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Study on the sugar content of blue-veined "Gorgonzola" PDO cheese

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ABSTRACT

A chromatographic method, HPAEC-PAD, was applied to 49 samples of Gorgonzola PDO cheese to quantify residual sugars. Very low levels were detected in both sweet and piquant categories: values were, respectively, 1.24 ± 1.30 and 0.69 ± 0.11 mg 100 g^{-1} for lactose, 1.21 ± 0.60 and 2.07 ± 1.77 mg 100 g^{-1} for galactose and 5.41 ± 4.58 and 4.46 ± 4.09 mg 100 g^{-1} for glucose. Analysis of cores, taken from the same cheese at different days of ripening, showed that sugars had already been completely metabolised 10 days after production. These results allow the use of term "naturally lactose free" for Gorgonzola PDO cheese. The chromatographic profile showed the presence of other peaks, which were tentatively identified as polyols (sugar alcohols), probably derived by metabolism and catabolism of microflora.

1. Introduction

Gorgonzola is a blue-veined cheese produced in a defined geographical area in the North of Italy from pasteurised whole cows' milk. The cheese gained the Protected Designation of Origin (PDO) certification in 1996 (Commission Regulation 96/1107/EC; EC, 1996), recently amended (Commission Regulation 2017/1595/EU; EU, 2017). The production process is strictly regulated by the Standard Specifications, issued by the Consortium for the protection of Gorgonzola cheese, defining the area of milk collection, the production, the ageing, and the standards of manufacturing, to ensure quality and authenticity. This cheese exists in two varieties, sweet and "piccante" (piquant).

Selected cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus* and a suspension of *Penicillium* spores including selected yeasts (*Saccharomyces cerevisiae*) are inoculated into the pasteurised milk. Milk is coagulated with liquid calf rennet at 28–36 °C; after extraction and draining of the coagulum, curd is transferred into cylindrical plastic moulds and stored in a sweating warm room at 18–24 °C from two to seven days, according to the variety of Gorgonzola. During this period, the cheese is regularly dry-salted. Then, it is ripened at a temperature of 3–7 °C and 85–100% relative humidity, for at least 50 days for sweet, and more than 80 days for piquant Gorgonzola. To promote the development of *Penicillium*, which gives Gorgonzola its characteristic blue-green veining, the cheese is skewered, with steel needles, two times during ripening (Gobbetti, Burzigotti, Smacchi, Corsetti, & De Angelis, 1997; Mucchetti & Neviani, 2006).

Characteristics of Gorgonzola are the result of the action of the complex microflora and enzymes, especially on proteins and fat, during ripening. Contarini and Toppino (1995), Gobbetti et al. (1997) and Fernández-Salguero (2004) conducted studies to characterise proteolytic and lipolytic processes occurring in Gorgonzola cheese. To our knowledge, no studies are available on the composition of the carbohydrate fraction, probably because it was considered negligible, due the strong fermentation processes taking place from the first hours of cheese making. Only Del Piano, Tari, and Carmagnola (2012) studied residual lactose content in 26 samples of Gorgonzola, and, in only one sample, detected lactose at a concentration higher than the limit of detection (0.035 g 100 g^{-1}).

Recently, carbohydrate, in particular lactose, has gained special attention, due to widespread lactose malabsorption in the population, leading to considerable reduction in dairy-based foods consumption (Silanikove, Leitner, & Merin, 2015).

A legal definition for the term "lactose free" is not universally accepted, and only some EU member states have set thresholds at a national level, varying from 0.01 to 0.1 g 100 g^{-1} of final product (EFSA, 2010).

While waiting for harmonisation at the European level, the Italian Health Ministry issued a Circular (IHM, 2015) that allowed

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the adoption of the term “lactose free” for dairy products containing less than 0.1% (w/w) of lactose. In 2016, a second Circular (IHM, 2016) allowed use of term “naturally lactose free” for dairy products, mainly cheese, in which reduction of lactose is only due to microbial fermentation occurring during cheese-making and ripening. Interest in a precise determination method for lactose in dairy products is demonstrated by studies published in the last two years, aiming to develop new and more sensitive methods (Gambelli, 2017; Garballo-Rubio, Soto-Chinchilla, Moreno, & Zafra-Gómez, 2018; Idda et al., 2016; Kučerová, Komenská, Tomková, Skopalová, & Barták, 2017; Monti et al., 2017; Perati, de Borba, & Rohrer, 2016; Trani et al., 2017; van Scheppingen, van Hilten, Vijverberg, & Duchateau, 2017; Vaskova & Buckova, 2016). In addition, EU rules on food labelling (EU, 2011), in force from the end of 2016, stimulated food companies to accurately verify the composition of their products.

