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Note

Improved sugar separation by high-performance liquid chromatography using porous microparticle carbohydrate columns

M. T. YANG*, L. P. MILLIGAN and G. W. MATHISON
Department of Animal Science, University of Alberta, Edmonton, Alberta T6G 2E3 (Canada)
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Analysis of carbohydrates is of growing importance in the field of biology, biomass processing, and food industry. Traditionally, sugars are separated by paper, ion-exchange, thin-layer and gel filtration chromatography with good resolution yet the methods are time-consuming. Although automated analysis of wood polysaccarides by ion-exchange chromatography is available for complete separation of L-arabinose, D-xylose, L-mannose, L-galactose, L-glucose, uronic acids and saccharose[1], the apparatus is expensive and the operational procedures are designed for large numbers of specimens with fast throughput and not for a research laboratory. Gas–liquid chromatography (GLC) provides rapid separation of sugars only after they are derivatized to volatile compounds. Usually polysaccharides are acid hydrolyzed to component monosaccharedes[2], which are then derivatized as trimethylsilyl (TMS) ethers[3]. Component sugars can alternatively be reduced to alditols then acetylated[4]. Alditol acetates of monosaccharides and methylated monosaccharides can be isolated by preparative GLC and identified by melting points, circular dichroism, optical rotatory dispersion, nuclear magnetic resonance and mass spectrometry[5,6], while the TMS derivatives can be characterized by mass spectrometry[7].

Recently, high-performance liquid chromatography (HPLC) using column material made of porous microparticles derivatized with amino groups has been developed which provides for rapid separation of sugars[8–10]. Without derivatization and with minimal sample preparation, simultaneous quantitation of mono- and oligosaccharides was achieved. The separated sugars can be recovered from the outlet for further identification.

This rapid procedure is particularly suitable for quality control of food products[8,11] such as honey, syrup, fruit juices, jams, soft drinks, soya bean extracts and molasses. HPLC is also useful in preparative isolation of oligosaccharides from physiological and other natural sources, using high-water-content isocratic eluent[12] or solvent gradient accompanied by UV detection at 192 nm[13,14]. In study of polysaccharide structures, the use of HPLC has been severely limited by incomplete separation of commonly occurring monosaccharides, notably D-mannose and D-glucose, D-glucose and D-galactose, and less seriously, D-xylose and L-arabinose. The objective of the present study was to improve the resolution of these common monosaccharides. The results show that HPLC possesses resolution similar to that of GLC in the separation of common sugars.

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EXPERIMENTAL

The following instruments were used: Varian (Palo Alto, CA, U.S.A.) Model 5000 liquid chromatograph; Aerograph differential refractive index (RI) detector, sensitivity $\pm 1 \cdot 10^{-7}$ RI units; Varian UV-50 variable-wavelength detector; Spectra-Physics Autolab Minigrators (linear integrators) linked to a Fisher dual recorder. Tap water (15°C) was used to maintain the temperature of the detectors. In a single-column separation, a Varian MicroPak NH_2-10 bonded-phase column 30 cm × 4 mm I.D., was used, which was preceded with a guard column of (4 cm × 4 mm I.D.)

