A RELIABLE GAS CAPILLARY CHROMATOGRAPHIC DETERMINATION OF LACTULOSE IN DAIRY SAMPLES

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Abstract

A gas capillary chromatography method for the determination of lactulose has been developed. The method has been evaluated for precision and accuracy using phenyl-β-D-glucoside as internal standard with satisfactory results and, then, applied to 27 commercial milk samples (pasteurized, UHT, sterilized, powder, condensed and chocolate-based milks). Results showed that it was suitable for the determination of lactulose in milks subjected to heat treatments of different intensity, giving good chromatographic resolution, as well as precise and reproducible results. Thus, lactulose levels found in pasteurized, UHT, sterilized and reconstituted powder milks were similar to those previously reported. In addition, this method provided a good separation between sucrose and lactulose/lactose peaks which allowed the suitable quantification of lactulose in samples such as condensed and chocolate-based milks which contain a high concentration of sucrose.

Introduction

Lactulose (4-O-β-D-galactopyranosyl-D-fructofuranose) is a disaccharide formed by isomerization of lactose in basic media and during heat treatment of milk [1]. It has been proposed as a chemical indicator capable of distinguishing UHT and sterilized milks [2, 3]. Also, having a sensitive analytical method for its detection, lactulose could be used for differentiating between UHT and pasteurized milks [4-6], as well as within different types of UHT and pasteurized milks either in combination with other indicators [7] or by itself [8].

The main analytical methods used for determination of lactulose are based on gas [9] and liquid [10] chromatography and enzymatic procedures [11, 12]. The main drawback of the official enzymatic method [11] is the requirement of six different enzymes to avoid the effects of glucose interference making the assay very time-consuming and expensive. Recently, other enzymatic methods exhibiting a higher sensitivity have been developed [8, 12]. Nevertheless, the main difficulty for the accurate measurement of low levels of lactulose using these methods is the potential interference from any free fructose which might originally be present in some types of milk. This point is particular important in special milks such as chocolate-based milks or condensed milks which contain considerable amount of sucrose and, consequently, may contain fructose.

As carbohydrates have neither chromophore nor fluorophore groups, lactulose determination by liquid chromatography is normally attained by isocratic separations with refractive index detection giving, thus, a relatively high detection limit [10]. Also, very time-consuming HPLC methods using a post-column labelling of lactulose

compatible with gradient elution have been developed [13, 14]. Although derivatization is necessary, gas chromatography provides very good separation of lactulose from other carbohydrates and the sensitivity can be higher than in HPLC methods [15]. Despite capillary columns provide higher efficiency, the presence of huge amounts of lactose in milk can explain the fact that laboratory-prepared packed and micropacked columns, which have a larger sample capacity, have been widely used for lactulose determination [9, 16-19]. Nevertheless, the GC quantitative determination of low amounts of lactulose, as those present in pasteurized and powder milks, using these micropacked columns can be inaccurate as consequence of the high content of lactose which can led to an overlapping with the lactulose peaks. Thus, a 48 hours treatment with ethanol of milk samples for partial removal of lactose was proposed prior to analysis [20].

In this paper we describe a reliable, sensitive and highly selective gas chromatographic method using a commercial capillary column for lactulose determination in milk samples, including special milks, subjected to different heat treatments.

Experimental

Materials

Chemicals

Reagents employed for GC analysis including sugar standards (lactose, lactulose, sucrose), internal standard (β-phenyl-glucoside) and derivatising reagent (*N*-trimethylsilylimidazole) were obtained from Sigma (St. Louis, USA). N,N-Dimethylformamide 99% was from Merck (Darmstadt, Germany). Ultrapure water quality with 1 – 5 ppb TOC and < 0.001 EU/mL pyrogen levels (Milli-Q) was produced in-house using a laboratory water purification Milli-Q Synthesis A10 system (Millipore, Bellerica, Mass., USA) and was used throughout.

Milk samples

A total of 27 commercial samples were purchased from local stores: 10 UHT, 6 pasteurized, 3 chocolate-based, 3 condensed, 2 sterilized, 2 powder milks and 1 powder dairy product. All samples were analysed before the recommended expire date. Powder milk samples were reconstituted with deionised water to 10% total solids before treatment. In addition, two samples of bovine raw milk, obtained from a local farm, were also analysed. The pH of all samples was measured in a MP 225 pH meter with glass electrode (Mettler-Toledo GmbH, Schwerzenbach, Switzenbach, Switzerland).

