

An Automated Procedure for Separation of Monosaccharides on Ion Exchange Resins

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The separation of 16 monosaccharides was studied by partition chromatography on anion exchange resins using an automated procedure. Most separations were quantitative. A sample containing several monosaccharides can be analyzed within a few hours.

In quantitative determinations of the monosaccharides present in wood hydrolyzates partition chromatography on ion exchange resins combined with automatic recording of the eluate concentration has proved to be a more reliable technique than paper chromatography.¹ The aim of this work is to elucidate the possibilities of this technique within other fields of carbohydrate chemistry. The work was started with the monosaccharides known to be present in human gastric mucopolysaccharides² (fucose, mannose, galactose, glucose) but has been extended to a number of other monosaccharides. Among these are the monosaccharides usually present in hydrolyzates of soils (rhamnose, fucose, ribose, arabinose, xylose, mannose, galactose, glucose).

EXPERIMENTAL

All experiments were carried out with strongly basic anion exchange resins in the sulfate form. One of the chromatograms reproduced in this paper is taken from experiments with a commercial resin (Dowex 21 K, 1-16 μ) obtained from Bio-Rad Laboratories, Richmond, Calif. This resin consisted of irregular crushed particles and a very high pressure drop occurred in the column even at very low flow rates. When this column was started, very broad and irregular elution curves were observed but it was found that improved results were obtained after several runs had been carried out. After a couple of months very sharp elution curves were observed with this column (*cf.* Fig. 1).

All other results reported in this paper were obtained with a spherical macroporous resin specially prepared for our purposes (exchange capacity 3.7 mequiv./g chloride resin, particle size 10-35 μ). Even with this resin certain improvements of the elution curves were observed after the column had been used for some time, indicating that the packing of the resin is an important factor.

Jacketed columns were used. These were kept at 75° by means of water circulated from a thermostat. The eluant was fed onto the column by a piston-type pump (*cf.* Ref. 3)

and preheated to 75° before entering the column. The eluate was analyzed automatically by means of a Technicon AutoAnalyzer using the orcinol method described in a previous paper.¹

DISTRIBUTION COEFFICIENTS OF VARIOUS MONOSACCHARIDES

In order to establish the possibilities to separate various monosaccharides from each other the positions of the elution peaks were determined at various ethanol concentrations. From the positions of the elution peaks the distribution coefficients (D_v) were calculated as usual.³ Small variations in the peak elution volumes were obtained in some duplicate runs, which can be ascribed to slight fluctuations in flow rate, eluant concentration, and elution temperature. The ratios between the distribution coefficients in various runs were, however, unchanged. In order to compensate for such variations, xylose was included as a standard in each run and the separation factor relative to xylose calculated for each individual sugar. As usual³ the separation factor is defined as the ratio of the distribution coefficient of a given sugar to that of xylose both determined at the same eluant concentration.

Table 1. Distribution factors of xylose at various ethanol concentrations.

86 %	88 %	89 %	90 %	92 %
7.38	9.43	10.68	12.09	18.19

Table 2. Separation factors relative to xylose at various ethanol concentrations (% by weight).

	86 %	88 %	89 %	90 %	92 %
D-Digitoxose	0.10 (0.07)	0.09	0.08	0.07	0.05
2-Deoxy-D-ribose	0.17 (0.13)	0.16	—	0.13	0.11
2-Deoxy-D-galactose	— (0.28)	0.28	0.28	0.27	0.24
2-Deoxy-D-glucose	0.38 —	0.36	0.35	0.34	0.32
L-Rhamnose	0.38 (0.35)	0.36	0.35	0.34	0.32
L-Fucose	0.44 (0.40)	0.43	—	0.41	0.39
D-Ribose	0.55 (0.51)	0.50	0.48	0.48	0.46
D-Lyxose	0.76 (0.70)	0.74	0.73	0.72	0.70
D-Arabinose	0.85 (0.79)	0.83	0.82	0.81	0.80
D-Xylose	1.00 (1.00)	1.00	1.00	1.00	1.00
D-Fructose	1.09 —	1.10	1.11	1.14	1.19
D-Tagatose	1.09 —	1.10	1.11	1.14	1.19
L-Sorbose	1.21 —	1.24	1.25	1.29	1.34
D-Mannose	1.27 (1.30)	1.34	1.39	1.40	1.46
D-Galactose	1.76 (1.85)	1.92	1.97	2.02	2.16
D-Glucose	2.02 (2.21)	2.29	2.41	2.44	2.71

Values within parentheses refer to Dowex 21 K.

