ASSESSMENT OF BROWNING INDEX OF MAILLARD REACTION FOR DIFFERENTIATION OF FISH, BOVINE AND PORCINE GELATIN WITH PRINCIPAL COMPONENT ANALYSIS (PCA)

BY

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ABSTRACT

Gelatin is commonly used as the ingredients in food, beverages, pharmaceutical, personal care and many more. It can be extracted from animal's bones and hides. However, *Halal* and *Haram* issues on the source of gelatin are widely debates among Muslim scholars. Scientists have made a lot of effort to detect the origin of gelatin sources by using various methods. Therefore, the assessment of browning index from Maillard reaction of different sources of gelatin have been performed with Principal Component Analysis (PCA) as the latest reliable chemical approach in gelatin authentication. The objectives of this study are to optimize the factors affecting the browning index of Maillard reaction of fish, bovine and porcine gelatin by one-factor-at-a-time and Response Surface Methodology, to differentiate the browning index of Maillard reaction using UV-Vis spectroscopy combined with PCA and to characterize the browning compound from Maillard reaction of different sources of gelatin with FTIR and GC-TOF/MS. All factors affecting Maillard reaction such as pH, type and concentration of reducing sugar, type and concentration of metal ions, reaction temperature and time were investigated based on one-factor-at-a-time experiment. Response Surface Methodology was also used to optimize the Maillard reaction. The absorbance of browning compound of each sample was obtained by using UV-Vis spectroscopy in the range of 200 nm to 600 nm and converted into browning index. Next, the browning compound was characterized by using FTIR and GC-TOF/MS to compare the gelatin sample before and after subjected to Maillard reaction. Finally, the differentiation of browning index from fish, bovine and porcine gelatin were established through PCA. In general, the result showed high browning index can be obtained from 0.5 M of xylose at high temperature and long reaction time with the presence of metal ions Cu^{2+}. However, the effect of pH was insignificant and can be ignored in Maillard reaction of gelatin. From optimisation experiment, reaction temperature at 95°C for 9 hours were suitable for all types of gelatin but 5 mM of metal ions Cu^{2+} only required for bovine gelatin. In comparison with the raw gelatin standard, the browning compound in Maillard reaction of gelatin was successfully characterized by FTIR and GC-TOF/MS. In conclusion, the result from PCA revealed a notable differentiation between the browning index of fish, bovine and porcine gelatins after undergoing Maillard reaction. All the results demonstrated the success of the proposed combination methods of browning index and PCA analysis in achieving the objectives of this study.

مقدمّة البحث

يشيع استخدام الجيلاتين كمكونات في الأغذية والمشروبات والأدوية والعناية الشخصية وغير ذلك. يمكن استخراجها من عظام الحيوانات وجلودها. ومع ذلك ، فإن قضايا الحلال والحرام على مصدر الجيلاتين تناقش على نطاق واسع بين العلماء المسلمين. لقد بذل العلماء الكثير من الجهد للكشف عن أصل مصادر الجيلاتين باستخدام طرق مختلفة. لذلك ، تم إجراء تقييم مؤشر التحمير من تفاعل Maillard لمختلف مصادر الجيلاتين باستخدام Principal Component Analysis (PCA) باعتباره أحدث نهج كيميائي موثوق به في مصادقة الجيلاتين.تهدف هذه الدراسة إلى تحسين العوامل المؤثرة على مؤشر التسمير لتفاعل Maillard للسمك والبقري وجيلاتين الخنازير بواسطة منهجية عامل واحد في وقت منهجية سطح الاستجابة ، للتمييز بين مؤشر التسمير لتفاعل Maillardباستخدام UV- يقابل الطيف البصري مع PCA ولتمييز مركب التسمير من تفاعل Maillard من مصادر مختلفة للجيلاتين مع FTIR و .MS/TOF-GC تم فحص جميع العوامل التي تؤثر على تفاعل Maillard مثل درجة الحموضة ونوع وتركيز تقليل السكر ونوع وتركيز أيونات المعادن ودرجة حرارة التفاعل والوقت من خلال تجربة عامل واحد في المرة. تم استخدام منهجية سطح الاستجابة أيضًا لتحسين تفاعل .Maillard تم الحصول على امتصاص مركب التسمير من كل عينة باستخدام التحليل الطيفي للأشعة فوق البنفسجية في حدود 200 نانومتر إلى 600 نانومتر وتحويلها إلى مؤشر التسمير. بعد ذلك، تم تمييز مركب التحمير باستخدام FTIR و MS/TOF-GC لمقارنة عينة الجيلاتين قبل وبعد تعرضها لتفاعل .Maillard أخيرًا، تم تحديد التمايز بين مؤشر التسمير البني للأسماك والبقر والجيلاتين المشتق من الخنزير من خلال .PCA بشكل عام ، أظهرت النتيجة في هذه التجربة أنه يمكن الحصول على مؤشر اللون البنيفي0.5 مل من الزيلوز في درجة حرارة عالية وفترة تفاعل طويلة مع وجود أيونات المعادنCu^{2+}. ومع ذلك، فإن تأثير درجة الحموضة كان ضئيلاً ويمكن تجاهله في تفاعل Maillard من الجيلاتين. من تجربة التحسين ، كانت درجة حرارة التفاعل عند 95 درجة مئوية لمدة 9 ساعات مناسبة لجميع أنواع الجيلاتين ولكن 5 مل من أيونات المعادن Cu^{2+} مطلوبة فقط للجيلاتين البقري. بالمقارنة مع معيار الجيلاتين الخام ، فإن مركب التحمير في تفاعل Maillard للجيلاتين يتميز بنجاح بـ FTIR و .MS/TOF-GC تكشف نتيجة PCA عن وجود تمايز ملحوظ بين مؤشر التسمير للأسماك والبقر وجيلاتين الخنزير بعد الخضوع لتفاعل .Maillard توضح جميع النتائج نجاح طريقة الجمع المقترحة لمؤشر التسمير وتحليل PCA في تحقيق أهداف هذه الدراسة.

