Quantification of ovine and bovine caseins in Slovakian bryndza ewes' cheese by isoelectric focusing

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Summary
Isoelectric focusing on ReadyStrips with immobilized pH gradient was modified for the purpose of quantification of ovine and bovine protein proportion in the Slovakian bryndza ewes' cheese. This preliminary study provided reliable results when the ovine and bovine γ_2- and γ_3- casein ratios were applied for calibration purposes and worked well for higher percentages of ewes' and cows' lump cheeses, respectively. No need was found for extra calibration for summer and winter types of bryndza. The results of ewes' lump cheese determination in commercial bryndza cheeses showed that most cheesemakers produced bryndza of both summer and winter type with about 50% contents of ewes' lump cheese, which is different from the traditional recipe of Slovakian summer bryndza cheese that was wholly made just from ewes' lump cheese.

Keywords
isoelectric focusing; densitometry; bryndza; γ–casein

The authenticity of dairy products has become a focal point attracting the attention of scientists, producers, consumers and policy makers. Among others, some of the practices not allowed in milk and dairy products are the substitution of a part of the fat or proteins, admixtures of milk of different species, additions of low-cost dairy products (mainly whey derivatives) or mislabeling of products protected by denomination of origin [1]. Adulteration of ewes‘ milk with cows‘ milk is relatively common due to seasonal fluctuations of the availability of ewes‘ milk, the higher price of ewes‘ milk compared to cows‘ milk and opportunity to use the overproduction of cows‘ milk [2]. Adulteration of ewes‘ milk with cows‘ milk represents a problem also with bryndza cheese, where ewes‘ lump cheese is often replaced by cheaper cheese made from cows‘ milk and this fact is not declared.

Bryndza is a natural, white, mature, spreadable cheese in granular form, manufactured in Slovakia according to the traditional method, by milling a lump of matured ewes‘ cheese or by milling a mixture of ewes‘ lump cheese and cows‘ lump cheese. Traditional bryndza is 100% ewes‘ cheese, but its production is nowadays very rare. Bryndza of winter type is legally produced as a mixture of ewes‘ and cows‘ lump cheeses. Current commercially available bryndza according to the Slovakian Food Codex [3], or Commission Regulation (EC) No 676/2008 [4] concerning the „Slovakian bryndza“ as a registered PGI product, have to contain more than 50% of ewes' lump cheese. In order to avoid the possible fraudulent substitution of ewes' cheese with cows' cheese, it is necessary to develop analytical procedures able to quantitatively determine the proportion of these components in bryndza cheese, or to control the over-limited content of cows' lump cheese in bryndza.

In recent years, a range of analytical methods to detect frauds have been developed, modified and continually re-assessed to be one step ahead of manufacturers who pursue these illegal activities [1]. Numerous methods were reported in the literature for the identification of milk and cheese adulteration [5]. Most of the methods described are related to the qualitative detection of low quantities of cows' milk in ewes' milk cheeses by different analytical techniques [6]. These analyses have been carried out using polyacrylamide gel electrophoretic techniques with urea (Urea–PAGE) [7–11] or sodium dodecylsulphate (SDS–PAGE) [12, 13]. Isoelectric focusing (IEF) [14–17] and capillary electrophoresis [2, 6, 18–22] have been successfully used to analyse milk pro-

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teins. High-performance liquid chromatography in their different modes [11, 23–25] and immunological methods [26–28] have also been applied for protein analysis. To a lesser extent, problems of protein species quantification in cheese mixtures have been dealt with [2, 14, 16, 17, 20, 22]. More possibilities for different animals' milk detection and determination in milk and dairy products have been extensively reviewed by RECIO et al. [29] and by BORKOVÁ and SNÁŠELOVÁ [30].

Principal component regression (PCR), partial least squares (PLS-1 and PLS-2) regression and multiple linear regressions (MLR) are multivariate statistical techniques that have been applied in different sciences to obtain calibration models as an alternative to linear regressions. Capillary electrophoresis was applied to the determination of the casein fraction from Panela cheese manufactured with milk mixtures, and several multivariate techniques were employed in order to provide predictive models for the determination of cows', ewes' and goats' milk percentages from this cheese. It was found that MLR led to more precise predictions than the other multivariate calibration methods with a root square error under 2.2% [31].

