol.

Residue	*RRT*
Rhamnose	0.447
Fucose	0.482
Arabinose	0.587
Xylose	0.698
Mannose	0.781
Galactose	0.809
Glucose	0.877
myo-Inositol	1.000

dards were given a relative retention time (RRT) by reference to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylmannitol (terminal mannose).

GC–MS analyses were made on a Pye 104 Series gas chromatograph interfaced with an Associated Electrical Industries MS 30 double-focussing mass spectrometer. The total ion current (TIC) and ion masses were recorded on cross-scan report

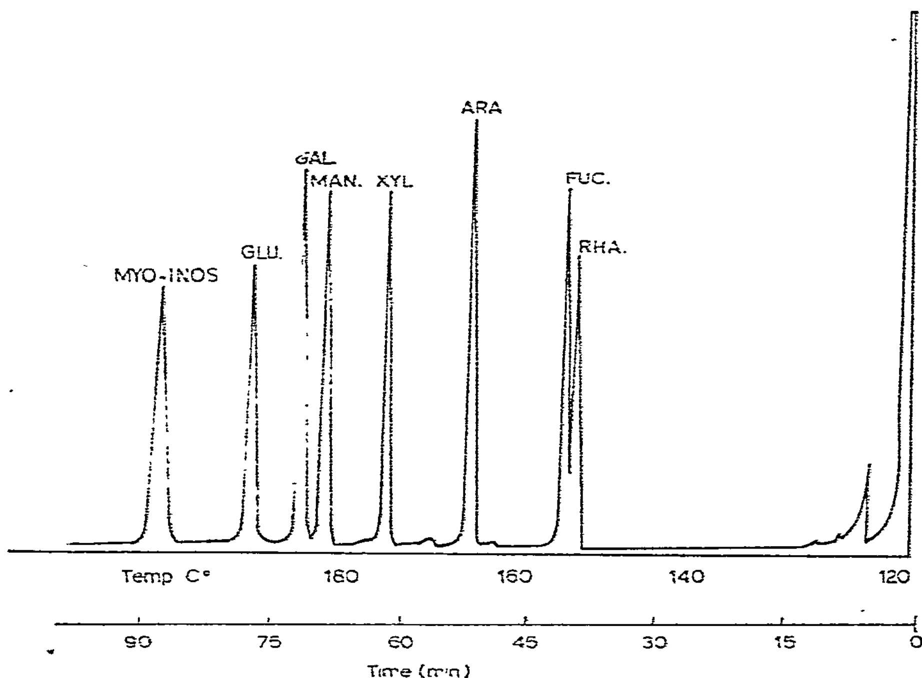

Fig. 1. Gas–liquid chromatogram showing separation of the alditol acetate derivatives. The amount of sample injected was 1 μl from a 1-ml standard solution containing 1.7–2.3 mg of each standard sugar. GC conditions: temperature, 120° held for 5 min, then raised at 1°/min to 180°: carrier gas (nitrogen flow-rate, 50 ml/min: recorder range, 10 mV; chart speed, 120 mm/h.

TABLE II

RELATIVE RETENTION TIMES OF PARTIALLY METHYLATED ALDITOL ACETATES ON THE OV-275–XF-1150 COLUMN

5-mg amounts of the standard sugars were methylated, then hydrolyzed, reduced and acetylated. 4 μl amounts were injected from 100-μl samples. Other conditions as in Table I.

