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Note

Total characterization of polysaccharides by gas–liquid chromatography

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The qualitative and quantitative analysis of simple or complex carbohydrates has been simplified over the past decade by the introduction of a variety of analytical techniques based on gas–liquid chromatography (g.l.c.) of appropriate, volatile derivatives. Early procedures[1–4] relied mainly on the formation of trimethylsilyl derivatives, but the formation of multiple products from each aldose, the instability of the derivatives, and the special reagents needed made quantitative work difficult. These difficulties were partially alleviated by the introduction of alditol acetate derivatives that are formed as a single product per aldose, and the alditol acetates are now extensively employed[5–8] for carbohydrate analysis. The synthesis of per-*O*-acetylated aldononitriles was found to be a rapid, simple means of obtaining from each aldose a single derivative that was readily analyzed[9–14] by g.l.c. However, g.l.c. analysis of these derivatives entailed the use of a variety of stationary phases in the column, or derivatizing reagents, or both.

The g.l.c. procedures now presented consist of a consolidation and modification of existing methods, as applied in our laboratory, that allow the analysis of glycoses, hexosamines, Smith-degradation products, and *O*-acetyl content on a single, stationary phase by the selection of simple, derivatization techniques and appropriate, temperature parameters.

EXPERIMENTAL

Materials. — The reagents used in this study were available commercially. Standard carbohydrates were purchased from Sigma Chemical Co. Polysaccharides were research samples available in the laboratory. The stationary phase was 3% of OV-225 on Gas-Chrom Q (100–120 mesh), a product of Applied Science Labs, Inc.,

Apparatus. — The g.l.c. analyses were performed with a Perkin–Elmer Sigma 1 gas chromatograph equipped with dual flame-ionization detectors. The analyzer

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was fitted with a glass column (2 m × 2 mm) packed with 3% of OV-225 on Gas Chrom Q (100–120 mesh). Nitrogen was used as the carrier gas at a flow rate of 30 mL/min.

Preparation of per-O-acetylated aldononitriles (PAAN). — A method modified from procedures previously reported[9–14] was used for preparing the per-*O*-acetylated aldononitriles. A dried sample in pyridine (0.5 mL) was treated with hydroxylamine (60% of the sugar by weight). The mixture was placed in a tube that was then sealed, and kept for 20 min in a bath at 60–65°. Acetic anhydride (0.5 mL) was then added, and the solution was heated, and mixed periodically, for 20 min, cooled, and extracted with 1:2 chloroform–H_2O (3 mL). The extract was washed with water (3 × 2 mL), 2,2-dimethoxypropane (~0.5 mL) was added, and the solution was kept for 15–20 min (to remove traces of water). The solution was evaporated under diminished pressure, the residue was dissolved in chloroform (~0.2 mL), and an aliquot (1–3 μL) was then injected into the analyzer, which was programmed as follows: 170 → 180°, at 1.0°/min; 180 → 200° at 2.0°/min; and hold at 200° for 10 min.

Deamination of hexosamines. — Samples containing hexoamines dissolved in 2M trifluoroacetic acid (1 mL) at 0° were deaminated by adding an equal, millimolar amount (150 mg) of sodium nitrite. After 1 h, residual nitrous acid was removed by placing the sample in a boiling-water bath for 5 min, cooling, and evaporating to dryness under diminished pressure. The PAAN derivative was prepared as already described; however, the salt formed during the deamination reaction made necessary the use of 1 mL of each of the reagents (instead of 0.5 mL). The temperature program was the same as that described for the PAAN derivative.

Analysis of alditol acetates. — Alditol acetate derivatives of six aldoses were analyzed by using the following temperature program: hold at 180° for 8 min; 180 → 200° at 2°/min; hold at 200° for 4 min; 200 → 230° at 6°/min; hold at 230° for 5 min; 200 → 235° at 1°/min; and hold at 235° for 5 min.

O-Acetyl determination. — The *O*-acetyl content of a polysaccharide was determined as benzyl acetate, with respect to an internal standard of propanoic acid, by a modification of the procedure of Bethge and Lindström[15].