The distribution coefficients of xylose at various ethanol concentrations are given in Table 1. The results confirm the earlier observation that the distribution coefficients are extremely sensitive towards changes in eluant concentration, and increase with an increased ethanol concentration.⁴

The separation factors relative to xylose for 15 monosaccharides at various ethanol concentrations are given in Table 2. Using the values given in the two tables the peak elution volume of all sugars can be easily calculated at any column dimension.³ From Table 2 it is seen that within the range of interest in chromatographic work, the order of elution of all monosaccharides is independent of the ethanol concentration. Similarly, the order of elution is the same with both resins. The selectivity is, however, somewhat better with Dowex 21 K than with the other resin, which is explained by a higher exchange capacity which, as shown previously, results in an increased distribution coefficient.⁵ In 86 % ethanol the distribution coefficient of xylose on Dowex 21 K was equal to 14.05 which is almost twice as high as the value observed with the other resin. Hence, these results confirm our earlier observation that an enhanced selectivity is obtained with resins of high exchange capacity.⁴

As a first approximation the sorption of the sugars is explained by the higher solubility in the water-rich resin phase than in the less polar external solution.⁶ The distribution coefficient should, therefore, be expected to (a) increase with an increased number of hydroxyl groups in the sugar and (b) decrease when non-polar groups such as methyl groups are present.

In agreement with the first rule, pentoses exhibit lower distribution coefficients than hexoses. It can also be mentioned that the tetrose, erythrose, is held less firmly than the pentoses. The commercial sample of erythrose used in this investigation was found to contain some impurity and in addition it was found that this sugar was not quite stable during the chromatographic run (75°). The values obtained with erythrose have, therefore, not been included in the table.

In agreement with the second rule the deoxysugars derived from hexoses, 2-deoxy-galactose, 2-deoxy-glucose, rhamnose, and fucose exhibit distribution coefficients which are lower than those of the pentoses. Similarly 2-deoxy-ribose appears ahead of erythrose. As expected from these rules digitoxose (2,6-dideoxy-ribo-hexose) shows the lowest distribution coefficient of all sugars investigated. Fructose is known to be more soluble in ethanol than glucose and it can be anticipated that fructose and other ketohexoses should appear ahead of the aldohexoses. This is confirmed by the results given in Table 2.

The order of elution of the sugars within each group, *e.g.* of the pentoses, cannot be predicted from these simple rules. In an earlier paper⁶ it was shown that an additional factor which affects the equilibrium uptake of sugars is the interaction between the ions in the resin phase and the sugar. This has been confirmed in a recent work in which a sulfonic acid resin in its potassium form was used instead of an anion exchange resin. With this resin the order of elution of arabinose and xylose was found to be reversed.⁷ More experimental results seem to be required if it should be possible to derive any rules about the positions of the individual sugars within each group.

SEPARATION OF VARIOUS MONOSACCHARIDES

From Table 2 it is seen that the separation factors of most sugars differ significantly from each other and that the selectivity increases with an increased ethanol concentration. As shown previously⁴ a satisfactory separation of glucose and galactose can be achieved in 88 % ethanol although an improvement is observed at higher ethanol concentration. At 88 % ethanol the separation factor is equal to $2.29/1.92=1.19$. With most of the monosaccharides investigated in this work the separation factor is higher, and it can be concluded that most of them can be separated quite easily. An inspection of Table 2 shows, however, that there are some exceptions. Hence, 2-deoxy-glucose and rhamnose exhibit an almost identical behavior and cannot be separated from each other under the conditions used in this work. The same holds true for fructose and tagatose.

