Profiling of caseins in cows', goats' and ewes' milk and dairy products by reversed-phase high-performance liquid chromatography

LENKA RUPRICHOVÁ – GORAZD TOMPA – MICHAELA KRÁLOVÁ – IVANA BORKOVCOVÁ – IVETA BEDÁŇOVÁ – LENKA VORLOVÁ

Summary

The aim of this study was to evaluate the composition of caseins in milk and dairy products employing a fast and effective method of reversed-phase high-performance liquid chromatography (RP-HPLC). An additional task was to separate and identify α_{S1}- and α_{S2}-caseins by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and thus confirm their presence in the analysed samples. A total of 120 milk samples (cows', goats' and ewes') and 46 samples of dairy products (cheese, yoghurt) obtained from farms and milk bars in Czech Republic were analysed by RP-HPLC. All main casein components (α_{S1}-, α_{S2}-, β- and κ-casein) were identified and quantified. Statistically significant differences between individual caseins in various samples of milk and dairy products were detected. RP-HPLC profiling of samples showed that the presence of individual caseins depends primarily on the animal source of milk but also on the technological treatment of dairy products. The quantity of α_{S1}-casein, which has the most pronounced allergenic potential, varied significantly between different samples. The current study demonstrated good performance of the novel approach for RP-HPLC analysis of α_{S1}- and α_{S2}-caseins utilizing in-house prepared analytical standards as well as provided valuable data regarding the casein profile of milk and dairy products on the market.

Keywords

milk; cheese; yoghurt; casein; chromatography

Casein constitutes over 80% of the total protein in milk. The main casein components involve α_{S1}-casein, α_{S2}-casein, β-casein and κ-casein [1]. Identification 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]. Composition of milk is influenced by the animal, its breed, nutrition, stage of lactation as well as condition of the animal's health [1, 3–5]. Ewes' and goats' milk differ from cows' milk not only in the distribution of individual caseins, but also in their allergenic potential, with α_{S1}-casein being considered as one with the most pronounced allergenic potential [6, 7]. Prevention against milk allergies is primarily based on elimination of all food products containing caseins with allergenic potential from one's diet [6, 8, 9]. Therefore, it is important to establish the composition of individual caseins not only in milk but also in dairy products.

This study provides comprehensive data on the presence of individual casein components in milk and dairy products obtained by employing a fast, effective and accessible method of reverse-phase high-performance liquid chromatography (RP-HPLC). Applicability of liquid chromatography/tandem mass spectrometry (LC-MS/MS) technique to separate and identify α_{S1}- and α_{S2}-caseins was also elucidated.

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MATERIALS AND METHODS

Analytical standards

In order to evaluate individual caseins in samples, analytical standards of bovine α_S-casein (≥ 70% purity), β-casein (≥ 98% purity) and κ-casein (≥ 70% purity) from Sigma Aldrich (St. Louis, Missouri, USA) were used.

Samples

For each sample, 2 parallel quantifications were performed. The samples were stored at –18 °C until analysed.

Milk samples

A total of 120 samples of cows', goats' and ewes' milk were collected from two farms and milk bars in the Czech Republic. Cows' milk samples (n = 40) from Holstein and Czech Fleckvieh breed (including crossbreed) were collected from milk bars from April to June 2010. Goats' milk (n = 40) from White Shorthaired goats was collected from one goat farm in the Southern Region of Czech Republic from May to June 2010. Samples of ewes' milk (n = 40) were collected from one sheep farm in the Zlín Region from May to June 2010. The sheep breeds were mainly Lacaune (87.5%) with minor proportion of Improved Wallachian and East Friesian sheep.

Cheese samples

Cheese samples were obtained from the market and from farms in the Czech Republic. Selection of samples was based on the animal source of milk from which the cheese had been produced, i.e. from cows' (n = 20), goats' (n = 9) and ewes' milk (n = 7).

Yoghurt samples

Yoghurt samples (n = 10) from cows' milk were obtained from the market in the Czech Republic.

Preparation of analytical standards

An amount of 10 mg of each bovine casein analytical standard (Sigma Aldrich) was weighed into a 10 ml volumetric flask, approx. 7 ml of 0.25 mol·l^{-1} of Tris-HCl (pH 6.8) was added, followed by the addition of 0.1 ml of 2-mercaptoethanol and filled up to the volume of 10 ml with Tris-HCl (pH 6.8) to obtain 1 mg·ml^{-1} solution.

Preparation of Tris-HCl (pH 6.8) solution

An amount of 3 g of Tris (hydroxymethyl)-aminomethane (Tris; Bio-Rad Laboratories, Richmond, California, USA) was dissolved in 50 ml of deionized water (dH_2O), titrated with concentrated HCl (Penta, Praha, Czech Republic) to pH 6.8 and dH_2O was supplemented to the volume of 100 ml in a volumetric flask to obtain 0.25 mol·l^{-1} solution.

Sample preparation

Preparation of milk samples

Isolation and lyophilization of caseins were done according to López-Fandiño et al. [10]. Milk samples were centrifuged (at 3000 ×g for 15 min), fat was removed from the surface and the defatted milk was adjusted to pH 4.6 using a 10% solution of acetic acid (Penta), which resulted in casein precipitation. After this step, milk was centrifuged again and the supernatant was separated from the casein. The caseins were rinsed with a dichloromethane-water mixture (1:1) and lyophilized according to López-Fandiño et al. [10] using Lyovac GT 2 lyophilizer (Amsco/Finn-Aqua, Hürth, Finland). Before HPLC determination, the lyophilized casein (0.04 g) was dissolved in approx. 7 ml of 0.25 mol·l^{-1} Tris-HCl (pH 6.8) and 0.1 ml of 2-mercaptoethanol, and subsequently filled up to the volume of 10 ml in a volumetric flask with Tris-HCl (pH 6.8) to obtain 4 mg·ml^{-1} solution. Finally, casein samples were filtered through a nylon membrane filter (pore size, 0.22 μm) into HPLC vials.

Preparation of cheese samples

The methodology for preparation of cheese samples was based on the procedure of Rodríguez et al. [11] with some modifications. The protein fractions were obtained from cheese samples (5 g) by extraction with water (15 ml) and sonication (Bandelin, Bernau near Berlin, Germany) for 10–15 min. The mixture was precipitated by addition of 10% acetic acid until pH = 4.6 and centrifuged at 4000 ×g for 10 min. Caseins were rinsed with a dichloromethane-water mixture (1:1) and lyophilized according to López-Fandiño et al. [10] using freeze-dryer Alpha 1–4 LSC (Martin Christ, Osterode am Harz, Germany). Before HPLC determination, the cheese casein samples (4 mg·ml^{-1}) were prepared in the same way as described for the preparation of milk samples.

Preparation of yoghurt samples

An amount of 5 g of yoghurt was placed into a tube and centrifuged (at 4000 ×g for 10 min), after which the supernatant was separated from the sedimented casein. Casein was rinsed by an aqueous mixture of dichloromethane (1:1) and lyophilized according to López-Fandiño et al. [10], while the subsequent part of the procedure

was the same as that for the preparation of cheese samples. Finally, pH value of the yoghurt was measured.

