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Approach to the quantification of milk mixtures by partial least-squares, principal component and multiple linear regression techniques

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Abstract

Four of the most widely employed multivariate calibration methods, partial least-squares regressions (PLS-1 and PLS-2), principal component regression (PCR) and multiple linear regression (MLR) were applied to predict the percentages of ternary mixtures of cow's, ewe's and goat's milk based in the analysis of casein fraction by capillary electrophoresis. The prediction models were calculated by using three batches of 10 milk mixtures each prepared in three different seasons and were validated by applying them to the analysis of nine milk mixtures. All the models were good for the prediction of percentages of milk of each species. However, it was found that MLR led to more precise predictions than the other multivariate calibration methods with a root square error under 1.2%.

Keywords: Capillary electrophoresis multivariate; Milk mixtures; Regression analysis

1. Introduction

Analysis of milk proteins has been carried out using classical gel electrophoresis methods, immunological methods, analysis of ADN, isoelectric focusing and ion-exchange, hydrophobic interaction or reversed-phased HPLC among others (Mafra, Ferreira, Faria, & Oliveira, 2004; O'Donnell, Holland, Deeth, & Alewood, 2004). However, over the past decade capillary electrophoresis (CE) has been successfully used to analyse milk proteins, and indeed its speed and ease of use make it a highly competitive technique for the study of dairy products (de Frutos, Molina, & Amigo, 1996; Paterson, Otter, & Hill, 1995; Recio, Amigo, & López-Fandiño, 1997). In this sense, CE has considerable potential to solve different problems in dairy technology related to the assessment of changes that occur during the technological processes implemented and to the quality and adulteration of dairy products.

The substitution of cow's milk for ewe's and goat's milk is a fraudulent practice in the dairy industry (Mayer, Heidler, & Rockenbauer, 1997) and it is a major issue concerning the quality control of milk, cheese and other dairy products. On the other hand, the origin of the milk used to manufacture cheese must be declared by the producer, especially in the case of the protected denomination of origin cheeses (Veloso, Teixeira, Peres, Mendonça, & Ferreira, 2004). The adulteration of goat's and ewe's milk cheeses with cow's milk is done because of the seasonal fluctuations in ewe's and goat's milk production, as well as the higher market prices of these kinds of milk (Recio, Amigo, & López-Fandiño, 2001).

Several methods for the detection of the adulteration of dairy products from different species have been based on the analysis of the milk proteins. The quantitative determination of ternary milk mixtures has been realized

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by isoelectric focusing and cation-exchange HPLC of γ-casein (Mayer et al., 1997) and *para*-κ-casein (Mayer et al., 1997; Moatsou, Hatzinaki, Psathas, & Anifantakis, 2004) and by reversed-phase high-performance liquid chromatography of β-lactoglobulins (Ferreira & Caçote, 2003). Other authors have selected ratios between casein peak areas of the hydrophobic interaction chromatograms in order to detect binary milk mixtures (Bramanti, Sortino, Onor, Beni, & Raspi, 2003). Other approaches, based on the capillary electrophoresis separation of whey proteins (Cartoni, Coccioli, Jasionowska, & Masci, 1999; Herrero-Martínez, Simó-Alfonso, Ramis-Ramos, Gelfi, & Righetti, 2000; Molina, Ramos, & Martín-Álvarez, 1995) and casein fraction (Cattaneo, Nigro, & Greppi, 1996; Molina & Martín-Álvarez, 1996; Molina, Martín-Álvarez, & Ramos, 1999) in binary and ternary milks have been published.

Principal component regression (PCR), partial least-squares (PLS-1 and PLS-2) regressions and multiple linear regression (MLR) are multivariate statistical techniques that have been applied to different sciences to obtain calibration models as an alternative to linear regressions. These statistical methods have provided good predictive models for the simultaneous analysis of multi-mixtures in pharmaceutical formulations (Dinç, Yücesoy, & Onur, 2002; Dinç, Yücesoy, Palabıyık, Üstünda, & Onur, 2003; Ferraro, Castellano, & Kaufman, 2004; Nepote, Damiani, & Olivieri, 2003; Ragno, Ioele, & Risoli, 2004) and in foods (Moreno, Merkoçi, Alegret, Hernández-Cassou, & Saurina, 2004; Poveda, García, Martín-Alvarez, & Cabezas, 2004; Rodriguez-Saona, Fry, McLaughlin, & Calvey, 2001; Skarpeid, Moe, & Indahl, 2001). The theoretical relationship among these chemometric techniques has been treated extensively in the literature (Adams, 1995; Beebe & Kowalski, 1987; Kalivas, 2001; Kramer, 1998; Lorber, Wangen, & Kowalski, 1987; Phatak & de Jong, 1997).

