International Dairy Journal 28 (2013) 32–35

Contents lists available at SciVerse ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Short communication

High resolution melting analysis for quantitative detection of bovine milk in pure water buffalo mozzarella and other buffalo dairy products

Ioannis Sakaridis [a], Ioannis Ganopoulos [a,b], Anagnostis Argiriou [a], Athanasios Tsaftaris [a,b,*]

[a] Institute of Applied Biosciences, CERTH, 6th km Charilaou-Thermis Road, Thermi, Thessaloniki 570 01, Greece
[b] Department of Genetics and Plant Breeding, School of Agriculture, Aristotle University of Thessaloniki, Thessaloniki 54 124, Greece

ARTICLE INFO

Article history:
Received 10 May 2012
Received in revised form
30 July 2012
Accepted 14 August 2012

ABSTRACT

Identification of buffalo dairy products has become an important issue to ascertain product quality, consumer rights and absence of food-borne allergic reactions. A polymerase chain reaction (PCR) followed by a high resolution melting (HRM) analysis was developed and applied for species specific detection of bovine milk in nine different commercial buffalo dairy products. A specific buffalo 12S rRNA and a bovine D-loop primer pair, targeting the mitochondrial genome, were employed in a duplex PCR assay. The analysis developed was found capable of identifying the presence of bovine milk down to 1% in commercial buffalo milk products and also of quantifying the ratio of bovine into buffalo milk. HRM was proven to be a fast and accurate technique for a routine authentication testing of mozzarella and other buffalo milk products.

1. Introduction

The authenticity of animal products has become a major issue to ascertain consumers' rights with respect to accurate product information. The labelling of animal products should provide enough information on its composition, ingredients, production technology, genetic identity and possible allergic pathologies that may occur. Milk products are often prone to adulteration practices, since milk is a fairly expensive raw material and can be easily replaced in part by other dairy or non-dairy ingredients. High grade cheeses or other dairy products and especially those that are registered by European law with a Protected Designation of Origin (PDO) should therefore be subjected to frequent fraud control.

Several immunological, electrophoretic and chromatographic methods have been proposed for the identification of species of origin for milk and milk products. However, the official control method, which is suggested by the European Union, to detect bovine proteins in dairy products relies on isoelectrofocusing (IEF) of γ-caseins after plasminolysis (Commission Regulation, 2001). Although this method is considered reliable, some researchers question its consistency in cases of severe heat treated milk or weak adulterations (Bottero, Civera, Anastasio, Turi, & Rosati, 2002).

Recently, DNA based methods became very popular, since they are considered more reliable as a result of DNA stability under high temperatures, pressures and chemical treatments used during processing of food products (Behrens, Unthan, Brinkmann, Buchholz, & Latus, 1999). The polymerase chain reaction (PCR) is the most widely used molecular technique thanks to its simplicity, sensitivity and reproducibility. Ruminant milk can be easily used as a source of DNA, since it has a large amount of somatic cells, mostly leucocytes but also epithelial cells from the milking mother, which contain genomic DNA suitable for PCR amplification (Bottero et al., 2003). PCR amplification of various regions of mitochondrial genome (Abdel-Rahman & Ahmed, 2007; De et al., 2011; Feligini et al., 2005; Pegels et al., 2011), 12S rRNA (López-Calleja et al., 2005), growth hormone (GH) gene (Lopparelli, Cardazzo, Balzan, Giaccone, & Novelli, 2007) and PCR–RFLP (Abdel-Rahman & Ahmed, 2007; Sun & Lin, 2003) have been reported and proved the sensitivity of DNA based methods and their reliability for species identification in a wide range of dairy products.

In the recent years a new alternative method, the high resolution melting (HRM) analysis of PCR products (Wittwer, Reed, Gundry, Vandersteen, & Pryor, 2003) has been applied for the identification of different processed food products. HRM is a DNA based method that allows genotyping and fingerprinting by discriminating DNA sequence variants such as single nucleotide polymorphisms (SNPs) and small insertion and deletions (indels) based on the shape of melting transitions (Tm) of real-time PCR products (Wittwer, 2009; Wittwer et al., 2003; Zhou, Wang, Palais,

* Corresponding author. Tel.: +30 2310 498271.
E-mail address: tsaft@certh.gr (A. Tsaftaris).

