



Communication

[Comunicação]

Correlating mesophilic counts to the pseudo-CMP content of raw milk

[Correlação entre contagem de mesófilos e índice de Pseudo-CMP em leite cru]

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Trading uninspected raw milk directly to consumers is prohibited in Brazil, contrary to other countries in Europe, such as Italy, where sales of raw milk are permitted since 2004 (Giacometti *et al.*, 2012). Adulterations, such as the addition of whey, still are among the main problems related to milk. One of the most popular methods for the detection of whey is based on the casein macropeptide (CMP). The addition of 1% whey solids to milk usually yields a concentration of 6pg CMP/mL (Riel & Olieman, 1995). On the other hand, pseudo-CMP (p-CMP) can be produced by the cleavage of casein by bacterial proteases. Since CMP and p-CMP differ by a single amino acid, their differentiation is required to indicate clearly how adulterated milk is or if it is due to intense proteolysis resulting from poor bacterial quality (Motta *et al.*, 2014; Riel & Olieman, 1995).

Raw milk may present low microbiological quality, as determined by standard plate counts of mesophilic microorganisms, also called as total bacterial count (TBC), which has been settled as the reference method to determine milk quality in Brazil (Bersot *et al.*, 2010). Given that direct trade of raw milk to consumers is still commonly performed in Brazil, the possibility of adulterations, and the microbiological quality of this product were assessed in this work. Thus, the main goals of this work were to quantify the casein macropeptide (CMP), as well as to differentiate CMP (due to adulteration with whey) from pseudo-CMP (due to bacterial proteolysis) in raw milk samples, and to correlate total bacterial counts and the CMP concentration.

CMP (91.3% of purity) was obtained from Davisco Foods (Eden Prairie, MN, USA).

Acetonitrile HPLC grade (ACN) and acetic acid were obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA), trifluoroacetic acid (TFA) and glycine were obtained from Vetec (Sigma-Aldrich Co. LLC, Duque de Caxias, Brazil) and trichloroacetic acid (TCA) was from Merck KGaA (Darmstadt, Germany). Pepsin A (EC 3.4.23.1), obtained from swine gastric mucous, and formic acid were supplied by Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). The synthetic peptides MAIPPCKKNQDKTEIPTINT and AIPPCKKNQDKTEIPTINT were obtained from Mimotopes Pty Ltd. (Notting Hill, Australia), with purity of 96.0% and 95.0%, respectively.

Uninspected raw milk samples were obtained from different households in five cities of relevant dairy production in the state of Paraná, southern Brazil. The samples (n= 46) were transported and kept under refrigeration until analysis. Samples for the analysis of CMP were immediately frozen and kept at -18°C until analysis.

Samples were diluted in 0.85% saline solution, and 1mL of each dilution was used for plate count agar (Merck KGaA, Darmstadt, Germany) pour plates in sterile Petri dishes. The dilutions employed in this study were 10⁻² to 10⁻⁵. Plates were incubated at 35-37°C for 48h. Readings were carried out in a plate counter and expressed in CFU/mL of sample, according to the ISO 4833 method (International..., 2003).

CMP has been quantitated by high performance liquid chromatography (HPLC) in accordance to MAPA (Brasil, 2010). Briefly, samples of milk were precipitated with trichloroacetic acid 24%, followed by a rest period for sedimentation and

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filtration, and analyzed on an Alliance 2695 pump (Waters Corporation, Milford, MA, USA) in isocratic mode, with phosphate buffer (pH 6.4) as mobile phase. Chromatographic separation was carried out using gel filtration columns (D-55122 Mainz, SI Analytics GmbH; 250 x 9.4mm²; particle size from 4 to 4.5µm), followed by diode-array detection at 205nm. Matrix-matched analytical curves were prepared in the range of 15-100mg/L for samples quantitation. Analyses were carried out in duplicate, and results were expressed as arithmetic means in mg/L.

