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Analytical methods for the species identification of milk and milk products

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Summary

The objective of this article is to point out the importance of milk and dairy product authentication with particular focus on the application of analytical methods to detect adulteration.The production of sheep and goat milk has considerable economic importance resulting from the widespread acceptance of traditional cheeses, many made exclusively of pure sheep milk. Fraudulent incorporation of nondeclared kind of milk during technological processing is a common practice that can cause a problem for reasons related to intolerance or allergy, religious, ethical or cultural objections, and legal requirements. Unfortunately, fraudulent substitution of sheep and goat milk with the cheaper cow milk is a common practice and for the detection of mutual adulteration various methods have been reviewed, such as immunological, electrophoretic, chromatographic, and PCR techniques.

Key words: analytical methods, milk, cheese, adulteration

Introduction

The extensive consumption of milk and dairy products makes these foodstuffs targets for potential adulteration with financial gains for unscrupulous producers (Nicolaou et al., 2011). Common adulterations of dairy products are the substitution of higher value milk by nondeclared milk or the omission of a declared milk species. Thus, the detection of milk species is important in cheese producing branch, especially those made from one pure species and with protected designation of origin (PDO), such as pure sheep or pure goat cheeses (Bottero et al., 2002).

In Commission Regulation (EC) No 676/2008 of 16 July 2008 certain names of protected designations of origin and protected geographical indications (PGI) are registered, among them also the third Slovak product "Slovak sheep cheese - bryndza" with PGI designation.

Zeleňáková et al. (2009) described current situation in adulteration of the sheep milk and sheep milk products in Slovakia as well as in some countries in the EU. The results were evaluated according to the requirements of the valid legal standards. From 70 samples, 20 were adulterated with nondeclared cow's milk. Impact of environment and breed affilitation were described by Siviková and Buleca (1999), Popelka et al., (2001), Buleca et al. (2002a, 2002b), Dudríkova et al. (2007) and Židek et al. (2008).

To avoid the possible fraudulent substitution of goat and sheep milk with cow's milk, it is necessary to develop analytical procedures able to detect such frauds and protect the consumers from misleading labelling (De la Fuente and Juárez, 2005).

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Analytical methods for the detection of milk and milk products adulteration

Authenticity testing of food products, such as meat, milk or fish, is important for labeling and assessment of value and is therefore necessary to avoid unfair competition and assure consumers protection against fraudulent practices commonly observed in the food industry (Xue et al., 2010). The majority of dairy products' authenticity identification methodologies are based on major milk proteins analysis (Stanciuc and Rapeanu, 2010).

Different analytical approaches have been applied for identification purposes; among these, immunological (Xue et al., 2010; Zeleňáková et al., 2008; Hurley et al., 2004), electrophoretical (Mayer, 2005), chromatographic (Enne et al., 2005) and PCR (Mafra et al., 2007) are worth mentioning. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-ToF-MS) is a potentially useful technique, with proven abilities in protein identification and more recently through the use of internal standards for quantification purposes of specific proteins or peptides (Nicolaou et al., 2011).

Recently Fourier transform infrared (FT-IR) spectroscopy combined with chemometric methods have been described as rapid methods for adulteration detection (Nicolaou et al., 2010).

The present European Community reference method for detection of cow milk and caseinate in cheeses made from ewe milk, goat milk, buffalo milk or mixtures of ewe, goat and buffalo milk is isoelectric focusing of γ-caseins after plasminolysis (Commission regulation (EC) No 273/2008).

Chromatography

Chromatography is a very well known unit operation in downstream processing of protein mixture. In chromatographic techniques, the principle separation occurs due to the different migration of the component of interest between the stationary phase (i.e. matrix phase) and continuous phase (i.e. solvent) in the system. Chromatography media (i.e. stationary phase) is normally packed into a column depending on the process scale. Various types of chromatography mode or interaction are available, such as size exclusion, ion exchange, hydrophobic interaction and reverse phase chromatography. They differ in terms of the separation mechanism and selection of stationary and continuous phase (Ghosh, 2002; Kawai et al., 2003).

