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Prediction of the Ripening Times of Ewe's Milk Cheese by Multivariate Regression Analysis of Capillary Electrophoresis Casein Fractions

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The effect of the ripening time on the proteolytic process in cheeses made from ewe's milk during a 139-day ripening period was monitored by the use of capillary electrophoresis of pH 4.6 insoluble fraction. Totals of 18 and 21 peaks were recognized and matched in the electropherograms obtained with a fused-silica capillary and a neutral capillary (hydrophilically coated), respectively. These peaks correspond to intact ovine caseins and their hydrolysis products (α_{s1}-casein I, α_{s1}-casein II, α_{s1}-casein III, α_{s2}-casein, β_1-casein, β_2-casein, *p*-κ-casein, α_{s1}-I-casein, γ_1-casein, γ_2-casein, and γ_3-casein). The α_s-caseins (α_{s1}- and α_{s2}-casein) displayed similar degradation pattern to one another, but different from those of β-caseins (β_1- and β_2-casein). β-Caseins were very much undergoing lesser degradation during the ripening time than α_s-casein. Finally, partial least-squares regression and principal components regression were used to predict the ripening time in cheeses. The models obtained yielded good results since the root-mean-square error in prediction by cross validation was <8.6 days in all cases.

KEYWORDS: Capillary electrophoresis; ewe's milk cheese; multivariate regression analysis; ripening time.

INTRODUCTION

Cheese ripening involves a series of physicochemical changes brought on by lipolytic, proteolytic, and glycolytic reactions, viewed by some workers as the most important phenomena involved in the development of sensory characteristics during cheese ripening (*1*−*3*). Of these biochemical phenomena, proteolysis may be the most important change taking place during aroma, flavor, and texture development (*4*, *5*). Attempts to monitor this process have led to the development of objective physicochemical indices which will eventually be useful in predicting the final quality of cheese. For most hard and semihard cheese varieties proteolysis is the most commonly used index of maturation (*6*). The proteolysis of cheeses manufacturated with ovine milks has been described by Sousa and Malcata (*7*) and Freitas and Macalta (*8*).

Recent studies (*9*−*12*) have shown that capillary electrophoresis is an efficient method for monitoring the hydrolysis of caseins in cheese. Transformations of caseins due to the action of proteinases in milk and cheese have been mainly studied in bovine products (*9*, *10*, *13*, *14*). However, despite the great importance of cheeses made from ewe's milk, there are few studies of the use of CE in analyzing the caseins in ewe's milk cheese (*12*, *15*, *16*).

On the other hand, a promising approach in the area of cheese ripening is the application of multivariate analysis (e.g., principal component analysis (PCA), principal component regression (PCR), and partial least-square regression (PLS)) to proteolysis patterns to model quantitative relationships (*17*, *18*). A range of proteolysis parameters has been used for characterization of Spanish Manchego cheese (*19*), as well as for predicting the ripening time by means of partial least-squares regression and other multivariate regression techniques (*20*). However, despite the literature covering chemometrical analysis of chromatographic or electrophoretic profiles from research on cheese ripening being extensive, there are few studies that use CE peptide profiles. Thus, Herrero-Martínez et al. (*21*) performed PLS of CE analysis of the ethanol−water protein fraction to predict the ripening time in goat and ewe cheeses. Recently, in our laboratory a chemometrical analysis of the CE data was used to predict the ripening time of cow's and ewe's mixture milk cheeses (*22*).

The aim of this work was to study, by means of CE analysis of the pH 4.6 insoluble fraction, the proteolytic process during

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the ripening of commercial cheeses manufactured from raw ewe's milk. Additionally, multivariate regression techniques of the CE data were used to predict ripening times of cheeses.

MATERIALS AND METHODS

Materials and Reagents. Citric acid, hydrochloric acid, dichloromethane, phosphoric acid, sodium hydroxide, sodium citrate, and urea were of analytical grade and obtained from Merck (Darmstadt, Germany). Dithiothreitol (DTT), hydroxypropyl methyl cellulose (HPMC), 3-morpholinopropanesulphonic acid, and plasmin (EC 3.4.21.7, from bovine plasma) were from Sigma Chemical Co. (St. Louis, MO). All solutions were based on highly purified water (Milli-Q Plus grade).

Buffer solutions were filtered through 0.45 μm HAWP and 0.22 μm GSWP filters (Millipore Co., Billerica, MA) before use.

Cheese Samples. Cheeses, manufactured from raw ewe's milk, were supplied by Quesos Frías, S.A. (Burgos, Spain). Caseins were extracted from cheese ripened for 0, 14, 42, 69, 102, and 139 days. Two separate batches of cheese were processed.

Isolation of Caseins. Isoelectric caseins were obtained by precipitation from whole ewe's milk by adding 2 M HCl to pH 4.6, followed by centrifugation at 3500 rpm for 15 min. To isolate the casein fraction completely from whey and eliminate the remaining fat, it was washed once with 1 M sodium acetate buffer (pH 4.6) and three times with dichloromethane/1 M sodium acetate buffer (pH 4.6) (1:1, v/v). The casein fractions obtained were lyophilized and stored at −20 °C.

Caseins from cheese were obtained by precipitation from 5 g of homogenized cheese in 30 mL of water at pH 4.6 and washed and lyophilized as above.

