Food Chemistry 156 (2014) 165–169

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Development and validation of RP-HPLC method for the quantitative estimation of αs_1-genetic variants in goat milk

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ARTICLE INFO

Article history:
Received 9 January 2013
Received in revised form 6 June 2013
Accepted 26 January 2014
Available online 5 February 2014

Keywords:
Genetic variants
αs_1-Casein
HPLC
Goat milk

ABSTRACT

A high-performance liquid chromatographic (HPLC) method was developed and validated for separation and quantification of the most common genetic variants of αs_1-casein in goat's milk, to evaluate the effect of αs_1-casein polymorphisms on casein content.

Chromatography was carried out by binary gradient technique on a reversed-phase C8 Zorbax column and the detection was made at a wavelength of 214 nm. The procedure was developed using individual raw milk samples of Girgentana goats. For calibration experiments, pure genetic variants were extracted from individual milk samples of animals with known genotypes, considering that commercial standards for goat genetic variants were not available. The data obtained for Girgentana goat breed showed that A, B, F variants were alleles associated with a content of αs_1-casein in milk of 3.2 ± 0.4, 5.4 ± 0.5 and 0.7 ± 0.1 g/L, respectively, whereas N variant was a 'null' allele associated with the absence of αs_1-casein in milk.

1. Introduction

In the milk of ruminants, more than 95% of proteins are synthesized by six structural genes, four caseins (αs_1-, β-, αs_2- and κ-caseins) and two whey proteins (α-lactalbumin and β-lactoglobulin). Among Ca-sensitive caseins (αs_1, β, and αs_2), the αs_1-casein fraction is the most extensively investigated in goat species (Martin, Szymanowska, Zwierzchowski, & Leroux, 2002; Rijnkels, 2002). The extensive polymorphism at αs_1-casein locus has been shown to affect not only the quantity of casein in goat milk, but also the structural and nutritional characteristics and technological properties of milk. In fact, polymorphism associated with a quantitative variability in casein synthesis has a significant effect on coagulation properties, micelle size and mineralsation, cheese yield, and sensory attributes (Ramunno et al., 2007). So far, at least 17 codominant alleles have been identified at DNA level, which are associated with different expression levels of αs_1-casein in milk. A first group of alleles (A, B1, B2, B3, B4, C, H, L and M) are associated with a high content of αs_1-casein (about 3.5 g/L), alleles I and E are associated with an intermediate content (about 1.1 g/L), and alleles D, F, and G with a low level (about 0.45 g/L) of this protein in milk. Alleles αs_1-casein N, 01 and 02 are 'null' alleles and have been associated with the absence of αs_1-casein in milk (Bevilacqua et al., 2002; Chianese, Ferranti, Garro, Mauriello & Addeo, 1997; Grosclaude, Mahé, Brignon, Di Stasio, & Jeunet, 1987; Martin, Ollivier-Bousquet, & Grosclaude, 1999; Ramunno et al., 2005). The presence of alleles associated with "low" and "null" content of αs_1-casein in goat milk, may be interesting considering that very low levels of αs_1-casein were found to be less allergenic than milk characterised by high level of αs_1-casein (Haenlein, 2004). Hence, the quantification of different genetic variants at αs_1-casein locus became very important for the quality of milk and also for the possible valorization of the products that are linked to a specific breed (i.e. mono-breed labeled cheeses). Nowadays, a great variety of methods have been developed to analyse milk protein fractions: alkaline urea polyacrylamide gel electrophoresis (urea-PAGE) and RP-HPLC (Reversed Phase-High Performance Liquid Chromatography) for whole caseins analysis and Cation-Exchange Chromatography (CEC) of whole casein for the fractionation of the lyophilized casein (Moatsou, Samolada, Panagiotou, & Anifantakis, 2004); Capillary Zone Electrophoresis (CZE) (Brambilla, Feligini, & Enne, 2003; Valenti, Pagano, & Avondo, 2012), RP-HPLC (Bonfatti, Grigoletto, Cecchinato, Gallo, & Carnier, 2008; Clark & Sherbon, 2000), SDS–polyacrylamide gel electrophoresis (SDS–PAGE) to identify allelic polymorphisms and Rocket Immunoelectrophoresis to estimate the contents of individual caseins (Grosclaude et al., 1987); Isoelectric focusing (IEF) and RP-HPLC/Electrospray Ionisation Mass Spectrometry (ESI-MS) to analyse the protein fractions and polymorphism of caseins of goat milk (Moatsou, Moschopoulou, Mollé, Kandarakis, & Léonil, 2008;