Hydrophobic interaction chromatography (HIC) was applied to commercial casein mixture and to the qualitative and quantitative analysis of casein fractions in unprocessed, raw cows', goats' and ewes' milk (10 samples analyzed for each species), in one sample of unprocessed buffalos' milk and in commercial cheeses (mozzarella, robiola, ricotta and stracchino). The precision of the method was evaluated, the coefficient of variation for alpha-, beta- and kappa-casein determination ranged between 3 and 6 % (Bramanti et al., 2003).

Ferreira and Caçote (2003) have used the same technique (RP-HPLC) to detect and quantify cows', sheep' and goat' milk percentages in milks and in Portuguese protected enomination cheeses. The chromatographic profiles of β-lactoglobulin and α-lactalbumin extracted from the investigated milks were very different. Additionally, different cheeses were manufactured using different proportions of cows', sheep' and goat' milk: mixtures of 20 % of cow milk and 80 % of sheep milk; 50 % of cow milk and 50 % of goat milk; and 50 % of sheep milk and 50 % of goat milk. All these milk mixtures were firstly analysed by RP-HPLC and then used to produce cheeses. The authors concluded that the RP-HPLC is a very sensitive and accurate method for studying milk percentage as well as fresh and ripened cheeses made from binary mixtures of cow, sheep or goat raw milk.

Urbanke et al. (1992) have also used RP-HPLC for control of the milk adulteration. A reversed-phase HPLC method for the identification of cow's milk has been developed. It enables the detection of 1 % cow milk in human milk by bovine β-lactoglobulin (AB), bovine α-lactalbumin in the whey fraction and κ-casein in the casein fraction.

The aim of research carried out by Stanciuc and Rapeanu (2010) was to detect the presence of cow milk in sheep and goat cheeses which are sold in the retail markets of Romania. For this purpose, a total of 73 sheep and goat cheese samples were purchased randomly from different markets. An immunochromatographic test kit was used to detect

the presence of cow milk in sheep and goat cheeses. No adulteration was found in 32.6 % and 20.3 % of sheep and goat cheese samples, respectively, while the presence of cow's milk was detected in 67.3 % and 79.7 % of samples, respectively.

Colak et al. (2006) have used immunochromatographic test for the detection of cow milk in sheep cheese presence. For this purpose, a total of 100 sheep cheese samples were purchased randomly from different markets. Immunochromatographic test kit was used to detect the presence of cow milk in sheep cheeses. While no adulteration was found in 52 % of cheese samples, cow milk was detected in 48 % of cheese samples.

Electrophoresis

A method based on isoelectric focusing and cation-exchange HPLC of *p*-casein (Mayer, 2005) has been proposed for quantitative analyses. However, as the estimated percentage of bovine milk in mixed cheese is strongly affected by the casein content of milks used for cheese manufacture, the results were approximate. On the other hand, methods for milk species quantification based on the whey protein fraction suffer from a shortcoming, as that fraction is more sensitive to heating than the casein fraction. Thus, such methods can cause false negatives when sterilized or powdered milk has been used in the cheese manufacture. Excessive proteolysis during cheese ripening can also be disadvantageous for quantification.

Cartoni et al. (1999) have developed capillary zone electrophoresis to determine the adulteration of cow milk in goat milk products. The detection and quantification of cows' milk was based on the presence of the specific whey proteins by the relative calibration curve. The minimum amount of detectable cow milk was 2 % in milk mixtures and 4 % in cheeses. Restrictions due to genetic variability and possible heat treatments, on only one of the two types of milk employed, are taken into account.

Molina et al. (1999) have carried out analysis of cows', sheep' and goat' milk mixtures by capillary electrophoresis. Adultered amount have been quantificated by multivariate regression analysis.

ELISA

ELISA is the most widely used form of immunoassay in milk analysis and has advantages of high sensitivity, low cost and fast application. It is easy to use, reliable, rapid and readily automated (Bottero et al., 2002; Popelka et al., 2002).

The presence of undeclared milk in other species milk or cheese, in principle, can be detected through using two basic ELISA methods: sandwich ELISA and indirect ELISA, including their different variations. The development of immunoenzymatic methods and their practical use depends mainly on the selection of the immunogenes, experimental animals, way of immunization, quality of used antiserum, or possibly used antibodies and specificity as well as sensitivity of the evidencing system (Levieux and Venien, 1994; Haza et al., 1999).

