Onderstepoort J. vet. Res., 53, 225–229 (1986)

A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE FLUORIMETRIC DETERMINATION OF LACTOSE, GALACTOSE AND GLUCOSE IN NORMAL AND ABNORMAL MILK OF COWS

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ABSTRACT

KOWALSKI, Z. E. & GIESECKE, W. H., 1986. A high performance liquid chromatographic method for the fluorimetric determination of lactose, galactose and glucose in normal and abnormal milk of cows. *Onderstepoort Journal of Veterinary Research*, 53, 225–229 (1986).

A high performance, liquid chromatographic, gradient method with post-column derivatization and highly sensitive fluorescence detection has been developed for the determination of reducing sugars, such as lactose, galactose and glucose, in normal and abnormal milk of cows. The method requires little sample preparation, is applicable to a fully automated HPLC system and has been found suitable for the processing of hundreds of samples. Peaks of minor constituent components of samples showed retention times identical with those of ribose, cellobiose, fucose, mannose and xylose.

Further research with the HPLC technique, supplemented with modern gas chromatography and mass spectrometry, is considered necessary for elucidating the role of minor carbohydrates in the physiology and physiopathology of the bovine udder.

INTRODUCTION

Carbohydrates play an essential role in the physiology of the bovine mammary gland (Linzell & Peaker, 1971; Bauman & Davis, 1974; Davis & Bauman, 1974; Ebner & Schanbacher, 1974; Saacke & Heald, 1974; Ahrne, Björck & Claesson, 1983). Their lacteal levels reflect the physiopathological status of the bovine udder (Mackie, Giesecke, Lück & De Villiers, 1977; Giesecke & Van den Heever, 1981; Giesecke, Durand & Petzer, 1984). The importance of the lacteal level of glucose to the functioning of the leucocytic udder barrier has been widely discussed (Jain & Lasmanis, 1978; Giesecke & Van den Heever, 1981; Giesecke *et al.*, 1984), and is apparently related to stress (Giesecke *et al.*, 1984; Giesecke, 1985) thought to affect udder health (Paape, Gwazdauskas, Guidry & Weinland, 1981; Roth, Kaeberle & Hsu, 1982; Giesecke *et al.*, 1984; Giesecke, 1985).

However, a detailed study of the latter aspect requires sophisticated laboratory technology. Because of the limitations of the enzymatic method used routinely at our laboratory for the determination of glucose in milk, non-enzymatic evaluation by means of HPLC was tried as an alternative method.

Preliminary experimental work indicated clearly that the sensitivity of refractive index measurements is satisfactory for lactose determination. It is not sufficient, however, for the quantitation of galactose, glucose and other reducing sugars which are present in minute quantities when non-concentrated deproteinized milk samples are processed. To solve this problem, one of the post-column derivatization procedures with highly sensitive fluorimetric detection (Honda, Matsuda, Takahashi & Kakehi, 1980; Kato & Kinoshita, 1980, 1982; Kato, Iinuma & Kinoshita, 1982; Mikami & Ishida, 1983) was employed. On the advice of Mr E. Okada[1] we used the Shimpack ISA-07/S2504 HPLC column[2], which had been successfully employed for the analysis of reducing sugars in human milk, using 5 % monoethanolamine in a 3 % boric acid solution as a derivatizing reagent (Shimadzu Application Notes 35 and 64). Because of the better linearity of the results obtained with arginine in the boric acid solution, we followed the derivatization procedure originally described by Mikami & Ishida (1983) for sugars separated on the Shimpack SCR-101 HPLC column with water as a mobile phase. It was further adapted as described below for the ISA-07/S2504 Shimpack column[2], using boric acid buffers as mobile phases in gradient elution.

Received 1 July 1986—Editor

MATERIALS AND METHODS

Experimental design

Work on the fluorimetric HPLC technique was conducted in several consecutive steps: (1) Preliminary chromatography under different HPLC conditions of single sugar standards at a concentration of 10 μM per mℓ each; (2) preliminary chromatography under different HPLC conditions of a combination of such standards mixed in equal proportions; (3) chromatography under definitive HPLC and derivatization conditions of the single sugar standards, one sample of each being analysed in 5 consecutive runs to determine the repeatability of retention times; (4) chromatography under the definitive HPLC and derivatization conditions of 4 deproteinized milk samples, each of which was analysed by means of 3 consecutive runs to determine the mean value, standard deviation and coefficient of variation of the retention times of the various identifiable sugars present and (5) practical testing of the technique on close to 1 000 milk samples processed for the purpose of research on bovine udder health. This will be discussed elsewhere.

