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Note

Complete separation and quantification of neutral sugars from plant cell walls and mucilages by high-performance liquid chromatography

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The analysis of complex sugar mixtures is of considerable importance. Sugars have been analysed by paper, thin-layer, gel filtration, ion exchange and gas–liquid chromatography (GLC) and by enzymic analysis. Each of these methods has certain advantages for specific problems. Some of them, however, suffer from difficulties in qualitative and quantitative determinations of complex sugar mixtures, some are time consuming and may require derivatization of the sugars prior to separation and others are unsuitable for trace analysis.

High-performance liquid chromatography (HPLC) offers the possibility of a rapid separation and quantification of underivatized mono- and oligosaccharides[1,2]. Mainly two methods have been employed for the separation of sugars by HPLC: cation-exchange chromatography with water as eluent[1] and partition chromatography on amino bonded phases with acetonitrile–water as eluent[1,2]. Both methods, however, are unsatisfactory for the separtion of complex neutral monosaccharide mixtures, which may result from the hydrolysis of either plant pectins, hemicelluloses, gums or mucilages.

In this work a mixture consisting of L-rhamnose, L-arabinose, D-xylose, D-mannose, D-galactose and D-glucose was baseline separated by HPLC on an amino bonded phase and on a cation-exchange resin. The practical use of the method was demonstrated by the analysis of sugar mixtures obtained after hydrolysis of tobacco cell walls and lime flower mucilage. The reliability of the results was examined by comparison with the GLC data for corresponding mixtures of peracetylated carbohydrates[3].

EXPERIMENTAL

High-performance liquid chromatography

The separation of monosaccharide mixtures was performed using a Gynkotek 600/200 pump with a Melz LCD 201 refractometer for monitoring the effluent. The RI detector was maintained at 30 ± 0.01°C by a Lauda NB-S15/12 ultrathermostat with a Lauda PTR R20/2 temperature controller. Peak areas were calculated with a Shimadzu Chromatopac C-R1 A data processor.

Sugar mixtures containing 10–500 μg each of L-rhamnose, L-arabinose, D-xylose, D-mannose, D-galactose and D-glucose in 20 μl of water were partially separated in a first run on an amino bonded phase. Chromatography was carried out

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with a 250 × 4.6 mm I.D. stainless-steel column packed with APS-Hypersil, 5 μm (Shandon, London, Great-Britain), and a 10 × 4.6 mm I.D. guard column packed with the same material. Acetonitrile–water (75:25; v/v) was used as the eluent at a flow-rate of 1 ml/min (*ca.* 12 bar). On this column a separation into rhamnose and two incompletely separated fractions containing xylose–arabinose and mannose–glucose–galactose could be achieved.

The two semi-separated fractions were collected, evaporated to dryness under reduced pressure and the residues dissolved in water. The individual sugars in each fraction were completely separated on a 300 × 7.8 mm I.D. stainless-steel column packed with the cation-exchange resin Aminex HPX-87P, Heavy Metal, 9 μm (Bio-Rad Labs., Richmond, CA, U.S.A.). A 20 × 7.8 mm I.D. guard column packed with Aminex Q 150S, 27 μm (Bio-Rad Labs.) was used. The column was maintained at 80 ± 0.1°C with a Lauda Compact-Thermostat S1. Distilled, degassed (80°C) water was used as the eluent at a flow-rate of 0.6 ml/min (*ca.* 10 bar).

Analysis of cell wall material

Cell wall material was isolated from suspension cultured cells of *Nicotiana tabacum* L.cv. *xanthi* according to methods described previously[4]. The cell wall material was hydrolysed with 2 *M* trifluoroacetic acid (TFA) (1 h, 120°C), releasing sugars from non-cellulosic cell wall polysaccharides only[4]. The insoluble residue was removed by filtration through a glass-fibre filter. The filtrate was evaporated to dryness under reduced pressure, and the residue was freeze-dried, dissolved in water and passed through a 100 × 0.6 mm I.D. column packed with the mixed-bed ion-exchange resin MB-2 (20–50 mesh) (Serva, Heidelberg, G.F.R.) with water as the eluent. The eluate was concentrated under reduced pressure and directly subjected to HPLC analysis or derivatized for GLC.