Fractionation of α_S-casein analytical standard

An amount of 10 mg of bovine α_S-casein analytical standard (Sigma Aldrich, product number C6780) was dissolved in 1 ml of buffer solution prepared according to FELIGINI et al. [12]. The solution of α_S-casein analytical standard was separated using liquid chromatograph Prominence 20-AP (Shimadzu, Kyoto, Japan) equipped with Prominence SPD–M20A detector (Shimadzu), fraction collector LC-10A (Shimadzu) and Zorbax 300 SB C18 column (Agilent Technologies, Santa Clara, California, USA) to obtain three α_S-casein fractions, presumably containing α_{S1}- and α_{S2}-caseins, which were further subjected to LC-MS/MS analysis for protein identification. Mobile phase A contained water-acetonitrile (Merck, Darmstadt, Germany)-trifluoroacetic acid (TFA; Sigma Aldrich) in a ratio of 95 : 5 : 0.1 (v/v/v), and mobile phase B contained water-acetonitrile-TFA in a ratio of 5 : 95 : 0.1 (v/v/v). Mobile phase flow rate was set at 1 ml·min^{-1} and gradient elution was used for separation. The gradient was generated 1 min after injection of 5 μl of the sample by increasing the proportion of mobile phase B linearly from 20% to 40% in 9 min, from 40% to 50% in 15 min and from 50% to 60% in 2 min, followed by an isocratic elution at 60% for 3 min and final decrease of the mobile phase B to 20% in 3 min. Total time of the chromatographic analysis was 40 min. Detection of caseins was monitored at 205 nm and the column temperature was set at 45 °C. Quantitative evaluation of chromatographic data was performed using Lab Solutions software, version 5.32 (Shimadzu).

Identification of α_{S1}- and α_{S2}-caseins in α_S-casein fractions by LC-MS/MS analysis

Five microlitres of α_S-casein fraction were diluted in 50 μl of denaturation buffer (6 mol·l^{-1} urea, 100 mmol·l^{-1} ammonium bicarbonate), reduced by addition of dithiothreitol (10 mmol·l^{-1} final concentration; incubation for 1 h at room temperature) and alkylated by addition of iodoacetamide (40 mmol·l^{-1} final concentration; incubation for 1 h at room temperature in the dark). Concentration of urea was lowered to 1 mol·l^{-1} by addition of water and 0.2 μg of sequence grade trypsin (Promega, Fitchburg, Wisconsin, USA) was added. After overnight incubation at 37 °C, trypsin was inactivated by addition of formic acid (0.5% final concentration) and resulting peptides were isolated on a C18 spin column (Sartorius, Göttingen, Germany). The LC-MS/MS analysis was performed using liquid chromatograph Agilent 1260 Infinity Nanoflow HPLC coupled with the XCT 6310 Ultra ion-trap mass spectrometer (Agilent Technologies). Peptides were separated on a Prot I. D. chip C18 column (Agilent Technologies) using 40 min acetonitrile gradient (5–46% organic phase) and a flow rate of 300 nl·min^{-1}. MS/MS spectra were obtained by collision-induced dissociation fragmentation of the nine most intense precursor ions from the full MS scan. Database search was performed using Mascot (version 1.3.0.339; Matrix Science, London, United Kingdom) set up to search NCBI non-redundant (nr) Protein Sequence Database (National Center for Biotechnology Information, National Institutes for Health, Bethesda, Maryland, USA) assuming the digestion enzyme was trypsin. The search was performed with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 0.001%.

Criteria for peptide and protein identification

Scaffold (version_3.6.2, Proteome Software, Portland, Oregon, USA) was used to validate MS/MS-based peptide and protein identification. Peptide identification was accepted if it could be established at a greater than 95.0% probability as specified by the Peptide Prophet algorithm [13]. Protein identification was accepted if it could be established at a greater than 99.9% probability and contained at least 2 identified peptides as specified by the Protein Prophet algorithm [14].

RP-HPLC analysis

Samples of milk and dairy products were analysed by RP-HPLC, analyses being repeated twice per sample. Separation of caseins was performed by liquid chromatograph Alliance 2695 with PDA 2996 detector (Waters, Milford, Massachusetts, USA) and XBridge TM C18 column, 150 mm × 3.0 mm, particle size 3.5 μm (Waters). Mobile phase A consisted of water-acetonitrile-TFA in a ratio of 95 : 5 : 0.1 (v/v/v), mobile phase B consisted of water-acetonitrile-TFA in a ratio of 5 : 95 : 0.1 (v/v/v). The gradient elution was applied 1 min after sample injection at a flow rate of 0.4 ml·min^{-1} by increasing the proportion of mobile phase B linearly from 20% to 60% in 19 min, and decreasing the proportion of mobile phase B linearly from 60% to 20% in 3 min. Injection volume was 5 μl, the column temperature was set to 45 °C and total analysis run time was 25 min. The detection was at 205 nm. Collection and evaluation of data were performed by Empower 2 software (Waters).

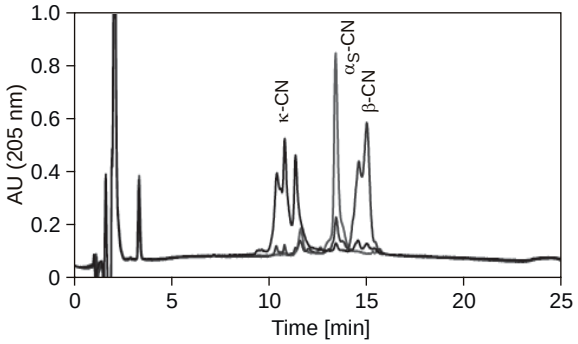

Fig. 1. RP-HPLC chromatogram of analytical standards of bovine caseins.

αS-CN – αS-casein, β-CN – β-casein, κ-CN – κ-casein.

Validation and optimization of RP-HPLC

Optimization of HPLC analysis was performed using standard solutions of αS-casein, β-casein and κ-casein analytical standards (Sigma Aldrich). Individual peaks were processed together as one peak (timed groups – sum of peaks quantitation). In the case of κ-casein, peaks were summarized in the time range 10.00–12.50 min, for αS-casein in the time range 13.20–14.40 min, and for β-casein in the time range 14.10–16.00 min. The repeatability of the procedure was determined from the results of multiple measurements per sample ($n = 6$) and was determined as relative standard deviation (*RSD*), which was 4.6% for αS-casein, 6.7% for β-casein and 0.7% for κ-casein. The limit of detection was 0.0045 mg·ml^{-1} and the limit of quantification was 0.015 mg·ml^{-1} for αS-casein, β-casein and κ-casein. Evaluation was performed using external standard and quantification was performed using timed groups. RP-HPLC chromatogram of analytical standards of bovine caseins is shown in Fig. 1.

