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Validation of capillary electrophoresis in the analysis of ewe's milk casein

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Abstract

Recent investigations have shown that capillary electrophoresis (CE) can be an alternative to other techniques such as polyacrylamide gel electrophoresis (PAGE) or sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in the qualitative analysis and separation of the different casein fractions in cow's and ewe's milk. However, past work has not yet clarified whether that method can achieve good quantifications. The present study has used a commercial whole ovine casein standard and a mixture of the standard and whole casein extracted from ewe's milk cheese to test the reliability of the technique. The results show that CE was able to quantify the ewe's milk caseins. The areas under four of the most representative peaks on the electrophoretogram for two α and two β-caseins (designated α-casein1$_{CE}$, α-casein2$_{CE}$, β-casein1$_{CE}$, and β-casein2$_{CE}$ in order of elution) were used to validate the method. In relation to linearity, coefficient of determination (r^2) values greater than 99% were obtained for the regressions of each of the caseins. Moreover, each casein yielded response factors with a relative standard deviation (R.S.D.) of less than or equal to 5. The coefficients obtained in the day-to-day reproducibility analysis were higher than those for the same-day repeatability, but all the values were within acceptable limits. In the study of accuracy, the percentage recovery rates for the α-casein fractions were higher than those for the β-casein fractions, hence quantification of the latter using this technique would appear to be more accurate under the conditions employed.

Keywords: Milk; Food analysis; Casein; Proteins

1. Introduction

Cheese ripening encompasses a series of physico-chemical 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 [4–6].

Breakdown of the different caseins and changes occurring in the caseins during ripening, along with the formation of peptides and other breakdown products of casein hydrolysis, have been studied in depth, to detect between-species variants and genetic differences [7], trace the formation of macro- and oligopeptides arising during normal ripening [8], and analyse the proteolytic activity of certain enzymes or microorganisms added to accelerate ripening [9–11].

Capillary electrophoresis (CE) is the first high-

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resolution analytical technique that requires simple sample preparation capable of determining whey proteins and caseins simultaneously [12,13]. CE analysis of the whey proteins in cow's milk yielded a level of reproducibility of the results comparable to that achieved with high-performance liquid chromatography (HPLC) [14] and better than that achieved with polyacrylamide gel electrophoresis (PAGE) [15]. CE also affords good quantification of casein macropeptide [16] and its genetic variants A and B [17], which may prove useful in studying the kinetics of the enzymatic hydrolysis of κ-casein during milk clotting caused by the action of rennet during cheese manufacturing. The main milk proteins (α-lactalbumin, β-lactoglobulin, α- and β-caseins) can be separated by CE in less than 10 min and quantified [18].

To date, very little research has dealt with the application of this technique to the analysis of caseins, and most has dealt with cow's milk caseins. Recently, the main protein components of ewe's milk were separated and identified using CE [13]. However, to our knowledge no work has been published regarding the use of CE in analyzing the caseins in ewe's milk cheese.

The object of the present study was to ascertain the reliability of CE in analyzing ovine casein fractions by testing for linearity, repeatability, reproducibility and accuracy.

2. Experimental

2.1. Samples and sample preparation

Ewe's milk cheese from the Ossau-Iraty-Brebis Pyrénées Appellation of Origin manufactured by Onetik-Berria (Macaye-Hazparren, France) that had been ripened for 2 d was used to obtain isoelectric ovine casein. 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 using an Ultra-Turrax blender and 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 dried powdered casein was stored in a refrigerator at 3–4°C until analysis. The whole ovine casein used as a standard was purchased from Sigma (St. Louis, MO, USA).

The reagents employed to prepare the buffers were electrophoresis grade dissolved in Ultrapure water (Milli-Q quality). The reagents trisodium citrate dihydrate, anhydrous citric acid, DL-dithiothreitol and urea were from Sigma.

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.

Both these solutions were filtered through GV-type hydrophilic membranes (Millipore) with a pore size of 0.45 μm and degassed by sonication in a water bath for 10 min.