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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This work is dedicated to my first love, my teacher of life, my late beloved father, Hamid bin Ahmad whom continue to shower me with encouragement and knowledge.

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LIST OF ABBREVIATIONS

µg	Microgram
µL	Microliter
µm	Micrometer
BG	Bovine gelatin
cm^{-1}	Reciprocal centimetres
FG	Fish gelatin
g	Gram
m	Metre
mL	Millilitre
mL^{-1}	Reciprocal millilitre
mM	Millimolar
mm	Millimetre
nm	Nanometre
PG	Porcine gelatin
UV-Vis	Ultraviolet–visible
w/w	Weight per weight

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Gelatin is one of the most versatile food ingredients.The Oxford Dictionary defined gelatin as a virtually colourless and tasteless water-soluble protein prepared from collagen which is used in food preparation, photographic process and glue. It is also known as a co-product of collagen hydrolysation from animals, mainly mammal's hides, bones and skins (Karim & Bhat, 2008). The properties and functions of gelatin in the pharmaceutical and food industries are very special, unique and related to each other (Abdullah *et* al., 2017). For instance, gelatin is used to make soft and hard capsule purposely to cover the medicine from moisture and to lengthen the shelf life. This is because gelatin is transparent, thermos-reversible, melt at body temperature and compatible with human tissues. Meanwhile, other multiple functional roles including gelling, stabilizing, foaming, thickening and water-binding are important properties that need to be considered in the making of marshmallow, jelly bean and pudding (Abdullah *et* al., 2018).

However, Muslims are not allowed to consume pigs and improper slaughtered animals. In Islam, humankind is reminded to consume what is permissible and wholesome to the body. Therefore, the consumption of gelatin from the permissible source is very important unless under certain circumstances whereby the necessities may overrule the prohibitions (Shah & Yusof, 2014). The lawful or permitted things referred in Quranic term as 'halal'. This fundamental concept and understanding can be pertained from the Holy Quran which clearly mentioned:

"He has only forbidden to you dead animals, blood, the flesh of swine, and that which has been dedicated to other than Allah. But whoever is forced [by necessity], neither desiring [it] nor transgressing [its limit], there is no sin upon him. Indeed, Allah is Forgiving and Merciful."

(Surah Al-Baqarah:173)

Although the unlawful sources undergo several extraction processes in order to obtain the gelatin, its characteristics remain chemically unchanged. Thus, Muslim jurists reject the Istihalah or transformation process in gelatin from the prohibited sources since it is not completely occurred (Jamaludin *et* al., 2011). In Malaysia, the issues of non-halal gelatin started to arise in 2011 where the gelatin powder was found to be mixed with coffee at a very famous local cafe in Johor. The Council of Islamic State, JAKIM made the investigation onsite upon a complaint from citizens and analysis was confirmed by the Chemistry Department of Malaysia (Ariffin *et* al., 2016; Yazam *et* al., 2011). This issue showed that the utilization of halal food such as gelatin in Malaysia is controlled by the customer demand, manufacturer transparency, government enforcement and scientific analysis. Although, the Ministry of Health counteracts with the hygienic issues of food, but the halal part is excluded. Since the permissibility (halal) concept comes along with the wholesome (toyyiban) part, the production and consumption of gelatin should abide by the rules in Malaysian Standard 1500: 2009 (Ahmad *et* al., 2018; Majid *et* al., 2015). Therefore, the declaration and detection of the gelatin sources are very important to the manufacturers in the food industry.

