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Quantitative determination of α_{s2}- and α_{s1}-casein in goat's milk with different genotypes by capillary electrophoresis☆

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Abstract

A capillary electrophoresis (CE) method has been applied for the quantitative determination of α_{s1}- and α_{s2}-CN in goat's milk. Several analytical parameters were evaluated showing the reliability of this CE method. Coefficients of determination (R^2) greater than 99% were obtained and determination limits of 1.23 and 0.98 mg/ml were achieved for α_{s1}- and α_{s2}-CN, respectively. The analytical parameters studied in terms of accuracy, precision and recovery were within acceptable limits. Among 18 samples of 4 different genotypes (BB, EE, BF and FF) for α_{s1}-CN were analysed, different amounts were obtained from the genotypes.

Keywords: Quantitative determination; α_{s1}- and α_{s2}-casein; Goat's milk

1. Introduction

The production and consumption of goat's milk and its by-products are increasing worldwide. This is reflected in the fact that the number of goats has increased over the last 20 years [1]. The protein and fat contents are variable among the different caprine breeds and they are genetically controlled, especially by the α_{s1}-casein locus, which exhibits a high degree of polymorphism [2]. Eight of the currently identified alleles (A, B_1, B_2, B_3, B_4, C, H and L) are associated with a high level of α_{s1}-CN (3.5 g/l per allele), two (E and I) with a medium level (1.1 and 1.7 g/l) and two (F and G) with a low level (0.45 g/l). The O_1 and O_2 are null alleles and produce no α_{s1}-CN [2–5]. In addition, α_{s2}-CN also presents seven alleles associated with three different synthesis levels: A, B, C, E and F alleles related with a "normal" α_{s2}-CN content (2.5 g/l), the D allele related with a reduced α_{s2}-CN content and the O allele related with a non-detectable amount of this casein in milk [6].

There are no studies about the influence of the α_{s2}-CN polymorphism on the technological properties of milk, and only some studies have been carried out to evaluate the effect of this protein on the potential allergenicity of goat's milk [6]. However, the α_{s1}-CN polymorphism is correlated with milk composition and some technological properties of milk, for instance, coagulation rate, mean micellar size, lipolysis and the proportion of fatty acids in milk, total solids, fat recovery and gross yield in the production of goat's cheese as well as on the flavour of these cheeses (for a review see [7]).

The separation and quantification of the different casein fractions is difficult to achieve. In addition, although there are different methods providing valuable information from a qualitative viewpoint (for instance SDS–PAGE), these methods only offer semiquantitative data. In the literature, the main cited values on the different levels of goat's casein synthesis associated with allele variability (mainly in α_{s1}-CN) are from the eighties, and were obtained by rocket inmunoelectrophoresis [2]. These values have been constantly cited, but no further studies have been carried out using other techniques to quantify the allele variability. Capillary electrophoresis (CE) was shown to be useful for the separation and quantification of whey proteins [8,9] and caseins from different species [9–11]

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The aim of this work was to quantify α_{s2}- and α_{s1}-CN contents in goat milk with different genotypes. A Fast Protein Liquid Chromatography (FPLC) method has been optimised in order to separate and prepare fractions of these proteins. Data obtained were used to calibrate a NIR equipment used to control goat's milk production.

2. Experimental

2.1. Samples

Eighteen samples of goat's milk previously genotyped by PCR were analysed. These samples belonged to different α_{s1}-CN genotypes: five samples corresponding to genotype BB (high level of protein), five samples to genotype EE (medium level of protein), six samples to genotype BF, and two samples to genotype FF (low level of protein). Initially, whole milk samples were skimmed. Fat was removed by centrifugation (3500 × *g*, 30 min) at room temperature followed by incubation of the samples in an ice-water bath for 30 min and filtration through glass wool. Isoelectric casein was prepared by precipitation of milk with 2 M HCl to pH 4.6, followed by centrifugation (4500 × *g*, 5 °C, 15 min). The casein precipitates were washed three times with a buffer made of 1 M sodium acetate and 100 ml/l acetic acid, pH 4.6, and twice with a mixture 1:1 of buffer:dichloromethane, and centrifuged after each wash (4500 × *g*, 5 °C, 5 min). Finally, the washed precipitates were lyophilised. Bovine α_s-CN was purchased from Sigma (St. Louis, MO, USA).

