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A SIMPLE AND RAPID PREPARATION OF ALDITOL ACETATES FOR MONOSACCHARIDE ANALYSIS

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ABSTRACT

A simple and rapid method is described for the preparation of alditol acetates from monosaccharides. It can be performed in a single tube without transfers or evaporations. Monosaccharides are reduced with sodium borohydride in dimethyl sulphoxide and the resulting alditols acetylated using 1-methylimidazole as the catalyst. Removal of borate is unnecessary and acetylation is complete in 10 min at room temperature. Monosaccharides are quantitatively reduced and acetylated by this procedure. The alditol acetates are completely separated by glass-capillary, gas–liquid chromatography on Silar 10C. The method has been applied to the analysis of monosaccharides in acid hydrolysates of a plant cell-wall.

INTRODUCTION

Gas–liquid chromatography of alditol acetates is widely used for determining the composition of monosaccharide mixtures, especially those resulting from the hydrolysis of polysaccharides[1]. Alditol acetates are better resolved than other commonly used derivatives. However, current methods for preparing alditol acetates involve relatively long acetylation-times at elevated temperatures (100–120°). We have therefore attempted to improve the method for preparing alditol acetates.

In the preparation of alditol acetates, monosaccharides are first reduced to alditols with sodium borohydride and then acetylated. Borate, formed from sodium borohydride, complexes with the alditols and interferes with subsequent acetylation. Borate is usually removed as the volatile trimethyl borate by evaporation with methanol[2]. This method is effective only under completely anhydrous conditions, as water converts methyl borate into methanol and boric acid. Repeated slow and tedious evaporations are necessary to remove all of the borate. Acetylation requires the presence of such catalysts as pyridine[3] or sodium acetate[4], which are effective only in the absence of borate. Acid-catalysed acetylation, although apparently successful

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in the presence of borate[5] produces artifacts having retention times similar to alditol acetates[1]. Connors and Pandit[6] introduced the use of 1-methylimidazole as a catalyst for the rapid analytical acetylation of polyhydroxy compounds at 45 . This catalyst has been used in the acetylation of alditols, following the removal of borate[7], and for the acetylation of aldononitriles[8].

We now report the successful use of 1-methylimidazole as a catalyst for the quantitative acetylation of alditols in the presence of borate. The resulting alditol acetates were separated by capillary g.l.c. on Silar 10C. The method has been used for analysis of monosaccharides in acid hydrolysates of a plant cell-wall preparation.

MATERIALS AND METHODS

Reagents. — Dichloromethane (cat. no. 6050) and dimethyl sulphoxide (cat. no. 802912, stored over molecular sieve type 4A) were obtained from Merck, Darmstadt, Germany. 1-Methylimidazole was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Monosaccharides and alditols were obtained from commercial sources except for L-sorbose, which was the kind gift of Professor V. M. Trikojus, University of Melbourne. A sample containing apiose was obtained by hydrolysis of crude apiin[9,10] with 0.05M sulphuric acid for 30 min at 100 . *myo*-Inositol hexa-acetate (m.p. 217) and glucitol hexa-acetate (m.p. 102) were prepared from *myo*-inositol and glucitol (5 g) by suspending them in 1-methylimidazole (5 mL) and adding acetic anhydride (50 mL) slowly, with mixing. After 15 min, water (200 mL) was added to decompose the excess of acetic anhydride. The acetylated alditols were extracted into dichloromethane (200 mL), and the extract was decolourised with carbon (5 g), filtered, and the filtrate evaporated to dryness (80). The residues were dissolved in acetone (200 mL) and the acetates precipitated by the addition of cold water (800 mL). The reprecipitated acetates were dried, dissolved in dichloromethane, and crystallized under vacuum.

All other reagents were of analytical grade.

Plant cell-walls. — Suspension cultures of endosperm cells of Italian ryegrass (*Lolium multiflorum* Lam.) were grown in modified White's medium containing 4% (w/v) of sucrose[11]. Cultures were used at mid-log phase of growth, 5 days after subculturing. The cells were broken by two passages through a French pressure-cell at 0.62×10^7 Nm $^{-2}$ and the cell walls were collected and washed successively with water, ethanol, methanol, and pentane on a nylon mesh (pore size, 10 μm).

