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Short communication

Quantitative determination of casein genetic variants in goat milk: Application in Girgentana dairy goat breed

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ABSTRACT

The study was conducted to develop a high-performance liquid chromatographic (HPLC) method to quantify casein genetic variants (αs_2-, β-, and κ-casein) in milk of homozygous individuals of Girgentana goat breed. For calibration experiments, pure genetic variants were extracted from individual milk samples of animals with known genotypes. The described HPLC approach was precise, accurate and highly suitable for quantification of goat casein genetic variants of homozygous individuals. The amount of each casein per allele was: αs_2-casein A = 2.9 ± 0.8 g/L and F = 1.8 ± 0.4 g/L; β-casein C = 3.0 ± 0.8 g/L and C1 = 2.0 ± 0.7 g/L and κ-casein A = 1.6 ± 0.3 g/L and B = 1.1 ± 0.2 g/L. A good correlation was found between the quantities of αs_2-casein genetic variants A and F, and β-casein C and C1 with other previously described method. The main important result was obtained for κ-casein because, till now, no data were available on quantification of single genetic variants for this protein.

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1. Introduction

The production and consumption of goat milk and its dairy products are increasing worldwide. Goat milk is a valuable source of protein in many countries, including a large number of African, Asian and European countries such as Norway, France, and Italy. The most abundant proteins in goat milk, as in other ruminant milks, are caseins. Goat caseins show high quantitative variability, caused by difference in protein expression, qualitative variability due to structural polymorphism of casein genes, differential splicing patterns and post-translation modifications (Marletta, Criscione, Bordonaro, Guastella, & D'Urso, 2007).

Goat breeds have been widely investigated for polymorphisms of milk proteins which have been related to milk chemical composition, cheese-making properties (coagulation properties, micelle size and mineralization, cheese yield, and sensory attributes), structural, biological and nutritional characteristics (Ramunno et al., 2007). Another important aspect for goat milk was the study of its nutritional and metabolic properties especially for potential use in infants and patients with cow's milk protein intolerance (Lamblin, Bourier, Orlanodo, Sauvage, & Wallaert, 2001).

A lot of methods have been developed to analyze milk casein fractions such as Rocket Immunoelectrophoresis (Grosclaude, Mahé, Brignon, Di Stasio, & Jeunet, 1987), Hydrophobic Interaction Chromatography (HIC) (Bramanti, Sortino, Onor, Beni, & Raspi, 2003), and Capillary Electrophoresis (CE) (Gómez-Ruiz, Miralles, Agüera, & Amigo, 2004) in order to quantify and separate the different caseins. In literature, there are no data regarding the quantitative chemical analysis of single casein genetic variants in goat milk probably because commercial standards for goat casein were not available, therefore, quantification is difficult to achieve. Only recently, some data for quantification of allelic variants of αs_1-casein in Girgentana goat milk was proposed by Montalbano, Tortorici, Mastrangelo, Sardina, and Portolano (2014).

The αs_2-casein (*CSN1S2* gene) comprises strong alleles associated with a normal content (about 2.5 g/L per allele) of this protein in milk (Boulanger, Grosclaude, & Mahè, 1984; Lagonigro, Pietrola, D'Andrea, Veltri, & Pilla, 2001; Ramunno, Cosenza, et al., 2001), an intermediate allele (about 1.5 g/L per allele), and a null allele which causes no detectable amount (Ramunno, Cosenza, et al., 2001; Ramunno, Longobardi, et al., 2001). Moreover, Erhardt, Jager, Budelli, and Caroli (2002) reported another variant associated with a normal content of αs_2-casein, typed at protein level by isoelectric focusing (IEF), but not characterized at molecular level.

The β-casein (encoded by *CSN2* gene) presents alleles associated with a normal content (5.0 g/L/allele) and null alleles associated with the absence of this casein fraction in milk (Chessa, Rignanese, Küpper, et al., 2008).

