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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF SUGARS ON A MIXED CATION-EXCHANGE RESIN COLUMN

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SUMMARY

The separation of mono-, di-, and trisaccharides on a cation-exchange 6% cross-linked resin column in the silver form (HPX-65A) is described. Acid-catalysed hydrolysis of sugars at elevated column temperatures could be suppressed by partially converting the column into the lead(II) form. The selectivity can be influenced by varying the ratio of the counter-ions. The use of this column connected in series with a cation-exchange 8% cross-linked resin column in the lead(II) form enabled the separation of sucrose, maltose, lactose, glucose, galactose, and fructose. The presence of the combinations maltotriose–sucrose and lactulose–glucose requires additional analysis on an unmodified HPX-65A column.

INTRODUCTION

The most prominent carbohydrate of milk is lactose, but traces of the monosaccharides glucose and galactose and other (poly)saccharides are also present. In dairy products lactose may be hydrolysed to D-glucose and D-galactose by enzymes. The determination of these three sugars by high-performance liquid chromatography (HPLC) should be performed on diol-modified silica or cation-exchange resin columns[1]. The accuracy and reproducibility of the determination of lactose in milk have been studied, and the results obtained by HPLC were compared with those found by polarimetry, enzymatic assay and reductometry (Luff-Schoorl)[2]. Since HPLC gave the same levels for lactose in milk as the enzymatic assay and is capable of measuring several sugars in one analysis, it is the method of choice.

In addition to the carbohydrates naturally present in dairy products, other sugars or sugar-containing components may be added in order to give the food product more desirable properties. The sugars encountered most frequently are sucrose, fructose, maltose and maltodextrins. The HPLC analysis of sugars in foods such as dairy products has been studied by several groups[3–6]. In these cases only a limited number of sugars was investigated or a marginal separation between some types of sugar was obtained. Diol-modified silica columns fail to give sufficient resolution for galactose and glucose, whereas the cation-exchange columns show insuf-

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ficient separation between the disaccharides. This implies that foods with an unknown or complicated sugar composition should be analysed on a diol-modified silica column and also on a cation-exchange resin column. The use of an acetonitrile-rich solvent in combination with a cation-exchange resin column gives sufficient resolution of sucrose, maltose and lactose[7]. However, many commercial cation-exchange resin columns do not tolerate the high content of acetonitrile (70–80%) in the eluent. Moreover, the sensitivity is considerably reduced, owing to the unfavourable refractive index of acetonitrile and to the high pressure-dependence of this index, giving a noisy baseline, caused by pump pulsation. The toxic properties of acetonitrile and the limited solubility of sugars in this eluent also make it less attractive.

Scobell and Brobst[8] described the preparation and application of a silver-loaded cation-exchange resin column with an intermediate cross-linking of 6%. Silver ions form monodentate complexes, which are stronger with disaccharides than with monosaccharides. This type of column is able to separate the disaccharides and to give reasonable separations for the monosaccharides.

Recently, a commercial version of this column became available and this prompted us to investigate its usefulness for the analysis of sugars, especially in dairy products.

TABLE I

RETENTION TIMES (min) OF SUGARS ON CATION-EXCHANGE RESIN COLUMNS AT VARIOUS TEMPERATURES

D = decomposition.

Sugar	*HPX-65A column*				*HPX-87P column*		
	25°C	*45°C*	*65°C*	*85°C*	*65°C*	*85°C*	*95°C*
Maltotriose	11.33	10.66	10.23	9.87	10.42	9.86	9.70
Stachiose	10.60	9.92 (D)	D	D	8.99	8.78	8.78
Gentiobiose	11.75	11.46	11.18	11.01	9.69	9.62	9.55
Cellobiose	12.12	11.71	11.39	11.17	10.19	10.06	9.98
Maltose	12.30	11.87	11.56	11.31	10.99	10.66	10.57
Melezitose	10.02	9.76 (D)	D	D	9.15	9.08	9.08
Raffinose	11.18	10.64 (D)	D	D	9.59	9.40	9.37
Melibiose	14.06	13.31	12.82	12.46	11.54	11.20	11.10
Lactose	14.18	13.42	12.87	12.47	11.25	10.97	10.80
Lactulose	17.10	15.38	14.26	13.40	13.74	12.70	12.37
Sucrose	11.44	11.23 (D)	D	D	10.29	10.16	10.12
Glucose	13.88	13.76	13.59	13.47	12.37	12.24	12.17
Tagatose	13.52	13.74	13.71	13.70	23.07	21.02	20.08
Xylose	14.30	14.16	13.94	13.82	13.54	13.26	13.13
Fructose	17.09	16.06	15.27	14.71	17.92	16.27	15.68
Galactose	16.78	16.04	15.48	15.06	14.60	14.10	13.88
Mannose	16.66	16.10	15.57	15.22	16.75	15.82	15.50
Arabinose	17.60	16.77	16.11	15.59	16.22	15.27	14.85
Fucose	18.21	17.84	17.32	16.83	16.12	15.48	15.15
Rhamnose	14.72	14.80	14.66	14.56	14.67	14.34	14.17

