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Separation and quantification of the major casein fractions by reverse-phase high-performance liquid chromatography and urea–polyacrylamide gel electrophoresis Detection of milk adulterations

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Abstract

The separation and quantification of bovine κ-, α- and β-caseins by HPLC–UV using an RP column which contained polystyrene–divinilbenzene copolymer based packing was optimized and validated. Gradient elution was carried out at a flow-rate of 1 ml/min and a temperature of 46 °C, using a mixture of two solvents. Solvent A was 0.1% trifluoroacetic acid in water and solvent B was acetonitrile–water–trifluoroacetic acid (95:5:0.1). The effluent was monitored by a UV detector at 280 nm. The determinations were performed in the linear range of 0.038–0.377 mg/ml for κ-casein, 0.188–1.883 mg/ml for α-casein and 0.151–1.506 mg/ml for β-casein. The detection limits were 0.006, 0.019 and 0.015 mg/ml for κ-casein, α-casein and β-casein, respectively. The validity of the method was verified. The recoveries ranged from 91 to 100% for bovine milk. The precision of the method was also evaluated, the RSD being less than 3.67%. The same HPLC procedure was used for the separation of caprine and ovine caseins. Different chromatographic profiles were obtained for bovine, ovine and caprine milks, although it was only possible to detect and quantify additions of 5% or more of bovine milk to caprine milk. With respect to detection of milk adulterations, electrophoresis using urea–polyacrylamide gel electrophoresis (PAGE) analysis was more sensitive. The evolution of casein proteolysis in cheeses made from bovine milk and cheeses made from ovine milk, during 30 days of ripening was followed by HPLC–UV and urea–PAGE methodologies. The results obtained by these techniques were similar.

Keywords: Milk; Cheese; Food analysis; Caseins; Proteins

1. Introduction

Milk is a biologically complex fluid, constituted mainly of water, proteins, lactose, fat and inorganic compounds, the majority of these substances having important nutritional and technological value. According to its solubility at pH 4.6 and 20 °C, the protein fraction can be divided into caseins, insoluble at this pH, and whey proteins which are soluble. Caseins are, quantitatively, the most important milk

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protein components. This protein complex, known as a micelle, comprises four different caseins (α_{s1}-, α_{s2}-, β- and κ-caseins) which are held together by non-covalent interactions and appear as a highly stabilized dispersion in milk [1]. During the classical cheesemaking process it is the casein fraction which constitutes the cheese curd after the enzyme-triggered milk coagulation step.

The determination of individual caseins, and their degradation products in milk, cheese and other dairy products has been a major task for several years, since it can provide valuable information [2].

In recent years, the methods performed to analyse the casein fractions were: (i) electrophoretic techniques using polyacrylamide gels with urea (urea–PAGE) [3–5] or sodium dodecylsulphate (SDS–PAGE) [6,7] and isoelectric focusing (IEF) [8], (ii) high-performance liquid chromatography (HPLC) by ion-exchange [9,10], hydrophobic interactions [11,12], gel filtration [9,13] and reversed-phase [14–16] modes, (iii) immunological methods [17,18] and more recently capillary electrophoresis [2,19–21]. Although each method has its own merits, the use of HPLC has resulted in the development of rapid, automated and quick analyses with good separations and high resolutions, which give accurate and reproducible results.

In this work, an RP-HPLC method for simultaneous qualitative and quantitative analysis of bovine caseins was optimized and validated. This technique was also used for the separation of caprine and ovine caseins. The analytical utility of the HPLC method to detect adulterations of caprine and ovine milks with bovine milk and to study the evolution of proteolysis was evaluated. The RP-HPLC results were compared with those obtained with urea–PAGE.

2. Experimental

2.1. Sampling

Bovine, ovine and caprine raw milks (from Frísia, Churra and Serrana's breeds, respectively) were obtained directly from the producers.

Ultra-high-temperature (UHT), dry milk powder and pasteurised bovine milks, were purchased from the market and were also analysed. Powered milk was reconstituted with deionized water according to the manufacturer's instructions.

In this study, ten different cheeses were also analysed: five from bovine milk and the others from ovine milk. Cheese samples were analysed after 5, 10, 15, 20 and 30 days of ripening.

