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Evaluation of the effect of rennet type on casein proteolysis in an ovine milk cheese by means of capillary electrophoresis

Aurora Irigoyen*, Jesús María Izco, Francisco Clemente Ibáñez, Paloma Torre

Area de Nutrición y Bromatología, Departamento de Ciencias del Medio Natural, Universidad Pública de Navarra, 31006 Pamplona, Spain

Abstract

Capillary electrophoresis was used to study the evolution of casein throughout the ripening process of Roncal Denomination of Origin ewe's milk cheese and to assess the type of rennet in its hydrolysis. Two manufactures were prepared, each with four vats; two of them had added lamb artisan rennet, batch A [clotting activity of 97.54 rennet units (RU) ml^{-1}] and batch B [clotting activity of 16.26 RU ml^{-1}]; one vat included calf industrial rennet, batch I (clotting activity of 45.70 RU ml^{-1}); and the fourth vat had added mixed rennet, batch M, a 50:50 mixture of lamb (batch A) and calf (batch I) (clotting activity of 77.53 RU ml^{-1}). The content of casein nitrogen in fractions α-casein1_{CE}, α-casein2_{CE}, β-casein1_{CE} and β-casein2_{CE} was quantified in cheese after 1, 15, 30, 60, 120 and 180 days of ripening. β-Casein fractions undergo lesser degradation during the ripening time than α-casein proteins. The degradation of α-caseins is very much influenced by the clotting activity of the rennet used, so that the more active the clotting activity the greater the hydrolysis of those caseins. Nevertheless, it is at the level of β-caseins that we observe the evidence of the influence of the type of rennet, thus noting a less intense proteolytic activity in the batch made with calf rennet, batch I. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Cheese; Food analysis; Caseins; Proteins

1. Introduction

Throughout the ripening process of cheese, proteolysis is considered to be the most complex and most important transformation to develop aroma, flavour and texture [1–3]. The main proteolytic agents in the ripening process are: [4] the natural proteases of milk (plasmin and cathepsin D); the rennet or clotting enzymes retained in the curd; the proteases and peptidases from starter microorganisms and the enzymes from non-starter bacteria.

During the elaboration and ripening of the cheese, the caseins are degraded several grades, and their hydrolysis is one of the main determinants of the texture and flavour of cheese [5,6]. The degradation of the main polypeptidic chains that results in peptides with an intermediate and high molecular mass is known as primary proteolysis [7]; that is when rennet acts most upon α_{s1}-casein and plasmin upon β-casein [7,8]. According to Jaubert and Martin [9], caseins represent around 83% of the total protein content in ewe's milk. During the cheese manufacturing process, κ-casein is degraded due to the rennet, producing κ-casein and casein macropeptides which is eliminated with the whey. The α_{s1}-casein is hydrolysed by the rennet in the first ripening stages at the Phe_{23}–Phe_{24} binding level; this reaction produces texture alterations at the beginning of the maturing process, which is one of the most important

*Tel.: +34-948-169-141; fax: +34-948-169-187.
E-mail address: lablac@unavarra.es (A. Irigoyen)

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events that takes place in most cheese manufacturing [5]. The clotting activity of the rennet used is one of the factors that has an influence upon the greater or lesser degradation of caseins; similarly, the origin of the clotting enzyme used (animal, microbial or vegetal) will condition the proteolysis degree. Vegetal and microbial clotting enzymes degree β-casein fractions more than clotting enzymes of animal origin [10–13]. In spite of the wide knowledge available about the influence of the type of rennet on the proteolysis of cheese, the few studies carried out using lamb rennet do not analyse its effects on casein degradation [14,15].

Roncal cheese, made of raw ewe's milk, was the first Spanish cheese to opt for a Denomination of Origin (OAC) in 1981. It is an uncooked, pressed paste cheese, with a minimum ripening time of 4 months. Its regulations allow the possible addition of artisan lamb or commercial calf rennet; thus, the OAC has shown great interest in a wider analysis of the subject that would facilitate the optimisation of the elaboration process.

