DETECTION OF PORCINE IN GELATIN

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Alma Ata University Press

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An inspirational book for the halal industry

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List of Abbreviations

ATR	Attenuated Total Reflectance
Bp	Base pairs
BLAST	Basic Local Alignment Search Tool
Ct	Cycle Threshold
CTAB	Cetyl Trimethylammonium Bromide
CYT b	*Cytochrome* b
dATP	Deoxyadenosine Triphosphate
dCTP	Deoxycytidine Triphosphate
dGTP	Deoxyguanosine Triphosphate
dTTP	Deoxthymidine Triphosphate
dNTPs	Deoxyribonucleasides Triphosphate
DNA	Deoxyribonucleic Acid
dsDNA Double	Stranded Deoxyribonucleic Acid
EB	Elution Buffer
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
FTIR	Fourier Transform Infrared
GC	Guanine Cytosine
gDNA	Genomic Deoxyribonucleic Acid
GMIA	Gelatin Manufactures Institute of America
HPLC	High Performance Liquid Chromatography
HRM	High Resolution Melt
ICH	International Conference on Harmonization
ISO	International Organization of Standardization
LoD	Limit of Detection
LoQ	Limit of Quantification
mRNA	Messenger Ribonucleic Acids
MS	Mass Spectrometric
mtDNA	Mitochondria Deoxyribonucleic Acid
NCBI	National Center for Biotechnology Information
NTC	No Template Control
PB	Phosphate Buffer
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
qPCR	Quantitative Polymerase Chain Reaction
RNase	Ribonuclease
RSD	Relative Standard Deviation
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SNPs	Single Nucleotide Polymorphisms
STR	Short Tandem Repeats
TAE	Tris Acetate EDTA
TE	Tris-EDTA
Tm	Melting Temperature
Ta	Annealing Temperature
WHO	Word Health Organization

Preface

We would like to extend my praise and gratitude to Allah SWT for His abundance of mercy and grace that we could complete this book. The title which has been chosen namely, *Detection of Porcine in Gelatin*.

The gelatin is a polypeptide that contains a high molecular weight acquired by hydrolysis of water-insoluble fibrous protein collagen which is the primary protein component of mammalian and fish skins, bones, and connective tissues.

This book contains 9 chapters. Chapter 1 and 2 provide various information about the introduction of gelatin in the halal industry while Chapter 3 focuses on DNA detection in gelatin capsules. Next, the analysis of gelatin capsules and validation methods are discussed in Chapters 4 and 5. Furthermore, the procedure of gelatin detection and validation of conventional PCR are described in Chapters 6 and 7. Lastly, the isolation of DNA is discussed in Chapter 8 and concluded in Chapter 9 as a conclusion of this book.

Infinite gratitude goes out to my beloved father, Dr. Badrul Munir Muhammad Nur, my beloved mother, T. Asmah Zatun Matabin, brothers and sisters, Maryam, Muhammad Abdurrahman, Hamid Alkhair, Abdul Jawwad, Tahani, Dalaal Mahmudah, and Ahmad Abdul Aziz for the prayers and the never-ending courage. Thank you also to my colleagues, especially those under the same supervision for the spirit and support given during the study. I hope that this book will be a helpful reference for the ummah.

AHLAM INAYATULLAH
HAMID ALKHAIR BADRUL
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Chapter 1

Introduction

Since the early days of gelatin manufacture, great strides have been made both in the engineering and technological spheres, which has been re-customized in the preparation of high-quality gelatins that are available at present.

Gelatin is not a naturally occurring protein, but a high molecular weight polypeptide obtained by hydrolysis of water-insoluble fibrous protein collagen which is the primary protein component of mammalian and fish skins, bones, and connective tissues (Cai et al., 2011; Nhari et al., 2012). The total worldwide consumption of gelatin was estimated to be 120,000 tons in 1976, and approximately 140,000 tons in 1982. The demand for gelatin has been increasing at a steady rate of approximately 2% per annum (Shyni et al., 2013), thus resulting in high prices for gelatin. This is due to the shortage of the primary raw materials mostly cattle hides, bones, and pigskins. This shortage has resulted from the competition by a growing legion of users including leather tanners, expanding snack industry, and the use of gelatin capsules in supplement and pharmaceutical products (GMIA, 2012).

In the market, porcine gelatin is cheaper than bovine gelatin or other gelatin produced from *halal* sources (Widyaninggar et al., 2012). Any products containing pig derivatives such as porcine gelatin is not allowed to be consumed according to hadith and the holy Quran not stated hereafter. Therefore, the tool to detect the presence of porcine gelatin is a necessity to ensure the *halalness* of the products (Rohman and Man, 2012).

***From Abu Abdillah Nu'man ibn Bashir radhiallahuanhu he said, I heard Rasulullah shallallahu`alaihi wa sallam said, "Verily, halal is clear and haram is clear. In between, some cases are doubtful (vague) that are not known by many people. So who's afraid of doubtful meaning he has saved his religion and honor. And who is mired in matters doubtful, it would fall in a case that is haram (forbidden). As herders who graze herds around (fields) that are prohibited to enter then gradually they would enter it. Know that every king has restrictions and prohibition of Allah is what he forbade. Know that in this self-contained piece of flesh, if it is good then the whole body is and if it is bad, then the Evil entire body; know that it is the heart*"(Bukhari and Muslim.**

Identifying the source of gelatin is of importance due both to concerns regarding possible disease transmission to humans, as well as religious concerns in Muslim countries (which strictly forbid porcine products). Several reports have been published concerning analytical methods capable of distinguishing porcine and bovine gelatins. Methods that rely on physicochemical properties such as infrared spectroscopy coupled with chemometrics of principal component analysis (PCA) (Hashim et al., 2010) and those with fish gelatin (Cebi et al., 2016), have been proven unsuitable for differentiating a mixture of gelatin (i.e., bovine/porcine mixtures) mainly because of the similarities in structure and physicochemical properties of gelatin derived from different sources.

Several molecular techniques can be used to identify the origin of gelatin products such as high-performance liquid chromatography coupled with a fluorescence detector and chemometrics of PCA (Nemati et al., 2004; Raraswati et al., 2013), enzyme-linked immunosorbent assay (ELISA) (Doi et al., 2009; Venien and Levieux, 2005) and DNA-based techniques. It is reported that protein-based analytical techniques for species identification in mixed samples are significantly less sensitive

than DNA-based techniques for the evaluation of thermally processed materials (i.e., gelatin) because of specific epitope alterations. The methods used for the processing and production of gelatin include acid/ base connective tissue hydrolysis, high-temperature extraction using water, and sterilization. Hence, gelatin contains very small amounts of highly degraded DNA (Boran et al., 2010). DNA is a relatively stable molecule, which can better withstand heat processing and can be detected even though it will be in a fragmented form (Hsieh et al., 2016). Detection and quantification of trace DNA can be performed using polymerase chain reaction (PCR)-based methods which have had the greatest success due to higher sensitivity, specificity, rapidity, and reproducibility.

On the other hand, extraction of high-quality DNA is an important prerequisite for PCR-based techniques, which could be a potential problem if there is extensive damage to DNA following heat processing (Mohammad et al., 2016; Avise et al., 2012). Many primers have been developed based on both mitochondrial and nuclear genes to trace species-specific DNA. Mitochondrial DNA analysis using PCR offers a series of advantages. The mtDNA genes are present in thousands of copies per cell; thus, the large variability of mtDNA allows reliable identification of precise species in mixtures. Although nuclear DNA (linear) is more powerful, mtDNA (circular) is more stable over time/and may also present intracellularly. The mtDNA of most animals codes for 37 genes; one of which is the gene for *Cytochrome* b (CYT b) (Mohammad et al., 2016).

The purpose of writing this book was to introduce a suitable and sensitive technique to simultaneously detect bovine and porcine DNA in gelatin-containing products especially in soft and

hard gelatin capsules in supplement products. Agilent detection porcine kit was selected as the method of DNA isolation from gelatin capsules (Agilent, 2015). Two sets of the primers of porcine and bovine DNAs (Tanabe et al., 2007) were evaluated in the NCBI website Primer-BLAST. The BLAST has a function to discover what a specific organism the primers designed with the information from the NCBI database. SYBR green is a reagent widely used to monitor the amplification that occurs during the cycle of real-time PCR. SYBR green is considered a convenient reagent because no design of probe is required (Bio-Rad, 2006). Furthermore, specific primers can be validated with high specificity, sensitivity, linearity, and repeatability. Finally, results from real-time PCR can be further validated using other methods to determine the *halal* status of the supplement products. To confirm the reliability of the results obtained using real-time PCR, the comparison was done with conventional PCR. In this book, further measurement to confirm the results from PCR was done using Fourier transform infrared spectroscopy and chemometrics.

The *halal* assurance in food or beverage product is something that must be enforced to provide a sense of security and confidence to the consumers (especially Muslims). At present, *halal* status is not a priority in some pharmaceutical industries. As a result, some pharmaceutical manufacturers are still using pig and non-*halal* materials as ingredients to formulate gelatin capsules. The false declaration about *halalness* of gelatin products is usually made for profit gaining purpose and has a great impact on Muslim consumers. Therefore the development of analytical methods for *halal* authentication in gelatin capsules will be very beneficial for the Muslim community and other consumers.

Challenge

Detection of porcine DNA using real-time PCR is common for *halal* authentication, however, a powerful method to distinguish the DNAs of different meat samples is required. Gelatin is a highly processed food and special skills are needed to extract and isolate DNA from gelatin even when a special kit such as the Agilent detection porcine kit is used. Therefore there is a need to further validate results from real-time PCR to improve the sensitivity, specificity, efficiency, and repeatability of the results.

The objectives of this book are to compare results obtained from the measurement of gelatin using real-time PCR and conventional PCR and to validate the *halal* status of gelatin capsules using FTIR spectroscopy and chemometrics.

Scope of This Book

The current research on *halal* authentication of gelatin capsules focused on the detection of porcine DNA in the capsules of the supplement products. The samples were collected around Nilai, Negeri Sembilan with total a total of 20 samples. The real-time PCR method was used to determine the *halal* status of gelatin capsules which detects changes in the fluorescent dye, SYBR green, and annealing temperature of specific species primer from the CYT b gene. As mentioned before, the proficiencies of real-time PCR with conventional PCR will be determined in terms of specificity sensitivity, efficiency, and repeatability. This is followed by validation using FTIR spectroscopy and the chemometrics method. The methods with finger printings corresponding to the *halal* status of the products were obtained. This can be further applied for *halal* authentication of other products.

Chapter 2

Gelatin

The animal kingdom has the structural pledge and most common protein and is called gelatin that is a primarily pure protein food ingredient and obtained by the thermals denaturation of collagen (Bailey and Paul, 1998). Water is the major component in gelatin structure so the gelatin is water-soluble and solubilization involves the destruction of the tertiary, secondary and to some extent, the primary structure of native collagens (Fernandez-Diaz et al., 2001), specifically by the partial hydrolysis of collagen derived from the skin, bones, hides skin, and connective tissue of animal (Morrison et al., 1999). Gelatin has benefits as a gelling and thickening agent and it is used in a wide range of food products. Gelatin is composed of various elements such as carbon (50.5%), hydrogen (6.8%), nitrogen (17%), and oxygen (25.2%) (GMIA, 2012). The main component of gelatin is similar to the complex polypeptides collagen so the gelatin has a high molecular weight polypeptide and an important hydrocolloid. The hydrocolloid system in gelatin differs from other hydrocolloids because most of them are polysaccharides, where gelatin is a digestible protein containing all the essential amino acids (except tryptophan) (Ladislaus et al., 2007).

The source of gelatin can be found in many parts of the animal body. The parts of the animal that usually contain gelatin are bones, skin, and hides. Gelatin could not be derived from the horns, hooves, and other non-collagenous parts of vertebrate animals. In the 17th century, commercial gelatin was produced on a large scale. In the early 19th century, the commercial production

method was gradually developed to obtain the extract of collagen with high molecular weight and good quality of gelatin gel. Lately, many studies have been designed to find an alternative source of gelatin (Abdelfadeel, 2012).

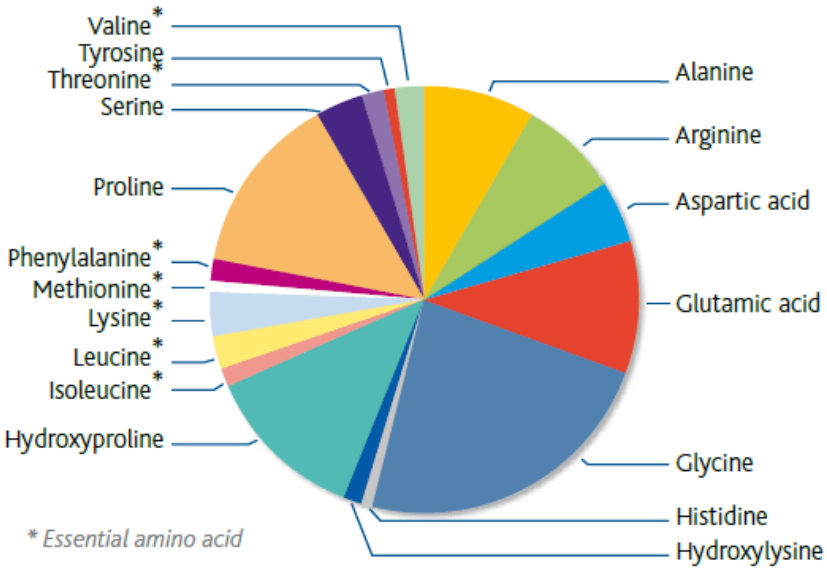


Figure 2.1: Composition of Amino Acid in Gelatin (GMIA, 2012)

Gelatin is prepared by the thermal denaturation and physical and chemical degradation of collagen. The gelatin in a dry form consists of 98-99% protein. The molecular weight of these large protein structures typically ranges between 20,000 and 250,000 g/mol, with some aggregates weighting in the millions. The chemical structure of gelatin is described by a linear sequence of amino acids. It is always written from the -NH2 end to the -COOH end. The predominant amino acids are *glycine*, *proline*, and *hydroxyproline*. As a result, gelatin contains relatively high levels of these amino acids: *glycine* 26-34%; *proline* 10-18%, and *hydroxyproline* 7-15%. Other significant amino acids include *alanine* 8-11%; *arginine* 8-9%; *aspartic acid* 6-7% and *glutamic acid*

10-12%. The water content will vary between 6-9% and the ash content varies between 0.1% and 3.25% (Figure 2.1) (GMIA, 2012).

Gelatin Sources

Gelatins can be produced from different animal sources. The principle of gelatin manufacture is that the source must be rich in collagen. It is one of the most abundant sources present in mammals, including humans. About 25% of the total amount of protein in the mammalian body contains collagen which can be found in the skin and bone of animals.

Animals that are typically used as the sources of gelatin are a pig, beef, fish, insect, and other vertebrate animals. There are alternative sources of gelatin from plants. But this is not gelatin because there is no evidence of the chemical relationship between gelatin and other plant-based materials that are referred to as vegetable gelatin, such as seaweed extracts (GMIA, 2012).

1. Mammalian gelatin

Mammalian gelatin is derived from collagen which is the principal constituent of connective tissues and bones of vertebrate animals. The often chosen animal as the sources of gelatin are buffalo, bow, sheep, goat, and pig but the most common animals used for gelatin production are cow and pig. In terms of the manufacturing process, gelatins are derived into two types. Type A gelatin is usually derived from an acidic process while Type B gelatin is from alkaline process bases. The structure of bovine and porcine gelatin is affected by the correlation between the average

molecular weight and the gel strength which determines their strengths based on the isoelectric and melting point. From the previous report, gelatin from cows and pigs had shown high strength (Lim and Mohammad, 2011). Therefore, mammalian gelatins (porcine and bovine) are the most popular and widely used because the raw material needed for the manufacture of gelatin from pigs and cattle is very easy to produce (Karim and Bhat, 2009).

2. Fish gelatin

The exploration of gelatin extracted from fish has been reported for hake (Montero et al., 1999), megrim (Montero and Gómez-Guillén, 2000), and tilapia (Grossman and Bergman, 1992; Jamilah and Harvinder, 2002). The main difference between fish gelatin and mammal gelatin such as pigs and cows is: Fish gelatin has gel strengths and gelling point that is lower but has a relatively higher viscosity than mammalian gelatin. Fish gelatin has gel strengths and melting temperatures that are associated with the place the fish lived. Generally, collagen derived from fish living in a lower temperature environment has a lower content of amino acids (*proline* and *hydroxyproline*) than the species that live at higher temperatures.

Thus, gelatin produced from the collagen of low temperatures fishes has a lower number of hydrogen bonds in water solution and lower melting points than the gelatin made from mammals (Montero and Gómez-Guillén, 2000).

3. Insect gelatin

The use of insects as raw material to produce gelatin is relatively unknown for many people. Not many countries produce gelatin from insects, because the source of insects is limited. In Sudan, gelatin has been extracted from insects. The types of insects such as *A. viduatus* and *A. pubescens* were used as raw materials to produce the gelatin. There are three different methods to extract the gelatin from *A. viduatus* and *A. pubescens* namely, mild acid and distilled water extraction method; distilled water extraction method; and extraction with hot water. From the results obtained, extraction of insect gelatin using hot water gave a higher yield of up to 3.0% followed by mild acid extraction (1.5%) and distilled water extraction (1.0%), respectively. The yields of gelatin extracted from *A. viduatus* and *A. pubescens* using distilled water with NaOH pretreatment at concentrations of 0.1, 0.2, and 0.5 mol/L was low compared to the results obtained for gelatin extracted using mild acid and distilled water (Abdelfadeel, 2012). Mariod et al., 2011 reported that the alkaline and acid treatments during insect gelatin extraction showed positive effects on removing non-collagenous proteins with minimum collagen loss. The gelatin obtained from insects was characterized using FTIR spectroscopy, and the spectra seemed to be similar to commercial gelatin. From the measurement, both insects showed 8.3% and 7.6% of moisture, 27.0% and 28.2% of crude protein, 54.2% and 57.3% fat and, 3.5% and 2.5% of ash in dry conditions, respectively. The two bugs proteins contained 16 essential amino acids when compared to the amino acid profile recommended by FAO/ WHO, the bug protein was of medium quality due to its moderate content of essential amino acids (Mariod et al., 2011).

Gelatin Structure

Gelatin consists of amino acids and so the composition of amino acids will affect the chemical structure of gelatin. The effect is also influenced by animal species and their connective tissues (Zhou and Regenstein, 2006). Gelatin contains *glycine*, *proline*, and 4-*hydroxyproline* residues. Gelatin is an amphoteric protein with iso-ionic points between 5 and 9 depending on raw material and method of manufacture. The only other animal product containing *hydroxyproline* is elastin and then at a very much lower concentration, so *hydroxyproline* is used to determine the collagen or gelatin content of foods. In brief, the protein is made up of peptide triplets, *glycine* - X - Y, where X and Y can be any one of the amino acids but *proline* has a preference for the X position and *hydroxyproline* the Y position. Approximately 1050 amino acids produce an alpha-chain with the left-handed proline helix conformation. Gelatin has a definite molecular weight distribution pattern and is not completely polydispersed, which corresponds to the α-chain and the oligomers (Rbii et al., 2011) (Figure 2.2).

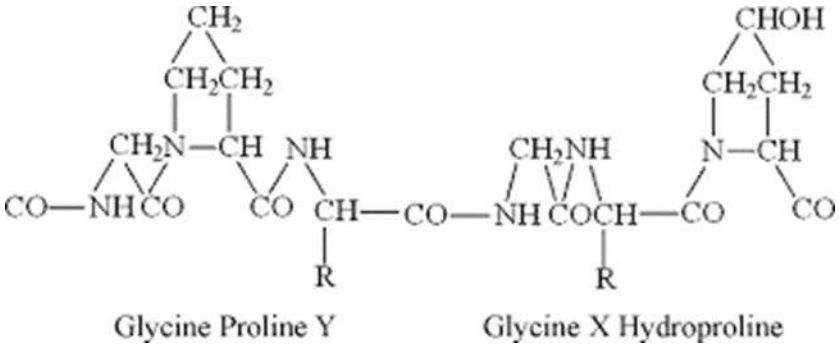


Figure 2.2: The Chemical Structure of Gelatin (Remawati, 2016)

Gelatin is the product of the denaturation or disintegration of collagen. Initially, the alpha-chains of collagen are held together with several different but easily reducible cross-links. As the collagen matures, so the cross-links become stabilized (Baily and Light, 1989). Then as time progresses the eta-amino groups of lysine become linked to *arginine* by glucose molecules (*Maillard* reaction) to form the *pentosidine* type cross-links which are extremely stable.

Hence when the alkaline processing is used on young animal skin the alkali breaks one of the initial (*pyridinoline*) cross-links and as a result, on heating, the collagen releases, mainly, denatured alpha-chains into solution. Once the *pentosidine* cross-links of the mature animal have formed in the collagen, the main process of denaturation has to be thermal hydrolysis of peptide bonds resulting in protein fragments of various molecular weights i.e. polydisperse protein fragments. With the acid process, the collagen denaturation is limited to the thermal hydrolysis of peptide bonds with a small amount of alpha-chain material from acid-soluble collagen in evidence (Cole and Roberts, 1996; Cole and Roberts, 1997). Nutritionally, gelatin is not a complete protein food because the essential amino acid *tryptophan* is missing and *methionine* is present only at a low level (Figure 2.3) (Ofori, 1999).

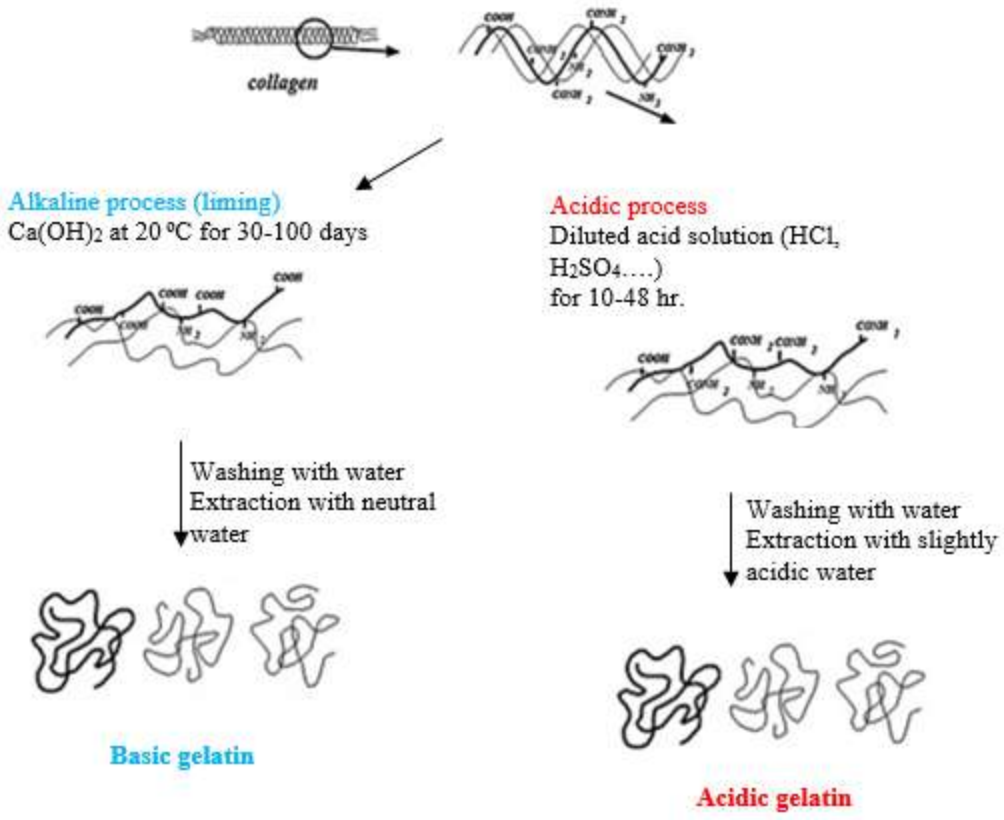


Figure 2.3: Process of Collagen Conversion to Gelatin (Ofori, 1999)

The functional properties of gelatin are related to its chemical characteristics. The gel strength, viscosity, setting behavior, and melting point of gelatin depend on their molecular weight distribution and the amino acid composition. It is generally recognized that amino acids like *proline* and *hydroxyproline* are important in the renaturation of gelatin subunits during gelling.

As a result, gelatin with high levels of amino acids tends to have higher gel strength and melting point. The molecular weight distribution is also important in determining the gelling behavior of gelatin. The sum of intact α and β fractions together with their peptides is proportional to the gel strength while the viscosity, setting rate, and melting point increase with the increase in the

amount of the high molecular weight fraction (Johnston-Banks, 1990).

Gelatin Uses and Applicants in Food and Pharmaceutical

Gelatin can stabilize colloidal solutions. The protein molecule moves towards the interface of the two phases and forms a monomolecular film around the micelles. Moreover, it increases the viscosity of the aqueous phase and promotes the formation and stability of suspensions or emulsions. In terms of gelling and stabilizing properties, gelatin plays a key role in food, pharmaceutical, and photographic products, and in addition to this, has many other technical applications (GMIA, 2012).

Gelatin has a considerable number of food applications and uses (Table 2.1). Gelatin has been used in foods as a beverage clarifier, a fining agent for white wine, as a beer clarifier, and to clarify fruit and vegetable juice (especially for clarified apple juice and pear juice). Gelatin is used in desserts at 8-10% of the dry weight, in yogurt at 0.3-0.5% as a thickener, in ham coatings at 2-3%, and confectionery and capsules (vitamin supplements) at 1.5-2.5%. Further uses include fruit toppings for pastry, instant gravy, instant sauces and soups, edible films for confectionery products, and as a stabilizer in ice cream, cream cheese, and cottage cheese as well as in food foams and fruit salads. Overall functional uses include a stabilizer, thickener, and texturizer (Cheng et al., 2009; Tyburcy et al., 2010).

