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IMPROVED COLUMN EFFICIENCY IN CHROMATOGRAPHIC ANALYSIS OF SUGARS ON CATION-EXCHANGE RESINS BY USE OF WATER–TRIETHYLAMINE ELUENTS

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SUMMARY

An improvement in the chromatographic separation of sugars and sugar alcohols on cation exchangers (Ca^{2+}) with water as eluent is presented. Addition of 0.001 M triethylamine to the eluent catalyses the mutarotation of reducing sugars, and results in reduced peak widths. Complete resolution is obtained for glucose, mannose and fructose in $3\frac{1}{2}$ min, for glucose, mannose, fructose, mannitol and glucitol in 9 min and for sucrose, glucose and fructose in $3\frac{1}{2}$ min. A nearly complete resolution of lactose, glucose and galactose is achieved in 3 min. The influence of the triethylamine content, column temperature and eluent flow-rate has been determined. Procedures are given for column preparation, activation and regeneration.

INTRODUCTION

Liquid chromatography of sugars using ion exchangers has become routine. The use of anion exchangers with borate buffers[1] and with ethanol–water[2], and the use of cation exchangers with ethanol–water[3], as eluent have been reported. Many improvements have been achieved, and a comprehensive review is provided by Jandera and Churáček[4]. The methods given by Samuelson and co-workers[2,3] result in a low column efficiency and are not in common use. However, in combination with borate buffers, anion exchangers are frequently employed for the separation of simple mixtures of sugars[5–10]. Although this method also leads to low column efficiencies, it has the advantage of a tailor-made eluent through variation of the buffer concentration and pH[7].

Two other systems are commonly employed: alkylamine-modified silica together with acetonitrile–water, and cation exchangers (Ca^{2+}) with pure water as the eluent. Silica can be modified with alkylamine both chemically[11–13] and physically[14–16]. It has the advantage of being resistant to high pressures, enabling the use of high eluent flow-rates. Separations can also be carried out at room temperature with simple apparatus. In the "Ca-column" system, the main function of the ion exchanger is to immobilize Ca^{2+}, while separation is the result of the different complexing

abilities of the polyols with Ca^{2+}. However, the degree of cross-linking is also important, polymeric sugars being better separated at low degrees and mono- and dimeric sugars at high degrees. Advantages of this system are cheap eluent, high column capacity and stability and complete elution of all sugars injected[17].

In contrast with the borate–anion exchanger system, the elution sequence cannot be changed in the modified silica system nor in that of the cation exchanger (Ca^{2+}). There is a remarkable difference between the elution sequences of the last two systems: on alkylamine-modified silica, sugars are eluted in order of increasing degree of polymerization, first monomeric then oligomeric and polymeric; the opposite is true for the cation exchanger (Ca^{2+}) system. Choice of the appropriate method will, therefore, depend on the nature and quantity of the sugars to be separated. Although the use of silica columns is increasing, this method will not supersede the two ion-exchange methods because of the different elution sequences.

In this paper we describe an extension of the applicability of the cation exchanger (Ca^{2+}). Addition of a small amount of triethylamine (TEA) to the eluent catalyses the mutarotation of reducing sugars, resulting in reduced peak widths without affecting the elution times. The separations are thus possible at room temperature, with no separate peaks for sugar anomers. The overall result is a greatly improved method of analysis.

EXPERIMENTAL

Apparatus

The column comprised a 316 stainless-steel Lichroma tube (Chrompack, Middelburg, The Netherlands), 250 mm × 4.6 mm I.D., fitted with filters (pore size 2 μm) and thermostated in a water-bath. The eluent was deaerated by heating it to 10°C above the column temperature; it was pumped, after passage through a 2-μm filter, by a solvent delivery system (Model 6000 A, Waters Assoc.). Injection was performed with a Rheodyne Type 7010 sample valve, fitted with a 10-μl loop, and filled by suction using a peristaltic pump. Detection was by a differential refractometer (Type R401, Waters Assoc.). The specifications of the cation-exchange resin Aminex A-5 (Bio-Rad Labs., Richmond, CA, U.S.A.) were as follows: matrix, polystyrene and divinylbenzene with 8 % cross-linking; functional group, sulphonic acid; particle diameter, 13 ± 2 μm; exchange capacity, 1.7 mequiv./ml; maximum allowable temperature, 150°C.