Sixteen allelic variants have been identified and characterized at molecular level in goat κ-casein (*CNS3* gene) (Prinzenberg, Gutscher, Chessa, Caroli, & Erhardt, 2005) with two new genetic

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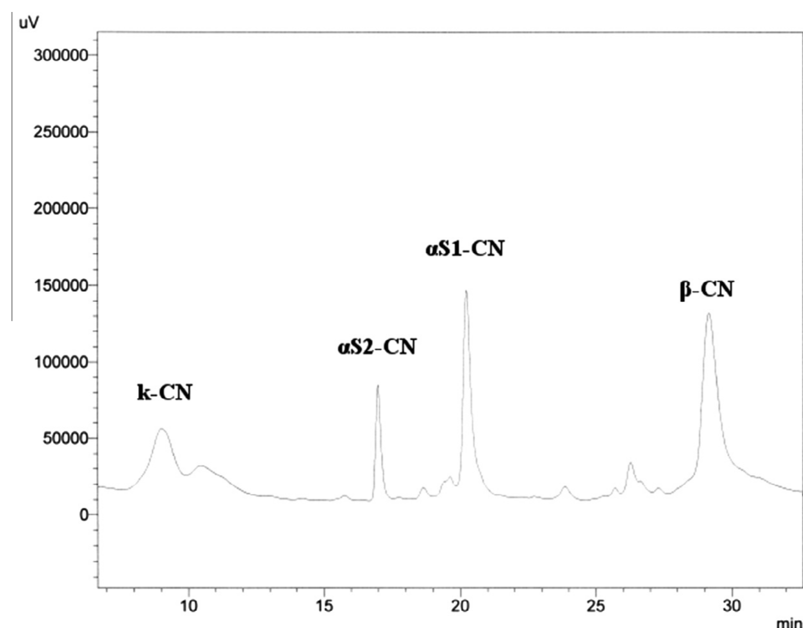

Fig. 1. Chromatograms relative to individual milk sample from a homozygous animal obtained using the optimized condition: Zorbax 300SB-C8 RP (Agilent Technologies), binary gradient, flow rate 0.5 ml min^{-1} at 45 °C, UV detection at 214 nm.

variants in Girgentana goat breed (Di Gerlando et al., 2015). These variants have been clustered into two groups by IEF of milk samples: A^{IEF} group (isoelectric point = 5.53) and B^{IEF} group (isoelectric point = 5.78).

The Girgentana goat is a Sicilian autochthonous breed reared for its good dairy production.

The endangered status of this breed is linked to the following peculiarities: in 1983 the population consisted of 30,000 goats, whereas 10 years later almost 98% of the pre-existing population disappeared. In 2001, only 252 mature goats participated in the national milk recording system and nowadays about 1500 heads are enrolled in the Herd Book (ASSONAPA, 2014). Therefore, this breed has become almost extinct, in part as a consequence of the marked decrease in fresh goat milk consumption (Mastrangelo, Sardina, Tolone, & Portolano, 2013).

The aims of this work was the quantification of the most common genetic variants of αs_2-, β-, and κ-casein in milk of homozygous individuals of Girgentana dairy goat breed, to evaluate the effect of each allele on casein content, using a high-performance liquid chromatographic (HPLC) method.

2. Experimental

2.1. Reagents, standards and samples

A total of 200 individual milk and blood samples of lactating goats of Girgentana breed were randomly collected in 15 different flocks located in different areas of Sicily.

Table 1
Casein (CN) content per allele (g/L).

Genetic variant	g/L for allele	Samples (n)[a]
αs_2-CN_A	2.9 ± 0.8	7
αs_2-CN_F	1.8 ± 0.4	6
β-CN_C	3.0 ± 0.8	9
β-CN_{C1}	2.0 ± 0.7	7
K-CN_A	1.6 ± 0.3	8
K-CN_B	1.1 ± 0.2	8

