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THE SEPARATION AND QUANTITATION OF CARBOHYDRATES ON CATION-EXCHANGE RESIN COLUMNS HAVING ORGANIC COUNTERIONS

J. S. HOBBS AND J. G. LAWRENCE
Unilever Research, Colworth/Welwyn, Sharnbrook, Bedford (Great Britain)

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SUMMARY

The influence of organic base counterions and their degree of substitution on the separation of carbohydrates on strongly acidic cation-exchange resins with ethanol–water as mobile phase is described. Contrary to previous findings with inorganic counterions, increasing counterion size reduced the capacity ratios for the carbohydrates. The trimethylammonium form of the resin was found to give the best separations and the optimization of these separations with respect to mobile phase composition and column temperature is described. The common naturally occurring mono- and disaccharides (rhamnose, xylose, arabinose, mannose, glucose, galactose, maltose and lactose) can be rapidly separated and a quantitative method for their determination based on the modified moving wire/flame ionization detector is discussed.

INTRODUCTION

As the technological significance of the polysaccharide and individual carbohydrate composition of food products has become more apparent, the requirement within our laboratory for rapid qualitative and quantitative methods for the characterization of the carbohydrate components of food has increased. The upsurge of interest in liquid chromatographic techniques, with its attendant developments in pump, column packing, and detector technology, has led to many advances in the separation of low-molecular-weight carbohydrates. SAMUELSON and coworkers[1–3] have described separation methods based on both cation- and anion-exchange resins with ethanol–water as mobile phase, while other workers[4,5] have described the separation of borate complexes of simple carbohydrates on anion-exchange resins. Initial experiments in our laboratory with anion- (using both ethanol–water and borate techniques) and cation-exchange resins[6] suggested better and more rapid separations with the cation-exchange resins. The effect of counterion size on the separations indicated that organic bases might have useful properties as counterions. The investigation of the effects of organic bases as counterions with cation-exchange resins for the separation of carbohydrates is described.

A number of methods of detecting carbohydrates in a column effluent have been

TABLE I

EFFECT OF ORGANIC BASES ON THE CAPACITY RATIOS FOR CARBOHYDRATES

Aminex A-6 strongly acidic cation-exchange resin, 50 × 0.4 cm I.D. column; column temperature, 65°; mobile phase composition, 85% (w/w) ethanol in water.

Counterion	*Rhamnose*	*Ribose*	*Xylose*	*Arabinose*	*Fructose*	*Mannose*	*Glucose*	*Galactose*	*Sucrose*	*Maltose*	*Lactose*
Ammonium	4.19	6.49	7.04	9.47	10.73	11.50	11.43	14.10	[a]	21.08	37.62
Methyl ammonium	3.86	5.82	6.59	9.18	9.38	10.70	11.05	13.39	10.61	15.97	25.86
Dimethyl ammonium	2.77	4.16	4.91	6.06	6.06	6.96	7.41	8.62	6.36	9.27	14.11
Trimethyl ammonium	2.44	3.60	4.59	5.14	4.95	5.76	6.53	7.15	6.01	7.34	10.72
Tetramethyl ammonium	1.90	2.90	4.31	4.62	3.97	4.96	6.12	6.30	dec.[b]	7.24	10.46
Tetraethyl ammonium	1.92	2.86	3.68	3.68	3.46	4.24	4.56	4.92	4.59	5.01	6.17
Piperidinium	1.83	2.59	2.95	3.36	3.27	3.84	3.89	4.39	3.02	3.77	5.21
Hydroxy ammonium	3.27	4.13	4.63	5.00	7.48	7.17	8.93	9.43	[a]	16.30	26.03

[a] Not run.
[b] dec. = decomposition.

described. The most common, most complicated and probably most sensitive technique is based on a reaction with anthrone, orcinol, or cysteine, the carbohydrate derivative being monitored colorimetrically[4,5]. Other methods include direct weighing of the eluted carbohydrate[7], refractometric monitoring[8], direct combustion in a flame ionization detector[9], and discontinuous monitoring of carbon content[10]. HOBBS AND LAWRENCE[6] have described a quantitative method for the determination of lactose in milk. This method, based on the modified moving wire/flame ionization detection system[11], was used in the work described below.

EXPERIMENTAL

Apparatus

A standard liquid chromatographic system consisting of a solvent reservoir, pump, thermostatted column and continuous detector was used.

