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Determination of cow's milk in non-bovine and mixed cheeses by capillary electrophoresis of whey proteins in acidic isoelectric buffers

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Abstract

An improved method for the determination of cow's milk in non-bovine cheese is reported: electrophoresis of whey proteins in acidic, isoelectric buffers. Two background electrolytes (BGEs) have been tested: (i) 50 m*M* iminodiacetic acid (pH=isoelectric point=2.30 at 25°C), 0.5% hydroxyethylcellulose, 0.1% Tween 20 and 6 *M* urea (apparent pH 3.1), E=300 V/cm, for the separation of α-lactalbumins (α-LAs); (ii) a BGE with the same composition, but supplemented with 10% Tween 20, E=450 V/cm, for the fractionation of β-lactoglobulins (β-LGs). Surfactants have a discriminating effect on the retention behaviour of the bovine α-LA and β-LG proteins, owing to the different strength of the protein–surfactant association complexes, and are needed for separating these two proteins from small peaks in the electropherograms generated by degradation of casein during cheese ripening. Novel equations are given for deriving the ratio of the area (or height) of bovine α-LA, or β-LG, to the area (or height) of ovine or caprine α-LA or β-LG (such ratios being typically used to determine the percentage of cow's milk in dairy products), since previous equations had marked drawbacks, such as non-linearity of the plots with increasing slopes at high cow's milk percentages, and too broad confidence limits at high cow's milk contents, where the peak area (or height) ratio tends asymptotically to infinite. With the novel procedures reported, contents of cow's milk as low as 1% can be quantified in goat's and ewe's cheeses. The present protocols give lower detection limits, are cheaper and more rapid than any other methodology reported in the literature, and can be easily applied to the routine quality control of binary and ternary cheeses.

Keywords: Cheese; Food analysis; Proteins; Whey proteins

1. Introduction

Dairy products, which constitute a major component of human food, are protected in almost all countries by specific laws. According to law, the animal origin of the milk used for manufacturing cheese must be declared by the producer; however, adulteration of goat and ewe cheese with cow's milk is relatively common, owing to: (i) seasonal fluctuations in the availability of goat's and ewe's milk; (ii) higher price of goat's milk, and particularly ewe's

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milk, in comparison to cow's milk; (iii) opportunity to use the overproduction of cow's milk without loss of profit. Therefore, reliable, rapid and sensitive control methods must be available to check the composition of milk, cheese and other dairy products.

This problem has been addressed by enzyme-linked immunosorbent assay (ELISA), slab gel electrophoresis and column liquid chromatographic methods. Richter et al. [1] developed an indirect competitive ELISA technique based on the evaluation of caseins and glycomacropeptides in milk. Beer et al. [2] also used ELISA for estimating bovine β-lactoglobulin in milk.

Gel isoelectric focusing has been used for analysing whey proteins [3–5] and caseins [6,7]. Molina et al. [8] predicted the percentages of cow's, goat's and ewe's milk in ''Iberico'' cheese by polyacrylamide gel electrophoresis (PAGE) of whey proteins, and Ramos and Juarez [9] evaluated the applicability of electrophoresis and immunoassay in milk mixtures. Both ELISA and slab gel electrophoresis provide semiquantitative valuable information, but they are slow, labour intensive and cannot detect small amounts of cow's milk in goat and ewe products. Chromatographic techniques in their different modes (reversed-phase, ion-exchange and size-exclusion) have also been applied to the evaluation of whey proteins [10–12]. Reversed-phase high-performance liquid chromatography has been used to determine cow's milk in buffalo mozzarella [13] as well as in goat [14] and ewe cheeses [15]. Chromatography has proved to be a valuable quantitative technique for protein analysis in dairy products; however, separation time is usually within the 30–50 min range, which is rather long for some routine quality control purposes.

Recently, a number of studies have demonstrated that high-performance capillary electrophoresis (HPCE) is a suitable technique for analysing milk proteins [16] by using uncoated [17–27], and coated capillaries [28–38]. Cattaneo et al. [34] have demonstrated the capability of an HPCE mode, i.e., capillary zone electrophoresis (CZE), to determine up to an 8% level of cow's milk in milk mixtures. Recently, Cartoni et al. [37,38] employed methyl silanized capillaries in the determination of down to 2% cow's milk in ewe and buffalo cheeses.

Righetti and co-workers [39–45] have developed fast CZE procedures using isoelectric buffers, which, due to their much reduced conductivities, are compatible with high voltage gradients, thus favouring high resolution with short migration times. These authors have used isoelectric acidic buffers for separating peptides [40,41], oligonucleotides [42,43] and wheat [44] and maize proteins [45].

We report here the fast identification and quantitation of cow's milk in goat and ewe cheeses on the basis of the CZE separation of whey proteins. Isoelectric buffers containing iminodiacetic acid and different concentrations of a non-ionic surfactant (Tween 20) were used.

