FOOD COMPOSITION AND ADDITIVES

A Novel Correlation for Rapid Lactose Determination in Milk by a Cryoscopic Technique

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Residual lactose in special milk was systematically determined for people with lactose intolerance by means of a rapid on-line measurement of the cryoscopic point. A proposed cryoscopic procedure was compared to 2 conventional yet highly laborious methods: the enzymatic procedure with spectrophotometric control and the polarimetric method. Several experiments with different mixtures of both semi-skimmed and low-lactose milk were performed. A lineal relationship was found between lactose concentration and freezing point, the analytical equation for which shows a close relationship regarding the 3 methods used. The advantages of the cryoscopic procedure include speed in obtaining results and operational simplicity at a low cost, better monitoring of enzymatic hydrolysis kinetics, and greater control over the production process for delactosed milk. The equation obtained also enables prediction of the lactose percentage in commercial milk by a simple measurement of freezing point.

In recent years, concern for the health of people with special needs has increased considerably. Because a large part of the population is in fact intolerant to lactose, major dairy companies have intensified research into reducing lactose levels in milk (1, 2). The traditional approach to conversion of lactose by its hydrolysis to the constituent monosaccharides has been practiced industrially for almost 20 years. In long-life milks, the enzyme is generally added to the milk after sterilization, and the product is released for sale after a period once the level of lactose has decreased (2).

The production of low-lactose milk has been analyzed by various authors (3, 4). The dairy industry has traditionally used conventional procedures and specific equipment, which entail a considerable delay between sampling and final results. One such procedure is the enzymatic method proposed by Luhu and Quigshang in 2001 (5). Fuxia and Xiaodong (6) later studied the characteristics of lactase (enzyme β-D-galactosidase) for hydrolyzing lactose to produce low-lactose milk. Alternatively, an enzymatic method using thio-NAD instead of the dinucleotide nicotinamide-adenine (NAD) and measurement at 405 nm rather than 340 nm has been used in the determination of lactose and galactose (7).

A simple and sensitive liquid chromatographic (LC) method for the separation and quantification of mono- and disaccharides in raw and processed milk was described by Cataldi et al. (8). In 2003, Giardina et al. (9) used near-infrared (NIR) and fourier transform infrared (FTIR) spectroscopy to analyze the structural modifications of low-lactose milk components in relation to storage time and temperature effects, comparing the results to the final quality of delactosed milk samples.

Recent research has been focusing on the determination of residual lactose in delactosed milk by calculating its freezing point. Cryoscopic reduction observed in milk depends mainly on the lactose and mineral salts present in solution. Fats and proteins do not significantly influence this property. The effect of fat content on the freezing point of milk was found to be minimal (10). For example, if a milk sample has a freezing point of –0.540°C, and the fat is removed from that milk, the remaining skim milk, which contains no fat, still has the same freezing point (11).

Buchberger and Klostermeyer (12) determined freezing points using the System 4000 MilkoScan (MoS), and they compare with measurements from standard cryoscopes (CryoStar III and IV Instruments; CoR). Results showed that repeatability of the MoS was at least as good as that of the CoR reference method.

The International Organization of Standardization (13), as well as the Institute of Belgian Normalization, the Association of French Normalization, and the Spanish Association of Normalization and Certification, among others, have described cryoscopic methods to determine the freezing point of milk. The reference is ISO 5764:2002.

In 1998, the Institute of Normalization of the Czech Republic published a standard that specified the conditions to determine the freezing point of fresh cow's milk as well as treated milk in a dairy using an automated or semi-automated approach, where crystallization is induced by mechanical vibrations and the freezing point is rapidly reached (14).

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Measurement of the freezing point of milk was discussed by Kirsanov (15) with particular reference to development of cryoscopic equipment for determination of milk's freezing point and economic aspects.

Our previous studies have often dealt with correlation, some of them to estimate approximate stability constants and to predict overall equilibrium constants (16), and more recently, the relationship among structurally similar compounds and their physicochemical properties, such as acidity. Final results may contribute to an effective control of production processes in the brewing industry (17). Therefore, we propose, as our first objective in this study, to establish a correlation for indirect determination of the residual lactose of low-lactose milk by means of cryoscopic measurements. Our final goal is an in situ control of the lactose levels of low-lactose milk during processing.