2.2. Sample preparation

Skim milks were prepared by separating the fat from the whole milk by centrifugation at 700 *g*, at 4 °C, for 10 min, and stored at −20 °C until use. Caseins were obtained from skim milks and cheeses by precipitation at pH 4.3, 20 °C, by the addition of 1 *M* ammonia–acetate buffer. The acidified milk was centrifuged for 15 min at 3000 *g*, at 20 °C, to recover the precipitated caseins. The caseins were dispersed in 1 m*M* ammonia–acetate buffer (pH 4.3), precipitated again and centrifuged for 10 min, at 3000 *g*, at 20 °C. This procedure was repeated twice. In order to eliminate the remaining fat, the sample was washed with acetone and left to dry in a fume hood at room temperature. Finally, the dried powdered casein was stored in a desiccator at 8 °C until analysis.

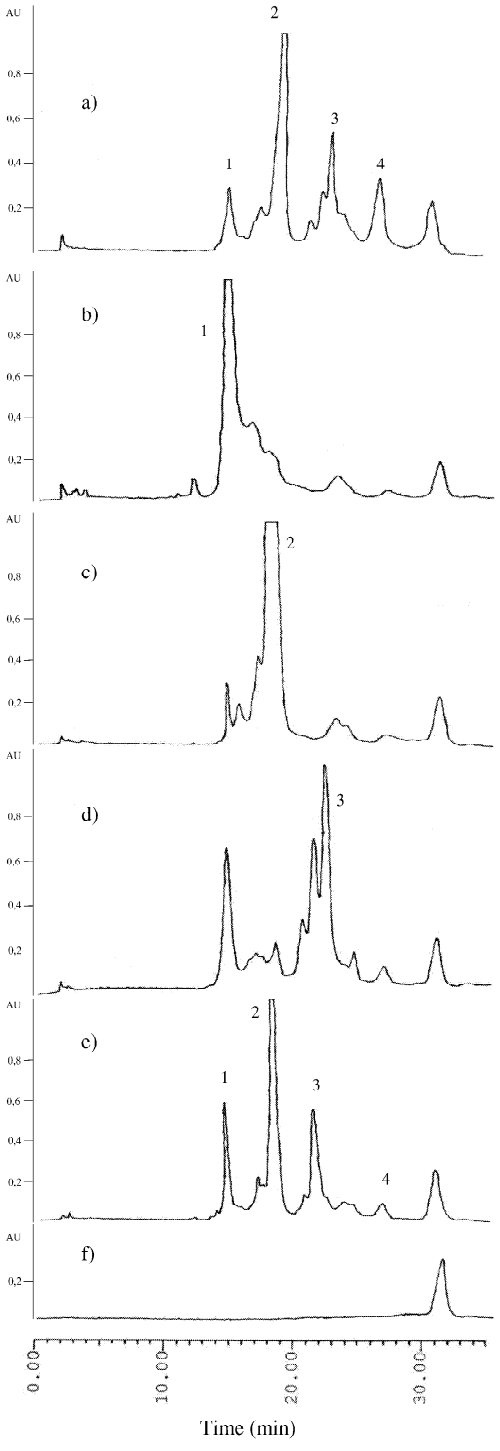
2.3. Reagents and protein standards

All reagents used were of analytical grade purity. Buffers for HPLC were filtered through 0.22-μm NL 17 filters and degassed under vacuum for at least 15 min before use. All reagents used in the electrophoresis had an adequate purity for this experiment and were used without any further purification.

Bovine milk casein, with a minimum purity of 75%, determined by the Bradford method [22], was supplied by Sigma. Purified α-, β- and κ-caseins, ovine casein and bovine serum albumin (BSA) were also obtained from Sigma and presented a minimum purity of 85, 90, 80, 85 and 98% (according to Sigma), respectively.

2.4. Reversed-phase HPLC separation

The HPLC equipment consisted of a Gilson chromatograph (Gilson Medical Electronics) equipped with a type 302 pump, a type 305 pump and a type 7125 Rheodyne Injector with a 20-μl loop. A Gilson 118 variable-wavelength longwave



ultraviolet detector was also used. The equipment was controlled by Gilson 712 software which controlled the solvent gradient, data acquisition, and data processing. The column was a reversed-phase Chrompack P 300 RP column that contains polystyrene–divinylbenzene copolymer-based packing (8 μm, 300 Å, 150×4.6 I.D.). A Chrompack P RP (24×4.6 mm I.D.) was used as a pre-column. Gradient elution was carried out with a mixture of two solvents. Solvent A consisted of 0.1% trifluoroacetic acid (TFA) in water and solvent B was acetonitrile–water–TFA (95:5:0.1, v/v). Proteins were eluted with a series of linear gradients increasing the proportion of solvent B, from 29 to 100% over 35 min: 1–5 min, 29% B; 5–10 min, 29–37% B; 10–12 min, 37–41% B; 12–14 min, 41–42.5% B; 14–16 min, 42.5% B; 16–17 min, 42.5–43% B; 17–19 min, 43% B; 19–21 min, 43–47% B; 21–23 min, 47% B; 23–25 min, 47–54% B; 25–27 min, 54% B; 27–28 min, 54–100% B; 28–30 min, 100–29% B; 30–35 min, 29% B. The flow-rate was 1 ml/min, the column temperature was 46±0.1 °C and the detection was made at a wave-length of 280 nm.

