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Comparative study of the protein fraction of goat milk from the Indigenous Greek breed and from international breeds

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Abstract

Individual goat milk samples, taken from animals of the Indigenous Greek breed and from the international breeds Saanen and Alpine, were studied by RP-HPLC regarding the qualitative and quantitative characteristics of their proteins. Thirty-two samples from the Indigenous Greek breed and 17 from the international breeds were characterised further by RP-HPLC/ESI-MS. The mean total protein content of the milk samples from the Greek breed was higher 38.8 g/l, compared to 31.9 g/l of those from international breeds, due to the great difference between their mean αs1-Cn contents (6.90 and 3.02 g/l, respectively). In the milk samples of the Greek breed, the strong αs1-Cn variants B3, B4 and As/B1 predominated, whereas in the milk samples from international breeds the medium variant E and the defectives F and null predominated. Variant A of αs2-Cn followed by variant C and the κ-Cn D (former B) were the most abundant in both groups. αs2-Cn F and the rare κ-Cn variant C/B were observed in the milk samples from the Greek breed. The β-caseins A and C were present in both groups of samples. Finally, the level of phosphorylation of the different genotypes is showed.

Keywords: Goat proteins; Casein variants; Phosphorylation; Protein quantification; Indigenous goat breed; RP-HPLC/ESI-MS

1. Introduction

Goat caseins show a complex qualitative and quantitative variability, mainly due to several genetic polymorphisms and multiple post-translational modifications. Alleles associated with low or null amounts of certain goat caseins have been reported. Apart from silent alleles, no protein variants of goat whey proteins have been characterised.

The unusual polymorphism of αs1-casein locus consists of 17 protein variants including three null alleles and influences the milk composition and technological behavior (Bevilacqua et al., 2002; Martin, Ollivier-Bousquet, & Grosclaude, 1999). The variability of goat αs2-casein locus is related to six alleles named A, B, C, D, E and F, to a null allele (Bouniol, Brignon, Mahé, & Printz, 1994; Lagonigro, Pietrola, D'Andrea, Veltri, & Pilla, 2001; Ramunno, Longobardi, et al., 2001; Ramunno, Cosenza, et al., 2001) and to one additional genetic variant G showed by isoelectric focusing (Erhardt, Jäger, Budelli, & Caroli, 2002). Moreover, the variability of goat αs1- and αs2-caseins is enhanced by the presence of deleted forms, resulting from alternative skipping (Cunsolo, Muccilli, Saletti, Marletta, & Foti, 2006; Ferranti, Lilla, Chianese, & Addeo, 1999).

Four genetic variants of β-casein named A, B, C and D have been reported. Three of them have been fully characterised with 5P and 6P (Galliano et al., 2004; Neveu, Mollé, Moreno, Martin, & Léonil, 2002; Roberts, Di Tullio, Vitale, Hehir, & Gordon, 1992). Variant B has been detected by IEF (Mahé & Grosclaude, 1993) but DNA or protein sequences have not been determined. Moreover, two null alleles (Cunsolo et al., 2005; Mahé & Grosclaude, 1993; Persuy, Printz, Medrano, & Mercier, 1999) and one silent allele (Cosenza, Pauciullo, Gallo, Di Berardino, & Ramunno, 2005) have been detected. The new

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developments in molecular knowledge have demonstrated that there is a high degree of polymorphism at the κ-casein locus of goats, consisting of 13 variants at the protein level and three silent mutations. Most of them have been recently identified in the blood of indigenous goats (Jann, Prinzenberg, Luikart, Caroli, & Erhardt, 2004; Prinzenberg, Gutscher, Chessa, Caroli, & Erhardt, 2005; Yahyaoui, Angiolillo, Pilla, Sanchez, & Folch, 2003). Another source of heterogeneity of κ-casein is the differences in the carbohydrate content (Moreno, Recio, Olano, & López-Fandiño, 2001).

During the last years, several studies based on molecular aid techniques have pointed out that the goat casein alleles show marked differences among breeds, as well as regional tendencies (e.g. Chessa et al., 2005; Chessa, Budelli, Gutscher, Caroli, & Erhatdt, 2003; Jann et al., 2004; Marletta, Bordonaro, Guastella, & D'Urso, 2004; Marletta, Bordonaro, Guastella, Criscione, & D'Urso, 2005; Prinzenberg et al., 2005; Sacchi et al., 2005; Zullo et al., 2005). The inclusion of the casein genotype, especially of the αs1-genotype as an additional selection criterion for dairy goat to improve the protein traits, in particular the protein content, is recommended (Martin et al., 1999; Sànchez, Ilahi, Manfredi, & Serradilla, 2005). Furthermore, it is under investigation how the genetic polymorphism of casein and the post-translational modifications are related to the nutritional properties of goat milk (Bevilacqua et al., 2001; Marletta et al., 2004). However, only a few studies have been reported, regarding the assessment of the genetic polymorphism and its relation to gross composition and to the quantity of different caseins in the milk of different breeds (Clark & Sherbon, 2000a, 2000b; Pierre et al., 1998; Remeuf, Ricordeau, Brignon, & Grosclaude, 2001; Zullo et al., 2005).

In Greece, there are many indigenous goat populations that have low milk production, live in general in small flocks under extensive breeding systems and are very well adapted to the climate and the mountainous topography of the country. A majority of them are considered as representatives of the Indigenous Greek breed. The aim of this research work was to study the protein fraction of the milk of the Indigenous Greek goat breed in comparison to that of the milk from the international breeds Saanen and Alpine. This study was triggered off by previous findings about the higher protein and fat contents of the milk from the Greek Indigenous breed compared to that of international highly selected breeds (Anifantakis & Kandarakis, 1980; Morgan et al., 2003; Simos, Voutsinas, & Pappas, 1991). For this purpose, individual milk samples were taken from goats of the Indigenous Greek breed from the Attiki region and from Saanen and Alpine goats (international breeds), which were analysed by RP-HPLC, using as standards the casein fraction of milk samples of known genotypes. A number of samples with particular qualitative (number of peaks, retention time) and quantitative characteristics (quantity of individual proteins) were chosen to be further analysed by IEF and RP-HPLC/ESI-MS.

2. Materials and methods

2.1. Milk samples

Two groups of individual milk samples were studied. Sixty individual milk samples were randomly collected after the complete morning milking of goats of the Indigenous Greek breed in the Attiki region. Similarly, 60 individual milk samples were collected from goats of the international Alpine and Saanen breeds (30 from each breed). After the addition of sodium azide (0.4 g/l), the milk samples were cooled down. The total protein contents of the milk samples were determined by means of infrared spectroscopy (Milkoscan 133 A/S N. Foss Electric, Denmark), which was calibrated against the reference methods using goat milk samples with different composition. Milk samples were skimmed by centrifugation at 2000*g* for 30 min at 4 °C. An aliquot of skim milk was used for chromatographic analysis and the rest was lyophilised.

