Lactose quantification in UHT milk by high-performance liquid chromatography and cryoscopy (freezing point depression)

Quantificação de lactose em leite UHT por cromatografia líquida de alta eficiência e crioscopia (depressão do ponto de congelamento)

Cuantificación de lactosa en leche UHT mediante cromatografía líquida de alta resolución y crioscopia (depresión del punto de congelación)

Cleiver Júnio Martins Costa
ORCID: https://orcid.org/0000-0002-4215-5428
Universidade Federal de Uberlândia, Brazil
E-mail: cleivervzt@hotmail.com
Camila Alves Moreira
ORCID: https://orcid.org/0000-0001-7805-0575
Universidade Federal de Uberlândia, Brazil
E-mail: camilamoreira13@hotmail.com
Ricardo Corrêa de Santana
ORCID: https://orcid.org/0000-0002-6022-5821
Universidade Federal de Uberlândia, Brazil
E-mail: ricardocs@ufu.br
Amado Jésus Silva
ORCID: https://orcid.org/0000-0003-1178-4130
Cooperativa Central Mineira de Laticínios, Brazil
E-mail: amado.jesus@cemil.com.br
Juliana Karla de Souza Teixeira Almeida
ORCID: https://orcid.org/0000-0001-7402-1881
Universidade Federal de Uberlândia, Brazil
E-mail: juliana.karla@cemil.com.br
Milla Gabriela dos Santos
ORCID: https://orcid.org/0000-0002-2708-4158
Universidade Federal de Uberlândia, Brazil
E-mail: milla@ufu.br

Abstract
Due to the large number of people with lactose maldigestion, the dairy industries have increased production and diversity of low lactose and lactose-free foods. Consequently, the need to control the lactose hydrolysis process has also risen. This study aimed to correlate freezing point depression (cryoscopy) and lactose concentration, quantified by high-performance liquid chromatography (HPLC), in UHT milk. To accomplish this, UHT milk samples were subjected to seven lactose hydrolysis treatments, using lactase enzyme, resulting in different lactose concentrations. All samples were subjected to HPLC analysis and freezing point measurement, using a cryoscope. The results were plotted on a graph and a linear regression was performed. There was a strong correlation between lactose concentration and freezing point ($R = 0,9973$) and the coefficient of determination (R^2) was $0,9946$, which means that $99,46\%$ of the variability of the response data is explained by the linear regression model. Therefore, the results point to the feasibility of estimating the lactose concentration in milk during the hydrolysis process for the production of low lactose milk, by cryoscopy, a quick analysis, with lower cost compared to HPLC and that is already among the analyses commonly performed in dairy industries.
Keywords: Milk; *β-galactosidase*; Lactose-free; Lactose maldigestion.

Resumo
Devido ao grande número de pessoas com má digestão de lactose, as indústrias de laticínios aumentaram a produção e a diversidade de alimentos com baixo teor de lactose e sem lactose. Consequentemente, a necessidade de controlar o processo de hidrólise da lactose também aumentou. Este estudo teve como objetivo correlacionar depressão de ponto de congelamento (crioscopia) e concentração de lactose, quantificada por cromatografia líquida de alta eficiência (CLAE), em leite UHT. Para isso, amostras de leite UHT foram submetidas a sete tratamentos de hidrólise da lactose, utilizando a enzima lactase, resultando em diferentes concentrações de lactose. As amostras foram submetidas a análises de CLAE e determinação de ponto de congelamento, utilizando um crioscópio. Os resultados foram plotados

em um gráfico e uma regressão linear foi realizada. Houve uma forte correlação entre concentração de lactose e ponto de congelamento (R = 0,9973) e o coeficiente de determinação (R^2) foi de 0,9946, o que significa que 99,46% da variabilidade dos dados de resposta são explicados pelo modelo da regressão linear. Portanto, os resultados apontam para a viabilidade de estimar a concentração de lactose no leite durante o processo de hidrólise para produção de leite com baixo teor de lactose, por meio da crioscopia, uma análise rápida, com menor custo em relação à análise de CLAE e que já se encontra entre as análises comumente realizadas em indústrias de laticínios.
Palavras-chave: Leite; *β-galactosidase*; Zero Lactose; Má digestão de lactose.

