Identification and Quantification of #, #, #, and #-Caseins in Water Buffalo Milk by Reverse Phase-High Performance Liquid Chromatography and Mass Spectrometry

Maria Feligini, Ivan Bonizzi, Joanna Natalia Buffoni, Gianfranco Cosenza, and Luigi Ramunno

J. Agric. Food Chem., **2009**, 57 (7), 2988-2992• DOI: 10.1021/jf803653v • Publication Date (Web): 03 March 2009

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Identification and Quantification of α_{S1}, α_{S2}, β, and κ-Caseins in Water Buffalo Milk by Reverse Phase-High Performance Liquid Chromatography and Mass Spectrometry

MARIA FELIGINI,*,† IVAN BONIZZI,† JOANNA NATALIA BUFFONI,† GIANFRANCO COSENZA,‡ AND LUIGI RAMUNNO‡

Istituto Sperimentale Italiano Lazzaro Spallanzani, Via Einstein, Località Cascina Codazza, 26900 Lodi, Italy, and Department of Soil, Plant, Environment and Animal Production Sciences, Agriculture Faculty, University of Naples "Federico II", Italy

A method for the simultaneous quantitation of α_{S1}, α_{S2}, β, and κ-caseins in water buffalo (*Bubalus bubalis*) milk using reverse phase high-performance liquid chromatography was developed. The molecular masses of the peaks separated by the described chromatographic protocol were determined by ESI-MS. α_{S1}- and κ-caseins were found to be heteromorphic in several individual milk samples. In particular, α_{S1}-casein showed two peaks with a molecular mass of 23,490 Da and 23,516 Da, and κ-casein showed three peaks with molecular masses of 19,165 Da, 19,177 Da, and 19,247 Da. Only one form for β-casein (24,033 Da) and α_{S2}-casein (22,741 Da) were detected. The mean values of casein fraction concentration observed throughout the individual samples were 8.89 gL^{-1} with a relative standard deviation (RSD) of 20% for α_{S1}-casein, 5.08 gL^{-1} with a RSD of 25% for α_{S2}-casein, 20.91 gL^{-1} with a RSD of 16% for β-casein, and 4.13 gL^{-1} with a RSD of 24% for κ-casein. Linear and second-order polynomial correlations with total nitrogen were calculated for all casein fractions.

KEYWORDS: Buffalo milk; casein; high-performance liquid chromatography; mass spectrometry

INTRODUCTION

Caseins (α_{S1}, α_{S2}, β, and κ) are the predominant phosphoproteins in the milk of ruminants, accounting for about 80% of total protein. In the bovine species, α_{S1}-casein constitutes up to 40% of the casein fraction. Its primary structure has been reported by Grosclaude et al. (*1*); it consists of 199 amino acids with a calculated molecular weight of 23,615 Da (*2*). α_{S2}-Casein accounts for up to 10% of the casein fraction and consists of 207 amino acids (*3*−*6*) with a calculated molecular weight of 25,266 Da. β-Casein represents about 45% of the casein; its reference sequence consists of 209 amino acids with a molecular weight of 23,983 Da (*7*). Finally, the fraction κ-casein represents about 5% of the total casein; its primary structure consists of 169 amino acids with a calculated molecular weight of 19,037 Da (*8*). The heterogeneity of casein pointed out by different purification procedures is further complicated by genetic variations as each of the fractions exists in genetically variable forms (*9*). Casein has been extensively studied in order to investigate the aspects affecting its qualitative and quantitative characteristics and to achieve an improvement in the yield of the cheese productions. It has been demonstrated that genetic, environmental, and management-related factors represent the most prominent sources of variability in milk protein content (*10*−*13*). Studies have also been carried out on buffalo milk proteins, although not as extensive as those concerning cattle. The heterogeneity of buffalo milk proteins has been shown, and it has been reported since 1963 that the relative proportions of α_S, β, and κ-caseins are different from those of bovine milk (*14*). The primary structures of Italian water buffalo α_{S1}- and β-caseins have been reported by Ferranti et al. (*15*); as in bovine, α_{S1}-casein is made up of 199 amino acids with a theoretical molecular weight of 22,798 Da (dephosphorylated form), and β-casein consists of 209 amino acids with a theoretical molecular weight of 24,047 Da and 23,981 Da (variants A and B, respectively). Recently, the coding region sequences (including the signal peptide) of κ- and α_{S2}-casein genes, corresponding to 190 amino acids (GenBank accession no.AM900443 (*16*)) and 222 amino acids (GenBank accession no. DQ133467), respectively, have been elucidated. During the last few years, worldwide buffalo population has doubled, possibly because this species shows some potential at meeting the growing commercial demands of animal protein at a global level. During the last fifty years, the Italian population of Mediterranean water buffalo has grown more than 10-fold; most of the produced milk is devoted to the manufacturing of Mozzarella cheese, a protected denomination of origin (PDO) pasta filata cheese (*17*). It is essential to monitor the milk's casein composition as it