The other monosaccharides have been separated completely or, in less favorable systems, sufficiently well for a fairly accurate quantitative determination. Working at a comparatively low ethanol concentration has the advantage that all sugars appear in the eluate within a moderate eluate volume but on the other hand the disadvantage that the separation factors are lower than at high concentration.

A chromatogram from a run with Dowex 21 K in 86 % ethanol is reproduced in Fig. 1. It is seen that despite the low ethanol concentration the

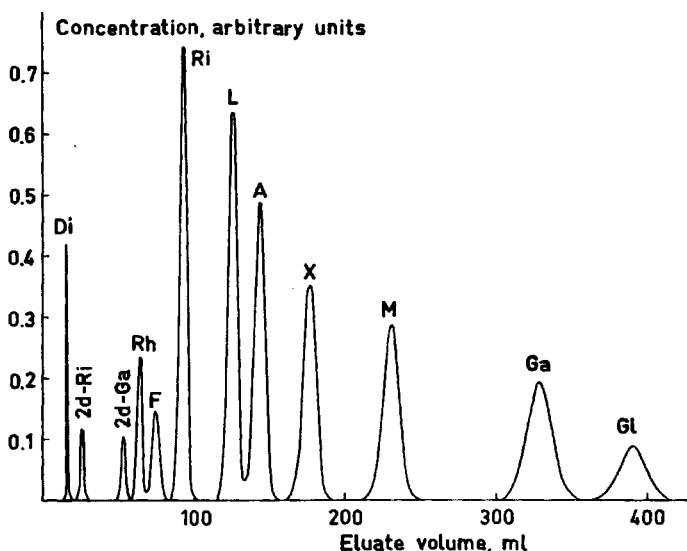


Fig. 1. Separation of various monosaccharides in 86 % ethanol. Resin bed 6×440 mm. Dowex 21 K, $1-16 \mu$. Flow rate $0.9 \text{ ml cm}^{-2} \text{ min}^{-1}$.

Di = Digitoxose, 0.05 mg; 2d-Ri = 2-Deoxy-ribose, 0.10 mg; 2d-Ga = 2-Deoxy-galactose, 0.05 mg; Rh = Rhamnose, 0.05 mg; F = Fucose, 0.05 mg; Ri = Ribose, 0.10 mg; L = Lyxose, 0.10 mg; A = Arabinose, 0.10 mg; X = Xylose, 0.10 mg; M = Mannose, 0.20 mg; Ga = Galactose, 0.30 mg; Gl = Glucose, 0.30 mg.

twelve sugars included in this run can be separated well enough for a quantitative determination of each sugar. The slight overlapping observed between the curves corresponding to rhamnose and fucose (separation factor: 1.14) and between lyxose and arabinose (separation factor: 1.13) does not interfere but can of course be eliminated by using an increased ethanol concentration or an increased column length which, however, results in a greater eluate volume.

Excepting the two pairs of sugars which could not be distinguished from each other the smallest separation factors are those of fructose:xylose (1.11) and mannose:sorbose (1.11). Similarly the separation factor sorbose:fructose (1.13) is unfavorable. The values refer to 89 % ethanol which is the concentration chosen in the chromatogram reproduced in Fig. 2. It is seen that a clear cut separation was achieved with the other sugars included in this run whereas an overlapping of the curves representing these sugars was obtained. The separation is good enough to permit an accurate determination of xylose and fructose in the complicated mixture whereas the determination of sorbose and mannose is less accurate. If a calibration run is made with about the same amounts as present in an unknown sample the error in the determination of the two last-mentioned sugars should not exceed 10 %.