Quantification of individual proteins in cheese mixtures is complicated by the strong protein fraction variation due to existence of genetic and non-genetic polymorphism, and by thermal denaturation or proteolysis that is common at manufacturing of milk products. The present reference European Community method (EC) No 273/2008 for the detection of cows' milk in ewes' and goats' milk products is based on isoelectric focusing (IEF) of γ-caseins, peptides originated from β-casein after enzymatic proteolysis by bovine plasmin [32]. The present work refers to the modification of this IEF reference method to quantitative determination of cows' cheese proportion in the Slovak bryndza ewes' cheese using ReadyStrip with immobilized pH gradient.

MATERIALS AND METHODS

Reagents and samples

All reagents used were of analytical grade and all solutions and buffers were prepared using demineralized water (0.02 $\mu S \cdot cm^{-1}$). Acetic acid, dichloromethane, acetone, glycerol, urea, dithiothreitol, ammonium carbonate, ε-aminocaproic acid, ethanol, methanol, trichloroacetic acid, Coomassie Brilliant Blue as reagents for protein isolation, preparation of dissolving buffers and protein staining solutions were purchased from Merck (Darmstadt, Germany). Ampholytes (pH 3–11) and EDTA were obtained from Sigma Aldrich (St. Louis, Missouri, USA). Bovine plasmin reagent for cleavage of caseins was purchased from Roche (Roche Diagnostics, Indianapolis, Indiana, USA).

Immobilized pH gradient (IPG) strips ReadyStrip with linear pH gradient range 3–10 (strip length 11.8 cm, gel length 11 cm, gel thickness 0.5 mm) were purchased from Bio-Rad (Hercules, California, USA).

Certified reference material BCR-599, a freeze-dried 50/50 mixture of ewes' and goats' curds with 0% cows' milk [33] was obtained from European Commission – Joint Research Centre (Geel, Belgium). Standard bryndza cheese as a secondary reference material was manufactured according to in-house bryndza cheesemaking procedure by Bryndziareň a syráreň, Zvolenská Slatina, Slovakia, using the mixtures of ewes' and cows' lump cheeses at proportions of 0 : 100; 20 : 80; 30 : 70; 40 : 60; 50 : 50; 60 : 40; 70 : 30; 80 : 20; and 100 : 0.

Commercial bryndza cheese samples were summer type bryndza cheeses originating from 6 Slovakian and 1 Czech cheesemakers, and winter type bryndza from 6 Slovakian cheesemakers. The bryndza cheeses originated from the producers Bryndziareň, Turčianske Teplice, Slovakia; Agrofarma, Červený Kameň, Slovakia; Brysyrt, Tisovec, Slovakia; Liptovská mliekáreň, Liptovský Mikuláš, Slovakia; Milk Trade, Český Těšín, Czech republic; Bryndziareň a syráreň, Zvolenská Slatina, Slovakia; Poľnohospodárske družstvo, Kluknava, Slovakia; and Tatranská mliekareň, Kežmarok, Slovakia. Samples were analysed with three sample replications.

For quantification of ovine and bovine γ_3- and γ_2-caseins in bryndza cheese, the reference qualitative method of Commission Regulation (EC) No 273/2008 [32] for detection of cows' milk and caseinate in cheeses was modified as stated below.

Isolation of caseins and plasmin treatment

For isolation of casein, an amount of 5 g of bryndza cheese was weighed, 60 ml distilled water was added and the mixture was homogenized using an Ultra Turrax T25 blender (Ika, Staufen, Germany). Isoelectric casein was obtained by precipitation at pH 4.6 with diluted acetic acid (25 ml of glacial acetic acid made up to 100 ml with distilled water), and then the precipitate was washed several times by water and dichloromethane with the aim to be desalted and defatted. The final casein was washed with acetone, left to dry overnight in the laboratory air, finely pulverized in a mortar, and stored in a refrigerator at –18 °C until analysis. For plasminolysis, 25 mg of the isolated casein

were dispersed in 0.5 ml of 0.2 mol·l^{-1} ammonium carbonate buffer and homogenized for 20 min by ultrasonic treatment, then mixed and incubated for 1 h at 40 °C with 10 μl of bovine plasmin.