* Corresponding author. Phone: 0039(0)37135118. Fax: 0039(0) 371437070. E-mail: maria.feligini@isils.it.
† Istituto Sperimentale Italiano Lazzaro Spallanzani.
‡ University of Naples "Federico II".

10.1021/jf803653v CCC: $40.75
Published on Web 03/03/2009

Table 1. Dilution Scheme and Final Concentration of the Casein Mixed Standards

	dilution scheme		fraction concentration (gL^{-1})				
standard level	mixed mother sol. (mL)	urea sol.[a] (mL)	κ-CN (purity 80%)	β-CN (purity 90%)	α_s-CN (purity 70%)	α_{s1}-CN[b]	α_{s2}-CN[b]
1	0.2	0.8	0.37	1.20	1.68	1.34	0.34
2	0.4	0.6	0.75	2.40	3.36	2.69	0.67
3	0.6	0.4	1.12	3.60	5.04	4.03	1.01
4	0.8	0.2	1.49	4.80	6.72	5.38	1.34
5	1.0	No	1.87	6.00	8.4	6.72	1.68

[a] Refer to milk sample preparation for detailed composition. [b] The values in this column are calculated from α_s-CN by applying the 4:1 proportion between the α_{s1} and α_{s2}-fractions reported in the literature (*30*).

affects the micelle size and structure, hence the industrial milk processing at dairies and, ultimately, the cheesemaking yield. The most reliable method for milk protein analysis is by far high performance liquid chromatography (HPLC) (*18*−*21*). In particular, reverse phase-HPLC has been widely used to achieve information on protein qualitative and quantitative heterogeneity because of its inherent analytical performance and its capability to be directly coupled to mass spectrometric analysis; a widespread instrumental setup of this sort relies on the electrospray ionization interface (HPLC-ESI-MS). In the present article, we describe a RP-HPLC method capable of separating and quantifying α_{S1}, α_{S2}, β, and κ-caseins for use in routine quality control laboratories. In addition, we investigate the correlation among total protein, total casein, and the milk content of each casein fraction.

MATERIALS AND METHODS

Reagents. Trifluoroacetic acid (TFA), R Chromasolv acetonitrile, sodium citrate (purity of 99.9%), TRIS (99.8%), and 2-mercaptoethanol (98%) were purchased from Sigma-Aldrich, (St. Louis, MO, USA). Urea was purchased from VWR International (West Chester, PA, USA). All standard solutions and aqueous solvents were prepared with Chromanorm water for HPLC (VWR International).

Milk Sample Preparation. Ninety individual raw milk samples from Mediterranean water buffalo (*Bubalus bubalis*) were collected at 3 farms located in the Campania region (Southern Italy); all animals were sampled at 120 days after birth. After collection, samples were immediately frozen and kept at −20 °C until analysis. Prior to HPLC determinations, milk was thawed overnight at 4 °C and defatted by centrifugation at 1000*g* for 10 min. Samples were prepared for analysis by diluting 400 μL of skimmed milk with 1.6 mL of urea solution (8 M urea, 165 mM Tris, 44 mM sodium citrate, and 0.3% v/v β-mercaptoethanol) (*22*). The diluted samples were filtered through a 0.45 μm-pore cellulose membrane (Phenomenex, Torrance, CA, USA) and analyzed by RP-HPLC in two repeats per sample.

Standard Solutions of Bovine Caseins. Purified α-, β-, and κ-casein fractions from cows (*Bos taurus*) were purchased from Sigma-Aldrich. Purities of these commercial caseins were 70%, 90%, and 80%, respectively. Each purified fraction was weighed (72 mg α, 40 mg β, 14 mg κ) and dissolved in 2 mL of the same urea solution used for sample preparation. These mother solutions were mixed together in equal proportions (1 mL each), then diluted with urea solution according to the scheme reported in **Table 1** to prepare a set of five mixed concentration standards. The standard solutions were analyzed in 10 repeats each to construct calibration curves for all of the casein fractions by linear regression.

