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Separation and determination of denatured α_{s1}-, α_{s2}-, β- and κ-caseins by hydrophobic interaction chromatography in cows', ewes' and goats' milk, milk mixtures and cheeses

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Abstract

Caseins α_{s1}-, α_{s2}-, β- and κ- from raw cows', ewes' and goats' milk were separated and determined by hydrophobic interaction chromatography (HIC) by using a Propyl column (Eichrom) in the presence of 8.0 *M* urea in the mobile phase. The method is based on fast and easy solubilization of real raw samples by 4.0 *M* guanidine thiocyanate followed by the HIC analysis, without any preliminary precipitation or separation of the casein fraction. Elution conditions have been optimized by analyzing commercial single bovine standard caseins and their mixture. In the optimized chromatographic conditions the four casein fractions were separated in less than 45 min. A linear relationship between the concentration of casein and peak area (UV absorbance detector at 280 nm) has been obtained over the concentration range of 0.5 to 40 μ*M*. The detection limit for α-, β- and κ-caseins ranged between 0.35 and 0.70 μ*M*. The precision of the method was evaluated, the coefficient of variation for α-, β- and κ-casein determination ranging between 3.0 and 6.0%. The method has been validated by the analysis of reference skim milk powder (BCR-063R) certificated for total nitrogen content. The method was applied to commercial casein mixture and to the qualitative and quantitative analysis of casein fractions in unprocessed, raw cows', goats' and ewes' milk (10 samples analyzed for each species), in one sample of unprocessed buffalos' milk and in commercial cheeses (mozzarella, robiola, ricotta and stracchino). Binary mixtures of milk (cow/goat and cow/ewe) were also analyzed and the ratio between casein peak areas (α_{s1}/κ, α_{s2}/β, β/κ and α_{s2}/α_{s1}) of the HIC chromatograms was proposed and discussed in order to evaluate a possible application of this method to detect milk adulteration.

Keywords: Milk; Cheese; Food analysis; Hydrophobic interaction chromatography; Caseins; Proteins; Whey proteins; Urea; Guanidine thiocyanate

1. Introduction

The caseins are the predominant milk proteins of almost all mammalian species [1]. They constitute a heterogeneous group of phosphoproteins present as stable calcium phosphate protein complexes termed micelles.

The knowledge of the casein composition in the various species is quite important for two major reasons. First, casein composition affects micelle size

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and structure, aggregation process [2], the action of proteolytic enzymes [3] and, thus, the milk processing in dairy industry. Second, a reliable identification and quantification of the major milk proteins offers the possibility of setting-up methods for the assessment of milk adulterations [4]. Casein, in particular, are less affected by heat treatment with respect to whey proteins [5].

Although the production of milk from cow prevails in the world, the production of milk from other species plays an important economic role in several countries [6–8].

The substitution of cows' milk for ewes' and goats' milk is a fraudulent practice in the dairy industry because of the low price of cows' milk and the much lower milk yield of ewes and goats.

In recent years, several analytical techniques for detecting mixtures of milk from different species have been developed [9]. Most of the analytical techniques described are concerned with the qualitative detection of low quantities of cows' milk in ewes' milk cheeses with 'appellation d'origine' such as Manchego, Roquefort or Pecorino [10]. A European Union (EU) reference method has been developed for detecting bovine casein in cheeses made from ovine and caprine milk by isoelectric focusing of γ-caseins [11]. Other approaches, based on immunological, Western blotting methods [12,13], enzyme-linked immunosorbent assay (ELISA) methods [14–16], or DNA techniques [17], have been recently published. In several papers the products of proteolytic activities of enzymes on casein fraction from different species have been also studied [18,19].

Despite the large number of techniques developed for detecting low quantities of cows' milk, only a few studies are concerned with detection of high percentages of adulterating milk [5,10,20].

In the last decade, capillary electrophoresis (CE) has been used both for evidencing the polymorphism of bovine, ovine and caprine milk proteins [21,22], and for the separation, analysis and determination of the percentages of milk from different species [23,24]. On the basis of the differences between the CE patterns of the casein fraction from the whole milk of each species several authors performed the identification and quantitative determination of milk in binary and ternary mixtures by principal components regression and partial least-squares regression [25].

Separation of casein from bovine, ovine and caprine milk using various high-performance liquid chromatography (HPLC) procedures has also been reported [5,26–31].

In previous recent papers we proposed and validated a chromatographic method for the separation and the quantitative determination of α-, β- and κ-casein by hydrophobic interaction chromatography (HIC) in the presence of 8.0 *M* urea in the mobile phase [32–34]. The method is based on fast and easy solubilization of commercial and real samples by 4.0 *M* guanidine thiocyanate (GdmSCN) without any other pre-treatment or preliminary separation. This method was accurate and reproducible and was successfully applied to the analysis of various real, raw samples.

In this paper we propose first the optimized chromatographic conditions for the separation and quantitative determination of denatured casein fractions by an Eichrom Propyl HIC column. The method has been optimized by analyzing commercial α-, β- and κ-casein samples and their commercial mixture. The Propyl column is characterized by a relative high hydrophobicity, comparable with a Phenyl TSK-gel column. For this reason it is suitable for the analysis of real samples of unknown composition, in which overlapping of peaks due to components with similar hydrophobicity could occur. The method has been validated by the analysis of reference skim milk powder (BCR-063R) certificated for total nitrogen content. At present no certificated material for α-, β- and κ-casein content is available. The content of α-, β- and κ-casein found in BCR-063R was compared with that found by analyzing the same material with the same method by a different HIC column (TSK Gel Phenyl) [33] and the data obtained by other authors with reversed phase (RP)–HPLC [4,30]. Accuracy was also verified by determining α-, β- and κ-casein content in a commercial casein mixture (Fluka) previously analyzed [34].

Second, the optimized method is applied to the qualitative and quantitative analysis of casein fractions in unprocessed, raw cows', goats' and ewes' milk (10 samples examined for each species) and in one sample of unprocessed buffalos' milk. Reproducible, different chromatographic elution pat-

terns were found which suggested the extension of the application of this method in food quality control for the detection of fraudulent manipulations of milk. Thus, the casein fraction in the HIC chromatograms of binary milk mixtures (cow/ewe and cow/goat) was analyzed, as a preliminary study, in order to evaluate the possible application of the proposed method to the quantitative determination of cows' milk percentage of such mixtures. Selected ratios between casein peak areas (α_{s1}/κ, α_{s2}/β, β/κ and α_{s2}/α_{s1}) of the HIC chromatograms have been proposed and discussed in order to evaluate a possible application of this method to detect milk adulteration.

Finally, the qualitative and quantitative results of the chromatographic analysis of five cheeses derived from cows', ewes' and buffalos' milk (robiola, stracchino and mozzarella cheese from cows' milk, mozzarella from buffalos' milk, ricotta from ewes' milk) have been reported and discussed.

The fast sample preparation procedure, the employment of common, low-cost instrumentation (HPLC) and the simple data processing based on peak area ratios make the proposed method novel and of easy application in quality control laboratories.

