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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 $\mu g\ mL^{-1}$ and quantification limit of 5.0 $\mu g\ mL^{-1}$. Specificity, matrix effects and applicability to real samples analysis were also performed and discussed.

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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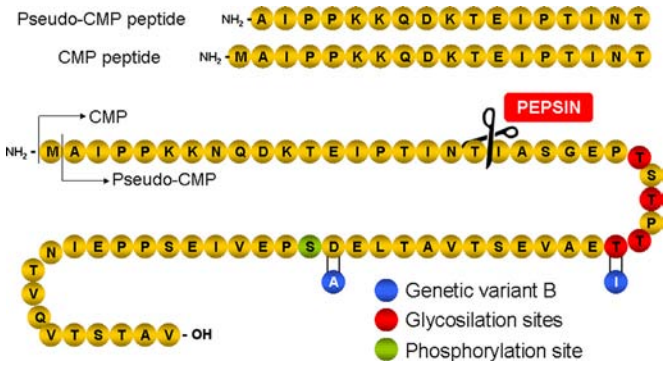

Fig. 1. Cleavage sites for enzymatic digestion of CMP and pseudo-CMP with pepsin. Two peptides are generated: MAIPPKKNQDKTEIPTINT (MAI-INT, 19 amino acids and MW=2140 Da) originated from CMP and AIPPKKNQDKTEIPTINT (AI-INT, 18 amino acids and MW=2008 Da) released from pseudo-CMP cleavage.

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.

2. Materials and methods

2.1. Chemicals and reagents

CMP (91.3% of purity) was obtained from Davisco Foods (Eden Prairie, MN, USA) and working standard solutions of 1 mg mL^{-1} were prepared with deionized water produced by a Milli-Q apparatus (Millipore, Bedford, MA, USA). Acetonitrile HPLC grade (ACN) and acetic acid were obtained from J.T. Baker, trifluoroacetic acid (TFA) and glycine were obtained from Vetec (Duque de Caxias, RJ, Brazil) and trichloroacetic acid (TCA) was from Merck. Formic acid was obtained from Sigma-Aldrich.

Pepsin obtained from swine gastric mucous was purchased from Sigma-Aldrich. The synthetic peptides MAIPPKKNQDKTEIPTINT and AIPPKKNQDKTEIPTINT were obtained from Mimotopes (Australia), with purity of 96.0% and 95.0%, respectively.

Pepsin stock solution (1 mg mL^{-1}) and glycine 1 mol L^{-1} were prepared with ultrapure water. Pepsin stock solution was divided in aliquots of 1.5 mL and stored at −20 °C, pepsin work solution was prepared before analysis. Solutions of synthetic peptides were prepared as per se manufacture recommendations, to a final concentration of 10 mg mL^{-1}.

2.2. Sample preparation

An aliquot of 1 mL of milk was taken and placed in a microcentrifuge tube (1.5 mL) and 0.5 mL of TCA 24% was added. The final concentration of TCA in the extract was 8%. Following, tubes were vigorously stirred for approximately 5 s in a vortex and then sonicated for 30 min in an ultrasonic bath from Unique (USC 1800, 40 kHz, 120 W) (Santo Amaro, SP, Brazil). The samples were centrifuged for 10 min at 12,000*g* (Heraeus Fresco 17, Thermo Fisher, Germany). For each batch, a calibration curve prepared with blank matrix (bovine raw milk) was performed, by adding several volumes of standard solution of CMP 1.0 mg mL^{-1} (Table 1).

2.3. Pepsin digestion

After sample centrifugation, an aliquot of supernatant (200 μL) was taken and placed into a glass vial. Glycine solution (1.0 mol L^{-1}), in ratio of 1:1, was added to provide a less acidic medium for digestion. The amount of enzyme was defined taking into account the highest concentrated level of the calibration curve. Protein cleavage was obtained by adding 10 μL of pepsin work solution (10 μg mL^{-1}) with deionized water into each sample to a final volume of 1 mL. Sample volume and digestion time were optimized, as explained in Section 3. Tubes were incubated in a dry heater at 37 °C and after incubation subsequently analyzed by LC–MS/MS.