An amount of 2 ml of the sample buffer was added to the casein sample. The suspension was mechanically shaken and sonicated in a water bath for 30 min. During that time the vial was occasionally shaken mechanically to enhance dissolution and then returned to the ultrasound bath. 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 Millenium 2010 software (Waters). The separation was carried out using a 50 cm×50 μm I.D. Celect P1 hydrophilically coated fused-silica capillary (Supelco, Bellafonte, PA, USA). The sample was injected hydrostatically at the anodic end of the capillary during 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 proteinaceous components were detected by UV absorbance at 214 nm. Before each injection the capillary was washed with ultrapurified water (Milli-

Q quality) for 5 min and equilibrated by purging with running buffer for another 5 min.

2.2. Validation conditions

Identification of the caseins was based on the results of Cattaneo et al. [13]. The areas under the four most representative peaks on the electrophoretogram (for α- and β-caseins), designated α-casein1$_{CE}$, α-casein2$_{CE}$, β-casein1$_{CE}$ and β-casein2$_{CE}$ in order of elution (Fig. 1), were used to validate the method.

Calculation of the validation parameters was based on the method of Castro et al. [19], and the results were processed using Statview 4.0 software (FPU Version 1992, Abacus Concepts). The analytical conditions employed in the validation method were the same as given above, the sample being dissolved in 2 ml of sample buffer.

The linearity determinations were repeated on three consecutive days, yielding three calibration curves for each of the four peaks: $y=a+bx$, $y'=a'+b'x$, and $y''=a''+b''x$. To calculate the regression lines, 20, 40, 60 and 80 mg of whole ovine casein standard was weighed out in duplicate. The regression line was calculated as $y=a+bx$, where x was casein concentration (in this case, since there were no commercial standards for the different ovine casein fractions, the concentration of each casein was taken as the total concentration of the whole casein in the sample buffer) and y was the response (peak area expressed as μVabs×s).

The first day, the coefficients of correlation (r) and determination (r^2), and confidence limits for the slope of the regression line and the independent term were calculated. The linearity of each peak was analyzed on the basis of the relative standard deviation (R.S.D.) value for the corresponding response factor.

The second and third days, the slopes and values of the independent term of the corresponding regression lines ($y'=a'+b'x$, and $y''=a''+b''x$) were tested to ascertain whether they were within the confidence limits calculated for the first day, in order to determinate if all the curves were statistically similar.

The reproducibility analysis employed the results for the calibration curves obtained on the three consecutive days. Reproducibility was calculated for each peak on the basis of the standard error and the R.S.D. for the areas under that peak for a known concentration on each of the three days.

Repeatability of the method was tested by weighing out 25 mg of isoelectric casein extracted from a single cheese and mixing it with 25 mg of whole ovine casein standard. Six replications were performed on the same day. The standard error and R.S.D. were calculated for the areas under each of the four peaks in each of the six replications.

Accuracy was determined using an added external standard. An amount of 20 mg of isoelectric casein extracted from a single cheese was taken as the blank. Three known quantities of whole ovine casein standard were then added, one quantity to each of three aliquots of the cheese casein extract (three replicates of each), to yield final quantities of: 20 mg casein$_{cheese}$ ($n=3$), 20 mg casein$_{cheese}$+30 mg casein$_{Sigma}$ ($n=3$), 20 mg casein$_{cheese}$+50 mg casein$_{Sigma}$ ($n=3$), and 20 mg casein$_{cheese}$+70 mg casein$_{Sigma}$ ($n=3$).

The R.S.D. for the responses (areas) for each peak was calculated, and the percentage recovery rate was established from the experimental response values [(blank+analyte)−blank] and the values read from the calibration curves for that same quantity of analyte using the R.S.D. values for the recovery rates. Student's t-test was applied to ascertain whether recovery was satisfactory.

3. Results and discussion

Fig. 1 shows the electropherograms of a mixture of: (a) standard and cheese whole casein, (b) cheese whole casein and (c) standard whole casein. Baseline has been forced by time (from minute 18 to 36) and peak integration was done clearing shoulders off the integrated peak areas. As it has been mentioned previously, identification of peaks was based on the results of Cattaneo et al. [13].

