CHAPTER III

METHODOLOGY

3.1 Materials and Instruments

3.1.1 Chemicals and Reagent

NO	Chemicals / Reagent	Manufacture
1	Genomic DNA of Cattle	Eurofins
2	Genomic DNA of Pig	Eurofins
3	Sso advanced SYBR green	Bio-rad
4	Porcine Primer	IDT Technologies
5	Bovine Primer	IDT Technologies
6	TAE Buffer	Fisher Scientific
7	Agarose Gel	Fisher Scientific
8	Green Nucleic Acid Stain Gel	Biotium
9	Porcine Detection Kits	Agilent
10	DNeasy Mericon Food Kit	Qiagen
11	PowerPrep DNA Extraction from Food and Feeed Kit	Kogenebiotech
12	Ethanol	Systerm
13	Iso-Propanol	QRec
14	Chloroform	QRec

3.1.2 Instrumentation

NO	Instruments	Model, Brand
1	Conventional PCR	T100 Thermal Cycler, BioRad
2	Gel Documentation	UVIDOC HD6, Uvitec
3	Gel Caster	Sub-Cell GT Agarose Gel Electrophoresis Systems, BioRad
4	Analytical Balance	ME 103E, Mettler Toledo
5	Shaker Incubator	Thermomixer Comfort 1.5 mL tubes, Eppendorf
6	Real-Time PCR	Applied Biosystem StepOnePlus, Life Technologies
7	Microcentrifuge	MiniSpinPlus, Eppendorf
8	UV/Vis Spectrophotometer	BioDrop µLiTE, BioDrop
9	FTIR Spectroscopy	Varian 640-IR, Varian
10	Micro Tube	1.5 mL, Eppendorf
11	Pipettes	1000 µl; 200 µl; 10 µl, Eppendorf
12	Tips	1000 µl; 200 µl; 10 µl No Filter, Eppendorf

3.2 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 analysis.

3.3 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.

3.3.1 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).

3.3.2 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 ^{o}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 ^{o}C (Qiagen, 2010).

3.3.3 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 ^{o}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 ^{o}C (Kogene, 2014).

3.4 Analysis of DNA Isolated using Biodrop

The DUO Biodrop instrument with touch screen system was used in this analysis. 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).

3.5 Polymerase Chain Reaction Analysis

PCR amplifications were conducted by mixing the reagent and diluted porcine and bovine DNAs to a total volume of 20 µl (**Table 3.1**).

TABLE 3.1: The Mixture of Reagent in PCR Analysis (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 3.2**).

TABLE 3.2: 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'	

3.5.1 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.

3.5.2 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 analysis.

3.6 FTIR Analysis

FTIR was used to determine the chemical bonds from the vibration of the compound at a particular wavelength. FTIR analysis of gelatin can be done in two ways (1) is done by comparing the spectral pattern of gelatin standard (**Figure 2.8**) 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 analysis was made on the frequency 4000-650 cm^{-1}. The all value of absorbance in the FTIR spectrum would be selected for the cluster analysis using PCA.

3.7 Chemometrics Analysis

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.