2. Experimental

2.1. Chemicals

Caseins α-, β- and κ- and a casein mixture (product no. 22078) were purchased from Fluka Chemie (Buchs, Switzerland Biochemika) (22084 α-caseins ≥90%, 22086 β-caseins ≥80%, 22087 κ-caseins ≥70%). The buffer solutions were prepared from NaH_2PO_4 and Na_2HPO_4, (BDH, Poole, UK), ammonium sulfate (Bio-Rad Labs., Hercules, CA, USA), urea (SigmaUltra) and guanidine thiocyanate, abbreviated as GdmSCN (Sigma, St Louis, MO, USA). The phosphate-buffered saline (PBS) buffer solutions contained 0.1 *M* phosphate at a pH of 7.2. Water deionized with a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout. A Propyl HIC column (Eichrom Europe, Paris, France) with dimensions of 10 cm×4.6 mm I.D. (particle size 6.5 μm, porosity 300 Å) was used for all the experiments.

2.2. Instrumentation

A narrow-bore HPLC gradient pump (P2000, ThermoQuest) equipped with a mechanical degassing system (SC1000, ThermoQuest) was connected to a diode array detector (UV6000, ThermoQuest). The UV detector was operated at 280 nm. Samples were introduced via a 10-port injection valve (Rheodyne PR700-102-1 Cotati, CA, USA) with a poly ether ether ketone (PEEK, Upchurch, Oak Harbor, WA, USA) injection loop of 100 μl was used for all experiments. Absorbance measurements were performed using a Varian DMS 300 spectrophotometer.

Chromatograms were processed by ChromQuest 3.0 (ThermoQuest). Where not well resolved, chromatographic peaks were fitted by gaussian profiles.

2.3. Chromatographic conditions

In all the experiments 8.0 *M* urea was kept constant in the mobile phase in order to prevent casein aggregation. The elution conditions were the following: 32 min linear salt gradient from 100% high salt buffer (PBS, 1.8 *M* ammonium sulfate, 8.0 *M* urea) to 100% lower salt buffer (PBS, 8.0 *M* urea) at 20±1 °C. A flow-rate of 0.5 ml/min was used during the chromatographic run. A higher flow-rate (until 1.5 ml/min) was used for equilibrating the column in a shorter time. In fact, differently from HIC TSK gel columns, the silica packing of the Propyl column is compatible with the high back pressure given by the employment of 8 *M* urea in the eluent phase. The mobile phase was filtered and sonicated (10 min) before using. All the solutions were filtered by a 0.45 μm cellulose acetate filter (Millipore). Lipid content in milk samples and centrifuged cheese samples does not affect column performance and retention time reproducibility probably because in the presence of 8.0 *M* urea in the eluent buffers lipids elute in the void volume of the column, keeping the column clean.

2.4. Standard solutions

All stock solutions of caseins from Fluka were

prepared by dissolving lyophilized powder (a suitable amount in order to obtain an approximate protein concentration of 7 mg/ml) in PBS and 4.0 *M* GdmSCN. Unprocessed milk samples (10 cows' milk, 10 goats' milk and 10 ewes' milk) were obtained from a breeder in Tuscany. Only one buffalos' milk sample was possible to obtain from a breeder in Capua, near Naples. Pooled samples were prepared for each species by mixing 1 ml of each of the 10 samples. A volume of 100 μl of each milk sample and of the pooled ones were diluted in 1 ml of PBS, 4.0 *M* GdmSCN within 24 h of milking. Total nitrogen content in cows', goats' and ewes' pooled milk samples and in the buffalos' milk sample was determined by the Kjeldhal method (three replicates).

Cheese samples were commercial products purchased in a local supermarket. Suitable amounts of cheeses were weighed, in order to obtain an approximate protein concentration of 7 mg/ml, dissolved in PBS and 4.0 *M* GdmSCN and centrifuged at 4500 *g* for 10 min prior to HIC analysis.

Stock solutions of binary mixtures of milk (cow/ewe and cow/goat) were prepared from sample no. 3, arbitrarily chosen, of cows', goats' and ewes' milk, containing 10, 25, 50, 75, 90% of bovine milk (10 ml total volume) and diluting 100 μl of each milk mixture in 1 ml of PBS, 4.0 *M* GdmSCN.

Milk samples for injection (injection volume 100 μl) were prepared by diluting 100 μl of the stock solutions in 900 μl of the mobile phase (PBS, 1.8 *M* ammonium sulfate, 8.0 *M* urea). For the other samples a suitable volume of the stock solutions was diluted in the same mobile phase. Concentration of stock solutions of caseins from Fluka was determined by spectrophotometry [35,36].

2.4.1. Stability

All stock solutions of commercial caseins and real samples dissolved in GdmSCN were stable in a refrigerator (2–8 °C). Injection of sample solutions diluted from fresh stock solutions gave results not significantly different from those obtained from stock solutions 5 months aged or less.

2.5. Purified proteins

As the other caseins and whey proteins are present as impurities in the commercial samples of α- and β-caseins used here as standards, pure α- and β-caseins were prepared for calibration experiments as previously reported [33,34]. For this purpose, the same column (Eichrom Propyl), the same elution conditions and a 500 μl injection loop was used in semi-preparative experiments by collecting the major peaks (α_{s1}- and β-casein peaks). For quantitation of α_{s2}- and γ-casein (see below) the same slope of α- and β-casein, respectively, was employed. Concentration of α- and β-caseins in the collected fractions was determined spectrophotometrically, as previously reported [32]. The κ-casein sample was used without further purification.

2.6. Purification of whey proteins from processed cows' milk

Whey proteins were obtained from 10 ml of unprocessed cows' milk by precipitation at pH 4.6 with 1 *M* HCl, followed by centrifugation at 4500 *g* for 20 min. The soluble fraction was diluted 1:10 in 1.8 *M* ammonium sulfate, 0.1 *M* PBS and injected.

2.7. Certificate reference material BCR-063R

BCR-063R was kindly supplied by Dr J. Pauwels of the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium), and was prepared from skimmed raw milk, as previously reported [33,37]. The certified value for *N* total is 62.3±0.8 mg/g. Five amounts of BCR-063R (sample identification no. 0446) were weighed and dissolved in PBS and 4.0 *M* GdmSCN. Three independent replicate determinations on each different weighed amount of BCR-063R were performed.

3. Results

3.1. HIC analysis of commercial caseins and of certificate reference material (BCR-063R) method validation

Caseins α-, β- and κ- solubilized in 4.0 *M* GdmSCN and diluted by the gradient starting buffer were injected into a Propyl HIC column and eluted keeping a 8.0 *M* concentration of urea constant in the

mobile phase. Fig. 1 shows HIC chromatograms of commercial α-, β- and κ-casein and Fluka casein mixture in parts A, B, C and D, respectively. As in the TSK-Gel® Ether-5PW HIC column [34], the Propyl column allows the separation of commercial α-casein in two peaks attributed to α_{s1}- (40.9 min) and α_{s2}-casein (35.9 min) fraction. Two peaks have been obtained also by injecting the α-casein fraction collected after separation in the TSK-Gel Phenyl-5PW HIC column (peak at 45.3 min) [33]. Assignment has been made on the basis of α_{s1}-/α_{s2}-casein ratio, α_{s2}-casein representing about 10–12% of total and on the basis of α_{s1}- and α_{s2} hydrophobicity [38]. The minor peak at 45.8 min present in the α-casein chromatogram is at present unassigned. The β-casein chromatogram has a major peak at 34.8 min and a minor peak at 37.8 min that could be assigned to γ-casein, described in the next paragraph, as well as to contamination of κ-casein. As explained in the Experimental section, the α- and β-casein standards used later in calibration studies were the major peaks at 40.9 and 34.8 min, respectively, isolated from these commercial samples. Recovery of commercial α-, β- and κ-caseins obtained by comparison of peak areas, after re-injection in the Propyl column of collected fractions from the same column, was 98.0%, 99.1% and 97.2%, respectively.