Reagents

Analytical grade arginine, boric acid, glucose, lactose, xylose, potassium hydroxide, activated carbon and ethanol of LiChrosolv grade were obtained from Merck[3]. The standards of other sugars, such as ribose, fucose[4], D-maltose[5], galactose[6], cellobiose[7], rhamnose[8] and mannose[9], were products from various manufacturers.

HPLC grade water with a resistance of 18 mega-ohm was produced by means of the Barnstead[10] reverse osmosis and the NANO pure II four cartridge water purifi-

[1] Shimadzu LC Analytical Application Laboratory, P.O. Box 209, Shimjuku-Mitsui, Tokyo 160, Japan;

[2] Shimadzu, Tokyo

[3] Merck, Darmstadt; [4] Fluka, Buchs; [5] Riedel de Haën, Hannover; [6] BDH, Poole; [7] Sigma, St. Louis; [8] Eastman Organic Chemicals, Rochester, N.Y.; [9] Nutritional Biochemical Comp., Cleaveland, Ohio; [10] Barnstead, Boston; [11] Millipore, Bedford; [12] Waters Associates, Milford

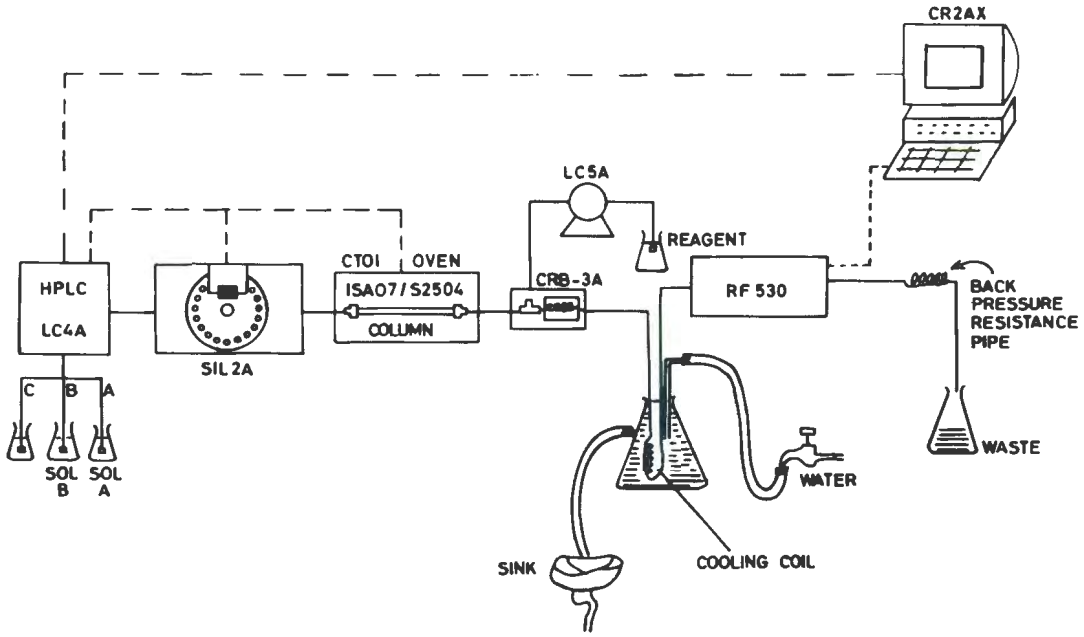

FIG. 1 Diagram of the instrumental configuration and flow of sample

cation system. The water was used for the final cleaning of glassware and the preparation of reagents.

