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Detection of cheese whey and caseinomacropeptide in fermented milk beverages using high performance liquid chromatography

[*Detecção de soro lácteo e caseinomacropeptídeo por cromatografia líquida de alta eficiência em bebidas lácteas fermentadas*]

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ABSTRACT

Cheese whey level and caseinomacropeptide (CMP) index of fermented milk beverages added with four levels of cheese whey (0, 10, 20, and 40%) and stored at 8-10°C for 0, 7, 14 and 21 days were determined by high performance liquid chromatography-gel filtration (HPLC-GF). Additionally, the interference of the starter culture and the storage time on the detection of cheese whey and CMP were investigated. Refrigerated storage up to 21 days did not affect ($P>0.05$) cheese whey and CMP amounts in milk (0% of cheese whey) and in fermented milk beverages added with 10 and 20% of cheese whey ($P>0.05$). However, cheese whey and CMP amounts were higher than expected ($P<0.05$) in fermented milk beverages added with 40% of cheese whey and stored for 21 days.

Keywords: fermented milk beverages, cheese whey, CMP, HPLC

RESUMO

O presente trabalho teve como objetivos quantificar o teor de soro e o índice de caseinomacropeptídeo (CMP) de bebidas lácteas fermentadas preparadas em laboratório, adicionadas de diferentes concentrações de soro (0, 10, 20 e 40%), fermentadas e armazenadas em refrigeração (8-10°C) por tempos distintos (zero, sete, 14 e 21 dias), por cromatografia líquida de alta eficiência-filtração em gel (CLAE-FG), bem como verificar a interferência da cultura utilizada no preparo das bebidas lácteas fermentadas e do tempo de armazenamento na detecção de soro lácteo e CMP. Quando os teores de soro lácteo e os índices de CMP obtidos por CLAE-FG de bebidas lácteas fermentadas foram analisados ao longo do tempo de armazenamento, verificou-se que não houve diferença (p>0,05) para o leite (0% de soro) e as bebidas lácteas com 10 e 20% de soro nos tempos de zero, sete, 14 e 21 dias de armazenamento. No entanto, para a bebida láctea fermentada adicionada de 40% de soro, foi observada diferença para o tempo de armazenamento de 21 dias (p<0,05), em que o teor de soro e o índice de CMP obtidos foram maiores que os demais, que se mostraram equivalentes entre si (p>0,05) para os tempos de zero, sete e 14 dias.

Palavras-chave: bebidas lácteas fermentadas, soro lácteo, CMP, CLAE-FG

INTRODUCTION

Cheese whey is a byproduct of cheesemaking and caseinate processing. It represents 80 to 90% of milk volume, and 50 to 55% of milk solids (Kosikowski, 1979; Furtado and Lourenço Neto, 1994; Camargo *et al.*, 2000). However, some of the milk constituents, such as protein, fat, and calcium are found in lower concentration in cheese whey than in milk. Although the use of cheese whey in some products is allowed in Brazil, it is often misused as an adulterant of pasteurized, UHT, and dry milk (Brasil, 1997).

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In Brazil, milk beverage production is one of the main options for rational use of cheese whey, a product with excellent nutritional value. The most marketed are the fermented milk beverages, with sensorial characteristics similar to yoghurt, and non-fermented ones (Santos *et al*., 2008).

Brazilian standards of identity define fermented milk beverage as the product obtained by mixing milk and cheese whey, with optional addition of other food products, vegetable fat, fermented milk, selected lactic cultures, and other dairy components. Fermentation is an optional step, and may be reached by dairy starter addition and incubation or by mixing to pre fermented milk beverages (Brasil, 2005).

During cheesemaking, proteolysis of κ-casein by chymosin will result in the bond cleavage between amino acids phenylalanine (position 105) and methionine (position 106). The resultant products are the insoluble para-κ-casein (105 amino acids) which will be retained in the cheese and the soluble glycomacropeptide called caseinomacropeptide (CMP) (polypeptide amino acids 106-169) which will be found in the whey (Mollé and Leónil, 1995). Thus, finding CMP in milk is unusual, unless abnormal conditions are met or adulteration of milk is happening. CMP may be quantified by chromatographic methods, particularly gel filtration chromatography (Carvalho *et al*., 2007).

The standard for milk beverage, "Regulamento Técnico de Identidade e Qualidade (RTIQ) de Bebida Láctea" (Brasil, 2005) does not establish the maximum amount of whey to be added in commercial milk beverages. This amount is constrained only by the minimum protein and fat required concentration. Consequently, the amount of whey found in these beverages is largely variable (Almeida *et al*., 2001).

Since one of the issues related to milk beverages is the amount of added whey, a quantification technique would allow more strict processes, mainly when using these beverages as ingredients and for process control.

