Research Article

Simultaneous Determination of Lactulose and Lactose in Conserved Milk by HPLC-RID

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Heat treatment is applied to dairy products to ensure microbiological quality and increase the shelf life. However, a suitable control of this process is necessary to guarantee nutritional and sensory quality. The aim of this study is to adapt the high performance liquid chromatography (HPLC) method for determination of lactulose and lactose content in commercial samples of UHT and sweetened condensed milk. The HPLC method used showed a good resolution of the analytes evaluated. The analyzed UHT milk samples presented levels for lactulose in accordance with the limit recommended by the International Dairy Federation. There was no significant variation in lactulose concentration for sweetened condensed milk samples. However, one sweetened condensed milk sample showed lactose level lower than the established values (10–12%).

This paper is dedicated to the memory of Professor Joab Trajano Silva, Ph.D.

1. Introduction

According to Brazilian legislation, milk is a product obtained from full and uninterrupted milking, in conditions of hygienic, healthy, well-fed, and rested cows [1]. It is a highly nutritious food to ensure the supply of essential nutrients, especially for children. Since the most remote times, dairy animals have been domesticated for this purpose [2]. According to national and international organizations, Brazil is the fourth largest milk producer in the world, with production of 32,091 billion liters in 2011, staying behind only the United States, India, and China [3, 4].

One of the challenges of the milk industry is the conservation. Nowadays many technologies to achieve this purpose exist. However, the ultrahigh temperature (UHT) treatment is applied in most dairy products to guarantee their microbiological safety and increase the shelf life [5]. Another method commonly used is the concentration; in this method, the milk is preserved with a considerable reduction of water content by evaporation. If a large quantity of sucrose is added, the product is called sweetened condensed milk [6, 7].

A proper control of the thermal process in both methods is important to ensure their nutritional and sensory quality since their effect on milk constituents is fundamental in

FIGURE 1: Basic structure of lactose and lactulose.

the characterization of the final product [8, 9]. The severity of thermal treatment can lead to degradation of milk constituents like proteins, enzymes, and vitamins; some of these substances could be indicators to assess the thermal damage in milk [8, 10].

Among the carbohydrates, lactose, originating from the blood glucose in the mammary gland, is one of the main constituents of milk [11]. However, when lactose is subjected to moderate heating, its isomerization can occur with the lactulose formation (glucose/fructose) via Lobry de Bruyn-Alberda van Ekenstein reaction, through the intermediate compound 1,2-enediol [12–14]. Consequently, the quantity of lactulose is directly proportional to the intensity of the heat treatment applied [15] and could be useful such as indicators of the quality of milk processing [8]. Lactose and lactulose chemical structures are shown in Figure 1. According to the International Dairy Federation (IDF), UHT milk contains less than 0.06 g/mL of lactulose, while the hydrostatically sterilized milk presents level of lactulose higher than this value [16]. A precise analysis of lactose and lactulose in food is interesting because both substances exert dose-dependent effects upon intake (lactose maldigestion and prebiotic or laxative action of lactulose) [17].

Early methods of lactose analyses such as polarimetric, gravimetric, Lane-Eynon, and Chloramine-T methods are no more useful because all of them assumed that lactose is the only carbohydrate present in milk and this is a problem in dairy products (fermented milk, cheese, etc.) with appreciable amounts of monosaccharides [18]. More recently, infrared spectroscopic [19], chromatography [20, 21], enzymatic colorimetric [22–24], and capillary electrophoresis [25] have been used for the detection and quantification of lactose and lactulose. From these, high performance liquid chromatography (HPLC) is a very promising technique and considerable research was carried out on the quantitative determination of lactulose [26]. Over all detectors coupled to HPLC, the refractive index detector (RID) is the most widely used for sugars because no fluorophore (fluorescence detector) or chromophore (UV detector) is necessary; in other words, no derivatization is required. RID operates under the principle that the refractive index changes depending upon the refracting or light bending properties of liquids [27]. The aim of

this paper was the development of a modified HPLC-RID method for simultaneous determination and quantification of lactulose and lactose in commercial UHT and sweetened condensed milk.

2. Materials and Methods

2.1. Sample Collection. Commercial brands of skimmed UHT milk (UHT) ($n = 5$) and sweetened condensed milk (COND) ($n = 5$) were obtained in markets located in the city of Rio de Janeiro, Brazil. Samples were transported to the laboratory in insulated polystyrene boxes on ice during 1 h.

2.2. Chemicals and Reagents. The standard of lactose and lactulose (both with 98% of purity) was purchased from Sigma-Aldrich (Sao Paulo, Brazil). The HPLC grade reagents used were acetonitrile (Tedia, RJ, Brazil) and all other reagents like methanol (Tedia, RJ, Brazil), zinc sulphate solution (Carrez I), and potassium hexacyanoferrate solution (Carrez II) were of analytical grade. Ultrapure water was obtained from Simplicity System (Millipore, Molsheim, France).

