Eventual limits of the current EU official method for evaluating milk adulteration of water buffalo dairy products and potential proteomic overcoming solutions

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Abbreviations: B, bovine; CN, casein; ERM, European reference method; HA, hydroxyapatite; IEF, isoelectric focusing; LC, liquid chromatography; MALDI-TOF-MS, matrix-assisted laser desorption ionisation/time-of-flight; MS, mass spectrometry; nanoESI-Q-TOF, nano-electrospray ionisation-quadrupole/time-of-flight mass; UTLIEF, ultra-thin-layer polyacrylamide isoelectric focusing; WB, water buffalo.

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Abstract

The European reference method (ERM) recognizes the fraudulent addition of bovine (B) milk in water buffalo (WB) milk/dairy products based on concomitant isoelectric focusing (IEF) detection of B γ_2- and γ_3-CN fragments after corresponding plasminolysis. We here used proteomics to characterize false positive results occurring in the ERM as due to WB β-CN(f100-209), which is formed after plasminolysis also of genuine WB milk/dairy products and comigrates in IEF with B γ_2-CN. These ERM limitations were overcome by a dedicated proteomic procedure based on loading of B/WB milk/cheese CN extracts on a hydroxyapatite column, *in situ* trypsinolysis and elution of B β-CN(f1-25)4P and WB β-CN(f1-28)4P proteotypic peptides. Based on their similar ionization properties and resolution in MALDI-TOF-MS, these phosphopeptides were identified as suitable markers for detection of B material in WB milk/dairy products to a detection limit of 0.8% v/v. This proteomic procedure is here proposed as integrative/alternative to the ERM.

1. Introduction

A large number of Protected Designation of Origin (PDO) cheeses under European Union (EU) regulations are manufactured with non-bovine milks. In this context, fraudulent practices are generally due to adulterations, where ovine, caprine or water buffalo (WB) milk is partially/fully substituted with cheaper, readily available bovine (B) counterpart. In addition to economic fraud, when non-declared B milk is added to raw non-bovine material, safety concerns arise because sensitive consumers may develop allergic reactions toward dairy products and stomach irritations. Thus, enforcement agencies and analytical laboratories responsible for food control activities became aware of these fraudulent practices; they promoted the development of descriptive assays to detect and to quantify milks from different species in dairy product samples. For example, analytical methods revealing specific DNA sequences allowed establishing the presence/absence of non-declared B material in ovine, caprine and WB dairy products, even in trace amounts. Thus, cow milk levels as low as 0.5% in 3-week-old Camembert model cheeses made from mixtures of bovine and ovine milk were detected (Mayer, 2005). Moreover, real-time PCR assays incorporating a fluorogenic probe allowed the quantification of the amount of B milk in bovine/ovine milk blends (Lopez-Calleja et al., 2007). Similarly, ^{13}C- and ^{1}H-NMR approaches were used to quantify adulterations in milk mixtures based on corresponding triacylglycerol and water-soluble compound contents (Andreotti et al., 2002; Lamanna et al., 2011).

Recently, protein-centred methods have become widely used for the speciation of milk and dairy products (De la Fuente et al., 2005). In this context, chromatographic, electrophoretic and immunological approaches have allowed the accurate detection of non-declared B milk proteins in ovine, caprine and WB cheeses with relatively simple instrumentations. Chromatographic methods generally focused on whey proteins (Ferreira & Caçote, 2003; Enne et al., 2005). The remaining methods were generally directed to detect casein (CN), which was widely recognised as a more useful protein marker than heat-sensitive whey counterparts (EU, 2008; Addeo et al., 2009; Moio et al., 1998). Based on its rapidity, reduced cost and technical easiness, gel isoelectric focusing (IEF) of CN plasminolysis products was chosen as the European reference method (ERM) for the detection of B milk in non-bovine dairy products (EU, 2008). In fact, B β-CN is very sensitive towards hydrolysis by plasmin, generating the fragments reported in Fig. 1. Plasmin primary cleavage sites in B β-CN occur at Lys^{28}, Lys^{105}, and Lys^{107}, leading to the formation of: i) N-terminal proteose-peptone β-CN(f1-28) (PP 8-fast), (f1-107) and (f1-105) (PP 5); ii) inner peptides β-CN(f29-105) and (f29-107) (PP 8-slow); iii) C-terminal β-CN(f29-209) (γ_1-CN), (f106-209) (γ_2-CN) and (f108-209) (γ_3-CN). Additional cleavage sites include Lys^{113} and Arg^{183}, yielding β-CN(f114-209) and (f184-209), respectively (O'Malley et al., 2000; Somma et al., 2008). Depending on reaction conditions, hydrolysis at other amino acids was also reported (Visser et al., 1989). In WB β-CN, primary cleavage sites occur at the above-mentioned sites, plus Lys^{68}, generating identical fragments, plus the inner peptides β-CN(f29-68), (f69-105) and (f69-107), and C-terminal β-CN(f69-209) (γ_4-CN) (Fig. 1) (Bastian & Brown, 1996; Somma et al., 2008; Di Luccia et al., 2009). Being easily distinguishable by IEF, resulting B and WB γ_2-, and γ_3-CN constitute signature polypeptides allowing the detection of B milk addiction in more precious material counterpart, such in the case of WB dairy products. Since more plasminolysis products can be generated in parallel, among which some are not valuable for adulteration detection by IEF, the ERM is based on using specific enzyme/substrate w/w ratio and timing values able producing equal amounts of B γ_2- and γ_3-CN during the assay (EU, 2008). Under these specific conditions, the generation of γ_2- and γ_3-CN occurs in parallel independently, and plasminolysis is limited to produce a reduced number of products. Then, B milk content evaluation is based on a visual (eventually densitometric) analysis of the combination of the intensity of B γ_2- and γ_3-CN bands after IEF, which are compared to reference WB samples containing variable, known B adulteration levels (EU, 2008). Due to the possible conversion of γ_2-CN into γ_3-CN under non-controlled assay conditions, the ERM is based on an experimental set able evaluating simultaneously two and not one of γ_2-/γ_3-CN products (EU, 2008).

To date, the above-mentioned ERM has been reportedly able to detect very low levels (0.5%) of B milk substitution in WB milk/cheese. However, literature data indicate that the formation of the γ-CN in samples with adulteration levels above 1% may correspond to false positive results upon application of ERM (Addeo et al., 2009). In fact, it was demonstrated that plasminolysis of WB casein/cheese might release a peptide focusing similarly to B γ_2-CN. This phenomenon was independent from the IEF technology used, since it occurs either in large- (EU, 2008) and mini-IEF gels (Moio et al., 1998). Attempts to avoid this undesired artefact by controlling plasminolysis kinetics were generally unsuccessful. Complete understanding of this artefactual phenomenon was hampered by the number of polypeptides formed upon digestion of α_{s2}- and α_{s1}-CN with plasmin (Le Bars & Gripon, 1989; Le Bars & Gripon, 1993).

To investigate on the occurrence of this plasminolysis product in WB CN under the experimental conditions used during the application of ERM and to fully elucidate its nature, thus providing a rationale to the limits presented by this official method, a dedicated proteomic investigation was undertaken. To this purpose, IEF was used in combination with various mass spectrometry-based techniques. We here demonstrate that the plasmin-generated WB β-CN(f100-209) was the fragment isoelectrofocusing together with B γ_2-CN. To circumvent this methodological drawback, the application of nanoESI-Q-TOF-MS and MALDI-TOF-MS procedures was here evaluated for the confident assignment of B γ_2- and γ_3-CN, β-CN(f1-28)4P or β-CN(f1-25)4P levels in adulterated WB milk/cheese samples subjected to treatment with plasmin or trypsin.

