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Detection and quantification of bovine, ovine and caprine milk percentages in protected denomination of origin cheeses by reversed-phase high-performance liquid chromatography of beta-lactoglobulins

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Abstract

A method for detecting and quantifying bovine, ovine and caprine milk mixtures in milk and cheeses by means of reversed-phase high-performance liquid chromatography (RP-HPLC) of β-lactoglobulins is described. Gradient elution was carried out with a flow rate of 0.5 ml/min and a temperature of 45 °C, using a mixture of two solvents: solvent A (0.1% TFA in water) and solvent B (0.09% TFA in 80% aqueous acetonitrile, v/v). The effluent was monitored at 215 nm. Under the conditions used different chromatographic patterns were obtained for bovine, ovine and caprine whey proteins. Each milk type presented different retention times for β-lactoglobulin peaks. Binary mixtures of bovine and ovine or bovine and caprine raw milks containing 1, 2, 5, 10, 20, 30, 50, 75 and 95% (v/v) of bovine milk, as well as ovine and caprine milk mixtures containing 1, 2, 5, 10, 20, 30, 50, 75 and 95% (v/v) of ovine milk were used for cheese making. Cheeses were prepared and ripened, according to traditional methods. Milk mixtures, fresh and ripened cheeses were analyzed. A linear relationship was established between log 10 of β-lactoglobulin peaks ratio (calculated as peak area values ratio) and log 10 of the relative percentage of bovine or ovine milk. The ratio between β-lactoglobulin peaks was not affected by the degree of ripening. Thus, enabling the quantification of milk type percentage, with a detection limit of 2%. This technique allowed quantification of milk species within the concentration range of 5–95%. The method was successfully applied for authenticity evaluation and quantitative determination of ovine and caprine milk percentages of commercial protected denomination of origin (PDO) cheeses.

Keywords: Cheese; Milk; Food analysis; β-Lactoglobulin; Proteins

1. Introduction

The substitution of bovine milk for ovine and caprine milk is a fraudulent practice in the dairy industry. The seasonal oscillations and the much lower milk yield of ewes and goats, together with the much

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lower price of bovine milk are the main reasons for this adulteration. As a consequence adequate control methods are required to verify milk and cheese authenticity, namely for protected denomination of origin (PDO) cheeses, which are object of special legislation from European Union [1].

A reference method for the detection of bovine milk and caseinate in cheese made from milks of other species based on isoelectric focusing (IEF) of γ-caseins have been published by EU [2]. Using two reference standards of milk (with 0 and 1% cow's milk, respectively) a sample is judged as being positive if both bovine γ_2-casein and γ_3-casein are equal to or great than the level in the 1% standard (ewe, goat). Additionally, several new methods, either, immunological [3–7], electrophoretical [8,9,17], chromatographic [10–14] or DNA-based [15,16], have been published. However, some of these methods for species identification may fail after excessive proteolysis.

In addition, some PDO cheeses are manufactured from defined amounts of each type of milk. Thus, authenticity issues in cheese analyses are challenged not only by milk species identification (e.g. the qualitative detection of bovine milk) but also by the need for quantitative determination of bovine, ovine and caprine milk in mixed cheeses. A method based on IEF and cation-exchange high-performance liquid chromatography (HPLC) of *para*-k-casein [14] has been proposed for quantitative analyses, however, as the estimated percentage of bovine milk in mixed cheese is strongly affected by the casein content of milks used for cheese making the results were approximate. Electrophoretic analysis of whey proteins was used to determine the presence and amount of bovine milk in ovine milk cheeses [17]. Various combinations of types of whey proteins were compared; using the ratio of bovine β-lactoglobulin A and B to bovine serum albumin was most accurate.

Methods for milk species quantification based on the whey protein fraction suffer from a shortcoming as that this fraction is more sensitive to heating than is the casein fraction, such methods can cause false negatives when sterilized milk has been used in the cheese manufacture [17,18]. However, when applied to PDO cheeses, which are made in farms, using raw milk and traditional methods, the analyses of whey proteins afford an advantage in that these proteins are less susceptible to proteolysis than caseins.

The objective of the present study was the separation and identification of homologous whey proteins in binary milk mixtures and determination of bovine, ovine and caprine milk percentages in ripened raw milk cheeses by HPLC analyses of β-lactoglobulins.

