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New chromatographic method for separation and determination of denatured α_{s1}-, α_{s2}-, β- and κ-caseins by hydrophobic interaction chromatography

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Abstract

Separation and determination of denatured α-, β- and κ-caseins by hydrophobic interaction chromatography (HIC) was improved by using a TSK-Gel Ether-5PW column (Tosoh Biosep). The method, already proposed and performed by a TSK-Gel Phenyl-5PW column (Tosoh Biosep), is based on fast and easy solubilization of commercial and real samples by 4.0 *M* guanidine thiocyanate and HIC analysis in the presence of 8.0 *M* urea in the mobile phase. Employment of the less hydrophobic ether phase had the main advantage of separating casein fractions in less than 22 min and, additionally, of separating α-casein in α_{s1}- and α_{s2}-casein fractions. The method has been validated by the analysis of reference skim milk powder (BCR-063R) certified for total nitrogen content. 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 0.5–40 μ*M*. The detection limit for α-, β- and κ-caseins ranged between 0.33 and 0.65 μ*M*. The precision of the method was evaluated, the RSDs for α_{s1}-, α_{s2}-, β- and κ-casein determination ranging between 2.3 and 5.5% for standard solutions and between 4.4 and 6.2% for real sample solutions. The mean value of casein content found in eight aliquots of BCR-063R calculated with respect to the total protein content (estimated on the basis of certified total nitrogen content) was 78.3±6.1%. Results of linear fitting of standard additions data of α_{s1}-, α_{s2}-, β- and κ-caseins to BCR-063R were compared with linear fitting of α_{s1}-, α_{s2}-, β- and κ-casein calibration data. The method was applied to commercial caseins and to 30 real, raw samples. A statistical comparison was performed between results on quantitation of α-, β- and κ-caseins obtained by TSK-Gel Ether-5PW and TSK-Gel Phenyl-5PW HIC columns, showing more accurate results for chromatographic analysis performed by the ether column.

Keywords: Hydrophobic interaction chromatography; Milk; Cheese; Food analysis; Caseins; Proteins

1. Introduction

Caseins represent ~80–85% of milk proteins, and play a key role in human nutrition, in the dairy industry and as additives in food, paints and glues because of their emulsifying properties [1–5]. Caseins are classified as α_{s1}-, α_{s2}-, β- and κ-caseins, representing about 38, 10, 36 and 13%, respectively, of the casein fraction. The B variant of α_{s1}-casein is predominant, containing 8 mol P/mol protein and is

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characterized by largely hydrophobic 14–26 amino acidic residues [6]. α_{s2}-Casein is characterized by high phosphate content (~10–13 mol P/mol protein) and by two –SH groups, being the most hydrophilic of the α-casein fractions [6]. The most common A variant of β-casein is characterized by 5 mol P/mol protein, all located in the hydrophilic N-terminal region of 47 amino acids, and by a hydrophobic tail. This division of two functional domains confers surfactant properties on β-caseins. κ-Casein is characterized by 1 mol P/mol protein, a hydrophilic C-terminal peptide with a high negative charge density and a predominantly hydrophobic remainder that has a small positive charge at near-neutral pH. The reciprocal ratio between these four fractions strongly affects micelle size, size distribution, voluminosity and, in general, the chemical-physical properties of milk. It is known for example that the proportion of κ-casein increases as the average size of the micelles decreases, and also that the proportion of α_{s2}-casein increases significantly with ease of sedimentation [6]. As many of these properties are fundamental in milk processing and the dairy-food industry, the development of methods for fast separation and determination of α_{s1}-, α_{s2}-, β- and κ-casein fractions is of interest.

Casein quantitation in real, raw samples presents two major problems: (i) their poor solubility in water, as poor solubility of caseins makes their analysis by separation techniques difficult; and (ii) interferences of whey proteins which represent the other 15–20% of milk proteins. An overview of casein determination chromatographic methods has been previously reported [7].

In previous papers we proposed and validated a chromatographic method for separation and determination of α-, β- and κ-caseins by hydrophobic interaction chromatography (HIC) [7,8]. The method, performed by a TSK-Gel Phenyl-5PW column (Tosoh Biosep), is based on fast and easy solubilization of commercial and real samples by 4.0 *M* guanidine thiocyanate (GdmSCN) and HIC analysis in the presence of 8.0 *M* urea in the mobile phase. The method was successfully applied to the analysis of various real, raw samples in order to quantitatively determine α-, β- and κ-caseins. The method resulted in accurate, reproducible sample preparation and required only an easy solubilization in GdmSCN without any other pre-treatment.

In this paper we propose an improvement of this method based on a less hydrophobic phase, a TSK-Gel Ether-5PW column (Tosoh Biosep). Employment of the less hydrophobic ether phase had several advantages over the TSK-Gel Phenyl-5PW column: (i) separation of caseins in less than 22 min; (ii) separation of α-casein fraction into α_{s1}- and α_{s2}-casein fractions; (iii) elution of whey protein fraction with solvent front. Furthermore, the moderately increased back pressure in this column, also in presence of high salt concentration, allowed a higher eluent flow-rate (0.8 instead of 0.5 ml/min), thus reducing the time of equilibration of the column.

