

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Introduction

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**).

4.2 Results and Discussion

4.2.1 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/ μ l (**Figure 4.1; 4.2**).

1. Specificity

The specificity of the porcine and bovine specific primer PCR assay was assessed with 1 ng/ μ l 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.

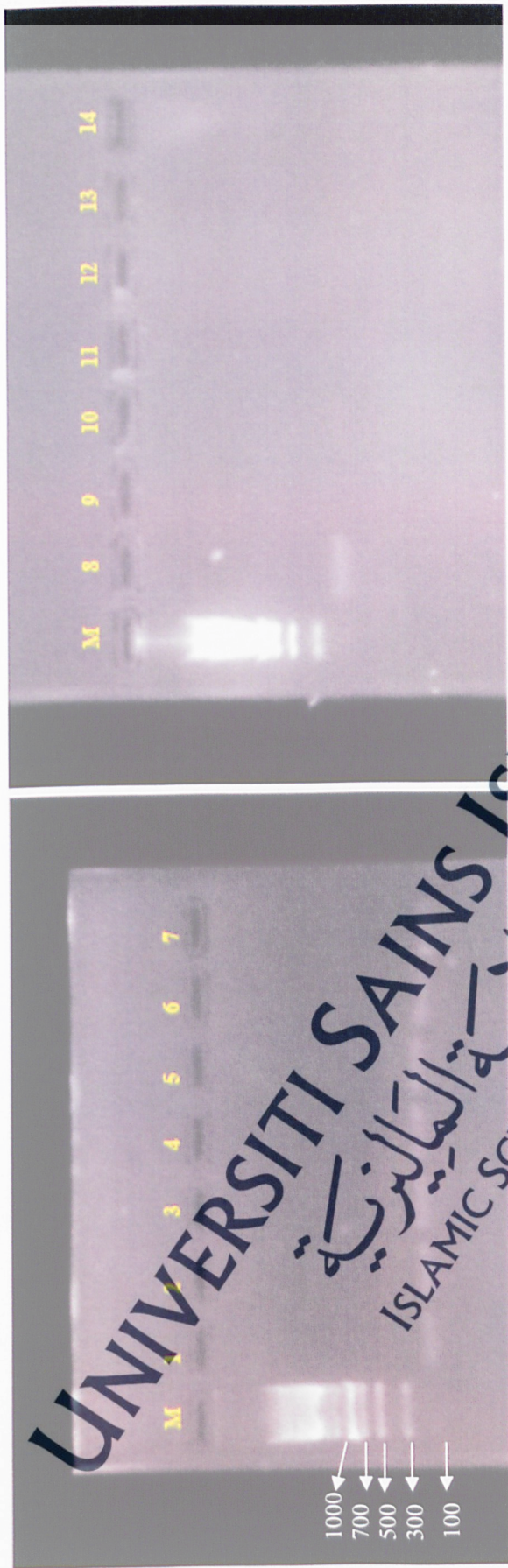
According to the mentioned figures, there isn't observed cross reaction of our interested primers with genomic DNA from cow that confirms the high specificity of the assay (**Figure 4.1**). Tanabe et al. (2007) with bovine primer showed that the sequence was able to detect bovine DNA specifically. However, 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 4.1: 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/ μ l; Lanes 3-4: 0.01 ng/ μ l; Lanes 5-6: 0.001 ng/ μ l; Lanes 7-8: 0.0001 ng/ μ l; Lanes 9-10: 0.00001 ng/ μ l; Lanes 11-13: negative control; Lane 14: blank.

FIGURE 4.2: Visualization of Conventional PCR Product with Specific Porcine Primer



Gel analysis of the Conventional PCR products of 2-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: 0.4 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; Lane 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/ μ l) are used. Porcine DNA could still be amplified up to 10^{-5} ng/ μ l, while at 10^{-5} ng/ μ l, bovine DNA was not amplified. These results were shown in **Figure 4.1** and **Figure 4.2**, where it could be seen that samples of bovine DNA containing 10^{-5} ng/ μ l (Lanes 9-10) were all found to be negative (**Figure 4.1**), while at the 10^{-5} ng/ μ l 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.