Column preparation and quality

Several methods of column preparation are given in literature. Rapp *et al.*[18] pumped a slurry of the resin in ethanol–water (75:25) into the column without any pretreatment. Ladisch *et al.*[19] carried out stepwise washings and extractions in order to purify the resin and to convert it into the appropriate ionic form. Scobell *et al.*[20] used the same procedure, but with the resin inside the column, which is less laborious. Ladisch and Scobell also employed sedimentation to obtain a more uniform particle size. We did not use sedimentation as we supposed the resin Aminex A-5 to be within 13 ± 2 μm. Our method, resembling that of Scobell *et al.*[20], is as follows.

(1) A weighed amount of Aminex A-5 (*ca.* 1.5 g per ml column content) was placed into a column and the eluent pump was connected;

(2) The resin was washed with 100 ml distilled water, then with 300 ml of 0.2 M $CaCl_2$ in distilled water and finally with 100 ml distilled water;

(3) The resin was pumped out of the column into a beaker together with four times its volume of water;

(4) The analysis column was fitted with a stainless-steel filter at the bottom and a filling column at the top, and then filled with distilled water;

(5) The resin was slurried over 5 min using an ultrasonic bath and poured into the filling column, the residual volume of which was filled with water. The top of this column was connected to the eluent pump. This procedure is carried out quickly in order to avoid selective sedimentation;

(6) During 1 h, water was pumped through the column, maintaining a pressure drop of 50 p.s.i. per cm of bed height;

(7) The filling column was disconnected, the analysis column filled and levelled and fitted with a stainless-steel filter.

The flow direction was marked on the column in order to avoid disturbance of the column packing. (The resin resists applied pressure; Rapp et al.[18] even used up to 20 % higher pressure drops. The procedure is carried out at room temperature.)

The column, prepared in this way and used at 85°C with an eluent flow-rate of 0.5 ml/min, gave a plate height for the reference substance D-mannitol of 80 μm (6 × particle diameter). It has been used for 3 months under various conditions: temperatures from 15 to 95°C; eluent changes between distilled water, 0.001 M TEA, KOH solution at pH 11 and 0.2 M $CaCl_2$ in water; and with longer stops of eluent flow and heating. The column performance, with reference to D-mannitol was unaltered over this period, in accordance with the experience of Angyal et al.[17].

Standard conditions

Column: 250 × 4.6 mm stainless steel filled with Aminex A-5 (Ca^{2+}); temperature, 45°C. Eluent: 0.001 M triethylamine in water; flow-rate, 0.5 ml/min. Injected amount: 0.1 mg of each sugar. Attenuation of the refractometer: × 32.

RESULTS AND DISCUSSION

Separation of sugars with this cation exchanger (Ca^{2+}), with water as eluent, is generally performed at elevated temperatures[19,20]. Owing to a higher rate of mass transfer, the peak widths decrease. At lower temperatures, double peaks of the different sugar anomers occur[21], which fuse at higher temperatures due to the increased mutarotation rate[17]. (The same behaviour is observed in the chromatography of sugars on anion exchangers with ethanol–water as eluent[22]). On the other hand, components are more strongly adsorbed (exothermic adsorption) at lower temperatures and, therefore, the resolution can be improved if the influence of the lower mass-transfer rate is small and the effect of mutarotation is suppressed.

TEA as a catalyst for mutarotation

Since mutarotation is catalysed by hydroxide ions, the use of an eluent having a high pH should improve the chromatographic separation[17]. The use of an amine, especially TEA, is preferred for several reasons: stainless steel is more resistant to corrosion in amines than in alkalis[23]; with TEA, the eluent pump and sample valve

will not be blocked by evaporation residues during shut-down periods; TEA has a boiling point which is relatively high in comparison to the column temperature; and TEA, unlike polyamines, does not result in too high a viscosity. To elucidate the effect of TEA addition, several experiments have been carried out at room temperature.