2.2. Isoelectric focusing (IEF) of goat casein samples

To verify the genotypes present in the different goat milks, the caseins were analysed by (IEF) using a PhastSystem (Pharmacia, Uppsala, Sweden) following the method described by Bovenhuis and Verstege [12] for cow's milk samples. Identification of the different protein fractions and genetic variants was carried out according to Recio et al. [13].

2.3. Fast protein liquid chromatography

In order to obtain individual α_{s1}-CN and α_{s2}-CN fractions, goat's milk with α_{s1}-CN BB and α_{s2}-CN AA genotype (high amount of protein) was analysed by cation-exchange FPLC (Pharmacia LKB) at 20 °C on a HiLoad™ 26/10 SP Sepharose Fast Flow column (Pharmacia LKB). We initially followed the method described by Law and Tziboula [14]. However, several modifications in the buffer composition and gradient were necessary to improve the separation of α_{s1}- and α_{s2}-CN in the column (preparative scale) used in this work. The best separation was obtained with Buffer A (6 M urea, 64 μM DL-dithiothreitol (DTT), 75 mM Na-formate) adjusted to pH 4. Buffer B had the same composition with the addition of 1 M NaCl. Casein fractions were eluted with this gradient: 0% B for 18.70 min, 2.5% B at 18.80 min, 9% B at 20 min, 16% B at 65 min and 30% B at 120 min. The flow rate was 5 ml/min and the absorbance was recorded at 280 nm.

Before the analysis, the lyophilised goat's casein (3 g) was dissolved in 100 ml of buffer A. The pH was adjusted to 7, and 2 ml of 2-mercaptoethanol were added. After 1 h, the sample solution was adjusted to pH 4 and different volumes (15–20 ml) were injected into the column using a Superloop™ (Pharmacia LKB). The different casein fractions were manually collected from the FPLC equipment, pooled and lyophilised after dialysis.

2.4. Capillary electrophoresis

CE was carried out using a Beckman P/ACE System 2050 controlled by a System Gold Software data system version 810 (Beckman Instruments, Inc., Fullerton, CA, USA). The separations were performed using a hydrophilic-coated fused-silica capillary column, Supelco CElect P1 (Supelco, Bellefonte, PA, USA), 57 cm × 50 μm i.d., with a slit opening of 100 μm × 800 μm. The distance between detection window and outlet was 100 mm, resulting in an effective capillary length of 500 mm. CE buffers were prepared and optimised according to the methods of De Jong et al. [15] and Recio and Olieman, [16]. The sample buffer (pH 8.6 ± 0.1) consisted of 167 mM Tris (reagent grade from Sigma), 42 mM 3-(*N*-morpholino)-2-hydroxypropane (Biochemika MicroSelect, Fluka, Buchs, Switzerland), 67 mM EDTA (Merck, Darmstadt, Germany), 17 mM DTT (Sigma), 6 M urea (Sigma) and 0.05% methylhydroxyethyl cellulose 30000 (MHEC) (Serva, Heidelberg, Germany). The electrophoresis buffer (pH 3.0 ± 0.1) was 0.32 M citric acid, 20 mM sodium citrate, 6 M urea and 0.05% MHEC. Sample buffer (700 μl) and water (300 μl) were added to 18 mg of casein or casein fractions. After 1 h incubation at room temperature, the reduction reaction was finished and the sample was injected without further preparation. Separations were performed in duplicate as described by Recio and Olieman, [16] with a final voltage of 25 kV in 3 min and a final current of around 52 μA. The injection was carried out at the anode with N_2 pressure for 18 s at 3.4 kPa. Detection was at 214 nm.