Experimental

Materials

Two types of commercial milk were used: low-lactose and semi-skimmed ultrahigh temperature (UHT). These are 2 types of semi-skimmed milks that differ only in lactose content and allow preparation of mixtures covering a wide range of lactose content.

Two types of samples were used in different experiments: (*1*) *Low-lactose milks*.—Random TetraPak© samples with different expiry dates were taken for calculating residual lactose content by means of high-performance LC (HPLC), polarimetry, or spectrophotometry. (*2*) *Dilutions of low-lactose milk with semi-skimmed milk*.—Increased volumes of semi-skimmed UHT milk were added to 100 mL low-lactose milk until reaching 500 mL, obtaining lactose concentrations between 1 and 5 g/100 mL by using the same methodology as in the former samples, including an MoS for FTIR measurements.

Mercury iodide, trichloracetic acid, and potassium iodide were obtained from Merck (Darmstadt, Germany; GR for analysis). Stock solutions were standardized using recognized procedures. All other materials were of reagent grade.

Methods

Two reference methods were used to analyze the residual lactose of different samples of milk: the enzymatic procedure (using UV spectrophotometry) and the polarimetric procedure. Once the lactose concentration was determined, the cryoscopic method was used to calculate the freezing point of each sample.

HPLC-MoS Method

Conventional HPLC (model milk line "WATERS") and MoS (Model *FT120)* measurements of low-lactose and semi-skimmed UHT milks were made. The freezing point was calculated by means of a termistancie cryoscope, *Advanced™ Cryoscope* Model 4D3. The instrument is totally automated, in that a sensor performs all the super freezing, freezing, thermal retention, and final digital reading operations. In order to ensure working with perfectly homogenized samples, each carton of milk was placed for 20 min in water at 45°C prior to opening.

Polarimetric Method

This method calculates the rotational power of a lactose solution resulting from previous separation of fats and proteins of the milk by precipitation to eliminate interference in the experiment (18). For this, 75 mL milk and 7.5 mL mercuric potassium iodide solution in sulfuric medium were taken and filled to 100 mL with distilled water. After stirring and resting for 15 min, it was filtered to separate fats and proteins. The filtrate, kept at 20°C, was placed in a 200 mm cell inside a polarimeter POLAX-D, avoiding air bubbles.

The rotational power of the resulting solution was measured (α_t), which corresponds to the rotational powers of lactose, glucose, and galactose. An adjustment enabled us to determine the actual lactose rotational power (α_r; 18), and finally to calculate lactose content according to Equation 1:

$$\text{Lactose (g/100 mL}_{\text{milk}}) = \alpha_{\text{real}} \cdot 0.9518 \cdot \frac{100}{75} \cdot 0.96 \qquad (1)$$

Enzymatic Method

This method is based on the hydrolysis of lactose by means of β-D-galactosidase and the subsequent determination of liberated galactose by means of an enzymatic kit (19).

For this purpose, a Boehringer Mannheim Test-Combination Lactose/D-Galactose Cat. No. 176303 was used, where D-galactose produced is oxidized at pH 8.6 by the dinucleotide NAD to D-galactonic acid in the presence of the enzyme β-galactose dehydrogenase (Gal-DH) according to Equation 2.

$$\text{D-galactose} + \text{NAD}^+ \xrightarrow[\text{pH = 8.6}]{\text{Gal-DH}} \text{D-galactonic acid} + \text{NADH} + \text{H}^+ \qquad (2)$$

The amount of NADH that stoichiometrically corresponds to the quantity of lactose in the sample is measured at 340 nm by means of a Spectronic-20 GENESIS spectrophotometer.