2.4. LC–Ms/Ms

Analysis was based on N-terminal fragments obtained after CMP or pseudo-CMP digestion, i.e., MAIPPKKNQDKTEIPTINT (MAI-INT) for CMP and AIPPKKNQDKTEIPTINT (AI-INT) for pseudo-CMP. Before mass spectrometry analysis optimization, theoretical digestion prediction was done using the in silico digestion performed by the Skyline software (MacCoss Lab, University of Washington).

Analysis and separation of pepsin digestion products were performed in a LC–MS/MS system API 5000 AB Sciex (Foster City, CA, USA) coupled with a liquid chromatography 1100 Series (Agilent). The column was a PLRP-S (polystyrene-divinylbenzene), 150 × 4.6 mm^2, 300 Å (Polymer Technologies, Varian). Quantitative analysis was obtained in MRM mode, using at least two transitions for each molecular ion. Mass spectrometer parameters for ionization and fragmentation were optimized using synthetic peptides standards injection by infusion followed by flow injection analysis (FIA). Mobile phase was composed by ultra-pure water (A) and acetonitrile (B), both with 0.1% of formic acid. Mobile phase flow was 600 μL min^{-1} and a gradient mode was used. Initial conditions were 10% of B in A, increasing to 60% from 2 to 5 min, holding for 5 min and returning to original composition in 2 min, for a total analysis time of 15 min. Equilibrium time was 2 min.

A Triple quadrupole mass detector with electrospray ionization source (ESI) in positive mode was used for detection and quantification of targeted fragments. Turbo ion spray voltage was optimized at 5500 V and temperature source was 650 °C. Other optimized parameters are: EP = 10 V; CAD = 12 V; CUR = 10 V; GS1 = 45 psi; GS2 = 55 psi Multiple reaction monitoring (MRM) conditions, typical retention time and optimal declustering potential (DP), collision energies (CE), collision cell exit potential (CXP) in the MS/MS mode for the product ions generated, are shown in Table 2.

2.5. Samples

Samples of raw milk were collected from dairy farms from several Brazil regions by Federal Inspectors from the Ministry of Agriculture, Livestock and Food Supply (MAPA). Samples were taken directly from refrigerated tanks in the dairy farms, in which recent milk was placed (less than 4 h). To avoid protein degradation, with further CMP-like peptides release, samples were immediately frozen after collection and sent to the laboratories where they remained in the freezer conditions (−10 to −30 °C) until the day of the analysis [27].

2.6. Validation procedure

Brazil has an official methodology for CMP analysis, which is currently performed in our laboratory. In the official method, to 10 mL of milk 24% of TCA is added under gentle agitation and dripping. In the present work, the sample preparation was modified to a micro-scale and the protein precipitation step was replaced by ultrasonic bath preparation. This new methodology

Table 1
Matrix-matched calibration curve preparation.

CMP concentration level (μg mL^{-1})	Milk (μL)	CMP standard solution (μL)
0	1000	0
5	995	5
10	990	10
30	970	30
60	940	60
90	910	90

Table 2
Mass spectrometry parameters for pepsin digest products analysis.

Peptide	Ion	Parent ion (*m*/*z*)	Daughter ion (*m*/*z*)	DP[a] (V)	CXP[b] (V)	CE[c] (V)
MAI-INT	$[M+3H]^{3+}$	713.8	797.9	120	27	24
			896.9	120	27	24
			953.5	120	27	24
AI-INT	$[M+3H]^{3+}$	670.0	732.4	120	27	24
			888.4	120	27	24
			912.4	120	27	24

[a] DP = declustering potential.
[b] CXP = collision cell exit potential.
[c] CE = collision energy; unit: Volt (V)

was validated considering the linearity, precision (intra and inter-day) and accuracy in order to replace the currently official method [14,18,19].