ELISA is able to detect cows' and goats' milk in milk mixtures by polyclonal and monoclonal antibodies produced to combat whey proteins, caseins or short-string peptides from milk proteins. The caseins which represent the main part of the protein fraction, feature advantage in being more or less stable under high temperature conditions. Therefore they can be successfully used as the main antigens in the heat treatment (pasteurization, UHT) of milk and dairy products. Their major disadvantage is weak immunogenicity and higher sensitivity to protheolytic degradation. Whey proteins, contrary to casein, are much better immunogens and they are protheolytically degradable only in minimal quantity. In respect of high temperatures, whey proteins are less resistant. At present, there are a small number of ELISA tests with really sufficient sensitivity for detection of additives in the heat treated milk (Zeleňáková et al., 2008).

An indirect enzyme-linked immunosorbent assay (ELISA) was developed for the detection and quantification of bovine milk adulteration in goat's milk. The polyclonal antibodies have been modified by mixing with goat's milk for the assay purposes. The absorbance at 450 nm in indirect ELISA revealed a linear relationship with the concentration of adulterated bovine milk at the range of 4 % - 50 %. Detection limit was 4 % for mixed milk samples. The assay was characteristics of high reproducibility with intra- and inter-assay variation coefficients less than 5 %. Therefore, the ELISA can be successfully used

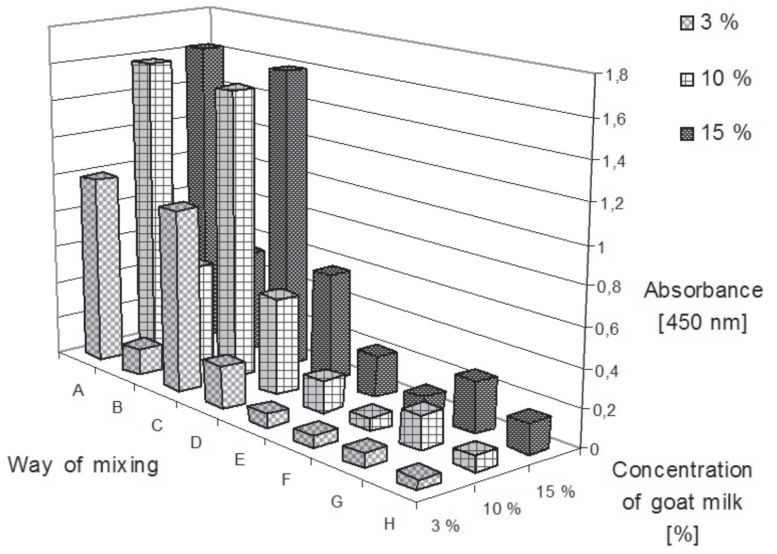

Figure 1. Impact of the thermal treatment of milk on the absorbance of adulterated amounts (3; 10; 15 % goat milk in sheep milk) (Zeleňáková and Golian, 2008)

Table 1. One - way ANOVA of absorbance values of studied milk amounts (Zeleňáková et al., 2008)

Goat milk conc.	Sources of variability	SS	Value P	F	F crit
3 %	Mixing	2.28511	0.000	14193	3.5
	Total	2.2853			
10 %	Mixing	5.57804	0.000	9252	3.5
	Total	5.57873			
15 %	Mixing	5.47566	0.000	56125	3.5
	Total	5.47577			

SS - Sum of squares; Value P - level of significance (P>0.05; *P<0.05; **P<0.01; ***P<0.001); F - testing criterion of F test; F crit - critical value of the Fischer-Snedecor distribution

to determine the adulteration of milk samples and it is suitable for developing a kit in routine inspection of milk (Xue et al., 2010).

An approach to estimate the proportion of goat's milk in sheep milk is described by Zeleňáková and Golian (2008) where these authors have focused on laboratory testing and evaluation of quality parameters of the ELISA tests. The detection and quantification of goat milk was based on the presence of the specific immunoglobulins (IgG). For the evaluation of the studied indicators the tests were applied to total of 43 samples corresponding to 43 combinations of goat and sheep milk mixtures, while there 86 measurements were performed. Using the laboratory testing of specificity and sensitivity of the ELISA tests applied it was established that the quality of adulterated milk detection is being impacted by a standard curve with a specific detection range. It was found that heat treatment of milk (71.7-77 °C for 20 sec.) had an influence on identification of the adulteration. The samples pasteurized in different combinations gave lower optical density responses than those prepared from the raw milk (Fig. 1).

