P64: 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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Abstract – The European reference method (ERM) identifies the fraudulent addition of bovine (B) milk in water buffalo (WB) milk/dairy products by concomitant isoelectric focusing (IEF) detection of B γ2- and γ3-CN fragments after plasminolysis. False positive results could occurr as the WB β-CN (f100-209), also formed after plasminolysis of WB milk/dairy products comigrates with B γ2-CN. These ERM limitations were overcome by a phosphopeptides enrichment procedure followed by trypsinolysis and the elution of B β-CN(f1-25)4P and WB β-CN(f1-28)4P peptides identified as suitable specie/specific markers with a detection limit of 0.8% v/v.

Keywords: γ_2- and γ_3-casein; peptide markers; milk; cheese; proteomics

1. INTRODUCTION

A large number of Protected Designation of Origin (PDO) cheeses under European Union (EU) regulations are manufactured with non-bovine milks. Common fraudulent practices consist generally in ovine, caprine or water buffalo (WB) milk partially/fully substitution with cheaper, readily available bovine (B) counterpart. In addition, when non-declared B milk is added to raw non-bovine material, safety concerns arise because of allergic reactions. Thus, enforcement agencies and analytical laboratories responsible for food control promoted the development of assays to detect and to quantify milks from different species in dairy product samples. Analytical methods based on specific DNA sequences [1] or Real-time PCR assays allowed the quantification of the fraudulent B milk adjunction [2]. 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 [3]. The method is based on B β-CN sensitivity towards plasmin enzyme, generating the fragments 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) (γ1-CN), (f106-209) (γ2-CN) and (f108-209) (γ3-CN). WB β-CN primary cleavage sites, at low enzyme/substrate ratio, occur at the above-mentioned sites, plus Lys68, generating identical fragments, plus the inner peptides β-CN(f29-68), (f69-105) and (f69-107), and C-terminal β-CN(f69-209) (γ4-CN) [4]. Being easily distinguishable by IEF, resulting B and WB γ2-, and γ3-CN constitute signature polypeptides allowing the detection of B milk addiction in WB milk/cheese at very low levels (0.5%). 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 [5]. It was demonstrated that plasminolysis of WB casein/cheese might release a peptide focusing similarly to B γ2-CN. To investigate on the occurrence of this plasminolysis product in WB CN a dedicated proteomic investigation was undertaken. We here demonstrate that the plasmin-generated

WB β-CN(f100-209) was the fragment isoelectrofocusing together with B γ2-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 γ2- and γ3-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. EXPERIMENTAL

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 and mozzarella cheese 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/cheese (~100 g final weight).

2.2 Plasmin Hydrolysis. B or WB milk/cheese (1 mL/200µg was added of 1 mL 0.2 M NH4HCO3 (pH 8.0); 500 uL of the resulting solution was 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. 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. After washing procedure, the pellets were air-dried, and kept at -20 °C until further analysis.

2.3 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 [3]. After migration, gels were stained using Coomassie Brilliant Blue G-250 (CBB). The gel bands were cut, destained washed and suspended in 0.25 mM NH4HCO3 pH 8.0, added 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. The supernatant after centrifuge evaporation to half of the initial volume, was directly submitted to LC-nanoESI-Q-TOF-MS or MALDI-TOF-MS analysis.

2.4 MALDI-TOF mass spectrometry. MALDI-TOF mass spectra were obtained on a Voyager-DE PRO instrument (Applied Biosystems, Framingham, MA). Sample (1µL) was mixed with an equal volume of matrix solution (10 mg of sinapinic acid in 1 mL of 0.1% TFA, 50% ACN) applied to a stainless steel sample plate and allowed to dry. Sample ionisation was carried out in positive linear mode.

2.5 Endoproteinase Asp-N. 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.

2.6 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. Samples were separately submitted to microgranular hydroxyapatite (HA) enrichment procedure according to Pinto et al., **[1].** The HA-CN bound phosphopeptides were deposited onto the MALDI plate, covered with the matrix solution (0.5 µL) containing 10 mg/mL 2,5-dihydroxybenzoic acid (Sigma-Aldrich).