For LC–MS/MS and pepsin digestion protocol, the validation was performed using the linearity, detection limit (LOD), quantitation limit (LOQ), matrix effect, specificity, precision (intra and inter-day) and accuracy. Acceptance criteria were based on the European Commission Decision 2002/657/EC [33]. For linearity evaluation, LOD and LOQ determination, a calibration curve in the concentration range from 1 to 120 $\mu g\,mL^{-1}$ was prepared from synthetic peptides. LOD was established as the analyte concentration that has a signal three times above the signal/noise ratio and LOQ as 10 times above the signal/noise ratio. Matrix effect was evaluated to verify enhancement or suppression of signal. The procedure was based on the analysis of three calibration curves: (I) curve prepared in solvent; (II) matrix-matched curve with samples spiked before extraction and (III) matrix-matched curve with samples spiked after extraction. The absence of compounds which may interfere with the analytes, e.g. by co-elution or because they present the same m/z, was evaluated to verify the method specificity. In this case, blank samples without analyte and/or without matrix presence were analyzed. CMP standard without pepsin digestion was also evaluated. Precision and accuracy were studied by the analysis of samples fortified in three different levels of concentration (15, 30 and 60 $\mu g\,mL^{-1}$). The intra-day precision was determined with six measurements in replicate in the three levels of concentration while the inter-day precision test was performed during the execution of three batches into three distinct and consecutive days [33].

3. Results and discussion

3.1. Analytical conditions

After pepsin digestion, peptides were converted into smaller peptides, as predicted by theoretical digestion. The markers (MAI-INT for CMP; AI-INT for pseudo-CMP) were chosen based on their molecular size so that they were more feasible to be routinely analyzed by LC–MS/MS using triple quadrupole and electrospray ionization. Moreover, it is the N-terminal fraction that provides differentiation between the two peptides and also eliminates glycosylated and phosphorylated isoforms diversification. Similarly, amino acids variation between genetic variants A and B was not taken into account, since both variants have the same N-terminal structure [34].

Commonly, peptides and proteins have more than one ionization site. In the case of ESI in positive mode, the N-terminal plus side chains of basic amino acids are able to ionize. Thus, the same peptide could be present in $[M+H]^+$, $[M+2H]^{2+}$ or $[M+3H]^{3+}$ forms. The m/z transitions selected for monitoring were preferentially the more charged ions. The use of ions with 2 or 3 charges increase the method selectivity, since the quasi-molecular ion is fragmented, ions with less charge are produced. Those fragments ($z=2$ or 1) have an m/z value higher than the precursor ($z=2$ or 3) and this lead to higher specificity for MRM analysis, because more frequent interference compounds had one charge ($z=1$) in both precursor and daughter ions [35]. For MAI-INT, the transitions $713.8 > 798.0$, $713.8 > 953.7$ and $713.8 > 897.2$ were chosen, as $713.8 > 798.0$ is the most intense and was selected for quantitative purpose. For AI-INT, $670.0 > 732.8$, $670.0 > 888.5$ and $670.0 > 913.0$ MRM transitions were optimized and monitored.

Moreover, despite the fact that intact CMP has a high mass value, the use of enzymatic digestion and the use of ions with charge of +2 and/or +3 permits the use of mass spectrometry systems with lower mass range to perform the present method.

Using MAI-INT and/or AI-INT as target, differential analysis between CMP originated by cheese-making and CMP-like peptides released by bacteria activity was possible using mass spectrometry.

Using pepsin, the enzyme could be added to the extract just after protein precipitation with TCA. To optimize pepsin activity, pH of the extract was adjusted to a less acidic condition, from 1.5 to 2.5. The adjustment was performed through the addition of equivalent amounts of glycine solution to the extract.

Chromatographic separation was achieved using a classic scheme for peptides / proteins analysis: a slow gradient of water and acetonitrile. Firstly, two reverse-phase columns were evaluated, with C18 and C4 as solid support. In both procedures, unsatisfactory separation with low repeatability of retention time and tailing peaks were obtained. In order to investigate the role of stationary phase in the separation, a non-silica based column was tested. Results obtained with a polystyrene-divinylbenzene (PLRP-S) column were more efficient and reproductive, besides showing better resolution in comparison with C18 and C4 columns. Thus, PLRP-S was chosen to perform the analysis.

Extraction procedure was changed to provide faster analysis. The official method used an extraction procedure based on selective precipitation of milk proteins using TCA with a final acid concentration of 8%. In the original method, TCA was added drop by drop, in order to avoid co-precipitation of water-soluble peptides (as CMP and pseudo-CMP) together with milk proteins. We evaluate extraction with TCA addition in one-step, adding 5 mL of TCA in just one aliquot. After that, samples were incubated in an ultrasonic bath for 20 min. This procedure showed satisfactory results, without loss of analytes and maintaining recovery values of the official method. After that, in order to downsize the reagents and sample amounts, a new procedure was implemented, using only 1.0 mL of milk and 0.5 mL of TCA 24% for sample preparation. All extraction procedures were done in a micro-centrifuge tube. After TCA addition, samples were mixed and placed in an ultrasonic bath to promote adequate and selective protein precipitation. Following, the same tubes were centrifuged and the supernatant was used for digestion, without the need of a filtration step. This new procedure of sample preparation and extraction was validated and allows analysis of several samples in 40 min, while the official method requires at least 120 min.

