UNIVERSITI PUTRA MALAYSIA

LARD TRACEABILITY IN LARD-ADULTERATED STARCH-BASED FOOD USING qPCR AND EVALUATION OF CHEMOMETRICS AND RANDOM FOREST ON GC-MS CHROMATOGRAM

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By

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October 2021

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The adulteration of lard in food materials can go undetected or encapsulated, increasing the difficulties in identifying food components within. Therefore, a reliable technique for detecting lard adulteration in starch-based foods is required to protect consumers from potential food adulteration. This research aimed to detect lard in lard-adulterated starch-based food samples using real-time PCR (qPCR) and gas chromatography-mass spectrometry (GC-MS) assisted by chemometrics and random forest. For the DNA-based detection method, CTAB and enzyme-CTAB were used to extract DNA from samples of tapioca starch and wheat flour spiked with different percentages of lard; 0%, 3%, 5%, 10% and 50% (v/w). The application of enzymatic treatment using starch-hydrolysing enzymes (α-amylase and amyloglucosidase) on lard-adulterated wheat flour increased the range of extracted DNA concentration from 1.80 ng/μL - 11.23 ng/μL (CTAB) to 3.60 ng/μL - 17.77 ng/μL (enzyme-CTAB) meanwhile, the DNA concentration from lard-adulterated tapioca starch slightly increased from 0.23 ng/μL - 0.33 ng/μL (CTAB) to 0.20 ng/μL to 0.60 ng/μL (enzyme-CTAB). However, the detection of lard in the samples using real-time PCR was unsuccessful. The application of enzymatic treatment in the DNA extraction protocol was ineffective for lard detection.

As an alternative, another method was explored by targeting the fatty acid content of wheat biscuits adulterated with 3%, 5%, 10% and 50% lard using GC-MS assisted by chemometrics and random forest. Chemometric analysis of the GC-MS profiles successfully distinguished the unadulterated wheat biscuits from lard and lard-adulterated wheat biscuits. Principal component analysis (PCA) and hierarchical clustering analysis (HCA) analysis clustered all samples into three distinct groups and further confirmed by the partial least squares – discriminant analysis (PLS-DA) and random forest. The random forest model outperformed PLS-DA with a prediction accuracy of 1.0, proposing C18:3n6 as a biomarker for

lard in discriminating unadulterated and lard-adulterated wheat biscuits based on their abundance which was proportionately affected by the increment of lard added. The outcomes of this study can serve as preliminary information in halal authentication to determine lard adulteration in starch-based food products.

Keywords: Chemometrics, GC-MS, Lard, Real-time PCR, Starch

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KEBOLEHJEJAKAN LEMAK KHINZIR DALAM MAKANAN BERASASKAN KANJI YANG DIADUKKAN LEMAK KHINZIR MENGGUNAKAN qPCR DAN PENILAIAN KEMOMETRIK DAN HUTAN RAWAK KE ATAS KROMATOGRAM GC-MS

Oleh

NUR INANI BINTI AZIZAN

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Pengadukan lemak khinzir dalam produk makanan Oleh itu, satu teknik pengesanan pengadukan lemak khinzir dalam makanan berasaskan kanji diperlukan bagi melindungi pengguna daripada isu pengadukan makanan. Kajian ini bertujuan untuk mengesan lemak khinzir dalam makanan berasaskan kanji yang telah diadukkan dengan lemak khinzir menggunakan PCR masa nyata (qPCR) dan GC-MS dibantu oleh kemometrik dan hutan rawak (*random forest*). Dalam teknik pengesanan berdasarkan DNA, kaedah CTAB dan enzim-CTAB telah digunakan untuk mengekstrak DNA daripada sampel tepung ubi kayu dan tepung gandum yang diadukkan dengan peratusan lemak khinzir yang berbeza; 0%, 3%, 5%, 10% and 50% (v/w). Penggunaan enzim kanji-hidrolisis (α-amilase and amiloglukosidase) ke atas tepung gandum yang diadukkan dengan lemak khinzir meningkatkan hasil ekstraks DNA daripada 1.80 ng/µL – 11.23 ng/µL (CTAB) kepada 3.60 ng/µL – 17.77 ng/µL (Enzim-CTAB) manakala ekstraks DNA daripada tepung ubi kayu yang diadukkan dengan lemak khinzir meningkat dengan kadar minima daripada 0.23 ng/µL – 0.33 ng/µL kepada 0.20 ng/µL – 0.60 ng/µL (Enzim-CTAB). Walau bagaimanapun, pengesanan lemak khinzir dalam sampel menggunakan qPCR tidak berjaya. Penggunaan enzim dalam protokol pengekstrakan DNA tidak berkesan untuk pengesanan lemak khinzir.