The method has been validated by the analysis of a reference skim milk powder (BCR-063R) certified for total nitrogen content. The method was then applied to commercial caseins and to 30 real, raw samples (processed cow's milk, both pasteurized and ultra-high temperature (UHT) treated, as well as follow-up milk powders, cream, cheeses, and casein-free infant formulae) with the aim of showing the wide applicability of the method in order to quantitatively determine α_{s1}-, α_{s2}-, β- and κ-caseins. A statistical comparison was performed between results obtained by TSK-Gel Ether-5PW and TSK-Gel Phenyl-5PW HIC columns.

2. Experimental

2.1. Chemicals

The α-, β- and κ-caseins and a casein mixture (product 22078) were purchased from Fluka (Buchs, Switzerland) (22084 α-caseins ≥90%, 22086 β-caseins ≥80%, 22087 κ-caseins ≥70%). Processed cow's milk, cheeses and baby food samples were commercial products purchased in a local supermarket. Follow-up milk powder samples and special baby formulae were kindly supplied by Dr A. Repetti, paediatrician. The buffer solutions were prepared from monobasic monohydrate sodium phosphate, dibasic anhydrous sodium phosphate (BDH, Poole, UK), ammonium sulphate (Bio-Rad Labs, Hercules, CA, USA), urea (SigmaUltra) and GdmSCN (Sigma, St. Louis, MO, USA). The buffer solutions contained 0.1 *M* phosphate, pH 7.2 (PBS). Water deionized with a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout. A TSK-

Gel Ether-5PW column (Tosoh Biosep, Stuttgart, Germany) of 7.5 cm×7.5 mm I.D. was used for all the experiments.

2.2. Instrumentation

A Twincle Jasco HPLC System One, equipped with a Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA) and a 100-μl injection loop was connected to a variable-wavelength monitor (Jasco UVIDEC 100-III). The UV detector was operated at 280 nm. The output from the detector was displayed on a 2221 Chromatopac C-R3A DANI integrator (Shimadzu).

Absorbance measurements were performed using a Varian DMS 300 spectrophotometer.

2.3. Chromatographic conditions

In all experiments 8.0 *M* urea was kept constant in the mobile phase in order to prevent casein aggregation. The elution conditions were as follows: 15-min linear salt gradient from 100% high salt buffer (PBS, 1.45 *M* ammonium sulphate, 8.0 *M* urea) to 100% lower salt buffer (PBS, 0.54 *M* ammonium sulphate, 8.0 *M* urea) at 20±1 °C. A flow-rate of 0.8 ml/min was used. 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 or retention time reproducibility probably because 8 *M* urea in the eluents elutes lipids in the dead volume of the column, keeping the column clean.

2.4. Standard solutions

All stock solutions of caseins from Fluka and lyophilized real sample powders (follow-up milks, casein free milks, milky mush powder) 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. A 100-μl aliquot of processed cow's milks, yoghurt and cream were diluted in 1.0 ml of PBS and 4.0 *M* GdmSCN. Suitable amounts of cheeses were weighed, in order to obtain an approximate protein concentration of 7 mg/ml, and dissolved in PBS and 4.0 *M* GdmSCN. Cheese and cream samples were centrifuged at 4500 *g* for 10 min (IEC Centra-4B centrifuge, International Equipment, MA, USA) prior to HIC analysis.

Samples injected (injection volume: 100 μl) were prepared by diluting a suitable volume of stock solutions in the mobile phase (PBS, 1.45 *M* ammonium sulphate, 8.0 *M* urea). Concentration of stock solutions of caseins from Fluka was determined by spectrophotometry [9,10].

2.5. Stability

All stock solutions of commercial caseins and real samples dissolved in GdmSCN were stable in a refrigerator (2–8 °C) for 5 months. All the solutions injected were diluted from the stock solutions prepared for the analysis on TSK-Gel Phenyl-5PW column [8]. Injection of sample solutions diluted from fresh stock solutions gave results not significantly different from those obtained from stock solutions aged 5 months or less.

2.6. 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. For this purpose, the same column (TSK-Gel Ether-5PW), the same elution conditions and a 500-μl injection loop were used in semi-preparative experiments by collecting the major peaks. Unlike the phenyl column, the ether column allowed the collection of α_{s1}- and α_{s2}-casein peaks. Concentration of α- and β-caseins in the collected fractions was determined spectrophotometrically, as previously reported [7]. Concentration of stock solutions of α_{s2}-casein were determined spectrophotometrically, taking the extinction values at 280 nm $E^{1\%}_{1\ cm}=10.7$ [9]. The κ-casein sample was used without further purification.

2.7. Purification of whey proteins from processed cow's milk

Whey proteins were obtained from 10 ml of UHT 1.5% fat cow's milk by precipitation at pH 4.6 with 1 *M* HCl, followed by centrifugation at 4500 *g* for

20 min. Soluble fraction was diluted 1:10 in 1.45 *M* ammonium sulphate, 0.1 *M* PBS and injected.