4.2.2 Validation of Real-Time PCR

The so 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/ μ l 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 4.1: Mean Ct Values Obtained with the Real-Time PCR

Concentration (ng/ μ l)	Porcine Primer		Bovine Primer	
	Mean $\pm$ SD	% RSD	Mean $\pm$ SD	% RSD
10^{-1}	18.14 $\pm$ 0.79	3.99	19.84 $\pm$ 1.58	8.69
10^{-2}	21.92 $\pm$ 1.07	4.60	23.22 $\pm$ 2.13	9.70
10^{-3}	24.77 $\pm$ 1.31	4.90	26.79 $\pm$ 1.14	4.60
10^{-4}	28.11 $\pm$ 2.07	7.12	29.02 $\pm$ 0.37	1.31
10^{-5}	31.94 $\pm$ 0.66	1.90	34.46 $\pm$ 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 Applied Biosystem 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 4.1). 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).

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: $\text{Efficiency} = 10^{(-1/\text{slope})} - 1$.

FIGURE 4.3: Standard Curves of 5-fold Dilutions of Porcine and Bovine gDNA



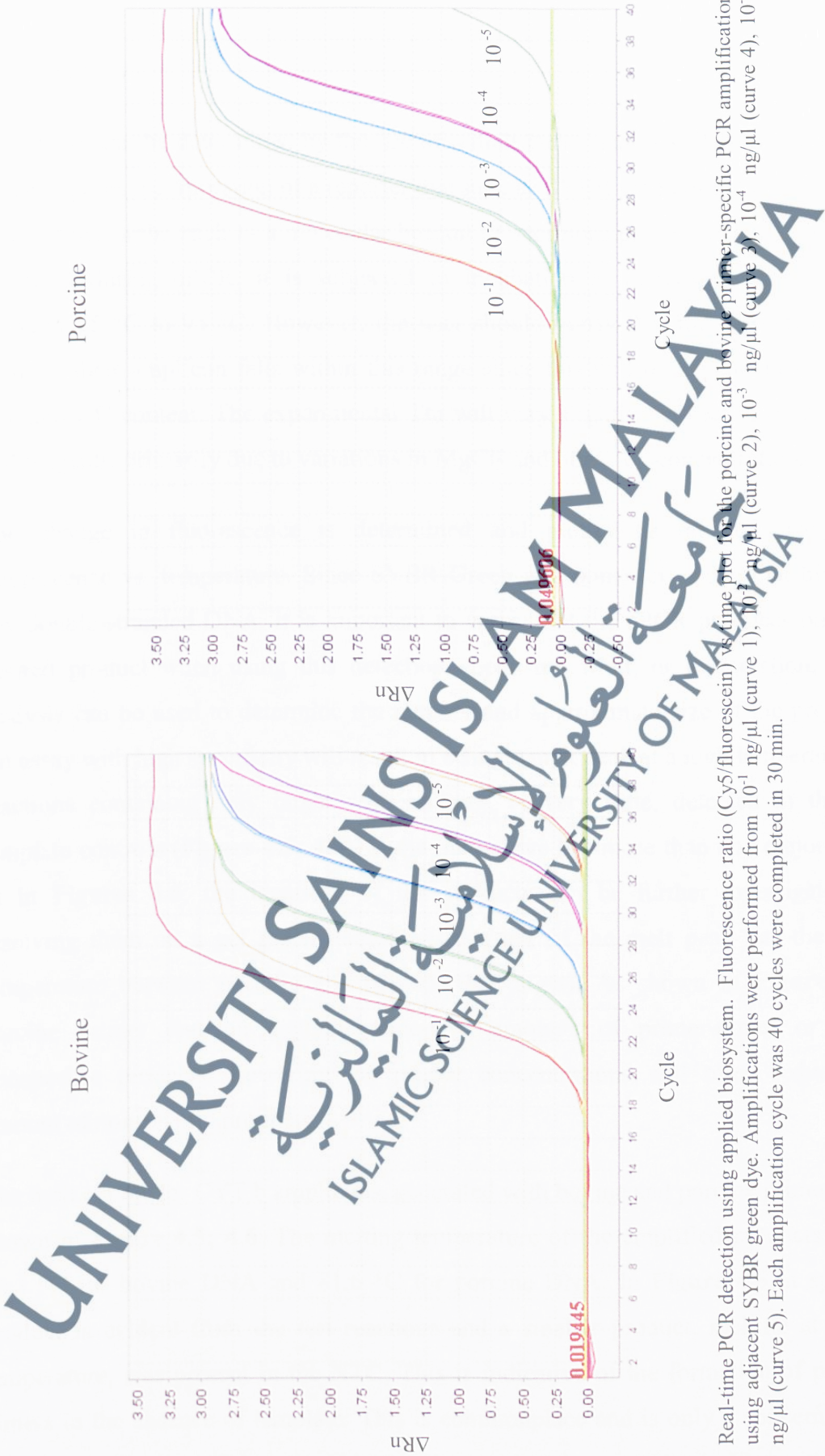
A standard curve was generated using a 5-fold dilution of a template amplified on the StepOnePlus real-time system. Standard curve with the Ct plotted against the log of the starting quantity of template for each dilution. The equation for the regression line and the r value are shown above the graph.