2. Materials and methods

2.1. Reagents

Iminodiacetic acid (IDA), urea and bovine standards of α-lactalbumin (α-LA) and β-lactoglobulins (β-LGs) A and B were obtained from Sigma (St. Louis, MO, USA). The caprine and ovine α-LA and β-LG used as standards were purified in the laboratory by slab gel isoelectric focusing. Acetic acid and tris(hydroxymethyl)aminomethane (Tris) were from Fluka (Buchs, Switzerland). Hydroxyethylcellulose (HEC; average molecular mass, 27 000) was from Polysciences (Warrington, PA, USA). A buffer containing 0.3 *M* acetic acid and the necessary 2 *M* Tris for reaching pH 4.6 was prepared. Deionized water was used throughout (Barnstead deioniser, Sybron, Boston, MA, USA).

2.2. Apparatus

Fused-silica uncoated capillaries (33.5 cm×50 μm I.D.×375 μm O.D.) were obtained from Supelco (Bellefonte, PA, USA). The CZE experiments were carried out with an HP^{3D} capillary electrophoresis system (Hewlett-Packard, Palo Alto, CA, USA) equipped with a diode array spectrophotometric detector.

2.3. Origin and preparation of samples

Samples of cow, goat and ewe cheeses of guaran-

teed composition, including cheeses of a single animal origin, and binary and ternary mixtures, were supplied by Mercadona and Lácteas Toledo (Spain). Cheeses of guaranteed single animal origin were used to prepare several binary and ternary mixtures to be used as standards. For this purpose, fresh and ripened cow, goat and ewe cheeses provided by several manufacturers were used. Other cheese samples, elaborated with binary and ternary milk mixtures, were also used as standards and for evaluation purposes. In the latter case, both the composition declared by the manufacturer or sodium dodecyl sulfate (SDS)–PAGE data were available (Table 1). Sample preparation was similar to that proposed by Pellegrino et al. [14] but with some modifications. Thus, 0.5 g cheese samples were mixed with 1 ml distilled water and shaken at room temperature for 5 min in a vortex mixer. The extracts were centrifuged at 12 000 rpm (16 500 *g*) for 15 min at 4°C. A 500-μl aliquot of the supernatant was diluted with 250 μl of the acetic acid–Tris buffer of pH 4.6, left to stand for 20 min, and centrifuged again for 15 min. Desalted and preconcentrated samples were obtained with Microcon membranes of 10 000 M_r cut-off (Amicon, Millipore, Bedford, MA, USA). After centrifugation on the Microcon tubes at 8 000 rpm (11 000 *g*) for 30 min, the extracts were stored at −20°C until needed.

2.4. Recommended CZE procedures

New capillaries were conditioned at 60°C as follows: 5 min with 1 *M* NaOH, 5 min with 0.1 *M* NaOH, and 10 min with water. At the beginning of each working session the capillary was rinsed for 30 min with the running background electrolyte (BGE) at 25°C. No alteration of the baselines were observed using a rinse time of 2 min between samples. The two following BGEs and electric fields are recommended:

(i) For the separation of α-LAs: 50 m*M* IDA (pH=p*I*=2.30 at 25°C, where p*I*=isoelectric point), 0.5% HEC, 0.1% Tween 20 and 6 *M* urea (apparent pH 3.1). *E*=300 V/cm.

(ii) For the separation of β-LGs: a BGE with the same composition, but supplemented with 10% Tween 20. *E*=450 V/cm.

All buffers and samples were pressed through 0.45-μm Millipore filters, and loaded in the capillary using hydrostatic pressure (50 mbar) for 3 s. The signal was monitored at 214 nm.

3. Results and discussion

3.1. Separation of α-LAs of different animal origin

A BGE containing 50 m*M* IDA, 0.5% HEC and 6 *M* urea, and an electric field of 700 V/cm, was first used. The electropherograms of the fractions of ripened cheeses of a single animal origin, which were retained by the Microcon membranes, are shown in Fig. 1A–C. The α-LA and β-LG peaks were identified in all cases by spiking the cheese

Table 1
Declared and found cow's milk contents (in percentages) for a series of commercial Spanish cheeses

Manufacturer[a]	Reference	Found from α-LAs	Found from β-LGs
Entrepinares (H)	70–90[b]	73±3	67±5
Puente Cesta (H)	50–75[b]	60±2	57±4
Flor de Ronda (L)	80–95[b]	92±3	90±6
Flor de Ronda (H)	80–95[b]	96±3	92±6
Roncal (H)	Pure ewe[c]	ND	ND
Don Bernardo (H)	Pure ewe[c]	10±2	8±4
Alquerías del Niño Perdido (H)	Pure goat[c]	30±3	28±5
Lácteas Toledo (L)	Pure goat[c]	ND	ND
Lácteas Toledo (H)	60[c]	60±2	55±4

[a] Ripening time in parentheses: L, fresh cheese; H, ripened cheese.
[b] Obtained by SDS–PAGE according to Ref. [4] in another laboratory.
[c] Declared by the manufacturer.
ND, Not detected.

