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Analytical Methods

A novel closed-tube method based on high resolution melting (HRM) analysis for authenticity testing and quantitative detection in Greek PDO Feta cheese

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ABSTRACT

Animal species identification of milk and dairy products has received increasing attention concerning food composition, traceability, allergic pathologies and accurate consumer information. Here we sought to develop an easy to use and robust method for species identification in cheese with emphasis on an authenticity control of PDO Feta cheese products. We used specific mitochondrial DNA regions coupled with high resolution melting (HRM) a closed-tube method allowing us to detect bovine, ovine and caprine species and authenticate Greek PDO Feta cheese. The primers successfully amplified DNA isolated from milk and cheese and showed a high degree of specificity. HRM was proven capable of accurately identifying the presence of bovine milk (not allowed in Feta) down to 0.1% and also of quantifying the ratio of sheep to goat milk mixture in different Feta cheese commercial products. In conclusion, HRM analysis can be a faster, with higher resolution and a more cost effective alternative method to authenticate milk and dairy products including PDO Feta cheese and to quantitatively detect its sheep milk adulterations.

1. Introduction

Species identification of milk and dairy products has a remarkable importance regarding food composition, traceability, allergic pathologies and accurate consumer information. In the dairy industry, commercial adulteration sometimes involves the replacing of expensive high quality milk with cheaper and lower quality milk from other species (Locci et al., 2008; Lopparelli, Cardazzo, Balzan, Giaccone, & Novelli, 2007; Woolfe & Primrose, 2004). It has been reported that cow's milk is frequently admixed with sheep's milk during the manufacture of ovine cheeses due to the lower yield of ewes combined with the much lower price of bovine milk (López-Calleja et al., 2005). Fraud control is therefore needed, especially for high-grade cheeses made entirely from sheep's or goat's milk or mixture thereof which are registered by European law with a Protected Designation of Origin (PDO) (Bottero et al., 2003).

Feta (Greek: φέτα) is a soft white cheese ripened in brine that is traditionally made in Greece (Mauropoulos & Arvanitoyannis, 1999). The European Commission (IP/02/866, 2002) adopted Feta as a PDO. Only those cheeses produced in the mainland of Greece and the island of Lesbos, and are made primarily from expensive sheep milk, or from a mixture of sheep's milk and up to 30% goats' milk from the same area, may bear the name Feta. In accordance with government regulation, Feta cheese can only be commercialized after a period of ripening that lasts at least 60 days (Mauropoulos & Arvanitoyannis, 1999). Feta cheese was traditionally made from non pasteurized milk in small family premises but nowadays for reasons of public health it is produced from pasteurized milk in organized cheese dairies (Manolopoulou et al., 2003). Similar white brined cheeses are found in many countries of the eastern Mediterranean Sea, which are often made partly or wholly of cow's milk, and cannot legally be called Feta.

A variety of methods are available for the identification of the species of origin for milk and dairy products, which include immunological, electrophoretic and chromatographic techniques. These techniques are rather effective in detecting species specific differences in milk proteins or fatty acids but have some limitations. Immunological and electrophoretic methods, for instance, are not always capable of distinguishing milk from closely related species, like sheep and goats, and are often not suitable for detection of heat treated material, whereas chromatographic methods, being

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rather laborious, are difficult for application in every day numerous sample numbers (López-Calleja et al., 2005).

In comparison with proteins and fatty acids, DNA based methods are considered more reliable as a result of the stability of the DNA under high temperatures, pressures and chemical treatments used during the processing of certain food products (Behrens, Unthan, Brinkmann, Buchholz, & Latus, 1999). Among them, PCR is the most popular and widely used molecular technique on account of its sensitivity, reproducibility and simplicity. As far as milk and milk products are concerned, it has been reported that ruminant milk can be 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). There are several reports that are based on PCR amplification of various regions of mitochondrial genome (Abdel-Rahman & Ahmed, 2007; De et al., 2011; Pegels et al., 2011), 12S rRNA (López-Calleja et al., 2005), growth hormone (GH) gene (Lopparelli et al., 2007) and PCR-RFLP (Abdel-Rahman & Ahmed, 2007; Sun & Lin, 2003) and have shown that DNA based methods are more sensitive than the protein or fatty acid based methods and therefore can be applied reliably for species identification in a wide range of dairy products (see Bottero & Dalmasso, 2011 for a recent review and references therein).