The pump, which was developed in our laboratory, was continuous, pulse-free, and capable of operating at up to 68 atm. The column flow-rate was temperature and composition dependent but was, typically, for a 100-cm column, 0.45 ml min^{-1} at a column temperature of 75° and inlet pressure of 30 atm with 85% ethanol in water as mobile phase.

The column (50 or 100 × 0.4 cm I.D.) was glass, fitted with a Pye GLC injection head (Pye Unicam, Cambridge). The column inlet was a 6.35-mm Kovar-to-glass seal, the injection head being slightly modified to take a compression fitting to the Kovar. The column outlet was a standard Pye GLC fitting connected directly to the detector. A water jacket, maintained at a constant temperature in the range 65 to 85° by circulation from a constant temperature water-bath, enclosed the column. A small cooling coil was wound round the column outlet connection to reduce solvent evaporation at the detector inlet.

A modified Pye moving wire detector[11] provided a direct trace of the column eluent composition on a potentiometric recorder, there being no necessity for coloured derivative formation as in methods previously described[2,4].

Materials

The carbohydrates used as standards were AnalaR grade (Hopkin and Williams, Chadwell Heath, Essex). The organic bases were obtained from B.D.H. (Poole, Dorset).

The resin (Aminex A-6, 17.5 μm particle size; Bio-Rad Laboratories, St. Albans, Herts.) was converted to the forms investigated (Table I) by repeated washings with 2 *N* solutions of the appropriate organic base. The packing procedure was to allow a slurry of the resin in the developing solvent to settle under gravity, aliquots of the slurry being added as necessary to fill the column.

Operating conditions

85% (w/w) ethanol in water was used as mobile phase to compare the effects of the organic base counterions. This composition was suggested by previous work (ref. 1, and Fig. 1 of ref. 6). A column temperature of 65° was chosen to compromise between carbohydrate solubility and column operating pressure. The shorter 50-cm column was used in each case.

For the fuller investigation of the trimethylammonium form of the resin, the column temperature was varied from 65 to 85° at mobile phase compositions from 80 to 87.5% (w/w) ethanol in water at both high and low flow-rates. The lower temperature limit was set by the mobile phase viscosity, the column inlet pressure to achieve adequate flow-rates at lower temperatures tending towards the safe limit for the column. Injection was from a 10-μl syringe, with the column flow stopped, into a 4-cm layer of fine glass beads above the column packing to give an even application to the resin.

RESULTS

Table I illustrates the effect of the resin form on the capacity ratios for the individual mono- and disaccharides. The capacity ratio (k') is related to the distribution volume (D_V, see ref. 1) and is given by:

$$k' = (V_R - V_0)/V_0$$

where V_R is the peak retention volume and V_0 is the column dead volume, which was the elution volume of the non-retained compound methyl palmitate.

The relative retentions of the individual carbohydrates on each resin form, the observed separations achieved, the total analysis times, and the effects of resin form on band broadening in the column showed that the best separation of mono- and disaccharides was achieved on the trimethylammonium form of the resin (Fig. 1).

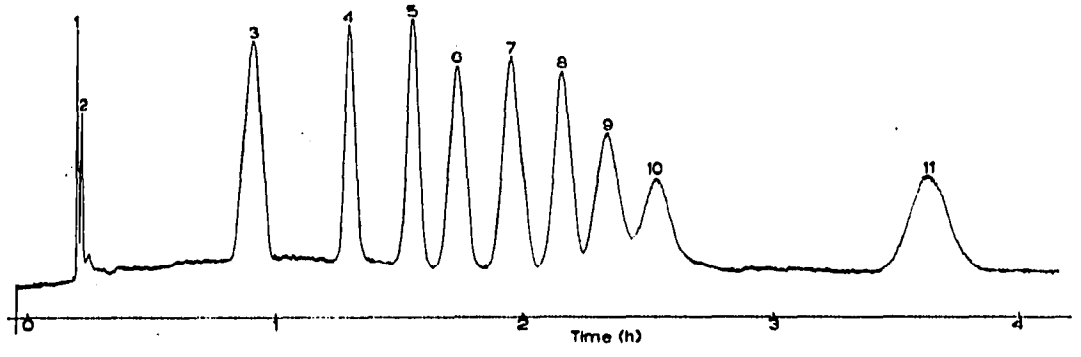

Fig. 1. Separation of mono- and disaccharides. Column, 100 × 0.4 cm I.D. Aminex A-6, trimethylammonium form; eluent, 85% (w/w) ethanol in water; temperature, 65°; flow-rate, 0.266 ml min^{-1}. Each peak corresponds to approximately 150 μg carbohydrate. 1 = Methyl palmitate; 2 = tetramethylglucose; 3 = rhamnose; 4 = ribose; 5 = xylose; 6 = arabinose; 7 = mannose; 8 = glucose; 9 = galactose; 10 = maltose; 11 = lactose.

