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Evaluation of cheese authenticity and proteolysis by HPLC and urea–polyacrylamide gel electrophoresis

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Abstract

Chromatographic and electrophoretic methods have been established as useful tools in characterising cheese ripening and in the detection of milk adulteration. The purpose of this work was to evaluate casein proteolysis of cheeses made from bovine, ovine or mixtures of bovine and ovine milks, as well as ovine cheese authenticity, for 30 days of ripening by HPLC and urea–polyacrylamide gel electrophoresis.

Complementary information was obtained by both techniques when applied to the study of casein proteolysis during 30 days of ripening of ovine milk cheeses, ovine milk cheeses with 10% and 20% of bovine milk and bovine milk cheeses, manufactured according to the traditional Terrincho technology. For ovine cheeses, α-casein was the fraction that showed the higher degradation during cheese ripening. A similar behaviour was observed for ovine milk cheese with 10% of bovine milk. The profile for ovine milk cheese with 20% of bovine milk was more similar to that obtained for bovine cheese. Concerning bovine milk cheeses, electrophoresis was the most sensitive technique for the evaluation of proteolysis in these cheeses.

Ten and 20% of bovine milk could be detected in ovine milk cheeses by urea–polyacrylamide gel electrophoresis and HPLC, respectively, even after 30 days of ripening.

Keywords: Cheese; Caseins; Proteolysis; Adulteration; HPLC; Urea–PAGE

1. Introduction

The origin of the milk used to manufacture cheese must be declared by the producer, especially in the case of protected denomination of origin (PDO) cheeses. However, adulteration of ovine milk with bovine milk is relatively common due to: (i) seasonal fluctuations of the availability of ovine milk; (ii) the higher price of ovine milk compared to bovine milk; and (iii) opportunity to use the overproduction of bovine milk without loss of profit (Herrero-Martínez, Simó-Alfonso, Ramis-Ramos, Gelfi, & Righetti, 2000). Therefore, the development of reliable detection methods that can be applied to cheeses after ripening is of great interest in countries that produce or import ovine milk cheeses.

In recent years, several methods were reported in the literature for the detection of milk and cheese adulteration based on the analysis of casein fractions or whey proteins, and the study of cheese ripening: (i) electrophoretic techniques using polyacrylamide gels with urea (urea–PAGE) (Carretero, Trujillo, Mor-Mur, Pla, & Guamis, 1994; Farkye, Kiely, Allshouse, & Kindstedt, 1991; Gobbetti et al., 2002; Mayer & Hörtner, 1992; Veloso, Teixeira, & Ferreira, 2002) or SDS (SDS–PAGE) (Basch, Douglas, Procino, Holsinger, & Farrell, 1985; Jin & Park, 1996) and isoelectric focusing (IEF) (Kim & Jimenez-Flores, 1994), (ii) high-performance liquid chromatography (HPLC) by ion-exchange (Andrews, Taylor, & Owen, 1985; Kaminarides & Anifantakis, 1993), hydrophobic interactions (Bramanti

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et al., 2001), gel filtration (Andrews et al., 1985; Gupta, 1983) and reversed-phase modes (Bobe, Beitz, Freeman, & Lindberg, 1998; Ferreira, Mendes, Marques, & Ferreira, 2000; Ferreira, Mendes, & Ferreira, 2001; Michaelidou, Alichanidis, Urlaub, Polychroniadou, & Zerfiridis, 1998; Veloso et al., 2002; Visser, Slangen, & Rollema, 1991), (iii) immunological methods (Haza et al., 1996) or more recently capillary electrophoresis (Cartoni, Coccioli, Jasionowska, & Masci, 1999; Herrero-Martínez et al., 2000; Miralles, Ramos, & Amigo, 2000; Molina, Frutos, & Ramos, 2000; Recio, Amigo, & López-Fandiño, 1997). However, in view of the wide variety of cheeses with different microbiological characteristics and the extent of proteolysis, some of these methods are not suitable for ripened cheeses.

