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Note

Separation of monosaccharides by high-performance liquid chromatography: comparison of ultraviolet and refractive index detection

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For the analysis of carbohydrates, various chromatographic methods, such as paper, thin-layer, liquid and gas chromatography, are in use. The various possibilities of liquid chromatography were reviewed by Jandera and Churáček[1]. Through the use of liquid chromatography, good separations can be achieved, but they are very time consuming compared with high-performance liquid chromatography (HPLC). Gas chromatography also permits rapid separations of sugars, but has the disadvantage of requiring derivatization. Quantitation is difficult because of cleavage of the anomers.

Paper and thin-layer chromatography are cheap methods, but the resolution is often poor and the quantitative evaluation is very limited.

Rapid separations of monosaccharides, which are easy to quantitate, are possible by HPLC. Until now, predominantly μBondapak–carbohydrate columns[2–5] (Waters Assoc., Milford, Mass., U.S.A.) and chemically bonded aminopropyl groups[6,7] have been used as stationary phases.

In this paper, the applicability of a chemically bonded amino stationary phase, and a comparison of UV and refractive index (RI) detection in the separation of some monosaccharides, are presented.

EXPERIMENTAL

Equipment

The HPLC system is based on the modular principle, built up with commercially available components. By the means of two piston pumps (Model 110, Altex, Berkeley, Calif., U.S.A.), which were controlled by a solvent programmer (Model 400, Altex), the solvent was delivered over a mixing chamber to the top of the column. Injection was performed by a sample loop valve (Model 70-11, Rheodyne, Berkeley, Calif., U.S.A.) fitted with a 20-μl loop. Stainless-steel columns (No. 103.07, Knauer, Oberursel, G.F.R.) (250 × 4.6 mm I.D.) were used. For monitoring the sugars, a UV detector (Model SF 770, Spectra Physics, Santa Clara, Calif., U.S.A.) and a series-connected RI detector (Model 6100 UV/RI detector, Knauer) were employed. The chromatograms were evaluated by an integrating unit (Minigrator, Spectra Physics).

Materials

Acetonitrile–water mixtures were used as the elution solvent. The acetonitrile (HPLC Grade S) was purchased from Rathburn Chemicals (Walkerburn, Great Britain). Silica gel with chemically bonded amino groups (No. 711120, Macherey, Nagel & Co., Düren, G.F.R.) with a particle diameter of 5 μm was utilized as the packing material. As reference substances, sugars for chromatographic comparisons (No. 8002 and 8005, E. Merck, Darmstadt, G.F.R.) were employed.

Column packing

The column was filled by the slurry-packing technique, using the method of Jones *et al.*[7]. For this purpose, one of the above-mentioned piston pumps was connected over a slurry reservoir to the column, which was terminated with a 2-μm mesh disc. A 2.7-g amount of the packing material was dispersed in the slurry medium (water) by use of an ultrasonic bath, poured into the slurry reservoir and pressed in the column by pumping acetonitrile–water (4:1) through the packing assembly at a flow-rate of 10 ml/min and a pressure of 5000 p.s.i. By this method it was possible to achieve 800 theoretical plates per metre more than by using a common packing procedure.

RESULTS AND DISCUSSION

Suitable wavelength for UV detection

As carbohydrates absorb only at wavelengths lower than 200 nm, very high demands are placed on the purity of the elution solvent, because the acetonitrile itself begins to absorb very strongly in this region. In order to optimize the analysis, it seemed appropriate to investigate the dependence of the UV response of a standard mixture of sugars (fructose, rhamnose, xylose, arabinose and glucose) on wavelength (Fig. 1). For all sugars, the absorption maximum lies between 187 and 188 nm. At a