EXPERIMENTAL

Chemicals and materials

All reference standard solutions were prepared from analytical-reagent grade chemicals (Fluka, Buchs, Switzerland; Merck, Darmstadt, F.R.G.; Sigma, St. Louis, MO, U.S.A.; BDH, Poole, U.K.). For injection the samples were dissolved in doubly-distilled water. The compounds investigated are listed in Table I. All chemicals used in sample clean-up were of reagent-grade quality. Analytical grade cation-exchange resin (H^+, AG 50W-X4, <400 mesh; Bio-Rad Labs., Richmond, U.S.A.) and anion-exchange resin (base form, AG 3-X4A, 200–400 mesh; Bio-Rad) were used to pack the pre-column. The clean-up solution of Biggs and Szijarto[9] contained 91.0 g of zinc acetate dihydrate, 54.6 g of phosphotungstic acid 24-hydrate and 58.1 ml of glacial acetic acid per litre of doubly-distilled water.

Apparatus

A Waters Assoc. Model 6000 A pump with a Wisp 710B automatic sample injector was used in combination with an Erma Optical Works ERC-7510 refractive index detector and a Kipp Analytica 9222 column oven. Chromatograms were recorded and integrated with a Spectra-Physics SP4200 data system. The separations

TABLE II

RETENTION TIMES (min) OF SUGARS ON A MIXED SILVER–LEAD ION-EXCHANGE RESIN COLUMN AT VARIOUS TEMPERATURES AND SILVER–LEAD RATIOS

Sugar	*Percentage lead in column regenerating solvent: % (mol Pb/mol Ag)*					
	7.5	*3*	*1.5*			
	65°C	*65°C*	*25°C*	*45°C*	*65°C*	*85°C*
Maltotriose	12.37	12.13	13.86	12.83	12.10	11.60
Stachiose	13.35	11.40	12.67	11.83	11.33	10.98
Gentiobiose	12.75	12.30	12.86	12.47	12.27	12.03
Cellobiose	12.97	12.58	13.31	12.87	12.58	12.28
Maltose	13.25	12.97	13.89	13.37	12.93	12.63
Melezitose	11.18	10.97	11.44	11.13	10.93	10.82
Raffinose	14.02	11.85	12.90	12.20	11.78	11.52
Melibiose	14.87	15.83	15.60	14.83	14.28	13.85
Lactose	14.67	14.15	15.31	14.63	14.08	13.65
Lactulose	16.78	16.43	20.55	17.85	16.38	15.27
Sucrose	12.43	12.20	12.73	12.37	12.17	12.00
Glucose	14.78	14.52	14.54	14.60	14.47	14.30
Tagatose	19.00	20.53	23.20	21.32	20.57	19.22
Xylose	15.20	15.03	15.56	15.23	15.05	14.78
Fructose	17.77	17.95	21.83	19.37	17.98	16.87
Galactose	17.05	16.75	18.30	17.30	16.72	16.17
Mannose	18.00	18.00	20.15	18.78	18.02	17.23
Arabinose	17.73	17.58	19.93	18.50	17.58	16.78
Fucose	18.55	18.17	19.38	18.67	18.17	17.53
Rhamnose	16.12	16.07	16.45	16.20	16.08	15.77

were performed on a HPX-65A (11 μm) and a HPX-87P (9 μm) (30 × 0.78 cm, Bio-Rad Labs.), used individually or connected in series. A pre-column from Waters Assoc. having an enlarged I.D. of 7 mm and equipped with flow-distributors was packed with a dry mixture of cation- and anion-exchange resins (10:15, mequiv.) and placed outside the column oven. The eluent reservoir was kept at 85°C. The eluent was filtered through a 0.45-μm Millipore filter.

Procedures

The sample clean-up according to Biggs and Szijarto[9] was carried out as follows. To a weighed amount of dairy product (*ca.* 2 g), 80 ml of distilled water and 25 ml of the Biggs solution were added, after which the volume was made up to 200 ml with water. The precipitate was filtered off (Whatman No. 40), the first 20 ml of the filtrate were discarded and 15 μl of the filtrate were injected.

