GAS–LIQUID CHROMATOGRAPHY OF ALDITOL ACETATES AND ITS APPLICATION TO THE ANALYSIS OF SUGARS IN COMPLEX HYDROLYSATES

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INTRODUCTION

Three main groups of derivatives have been successfully used for gas–liquid chromatography (GLC) of sugars, *viz.* methyl ethers, trimethylsilyl (TMS) ethers and acetates[1]. Although O-methylated sugars can be prepared practically quantitatively, the technique does not lend itself to routine handling of hydrolysates and will probably be reserved for structural studies of polysaccharides. Both TMS ethers and acetates are easy to prepare but the TMS ethers cannot be prepared quantitatively; SWEELEY *et al.*[2] quote 90 % conversion for glucose; PERRY[3], 85 % conversion for glucosamine. In addition, many of the TMS derivatives cannot be obtained in crystalline form. Thus the acetates are the most suitable derivatives for investigating complex hydrolysates as most can be obtained in crystalline form and characterised by melting point as well as by GLC. The work of GUNNER, JONES AND PERRY[4, 5] established the thermal stability of alditol acetates and they also succeeded in separating some of these compounds. Much improved separations have since been reported by SAWARDEKER, SLONEKER AND JEANNES[6]. It is essential to reduce the sugars to the corresponding alcohols to avoid the multiplicity of peaks which can arise with a hydrolysate containing a range of sugars each capable of producing two anomeric and two ring isomers, all of which may be separated during GLC.

The technique described in this paper was developed primarily to deal with soil hydrolysates. Such hydrolysates contain a wide range of sugars as shown by paper chromatography, and in addition a vast range of other organic and inorganic materials. The carbohydrate content of soils may be less than 0.1 % in mineral soils and as high as 20 % in peats. A technique which can be applied successfully to soil hydrolysates can probably be used with most other complex hydrolysates.

METHODS

Gas–liquid chromatography was carried out using a Perkin Elmer 801 gas chromatograph with dual flame ionisation detector and controlled heating of both injection port and detector. Glass columns 1.84 m long by 0.32 cm internal diameter were used.

Materials

Solid supports. Embacel (May and Baker) acid washed Chromosorb, 60 to 100 mesh, and hexamethyldisilazane (HMDS) coated Chromosorb W, 60 to 80 mesh, (Johns Mansville) were used.

Liquid phases. A range of liquid phases were investigated including those described by GUNNER, JONES AND PERRY[4,5] but only the following were found useful.

LAC IR 296 (Cambridge Laboratories, England)—a polymer of diethylene glycol adipate.

DD 071 (Perkin Elmer)—as for LAC IR 296 but reported to possess improved thermal stability.

ECNSS-M (Applied Science Laboratories, Pa., U.S.A.)—a medium polarity liquid phase based on a chemical combination of a cyanoethyl silicone polymer with ethylene glycol succinate polyester.

The best results in terms of peak shape, resolution and useful life of columns were obtained with 10 % w/w liquid phase on the support. Smaller amounts tended to result in peak tailing and larger amounts caused severe liquid phase bleed. Conditioning the 10 % columns for several days at 220° in a stream of nitrogen was necessary for satisfactory results and resolution gradually improved during the first two or three weeks of continual use. Cooling below about 100° even for a few minutes and then reheating to working temperature resulted in broad peaks which only became sharp after overnight heating at 220°. Retention volumes gradually decreased over a period of several weeks.

Separations of the alditol acetates were possible using carrier gas flow rates from 30 to 100 ml nitrogen per minute at temperatures from 180 to 220°. The best combination of these two parameters was found to be a flow rate between 30 and 50 ml nitrogen per minute and a linear temperature programme from 170 to 220° at 0.8°/min. The temperature programme was commenced immediately after the injection.