Plasmin Treatment of Caseins. Half-milliliter lyophilized cheese casein solutions (20 mg mL^{-1} in 0.05 M phosphate buffer, pH 7) and 0.5 mL of plasmin preparation (0.152 unit mL^{-1} in 0.05 M phosphate buffer, pH 7) were incubated at 37 °C for 240 min. The enzymatic reaction was stopped by heating to 85 °C for 10 min. The final hydrolyzates were centrifuged at 2600 rpm for 20 min at 4 °C, and the pH in the supernatants was decreased to 4.6 with 0.5 M acetic acid. After equilibration for 1 h, centrifugation was repeated, and the precipitates were stored frozen until analysis.

Sample Pretreatment for CE. To dissociate the caseins, all samples were dissolved in a sample buffer (0.05 M phosphate buffer) containing 8 M urea and 10 mM dithiothreitol (DTT) at pH 8 and left for at least 1 h at room temperature before filtration (0.22 μm Millex-GV$_{13}$, Millipore Co.) and CE analysis. Both caseins from milk and cheese were dissolved at 10 mg mL^{-1}, and the precipitate obtained from cheese caseins treated with plasmin was dissolved in 1 mL of the sample buffer.

CE Separations. CE was carried out with a Beckman P/ACE System 2200 (Beckman Instruments Inc., San Ramon, CA) controlled by a System Gold Software data system version 810. The separations were performed using two 50 μm i.d. capillaries, a fused-silica capillary (eCap, Beckman Instruments) of 57 cm (50 cm to the detector window) with negatively charged silanol sites on its surface, and a neutral capillary (eCAP Neutral Capillary, Beckman Instruments) of 45 cm (33 cm to the detector window) that utilizes a polyacrylamide layer covalently linked onto the inner wall to generate a hydrophilic surface. All experiments were carried out in the cationic mode (anode at the inlet and cathode at the outlet). The sample introduction was achieved by pressure injection for 5 s at 0.5 psi.

The analysis with the fused-silica capillary was performed according to the method of Ortega et al. (*23*). The run buffer was 50 mM sodium phosphate with 6 M urea (pH 3.0) containing 0.05% (w/v) HPMC. During sample analysis a constant voltage of 18.5 kV was applied, and the temperature of the separation was kept at 23 °C with circulating coolant surrounding the capillary. Between runs the capillary was purged for 5 min with run buffer.

The CE analysis carried out with the neutral capillary was performed following the method described by Albillos et al. (*22*). The run buffer was 0.32 M citric acid/0.02 M sodium citrate with 6 M urea (pH 3.0) containing 0.05% (w/v) HPMC. Separation was performed at 21 °C and a constant voltage of 25.09 kV. The capillary was rinsed sequentially between successive electrophoretic runs, with 0.1 M HCl (2 min), ionized water (2 min), and the rinsed buffer (pH 3.0) which contained 0.32 M citric acid, 0.020 M sodium citrate, 6 M urea, and 0.042 M 3-morpholinopropanesulphonic acid (5 min).

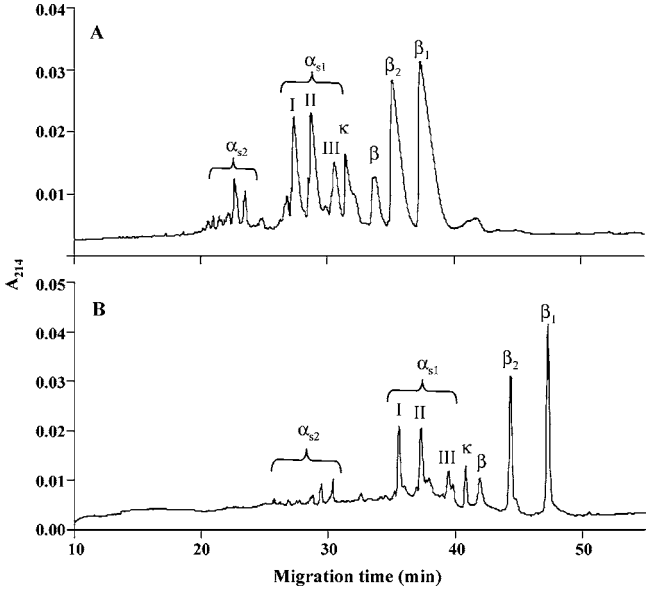

Figure 1. CE analysis of ewe's milk caseins. Separations were performed in a fused-silica capillary at 18.5 kV (∼35 mA) (**A**) and a neutral capillary at 25.09 kV (∼50 mA) (**B**). Other conditions are described in the Materials and Methods section. α_{s1}, α_{s1}-CN; α_{s2}, α_{s2}-CN; κ, κ-CN; β, β-CN; β_1, β_1-CN; β_2, β_2-CN.

For all experiments, detection was carried out at 214 nm (data collection rate of 5 Hz). The first electropherogram in a series was always discarded.

The repeatability of peak areas was assessed by replicate injections ($n = 10$) of a mixture of α_s-casein, β-casein, and κ-casein at 5 mg mL^{-1}. Relative standard deviations were lower than 6.2 and 4.9% for the fused-silica capillary and the neutral capillary, respectively.