2. Materials and Methods

2.1 Milk and cheese samples. WB and B milk samples were taken from herds located within the Campania region of Italy. Mixtures of WB milk containing 25%, 12.5%, 6.2%, 3.12%, 1.56%, and

0.78% v/v B milk were prepared on a laboratory scale by weighing the exact amount of milk (~100 g final weight) to ±0.01 g for higher volumes, and to ±0.001 g for lower volumes. Milk samples were stored at 4 °C and the day after milking treated for cheese making according to the PDO disciplinary document (PDO, 1996). Samples of Mozzarella cheese from pure B milk, WB milk, and mixtures of the latter containing 10%, 5%, 2%, 1%, 0.5%, and 0.25% v/v B milk were obtained from a cheese plant in separate vats. Analogous WB Mozzarella cheese samples made from WB milk containing 50%, 25%, 12.5%, 6.2%, 3.12%, 1.56% and 0.78% v/v B milk were also prepared. Mozzarella cheese samples were divided into different lots and stored at -20 °C, until further analysis. Reference CN samples were obtained from skimmed milk by isoelectric precipitation and centrifugation for 10 min at 4500 × *g*, according to the procedure described by Aschaffenburg & Drewry (Aschaffenburg & Drewry, 1959). Protein concentration in the samples was determined by the Biuret method according to Bradford (Bradford, 1976), using a protein assay dye reagent (Bio-Rad, Hercules, CA).

2.2 B and WB β-CN isolation. B or WB β-CN were obtained by fractionating whole CN from skimmed raw B or WB milk on a Q-Sepharose Fast Flow anion exchanger (Pharmacia, Uppsala, Sweden) (Addeo et al., 1977). Preparations were checked for purity by both gel isoelectric focusing and ESI-Q-TOF-MS analysis (data not shown).

2.3 Preparation of milk/cheese sample plasminolysates. B or WB milk (1 mL) was added to an equal volume of 0.2 M NH_4HCO_3 (pH 8.0); the resulting solution was shaken for few sec and then allowed to stand for 5 min. A 500 µL aliquot was transferred to a plastic culture tube (15 mm × 10 mm), added with 10 µL of plasmin suspension (5 U mL^{-1}) (EC. 3.4.21.7, Boehringer, Mannheim, Germany), and incubated for 1 h, at 37 °C. Then, an equal volume of 24% TCA w/v was added, and the protein pellet was recovered by centrifugation at 4500 × *g*, for 10 min, at room temperature. The pellet was solved by the addition of 9 M urea (250 μL). Similarly, Mozzarella cheese (200 mg) was added with 0.2 M NH_4HCO_3 at pH 8.0 (1.5 mL) and shaken on a vortex until CN was solved (~3 min). Next, a 500 μL aliquot was processed using the same method reported above for milk. Finally, plasminolysis of WB β-CN (100 mg) was performed on the protein solved in 50 mM NH_4HCO_3 pH 8.5 (2 mL), which was added with 10 μL of the above-mentioned plasmin suspension. After 1 h of incubation, the peptides were precipitated with TCA (12% final concentration), the supernatant was discarded, and the pellet was washed three times with 1 mL of dry acetone, which had been kept at -20 °C. After each wash, the supernatant was discarded and the pellets were washed twice with 2 mL of diethyl ether. Finally, the peptides were air-dried, and kept at -20 °C until further analysis.

2.4 Isoelectric focusing of CN plasminolysates and their extraction from the gel. Isoelectric focusing on ultra-thin-layer polyacrylamide isoelectric focusing gel (UTLIEF) was carried out according to the ERM (EU, 2008); the PhastSystem isoelectric focusing (FIEF) apparatus was also used to the same purpose (Moio et al., 1998). In both cases, gel composition and pH gradient were the same. After migration, gels were stained using Coomassie Brilliant Blue G-250 (CBB), according to the procedure of Neuhoff and coworkers (Neuhoff et al., 1988). Then, gel bands were cut with a razor blade, disengaged from the polyester backing and destained by the addition of 50% acetonitrile, with 1 h of shaking. The coloured supernatant was discarded, and the process was repeated until the supernatant was almost colourless. After 2 washes with distilled water (100 μL) for 15 min, each gel piece was suspended in 0.25 mM NH_4HCO_3 pH 8.0, added with an equal volume of 0.1% trifluoroacetic acid (TFA) in acetonitrile, and sonicated for 1 h. The suspension was centrifuged at 12,000 × *g* for 1 min, at 4 °C, and the supernatant transferred to a clean vial; after centrifuge evaporation to half of the initial volume, the sample was directly submitted to LC-nanoESI-Q-TOF-MS or MALDI-TOF-MS analysis.

2.5 nanoESI-Q-TOF mass spectrometry. Electrospray mass spectra were acquired using a Q-TOF Ultima quadrupole-TOF hybrid mass spectrometer (Waters, Milford, MA) equipped with a nano Z-spray source operating in positive ion mode. The ionisation conditions used included: i) a capillary voltage of 2.6 kV; ii) a cone voltage and RF1 lens of 100 V and 40 V, respectively; iii) a collision energy of 12 eV. The source temperature was set to 80 °C, and the cone gas was N_2 (at a flow of 40 L h-1); no nebulising gas was used to obtain the spray. External calibrations with equine apomyoglobin (A8673 Sigma Aldrich, Germany) and human (Glu1)-fibrinopeptide B (GFP) (F3261 Sigma Aldrich) were performed over mass ranges of 600 to 2000 and 400 to 1600 *m/z*, respectively. All spectra were acquired with the TOF analyser in “V-mode” (9.1 kV TOF), and the MCP voltage set at 2000 V.

2.6 Reversed-phase capillary chromatography. Reversed-phase capillary chromatography was performed using a Waters CapLC ternary pump system connected through a stream-select valve module to the nano Z-spray source of the mass spectrometer. The sample was injected into the system through the CapLC autosampler using the “microliter pickup” injection method. Solvents A and C consisted of 0.1% TFA, and solvent B consisted of 0.1% TFA in acetonitrile. The sample was pre-concentrated and desalted on a pre-column cartridge packed with Atlantis dC_{18} resin (Waters) and connected to pump C through the stream-select valve module. The pre-concentration/desalting step was conducted at 20 µL/min over 3 min, using pump C. After switching to pumps A and B, a gradient was applied to the pre-column cartridge and then used to elute the peptides directly into the mass spectrometer’s source. The column was equilibrated with 5% of solvent B, and a linear gradient from 5% to 50% of this solvent was applied over 13 min, at a flow rate of 0.5 µL/min, using a pre-column split with a pump delivering at 2 µL/min. TOF-MS analysis was conducted *on-line* with chromatographic separation of peptides, using the instrument reported above. Mass spectra were acquired over a mass range of 400-1600 *m/z*, at a scan speed of 2.4 s/scan. Mass values were calculated using the MaxEnt 3 deconvolution algorithm (Waters).

2.7 MALDI-TOF mass spectrometry. MALDI-TOF mass spectra were obtained on a Voyager-DE PRO instrument (Applied Biosystems, Framingham, MA) equipped with a 337-nm pulsed N_2 laser. The matrix solution was prepared by dissolving 10 mg of sinapinic acid in 1 mL of 0.1% TFA, 50% ACN. Samples were desalted on a ZipTip® pipette tips according to manufacturer's protocol. The eluate was mixed with an equal volume of matrix solutions; 1 μL of the resulting solution was applied to a stainless steel sample plate and allowed to dry. Sample ionisation was carried out in positive linear mode, and the generated ions were accelerated to 25.0 keV of kinetic energy. The following extraction delay conditions were used: grid voltage = 95%; guide wire = 0.2%; extraction delay time = 300 ns. Mass spectra were obtained by averaging 100 laser shots.

2.8 Polypeptide hydrolysis with endoproteinase Asp-N. Polypeptide hydrolysis with endoproteinase Asp-N (sequencing grade, Boehringer, Mannheim) was carried out overnight at an enzyme/substrate ratio of 1/100 w/w in 50 mM ammonium bicarbonate, pH 8.5, at 37 °C. Then the reaction mixture was cooled in an ice bath followed by freeze-drying.