The complicated cheese casein preparation yield, together with intact casein fractions, a number of non-identified peaks which represent the peptides released from the breakdown of caseins. These peaks appear in the electropherogram and in some cases they can interfere with the identified peaks.

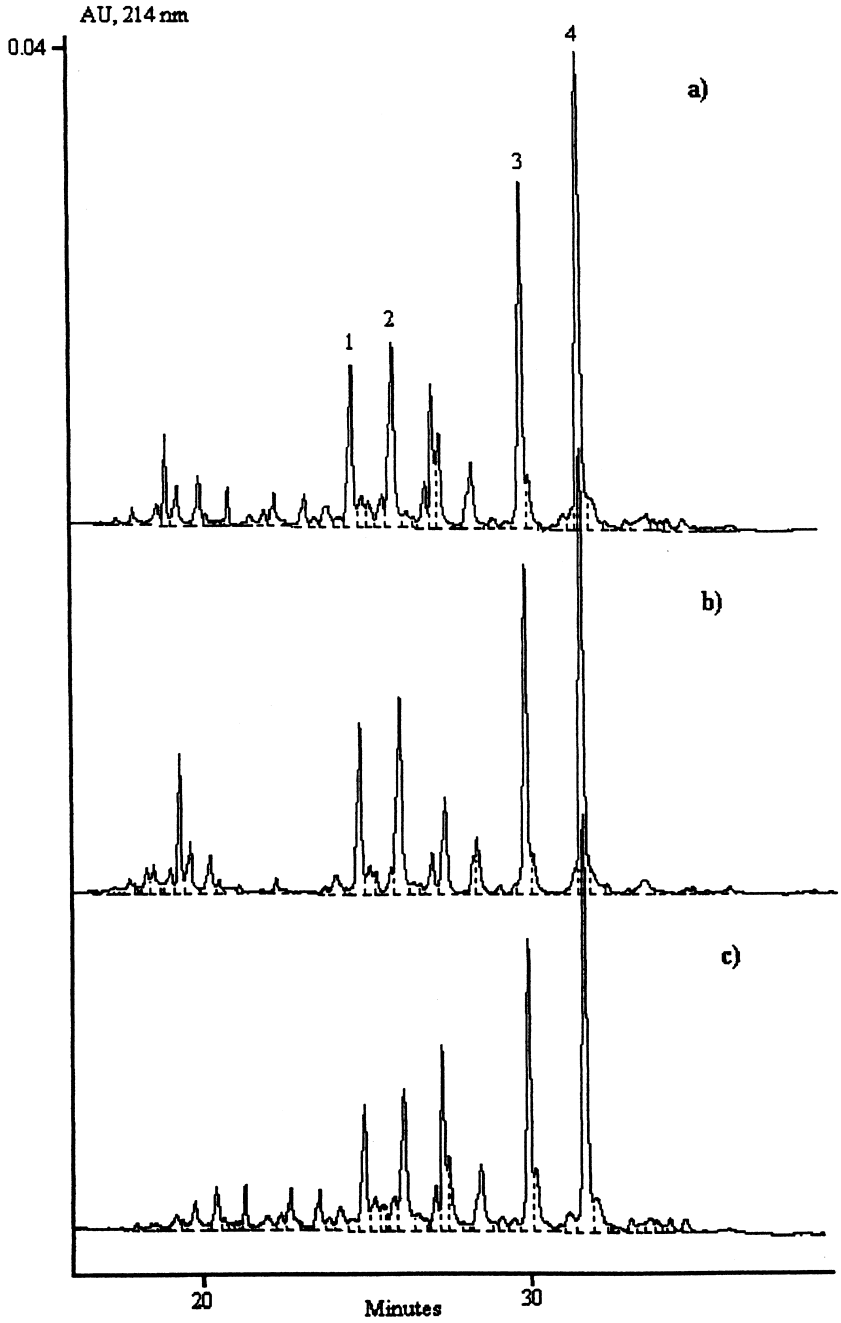

Fig. 1. CE analysis of: (a) a mixture of standard (15 mg/ml) and cheese whole ovine casein (10 mg/ml), (b) cheese whole ovine casein (20 mg/ml), (c) standard whole ovine casein (20 mg/ml). Identification of caseins according to Ref. [13] and peak designations in order of elution [hydrophilically coated fused-silica capillary, 50 cm×50 μm I.D.; buffer pH: 3.1; hydrostatic injection during 40 s; voltage applied: 25 kV; T=40°C; UV detection at 214 nm (see Section 2 Section 3 for details)]. Peaks: 1=α-casein1$_{CE}$; 2=α-casein2$_{CE}$; 3=β-casein1$_{CE}$; 4=β-casein2$_{CE}$.