In this sense, the aim of the present paper is to compare different multivariate statistical tecniques (PLS-1, PLS-2, PCR and MLR) to provide predictive models for the quantification of bovine, ovine and caprine milk percentages from milk mixtures.

2. Material and methods

2.1. Chemicals and reagents

Sodium hydroxide and phosphoric acid were of analytical grade. Hydroxylpropyl cellulose (HPMC), dithiothreitol (DTT) and urea were obtained from Sigma (St. Louis, MO, USA). All solutions were prepared with purified water (Milli-Q system, Millipore Corp., Bedford, MA, USA). Standards of α_s-casein (C-6780), β-casein (C-6905) and κ-casein (C-0406) from bovine milk were all supplied by Sigma (St. Louis, MO, USA).

2.2. Experimental design

Three farms of the Hidalgo State (Mexico) were selected for this study. In each farm, only one species of livestock (cow, ewe or goat) is bread. Cow's milk from Hostein and Jersey breeds, ewe's milk from black face criolla breed and goat's milks from Anglo-Nubia and Alpine breeds milks were collected from these farms in three different seasons (spring, summer and autumn). Ten milk mixtures (T1–T10) with different percentages of bovine, ovine and caprine milks were prepared by measuring the appropriated volume of each milk kind in our pilot plant and they were considered as calibration samples. The trials of the calibration samples correspond to a simplex-centroid design, which enables us to analyse six levels of concentration (0.0%, 13.7%, 33.3%, 50.0%, 66.7% and 100.0%) (see Table 1). Each one of the 10 milk mixtures was realized in duplicate with milks recollected in the same livestocks but with 15 days of difference. This process was realized with milk from three seasons (spring, summer and autumn), to obtain the total training set (60 milk samples). For the validation of the prediction models, three different mixtures (P1–P3) were prepared by duplicated from milk recollected in the three seasons (18 milk samples) following the same considerations that the training samples. Each blend was kept at −18 °C prior to its use.

A total of 12 variables were selected for the chemometric analysis of the chromatographic data. Specifically, the relative peak areas for β-A^1, β-A^2, κ-, α_{s1}-9P and α_{s1}-8P casein from bovine milk; κ-, α_{s1}-casein I and II from ovine milk; and for κ-casein from caprine milk and the combinations of ovine and caprine β_1- and ovine and caprine β_2-casein were selected. In some blends were not possible to separate bovine β-caseins A^2 from ovine and caprine β_2-caseins adequately, so the combination of these three peaks was also included as a new variable in the prediction model. All variables were initially autoscaled to zero mean and unit variance

Table 1
Composition of the training (T) and prediction (P) sets of milk mixtures of three types of milk

Standard no.	Cow (%, v/v)	Ewe (%, v/v)	Goat (%, v/v)
T1	100.0	0.0	0.0
T2	0.0	100.0	0.0
T3	0.0	0.0	100.0
T4	50.0	50.0	0.0
T5	0.0	50.0	50.0
T6	50.0	0.0	50.0
T7	33.3	33.3	33.3
T8	66.7	16.7	16.7
T9	16.7	66.7	16.7
T10	16.7	16.7	66.7
P1	50.0	30.0	20.0
P2	50.0	20.0	30.0
P3	30.0	20.0	50.0

because it provides better mathematical accuracy in calculating the multivariate models.

The design of the statistical experiments and the evaluation were performed using the computer program Statgraphics® Plus for Windows 4.0 (Statistical Graphics Corp., Rockville, MD 20852-4999, USA). Unscrambler program v. 7.01 (Camo, ASA, Trondheim, Norway) was used for the application of the PLS-1, PLS-2, PCR and MLR methods.