Starting from a request by the Consortium for the Protection of the Gorgonzola Cheese, the aim of this work was to accurately quantify lactose, glucose and galactose remaining in Gorgonzola at commercial sale, using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

2. Materials and methods

2.1. Chemicals and reagents

Methanol (analytical grade, >99%), potassium hexacyanoferrate (III) (purity 99%), zinc acetate (purity 99%), sodium acetate and carbonate-free 50% NaOH solution for ionic chromatography were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Deionised water was obtained using a Milli-Q® system (Merck KgaA, Darmstadt, Germany).

Carrez solutions I and II were prepared by dissolving 15.0 g potassium hexacyanoferrate (III) and 22 g zinc acetate in 100 mL deionised water, respectively. Pure standards of sugars (Sigma Aldrich Chemical Co.) were used to quantify glucose, galactose and lactose, according to Monti et al. (2017). Pure standards of glycerol, mannitol, arabinitol, xylitol, erythritol, ribitol, dulcitol, myo-inositol and sorbitol (Alfa Aesar, Heysham, UK) were injected to verify presence of polyols.

2.2. Sampling

The Consortium for the Protection of Gorgonzola (Novara, Italy) supplied slices of forty-nine samples of Gorgonzola PDO cheese at commercial stage (approximately 250 g each): forty-four at 50–60 days of ripening, belonging to the category “sweet” and five at 80–90 days of ripening, to the category “piquant”. The small number of piquant category was due to the low number of cheese factories producing this type of Gorgonzola PDO cheese compared with the sweet type. In addition, samples at 10, 20, 30, 40 and 65 days of ripening, were collected in one cheese factory.

2.3. Sample preparation

Samples of Gorgonzola PDO cheese were analysed by an in-house validated HPAEC-PAD chromatographic method, developed for the quantification of low sugar levels in hard cheese (Monti et al., 2017). An altered extraction procedure was necessary, due to the peculiar characteristics of the paste, and, in particular, to the presence of moulds. After removing the rind (0.5 cm), the entire cheese was blended, and 10 g were put in a 100 mL flask, together with 30 mL deionised water. The flask was heated in microwave and, when boiling started, it was immediately cooled in ice. The sample was then subjected to 3 cycles of sonication by probe (9.5 mm tip diameter, 23 kHz output frequency, 22% amplitude; Soniprep 150, MSE, London, UK) for 1 min with 30 s gaps between each cycle and then homogenised by Ultraturrax (IKA-Werke GmbH & Co, Staufen, Germany) for 2 min. The sample was then quantitatively transferred into a 50 mL volumetric flask, with addition of 1 mL and 1.5 mL of Carrez I and II, respectively, and diluted to mark with deionised water. After 15 min, sample was poured into centrifuge tubes, centrifuged at 6000 $\times g$ for 30 min at 4 °C, and filtered through a filter paper. Twenty millilitres of clear filtrate were taken and diluted to 50 mL with deionised water. Four millilitres of the diluted extract were loaded onto a SPE sulphonic 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 methanol and 10 mL deionised water. The first millilitre of eluate was discarded, and the next 3 mL were recovered and filtered through a 0.45 μm nylon membrane; 25 μL of this final clarified extract was injected into the HPAEC-PAD system for analysis.

2.4. HPAEC-PAD analysis

Lactose, glucose and galactose were analysed according to Monti et al. (2017), using a CarboPac PA20 column (Thermo Scientific, Sunnyvale, CA, USA).

2.5. Statistical analysis

The method was in-house validated in a previous work (Monti et al., 2017), on hard and long ripened cheese, characterised by an average content of 61 g dry matter 100 g^{-1} cheese. The different texture of Gorgonzola cheese (about 49 and 55 g dry matter 100 g^{-1} cheese for sweet and piquant type, respectively) and the scarce homogeneity of its paste due to presence of moulds required repeatability parameters to be verified, according to the indications of the Eurachem Laboratory Guide to Method Validation (Magnusson & Örnemark, 2014).