The largest proportion of gelatin procured by the pharmaceutical industry is used mainly for hard and soft gelatin capsules and for binding tablets, where it helps prevent oxidation and makes the preparation more palatable. The capsules are formed

on mould pins on the surface of which carry a lubricant to facilitate the subsequent removal of the capsule (GMIA, 2012).

Table 2.1: The Function and Uses of Gelatin in Food

Functions	Area Used
Gel former	Gelled desserts, lunch meats, confectionery, pate, consommé, aspics
Whipping agent	Marshmallows, nougats, mousses, soufflés, chiffons, whipped cream
Protective colloid	Confectionery, icings, ice creams, frozen desserts and, confections
Binding agent	Meat rolls, canned meats, confectionery, cheeses, dairy products
Clarifying agent	Beer, wine, fruit juices, vinegar
Film former	Coating for fruits, meats, deli items
Thickener	Powdered drink mixes, bouillon, gravies, sauces, soups, puddings, jellies, syrups, dairy products
Process aid	Microencapsulation of colors, flavors, oil and, vitamins
Emulsifier	Cream soups, sauces, flavorings, meat pastes, whipped cream, confectionery, dairy products
Stabilizer	Cream cheese, chocolate milk, yogurt, icings, cream fillings, frozen desserts
Adhesive agent	Affix nonpareils, coconut and, other items to confections, to bond layered confections together, to bind frostings to baked goods, to bind seasonings to meat products.

(Turner, 1988)

Gelatins with bloom in the range of 0-140 are offered for the microencapsulation of vitamins A, D, and E. High Bloom gelatin (between 200 and 260 Bloom) is used for the production of hard gelatin capsules. Bloom is also known as the gel strength and is one of the most important properties of gelatin. The need to commercially characterize gelatin gels has resulted in the concept of gel strength. Commercial gelatins vary from 50 to 300 blooms. Hydrolysis of gelatin gels can be initiated by numerous factors, including pH, temperatures, enzymes, acids, bases as well as bacteria. These cause a reduction in the gelling properties of the gelatin (GMIA, 2012).

Another use of gelatin is the percipients in pharmaceutical formulations, including vaccines, and is used as a binder for tablets and for excipients (material other than the active substance that is added in the formulation of preparation for various purposes or functions) and may be originating from quite distinct sources, including gelatin (Muyonga et al., 2004a).

Chapter 3

Detection of DNA in Gelatin Capsule

A reliable analytical method needs to be developed to detect the absence or presence of certain DNA in gelatin products. Several methods have been reported so far, based on chemisorption, chromatographic, immunochemical, mass spectrometric, spectroscopic, and molecular techniques.

1. Chemisorption Method

In the chemisorption method, it was reported by Hunter et al. (1986) that collagen, gelatin, and agarose gels promote the formation of hydroxyapatite from amorphous calcium phosphate. Hydroxyapatite is a compound that is formed by the chemisorption of gelatin. Termine et al. (1970) reported that the presence of collagen or gelatin enhanced ACP formation, suggesting that the interaction between gelatin and calcium phosphate precipitation may be used to analyze the source of gelatins. Based on this fact, Hidaka and Liu (2003) presented a new method distinguishing bovine bone gelatin from porcine skin gelatin using the in vitro formation of calcium phosphate precipitates. In their study, the reaction of calcium phosphate precipitation was found to be useful in distinguishing bovine gelatin from other gelatin products. However, it was stated that further study was needed to clarify these effects.

2. Chromatographic Methods

In the use of chromatographic methods, Raraswati et al. (2012) carried out HPLC-based separation and determination of amino acids in gelatin using principal component analysis. In their study, 7 samples of porcine and bovine gelatins purchased from Sigma Aldrich, USA, and 5 laboratories prepared soft candy gelatins were examined using the analytical procedure following complete hydrolysis of samples by conventional acid hydrolysis to promote the release of amino acid residues. Separation and determination of amino acids were achieved by reversed-phase HPLC (RP-HPLC) following pre-column derivatization. The 16 peaks resulting from HPLC measurement concluded that one sample was very typical of bovine gelatin (Raraswati et al., 2012). In this study, PCA was used to extract the significant variables from parameters of peak height percentage for each amino acid.

The result of PCA is the principal component (PC), which contains information to a certain amount of data variability. PC1 (first principal component) accounts for the most variation among data, while PC2 explains for the next largest variation and so on (Raraswati et al., 2012).

PCA score plot for classification of porcine and bovine gelatins and laboratory prepared soft candy from bovine and porcine gelatins. 1= porcine gelatin; 2 = bovine gelatin; 3= laboratory prepared soft candy from porcine gelatin; 4 and 5 = laboratory prepared soft candy from bovine gelatin; 6 and 7 = laboratory prepared soft candy from porcine and bovine gelatins. Figure 3.1 shows the PCA score plot of porcine and bovine gelatins coming from Sigma and laboratory prepared soft candy. The horizontal axis is the scores for the first PC, and the vertical axis is for the second PC. Bovine and porcine gelatins, both in standard or in

soft candy, were separated. However, the figure could not give the classification of porcine and bovine. The aforementioned physicochemical methods based on HPLC and calcium phosphate precipitation have not been proved yet to be able to detect bovine and porcine in commercial food products.

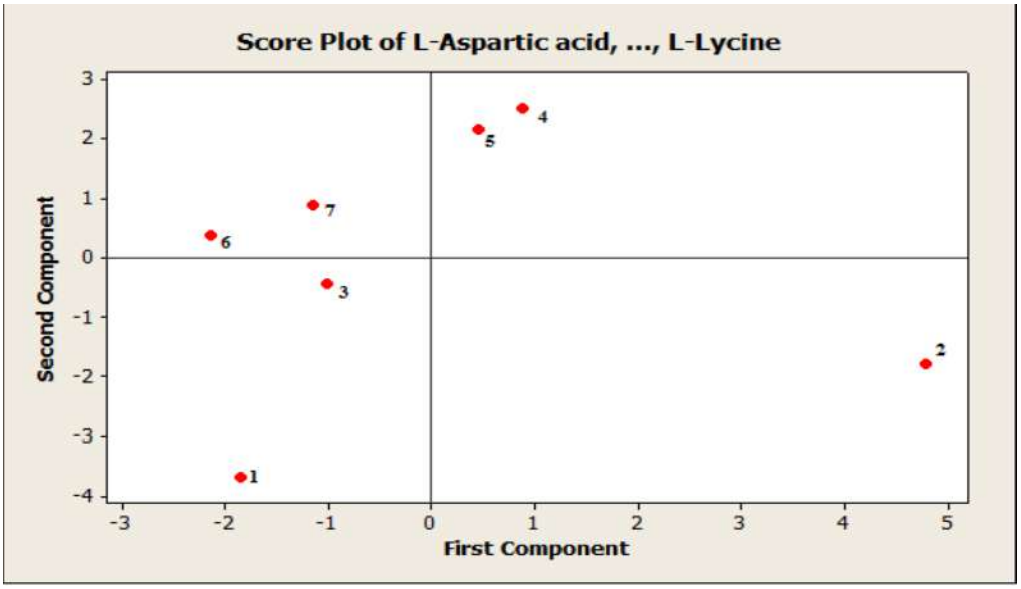


Figure 3.1: The Score Plot of Soft Candy Samples (Raraswati et al., 2012)

3. Immunochemical Methods

As for the immunochemical methods, Hofmann et al. (1999) examined an ELISA kit for its applicability to species identification in gelatin and gelatin-containing products, such as gum confectionery, processed turkey and chicken.

However, results were influenced by gelatin type, gelatin quality and concentration used and, in some cases, led to false negative or positive readings. The very high homology between

collagen sequences of mammals' makes their immunochemical differentiation difficult when polyclonal antibodies raised against the whole molecule are used (Venien and Levieux, 2005). To overcome the challenge, Venien and Levieux (2005) produced highly specific antibodies, immunized rabbits against putative species-specific sequences of the bovine collagen alpha 1 (I) chain for differentiation of bovine from porcine gelatins However, such antibodies were found to be very sensitive to the alkaline or acidic process used for the gelatin production and not enough species-specific to allow sensitive detection of a mixture of low concentration of bovine gelatin in porcine gelatin (Hashim et al., 2010).

4. Mass Spectrometric Method

Within the context of the mass spectrometric methods, Ocana et al. (2004) reported that some species-specific ions could be detected using mass spectroscopy after bovine gelatin was hydrolyzed with 3 mol/L HCl and could be used for the detection of bovine gelatin. However, the content of target ions might be influenced by the hydrolysis time and temperature. In this respect, Zhang et al. (2009) have developed a new method to differentiate bovine and porcine gelatin based on the detection and identification of marker peptides in digested gelatins. In their study, the gelatins were digested by *trypsin*, and the resulting peptides were analyzed by high-performance liquid chromatography/tandem mass spectroscopy (HPLC–MS/MS). Using this methodology, they could successfully detect the marker peptides specific for bovine and porcine in the digested bovine and porcine gelatin, respectively. Another important result that was reached by this study was that the peptide identification was remarkably influenced by *proline*

hydroxylation. However, it was also reached to a conclusion that it was necessary to manually verify the sequence for peptides (GPPGSAGSPGK and GPPGSAGAPGK detected in digested bovine and porcine gelatin, respectively) since there might be a risk to confuse the *proline* hydroxylation with the mass difference between Ser and Ala residues. They concluded that detection of marker peptides in the digested gelatin sample using HPLC–MS/ MS was an effective method to differentiate between bovine and porcine gelatin. However, the method also has some drawbacks (Zhang et al., 2009).

During MS/MS data processing, the threshold for specific peptide identification might be different from one species to another (Li Chen et al., 2007; Shadforth et al., 2005). More clearly, it is very difficult to detect marker peptides in the digested collagens because of the very high homology between the collagen sequences of mammals using this method. Furthermore, *proline* hydroxylation is another challenge making the identification of peptides more difficult than that most proteins (Zhang et al., 2009).

5. Spectroscopic method

In the spectroscopy method, the FTIR spectroscopy together with attenuated total reflectance (ATR) or transmission accessories has been used to determine gelatin and intermolecular cross-linking study of collagen and proteins (Cao and Xu, 2008; Muyonga et al., 2004). Hashim et al. (2010) have used the Fourier Transform Infrared (FTIR) spectroscopy for the differentiation of bovine and porcine gelatins. In their study, in order to determine unknown gelatin sources, deformation of N–H bonds found in the range 3290–3280 and 1660–1200 cm^{-1} within infrared spectra of all gelatin

samples were analyzed using discriminant measurement. These regions were found to give information about the origin of the gelatin. However, this method was concluded to need repeated results (Hashim et al., 2010).

6. Molecular technique

DNA techniques or molecular techniques have become very important and are widely used nowadays compared to protein techniques. Advantages of DNA analyzing methods are manifold: DNA is a relatively stable molecule easily allowing measurement of processed and heat treated food products. Due to the ubiquity of DNA in every type of cell, all kinds of tissue can be analyzed. Early methods based on hybridization of specific probes (Chikuni et al., 1990; Wintero et al., 1990) were complicated, time-consuming, and insufficient for complex matrices. The polymerase chain reaction (PCR) proved to be an adequate technique for the detection of small amounts of DNA, specifically amplifying a target region of template DNA rapidly and sensitively (Chikuni et al., 1990).

PCR systems used today only allow a qualitative detection of animal species. Because even trace amounts of less than 0.1% of one species lead to positive PCR results (Behrens et al., 1999), it is necessary to differentiate between technically unavoidable contamination or intentional admixture. Therefore, adequate quantitative detection methods for small amounts of DNA must be available. PCR measurement can be used as a quantitative method if internal standards (competitors) are co-amplified with the target DNA (Section 2.4).

Cai et al. (2011) have used real-time PCR for the detection and quantization of porcine and bovine DNA in gelatin mixtures and gelatin capsules. In this study, two species-specific qPCR assays were developed based upon repetitive elements. They allowed sensitive detection of porcine and bovine DNA at as low as 1 pg/mL. The lack of cross-reactivity when the sets were used to amplify DNA from the other species indicates the high specificity of the assay. When binary gelatin blends containing various amounts of porcine and bovine gelatin were prepared and analyzed by the qPCR assays, the determined ratios of porcine material to bovine material were very close to their theoretical values, and a contamination level as low as 1% of the other species in the gelatin blends could be determined. When evaluated in gelatin capsules, although significantly less DNA was detected, determination of porcine and bovine species identities and estimation of the relative abundance of each species was possible. Therefore, the porcine and bovine species-specific qPCR assays described here represent simple, reliable, and sensitive DNA-based tests for the determination and quantization of the species of origin from highly processed products (Cai et al., 2011).

DNA Isolation Methods for Gelatin

The isolation of genomic DNA is a fundamental requirement for many analytical procedures. Although proper collection and stabilization of the sample are crucial, purification of the DNA is often the key step for success in downstream measurement. Ideally, in an analytical environment, an effective DNA extraction procedure should be as simple, safe, and cost and time-efficient as possible. It should also reproducibly provide DNA of sufficient

quality and yield to allow subsequent measurement (Ricci et al., 2013).

Generally, there are three important factors that affect the yield of the result of suitable analytical method of isolated DNA as an analyte for a given technique, namely: (i) Concentration of the DNA (ng/µl), (ii) Purity of the DNA (1.8-2.0) and (iii) Stability. The three factors can be influenced by how the technique is engaged and, in turn, in subsequent measurement can impact the validity of techniques applied (Ricci et al., 2013).

The result of yields and purity of DNA give any difference if the extraction method is engaged differently. From the previous study extraction methods have been systematically evaluated for specific applications with the different samples such as soil and sediment (Collen, 2011), human microbiome (Yuan et al., 2012), and fecal (Claassen et al., 2013; Kennedy et al., 2014; Peng et al., 2013) and the study had been concluded that the use of a modern method such as silica-based technologies, magnetic separation, anion exchange technologies give the optimum result if we look based on the purity and the yield of the DNA.

Silica-based technologies with micro spin tubes are already the method that has been used for many isolation kits. Qiagene, Kogene, and Agilent also use the method to isolate the DNA from samples. The basis for most of the products related to nucleic acid purification is the unique properties of silica matrices for selective DNA binding. Types of silica materials include glass particles, such as glass powder, silica particles, and glass microfibers prepared by grinding glass fiber filter papers, and including diatomaceous earth. Hydrated silica matrix, which was prepared by refluxing silicon dioxide in sodium hydroxide or potassium hydroxide at a molar ratio of about 2:1 to 10:1 for at

least about 48 hours, had been introduced in DNA purification (Padhye et al., 1997; Woodard et al., 1994).

The principle of silica matrices purification is based on the high affinity of the negatively charged DNA backbone towards the positively charged silica particles. Sodium plays a role as a cation bridge that attracts the negatively charged oxygen in the phosphate backbone of nucleic acid. Sodium cations break the hydrogen bonds between the hydrogen in water and the negatively charged oxygen ions in silica under high salt conditions (pH ≤ 7). The DNA is tightly bound, and extensive washing removes all contaminations (Arnolds et al., 2005).

The purified DNA molecules can be eluted under low ionic strength (pH ≥ 7) later by using TE buffer or distilled water (Esser et al., 2006). Besides silica matrices, nitrocellulose and polyamide membranes such as nylon matrices are also known to bind with nucleic acids, but with less specificity (Arnold et al., 2005).

The first stage in DNA isolation is the process of destruction of the membrane and cell wall. The destruction cell (lysis) is a stage of initial isolation of DNA that aims to remove the contents of the cell (Holme and Hazel, 1998). The destruction of cells or tissues has several methods namely physically, chemically, and enzymatic. Techniques destruction of cell or tissue in this research is the enzymatic technique, which as many as 220 µl proteinase K working solution (200 µl of proteinase K digestion buffer and 20 µl of proteinase K) put in the sample and incubated at 65° C for 1 hour. Extraction using Proteinase K working solution causes the protein to lose solubility and undergo further precipitation that could be separated from the DNA by centrifugation (Karp, 2008). Bettelheim and Landesberg (2007)

stated that after centrifugation would be formed two phases which is the organic phase on the bottom layer and the phase aqueous phase (water) on the top layer DNA would be in phase aqueous after centrifugation, the protein that was denatured would be in inter-phase and lipids would be in the organic phase.

After extraction process, the DNA had been obtained was concentrated by the precipitation stage. In general, the solution used at this stage is ethanol or isopropanol. The two solutions would precipitate DNA in the aqueous phase so that DNA clumping to form fiber structure and after it is formed pellet (centrifuge) (Switzer and Liam, 1999). In this precipitation stage, DNA had been separated from the residues of RNA and protein that remained. At the time ethanol or isopropanol had been discarded and the pellet was dried in a tube, the pellets were remaining in the tube is concentrated DNA. The process of re-precipitation with ethanol or isopropanol before the dried pellets could increase the degree of purity of DNA isolated (Bettelheim and Landesberg, 2007). Keller and Mark (1989) explained that washing back the pelleted precipitated using ethanol or propanol was intended to eliminate the residues of salt remaining (Keller and Mark, 1989).

The salts that were involved in the extraction process are less soluble in isopropanol so could be precipitated along with the DNA, and therefore is required ethanol after precipitation with isopropanol to remove residual salt (Ausubel et al., 2003). After precipitation and washing with ethanol, the ethanol solution which has been used was discarded and the pellet was dried by centrifuging the pellet without any treatment. The stage was aimed to remove residual ethanol from pellet DNA. After the DNA pellet had been dried, the next step was the addition of the elution buffer into tubes that contained pellets and then stored in a

freezer with temperatures at around -20°C. Verkuil et al. (2008) suggested that the elution buffer and temperature storage at -20°C were intended the DNA that had been extracted could be kept up for weeks (Ausubel et al., 2003).

Traditional DNA extraction methodologies employing chemicals such as SDS, proteinase K, and phenol are now reasonably well established (Sambrook and Russell, 2001). These methods tend, however, to be time-consuming and involve multiple liquid transfer operations. Alternative commercial kits often offer reduced hands-on time and cleaner approaches to extractions; however, they can be more expensive and limited to very specific applications. In an analytical environment, neither of the approaches mentioned may be ideal and the situation can be further complicated by the sample matrix composition. Whilst simple approaches may be preferable, complex matrices and non-ideal samples may demand additional clean-up procedures. Measurement of DNA yield in itself is not sufficient to determine the suitability of an extraction methodology (Sambrook and Russel1, 2001).

The quality, encompassing purity and integrity, of the DNA analyte can also be important in ensuring suitability for downstream measurement. An inappropriate choice or a sub-optimal extraction methodology could have significant consequences for subsequent analyses, which may have to be repeated, or produce false-negative results. Automation of the entire extraction procedure increases throughput and reduces analyst errors, often resulting in a more accurate and cost-effective DNA isolation step in the laboratory workflow. Validation of sampling procedures, sample storage, sample preparation, and DNA extraction should all be considered vital to the production of quality data in subsequent

analyses whether these are accomplished manually or on automated platforms (Sambrook, 2001).

Chapter 4

Measurement of Gelatin Capsule

Polymerase Chain Reaction

PCR (polymerase chain reaction) is one of the techniques to learn about molecular biology and it is an enzymatic method that was used to multiply exponentially a nucleotide sequence-specific in vitro way. The method is very sensitive, so it can be used to multiply the DNA molecule (Rosenthal, 1992).

The original concept of PCR technology requires that certain parts of the DNA sequence be multiplied should be known before the multiplication process can be done. The known sequence is important to provide a primer, which is the short oligonucleotide sequence that the function initiate synthesis of DNA in the polymerase chain reaction. Further development of the PCR method allows the multiplication of a DNA fragment of the unknown sequence, for example with the Alu-PCR method (Rosenthal, 1992). Alu is a DNA sequence (length approximately 300 bp), which is widely available throughout the human genome (repetitive DNA sequences). Alu-PCR is a PCR method that utilizes the Alu sequences as a basis for primers to multiply a DNA fragment of an unknown sequence that contained between two Alu sequences (Rosenthal, 1992).

Target PCR is a nucleic acid (DNA) double-stranded that was extracted from the cells and denatured into single-stranded nucleic acids. The components of PCR reaction consists of a form of specific oligonucleotide primers for the target genes selected, enzymes (generally Taq polymerase, thermostable and

thermoactive enzyme derived from *Thermus aquaticus*) and triphosphate deoxynucleoside (dNTP) that is used to amplify the target gene replicated exponentially with multiply the initial target. The reaction is carried out in a heating machine that is programmed automatically and called a thermocycler. The machine provides thermal conditions that are necessary for the amplification process (Nollet and Toldrá, 2011). The process that occurs in the PCR machine includes three main stages, namely is denaturation (the separation of double-stranded DNA), annealing, and extension (primer elongation). The process that is started from denaturation, annealing, and extensions was referred to as one cycle. The PCR products can be directly visualized through a process of electrophoresis and used for further measurement (Weissensteiner et al., 2004).

PCR techniques began to develop after the discovery of the DNA polymerase enzyme, which can replicate DNA. The technique was initially developed using a Klenow fragment of DNA polymerase I that was derived from Escherichia coli. Klenow fragment is DNA polymerase that has eliminated the exonuclease activities. The enzyme has some weaknesses in the highest temperature, the medium of polymerization medium, and the process is low (Ali et al., 2012).

The low process of this reaction indicates a low ability of the enzyme to incorporate nucleotide polymerase with a primer continuously without experiencing dissociation of a complex of primer-template DNA. PCR method was first developed in 1985 by Kary B. Mullis, the researcher at the company Cetus Corporation. In the early development of PCR method is only used to multiply the DNA molecule, but then it was developed further so that it could also use to multiply and perform quantization of mRNA

molecules. This time PCR method has been widely used for a variety of genetic manipulation and measurement (Ali et al., 2012).

PCR process involves four main components: (1) DNA template, the DNA fragment to be duplicated, (2) oligonucleotide primer, which is short sequences of oligonucleotide (15-25 nucleotide bases) that initiate the synthesis of DNA chains, (3) deoxyribonucleotides triphosphate (dNTPs) that is consisting of dATP, dCTP, dGTP, dTTP, and (4) DNA polymerase enzyme, which is a reaction catalyst synthesis of DNA chains. PCR process consists of three stages: denaturation, annealing, and amplification. At the stage of denaturation, a DNA fragment (double strand) was heated at 95 °C for 1-2 minutes so it will separate into a single chain (single-strand). The annealing stage is started at a temperature of 55 °C for 1-2 minutes, the oligonucleotide primer is attached to the template DNA that complementary with the primer sequences. After the annealing process, the temperature was raised to 72 °C for 30 seconds. At this temperature, the DNA polymerase enzyme will do the poses polymerase, the DNA chain that will form a hydrogen bridge with the template DNA. The process is called amplification (Yuwono, 2006).

The advent of PCR during the mid-1980s enabled measurements of target DNA across a wide dynamic range and is sensitive to as little as a few copies (Saiki et al., 1988).

Conventional PCR measurement involves and-point detection of the products formed, and as the rate of product generation is not linear over the course of the reaction then extrapolating from the final amount of product to be the initial amount of starting material is not straightforward. However, by defining the sensitivity of the reaction or by the use of reference samples, comparative standards, or competitive mimics it is possible to

generate data ranging from qualitative to semi-quantitative and quantitative (Saiki et al., 1988).

Such strategies all involve the detection or quantifications of PCR products, using a range of methodologies including agarose gel in the presence of ethidium bromide, capillary electrophoresis, and mass spectrometry. Detection methods vary in the accuracy and precision of measurement. More recently the development of quantitative real-time PCR has enabled highly accurate quantification using appropriate calibration standards, and this will be detailed out in the next section (Section 2.4.2) (Higuchi et al., 1993).

Real-Time PCR

The sensitivity of measurement achievable with PCR has led to the technology being adopted across a range of sectors. For many applications, a quantitative result is required, which has driven the development of arrange of strategies to determine the amount of starting material in a sample. Approaches such as competitive PCR (Gilliland et al., 1990) and limiting dilution measurement (Levinson et al., 1994) have been used as routes to quantification although the variable nature of the PCR process and the amplification of the target to a maximal level irrespective of the starting amount of target limit the accuracy of these methods (Alvares et al., 2000).

The development of the PCR method that is currently used is real-time PCR. The detection in real-time PCR is underway in a single stage because the accumulation of specific products is recorded continuously during the cycle and it could not be

performed on a standard PCR (conventional PCR) that still relied on agarose gel electrophoresis to quantify the amplicons.

The detection of Real-time PCR products is quantitatively different from the standard PCR that the quantization of the amplicon based on the length of base or molecular weight (Dharmaraj, 2009).

The quantity of real-time PCR products is calculated based on the threshold cycle (Ct) which is the fluorescent intensity is greater than the fluorescence that was induced by noise (background fluorescence). Noise can be caused by the attachment of a solution of DNA isolates along with PCR reagents in the tube wall micro-well. The advantages of real-time PCR are the detection was measured precisely when the target amplification was first detected in each cycle (exponential phase) and not in the final phase of amplification (the plateau phase) as occurs in standard PCR. The more amplification cycles that are performed, the higher the yield of the reaction, as long as the components of the reaction are not completely limiting and the enzyme retains some activity. Through the repeated denaturation cycles of the PCR the enzyme activity does become depleted, and in practice running more than 40 cycles does not appreciably increase yield. It is also important to balance speed/throughput with overall efficiency, so it may be effective to use a smaller number of amplification cycles if this allows an additional run to be performed within the working day (Montowska and Pospiech, 2010).

According to Edwards et al. (2004), the application of real-time PCR technology reduces the time of treatment or testing and improves the accuracy of PCR quantification methods. Thus, the use of real-time PCR technique is more efficient and effective than standard PCR (Edwards et al., 2004). Quantification of real-time

PCR can be performed by two techniques: (1) the use of fluorescent dye that binds to double-stranded DNA and (2) the use of a modified DNA oligonucleotide probes that emit fluorescence when hybridized with a complementary DNA.