Whey proteins (WPs) co-elute in the Propyl column before all the casein fraction, not interfering with casein separation and determination. This has been verified, by injecting both a 1:10 dilution of WPs prepared as described in the Experimental section and standard solution of α-lactalbumin and β-lactoglobulin, which are the major WPs in milk. WPs are eluted in our operating conditions before all the casein fractions, at about 32.5 min. Thus, with this separation method, no preliminary separation or precipitation procedure of the casein fraction is required.

The method has been straightforwardly validated

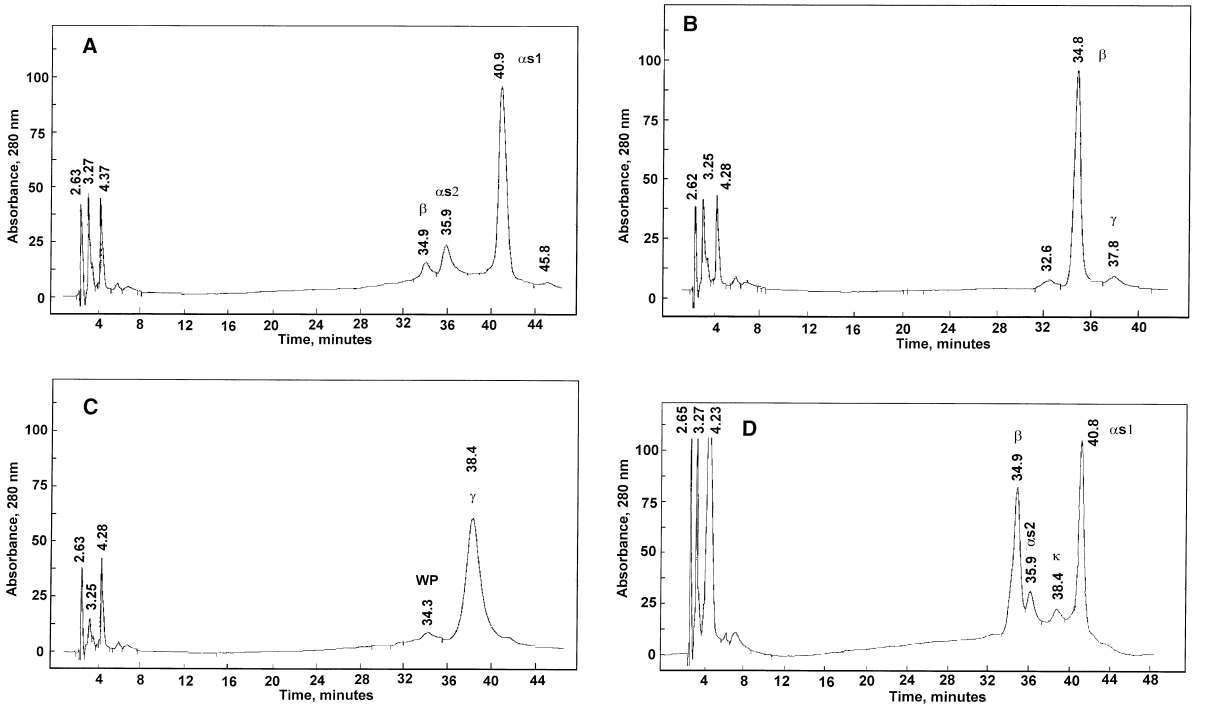

Fig. 1. HIC chromatograms of denatured commercial caseins. (A) α-casein (34.5 μ*M*) with a retention time of 40.9 min (α_{s1}) and 35.9 min (α_{s2}); (B) β-casein (37.1 μ*M*) with a retention time of 34.8 min; (C) κ-casein (71.0 μ*M*) with a retention time of 38.4 min; (D) commercial casein mixture (Fluka) (0.55 mg/ml) $\alpha_{s1}=\alpha_{s1}$-casein ($t_{\alpha_{s1}}=40.8$ min). $\alpha_{s2}=\alpha_{s2}$-casein ($t_{\alpha_{s2}}=35.9$ min). β=β-casein ($t_{\beta}=34.9$). κ=κ-casein ($t_{\kappa}=38.4$ min). WP, whey proteins. Chromatographic conditions: see the Experimental section.

Table 1
Results of linear fitting of calibration data of α_{s1}-, β- and κ-caseins (standard commercial samples from bovine milk)

Calibration data	α_{s1}-Casein	β-Casein	κ-Casein
Injected concentration range (μM)	0.5–50.0	0.5–50.0	0.5–60.0
R	0.9996	0.9988	0.9971
Number of points	7	6	7
Slope (μM^{-1})	$1.440 \pm 0.058 \cdot 10^6$	$0.843 \pm 0.026 \cdot 10^6$	$1.340 \pm 0.094 \cdot 10^6$
RSD (%)[a]	3.0	4.2	6.0
LOD (μM)[b]	0.4	0.7	0.4

[a] Average value of five replicate determinations for a standard solution whose protein concentration injected was 10 μM.
[b] LOD, 3σ/slope, where σ has been estimated on the basis of baseline noise.

by the analysis of the available certificate reference material (BCR-063R). Quantitative analysis of α_{s1}, α_{s2}-, β- and κ-caseins in BCR-063R has been performed using calibration curves of α_{s1}-, β- and κ-caseins. Quantitation of α_{s2}-casein has been performed by using the same slope of the calibration curves of α_{s1}-caseins. Table 1 shows the results of linear fitting of calibration curves of α_{s1}, β- and κ-caseins, obtained by plotting integrated area of chromatographic peaks as a function of casein concentration. In the same table, the limit of detection (LOD) values are also reported. The total casein percentage found has been calculated on the basis of the protein concentration injected, and estimated by total nitrogen certified injected, as previously described [33], on the basis of the equation [39]:

$$[\text{Protein total}] = N_{\text{total}} \cdot 6.38 \cdot 0.97$$

Table 2 summarizes the quantitative results obtained on five different stock solutions of the same BCR-063R lot. BCR-063R analysis gave that total caseins represent 74±5% (average value) of the estimated protein content, with α_{s1}, α_{s2}-, β- and κ-caseins representing 32±2%, 13±2%, 48±2% and 7±1%, respectively. In the range of linearity the casein recovery appears to be independent of the protein concentration of BCR-063R injected.

3.1.1. Specificity

No interfering peaks at the retention times where the α_{s1}-, α_{s2}-, β-, γ- and κ-caseins were detected. The analysis by this method of casein-free samples,

Table 2
Results of quantitative determination of α_{s1}-, α_{s2}-, β- and κ-caseins (CNs) in five amounts of certificated skim milk powder (BCR-063R)

Sample no.	N_{total} conc. injected[a] (μg/ml)	Protein conc. injected[b] (μg/ml)	α_{s1}-CN (μg/ml)		α_{s2}-CN (μg/ml)		β-CN (μg/ml)		κ-CN (μg/ml)		Total caseins (μg/ml)		Total casein found (%)	
			Mean	SD[c]	Mean	SD[c]	Mean	SD[c]	Mean	SD[c]	Mean	SD[c]	Mean	SD[c]
1	168	1041	237	15	83	6	370	19	58	5	748	25	72	3
2	158	977	254	16	104	9	332	18	41	4	732	26	75	4
3	89	550	122	9	42	3	185	11	28	3	378	14	69	4
4	57	350	82	5	33	3	122	7	18	2	256	9	73	4
5	37	230	54	4	26	2	93	6	14	1	187	7	82	4

Mean±SD of means is 74±5%.
[a] Calculated on the basis of N total certificated value.
[b] Estimated on the basis of the equation $[\text{Protein total}] = N_{\text{total}} \cdot 6.38 \cdot 0.97$ [39].
[c] $n = 3$.

which contain hydrolyzed proteins, did not show any peaks at the elution time examined.