Recent studies [16–19] have shown that capillary electrophoresis is an efficient method for monitoring the hydrolysis of caseins in cheese. The advantages of this method compared to more traditional techniques (HPLC and polyacrylamide gel electrophoresis, PAGE) are high sensitivity, resolution and repeatability, as well as a high separation velocity.

Due to the importance of rennet in the ripening of the cheese, the objective of the present paper is to study the effect of the type of rennet (lamb and calf) used in its preparation upon the hydrolysis of the caseins, studied by means of capillary electrophoresis.

2. Experimental

2.1. Preparing the lamb rennet samples

The lamb rennet was prepared following the method recommended by the Roncal Denomination of Origin and Santamaria et al. [20]. To do so, the milking lamb rennet collected was dried in a ventilated place protected from light. Once dry, the fat was removed from the external surface and cut into little cubes, which were mixed with salt and kept for later use in darkness at 4°C. To use them in the elaboration of cheese, they were dissolved in water and filtered. The resulting filter was preserved in a cold, dry and dark area until use. The clotting activity was established using a modification of the Berridge method [21], based on the visual determination of the flocculation point of a milk substrate after adding the clotting enzyme. The rennet meets the microbiological standards established in the general identity and purity standard for rennet [22].

2.2. Cheese samples

Two trials were made. In each trial, four different batches of cheese (A, B, M and I, see Table 1) were manufactured from raw ewe's milk in accordance with the regulations of the Regulatory Board of the Roncal Cheese Denomination of Origin [23]. Milk was incubated with starters (1.2 doses/100 l, Ezal, Dangé Saint-Romain, France) at 22°C. After 30 min and at a temperature of 32°C, the corresponding type of rennet (Table 1) was added to each vat in doses of 12 ml/40 l. After 40–50 min, the curds were cut, shaken for 30 min and reheated at 38°C to favour the dripping of curds; the curds were moulded and pressed for 3 h at 2.5 kg cm^{-2} and 20°C. Then they were salted by soaking them in a sodium chloride saturated brine at 13°C for 18 h. The cheeses were then moved to an airing chamber to be kept there for two more weeks at 10°C and 75% relative humidity. Later they were transferred to a maturing chamber at 10°C and 85% relative humidity until analysis.

For each manufacturing process, two cheeses were taken from each vat after 1, 15, 30, 60, 120 and 180 days of ripening for analysis; this amounts to 96 cheeses. All the analysis carried out was duplicated so that eight analyses were determined for each type of cheese on each of the collection dates.

Table 1
Type of rennet used in each vat[a]

Vat	Type of rennet	Clotting activity
A	Lamb	97.54 RU ml^{-1}
B	Lamb	16.26 RU ml^{-1}
M	Lamb and calf (50:50)	77.53 RU ml^{-1}
I	Calf	45.70 RU ml^{-1}

[a] RU: rennet unit.

2.3. Physicochemical analysis

The total nitrogen (TN) content was estimated (Kjeldahl method) according to IDF standard No. 25 for cheese [24]. The soluble nitrogen (SN) fraction was determined by precipitating out the insoluble nitrogen fraction (caseins) in a buffer solution of acetic acid–acetate at pH 4.6 as per Basch et al. [25]. The casein nitrogen was determined by the difference between the TN and the SN.

The caseins were extracted by the Ibáñez et al. method [26]. An amount of 10 g of comminuted cheese was weighed out, and 1 *M* sodium acetate buffer, pH 4.6, was added. The mixture was homogenized and after that centrifuged at 4500 *g* for 15 min. The lipid fraction remaining in the casein precipitate was removed by washing with dichloromethane–acetate buffer (1:1, v/v). The precipitate was washed with acetone and left to dry in a muffle oven at 30°C. Finally, the casein was comminuted to the smallest possible grain size. The extracted caseins were kept refrigerated at 3–4°C until analysis.

