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Detection of cheese whey in raw milk preserved with bronopol® through high performance liquid chromatography

[*Detecção de soro lácteo por cromatografia líquida de alta eficiência em amostras de leite cru conservadas por bronopol®*]

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ABSTRACT

High performance liquid chromatography was used in order to detect cheese whey in samples of raw milk preserved with Bronopol®. Six samples were collected and divided in 45 aliquots of 40mL. From these, 15 were used as control and stored frozen, 15 were added with Bronopol® and stored at 7ºC, and the other 15 were added with Bronopol® and stored at 30ºC. In all groups, five levels of cheese whey addition (0, 2, 5, 10, and 20%) were tested. The samples were submitted to high performance liquid chromatography on the 2nd, 4th, and 8th days of storage. A completely random design was used, following the factorial scheme (5x3x3) and the results were compared through the non-parametric Kruskal-Wallis test. There was no difference among the treatments (P>0.05), which allows the conclusion that raw milk preserved with Bronopol® may be used for the determination of cheese whey addition in milk through high performance liquid chromatography.

Keywords: raw milk, Bronopol, cheese whey detection, HPLC

RESUMO

O objetivo deste trabalho foi avaliar a viabilidade do uso de amostras de leite cru conservadas com Bronopol® na pesquisa de soro de queijo por cromatografia líquida de alta eficiência (CLAE). Seis amostras foram coletadas e subdivididas em 45 alíquotas de 40mL. Destas, 15 compuseram o grupo controle e foram armazenadas sob congelamento, 15 amostras foram adicionadas de Bronopol® e armazenadas a 7ºC e outras 15 foram adicionadas de bronopol e estocadas a 30º C. Em todos os grupos, cinco porcentagens de soro de queijo foram adicionados, 0, 2, 5, 10 e 20%. As amostras foram submetidas à cromatografia líquida de alta eficiência no segundo, quarto e oitavo dias de armazenamento. Foi utilizado o delineamento inteiramente ao acaso, com os tratamentos em esquema fatorial 5x3x3 e os resultados comparados por meio do teste não paramétrico de Kruskal Wallis. Não houve diferença (P>0,05) entre os tratamentos, concluindo-se que é possível utilizar amostras de leite cru conservadas com Bronopol® para pesquisa de soro de queijo em leite por CLAE.

Palavras-chave: leite cru, bronopol, detecção soro de queijo, CLAE

INTRODUCTION

The adding of cheese whey to pasteurized, ultra high temperature (UHT), sterilized or powder milk is considered worldwide as a fraud and must be detected in order to avoid decrease in yielding at dairy plants and nutritional drawbacks to consumers.

A milk-clotting agent acts on κ-casein during cheese making, specifically on the binding between amino acids 105-106 (Phe-Met),

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releasing an hydrophilic macro peptide (from amino acids 106 to 169) bound to N-acetylneuraminic acid (NANA), that may be found in cheese whey. The other κ-casein peptide fraction (from amino acids 1 to 105) and the other fractions of casein form the para-κ-caseinate under the influence of calcium ions.

The polypeptide released during the primary phase of clotting is called caseinmacropeptide (CMP). Its amino acid profile coupled with carbohydrate content make it highly polar. Consequently, it turns soluble CMP into cheese whey after curd separation (López-Fandino and Ramos, 1992).

CMP molecular weight is approximately 8,000D and may be separated from the other peptides and proteins through gel filtration HPLC. Contrarily, the para-κ-caseinate residuum exhibits hydrophobic nature (Alvim, 1992).

CMP determination is an excellent index of fraud since it is not expected to be found in milk but in cheese whey. Analytical methods, such as high performance liquid chromatography (HPLC), are important tools to search for this adulteration of milk.

The Regulatory Instruction (RI) nº68, issued by the Brazilian Ministry of Agriculture (Brasil…, 2006a), established the determination of CMP index through HPLC and the RI nº69 (Brasil…, 2006b) stated thresholds for this index and the industrial processing of the samples according to the category in which they are fitted.

Bronopol (2-brome-2-nitropropane-1,3-propanediol) is highly used as an antimicrobial in cosmetics, medicines for external use, shampoos, and other hygiene products (Legin, 1996; Wang *et al*., 2002). In Brazil, Bronopol® has been regularly used as a preservative for milk samples directed to analyses regarding composition and somatic cell count (SCC) (Fonseca *et al*., 2004; Leite *et al*., 2004; Cassoli, 2005; Fonseca *et al*., 2005.; Leite *et al*., 2005; Souza *et al*., 2005; Leite, 2006).

Wang *et al*. (2002) proposed a method of separation by reverse phase HPLC for Bronopol® determination and its degradation products and observed that the best chromatographic profile was obtained when the sample had been dissolved in methanol. In the same study, column C18 with mobile phase with methane/water/orthophosphoric acid and wavelength of 210nm was used. Hence, it is expected that Bronopol® does not interfere in the determination of cheese whey addition in milk through gel filtration HPLC with phosphate buffer mobile phase and detection at wavelength of 205nm (Brasil…, 2006a).

