

THESIS

SPECIES SPECIFIC DETECTION OF ADULTERATION OF WATER BUFFALO MILK
WITH COW MILK USING HIGH RESOLUTION MELTING ANALYSIS

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ABSTRACT

SPECIES SPECIFIC DETECTION OF ADULTERATION OF WATER BUFFALO MILK WITH COW MILK USING HIGH RESOLUTION MELTING ANALYSIS

Economically motivated adulteration, a subset category of food fraud, was defined in 2009 by the Food & Drug Administration (FDA) as “the fraudulent, intentional substitution or addition of a substance in a product for the purpose of increasing the apparent value of the product or reducing the cost of its production,” and can often encompass public safety effects through the unknown addition of allergens, toxins, and hygienic risks (Wheatley & Spink, 2013). According to the FDA database which tracks scholarly records of adulterated foods, dairy products are the second most reported category of adulterated food products (Moore, Spink, & Lipp, 2012). Water buffalo milk represents the second most produced milk worldwide and higher value dairy raw products make it subject to economically motivated adulteration by means of dilution with cow milk (Bonfatti, Giantin, Rostellato, Dacasto, & Carnier, 2013). It is of interest to consumers, manufacturers and governing bodies to have a simple, fast, accurate, and sensitive method to detect adulteration by cow’s milk.

Several methods have been developed to detect species adulteration in dairy products including immunological, chromatography, and molecular methods (Mayer, 2005). In this investigation a real-time PCR assay was developed to specifically detect and relatively quantify cow milk adulteration in water buffalo milk utilizing high resolution melting analysis. A duplex real-time PCR reaction was performed targeting mitochondrial DNA in both species of interest and the resulting amplified products were analyzed after a melt cycle was performed. Results

indicated that the assay was specific, efficient, and validated the use of real-time PCR for analysis. The use of high resolution melting analysis allowed for the discrimination of dilution of water buffalo milk with cow milk down to 0.1%. This study indicates the feasibility of real-time PCR and high resolution melting analysis in the detection of adulteration in dairy products.

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Chapter I

Introduction

Food fraud for economic gain has been prevalent throughout history for as long as food has had economic value as commodities. Economically motivated adulteration, a subset category of food fraud, was defined in 2009 by the Food and Drug Administration (FDA) as “the fraudulent, intentional substitution or addition of a substance in a product for the purpose of increasing the apparent value of the product or reducing the cost of its production,” and can often encompass effects public safety through the unknown addition of allergens, toxins, and hygienic risks (Wheatley & Spink, 2013). The FDA is increasingly is focusing on prevention of food fraud, and as a result has started funding research to define this threat to public health as well as analyze trends of its manifestations. Milk was the second most adulterated food reported in the scholarly records with 14% (Moore, Spink, & Lipp, 2012).

Several methods have been developed to detect species adulteration in dairy products including immunological, chromatography and molecular methods (Mayer, 2005). Many of these methods can be time consuming, require technical expertise, and are expensive. Development of a fast, accurate, inexpensive method that requires minimal technical knowledge would be valuable to the dairy industry in the stewardship of the value, quality, and safety of its products in the interest of public health. Of particular interest to a manufacturer would be a method to detect adulteration in the raw commodity.

Many of these methods are based on the properties of proteins found in the milk of dairy species. Methods identified are electrophoretic (Mayer, 2005; Moio, Chianese, Rivemale, & Addeo, 1992; Molina, Martin-Alvarez, & Ramos, 1999), chromatographic (Chen, Chang, Chung,

Lee, & Ling, 2004; Cozzolino, Passalacqua, Salemi, & Garozzo, 2002; Mayer, 2005), immunological (Hurley, Ireland, Coleman, & Williams, 2004), and molecular (Mafra, Ferreira, & Oliveira, 2008). Isoelectric focusing (IEF) of proteins has historically been the standard method of detecting species-specific adulteration in milk and dairy products, and the European community has established an official method for detecting bovine milk in water buffalo milk using Υ_2 - and Υ_3 - casein (CN) protein fractions from the plasmin hydrolysis of β -CN (Addeo, Pizzano, Nicolai, Caira, & Chianese, 2009; Addeo et al., 1983). This method is based on the distinct separation of the Υ -CN fractions and their different migration patterns through polyacrylamide gel during isoelectric focusing.

In an attempt to further refine techniques to detect adulteration in dairy products with substitution of milks from different species, a variety of immuno assays have been developed over the years including enzyme-linked immunosorbent assays (Hurley et al., 2004). The use of proteomic technology platforms have recently been reviewed and highlight the use of chromatography and mass spectrometry platforms such as matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOFMS), liquid chromatography coupled with mass spectrometry (LC-MS), and capillary electrophoresis coupled with mass spectrometry (CE-MS) to detect adulteration in dairy products (Abd El-Salam, 2014). Often the target proteins for analyses are the casein and whey proteins that have been useful in IEF and immunoassays. Other chromatography methods such as high performance liquid chromatography (HPLC), reverse-phase high performance chromatography (RP-HPLC), and ion exchange chromatography have been used to separate proteins in dairy products (De la Fuente & Juarez, 2005).

Deoxynucleic acids (DNA) or biomolecule based methods for species identification and authentication have gained popularity due to DNA molecule's presence in most tissues, DNA's

stability and ability to survive processing like thermal treatment. As well it has potential to be fast, simple, and specific (Mafra et al., 2008). A growing number of DNA based methods utilize the specific amplification of target sequences by means of polymerase chain reaction (PCR) in species identification (Mafra et al., 2008). Mitochondrial DNA (mtDNA) has been targeted due to being present in multiple copies in the cell and its ability to quickly accumulate mutations (Bottero & Dalmaso, 2011). In dairy products, in particular, it is known that DNA and mtDNA is present in the somatic cells found in the milk (De la Fuente & Juarez, 2005).

Mafra et al. (2007) applied the PCR technique to quantify the adulteration of cow milk in goat milk and described that the intensity of the band in agarose gel is proportional to the contamination in the sample. The researchers developed a duplex assay, which targeted the 12S rRNA gene in mtDNA from both species. This investigation is an early example of when initially PCR amplification assays applied in a qualitative manner. In the past it was difficult to obtain accurate quantitative information until the development of fluorescence based real-time PCR. It has its foundation in dyes, which bind to double-stranded DNA molecules and can be detected (qPCR) (Mafra et al., 2008).

In recent years the analysis of melt curves of amplicon products from the amplification of target templates has been gaining interest as an approach in the analysis of foodstuffs (Druml & Cichna-Markl, 2014). Melting curve analysis has been used in qPCR assays to check if the correct products were formed in the reaction. High resolution melting (HRM) analysis is based in the theory that although double stranded DNA is very stable at room temperature it will begin to disassociate when the temperature is slowly increased (Vossen, Aten, Roos, & den Dunnen, 2009). The resulting curves from the data allow for the discrimination of subtle changes in the amplicons present in the products of the reaction, including the presence of adulterant DNA in

food matrices. Mader et al. (2011) utilized this technology for this application and proposed that the relative fluorescence of the melt curve at a defined temperature was correlated to the extent of adulteration in the sample.

In the present investigation raw cow milk samples were collected from a local farm (Brighton, CO) from three different milking days. Raw water buffalo milk samples were collected from a dairy in Cañon City, CO over three different milking days. DNA was eluted from samples using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for tissue. Primers were designed to amplify target sequences from mitochondrial DNA in cow and water buffalo species. Sets of forward and reverse primers for both bovine (C12S1) and water buffalo (WB12S1) were designed for the polymerase chain reaction (PCR) assay using the National Center for Biotechnology Information (NCBI) database primer design tool PrimerBLAST (Ye et al., 2012). A second set of primers for bovine and water buffalo species was chosen from the literature (Lopez-Calleja et al., 2005; Pegels et al., 2011).

PCR conditions were screened in singleplex reactions in order to optimize the reaction for the primers used, and then confirmed in a duplex assay. Each primer was challenged for specificity to the target with template DNA from the non-target species and confirmed using 2% agarose gel electrophoresis. Once specificity was confirmed and conditions optimized, the assay was repeated for multiple samples over multiple testing days to assess the efficiency and validate the reaction. In this investigation validation of the assay was defined as the development and demonstration of the assay to easily reproduce accurate and sensitive results from multiple samples on multiple days of analysis. Once validated the assay was set up to test for correlation between the amount of cow milk adulteration and relative fluorescence by constructing calibration curves using two methods. One method used pure cow DNA to adulterate pure water

buffalo DNA in defined ratios, and the other mixed pure cow milk with pure water buffalo milk at defined ratios in order to simulate adulteration in a dairy. The assay and HRM analysis was performed. The resulting data of the relative fluorescence were plotted on a graph over the negative log of the ratio of adulteration.

All primers resulted in specific reactions and amplification of products based on melting analysis at all temperatures in the gradient. The melt temperatures for WB12S1 and C12S1 amplicons were 78.8°C and 76.2°C, respectively. The BDLOOP and WB12S2 amplicons resulted in melting temperatures of 75.8°C and 80.0°C, respectively. The BDLOOP and WB12S2 set was chosen for further analysis due to a wider gap between melting temperatures of amplicons from each primer pair. These primers were used for further validation of the PCR assay by repeating the assay for 6 samples analyzed on 18 days. The assay was validated to be sufficiently efficient, specific, and sensitive. However, it was found that the day of the analysis had more of an effect on efficiency than analysis of different samples. Thus, standard curves were constructed from serial dilutions of the sample on each day of analysis. Results from the HRM analysis of the calibration curves of known dilutions of adulterated water buffalo samples indicated strong correlations between relative fluorescence at the predefined temperature and the level of contamination. It was observed that stronger correlation was found when pure DNA samples were admixed compared to when milk was admixed and DNA isolated.

In the following investigation using real-time PCR and HRM analysis, an assay was successfully designed to detect bovine adulteration in water buffalo milk. A relatively quick, accurate, and sensitive assay for the specific detection of adulteration of water buffalo milk with cow milk was developed utilizing these technologies. The use of the method could be applied in the detection and relative quantification of adulterations in other food matrices. This method

could potentially be used to verify the authenticity of food products from a variety of matrices as well as herbal supplements and other plant and animal derived products. It could be useful in detecting DNA of allergens such as wheat, soy, peanuts, and tree nuts in food matrices. The assay should be further investigated to understand which method used to construct the calibration curve is more accurate in the prediction of the adulteration in unknown samples. This would further validate the assay making it a more useful tool in the detection of bovine adulteration in water buffalo milk. The effect of thermal processing conditions on the accuracy and sensitivity of the assay should be determined. In addition, statistics should be analyzed to understand the effect of samples and the day the sample was run on the accuracy of the calibration curve. This would help to understand the limitations of the assay.

Chapter II

Review of Literature

2.1 Introduction

Food fraud for economic gain has been prevalent throughout history for as long as foods have had economic value as commodities. Issues that arise from food fraud can often encompass deception of the consumer, inequitable competition against food producers operating lawfully, threats to public safety, and a burden on regulatory agencies. Dairy products constitute some of the most significant foods bought and sold in the history of human society, and most likely one of the earliest foodstuffs to be subject to adulteration and misrepresentation. The most recognized documented case in recent history was the 2007 incident of melamine addition to milk products, which resulted in death (Everstine, Spink, & Kennedy, 2013). Economically motivated adulteration, a subset category of food fraud, was defined in 2009 by the FDA as “the fraudulent, intentional substitution or addition of a substance in a product for the purpose of increasing the apparent value of the product or reducing the cost of its production,” and encompasses effects of public safety through the unknown addition of allergens, toxins, and hygienic risks (Wheatley & Spink, 2013). The FDA focuses on prevention of food fraud, and as a result has started funding research to define this threat to public health as well as analyze trends of its manifestations.

