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A fast and accurate method for controlling the correct labeling of products containing buffalo meat using High Resolution Melting (HRM) analysis

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ABSTRACT

The substitution of high priced meat with low cost ones and the fraudulent labeling of meat products make the identification and traceability of meat species and their processed products in the food chain important. A polymerase chain reaction followed by a High Resolution Melting (HRM) analysis was developed for species specific detection of buffalo; it was applied in six commercial meat products. A pair of specific 12S and universal 18S rRNA primers were employed and yielded DNA fragments of 220 bp and 77 bp, respectively. All tested products were found to contain buffalo meat and presented melting curves with at least two visible inflection points derived from the amplicons of the 12S specific and 18S universal primers. The presence of buffalo meat in meat products and the adulteration of buffalo products with unknown species were established down to a level of 0.1%. HRM was proven to be a fast and accurate technique for authentication testing of meat products.

1. Introduction

In recent years, species identification of meat and meat products has gained increased attention. The substitution of high priced meats meat from low priced ones, along with the fraudulent labeling of meat products has become common in the meat industry and raised a number of concerns regarding health (e.g. absence of allergens), diet (e.g. nutritional value and calories), religion (e.g. absence of pork from some diets) and lifestyle (e.g. vegetarianism and organic food) issues (Ballin, 2010). Fraud control is therefore needed in order to support fair trade and protect consumer rights.

Buffalo meat is one of the products frequently implicated in meat adulterations (Mane, Mendiratta, Tiwari, & Bhilegaokar, 2011). In India for instance, where it is found in abundance and its price is low, buffalo meat is often used to replace costlier meats such as goat and sheep meats (Karabasanavar et al., 2011). Moreover, sometimes buffalo meat exported from India is allegedly partially or totally replaced by beef, since slaughter of cows and export of beef is prohibited (Girish, Haunshi, Vaithiyanathan, Rajitha, & Ramakrishna, 2011). In European and other countries, like Egypt, buffalo meat is considered a delicacy and possesses high commercial value, making buffalo products prone to adulteration. Therefore, there is a need for rapid and reliable methods of authentication of buffalo meat and products.

Numerous conventional methods, i.e., anatomical, histological, microscopic, organoleptic, or other analytical methods i.e. chemical, electrophoretic, chromatographic and immunological have been applied for species specific identification of buffalo meat, with each method having its own limitations (Karabasanavar et al., 2011). In parallel, over the last few years DNA based methods have become popular as replacements of conventional methods in meat identification assays. The stability of DNA under high temperatures, pressures and chemical treatments used during processing ascertains the specificity and reliability of DNA based methods (Behrens, Unthan, Brinkmann, Buchholz, & Latus, 1999). Among them, PCR is the most widely used molecular technique as a result of its sensitivity, reproducibility and simplicity. Its accuracy in identifying meat products from closely related species can be improved further by using mitochondrial DNA due to its higher mutation rate and number of copies in each cell compared to genomic DNA (Girish et al., 2005). Several reports have proven the reliability of PCR amplification of various regions of the mitochondrial genome as a tool for identification of buffalo meat and products (Girish et al., 2011; Karabasanavar et al., 2011; Mane et al., 2011).

HRM analysis is a novel PCR 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, Reed, Gundry, Vandersteen, & Pryor, 2003; Zhou, Wang, Palais, Pryor, & Wittwer, 2005). High Resolution Melting (HRM) analysis (Wittwer et al., 2003) has been developed as an alternative approach to post PCR processing enzyme restriction and electrophoresis, labeled probes for SNP detection sequencing and TaqMan-probe-based real-time PCR. Besides 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. This

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Table 1
Commercial samples analyzed and Syto9® real-time PCR assay for buffalo detection in meat products (Ct values obtained from 20 ng DNA).