2.9 Peptide assignment based on mass spectrometry data. Peak signals recorded after mass spectrometric analysis of the CN peptide mixtures generated following plasmin, trypsin and endoprotease Asp-N digestion were assigned to specific components based on: i) B β-CN (P02666), α_{s2}-CN (P02663), α_{s1}-CN (P02662) and κ-CN (P02668) sequence and corresponding variants; ii) WB β-CN (Q9TSI0), α_{s2}-CN (B6VPY2), α_{s1}-CN (O62823) and κ-CN (P11840) sequence and corresponding variants; iii) theoretical mass values obtained by digesting each protein with FindPept tools (*www.expasy.ch*); iv) literature data.

2.10 Preparation of B β-CN (f1-25) and WB β-CN (f1-28) phosphopeptides after trypsinolysis of WB and B CN mixtures and corresponding quantitative analysis. Mixtures of WB and B CN from milk and/or mozzarella cheese samples were prepared as described in section 2.1. They were separately adsorbed onto microgranular hydroxyapatite (HA) (100 mg) columns, which were previously equilibrated with the loading buffer (50 mM Tris–HCl, 0.2 M KCl, 4.5 M urea, and 10 mM DTT, pH 8.0). After incubation for 15 min at room temperature, each HA-protein complex was recovered by centrifuging for 3 min at 4,000 × *g* for 2 min. Each resin sample was successively washed with three different buffers: loading buffer (1 mL), 50 mM Tris–HCl at pH 8.0 (1 mL), and 20 mM Tris–HCl in 20% v/v ACN at pH 8.0 (1 mL). Then, it was washed with Milli-Q water (1 mL), and freeze-dried in a vacuum centrifuge (Thermo Electron, Milford, MA). HA-bound CN phosphoproteins were submitted to *in situ* trypsinolysis as previously described (Pinto et al., 2010). Corresponding HA-bound CN phosphopeptides were eluted and deposited onto the MALDI plate, covered with the matrix solution (0.5 µL) containing 10 mg/mL 2,5-dihydroxybenzoic acid (Sigma-Aldrich) in H_2O/ACN/phosphoric acid 49:50:1 analyte/matrix pairs that promote co-crystallisation by air-drying at room temperature.

3. Results

3.1 IEF behaviour of WB and B CN plasminolysates

Reference samples of B or WB milks, and their mixtures were submitted to plasmin action. Based on the ERM, it was expected that corresponding β-CN formed two distinct bands for γ_2- and γ_3-CN. FIEF analysis confirmed this result and showed a number of additional peptide bands in B and WB CN plasminolysates (Supplementary Fig. S1). As already described (Addeo et al., 2009), WB CN plasminolysis products showed two evident bands in the region of B γ_2-CN, among which one having an almost identical isoelectric focusing behaviour to the bovine fragment. The occurrence of these components was observed either in WB milk or in derived WB Mozzarella cheese (Supplementary Fig. S1A and S1B, lane 8). Their presence was also observed in WB milk samples adulterated with B milk, and in cheese samples deriving from WB milk adulteration with the B counterpart (Supplementary Fig. S1A and S1B, lanes 2-7); in these cases, no distinction of the above-mentioned WB CN-derived component was possible with respect to B γ_2-CN. In order to further investigate on the nature of this WB CN plasminolysis product, thus providing a rationale to the limits due to false-positive results deriving from the application of the ERM, a dedicated proteomic investigation was undertaken.

3.2 Characterization of WB and B CN plasminolysate IEF bands by MALDI-TOF-MS analysis

To characterize the nature of the WB and B CN bands comigrating in IEF, the following samples were submitted to plasminolysis as described in the experimental section: i) whole B CN; ii) whole WB CN, iii) WB Mozzarella cheese; iv) WB β-CN. Plasminolytic products were analysed by UTLIEF on large gels (EU, 2008), which allowed a better resolution of WB CN bands migrating similarly to B γ_2-CN (Fig. 2). Then, the numbered CBB-stained gel bands from all B and WB samples were excised from the gel and directly subjected to MALDI-TOF-MS analysis. Identical results were obtained for all WB samples (data not shown). Signal mass values and shapes, which were suggestive for the occurrence of multiple poorly resolved, oxidized species in each band (data not shown), allowed tentatively to assign major β-CN products in each gel lane (Tab. 1). This was due to the resolution characteristics of the MALDI-TOF-MS instrument used. Thus, main bands in B and WB samples were associated with the expected B γ_2- (band 3) and γ_3-CN (band 7), and WB γ_4- (band 2), γ_2- (band 6) and γ_3-CN (band 8) (Tab. 1, Fig. 3 and Supplementary Fig. S3-S6). In some cases, minor compounds were also observed. The ERM assumes that plasminolysis in 1 h fully converts both WB and B β-CN into the corresponding γ_2-CN and γ_3-CN. We verified that this assumption was partially correct, since additional peptide fragments were also detected. They were tentatively assigned to oxidized B β-CN(f108-207) (band 1) and WB β-CN(f100-209) (band 4, here named γ_x-CN) and (f106-176) (band 5) (Fig. 3, Tab. 1 and Supplementary Fig. S2). The WB component corresponding to band 4 was the one determining the false-positive results observed during the application of the ERM, based on its co-migration properties with B γ_2-CN in IEF. In conclusion, above-mentioned results suggested the nature of WB CN plasminolytic products migrating in electrophoretic bands reported in the ERM (EU, 2008).

3.3 Definitive assignment of the WB β-CN fragments migrating in the region of B γ_2-CN

Products of protein oxidation at Met and Trp residues may occur *in vivo* or can be generated *in vitro* as result of sample digestion and/or electrophoresis (Bachi et al., 2013). The occurrence of multiple oxidized species in some WB β-CN fragments visualized as IEF bands (Fig. 2) was confirmed by direct nanoESI-Q-TOF-MS analysis of corresponding gel extracts. For example, bands 2, 4 and 6 showed the presence of multiple components differing for about 16 Da (Supplementary Fig. S7). Their mass values were compatible with the tentative assignments reported in Tab. 1; similarly, their number in each band was coherent with the number of *in silico* oxidable Met and Trp residues present in each peptide sequence. Indeed, recorded mass signals were all compatible with the occurrence of a common Trp residue, plus 6, 5, and 4 methionines in WB γ_4-CN (band 2), γ_x-CN (band 4) and γ_2-CN (band 6), respectively. Most of these oxidized fragments were not formed when WB CN plasminolysates were subjected to direct nanoESI-Q-TOF-MS or MALDI-TOF-MS analysis without preliminary IEF separation (Supplementary Fig. S8 and S9), thus suggesting that their occurrence was the result of the preventive electrophoretic step. On the other hand, the latter analyses easily allowed revealing B γ_1-, γ_2- and γ_3-CN, and WB γ_2-, γ_3- and γ_4-CN in a single mass measurement.

A definitive assignment of each WB CN peptide fragment present in band 4, 5 and 6 was obtained after their digestion with endoprotease Asp-N and subsequent MALDI-TOF-MS analysis.

Tab. 2 reports the results obtained for each digest. These experiments definitively assigned band 4, 5 and 6 to WB β-CN(f100-209) (γ_x-CN), (f106-176) and γ_2-CN, respectively, also demonstrating the nature of minor components present in each sample as result of an undesired smearing of abundant polypeptides during IEF, likely due to an incomplete gel polymerization and/or sample overload, or to possible imprecision in cutting individual gel bands.