Statistical Procedures. Partial least-squares regression (PLS), principal component regression (PCR), and principal component analysis (PCA) were applied to peak area obtained by CE for the prediction of the ripening time of the cheese. Data were autoscaled prior to model calculations. This normalization involved the substraction of the mean and then the divison of each value of a given variable by the standard deviation of all the values for this variable over the entire sample collection period (*24*). After normalization, all variables had the same weight because they had a mean of zero and unitary variance.

The root-mean-square error of prediction (RMSECV) obtained by cross validation used a measure of the ability of the two models to furnish accurate predictions and was calculated using the equation

$$\mathrm{RMSECV} = \left(\sum_{i=1}^{n}(t_i - t_{(i)})^2/n\right)^{1/2}$$

where t_i is the real ripening time for the ith sample of the standard cheese, $t_{(i)}$ is the predicted ripening time obtained with the model constructed without the ith sample, and n was the number of standard cheeses used in the calibration model ($n = 12$). This procedure is commonly called leave-one-out cross validation, and the RMSECV value can be considered an approximation of the prediction error (*20*, *25*).

Calculations were performed using the Q-PARVUS 3.0 package (*26*).

RESULTS AND DISCUSSION

Very similar casein patterns were obtained when ewe's milk caseins were analyzed using a fused-silica capillary and a neutral capillary (**Figure 1**). The only clearly visible difference between electropherograms obtained with both capillaries was the narrower peaks for the neutral capillary. Peak broadening was

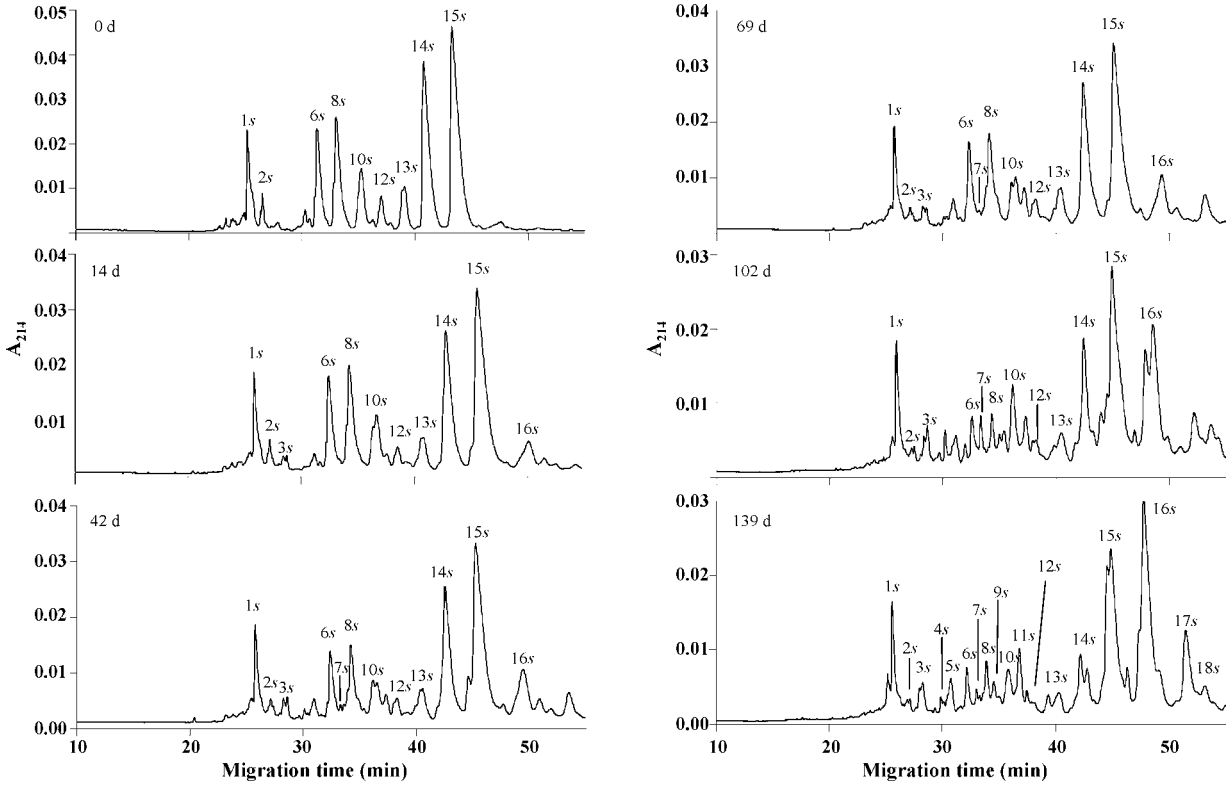

Figure 2. CE analyses of the pH 4.6 insoluble fraction of ewe's milk cheeses after different days of ripening. Separations were performed in a fused-silica capillary at 18.5 kV (~35 μA). Other conditions are described in the Materials and Methods section. Peak identification: 1*s*: *p*-κ-CN + α_{s2}-CN; 2*s*: α_{s2}-CN; 3*s*: unidentified; 4*s*: unidentified; 5*s*: γ_2-CN; 6*s*: α_{s1}-CN I; 7*s*: γ_1-CN; 8*s*: α_{s1}-CN II; 9*s*: unidentified; 10*s*: α_{s1}-CN III + γ_3-CN; 11*s*: unidentified; 12*s*: P; 13*s*: β-CN; 14*s*: β_2-CN; 15*s*: β_1-CN + P; 16*s*: α_{s1}-I-CN; 17*s*: P; 18*s*: P. P represents peptides from the action of plasmin on caseins.

likely caused by nonspecific interaction between casein and the charged inner surface of the fused-silica capillary. In fact, neutral surfaces are designed to coat the exposed silanol groups on the fused silica and serve two major functions: (i) to reduce electroosmotic flow and (ii) to prevent wall interaction with the sample.