2.3. Capillary electrophoresis

Capillary electrophoresis was carried out using a Beckman P/ACE™ system MDQ, equipped with an UV detector, a temperature-controlled capillary compartment and an autosampler. Separations were performed using a fused-silica capillary column eCap™ (Beckman Instruments, Fullerton, CA, USA) of 60 cm × 50 μm ID (50 cm to the detector window). Sample solutions were injected for 5 s at 0.5 psi. The separations were conducted at constant voltage (20 kV) and the separation temperature was kept constant (30 °C). UV-detection was performed at 214 nm. Before each injection, the capillary was washed with 0.1 M NaOH (5 min), deionised water (5 min), and 1 M HCl (5 min) and equilibrated with the running buffer (5 min). The running buffer (50 mM) was prepared by mixing 14.7 M H_3PO_4 (847 μL) and 0.05% HPMC with 6 M of urea solution (250 mL); the pH was adjusted at 3.0 with 2 M NaOH (Rodríguez-Nogales, Revilla, & Vivar-Quintana, 2003).

The milk mixtures were skimmed by low-speed centrifugation (3000*g* for 20 min). The sample solutions were easily prepared dissolving 300 μL of skim milk (or milk mixtures) in 1 mL of sample buffer. Sample buffer (pH 8) consisted of 10 mM H_3PO_4, 8 M urea and 10 mM DTT. The sample solutions were filtered through a 0.20 μm filters (Millex-GV_{13}, Millipore, Molsheim, France) before analysis by capillary electrophoresis. Standard proteins were dissolved in sample buffer at 10 mg mL^{-1} for α-casein and β-casein and at 5 mg mL^{-1} for κ-casein. Each sample was analyzed three times ($n = 3$) by capillary electrophoresis and the average of the relative area of each peak was calculated.

3. Results and discussion

3.1. Capillary electrophoresis of different milk blends

Fig. 1 shows the electrophoretic profiles of the cow's, ewe's and goat's milk. Identification of caseins was established by comparing the migration times of standard proteins for bovine milk and comparing electro-