0958-6946/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.idairyj.2012.08.006

Pryor, & Wittwer, 2005). It is considered to be a powerful, fast and accurate technique, which is cheaper and simpler than alternative approaches requiring post-PCR processing enzyme restriction and electrophoresis, or labelled probes for SNP detection sequencing or TaqMan-probe-based real-time PCR (Reed, Kent, & Wittwer, 2007). Lately, HRM analysis was applied by our group for the authentication and accurate quantitation of adulterants in commercial food products (Bosmali, Ganopoulos, Madesis, & Tsaftaris, 2012; Ganopoulos, Madesis, Darzentas, Argiriou, & Tsaftaris, 2012; Madesis, Ganopoulos, Argiriou, & Tsaftaris, 2012).

The goal of this study was to develop and test an HRM based method using specific mitochondrial primers for the rapid detection, quantification and adulteration measurements of bovine milk in buffalo milk products. HRM was proven capable of identifying the presence of bovine milk down to 1% in PDO Mozzarella cheese and other commercial buffalo milk products and also of quantifying the range mixture of bovine into buffalo milk.

2. Materials and methods

2.1. Buffalo milk products

The buffalo milk products, containing mozzarella di bufala campana, burro di bufala, buffalo butter, buffalo cream, buffalo yoghurt, buffalo rice pudding, kazan dipi (a traditional sweet made entirely from buffalo milk), buffalo milk and buffalo ariani (fluidised yoghurt) were purchased from local shops and super markets. Five samples from each commercial product were used (45 samples in total). Dilutions of bovine milk in buffalo milk (50%, 30%, 20%, 10%, 5% and 1%) were also prepared.

2.2. DNA isolation

Milk samples (50 mL) were initially centrifuged at 1500 × *g* for 15 min to collect somatic cells. The pellets were rinsed three times in 1 mL of Phosphate Buffered Saline (PBS), centrifuged at 12,000 × *g* for 5 min and finally resuspended in 200 μL of PBS. Total DNA isolation using 25 mg of buffalo milk products were performed using Nucleospin Food kit (Macherey-Nagel, Duren, Germany) as described in the instructions given by the manufacturer. The DNA concentration was estimated by spectrophotometric analysis using BioPhotometer plus UV/Vis Photometer (Eppendorf, Hamburg, Germany) at 260 nm. The extracted DNA solution was stored in 4 °C until further use.

2.3. PCR amplification and HRM analysis

Buffalo species identification by real time PCR with fluorescent Syto9® dye was performed using one primer-pair, which has been reported previously to be specific for buffalo DNA sequences (Pegels et al., 2011).

The 12S region has been a well-accepted marker for buffalo milk and for specific detection of milk products (López-Calleja et al., 2005; Pegels et al., 2011). For the detection of adulteration with bovine milk, a duplex PCR assay was developed according to the approach of Mader, Ruzicka, Schmiderer, and Novak (2011), containing a buffalo specific primer for the 12S rRNA gene and a bovine specific primer for the D-loop mitochondrial gene (Table 1). An alternative 12S rRNA bovine primer was also used. Here a ratio of specific primers of 1:1 was used after optimisation (data not shown).

PCR amplification, DNA melting and end point fluorescence level acquiring PCR amplifications were performed in a total volume of 15 μL on a Rotor-Gene 6000 real-time 5Plex HRM PCR Thermocycler (QIAGEN GmbH, Mannheim, Germany) according to Ganopoulos, Argiriou, and Tsaftaris (2011).

Table 1
List of buffalo dairy product used for real-time PCR analysis with Syto9 dye and HRM analysis.[a]

Buffalo dairy product	Buffalo specific 12S	Bovine specific D-loop	Bovine specific 12S
Mozzarella di bufala campana	+	−	−
Burro di bufala	+	+	+
Buffalo butter	+	−	−
Buffalo cream	+	−	−
Buffalo yoghurt	+	+	+
Buffalo rice pudding	+	−	−
Kazan dipi	+	−	−
Buffalo milk	+	−	−
Buffalo ariani	+	−	−

[a] Specificity test for buffalo specific 12S, bovine specific D-loop and bovine specific 12S primers was performed on genomic DNA; +, positive result (corresponding to a peak of predicted Tm); −, negative result (corresponding to absence of peak).

A rapid PCR protocol and HRM analysis were conducted in a 36-well carousel and were performed as described previously by Ganopoulos et al. (2011). PCR products were analysed on a 2% agarose gel to ensure the amplification of the correct size products (data not shown). All experiments were repeated 3 times with 3 sets of independent samples for testing the reproducibility of the results.

