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Analysis of mono- and disaccharides in milk-based formulae by high-performance liquid chromatography with refractive index detection☆

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Abstract

A simple and reproducible method for the qualitative and quantitative analysis of free mono- and disaccharides (fructose, glucose, galactose, sucrose, lactulose and lactose) in milk-based formulae by high-performance liquid chromatography (HPLC) with refractive index (RI) detection was developed and validated. The method showed good linearity with determination coefficients exceeding 0.99. The limits of detection (DL) in these sugars were 0.17, 0.13, 0.06, 0.16, 0.05 and 0.25 mg/ml, respectively; and the limits of quantification (QL), 0.27, 0.24, 0.20, 0.26, 0.22 and 0.38 mg/ml. The relative standard deviations (R.S.D.s) for repeatability in fructose, sucrose, lactulose and lactose were 0.78, 0.99, 2.91 and 0.46 and the R.S.D.s for reproducibility were 4.8, 6.15, 7.04 and 2.49, respectively. Recoveries in all sugars were between 93 and 113%.
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1. Introduction

The industry has deployed considerable technological resources to bringing the composition of infant formulae closer to that of human milk [1]. Besides, it has developed several milk-based formulae for adults, e.g. for pregnant women [2–6]. Milk-based formulae can be based on any appropriate blend of proteins, carbohydrates, fats, minerals and vitamins. Milk powders are usually free-flowing agglomerates formed by spray drying, which extend the shelf-life of dried milk from several days to 18–24 months [7,8].

One of the industry's main problems is to control the stability of milk-based formulae, because they contain a lot of components that may interact. Milk powders are especially sensitive to Maillard reaction, as they contain a relatively high concentration of lactose and proteins with a high lysine level, besides the high temperature applied during the manufacturing process and their storage for long periods of time [9]. During heat treatment, lactose undergoes the Lobry de Bruyn-Alberda van Eckenstein rearrangement, which gives rise initially to isomeric disaccharides, mainly lactulose. As lactulose is not known to occur naturally in milk and is only formed in heated dairy products [10–14], it is a good indicator of heat damage in milk products. Changes may occur during the formulae powders' long periods of storage, even under appropriate storage conditions, and may even be greater than those caused by heat treatment in the production process. The result is, an unacceptable product. Many milk-based formulae contain sugars besides lactose, the evolution of mono- and disaccharides needs to be evaluated. Observation of product stability will help determine whether there are any differences between the same formulae during storage time and the shelf-life.

Many techniques have been developed in order to evaluate sugar fraction during the Maillard reaction. One of the methods is spectrophotometric [15,16], also in some studies lactulose is identified by enzymatic method [17]. The major disadvantage of these consists in the difficulty to evaluate simultaneously different sugars.

Another method developed to evaluate damage in milk powders is the capillary electrophoresis [9], which consist in monitoring the β-lactoglobuline of the whey pro-

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tein fraction. In spite of the promising use, for preparation of sample the caseins need to be precipitated with HCl overnight at 4 °C this mean a lot of time in analysis sample. Yet another method is the high-pH anion-exchange separation with pulsed amperometric detection (AEC–PAD) for evaluating monosaccharides as glucose, fructose and disaccharides as lactulose, lactose, sucrose and maltose. Kaine and Wolnik [18] studied sugars in infant formulae by high pH AEC–PAD, Cataldi et al. [19] gave a comprehensive overview of analytical applications in food for carbohydrate analysis by high-pH AEC-PAD.

One of the methods commonly used in sugar analysis is the gas chromatography (GC). Troyano et al. [20,21] developed a GC method. With this is possible quantifying glucose, galactose, *myo*-inositol, lactulose, *N*-acetylglucosamine, *N*-acetylgalactosamine and other derivatives. GC has been used in the study of milk [20,22], dried skim milk [23], in model systems containing protein-bonded lactose [24] and in pasteurized milk [25]. Valero et al. [13] determined the intensity of the heat treatment in milk pasteurized for the amount of lactulose formed by GC of the trimethylsilyl derivatives of the free sugar, besides monosaccharides were determined. Also in UHT milk [26] and in milk permeate GC has been used [27]. In spite of GC is a sensitive method for sugar analysis, sample preparation is laborious. Besides in the CG procedure the anomeric composition of α- and β-anomers is obtained which mean more than one area peak for each compound. The procedure is tedious to be used routinely.