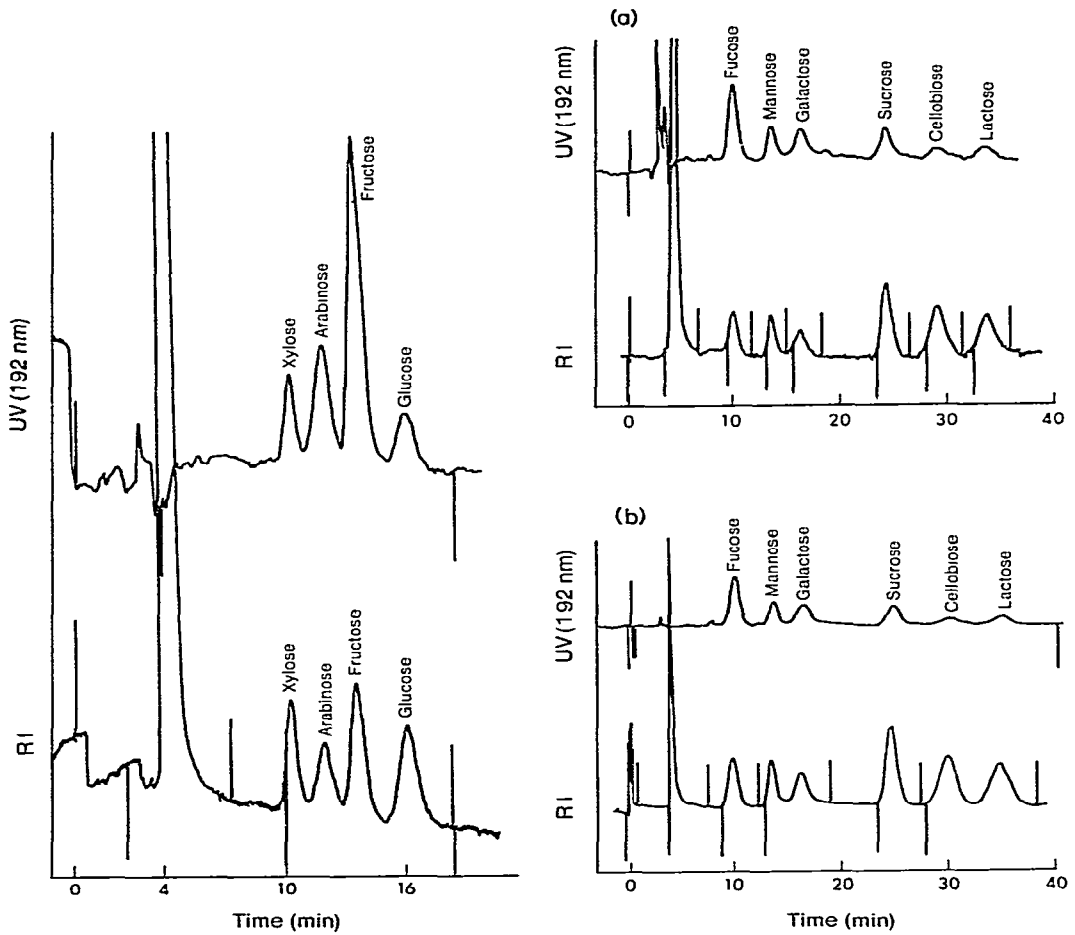


Fig. 1. Separation of D-xylose, L-arabinose, D-fructose and D-glucose, 18 μg each in a 10-μl solution. The effluent first enters the UV detector and then the RI detector; recording is simultaneous using the dual recorder. Column: MicroPak NH_2-10. Detectors: UV and RI. Composition: water–acetonitrile (20:80). Flow-rate, 0.6 ml/min; instrument, LC 5000.

Fig. 2. (a), Separation of L-fucose, D-mannose, D-galactose, sucrose, cellobioase and lactose. An 18-μg amount each in 10 μl solution. Flow-rate, 0.6 ml/min. (b), Separation of the same sugars. Ten times concentrated.

