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Short communication

High-performance liquid chromatography of governing liquid to detect illegal bovine milk's addition in water buffalo Mozzarella: Comparison with results from raw milk and cheese matrix

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Abstract

A method to detect fraudulent addition of bovine milk in water buffalo Mozzarella cheese by gradient high-performance liquid chromatography (RP-HPLC), relying on the measurement of quantity ratios within β-lactoglobulin protein family, is described. Analyses were performed on raw milk, cheese matrix and cheese governing liquid using a C_4 column and UV detection. This work demonstrated that bovine milk addition during cheesemaking can be detected in governing liquid of Mozzarella down to the EU law limit of 1% as well as in raw milk and cheese matrix. A significant lowering of peaks' areas and heights was observed in cheese matrix and governing liquid samples in comparison with the corresponding milk ones, possibly due to proteins' degradation during the cheesemaking process. The results show that, unlike previous works reported, the use of a matrix-specific calibration curve is essential in order to achieve a proper quantitation of β-lactoglobulin proteins, thus allowing a reliable estimation of bovine milk addition.

Keywords: Cheese; Milk; Governing liquid; Food analysis; Whey proteins; Adulteration

1. Introduction

Italian Mozzarella is a typical fresh "pasta filata" cheese made from raw water buffalo's milk only using natural whey cultures as fermentation starters. It received the European protected denomination of origin (PDO) certification under the designation "Mozzarella di Bufala Campana" [1].

The seasonal increase in market demand occurring every summer and, on the other hand, the limited productions of buffalo milk may induce fraudulent addition of bovine milk during manufacturing of Mozzarella. A number of protein-based methods was developed to spot this fraud and to assure products' genuinity to both producers and consumers. The current legislation in EU [2] and in Italy [3], based on official control methods which rely on the isoelectrofocusing (IEF) of γ-caseins after plasminolysis [4] and on HPLC analysis [5], respectively, tolerates a maximum addition of 1% bovine milk. In addition, several other techniques to detect bovine milk in water buffalo mozzarella were developed. Identification of bovine β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) was performed by a capillary zone electrophoresis procedure for basic proteins [6]; this method is limited by the presence of interfering peaks in the area of α-LA and the variability of β-LG A/β-LG B ratio. Immunoblotting analysis to determine the presence of bovine milk in water buffalo Mozzarella was optimized by Addeo et al. [7] and consists in detecting the bovine γ-casein and peptides by the use of polyclonal antibodies against β-casein. This analysis confirmed the results obtained by IEF [4]. Recently, a new method based on the determination of protein molecular masses was developed [8]. Bovine whey protein (α-LA and β-LG) were detected in water buffalo Mozzarella by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. On the other hand, DNA-based methods relying on conventional PCR technology [9–12] are very reliable and suitable to routine detection of bovine DNA but, since these techniques are not quantitative assays, they're not appropriate to provide information on the observance of the 1% law threshold, notwithstanding a quantitative PCR method was recently developed to detect fraudulent addition of bovine milk in ovine cheeses [13]. The aim of this work is to detect fraudulent addition of bovine milk

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in Italian Mozzarella cheese by using gradient reversed-phase HPLC to assess the presence of and the quantity relationships between bovine- and buffalo-specific whey proteins. The method we propose focuses on the detection of cow milk relying on the analysis of the β-LG A and B, as reported by Urbanke et al. [14]. There are, however, other ways to adulterate cheese involving the use of industrial derivatives, for instance caseinate, instead of milk. The detection of illegally added caseinate is, therefore, not possible by this method but, besides, it appears of interest for future work since it's not directly related to the method we present. A recent method for the detection of caseinate in cheese relied on quantifying the intact, non-glycosylated κ-casein by capillary electrophoresis [15]. Another possible way to carry out a fraud in manufacturing Mozzarella is represented by the addition of previously heated bovine milk although, given the production context, it seems less probable than the addition of raw cow milk from cows raised in neighboring farms. The addition of UHT milk featuring a reduced content of β-LG might possibly lead to an underestimation of the addition itself but doesn't prevent the application of our method unless the cow-specific β-LG A is completely destroyed; this is never observed even in heavily heat-treated milks due to first-order kinetics of β-LG denaturation [16,17], nor it is reported in long-ripened cheeses [18]. Besides, a number of thermal treatment markers can be used to detect the presence of heated milk regardless of species [19]. The original experimental approach here described focuses on the possibility to directly analyze the governing liquid (i.e. the pickle in which Mozzarella is packaged and sold), thus avoiding the extraction of soluble fraction from cheese matrix which has a critical effect on protein integrity [20] and significantly increases the protocol's duration. Special attention was dedicated to the comparison between the results obtained from governing liquid, cheese matrix and the raw milk to evaluate the method's reliability. The practical aspects of this new investigative approach are also discussed.