2.4. Capillary electrophoresis

To prepare the sample and separate the caseins by capillary electrophoresis, the method used by Izco et al. [27] was used. The buffer used to run the samples was citrate buffer containing 10 m*M* trisodium citrate dihydrate, 135 m*M* anhydrous citric acid, and 0.05% methylhydroxyethyl cellulose (Tylose MHB 30.000, Hoechst Ibérica, Barcelona, Spain) in 8 *M* urea to a pH of 3.1. The sample buffer was 5 m*M* trisodium citrate dihydrate, and 5 m*M* DL-dithiothreitol in 7 *M* urea to a solution pH of 8.0. An amount of 2 ml of the sample buffer was added to 50 mg of the casein sample. The suspension was mechanically shaken and sonicated in a water bath for 30 min. An amount of 100 μl was filtered through a type-HV hydrophilic filter (Millipore) with a pore size of 0.45 μm. Two to three drops (approximately 25 μl) of the filtrate was a sufficient volume for transfer to the siliconized vial for injection in the capillary system.

Electromigration was performed using a Quanta 4000E capillary electrophoresis system (Waters, Milford, MA, USA) operated using Millennium 2010 software (Waters). The separation was carried out using a 50 cm×50 μm I.D. Celect P1 hydrophilically coated fused-silica capillary (Supelco, Bellefonte, CA, USA). The sample was injected hydrostatically at the anodic end of the capillary for 40 s. The caseins were separated by applying a constant voltage of 25 kV, equivalent to an intensity of 30–32 μA. The temperature was held at 40±0.1°C and the casein fractions were detected by UV absorbance at 214 nm. Casein identification is based on the Cattaneo et al. results [28], i.e. α-casein1_{CE}, α-casein$2_{CE,}$ β-casein1_{CE} and β-casein2_{CE} depending on the elution order (Fig. 1). To obtain the relative concentration of each component in whole casein (insoluble protein fraction at pH 4.6), the normalised area percentage of each peak was calculated with reference to the total sum of normalised areas. Knowing the amount of casein nitrogen present in the cheese, it was possible to estimate the grams of the casein fraction with reference to 100 g of cheese.

2.5. Statistical analysis

The SPSS statistical package version 6.1 (SPSS, Chicago, IL, USA) was used for treating the results. We performed a factorial analysis of the variance (ANOVA) of two factors (α=0.05), rennet type and ripening time, to see what effect they had on the variables under study

3. Results and discussion

Fig. 1 shows the electrophoregram for the casein extracted from batch M cheeses, after 1, 30, 60 and 180 days of ripening. We observe a significant reduction in the α- and β-casein content depending on the maturing time. This is due to the action of residual rennet, together with the action of the hydrolytic enzymes of the microorganisms present in the cheese [29]. As explained by Izco et al. [27], the complicated cheese casein preparation yields, together with intact casein fractions, a number of non-identified peaks which represent the peptides released from the breakdown of caseins. The variance analysis (Table 2) illustrates significant differences both in the effect due to the type of rennet, as well as to the ripening time of all quantified frac-

