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Proving the adulteration of ewe and goat cheeses with cow milk using the reference method of isoelectric focusing of γ-casein

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Summary

The aim of this study was to introduce a reference method for the detection of cow milk in ewe and goat cheeses (EC 273/08) in order to protect manufacturers and consumers from adulterations and imitations, and to ensure alignment with the demands of domestic and EU markets. The method includes isolation of casein from cheese, isoelectric focusing of γ_2- and γ_3-casein originating from the hydrolysis of β-casein by plasmin, the detection and quantitative determination of γ-casein in cow, ewe and goat cheese by densitometry. Ewe or goat cheese products with a minimum of 1 % of cow milk are considered to be adulterated. For the quantitative determination of cow, ewe and goat milk in cheeses, standard mixtures of cow-ewe and cow-goat milk were made by adding 0; 0.5; 1; 2; 5; 10; 25; 50; 75 and 100 % (v/v) of cow milk. The quantification was performed by determining the peak area ratio of cow γ-casein in comparison to ewe/goat casein in prepared standard cheeses. The calibration curves were calculated based on the relation of the peak area ratio of cow γ-caseins (calculated as the percentage of total γ-caseins) in contrast to the relative content (%) of cow milk in the mixture. The method proved to be adequate for the detection of raw and heat-treated cow milk in fresh and ripened cheeses made from ewe or goat milk, or a mixture of ewe and goat milk.

Key words: cheese adulteration, isoelectric focusing (IEF), γ-casein, identification of milk types, ewe cheese, goat cheese

Introduction

One of the main problems in cheese production is the adulteration of cheese produced from ewe and goat milk with less expensive cow milk, the presence of which is however not stated on the product declaration. Cow milk is most often used to adulterate cheeses made from ewe and goat milk due to its lower price and availability throughout the year. The addition of cow milk does not only change the organoleptic properties of the final product, but also affects its quality related to its suitability in terms of health intolerance, and thus deceives the consumers (Spuergin et al., 1997; Borková and Snášelová, 2005). Furthermore, the adulteration of products creates negative social consequences because it erodes the economic interests of consumers. Although mixing different types of milk is permitted by law, problems arise when the final product is not properly labelled. Namely, food placed on the Croatian market must be labelled, advertised and presented in adherence to the provisions of the Food Act (NN 46/07, 84/08, 155/08 and 55/11) and other regulations concerning foodstuffs. The labelling of foodstuffs must not deceive the final consumer about the characteristics of the food, particularly its nature, identity, properties, composition, quantity, expiration period, origin, and method of production. Therefore, determining the types of milk contained in cheese is of great importance for cheeses produced exclusively from one

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type of milk (ewe or goat), as well as for cheeses bearing the quality symbol of: protected designation of origin (PDO) (Samaržija et al., 2006; Commission Regulation, 2008; Zachar et al., 2011). Over the past thirty years, the applicability of the reference method (EC 273/08) used in this study has been proven for cheeses in verifying the content of cow casein in quantities greater or less than 1 % in cheeses produced from ewe or goat milk (Mayer, 2005). The cheese sample, or product of ewe/goat milk, is considered adulterated if both cow γ_2- and γ_3- caseins, or the corresponding peak area ratios, are equal to or greater than the level of the 1 % reference standard (Commission Regulation, 2008). The determination of adulterants i.e. the detection of cow milk in cheeses produced from other types of milk, using the reference method, is based on the differentiation of isoelectric points of cow γ_2- and γ_3-caseins, originating from the hydrolysis of β-casein by plasmin, in comparison to the value of the isoelectric points of the same proteins from ewe or goat milk (Commission Regulation, 2008). The pH value of the isoelectric points for cow γ_2-casein is 7.0; for γ_3-casein 6.5; as opposed to ewe or goat γ_2-casein at 7.2 and 6.7 for γ_3-casein (Sienkiewicz et al., 2006). Because of the same pH values of the isoelectric points of ewe and goat caseins, this method cannot be used to determine the content of ewe and goat milk in cheese (Addeo et al., 1990; Mayer et al., 1997).