2. Experimental

2.1. Sampling

Bovine and water buffalo raw milks were obtained from the producers in northern Italy. Mozzarella cheeses were made according to traditional manufacture, therefore exclusively using raw milk. During manufacturing, bovine milk was added in known v/v percentages as follows: 0.5% (mixture 1), 1% (mixture 2), 5% (mixture 3), 10% (mixture 4), 20% (mixture 5) and 30% (mixture 6). Milk samples were prepared by mixing bovine and water buffalo raw milks in the same ratios as above. Reference samples of raw milk and cheese from cow only and from water buffalo only were also prepared. The cheese and governing liquid samples were then preserved at 4–6 °C until analysis.

2.2. Whey protein extraction

The whey protein fractions were extracted from cheese as follows: samples (20 g) were homogenized (2 cycles of 1 min each) in doubly distilled water (30 ml) with an Ultraturrax homogenizer (Ika®-Werke, Staufen, Germany). Since this step is heat-producing, a strict time optimization was required to avoid whey proteins' degradation. The homogenized samples were skimmed by centrifugation (2000 × *g* for 15 min at 4 °C). Casein was precipitated at its isoelectric point by adding 1M hydrochloric acid and centrifuging at 2500 × *g* for 10 min at 4 °C.

Whey proteins were separated from raw skimmed milk (25 ml) by isoelectric precipitation (at pH 4.2–4.6) of casein and centrifugation. All samples were stored at −20 °C, thawed at room temperature and filtered through a 0.45 μm membrane (Millipore Corp., Bedford, MA, USA) before chromatographic analysis.

2.3. Governing liquid preparation

Fifty milliliter-aliquots of each Mozzarella's governing liquid were poured directly into sterile tubes and frozen at −20 °C. Samples were thawed at room temperature and subsequently filtered through 0.45 μm membrane (Millipore) before HPLC analysis.

2.4. Instrumentation and separation conditions

The HPLC system consisted of two pumps (model 515 Waters, Milford, MA, USA), a manual injector (Rheodyne, Cotati, CA, USA), an UV detector (2487, Waters). The instrument was controlled by the Millenium® 32 software (Waters) for data acquisition and processing. The separation was performed on a C_4 column (250 mm × 4.6 mm) with 300 Å pores and 5 μm-sized particles (Phenomenex®, Torrance, CA, USA) kept at room temperature; the detection wavelength was 205 nm. A 50-μl loop was used to load milk and cheese matrix soluble fractions, whilst a 200-μl loop was used to load governing liquid samples. Analysis was carried out applying a gradient of mobile phase's composition. Eluant A was HPLC-grade water (Nova Idrochimica, Milano, Italy) containing 0.1% trifluoroacetic acid (TFA; Merck, Darmstadt, Germany); eluant B was acetonitrile (Merck) containing 0.1% TFA. The elution gradient was set as follows: 0–1 min 35% B, 1–8 min 35–38% B, 8–16 min 38–42% B, 16–22 min 42–46% B, 22–24 min 46–90% B, 24–25 min 90% B, 25–30 min 90–35% B, 30–35 min 35% B; the flow rate was 1.0 ml min^{-1}.