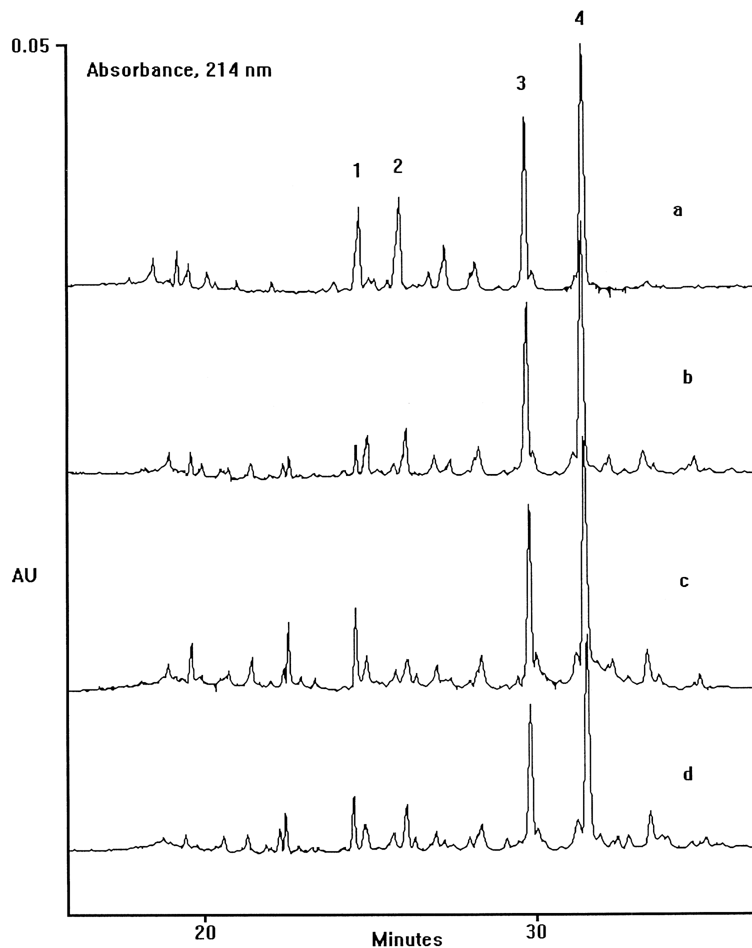

Fig. 1. Electrophoregrams for the cheese whole casein fraction from batch M after ripening for: (a) 1 day; (b) 30 days; (c) 60 days; and (d) 180 days. 1, α-casein1_{CE}; 2, α-casein2_{CE}; 3, β-casein1_{CE}; 4, β-casein2_{CE}.

Table 2
F values obtained from the factor variance analysis of the caseins determined by capillary electrophoresis[a]

	Type of rennet	Ripening time (days)	Interaction
α-CN1	26.53***	132.98***	3.18**
α-CN2	81.21***	276.45***	5.81***
β-CN1	22.11***	144.41***	4.72***
β-CN2	17.74***	62.93***	1.60 NS

[a] *, $p<0.05$; **, $p<0.01$; ***, $p<0.0001$; NS, not significant. α-CN1, α-casein1_{CE}; α-CN2, α-casein2_{CE}; β-CN1, β-casein1_{CE}; β-CN2, β-casein2_{CE}.

tions. The results of the Fisher analysis (not shown) for α-caseins indicate that those proteins are significantly degraded up to 120 days of ripening, after which they stabilise. Nevertheless, β-caseins present significant differences in all periods of time studied, which implies that their degradation is prolonged to the 180 days of ripening.

Fig. 2 shows the electrophoregram for the caseins obtained in the four batches of elaborated cheese, after 30 days of ripening. In that period, the α_s-caseins fractions are hydrolysed more quickly than those of the β-casein. Thus, at 30 days of ripening, the hydrolysis percentages for the α-casein1_{CE} and α-casein2_{CE} fractions are 52% and 49% (batch A),