The casein powder was dissolved in a mixture of solvent A and solvent B (70:30, v/v). The solution was filtered through a 0.45-μm TR-200104 filter, made of a mixture of cellulose esters (Teknokroma), and stored at −20 °C until use. For each analysis 20 μl of the solution were applied to the HPLC column.

The validation of the method for the determination and quantification of the caseins fraction of bovine raw milk was accomplished by testing the linearity, the precision (repeatability and reproducibility) and the accuracy.

2.5. Urea–PAGE separation

Polyacrylamide gel electrophoresis of casein samples was undertaken according to the method of

Fig. 1. Chromatographic profiles of bovine milk caseins obtained by RP-HPLC at 280 nm, 20-μl injection volume: (a) whole casein standard solution (concentration 3.76 mg/ml); (b) κ-casein standard solution (concentration 1.50 mg/ml); (c) α-casein standard solution (concentration 1.51 mg/ml); (d) β-casein standard solution (concentration 1.51 mg/ml); (e) whole casein of bovine milk (concentration 3.61 mg/ml); (f) baseline. Peak identification: 1=κ-casein; 2=α-casein; 3 and 4=β-casein.

Table 1
Calibration curves determined by the external standard method

Casein	Concentration range (mg/ml)	n[a]	Slope[b] (area counts/mg)	Intercept[b] (area counts)	r[c]
Whole casein	0.377–3.76	6	3.42 ($\pm$0.07)·10^6	7.1 ($\pm$1.4)·10^5	0.9998
κ-Casein	0.038–0.377	6	2.77 ($\pm$0. 15)·10^6	0.67 ($\pm$2.9)·10^5	0.9988
α-Casein	0.188–1.88	6	3.51 ($\pm$0.07)·10^6	3.2 ($\pm$0.07)·10^5	0.9998
β-Casein	0.151–1.51	6	3.48 ($\pm$0.18)·10^6	3.2 ($\pm$1.4)·10^5	0.9988

[a] Number of points considered for the regression. Each point represents the average of three injections of each standard solution.
[b] Standard deviation in the slope and intercept of the regression line are given in parentheses.
[c] Correlation coefficient.

Table 2
Repeatability and reproducibility of RP-HPLC, expressed as the relative standard deviation (RSD), determined from the analysis of precipitated bovine casein

Assay	n_1[a]	n_2[b]	κ-Casein		α-Casein		β-Casein		Whole casein
			t_R, RSD (%)	Area, RSD (%)	t_R, RSD (%)	Area, RSD (%)	t_R, RSD (%)	Area, RSD (%)	Area, RSD (%)
Same day	6	16	0.58	3.67	0.44	2.26	0.64	2.69	1.94
Between days[c]	12	34	0.65	4.28	0.41	4.46	0.53	4.18	3.64

[a] Number of aliquots analysed from the same milk sample.
[b] Total number of injections made (two or three injections per aliquot).
[c] Two different days.

Table 3
Method recovery assays in bovine milk sample with RP-HPLC

Caseins	Initial content		Addition (mg/15 ml)	Measured content		Recovery (%)
	mg/15 ml	RSD (%)		mg/15 ml	RSD (%)	
Whole casein	246	0.97	57.0	294	2.17	97
κ-Casein	40.6	0.52	5.70	45.3	1.69	98
α-Casein	130	1.24	28.5	151	2.25	95
β-Casein	78.7	3.82	22.8	102	2.45	100
Whole casein	246	0.97	113	333	1.83	93
κ-Casein	40.6	0.52	11.3	50.2	0.97	97
α-Casein	130	1.24	56.5	168	3.22	91
β-Casein	78.7	3.82	45.2	118	1.67	95
Whole casein	246	0.97	169	395	1.19	95
κ-Casein	40.6	0.52	16.9	55.1	2.27	96
α-Casein	130	1.24	84.5	205	1.90	96
β-Casein	78.7	3.82	67.6	139	1.28	95

Andrews [23] with some modifications. The assays were carried out in a vertical vat (SE 280 Hoefer Scientific Instruments), using a Unipack 2000 power supply (UniQuip).