2. Experimental

2.1. Sampling

Bovine, ovine and caprine raw milks (from Frísia, Churra and Serrana's breeds, respectively) were obtained directly from the producers in North Portugal.

Standard binary mixtures of bovine and ovine or bovine and caprine milks containing 1, 2, 5, 10, 20, 30, 50, 75 and 95% (v/v) of bovine milk, as well as ovine and caprine milk mixtures containing 1, 2, 5, 10, 20, 30, 50, 75 and 95% (v/v) of ovine milk were used for cheese making. Cheeses were prepared and ripened, according to Terrincho cheese traditional manufacture [19]. Cheese samples for HPLC analysis were taken immediately after preparation and after 4 weeks of ripening. The respective milk standard mixtures were also analyzed.

PDO commercial cheeses analyzed included: two "Serra da Estrela" cheeses made from milk of Bordaleira ewe's race, five "Terrincho" cheeses made from milk of Churra ewe's race, two "Transmontano" cheeses made from Serrana goat's race, three caprine/ovine "Picante da Beira Baixa" cheeses. All these cheeses were made from raw milk, their ripening time ranged between 45 and 60 days, and were sealed by the respective PDO entities.

2.2. Sample preparation

The whey protein fractions were obtained from the cheese samples (5 g) by extraction with water (15 ml), in a sonicator prior to centrifugation (2000 g, 10 min) or from 15 ml of skimmed milk. The supernatant was precipitated by addition of hydrochloric acid 1 M until pH 4.6. After centrifugation (2000 g, 10 min) the resulting supernatant was filtered, before HPLC analysis. All the samples were stored at 4 °C.

2.3. Reagents and proteins standards

All reagents used were of analytical grade purity. Solvents for HPLC were filtered through a 0.22 μm NL 17 filters and degassed under vacuum for at least 15 min before use. Purified bovine standards of β-lactoglobulin (β-lg) and α-lactalbumin (α-la) were supplied by Sigma Chemical Co. Both β-lg and α-la standards were dissolved in a mixture of 70% of water and 30% of acetonitrile (v/v).

2.4. HPLC separation

2.4.1. Instrumentation

The chromatographic analysis was carried out in an analytical HPLC unit (Jasco) equipped with two types of PU-980 pumps, one type is UV-970 detector and another type is 7125 Rheodyne Injector with a 20 μl loop. A Borwin PDA Controller Software (JMBS Developements) was also used. Peak integration was made on horizontal mode (a common baseline and drop perpendiculars).

The column was a reversed-phase column *Chrompack* P 300 RP that contains a polystyrene–divinylbenzene copolymer-based packing (8 μm, 300 Å, 150 × 4.6 i.d.). A *Chrompack* P RP (24 mm × 4.6 mm i.d.) was used as a pre-column.

2.4.2. Separation conditions

Gradient elution was carried out with a mixture of two solvents. Solvent A consisted of 0.1% trifluoroacetic acid (TFA) in water and solvent B consisted of 0.1% TFA in 80% aqueous acetonitrile (v/v). Proteins were eluted with a series of linear gradients increasing the proportion of solvent B, from 36 to 56% of B over 20 min, 56 to 60% of B over 10 min, finishing with 60 to 36% of B in 5 and 3 min for column re-equilibration. The flow rate was 0.5 ml/min, the column temperature was 45 ± 0.1 °C and the detection was made at a wavelength of 215 nm.

3. Results and discussion

3.1. Separation of β-lg and α-la of bovine, ovine and caprine milks

The chromatographic system was calibrated by the external standard method with solutions that contained bovine β-lg in the range of 0.02–2.5 mg/ml and bovine α-la in the range of 0.01–1.5 mg/ml. A linear relationship between the concentrations of bovine β-lg and α-la and the UV absorbance at 214 nm was obtained.

The HPLC conditions were optimized for mobile phase composition, gradient, operating temperature and flow rate in order to obtain the best separation of bovine, ovine and caprine milk whey proteins. Under the conditions adopted the β-lg and α-la fractions of bovine, ovine and caprine raw milks were well separated and different chromatographic patterns were obtained as shown in Fig. 1. All samples of ovine milk analyzed presented β-lg A and B genetic variants. Caprine milk presented only β-lg B, this was excepted since no genetic variants for caprine β-lg are described. Bovine milk samples obtained from different producers of North of Portugal were analyzed, these samples contained only β-lg B, the prominent variant of occidental cattle [20]. However, if bovine milk samples contained also β-lg A, as described by other authors [10,11], this peak would separated from others, presenting higher retention time than β-lg B. That was verified by the addition of standard bovine β-lactoglobulin containing A and B variants to bovine milk and is in good agreement with results obtained by other authors [10].