2.5. Protein determination

The protein content of the different goat's milk was determined by the Kjeldahl method following the IDF Standard 20 B [17]. The protein content in the casein fractions collected from FPLC was analysed by a spectrophotometric technique based on measurement of the extinction coefficients at 280 nm [18]. This method was carried out by dissolving the samples in 0.02 M phosphate buffer pH 6.5 containing 6 M guanidine hydrochloride (Sigma) and monitoring the absorbance at 280 nm.

2.6. Statistical methods

Linear regression analyses were established using Microsoft Excel 7.0. The determination signal and determination limit were calculated using the Detarchi program, as described by Ortiz and Sarabia [19].

3. Results and discussion

3.1. Isolation of α_{s1}- and α_{s2}-CN of goat's milk

Several runs of the preparative FPLC were carried out on Sepharose Fast Flow column to obtain large amounts of α_{s1}- and α_{s2}-CN. The different proteins in each fraction were identified by CE. Fig. 1 shows the chromatographic profile of a goat's casein analysed by FPLC using the method described by Law and Tziboula [14]. In this analysis, β- and κ-CN (F1 and F2) were separated, but α_{s1}- and α_{s2}-CN were co-eluted. Despite the gradient optimisation, two fractions were not separated, and thus different buffer compositions were used. In this way, 20 mM acetate–urea buffer was changed to 75 mM formate–urea buffer and the pH from 5 to 4. After the sample was treated, previously to the injection, with mercaptoethanol to break up disulfide-linked polymers of κ- and α_{s2}-CN, the addition of DTT in buffer A and B improved the separation. Fig. 2 shows the results of FPLC analysis with the improved method. After the CE analysis of each fraction, β- and κ-CN were identified in F1, α_{s1}-CN in F2 and α_{s2}-CN in F3. Therefore, we could collect α_{s1}- and α_{s2}-CN separately to study the reliability of the CE method for quantitative determination.

The CE analysis showed that the α_{s1}-CN fraction resolved as three peaks with migration times (M_t) between 24 and 29 min (Fig. 2 F2) and α_{s2}-CN resolved as several peaks with short M_t (between 16 and 21 min) (Fig. 2 F3). The elution order obtained by cation-exchange chromatography was consistent with previous results [20].

