DETECTION OF BOVINE AND PORCINE GELATINE IN DENTAL MATERIALS

BY

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A thesis submitted in fulfillment of the requirement for the degree of Master of Science (Halal Industry Science)

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ABSTRACT

The rapid innovation of technology leads to the availability of a variety of products, including medical and dental products which may contain ingredients that are from prohibited sources. The presence of pig derivatives, such as porcine gelatine, in any products is prohibited to be consumed by Muslim. The presence of gelatine is not limited to food products but has also been found in medical and dental products. Most dental materials available in Malaysia are imported from other countries and might contain gelatine which is a protein derived either from porcine, bovine or other animal sources. Authentication of gelatine is crucial due to religious and health concerns. Therefore, this study aimed to detect porcine and bovine gelatine in dental materials using attenuated total reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) combined with Principal Component Analysis (PCA) and Polymerase Chain Reaction (PCR). Forty-two dental material samples were analyzed in this study. ATR-FTIR analysis was carried out in order to distinguish the spectra between gelatine bovine and porcine gelatine standards. These spectra were then compared to the spectra of the dental material samples. All acquired data from ATR-FTIR were subjected to data pre-processing followed by PCA. Detection and discrimination of gelatine standards (bovine and porcine) and gelatine in dental materials were successfully achieved using ATR-FTIR combined with PCA within a small region, which is between wavenumber 1756-1584 cm^{-1} (Amide I and Amide II). Detection and discrimination of porcine and bovine gelatine were also carried out using conventional PCR. PCR methods were run to verify the presence of porcine and bovine in dental materials. Species-specific primers targeting the 212 bp porcine Cytochrome *b* gene and 271 bp bovine Cytochrome *b* were used to amplify both DNA in nine dental material samples. The species-specific primers were found to be specific and sensitive. The detection limit of DNA concentration was 0.001 ng/µL and 0.0001 ng/µL for bovine and porcine gelatines, respectively. Using PCR, one sample, BDM 01 was found to contain porcine DNA while two samples (BDM 01 and BDM 14) were found to be positive for bovine DNA. The presence of porcine DNA was also evaluated using commercial *Mericon* pig kit. However, the pig kit did not detect the presence of porcine DNA in all dental material samples. The findings suggested that ATR-FTIR combined with PCA and conventional PCR are applicable in the identification of porcine and bovine gelatine in highly processed products such as dental materials.