Meat product	Species labeled	12S Buffalo specific	Ct
Raw buffalo	Buffalo	+	10.51 ± 0.04
Fillet slices	Buffalo	+	12.82 ± 0.04
Kavourmas	Buffalo:porcine:ovine	+	13.03 ± 0.05
Smoked salami	Buffalo:porcine	+	12.21 ± 0.06
Sausage	Buffalo:pork	+	13.84 ± 0.07
Sausage with cheese	Buffalo:pork	+	12.97 ± 0.05

method has been adapted recently 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, 2012; Madesis, Ganopoulos, Argiriou, & Tsaftaris, 2012).

In this study, HRM was employed using specific and universal rRNA primers for developing and testing a method for the rapid detection and adulteration control of buffalo meat. HRM was proven to be a fast and accurate technique, capable of identifying the presence of buffalo meat and adulteration of buffalo products from unknown species down to 0.1%.

2. Materials and methods

2.1. Meat samples

The buffalo meat samples, containing raw buffalo meat, fillet slices, kavourmas (a local traditional product), smoked salami, sausage and sausage with cheese were purchased from local markets in Kerkini lake in Serres, Greece. Five samples from each commercial product were used (30 samples in total). Furthermore, certified samples from buffalo, bovine, pork, sheep and goat meat were obtained. All samples were stored at −20 °C until use to prevent enzymatic degradation of DNA.

2.2. DNA isolation

Total DNA isolations using 25 mg samples were performed using Nucleospin Tissue (Macherey–Nagel) as described in the instructions given by the manufacturer. The DNA concentration was estimated by spectrophotometric analysis using (BioPhotometer plus UV/Vis Photometer, Eppendorf, Germany) at 260 nm. The extracted DNA solution was stored in 4 °C until 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 to be specific for buffalo DNA sequences (López-Calleja et al., 2005). The 12S and 18S rRNA region is a well-accepted marker for buffalo meat and for specific detection of meat products (López-Calleja et al., 2005; Pegels et al., 2011). As the functionality and specificity of 12S and 18S primer-pairs had been already proven, these primers were used for qPCR experiments. In this study, a genotyping method based on 220 bp and 77 bp fragments respectively, using HRM analysis was developed. For the detection of adulteration with an unknown species, a duplex PCR assay was developed according to Mader, Ruzicka, Schmiderer, and Novak (2011), containing a buffalo specific primer for the 12S rRNA gene and a universal primer for 18S rRNA. Here a ratio of specific primers of 1:1.5 was used after optimization (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 (Corbett Research, Sydney, Australia) according to Ganopoulos, Argiriou, and Tsaftaris (2011). 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, 1.5 mM Syto9® green fluorescent nucleic acid stain, and 1 U Kapa Taq DNA polymerase (Kapa Biosystems, USA). A third generation DNA intercalating dye, Syto9®, that at high concentrations can saturate all available sites within double stranded DNA was used. Syto9® fluorescence provides