The 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: 4.3**), bovine primer shows R^2 value: 0.995; efficiency: 90.6%, slope: -3.569 (**Figure: 4.3**).

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^{-3} ng/ μ l (**Figure 4.4**). 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 primer-specific PCR amplification using adjacent SYBR green dye. Amplifications were performed from 10^{-1} ng/ μ l (curve 1), 10^{-2} ng/ μ l (curve 2), 10^{-3} ng/ μ l (curve 3), 10^{-4} ng/ μ l (curve 4), 10^{-5} ng/ μ l (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 T_m 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 4.5; 4.6**. The melting temperature of the amplified products were; 80.3 °C for bovine DNA and 81.6 °C for porcine DNA. In **Figure 4.5**, 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 primerdimer products are evident in the test samples as shown in **Figure 4.5**.

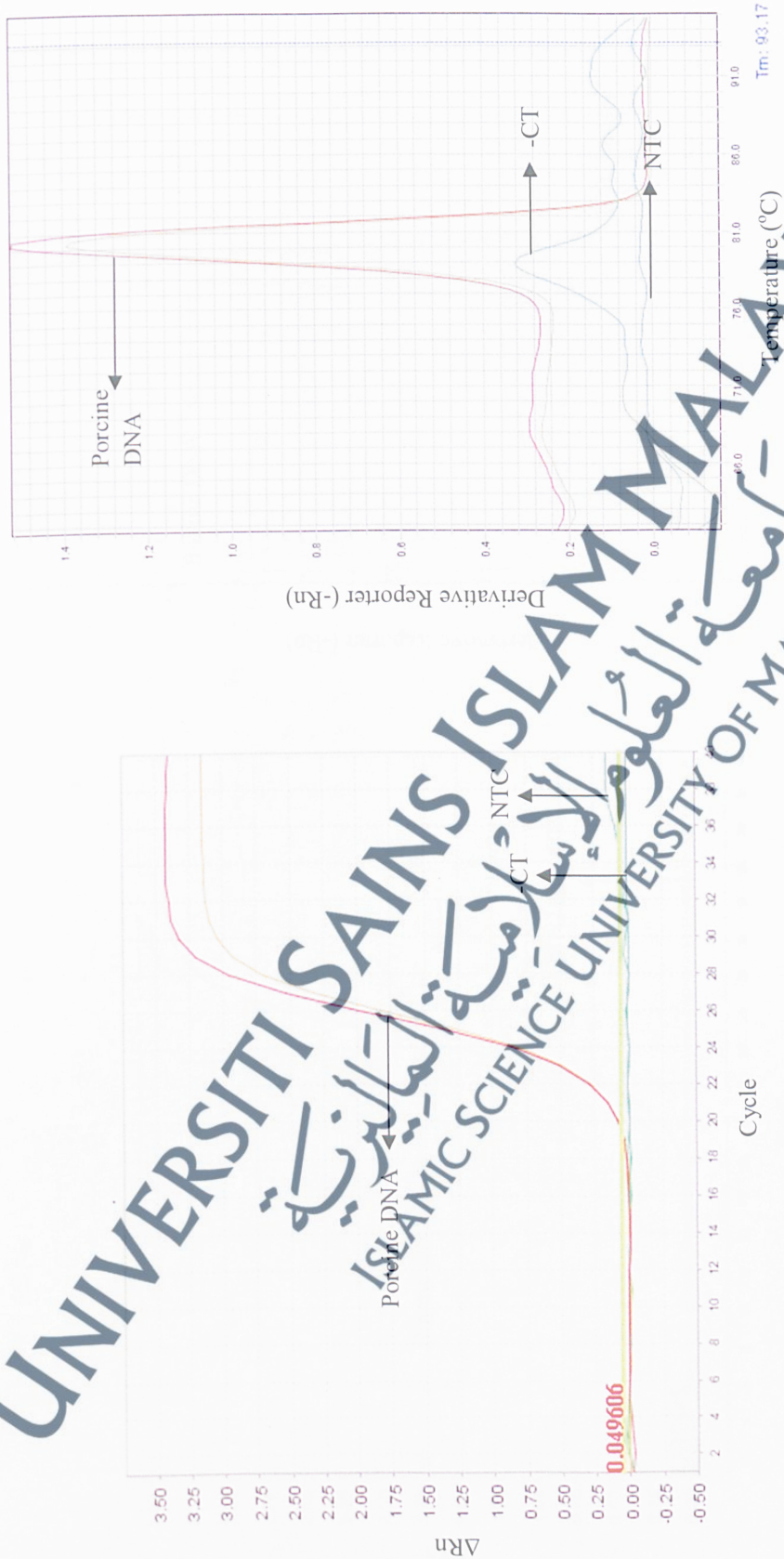
The illustrated in **Figure 4.5** and **Figure 4.6** showed detection of the gDNA was distinguished by their melt profile with the gDNA product melting at a lower temperature compare to non-specific product that have a higher temperature ($\pm 90^{\circ}\text{C}$).