خلاصة البحث

أدى الابتكار السريع للتكنولوجيا إلى توفر مجموعة متنوعة من المنتجات، بما في ذلك المنتجات الطبية والأسنان التي قد تحتوي على مكونات من مصادر محظورة. يحظر على المسلمين تناول مشتقات الخنزير، مثل جيلاتين الخنازير في أي من المنتجات. لا يقتصر وجود الجيلاتين على المنتجات الغذائية فقط، لكن تم العثور عليه أيضًا في المنتجات الطبية والأسنان. يتم استيراد معظم مواد طب الأسنان المتوفرة في ماليزيا من بلدان أخرى وقد تحتوي على جيلاتين وهو بروتين مشتق جلود و عظام الخنازير أو الأبقار أو غيرها من المصادر الحيوانية. مصادقة الجيلاتين أمر بالغ الأهمية بسبب المخاوف الدينية والصحية. لذلك، تهدف هذه الدراسة إلى الكشف عن الجيلاتين الخنازير والبقري في مواد طب الأسنان باستخدام التحليل الطيفي للأشعة تحت الحمراء لتحويل فورييه الطيفي (ATR-FTIR) جنبًا إلى جنب مع تحليل المكونات الرئيسية (PCA) وتفاعل سلسلة البوليميريز (PCR). تم تحليل 42 عينة من مواد طب الأسنان في هذه الدراسة. تم إجراء تحليل ATR-FTIR من أجل التمييز بين الأطياف بين الجيلاتين البقري ومعايير الجيلاتين الخنازير. ثم تمت مقارنة هذه الأطياف بأطياف عينات المواد السنية. تعرضت جميع البيانات المكتسبة من ATR-FTIR للمعالجة المسبقة للبيانات تليها PCA. تم الكشف والتمييز في معايير الجيلاتين (الأبقار والخنازير) والجيلاتين في مواد طب الأسنان بنجاح باستخدام ATR-FTIR مع PCA داخل منطقة صغيرة، والتي تقع بين طيف الموجة 1756-1584 سم$^{-1}$ (أميد Am وأميد II). كما تم الكشف والتمييز من الخنازير والجيلاتين البقري خارج باستخدام PCR التقليدية. تم تشغيل طرق PCR للتحقق من وجود الخنازير والبقري في مواد طب الأسنان. تم استخدام البوليميراز الخاص بالأنواع التي استهدفت الجين Cytochrome b ذي 212 نبضة و 271 bp البقري Cytochrome b لتضخيم كلا الحمض النووي في تسع عينات من مواد طب الأسنان. تم العثور على البوليميراز الخاص بالأنواع لتكون محددة وحساسة. كان حد الكشف عن تركيز الحمض النووي 0.001 نانوغرام / ميكرولتر و 0.0001 نانوغرام / ميكرولتر للجيلاتين البقري والخنزيري، على التوالي. باستخدام PCR، عينة واحدة، وجد أن BDM 01 يحتوي على DNA الخنزيري، بينما تم العثور على عينتين (BDM 01 و BDM 14) إيجابية بالنسبة للحمض النووي البقري. تم تقييم وجود الحمض النووي للخنزير أيضا باستخدام عينة خنزير *Mericon* التجارية. ومع ذلك، فإن مجموعة الخنازير لم تكتشف وجود الحمض النووي الخنازير في جميع عينات مواد معاجة الأسنان . وتشير النتائج إلى أن ATR-FTIR جنبا إلى جنب مع PCA و PCR التقليدية كانت قابلة للتطبيق في تحديد الجيلاتين الخنازير والبقري في المنتجات فائقة التصنيع مثل مواد طب الأسنان.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Halal Industry Science).

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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This thesis is dedicated to my beloved parents, Irfan Kamaruddin and Ita Irawati, family, lecturers and friends.

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LIST OF ABBREVIATIONS

%	Percentage/ proportion
±	Plus-minus
ºC	Degree Celsius
µL	Microlitre
µM	Micromolar
ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infrared
bp	Base pair
cm	Centimeter
Cq	Quantification cycle
Cyt b	Cytochrome b
g	Gravitational force
DNA	Deoxyribonucleic acid
et al	*(et alia)*: and other
etc.	(*et cetera*): and so forth
g	gram
IIUM	International Islamic University Malaysia
IR	Infrared
L	Litre
M	Molar
mg	milligram
min	Minute
mL	millilitre
mtDNA	Mitochondrial DNA
ng//µL	Nanogram per microlitre
OD	Optical Density
PCR	Polymerase chain reaction
rpm	Revolutions per minute
S.D	Standard deviation
TAE	Tris-Acetate-EDTA
Taq	*Thermus aquaticus*
WHO	World Health Organization

CHAPTER ONE
INTRODUCTION

1.1 BACKGROUND OF THE STUDY

In recent years, there has been growing awareness among Muslims towards *Halal* integrity not only in meat-based products but also in a wider range of products, including cosmetics, personal care products, pharmaceuticals and dental products. It has been reported that more than 20% of Muslim consumers are concerned about the *Halal* status of products that they are using (Hunter, 2012). The rapid innovation of technology leads to the availability of a variety of products, including medical and dental products which may contain ingredients that are from permissible (*Halal*) or prohibited (*Haram*) sources. For the Muslim consumer, it is important that these innovations and their products should fall under Islamic principles.