The blood samples were used to extract genomic DNA from buffy coats of nucleated cells using a salting out method (Miller, Dykes, & Polesky, 1988). At *CSN1S2* locus alleles B and C/E were detected by multiplex AS-PCR (Vacca et al., 2009). The allele E was identified by PCR-RFLP protocol of Lagonigro et al. (2001) using primers by Chessa, Rignanese, Chiatti, et al. (2008). The alleles D, 0, and F were genotyped by PCR-RFLP (Ramunno, Cosenza, et al., 2001). The allele A at this locus was assigned when all the other alleles were not present. The *CSN2* A/A1, C, C1, E, and 0' alleles were identified using PCR protocols of Chessa et al. (2005) and Chessa, Rignanese, Küpper, et al. (2008), amplifying part of exon 7-intron 8, and exon 8-intron 9, followed by sequencing of amplified fragments with ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems). Moreover, PCR-RFLP protocol proposed by Cosenza, Paciullo, Gallo, Di Berardinno, and Ramunno (2005) was used to discriminate allele A to A1. The several alleles at *CSN3* locus were identified by PCR protocol described by Prinzenberg et al. (2005) with primers by Di Gerlando et al. (2015), amplifying exon 4, followed by sequencing of amplified fragments with ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems). Purified αs-, β-, and κ-casein standards from bovine milk were purchased from Sigma–Aldrich (Milano, Italy) and all other chemicals were purchased as described Montalbano et al. (2014).

A subset of 45 samples was used for validation and quantification procedures and all samples, previously genotyped, were analyzed by RP-HPLC method. Goat casein genetic variants, used as standards for calibration, were obtained by extraction and lyophilization from individual milk samples with homozygous genotypes. In particular, seven samples with AA genotype and six samples with FF genotype at αs_2-casein; nine samples with CC genotype and seven samples with C1C1 genotype at β-casein, and eight samples with AA genotype and eight samples with BB genotype at κ-casein were used.

All goat milk samples were lyophilized and frozen at −20 °C until analysis. Before analysis, the lyophilized milk samples were solubilized by adding a corresponding volume of ultrapure water. Milk samples were prepared following the method proposed by Bobe, Beitz, Freeman, and Lindberg (1998). In particular, sample aliquots of 500 μl of whole milk were immediately frozen at

Table 2
Parameters of regression equations for calibration curves, response factors, and limit of detection (LOD) for single casein (CN) genetic variants.[a]

Allelic variant	Intercept ± SE[b]	Slope ± SE[b]	R^2	Response ± SD[c] (μg/area) 10^5	LOD (μg)[c]	Injected amount (μg)[d]	Theoretical plates ($N \cdot 10^3$)[e]
αs_2-CN_A	−12,825 ± 6750	19870 ± 251	0.9979	5.4 ± 0.4	1.3	3.3–52.0	36.14
αs_2-CN_F	−46,528 ± 9093	102,320 ± 801	0.9992	1.1 ± 0.1	0.3	1.4–22.0	34.26
β-CN_C	12,516 ± 11,977	36,365 ± 748	0.9949	2.7 ± 0.3	1.2	1.9–30.0	12.12
β-CN_{C1}	54,276 ± 10,352	17,642 ± 236	0.9979	4.8 ± 0.7	2.2	5.1–82.0	16.47
κ-CN_A	−433,648 ± 218,151	210,486 ± 6870	0.9874	0.54 ± 0.06	3.9	3.7–60.0	–
κ-CN_B	−162,912 ± 106,761	222,446 ± 2946	0.9977	0.47 ± 0.03	1.9	4.4–70.2	–

[a] Separated solutions of purified casein allelic variants injected at volume of 5, 10, 20, 40, and 80 μl in triplicate.
[b] Standard error.
[c] Computed as LOD = 10 × (3 × SD) where SD is the standard deviation of the background noise.
[d] Different injected amounts were used respect to the average proportions of proteins in milk.
[e] For computation of the number of theoretical plates, peak width at the baseline was obtained by tangential lines drawn at half-height.

Table 3
Relative standard deviation (RSD) of retention times and peak areas for milk protein fractions or genetic variants obtained in analysis of repeatability and reproducibility.

Allelic Variant	Repeatability[a]		Reproducibility[b]		Samples (n)
	Retention time RSD (%)	Area RSD (%)	Retention time RSD (%)	Area RSD (%)	
αs_2-CN_A	0.12	0.90	0.17	4.43	8
αs_2-CN_F	0.06	1.04	0.15	5.08	7
β-CN_C	0.21	0.52	0.22	4.11	10
β-CN_{C1}	0.05	0.41	0.24	4.12	9
κ-CN_A	0.23	0.43	0.40	1.71	8
κ-CN_B	0.20	0.40	0.43	2.96	9

[a] Ten aliquots of the same individual goat milk sample were injected consecutively.
[b] A sequence of 10 individual goat milk samples was injected over 4 days.