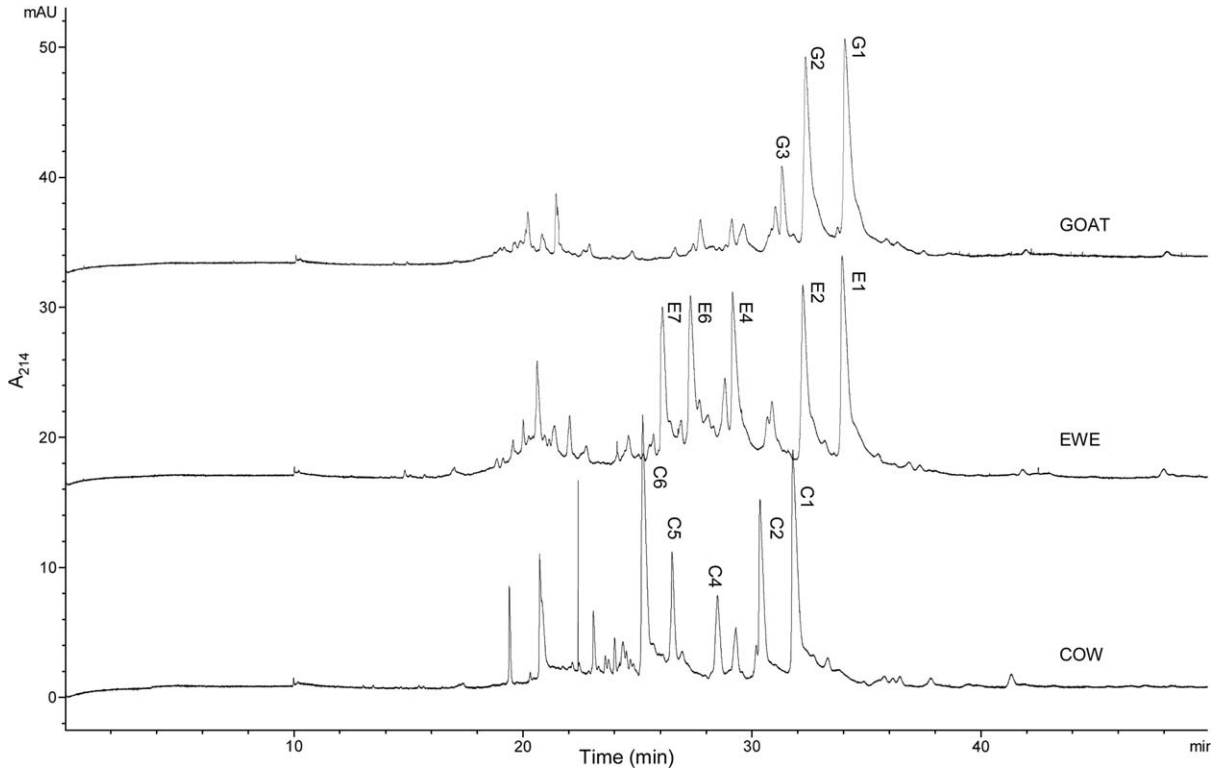

Fig. 1. Capillary electropherograms of cow's, ewe's and goat's milk. Experimental conditions: 20 kV; 30 °C; running electrolyte: 50 mM H_3PO_4, 6 M urea, 0.05% HPMC, pH 3. Cow's milk: C1 = β-CN A^2, C2 = β-CN A^1; C4 = κ-CN; C5 = α_{s1}-CN, 9P; C6 = α_{s1}-CN, 8P. Ewe's milk: E1 = β_1-CN; E2 = β_2-CN; E4 = κ-CN; E6 = α_{s1}-CN II; E7 = α_{s1}-CN I. Goat's milk: G1 = β_1-CN; G2 = β_2-CN; G3 = κ-CN.

pherograms from previous reports (Cartoni et al., 1999; Izco, Ordóñez, Torre, & Barcina, 1999; Recio et al., 1997) for ovine and caprine milks.

Peaks corresponding to β_1-, β_2-, α_{s1}- (I and II) and κ-casein from ovine milk and to β-A^1, β-A^2, α_{s1}-9P, α_{s1}-8P and κ-casein from bovine milk could be clearly identified in the chromatogram of an ovine-bovine milk blend (Fig. 2). When a blend of ovine and caprine milk was injected, neither the β_1-caseins nor the β_2-caseins from ovine and caprine milk were resolved as individual peaks. The electropherogram of a blend of bovine and caprine milk showed that β_1-, β_2- and κ-casein for goat milk and β-A^1, β-A^2, κ-, α_{s1}-9P and α_{s1}-8P-casein for bovine milk can be distinguished as individual peaks. In the case of ternary milk mixtures (Fig. 3), separate peaks were clearly identified for: β-A^1, β-A^2, κ-, α_{s1}-9P and α_{s1}-8P-casein from bovine milk; κ-, α_{s1}-casein I and II from ovine milk; and for κ-casein from caprine milk.

A cluster analysis was carried out to find the most characteristic peaks, which provide the most information on the differences between all analyzed caseins of the milk mixtures (data not showed). The peaks were selected base on the Euclidean distances. Among all the analyzed peaks, the area of the 13 selected casein peaks were found to be correlated to the greatest degree with the percentage content of a given milk preparation. The peaks selected are showed in Fig. 1.

The repeatability and precision of the method were assessed by carrying out eight consecutive injections of a mixture of 1/3 each of cow's, ewe's and goat's milk (sample T7). For all proteins, the relative standard deviation (RSD) was lower than 1.8% for the migration time, and less than 1.4% for the relative migration time. On the other hand, RSD values of around 3.3% were obtained for peak areas.

3.2. Chemometric analysis of chromatographic data

With the aim of predicting the percentage of milk of different species included in a mixture, several different chemometric approaches were evaluated (PLS-1, PLS-2, PCR and MLR). It is very difficult to generalise the superiority of one method over another, because the relative performance of the methods often dependents on the particular data set analysed. Multivariate calibration methods require a suitable experimental design to define the calibration set, in order to keep a lowest number of samples and to arrange theses homogeneously in the experimental design (Ragno et al., 2004). In this sense, a simplex-centroid design was used to optimise the calibration set.