Standard alditol acetates. Alditol acetates were obtained by reduction of the parent sugars with sodium borohydride in aqueous solution overnight. Excess borohydride was destroyed by addition of acetone and sodium removed from the reaction mixture by passage through H^+ Dowex-50. Borate was removed as far as possible by continued addition and removal of methanol, using a rotary evaporator. The alditols were acetylated at room temperature overnight using acetic anhydride and redistilled pyridine (50/50). The alditol acetates were recrystallised from ethanol or methanol until only a single peak was given during GLC. The acetates which could not be obtained in crystalline form were purified by vacuum distillation onto a cold finger and by the column procedure, described below, for the purification of alditol acetates obtained from hydrolysates. A few preparations were obtained by acidic acetylation (2 % v/v concentrated H_2SO_4 in acetic anhydride, 60° for 10 min) of the alditols without removal of sodium or borate as described for the treatment of hydrolysates.

Treatment of soil hydrolysates

3 g of air dry soil were mixed to a slurry with 4 ml 72 % H_2SO_4 (v/v) and allowed to stand 2 h at room temperature. The acid concentration was reduced to 1 *N* and the suspension refluxed for 16 h. The clear supernatant obtained by centrifugation was decolourised by charcoal and the carbohydrate content determined with an-

throne[7]. The clarified solution was passed through a column of Na^+ Dowex-50 to remove iron and the eluate of about 300 ml adjusted to pH 7 with sodium bicarbonate before evaporation just to dryness in a rotary evaporator. The sugars were quantitatively extracted with methanol (6 × 50 ml) and the volume reduced to about 20 ml. About 1 mg of solid sodium borohydride was added and the solution allowed to stand overnight. A few drops of acetone were added, the sample evaporated to dryness and the reduced sugars acetylated using 2.5 % (v/v) concentrated H_2SO_4 in acetic anhydride at 60° for 10 min. The reaction mixture was cooled in ice, water added cautiously and then in sufficient quantity to render the reaction mixture immiscible with chloroform. The alditol acetates were extracted using chloroform. The bulked chloroform extracts were evaporated to dryness, and the sample taken up in a few drops of chloroform–methanol (50/50) and transferred quantitatively to a small wad of cotton wool. The solvents were *completely* removed in a stream of warm air when the sample on cotton wool was placed on top of a column (2 cm × 0.5 cm diameter) of Silica Gel G poured in benzene. At least 6 ml benzene were passed through the column under pressure. The alditol acetates, which remained on the column during the benzene washing were quantitatively eluted using several ml of chloroform–methanol (50/50). The solvent mixture was evaporated to dryness and the residue taken up in a known volume of chloroform for injection into the gas chromatograph. When possible the volume of solvent injected into the GLC column was kept to 1 μl or less to avoid liquid phase bleed.

RESULTS AND DISCUSSION

Separation of standard alditol acetates

Table I shows the separations obtained by gas–liquid chromatography of mixtures of standard alditol acetates on columns packed with 10 % w/w DD 071 and 10 % w/w ECNSS-M on HMDS coated Chromosorb W and the separations obtained by chromatography of the same mixtures of standards on a column packed with 5 % DD 071 plus 5 % ECNSS-M on HMDS coated Chromosorb W. The liquid phases based on diethylene glycol adipate were particularly useful for the separation of tetritol, pentitol and 6-deoxy hexitol acetates. However, this system did not resolve galactitol and glucitol acetates and thus has limited use for the study of complex hydrolysates. Conversely the other liquid phase (ECNSS-M) was useful for separation of hexitol acetates but did not resolve satisfactorily arabitol from ribitol and rhamnitol from fucitol acetate. A useful separation of all the alditol acetates was given by the mixed liquid phase system. The separation of hexitol acetates was favoured by an increase in the proportion of ECNSS-M while the total liquid phase concentration was maintained at 10 % w/w and *vice versa*. Thus the two liquid phases can be used separately to produce the best resolution within the group of sugars in which the experimenter is most interested or a mixture used to give reasonable separations over the complete range of compounds from glycerol to iditol acetate.