Cheese quality is greatly influenced by the levels of peptides, amino acids and free fatty acids resulting from proteolysis and lipolysis. Proteolysis is probably the most important biochemical event, having a major impact on the flavour and texture of most cheese varieties. Proteolysis of cheeses in general is influenced by several factors, including plasmin, chymosin, proteinases and peptidases from the starter and non-starter bacteria, pH and moisture levels of the curds, ripening time and temperature, salt content, salt-to-moisture ratio, and humidity (Fox, 1989; Park, 2001). Cheese quality is also influenced by the enzymatic catabolism of amino acids. In this work ovine cheese adulteration with bovine milk, as well as proteolysis of ovine and bovine milk cheeses during ripening were investigated. Terrincho cheese, a PDO cheese from the Northeast of Portugal, made from raw ovine milk and rennet whey, sold after a minimum of 30 days of ripening was chosen. HPLC and urea–PAGE methodologies previously validated for the separation and quantification of α-, β- and κ-caseins in bovine milk and applied to the separation of homologous caseins from ovine and caprine milks, as well as in the detection of bovine milk in ovine or caprine milk (Veloso et al., 2002) were used.

2. Materials and methods

2.1. Sampling

Raw bovine and ovine milks with more than three months of lactation (from Friesian and Churra breeds, respectively) were obtained directly from the producers.

Twenty different cheeses were manufactured: five bovine milk cheeses (BCH), five ovine cheeses (OCH), five ovine cheeses with 10% of cow milk adulteration (OCH-10-BM) and five ovine cheeses with 20% of cow milk adulteration (OCH-20-BM). One cheese of each was picked with 5, 10, 15, 20 and 30 days of ripening. Caseins from each cheese were extracted twice and analysed in duplicate. Percentages of cows' milk were set equal to 10% and 20% since: (i) taking into account the dimension of the traditional Terrincho cheese for producers an adulteration lower than 10% will not be economically interesting; and (ii) an adulteration higher than 20% will be easily detected by the consumer. The 20 cheeses were made according to Terrincho cheese traditional method (Despacho Normativo, 1993). Nevertheless, it should be noted that, although commercial Terrincho cheeses have an average weight of 1 kg, the cheeses manufactured for this study weighed from 60 to 80 g.

2.2. Caseins extraction

Caseins were prepared from skim milks according to the procedure described in a previous paper (Veloso et al., 2002). Briefly, caseins from cheeses were obtained from 2.5 g or 1 g for HPLC and urea–PAGE assays, respectively. The samples were homogenized in a mortar and, after addition of 1 M ammonia-acetate buffer at pH 4.3, in a Potter homogeniser. The resulting suspension was held at 8 °C for 20 min. The suspension was centrifuged at 4 °C, for 15 min at 3000*g*, to recover the precipitated caseins. The caseins were dispersed in 1 mM ammonia-acetate buffer (pH 4.3), precipitated again and centrifuged at 3000*g* for 10 min, at 4 °C. This procedure was repeated twice. In order to remove the remaining fat, the sample was washed with acetone and left to dry in a fume hood at room temperature. Finally, the dried powdered casein was stored in a desiccator at 8 °C until analysed.

2.3. Sample preparation

The casein powder was dissolved in a mixture of solvent A and solvent B (70:30, v/v) for HPLC analyses. The solution was filtered through a 0.45 μm TR-200104 filter, made of a mixture of cellulose esters (Teknokroma), and stored at −20 °C until use.

The casein samples for electrophoresis were dissolved in a diluted NaOH solution at pH 9. The samples were stirred vigorously and then placed on an ultrasound bath for complete dissolution. In order to remove the fat and the particles in suspension, the sample was centrifuged at 3000*g*, at 4 °C, for 10 min. The casein concentration was determined using the Bradford method (1976), with BSA as standard. The samples were mixed with the sample buffer (0.12 M Tris, 8.2 M urea, 2.5 mM EDTA, 0.2 M β-mercaptoethanol, 0.01% of bromophenol blue, pH 6.8) and 20 μg of proteins were applied in the wells.

