NON-TARGETED ^{1}H NMR METABOLOMIC FINGERPRINTING AND CHEMOMETRIC APPROACH IN DIFFERENTIATING MEAT SPECIES FOR POTENTIAL HALAL AUTHENTICATION

By

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

September 2023

IPPH 2023 3

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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September 2023

Chairman : Professor Azizah binti Abd Hamid, PhD
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Food fraud, driven by unethical practices for quick financial gain, has become a global concern. Meat and meat products are particularly vulnerable to fraudulent activities due to their ability to blend without noticeable changes in appearance or texture. However, the consequences of meat fraud extend beyond financial losses, affecting consumers who adhere to dietary restrictions, such as Muslims and Jews avoiding pork or Hindus abstaining from beef, leading to significant emotional distress.

Halal food fraud has gained significant attention, encompassing practices such as the misuse of halal labels, falsification of halal certificates, product mislabeling, and the introduction of non-halal ingredients into halal products. These fraudulent activities often include the substitution or dilution of one meat species for another, including the use of different body parts of the same species. Furthermore, non-meat ingredients, such as plant or dairy fillers in processed meat products, can inadvertently introduce prohibited substances into the supply chain, raising concerns among Muslim consumers about contamination with prohibited (haram) substances during food processing and logistics. This highlights the urgent need for improved meat authentication and traceability measures.

Meat authentication faces challenges due to the similarity in characteristics and appearances among different meat types, and the complexity of processed foods makes visually detecting pork adulteration nearly impossible. To overcome these obstacles, a combination of targeted and untargeted analytical methods has been developed. Conventional targeted approaches like protein and DNA analysis are effective for known markers but may miss unexpected ones. Non -

targeted metabolomics, a cutting-edge technique, measures a wide range of metabolites without altering the sample, providing a comprehensive analysis of meat composition. When coupled with proton nuclear magnetic resonance (^{1}H NMR), it generates distinct fingerprint patterns for each sample, enabling accurate and efficient food authenticity verification.

This study aimed to qualitatively differentiate between five types of meat samples—beef, buffalo, chicken, mutton, and pork—based on their metabolites. Metabolites were extracted using perchloric acid and bi-phase methanol-chloroform, for polar metabolites and chloroform for non-polar metabolites. Analysis of spectral data acquired through ^{1}H NMR revealed 23 important metabolites in the polar fraction and 26 compounds in the lipophilic fraction that significantly contributed to the differentiation of meat types. Chemometric analysis, employing the Principal Component Analysis (PCA) as an unsupervised method and Partial Least Squares Discriminant Analysis (PLS DA) as a supervised method, demonstrated complete differentiation between meat types. Notable metabolites, including lactate, betaine, glutathione, myo inositol, IMP, carnosine, and acetate from the polar fraction, played a crucial role in pork detection. Additionally, linoleic acid from the lipophilic fraction significantly discriminated pork from other meat types. The metabolic fingerprinting approach successfully distinguished between pure and adulterated meat samples, even at a 10% adulterant level (pork), and effectively differentiated pure meat from meat-based products.

In conclusion, the integration of NMR-based metabolomics with chemometric analysis offers a significant and dependable method for authenticating meat species. The specificity and selectivity of this approach enable the identification of essential metabolite markers, which could be used in rapid test kits to boost food safety and consumer trust. This scientific advancement plays a crucial role in addressing the growing issue of meat fraud and ensuring the authenticity of meat and meat-based products.

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

BUKAN – SASAR CAP JARI METABOLOMIK ^{1}H NMR DAN PENDEKATAN KIMOMETRIK DALAM PEMBEZAAN SPESIES DAGING UNTUK POTENSI PENGESAHAN HALAL

Oleh

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Penipuan makanan, yang didorong oleh amalan tidak beretika demi keuntungan segera, telah menjadi keprihatinan antarabangsa. Daging dan produk daging adalah lebih rentan kepada aktiviti penipuan kerana keupayaannya untuk dicampur tanpa perubahan yang ketara dalam penampilan atau tekstur. Walau bagaimanapun, akibat penipuan makanan ini melampaui kerugian, ia juga memberi kesan kepada pengguna yang mematuhi pembatasan diet, seperti orang Muslim dan Yahudi yang mengelakkan produk berunsurkan daging babi atau orang Hindu yang menjauhi produk daging lembu, menyebabkan kemudaratan emosi yang signifikan.