Rehydration of IPG ready strips and sample application [34]

The rehydration buffer was prepared by mixing of 48 g urea, 1 ml Triton X-100 and 800 μl Coomassie Briliant Blue solution (0.3 g in 100 ml of 90% methanol), and filling up to 100 ml with deionized water. The aliquot of the plasmined sample solution (45 μl) mixed with 1.5 ml rehydration buffer was placed into the rehydration/equilibration tray and used to passively rehydrate individual IPG ready strips (overnight, 14–16 h).

Isoelectric focusing and densitometric scanning

Following rehydration, the strips were electrophoresed on a water-cooled (14 °C) IF-SYS flatbed apparatus (Scie-Plas, Cambridge, United Kingdom). Electrophoresis was performed at constant voltage, first at 700 V for 20 min, then at 1500 V for 7 h until the tracking Coomassie Briliant Blue dye migrated to the IPG strip edge. The strips after isoelectric focusing were washed with fixative solution (15% w/v trichloroacetic acid), stained with Coomassie Brilliant Blue G-250 (0.1% w/v in methanol–water 90 : 10, v/v) solution mixed immediately prior strip dyeing equally with copper sulphate pentahydrate solution (0.5% w/v in 20% v/v acetic acid) and several times destained with copper sulphate solution (1 g copper sulphate pentahydrate + 25 ml methanol + 10 ml glacial acetic acid filled up to 100 ml with deionized water). The strips were two times washed with distilled water and dried on the air overnight. For more accurate analytical results, commercial bryndza samples were analysed simultaneously in a IF-SYS flatbed apparatus with reference standards.

Densitometric measurements were performed by Gel-Scanner (Carl Zeiss, Jena, Germany) in connection with a UV-Vis spectrophotometer Specord M40 (Carl Zeiss). The IPG transparent strips were scanned with a monochromatic light beam at 634 nm (λ_{max} of Coomassie Briliant Blue), the absorption being recorded along the entire length of the strip at a scanning rate of 30 mm·min^{-1}. The following spectrometric settings were used: spectral bandwidth 15 cm^{-1}; integration time 1 s; gain 0 and speed of plotter 2 mm·s^{-1}.

Statistics

Calibration curves and regression analysis were processed using the Microsoft Office Excel 2003 (Microsoft, Redmond, Washington, USA). The Q.C. Expert, v. 2.5 (Trilobyte, Pardubice, Czech Republic) statistical programme was applied to determination of the limit of detection (LOD) and limit of quantification (LOQ) of the ewes' lump cheese content in bryndza. Unistat v. 5.6 Statistical Package for Windows (Unistat, London, United Kingdom) was used for data visualization and differentiation by PCA. Canonical discriminant analysis (CDA) was applied to data discrimination and classification. Furthermore, Unistat statistical package was used for both non-parametric testing (multisample median test by method of 95% Tukey-HSD interval) and heterogeneity of regression lines testing by ANOVA.

RESULTS AND DISCUSSION

Modification of the EU isoelectric focusing reference method for quantification of ewes' and cows' protein proportion in Slovak bryndza cheese was based on the determination of four quantitative markers of ovine and bovine γ_3- and γ_2-caseins (γ_3-O, γ_2-O, γ_3-B and γ_2-B caseins) on IPG ReadyStrips.

Fig. 1 shows the isoelectrophoregrams of plasmin-treated caseins from bryndza cheese performed with secondary reference materials of defined mixtures of ewes' and cows' lump cheeses (from 0% to 100% of ewes' lump cheese in cows' lump cheese). As is visible, the most intensive electrophoretic bands were located in the middle of the IPG strips, at the pH range of about 6.5–7.5 corresponding to the isoelectric points of ovine and bovine γ_2- and γ_3-casein fractions. The isoelectric protein pattern was qualitatively evaluated by visual comparison with the isoelectrophoregrams previously published by Recio et al. [18] and Miralles et al. [21]. The differences of the bovine and ovine casein fractions in this range of pH constituted the basis for determination of the presence and proportion of cows' and ewes' cheeses used for the bryndza cheesemaking. The different position of ovine and bovine casein fractions is well demonstrated by superposition of densitograms of the IPG strips of standard 100% bovine and ovine bryndza cheeses of summer type (Fig. 2). In Fig. 1 and 2, some weak bands of reciprocal contamination of standard 100% ewes' and 100% cows' bryndza cheeses are noticeable, which might have been a result of not fully contained standard bryndza cheese preparation in normal cheesemaking technological devices. This study only presents introductory results and the following work will seek and demand to avoid such problem. In spite of this, the found recovery of 101.5% for the ovine