Analysis of lime flowers mucilage

Dried and powdered lime flower (*Tiliae flos, pulvis*, DAB 8; Müggenburg, Hamburg, G.F.R.) were pre-treated with 96% ethanol in a Soxhlet apparatus (1 h). After extraction with 20 volumes of water (40°C, 48 h) the polysaccharides were precipitated with 4 volumes of ethanol (4°C), redissolved in water, dialysed and lyophylized to yield the crude lime flower mucilage. The conditions of hydrolysis and preparation for HPLC or GLC analysis were the same as for the tobacco cell wall material.

Gas–liquid chromatography

Reduction and acetylation of the sugars were performed according to methods described previously[3,4]. Separation was achieved on a 20 m × 0.25 mm I.D. WCOT glass capillary column coated with OV-225 (Chrompack) in a Varian 3700 gas chromatograph. The temperature was programmed linearily from 170 to 210°C at a rate of 2°C/min, and nitrogen was used as the carrier gas (0.7 bar). Peak areas were calculated with a Varian CDS-111 data processor.

RESULTS AND DISCUSSION

The chromatogram in Fig. 1 shows the separation of a mixture of 400 μg each

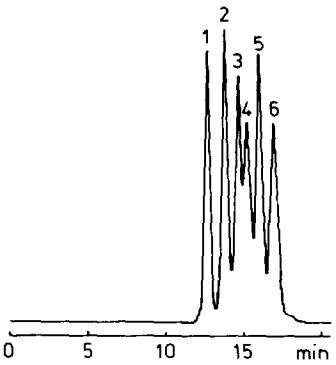

Fig. 1. Chromatography of 20 μl of a sugar mixture containing 400 μg each of (1) glucose, (2) xylose, (3) galactose, (4) rhamnose, (5) arabinose and (6) mannose on Aminex HPX-87P with water as eluent.

of rhamnose, arabinose, xylose, mannose, galactose and glucose on Aminex HPX-87P. The retention times of the individual sugars are presented in Table I. No satisfactory baseline separation of equal amounts of all of these sugars, which represent the main neutral constituents of most plant cell wall or mucilage polysaccharides, can be achieved on this column.

When the same sugar mixture was chromatographed on a Hypersil column, again the six sugars could not be completely separated (Fig. 2A). The retention times of the individual sugars are listed in Table I. On this column, however, a baseline separation of rhamnose, one fraction containing xylose and arabinose and a second fraction containing mannose, glucose and galactose can be obtained. By decreasing the amount of water in the eluent, separation of arabinose and xylose is possible (results not shown here). Chemically bonded amine columns, however, tend to deteriorate with prolonged use, possibly owing to losses of amine functions by formation of Schiff bases[5]. However, also with poorly working Hypersil columns a separation can be obrained as shown in Fig. 2A. The carbohydrates in the two sugar fractions can now be fully separated by two subsequent runs on the cation-exchange resin (Fig. 2B and C). By using this two-step procedure a baseline separation of the six

TABLE I

RETENTION TIMES OF INDIVIDUAL SUGARS ON AN AMINO-BONDED PHASE (HYPERSIL) OR CATION-EXCHANGE RESIN (AMINEX)

Hypersil column		*Aminex column*	
Sugar	*Retention time (min)*	*Sugar*	*Retention time (min)*
Rhamnose	4.42	Glucose	12.68
Xylose	5.04	Xylose	13.64
Arabinose	5.36	Galactose	14.47
Mannose	6.45	Rhamnose	15.06
Glucose	6.91	Arabinose	15.89
Galactose	7.06	Mannose	16.74