2.2. RP-HPLC of milk samples

The defatted milk samples were analysed by RP-HPLC on a Vydac C4 214 TP 5415 column (Separation Group, Hesperia, CA 92345, USA). The HPLC system consisted of the Waters 600E pump (Waters, 34 Marple Street, Milford, MA, 01757, USA) a diode array UV/Vis detector (Waters 996), a helium degasser, a Rheodyne injector (model 7125, Rheodyne Inc., Cotati, California, USA) and Millenium v. 3.05.01 software (1998, Waters). Solvent A was 1.06 ml/l trifluoroacetic acid in ultra pure water and Solvent B was 1 ml TFA, 800 ml acetonitrile and 200 ml ultra pure water. The flow rate was 1 ml/min, the analyses were carried out at 40 °C and the eluent was monitored at 214 nm. A linear gradient from 350 to 620 ml/l Solvent B, within 54 min, was applied (Jaubert & Martin, 1992; Neveu et al., 2002). Samples were prepared as follows: 0.5 ml of defatted milk was dissolved in 1 ml buffer, pH 7.0 (100 mM Tris–HCl, 8 M urea, 13 g/l trisodium citrate, 20 mM dithiothreitol). After 1 h at 37 °C, 10 ml of Solvent A containing 6 M urea was added to the sample solution and the pH was adjusted to 2.1–2.2 by the addition of 0.5 ml of a TFA solution (100 ml/l). After filtration through 0.45 μm filter (Millipore Corporation, Bedford, MA 01730, USA), 50 μl of sample was injected. Two independent preparations of each sample were analysed and the profiles were compared to those obtained from the analyses of whole casein standards of known casein genotypes (κ-casein AA, αs2-casein AA, αs1-casein AA, EE and O1O1, and αs1-casein CC) (Neveu et al., 2002).

2.3. RP-HPLC/ESI-MS of certain milk samples

After the assessment of the chromatographic profiles, 32 samples were selected for further analysis by RP-HPLC/ESI-MS. The selection was based on the retention time and on the quantification data obtained after the

integration of their protein profiles. A Vydac C4 column 214 TP 5215 was used in a system coupling on-line RP-HPLC and ESI-MS. RP-HPLC was carried out on a Hewlett Packard 1100 system (Agilent Technologies, Massy, France) at a flow rate of 0.25 ml/min at 40 °C and the detection was by absorbance at 214 nm and by total ion current. Solvents A and B were as previously described. The elution conditions were as follows: 370 ml/l Solvent B for 5 min, then a linear gradient from 370 to 570 ml/l Solvent B within 37.5 min was applied. The column was directly interfaced with a Sciex API III Plus mass spectrometer (Perkin–Elmer–Sciex, Thornhill, Ontario, Canada), through a post-flow splitter permitting to introduce only 1/10 of the HPLC eluate into the mass spectrometer. The ion source voltage and the orifice voltage were set at 4–5 kV and 70/90 V, respectively. Positive ion mode was used and mass scans were acquired over a m/z range of 500–2400 with a step size of 0.3 Da and a dwell time of 1 ms per step. The charge number of the multicharge ions, the deconvoluted mass spectra and the proteins M_r (mass) determination were obtained using the BioMultiView software 1.3.1. (PE-SCIEX). Fifty milligrams of lyophilised milk was dissolved in 800 ml buffer, pH 7.0 (100 mM Tris–HCl, 8 M urea, 13 g/l trisodium citrate, 20 mM dithiothreitol). After 1 h at 37 °C, 3 ml of Solvent A containing 4 M urea was added to the sample solution and the pH was adjusted to 2.1–2.2 with the addition of 150 μl of a TFA solution (100 ml/l). After filtration through 0.45 μm filter (Millipore Corporation, Bedford, MA 01730, USA), 50 μl of sample was injected.

2.4. Isoelectric focusing (IEF) of whole casein

Whole casein of samples, with characteristic protein profiles prepared by acidification at pH 4.2 and whole casein of the standard milks of known genotypes, were simultaneously analysed by isoelectric focusing on ultrathin (0.20 mm) polyacrylamide gels, as described by Moatsou, Samolada, Katsabeki, and Anifantakis (2004). The gel layers were prepared with a mixture of ampholytes consisting of Ampholine™ pH 2.5–5.0, Pharmalyte™ pH 4.5–5.4 and Ampholine™ pH 4.0–6.5 (Amersham Pharmacia Biotech) in a 1.6:1.4:1 ratio, according to Ferranti et al. (2001). Analysis was carried out in a LKB 2117 Multiphor II Electrophoresis Unit (Amersham Pharmacia Biotech). Fixation was carried out by immersing the gels in a 15% trichloroacetic acid solution and staining in a methanolic dilution of Comassie brilliant blue G250 as described in detail in the Commission Regulation, EEC No 213/2001.

2.5. Statistical analysis

The software Statgraphics Plus for Windows 2.1 (Manugistics, Inc., Rockville, MA 20852, USA) was used for the statistical analysis. Principal component analysis (PCA) was applied with the aim to group the milk samples of each breed. The factors with eigenvalues >1 were retained.

3. Results and discussion

3.1. Quantitative characteristics of the protein profiles

Some characteristic RP-HPLC profiles of the defatted goat milks from the Indigenous Greek breed are presented in Fig. 1. The main peaks were κ-Cn (1), αs2-Cn (2), αs1-Cn (3), γ-Cn (4), β-Cn (5), α-La (6) and β-Lg (7). The position of the whey proteins on the chromatograms was confirmed by analysing goat acid whey and individual whey proteins prepared by gel filtration. The elution order of γ-Cn was determined by the analysis of a plasmin hydrolysate of goat β-Cn, prepared by cation-exchange chromatography (Moatsou et al., 2004). Finally, to check the efficiency of separation, mixtures of β- and γ-Cn and mixtures of β-Cn with whey proteins were also injected. The quantification data based on the peak areas of the chromatographic profiles are presented in Table 1.

The retention time (volume) of individual caseins changed from day to day, but changes in the elution order or loss of resolution were not observed. The β-casein was resolved in a single peak and it was the most abundant individual casein of the goat milk samples, being $45 \pm 3.5\%$ and $49 \pm 5\%$ of total casein for Greek and international breeds, respectively. Therefore, the retention times of the peaks were calculated relatively to that of β-Cn. The individual caseins of standard genotypes had different relative retention times (rRTs) with the exception of the variants of κ-Cn, which co-eluted each-other.

