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DETECTION OF OVINE MILK ADULTERATION USING TAQMAN REAL-TIME PCR ASSAY

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ABSTRACT

Food safety, quality and composition have become the subjects of increasing public concern. To prevent fraud and enhance quality assurance, credible analysis of dairy products is crucial. Bovine milk is more widely available and cheaper than milk of sheep and goat. Bovine milk is also processed in large quantities to produce a range of dairy produce. DNA-based methods have proven to be more reliable, because of the stability of DNA under the conditions of high temperature, high pressure, and chemical treatment used during the processing of some food products. The commercial InnuDETECT cheese assay based on the principle TaqMan real-time PCR systems have been tested for the identification and quantification of bovine DNA in ovine milk samples. DNA was extracted using the InnuPREP DNA Mini Kit and quantified by the QuantiFluor dsDNA system. The assay showed good linearity, with correlation coefficient of $R^2 = 0.983$ and efficiency of 86%. The internal control amplified fragment from different mammalian species (cow, sheep and goat), with similar C_T values. Detection of bovine DNA in milk mixtures was achieved even in samples containing 0.5% of bovine milk. The InnuDETECT cheese assay has been successfully used to measure bovine DNA in ovine milk, and will prove useful for bovine species identification and quantitative authentication of animal-derived products.

Keywords: TaqMan PCR; Cow; Sheep; Milk adulteration

INTRODUCTION

Nowadays, consumers are more and more interested in the topic of wholesomeness and authenticity of food, so the identification of the animal species is gaining increasing importance **(Dalmasso, Sacchi and Bottero, 2012)**. Dairy field is subjected to growing number of frauds **(Kemal Seçkin, Yilmaz and Tosun, 2017)**.

Differences in price and seasonal availability might make it attractive for farmers to adulterate expensive ovine milk with cheaper bovine milk **(López-Calleja et al., 2007)**. Besides, milk origin is important in cheese making, especially those made from one pure species and with protected designation of origin, such as pure ovine or pure caprine cheese. In addition, some cheeses are manufactured with defined amounts of each type of milk **(Mafra, Ferreira and Oliveira, 2007)**.

European Regulations require that producers declare the type of milk used in manufacturing. Unintentional mislabelling may also occur when several species are handled on the same manufacturing equipment **(Di Domenico et al., 2017; Kemal Seçkin, Yilmaz and Tosun, 2017)**. Whether fraudulent or unintentional, compliance of dairy products with the Regulations is mandatory since mislabelled products give rise to economic loss and possible dangers to public health because milk proteins from any animals (most commonly bovine) are potential allergens **(Agrimonti et al., 2015; (Di Domenico et al., 2017)**.

Unfortunately, in the market, milk origin cannot be identified by consumers looking at the name of the product. Therefore, there has been huge effort to develop and improve analytical methods for milk authentication. The official EU reference method, which is based on the isoelectrofocusation of γ-caseins, is an appropriate tool to detect bovine milk in products made from milk of other species (detection limit ≤0.5%) **(Mayer, Bürger and Kaar, 2012)**. Other protein-based approaches include ELISA **(López-Calleja et al., 2007; Costa et al., 2008; Zeleňáková et al., 2016)** and HPLC **(Mayer, 2005; Motta et al., 2014)**.

In recent years, significant attention has been turning towards DNA-based approaches, which have proven to be reliable, sensitive and fast for many aspects of food authentication **(Mayer, 2005)**. The amount of DNA recoverable from milk and milk products is directly related to the somatic cell content of the raw milk and to the strength of the technique used to process the product,

because this can influence the integrity and the extractability of the DNA **(Rea et al., 2001)**.

Traceability of foods has become very important problem respect to food quality and typicalness of foods. Milk origin cannot be identified by the consumer and they are sold at different prices under various product names. Recently, this has caused the problem of 'adulteration' **(Kemal Seçkin, Yilmaz and Tosun, 2017)**.

Among them, the polymerase chain reaction (PCR) is undoubtedly the most common genetic technique used for tracing the species origin in food because DNA is extremely persistent during food processing and can retain sequence-specific information retrievable after an amplification **(Agrimonti et al., 2015)**.

As DNA can be obtained from thermally treated milks such as, pasteurised milk, ultra-pasteurised and powder milks, as well as bovine milk caseinates, cheese adulterations by partial or total substitution of non-declared milk species can be easily detected by PCR techniques **(Mafra, Ferreira and Oliveira, 2007)**. If the somatic cell content in a mixture (milk or cheese) is similar in the two species, it should be possible to quantitatively trace the amount of undeclared milk by comparing the sample to well-known standard DNA mixtures **(Rea et al., 2001)**.