3. Results and discussion

An HRM method was developed and tested for the detection and quantitative determination of bovine milk in buffalo milk products. The 12S rRNA gene was used initially to examine the specificity of the buffalo primers and to verify the presence of specific buffalo DNA amplicons (López-Calleja et al., 2005). The D-loop mitochondrial gene was used, as described by Pegels et al. (2011), to specifically detect bovine species in buffalo milk products, along with a specific 12S rRNA bovine primer-pair (López-Calleja et al., 2005). As the functionality and specificity of D-loop primer-pair for bovine had been already proven (Pegels et al., 2011), these primers together with the 12S rRNA buffalo primers were preferred for qPCR experiments. The efficiency of the method was evaluated by Syto9® dye using the DNA template extracted from an authentic buffalo and bovine reference milk.

Table 1 depicts the results of the Syto9® assay for bovine milk detection in buffalo milk products. The DNA extracted from all commercial buffalo milk products yielded specific amplification products with the 12S rRNA buffalo primers. However, the DNA extracted from two commercial buffalo milk products, burro di bufala and buffalo yoghurt, also yielded specific amplification products with the D-loop bovine primers. Specific amplification products were also observed using the 12S rRNA bovine primers. Therefore, it can be concluded that there was an admixture of bovine and buffalo milk during the processing of these two products, in contrast with their label claims. Although the whole experiment was setup to detect the absence or presence of bovine contamination and not the percentage on contamination it could be possible to detect the level of contamination using reference material with known level of contamination and extrapolating the contamination level of the unknown samples.

Furthermore, the application of duplex HRM analysis revealed that these samples (burro di bufala and buffalo yoghurt) presented two specific melting peaks and the ratio of buffalo milk adulteration obtained from the melting curve profiles were approximately 5 and 10%, respectively (data not shown). The HRM curves of the artificial admixtures of bovine milk into buffalo milk (1%, 5%, 10%, 20%, 30%, 50%) are illustrated in Fig. 1. The results in Fig. 1 depict the analysis for one experiment, because all 3 experiments gave similar

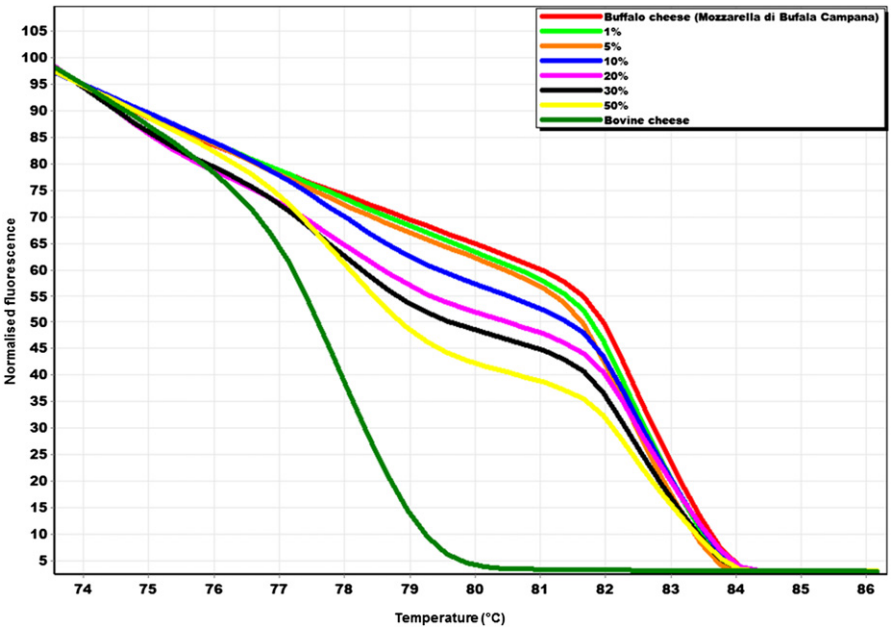

Fig. 1. Melting curves obtained by high resolution melting analysis of the two species specific amplicons and applied to reference mixtures containing 50, 30, 20, 10, 5 and 1% of bovine milk in buffalo milk. Data are from a single experiment.

results thus showing very good reproducibility. As shown in this Fig. 1, the PCR products of pure buffalo sample showed melting curve with a single inflection point at a Tm value of 83 °C corresponding to the mid-point of the inflection. The curve of pure bovine sample also depicted one inflection point at 78 °C. All mixed samples showed both melting domains in their curves, resulting in two inflection points. As stated by Mader et al. (2011), the level of fluorescence after dissociation of the adulterant's amplicon can be regarded as a quantitative measure of contamination with adulteration. As shown in Fig. 2, the highly significant correlation between the level of fluorescence at a predefined temperature and the contamination percentage allowed the development of a standard curve ($R^2 = 0.988$). This R^2 value suggests high correlation of the fluorescence mean with the artificial samples used. Although, in our case, points at low level of contamination seems to be slightly away from line of best fit a relative limit of detection and quantification of up to 1% could be obtained with real-time PCR.