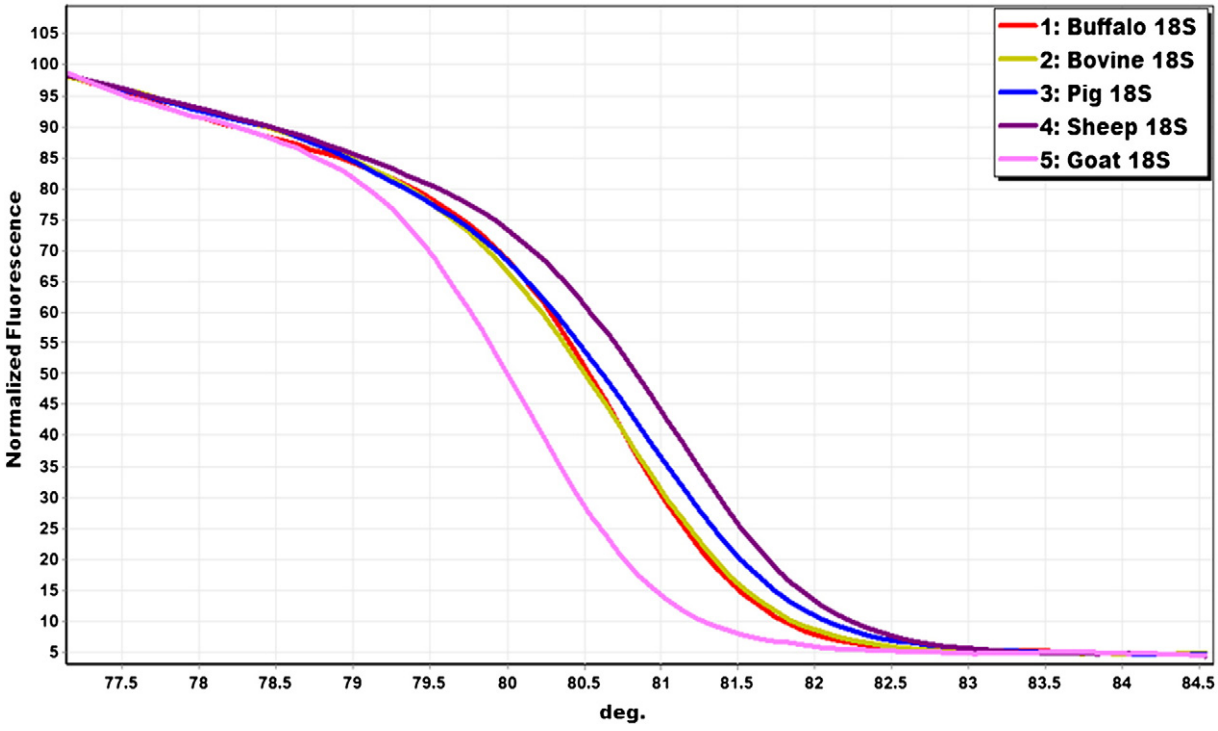

Fig. 1. HRM melting profile of the 18S region of the five animal species analyzed.

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, 63 °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 et al. (2011). 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 Tm, 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 (Fig. S2).

The efficiency of the method was evaluated by real-time PCR with Syto9® dye using the DNA template extracted from a reference buffalo meat. To verify again the specificity and the sensitivity of the above method, an increasing quantity of standard buffalo DNA extract was added to a mixture of DNA extracts of certain animal species (bovine, pork, ovine, caprine) (0.1%, 1%, 2%, 5%, 10%, 20% and 30%). In parallel, in order to verify the limit of detection of adulteration of buffalo meat products, an increasing quantity of the bovine DNA extract was added to the standard buffalo DNA extract (0.1%, 1%, 5%, 10%, 20%, 30% and 50%).