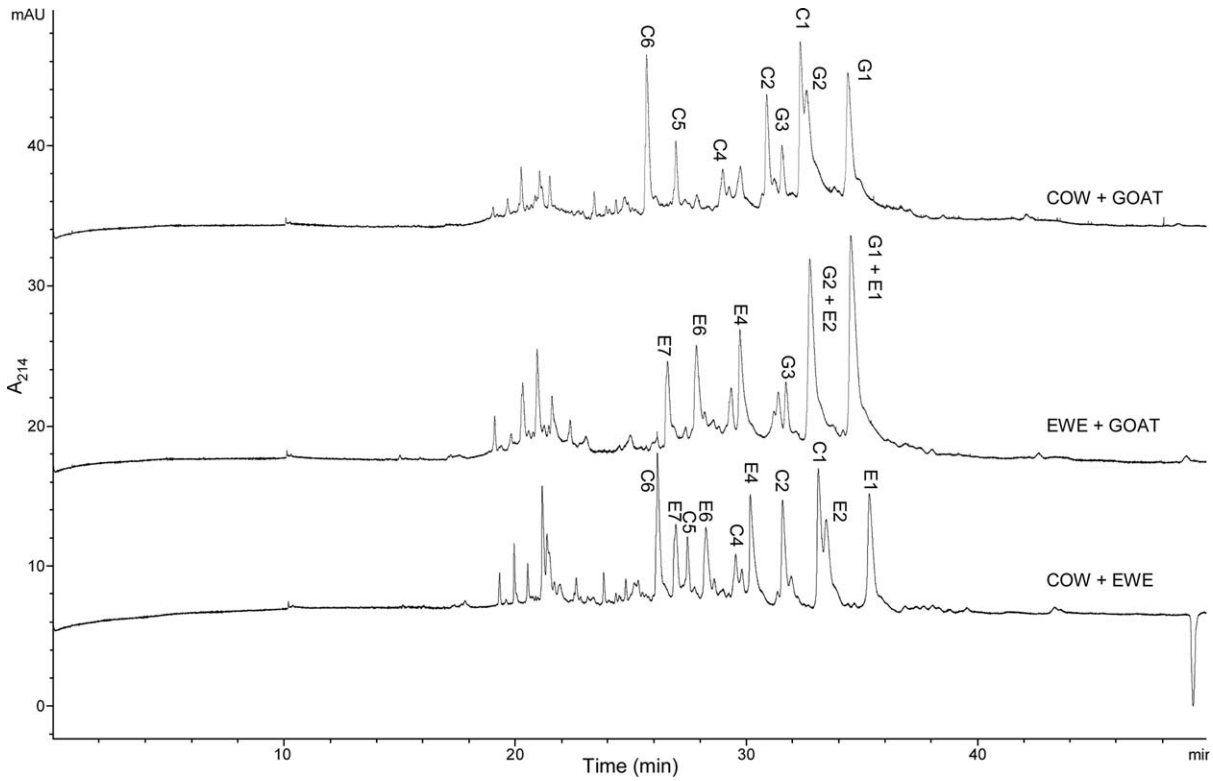

Fig. 2. Capillary electropherograms of binary mixtures of cow's, ewe's and goat's milk. Experimental conditions: 20 kV; 30 °C; running electrolyte: 50 mM H_3PO_4, 6 M urea, 0.05% HPMC, pH 3. Cow's milk: C1 = β-CN A^2, C2 = β-CN A^1; C4 = κ-CN; C5 = α_{s1}-CN, 9P; C6 = α_{s1}-CN, 8P. Ewe's milk: E1 = β_1-CN; E2 = β_2-CN; E4 = κ-CN; E6 = α_{s1}-CN II; E7 = α_{s1}-CN I. Goat's milk: G1 = β_1-CN; G2=β_2-CN; G3 = κ-CN.