The conversion of the HPX-65A column into the mixed-ion mode was carried out by pumping 400 ml of a solution of silver and lead(II) nitrate (total concentration $\approx$0.1 M) in the desired ratio (see Table II) through the column at room temperature. If the column developed a void at the top, it was carefully filled with a thick slurry of resin from an old HPX-87P column and converted again with the appropriate solution of silver and lead(II) nitrate.

RESULTS AND DISCUSSION

Since the determination of sugars is of interest not only for the analysis of dairy products, we have investigated the retention of twenty sugars on a silver-loaded intermediate cross-linked HPX-65A column at different column temperatures (Table I). Fig. 1 shows a separation at 85°C. Lower temperatures can be used with this type of column, because α and β anomers of glucose (oligosaccharide) are not resolved. Stachiose, melezitose, raffinose and sucrose showed multiple or broad peaks at temperatures above 25°C, owing to acid-catalysed hydrolysis due to sulphonic groups in the hydrogen form. Regeneration of the column with 0.1 *M* silver nitrate solution did not eliminate the decomposition of these sugars (Fig. 2). The analysis of samples containing one or more of these acid-sensitive sugars, can be performed only at room temperature. Under these conditions rather broad peaks are observed, owing to slower mass transfer. Moreover, the frequently occurring combinations lactose–glucose and galactose–fructose are not resolved.

For comparison we have analysed the same sugasrs on an HPX-87P column in the temperature range 65–95°C (Table I). Lower temperatures are not practical, because of increased separation of α and β anomers. The HPX-65A and the HPX-87P columns show different selectivities, and we have therefore investigated the possibility of using both columns connected in series. As a test sample a mixture of maltose, lactose, sucrose, glucose, galactose and fructose was used. The temperature of the HPX-65A was fixed at 25°C, but that of the HPX-87P was varied between 65°C and 95°C. Calculation of the resolution for critical pairs of sugars with the data of Table I and plate numbers did not suggest a satisfactory separation, which was confirmed by experiment.

We reasoned that the problem of acid-catalysed hydrolysis could be solved if the residual sulphonic groups in the hydrogen form of the HPX-65A column could

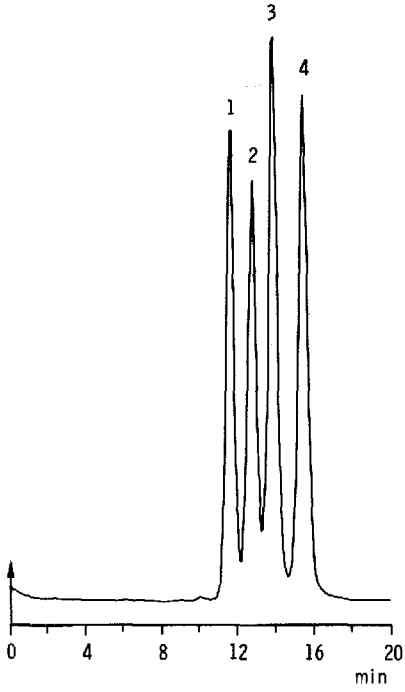

Fig. 1. Separation of maltose (1), lactose (2), glucose (3) and galactose (4) on an unmodified HPX-65A column at 85°C; flow-rate, 0.6 ml/min.

be converted into a neutral form, enabling its use at elevated temperatures for acid-sensitive sugars. The HPX-87P column does not display any sulphonic groups in the hydrogen form. Assuming that both resins are sulphonated in a similar way, resulting in the same number and distribution of sulphonic groups, the HPX-65A column should contain double the amount of silver ions in HPX-87P column, which contains lead(II) ions. The ionic radii of silver(I) and of lead(II) ions are comparable, which implies that the resin in the silver form contains twice the volume fraction, occupied by the ions, compared with the resin in the lead form, assuming that each lead(II) ion needs two sulphonic residues. Although the cross-linking of the resin used for the

