beverages

MDPI

Short Note

Milk and Its Sugar-Lactose: A Picture of Evaluation Methodologies

Loretta Gambelli

Council for Agriculture Research and Analysis of the Agrarian Economy, Research Center CREA-Food and Nutrition, Via Ardeatina 546, 00178 Rome, Italy; loretta.gambelli@crea.gov.it; Tel.: +39-065-149-4411

Academic Editor: Alessandra Durazzo
Received: 30 May 2017; Accepted: 26 June 2017; Published: 13 July 2017

Abstract: Lactose is the major disaccharide found in milk, and is catabolized into glucose and galactose by the enzyme lactase. Lactose is an important energy source and ssometimes it is referred to simply as milk sugar, as it is present in high percentages in dairy products. Lactose is the primary source of carbohydrates during mammal development, and represents 40% of the energy consumed during the nursing period. Lactose-intolerance individuals have a lactase deficiency; therefore, lactose is not completely catabolized. Lactose intolerance is a significant factor in the choice of diet for many sick people, therefore its content in foods must be monitored to avoid disorders and illnesses. This has created the need to develop simple methods, such as polarimetry, gravimetric, middle infrared, differential pH and enzymatic monitoring, but all these methods are time-consuming, because they required extensive sample preparation and cannot differentiate individual sugars. In order to quantify low levels of lactose, new and more accurate analytical methods have been developed. Generally, they require equipment such as HPLC or High Performance Anion Exchange with Pulsed Amperometric Detection (HPAE-PAD).

Keywords: lactose; analytical methodologies; nutrition

1. Lactose and Its Peculiar Role Nutrition

Milk and dairy products represent a food category of absolute centrality in the diet of the Italian population. The Italian Food Pyramid has entered these products at an intermediate level, attributing to milk, yogurt and cheese a consumption mode in the order of 2–3 servings per day.

Milk is a complex fluid foodstuff secreted by the breast glands of female mammals. It is a complete food, since it contains nearly all the necessary nutrients to sustain the life and growth of a newborn.

Even if different types of milk can be used for human consumption, such as sheep, goat, donkey and other infant formula milks, in addition to breast milk, in the first period of life, usually when we speak of milk we are referring to cow's milk [1].

Among the nutrients contained in milk, saccharides play a key role. They are present in amounts of up to ca. 10%, depending on the mammal [2], and they are represented almost exclusively by lactose (98% of the sugars present in milk), which is not found in any other food, and is important for the development of the nervous tissue in the first few months of life. The main role of lactose is to provide the newborn with galactose for the synthesis of the nerve structures (myelin sheaths).

D-lactose is present in milk in two anomeric forms, α-lactose and β-lactose (ratio 2:3), depending on the pyranose form (α or β) of glucose. Galactose is always present in the β-pyranose form.

These anomers also have different physical properties, such as melting point and, primarily, solubility in water, as β-lactose is much more soluble than α-lactose [3,4].

Lactose is synthesized in the mammary tissue from the conversion of a part of the glucose present in the blood to galactose. This synthesis involves the complex lactose synthetase, which is provided by the galactosyltransferase and α-lactalbumin [5].

2. Methodologies for the Determination of Lactose

Detection of lactose is very important because of lactose intolerance diseases. Small quantities of it are present in many foods—not only in milk and dairy products—and therefore, traces of lactose may be present in food under the following headings: milk solids, whey, curds, skim milk powder, and skim milk solids, meaning that lactose is present, and must be reported on the food label. Lactose intolerance is a significant factor in the choice of the diet of people sensitive or intolerant to this sugar, so its content in foods must be monitored to avoid disorders and diseases [6,7]. It is therefore important to quantify lactose with precision and accuracy in these products. The chosen method should be one that is economical, rapid, and sensitive.

A considerable number of methods for determining carbohydrates in milk have been developed, including older and less sensitive ones, such as gravimetric, polarimetric, enzymatic, or spectrophotometric analysis, as well as more specific and sensible methods such as HPLC-RI and HPAEC-PAD.