2.3. Standard Preparation and Calibration Curve. Linearity of UHT milk was performed injected six lactose (range of 0.625 to 20 mg/mL) and six lactulose standards (range from 0.0625 to 1 mg/mL). On the other hand, the linearity of sweetened condensed milk was performed with six levels of lactulose standard (range of 1 to 100 mg/mL) and the same range for lactose. The standard solutions were dissolved in ethanol:water (1:1) and filtered on PTFE membrane with pore size of $0.45\ \mu\text{m}$ and $\varnothing = 13\ \text{mm}$ (Millipore, USA). Solutions were kept at 4°C until the injection in the chromatography system.

2.4. Milk Sample Preparation. The methods proposed by Chávez-Servín et al. [28] and Schuster-Wolff-Bühning et al. [17] were adapted for our experiment. In brief, a protein precipitation was achieved with a sequential mixture of 1.5 mL of the skimmed UHT milk, $30\ \mu\text{L}$ Carrez I solution, and the same volume of Carrez II solution. The resulting volume was centrifuged (Hermle Z 360 K, Germany) at 12000 RPM for $4^\circ\text{C}/30\ \text{min}$. Afterwards, the solution was

filtered in the sterile polyethersulfone membrane with pore size of $0.22\ \mu\text{m}$ and $\varnothing = 33\ \text{mm}$ (Millipore, USA). Before the chromatograph injection, the extract obtained was diluted in ethanol:water (1:1). Sweetened condensed milk (0.60 g) was weighted and diluted with 1.5 mL of ultrapure water. The solution was stirred in mechanical agitator (Biotech International, Melsungen, Germany), during 30 min. After dilution of the sample, the resulting solution followed the same procedure described for the skimmed UHT milk.

2.5. Chromatographic Conditions. Milk samples and standard solution of lactose and lactulose were analyzed from the HPLC system (Shimadzu) provided by degasser (DGU-20A3), binary pump (LC-20AD), automatic injector sample (SIL-20AC), column oven (CTO-20A), and refractive index detector (RID 10A). An isocratic HPLC method involves a mobile phase of acetonitrile:water (75:25, v/v), a Prevail Carbohydrate ES precolumn ($5\ \mu\text{m}$, $7.5\ \text{mm} \times 4.6\ \text{mm}$), and a Prevail Carbohydrate ES column ($5\ \mu\text{m}$, $250\ \text{mm} \times 4.6\ \text{mm}$). The system used a flow of $1.1\ \text{mL/min}$, an oven temperature of 30°C , an injection volume of $20\ \mu\text{L}$, and a running time of 20 min. Lactose and lactulose were identified by retention time and were quantified by peak area.

2.6. Experimental Design and Statistical Analyses. Data collected in this study were analyzed using GraphPad Prism 5.00 package [29] for Windows by one-way ANOVA, and the means were compared with Tukey's test ($P < 0.05$).

3. Results and Discussion

3.1. Validation Parameters. A linearity of the chromatography method was verified with the coefficient of determination (R^2). According to the results, values of R^2 for lactose and lactulose (UHT and sweetened condensed milk) were superior to 0.99 (Figure 2). These results were possible due to the use of six different levels for the construction of the calibration curve, which showed a best-fit linear regression model. The legislation of the European Community recommends at least five concentration levels for the construction of calibration curves [30]. Our results are consistent with Brazilian legislation. Health Surveillance Agency (ANVISA) and National Institute of Metrology, Quality and Technology (INMETRO) consider 0.99 and 0.90 excellent R^2 values, respectively [31, 32].

A separation performance of lactose and lactulose is represented in Figure 3. A standard of (I) lactulose and (II) lactose showed a retention time of 13.6 and 17.2 min, respectively. The same retention time was observed in milk samples. This retention time was similar to that reported by Corzo et al. [18] who used similar chromatography conditions to ours except for flow rate ($0.9\ \text{mL/min}$). These authors remarked the advantage of Prevail column in terms of clear peak resolution and short time analysis for lactose and lactulose. Our result showed that a highest content of lactose in UHT milk and of lactulose in condensed milk has been verified. Food samples may contain components that interfere with performance measurement and may increase or decrease the signal detector [33]. It is important to remark

TABLE 1: Concentrations of lactose and lactulose found in five different commercial brands of UHT milk.

Brand codification	Concentration in UHT milk (g/100 mL)	
	Lactose	Lactulose
UHT ₁	5.66 ± 0.01^a	0.04 ± 0.00^a
UHT ₂	5.50 ± 0.51^a	0.02 ± 0.00^b
UHT ₃	6.64 ± 0.35^a	0.05 ± 0.00^c
UHT ₄	5.57 ± 0.21^a	0.02 ± 0.00^b
UHT ₅	5.47 ± 0.11^a	0.06 ± 0.00^c

UHT_n: ultrahigh temperature milk brand.

Values with the same letter in the column indicate that there is no statistically significant difference between the samples to the level of 5%.