The process or method of providing or showing something to be true, genuine or valid are conducted in order to protect the consumers from food frauds which is the main objective of the authentication (Hameed *et* al., 2018). Thus, verification of

gelatin from farm to fork that covers the aspect of its permissible sources by a specific method is called as halal authentication. Malaysia is one of Muslim countries that technically committed on Halal issues. This halal authentication is very important to facilitate manufacturers and consumers to have the access to the high quality and services not only in food industry but also in the pharmaceutical niche (Ali *et* al., 2017).

In this research, effort has been made to develop a new method based on UV-Vis spectroscopic of browning index during Maillard reaction of gelatin. Maillard reaction is a non-enzymatic browning reaction between protein and reducing sugar. Browning index is a measurement taken in the final phase of Maillard reaction through the differences in the absorbance of sample and reference at 420 nm. It is very useful to monitor the reaction process of brown macromolecules formation in this non-enzymatic browning reaction (Yu *et* al., 2018; Stevenson *et* al., 2019). This study focused on the differentiation of animal gelatin source and investigated the factors affecting the Maillard reaction of gelatin such as pH, reaction time, temperature, type of sugar and presence of metal ions. Furthermore, the data analysis demands suitable tools such as statistic or chemometric techniques. The Principal Component Analysis (PCA) is the common chemometric techniques that can sort numerous data from different parameters into specific category including the sources of sample. Otherwise, the data can be evaluated by using cluster analysis, discriminant analysis or classification techniques which are also based on the chemometric techniques.

1.2 STATEMENT OF THE PROBLEM

The problem arises when the source of gelatin in this vast application is very ambiguous and difficult to be distinguished. This is because gelatin is mainly derived from animals and exposes to higher chances of the adulteration of *haram* (porcine) and *shubhah* (bovine species without proper slaughtering) status which are forbidden for Muslim consumption. Several studies have been conducted to develop some authentication protocols of gelatin in food and non-food products. The detection and characterization methods generally rely on the protein analysis or physicochemical properties such as chemisorption (Hidaka & Liu, 2003), chromatographic-chemometric method (Nemati *et al*., 2004), electrophoretic analysis (Hermanto *et* al., 2013), Fourier Transform Infrared Spectroscopy (FTIR) (Cebi *et* al., 2016; Hermanto *et* al., 2013), High-Performance Liquid Chromatography-Mass Spectrometric Method (HPLC/MS) (Grundy *et* al., 2016;Yilmaz *et* al., 2013, Zhang *et* al., 2009), Enzyme-Linked Immunosorbent Assay (ELISA) (Nur Azira *et* al., 2016a; Venien & Levieux, 2005) and Polymerase Chain Reaction (PCR) (Ali *et* al., 2014; Mutalib *et* al., 2015; Shabani *et* al., 2015) have been applied to differentiate bovine gelatin from porcine gelatin.

The methods mentioned above involved with the difficult experimental procedure and only focused on the differentiation of two origins of gelatin which were porcine and bovine. Hassan *et* al. (2018) also stated that some limitations of the analytical techniques are due to the inconsistent level of amino acids in gelatin, large similarities of gelatin spectra, long profiling time, tedious marker peptides identification and missing of amplification signal due to DNA denaturation. Therefore, the need to have a new approach of halal authentication method could be overcome by the assessment of Maillard reaction in gelatin with combination of chemometrics.

Currently, to the best of our knowledge, there is only one research on the differentiation of the gelatin sources by Maillard reaction which conducted by Tan *et* al. (2012). However, their study only focused on the discrimination between porcine and bovine gelatin in the addition of ribose sugar. Therefore, this research tried to make a distinction of another more type of gelatin that is available in the industry, which is fish gelatin and with the presence of xylose sugar.