Since the European Commission adopted regulations and rules for cheese produced exclusively from ewe or goat milk, it was necessary to develop a method that would enable adequate control of product composition. Therefore the European Commission adopted in 1992 the reference method of detecting cow milk and casein in cheeses made from ewe, goat and buffalo milk, or mixtures of ewe, goat and buffalo milk (Commission Regulation 1992, 2008).

The aim of this study was to introduce the reference method of detecting the presence of cow milk in ewe and goat cheeses (Commission Regulation, 2008) into the work of the Reference Laboratory for Milk and Dairy Products at the Faculty of Agriculture, University of Zagreb. The application of this method might help protect domestic producers who abide by good manufacturing practice and prevent the deception of consumers by adulteration and imitations of ewe and goat cheeses. It might also help to ensure alignment with the requirements of domestic and EU markets.

Materials and methods

Cheese samples

The calibration cheeses with known composition, produced from a mixture of cow-ewe or cow-goat milk with 0; 0.5; 1; 2; 5; 10; 25; 50; 75 and 100 % (v/v) cow milk, were prepared at the Lončar farm (Bedenica, Croatia). The cheeses, declared as cheeses produced exclusively from ewe or goat milk, were purchased from retail chains. All cheese samples were cool stored at -20 °C until analysis.

Isolation of casein

A quantity equivalent to 5 g cheese dry matter, or the reference standard BCR 599 (with 0 and 1 % cow milk, *Certified Reference Material* BCR-599, Geel, Belgium), was weighed into a 120 mL test tube. 60 mL of distilled water were added and the mixture was homogenised using a homogeniser (IKA T25 Ultraturax, Labortechnik, Staufen, Germany) at 10,000 rpm. The homogenised suspension was adjusted to a pH value of 4.6 using 25 % acetic acid. The suspension was then centrifuged for 5 min at 3000×g (Centra GP8R, Thermo IEC, USA) at room temperature and thereafter fats and whey were decanted. The precipitate was transferred to a clean test tube and homogenised at 20,000 rpm in 40 mL distilled water adjusted to pH 4.5 with 25 % acetic acid. After the addition of 20 mL dichloromethane, the suspension was re-homogenised and centrifuged.

The aqueous and organic phases were separated by decanting, and the casein layer was transferred to a clean test tube, re-homogenised in 40 mL of distilled water (pH 4.5) and 20 mL dichloromethane, and centrifuged. The procedure was repeated until both extraction phases (minimum two to three times) were colourless. The casein layer was homogenised in 50 mL acetone, and the suspension filtered through a medium flow filter paper (Schleicher & Schüll, Dassel, Germany). The casein residue remaining on the filter paper was washed with two separate 25 mL portions of acetone, allowing each time to dry in the air. The casein was pulverized in a mortar and kept at -20 °C until further analysis.

Hydrolysis of β-casein with plasmin

The isolated casein was dissolved in 0.2 M ammonium-carbonate buffer, pH 8.0 with 0.05

M EDTA to a final concentration of 20 mg/mL in a 2 mL Eppendorf test tube and homogenised for 20 min in an ultrasonic bath (Lab Sonic DLS 490, Donaulab, Switzerland). The reaction mixture was pre-incubated for 5 min at 38-39 °C, and then 20 μL of plasmin was added (EC 3.4.21.7) (5 U/mL, Roche Diagnostics, Indiana, USA). The suspension was incubated for 1 hour at 38-39 °C with continuous shaking in a thermostatic water bath. The reaction was stopped by the addition of 40 μL ε-aminocaproic acid. Samples were lyophilised using the *Alpha* 1-2 LDplus (Christ, Osterode am Harz**,** Germany). Prior to isoelectric focusing (IEF), lyophilised samples were dissolved for at least 1 hour at room temperature in a denaturing solution: 8 M urea, 7.9 % v/v ethylene glycol, mixture of Pharmalyte pH 3.5-9.5 and pH 5.0-8.0 in the ratio 1.2:1 (v/v) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and 0.032 M dithiothreitol (Sigma Aldrich, Steinheim, Germany).