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http://dx.doi.org/10.1016/j.foodchem.2014.01.091
0308-8146/

Moatsou, Vamvakaki, Mollé, Anifantakis, & Léonil, 2006) RP-HPLC/ESI-MS and Matrix-Assisted Laser Desorption/ionisation Mass Spectrometry (MALDI-MS) to identify and characterise caseins (Cunsulo et al., 2005; Cunsulo, Muccilli, Saletti, Marletta, & Foti, 2006); Hydrophobic Interaction Chromatography (HIC) to separate and determine caseins (Bramanti, Sortino, Onor, Beni, & Raspi, 2003); Capillary Electrophoresis (CE) for quantitative determination of caseins (Gómez-Ruiz, Miralles, Agüera, & Amigo, 2004).

Separation and quantification of the different αs_1- genetic variants were difficult to achieve. In literature, the main cited values on the different levels of allelic casein content were obtained by Rocket Immunoelectrophoresis (Grosclaude et al., 1987).

Capillary Electrophoresis (CE) was used for αs_1- quantitative determination confirming the results of Grosclaude et al. (1987) for the analysed genotypes. However, till now, there were not data in literature regarding the quantitative chemical analysis of individual genetic variants of αs_1-casein in goat milk. The Girgentana goat is a Sicilian autochthonous breed reared for its good dairy production. Due to sanitary policies the size of the Girgentana goat breed decreased of almost 90% in 20 yrs. In 1983, the population consisted of 30,000 individuals but, nowadays, only 522 lactating goats in 25 farms are present in Sicily (AIA, 2012). The aims of this work were to separate and quantify the most common genetic variants of αs_1-casein in milk of Girgentana goat breeds, to compare our results with the quantitative data proposed by Grosclaude et al. (1987) and to evaluate the effect of each allele on αs_1-casein content. Moreover, it could be interesting to evaluate the possibility of revitalizing interest in the milk produced by Girgenatana goat breed in order to regain an important economic role in the production of "drinking-milk" requested for particular food products, such as milk for infants, using weak and null genotypes, and in the production of niche products, using strong genotypes.

2. Experimental

2.1. Reagents, standards and samples

Acetonitrile and Water ultra Plus (Carlo Erba Reagents, Italy) were of HPLC grade, Trifluoroacetic acid (TFA) was from Romil Pure Chemistry (Cambridge, United Kingdom). All other chemicals were of analytical grade. BisTris buffer, Dithiothreiol (DDT), Guanidine hydrochloride (GdnHCl), Sodium citrate were from Sigma–Aldrich (Milano, Italy). Purified αs-casein standard from bovine milk was purchased from Sigma–Aldrich (Milano, Italy). 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. Samples were collected from 10–15 unrelated individuals per herd. A subset of 40 samples were used for validation and quantification procedure and a total of 100 individual goat milk samples, previously genotyped, was analysed by RP-HPLC method. Goat αs_1-casein genetic variants, used as standards for calibration, were obtained by extraction and lyophilization from individual milk samples with homozygous genotypes. The samples belonged to different αs_1-casein genotypes: four samples corresponding to genotype AA, four samples to genotype BB, five samples to genotype AB, five samples to genotype FF, six samples to genotype AF, five samples to genotype BF, one sample to genotype NN, five samples to genotype AN, two samples to genotype BN, and finally three samples to genotype FN.

All goat milk collected samples were lyophilized and frozen at −20 °C until analysis. Before analysis, the lyophilized milk sample was solubilised by adding a corresponding volume of ultrapure water. Milk samples were prepared following the method proposed by Bobe, Beitz, Freeman, and Lindberg (1998). The diluted samples were analysed by direct chromatographic.

2.2. HPLC equipment

The chromatographic system (Shimadzu, Kyoto, Japan) used to perform the analyses consisted of a model LC-20AT liquid chromatographer, a model DGU-20A 5 degasser, a model CTO-20A column oven, a model SPD-20A UV/VIS detector and a model 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 a 100 μl was used.

2.3. Chromatographic conditions

The analyses were carried out applying a binary gradient profile to the mobile phase composition using two solvents. 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 et al. (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.

2.4. Purified proteins

Pure αs_1-casein genetic variants were extracted for calibration experiments considering that commercial standards for goat were not available. Each variant was purified by RP-HPLC, starting from individual milk samples of DNA-genotyped animals, and then lyophilized and weighted.