The XLSTAT© 7.5 package (Addinsoft, France) was used for calculation.

3. Results and discussion

3.1. Sample preparation

Samples to be analysed were at first prepared following the protocol for hard and long ripened cheese, as described by Monti et al. (2017). However, the soft and creamy texture of sweet type, and crumbly consistency of the piquant, together with the presence of mould, affected the extraction procedure. Filtration of the water-diluted cheese was not feasible. Consequently, both the deproteinisation and centrifugation steps were prerequisite, compared with the original protocol. Moreover, the quantity of Carrez solutions and the time of centrifugation were altered to obtain a clear filtrate.

3.2. Repeatability

Six independent sugar measurements were performed on the same 250 g slice of cheese by a single analyst, using the same equipment, in a short time frame. The sample was homogenised as carefully as possible, using an electric blender. The repeatability limits, expressed as mg 100 g^{-1}, were 0.42 for lactose, 0.20 for galactose and 0.62 for glucose. As expected, the repeatability calculated for the three sugars was higher than previously obtained (Monti et al., 2017) for hard and long ripened cheese, i.e., 0.04, 0.12 and 0.05 mg 100 g^{-1} for lactose, galactose and glucose, respectively.

To confirm if the presence of moulds really affected these results, an experiment was conducted analysing, separately, the paste appearing with (B) and without (W) blue mycelium (Fig. 1).

The analyses were carried out on only one slice of cheese, sampling manually four portions of each W and B area. The four samples were carefully blended and 5 replicate analyses were carried out on each sample. The results, statistically evaluated by applying the *t* test (Table 1), confirmed that the presence/absence of the mycelium greatly affected the sugar content.

Lactose and glucose, in particular, provided the highest differences between the two areas (1.45 versus 0.57 and 0.96 versus 0.26 mg 100 g^{-1}, for lactose and glucose, respectively). The same two sugars showed higher repeatability limits (0.42 and 0.62 mg 100 g^{-1} for lactose and glucose, respectively) than galactose (0.20 mg 100 g^{-1}).

3.3. Sugar concentration in Gorgonzola PDO cheese

Forty-nine samples of Gorgonzola PDO cheese were analysed by HPAEC-PAD to quantify lactose, galactose and glucose content. All the samples were analysed in duplicate, under repeatability conditions. Descriptive statistics indices for each sugar are reported in Table 2. The small number of piquant cheese samples did not allow for application of statistical tests useful to detect significant differences between the two Gorgonzola PDO categories.

Independent of category, glucose was more concentrated (5.33 ± 4.49 mg 100 g^{-1}) than both galactose (1.29 ± 0.81) and lactose (1.21 ± 1.26). However, lactose showed the highest variability. Distribution values for each sample (Fig. 2), allowed for interesting comparisons to be made.

In three samples of sweet category and in two samples of the piquant one, lactose was not quantifiable, as it was below LOD (0.25 mg 100 g^{-1}) or LOQ (0.41 mg 100 g^{-1}) values, calculated in previous work (Monti et al., 2017).

Among the other samples, the piquant samples, together with the 61% of the samples in the sweet category, showed a lactose content lower than 1 mg 100 g^{-1} (Fig. 2). The lactose concentration of remaining sweet samples ranged from 1 to 3 mg 100 g^{-1}, except for two samples with higher values (7.58 and 4.85 mg 100 g^{-1}). These two samples contributed to the high standard deviation (1.21 ± 1.26) reported in Table 2; re-calculation excluding these samples gave a lower value (0.97 ± 0.53). However, all Gorgonzola PDO samples were always far below the 0.1 g 100 g^{-1} Italian limit for lactose-free labelled products. In addition, since the low lactose concentration was due to the action of natural microflora, the Gorgonzola PDO cheese can adopt the term "naturally lactose free".