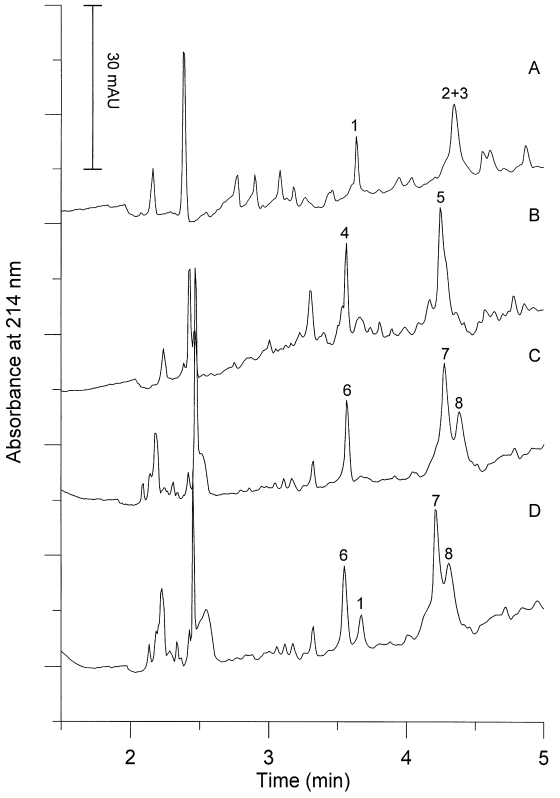

Fig. 1. Electropherograms of whey from (A) cow (B) goat and (C) ewe ripened cheeses. Electropherogram (D): ewe's extract spiked with 200 mg/l of bovine α-LA. Capillary: 33.5 cm (effective length 25 cm)×50 μm I.D.; hydrodynamic injection: 50 mbar×3 s; running buffer: 50 m*M* IDA, 6 *M* urea and 0.5% HEC; electric field: 700 V/cm (resulting in a current of 27 μA); detection at 214 nm. Peak identification: 1=bovine α-LA; 2=bovine β-LG A; 3=bovine β-LG B; 4=caprine α-LA; 5=caprine β-LG; 6=ovine α-LA; 7=ovine β-LG A; 8=ovine β-LG B.

extracts with the protein standards. Fresh cheeses gave similar electropherograms, which showed the same main peaks but with a simpler baseline. Retention of the proteins by the membranes was checked by also injecting the filtrates; in all cases, the α-LA and β-LG peaks were not present (electropherograms not shown). In addition, the electropherograms of the retained fractions were also injected after dilution to 50% with water. The peak areas showed a 50% reduction, which indicated the absence of non-linear effects, such as those associated to sample viscosity.

The α-LAs appeared at the same migration time for the goat and ewe samples (Fig. 1B and C, peaks 4 and 6, respectively), but at a different time for the cow's samples (Fig. 1A, peak 1). Unfortunately, in ripened cheeses, the cow's α-LA overlapped with a small peak of the goat and ewe samples, adjacent to the corresponding α-LA peak. This is shown in Fig. 1D, which corresponds to a ewe extract spiked with 200 mg/l bovine α-LA. Small peaks adjacent to α-LA peaks are usually due to casein degradation products during cheese ripening [14]. These small peaks were absent in fresh cheeses. On the other hand, the β-LG peaks appeared at similar migration times in all samples. Thus, a peak for the adequate evaluation of goat–ewe mixtures was not present in these electropherograms, and the evaluation of small amounts of cow's milk in ripened ewe cheeses was subjected to bias owing to overlapping of the cow's α-LA peak with a low but not negligible peak of the goat and ewe profiles.

Xu et al. [46] found that Tween 20 and other non-ionic surfactants have a discriminating effect on the retention behaviour of the bovine α-LA and β-LG proteins owing to the different strength of the protein–surfactant association complexes. Therefore, a 0.1% Tween 20 was added to the BGE. Also, the electric field was decreased to 300 V/cm. As shown in Fig. 2, in the presence of surfactant, simpler baselines, higher efficiencies and differential shifts in the peaks of the bovine, caprine and ovine α-LAs were produced. The peaks of the three α-LAs appeared at different migration times, thus making possible the determination of binary and ternary mixtures. Furthermore, as shown in Fig. 2D, the bovine α-LA peak (added to a ewe sample) appeared in a flat background region of the ewe electropherograms, well resolved from the small peak exhibited by the samples of ripened ewe cheese at a migration time slightly over the ovine α-LA peak. Similarly, the bovine α-LA peak appeared at a migration time between the caprine α-LA peak (number 4 in Fig. 2) and the small peak showed by the ripened goat cheeses at a larger migration time (Fig. 2B), well resolved from the former and satisfactorily resolved from the latter (results not shown).