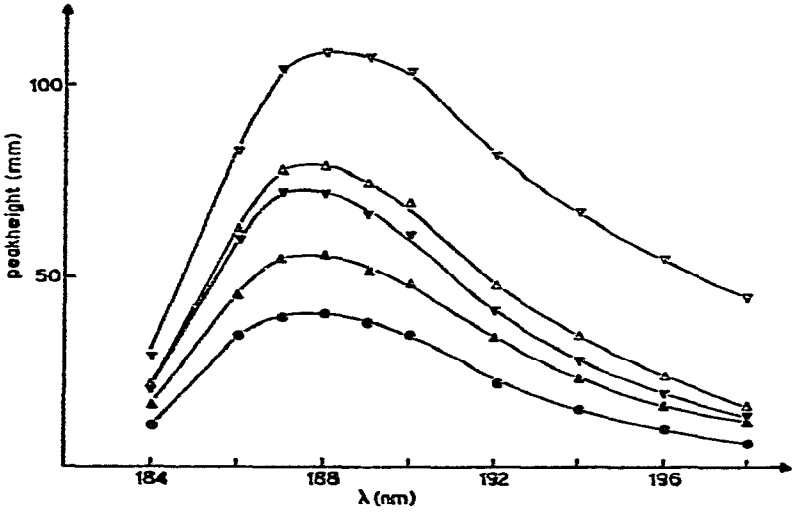

Fig. 1. Dependence of the UV response of fructose (▽), rhamnose (△), xylose (▼), arabinose (▲) and glucose (●) on wavelength. NH_2 column, 250 × 4.6 mm I.D.; solvent, acetonitrile–water (75:25); flow-rate, 2.0 ml/min.

wavelength of less than 187 nm, the influence of the strong absorption of acetonitrile becomes noticeable. When working in this wavelength range, it is advantageous to fill the reference cell of the detector with water or nitrogen in order not to decrease the transmittance too much. When decreasing the wavelength, the noise of the detector signal rapidly increases. Therefore, it is advisable to work at 188 nm or even at higher wavelengths, provided that the concentration of the solution permits this.

Influence of the mixing ratio of acetonitrile and water

The acetonitrile–water composition of the elution solvent was varied from 70:30 to 80:20 at a wavelength of 188 nm. Whereas at a ratio of 70:30 mannose and fructose could not be separated and glucose and galactose showed poor resolution, with higher proportions of acetonitrile, *e.g.*, 75%, baseline separation could be achieved for nearly all of the sugars used (rhamnose, xylose, arabinose, fructose, mannose, glucose and galactose). Only for the pair glucose–galactose was it not possible to obtain a baseline separation, even with high proportions of acetonitrile, but the resolution was high enough to make quantitation practicable. Fig. 2 shows the change in the capacity factor (k') with variations in the acetonitrile-water composition.

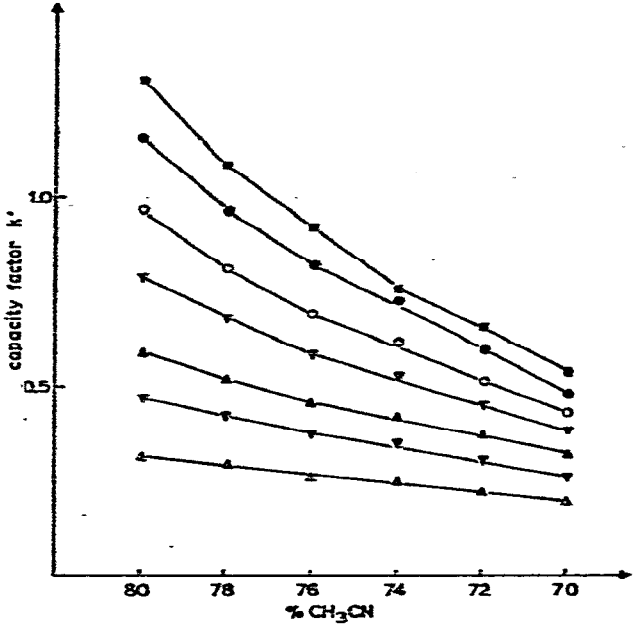

Fig. 2. Change in capacity factor, k', for galactose (■), glucose (●), mannose (○), fructose (▽), arabinose (▲), xylose (▼) and rhamnose (△) with the mixing ratio of acetonitrile and water. NH_2 column, 250 × 4.6 mm I.D.; flow-rate, 2.0 ml/min; UV detection at 188 nm.