2.8. Certified 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 [8,11]. The certified value for *n* total is 62.3±0.8 mg/g. Eight samples 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 and discussion

3.1. HIC behavior of commercial caseins in the TSK-Gel Ether-5PW column

The α-, β- and κ-caseins solubilized in 4.0 *M* GdmSCN and diluted by the gradient starting buffer were injected into a TSK-Gel Ether-5PW HIC column and eluted keeping a 8.0 *M* concentration of urea constant in the mobile phase. Fig. 1 shows the HIC chromatograms of commercial α-, β- and κ-casein in parts A, B and C, respectively. Using the adopted separation conditions, α-, β- and κ-casein showed different retention times which allow their separation (two peaks for α-casein at 18.4 and 22.5 min, and one peak for β-casein at 19.2 min and for κ-casein at 16.7 min). It is interesting to observe that in this column commercial α-casein is separated in two peaks attributed to α_{s1}- (22.5 min) and α_{s2}-casein (18.4 min) fractions. 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) [7]. Assignment has been made on the basis of α_{s1}-/α_{s2}-casein ratio, α_{s2}-casein representing ~10–12% of the total and on the basis of α_{s1}- and α_{s2}-hydrophobicity [6]. As explained in the Experimental section, the α- and β-casein standards used later in calibration studies were isolated from these commercial samples. Recovery of commercial α-, β- and κ-caseins obtained by comparison of peak areas, after re-injection in the ether column of collected fractions from the same column, was 95.3, 98.6 and 103%, respectively.

Whey proteins do not interfere with casein separation. This has been verified by injecting both a 1:10 dilution of whey proteins prepared as described in the Experimental section and standard solution of α-lactalbumin and β-lactoglobulin, which are the major whey proteins in milk. Experiments showed that whey proteins are eluted in our operating conditions with the dead volume of the TSK-Gel Ether-5PW HIC column. Thus, with this separation method, no preliminary separation or precipitation procedure of the casein fraction is required.

The method has been straightforwardly validated by analysis of the available certificate reference material and successfully applied to the separation and determination of caseins in raw samples.

3.2. BCR-063R analysis

Fig. 2 shows the HIC profile of BCR-063R solubilized in 4.0 *M* GdmSCN, diluted by the gradient starting buffer and injected into a TSK-Gel Ether-5PW HIC column using the adopted separation conditions. The chromatogram shows peaks of κ- (16.7 min), α_{s2}- (18.4 min), β- (19.2 min), and α_{s1}-casein (22.5 min).

Quantitative analysis of α_{s1}-, α_{s2}-, β- and κ-caseins in BCR-063R has been performed using calibration curves of α_{s1}-, α_{s2}-, β- and κ-caseins. The marks on the elution profiles are integration markers indicating the vertical drop from the valley to the interpolated baseline (from 15 to 24.5 min). Table 1 shows the results of linear fitting of calibration curves of α_{s1}-, α_{s2}-, 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 and results of linear fitting of data obtained by standard addition of α_{s1}-, α_{s2}-, β- and κ-caseins to BCR-063R (sample No. 1) have been reported. Comparison of slopes of linear fitting of calibration data and of linear fitting of data obtained by standard addition of α_{s1}-, α_{s2}-, β- and κ-caseins to BCR-063R gives a good estimation of mean recovery of α_{s1}-, α_{s2}-, β- and κ-caseins added singly. Data show that for α_{s1}-, α_{s2}-, β- and κ-caseins, the slopes in the real matrix are 76.1, 85.0,