The aims of the present study were to evaluate the use of raw milk samples preserved with Bronopol® for the determination of cheese whey addition in milk through HPLC, substituting the need of sample freezing and to verify the interferences of storage time and temperature on the samples.

MATERIAL AND METHODS

Six samples of raw milk were collected in six farms from Pedro Leopoldo municipality, Minas Gerais State. They were preserved under refrigeration and sent to the LabUFMG where they were analyzed. Each sample, corresponding to 2L of milk, was divided in 45 aliquots of 40mL. From these, 15 were used as control and stored frozen, 15 were added with Bronopol® pills and stored at 7ºC, and the other 15 were added with Bronopol® pills and stored at 30ºC. The pills were made of bronopol 8mg and natamycin 0.3mg (D&F Control Systems Inc.). In all groups, five levels of cheese whey addition (0, 2, 5, 10, and 20%) were tested. The samples were submitted to HPLC on the 2nd, 4th, and 8th days of storage.

One milliliter of liquid clotting agent (clotting ratio 1:3,000) was added in 500mL of raw milk, origin and quality controlled, and kept in a water-bath at 32ºC. After 60min, the curd was cut and shaken. The cheese whey was drained, collected in 500mL Erlenmeyer and heated until boiling in order to denature its enzyme (Brasil…, 2003).

The calibration curve of the HPLC device was prepared weighting 20g of free-cheese whey milk in a 50mL Becker, representing the blank sample, and aliquots of 19.6, 19, 18, and 16g of the same milk in another 50ml becker plus 0.4, 1, 2, and 4g of cheese whey to each one, respectively. Then the 0, 2, 5, 10, and 20% patterns of cheese whey addition were achieved.

After weighting milk and cheese whey, the patterns were precipitated by adding 10mL of 24% tri-chloride acetic acid under constant shaking, in a 2min interval. The mixture was kept stable for 60min at room temperature and then filtered through qualitative paper (Brasil…, 2003). From each filtered material, 20μL were injected in the chromatograph (Shimadzu CLASS VP 6.1) with a column (Zorbax GF Bioseries from Agilent) in which the separation principle is based on the molecular exclusion, at a 1.5mL per min mobile phase flux (isocratic pumping of phosphate buffer pH 6.0) and detection by UV visible at 205nm wavelength. The graph of cheese whey percent versus intensity of detector signal was plotted and the regression line was calculated accepting values of $R \geq 0.95$. This procedure was repeated weekly using milk of known origin and quality (collected at the Fazenda Modelo – UFMG).

The same procedures were used for the precipitation and injection of the samples (Brasil…, 2003). The chromatograms were compared to that of the calibration curve and the peak with similar retaining time was identified. The cheese whey percent in the sample was figured out through the interposing of signals on the regression line of milk added with cheese whey, using the following equation obtained by the injection of calibration curve patterns: $y = ax + b$.

The study was a completely random design, and the treatments followed the factorial scheme 5x3x3 (Sampaio, 2002). The results were compared by the non-parametric Kruskal-Wallis test using InfoStat software (*Universidad de Córdoba – Argentina*).

RESULTS AND DISCUSSION

The mean values of each treatment according to the percent of cheese whey added are shown in Table 1. According to the data, there was no difference ($P>0.05$) among treatments. Samples added with Bronopol® and stored at 7 or 30ºC for up to eight days may be analyzed by HPLC in order to determine cheese whey in milk without altering the results when compared to the Brazilian Ministry of Agriculture recommendations for sending the samples frozen and without preservatives.

Representative chromatograms in Fig. 1 (a) (b) of samples added with bronopol and five levels of whey cheese demonstrate the efficiency of the preservative. None of the temperatures and times adopted in the storage showed interfering signals at the retention time and areas at the chromatograms. The obtained data also demonstrated neither microbial growth nor proteolytic enzyme production, which would increase the values and produce false-positive results.

The use of Bronopol® has been studied as a preservative for milk samples sent to analyses of milk composition and somatic cell count and its action has been recorded at different temperatures and times of storage (Cassoli, 2005; Sanchez *et al*. 2005; Souza *et al*., 2005; Leite, 2006).

The chromatographic profile of Bronopol® may be detected in phase reverse column in samples dissolved in methanol and using the mobile phase composed by methanol/water/ orthophosphoric acid. In the present study, it was not detected even using molecular exclusion column, tri-chloride acetic acid for preparation of the samples, and mobile phase with phosphate buffer solution pH 6.0.