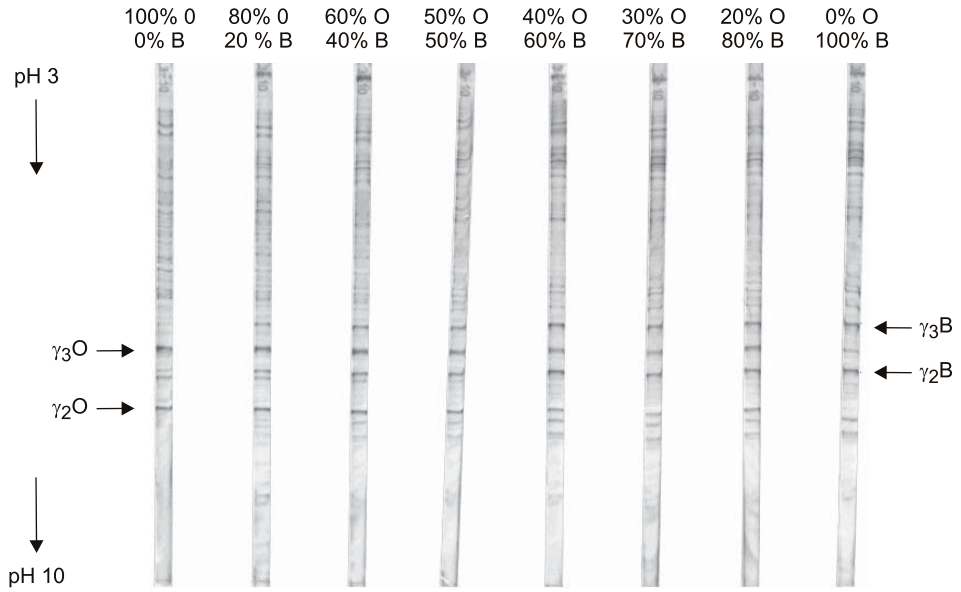

Fig. 1. Results of isoelectric focusing on IPG ReadyStrip of plasmin-treated standard bryndza cheese caseins.
γ_2-, γ_3-caseins: O – ovine, B– bovine.

γ_3-casein marker confirmed acceptable agreement between the certified BCR ovine reference material and standard ewes' bryndza prepared in manufactory conditions.

Calibration curves were constructed by plotting the relative densitometric peak areas of the four γ-casein markers versus the percentages of ewes' or cows' lump cheeses in standard bryndza cheese, but simple linear calibration curves of the ovine and bovine γ_2- and γ_3-peaks alone did not provide acceptable results for the determination of the proportion of ewes' and cows' lump cheese in bryndza (R^2 ranged from 0.6809 to 0.9143). For purposes of the present study, the ratios of bovine/ovine γ_3-casein (Fig. 3A) and of bovine/ovine γ_2-casein (Fig. 3B) were used, which resulted in improved characteristics of calibration curves (R^2 = 0.9911; R^2 = 0.9971), which were well described by quadratic model. The ratios of casein fractions were also successfully used by other authors [16, 35].

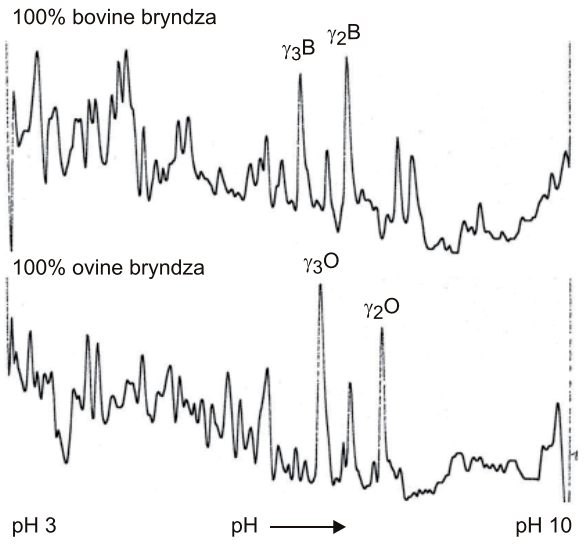

Fig. 2. Superposition of densitograms of standard 100% cows' and 100% ewes' summer type bryndza cheeses.
γ_2-, γ_3-caseins: O – ovine, B – bovine.