Based on one factor analysis of variance (Tab. 1) it was proven that the absorbance of the defined adulterated amounts was influenced by the mode of mixing of the goat and sheep milk (including the heat treatment of milk). This fact is documented either by the P value or by the value of the F criterion tested.

The significance level was set at P<0.05, thus indicating that within the measured absorbance val-

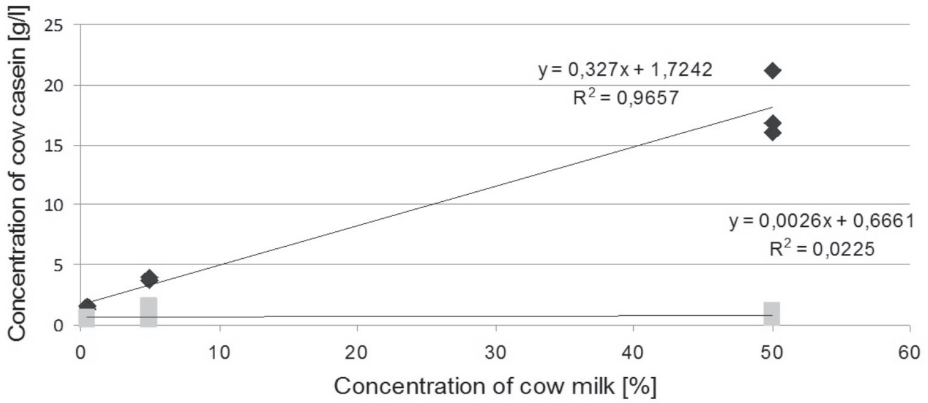

Figure 2. Trend of detected cow casein in milk and cheese [g/L] (Zeleňáková et al., 2010a)

ues of different adulterated amounts there were statistically significant differences.

The value of the test F criterion was in all the cases higher than F crit. (F > F crit.), thus indicating that the variation factor studied influenced the absorbance dispersion considerably (Zeleňáková et al., 2008).

In order to take any decision about potential frauds, the industries must ensure that the milk-testing laboratories have the analytical techniques validated and their uncertainty calculated. It is very important to realize evaluation of a commercial ELISA method for the quantitative detection of milk and cheese adulteration (Costa et al., 2008; Zarranz and Izco, 2007).

Zeleňáková et al. (2010a) have realized laboratory testing of ELISA kit (casein ELISA Set) for detection of cow casein in sheep milk in order to obtain high-quality, reliable and economically beneficial method suitable for routine application in practice. The results (Fig. 2) showed that this assay takes only about three hours and is suitable for detection of lots of sheep milk adulterated with 0.5 to 50 % cow milk (regression equations with R^2 determination coefficient: R^2=0.965). The experiments have shown that used ELISA test is not suitable to reliably detect the presence of cow milk casein in sheep cheese (R^2=0.022).

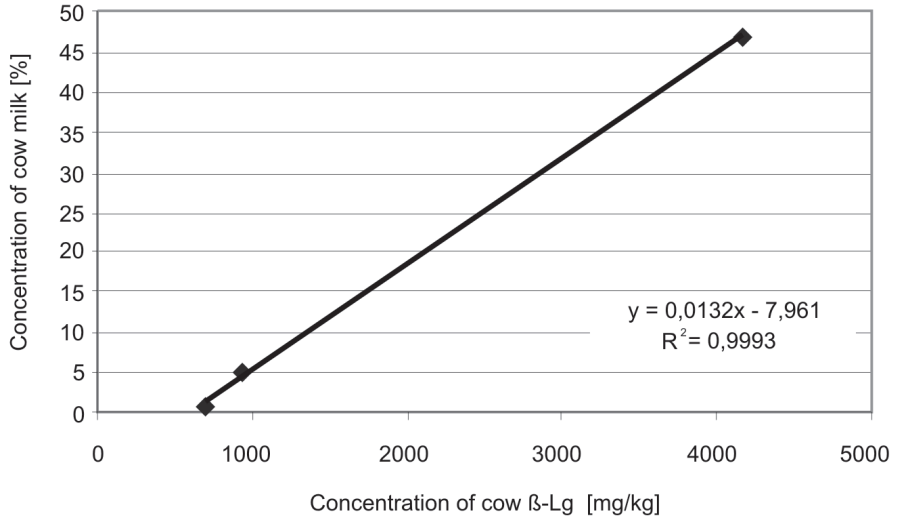

Figure 3. Linear function with the regression equations for concentration conversion of raw cow milk β-Lg in sheep milk samples (Zeleňáková et al., 2010b)