1.3 PURPOSE OF THE STUDY

The proposed method is based on the browning index from Maillard reaction of gelatin by UV-Vis spectroscopy. UV-Vis spectroscopy served as a straightforward method for halal authentication of gelatin. Many UV-Vis spectroscopic analytical procedures have been found to be useful for food analyses such as detection of melamine in fish and phenolic compounds in olive oils (Munjanja & Sanganyado, 2015).

Recently, browning value from melanoidins that formed in Maillard systems has been widely used as an indicator of reaction progress (Extabide *et* al., 2015). The absorption of the browning compound from Maillard reaction products can be measured at 420 nm as a single spectrum. Another researcher also considered a correction for any turbidity in the samples by measuring the absorption at 550 nm (Tan *et* al., 2012).

1.4 RESEARCH OBJECTIVES

The main objective of this study is to access the browning index from Maillard reaction of gelatin from fish, bovine and porcine for its differentiation. Therefore, the study aimed to achieve the following specific objectives:

i. To optimize the factors affecting the browning index of Maillard reaction of fish, bovine and porcine gelatin by one-factor-at-a-time (OFAT) and Response Surface Methodology (RSM).

ii. To differentiate the browning index of Maillard reaction using UV-Vis spectroscopy combined with Principal Component Analysis (PCA).

iii. To characterize the browning compound from Maillard reaction of different sources of gelatin with FTIR and GC-TOF/MS.

1.5 RESEARCH QUESTIONS

1. What are the optimum conditions for color development during Maillard reaction of gelatin from different sources?
2. Does principal component analysis classify the browning index of gelatin from different sources?
3. What are the characteristics of Maillard Reaction Products (MRPs) that formed in the gelatin which contribute to the color formation?

1.6 RESEARCH HYPOTHESIS

The Maillard reaction of the fish, porcine and bovine gelatin have different browning index under the optimum condition for color development via UV-Vis Spectroscopy in combination of Principal Component Analysis. The characterization of browning compound after Maillard reaction leads to a better understanding when comparing with the raw gelatin.

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

The advancement of technology and its emergence in the food industry particularly manufacturing of gelatin have created a great impact to the consumers. However, the arising of halal issue on gelatin sources has awakened the concern among the Muslim all over the world. Thus, knowing-how on the gelatin production, composition and detection are very essential. The involvement of the analytical instruments and chemometrics are very important for halal authentication of the food (Hassan *et* al., 2018; Nurrulhidayah *et* al., 2011a; Nurrulhidayah *et* al., 2014a; Nurrulhidayah *et* al., 2015). It provides empirical evidence, scientific reasoning and valid proof to any arising issues of halal authentication.

Basically, the DNA testing has the best result for the confirmation of any cross-contamination issues of halal and non-halal sources as compared to the other methods (Mutalib *et* al., 2015). Next, the sample temperature sensitivity should be observed properly especially for the compound that is easily denatured above 60^0C. Then, the integration of internal standard in high-end instrument should be included to reduce the matrix interference. Subsequently, the method validation of internal standard and method verification of sample can be performed as easy as a test kit. In addition, the data analysis demands of suitable tools such as statistic or chemometric techniques. The Principal Component Analysis (PCA) is the common chemometric techniques that can sort numerous data from different parameters into specific category (Nurrulhidayah *et* al., 2014b). Otherwise, the data can be evaluated by using cluster analysis, discriminant analysis or classification techniques.

2.2 GELATIN

2.2.1 Gelatin Extraction and Composition

Gelatin is a high molecular weight polypeptide of collagen partial hydrolysation from animals mainly mammal's hides, bones and skins. Acids and alkaline treatments are being used on the animal's connective tissue and skins to transform the collagen helical structure into random coils (Nhari *et* al., 2012). The acidic treatment is known as gelatin Type A which applicable for porcine skin to avoid the saponification of higher fat content on the skin. Fish skin also undergoes mild acidic treatment for gelatin extraction from both cold and warm-water fish species (Gómez-Guillén et al., 2009). Meanwhile, gelatin Type B represents the alkaline treatment to the animal tissue that has a lesser degree of covalent bonding in collagen as bovine skin. In comparison of protein content, the crude protein content reported from different species of fish skin gelatin was in the range of 87–89% which was slightly lower than porcine and bovine gelatin which was around 91% (Jongjareonrak *et* al., 2006; Muyonga *et* al., 2004).