The precision of the method was measured using bovine, ovine and caprine cheeses, by analyzing eight injections of each sample. Values of relative standard deviation (R.S.D.) for measurements of retention time and peak area are given in Table 1. The reproducibility of retention times and peak areas between days is

Table 1
Repeatability and reproducibility of the method expressed as the relative standard deviation (R.S.D.)

Ripened cheese sample	Assay	n[a]	β-lg B	
			Time	Area
Bovine	Same day	8	1.4	3.1
	Between days[b]		3.6	4.9
Ovine	Same day	8	2.3	4.1
	Between days[b]		4.6	5.9
Caprine	Same day	8	1.5	4.2
	Between days[b]		3.2	6.7

[a] Number of consecutive runs on the same day.
[b] Ten analyses performed on the same sample on two different days.

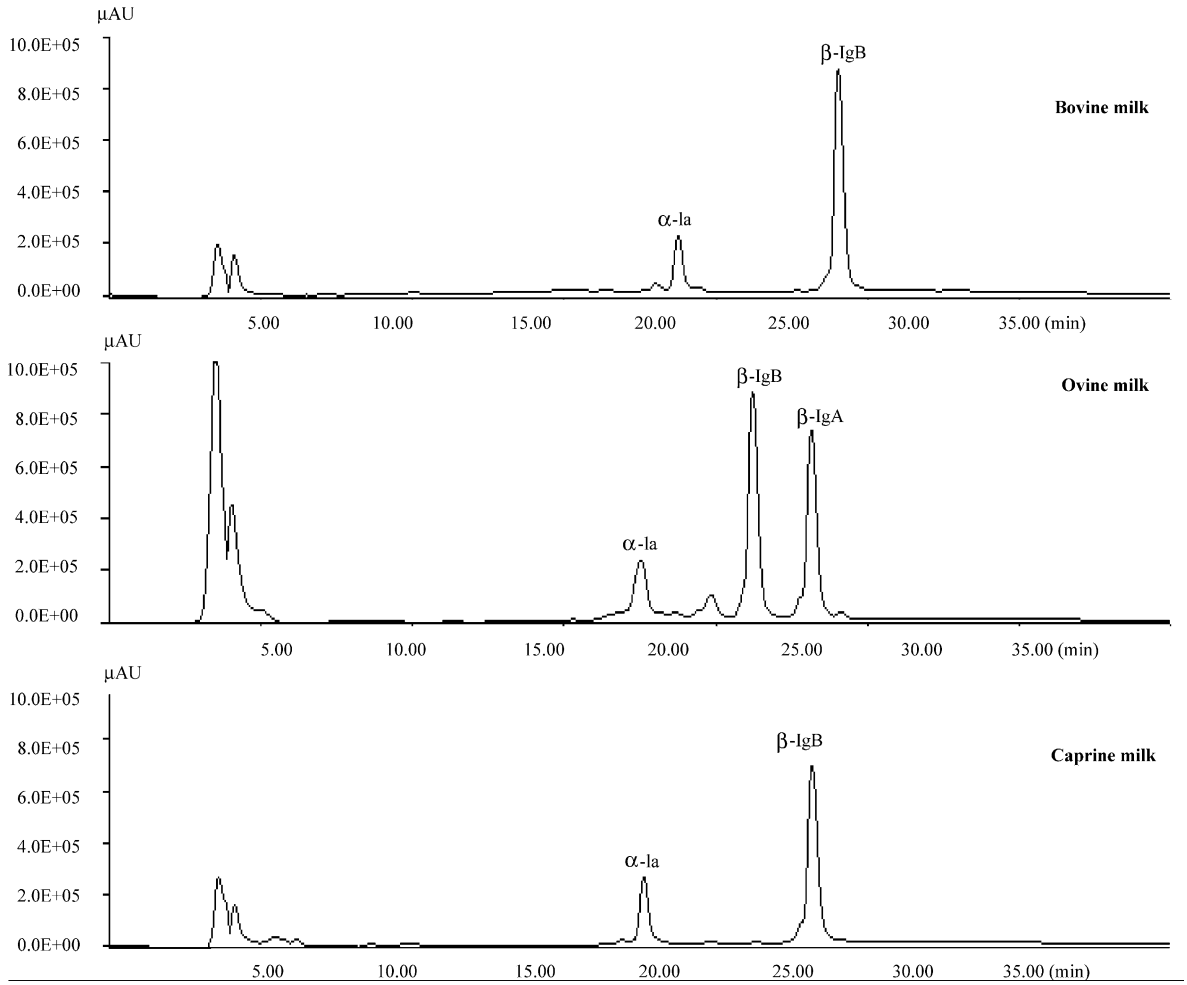

Fig. 1. Chromatographic profiles of β-lg and α-la proteins extracted from bovine, ovine and caprine raw milks.

acceptable (R.S.D. <7–8%). On the other hand, the within-day precision of the method seems to be good (R.S.D. <4.2%).

3.2. Determination of bovine, ovine and caprine milk mixtures in cheeses

The analysis of β-lg in binary mixtures containing defined amounts of bovine, ovine or caprine raw milks, and in the respective cheeses, fresh and 4 weeks ripened, provided interesting results. As expected different chromatographic profiles were obtained for each type of milk binary mixtures as shown in Figs. 2–4. Similar chromatographic profiles were obtained for each milk mixture and the respective fresh and ripened cheeses.

The effect of proteolysis was observed in ripened cheeses, however the products of proteolysis did not interfere with β-lg peaks. In addition, the ratio between β-lactoglobulin peaks of each binary milk mixture was not affected by the degree of ripening, enabling the quantification of different milk type percentages (Tables 2–4).

Table 2 depicts the relationship between log 10 (ovine β-lg A peak area/bovine β-lg B peak area) ratio and log 10 of bovine milk percentage. There were no significant differences ($P > 0.05$) between the regression lines of milk mixtures, and respective fresh

