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STUDY OF THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF REDUCING SUGARS, APPLIED TO THE DETERMINATION OF LACTOSE IN MILK

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SUMMARY

The loss of reducing sugars by formation of Schiff bases on amino-bonded silica or on dynamically coated amino-silica columns was investigated at two column temperatures. Depending on the type, age and temperature of the column, these losses were in the range of 0–100%. A dimethylamino-bonded silica column did not cause loss of reducing sugars, but the retention of sugars was too weak to allow separation. The analysis of reducing sugars can be carried out on diol-modified silica with diisopropylethylamine in the eluent to enhance mutarotation. It is also possible to use a cation-exchange resin (Ca^{2+}) column, equipped with a pre-column packed with a mixed-bed ion-exchanger to remove interfering salts and acids. In combination with an acetate–acetonitrile sample clean-up, this method results in coefficients of variation of less than 1% for the determination of lactose in skim-milk.

INTRODUCTION

High-performance liquid chromatography (HPLC) is applied routinely in many laboratories for the determination of sugars in food products[1,2]. Separations are performed mainly on silica, modified chemically with γ-aminopropyl groups[3], silica with[4–6] and without[7] an amine modifier and on cation-exchange resins (form mainly Ca^{2+}, with water as the eluent)[8–10]. The retention of and selectivity for sugars differ strongly between the cation-exchange resins and the columns based on silica. Resins separate on the basis of exclusion, complexation and hydrophobic adsorption mechanisms, resulting in the elution of higher saccharides before the lower ones. The (modified) silica columns separate by partition of the sugars between two liquid phases[11,12], leading to elution of the monosaccharides before the higher ones.

The use of HPLC for the accurate determination of sugars, *e.g.*, lactose, requires a stable chromatographic system giving low coefficients of variation. The determination of lactose in milk and milk products is normally carried out after removal of proteins and fat by precipitating agents[7,13,14]. The method of clean-up may lead to complications in the chromatographic separation, owing to the (in)organic ions

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of the buffer used. Reducing sugars like lactose, glucose and galactose, which may be present in dairy products, are able to react with the primary amino groups of amino-bonded silica and amine modifiers to give Schiff bases[15]. This reaction might be responsible for the comparatively short life of amino-bonded silica columns, depending on the type and the amount of sugars analysed.

The coefficient of variation of the HPLC analysis of lactose in dairy products has ranged from 2 to 5%[7,13,14,16,17]. We have tested the most promising methods, using the necessary precautions regarding detector noise[18]. It proved to be very difficult to achieve a coefficient of variation of less than 2%. If it is desired to use HPLC as a reference method, *e.g.*, for the calibration of infra-red milk analysers, not only a coefficient of variation of less than 1% but also an accurate level (*i.e.*, no systematic errors) is required.

This prompted us to re-investigate the merits of HPLC analysis of reducing sugars on six stationary phases in combination with two milk clean-up methods.

EXPERIMENTAL

Chemicals and materials

Reagent-grade acetonitrile (Chroma; Mallinckrodt, St. Louis, MO, U.S.A.) was used and mixed on a volume-to-volume basis with double-distilled water. 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, CA, U.S.A.) and anion-exchange resin (base form, AG 3-X4A, 200–400 mesh; Bio-Rad) were used to pack the pre-columns, and to desalt the samples. Tetraethylenepentamine (BDH, Poole, Great Britain) and N,N-diisopropylethylamine (Aldrich, Milwaukee, WI, U.S.A.) were used as received.

The clean-up solution of Biggs and Szijarto[19] 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.

The acetate–acetonitrile clean-up method made use of an acetate buffer, 0.01 *M* sodium acetate and 0.01 *M* acetic acid, adjusted to pH 4.60.