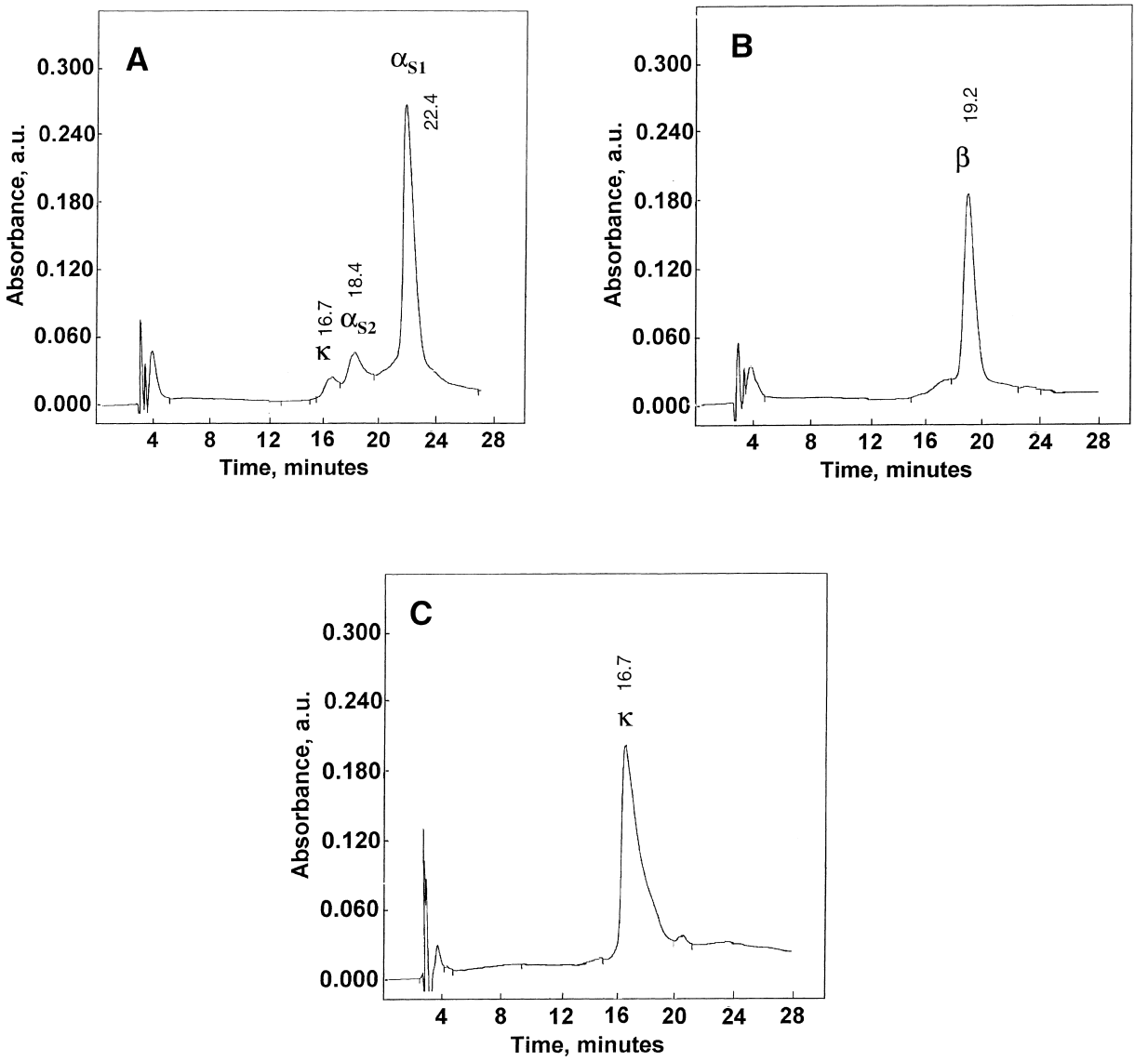

Fig. 1. HIC chromatograms of denatured commercial caseins. (A) α-Casein (42.6 μ*M*) with retention times of 22.4 min (α_{s1}) and 18.4 min (α_{s2}); (B) β-casein (41.7 μ*M*) with a retention time of 19.2 min; (C) κ-casein (52.6 μ*M*) with a retention time of 16.7 min. For chromatographic conditions, see Experimental section. a.u., absorbance units.

87.9 and 65.5%, respectively, of the slope in calibration experiments, showing that in this type of column the matrix effect is more significant than in the phenyl column. Table 2 summarises the quantitative results obtained on eight different stock solutions of the same BCR-063R lot. In the same table quantitative results on BCR-063R sample No. 1 obtained by applying standard addition method are also reported.

The total casein percentage found has been calculated on the basis of the protein concentration injected, and estimated by total certified nitrogen injected, as previously described [8], on the basis of the equation [12]:

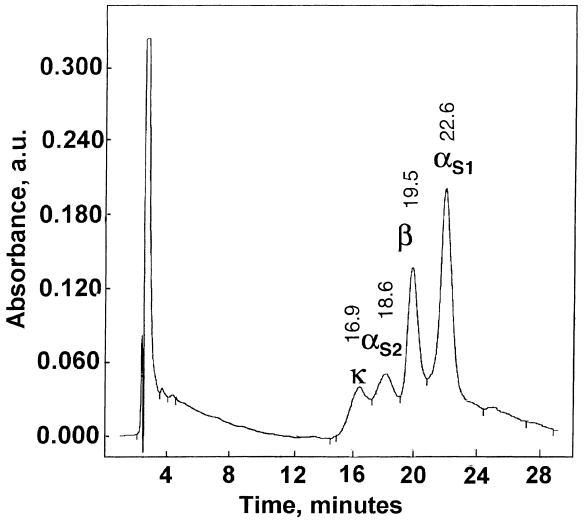

Fig. 2. HIC chromatogram of certified skim milk powder (BCR-063R) denatured in 4.0 *M* GdmSCN. α_{s1}: α_{s1}-Casein ($t_{\alpha s1}=22.6$ min); α_{s2}: α_{s2}-casein ($t_{\alpha s2}=18.6$ min); β: β-casein ($t_{\beta}=19.5$); κ: κ-casein ($t_{\kappa}=16.9$). For chromatographic conditions, see Experimental section.

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

We found that total caseins represent 78.3±6.1% (average value) of the estimated protein content, with α_{s1}-, α_{s2}-, β- and κ-caseins representing 34.4±1.4, 9.3±0.9, 49.0±0.8 and 7.7±1.7%, respectively. In the range of linearity the casein, recovery appears to be independent of the protein concentration of BCR-063R injected.