Statistics

Basic statistical characteristics, namely, mean, standard deviation (*SD*) and standard error of the mean (*SEM*) were calculated using Microsoft Excel (Microsoft, Redmond, Washington, USA). The results were analysed using the statistical package Unistat 5.1. (Unistat, London, United Kingdom). For all variables tested, normality was checked by means of the Shapiro-Wilk test [15] and homogenity of variances among groups was tested by means of the Bartlett-Box test [15]. Data were subjected to a one-way ANOVA with the animal source of milk as the main effect with three levels (cows', goats', ewes') and, subsequently, to the Tukey-HSD test [15] for multiple comparisons in order to assess the statistical significance of differences between all possible pairs of groups. *p*-value of 0.05 was considered a limit for statistical significance. To assess correlations in the experiment, Pearson's correlation coefficients were calculated between the contents of individual caseins for each type of milk.

RESULTS AND DISCUSSION

Fractionation of αS-casein standard

Separation of αS-casein by RP-HPLC facilitated collection of individual fractions on the basis of retention time. Three fractions were prepared: Fraction 0 (assuming the presence of αS2-casein), Fraction 1 (first part of the peak assumed as αS1-casein) and Fraction 2 (the largest part of the peak assumed as αS1-casein). Chromatogram of the obtained fractions of αS-casein standard is shown in Fig. 2.

Identification of αS1- and αS2-caseins in αS-casein fractions by LC-MS/MS analysis

LC-MS/MS analysis revealed different distribution of individual types of casein in the collected fractions of αS-casein. As shown in Tab. 1, the dominant protein present in Fraction 0 was αS2-casein. Forty percent (89 out of 222 amino acids) coverage of the αS2-casein amino acid sequence was observed by LC-MS/MS analysis utilizing 16 out of 30 identified peptide spectra. The same dominant protein was observed also in Fraction 1 with 39% (86 out of 222 amino acids) coverage of the αS2-casein amino acid sequence utilizing 15 out of 31 identified peptide spectra. Five peptide spectra of αS1-casein were observed in Fraction 0, indicating its presence only in traces, however, αS1-casein was more abundant in Fraction 1 compared to Fraction 0, totalling 13 peptide spectra. The dominant protein in Fraction 2 was shown to be αS1-casein with 45% (87 out of 195 amino acids) coverage of the αS1-casein amino acid sequence utilizing 69 out of 87 identified pep-

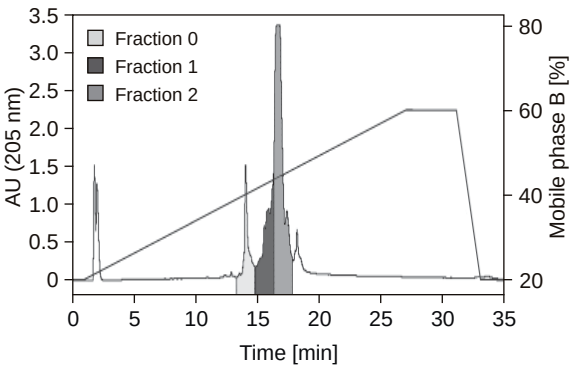

Fig. 2. RP-HPLC chromatogram of bovine αS-casein analytical standard.

Tab. 1. Results of LC-MS/MS analysis.

Identified protein	NCBI Accession No.	Molecular mass [kDa]	Number of identified MS/MS peptide spectra		
			Fraction 0	Fraction 1	Fraction 2
α_{S1}-CN [*Bos taurus*]	ABW98949	22	5	13	69*
α_{S2}-CN [*Bos taurus*]	NP_776953	26	16*	15*	18
κ-CN [*Bos taurus*]	ABN42697	18	9	3	0

* – Values indicate the most abundant type of casein present in the corresponding RP-HPLC fractions of α_S-casein according to the number of identified MS/MS peptide spectra.

tide spectra, whereas α_{S2}-casein was represented only with 18 peptide spectra. Additionally, a few peptide spectra of κ-casein were also observed in Fraction 0 and Fraction 1, but not in Fraction 2. All types of casein in the corresponding RP-HPLC fractions were identified with 100% probability. Representative MS/MS peptide spectra of identified caseins in RP-HPLC fractions are shown in Fig. 3.

Although the proportions of identified MS/MS peptide spectra do not correspond directly to the content of each type of casein in the fraction, they can be viewed as an indication that one type of casein is more abundant in one fraction than another (Tab. 1). For instance, in Fraction 2 proportionally more peptide spectra of α_{S1}-casein than peptide spectra of α_{S2}-casein were identified, and it was apparent that proportionally more peptide spectra of α_{S2}-casein than peptide spectra of α_{S1}-casein were represented in Fraction 0. In Fraction 1, both types of casein were approximately equally represented. Considering the fact that α_{S2}-casein was represented most abundantly in Fraction 0, this fraction was used indicatively as an in-house standard of bovine α_{S2}-casein when performing RP-HPLC analyses of caseins. Similarly, Fraction 2 was used indicatively as an in-house standard of bovine α_{S1}-casein, because analytical standards for bovine α_{S1}- and α_{S2}-caseins are currently not commercially available.

An interesting result obtained by MS/MS analysis was also the presence of κ-casein in the α_S-casein standard, which was more abundant in Fraction 0 than in Fraction 1, but not detected in Fraction 2. The commercially available analytical standard of bovine α_S-casein (product number C6780) from Sigma Aldrich contains about 70% of α_S-casein. According to our results, the rest are probably the remains of κ-casein. This observation is in accordance with the results of Feligini et al.

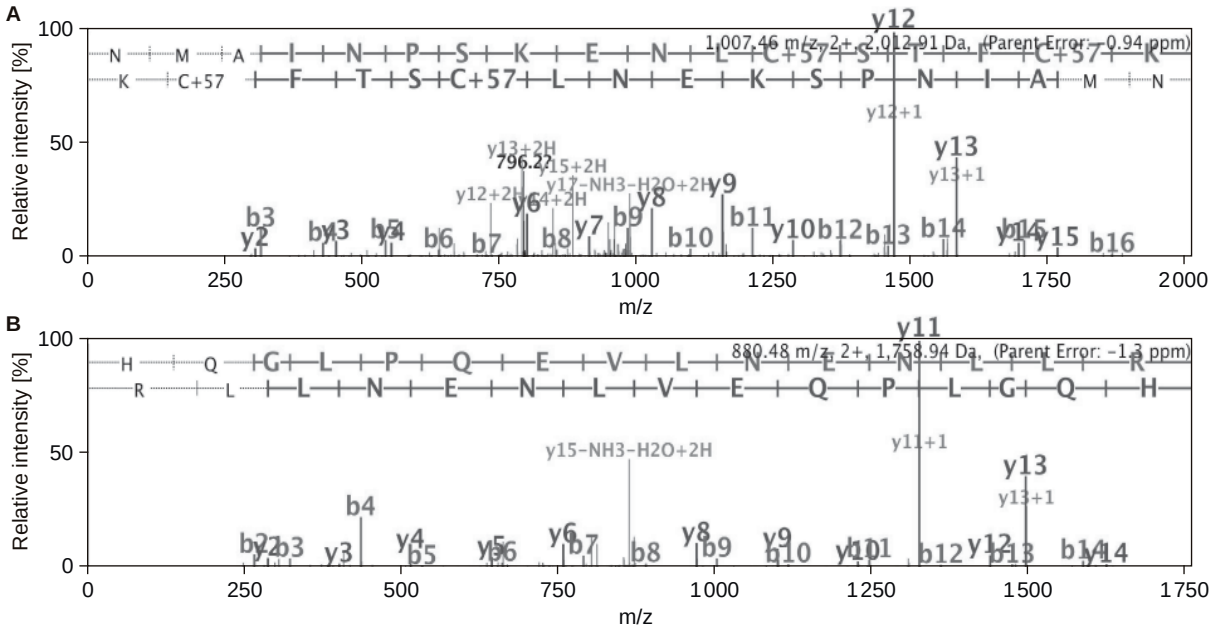

Fig. 3. Representative MS/MS peptide spectra of identified caseins in RP-HPLC fractions.