Collection and preparation of milk samples

Foremilk samples were collected aseptically from udder quarters by means of a technique already described elsewhere (Giesecke & Viljoen, 1974). Immediately after collection each milk sample was treated as follows: a portion of 0,4 mℓ of milk was transferred to a plastic tube pre-filled with 0,6 mℓ of ethanol, vortexed for 2 min, sealed with a cap fitted to the tube, and stored at −20 °C for further processing. The final preparation of each sample involved the following steps: thawing at room temperature, vortexing for 1 min, centrifugation for 5 min at 12 000 rpm, transferring of 0,5 mℓ of supernatant to a clean tube, mixing with 0,025 g of activated carbon for absorbing lipophilic substances, vortexing for 2 min, standing for 5 min and re-centrifugation, removal of supernatant with a disposable syringe and filtration through a Millex HV filter[11] fitted to the syringe. The filtrate was then pipetted into a low volume insert[12] held by a spring in the automatic injector vial which was closed with a disposable teflon septum and cap.

After such routine preparations each sample was analysed by means of HPLC.

HPLC configuration

A range of HPLC equipment from the same manufacturer[2] was used in a configuration consisting of an LC4A chromatograph equipped with: micro-processor-controlled CTO-1 column oven holding a Shimpack ISA-07/S2504 column [25 cm long with an internal diameter (ID) of 4 mm]; high sensitivity dampener; SIL-2A automatic injector; dual monochromator fluorescence flow monitor RF-530; post-column reaction unit CRB-3A; LC-5A pump; Chromatopac CR-2AX data processor with chromatogram memory.

HPLC conditions

The flow rate of mobile phase = 0,5 mℓ per min; temperature of oven = 60 °C; composition of mobile phase (i) solvent A: 0,15 M of boric acid in water adjusted with KOH to pH 8,0 and (ii) solvent B: 0,45 M of boric acid in water adjusted with KOH to pH 9,0; auto-injection of 25 μℓ per sample.

Linear gradient elution programme

Time of run (min)	Concentration (%) of solvents in mobile phase A		B	
From 0	From	100 %	and	0 %
to 45	to	55 %	and	45 %
to 50	to	0 %	and	100 %
to 90*	to	100 %	and	0 %
to 120*		0 %		0 %

* From 90 to 120 min isocratic re-equilibration of column

Fluorescence monitor settings

Excitation 320 nm, emission 430 nm, high sensitivity.

Flow diagram of the HPLC system and conditions of derivatization

Effluent from HPLC column ISA-07/S2504 was transported by the LC-4A pump to the CRB-3A post-column derivatization unit where it was mixed with derivatizing reagent (2 % arginine, 5 % boric acid in water) delivered by the LC-5A pump at a flow rate of 0,5 mℓ per min; the reaction between sequentially eluted sugars and derivatizing reagent occurred in the temperature controlled (150 °C) reaction bath of the unit.

The mixture was then cooled in the cooling coil (1 m stainless steel tubing with ID of 0,3 mm) and directed through the fluorescence detector RF-530. The formation of bubbles in the tubing and in the detector was prevented by the action of a coiled, back pressure, resistance pipe (2 m standard HPLC tubing).

HPLC analysis

The complete HPLC system (Fig. 1) was set up, equilibrated and stabilized. The blank baseline of the gradient was memorized in the CR-2AX processor. The blank gradient values recorded were subsequently subtracted automatically from the values of each sample analysis. Graphs of the corrected analytical values therefore indicated the contents in the sample of the reducing substances, excluding changes in the solvent composition (due to gradient elution) and changes in the fluorescence intensity (due to borate concentration and solvent impurities). The system was calibrated at the start of