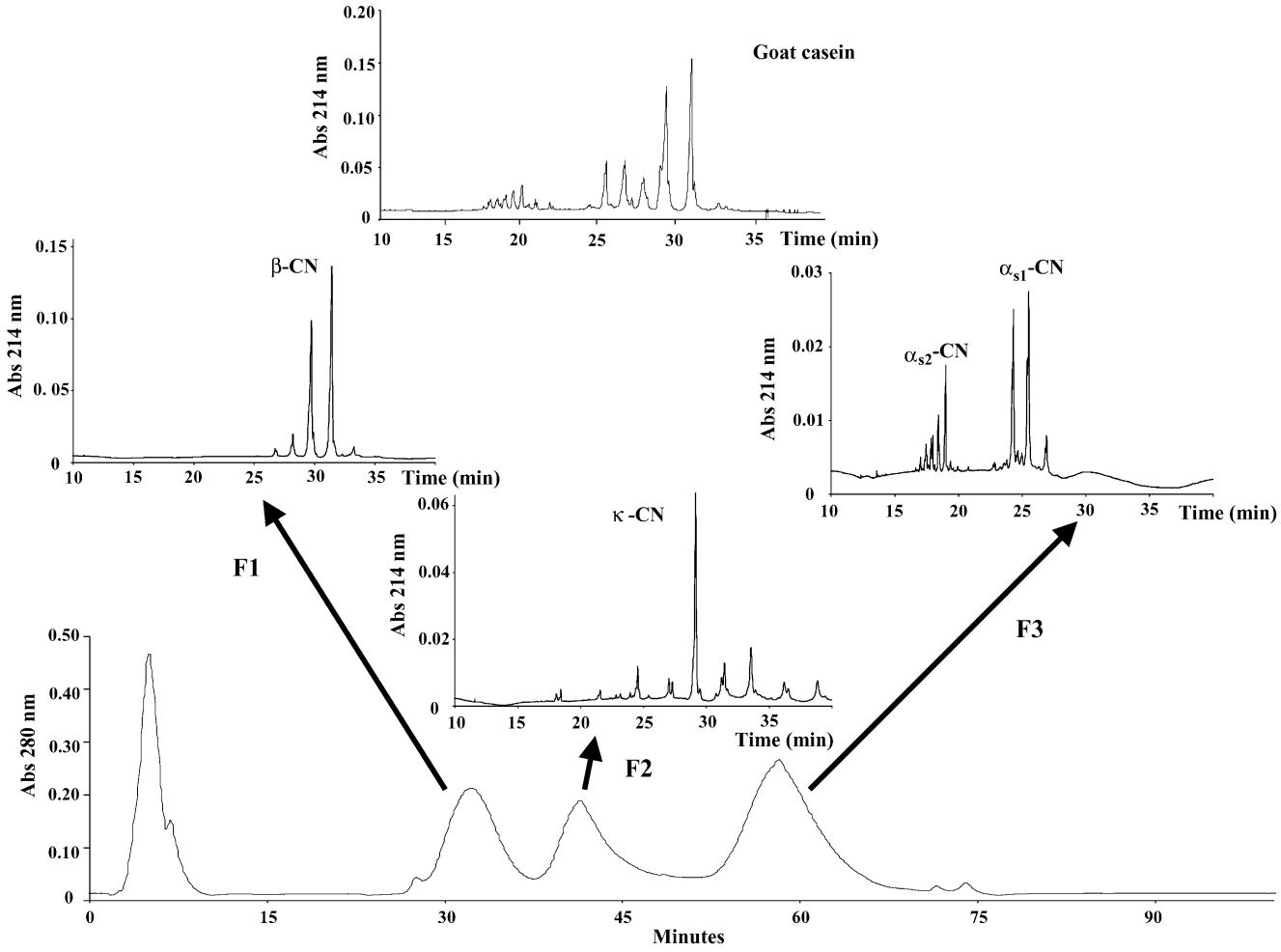

Fig. 1. FPLC analysis of goat's caseins and electrophoregrams of goat's caseins and the three fractions collected from FPLC. FPLC conditions were: Buffer A (6 M urea and 0.02 M acetate, pH 5); Buffer B (Buffer A + 1 M NaCl) were used in FPLC condition. Electrophoresis buffer (6 M urea, 0.32 M citric acid, 20 mM sodium citrate, 0.05% MHEC at pH 3.0 ± 0.1) and separation voltage of 25 kV were used for the CE analysis.