EVALUATION OF CHROMATOGRAMS FOR QUANTITATIVE PURPOSES

In most work reported in this paper, part of the eluate as well the reagents were fed into the analyzing system by means of a peristaltic pump (proportioning pump). This technique has two great advantages. Firstly, part of the eluate can be saved for identification purposes. Secondly, the conditions

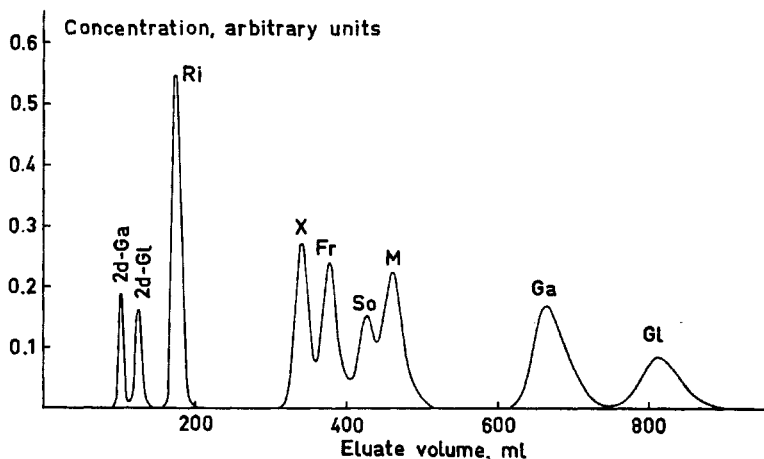


Fig. 2. Separation of various monosaccharides in 89 % ethanol. Resin bed 6×760 mm. Flow rate $1.8 \text{ ml cm}^{-2} \text{ min}^{-1}$. 2d-Ga = 2-Deoxy-galactose, 0.08 mg; 2d-Gl = 2-Deoxy-glucose, 0.08 mg; Ri = Ribose, 0.08 mg; X = Xylose, 0.08 mg; Fr = Fructose, 0.30 mg; So = Sorbose, 0.30 mg; M = Mannose, 0.20 mg; Ga = Galactose, 0.23 mg; Gl = Glucose, 0.23 mg.

Table 3. Comparison between the areas of the elution curves in two runs (I and II).

	Area of elution curve		Area relative to that of ribose		Deviation from mean %
	I	II	I	II	
Ribose (0.10 mg)	13.82	15.00	—	—	—
Fucose (0.10 mg)	6.19	6.53	0.448	0.435	1.5
Mannose (0.20 mg)	11.76	12.55	0.851	0.837	0.8
Galactose (0.30 mg)	14.22	14.91	1.029	0.994	1.7
Glucose (0.30 mg)	6.45	6.89	0.467	0.459	0.9

in the analyzer are not affected when such variables as column diameter and flow rate are altered. One drawback is that the tubings used for feeding the eluate are exhausted very rapidly, which can interfere with the evaluation of the chromatograms for quantitative purposes.

To avoid this difficulty the whole eluate pumped through the column was, in the runs described in this paragraph, mixed with the reagents after the peristaltic pump.

The reproducibility of the method is demonstrated by the results (Table 3) from two experiments (I and II) made under identical conditions. A comparison between the first two columns shows that the areas of the elution curves corresponding to all monosaccharides are larger (5–9 %) in run II than in run I. This is explained by variations in the analyzing system (rate of feed of reagent solutions and other undesired variations). Columns 3 and 4 in the same table show the values recalculated using ribose as an internal standard. It is seen that in this method of calculation the variations are largely compensated for. The deviations from the mean amount to 0.8–1.7 %.

Similar calculations have been carried out in other experiments. In an experiment with the same sugars, mannose was chosen as internal standard. In two runs the area corresponding to the internal standard differed by a factor of 1.162. The maximum deviation from the mean, observed with ribose, was 0.9 %, whereas glucose exhibited a deviation of 0.1 %. These results show that an excellent compensation for variations in the analyzing system can, in favorable systems, be attained by using a suitable internal standard.

