CHAPTER I

INTRODUCTION

1.1 Background of Study

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 available at the present time. Gelatin is not a naturally occurring protein, but a high molecular weight polypeptide obtained by hydrolysis of a 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 competition by a growing legion of users including leather tanners, an expanding snack industry and the use of gelatin capsule in supplement and pharmaceutical product (GMIA, 2012).

In the market, porcine gelatin is cheaper that 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 no 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 there are cases that 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 he 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 with respect to 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. There are a number of molecular techniques that can be used to identify the origin of gelatin products such as high performance liquid chromatography coupled with fluorescence detector and chemometrics of PCA (Nemati et al., 2004; Rohman et al., 2013), enzyme-linked immuno-sorbent assay (ELISA) (Doi et al., 2009; Venien and Levieux, 2005) and DNA-based techniques. It is reported that protein-based analytical techniques for the species identification in mixed samples are significantly less sensitive than DNA-based techniques for 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). In fact, DNA is a relatively stable molecule, which can better withstand heat processing and can be detect even though it will be in 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 this study 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. In this study, 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 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 as 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, comparison was done with conventional PCR. In this study, further analysis 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 ingredient to formulate gelatin capsules. The false declaration about *halalness* of gelatin products are usually made for profit gaining purpose and have a great impact to Muslim consumers. Therefore development of analytical methods for *halal* authentication in gelatin capsules will be very beneficial for Muslim community and other consumers.

1.2 Problem Statement

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 validated results from real-time PCR to improve sensitivity, specificity, efficiency and repeatability of the results.

1.3 Objectives

The objectives of this research are:

i. To compare results obtained from analysis of gelatin using real-time PCR and conventional PCR.

ii. To validate the *halal* status of gelatin capsules using FTIR spectroscopy and chemometrics.

1.4 Scope of Study

The current research on *halal* authentication of gelatin capsule 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 fluorescent dye, SYBR green, and annealing temperature of specific species primer from 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 chemometrics method. The methods with finger printings corresponding to *halal* status of the products were obtained. This can be further applied for *halal* authentication of other products.