GC analysis

Sample preparation

1 mL of milk sample was added with methanol up to 10 mL in a volumetric flask to remove proteins and fats. Mixtures were gently stirred followed by standing for at least 1 hour at room temperature until the supernatant became transparent. Supernatants were employed for carbohydrate analysis and a solution of 0.01% (w:v) phenyl-β-D-glucoside in methanol/water (70:30, v/v) was added as internal standard. Prior to derivatization equal volumes (0.5 mL) of supernatant and internal standard were mixed and dried at 38-40ºC in a rotavapor.

Derivatization and GC analysis

0.1 mL of N,N-Dimethylformamide were added to the dried mixtures and held at 65ºC for 1 hour to obtain a constant anomeric composition. Then, 100 μL of *N*-trimethylsilylimidazole were added to sylilate the carbohydrates and the reaction was completed in 30 minutes at 65ºC. The reaction is stopped by cooling and the sylilated carbohydrates extracted with 0.1mL of hexane and 0.2 mL of water. Volumes in the range of 1-2 μL of the organic phase containing sylil derivates were injected onto the column. To study the response factor relative to the internal standard, standard solutions containing lactulose and lactose were prepared. The identity of lactulose and lactose present in milk samples was confirmed by comparison with relative retention times of standard samples.

The trimethylsilyl ethers were separated using a commercial 30 m x 0.32 mm inside diameter, 0.5 μm film fused silica capillary column SPB^{TM}–17, bonded, crosslinked phase poly (50% diphenyl/50% dimethylsiloxane) (Supelco, 595 North

Harrison Road, Bellefonte, PA, USA). Separation was performed at 235ºC for 9.5min, followed for an increase up to 270ºC at rate of 20ºC/min and keeping this temperature for 5 minutes. Temperature of injector and detector was 300ºC during the analysis. Injections were carried out in split mode (1:50-1:60). Data was acquired by means of HP ChemStations (Hewlett Packard, Wilmington, DE. USA).

Results and Discussion

Fig. 1 illustrates the GC profiles of raw, raw with added lactulose (20.8 mg L^{-1}) and chocolate-based milks. In all samples analysed, the separation was accomplished in 12 minutes with excellent chromatographic resolution of the lactulose and lactose peaks. The signal corresponding to lactulose comprised a narrow and symmetrical peak preceded by a minor and very broad peak (Fig. 1C), indicative of the presence of several anomeric forms not resolved by GC [6]. However, the equilibration with N,N-dimethylformamide before derivatization allowed to obtain a constant anomeric composition. Another advantage of this method is the separation of sucrose from lactulose and lactose peaks (Figs. 1C). The GC peaks of these carbohydrates frequently overlapped using the methods described in the literature [6, 9, 16, 21].

Prior to quantification of the lactulose and lactose in milk samples, the suitability of the method was evaluated. The response factors obtained for each disaccharide relative to the internal standard determined over the expected operating range of lactose (50 g L^{-1}) and lactulose (range 10.4 mg L^{-1} – 2080 mg L^{-1}) were $0.86_{\pm 0.07}$ and $1.08_{\pm 0.09}$, respectively. The precision of the entire method was determined by analysing the lactulose content in the same UHT milk sample within the same day (n=12) and in 5 different days (n=10), obtaining a relative standard deviation of 3.7% and 5.1%, respectively. Finally, to evaluate the accuracy of the method, known amounts of lactulose in the range of 10.4 to 520mg L^{-1} were added to raw milk samples. As shown in Table 1, satisfactory recoveries were obtained for the whole range studied.

Table 2 summarises the concentration of lactulose and lactose found in the commercial samples analysed. This method was sensitive enough to detect and quantify

lactulose in all samples, including pasteurized milks. A clear differentiation was obtained among pasteurized, UHT and sterilized milks with levels ranged from 13.0 mg L^{-1} to 32.1 mg L^{-1}, 95.6 mg L^{-1} to 437.1 mg L^{-1} and above 622.7 mg L^{-1}, respectively. These contents were within the ranges reported previously [4, 6, 20, 22-28].