The experiments were also performed at 500 V/cm, but a better resolution was obtained at 300 V/cm. With this electric field and using the recommended BGE (i), cheese samples elaborated with

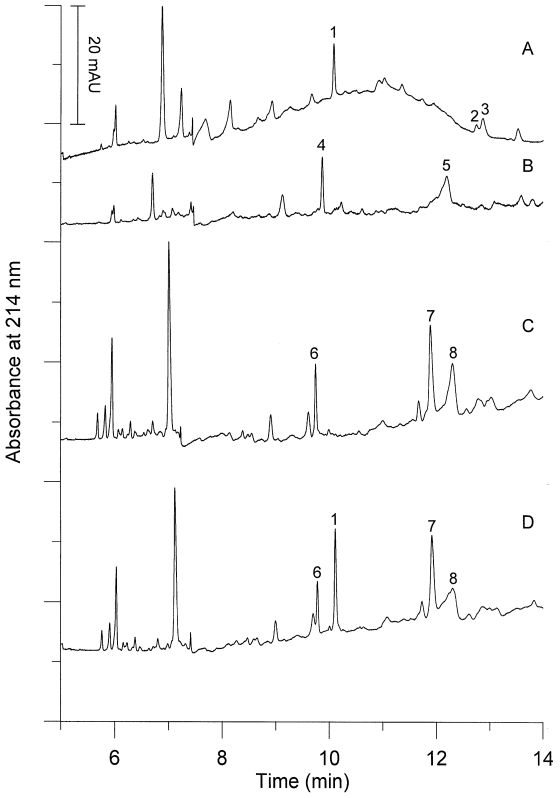

Fig. 2. Electropherograms of whey from (A) cow, (B) goat and (C) ewe ripened cheeses. Electropherogram (D): ewe's extract spiked with 200 mg/l of bovine α-LA. Running buffer: as in Fig. 1 but supplemented with 0.1% Tween 20 [BGE (i)]; electric field: 300 V/cm (resulting in a current of 11 μA). Peak identification and other conditions as in Fig. 1.

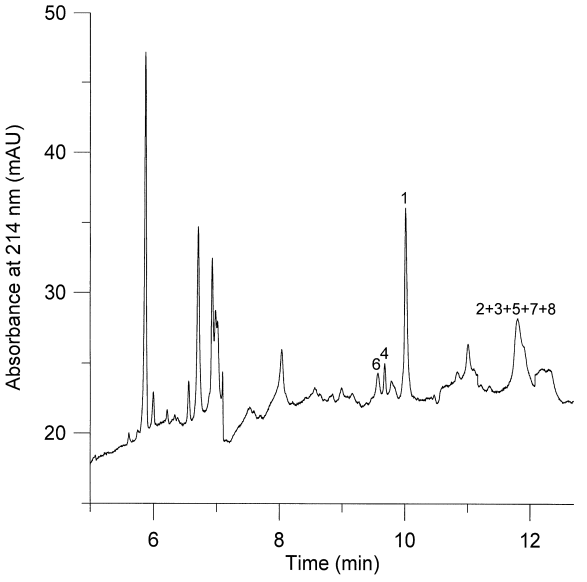

Fig. 3. Electropherogram of whey from a ternary cheese (Table 1, Entrepinares, ripened) with the following composition: *C*, 70%; *G*, 15%; *E*, 15%. Separation conditions as in Fig. 2 and peak identification as in Fig. 1.

mixtures of goat–cow and ewe–cow milk, and with a mixture of the three types of milk, were injected. As shown in the electropherogram of Fig. 3, excellent separation among the three α-LAs was achieved (peaks 6, 4, 1 representing α-LAs from ewe, goat and cow, respectively). The peak located between peaks 4 and 1 appeared only in ripened cheeses, and in the electropherogram showed in Fig. 3 this peak was particularly large, thus it was attributed to a degradation product. To measure the peak areas, the baseline was drawn by connecting the valley bottoms.

3.2. Separation of β-LGs of different animal origin

The possibility of using not only the α-LAs, but also the β-LGs, for developing improved procedures for the determination of the composition of binary and ternary cheeses, was studied. In order to achieve a better resolution of the β-LGs of different animal origin, the BGE composition was modified. First, the influence of the urea concentration within the 6–9 *M* range was examined. When the concentration of urea increased, the pH of the BGE also increased (up to 3.3 with 9 *M* urea), and the resolution improved up to 8 *M*, but remained unaffected at a higher urea concentration. Resolution of the bovine, ovine and caprine β-LGs also improved by increasing the Tween 20 concentration up to 15%, but sensitivity decreased by more than 10%. As shown in Fig. 4, with a 10% Tween 20, the peaks of the three β-LGs appeared at different migration times, thus making possible the determination of mixtures. A drawback was the progressive loss of resolution between the caprine and ovine α-LA peaks as the Tween 20 concentration increased. This confirmed that the optimisation of the separation of the α-LAs and the β-LGs of different origin in two independent procedures, was a better choice than the joint optimisation of the separation of the α-LA and β-LG