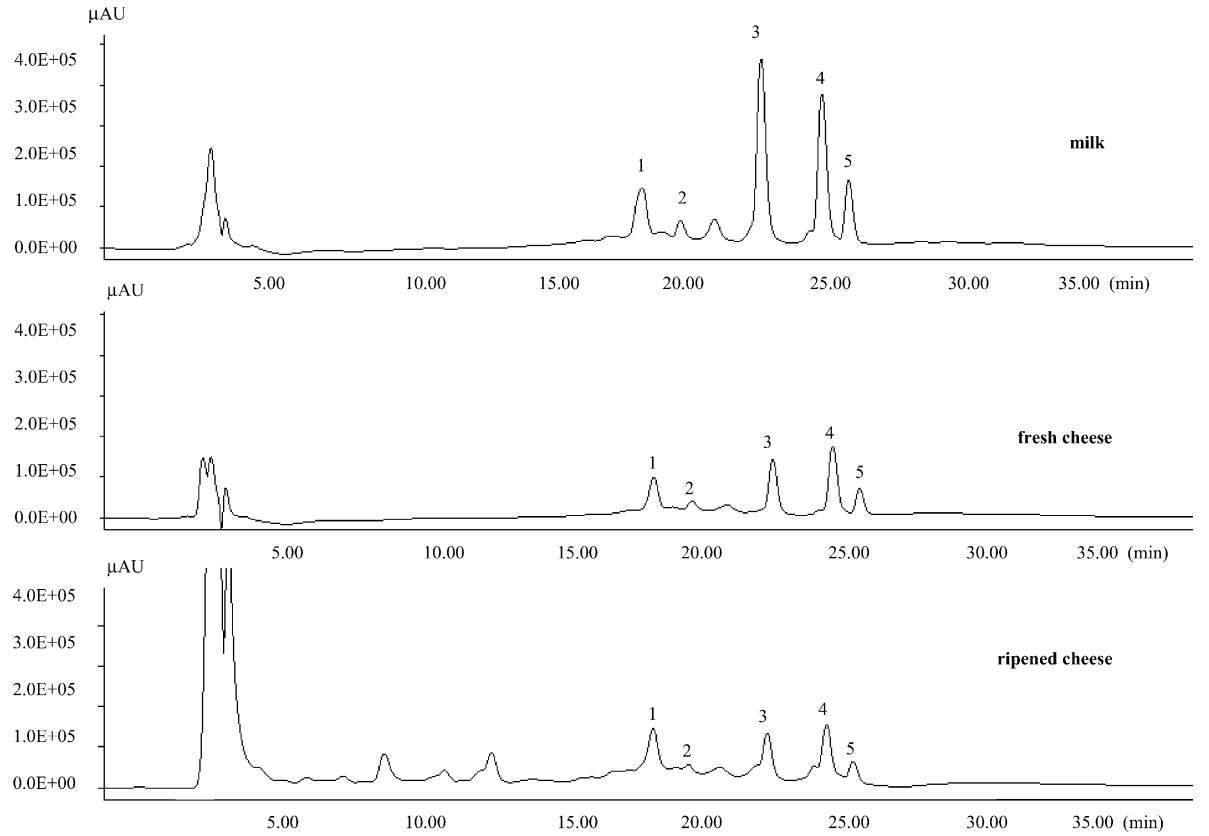

Fig. 2. Chromatographic profiles obtained for a mixture of 20% of bovine milk and 80% of ovine milk and respective fresh and ripened cheeses. (1) ovine α-la, (2) bovine α-la, (3) ovine β-lg B, (4) ovine β-lg A, (5) bovine β-lg B.

Table 2
Linear regressions between log 10 of ovine β-lg A peak area/bovine β-lg B peak area ratio and log 10 of bovine milk percentage (calculated as peak areas)

	Equation	r^2 value
Milk mixtures	$y = -1.283x + 2.072$	0.9985
Fresh cheeses	$y = -1.253x + 2.035$	0.9971
Ripened cheeses	$y = -1.255x + 2.042$	0.9975

Table 3
Linear regressions between log 10 of caprine β-lg B peak area/bovine β-lg B peak area ratio and log 10 of bovine milk percentage (calculated as peak areas)

	Equation	r^2 value
Milk mixtures	$y = -1.318x + 2.057$	0.9992
Fresh cheeses	$y = -1.290x + 2.043$	0.9987
Ripened cheeses	$y = -1.320x + 2.042$	0.9993

and ripened cheeses. Similar results were reported by Addeo et al. [9] for determination of β-lg by IEF in Peccorino cheese. HPLC method allowed detection of 2% of bovine milk in ovine cheese. Identification of bovine β-lactoglobulin in ripened cheeses prepared with 1% of bovine milk was not possible. Quantification was carried out within the range of 5–95%. Analyses were carried out in triplicate and the %CV was less than 4.3%.

Table 4
Linear regressions between log 10 of ovine β-lg B peak area/ovine β-lg A + caprine β-lg B peak area ratio and log 10 of ovine milk percentage (calculated as peak areas)

	Equation	r^2 value
Milk mixtures	$y = 0.897x - 1.924$	0.9997
Fresh cheeses	$y = 0.954x - 2.026$	0.9996
Ripened cheeses	$y = 0.889x - 1.899$	0.9988