High specificity of this method is also reflected in the concentration amounts, in which ELISA results are expressed worldwide, and thus either in ppm (mg/kg) or ppb (μg/kg). The main advantages are processing of a large number of samples, creation of calibration curve and measuring of blind samples simultaneously on one microtitration plate, which eliminates the impact of changing conditions during determination. ELISA detects proteins as food allergens. ELISA is sufficiently sensitive and specific for detection of food allergen residues. ELISA can also be produced in formats that are compatible with the industrial food processing environment. However, ELISA also have disadvantages that should be carefully evaluated and widely recognized. (Taylor et al., 2009).

For better quality determination, especially of low concentrations, it is necessary to find an appropriate dilution for various concentrations of cow milk in the context with the way of its thermal treatment as well as further technological processing. The starting point for obtaining relevant data was to create separate regression curves (Fig. 3) with high determination coefficient, which allowed quick and easy detection of cow milk additions in sheep milk in cloddish cheese and Slovak sheep cheese (Zeleňáková et al., 2010b).

PCR

In relatively recent years molecular biology techniques have been used for species identification in food of animal origin. These techniques take advantage of the high specificity and sensitivity of polymerase chain reaction (PCR)-based methods to detect very low amounts of cow milk, such as 0.5 % (Feligini et al., 2005) or 0.1 % (Lopez-Calleja et al., 2005a).

Moreover, the DNA based methods like PCR have been successfully applied to matured cheeses (Mayer, 2005) and heated dairy products (López-Calleja et al., 2005b), in comparison with protein-based methods, which are not always applied and have to be chosen carefully. However, attempts to use PCR as a quantitative tool for food authentication are still very scarce.

Maudet and Taberlet (2001) have described a very simple approach to estimate the addition of cow milk in goat cheese, although this method lacks control over the variations associated with sample and gel preparation.

Mafra et al. (2004) have developed another approach to quantify the addition of cows' milk to sheep milk cheese by means of a duplex PCR technique, which used a normalised calibration curve to control the problems associated with DNA extraction and gel preparation. Duplex polymerase chain reaction allow the detection of partial or even total substitution of cow milk for buffalo milk, in some cases in samples of cheese misleadingly labeled "pure buffalo" mozzarella.

Bottero et al. (2002) have applied the primers proved to be species-specific, giving rise to 279-bp (bovine) and 192-bp (buffalo) amplified fragments. The results, carried out by Rea et al. (2001) indicate the applicability of this method, which showed an absolute specificity for the two species and a high sensitivity even down to low DNA concentrations (1 pg).

Mašková and Paulíčková (2006) have used PCR method for detecting cow's milk in goat and sheep cheeses. DNA was isolated from the cheeses using the isolation kit Invisorb Spin Food I by Invitek Co., designed for the samples of animal origin. The PCR method applied utilizes the sequence of the mitochondrial gene coding cytochrome b which is specific for mammals. It uses the common forward primer and the reverse primer species- specific. After electrophoresis, cow DNA was characterised by the fragment of the size of 274 bp, goat DNA by the fragment of 157 bp, and sheep DNA by the fragment of 331 bp. The detection limit of the described PCR method (1 %) was determined with model samples made from pure goat cheese with a defined addition of cheese made from cow's milk. The validated method was applied in the analysis of 17 goat cheeses and 7 sheep cheeses obtained from retail trade. Products of Czech, Slovak, French, Dutch, and Italian origin were examined. The presence of undeclared cow milk was detected in three kinds of goat cheese and in one of sheep cheese.