Penipuan makanan halal telah menarik perhatian yang besar, merangkumi amalan seperti penyalahgunaan label halal, pemalsuan sijil halal, penamaan produk yang palsu, dan pengenalan bahan bukan halal ke dalam produk halal. Aktiviti penipuan ini sering melibatkan penggantian atau pencairan satu spesies daging dengan spesies lain, termasuk penggunaan bahagian tubuh yang berbeza dari spesies yang sama. Selain itu, bahan bukan daging, seperti bahan pengisi tumbuhan atau susu dalam produk daging yang diproses, secara tidak sengaja boleh memperkenalkan bahan yang dilarang ke dalam rangkaian bekalan, menimbulkan kebimbangan di kalangan pengguna Muslim tentang pencemaran dengan bahan yang dilarang (haram) semasa pemprosesan makanan dan logistik. Ini menekankan keperluan mendesak untuk meningkatkan langkah-langkah pengesahan daging dan penjejakan yang lebih baik.

Pengesahan dan kebolehkesanan daging menghadapi cabaran disebabkan persamaan dalam ciri-ciri dan penampilan di antara pelbagai jenis daging yang

berbeza, dan kerumitan makanan yang diproses menjadikan pengesanan penipuan daging babi secara visual hampir mustahil. Bagi mengatasi halangan ini, gabungan kaedah analisis yang sasar dan bukan sasar telah dibangunkan. Pendekatan sasar konvensional seperti analisis protein dan DNA adalah berkesan untuk penanda yang diketahui tetapi mungkin terlepas penanda yang tidak dijangka. Metabolomik bukan sasar, kaedah yang canggih, mengukur pelbagai metabolit tanpa mengubah sampel, menyediakan analisis menyeluruh mengenai komposisi daging. Apabila digabungkan dengan resonans magnetik proton nuklear (^{1}H NMR), ia menghasilkan corak cap jari yang unik untuk setiap sampel, membolehkan pengesahan ketepatan dan keberkesanan makanan yang sah.

Kajian ini bertujuan untuk mengasingkan secara kualitatif antara lima jenis sampel daging - daging lembu, kerbau, ayam, kambing, dan babi - berdasarkan metabolit mereka. Metabolit diekstrak menggunakan asid perklorik dan metanol-kloroform dua fasa untuk metabolit polar dan kloroform untuk metabolit bukan polar. Analisis data spektral yang diperoleh melalui ^{1}H NMR mendedahkan 23 metabolit penting dalam fraksi polar dan 26 sebatian dalam fraksi lipofilik yang memberi sumbangan yang signifikan dalam pembezaan jenis daging. Analisis kemometrik, menggunakan Analisis Komponen Utama (PCA) sebagai kaedah tanpa selia dan Analisis Pembezaan Kuadrat Terkecil Separa (PLS DA) sebagai kaedah selia, menunjukkan pembezaan lengkap antara jenis daging. Metabolit yang ketara, termasuk laktat, betaina, glutathione, myo inositol, IMP, karnosin, dan asetat dari fraksi polar, memainkan peranan penting dalam pengesanan babi. Selain itu, asid linoleik dari fraksi lipofilik membezakan babi secara signifikan daripada jenis daging yang lain. Pendekatan cap jari metabolik berjaya membezakan antara sampel daging tulen dan dicemari, bahkan pada tahap pencampuran 10% (daging babi), dan berkesan membezakan daging tulen daripada produk daging.

Sebagai kesimpulan, integrasi metabolomik berasaskan NMR dengan analisis kemometrik menawarkan kaedah yang signifikan dan dapat dipercayai untuk mengesahkan spesies daging. Kebolehan kekhususan dan selektiviti yang diberikan oleh pendekatan ini membolehkan pengenalpastian penanda metabolit penting, yang boleh digunakan dalam kit ujian cepat untuk meningkatkan keselamatan makanan dan kepercayaan pengguna. Kemajuan saintifik ini memainkan peranan penting dalam menangani isu penipuan daging yang semakin berkembang dan memastikan keaslian daging dan produk berdasarkan daging.

ACKNOWLEDGEMENTS

I would like to express my most sincere gratitude and appreciation to my supervisory committee chairman, Professor Dr. Azizah Binti Abd Hamid, for her valuable guidance, advice, and constant encouragement throughout the course of my research study. Appreciation also goes to the members of my supervisory committee, Prof. Dr. Amin Bin Ismail and Prof. Dr. Shuhaimi Bin Mustafa, for their support and invaluable suggestions to guide me during my study.

I wish to express my gratitude to all members of the Halal Products Research Institute, UPM and Institute of Biosciences, IBS for providing the research facilities and technical assistance during my graduate study. Acknowledgement is also all due to all my friends and those who have given me the moral encouragement and support to complete my graduate study.