A Brazilian Standard (Instrução Normativa n° 68/2006, Ministério da Agricultura, Pecuária e Abastecimento) establishes the CMP index quantification by high performance liquid chromatography-gel filtration (HPLC-GF) as the official method to detect cheese whey addition to the milk. However, there is no report describing the use of this analytical method to quantify whey in milk beverages.

Based on the need for a method for cheese whey quantification in milk beverages, the aim of this work was to evaluate the use of HPLC-GF quantification of cheese whey and CMP index in fermented milk beverages, added with different whey concentrations, dairy starters, and stored for up to 21days.

MATERIAL AND METHODS

Raw milk samples (n = 6) were obtained from the university experimental farms, and immediately sent, under refrigerated conditions, to the Laboratory of Microbiology (School of Veterinary Medicine, Universidade Federal de Minas Gerais, Brazil). Fermented milk beverages were then processed by mixing milk, cheese whey and dairy starter. Each milk sample was split into four aliquots, and each aliquot was added of cheese whey (0, 10, 20, and 40%). Final products, fermented beverage milk (10, 20, and 40% of whey addition), and fermented milk (0% of whey added) were stored at $9^oC\pm1^oC$ for 7, 14, and 21 days.

After fermentation, gel was manually agitated during 30 seconds, and 50mL aliquots were aseptically taken and frozen at -18^oC. The remaining was refrigerated at $9^oC\pm1^oC$ for subsequent sampling of 50 mL aliquots after 7, 14, and 21 days of refrigerated storage, which totalized 96 aliquots. Each aliquot was immediately frozen at -18^oC for subsequent chromatographic analysis in the Chromatography Laboratory (School of Veterinary Medicine, Universidade Federal de Minas Gerais, Brazil).

The starter culture was prepared using nonfat dry milk (Molico, Nestlé®, Araçatuba, SP, Brazil) reconstituted to 12% (w/v) in distilled water, followed by sterilization (110^oC/10min), and cooling. Freeze dried lactic culture containing *Streptococcus thermophilus* subsp. *salivarius* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (YF-L811; Christian Hansen, Valinhos, SP, Brazil), was aseptically dissolved, and 25mL aliquots were distributed in sterilized 50mL bottles, and kept frozen (-18^oC) for later use.

Partially demineralized cheese whey (Alibra Ingredientes Ltda, Campinas, SP, Brazil) was reconstituted to 8.3% (w/v) in distilled water, and mixed with milk to a final volume of 200mL, which was pasteurized (85°C/30 min), and cooled to 43°C. 800µL of yoghurt lactic acid culture were then added to each sample, and incubated at 44.5°C for about four hours (final pH of 4.6) (Brasil, 2006).

Chromatographic analysis was done by HPLC-GF, using cheese whey and CMP (89.72%) as standards, added to non-fat dry milk (Molico, Nestlé®, Araçatuba, SP, Brazil) reconstituted to 10% (w/v) in distilled water.

The cheese whey standard curve was obtained using partially demineralized cheese whey (Alibra Ingredientes Ltda, Campinas, SP, Brazil) reconstituted to 10% with distilled water, and added to milk for a final concentration of 0, 5, 10, 20, 40, and 50% of whey. For the CMP standard curve, CMP (89.72%) was dissolved in milk to obtain final concentrations of 0, 15, 30, 45, 60, 75, and 90mg/L.

To prepare both curves, for cheese whey and CMP, 10mL of each standard was added with 5mL of trichloroacetic acid (TCA) 24%, under agitation during two minutes, followed by standing during 60 minutes at room temperature. This material was filtered in qualitative paper, discarding the first drops, and 20uL was injected in the HPLC chromatograph (Shimadzu CLASS VP 6.1) with GF column (Zorbax GF 250 Biosséries, Agilent). Flow was set at 1.5mL/minute, with phosphate buffer (pH 6.0), and UV detection at 205nm. The amount of whey and CMP concentration in milk were plotted against detector signal intensity. The regression equation was considered satisfactory when R^2 was higher than 0.95 (Brasil, 2006).

For sample analysis the same procedure was used for preparation of standard curve based on CMP or whey concentration (Brasil, 2006).

Sample chromatograms were compared to the calibration curves, with identification of the peak with similar retention time. Results of whey percentage and CMP concentration of the samples were calculated through interpolation of the signal results with the regression curves obtained with whey and CMP, using the regression equation:

$y = ax + b$

where x = concentration (Whey % or mg/L of CMP), y = peak area, a = slope, b = y-intersection

The final formula is:

$$\text{Whey (\%) or CMP (mg/L)} = \frac{\text{peak area - b}}{\text{a}}$$

Statistical analysis was based on randomized block design, considering milk samples (n = 6), whey concentrations (0, 10, 20, and 40%) and storage times (0, 7, 14, and 21 days) as variables. Treatment averages were compared using the Student-Newman-Keuls test (p = 0.05) (Sampaio, 2002). SISVAR 5.0 (Universidade Federal de Lavras, Lavras, MG, Brazil) software was used for statistical analysis.