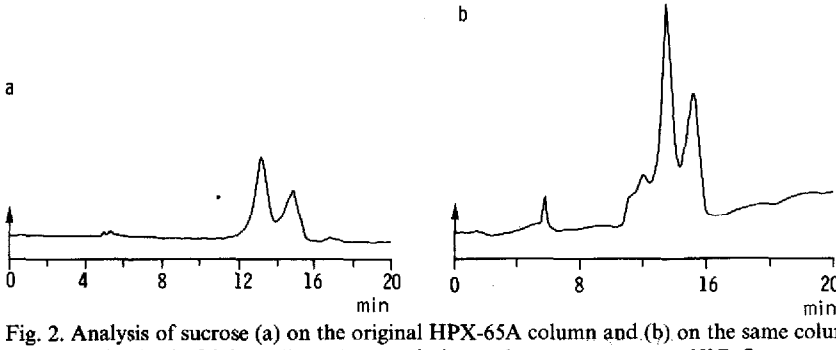

Fig. 2. Analysis of sucrose (a) on the original HPX-65A column and (b) on the same column after regeneration with 15 ml of 0.1 *M* silver nitrate solution; column temperature, 65°C; flow-rate, 0.6 ml/min.

HPX-65A is 6%, which is lower than for the HPX-87P (8%), the physical space might be insufficient to accommodate all the silver ions. Converting a cation-exchanger, which is necessarily partly in the silver form, by a small fraction into the lead form, could eliminate the presence of sulphonic groups in the hydrogen form, while maintaining the positive effects of the silver ions on the retention mechanism. This was accomplished by treating the HPX-65A column with mixtures of lead(II) and silver(I) nitrate solutions. Table II shows the retention of the sugars as a function of the column temperature and as a function of the percentage of lead(II) nitrate in the regenerating solvent. Acid-sensitive sugars such as sucrose and raffinose gave single, sharp peaks at 85°C, which proves the virtual absence of sulphonic residues in the hydrogen form. At 25°C some sugars show broad or double peaks, due to α and β anomer separation, which limits the use of the column at this temperature. The HPX-65A column, thus modified, remains stable for one week of continued use, provided that a mixed-bed ion-exchanger with excess anion-exchanger (see Experimental) is used. With the pre-column installed, a column temperature of 70°C and a flow-rate of 0.6 ml/min, a plate count of 3005 and an asymmetry factor of 0.85

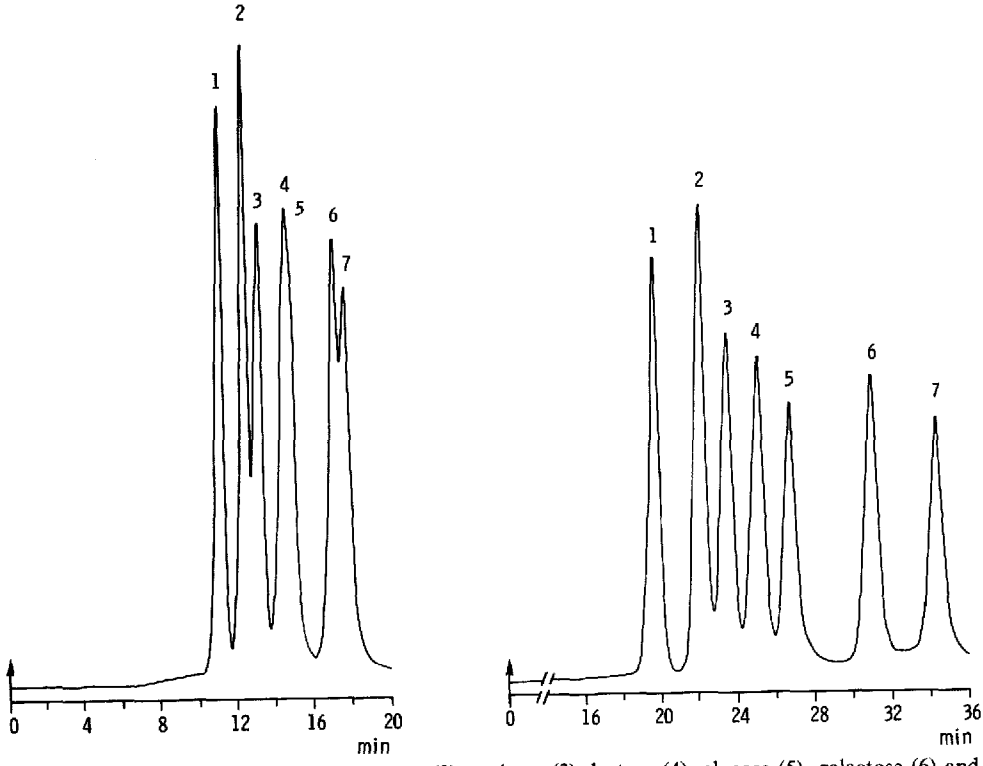

Fig. 3. Separation of melezitose (1), sucrose (2), maltose (3), lactose (4), glucose (5), galactose (6) and fructose (7) on a HPX-65A column, modified with 1.5 mol% lead(II)–silver nitrate solution; column temperature, 70°C; flow-rate, 0.6 ml/min.