For this purpose, 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 × 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 solubilised in a solution containing 4.5 M GndHCl and solvent A, and stored at −20 °C.

2.5. DNA genotyping

For our study, 200 blood samples of Girgentana goat breed were randomly collected and genomic DNA was extracted from buffy coats of nucleated cells using a salting out method (Miller, Dykes, & Polesky, 1988). The αs_1-caseinA*/01, B*/E, F and N alleles were simultaneously investigated by PCR-RFLP using *Xmn*I restriction enzyme (Ramunno et al., 2000). This protocol allowed the identification of F and N alleles, but not distinguish allele A* from 01, and allele B* from E. Allele Specific-PCR was used for the detection of the αs_1-casein E (Dettori et al., 2009) and αs_1-casein 01 alleles (Cosenza et al., 2001; Cosenza et al., 2003). The A* indicated A, G, I, and H alleles while B* indicated B1, B2, B3, B4, and C alleles.

2.6. Validation

In validation tests, ten individual milk samples from Girgentana goats were used. Linearity was tested by running the same sample

at increasing injecting volume 5–80 μl in triplicate. To estimate the precision of method, the repeatability and the reproducibility were evaluated. Repeatability was established by consecutive injections of samples while reproducibility by analysing 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 analysed in duplicate.

For each genetic variant of αs_1-casein, 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 αs_1-casein peak in milk samples was confirmed by comparison with commercial standard that consisted of purified genetic variants from bovine milk. Since in commercial standards αs_1 and αs_2 are not available as single proteins, assignment was made on the basis of the 4:1 proportion known for cow milk (Alais, 1984). The identification of αs_1-casein genetic variants of Girgentana goat breed was confirmed by comparison with chromatograms of individual milk samples of animals with homozygous genotypes. For homozygous animals, genetic variants gave rise to a single peak. The assignment of peaks of αs_1-casein genetic variants was made by comparing the chromatograms of individual milk samples of homozygous animals with those of heterozygous animals (Figs. 1 and 2,).

In this study, A and B genetic variants of αs_1-casein were perfectly resolved with the current method; in fact, the resolution between these two peaks exceeded 1.05 which is considered satisfactory value in chromatographic separation. Despite the gradient optimisation, B and F genetic variants co-eluted and this made impossible to quantify the genetic variants in the heterozygous condition (BF), whereas, A and F variants were perfectly resolved. Nevertheless, the validity of this method was confirmed by the fact that the most frequent genotype at this locus in Girgentana breed was AF (0.365) followed by AA (0.340) and FF (0.090), whereas the frequencies for BF genotype was very low (0.015) (Mastrangelo, Sardina, Tolone, & Portolano, 2013). Chromatographic analysis confirmed that N was a 'null' allele associate with the absence of αs_1-casein in milk (Ramunno et al., 2005).

In the group of sampled animals, no individuals carrying E and 0' were found therefore, assessment for these two variants was not feasible.

To analyse genetic polymorphism of caseins several electrophoretic techniques have been used but none of them appears to be fully satisfactory for resolution of αs_1- and αs_2 caseins and the identification of the relevant variants (Boulanger, Grosclaude, & Mahè, 1984; Grosclaude et al., 1987; Russo, D'Avoli, Dall'Olio & Tedeschi, 1986). As alternative, chromatographic techniques such as RP-HPLC, have been shown to achieve genetic variants of casein fraction of bovine milk (Bonfatti et al., 2008). Consequently, the comparison of our results with other reported in literature was impossible. However, it was possible to compare our results with those reported by Bonfatti et al. (2008) on bovine milk proteins. In fact, while their data for αs_1-casein genetic variants showed a co-eluting of the two found variants B and C, our data on a wider number of genotypes showed a separation of αs_1-casein genetic variants with very high resolution.