I would like to acknowledge the financial support provided by Universiti Putra Malaysia (Grant No. Research University Grant Scheme: 9411900) awarded to Professor Dr. Azizah Binti Abd Hamid for this study.

I would like to express my profound gratitude to my parents, whose unconditional love, support, and sacrifices have been instrumental in my academic journey. To my spouse and children, your unwavering encouragement and understanding have sustained me through the challenges of pursuing a Ph.D. I am deeply appreciative of everyone who has played a part, directly or indirectly, in helping me achieve this milestone.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

ADP	Denosine Diphosphate
AMSA	American Meat Science Association
ATP	Adenosine Triphosphate
CE	Capillary Electrophoresis
CFDAR	Canadian Food and Drugs Act and Regulations
CLA	Conjugated Linoleic Acid
dCTP	Deoxycytidine Triphosphate
DNA	Deoxyribonucleic Acid
dTTP	Deoxythymidine Triphosphate
ELISA	Enzyme-Linked Immunosorbent Assay
GC	Gas Chromatography
GC-MS	Gas Chromatography–Mass Spectrometry
glog	generalized log transformation
GTP	Guanosine-5'-Triphosphate
HC	Hierarchical Clustering
HCA	Hierarchical Clustering Algorithms
HMDB	Human Metabolome Database
HPLC	High Performance Liquid Chromatography
kDa	Kilodaltons
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Liquid Chromatography
LC-MS	Liquid Chromatography -Mass Spectrometry
MetPA	Metabolic Pathway Analysis
MHz	Megahertz
mRNA	Messenger RNA
MS	Mass Spectroscopy
MVA	Multivariate Data Analysis
NADH	Nicotinamide Adenine Dinucleotide
NADP+	Nicotinamide Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance
PCA	Principal Component Analysis

PCR	Polymerase Chain Reaction
PLS	Partial Least Squares
PLS-DA	Partial Least Squares Discriminant Analysis
PUFA	Polyunsaturated Fatty Acid
RAPD	Randomly Amplified Polymorphic DNA
rRNA	Ribosomal Ribonucleic Acid
SDS–PAGE	Sodium Lauryl Sulfate Polyacrylamide Gel Electrophoresis
SSCP	Single-Stranded Conformation Polymorphism
TAG	TriAcylGlyceride
UPLC	Ultra Performance Liquid Chromatography
VIP	Variable Importance Plot

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Food fraud is a global problem that has been around for a long time. Food fraud is immoral behavior motivated by greed and committed with a desire to maximise profits. Food fraud involves 'the deliberate and intentional substitution, addition, tampering, or misrepresentation of food, food ingredients, or packaging; or false or misleading statements made about a product (Soon, 2022). This fraudulent activity may include products that are marketed as "halal" but may, in fact, contain haram ingredients.

Halal food is an important part of food selection, as it is essential for Muslims. Hence, halal food fraud is a major concern among Muslim consumers and the authenticity of halal food is rapidly gaining public attention. Halal food production industry involves a variety of processes that cover farm-to-table operations such as slaughtering, storage, display, preparation, hygiene, sanitation, and logistics. Many cases of halal food fraud involve the misuse of the halal logo and certificate, product mislabeling, and the adulteration of halal products with non-halal ingredients (Ruslan et al., 2017).

The issues of halal food fraud involving meat and meat-based products, such as adulteration, substitution, stolen livestock, smuggling, misrepresentation, and mislabelling, have recently received more attention from the general public. In general, the halal status of meat products is determined by several factors; derived from halal meat, such as poultry, lamb, and beef, free from contaminated with any pork or its derivatives and the slaughtering process must be performed in a Shariah compliant manner. However, during the processing of meat, adulteration may occur whereby the meat is mixed or substituted with alternative meats that are cheaper and more easily available, such as pork (Ruslan et al., 2017). Moreover, various treatments can alter the original flavor, structure, and texture of the meat (Lubis et al., 2016). As a result, this becomes even more of a concern because it is difficult to determine the halal status of some products, especially meat-based ones, based on visual inspection when they are pre-packaged or processed (Zakaria, 2008). In other cases, some irresponsible food industry players neglect the concept of halal food and exploit the halal food market by using the unauthorized halal logo.

As a result, authenticating the halal status of meat products is crucial and requires the use of appropriate analytical techniques, especially since it can be challenging to analyze products that have undergone extensive processing. Food science and technology have advanced to such an extent where they have

become increasingly complex. Foods contain a wide range of ingredients, many of which are unfamiliar to consumers unless they are directly involved in the field (Nurull, 2012). In addition, the process of halal authentication necessitates knowledge of food science and technology, chemistry, and veterinary science in addition to expertise from shari'ah (Mohd Zin et al., 2021). Additionally, the most up-to-date, high-tech analytical instrumentation is now required for halal authentication, which no longer relies solely on physical inspection and documentation.