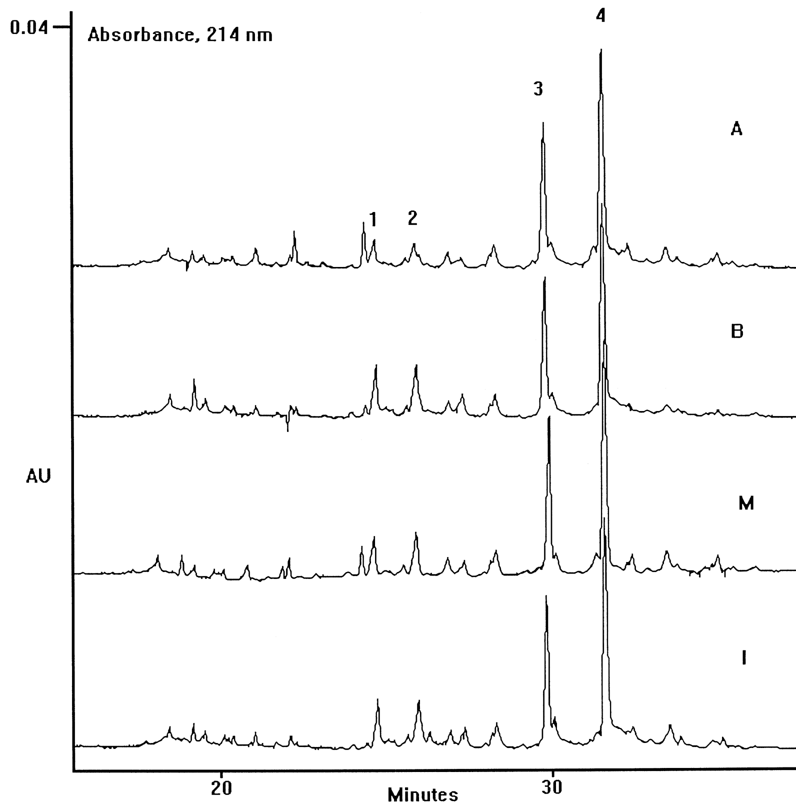

Fig. 2. Electrophoregrams for the cheese whole casein fraction from the four batches (A, B, M and I) at 30 days of ripening. 1, α-casein1_{CE}; 2, α-casein2_{CE}; 3, β-casein1_{CE}; 4, β-casein2_{CE}.

13% and 12% (batch B), 29% and 33% (batch M), and 29% and 31% (batch I). On the other hand, the degradations of β-casein1_{CE} and β-casein2_{CE} fractions after 60 days of ripening are 28% and 26% (batch A), 6% and 6% (batch B), 14% and 8% (batch M), and 13% and 20% (batch I). α_s-casein is hydrolysed during the first stages of ripening which, according to Creamer and Olson [5], results in important alterations in the texture of the cheeses. During the first stages of the ripening, the hydrolysis of casein is more acute in the batch made with ewe's milk with the highest clotting activity, batch A, although those differences decrease throughout the ripening process (see batch M, Figs. 3 and 4).

The hydrolysis percentages for α-casein1_{CE} and α-casein2_{CE} at 180 days of ripening are 67% and 63% (batch A), 49% and 50% (batch B), 79% and 66% (batch M), and 66% and 63% (batch I). Batch B, made with the least clotting activity rennet, is the one with the smallest level of proteolysis for both fractions. Contrary to what was pointed out for the Ossay Iraty cheese [19], the residual level for α-casein2_{CE} in Roncal cheese is a lot higher than the residual level for α-casein1_{CE} in all batches (Fig. 3).

The hydrolysis percentages at the end of the ripening process for the β-casein1_{CE} and β-casein2_{CE} fractions are 46% and 48% (batch A), 32% and 30% (batch B), 52% and 52% (batch M), and 37% and 44% (batch I). The fractions that correspond to the β-casein region are less degraded than those that correspond to the α-caseins, as observed in all batches studied (Fig. 4). That greater resistance of β-caseins to enzyme hydrolysis was already pointed out by several authors [4,29,30]. Those β-casein levels coincide with those found in ewe's milk Ossau Iraty cheese [19].

The multiple comparison analysis of the effect of rennet type (Table 3) reveals that at the α-casein