The most common methods used to quantify lactose in milk are described below:

2.1. Polarimetric

Polarimetric methods are based on the measurement of the specific rotation of polarized light by chiral molecules such as lactose in a skimmed and deproteinized milk filtrate [8,9].

2.2. Gravimetric

Gravimetric methodologies are founded on the decrease of copper sulfate to cuprous oxide precipitated by the addition of potassium hydroxide in the presence of aldoses and ketoses. The lactose content is calculated after weighing the cuprous oxide formed, by using empirical tables that allow the conversion of the cuprous oxide formed in terms of lactose [8,10].

2.3. Mid-Infrared

Infrared Spectroscopic Methods are based on the absorbance of infrared energy by the hydroxyl groups (OH) of lactose molecules. Lactose determination in mid-infrared (MIR) spectroscopy is carried out at 1042 cm^{-1} [11]. Early instruments were entirely filter-based, using pairs (sample and reference) of optical filters to select a band of wavelengths for the measurement of fat, protein, and lactose.

Now, more recent instruments utilize an interferometer to acquire complete spectrum information within the MIR region using Fourier Transform Infrared Spectroscopy (FTIR); in this way, it is possible to obtain an extensive computing and data manipulation capabilities [12–15].

2.4. Enzymatic

A large number of enzymatic methodologies able to quantify lactose have been reported [16–19]. They have been characterized by the common reaction of enzymatic hydrolysis of lactose to glucose and galactose, followed by the enzymatic determination of one of the liberated monosaccharides. The amount of lactose in the sample is given by the difference between monosaccharide content before and after hydrolysis.

2.4.1. NAD Enzymatic

In my opinion, the most used enzymatic method to measure galactose is based on its oxidation by β-galactose dehydrogenase to galacturonic acid in the presence of nicotinamide-adenine dinucleotide (NAD) that is reduced to NADH, as reported by the following reaction:

$$\beta\text{-D-Galactose} + NAD^+ \ \beta\text{-D-Galactose Dehydrogenase} \rightarrow \text{D-Galactono-}\gamma\text{-Lactone} + NADH + H^+$$

Absorbance of NADH at 340 nm is calculated as the difference between the readings before and after the addition of the enzyme, galactose dehydrogenase [20–22]. Although this UV method is specific and accurate, as the measurements of NADH require reading in the UV range, replacement of NAD by thio-NAD and measurement in the visible range at 405 or 415 nm can also be done. This variation allows the simultaneous quantification of D-galactose concentrations in several samples using microplate-readers, rather than UV spectrophotometers [16–19].

2.4.2. Differential pH

The differential pH technique for determining of lactose and lactulose in milk samples is based on changes in the pH owing to enzymatic reactions. Lactose determination is performed by measuring the pH change caused by the reaction of glucose and ATP in the presence of hexokinase (HK) before and after treatment of the sample with β-galactosidase. The determination of lactulose is carried out by treating the sample with a mixture of β-galactosidase and glucokinase in the presence of ATP. After 3 h, the pH change is measured, HK is added, and the pH change is monitored for 4 h to observe the d-fructose-6-phosphate formation [23].

2.5. HPLC RI/HPAEC-PAD

HPLC remains as one of the most extensively used techniques. It has been widely used for separating a large variety of carbohydrates, especially in foods, as it is particularly advantageous in terms of the speed and simplicity of sample preparation. HPLC allows direct detection of carbohydrates, as they can absorb low UV wavelengths.

However, detection in this spectrum area (below 200 nm) is difficult due to its low sensitivity and selectivity; it also requires the use of high-quality and expensive reagents. The most common sugar-detection system after HPLC separation is the refractive index; however, the response of this detector is very poor and non-specific, quite sensitive to changes in temperature, pressure, and solvent composition, and does not allow gradients. If a refractive index (RI) is used for detection, the analysis is straightforward, but not very sensitive, with a Limit of Detection (LOD) of 250 mg/L and a Limit of Quantification (LOQ) of 380 mg/L having been reported [24].