The order of appearance of the alditol acetates from the column is of some interest. BISHOP[1] stated that there appeared to be no rationale between the retention volumes of aldose derivatives and corresponding structures[4,5]. The sequence of alditol acetates shown in Table I under ECNSS-M has been obtained by SAWARDEKER, SLONEKER AND JEANNES[6] and also by WILLIAMS AND JONES[8] for acetylated iso-

TABLE I

RELATIVE RETENTION TIMES OF ALDITOL ACETATES AND RELATED COMPOUNDS[a]

	Melting[b] *point*	*Liquid phase*		
		10% LAC IR 296 or 10% DD 071	*10% ECNSS-M*	*5% DD 071 + 5% ECNSS-M*
Tri-O-acetyl-D-glycerol	Syrup	10	13	11
Tetra-O-acetyl-D-erythritol	85–86	39	42	40
Tetra-O-acetyl-D-threitol	Syrup	45	50	46
Tetra-O-acetyl-2-deoxy-D-ribitol	Syrup	57	60	58
Penta-O-acetyl-6-deoxy-L-mannitol (rhamnitol)	Syrup[c]	68	66	67
Penta-O-acetyl-6-deoxy-L-galactitol (fucitol)	126.5–127.5	73	69	72
Tetra-O-acetyl-pentaerythritol	74–75	70	72	72
Penta-O-acetyl-D-ribitol	Syrup	84	83	84
Penta-O-acetyl-L-arabitol	73–75	88	87	88
Penta-O-acetyl-D-xylitol	60.5–62	100[d]	100	100
Hexa-O-acetyl-D-allitol	62.5–64.5	128	120	126
3-O-methyl-penta-O-acetyl-D-glucitol	Syrup[e]	130	124	129
Hexa-O-acetyl-D-mannitol	123.5–124	137	126	134
Hexa-O-acetyl-D-altritol	Syrup	136	127	134
Hexa-O-acetyl-D-galactitol	165–166.5	146	133	142
Hexa-O-acetyl-D-glucitol	97.5–98.5	146	141	146
Hexa-O-acetyl-D-iditol	121.5–122.5	153	155	155
Hexa-O-acetyl-myoinositol	216.5–217.5	152	157	155

[a] Using linear temperature programme from 170 to 220° at 0.8° per minute.
[b] Corrected melting points obtained using a Kofler hot stage microscope.
[c] Crystals appeared but were unmanageable.
[d] The retention time for xylitol acetate was from 35 to 55 min depending on the carrier gas flow rate and age of the column.
[e] Contained several percent of glucitol acetate.

deoxyalditols. However, on other liquid phases glucitol and galactitol acetates have a reversed order of appearance[4,5], and Table I shows that this is also true of altritol and mannitol acetates on different liquid phases. GLC of the TMS ethers of the alditols has yielded a quite different sequence of retention times and confirms that at present there is no complete relationship between retention times and structure. With the exception of mannitol and galactitol acetates those alditols with the most groups in *cis* relationship on one side of the conventional zigzag planar chain have the longest retention times, *i.e.* those molecules with the closest approach of the acetyl groups remain on the column longest. It is possible that the zigzag planar carbon chain is not maintained when O-acetyl or more particularly O-trimethylsilyl groups are present on the molecule at temperatures near 200°, particularly when these groups have a *cis* relationship on alternate carbon atoms in the chain.

Application of the method to complex hydrolysates

Removal of salts and reduction. The clarification of hydrolysates and removal of iron by Dowex-50 were not necessary when dealing with hydrolysates of purified polysaccharides. However, with soil hydrolysates removal of iron was particularly

necessary otherwise considerable losses (75 %) of sugars occurred during rotary evaporation to dryness due to reduction of iron and consequent oxidation of some of the sugars. Similarly the methanol extractions can be omitted when the hydrolysate contains higher ratios of sugars to acid than can be obtained with soil hydrolysates. Large amounts of salt caused problems during acetylation when only microgram quantities of sugar were present. The borohydride reduction can be carried out directly in aqueous solution after neutralisation of the hydrolysate with sodium bicarbonate. The rate of reduction of the sugars at room temperature was followed using the anthrone procedure and was not entirely complete after 2 h, but no anthrone reaction could be detected after 5 or 6 h. Normally the reduction was allowed to proceed overnight.