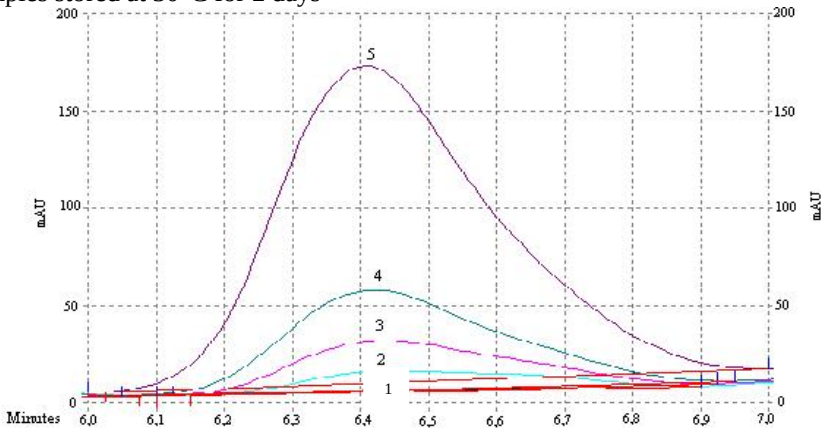
The alternative storage of milk samples added with Bronopol®, under room temperature or cooling for up to eight days may be considered very important, especially considering situations in which it would not be possible to analyze the samples immediately after their arrival at the lab. These cases have been presented logistic difficulties either for dairy plants or the labs. Milk samples may then be stored under a wider temperature range and for longer times feasible to the collection needs, transported to the laboratory and undergo analysis procedures without compromising the results.

Table 1. Mean concentration of cheese whey through HPLC in milk samples added with five levels of cheese whey regarding the addition of preservatives and temperature and time of storage.

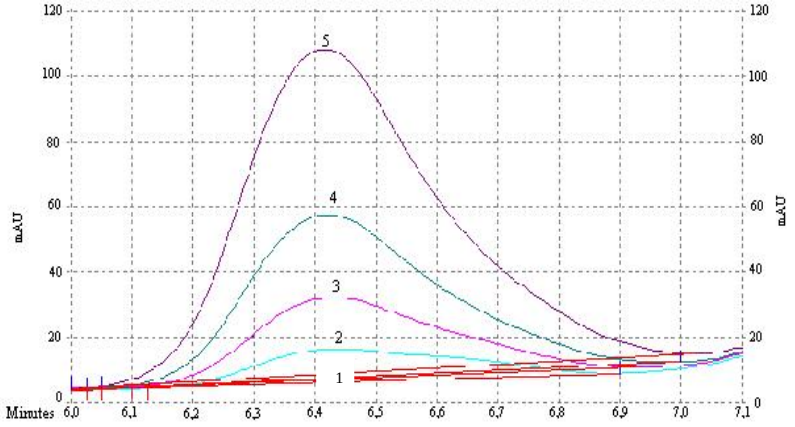
Treatment	% of cheese whey added				
	0	2	5	10	20
Control, freezing for 2 days	-0.13A	1.58A	4.74A	9.85A	20.02A
Control, freezing for 4 days	-0.08A	1.48A	4.49A	9.64A	19.45A
Control, freezing for 8 days	-0.18A	1.46A	4.48A	9.42A	19.60A
Bronopol®, 7º C for 2 days	-0.10A	1.62A	4.93A	10.07A	20.60A
Bronopol®, 7º C for 4 days	-0.11A	1.76A	4.82A	10.17A	20.91A
Bronopol®, 7º C for 8 days	0.05A	1.78A	5.04A	10.34A	21.30A
Bronopol®, 30º C for 2 days	0.24A	2.06A	5.09A	10.47A	23.26A
Bronopol®, 30º C for 4 days	0.80A	2.26A	5.32A	10.39A	23.25A
Bronopol®, 30º C for 8 days	1.67A	2.98A	5.95A	11.18A	24.16A

[A] Averages followed by same letter do not differ by Kruskal Wallis test (P > 0.05).

(a) Samples stored at 30°C for 2 days



(b) Samples stored at 30°C for 8 days



1. sample without cheese whey
2. sample with 2% of cheese whey
3. sample with 5% of cheese whey
4. sample with 10% of cheese whey
5. sample with 20% of cheese whey

Figure 1. Chromatograms of samples added with bronopol and five levels of cheese whey, stored at 30°C for 2 (a) and 8 (b) days.

Fukuda (2003) studied the effects of freezing on raw milk and storage on UHT milk and observed different CMP values between one-day frozen raw milk and not frozen samples. The tendency was for CMP values to decrease during storage while frozen. For UHT milk, a soft and continuous decrease tendency was observed.

These results are alarming, since most analyzed samples in Brazil are stored frozen for long periods, which may reach months until the onset of the analyses. In those situations, underestimated cheese whey values in adulterated samples may be detected. Hence, the results of the present study represent a gain for the legislation, the labs, and the dairy plants since they allow the use of milk samples preserved with Bronopol®, replacing the freezing for researches of cheese whey addition on fluid milk.

CONCLUSIONS

Samples of raw milk added with Bronopol® may be stored for up to eight days at 7 or 30ºC without altering the detection of cheese whey in milk through HPLC.

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