Economically motivated adulteration of food and its risks and concerns has gained attention and awareness in recent years (Everstine et al., 2013; Wheatley & Spink, 2013). Moore, Spink and Lipp (2012) developed a database for the existing data on food fraud and economically motivated adulteration. The meta-analysis of the current literature, media reports, and other

sources was organized into a database and analyzed to identify tendencies. Milk was found to be the second most commonly reported in the scholarly records (14% of records), and fifth most in media and other reports at three percent of the total records.

Water buffalo milk represents the second most produced milk worldwide (Bonfatti, Giantin, Rostellato, Dacasto, & Carnier, 2013). Milk from this species is economically relevant as it has a higher market price and is used to make high value cheeses, such as “mozzarella di bufala campana,” with protected origins and strict quality regulations (Addeo et al., 1983). Milk from this species is a target for economically motivated food adulteration by partially substituting or diluting the milk with bovine milk (Lopez-Calleja et al., 2005). Several methods have been developed to detect species adulteration in dairy products including immunological, chromatography and molecular methods (Mayer, 2005). Many of these methods can be time consuming, require technical expertise, and expensive. Development of a fast, accurate, inexpensive method that requires minimal technical knowledge would be valuable to the dairy industry in the stewardship of the value, quality, and safety of its products in the interest of public health. Of particular interest to a manufacturer would be a method to detect adulteration with cow milk in the raw commodity.

2.2 Analytical Methods for Species Detection in Dairy Products

2.21 Isoelectric-Focusing Methods

In recent years several analytical methods for species identification in foods have been developed. Species identification of milk and dairy product sources is important because of the risk for adverse reactions to milk proteins as well as to detect fraudulent practices and unfair economic gains. Many of these methods are based on the properties of proteins found in the milk of dairy species. Methods identified include electrophoretic (Mayer, 2005; Moio et al., 1992;

Molina et al., 1999), chromatographic (Chen et al., 2004; Cozzolino et al., 2002; Mayer, 2005), immunological (Hurley et al., 2004), and molecular (Mafra et al., 2008). It is important to define the scope of the detection method with respect to specificity and accuracy of quantification to understand the advantages and drawbacks of any analytical method used for species detection in dairy products.

Isoelectric focusing (IEF) of proteins has been the standard method of detecting species-specific adulteration in milk and dairy products, and the European community has established an official method for detecting bovine milk in water buffalo milk using Υ_2 - and Υ_3 - casein (CN) protein fractions from the plasmin hydrolysis of β -CN (Addeo et al., 2009; Addeo et al., 1983). This method is based on the distinct separation of the Υ -CN fractions and their different migration patterns through polyacrylamide gel during isoelectric focusing. The plasminolysate is stained with Coomassie Brilliant Blue G-250 stain, and the concentration of the band is relative to the amount of adulterant CN fraction present in the sample. Addeo et al. (1983) first demonstrated the usefulness of this technique by preparing standard mixtures of bovine milk in water buffalo milk and reference samples of 0 and 1% bovine milk. Protein was extracted from the milk and plasmolyzed before IEF was performed on an ultrathin polyacrylamide gel in a pH gradient of 3.5-10. Migration of the Υ_2 - and Υ_3 -CN peptides from bovine and water buffalo were distinct. However, it was found that standard bovine samples must be used to determine if the unknown sample is adulterated with greater than or equal to 1%. Recently this method has been reported to lack specificity and high resolution and has shown false positives in identifying bovine adulteration in water buffalo mozzarella cheese due to a bovine-like CN fraction in water buffalo milk cheese (Addeo et al., 2009).

2.22 Immunological Methods

In an attempt to further refine techniques to detect adulteration in dairy products with substitution of milks from different species, a variety of immuno assays have been developed over the years including enzyme-linked immunosorbent assays (Hurley et al., 2004). The basis of this technology can be applied in a multitude of formats contingent on the method used to detect the antigen using a labeled antibody. Antibodies can be synthesized as monoclonal or polyclonal. The type of antibody used will affect the function of this type of assay, with polyclonal antibodies usually being less specific than their monoclonal counterpart, with more occurrence of cross reactivity to unintended targets (Hurley et al., 2004).

Beer et al. (1996) demonstrated the ability of an ELISA assay to be specific and improve on the resolution of adulteration detection limit by evaluating bovine milk adulteration in sheep or goat cheese. An indirect competitive assay format was designed utilizing polyclonal antibodies in the investigation. Indirect assays use as its basis a labeled antibody attached to the primary binding antibody. The antigen the researchers targeted was the whey protein, native and heat-denatured bovine β -lactoglobulin. Researchers demonstrated that bovine milk adulteration could be detected in the cheeses to the level of 0.1 and 0.2%, respectively, for native and heat-denatured β -lactoglobulin. Garcia et al. (1993) developed a format that utilized a plate bound antibody to first bind to the antigen followed by the labeled antibody to detect caprine milk in ovine milk samples. The researchers targeted goat whey proteins, and utilizing polyclonal antibodies they were able to reliably detect adulteration down to 1%.

Recently Ren et al. (2014) developed an indirect competitive ELISA assay to detect bovine milk in yak milk. Researchers utilized highly specific monoclonal antibodies to detect the β -CN in bovine milk that was further assessed with standard adulterated milk mixtures to determine the

effect of different treatments of milk on the reliability and sensitivity of the assay. Heat treatments included pasteurization at 65°C for 10 minutes, ultra-high temperature pasteurization at 138°C for 4 seconds, and spray drying by means of heating to 150-160°C for 10-20 seconds to simulate common milk processing parameters. Milk underwent acidification treatment by adding hydrochloric acid to pH endpoints 6.3, 5.8, 5.0, 4.6, which are common to dairy products. Lastly, to simulate cheese production a rennet treatment was prepared as well. The assay demonstrated specific detection of cow milk in yak milk down to 1% with cross reactivity below 1%. Heat treatment of bovine and yak milk mixtures had no effect on the reliability of the assay, indicating that β -CN target is not severely denatured during intense processing. Acidification and rennet treatments were found to have no effect on the performance of the ELISA assay.

Addeo et al. (2009) sought to improve upon the specificity and resolution capability of IEF and immunoassay antipeptide antibodies simultaneously by means of immunoblotting. The investigators synthesized three antibodies against specific short amino acid sequences in the Υ_2 -CN peptide in order to investigate their ability to distinguish bovine versus water buffalo IEF protein bands. Standard mixtures of milk and cheese samples were prepared by plasmin hydrolysis followed by electrophoresis through an ultrathin polyacrylamide gel and subsequent transfer of the proteins on nitrocellulose paper for immunostaining. The initial results for the IEF of milk admixtures presented the bovine-like Υ_2 -CN peptide in the pure water buffalo hydrosylate as predicted. The antipeptide antibody that was prepared to bind to the bovine β -CN (106-110) amino acid sequence was able to recognize both bovine and water buffalo Υ_2 -CN peptide but did not cross react with the bovine-like peptide, demonstrating specificity. The investigators then applied this assay to the standard admixtures of mozzarella cheese and were able to achieve the same results with an effective resolution down to 0.25% adulteration.

2.23 Proteomic Methods

The use of proteomic technology platforms has recently been reviewed and highlights the use of chromatography and mass spectrometry platforms such as matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOFMS), liquid chromatography coupled with mass spectrometry (LC-MS), and capillary electrophoresis coupled with mass spectrometry (CE-MS) to detect adulteration in dairy products (Abd El-Salam, 2014). Usually the target proteins for analyses are the casein and whey proteins that have been established as useful in IEF and immunoassays. Other chromatography methods such as high performance liquid chromatography (HPLC), reverse-phase high performance chromatography (RP-HPLC), and ion exchange chromatography have been used to separate proteins in dairy products (De la Fuente & Juarez, 2005). De la Fuente and Juarez (2005) reviewed chromatography methods used to authenticate dairy products and noted that in general the chromatograms of native proteins could become convoluted from the biological and chemical processes that occurred during proteolysis and cheese ripening in certain dairy products. It was stated that if the method was based on heat-sensitive whey proteins the same complex chromatograms could be produced. This called into question the sensitivity and accuracy of such techniques to the detection of adulteration in some dairy products.

Cozzolino et al. (2001) investigated detection of cow milk in either ewe or water buffalo milk, as well as the addition of powdered cow milk to raw ewe or water buffalo milk using MALDI-TOFMS. The investigators sought to develop an analytical method that would be sensitive as the 1% level achieved by the standard IEF method and could be used to detect thermally treated milk in adulterations by examining patterns of whey proteins α -lactalbumin and

β -lactoglobulin as biomarkers. They theorized that the different molecular weights of these proteins for each respective species would be distinct on the resulting mass spectrum.

Milk samples were diluted with trifluoroacetic acid to precipitate casein and decrease the background signal, and sinapinic acid in acetonitrile-water was used for the matrix. Analysis of whey proteins for cow and ewe milk showed lactoglobulins mass signals differed enough to be considered a biomarker. Standard mixtures of cow and ewe milk were analyzed; researchers showed cow β -lactoglobulins could be detected down to 5%. Water buffalo milk was analyzed to determine a suitable biomarker for cow milk adulteration of this species milk. Both α -lactalbumin and β -lactoglobulin showed signals at different masses on the spectra, but α -lactalbumin in cow milk showed a larger mass difference than the buffalo milk counterpart. Thus the evidence showed that α -lactalbumin suitable choice as a biomarker in the detection of cow milk adulteration. Again, standard water buffalo milk mixtures were analyzed and cow milk was detectable to 5% adulteration. In the case of the addition of powdered milk it was possible to observe whey protein adjunct formation due to the Maillard reaction as it was observed as predicted on the mass spectra. Cozzolini et al. (2002) would later apply this method to detection of cow milk and ewe milk adulteration in mozzarella cheese. They were able to show that even though the biomarker whey proteins are soluble in water they are not completely lost from the cheese matrix during processing. Thus they are suitable biomarkers in analysis of mozzarella cheese. Both α -lactalbumin and β -lactoglobulin were suitable for detecting ewe whey proteins in water buffalo mozzarella down to 2%. The same results were achieved for cow and water buffalo milk mixtures made into mozzarella cheese. However, the detection limit was found to be higher at 5%, because of the lower concentration of proteins in cow milk compared to ewe milk. The researchers theorized that the relative area of the biomarkers peaks could be determined and

plotted against the known ratio of milks in the cheese to be used as a curve for the quantification of the adulterant. The researchers showed a relatively strong correlation for both ewe and cow milk adulteration respectively.

Recently a LC-MS/MS method was applied to cow milk to determine adulteration with whey caseinmacropeptide (CMP) as a biomarker (Motta et al., 2014). Samples were prepared and digested with pepsin before analysis and separation with LC-MS/MS. A triple quadrupole electron spray ionization source in positive mode was used to detect the mass of the peptides. Researchers showed that CMP was suitable as a biomarker for whey additions to milk with a limit of detection $1\mu\text{g mL}^{-1}$, and a limit of quantification of $5\mu\text{g mL}^{-1}$. The researchers noted a satisfactory precision and demonstrated the specificity of the method.

Whey proteins were the focus of an investigation in constructing a method to detect cow milk in either ewe or caprine milk using CE-MS (Muller et al., 2008). Muller et al. (2008) sought to detect bovine β -lactoglobulin in standard milk mixtures of cow in ewe milk, and cow in caprine milk. The investigators found that using a negatively charged fused-silica surface for the capillary led to poor adsorption, and consequently poor resolution and quantification of the peaks in the electropherogram. Therefore, the researchers used a non-internal coated fused silica capillary with a low pH and high ionic running buffer, which increased adsorption and resolution. It was found that the peaks of β -lactoglobulin could be separated and detected for all three species in the electropherograms. The investigators were able to quantitatively analyze the data by coupling CE with MS and plotting the ratio of the corresponding β -lactoglobulin bovine and ovine, or bovine and caprine peaks against the known dilution of the mixture. In another study that combined CE with MS techniques the researchers attempted to utilize plasmin hydrolysis products of β -CN from bovine milk in the detection of adulteration with water buffalo

milk (Somma, Ferranti, Addeo, Mauriello, & Chianese, 2008). The researchers were successful in separating the hydrosylates to confirm the presence of bovine milk in water buffalo milk down to 5%.