The slab gels consisted of a 4% stacking gel and a 10% resolving gel. The stacking gel buffer was 0.06 *M* tris(hydroxymethyl)aminomethane (Tris), 4.5 *M* urea at pH 7.6, and the resolving gel buffer was 0.76 *M* Tris, 9 *M* urea at pH 8.9. The electrophoresis buffer was a solution of 0.02 *M* Tris, 0.19 *M* glycine. The run was performed at 4 °C, at 20 mA until the end of the stacking gel, followed by a current of 30 mA. The gels were stained with Coomassie brilliant blue R250. Protein band density was determined using a laser densitometer (Vilbert Lourmat). Quantitative determination of caseins was made by peak area integration of the densitometer traces.

The casein samples for electrophoresis were dissolved in a diluted NaOH solution at pH 9. The samples where vigorously stirred and then placed on an ultrasound bath until total dissolution. In order to remove the fat and the particles in suspension, the sample was centrifuged 3000 *g*, at 4 °C, for 10 min. The casein concentration was determined using the Bradford method with BSA as the standard [22]. The samples were mixed with the sample buffer (0.12 *M* Tris, 8.2 *M* urea, 2.5 m*M* EDTA, 0.2 *M* β-mercaptoethanol, 0.01% of bromophenol blue, pH 6.8) and 20 μg of proteins were applied in the wells.

3. Results and discussion

3.1. Separation and quantification of caseins in bovine milk by HPLC–UV

In this study the HPLC conditions were optimised for mobile phase composition, gradient, operating temperature and flow-rate.

As shown in Fig. 1, retention times of the major eluted peaks coincide with the retention times of standard casein fractions. Furthermore, peak areas of skim milk chromatograms are proportional to known relative abundance of caseins in bovine milk: 10:50:40, for κ-, α- and β-caseins, respectively [14,15,18]. However, it should be noticed that the chromatographic profile obtained for β-casein shows a peak in the region of κ-casein, which is due to the contamination of that standard. This fact was confirmed in the electrophoretic assays. Therefore, it was established that the milk caseins eluted in the following order: κ-, α-, and β-caseins. Typical chromatograms depicting separation of standard solutions of milk caseins are shown in Fig. 1a–d, which also presents the chromatographic profile

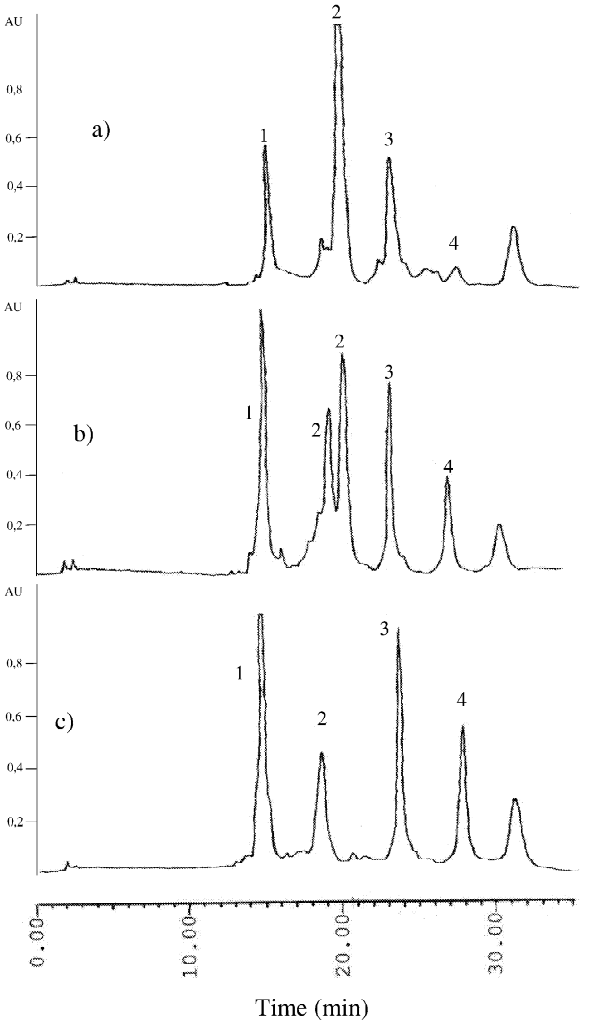


Fig. 2. Chromatographic profiles of bovine, ovine and caprine whole casein obtained by RP-HPLC at 280 nm, 20-μl injection volume: (a) raw bovine milk (concentration 3.61 mg/ml); (b) raw ovine milk (concentration 3.71 mg/ml); (c) raw caprine milk (concentration 3.67 mg/ml). Peak identification: 1=κ-casein; 2=α-casein; 3 and 4=β-casein.