1.2 Problem Statement

Conventionally, most analytical approaches for halal authentication have been based on targeted analysis. Since these approaches only detect one compound at a time, they often provide limited information and may not fully safeguard consumers against halal food fraud and adulteration. This approach becomes ineffective as an authentication strategy when dealing with the thousands of contaminants that could be added to food unless a specific contaminant is suspected.

Recently, there has been a rapid increase in the use of non-targeted analytical methods for food authentication. Non-targeted analysis, often referred to as 'fingerprinting,' enables the simultaneous detection of a significant number of unspecified targets or data points, often exceeding 100. This approach is particularly valuable when there are no defined or readily accessible primary or secondary markers. Non-targeted methods yield essential information in the form of a fingerprint, acknowledging the intricacies of contemporary food authentication.

Through the application of chemometric analysis, fingerprinting can identify numerous subtle changes in food products, extracting valuable information from these variations. Typically, a fingerprinting method is qualitative and necessitates the creation and utilization of a suitable database for comparison with genuine reference samples. In situations where a database is unavailable, non-targeted analysis can also be employed for sample-to-reference comparisons.

The abundance of unidentified targets or data points minimizes the likelihood of random similarities between different samples, making this approach a highly valuable strategy.

In the area of food analysis, food fingerprinting is the non-targeted chemical analysis of food products followed by chemometric analysis is based on the concept of "metabolomics" (Esslinger et al., 2014). The focus of metabolomic is on the study of low molecular weight molecules (less than 1000 Da). Because

chemicals, as opposed to proteins or genetic markers, are unique identifiers of food components, metabolomics is utilized in the context of food analysis to investigate and characterize food constituents, resulting in a detailed and comprehensive metabolic chemical profile of the food. As a result, non-targeted metabolomics is an excellent method for identifying food markers. The majority of metabolomic studies involve either the creation of statistical models that are able to classify samples and predict class memberships (predictive metabolomics) or the detection of metabolites (biomarkers) that are able to distinguish between sample populations (discriminative metabolomics).

One of the most common methods for non-targeted metabolomic approaches is proton nuclear magnetic resonance (^{1}H NMR) spectroscopy because it is the "gold standard" for identifying novel compounds and provides a quick and comprehensive overview of the metabolite content of food matrix with minimal or no sample preparation. Furthermore, compared to LC-MS or GC-MS, NMR makes automated high-throughput metabolomics studies much more feasible and reliable because it is extremely reproducible and easy to automate. In addition to these advantages, NMR is particularly adept at identifying and characterizing compounds, such as sugars, organic acids, alcohols, polyols, and other highly polar compounds, that can be challenging for LC-MS analysis.

However, much of this approach's potential remains unexplored, especially regarding the authentication of meat species origin and detection of meat adulteration in meat-based products. Hence, this study was conducted to determine pork specific metabolites from muscle's extract using NMR – based metabolomics approach. With the help of chemometric analysis and metabolomic fingerprint strategy, it could be conceivably hypothesized that muscle's metabolites are species-specific and could provide reliable biological information to detect species origin in foods. Moreover, this study can be an alternative authentication method and rapid screening method that compliment with other omics-based method (genomic and proteomic). From this study, the established metabolites markers for pork can be specifically to detect pork for meat speciation and assist any enforcement laboratory to authenticate meat species routinely. In addition, combination of metabolomic and multivariate analysis allows comparison of overall metabolite fingerprints and discrimination of redundant information resulting rapid visualization differences of metabolites among the meat samples and make it as a useful technique for identifying biomarkers for distinguishing halal and haram meats.

1.3 Aims

The overall aim of this study is to authenticate meat and meat-based products found in Malaysian market from the perspective of halal authentication through NMR – based metabolomics approach. In order to achieve the main objective, the specific objectives of this study were;

1. To profile the interspecies differences in polar metabolites, present in five different species (beef, buffalo, chicken, mutton and pork) extracted using two different types of solvent systems
2. To conduct non - targeted NMR - based lipidomics that are significant in species differentiation
3. To evaluate the applicability of polar and lipophilic metabolites as a new approach to monitor adulteration
4. To verify presence of adulterant in commercialized meat – based products using semi – targeted NMR – based metabolomic coupled with chemometric analysis

In summary, non-targeted ^{1}H NMR-based metabolomic analysis was employed to evaluate metabolites in animal tissues that could potentially serve as markers for distinguishing meat species. Figure 1.1 provides an overview of this comprehensive analysis. This method ensures the integrity of halal food products through an exploratory analysis, encompassing the extraction and assessment of both polar and non-polar metabolites. Additionally, this approach involves the utilization of potential biomarkers derived from polar and non-polar metabolites as indicators for monitoring adulteration in mixed (adulterated) samples. Finally, practical applications of this approach to real-world samples were conducted to assess its effectiveness in detecting adulterants.