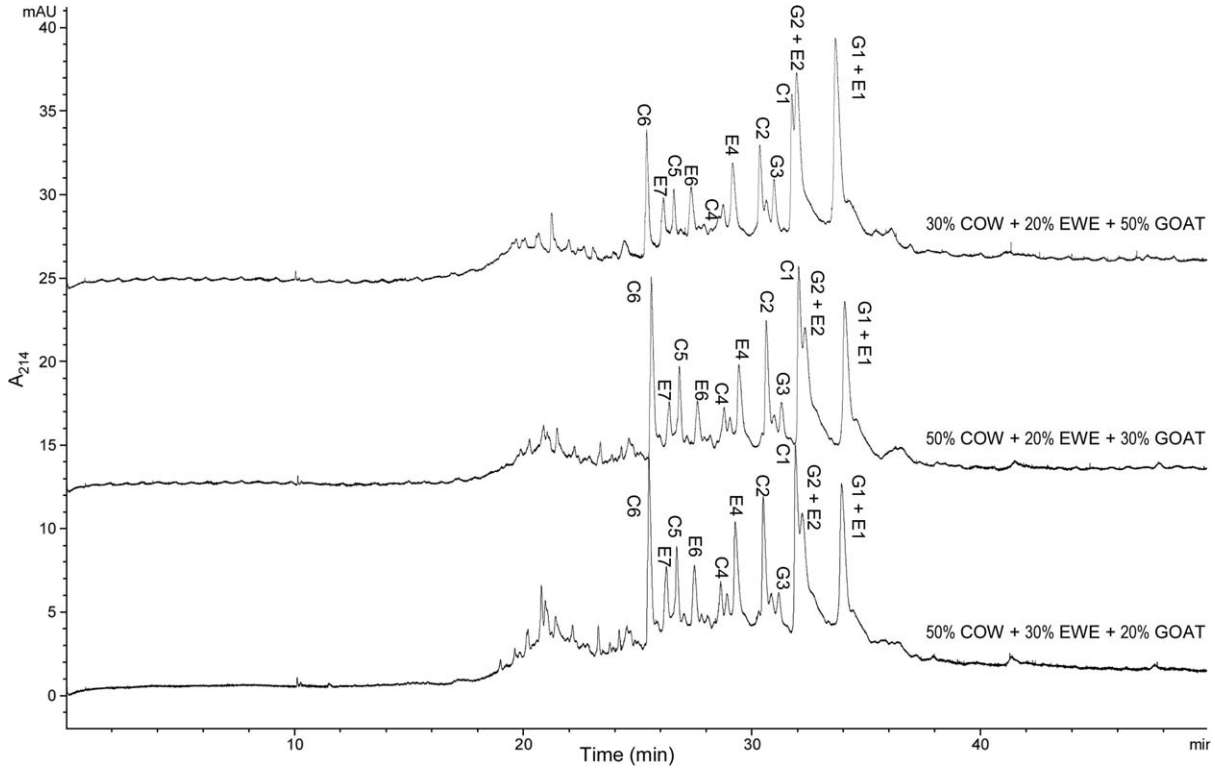

Fig. 3. Capillary electropherograms of ternary mixtures of cow's, ewe's and goat's milk. Experimental conditions: 20 kV; 30 °C; running electrolyte: 50 mM H_3PO_4, 6 M urea, 0.05% HPMC, pH 3. Cow's milk: C1 = β-CN A^2, C2 = β-CN A^1; C4 = κ-CN; C5 = α_{s1}-CN, 9P; C6 = α_{s1}-CN, 8P. Ewe's milk: E1 = β_1-CN; E2 = β_2-CN; E4 = κ-CN; E6 = α_{s1}-CN II; E7 = α_{s1}-CN I. Goat's milk: G1 = β_1-CN; G2 = β_2-CN; G3 = κ-CN.

To select the number of factors used to build PLS-1, PLS-2 and PCR models and in order to model the system without overfitting the percentage data, a full cross-validation method, leaving out one sample a time, was used (Frenich, Liebanas, Mateu-Sanchez, & Martínez, 2003; Lomillo, Renedo, & Martinez, 2001; Skarpeid et al., 2001). In the case of MLR model, full cross-validation was also employed. It consists on removing one sample at time from the calibration step and performing the calibration with the rest of the samples. The response of the sample removed is then predicted with the obtained model. This step is in turn repeated for each sample considered (Ragno et al., 2004). The root mean square error of calibration (RMSEC) was chosen as an optimizing criterion to select the optimal number of factors. Its value depends on the number of factors used for the calibration. The maximum number of factors used to calculate the optimum RMSEC was selected as 12. RMSEC is an indicator of the average error in the analysis for each component and how well the model fits to the data. RMSEC is defined by the following formula:

$$\mathrm{RMSEC} = \sqrt{\sum_{i=1}^{n} \frac{(y_{Ci} - y_i)^2}{n}},$$

where y_{Ci} is the predicted percentage of milk in each species in calibration sample i, y_i is the real percentage in calibration samples i and n is the number of calibration samples.