Isoelectric focusing (IEF) of γ-casein

The IEF procedure for γ-casein was performed according to the modified EU reference method (Commission Regulation, 2008) using a Multiphor II with an Electrophoresis Power Supply EPS 3501 XL (GE Healthcare Bio-Sciences AB) on horizontal ultrathin-layer polyacrylamide gels, CleanGel IEF (5 % T, 3 % C) dimension 245×125×0.5 mm after re-hydration. The gel was equilibrated with gently stirring for approximately 2 hours at room temperature, in a solution containing: 8 M urea, 7.9 % v/v ethylene glycol and mixture of Pharmalyte pH 3.5-9.5 and pH 5.0-8.0, in the ratio 1.2:1 (v/v) (GE Healthcare Bio-Sciences AB). All chemicals for the IEF were of electrophoretic purity. The gel was pre-focused for 30 min at a charge of 2000 V and current strength of 20 mA. Sample volumes of 18 μL were applied using filter paper for sample application dimension 0.5×1 cm (GE Healthcare Bio-Sciences AB) laid onto the surface of the gel. The focusing conditions at the time entrance of the sample into the gel were: charge 2000 V and current 20 mA. After 1 hour, the filter papers were removed from the gel and focusing continued under the following conditions: 2000 V and current 30 mA for 30 min, then 2000 V and current 40 mA for 1 hour. To ensure the best possible focusing of proteins for the last 30 min, the conditions were adjusted to higher values. The charge was increased up to 2500 V, and the current to 40 mA. After focusing, proteins were visualised with Coomassie Brilliant blue G-250 (Sigma Aldrich, Steinheim, Germany) overnight with gentle shaking (Westermeier, 2005). After staining, the gel was rinsed with re-distilled water for 2 hours, transferred for 10 min to a solution of 2.5 % glycerol and air-dried overnight. All electrophoretic analyses were performed in triplicate.

Densitometric estimation

Evaluation was performed by comparing the protein patterns of the unknown sample with reference standards on the same gel. An ImageScanner III (GE Healthcare) was used to scan the gels after the IEF. Densitometric evaluation of γ_2- and γ_3-caseins in the polyacrylamide gels was performed using the program ImageQuant TL (GE Healthcare) for analysis of the 1D electrophoretic image**.** Determination was based on measurements of the peak area ratio of γ-casein in comparison to the reference standard for 1 % cow milk.

The standard mixture of cow-ewe or cow-goat milk, with 0; 0.5; 1; 2; 5; 10; 25; 50; 75 and 100 % (v/v) cow milk, was used to obtain the calibration curve, showed by calibration curves calculated from the relation of the peak area ratio of cow γ-caseins (expressed as a percentage of total γ-caseins) in contrast to the relative content (expressed in % (v/v)) of cow milk in the calibration cheeses made from various mixtures of a known composition (Graph 1 and 2).

Results and discussion

Isoelectric focusing of γ-casein

After isolation and hydrolysis of casein with plasmin according to the reference method (Commission Regulation, 2008), cheese samples and reference standards (BCR-599) were analysed using isoelectric focusing. After focusing, the clearly separated γ_2- and γ_3-caseins of cow milk were clearly differentiated from the same caseins originating from ewe or goat milk on the urea-polyacrylamide gels.

Figure 1 shows the results of the electrophoretic separation of γ-casein in calibration cheeses of cow-ewe milk (0-100 % cow milk) and commercial cheese samples (positions on gel: cheese samples lines 1-5).