With this system it was not possible to separate rhamnose from ribose, and fructose from sorbose. Only by the application of the more apolar ternary mixture ethyl acetate–ethanol–water (40:50:10) could differentiation of these sugars be achieved on the amino phase (Fig. 3).

For mixing the acetonitrile with water it was advantageous to use both pumps, one of which delivered the acetonitrile and the other one the water, because even small

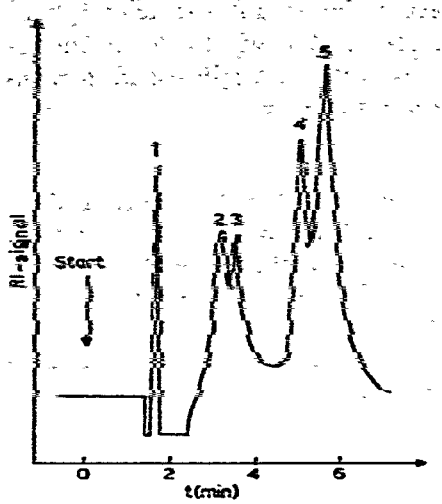

Fig. 3. Separation of rhamnose, ribose, fructose and sorbose using ethyl acetate–ethanol–water (40:50:10) as mobile phase at 2.0 ml/min. NH_2 column, 250 × 4.6 mm I.D. Peaks: 1 = solvent; 2 = rhamnose; 3 = ribose; 4 = sorbose; 5 = fructose.

changes in the mixing ratio influences the chromatographic separation. In this way it is always possible to obtain reproducible mixtures of any desired acetonitrile–water ratio simply by setting the corresponding mixing ratio on the solvent programmer.

Optimization of the flow-rate

The flow-rate was optimized with a 75:25 acetonitrile–water mixture at a wavelength of 188 nm. Fig. 4 shows the change in H/u (where H is the height equivalent to a theoretical plate) with variation in the linear velocity, u. H/u decreases very

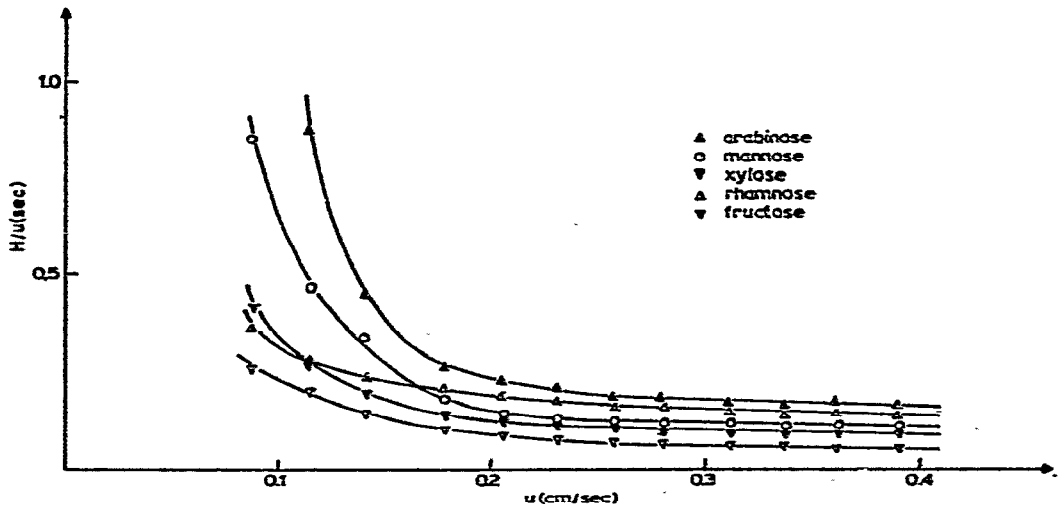

Fig. 4. Graph of H/u *versus* linear velocity, u. NH_2 column, 250 × 4.6 mm I.D.; solvent, acetonitrile–water (75:25); UV detection at 188 nm.

rapidly up to a linear velocity of 0.26 cm/sec, corresponding to a flow-rate of 1.8 ml/min. Therefore, a flow-rate of 2.0 ml/min was chosen for the separation of monosaccharides, because a further increase would only result in a negligible gain in separation time, but would cause a considerable increase in pressure.