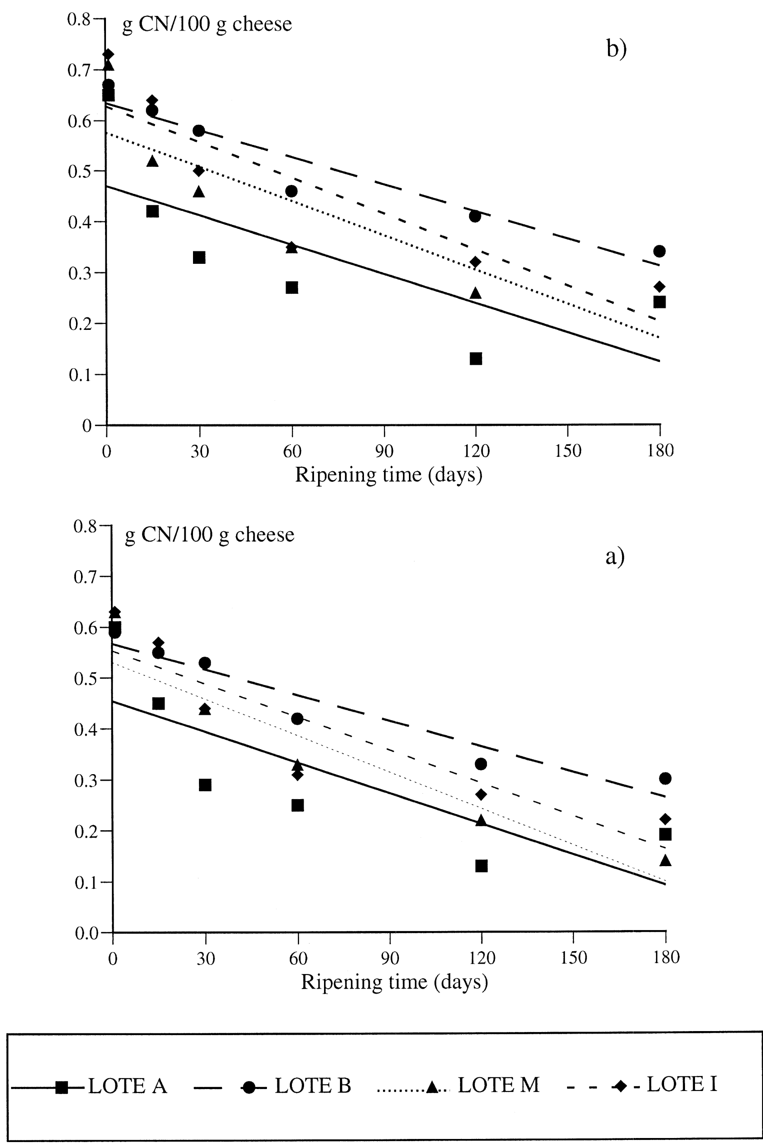

Fig. 3. Casein nitrogen content (g/100 g cheese) of (a) α-casein1_{CE} and (b) α-casein2_{CE} fractions throughout the ripening time, depending on the type of rennet used.

level there are significant differences between all the batches studied; nevertheless, there are no differences between batches B, M and I in the β-casein1_{CE} fraction, as there are none either in the β-casein2_{CE} (p=0.7256) between batches B and I. This fact indicates that the proteolysis of β-caseins is not as influenced by the clotting activity of rennet as α-caseins are.

Some authors [12,31,32] observed that curdling enzymes of vegetal origin present more proteolytic activity on α- and on β-casein fractions than curdling enzymes of animal origin. According to Ref. [11], independently of the type of curdling enzymes used (calf rennet or curdling enzymes of microbial origin), the degradation of α_{s1}-casein was very similar in both cases; however, proteolysis of the β-casein