Several chromatographic methods are available for the separation of carbohydrates, reverse phase systems and cation exchange are the most widely used [25–29].

The separation in reverse-phase partition chromatography is based on the principle of hydrophobic interactions derived from the repulsive forces among relatively polar solvents, nonpolar analytes, and nonpolar stationary phases. Alkylated and aminoalkylated silica gels are most frequently used as stationary phases, in combination with aqueous methanol or aqueous acetonitrile as mobile phases, and the separation is carried out by hydrophobic and polar interactions and partition [24,30,31].

Traditional adsorption chromatography was almost universally replaced by Ion-Exchange Chromatography (IEC). Carbohydrates are separated by charge differences using two types of ion-exchanger—anionic and cationic—where the compounds are negatively and positively charged, respectively [32,33].

Several methodologies based on cationic-exchange HPLC chromatography have been optimized to quantify carbohydrates in a lot of dairy products, using different stationary and mobile phases, such as Amine with calcium as counterion, and Sugar Pak [27–29].

A second, more sensitive method is High-Performance Anion-Exchange Chromatography (HPAEC) with Pulsed Amperometric Detection (PAD). High-performance anion-exchange chromatography (HPAEC) coupled with PAD is an alternative analytical technique that presents high sensitivity and good resolution compared with non-derivatized carbohydrates.

Carbohydrate separation and elution order is based on the differences in their pKa values, in fact HPAEC takes advantage of the weakly acidic nature of carbohydrates to give highly selective separations at high pH using a strong anion exchange stationary phase. Columns are packed with poly(styrene-divinylbenzene)-based stationary phases functionalized with alkyl quaternary

ammonium groups [34]. The possible co-elution of closely related carbohydrates with very similar retention times represents the main problem of HPAEC-PAD. Cataldi et al. [35] optimized a rapid and sensitive HPAEC-PAD method using 10–12 mM NaOH modified with 1–2 mM barium acetate in heated milks; in this way, the separation and quantification of lactulose and lactose along with other carbohydrates was obtained in milk and milk products [35,36].

In one recent application note, 248, the Dionex Corporation, now part of Thermo Fisher, suggested using this technique to quantify lactose in lactose-free products, and indicated a LOD lower than 1 mg/L [37,38].

Obviously, all the methods above describe present advantages and disadvantages, but in my opinion this last method presents a good cost/benefit ratio.

3. Conclusions

Lactose, a very important nutrient during the neonatal years, is not always tolerated in adults. In this short review, various methods, from the oldest to the more modern and innovative, for determining lactose in milk have been reported. Considering how widespread lactose intolerance is, today lactose analysis should be considered as routine analysis. In order to choose the most suitable method, important parameters such as sensitivity, precision, accuracy, and speed of analysis have been taken under consideration.