Above mentioned experiments definitively identified WB β-CN(f100-209) (γ_x-CN) as the component co-migrating with B γ_2-CN in IEF, thus responsible for false-positive results sporadically observed during the application of the ERM. They also demonstrated that this plasminolysis product (plus other minor ones) is poorly detectable in the spectra resulting from direct nanoESI-Q-TOF-MS or MALDI-TOF-MS analysis of B and WB plasminolysates (Supplementary Fig. S8 and S9). In the whole, these results emphasized the low sensitivity of direct MS measurements of CN plasminolysates from B and WB milk/dairy products in the detection of minor components, as result of ion suppression phenomena or their relative concentration. More importantly, they also highlighted that the ERM, although more sensitive than a direct MS-based assay in revealing minor components, is not suitable for evaluating illegal milk addictions, based on the electrophoretic co-migration phenomena here characterized in detail. A modification of the ERM toward the unique analysis of B γ_3-CN should also be considered (although out of the scope of this work). However, this novel IEF assay may eventually suffer of the limitations reported above on the control of the plasminolysis reaction toward production of a limited number of polypeptide products, among which one (γ_2-CN) may eventually be transformed into the analyte of interest (γ_3-CN) over the time. On this basis, we investigated on the development of novel and rapid analytical procedures based on another proteolytic enzyme and MS measurements for revealing WB milk/cheese adulterations due to the addition of B milk.

3.4 Combining affinity-trapping procedures with MALDI-TOF-MS analysis of CN trypsinolysates reveals even limited adulteration levels of B milk in WB dairy products

Relative and absolute quantification of adulterations in foods by MS procedures generally require the use of an internal standard (IS), which is present/added to the sample to compensate eventual fluctuations in the analyte ion signal due to subtle changes in the experimental performances of the instrument and/or in the analyte content due to sample manipulation (Brönstrup, 2004). When the MS measurement focus on relative quantification of proteins, it generally involves the use of signature protein/peptide ISs, which are already present in the sample and/or generated following its treatment, *e.g.* enzymatic digestion. In the case of absolute quantification, stable isotope-labelled protein/peptide analogue ISs are generally added to the sample before processing. In both cases, ISs must present high ionization efficiencies to facilitate the detection of the analyte, even when it occurs at low concentrations. Unavailability of both standard and labelled signature products is a serious drawback in the development of a robust quantitative procedure. As a first attempt to develop a MS-based procedure alternative to the ERM, we verified the possible use of B and WB γ_2- and γ_3-CN from CN plasminolysates as signature polypeptides for the relative quantification of B milk in WB milk/cheese products by LC-nanoESI-Q-TOF-MS measurements. However, we verified that no concomitant linearity, statistical significance and level of detection was observed for both B γ_2- and γ_3-CN signals, when WB Mozzarella samples were prepared by using various amounts of B milk (see Supplementary Material for details); accordingly, no B milk adulterations lower than 10% were detectable when using both signature polypeptides. A modification of this procedure to the unique analysis of the WB and B γ_3-CN signal couple was also considered, but it was not further investigated as result of the limitations reported above on the control of the plasminolysis reaction.

In the last decade, a number of affinity-trapping procedures have been developed to selectively enrich molecules bearing a specific chemical signature, thus simplifying the subsequent analysis of complex peptide mixture by MS techniques (Xiao & Wang, 2016; Lai et al., 2013). In this context, various functionalized polymers have been used to enrich phosphopeptides from proteome digests (D'Ambrosio et al., 2007; Thingholm et al., 2009). We previously reported on the good performances of ceramic HA as a solid-phase adsorbent to efficiently capture and release CN phosphoproteins/phosphopeptides (Pinto et al., 2010). Accordingly, we decided to use this material in combination with direct MALDI-TOF-MS measurements for the relative evaluation of WB β-CN(f1-28)4P and B β-CN(f1-28)4P in CN plasminolysates from WB dairy products adulterated by B milk addition. However, when this approach was applied to WB milk samples containing reduced amounts of B milk, detection of the above-mentioned B phosphopeptide was strongly hampered by the intensity of the signal of the WB counterpart presenting a very close mass value. Additional problems observed are reported in Supplementary Material.

To overcome above-mentioned difficulties and develop a more robust assay, we used HA for the selective trapping of CN from a 1:1 v/v WB/B milk mixture, protein *in situ* trypsinolysis and selective elution of resulting casein phosphopeptides, which were then directly subjected to MALDI-TOF-MS analysis. In this case, well resolved ($\Delta M = +336$ u), intense signals associated with the proteotypic phosphopeptides WB β-CN(f1-28)4P and B β-CN(f1-25)4P were observed in the spectrum (Fig. 4); their intensity was comparable as result of a similar ionization efficiency. No signals related to other phosphopeptides occurred at the corresponding mass values. When this approach was applied to the analysis of five Mozzarella cheese samples made with WB milk mixed to various amounts of B milk (0-50%, v/v), detection of very low amounts of the B phosphopeptide was possible (Supplementary Fig. S12). In order to provide information on the signal response in the presence of different amounts of B milk, WB milk samples containing a variable degree of B counterpart adulteration were analysed in triplicate and corresponding results were subjected to linear regression analysis. A linear relationship was established between the area of the WB β-CN(f1-28)4P peptide signal and the adulterant B β-CN(f1-25)4P peptide ratio against percentage of

B milk (y = 0.018x – 0.0182, R2 = 0.9998). Using this calibration curve, the actual percentage of B milk present in adulterated WB milk samples was calculated with a detection limit of 0.8% (Fig. 5). Information on the robustness of the whole proteomic procedure is provided in Supplementary Tab. S1, where data from triplicate independent (regarding CN isolation, HA loading, *in situ* proteolysis and phosphopeptide elution) experiments on the same samples were performed. On the basis of its sensitivity and robustness, and the possibility to avoid false-positive results, direct MALDI-TOF-MS analysis of enriched proteotypic phosphopeptides WB β-CN(f1-28)4P and B β-CN(f1-25)4P from CN tryptic digests is proposed here as a promising complementary or alternative method to the actual ERM (EU, 2008).

When this procedure was used to evaluate in blind experiments the adulteration of WB Mozzarella cheese samples eventually made also with the addition B milk (Supplementary Fig. S13), which were stored for 7 days at 4 °C, a very good agreement of the ascertained percentage of corruption (5.9%) was verified with respect to the real condition (6.0%). These experiments demonstrated the good applicability of the above-mentioned proteomic method to a routine evaluation of commercial dairy product samples.

4. Discussion

Based on their high-resolution power able to discriminate molecules according to their molecular mass value, MS instrumentations have found a widespread application in the quali-quantitative analysis of food products, especially when combined with separation procedures. Accordingly, various MS techniques and related protein-centered approaches have been used for the analysis of milk/dairy products and, in particular, for speciation, with the aim to detect illegal adulteration of O, C and WB milk/cheeses with cheaper/readily available B counterpart (Cozzolino et al., 2002; Cuollo et al., 2010; Guarino et al., 2010; Ansari et al., 2011; Nicolaou et al., 2011; Cunsolo et al., 2013; Russo et al., 2012; Di Girolamo et al., 2014; Bernardi et al., 2015; Sassi et al., 2015; Russo et al., 2016; Camerini et al., 2016). These procedures were proposed in the recent years, also due to the observation that the actual ERM presents false-positive results during analysis of WB dairy products (EU, 2008; Addeo et al., 2009; Moio et al., 1998). In this study, we provided a rationale to this phenomenon, demonstrating that WB β-CN(f100-209) is responsible for its occurrence. This observation confirmed the expectation that β-CN plasminolysis does not proceed solely by forming the proteotypic peptides γ_2- and γ_3-CN, without passing through various peptide intermediates, but generates additional products that are due to hydrolysis at non-primary protein sites. This is known since three decades, since 🚲-CN was found to be cleaved by plasmin at 15 proteolytic sites, among which 5 are internal to the γ_2-/γ_3-CN sequence (Visser et al., 1989). Based on these findings, a modification of the ERM toward the unique evaluation of the transient γ_3-CN may eventually suffer of the limitations reported above on the strict control of the plasminolysis reaction, which we verified generates a number of polypeptide products.