Table 1
Results of the analysis of linearity for each of the selected peaks

Quantity	α-Casein1$_{CE}$		α-Casein2$_{CE}$		β-Casein1$_{CE}$		β-Casein2$_{CE}$	
	Response	f	Response	f	Response	f	Response	f
10	$51.7\cdot10^3$	$5.2\cdot10^3$	$67.7\cdot10^3$	$6.8\cdot10^3$	$118.5\cdot10^3$	$11.8\cdot10^3$	$191.3\cdot10^3$	$19.1\cdot10^3$
10	$48.7\cdot10^3$	$4.9\cdot10^3$	$63.6\cdot10^3$	$6.4\cdot10^3$	$108.4\cdot10^3$	$10.8\cdot10^3$	$178.2\cdot10^3$	$17.8\cdot10^3$
20	$104.0\cdot10^3$	$5.2\cdot10^3$	$140.1\cdot10^3$	$7.0\cdot10^3$	$228.4\cdot10^3$	$11.4\cdot10^3$	$356.7\cdot10^3$	$17.8\cdot10^3$
20	$105.5\cdot10^3$	$5.3\cdot10^3$	$140.8\cdot10^3$	$7.0\cdot10^3$	$229.6\cdot10^3$	$11.5\cdot10^3$	$361.7\cdot10^3$	$18.1\cdot10^3$
30	$157.4\cdot10^3$	$5.2\cdot10^3$	$209.1\cdot10^3$	$7.0\cdot10^3$	$329.1\cdot10^3$	$11.0\cdot10^3$	$502.3\cdot10^3$	$16.7\cdot10^3$
30	$159.3\cdot10^3$	$5.3\cdot10^3$	$211.5\cdot10^3$	$7.1\cdot10^3$	$334.7\cdot10^3$	$11.2\cdot10^3$	$511.9\cdot10^3$	$17.1\cdot10^3$
40	$220.9\cdot10^3$	$5.5\cdot10^3$	$289.2\cdot10^3$	$7.2\cdot10^3$	$459.5\cdot10^3$	$11.5\cdot10^3$	$691.0\cdot10^3$	$17.3\cdot10^3$
40	$206.2\cdot10^3$	$5.2\cdot10^3$	$271.5\cdot10^3$	$6.8\cdot10^3$	$429.2\cdot10^3$	$10.7\cdot10^3$	$654.5\cdot10^3$	$16.4\cdot10^3$
f [a]	$5.2\cdot10^3\pm0.1\cdot10^3$ (3.5)[b]		$6.9\cdot10^3\pm0.1\cdot10^3$ (3.8)		$11.2\cdot10^3\pm0.1\cdot10^3$ (3.4)		$17.5\cdot10^3\pm0.3\cdot10^3$ (5.0)	

In all cases the quantity has been expressed in mg of whole ovine casein standard/ml; response as μV×s; and response factor (f) as response/quantity.
[a] Arithmetic mean of the f values for each casein.
[b] R.S.D. (%) of f in parentheses.

3.1. Linearity

Table 1 shows the results of the analysis of linearity for each of the selected peaks.

The R.S.D. values for the response factors were in the range of 0–5%, considered adequate for verifying the linearity of regression lines [19]. The β-casein2$_{CE}$ presented a higher response per quantity of casein analyzed. This may be because it was present in proportionately larger amounts in the commercial casein or because its slower migration rate yielded higher response rates. Additionally this casein showed a higher R.S.D. value of the response factors.