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).

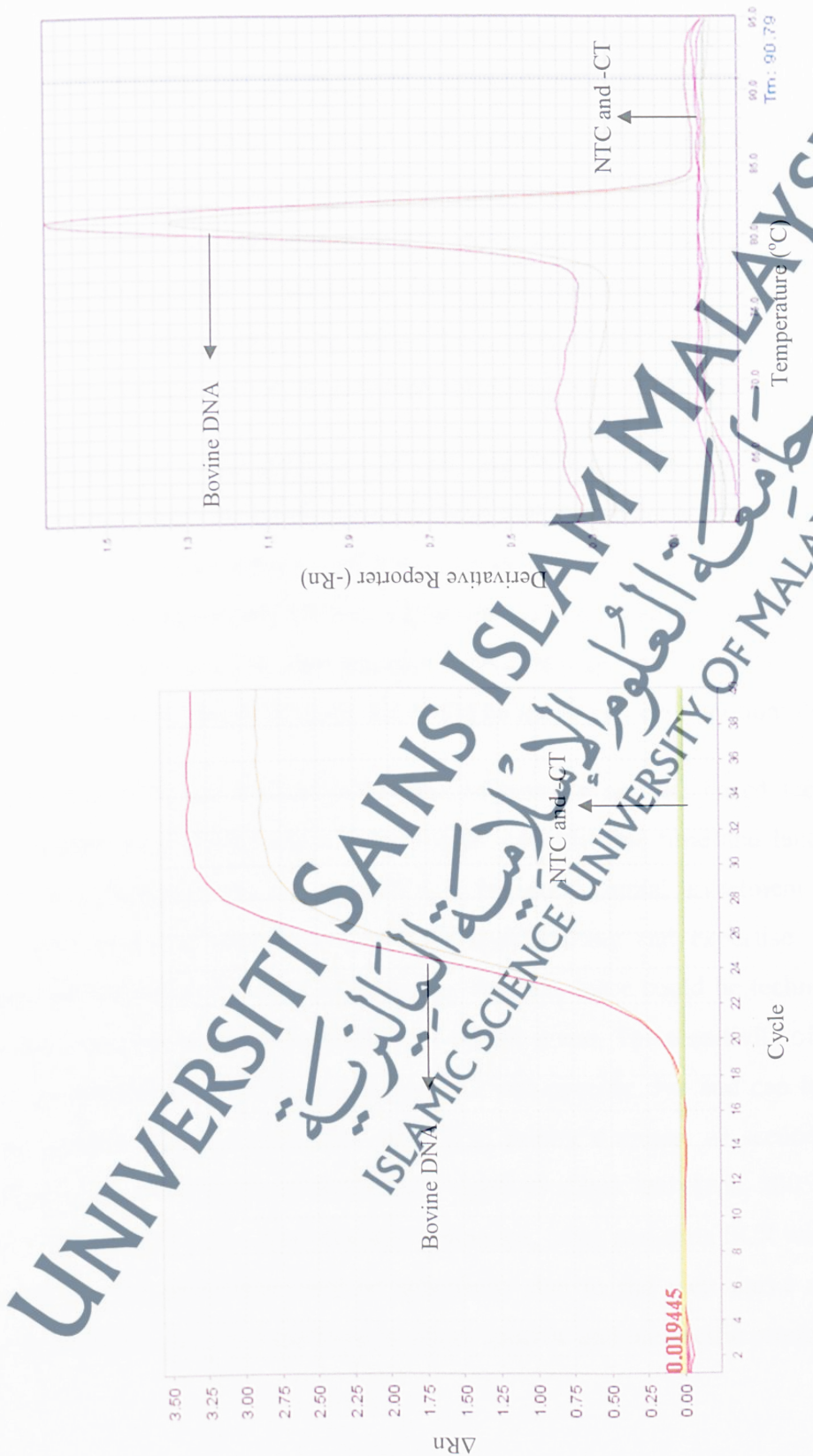
The main advantages of SYBR green assays over other real-time PCR detection formats are: 1) SYBR green is a low-cost fluorochrome; 2) SYBR green assays are simpler to use, especially in regard to primer design and optimization procedures; and 3) artifacts commonly observed in specific probes, particularly 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 4.5: The Amplification Plot and Melt Curve Analysis for Porcine