High resolution melting (HRM) analysis (Wittwer, Reed, Gundry, Vandersteen, & Pryor, 2003) is a novel 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 (T_m) of real-time PCR products (Wittwer, 2009; Wittwer et al., 2003; Zhou, Wang, Palais, Pryor, & Wittwer, 2005). Along with allele discrimination by targeting well-characterized SNPs, HRM analysis can also be applied for screening for the existence of unknown sequence variations without a sequencing process. HRM is therefore a powerful, fast and accurate technique that is cheaper and simpler than alternative approaches requiring post-PCR processing, enzyme restriction and electrophoresis, labeled probes for SNP detection sequencing or TaqMan-probe based real-time PCR (Reed, Kent, & Wittwer, 2007). Very recently, a new method which combines plant barcoding with HRM analysis (Bar-HRM) was developed for the authentication of berry species by Jaakola, Suokas, and Häggman (2010) and by our group for the authentication of plant species and their PDO products and for the accurate quantitation of adulterants in commercial food products (Bosmali, Ganopoulos, Madesis, & Tsaftaris, 2012; Ganopoulos, Madesis, Darzentas, Argiriou, & Tsaftaris, in press; Madesis, Ganopoulos, Argiriou, & Tsaftaris, 2012).

Herein, we developed and tested an HRM based method using specific mitochondrial primers for the rapid detection, quantification and adulteration measurement of bovine, ovine and caprine species in cheeses. HRM was proven capable of identifying the presence of bovine milk down to 0.1% in putative Feta and also of quantifying the range mixture of sheep to goat milk in different Feta cheese commercial products.

2. Materials and methods

2.1. Samples

Five sheep (*Ovis aris*), 5 goat (*Capra hircus*) and 5 bovine (Bos taurus) blood samples were used as positive control to set up the assay. Similarly, five samples of sheep milk and five samples of goat's milk were tested. Cheese samples included eight samples of commercial Feta PDO, three samples of sheep–goat's cheese (unknown mixture of sheep's and goat's milk – no PDO) and one sample allegedly made only with goat milk (no PDO). A Feta cheese made entirely of ovine milk and another one of a known mixture of ovine and caprine milk (70:30, respectively) were also prepared following the traditional technology, described by Anifantakis (1991), to be used as references. Successively, dilutions of goat's milk mixtures in sheep milk (50%, 40%, 30%, 15%, 5% and 1%) were prepared, together with dilutions of cow's milk mixtures in sheep/goat (70:30) milk (20%, 10%, 5%, 2%, 1% and 0.1%). The samples of commercial Feta and of sheep–goat's cheese, produced with the traditional technique of Feta, were obtained from local supermarkets and dairy shops or directly from cheese manufacturers.

2.2. DNA isolation

To extract the DNA from blood, heparinized samples were subjected to centrifugation at 3000*g* for 20 min and 1 ml of buffy coat was withdrawn and stored at −20 °C. DNA was extracted from 50 μl of buffy coat using Dneasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for blood samples. Milk samples (50 ml) were centrifuged at 1500*g* for 15 min to collect somatic cells. The pellets were rinsed three times in 1 ml of PBS, centrifuged at 12,000*g* for 5 min and finally resuspended in 200 μl of PBS. DNA was extracted following the protocol of Nucleospin Tissue kit (Macherey–Nagel). (DNA from cheese samples was extracted following the animal tissue protocol of Nucleospin Tissue kit (Macherey–Nagel). The DNA concentration was estimated by standard spectrophotometric methods at 260 and 280 nm UV lengths by an Eppendorf BioPhotometer and the integrity by gel electrophoresis in a 0.8% agarose gel. Samples were then diluted to 20 ng/μl work concentration.