Apparatus

A Waters Model 6000 A pump with a Wisp 710B automatic sample injector was used in combination with a Model R401 refractive index detector and a Kipp Analytica 9222 column oven. Columns packed with silica were fitted in an aluminium block in the oven to equalize temperature fluctuations. Chromatograms were recorded and integrated with a Perkin-Elmer Sigma 15 B data system. The refractive index detector was held at constant temperature with a Haake F3 circulating waterbath at 35°C. The following columns were used: LiChrosorb-NH_2 and LiChrosorb-DIOL (25 × 0.46 cm I.D., 10 μm, Merck), Partisil PAC (25 × 0.46 cm I.D., 10 μm, Whatman) and Nucleosil 10 $N(CH_3)_2$ (25 × 0.46 cm I.D., 10 μm, Macherey, Nagel & Co.), all packed by Chrompack (Middelburg, The Netherlands); μBondapak carbohydrate (30 × 0.39 cm I.D., 10 μm) and a Radial Pak B cartridge in combination with a RCM-100 Module, all from Waters Assoc. (Milford, MA, U.S.A.); a HPX-87C (30 × 0.78 cm, 9 μm. Bio-Rad Labs.). The last column was used in combination with a pre-column from Waters Associates having an enlarged I.D. of 7 mm, packed

with a dry mixture of the equivalent amounts (mequiv.) of cation- and anion-exchange resins (see Chemicals and materials) and placed outside the column oven. The eluent reservoir for the HPX-87C column was kept at 85°C. The eluents were filtered through a 0.45-μm Millipore filter.

Procedures

The sample clean-up according to Biggs and Szijarto[19] was carried out as follows. To a weighed amount of milk ($\approx$70 g), 80 ml of water and 25 ml of the Biggs solution were added, and then made up to a volume of 200 ml with water. The precipitate was filtered off (Whatman No. 40), the first 20 ml of the filtrate were discarded and 20 μl of the filtrate were injected on silica-containing columns and 10 μl on the HPX-87C column.

The acetate–acetonitrile sample clean-up method was performed on a weighed amount of milk ($\approx$2 g), to which 10 ml of water and 15 ml of the acetate buffer were added. The volume was made up to 50 ml with acetonitrile. After standing for 15 min, the precipitate was filtered off (S & S 589.3), the first few millilitres of filtrate were discarded and 50 μl of the filtrate were injected.

RESULTS AND DISCUSSION

Separation of sugars

The analysis of reducing sugars on amino-modified silica is often accompanied by on-column formation of Schiff bases. This reaction might be the cause of coefficients of variation of 2% or more, and also of a rapid change in response factors. To study these phenomena we analysed a mixture of galactose, lactose and sucrose on LiChrosorb-NH_2, μBondapak carbohydrate and Partisil PAC columns at two column temperatures. The last two columns have been developed especially for the analysis of sugars and they might contain primary amino groups, but their exact composition is proprietary.

From Fig. 1 it is seen that the peak areas of the reducing sugars galactose and lactose decreased considerably on LiChrosorb-NH_2 with increasing column temperature. Formation of Schiff bases is more rapid at higher temperatures and, as expected, the peak area of the non-reducing sugar sucrose was not affected by a change in column temperature. Assuming on the basis of mass that the response factors are

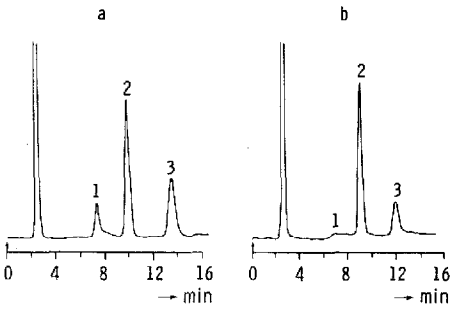

Fig. 1. Separation of galactose (1), sucrose (2) and lactose (3) on a LiChrosorb-NH_2 column at 30°C (a) and 40°C (b). Flow-rate: 1.5 ml/min. Eluent: acetonitrile–water (75:25).