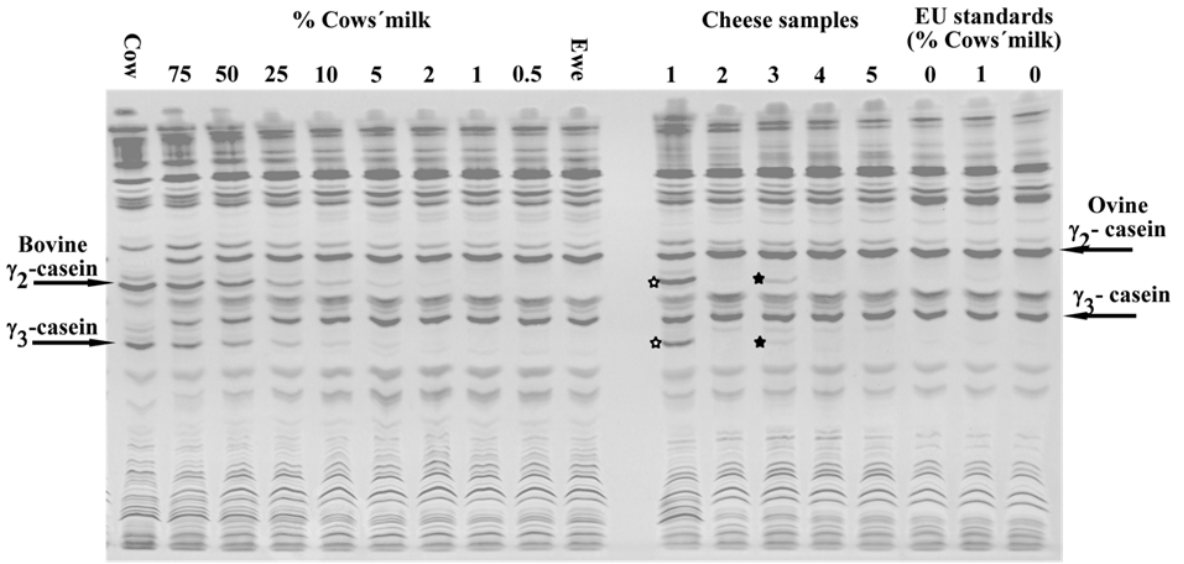
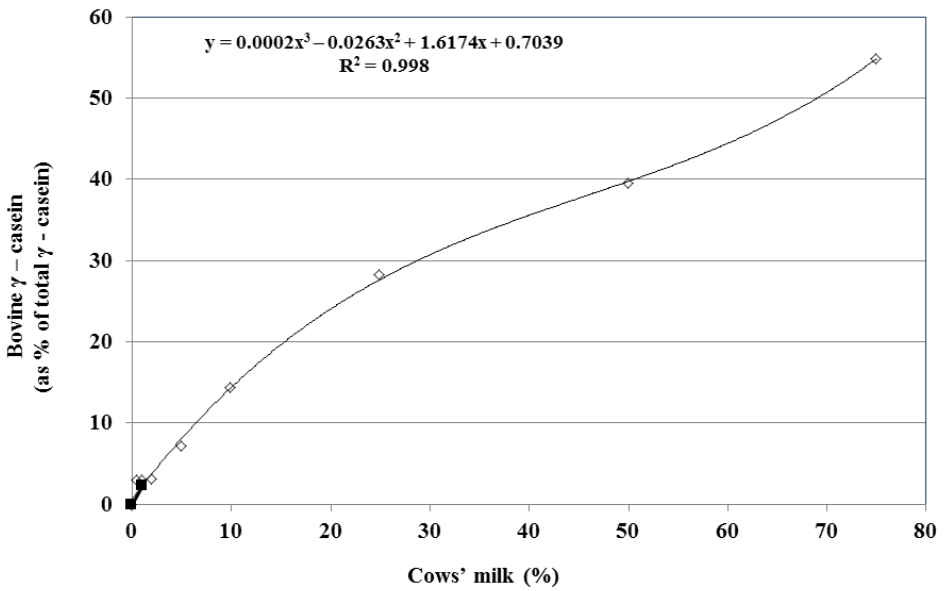