The milk samples which were different from the profiles of the standards (peak shape or retention time) as well as those with marginal quantitative characteristics (contents of total protein, of total casein and of individual caseins) were chosen to be further analysed by IEF and RP-HPLC/ESI-MS (15 from the Indigenous Greek breed and 17 from the international breeds). Finally, four samples of the Greek goats were not included in the study due to proteolysis. The experimental masses of the individual casein peaks were assigned to the known theoretical masses, when their difference was lower than 0.03%. For the assignment to the known variants the rRT and the quantity of the corresponding peak was also taken into consideration, since several variants sharing very similar molecular masses and having the same rRT, differ in their levels of expression.

According to Table 1, there was a great difference between the mean αs1-Cn contents of the two groups of samples (6.90 ± 1.57 and 3.02 ± 2.35 g/l), whereas there was a great standard deviation of the mean of the milk samples from international breeds. On the other hand, αs2- and κ-casein contents were similar in both groups of samples.

An individual variability in β-Lg content was observed as shown in Table 1. The β-Lg/α-La ratio estimated from 0.81 to 2.09 (mean value 1.39 ± 0.32) within the samples from Indigenous Greek breed, the majority of them (32 out of 56) being from 1.2 to 1.7. The respective values

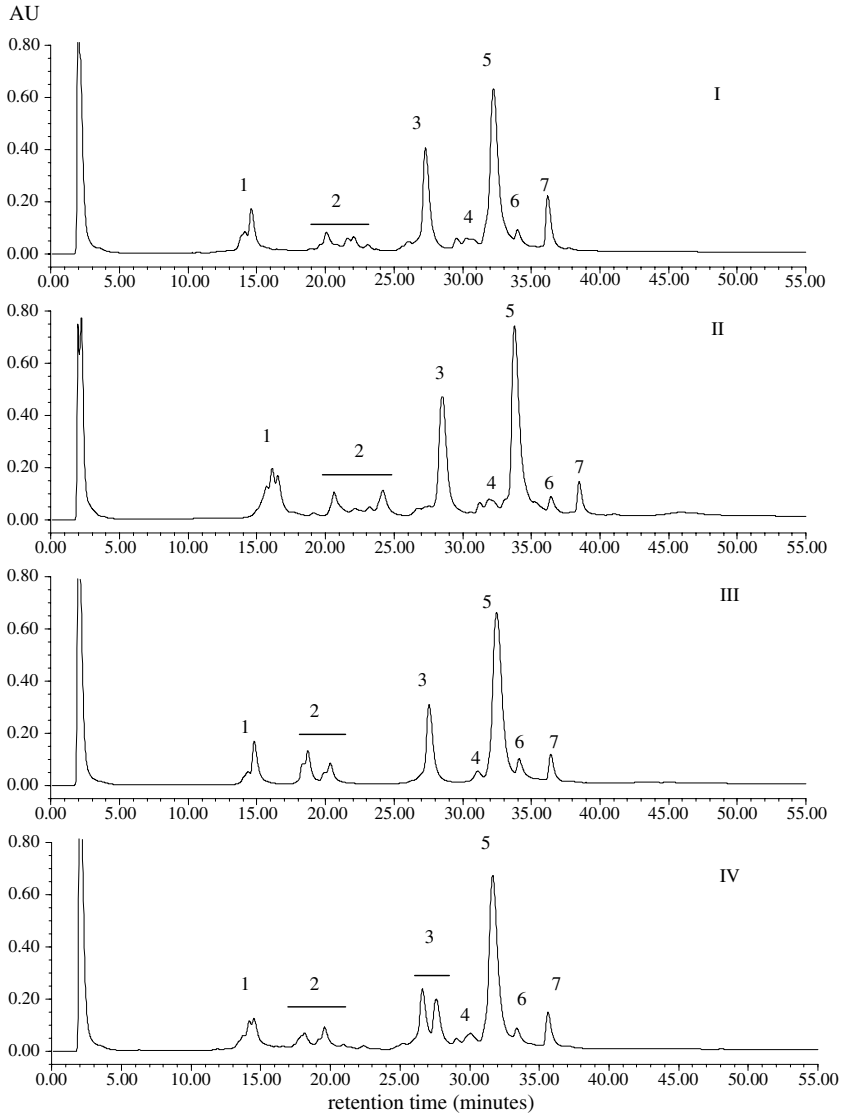

Fig. 1. Characteristic RP-HPLC profiles (A214) of individual goat milk samples from the Indigenous Greek breed. Column: Vydac C4 214 TP 5415; Elution conditions: as described in Section 2.2. The variants of caseins were determined by RP-HPLC/ESI-MS. I: κ-Cn D C/B, αs2-Cn C F, αs1-Cn B4 B4, β-Cn A A (high αs1-Cn content, 8.1 g/l); II: κ-Cn A C/B, αs2-Cn A F, αs1-Cn B3 B4, β-Cn A A (high αs1-Cn content, 8.1 g/l); III: κ-Cn D D, αs2-Cn A C, αs1-Cn B3 E, β-Cn A C (medium/high αs1-Cn content, 5.8 g/l); IV: κ-Cn D G, αs2-Cn A C, αs1-Cn B3 As/B1, β-Cn C C (high αs1-Cn content, 8.1 g/l).

within those from international breeds were from 0.49 to 2.38 (mean value 1.35 ± 0.35), the majority of them (48 out of 60) being from 0.8 to 1.6. Chianese et al. (2000) have also detected differences in β-Lg content ranging from 43% to 63% of the major whey proteins.

3.2. κ-Casein

In Table 2, the types of κ-Cn including variants and phosphorylation levels of the samples analysed by RP-HPLC/ESI-MS are presented. The front part of the peaks included the glycosylated forms (Moreno et al., 2001). The presentation is according to the most recent nomenclature, proposed by Jann et al. (2004) in which the former B variant (Yahyaoui et al., 2003) has been renamed to D and vice versa.

Most of the milks from the Greek breed (Table 2) contained variants A and D (former B). Especially variant D was the most abundant observed in 13 samples out of 15, followed by variant A, as previously reported for most of the European goat breeds (Chessa et al., 2003; Sacchi et al., 2005; Yahyaoui et al., 2003) with frequencies ranging from 0.40 to 0.78.