RP-HPLC-UV Analysis. To separate and quantify the casein fractions, a HPLC-UV system (Waters, Milford, MA, USA) was used. This consisted of two pumps (model 515, Waters), a manual injector (Rheodyne, Cotati, CA, USA) equipped with a 20-μL loop, and a UV detector (model 2487, Waters); it was operated by means of the Empower 2 software (Waters). The chromatographic separation was performed in reversed-phase mode using a Jupiter C4 column (250 × 4.6 mm, 300 Å-sized pores, 5 μm-sized particles; Phenomenex); elution was performed at room temperature with a linear gradient of eluent B (CH_3CN + 0.1% TFA) in A (H_2O + 0.1% TFA) from 35 to 55% in 40 min (*22*) at a flow rate of 0.8 mL/min. Peaks were detected by their UV absorption at 220 nm.

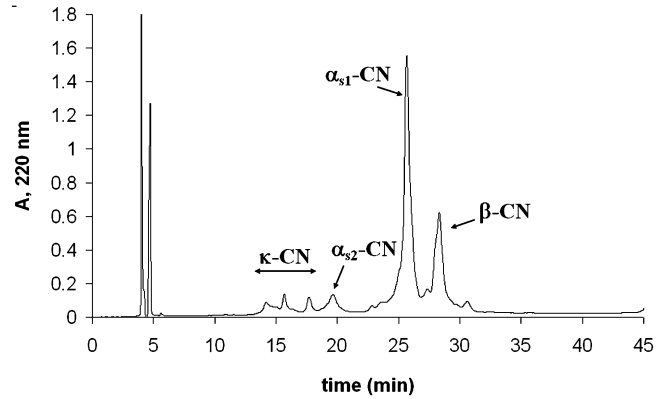

Figure 1. RP-HPLC chromatogram of commercial bovine caseins. α_s-Casein (3.36 gL^{-1}) with a retention time of 25.68 min (α_{S1}) and 19.65 (α_{S2}); β-casein (2.40 gL^{-1}) with a retention time of 28.32 min; κ-casein peak system (0.75 gL^{-1}) with retention times of 14.30, 15.73, and 17.82 min.

RP-HPLC/ESI-MS Analysis of Casein Fractions. RP-HPLC was carried out on an Agilent 1100 series HPLC (Agilent, Santa Clara, CA, USA) as described above. The column was directly interfaced to a Micromass Q-TOF mass spectrometer (Waters) equipped with a nanospray source. Experiments were run in positive ion mode applying a capillary voltage of 2.5 kV−3 kV and a cone voltage of 30−35 V. The casein M_r (mass) determinations were obtained using the Masslinks version 4.1 (Waters) software. Determinations were carried out on two individual samples and were repeated 2 times each.

Determination of Milk Nitrogen Content. Milk total nitrogen (TN), noncasein nitrogen (NCN), and nonprotein nitrogen (NPN) contents were determined by the Kjeldhal method (*23*). Nitrogen values were converted to equivalent protein using the coefficients 6.38, 6.25, and 3.60, respectively; casein nitrogen (CN) was calculated as [CN] = [TN] − [NCN] (*24*).

Storage Stability of Caseins. To check the stability of the samples in the chosen preservation conditions, the duplicate HPLC analysis of 8 milk samples with different α_{S1}, α_{S2}, β, κ-casein contents was repeated after 3 months by RP-HPLC according to the same methods. The differences between the two sets of calibrated concentration values were tested for statistical significance by a *t*-test.