Quantitative evaluation and detection limit

Often only very dilute solutions of sugars are available, so the detection limit of this system is therefore of interest. Fig. 5 shows the calibration graphs for fructose, rhamnose, xylose, arabinose and glucose, which were established with reference solutions of different concentrations, using the UV detector at a wavelength of 188 nm. The evaluation was made by the peak-height method. It can be seen that there are linear relationships between peak height and concentration in the investigated region from about 10 to 600 μg of injected sample.

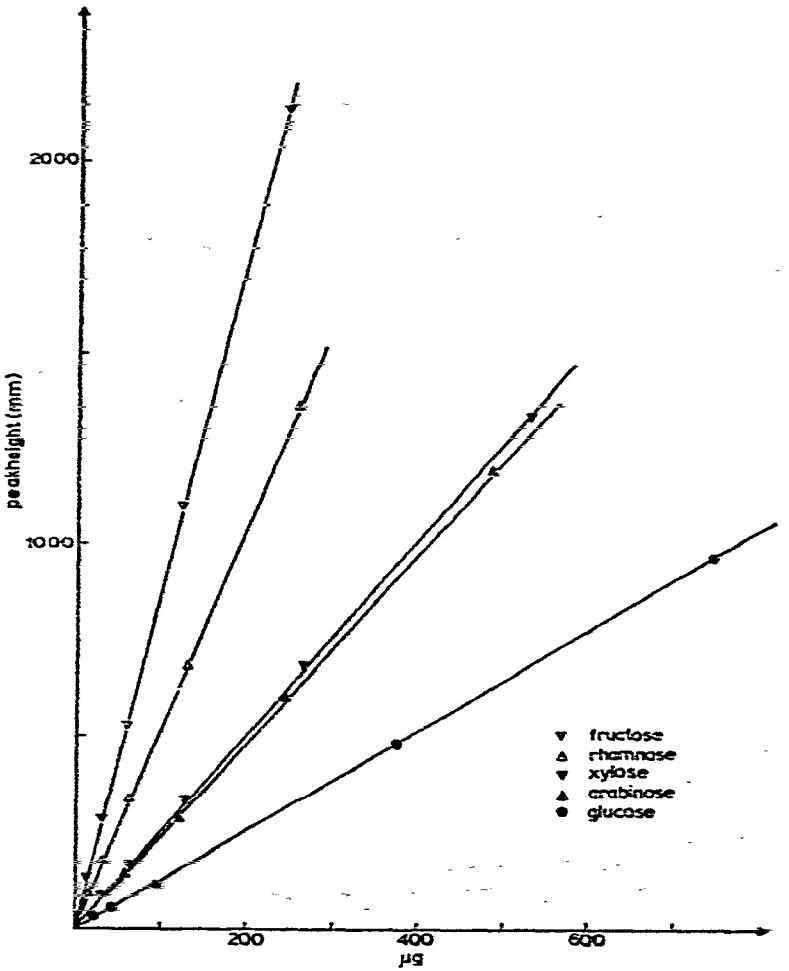

Fig. 5. Calibration graphs constructed by the peak-height method. NH_2 column, 250 × 4.6 mm I.D.; solvent, acetonitrile–water (75:25); flow-rate 2.0 ml/min; UV detection at 188 nm.