2.5. Quantification of whey proteins

Samples obtained from milk mixtures and Mozzarella cheeses were analyzed to construct a calibration curve for each matrix (milk, cheese and governing liquid). Purified bovine standards of β-LG A and B and α-LA (Sigma–Aldrich, St. Louis, MO, USA) were used to identify the chromatographic peaks corresponding to bovine whey proteins; it was also found a peak corresponding to the Bx compound, as reported by literature [5], in water buffalo samples. The areas of proteins' chromatographic peaks were subsequently measured in mixture samples. β-LG A/β-LG B and β-LG A/Bx peak area ratios were calculated; calibration curves were then constructed by plotting the two ratios' values against added bovine milk percentage. Values

of β-LG A/β-LG B and β-LG A/Bx ratios were taken as the mean of three repeats.

3. Results and discussion

3.1. Separation and identification of whey proteins in cow and water buffalo milk

The described analytical conditions allowed an effective separation of the whey proteins in cow and water buffalo raw milk (Fig. 1). The elution patterns observed in cow milk, obtained using a C_4 column, showed good accordance with the ones reported by Resmini et al. [21] resulting from separation on a C_8 column, being the retention times of the major peaks (corresponding to α-LA, β-LG A and β-LG B) coincident or very similar. Furthermore, the proportion relationships between the α-LA and β-LG peaks were overall the same whereas, within the β-LG family, a slightly different ratio between A and B variants was observed. This difference was probably due to quantitative variations in the allelic expression of the variants [22,23]. The reason for the low rate of synthesis of BLG B in some milks should be at the gene level [24], but it's currently unknown and investigations are required to better describe this phenomenon. Studies so far showed a slightly higher frequency of BB genotype compared to AA but, having the A allele a higher expression level than the B one [25], a substantial balance is observed in milk since the expression levels compensate for the respective frequencies. A variety of studies indicates instead the same distribution of the three genotypes (AA, AB and BB) in Friesian and its crossbred cattles [26–31], whereas higher frequencies for B allele were observed in some autochthonous breeds [32,33]. An exception to this is represented by the Simmenthal cow, featuring a higher frequency for A allele [34]. Given this, the only case in which β-LG A could not be detected is if individual cow milk featuring BB genotype was used to adulterate the cheese. Some authors [35] achieved the separation of all the major bovine milk proteins at the same time by using a C_4 column and a purposely designed gradient of mobile phase's composition, to improve the caseins' resolution. These conditions allowed the separation of casein variants, but provided lower resolving capability for whey proteins. An analytical protocol based on the use of a polystyrene-divinylbenzene copolymer column to better separate the whey proteins only was recently developed [18], proving capable to effectively analyze binary mixtures of bovine, ovine and caprine milks. A comparison between the chromatographic profiles obtained from water buffalo and bovine raw milk, shown in Fig. 1, clearly pointed out the lack of peaks referable to Bx in the milker's one and the absence of a peak corresponding to β-LG A in water buffalo's milk, according to Pellegrino et al. [5]. To date, no water buffalo β-LG polymorphic variants are known whilst the existence of a novel α-LA variant, characterized by a single amino acid change, was recently reported [36].

3.2. Separation and identification of whey proteins in cow and water buffalo cheese

The analysis of whey protein fractions extracted from reference cheese matrix samples (made from raw cow and water buffalo milk, respectively) led to results qualitatively similar to milk samples, as shown in Fig. 2. The absence of β-LG A in water buffalo Mozzarella and of Bx compound in cow cheese was evident. From a quantitative standpoint, a significant lowering of peaks' areas and heights was observed in comparison with milk, possibly due to proteins' degradation during the cheese-making process. Ferreira and Caçote [18] observed the effect of proteolysis in fresh and ripened cheese, although the proteolysis products did not interfere with β-LG peaks. Moreover, the proteolysis degree did not affect peak ratios, and hence quantitation, in binary mixtures.