Fig. 4. Separation of melezitose (1), sucrose (2), maltose (3), lactose (4), glucose (5), galactose (6) and fructose (7) on a dual column system, consisting of a modified HPX-65A column (see Fig. 3) and a HPX-87P column; column temperature, 70°C; flow-rate, 0.6 ml/min.

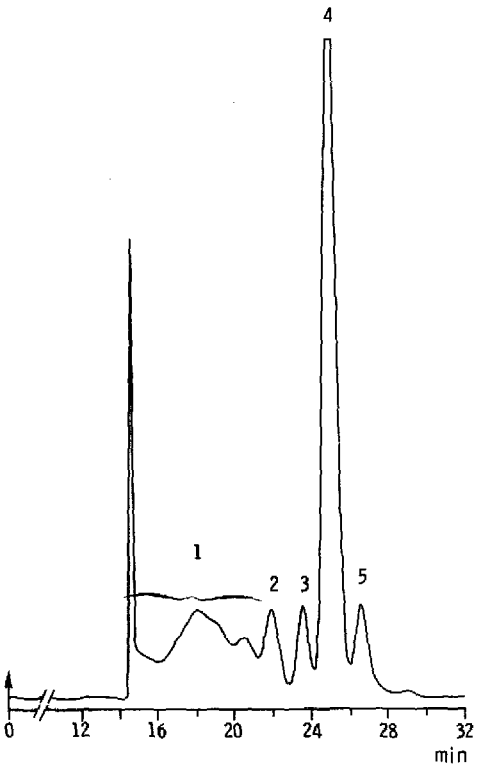

Fig. 5. Analysis of a baby-food containing lactose (4) and maltodextrins: malto-oligosaccharides (1), maltotriose (2), maltose (3) and glucose (5); 15 μl injection of a Biggs filtrate; for conditions see Fig. 4.

were obtained for lactulose. Without the pre-column these values were 3884 and 1.06, respectively. In this case the pre-column causes the peaks to front.

Fig. 3 shows a chromatogram of the separation of a mixture of sugars that occur frequently in dairy products. Melezitose was included as a suitable internal standard. Lactose and glucose are not resolved, and galactose and fructose are only partly separated. This cannot be improved by changing the column temperature (Table II). Although the selectivity for galactose and fructose increases with decreasing temperatures, the resolution does not increase, owing to peak broadening. The use of other ratios of silver to lead(II) did not improve the situation (Table II).

The problem of co-eluting pairs of sugars can be solved by connecting an HPX-87P column in series with a modified HPX-65A column, as was predicted by summing the retention times of the sugars on the individual columns (Tables I and II). If both columns are installed in the same oven, then the useful temperature range is dictated by that of the HPX-87P, being 65–85°C. The same set of sugars can be separated on this dual column system (Fig. 4) within 40 min. Two interferences might occur in the analysis of dairy products. Firstly, the presence of lactulose, which co-elutes with glucose. Lactulose is formed on strong heating of lactose-containing products. This reaction is strongly dependent on the pH. The presence of lactulose in dairy products can be determined with an HPX-87P column (Table I). Secondly, the

presence of maltotriose in sucrose-containing products. These sugars co-elute on the dual column system, as well as on the individual columns. However, maltotriose is well separated from other sugars (including maltotetraose) on an unmodified HPX-65A column. Fig. 5 shows the analysis of a baby-food, containing maltodextrins. For sample clean-up, the method of Biggs and Szijarto[9] has to be used since acetonitrile is retained strongly on the silver-loaded resin column.

CONCLUSION

We have found that a cation-exchange resin in the silver form with a cross-linking of 6% is suitable for the analysis of mono- and disaccharides. The column can be used at elevated temperatures, provided that sugars, which are sensitive to acid-catalysed hydrolysis, are absent. The use of this column in a mixed cation form can eliminate the adverse effects of sulphonic groups in the hydrogen form on acid-sensitive sugars. Moreover, it offers the additional possibility of influencing the selectivity of sugar separations by altering the ratio of the cations on the column. The analysis of sugars in foods has been improved by the use of a cation-exchange resin column in the silver–lead(II) form connected in series with a cation-exchange resin column in the lead(II) form. The presence of the combination of maltodextrins and sucrose, however, requires a separate analysis on an unmodified HPX-65A column. This is also the case when lactulose is present in heated dairy products, because it co-elutes with glucose.

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