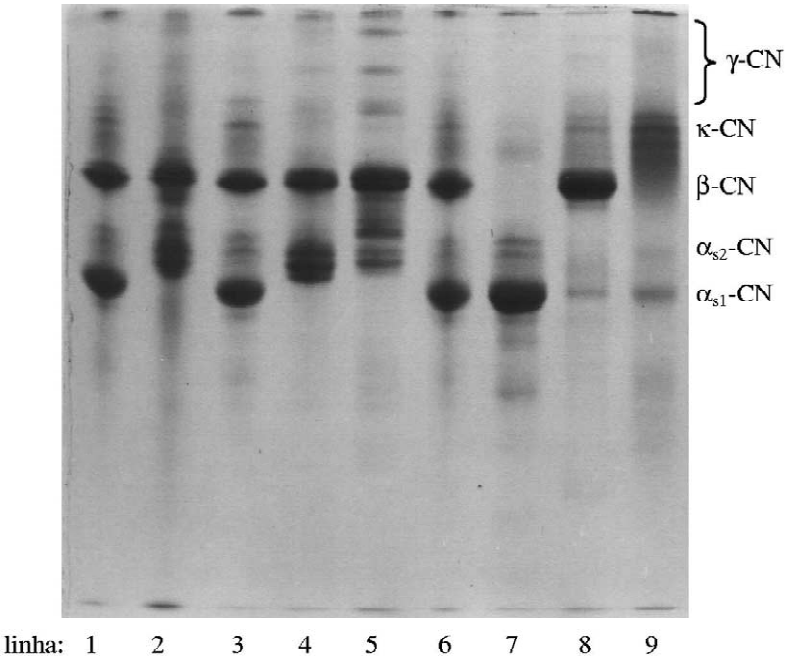


Fig. 3. Casein profiles obtained by urea–PAGE: (1,6) bovine casein standard; (2) ovine casein standard; (3) raw bovine milk; (4) raw ovine milk; (5) raw caprine milk; (7) α-casein bovine; (8) β-casein bovine; (9) κ-casein bovine.

obtained for a blank run (injection of 20 μl of solvent). In the latter a slight rise of the baseline was observed which appeared in the chromatograms of all the analysis performed throughout this work. This fact is likely due to the elution of small amounts of TFA, which was adsorbed to the stationary phase in polar solvent and eluted in the increasingly organic phase. A similar behavior was observed by Elgar et al. [24] when studying the major bovine whey proteins by reversed-phase HPLC on a polystyrene–divinylbenzene column.

The external standard method was used to calibrate the chromatographic system for the protein quantification. For this purpose standard solutions of whole bovine casein with concentrations ranging from 0.377 to 3.765 mg/ml (corrected according to the standard purity), were used. Each solution was analysed in triplicate. The linearity of the method

Table 4
Casein content in bovine milk: comparison between experimental values obtained in this work and those obtained by Walstra and Jenness [28] and by Bobe et al. [14]

Casein	Casein composition (%)[a]					
	Current study				Literature	
	Raw milk[b]	Pasteurised milk[b]	UHT milk[b]	Dry milk powder[b]	Raw milk [29]	Raw milk [14]
κ-Casein	16.4	15.5	7.02	10.3	12.7	19.7
α-Casein	52.5	49.8	50.3	50.2	48.6	46.9
β-Casein	32.4	35.8	42.1	39.5	38.7	33.4

[a] The symbol % is the mass percentage of each casein fraction in total.
[b] Values expressed as the mean of two determinations.

was checked through the calibration curves, which were calculated for the whole casein and for each casein fraction, and obtained by linear regression of the peak area versus concentration. The calibration curves for the caseins fractions were determined taking into account the area of the respective peaks (peak identification given in Fig. 1) and the concentration of each fraction in the injected solution. The values of the slope, intercept and correlation coefficient are given in Table 1.