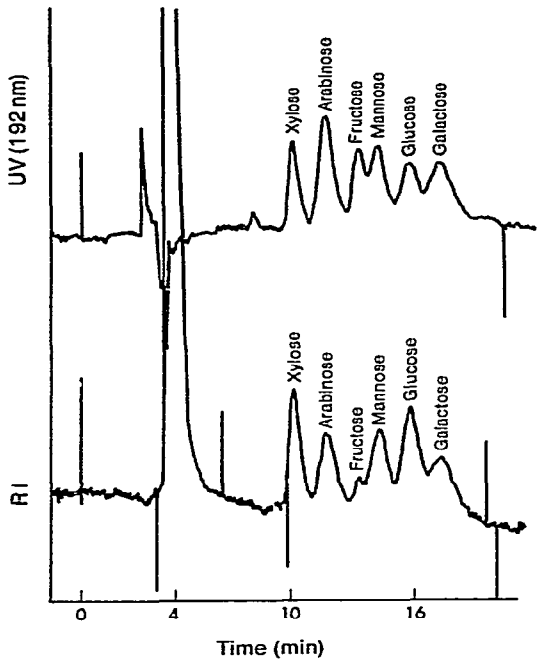


Fig. 3. Separation of D-xylose, L-arabinose, D-fructose, D-mannose, D-glucose and D-galactose. Conditions are identical to Fig. 1, except that the concentration of D-fructose was one-fifth of the other sugars.

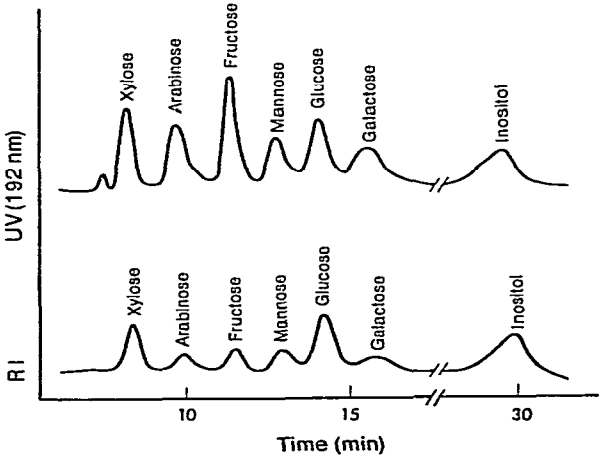


Fig. 4. Double column separation of D-xylose, L-arabinose, D-fructose (one-fourth the concentration of other sugars), D-mannose, D-glucose, D-galactose, and myo-inositol, 180 μg each in a 10-μl solution. Columns: Varian MicroPak NH_2-10 and Waters μBondapak carbohydrate column. Flow-rate, 1.7 ml/min. Other conditions identical to Fig. 1.

packed with Vydac SC polar phase. In double-column separation, a Waters Assoc. (Milford, MA, U.S.A.) μBondapak carbohydrate column of 30 cm × 3.9 mm I.D. was connected to the Varian MicroPak NH_2-10 column.

Operation conditions: premix 20% water in acetonitrile (both LC grade; Fisher Scientific, Pittsburgh, PA, U.S.A.) except for a few instances of 30% water in acetonitrile to increase the polarity of the eluent to shorten the retention time of oligosaccharides. The effluent flowed into the UV detector first then the RI detector. The detectors were connected to separate integrators and responses monitored by a dual recorder. For the single column analysis, the flow-rate was 0.6 ml/min, the sensitivity of the UV detector (192 nm) was set at 0.2 a.u.f.s., with 1-cm pathlength, while the sensitivity of RI detector was $1 \cdot 10^{-7}$ RI units, attenuation 1–32. For double column analysis, the flow-rate was 1.7 ml/min and the attenuation of the RI detector was set at 8.

Myo-inositol was added to forage and silage before hydrolysis as the internal standard for the analysis of hemicelluloses[15]. The component sugars were collected in the LC effluent and identified by paper chromatography and by GLC.

RESULTS AND DISCUSSION

Typical separations of sugars using a single column are shown in Figs. 1–3. The relative retention times with respect to D-glucose and D-xylose of common sugars and alditols and their molar response factors with respect to D-glucose determined by RI and UV at 192 nm are listed in Table I.

The minimal identifiable level of sugars on LC is approximately 4 μg. Accurate quantitation requires 18 μg or more for each sugar.