Figure 1. Isoelectric focusing of γ-casein of calibration cheeses made from a mixture of **cow-ewe milk**, reference standards BCR-599 (0 % and 1 % cow milk) and samples of ewe cheese (1-5) from retail shops declared as exclusively ewe cheese. Approximately 360 μg of protein was applied to each line on the gel. Proteins were visualized using Coomassie Brilliant blue G-250 (asterisk * indicates the presences of cow γ_2- and γ_3-caseins)



Graph 1. Calibration curve of the content of cow γ-casein (expressed as % of total γ-casein) in contrast to the relative % of cow milk in prepared calibration cheeses of a known composition of **cow-ewe milk** (♦). EU standards of 0 and 1 % cow milk (■)

For commercial cheese samples declared as cheese products made exclusively from ewe's milk, the isoelectric focusing detected adulteration, i.e. the addition of cow milk of approximately 55 % (position on gel: cheese samples line 1), or ~5 % (position on gel: cheese samples line 3), while cheese declared to be exclusively from ewe milk in positions on gel: cheese sample 2, 4 and 5 proved to not be adulterated.

Electrophoretic separation of γ-casein was applied for the calibration of cheeses produced from goat and cow milk (0-100 % cow milk) and commercial cheese samples. Based on the results of the analysis, the commercial cheese sample (Figure 2), declared as a product made exclusively from goat milk, was confirmed to have an addition of cow milk of ~30.0 % (position on gel: cheese sample line 6).

Graphs 1 and 2 show the calibration curves of cow γ-casein (as % of the total γ-casein) and the relative % of cow milk in the prepared calibration cheeses of a known composition. By using these relations the exact values of the content of cow milk in the cheese samples were determined. In order to obtain the most reliable results, a semi-quantitative determination of the cow milk content is essential. This requires a time-consuming densitometric evaluation of gels, where samples of unknown composition are analysed simultaneously with the reference standards on the same gel. The IEF method proved to be appropriate for the simultaneous determination of a large number of samples. To verify the results obtained in this study, the analysed cheese samples were sent to two European laboratories (Laboratory 1 and Laboratory 2). Table 1 shows the results of the collaborative study.

The results of the applied method (Commission Regulation, 2008) were determined visually in Laboratory 1. In Laboratory 2 as well as in the Reference Laboratory for Milk and Dairy Products at the Faculty of Agriculture, University of Zagreb the results were determined employing densitometry. Each of the mentioned laboratories used the standards with defined contents of cow milk for the analyses of ewe cheeses, and these were thus expressed as approximate values. The experimental results obtained in these two independent European laboratories corroborate the values obtained by the Reference Laboratory for Milk and Dairy Products. Hence it can be concluded that the method was well implemented in the work of the Laboratory, and that it can be considered