Resumen
Debido a la gran cantidad de personas con mala digestión de lactosa, las industrias lácteas han aumentado la producción y diversidad de alimentos con bajo contenido de lactosa y alimentos sin lactosa. En consecuencia, también ha aumentado la necesidad de controlar el proceso de hidrólisis de lactosa. Este estudio tuvo como objetivo correlacionar la depresión del punto de congelación (crioscopia) y la concentración de lactosa, cuantificada por cromatografía líquida de alta resolución (CLAR), en leche UHT. Para ello, las muestras de leche UHT fueron sometidas a siete tratamientos de hidrólisis de lactosa, utilizando la enzima lactasa, dando como resultado diferentes concentraciones de lactosa. Las muestras se sometieron a análisis por CLAR y determinación del punto de congelación, usando un crioscopio. Los resultados se representaron en un gráfico y se realizó una regresión lineal. Hubo una fuerte correlación entre la concentración de lactosa y el punto de congelación (R = 0,9973) y el coeficiente de determinación (R^2) fue 0,9946, lo que significa que el 99,46% de la variabilidad de los datos de respuesta se explica por el modelo de regresión lineal. Por tanto, los resultados apuntan a la viabilidad de estimar la concentración de lactosa en la leche durante el proceso de hidrólisis para la producción de leche con bajo contenido en lactosa, mediante crioscopia, un análisis rápido, con menor costo en comparación con el CLAR y que ya está entre los análisis comúnmente realizado en industrias lácteas.
Palabras clave: Leche; *β-galactosidase*; Cero lactosa; Mala digestión de lactosa.

1. Introduction

Milk and its derivatives are very nutritious foods and have a prominent role in human nutrition, providing high-quality proteins, minerals and vitamins. For this reason, the consumption of these products is especially important in all age groups (Mckinley, 2005; National Center of Public Health Protection [NCPHP], 2006; Ordóñez, 2005). Cow's milk is the most consumed worldwide, representing 81% of production (Food and Agriculture Organization of the United Nations [FAO], 2020).

Among the carbohydrates in milk, lactose is the most abundant and is present at a concentration of approximately 5% (Churakova et al., 2019; NCPHP, 2006; Ordóñez, 2005). To be digested, lactose must first be hydrolyzed to the monosaccharides (glucose and galactose), by the action of the enzyme lactase (Churakova et al., 2019; Ingram et al., 2009; Tomar, 2014). Approximately 70% of the world population has difficulty digesting lactose due to deficiency in the production of this enzyme, promoting a growing increase in the supply of products with low content or without lactose by the food industry (Lule et al., 2016; Neves & Oliveira, 2020; Tomar, 2014).

We can find the lactase enzyme naturally in the human body. However, as already mentioned, a given portion of the population is deficient in its production. When this occurs, the lactose present in dairy products is not properly hydrolyzed, and when it reaches the large intestine, microorganisms ferment it, and produces carbon dioxide, hydrogen and methane, which can cause discomfort such as bloating, abdominal pain, flatulence and diarrhoea (Churakova et al., 2019; Lule et al., 2016).

To prevent people with deficiency lactose digestion from stopping ingesting milk nutrients, especially calcium and high-quality proteins, the global dairy industry started the development products with low content and lactose-free, in which the lactose is hydrolyzed by the addition of exogenous lactase, *β-galactosidase* (E.C. 3.2.1.23) (Churakova et al., 2019).

Currently, there is no international standard for lactose concentrations in foods to be classified as products with low content or lactose-free (Churakova et al., 2019; European Food Safety Authority [EFSA], 2010). In Brazil, dairy products with a lactose content greater than 0,1% (w/w or w/v) and less than or equal to 1,0% are classified as "low lactose" and those with a maximum content of 0,1% are classified as "lactose-free" (Brasil, 2017).