Milk samples that yielded results over 30mg/L of CMP underwent the peptide identification by LC-MS/MS according to Motta *et al.* (2014). Samples of milk were submitted to precipitation with trichloroacetic acid 24%, followed by a rest period and filtration. Then, 200µL of the supernatant were transferred to vials; after that, 200µL glycine 1mol/L, 550µL of ultrapure water and 50µL pepsin 10µg/mL were added to each vial, which were incubated at 37°C for at least 8h. Analysis of digestion products was carried out by injection in the API 5000 mass spectrometer with electrospray ionization in positive mode (Sciex, Foster City, CA, USA), coupled to a 1100 Series liquid chromatography system (Agilent Technologies, Inc., Santa Clara, CA, USA). Chromatographic separation was achieved in a PLRP-S (polystyrene-divinylbenzene) column, 150 x 4.6mm², 300 Å (Polymer Technologies, Varian). For CMP, the transitions (*m/z*) 713.8 > 798.0, 713.8 > 953.7 and 713.8 > 897.2 were chosen. The 713.8 > 798.0 transition was the most intense and was selected for quantitative purpose. For p-CMP, the transitions (*m/z*) 670.0 > 732.8, 670.0 > 888.5 and 670.0 > 913.0 were monitored. Matrix-matched analytical curves were prepared in the range of 5-90mg/L for samples quantitation.

The correlation ($P < 0.001$) between the results of Total Bacterial Count (TBC) and CMP index was determined using the SAS 9.2 software (SAS Institute, Cary, NC, USA).

Mean TBC was 5,200,000CFU/mL, which is much higher than the regulatory limit of 100,000CFU/mL settled by current Brazilian regulation (Brasil, 2011). Besides, 60.9% of the samples were non-compliant with official

regulations. The chromatographic analysis of milk samples led to typical size-exclusion chromatograms, where the elution of the analyte occurred around 10min (Figure 1). Ten samples (21.7%) were non-compliant with the CMP official regulation (maximum 30mg/L) (Table 1). Mean concentration of CMP was 57.18mg/L. Moreover, all samples were non-compliant to TBC regulatory limit. Milk samples that were non-compliant to the maximum CMP regulatory level of 30mg/L settled by Brazilian regulation (Brasil, 2006) were all positive for p-CMP at LC-MS/MS confirmation. The reliability of the CMP analysis of milk is only possible soon after packaging, and sample freezing is requested if immediate analysis is not possible (Villanoeva *et al.*, 2014).

High TBC in the samples that showed non-compliance with the regulations presented a positive correlation ($P = 0.0067$) with the respective CMP levels, indicating a directly proportional relationship between CMP index and TBC ($r = 0,50061$). Considering all samples of uninspected raw milk analyzed ($n = 46$), no matter their compliance with the official regulations considered in this study, there was a positive correlation ($P < 0.0001$) between TBC and the concentration of p-CMP index. Thus, a high correlation between TBC and the existence of proteolytic bacteria could be concluded.

Values over 30mg/L CMP may indicate either intentional addition of whey or changes in raw milk caused by proteolytic enzymes produced by high loads of contaminant microorganisms during the refrigerated storage (Hantisis-Zacharov & Halpern, 2007; Motta *et al.*, 2014). An important requirement of any assessment of CMP in milk is the capability to differentiate CMP from p-CMP, to prevent falsely adulterated results (Riel & Olieman, 1995). CMP is a phosphorylated hydrophilic peptide, with three genetic variations (Smith *et al.* 2002). Due to the presence of a high degree of glycosylation sites, CMP is also known as glycomacropptide (GMP). Since the multiple reaction monitoring (MRM) was used to monitor products of CMP digestion with pepsin, glycosylation and phosphorylation sites and genetic variations did not interfered the LC-MS/MS analysis, because they remained in non-monitored fragments (Motta *et al.*, 2014).