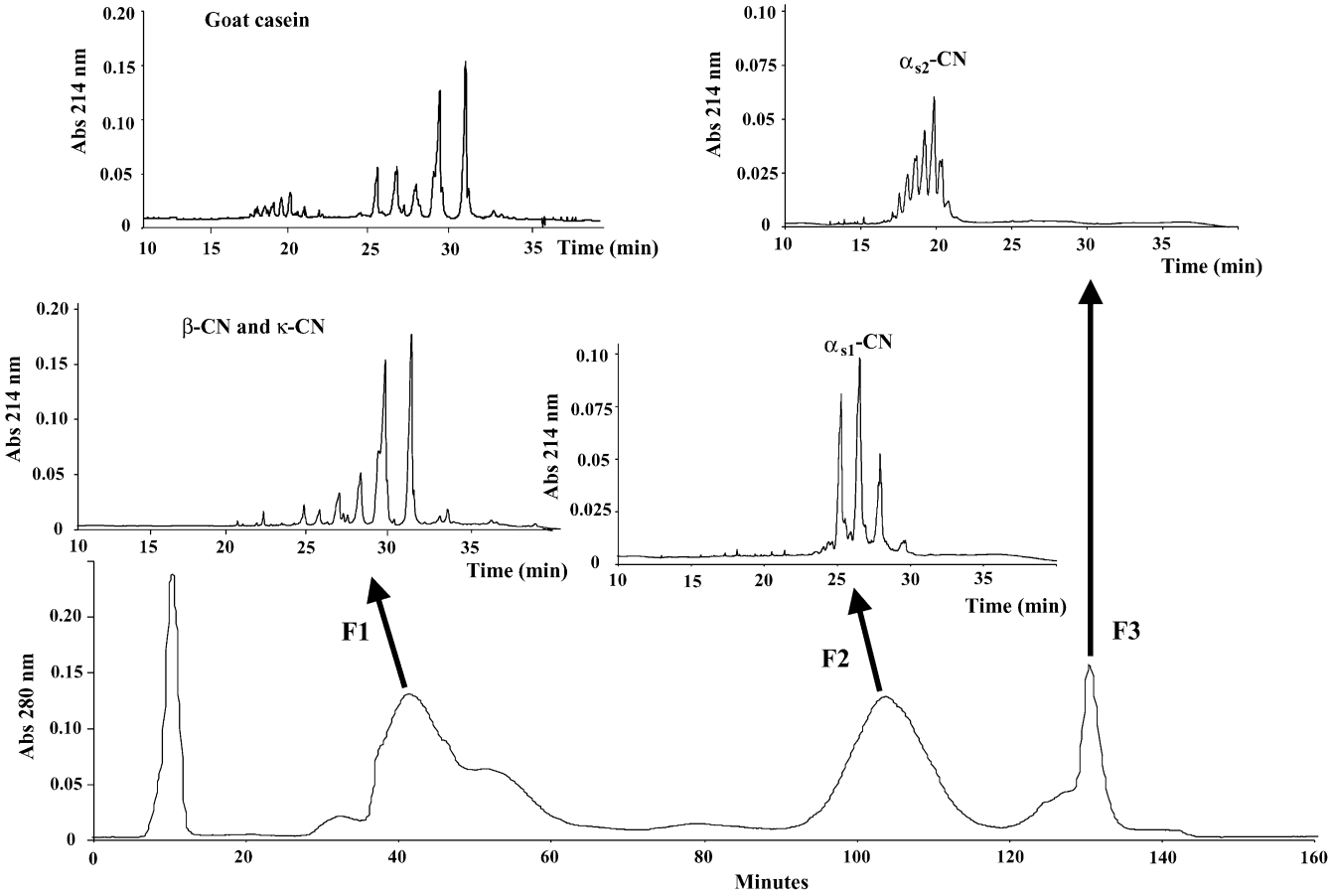

Fig. 2. FPLC analysis of goat's caseins and electrophoregrams of the three fractions collected from FPLC. FPLC conditions were: Buffer A (6 M urea, 64 μM DL-dithiothreitol (DTT) and 75 mM Na-formate adjusted to pH 4) and Buffer B (Buffer A + 1 M NaCl) were used for the FPLC condition. Electrophoresis buffer (6 M urea, 0.32 M citric acid, 20 mM sodium citrate, 0.05% MHEC at pH 3.0 ± 0.1) and separation voltage of 25 kV were used for the CE analysis.

3.2. Quantitative analysis

The protein contents determined were 88.3 and 76.3% for α_{s1}- and α_{s2}-CN, respectively. To calculate calibration curves for α_{s1}- and α_{s2}-CN, concentrations of α_{s1}-CN in the range 0.25–15.89 mg/ml and of α_{s2}-CN in the range 0.42–13.73 mg/ml were analysed in duplicate. Table 1 shows the different parameters of the quantitative determination of α_{s1}- and α_{s2}-CN by CE. Plots of velocity-corrected peak area [(area × distance between capillary inlet and detection window in mm)/M_t] versus concentration in mg/ml demonstrated that the regression analysis was linear in this range of concentrations. Determination limits of 1.23 and 0.98 mg/ml and determination signals of 10.6 and 15.4 peak area units for α_{s1}- and α_{s2}-CN, respectively, were obtained. The mean accuracies were 105.6 and 90.2% for quantification of α_{s1}- and α_{s2}-CN, respectively.