Four samples from the Indigenous Greek breed contained variants C or B, symbolised as C/B. These two variants have the same molecular mass due to their amino acid sequence (Yahyaoui et al., 2003) and they cannot be distinguished. Furthermore, since they belong to the same group regarding their isoelectric point, they were undistinguishable by IEF analysis (Prinzenberg et al., 2005). Variant B has been found in European and African breeds at frequencies ranging from 6.5% to 67%, but variant C is in general

Table 1
Quantitative characteristics of the protein fraction (expressed in g/l) of individual goat milk samples from the Indigenous Greek breed and from international breeds

	Total protein[a]	κ-Cn[b]	αs2-Cn[b]	αs1-Cn[b]	γ-Cn[b]	β-Cn[b]	Total CN[b]	α-LA[b]	β-Lg[b]
Indigenous Greek breed									
n	56	56	56	56	56	56	56	56	56
Mean	38.8[b]	3.93	3.93	6.90[b]	1.51	13.2[b]	29.5[b]	2.49[b]	3.35[b]
SD	2.93	0.72	0.86	1.57	0.31	1.18	2.33	0.61	0.65
Min	33.1	2.50	1.57	3.62	0.94	10.9	24.8	1.51	2.22
Max	46.8	5.56	6.14	9.58	2.12	16.2	34.5	4.38	5.63
International breeds									
n	60	60	60	60	60	60	60	60	60
Mean	31.9[a]	3.77	3.84	3.02[a]	1.37	11.5[a]	23.5[a]	2.25[a]	2.94[a]
SD	3.5	0.59	0.68	2.35	0.30	1.42	3.17	0.44	0.64
Min	24.6	2.65	1.67	0	0.74	8.42	15.8	1.40	1.59
Max	43.5	5.29	5.47	7.69	2.2	15.0	31.4	3.26	4.83

The respective means with different superscripts were significantly different (*t*-test at the 95% confidence level).
[a] Estimated by Milkoscan (Section 2.1).
[b] Estimated by the area of the chromatographic peaks and by the compositional data (Section 3.1).

Table 2
Types of κ-Cn in individual goat milk samples from the Indigenous Greek breed and from international breeds determined by RP-HPLC/ESI-MS

Types of κ-casein	*N*[a]	Peaks[b]	κ-Cn, g/l[e]	κ-Cn/total Cn[e]
Greek Indigenous breed				
D[c] 1P **2P**	4	1	3.58 ± 0.53	0.13 ± 0.01
A 1P **2P** + D[c] 1P **2P**	6	1	3.77 ± 0.78	0.12 ± 0.02
C/B[d] 1P **2P**	1	1	5.06	0.17
D[c] 1P **2P** + C/B[d] 1P **2P**	2	1	4.40	0.15
A **2P** + C/B[d] **2P**	1	2	5.56	0.17
D[c] 1P **2P** + G 1P **2P**	1	2	4.21	0.13
International breeds				
A (1P) **2P**	1	1	3.25	0.18
D[c] (1P) **2P**	10	1	3.15 ± 0.29	0.13 ± 0.02
A (1P) **2P** + D[c] (1P) **2P**	4	1	4.03 ± 0.71	0.17 ± 0.03
A **2P** + G **2P**	1	2	4.15	0.15
D[c] (1P) **2P** + G (1P)**2P**	1	2	3.96	0.14

Phosphorylations in parenthesis were rare and the most abundant of them are typed in bold.
[a] Number of samples with this certain type of κ-Cn.
[b] Number of peaks in the RP-HPLC profile.
[c] Former B variant, according to the nomenclature proposed by Jann et al. (2004).
[d] Former D variant according to the nomenclature proposed by Jann et al. (2004).
[e] Estimated as described in Section 3.1.

rare observed in Saanen goats at frequencies from 3% to 13% (Chessa et al., 2003; Prinzenberg et al., 2005; Sacchi et al., 2005; Yahyaoui et al., 2003). In the present study, the variant C/B was present in four samples out of 15 from the Greek breed and it was not observed in the samples from the international breeds. Finally, in one milk sample from the Indigenous Greek breed and in two from the international breeds, a mass corresponding to κ-Cn variant G was detected which has been found in Italian and Turkish breeds at low frequencies (Prinzenberg et al., 2005; Yahyaoui et al., 2003).

In all cases the major form of κ-Cn was the 2P form. Variants C/B and G were separated from variants A and D, respectively, on the C4 RP-HPLC column although not at the baseline, whereas the most abundant A and D variants co-eluted with each-other (Fig. 1, Table 2).

3.3. αs2-Casein

Table 3 shows the characteristics of αs2-Cn of the total of 116 individual milk samples from both groups of goats, based on the assignment of the peaks of αs2-Cn in the RP-HPLC profiles (e.g. Fig. 1). Each αs2-Cn peak was attributed to a particular variant by considering: (i) the rRT of αs2-Cn peaks of the profiles in comparison to that of the standard genotypes analysed by the same method, (ii) the RP-HPLC/ESI-MS results (experimental masses of particular samples), which indicated that αs2-Cn A, C and F were very clearly separated as shown in Fig. 1, (iii) the quantification data, (iv) the IEF profiles, although their interpretation was sometimes uncertain due to the multiple bands of αs2-Cn in IEF gels, and (v) the results of previous studies.

The variable contents in αs2-Cn (Table 1), which were in part a result of the different protein content of the samples and from their variable αs1-Cn contents, made the interpretation of quantification data complex. Therefore, to classify milk samples according to their αs2-Cn content, a principal component analysis (PCA) was performed (Fig. 2). Apart from the αs2-Cn content (g/l), also the β-Cn content (g/l) and the ratio β-Cn/αs2-Cn were used as original variables, because β-Cn presented the lowest variability (Table 1). Two components with eigenvalues >1 explained 97.6% and 97.7% of the total variance in the Greek and international breeds, respectively.