3.2. Ultrasonic sample preparation validation

The Ultrasonic preparation sample method (UsM) performance was compared with the official preparation sample protocol (OfM). Three batches of samples spiked at three distinct levels, with the maximum limit (ML) as central level (15, 30 and 45 $\mu g\,mL^{-1}$) were analyzed for three consecutive days, using a matrix matched calibration curve prepared by the UsM method. For each level, seven replicates were analyzed.

The UsM method shows variation coefficients $< 15\%$, in agreement with the acceptance criteria proposed by the Commission Decision 657/2002/EC for the concentration levels studied [33]. Tables 3 and 4 show the results obtained in precision and accuracy investigation.

Otherwise, matrix matched calibration curves prepared by both methods (UsM and OfM) were overlapped, demonstrating the equality between the procedures. Moreover, variance between UsM and OfM, intercept and slope obtained with the calibration curve of the proposed method (UsM) must be between the regression analyses limits of the OfM calibration curve (Table 5). As the statistical analysis shows no significant difference between the methods that were observed. Thus, the UsM method has the same fitness to purpose as demonstrated by the OfM.

3.3. Enzymatic digestion optimization

For peptides and/or proteins digestion, the amount of sample is a very important parameter. Small volumes will influence method sensitivity and excessive sample amounts could lead to a high matrix effect with undesirable interference in further analytes ionization by LC–MS/MS. To optimize the sample amount in the digested step, matrix-matched calibration curves were prepared using 50, 100 and 200 μL of sample extracts (Table 6). The most intense signal was obtained with 200 μL of sample and with negligible matrix effect. Fig. 2 shows the data obtained in this experiment. All optimization experiments were performed taking into account the signal obtained for MAI-INT (transition 713.8 > 798.0).

To optimize the digestion time, 14 tubes containing CMP standard solution (30 mg mL^{-1}) were added with 10 mL of pepsin solution (10 μg mL^{-1}) and incubated at 37 °C until analysis. Each tube was removed from incubation at a distinct period: 1, 2, 3, 4, 5, 6, 7, 8, 10, 16, 24, 36 and 48 h of digestion and was evaluated. As revealed in Fig. 3, the intensity of the fragment MAI-INT is relatively constant, especially inside the interval between 5 and 24 h. The digestion time was established as 5 h. Enzyme amount and temperature were also optimized, but the results show no relevant impact on the analytes response. Amounts of pepsin in a range from 0.4 to 1.0 μg were evaluated and the data show similar signal for all experiments with a coefficient of variation of 4.6%.

3.4. LC–MS/MS method validation

3.4.1. Linearity, LOD and LOQ

The method shows linearity in the range of 1–120 μg mL^{-1} with satisfactory linear correlation coefficient values ($R^2 > 0.99$) using synthetic peptides (MAI-INT and AI-INT). The same calibration curve, analyzed in 3 replicates, was used to determine LOD and LOQ. The LOD was established as 1 μg mL^{-1} that corresponds to the lower point of the curve. Signal-to-noise ratio of this concentration level was 176 and 197 for MAI-INT and AI-INT, respectively. LOQ was established as 5 μg mL^{-1} that corresponds to the second point of the calibration curve. Signal-to-noise ratio was 1954 (MAI-INT) and 3661 (AI-INT). Fig. 4 shows chromatograms for the two peptides at LOD and LOQ level. Using mathematical parameters, LOD and LOQ should be lower, e.g., LOD of MAI-INT as 0.017 μg mL^{-1}. However, these values generally are irreproducible in the laboratory routine. Thus, LOD and LOQ were focused in levels included in the calibration curve.

3.4.2. Matrix effect

The analysis of the 3 calibration curve types shows interference in the analytes response when the matrix was present. However,

Table 3
Proposed method performance (UsM) in comparison with the official method (OfM).