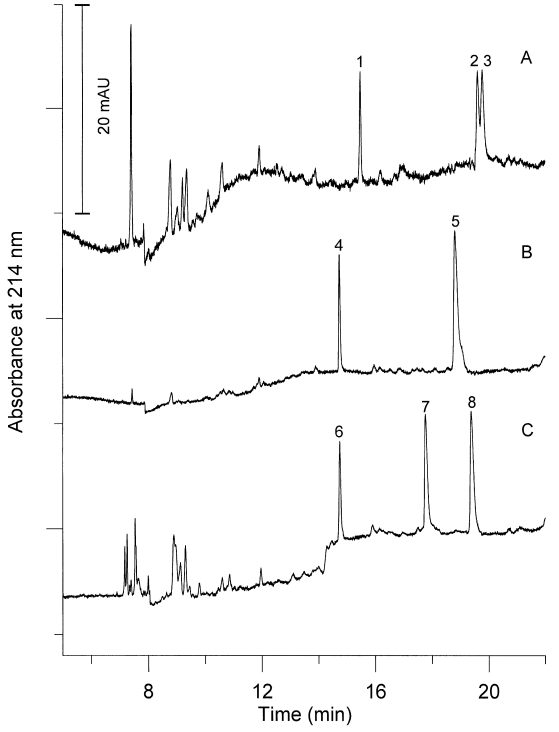

Fig. 4. Electropherograms of whey from (A) cow, (B) goat and (C) ewe ripened cheeses. Running buffer: 50 m*M* IDA, 8 *M* urea, 0.5% HEC and 10% Tween 20; electric field: 450 V/cm (resulting in a current of 10 μA); detection at 214 nm. Peak identification and other conditions as in Fig. 1.

peaks. The high Tween 20 concentration also led to an undesirable increase of the β-LG migration times. In order to offset this effect, thus reducing analysis time, the electric field was increased. Thus, a BGE containing 8 *M* urea and 10% Tween 20, and an electric field of 450 V/cm, was selected.

An electropherogram of a ternary mixture obtained under these conditions is shown in Fig. 5. As can be observed, the peaks of the β-LGs of different animal origin, including the bovine and ovine A and B genetic varieties (as confirmed by spiking the extracts with the corresponding standards), were resolved (see peaks 8, 7, 5, 2 and 3 in the 17 to 20 min elution window).

3.3. Quantitation studies

The ratio of the area (or height) of bovine α-LA, or β-LG, to the area (or height) of ovine or caprine

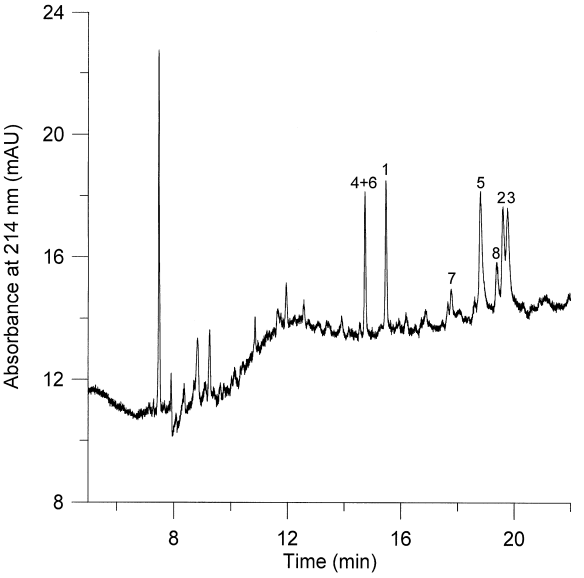

Fig. 5. Electropherogram of whey from a ternary cheese. Running buffer and other conditions as in Fig. 4.

α-LA or β-LG, have been used to determine the percentage of cow's milk in dairy products [12–15,37,38]. Drawbacks of using these ratios for constructing the calibration curves are non-linearity, with increasing slopes as the cow's milk percentage increases, and large confidence limits at high cow's milk contents, where the peak area (or height) ratio tends asymptotically to infinite. The curves are approximately linear up to 20% cow's milk, but the slope increases progressively, being very large over a ca. 60% cow's milk. A different approach which overcomes these limitations is proposed in this work. First, binary mixtures of cheeses were prepared and used as standards, for establishing the bovine/caprine and bovine/ovine sensitivity ratios. These were defined, respectively, as:

$$\frac{A_c}{A_g} = f^{\alpha}_{c,g} \cdot \frac{C}{G} \quad (1)$$

$$\frac{A_c}{A_e} = f^{\alpha}_{c,e} \cdot \frac{C}{E} \quad (2)$$

where A_c, A_g and A_e are the areas of the bovine, caprine and ovine α-LA peaks, and *C*, *G* and *E* are the percentages of cow, goat and ewe's milk in binary cheeses, respectively; finally, $f^{\alpha}_{c,g}$ and $f^{\alpha}_{c,e}$ are