2.3. PCR amplification and HRM analysis

Bovine 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 bovine DNA sequences (Pegels et al., 2011).

The D-loop region and $tRNA^{Lys}$ has been a well-accepted marker for caprine and ovine specific detection in dairy products (Krcmar & Rencova, 2003; Pegels et al., 2011). In this study we have developed a genotyping method based on 77 and 145 bp fragments, respectively using HRM analysis. For the detection of a contamination with a known species, a duplex PCR assay was developed according to Mader, Ruzicka, Schmiderer, and Novak, 2011 approach, containing a caprine specific primer for the D-loop gene and an ovine specific primer for $tRNA^{Lys}$ (Table 2). Here a ratio of specific primers of 1:1 was used after optimization (data not shown).

Table 1
Syto9® real-time PCR assay for cow milk detection in cheese (Ct values obtained from 20 ng DNA).

No.	Cheese	BDLOOP	Ct
1	Sheep	–	–
2	Goat	–	–
3	Cow	+	16.07 ± 0.04
4	Feta1	–	–
5	Feta2	–	–
6	Feta3	–	–
7	Feta4	–	–
8	Feta5	–	–
9	Feta6	–	–
10	Feta7	–	–
11	Feta8	–	–
12	Sheep–goat cheese1	+	23.32 ± 0.03
13	Sheep–goat cheese2	–	–
14	Sheep–goat cheese3	–	–
15	Goat commercial	–	–

Table 2
Species-specific primers used in this study.

Primers	Forward (5′–3′)	Reverse (5′–3′)	T_m	References
BDLOOP	AACCAAATATTACAAACACCACTAGCT	CCTTGCGTAGGTAATTCATTCTG	58	Pegels et al. (2011)
CDLOOP	CCCAATCCTAACCCAACTTAGATACC	TGTGTAGGCGAGCGGTGTAA	58	Pegels et al. (2011)
OvtRNA	ACACAACTTCTACCACAACCC	AAACAATGAGGGTAACGAGGG	58	Krcmar and Rencova (2003)

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 5P HRM PCR Thermocycler (Corbett Research, Sydney, Australia) according to (Ganopoulos, Argiriou, & Tsaftaris, 2011b). More specifically the reaction mixture contained 20 ng genomic DNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 300 nM forward and reverse primers (Table 2), 1.5 mM Syto® 9 green fluorescent nucleic acid stain, and 1 U Kapa Taq DNA polymerase (Kapa Biosystems, USA). A third generation DNA intercalating dye, Syto® 9, that at high concentrations can saturate all available sites within double stranded DNA was used. Syto® 9s' fluorescence provides a more accurate assessment of DNA melt status compared to SYBR Green I (Monis, Giglio, & Saint, 2005) and can be used to monitor both the accumulation of the amplified product during PCR and also the subsequent product melting on the RotorGene 6000 (software version 2.0.2 Corbett Life Science, Cambridge, UK).

A rapid PCR protocol was conducted in a 36-well carousel using an initial denaturing step of 95 °C for 3 min followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, then a final extension step of 72 °C for 5 min. The fluorescent data were acquired at the end of each extension step during PCR cycles. HRM was performed as described previously by Ganopoulos, Argiriou, and Tsaftaris (2011a). The Rotor-Gene 6000 proprietary software (version 2.0.2) was used to genotype the different species. The negative derivative of fluorescence (F) over temperature (T) (dF/dt) curve primarily displaying the T_m, the normalized raw curve depicting the decreasing fluorescence vs increasing temperature, and difference curves (Wittwer et al., 2003) were mainly used. PCR products were analyzed on a 2% agarose gel in order to ensure the amplification of the correct size products (data not shown).