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

FIGURE 4.6: 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 dimers 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 (**section 4.2**) 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 4.3**). For confirmation result, we conducted FTIR technique and chemometrics using software Unscrambler X.

4.2.3 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**).

In this study 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 4.2: The Chemical Reagents Used in Agilent, Qiagen and Kogene Porcine Detection Kits

Stage	Agilent	Qiagen Reagent	Kogene
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 Rb	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 4.3, 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 study (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 4.3).

TABLE 4.3: 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 study 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.

4.2.4 Real-Time PCR Analysis

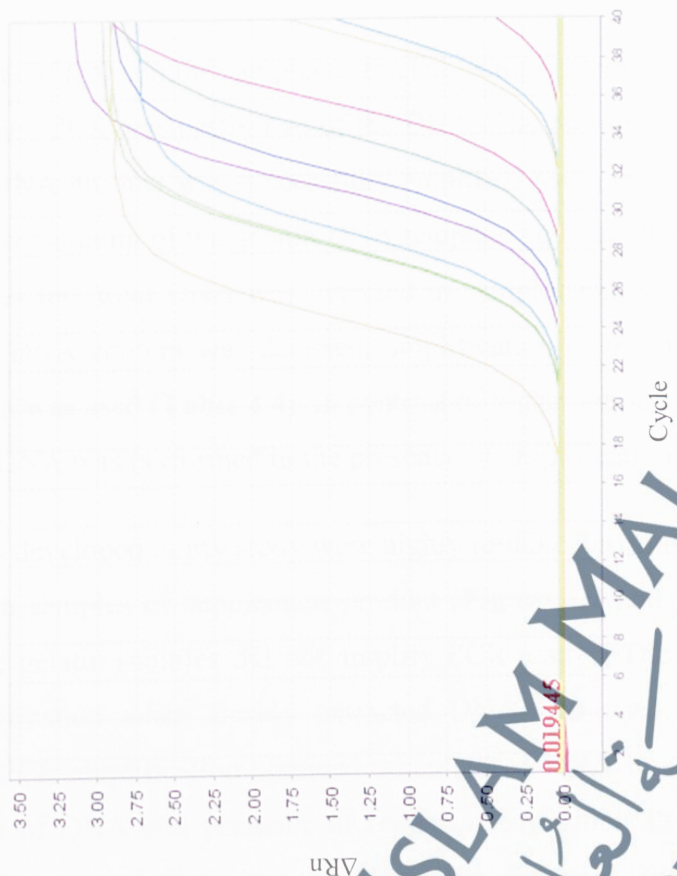
The qPCR assays (section 4.2.2) were further evaluated for species determination and quantization from gelatin capsules. The extracted DNA of isolated gelatin from 20 supplements was tested by three replicates of each sample and in addition to a negative control was tested in each run. 4 gelatin samples were positive for bovine primer, 2 samples were positive for porcine primer, and 6 samples were positive for both primers (Figure 4.7).