The detection limit values were calculated as the concentration corresponding to three times the standard deviation of the background noise and were 0.0188 mg/ml for α-casein, 0.0150 mg/ml for β-casein (determined after the injection of a standard solution of whole casein with 0.0375 mg/ml) and 0.0060 mg/ml for κ-casein (determined after the injection of a standard solution of whole casein with 0.0600 mg/ml).

The precision of this method was evaluated taking into account its repeatability and reproducibility. The RSD values for the retention time and peak areas are given in Table 2. The RSD values are similar to those reported in the literature for within-day variation and between-day variation [14,25–27]. Therefore, this method is able to quantify casein of milk samples with good precision and shorter analysis time.

Recovery studies were carried out to determine the accuracy of the method. It was found that recoveries ranged between 93 and 97% for the whole casein contents (Table 3).

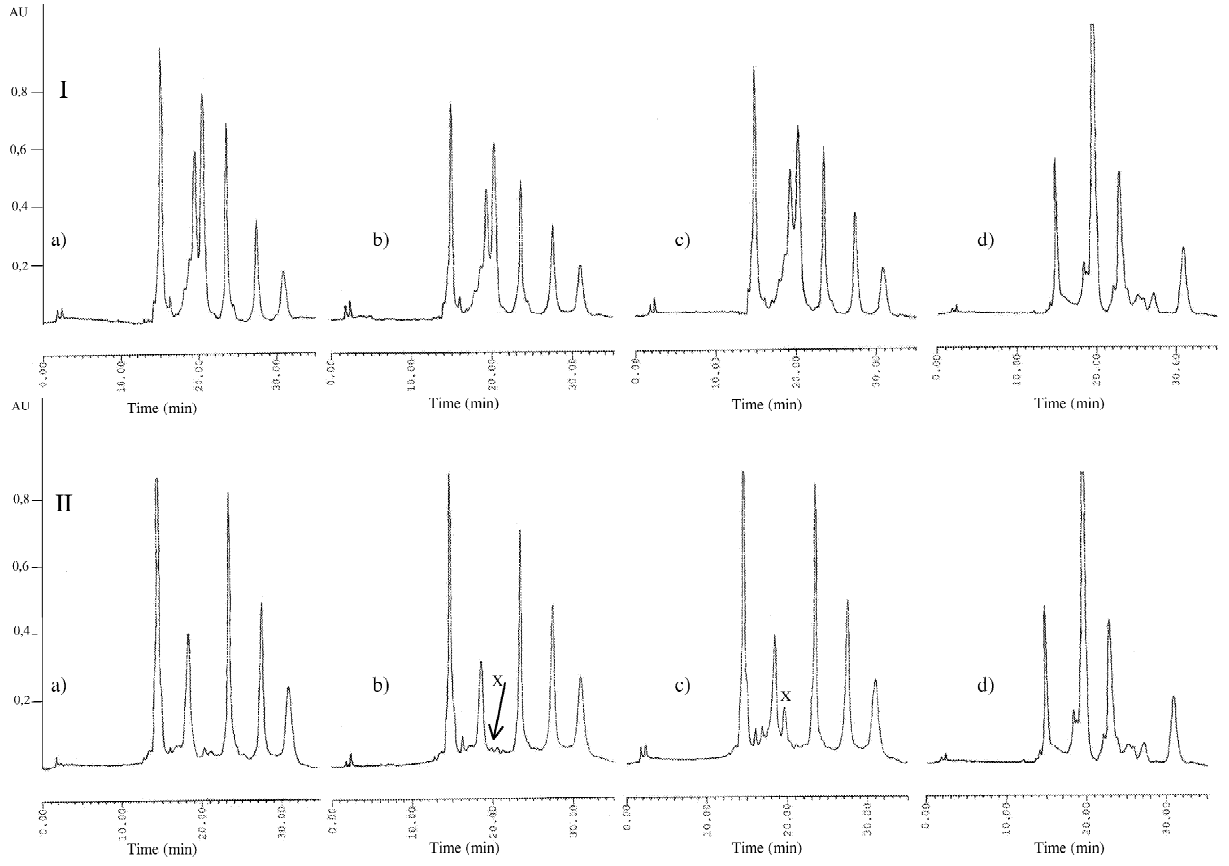


Fig. 4. (I) Chromatographic profiles of bovine and ovine whole casein obtained by RP-HPLC at 280 nm, 20-μl injection volume: (a) raw ovine milk; (b) 5% adulteration; (c) 20% adulteration; (d) raw bovine milk. (II) Chromatographic profiles of bovine and caprine whole casein obtained by RP-HPLC at 280 nm, 20-μl injection volume: (a) raw caprine milk; (b) 5% adulteration; (c) 20% adulteration; (d) raw bovine milk.