The use of fluorescent dye is also known as SYBR green method. SYBR Green-based detection is the least expensive and easiest method available for real-time PCR. Other methods (such as TaqMan) require an expensive third primer labeled with a dye and a quencher. Most real-time systems detect and accommodate SYBR Green making the method very flexible; however, some instrumentation may also require the simple addition of a reference dye to normalize the system's optics.

SYBR Green specifically binds double-stranded DNA by intercalating between base pairs and fluoresces only when bound to DNA. Detection of the fluorescent signal occurs during the PCR cycle at the end of either the annealing or the extension step when the greatest amount of double-stranded DNA product is present (Soares et al., 2013). However, SYBR Green detects any double-stranded DNA non-specifically. Therefore, the reaction must contain a combination of primers and a master mix that only generates a single gene-specific amplicon without producing any non-specific secondary products (Soares et al., 2013).

The data generated can be analyzed by computer software that is connected with a thermal cycler to calculate the number of copies of DNA or the threshold cycle (Ct) of certain pathogens in food samples. Amplification is carried out in a thermal cycler was shown in the form of a graph on a computer screen with software that is applicable against thermal cycler, for example is a software steponeplus (applied biosystem). The graphics are obtained in the form of graphs amplification, standard curves, and

melt curve (only for the SYBR green method). The graph is used to evaluate the performance of real-time PCR amplification (Wang et al., 2010).

Graph amplification is formed since the amplification process is started. The graph serves to determine whether the thermal cycler was amplification or not. Graph amplification could be seen in Figure 4.1. Amplification is determined based on fluorescence intensity. The more the amplification product is produced the greater the accumulation of fluorescent is legible. The increase in fluorescence is characterized by the formation of graph sigmoidal (red line). The sigmoid graph would be intersected with the baseline threshold that has been determined automatically by the program. The intersection point between the sigmoid graph and baseline threshold if reflected in the X-axis (Cycle) is called the threshold cycle (Ct) for the samples that were amplified. The blue line will be formed if there is no amplification in a thermal cycler. Ct value is the cycle above the noise product which is the product of accumulation (2^n, n is the number of repetition cycles of amplification) was first legible in the exponential phase. The exponential phase ended into the plateau phase when PCR reagents in the reaction mixture had completely reacted (Kordo et al., 2013).

The model software will construct amplification plots from the extension phase fluorescent emission data collected during the PCR amplification. The standard deviation is determined from the data points collected from the baseline of the amplification plot. Ct values are calculated by determining the point at which the fluorescence exceeds a threshold limit (usually 10 times the standard deviation of the baseline) (Kordo et al., 2013).

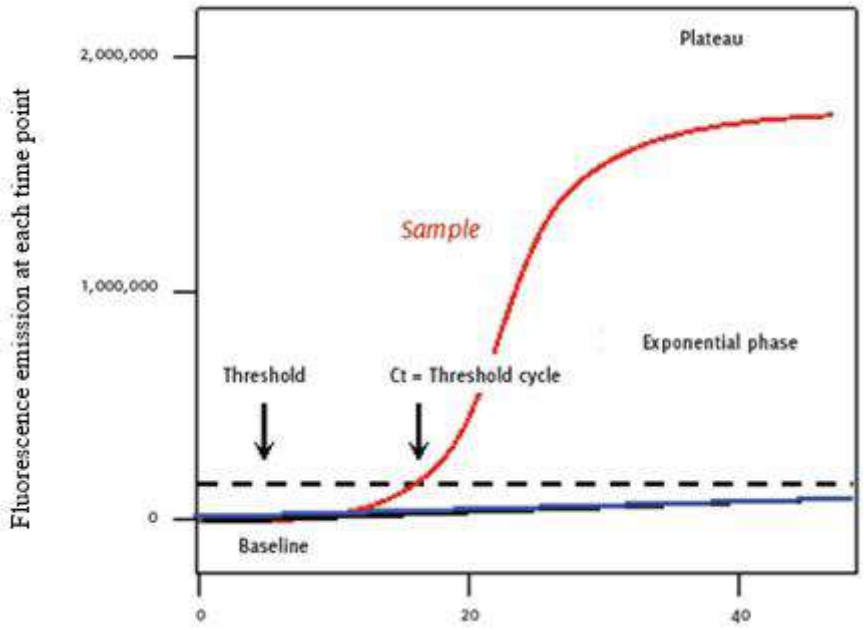


Figure 4.1: The graphs Amplification in *Real-Time* PCR (Kordo et al., 2013)

The fluorescent conversion factor can be determined by means of a calibration graph, on which the fluorescence values from a dilution series of DNA standard has been plotted against known concentration (Figure 4.2). The variations in fluorescence with concentration should be linear provided the DNA concentration is not excessively high and sufficient dye has been used to saturate all possible intercalation sites. It is important to ensure that the concentration ranges of the standards give fluorescence values that are within the dynamic range of the fluorometer or fluorescence plate reader being used for the assay. The gradient of the calibration graph can then be used as a conversion factor to determine sample DNA concentrations (Alexander et al., 2013).

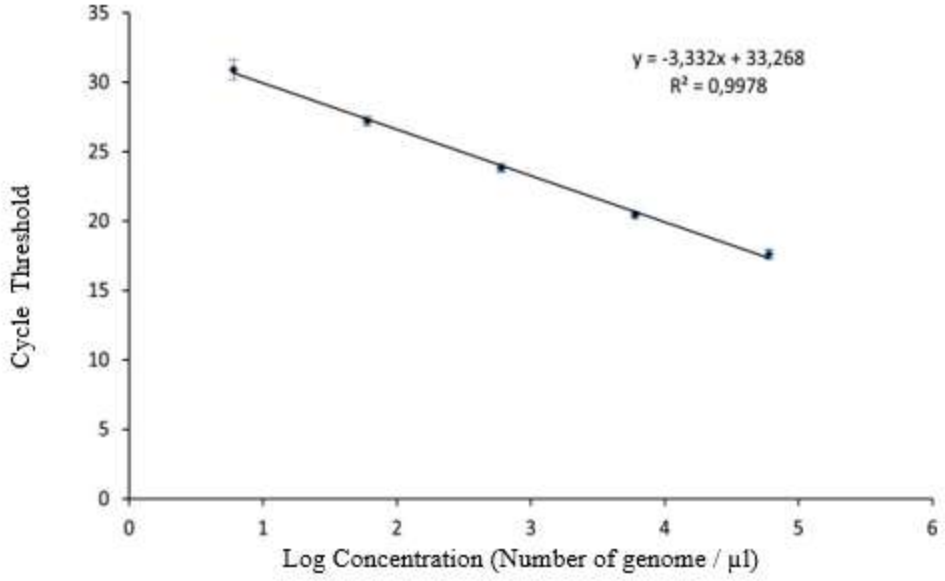


Figure 4.2: Calibration Curve of DNA Standard (Alexandra et al., 2013)

Concentration values of the standard could have been measured by another established methodology such as UV spectroscopy, or supplied by a manufacturer. The accuracy of the quantification process is significantly influenced by the quality of the standards used, and thus consistency in the source and use of standards is central to acceptable method performance. Further, as determination of absolute DNA quantities remains technically challenging, then the accuracy of any assigned standard values must be critically assessed to determine the reliability of standards used for fluorescent spectroscopy (Wilhelm et al., 2003).

In addition to measuring the increase in product at each cycle, a measurement of the products generated in the reaction may be performed at the end of the amplification process. This is termed 'melt measurement' and is compatible with both intercalating dye reporter system and those where the primers binds

to the PCR product to achieve a change in fluorescence intensity. To perform the measurement the fluorescent signal is monitored as the temperature is gradually increased from around 50 to 95 °C, which results in an increase in fluorescence as the double stranded DNA or primer: product complex is dissociated. The change in fluorescence against temperature is usually plotted by instrument software, yielding peaks corresponding to the denaturation maxima of each double-stranded species present in the system (Figure 4.3).

The dissociation curve for the measurement was showing a single, sharp peak, suggesting that only a specific PCR product was generated with this set of primers. Primers-dimmers, non-specific or mismatched sequences will generally have a lower melting temperature than the specific product of the reaction, and can be distinguished by this post-PCR measurement (Cubie et al., 2001).

Recently, an extension of melt curve measurement have been developed, termed High Resolution Melt (HRM) (Corbett Life Science), enabled by improvements in real-time PCR instrument capabilities and the dye used in measurement. Rapid data collection is required, with very high precision thermal resolution (down to 0.02 °C) and dedicated measurement software. Samples are characterized based on very detailed measurement of their disassociation kinetics, and with more detailed melt profiles samples may be discriminated by length, GC content and sequence. Even single base-pair mismatched can be distinguished, allowing application of the method to detailed genotyping measurement, and thus this approach could potentially be exploited to replace the use of more complex probe reporter system (Dufresne et al., 2006).

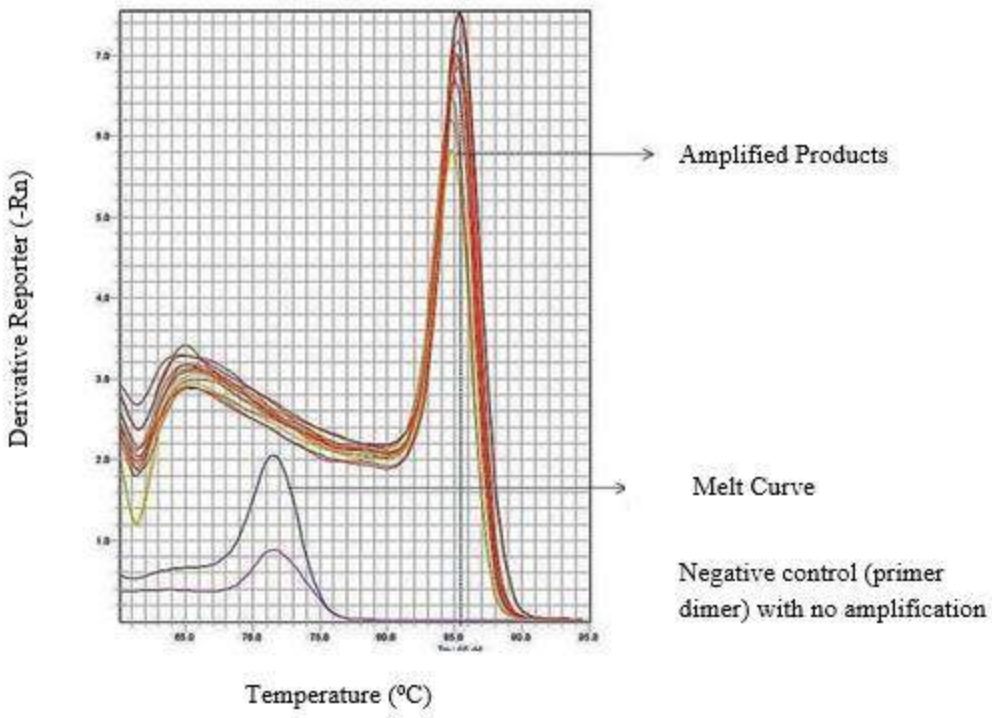


Figure 4.3: Melt Peaks of DNA Amplification Process (Kumar et al., 2013)

Fourier Transform Infrared Spectroscopy

In infrared spectroscopy, infrared light is passed through the sample and then was measured the fraction of absorbed radiation in the wavelength range and produces a spectrum in which shows the qualitative information from protein. The chemical structure and forms a molecular bond with certain functional groups of samples tested becomes the basis of the spectrum form to be obtained from the result of measurement. Thus it can be used for testing qualitatively and quantitatively. Infrared spectroscopy is a method to observe the interaction of molecules with electromagnetic radiation in the wavelength of 0.75 to 1000 µm, or in wave numbers 13000-10 cm^{-1} (Rohman et al., 2012).

Infrared is an electromagnetic ray that the wavelength is more than the visible light and less than the microwave (between 700 nm and 1 mm). The wavelength region in infrared spectroscopy in the mid-infrared region, which is at wavelengths from 2.5 to 50 µm or at wave number 4000-200 cm^{-1} (Petibois and Deleris, 2006).

Table 4.1: Specific Functional Groups on a Particular Wave Numbers

Group	Specific compound	Absorption (cm^{-1})
C-H	Alkane	2960, 2850, 1350
C-H	Alkenes	3080-3020, 870-675
C-H	Aromatic	3100-3000, 870-675
C-H	Alkynes	3300
C=C	Alkenes	1680-1640
C=C	Aromatic	1600-1500
C-O	Alcohols, Ethers, Carboxylic Acids, Esters	1300-1080
C=O	Aldehydes, Ketones, Carboxylic Acids, Esters	1760-1690
O-H	Alcohol, Phenol (Monomer)	3640-3610
O-H	Alcohol, Phenol ('H' Bond)	3600-2000
O-H	Carboxylic Acid	3600-3000
N-H	Amine	3500-3310
C-N	Amine	1360-1180
NO_2	Nitro	1560-1515, 1386-1345

(Sastrohamidjojo, 1992)

The vibration that was used for identification is the vibration bends, particularly rocking, which is located in the area of wave number 2000-4000 cm^{-1} (functional groups). Therefore, the region 2000-400 cm^{-1} of each organic compound has a unique absorption, so that the area is often referred to as the

fingerprint region. The principle work of infrared spectroscopy is the ray of infrared will be passed through the sample and the wave will be transmitted by the sample and forwarded to the detector that was connected to the computer (spectrum). The chemical structure and forms of molecular bond with certain functional groups in the sample tested become the basic shape of the spectrum to be obtained from the measurement. Thus, the FTIR can be used for testing qualitatively and quantitatively. The chemists have set a thousand infrared spectra and determined the wavelength of absorption of each functional group (Chongjun et al., 2010). The vibration of a specific group at a particular wavenumber is shown in Table 4.1.

Generally, bovine gelatin and porcine gelatin have absorption peaks at almost identical wave numbers. However, if we look carefully at the curves, there are some differences between the bovine and porcine gelatins. In Figure 4.4, the spectrum showed that bovine gelatin has relatively higher peaks compared to the spectrum of porcine gelatin as shown in amide I and II regions (1656-1644 cm^{-1} and 1560-1335 cm^{-1}). The region between 3290-3280 cm^{-1} is related to the N-H bond stretching and intramolecular hydrogen bonds of the amine group in the amino acid chains. The absorption corresponded to the N-H bonds, showing the interactions of hydrogen bonds in the alpha-helical structures of the gelatin. The resulted peaks can be shifted to lower frequencies when the hydrogen bonds strength increases (Hafidz et al., 2011).

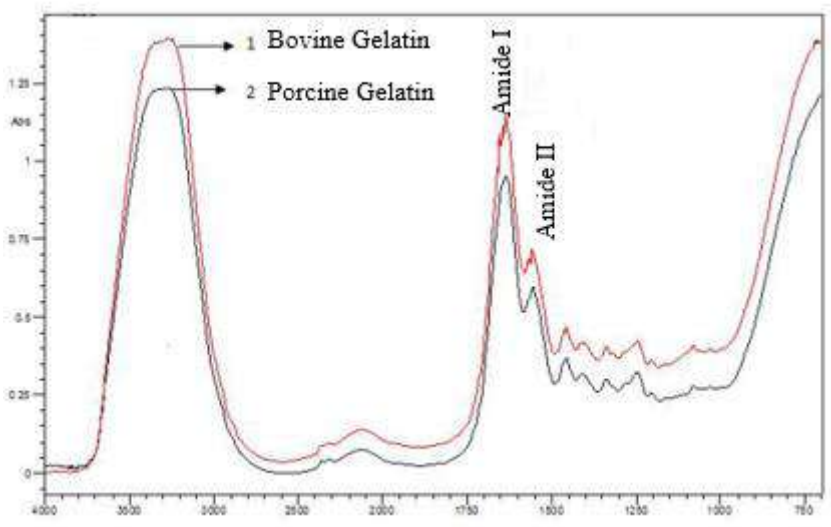


Figure 4.4: FTIR Spectra for (1) Bovine Gelatin and (2) Porcine Gelatin (Fathimah, 2010)

The stretching of the carbonyl (C=O) group, appeared in the area between 1660 to 1620 cm^{-1} which referred to the existence of amide I. Peaks in the range between 1660-1650 cm^{-1} represents the alpha helical structures and 1640-1620 cm^{-1} represents as the beta-sheet structure. The existence of amide II in depicted by peaks in region between 1550-1520 cm^{-1} which corresponded to the NH bound deformation in the structure of alpha helices (1550-1540 cm^{-1}) and the beta-sheet structures (1525-1520 cm^{-1}) (Fischer et al., 2005). The peaks at 1500-1200 cm^{-1} are a representation of the CH_2. This is related to the multiple hydrocarbon groups contained in macromolecular compounds such as fatty acids, proteins and polysaccharides (Figure 4.4) (Fatimah, 2013).

Chemometrics Measurement using Unscrambler

The function of the software is to assist in analyzing multivariate data and form a design of the experiment. One abilities of Unscrambler is to classify unknown samples systematically.

Classification aims to find new sample similar to the categorization of samples that have been used to create the model. If the new sample in accordance with a model that has been created, it can be seen that sample category (Citrasari, 2015). Chemometrics is multidisciplinary involving multivariate statistical mathematical modeling and information technology. Multivariate measurement is statistical measurement that used on the data that consists of many variables, and between the correlated variables. Some of the method included in the measurement group is the principal component measurement (Rohman et al., 2012)

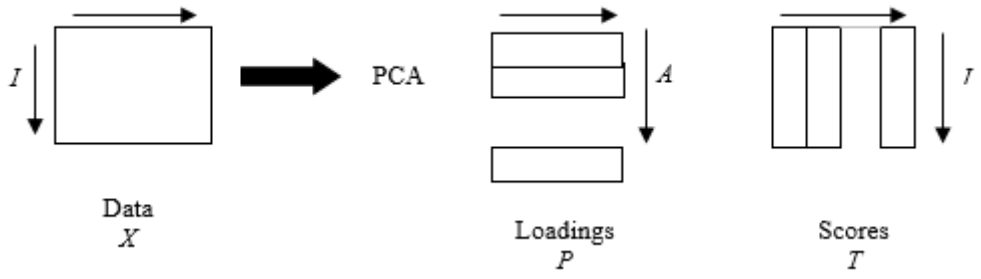


Figure 4.5: PCA Principle (Kautsar, 2012)

Principal component analysis (PCA) is a technique for building new variables that are linear combinations of the original variables. The maximum number of new variables will be equal to the number of old variable and each of them is not correlated. The Excess PCA which can eliminate the correlation, not reduce the number of original variables and more accurate than the use of

other methods (Kautsar, 2012). The working principle of PCA can be seen in Figure 4.5.

The principle of PCA is to find the main component that is a linear combination of the original variables. The selected the major components to become the first principal component with the greatest variance in the data group, while the second main component perpendicular to the first principal component and has the next largest variant. PCA technique works on the data matrix X (J × I) into two matrices T (I × A) and matrix P (J × A) that perpendicular to each other (Figure 4.5) (Kautsar, 2012) T matrix called the matrix score that describes the variation in the object, while the loading matrix describes the effect of loading on the main components. Loading matrix composed of the original data in the new coordinate system (Nurcahyo, 2015).

The error of the model forms expressed in E, while the value of A is the number of PCs that are used to create the model (Kautsar, 2012). In matrix representation, the model with a given number of components has the following equation:

$$\mathrm{X} = \mathrm{TP}^{\mathrm{T}} + \mathrm{E} \qquad (4.1)$$

Where *T* is the scores matrix, *P* the loadings matrix and *E* the error matrix, The combination of scores and loadings is the structured part of the data: the part that is most informative, what remains is called error or residual, and represents the fraction of variation that cannot be modeled well. Most of the chemometrics publication is using the same terminology to discuss the data of PCA. From the unscrambler, it can be concluded that 4 terminologies can be explained namely; Scores plots, loading plots, explained variation and influence (Kong et al., 2007).

1. Scores plot

Scores also called component scores in PCA; are the scores are the scores of each sample (row) on each factor (column). To compute the factor score for a given case for a given factor, one takes the case's standardized score on each variable, multiplies by the corresponding factor loading of the variable for the given factor, and sums these products. Scores are estimated in bilinear modeling methods where information carried by several variables is concentrated onto a few underlying variables. Each sample has a score along each model component. The scores show the locations of the samples along each model component, and can be used to detect sample patterns, groupings, similarities or differences (Kong et al., 2007).

2. Loading plot

The plot shows the importance of the different variables for the two components specified. Variable with loadings to the right in the loadings plot will be variables which actually have high values for samples to the right in the score plot. Variables close to each other in the loadings plot will have a high positive correlation if the two components explain a large portion of the variance of X. The same is true for variables in the same quadrant lying close to a straight line through the origin. Variables in diagonolly opposed quadrant will have a tendency to be negatively correlated.

In geometrical terms, a loading is the cosine of the angle between the variable and the current PC: the smaller the angle (i.e. the higher the link between variable and PC), the larger the loading. It also follows that loadings can range between −1 and +1. The

correlation r between two variables (vectors), x and y, is defined as,

$$r(x,y) = \frac{Cov\,(x,y)}{s_x\, s_y} \qquad (4.2)$$

Where, Cov is the covariance between x and y (Kong et al., 2007).

3. Explained Variances

The plot gives an indication of how much of the variation in the data is described by the different components. Total residual variants are computed as the sum of squares of the residuals for all the variables, divided by the number of degrees of freedom. Total explained variance is then computed as:

100*(Initial Variance – Residual Variance) / (Initial Variance)

It is the percentage of the original variance in the data that is taken into account by the model.

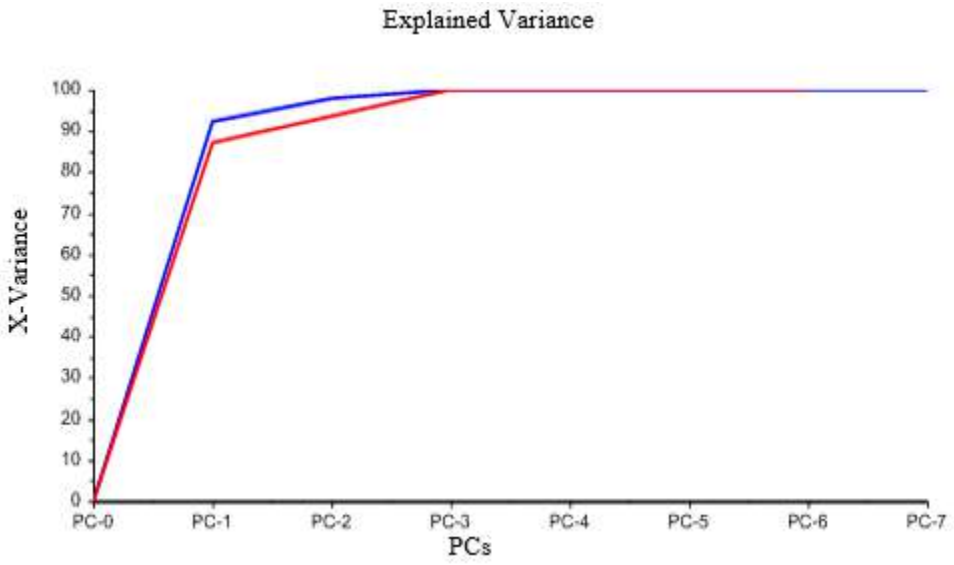


Figure 4.6: Total Explained Variance Curve (Kong et al., 2007)

Calibration variance is based on fitting the calibration data to the model. Validation variance is computed by testing the model on data that were not used to build the model. Based on the Figure 4.6, if they differ significantly, there is good reason to question whether either the calibration data or the test data are truly representative. The figure shows a sitution where the explained validation variance is much similar than the explained calibration variance. This means that the calibration data are well fitted and the models also describe the new data well. But if the explained variance are not close together the model does not represent the data (Kong et al., 2007).

4. Influence

The plot shows the Q-residual, X-variance or F-residual vs. Leverage or Hotelling's T^2. It is most useful for detecting outliers, influential samples and dangerous outliers. Samples with high residual variance, i.e. lying to the top of the plot, are likely outliers. Sample with high leverage, i.e. lying to the right of the plot, are influential. This means that they attract the model so that it describes them better. Influential samples are not necessarily dangerous, if they obey the same model as more "average" samples. A sample with both high residual and high leverage is dangerous outlier (Syahariza et al., 2005).

It is not well described by a model which correctly describes most samples, sand it distorts the model so as to be better described, which means that the model then focused on the difference between that particular sample and the others, instead

of describing more general features common to all samples (Hidaka and Liu, 2003).

The difference between Leverage and Hotelling's T^2 is only a scaling factor but the critical limit for Leverage is based on an ad-hocrule whereas the Hotelling's T^2 critical limit is based on assumption of a student-t distribution. Note that the F-residuals are available for both calibration and validation. If the residual x-variance from validation is much higher than for calibration one should investigate the residuals in more detail. The validated residuals reflect the scheme chosen in the validation and may give a more realistic view of the residuals than the Q-residuals which are only available for calibration (Kong et al., 2007).

Chapter 5

Parameters for Analytical Methods Validation

Method validation is the practical process of determining the suitability of a method for providing analytical data that is fit for the intended purpose. Method validation is defined in ISO/IEC 17025:2005 as:

'*Confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled*'

For any method to produce meaningful and reliable data, some performance checks should be made before the method is applied to real samples. The validation process typically entails firstly understanding the reason the measurement are being made and the performance of the method that is required to produce data that are fit for that intended purpose. Secondly experiments are planned and performed to evaluate the performance of the method. The observed performance is then compared with the required performance of the method, and relevant/specified criteria are used to determine whether the performance is adequate. The actual level of assessment and validation that is undertaken will depend on the intended use of the method and the importance of the data produced (Ali et al., 2012).

There are many performance characteristics that can potentially be investigated for a particular method, some of which are listed in Table 5.1, and described in more detail in the following sections. Various performance parameters are important

depending on the type of measurement being made and the reasons for making the measurements, so choosing the characteristics to be investigated is a crucial part of the validation process. Accuracy (comprising both precision and bias) may be important for calculating absolute values of properties or analytes, for example, whereas precision is more significant in comparative studies.