3.3. HPLC analysis of experimental mixtures

Fig. 3A–C report the chromatograms resulting from the analysis of raw milk, cheese matrix and governing liquid from experimental mixtures, respectively. The presence of cow's milk

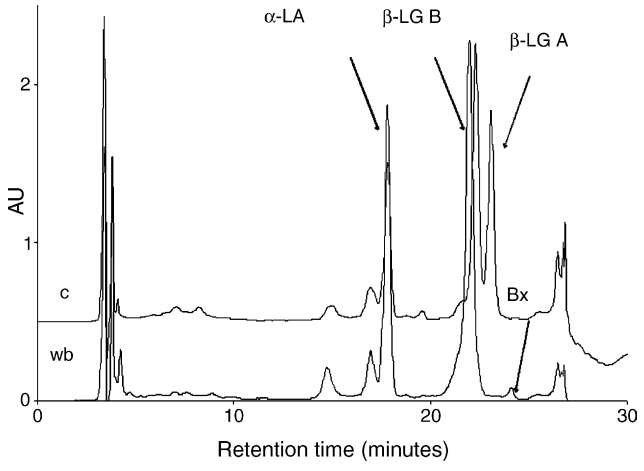

Fig. 1. Chromatographic profiles of whey proteins from cow (c) and water buffalo (wb) bulk milk. Bovine α-LA, β-LG A and B peaks, and water buffalo a-LA and Bx peaks are indicated.

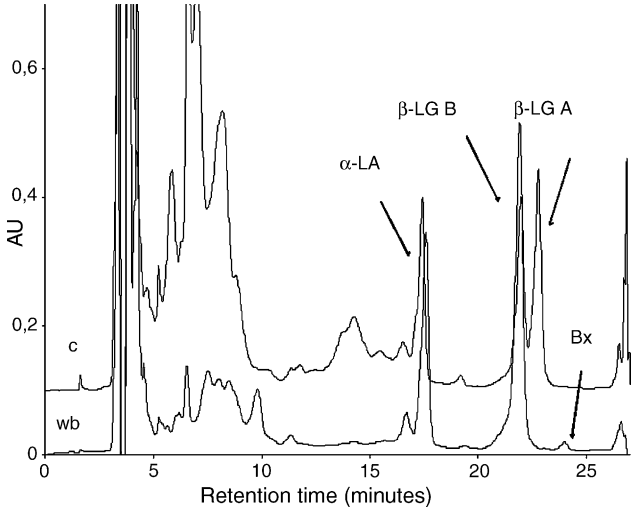

Fig. 2. HPLC patterns of soluble fraction from bovine (c) and water buffalo (wb) Mozzarella cheese.

