Food Chemistry 220 (2017) 18–24

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Lactose, galactose and glucose determination in naturally "lactose free" hard cheese: HPAEC-PAD method validation

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ARTICLE INFO

Article history:
Received 30 May 2016
Received in revised form 28 September 2016
Accepted 28 September 2016
Available online 29 September 2016

Chemical compounds studied in this article:
Lactose (PubChem CID: 440995)
Galactose (PubChem CID: 6036)
Glucose (PubChem CID: 5793)

Keywords:
Lactose
Galactose
Glucose
HPAEC-PAD
Method validation
Grana Padano PDO cheese

ABSTRACT

A chromatographic method by HPAEC-PAD was developed and in-house validated for the quantification of low sugar levels in hard cheese, specifically Grana Padano PDO cheese. Particular attention was paid to the extraction procedure, due to residual microbial and enzymatic activities. Specificity in detection and linearity were verified. Recoveries ranged from 93% for lactose to 98% for glucose and galactose. The obtained LOD and LOQ values were, respectively, 0.25 and 0.41 mg/100 g for lactose, 0.14 and 0.27 mg/100 g for galactose, and 0.16 and 0.26 mg/100 g for glucose. The method was applied to 59 samples of Grana Padano PDO cheese: galactose showed the highest concentration and variability among the samples (1.36 ± 0.89), compared to both lactose (0.45 ± 0.12) and glucose (0.46 ± 0.13). Considering the very low levels of sugars detected, authentic PDO Grana Padano could be safely included in the diet of people suffering from lactose intolerance.

1. Introduction

The inability to metabolise lactose, due to the absence or reduced production of the enzyme lactase, is a widespread condition. The frequency of lactose intolerance varies considerably between different ethnic groups and populations, the lowest rates in North European, North American and Australasian people (5–18%) and the highest ones in South America, Africa and Asia with approximately 50% of the population affected and almost 90% in some Far East countries (Lomer, Parkes, & Sanderson, 2008).

The alteration or the reduction in the expression of the lactase gene is the cause of primary lactase deficiency, while damage of the epithelium of the small intestine, due to different intestinal diseases, is responsible for the secondary lactase deficiency. The latter is often reversible with the correction of the underlying disease (EFSA, 2010). As a consequence, lactose tolerance varies widely among individuals with lactose maldigestion. A single threshold of lactose for all lactose intolerant subjects cannot be determined owing to the great variation in individual tolerances.

In the last decades, in order to allow the consumption of dairy products also by people suffering from lactose intolerance, without experiencing discomfort, lactose-free or lactose-reduced dairy products have been developed.

In dairy products, lactose content can be reduced by both lactic acid fermentation and enzymatic hydrolysis by lactase (Harju, Kallioinen, & Tossavainen, 2012). The enzymatic process leads to the reduction of lactose through its hydrolysis to glucose and galactose, thus increasing the sweetness of the product. The lactic acid microbial fermentation determines the reduction not only of lactose, but also of galactose and glucose, which are metabolized by microflora. As a result, long fermented products, as ripened hard cheese, contain very low amounts of all the sugars.

There is no legal definition for the terms "lactose free" or "lactose-reduced", either in USA or in EU legislation, except for

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http://dx.doi.org/10.1016/j.foodchem.2016.09.185
0308-8146/

infant and follow-on formula in which lactose should be ⩽10 mg/100 kcal (Commission Directive, 2006). Some EU Member States have set thresholds at national level for the use of the terms "lactose-free", "very low lactose" and "low lactose" for foodstuffs other than products intended for infants. These threshold levels vary from 0.01 to 0.1 g/100 g of final product (EFSA, 2010).

Unfortunately, to our knowledge, no official methods are available for the determination of low concentration of lactose in dairy products, and particularly in long ripened cheese. The Standard method 22662 (ISO, 2007) reports the reference HPLC method for the determination of lactose content of raw milk, heat-treated milk, dried milk and raw and pasteurised cream and the precision parameters are referred to a lactose content varying from 1.5–50 g/100 g of product. In addition, both ISO 26462 (ISO, 2010) and ISO 9622 (ISO, 2013), based on differential pH measurement and infrared spectroscopy, respectively, are only applicable to milk and liquid milk products with full lactose content. The field of application of the ISO enzymatic methods (ISO, 2002a and ISO, 2002b) is likewise restricted to dried milk and ice-mixes in dry form (IDF Bulletin International Dairy Federation., 1993) having a lactose concentration 10–50 g/100 g.

Traditional approach to the analysis and detection of milk sugars is HPLC coupled with Refractive Index (RI) detector (Chavez-Servin, Castellote, & Lopez-Sabater, 2004; Pirisino, 1983; Pereira da Costa & Conte-Junior, 2015; Silveira et al., 2015) because neither fluorophore nor chromophore is necessary. However, RI has some disadvantages: it is non-specific, quite sensitive to changes in temperature, pressure, and solvent composition, and it does not allow gradients. Moreover, this detector has low sensitivity when compared to other detection methods. In order to overcome some of the above-cited disadvantages, the anion exchange chromatography coupled with the pulsed amperometric detection (HPAEC-PAD) was successfully applied to the sugar determination, also in milk and dairy products (Cataldi, Angelotti, & Bianco, 2003; Gopal & Richardson, 1996; Mullin & Emmons, 1997; Perati, De Borba, & Rohrer, 2014; Pollman, 1989; Van Calcar et al., 2014; Van Riel & Olieman, 1991). Together with chromatographic methods, the spectrophotometric/enzymatic measurement was applied to lactose and galactose determination (Lynch, Barbano, & Fleming, 2007; Portnoi & MacDonald, 2009, 2011, 2013).