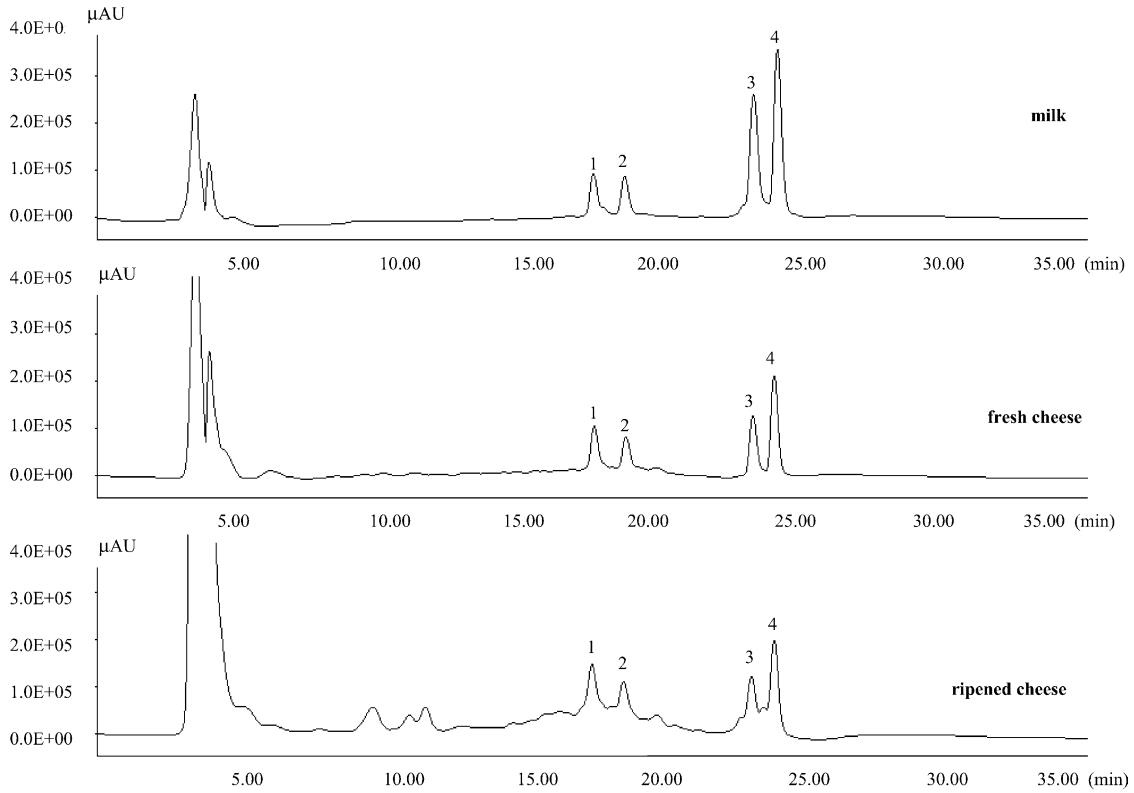

Fig. 3. Chromatographic profiles obtained for a mixture of 50% of bovine milk and 50% of caprine milk and respective fresh and ripened cheeses. (1) caprine α-la, (2) bovine α-la, (3) caprine β-lg B, (4) bovine β-lg B.

Similar results were obtained for the relationship between log 10 (caprine β-lg B peak area/bovine β-lg B peak area) ratio and log 10 of bovine milk percentage as shown in Table 3. Detection limit for bovine milk added to caprine cheese and quantification ranges were similar to those of bovine milk added to ovine cheese.

The differences between the actual percentages of bovine milk present in the cheese samples and those calculated using the regression equations were less than 5% for ovine/bovine and caprine/bovine cheeses analyzed.

With respect to cheeses made from binary mixtures of ovine and caprine milks, ovine β-lg A peak was overlapped with caprine β-lg B peak. Similar profile was reported by Torre et al. [11]. Table 4 depicts the relationship between log 10 (ovine β-lg B peak area/ovine β-lg A + caprine β-lg B peak areas) and log 10 of ovine milk percentage. Similar regression lines were obtained for ovine/caprine milk mixtures, and respective fresh and ripened cheeses.

3.3. Authenticity evaluation of commercial PDO cheeses

The method was applied for evaluation of authenticity of PDO cheeses made exclusively from ovine milk, from caprine milk and from mixtures of ovine and caprine milks. Table 5 presents the results obtained.

As expected bovine and caprine milks were not detected in PDO cheeses made from raw ovine milk and only caprine milk was detected in PDO cheeses made from raw caprine milk. “Picante da Beira Baixa” cheeses contained only ovine and caprine milks ranging between 40 and 49% of ovine milk, and 60 and 51% of caprine milk. These results were in good