Sample preparation method	Calibration curve level (μg mL^{-1})	Signal	Calculated concentration (μg mL^{-1})	Average	SD[a] (%)	CV[b] (%)
OfM	0	0.00	0.00	0.00	0.00	0.00
	0	0.00	0.00			
	25	895,104	29.33	29.08	0.35	1.21
	25	883,134	28.83			
	50	1,458,116	52.79	52.57	0.31	0.60
	50	1,447,467	52.35			
	75	2,045,389	77.26	77.25	0.02	0.02
	75	2,044,768	77.24			
	100	2,644,146	102.21	102.13	0.11	0.11
	100	2,640,284	102.05			
	200	4,918,546	196.99	196.94	0.07	0.04
	200	4,916,147	196.9			
UsM	0	0.00	0.00	0.00	0.00	0.00
	0	0.00	0.00			
	25	854,167	30.51	30.40	0.16	0.52
	25	848,974	30.29			
	50	1,403,370	53.94	53.88	0.09	0.16
	50	1,400,436	53.81			
	75	1,989,788	78.96	78.85	0.16	0.20
	75	1,984,606	78.74			
	100	2,288,314	91.69	91.78	0.13	0.14
	100	2,292,651	91.88			
	200	4,849,953	200.97	201.02	0.07	0.04
	200	4,852,368	201.08			

[a] Standard deviation.
[b] Coefficient of variation.

Table 5
Calibration curve data regression analysis for both official method (OfM) and ultrasonic method (UsM) methods at 95% confidence level.

	UsM curve	OfM curve	*p*-Value	Lower limit 95.0%	Upper limit 95.0%
Linear coefficient	138,959	191,248	0.002895849	82,367	300,129
Angular coefficient	23,441	23,998	3.28675E−13	22,898	25,098

Table 6
Sample amounts evaluated in the optimization of enzymatic digestion protocol.

Sample amount (μL)	Glycin 1 mol L^{-1} (μL)	Pepsin 1 μg mL^{-1} (μL)	Ultra-pure water (μL)
50	50	10	890
100	100	20	780
200	200	40	560

Table 4
Validation data for UsM sample preparation method for precision.

Calibration curve	Angular coefficient	Intercept	Linear correlation coefficient	
Day 1 (A1)[a]	1.91E+04	93,212	R^2=0.9930	
Day 2 (A1)	2.08E+04	44,554	R^2=0.9857	
Day 3 (A2)	2.28E+04	38,761	R^2=0.9990	
Concentration level	Average signal	Intra-day precision (%)	Inter-day precision (%)	CV[b] (%)
15 μg mL^{-1}	460,243.0	5.4	1.1	5.5
30 μg mL^{-1}	749,452.6	4.6	3.0	5.4
45 μg mL^{-1}	1,006,475.1	6.8	2.2	7.1

[a] Analyst.
[b] Coefficient of variation.

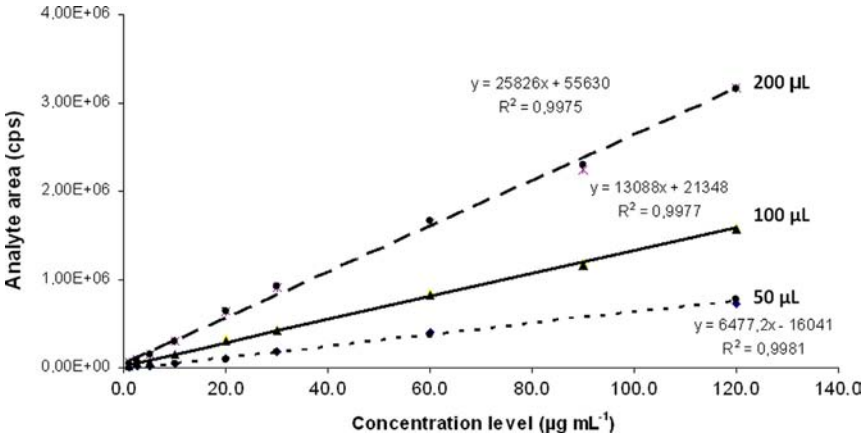

Fig. 2. Graphical plot of the signal obtained for MAI-INT using distinct amounts of sample.