MS/MS spectra with the highest Mascot Ion Score are shown: A – Fraction 0 and Fraction 1: peptide NMAINPSKENLCSTFCK (α_{S2}-casein), Mascot Ion Score 87.3; B – Fraction 2: peptide HQGLPQEVLNENLLR (α_{S1}-casein), Mascot Ion Score 97.3.

[12], who found elutions of α_{S2}- and κ-casein to be always very close and those two caseins were difficult to separate [16]. RP-HPLC chromatogram obtained by the analysis of α_S-casein (≥ 70%) and fractions of α_S-casein using the liquid chromatograph Alliance 2695 (Waters) and the X Bridge TM C18 column (Waters) is shown in Fig. 4.

RP-HPLC analysis of samples

Milk

RP-HPLC chromatograms of caseins from milk of different origin are shown in Fig. 5. The detected casein profile for cows' milk was the following: α_{S1}-casein 28.3% ± 0.2%, α_{S2}-casein 6.0% ± 0.1%, β-casein 57.6% ± 0.2% and κ-casein 8.1% ± 0.1%. Veloso et al. [2] compared the results of other authors, which were 48.6% and 46.9% of α_S-casein, 38.7% and 33.4 % of β-casein, 12.7% and 19.7% of κ-casein. Bramanti et al. [17] reported the following percentage of casein components in raw milk from literature: 37.6–39.5% of α_{S1}-casein, 7.8–12.1% of α_{S2}-casein, 33.4–44.6% of β-casein and 9.5–19.7% of κ-casein. Nevertheless, the results obtained by Bramanti et al. [17] were 37% ± 7% of α_{S1}-casein, 7% ± 1% of α_{S2}-casein, 42% ± 8% of β-casein and 6% ± 2% of κ-casein, which are comparable with the data from literature, the only difference being the lower percentages of κ-casein, which is in accordance with the results obtained in our study.

From more recent sources, quantification of casein components in cows' milk was reported by Selvaggi and Tufarelli [5], with the following data: 38% of α_{S1}-casein, 12% of α_{S2}-casein, 36% of β-casein and 14% of κ-casein. The nearest values to our results (after re-calculation from the original grams per litre to the percentage of individual caseins) were determined by Bonizzi et al. [18], with the following casein profile: α_{S1}-casein 37.0%, α_{S2}-casein 6.2%, β-casein 44.7% and κ-casein 12.1%. From the overview of the literature we can conclude that the range of detected values varies considerably across individual casein components. Above all, our determined values differ from the published results in lower contents of α_{S1}-casein and, on the other hand, in higher contents of β-casein. Possible differences can be attributed to the fact that, for comparable data, it was not always clearly stated whether the potential impurity of the standard (up to 30%) was included in the calculation or not.

According to our results, goats' milk contained 5.8% ± 0.1% of α_{S1}-casein, 7.7% ± 0.2% of α_{S2}-casein, 71.6% ± 0.4% of β-casein and 15.0% ± 0.1% of κ-casein. Tziboula-Clarke [3] reported the presence of individual caseins in

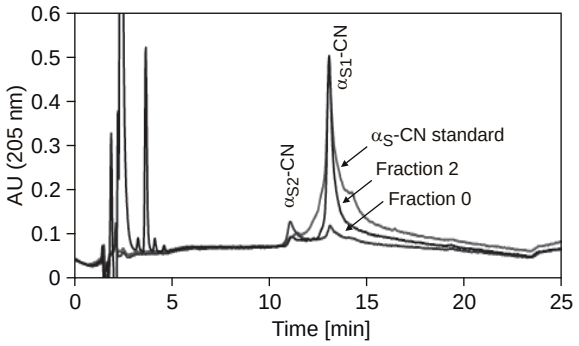

Fig. 4. RP-HPLC chromatogram of bovine α_S-casein analytical standard and isolated α_S-casein RP-HPLC fractions.

α_S-CN – α_S-casein, α_{S1}-CN – α_{S1}-casein, α_{S2}-CN – α_{S2}-casein.

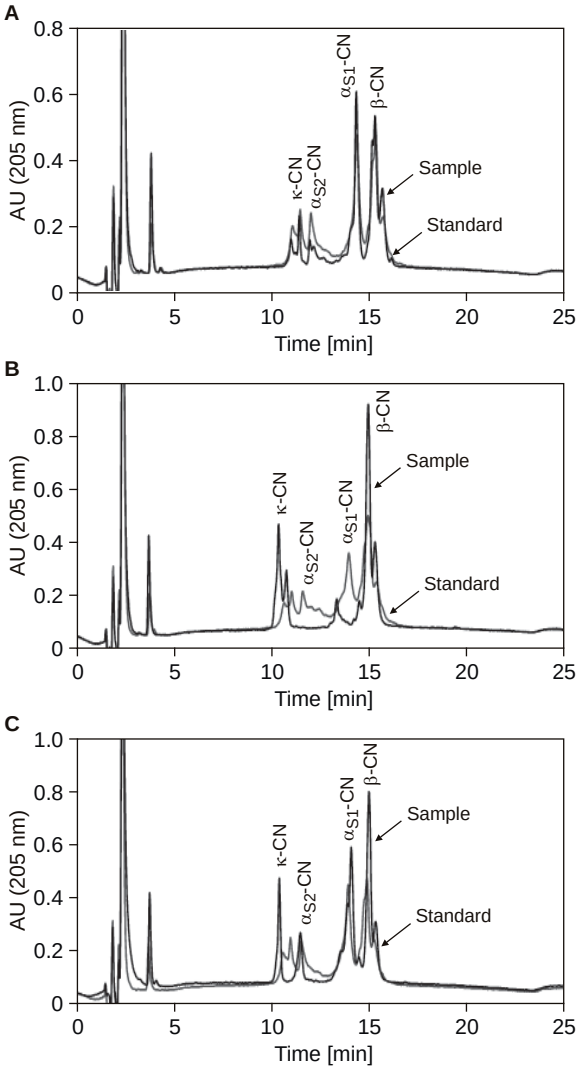

Fig. 5. RP-HPLC chromatograms of analytical standards and samples of milk.