D-Glucose solutions in water and in 0.001 M TEA were analysed at set times after preparation, using our column with pure water as eluent and under circumstances in which the anomers are separated (45°C, flow-rate 0.5 ml/min). Freshly prepared solutions contain mainly the α-form, while at equilibrium the β-form is predominant, as confirmed by polarimetry. Plots of peak areas for the α- and β-forms *versus* time elapsed after preparation of the solutions are presented in Fig. 1. Apparently the mutarotation rate is increased by a factor of *ca.* 20 by the addition of 0.001 M TEA. Other results of this experiment are that β-glucose is eluted before α-glucose, and that both forms give the same quantitative response. For quantitative analysis, therefore, it does not matter whether an equilibrium mixture is injected or not.
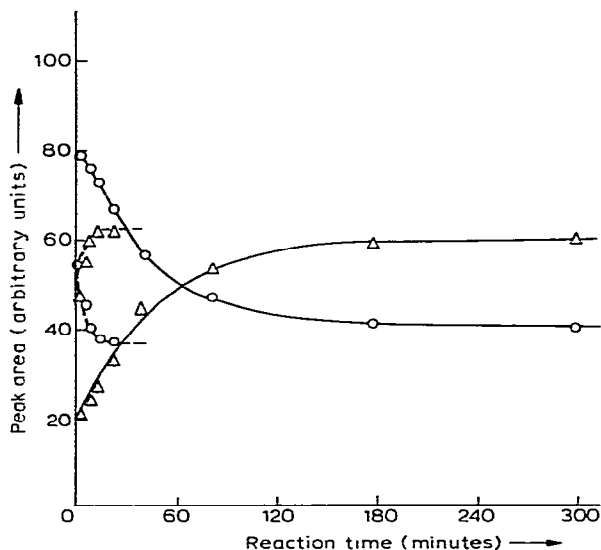

Fig. 1. Time required for equilibrium between α-glucose ($\bigcirc$) and β-glucose ($\triangle$) at 22°C: ————, a freshly prepared solution of glucose in water; – – –, the same solution but after addition of 0.001 M triethylamine.

Influence of TEA concentration

To determine the optimum TEA concentration for analysis, a reference sample was injected using eluents comprising various amounts of TEA in water. First, suf-. ficient eluent had to be pumped through in order to reach a "steady state". The results are presented in Table I. Retention times are almost independent of the TEA concentration; however, the peak width of glucose decreases with increasing TEA content. Concentrations of TEA higher than 1 mM do not further improve the analysis.

TEA from the eluent will gradually displace Ca^{2+} from the ion exchanger, resulting in decreased retention times and in low separation efficiency. This effect is illustrated in Table II. Concentrations higher than 0.001 M TEA should not be used,

TABLE I

ELUTION TIME AND PEAK WIDTH AS A FUNCTION OF TRIETHYLAMINE CONCENTRATION IN THE WATER ELUENT

t_R = Elution time in minutes; $w_{\frac{1}{2}h}$ = peak width at half height in minutes.

Sugar**	Triethylamine concentration (mM)*							
	0.2 (9)		0.4 (5)		0.6 (3)		1.0 (3)	
	t_R	$w_{\frac{1}{2}h}$	t_R	$w_{\frac{1}{2}h}$	t_R	$w_{\frac{1}{2}h}$	t_R	$w_{\frac{1}{2}h}$
Glucose	3.63	0.37	3.63	0.31	3.61	0.27	3.59	0.26
Fructose	5.07	0.34	5.07	0.34	5.07	0.33	5.05	0.32
Mannitol	6.89	0.44	6.93	0.44	6.91	0.43	6.85	0.42
Glucitol	9.23	0.57	9.23	0.56	9.15	0.54	9.13	0.54

 * The column conditioning time is given in parentheses.
 ** All sugar products mentioned in this paper belong to the D-series.

TABLE II

COLUMN EFFICIENCY BEFORE AND AFTER 2000 ml OF 0.001 M TRIETHYLAMINE ELUENT HAS BEEN PUMPED THROUGH

N = Number of theoretical plates. t_R and $w_{\frac{1}{2}h}$ as in Table I.

	Ca^{2+}-column (freshly regenerated)–water	Ca^{2+}-column (after 2000 ml 0.001 M TEA eluent)– 0.001 M TEA	TEA-column*–water
Mannitol			
t_R	7.09	6.71	4.03
$w_{\frac{1}{2}h}$	0.41	0.38	0.31
N	1656	1728	936
Glucitol			
t_R	9.59	8.85	4.95
$w_{\frac{1}{2}h}$	0.54	0.50	0.36
N	1747	1736	1047
Resolution (mannitol–glucitol)	2.63	2.43	1.37

 * Realized by pumping of 150 ml of 0.1 M TEA · HCl.

and, depending on the resolution needed, the column should be regenerated after $\geqslant 2$ l of eluent have been pumped through it. Column regeneration can be performed as described below.