But unfortunately, this is not always possible, because the high cost of scientific instruments, reagents, laboratories and the adequate training of technicians in some situations, for example, in undeveloped countries, makes it difficult to perform sophisticated analysis. However, under working conditions, each operator must choose the best methods available. That is the principal aim of this review.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Nickerson, T.A. Lactose. In *Fundamentals in Dairy Chemistry*, 2nd ed.; Webb, B.H., Johnson, A.H., Alford, J.A., Eds.; Avi Publishing Co.: Westport, CT, USA, 1974; Chapter 6.
2. Fox, P.F.; Mcsweeney, P.L.H. *Dairy Chemistry and Biochemistry*; Blackie Academice Professional: London, UK, 1998.
3. Ganzle, M.G. Enzymatic synthesis of galacto-oligosaccharides and other lactose derivatives (hetero-oligosaccharides) from lactose. *Int. Dairy J.* **2012**, *22*, 116–122. [CrossRef]
4. Idda, I.; Spano, N.; Ciulu, M.; Nurchi, V.M.; Panzanelli, A.; Pilo, M.I.; Sanna, G. Gas chromatography analysis of major free mono- and disaccharides in milk: Method assessment, validation, and application to real samples. *J. Sep. Sci.* **2016**, *39*, 4577–4584. [CrossRef] [PubMed]
5. Harju, M.; Kallioinen, H.; Tossavainen, O. Lactose hydrolysis and other conversions in dairy products: Technological aspects. *Int. Dairy J.* **2012**, *22*, 104–109. [CrossRef]
6. EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies). Scientific opinion on lactose thresholds in lactose intolerance and galactosaemia. *EFSA J.* **2010**, *8*, 29. [CrossRef]
7. Brown-Esters, O.; Namara, M.P.; Savaiano, D. Dietary and biological factors influencing lactose intolerance. *Int. Dairy J.* **2012**, *22*, 98–103. [CrossRef]
8. Lactose. In *Official Methods of Analysis*, 15th ed.; Helrich, K., Ed.; Association of Official Analytical Chemists (AOAC): Washington, DC, USA, 1990; p. 810.
9. Official Method 896.01. Lactose in Milk. Polarimetric Method. In *Official Methods of Analysis of AOAC International*; AOAC: Washington, DC, USA, 2005.
10. Official Method 930.28. Lactose in Milk. Gravimetric Method. In *Official Methods of Analysis of AOAC International*; AOAC: Washington, DC, USA, 2005.
11. Lactose. Infrared Milk Analysis. In *Official Methods of Analysis*, 18th ed.; Horwitz, W., Ed.; AOAC: Washington, DC, USA, 2005.
12. Linch, J.M.; Barbano, D.M.; Schweisthal, M.; Fleming, J.R. Precalibration evaluation procedures for mid-infrared milk analyzers. *J. Dairy Sci.* **2006**, *89*, 2761. [CrossRef]