RESULTS AND DISCUSSION

Chromatographic Separation. The described protocol allowed an effective and repeatable separation of all of the bovine casein fractions in about 30 min, providing good accordance with other recent protocols for the chromatographic analysis of bovine caseins (*18*) (**Figure 1**). Similar analytical performance in terms of peak resolution and separation speed was achieved when analyzing buffalo milk samples as well. The peak structure observed in the buffalo chromatograms was qualitatively different from the bovine casein fractions as two peaks seemingly belonging to κ-casein eluted after the α_{s2} fraction (**Figure 2**). Furthermore, a number of distinct peak patterns was observed consistently over all samples (**Figures 2A**−**D**). In particular, only one of these patterns (**Figure 2A**) featured all of the observed peaks simultaneously. The standard deviation of retention times was about 1% or less for all peaks (**Table 2**), thus ensuring a reliable and straightforward recognition of all peaks and corresponding chromatographic patterns. The high quality of separation allowed us to simultaneously detect all casein fractions and, for the first time, variants for both α_{S1} and

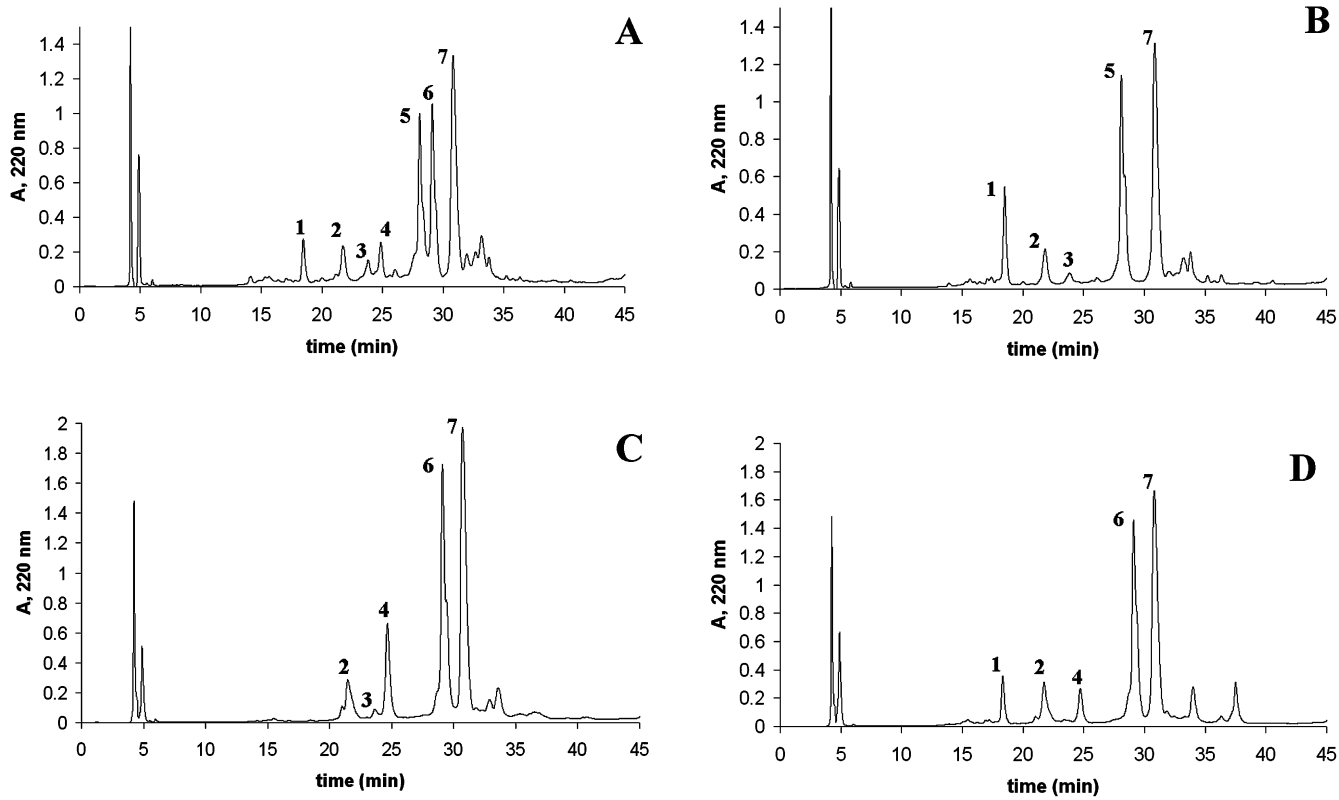

Figure 2. RP-HPLC chromatograms (**A**, **B**, **C**, and **D**) of different buffalo milk samples. Peaks 1 to 7: casein fractions are identified in **Table 2**.