Hydrolysis of cell walls. — Cell walls (10 mg) were treated with 72% (w/w) sulphuric acid (125 μL) under argon in glass tubes fitted with Teflon-lined screw caps. Dissolution of the walls in the acid was aided by agitation on a vortex mixer. After 45 min at room temperature, the acid was diluted with water (1.35 mL) to give M sulphuric acid and heated under argon: (*a*) for 1 h at 121 , (*b*) for 2 h at 100 , and (*c*) for 3 h at 100 . After cooling, the solutions were made neutral and made M with respect to ammonia by adding 15M ammonia solution (0.32 mL). *myo*-Inositol

(0.05 mL of a 20 mg/mL solution) was added as an internal standard. Aliquots (0.1 mL) were reduced and acetylated as described.

Reduction of monosaccharides. — Monosaccharides were reduced with a solution of sodium borohydride in dimethyl sulphoxide prepared by dissolving sodium borohydride (2 g) in anhydrous dimethyl sulphoxide (100 mL) at 100°. Monosaccharides were routinely reduced for 90 min at 40° by adding 1 mL of the sodium borohydride solution to 0.1 mL of the monosaccharide mixture in M ammonia. After reduction, the excess of sodium borohydride was decomposed by the addition of 18M acetic acid (0.1 mL).

Acetylation. — 1-Methylimidazole (0.2 mL), followed by acetic anhydride (2 mL), were added to the reduced monosaccharides and the components were mixed. After 10 min at room temperature, water (5 mL) was added to decompose the excess of acetic anhydride. When cool, dichloromethane (1 mL) was added and the mixture was agitated on a vortex mixer. After the phases had separated, the lower one was removed with a Pasteur pipette and stored in a 1-mL, septum-cap vial at −20°.

Gas–liquid chromatography. — The alditol acetates were separated on a SCOT glass-capillary column (28.5 m × 0.5 mm, i.d., Silar 10C) S.G.E. Pty. Ltd., Melbourne, Australia) fitted to a Hewlett–Packard 5710A chromatograph equipped with a flame-ionization detector, and a S.G.E. "Unijector" capillary injection-system used in the split mode. High-purity hydrogen was used as the carrier gas at a flow rate of 77 cm/sec (determined by using dichloromethane). Routinely, 2-μL samples were injected. The oven temperature was kept for 4 min at 190° following injection and then raised at 4°/min to 230°, where it was kept for 8 min. The injection port and detector were heated to 250 and 300°, respectively.

RESULTS AND DISCUSSION

Separation of alditol acetates. — Separations of alditol acetates by g.l.c. using packed columns have utilized polar stationary-phases (such as ECNSS-M, SP2330, SP2340, and OV-275)[1,12]; OV-275 has also been used for separations of alditol acetates on a capillary column[13]. Hirase *et al.*[14] introduced a new polar phase, Silar 10C, for the separation of alditol acetates on packed columns, and partially methylated alditol acetates have recently been separated on a glass-capillary column coated with this phase[15]. Silar 10C is reported to have excellent thermal stability and good resolving power for the g.l.c. analysis of alditol acetates. We have found that a capillary column of Silar 10C gives baseline resolution of 13 alditol acetates (Fig. 1). Each peak in Fig. 1 corresponds to 3 μg of monosaccharide. The order of elution (Table I) was the same as that reported by Okahira *et al.*[16] using a column packed with Silar 10C/Chromosorb W.

Reduction of monosaccharides with sodium borohydride. — The use of dimethyl sulphoxide as the solvent in the reduction of monosaccharides has two advantages. Firstly, solutions of sodium borohydride in anhydrous dimethyl sulphoxide are stable, whereas aqueous solutions must be freshly prepared. Secondly, dimethyl