B-lactoglobulin whey proteins were the attention of an investigation in another proteomic approach to detect and quantify of bovine in ewe or bovine in caprine milk using RP-HPLC (Ferreira & Cacote, 2003). The study analyzed the effect of ripening and proteolysis on the method that was developed by analyzing cheeses manufactured from standard mixtures. Analysis was performed after preparation, after four weeks of ripening, and commercial cheeses with ripening times between 45 and 60 days were analyzed using the RP-HPLC method. The researchers found that the technique produced reproducible and precise results in the separation and identification of whey proteins, including β -lactoglobulin, from all species. As expected, similar chromatography profiles were obtained for milk mixtures and the corresponding fresh cheese mixture. Although it was observed that ripening affected the products obtained on the chromatogram, β -lactoglobulin peaks were discernible. The method was used to quantitatively determine the presence of bovine milk in ewe and bovine in caprine milk cheeses to a detection limit of 2%. When the method was applied to commercial cheeses the results indicated that all cheeses examined were produced in accordance with standards of identity for that product. In another study utilizing lactoglobulins investigators coupled HPLC with MS in order to detect and quantify bovine milk in caprine milk (Chen et al., 2004). The use of mass spectrometry allowed for the mass determination of whey proteins and lead to positive identification of peaks on chromatograms. The researchers used the relative ratio of the peak area of bovine β -lactoglobulin to total β -lactoglobulin for both species and plotted it against known dilutions of milk to create a standard curve. They showed a strong correlation down to 5% adulteration.

2.24 Review of Multiple Methods

In another study designed to evaluate several methods of species-specific detection Mayer (2005) applied several different methods to the identification of animal species in milk and several different types of cheese products including cheeses known to undergo extensive proteolysis. Mayer made standard mixtures of cows' and ewes' or cows and goats' milk (w/w) for analysis, and for pilot scale cheese production of Camembert and Tilsit cheeses. Camembert cheeses were aged for three and seven weeks and Tilsit for two, five, and nine weeks to examine the effect of proteolysis on each of the analytical methods chosen. Kashkaval cheese, a semi-hard cheese, made with standard mixtures of cows' and ewes' milk was obtained after ripening for one, two and three months. Cheese and milk samples were prepared for analysis with techniques that included urea-polyacrylamide gel electrophoresis (urea-PAGE), anion-exchange and cation-exchange high performance (HPLC) of CN, isoelectric focusing (IEF), and polymerase chain reaction (PCR).

Results of urea-PAGE of CN demonstrated that α_{s1} -CN could be successfully used as a marker for bovine milk in milk mixtures, but were not quantified. However, the marker could no longer be distinguished when applied to cheese due to the proteolysis that occurs during ripening. When IEF of γ - and para- κ -CN was applied to milk samples, γ -CN yielded an electrophoretic pattern in bovine milk that was distinguishable from caprine milk but not ovine milk. Semi-quantitative analysis could be performed in post processing of the gel. In all of the cheese samples no interfering bands were detected, but Mayer noted that excellent separation was necessary to minimize risk of false positives. Para- κ -CN showed potential as a marker for adulteration in milk and all cheese samples.

Anion-exchange HPLC of CN was conducted on milk samples and α_{s1} -CN was found to be a likely candidate as a marker (Mayer, 2005). Standard mixtures of cow milk were shown to accurately estimate the percentage of adulteration in samples. Because of the proteolytic nature of cheese ripening, this method was unable to successfully be applied to cheese. Cation-exchange HPLC was applied to para- κ -CN isolate from IEF and chromatograms showed distinct peaks in all cheese samples for semi-quantitative analysis. Native PAGE of whey proteins demonstrated the higher electrophoretic mobility of bovine β -lactoglobulin compared to whey proteins of other species milk. When IEF was applied to whey proteins α -lactalbumin and β -lactalbumin both were shown to be markers of bovine milk in other species milk. PCR was used to analyze three samples of feta cheese that had conflicting results between IEF and an ELISA assay. Two different bovine specific primers were used to detect bovine milk in mixed Camembert cheeses and were able to detect to 0.5% adulteration. To determine the effect of proteolysis on qualitative detection of bovine adulterant in PCR results, Mayer applied the assay to fresh, ripe and overripe Camembert and found no differences. Next the PCR assay was applied to the feta cheese and was found to confirm the results from IEF.

2.25 Molecular Methods

Deoxynucleic acids (DNA) or biomolecule based methods for species identification and authentication have gained popularity due to DNA molecule's presence in most tissues, DNA's stability and ability to survive processing like thermal treatment. As well it has potential to be fast, simple, and specific (Mafra et al., 2008). A growing number of DNA based methods utilize the specific amplification of target sequences by means of polymerase chain reaction (PCR) in species identification (Mafra et al., 2008). Many PCR methods have been exploited and protocols developed for species analysis of several food matrices including those from meat,

dairy, fish, plant, feed, and matrices subject to severe treatments (Bottero & Dalmaso, 2011). These assays have the advantage of having improved sensitivity, and performance over protein-based assays, as well as are able to be automated to rapidly process large sample numbers (De la Fuente & Juarez, 2005).

DNA based assays are constructed using critical elements that include the selection of a DNA marker or target sequence, DNA extraction, efficient amplification, and detection (Bottero & Dalmaso, 2011). Post-processing procedures are used in the detection of a target sequence. Choosing a target sequence and the development of specific primers to amplify the target is of supreme importance to any PCR based assay. It can ensure the specificity of the assay, and in many cases, has shown that closely related species can be identified. Mitochondrial DNA (mtDNA) has been targeted due to being present in multiple copies in the cell and its ability to quickly accumulate mutations (Bottero & Dalmaso, 2011). In dairy products in particular it is known that DNA is present in the somatic cells found in the milk (De la Fuente & Juarez, 2005). However, many elements of the food matrix, such as proteins and lipids, can inhibit the reaction that allows the amplification of target sequences and can negatively affect the assay. This demonstrates the importance of DNA extraction, and is why numerous commercial kits are now available (Mafra et al., 2008). Several different protocols can be used, including commercial kits and non-commercial protocols, for DNA extraction. Many of these were recently investigated for purity, yield, reproducibility, and labor intensity (Volk et al., 2014). The researchers concluded that these kits often provided the best results.

Denaturing the double stranded DNA to a single strand, the annealing primers (which flank the target sequence) to the single stranded DNA, are required to amplify of the target sequence. Extension of the target sequences occurs with the enzyme DNA polymerase. The product is

detected and discriminated by post processing procedures such as species specific enzymes or visualization by ethidium bromide after agarose gel electrophoresis (De la Fuente & Juarez, 2005). Other discrimination procedures that do not involve post processing have been used such as restriction fragment length polymorphism (RFLP), and specific fluorescent probes which have allowed the simultaneous detection of multiple organisms and leading to the development of multiplex and qPCR assays (Bottero & Dalmaso, 2011).

One investigation looked into the qualitative detection of bovine milk in water buffalo milk and cheese using PCR and species specific primers (Lopez-Calleja et al., 2005). Investigators targeted the 12S rRNA gene from mtDNA from both species, which yielded a 346 base pair (bp) fragment from bovine mtDNA and 220 bp fragment from water buffalo mtDNA. All primers were tested against the other respective species, as well as a goat milk sample, to confirm specificity to the target species. DNA was extracted from milk by using milk clearing solution then a protocol using guanidine hydrochloride. DNA was extracted from cheese by performing a guanidine hydrochloride protocol as well. Both DNA extractions then went through a DNA cleanup kit to further purify the DNA from lipids and proteins. PCR products were confirmed using agarose gel electrophoresis. The researchers found that the primer pairs were specific to their respective species. The investigators made standard dilutions of raw and pasteurized milk and mozzarella cheese to find the limit of detection of the assay as well to assess processing effects on the assay. Researchers showed that the limit of detection could be confirmed down to 0.1% bovine milk adulteration, and processing into cheese had no effect on the sensitivity or accuracy of the assay. The investigators noted that when visualizing the PCR products on agarose gel the amount of contamination was proportional to the luminescence detected. Furthermore, the researchers tested the raw, pasteurized, and sterilized bovine milk samples for

somatic cell count and found that the pasteurized and sterilized samples were similar in having half the somatic cells at $85,000 \text{ cells mL}^{-1}$ when compared to raw milk. Thus, reporting that even extreme thermally processed milk contains somatic cells and, therefore, DNA from the species of origin.

Mafra et al. (2007) applied this technique to quantify the adulteration of cow milk in goat milk and exploited the observation that the intensity of the band in agarose gel is proportional to the contamination in the sample. The researchers developed a duplex assay, which targeted the 12S rRNA gene in mtDNA in both species. Reference cheese samples were made from pure cow and pure goat milk as well as from binary mixtures ranging from 0.1 to 60%, fresh cheeses and cheeses after two weeks of ripening were used as samples for analysis and 0.1% adulteration could be detected in both cheese samples. Once the reaction conditions were optimized the investigators created a calibration curve by resolving the PCR products in agarose gel electrophoresis and measuring the intensity of luminescence using software. In order to account for variation in sample processing the luminescence was normalized by calculating the ratio of the band intensity of the cow with the intensity of the cow and goat bands. The assay and calibration curve was applied to two lab produced blind samples and several from commercial manufacturers. The assay was found to be accurate for several of the samples, but not all of the samples using this technique.

In another qualitative study researchers designed a multiplex PCR assay to simultaneously detect bovine, ewe, and caprine milk in dairy products using PCR-RFLP (Bottero et al., 2003). Bottero et al. (2003) used the 12S rRNA gene from mtDNA for identification of each species. Species-specific primers were chosen and first challenged against blood samples from respective species in a simplex PCR assay to confirm specificity. The investigators were able to confirm the

specificity of the primers yielding 256 bp, 326 bp, and 172 bp fragments for bovine, caprine, and ewe samples respectively and a multiplex assay was designed. To test the sensitivity of the assay, serial dilutions of DNA and mixtures of bovine in caprine DNA were tested. It was found that the limit of detection for the assay was 0.125 ng of template DNA, and bovine template could be detected in a bovine/caprine sample mixture down to a level of 0.5%. All results were confirmed on agarose gel electrophoresis with ethidium bromide. The multiplex assay was applied to cheeses that were lab made and retail dairy products were purchased. Those samples that did not agree with the label statements were subjected to PCR-RFLP confirmatory test. RFLP is a DNA fingerprinting technique that allows the discrimination of species that are closely related by utilizing species-specific enzymes that digest the amplicon at enzyme restriction sites yielding known fragments of length for each species which can be visualized using ethidium bromide in agarose gel electrophoresis (De la Fuente & Juarez, 2005). Bottero et al. (2003) found that four of the retail samples needed confirmatory testing after testing positive for adulteration in the multiplex assay and confirmed the presence of unlabeled species in subsequent PCR-RFLP assay. In a follow up study by Abdel-Rahman & Ahmed (2007) they were able to confirm the use of this technique utilizing the cytochrome-b gene for bovine, ewe, and caprine mtDNA. Thus, it was shown the RFLP technique was useful in the application on closely related species in PCR assays with different molecular markers.