13. Official Method 972.16 Lactose in Milk. Mid-Infrared Method. In *Official Methods of Analysis of AOAC International*; AOAC: Washington, DC, USA, 2005.
14. IDF. *International Standard 141C. Whole Milk-Determination of Milkfat, Protein and Lactose Content, Guidance on the Operation of Mid-Infrared Instruments*; Int. Dairy Fed.: Brussels, Belgium, 2000.
15. Kittivachra, R.; Sanguandeekul, R.; Sakulbumrungsil, R.; Phongphanphanee, P.; Srisomboon, J. Determination of essential nutrients in raw milk. *Songklanakarin J. Sci. Technol.* **2006**, *28*, 115–120.
16. Shapiro, F.; Shamay, A.; Silanikove, N. Determination of lactose and D-galactose using thio-NAD+ instead of NAD+. *Int. Dairy J.* **2002**, *12*, 667. [CrossRef]
17. *ISO 5765-1:2002. Dried Ice-Mixes and Processed Cheese—Determination of Lactose Content—Part 1: Enzymatic Method Utilizing the Glucose Moiety of the Lactose*; International Organization for Standardization: Geneva, Switzerland, 2002.
18. *ISO 5765-2:2002. Dried Ice-Mixes and Processed Cheese—Determination of Lactose Content—Part 2: Enzymatic Method Utilizing the Galactose Moiety of the Lactose*; International Organization for Standardization: Geneva, Switzerland, 2002.
19. Official Method 984.15. Lactose in Milk. Enzymatic Method. In *Official Methods of Analysis of AOAC International*; AOAC: Washington, DC, USA, 2005.
20. Coffey, R.G.; Reithel, F.J. An enzymic determination of lactose. *Anal. Biochem.* **1969**, *32*, 229. [CrossRef]
21. Lynch, J.M.; Barbano, D. Determination of the lactose content of fluid milk by spectrophotometric enzymatic analysis using weight additions and path length adjustment: Collaborative study. *J. AOAC Intern.* **2007**, *90*, 196.
22. Kleyn, D.H. Determination of lactose by an enzymatic method. *J. Dairy Sci.* **1985**, *68*, 2791–2798. [CrossRef]
23. *Milk: Determination of Lactose Content. Enzimatic Method Using Difference in pH. ISO Standard 26462*; International Organization of Standardization: Geneva, Switzerland, 2010.
24. Chavez-Servin, J.L.; Castellote, A.I.; Lopez-Sabater, M.C. Analysis of mono-and disaccharides in milk-based formulae by high-performance liquid chromatography with refractive index detection. *J. Chromatogr. A* **2004**, *1043*, 211–215. [CrossRef] [PubMed]
25. Richmond, M.L.; Barfuss, D.L.; Harte, B.R.; Gray, J.I.; Stine, C.M. Separation of carbohydrates in dairy products by high performance liquid chromatography. *J. Dairy Sci.* **1982**, *65*, 1394. [CrossRef]
26. West, L.G.; Llorente, M.A. High performance liquid chromatographic determination of lactose in milk. *J. Assoc. Off. Anal. Chem.* **1981**, *64*, 805. [PubMed]
27. Pirisino, J.F. High performance liquid chromatographic determination of lactose, glucose and galactose in lactose-reduced milk. *J. Food Sci.* **1983**, *48*, 742. [CrossRef]
28. Mullin, W.J.; Emmons, D.B. Determination of organic acids and sugars in cheese, milk and whey by high performance liquid chromatography. *Food Res. Int.* **1997**, *30*, 147. [CrossRef]
29. Elliot, J.; Dhakal, A.; Datta, N.; Deeth, H.C. Heat-induced changes in UHT milks. Part 1. *Aust. J. Dairy Technol.* **2003**, *58*, 3.
30. Honda, S. High-performance liquid chromatography of mono- and oligosaccharides. *Anal. Chem.* **1984**, *140*, 1. [CrossRef]
31. Manzi, P.; Pizzoferrato, L. HPLC determination of lactulose in heat treated milk. *Food Bioprocess Technol.* **2013**, *6*, 851–857. [CrossRef]
32. Kennedy, J.F.; Pagliuca, G. Oligosaccharides. In *Carbohydrate Analysis. A Practical Approach*; (The Practical Approach Series); Chaplin, M.F., Kennedy, J.F., Eds.; Oxford University Press: New York, NY, USA, 1994; Chapter 2.
33. Macrae, R. Applications of HPLC to food analysis. In *HPLC in Food Analysis*; Macrae, R., Ed.; Academic Press: San Diego, CA, USA, 1988; Chapter 2.
34. Cataldi, T.R.I.; Campa, C.; De Benedetto, G.E. Carbohydrate analysis by high performance anion-exchange chromatography with pulsed amperometric detection: The potential is still growing. *Fresen. J. Anal. Chem.* **2000**, *368*, 739. [CrossRef]
35. Cataldi, T.R.I.; Angelotti, M.; Bufo, S.A. Method development for the quantitative determinationof lactulose in heat-treated milks by HPAEC-with pulsed amperometric detection. *Anal. Chem.* **1999**, *71*, 4919. [CrossRef] [PubMed]
36. Cataldi, T.R.I.; Angelotti, M.; Bianco, G. Determination of mono-and disaccharides in milkand milk products by high performance anion-exchange chromatography with pulsed amperometric detection. *Anal. Chim. Acta* **2003**, *485*, 43. [CrossRef]

37. Monti, L.; Negri, S.; Meucci, A.; Stroppa, A.; Galli, A.; Contarini, G. Lactose, galactose and glucose determination in naturally "lactose free" hard cheese: HPAEC-PAD method validation. *Food Chem.* **2017**, *220*, 18–24. [CrossRef] [PubMed]
38. Determinatio of Lactose in Lactose-Free Milk products by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection. Available online: https://tools.thermofisher.com/content/sfs/brochures/AN-248-Lactose-Milk-Products-HPAE-PAD-AN70236-EN.pdf (accessed on 13 July 2017).