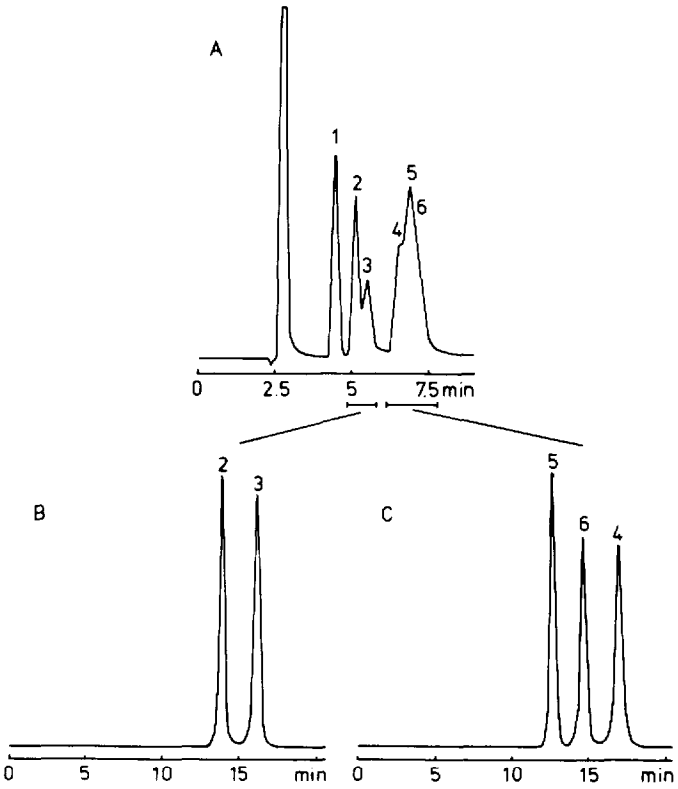

Fig. 2. Chromatography of 20 μl of a sugar mixture containing 400 μg each of (1) rhamnose, (2) xylose, (3) arabinose, (4) mannose, (5) glucose and (6) galactose on APS-Hypersil with acetonitrile–water (75:25) as eluent (A). The peak fractions containing xylose–arabinose and mannose–glucose–galactose were collected and chromatographed on Aminex HPX-87P with water as eluent (B,C).

sugars at concentrations of 10–500 μg could be obtained. In order to obtain exact separations, the Aminex column is normally used at an elevated temperature (80°C). Its capacity to separate arabinose from xylose and mannose from galactose and glucose, however, is so good that it can even be run for this purpose at room temperature (results not shown here). In this instance the retention times of the individual sugars increase and the peaks broaden, but are still well separated.

The method described makes it possible to achieve a complete separation of neutral sugar mixtures as they usually occur in plant cell walls or mucilages, even under non-optimal conditions (*e.g.*, poor columns after prolonged use). This could be demonstrated in a first example by the analysis of cell wall material from suspension cultured tobacco cells. The composition of the neutral non-cellulosic sugars obtained by TFA hydrolysis of these cell walls was analysed and quantified by HPLC. The results were compared with the GLC results for the peracetylated sugar mixture from the same source.

Fig. 3A shows the first separation step on the Hypersil column and Fig. 3B and C the subsequent separations of the two sugar fractions on the cation-exchange resin. All of the chromatograms were followed by a calibration run with a known amount

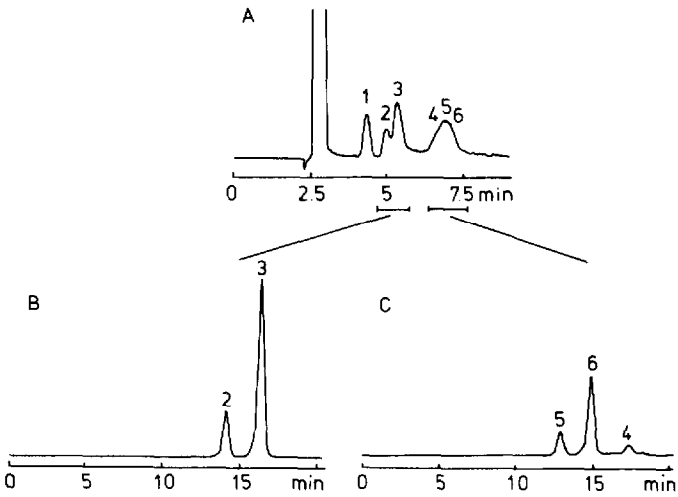

Fig. 3. Chromatography of 20 μl of a cell wall hydrolysate from suspension cultured tobacco cells on APS-Hypersil (A). The peak fractions containing xylose–arabinose and mannose–glucose–galactose were collected and chromatographed on Aminex HPX-87P (B,C). Peaks: 1 = rhamnose; 2 = xylose; 3 = arabinose; 4 = mannose; 5 = glucose; 6 = galactose.

of the corresponding sugars in order to calculate the absolute amounts of sugars. For GLC analysis a known amount of myo-inositol was added to the sugar mixture as an internal standard before derivatization. The GLC analysis of the cell wall sugar constituents is shown in Fig. 5A. HPLC and GLC yielded comparable results (Table II) for the amounts of individual sugars.