Taking into account the above-mentioned data and the PCA plot, it had been concluded that within the samples from both groups of breeds there were two levels of αs2-Cn content: low and high. According to rRTs and RP-HPLC/ESI-MS results, there were three genotypes

Table 3
Characteristics of αs2-casein of individual goat milk samples from the Indigenous Greek breed and from international breeds

Groups	αs2-Cn peaks	rRT[a]	αs2-Cn content	N[b]	αs2-Cn, g/l[c]	αs2-Cn/total Cn[c]	Types of αs2-casein	Samples analysed by RP-HPLC/ESI-MS[d]
Indigenous Greek breed								
A	1	13.80 ± 0.42	Low	4	2.48 ± 0.50	0.08 ± 0.01	A 6P 7P 8P 9P 10P **11P**	2
			High	17	4.24 ± 0.68	0.15 ± 0.02	A 6P 7P 8P 9P 10P **11P** or A 5P **6P 7P** 8P 9P 10P 11P	3
B	1	12.17 ± 0.27	Low	2	1.98	0.07		–
			High	5	3.24 ± 0.28	0.12 ± 0.01	C 8P 9P 10P **11P**	1
C	2	13.78 ± 0.27/12.14 ± 0.27	High	24	4.17 ± 0.55	0.14 ± 0.02	A(6P) 7P 8P 9P **10P 11P**/C 7P 8P 9P 10P **11P**	6
D	2	13.66 ± 0.28/10.08 ± 0.31	High	3	4.58 ± 1.10	0.15 ± 0.03	A 7P 8P 9P **10P 11P**/F (6P) 7P 8P 9P 10P **11P**	2
E	2	12.25/10.26	High	1	3.85	0.13	C 6P 7P 8P 9P 10P **11P**/F 7P 8P 9P 10P **11P**	1
International breeds								
A	1	13.52 ± 0.51	Low	5	2.36 ± 0.58	0.09 ± 0.02	A (8P) 9P 10P **11P**	1
			High	35	3.92 ± 0.55	0.17 ± 0.03	A (7P) 8P 9P 10P 11P or A 9P 10P **11P** + A/B 7P 8P 9P **10P**	9
B	1	12.06	High	1	3.76	0.16		–
C	2	13.72 ± 0.55/12.01 ± 0.64	High	19	4.08 ± 0.43	0.17 ± 0.03	A (7P) 8P 9P 10P **11P**/C (7P) 8P 9P 10P **11P** or A/B 6P 7P 8P 9P **10P**/C 7P 8P 9P 10P **11P**	7

The identification of the variants was based on the experimental masses, rRT and IEF profiles (Section 3.3). Phosphorylations in parenthesis were rare and the most abundant of them are typed in bold.

[a] Retention time relative to that of β-Cn.
[b] Number of samples in each group.
[c] Estimated as described in Section 3.1.
[d] Samples of each group analysed by RP-HPLC/ESI-MS.

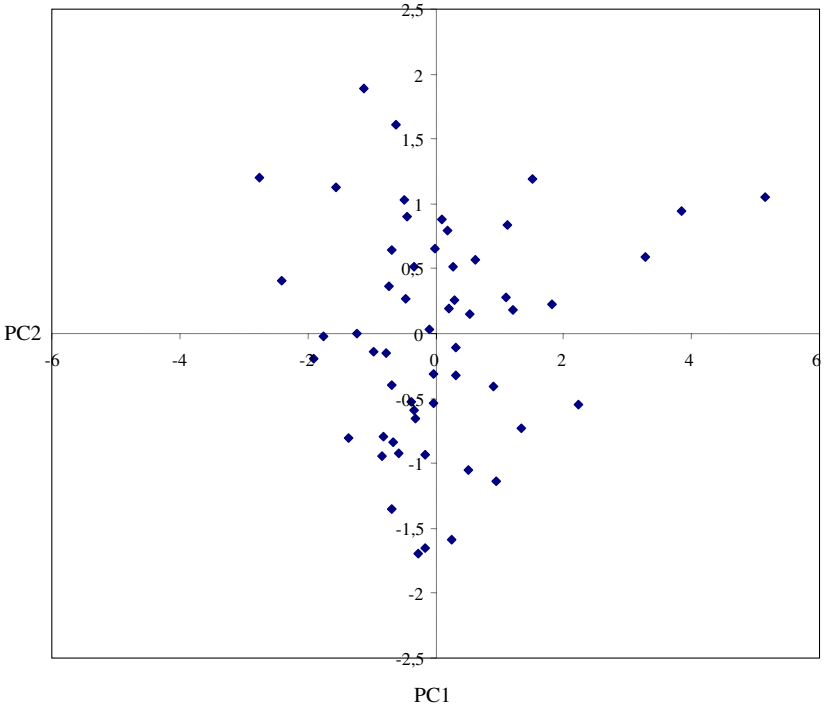

Fig. 2. Plot of PCs based on αs2-Cn (g/L), β-Cn (g/L) and on β-Cn/αs1-Cn ratio.

assigned to variants A (or B), C and F in the samples from the Indigenous Greek breed that were clearly separated using the RP-HPLC system (Fig. 1). The discrimination between αs2-Cn A and B is difficult since they almost co-elute with each-other and they differ by 1 mass unit. However, variant B has 1P lower than A, due to the amino acid

substitution Glu64 → Lys64, that affects the phosphorylation site Ser62 (Neveu et al., 2002). In addition to that, it has a different IEF pattern. According to the IEF patterns and the phosphorylation level observed, the αs2-Cn B was not found in the milks from the Indigenous Greek breed.

The individual milk samples from the Indigenous Greek breed were classified in five different groups regarding the rRT of their αs2-Cn peaks as presented in Table 3. The goat αs2-casein variants are associated with the normal production level of 2.5 g/l and they differ regarding amino acid substitutions, with the exception of variant D. This variant is a deleted form, 205 amino acids long, associated with a decreased synthesis of αs2-casein (Ramunno, Cosenza, et al., 2001). The profiles of the samples with low αs2-Cn content had only one αs2-Cn peak and considering the ratio of low to high αs2-content, it can be assumed with reasonable certainty that they are heterozygotes with a null variant. The most frequent was the variant A, found in 48 samples out of 56 (milk samples of group A with high αs2-Cn content, milks of group A with low αs2-Cn content, milk samples of group C and D). The second most frequent variant was αs2-Cn C followed by the null allele in the heterozygote condition with A or C variant as discussed above. Finally, the less frequent was variant F that was present only in the heterozygote condition with A and C variants.

The milk samples from the international breeds were classified, following the same strategy, into three groups (Table 3). In this case, variant B was observed following the above-mentioned discrimination criteria in four out of the 17 samples analysed by RP-HPLC/ESI-MS and by IEF. The low αs2-Cn content of five samples implied the existence of a null allele. Finally, three αs2-Cn variants A, B and C were observed in these samples, the most abundant being the variant A followed by variant C. Variant F was not observed in this case.

