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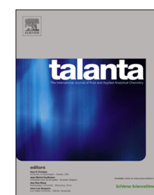

Detection and confirmation of milk adulteration with cheese whey using proteomic-like sample preparation and liquid chromatography–electrospray–tandem mass spectrometry analysis

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ABSTRACT

Caseinomacropeptide (CMP) is a peptide released by chymosin in cheese production, remaining in whey. Thus, CMP can be used as a biomarker to fluid milk adulteration through whey addition. Commonly, CMP is analyzed by reversed phase (RP-HPLC) or size-exclusion chromatography (SEC). However, some psychrotropic microorganisms – specially *Pseudomonas fluorescens* – when present in storaged milk, can produce, by enzymatic pathway, a CMP-like peptide generally called pseudo-CMP. These two peptides differ from each other only by one amino acid. RP-HPLC and SEC methods are unable to distinguish these two peptides, which demand development of a confirmatory method with high selectivity. Considering the several degrees of glycosilation and phosphorylation sites in CMP, allied with possible genetic variation (CMP A and CMP B), analytical methods able to differentiate these peptides are extremely complex. In the present work, we developed a proteomic-like technique for separation and characterization of these peptides, using liquid chromatography coupled to mass spectrometry with electrospray ionization able to differentiate and subsequently quantify CMP and pseudo-CMP in milk samples in order to identify adulteration or contamination of these products. The method shows satisfactory precision ($<11\%$) with a detection limit of 1.0 μg mL^{-1} and quantification limit of 5.0 μg mL^{-1}. Specificity, matrix effects and applicability to real samples analysis were also performed and discussed.

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1. Introduction

Cheese making is based on enzymatic cleavage of k-casein through chymosin action. This enzyme shows a high degree of specificity for its cleavage site, breaking the peptidic bond between the amino acids phenylalanine and methionine, in positions 105 and 106 of k-casein, respectively (Phe_{105}-Met_{106}) [1–3]. From the cleavage, two peptides are released: a hydrophobic N-terminal polypeptide, named κ-paracasein (residues 1–105 from original k-casein) and a hydrophilic phosphorylated and partially glycosylated C-terminal polypeptide (residues 106–169 from original k-casein), called caseinomacropeptide (CMP). This peptide, also known as glycomacropeptide (GMP), presents a high degree of glycosilation sites, and is a water-soluble peptide that remains in the rennet whey [4–6].

There are four major types of casein in bovine milk: αS1-, αS2-, β-, and k-casein [7]. These four caseins are phosphorylated on specific seryl residues and in addition k-casein can be also glycosylated in several sites [8]. Thus, in one same milk sample, CMP can be present in several forms depending on the number of post-translational modifications (phosphorylation and/or glycosylation sites). The possible sites for glycosylation and phosphorylation in a CMP primary structure are shown in Fig. 1. Moreover, three genetic variants of CMP have been identified, originated from the precursor k-casein A, B and E [9]. Variants A and B are the most frequent forms in bovine milk. Both variants existing in singly and doubly phosphorylated forms [9]. Although phosphorylation sites in CMP are a relatively homogeneous post-translational modification, glycosylation can show a high grade of heterogeneity, not only by the bindings sites variation, but also because several kinds of carbohydrates can be linked with the CMP. The sugars more frequently observed are galactosyl (Gal), N-acetylgalactosaminitol ($GalNAc_{OH}$) and N-acetylneuraminic acid (NaNA) [5,10]. The latter had enough specificity to be considered as an adequate marker for CMP detection and quantitation [11]. Currently, besides high number of interferences and false positive

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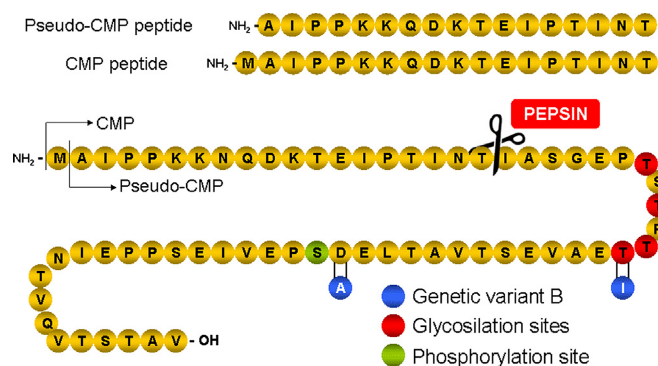

Fig. 1. Cleavage sites for enzymatic digestion of CMP and pseudo-CMP with pepsin. Two peptides are generated: MAIPPKKNQDKTEIPTINT (MAI-INT, 19 amino acids and MW=2140 Da) originated from CMP and AIPPKKNQDKTEIPTINT (AI-INT, 18 amino acids and MW=2008 Da) released from pseudo-CMP cleavage.

and negative results, NaNA analysis is still used as a method for CMP determination [12,13].

Milk adulteration is a fraud recently observed in Brazil, which has a specific program to monitor and detect this kind of crime [14]. Fraud in milk production included water, glucose or other sugar addition, pH adjustment, as well as other substances addition to correct protein and/or density values, as melamine, maltodextrose and others [15]. As cheese whey is a by-product of cheese production, adulteration of milk with cheese whey can be considered an attractive solution for the destination of this by-product. Besides, in recent years, several cases of whey addition in milk were detected in Brazil and other countries and the traditional methods – as Kjeldahl based methods for nitrogen determination – have been suffering criticism regarding their effectiveness [15,16].