To the polysaccharide (~10 mg) was added 0.25M sodium ethoxide (2 mL), and the solution was mixed periodically for 30 min. Water (6 mL) was then added, to make a 25% ethanol solution, and the sample was mixed for an additional 30 min. The solution was applied to a column (85.0 × 6 mm) of Dowex-50 (H^+) resin (20–50 mesh) that had been pre-washed with deionized water (50 mL) and 25% ethanol (2 × 5 mL), and the column was eluted with 25% ethanol (15 mL). The eluate was titrated with 0.03M tetrabutylammonium hydroxide to pH 8.1. A standardized solution of propanoic acid (V_P) was added as the internal standard according to the following formula.

$$V_P = (C_t V_t) \cdot 10^3 / C_P,$$

where C_t = concentration of tetrabutylammonium hydroxide titrant in mol.L^{-1}, V_t = volume of titrant required, and C_P = concentration of propanoic acid in mol.L^{-1}.

The pH was re-adjusted to 8.1, and the solution was evaporated in a conical tube. The residue was dissolved in acetone (~1 mL), and benzyl bromide (V_b) was added to the solution according to the formula $V_b = 120\ C_t V_t$. An aliquot for g.l.c. analysis was injected after reaction for 10 min. The acetyl content was then calculated by the following formula.

$$\text{Acetyl}(\%) = (R_f)\ (Aa)\ C_P\ V_P \cdot 43/Ap \cdot Wt \cdot 10,$$

where Aa = area of acetic acid, Ap = area of propanoic acid, Wt = weight of sample in mg, and R_f = molar response factor of benzyl acetate relative to benzyl propanoate. The response factor was determined by combining 0.1M acetic acid and 0.1M propanoic acid standard (100 μL each), diluting to 10 mL, and titrating with tetrabutylammonium hydroxide. The solution was evaporated *in vacuo*, and the residue was dissolved in acetone, and derivatized and analyzed as already described. The temperature program was as follows: hold for 5 min at 90°; 90 → 100° at 1.0°/min; and hold for 5 min at 100°.

Smith degradation. — Smith-degradation products, glyceraldehyde, glycerol, and erythritol, were analyzed by first converting them into their respective per-*O*-acetylated aldononitrile or alditol acetate derivatives by using the PAAN procedure already described. The program for the Smith-degradation products was as follows: hold for 5 min at 120°; 120 → 180° at 3°/min; and hold for 5 min at 180°.

Analysis of carbohydrate composition of polysaccharides. — Samples known not to contain hexosamines were hydrolyzed in 2M trifluoroacetic acid (0.5 mL) for 5 h. After the addition of the internal standard (ribose, in most cases), the hydrolyzate was passed through a column (85 × 6 mm) of Dowex-1 X-2 (acetate) resin. The column was washed with water (20 mL), and the effluent and washings were combined, evaporated to dryness, and dried over KOH *in vacuo*. The PAAN derivatives were prepared, and analyzed, as already described.

Samples containing hexosamines were hydrolyzed in 2M trifluoroacetic acid (1 mL) for 16 h. Ribose was added as the internal standard and the sample deaminated as already described. The PAAN derivatives were prepared, and analyzed, as already described.

RESULTS AND DISCUSSION

A chromatogram illustrating the g.l.c. analysis of the PAAN derivatives of a standard mixture of neutral monosaccharides is shown in Fig. 1. Baseline separation was achieved in this case, except for glucose and galactose. Of all the monosaccharide combinations tested, only fucose and ribose were not resolvable when present concurrently. The analyzer time was less than 30 min. The extension of the procedure to include amino sugars was achieved, without affecting the analysis time, by deaminating the amino sugars prior to applying the usual, PAAN-derivatization procedure. This avoided the extended time periods required both for formation of the oximes of the amino sugars and their elution from the column, as the retention times

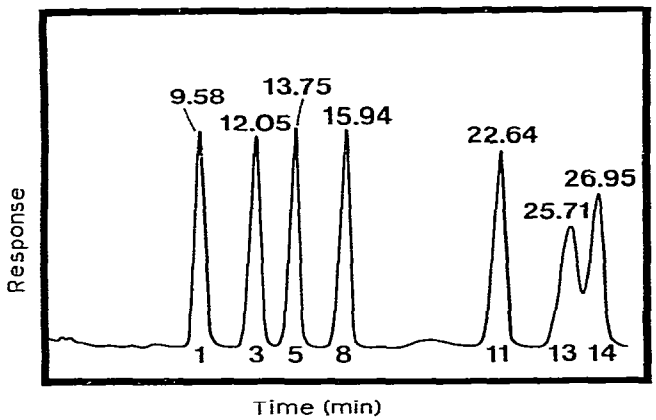

Fig. 1. G.l.c. separation of the PAAN derivatives of a mixture of neutral sugars. [Key: 1 = rhamnose; 3 = ribose; 5 = arabinose; 8 = xylose; 11 = mannose; 13 = glucose; and 14 = galactose.]