Table 2. Retention Time and Molecular Mass of Buffalo Casein Fractions Eluted Using RP-HPLC

		retention time (min)			
casein fraction	peak	mean	SD	*n*	molecular mass (Da)
κ-	1	18.22	0.144	59	19,165
α_{S2}-	2	21.46	0.234	65	22,741
κ-	3	23.38	0.235	38	19,247
κ-	4	24.56	0.184	43	19,177
α_{S1}-	5	27.93	0.133	57	23,490
α_{S1}-	6	29.03	0.117	41	23,516
β-	7	30.65	0.151	65	24,033

Table 3. Calibration Curve Equations and Statistical Parameters

				standard errors	
casein	equation[a]	r^2	RSD %[b]	slope	intercept
κ-	$y = 1.71 \times 10^7 x - 1.51 \times 10^5$	0.999	1.53	2.46×10^5	3.04×10^5
α_{S2}-	$y = 1.25 \times 10^7 x + 2.77 \times 10^5$	0.999	1.56	1.89×10^5	2.11×10^5
α_{S1}-	$y = 2.30 \times 10^7 x + 4.52 \times 10^6$	0.998	2.15	4.91×10^5	2.19×10^6
β-	$y = 1.26 \times 10^7 x + 3.66 \times 10^4$	0.999	0.75	8.94×10^4	3.56×10^5

[a] *y* is the peak area, and *x* is the concentration (gL^{-1}). [b] Relative standard deviation of *y* ($n = 10$ for each concentration level).

κ-casein, which were characterized at the genetic level (data not shown); the presence of two α_{S1} variants was recently established by Chianese et al. (*25*).

Determination of the Molecular Masses of Casein Peaks. The molecular masses of the peaks separated by the described chromatographic protocol were determined by ESI-MS. The comparison between the measured molecular masses and the values obtained from the amino acid sequences of the buffalo proteins ((*15*) GenBank accession DQ133467 (*16*)) allowed us to clearly identify the peaks corresponding to each casein fraction once the potential posttranslational modifications, in particular phosphorylations, were taken into account. It was possible to identify the peaks shown in **Figure 2** as α_{S1} (peaks 5 and 6), α_{S2} (peak 2), β (peak 7), and κ-casein (peaks 1, 3, and 4), respectively, based on the results reported in **Table 2**. The mass values observed for α_{S1} and β- caseins are in agreement with those reported by Ferranti et al. (*15*) but two polymorphic forms (A and B) of α_{S1}-casein were found, featuring a molecular mass of 23,490 Da (peak 5) and 23,516 Da (peak 6), respectively, whereas only one form for β-casein with a molecular mass of 24,033 Da (peak 7) was detected. Recently, Chianese et al. (*25*) found two polymorphic variants of α_{S1}-casein with molecular masses ranging from 23,279 and 23,439 Da at various degrees of phosphorylation. Similarly, we found α_{S1}- and κ-caseins heteromorphic in 50 and 51 out of 90 individual milk samples, respectively. Molecular mass analysis confirmed that peaks 1, 3, and 4 correspond to κ-casein, although the elution order of the peaks belonging to κ and α_{s2} fractions differs from the one obtained by Bordin et al. (*18*) for cow's milk. The molecular mass measured for α_{S2}-casein (22,741 Da) was lower than expected in comparison with that of the bovine sample (25,226 Da) as reported by Farrell et al. (*9*). Thus, in order to further confirm the identity of the α_{S2}-casein fraction, the first 10 positions of the N-terminal amino acid sequence of peak 2 were determined (data not shown). The results support the identification of peak 2 as α_{S2}-casein.

Quantification of Casein Fractions. Calibration curves were constructed according to the external standard method, using bovine casein fractions in mixed standard solutions at five concentration levels. The mean peak area corresponding to each fraction (the sum of peak areas in the case of κ-casein) was linearly regressed over fraction concentration, yielding the equations and the associated statistical parameters presented in **Table 3**. The instrumental response was linear throughout the chosen protein concentration ranges with r^2 coefficients scoring at least 0.998 for all of the fractions, allowing reliable quantification of samples. The mean values of the casein fraction concentration observed throughout the 90 individual samples