RESULTS AND DISCUSSION

The average values of whey concentration and CMP index in milk and fermented milk beverages, according to the cheese whey addition and days of storage at 9°C±1°C are presented in Table 1 and 2, respectively. Average values were different (p<0.05) for different concentrations considering the same storage time.

Table 1. Average results of whey (%) for milk (0% of whey addition) and fermented milk beverages (FMB) (with 10, 20, and 40% of whey addition) during storage for 21 days, obtained by HPLC-GF

Product	Storage time (days)			
	0	7	14	21
Milk (0% whey)	1.66Ad	1.62Ad	1.61Ad	1.63Ad
FMB (10% whey)	12.52Ac	12.40Ac	13.02Ac	13.20Ac
FMB (20% whey)	25.46Ab	24.53Ab	25.27Ab	25.49Ab
FMB (40% whey)	52.09Ba	51.83Ba	52.95Ba	55.63Aa

Different lower case letters in the same column indicate different values (SNK test; P<0.05)
Different capital letters in the same line indicate different values (SNK test; P<0.05)

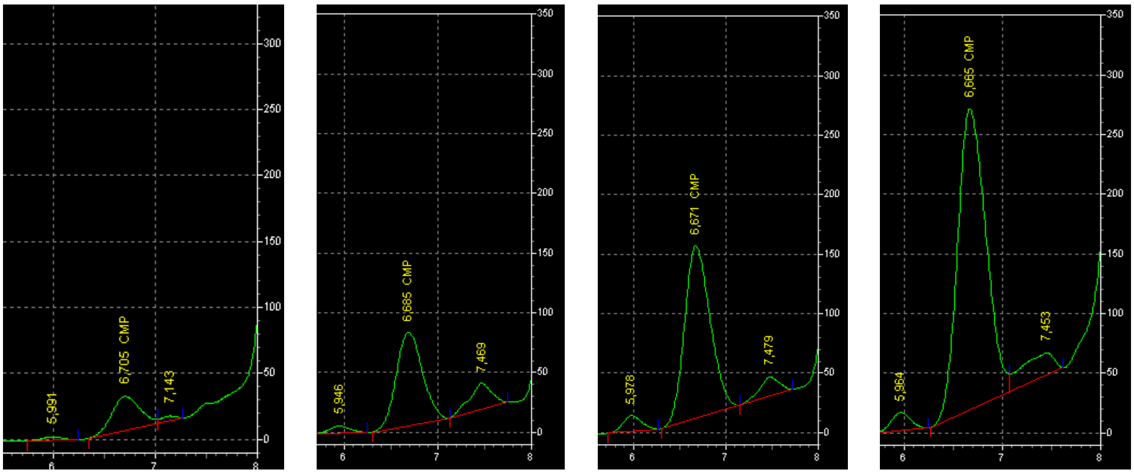
There was no difference for whey concentration as determined by HPLC-GF for milk and fermented milk beverages added with 10 and 20% of cheese whey during the storage period. However, for samples added with 40% of cheese whey, there was an increase in whey concentration in the 21st day of storage ($p<0.05$), but not for day 7 or 14.

Average CMP indexes repeated the same trend of whey concentration in the samples (Table 2). Figure 1 shows the chromatogram profiles obtained for milk (0% of whey) and for fermented milk beverages (with 10, 20, and 40% of cheese whey added).

Table 2. Average results of CMP index (mg/L) for milk (0% of whey addition) and fermented milk beverages (FMB) (with 10, 20, and 40% of whey addition) during storage for 21 days, obtained by HPLC-GF

Product	Storage time (days)			
	0	7	14	21
Milk (0% whey)	26.83Ad	26.30Ad	25.83Ad	24.91Ad
FMB (10% whey)	194.85Ac	190.11Ac	202.62Ac	205.25Ac
FMB (20% whey)	393.54Ab	379.74Ab	387.16Ab	390.92Ab
FMB (40% whey)	801.48Ba	798.98Ba	810.73Ba	850.53Aa

Different lower case letters in the same column indicate different values (SNK test; P<0.05). Different capital letters in the same line indicate different values (SNK test; P<0.05).



(a) **(b)** **(c)** **(d)**

Figure 1. Chromatograms of milk (a), and fermented milk beverages added with whey at levels of 10% (b), 20% (c), and 40% (d), showing caseinomacropeptide peak, obtained by HPLC-GF.