According to the literature, variant A is in general predominant in the milk of most goat breeds. The frequencies of goat αs2-Cn A, B and C in the French dairy breed Alpine and Saanen are 0.85, 0.04 and 0.11, respectively (Bouniol et al., 1994). Erhardt et al. (2002) report that variant A is predominant in German and Italian breeds (from 0.662 to 0.922), while the variant B occurs at frequencies lower than 10% or at zero frequencies and the variant C occurs with higher frequencies in Italian breeds than in German breeds. The abundance of variant A in Italian goat breeds has been reported also by Ramunno, Longobardi, et al. (2001), Marletta, Bordonaro, Guastella, and D'Urso (2004) and Sacchi et al. (2005). According to their findings, variant F is also abundant followed by variant C, whereas variants B, D and null are the most rare. Lagonigro et al. (2001) have characterised αs2-Cn E and report that its frequency is 10.6%, 6.7%, 0%, 0% and 0% in goats of three different Italian breeds and of Alpine and Saanen goats, respectively, in agreement to Sacchi et al. (2005) who report very low frequencies from 0 to 0.171 in five Italian breeds. Finally, Erhardt et al. (2002) report αs2-Cn G separated by IEF with an isoelectric point between A and C that occurs at very low frequencies (from 0.004 to 0.068) in two German and one Italian breed.

The αs2-Cn was present in several phoshorylated forms as it is the most phosphorylated casein with the level of phosphorylation ranging from 6 to 11P, in accordance with the findings of Neveu et al. (2002) and Pierre et al. (2001). Experimental masses corresponding to the 12P and 13P types reported by Trujillo, Casals, and Guamis (2000) were not observed. In most cases, the most abundant types were that of 11P followed by that of 10P.

3.4. αs1-Casein

Among the 14 protein variants of αs1-Cn detected up to now in addition to three null alleles (Bevilacqua et al., 2002; Martin et al., 1999; Ramunno et al., 2002), there are strong variants associated to an αs1-Cn content of 3.6 g/l per allele (A, B1, B2, B3, B4, C, L, M) and 4.2 g/l (H), intermediate variants associated with 1.6 g/l αs1-Cn per allele (E, I), weak variants (D, F, G) associated with 0.6 g/l and the null variants (O1, O2, N). Apart from genetic polymorphism, mature goat αs1-Cn exists as a mixture of at least seven molecular species with different peptide chain lengths as a result of alternative skipping. The main component corresponds to the 199-residue-long-form and the deleted proteins differ from the complete one by the absence of peptides 141–148, 110–117 or Gln78 or a combination of such deletions (Ferranti et al., 1999).

For the interpretation of the complex results regarding αs1-Cn, a strategy similar to that for αs2-Cn was followed. The outcome is shown in Tables 4 and 5 (similar to Table 3) and it was based on the following: (i) the quantification data of Table 1, (ii) the rRT of αs1-Cn peaks in the RP-HPLC profiles in comparison to that of the standard genotypes analysed by the same method (Table 2), (iii) the elution order of αs1-Cn, which according to Jaubert and Martin (1992), Clark and Sherbon (2000a, 2000b) and Bevilacqua et al. (2002) is: D < M, F, E, B3, B4 < C, A, I < B1, B2, (iv) the RP-HPLC/ESI-MS results, (v) the IEF profiles, (vi) the PCA plots of the two principal components based on αs1-Cn and β-Cn contents (g/l) and on the β-Cn/αs1-Cn ratio that accounted for 99.2% of the variability within the samples from Indigenous Greek breed and for 92.9% within the samples from international breeds (Fig. 3), (vii) the fact that there are two couples of αs1-Cn variants (As and I, B4 and E) with the same amino acid composition – therefore undistinguishable in IEF and RP-HPLC profiles – corresponding to different αs1-Cn production, and (viii) the fact that variants A and B1 having similar elution order and the same expression level, differing by 1 mass unit. However in variant A there is a potential tenth phosphorylation site at Ser75 (Mercier, 1981) which is eliminated in variant B1 due to Glu/Gln replacement at position 77.

In Table 4, the milk samples from the Indigenous Greek breed were allocated in the three groups according to the

Table 4
Characteristics of αs1-casein of individual goat milk samples from the Greek Indigenous breed

Groups	αs1-Cn peaks	rRT[a]	αs1-Cn content	N[b]	αs1-Cn g/l[c]	αs1-Cn/ total Cn[c]	Types of αs1-casein	Samples analysed by RP-HPLC/ESI-MS[d]
A	1	5.24 ± 0.21	Medium	5	4.40 ± 0.59	0.16 ± 0.01	E **8P** 9P (10P)	3
			High	15	7.61 ± 1.04	0.25 ± 0.02	B4 8P **9P**(10P) or B4 8P **9P** (10P) + B3 6P 7P **8P** 9P (10P)	4
			Medium/ high	3	5.82 ± 0.05	0.20 ± 0.02	E **8P** 9P + B3 **8P** 9P	1
B	1	4.32 ± 0.23	Medium	6	4.54 ± 0.73	0.16 ± 0.01	I **7P** 8P 9P	1
			High	6	7.56 ± 1.09	0.26 ± 0.02	As/B1 **7P** 8P 9P	1
			Medium/ high	2	5.95 ± 0.02	0.21 ± 0.02	A/B1 **7P** 8P 9P (10P) + I **7P** 8P	1
C	2	4.30 ± 0.17/5.35 ± 0.21	High	19	7.80 ± 0.91	0.26 ± 0.02	B3 **8P** 9P (10P)/As/B1 **7P** 8P (9P) or	4
		5.35 ± 0.21					B4 8P **9P** (10P)/As/B1 **7P** 8P (9P)	

The identification of the variants was based on the experimental masses, rRT and IEF profiles (Section 3.4). Phosphorylations in parenthesis were rare and the most abundant of them are typed in bold.
[a] Retention time relative to that of β-Cn.
[b] Number of samples in each group.
[c] Estimated as described in Section 3.1.
[d] Samples of each group analysed by RP-HPLC/ESI-MS.

Table 5
Characteristics of αs1-casein of individual goat milk samples from international breeds

Groups	αs1-Cn peaks	rRT[a]	αs1-Cn content	N[b]	αs1-Cn, g/ l[c]	αs1-Cn/ total Cn[c]	Types of αs1-casein	Samples analysed by RP-HPLC/ESI-MS[d]
Null				8				1
A	1	5.12 ± 0.26	Medium/ low	8	1.92 ± 0.61	0.09 ± 0.03	E 8P **9P**	1
			Medium	10	4.08 ± 0.38	0.17 ± 0.01	C 7P 8P 9P **10P** 11P or E/B4 7P 8P **9P** (10P)	3
			High	5	6.81 ± 0.64	0.27 ± 0.02	B3 8P **9P**	1
			Medium/ high	1	5.43	0.20		–
B	1	4.10 ± 0.20	Medium	6	4.41 ± 0.31	0.17 ± 0.01	As/B1 **7P** **8P** 9P	2
			High	1	6.86	0.24	As/B1 **7P** **8P** 9P	1
C	1	5.72 ± 0.18	Very low	9	0.57 ± 0.02	0.02 ± 0.004	F 1P **2P** 3P	1
D	2	4.17/5.18	High	2	7.59	0.26	As/B1 7P **8P**/C 9P **10P** or As/B1 7P **8P** 9P/B3 8P **9P**	2
E	2	5.00 ± 0.27/ 5.97 ± 0.56	Medium	3	4.68 ± 0.59	0.17 ± 0.006	B3 6P 7P **8P** 9P 10P/F 1P **2P**	2
			Medium/ low	4	1.94 ± 0.51	0.09 ± 0.02	E 8P 9P/F 1P **2P**	3
F	2	4.00/5.68	Medium	2	4.98	0.19		–