The selected factors and the corresponding RMSEC values are listed in Table 2. The square of the correlation coefficient (R^2), which indicates the fraction of the total variance explained by the models, is also reported and, in all cases, it is always more than 0.9895. It also shows, in most of the cases, the optimum number of factors was greater than the number of the components in the mixtures for PLS-1, PLS-2 and PCR methods. On the other

Table 2
Statistical parameters of validation from data of calibration set

Terms	Cow				Ewe				Goat			
	PLS-1	PLS-2	PCR	MLR	PLS-1	PLS-2	PCR	MLR	PLS-1	PLS-2	PCR	MLR
Number of factors	4	2	6		5	8	8		5	10	8	
R^2	0.9971	0.9968	0.9970	0.9980	0.9951	0.9989	0.9972	0.9940	0.9955	0.9895	0.9895	0.9960
RMSEC	2.1857	2.5410	2.0686	1.4735	3.1763	1.3276	2.1836	2.448	2.9767	4.5621	4.5621	1.7811

hand, lower values of RMSEC were obtained from the percentages of cows' and goats' milk by using MLR model and for ewe's milk by using PLS-2 regression. The regression coefficients of the four models constructed for each milk type are shown in Table 3.

It is well known that the real predictive ability of any calibration models cannot be judged solely by using internal validation. It has to be validated on the basis of predictions for samples not included in the calibration test (Boeris, Luco, & Olsina, 2000). In order to test the quality of the proposed models, they were applied to the prediction of the percentages of each milk type in the prediction set (see Table 1, P1–P3). Table 4 shows the results obtained. In this case, root mean square error of prediction (RMSEP) was chosen as an optimizing criterion to validate the built calibrations, which is given by the following formula:

$$\mathrm{RMSEP} = \sqrt{\sum_{i=1}^{m} \frac{(y_{\mathrm{P}i} - y_i)^2}{m}},$$

where $y_{\mathrm{P}i}$ is based on the previously developed calibration models, y_i is the real percentage in calibration samples i and m is the number of evaluation samples. The results were also expressed as recovery values (%). Good results were achieved in all cases with RMSEP ranging from 1.00 to 3.33 and recoveries ranging from 95.3 to 104.0 (Table 4). These values show us that four statistical techniques are suitable for the prediction of percentages of milk of each species included in a mixture. However, the best results were found for MLR models with RMSEP values of 1.0%, 1.2% and 1.1% for the percentages of bovine, ovine and caprine milk, respectively. In order to compare the performances of the chemometric techniques according to the real values, we applied Tuckey test at the values of recovery obtained. The mean recovery obtained for cow's milk was slightly higher with PLS-1 compared to those obtained with PLS-2, PCR and MLS methods (which means they were not significantly different). For ewe's and goat's milk,

Table 3
The regression coefficients estimated in the PLS-1, PLS-2, PCR and MLR models

Terms	Cow (%)				Ewe (%)				Goat (%)			
	PLS-1	PLS-2	PCR	MLR	PLS-1	PLS-2	PCR	MLR	PLS-1	PLS-2	PCR	MLR
C6	0.098	0.076	0.159	0.087	−0.045	−0.068	−0.008	0.018	−0.214	−0.086	−0.152	−0.104
E7	−0.006	−0.009	0.017	−0.097	0.499	0.295	0.381	0.638	−0.349	−0.216	−0.375	−0.540
C5	0.027	0.023	0.037	0.194	0.041	−0.058	0.045	−0.777	−0.049	0.046	−0.065	0.583
E6	−0.015	−0.010	−0.017	−0.174	0.151	0.164	0.227	0.005	−0.192	−0.239	−0.210	0.169
C4	0.032	0.021	0.036	0.118	0.002	−0.004	0.073	0.139	−0.061	−0.090	−0.139	0.257
E4	−0.013	−0.008	−0.026	0.156	−0.045	0.030	0.081	−0.133	−0.084	0.024	−0.057	−0.023
C2	0.057	0.049	0.042	0.154	−0.077	0.002	−0.125	−0.047	−0.012	−0.102	0.171	−0.080
G3	−0.026	−0.015	−0.030	−0.197	−0.503	−0.653	−0.187	−0.700	0.333	0.821	0.262	0.897
C1	0.099	0.086	0.065	−0.007	−0.052	−0.060	0.014	−0.017	−0.015	0.053	−0.107	0.024
E2 + G2	−0.057	−0.069	−0.042	−0.019	0.046	0.009	0.028	−0.003	−0.045	0.010	0.028	0.022
E1 + G1	−0.053	−0.080	−0.056	−0.044	−0.080	−0.016	−0.033	−0.086	0.092	0.088	0.087	0.130
C1 + E2 + G2	0.025	0.040	0.211	0.016	−0.005	−0.001	−0.004	0.008	−0.005	−0.027	−0.014	−0.025
Intercept	26.565	32.381	23.867	24.480	38.331	30.868	1.240	61.484	69.262	34.525	57.205	14.036