Dalmasso et al. (2011) utilized fluorescent-based PCR in the development of an assay exploiting an allelic discrimination technique to detect bovine and water buffalo milk simultaneously in mozzarella cheese. Allelic discrimination technique uses TaqMan minor groove binding (MGB) in which a fluorescent probe, which can be designed with 100% specificity, binds to a amplicon target by exploiting a variation in the sequence of closely related

species (Bottero & Dalmaso, 2011). This eliminates the need for post processing steps and only one pair of primers was needed. These primers amplify products from both species and discriminate by their respective probes. Analysis of the data can be done on software by plotting the fluorescence of each probe on their corresponding axis. The researchers were able to show that the assay could successfully discriminate between pure water buffalo and bovine samples, whereas cheese made from mixtures of the two species plotted between those two areas. The sensitivity of the test was accurate in up to 2% bovine milk addition to water buffalo milk in mozzarella cheese. The assay was performed on retail purchased samples and those samples which showed positive results that identified adulterated products, and were confirmed with a duplex PCR.

Initially PCR amplification assays were applied in a qualitative manner and it was difficult to obtain quantitative information until the development of fluorescence based real-time PCR. It has its foundation in dyes, which bind to double-stranded DNA molecules and can be detected (qPCR) (Mafra et al., 2008). Loparelli et al. (2007) sought to quantifiably detect cow milk adulteration in water buffalo cheese using SYBR green dye and MGB reporter probes in qPCR assays. The researchers designed two pairs of bovine primers, one from the mRNA growth hormone gene as a reference gene and the other a species-specific gene from cytochrome B gene in mtDNA. Blood, milk, and cheese were collected as standard samples from the representative species. Cheeses were lyophilized and binary mixtures were made on a weight-by-weight basis from 0.1 to 20%. Primers were checked for specificity and efficiency in the reaction. Standard dilutions of each sample were made and analyzed on a real-time fluorescence detecting PCR platform. The fluorescence signal in each sample was plotted against the cycle number crossing threshold. It was reported that the SYBR green dye had an efficiency of 114% for cheese

samples, as opposed to 92% for the MGB reporter. The application of the validated assay was applied for absolute quantification on lab produced and commercial cheese. Researchers showed that the lowest detectable limit of adulteration was 0.1% but could only quantify adulteration as low as 0.6%.

Pegels et al. (2011) developed a real-time PCR assay utilizing TaqMan fluorescent probes to detect bovine, ovine, and caprine genetic material in highly processed rendered feedstuffs. Because of the high temperature and pressure processing parameters of the samples, the researchers targeted short sequences in the D-loop region of mtDNA for the specific detection of each species when the designing the primers. They challenged each primer pair against 31 animal tissues to check the specificity of each primer to its species. They tested the sensitivity of the assay using blind samples of known adulterated feeds manufactured commercially and reported 100% positive detection threshold of 0.1%. The investigators then constructed a standard curve using reference samples with known amount of the adulterant species and plotted it against the cycle number the fluorescence was detected above background level. Researchers made the observation that the correlation between the variables of the crossing point and concentration were logarithmic. However, when they applied the calibration curves to blind samples from industry it was found that conflicts between the detected amount and declared amount of target species content existed. The investigators cited issues regarding possible heterogeneous processing conditions and variable number of cells and target template in different tissues used in feedstuffs.

In recent years the analysis of melt curves of amplicon products from the amplification of target templates has been gaining interest as an approach in the analysis of foodstuffs (Druml & Cichna-Markl, 2014). Melting curve analysis has been used in qPCR assays to check if the

correct products were formed in the reaction. High resolution melting (HRM) analysis is based in the theory that although double stranded DNA is very stable at room temperature it will begin to disassociate when the temperature is slowly increased (Vossen et al., 2009). The melting temperature is defined as the point where 50% of the amplicon has melted, and depends on the length and guanine-cytosine (GC) content of the product (Druml & Cichna-Markl, 2014). What distinguishes HRM analysis from melt curve analysis is the use of saturating double stranded DNA binding dye, a melting protocol that raises the temperature in smaller more precise increments, and software that analyzes the data obtained at a higher resolution by normalizing the fluorescence levels detected and creating difference plots (Wittwer, 2009). Using HRM has allowed researchers to detect even single nucleotide polymorphisms (Zhou, Wang, Palais, Pryor, & Wittwer, 2005). A benefit of using HRM in the analysis of qPCR products for food authentication is that post processing steps are no longer needed and thus the risk of contamination and other confounding factors is reduced.

Druml (2014) recently reviewed several important aspects of the design of a HRM method for food analysis. As with all qPCR assays the specific and efficient amplification of a target template is essential for obtaining consistent and reproducible melting profiles, and thus primer design and optimization must be done carefully. Non-specific products, such as primer dimers, must be avoided. Short amplicon products should be targeted at less than 300 bp because the length of the amplicon affects the melting profile. Amplicons longer than this can contain multiple melting domains, which can then result in complex melting profiles.

HRM has been used in food analysis to detect closely related species for food authentication, genetically modified organisms (GMO), allergens, and to serotype and genotype pathogens (Druml & Cichna-Markl, 2014). Recently Mader et al. (2011) developed a HRM method to

identify common plant species used in the substitution of herbal medicines. The researchers were interested in using their method to be able to quantify the level of adulteration and theorized that if their target amplicons had different melting temperatures then the normalized fluorescence level after the disassociation of the adulterant amplicon could be used as a measure of the level of contamination. They made admixtures of DNA elutions in known amounts down to 0.05 % of adulterant. Samples were analyzed on qPCR platform with melting protocol and the analysis of the data clearly showed admixtures had two inflections points at the melting temperature of the adulterant and target. A predefined temperature was chosen after the disassociation of the adulterant and the level of fluorescence of the admixtures was plotted against the logarithm of the known contamination to create a standard curve. The correlation between the contamination and fluorescence level was highly significant at 0.9806. The sensitivity reported for the method was 0.1%

Other researchers then followed up on the quantitative method of HRM analysis Mader had developed to detect bovine milk in water buffalo dairy products (Sakaridis, Ganopoulos, Argiriou, & Tsafaris, 2013). The investigators chose primers that amplified a target amplicon in the 12s rRNA region on the mtDNA for both species. Milk admixtures were made from bovine and water buffalo species at known concentrations. DNA was isolated from milk samples and from commercially made water buffalo products purchased at local markets. A duplex assay was developed and samples were tested for the presence of bovine DNA. Two commercial made products tested positive for bovine DNA and indicated adulteration. The resulting melt curve profiles for the milk admixtures clearly showed two inflection points indicating products from both species were amplified. A calibration curve was constructed from mixing pure DNA extractions from pure milk of both species. The fluorescence level from the normalized data at a

predefined temperature after the melting of the bovine amplicon was used to plot against the known level of contamination. A high correlation was found between the known contamination and the fluorescence. However the investigators noted that the data points below 1% contamination strayed away from the line of best fit and concluded that the method could detect and quantify bovine adulteration in water buffalo dairy products.

It is currently unknown if this method of analysis can quantify adulteration below the 1% that has been previously reported for detecting undeclared species in milk. Although pure DNA sample admixtures show a very strong correlation in the construction of calibration curves, to date it could not be found in the literature if the correlation between fluorescence level and known concentration of adulteration in premixed milk samples exists. This would more closely resemble the reality of adulteration in a processing situation.

The goal of the current research is to develop a method to quickly and efficiently detect bovine adulteration in water buffalo milk below 1% using HRM analysis by developing a bovine primer that produces a product with a distinctly lower melt temperature than the water buffalo product. Methods for the determination of adulteration in milk exist as chromatographic, immunological, and molecular. However, no methods in the literature describe quantitatively detecting bovine milk in water buffalo milk using high resolution melting analysis.

Chapter III

Materials & Methods

3.1 Sample Collection

Raw cow milk samples were collected from a local dairy farm (Brighton, CO) from a herd of Holstein, Jersey, and Brown Swiss cows over three different milking days. Samples were collected off the bulk tank into 0.5 gallon jars and immediately placed on ice in a cooler and transported to the lab. Raw water buffalo milk samples were collected from another dairy (Cañon City, CO) from the bulk tank and aseptically collected in 50 pound bags over three different milking days. Milk samples were aliquoted into 500 g non-sterile samples bags and frozen at -20°C until further processing for DNA extraction.

3.2 DNA Extraction

Milk samples were thawed to room temperature in cold water and further divided into 2 mL. One aliquot was centrifuged at 14,000 x g at 4°C for 30 minutes resulting in a cell pellet at the bottom, supernatant in the middle and a fat pad at the top of the tube. The fat pad was carefully removed and the supernatant discarded. A sterile cotton swab was used to remove any remaining fat on the tube. The cell pellet was washed and suspended 200 µL phosphate buffered saline (PBS) and centrifuged at 14,000 x g at 4°C for 15 minutes. The wash step was repeated twice more. The result was a pellet with clear supernatant, and the supernatant was discarded. The DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) then was used to isolate, purify and elute DNA from the remaining cell pellet following the manufacturer's protocol for tissue. The resulting DNA elution was measured for concentration and purity using the Q3000 UV

spectrophotometer (Quawell, San Jose, California, USA). DNA elutions were normalized to 2 $\eta\text{g}/\mu\text{L}$ in molecular grade water and frozen at -20°C until further analysis.

3.3 Primer Design

A set of forward and reverse primer for bovine (C12S1) and water buffalo (WB12S1) was designed using the National Center for Biotechnology Information (NCBI) database primer design tool PrimerBLAST (Ye et al., 2012). Bovine mitochondrial 12S rRNA gene fragment from accession number AJ849533 has been a reported target for amplification as well as water buffalo 12S rRNA from accession number AJ846850 (Lopez-Calleja et al., 2005). The specificity of the primer set were first checked using the Genbank database FASTA program (Ye et al., 2012) and later by challenging it with milk samples from the different species in a real-time PCR assay.

A second set of primers for bovine and water buffalo species was chosen from the literature (Lopez-Calleja et al., 2005; Pegels et al., 2011). Lopez-Calleja et al. had previously reported the optimization and specific detection of water buffalo mitochondrial 12S rRNA fragment in an assay used to identify adulteration of water buffalo milk with cow milk using a primer set (WB12S2) with a forward primer 5'-CTAG-AGGAGCCTGTTCTATAATCGATAA-3'' and reverse primer 5'-TTCATAATAACTTTCGTGTTGGGTGT-3'. Pegels et al. (2011) have demonstrated the optimization and specific detection the bovine mitochondrial D-Loop fragment using a primer set (DLOOP) with the forward primer 5'-AACCAAATATTACAAACACCACTAGCT-3' and the reverse primer 5'-CCTTGCGTAGGTAATTCATTCTG-3'. The specificity of the primers would later be verified by challenging them with milk samples of the different species in the real-time PCR assay.

3.4 Real Time PCR Assay Primer Optimization

All reactions were performed on Bio-Rad CFX96 thermal cycler (Hercules, CA, USA). An EvaGreen Precision Melt Supermix (Bio-Rad, Hercules, CA, USA) real-time PCR assay was screened for optimal concentration and annealing temperature for the water buffalo (WB12S1) and bovine primers (C12S1) in separate reactions. DNA template loading quantity was 10 ng and all reaction volumes were 20 µL. Primer concentrations assessed were 200 µM, 300 µM, and 400 µM and cycling conditions were 95°C for 2 minutes followed by 95°C for 10 seconds and 57.6, 59.2, 61.1, 62.7, 63.6, and 64°C for 30 seconds each with fluorescent detection for 40 cycles. To determine product purity, melt temperatures, and for further analysis of the amplification products a high resolution melt protocol was performed as follows. A heating step of 95°C for 30 seconds to denature products, cooling to 60°C for 1 minute to form heteroduplexes, finally a gradual heating step from 65 to 95°C in 0.2°C increments in 10 seconds intervals with fluorescence detection (Bio-Rad, Hercules, CA, USA). All reactions were performed in triplicate. Amplified products were resolved on 2% agarose electrophoresis gel carried out in Tris acetate EDTA buffer for one hour at 75 volts, stained with ethidium bromide at 1µg/mL and destained in deionized water for 20 minutes. The agarose gel was visualized using bioluminescence and a digital image was obtained using a digital camera (UVP, Upland, CA, USA).