3.3. Raw sample analysis

The proposed method has been extensively applied to the real, raw samples (dairy products, processed cow's milks, follow-up milk powder, special baby food and No. 22078 casein mixture from Fluka), the same samples analyzed previously by a TSK-Gel Phenyl-5PW column [8]. These experiments had the two aims of: (i) testing the goodness and reliability of the ether column for the analysis of real samples; and (ii) comparing the quantitative results obtained by the ether and phenyl columns. Table 3 shows the results of the quantitative determination of α_{s1}-, α_{s2}-, β- and κ-caseins in the considered samples. While for total, α- and β-casein, correlation of quantitative data obtained by analysing the same sample in the two columns is good (for total caseins the slope of the linear regression is 1.04±0.02 with $R=0.829$, n (number of points considered in the correlation)=28; for total α-casein the slope is 1.01±0.02 with $R=0.707$, $n=28$; for β-casein the slope is 0.958±0.01

Table 1
Comparison of results of linear fitting of calibration data of α_{s1}-, α_{s2}-, β- and κ-caseins with results of linear fitting of standard addition data of α_{s1}-, α_{s2}-, β- and κ-caseins to BCR-063R (sample No. 1, 69.5 μg/ml injected)

	α_{s1}-Casein ($t_R=22.5$ min)	α_{s2}-Casein ($t_R=18.4$ min)	β-Casein ($t_R=19.2$ min)	κ-Casein ($t_R=16.7$ min)
Calibration data				
Injected concentration range (μ*M*)	0.5–40	0.5–10	0.5–40	0.5–40
R	0.9992	0.9889	0.9883	0.9880
Number of points	7	6	6	4
Slope (μM^{-1})	9171±98	10102±413	5135±172	9795±616
RSD (%)[a]	2.3	5.5	3.2	5.0
LOD (μ*M*)[b]	0.33	0.40	0.65	0.40
Standard addition data				
Injected concentration range (μ*M*)	0.0–25.0	0.0–5.0	0.0–25.0	0.0–15.0
R	0.9995	0.9995	0.9995	0.9993
Number of points	4	4	4	3
Slope (μM^{-1})	6982±154	8577±186	4512±102	6414±246
RSD (%)[c]	5.2	6.2	4.4	6.1

[a] Average value of five replicate determinations for a standard solution whose injected protein concentration was 10 μ*M*.
[b] LOD=3σ/slope, where σ has been estimated on the basis of baseline noise.
[c] Average value of three replicate determinations for standard solution whose added protein concentration was 5.0 μ*M*.

Table 2
Results of quantitative determination of α_{s1}-, α_{s2}-, β- and κ-caseins (CN) in eight samples of certified skim milk powder (BCR-063R)

Sample No.	N_{total} conc. injected[a] (μg ml^{-1})	Protein conc. injected[b] (μg ml^{-1})	α_{s1}-CN (μg ml^{-1})		α_{s2}-CN (μg ml^{-1})		β-CN (μg ml^{-1})		κ-CN (μg ml^{-1})		Total caseins (μg ml^{-1})		Total casein found (%)	
			Mean	SD[c]	Mean	SD[c]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	69.5	429.9	121.9	10.1	35.3	5.6	168.5	5.1	21.2	2.2	347.0	12.8	80.7	3.0
2	190.9	1181.7	296.6	16.5	69.4	3.0	428.7	12.8	72.9	4.1	867.6	21.5	73.4	1.8
3	205.0	1268.5	353.9	19.2	80.5	3.2	481.9	16.8	78.9	5.2	995.2	26.2	78.5	2.1
4	126.6	783.5	210.0	20.3	58.4	4.8	307.7	21.4	41.5	6.0	617.6	30.5	78.8	3.9
5	63.4	392.6	114.6	8.7	27.8	11.1	153.1	5.3	15.1	2.0	310.5	15.2	79.1	3.9
6	106.5	659.3	155.5	9.5	44.3	2.1	227.9	14.0	41.9	2.5	469.6	17.2	71.2	2.6
7	132.1	817.4	194.4	14.5	59.0	5.0	287.0	6.5	59.6	2.7	600.0	16.9	73.4	2.1
8	171.3	1060.3	329.5	32.8	97.5	3.1	481.7	6.3	55.4	1.5	964.1	33.6	90.9	3.2
1[d]	69.5	429.9	159.6	8.4	29.3	1.3	151.6	8.8	63.1	7.0	403.7	14.1	93.9	3.3

Mean±SD of means is 78.3±6.1%. Standard error calculated on the basis of linear fitting results.
[a] Calculated on the basis of *n* total certified value.
[b] Estimated on the basis of the equation: [Protein total] = $N_{total} \cdot 6.38 \cdot 0.97$ [12].
[c] $n = 3$.
[d] Data obtained on sample No. 1 by standard addition method.

with $R = 0.668$, $n = 28$), correlation for κ-casein quantitative data is poor (slope = 0.540±0.14 with $R = 0.2730$, $n = 28$). This is probably due to an overlap of κ-casein peak in the phenyl column at $t_R = 42.6$ min with the large peak of α-casein at $t_R = 43.9$ min. In the phenyl column overlapping of peaks makes the κ-casein undetectable in real samples whenever the ratio $\alpha/\kappa \geq 5$. The better separation of κ-casein in the ether column also makes the κ-casein peak detectable and quantifiable in real samples at low concentration levels.