Identification of peaks was based on the results of Cattaneo et al. (*27*) and Recio et al. (*28*, *29*). α_{s2}-Casein appeared at the early part of the electropherograms, giving two main peaks and a number of minor peaks. α_{s1}-casein was resolved as three peaks: I, II, and III. The β-casein fraction gave two major peaks (β_1- and β_2-caseins) and another minor peak (called β-casein) with a shorter migration time. Ovine β_1-casein has larger migration times because it has more phosphate groups than its respective β_2-casein (six vs five (*30*, *31*)). The κ-casein fraction was resolved by CE as one major peak.

Proteolysis of the Casein Fraction. Electropherograms of the pH 4.6 insoluble fraction throughout a 139-day ripening period (with sampling at 0, 14, 42, 69, 102, and 139 days) using a fused-silica capillary and a neutral capillary are shown in **Figures 2** and **3**. The peaks were indicated on the electropherograms with serial numbers (in order of migration time) followed by the letter *s* (fused-silica capillary) or *n* (neutral capillary). Identification of peaks is detailed in the figure caption.

In general, good resolution between intact casein (α_{s1}-casein, α_{s2}-casein, and β-caseins) and breakdown product was achieved at day 0. Electrophoretic profiles from 0-day-old cheese were very similar to the pH 4.6 insoluble fraction of cheese milk counterparts (**Figure 1**). However, the peak belonging to κ-casein disappeared with the concomitant appearance of a new peak corresponding to *p*-κ-casein (peak 1*s* and 1*n* in **Figures 2** and **3**, respectively). The *p*-κ-casein showed the same migration time as the major α_{s2}-casein component in the separation obtained with the fused-silica capillary (peak 1*s*). This overlapped due to the charged surface of this capillary not being detected when the neutral capillary was used (peaks 1*n* and 2*n*). κ-Casein is the principal casein fraction affected by chymosin in the primary phase of the milk clotting process. During this enzymatic reaction, the Phe_{105}-Met_{106} bond is specifically cleaved in bovine as well as in ovine κ-caseins (*32*), realizing *p*-κ-casein or κ-casein f(1-105) and κ-macropeptide or κ-casein f(106-171).

Throughout the ripening time α_{s1}-casein was extensively decreased (**Figures 2** and **3**). The hydrolysis of α_{s1}-casein, giving rise to α_{s1}-I-casein or α_{s1}-casein f(24-191), is one of the most important events during the ripening of cheese because of this role in the development of texture and flavor (*33*). The bond Phe_{23}-Phe_{24} has also been found to be the primary cleavage site by chymosin in a solution of ovine α_{s1}-casein (*34*). As α_{s1}-I-casein has a low electropositive charge due to its eight phosphorylated serines (*35*), this peak must be a large migration time. Thus, the peaks assigned as 16*s* (**Figure 2**) and 19*n* (**Figure 3**) are supposed to be α_{s1}-I-casein.

Another important change was the presence of a few peaks located on the intermediate zone of the electropherograms between α_{s1}- and α_{s2}-caseins. On the basis of their relative migration times on the electropherograms shown by different authors (*16*), they could be recognized as γ-caseins. It is known that plasmin acts mainly on β-casein, giving rise to γ_1-, γ_2-, and γ_3-casein (fractions 29-209, 106-209, and 108-209, respectively). From its structures, the migration times in CE should be γ_2-casein < γ_1-casein < γ_3-casein (*16*). In ovine milks the γ_1-casein from β_1-casein and the γ_1-casein from β_2-casein have the same migration time because the only difference between