3.1.2. Accuracy

Quantitative data obtained on BCR-063R (74±5% total casein found) are in agreement with the expected range of casein content in skim milk (75–85%) [39], and with the data obtained previously by the TSK gel Phenyl and Ether columns (79±3% and 78±6% total casein found, respectively). These data are different from those found by Cordeiro et al. on BCR-063R by RP-ion pair HPLC (total caseins 91%, with α_{s1}-, α_{s2}-, β- and κ-caseins representing about 45.3±5.9%, 5.8±0.9%, 35.3±2.3% and 13.6±1.8%, respectively) [4,30].

Quantitative data obtained on commercial casein mixture from Fluka (550 μg/ml injected) gave a total casein content of 98±4% with α_{s1}-, α_{s2}-, β- and κ-caseins representing 44±3%, 9±1%, 38±3% and 9±1%, respectively.

In the case of real samples (see below) we found that casein recovery was 83±10% for bovine, 99±12% for caprine, 93±10% for ovine and 90±5% for buffalos' raw milk with respect to the value obtained by Kjeldhal method. In cheese samples total casein recovery ranges between 72 and 93% with respect to the value indicated in the label, except in ewes' milk ricotta (unlabeled) in which total casein recovery is 63%.

The mean recovery of α_s-, β- and κ-casein standard solutions singly added to the BCR-063R sample and to unprocessed raw milk samples was 86.1 for α_{s1}-, 85.0 for α_{s2}-, 99.5 for β- and 75.5% for κ-caseins, showing a 'matrix effect'.

3.2. Applications

3.2.1. HIC analysis of unprocessed raw cows', goats', ewes' and buffalos' milk, their mixtures and cheeses

Protein milk composition varies considerably among species and within a species, too, depending on breed, stage of lactation [40]. While bovine caseins have been extensively studied (for reviews see Refs. [41,42]) and their percentages are well known in raw and processed milk, only few studies have been performed on casein from other species (goat, ewe, buffalo) which give quantitative information on the single casein fractions. It is known that total protein percentage in milk is from ewe (5.5)>buffalo (4.9 mean value, range 3.6–6.0)> cow (3.6 mean value, range 3.1–3.9)≥goat (3.1)> human (1.1), casein representing 82%, 87%, 80%, 77% and 25–40%, respectively [40]. Table 3 resumes data available in literature on casein fraction composition in cows' and goats' milk. Buffalos' milk is not included because to our knowledge no data are reported. Ewes' milk casein fractions have been identified [43] and quantified [22] also by CE by several authors, who found two peaks assigned to α-casein (defined α-casein_{CE}) and two peak assigned to β-casein (β-casein_{CE}). However since these frac-

Table 3
Summary of literature data on casein distribution of bovine, ovine, caprine and buffalo casein fractions from unprocessed raw milk

	Casein type				Minor (%)	Casein (%)	Ref.
	α_{s2} (%)	α_{s1} (%)	β (%)	κ (%)			
Bovine	12.1	39.5	37.2	11.2	–	–	[51]
	9.3	37.6	33.4	19.7	–	85.7	[52]
	10.1	38.6	38.7	12.7	–	84.3	[53]
	7.8	38.1	44.6	9.5	–	81.6	[4,30]
Caprine[a]	13.6	18.4	54.0	12.4	1.6	–	[54]
	11.0	21.0	48.0	15.0	5.0	–	[49]
Caprine[b]	19.3	1.9	58.5	14.9	5.4	–	[54]
	14.0	0.0	60.0	20.0	6.0	–	[49]
Ovine	8.0	35.0	38.0	17.0	–	–	[55–57]

[a] α_{s1}-casein high genotype.
[b] α_{s1}-casein low genotype.

tions have not been assigned in terms of α_{s1}-, α_{s2}- or β-casein these data are not included in the table.

The proposed HIC method has been applied to the analysis of unprocessed, raw milk from cow, goat, ewe and buffalo, several binary milk mixtures (cow/goat and cow/ewe) and to five cheeses processed with cows' (mozzarella, robiola, stracchino), ewes' (ricotta) and buffalos' milk (mozzarella). No sample precipitation or separation of casein fraction, is required prior to injection of the sample.

These experiments had the first aim of showing different, reproducible elution patterns for the casein fraction of milk from different species. Second, a quantitation of the different casein fractions was performed and compared with literature data. Data obtained on buffalos' milk have to be considered indicative because they are obtained on a single sample. Third, the analysis of milk mixtures showed a possible application of the method to detection of milk adulteration. Finally, the qualitative and quantitative results obtained on milk and the derived cheeses is discussed.