The identification of the variants was based on the experimental masses, rRT and IEF profiles (Section 3.4). Phosphorylations in parenthesis were rare and the most abundant of them are typed in bold.
[a] Retention time relative to that of β-Cn.
[b] Number of samples in each group.
[c] Estimated as described in Section 3.1.
[d] Samples of each group analysed by RP-HPLC/ESI-MS.

rRT of their αs1-Cn peaks. Three different levels of expression were detected within the samples. The majority of the samples were included in the group of high αs1-Cn content (about 7.7 g/l), while 11 samples were in the group of medium αs1-Cn content (about 4.5 g/l). Five samples constituted the group with medium/high content (about 6 g/l) which implies the existence of a strong and a medium variant in the same peak (Fig. 1). No milk samples with a null or a weak variant (D, F, G) in the homozygous condition were found within this population. Therefore, the possibility that the samples with medium αs1-Cn content to be heterozygotes (strong + null variant) was not taken into consideration.

On the contrary, eight out of 60 samples from the international breeds were homozygous for the null αs1-Cn allele (Table 5), whereas the weak variant F was present in nine samples out of 60, according to rRT and RP-HPLC/ESI-MS analyses. Therefore, these samples were allocated

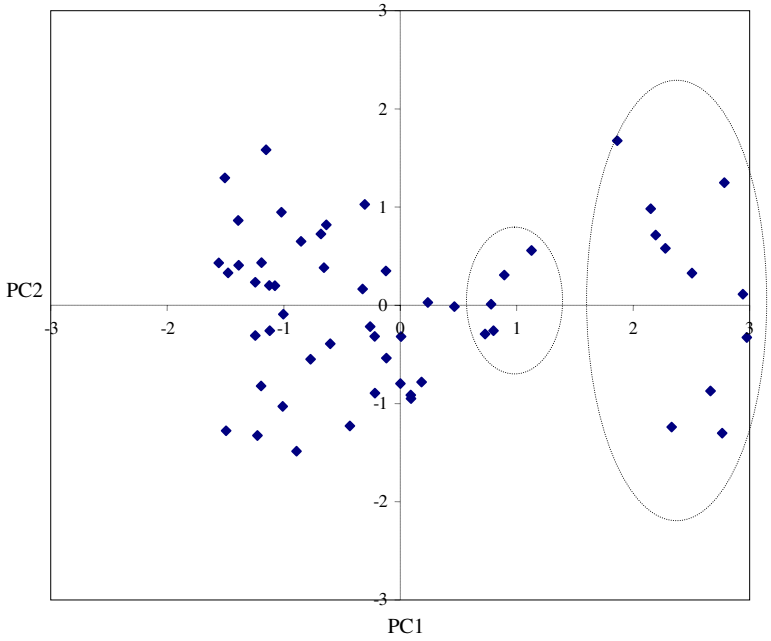

Fig. 3. Plot of PCs based on αs1-Cn (g/L), β-Cn (g/L) and on β-CN/αs1-Cn ratio.

according to the characteristics of their αs1-Cn in several groups as presented in Table 5. The majority of these samples (21 out of 60) had a medium αs1-Cn content (about 4.3 g/l). Unlike the samples from the Greek breed, the milks with high αs1-Cn content (about 7.0 g/l) were a minority within these samples.

From the data of Table 4, it was obvious that the strong αs1-Cn variants B3 and B4 were predominant in the milk of the Greek Indigenous goat breed (in 37 samples out of 56) followed by variants As or B1 (in 27 samples out of 56). The opposite was true for the milk samples from international breeds, where the medium variant E, the weak variant F and the null variant predominated. Variant C was found only in this group of samples.

The above-mentioned results were in accordance with the literature. According to Martin et al. (1999), Sacchi et al. (2005) and Marletta et al. (2005), highly expressed alleles predominate in breeds from the Mediteranean area and in breeds originating from Africa, while the medium allele E is by far the most frequent in French, Spanish and Swiss breeds and in breeds from Nothern Itlay, followed by "weak" and null alleles.

Variant B3 had up to five phosphorylation levels, from 6 to 10P, the major form being 8P, whereas B4 presented up to three phosphorylation levels from 8P to 10P, the major form being 9P. The masses of the variants As/B1 and I were assigned to the phosphorylated forms 7P, 8P and 9P, the major form being 7P and variant E to the forms 8P, 9P and 10P, the major form being 8P, in accordance with the findings of Neveu et al. (2002), Trujillo et al. (2000) and Pierre et al. (2001). Finally, variant F was present with 1-3P according to Brignon, Mahé, Ribadeau-Dumas, Mercier, and Grosclaude (1990).

In all samples analysed by RP-HPLC/ESI-MS, the deleted form differing from the respective complete form by Gln78 was found at different levels of phosphorylation with the exception of variant F. No other deleted forms were detected in the profiles probably due to their low quantities.

3.5. β-Casein

The characteristics of the β-Cn fraction of the samples are presented in Table 6. There are six genetic variants of β-Cn: A, B, C associated with a normal β-Cn content in milk, the recently found variant D and two null alleles O and O′. They co-elute with each-other in the RP C4-HPLC column and they are also undistinguishable by IEF, since the mono amino acid substitutions in variants A, C and D are neutral (Chessa et al., 2005). The 32 samples analysed by RP-HPLC/ESI-MS are shown in Table 6. The contents of β + γ-Cn of the samples were also presented, because the latter results from the hydrolysis of the former.