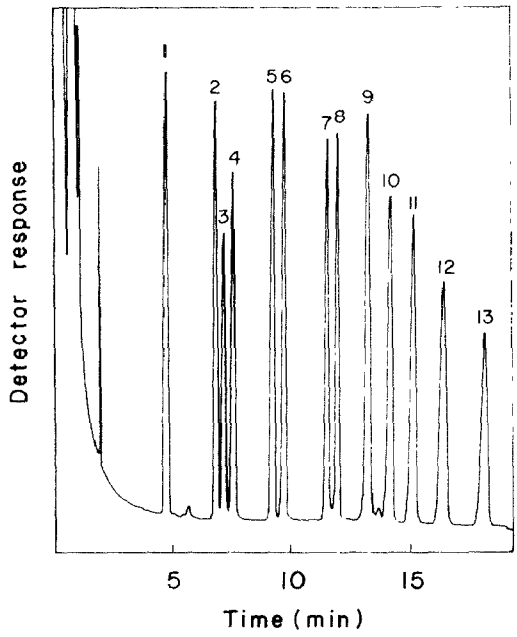

Fig. 1. Separation of alditol acetates by gas–liquid chromatography on a Silar 10C glass-capillary column. The temperature was kept at 190° for 4 min and then increased to 230° at 4°/min. 1, erythritol; 2, 2-deoxy-*erythro*-pentitol; 3, rhamnitol; 4, fucitol; 5, ribitol; 6, arabinitol; 7, xylitol; 8, 2-deoxy-*arabino*-hexitol; 9, allitol; 10, mannitol; 11, galactitol; 12, glucitol; and 13, *myo*-inositol.

sulphoxide, unlike water, does not readily consume acetic anhydride during acetylation. A high concentration of sodium borohydride (20 mg/mL) was chosen in an attempt to increase the rate of reduction. Although it was necessary to decompose unreacted sodium borohydride by adding an excess of acetic acid (0.1 mL), the addition of too much acid (for instance, 1.0 mL) was found to interfere with acetylation.

Most monosaccharides were reduced rapidly at room temperature, but glucose and rhamnose were reduced more slowly (Table II). Reduction at 40° was faster than at room temperature and no epimerization was observed. Reduction of glucose at 40° reached a maximum in 90 min and was unchanged after a further 90 min. At 60°, there was vigorous evolution of gas and reduction was less successful, possibly because of instability of the reduction mixture at this temperature. After very short reduction-times (0.5 min, Table II), additional peaks attributed to acetylated, unreduced monosaccharides were observed. These peaks were not present after longer times of reduction.

In an attempt to determine the absolute extent of reduction, glucose reduced for 90 min at 40° was compared with a standard of glucitol hexa-acetate, both relative to an internal standard of *myo*-inositol hexa-acetate. If all losses are attributed to

TABLE I

RETENTION TIMES OF ALDITOL ACETATES ON A SILAR 10C GLASS-CAPILLARY COLUMN[a]

Alditol acetate	*Retention time (min)*	*Relative retention time (myo-inositol = 1.00)*
Glycerol	0.91	0.08
Erythritol	4.66	0.26
2-Deoxy-*erythro*-pentitol	6.70	0.37
Rhamnitol	7.08	0.39
Fucitol	7.45	0.41
Ribitol	9.12	0.50
Arabinitol	9.68	0.53
Xylitol	11.40	0.63
2-Deoxy-*arabino*-hexitol	11.90	0.66
Allitol	13.0	0.72
Apiitol	13.8	0.76
Mannitol	14.2	0.78
Talitol	14.3	0.79
Galactitol	15.1	0.83
Glucitol	16.2	0.90
Inositol	18.1	1.00
Iditol[b]	18.6	1.03

[a]Temperature program as in Fig. 1. [b]By reduction of L-sorbose.

TABLE II

REDUCTION OF MONOSACCHARIDES WITH SODIUM BOROHYDRIDE[a]

Sugars	*Relative peak heights*[b]												
	Reduction time (min) at 23°					*Reduction time (min) at 40°*				*Reduction time (min) at 60°*			
	0.5	*15*	*30*	*60*	*90*	*15*	*30*	*60*	*90*	*15*	*30*	*60*	*90*
Rhamnose	0.47	0.59	0.71	0.77	0.98	0.86	0.85	1.02	1.05	0.79	0.70	0.77	0.80
Fucose	0.69	0.84	0.95	0.98	1.13	1.01	1.04	1.17	1.19	0.91	0.82	0.93	0.94
Arabinose	1.10	1.16	1.19	1.07	1.26	1.13	1.15	1.28	1.30	0.99	0.95	1.07	1.07
Xylose	0.91	1.05	1.13	1.07	1.22	1.12	1.17	1.27	1.32	0.99	0.94	1.07	1.05
Allose	0.75	0.82	0.87	0.86	0.86	0.85	0.97	0.95	1.06	0.76	0.75	0.86	0.81
Mannose	0.68	0.74	0.79	0.72	0.75	0.74	0.84	0.81	0.91	0.66	0.65	0.74	0.71
Galactose	0.64	0.72	0.77	0.66	0.74	0.70	0.80	0.76	0.85	0.63	0.62	0.71	0.67
Glucose	0.26	0.38	0.50	0.55	0.73	0.58	0.69	0.65	0.74	0.51	0.52	0.58	0.55