The bovine (DLOOP) and water buffalo (WB12S2) primer set chosen from the literature were optimized separately, using EvaGreen Precision Melt Supermix. Primer concentration in the reaction was 300 µM with a template loading quantity of 10 ng/reaction and reaction volume of 20 µL. Thermal cycling conditions were 95°C for 2 minutes followed by 95°C for 10 seconds and 61.1°C for 30 seconds with fluorescent detection for 40 cycles. To determine product purity,

melt temperatures, and for further analysis of the amplification products a high resolution melt protocol was performed as detailed above. A standard curve of loading template quantity was made from 10^1 ng- 10^{-3} ng to confirm the efficiency of the reaction. All reactions were analyzed in triplicate. Amplified products were resolved on 2 % agarose electrophoresis gel carried out in Tris acetate EDTA buffer for one hour at 75 volts, stained with ethidium bromide at 1 μ g/mL and destained in deionized water for 20 minutes. The agarose gel was visualized and a digital image taken using bioimaging on EpiChem³ DarkRoom (UVP, Upland, CA, USA)

3.5 Duplex Assay Optimization and Validation

Once products were confirmed to be of expected size and specific to the sample template a duplex reaction was optimized for the WB12S2 and DLOOP primers for both species. A standard curve of the template quantity was prepared using equal concentrations of both templates from 10^1 - 10^{-3} ng/reaction to assess the efficiency of the reaction. Thermal cycling conditions for the optimized reaction was 95°C for 2 minutes followed by 95°C for 10 seconds and 61.1°C for 30 seconds with fluorescent detection for 40 cycles. To determine product purity, melt temperatures, and for further analysis of the amplification products a high resolution melt protocol was performed as follows. First, a heating step of 95°C for 30 seconds was used to denature products, then cooling to 60°C for 1 minute to form heteroduplexes, and finally a gradual heating step from 65 to 95°C at 0.2°C in 10 seconds intervals with fluorescence detection. All reactions were performed in triplicate.

3.6 Construction of Calibration Curve for Adulteration Quantification using High Resolution Melt Analysis

A calibration curve for the prediction of the cow milk adulterant was constructed according to the method of Mader et al. (2011), Sakardis et al. (2013), and Ganopoulos et al. (2013) with some modifications. Known dilutions of cow milk in water buffalo milk were mixed at 50, 40, 30, 20, 10, 1, and 0.1% v/v to simulate adulteration of water buffalo milk with cow milk in a dairy setting. Adulterated samples then were processed using the previously mentioned DNA extraction method. Samples were loaded in triplicate and thermal cycling conditions were as follows for the duplex real-time PCR assay: 95°C for 2 minutes followed by 95°C for 10 seconds and 61.1°C for 30 seconds with fluorescent detection for 40 cycles. To determine product purity, product melt temperatures, and for further analysis of the amplification products a high resolution melt protocol was performed as follows. First, a heating step of 95°C for 30 seconds was used to denature products, then cooling to 60°C for 1 minute to form heteroduplexes, and finally a gradual heating step from 70 to 85°C at 0.2°C in 10 seconds intervals with fluorescence detection. The gradual heating step was optimized to the melt temperatures of the respective products of the duplex reaction and allowed for shorter analysis time. Each of the milk samples from the three different milking days from the respective species was analyzed in the assay in triplicate on three different days.

Data from the real-time PCR assay were analyzed using Precision Melt Analysis Software (Bio-Rad, Hercules, CA, USA). Fluorescence levels of the melt curves were normalized so that melt curve relative fluorescence started melt curve analysis at 1 and finished at 0.0 after complete disassociation. Creation of the calibration curve was done by simple linear regression.

The normalized fluorescence value of each adulterated sample at 78.8°C was plotted on the y-axis over the known level of adulteration for that sample on the x-axis.

3.7 Statistical Analysis

Statistical analysis was carried out using SAS software release 9.4 with SAS/STAT release 13.1 (SAS Institute Inc., Cary, NC, USA). Variances for samples and days were computed using STAT/VARCOMP. Fixed effects and random effects of data generated between samples and between dates within samples were analyzed using STAT/MIXED. This procedure was used to determine overall estimates, standard errors, and confidence intervals using the least square means estimate for all data. STAT/MEANS was used to determine overall means estimates, standard deviations, standard error estimates and confidence intervals for data within samples and for overall data.

Chapter IV

Results

4.1 Primer Optimization

Water buffalo and bovine milk were collected on three separate milking days, and DNA was extracted and standardized to 10 ng/μl. Two set of primers were designed to specifically and efficiently amplify mtDNA sequences for both species. The first set was primer pairs never reported before, and the second set was two pairs that were chosen from the literature and reported as specific for both bovine and water buffalo species (Lopez-Calleja et al., 2005; Pegels et al., 2011). Samples were first assayed in singleplex reactions with each pair of primers for its respective species to confirm efficiency and specificity. Next, DNA samples from each species were assayed in duplex reactions with each set of primer pairs in order to validate the efficiency and specificity. Once the duplex reaction was optimized it was validated by assaying each different milking day sample on six independent days and checking for statistical differences in the results. Once validated, water buffalo milk samples were diluted with cow milk at known rates to simulate adulteration and DNA extractions were performed. DNA from water buffalo reference samples was diluted with DNA from reference bovine samples at known concentrations and used to construct calibration curves for the quantification of adulteration utilizing HRM analysis.

The primer set WB12S1 and C12S1 was screened for reaction specificity and melting temperature profiles using a temperature gradient. The primer set BDLOOP and WB12S2, designed from the literature, was screened for specificity and melting temperature profile at the annealing temperature described previously by Pegels et al. (2011). All primers resulted in

specific reactions and amplification of products based on melting analysis at all temperatures in the gradient. The melt temperatures for WB12S1 and C12S1 amplicons were 78.8°C and 76.2°C, respectively. The BDLOOP and WB12S2 amplicons resulted in melting temperatures of 75.8°C and 80.0°C, respectively. The BDLOOP and WB12S2 set was chosen for further analysis due to a wider gap between melting temperatures of amplicons from each primer pair. This point is illustrated in the melting curve profiles plotted on the same graph for WB12S1 and C12S1 amplicons in Figure 8 in the appendix.

4.2 Primer Validation

Once specificity and melting profile was confirmed standard curves were constructed for each primer set in singleplex reactions by serial dilutions (Figure 1). The reaction was able to amplify enough product to quickly detect fluorescence above background as illustrated in Figure 1.1. The melt curve of the reaction indicated no primer dimer or non-specific product formation as seen by the single inflection in Figure 1.2. DLOOP primer standard curve showed an acceptable efficiency of 107.5% with a slope of -3.152, and $r^2 = 0.994$ as illustrated in Figure 1.3.

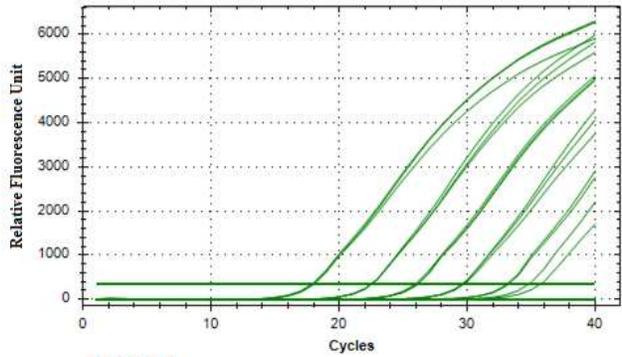


Figure 1.1

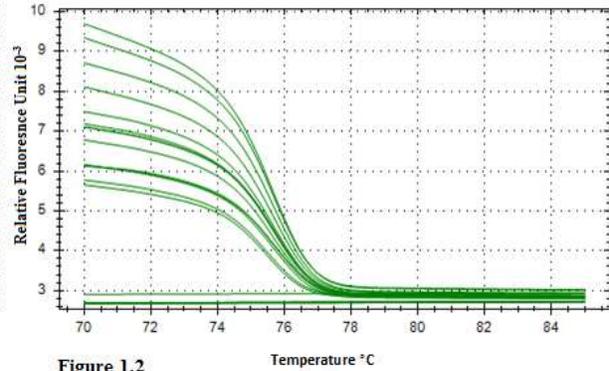


Figure 1.2

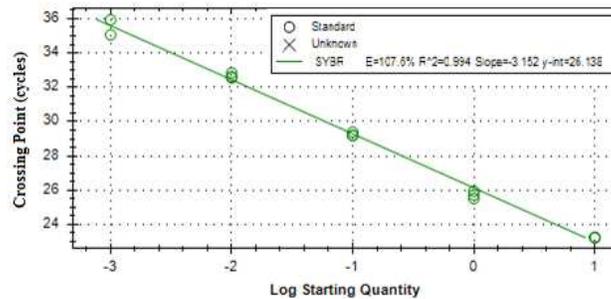


Figure 1.3

Figure 1. PCR singleplex reaction for a dilution series of DNA isolated from bovine milk samples using the DLOOP primer pair. Figure 1.1: amplification curve; Figure 1.2: melt curve profile; Figure 1.3: standard curve

Figure 2 demonstrates the usefulness of the water buffalo primer pair that was chosen for further analysis. The primer pair for WB12S2 in the singleplex reaction was able to quickly amplify products so that fluorescence could be detected above background as seen in Figure 2.1. The reaction with the primer pair made the intended target product and no primer dimers or non-specific products as indicated by the melt curve profile with a single inflection point as seen in Figure 2.2. The standard curve from serial dilutions from the reaction with the primer pair of WB12S2 resulted in 92.2% efficiency with a slope of -3.524, and $r^2 = 0.990$ as shown in Figure 2.3.

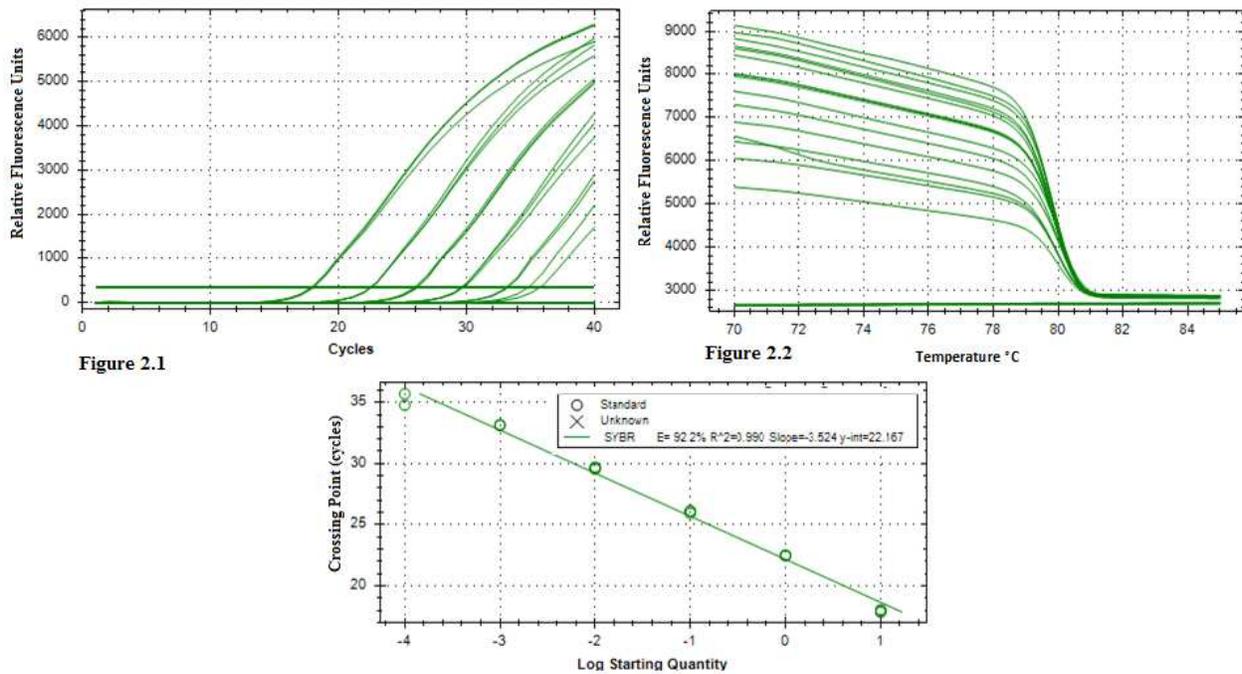


Figure 2.3

Figure 2. PCR singleplex reaction for a dilution series of DNA isolated from water buffalo milk samples using the WB12S2 primer pair. Figure 2.1: amplification curve; Figure 2.2: melt curve profile; Figure 2.3: standard curve