When whey is added in the milk it does not necessarily correct the proteins, pH, among others, since matrix has the same origin. Thus, one way to detect this fraud is by the use of CMP as a marker, since it is proportional to whey addition in milk.

Olieman et al. [8] proposed for the first time the use of CMP to detect frauds by whey addition in milk [17]. They proposed selective milk proteins precipitation with trichloroacetic acid (TCA) to obtain only CMP in the final acid extract. The separation and identification of CMP were performed using a SEC method, with UV detection (205 nm). Although CMP quantitation was easily performed, TCA caused high intensity interference in the chromatograms background. The method was adopted in the European Community as an official method for milk adulteration with whey. Since no commercial standard of CMP was available at that moment, calibration curve was prepared from in-house produced whey cheese. For every batch of analysis, a small scale cheese making process was performed in their own laboratory. Following, whey produced by enzymatic cleavage was added in blank milk in order to produce several concentrations of whey. The concentration was expressed as percentage (%) of whey in milk. As a consequence, regulations related to the permitted level of CMP (assumed as whey percentage in milk) deal with limits from 1% (European Community) to 5% (Brazil), taking into account climate and livestock characteristics [8,18,19].

However, this calibration curve approach has a serious lack of reproducibility, since "in-lab" cheese making process can suffer intense variations from one batch to another, originated from blank milk, coagulation agent, process parameters (temperature, time) and others sources. In the same way, CMP is a minor component of whey, representing a variable fraction between 1 and 5% of the total whey composition depending on several factors such as bovine breed, genetic variety, fat content, temperature, and time of storage. For this reason, uncertainty inherent to this method is unacceptable to regulatory methods.

In Brazil, the SEC–UV method was applied as a monitoring method, using similar parameters to express the analysis results [18,19]. After a critical analysis of this method, carried out by our laboratory, calibration curves with whey were replaced by purified CMP, used as any other chemical standard. From this moment, percentage was replaced by a "CMP-index", established based on endogenous CMP level found in raw milk which was analyzed immediately after milking. A level of 30 mg L^{-1} was established as the tolerance level for milk [14,19,20]. This CMP-index was correlated to the cheese whey addition. As CMP is present in cheese whey in a concentration range of 1.2–1.5 g L^{-1}, 30 mg L^{-1} of CMP in fluid milk is approximately equivalent to 2–4% of the cheese whey [21].

Moreover, until that moment, proteolysis activity caused by psychrotrophic bacteria in milk was not considered in CMP methods. It is well established that psychrotrophic bacteria, especially *Pseudomonas* sp., are able to produce lipases and proteases, which cause organoleptic alterations in milk [7].

Within the *Pseudomonas* genus, *Pseudomonas fluorescens* is the most frequent psychrotrophic bacteria found in milk [7]. Lipases and proteases are secreted into the extra-cellular medium to provide more easily disposable nutrients to the bacteria. *P. fluorescens* proteases produce cleavage of k-casein in a very similar way to chymosin, mostly between amino acids residues 106 and 107 (Met_{106}-Ala_{107}) [7]. Water-soluble peptides released by this cleavage are called pseudo-CMP. Moreover, *Pseudomonas* proteases are thermally stable and their activity in milk remains even after bacteria elimination by pasteurization or ultra-high temperature treatment [17]. For these reasons, pseudo-CMP presence leads to false-positive results in CMP determination by SEC because this analytical technique cannot distinguish between CMP and pseudo-CMP [22].

This lack of specificity leads several researchers to explore other techniques capable to differentiate CMP and similar peptides. Methods dealing with reversed phase chromatography, mass spectrometry, capillary electrophoresis, SDS-PAGE, immunochemical assays were reported in last decades [23–29]. First separation of CMP and pseudo-CMP was obtained by Recio et al. using capillary electrophoresis with UV detection [25]. However, electropherograms show a multi-peaks profile and a noisy background, probably because of several forms of CMP, depending on post-translational modifications. Later, Hernández-Ledesma and co-workers introduced mass spectrometry analysis of whole and partially fragmented CMP and others milk proteins [30].

Considering that the only difference between CMP and pseudo-CMP is the N-terminal sequence, the application of a proteome-like protocol allows peptide fragmentation into a predictable pattern. To achieve this goal, pepsin (EC 3.4.23.1) is a logical choice, since it provides specific cleavages in the carboxyl linkage between the amino acids tyrosine and isoleucine. Thus, pepsin digestion of CMP and/or pseudo-CMP provides a short and specific N-terminal sequence that can be identified by means of high-selective methods such as mass spectrometry [31,32]. Fig. 1 shows the cleavage points obtained with pepsin digest in CMP and pseudo-CMP, which generate N-terminal peptides with molecular mass of 2140 and 2008 Da, respectively.

Considering the great importance of milk in the population diet, mainly for children, the overall aim of this work was the development of an analytical method to provide confirmatory results for CMP analysis, using a sample preparation protocol based on proteomic techniques. Mass spectrometry was used to obtain unequivocal identification of specific sequence of amino acids originated from CMP or pseudo-CMP digestion.

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