The coefficient of correlation (r) values (Table 2) for the four caseins were all very close to one, hence there was a close relationship between the amount of casein and detector response for all the peaks. In all cases there was a positive correlation greater than 99.8%. Also, it can be observed (r^2) that the variance in the response as measured by the detector was caused by variations in casein concentration in 99.6% of cases (99.5% in case of β-casein$_{1CE}$). These results were similar to those reported for CE analysis of purine, which yielded r values greater than 0.999 [20], analysis of milk whey proteins [21], and analyses of other proteins like insulin [22] and low-molecular-mass peptides [23].

Except in the case of β-casein2$_{CE}$, the value of 0 fell within the confidence limits for the independent term, hence the method is unbiased. However, in all cases there was a high probability (68.56, 59.64, 47.77 and 95.03%, respectively) that the regression line would not pass through the origin (Table 2), and hence there would appear to be an intrinsic systematic error built into the method. Probably the small bias could be caused by a small integration error.

Table 2
Regression equations for the calibration curves for the four caseins analyzed by CE

Casein	Regression equation	r	r^2	b			a		
				Lower limit	Upper limit	p	Lower limit	Upper limit	p
α-Casein1$_{CE}$	$y=-4194.5+5436.6x$	0.998	0.996	$5.1\cdot10^3$	$5.8\cdot10^3$	<0.0001	$-13.5\cdot10^3$	$5.2\cdot10^3$	0.3144
α-Casein2$_{CE}$	$y=-4286.8+7139.9x$	0.998	0.996	$6.7\cdot10^3$	$7.6\cdot10^3$	<0.0001	$-16.0\cdot10^3$	$7.4\cdot10^3$	0.4036
β-Casein1$_{CE}$	$y=5795.3+10\,955.2x$	0.998	0.995	$10.2\cdot10^3$	$11.7\cdot10^3$	<0.0001	$-15.1\cdot10^3$	$26.7\cdot10^3$	0.5223
β-Casein2$_{CE}$	$y=27\,990+16\,119.1x$	0.998	0.996	$15.1\cdot10^3$	$17.1\cdot10^3$	<0.0001	$0.1\cdot10^3$	$55.9\cdot10^3$	0.0497

b=Slope; a=independent term.

Table 3
Equations for the two additional regressions calculated subsequent to the initial calculation on each of the two days following the first day in the consecutive three-day study

Casein	Regression equation 2	Regression equation 3
α-Casein1$_{CE}$	$y'=-4914.3+5557.1x$	$y''=-3423.3+5550.3x$
α-Casein2$_{CE}$	$y'=-4662.5+7267.4x$	$y''=-113.0+7199.8x$
β-Casein1$_{CE}$	$y'=10\,755.0+10\,997.3x$	$y''=21\,058.8+10\,696.4x$
β-Casein2$_{CE}$	$y'=32\,418.5+16\,301.9x$	$y''=51\,726.0+15\,584.2x$

The slope and the independent term for the two additional regression lines calculated on each of the following two days (Table 3) in the three-day reproducibility analysis fell within the confidence limits calculated for the regression lines for the four caseins calculated on the first day of the analysis (Table 2). Therefore, there were no significant differences between the slope of the regression line and the *y*-intercept in any of the cases. The three calibration curves calculated for each of the caseins were statistically similar.

3.2. Reproducibility

The data points used to construct the three calibration curves referred to above were also employed in the analysis of reproducibility, also termed day-to-day repeatability. Table 4 shows that the R.S.D. values for all the response rates (duplicate readings taken on three different days; $n=6$) ranged between 1.7 and 5.4%. These values were slightly higher than those recommended by Castro et al. [19], who noted that results with R.S.D. values of less than 1–2% were usually acceptable for analyses of precision (reproducibility+repeatability) of instrumental systems. Nevertheless, those same workers also accepted values lower than 4–5% when examining reproducibility for analytical methods on an overall basis. In any case, the values obtained were similar to and in some cases even lower than those obtained in the CE analysis of milk whey proteins [14]. The differences observed with respect to these aforementioned results may have been due to the fact that those workers used proteins extracted from milk while in the present study we have used a commercial ovine casein standard.