Table 4. Determination of Nitrogen Content by the Kjeldhal Method in Eight Individual Buffalo Milk Samples, Expressed as Equivalent Protein (mean ± SD) and Literature Data on Buffalo and Cow Milk Samples

		literature data		
	buffalo milk ($n = 8$)	buffalo milk[a] ($n = 3$)	buffalo milk[b] ($n = 3$)	cow milk[b] ($n = 3$)
TN (g/kg)	47.2 ± 4.3	43.4 ± 12.5	43.5 ± 3.4	33.5 ± 0.3
NCN (g/kg)	10.1 ± 1.6		8.9 ± 1.6	7.4 ± 0.5
NPN (g/kg)	1.6 ± 0.4		1.0 ± 0.4	0.9 ± 0.02
CN (g/kg)	37.0 ± 5.6	32.6 ± 14.4	34.6 ± 1.1	26.1 ± 0.8
sum of HPLC casein fractions (g/kg)	37.85 ± 5.18			

[a] Ref *28*. [b] Ref *24*.

Table 5. Correlation between Casein Fraction Concentration and Kjeldhal Total Nitrogen under Linear and Second-Order Polynomial Regression

	linear model		2nd order model	
casein	r^2	*p*-value	r^2	*p*-value
κ-	0.57	0.0297	0.58	0.1184
α_{S2}-	0.08	0.4886	0.27	0.4516
α_{S1}-	0.64	0.0169	0.78	0.0230
β-	0.65	0.0150	0.66	0.0686

were 8.84 gL^{-1} with relative standard deviation (RSD) of 21% for α_{S1}-casein, 5.12 gL^{-1} with RSD of 26% for α_{S2}-casein, 20.86 gL^{-1} with RSD of 17% for β-casein, and 4.10 gL^{-1} with RSD of 25% for κ-casein. These results are in accordance with the ones reported in recent literature (*26*); it has to be noted, however, that the observed mean concentration of β-casein roughly corresponds to the sum of β-casein and γ-caseins reported in the work by Bramanti et al. (*26*). This is consistent with the known role of β-casein as the main source of γ-caseins in cow and buffalo milk, due to plasmin activity (*27*).

Determination of Nitrogen Content. The Kjeldhal determination of nitrogen content was performed on 8 samples, leading to the mean results reported in **Table 4**. The value of casein nitrogen, expressed as equivalent protein, and the sum of the casein fraction concentrations determined by HPLC were checked for significance of difference between means by paired *t*-test, after correcting for the buffalo milk relative density value (1.07) as reported by Imran et al. (*28*). The result did not support the presence of a significant difference (*p*-value of 0.4009). The observed values show good consistency with other research (**Table 4**) and confirmed a substantial difference from cow's milk (*29, 24, 28*). The strength of the relationship between the casein fraction concentrations obtained by HPLC analysis for the same samples, the sum of which is also presented in **Table 4**, and the total nitrogen content of milk was evaluated by calculating the coefficient of determination r^2 under linear regression models. The resulting values are shown in **Table 5**. The linear regressions showed statistically significant (i.e., p-values <0.05) correlation values around 0.6 for α_{s1}-, β-, and κ-caseins and, by contrast, a very low correlation between the α_{s2} fraction and total nitrogen. A substantial improvement of the r^2 value, however, could be observed for the α_{s1} fraction by second-order polynomial regression (also shown in **Table 5**), suggesting that a nonlinear (i.e., quadratic) model is more suitable to describe the relationship between this fraction and total nitrogen in samples. However, no significant correlation with total nitrogen could be detected for α_{s2}-casein.

Storage Stability of Caseins. To assess whether storage at −20 °C allows long-term stable preservation of buffalo milk for analysis purposes, a subset of 8 samples was analyzed again by the described HPLC protocol after 3 months in duplicate, checking the significance of the differences between the fraction concentrations measured before and after storage by paired *t*-test. Out of 8, only one sample showed statistically significant differences in α_{S1}-, α_{S2}-, and β-caseins (*p*-values of 0.0060, 0.0238, and 0.0099, respectively), whereas κ-casein was not significantly different. The average values of the significant differences, however, were as small as 1.2 gL^{-1} for α_{S2}-, 1.6 gL^{-1} for α_{S1}-, and 2.9 gL^{-1} for β-casein. These results suggest that preservation can negatively affect the analytical determinations after three months of preservation.

As different peak patterns emerged from the analyses, the quantitation results were checked for the statistical significance of the between-pattern differences in the fractions' mean concentration by *t*-test. No significant differences were observed.