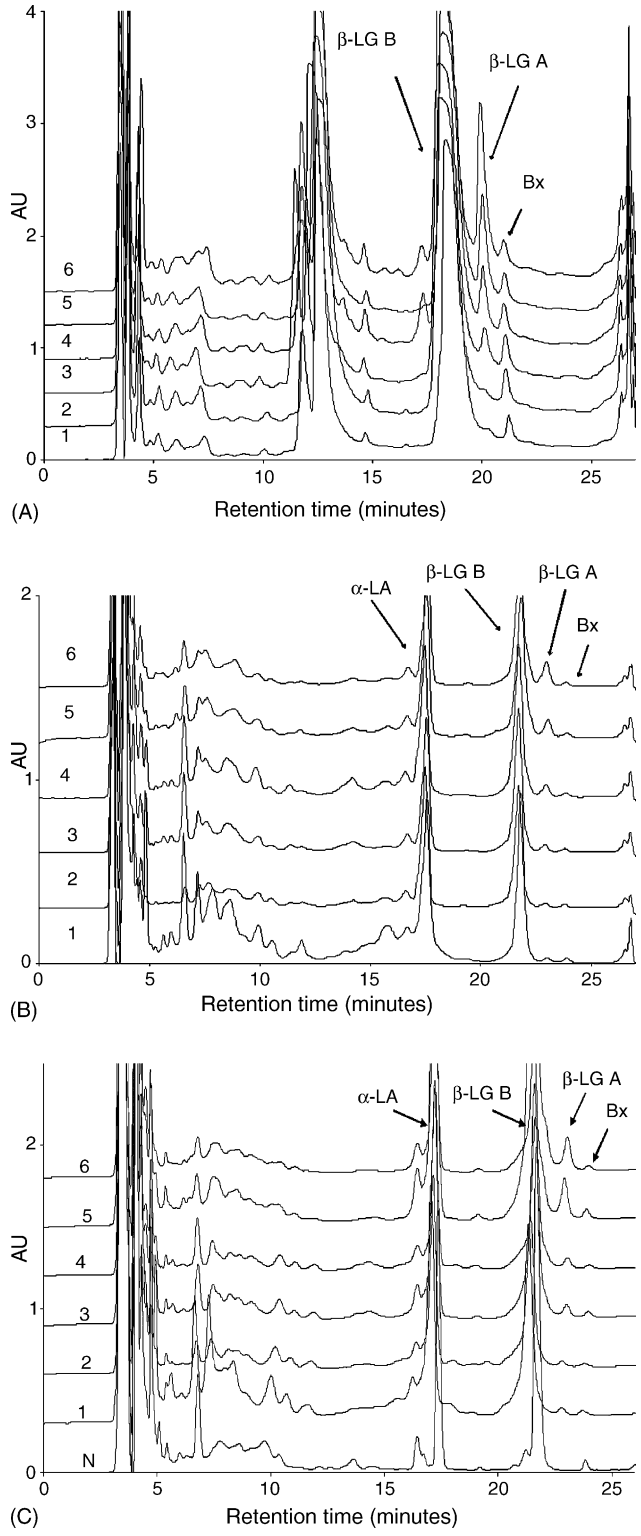

Fig. 3. HPLC patterns of milk (A), soluble fraction (B) and governing liquid (C) from experimental mixtures. Samples 1, 2, 3, 4, 5 and 6 contain 0.5, 1, 5, 10, 20 and 30% cow milk, respectively. Sample *N* is a pure-water buffalo negative control.

was always detected down to 0.5% in all matrices, demonstrating the actual applicability of this method to routine control since this value is below the law limit of 1% [2]. The peaks of interest, β-LG A and Bx, were always well resolved in each matrix, and

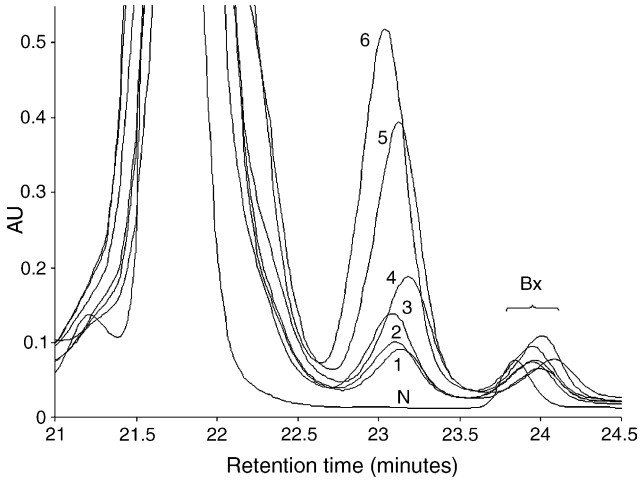

Fig. 4. Detail of P-LG A and Bx peaks in Fig. 3C. The relationship between β-LG A peak area and percentage of added bovine milk is shown, whilst the Bx peak area is not significantly affected by the addition itself.

the former clearly increased along with percentage of added cow milk. In addition, the lowering of peaks' areas from raw milk to cheese matrix and governing liquid was prominent, due to the whey proteins' degradation during cheesemaking, according to Ferreira and Caçote [18].

Fig. 4 shows a detail of the comparison between the β-LG A and Bx peaks obtained from the governing liquid samples of experimental mixtures (Fig. 3C). An evident positive relation between the β-LG A peak's area and the added bovine milk percentage in governing liquid as well as in the other matrices was observed, while the Bx peak's area showed only slight variations. This, however, seemed to be due to the fact that water buffalo always represented the major contribution to samples' composition (at least 70% in mixture 6), so that the dilution effect was scarcely important. The abovementioned results indicated that the chosen analytical conditions, consisting in the use of a C_4 column in conjunction with a specifically designed mobile phase elution gradient programme and UV detection at 205 nm, were optimal to ensure an effective separation of the whey proteins from both cow and water buffalo; in particular, the quantitative relationships involving the two species-specific proteins (i.e. β-LG A and Bx) could be measured.