The calibration curves obtained in this work for the bovine whole casein and the respective fractions were used to quantify the contents of bovine caseins in raw and processed milks (UHT, dry powder and pasteurised). The results obtained in this study for the milk casein composition were similar to literature values, as can be seen in Table 4. Differences in milk casein composition reported by different authors can be partly explained by factors such as animal breed, season of the year, and diet composition [14,28].

Slightly different contents of κ-, α- and β-caseins obtained for raw and processed milks, as shown in Table 4, can also be due to heat treatment. Indeed, above 65 °C the whey proteins denature, leading to aggregation and coagulation. Moreover, the decrease of the κ-casein could be due to the formation of complexes between β-lactoglobulin and the κ- and α_{s2}-caseins as a result of the heat treatment. These complexes coagulate together with the caseins. This fact is in agreement with the results obtained by Douglas et al. [29] and Parris et al. [30], who observed by electrophoresis and/or RP-HPLC, a similar decrease in κ-casein and the appearance of a β-lactoglobulin–κ-casein complex in UHT milks in comparison to raw milk.

3.2. Casein profiles of bovine, ovine and caprine milk by HPLC–UV and urea–PAGE: detection of milk adulterations

The methods developed in this work have also been used for separation of homologous caseins from ovine and caprine milks. It was observed that HPLC–UV separation of the casein fraction in the three kinds of milk under the chromatographic conditions used gave a good separation. Different chromatographic profiles were obtained as shown in Fig. 2. Urea–PAGE also produced a good separation profile for the milk samples used (Fig. 3).

The different chromatographic and electrophoretic profiles obtained could be used to detect and determine bovine milk in ovine and caprine milks. Binary mixtures (bovine/ovine and bovine/caprine) containing different proportions were prepared and analysed by HPLC–UV and urea–PAGE.

Typical chromatograms are presented in Fig. 4.

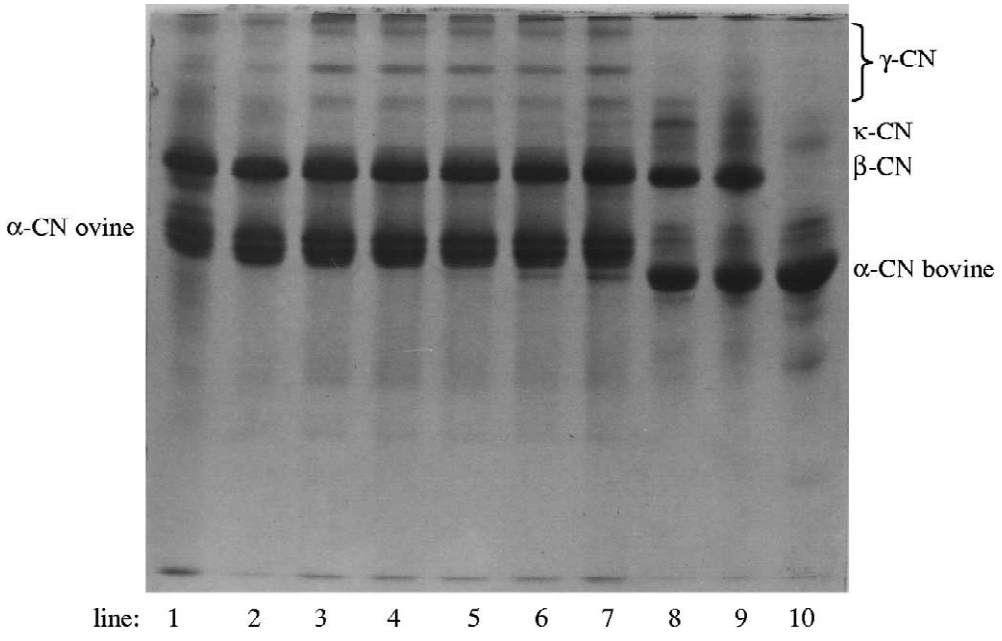


Fig. 5. Casein profiles obtained by urea–PAGE for adulterations of ovine milk with bovine milk: (1) ovine casein standard; (2) raw ovine milk; (3) 1% of bovine milk in ovine milk; (4) 2% of bovine milk in ovine milk; (5) 5% of bovine milk in ovine milk; (6) 10% of bovine milk in ovine milk; (7) 20% of bovine milk in ovine milk; (8) raw bovine milk; (9) bovine casein standard; (10) α-casein.