the sensitivity ratios. For determining the sensitivity ratios for the α-LA and β-LG peaks, two series of binary mixtures, containing from 0 to 70% of cow's cheese and goat or ewe cheese, were prepared and analysed using the two recommended BGEs. Each series contained seven binary mixtures of fresh cheeses and seven binary mixtures of ripened cheeses. As shown in Figs. 6 and 7, straight-lines were obtained for the A_c/A_g versus C/G and A_c/A_e versus C/E plots, thus indicating that $f^{\alpha}_{c,g}$ and $f^{\alpha}_{c,e}$ were independent of the cow's milk contents. A linear behaviour was also observed for the $f^{\beta}_{c,g}$ and $f^{\beta}_{c,e}$ sensitivity ratios, which were defined by using expressions analogous to Eqs. (1) and (2), but by utilising the corresponding β-LG peaks. For the bovine and ovine β-LGs, the sum of the areas of the peaks 2 and 3, and 7 and 8, respectively, were used. Furthermore, in all cases, and within the experimental error range, the points obtained with fresh and ripened cheeses coincided on the same straight-lines. Experimental values of the sensitivity ratios were: $f^{\alpha}_{c,g}=0.71$, $f^{\alpha}_{c,e}=0.71$, $f^{\beta}_{c,g}=1.09$ and $f^{\beta}_{c,e}=0.82$. The differences from unity could be due to diverse molar absorptivities of the proteins at 214 nm, or maybe also to the various percentages of whey proteins of milk of different animal origin retained by cheese. The linear relationships thus obtained indicated that the A_c/A_g, A_c/A_e and A_g/A_e ratios can be directly used for establishing the composition in binary cheeses. Thus, according to Eqs. (1) and (2), the C/G and C/E ratios of cow–goat and cow–ewe cheeses are given by the A_c/A_g and A_c/A_e peak ratios divided by $f^{\alpha}_{c,g}$ and $f^{\alpha}_{c,e}$, respectively. Furthermore, the G/E ratio in goat–ewe binary cheeses is given by the A_g/A_e ratio divided by $f^{\alpha}_{c,e}/f^{\alpha}_{c,g}$. The same applies to the β-LG peaks. In order to directly calculate the cow, goat and ewe contents in binary and ternary cheeses using either the α-LA or the β-LG peak areas, the following expressions can also be used:

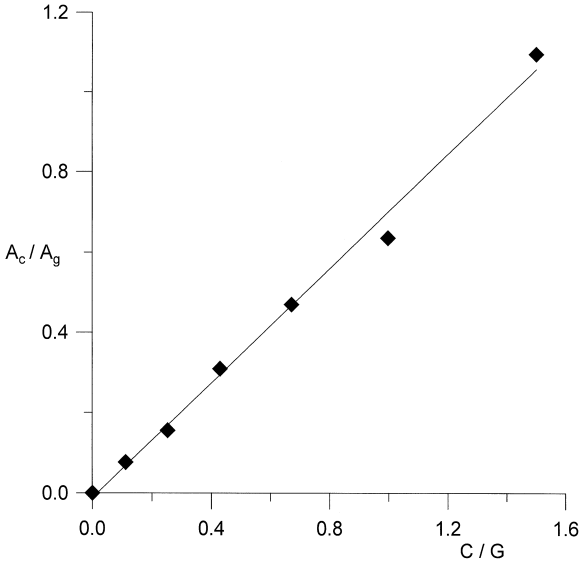

Fig. 6. A_c/A_g versus C/G for the α-LA peaks of a series of binary mixtures of cow and goat ripened cheeses.

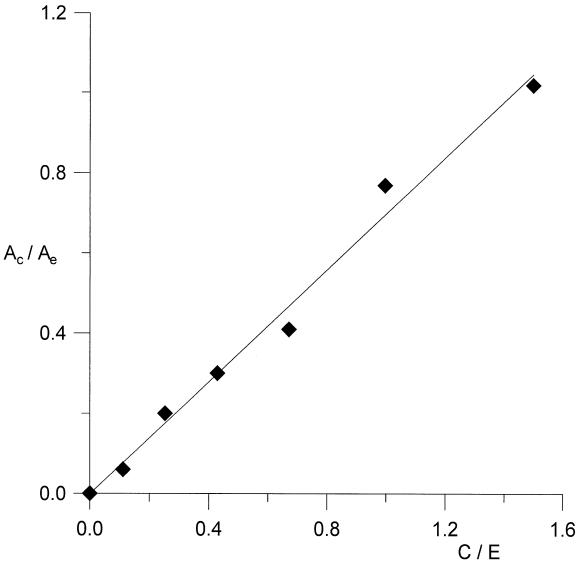

Fig. 7. A_c/A_e versus C/E for the α-LA peaks of a series of binary mixtures of cow and ewe ripened cheeses.