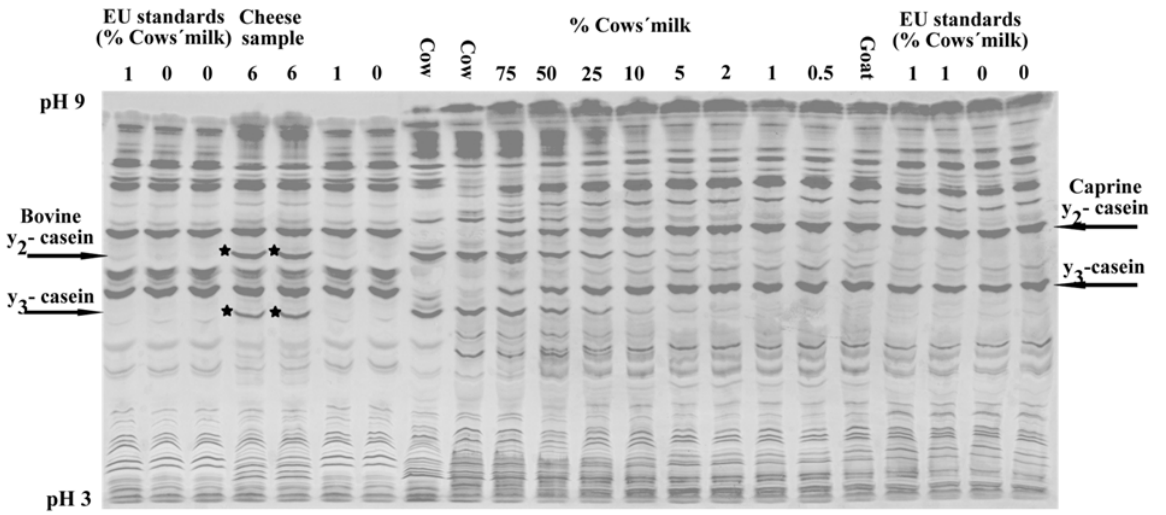
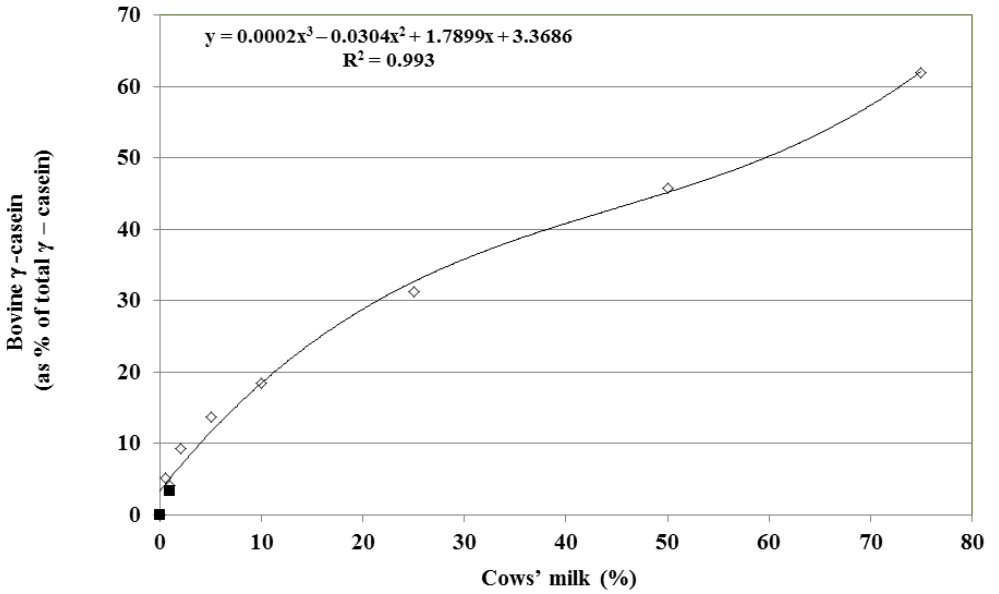


Figure 2. Isoelectric focusing of γ-casein of calibration cheeses made from a mixture of **cow-goat milk**, EU standards (0 % and 1 % cow milk) and sample of goat cheese (6) from retail shops declared as exclusively goat cheese. Approximately 360 μg of protein were applied to each line on the gel. Proteins were visualized using Coomassie Brilliant blue G-250 (asterisk * indicates the presence of cow γ_2- and γ_3-caseins)



Graph 2. Calibration curve of the content of cow γ-casein (expressed as % of total γ-casein) in contrast to the relative % of cow milk in prepared calibration cheeses of a known composition of **cow-goat milk** (♦). EU standards of 0 and 1% cow milk (■)