The effects of column temperature, mobile phase composition, and flow-rate on the separation of carbohydrates on a 100-cm column of the trimethylammonium form of the strongly acidic cation-exchange resin were therefore investigated in order to optimize the separations obtained. The results obtained are summarized in Table II.

The effect of flow-rate was also considered. It was found that, within experimental error in the range 0.15 to 0.45 ml min^{-1}, the capacity ratio for each carbohy-

TABLE II

EFFECT OF SOLVENT COMPOSITION AND TEMPERATURE ON THE CAPACITY RATIOS FOR CARBOHYDRATES

100 × 0.4 cm I.D. column of trimethylammonium form of strongly acidic cation-exchange resin.

Carbohydrate	*Solvent composition % ethanol (w/w)*[a]											
	80.0			*82.5*			*85.0*			*87.5*		
	65°	*75°*	*85°*	*65°*	*75°*	*85°*	*65°*	*75°*	*85°*	*65°*	*75°*	*85°*
Rhamnose	2.06	1.93	1.86	2.20	2.17	2.08	2.44	2.30	2.23	3.30	3.17	3.04
Fucose	2.66	2.39	2.25	2.87	2.64	2.42	3.28	2.95	2.69	4.32	3.86	3.65
Ribose	3.02	2.83	2.66	3.22	3.14	3.01	3.60	3.33	3.20	4.91	4.62	4.33
Xylose	3.73	3.48	3.29	4.01	3.94	3.77	4.59	4.25	4.08	6.31	5.96	5.59
Arabinose	4.18	3.84	3.54	4.49	4.32	4.06	5.14	4.66	4.38	7.07	6.52	6.01
Mannose	4.40	4.17	3.94	4.89	4.83	4.64	5.76	5.36	5.15	8.30	7.91	7.42
Glucose	4.90	4.61	4.33	5.48	5.37	5.12	6.53	6.01	5.75	9.35	8.84	8.18
Galactose	5.33	4.94	4.55	5.94	5.70	5.34	7.15	6.33	5.99	10.08	9.36	8.67
Fructose	3.82	3.49	3.29	4.26	3.96	3.62d	4.95	4.51	4.09d	6.84	6.14d	dec.
Sucrose	3.80d	dec.	dec.	4.55d	4.44d	dec.	6.01	5.88d	dec.	9.26	8.38	8.04
Maltose	4.73	4.39	4.14	5.68	5.42	5.06	7.34	6.84	6.44	11.92	10.72	10.24
Lactose	6.71	6.01	5.56	8.23	7.56	6.86	10.72	9.55	8.80	17.84	15.36	14.02

[a] dec. or d. = total or partial decomposition.