Despite both HPAEC-PAD and spectrophotometric/enzymatic methods improved the sensitivity (Cataldi, Campa, Angelotti, & Bufo, 1999; Cataldi et al., 2003; Portnoi & Macdonald, 2011), it still remains the problem of a precise sugar measurement in dairy products naturally containing very low amount of glucose, galactose and lactose, i.e. medium and long ripened cheeses. The aim of this research was the development and the validation of a method able to determine the real content of sugars in ripened hard cheese. This method could be successfully adopted to define the concentration limits allowing the term "naturally lactose free" to be used for labelling purposes. Moreover, it could provide more precise data on the composition of Grana Padano PDO cheese and its possible inclusion in the diet of people suffering from galactosemia, which unlike lactose intolerance, causes permanent and severe damages (EFSA, 2010).

2. Materials and methods

2.1. Chemicals and reagents

Methanol, (analytical grade, >99%), potassium hexacyanoferrate (III) (purity 99%), zinc acetate (purity 99%), and the carbonate-free 50% NaOH solution for ionic chromatography were purchased from Sigma Aldrich Chemical Co., St. Louis, MO, USA. The deionized water was always obtained by Milli-Q® system (Merck KgaA, Darmstadt, Germany). The three stock standard solutions of glucose, galactose and lactose, were prepared by diluting 0.1 g of each sugar (99% purity-Sigma Aldrich Chemical Co.) in 1 L of deionized water.

The Carrez solutions I and II were prepared by dissolving 15.0 g of potassium hexacyanoferrate (III) and 22 g of zinc acetate in 100 mL of deionized water, respectively. The mobile phase for anionic exchange chromatography, consisting of NaOH 200 mM, was prepared as follow: one liter of deionized water, in a plastic bottle, was sonicated for 30 min and then 10.4 mL were removed and substituted with the same amount of the 50% NaOH solution.

Single standards, containing different concentrations of glucose and galactose, were prepared by diluting the corresponding stock solution to yield concentrations of 0.125, 0.25, 0.5, 1, 2 and 5 mg/L. Due to high variability observed in the preliminary analyses of the lactose standard solution at a concentration of 0.125 mg/L, it was excluded from the calibration data set.

2.2. Samples

To develop and validate the analytical procedure, six samples of Grana Padano PDO cheese were purchased at the local market. After removing the rind (3 mm) in such a way as to provide a sample representative of the cheese as it is usually consumed, the cheese was grinded.

After the validation of the method, several Grana Padano PDO cheese samples (slices of about 1 kg each) were taken directly at producers, by the technicians of the Grana Padano Protection Consortium. All the samples belonged to the categories excellent (E) and good (G), i.e. all the cheeses met the requirements requested by the Product Specifications and were fire-branded with the PDO mark by the technicians of the Consortium.

The samples ranged from 9 (period at which the cheese can be commercialized) to 23 months of ripening. Moreover, to verify the possible influence of lysozyme, additive usually adopted in the production of Grana Padano to prevent the Clostridia development, some samples produced without this additive (EnL) were collected, as well.

2.3. Sugar extraction

Thirty milliliters of deionized water were added to 10 g of grinded cheese in a 100 mL flask. The flask was heated in microwave and, when the boiling started, it was immediately cooled under fresh water. The sample was then submitted to 3 cycles of sonication by probe (9.5 mm tip diameter, 23 kHz output frequency, 22% amplitude; Soniprep 150, MSE, UK) for 1 min with 30 s gaps between each cycle and homogenized by Ultraturrax (IKA-Werke GmbH & Co, Staufen, Germany) for 2 min. The sample was then quantitatively transferred into a 50 mL volumetric flask and diluted to mark with deionized water.

After filtration and centrifugation at 6000*g* for 10 min at room temperature, 20 mL of supernatant were mixed with 400 μL of Carrez I and 500 μL of Carrez II and diluted to mark (50 mL) with deionized water. At the appearance of a thick precipitate, usually 15 min, the sample was filtered and 4 mL were loaded onto a SPE sulfonic acid bonding column (Discovery DSC-SCX, 6-ml volume, 1 g sorbents, 50 μm particle size, Sigma Aldrich Chemical Co), previously washed with 5 mL of methanol and 10 mL of deionized water. The first milliliter of eluate was discarded and the next 3 mL were recovered, filtered through a 0.45 μm nylon membrane and 25 μL of this final clarified extract was injected into the HPAEC-PAD system for analysis.

2.4. HPAEC-PAD analysis

A UltiMate 3000 chromatography system (Dionex, Sunnyvale, CA) consisting of a LPG 3400 SD pump and an ECD 3000 RS