In this study, a novel method was also developed to overcome the above-mentioned ERM limits. This method is based on a procedure able to capture WB and B CN from milk and solid/liquid dairy product samples on solid-phase HA-based adsorbents, to hydrolyze phosphoproteins with trypsin, to ispecies WB β-CN(f1-28)4P and B β-CN(f1-25)4P by MALDI-TOF-MS analysis. No eventual addition to samples of unlabelled or stable isotope-labelled peptide analogue is necessary to generate final quantitative information on the eventual adulteration of WB dairy products with B milk. When compared with other methods based on the use of MS instrumentations for the detection of milk adulteration (see below), this procedure is relatively simple and is based on the use of low-resolution, user-friendly MALDI-TOF-MS equipment having a relatively low cost, in agreement with the general standard request of the EU legislation. Accordingly, this method can integrate the above-mentioned MS-based procedures in quantifying illegal adulterations of ovine, caprine and WB milk/dairy products with B milk. In fact, the latter methodologies present the limitations of being exclusively dedicated to the analysis of raw milk (Cuollo et al. 2010; Nicolaou et al., 2011; Cunsolo et al., 2013; Di Girolamo et al., 2014; Sassi et al., 2015) or whey-based dairy products (Russo et al., 2016) through the use of user-friendly, low-resolution MALDI-TOF-MS instrumentations, or to the analysis of both milk and cheese products by: i) user-friendly, low-resolution MALDI-TOF mass spectrometers following the addition of unlabelled peptide analogue ISs (Cuollo et al., 2010), or ii) LC-ESI-MS/MS-based single (SRM) (Guarino et al., 2010) or multiple (MRM) (Ansari et al., 2011; Russo et al., 2012; Bernardi et al., 2015; Camerini et al., 2016) reaction monitoring procedures, which request dedicated, sophisticated instrumentations, having a relatively high cost, and requiring well-trained personnel. Worth mentioning is also the fact that more than a third of the above-mentioned procedures are not directed to evaluate heat-resistant indicators, such as CN molecules, but focus on whey proteins that are non-optimal milk markers, based on their high propensity for denaturation during heat and high-pressure processing.

5. Conclusions

In conclusion, the MALDI-TOF-MS-based methodology proposed here can be integrated in the actual ERM in evaluating adulterations of WB products due to the limited addition of B milk, by providing confirmatory results, or can be proposed as a second-generation ERM for authenticating pure WB milk/cheese. Work is now in progress to broaden the application of this or related methodologies to milk and dairy products from other species. Considerations reported above on the limits of the actual ERM, as well as linearity, statistical significance and detection limit performances of the MS-based procedures recently developed to quantify illegal adulterations in milk/dairy products strongly suggest the introduction of the latter in a novel ERM. In this context, dedicated facilities with state-of-art instruments and well-trained personnel from control institutions should be considered in routinely performing these analyses.

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References

Addeo, F., Chobert, J.-M., & Ribadeau-Dumas, B. (1977). Fractionation of whole casein on hydroxyapatite. Application to a study of buffalo κ-casein. *Journal of Dairy Research*, *44*(1), 63.

Addeo, F., Pizzano, R., Nicolai, M. A., Caira, S., & Chianese, L. (2009). Fast isoelectric focusing and antipeptide antibodies for detecting bovine casein in adulterated water buffalo milk and derived mozzarella cheese. *Journal of Agricultural and Food Chemistry*, *57*(21), 10063–10066.

Andreotti, G., Lamanna, R., Trivellone, E., & Motta, A. (2002). 13C NMR spectra of TAG: An easy way to distinguish milks from different animal species. *Journal of the American Oil Chemists' Society*, *79*(2), 123–127.

Ansari, P., Stoppacher, N., Rudolf, J., Schuhmacher, R., & Baumgartner, S. (2011). Selection of possible marker peptides for the detection of major ruminant milk proteins in food by liquid chromatography-tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, *399*(3), 1105–1115.

Aschaffenburg, R., Drewry, J. (1959). New procedures for the routine determination of the various non-casein proteins of milk. *Proceedings of the 15th International Dairy Congress*, London, 3 1631–1637.

Bachi A., Dalle-Donne I., Scaloni A. (2012). Redox proteomics: chemical principles, methodological approaches and biological/biomedical promises. *Chemical Reviews*, *113*(1), 596–698.

Bastian, E. D., & Brown, R. J. (1996). Plasmin in milk and dairy products: An update. *International Dairy Journal*, *6*(5), 435-457.

Bernardi, N., Benetti, G., Haouet, N. M., Sergi, M., Grotta, L., Marchetti, S., … Martino, G. (2015). A rapid high-performance liquid chromatography-tandem mass spectrometry assay for unambiguous detection of different milk species employed in cheese manufacturing. *Journal of Dairy Science*, *98*(12), 8405–8413.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, *72*, 248–254.

Brönstrup, M. (2004). Absolute quantification strategies in proteomics based on mass spectrometry. *Expert Review of Proteomics*, *1*(4), 503–12.

Camerini, S., Montepeloso, E., Casella, M., Crescenzi, M., Marianella, R. M., & Fuselli, F. (2016). Mass spectrometry detection of fraudulent use of cow whey in water buffalo, sheep, or goat Italian ricotta cheese. *Food Chemistry*, *197*(Pt B), 1240–1248.

Cozzolino, R., Passalacqua, S., Salemi, S., & Garozzo, D. (2002). Identification of adulteration in water buffalo mozzarella and in ewe cheese by using whey proteins as biomarkers and matrix-assisted laser desorption/ionization mass spectrometry. *Journal of Mass Spectrometry*, *37*(9), 985–991.

Cunsolo, V., Muccilli, V., Saletti, R., & Foti, S. (2013). MALDI-TOF mass spectrometry for the monitoring of she-donkey's milk contamination or adulteration. *Journal of Mass Spectrometry*, *48*(2), 148–153.

Cuollo, M., Caira, S., Fierro, O., Pinto, G., Picariello, G., & Addeo, F. (2010). Toward milk speciation through the monitoring of casein proteotypic peptides. *Rapid Communications in Mass Spectrometry*, *24*(11), 1687–1696.

D'Ambrosio, C., Salzano, A. M., Arena, S., Renzone, G., & Scaloni, A. (2007). Analytical methodologies for the detection and structural characterization of phosphorylated proteins. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *849*(1-2),163-180.

De la Fuente, M. A., & Juárez, M. (2005). Authenticity assessment of dairy products. *Critical Reviews in Food Science and Nutrition*, *45*(7–8), 563–85.

Di Girolamo, F., Masotti, A., Salvatori, G., Scapaticci, M., Muraca, M., & Putignani, L. (2014). A sensitive and effective proteomic approach to identify she-donkey's and goat's milk adulterations by MALDI-TOF MS fingerprinting. *International Journal of Molecular Sciences*, *15*(8), 13697–13719.

Di Luccia, A., Picariello, G., Trani, A., Alviti, G., Loizzo, P., Faccia, M., & Addeo, F. (2009). Occurrence of β-casein fragments in cold-stored and curdled river buffalo (Bubalus bubalis L.) milk. *Journal of Dairy Science*, *92*(4), 1319–1329.

Enne, G., Elez, D., Fondrini, F., Bonizzi, I., Feligini, M., & Aleandri, R. (2005). 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. *Journal of Chromatography A*, *1094*(1–2), 169–174.

EU, 2008, Commission Regulation (EC) No 273/2008, 2008 Official Journal of the European Union, L 88, 53–115.

Ferreira, I. M. P. L. V, & Caçote, H. (2003). Detection and quantification of bovine, ovine and caprine milk percentages in protected denomination of origin cheeses by reversed-phase high-performance liquid chromatography of beta-lactoglobulins. *Journal of Chromatography A*, *1015*(1–2), 111–118.

Guarino, C., Fuselli, F., la Mantia, A., Longo, L., Faberi, A., & Marianella, R. M. (2010). Peptidomic approach, based on liquid chromatography/electrospray ionization tandem mass spectrometry, for detecting sheep's milk in goat's and cow's cheeses. *Rapid Communications in Mass Spectrometry*, *24*(6), 705–713.

Lai, Z. W., Nice, E. C., & Schilling, O. (2013). Glycocapture-based proteomics for secretome analysis. *Proteomics*, *13*(3-4), 512-525.