Apparently, the composition of amino acid in gelatin is the main reason of gelatin distinctive properties. Glycine is the major occupancy of amino acid in gelatin either mammalian (porcine and bovine) or fish sources as shown in Table 2.1. However, mammalian gelatin contain high amount of hydroxyproline and proline compared to fish gelatine (Azilawati *et* al., 2015). These two type of amino acids content also lower in fish collagens in comparison with mammalian collagen. Previous studies have shown that the amino acid profiles and film properties of different gelatin sources vary most especially methionine and histidine (Gómez-Guillén*et* al., 2009).

Table 2.1: The composition of amino acid in bovine, porcine and fish gelatin expressed in percentage weight per weight (% w/w)

Amino acid	Mean and standard deviation result of $n = 10$ from each gelatin source (expressed in % w/w)		
	Bovine gelatin	Porcine gelatin	Fish gelatin
Hydroxyproline	10.85 ± 0.54	10.91 ± 0.61	7.01 ± 0.37
Aspartic acid	4.92 ± 0.39	5.47 ± 0.33	5.55 ± 0.37
Serine	3.98 ± 0.16	4.27 ± 0.20	6.91 ± 0.23
Glutamic acid	9.26 ± 0.58	9.67 ± 0.58	9.23 ± 0.53
Glycine	23.05 ± 1.33	21.64 ± 2.70	22.37 ± 2.28
Arginine	8.80 ± 0.52	8.67 ± 0.47	9.23 ± 0.45
Threonine	2.34 ± 0.12	2.18 ± 0.15	3.18 ± 0.15
Alanine	8.37 ± 0.73	8.05 ± 0.76	8.43 ± 0.73
Proline	11.75 ± 0.53	11.21 ± 0.56	9.58 ± 0.38
Tyrosine	0.63 ± 0.04	1.01 ± 0.07	0.72 ± 0.05
Valine	2.47 ± 0.32	2.33 ± 0.09	2.05 ± 0.05
Methionine	1.12 ± 0.20	1.51 ± 0.13	2.64 ± 0.11
Lysine	3.25 ± 0.27	3.78 ± 0.19	3.38 ± 0.27
Isoleucine	1.63 ± 0.13	1.56 ± 0.06	1.26 ± 0.04
Leucine	3.10 ± 0.12	3.10 ± 0.09	2.50 ± 0.06
Phenylalanine	2.38 ± 0.15	2.22 ± 0.15	2.31 ± 0.10

Another report showed that properties of gelatin film depended on the source of the gelatin such that fish gelatin exhibited the lowest water vapor permeability while pork gelatin exhibited the least water solubility (Hanani *et* al., 2012). Therefore, there is a possibility that variation in degree of browning in Millard reaction of different gelatin sources can be observed. The degree of browning can be detected by UV-spectroscopy and the readings can then be used to differentiate gelatin of varying sources (Tan *et* al., 2012).

2.2.2 Gelatin Authentication Method

The analysis of Halal authentication of porcine gelatin from other sources of gelatin has been studied over the years. Various applications of analytical methods involving spectroscopic, chemical precipitation, liquid chromatography and

immunochemical techniques involving eight studies have been published from 2003 to 2010 (Hafidz *et* al., 2012). To the best of found knowledge, the expansion of the authentication methods of gelatin from 2010 onwards have resulted in more studies as shown in Table 2.2.

Table 2.2: The method of halal authentication of gelatin

Method	Technique/ Instrument	Biomarker/ Principle	References
DNA-based	PCR	DNA sequence	(Demirhan *et* al., 2012; Mutalib *et* al., 2015)
Protein-based	ELISA	Anti-peptide polyclonal antibody	(Nur Azira *et* al., 2016a)
	SDS-PAGE	Polypeptide	(Hermanto *et* al., 2013; Nur Azira *et* al., 2012; Nur Azira *et* al., 2014; Nur Azira *et* al., 2016b)
Analytical	HPLC	Peptide/ Chromatography	(Azilawati*et* al., 2015; Yilmaz *et* al., 2013)
	FTIR	Spectroscopy	(Cebi*et* al., 2016; Hermanto *et* al., 2013)
	UV-Vis	Spectroscopy	(Hermanto*et* al., 2013)
Chemical reaction assessment	Maillard reaction	Chemical compound	(Tan *et* al., 2012)

The variation of authentication methods is according to the principles, advantages and limitation of each method. Most of the methods used Principal Component Analysis (PCA) to facilitate the discriminant analysis of different sources of gelatin. Besides, nearly all methods utilized two types of samples which are the raw material of gelatin and food product that contained gelatin for validation of the methods in the industry later for both samples; the raw ingredient and end-product.