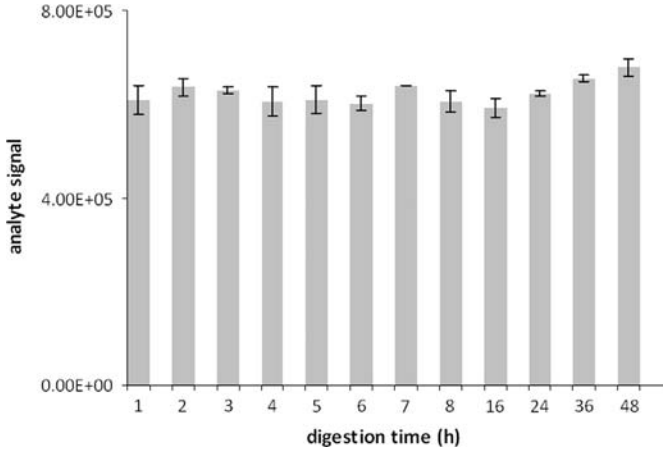

Fig. 3. MAI-INT signal intensity vs digestion time.

comparison between curve type I (solvent) and type III (fortified after extraction) exhibits high concordance, with plot almost overlapped. Curve type II (fortified before extraction) shows non-similarity in comparison with the other two curves, with differences in slope and inclination [36,37]. As a consequence, matrix-matched calibration curve, spiked before extraction, was elected to use in routine analysis, in order to avoid the influence of matrix effect in the determinations. Fig. 5 shows graphical plot of the 3 calibrations curves.

3.4.3. *Specificity*

When CMP samples without digestion with pepsin are analyzed, an interference signal can be observed (Fig. 6). This interference is present in a very low intensity and was considered negligible to the quantitation performance of the method. When a reagent blank without CMP standard addition was analyzed (Fig. 7), no interfering peaks were observed.

3.4.4. *Precision*

Repeatability, in terms of intra-day and inter-day precision, presented in Table 7 were obtained by the analysis of 3 validation batches. The method is in agreement to the criteria of the Commission Decision 657/2002/EC ($<15\%$) [33].

3.5. *Method applicability*

The present method was used for the analysis of raw milk samples from 43 independent producers, collected from all Brazil regions. The objective was to establish the level of endogenous CMP. Results show an average value of 5.87 $\mu g\ mL^{-1}$. Fig. 8 shows the results for endogenous CMP estimation [20]. With the exception of 2 outlier samples, no significant difference was observed in the results, related to geographical localization or other parameters.

4. Conclusions

To evaluate the validation data, criteria parameters proposed in the European Commission Decision 657/2002/CE were adopted. The guide was proposed for contaminants and veterinary drugs residue analysis in food matrices. However, the acceptance criteria for the validation data proposed in the Directive were adopted because they have straight limits for food matrices, which are adequate for the confirmatory purpose of the present study.

The method was validated as a quantitative method for CMP analysis. However, for pseudo-CMP, only qualitative results can be obtained currently. Since pseudo-CMP is not commercially available, the method was developed and validated using synthetic peptide that corresponds to pseudo-CMP digestion (AI-INT). Further experiments will be carried out to produce pseudo-CMP in vitro, using raw milk incubation with *P. fluorescens*.

Method applicability for real samples obtained from recent milking dairy cows shows a variable amount of endogenous CMP in milk. Thus, that CMP is not originated from whey cheese addition or a product of bacterial proteases. Hypothetically, a CMP basal value should be estimated caused probably by natural errors in protein synthesis associated to endogenous proteolysis and physiological responses related to feed, climate other factors. This basal value is very important for taking into account a revision of the limits and parameters adopted for CMP as a marker for milk quality.

The method also shows a high throughput capability: batches of 20–30 samples plus quality control samples could be prepared in a day of work. From the sample receipt to the final result, including sample defrost, extraction, enzymatic digestion, chromatographic run and data analysis, a maximum of 3 days are expended.