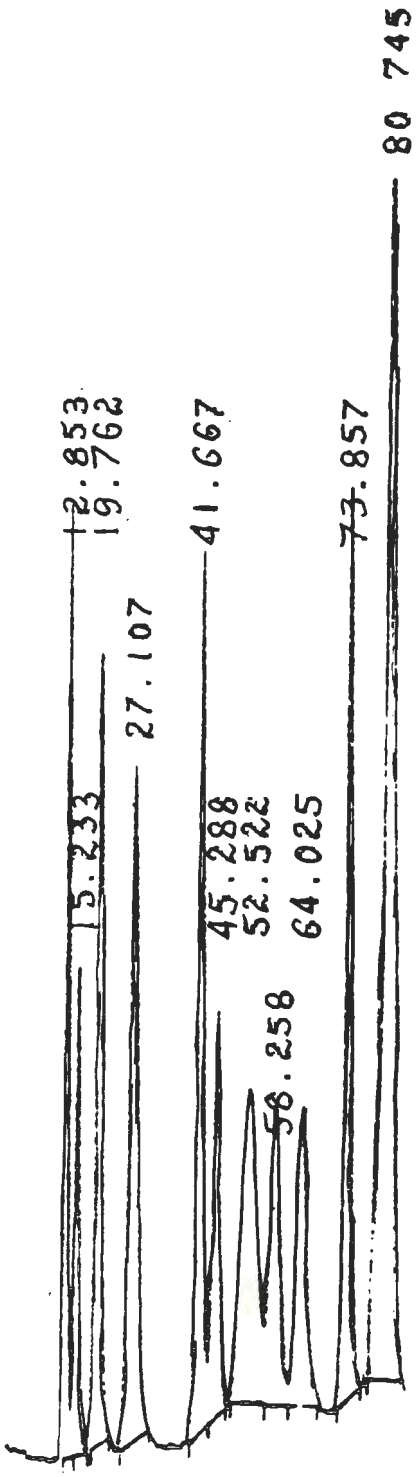

FIG. 2 Chromatogram of mixed sugar standards

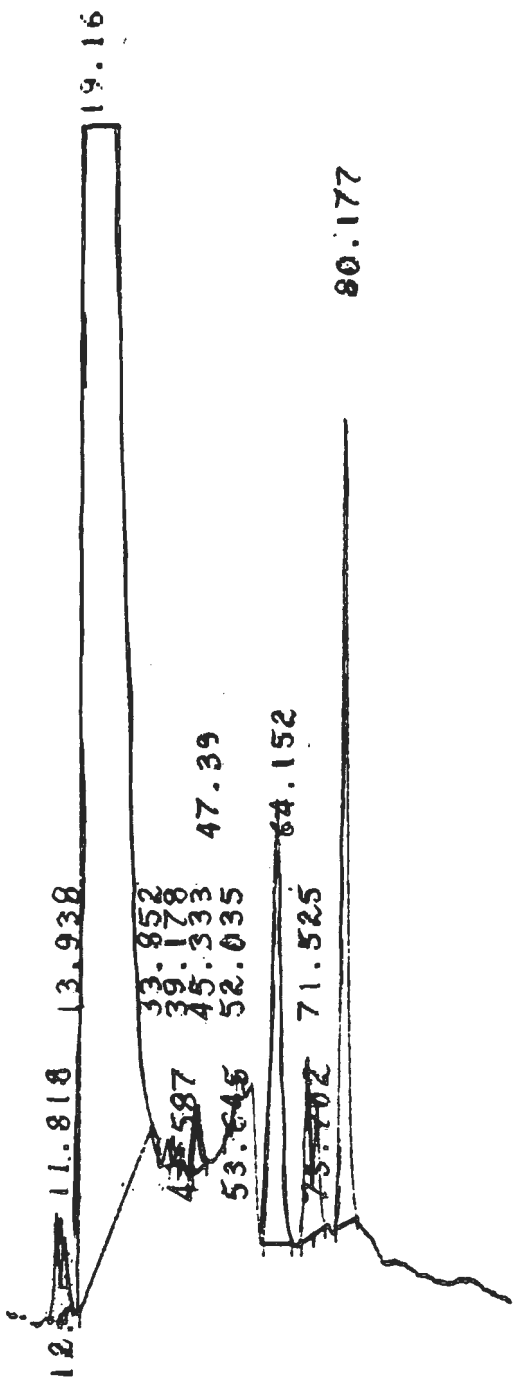

FIG. 3 Typical chromatogram of reducing sugars in a field sample of milk

each new batch of solvent and after each batch of 12 samples. The external standard method for quantitative analysis was used for determining lacteal levels of lactose, glucose and galactose.

RESULTS

Elution pattern and retention times of sugar standards

The elution pattern of the mixture of sugar standards (Fig. 2) shows that the sugars were distinctly separated. The retention time of each sugar eluted (Fig. 2) was within the range of the corresponding mean retention time determined for each single sugar standard (Table 1).

The results of the elution (Fig. 2) and the retention times (Table 1) of the sugar standards indicate that the HPLC technique under investigation may be regarded as methodologically satisfactory.