2.4. Reagents and proteins standards

All reagents used were of analytical grade purity. Buffers for HPLC were filtered through 0.22 μm NL 17 filters and degassed under vacuum for at least 15 min before use. All reagents used for the electrophoresis had

an adequate purity for this experiment and they were used without further purification.

Bovine casein, with a minimum purity of 75%, determined by the Bradford method (1976) was supplied by Sigma Chemical Co. Purified α-, β- and κ-casein, ovine casein and bovine serum albumin (BSA), were also obtained from Sigma Chemical Co., and had a minimum purity of 85%, 90%, 80%, 85% and 98% (according to Sigma), respectively.

2.5. HPLC analyses

The HPLC equipment consisted on a Gilson chromatograph (Gilson Medical Electronics) equipped with a type 302 pump, a type 305 pump and a type 7125 Rheodyne Injector with a 20 μl loop. A Gilson 118 variable-wavelength ultra violet detector was used. The equipment was controlled by Gilson 712 software that controlled the solvent gradient, data acquisition and data processing. The column was a reversed-phase *Chrompack* P 300 RP column that contains polystyrene–divinylbenzene copolymer-based packing (8 μm, 300 Å, 150 × 4.6 I.D.). A *Chrompack* P RP (24 × 4.6 mm I.D.) was used as a pre-column. 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 was acetonitrile–water–TFA (95:5:0.1, v/v). Proteins were eluted with a series of linear gradients increasing the proportion of solvent B, from 29% to 100% over 35 min: 1–5 min, 29% B; 5–10 min, 29–37% B; 10–12 min, 37–41% B; 12–14 min, 41–42.5% B; 14–16 min, 42.5% B; 16–17 min, 42.5–43% B; 17–19 min, 43% B; 19–21 min, 43–47% B; 21–23 min, 47% B; 23–25 min, 47–54% B; 25–27 min, 54% B; 27–28 min, 54–100% B; 28–30 min, 100–29%; 30–35 min, 29% B. The flow-rate was 1 ml/min, the column temperature was 46 ± 0.1 °C and the eluate was monitored at 280 nm.

2.6. Statistical analyses

Statistical analysis was used to study the influence of ripening, adulteration and the interaction of these two factors, by analysis of variance (ANOVA), accordingly to the GLM algorithm (*General Linear Model*), of the SAS program, version 8.0 (*Statistical Analysis Systems Institute*, *1999*). Significant differences were considered for $p < 0.05$. The differences between individual average were tested using Tukey's method.

2.7. Urea–PAGE analyses

Polyacrylamide gel electrophoresis of casein samples was performed according to the method of Andrews (1983) with some modifications. The assays were carried out in a vertical vat (SE 280 Hoefer Scientific Instruments), using a Unipack 2000 power supply (UniQuip). The slab gels consisted of a 4% stacking gel and a 10% running gel. The stacking gel buffer was 0.06 M tris(hydroxymethyl)aminomethane (Tris)–4.5 M urea at pH 7.6, and the resolving gel buffer was 0.76 M Tris–9 M urea at pH 8.9. The electrophoresis buffer was a solution of 0.02 M Tris and 0.19 M glycine. The run was performed at 4 °C, at 20 mA until the end of the stacking gel, followed by a current of 30 mA. The gels were stained with Coomassie brilliant blue R250. Protein band density was determined using a laser densitometer (Vilbert Lourmat). Quantitative determination of caseins was made by peak area integration of the densitometer traces.

3. Results and discussion

3.1. HPLC analyses

The chromatographic profiles of the casein fraction of Terrincho cheese after 5, 10, 15, 20 and 30 days of ripening are shown in Fig. 1.

As shown in Fig. 1(A), the chromatographic profile of caseins from ovine milk cheese changed during ripening time, especially the second peak (numbered 2a, 2b), which corresponds to the α-caseins fraction (Veloso et al., 2002). This was expected since, as described in the literature, it is this casein that undergoes greatest proteolysis during cheese ripening (Grappin, Rank, & Olson, 1985). Moreover, after 30 days of ripening it was observed an inversion of these two peaks, being peak '2a' bigger than peak '2b'. This inversion is probably due to the formation of α_{s1}-I peptide as suggested by Christensen, Kristiansen, and Madsen (1989).