Fig. 3 shows chromatograms of (a) Robiola, (b) ewe's milk ricotta (cottage), (c) cow's milk mozzarella and (d) buffalo's milk mozzarella cheeses, solubilized in 4.0 *M* GdmSCN, centrifuged, diluted in the gradient starting buffer and injected. No complex sample preparation, such as precipitation or separation of casein fraction, is required prior to injection of the sample. In Fig. 3, HIC profiles and the proportions of α_{s1}-, α_{s2}-, β- and κ-casein are different depending on the cheese analyzed, suggesting successful employment of the proposed procedure for detecting fraudulent addition of cow's milk to goats' or ewes' milk in dairy products.

It is interesting to discuss the elution pattern of caseins of buffalo's milk mozzarella cheese. The analysis of the sample by the TSK-Gel Ether column revealed only three peaks at retention times of 18.2, 19.9 and 22.4 min, assigned to α_{s2}-, β- and α_{s1}-casein, respectively, by the addition of standard solutions of the three caseins. Addition of κ-casein standard solution gave a typical peak at 16.5 min. These results can explain the chromatographic data obtained previously by the TSK-Gel Phenyl column with caseins of mozzarella from buffalo's milk [8]. For that sample the assignment in the elution pattern of the peak at 42.0 min to κ-casein was uncertain. On the basis of current data, that peak has to be assigned to α_{s2}-casein. It can be suggested that α_{s2}-casein peak in the phenyl column is not generally separated from the β-casein peak, giving a consequent overestimation of β-casein and underestimation of total α-casein content. The β-casein/α_{s2}-casein ratio is generally ≥3.5 [3], ranging in the real samples examined between 3.4 and 6.8. mozzarella cheese from buffalo's milk has a higher content of α_{s2}-caseins, the β-casein/α_{s2}-casein ratio being 2.6. For this reason, α_{s2}-casein peak is probably quantifiable also in the phenyl column.

3.4. Specificity

There were no interfering peaks at the retention times where the α_{s1}-, α_{s2}-, β- and κ-caseins were detected. As reported above, in the the TSK-Gel Ether column, in the selected elution conditions, whey proteins are eluted in the dead volume of the

Table 3
Results of quantitative determination of α_{s1}-, α_{s2}-, β- and κ-caseins (CN) in commercial raw samples[a]