$$C = \frac{A_c}{A_c + f^{\alpha}_{c,g}A_g + f^{\alpha}_{c,e}A_e} \cdot 100\% = F_c 100\% \quad (3)$$

$$G = \frac{A_g}{A_c/f^{\alpha}_{c,g} + A_g + (f^{\alpha}_{c,e}/f^{\alpha}_{c,g})A_e} \cdot 100\% = F_g 100\% \quad (4)$$

$$E = \frac{A_e}{A_c/f^{\alpha}_{c,e} + (f^{\alpha}_{c,g}/f^{\alpha}_{c,e})A_g + A_e} \cdot 100\% = F_e 100\% \quad (5)$$

Eqs. (3), (4) and (5) are easily demonstrated by substituting ($f^{\alpha}_{c,g}$ A_g), ($f^{\alpha}_{c,e}$ A_e), ($A_c/f^{\alpha}_{c,g}$), [($f^{\alpha}_{c,e}/f^{\alpha}_{c,g}$)$A_e$], ($A_c/f^{\alpha}_{c,e}$) and [($f^{\alpha}_{c,g}/f^{\alpha}_{c,e}$)$A_g$] by their values obtained from Eqs. (1) and (2), and reorganising. Eqs. (3), (4) and (5) eliminate the need of constructing calibration curves other than those previously used to evaluate the sensitivity ratios. The equations can also be indistinctly used with binary and ternary cheeses. Furthermore, expressions analogous to Eqs. (3), (4) and (5) can be derived for the β-LG peaks, taking into account that in this case, and for the bovine and ovine β-LGs, the sum of the areas of the peaks 2 and 3, and 7 and 8, respectively, should be used. Sensitivity ratios can change with cheeses of different type, manufactured with a different procedure, or with milk of the same animal species but not of the same breed. For this reason, Eqs. (3), (4) and (5) could lead to small systematic errors when used with tabulated sensitivity ratios. In this case, experiments to establish the sensitivity ratios with cheeses of the same type as the problems to be analysed should be performed.

Alternatively, the standards can be used to plot F_c, F_g and F_e (calculated using the tabulated values of the sensitivity ratios) against C, G and E, respectively. As shown in Fig. 8, this gives almost linear or low curvature relationships, with zero intercepts and slopes close to the unity. In case of non-linearity, a least-squares fitting to the quadratic equation ($F_c = a+bC+cC^2$) gives accurate results. Several series of experimental data were also plotted in Fig. 8. As it can be observed, no significant deviations from the predicted linear behaviour were produced. An analogous plot was obtained with β-LG peaks.

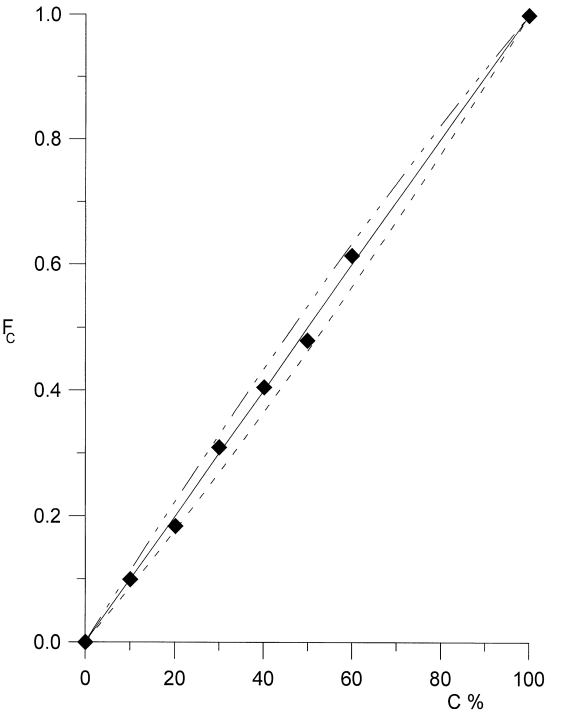

Fig. 8. Plot of F_c, calculated as defined in Eq. (3), and using $f^{\alpha}_{c,g}=f^{\alpha}_{c,e}=0.71$, against C. Slope of the regression straight-line (continuous line), 1.00. Dashed lines obtained by plotting F_c calculated using $f^{\alpha}_{c,g}=f^{\alpha}_{c,e}=0.60$ (upper line) and $f^{\alpha}_{c,g}=f^{\alpha}_{c,e}=0.80$ (lower line) for the same C values.