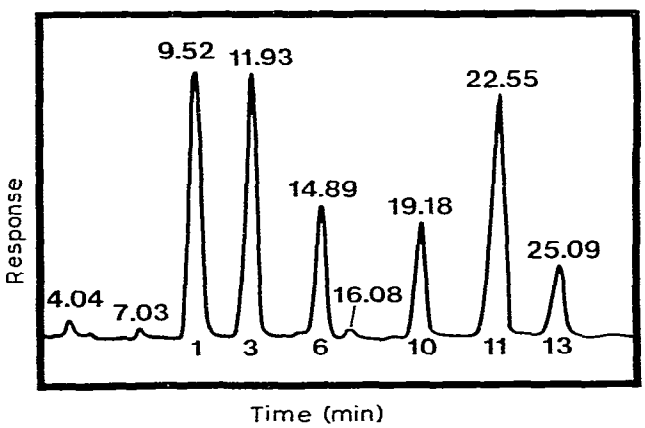

Fig. 2. G.l.c. separation of the PAAN derivatives of a mixture of neutral sugars and deaminated amino sugars. [Key: 1 = rhamnose; 3 = ribose; 6 = 2,5-anhydromannose (2-amino-2-deoxyglucose); 10 = 2,5-Anhydrotalose (2-amino-2-deoxygalactose); 11 = mannose; and 13 = glucose (2-amino-2-deoxymannose).]

of the deamination products resembled those of neutral monosaccharides. Fig. 2 shows the results of applying this technique to a mixture containing both neutral and amino sugars. The products of deamination of 2-amino-2-deoxy-glucose and -galactose formed derivatives having retention times that did not interfere with the detection of the neutral sugars (see Table I). However, ribitol and 2-amino-2-deoxyglucose (as 2,5-anhydromannose) had almost identical retention-times (see Table I). The deamination of 2-amino-2-deoxymannose gives rise to glucose, and, obviously, these two sugars cannot be determined simultaneously. However, by determining the concentration of glucose before and after deamination, both of these constituents can be evaluated when present in the same mixture. The relative retention-times,

TABLE I

RELATIVE RETENTION-TIMES AND RESPONSE FACTORS FOR THE PAAN DERIVATIVES

Carbohydrate		*RRT*	±[a]	*RF*	±[a]
1	Rhamnose	0.80	0.01	1.11	0.05
2	Fucose	0.98	0.02	0.98	0.1
3	Ribose	1.00		1.00	
4	Lyxose	1.08	0.01	0.90	0.1
5	Arabinose	1.15	0.02	1.02	0.04
6	2-Amino-2-deoxyglucose	1.25	0.01	1.9	0.3
7	Ribitol	1.26	0.01	0.62	0.03
8	Xylose	1.34	0.01	0.94	0.1
9	Xylitol	1.54	0.07	0.58	0.03
10	2-Amino-2-deoxygalactose	1.60	0.02	2.4	0.3
11	Mannose	1.88	0.02	0.96	0.06
12	3-*O*-Methylglucose	1.96	0.02	1.1	0.2
13	Glucose	2.14	0.02	0.93	0.08
	2-Amino-2-deoxymannose	2.10	0.02	3.0	0.1
14	Galactose	2.23	0.02	1.06	0.05

[a]Confidence limit, 95%.

and their response factors relative to ribose, for the PAAN derivatives evaluated in this study are shown in Table I. The data presented are for mixtures that had been deaminated prior to derivatization. The retention times and response factors for PAAN derivatives formed from non-deaminated samples were within the experimental errors stated in Table I. It was observed that any two sugar derivatives were resolved, with baseline separation, if the difference in their relative retention-times was 0.1 or greater.