TABLE I

LOSS (%) OF REDUCING SUGARS ON VARIOUS COLUMNS AT 30°C AND 40°C

Column	*Eluent, acetonitrile–water*	*Loss of galactose*		*Loss of lactose*	
		30°C	*40°C*	*30°C*	*40°C*
LiChrosorb-NH_2	75:25	67	95	37	39
μBondapak carbohydrate (new)	75:25	94	100	62	87
μBondapak carbohydrate (old)	75:25	2	5	<1	13
Partisil PAC	80:20	36	46	15	31
Radial Pak B	80:20*	88**		70**	
LiChrosorb-DIOL	85:15***	<1	<1	<1	<1

* The eluent contained 0.01% TEPA.
** Column temperature was 25°C.
*** The eluent contained 0.1% DIPEA.

approximately equal for these sugars, which was confirmed by experiments on diol-bonded silica (see below), we have calculated the loss of the reducing sugars (Table I). Obviously, these losses are the same for calibration samples, but sample matrix components and variations of the column temperature might adversely affect precision and accuracy. On old, much used columns the losses are considerably less (Table I). The much used combination of a silica column with an amine modifier in the eluent, such as tetraethylenepentamine (TEPA), also leads to considerable losses of galactose and lactose (Table I). TEPA contains two primary amino groups per molecule and reaction with reducing sugars can also occur in the eluent. Column performance does not decrease, however, because the surface amino groups are renewed continuously.

In order to eliminate the formation of Schiff bases we have investigated the separation of sugars on a silica column modified with non-reactive tertiary amino groups [Nucleosil $N(CH_3)_2$]. However, sucrose and lactose could not be separated with acetonitrile–water (90:10, $k' \approx 1.5$) as the eluent. Eluents having a higher acetonitrile to water ratio should not be used, owing to the very low solubility of sugars in these solvent mixtures.

The reaction of reducing sugars with amino groups can be avoided by using a silica column modified with diol groups. Fig. 2a shows the separation of the test

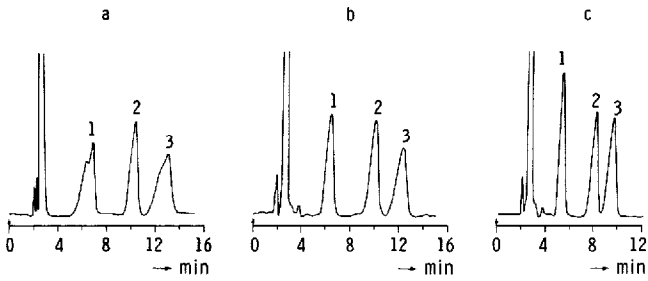

Fig. 2. Separation of galactose (1), sucrose (2) and lactose (3) on a LiChrosorb-DIOL column with acetonitrile–water (85:15) as the eluent; flow-rate 1.5 ml/min. a, 30°C; b, 30°C and 0.1% DIPEA in the eluent; c, 40°C and 0.1% DIPEA in the eluent.

mixture on LiChrosorb-DIOL. The peaks of galactose and lactose are, however, broad, due to mutarotation of these sugars. Mutarotation can be accelerated by increasing the pH[11] and the temperature. The limited stability of silica towards bases restricts its use to eluents with pH values below 9, depending on the nucleophilicity of the base present and the temperature. The optimum conditions regarding the enhancement of the mutarotation and column life were found for addition to the eluent of 0.1% of the tertiary amine diisopropylethylamine (DIPEA), a base having a very low nucleophilicity[20] and a column temperature of 35°C. Fig. 2b and 2c demonstrate that mutarotation is accelerated sufficiently without greatly affecting the retention times. Moreover, the peak areas of the reducing sugars galactose and lactose are not affected by a change of the column temperature (Table I). The response factors based on the masses of the sugars differed by less than 5%. The performance of this column under these conditions did not decrease significantly during several months of use, which implies that the silica is sufficiently stable towards this eluent and temperature.

Table II gives the capacity factors of several sugars on different columns. The LiChrosorb-DIOL column shows changes of selectivity compared with amine-bonded silica columns. Its use in combination with the eluent described resulted in a coefficient of variation of $<0.9\%$ ($n = 10$, lactose standard). If lactose is the only sugar present in the sample, then its retention can be decreased by increasing the amount of water in the eluent to 20%, and the use of DIPEA is then not necessary (Fig. 3). The inorganic salts in the sample were eluted before the peaks of interest.