Lamanna, R., Braca, A., Di Paolo, E., & Imparato, G. (2011). Identification of milk mixtures by 1H NMR profiling. *Magnetic Resonance in Chemistry*, *49*(SUPPL. 1).

Le Bars, D., Gripon, J.C. (1989). Specificity of plasmin towards bovine alpha S2-casein, *Journal of Dairy Research*, *56 (5)*, 817–821.

Le Bars, D., Gripon, J.C. (1993). Hydrolysis of α_{s1}-casein by bovine plasmin. *Lait, 73*, 337–344.

López-Calleja, I., González, I., Fajardo, V., Martín, I., Hernández, P. E., García, T., & Martín, R. (2007). Real-time TaqMan PCR for quantitative detection of cows' milk in ewes' milk mixtures. *International Dairy Journal*, *17*(7), 729–736.

Mayer, H. K. (2005). Milk species identification in cheese varieties using electrophoretic, chromatographic and PCR techniques. In *International Dairy Journal* (Vol. 15, pp. 595–604).

McSweeney, P. L. H., Olson, N. F., Fox, P. F., Healy, A., & Hojrup, P. (1993). Proteolytic specificity of plasmin on bovine α s1 Casein. *Food Biotechnology*, *7*(2), 143–158.

Moio, L., Chianese, L., Addeo, F. (1998). Use of PhastGel dry IEF for rapid detection of bovine milk in ovine, caprine and water buffalo milk or cheese. Application Note 374, Amersham Biosciences: Wilkstroms, Sweden.

Neuhoff, V., Arold, N., Taube, D., & Ehrhardt, W. (1988). Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis*, *9*(6), 255–262.

Nicolaou, N., Xu, Y., & Goodacre, R. (2012). Detection and quantification of bacterial spoilage in milk and pork meat using MALDI-TOF-MS and multivariate analysis. *Analytical Chemistry*, *84*(14), 5951–5958.

O'Malley, A. M., Mulvihill, D. M., & Kumar Singh, T. (2000). Proteolysis in rennet casein-based cheese analogues. *International Dairy Journal*, *10*(11), 743–753.

PDO (1996). Registration of Mozzarella di Bufala Campana DOP as Protected Designation of Origin (PDO) product. Commision Regulation EC n. 1107/1996.

Pinto, G., Caira, S., Cuollo, M., Lilla, S., Fierro, O., & Addeo, F. (2010). Hydroxyapatite as a concentrating probe for phosphoproteomic analyses. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *878*(28), 2669–2678.

Russo, R., Severino, V., Mendez, A., Lliberia, J., Parente, A., & Chambery, A. (2012). Detection of buffalo mozzarella adulteration by an ultra-high performance liquid chromatography tandem mass spectrometry methodology. *Journal of Mass Spectrometry*, *47*(11), 1407–1414.

Sassi, M., Arena, S., & Scaloni, A. (2015). MALDI-TOF-MS platform for integrated proteomic and peptidomic profiling of milk samples allows rapid detection of food adulterations. *Journal of Agricultural and Food Chemistry*, *63*(27), 6157–6171.

Russo, R., Rega, C., & Chambery, A. (2016). Rapid detection of water buffalo ricotta adulteration or contamination by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry*, *30*(4), 497–503.

Somma, A., Ferranti, P., Addeo, F., Mauriello, R., & Chianese, L. (2008). Peptidomic approach based on combined capillary isoelectric focusing and mass spectrometry for the characterization of the plasmin primary products from bovine and water buffalo β-casein. *Journal of Chromatography A*, *1192*(2), 294–300.

Thingholm, T. E., Jensen, O. N., & Larsen, M. R. (2009). Analytical strategies for phosphoproteomics. *Proteomics*, *9*(6), 1451-1468.

Visser, S., Noorman, H. J., Slangen, C. J., Rollema, H. S. (1989). Action of plasmin on bovine β-casein in a membrane reactor. *Journal of Dairy Research*, *56*, 323-333.

Xiao, Y., & Wang, Y. (2016). Global discovery of protein kinases and other nucleotide-binding proteins by mass spectrometry. *Mass Spectrometry Reviews*, *35*(5), 601-6019.

Table 1. MALDI-TOF-MS analysis of β-CN polypeptide components present in the UTLIEF bands reported in Fig. 2. Identification was hypothesized based on theoretical and measured molecular mass values (Fig. 3 and Supplementary Fig. S2-S6), taking also into account the number of oxidable Met and Trp residues. In each band, signals are reported according to their relative intensity (Fig. 3). [†] B. [‡] WB.

UTLIEF band	**Molecular mass (Da)**								**Identified Peptide**
	Theor.	***Theor.* Ox**	***Theor.* 2 Ox**	***Theor.* 3 Ox**	***Theor.* 4 Ox**	***Theor.* 5 Ox**	***Theor.* 6 Ox**	**Measured**	
1 (I)	11346.3	11362.3	11378.3	11394.3	11410.3			11403.4	β-CN (f108-207)[†]
2 (II/III/IV)	15748.7	15764.7	15780.7	15796.7	15812.7	15828.7	15844.7	15810.2	β-CN (f69-209)[‡]
3 (I)	11823.9	11839.9	11855.9	11871.9	11887.9			11856.5	β-CN (f106-209)[†]
4 (II/III/IV)	12411.7	12427.7	12443.7	12459.7	12475.7	12491.7		12455.4	β-CN (f100-209)[‡]
	15748.7	15764.7	15780.7	15796.7	15812.7	15828.7	15844.7	15812.3	β-CN (f69-209)[‡]
5 (II/III/IV)	8079.5	8095.5	8111.5	8127.5				8125.4	β-CN (f106-176)[‡]
	11783.9	11799.9	11815.9	11831.9	11847.9			11829.9	β-CN (f106-209)[‡]
	15748.7	15764.7	15780.7	15796.7	15812.7	15828.7	15844.7	15809.1	β-CN (f69-209)[‡]
6 (II/III/IV)	11783.9	11799.9	11815.9	11831.9	11847.9			11826.9	β-CN (f106-209)[‡]
	8079.5	8095.5	8111.5	8127.5				8126.9	β-CN (f106-176)[‡]
7 (I)	11558.6	11574.6	11590.6	11606.6	11622.6			11590.7	β-CN (f108-209)[†]
8 (II/III/IV)	11518.6	11534.6	11550.6	11566.6	11582.6			11567.5	β-CN (f108-209)[‡]

Table 2. MALDI-TOF-MS identification of WB β-CN polypeptide components present in the UTLIEF bands 4, 5 and 6 (Fig. 2). Identification hypothesized in Tab. 1 and reported on the left side of this table was confirmed based on MALDI-TOF-MS data on fraction endoproteinase Asp-N digests. Reported are the measured mass values. Asterisks indicate the number of oxidized residues, *i.e.* determining the mass increase of +16.0 (*) or +32.0 Da (**), respectively.

UTLIEF				**Endoprotease Asp-N**					
			Peptide	**(f69-128)**	**(f100-128)**	**(f106-128)**	**(f129-176)**	**(f129-183)**	**(f184-209)**
Band	**Peptide**	**Mass (Da)**	**Theoretical**	6671.9	3334.9	2707.1	5390.4	6202.3	2910.5
4	β-CN (f100-209)		**Measured**		3350.9*			6219.1*	2926.8*
	β-CN (f69-209)			6704.1**				6219.1*	2926.8*
5	β-CN (f106-176)					2723.3*	5422.5**		
	β-CN (f106-209)					2723.3*		6219.6*	2926.7*
	β-CN (f69-209)			6704.2**				6219.6*	2926.7*
6	β-CN (f106-209)					2723.1*		6219.8*	2926.5*
	β-CN (f106-176)					2723.1*	5422.3**		

Figure Legends

Figure 1. Amino acid sequence of B and WB β-CN. Arrows indicate peptide bonds sensitive to plasmin whose cleavage generate γ_1-, γ_2-, γ_3- and γ_4-CN. Labeling β-CN(f114-209) and β-CN(f183-209) is out of the current casein nomenclature. Arg^{25} in B 🚲-CN is boxed.