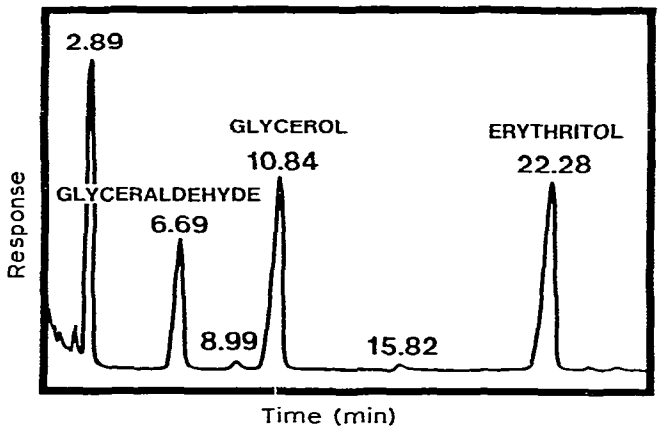

Fig. 3. G.l.c. separation of the PAAN derivatives of the products obtained after Smith degradation. (The response at 2.89 min is a reagent peak.)

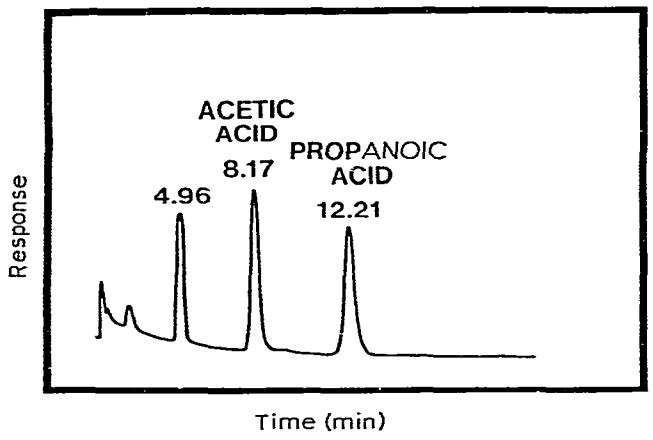

Fig. 4. G.l.c. quantitation of the *O*-acetyl content of a polysaccharide, as benzyl acetate, with benzyl propanoate as the standard. (The peak at 4.96 min is due to benzyl bromide.)

Glyceraldehyde, glycerol, and erythritol (the common, Smith-degradation products) were analyzed by following the PAAN procedure for neutral sugars and simply decreasing the temperature program to accommodate these derivatives of lower molecular weight (see Fig. 3). Acetic acid, resulting from the saponification of a polysaccharide, was determined as its benzyl ester (see Fig. 4). Acetic acid was found to have a response factor of 0.918, relative to propanoic acid as unity.

The procedures described herein have numerous advantages over other methods generally used for carbohydrate analysis. Per(trimethylsilyl)ation results in the formation of as many as four discrete products, which arise from the α- and β-pyranose and α- and β-furanose forms of the aldoses investigated. This prohibits baseline separation of many constituents of a multicomponent system. The (trimethylsilyl)ation reagents are relatively expensive, and are very sensitive to moisture, and the per(trimethylsilyl) derivatives are not very stable; the derivatives and the reagents leave a residue that can affect the response, and the life expectancy, of the flame-ionization detector. The residue has to be periodically removed, and this requires shutdown of the instrument and dismantling of the detectors.

The analytical procedures using alditol acetates eliminate the problem of multiple products, but they do not permit separation of the enantiomeric forms produced by reduction of some pairs of aldoses, *e.g.*, lyxose and arabinose. In addition, certain naturally occurring alditols could not be distinguished from the parent aldoses, *e.g.*, ribitol and ribose. The alditol acetates are stable, except for those of the amino sugars (which are susceptible to thermal degradation upon injection). The quantity of residue in the injector dramatically affects the recovery of the aminohexitol acetates. A chromatogram of a standard mixture of alditol acetates is shown in Fig. 5; this separation required an increase in both the programmed temperature and the time, and the increased temperature resulted in a shortened column-life. The total time for an analysis, that is, for preparation of the derivative, and the g.l.c. separation for the PAAN derivatives (3 h) is much less than for the alditol acetates (2 days).