Influence of column temperature

The results of experiments carried out to determine the influence of column temperature on the resolution are given in Table III. The eluents pure water and 0.001

M TEA are compared. While at low column temperatures, using water, double peaks occur for the reducing sugars, single peaks are obtained under all circumstances using TEA. At higher temperatures the use of TEA results in a substantial decrease of peak width for these sugars. The peak widths for the sugar alcohols, which do not mutarotate, are only slightly affected. The mutarotation rate decreases in the sequence: fructose > mannose > glucose, as can be seen from the occurrence of double peaks. The most significant improvement of the analytical result by adding TEA is obtained for the slowly mutarotating sugars. Aitzetmüller[14] reported improved resolution, apart from increased elution times, upon adding polyamine to an acetonitrile–water eluent using silica columns. This improved resolution can also be explained by increased mutarotation.

TABLE III

ELUTION DATA FOR SUGARS AT VARIOUS COLUMN TEMPERATURES WITH (+) AND WITHOUT (−) 0.001 *M* TEA

t_R and $w_{\frac{1}{2}h}$ as in Table I.

Sugar	Column temperature (°C)						
	25 (+/−)	35 (+/−)	45 (+/−)	55 (+/−)	65 (+)	75 (+)	85 (+/−)
Glucose							
t_R	3.67/ 3.65*	3.59/ 3.65*	3.61/3.63*	3.66/3.67*	3.63	3.57	3.68/3.69
$w_{\frac{1}{2}h}$	0.42/ −**	0.30/ −	0.23/ −	0.20/ −	0.19	0.17	0.17/0.24
Mannose							
t_R	4.35/ 4.37*	4.35/ 4.32*	4.25/4.34*	4.23/4.29	4.25	4.14	−/4.21
$w_{\frac{1}{2}h}$	0.31/ −	0.27/ −	0.25/ −	0.22/0.51	0.20	0.19	−/0.23
Fructose							
t_R	5.69/ 6.03*	5.31/ 5.61*	5.07/5.29	4.93/4.99	4.75	4.51	4.51/4.53
$w_{\frac{1}{2}h}$	0.43/ −	0.35/ −	0.30/0.94	0.25/0.60	0.22	0.22	0.20/0.24
Mannitol							
t_R	7.93/ 8.21	7.31/ 7.61	6.85/7.09	6.53/6.69	6.21	5.85	5.79/5.87
$w_{\frac{1}{2}h}$	0.58/ 0.62	0.47/ 0.49	0.40/0.41	0.32/0.35	0.28	0.26	0.24/0.25
Glucitol							
t_R	11.4 /12.1	10.1 /10.7	9.09/9.59	8.41/8.75	7.79	7.19	6.99/7.11
$w_{\frac{1}{2}h}$	0.78/ 0.87	0.60/ 0.66	0.51/0.54	0.41/0.44	0.34	0.30	0.28/0.28

* Elution time is calculated according to $t_R = t_0 (1 + k_z' f_z + k_\beta' f_\beta)$, where t_0 is the elution time in minutes of an unretained compound, k_z' and k_β' are the capacities of anomers α and β, respectively, and f_z and f_β are the corresponding mol fractions.
** Double peak.

Influence of eluent flow-rate

Results of experiments at different eluent flow-rates are presented in Table IV. Peak widths decrease with decreasing eluent flow-rate, while elution volumes hardly change. The improvement in column efficiency, however, is not sufficient to justify the longer elution times.

TABLE IV

ELUTION DATA FOR SUGARS AT VARIOUS ELUENT FLOW-RATES

V_R = Elution volume in ml; $w_{\frac{1}{2}h}$ = peak width at half height in μl.