3.4. Quantification of whey proteins

Calibration curves were constructed for milk, cheese matrix and governing liquid by measuring β-LG A, β-LG B and Bx peak areas (as the mean of three repeats each), calculating the β-LG A/β-LG B and β-LG A/Bx peak area ratios for each mixture sample [5] and plotting the resulting values against the percentage of added bovine milk; the obtained graphs featured a good linear trend. The corresponding equations and regression analysis data are listed in Table 1. The β-LG A/β-LG B ratio was significantly higher in milk mixtures than in cheese matrix and governing liquid ones; as it represents the proportion between a protein from cow only and the sum of co-eluted ones from both species, the difference between milk and its products

Table 1
Linear regression analysis for β-LG A/β-LG B and β-LG A/Bx peak area ratios in raw milk, cheese matrix and governing liquid mixtures

Sample	Equation	Standard errors			r^2
		Slope	Intercept	y	
β-LG A/β-LG B					
Raw milk	$y = 0.00732x + 0.0183$	0.000158	0.00243	0.00414	0.998
Cheese matrix	$y = 0.00354x + 0.0146$	0.0000865	0.00133	0.00227	0.998
Governing liquid	$y = 0.00364x + 0.0201$	0.000271	0.00418	0.00711	0.978
β-LG A/Bx					
Raw milk	$y = 0.193x + 0.436$	0.00408	0.0630	0.107	0.998
Cheese matrix	$y = 0.274x + 0.851$	0.0283	0.436	0.742	0.959
Governing liquid	$y = 0.235x + 1.13$	0.0193	0.298	0.507	0.974

Equations are based on six data points, corresponding to different percentages of added bovine milk. Each data point was taken as the mean of three repeats.

must be due to the effects of processing, i.e. to the degradation of β-LG A during cheesemaking. Moreover, the β-LG A/Bx ratio, representing the proportion between a cow-specific protein and a buffalo-specific one, was lower in milk than in the other matrices thus confirming the effect of processing on whey proteins' degradation. The obtained results showed that using a calibration curve based on mixed milk samples for quantitative detection of illegal bovine milk addition during water buffalo Mozzarella's cheesemaking doesn't take in account the effect of manufacturing on whey proteins. In this work, the quantitation of bovine milk addition in processed matrices such as Mozzarella's cheese matrix and governing liquid was performed by the use of a calibration curve constructed on the same matrix. This proved essential to achieve proper quantification since the processing induces important differences in the specific proteins' content compared to raw milk.

4. Conclusions

The described HPLC analytical protocol for the separation and quantification of whey proteins is appropriate for the detection of low amounts of bovine milk in Italian PDO water buffalo Mozzarella. The presence of milk proteins (caseins and whey proteins) in governing liquid is due to the cheese matrix's exfoliation that occurs since the early hours of the preservation period, yielding detectable amounts of proteins already after six hours (data not shown). Results showed that bovine β-LG could be detected in raw milk, cheese matrix and governing liquid down to 0.5% of added cow milk. The use of a matrix-specific calibration curve allows taking into account the effect of manufacturing on whey proteins' degradation, thus improving the analytical reliability by assuring proper quantification of the relevant proteins. The analytical strategy we describe in this work allows detecting the fraudulent addition of bovine milk in different matrices using a calibration curve constructed on the same matrix to be analyzed. This work demonstrated the importance of using the proper calibration curve to eliminate matrix-dependent biases. The possibility to directly analyze governing liquid samples, thus bypassing whey proteins' extraction from cheese matrix, considerably sped up the procedure and avoided samples' thermal degradation during homogenization. A possible disadvantage of the application to governing liquid is that its applicability is limited to cheeses prepared and sold with a sufficient pickle volume. The abovementioned features make the procedure here described capable to accomplish routine control requirements in terms of sensitivity, speed and accuracy.

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