Molecular techniques using DNA technology to combat fraud, improve traceability and distinguish between closely related species are being increasingly utilised in food forensic analysis **(Caldwell, 2017)**.

A successful PCR assay depends largely on the quality of extracted DNA; thus, extraction of high-quality DNA has been a crucial step in the authentication process. Numerous DNA extraction methods have been used for the preparation of DNA from milk, including research protocols and commercial kits **(Liu et al., 2014; Liao et al., 2017; Pokorska et al., 2016)**.

In the study, a commercial kit for real-time PCR was used to test reliability of quantification of bovine milk in prepared milk mixtures.

MATERIAL AND METHODOLOGY

Sample preparation

Fresh and processed commercial cow and sheep milks were purchased from several national food retailers and/or producers In Nitra, SVK. Samples were transported to the laboratory and stored at 4 °C. Milk mixtures of cow's milk in sheep milk were prepared for further DNA extraction and PCR analysis. Five different mixtures, containing 50, 10, 5, 1, and 0,5% (v/v) cow's milk, were prepared in a final volume of 1 mL.

DNA extraction

DNA was extracted using the InnuPREP DNA Mini Kit (Analytik Jena, Jena, Germany) rendering an elution volume fo 250 µL DNA according to the manufacturer´s instruction. DNA samples were quantified using the QuantiFluor dsDNA system (Promega) with Quantus™ Fluorometer (Promega).

Real-time PCR reaction

With the InnuDETECT Cheese Assay, cattle and sheep species were identified by using specific primers complementary to the cattle and sheep species. An internal positive control was incorporated in the InnuDETECT Cheese Assay kit. The internal control coamplified with the primers used for the qPCR reaction. PCR amplification was performed according to the manufacturer´s recommendations by adding 10 µL 2x MasterMix, 3 µL Primer/Probe Mix cattle (sheep), 1 µL Internal control, 5 µL of sample and the mixture was filled up to 20 µL. Real-time qPCR assay was performed with a LightCycler (Roche, Germany) based on the TaqMan principle. Cattle and sheep DNA have been detected in separated tubes (FAM channel) in order to reach the maximum sensitivity. Internal Control was used as an amplification control (HEX channel). Real-time PCR cycling parameters were optimized based on manufacturer´s manual: Initial denaturation 95 °C, 120 s, followed 40 by cycles of 95 °C 10 s of denaturation, 62 °C 45s of annealing/elongation and finally, absolute quantification analysis.

Data analysis

Primary real-time PCR data were analysed by the LightCycler Software 4.1.1.21 (Roche, Germany) and the threshold cycle (C_T) was calculated. C_T values of standard curve replicates (Y) and $\log_{10}$ (DNA amount) (X) were analysed using XLSTAT (Addinsoft, 2016) software and a linear regression equation of the C_T value plotted against the $\log_{10}$ (DNA amount) was calculated.

RESULTS AND DISCUSSION

Sensitivity and efficiency of qPCR assay

DNA extracted from a sample of 100% cow milk was used for the sensitivity and efficiency determination of the TaqMan real-time PCR assay. Linear range of positive amplification for the cow milk assay was achieved over five log units, which extended from 10 ng to 0,001 ng bovine DNA (Figure 1, Table1).

Parameters of the model for calibration curve are shown in Table 2. The assay showed good linearity, with correlation coefficient of R^2 = 0.983 and efficiency of 86%. **López-Calleja et al. (2007)** observed the correlation between the two variables, C_t and logarithm of cow´s DNA concentration, using the plasmid cow DNA as standard a determination coefficient value of 0.9955. The initial somatic cell content was not known for ovine milk

Table 1 Sensitivity of cow-specific assay.

Dillution (%)	DNA amount (ng)	$\log_{10}$(DNA amount)	Mean C_T ±SD
100	10	1.00	23.36 ±0.23
10	1	0.00	26.54 ± 0.08
1	0.1	-1.00	32.09 ± 0.37
0.1	0.01	-2.00	34.90 ±0.16
0.01	0.001	-3.00	37.80 ±0.21

Table 2 Parameters of the model.