A similar behaviour was observed for OCH-10-BM (Fig. 1(B)). On the other hand, the profile for OCH-20-BM (Fig. 1(C)) was more similar to that obtained for BCH (Fig. 1(D)).

Table 1 shows the variation of the relative area of peaks '2a' and '2b' during the ripening period for each cheese studied. These results reinforce the differences of the chromatographic profiles obtained for the four kinds of cheeses analysed. It could be observed that OCH and OCH-10-BM presented higher variation of the α-casein fractions during the 30 days of ripening. In fact, when compared the relative areas of peaks '2a' and '2b' using ANOVA methodology significant differences were observed between the various ripening days for these cheeses ($p < 0.0001$; $F = 350.16$ and $F = 307.90$ for peaks '2a' and '2b', respectively). For BCH-20-BM and BCH the behaviour of peaks 2a and 2b was similar, however, the whole chromatographic profile appeared to be very distinct.

For five days of ripening OCH-10-BM were significantly different from OCH ($p < 0.0001$). Fig. 2 shows the evolution of ratio of peaks 2a and 2b for 30 days of

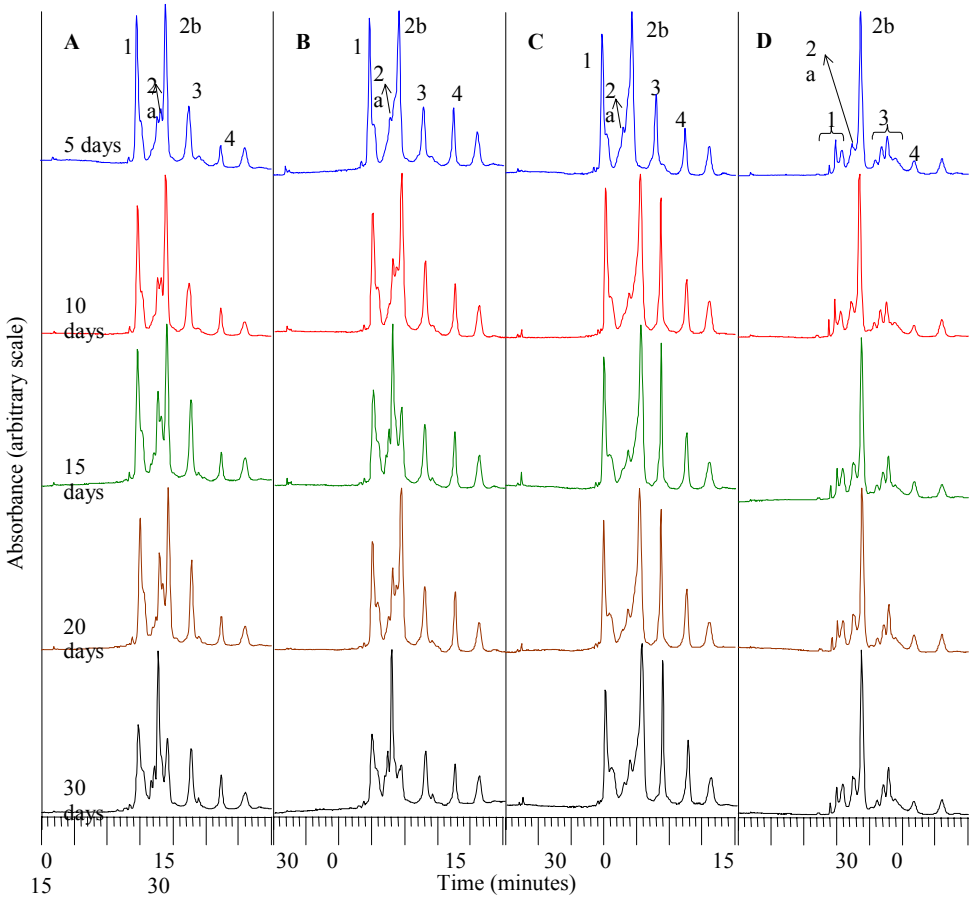

Fig. 1. HPLC chromatographic profiles at 280 nm of the casein fraction of cheese during ripening: (A) OCH; (B) OCH-10-BM; (C) OCH-20-BM; (D) BCH. (1) κ-casein; (2) α-casein; (3) and (4) β-casein.