The basic principle in Islam is that all things created by God (Allah) are permitted, with few exceptions that are specifically prohibited (non-*Halal*, or *Haram*). The foundation for prohibition in Islam is purely and firmly Quranic guidance (The divine book) from Allah (The Creator) to Prophet Mohammed S.A.W. for all mankind. This is stated in Chapter II, verse 172-173, Chapter V, verse 3-5 and Chapter VI, verse 145 of the Quran. Muslims of all ethnic and geographic backgrounds should stringently observe these commandments. In Islam, *Halal* products must not be derived from any doubtful source, such as porcine. Furthermore, *Halal* animals can be rendered *Haram* if they are not slaughtered according to Islamic laws. In addition to Muslims, Jews also have their dietary law called kosher law which forbids them from consuming porcine-derived products, while Hindus abstain from the consumption of bovine or cow-based products (Cai et al., 2012).

Gelatine is a protein derived from the hydrolyzed collagen of bones, hides and skins from various animals such as cattle, pig, fish and chicken (Liu et al., 2015). Gelatine is one of the most commonly used raw material in foods, pharmaceuticals (gelatine capsules), and cosmetic products (cream, facemasks and lotions). Gelatine is a hydrocolloid product with numerous functions in a wide range of applications across different industries. It is used as a gelling and foaming agent, thickener, plasticizer, emulsifier, moisture retainer, texture improver and binding agent. It was previously reported that gelatine derived from porcine (pig) skin is more abundant compared to gelatine derived from other animals such as cattle and fish (GMIA, 2012). Globally, 80% of gelatine manufactured is derived from pork by-products (Boran & Regenstein, 2010). Authentication of gelatine is crucial not only due to religious reasons but also for health concerns. Bovine sources, for example, have previously been associated with the outbreak of Bovine Spongiform Encephalopathy (BSE) or mad cow disease which is transmitted through infected cattle (OIE, 2011).

Dental materials are materials used in both laboratory and practices dentistry and can be categorized as preventive materials, restorative materials or auxiliary materials. Preventive dental materials are materials which contain antibacterial, fluoride or other therapeutic agents protecting against dental caries. Materials that can be applied in repairing or replacing the tooth structure can be categorized as restorative dental materials which mainly consist of synthetic components (Powers & Wataha, 2013). Meanwhile, materials which are used in fabricating dental prostheses are known as auxiliary dental materials. An example of auxiliary dental material is impression material (Anusavice et al., 2013). Most dental materials available in Malaysia are imported from other countries and might contain gelatine. There are few examples of dental materials that may contain gelatine such as dental haemostatic agent, dental

prophylaxis paste and toothpaste. The exact composition of these products is not clear since their labels do not mention the excipients used, which could include gelatine. It is essential for Muslim patients to seek treatment with *Halalan-Toyyiban* dental materials. Meanwhile, dental practitioners need to ensure that they treat their patients with *Halalan-Toyyiban* products as part of their professional and religious responsibility.

Most published authentication methods have focused on meat species identification rather than gelatine identification. Various analytical methods to detect the source of gelatine have been reported by researchers. These methods include amino acid analysis (Raja Mohd Hafidz, Yaakob, Amin, & Noorfaizan, 2011; Raraswati, Triyana, & Rohman, 2014), spectroscopic analysis (Hashim et al., 2010; Hermanto et al., 2013; Cebi et al., 2016; Azrul Hafiz et al., 2017) and DNA analysis (Tasara et al., 2005; Cai et al., 2012; Demirhan et al., 2012; Sahilah et al., 2012; Mutalib et al., 2015; Shabani et al., 2015; Wardani et al., 2015; Lee et al., 2016; Mohamad et al., 2016; Muñoz-Colmenero et al., 2016; Nikzad et al., 2017). Most studies report the detection of raw gelatine or gelatine present in processed foods or capsules. Meanwhile, studies reporting authentication of dental materials are limited since the authentication was done in only one specific type of dental material which was orthodontic dental material using ATR-FTIR (Azrul Hafiz et al., 2017). Hence, the present study aimed to detect porcine and bovine gelatine in other types of dental materials using attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) combined with Principal Component Analysis (PCA) and verified by Polymerase Chain Reaction (PCR).