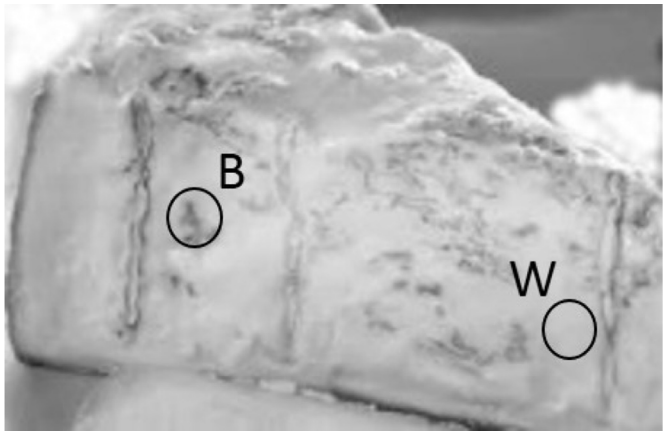

Fig. 1. Slice of Gorgonzola cheese: circles highlight portions with (B) and without (W) visible development of *Penicillium* mycelium.

Table 1
Sugar concentration of samples taken from portions of Gorgonzola PDO cheese with (B) and without (W) evident mycelium.[a]

Sugar	B zone	W zone
Lactose	1.45 ± 0.12[a]	0.57 ± 0.21[b]
Galactose	0.52 ± 0.17[a]	0.72 ± 0.07[b]
Glucose	0.96 ± 0.23[a]	0.26 ± 0.04[b]

[a] Values (mg 100 g^{-1}) are means and standard deviation (n = 5); means in a row with different superscript letters are significantly different ($p < 0.05$).

In both sweet and piquant type, galactose showed similar minimum and maximum values, and only in four samples, did its concentration exceed 2 mg 100 g^{-1}. Surprisingly, glucose, which is the carbohydrate preferentially used by microorganisms (Gorke & Stulke, 2008), was the most concentrated sugar in both categories of Gorgonzola PDO cheese, with higher values in sweet samples. These results led to the hypothesis that this sugar was derived not only from lactose, but also from hydrolysis of other carbohydrates. Some fermentable polysaccharides consisting of glucose units can be present in blue veined cheese, as is the case for maltodextrins that are commonly used as cryoprotectants and preservatives in commercial mould starters, and gluco-mannans that are the main constituents of yeast and mould wall and mycelium (Andriyanova et al., 2011). The hypothesis seemed to be supported by the results obtained on the samples analysed during the ripening period (Fig. 3).

It is worth noting that, because of the action of lactic and non-lactic microflora, at 10 days of ripening, the contents of both lactose and galactose were already negligible (about 1.5 mg 100 g^{-1}) and did not show further variation. In contrast, glucose was lower than LOQ (0.26 mg 100 g^{-1}) during the first 30 days, but constantly increased up to 8.8 mg 100 g^{-1} over the remaining ripening time. This behaviour seemed to demonstrate that glucose deriving from lactose hydrolysis was immediately metabolised by microflora, with the increase being due to the progressive death and lysis of the mould cells resulting in release of hydrolytic enzymes which acted on the polysaccharides of the cell walls (White, McIntyre, Berry, & McNeil, 2002).

The profile of HPAEC-PAD analysis (Fig. 4) showed, in addition to galactose, glucose and lactose, the presence of other detectable peaks. The highest ones eluted before galactose, at retention times ranging from 1 to 3 min, and others between glucose and lactose. Comparison with literature data (Cataldi, Campa, Casella, & Bufo, 1999; Corradini, Cristalli, & Corradini, 1993; Rohrer, 2013) indicated that some polyols in similar chromatographic conditions elute before glucose, suggesting the possible presence of sugar alcohols. The straight-chain sugar alcohols belong to the class of easy mobilisable carbohydrates used in fungi metabolism as the main energy-storage compounds (Walker & White, 2005). Among the different polyols, literature data on *Penicillium* and *Saccaromyces* strains report the presence of glycerol (Ferreira et al., 2005; Lewis & Smith, 1967; Nevoigt & Stahl, 1997; Rast & Pfyffer, 1989), mannitol (Mioso, Marante, Laguna, Bravo de González, & Rodríguez, 2014; Nguyen Van Long et al., 2017), arabinitol (Beuchat, 1986), xylitol (Hansa & Mulay, 2014), erythritol (Shindou & Ishizuka, 1996), ribitol (Deacon, 2006), dulcitol (Rast & Pfyffer, 1989) and myo-inositol (Zhao, Liu, Zhang, & Zhang, 2010).