Table 1
Relative area of peaks '2a' and '2b' during 30 days of ripening

		Relative areas % ± SD[a]				
Cheese type	Peak	5 days	10 days	15 days	20 days	30 days
OCH	2a	36.8 ± 0.2	38.4 ± 0.6	48.8 ± 0.03	48.2 ± 1.6	72.5 ± 0.2
	2b	63.2 ± 0.2	61.5 ± 0.5	51.2 ± 0.03	51.8 ± 1.5	27.5 ± 0.1
OCH-10-BM	2a	16.7 ± 0.3	41.8 ± 0.5	66.2 ± 0.26	44.6 ± 0.4	79.5 ± 0.2
	2b	83.3 ± 0.3	58.2 ± 0.5	33.2 ± 0.03	55.4 ± 0.4	20.5 ± 0.2
OCH-20-BM	2a	16.2 ± 0.2	16.5 ± 0.01	17.0 ± 0.4	17.0 ± 0.4	19.4 ± 1.0
	2b	83.8 ± 0.2	83.5 ± 0.04	82.6 ± 0.04	82.6 ± 0.3	80.5 ± 1.0
BCH	2a	19.8 ± 0.6	19.1 ± 0.2	20.5 ± 0.2	20.3 ± 0.2	21.4 ± 0.2
	2b	80.1 ± 0.6	80.9 ± 0.2	79.5 ± 0.1	79.7 ± 0.2	78.5 ± 0.2

[a] Results are expressed as relative areas % ± standard deviation.

ripening. At five days this ratio was similar for OCH-10-BM, OCH-20-BM and BCH. However, for 15, 20 and 30 days of ripening OCH and OCH-10-BM presented a similar behaviour. For 30 days of ripening, OCH-10-BM was not significantly different from OCH ($p = 0.4591$). Nevertheless, these two cheeses were significantly different from OCH-20-BM ($p < 0.0001$). These results suggest that the HPLC methodology reported could be applied to follow cheese proteolysis and to detect adulterations of Terrincho cheese, (equal or higher than 20%, after 30 days of ripening) as shown in Fig. 2.

3.2. Urea–PAGE analyses

A good resolution of the caseins extracted from the cheeses under study was obtained by Urea–PAGE. Figs. 3 and 4 show the proteolysis profiles obtained during ripening of OCH and BCH.

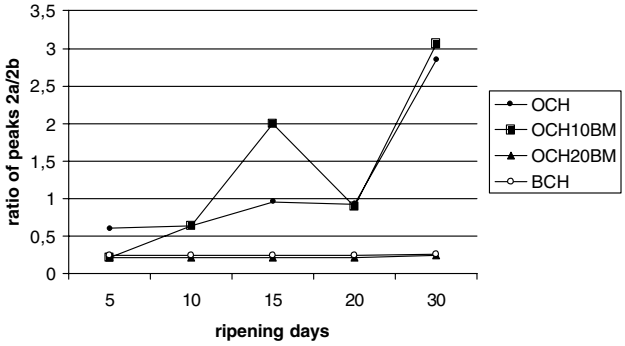

Fig. 2. Changes in the ratio of peaks '2a' and '2b' for cheeses OCH, OCH-10-BM, OCH-20-BM, and BCH during ripening (5, 10, 15, 20 and 30 days).