Sample	Protein conc. injected (labelled) (μg ml^{-1})	α_{s1}-CN (μg ml^{-1})		α_{s2}-CN (μg ml^{-1})		β-CN (μg ml^{-1})		κ-CN (μg ml^{-1})		Total caseins found (μg ml^{-1})		Total casein found (%)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Processed cow's milk													
Pasteurized 3.5% fat cow's milk (A)	286.4	96.9	3.5	23.8	2.0	112.9	2.0	12.1	1.2	245.7	4.7	85.8	1.6
Pasteurized 3.5% fat cow's milk (D)	281.8	80.2	4.2	28.2	8.0	115.5	6.5	16.5	3.4	240.3	11.6	85.3	4.1
Pasteurized HQ 3.5% fat cow's milk (D)	291.0	109.0	10.4	23.2	3.7	113.5	2.4	14.8	1.9	260.6	11.5	89.6	3.9
Pasteurized 1.5% fat cow's milk (A)	294.6	87.5	5.2	31.9	4.1	132.2	0.6	16.3	5.7	267.8	8.8	90.9	3.0
UHT 3.5% fat cow's milk (A)	286.4	127.7	8.6	19.7	2.0	97.2	2.1	2.0	0.5	246.6	9.1	86.1	3.2
UHT 1.5% fat cow's milk (B)	309.2	116.7	11.9	29.4	10.5	113.7	10.8	2.0	2.8	261.7	19.4	84.6	6.3
UHT 3.5% fat cow's milk (C)	291.0	91.0	9.1	29.1	7.9	116.1	4.2	13.1	2.2	249.2	12.9	85.7	4.5
UHT 1.5% fat cow's milk (C)	150.0	53.1	0.1	8.6	3.2	56.3	4.2	1.0	0.1	118.9	5.3	79.3	3.5
UHT 0.05%fat cow's milk (E)	291.0	97.4	5.2	34.2	3.2	112.1	3.5	5.5	1.0	249.1	7.1	85.6	2.4
UHT 3.5% fat cow's milk (F)	281.8	91.0	9.1	29.5	3.6	118.6	1.1	12.1	3.9	251.1	10.6	89.1	3.8
UHT 1.5% fat cow's milk (F)	281.8	90.4	1.1	32.8	0.5	122.7	0.8	8.1	3.1	254.0	3.4	90.1	1.2
UHT 1.5% fat cow's milk (G)	281.8	128.1	7.5	33.2	2.5	106.1	3.2	0.0	0.0	267.4	8.5	94.9	3.0
Follow-up milk powder and special baby food													
Follow-up milk powder (A)	320.0 (80% caseins)	125.8	0.2	22.8	4.0	125.1	11.0	17.9	2.3	291.6	11.9	91.1	3.7
Follow-up milk powder (B)	294.0 (79% caseins)	81.6	5.6	15.3	1.1	93.6	2.3	12.4	1.5	202.8	6.3	69.0	2.2
Dietary powder food for diarrhea (A)	353.9 (94% caseins)	105.2	11.0	37.3	8.1	171.6	7.3	19.6	0.8	333.6	15.5	94.3	4.4
Dietary powder food for diarrhea (B)	361.5	98.7	2.3	0.0	0.0	96.1	1.5	0.0	0.0	194.8	2.7	53.9	0.8
Dietary powder food for gastric-esophageal illness	365.1 (79.5% caseins)	102.5	5.6	39.7	4.0	149.1	5.6	16.8	1.5	308.0	9.0	84.4	2.5
Casein free milk (A)	705.6	–	–	–	–	–	–	–	–	–	–	–	–
Casein free milk (B)	860.4	–	–	–	–	–	–	–	–	–	–	–	–
Casein free milk (C)	748.5	–	–	–	–	–	–	–	–	–	–	–	–
Milky mush powder	801.9	293.9	20.1	75.8	6.2	334.4	15.2	47.1	5.0	751.2	26.4	93.7	3.3
Dairy products													
Condensed milk	364.9	139.6	9.6	16.2	1.5	109.2	2.4	7.6	1.0	272.5	10.1	74.7	2.8
Cream	798.5	143.5	8.9	49.6	3.5	211.6	5.2	12.2	1.2	416.9	11.0	52.2	1.4
Stracchino cheese 21% fat	873.8	234.1	41.1	114.5	23.6	446.9	26.0	88.3	34.9	883.7	64.3	101.1	7.4
Philadelphia cheese 30% fat	721.9	206.8	38.8	79.3	5.4	278.3	2.1	22.2	1.8	586.4	39.3	81.2	5.4
Robiola cheese 21.5% fat	284.0	97.7	8.4	11.0	0.6	97.6	8.8	8.9	0.5	215.2	12.2	75.8	4.3
Ewe's milk ricotta cheese 13% fat	787.6	204.7	22.0	39.4	3.4	218.7	34.4	7.1	2.3	469.8	41.0	59.7	5.2
Cow's milk mozzarella cheese 15% fat	620.0	149.4	10.8	30.9	2.4	213.2	12.5	0.0	0.0	393.5	16.7	63.5	2.7
Buffalo's milk mozzarella cheese 15% fat	483.0	193.2	20.7	51.0	5.4	137.1	6.1	18.5	7.4	399.8	23.4	82.8	4.9
Yogurt	336.4	111.6	5.4	24.3	2.0	163.8	2.6	20.8	1.6	320.5	6.5	95.3	1.9
Commercial casein mixture													
Casein mixture (product No. 22078, Fluka)	275.0	97.9	6.5	23.1	0.1	110.0	2.9	15.3	2.7	246.2	7.6	89.5	2.8

[a] $n = 3$.

column. The analysis by this method of casein-free samples, which contain hydrolyzed proteins, did not show any peaks at the elution time examined.