Sugar	Eluent flow-rate (ml/min)						
	0.1	0.2	0.3	0.4	0.5	0.6	0.7
Sucrose							
V_R		1.53	1.52	1.52	1.49	1.47	1.41
$w_{\frac{1}{2}h}$		93	100	104	112	110	118
Lactose							
V_R	1.68	1.62	1.59	1.57	1.58	1.56	1.57
$w_{\frac{1}{2}h}$	83	98	106	112	120	120	126
Glucose							
V_R	1.92	1.86	1.83	1.82	1.81	1.80	1.81
$w_{\frac{1}{2}h}$	77	90	98	102	106	110	112
Galactose							
V_R	2.18	2.10	2.07	2.05	2.06	2.03	2.03
$w_{\frac{1}{2}h}$	79	93	100	109	112	113	118
Mannose							
V_R		2.15	2.13	2.12	2.10	2.08	2.08
$w_{\frac{1}{2}h}$		91	106	107	112	113	120
Fructose							
V_R	2.56	2.47	2.43	2.42	2.41	2.40	2.41
$w_{\frac{1}{2}h}$	88	102	116	120	128	130	134
Mannitol							
V_R	3.39	3.27	3.23	3.21	3.20	3.18	3.18
$w_{\frac{1}{2}h}$	108	124	143	156	165	168	179
Glucitol							
V_R	4.33	4.19	4.14	4.11	4.09	4.07	4.08
$w_{\frac{1}{2}h}$	135	150	175	192	200	211	227

Addition of 0.001 M TEA to the eluent causes the pH to rise to 10.8, and during the elution isomerization and/or degradation of the sugars may occur. We studied this possibility by determining the quantitative response, peak form and elution volume of the different sugars as a function of the eluent flow-rate. No influence could be found and, therefore, isomerization and degradation of the sugars at high pH can be neglected.

Column regeneration and activation

Column regeneration and activation can be performed by pumping through the column successively: 100 ml distilled water, 100 ml 0.2 M CaCl$_2$ in water, 100 ml

distilled water and 50 ml of 0.001 M TEA eluent. Regeneration can be carried out overnight, and, therefore, the procedure has not been optimized. The frequency of regeneration will depend on the resolution needed. On the basis of an average elution volume of 2 ml and a regeneration step after 2000 ml eluent, 1000 analyses can be carried out between two steps.

For reactivation and deactivation of the column, some time is needed. This is illustrated in Table V. Six column volumes of TEA eluent must be pumped through in order to attain a "steady state". This amount of amine corresponds to only 1/300 of the total column capacity. The increased rate of mutarotation, is therefore not due to the column being in the amine form, but merely to the higher pH throughout the column. For deactivation (not regeneration) of the column, which means lowering the pH inside the resin, even more time is needed. A hundred column volumes of water eluent have to be pumped through to convert an activated column into a "normal" column (Ca^{2+}).

TABLE V

COLUMN ACTIVATION

t_R and $w_{\frac{1}{2}h}$ as in Table I.

Sugar	Column volumes of eluent				
	0	2.4	4.8	6.0	7.5
Glucose					
t_R	3.51*	3.51*	3.67	3.71	3.69
	3.95*	3.95*			
$w_{\frac{1}{2}h}$	—	—	0.29	0.24	0.24
Fructose					
t_R	5.19	5.19	5.19	5.19	.5.19
$w_{\frac{1}{2}h}$	0.70	0.66	0.38	0.32	0.31
Mannitol					
t_R	7.01	7.01	6.99	7.01	6.99
$w_{\frac{1}{2}h}$	0.41	0.40	0.40	0.40	0.40
Glucitol					
t_R	9.35	9.29	9.29	9.33	9.27
$w_{\frac{1}{2}h}$	0.52	0.52	0.53	0.53	0.52

* Double peak.

Application of the method to some common sugar mixtures

The addition of TEA to the eluent results in a large reduction in peak widths of the mutarotating sugars. Therefore, better separations within shorter analysis times are obtained, as compared with previous methods. The separations of some common sugar mixtures are now presented to illustrate the method.

Glucose, mannose, fructose. This product mixture is obtained from the iso-merization of glucose to fructose, where mannose is a side-product. The "isomerose" mixture is used as a sweetener in the food industry, as an alternative to sucrose and "invert-sugar". Analysis can be performed using an anion exchanger with borate as

eluent[6], a cation exchanger (Ca^{2+}) with water as eluent[19] or unmodified silica with acetonitrile containing a trace of water[24]. In these three systems the analysis time needed is *ca.* 30 min and only the first system yields complete separation. No literature data are available for the analysis of this mixture using propylamine-modified silica with acetonitrile–water, and interpretation of other data suggests that separation will be difficult.