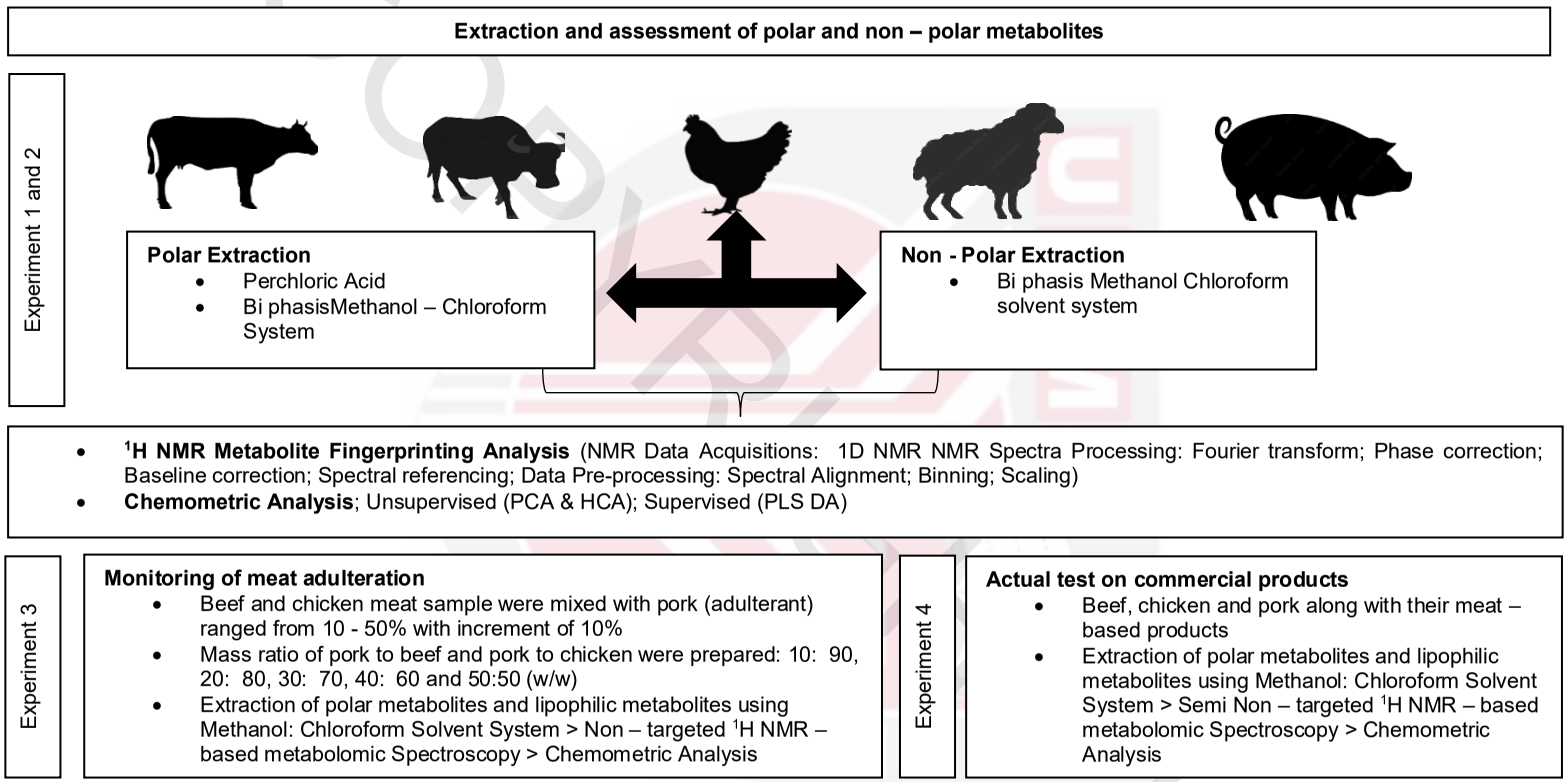

Figure 1.1: Non – targeted ^{1}H NMR based metabolomic and chemometric analysis in meat species differentiation
The infographic of the application of non – targeted 1H NMR based metabolomic fingerprinting and chemometric approach in differentiationg meat species for potential halal authentication.

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