3.2.2. HIC analysis of unprocessed raw cows', goats', ewes' and buffalos' milk

Fig. 2 shows chromatograms of (A) cows' milk,

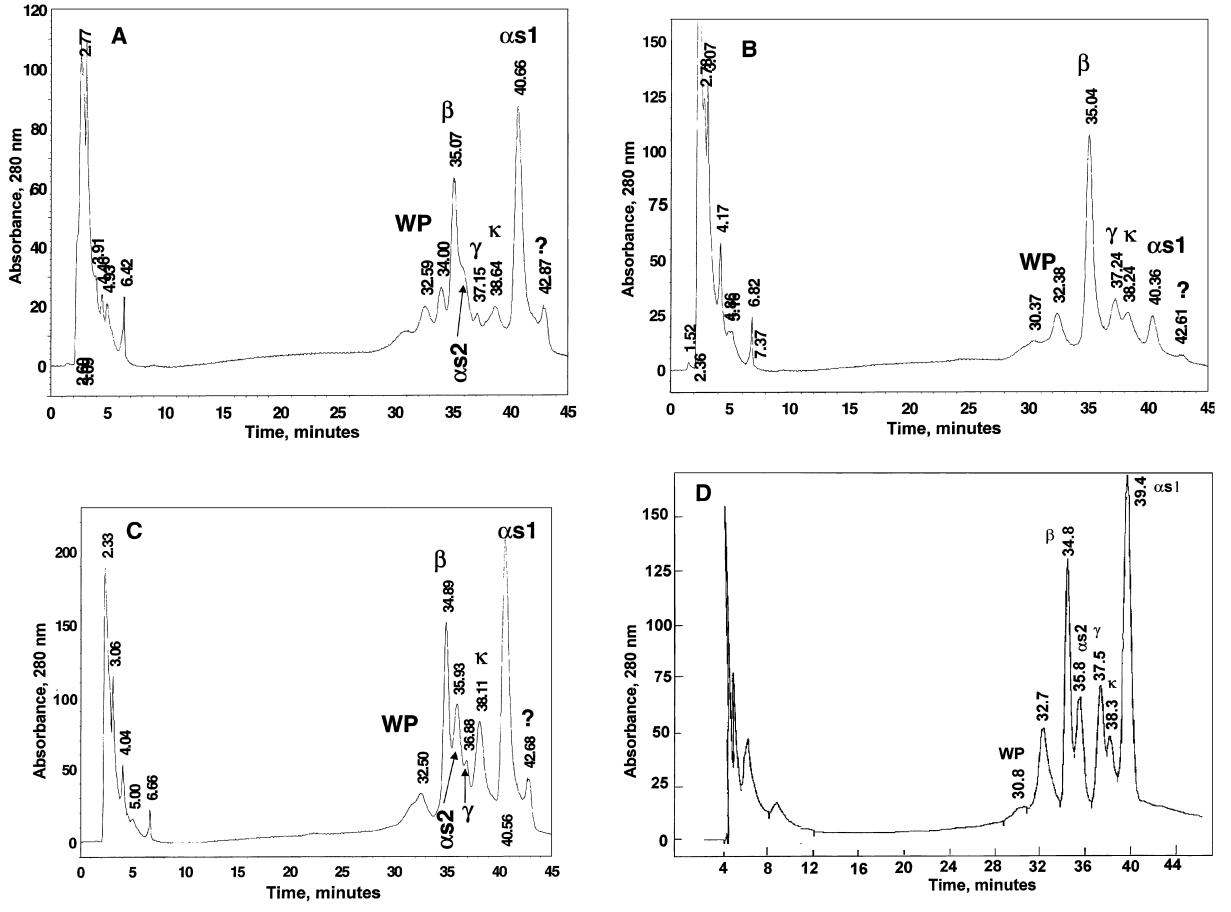

Fig. 2. HIC chromatograms of unprocessed milk samples. (A) Cows' milk (retention time $t_{\alpha_{s1}}=40.7$, $t_{\beta}=35.1$, $t_{\kappa}=38.6$, $t_{\gamma}=37.2$ min). (B) Goats' milk (retention time $t_{\alpha_{s1}}=40.4$, $t_{\gamma}=37.2$, $t_{\beta}=35.0$, $t_{\kappa}=38.2$ min). (C) Ewes' milk (retention time $t_{\alpha_{s1}}=40.6$, $t_{\alpha_{s2}}=35.9$, $t_{\beta}=34.9$, $t_{\kappa}=38.1$, $t_{\gamma}=36.9$ min). (D) Buffalos' milk (retention time $t_{\alpha_{s1}}=39.4$, $t_{\alpha_{s2}}=35.8$, $t_{\beta}=34.8$, $t_{\kappa}=38.3$, $t_{\gamma}=37.5$ min). Chromatographic conditions: see the Experimental section.

(B) goats' milk, (C) ewes' milk and (D) buffalos' milk. It is interesting to observe that in addition to α_{s1}-, α_{s2}-, β- and κ-casein peaks one more peak around 37.2 min is well detectable in all the raw milk samples. This peak has been assigned to γ-casein fraction which may derive from hydrolysis of β-casein by indigenous plasmin [44]. In ewes' milk this component almost co-elutes with α_{s2}-caseins; in buffalos' milk it shifts toward κ-casein peak.

Table 4 shows the retention time of peaks and the results of the quantitative determination of α_{s1}-, α_{s2}-, β-, γ- and κ-caseins in the considered samples. In the same table the assignment of major peaks is reported, based on the addition of bovine standard solutions of α-, β- and κ-casein. The total casein percentage found has been calculated, analogously to BCR-063R, on the basis of the protein concentration injected, and estimated by total nitrogen injected determined by the Kjeldahl method. The peak at 42.7 min detectable in cows' and ewes' milk, at present unassigned, has not been included in quantitation. Because of the high hydrophobicity of this component, extrapolated from the long retention time, we can hypothesize it is due to dimeric κ-casein or α_{s1}-casein variant isomer [3]. Standard deviation of data obtained on ewes', cows' and goats' milk pooled samples and on buffalos' milk samples takes into account the variability due to the analysis procedure; the standard deviation of the mean of mean values obtained for the 10 samples of cows', goats' and ewes' milk ($n=3$ replicates), takes into account both the variability due to the analysis procedure and the biological variability which exists between subjects also within the same species [45,46].

Data show that both total casein content and the proportion between α_{s1}-, α_{s2}-, β-, γ- and κ-casein are different depending on the milk species analyzed. The total protein content of raw milk from the different species analyzed was estimated on the basis

Table 4
Results of quantitative determination of α_{s1}-, α_{s2}-, β-, γ- and κ-caseins (CNs) in unprocessed raw milk

Sample	Protein conc. injected[a] (μg/ml)	α_{s1}-CN (μg/ml)		α_{s2}-CN (μg/ml)		β-CN (μg/ml)		γ-CN (μg/ml)		κ-CN (μg/ml)		Total casein found		Total casein found (%)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Bovine milk		$t_R=40.7\pm0.1$		$t_R=35.6\pm0.1$		$t_R=35.1\pm0.1$		$t_R=37.2\pm0.1$		$t_R=38.6\pm0.1$					
Pooled sample[b]	253±20	77	5	20	1	100	1	12	0.2	15	1	223	5	88	7
Mean of means[c]		79	12	15	2	87	14	11	4	18	8	210	21	83	10
Caprine milk		$t_R=40.4\pm0.1$		-----		$t_R=35.0\pm0.1$		$t_R=37.2\pm0.1$		$t_R=38.2\pm0.1$					
Pooled sample[b]	304±15	24	5	0	0	176	15	47	6	24	5	271	18	89	7
Mean of means[c]		31	17	0	0	190	25	55	9	25	6	300	33	99	12
Ovine milk		$t_R=40.6\pm0.1$		$t_R=35.9\pm0.1$		$t_R=34.9\pm0.1$		$t_R=36.9\pm0.1$		$t_R=38.1\pm0.1$					
Pooled sample[b]	540±30	172	2	63	1	142	4	45	7	67	8	489	11	91	5
Mean of means[c]		167	39	69	7	152	20	46	5	72	9	505	45	93	10
Buffalos' milk[b]		$t_R=39.4\pm0.1$		$t_R=35.8\pm0.1$		$t_R=34.8\pm0.1$		$t_R=37.5\pm0.1$		$t_R=38.3\pm0.1$					
	447±21	125	7	52	2	112	6	88	4	26	3	402	10	90	5

[a] Estimated on the basis of the equation [Protein total] = $N_{total} \cdot 6.38 \cdot 0.97$ [39], where N_{total} has been determined by the Kjeldhal method.
[b] Mean value±SD of $n=3$ injections.
[c] Mean±SD of the mean values obtained for the 10 samples examined, each repeated three times.

of the total nitrogen determined by the Kjeldhal method. We found 59.4±3.3 mg/ml of proteins in ewes' milk, 49.2±1.9 mg/ml in buffalos' milk, 33.4±1.6 mg/ml in goats' milk and 27.8±2.2 mg/ml in cows' milk, according to literature values [40].

Casein composition of raw bovine milk determined by our method is in agreement with data reported in the literature, total casein representing 83±10% with α_{s1}-, α_{s2}-, β-, γ- and κ-caseins representing 37±7%, 7±1%, 42±8%, 6±2% and 9±4%, respectively.