Figure 2. UTLIEF profile of plasminolysate samples from B CN (lane I), WB Mozzarella cheese (lane II), purified WB β-CN (lane III), and WB CN (lane IV). Staining was carried out with CBB R-250. Gel bands formed by plasminolysis of B and WB β-CN were numbered and extracted for further MALDI-TOF-MS-based characterization of present polypeptides, as described in the experimental section. Corresponding results provided useful information for the evaluation of the actual ERM.

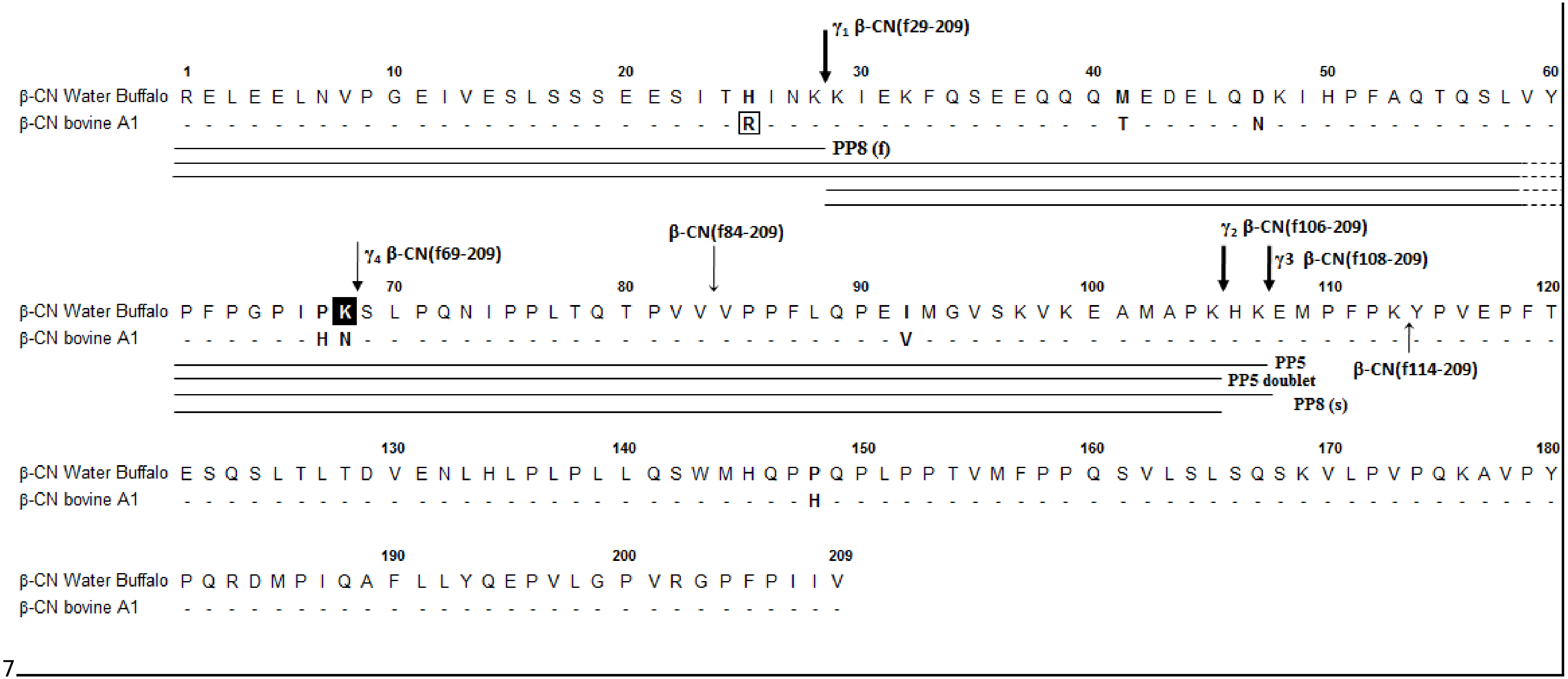
Figure 3. MALDI-TOF mass spectra and tentative signal assignment of plasmin-generated peptides occurring in the 3 electrophoretic bands numbered as 4, 5, and 6 in Fig. 2.

Figure 4. MALDI-TOF-MS-based identification of the proteotypic species WB β-CN(f1-28)4P and B β-CN(f1-25)4P as deriving from the CN fraction of WB milk containing 50% v/v B counterpart, which was preventively subjected to HA-based phosphoprotein enrichment and trypsinolysis. Reported is a partial view of the mass spectrum, showing well resolved (ΔM = +336 u), intense signals associated with the proteotypic species. WB β-CN(f1-28)4P (*theor.* $MH^+ = 3460.3$); B β-CN(f1-25)4P (*theor.* $MH^+ = 3124.3$).

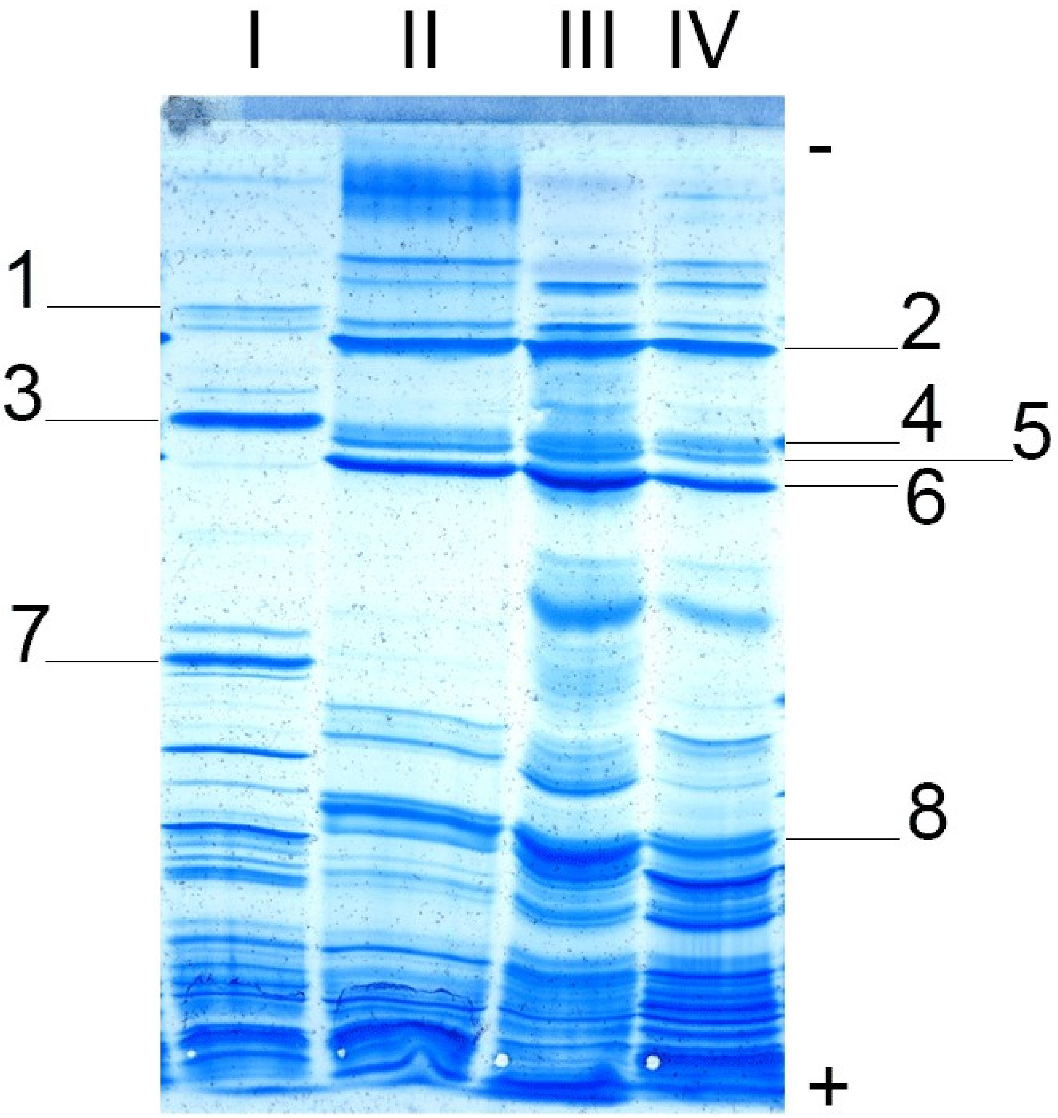
Figure 5. Calibration curve obtained by plotting the ratio of the area of the signals related to the proteotypic phosphopeptides B β-CN(f1-25)4P and WB β-CN(f1-28)4P against the composition of

adulterated WB milk used to prepare Mozzarella cheese samples. Ascertained bovine to water buffalo peak area ratio values are reported.