There was a high correlation of CMP index and whey concentration detected by HPLC-GF, with a correlation coefficient of 99.5% ($p<0.0001$). This confirms a strong association between these variables. The following linear regression model was calculated for association between CMP index and whey concentration levels detected by HPLC-GF, based on obtained data:

$Y = 15.33408X + 1.39933$, where:

$R^2= 99.05\%$

Y= CMP index (mg/L)

X= whey level detected (%)

Figure 2 shows the CMP index of milk and fermented milk beverages according to the amount of cheese whey added.

These results indicate that CMP index determination by HPLC-GF is a feasible technique to be used in fermented milk beverages, since there was no effect on the HPLC chromatograms. However, for products added with 40% of whey and stored for longer periods, results may deviate from normal. This method should be indicated for fermented milk beverages composed of milk, cheese whey, and yoghurt starters, for up to 14 days of storage.

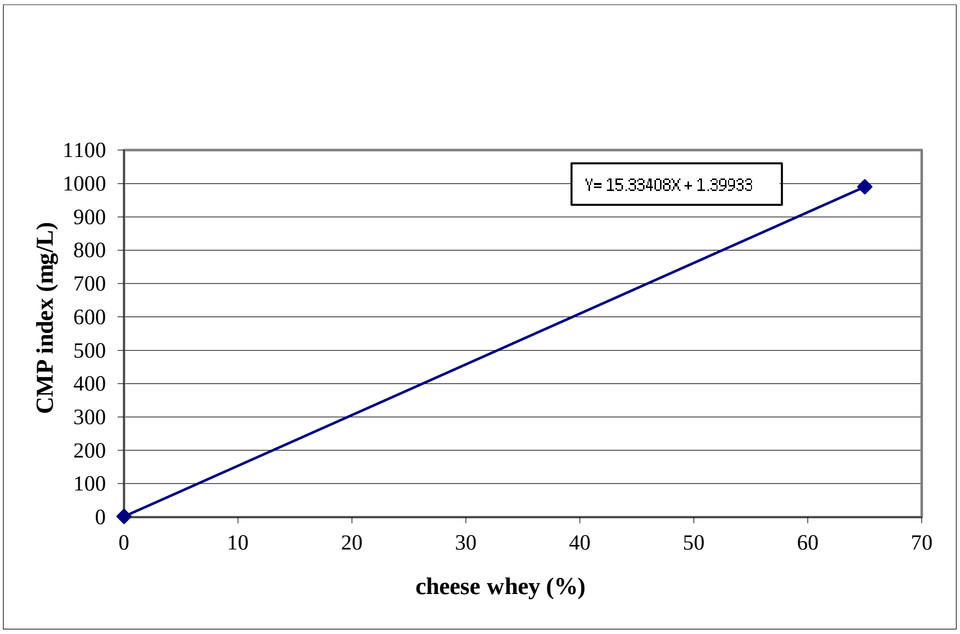


Figure 2. CMP index (mg/L) of milk and fermented milk beverages according to the amount of cheese whey added (%).

The levels of CMP index show that the starter bacteria culture used for fermentation, composed of *Streptococcus thermophilus* subsp. *salivarius* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, did not hydrolyze the κ-casein bond between amino acids 105 and 106, so there was no increase in CMP index at the end of the fermentation process. The increase of CMP index for the fermented milk beverage added with 40% of cheese whey, and stored during 21 days might be due to nonspecific proteolysis of the κ-casein, due to the microorganism components of the starter culture, mainly *Lactobacillus* sp, which is proteolytic, and obtain amino acids by casein breakdown (Martins and Luchese, 1988) with consequent increase in CMP levels. Although these microbial enzymes do not cleavage specific bonds, the resulting polypeptides may have the same size as the CMP, or a similar size, when hydrolysis is in the bond between metionine (position 106) and alanine (position 107). This last polypeptide is called pseudo-CMP, and it is not differentiated from CMP by using only HPLC (Magalhães, 2008).

Olieman and van Riel (1989) observed that fermentation of non-fat milk with different lactic cultures used in butter making, in distinct storage conditions, did not lead to CMP formation detectable by HPLC reverse phase. These results show that starter cultures used for butter making do not contain proteolytic enzymes with similar specificity to chemosin.

Similar findings were reported by Alvim (1992), who inoculated *Streptococcus thermophilus* subsp. *salivarius* and *Lactobacillus delbrueckii* subsp. *bulgaricus* in sterilized non-fat milk. After fermentation these samples were stored at 5^{o}C for 10 and 15 days. No increase in CMP index, obtained by HPLC, was found. The same result was observed in milk inoculated with *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*.

CONCLUSIONS

CMP index determination is a feasible technique for use in fermented milk beverages, added with up to 40% of cheese whey, and stored during 14 days under refrigeration.

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Capes/PROEX and FAPEMIG

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