A – cows' milk, B – goats' milk, C – ewes' milk.
α_{S1}-CN – α_{S1}-casein, α_{S2}-CN – α_{S2}-casein, β-CN – β-casein, κ-CN – κ-casein.

the range of 0–28% for α_{S1}-casein, 10–25% for α_{S2}-casein, 0–64% for β-casein and 15–29% for κ-casein. The values of caseins reported by other authors were within the ranges given by TZIBOULA-CLARKE [3], only BRAMANTI et al. [17] stated a lower proportion for κ-casein (12.4%). Specific percentages of caseins were published by, for example, SELVAGGI and TUFARELLI [5], and were 5.6% for α_{S1}-casein, 19.2% for α_{S2}-casein, 54.8% for β-casein and 20.4% for κ-casein. Taking into account the latter, we can conclude that our results are comparable with the literature. The results determined by BRAMANTI et al. [17]: 10% ± 6% for α_{S1}-casein, 63% ± 11% for α_{S2}-casein, 18% ± 4% for β-casein and 8% ± 2% for κ-casein, show the diversity of goats' milk, which, depending on the genotype, can be rich in α_{S1}-casein or, on the contrary, does not contain α_{S1}-casein at all [19–21].

Specific percentages of individual casein components determined in ewes' milk were 25.0% ± 0.6% for α_{S1}-casein, 5.3% ± 0.2% for α_{S2}-casein, 59.5% ± 0.8% for β-casein and 10.2% ± 0.1% for κ-casein. BRAMANTI et al. [17] published the following percentages of casein components: 35% for α_{S1}-casein, 8% for α_{S2}-casein, 38% for β-casein and 17% for κ-casein. On the other hand, SELVAGGI and TUFARELLI [5] reported values of 6.6% for α_{S1}-casein, 22.8% for α_{S2}-casein, 61.6% for β-casein and 8.9% for κ-casein. To conclude, the results of analyses performed by our group are comparable with data obtained from the literature.

Fig. 6 demonstrates the differences in composition of individual caseins between cows', goats' and ewes' milks. Goats' milk had the highest content of β-casein, α_{S2}-casein and κ-casein but, on the other hand, the lowest content of α_{S1}-casein. On the contrary, cows' milk had the largest proportion of α_{S1}-casein and the lowest proportion of β-casein as well as κ-casein compared to goats' and ewes' milks. The lowest content of α_{S2}-casein was found in ewes' milk.

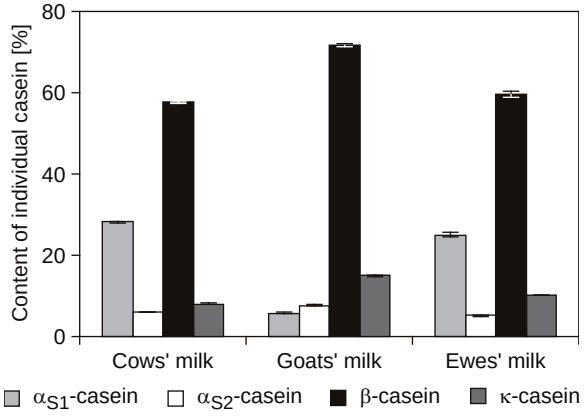

Fig. 6. Distribution of individual caseins in milk samples of different animal origin.

Cows' milk (n = 40); goats' milk (n = 40); ewes' milk (n = 40). Mean values ± standard error of the mean are presented.

By means of the Tukey-HSD test it was found that the content of α_{S1}-casein in cows' milk was significantly higher ($p < 0.001$) than in goats' milk. The content of α_{S1}-casein for ewes' milk was significantly higher ($p < 0.001$) than in goats' milk and significantly lower ($p < 0.001$) than in cows' milk. Significant difference ($p < 0.001$) was found for goats' milk, which contained more α_{S2}-casein than cows' and ewes' milks. The content of α_{S2}-casein in cows' milk was significantly higher ($p = 0.0101$) in comparison with ewes' milk.

In goats' milk, a significantly higher ($p < 0.001$) content of β-casein was observed than in cows' milk and ewes' milk. On the other hand, the content of β-casein in cows' milk was significantly lower ($p = 0.0275$) in comparison with ewes' milk. Significantly ($p < 0.001$) higher proportion of κ-casein was demonstrated in goats' milk in comparison with cows' milk and ewes' milk. In ewes' milk, a significantly higher ($p < 0.001$) content of κ-casein was found compared to cows' milk.

The results of the study have further shown a significant positive correlation between the contents of α_{S1}-casein and α_{S2}-casein in cows' ($r = 0.3809$; $p = 0.0077$), goats' ($r = 0.6553$; $p < 0.001$) and ewes' ($r = 0.7457$; $p < 0.001$) milks. By statistical inference of the data for cows' milk it was also established that β-casein was significantly negatively correlated with α_{S1}-casein ($r = –0.8575$; $p < 0.001$), α_{S2}-casein ($r = –0.6446$; $p < 0.001$) and κ-casein ($r = –0.4630$; $p = 0.0013$).

The content of β-casein in goats' milk was proven to be significantly negatively correlated with α_{S1}-casein ($r = –0.8307$; $p < 0.001$), α_{S2}-casein ($r = –0.9058$; $p < 0.001$) and κ-casein ($r = –0.7008$; $P < 0.001$). Similar to cows' and goats' milks, the content of β-casein in ewes' milk was significantly negatively correlated with the contents of α_{S1}-casein ($r = –0.9676$; $p < 0.001$), α_{S2}-casein ($r = –0.8602$; $p < 0.001$) and κ-casein ($r = –0.4483$; $p = 0.0019$).

Monitoring the dependence of κ-casein in goats' milk, we further found that it was significantly positively correlated with α_{S1}-casein ($r = 0.4941$; $p = 0.0006$) and α_{S2}-casein ($r = 0.3939$; $p = 0.0060$), while a significant positive correlation was demonstrated in ewes' milk between the contents of κ-casein and α_{S1}-casein ($r = 0.2757$; $p = 0.0425$), and between κ-casein and α_{S2}-casein ($r = 0.3602$; $p = 0.0112$). In cows'