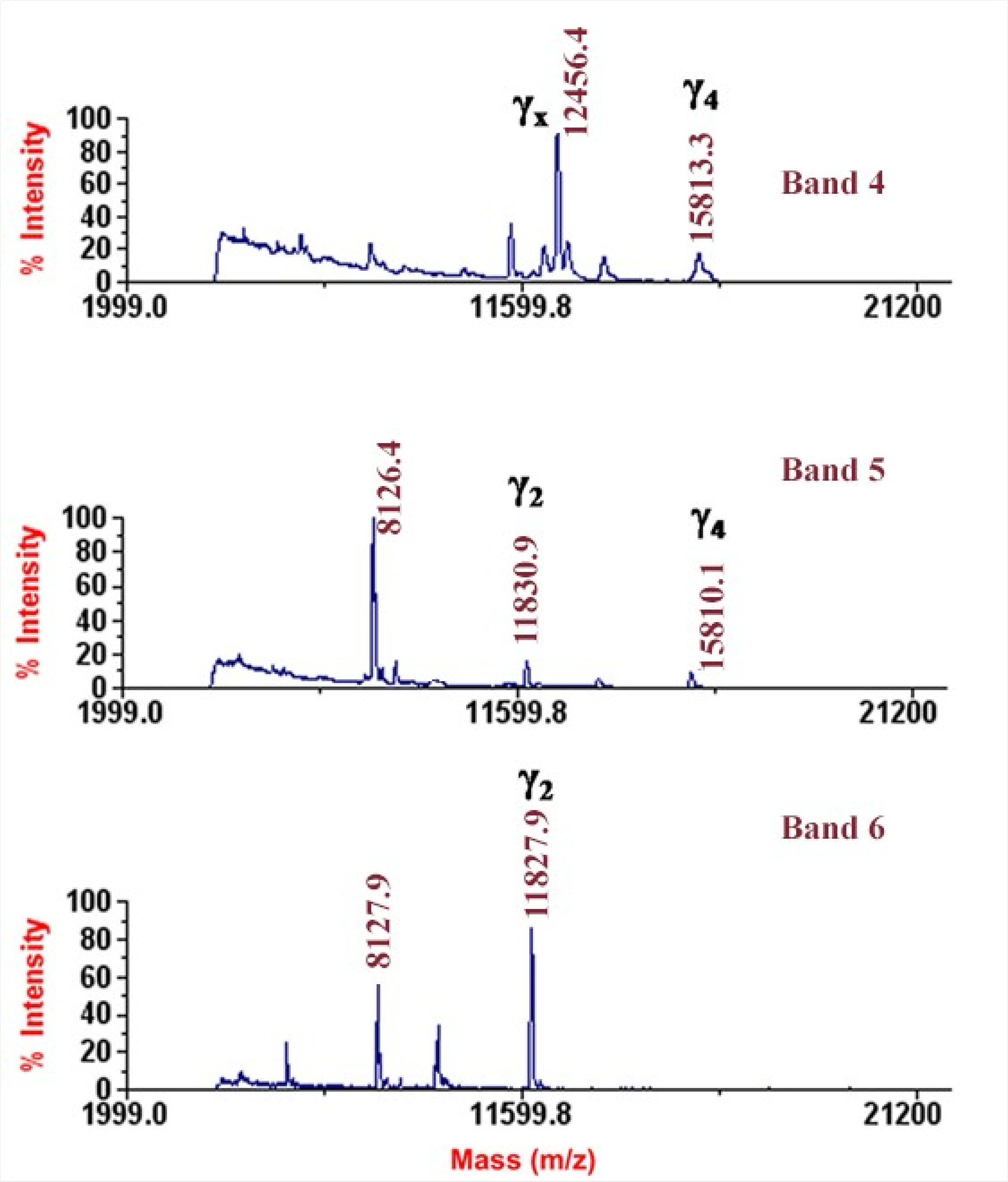
Caira et al., Figure 1



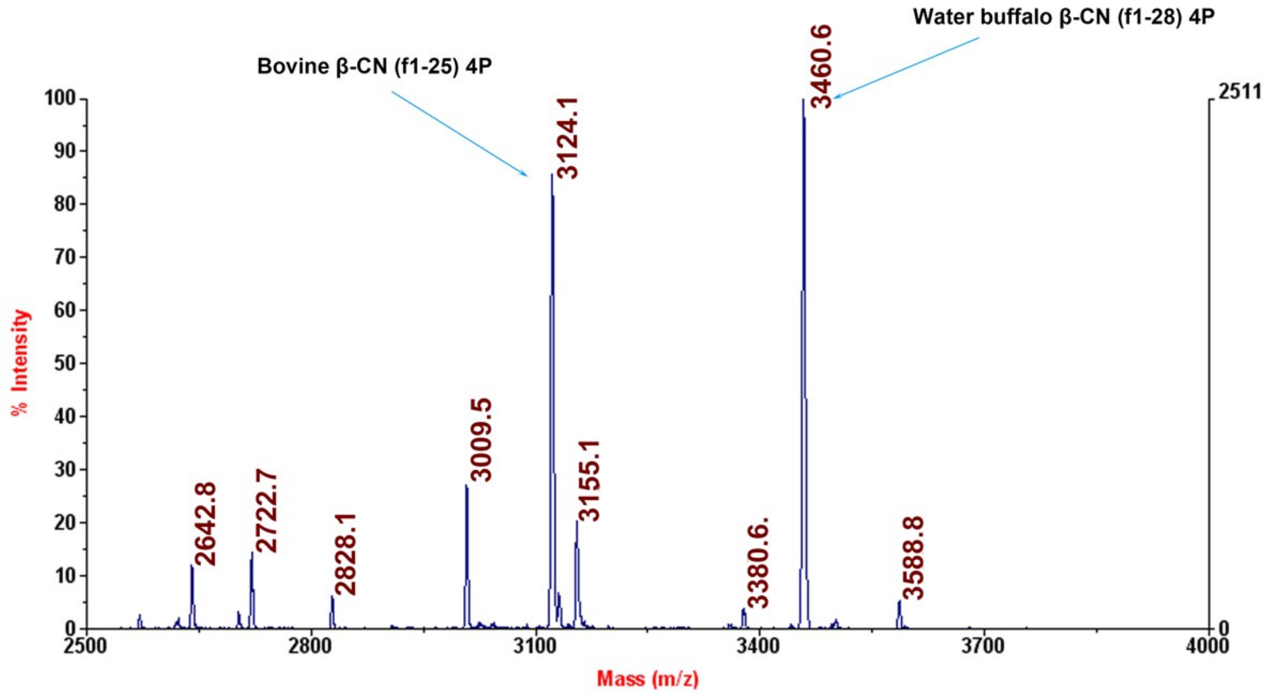
Caira et al., Figure 2



Caira et al., Figure 3



Caira et al., Figure 4



Caira et al., Figure 5