3.3. Repeatability

The results for the analysis of repeatability are shown in Table 5.

The R.S.D. values calculated were similar to those reported for CE analysis of proteins [22,24] and oligopeptides [23]. The R.S.D. values for an analysis of commercial bovine milk whey protein standards carried out under basic pH conditions ranged be-

Table 5
Results of the analysis of repeatability

Casein	Response ($n=6$)	R.S.D.
α-Casein1$_{CE}$	$143.8\cdot10^3\pm1.8\cdot10^3$	3.0
α-Casein2$_{CE}$	$191.8\cdot10^3\pm1.6\cdot10^3$	2.0
β-Casein1$_{CE}$	$290.6\cdot10^3\pm2.4\cdot10^3$	2.0
β-Casein2$_{CE}$	$443.8\cdot10^3\pm3.8\cdot10^3$	2.1

The response has been expressed as μV×s.
R.S.D.=Relative standard deviation (%).

Table 4
Results of the analysis of reproducibility

	α-Casein1$_{CE}$		α-Casein2$_{CE}$		β-Casein1$_{CE}$		β-Casein2$_{CE}$	
Quantity	Response ($n=6$)	R.S.D.	Response ($n=6$)	R.S.D.	Response ($n=6$)	R.S.D.	Response ($n=6$)	R.S.D.
10	$49.8\cdot10^3\pm0.5\cdot10^3$	2.2	$66.7\cdot10^3\pm0.8\cdot10^3$	2.9	$118.0\cdot10^3\pm2.6\cdot10^3$	5.4	$190.0\cdot10^3\pm3.3\cdot10^3$	4.2
20	$107.8\cdot10^3\pm1.4\cdot10^3$	3.2	$143.8\cdot10^3\pm1.7\cdot10^3$	2.9	$235.5\cdot10^3\pm2.6\cdot10^3$	2.7	$368.7\cdot10^3\pm4.0\cdot10^3$	2.6
30	$161.4\cdot10^3\pm1.5\cdot10^3$	2.3	$214.6\cdot10^3\pm2.1\cdot10^3$	2.3	$338.7\cdot10^3\pm2.4\cdot10^3$	1.8	$517.2\cdot10^3\pm3.6\cdot10^3$	1.7
40	$215.8\cdot10^3\pm2.5\cdot10^3$	2.8	$280.3\cdot10^3\pm3.1\cdot10^3$	2.7	$446.3\cdot10^3\pm5.0\cdot10^3$	2.7	$673.9\cdot10^3\pm6.8\cdot10^3$	2.5

Six replications were performed for each concentration measurement. The quantity has been expressed in mg of whole ovine casein standard/ml and the response as μV×s. R.S.D.=Relative standard deviation (%).

tween 1.9 and 2.4% [14]. For whey proteins extracted from raw and UHT milk, the R.S.D. values were higher. On the other hand, R.S.D. values of around 1% have been reported in the analysis of milk whey proteins under acid pH conditions [21].

The values calculated in this study were lower than those obtained by Chen and Zang [18] for different analytical conditions and were equivalent to those reported by Jong et al. [12], who found R.S.D. values of between 2 and 4% for simultaneous analysis of bovine milk whey proteins and caseins using the same technique employed in our study.

3.4. Accuracy

In the absence of commercial standards for each of the caseins, the accuracy was analyzed by addition of a whole-casein standard, as explained in Section 2. A cheese casein sample was used as a blank, with one of three different quantities of casein added to aliquots of the blank. Three replications were performed for the blanks and for each quantity. The mean recovery rate and the R.S.D. values for the recovery rates were calculated for each peak. The results are shown in Table 6.

The highest R.S.D. values and lowest recovery rates were for the α-casein1$_{CE}$ and α-casein2$_{CE}$. The reason for this was that the percentage recovery calculated for the lowest concentration (see Table 6) was 110.4 and 113.0% for both these caseins, which increased the mean value. However, applying Student's *t*-test to each of the four caseins failed to yield any significant differences in the percentage recovery rates recorded.