Table 5.1: Parameters and Approaches to Consider in Assessing qPCR Performance

Performance characteristic	qPCR performance	Experimental procedure
Dynamic range	Range of sample concentrations over which the assays remains linear	Dilution series from a known concentration DNA analyzed
Repeatability	Variability of result under closely controlled conditions	Same measurement on same sample repeated by same analyst
Reproducibility	Variability of result under differing conditions	Same sample measurement repeated by different measurement using different instruments, different laboratories or over time
Bias	Consistent over-or underestimation of the true result	Average measured value of a reference material compared to the assigned value
Specificity	Ability of the assay to detect the target but not other potential analytes present in the sample	Assay performed with a variety of related targets to check for false positive signals
Sensitivity (LoD / LoQ)	The lowest amount of the target that is detectable/reliably quantifiable	Assay performed with increasing dilutions of analyte to determine the limit of detection/ linearity

(ISO/IEC, 2005)

Random errors in the method are reflected in the precision of the results, whereas systematic errors (such as out-of-calibration instrumentation or consistently low recovery rates during sample preparation) give rise to method bias. Working range will be of some interest in most cases. For trace work, limits of detection (LoD) and quantification (LoQ) may be relevant, but for planning

calibration strategies it may be more useful to know the range over which the method response is linear (Watson et al., 1987).

Selectivity (Sensitivity)

The selectivity or specificity is the ability to measure only certain substances carefully and thoroughly with the other components that may be present in the sample matrix. Selectivity can often be expressed as the degree of bias. The selectivity of the method was determined by comparing the results of the measurement of samples containing contamination, the result of dust, similar compounds, other foreign compounds or carrier placebo with sample measurement results without the addition of these materials (Vessman et al., 2001). Selectivity and specificity are often used synonymously; specificity can be termed the ultimate in selectivity as if a process is specific it is by definition wholly selective. The selectivity of an analytical method may be affected by many factors, including the presence of impurities, degraded components and possible inhibitors or enhancers of the reaction, and physical parameters such as temperature, ionic strength or pH (Brown, 2005).

Poor selectivity of a reaction indicates that other substances can interfere with the measurement, and selectivity may change as assay conditions are altered. For example, a PCR at the optimized annealing temperature will produce a single, specific amplification product but, if the annealing temperature is changed and the reaction made less stringent, multiple non-specific products may be generated. In clinical diagnostics the selectivity of an assay is often reported, and is calculated as the percentage of true negative results obtained in testing a number of known negative samples (Sambrook et al., 1989).

Limit of Detection (Sensitivity)

It should be noted that the biological definition of sensitivity given here varies from the ISO chemical definition. Detection limit terminology is inconsistent and confusing, so it is important to try and follow specific sector guidelines where available. In most biological measurement, sensitivity is used to describe the lowest level of an analyte that can be measured. However, in fields outside of biological measurement the term 'sensitivity' has different definitions (ISO, 2005).

For example, in chemical measurement, sensitivity is usually defined as 'the change in the response of a measuring instrument divided by the corresponding change in the stimulus (ISO, 1993). In clinical applications sensitivity again has a slightly different meaning, and is often expressed as the percentage of tests that give the correct positive result in testing a number of known positives. Often two limits are defined for a quantitative assay, firstly the limit of detection (LoD), which is the lowest amount of a target which can be reliably detected and distinguished from zero results and background, signals with confidence. The second is the limit of quantification (LoQ), which is the lowest concentration of analyte that can be quantitatively measured with an acceptable level of uncertainty (Lovatt, 2002).

Practically there are several ways of determining the sensitivity of a method. In qualitative measurement the analyte is typically diluted serially until it can no longer be detected reliably using the method (usually once the percentage detection falls below a specific level, often 95 or 100%) (Drosten et al., 2001).

A variety of approaches is used in quantitative determinations, but most approaches use the result of repeated measurement of a negative sample or zero calibrator (a sample known not to contain the analyte of interest). The zero calibrator is analysed between 10 and 20 times, and the mean and standard deviation of the data obtained. Usually the limit of detecting, or analytical sensitivity, is set as the mean signal +2 standard deviations while the limit of quantification is set as the mean +10 standard deviation. In quantitative PCR measurement, for example, where negative result do not yield a meaningful value, the definition of LoD and LoQ is more difficult. One developed approach is to define the LoD as the input analyte level giving a (for example) 95% probability of a positive PCR result, calculated using probit regression measurement of dilution series data (Drosten et al., 2001). Sensitivity can be expressed in many ways depending on the assays, for example:

1. Number of cells per mass of matrix that is detectable;
2. Percentage of adulterant in a matrix that is detectable;
3. Mass of DNA required for reliable qualitative measurement (such as STR profiling);
4. Amount or copy number of gene, genome or DNA target that is detectable, per volume or mass (Madej, 1991).

Sensitivity should be assessed across the range of analyte levels and sample masses that may be routinely used, as the ratio of detectable target within a given sample cannot always be linearly extrapolated. For example if 100 microbial cells can be detected in 1 gram of soil by PCR, 10 cells may not be detected if only 0.1 g of soil is tested. Sensitivity is often determined an indication of the lower operating limits of the test method such as a replicated sequence of experiments on low level samples, blanks and low-level spiked materials or standards. Therefore, the

results of a measurement are often close to the lower operating limit to perform regular assessment of the LoD. This ensures that negative results are being interpreted and reported correctly with reference the detection or quantification limit of the method (Persing and Tenover, 2004).

Efficiency

In kinetic real time PCR each individual reaction position is excited and detected independently based on the fluorescence history. The former shapes of amplification curves differ in the steepness of any increasing fluorescence and in the absolute fluorescence levels at plateau depending on background fluorescence levels. The PCR efficiency has a major impact on the fluorescence history and the accuracy of the calculated expression result and is critically influenced by PCR reaction components. Efficiency evaluation is an essential marker in real-time gene quantification procedure (Liu and Saint, 2002a; 2002b; Rasmussen et al., 2001; Tichopad et al., 2003a; 2003b; 2004). The condition of amplification efficiency in all compared samples must be in constant state because it is one of crucial factors for reliable comparison between samples. The factors also become crucially important from analyzes the relationship between an unknown sample (DNA) and a standard sample, which is performed in all relative quantification models (Atlas et al., 2006).

The threshold method is most commonly used for the quantification of unknowns, and as illustrated in Figure 2.6 utilizes information from the points at which known DNA standards reach a specified fluorescence threshold to construct a standard curve of crossing threshold against target level. As described, the reaction efficiency is determined from the slope of the

standard curve (Figure 2.6). The threshold level should be set in the exponential phase of the amplification, and most instruments calculate an optimal level setting. Manually setting the level is possible, but is subjective and may also introduce variability between runs of the assay. Advantages are that the method is simple, and the quality of the assay may be monitored using the parameters of the standard curve. Disadvantages are that the dilution series used to construct the standard curve is prone to errors, and the assumption that the reaction efficiency is a constant in the exponential phase of the reaction is not always valid (Rasmussen et al., 2001).

The overcome the problems in using dilution series, alternative methods based on estimating the amplification efficiency from single reactions have been developed (Wittwer and Garling, 1991).

The rate of change of fluorescent signal within a single reaction may be monitored, ideally within the linear phase of signal increase, to determine the efficiency of each reaction. The second derivative maximum option in the Applied Biosystem StepOnePlus software similarly calculate the maximum rate of change of the signal in the reaction, and utilizes the peak to determine the fluorescence at the maxima, and hence the initial number of copies in the reaction (Wittwer and Garling, 1991). The PCR process generates anywhere between an average of 0 to 1 copy of each target in each reaction cycle, so that for any cycle the number of molecule is:

$$N_c = N_{C0} \times (E + 1)^c \qquad (5.1)$$

Where N_C is the number of molecules at cycle N, N_{C0} is the number of molecules at cycle 0, E is efficiency of the reaction and C is

the cycle number. Making assumptions that the efficiency of the amplification is constant in the early exponential stages of the reaction, and that all standards possess the same number target molecules at the point at which their signal crosses the determined threshold level, then the equation may be simplified to allow determination of the reaction efficiency (Birren et al., 1997).

$$\mathrm{E_s} = \left(\left(10^{(-1/-slope)}\right) - 1\right) \qquad (5.2)$$

The number of molecules at the threshold point, N_t, can also be determined from the standard curve (Muller et al., 2002).

$$\mathrm{N_t} = 10^{intercept} \qquad (5.3)$$

Chapter 6

Procedure of Detection of Gelatin

Fatty acids in biological sample material are traditionally analyzed with gas chromatography (GC) after being extracted and trans methylated to their fatty acid methyl ester (FAME) derivates (71, 96).

Samples and Reagents

Sodium hydroxide, hexane, methanol, boron trifluoride in methanol (20 % w/v) and chloroform. Butylated hydroxytoluene (BHT) and boron trichloride in methanol (14 %). FAME standards, the nonadecanoic acid methyl ester (C19:0) and De-ionized water. The several commercial fats obtained from a local market is Butter (Cow, Buffalo, Goat), margarine, Vegetable Oil (Palm Oil, Cocoa Oil), Oil (Lard, Buffalo, Goat and Cow). The fats will be designated as F1, F2, F3, F4, F5, F6, F7, F8, F9 and F10 respectively.

Fatty Acids Methyl Esters Preparation

The FAME preparation protocol has been published elsewhere. Briefly, 50 mg of sample are mixed with 2 ml BF3/CH3OH and 5 mg of C19:0 internal standard. The mixture is heated at 100 °C for 1 h and cooled down to room temperature. Aliquots of 1 ml of hexane and 2 ml of H2O are added, vortex-mixed for 15 seconds, placed in a centrifuge at 3000 rpm for 2 min and the FAME are then extracted from the upper hexane phase. Depending on the fat content the sample is either concentrated under nitrogen or diluted with hexane and subsequently subjected to GC-MS measurement.

Sample Preparation

The samples from the genomic DNA of cattle (*Bos taurus*) and the genomic DNA of pig (*Sus scrofa*) were tested using real-time and conventional PCR with the series of concentrations was 0.1; 0:01; 0001; 0.0001; 0.00001 ng / µl. The dilution of the initial concentration of DNA was done with distilled water.

Gelatin capsules from supplement products with *halal* logo from different countries were purchased and used in this study. The capsules were cleaned using distilled water, dried and weighted to 0.65 g and put the capsules in centrifuge tube for PCR measurement.

DNA Extraction and Isolation

The DNAs were extracted using Agilent porcine detection kit, Qiagen DNeasy mericon food, and Kogene powerprep DNA extraction kit from food and feed kit. The methods have been modified accordingly with additional reagents followed by decrease or increase in temperature from the original method.

Agilent Porcine Detection Kit

Proteinase K working solution (220 µl) was added to samples and incubated at 65 °C for 1 hr. Each supernatant (150 µl) was transferred into a fresh 1.5 mL tube and 500 µl of nucleic acid binding buffer was added to each of supernatants. The solutions were homogenized and transferred into a separate DNA binding spin cup and the samples were spun for 1 minute at 13,200 rpm. The spin cup was retained while the filters were discarded.

High Salt Wash Buffer (500 µl) was added and tube containing samples were spun at 10,000 rpm. Removed and retained the spin cups like before and added 500 µl of 80% ethanol. The samples were

spun at 10,000 rpm and remove and retain the filters. Repeated the alcohol stage for three times and repeated again the process without any additional to remove any contaminant for 3 minutes at 13,200 rpm. The spin cup was transferred to fresh 1.5 mL collection tubes. Added 75 µl of elution buffer (pre-warmed to 65 °C) and incubate at room temperature for 1 minute and spun the samples in a microcentrifuge at 13,200 rpm for 1 minute. Discarded the spin cups and cap the tubes. The sample extracts stored at -20 °C (Agilent, 2015).

Qiagen DNeasy Mericon Food

Food Lysis Buffer (1 mL) was added to 2.5 µl Proteinase K solution and incubated at 60 °C for 30 min and continued with centrifugation for 5 min at 2,500 x g. In the new 2 mL microcentrifuge tube, 500 µl of chloroform was added and the lid was closed and let to stand for a moment. Transferred the clear supernatant from previous step until the maximum volume and was continued with vortex, incubated and centrifuged with the same time and temperature like the first step.

The 700 µl of supernatant was transferred to the microcentrifuge tube containing the chloroform. The mixed solution was vortex vigorously for 15 second and centrifuge at 14,000 x g for 15 min. Pipette 1 ml Buffer PB into a fresh 2 ml microcentrifuge tube, and added 250 µl of the upper from the previous step and mixed thoroughly. Pipette 600 µl of the mixture into the Qiaquick spin column and placed in a 2 mL collection tube. Centrifuge at 17,900 x g for 1 min and discarded the flow through. Reused the collection tube for the next step and repeated the previous step with remaining sample and discarded flow through. Reused the collection tube and centrifuged again at 17,900 x g for 1 minute

to dry the membrane. The QIAquick spin column was transferred to a 1.5 ml or 2 ml microcentrifuge tube and pipet 100 µl buffer EB directly onto the Qiaquick membrane. The tube was Incubated for 1 minute at room temperature (15-25 °C) and then centrifuge at 17,900 x g for 1 minute to elute. Discarded the spin cups and cap the tubes. The sample extracts stored at -20 °C (Qiagen, 2010).

Kogene Powerprep DNA Extraction Kit from Food and Feed Kit Added 400 µl Lysis Buffer A and 40 µl Lysis Buffer B; 10 µl Proteinase K and 10 µl RNase A and mixed well by vortex Incubated at 65 °C for 1 hr. Added 400 µl Chloroform and close the lid and mixed well by vortex and centrifuged at 12,000 rpm for 15 min. Carefully transferred 200 µl the supernatant from the last step to a new 1.5 ml tube and added 200 µl binding buffer and 200 µl Iso-propanol and gently mix well. Transferred 600 µl mixed solution to DNA binding column tube. Close the lid and centrifuge at 12,000 rpm for 2 min and removed the flow-through. Carefully opened the DNA Binding Column and added 600 µl 75% ethanol without wetting the rim and centrifuge at 12,000 rpm for 1 min. Removed the flow – through from the tube and centrifuged at 12,000 rpm for 3 min to dry the membrane completely. Placed the DNA binding column in a clean 1.5 ml tube and discarded the collection tube containing the flow – through. Carefully opened the lid and applied 100 µl TE Buffer. Close the lid and centrifuged at 8,000 rpm for 3 min. The spin cup was removed and stored the tubes at -20 °C (Kogene, 2014).

Measurement of DNA Isolated using Biodrop

The DUO Biodrop instrument with touch screen system was used in this measurement. Elution buffer was measured as a blank sample before DNA sample. Measurement was performed at wavelength 260 nm

– 280 nm. The result obtained was in the form of DNA concentration data (ng / µl) and data of purity DNA (ratio A260/A280).

Polymerase Chain Reaction Measurement

PCR amplifications were conducted by mixing the reagent and diluted porcine and bovine DNAs to a total volume of 20 µl (Table 6.1).

Table 6.1: The Mixture of Reagent in PCR Measurement (Afifah, 2014)

No.	Component	Concentration (µM)	Volume (µl)
1	SYBR Green Master Mix	-	10.4
2	Primer Forward	10	0.4
3	Primer Reverse	10	0.4
4	Distilatted water	-	6.8
5	DNA template	-	2
Total Volume			20

The same sequences of primers as reported by Tanabe et al. (2007), which have been blasted in NCBI Blast (Appendix 2), were used (Table 6.2).

Table 6.2: The Sequences of Porcine and Bovine Primers Used

Primer		Sequence	Length
Bovine	Forward	5'-CCCGATTCTTCGCTTTCCAT-3'	120 bp
	Reverse	5'-CTACGTCTGAGGAAATTCCTGTTG-3'	
Porcine	Forward	5'-CTTGCAAATCCTAACAGGCCTG-3'	131 bp
	Reverse	5'-CGTTTGCATGTAGATAGCGAATAAC-3'	

Conventional PCR

Amplification was carried out at 95 °C for 7 minutes and was continued with denaturation stage at the same temperature for 30 seconds. Second stage was annealing stage, where primer designed would anneal the single-stranded DNA target. For porcine primer, the annealing stage was at 63 °C while the annealing stage for bovine primer was at 61 °C. The stage was repeated for 40 cycles. The third stage was elongation that was allowed to occur at 72 °C and was continued with the final elongation at the same temperature for 7 minutes. The PCR products were then analyzed by electrophoresis in 1% agarose gels in 1x Tris-Acetate-EDTA buffer followed by gel green staining and visualization under UV light transillumination. The 1 kb DNA ladder marker was used to estimate the size of all DNA fragments.

Real-Time PCR

Real-time PCR assay was performed using Sso advanced SYBR green (BioRad) following the instructions by Applied Biosystem Real Time System StepOnePlus. Amplification was carried out at 95 °C for 10 minutes and was continued at 95 °C for 10 seconds. Annealing and elongation stages were conducted simultaneously. Annealing stage for porcine primer was at 63 °C while annealing stage for bovine primer was at 61 °C for 45 seconds. A melting curve was recorded by holding the temperature was at 95 °C for 15 seconds then cooled to 60 °C for 30 seconds and heated at 95 °C for 15 seconds. The results were depicted in amplification plot and melt curve measurement.

FTIR Measurement

FTIR was used to determine the chemical bonds from the vibration of the compound at a particular wavelength. FTIR measurement of gelatin can be done in two ways (1) is done by comparing the spectral pattern of gelatin standard (Figure 4.4) with a spectrum pattern of gelatin capsule generated. (2) By measuring the absorbance values obtained from the peaks of the spectrum produced. The working principle of FTIR spectroscopy is the interaction of energy and matter. The measurement was made on the frequency 4000-650 cm^{-1}. The all value of absorbance in the FTIR spectrum would be selected for the cluster measurement using PCA.

Chemometrics Measurement

The data were analyzed with PCA techniques using the Unscrambler X 10.3 software. The main objective of PCA technique is to distinguish the amino acid composition of gelatin capsule in supplement product. The data were pre-processed before PCA was performed. The absorbance data from FTIR were pre-processed using *Savitsky-Golay* smoothing technique and continued with normalization by peak normalize before subjected to PCA.

Chapter 7

Validation of Conventional PCR

The purpose of a PCR-Polymerase Chain Reaction is to increase the number of copies of known fragments of DNA. In the non-limiting phase of the reaction the increase in the number of copies of the fragment is exponential. The improvement of the efficiency in PCR analysis, reaction conditions is needed to be optimized to avoid nonspecific amplification products. Nonspecific products can be detected as smears or faint bands in addition to the bands that are of interest. Non-specific bands can have a negative effect on electrophoresis analysis because they appear as extra peaks. PCR optimization was performed by modifying reaction conditions including the annealing temperature, concentration of magnesium chloride ($MgCl_2$), PCR reaction volume and the source of DNA polymerase. For this study, we focused on the annealing temperature.

The sequence and length of PCR primers generally determine the annealing temperature of the thermal cycling reaction for a specific assay. Although primers are usually supplied with theoretical melting temperatures, these can be calculated in different ways which may give widely varying values. The calculations have set the annealing temperature of bovine primer is 60 °C and porcine primer is 61 °C (Appendix 2). Initially the PCR conditions used by Rahmawati (2012) for the same primers were used. The results showed that the annealing temperature for bovine primer and porcine primer was 60 °C. However, the results obtained

by using that conditions were not satisfactory, therefore the PCR was validated further (detailed below). The designed primers were verified by NCBI blast analysis to ensure porcine and bovine specificity (Appendix 1).

Validation of Conventional PCR

The PCR targeted a sequence corresponding to a region of the CYT b. The obtained PCR products of 120 bp for bovine primer and 131 bp for porcine primer in length were separated on a 1.5% agarose gel stained with gel green staining. PCR products were detectable at a dilution of 10^{-5} ng/µl (Figure 4.1; 4.2).

1. Specificity

The specificity of the porcine and bovine specific primer PCR assay was assessed with 1 ng/µl of DNA standard from the fresh muscle tissues of 2 meat-producing terrestrial (cow and pig) animal species. Species specific primers used under the selected conditions amplified cow and pig genes with expected bands of below than 300 bp which is 120 bp for bovine and 131 bp for porcine, respectively (Appendix 2). But, different result was obtained where primers with genomic DNA from pig.

The gel electrophoresis results for the PCR amplified products revealed expected bands of 131 bp and 120 bp for porcine and bovine gelatin, respectively (Figure 4.1 and Figure 4.2). The specificity of the method was tested using DNA obtained from DNA standard of bovine and porcine origin. The specificity of PCR assay is demonstrated by its negative results for non-positive control DNA.

According to the mentioned figures, there isn't observed cross reaction of our interested primers with genomic DNA from cow that confirms the high specificity of the assay (Figure 4.1). Tanabe et al. (2007) with bovine primer showed that the sequence was able to detect bovine DNA specifically. However, among the lanes (11, 12) of porcine primer, two samples of negative control was detected target DNA (Figure 4.2).

A highly specific PCR would generate one and only one product of the correct size. However, it is not unusual to observe a series of bands, especially when a new target sequence and/or primers are utilized for the first time (Weissensteiner et al., 2004). Appearance of unspecific amplification products can be attributed to a number of factors. First, primers may be annealing to unspecific sites in template DNA.

In this case, one may be able to increase the specificity of PCR by changing reaction mixture that would make it more difficult for primers to anneal to unspecific sites in the sample. These include addition of glycerol, or form amide, reduced pH, or lowering concentrations of primers, dNTPs and $MgCl_2$. One may also try altering the annealing temperature and/or the duration of the annealing and extension steps. In general, higher temperature, and shorter annealing and extension periods confer higher specificity. Alternatively, the unspecific bands may have resulted from over amplification (Weissensteiner et al., 2004).

Furthermore, we changed the annealing temperature for porcine primer at 62 °C and 63 °C (previously 61 °C). The reaction mixture was still had the DNA template on negative control. If bands still seen after PCR, they are either contaminants or primer-dimers. It has been shown that species specific PCR technique described here is not suitable enough for authentication *halal* gelatin. The

results of this study showed that, specific porcine primer was not able to distinguish of target species which confirms less specificity of conventional PCR technique for gelatin adulterant identification.

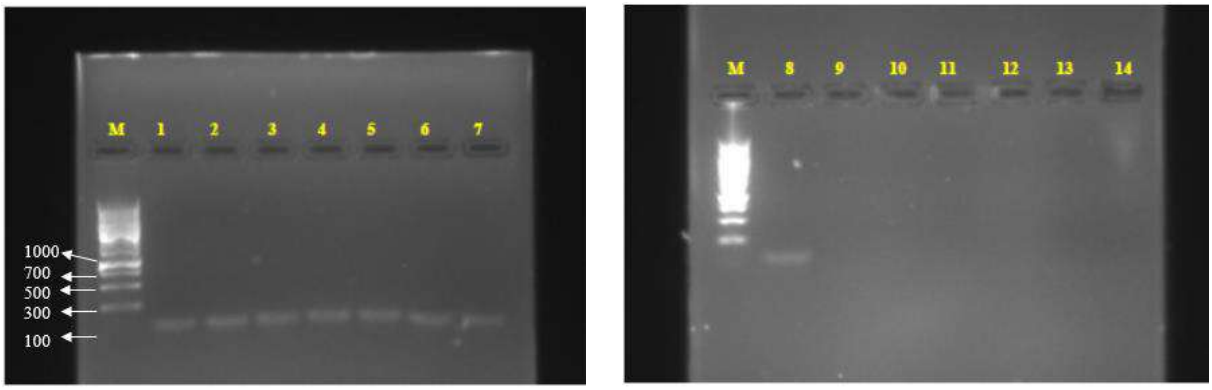


Figure 7.1: Visualization of Conventional PCR Product with Specific Bovine Primer

Gel analysis of the Conventional PCR products of 5-fold dilutions of porcine and bovine gDNA to determine sensitivity and specificity. Lane M, Amplisize 300-10,000 base pairs (bp) in 1 Kb increments. Lanes 1-2: 0.1 ng/µl: Lanes 3-4: 0.01 ng/µl; Lanes 5-6: 0.001 ng/µl; Lanes 7-8: 0.0001 ng/µl; Lanes 9-10: 0.00001 ng/µl; Lanes 11-13: negative control; Lanes 14: blank.

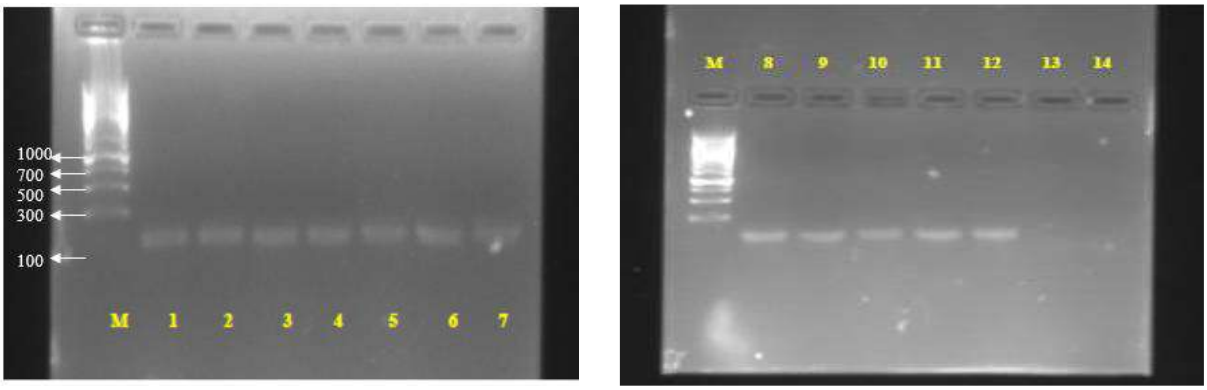


Figure 7.2: Visualization of Conventional PCR Product with Specific Porcine Primer

Gel analysis of the Conventional PCR products of 5-fold dilutions of porcine and bovine gDNA to determine sensitivity and specificity. Lane M, Amplisize 300-10,000 base pairs (bp) in 1 Kb increments. Lanes 1-2: 0.1 ng/µl: Lanes 3-4: 0.01 ng/µl; Lanes 5-6: 0.001 ng/µl; Lanes 7-8: 0.0001 ng/µl; Lanes 9-10: 0.00001 ng/µl; Lanes 11-13: negative control; Lanes 14: blank.