The determined molecular masses were assigned to variants A and C (Table 6). In addition to this an unknown mass of 23 876 or 23 878 was detected in the heterozygous condition in 2 (out of 15) Greek samples, in at least two levels of phosphorylation, which is under investigation. The major phosphorylation levels were 5P and 6P in both groups, but forms with 3P and 4P existed in low quantities in the milk samples from Indigenous Greek breed. It is noteworthy that in some samples, there were 7P forms of both A and C variants. Goat β-Cn with 7P has not being reported to our knowledge, although there are seven potential phosphorylation sites on its amino acid sequence according to the consensus tripeptide sequence Ser/Thr-X-A, where X represents

Table 6
Types of β-Cn in individual goat milk samples from the Indigenous Greek breed and from international breeds determined by RP-HPLC/ESI-MS

Variant	N[a]	β-Cn, g/l[b]	β + γ Cn, g/l[b]	β-Cn/total Cn[b]
Indigenous Greek breed				
C (3P 4P) **5P** 6P or C (3P 4P) **5P** 6P 7P	3	15.20 ± 0.97	16.73 ± 1.15	0.47 ± 0.02
A (3P 4P) **5P 6P** or A (3P 4P) **5P 6P** 7P	3	13.32 ± 1.15	14.81 ± 1.18	0.43 ± 0.04
[A (3P 4P) **5P 6P**or A (3P 4P) **5P 6P** 7P] + [C (3P 4P) **5P** 6P or C (3P 4P) **5P** 6P 7P]	7	13.14 ± 1.45	14.51 ± 1.44	0.47 ± 0.04
A (3P 4P) **5P 6P** + unknown (4P) **5P 6P**	2	13.48	14.96	0.47
International breeds				
C 5P **6P** or C **5P 6P** or C 4P **5P** 6P	7	11.46 ± 1.57	12.95 ± 1.75	0.45 ± 0.09
[A **6P** 7P or A 5P **6P** or A **5P 6P**] + [C 5P **6P** or C **5P 6P**]	10	11.64 ± 1.56	13.04 ± 1.61	0.49 ± 0.05

Phosphorylations in parenthesis were rare and the most abundant of them are typed in bold.
[a] Number of samples with this type of β-Cn.
[b] Estimated as described in Section 3.

any amino acid residue and A is an acidic residue Glu or Asp or a phosphorylated Ser (Bevilacqua et al., 2002; Mercier, 1981). According to Neveu et al. (2002) and Galliano et al. (2004), the five phosphorylation sites are Thr12 and Ser15, 17, 18, 19 and the sixth is Ser 35, whereas Trujillo, Guamis, and Carretero (1997) suggest that Thr 41 is phosphorylated instead of Thr12. In conclusion, β-Cn A and C predominated in the milk samples from the Greek Indigenous breed and variant C predominated in the international breeds. The results of Table 6 showed that the β-casein genotypes were not related to differences in the β-Cn content of the milks.

The information about the frequencies of goat β-Cn alleles is rare. The O′ allele was found in goat breeds reared in Italy at frequencies from 0% to 0.1% (Chessa et al., 2005) and the O allele has been found in Creole goats and in Pyrenean breed with a frequency of 0.2 and 0.12, respectively (Mahé & Grosclaude, 1993; Persuy et al., 1999). Recently, Marletta et al. (2004) and Sacchi et al. (2005) have reported only the alleles A and O in some Italian breeds; the latter with frequencies up to 0.092. However, according to the findings of Chessa et al. (2005), the allele C predominates in Italian goat populations with frequencies ranging from 0.700 to 0.975, whereas the frequencies of alleles A and C in Saanen breed are 0.317 and 0.507, respectively.

3.6. Whey proteins

The α-lactalbumin (α-La) mass detected by RP-HPLC/ESI-MS analyses was 14198 ± 1 which corresponds to theoretical mass of goat α-La (14192.2). The mass 18197 ± 1 was detected in the β-Lactoglobulin (β-Lg) peaks of the milk samples of the present study that corresponds to the theoretical mass of this goat protein 18191.3. In some samples a mass of 18523 ± 1 was detected in the front part of β-Lg peak which is consistent with a covalent linkage of lactosyl residue to the protein (Léonil et al., 1997; Trujillo et al., 2000). No protein variants were observed in accordance to previous reports. A silent allele has been reported at the α-La locus by Cosenza et al. (2003) and different β-Lg polymorphisms have been reported in the goats of different breeds that do not produce any amino acid substitutions in the protein and have not been correlated with the β-Lg quantity of milk (Ballester, Sánchez, & Folch, 2005; Graziano, D'Andrea, Angiolillo, Lagonigro, & Pilla, 2003).

4. Conclusions

According to the findings of the present study, there were marked differences between the two groups of individual goat milk samples with respect to the characteristics of their protein fraction. The κ-Cn D (former B) predominated in both groups of milks and the rare variant C or B (former D) was present only in the samples from the Greek breed. The two levels of αs2-Cn content (about 4 and 2.3 g/l) observed within both groups of milk samples (the Indigenous Greek and the international breeds) implied the existence of a null αs2-Cn allele. Variant A was the most abundant followed by variant C in both cases, whereas αs2-Cn F was only observed in the milks from the Indigenous Greek breed. The level of phosphorylation ranged from 6 to 11P, the most abundant being 11P.

The milks from the Indigenous Greek breed were characterized by the existence of the strong αs1-Cn variant, especially B3 and B4, followed by As or B1 and the absence of weak variants. On the contrary, in the milk samples from international breeds predominated the medium variant E followed by the defective F and the null. These findings explain the great difference between the mean αs1-Cn contents of the two groups of samples (6.90 ± 1.57 and 3.02 ± 2.35 g/l) that resulted in higher mean total protein content of the milks from the Greek breed. The great standard deviation of this mean in the milk samples from international breeds was also a result of this situation, due to the combination of more types of alleles (medium, weak, null, high). In conclusion, it was clear that the higher αs1-Cn content was the reason for the higher mean total protein content of the milk samples from the Greek breed that was 38.8 g/l compared to 31.9 g/l of those from international breeds. There were no pronounced differences with respect to β-Cn content. Variants A and C were present in both groups of samples, C being predominant in milks from international breeds. However, it has to be further studied the potential effect of the forms with 3P

and 4P observed in the Greek breed on the properties of milk (e.g. technological behavior).

An individual variability in the quantities of major whey proteins α-La and β-Lg was observed within both groups of milk samples but no protein variants were detected.

The information obtained from the present study, pointed out that the milk from the Indigenous Greek breed has the main advantage of the abundance of the strong αs1-casein genotypes. Taking into consideration that in Greece most of this kind of milk is transformed to cheese, the quality of its protein fraction has apart from scientific interest also an economic importance. Finally, the strategy followed in the present paper for the interpretation of RP-HPLC profiles using the retention times of standard genotypes and the quantity of the individual caseins, could be a way for a rapid profiling and screening of the protein fraction of unknown goat milk samples with reasonable certainty.

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