A duplex reaction of serial dilutions of DNA from both bovine and water buffalo samples with primer pairs for both species was performed in parallel to the singleplex reactions. Figure 3.1 demonstrates that the crossing point value of the duplex reaction was between the crossing point values for each of the singleplex reactions (Figure 3). This indicates that the ability of the primers to bind to template in the reaction was not impaired by competition. The melt curve profile of the duplex reaction shows two inflection points at their predicted temperatures in Figure 3.2. Figure 3.3 illustrates the acceptable efficiency of the duplex reaction at 95.0% with a slope of -3.448, and $r^2 = 0.998$.

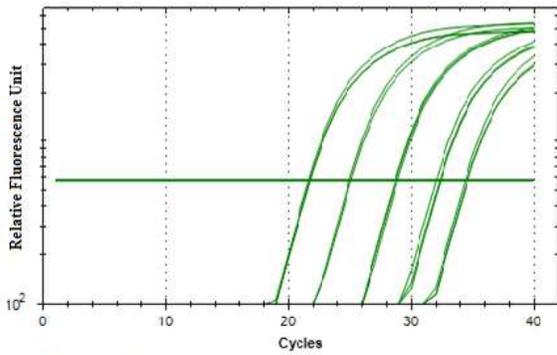


Figure 3.1

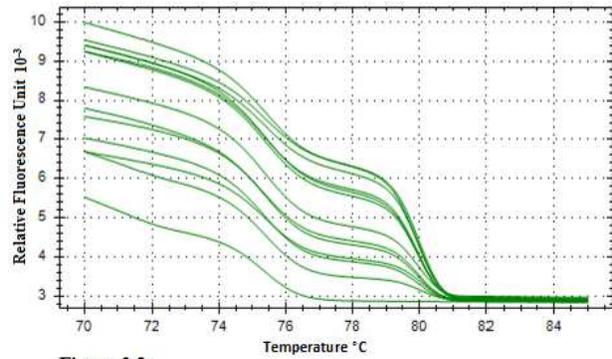


Figure 3.2

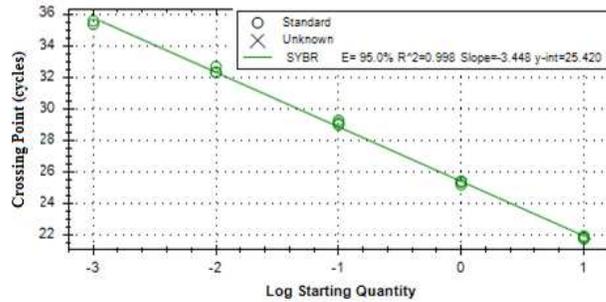


Figure 3.3

Figure 3. PCR duplex reaction for a dilution series of DNA isolated from bovine and water buffalo milk 1:1 (w/w) samples using the DLOOP primer pair. Figure 3.1: amplification curve; Figure 3.2: melt curve profile; Figure 3.3: standard curve

The results from 2% agarose gel electrophoresis are seen in Figure 4. Lane 1 is the reference gene ladder used to corroborate the size of the amplicons from the reactions. Lane two shows the product of the bovine sample using DLOOP primer was the expected size of 77 bp. Lane 3 confirms the specificity of the DLOOP primer to amplify the target sequence and have no cross reactivity with water buffalo DNA samples. Lane 4 confirms that the DLLOP primer created no primer dimers or unspecific products from a no template control reaction. Lane 5 confirms size of the product from the singleplex reaction with WB12S2 was 220 bp as expected. Lane 6 corroborates the specificity of the primer when challenged with bovine DNA sample. Lane 7 shows the predicted products were amplified in the duplex reaction. Lane 8 is a no template

control reaction sample and no resulting band indicates that the specificity of the primers that only amplify the intended products and do not cross react with each other in the duplex reaction.

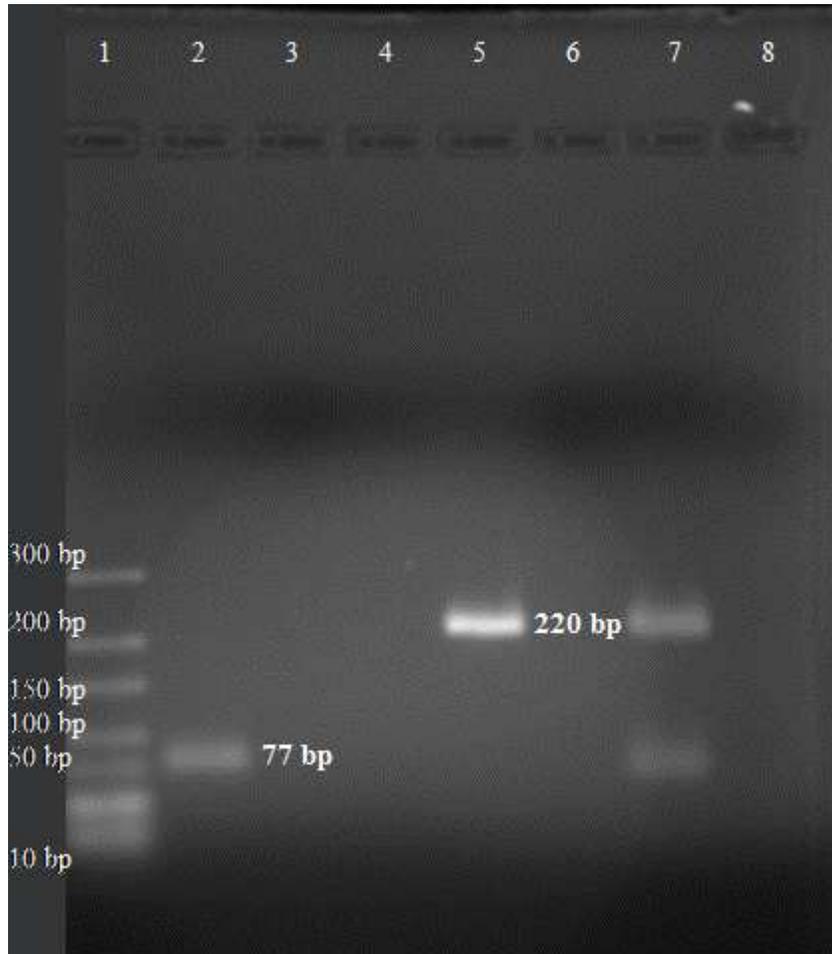


Figure 4. Two per cent agarose gel electrophoresis of PCR products amplified with 40 cycles from DNA of bovine and water buffalo milk samples. Lane 1: O'GeneRuler ultra low range (Thermo Scientific, Waltham MA, USA) ; Lane 2: bovine milk sample singleplex reaction with the DLOOP primer; Lane 3: bovine milk sample singleplex reaction with the WB12S2 primer set; Lane 4 no template control for the DLOOP primer set, Lane 5: water buffalo milk sample singleplex reaction with the WB12S2 primer set; Lane 6: water buffalo milk sample singleplex reaction with the DLOOP primer set; Lane 7: bovine and water buffalo milk samples duplex reaction with DLOOP and WB12S2 primer set, Lane 9: no template control for the duplex reaction with DLOOP and WB12S2 primer set

Table 1 summarizes analysis of variance between samples and between days within samples that the assay was performed. The variance among samples was found to be negative for both efficiency and slope of the standard curves for the duplex reaction, suggesting that different samples had no effect on efficiency or slope of the reaction. The variance of efficiency and slope of the standard curves constructed from the assay on days within samples showed a variance of 20.3 and 0.010, respectively.

Table 1 Variance of the slope and efficiency of PCR standard curves between samples, and on different days within samples

Variance component	Efficiency	Slope
Sample n=3	-5.56	-.001
Date(sample) n=6	20.3	0.010

Table 2 illustrates the covariance of the date within samples on the slope and efficiency of each sample analyzed on the assay. Efficiency is calculated using the formula $E=10^{-1/\text{slope}}$. It is based on the concept that ideally for every thermal cycle in the assay the product doubles and therefore so does the fluorescent signal. Therefore when making serial dilutions of known concentrations of template DNA, when the values for the number of cycles it takes for fluorescence to reach above background level is plotted against the dilution a linear association should be apparent. This can then be used as a tool to assess the efficacy of the primers and reaction environment in the assay. The theoretical optimum slope for a dilution series would be -3.320. In order to assess usefulness of the assay, day of analysis was taken into consideration and it was apparent that the covariate for day of analysis on different samples was different for each

sample. This indicates that the day of analysis had a significant effect on the efficiency and slope of the standard curve analyzed in the assay for the same sample.

Table 2. Covariance estimates of the date within samples on slope and efficiency of the standard curves constructed from dilutions of DNA mixtures (1:1 w/w) of bovine and water buffalo milk samples in the duplex PCR assay

Covariance parameter	Sample	estimate on slope	estimate on efficiency
Date(sample) n=6	B0227C0227	0.009	28.3
Date(sample) n=6	B0225C0310	0.000	10.5
Date(sample) n=6	B0302C0226	0.004	19.2

Table 3 illustrates the validation of the assay by summarizing the means of the efficiency of the duplex reaction and slope of the standard curve for each of the samples (n=3) and all of the samples on each of the six days the assay was performed (n= 18). No significant differences were found among means of the slope or efficiency of the reactions on the different samples. This indicates that the assay is valid for the daily use of detection of adulteration of water buffalo milk with bovine milk and the results of the assay are reproducible.

Table 3. Confidence intervals for duplex PCR assay using DNA binary mixture (1:1 w/w) dilutions from bovine and water buffalo milk samples for efficiency and slope of standard curves

Sample	Variable	Mean	Standard Deviation	Standard Error	Lower 95% CL for Mean	Upper 95% CL for Mean
B0227C0227	Efficiency	101.73	5.4107	2.2089	96.060	107.41
	Slope	-3.2863	0.12513	0.05108	-3.4176	-3.1550
B0302C0226	Efficiency	100.14	4.4874	1.8319	95.436	104.85
	Slope	-3.3220	0.10129	0.04133	-3.4283	-3.2158
B0310C0225	Efficiency	100.19	3.3910	1.3843	96.632	103.74
	Slope	-3.3194	0.08203	0.03348	-3.4055	-3.2334
Overall (n=18)	Efficiency	100.69	4.3007	1.0136	98.553	102.83
	Slope	-3.3093	0.09939	0.02342	-3.3587	-3.2598

P>.05

4.3 High Resolution Melting Analysis

Results from the HRM analysis of melt curve data of the duplex reactions from samples of binary mixtures of pure DNA elutions is shown in Figure 5. The level of resolution clearly shows a distinct melt pattern at 0.1% adulteration of water buffalo DNA with bovine DNA (Figure 5.1). The sensitivity of the method is further demonstrated in the difference graph in Figure 5.2.