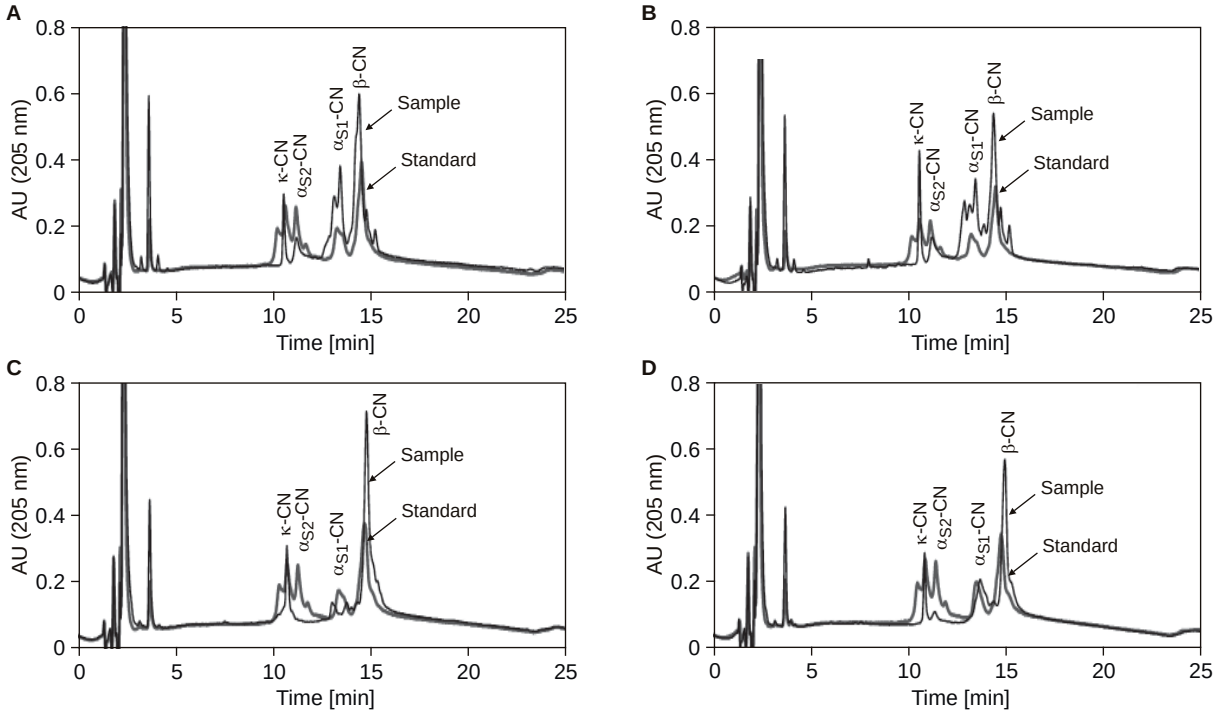

Fig. 7. RP-HPLC chromatograms of analytical standards and samples of cheese.

A – Koliba cows'-milk cheese, B – Mozzarella premium cows'-milk cheese, C – goats'-milk cheese, D – ewes'-milk cheese. α_{S1}-CN – α_{S1}-casein, α_{S2}-CN – α_{S2}-casein, β-CN – β-casein, κ-CN – κ-casein.

milk, no statistically significant correlation was found between the contents of κ-casein and α_{S1}-casein, and between κ-casein and α_{S2}-casein.

Cheese

A total of 36 cheese samples were examined by RP-HPLC, namely, 20 samples from cows' milk, 9 samples from goats' milk, and 7 samples were produced from ewes' milk. The majority of cheese samples from cows' milk belonged to the category of traditional cheeses. Cheese samples from cows' milk were represented mainly by ripened cheese, unlike samples of cheese from goats' milk, which were fresh cheeses. Similarly, most of ewes' cheeses were fresh cheeses, just one sample of ewes' cheese (Gouda) was ripened. Examples of RP-HPLC chromatograms for casein standards and cheese samples are shown in Fig. 7.

In the scientific literature, we managed to find only one cheese (Mozzarella cheese) for which the individual casein quantification was carried out. It was a cheese made from cows' milk, with 15% of fat in dry matter and was also included in our study. The percentages of caseins, as determined by the method of hydrophobic interaction chromatography (HIC), were 56% of α_{S1}-casein, 7% of α_{S2}-casein, 32% of β-casein, 2% of κ-casein and 4% of γ-casein [17]. In comparison, the sample of Mozzarella premium cheese from our study, with 44% of fat in dry matter (Fig. 7B), contained 30.4% of α_{S1}-casein, 6.2% of α_{S2}-casein, 57.2% of β-casein and 6.2% of κ-casein.

The composition of individual caseins in cheeses produced from goats' milk could not be found in the scientific literature. Publications concerning milk proteins often report solely the total protein or casein percentage but do not evaluate individual casein components. Similarly to goats' cheeses, finding publications containing information on the presence of individual caseins in cheeses from ewes' milk was problematic as well [22–24]. Nevertheless, BRAMANTI et al. [17] provided the following casein composition in a Ricotta cheese sample (13% of fat in dry matter) produced from ewes' milk: 41% for α_{S1}-casein, 11% for α_{S2}-casein, 37% for β-casein and 11% for γ-casein. The aforementioned cheese, however, was not in our product line of examined samples from ewes' milk.

The percentages of individual caseins present in our cheese samples produced from cows' milk were as follows, 27.5% ± 1.6% for α_{S1}-casein, 5.4% ± 0.7% for α_{S2}-casein, 61.6% ± 2.2% for β-casein and 5.6% ± 0.7% for κ-casein. The obtained percentages roughly correspond to the values for caseins in cheeses from ewes' milk:

24.5% ± 2.6% for α_{S1}-casein, 3.7% ± 1.6% for α_{S2}-casein, 65.0% ± 3.08% for β-casein and 7.2% ± 1.1% for κ-casein. Our results for samples of goats' cheeses (11.0% ± 0.7% for α_{S1}-casein, 1.3% ± 0.3% for α_{S2}-casein, 77.2% ± 0.8% for β-casein and 10.5% ± 0.6% for κ-casein) differed from those of cheeses produced from cows' milk, however, they were not statistically significantly different from ewes' milk cheeses.

Differences in the casein composition of cheeses can be attributed to different types of cheese and technological process, types of animal breed, nutrition, stage of lactation, condition of animal's health, season, geographical situation and other factors [1, 3–5, 25]. Lower contents of κ-casein could have been the result of complexes forming between β-lactoglobulin, κ-casein and α_{S2}-casein, due to thermal treatment that causes changes in the stratification of caseins [2]. Bramanti et al. [17] stated that the reason for low values of κ-casein can be attributed to partial creation of dimers and polymers of κ-caseins via S-S bridges [17]. From the graphs representing the average percentage values of individual caseins in cheeses (Fig. 8) it can be concluded that the content of β-casein was unambiguously the highest, followed by α_{S1}-casein. Only minor proportion of the cheese matrix was formed by κ-casein and α_{S2}-casein.

Using analysis of variance (ANOVA) with multiple comparisons for cheeses from cows' milk, we have proven a significantly higher difference for α_{S1}-casein (p = 0.0029) and significantly lower difference for κ-casein (p = 0.0015), compared to the cheeses from goats' milk. The presence of α_{S2}-casein was significantly higher (p = 0.0134) in cheeses from cows' milk, compared to cheeses from goats' milk. For β-casein, no statistically significant difference between various kinds of cheeses was observed. Similarly, when comparing ewes' milk cheeses with goats' milk and cows' milk cheeses, no statistically significant differences were detected.

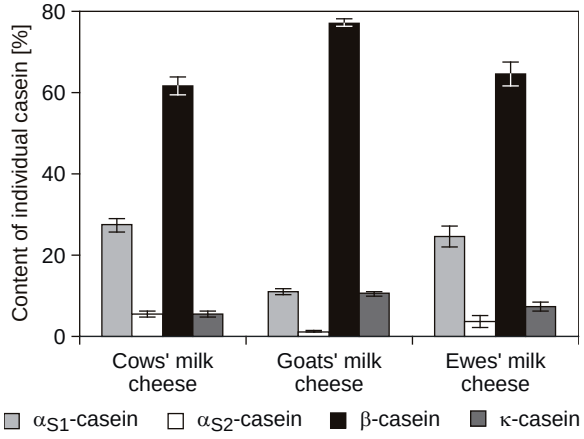

Fig. 8. Distribution of individual caseins in samples of cheese produced from milk of different animal origin.