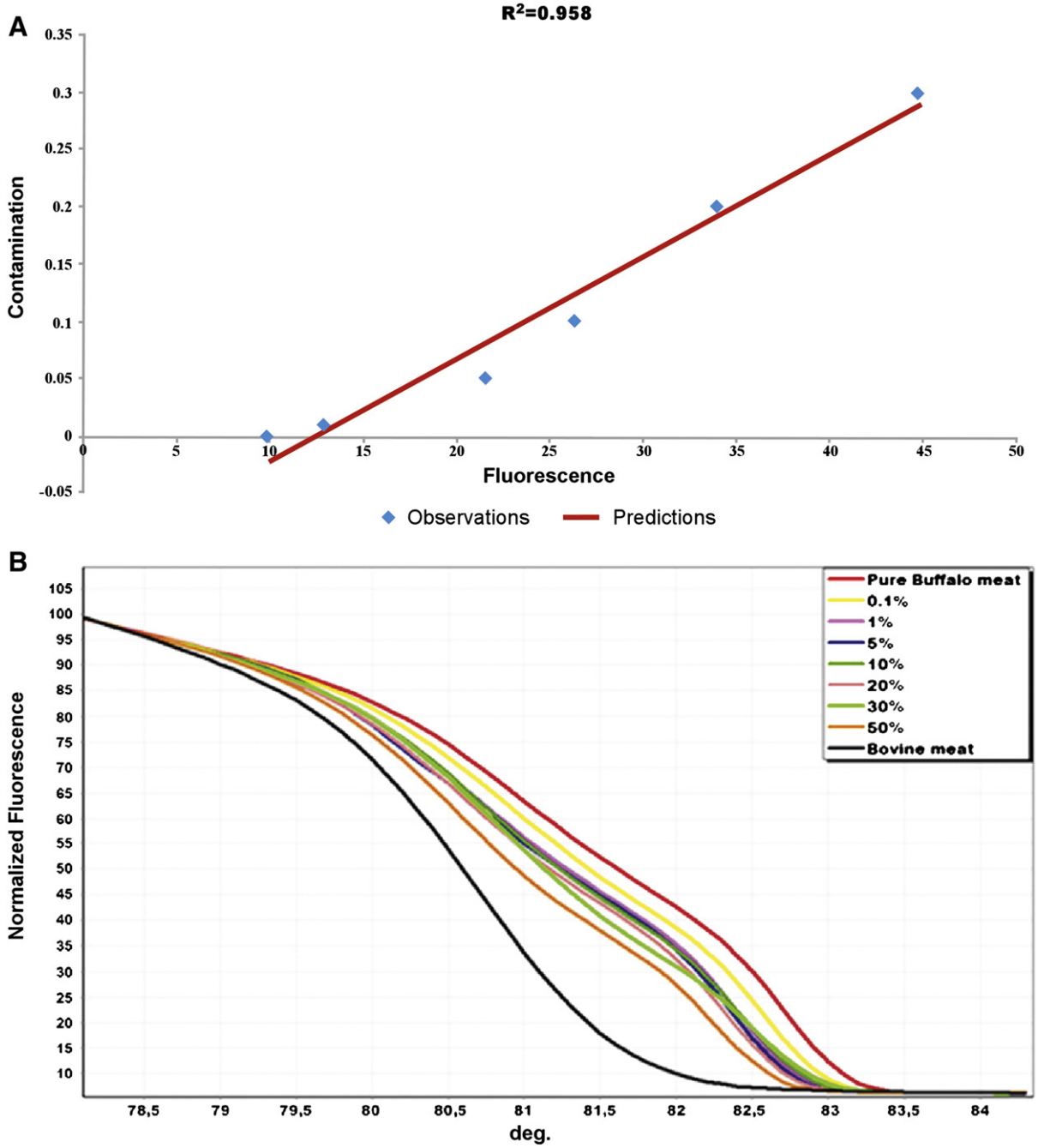

Fig. 2. A) Pure buffalo meat DNA was mixed with bovine in progressive proportions (0.1%, 1%, 5%, 10%, 20%, 30% and 50%). Values of fluorescence were plotted against the percentage of adulteration of each dilution to generate a typical standard curve. All the experiments were performed in duplicate. B) Melting curves obtained by HRM analysis of the two species amplicons and applied to reference mixtures containing 0.1%, 1%, 5%, 10%, 20%, 30% and 50% of bovine DNA in pure buffalo.

3. Results and discussion

A real-time PCR protocol was applied for the identification and quantitative determination of buffalo meat in commercial meat products. Table 1 depicts the results of the Syto9® assay for the detection of adulteration in buffalo meat products. The DNA extracted from all commercial buffalo products yielded a specific amplification product with 12S primers.

The efficiency of the method was confirmed by adding an increasing quantity of standard buffalo DNA extract to a mixture of DNA extracts of certain animal species (bovine, pork, ovine, caprine). A standard curve was obtained by real-time PCR with Syto9® dye amplification targeting the 12S gene of buffalo and applied to reference mixtures containing 10%, 5%, 0.5%, 0.2%, 0.1% of buffalo. A relative limit of detection and quantification of 0.1% with high correlation coefficient ($R^2 = 0.978$) was obtained (data not shown).