The measurement of lactose has been carried out using a range of methods including IR spectroscopy, polarimetry, gravimetry method along with various types of chromatographic and enzymatic methods (Mangan et al., 2018). High-performance liquid chromatography with detection of refractive index (HPLC-RI) is widely used in the quantification of carbohydrates, as it is an accurate method, with good ability to separate the compounds to be analysed, with agility (Chávez-Servín et al., 2004), however, it has a high cost of implementation and operation.

The cryoscopic index or freezing point of milk has a direct relation to the concentration of water-soluble substances present in milk. For this reason, it is commonly used to detect fraud by dilution (Costa et al., 2019a; Hanuš et al., 2015). Variations in the composition of milk can occur due to several factors, including the breed of cattle and their feed, as well as the lactation period and health condition (Fox, 2011; Hanuš et al., 2015). However, this variation should not be significant, which leads to the establishment of standards to which milk must follow to guarantee its quality and food safety. Regarding the cryoscopic index, Brazilian legislation, for example, determines a range of -0,530 ºH to -0,555 °H (Hortvet degree) for refrigerated raw milk, that is, milk that arrives in the dairy industries to be industrialized. These values correspond to -0,512 ºC and -0,536 ºC, respectively (Brasil, 2018).

A comparison between classical methods and a well-established HPLC method help to evaluate whether these classical methods can be applied in specific dairy products (Silva et al., 2020). The analysis of the freezing point of milk, using cryoscope equipment, is routine in dairy industries; therefore, industries can use this same equipment to measure lactose hydrolysis indirectly. Thus, as lactose is hydrolyzed, there is a change in the freezing temperature of the milk. In this sense, when compared to methods of direct measurement of lactose concentration, such as HPLC, the application of cryoscopy is an economically more viable resource, without the need for additional costs with the implementation of a chromatographic system, in addition to the shorter analysis time. Therefore, the objective of this study was to simulate an industrial lactose hydrolysis process and to compare two lactose quantification methods, one direct (HPLC-RI) and the other indirect (cryoscopy), to obtain a relation between lactose concentration and milk freezing point.

2. Methodology

This study consists of an experimental, applied and quantitative research (Gil, 2008; Köche, 2011; Pereira et al., 2018) developed by the first and second authors. The other authors provided methodological support. The data collected were treated statistically, as detailed in item 2.5.

2.1 Equipment

Benchtop digital electronic cryoscope (MK 540 Flex II, ITR, Esteio, RS), centrifuge (Neofuge 15R, Heal Force, Shanghai, China), High-Performance Liquid Chromatograph (LC-20 AT Prominence) with refractive index detector (Shimadzu Corporation, Kyoto, Japan).

2.2 Sample

The samples of whole UHT milk, with 3% fat (w/v), were supplied by a dairy industry located in Patos de Minas - MG, Brazil. The samples were collected from three different production batches.

2.2.1 Preparation of samples

UHT milk was added of lactase to obtain samples with different concentrations of lactose (between 0 and 5 g/100 mL). For this, the pure lactase enzyme (LACTLOW L, Granolab/Granotec) from *Bacillus licheniformis* was used. Hydrolysis was carried out in a volume of 600 mL of milk, which was heated in an ultra-thermostatic bath at 40 °C (optimal temperature

for the enzyme, according to the manufacturer's recommendations). The added enzyme concentration was 0,12% (0,12 mL for every 100 mL of milk) and the hydrolysis times were 5, 25, 45, 65, 85, and 105 min. After hydrolysis, 60 mL aliquots were transferred to glass tubes to inactivate the enzyme. The tubes were immersed in boiling water for 3 minutes, followed by immersion in an ice bath, promoting thermal shock. In addition, a milk sample without the addition of the enzyme, and therefore, without lactose hydrolysis, was separated and used as a control sample. All samples were stored under refrigeration (7 °C) until the time of analysis.