Cifuentes et al. [14] calculated similar R.S.D. values in CE analysis of milk whey proteins and concluded that the accuracy was slightly lower than yet still equivalent to that of HPLC.

For the β-caseins$_{CE}$ the percentage recovery rates were close to 100% for the three concentrations

Table 6
Results of the analysis of accuracy

	Casein			
	α-Casein1$_{CE}$	α-Casein2$_{CE}$	β-Casein1$_{CE}$	β-Casein2$_{CE}$
Casein$_{cheese}$ (20 mg); (A)	$54.6\cdot10^3$	$75.0\cdot10^3$	$108.3\cdot10^3$	$166.3\cdot10^3$
Casein$_{cheese}$ (20 mg)+casein$_{Sigma}$ (30 mg); (B)	$140.4\cdot10^3$	$191.8\cdot10^3$	$284.2\cdot10^3$	$443.6\cdot10^3$
Casein$_{Sigma}$ (30 mg); (C)=[(B)−(A)]	$85.9\cdot10^3$	$116.8\cdot10^3$	$176.0\cdot10^3$	$277.3\cdot10^3$
Casein$_{Sigma}$ (30 mg); (D) [from calibration curves]	$77.8\cdot10^3$	$103.4\cdot10^3$	$172.6\cdot10^3$	$273.0\cdot10^3$
R_1=[100·(C)/(D)]	110.4	113.0	101.9	101.6
Casein $_{cheese}$ (20 mg)+casein$_{Sigma}$ (50 mg); (E)	$189.1\cdot10^3$	$255.7\cdot10^3$	$382.0\cdot10^3$	$605.1\cdot10^3$
Casein$_{Sigma}$ (50 mg); (F)=[(E)−(A)]	$134.5\cdot10^3$	$180.8\cdot10^3$	$273.7\cdot10^3$	$438.8\cdot10^3$
Casein$_{Sigma}$ (50 mg); (G) [from calibration curves]	$132.7\cdot10^3$	$175.4\cdot10^3$	$282.4\cdot10^3$	$435.1\cdot10^3$
R_2=[100·(F)/(G)]	101.4	103.0	96.9	100.9
Casein$_{cheese}$ (20 mg)+casein$_{Sigma}$ (70 mg); (H)	$253.5\cdot10^3$	$336.8\cdot10^3$	$504.4\cdot10^3$	$784.9\cdot10^3$
Casein$_{Sigma}$ (70 mg); (I)=[(H)−(A)]	$198.9\cdot10^3$	$261.9\cdot10^3$	$396.1\cdot10^3$	$618.6\cdot10^3$
Casein$_{Sigma}$ (70 mg); (J) [from calibration curves]	$187.9\cdot10^3$	$247.5\cdot10^3$	$392.2\cdot10^3$	$597.2\cdot10^3$
R_3=[100·(I)/(J)]	105.9	105.8	101.0	103.6
R, (total recovery rate)	105.9	107.3	99.9	102.0
R.S.D.	4.3	4.8	2.7	1.4
$t_{Student}$ = [(|100−*R*|·$\sqrt{n}$)/R.S.D.]	2.38	2.63	0.03	2.53
$t_{(0.05,\ 2)}$	4.30	4.30	4.30	4.30

(A), (B), (E), (H): response from three replicates (n=3); (A–J): response as μV×s.
R_1, R_2, R_3 and R=Recovery rate (%).
R.S.D.=relative standard deviation of R (%).

analyzed (see Table 6). For the α-caseins, in contrast, the percentage recovery rates closest to 100% were recorded for the intermediate concentration situated midway along the interval used for the regression line, that is, in the region of the line where the confidence interval narrowed; in other words, in the conditions employed the technique would appear to afford greater accuracy for the β-caseins.

4. Conclusions

CE is extremely well-suited to the analysis and quantification of ewe's milk casein fractions. Ovine casein fractions can be satisfactorily resolved in less than 40 min. CE has been shown to achieve adequate separations and to be appropriate for quantification of the casein fractions extracted from ewe's milk cheese. The reliability of the technique was verified by a preliminary analysis of the linearity, reproducibility, repeatability and accuracy. The lack of individual casein standards makes difficult the accuracy determination. Nevertheless, by using the addition of a whole-casein standard it has been obtained an approximation of the accuracy of the method.