In order to prepare the sample to be analyzed in the spectrophotometer, 10 mL milk at 20°C was diluted to 250 mL with distilled water. Trichloracetic acid (3 M) is added to a pH 4.3 to induce protein precipitation. After 10 min, the sample is filtered, and 1 M NaOH is added to the filtrate until

Table 1. Average values of fat, nonfat solid, protein, and total solids in TetraPak samples of low-lactose and semi-skimmed UHT milks expressed as percentage

TetraPak samples	Fat	Nonfat solids	Protein	Total solids
Low-lactose	1.72	8.58	3.23	10.46
Semi-skimmed	1.52	8.68	3.13	10.24

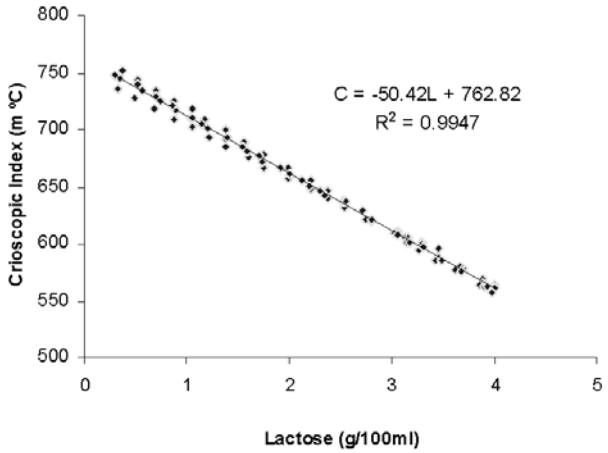

Figure 1. Cryoscopic index in terms of lactose concentration measured by HPLC and MilkoScan.

the initial pH of the milk is re-established (ca 6.6). Finally the transparent sample is refrigerated (4°C). Following the Boehringer Mannheim Test-Combination, the mixtures to be measured spectrophotometrically in a glass cell (1 cm path-length) are prepared.

Results and Discussion

To develop the cryoscopic methodology that allows a relationship between residual lactose and cryoscopic index to be established, we used delactosed semi-skimmed milk and a semi-skimmed milk with the characteristics expressed in Table 1.

A 100 mL volume of low-lactose milk whose lactose concentration had been previously measured by means of HPLC was taken, to which were volumes of between 1 and 450 mL semi-skimmed UHT milk whose lactose concentration had been previously measured by means of MoS. The resulting cryoscopic index was measured for each sample. We aimed to obtain a wide range of lactose concentrations close enough to those of semi-skimmed milk.

To obtain a wide range in the cryoscopic index against lactose concentration, these results were merged with those of 5 other experiments; in all cases delactosed and semi-skimmed milk with different expiry dates were used. The results obtained are shown in Figure 1.

Polarimetric Method

To verify the validity of the previous method, a polarimetric approach was used. As in the previous cryoscopic method, a series of semi-skimmed and delactosed mixtures were prepared. The optical rotation was measured for each mixture. By using Equation 1, lactose concentration was determined for each mixture of milk. Parallel to these measurements, a second sample of each mixture was placed in the cryoscope to measure the freezing point. The results obtained in the polarimeter and in the cryoscope for the different mixtures of milk, in 2 different series, are shown in Figure 2.

Enzymatic Method

In this case and to assess the validity of the cryoscopic method by HPLC and MoS lactose determinations, the amount of lactose in each milk sample was determined by the enzymatic procedure described in the *Experimental* section, above. A series of mixed solutions of semi-skimmed and delactosed milk was analyzed.

Each sample analyzed was prepared with 100 mL low-lactose milk with increasing quantities of semi-skimmed milk added to cover a wide range of lactose concentrations. By subsequently applying Equation 2, the quantity of residual lactose analyzed was obtained and its cryoscopic index, measured. The results obtained are shown in Figure 3.

When all the methods were considered, significant agreement in results were found. Results of direct lactose measurements by HPLC in delactosed stabilized milk and those obtained by MoS for semi-skimmed UHT milk

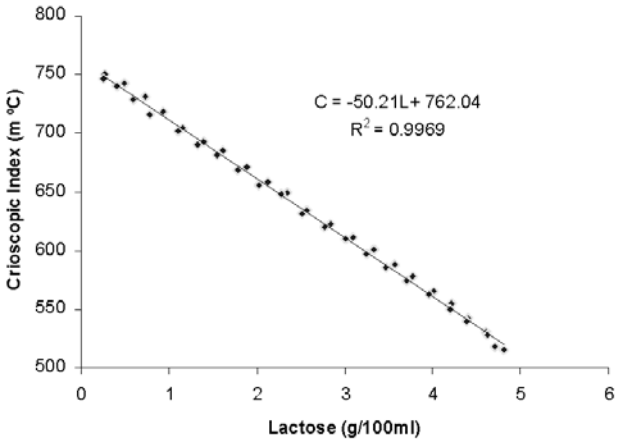

Figure 2. Cryoscopic index in terms of lactose content measured by polarimetry.