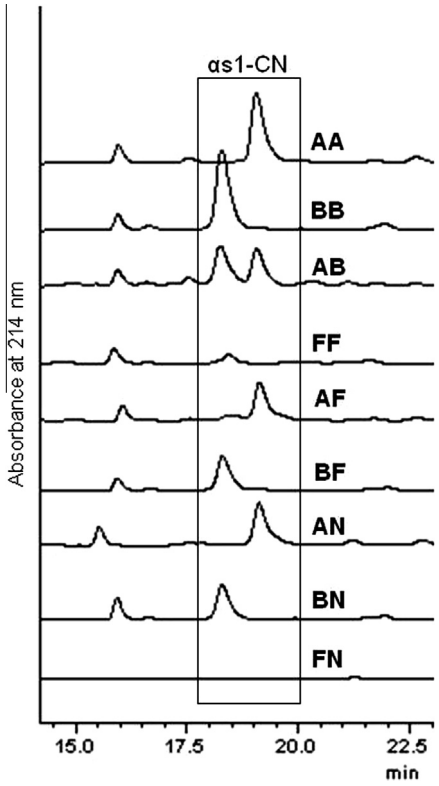

Fig. 1. Chromatograms relative to individual milk samples with different αs_1-casein (αs_1-CN) genotypes obtained using the optimised condition: Zorbax 300SB-C8 RP (Agilent Technologies), binary gradient, flow rate 0.5 ml/min at 45 °C, UV detection at 214 nm.

3.2. Quantitative analysis

Quantification by RP-HPLC was performed for individual milk samples. The external standard method was used to calibrate the chromatographic system for αs_1-casein genetic variants quantifications. Five points calibration curves were generated for each genetic variant by estimating parameters of the linear regression of the peak area on the amount injected, with increasing injection volume of each standard solution (5, 10, 20, 40 and 80 μl). Each solution was analysed in triplicate.

The data obtained for Girgentana goat breed showed that A and B variants were strong alleles associated with a high content of αs_1-casein with some quantitative differences respect to Grosclaude et al. (1987), and that F variant was a weak allele associated with a low level of αs_1-casein in milk (Table 1). In our study, quantification data of B genetic variant compared to A showed that the expression of this allele determines a higher content of αs_1-casein in milk.

3.3. Linearity, repeatability, reproducibility and recovery

The linearity of method was evaluated by the least square regression method using unweighted calibration data. The linear relation was estimated between peak area and injected amount of genetic variants of αs_1-casein ($R^2 > 0.999$; data not shown). Parameters of calibration curves are 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 between-days variation for genetic variants in bovine milk (Martin et al., 1999; Moatsou et al., 2004). Results indicate that the precision of the method was acceptable. The RSD values for retention times were below 0.22% within analytical day (repeatability) and below 0.60% across analytical days (reproducibility). Values of RSD for