The use of a cation-exchange resin (Ca^{2+}) column for the separation of various sugars is straightforward. No problems are encountered concerning the solubility of the sugars in the eluent used, *i.e.*, water. Moreover, due to the refractive index of water, the signal-to-noise ratio is improved by a factor of ten. For lactose a detection limit of 0.5 μg was obtained. The retention time and response factors showed very little variation ($<2\%$) during several months. Coefficients of variation for lactose of $<0.8\%$ could be obtained (ten injections). It is obvious that, to reach such low

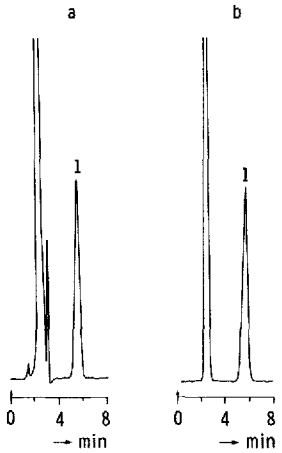

Fig. 3. Determination of lactose (1) in skim-milk. a, 10-μl injection of a Biggs filtrate (RI, × 16); b, 40-μl injection of an acetate–acetonitrile filtrate (RI, × 8). Column: LiChrosorb-DIOL. Eluent: acetonitrile–water (80:20). Column temperature: 35°C. Flow-rate 1.5 ml/min.

TABLE II

CAPACITY FACTORS, k', OF SUGARS ON VARIOUS COLUMNS

	LiChrosorb-NH_2	*μBondapak Carbohydrate (new)*	*Partisil PAC*	*LiChrosorb-DIOL*	*Radial Pak B*	*HPX-87C**
Column temperature (°C)	30	30	30	40	25	85
Eluent, acetonitrile–water	75:25	75:25	75:25	85:15 (+0.1% DIPEA)	80:20 (+0.01% TEPA)	0:100
Fructose	1.33	1.44	0.74	1.01	3.6	2.29
Glucose	1.75	1.92	0.84	1.37	5.2	1.59
Galactose	1.90	2.04	0.95	1.33	5.6	1.92
Sucrose	2.89	3.13	1.22	2.56	9.7	1.11
Lactulose	3.68	3.74	1.45	2.69	—**	1.42
Maltose	3.77	4.12	1.39	3.07	13.7	1.13
Lactose	4.23	4.52	1.58	3.21	15.8	1.22
Sorbitol	1.59	1.69	0.94	1.33	4.6	4.47
Mannitol	1.65	1.77	0.96	1.40	4.7	3.40

* For V_0 the volume of exclusion (4.13 ml) was chosen.

** Not determined.

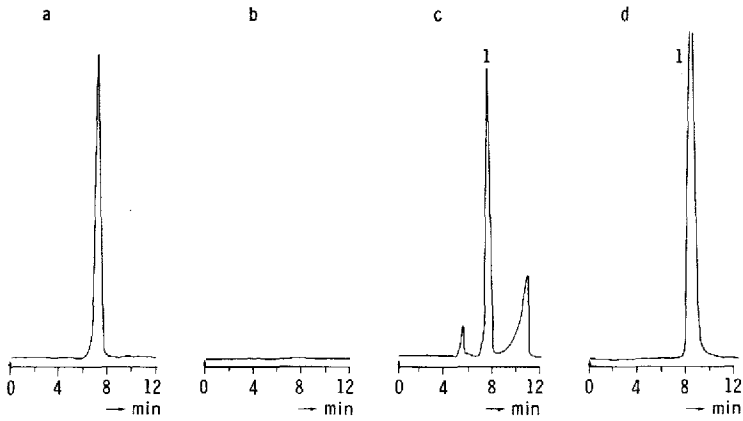

Fig. 4. a, 5-μl injection of 0.6% (w/w) sodium chloride solution on a HPX-87C column at 85°C, flow-rate 0.6 ml/min (water). b, 25-μl injection of the same solution, conditions as in a, but with a pre-column packed with a mixed bed ion-exchanger. c, 5-μl injection of a Biggs filtrate of skim-milk (1 = lactose), conditions as in a. d, 25-μl injection of the solution from c, conditions as in b.

coefficients of variation, the injector, pump and detector must be in excellent condition and the integration parameters should be chosen after careful evaluation.