Acetylation. Initially it was intended to carry out acetylation of the reduced sugars using acetic anhydride and pyridine. However, this was not possible presumably due to the fact that borate could not be completely removed from the reaction mixture even after exhaustive addition and evaporation of methanol after treatment with H^+ Dowex-50 to remove sodium. The same problem was encountered when 100 μg quantities of individual sugars were subjected to the reduction and basic acetylation procedure; alditol acetates were not detected in the final reaction mixtures until considerably larger quantities of starting material were used. These difficulties were overcome by the use of an acid catalyst during the acetylation and microgram quantities of sugar were reduced and acetylated by this method in the presence of large excesses of borate. The method is not particularly suitable for the acetylation of glycerol and the tetritols as other minor components appear in the reaction mixture. The amounts of these components is minimal (< 5 %) if the acetic anhydride contains only 2.5 % (v/v) concentrated H_2SO_4.

Extraction of acetylated products. The acid acetylation led to the formation of a number of artefacts which were produced by heating 2.5 % (v/v) concentrated H_2SO_4 in acetic anhydride in the absence of other materials. These artefacts ran with the alditol acetates during GLC so that their removal was essential. This was readily accomplished since they ran faster than alditol acetates during thin layer chromatography (TLC) on Silica Gel G nach Stahl using methanol in benzene (4 % v/v). Acetates were detected by the ferric hydroxamate spray of TATE AND BISHOP[9]. The position of the artefacts was determined by removal of successive bands of the thin layer ahead of the alditol acetates and extraction of any materials present by chloroform which was then concentrated and injected into the gas chromatograph. The separation of the alditol acetates from the artefacts was improved by repeated TLC in benzene as the acetates remained stationary in this solvent but the artefacts moved slowly. This information led to the development of the column procedure described. The elution of the artefacts from such columns using benzene was confirmed by concentration and injection of the benzene washings into the gas chromatograph. In addition to the removal of a number of peaks due to artefacts this procedure also removed materials which caused serious tailing after injection of the sample. Such materials ran near the front during TLC in methanol in benzene (4 % v/v) and were detected by iodine staining. Similar tailing after injection into the gas chromatograph often occurs with sugar acetates produced using pyridine and acetic anhydride. The column procedure removed the materials responsible for this tailing.

The chromatograms shown in Fig. 1 represent the monosaccharide composition

of complex carbohydrates from partly decomposed plant remains, a soil polysaccharide, the whole soil before removal of these two fractions and finally a peat.

Myoinositol may be used as an internal standard as it is commercially available in relatively pure form and was well separated from the hexitols under the conditions described. This necessitates two GLC runs on each hydrolysate because myoinositol was present in all the soils investigated. However, if this is done with the two different liquid phases, some predictions can be made concerning the identity of sugars responsible for some of the unlabelled peaks present on the chromatograms.

The chromatogram which shows the peat sugars in Fig. 1 is the result of addition of a known quantity of myoinositol to the hydrolysate of the peat soil. The relative