Achievement of complete separation of D-xylose, L-arabinose and D-fructose and near-baseline separation of D-mannose, D-glucose and D-galactose (Fig. 4) using double column technique provides an opportunity for application of this method to analyse accurately the composition of oligo- and polysaccharides, using a simple premixed eluent composed of water–acetonitrile (20:80).

Rigourous temperature control is necessary for the RI detector as the RI is highly sensitive to temperature fluctuations; the UV absorbancy is much less temperature dependent. However, the solvent (acetonitrile) absorbs strongly at the wavelength (192–195 nm) and the sugars lack UV sensitive chromophores. As a result, the sensitivity of the UV detector is drastically reduced. UV detection was slightly more sensitive than RI for monosaccharides and less sensitive toward disaccharides. D-fructose, L-fucose, L-rhamnose and alditols have distinctly higher UV responses at 192 nm as compared to RI (Table I).

UV detection can only be applied to relatively pure sugar solutions. Interfering substances must be removed from the solution by absorbents which do not retain sugars thus maintaining the true sugar ratio in the specimen. For analytical or preparative separation of a mixture of mono- and oligsaccharides, one can use a RI detector with an eluent of high water content[11] or use UV at 192 nm with a solvent gradient of increasing polarity[13]. RI is not suitable for the solvent gradient.

The authors had difficulty quantitating the composition of an hydrolysate of hemicellulose from a mixed hay using a single column (Fig. 5), although qualitative identification of constituent sugars was excellent. The identical specimen was subject-

TABLE I

RELATIVE RETENTION TIMES (RRT) AND MOLAR RESPONSE FACTORS (BOTH UV AND RI) OF COMMONLY OCCURRING MONOSACCHARIDES, ALDITOLS AND DISACCHARIDES

Actual retention time of D-glucose: (a) 16 min on a single column; flow-rate, 0.6 ml/min; eluent, water–acetonitrile (20:80). (b) 14.5 min on double columns; flow-rate, 1.7 ml/min; same eluent as in (a).

	RRT with respect to		*Molar response factor with respect to* D-*glucose*	
	D-*glucose*	D-*xylose*	*UV at 192 nm*	*RI*
L-Rhamnose	0.53	0.84	2.15	0.71
D-Ribose	0.55	0.87	1.47	0.36
D-Xylose	0.65	1.00	0.94	0.68
L-Fucose	0.66	1.02	3.17	0.88
L-Arabinose	0.73	1.15	1.50	0.53
D-Fructose	0.83	1.31	4.30	0.97
D-Mannose	0.92	1.46	0.76	0.43
D-Glucose	1.00	1.58	1.00	1.00
D-Sorbitol	1.04	1.60	1.97	0.87
D-Mannitol	1.05	1.62	2.69	0.89
Dulcitol	1.07	1.65	2.43	0.96
D-Galactose	1.09	1.73	1.01	0.48
Sucrose	1.64	2.59	1.91	2.10
Myo-inositol	2.01	3.18	0.80	1.17
Cellobiose	2.06	3.26	0.89	1.84
Maltose	2.07	3.27	0.04	0.25
Lactose	2.35	3.63	1.09	1.59