2. Sensitivity

The sensitivity of real-time PCR using CYT b was expressed by limit of detection (LoD). For determination of LoD, dilution series (10^{-1} to 10^{-5} ng/µl) are used. Porcine DNA could still be amplified up to 10^{-5} ng/µl, while at 10^{-5} ng/µl, bovine DNA was not amplified. These results was shown in Figure 4.1 and Figure 4.2, where it could be seen that samples of bovine DNA containing 10^{-5} ng/µl (Lanes 9-10) were all found to be negative (Figure 4.1), while at the 10^{-5} ng/µl of porcine DNA were all found to be positive (Figure 4.2). Thus, for any sample of gelatin designated as *halal*, if any cross-contamination of raw materials such as incorporation of some pig skins had occurred during manufacture, the test would be sufficiently sensitive to detect the small concentration of adulteration.

The detection limit obtained by the conventional PCR assay was similar to that obtained by other studies (Sahilah et al., 2015; Hui cai et al., 2012; Demirhan et al., 2012). Although in vitro sensitivity found is enough to detect very low amounts of DNA, the result obtained in detecting the CYT b gene from standard DNA (bovine and porcine) did not show adequate sensitivity to justify the adoption of this test as a tool for determination the *halal* status of gelatin capsule. Several factors are involved in the decrease of sensitivity of the conventional PCR. The first factor is the quantification of DNA target that have been amplified. We have to compare the sample with the marker qualitatively which is less accurate. The second is the specific species primer of porcine DNA that less sensitive to detect only the positive control. On the other hand, conventional PCR is not the suitable method for determination *halal* gelatin.

Validation of Real-Time PCR

The sso advanced SYBR green master mix could detect all DNA target except for sample blanko and negative control where real-time PCR assay could not detect the target (no amplification plot). Since real-time quantification is based on the relationship between initial template amount and obtained Ct (cycle threshold) value during amplification, a validated qPCR assay is essential for accurate quantification of samples. A powerful way to determine the validation of a qPCR assay is to run serial dilutions of a template and use the results to generate a standard curve. The amplification curve is built up by plotting the log of the starting quantity of template against the Ct value obtained during amplification of each dilution (Demirhan et al., 2012). The amplification plot shows the fluorescent signals (dRn) against the cycle number. In this experiment 5 dilutions were prepared with the dilution factor of 2 for selected primers (bovine primer and porcine primer). As it shown in Figures 4.3; 4.4., the highest chosen concentration was 0.1 ng/µl for this purpose. In perfect amplification cycle, spacing of fluorescence curves is determine by equation 2^n, where n is the number of cycles between curves at the fluorescence threshold (Mullis, 1990).

1. Precision (Repeatability)

ICH defines the precision of an analytical procedure as the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility (ISO, 2003).

- Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.
- Intermediate precision expresses variations within laboratories, such as different days, different analysts, different equipment, and so forth.
- Reproducibility expresses the precision between laboratories (collaborative studies usually applied to standardization of methodology) (ISO, 2003).

The ICH requires repeatability to be tested from at least six replications measured at 100 percent of the test target concentration or from at least nine replications covering the complete specified range.

Table 7.1: Mean Ct Values Obtained with the Real-Time PCR

Concentration (ng/µl)	Porcine Primer		Bovine Primer	
	Mean ± SD	% RSD	Mean ± SD	% RSD
10^{-1}	18.14 ± 0.79	3.99	19.84 ± 1.58	8.69
10^{-2}	21.92 ± 1.07	4.60	23.22 ± 2.13	9.70
10^{-3}	24.77 ± 1.31	4.90	26.79 ± 1.14	4.60
10^{-4}	28.11 ± 2.07	7.12	29.02 ± 0.37	1.31
10^{-5}	31.94 ± 0.66	1.90	34.46 ± 0.75	2.36

SD: standard deviation; RSD: relative standard deviation

The relative standard deviation (RSD) is often times more convenient. It is expressed in percent and is obtained by multiplying the standard deviation by 100 and dividing this product by the average. To confirm accuracy and repeatability of real-time PCR the inter-assay precision was determined in six repeats within one AppliedBiosystem run. Inter-assay variation was investigated in the same experimental runs performed on 3 days. Test repeatability was low in inter-test experiments (< 25%) (Table 7.1). The calculation of test precision and test variability is based on the Ct variation from the Ct mean value. Day had a statistically significant effect upon the Ct value of the sample. Repeatability conditions are conditions under which the independent results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time (ISO 24276, 2006). Moreover, it should not be necessary because the most important differences between the methods are the sequences of the primers and probes. The choice for a certain DNA isolation method is based on the matrix, not on the subsequent gelatin detection methods.

2. Efficiency and Limit of Detection

The most effective means to measure assay performance is via the construction of a standard curve from a serial dilution of template. Assay efficiency can be measured as a factor of the standard curve gradient. A wide range of sample concentrations is run, ensuring that these reach a limiting dilution, thus allowing determination of the technical assay dynamic range from the same experiment (Hofmann et al., 1999).

Any suitable template material is appropriate for these technical determinations of assay performance. Selection of a standard, transferable reference material allows for inter- and intra-laboratory validation. Therefore, this stage of validation can be carried out on linearized or nicked plasmid (super coiled DNA does not amplify efficiently and results in low reproducibility), cloned fragment or synthetic oligo. However, it must be recognized that validation on these targets is a measure of the assay function and does not accommodate variability introduced by the complexity of a biological sample (Li et al., 2007).

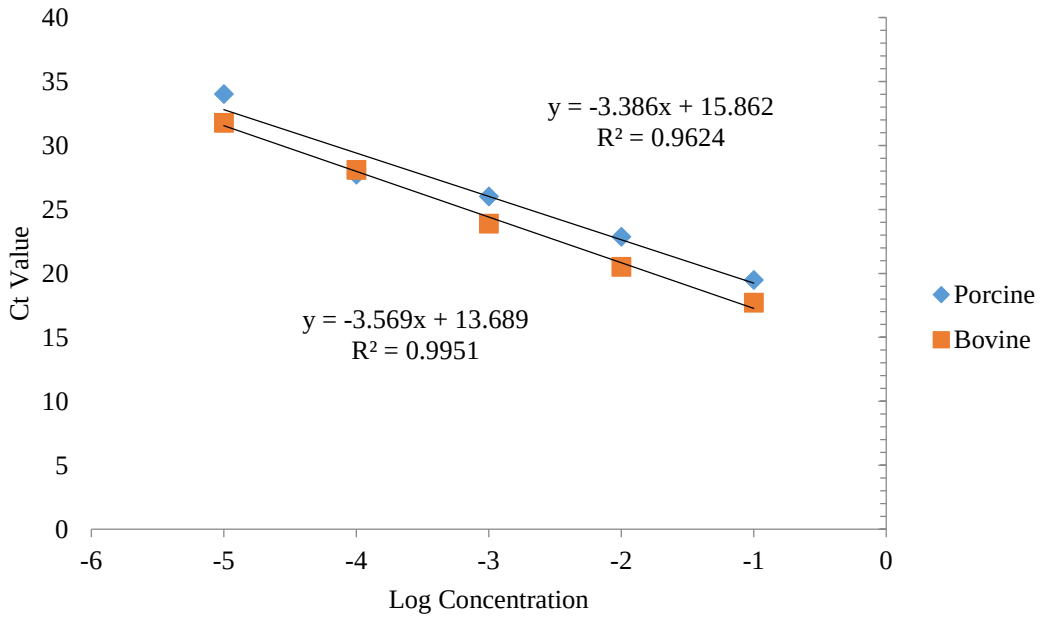


Figure 7.3: Standard Curves of 5-fold Dilutions of Porcine and Bovine gDNA

A standard curve was generated using a 5-fold dilution of a template amplified on the StepOnePlus real-time system. Standard curve with the Ct plotted against the log of the starting quantity of template for each dilution. The equation for the regression line and the r value are shown above the graph.

The determination of the technical dynamic range and efficiency of an assay from a standard curve was illustrated in Figure 4.3. In this study, the template has been diluted through a 5-fold series and demonstrated as 2 copies/day (Appendix 3), assay efficiency is determined by measurement of the gradient of a standard curve that is a plot of the log of the target concentration against the Ct (Figure 4.3). Efficiency can be calculated according to the equation: Efficiency = $10^{(-1/slope)} -1$.

The equation of linear regression line was used to evaluate qPCR assay validation. The coefficient of determination (R^2) of standard curve represents that whether the experimental data is fit the regression line. Linearity, gives a measure of variability across replicates and whether the amplification efficiency is the same for different starting template. Slopes between -3.1 and -3.6 result in efficiencies between 90% and 110% and are typically accepted, but it is important to strive for as close to 100% as the assay will permit (Higuchi, 1992). In this study, porcine primer represents R^2 value: 0.962; efficiency: 97.4%; slope: -3.389 (Figure: 7.3), bovine primer shows R^2 value: 0.995; efficiency: 90.6%, slope: -3.569 (Figure: 7.3).

The LOD corresponds to the smallest target DNA concentration for which six replicates give a positive result. The threshold sensitivity of this method in targeting the presence of porcine and bovine DNA and also quantification is given by the lower DNA concentration detected in the linear zone (at least 95% of 6 tested replicates), and corresponds to the LOD of porcine and bovine DNA of 10^{-5} ng/µl (Figure 7.4). As it shown in Figures 4.3 and Figure 4.4, the highest concentration has the lowest Ct value which means that amplification starts earlier compare to the lower concentration.

The standard curves generated by real-time PCR in bovine and porcine primer demonstrated excellent coefficients of correlation for the primers and fluorescent dye used. Furthermore, both standard curves were stackable indicating that the DNA analysis procedure for samples with a smallest concentration was satisfactory. Alternative approaches to standard curve efficiency calculation have been proposed. These methods report the efficiency of single reactions within the tube. These approaches rely on algorithms to model the amplification plot curves and so are dependent on the number of cycles over which there is an increase in fluorescence. While this type of approach potentially offers an ideal alternative to standard curves, the latter is still the more common method used for assay evaluation. This is because standard curves not only provide an estimation of efficiency, but also provide additional information about working dynamic range, sensitivity and reproducibility and are conceptually easier to apply (Ruijter et al., 2013).

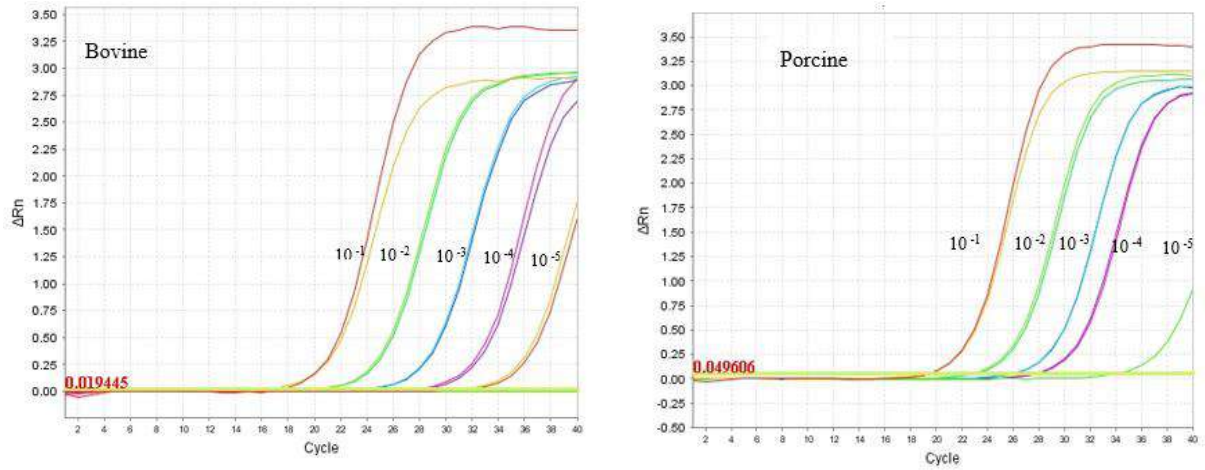


Figure 4.4: Amplification Plot of 5-fold Dilutions of Porcine and Bovine gDNA

Real-time PCR detection using applied biosystem. Fluorescence ratio (Cy5/fluorescein) vs time plot for the porcine and bovine premier-specific PCR amplification using adjacent SYBR green dye. Amplifications were performed from 10^{-1} ng/µl (curve 1), 10^{-2} ng/µl (curve 2), 10^{-3} ng/µl (curve 3), 10^{-4} ng/µl (curve 4), 10^{-5} ng/µl (curve 5). Each amplification cycle was 40 cycles were completed in 30 min.

3. Specificity

Specificity can be determined by the use of a melt curve analysis. Performing a melt curve requires incorporation of a reporter dye such as SYBR green or the use of a non-hydrolyzing probe such as a molecular beacon or scorpions probe. The amplicon was produced during qPCR, it is subjected to incubation at increasing temperatures, between 55 °C to 95 °C. However, the user should verify that the theoretical melt point of their amplicon falls within this range since this will be dependent upon the size and GC content. The experimental Tm will vary slightly between different runs and reagents, primarily due to variations in $MgCl_2$ and other ion concentrations.

The change in fluorescence is determined and plotted as rate of change of fluorescence vs. temperature. Since SYBR Green is a nonspecific dye that binds to any double-stranded DNA, it is important to verify that the qPCR produces only the desired product when using this detection chemistry. Melt, or dissociation, curve analysis can be used to determine the number and approximate size of the products. An assay with high specificity will result in a single melt peak at a high temperature in reactions containing only target with nothing, or very little, detected in the no-template controls (Figure 4.5; 4.6). If the melt curve has more than one major peak, as in Figures 4.5, the identities of the products can be further investigated by resolving them on a gel green-stained agarose gel (if the melt peak has the same temperature between specific and non-specific target). As shown in Figures 4.5, porcine primer reaction contains excessive amounts of primer-dimer or other nonspecific products. Lowering the primer concentrations will often reduce the amount of nonspecific products.

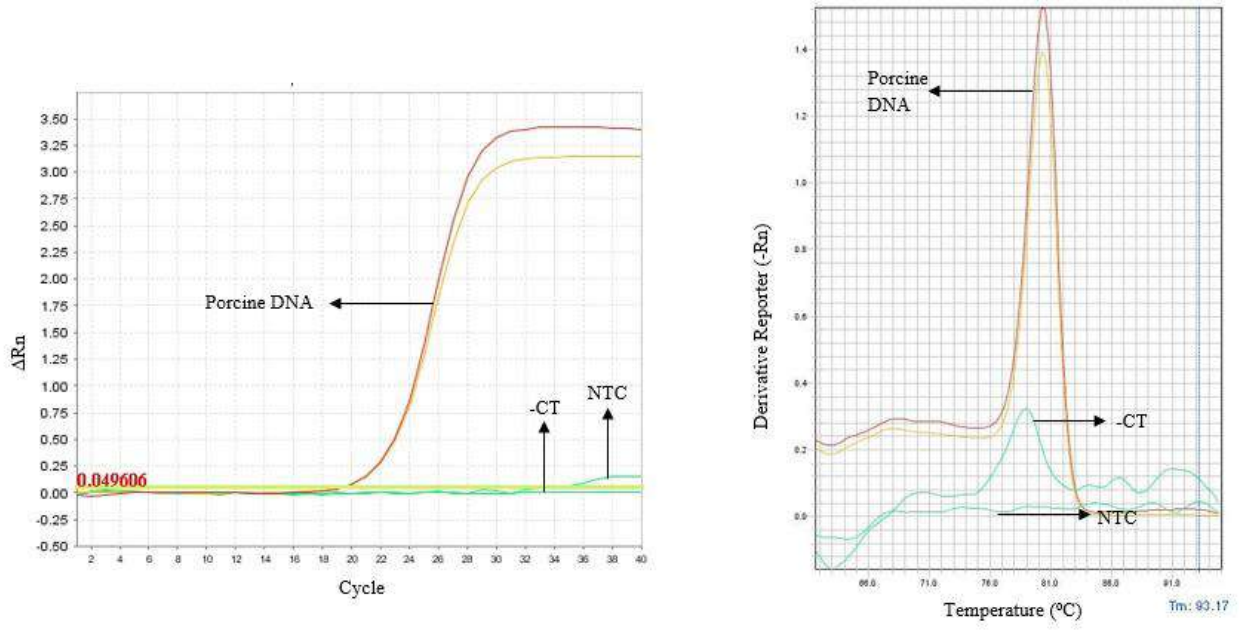
The melt curves for CYT b amplicons, generated with bovine and porcine primers was shown in Figure 7.5; 7.6. The melting temperature of the amplified products were; 80.3 °C for bovine DNA and 81.6 °C for porcine DNA. In Figure 7.5, a specific product is evident from the test reactions and a smaller product, melting at lower temperature, was present in the NTC. This is indicative of the formation of primer-dimers in the absence of template. This is commonplace and is only a concern when these primer dimerr products are evident in the test samples as shown in Figure 7.5.

The illustrated in Figure 7.5 and Figure 7.6 showed detection of the gDNA was distinguished by their melt profile with the gDNA product melting at a lower temperature compare to non-specific product that have a higher temperature (± 90 °C).

The specificity of primers and fluorescent dye have been demonstrated here with the real-time PCR assays carried out on genomic DNA samples belonging to the *S. scrofa* and *B. Taurus*. The melt curve analysis obtained the melting temperature that could different either specific or non-specific products. Specificity is critically important when designing assays for genotyping. These are often primer assays and require discrimination of a single base difference such as when differentiating between Single Nucleotide Polymorphisms (SNPs). In this case, it is critical to test each primer in a single reaction against a template that is known to contain the specific matched sequence and against the mismatched sequence (Raraswati et al., 2012).

Melting curve analysis can only be performed with real-time PCR detection technologies where the fluorophore remains associated with the amplicon. Amplifications that have used SYBR green dye can be subjected to melting curve analysis. Dual-labeled probe detection systems such as TaqMan probes are not compatible

because they produce an irreversible change in signal by cleaving and releasing the fluorophore into solution during the PCR. However, the increased specificity of this method makes this less of a concern. The level of fluorescence of both SYBR dyes significantly increases upon binding to double stranded DNA. By monitoring the dsDNA as it melts, a decrease in fluorescence will be seen as soon as the DNA becomes single-stranded and the dye dissociates from the DNA (Zhang et al., 2009).



The melt curves (right) for this analysis showing a double peak, which one is suggesting that only a primer-dimmer or mispriming product was generated with this set of primers.

Figure 7.5: The Amplification Plot and Melt Curve Analysis for Porcine

The main advantages of SYBR green assays over other real-time PCR detection formats are: 1) SYBR green is a low-cost fluorochrome; 2) SYBR green assays are simpler to use, especially in regard to primer design and optimization procedures; and 3) artifacts commonly observed in specific probes, particularly at

amplification cycles beyond the 30 cycle, are minimal and can be ruled out by melting curve analysis (Aldea et al., 2004). The assay described here is therefore ideal for establishing porcine adulteration in Muslim countries such as Malaysia.

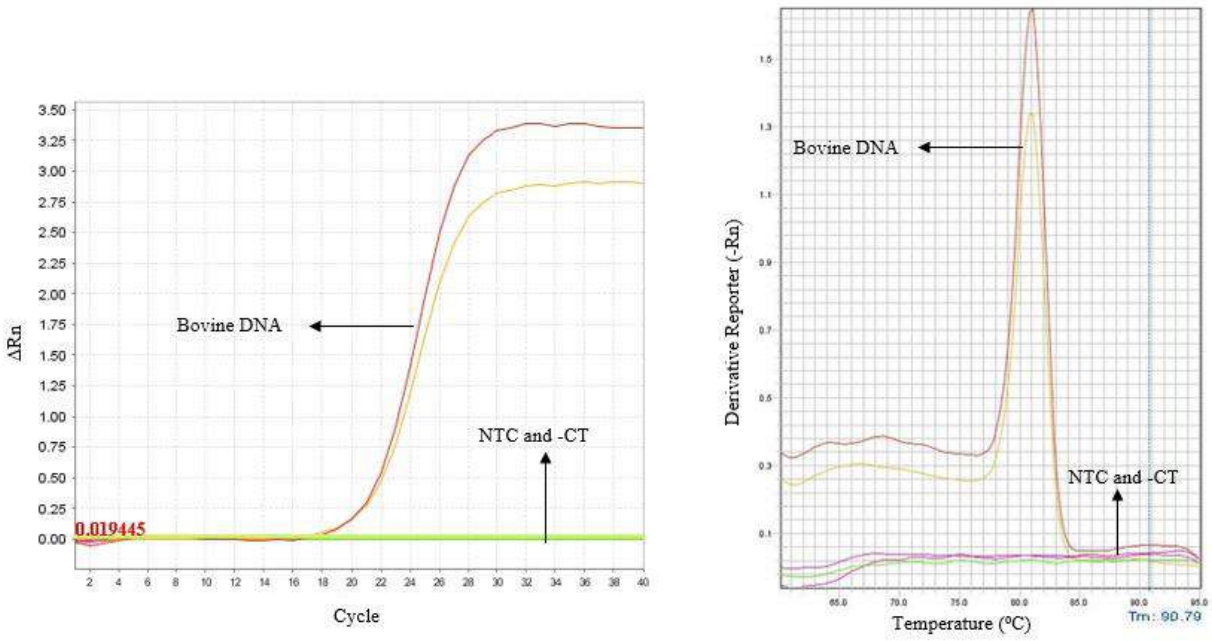


Figure 7.6: The Amplification Plot and Melt Curve Analysis for Bovine

Amplification plot (left) illustrating the increase in fluorescent reporter signal (*y*-axis, note the log scale) with each PCR cycle (*x*-axis). The *y*-axis units (ΔRn) actually reflect the reporter signal normalized to a passive reference dye in the reaction buffer. The curves seen with a no-template control (NTC), which lacks added DNA, show that the primers alone do not generate a signal and that the reagents used in this assay showed no DNA contamination. Dissociation curve (right) for this analysis showing a single, sharp peak, suggesting that only a specific PCR product was generated with this set of primers.

The benefits of the real-time PCR assays in adulteration issue have also been reported earlier. However, the evaluation of real-time PCR in diagnosis of gelatin capsule is limited. In the present study, a real-time PCR assay using SYBR green methodology has been evaluated for the specific identification of porcine and bovine DNA from gelatin capsule samples for *halal* authentication. Real-time PCR assay provided sensitivity and specificity. These results when compared to those of conventional PCR assay showed high

concordance, which shows that molecular tests are well suited for *halal* authentication of adulteration issue. Real-time PCR assay also provided correct diagnosis for primer-dimer and mispriming cases of melt curve analysis, which were missed by conventional PCR; although sensitivity was similar in either case.

The reasons for better sensitivity of real time PCR assay is that the fluorescent dyes used in the system are much more sensitive and can detect as little as a 2-fold change in DNA load, while agarose gel based system of conventional PCR stained with gel green dye can detect only 10-fold differences in DNA load (Parashar et al., 2006). It is thus very much possible that samples having less quantity of gelatin DNA may get detected by real-time PCR assay but could be missed by conventional PCR assay.

Real-time PCR has distinct advantage of being a non-gel based technique having automated data interpretation, which saves considerable time and labor. However, a major limitation of the real-time PCR is the initial capital investment for equipment, as well as the investment required for staff training and expertise, which may be beyond the means of many laboratories. Besides, there could be technical limitations when using non-specific dyes, such as, SYBR green. The sensitivity of detection may be compromised as SYBR green dye is a non-specific dye and can incorporate into any double stranded structures either it is primer dimmers or secondary structures. This factor could register a fluorescent signal (Valasek and Repa, 2005) and may lead to false positivity. To overcome the limitations, other real-time PCR with SYBR green method offer additional level of specificity due to the melt curve analysis. These assays on this study have proven to detect specific and unspecific product.

The qPCR assays (latter section) were further evaluated for species determination and quantization from gelatin capsules. Significantly less DNA was detected in gelatin capsules, possibly due to the further degradation of DNA during gelatin capsule manufacture (Table 7.3).For confirmation result, we conducted FTIR technique and chemometrics using software Unscrambler X.

Chapter 8

DNA Isolation

The three most commonly used DNA extraction methods for food include: 1) a surfactant based method known as CTAB: 2) silica based method: and 3) a commercially available food extraction kits. The use of a commercially available kit involves silica-spin column method. Agilent, Qiagen and Kogene are the extraction kits that using silica-spin column method to remain DNA bound. Unfortunately, Qiagen and Kogene were not successfully producing consistent result for gelatin samples. One of the limitations of this extraction method is the numerous buffer and reagents were used by the method (Table 4.2).

The samples of gelatin all methods from isolation kits were digested with protolytic enzyme such as proteinase-K, since eukaryotic DNA is not free but it is assembled in to chromatin in association with basic protein histones (Watson, 1977). The key step in purification of nucleic acid is the removal of most protein. For DNA purification, Qiagen and Kogene kits used chloroform for extraction followed by buffer (Qiagen) and isopropanol (Kogene) precipitation.

The initial comparison showed that although chloroform extraction from the commercial Kogene and Qiagen were efficient at isolating DNA from meat sample, the kits were not efficient with gelatin samples (Kesmen et al., 2012). Unlike Agilent kits, non-addition of chloroform in remove contaminant step was sufficient to overcome the DNA from gelatin. The organic chloroform method relies on organic and water-soluble phase separation and DNA is

extracted within the aqueous phase during the phase separation step (Collen et al., 2011).

The mode of DNA isolation used by the Agilent, Kogene and Qiagen kit is most likely based on the charge differences between DNA molecules and the silica used for the DNA binding step where positively charged silica spin column use the negative charge of DNA molecules for the binding (Holme and Hazel, 1998). However, the presence of DNA in gelatin capsule is lower which creates a problem with binding of DNA using these DNA extraction techniques with chloroform. The use of chloroform might have removed not only protein or contaminants but also the DNA itself, thus resulting non-detected DNA in sample.