The quadratic model expression of calibration lines was performed for calculation of limit of detection (LOD) and the limit of quantification (LOQ) of this method. Using the calibration data of bovine and ovine γ_3-caseins (B3/O3) for the boundary values calculation resulted in LOD of 6.9% and LOQ of 11.2%. In the case of γ_2-casein markers (B2/O2), LOD of 5.4% and LOQ of 8.6% were determined. These parameters are sufficient for the determination of ewes' cheese proportion in bryndza, as long as they are substantially lower than the minimal ewes' cheese content in bryndza of 50% (w/w) in dry matter quoted by legislative requirements [3, 36].

According to the non-parametric (multisample median test by method of 95% Tukey-HSD interval) and heterogeneity testing (ANOVA) of regression lines, there were no statistical differences between the standard bryndza cheese calibration data of the summer and winter types of standard bryndza. It follows that the bryndza cheese analysis could be done by a simpler, less laborious man-

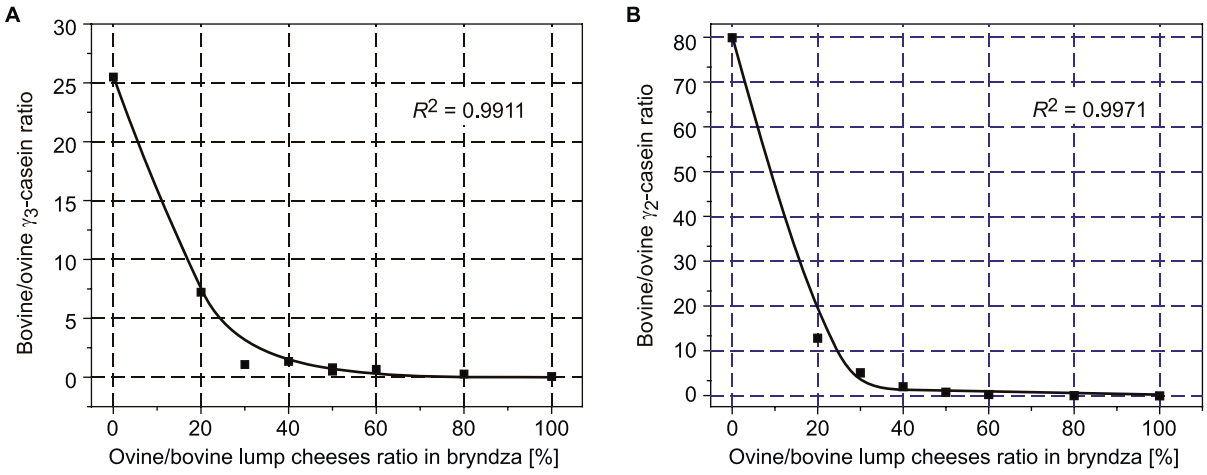

Fig. 3. Calibration curve plots for ewes'/cows' cheese content in bryndza *vs* ratio of bovine/ovine γ-casein. A – γ_3-casein, B – γ_2-casein.

ner without the need to perform extra calibration for winter and summer types of bryndza. On the other hand, multivariate analysis of these calibration data found differences between the samples subjected to plasmin treatment and without this procedure. From the plot of the principle components (Fig. 4) it can be seen that PCA using all the individual and ratioed casein markers significantly segregate samples with plasmin treatment from untreated samples thanks mainly to the variability and contribution of ratioed marker caseins B2/O2.