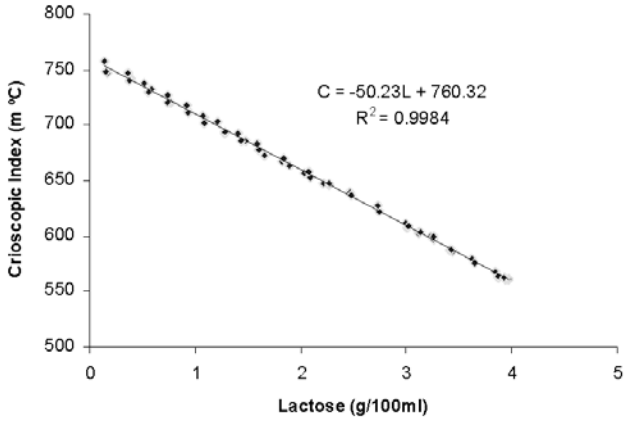

Figure 3. Cryoscopic index in terms of lactose content measured by the enzymatic procedure.

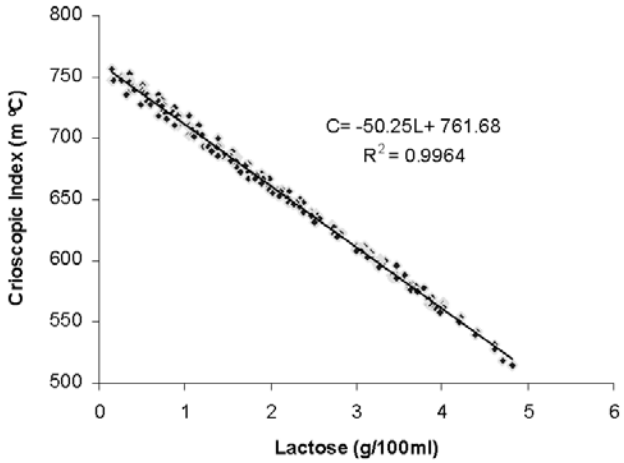

Figure 4. Obtained fitting mode line for lactose determination in low lactose milks including all the results.

correlated quite well with the cryoscopic index. Polarimetry also led to similar results when optical rotations of lactose in mixtures of low-lactose and semi-skimmed milk were measured. When enzymatic hydrolysis of the lactose of the milk was caused, the results obtained were also perfectly interpolative with those obtained by the first method proposed.

The regression obtained considering all the results of the 3 methods (Figure 4) yielded the following lineal equation:

$$L = 15.16 - 0.02\ C \qquad (3)$$

where L represents the lactose concentration in g/100 mL and C the cryoscopic index expressed in m°C.

Bearing in mind the quality of the obtained lineal relationship calculated (R^2 = 0.9959), the residual lactose contained in low-lactose milk can be determined by rapid and prior calculation of the freezing point of the milk analyzed.

The real advantages of this method may be summed up as follows: (*1*) It is faster in obtaining results, leading to tighter control during low-lactose milk production, thus enabling control over the kinetics of enzymatic hydrolysis at an industrial scale; (*2*) it offers low-cost analysis of milk samples; and (*3*) its simplicity compared to the complex operations involved in the HPLC, enzymatic, or polarimetric procedures seems apparent.

Finally, with a tool such as the one described in this work, by a simple measurement of the freezing point and the proposed equation, a quick lactose test is available for the large numbers of people throughout the world with lactose intolerance. Furthermore, because the effect of fat content on the freezing point of milk is minimal (10), it would be possible to determine lactose content in whole and skim milk using the same method.

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