Finally, in many studies, HPLC is used for its accuracy, separation abilities and rapidity [28,29]. It appeared more then 20 years ago, but remains one of the most widely used techniques. HPLC with refractive index (RI) detection is a powerful technique for quantifying various types of carbohydrate compounds. HPLC–RI was used for determining sugars (glucose, fructose and sucrose) in apple juice [30], disaccharides in whey permeate (lactose, galactose and lactulose) [31], oligosaccharides (fructose, glucose, sucrose, maltose and lactose) in plain cereals, sugar coated cereals, canned fruits, canned vegetables, crackers cookies [32]. HPLC–RI has also been used for determining sugars (sucrose, glucose and fructose), in fruit and drink samples [33], sugars in meat products [34], oligosaccharides in lactose–sucrose systems for determining sucrose inversion by invertase [35] and in sugar casein systems [36]. Martins et al. [37] studied the kinetic modelling of amadori *N*-(1-deoxy-D-fructos-1-yl)-glycine (DPG; intermediate in the early stages of the Maillard reaction) pathways in aqueous model systems, the quantification of D-glucose and D-manose was made by HPLC using an ion-exchange column (ION-300), and sugars were detected by monitoring the refractive index.

Although difficulties of using eluent gradients and relatively poor sensitivity associated with refractometry, HPLC–RI appears to be an economical, simple and fast method for determination of sugars. The aims of this study were to design and to validate an easy HPLC–RI method that separates the free sugar fraction from components such as proteins and other macromolecules that could create interference in the system; and to analyze qualitative and quantitative free mono- and disaccharides in milk-based formulae.

2. Experimental

2.1. Reagents and standards

The chemicals used for sample preparation were of analytical reagent grade: HPLC-grade, SDS acetonitrile and methanol (Peypin, France), HPLC-grade, Panreac absolute ethanol, Carrez I and Carrez II reagents (Barcelona, Spain), deionised water purified through a Milli-Q system (Millipore, Bedford, MA, USA). The standard sugars (fructose, glucose, galactose, sucrose, lactulose and lactose) came from Sigma (St. Louis, MO, USA), were >99% pure and were stored in a vacuum desiccator, with silica gel as desiccant, until use.

2.2. Samples

The method can be applied to any kind of milk-based formula: infant formulae, formulae for pregnant women, etc. In this paper, the samples used were an experimental formula for pregnant women, which contained according to the label milk powder, animal fat, fructose, sucrose, minerals and artificial aroma, and an infant formula (58% carbohydrates), whose ingredients were whole milk powder, lactose, minerals and vitamins.

Both were obtained from a firm in Barcelona, Spain. The formulae were stored at room temperature (25 °C).

2.3. Instrument

The chromatographic analyses were carried out in a Shimadzu high-performance liquid chromatograph equipped with a LC-10AD double pump, a 7725 Rheodyne manual injector (Cotati, CA, USA) with a 20 μL loop, a RID-6A Shimadzu refractive index detector and a C-R6A chromatopac integrator. Chromatographic separation was achieved with a Tracer carbohydrates column (5 μm particle size; 250 mm × 4.6 mm i.d.), and an NH_2 precolumn (13 mm × 3 mm i.d.), both from Tracer (Teknokroma, Barcelona, Spain).

2.4. Sample preparation

Six hundred milligrams of milk-based formula was weighed and transferred to a 25 ml volumetric flask. The sample was dissolved in approximately 10 ml ethanol–water (1:1, v/v). It was placed in a 60 °C water bath and stirred for 25 min until it dissolved completely. After cooling at room temperature, 250 μl Carrez I solution (stirred 1 min)

and 250 μl Carrez II solution (stirred 1 min) were added. Five milliliters of acetonitrile (HPLC-grade) was added. These reagents were used to precipitate the protein and non-sugar fraction. The solution was made up to 25 ml with ethanol–water (1:1, v/v) in a volumetric flask, then was left for 1 or 2 h until complete formation and precipitation of protein clot. The resulting solution was filtered through filter paper and passed through a C_{18} Sep-Pak Plus cartridge Waters (Milford, MA, USA) previously conditioned with 10 ml of methanol (HPLC-grade) and 10 ml of Milli-Q water. This filtered extract was forced through a 0.45 μm nylon filter Tracer (Barcelona, Spain) and was injected into the HPLC system.

2.5. HPLC–RI conditions and quantification

Chromatographic separation was undertaken with an isocratic elution mobile phase of acetonitrile–water (75:25, v/v) and degassed before use. The flow-rate of this eluent was 1.8 ml/min and the volume of the sample injected was 20 μl (filling the loop completely). Column temperature was maintained at 25 °C. Peaks were identified by comparing retention times with sugar standards. The respective peak areas were used for the quantitative analysis. Calibration curves for each sugar were prepared at seven levels, from 0.5 to 10 mg/ml for fructose, glucose, galactose and sucrose; 2–15 mg/ml for lactose; and 0.25–3 mg/ml for lactulose, all dissolved in ethanol–water (1:1, v/v).