2.3 High-Performance Liquid Chromatograph

2.3.1 Preparation of samples for HPLC analysis

All samples obtained in section above were purified before HPLC analysis. Methodology performed by Cataldi et al. (2003) and AOAC Official Method 997.05 (Association of Official Analytical Chemists [AOAC], 2003) were used, with some adjustments. The samples were diluted in ultra-filtered water in a 1:4 ratio and for every 100 mL of the dilution, 1 mL of Carrez I solution ($K_4[Fe(CN)_6].3H_2O$) and 1 mL of Carrez II solution ($Zn(OAc)_2.2H_2O$) were added. Then, the samples were shaken and after that, centrifuged for 30 min at 4 °C and 4000 rpm. The samples were filtered using a Millipore system with 0,22 μm membrane filters (13 mm).

2.3.2 HPLC Analysis

The samples obtained in section above were injected into the chromatography system for the quantification of lactose. For the chromatographic analysis, HPLC grade lactose standard (Sigma-Aldrich, Saint Louis, USA), Hi-Plex Ca 8 μm column (Agilent Technologies, Santa Clara, USA), ultra-filtered water with a flow rate of 0,6 mL/min (eluent), column pressure of 16 bar, injection volume of 20 μL and oven temperature of 85 °C were used. The results were obtained by reading the peak lactose area, used to calculate the lactose concentration, considering the sample dilution factor.

2.4 Cryoscopy (freezing point depression)

First, the cryoscope was calibrated with standard freezing point samples. Then, 2,5 mL of the samples, obtained in section 2.2.1, was used for measurement.

2.5 Statistical analysis

The values obtained in the analyses were expressed as the mean (n = 9; 3 batches and 3 analyses for each batch). One-way ANOVA and Tukey test, at 5% significance level, were used to verify if there was significant difference between the results obtained for each time of hydrolysis, for both methods, HPLC and cryoscopy, using the Statistica 7.0 software (StatSoft Inc, Tulsa, OK, USA). A linear regression was performed to verify the correlation between freezing point and lactose concentration.

3. Results and Discussion

The results of the lactose quantification by HPLC-RI and the freezing point values of the milk as a function of the hydrolysis time are shown in Table 1. The cryoscopic analysis, using bench cryoscope for milk analysis, showed precision in the measurement, as observed by the low standard deviations of the cryoscopic indexes (freezing point).

Table 1. Lactose quantification by HPLC-RI and freezing point values in milk samples with different lactose contents.

Hydrolysis time (min)	Lactose concentration (g/100 mL)		Freezing point (ºC)	
	Mean	SD*	Mean	SD*
0	4,853[a]	0,426	-0,524[a]	0,001
5	3,419[b]	0,144	-0,640[b]	0,019
25	1,816[c]	0,273	-0,755[c]	0,005
45	0,785[d]	0,243	-0,811[d]	0,007
65	0,250[e]	0,021	-0,837[e]	0,011
85	0,088[e]	0,004	-0,850[e]	0,013
105	0,000[e]	0,000	-0,867[f]	0,011

*SD: standard deviation (n = 9); Values with different superscript letters within a column are significantly different (P < 0,05).
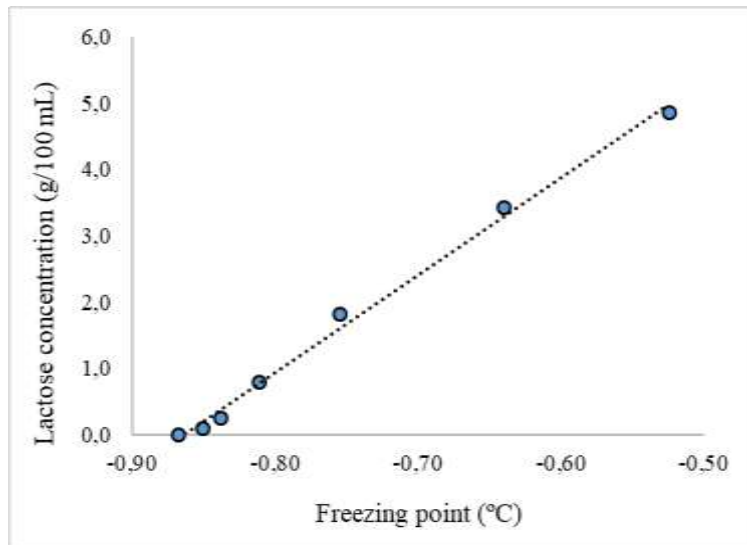Source: Authors (2021).