Table 1. Overview of results of the collaborative study to determine the adulteration of cheeses according to the EC method "*Reference method for the detection of cows` milk and caseinate in cheeses from ewes' milk, goats' milk and buffaloes' milk or mixtures of ewes', goats' and buffaloes' milk*" (Commission Regulation, 1996; 2001; 2008)

Sample	Reference Laboratory for Milk and Dairy Products	Laboratory 1	Laboratory 2
	Cow´s milk percentage approximately (%)		
1	~5	~5	Not determined
2	~25	~20	~25
3	~10	Not determined	~10
4	~51.5	Not determined	~53

reliable. The introduction of this method for the control of adulteration will enable the improvement of the food quality control (cheeses made from ewe and goat milk) and might help confirm the standard quality of Croatian products. It might also be a good tool for improving the labelling of cheese in order to ensure better alignment with the national legislation in the area of product quality and suitability. Additionally, it could also help to enhance the level of competitiveness of Croatian products on the market and improve consumer protection. The application of this method will also enable those manufacturers who abide by good manufacturing practices to improve their competitiveness over those manufacturers who do not abide by such practices.

Conclusion

The reference method for determining the presence of cow milk in ewe and/or goat cheeses (Commission Regulation, 2008) is based on the detection of cow γ_2- and γ_3-caseins from cheese after electrophoretic separation on urea-polyacrylamide gels from the homologous proteins of ewe or goat milk. This method has proven to be reliable and sensitive in detecting raw and heat-treated cow milk in fresh and ripened cheeses produced from ewe or goat milk, or mixtures of ewe and goat milk. The method for controlling cheese adulterations has successfully been implemented and is applied within the frame of the regular activities and analyses performed by the Reference Laboratory for Milk and Dairy Products.

Dokazivanje patvorenja ovčjih i kozjih sireva s kravljim mlijekom referentnom metodom izoelektričnog fokusiranja γ-kazeina

Sažetak

Cilj rada bio je uvesti referentnu metodu za dokazivanje kravljeg mlijeka u ovčjim i kozjim sirevima (EC 273/08), radi zaštite proizvođača i potrošača od krivotvorenja i imitacija, te usklađivanja sa zahtjevima domaćeg i EU tržišta. Metoda obuhvaća izolaciju kazeina iz sira, izoelektrično fokusiranje γ_2- i γ_3-kazeina dobivenih hidrolizom β-kazeina s plazminom, te detekciju i kvantitativno određivanje γ-kazeina kravljeg, ovčjeg i kozjeg mlijeka denzitometrijom. Proizvod od ovčjeg ili kozjeg mlijeka smatra se patvorenim ukoliko se dokaže prisutnost od minimalno 1 % kravljeg mlijeka. U svrhu kvantitativnog određivanja kravljeg, ovčjeg i kozjeg mlijeka u sirevima, pripremljene su standardne smjese kravljeg i ovčjeg, odnosno kravljeg i kozjeg mlijeka s 0; 0,5; 1; 2; 5; 10; 25; 50; 75 i 100 % (v/v) kravljeg mlijeka. Kvantifikacija je provedena određivanjem odnosa površina vrhova kravljih γ-kazeina prema ovčjim/kozjim, u pripremljenim standardima sireva. Izračunate su kalibracijske krivulje odnosa površina vrhova kravljih γ-kazeina (izračunatih kao postotak od ukupnih γ-kazeina) u odnosu na relativni postotak kravljeg mlijeka u smjesi. Metoda se pokazala prikladnom za detekciju sirovog i toplinski obrađenog kravljeg mlijeka u svježim i zrelim sirevima, proizvedenih od ovčjeg ili kozjeg mlijeka ili mješavine ovčjeg i kozjeg mlijeka.

Ključne riječi: patvorenje sira, izoelektrično fokusiranje (IEF), γ-kazein, identifikacija vrste mlijeka, ovčji sir, kozji sir

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