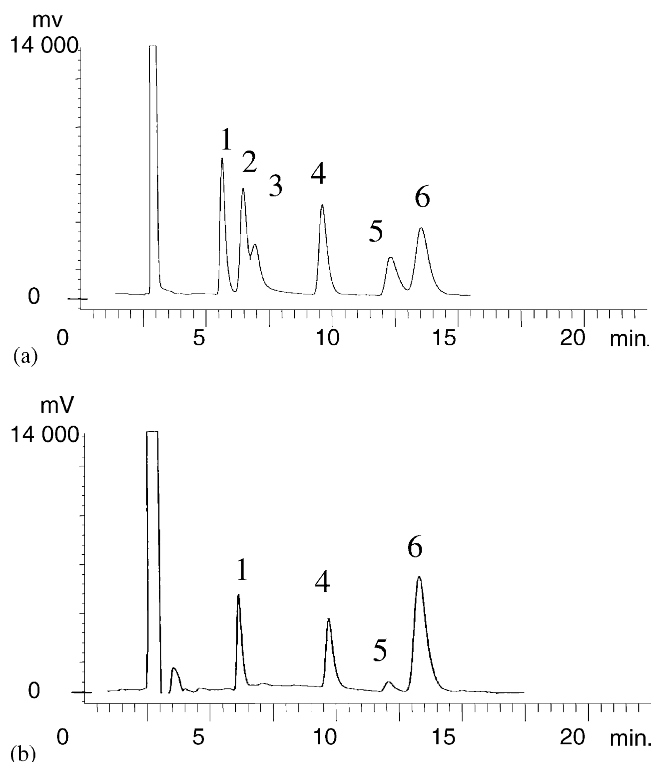

Fig. 1. Typical chromatogram of sugar analysis by the HPLC–RI method. See conditions in Section 2.5. Sugar peaks: 1, fructose; 2, glucose; 3, galactose; 4, sucrose; 5, lactulose; 6, lactose. (a) Saccharide standard content: 3 mg/ml fructose, glucose and galactose, respectively, 2 mg/ml sucrose, 1 mg/ml lactulose and 3 mg/ml lactose. (b) Formula for pregnant women sample, see contents in Table 3.

3. Results and discussion

HPLC–RI detection was used to determine fructose, glucose, galactose, sucrose, lactulose and lactose. Folks and Jordan [38] suggested as an appropriate mobile phase acetonitrile–water in the range 75:25 to 85:15. We experimented with 75:25, 80:20, 85:15 and 90:10 and found that with a 75:25 (v/v) the sugars eluted rapidly and the mobile phase provided better peak symmetry and acceptable separation peaks, except from glucose and galactose which were overlapped. Although this, glucose and galactose showed acceptable recoveries. Besides the analyzed samples not contain this sugars. Addition of galactose in milk-based formulae is not usual. Ferrerira et al. [1] determined sugars in infant formulae, follow-up milks and human milk, no galactose were founded in 50 samples studied.

The flow rate was 1.8 ml/min, with the following retention times: fructose, 5.8 min; glucose 6.8 min; galactose 7.4 min; sucrose 9.8 min; lactulose, 11.7 min and lactose 13.7 min (Fig. 1).

Ethanol is used to extract sugars in several analytical methods. We used a mixture of ethanol–water (50:50, v/v). The ethanol extracts contained high amounts of soluble non-sugar components. For this reason, reagents such as the Carrez solutions are needed to precipitate the compounds.

In previous analyses, when the samples were injected into the HPLC system, the mobile phase (acetonitrile 75%) precipitated the remains of the non-sugar component in spite of the addition of Carrez reagents. This caused, after injection into the HPLC system, a slight interference in the sugar peaks. For this reason, 5 ml of acetonitrile were added to the sample in order to completely precipitate all the substances which may interfere with the mobile phase (acetonitrile–water, 75:25, v/v) after HPLC injection.

In addition, Carrez solutions were reduced from 1 to 0.25 ml because the smaller volume is enough to precipitate and rid the solution sample of substances that might interfere with the sugar analysis. An excess of Carrez solution causes instability baseline after injection of several samples into the HPLC system, which interferes with the saccharide analysis.

3.1. Validation of proposed method

3.1.1. Linearity

Under the chromatographic conditions described above, a linear relationship between the concentrations of sugars (fructose, glucose, galactose, sucrose, lactulose and lactose) and RI was found. For all these sugars, the r^2 values were >0.99 at seven levels (Table 1).