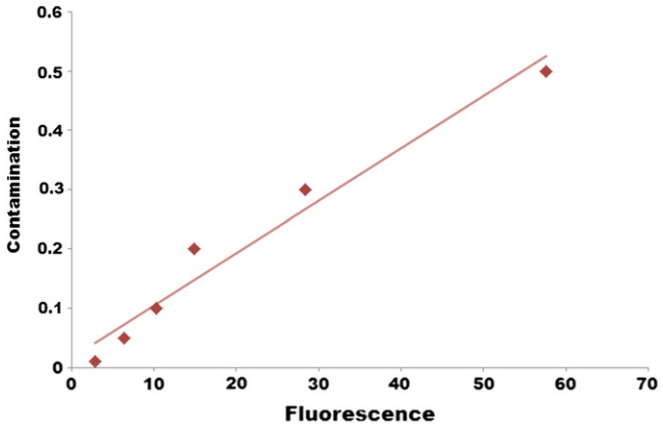

Fig. 2. Typical standard curve ($R^2 = 0.988$) of fluorescence plotted against the percentage of adulteration of each dilution; pure buffalo milk DNA was mixed with pure bovine milk DNA in progressive proportions (1%, 5%, 10%, 20%, 30%, and 50%). Data represent the average of three independent experiments.

In other studies, Dalmasso, Civera, La Neve, and Bottero (2011) applied a Real-Time PCR Allelic Discrimination assay. Using specific primers and probes, this method showed a detection threshold of 2% addition of bovine milk in buffalo milk, which was higher than the detection threshold of our method. Likewise, Lopparelli et al. (2007) detected and quantitated the presence of cow DNA in pure water buffalo mozzarella cheese using real-time TaqMan polymerase chain reaction and targeting the mitochondrial cytochrome *b* (cyt *b*) and growth hormone (GH) gene. The downside of this method was its high cost, especially when compared with HRM.

There are several advantages of HRM analysis over traditional SNP and quantitative probe methods (Bai, Wang, Yin, Tian, & Li, 2012): (a) the HRM can analyse a large number of samples at the same time; (b) its sensitivity can reach 1%–0.1%; (c) the specificity of PCR products approaches 100%; (d) there is no need for further processing, it is a closed tube technique which avoids cross-contamination of the samples; (e) there is no need for specific probes and sequencing analysis; (f) the sample genotype can be evaluated completely by HRM curve directly; (g) the presence of toxic reagents like ethidium bromide is avoided, since gel-electrophoresis of PCR products is unnecessary. Therefore, HRM analysis can be used in a routine basis since it is a low cost method, it simplifies the procedure and significantly reduces the time of analysis.

4. Conclusion

The results obtained in this study suggests that HRM is a fast, accurate and highly reproducible post-PCR method that permits the detection of bovine milk in mozzarella and other buffalo dairy products via the use of specific mitochondrial genetic regions. It was found effective to accurately detect down to 1% of bovine milk addition in buffalo products and to quantify the ratio of adulteration of bovine milk into buffalo milk used for processing of these products. Therefore, HRM analysis could be applied as a simple and affordable technique for routine authentication testing of buffalo dairy products.

Acknowledgement

This research was funded by the European territorial cooperation programme Greece–Bulgaria 2007–2013 "AGRI-GENO-TRANS/Agri-Genomics Transfer and Dissemination Between Greece & Bulgaria".

References

Abdel-Rahman, S. M., & Ahmed, M. M. M. (2007). Rapid and sensitive identification of buffalo's, cattle's and sheep's milk using species-specific PCR and PCR-RFLP techniques. *Food Control, 18*, 1246–1249.

Bai, M., Wang, C., Yin, H., Tian, Y., & Li, J. (2012). Evaluation of different reaction systems for HRM analysis in apple. *Bioscience Methods, 3*, 1–6.

Behrens, M., Unthan, M., Brinkmann, Y., Buchholz, R., & Latus, N. (1999). Identification of animal species heated and complex meat products using specific PCR reactions. *Fleischwirtschaft, 79*, 97–100.

Bosmali, I., Ganopoulos, I., Madesis, P., & Tsaftaris, A. (2012). Microsatellite and DNA-barcode regions typing combined with High Resolution Melting (HRM) analysis for food forensic uses: a case study on lentils (*Lens culinaris*). *Food Research International, 46*, 141–147.