Mafra et al. (2007) identified cow milk in sheep and goat milk by using primers targeting the mitochondrial 12S rRNA gene. The technique allowed the detection of 0.1 % of cow milk with a 35-cycle duplex PCR and quantification in the range

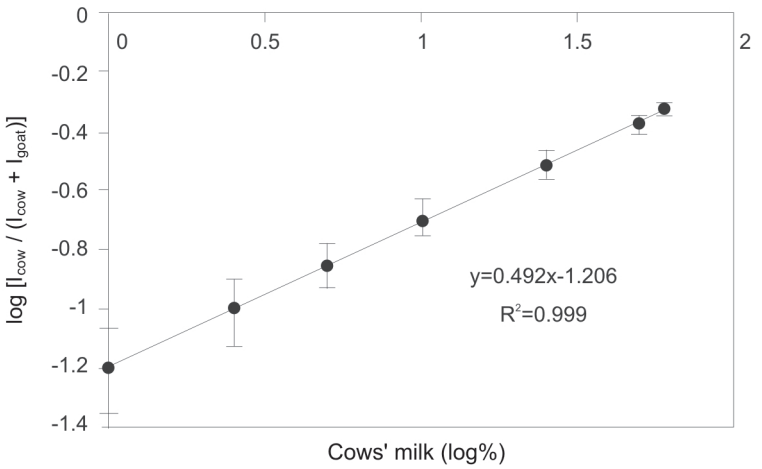

Figure 4. Normalised calibration curves for quantification of bovine milk in ovine milk cheeses obtained with a 30 cycles-polymerase chain reaction (PCR) (Mafra et al., 2007)

of 1-60 % with a 30-cycle duplex PCR by means of a normalised calibration curve that was successfully applied to known cheeses (Fig. 4).

Bobková et al. (2009) have applied PCR method for detection of sheep milk adulteration by cow milk. In accordance with the stated objective was performed analysis of 8 intentionally falsified sheep milk samples. Detection limit was 0.01 % of cow milk.

Also Zeleňáková et al. (2009) have used PCR method for the analysis of 70 milk and cheese samples. From the total number (20) of the analyzed sheep milk samples, cow milk occurrence was detected in 8 samples. From the 30 samples of sheep cheese, 12 samples contained a mixture of the cow milk. The exceptions were the samples of Slovak sheep cheese (bryndza), such as traditional dairy products, which are composed of several kinds of milk (min 50 % sheep milk, which is declared on the label). Exact quantification of samples was not made because of the use of a simple PCR.

Conclusions

This article deals with topic that is of great interest for broader scientific public as well as for dairy industry and regulatory bodies in search for critical evaluation of available methods for detecting adulteration. The importance of this topic is clearly illustrated by existence of EC reference method for detection of cow milk in ewe, goat, buffalo milk or their mixtures.

A variety of analytical methods are potentially available for use in milk authentication. They vary in their complexity and cost and both of these factors are liable to influence the uptake of such tests by food control laboratories. With the likelihood of increased regulation of food products, in response to consumer concern, tests such as those described here, many of which are used routinely in a research environment, could be adopted for use in the marketplace. However, before this can occur, strict processes of validation would need to be undertaken. Indeed, examination as to the reliability and reproducibility of such protocols for more general use is already underway and some of the methods described above are currently in practice. The use of high quality ELISAs and PCR methods in combination will ensure the food analyst can gain sufficient evidence to enforce European Commission legislation and control adulteration in dairy products.

Acknowledgement

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Analitičke metode u identifikaciji vrsta mlijeka i mliječnih proizvoda

Sažetak

Svrha ovog rada bila je prikazati važnost provjere autentičnosti mlijeka i mliječnih proizvoda, s posebnim naglaskom na primjenu analitičkih metoda za otkrivanje patvorenja. Nedopušteno dodavanje jeftinijih vrsta mlijeka tijekom tehnološkog procesa proizvodnje postala je ustaljena praksa koja može prouzročiti probleme u smislu netolerancije ili alergija, vjerskih, etičkih i kulturoloških prigovora te propisanih zahtjeva kakvoće. U radu su prikazane i raspravljene različite metode identifikacije vrsta mlijeka i mliječnih proizvoda, poput elektroforetskih, kromatografskih, PCR i imunoloških tehnika.

Ključne riječi: analitičke metode, vrste mlijeka, sir

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