Table 8.1: The Chemical Reagents Used in Agilent, Qiagen and Kogene Porcine Detection Kits

Stage	Agilent	Qiagen	Kogene
	Reagent		
Lysis	Proteinase K Proteinase K Digestion Buffer	Food Lysis Buffer Proteinase K	Lysis Buffer A Lysis Buffer B Proteinase K
Remove contaminant	-	Chloroform	Chloroform
Binding	Nucleic Acid Binding Buffer	Buffer Pb	Binding Buffer Iso-propanol
Wash	High Salt Wash Buffer Ethanol 80%	Buffer AW2	Ethanol 75%
Eluted	Buffer EB	Buffer EB	Buffer TE

The quantity and purity of the DNA extracts from 0.6 g minced hard/soft-gelatin capsule shells (from supplement-containing capsules) were examined by UV biodrop at 260 nm and 280 nm. As shown in Table 8.2, the DNA concentration ranged from 1.9 to 6.14

ng/µl. The ratio of optical density readings at 260 nm to that at 280 nm which represented the purity of samples, ranged from 0.9 to 2 ng/µl. Despite the fact that Agilent method has been shown to successfully extract DNA from gelatin capsule it were unsuccessful at purifying DNA from protein in this book (lower than 1.8-2.0). The reason behind the failure to extract DNA of sufficient purity, possibly the process of gelatin that already degraded the DNA structure caused the experiments to fail at extraction of high purity DNA (Table 8.2).

Table 8.2: DNA Concentration of Agilent Detection Porcine Kit

Sample	Concentration (ng/µl)	Purity (A260/A280)
	Mean ± SD	Mean ± SD
S1	4.85 ± 0.21	1.60 ± 0.05
S2	3.23 ± 0.34	1.43 ± 0.10
S3	2.95 ± 0.05	1.48 ± 0.05
S4	3.90 ± 0.02	1.29 ± 0.05
S5	5.08 ± 0.06	2.21 ± 0.19
S6	6.14 ± 0.82	1.46 ± 0.06
S7	4.61 ± 1.07	2.19 ± 0.11
S8	4.41 ± 0.91	1.42 ± 0.04
S9	2.50 ± 0.17	0.90 ± 0.34
S10	3.23 ± 0.01	0.66 ± 0.34
S11	5.01 ± 0.14	1.69 ± 0.06
S12	4.72 ± 0.16	1.75 ± 0.05
S13	4.43 ± 0.01	1.94 ± 0.11
S14	4.67 ± 0.01	1.71 ± 0.04
S15	3.05 ± 0.41	1.50 ± 0.06
S16	2.16 ± 0.18	1.64 ± 0.06
S17	1.94 ± 0.07	1.73 ± 0.05
S18	3.78 ± 0.54	1.50 ± 0.04
S19	4.82 ± 0.33	1.32 ± 0.02
S20	4.30 ± 1.02	1.39 ± 0.08

The most important factor among PCR amplification is the DNA extraction from samples. It has to yield an accurate DNA that does not include inhibitory substance and is purified from cell structure for successful PCR test. DNA based methods are useful for taxonomy at the level of genus species and subspecies identification. Uses of such method often require careful attention to prepare pure DNA in adequate (Sharbatkhori et al., 2009).

Gelatin is one of the most difficult samples to extract PCR-ready DNA from, where the significant amount of non-target DNA present is not the only issue encountered. Gelatins are the highly processed food that contains 18 different amino acids. Thus, effective DNA extraction methods for gelatin that can extract the small quantity of sample DNA are highly desirable. Moreover, the ideal method would also be suitable for automation, eliminating human error as well as potential cross contamination associated with sample processing (Karim and Bhat, 2007).

DNA extraction from gelatin capsule in the present report had threefold objectives; lysis of representative protein within the sample, obtaining high molecular weight intact DNA and removal of inhibitors from the extracted DNA for subsequent molecular manipulations (Ausubel et al., 2003). As mentioned previously, Agilent porcine detection kit is most efficient to extract the DNA from gelatin capsule. The total amount of DNA using this kit was far higher than those seen using Qiagen and Kogene (-value). However, the Agilent detection porcine kit based DNA extraction technique unsuccessfully remove protein across the whole sample range, even when proteinase K and silica spin column was used for the DNA binding step.

Real-Time PCR Measurement

The qPCR assays were further evaluated for species determination and quantization from gelatin capsules. The extracted DNA of isolated gelatin from 20 supplements was tested by three replicates of each sample and in addition to a negative control was tested in each run. 4 gelatin samples were positive for bovine primer, 2 samples were positive for porcine primer, and 6 samples were positive for both primers (Figure 8.1).

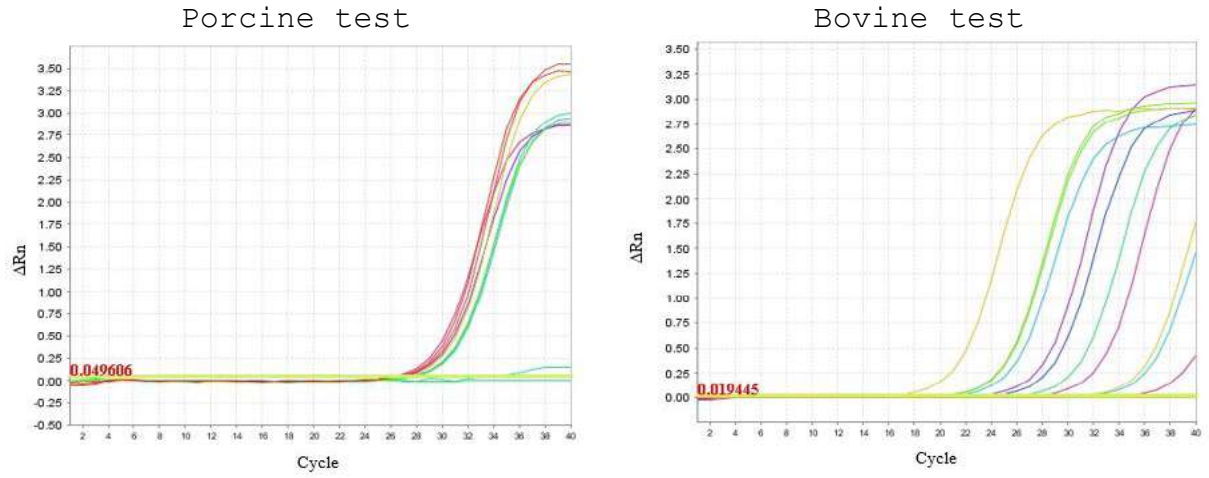


Figure 8.1: Amplification Plot of Capsule Gelatin in Supplement

SYBR green real-time PCR amplification plot derived from DNA of porcine primer and bovine primer. The sample that was amplified by porcine primer (left) is S1, S4, S5, S6, S9, S11, S12, S18. The sample was amplified by bovine primer (right) is S1, S2, S4, S9, S11, S12, S13, S14, S17, S18.

The amounts of DNA in the samples extracted using Agilent detection porcine kit was detected when PCR was performed of the DNA. The fact that the porcine and bovine CYT b mtDNA product was successfully amplified using the isolated DNA suggests an insufficient amount of the gelatin DNA template no being the cause of PCR failure. The fact that less total DNA was detected in samples extracted and had significant increase in DNA content was detected, amplification of the product occurred when SYBR green

was used (Table 8.3). In contrast of higher amount was not present when binding of DNA was performed in the presence of amplification plot in PCR assay.

PCR assays developed in this book were highly reliable due to positive result obtained from gelatin samples of supplement product (Figure 8.1 and Table 8.3). However, most of the gelatin samples did not amplify PCR assays. The figure showed higher PCR amplification when freshly extracted DNA was used. However, the lower successful PCR amplification obtained when stored DNA was used for PCR due to degradation of DNA and presence of reagents that inhibit PCR amplification. The entire DNA extracted from hair samples were degraded, therefore failed to PCR amplification. On the other hand, DNA extract from gelatin capsule did not amplify PCR program due to poor DNA quality and also quantity.

The standard curve of threshold cycle (Ct) approach generated from 10^{-1} to 10^{-5} ng/µl of porcine and bovine DNA was used for determining unknown amounts of porcine and bovine target DNA in gelatin capsules. The Equation is $\mathrm{Ct} = 13.689 - 3.569\mathrm{C}$ for bovine DNA and $\mathrm{Ct} = 15.862 - 3.386\mathrm{C}$ for porcine DNA, where Ct is the cycle threshold values obtained from real-time PCR and C is the concentration values to be determined. The concentration of porcine and bovine DNA in the specimens collected from retail markets were seen in Table 8.3.

Table 8.3: Result of Real-Time PCR for Gelatin Capsules

Sample	Concentration (ng/µl)	Ct Value		Remarks
		Porcine	Bovine	
	Mean ± SD	Mean ± SD	Mean ± SD	
S1	4.85 ± 0.21	26.97 ± 0.38	20.54 ± 1.28	Mixture
S2	3.23 ± 0.34	-	26.20 ± 0.46	Bovine
S3	2.95 ± 0.05	-	-	-
S4	3.90 ± 0.02	26.41 ± 0.83	23.07 ± 0.76	Mixture
S5	5.08 ± 0.06	27.20 ± 0.74	-	Porcine
S6	6.14 ± 0.82	26.57 ± 0.55	-	Porcine
S7	4.61 ± 1.07	-	-	-
S8	4.41 ± 0.91	-	-	-
S9	2.50 ± 0.17	27.69 ± 0.81	20.52 ± 1.02	Mixture
S10	3.23 ± 0.01	-	-	-
S11	5.01 ± 0.14	27.73 ± 0.53	21.34 ± 0.68	Mixture
S12	4.72 ± 0.16	26.89 ± 0.47	16.67 ± 0.53	Mixture
S13	4.43 ± 0.01	-	24.23 ± 0.53	Bovine
S14	4.67 ± 0.01	-	31.49 ± 0.75	Bovine
S15	3.05 ± 0.41	-	-	-
S16	2.16 ± 0.18	-	-	-
S17	1.94 ± 0.07	-	31.38 ± 1.35	Bovine
S18	3.78 ± 0.54	27.8 ± 0.28	27.81 ± 0.29	Mixture
S19	4.82 ± 0.33	-	-	-
S20	4.30 ± 1.02	-	-	-
-CT	1.00	-	-	-

The results of a small survey of gelatin products were shown in Table 8.3. Of 12 retail products purchased in Nilai area, 6 samples were found to contain porcine-bovine gelatin, 2 samples were found to contain only porcine gelatin, with the remaining 4 samples being bovine gelatin. The 12 positive samples had lower concentration values (Table 8.4).

Table 8.4: Concentration of Porcine and Bovine DNA in Gelatin Capsules

Sample	Concentration (ng/µl)	
	Porcine	Bovine
	Mean ± SD	Mean ± SD
S1	$5.24 \times 10^{-4} \pm 0.38$	$1.20 \times 10^{-2} \pm 1.28$
S2	-	$3.12 \times 10^{-4} \pm 0.46$
S4	$7.67 \times 10^{-4} \pm 0.83$	$2.35 \times 10^{-3} \pm 0.76$
S5	$4.48 \times 10^{-4} \pm 0.74$	-
S6	$6.68 \times 10^{-4} \pm 0.55$	-
S9	$3.21 \times 10^{-4} \pm 0.81$	$1.21 \times 10^{-2} \pm 1.02$
S11	$3.13 \times 10^{-4} \pm 0.53$	$7.18 \times 10^{-3} \pm 0.68$
S12	$5.53 \times 10^{-4} \pm 0.47$	$1.46 \times 10^{-1} \pm 0.53$
S13	-	$1.11 \times 10^{-3} \pm 0.53$
S14	-	$1.03 \times 10^{-5} \pm 0.75$
S17	-	$1.10 \times 10^{-5} \pm 1.35$
S18	$2.98 \times 10^{-4} \pm 0.28$	$1.11 \times 10^{-4} \pm 0.29$

The mitochondrial gene encoding of the CYT b was chosen as a target for porcine and bovine DNA quantification. As shown in Figure 8.1, different concentrations of gelatin capsules were detected based on the threshold cycle (Ct) of each sample which is the cycle number where the samples fluorescent curve jumps sharply upward and corresponds to the initial concentration of DNA. In general, the higher the Ct is, the lower the initial concentration of DNA will be. The technique was robust enough to detect up to 10^{-5} ng/µl of the pig and bovine tissues in experimentally made gelatin capsule (Figure 8.1). Whilst this survey was very limited in scope, the clear discrimination between positive and negative samples of differing compositions shows its robustness. The detection of a gelatin capsule in supplement product, which was not *halal*, clearly shows the need for further surveillance of retail gelatin-containing foods and possible regulatory action by the authorities.

FTIR and Chemometrics Measurement

The importance of IR spectroscopy for the qualitative measurement comes from much information contents obtained and the possibility to assign certain absorption bands related to the functional groups. In gelatin, most of the peaks and shoulders of the spectrum are attributable to specific functional groups (Bendini et al., 2007). Figure 8.2 showed FTIR spectra of gelatin capsule from previous study (Chapter 7). The spectra look very similar and show a typical absorption band of gelatin structure (Figure 2.8). Four regions involves are 3600-2300 cm^{-1} (Amide A), 1656-1644 cm^{-1} (Amide I), 1560-1335 cm^{-1} (Amide II) and 1240-670 cm^{-1} (Amide III). A typical gelatin capsule spectrum showed low intensities of Amides A, I, II and III bands, with the Amide III band almost non-existent of a few samples (Figure 8.2). This is consistent with changes

expected because of denaturation of collagen to gelatin. A very low intensity showed for Amide III region is associated with loss of triple helix state during high temperature gelatin extraction (Muyonga et al., 2004b).

The Amide A (3600-2300 cm^{-1}) region is donated by N–H bond-stretching mode of hydrogen bonded amide groups. The absorption is polarised parallel to N–H bond, which is parallel to the helix axis in a-helical structures and perpendicular to the polypeptide chain in b-sheets. The band might be shifted to lower frequency when the hydrogen bonding strength increases (Krimm and Bandekar, 1986). The carbonyl C=O double bond-stretching mode, with contributions from in-phase bending of the N–H bond and stretching of the C–N bond, occurs in frequency range 1660-1620 cm^{-1} region which is often referred to as Amide I band. The frequency range 1660–1650 cm^{-1} was known as a-helical and 1640-1620 cm^{-1} as b-sheets structures. The frequency range of 1550-1520 cm^{-1} is due to Amide II with a-helical structure between 1550-1540 cm^{-1} and b-sheets at 1525-1520 cm^{-1}. The Amide II vibration is caused by deformation of the N–H bonds. Fischer et al. (2005) and Lagant et al. (1983) attributed 1500-1200 cm^{-1} to CH_2 deformation. It is known that this region contains vibrations corresponding to groups present in fatty acids, proteins, polysaccharides and phosphate derivatives.

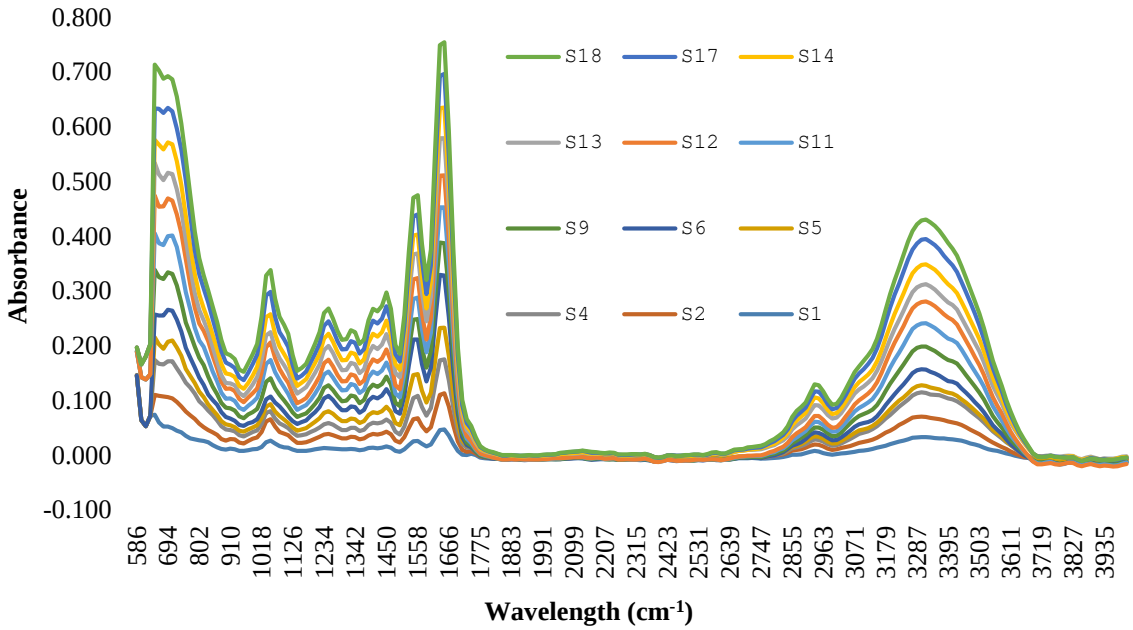


Figure 8.2: FTIR Spectra of Gelatin Capsules

The figure showed the enlarged FTIR spectra at fingerprint regions. The different peaks in terms of peak intensity were used for selecting the spectral regions for the quantification and classification of gelatins capsule in supplement product.

Spectral region (frequency) selection is the major problem in FTIR measurement because the chosen frequency regions must be chosen in such a way that the ones describe the most characteristics analytes to be determined and to provide non-interfered data for the analytes. The FTIR spectra showed that the maximum absorption in gelatin capsules is at Amide A and Amide I region. This can be easily used to differentiate these compounds and identify the chemical forms of the gelatin ingredients porcine and bovine in supplements. Gelatins with porcine and bovine were classified using chemometrics of Principal Component Analysis (PCA). The wave number regions for PCA were also optimized based on its capability to separate between pig and aduleration present in gelatin capsules. The optimal wave numbers used for quantitative measurement (Amide A, I and II), was chosen for PCA (Table 8.5).

Table 8.5: The Absorbance Value of FTIR Spectroscopy in Selected Regions

Sample	Wavelength (cm^{-1})							
	694	1250	1342	1404	1450	1558	1636	3271
S1	0.053	0.013	0.011	0.014	0.016	0.026	0.046	0.032
S2	0.054	0.026	0.022	0.025	0.027	0.041	0.064	0.037
S4	0.066	0.020	0.017	0.021	0.022	0.041	0.064	0.040
S5	0.035	0.022	0.016	0.019	0.023	0.040	0.059	0.013
S6	0.059	0.028	0.022	0.028	0.032	0.063	0.097	0.028
S9	0.068	0.019	0.020	0.022	0.023	0.037	0.059	0.040
S11	0.066	0.025	0.020	0.024	0.026	0.040	0.064	0.041
S12	0.069	0.022	0.017	0.022	0.024	0.035	0.058	0.038
S13	0.047	0.026	0.022	0.024	0.028	0.045	0.068	0.029
S14	0.055	0.022	0.019	0.022	0.025	0.035	0.055	0.035
S17	0.063	0.023	0.020	0.023	0.026	0.037	0.059	0.043
S18	0.058	0.023	0.019	0.022	0.025	0.035	0.056	0.034

Many times highlights from spectra are made by direct observations, but statistical comparison of spectra can also be helpful. There are a number of multivariate tools to allow mathematical and statistical comparison of spectra of the same material under contrasting conditions. One of the limitations of FTIR in making quantitative conclusions is overlapping band components and difficulties of band assignments from various complex amino acids such as *proline*, *hydroxyproline* and *glysine*.

One way of resolving this problem and analyzing the spectra is the use of principal component analysis (PCA). PCA is a multivariate technique in which a number of related variables, several spectral data points are transformed into a smaller number

of dimensions (Jackson, 2003). PCA is a chemometric and factor based technique. The factors in this present case would be the relevant wavenumbers (Table 4.6).

Infrared spectroscopy is employed to study structural properties of polymeric compounds such as gelatin capsules. In many cases however, determining the wavelength and absorbance of individual bands becomes difficult due to the presence of other interfering bands. Such problems can be overcome by differentiating IR spectra. The most frequently used preprocessing tools for multivariate measurement or calibration is the use of smoothing and normalization data. There are several approaches to mathematically differentiating a spectrum. The most popular and common calculation of the smoothing is via the *Savitzky-Golay* method. The method proposed and described by Savitzky and Golay (1964) calculates up to the ninth derivative and adjusts a convoluting function to give a desired derivative order and degree of smoothing. These different derivatives of all the spectra lay a foundation, or hold some significance, to be used as a reference and guide for input of data into statistical programs such as PCA. The normalization of the data to the area under the entire spectrum was applied due to the nonexistence of a most intense, yet static spectral band during the course of treatment as well as varying concentrations of gelatin capsule. Normalization is a common pre-processing step and required for adequate PCA results. This treatment is employed because it deletes differences between spectra due to different amounts of sample and path length variation (Mariey et al., 2001; Kher et al., 2007). Normalizing spectra does not substantially affect the relative intensities within a spectrum.

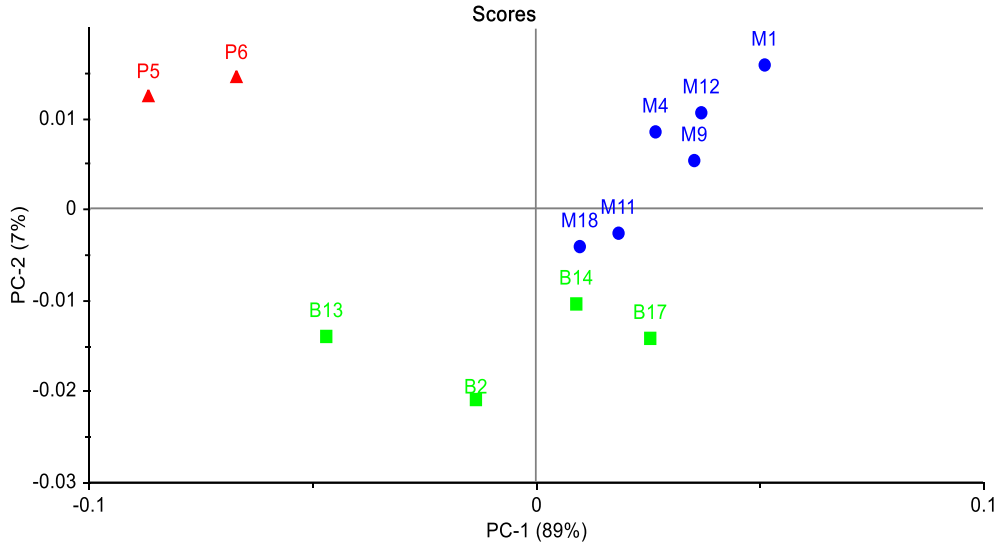


Figure 8.3: PCA Scores Plot of FTIR Spectra of Gelatin Capsule Supplements

PC1 vs. PC2. PCA was pre-treatment with *Savitsky-Golay* and area normalization and the S code would be changed to the specific classification where number: P is code for porcine; B is code for bovine; and M is coded for mixture.

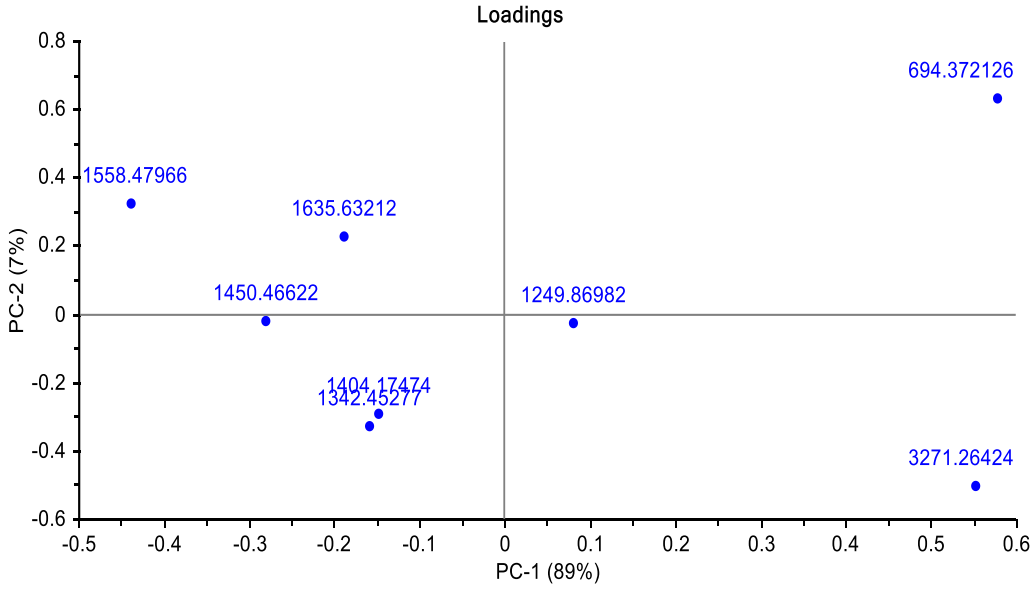


Figure 8.4: PCA loading plot (PC1 vs. PC2)

Normalization and smoothing of the FT-IR spectrum of gelatin capsule was shown in Figure 8.3. PCA manipulates the data of variables (peak height of amino acids) in the way that these variables can be displayed on an x, y coordinate system. PCA did this by calculating principal components (PC1) which are linear combination of original variables. PC1 (first principal component) explained the most variation among data, while PC2 or second principal component described the second largest variation among data. PC1 was orthogonal to PC2.

Figure 8.3 exhibited the PCA score plot of porcine and bovine gelatins coming from commercial capsule shells. Bovine and porcine gelatins were clearly separated. PC1 described 89% variation of data, while PC2 and PC3 account for 7% and 3% variations, respectively. Therefore, more than 90% of variation can be described only by three PCs.