FIGURE 4.7: Amplification Plot of Capsule Gelatin in Supplement

Porcine Test



Bovine Test



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 4.4**). 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 my study were highly reliable due to positive result obtained from gelatin samples of supplement product (**Figure 4.7** and **Table 4.4**). 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/ μ l of porcine and bovine DNA was used for determining unknown amounts of porcine and bovine target DNA in gelatin capsules. The Equation is $Ct = 13.689 - 3.569C$ for bovine DNA and $Ct = 15.862 - 3.386C$ 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 4.4**.

TABLE 4.4: 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 ± 0.25	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	100	-	-	-

The results of a small survey of gelatin products were shown in **Table 4.4**. 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 4.5**).

TABLE 4.5: Concentration of Porcine and Bovine DNA in Gelatin Capsules

Sample	Concentration (ng/ μ l)	
	Porcine Mean $\pm$ SD	Bovine Mean $\pm$ 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	-	$1.10 \times 10^{-3} \pm 1.35$
S18	$2.98 \times 10^{-4} \pm 0.28$	$1.11 \times 10^{-3} \pm 0.29$

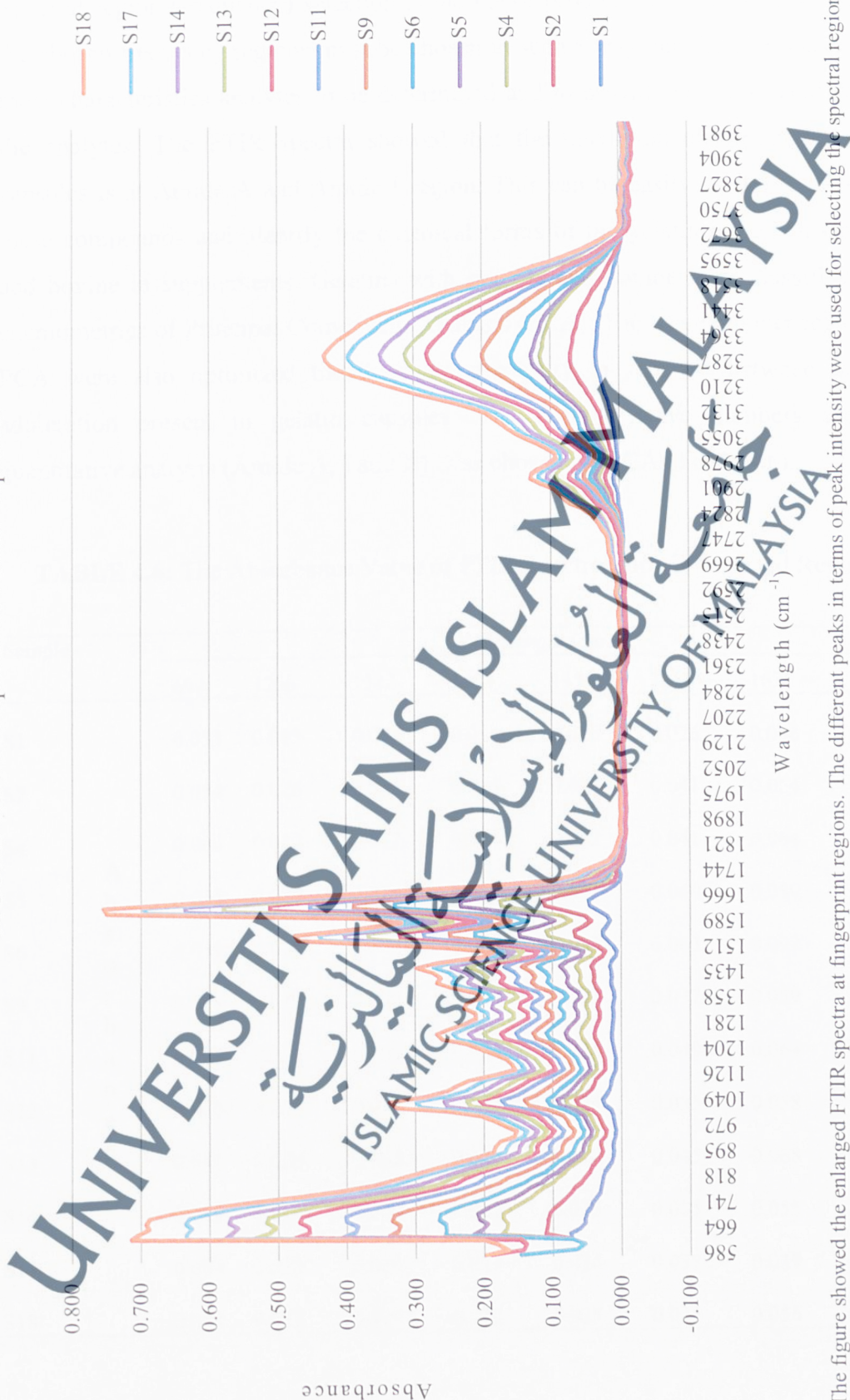
The mitochondrial gene encoding of the CYT b was chosen as a target for porcine and bovine DNA quantification. As shown in Figure 4.7, different concentrations of gelatin capsules were detected based on the threshold cycle (Ct) of each sample which is the cycle number where the samples fluorescent curve 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/ μ l of the pig and bovine tissues in experimentally made gelatin capsule (Figure 4.7). 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.