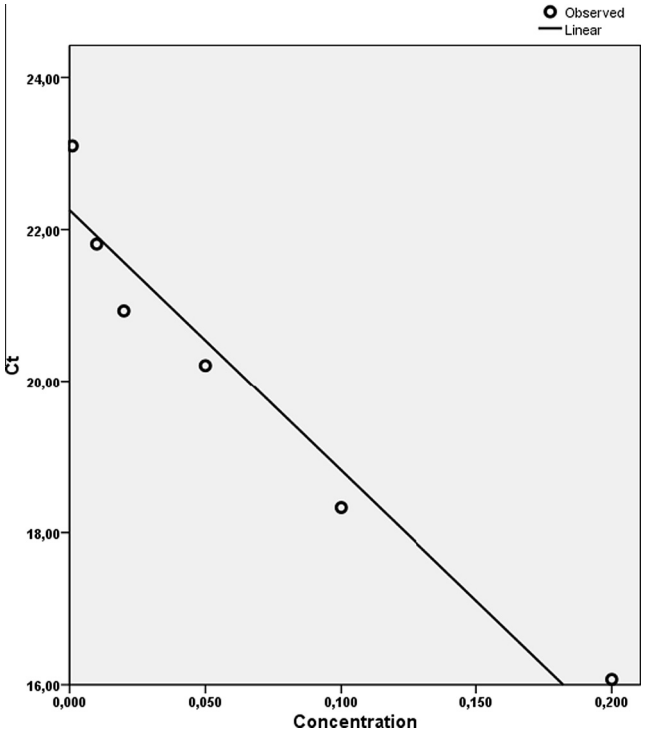

Fig. 1. Standard curve of the bovine specific D-loop assays with fluorescent Syto9® dye on cheese templates serially diluted.

3. Results and discussion

A real-time PCR protocol was applied for the quantitative determination of cow's milk percentage in Feta cheese. The D-loop mitochondrial gene was used, as described by Pegels et al. (2011), to specifically detect bovine species in Feta cheeses. As the functionality and specificity of D-loop primer-pair for bovine had been already proven (Pegels et al., 2011), these primers were used for qPCR experiments. The efficiency of the method was evaluated by Syto9® dye using the DNA template extracted from an authentic bovine reference cheese. To verify again the specificity of the above primers and their applicability to real-time PCR amplification using the fluorescent dye Syto9®, the DNA extracts of sheep's and goats' milk mixtures containing known amounts of bovine milk (20%, 10%, 5%, 2%, 1% and 0.1%) were also used. As illustrated in Fig. 1, a relative limit of detection and quantification of 0.1% with high correlation ($R^2 = 0.951$) was obtained with real-time PCR. The respective melting curve analysis revealed the amplification of similar products since they exhibited the same melting temperature of 78.2 ± 0.3 °C (data not shown). In good accordance with the specifications of the EU reference method (LOD must be 0.5% or lower; Commission Regulation 2008), a limit of detection of about 0.1% cow's milk in mixed-milk was found in this study.

Table 1 depicts the results of the Syto9® assay for cow milk detection in cheese. The DNA extracted from cow's cheese and sheep–goat cheese 1 yielded bovine specific amplification product with D-loop primers. The Ct value of the cow's cheese template was 16.07, whereas the Ct value of the sheep–goat cheese 1 amplicon was found to be 23.32, revealing that there was an adulteration of bovine milk in the mixture of sheep and goats milk. However, the significant difference in Ct values suggested that there was only a small quantity of bovine milk that was added and the adulteration was probably unintentional. Mayer (2005) used four pairs of bovine-specific PCR primers for identification of the species of origin of three samples of Feta cheese and suggested that adulteration was detected in two out of three samples. In contrast, in our research, all Feta cheese samples were found unadulterated with bovine milk. However, extreme care should be taken to address the addition of bovine milk in sheep–goat's or in Feta cheese in order to avoid the misleading of consumers and possible occurrence of allergic pathologies.