3.5. Accuracy

Quantitative data obtained on BCR-063R

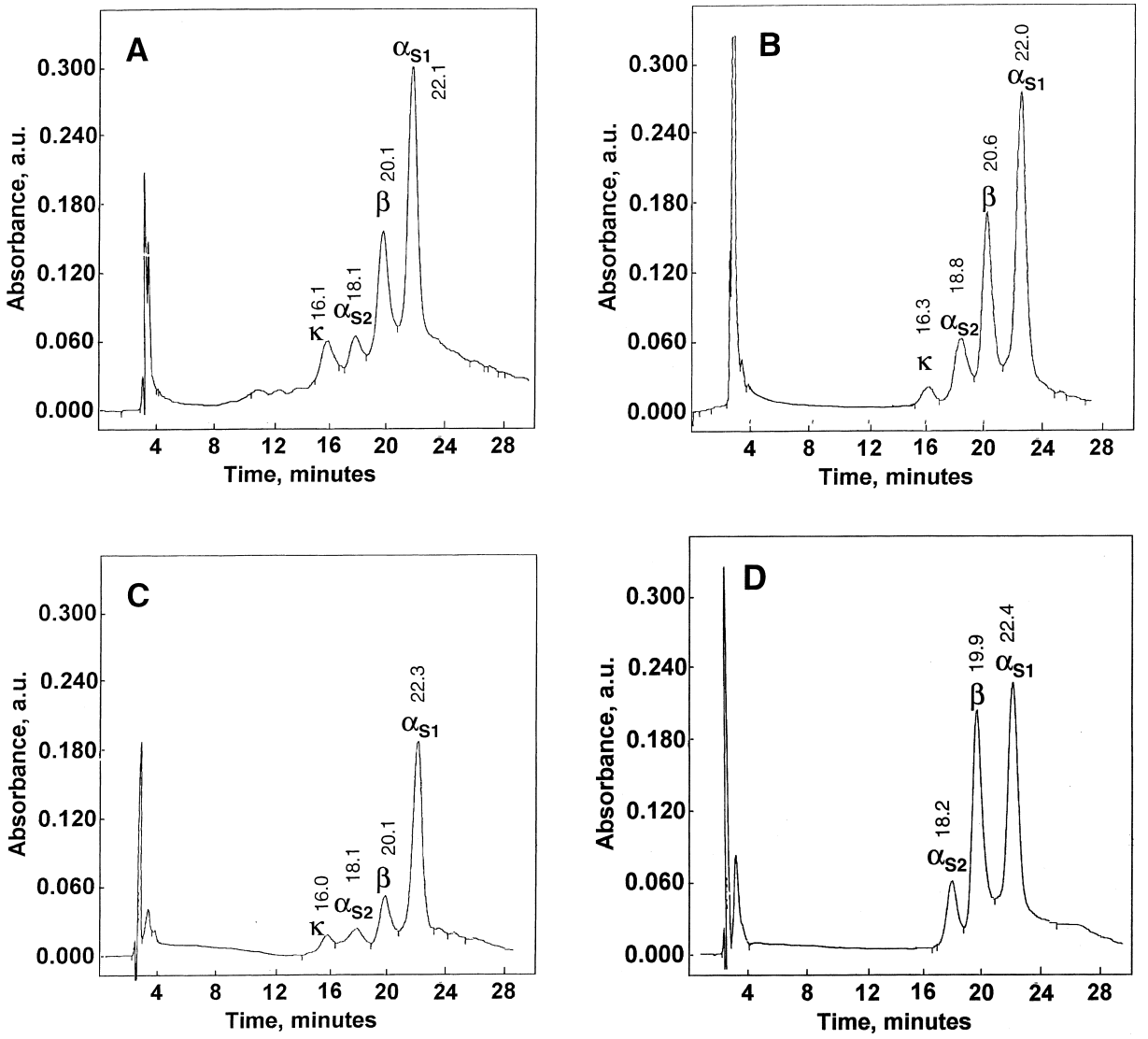

Fig. 3. HIC chromatograms of cheese samples dissolved in 4.0 *M* GdmSCN and centrifuged. (a) Robiola cheese (retention times: $t_{\alpha s1}=22.1$; $t_{\alpha s2}=18.1$; $t_{\beta}=20.1$; $t_{\kappa}=16.2$ min); (b) ewe's milk ricotta cheese (retention times: $t_{\alpha s1}=22.0$; $t_{\alpha s2}=18.8$; $t_{\beta}=20.6$; $t_{\kappa}=16.3$ min); (c) cow's milk mozzarella cheese (retention times: $t_{\alpha s1}=22.3$; $t_{\alpha s2}=18.1$; $t_{\beta}=20.1$; $t_{\kappa}=16.0$ min); (d) buffalo's milk mozzarella cheese (retention times: $t_{\alpha s1}=22.4$; $t_{\alpha s2}=18.2$; $t_{\beta}=19.9$ min). For chromatographic conditions, see Experimental section.

(78.3±6.1% total casein found) are in agreement with the expected range of casein content in skim milk (75–85%) [12], and with the data obtained previously by the TSK-Gel Phenyl column (79.1±2.7% total casein found). In the case of real samples we found that casein recovery ranges between 98.6 and 113.0% with respect to the value indicated on the label.

As reported above, the mean recovery of α_{s1}-, α_{s2}-, β- and κ-casein standard solutions added singly to BCR-063R sample was estimated by comparing the slopes of linear fitting of calibration data and of

standard addition data. Data show that for α_{s1}-, α_{s2}-, β- and κ-caseins the slope in real matrix is 76.1, 85.0, 87.9 and 65.5%, respectively, of the slope of calibration experiments, showing a ''matrix effect'' more significant than in the phenyl column.

3.6. Conclusions

HIC coupled with employment of strong denaturants (GdmSCN and urea) resulted in good separation and quantitation of α_{s1}-, α_{s2}-, β- and κ-caseins in commercial, raw samples. Chromatographic behavior of caseins injected in the TSK-Gel Ether-5PW column (a HIC phase less hydrophobic than the phenyl one) was substantially different from that observed in the TSK-Gel Phenyl-5PW column, allowing good separation of α_{s1}-, α_{s2}-, β- and κ-caseins in less than 22 min. Under the operating conditions of the ether column, whey proteins were eluted in the dead volume. In addition, the low back pressure together with the presence of high concentration of denaturants in the mobile phase, allowed the employment in this column of flow-rate $\geq$0.8 ml/min, significantly reducing the run and equilibrating times.

The method has been validated by the analysis of reference skim milk powder (BCR-063R) certified for total nitrogen content, obtaining a value for total casein of 78.3±6.1% of total ''true'' proteins injected in agreement with the expected range of casein content in skim milk (75–85%) and with the value previously found by analysis in the phenyl column [8,12]. The method has been widely applied for the quantitative determination of α_{s1}-, α_{s2}-, β- and κ-caseins in 31 real, raw samples. The method, faster than that previously proposed [8], versatile and specific, does not require any preliminary precipitation or separation of casein fraction, thus minimizing sample handling. Because of its characteristics, the proposed method can be suitably applied in many fields of dairy industry.

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