UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT AND VALIDATION OF AMINO ACID ANALYSIS METHODS IN GELATIN AND GELATIN-BASED PRODUCTS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

AZILAWATI MOHD ISMAIL

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By

AZILAWATI MOHD ISMAIL

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Doctor of Philosophy

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Chairman : Professor Amin B. Ismail, PhD, FNSM
Institute : Halal Products Research Institute

The similarity of physicochemical properties among gelatin especially the porcine and bovine gelatin has sparked skepticism among Muslim consumers towards gelatin-based commercial products. This study was aimed to develop and validate a reverse-phase high performance liquid chromatography (RP-HPLC) method of amino acid analysis in gelatin and differentiate the bovine, porcine and fish gelatin as an ingredient and in gelatin-based commercial products using the principal component analysis (PCA). The analytical method used was amino acid analysis that using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate as derivatization reagent and the chromatographic separation was determined by RP-HPLC coupled with a fluorescence detector. Method development was conducted according to ISO 17025 guidelines. In-house method validation revealed that the method was selectively performing a good chromatographic separation for 18 amino acids; the detection and quantitation limit were ranged from 5.68–12.62 and 36.0–39.0 pmol/µl, respectively; no matrix effect was observed and the linearity range was 37.5–1000 pmol/µl. Method precision revealed by HorRat values was significantly less than 2 and the method recoveries had a range of 80–115%. The uncertainty evaluation was estimated on the basis of the method validation data. The uncertainty of method precision, µ(P), method recovery, µ(R) and measurement of standard, µ(Std) for overall amino acids are within a concentration range of 0.024 to 0.113 pmol/µl, 0.006 to 0.10 pmol/µl and 0.010 to 0.0297 pmol/µl, respectively. PCA has assisted the process of distinguishing the bovine, porcine and fish gelatin. Data pre-treatments such as centering and area normalization were performed to reduce the variances of variables in dataset. Database for three gelatins were established through this work and were verified by samples from gelatin-based commercial products. Data analysis demonstrated that the fish gelatin was correlated to threonine, serine and methionine on the positive side of principal component (PC) 1; bovine gelatin was correlated to the non-polar side chains amino acids that were proline, hydroxyproline, leucine, isoleucine and valine on the negative side of PC1 and porcine gelatin was correlated to the polar side chains amino acids that were aspartate, glutamic acid, lysine and tyrosine on the negative side of

PC2. The lowest detection value for adulteration of porcine gelatin in bovine gelatin was determined at 0.05% (w/w) of porcine gelatin. The extraction of gelatin from gelatin-based commercial products was successful by samples clean-up using acetone solvent and modification on several parameters of amino acid analysis method specifically the sample digestion process. Gelatin used in the commercial products was verified using gelatin database and the results revealed that products made of porcine gelatin can be differentiated from the products that were made of bovine gelatin. This quantitative method was very useful as an alternative method for halal products authentication via laboratory testing.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PEMBANGUNAN DAN VALIDASI KAEDAH ANALISIS ASID AMINO DALAM GELATIN DAN PRODUK BERASASKAN GELATIN MENGGUNAKAN KROMATOGRAFI CECAIR PRESTASI TINGGI

Oleh

AZILAWATI MOHD ISMAIL

November 2016

Pengerusi : Profesor Amin B. Ismail, PhD, FNSM
Institut : Penyelidikan Produk Halal