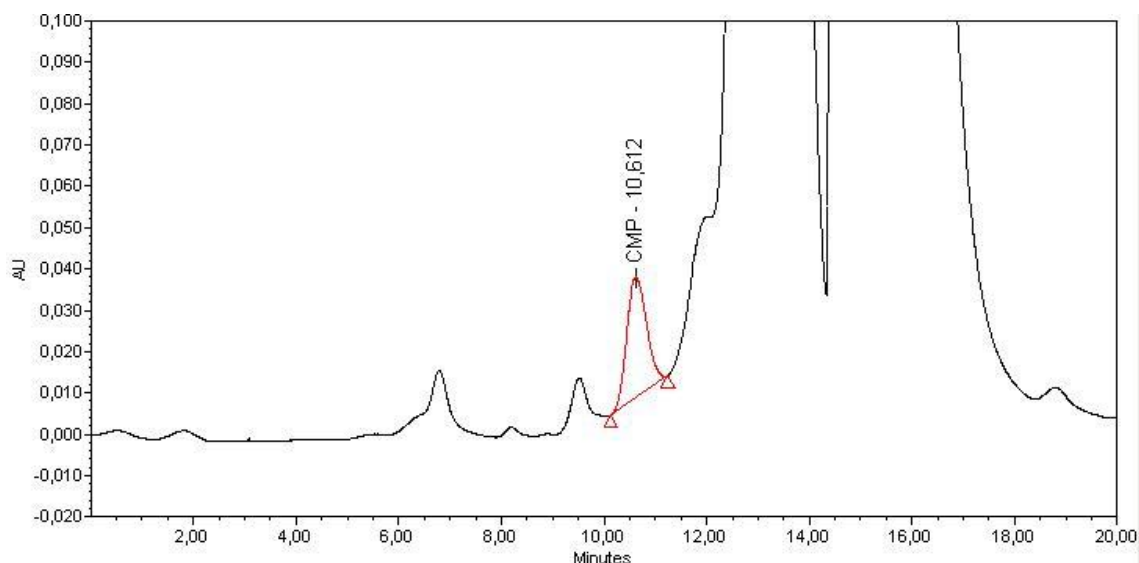


Figure 1. Size-exclusion chromatogram with diode-array detection at 205nm for the analysis of casein macropeptide (CMP) in raw milk.

Table 1. Casein macropeptide (CMP) size-exclusion chromatography (diode array detection at 205nm) results of non-compliant raw milk samples and respective total bacterial counts (n= 10)

Sample number	CMP* (mg/L)	TBC** (CFU/mL)
2	33.35	5,600,000
4	49.16	24,000,000
5	47.93	18,000,000
14	111.50	170,000,000
19	59.49	50,000,000
24	73.98	8,100,000
25	49.81	13,000,000
43	81.77	2,800,000
45	32.58	3,400,000
46	32.22	3,500,000

Brazilian regulatory limits: *CMP < 30mg/L; **TBC (total bacteria count) < 100,000CFU/mL.

The high CMP levels observed in milk were, in fact, of microbial origin. Therefore, TBC continues to be the most practical and fast method for the estimation of milk bacterial load. It can be inferred that the high P-CMP concentrations were due to the action of bacterial enzymes on milk casein, instead of the fraud by addition of whey. High microbial loads are well known to increase the concentration of p-CMP, being related to inadequate hygiene procedures and/or storage at inadequate temperatures (Bremer *et al.*, 2008). Psychrotrophic bacteria, especially *Pseudomonas* sp., produce lipases and proteases. *Pseudomonas fluorescens* is the most frequent psychrotrophic bacteria found in milk,

featuring the cleavage of k-casein in a very similar way to chymosin in the cheese making process (Recio *et al.*, 1996).

Our results also reinforce the need for hygienic practices during milking and milk storage to ensure proper quality. Good manufacturing procedures must not being adequately followed in the trade of raw milk to consumers. In conclusion, CMP quantitation and confirmation of p-CMP did not evidence the occurrence of adulteration of uninspected milk by the addition of whey. Therefore, screening milk for CMP by size-exclusion chromatography alone cannot be considered determinant for the confirmation of

adulteration by the addition of whey. The use of liquid chromatography coupled to mass spectrometry plays an essential role in this case. The p-CMP concentrations found in this study were positively correlated to mesophilic microbial load. Thus, TBC could be a reliable indirect indicator of the presence of proteolytic microorganisms and is both faster and easier to perform than the direct quantitation of proteolytic microorganisms.

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Keywords: chromatography, correlation, mesophilic counts, p-CMP, uninspected milk

RESUMO

A presente comunicação objetivou avaliar a quantificação do caseínomacropéptido (CMP), bem como diferenciá-lo (devido à adulteração com soro) do pseudo-CMP (devido à proteólise bacteriana) em amostras de leite cru coletadas nos domicílios do sul do Brasil. Os resultados reforçam a necessidade de práticas higiênicas durante a ordenha e estocagem do leite. As amostras de leite estudadas não estavam adulteradas por adição de soro, mostrando que a análise por cromatografia de exclusão por tamanho deve ser complementada a fim de revelar a identidade do peptídeo (CMP ou pseudo-CMP). A contagem bacteriana total (TBC) também se mostrou útil como indicador da contaminação do leite por microorganismos proteolíticos, uma vez que uma relação diretamente proporcional entre TBC e pseudo-CMP foi estabelecida.

Palavras-chave: cromatografia, correlação, contagem de mesófilos, p-CMP, leite sem inspeção

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