Cows' milk cheese (n = 20); goats' milk cheese (n = 9); ewes' milk cheese (n = 7). Mean values ± standard error of the mean are presented.

By statistical data inference, some significant correlations were acquired. For example, in cows' milk cheeses the content of α_{S1}-casein was significantly positively correlated with the contents of β-casein (r = 0.8778; p < 0.001), α_{S2}-casein (r = 0.6469; p = 0.0010) and κ-casein (r = 0.7087; p = 0.0002). These cheeses further showed a significant positive correlation between the contents of α_{S2}-casein and β-casein (r = 0.4189; p = 0.0330), and between α_{S2}-casein and κ-casein (r = 0.4489; p = 0.0236).

The content of α_{S1}-casein in goats' cheeses was significantly positively correlated with the contents of β-casein (r = 0.6335; p = 0.0335) and α_{S2}-casein (r = 0.7235; p = 0.0138). On the other hand, the content of κ-casein in goats' cheeses did not show any statistically significant correlation with the contents of α_{S1}-casein or α_{S2}-casein. Similarly insignificant was the correlation between the contents of α_{S2}-casein and β-casein. However, significantly positive correlation was found between the contents of κ-casein and β-casein in cheeses from cows' milk (r = 0.6754; p = 0.0005), goats' milk (r = 0.8448; p = 0.0021) and ewes' milk (r = 0.8833; p = 0.0042).

The content of α_{S2}-casein in ewes' cheeses did not correlate with the contents of κ-casein, α_{S1}-casein or β-casein. On the contrary, the content of α_{S1}-casein was significantly correlated with the contents of β-casein (r = 0.9086; p = 0.0023) and κ-casein (r = 0.8395; p = 0.0091) for ewes' cheeses. The results further showed that there were no statistically significant differences between ripened (n = 10) and fresh (n = 8) cheeses.

Yoghurts

A total of 10 yoghurt samples from cows' milk were analysed by RP-HPLC. Fig. 9 presents the casein RP-HPLC chromatogram of the sample of yoghurt produced from cows' milk, which correlates with the chromatogram of bovine casein analytical standards. Fig. 10 sums up the percentages of individual caseins in yoghurt samples. Values determined for yoghurts produced from cows' milk (α_{S1}-casein 17.3% ± 1.2%, α_{S2}-casein 4.2% ± 0.5%, β-casein 73.0% ± 2.0% and κ-casein 5.5% ± 0.5%) were lower for all caseins analysed

in cows' milk, with the exception of β-casein (α_{S1}-casein 28.3% ± 0.2%, α_{S2}-casein 6.0% ± 0.1%, β-casein 57.6% ± 0.2% and κ-casein 8.1% ± 0.1%). Unfortunately, data with the reference to the composition of individual caseins in yoghurts could not be found in the literature.

The content of α_{S1}-casein in yoghurts produced from cows' milk was found in a significantly positive correlation with the contents of α_{S2}-casein (r = 0.9386; p < 0.001), β-casein (r = 0.9713; p < 0.001) and κ-casein (r = 0.9648; p < 0.001). Similarly, the content of β-casein was found to be significantly positively correlated with the contents of α_{S2}-casein (r = 0.9451; p < 0.001) and κ-casein (r = 0.8997; p = 0.0002). Furthermore, the content of α_{S2}-casein was in a significantly positive correlation with the content of κ-casein (r = 0.8476; p = 0.0010).

CONCLUSIONS

On the basis of our results, it can be concluded that the lowest content of α_{S1}-casein (5.8% ± 0.1%), having the highest allergenic potential, was detected in goats' milk obtained from the White Shorthaired Goat breed. On the other hand, the highest content of α_{S1}-casein (28.3% ± 0.2%) was determined in cows' milk obtained from dairy cows of Czech Red Cattle and Holstein Cattle breeds (including hybrid dairy cows). In the case of ewes' milk, with the Laucane Ewe breed prevailing and minor proportion of Improved Wallachian Ewe and East Friesian Ewe breeds, the content of α_{S1}-casein was 25.0% ± 0.6%. The lowest content of α_{S1}-casein was found in cheeses from goats' milk (11.0% ± 0.7%), higher content of α_{S1}-casein was found in cheeses from ewes' milk (24.5% ± 2.6%) and the highest content of α_{S1}-casein was found in cheeses from cows' milk (27.5% ± 1.6%). The content of α_{S1}-casein in yoghurts from cows' milk was found to be 17.3% ± 1.2%. Our results show that casein profiles vary not only between milk from different animal origin, but also within the specific types of dairy products (cheese, yoghurt). Moreover, as the presence of α_{S1}-casein varies greatly between dairy products, their allergenic potential also differs, which allows consumers to avoid certain types of dairy products and choose the less problematic ones.

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Lenka Ruprichová and Gorazd Tompa contributed equally to this work.

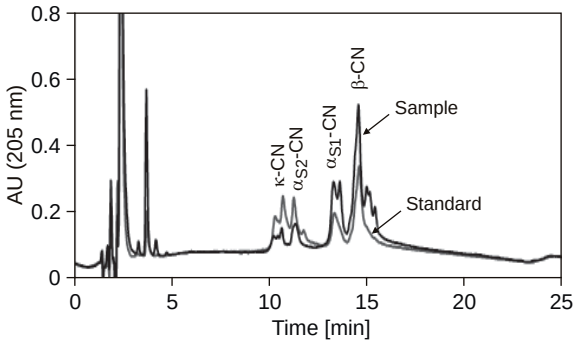

Fig. 9. RP-HPLC chromatogram of analytical standard and sample of yoghurt produced from cows' milk.

α_{S1}-CN – α_{S1}-casein, α_{S2}-CN – α_{S2}-casein, β-CN – β-casein, κ-CN – κ-casein.

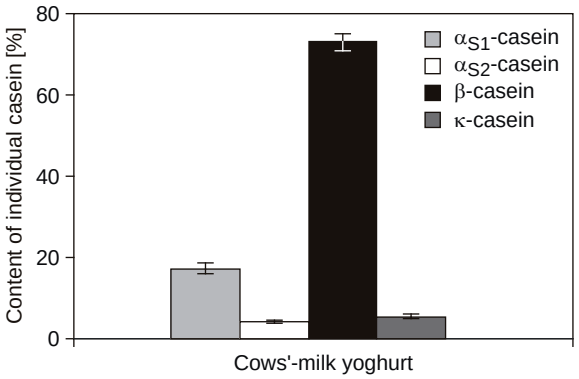

Fig. 10. Distribution of individual caseins in samples of yoghurt produced from cows' milk.

Mean values ± standard error of the mean are presented (n = 10).