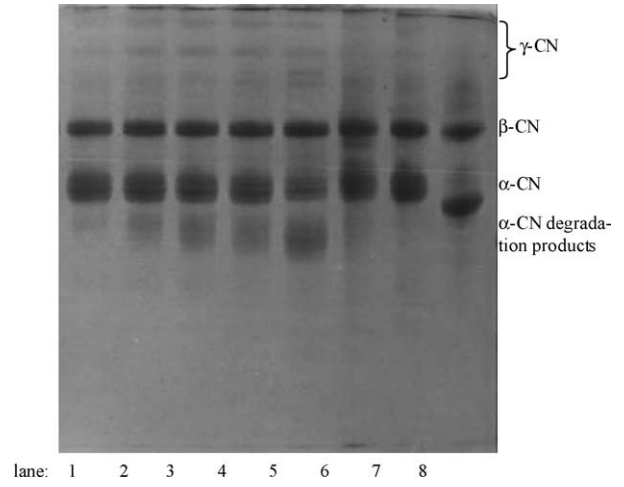

Fig. 3. Electrophoretograms of ovine cheese caseins during ripening for 5, 10, 15, 20 and 30 days ((1)–(5), respectively); ovine casein standard (6); raw ovine milk (7); bovine casein standard (8).

The α-casein band was still prominent in BCH and OCH, though a partial degradation of this casein was observed after five days of ripening. Therefore, when compared with the HPLC method, the urea–PAGE method was more sensitive, allowing earlier detection of proteolysis. Along the ripening time, an intensification of a band with higher electrophoretic mobility was observed, due to the proteolysis of α-casein. The electrophoretic profile revealed that, in all cheeses, the β-casein did not undergo as much proteolysis as α-casein, which was in agreement with other studies already reported in the literature (Jin & Park, 1996; Kim & Jimenez-Flores, 1994; Marcos, Esteban, León, & Fernandez-Salguero, 1979; Park, 2001; Pavia, Trujillo, Gusmis, & Ferragut, 2000; van Hekken & Thompson, 1992; Visser et al., 1991). These conclusions are enhanced by the results obtained by densitometric analysis summarised in Fig. 5.

Densitometric analyses of the electrophoretic results of ewe cheeses showed that α-casein decreased significantly during ripening, which is correlated with the

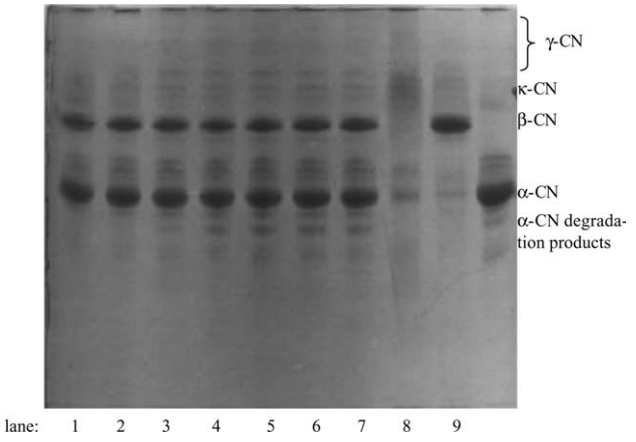

Fig. 4. Electrophoretograms of bovine cheese caseins during ripening for 5, 10, 15, 20 and 30 days ((3)–(7), respectively); bovine casein standard (1); raw bovine milk (2); κ-casein (8); β-casein (9); α-casein (10).

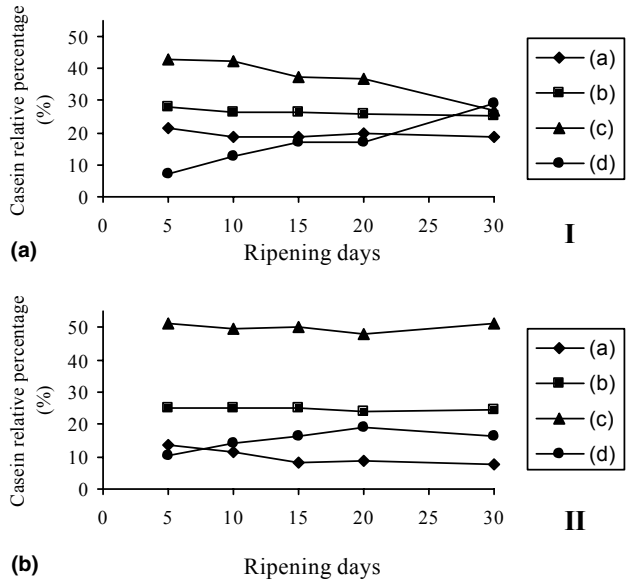

Fig. 5. Relative percentage of caseins during cheese ripening calculated from densitometry analysis: I: OCH; II: BCH. (a) γ-casein; (b) β-casein; (c) α-casein; (d) degradation products.

appearance of bands with higher electrophoretic mobility. Only a slight decrease of β-casein was observed during ripening. Bovine casein bands remained almost constant during 30 days of ripening. These results were in good agreement with the results obtained for HPLC analyses.