Caprine caseins, in contrast to bovine caseins, vary considerably in the types of casein present; some are rich in α_{s1}-casein, whereas some are poor, depending on the genotype [47,48]. The diminished level of expression of α_{s1}-casein was not counterbalanced by an increase in the levels of the other caseins, resulting globally in a lower casein content [49]. In the 10 samples examined by the proposed method we found that total casein represents 99±12% with α_{s1}-, β-, γ- and κ-caseins representing 10±6%, 63±11%,18±4% and 8±2%, respectively. While α_{s1}- and β-casein values are in agreement with literature data, taking into account the biological variability, we found different values for minor caseins, α_{s2}-, γ- and κ-. In fact, κ-casein percentage found by our method resulted similarly to that one present in other species (about 8% instead of 20% found in literature). It cannot be excluded that the low values found for the κ-casein percentage are due to the partial formation of dimers and polymers of κ-casein via S–S bridges. Moreover, no α_{s2}-casein was found in goats' milk samples, the peak at 37.2 min being assigned to γ-casein. In fact, by adding a standard solution of commercial bovine α-casein, this peak did not increase. Although it cannot be excluded that the peak assigned to γ-casein in goats' milk samples is the α_{s2}-casein peak shifted by 1 min to a higher retention time, it is more likely that the high percentage of α_{s2}-casein fraction found by other authors by HPLC methods (about 15%) can be due to a co-elution of α_{s2}, where present, and γ-casein fractions [48,49].

Values found for the 10 ewes' milk samples (total casein 93±10% with α_{s1}-, α_{s2}-, β-, γ- and κ-caseins representing 33±8%, 14±2%, 30±5%, 9±1% and 14±2%, respectively) are in good agreement with the casein composition reported by other authors, except for α_{s2}-casein percentage and for the presence of γ-casein, not previously described.

In the buffalos' milk sample we found the total casein representing the 90±5% with α_{s1}-, α_{s2}-, β-, γ- and κ-caseins representing 31±2%, 13±1%, 28±2%, 22±1% and 7±1%, respectively. Although the quantitative data obtained on just one sample can be considered only indicative, the qualitative comparison of the HIC chromatogram of buffalos' milk with that one of a cheese derived from milk of the same species, described in the next paragraph, can be interesting.

Fig. 3 compares the differences between the mean area±SD of α_{s1}-, α_{s2}-, β-, γ- and κ-casein peaks for the four species (10 milk samples for cow, goat and ewe; one milk sample for buffalo). It is worth noting that by using the proposed method the retention time of the peaks assigned to the single casein fractions in the various species are approximately the same, making the interpretation of data greatly simplified. Peak areas are considered instead of casein percentages because peak area analysis of HIC chromatograms can straightforwardly indicate differences between milk from the different species without needing any calibration.

In order to quantitate these differences the ratio of

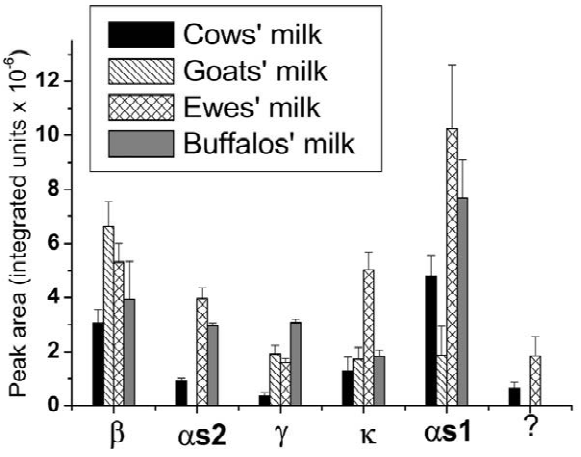

Fig. 3. Comparison of the area of peaks assigned to α_{s1}-, α_{s2}-, β-, γ- and κ-casein of HIC chromatograms of cows', goats', ewes' and buffalos' milk samples. The reported value for cows', ewes' and goats' milk is the mean±SD of the mean values obtained for the 10 samples examined; for buffalos' milk sample the mean±SD obtained for $n=3$ replicate injections of the same milk sample is reported.

the peak area of the major caseins, α_{s1}-, α_{s2}-, β- and κ-casein were evaluated. In fact, the evaluation of the area ratio of the peaks assigned to casein fractions, present in different proportion in milk from different species, could be a useful parameter for revealing milk adulterations. While for detecting a dilution as fraudulent processing of milk at least quantitation of total casein has to be performed, the alteration of the area ratio could indicate, in principle, the mixing of milk from different species without needing any calibration. Furthermore, ratio values should be at least in the linear dynamic range of quantitation of caseins, independent of the actual casein content or the casein recovery.

Four peak area ratios, α_{s1}/κ, α_{s2}/β, β/κ and α_{s2}/α_{s1}, out of the six values, have been considered in this study and are compared in Fig. 4. Despite the biological variability between animals of the same species (not valuable in this study for buffalos' milk and only indicative for the other three species because of the limited number of samples examined), affecting most of all cows' and goats' milk samples [53], the peak area ratios described above are significantly different and they could be suitable for detecting fraudulent addition of cows' milk to goats' or ewes' or buffalos' milk.

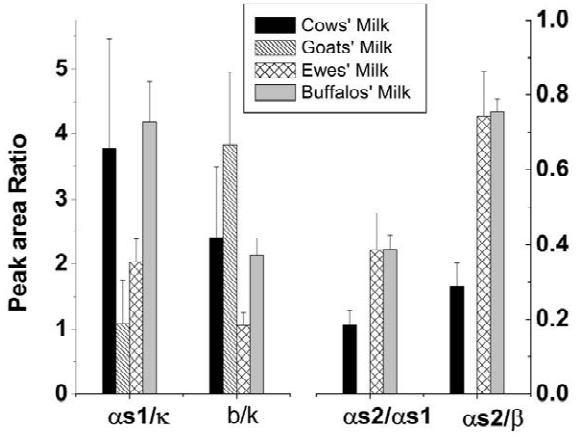

Fig. 4. Comparison of the α_{s1}/κ, α_{s2}/β, β/κ and α_{s2}/α_{s1} ratio values of peaks in HIC chromatograms of cows', goats', ewes' and buffalos' milk samples. The reported value for cows', ewes' and goats' milk is the mean±SD of the mean values obtained for the 10 samples examined; for buffalos' milk sample the mean±SD obtained for $n=3$ replicate injections of the same milk sample is reported.

3.2.3. HIC analysis of binary cow/goat and cow/ewe milk mixtures

In order to verify a possible application of the proposed method to the determination of the percentage of cows' milk in milk mixtures, a preliminary investigation was performed by analyzing binary milk mixtures. Fig. 5A and B show the α_{s1}/κ, α_{s2}/β, β/κ and α_{s2}/α_{s1} area ratios calculated from the HIC chromatograms of cow/goat and cow/ewe milk mixtures, respectively, as a function of cows' milk percentage in the mixture prepared as described in the Experimental section. Continuous and dotted lines indicate theoretical trends of the ratios as a function of cows' milk percentage in the mixture, resulting from the linear combination of the ratio values of the two pure milk sample constituting the mixture. A continuous line is computed from the peak area mean value of the three replicate chromatograms of the milk sample used for the mixture (the sample no. 3 for each species, arbitrarily chosen); the dotted line is computed from the mean of means of the peak areas of HIC chromatograms obtained for the 10 milk samples of each species (data of Fig. 4). The two theoretical trend-lines are compared in the figures in order to give an approximate idea of how much biological variability can affect these results. Although an accurate study should involve a greater number of milk samples from different animals of the same species, we can state that the described ratios calculated from HIC chromatograms of denatured casein from unprocessed raw milk of different species can be employed in the quality control of milk. In fact, the ratio values are dependent on milk mixture composition, they follow an additive model, revealing addition of cows' milk to goats' and ewes' milk ≥10%. More reliable results are, obviously, obtained by considering the ratio of peaks characterized by a good reproducibility, i.e. major peaks, and by a modest biological variability (e.g. the α_{s1}, α_{s2} and β casein peaks).