REFERENCES

1. Park, Y. W.: Goat milk – chemistry and nutrition. In: Park, Y. W. – Haenlein, G. F. W. (Ed.): Handbook of milk of non - bovine mammals. Ames, Iowa, Oxford : Blackwell, 2006, pp. 11–135. ISBN: 0-8138-2051-0.
2. Veloso, A. C. A. – Teixeira, N. – Ferreira, I. M. P. L. V. O.: Separation and quantification of the major casein fractions by reverse-phase high-performance liquid chromatography and urea–polyacrylamide gel electrophoresis: Detection of milk adulterations. Journal

of Chromatography A, *967*, 2002, pp. 209–218. DOI: 10.1016/S0021-9673(02)00787-2.
3. Tziboula-Clarke, A.: Goat milk. In: Roginski, H. – Fuquay, J. W. – Fox, P. F. (Ed.): Encyclopedie of dairy science. Amsterdam : Academic Press, 2003, pp. 1270–1279. ISBN: 0-12-227238-2.
4. Fox, P. F.: Milk: in overview. In: Thompson, A. – Boland, M. – Harjinder, S. (Ed.): Milk proteins: from expression to food. New York : Academic Press, 2009, pp. 1–54. ISBN: 978-0-12-374039-7.
5. Selvaggi, M. – Tufarelli, V.: Caseins of goat and sheep milk: analytical and technological aspects. In: Ventimiglia, A. M. – Birkenhäger, J. M. (Ed.): Casein: production, uses and health effects. New York : Nova Publishers, 2012, pp. 1–26. ISBN: 978-1-62100-129-4.
6. Monaci, L. – Tregoat, V. – van Hengel, A. J. – Anklam, E.: Milk allergens, their characteristics and their detection in food: A review. European Food Research and Technology, *223*, 2006, pp. 149–179. DOI: 10.1007/s00217-005-0178-8.
7. Chatchatee, P. – Jarvinen, K. M. – Bardina, L. – Beyer, K. – Sampson, H. A.: Identification of IgE- and IgG-binding epitopes on αs1-casein: Differences in patients with persistent and transient cow's milk allergy. Journal of Allergy and Clinical Immunology, *107*, 2001, pp. 379–383. DOI: 10.1067/mai.2001.112372.
8. Pajno, G. B.: Oral desensitization for milk allergy in children: state of the art. Current Opinion in Allergy and Clinical Immunology, *11*, 2011, pp. 560–564. DOI: 10.1097/ACI.0b013e32834cd298.
9. Passalacqua, G. – Landi, M. – Pajno, G. B.: Oral immunotherapy for cow's milk allergy. Current Opinion in Allergy and Clinical Immunology, *12*, 2012, pp. 271–277. DOI: 10.1097/ACI.0b013e3283535b93.
10. López-Fandiño, R. – Olano, A. – Corzo, N. – Ramos, M.: Proteolysis during storage of UHT milk: differences between whole and skim milk. Journal of Dairy Research, *60*, 1993, pp. 339–347. DOI: 10.1017/S0022029900027680.
11. Rodríguez, N. – Ortiz, M. C. – Sarabia, L. – Gredilla, E.: Analysis of protein chromatographic profiles joint to partial least squares to detect adulterations in milk mixtures and cheeses. Talanta, *81*, 2010, pp. 255–264. DOI: 10.1016/j.talanta.2009.11.067.
12. Feligini, M. – Bonizzi, I. – Buffoni, J. N. – Cosenza, G. – Ramunno, L.: Identification and quantification of α_{S1}, α_{S2}, β, and κ-caseins in water buffalo milk by reverse phase-high performance liquid chromatography and mass spectrometry. Journal of Agricultural and Food Chemistry, *57*, 2009, pp. 2988–2992. DOI: 10.1021/jf803653v.
13. Keller, A. – Nesvizhskii, A. I. – Kolker, E. – Abersold, R.: Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Analytical Chemistry, *74*, 2002, pp. 5383–5392. DOI: 10.1021/ac025747h.
14. Nesvizhskii, A. I. – Keller, A. – Kolker, E. – Abersold, R.: A statistical model for identifying proteins by tandem mass spectrometry. Analytical Chemistry, *75*, 2003, pp. 4646–4658. DOI: 10.1021/ac0341261.
15. Zar, J. H. (Ed.): Biostatistical analysis. 4th edition. New Jersey : Prentice Hall, 1999. ISBN: 0-13-084542-6.
16. Bordin, G. – Cordeiro Raposo, F. – De La Calle, B. – Rodriguez, A. R.: Identification and quantification of major bovine milk proteins by liquid chromatography. Journal of Chromatography A, *928*, 2001, pp. 63–76. DOI: 10.1016/S0021-9673(01)01097-4.
17. Bramanti, E. – Sortino, C. – Onor, M. – Beni, F. – Raspi, G.: 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. Journal of Chromatography A, *994*, 2003, pp. 59–74. DOI: 10.1016/S0021-9673(03)00483-7.
18. Bonizzi, I. – Buffoni, J. N. – Feligini, M.: Quantification of bovine casein fractions by direct chromatographic analysis of milk. Approaching the application to a real production context. Journal of Chromatography A, *1216*, 2009, pp. 165–168. DOI: 10.1016/j.chroma.2008.11.045.
19 Marletta, D. – Criscione, A. – Bordonaro, S. – Guastella, A. M. – D'urso, G.: Casein polymorphism in goat's milk. Lait, *87*, 2007, pp. 491-504. DOI: 10.1051/lait:2007034.
20. Sztankóová, Z. – Mátlová, V. – Kysel'ová, J. – Jandurová, O. M. – Říha, J. – Senese, C.: Short communication: Polymorphism of casein cluster genes in Czech local goat breeds. Journal of Dairy Science, *92*, 2009, pp. 6197–6201. DOI: 10.3168/jds.2008-1519.
21. Caroli, A. – Chiatti, F. – Chessa, S. – Rignanese, D. – Bolla, P. – Pagnacco G.: Focusing on the goat casein complex. Journal of Dairy Science, *89*, 2006, pp. 3178–3187. DOI: 10.3168/jds.S0022-0302(06)72592-9.
22. Guarino, C. – Fuselli, F. – La Mantia, A. – Longo, L. – Faberi, A. – Marianella, R. M.: Peptidomic approach, based on liquid chromatography/electrospray ionization tandem mass spectrometry, for detecting sheep's milk in goat's and cow's cheeses. Rapid Communications in Mass Spectrometry, *24*, 2010, pp. 705–713. DOI: 10.1002/rcm.4426.
23. Pandya, A. J. – Ghodke, K. M.: Goat and sheep milk products other than cheeses and yoghurt. Small Ruminant Research, *68*, 2007, pp. 193–206. DOI: 10.1016/j.smallrumres.2006.09.007.
24. Ren, D. – Chen, B. – Chen, Y. – Miao, S. – Liu, J.: The effects of κ-casein polymorphism on the texture and functional properties of mozzarella cheese. International Dairy Journal, *31*, 2013, pp. 65–69. DOI: 10.1016/j.idairyj.2013.01.005.
25. Karoui, R. – Baerdemaeker, J. D. A review of the analytical methods coupled with chemometric tools for the determination of the quality and identity of dairy products. Food Chemistry, *102*, 2007, pp. 621–640. DOI: 10.1016/j.foodchem.2006.05.042.

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