1.2 PROBLEM STATEMENT AND ITS SIGNIFICANCE

Gelatine can be found not only in food products and pharmaceutical products such as capsules but also in dental materials. This has given rise to consumer concern pertaining to the animal origin of the gelatine derived. It is reported that most commercial gelatine is manufactured from slaughterhouse by-products of animals such as pig and cattle. Gelatine sourced from pigskin is significantly more abundant compared to other animals (GMIA, 2012).

Labelling of ingredients in dental products remains poor; some products disclose only the active ingredients used. However, dental materials also contain inactive ingredients called excipients, which improve the physical qualities of the product, such as colouring agents, thickening agents, diluents and flavourings. The list of inactive ingredients present in dental materials may include gelatine (Aziz et al., 2014). Detection of gelatine is important for religious and safety reasons. Muslims and Jews are prohibited from consuming any porcine-based products, while Hindus are prohibited from consuming cow-based products since cows are considered sacred in their religion. Most dental materials applied during dental treatment come into contact with the patient's saliva and blood, especially during tooth extraction. This may be considered a point of consumption or contamination with the material source, which may be against the patients' religious requirements. This study may contribute to the knowledge of the *ummah* regarding ingredients used in dental products, especially among doctors and patients. Furthermore, such knowledge could protect the consumer's religious beliefs, especially Muslim consumers. Apart from religious reasons, the detection of porcine or bovine gelatine in dental materials is important for patients with gelatine allergies (Doi et al., 2009).

Studies on the detection of porcine gelatine in dental materials are limited. A previous study reported the authentication of *Halal* dental materials; however, the study was specific to orthodontic dental materials and was analyzed by means of ATR-FTIR (Azrul Hafiz et al., 2017). In the present study, the authentication of bovine and porcine gelatine was analyzed in other types of dental materials using ATR-FTIR combined with PCA and further verified with PCR. PCR can serve as a better alternative method in validating the presence of bovine or porcine gelatine since DNA is more stable which can withstand high temperature and pressure treatment (Soares et al., 2013). In addition, authentication of porcine and bovine gelatine by means of PCR has had a greater success due to higher specificity and sensitivity (Cai et al., 2012; Shabani et al., 2015; Lee et al., 2016; Nikzad et al., 2017) compared to ATR-FTIR. On the other hand, ATR-FTIR has been reported to be successful in authenticating raw gelatine, not in the mixture of gelatine (Hashim et al., 2010).

1.3 RESEARCH OBJECTIVES

1.3.1 General Objective

To detect porcine and bovine gelatine in dental materials using ATR-FTIR and PCR.

1.3.2 Specific Objectives

i. To detect the presence of porcine and bovine gelatine in dental materials using Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectroscopy.

ii. To discriminate between porcine and bovine gelatine in dental materials by means of chemometric analysis namely Principal Component Analysis (PCA).

iii. To verify porcine and bovine DNA in dental materials containing gelatine by means of PCR analysis.

1.4 RESEARCH QUESTIONS

i. Can porcine and bovine gelatine in dental materials be detected using Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectroscopy?

ii. Can porcine and bovine DNA be detected in gelatine and gelatine-containing dental materials?

1.5 RESEARCH SCOPE

The study design was based on an experimental study in order to develop a method for porcine and bovine gelatine detection in dental materials. Samples were limited to dental materials or products used in Polyclinic International Islamic University Malaysia, Kuantan, and Pahang. Most of the dental materials such as dental prophylactic paste, fluoride gel, impression materials, dental polymer and impression materials used in the Polyclinic Kuantan, Pahang were imported from other countries. These countries include the United States of America, Germany, United Kingdom, Switzerland, and France. The samples did not have any *Halal* label on the packaging. Dental materials that were *Halal* certified by Malaysian standards and commercially available in the market were not included in this study.