Values represent the mean $\pm$ SEM.

that HPLC method coupled to refractive index detector (RID) is preferred when lactose and lactulose are determinate simultaneously [14, 26]. The HPLC is one of the most extensively used techniques employed for the separation of a large variety of carbohydrates in foods [18], as it is particularly advantageous in terms of speed, simplicity of sample preparation (without a prior derivatization), and obtaining a high-resolved chromatogram in a short period of time (20 min).

3.2. Commercial Milks. The results obtained for quantification of lactose and lactulose in UHT milk are shown in Table 1. Brands did not present a significant difference ($P > 0.05$) for lactose values varied from 5.47 to 6.64 g/100 mL. Our results are similar to those reported by Walstra et al. [34]; milk presents the levels of 3.8 to 5.3 g/100 mL of lactose and these values may vary with environmental and biological conditions. On the other hand, concentrations of lactulose showed a significant difference among brands ($P < 0.05$) with values ranging from 0.02 to 0.06 g/100 mL.

When milk is heated, lactose may isomerize into lactulose. According to the IDF and the European Union (EU), the quantification of lactulose allows distinguishing the milk submitted to different thermal processes and can be used as an indicator of the intensity of the heat treatment [28, 35, 36]. Walstra et al. [34] recommend that UHT milk must contain less than 0.06 g/100 mL of lactulose but these values can vary according to the thermal process. The UHT milk obtained by direct system (injection and infusion) is heated by direct contact with the steam, keeping the high temperature for a short period of time, causing less damage to the product (lactulose values range of 0.005 to 0.011 g/100 mL). However, the indirect system is characterized by slow heating of the product in tubes or plates (lactulose values range of 0.010 to 0.065 g/100 mL) [37]. Although the UHT system was not showed in any label, the levels of lactulose were within the limit values and suggested that there was not an additional heating to UHT process. Our results are according to Morales et al. [8], Elliott et al. [38], Feinberg et al. [39], and Sakkas et al. [40] who found values of lactulose between 0.012 and 0.046 g/100 mL, 0.013 and 0.024 g/100 mL, 0.014 and 0.040 g/100 mL, 0.082 g/100 mL, respectively.

FIGURE 2: (a) Linearity of lactose by HPLC-RID for both samples. (b) Linearity of lactulose by HPLC-RID for UHT milk samples. (c) Linearity of lactulose by HPLC-RID for condensed milk.

Table 2 shows the values of lactulose and lactose in sweetened condensed milk samples. Lactulose levels ranging from 43.56 to 48.97 g/100 mL were similar in all brands ($P > 0.05$). These values were higher in contrast to UHT milk; this finding was expected because in the condensed process 60% of water is removed from the milk and all solid components increase including lactulose. However, lactose levels showed values between 9.96 and 13.86 g/100 mL. These values are similar to those reported by Muehlhoff et al. [2] who found lactose values between 10 and 14 g/100 mL in different sweetened condensed milks. However, these values were lowest compared with the range from 38 to 45 g lactose per 100 g water reported by Walstra et al. [41]. Our results showed slight variations in lactose values in all brands since sweetened condensed milk is elaborated following different methods and the process did not applied high temperature compared with the UHT milk. The determination of lactulose and lactose values in this milk product is not an important indicator for heat treatment.

TABLE 2: Concentrations of lactose and lactulose found in five different commercial brands of condensed milk.

Brand codification	Concentration in condensed milk (g/100 mL)	
	Lactose	Lactulose
COND ₁	13.86 ± 1.15 ^a	43.56 ± 3.28 ^a
COND ₂	10.63 ± 0.22 ^b	44.31 ± 1.75 ^a
COND ₃	9.96 ± 0.01 ^b	48.97 ± 1.22 ^a
COND ₄	13.60 ± 0.56 ^a	44.62 ± 1.18 ^a
COND ₅	12.36 ± 0.03 ^{a,b}	43.72 ± 0.02 ^a

COND_n: condensed milk brand.

Values with the same letter in the column indicate that there is no statistically significant difference between the samples to the level of 5%.

Values represent the mean ± SEM.

Although the heat treatment in UHT milk interferes with the levels of lactose and lactulose, other chemical indicators such as furosine, 5-hydroxymethylfurfural, galactosyl- β -pyranona, and lysinoalanine can be used to evaluate changes

FIGURE 3: Chromatogram by HPLC-RID: (a) standard lactose-lactulose; (b) UHT milk sample; (c) condensed milk sample. I: lactulose (13.6 min); II: lactose (17.2 min).

in the milk submitted to heat processing [38]. From both, lactulose is a better indicator for heat process but it does not represent itself as a distinguishing criterion. In fact, the combination of different indicators would be a better characterization [42].

4. Conclusion

The modified HPLC-RID method is useful in the detection and quantification of lactulose and lactose in dairy products simultaneously. Values of lactose were normal in both milk products. Although UHT milk included high thermal processing, lactulose values remained within the limit values. On the other hand, values of lactulose were high in sweetened condensed milk but this is not an important indicator because the process does not use high temperature. Lactulose is an important indicator of heat treatment; however, the correlation with other substances formed in the milk products after the heat process to define their application in the dairy industry is suggested.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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