Residue	*Glycosidic linkage*	*Positions of methoxy groups*	*RRT*
Arabinose	terminal	2,3,5	0.60
	terminal	2,3,4	0.74
	2	3,5	0.96
	3	2,5	1.06
	5	2,3	1.19
	2,5	3	1.65
	3,5	2	1.69
Xylose	terminal	2,3,4	0.76
	2 or 4	3,4 or 2,3	1.31
	3	2,4	1.12
	3,4 or 2,3	2 or 4	1.52
	2,4	3	1.84
Mannose	terminal	2,3,4,6	1.00
	2	3,4,6	—
	3	2,4,6	1.45
	4	2,3,6	1.40
	6	2,3,4	1.62
	2,6	3,4	2.09
	3,4	2,6	2.08
	4,6	2,3	—
Galactose	terminal	2,3,4,6	1.16
	2	3,4,6	1.82
	3	2,4,6	1.56
	4	2,3 6	1.59
	6	2,3,4	1.57
	2,4	3,6	2.56
	2,6	3,4	2.52
	3,4	2,6	2.34
	3,6	2,4	2.28
	4,6	2,3	2.12
Glucose	terminal	2,3,4,6	1.00
	2	3,4,6	1.91
	3	2,4,6	1.18
	4	2,3,6	1.65
	6	2,3,4	1.54
	2,3	4,6	—
	2,4	3,6	2.22
	3,4	2,6	1.86
	3,6	2,4	1.98
	4,6	2,3	2.09
	2,3,4	6	2.45

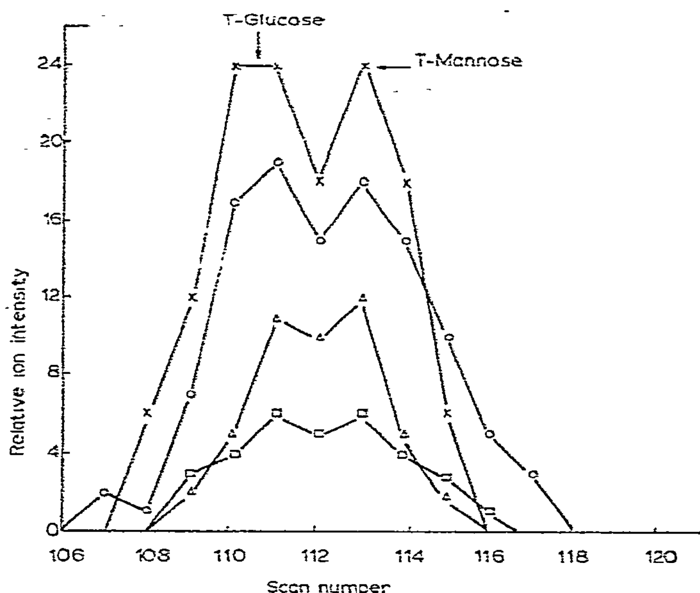

Fig. 2. Separation of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylmannitol (terminal mannose) and 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol (terminal glucose) using a cross-scan report obtained from GC–MS analysis. The results are taken from a complete cross-scan report of a mixture of partially methylated alditol acetates of starch and mannan. *m/e* values monitored for this separation: □—□, 45; ○—○, 145; and △—△, 161. ×—×, Total ion current.

print-outs. The conditions were as follows: resolution, × 1000; mass range, *m/e* 15–400; scan speed, 3 sec per decade; voltage, 24 eV; electron current, 300 μA; cage temperature, 180–200°; separator temperature, 165°; line temperatures, 200° (i), 190° (ii) and 160° (iii).

Qualitative and quantitative identification of each partially methylated alditol acetate component of a cell-wall hydrolysate was then possible using: (a) the substitution pattern (the position of the methoxy groups) for MS analysis and comparison with the spectra of standard derivatives; (b) the RRT to terminal mannose on a single GC column and (c) the sugar content of the methylated sample.

RESULTS AND DISCUSSION

The relative retention times of the standard alditol acetates are given in Table I. A complete separation of all the alditol acetates was obtained (Fig. 1); this included rhamnose and fucose which previously had proved difficult to separate completely. RRT values of the standard partially methylated alditol acetates are given in Table II. Previous columns failed to separate 2,3,6-methylated glucose and galactose; similarly, 3,6-methylated glucose or galactose were very difficult to separate by GC alone[1]. The GC system used here succeeded in completely separating both these types. Although terminal mannose and glucose gave apparently identical retention times, after GC–MS the cross-scan reports allowed the double peak to be resolved into its two components (Fig. 2).

The great advantage of this GC–MS system is that a single column can be

used to identify the alditol acetate components and all the partially methylated alditol acetates from a complete cell-wall sample, whereas previously at least two columns have been necessary for a complete analysis of any one sample. In the temperature range used, the column bleed is negligible and the necessity for a double-column GC analysis is avoided.

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