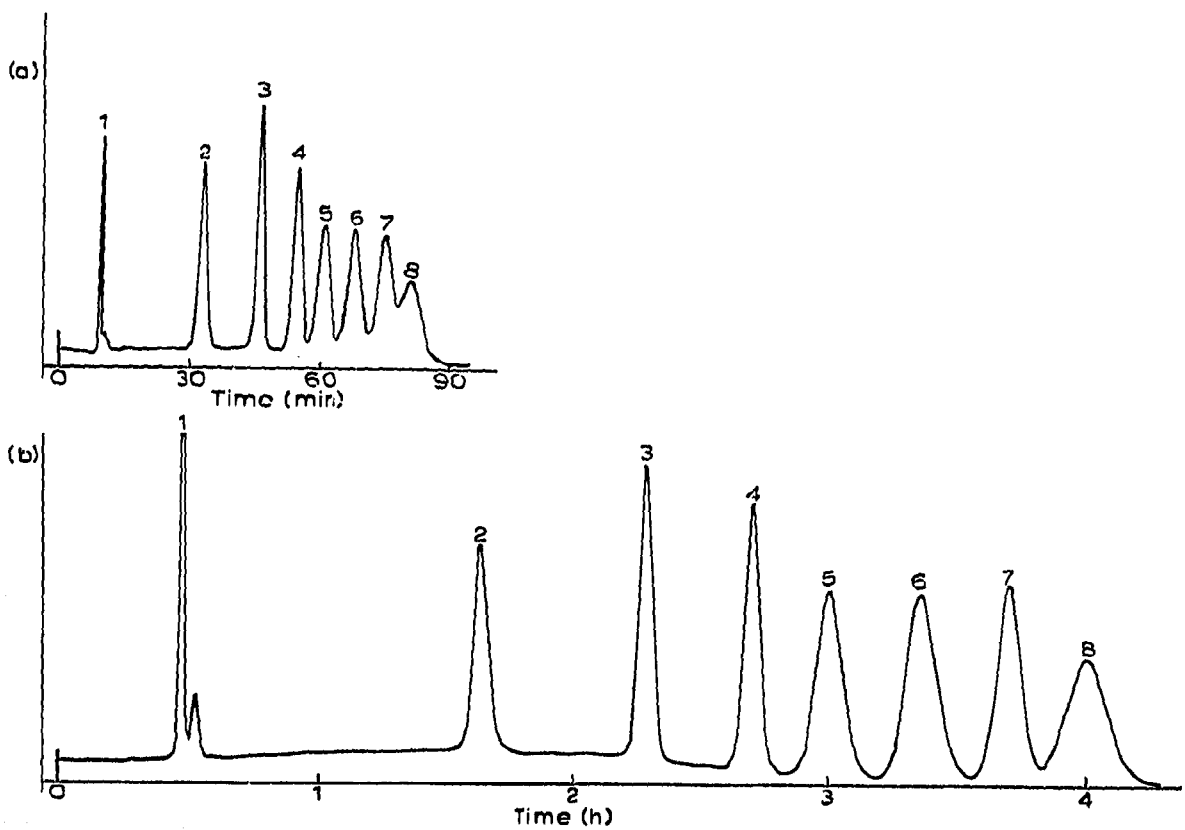

Fig. 2. Effect of flow-rate on the separation of monosaccharides. Flow-rate, (a) 0.49 ml min^{-1}; (b) 0.16 ml min^{-1}. For further conditions, see the legend to Fig. 1. 1 = Tetramethylglucose; 2 = rhamnose; 3 = ribose; 4 = xylose; 5 = arabinose; 6 = mannose; 7 = glucose; 8 = galactose.

drate was independent of flow-rate. However, it is apparent from Fig. 2 that flow-rate has a marked effect on resolution. This is particularly noticeable for glucose and galactose, it being possible to achieve their base-line separation if the flow-rate is low enough. The effect of temperature and mobile phase composition at constant flow-rate on the resolution of glucose and galactose is shown in Table III.

TABLE III

EFFECT OF SOLVENT COMPOSITION AND TEMPERATURE ON RESOLUTION OF GLUCOSE AND GALACTOSE
100 × 0.4 cm I.D. column of trimethylammonium form of strongly acidic cation-exchange resin.

Temperature (°C)	*Solvent composition % ethanol (w/w)*			
	80.0	*82.5*	*85.0*	*87.5*
65	1.238	1.183	1.187	1.143
75	1.238	1.112	1.113	1.037
85	1.058	1.007	0.965	0.854

Here, resolution $= (V_R^2 - V_R^1)/(P_W^2 + P_W^1)$, where the superscripts refer to individual peaks, V_R is defined above, and P_W is the peak width at 0.6065 of the peak height.

The mechanism of the separation system leads to chromatographically efficient columns, the theoretical plate height of the majority of carbohydrate peaks lying between 0.1 and 0.5 mm. Generally, sharper peaks are obtained at higher temperatures and higher water contents in the mobile phase.

DISCUSSION

Previous work[1,2] has shown that increasing the size of inorganic counterions used with strongly acidic cation-exchange resins increases the capacity ratios for individual mono- and disaccharides separated using them. However, the opposite trend is observed for alkyl-substituted ammonium ions as their size increases from ammonium, through the methylammonium forms to tetraethylammonium (Table I). The tetraethylammonium form gives a smaller difference from the tetramethylammonium form than might be expected from the change in ionic size. Another noticeable effect is that the hydroxyammonium form gives a good group separation of the pentoses, hexoses and disaccharides. As opposed to the inorganic counterions (including ammonium), the alkyl-substituted counterions separate mannose and glucose.