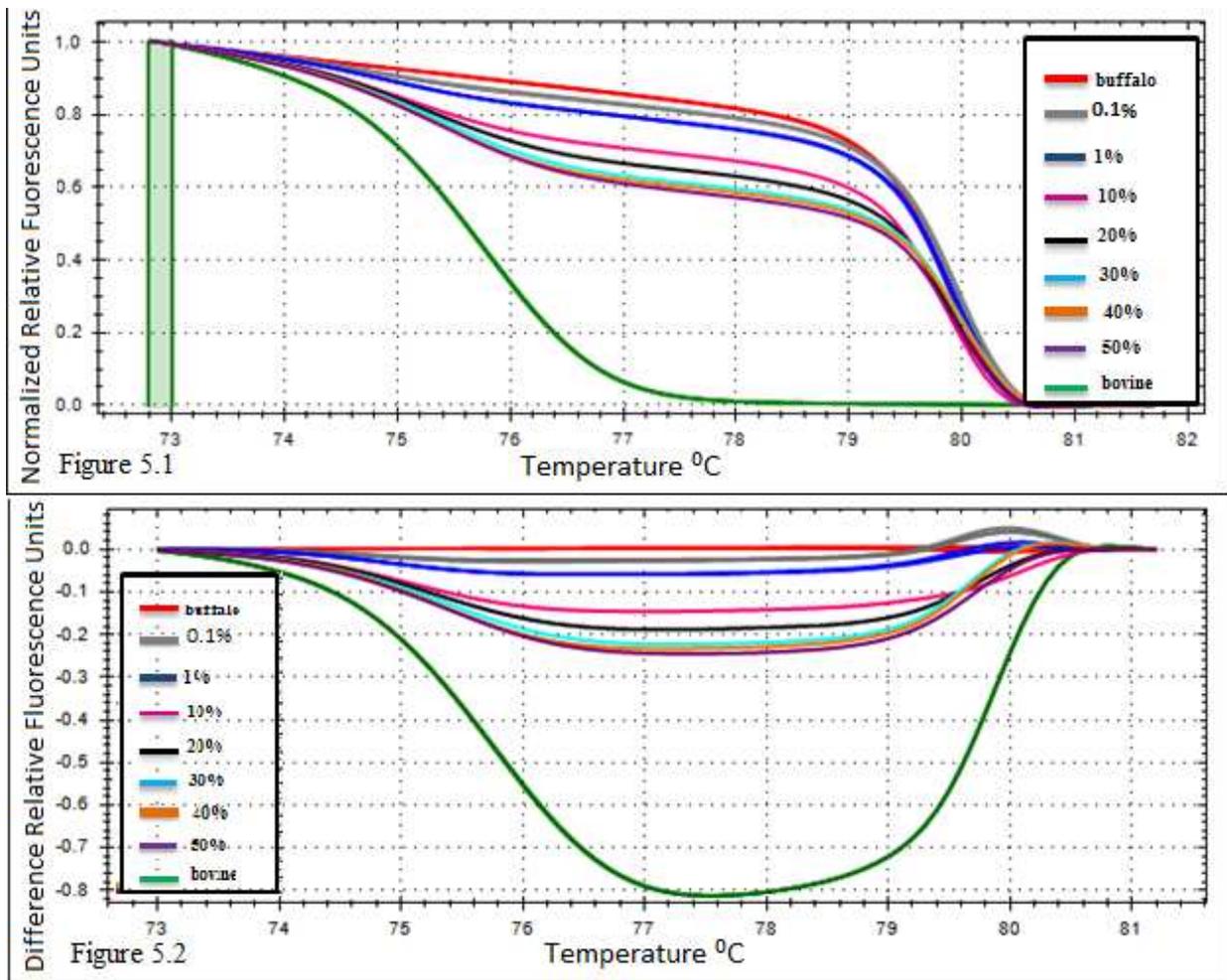


Figure 5. High resolution melt curves from binary mixtures of pure DNA from bovine and water buffalo milk samples; Figure 5.1: high resolution melt curve of buffalo reference, binary mixtures of bovine DNA in water buffalo DNA from 0.1% to 50%, and bovine reference; Figure 5.2: Difference curve for sample melting temperatures from pure water buffalo reference sample, bovine in water buffalo DNA binary mixtures, and pure bovine reference sample

Once again the level of resolution was shown to be sensitive enough to detect adulteration to 0.1% for the duplex reaction for eluted DNA samples from binary mixtures of bovine milk in water buffalo milk as shown in Figure 6. This is illustrated in the high resolution melt curve profile shown in Figure 6.1, where there is a clear distinction in the melt curve profile of the binary mixture adulterated with 0.1% bovine milk and the water buffalo reference sample. The difference graph in Figure 6.2 further reinforces the proof of concept for the method of analysis.

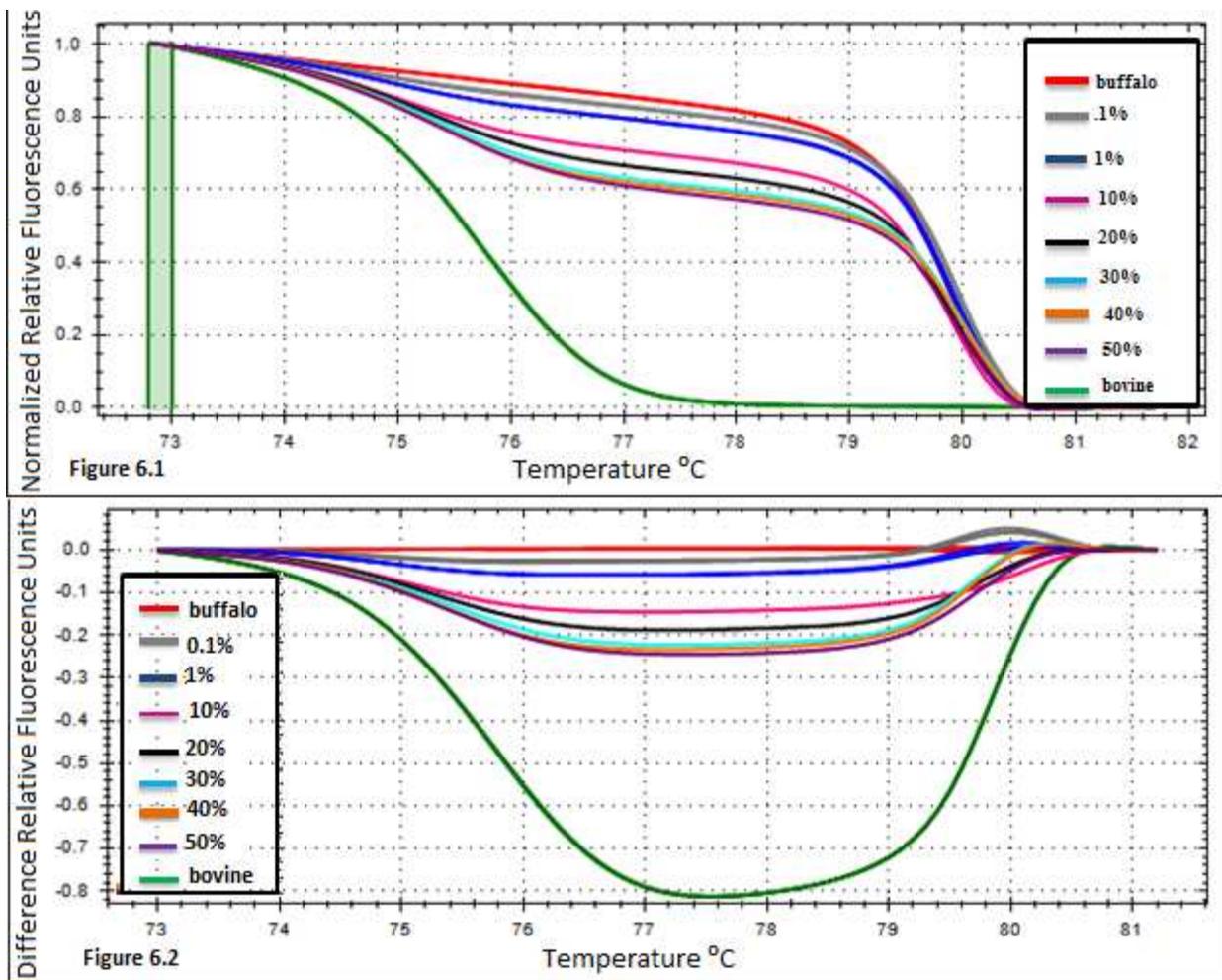


Figure 6. High resolution melt curves from binary mixtures of milk samples from bovine and water buffalo milk samples; Figure 6.1: high resolution melt curve of buffalo reference, binary mixtures of bovine milk in water buffalo milk from 0.1% to 50% v/v, and bovine reference; Figure 6.2: Difference curve for sample melting temperatures from pure water buffalo reference sample, bovine in water buffalo milk binary mixtures, and pure bovine reference sample

Calibration curves were constructed from the relative fluorescence units of the predefined temperature and plotted. Figure 7 illustrates the strong correlation between the level of fluorescence at the predefined temperature and the level of adulteration in the sample. Samples made from binary mixtures of DNA elution had an average $r^2 = 0.997$ as shown in Figure 7.1. It is clearly seen that the data points fit the line and little deviation can be seen. Figure 7.2 shows the calibration curve constructed from those samples of DNA eluted from binary mixtures of

bovine milk in water buffalo milk had an $r^2 = 0.948$. In contrast to the binary DNA mixtures it can be seen that the data points do not fit the line quite as closely, but never the less there was a strong correlation between relative fluorescence at the predefined temperature and the extent of adulteration. This indicates that either method of calibration curve could be used to accurately quantify bovine adulteration in water buffalo milk.

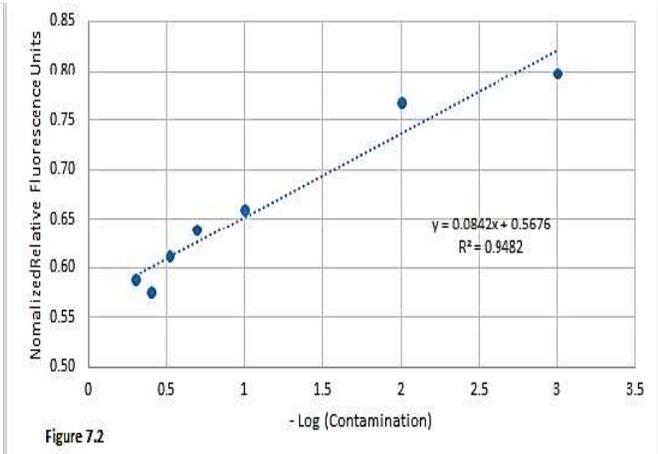
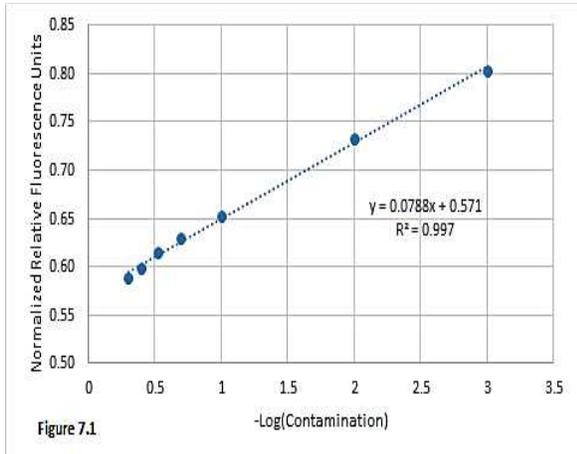


Figure 7. Linear regression calibration curves averaged from the data of three different samples analyzed on separate days; Figure 7.1: Calibration curve from the binary mixtures of bovine and water buffalo DNA samples isolate from milk from 0.1% to 50%; Figure 7.2: Calibration curve from the binary mixtures of bovine and water buffalo milk samples from 0.1% to 50%

Chapter V

Discussion

5.1 Primer Optimization

After screening the two sets of primer pairs for several characteristics such as specificity, efficiency, and melting temperature of products, the BDLOOP and WB12S2 primer pair sets were chosen for further use in the development of the assay. The BDLOOP primer pair in a singleplex reaction at a concentration of 300 μ M was found to amplify a 77 bp product. It produced no primer dimers or non-specific products, have no cross reactions with water buffalo template DNA, and was found be suitable for the development of a PCR assay to detect bovine mtDNA, which is in agreement with the results of Pegels (2011). The WB12S2 primer amplified a 220 bp product from the 12S rRNA region of the mtDNA of water buffalo and yielded no primer dimers, non-specific products, nor did it amplify product when challenged with bovine template, and was found to efficiently amplify the water buffalo template DNA at a concentration of 300 μ M. These findings are in agreement with the results of Lopez-Calleja (2005) when the researchers developed a PCR method to detect bovine adulteration in a PCR assay. Once each primer pair was demonstrated to be specific and efficient in a singleplex assay they were combined in a duplex assay to assess their efficiency and specificity to detect template from each species in a single reaction. Using a dilution series of starting template in the reaction, the efficiency of the reaction was found to be 95%. More importantly the melting curve profile showed two inflection points that corresponded specifically to the products expected indicating that neither primer inhibited the other.