3.4. Figures of merit

In order to establish the detection limits, two cheese samples containing a 2% cow's milk and 98% goat or ewe's milk, respectively, were analysed eight times per day during 3 days. Ripened cheeses were analysed using BGE (i) at 300 V/cm. The cow contents were calculated using the tabulated sensitivity ratios and Eq. (3). Average intra-day (as the average of the three intra-day values, 7×3 degrees of freedom) and inter-day standard deviations (as total standard deviation, 23 degrees of freedom) were calculated. According to the IUPAC recommendation [47,48], the detection limits were calculated as 3.29 times the average intra-day standard deviations of the C values. Using this criterion, at the detection limit the α and β errors are 5%, and the relative standard deviation of C is 30%. The same study was repeated with the same samples but by using BGE (ii) at 450 V/cm. The β-LG peaks and an expression analogous to Eq. (3) were also used. The detection limits given in Table 2 were obtained from the three series of eight determinations, as the average of the corresponding three relative standard deviations of C multiplied by 3.29. The relationship between the standard deviations of the peak areas and the standard deviation of C can also be established theoretically, by using the law of propagation of errors; however, it should be taken into account that the random errors of the peak areas used as input variables in Eq. (3) (as well as in Eqs. (4) and (5))

Table 2
Detection limits[a] and repeatability for the determination of the cow's milk contents, *C*, by the proposed procedure

Peaks measured	Detection limits, *C* (%)	Repeatability of peak area (RSD, %)		Repeatability of migration time (RSD, %)	
		Intra-day	Inter-day	Intra-day	Inter-day
α-LA[b]	1.1[b]	0.9	1.5	0.4	0.6
β-LG[b]	2.0[b]	1.2	1.8	0.7	0.9
β-LG[c]	2.1[c]	1.2	1.9	0.7	1.0

[a] As 3.29 times the standard deviation, as recommended by IUPAC [47].
[b] Using a sample with $C=2\%$ and $G=98\%$, and another sample with $C=2\%$ and $E=98\%$, to estimate the standard deviation in the vicinity of the detection limit.
[c] The same as above, but with $C=5\%$.

are strongly correlated. This largely reduces the standard deviation of *C* in relation to the standard deviations of the peak areas. In other words, the relative standard deviation of the peak areas is much larger than the relative standard deviation of the ratio of peak areas used to calculate *C*.

Owing to the large standard deviation of the β-LG peaks, this study was also repeated using samples containing 5% cow's milk. The results, also given in Table 2, confirmed the better repeatability and lower detection limits obtained with the α-LA peaks in comparison to those yielded by the β-LG peaks. This can be attributed to the larger errors associated to the quantification of the sum of two peak areas, as occurs with the bovine and ovine β-LGs, in comparison to integration of a single peak area with the α-LAs. Migration time repeatability exhibited a relative standard deviation (RSD) below 2% in all cases. Better peak area and migration time repeatabilities were achieved with fresh cheeses.

3.5. Analysis of samples

The recommended procedures were used for analysing the commercial cheese samples indicated in Table 1. Cow's milk should not be present in pure goat and ewe cheeses, and the adulteration of supposedly pure and mixed cheeses with cow's milk is economically significant when the actual cow's milk contents surpasses the declared value by 10% or more. As shown in Table 1, both the detection of a few parts percent of cow's milk in pure goat and ewe cheeses, and a cow's milk excess larger than 10% in the declared composition of a binary or ternary cheese, can be addressed by the proposed CZE method. The results (columns 3 and 4) coincided with the declared composition and with the SDS–PAGE data, as given by the manufacturers, within the experimental error. Notice, however, that the values found by integration of the β-LG peaks are consistently 5–8% lower than those obtained upon α-LA quantitation, and also exhibit larger standard deviations. As indicated above, this latter can be explained by the larger random error associated to the quantification of the sum of two peak areas in comparison to the integration of a single peak area. On the other hand, a systematic error can only be produced by factors affecting the samples showed in Table 2, but not the standards used to obtain the sensitivity ratios, or to construct the calibration curves. As indicated above, the standards were prepared by mixing cheeses of a single animal origin, whereas the different milks used to elaborate the samples were mixed at the beginning of the cheese manufacture process. Maybe due to the easier denaturation of the bovine β-LGs, to water solubility differences, or for any other unknown reason affecting the manufacture process, the use of the β-LG peaks lead to a slight underestimation of the cow contents.

4. Concluding remarks

A quick and simple capillary electrophoresis procedure for the separation and quantitation of the bovine, caprine and ovine α-LA and β-LG peaks in binary and ternary cheeses has been developed. With the β-LG peaks, the bovine and ovine A and B genetic variants can also be distinguished as in-

dividual peaks (see Figs. 4 and 5). In the proposed procedure, separation is performed in a very short time, thus for instance, the separation of α-LAs is completed in 11 min, and the between-sample rinsing time can be reduced to 2 min or less. This is significantly less than the 30 min which are required in the chromatographic procedures described in the literature. A calibration approach, which is simpler and gives a better precision and accuracy than the procedures used in the literature, has also been described. The present procedures give lower detection limits, are cheaper and more rapid than any other protocol reported in the literature, and can be easily applied to the routine quality control of binary and ternary cheeses. An additional, non-negligible advantage, is that our protocol does not require coated capillaries, as necessary in methods adopting alkaline buffers, thus substantially reducing analysis costs. Due to the low operative pH, the effective suppression of silanol ionization not only prevents protein binding to the wall, but also minimises the electroendoosmotic flow, with markedly improved run reproducibility.

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