4.2.5 FTIR and Chemometrics Analysis

The importance of IR spectroscopy for the qualitative analysis 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 4.8 showed FTIR spectra of gelatin capsule from previous study (Section 4.3.2). The spectra look very similar and show a typical absorption band of gelatin structure (Figure 2.8). Four regions involves are $3600\text{--}2300\text{ cm}^{-1}$ (Amide A), $1656\text{--}1644\text{ cm}^{-1}$ (Amide I), $1560\text{--}1335\text{ cm}^{-1}$ (Amide II) and $1240\text{--}670\text{ cm}^{-1}$ (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 4.8). 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 ($3600\text{--}2300\text{ cm}^{-1}$) 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 $1660\text{--}1620\text{ cm}^{-1}$ region which is often referred to as Amide I band. The frequency range $1660\text{--}1650\text{ cm}^{-1}$ was known as a-helical and $1640\text{--}1620\text{ cm}^{-1}$ as b-sheets structures. The frequency range of $1550\text{--}1520\text{ cm}^{-1}$ is due to Amide II with a-helical structure between $1550\text{--}1540\text{ cm}^{-1}$ and b-sheets at $1525\text{--}1520\text{ cm}^{-1}$. The Amide II vibration is caused by deformation of the N-H bonds. Fischer et al. (2005) and Lagant et al. (1983) attributed $1500\text{--}1200\text{ cm}^{-1}$ 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 4.8: 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 gelatin capsule in supplement product.

Spectral region (frequency) selection is the major problem in FTIR analysis 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 adulteration present in gelatin capsules. The optimal wave numbers used for quantitative analysis (Amide A, I and II), was chosen for PCA (Table 4.6).