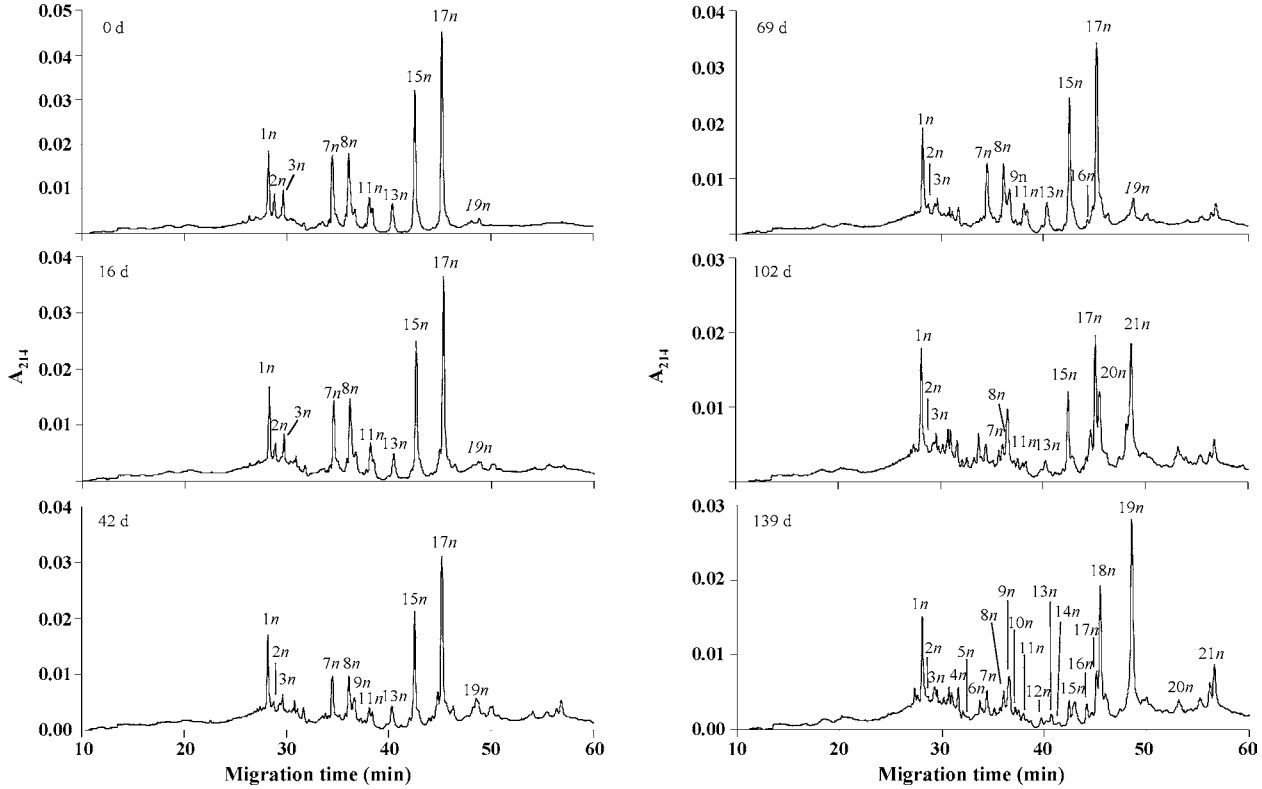

Figure 3. CE analyses of the pH 4.6-insoluble fraction of cheeses manufactured with ewe's milk after different days of ripening. Separations were performed in a neutral capillary at 25.09 kV (~50 μA). Other conditions are described under Materials and Methods. Peak identification: 1*n*: *p*-κ-CN; 2*n*: α_{s2}-CN; 3*n*: α_{s2}-CN + hydrolysis products; 4*n*: γ_2-CN; 5*n*: P; 6*n*: unidentified; 7*n*: α_{s1}-CN I; 8*n*: α_{s1}-CN II; 9*n*: γ_3-CN; 10*n*: P; 11*n*: α_{s1}-CN III; 12*n*: unidentified; 13*n*: β-CN; 14*n*: P; 15*n*: β_2-CN; 16*n*: P; 17*n*: β_1-CN; 18*n*: unidentified; 19*n*: α_{s1}-I-CN; 20*n*: P; 21*n*: P. P represents peptides from the action of plasmin on caseins.

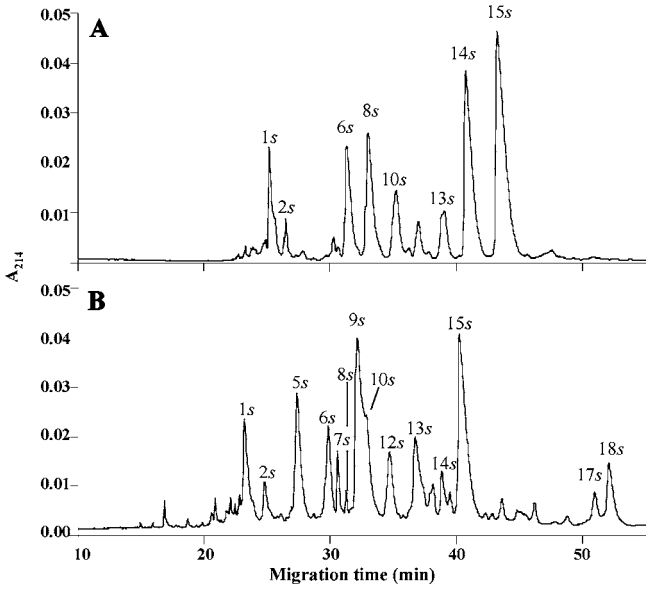

Figure 4. CE analyses of the pH 4.6 insoluble fraction of 0-day-old cheese (**A**) untreated and (**B**) treated with plasmin (0.076 U mL^{-1}, 240 min at 37 °C). Separations were performed in a fused-silica capillary at 18.5 kV (~35 mA). Other conditions are described in the Materials and Methods section. Peak identification: 1*s*: *p*-κ-CN + α_{s2}-CN; 2*s*: α_{s2}-CN; 5*s*: γ_2-CN; 6*s*: α_{s1}-CN I; 7*s*: γ_1-CN; 8*s*: α_{s1}-CN II; 9*s*: α_{s1}-CN III + γ_3-CN; 12*s*: P; 13*s*: β-CN; 14*s*: β_2-CN; 17*s*: P; 18*s*: P. P represents peptides from the action of plasmin on caseins.