Table 4
Prediction of results for percentages of milk by different chemometric methods

Standard no.	Cow (%)					Ewe (%)					Goat (%)				
	Real	PLS-1	PLS-2	PCR	MLR	*Real*	PLS-1	PLS-2	PCR	MLR	*Real*	PLS-1	PLS-2	PCR	MLR
P1	*50.0*	53.6	50.5	51.0	51.9	*30.0*	27.8	30.6	32.6	29.0	*20.0*	17.6	23.2	16.3	20.9
P1	*50.0*	53.1	50.4	50.4	50.3	*30.0*	28.2	28.4	30.5	29.3	*20.0*	20.1	23.5	15.6	20.5
P1	*50.0*	52.1	49.2	49.5	49.5	*30.0*	28.1	28.5	30.5	29.3	*20.0*	21.2	25.4	19.7	21.3
P2	*50.0*	54.8	51.5	51.7	51.2	*20.0*	18.0	17.4	20.4	18.6	*30.0*	29.9	33.1	28.2	30.2
P2	*50.0*	54.1	50.9	51.0	48.3	*20.0*	18.9	17.5	20.1	21.2	*30.0*	30.3	33.3	29.6	30.6
P2	*50.0*	53.9	50.9	50.8	50.0	*20.0*	19.2	16.6	19.4	21.8	*30.0*	31.5	33.1	30.3	28.3
P3	*30.0*	29.4	27.6	27.9	29.1	*20.0*	20.4	21.7	22.3	21.4	*50.0*	50.1	53.5	49.4	19.7
P3	*30.0*	26.7	28.1	27.9	29.9	*20.0*	21.0	20.9	21.6	19.5	*50.0*	50.4	51.8	50.6	50.7
P3	*30.0*	28.7	27.8	27.3	29.7	*20.0*	21.8	19.1	20.0	21.5	*50.0*	53.0	51.6	53.2	47.8
RMSEP		3.104	1.450	1.532	1.001		1.563	1.942	1.323	1.189		1.411	3.333	1.768	1.121
Mean recovery (%)[A]		104.0[a]	98.3[b]	98.5[b]	99.7[b]		97.4[a]	95.3[b]	103.4[a]	101.3[a]		100.8[a]	111.8[b]	97.5[a]	100.7[a]

[A] Different letters show a statistically significant difference at the 95% confident level.

PLS-1, PCR and MLS models appear better results than those with PLS-2.

Molina et al. (1999) applied two multivariate regression techniques (PLS and PCR) in order to predict the percentages of milk of cow's, ewe's and goat's milk which are included in a mixture. However, this study did not include a training set with milk mixtures from different period of the year in order to draw a conclusion with a more statistical basis. On the other hand, these authors did not check MLR regression, which is a standard technique used for fitting mixture data (Bautista, Aberásturi, Jiménez, & Jiménez, 1996; Pratiwi, Fawcett, Gordon, & Rades, 2002; Simmonsi & Pongsakul, 2004).

In conclusion, the simultaneous application of capillary electrophoresis and multivariate techniques (PLS-1, PLS-2, PCR and MLR) gave successful results for the chemometric determination the percentages of milk in binary and ternary mixtures. According to the RMSEP values, better results have been found with the application of multiple linear regression models with an RMSEP under 1.2%.

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