Source	Value	Standard error	t	Pr >|t|	Lower bound (95% CI)	Upper bound (95% CI)
Intercept	27.214	0.234	116.272	<0.01	26.708	27.720
Slope	-3.724	0.135	-27.558	<0.01	-4.016	-3.432

Table 3 Mean C_T values and mean absolute amount of catlle DNA in milk mixtures as predicted by the model.

cow/sheep mixture (v/v)	CT ±SD	DNA ±95% CI (ng)
50	28.81 ±0.25	4.15 ±1.47
10	30.86 ±0.30	0.65 ±1.34
5	31.28 ±0.05	0.15 ±1.29
1	32.23 ±0.16	0.05 ±1.30
0.5	37.21 ±0.22	0.0022 ±1.48

Note: CT – Cycle treshold; SD – Standard deviation; CI – Confidence interval.

because in European Union there is no maximum limit for the number of somatic cells. The maximum limit for the number of somatic cells in raw bovine milk is ≤400 000 per mL. according to Regulation (EC) No. 1662/2006 of the European parliament and of the council laying down specific hygiene rules for food of animal origin.

Rentsch et al. (2012) developed and interlaboratory validated two multiplex TaqMan real-time PCR assays to determine DNA of bovine, ovine and caprine in milk and cheese. For caprine DNA, milk and cheese assays showed amplification efficiency of 85% and 116%, respectively. Linear detection and quantification range was 0.32 – 32 ng of sheep DNA (R^2 = 0.97).

Specificity

Detection system was tested for its selectivity and cross reactions to other milk-producing species. The cow-specific system amplified fragment from cow DNA, whereas no amplification was obtained from sheep and goat DNA. The internal control amplified fragment from different mammalian species (cow, sheep and goat), with similar C_T values.

Quantification of bovine DNA in milk mixtures

Table 3 summarises mean CT values for individual milk mixtures and total bovine DNA content as predicted by linear regression model. Amount of amplified DNA in all mixtures corresponded to ~10 ng DNA. Detection of bovine DNA in milk mixtures was achieved even in samples containing 0.5% of cows' milk.

The authenticity assessment of dairy products is an important issue regarding the consumer's interests due not only to the economic point of view, but also to medical requirements, food allergies or religious practices **(Mafra, Ferreira and Oliveira, 2007)**.

Polymerase Chain Reaction (PCR), single-plex PCR and quadru-plex PCR, are suitable methods to detect animal species origin in milk and in dairy products. At the

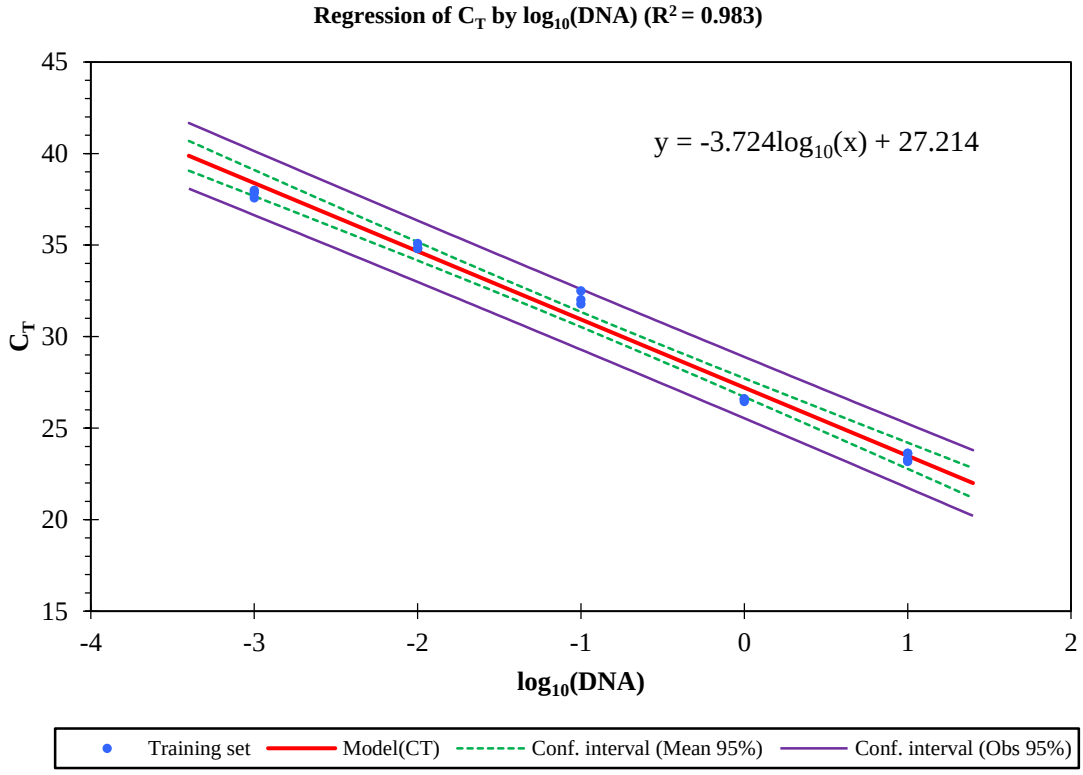

Figure 1 Calibration curve.

purpose, single-plex **(Agrimonti et al., 2015; Bania et al., 2001; Cozzolino et al., 2002)**, duplex **(Mafra et al., 2007)**, and triplex end-point PCR have been used **(Geng, 2014)**.