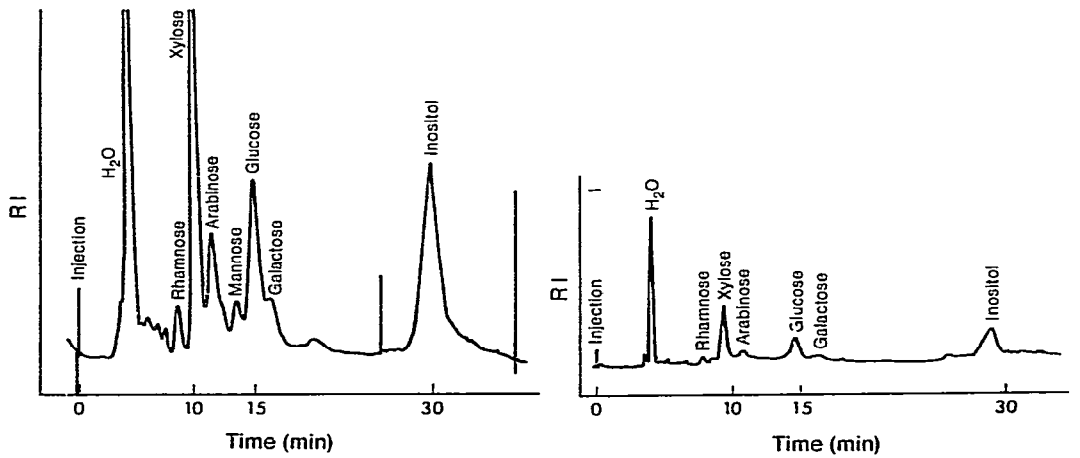
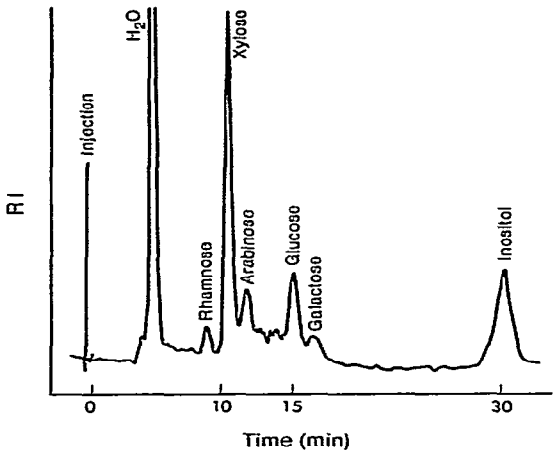


Fig. 5. Single column separation of the hydrolysate of hemicellulose from mixed hay. Column: MicroPak NH_2-10. Detector: RI. Composition, water–acetonitrile (20:80). Flow-rate, 0.6 ml/min.

Fig. 6. Double column separation of the hydrolysate in Fig. 5. Columns: Varian MicroPak NH_2-10 and Waters μBondapak carbohydrate column. Flow-rate: 1.7 ml/min. Same eluent composition as Fig. 5.



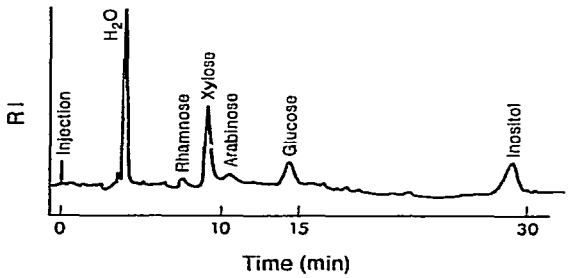


Fig. 7. Single column separation of the hydrolysate of silage hemicellulose prepared from the mixed hay in Fig. 5. Analytical conditions identical to Fig. 5.

Fig. 8. Double column separation of the hydrolysate of silage hemicellulose in Fig. 7. Analytical conditions identical to Fig. 6.

ed to a double column analysis and resulted in a well separated chromatogram with good reproducibility (Fig. 6). However there was a band broadening effect due to longer residence in the columns. There was a ratio of 0.36 for L-arabinose to D-xylose in the hemicellulose hydrolysate, while after ensiling, the ratio dropped to 0.22 (Figs. 7 and 8), indicating the loss of L-arabinose side chain units in the course of ensiling[16]. The data are comparable to the results of GLC analysis based on derivatized alditol acetates[17].

Double-column LC offers rapid quantitation of commonly occurring monosaccharides, thus potentiating HPLC as a powerful tool in structural elucidation of oligo- and polysaccharides. A column 60 cm long would provide approximately the same degree of resolution as two columns, 30 cm each; the increment of resolution is proportional to the square root of the increased length of the column[18]. However, a uniform packing of the porous microparticles into a 60-cm column is very difficult[19].

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