[a]A mixture of monosaccharides (total concentration 20 mg/mL) was reduced with sodium borohydride for various times at three different temperatures. The acetylated alditols were determined by gas–liquid chromatography following the addition of *myo*-inositol hexa-acetate (0.2 mg) as the internal standard. [b]Relative peak height is the ratio of peak height to the peak height of the internal standard (mean of two determinations).

incomplete reduction, 87% of the glucose was reduced. Glucose was chosen for this study because it was the sugar reduced at the lowest rate (Table II).

The apparent slow acetylation of glucitol and rhamnitol reported by Buchala *et al.*[17] and Selvendran *et al.*[18] may be due to slow, and hence incomplete, reduction of glucose and rhamnose. Maltby *et al.*[19] reported that, whereas reduction of partially methylated alditol acetates for 60 min at room temperature was incomplete, reduction was apparently complete in 90 min at 37 .

Acetylation of alditols in the presence of borate. – 1-Methylimidazole is a highly efficient catalyst for the acetylation of hydroxy compounds[20]. For alditols, we routinely used a 10-min acetylation at room temperature. No further reaction occurred when acetylation was continued for 90 min.

The concentration of 1-methylimidazole and acetic anhydride influences the acetylation of glucitol in the presence of borate. For the complete acetylation of 1 mg of glucitol (in 1.2 mL of acidified, reduction mixture), 0.2 mL of 1-methylimidazole and 2 mL of acetic anhydride was found to be sufficient. Bittner *et al.*[7] used much lower concentrations of acetic anhydride (0.2 mL), but under these conditions we found that borate interfered with acetylation. When either 1-methylimidazole or acetic anhydride, or both, were used at high concentrations (for instance, 1-methylimidazole 2 mL, acetic anhydride, 10 mL) less acetylated glucitol was recovered.

At the concentrations of catalyst and acetic anhydride routinely used, g.l.c. indicated that borate did not interfere with acetylation. The extent of acetylation was determined by using the titrimetric method of Connors and Pandit[6]. Glucitol, ribitol, xylitol, and *myo*-inositol were acetylated in the presence of borate by reagents in the same proportions as used in acetylation for g.l.c. Acetylation was complete, to the limits of precision of the titrimetric procedure ($100 \pm 2\%$)

Water does not interfere with the 1-methylimidazole-catalysed acetylation if an excess of acetic anhydride is present[20]. Interference by other hydroxy compounds, such as ethanol or methanol, may be overcome by adding more acetic anhydride.

Acetylation in the presence of borate has the advantage that no evaporation of the sample is necessary, thus obviating selective losses of more-volatile alditol acetates. It is also an obvious advantage where volatile, partially methylated sugars are to be reduced and acetylated. The selective loss of components during evaporation has limited the quantitative recovery of components in methylation analysis[1]. Dawson and Mopper[21] reported selective losses of monosaccharides during evaporation to dryness, which they attributed to adsorption on glass. The present method avoids this problem, as no concentration of the mixture is required, and reduction and acetylation occur in one tube.

Use of the method in quantitative monosaccharide analysis. – Equal amounts of 12 monosaccharides were reduced and acetylated at five concentrations. The relationship between monosaccharide concentration and detector response was linear (Table III).