The HRM assay, developed in this study for Feta cheese authentication, was based on species-specific primer pairs obtained from Pegels et al. (2011) and Krcmar and Rencova (2003) and HRM analysis designed on mitochondrial sequences. As stated by Pegels et al. (2011), mitochondrial (mt) DNA evolves faster than nuclear DNA, providing the degree of sequence variation required for identification of closely related species. Furthermore, mitochondrial genes are present in multicopy in the cells increasing the sensitivity of the PCR and contributing to the recovery of at least a few copies when the tissue is subjected to extreme processing conditions (Girish et al., 2004), like the ones during cheese making. As shown

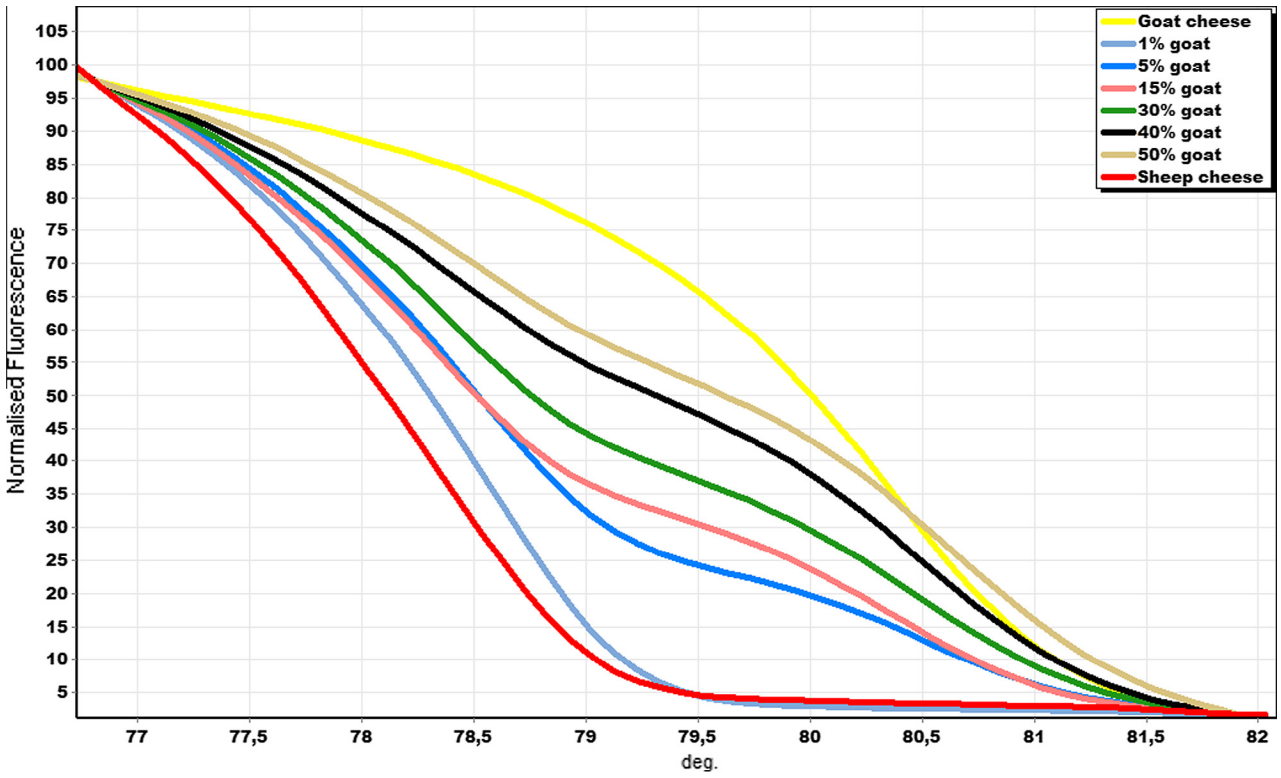

Fig. 2. Melting curves obtained by HRM analysis of the two species specific amplicons and applied to reference mixtures containing 50%, 40%, 30%, 15%, 5% and 1% of goat milk in cheese product.