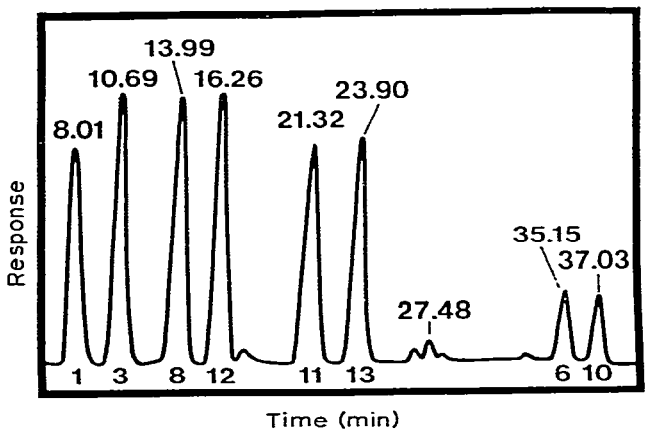

Fig. 5. G.l.c. separation of the alditol acetate derivatives of a standard mixture of sugars. [Key: 1 = rhamnose; 3 = ribose; 8 = xylose; 12 = 2-deoxy-*arabino*-hexose; 11 = mannose; 13 = glucose; 6 = 2-amino-2-deoxyglucose; and 10 = 2-amino-2-deoxygalactose.]

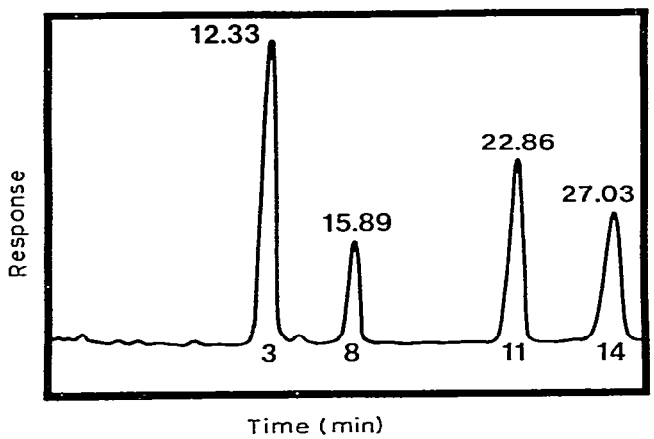

Fig. 6. G.l.c. analysis of a polysaccharide obtained from *Cryptococcus neoformans*. [Key: 3 = ribose (internal standard); 8 = xylose; 11 = mannose; and 14 = galactose.]

The chloroform–water extraction diminishes the extent of the solvent front, improves the baseline, and extends the useful life of the column from 2 to 6 months.

The standard methods were applied to polysaccharides currently under investigation in this laboratory. The g.l.c. analysis of a polysaccharide isolated from *Cryptococcus neoformans* is shown in Fig. 6. The molar composition was determined as xylose:mannose:galactose = 1:2:2. Excellent results were also obtained with a polysaccharide from *Clostridium perfringens* that contained 2-amino-2-deoxy-glucose and -mannose in the ratio of 1:1 (see Fig. 7).

The *O*-acetyl content of the *C. neoformans* polysaccharide was determined to be 5.4% (see Fig. 4).

The procedures described herein are inappropriate for the direct determination of uronic acids. However, reduction of the polysaccharide prior to hydrolysis results

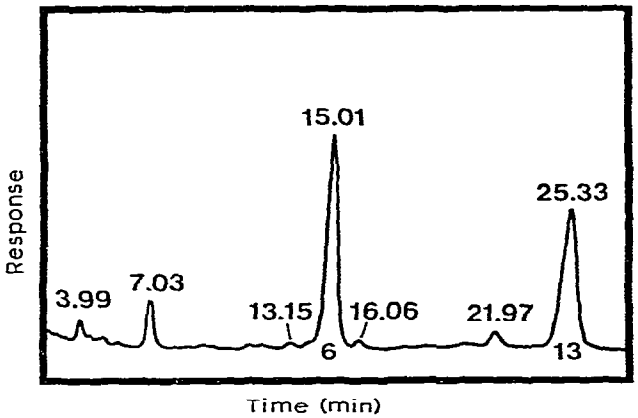

Fig. 7. G.l.c. analysis of a polysaccharide obtained from *Clostridium perfringens* type A. [Key: 6 = 2,5-anhydromannose (2-amino-2-deoxyglucose); and 13 = glucose (2-amino-2-deoxymannose).]

in the formation of the aldose corresponding to the parent uronic acid, which may then be determined by the PAAN procedure.

The procedures described offer the possibility of making multiple analyses (*e.g.*, for PAAN, alditol acetates, benzyl acetate, and Smith-degradation products) with the following advantages: (*1*) rapid and simple derivatization; (*2*) common, inexpensive reagents; (*3*) a single derivative for each sugar; (*4*) stable derivatives; (*5*) short analysis time; (*6*) good resolution; (*7*) minimal instrument maintenance; and (*8*) analysis of a wide range of carbohydrate varieties.

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