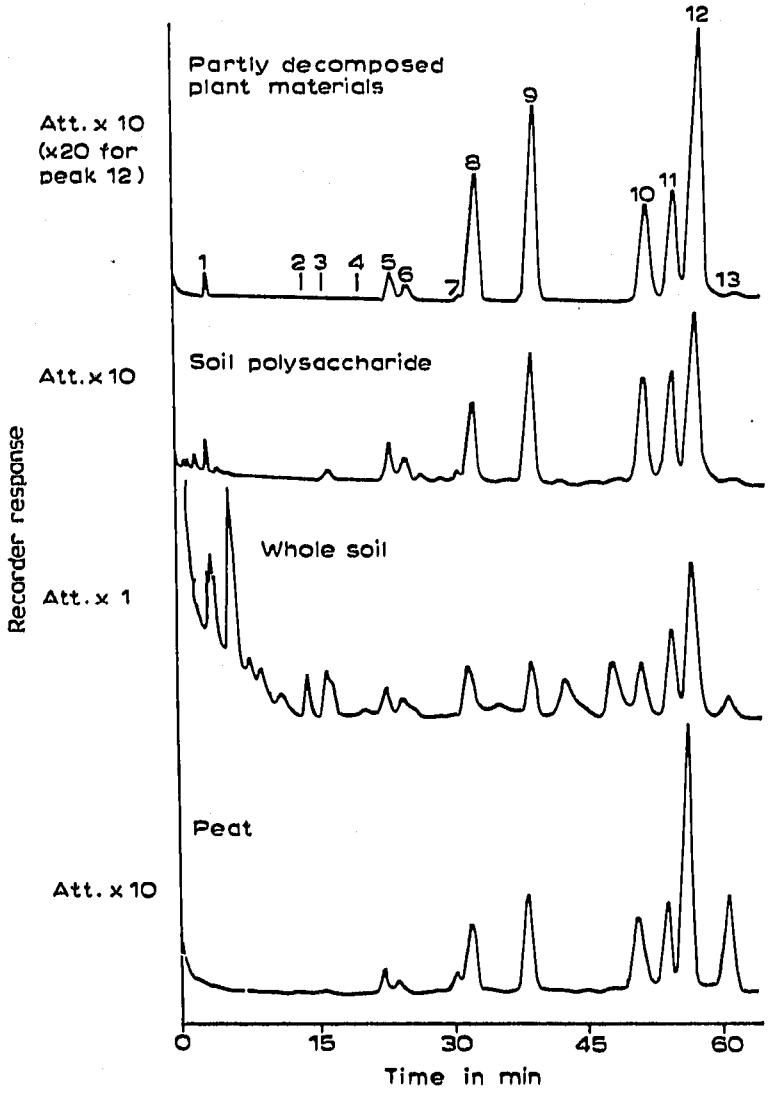

Fig. 1. Gas–liquid chromatography of alditol acetates from complex hydrolysates. Liquid phase: 10% w/w ECNSS-M on Chromosorb W coated with hexamethyldisilazane. Flow rate: 40 ml/min. Temperature: 170° by 0.8°/min to 220°. Attenuation: × 10; × 1 for whole soil sample; × 20 for glucitol acetate peak (peak 12) on the run for partly decomposed plant remains. Designation of peaks: 1 = Glycerol acetate; 2 = erythritol acetate; 3 = threitol acetate; 4 = 2-deoxy-ribitol acetate; 5 = rhamnitol acetate; 6 = fucitol acetate; 7 = ribitol acetate; 8 = arabitol acetate; 9 = xylitol acetate; 10 = mannitol acetate; 11 = galactitol acetate; 12 = glucitol acetate; 13 = myoinositol acetate. Other peaks have not yet been identified.

responses of the alditol acetates compared with myoinositol acetate are being determined and the procedure applied to the study of the fate of plant polysaccharides during decomposition in the soil and as a method for following the fractionation of complex mixtures of polysaccharides. The small amounts of sugar which can be quantitatively analysed render the technique particularly useful for monitoring polysaccharides from chromatographic columns.

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SUMMARY

A procedure is described for the quantitative conversion of sugars in a complex hydrolysate to the corresponding alditol acetates. The conditions which allow the separation of alditol acetates by gas–liquid chromatography are defined and several examples of the application of these techniques to natural complex polysaccharide materials are given.

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