Elution pattern and retention times of reducing sugars in field samples of milk

The chromatogram (Fig. 3) of reducing sugars in milk shows major peaks for lactose, galactose and glucose, and minor peaks for cellobiose, ribose, mannose, fucose and xylose. The general pattern of elution is similar to that of the mixed sugar standards, however, (Fig. 2) and the mean retention times of the reducing sugars in milk (Table 2) differed from those of the single sugars (Table 1) by only 1,35 % on average.

TABLE 1 Retention times of single sugar standards

Sugar	Statistical designations × values of the retention time of each sugar standard		
	Mean value (min)	Standard deviation (±)	Coefficient of variation (%)
Cellobiose	12,83	0,02	0,16
Maltose	15,18	0,05	0,33
Lactose	19,68	0,07	0,36
Rhamnose	26,83	0,27	1,01
Ribose	41,51	0,12	0,29
Mannose	45,17	0,11	0,24
Fucose	52,33	0,14	0,27
Galactose	63,9	0,13	0,20
Xylose	73,8	0,04	0,05
Glucose	80,7	0,04	0,05

TABLE 2 Retention times of reducing sugars in 4 milk samples each analysed 3 times

Sugar	Statistical designations × values of the retention time of reducing sugars			Time windows required for quantitative determination (%)
	Mean value (min)	Standard deviation (±)	Coefficient of variation (%)	
Cellobiose	12,73	0,08	0,63	0,31
Maltose	—	—	—	—
Lactose	19,13	0,04	0,21	2,80
Rhamnose	—	—	—	—
Ribose	41,60	0,05	0,12	0,22
Mannose	45,34	0,01	0,02	0,38
Fucose	52,72	0,21	0,40	0,19
Galactose	67,14	0,04	0,06	0,38
Xylose	73,71	0,06	0,08	0,12
Glucose	80,17	0,03	0,04	3,05

TABLE 3 The coefficients of variation (%) of the concentrations of the 3 major sugars in each of the 4 field samples of milk analysed by means of 3 consecutive HPLC runs each

Serial number of milk sample	The 3 major reducing sugar in milk × coefficients of variation (%)		
	Lactose	Galactose	Glucose
1	3,26	7,45	5,99
2	1,28	0,91	0,12
3	1,27	13,34	1,82
4	1,46	0,09	2,31

The chromatogram (Fig. 3) and retention times (Table 2) therefore indicate that the HPLC technique may also be considered acceptable for application to bovine milk. This conclusion is further supported by the small coefficients obtained for variation of the concentrations of reducing sugars in each of the 4 field samples of milk analysed (Table 3).

From the data (Table 3) it is apparent that the variation within each sample during 3 consecutive evaluations demonstrate an acceptable repeatability of the HPLC technique. Differences between the corresponding values of the various samples suggest that, depending on the milk samples assessed, sugar concentrations may vary considerably.

Further analysis of approximately 1 000 milk samples from normal and abnormal udder quarters has shown that the procedure of sample preparation is practical and economical, whereas the HPLC technique evaluated may be used successfully for qualitative and quantitative determinations on reducing sugars in deproteinized milk. Because the quantitative analyses, even of the lowest lacteal values of lactose, galactose and glucose, were feasible, it was not thought necessary to establish the detection limit of the HPLC technique. It seemed justifiable, instead, to assume that the detection limit would be similar to that determined by Mikami & Ishida (1983).

From the point of view of research the HPLC technique described above is considered practical and very reliable for the unattended automatic processing of a large number of milk samples.

DISCUSSION

Chromatography has been applied successfully in the past to the analysis of carbohydrates in milk and milk products. Reineccius, Kavanagh & Keeney (1970), using gas chromatography (GC) and mass spectrometry (MS), have published results on lacteal values of lactose, galactose and glucose. Their work was follwed by Newstead & Gray (1972), Jaynes & Asan (1973) and Adachi & Yamaji (1978), who were mainly interested in developing the GC-technique for determining lactose without particular attention to the minor carbohydrates in milk and milk products. Their technique achieved the determination of lactose at 95,37 % accuracy. Because gas chromatography requires a complicated derivatization procedure to produce volatile carbohydrates, it is not an easy technique, and attempts were thus made to employ high performance liquid chromatography.