The levels of lactulose found in powder milk samples were considerably lower than in UHT milks as previously reported [24] indicating the milder processing conditions to which powder milk is subjected in the industry. Moreover, the isomerization of lactose is relatively slow in the solid state [29], being the condensation with proteins via the Maillard reaction the predominating reaction of lactose during the storage of milk powders [24, 30, 31].

Regarding lactulose levels in condensed and chocolate-based milk samples, to our best knowledge, no data are available in the literature. This can be explained by the fact that sucrose and lactulose/lactose peaks were not resolved with the GC methods described for the lactulose determination [6, 9, 16, 21]. Thus, the lactulose levels determined for condensed milks were within the range observed for UHT milks, whereas the levels detected in chocolate-based milks were even higher than those found in the sterilized milks. This can suggest a very severe heat treatment, specially in samples no. 23 and 25 which are labelled as UHT products (Table 2). Interestingly, the pH of all milk samples was within the normal values (6.6-6.8) except for the sample no. 25 which had a pH value of 7.3 and, consequently, unusually high levels of lactulose (1955.3 mg L^{-1}). Martínez-Castro and Olano [18] indicated that at pH values above 7 the formation of lactulose increased markedly during heat treatment of milk.

In view of these results, it can be concluded that the proposed method allows a reliable quantitative determination of lactulose by gas capillary chromatography in

commercial milk samples subjected to different heat treatments. In addition, the separation of sucrose from lactulose and lactose enables the correct determination of lactulose in milks which contain a high amount of sucrose such as condensed and chocolate-based milks.

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Table 1. Recoveries (%) of different amounts of lactulose added to raw milk analysed by GC.

Added lactulose ($mg\ L^{-1}$)	Recovered lactulose ($mg\ L^{-1}$)	Recovery (%)
10.4	11.5 (14.2%)*	110.6
20.8	20.3 (6.5%)	97.6
52	46.8 (3.5%)	90.0
104	94.6 (6.9%)	91.0
208	186.4 (2.8%)	89.6
520	480.5 (2.0%)	92.4

* Relative standard deviation in brackets, n=4

Table 2. Lactulose and lactose contents of different commercial milk samples.

Type of milk	Samples	Lactulose (mg L^{-1})	Lactose (g L^{-1})
Pasteurized	1	14.2 (9.9%)*	44.9 (6.6%)
	2	14.1 (9.4%)	51.3 (6.8%)
	3	13.0 (11.8%)	52.2 (11.8%)
	4	20.2 (8.0%)	44.0 (7.9%)
	5	32.1 (7.6%)	46.9 (2.5%)
	6	27.7 (13.1%)	53.1 (9.2%)
UHT	7	437.1 (5.9%)	52.0 (5.3%)
	8	162.6 (5.8%)	47.3 (1.5%)
	9	307.0 (9.4%)	46.9 (7.4%)
	10	384.1 (6.1%)	51.6 (7.7%)
	11	178.1 (7.6%)	53.6 (9.1%)
	12	186.6 (4.0%)	46.9 (1.3%)
	13	331.7 (2.9%)	48.7 (1.1%)
	14	95.6 (5.9%)	51.1 (4.5%)
	15	327.9 (5.4%)	49.2 (4.7%)
	16	171.9 (3.7%)	50.1 (5.6%)
Powder dairy products	17	37.4 (8.8%)	80.8 (3.8%)
Powder (reconstituted)	18	24.2 (13.2%)	63.1 (4.8%)
	19	48.8 (1.0%)	53.1 (5.6%)
Condensed	20	207.5 (9.8%)	78.0 (3.7%)
	21	301.4 (9.4%)	50.7 (6.9%)
	22	313.3 (6.3%)	88.2 (14.2%)
Chocolate-based	23	1064.7 (7.9%)	45.7 (6.2%)
	24	758.2 (7.0%)	44.1 (5.3%)
	25	1955.3 (4.5%)	46.0 (6.8%)
Sterilized	26	705.2 (8.2%)	47.7 (8.4%)
	27	622.7 (2.5%)	48.7 (2.3%)

* Relative standard deviation in brackets, n=4

Legends of the figures

Figure 1. Gas cromatography profiles of trimethylsilyl derivatives of disaccharides from (A) raw milk, (B) raw milk with 20.8 mg L^{-1} of added lactulose and (C) chocolate-based milk. 1= phenyl-β-D-glucoside (internal standard), 2= lactulose, 3= sucrose, 4 and 5= lactose.

Figure 1. Montilla et al 2005

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