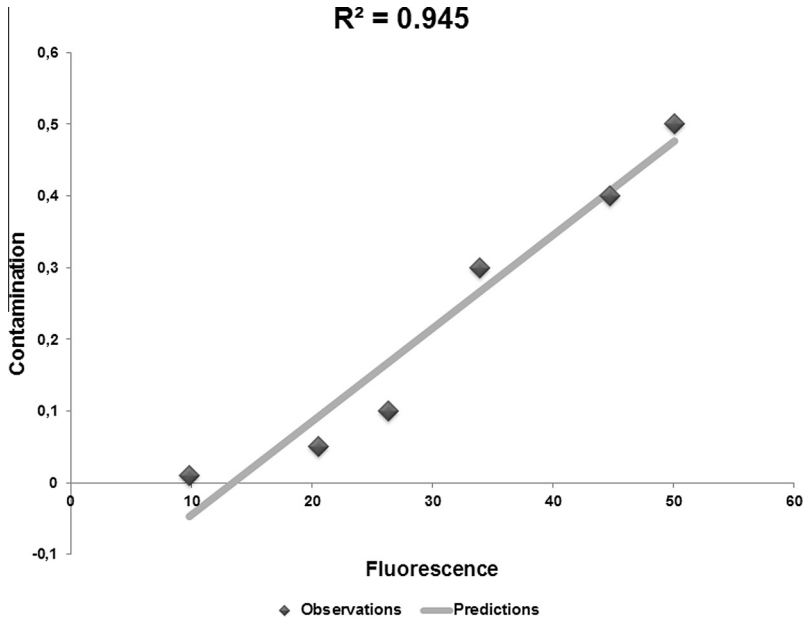

Fig. 3. Pure sheep milk was mixed with goat milk in progressive proportions (50%, 40%, 30%, 15%, 5% and 1%). Values of fluorescence were plotted against the percentage of adulteration of each dilution to generate a typical standard curve. All the experiments were performed in duplicate.

in Fig. 2, in the developed assay the PCR products of pure ovine samples showed melting curves with a single inflection point at a T_m value of 78.5 °C. The curves of pure caprine samples also depicted one inflection point at 80.6 °C. All mixed samples showed both melting domains in their curves, resulting in two inflection points. According to Mader et al. (2011) the level of fluorescence after dissociation of the ovine amplicon can be regarded as a quantitative measure of contamination with adulteration. 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 = 0.945). This R^2 value suggests high correlation of the fluorescence mean with the samples used (Fig. 3).

The high resolution melting curves of eight commercial Feta samples along with three samples of sheep–goat cheese, a commercial goat cheese, two standard Feta samples (100% sheep's milk