The effect of injection of the Biggs filtrate and of a sodium chloride solution on a cation-exchange resin column is shown in Fig. 4a and 4c. Obviously, an accurate determination of sugars is impossible, due to a coincidence of peaks. A pre-column packed with a mixed bed ion-exchanger effectively removed the salts[21], while the plate number for lactose dropped from 3100 to 2500 as a result of insufficiently small particles of the ion-exchange resin used (Fig. 4b). Since the weakly basic ion-exchanger possessed tertiary amino groups, no loss of reducing sugars was observed. The pre-column should be installed outside the oven, otherwise decomposition of some sugars such as sucrose occurs. Recently, a pre-column (Microguard Anion 16H cartridge) packed with a basic ion-exchanger (Aminex A-25) has become available from Bio-Rad. The use of this pre-column has been described by Richmond *et al.*[22]. These authors noticed some losses of certain sugars. This is expected, because the pre-column contains a quaternary ammonium base, to which sugars have a limited stability at room temperature.

Sample clean-up

The clean-up of milk and milk products can be carried out by various methods. The precipitating agent of Biggs and Szijarto[19] gave rise to interfering peaks on amino-bonded silica columns and on the cation-exchange column (HPX-87C). Mixing of the filtrate obtained in this way with acetonitrile caused precipitation, leading to clogging of the pre-column. The analysis of sugars on amino- or diol-modified silica columns requires an injection solution, which should consist of a mixture of acetonitrile–water (*e.g.*, 50:50) in order to avoid peak distortion at larger injection volumes (> 25 μl). Therefore, a method was developed which employs an acetate buffer (pH 4.6) combined with acetonitrile. Fats and proteins are rapidly and quantitatively precipitated by this method. The presence of acetate does not interfere with the analysis on amino- or diol-bonded silica columns (Fig. 3b). When using a cation-exchange column, however, a small peak of acetate coincided with that of lactose. A large solvent peak (t_R = 20 min) was observed after the elution of the sugars. A pre-column packed with the mixed bed ion-ex changer eliminates the acetate peak

and also enables the Biggs precipitating agents to be used.

Richmond *et al.*[22] recently used a complicated and laborious method to isolate sugars from dairy products. In our opinion this method is not suitable for the analysis of large numbers of samples.

Applying the acetate–acetonitrile sample clean-up technique in combination with the cation-exchange resin or the LiChrosorb-DIOL column we could obtain coefficients of variation of $< 1\%$ for the determination of lactose in skim-milk. The recovery of lactose in skim-milk is better than 99.5%. Detailed results of this investigation in comparison with an enzymatic and a polarometric method will be published in due course.

CONCLUSIONS

Chromatography of reducing sugars on amino-bonded silica columns at two column temperatures demonstrated that extensive formation of Schiff bases can occur between these sugars and the stationary phase. Therefore, the widespread use of amino-bonded silica columns for the analysis of reducing sugars seems of doubtful value. This study shows that a precise analysis of reducing sugars is possible on a diol-modified silica column in combination with (if necessary) DIPEA in the eluent to enhance mutarotation, and on a cation-exchange resin (Ca^{2+}) column. The latter is preferred, because the signal-to-noise ratio is approximately ten times better than that for the diol- (or amino-) modified silica column. Pure water is used as the eluent, and the retention times and response factors show very little variation over long periods. The separation of disaccharides, such as lactose, sucrose and maltose, is, however, not possible on such a column. In such cases the use of a diol-modified silica column is necessary, or an amino-bonded silica column for non-reducing sugars.

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