Meat samples from different animal species could be distinguished using HRM analysis and the universal primers 18S. The melting profiles of the 18S amplicons of 5 different animal species (buffalo, bovine, pork, sheep and goat) are illustrated in Fig. 1. A distinct melting curve was generated for each animal species presenting one inflection point. However, the melting profiles of buffalo and bovine meat were similar and revealed the need for the use of both specific 12S and universal 18S primers, in order for these meat samples to be clearly distinguished (Fig. S1).

The limit of detection of adulteration of buffalo meat products was evaluated by adding an increasing quantity of bovine DNA extract to the standard buffalo DNA extract. As shown in Fig. 2A, the highly significant correlation between the level of fluorescence at a predefined temperature and the contamination percentage allowed the development of a standard curve ($R^2 = 0.958$). This R^2 value suggests high correlation of the fluorescence mean with the artificial samples used. The melting profiles of the amplicons derived from the artificial mixtures are illustrated in Fig. 2B. The presence of increasing quantity of bovine DNA extract into the buffalo DNA alters the shape and shifts proportionally the melting curve, compared to the curve of pure buffalo DNA, thus revealing the adulteration.

As far as the commercial buffalo products are concerned, the normalized HRM curves for the amplicons, from the six products, based on HRM analysis with specific and universal primers are shown in Fig. 3. In the developed assay the PCR products of standard buffalo meat samples showed melting curves with two visible inflection points at 82.5 °C and at 80.5 °C derived from the amplicons of the 12S specific and 18S universal primers, respectively. The first two samples (raw buffalo meat and fillet slices) had identical melting curves with the standard buffalo meat and presented the same inflection points, even though one was raw and the other was thermally processed. The respective melting curve analysis revealed the amplification of similar products since they exhibited the same melting curve profile. On the contrary, the melting curves of the other buffalo meat products generated different melting profiles as they contained pork meat or mixture of pork and ovine meat or pork meat and buffalo cheese according to their labeling. Results obtained from the six different commercial meat products indicate that HRM can be effectively used to assess if a single known species or more than one has been used during the commercial production.

In other studies, Mane et al. (2011) used a pair of mitochondrial D-loop primers, restriction enzymes and electrophoretic treatment of PCR products for the detection of adulteration of meat products by buffalo meat at 1% level. Likewise, Karabasanavar et al. (2011) used a similar pair of mitochondrial D-loop primers for the authentication of buffalo meat and established the detection of adulteration of buffalo meat into non-buffalo meats down to 0.1%. Similarly, De et al. (2011) suggested a simplex and duplex PCR assay for detection of bovine and buffalo milk targeting a mitochondrial D-loop region. Previously, RAPD-PCR (Mane, Tanwar, Girish, Sonawane, & Sharma, 2008) and PCR-RFLP (Girish et al., 2005) were applied for the differentiation of buffalo meat from other species.