TABLE 4.6: The Absorbance Value of FTIR Spectroscopy in Selected Regions

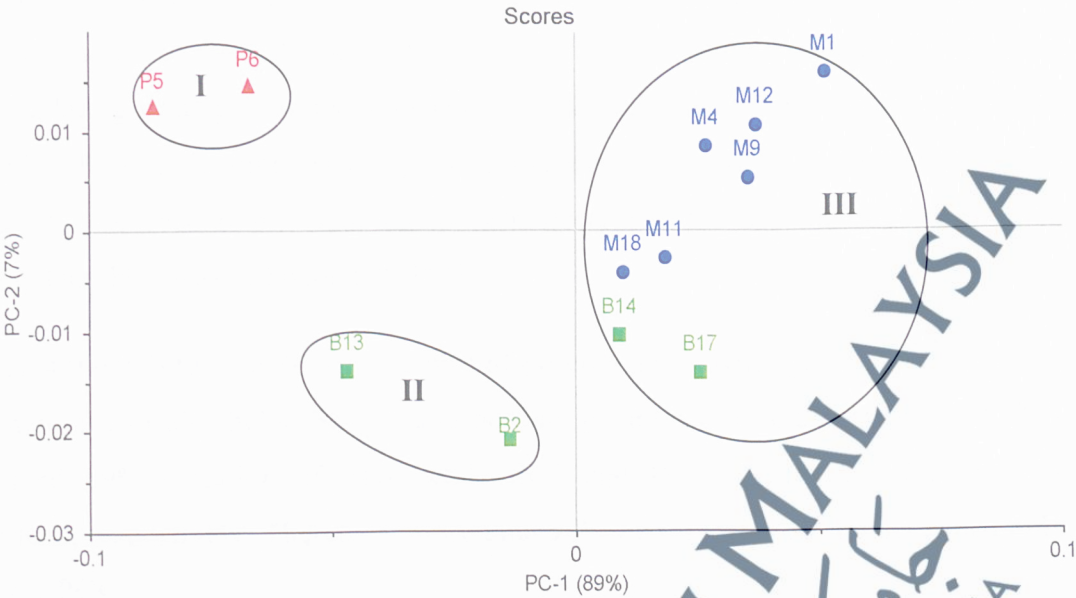
Sample	Wavelength (cm ⁻¹)							
	694	1250	1342	1404	1450	1558	1636	3271
S1	0.053	0.013	0.011	0.014	0.016	0.026	0.046	0.032
S2	0.054	0.026	0.022	0.025	0.027	0.041	0.064	0.037
S4	0.066	0.020	0.017	0.021	0.022	0.041	0.064	0.040
S5	0.035	0.022	0.016	0.019	0.023	0.040	0.059	0.013
S6	0.059	0.028	0.022	0.028	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 *glystine*.

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).

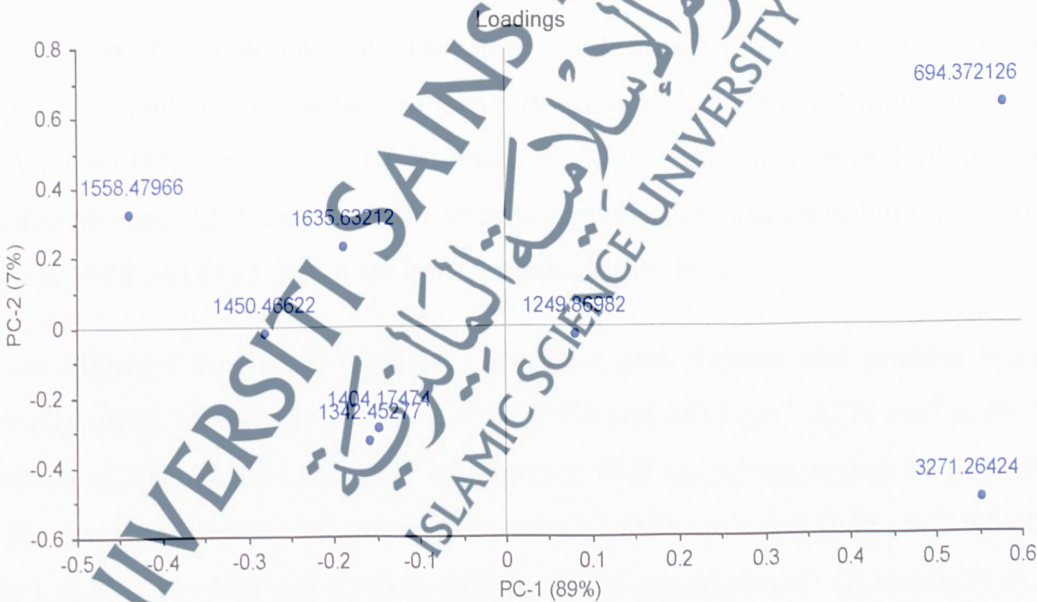
Infrared spectroscopy is employed to study structural properties of polymeric compounds such as gelatin capsules. In many cases however, determining the wavelength and absorbance of individual bands becomes difficult due to the presence of other interfering bands. Such problems can be overcome by differentiating IR spectra. The most frequently used preprocessing tools for multivariate analysis 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 4.9: 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 4.10: PCA loading plot (PC1 vs. PC2)



Normalization and smoothing of the FT-IR spectrum of gelatin capsule was shown in **Figure 4.9**. 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 4.9 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 4.10 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 wavenumber 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 4.10**, 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 4.10**, 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 analysis 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.