Pure standards of the eight above-mentioned polyols, together with sorbitol, were injected by applying the same HPAEC-PAD conditions adopted for sugar analysis. No defined separation was obtained among the polyols, which all eluted from 1 to 3 min and completely overlapped. This result was in accordance with

Table 2
Lactose, galactose and glucose concentration (mg 100 g^{-1} of cheese) in Gorgonzola PDO samples.

Cheese category	Sugar	Minimum	Maximum	Mean	Standard deviation
"Piquant" (n = 5)	Lactose	<LOQ	0.78	0.69	0.11
	Galactose	0.51	4.57	2.07	1.77
	Glucose	1.51	10.91	4.64	4.09
"Sweet" (n = 44)	Lactose	0.50	7.58	1.24	1.30
	Galactose	0.49	4.05	1.21	0.60
	Glucose	0.54	19.03	5.41	4.58
Total (n = 49)	Lactose			1.21	1.26
	Galactose			1.29	0.81
	Glucose			5.33	4.49

Corradini, Canali, Cogliandro, and Nicoletti (1997) reporting that, under the conditions applied for mono-and disaccharides analysis (type of column and sodium hydroxide concentration), the ionisation of alditols is weak and their separation is poor. However, the reasonable hypothesis that the carbohydrate fraction of Gorgonzola would also include sugar-alcohols may not be rejected.

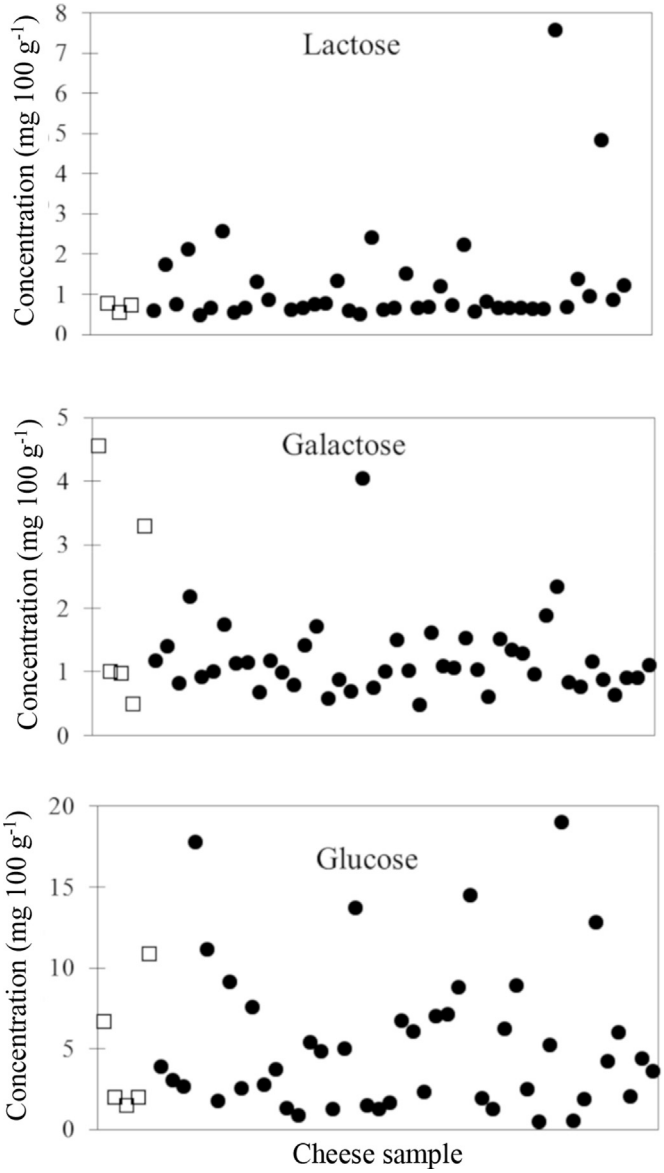

Fig. 2. Lactose, galactose and glucose content, expressed as mg 100 g^{-1}, in piquant (white squares, □) and sweet (black circles, ●) Gongonzola PDO cheese samples.