The mentioned calibration dissimilarities in plasmin-treated and untreated assays led to statistically significant differences in results achieved by applying this method on the commercial bryndza cheese samples. Fig. 5 compares densitograms of standard (50/50 ewes'/cows') and commercial summer type bryndza cheese. Overall results of ewes' lump cheese content determination (or cows' as a complementary value) in commercial winter and summer type bryndza cheeses treated and untreated with plasmin are presented in Tab. 1. First, the

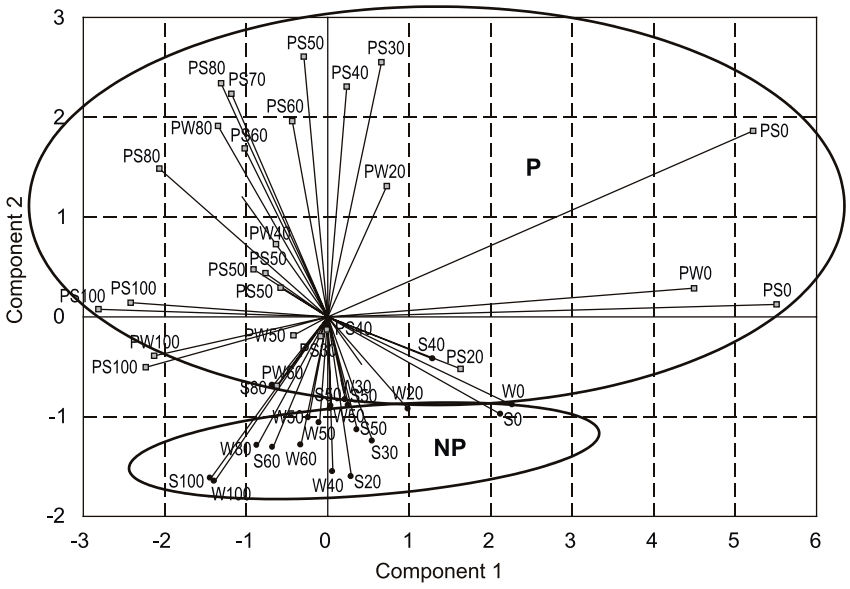

Fig. 4. Principal component analysis of calibration data of standard bryndza.
P – plasmin treatment; NP – no plasmin treatment; S – summer type bryndza, W – winter type bryndza with 0, 20, 40, 50, 60, 80 and 100% contents of ewes' lump cheese.
PCA using all individual ovine and bovine casein γ_2- and γ_3-markers and their ratios.

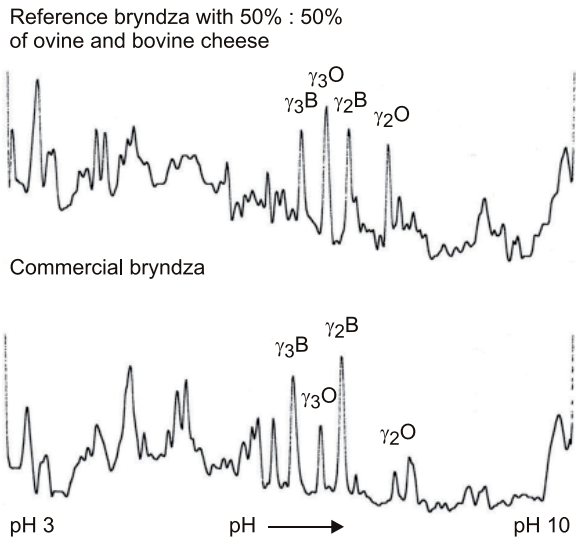

Fig. 5. Superposition of densitograms of 50/50 cows' and ewes' standard bryndza and commercial summer type bryndza cheese.
γ_2-, γ_3-caseins: O – ovine, B – bovine.

determinations of cheeses treated and untreated with plasmin were evaluated. The results show that the method without plasmin treatment resulted in higher recovery of ovine casein compared to the method with plasmin treatment. This can be explained by the fact that the enzymic treatment of bovine plasmin is used for cleaving of β-casein in order to intensify creation of bovine γ-casein, what is the objective of the original EU method for the detection of cows' protein addition to ewes' cheeses. Therefore, there arises a question whether it is necessary to use plasmin treatment in case of quantification of ovine and bovine caseins. Because plasmin treatment intensifies both ovine and bovine γ-casein bands, it may be useful in all cases. Furthermore, in the case when the traditional pure ewes' bryndza is produced, plasmin treatment should be used for the identification of potential adulteration of this cheese by cows' milk addition. Since too many variables affect the percentage of individual cheeses in mixed bryndza, the obtained quantitative results, as it was shown, should be considered only as approximate values in the adulteration control.