The urea–PAGE method was also applied to the study of adulterations of ovine cheeses manufactured according to the traditional Terrincho technology, during ripening. It was possible to detect the addition of 10% and 20% of bovine milk until 30 days of ripening based on the presence of the bovine α-casein (Fig. 6). Ramos and Juárez (1986) also detected a 10% adulteration of ovine cheese with bovine milk, however only in

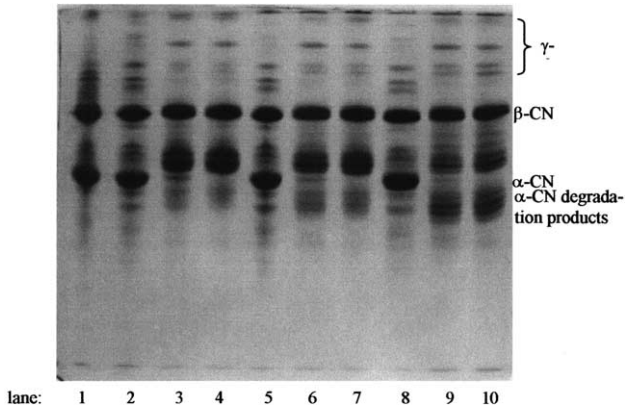

Fig. 6. Electrophoretograms for ewe cheeses with 10% of cow's milk during the 30 days of ripening: (1) bovine casein standard; (2) BCH, 10 days; (3) OCH-10-BM, 10 days; (4) OCH, 10 days; (5) BCH, 20 days; (6) OCH-10-BM, 20 days; (7) OCH, 20 days; (8) BCH, 30 days; (9) OCH-10-BM, 30 days; (10) OCH, 30 days.

the first 10 days of ripening. Therefore, the technique reported here presents an advantage since, for the same percentage of adulteration, bovine milk in ovine cheeses could be detected at least up to 30 days of ripening.

OCH-10-BM had a proteolytic pattern similar to that obtained for ewe cheese (Fig. 6). On the other hand, the casein profile of OCH-20-BM presented a lower level of proteins with electrophoretic mobilities higher than α-casein. This fact is in agreement with the results obtained by the HPLC methodology. Indeed, it was shown that this cheese has a greater similarity to the protein profile of BCH, which does not change significantly throughout ripening.

4. Conclusions

The results showed that both HPLC and urea–PAGE methods were successfully applied to evaluate proteolysis during ripening of OCH, OCH-10-BM, OCH-20-BM and BCH for 30 days, manufactured according to the traditional Terrincho technology. It was observed that α-casein was the fraction that underwent higher proteolysis during that period, while the other fractions remained almost unaltered, as reported before for other types of cheeses. Bovine cheeses manufactured according to the above-mentioned technology, it was observed that electrophoresis was the most sensitive technique in the evaluation of proteolysis. Both techniques were able to detect the presence of bovine milk in ovine cheese. However, based on the chromatographic profiles obtained, it was only possible to detect addition of 20% bovine milk by HPLC, while the electrophoretic technique could identify the 10% of cow's milk.

Both methodologies can play a key role in the analysis and quality control of ripened cheeses. Knowledge of cheese changes during ripening is of great technological relevance as it can lead to improved cheese quality. Especially for PDO cheese the guarantee of its quality and authenticity continues to be a challenge for cheese makers. Differences may occur in ripening relating to cheese size variations, native microflora, and others. For that reason, further studies with Terrincho cheeses of real dimensions will be performed. The method will also be applied to cheeses made from pasteurised milk, because pasteurisation causes minimal modifications on caseins.

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