Bottero, M. T., Civera, T., Anastasio, A., Turi, R. M., & Rosati, S. (2002). Identification of cows milk in "buffalo" cheese by duplex polymerase chain reaction. *Journal of Food Protection, 65*, 362–366.

Bottero, M. T., Civera, T., Nucera, D., Rosati, S., Sacchi, P., & Turi, R. M. (2003). A multiplex polymerase chain reaction for the identification of cows', goats' and sheep's milk in dairy products. *International Dairy Journal, 13*, 277–282.

Commission Regulation. (2001). Commission Regulation No. 213/01 of 9 January, 2001 lying down detailed rules for the application of Council Regulation (E.C.) No. 1255/1999 as regards methods for the analysis and quality evaluation of milk and milk products and amending Regulations (E.C.) No. 2771/1999 and (E.C.) No. 2799/1999. *Official Journal of the European Communities, L037*, 1–99.

Dalmasso, A., Civera, T., La Neve, F., & Bottero, M. T. (2011). Simultaneous detection of cow and buffalo milk in mozzarella cheese by real-time PCR assay. *Food Chemistry, 124*, 362–366.

De, S., Brahma, B., Polley, S., Mukherjee, A., Banerjee, D., Gohaina, M., et al. (2011). Simplex and duplex PCR assays for species specific identification of cattle and buffalo milk and cheese. *Food Control, 22*, 690–696.

Feligini, M., Bonizzi, I., Čubrić Čurik, V., Parma, P., Greppi, G. F., & Enne, G. (2005). Detection of adulteration in Italian mozzarella cheese using mitochondrial DNA templates as biomarkers. *Food Technology and Biotechnology, 43*, 91–95.

Ganopoulos, I., Argiriou, A., & Tsaftaris, A. (2011). Adulterations in Basmati rice detected quantitatively by combined use of microsatellite and fragrance typing with High Resolution Melting (HRM) analysis. *Food Chemistry, 129*, 652–659.

Ganopoulos, I., Madesis, P., Darzentas, N., Argiriou, A., & Tsaftaris, A. (2012). Barcode High Resolution Melting (Bar-HRM) analysis for detection and quantification of PDO 'Fava Santorinis' (*Lathyrus clymenum*) adulterants. *Food Chemistry, 133*, 505–512.

López-Calleja, I., González Alonso, I., Fajardo, V., Rodríguez, M. A., Hernández, P. E., García, T., et al. (2005). PCR detection of cows' milk in water buffalo milk and mozzarella cheese. *International Dairy Journal, 15*, 1122–1129.

Lopparelli, R. M., Cardazzo, B., Balzan, S., Giaccone, V., & Novelli, E. (2007). Real-time TaqMan polymerase chain reaction detection and quantification of cow DNA in pure water buffalo mozzarella cheese: method validation and its application on commercial samples. *Journal of Agricultural and Food Chemistry, 55*, 3429–3434.

Mader, E., Ruzicka, J., Schmiderer, C., & Novak, J. (2011). Quantitative high-resolution melting analysis for detecting adulterations. *Analytical Biochemistry, 409*, 153–155.

Madesis, P., Ganopoulos, I., Argiriou, A., & Tsaftaris, A. (2012). The application of Bar-HRM (Barcode DNA-High Resolution Melting) analysis for authenticity testing and quantitative detection of bean crops (Leguminosae) without prior DNA purification. *Food Control, 25*, 576–582.

Pegels, N., González, I., Martín, I., Rojas, M., García, T., & Martín, R. (2011). Applicability assessment of a real-time PCR assay for the specific detection of bovine, ovine and caprine material in feedstuffs. *Food Control, 22*, 1189–1196.

Reed, G. H., Kent, J. O., & Wittwer, C. T. (2007). High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics, 8*, 597–608.

Sun, Y.-L., & Lin, C.-S. (2003). Establishment and application of a fluorescent polymerase chain reaction β'restriction fragment length polymorphism (PCR-RFLP) method for identifying porcine, caprine, and bovine meats. *Journal of Agricultural and Food Chemistry, 51*, 1771–1776.

Wittwer, C. T. (2009). High-resolution DNA melting analysis: advancements and limitations. *Human Mutation, 30*, 857–859.

Wittwer, C. T., Reed, G. H., Gundry, C. N., Vandersteen, J. G., & Pryor, R. J. (2003). High-resolution genotyping by amplicon melting analysis using LCGreen. *Clinical Chemistry, 49*, 853–860.

Zhou, L., Wang, L., Palais, R., Pryor, R., & Wittwer, C. T. (2005). High-resolution DNA melting analysis for simultaneous mutation scanning and genotyping in solution. *Clinical Chemistry, 51*, 1770–1777.