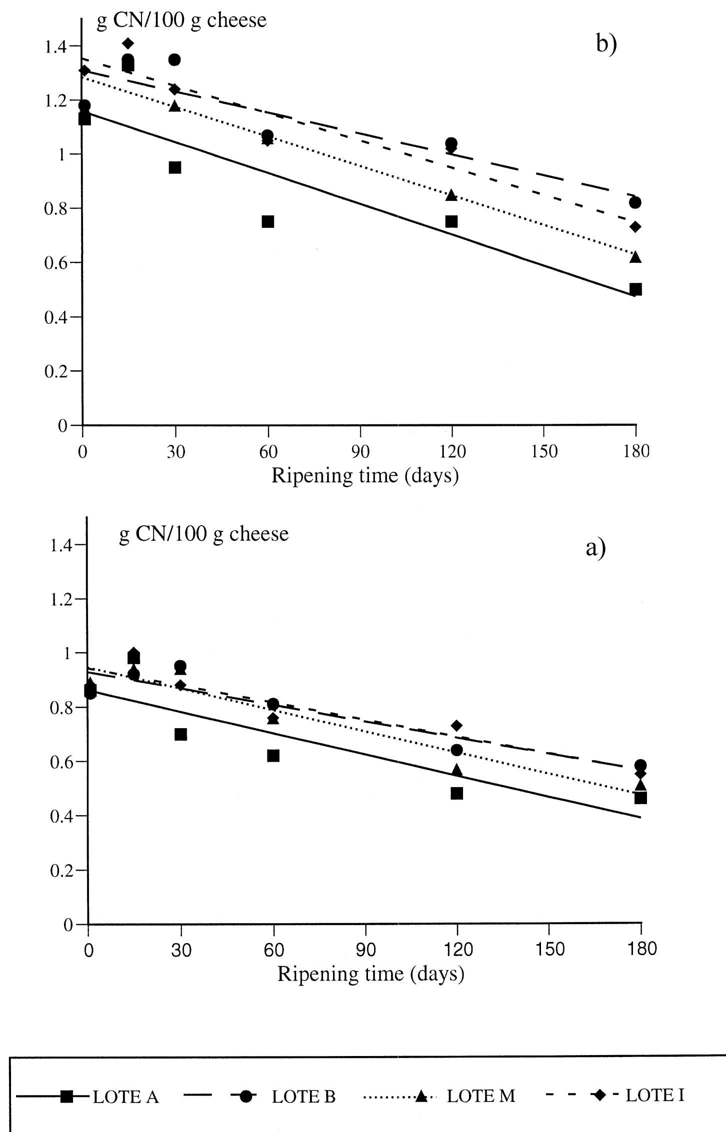

Fig. 4. Casein nitrogen content (g/100 g cheese) of (a) β-casein1_{CE} and (b) β-casein2_{CE} fractions throughout the ripening time, depending on the type of rennet used.

fractions was highest in cheeses with added calf rennet.

4. Conclusions

This paper illustrates that CE is a good method to determine the level of proteolysis in a cheese and to study the effect that several rennet types, with different clotting activities, have upon casein degradation.

Both the ripening time and the type of rennet used significantly influence the evolution of caseins determined by capillary electrophoresis. α-casein fractions are more acutely hydrolysed than β-caseins throughout the ripening process. The different degradation of α-caseins in the batches is due to the clotting activity of the rennet type used; thus, those

Table 3
Average multiple comparisons using the Fisher method of differences for variables: α-casein1$_{CE}$ (DC=0.031), α-casein2$_{CE}$ (DC=0.023), β-casein1$_{CE}$ (DC=0.032), β-casein2$_{CE}$ (DC=0.068)[a]

	Batches					
	A–B	A–M	A–I	B–M	B–I	M–I
α-CN1	−0.136*	−0.050*	−0.087*	0.086*	0.049*	−0.037*
α-CN2	−0.171*	−0.083*	−0.127*	0.089*	0.045*	−0.044*
β-CN1	−0.109*	−0.086*	−0.118*	0.023	0.009	−0.032
β-CN2	−0.219*	−0.126*	−0.231*	0.093*	0.012	−0.106*

[a] DC, Critical difference; factor, type of rennet; level of significance, 5%. α-CN1, α-casein1$_{CE}$; α-CN2, α-casein2$_{CE}$; β-CN1, β-casein1$_{CE}$; β-CN2, β-casein2$_{CE}$.

batches made with higher activity clotting rennet undergo a greater proteolysis in both fractions. Nevertheless, this does not happen the same way in β-caseins, where no difference is appreciated between batches B and I in spite of their different clotting activity. The use of lamb or calf rennet can be shown as a result of the lesser proteolytic level in the β-caseins of cheese made with calf rennet vs. cheese made with lamb rennet.

A bibliographic review was done and no articles evaluating the effect of rennet type on casein proteolysis in cheese using CE were found. PAGE and sodium dodecylsulfate-PAGE have been the more commonly used separation methods and one of the main drawbacks of those electrophoretic methods is that quantitative determinations are not very accurate; hence, the comparisons must be interpreted with caution. Also, works dealing with vegetables or microbial curdling enzymes and the influence on proteolysis do not consider their curdling activity. In this regard this paper contributes new data on rennet types and on curdling activities to help fill the gap in available knowledge on the effect on casein proteolysis in ewe's-milk cheeses.

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