The results of determination of ewes' lump cheese in the summer and winter types of bryndza cheese (Tab. 1) show also that the most bryndza cheesemakers keep to the Food Codex limit 50% of ewes' lump cheese in bryndza [3] as a principal value for both summer and winter types of bryndza. Thus, at present they do not use the traditional recipe of the Slovakian summer type of bryndza, which was wholly made just from ewes' lump cheese. Only in samples from two bryndza cheese producers, higher (up to 80%) contents of ewes' lump cheese was found (producer 1 and 5). In contrast, one sample (from producer 6) was found to contain much less ewes' lump cheese than required, but this low contents was declared on the product label.

Tab. 1. Results of ewes' lump cheese content determination in commercial bryndza cheeses according to ratioed ovine and bovine γ-3 and γ-2 casein fractions.

Cheesemaker	Ewes' cheese in bryndza [%] *						
	Plasmin treatment			No plasmin treatment			
	Bryndza (summer type)		Bryndza (winter type)	Bryndza (summer type)		Bryndza (winter type)	
	γ_3 B/O (s_x)	γ_2 B/O (s_x)	γ_3 B/O (s_x)	γ_3 B/O (s_x)	γ_2 B/O (s_x)	γ_3 B/O (s_x)	γ_2 B/O (s_x)
Producer 1	51.8 (0.71)	48.7 (1.13)		79.2 (9.05)	78.5 (3.67)		
Producer 2	44.3 (1.48)	43.0 (1.27)		55.6 (2.12)	51.1 (6.72)		
Producer 3	42.9 (1.48)	42.8 (1.91)	42.4 (5.30)	47.8 (3.74)	38.6 (4.10)	45.5 (1.62)	50.3 (0.63)
Producer 4	48.7 (0.11)	49.4 (1.27)	45.7 (2.23)	58.4 (3.25)	64.7 (0.14)	48.8 (7.52)	51.7 (10.8)
Producer 5	67.2 (2.96)	59.2 (1.69)	54.9 (3.59)	75.7 (1.82)	79.0 (1.13)	79.2 (2.68)	79.1 (5.09)
Producer 6	33.0 (5.65)	13.9 (5.16)	35.2 (6.98)	31.6 (0.97)	48.1 (1.41)	26.5 (1.97)	33.2 (4.81)
Producer 7	40.1 (2.76)	42.6 (1.91)		53.3 (3.37)	47.8 (5.93)		
Producer 8			41.7 (1.62)			49.2 (9.19)	49.3 (4.81)
Producer 9			29.7 (7.72)			50.8 (4.66)	44.8 (3.75)

* – content of cows'lump cheese is complementary to ewes' lump cheese in bryndza (ewes' lump cheese + cows' lump cheese = 100%).
B – bovine, O – ovine.

CONCLUSION

Isoelectric focusing on ReadyStrip with immobilized pH gradient was applied to the quantification of ovine and bovine protein proportion in the Slovak bryndza sheep cheese. The method proves reliable results when the peak area ratios of ovine and bovine γ-2 and γ-3 caseins were applied for determination. The same calibration data may be used for both, summer or winter type of bryndza, respectively. These preliminary results achieved are a good starting point for upholding the method for control purposes, but it would be appropriate to devote more attention for preparation of model standard bryndza cheese to avoid contamination problem and testing the robustness of the method in the cases of undeclared addition of some protein substrates of different origin, using of alternative sources of rennet and study the proteolysis effect to quantification of casein markers. In terms of the other research ambition it would be appropriate to address greater attention to the proteomics analysis using the modern instrumentation such as LC MS/MS, LC-FTICR (liquid chromatography – Fourier transform ion cyclotron resonance) or MALDI-TOF (Matrix-assisted laser desorption/ ionization – time-of-flight mass spectrometry) for the evolution of new progressive methods for controlling of food protein composition.

Acknowledgements

This study was supported by a project No APVV 0319-07 of Slovak Research and Development Agency. Authors thank to Dr. Bernadetta Krkošková for technical assistance.

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Received 9 February 2010; revised 10 March 2010; accepted 14 March 2010.