3. RESULTS AND DISCUSSION

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. As already described [7], WB CN plasminolysis products showed two evident bands in the region of B γ2-CN, among which one having an almost identical isoelectric focusing behaviour to the bovine fragment. The occurrence of these

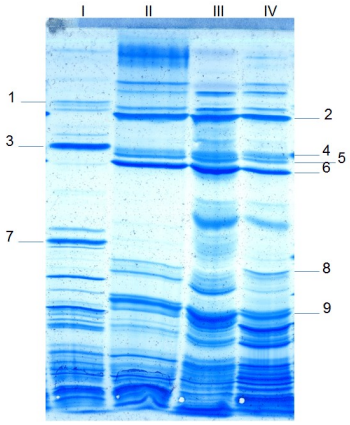

Figure 1. UTLIEF profile of plasminolysate samples from B CN (lane I), WB Mozzarella cheese (lane II), purified WB β-CN (lane III), and WB CN (lane IV). Gel bands were numbered and extracted for further MALDI-TOF-MS-based characterization of present polypeptides.

components was observed either in WB milk or in derived WB Mozzarella cheese. In order to characterize the nature of the WB and B CN bands comigrating in IEF, i) whole B CN; ii) whole WB CN, iii) WB Mozzarella cheese; iv) WB β-CN samples were submitted to plasminolysis and analysed by gels [3], (Fig. 1). 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. Thus, main bands in B and WB samples were associated with the expected B γ2- (band 3) and γ3-CN (band 7), and WB γ4- (band 2), γ2- (band 6) and γ3-CN (band 8). The ERM assumes that 1h-plasminolysis fully converts both WB and B β-CN into the corresponding γ2-CN and γ3-CN. We verified that this assumption was partially correct, since additional peptide fragments were also detected. They were assigned to B β-CN(f108-207) (band 1) and WB β-CN(f100-209) (band 4, here named γx-CN) and (f106-176) (band 5). 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, thus definitively confirming WB β-CN(f100-209) (γx-CN) as the component co-migrating with B γ2-CN in IEF responsible for false-positive results observed during the application of the ERM. In the whole, the results emphasize that the ERM, although more sensitive than a direct MS-based assay in revealing minor components, could give false-positive results for detecting B milk addictions, based on the electrophoretic co-migration phenomena. 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.

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

A MS-based procedure alternative to the ERM, was proposed exploiting also on the good performances of ceramic HA as a solid-phase adsorbent to efficiently capture and release CN phosphoproteins/phosphopeptides [8]. Accordingly, we decided to use this material in combination with direct MALDI-TOF-MS measurements for the selective binding of the tryptic casein phosphopeptides (CPP) from a 1:1 v/v WB/B milk mixture, and HA-bound CPP by MALDI-TOF-MS analysis. In this case, well resolved (ΔM = +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. 2);

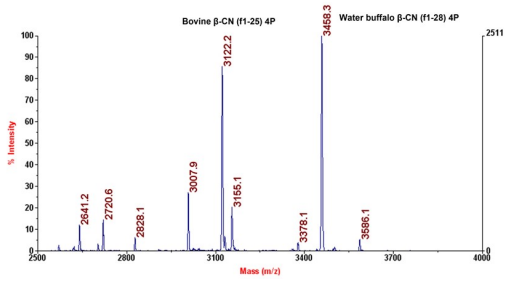

Figure 4. MALDI-TOF-MS-based identification of the proteotypic species WB β-CN(f1-28)4P and B β-CN(f1-25)4P as deriving from the CN fraction of WB milk containing 50% v/v B counterpart, which was preventively subjected to HA-based phosphoprotein enrichment and trypsinolysis. Reported is a partial view 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).

When this approach was applied to the analysis of five Mozzarella cheese samples made with WB milk mixed to various amounts of B milk (0-6.25%, v/v), detection of very low amounts of the B phosphopeptide was possible (Fig. 3).

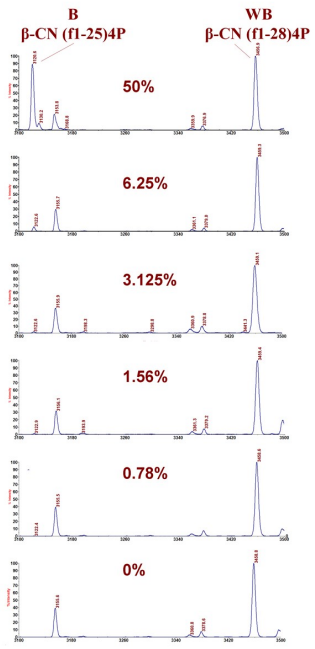

Figure 9. Identification of the proteotypic phosphopeptides WB β-CN (f1-28)4P and B β-CN (f1-25)4P in the tryptic digest of Mozzarella cheese samples made with WB milk mixed to various amounts of B milk (0-50%, v/v). Reported are a partial view of the MALDI-TOF mass spectra as obtained from the corresponding CN fractions, which were subjected to HA-based phosphoprotein enrichment and further trypsinolysis. Highlighted are the signals associated with the proteotypic components.

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%. 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 [3]. We confirmed the expectation that β-CN plasminolysis does not proceed solely by forming the prototypic peptides γ2- and γ3-CN, without passing through various peptide intermediates, but generates additional products that are due to hydrolysis at non-primary protein sites. 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. 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.

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