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Determination of residual lactose in lactose-free cow milk by hydrophilic interaction liquid chromatography (HILIC) coupled to tandem mass spectrometry

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ABSTRACT

Lactose is the major carbohydrate found in milk and dairy products. Lactose intolerance means the body cannot digest foods with this natural sugar in them. In this context, the lactose-free market has experienced a steep increase in recent years. A new method for the determination of residual lactose in lactose-free dairy products using liquid chromatography coupled to tandem mass spectrometry triple quadrupole (HPLC–MS/MS) has been developed. Hydrophilic interaction chromatography (HILIC) has been used for this purpose. An amide chromatographic column with an alkaline mobile phase were selected as optimal. In addition, a fast, cost-effective and reliable sample treatment has been developed for routine analytical laboratory use. The method has been validated by using matrix-matched calibration standards and a recovery assay on a lactose-free milk sample obtained by lactase hydrolysis of regular milk. The limit of quantification (LOQ) was 15 mg L^{-1}, while the recovery was close to 100% with relative standard deviation lower than 9% in all cases. The method was applied to several lactose-free products and the results showed that lactose values in these products are not always below the recommended maximum value of 100 mg L^{-1}.

1. Introduction

Lactose is a major component of milk from mammals and has important nutritional and prebiotic properties. Lactose concentration in human milk is relatively high (7.0%), while in cow milk is about 4.6% (Perati et al., 2016). In the small intestine, it is hydrolysed by the enzyme lactase (β-galactosidase) into glucose and galactose to allow absorption through the intestinal mucosa (van Scheppingen et al., 2017). Approximately 70% of the global adult population and 95% of Asian population have lactase deficiency and are unable to digest lactose (ADILAC, 2016; Schaafsma, 2008). This is known as lactose intolerance (LI). Newborn mammals subsist on milk over the first few months of life, and after weaning there is a genetically-programmed decrease in lactase expression. A large majority of humans show this typical lactase decrease early in life, therefore, adults are unable to properly digest lactose. These individuals are lactose intolerant and are said to have the trait of lactase non-persistence (LNP). Congenital absence of lactase due to a mutation in the gene that is responsible for producing the enzyme is a very rare cause of lactase deficiency, and the symptoms of this type of lactase deficiency begin shortly after birth (Ingram et al., 2009). LI is bothersome but usually not serious and symptoms include abdominal pain, diarrhea, abdominal bloating and distension which reflect the osmotic effects of the unassimilated lactose in the intestinal lumen, plus the fermentation products (such as hydrogen and methane) generated by bacteria in the large intestine.

The demand for lactose-free products is driven by the high prevalence of LI and the worldwide increase in incidences of food intolerances. The dairy industry is continually launching new lactose-free food products. Although the European Food Safety Agency sets the limit of residual lactose content for products labelled as “lactose-free” to 1 g L^{-1}, many dairy companies have set a lower value (0.01%) as a quality feature (Spanish Agency for Consumer Affairs, Food Safety and Nutrition, 2017). In addition, some dairy foods are marketed using the claim “low-lactose”. In that case, the concentration of lactose is < 5 g L^{-1} (Trani et al., 2017). The resulting dairy products contain varying amounts of residual lactose.

In this context, there is a clear need for simple analytical methods to monitor the amount of residual lactose in these products in routine quality control analysis. Different methods have been traditionally applied for the determination of residual lactose such as differential pH

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techniques, paper chromatography (Honer and Tuckey, 1953) and gas chromatography (Idda et al., 2016). The current methods recommended by the Association of Official Analytical Chemists (AOAC) are based on the enzymatic hydrolysis of lactose to glucose and galactose followed by different biochemical pathways. The detection of reaction products is done with uv–vis or fluorescence (Bankar et al., 2009; Essig and Kleyn, 1983; Lynch et al., 2007; Megazyme International Ireland, 2014; Trani et al., 2017). The main drawbacks of these methods are that they need large amounts of reagents and all them also require sample deproteinization because proteins are interfering compounds. Additionally, the presence of large amounts of glucose and galactose in hydrolysed milk may also result in interferences. An additional reduction with sodium borohydride is required to obtain non-interfering sugar alcohol derivatives (Megazyme International Ireland, 2014). To our knowledge, and due mainly to the interferences described above, these methods have been validated for regular milk and dairy products, but not for low-lactose or lactose-free products.

Normal-phase chromatography with refractive index detector has been commonly used for the determination of sugars (Chavez-Servin et al., 2004; Trani et al., 2017; Indyk et al., 1996). However, this detector lacks sensitivity and is not appropriate for low/free-lactose products. The use of ion pair in the mobile phase (Erich et al., 2012) or the derivatization for fluorescent or mass spectrometric detection have been also reported (Mopper, 1983; Zhang et al., 2010), but these procedures are tedious and expensive. More recently, a method based on the use of HILIC-HPLC coupled with mass spectrometry has been reported for residual lactose determination with good results (Trani et al., 2017). This method uses an amine stationary phase, but the use of amine for routine analysis produces stability problems due to the reactivity of reducing the sugar with the stationary phase. Additionally, the LOQ of this method is below the EFSA recommendations (EFSA, 2010) (100 mg L^{-1} for products labelled lactose-free). High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is the most commonly used chromatographic technique (Trani et al., 2017; QCL, 2010; van Scheppingen et al., 2017). The main drawback in this case is that the laboratory needs specific instruments capable of managing highly alkaline mobile phases. Moreover, the resolution obtained in most cases is low due to the presence of interferences that makes adequate quantification in routine analysis difficult. This usually occurs when the column has analysed several sets of samples.