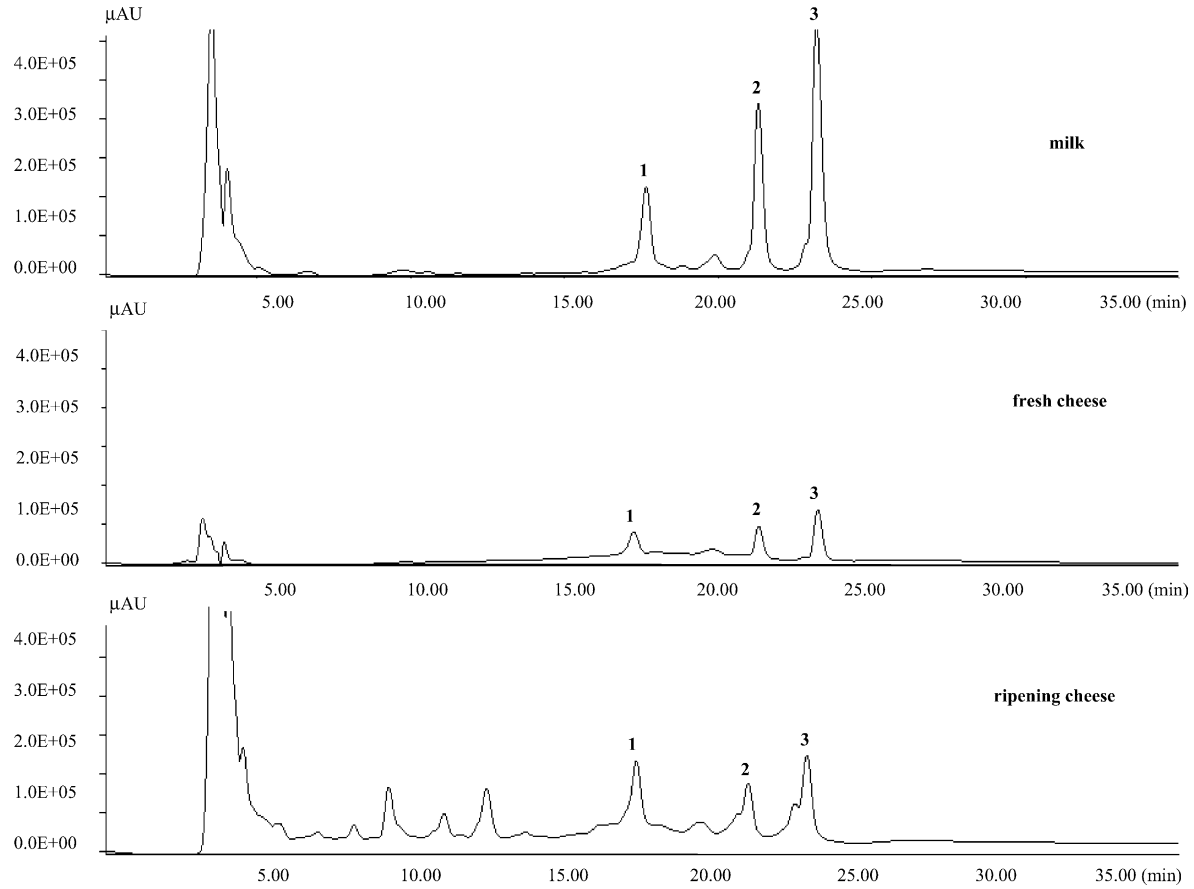

Fig. 4. Chromatographic profiles obtained for a mixture of 50% of ovine milk and 50% of caprine milk and respective fresh and ripened cheeses. (1) ovine + caprine α-la, (2) ovine β-lg B, (3) ovine β-lg A + caprine β-lg B.

Table 5
Results of evaluation of commercial PDO cheeses authenticity

Sample no.	PDO cheese	Milk type found
1	"Serra da Estrela"	Ovine milk
2	"Serra da Estrela"	Ovine milk
3	"Terrincho"	Ovine milk
4	"Terrincho"	Ovine milk
5	"Terrincho"	Ovine milk
6	"Terrincho"	Ovine milk
7	"Terrincho"	Ovine milk
8	"Serrano Transmontano"	Caprine milk
9	"Serrano Transmontano"	Caprine milk
10	"Picante da Beira Baixa"	Ovine/caprine milks (40/60%)
11	"Picante da Beira Baixa"	Ovine/caprine milks (49/51%)
12	"Picante da Beira Baixa"	Ovine/caprine milks (46/54%)

agreement with the information obtained from the respective PDO certification organisms.

4. Conclusions

HPLC analyses of β-lg are suitable for the identification of homologous proteins in mixtures of milks from different species. It proved to be a very sensitive and accurate method for studying milk type percentage of fresh and ripened cheeses made from binary mixtures of bovine, ovine or caprine raw milks. The method was applied with success to PDO cheeses, which are made in farms, using raw milk and traditional methods, the analyses of whey proteins afford an advantage in that these proteins are less susceptible to proteolysis than caseins.

Acknowledgements

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