To verify if the determination of α_{s1}- and α_{s2}-CN could be affected by the protein adsorbed on the capillary wall, analysis of the casein solution was performed before and after addition of different amounts of α_{s1}- and α_{s2}-CN. The results confirmed that no adsorption effect was found in quantification.

Table 1
Analytical parameters calculated for the quantitative determination of goat's α_{s1}-CN and α_{s2}-CN by CE[a]

	α_{s1}-CN	α_{s2}-CN
Linearity		
Regression analysis (n =14)	$a = -0.95 \pm 2.86$ $b = 18.06 \pm 0.41$ $R^2 = 0.994$	$a = 4.23 \pm 3.03$ $b = 22.19 \pm 0.47$ $R^2 = 0.996$
Sensibility		
Determination limit (mg/ml)	1.23	0.98
Determination signal (u.a.)	10.6	15.36
Accuracy (%)		
Mean accuracies (n = 14)	105.6	90.2
Precision		
Repeatability (n = 4)	M_t R.S.D. = 0.35 R.S.D. = 1.77	M_t R.S.D. = 0.46 C_a R.S.D. = 3.86
Reproducibility (n = 4)	M_t R.S.D. = 0.41 C_a R.S.D. = 2.28	M_t R.S.D. = 0.38 C_a R.S.D. = 6.42
Recovery		
0.5 mg/ml added	94%	88%
1.0 mg/ml added	102%	86%

[a] The conditions for the CE analysis were: electrophoresis buffer containing 6 M urea, 0.32 M citric acid, 20 mM sodium citrate, 0.05% MHEC at pH 3.0 ± 0.1. Separation voltage of 25 kV.

Table 2
Total protein concentration, casein protein, α_{s1}-, α_{s2}- and κ-CN genotypes and amount of α_{s1}- and α_{s2}-CN found in different goat's milk samples

Sample	Total protein (g/kg of milk)[a]	Casein protein (g/kg of milk)[a]	α_{s1}-CN (g/kg of milk)[b]	α_{s2}-CN (g/kg of milk)[b]	α_{s1}-CN genotype[c]	α_{s2}-CN genotype[d]	κ-CN genotype[d]
1	38.15	29.54	7.44 ± 0.01	3.1 ± 0.01	BB	AA	AA
2	24.82	10.98	4.74 ±0.09	1.7 ± 0.0.1	BB	AA	AA
3	46.44	37.13	11.43 ± 0.07	2.6 ± 0.01	BB	AA	AA
4	51.29	38.47	12.32 ± 0.14	2.7 ± 0.03	BB	AA	AA
5	42.04	33.87	11.19 ± 0.50	2.7 ± 0.01	BB	AA	AA
6	34.96	26.16	7.84 ± 0.12	2.8 ± 0.01	BF	AA	AA
7	37.06	29.72	7.94 ± 0.39	2.6 ± 0.01	BF	AA	AA
8	35.09	26.35	4.86 ± 0.08	2.4 ± 0.01	BF	AA	AA
9	40.38	30.36	4.56 ± 0.05	2.6 ± 0.01	BF	AC	AA
10	39.04	26.92	7.17 ± 0.12	2.5 ± 0.01	BF	AC	AB
11	27.69	17.87	4.31 ± 0.01	2.3 ± 0.01	BF	AA	AA
12	38.09	28.78	3.98 ± 0.32	2.5 ± 0.02	EE	AA	AA
13	25.26	16.78	2.85 ± 0.17	1.7 ± 0.01	EE	AA	AA
14	32.73	18.31	3.06 ± 0.31	1.7 ± 0.01	EE	AC	AA
15	30.94	22.26	3.20 ± 0.05	2.3 ± 0.01	EE	AA	AA
16	33.62	24.82	3.68 ± 0.37	2.5 ± 0.01	EE	AB	AA
17	24.43	11.73	1.17 ± 0.02	1.0 ± 0.01	FF	AA	AA
18	34.32	24.62	1.73 ± 0.02	2.7 ± 0.01	FF	AA	AA

[a] Calculated by Kjeldahl method.
[b] Calculated by CE method.
[c] Identified by PCR.
[d] Identified by IEF.