Plant gums and mucilages are an important group of plant constituents with pharmaceutical and technical uses. In contrast to other plant gums and mucilages[6], the sugar composition and structure of lime flower mucilage is still unknown. It was considered as a second example of the practical application of the HPLC method. The neutral sugar constituents of this mucilage were determined by HPLC analysis (Fig. 4) in comparison with GLC analysis (Fig. 5B). Similar results were obtained (Table II). The predomiannce of rhamnose, arabinose and galactose in the neutral

TABLE II

NEUTRAL SUGAR COMPOSITION (MOLE%) OF THE TOTAL NON-CELLULOSIC CELL WALL FRACTION OF SUSPENSION CULTURED TOBACCO CELLS AND OF LIME FLOWER MUCILAGE

Sugar	*Tobacco cell wall*		*Lime flower mucilage*	
	GLC	*HPLC*	*GLC*	*HPLC*
Rhamnose	11.73	9.73	26.7	28.4
Arabinose	46.47	45.73	23.21	25.94
Xylose	12.51	10.35	7.86	9.06
Mannose	2.01	2.82	3.28	2.67
Galactose	21.36	25.36	33.04	28.47
Glucose	5.93	6.04	5.92	5.41

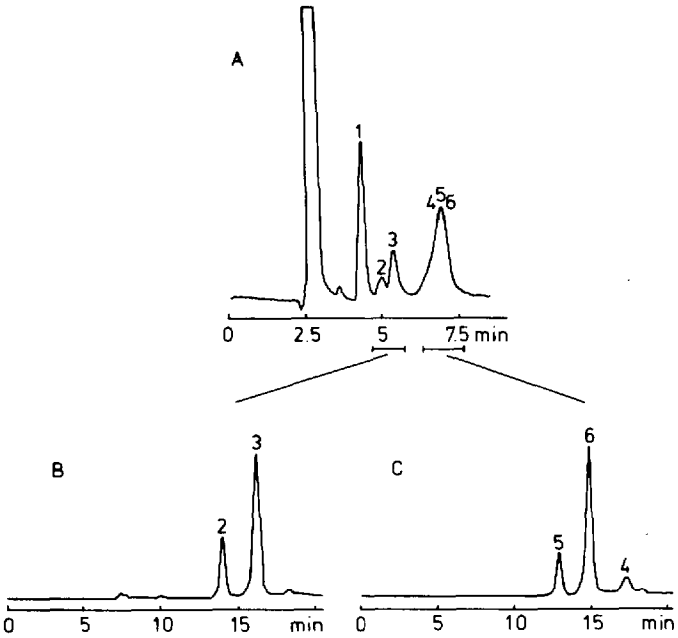

Fig. 4. Chromatography of 20 μl of a mucilage hydrolysate from lime flowers on APS-Hypersil (A). The peak fractions containing xylose–arabinose and mannose–glucose–galactose were collected and chromatographed on Aminex HPX-87P (B, C). Peaks: 1 = rhamnose; 2 = xylose; 3 = arabinose; 4 = mannose; 5 = glucose; 6 = galactose.

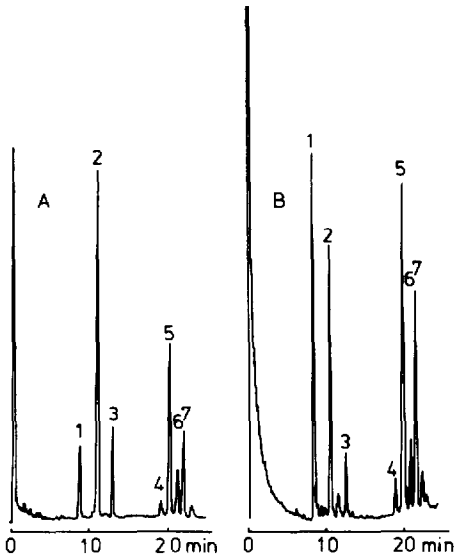

Fig. 5. GLC of a mixture of peracetylated sugars from (A) a cell wall hydrolysate from suspension cultured tobacco cells and (B) a mucilage hydrolysate from lime flowers. Peaks: 1 = rhamnose; 2 = arabinose; 3 = xylose; 4 = mannose; 5 = galactose; 6 = glucose; 7 = myo-inositol (internal standard).

sugar fraction and the high content of uronic acids (results not shown here) suggest that lime flower mucilage may be of the arabino-galactan and galacturono-rhamnan type. Further investigations on the composition and structure of these polysaccharides are in preparation.

The HPLC method seems to be a good completion to the well established GLC methods mainly used so far for the analysis of carbohydrate mixtures as they may occur in biological materials. The method is rapid, as no derivatization of the carbohydrates is necessary and the amount of each sugar can be measured with great accuracy. Further, the method offers advantages in biosynthetic tracer studies, because the individual sugars can easily be collected for isotope measurements.

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