The results of cheese analysis, discussed in the next paragraph are included in Fig. 5B. Although the model for the determination of milk composition is based on milk mixtures and not on the derived cheeses, it is surprising that the value of α_{s2}/β and α_{s2}/α_{s1} area ratios calculated from HIC chromatograms of ricotta from ewes' milk and the three

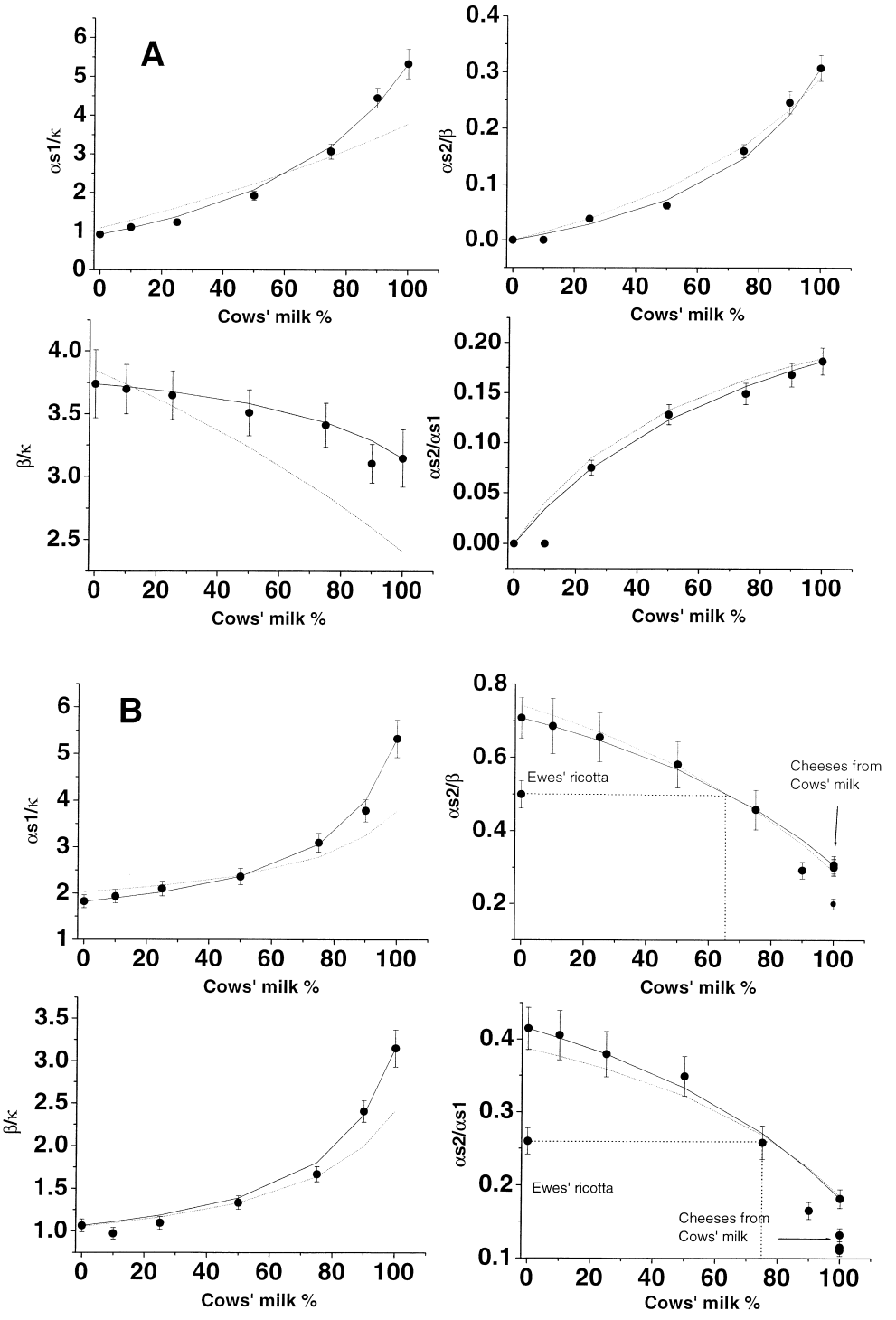

Fig. 5. α_{s1}/κ, α_{s2}/β, β/κ and α_{s2}/α_{s1} ratio values of peaks calculated from HIC chromatograms of binary milk mixture. (A) Cow/goat; (B) cow/ewe.

cheeses from cows' milk are in reasonable agreement with those of the corresponding milk (the other two ratios are not evaluated because κ-casein peak is not present in most of the cheese HIC chromatograms). The α_{s2}/α_{s1} ratio calculated for cheeses is systematically lower than that one calculated from milk chromatograms. The α_{s2}/β ratio values of cheeses from cows' milk are, instead, in agreement with those of the corresponding milk. However, α_{s2}/β ratio value calculated for ewes' ricotta is lower than expected. As this cheese had no 'appellation d'origine', it cannot be excluded that a cow/ewe milk mixture instead of 100% ewes' milk has been used for its production.

All these results, although preliminary, are encouraging for the extension of application of the proposed method to the detection of adulterations in dairy products.

3.2.4. HIC analysis of cheeses

Fig. 6 shows, as examples, HIC profiles of (A) stracchino cheese from cows' milk, (B) ewes' milk ricotta (cottage) and (C) buffalos' milk mozzarella cheeses, solubilized in 4.0 *M* GdmSCN, centrifuged, diluted in the gradient starting buffer and injected. Table 5 shows results of the quantitative determination of α_{s1}-, α_{s2}-, β-, γ- and κ-caseins in all the five cheese samples examined.

It is known that during cheese processing chymosin added partially hydrolyzes α- and κ-casein giving α_{s1} casein I and para-κ-casein, respectively, and indigenous plasmin gives γ_1- (29–209), γ_2-(106–209) and γ_3-casein (108–209) from β-casein [44]. It is also known that, once β-casein is hydrolyzed by plasmin to γ-caseins, these ones are not hydrolyzed anymore by chymosin [50]. After this primary proteolysis, further proteolysis occurs in