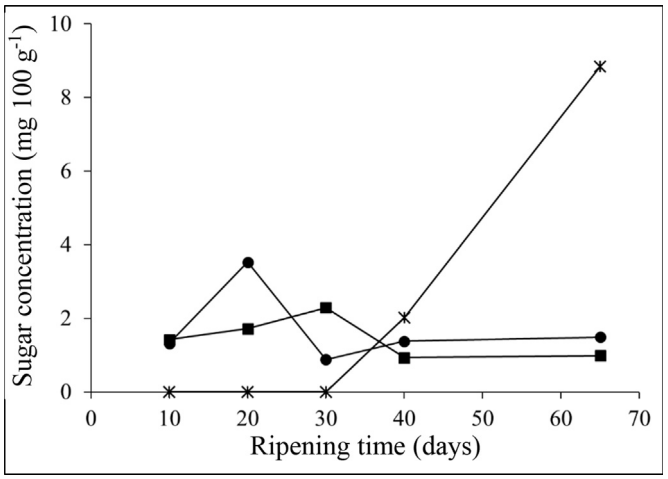

Fig. 3. Lactose (■), galactose (●) and glucose (⁎) content (mg 100 g^{-1} of cheese) in Gorgonzola cheese at different ripening times.

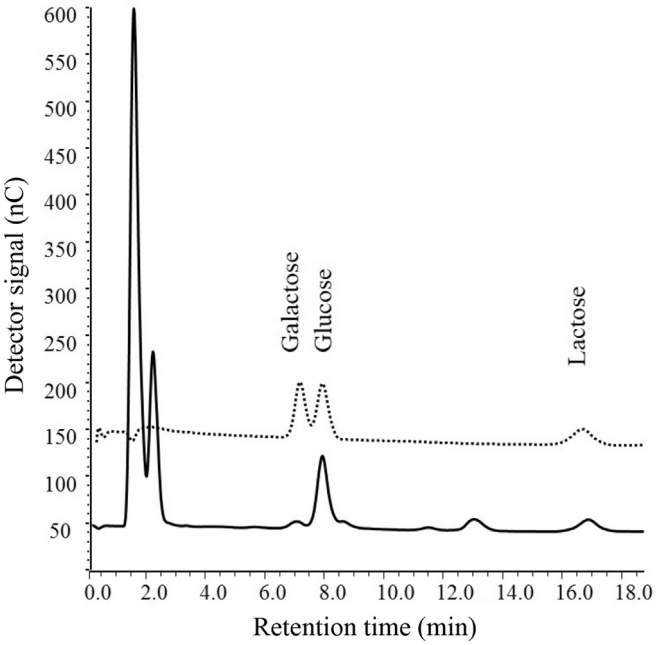

Fig. 4. HPAEC-PAD profile of both a Gorgonzola cheese sample (solid line) and a standard (dotted line) containing 1 mg 100 g^{-1} of galactose, glucose and lactose.

The next step of this research, already in progress, will be dedicated to the identification of the unknown peaks detected in the main sugar analysis, by using HPAEC-PAD conditions specific for polyols, followed by confirmation by GC/MS.

4. Conclusions

Application of the HPAEC-PAD method, previously developed for hard cheese, to blue-veined cheese, needed some modifications to the extraction procedure. The different texture of Gorgonzola PDO cheese, characterised by both higher moisture content and stronger proteolysis and lipolysis phenomena than hard cheese, caused some filtration difficulties. The problem was solved by moving the centrifugation/filtration step after the addition of the reagents (Carrez) useful to separate proteins and fat. In addition, the low homogeneity of the paste, due to the presence of regions with and without mycelium, increased repeatability values with respect to those obtained on hard cheese samples. However, the application of the HPAEC-PAD method to a consistent sampling of Gorgonzola PDO cheese allowed for verification that the lactose content was almost one hundred times lower (0.0012 g 100 g^{-1}) than the value that Italian legislation stated for cheese labelled as "naturally lactose free" (0.1 g 100 g^{-1}). The HPAEC-PAD profile showed the presence of peaks eluting before those of the three sugars quantified in this research. A preliminary evaluation suggested the possible presence of polyols.

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