In order to continue this work, it should be necessary to get standards for each of the ovine caseins to be able to establish the linearity ranges and the detection and quantification limits.

In forthcoming studies this technique will be used to analyse the casein fractions during the ripening of ewe's milk cheese. This will make it possible to achieve partial characterization of the cheese during ripening and evaluate the effect of changes in technological parameters during the cheese-making process, e.g., addition of proteolytic enzymes to accelerate cheese ripening, on the casein fractions.

References

[1] B.A. Law, Dairy Sci. Abstract 43 (1981) 143.
[2] P.F. Fox, J. Dairy Sci. 72 (1989) 1379.
[3] G. Urbach, Int. Dairy 3 (1993) 389.
[4] R. Grappin, T.C. Rank, N.F. Olson, J. Dairy Sci. 68 (1985) 531.
[5] P.F. Fox, Food Biotechnol. 2 (1988) 133.
[6] P.F. Fox, B.A. Law, Food Biotechnol. 5 (1991) 239.
[7] L. Chianese, R. Mauriello, L. Moio, N. Intorcia, F. Addeo, J. Dairy Res. 59 (1992) 39.
[8] F.J. Ibañez, M.I. Torres, A.I. Ordóñez, Y. Barcina, Chem. Mikrobiol. Technol. Lebensm. 17 (1995) 37.
[9] A. Vafopoulou, E. Alichanidis, G. Zerfiridis, J. Dairy Res. 56 (1989) 285.
[10] M. Núñez, A.M. Guillen, M.A. Rodríguez-Marín, A.M. Marcilla, P. Gaya, M. Medina, J. Dairy Sci. 74 (1991) 4108.
[11] A. Picon, P. Gaya, M. Medina, M. Nuñez, J. Dairy Sci. 78 (1995) 1238.
[12] N. Jong, S. Visser, C. Olieman, J. Chromatogr. A 652 (1993) 207.
[13] T.M.P. Cattaneo, F. Nigro, P.M. Toppino, V. Denti, J. Chromatogr. A 721 (1996) 345.
[14] A. Cifuentes, M. Frutos, J.C. Diez-Masa, J. Dairy Sci. 76 (1993) 1870.
[15] N.M. Kinghorn, C.S. Norris, G.R. Paterson, D.E. Otter, J. Chromatogr. A 700 (1995) 111.
[16] J. Otte, L. Midtgaard, K.B. Qvist, Milchwissenschaft 50 (1995) 75.
[17] J. van Riel, C. Olieman, Electrophoresis 16 (1995) 529.
[18] F.-T.A. Chen, J.-H. Zang, J. Assoc. Off. Anal. Chem. 75 (1992) 905.
[19] M. Castro, S. Gascon, M. Pujol, J.M. Sans, P. Vicente, in: Monografía de Asociación Española de Farmaceúticos de la Industria, Sección Catalana; Comisión de Normas de Buena Fabricación y Control de Calidad, Editada por Hewlett-Packard, Barcelona, 1989.
[20] C.W. Demarest, E. Monnot-Chase, J. Jiu and R. Weinberger, in: P.D. Grossman, J.C. Colburn (Eds.), Capillary Electrophoresis – Theory and Practice, Academic Press, London, 1992, Ch. 11, p. 301.
[21] J. Otte, K.R. Kristiansen, M. Zakora, K.B. Qvist, poster presented at the Proceedings of the 3rd International Symposium on Capillary Electrophoresis, University of York, UK, August 1994.
[22] P.D. Grossman, J.C. Colburn, H.H. Lauer, R.G. Nielsen, R.M. Riggin, G.S. Sittampalam, E.C. Rickard, Anal. Chem. 61 (1989) 1186.
[23] W. Bockelmann, T. Hoppe-Seyler, K.J. Heller, Milchwissenschaft 50 (1995) 13.
[24] M. Lookabaugh, M. Biswas, I.S. Krull, J. Chromatogr. 549 (1991) 357.