−20 °C, then a solution containing 0.1 M BisTris buffer (6.8 pH), 6 M GdnHCl, 5.37 mM sodium citrate, and 19.5 mM DTT (7 pH) was added directly to each aliquot in a 1:1 ratio (v:v) at room temperature. After thawing, each sample was shaken for 10 s, incubated for 1 h at room temperature, and centrifuged for 5 min at 16,000×*g*. Then, the fat layer was removed with a spatula and the remaining solubilized sample was diluted 1:3 (v:v) with a solution containing 4.5 M GdnHCl and solvent A, consisting of acetonitrile, water, and trifluoroacetic acid in a ratio 100:900:1 (v:v:v, 2 pH). The prepared samples were analyzed by direct chromatography.

2.2. HPLC equipment and chromatographic conditions

The chromatographic system (Shimadzu, Kyoto, Japan) used to perform the analyses consisted of LC-20AT liquid chromatographer, DGU-20A 5 degasser, CTO-20A column oven, SPD-20A UV/VIS detector, and FRC-10A fraction collector.

It was operated by means of the LC Solutions software which sets solvent gradient, data acquisition and data processing. Separations were performed on a reversed-phase analytical column C8 (Zorbax 300SB-C8 RP, Agilent Technologies) with a silica-based packing (3.5 μm, 300 Å, 150 × 4.6 I.D.). A security Guard Cartridge System (product No. 820999-901, Agilent Technologies) was used as pre-column (Zorbax 300SB-C8, Agilent Technologies).

The sample vial was injected via an auto-sampler (Shimadzu SIL-20A HT series). An injection loop of 100 μl was used. Analyses were carried out applying a binary gradient profile to the mobile phase composition using two solvents. In particular, solvent A consisted of 0.1% TFA in water and solvent B of 0.1% TFA in acetonitrile.

Separations were performed with the program proposed by Bonfatti, Grigoletto, Cecchinato, Gallo, and Carnier (2008) except for duration of the final re-equilibration condition under the starting conditions that was 13 min. Therefore, the total analysis time per sample was 50 min. This ensured the maintenance of chromatographic performance in sample run. The flow rate was 0.5 ml/min, the column temperature was kept at 45 °C and the detection was made at a wavelength of 214 nm. The injection volume consisted of 5 μl. Pure casein genetic variants were extracted for calibration experiments considering that goat commercial standards were not available. Each variant was purified by RP-HPLC, starting from individual milk samples of DNA-genotyped animals, and then lyophilized and weighted. The same elution conditions were used in semi-preparative experiments by collecting the correspondent peaks. A semi-preparative Zorbax 300SB-C8 (5 μm, 300 Å, 250 mm × 9.4 mm, Agilent Technologies) column and a fraction collector were used. The flow rate was 2 ml/min. After lyophilization, in order to obtain a standard solution, purified proteins were solubilized in a solution containing 4.5 M GndHCl and solvent A, and stored at −20 °C.

2.3. Validation

Individual data for each casein genetic variant was analyzed to test the linearity, repeatability, reproducibility, and accuracy of the method and for each of them calibration curves were computed. For this validation step, ten individual milk samples were used. Linearity was tested by running the same sample at increasing injecting volume 5–80 μl in triplicate. Repeatability was established by consecutive injections of samples while reproducibility by analyzing each sample on four different days. The accuracy was determined by quantifying each genetic variant in two samples and by repeating the quantification on different mixtures of them (at 75%, 50% and 25%). Each mixture was analyzed in duplicate. For each casein genetic variant, calibration curves were computed injecting increasing volume (5, 10, 20, 40, and 80 μl) of corresponding purified standard solution.

3. Results and discussion

3.1. Separation

The identification of caseins peaks in goat milk samples was confirmed by comparison with commercials standards that consisted of purified caseins from bovine milk. Therefore, it was ascertained that goat proteins eluted in the same order of bovine ones: κ-casein, αs_2-casein, αs_1-casein and β-casein (Fig. 1). In particular, Fig. 1 showed an example of chromatogram in which was possible to identify not only the major peaks relative to the four caseins (in homozygous condition) but also some minor peak near to the major one corresponding to glycosylated form (e.g. κ-casein). In particular, the boundaries of κ-casein peaks were from 8 to 12 min.