In this study we developed a LC–ESI-MS/MS method in which CMP and pseudo-CMP are completely distinguishable. By using MRM it is possible to monitor products of pepsin digestion without interfering the glycosylation and phosphorylation sites, besides genetic variations, which remain in non-analyzed fragments. Also, the extraction procedure was modified achieving a fast, cheap and easy protocol that used just 1 mL of the sample. This method presents unequivocal molecular mass identification of peptides. Characteristic fragments were proposed as markers to CMP and pseudo-CMP. Method was applied to real samples and proved to be

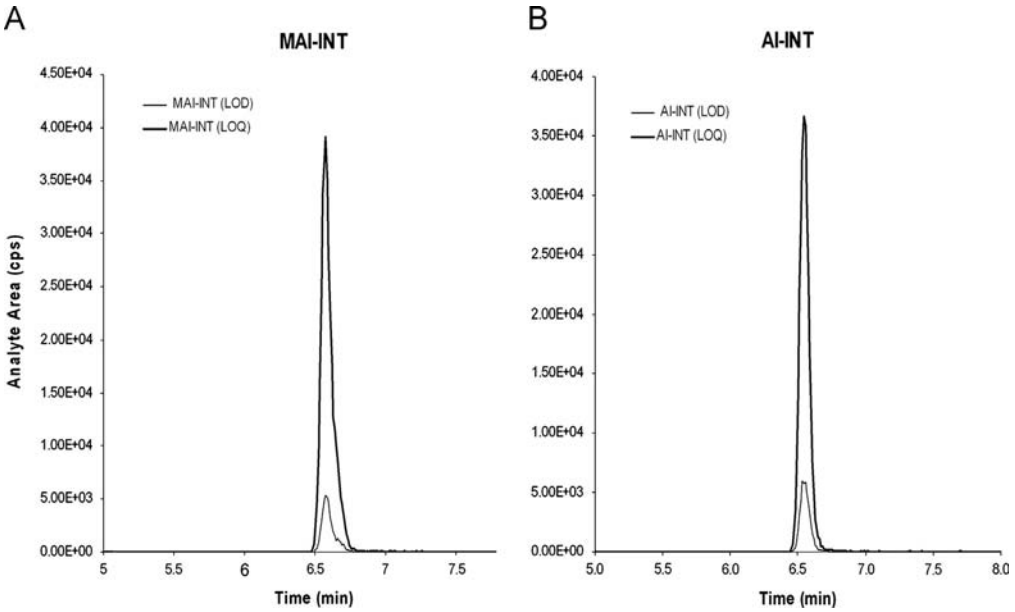

Fig. 4. Chromatograms of MAI-INT (A) and AI-INT (B) at LOD and LOQ level (1 μg mL^{-1} and 5 μg mL^{-1}).

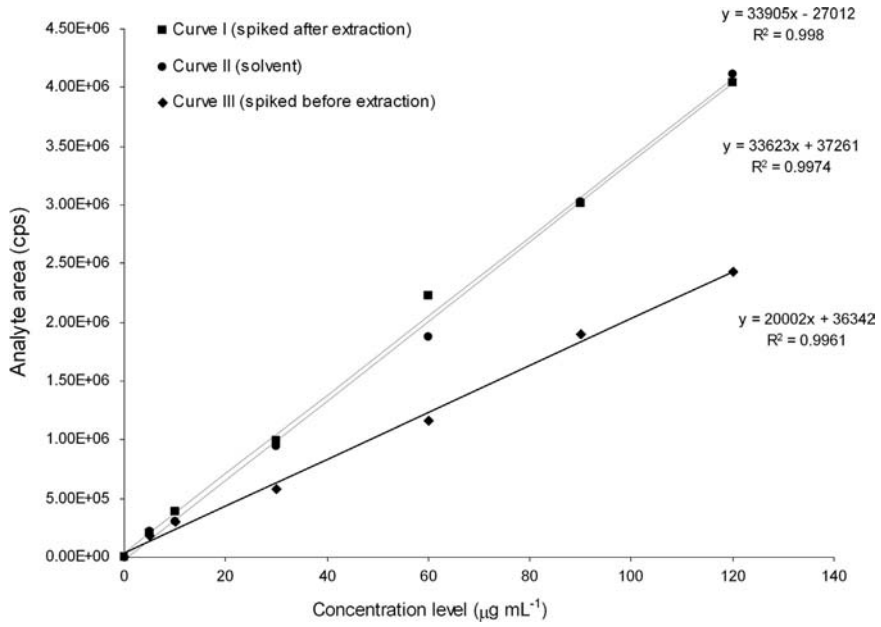

Fig. 5. Graphical plot of the 3 calibration curve types used to matrix effects evaluation. Concentration range from 1 to 120 μg mL^{-1}.