Figure 8.4 illustrated the loading plot for the determination of variables (wavenumber) contributing to the differentiation and separation of the samples. The PCA loading plot described the projection of variables in the same plane as the score plot. The absolute value of loading plot in wavenumuber explains the importance of the contribution of each amino acid. Therefore, the further away a wavenumber from the origin of variable point, the larger the contribution of that variable (amino acid) to the PCA model (Marina et al., 2010; Rohman et al., 2012). From Figure 8.4, it was known that 694 and 3271 cm^{-1} were the variables giving the most contribution toward PC1, while 1558 and 1635 cm^{-1} were more influencing on PC2.

Based on Figure 8.4, it was seen that the PCA plot showed that profiles were significantly different in the region 694, 3271, 1558 and 1635 cm^{-1}. 3271 cm^{-1} in PC1 contribution is indicate the

presence of aliphatic N-H stretching region in peptide bond. The second difference lies in the frequency of 1558 cm^{-1} and 1635 cm^{-1}, which indicate a C-N-H bending and C=O stretching of the peptide bonds (Rohman et al., 2012).

Some capsule shells available in numerous pharmacies were investigated by determining the level of amino acid contents and subsequently subjected to PCA. The results showed that PCA could not distinguish the adulteration in capsule shells as indicated by the irregular profile of PCA score plot of capsule shells samples, where the sample was very close with bovine gelatin. It could be explained that the capsule shells assayed in adulteration sample may be had the same level concentration of bovine gelatin so the adulteration have the same spot with bovine gelatin. As mentioned previously, the composition of the gelatin structure had similar vibrational frequencies indicating similar content. A decrease and increase in intensity would indicate less amino acid material. However, the spectra do contain fewer peaks due to gelatin indicating a decrease in amino acids content (Rohman et al., 2012). The most obvious feature is the comparison of the abaxial side to the adaxial side of the gelatin capsule. IR peak gelatin standard and thus gelatin capsule content seems incomparable. During manufacture of gelatin capsule, the chemical structure of polypeptide is degraded which may be caused by chemical and physical treatment (GMIA, 2012).

FTIR spectroscopy has facilitated the real-time measurement on the chemical structure. The identification of peaks due to chemical structure can give any confirmation between porcine gelatin and bovine gelatin. However, the technique is difficult for adulteration in gelatin capsules, since the same spots on the different sample result in different spectra.

Chapter 9

Conclusion

A real-time PCR assay with SYBR green method is suitable for the determination of adulterated pork in gelatin formulations. Two species-specific qPCR assays were optimized based upon repetitive elements of the porcine and bovine genomes and they allowed sensitive detection of porcine and bovine at concentrations as low as 10^{-5} ng/µl. In addition, the lack of cross reactivity when the sets were used to amplify DNA from the other species indicates high specificity of the qPCR assay for its own species. When binary gelatin blends containing various amounts of porcine and bovine gelatin were prepared and analyzed by the qPCR assays, the determined ratios of porcine material to bovine material were very close to their theoretical values, and a contamination level of as low as 1% of the other species in the gelatin blends could be determined. When evaluated in gelatin capsules, although significantly less DNA was detected, determination of porcine and bovine species identities and estimation of the relative abundance of each species was possible. The data reported in this book are from DNA samples that were potentially degraded during manufacturing of gelatin and gelatin capsules, which are highly processed products. Therefore, the porcine and bovine species-specific qPCR assays described here represent a simple, reliable and sensitive DNA-based test for determining the species of origin of highly processed products.

Using a commercial DNA extraction kit from Agilent porcine detection kit has been demonstrated that porcine DNA can be reliably detected in gelatin capsule. The amounts of DNA present

in isolated DNA using Agilent were higher than in Qiagen and Kogene kits. Therefore, the Agilent kit had successfully isolated DNA from gelatin capsules. However, the additional protocols on the purification is required in order to further reduce the amount of interfering protein.

The validation method of this research expects broad applicability of the assay with minor modifications to other food matrices as well. However, since the extent of DNA degradation that occurs during gelatin and gelatin capsule manufacture may vary and the copy number of repetitive elements between different animal subjects within the same species may vary (although the data generated from this book suggested, with careful qPCR assay design, there were minimal impact from amplifying repetitive elements on DNA quantization), the DNA quantity determined may not always represent the amount of porcine and bovine species-specific materials in gelatin and gelatin capsules. Thus the quantitative data obtained would only be an approximation.

The FTIR spectroscopic techniques can be used for rapid classification of gelatin. In order to ensure the result of real-time PCR, the formation of Amide A, I, II and III were analyzed using PCA measurement. These regions were found to give information about the origin of the gelatin. Two independent PCA models were calculated defining three separate classes of samples. However, PCA was not successful for classification of adulteration gelatin in capsule shells due to the similar chemical structure.

Since PCR based techniques are effective in identifying small pieces of DNA, they have received significant attention in recent years. The ideal PCR is the one with high specificity, sensitivity and efficiency and are influenced by the nature of target sequence, as well as by each component of PCR. Often, the conditions that

would permit maximum yield are not compatible with high specificity and conditions optimized in regard to specificity may adversely affect the efficiency. Thus, in setting up a PCR it is important to determine beforehand to attain the specificity, efficiency and sensitivity of the PCR that is required for the intended application.

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Appendices

Appendix 1: Calculation of annealing temperature in bovine and porcine primer

Primer	**Sequence**	
Bovine	Forward	5'-CCCGATTCTTCGCTTTCCAT-3'
	Reverse	5'-CTACGTCTGAGGAAATTCCTGTTG-3'
Porcine	Forward	5'-CTTGCAAATCCTAACAGGCCTG-3'
	Reverse	5'-CGTTTGCATGTAGATAGCGAATAAC-3'

$$Tm = 2^{o}C\ (A + T) + 4^{o}C\ (G + C)$$

Bovine Primer

Forward

$Tm = 2^{o}C\ (2+8) + 4^{o}C\ (2+8)$

Reverse

$Tm = 2^{o}C\ (5+8) + 4^{o}C\ (6+5)$

$Means = (60^{o}C + 70^{o}C) / 2$

$= 65^{o}C$

$Ta* = 60^{o}C$

Porcine Primer

Forward

$Tm = 2^{o}C\ (6+5) + 4^{o}C\ (4+7)$

Reverse

$Tm = 2^{o}C\ (8+7) + 4^{o}C\ (6+3)$

$Means = (66^{o}C + 66^{o}C) / 2$

$= 66^{o}$

$Ta* = 61^{o}C$

Appendix 2: Nucleotide BLAST

Bos taurus cytochrome b (cytb) gene, partial cds; mitochondrial

Sequence ID: gb|KF799994.1| Length: 704 Number of Matches: 1

Range 1: 517 to 636 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
217 bits(240)	2e-53	120/120(100%)	0/120(0%)	Plus/Plus

```
Query  1    CCCGATTCTTCGCTTTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCC  60
Sbjct  517  CCCGATTCTTCGCTTTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCC  576

Query  61   ACCTACTATTCCTCCACGAAACAGGCTCCAACAACCCAACAGGAATTTCCTCAGACGTAG  120
Sbjct  577  ACCTACTATTCCTCCACGAAACAGGCTCCAACAACCCAACAGGAATTTCCTCAGACGTAG  636
```

Sus scrofa isolate 1100 cytochrome b (cytb) gene, complete cds; mitochondrial

Sequence ID: gb|HM010472.1| Length: 1140 Number of Matches: 1

Range 1: 126 to 256 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
237 bits(262)	3e-59	131/131(100%)	0/131(0%)	Plus/Plus

```
Query  1    CTTGCAAATCCTAACAGGCCTGTTCTTAGCAATACATTACACATCAGACACAACAACAGC  60
Sbjct  126  CTTGCAAATCCTAACAGGCCTGTTCTTAGCAATACATTACACATCAGACACAACAACAGC  185

Query  61   TTTCTCATCAGTTACACACATCTGTCGAGACGTAAATTACGGATGAGTTATTCGCTACCT  120
Sbjct  186  TTTCTCATCAGTTACACACATCTGTCGAGACGTAAATTACGGATGAGTTATTCGCTACCT  245

Query  121  ACATGCAAACG  131
Sbjct  246  ACATGCAAACG  256
```

Appendix 3: Data Absorbance Gelatin Capsule

	B2	M1	M4	B13	M9	B14	M11	P5	M12	M18	B17	P6
586.3587	0	0.145879	0	0.007137	0.043815	0	0	0	0	0	0	0
601.7892	0	0.064129	0	0.023151	0.078303	0	0	0	0	0	0	0
617.2197	0	0.052764	0	0.042215	0.08545	0	0	0	0	0	0	0
632.6502	0	0.072037	0	0.055242	0.075463	0	0	0	0	0	0	0
648.0807	0.036042	0.074184	0.064037	0.05954	0.082218	0.042218	0.066205	0.041438	0.069431	0.080199	0.057154	0.041136
663.5111	0.04902	0.059784	0.058023	0.058565	0.070373	0.054295	0.061764	0.035718	0.067048	0.07056	0.065547	0.052581
678.9416	0.05465	0.052615	0.058544	0.047715	0.066939	0.055874	0.061791	0.028802	0.070917	0.062132	0.066964	0.060657
694.3721	0.053614	0.05254	0.065776	0.04706	0.0682	0.055018	0.066038	0.035431	0.068997	0.057929	0.063142	0.058586
709.8026	0.054101	0.049699	0.067794	0.048837	0.067011	0.054113	0.070223	0.038118	0.0636	0.058854	0.06019	0.054365
725.2331	0.052528	0.04499	0.061478	0.045804	0.062401	0.051416	0.070679	0.038051	0.059261	0.058029	0.059697	0.050523
740.6636	0.047773	0.041757	0.056797	0.041804	0.05247	0.045921	0.063253	0.03522	0.064195	0.047771	0.064485	0.044402
756.0941	0.044073	0.035608	0.053049	0.036442	0.045847	0.042876	0.055656	0.024926	0.060214	0.040055	0.0652	0.038853
771.5246	0.042172	0.030996	0.050805	0.030515	0.042875	0.040326	0.045269	0.020944	0.044922	0.03783	0.055204	0.033517
786.9551	0.03747	0.029268	0.045832	0.026549	0.038209	0.035376	0.034492	0.019605	0.036945	0.034321	0.042639	0.028293
802.3856	0.030087	0.027408	0.041347	0.024504	0.036071	0.028382	0.032081	0.017348	0.03076	0.029566	0.036935	0.02561
817.8161	0.02498	0.026188	0.039826	0.019586	0.036065	0.021557	0.033722	0.015143	0.029009	0.024331	0.035838	0.022266
833.2466	0.02185	0.02394	0.035906	0.017041	0.032698	0.020797	0.032786	0.011178	0.032533	0.021612	0.032781	0.017823
848.677	0.023793	0.018287	0.031462	0.016303	0.025042	0.02233	0.027193	0.009564	0.028672	0.022935	0.028598	0.01765
864.1075	0.022947	0.014197	0.026421	0.012945	0.019467	0.02236	0.023327	0.010152	0.023034	0.023552	0.026016	0.018501
879.538	0.018105	0.011647	0.023068	0.01199	0.016813	0.020739	0.01912	0.009558	0.019738	0.020285	0.023564	0.016329
894.9685	0.016211	0.010616	0.020163	0.010539	0.016602	0.017819	0.016694	0.009477	0.017862	0.017261	0.019664	0.013656
910.99	0.017557	0.011885	0.016562	0.00946	0.018587	0.016551	0.018215	0.008967	0.018167	0.017733	0.017325	0.01208
925.895	0.01806	0.010941	0.01473	0.0106	0.01847	0.01476	0.017947	0.007699	0.018148	0.016497	0.016656	0.01153
941.26	0.014824	0.008226	0.013824	0.011304	0.015267	0.012699	0.016058	0.007707	0.01586	0.014167	0.015592	0.011042
956.6905	0.013502	0.007844	0.013236	0.011111	0.014272	0.013082	0.015175	0.008279	0.01399	0.015422	0.015809	0.010759
972.121	0.016791	0.009209	0.012731	0.011929	0.016237	0.014708	0.016165	0.008517	0.014851	0.017314	0.018555	0.011002
987.5515	0.019503	0.01084	0.012307	0.015607	0.017682	0.016592	0.018393	0.008012	0.016231	0.018242	0.020954	0.010259

1002.982	0.021281	0.011424	0.013008	0.020663	0.018674	0.018993	0.020662	0.009239	0.017088	0.019559	0.021934	0.01081
1018.412	0.027518	0.014889	0.01469	0.023311	0.023616	0.025036	0.025667	0.010928	0.021961	0.025312	0.028394	0.012719
1033.843	0.037886	0.023722	0.01576	0.021575	0.032177	0.033257	0.032821	0.011231	0.030596	0.036311	0.039768	0.013439
1049.273	0.039295	0.026671	0.014688	0.018307	0.033182	0.033252	0.033316	0.012509	0.032176	0.039431	0.040826	0.014534
1064.704	0.03139	0.021278	0.014249	0.017648	0.026182	0.026387	0.028839	0.013268	0.025818	0.032873	0.03168	0.016414
1080.134	0.02618	0.016601	0.015752	0.019499	0.021558	0.021933	0.026389	0.0132	0.020488	0.027897	0.026319	0.017281
1095.565	0.024639	0.014708	0.014953	0.020773	0.020387	0.020699	0.023917	0.011086	0.019559	0.026245	0.026883	0.014629
1110.995	0.02306	0.013767	0.012925	0.019568	0.019909	0.020058	0.021229	0.009349	0.020303	0.024102	0.02591	0.011733
1126.426	0.018524	0.010392	0.012431	0.017412	0.01617	0.016983	0.016723	0.009092	0.016375	0.01841	0.018869	0.011719
1141.856	0.014997	0.007753	0.012757	0.017508	0.012614	0.014022	0.013386	0.009118	0.012023	0.014421	0.01328	0.012526
1157.287	0.014976	0.007815	0.013672	0.0193	0.012948	0.013722	0.014314	0.010456	0.011759	0.013883	0.012962	0.013534
1172.717	0.016183	0.007765	0.014578	0.020158	0.012612	0.014732	0.015013	0.011016	0.012939	0.013891	0.013898	0.014262
1188.148	0.017874	0.009082	0.015621	0.021246	0.013079	0.016521	0.016181	0.01308	0.015238	0.015965	0.015455	0.016074
1203.578	0.019045	0.010978	0.016216	0.021567	0.015156	0.017625	0.018477	0.014962	0.016644	0.018162	0.016987	0.017863
1219.009	0.021318	0.012213	0.016925	0.021488	0.016815	0.018975	0.021403	0.016928	0.018091	0.020251	0.019798	0.020748
1234.439	0.024534	0.013126	0.018763	0.023813	0.01904	0.021149	0.02434	0.021298	0.020941	0.023568	0.023108	0.026452
1249.87	0.025917	0.012864	0.019862	0.025531	0.019397	0.021732	0.024635	0.021995	0.021748	0.023459	0.0232	0.0281
1265.3	0.024582	0.012318	0.019311	0.024727	0.018163	0.020586	0.023031	0.019707	0.020146	0.020388	0.021442	0.02496
1280.731	0.022009	0.011659	0.017213	0.022672	0.0173	0.019029	0.021113	0.017614	0.017766	0.018808	0.020067	0.022114
1296.161	0.020131	0.010751	0.015332	0.020719	0.016352	0.017948	0.019691	0.016108	0.016072	0.017772	0.019751	0.020232
1311.592	0.02004	0.01072	0.015391	0.020346	0.017182	0.01794	0.019667	0.016001	0.016297	0.018014	0.020031	0.020327
1327.022	0.02208	0.01156	0.01663	0.021367	0.019566	0.018878	0.020785	0.016861	0.017594	0.019643	0.020966	0.02244
1342.453	0.022019	0.010997	0.016829	0.02151	0.019645	0.018669	0.020281	0.016292	0.017318	0.019206	0.020459	0.021806
1357.883	0.019977	0.009279	0.015228	0.020117	0.017415	0.017582	0.018649	0.014263	0.016085	0.017636	0.018088	0.019074
1373.314	0.020509	0.010304	0.016024	0.020472	0.017691	0.018108	0.019204	0.015377	0.016864	0.017385	0.018654	0.020874
1388.744	0.022981	0.013088	0.019281	0.023115	0.02092	0.020338	0.02178	0.018337	0.019805	0.019547	0.021447	0.025868
1404.175	0.025329	0.013637	0.020583	0.024476	0.02222	0.022422	0.023863	0.019237	0.022409	0.021846	0.02348	0.028095
1419.605	0.025332	0.012751	0.020113	0.023856	0.020799	0.022315	0.023493	0.018821	0.022116	0.022218	0.023926	0.026901
1435.036	0.025509	0.013987	0.02054	0.025396	0.021049	0.022901	0.024344	0.020587	0.022398	0.023432	0.024449	0.028466
1450.466	0.026993	0.016391	0.022228	0.028292	0.023082	0.024534	0.025858	0.022856	0.023818	0.025363	0.025991	0.032149

1465.897	0.024362	0.014025	0.021106	0.026612	0.02094	0.021889	0.022283	0.01996	0.020571	0.023327	0.023707	0.028745
1481.327	0.018818	0.007958	0.016622	0.022403	0.015395	0.016173	0.016821	0.015726	0.015289	0.016758	0.017997	0.021333
1496.758	0.016952	0.006368	0.01481	0.022112	0.013955	0.013407	0.015748	0.016622	0.01469	0.014405	0.015275	0.020985
1512.188	0.022134	0.011001	0.019343	0.028727	0.01904	0.017525	0.021452	0.023937	0.020548	0.020109	0.01914	0.032842
1527.619	0.031766	0.018397	0.028674	0.038569	0.027826	0.026478	0.030394	0.034503	0.02899	0.0277	0.027368	0.051016
1543.049	0.040737	0.025431	0.038325	0.045553	0.036128	0.034298	0.038066	0.04229	0.03547	0.034177	0.035175	0.064888
1558.48	0.04113	0.026356	0.040831	0.045148	0.037243	0.034551	0.03958	0.039936	0.035263	0.03542	0.036735	0.063186
1573.91	0.032857	0.02081	0.034361	0.038037	0.030459	0.028158	0.033372	0.030156	0.028338	0.030242	0.030781	0.048394
1589.341	0.027316	0.01613	0.029134	0.033124	0.025337	0.023603	0.028196	0.02364	0.023254	0.025609	0.026157	0.038535
1604.771	0.031887	0.019697	0.034678	0.037755	0.030524	0.027249	0.033166	0.028041	0.027757	0.029063	0.03035	0.045757
1620.202	0.048242	0.032991	0.05062	0.053285	0.045882	0.041506	0.049376	0.044448	0.043454	0.04297	0.044576	0.072382
1635.632	0.064435	0.045764	0.063513	0.068199	0.059158	0.055234	0.064345	0.058652	0.057918	0.056355	0.058865	0.09703
1651.063	0.06606	0.047233	0.062128	0.067734	0.059107	0.056358	0.065344	0.057893	0.058336	0.057466	0.061499	0.095217
1666.493	0.05291	0.036302	0.048278	0.054066	0.046933	0.045569	0.051575	0.044	0.046305	0.045471	0.050643	0.070866
1681.924	0.033899	0.020961	0.031316	0.036644	0.030197	0.029897	0.033339	0.027122	0.030165	0.028867	0.033605	0.042032
1697.354	0.017343	0.008301	0.0179	0.021758	0.015937	0.016118	0.018606	0.013784	0.016259	0.01574	0.01798	0.018996
1712.785	0.008724	0.00101	0.010675	0.013396	0.008344	0.008579	0.010252	0.006352	0.008502	0.009858	0.008909	0.006158
1728.215	0.005956	0.000245	0.007746	0.010299	0.004938	0.006618	0.00659	0.003461	0.005587	0.007493	0.006148	0.001123
1743.646	0.004618	0.002688	0.005848	0.010136	0.002896	0.006452	0.004559	0.002017	0.004518	0.005541	0.005344	-0.00079
1759.076	0.002897	0.000593	0.00323	0.009008	0.001353	0.004059	0.002837	0.001541	0.00258	0.003485	0.003843	-0.00224
1774.507	0.002008	-0.00395	0.002107	0.007135	0.000619	0.00172	0.002271	0.001569	0.001041	0.001767	0.003115	-0.0024
1789.937	0.00152	-0.00537	0.001923	0.006687	0.000656	0.001287	0.001987	0.0009	0.000833	0.000995	0.001887	-0.00228
1805.368	0.001151	-0.00588	0.001613	0.006618	0.000745	0.001036	0.001107	0.000744	0.000695	0.000932	0.001076	-0.00224
1820.798	0.001062	-0.00647	0.001665	0.006611	0.000819	0.000514	0.000646	0.000488	0.000345	0.001031	0.001451	-0.00256
1836.229	0.000929	-0.0061	0.001063	0.006587	0.000499	0.000215	0.000483	0.00018	1.96E-05	0.000764	0.000997	-0.00292
1851.659	0.000652	-0.00585	0.000195	0.00662	0.000335	-0.0002	1.57E-05	0.000331	-4.3E-05	0.000696	0.000711	-0.00321
1867.089	0.000472	-0.00605	0.000212	0.006719	0.000798	-0.00048	-7.2E-05	0.00049	0.000107	0.000622	0.000909	-0.00354
1882.52	0.000585	-0.00663	0.000739	0.006538	0.000751	-0.00025	-0.0001	0.000764	4.59E-06	-1E-05	0.001161	-0.00307
1897.95	0.00061	-0.00678	0.000655	0.006396	0.00054	-0.00011	-3E-05	0.000814	-0.00042	-0.00025	0.001302	-0.00293
1913.381	0.000611	-0.00703	0.000494	0.005857	0.000686	-0.00027	0.000311	0.000827	-0.00014	3.48E-05	0.001293	-0.00312

1928.811	0.000589	-0.00771	0.000485	0.005719	0.000768	-0.00034	9.22E-05	0.000915	0.000642	0.000328	0.001391	-0.00324
1944.242	0.000793	-0.00747	0.000376	0.00643	0.000612	1.85E-05	-0.00026	0.001037	0.000497	0.00037	0.00097	-0.00316
1959.672	0.00064	-0.0072	0.000827	0.006541	0.000132	-2.6E-06	-0.00013	0.001172	-1.5E-06	0.000619	0.000626	-0.00314
1975.103	9.85E-05	-0.00696	0.000953	0.006589	-0.00032	-0.00037	0.0005	0.001127	0.000214	0.000796	0.000763	-0.00331
1990.533	0.000253	-0.00622	0.000862	0.006977	-0.00063	-0.00016	0.000516	0.000722	0.000256	0.000474	0.001125	-0.00304
2005.964	0.000665	-0.00606	0.000971	0.007035	-0.00023	0.000162	0.000227	0.00017	-0.00021	0.000294	0.001305	-0.0028
2021.394	0.000984	-0.00671	0.001159	0.006858	0.000343	-0.00011	0.000662	-0.00026	0.000111	0.000206	0.000742	-0.00252
2036.825	0.001014	-0.00705	0.00168	0.006929	0.000161	-0.0004	0.000929	-0.00024	0.000771	0.000584	0.000757	-0.00274
2052.255	0.000918	-0.00663	0.001491	0.007452	0.000106	0.000125	0.001168	0.000221	0.000502	0.000806	0.001385	-0.00301
2067.686	0.000815	-0.00624	0.001165	0.007553	0.000603	0.000477	0.001178	0.000347	-0.00016	0.000536	0.001664	-0.00277
2083.116	0.000761	-0.00588	0.001358	0.007194	0.001383	0.000559	0.000757	0.000463	-0.00052	0.000648	0.00207	-0.00262
2098.547	0.001057	-0.00566	0.001455	0.006878	0.001335	0.001059	0.00061	0.000684	-0.00066	0.000708	0.002088	-0.00273
2113.977	0.001175	-0.00558	0.001552	0.007007	0.000833	0.001394	0.000962	0.000733	-0.00046	0.000588	0.002067	-0.00295
2129.408	0.001192	-0.00564	0.001436	0.0078	0.000787	0.000972	0.001368	0.000369	0.000113	0.000697	0.002542	-0.00288
2144.838	0.001111	-0.00612	0.001126	0.008107	0.00051	0.000504	0.001237	-0.00025	0.000576	0.001149	0.002412	-0.00312
2160.269	0.000697	-0.00614	0.000844	0.007531	0.00078	0.000753	0.001287	-0.00031	0.000771	0.001251	0.001825	-0.00322
2175.699	0.000171	-0.00592	0.001087	0.007348	0.001543	0.000138	0.001031	-3.9E-05	0.000866	0.000874	0.001471	-0.00287
2191.13	0.000116	-0.00596	0.001018	0.008056	0.0014	-0.00049	0.000176	0.000453	0.000665	0.000491	0.001292	-0.00264
2206.56	0.000583	-0.00581	-5.3E-05	0.008343	0.000282	0.00013	0.000184	0.001013	0.000261	0.000744	0.001302	-0.00295
2221.991	0.000837	-0.006	-0.0003	0.008233	-0.00019	0.000255	0.00111	0.001356	0.000238	0.001313	0.001271	-0.00318
2237.421	0.000837	-0.00688	0.000359	0.008263	0.000201	0.000135	0.001497	0.001038	0.000294	0.000949	0.000652	-0.00271
2252.852	0.000784	-0.00746	0.001047	0.007872	4.76E-05	9.83E-05	0.001025	0.000212	-0.00016	0.000931	0.000409	-0.00305
2268.282	0.001036	-0.00726	0.00107	0.007607	5.76E-05	-0.00051	0.000977	0.000335	-0.00039	0.001197	0.000932	-0.00354
2283.713	0.00127	-0.00679	0.000422	0.007555	0.000182	-0.00088	0.000676	0.000883	-0.00016	0.001158	0.000983	-0.00363
2299.143	0.001023	-0.00655	0.000479	0.007834	0.000264	-0.00033	-0.0001	0.00075	-6.8E-05	0.001166	0.001109	-0.00355
2314.574	0.000504	-0.00678	0.000551	0.008127	0.000841	-9.7E-06	-0.0001	0.000314	0.000201	0.00054	0.001234	-0.00319
2330.004	0.000243	-0.00654	7.84E-05	0.007808	0.001053	-5.2E-05	0.000237	0.000375	0.000598	0.000398	0.000874	-0.00323
2345.435	0.00053	-0.00617	-0.00034	0.008087	0.000985	-0.00021	0.000667	0.001088	0.000364	0.000662	0.000726	-0.00335
2360.865	5.2E-05	-0.00658	-0.0006	0.008636	0.000771	-0.00047	0.000813	0.00098	-0.00037	0.000363	0.000521	-0.00339
2376.296	-0.00034	-0.00693	-0.00046	0.008459	-0.00028	-5.3E-05	0.000435	0.000107	-0.00072	6.84E-05	0.000277	-0.0035