From the elution data in Table III, it is seen that the resolution is dependent on the column temperature. With increasing temperature, the glucose–mannose resolution improves, while that of mannose–fructose worsens. The optimum temperature is 45°C, and, using our standard analytical conditions, complete separation is obtained within 6 min (see Fig. 2). In serial analysis the time required can be reduced to $3\frac{1}{2}$ min.
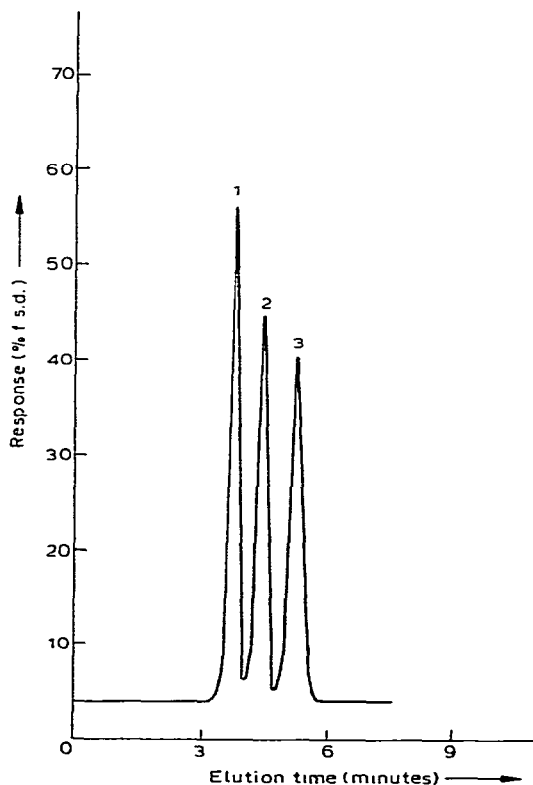

Fig. 2. Chromatogram of glucose (1), mannose (2) and fructose (3) under standard conditions. f.s.d. = Full scale deflection.

Peak heights remain constant within 2%. From the injection of different amounts of sugar, a linear calibration curve could be constructed. If the signal-to-noise ratio is higher than 10, the detection limit for our system is 1 µg sugar, corresponding to 0.1 g/l with an injection volume of 10 µl.

Glucose, mannose, fructose, mannitol, glucitol. This product mixture is obtained from the simultaneous isomerization and hydrogenation of glucose. The sugar al-

cohols obtained by hydrogenation are eluted after the sugars, and a complete separation is possible using the conditions described above. A typical chromatogram is shown in Fig. 3. The time needed to separate the five components is 12 min; in serial analysis, 9 min. The detection limit for the sugar alcohols is a factor of 2 higher than for the sugars owing to the longer elution times.
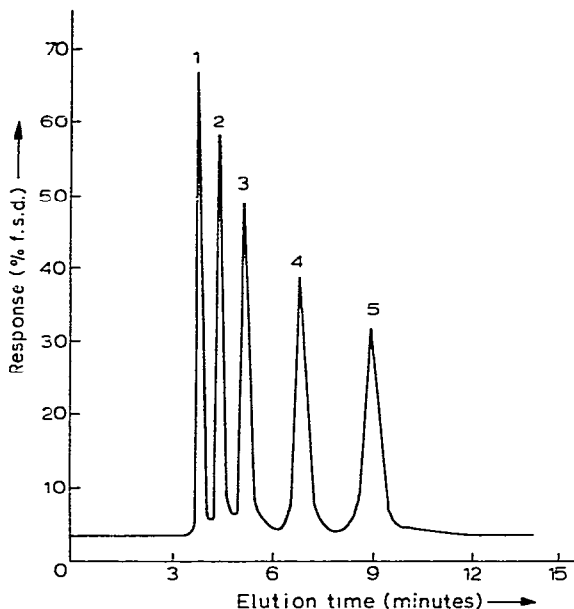

Fig. 3. Chromatogram of glucose (1), mannose (2), fructose (3), mannitol (4) and glucitol (5) under standard conditions.

Lactose, glucose, galactose. People suffering from lactose intolerance cannot digest lactose or "milk-sugar". Hydrolysis of lactose to the digestible sugars glucose and galactose is a useful method of overcoming this problem. In this reaction, some oligomeric sugars are formed as side-products. Chromatographic separation of the main components lactose, glucose and galactose can be achieved using an anion exchanger with borate as eluent[25] and a cation exchanger with water as eluent[19]. The time needed for the analysis is 30 min, which can be reduced in serial analysis to 20 and 10 min respectively for the two systems. No data are available on the use of modified silica with acetonitrile–water as eluent for the analysis of this sugar mixture. Such analysis will probably be difficult.