5.2 Duplex PCR Assay Validation

Once the duplex assay was confirmed to be specific, sensitive and efficient it was repeated for each of the three milk samples obtained on different milking days from both species, on six different days in order to validate the reproducibility of the method. The efficiency and slope summary statistics from the standard curves constructed from standard dilutions of the samples were analyzed for coefficient of variation to assess the influence of random events such as the day of analysis and sample. In agreement with the results of Pegels et al. (2011) that was significant covariance ($p > .05$) values on the samples analyzed on different days. This suggested that standard curves should be determined each day of analysis for a sample. No significant values were found for covariance between samples analyzed in the assay which indicates the validity of the precision of the assay. Confidence intervals for assay efficiency and slope were calculated for each sample analyzed on different days as well as for all samples for every day of analysis. No significant differences were found between the samples for slope and efficiency means. The result for the average slope of all samples analyzed on all days was -3.093, which is near -3.320, the slope resulting from 100% PCR efficiency. These results indicate the ability to obtain accurate, sensitive, and precise and reproducible results in the detection of bovine adulteration in water buffalo milk.

5.3 High Resolution Melting Analysis

The results of the high resolution melting analysis melt curves of binary mixtures of DNA elutions from reference milk samples showed that all curves had two inflection points. This indicated that both PCR products were amplified in the reaction. It is clearly visible that the shape of the curve changes with the level of contamination. Calibration curves constructed by plotting the known adulteration against the relative fluorescence at a predefined temperature

resulted in a highly significant correlation, which is in agreement with other reports (Ganopoulos et al., 2013; Mader et al., 2011; Sakaridis et al., 2013). It was noted that the assay could detect bovine milk adulteration at a lower limit of 0.1%. This indicates the sensitivity of the assay to be in agreement with Mader et al. (2011) as he reported a similar result in the detection of adulteration of plant material using known DNA binary mixtures.

The results for the calibration curve constructed from the HRM analysis data for DNA elutions from binary mixtures of milk were similar to that of those for DNA elutions from DNA binary mixtures. A strong correlation was found between the relative fluorescence at a predefined temperature and the known amount of adulteration in the sample. However, the r^2 value was 0.948 compared to 0.997. The lower limit of detection using this method to construct the calibration curve was comparable to that of binary mixtures of DNA elutions at 0.1%. This once again demonstrates the sensitivity. Sakaridis et al. (2013) reported a detection limit of 1% in the detection of species specific adulteration in cheese when binary mixtures of milk were used to make cheese from which DNA was extracted for analysis. This result indicates an improvement in the resolution of such assays. It should be noted that the data points deviated from the line of best fit using this method when compared to the binary DNA mixtures line of best fit, however, this method more accurately reflect what could be happening in the dairy setting.

Many factors could account for the deviations including discrepancies in copy number of the gene in each somatic cell, slight variation in concentration of somatic cells in the milk of each species, and recovery of DNA during extraction. Another point to consider is that the assay could show positive results of adulteration when in fact the adulteration of water buffalo milk may not have been done intentionally below 1%. For example, if the water buffalo milk were processed

on the same equipment as bovine milk it would be plausible that a small amount of bovine DNA could contaminate the product. It would not be financially motivating for a dairy to adulterate water buffalo milk at such a low level and therefore would not be commensurate with current theories on the motivating forces behind adulteration in the dairy industry (Everstine et al., 2013).

5.4 Advantages, Limitations and Implications

Limitations of this investigation did not allow for the examination of different processing technologies on the accuracy and sensitivity of this particular assay. This limits the applicability of the assay to raw milk before any processing. However, it could be assumed that the method is still valid considering the work of Giannopoulos et al. (2013) and Sakaridis et al. (2013). They applied a similar technique to detect adulteration in the cheese matrix with success. These researchers were not able to reach the same resolution in respect to the limit of detection of the assays in their investigations, nor the same level of correlation. This indicated that processing may have an effect on the accuracy of the assay and this should be further explored and quantified to validate the assay application to various dairy products.

Another limitation of the method employed in the design of a PCR assay for detection of adulteration in dairy products is that only milk from those species which are targeted are able to be identified. If adulteration occurs with milk from another species than that which was being screened in the assay then it may go unnoticed. However, the flexibility of the assay is that additional primers could be designed to detect other species but would most likely need to be analyzed in separate reactions in order to avoid complex melt profiles, which could convolute results. If an act of adulteration was committed with a substance other than that of a dairy

animal, such as plant based material, then the current assay would be unable to detect it due to its specific detection.

The advantage of this assay is that it is a relatively quick, accurate, and sensitive assay for the specific detection of adulteration of water buffalo milk with cow milk. Samples can be processed within a matter of hours, which is important in a dairy processing setting where the quality of milk deteriorates quickly and there is little opportunity for long storage of milk. This assay can be performed without advanced technical knowledge. This allows the assay to be applicable to the dairy industry where often quality control and quality assurance analysts may not have the skills required for other methods of detection, such as reading complex chromatographs or mass spectra. In addition, commercial kits for the isolation and purification of DNA from food matrices are available and easily performed. The necessary equipment in order to perform the assay is relatively low in cost, requires little technical expertise for operation, and the assay can be completed in a matter of hours.

Implications for this investigation include the use of the method applied for the detection and relative quantification of adulterations in other food matrices. This method could be used to verify the authenticity of food products from a variety of matrices as well as herbal supplements and other plant and animal derived products. It could be useful in detecting allergens such as wheat, soy, peanuts, and tree nuts in food matrices. The geographical source of food stuffs or ingredients could be verified for those foods and ingredients making such a claim. This method could possibly be used in the verification of foods claiming to contain no genetically modified organisms. Applications that could be especially useful are those that the target gene and species of the adulterant would be known.

Chapter VI

Conclusions

In this investigation using real-time PCR and HRM analysis, an assay was successfully designed to detect bovine milk adulteration of water buffalo milk. The assay was specifically designed using primers that amplified specific target templates in mtDNA in the closely related species of cow and water buffalo. Primers were chosen and proven to be specific and sensitive first in singleplex, and then duplex reactions. The assay was performed with multiple samples over multiple days to assess the impact of the sample and day on the assay in order to verify that accurate results could be obtained every time the assay was used in analysis. The investigation was successful in the validating the usefulness of the assay in the detection of bovine DNA in water buffalo milk over a variety of samples from day to day. However, it was determined that the day the sample is analyzed in the assay does have an impact on the results and a standard curve should be constructed each time.

The primers chosen for the assay were designed to melt at two distinctly different temperatures, with the adulterant product completely disassociating before the water buffalo product during a high resolution melt protocol. This design not only allowed for the visual distinction in melt curve profiles but to take advantage of the theory that the relative fluorescence measured in a sample after the complete disassociation of the adulterant product is correlated with the degree of adulteration in the milk. This theory was tested in the design of this assay using two different methods of constructing calibration curves. One method was designed to dilute known concentrations of pure DNA elutions of buffalo milk and known quantities of bovine milk elutions and use that to construct a calibration curve for the prediction of

adulteration. The other method was designed to extract DNA from known dilutions of water buffalo milk with cow milk and calculate calibration curves. It was concluded that both methods showed a strong correlation between relative fluorescence at the predefined temperature and the level of adulteration in the sample, and either method could be used in the detection and quantification of bovine milk adulteration in water buffalo milk. The assay proved to be fast, simple, and accurate. Analysis could be carried out the same day as the sample was collected and results could be obtained within hours of beginning analysis.

There are several considerations for further study from this investigation. The assay should be further investigated to understand which method used to construct the calibration curve is more accurate in the prediction of the adulteration in masked samples. This would further validate the assay making it a more useful tool in the detection of bovine adulteration in water buffalo milk. In addition, statistics could be analyzed to understand the effect of somatic cell count in each milk sample. This would help to understand the limitations of the usefulness of the assay.

The study of the effect of thermal processing conditions on the accuracy and sensitivity of the assay should be performed. There is several different processing parameters milk processors will follow in order to thermally treat milk. The scope of this investigation did not include this variable in milk processing. These future studies would be necessary in order to further validate the assay in applications beyond raw milk to low and high temperature pasteurization, ultra high temperature pasteurization and dried milk. It would be of interest to look at other dairy food matrices. These should be considered in order to expand the applicability of the assay beyond fluid milk to cheese, yogurt, butter, and other processed dairy products.

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Appendix

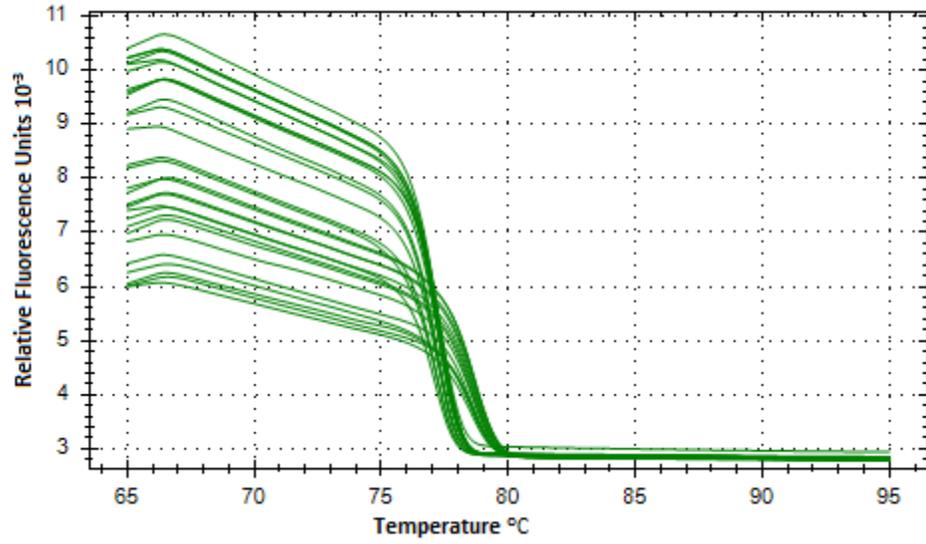


Figure 8. Melt curves from PCR assay of WB12S1 and C12S1