A number of effects may contribute to the separation of carbohydrates on ion-exchange resin columns. The most important are interaction with the counterion and partition between the water-rich stationary phase within the resin matrix and the ethanol-rich mobile phase[1,12]. Dispersion, electrostatic, and hydrogen bonding interactions with the resin matrix, the counterion, and the stationary phase may also influence the separation. The increasing hydrophobic character of the organic counterions with successive substitution of methyl or ethyl groups for the hydrogen of the ammonium ion will tend to reduce interaction with the hydroxyls of the carbohydrates and thus reduce capacity ratios. Similarly, the increasing hydrophobic character and decreasing charge density of the counterions will alter the relative amounts of ethanol

and water taken up by the resin, reducing water relative to ethanol and therefore decreasing the difference between the compositions of the mobile and stationary phases. The balance of the effects influencing the retention of the carbohydrates on the column will govern the separations obtained.

The effects of increasing water content of the mobile phase and increasing column temperature are to reduce the capacity ratios for the individual carbohydrates (Table II, trimethylammonium form). The effect of mobile phase composition changes is especially marked for disaccharides, which are generally less soluble in solutions of high alcohol content. The data in Table II may be used to predict the optimum conditions for the separation of most carbohydrate mixtures, base-line separation being achievable when the ratio of capacity ratios of any carbohydrate pair is greater than 1.08.

The implication of these temperature and mobile phase composition influenced variations in capacity ratios is that care must be taken in the initial selection of analysis conditions. If the carbohydrate mixture under analysis contains only monosaccharides, the mobile phase can have a much higher level of water, reducing the capacity ratios and giving a more rapid separation. However, if the disaccharides maltose and lactose are present, lower water contents are required to permit their elution after the monosaccharides (Table II). The advantages of higher water contents are evident from Table III, which illustrates that the resolution of the monosaccharide pair glucose and galactose increases with the water content of the mobile phase. In this case, the peaks become narrower more rapidly than their maxima approach and thus an enhancement in resolution is obtained.

It is evident from Fig. 2 that flow-rate influences peak resolution. However, in order to maximize the number of analyses, it is necessary to work at the highest possible flow-rate. This will be influenced by the resolution required between the carbohydrates present. For qualitative work, optimum resolution is not always necessary but for quantitative work it is advantageous to obtain complete resolution. The use of the modified moving wire detector, which permits the continuous monitoring of the column outflow and gives a reproducible, predictable response to carbohydrates, allows the carbohydrates present in a mixture to be readily quantified. The method of quantitation recommended is an extension of that described by HOBBS AND LAWRENCE[6], in which a carbohydrate not present in the mixture is added as an internal standard. In the majority of cases, ribose can conveniently be used. The use of this method with the moving wire detector gives an absolute measurement. The use of continuous refractive index monitoring for carbohydrate detection was studied but found to be less sensitive than the method described.

One minor disadvantage of the method described has been found to be ketose decomposition. This is particularly true for fructose at higher temperatures (>75°) and sucrose which hydrolysed at below 82.5% ethanol at all temperatures investigated. There are, however, a number of advantages in the method. The column itself, the trimethylammonium form of a strongly acidic cation-exchange resin, is the first cation-exchange resin column on which the separation of mannose and glucose has been reported, although it should be noted that similar separations were obtained with the other organic bases. The column system is flexible and gives rapid separations (*cf.* refs. 1, 4, and 5). Cation-exchange resins also have the advantage over anion-exchange resins that they are readily commercially available as smaller, more uni-

form resin particles (due to their use in amino acid analysers). They thus pack well and give narrow, chromatographically efficient peaks which are simple to quantify. Although cation-exchange resin columns have given superior results in our laboratory, other workers[13] have demonstrated equally fast and efficient separations of complex mixtures using anion-exchange resins in the sulphate form.

CONCLUSIONS

The most suitable of the organic base counterions for use with a strongly acidic cation-exchange resin for the separation of carbohydrates was trimethylammonium. This resin form gave the optimum compromise between elution volumes, peak widths and analysis times, permitting the rapid separation of the common naturally occurring mono- and disaccharides. The quantitation of the carbohydrate peaks is readily achieved with the modified moving wire/flame ionization detector.

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