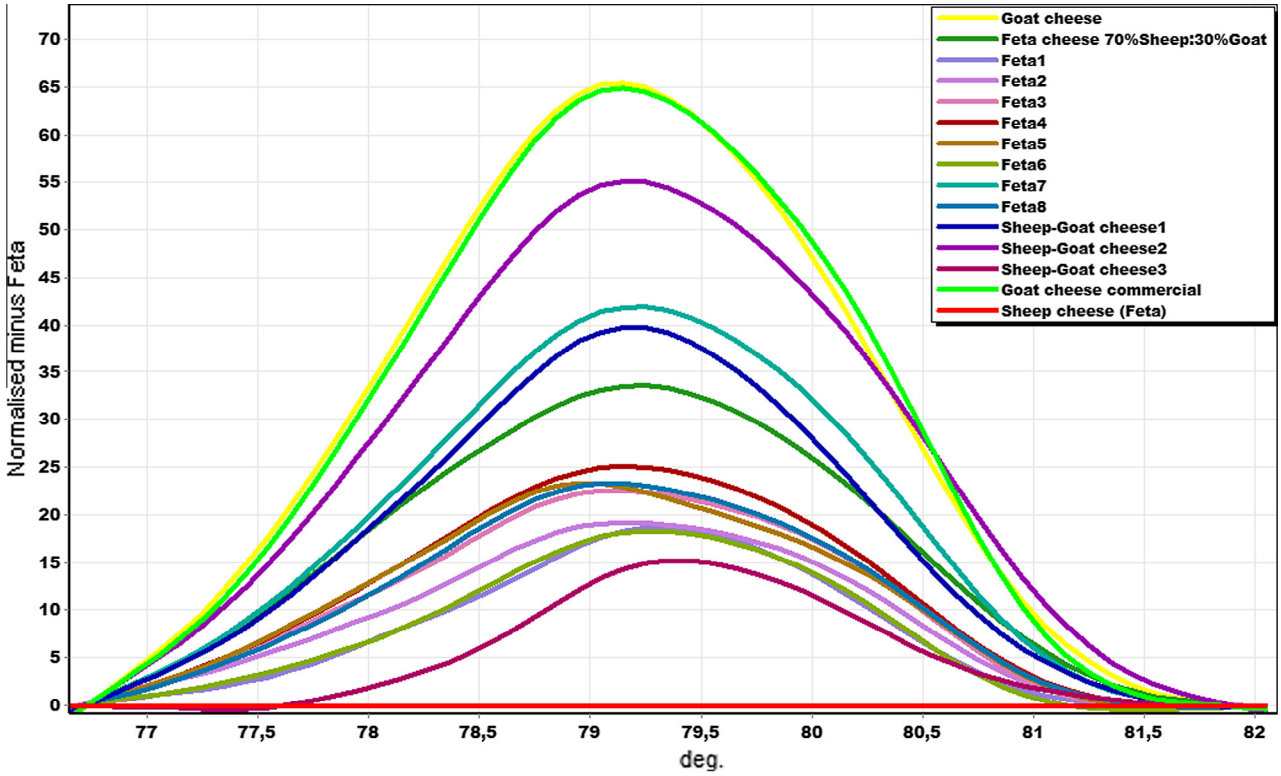

Fig. 4. Difference graph of commercial Feta cheeses using sheep cheese as reference genotype. Assigned genotypes using a cut off threshold of known Feta cheese (70% sheep/30% goat) melting profile. Color code table with the samples used.

and 70% sheep's–30% goat's milk) and a standard goat cheese are illustrated in Fig. 4. The melting profile of the second standard (Feta cheese with 70% sheep's–30% goat's milk) was used as a cut off in order to assign the genotypes. The HRM curves of all Feta samples were compared to this standard and were resulted as Feta cheese if the melting profile was below the threshold line or as no-Feta if it was above. Only one Feta sample, Feta 7, was found to present a melting profile above the threshold line in contrast to the other seven Feta samples. The melting curves of sheep–goat's cheese samples 1 and 2 were also found above the threshold line, which was an expected finding, as the mixture of milk in these cheeses is not defined and goat's milk might be in excess. Therefore, it could be suggested that commercial Feta 7 was probably prepared with more than 30% of goat's milk and should not be legally called Feta cheese. However, ovine and caprine DNA in cheese is derived from somatic cells of milk and variations of their number may occur among species, stage of lactation, season and management practices (Paape et al., 2007). Nevertheless, since Feta and other dairy products are processed from 'bulk' milk, errors in quantifying DNA could be minified (Bottero & Dalmasso, 2011) and the strict requirement for up to 30% goat's milk in Feta cheese should be applied with caution only when the HRM measurement is just above 30%.

4. Conclusion

The HRM method is a closed tube post PCR method which permits the rapid analysis of genetic variation in commercial food products via the use of mitochondrial regions. HRM analysis has already been used for the authentication of plant derived foods (Ganopoulos et al., in press, 2011a). This is the first report describing the development of an HRM method for milk and dairy products authentication. The method was proven to be effective and accurate detecting down to 0.1% of bovine milk addition in putative Feta and of quantifying the ratio of goat to sheep milk. Hence, HRM analysis could be applied as a useful means for a routine authentication testing of animal, milk and dairy products.

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