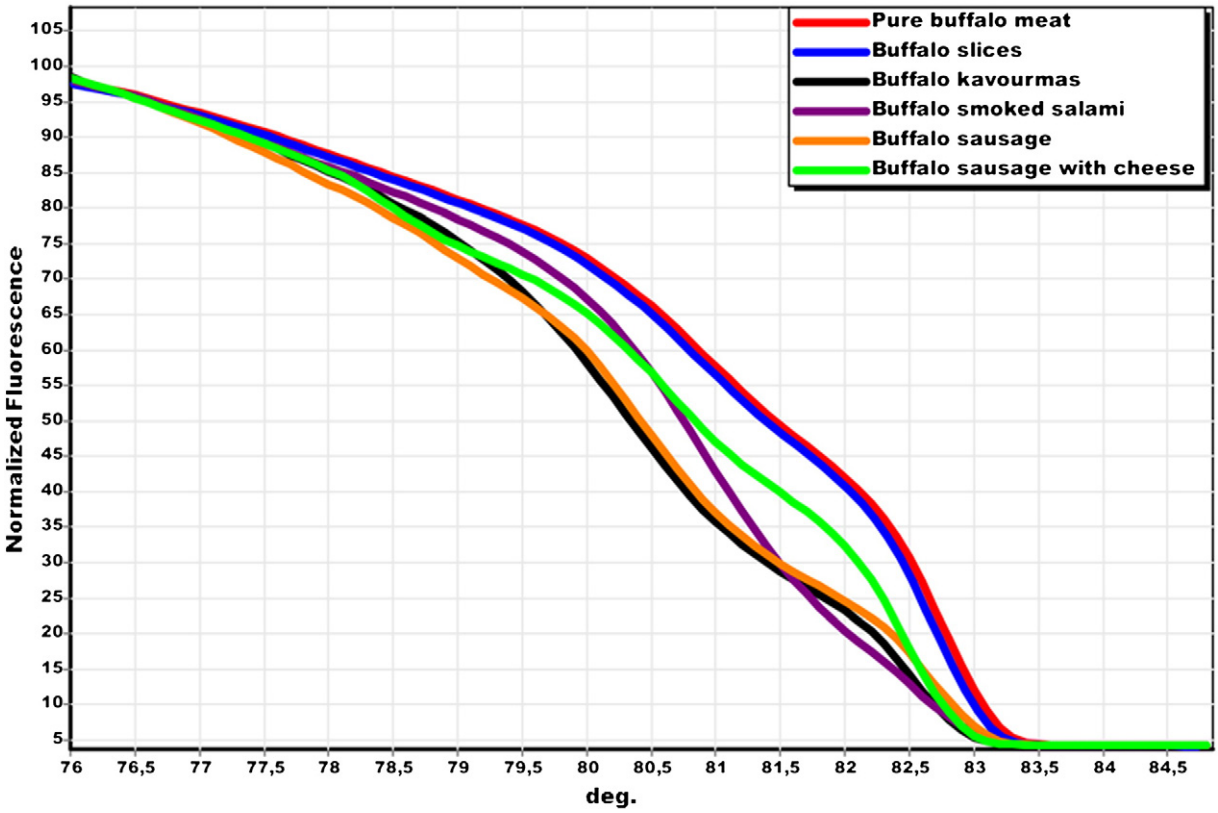

Fig. 3. Melting curves obtained by HRM analysis of the 18S universal gene and buffalo 12S specific amplicon when applied to buffalo meat and its processed meat products.

In comparison with the studies of Mane et al. (2011) and Karabasanavar et al. (2011), where only the presence of buffalo meat in meat products is detected, the present study reports the adulteration of buffalo products with an unknown mixture of meats along with the one of meat products with buffalo meat. Moreover, the number of different meat species and their percentage as contents of a product can be estimated in order to confirm the labeling claims. To achieve that, the results from the HRM analysis of the commercial buffalo products must be compared with those from the artificial admixtures of unknown meat extracts into the buffalo meat extract. The shape of the melting curve is proportional to the mixtures of meat in the product and the analysis of the curve can provide a quantitative measurement of the percentage of the selected meat species.

4. Conclusion

HRM analysis was proven to be a fast and accurate closed tube post PCR method which permits the identification of animal species via the use of specific (ribosomal or mitochondrial) genetic regions in commercial food products. This is the first report describing the development of an HRM method for adulteration testing of meat products. The method was proven to be effective and accurate detecting down to 0.1% of unknown meat addition to buffalo meat products and vice versa and of quantifying the ratio of meat species in a meat product. Therefore, HRM analysis could be easily used for rapid and low-cost authentication testing of meat products.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meatsci.2012.12.017.

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