Persamaan sifat fizikokimia di antara gelatin terutamanya gelatin porsin dan gelatin bovin telah mencetuskan keraguan di kalangan pengguna Muslim terhadap produk komersil berasaskan gelatin. Kajian ini bertujuan untuk membangunkan dan mengesahsahihan kaedah fasa-terbalik kromatografi cecair berprestasi tinggi (RP-HPLC) analisis asid amino dalam gelatin dan membezakan gelatin bovin, porsin dan ikan sebagai bahan ramuan dan di dalam produk komersil berasaskan gelatin menggunakan analisis komponen utama (PCA). Kaedah analitikal yang digunakan adalah analisis asid amino menggunakan 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate sebagai reagen *derivatization* dan pemisahan kromatografi ditentukan oleh RP-HPLC dengan pengesan berpendarfluor. Pembangunan kaedah dijalankan mengikut garispanduan ISO 17025. Pengesahsahihan kaedah secara dalaman menunjukkan bahawa kaedah ini adalah selektif dengan pemisahan kromatografi yang baik bagi 18 asid amino; had pengesanan dan had kuantitatif adalah dalam julat 5.68 – 12.62 dan 36.0 – 39.0 pmol/µl, masing-masing; tiada pemerhatian berkaitan kesan matriks dan julat kelinearan adalah 37.5 - 1000 pmol/µl. Ketepatan kaedah didedahkan oleh nilai HorRat yang nyata sekali kurang daripada 2 dan peratusan perolehan semula bagi kaedah adalah di antara 80-115%. Penilaian ketidakpastian telah dianggarkan berdasarkan data kaedah validasi. Ketidakpastian bagi ketepatan kaedah, µ(P), perolehan semula kaedah, µ(R) dan pengukuran standard, µ(Std) untuk keseluruhan asid amino adalah dalam julat kepekatan 0.024 - 0.113 pmol/µl, 0.006 - 0.10 pmol/µl dan 0.010 - 0.0297 pmol/µl, masing-masing. PCA telah membantu proses membezakan di antara gelatin bovin, porsin dan ikan. Pra-perlakuan (*treatment*) data seperti pemusatan dan normalisasi kawasan puncak dilakukan untuk mengurangkan varians di antara pembolehubah dalam set data. Pangkalan data untuk tiga gelatin telah dibangunkan dan ditentusahkan oleh sampel-sampel dari produk komersial yang berasaskan gelatin. Analisis data menunjukkan bahawa gelatin ikan berkorelasi dengan treonina, serina dan metionina pada posisi positif komponen utama (PC) 1; gelatin bovin berkorelasi kepada asid amino dengan rantai sisi tidak polar iaitu prolina, hidroksiprolina, leusina, isoleusina dan valina di posisi negatif PC1 dan gelatin porsin

berkorelasi kepada asid amino dengan rantai sisi polar iaitu aspartik, asid glutamik, lisin dan tirosina di posisi negatif PC2. Nilai pengesanan terendah untuk pencemaran gelatin porsin dalam gelatin bovin ditentukan pada 0.05% (w/w) gelatin porsin. Pengekstrakan gelatin daripada produk komersial berasaskan gelatin berjaya dilakukan melalui pembersihan sampel menggunakan larutan aseton dan pengubahsuaian beberapa parameter dalam kaedah analisis asid amino khususnya bagi proses pencernaan sampel. Gelatin daripada produk komersial telah ditentusahkan menggunakan pangkalan data gelatin dan keputusan menunjukkan bahawa produk yang diperbuat daripada gelatin porsin boleh dibezakan daripada produk yang diperbuat daripada gelatin bovin. Kaedah kuantitatif ini adalah sangat berguna sebagai satu kaedah alternatif bagi pengesahan produk halal melalui ujian makmal.

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I certify that a Thesis Examination Committee has met on 7 November 2016 to conduct the final examination of Azilawati Mohd Ismail on her thesis entitled "Development and Validation of Amino Acid Analysis Methods in Gelatin and Gelatin-Based Products using High-Performance Liquid Chromatography" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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- the research conducted and the writing of this thesis was under our supervision;
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LIST OF ABBREVIATIONS

AABA	L-Aminobutyric acid
AOAC	Association of Analytical Communities
AMQ	6-aminoquinoline
AQC	6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate
BSE	Bovine spongiform encephalopathy
CV	Coefficient of variation
CRM	Certified reference material
DA	Discriminant analysis
DCSI-MSMS	Doubly charged selected ion coupled with MS/MS fragments monitoring
DNA	Deoxyribonucleic acid
DSC	Disuccinimidyl carbonate
EDA	Exploratory data analysis
EMA	Economically motivated adulteration
FA	Factor analysis
FDA	Food and Drug Administration
FMOC-CL	Dansyl chloride and fluorenylmethyl chloroformate
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GMIA	Gelatin Manufacturers Institute of America
GUM	Guide to the expression of uncertainty in measurement
HCL	Hydrochloric acid
HPLC-MS/MS	High-performance liquid chromatography/tandem mass spectrometry