The HPLC allowed the detection of bovine milk in caprine milk, for percentages equal to or greater than 5% (v/v), based on the peak of α-casein bovine (peak X; Fig. 4, II). The amount of added bovine milk in adulterated samples could be calculated by integration of the bovine α-casein peak area, using a standard curve prepared previously with adulterated samples of known composition. However, the HPLC was not able to detect the addition of bovine milk to ovine milk as can be observed in Fig. 4, I.

Analysis of bovine/ovine and bovine/caprine mixtures (1, 2, 5, 10 and 20%, v/v) by urea–PAGE revealed that the protein profile of bovine α-casein was significantly different from all the other casein bands, as can be observed from Figs. 5 and 6.

Therefore, it was possible to detect percentages of adulteration of bovine milk in ovine milk equal to or higher than 5% (v/v) as shown in Fig. 5. Similar results were obtained by Ramos and Juárez [31]. The optimised urea–PAGE method also allowed detection of adulteration percentages of 5% (v/v) of bovine milk in caprine milk, based on the presence of the bovine α_{s1}-casein band in the bovine/caprine mixed milks, which was also achieved by Furtado [32]. However, with the present technique, adulterations of 2% can be detected due to the presence of a light band in the region of the bovine α_{s1}-casein. The performance of the urea–PAGE method optimised in this study is comparable to that obtained by Cattaneo et al. [33].

4. Conclusions

The described HPLC–UV procedure is suitable for routine separation and quantification of κ-, α- and β-caseins in raw and processed milks. Appropriate accuracy, precision and rapidity are characteristics of the optimised method. It can also be a useful tool to detect and quantify the adulteration of caprine milk.

The results obtained by the HPLC–UV method and by urea–PAGE are in good agreement. Nevertheless, electrophoresis is more sensitive for the detection of milk adulterations. However, HPLC is more efficient as regards quantitative results.

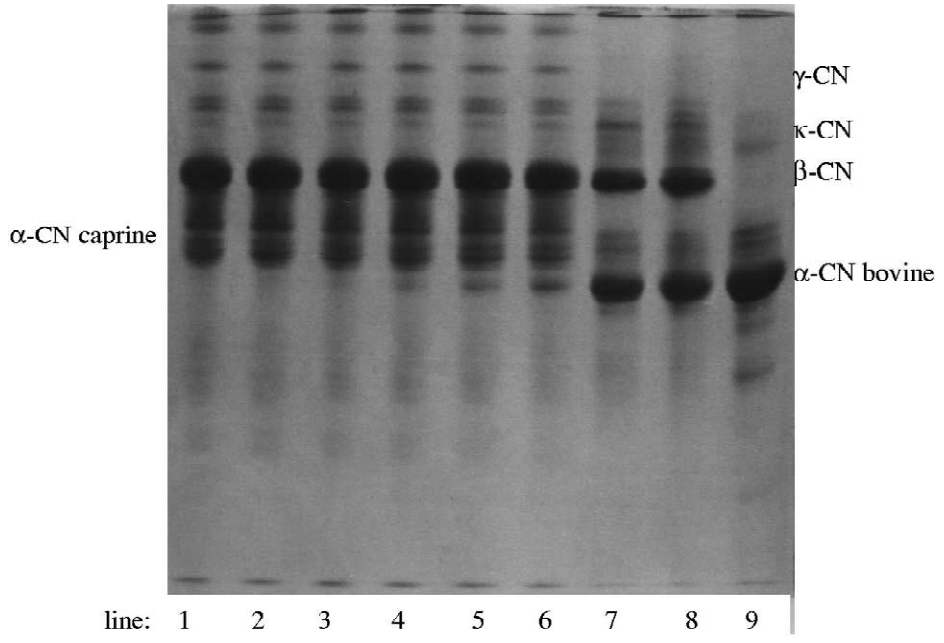


Fig. 6. Casein profiles obtained by urea–PAGE for adulterations of caprine milk with bovine milk: (1) raw caprine milk; (2) 1% of bovine milk in caprine milk; (3) 2% of bovine milk in caprine milk; (4) 5% of bovine milk in caprine milk; (5) 10% of bovine milk in caprine milk; (6) 20% of bovine milk in caprine milk; (7) raw bovine milk; (8) bovine casein standard; (9) bovine α-casein.

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