The initial lactose content in milk (4,85 g/100 mL) was compatible with the value obtained by Churakova et al. (2019), for semi-skimmed UHT milk (1,5% fat), using HPLC-RI (4,77 g/100 g). These values are very close to 5%, therefore, are in line with expectations (NCPHP, 2006).

The hydrolysis time, therefore, the reduction in the lactose concentration, leads to a decrease in the freezing point of the milk (P < 0,05), which tends to become as far from 0 °C (freezing point of water at 1 atm) the lower the lactose concentration, which is consistent with the results obtained by Costa et al. (2019a) and Costa et al. (2019b).

Trevisan (2008), analyzing samples of pasteurized semi-skimmed milk (~ 1% fat), found a difference in the cryoscopy index greater than 30% between samples of milk with and without lactose, corroborating the results found in the present study. This variation is due to the increase in the molar concentration of sugars in milk since lactose is hydrolyzed to glucose and galactose (Churakova et al., 2019; Trevisan, 2008).

Although lactose concentrations are statistically equal (P > 0,05) from 65 minutes of hydrolysis (0,250 g/100 mL), the maximum content required by many countries, including Brazil (0,1 g/100 g or 100 mL), for the product to be classified as "lactose-free" was only reached after this time, which corresponds to the range in which there is less variation in the freezing point as function of the lactose concentration, as can be confirmed by the slope of a straight line in Figure 1. On the other hand, the maximum concentration required by legislation in many countries for low lactose foods is within the range in which there is the greater variation in the freezing point as function of the lactose concentration, which points to a greater precision of the cryoscopy method for monitoring lactose hydrolysis in low lactose milk compared to lactose-free milk (Brasil, 2017; Churakova et al., 2019; EFSA, 2010).

Figure 1. Freezing point variation as a function of lactose concentration.

Source: Authors (2021).

Since a tendency to reduce the concentration of lactose and freezing point was observed as the hydrolysis time increased, and to obtain a relation between the two responses obtained, by HPLC-RI and cryoscopy, a linear regression was performed. The parameters of the fitted linear equation (1) as well as the R, R^2 and RMSE values are shown in Table 2.

$$y = b + a \cdot x \quad (1)$$

where y is the concentration of lactose (g/100 mL) and x is the freezing point (°C).

Table 2 - Parameters of the fitted linear equation (1), R, R^2 and RMSE values.

Equation	Parameters	R	R^2	RMSE
a	b			
14,6819	12,6864	0,9973	0,9946	0,2779

Source: Authors (2021).

There was a strong correlation between lactose concentration and freezing point (R = 0,9973) and the coefficient of determination (R^2) indicates that 99,46% of the variability of the response data is explained by the linear regression model. Therefore, there was a good fit of the linear model to the response data. This result indicates the possibility of estimating the concentration of lactose in low lactose milk, by measuring the freezing point, a quick indirect analysis, of lower cost compared to HPLC, and which is already commonly used in the dairy industries. However, attention is required to the need to evaluate the milk before hydrolysis, to verify if the cryoscopy index is within the expected range, avoiding errors in the interpretation of the estimated lactose concentration results.

Churakova et al. (2019), found results that point to an overestimation of the lactose concentration in milk by the indirect cryoscopy quantification method, which does not prevent relying on the cryoscopy index to estimate the lactose concentration, since the biggest problem would be to underestimate the concentration.

4. Conclusion

The good fit of the linear regression model to the data indicates the viability of using a linear equation to estimate the lactose concentration in milk during a hydrolysis process, based on its freezing point, using a cryoscope. However, according to the results obtained, the use of cryoscopy as an indirect method of quantifying lactose is indicated only for milk with low lactose content, since the maximum concentration required for lactose-free products is very low, demanding a very accurate and exact quantification method. The authors suggest for future studies, that new simulations of lactose hydrolysis in milk be carried out, with a larger number of samples (shorter hydrolysis time intervals), in order to obtain graphs with more points, therefore, equations with greater representation to more accurately estimate the amount of lactose by cryoscopy.

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