ICH	The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IR	Infrared spectroscopy
ISO	International Organization for Standardization
ISO/IEC 17025	General requirements for the competence of testing and calibration laboratories
IUPAC	International Union of Pure and Applied Chemistry
IRMS	Isotope ratio with mass spectrometry
JAIN	State Islamic Religious Department
JAKIM	Department of Islamic Development Malaysia
JCGM	Joint Committee for Guides in Metrology
K-NN	K-nearest neighbor classification
LOD	Limit of detection
LOQ	Limit of quantitation
MAIN	State Islamic Religious Council
MS	Mass spectrometry
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of flight mass spectrometry
MCE	2-mercaptoethanol
MPA	3-mercaptopropionic acid
NBD-Cl	4-chloro-7-nitrobenzofurazane
NHS	*N*-hydroxysuccinimide
NATA	National Association of Testing Authorities
NaOH	Sodium hydroxide
nanoUPLC-ESI-Q-TOF-MS	Ultra-performance liquid chromatography and electrospray ionization quadrupole time-of-flight mass spectrometry
OPA	Orthophthalaldehyde

PC	Principal component
PCR	Polymerase chain reaction
PCA	Principal component analysis
PEG	Polyethylene glycol
PLS-DA	Partial least squares-discriminant analysis
PITC	Phenylisothiocyanate
RP-HPLC	Reversed-phase high performance liquid chromatography
RSD	Relative standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SIMCA	Soft independent modeling class analogy
SLV	Single laboratory validation
VAM	Valid Analytical Measurement

CHAPTER 1

INTRODUCTION

Halal is an Arabic word which means permissible or lawful. The underlying principle for a Muslim's diet is that food intake not only has to be halal (permissible – Syariah compliant), but also toyyiban which means wholesomeness (healthy, safe, nutritious, quality). Allah has commanded in the Holy Quran for Muslims and all of mankind to eat only the halal things - "O mankind! Eat of that which is lawful and wholesome, and follow not the footsteps of the devil. Lo! he is an avowed enemy of you" (2:168). Halal food and drinks can be described as anything that man can eat or drink and there is no legal evidence prohibiting it and its constituents are free from any unlawful or impure elements. It shall be good and pure and its consumption brings no harm (Mahiah et al., 2014).

Some Islamic countries have established strict regulations for producers and importers to stamp their products with a halal certificate to distinguish them from non-halal products. Malaysia is one of the countries who implemented a halal certification to certify the products or services as pronounced by the shariah law and complying to the Halal Standard MS 1500:2009 (Halal Food – Production, Preparation, Handling and Storage – General Guidelines). The Malaysian halal certification and logo are issued at the federal level by Department of Islamic Development Malaysia (JAKIM) to certify the products for the domestic and international market and at the state level by the State Islamic Religious Department (Jabatan Agama Islam Negeri - JAIN) and State Islamic Religious Council (Majlis Agama Islam Negeri - MAIN) to issue the halal certificate for domestic market. Currently, the Halal certification is executed through the auditing of documents and at the field with regular monitoring to the manufacturers of certified halal products. However, the halal authentication based on halal laboratory analysis is not fully implemented due to lack of capabilities in terms of methods and facilities. At present, the Department of Chemistry Malaysia is appointed as the authority for performing the halal analysis specifically related to the enforcement tasks. In most cases, the halal authentication by laboratory analysis is confirmed by the DNA analysis. In this regard, development of reliable and sensitive analytical methods for evaluating halal authenticity is critically in demand.