In this study, a high resolution RP-HPLC method for simultaneous separation and quantification of casein fractions from buffalo milk was developed. This approach permits one to quickly separate all fractions and to detect casein variants at the same time. The obtained results provide new information about milk protein from the Mediterranean water buffalo population that may be useful for selection purposes.

LITERATURE CITED

(1) Grosclaude, F.; Mahé, M. F.; Ribadeau-Dumas, B. Structure primaire de la caseine αs1-et de la caseine β-bovine. *Eur. J. Biochem.* **1973**, *40*, 323–324.

(2) Mercier, J.-C.; Grosclaude, F.; Ribadeau-Dumas, B. Structure primaire de la caseine αs1 bovine. Sequence complete. *Eur. J. Biochem.* **1971**, *23*, 41–51.

(3) Brignon, G.; Ribadeau Dumas, B.; Mercier, J.-C.; Pelissier, J.-P.; Das, B. C. The complete amino acid sequence of bovine αS2 -casein. *FEBS Lett.* **1977**, *76*, 274–279.

(4) Mahé, M. F.; Grosclaude, F. Polymorphisme de la caseine αS2 des bovines: Characterization du variant C du yak (*Bos grunniens*). *Ann. Genet. Sel. Anim.* **1982**, *14*, 401–416.

(5) Stewart, A. F.; Bonsing, J.; Beattie, C. W.; Shah, F.; Willis, I. M.; Mackinlay, A. G. Complete nucleotide sequences of bovine αs2- and β-casein cDNAs: Comparisons with related sequences in other species. *Mol. Biol. Evol.* **1987**, *4*, 231–241.

(6) Groenen, M. A. M.; Dijkhof, R. E. M.; Verstege, A. J. M.; van der Poel, J. J. The complete sequence of the gene encoding bovine αS2-casein. *Gene* **1993**, *123*, 187–193.

(7) Ribadeau-Dumas, B.; Brignon, G.; Grosclaude, F.; Mercier, J.-C. Structure primaire de la casein β bovine. *Eur. J. Biochem.* **1972**, *25*, 505–514.

(8) Mercier, J.-C.; Brignon, G.; Ribadeau-Dumas, B. Structure primarie de la caseine κ-bovine B. Sequence complete. *Eur. J. Biochem.* **1973**, *35*, 222–235.

(9) Farrell, H. M.; Jimenez-Flores, R., Jr.; Bleck, G. T.; Brown, E. M.; Butler, J. E.; Creamer, L. K.; Hicks, C. L.; Hollar, C. M.; Ng-Kwai-Hang, K. F.; Swaisgood, H. E. Nomenclature of the proteins of cows' milk: Sixth revision. *J. Dairy Sci.* **2004**, *87*, 1641–1674.

(10) Jakob, E.; Puhan, Z. Technological properties of milk as influenced by genetic polymorphism of milk proteins: A review. *Int. Dairy J.* **1992**, *2*, 157–178.

(11) Martin, P.; Szymanowska, M.; Zwierchowski, L.; Leroux, C. The impact of genetic polymorphisms on the protein composition of ruminant milks. *Reprod. Nutr. Dev.* **2002**, *42*, 433–459.

(12) Summer, A.; Franceschi, P.; Bollini, A.; Formaggioni, P.; Tosi, F.; Mariani, P. Seasonal variations of milk characteristics and cheesemaking losses in the manufacture of Parmigiano-Reggiano cheese. *Vet. Res. Commun.* **2003**, *27*, 663–666.

(13) Van Knegsel, A. T. M.; Van den Brand, H.; Dijkstra, J.; Tamminga, S.; Kemp, B. Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle. *Reprod. Nutr. Dev.* **2005**, *45*, 665–668.

(14) Aschaffenburg, R.; Sen, A. Comparison of the caseins of buffalo's and cow's milk. *Nature* **1963**, *197*, 797–799.

(15) Ferranti, P.; Scaloni, A.; Caira, S.; Chianese, L.; Malorni, A.; Addeo, F. The primary structure of water buffalo alpha(s1)- and beta-casein identification of phosphorylation sites and characterization of a novel beta-casein variant. *J. Protein Chem.* **1998**, *8*, 835–844.

(16) Mukesh, M.; Mishra, B. P.; Kataria, R. S.; Sobti, R. C.; Ahlawat, S. P. Sequence analysis of UTR and coding region of kappa-casein gene of Indian riverine buffalo (*Bubalus bubalis*). *DNA Seq.* **2006**, *2*, 94–98.