With our system, separation at 55°C is achieved within 6 min as is shown in Fig. 4. The quantitative results are similar to those already mentioned for the glucose–mannose–fructose mixture. An advantage of our system over the modified silica system is that oligomeric sugars are eluted before the monomeric sugars, which enables accurate estimation.

Sucrose, glucose, fructose. Sucrose, glucose and fructose are the most commonly used sweeteners. A glucose–fructose (1:1) mixture obtained by hydrolysis of sucrose is called "invert-sugar". Many methods for chromatographic separation of this mixture are given in the literature: anion exchangers with borate as eluent[9,10,25],
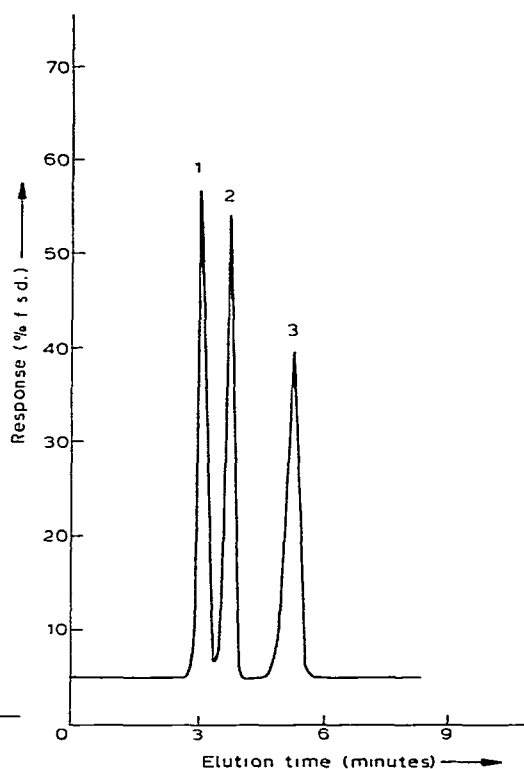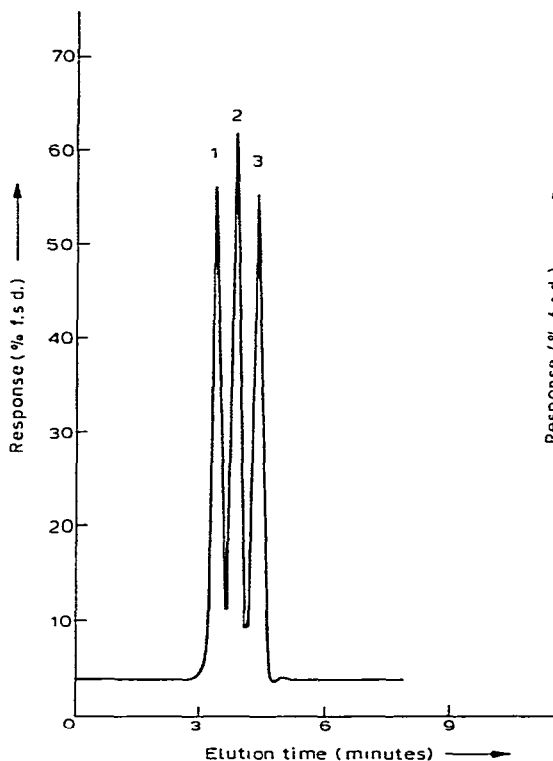
Fig. 4. Chromatogram of lactose (1), glucose (2) and galactose (3) under standard conditions, but with a column temperature of 55°C.

Fig. 5. Chromatogram of sucrose (1), glucose (2) and fructose (3) under standard conditions.

cation exchangers with water[18-20,26] and alkylamine-modified silica gel with acetonitrile–water[10,11]. For the anion- and cation-exchanger systems, the time required for a single analysis is 30 min, and in the latter case, as little as 15 min for serial analysis. With the silica gel system, 10 min are usually necessary, and the time cannot be less in serial analysis because of baseline disturbances caused by injection.

Sucrose, glucose and fructose can be separated under identical conditions to those used for glucose–mannose–fructose, as shown in Fig. 5. The analysis requires $3\frac{1}{2}$ min if carried out serially. The quantitative results are similar to those obtained for glucose–mannose–fructose. The three components could also be separated using only water as eluent at 85°C. The time needed for elution is the same, but addition of TEA to the eluent enables the use of a much lower column temperature.

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