The precision studies were composed of repeatability and reproducibility. The repeatability was established by four consecutive injections of α_{s1}- and α_{s2}-CN. The relative standard deviation values (R.S.D.) of α_{s1}-CN were 0.35% to migration time and 1.77% to corrected peak areas. The values obtained for α_{s2}-CN were 0.46 and 3.86% to migration time and corrected peak areas, respectively. The reproducibility, also termed day-to-day repeatability, was determined by analysing each sample on four different days. The R.S.D. values achieved for the migration times were of 0.41 and 0.38% for α_{s1}- and α_{s2}-CN samples, respectively. For the corrected peak areas, the R.S.D. values obtained were 2.28 and 6.42% for α_{s1}- and α_{s2}-CN, respectively. These results indicate that the present CE method can be used for quantitative analysis of α_{s1}- and α_{s2}-CN in goat's milk.

3.3. Application of the CE method to goat's milk samples

Eighteen samples of goat's milk previously genotyped by PCR were analysed. The CE analysis confirmed that: five samples corresponding to α_{s1}-CN genotype with high level of protein, eleven samples to α_{s1}-CN genotype with medium level of protein, and two samples to α_{s1}-CN genotype with low level of protein. In each sample the quantification of α_{s1}- and α_{s2}-CN by CE was carried out.

Table 2 lists the different genotypes analysed, contents of total protein and casein protein achieved by the Kjeldahl method and the concentrations of caprine α_{s1}- and α_{s2}-CN found in each sample by the CE method.

In general, a good correlation was found between the quantities of α_{s1}- and α_{s2}-CN described for each genotype in the literature and the quantities calculated with the CE method taking account that individual milk samples were analysed [2,5,6,21]. The main differences were found for samples with α_{s1}-CN genotypes BB and BF. For instance, sample 2 showed a low quantity of α_{s1}- and α_{s2}-CN. This sample showed genotype without β-CN, which affects the total amount of protein and the different casein fractions. On the contrary, samples 3, 4 and 5 presented a very high amount of α_{s1}-CN and the same behaviour was observed in samples 6, 7 and 10. In some of the electropherograms of the samples containing a high value of α_{s1}-CN, we observed a peak co-migrating with the peak with longest M_t of α_{s1}-CN. Recio et al. [13] had described the presence of the genetic variant B from κ-CN co-migrating with a peak corresponding to α_{s1}-CN. In order to confirm this observation, these samples were analysed by IEF. IEF allowed us to verify the presence of one sample with two different alleles for κ-CN (A and B) (sample 10), that explain the high value of α_{s1}-CN found in this sample. Therefore, the frequency of allele B for κ-CN was very low in the samples analysed and the high amount of α_{s1}-CN detected in samples without κ-CN B should be explained by other factors such as breed, age, stage of lactation or lactation number, etc. that eventually affected milk composition [22,23]. The low number of samples containing κ-CN B agreed with previous works carried out in our laboratory (results non-published), in which only 5% of almost 100 casein' goat samples showed κ-CN B. Therefore, the α_{s1}-CN concentration is only rarely overestimated by this CE method.

4. Conclusions

A CE method was successfully applied for the quantitative determination of α_{s1}- and α_{s2}-CN in goat's milk. The analytical parameters studied for the CE method (linearity,

sensibility, accuracy, precision and recovery) are suitable for the quantitative determination of caprine α_{s1}- and α_{s2}-CN.

A good correlation was found between the quantities of α_{s1}-CN described for each genotype in the literature and the quantities calculated with the CE method. The optimised CE method is currently being used after calibration of the NIR equipment to control goat's milk production at Cordoba University.

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