The issue of gelatin is alarming and sometimes controversial due to commercial gelatins were offered from various sources in the global market including the porcine gelatin. The situation has prompted consumers to be more cautious, especially when it comes to religious restrictions, health hazards and safety in the intake. In the mid 1980s, the world was shaken by the emergence of bovine spongiform encephalopathy ("mad cow disease") epidemic that swept the European countries. Since then, there has been much concern about using the gelatin from the infected animals (Karim & Bhat, 2008). Besides that, almost all Hindus abstain from cow in their dietary intake. They do not worship the cow but are deeply respecting the cow that became essential to their spiritual life (Grabenstein, 2013). In addition, Islam and Judaism have forbidden the consumption of porcine derived gelatin in food materials. It was stated

in the Holy Quran (Surat Al-'An`am, 6:145) that Muslims are prohibited from taking the flesh of swine and its derivatives as indeed it is impure. For Judaism, the Torah (Lev. 11:3; Deut. 14:6) has specifies that the pig is not kosher because it does not has cloven hooves and not chews the cud. Therefore, the religious and socio-cultural factors have influenced the need for other alternative sources such as fish and poultry to fulfill halal and kosher markets.

The similarity of the physicochemical properties of different sources gelatin made them difficult to be differentiated. Several methods have been reported based on different approaches including chemisorptions, chromatographic, immunochemical, mass spectrometric, spectroscopic and molecular techniques (Yilmaz et al., 2013). A distinct identification was offered by each approach to distinguish gelatin from different sources. For example, the formation of calcium phosphate precipitation using a pH drop method (Hidaka & Liu, 2003), a deformation of N-H bonds within the range 3290-3280 and 1660-1200 cm of infrared spectra (Hashim et al., 2010), identification of marker peptides in digested gelatin using high performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) (Zhang et al., 2009) and ultra-performance liquid chromatography and electrospray ionization quadrupole time-of-flight mass spectrometry (nanoUPLC-ESI-q-TOF-MS) (Yilmaz et al., 2013) had scientifically showed the ability of analytical methods to distinguish gelatin from various sources. However, these methods have to be confirmed and repeatability under laboratory collaborative study is recommended to assure the accuracy and quality of the reproducible results. Besides that, certain applications such using mass spectrometric is very costly, require highly skilled personnel to run the analysis and took a longer time for the profiling of peptides through chromatographic separation.

Apart from using direct methods to distinguish the gelatin, an additional of descriptive and predictive method through chemometric techniques (multivariate data analysis) was applied to facilitate discrimination among gelatins. Principal component analysis (PCA) has been the most commonly used as it is simple and revealing the internal structures of the data in a way that best explains the variance in the data. The technique has been used with analysis involving the fourier transform infrared (FTIR) spectroscopy (Hashim et al., 2010), a reversed-phase (RP) - high performance liquid chromatography (HPLC) (Nemati et al., 2004) and ultra-performance liquid chromatography-quadrupole-time of flight mass spectrometry (UPLC/Q-TOF-MS) (Cheng et al., 2012) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS)-PAGE (Nur Azira et al., 2014) in which differentiation among gelatins were performed. The researches indicated that bovine and porcine gelatins have large similarities in chemical/electrophoretic profiles, spectrum chromatography structure and having a very high homology between the collagen sequences at higher concentrations that make the ability to differentiate them by visual observation is impossible.

1.1 Objectives

The general objective of this study was to develop an RP-HPLC method of amino acid analysis that incorporated with PCA to differentiate the bovine, porcine and fish gelatins as an ingredient and in the gelatin-based commercial products.

The study encompassed of several important topics that related to a method development and uncertainty estimation for a quantitative analysis, a development of gelatin database, identification of adulteration between bovine and porcine gelatins and modification on method of amino acid analysis in order to improves the extraction of gelatin from gelatin-based commercial products. Therefore, the specific objectives of this study were:

1) To perform a method validation for determination of amino acid compositions in gelatin by application of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) as derivatization reagent using an RP- HPLC and estimating the uncertainty of measurement using Valid Analytical Measurement (VAM) Project 3.2.1 protocol that based on information from the validation data.
2) To determine the amino acid compositions in bovine, porcine and fish gelatins using amino acid analysis and differentiate the gelatins using a chemometric method specifically the principal component analysis (PCA) technique.
3) To determine the minimum detection value for adulteration of porcine gelatin in the bovine gelatin.
4) To develop a method that can enhance the gelatin extraction from gelatin-based commercial products through modification of several parameters in amino acid analysis method.

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