2391.726	0.000546	-0.00699	-0.00028	0.008105	-0.00115	-0.0003	-0.0001	9.17E-05	-0.00062	0.000338	2.56E-05	-0.00409
2407.157	0.00096	-0.00655	-5.9E-05	0.00802	-0.00046	-0.00079	-0.00066	0.000464	-0.00024	0.000463	5.81E-05	-0.0042
2422.587	0.000857	-0.00619	2.73E-05	0.008156	0.000542	-0.00053	-0.00074	0.000647	0.000319	-0.00029	0.000634	-0.00367
2438.018	0.00094	-0.00653	-0.00036	0.008667	0.000701	-0.00056	-0.0004	0.000403	0.000167	-0.00053	0.000861	-0.00365
2453.448	0.000587	-0.00683	-0.00064	0.009371	0.000531	-0.00071	-0.00025	-1.8E-05	-4.5E-05	-0.00022	0.000678	-0.00356
2468.879	0.000454	-0.00744	-0.00062	0.009125	0.000552	-0.00099	-0.00048	0.000552	0.000342	-4.6E-05	0.00106	-0.00329
2484.309	0.001119	-0.00806	-0.00062	0.008486	0.000629	-0.00095	-0.00027	0.000785	3.58E-05	0.000144	0.001829	-0.00297
2499.74	0.00146	-0.00792	-0.00046	0.008866	2.49E-05	-0.00069	0.000469	0.000357	-0.00026	0.000389	0.001477	-0.00308
2515.17	0.001046	-0.0074	-0.00024	0.009091	-0.00017	-0.0004	0.001024	0.000434	-0.00019	0.00067	0.000828	-0.0031
2530.601	0.000534	-0.0069	-0.00048	0.007936	0.000561	0.000242	0.001067	0.000613	-0.00036	0.000565	0.000737	-0.00294
2546.031	0.000715	-0.00674	-0.00077	0.007525	0.000852	0.000338	0.000242	0.000962	-0.00016	0.00057	0.000196	-0.00351
2561.462	0.001284	-0.00672	-0.00059	0.008744	0.000597	0.000108	-0.00029	0.001324	-9.4E-05	0.000892	0.000208	-0.00353
2576.892	0.0012	-0.00655	-0.00044	0.009941	0.000482	0.000381	0.000336	0.00113	-0.00014	0.001143	0.000778	-0.00328
2592.323	0.000778	-0.00626	-0.00052	0.010489	0.000335	0.000824	0.000742	0.00015	-6E-05	0.001082	0.001008	-0.00303
2607.753	0.000778	-0.0064	-0.00044	0.009868	-0.00027	0.000717	0.000167	-0.00023	2.9E-05	0.000798	0.000965	-0.00235
2623.184	0.001348	-0.00655	-0.0002	0.009398	-0.00033	0.000566	-1.6E-05	6.84E-05	0.000309	0.000624	0.000776	-0.00244
2638.614	0.001606	-0.00615	-5.1E-05	0.009611	0.000108	0.00068	0.00085	0.000109	0.000526	0.000928	0.001717	-0.00308
2654.045	0.001521	-0.00584	0.000208	0.00963	0.000397	0.000703	0.001317	0.000815	0.000861	0.001278	0.002476	-0.00316
2669.475	0.00173	-0.00562	0.000877	0.010353	0.000554	0.000292	0.001288	0.0011	0.000466	0.001011	0.001675	-0.00286
2684.906	0.001225	-0.00553	0.001246	0.010756	0.000611	-0.00018	0.001566	0.001021	0.000112	0.00151	0.001624	-0.00235
2700.336	0.000927	-0.00524	0.000592	0.010549	0.001139	0.000288	0.001708	0.001174	0.000709	0.002021	0.002472	-0.00201
2715.767	0.002198	-0.005	-1.2E-05	0.010621	0.001322	0.000773	0.001634	0.000805	0.000582	0.001983	0.002532	-0.00212
2731.197	0.002815	-0.00562	-0.00038	0.010708	0.001315	0.001379	0.001894	0.000634	0.000779	0.002605	0.001993	-0.00185
2746.628	0.002098	-0.006	-0.00089	0.010871	0.001736	0.002071	0.002354	0.001006	0.001221	0.002523	0.001776	-0.0015
2762.058	0.002421	-0.0058	-0.00054	0.011003	0.001955	0.002101	0.002702	0.001516	0.001164	0.002625	0.002138	-0.00094
2777.489	0.003395	-0.00542	0.000199	0.010809	0.002415	0.002245	0.002833	0.001435	0.001827	0.003746	0.003269	-0.00101
2792.919	0.003757	-0.00467	0.000707	0.010571	0.002795	0.002395	0.003176	0.001601	0.00201	0.004303	0.004517	-0.00146
2808.349	0.004341	-0.00397	0.001014	0.01137	0.002744	0.003216	0.004166	0.001969	0.001994	0.00438	0.004857	-0.0004
2823.78	0.005071	-0.00323	0.00147	0.011865	0.002961	0.004446	0.004123	0.00198	0.002699	0.004655	0.005364	0.000513
2839.21	0.005939	-0.00115	0.00304	0.012284	0.003613	0.005948	0.003659	0.002354	0.003893	0.005598	0.006466	0.00144

2854.641	0.007319	0.001071	0.004237	0.013908	0.004865	0.00804	0.004757	0.0024	0.005929	0.007066	0.00767	0.002449
2870.071	0.008829	0.000964	0.004703	0.014715	0.006808	0.009054	0.005769	0.00266	0.007337	0.008279	0.008767	0.002944
2885.502	0.009626	0.001462	0.005742	0.015296	0.007888	0.009562	0.006372	0.003364	0.007236	0.008838	0.008847	0.003818
2900.932	0.00981	0.004034	0.006612	0.015945	0.007649	0.010771	0.007101	0.004425	0.006833	0.009717	0.008794	0.004708
2916.363	0.010961	0.00656	0.007724	0.01742	0.00786	0.013038	0.00837	0.005503	0.008322	0.011466	0.009824	0.00647
2931.793	0.012026	0.007981	0.008981	0.019381	0.008798	0.01432	0.010278	0.005721	0.010869	0.012702	0.011248	0.007349
2947.224	0.012152	0.006744	0.009089	0.019282	0.009795	0.012953	0.010295	0.005852	0.010915	0.01214	0.011978	0.006933
2962.654	0.01197	0.004105	0.00866	0.018824	0.009669	0.01105	0.008696	0.005235	0.009418	0.010536	0.011664	0.007039
2978.085	0.010332	0.001983	0.008134	0.017877	0.008399	0.009075	0.007648	0.004544	0.008	0.009285	0.010462	0.006767
2993.515	0.008701	0.001267	0.007566	0.017164	0.007579	0.00792	0.007662	0.004098	0.007121	0.007857	0.009254	0.005974
3008.946	0.008873	0.002212	0.007532	0.017369	0.007502	0.008228	0.008409	0.003568	0.007613	0.007577	0.009337	0.005706
3024.376	0.009824	0.003265	0.008643	0.017345	0.008701	0.00879	0.009314	0.004278	0.008992	0.009219	0.010214	0.00675
3039.807	0.010825	0.004123	0.010656	0.018224	0.011206	0.009644	0.010286	0.005224	0.010202	0.010332	0.011513	0.007812
3055.237	0.011992	0.005057	0.01256	0.019687	0.013536	0.010687	0.011863	0.005832	0.011204	0.011171	0.013555	0.009192
3070.668	0.013548	0.006399	0.014421	0.02034	0.014277	0.011733	0.013587	0.0065	0.012847	0.011986	0.015196	0.01093
3086.098	0.014853	0.007778	0.016147	0.01977	0.014741	0.013194	0.014651	0.006738	0.014339	0.013023	0.016096	0.011266
3101.529	0.015694	0.008556	0.01693	0.019468	0.016579	0.014322	0.015886	0.00625	0.015173	0.014739	0.017289	0.01121
3116.959	0.016509	0.009771	0.017684	0.019741	0.018123	0.014809	0.017534	0.005522	0.01644	0.01547	0.019315	0.01119
3132.39	0.01684	0.011814	0.019148	0.020018	0.019122	0.015697	0.019538	0.005573	0.017653	0.016068	0.021325	0.010978
3147.82	0.018252	0.014009	0.020888	0.021238	0.020672	0.017195	0.021701	0.006108	0.019454	0.017844	0.02345	0.011986
3163.251	0.021272	0.015626	0.023025	0.022164	0.022806	0.019371	0.023408	0.006921	0.022789	0.02031	0.026417	0.014369
3178.681	0.023456	0.017665	0.025759	0.022806	0.025704	0.02215	0.025809	0.008314	0.025758	0.022682	0.029706	0.016298
3194.112	0.024698	0.020365	0.028732	0.02422	0.028841	0.024727	0.029144	0.010063	0.028121	0.024589	0.032991	0.017908
3209.542	0.026807	0.022947	0.031282	0.02561	0.031125	0.027066	0.031909	0.011071	0.030798	0.026726	0.035045	0.019491
3224.973	0.030248	0.025275	0.033443	0.026824	0.033094	0.029354	0.033768	0.010709	0.0328	0.028895	0.036707	0.021192
3240.403	0.032885	0.026755	0.035424	0.027995	0.036097	0.031661	0.036106	0.01067	0.034452	0.030947	0.039523	0.023748
3255.834	0.034832	0.02921	0.037476	0.028627	0.03875	0.03367	0.038919	0.011724	0.03649	0.032824	0.041658	0.026099
3271.264	0.037017	0.031811	0.040167	0.029387	0.040091	0.034719	0.0405	0.012736	0.037827	0.033904	0.043217	0.028287
3286.695	0.037504	0.032546	0.043116	0.030766	0.041073	0.035379	0.041185	0.012907	0.038377	0.03458	0.045154	0.029075
3302.125	0.037364	0.033001	0.04467	0.031363	0.041574	0.036298	0.042115	0.01283	0.039108	0.035167	0.046397	0.029049

3317.556	0.037151	0.032955	0.043654	0.031396	0.041938	0.036374	0.042795	0.013103	0.039907	0.035509	0.046319	0.029683
3332.986	0.036124	0.03239	0.042542	0.031068	0.042018	0.035726	0.042638	0.01268	0.040159	0.035421	0.045556	0.028952
3348.417	0.03562	0.03168	0.043257	0.030343	0.040918	0.036049	0.042151	0.011649	0.039851	0.034989	0.04526	0.027146
3363.847	0.035113	0.030982	0.043506	0.029562	0.039419	0.035913	0.041212	0.01072	0.039067	0.034724	0.04505	0.024992
3379.278	0.033505	0.03075	0.042552	0.028537	0.038624	0.03454	0.040202	0.01018	0.038607	0.034103	0.044237	0.023066
3394.708	0.031964	0.029938	0.041743	0.027674	0.03815	0.033286	0.039569	0.010646	0.038322	0.032308	0.042974	0.021388
3410.139	0.031454	0.028868	0.041615	0.027124	0.037559	0.031521	0.038806	0.010788	0.036818	0.030691	0.041071	0.021303
3425.569	0.0303	0.027934	0.040547	0.027308	0.036978	0.029951	0.037507	0.00981	0.034083	0.029982	0.03843	0.021998
3441	0.028554	0.026617	0.038081	0.026961	0.035404	0.029266	0.035573	0.009069	0.031346	0.02849	0.036106	0.01981
3456.43	0.026872	0.024453	0.036025	0.026155	0.032267	0.027715	0.033392	0.009071	0.030038	0.025983	0.034355	0.017843
3471.861	0.024741	0.021715	0.03412	0.026043	0.029865	0.025742	0.030091	0.00858	0.028772	0.02382	0.032341	0.016961
3487.291	0.022601	0.019826	0.031948	0.025834	0.028509	0.024107	0.027155	0.007042	0.026616	0.02227	0.029407	0.01567
3502.722	0.020621	0.017628	0.029408	0.024819	0.025973	0.022127	0.025752	0.006479	0.024478	0.021494	0.025338	0.014513
3518.152	0.018957	0.014112	0.026908	0.022817	0.023127	0.02025	0.023417	0.006415	0.021484	0.02027	0.022394	0.012533
3533.583	0.016552	0.011153	0.02406	0.021832	0.020247	0.017868	0.020323	0.004625	0.018507	0.016576	0.020336	0.010569
3549.013	0.013611	0.009489	0.020586	0.020966	0.016839	0.014756	0.017801	0.002938	0.016371	0.013426	0.017572	0.008367
3564.444	0.011063	0.008031	0.018335	0.019309	0.014012	0.012149	0.015697	0.003041	0.014476	0.011938	0.015413	0.006098
3579.874	0.009485	0.005758	0.015626	0.017898	0.011831	0.009693	0.012911	0.003011	0.01254	0.009544	0.013125	0.005224
3595.305	0.008165	0.003209	0.01257	0.016333	0.010117	0.007579	0.010045	0.001363	0.009668	0.007748	0.010428	0.003515
3610.735	0.005672	0.001326	0.011289	0.016071	0.008096	0.005857	0.008237	0.000379	0.006572	0.006558	0.008265	0.001002
3626.166	0.003962	-0.00048	0.008907	0.015932	0.006139	0.003497	0.006434	0.001673	0.004173	0.00471	0.006179	-6.6E-06
3641.596	0.002553	-0.00269	0.005921	0.015012	0.005173	0.002168	0.004293	0.002304	0.002598	0.003327	0.00385	-0.00099
3657.027	0.001285	-0.00425	0.00481	0.014571	0.003672	0.0017	0.00215	0.001101	0.0019	0.002828	0.002309	-0.00228
3672.457	0.000773	-0.00492	0.003033	0.014162	0.00202	0.000199	0.000384	4.26E-05	0.00059	0.002073	0.001969	-0.0033
3687.888	-0.00062	-0.00529	0.000189	0.013936	0.001454	-1.5E-05	-0.0011	-0.00069	-0.00113	0.000453	0.000695	-0.00416
3703.318	-0.00146	-0.00487	-0.00109	0.014212	0.000775	0.000516	-0.0021	-0.00127	-0.00136	-0.00041	-0.00051	-0.00488
3718.749	-0.0017	-0.00479	-0.00115	0.014729	0.000553	-0.00043	-0.00206	-0.00082	-0.00142	-0.00088	-6.4E-05	-0.00538
3734.179	-0.00192	-0.00591	-0.00052	0.014327	0.000955	-0.00141	-0.00114	0.000122	-0.00207	-0.001	0.000564	-0.00462
3749.609	-0.00174	-0.00662	0.000395	0.01395	0.000102	-0.00171	-0.00022	0.000174	-0.00196	-0.00011	0.000989	-0.00409
3765.04	-0.00156	-0.00652	-0.00084	0.014585	-0.00046	-0.00158	-0.00068	-0.00104	-0.00143	-0.00033	0.001267	-0.00424

3780.47	-0.00077	-0.00635	-0.0021	0.01476	-5.1E-05	-0.00157	-0.00117	-0.00227	-0.00026	-0.00074	0.00111	-0.00423
3795.901	-8E-05	-0.00689	-0.00124	0.013969	-0.00028	-0.00153	-0.0009	-0.00211	0.00028	-0.00056	-0.00068	-0.00417
3811.331	0.000215	-0.00728	-0.0007	0.012927	-0.00022	-0.0011	-0.00018	-0.00097	-0.00045	-0.0002	-0.00244	-0.00398
3826.762	0.000409	-0.00686	-0.00133	0.012871	0.000569	-0.0011	-0.00013	-0.00022	-0.00109	0.000346	-0.00164	-0.00531
3842.192	-0.00017	-0.00703	-0.0016	0.012714	0.000314	-0.00126	-0.00104	-0.00039	-0.00257	0.000224	-0.00142	-0.00562
3857.623	-0.00076	-0.00727	-0.00102	0.012663	-0.00087	-0.00125	-0.0012	-0.00119	-0.00307	3E-05	-0.00233	-0.0045
3873.053	-0.001	-0.00651	-0.00041	0.013614	-0.00102	-0.00152	-0.00122	-0.00067	-0.00223	-0.00041	-0.0019	-0.00482
3888.484	-0.00146	-0.00594	-0.00068	0.013753	-0.00075	-0.00184	-0.00097	0.000332	-0.00155	-0.00089	-0.00059	-0.00516
3903.914	-0.00104	-0.00686	-0.00172	0.013268	-0.00087	-0.00106	-0.00073	-0.00039	-0.00129	-0.00047	-0.00027	-0.00527
3919.345	-7.6E-05	-0.00718	-0.00232	0.013242	-0.00062	-0.00019	-0.0014	-0.0014	-0.0024	-0.00063	-0.00071	-0.00482
3934.775	-0.00015	-0.00657	-0.00121	0.013181	-0.00057	-0.00096	-0.00167	-0.00177	-0.0026	-0.00096	-0.00022	-0.00459
3950.206	-0.00054	-0.0066	-0.00079	0.013063	-0.00093	-0.00134	-0.00152	-0.0016	-0.00188	-0.00029	-0.00027	-0.00568
3965.636	-0.00039	-0.00668	-0.00194	0.014027	-0.00137	-0.00051	-0.00151	-0.00123	-0.0017	0.000272	-0.00165	-0.00564
3981.067	-0.00048	-0.00702	-0.00177	0.015111	-0.00124	-0.0006	-0.00203	-0.00139	-0.00173	0.000683	-0.00248	-0.00429
3996.497	-0.00084	-0.00656	-0.00118	0.014925	-0.0002	-0.00136	-0.00189	-0.00076	-0.00239	0.000636	-0.00186	-0.00362
4011.928	-0.00054	-0.00641	-0.00125	0.014063	0.000311	-0.00135	-0.00089	-0.00032	-0.0028	-0.00035	-0.00096	-0.00392

Appendix 4: Supplement Product

Appendix 5: Nucleotide BLAST

Porcine Reverse

Descriptions

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Sus scrofa isolate WBMoroc5 cytochrome b (CYTB) gene, complete cds; and tRNA-Thr gene, partial seq	44.1	44.1	100%	0.024	100%	KU664553.1
Sus scrofa isolate WBMoroc4 cytochrome b (CYTB) gene, complete cds; and tRNA-Thr gene, partial seq	44.1	44.1	100%	0.024	100%	KU664548.1
Sus scrofa isolate WBMoroc3 cytochrome b (CYTB) gene, complete cds; and tRNA-Thr gene, partial seq	44.1	44.1	100%	0.024	100%	KU664547.1
Sus scrofa isolate WBMoroc2 cytochrome b (CYTB) gene, complete cds; and tRNA-Thr gene, partial seq	44.1	44.1	100%	0.024	100%	KU664546.1
Sus scrofa isolate WBMoroc1 cytochrome b (CYTB) gene, complete cds; and tRNA-Thr gene, partial seq	44.1	44.1	100%	0.024	100%	KU608293.1
Sus scrofa isolate Vietnam_MBT4 mitochondrion, complete genome	44.1	44.1	100%	0.024	100%	KX982660.1
Sus scrofa isolate Vietnam_BV02 mitochondrion, complete genome	44.1	44.1	100%	0.024	100%	KX982659.1
Sus scrofa isolate Vietnam_BA19 mitochondrion, complete genome	44.1	44.1	100%	0.024	100%	KX982656.1
Sus scrofa isolate Vietnam_BA9 mitochondrion, complete genome	44.1	44.1	100%	0.024	100%	KX982654.1
Sus scrofa isolate Vietnam_BA7 mitochondrion, complete genome	44.1	44.1	100%	0.024	100%	KX982653.1
Sus scrofa isolate Vietnam_MCN16 mitochondrion, complete genome	44.1	44.1	100%	0.024	100%	KX982652.1
Sus scrofa isolate Vietnam_MCC09 mitochondrion, complete genome	44.1	44.1	100%	0.024	100%	KX982651.1
Sus scrofa isolate Vietnam_MCB40 mitochondrion, complete genome	44.1	44.1	100%	0.024	100%	KX982650.1
Sus scrofa isolate Vietnam_MCB27 mitochondrion, complete genome	44.1	44.1	100%	0.024	100%	KX982649.1

Porcine Forward

Descriptions

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Sus scrofa mRNA, clone: DCI010010A07, expressed in dendritic cells (immature)	50.1	50.1	100%	7e-04	100%	AK399531.1
Sus scrofa isolate WBMoroc4 cytochrome b (CYTB) gene, complete cds; and tRNA-Thr gene, partial sec	50.1	50.1	100%	7e-04	100%	KU664548.1
Sus scrofa isolate WBMoroc3 cytochrome b (CYTB) gene, complete cds; and tRNA-Thr gene, partial sec	50.1	50.1	100%	7e-04	100%	KU664547.1
Sus scrofa isolate WBMoroc2 cytochrome b (CYTB) gene, complete cds; and tRNA-Thr gene, partial sec	50.1	50.1	100%	7e-04	100%	KU664546.1
Sus scrofa isolate WBMoroc1 cytochrome b (CYTB) gene, complete cds; and tRNA-Thr gene, partial sec	50.1	50.1	100%	7e-04	100%	KU608293.1
Sus scrofa mitochondrial Cytb gene for cytochrome b, partial cds, note: 554_PHLEBO	50.1	50.1	100%	7e-04	100%	LC088167.1
Sus scrofa mitochondrial Cytb gene for cytochrome b, partial cds, note: 152_PHLEBO	50.1	50.1	100%	7e-04	100%	LC088166.1
Sus scrofa mitochondrial Cytb gene for cytochrome b, partial cds, note: 73_PHLEBO	50.1	50.1	100%	7e-04	100%	LC088165.1
Sus scrofa mitochondrial Cytb gene for cytochrome b, partial cds, note: 66_PHLEBO	50.1	50.1	100%	7e-04	100%	LC088164.1
Sus scrofa mitochondrial Cytb gene for cytochrome b, partial cds, note: 64_PHLEBO	50.1	50.1	100%	7e-04	100%	LC088163.1
Sus scrofa mitochondrial Cytb gene for cytochrome b, partial cds, note: 58_PHLEBO	50.1	50.1	100%	7e-04	100%	LC088162.1
Sus scrofa mitochondrial Cytb gene for cytochrome b, partial cds, note: 56_PHLEBO	50.1	50.1	100%	7e-04	100%	LC088161.1

Appendix 6: The Calculation of Annealing Temperature

Primer		Sequence
Bovine	Forward	5'-CCCGATTCTTCGCTTTCCAT-3'
	Reverse	5'-CTACGTCTGAGGAAATTCCTGTTG-3'
Porcine	Forward	5'-CTTGCAAATCCTAACAGGCCTG-3'
	Reverse	5'-CGTTTGCATGTAGATAGCGAATAAC-3'

$$Tm = 2\ ^{o}C\ (A + T) + 4\ ^{o}C\ (G + C)$$

Bovine Primer

Forward

$Tm = 2\ ^{o}C\ (2+8) + 4\ ^{o}C\ (2+8) = 60\ ^{o}C$

Reverse

$Tm = 2\ ^{o}C\ (5+8) + 4\ ^{o}C\ (6+5) = 70\ ^{o}C$

$Means = (60\ ^{o}C + 70\ ^{o}C) / 2$
$= 65\ ^{o}C$

$Ta = 60\ ^{o}C$

Porcine Primer

Forward

$Tm = 2\ ^{o}C\ (6+5) + 4\ ^{o}C\ (4+7) = 66\ ^{o}C$

Reverse

$Tm = 2\ ^{o}C\ (8+7) + 4\ ^{o}C\ (6+3) = 66\ ^{o}C$

$Means = (66\ ^{o}C + 66\ ^{o}C) / 2$
$= 66\ ^{o}C$

$Ta = 61\ ^{o}C$

Appendix 7: The Ct Value of 5-Fold Series of DNA Standard

DAY	Sample	Replicate	The Concentration series (ng/µl)				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Day 1	Bovine	1	17.68	20.65	24.08	27.86	31.25
		2	17.74	20.48	23.70	28.36	32.29
		Mean	17.71	20.52	23.89	28.09	31.77
	Porcine	1	19.23	22.21	26.01	27.24	33.87
		2	19.77	22.53	26.01	28.18	34.15
		Mean	19.50	22.87	26.01	27.71	34.01
Day 2	Bovine	1	16.26	20.25	23.96	27.68	30.84
		2	17.08	20.97	24.50	27.94	31.92
		Mean	16.67	20.61	24.23	27.81	31.38
	Porcine	1	19.04	22.76	26.01	28.19	33.84
		2	19.52	23.54	26.01	27.65	34.38
		Mean	19.28	23.15	26.01	27.92	34.11
Day 3	Bovine	1	19.82	24.16	26.06	28.13	32.73
		2	20.28	25.12	26.34	28.69	32.58
		Mean	20.05	24.64	26.20	28.41	32.79
	Porcine	1	21.19	25.19	29.16	32.71	35.26
		2	20.27	23.09	27.53	30.15	35.28
		Mean	20.73	24.14	28.35	31.43	35.27
σ		Bovine	1.577	2.128	1.138	0.368	0.754
		Porcine	0.791	1.068	1.311	2.066	0.655
RSD (%)		Bovine	8.691	9.698	4.595	1.308	2.361
		Porcine	3.987	4.601	4.895	7.118	1.902