1.6 OUTLINE OF THE THESIS

This thesis comprises five chapters. Chapter one of the thesis describes the general background of the research study, the specific objectives and the research scope. Chapter two presents the literature on previous studies, especially porcine and bovine gelatine detection in various products, analytical methods pertaining to authentication of gelatine, as well as information on gelatine and classification of dental materials. In Chapter Three, the materials and methods employed are explained in detail. Chapter four presents the results of the analysis and discussions on the results obtained compared with previous studies. Chapter five describes the outcome or conclusion based on the results obtained and the list of recommendations for future studies.

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CHAPTER TWO
LITERATURE REVIEWS

2.1 INTRODUCTION

Gelatine is used in various industries namely the food, pharmaceutical, cosmetic, and photographic industries. Gelatine is also reported as the most widely used biopolymer and is added to food, drugs, cosmetics, paints, matches, fertilizers and photographic products (Karim & Bhat, 2008). Gelatine is added to various products due to its gelling properties and its surface-active properties. The gelling properties include gel strength, gelling time, setting and melting temperatures, viscosity, and thickening, texturizing, and water-binding abilities. Meanwhile, the secondary properties of gelatine are associated with gelatine surface behaviours such as stabilization, emulsion, film and foam formation and adhesion/cohesion (Schrieber & Gareis, 2007). Most commercial gelatine is derived from the hides and bones of mammalian animals such as cows and pigs (bovine and porcine sources) (Shabani et al., 2015). The origin of gelatine has become a serious concern among Muslims, Jews, Hindus, vegans, and vegetarian communities (Hidaka & Liu, 2002; Boran & Regenstein, 2010). Hence, gelatine authentication is becoming a must in order to protect the consumer from non-halal ingredients, as well as for health and safety concerns. In this chapter, information on gelatine source, structure, production and application are discussed briefly. The classification of dental materials and previous reports on analytical methods for the detection of gelatine, especially bovine and porcine gelatines are also reviewed.

2.2 HALAL CONCEPT IN QURAN

Halal can be defined as permissible or lawful according to Islamic laws while *haram* can be referred to as unlawful, prohibited or forbidden. In Islam, Muslims are obligated to consume substances, which are *halal* according to Islamic laws. In fact, almost all foods of plant and animal origin are considered *halal* except those that have been specifically prohibited by the Quran and the Hadith (Riaz & Chaudry, 2004). It is mentioned in the following verses of the Quran:

> O mankind, eat from whatever is on earth (that is) lawful and pure. (Chapter 2: 168)
>
> O ye who believe! Eat of the good things wherewith We have provided you, and render thanks to Allah if it is (indeed) He whom ye worship. (Chapter 2: 172)
>
> He hath forbidden you only carrion, and blood, and swine flesh, and that which hath been immolated to (the name of) other than Allah. But he who is driven by necessity, neither craving nor transgressing, it is no sin for him. Lo! Allah is Forgiving, Merciful. (Chapter2:173)

According to al-Zamakhsyari (1998), the word 'swine flesh' or pork meat in the above verse of the Quran can also be interpreted as lard, pig derivatives and by-products. Similarly, this view is consistent with al-Qurtubi (2006) in *al-Jami'li Ahkam al-Quran* which mentions lard as being included as part of the meat. Ibn Hazm al-Zahiri views that pig bones and furs are also prohibited to be consumed. Meanwhile, Ibn Hayyan and Dawood advise that only the meat is prohibited, and not the lard and derivatives (al-Andalusia, n.d).

> Forbidden unto you (for food) are carrion and blood and swine flesh, and that which hath been dedicated unto any other than Allah, and the strangled, and the dead through beating, and the dead through falling from a height, and that which hath been killed by (the goring of horns, and the devoured of wild beasts saving that which ye make lawful (by the death-stroke) and that which hath been immolated unto idols. And (forbidden is it) that ye swear by the divining arrows. This is an abomination… (Chapter 5:3)