Klančnik et al. (2015) took an addition of >5% bovine milk as the limit for fraudulent addition of bovine milk to these caprine and ovine cheeses. We achieve detection limit for addition bovine milk in sheep milk 0.5%.

Fluorescence can be measured throughout the PCR, providing real-time analysis of the reaction kinetics and allowing quantification of specific DNA targets. Realtime PCR also offers a lower potential for contamination of the PCR mixture with target DNA because the reaction tubes remain closed throughout the assay. Moreover, the threshold cycle is observed when PCR amplification is still in the exponential phase and none of the reaction components is limited. This is the main reason why Ct is a more reliable measure of starting DNA copy number than an endpoint measurement of the amount of accumulated PCR product **(Rodríguez et al., 2004; López-Calleja et al., 2007)**.

Real-time analysis can facilitate quantification of the amount of cow DNA present in the sample by ascertaining when (i.e., during which PCR cycle) fluorescence in a given reaction tube exceeds that of a threshold (threshold cycle (Ct)). Comparison between reaction tubes and known standards allows quantification of cows' milk content present in a given sample **(López-Calleja et al., 2007)**.

The specificity and sensitivity of the real-time quantitative polymerase chain reaction (PCR), combined with its high speed, robustness, reliability, and the possibility of automation **(Heid et al., 1996; López-Calleja et al., 2007)**.

Mininni et al. (2009), developed a TaqMan real-time PCR assay to detect and quantify bovine milk in ovine and caprine cheeses, based on two target genes. The *cyt-b* gene of *Bos taurus* was used to detect and quantify bovine DNA. The nuclear gene *Myo*, mt*18S* rRNA and mt*16S* rRNA were used alternatively as universal reference markers. Caprine (n = 30) and ovine (n = 51) cheese samples were purchased and analysed and most were shown to be contaminated by bovine milk. Regarding the sensitivity, the limit of detection of cyt-b assay for bovine DNA corresponded to 0.2% (v/v) of bovine for standard caprine and ovine cheeses. The limit of detection of 16S assay for bovine DNA corresponded to 0.5% (v/v) for ovine cheese, the 0.5% (v/v) for ovine cheese obtained by the 16S assay, the 1% (v/v) for the 16S assay in caprine cheese. The limit of quantification of Myo and 18S assays was 1% (v/v) for both species.

Branciari et al. (2000) developed PCR-RFLP system for cytochrome b (*cyt-b*) gene to investigate the adulteration rate of feta cheeses, made from mixture of ovine and caprine milk, with less expensive bovine milk. The restriction enzymes *Hae*III and *Sau3A*I differentiated DNA of bovine, ovine, and caprine milk. The limit of detection of undeclared milk admixture was about 1% for all tested samples.

Zeleňáková et al. (2009) have analysed 70 milk and cheese samples by using PCR method. From twenty samples of the analysed sheep milk samples, cow milk occurrence was detected in eight samples. From the thirty samples of sheep cheese, eleven samples contained a mixture of the cow milk.

CONCLUSION

The uptake of real-time PCR system by the food industry depends on its technical advantages and relatively low cost. The TaqMan real-time PCR system for the identification of milks is sensitive, quick and safe. Its capability to detect low levels of bovine DNA will meet the standard required by many authentication measurements. If the somatic cell content in a mixture of bovine and ovine milk is similar, it should by be possible to quantitatively trace the amount of undeclared milk by comparing the sample to well-known DNA mixture standard. From practical point of view, in the mixtures of sheep and bovine milk the volume of both kind of milk can be different and also the concentration of somatic cells in both kind of milk can be different as well. This mean, this method ca is not suitable for quantification purposes because mainly due to the factor of different concentration of somatic cells in both kind of milk.

The InnuDETECT cheese array based on bovine and sheep specific primers and probes has been used to measure DNA amounts in commercial milks. The InnuDETECT cheese array reported herein gives reasonably accurate and reproducible estimates, it may be used to detect minimal amounts of cow's milk in milk mixtures, which is important for a variety of economic, religious and health reasons. We recommend to use this method for the purpose of qualitative determination only.

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