β_1-casein and β_2-casein is an extra phosphorylation site in the Thr_{12} residue of the β_1-casein (*30*), so both γ_1-caseins f(29-209) are identical. γ_2-Casein has the lowest migration time, although it has two more residues than γ_3-casein because these two residues correspond to His and Lys, both positively charged at pH 3, the pH of analysis. To identify the different ovine γ-caseins, the casein fraction extracted from 0-day-old cheese was treated with plasmin (**Figures 4** and **5**; electropherograms obtained using a fused-silica capillary and a neutral capillary, respectively). These figures show a decrease in the β-casein fraction and a concomitant increase in the peaks named 5*s*, 7*s*, and 10*s* (**Figure 4**) and 4*n*, 9*n*, and 11*n* (**Figure 5**). On the basis of literature data (*16*), the order of migration time of the ovine γ-casein and α_{s1}-casein must be γ_2-casein < α_{s1}-casein I < γ_1-casein < α_{s1}-casein II < γ_3-casein < α_{s1}-casein III. Thus, the peaks 5*s*, 7*s*, and 10*s* (**Figure 4**) should be γ_2-, γ_1-, and γ_3-caseins, and the peaks 4*n* and 9*n* probably correspond to γ_2- and γ_3-caseins (**Figure 5**), respectively. Therefore, the analysis of the cheese using the fused-silica capillary allowed identification of the three ovine γ-caseins, although the γ_3-casein and α_{s1}-casein III had similar migration times and were integrated in the peak named 10*s*. There were other peaks in addition to γ-casein in the electropherograms (marked P) with large migration times that probably corresponded to hydrophilic proteose peptone components (*16*). A number of nonidentified peaks which represent peptides releasing from the breakdown of caseins were also detected.

A total of 18 and 21 peaks were visually recognized and matched in the electropherograms performed in the fused-silica capillary (**Figure 2**) and neutral capillary (**Figure 3**), respectively.

The extent of breakdown of α_s- and β-caseins was expressed as a relative percentage of peak areas of 0-day-old cheeses (**Figure 6**). The α_s- and β-caseins contents decreased with ripening time with respect to those at 0 days as a consequence

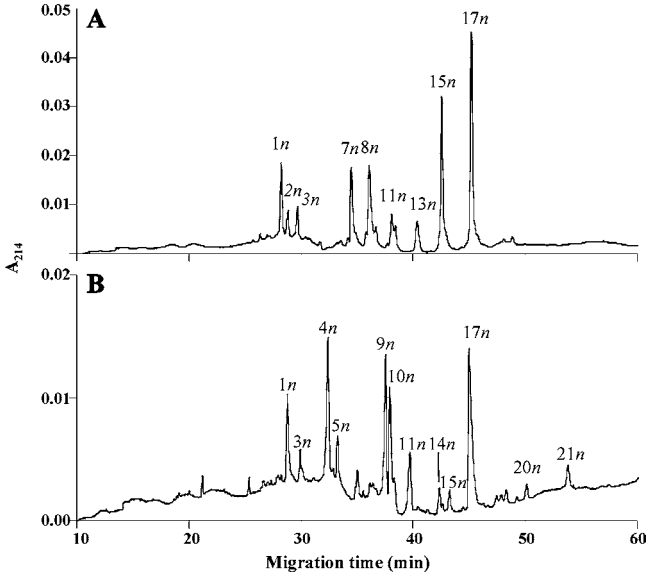

Figure 5. CE analyses of the pH 4.6 insoluble fraction of 0-days-old cheese (**A**) untreated and (**B**) treated with plasmin (0.076 U mL^{-1}, 240 min at 37 °C). Separations were performed in a neutral capillary at 25.09 kV (~50 mA). Other conditions are described in the Materials and Methods section. Peak identification: 1*n*: *p*-κ-CN; 3*n*: α_{s2}-CN; 4*n*: γ_2-CN; 5*n*: P; 9*n*: γ_3-CN; 10*n*: P; 11*n*: α_{s1}-CN III; 13*n*: β-CN; 14*n*: P; 15*n*: β_2-CN; 17*n*: β_1-CN; 20*n*: P; 21*n*: P. P represents peptides from the action of plasmin on caseins.