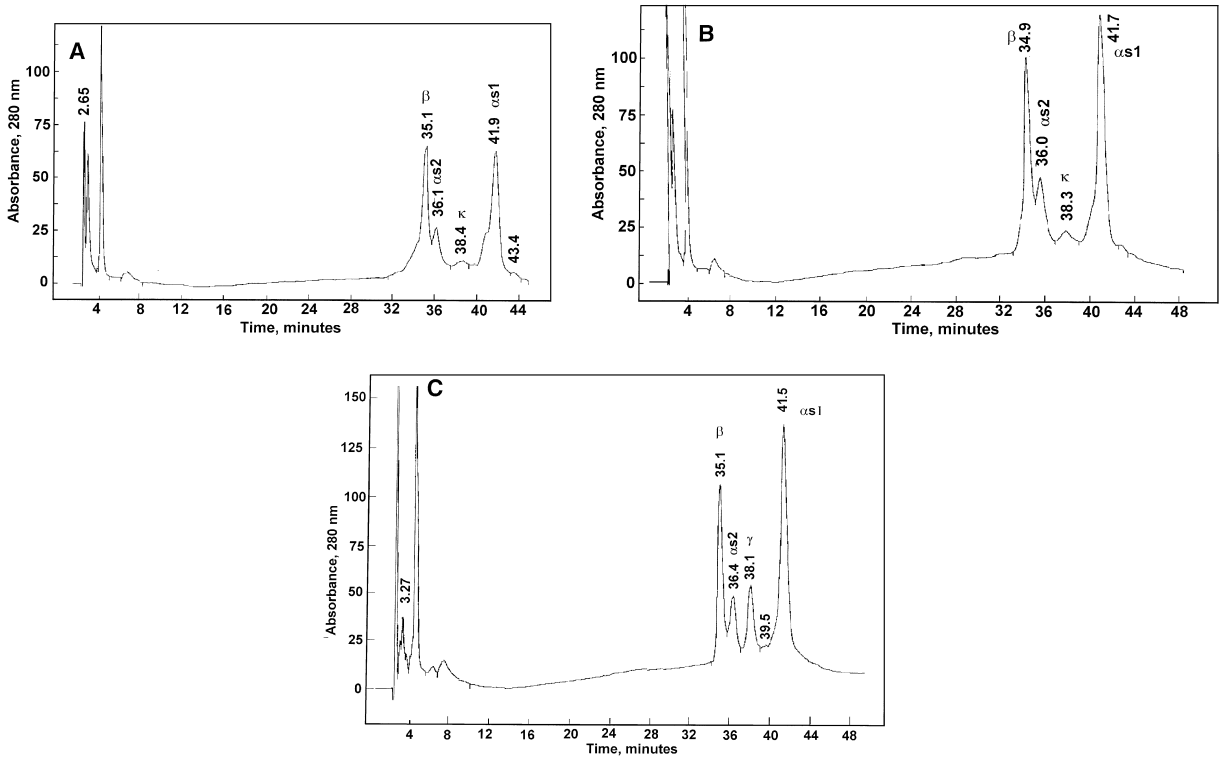

Fig. 6. HIC chromatograms of cheese samples dissolved in 4.0 *M* GdmSCN and centrifuged. (A) Stracchino cheese from cows' milk (retention time $t_{\alpha_{s1}}=41.9$, $t_{\alpha_{s2}}=36.1$, $t_{\beta}=35.1$, $t_{\kappa}=38.4$ min); (B) ewes' milk ricotta cheese (retention time $t_{\alpha_{s1}}=41.7$, $t_{\alpha_{s2}}=36.0$, $t_{\beta}=34.9$, $t_{\kappa}=38.3$ min); (C) buffalos' milk mozzarella cheese (retention time $t_{\alpha s1}=41.5$, $t_{\alpha s2}=36.4$, $t_{\beta}=35.1$, $t_{\gamma}=38.1$ min).

Table 5
Results of quantitative determination of α_{s1}-, α_{s2}-, β-, γ- and κ-caseins (CNs) in cheeses[a]

Sample	Protein conc. injected (labelled) (μg/ml)	α_{s1}-CN (μg/ml)		α_{s2}-CN (μg/ml)		β-CN (μg/ml)		γ-CN (μg/ml)		κ-CN (μg/ml)		Total caseins found (μg/ml)		Total casein found (%)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Buffalos' milk Mozzarella cheese 15% fat	483	186 (43)	12 (2)	39 (9)	3 (1)	118 (27)	7 (2)	83 (19)	6 (2)	8 (2)	1 (0.2)	433	15	90	4
Cows' milk Mozzarella cheese 15% fat	620	302 (56)	19 (2)	36 (7)	3 (1)	172 (32)	9 (2)	21 (4)	1 (0.3)	10 (2)	1 (0.2)	541	22	87	4
Robiola cheese 21.5% fat	568	267 (51)	17 (3)	38 (7)	2 (1)	213 (40)	12 (3)	12 (2)	1 (0.2)	–	–	529	21	93	4
Stracchino cheese 21% fat	1748	612 (49)	40 (3)	75 (6)	6 (1)	524 (42)	29 (3)	48 (4)	4 (0.3)	–	–	1259	50	72	4
Ewes' milk ricotta cheese 13% fat	788	203 (41)	13 (3)	56 (11)	5 (1)	183 (37)	10 (3)	54 (11)	4 (1)	–	–	497	18	63	4

Percentages are reported in brackets.
[a] Three replicates.

cheeses catalyzed by proteases and peptidases released from starter and other bacteria [44].

Despite all these complex chemical modifications of casein fraction in cheeses, the qualitative comparison of HIC chromatograms of cheeses with the respective HIC profiles of milk samples from the same species shows that elution pattern is approximately the same except for the absence of the peaks of WPs at 32.8 and the absence or the reduced intensity of the peak of κ-casein at 38.3 min. Furthermore, modified caseins in cheeses do not show retention times in HIC significantly different from those observed for non-proteolyzed caseins. This phenomenon, previously observed by HIC analysis of cheeses obtained from cows' milk with HIC TSK Gel 5PW-Phenyl [33] and Ether column [34], is now confirmed also for chromatographic elution pattern of cheeses obtained from cows', ewes' and buffalos' milk in the Propyl column. On this basis, the HIC method for the analysis of denatured caseins makes the recognition and determination of α_{s1}-, α_{s2}-, β-, γ- and κ-caseins direct and easier.

4. Conclusions

HIC coupled with employment of strong denaturants (GdmSCN and urea) resulted in a good tool for separation and quantitation of α_{s1}-, α_{s2}-, β-, γ- and κ-caseins in commercial samples, unprocessed raw milk and cheese samples. The method has been validated by the analysis of reference skim milk powder (BCR-063R) certificated for total nitrogen content, obtaining a value for total casein (73.9±4.6% of total 'true' proteins injected) in agreement with the expected range of casein content

in bovine skim milk (75–85%) [39] and with previous papers [33,34].

The method has been applied to the quantitative analysis of casein fractions of unprocessed raw milk from cow, goat, ewe and buffalo, their mixtures and cheeses. The method fast, versatile and specific, does not require any preliminary precipitation or separation of casein fraction, thus minimizing sample handling.

Differences were found between the HIC elution profiles of the casein fraction from cows', ewes', goats' or buffalos' milk, providing the basis for the selection of different peak characteristic of the presence of these milk types in mixtures. The quantitative and reproducible results obtained by HIC made it possible to analyze, in fact, as a preliminary study, two milk binary mixtures (cow/goat and cow/ewe), establishing a model of prediction of their composition. An advantage of the method is that modified caseins in cheeses show the same number of peaks as the non-proteolyzed caseins in milk. All the peaks are assigned and retention times of non-proteolyzed caseins do not significantly differ from those observed for modified caseins in cheeses. The ratio of the area of selected peaks of casein (α_{s1}/κ, α_{s2}/β, β/κ and α_{s2}/α_{s1}) was proposed to identify and predict the composition of the milk mixtures, suggesting a successful employment of the method for detecting fraudulent addition of cows' milk to goats' or ewes' milk. The alteration of the area ratio can indicate, in principle, the mixing of milk from different species without needing any calibration. At least in the linear dynamic range of quantitation of caseins, the ratios are also independent of the actual casein content or the casein recovery. All these facts and the employment of inexpensive, common HPLC instrumentation makes the proposed method a novel approach for the quality control of milk and also of easy and direct application for un-skilled end-users.

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