The recovery of alditol acetates by partitioning into dichloromethane containing *myo*-inositol hexa-acetate was investigated by repeated extraction. Three consecutive

TABLE III

RELATIVE PEAK HEIGHTS OF ALDITOL ACETATES[a]

Alditol acetate	*Relative peak height*[b]	*Correlation coefficient of standard curve*
Allitol	0.354	0.999
Arabinitol	0.441	0.999
2-Deoxy-*arabino*-hexitol	0.438	0.953
2-Deoxy-*erythro*-pentitol	0.479	0.999
Erythritol	0.499	0.998
Fucitol	0.425	0.996
Galactitol	0.280	1.000
Glucitol	0.259	0.999
Mannitol	0.311	0.998
Ribitol	0.443	0.999
Rhamnitol	0.369	0.998
Xylitol	0.447	0.999

[a]Mixtures of 12 monosaccharides at five concentrations up to a total of 20 mg/mL were reduced and then acetylated (in triplicate) and 2 mg of *myo*-inositol hexa-acetate was added as the internal standard. [b]Slope of standard curve (peak height relative to internal standard plotted against monosaccharide concentration in mg/mL).

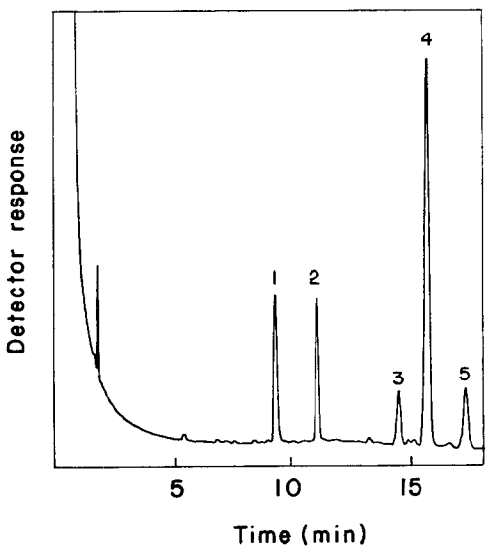

Fig. 2. Separation of alditol acetates produced by reducing and then acetylating the monosaccharides in an acid hydrolysate of the cell walls of rye-grass. The cell walls were dissolved in 72% (w/w) sulphuric acid at room temperature for 45 min and the acid diluted to M by the addition of water. Hydrolysis was continued at 100° for 2 h under argon. 1, arabinitol; 2, xylitol; 3, galactitol; 4, glucitol; and 5, *myo*-inositol (internal standard).

TABLE IV

DETERMINATION OF THE MONOSACCHARIDE COMPOSITION OF RYEGRASS CELL-WALL HYDROLYSATES[a]

Hydrolysis conditions	*Monosaccharide content (μg/mg of cell walls, mean of duplicates)*			
	Arabinose	*Xylose*	*Galactose*	*Glucose*
2 h, 100	128	137	63.6	562
3 h, 100	164	125	61.4	578
1 h, 121	100	99.6	56.5	545

[a]The cell walls were dissolved in 72% (w/w) sulphuric acid for 45 min and then diluted to M acid concentration with water

extracts contained 80, 19, and 1%, respectively, of the total alditol acetate recovered. Different alditol acetates were not extracted selectively. Correction for incomplete partitioning of alditol acetates may be made by including an internal standard in the dichloromethane.

Analysis of a plant cell-wall hydrolysate. — The separation of the alditol acetates derived from a hydrolysate is shown in Fig. 2. Four major peaks are present, corresponding to arabinose, xylose, galactose, and glucose derivatives. The proportions of these four components determined by the present method agree with those reported by Anderson and Stone[22]. A number of small peaks are also present, but these do not necessarily correspond to alditol acetates. One of these peaks, having a retention time slightly less than mannose, but resolved from mannose, was always present, even in samples containing no monosaccharides. A contaminant having a similar retention time has been reported and attributed to phthalic esters widely used as plasticisers[23].

Hydrolysis at 121° resulted in lower recoveries of arabinose and xylose than hydrolysis at 100°. The total monosaccharides, recovered after hydrolysis for 3 h at 100°, accounted for 93% of the dry weight of the cell walls (Table IV). As the cell walls contain protein and other minor components, these values are close to the expected total carbohydrate-content of the walls. Alditol acetates are often used only to determine the ratios of monosaccharides in polysaccharide hydrolysates[24], but the procedure described here offers a means of determining total monosaccharide content.

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