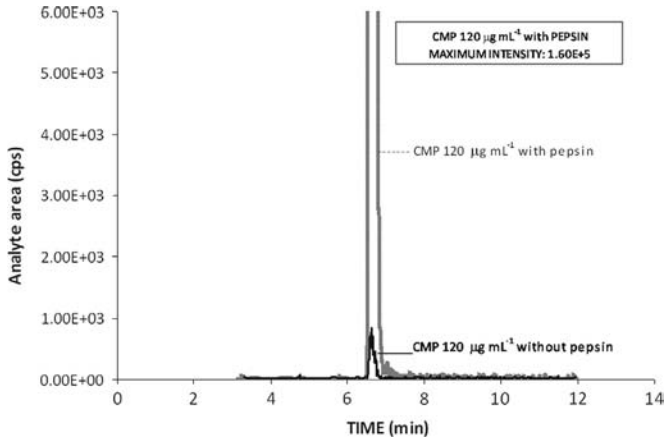

Fig. 6. Chromatogram of CMP standard without pepsin digestion and with pepsin addition.

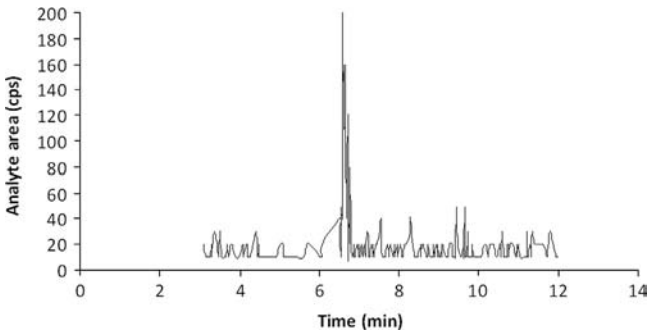

Fig. 7. Chromatogram of blank of reagents (pepsin and glycine), without CMP standard addition.

Table 7
Data for repeatability experiments for CMP analysis in bovine milk.

Calibration curve	Slope	Intercept	Linear correlation coefficient	
Day 1	3.17E+04	363,819	R^2=0.9891	
Day 2	2.99E+04	240,786	R^2=0.9911	
Day 3	2.65E+04	241,455	R^2=0.9959	
Level (μg mL^{-1})	Calculated concentration (μg kg^{-1}, average, n=21)	Intra-day CV[a] (%)	Inter-day CV (%)	Global CV (%)
15	13.7	7.4	3.8	8.3
30	26.2	11.5	2.2	11.7
45	40.5	8.0	5.3	9.6

[a] Coefficient of variation.

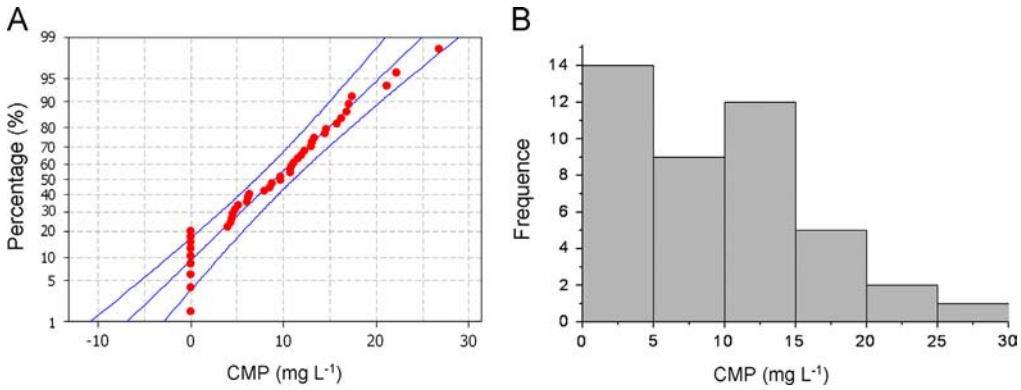

Fig. 8. CMP endogenous level determination. Values dispersion (a) and Pareto chart (b).

reliable and sensitive. Method was fully validated and will be applied in routine to monitor milk quality.

Acknowledgments

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