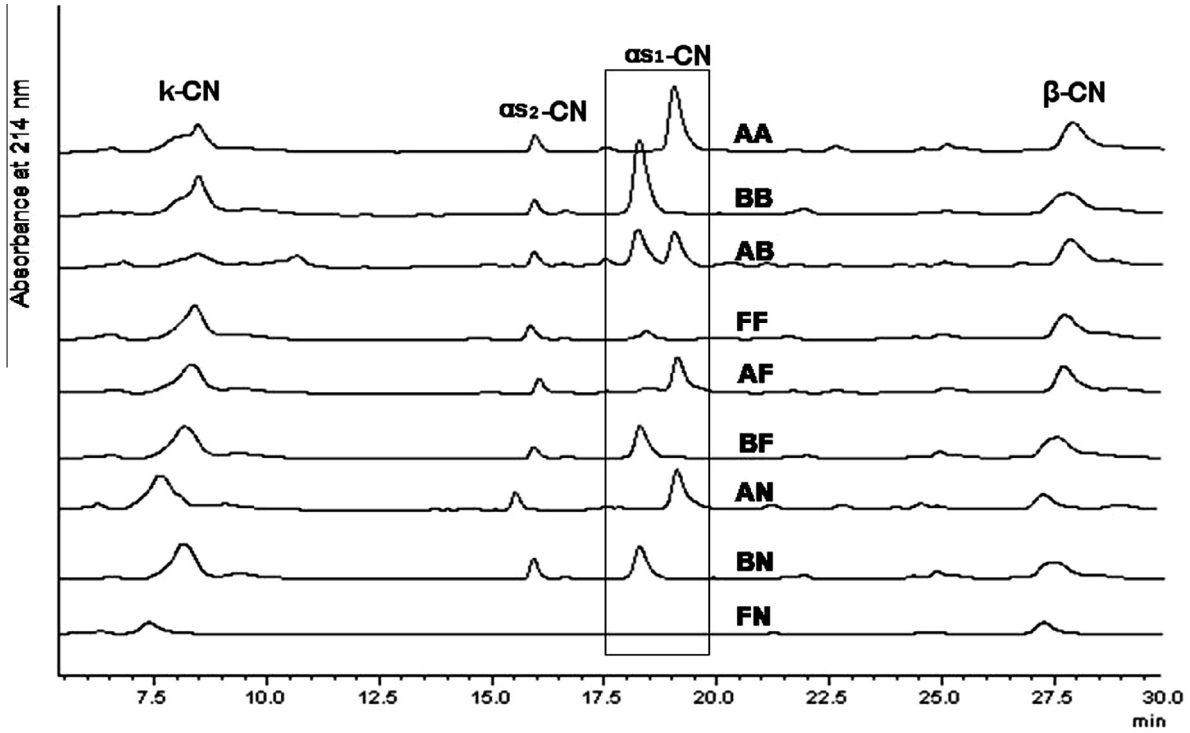

Fig. 2. Chromatograms relative to individual milk samples with different casein (CN) genotypes obtained using the optimised condition: Zorbax 300SB-C8 RP (Agilent Technologies), binary gradient, flow rate 0.5 ml/min at 45 °C, UV detection at 214 nm.

Table 1
Content (g/L) in αs_1-casein (αs_1-CN) for allele.

Genetic variant	g/L for allele	Samples (n)[a]
αs_1-CN_A	3.2 ± 0.4	8
αs_1-CN_B	5.4 ± 0.5	7
αs_1-CN_F	0.7 ± 0.1	9

[a] Homozygous and heterozygous analysed samples.

peak areas were below 0.77% within day and below 5.00% among days.

Pre-column conditions might have also affected the reproducibility of quantification of whey proteins. Thus, a frequent guard-cartridge turnover was advisable. In addition, a blank injection might be used after each sample run.

Recovery studies were carried out to determine the accuracy of the method (Table 4). Recoveries ranged from 99.33% to 103.13% and results of Student's t-test indicated that recovery rates were not significantly different from 100% at $P < 0.05$.

Table 2
Parameters of regression equations for calibration curves, response factors, and limit of detection (LOD) for single αs_1-casein (αs_1-CN) genetic variants.[a]

Allelic variant	Intercept ± SE[b]	Slope ± SE[b]	R^2	Response ± SD (μg/area) 10^5	LOD (μg)[c]	Injected amount (μg)[d]	Theorical plates (N 10^3)[e]
αs_1-CN_A	187031 ± 26856	187536 ± 964	0.9997	0.49 ± 0.03	0.5	3.4–54.0	20.00
αs_1-CN_B	66967 ± 20990	120428 ± 756	0.9995	0.80 ± 0.02	0.6	3.2–52.0	11.57
αs_1-CN_F	−44579 ± 4370	165551 ± 705	0.9998	0.66 ± 0.04	0.1	0.75–12.00	20.00

[a] Separated solutions of purified αs_1-CN allelic variants injected at volume of 5, 10, 20, 40 and 80 μl in triplicate.
[b] Standard error.
[c] Calculated on the basis of calibration curve slope.
[d] Different injected amounts were used in respect to the average proportions of the 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 of retention times and peak areas for milk proteins fractions or genetic variants obtained in the analysis of repeatability and reproducibility.

Allelic variant	Repeatability[a]		Reproducibility[b]		Samples (n)
	Retention time RSD (%)	Area RSD (%)	Retention time RSD (%)	Area RSD (%)	
αs_1-CN_A	0.13	0.47	0.57	5.00	10
αs_1-CN_B	0.22	0.77	0.57	4.72	10
αs_1-CN_F	0.07	0.67	0.60	4.50	7

[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.

Table 4
Results of the analysis of accuracy.[a]

Allelic variant	Recovery rate (%)	RSD (%)
s_1-CN_A	99.33	4.13
s_1-CN_B	103.13	4.35
s_1-CN_F	101.97	3.54

[a] Mixtures of two raw milk samples were obtained following relative proportions of 75%, 50% and 25%. Mixtures and whole samples were analysed in duplicate and recovery rates were calculated using expected areas provided by calibration curves and observed areas.

4. Conclusion

In this study, RP-HPLC method for separation and quantification of αs_1-casein genetic variants in goat milk was developed and validated. The proposed method was simple and selectively providing satisfactory accuracy with low limits of detection. It ensures a precise quantification of the αs_1-casein variants and could be a useful tool for studies on composition of goat milk proteins. The data obtained for genetic variants were in agreement with the only available data published by Grosclaude et al. (1987) but, in addition showed significant differences in the protein contents per allele. Finally, this chromatographic method appears to be particularly interesting, because it provides fractionation and resolution of several genetic variants of αs_1-casein goat milk.

Acknowledgement

This work was supported by PSR Sicilia 2007–2013 - Misura 1.2.4, CUPG66D11000039999.

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