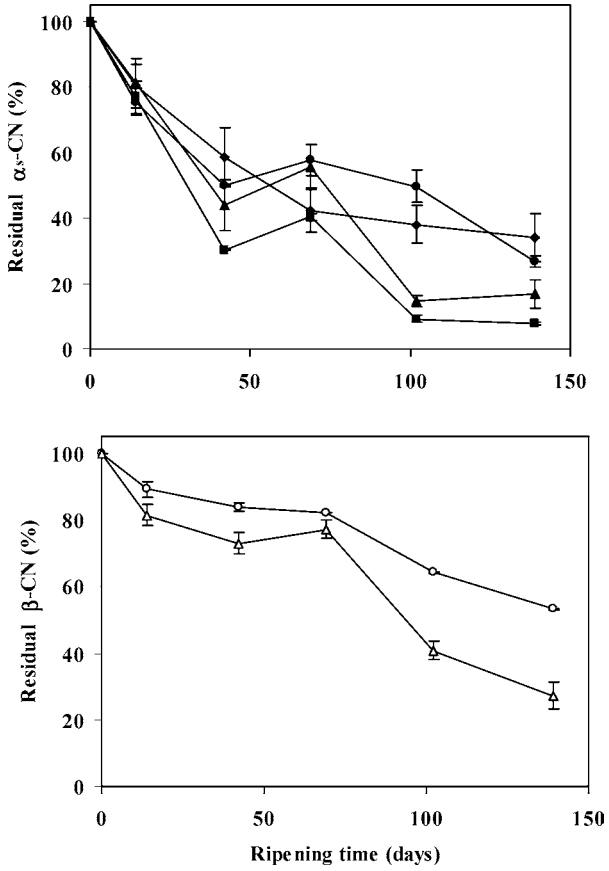

Figure 6. Changes in the residual α_s-CNs and β-CNs during ripening of ewe's milk cheese. The amount of residual proteins is expressed as a percentage of the amount in the corresponding 0-day-old cheese. Data obtained from electropherograms of fused-silica capillary. (▲) α_{s1}-CN I; (■) α_{s1}-CN II; (●) α_{s1}-CN III; (◆) αs_2-CN; (○) β_1-CN; (△) β_2-CN.

of the action of residual rennet, combined with the action of hydrolytic enzymes, released by microorganisms present in cheese (*36*). The α_s- and β-caseins were degraded up to 21 and 42%, respectively, by 19 days of ripening. The α_s-caseins variants (α_{s1}- and α_{s2}-caseins) displayed similar degradation patterns to one another, but different from those of β-caseins variants (β_1- and β_2-caseins). The β-caseins were broken down more slowly than α_s-caseins at early stages of ripening (59, 41, 11, and 19% of α_{s1}-, α_{s2}-, β_1-, and β_2-caseins, respectively, were degraded by 42 days of ripening). However, with a ripening time longer than 69 days, the β-caseins fractions were degraded faster, although the hydrolysis percentage at the end of the ripening period (139 days) was smaller than that of α_{s1}-casein. The casein that degraded to a higher degree was α_{s1}-casein II, with about 8% still intact at 139 days. On the other hand, α_{s2}-casein also showed a marked decrease up to 15 days, presenting a mean of intact protein on day 139 of 34%, compared to day 0.

Therefore, in this cheese made from ewe's milk, the fractions that correspond to the β-caseins region were less degraded than those that correspond to the α_s-caseins. That greater resistance of β-casein to enzyme hydrolysis was already pointed out by several authors (*15*, *36*, *37*). In contrast, Macedo and Malcata (*38*) observed similar degradation for α_s- and β-caseins after 35 days of ripening in Serra cheese manufactured from raw sheep's milk coagulated with a plant rennet.

Prediction of Ripening Time by Multivariate Regression Analysis. In a preliminary analysis, PLS regression was applied to the calibration samples with ripening times from 0 to 139 days ($n = 12$), using the areas of the visually recognized peaks, 18 and 21 peaks for the fused-silica capillary and neutral capillary, respectively (**Figure 2 and 3**), as the predictor variables. This first model, with the whole data set of the standard cheeses, indicated the peaks with a low modeling power of variables [peaks 3*s*, 7*s*, 17*s*, and 18*s* (fused-silica capillary) and peaks 11*n*, 13*n*, and 20*n* (neutral capillary)]. These peaks were discarded in the following analysis. Thus, **Table 1** shows the results when PLS and PCR regression were applied to the area of peaks selected (14 and 18 for the fused-silica capillary and the neutral capillary, respectively). These results include number of components selected by cross validation (*a*), the determination coefficient (R^2), the RMSECV, and the percentage of explained variance. The RMSECV was used as a diagnostic test for examining the errors in the predicted maturation time of the cheese samples (*20*). It indicates both precision and accuracy of prediction.

Table 1. Partial Least-Squares Regression (PLS) and Principal Components Regression (PCR) Results for the Prediction of the Ripening Times of Ewe's Milk Cheeses Obtained from the Capillary Electrophoresis Peak Areas

	fused-silica capillary		neutral capillary	
	PLS	PCR	PLS	PCR
a[a]	8	4	4	4
(R^2)[b]	0.9997	0.9823	0.9981	0.9975
RMSECV[c]	3.8	8.6	4.1	3.6
% var[d]	99.17	99.23	99.24	96.18

[a] Number of components selected by cross validation. [b] Determination coefficient. [c] Root-mean-square error of prediction (in days). [d] Percentage of explained variance.

In all regression methods the percentage of the explained variance for the model was >96%, yielding good correlation (R^2 values > 0.982) between the observed and calculated ripening times. The values of RMSECV obtained with the PLS

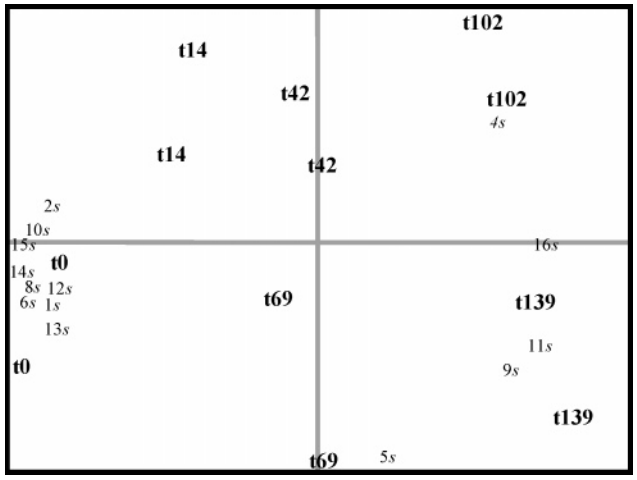

Figure 7. Biplot on the plane of the first and second eigenvector obtained by principal components analysis of peak area from electropherograms of fused-silica capillary. Training samples are represented by t_i (where i is the ripening time). The peak names represent the original variables, plotted according to the respective loading.