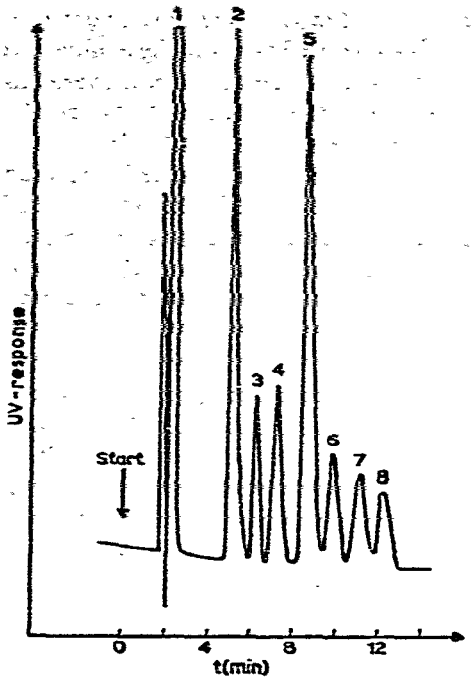

Fig. 6. Separation of sugars. Peaks: 1 = solvent; 2 = rhamnose; 3 = xylose; 4 = arabinose; 5 = fructose; 6 = mannose; 7 = glucose; 8 = galactose. NH_2 column, 250 × 4.6 mm I.D.; solvent acetonitrile–water (75:25); flow-rate, 2.0 ml/min; UV detection at 188 nm.

For the comparison of the sensitivity of the UV detector with that of the RI detector, the peak heights of the refractive index signals were also evaluated. Table I gives the detection limits for the investigated monosaccharides, using the UV detector, and the sensitivity ratio between UV and RI detection.

It can be seen that the detection sensitivity for fructose and arabinose using UV detection is much better than that with RI detection. The sensitivity for xylose and glucose is greater when measuring the refractive index. However, in connection with these figures, it should be stated that the disturbance caused by the pulsation of

TABLE I

DETECTION LIMITS OF MONOSACCHARIDES BY UV DETECTION AND SENSITIVITY RATIO BETWEEN UV AND RI DETECTORS

Sugar	*Detection limit*		*Sensitivity ratio (UV/RI)*
	μg injected	*mg/ml of solution*	
Rhamnose	4	0.2	1.26
Xylose	4	0.2	0.85
Arabinose	8	0.4	1.77
Fructose	2	0.1	2.15
Mannose	12	0.6	1.15
Glucose	12	0.6	0.68

the pumps is usually considerably greater for the RI than the UV detector. Therefore, the use of a UV detector with a variable wavelength is to be preferred for the evaluation of diluted solutions. Further, the measured sensitivity ratios cannot be considered as absolute figures, because various parameters such as the age of the UV lamp, the purity of the solvent and the filling of the reference cell have significant effects.

Fig. 6 shows a chromatogram of the monosaccharides investigated obtained using the optimal conditions of separation, *i.e.*, a flow-rate of 2.0 ml/min, 75:25 acetonitrile–water and a wavelength of 188 nm. It can be seen that pentoses are eluted before the hexoses. Only rhamnose is an exception, because it has the smallest retention time of those monosaccharides investigated, although it is a hexose. Concluding from this, it can be said that the adsorption between the amino groups of the stationary phase and the hydroxy groups of the sugars is derived from hydrogen bonding. As the capacity factor decreases with increasing water content of the elution solvent (Fig. 2), the water is responsible for the elution of the sugars because it is also capable of forming hydrogen bonds. Therefore, one can be explain why rhamnose, which possesses four hydroxy groups as do the pentoses, has a comparable retention time. Fructose, which is eluted between the furanoses and the pyranoses, can be considered as a link between these two groups, as it exists in an aqueous solution because of mutarotation as well as in the furanoside and the pyranoside form.

CONCLUSIONS

For the most commonly occurring monosaccharides such as rhamnose, xylose, arabinose, mannose, glucose and galactose, HPLC is a rapid separation method. Depending on the sugars in the sample, it is possible to reduce the analysis time by increasing the proportion of water in the elution solvent. If, for example, there are only xylose, fructose and glucose present in the sample, only 8 min are necessary in order to obtain a sufficient resolution on the described amino phase, using a flow-rate of 2.0 ml/min and 72:28 acetonitrile–water.

This system seems to be especially suitable for routine analysis, because, except for the separation of solids contained in the solution, no further treatment of the sample is necessary. Compared with the normally used detection by refractive index measurement, UV detection has the advantage of a lower detection limit because of its higher sensitivity and because of the smaller disturbances caused by the pulsation of the pump.

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