The aim of this work is to validate a simple, reliable, fast and cost-effective analytical method to determinate residual lactose in milk using HILIC-HPLC–MS/MS. The method has been applied to different commercial lactose-free milk samples, proving a useful tool for routine quality control analysis.

2. Materials and methods

2.1. Chemicals and reagents

Water (18.2 MΩ cm) was purified using a MilliQ system from Millipore (Molsheim, France). Analytical grade standards, lactose monohydrate and melezitose were purchased from Sigma-Aldrich (St. Louis, MO, USA). A total of 100 mg of lactose (105 mg of lactose monohydrate) were accurately weighted and diluted in 100 mL of purified water in a volumetric flask. The melezitose solution, used as surrogate, was prepared in a volumetric flask by dissolving 40 mg of the solid substance in a final volume of 100 mL of purified water. Mass spectrometry grade acetonitrile, ammonium hydroxide, phosphotungstic acid · $4H_2O$, zinc acetate · $2H_2O$, and glacial acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lactase Ha5200 was purchased from CHR-HANSEN Holding A/S (Hoersholm, Denmark). Phosphotungstic acid solution for protein and fat precipitation was prepared in a 100 mL volumetric flask by dissolving of 9.1 g of zinc acetate and 5.46 g of phosphotungstic in 70 mL of water. After complete dissolution, 5.8 mL of glacial acetic acid were added and the flask was filled to the mark with water (International Dairy Federation, 1998). The solution was stored in a dark and cool place for a maximum of five days.

2.2. Instrumentation and software

HPLC–MS/MS analysis of lactose was performed using an Agilent Technologies 1200 series HPLC instrument coupled to a 6460 triple quadrupole mass spectrometer (Palo Alto, CA, USA) with electrospray ionization (ESI) source. The control of the instrument was carried out with MassHunter WorkStation software vs. B.08.00. For HPLC–MS analyses, four type of columns were used: two propylamine columns: Carbohydrate-NH_2 (5 μm, 250 × 4.6 mm) purchased from Teknokroma (Barcelona, Spain) and Luna-NH_2 (5 μm, 250 × 4.6 mm) from Phenomenex (Torrance, CA, USA); a BEH Amide column (3.5 μm, 250 × 4.6 mm) from Waters (Milford, MA, USA); and a strongly acid cation exchange resin MCI®GEL CK08E (9.0 μm, 8 × 300 mm) from Mitsubishi Chemical (Tokyo, Japan), were tested as stationary phases. For HPAE-PAD analysis, assays were carried out with an chromatograph Dionex AMMS III–4 mm equipped with an AS50 autosampler, an AS50 column compartment, an ED50 electrochemical detector, and a GP50 gradient pump. The software employed was Chromeleon (v. 6.40). A strong anion-exchange column Dionex PA20 (150 × 3 mm) provided with a guard column (30 × 3 mm) from Thermo Fisher Sci (Sunnyvale, CA, USA) was tested. Other laboratory equipment such as an Eppendorf microcentrifuge (Eppendorf, Hamburg, Germany) and a vortex mixer (Heidolph, Schwabach, Germany) were also used. For calibration and statistical analyses of data, Statgraphics Plus software vs 5.0 (Manugistics Inc., Rockville, MD, USA) was used.

2.3. Extraction procedure

Based on the procedure previously proposed by Zafra-Gómez et al. (2008) an aliquot of 500 μL of homogenized milk sample (raw, whole, semi-skimmed and skimmed milk) was introduced into a 1.5 mL Eppendorf tube and 100 μL of the melezitose internal standard solution (0.4 g L^{-1}) was added. After vortex homogenization for 10 s, 600 μL of pure acetonitrile was added and vortexed for 1 min. The mixture was centrifuged for 5 min at 14.500 rpm (14.100*g*) and the supernatant was filtered through a 0.22 μm cellulose regenerated filter. The final extract was directly injected into the HPLC–MS/MS system. The analysis was carried out in triplicate.

2.4. Preparation of calibration standard and spiked samples

For calibration purposes, different volumes of standard solutions (250 μL, 500 μL, 750 μL, 1000 μL, 1250 μL and 1500 μL of a 1000 mg L^{-1} lactose standard solution) were transferred to 10 mL volumetric flasks. After filling to the mark with water, 500 μL of each standard solution underwent the previously described sample treatment procedure. For standard addition studies and matrix-matched calibration, different volumes of the standard solutions (250 μL, 500 μL, 750 μL, 1000 μL, 1250 μL and 1500 μL of a 1000 mg L^{-1} lactose standard solution) were transferred to 10 mL volumetric flasks filled to the mark with lactose-free milk. Finally, 500 μL of each standard solution were treated according the previously described sample treatment. The lactose-free milk was obtained by adding 1.6 mL of lactase to 250 mL of UHT milk, which was allowed to hydrolyze for 10 h at 25 °C. The hydrolysed milk was heated to the boiling point in order to completely deactivate lactase.

2.5. HPLC–MS/MS conditions

Four of the most common stationary phases used for the determination of sugars by liquid chromatography were tested in order to