The validated RP-HPLC method, that allows the quantification of content per allele (g/L) for αs_2-, β- and κ-casein was carried out by analyzing only milk samples of homozygous animals. For each casein the different genetic variants co-eluted and this made impossible to quantify the alleles in heterozygous individuals. However, the casein genetic variants quantified in our study were the most frequent in Girgentana goat breed; in fact, at *CSN1S2*, the most frequent alleles were A and F (Palmeri, Mastrangelo, Sardina, & Portolano, 2014), at *CSN2* were C and C1 (Tortorici, Di Gerlando, Mastrangelo, Sardina, & Portolano, 2014), and at *CSN3* gene were A and B (Di Gerlando et al., 2015). Comparing our data with those reported by Bonanno et al. (2013), which used a different chromatographic method, it can be possible to state that our applied chromatographic conditions allowed in less time running, run was 5 min shorter, a greater separation and resolution of caseins as higher distance between caseins peaks.

3.2. Quantitative analysis

The quantification of casein genetic variants was determined by RP-HPLC for individual milk samples. The calibration of chromatographic system for quantifications was performed using an external standard method proposed by Bonfatti et al. (2008). All standards were considered 100% pure.

The comparison of our data (Table 1) with other studies on αs_2-casein (Boulanger et al., 1984; Lagonigro et al., 2001; Ramunno, Cosenza, et al., 2001) showed a similar protein content for A and F alleles, that were defined "strong" alleles associated with a normal content of this protein in milk. The data obtained for β-casein showed a lower content of this protein associated to C and C1 alleles (3.0 ± 0.8 and 2.0 ± 0.7 g/L, respectively) compared with those published by Chessa, Rignanese, Küpper, et al. (2008), that report the *CSN2* A, A1, B, C, C1, D, E alleles associated with higher content (5.0 g/L/allele). The application of our analytical method allowed the separation of A and B alleles at κ-casein (Table 1), with the first associated with higher protein content than the second one (1.6 ± 0.3 and 1.1 ± 0.2). There were no data reported the quantification of single allelic variants for κ-casein. The only data available reported that B^{IEF} group represents the more favorable variant group in terms of κ-casein content (+0.5 g/L) respect to A^{IEF} group (Chiatti et al., 2007).

3.3. Linearity, repeatability, reproducibility and recovery

The linearity was evaluated by the least square regression method using unweighted calibration data. The linear relation was estimated between peak area and injected amount of casein genetic variants ($R^2 > 0.987$). Parameters of calibration curves were reported in Table 2. The precision studies were composed of repeatability and reproducibility and in Table 3 were shown the values of relative standard deviation (RSD) for retention times and peaks areas. All RSD values were similar to those reported in literature for within- and among-days variation for genetic variants in bovine milk (Martin, Ollivier-Bousquet, & Grosclaude, 1999; Moatsou, Samolada, Panagiotou, & Anifantakis, 2004). Results indicate that the precision of the method was similar to previous study (Montalbano et al., 2014). The RSD values for retention times were below 0.23% within analytical day (repeatability) and below 0.43% among analytical days (reproducibility). Values of RSD for peak areas were below 1.04% within day and below 5.08% among days.

Recovery studies were carried out to determine the accuracy of the method and results of Student's *t*-test indicated that recovery rates were not significantly different from 100% at $P < 0.05$ (data not show).

4. Conclusions

The RP-HPLC method was successfully applied for quantitative determination of αs_2-casein A and F, β-casein C and C1, κ-casein A and B genetic variants in milk of homozygous individuals of Girgentana goat breed. These data were obtained by methods and techniques with high precision and accuracy. The studied analytical parameters for HPLC method (linearity, repeatability, reproducibility and recovery) are suitable for caseins quantification in milk. A good correlation was found between the quantities of αs_2-casein genetic variants A and F, and β-casein C and C1 with other previously described method. The main important obtained result was for κ-casein because, till now, no data were reported for quantification of single genetic variants of this protein. The proposed RP-HPLC method was validated in milk of Girgentana goat breed but could be applicable to milk of other goat breeds.

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