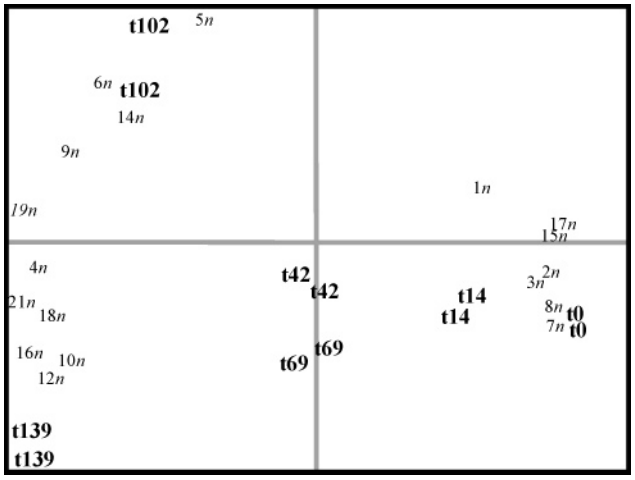

Figure 8. Biplot on the plane of the first and second eigenvector obtained by principal components analysis of peak area from electropherograms of neutral capillary. Training samples are represented by t_i (where i is the ripening time). The peak names represent the original variables, plotted according to the respective loading.

and PCR model were 3.8 and 8.6 days for the fused-silica capillary and 4.1 and 3.6 days for the neutral capillary, respectively.

The values of the RMSECV mentioned above can be considered to be relatively low. In fact, García-Ruiz et al. (*20*) and Poveda et al. (*39*) obtained values of 10.3 and 11.9 days, respectively, using PLS regression to calculate ripening time in standard Manchego cheeses bases on some physicochemical parameters and secondary proteolysis indices.

The equation that relates the ripening times predicted by cross validation ($t_{(i)}$) and real ripening times (t_i) obtained using PCR for the neutral capillary (the model with lowest RMSECV value) is

$$t_{(i)} = 0.9950t_i + 0.0682 \qquad (r^2 = 0.9917)$$

The fit for the prediction of the period between 0 and 139 days of ripening was good as shown by the values of r^2 obtained.

Additionally, principal component analysis (PCA) was applied to the area of peaks selected (except the peaks with a low modeling power of variance). When the fused-silica capillary was used to analyze the casein fractions of cheese, four principal components (PC) were obtained, accounting for 97% of the total variance. PC1 explained 77% of the total variance and the peak areas that correlated best with this PC were peak 1*s* (*p*-*κ*-casein and α_{s2}-casein), peak 6*s* (α_{s1}-casein I), peak 8*s* (α_{s1}-casein II), and peak 10*s* (α_{s1}-casein III and γ-casein). In fact, these caseins showed the major modification along the ripening time. A biplot showing the projection of the samples (scores) and the variables (loading) on the plane of the first and second eigenvector is given in **Figure 7**. It can be observed that samples appear separated according to their ripening time from left to right, although with the PC1 no separation occurred between samples of cheese at 42 and 69 days of ripening. PC2, which accounted for only 12% of the variation, distinguished the cheeses at 42 days of ripening from the cheeses at 69 days of ripening. On the other hand, similar analysis was carried out with the peak areas obtained from the neutral capillary. PC1 explained 71% of the total variance and the peak areas that correlated best with this PC were α_{s1}-casein I (peak 7*n*), α_{s1}-casein II (peak 8*n*), β_2-casein (peak 15*n*), β_1-casein (peak 17*n*), and peak 21*n*. **Figure 8** shows the distribution of the samples in the plane defined by PC1 and PC2 when a neutral capillary was used. PCA seem to distribute the cheese samples according to the level of ripening. However, in this case PC2, which explained 15% of the total variance, between cheese of 42 and 69 days of ripening distinguised worse than PC2 of the results of the PCA analysis applied to data obtained from the fused-silica capillary.

In conclusion, CE in coated and untreated capillaries under the present conditions has been shown to achieve adequate separation and to be appropriate to quantification of the casein fractions extracted from ewe's milk cheese throughout the ripening period. Using electrophoretic profiles and the two statistical methods tested, it was possible to predict the ripening times of commercial ewe's milk cheeses within approximately 4−8 days. The most accurate method, because it results in the lowest RMSECV, was the PCR model constructed with the peak areas of the neutral capillary electropherograms. However, it would be necessary to check out the predictive capability of the proposed equations with a larger number of commercial cheese samples and manufacturing standard cheese samples in different periods of the year to draw a firm conclusion. Additionally, chemometrical analysis of capillary electrophoresis proteolytic profiles has been shown to be a powerful method to get information on the biochemical process of proteolysis during the ripening of ewe's milk cheeses.

ABBREVIATIONS USED

CN, casein; CE, capillary electrophoresis; PCA, principal component analysis; PCR, principal component regression; PLS, partial least-squares regression; RMSECV, root-mean-square error of prediction by cross validation.

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