One of the first HPLC techniques for lactose estimations in milk and milk products was that developed by Euber & Brunner (1979), using the uBondapak carbohydrate column and acetonitrile/water as the mobile phase. Brons & Olieman (1983) introduced the HPLC column loaded with diol-modified silica or, alternatively, with cation exchange resin in Ca^{++} form, using acetate/acetonitrile and water, respectively, as mobile phase. Both techniques depend on a refractive index detector and are thus unsuitable for our work because prelinary experiments showed that this type of detector was not sufficiently sensitive for the detection in deproteinized milk samples of reducing sugars other than lactose. To improve the detection limit we assessed several derivatization techniques, including the one developed by Mopper & Grindler (1973) using bicinchoninic acid and copper salts as a post-column reagent and UV detection. Though highly sensitive, these techniques were found to be impractical because of problems such as instability of the derivatizing reagent proposed, bleeding of the column and possible blocking of the tubing with calcium salts.

Other methods considered were those developed in Japan, using 2-cyanoacetamide (Honda *et al.*, 1980), ethanolamine (Kato & Kinoshita, 1980), 2-aminopropionitrile (Kato & Kinoshita, 1982), taurine (Kato, Iinuma & Kinoshita, 1982) arginine in boric acid (Mikami & Ishida, 1983), and fluorescence detection. Only the derivatization procedure with ethanolamine has been used for the analysis of reducing sugars in human milk. Because of the better linearity of the results obtained with arginine in borate buffers, we decided to try the derivatization procedure described by Mikami & Ishida (1983). The promising results obtained with human milk assessed by means of the anion exchange column ISA-07/S2504 at the Shimadzu Application Laboratory[1], and the unsatisfactory resolutions obtained at our laboratory with the Ca^{++} loaded cation exchange fast carbohydrate column, prompted us to use the former column with borate buffers as mobile phase.

The high temperature of derivatization further limited the choice of the HPLC column to one suitable for water or water based buffers. We therefore had to use gradient elution to obtain a satisfactory separation of the compounds of interest.

Under these conditions, the selection of an internal standard became difficult because of its co-elution with

peaks of interest. Quantitative evaluations were thus performed, using the external standard method. It had the additional advantage of an elution pattern which differs from that obtained by gas chromatography. Hence, a broader spectrum of compounds can be analysed by means of a combination of the HPLC and GC techniques.

The HPLC technique developed gave accurate, precise and repeatable results and facilitated practical and reliable determinations of several major and minor reducing sugars in cow's milk.

The data from Ebner & Schanbacher (1974) suggest that lactose, galactose and glucose are the only significant free sugars in milk. Other sugars, such as mannose and fucose, which are structural components of the complex carbohydrates and glycoproteins in milk, have not been identified as free sugars in milk. Hence, questions arise as to the origin of the cellobiose, ribose, mannose, fucose and xylose found in milk during this investigation, and whether they are present in a free or bound form. The latter seems possible, because Reineccius *et al.* (1970), using very sophisticated GC-MS techniques, did not find such minor sugars in the milk examined. However, this negative result of the extensive work may also be related to factors such as: (i) incomplete separation by means of the GC packed column used; (ii) low recovery from the dialysis procedure; (iii) absorption on the ion exchange resin used for preliminary purification of the sample.

On the other hand, one cannot preclude the possibility that the minor sugars, in fact, are not free in milk but were released during this investigation from glycoproteins during the initial deproteinization with ethanol. Glycoproteins subjected to methanolysis (Tikhomirov, Khorlin, Voetter & Bauer, 1978) yielded carbohydrates, such as rhamnose, mannose, fructose, fucose, arabinose, xylose, melibiose, gentobiose and trehalose. Other workers have suggested that in humans the ratio of such sugars to blood protein increases in cases of carcinoma (Gehrke, Waalkes, Borek, Swartz, Cole, Kuo, Abeloff, Ettinger, Rosenheim & Young, 1979).

Because data on free carbohydrates other than lactose, galactose and glucose in bovine milk are almost non-existent, a satisfactory interpretation of the presence, importance and origin of the minor sugars determined during this investigation is impossible at present. Further research, using the above-described HPLC technique supplemented with modern GC-MS determinations, is required for elucidating the role of such carbohydrates in the physiology and physiopathology of the bovine mammary gland.

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