Table 1
Linearity in sugars by RI detection

Compound	Range (mg/ml)	r^2 [a]	Equation curve[b]
Fructose	0.5–10	0.998	$y = 563898x - 73672$
Glucose	0.5–10	0.998	$y = 514950x - 43672$
Galactose	0.5–10	0.997	$y = 413570x + 259.88$
Sucrose	0.5–10	0.999	$y = 570340x - 68877$
Lactulose	0.25–3	0.999	$y = 495929x - 10673$
Lactose	2–15	0.998	$y = 399859x - 66979$

[a] Determination coefficient.
[b] x: concentration (mg/ml); y: peak area.

Table 2
Detection (DL) and quantification limits (QL) in sugars by RI detection

Compound	DL (mg/ml)	QL (mg/ml)
Fructose	0.17	0.27
Glucose	0.13	0.24
Galactose	0.06	0.20
Sucrose	0.16	0.26
Lactulose	0.05	0.22
Lactose	0.25	0.38

3.1.2. Sensitivity

To check the sensitivity of this method both the detection limit (DL) and the quantification limit (QL) were studied according to the USP criteria [39]. DL and QL was determined by the chromatographic noise obtained for a blank of ethanol–water (1:1, v/v) through the method and injected under the HPLC conditions cited. The resulting standard deviation of areas was used to determine DL and QL. Results obtained showed acceptable sensitivity (Table 2).

3.1.3. Precision

To evaluate the repeatability of the method, six replicate determinations were carried out on the same day. For reproducibility, six determinations with the same reference (formula for pregnant women) sample on different days were done. The standard deviations and relative standard deviations (R.S.D.s) show good precision (Table 3) within the limits of acceptable variability in methods of analysis [40].

3.1.4. Recovery

The sugar standards fructose, sucrose and lactose which are the principal saccharides in the formula for pregnant women were added in a known mass at two levels in a previously analyzed formula. The trial was in duplicate and the samples injected in triplicate into the HPLC system (Table 4).

Table 3
Precision of the method

Compound	Repeatability ($n = 6$)			Reproducibility ($n = 6$)		
	Mean (g/100 g)	S.D.[a] (g/100 g)	R.S.D.[b] (%)	Mean (g/100 g)	S.D.[a] (g/100 g)	R.S.D.[b] (%)
Fructose	12.61	0.10	0.78	13.45	0.65	4.8
Sucrose	8.58	0.09	0.99	8.67	0.53	6.15
Lactulose	0.9	0.03	2.91	0.87	0.05	7.04
Lactose	16.39	0.08	0.46	16.04	0.40	2.49

Experimental formula for pregnant women. See chromatogram in Fig. 1.
[a] S.D.: standard deviation.
[b] R.S.D.: relative standard deviation.

Table 4
Results of the recovery of sugars

	Recovery (%)					
	Fructose	Glucose	Galactose	Sucrose	Lactulose	Lactose
Level 1	104 ± 4	110 ± 5	107 ± 4	99 ± 5	95 ± 4	110 ± 4
Level 2	108 ± 0.7	113 ± 5	103 ± 8	101 ± 1.8	95 ± 2.3	93 ± 3

Level 1 (mg/g): 27 of fructose, 6.25 of glucose, galactose and lactulose, respectively, 20 of sucrose and 40 of lactose. Level 2 (mg/g): 40 of fructose, 26 of glucose, galactose and lactulose respectively, 35 of sucrose and 70 of lactose. The results are expressed as mean values ± standard deviation ($n = 6$).

4. Conclusion

The results of sugar analysis in the experimental formula for pregnant women sample are given in Table 3, containing 13% of fructose, 9% of sucrose, 0.9% of lactulose and 16% of lactose. The infant formula sample analyzed only contain lactose (57.21 ± 0.2%). The establishment of thermal parameters, defined under specific temperature/time conditions, contributes to the classification of heat treated milks. These thermal parameters are mainly employed to identify and optimize processes, assess heat-loads and identify the degree of thermal damage. Lactulose was proposed by the International Dairy Federation [41] and the European Union [42] as parameter capable of differentiating between UHT milk and in-container sterilized milk. Both international bodies suggested 600 mg/L of lactulose as marker for distinguishing between the two milk types, so as to guarantee the quality of UHT milk and between 600 and 1400 mg/L for sterilized milk; experimental formula for pregnant women are in this last range. However, no limit to lactulose content in infant formulas and/or milk-based formulas has been established.

We developed a simple and reproducible HPLC–RI method to characterize and quantify the free sugar fraction. This method is suitable for routine analysis of mono- and disaccharides in milk-based formulae, in order to monitor the evolution of the compounds, and possible adulteration and stability in the sugar fraction of the formulae. The method provides acceptable precision, recovery and sensitivity.

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