(17) Commission Regulation (EC) No 213/01 of 9 January 2001 lying down detailed rules for the application of Council Regulation (EC) No 1255/1999 as regards methods for the analysis and quality evaluation of milk and milk products and amending Regulations (EC) No 2771/1999 and (EC) No 2799/1999. Official J. Eur. Comm. 2001, *L037*, 1−99.

(18) Bordin, G.; Cordeiro Raposo, F.; de la Calle, B.; Rodriguez, A. R. Identification and quantification of major bovine milk proteins by liquid chromatography. *J. Chromatogr., A* **2001**, *928*, 63–76.

(19) Veloso, A. C. A.; Teixeira, N.; Ferreira, I. M. P. L. V. O. Separation and quantification of the major casein fractions by reverse-phase high-performance liquid chromatography and urea-polyacrylamide gel electrophoresis. Detection of milk adulterations. *J. Chromatogr., A* **2002**, *967*, 209–218.

(20) Careri, M.; Mangia, A. Analysis of food proteins and peptides by chromatography and mass spectrometry. *J Chromatogr., A* **2003**, *1000*, 609–635.

(21) Bonfatti, V.; Grigoletto, L.; Cecchinato, A.; Gallo, L.; Carnier, P. Validation of a new reversed-phase high-performance liquid chromatography method for separation and quantification of bovine milk protein genetic variants. *J. Chromatogr., A* **2008**, *1195*, 101–106.

(22) Bonizzi, I.; Buffoni, J. N.; Feligini, M. Quantification of bovine casein fractions by direct chromatographic analysis of milk. Approaching the application to a real production context. *J. Chromatogr., A* **2009**, *1216*, 165−168.

(23) International Dairy Federation. Lait. Détermination de la teneur en azote. 4. Détermination de la teneur en azote non protéique. International Standard FIL-IDF 20B, 1993.

(24) Ahmad, S.; Gaucher, I.; Rousseau, F.; Beaucher, E.; Piot, M.; Grongnet, J. F.; Gaucheron, F. Effects of acidification on physico-chemical characteristics of buffalo milk: A comparison with cow's milk. *Food Chem.* **2008**, *106*, 11–17.

(25) Chianese, L.; Quarto, M.; Pizzolongo, F.; Calabrese, M. G.; Caira, S.; Mauriello, R.; De Pascale, S.; Addeo, F. Occurence of genetic polymorphism at the αs1-casein locus in Mediterranean water buffalo milk. *Int. Dairy J.*, **2009**, *19*, 181−189.

(26) Bramanti, E.; Sortino, C.; Onor, M.; Beni, F.; Raspi, G. Separation and determination of denatured alpha (s1)-, alpha(s2)-, beta- and kappa-caseins by hydrophobic interaction chromatography in cows', ewes' and goats' milk, milk mixtures and cheeses. *J. Chromatogr., A* **2003**, *994*, 59–74.

(27) Somma, A.; Ferranti, P.; Addeo, F.; Mauriello, R.; Chianese, L. Peptidomic approach based on combined capillary isoelectric focusing and mass spectrometry for the characterization of the plasmin primary products from bovine and water buffalo beta-casein. *J. Chromatogr., A* **2008**, *1192*, 294–300.

(28) Imran, M.; Khan, H.; Hassan, S. S.; Khan, R. Physicochemical characteristics of various milk samples available in Pakistan. *J. Zhejiang Univ. Sci. B* **2008**, *9*, 546–551.

(29) Kanwal, R.; Ahmed, T.; Mirza, B. Comparative analysis of milk collected from buffalo, cow, goat and sheep of Rawalpindi/Islamabad region in Pakistan. *Asian J. Plant Sci.* **2004**, *3*, 300–305.

(30) Alais, C. Physics and Physical Chemistry of Milk. Effects of Processing. In *Dairy Science [Scienza del latte]*, 3rd ed.; De Noni, I., Ed.; Tecniche Nuove, Milan, Italy, 1984; p 224.

Received for review November 27, 2008. Revised manuscript received February 2, 2009. Accepted February 11, 2009. This research was financially supported by the Italian Ministry for Agriculture and Forestry Policy, MiPAF (SELMOL project).

JF803653V