With certain mixtures of monosaccharides less favorable results were observed. In some experiments with the same hexoses but with arabinose instead of ribose it was found that the variations in the areas of the arabinose curves differed more than the areas of the other sugars. In an unfavorable series of experiments the areas of the mannose curves differed by a factor of 1.107. This variation can be considered as normal. Using mannose as internal standard the following deviations were observed with the other monosaccharides: fucose 0.6 %; arabinose 5.0 %; galactose 1.4 %, and glucose 0.6 %. A possible explanation is that variations in the relative amount of acid fed into the analyzing system occurred and that these variations have a different effect upon various monosaccharides. The choice of internal standard

or internal standards is, therefore, of great importance. No doubt further improvements are possible as far as the quantitative analysis of the eluates is concerned. Such possibilities are being studied at present.

INFLUENCE OF THE FLOW RATE UPON THE SEPARATION

No systematic study of the influence of the flow rate upon the chromatographic separation of monosaccharides has been reported earlier, the chief reason being that certain mechanical troubles, *e.g.* leakage of the pump used for feeding the eluant, occurred when attempts were made to run the elution at high speed. These difficulties have now been overcome.

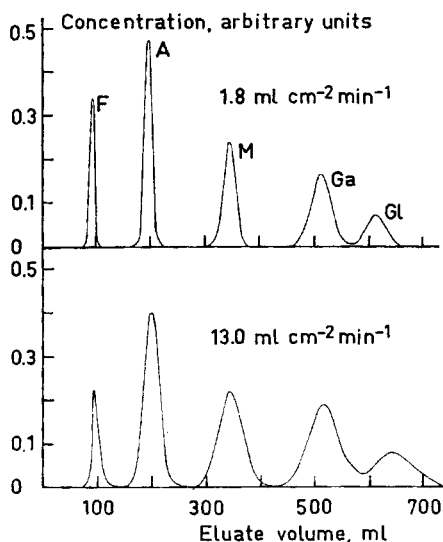


Fig. 3. Influence of the flow rate. Resin bed 6×430 mm. Ethanol concentration 92 %.

F = Fucose, 0.10 mg (0.25); A = Arabinose, 0.10 mg (0.25); M = Mannose, 0.20 mg (0.50); Ga = Galactose, 0.30 mg (0.75); Gl = Glucose, 0.30 mg (0.75).

Larger quantities were added in the run represented by the lower chromatogram. The amounts added in this run are given within parantheses.

Some typical results obtained with a sample containing fucose, arabinose, mannose, galactose, and glucose are reproduced in Fig. 3. The upper chromatogram shows that at low flow rate ($1.8 \text{ ml cm}^{-2} \text{ min}^{-1}$) there is under the working conditions chosen, a complete separation of the four first-mentioned sugars and a slight overlapping of the curves corresponding to galactose and glucose. An increased flow rate resulted in a successively increased overlapping of these two elution curves but as can be seen from the lower chromatogram fucose, arabinose, mannose are completely separated from each other even at a flow rate of $13.0 \text{ ml cm}^{-2} \text{ min}^{-1}$. The separation of galactose and glucose is still good enough to permit a quantitative determination of these sugars by evaluating the chromatograms according to the conventional method used in amino acid analysis.⁸ In the run represented by the upper chromatogram the time of elution of the last sugar was 21 h whereas the corresponding time at the increased flow rate was 3.5 h. At the higher flow rate the pressure drop in the column was about 60 atmospheres.

A prerequisite for successful separations at high flow rate is that the eluant be preheated to the elution temperature before it enters the resin bed. For this purpose a jacketed tube with water circulated from a thermostat was inserted before the column.

A few runs have been carried out at even higher flow rates. Successful separations were still observed. Due to the high pressure required to pump the solution through the column mechanical difficulties arose and the results were not reproducible. A better fractionation of the resin particles would no doubt permit reliable results even at higher flow rate. The results given here show that even with non-uniform resin particles reliable chromatograms permitting quantitative determinations of monosaccharides in complicated mixtures can be obtained within reasonable time.

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