Sebagai alternatif, satu lagi kaedah pengesanan telah diterokai dengan mensasarkan molekul asid lemak dalam biskut gandum yang telah diadukkan dengan 3%, 5%, 10% dan 50% lemak khinzir menggunakan GC-MS dibantu oleh kemometrik dan hutan rawak. Analisis kemometrik ke atas profil GC-MS berjaya membezakan biskut gandum yang tidak diadukkan dengan lemak khinzir dengan sampel lemak khinzir dan biskut gandum yang telah diadukkan dengan lemak khinzir. Analisis komponen utama (PCA) dan analisis kluster hieraki (HCA) membahagikan semua sampel kepada tiga kumpulan dan seterusnya disahkan

oleh analisis separa kuasa dua terkecil – diskriminan (PLS-DA) dan random forest. Model hutan rawak mengatasi PLS-DA dengan kadar ketepatan ramalan 1.0, dan mencadangkan C18:3n6 sebagai penanda bio untuk lemak khinzir bagi membezakan biskut gandum yang tidak dan telah diadukkan dengan lemak khinzir berdasarkan kuantiti sebatian yang dipengaruhi oleh penambahan lemak khinzir dalam sampel. Dapatan kajian ini boleh digunakan sebagai maklumat awal untuk pengesahan halal bagi menentukan pengadukkan lemak khinzir dalam produk makanan berasaskan kanji.

Kata kunci: Kemometrik, GC-MS, Lemak khinzir, PCR masa-nyata, Kanji

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

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
Bp	base pair
CTAB	cetyltrimethylammonium bromide
Cq	quantification cycle
Ct	threshold cycle
CV	cross-validation
DA	discriminant analysis
DNA	deoxyribonucleic acid
DSC	differential scanning colorimetry
ECD	electron capture detector
EDTA	ethylenediaminetetraacetic acid
FID	flame ionisation detector
FTIR	Fourier-transform infrared spectroscopy
FAME	fatty acid methyl ester
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GLA	gamma-linolenic acid
HCA	hierarchical cluster analysis
HPLC	high-performance liquid chromatography
^{1}H-NMR	proton nuclear magnetic resonance
IR	infrared spectroscopy
MAE	microwave-assisted extraction
MS	mass spectrometry
NaCl	sodium chloride

NaOAc	sodium acetate
NIST 11	National Institute of Standards and Technology Mass spectral Database
NMR	nuclear magnetic resonance
OOB	out-of-bag
PC	principal component
PCA	principal component analysis
PCR	polymerase chain reaction
PCR-RFLP	PCR-restriction fragment length polymorphism
PLS	partial least squares
PLS-DA	partial least squares-discriminant analysis
ppm	parts per million
PVP	polyvinylpyrrolidone
Q^2	predictive ability
qPCR	real-time PCR
R^2	determination coefficient
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SFE	supercritical fluid extraction
TAG	triacylglycerol
TCD	thermal conductivity detector
UFA	unsaturated fatty acid
SFA	saturated fatty acid
UV	ultraviolet
VIP	variable importance in projection
v/v	volume over volume

w/v	weight over volume
XRD	X-ray diffraction analysis
Σ	sum
λ	lambda

CHAPTER 1

INTRODUCTION

1.1 Overview

Food is one of the important components to sustain life. To cope with the increasing population worldwide, the food industry has been rapidly growing over time. As more foods are available in the market including processed food, the authentication of the food products has become indispensable. Nowadays, consumers opt for a convenient and easy lifestyle such as ready-to-eat food like biscuits, bread and pastries products which are easily available in any convenience stores and local supermarkets. one of the food adulteration issues concerning these starch-based products is the usage of shortening, butter or margarine made from pork fat or also known as lard.

The addition of porcine materials in food products without proper labelling has raised public concerns, particularly among Muslim consumers as Islam prohibits its followers from consuming food containing any porcine derivatives. This issue will harm the food industry specifically the halal food market as Muslim consumers must ensure that their food is compliant with the Syariah Law i.e. Halal. Aside from the religious issue, the addition of porcine materials is also undesirable among vegetarians who restrict themselves from consuming food containing any animal-based ingredients. Undeclared addition of ingredients in food products may jeopardise consumers' health as it can lead to potential allergic reactions and other food-related diseases. In addition, cross-contamination due to improper handling such as during processing, packaging and transporting of food products also may add to the respective problem.

Other than that, there are also issues where irresponsible food manufacturers substitute raw materials with a cheaper substance such as blending vegetable-based shortening with lard to reduce the production cost (Che Man et al., 2011). A previous study by Rosman et al. (2016) was unable to detect the presence of lard in lard-adulterated chocolate, probably due to the entrapment of lard in the cocoa powder matrix preventing deoxyribonuclease acid (DNA) release, thus, producing false-negative results (Do et al., 2011). Therefore, a strict authentication method needs to be developed to ensure complete transparency of food products marketed to consumers. The outcomes of the study can be beneficial in determining lard adulteration in starch-based food products. Methods established and applied in this research are expected to assist authorities in determining lard adulteration in food products for upkeeping the integrity of the halal supply chain.

1.2 Hypothesis

The hypotheses of this study are:

1. The application of enzymatic treatment will improve the DNA extraction by increasing the DNA yield.
2. The presence of porcine in lard-adulterated tapioca starch and wheat flour pastes can be detected using qPCR.
3. Fatty acid profile of lard-adulterated wheat biscuits can be distinguished from unadulterated wheat biscuits using chemometric and random forest, permitting lard detection.

1.3 Problem Statements

With the current advancement in food technology, the presence of adulterants can be undetected or encapsulated, increasing the difficulties in identifying food components within. Processed food products also have complex matrices due to the blending of various ingredients in the presence of several processing steps, altering the original composition structure of each ingredient.

1.4 Objectives

The objectives of this study are;

1. To determine the effects of starch-hydrolysing enzyme treatments integrated with the conventional CTAB method on DNA extraction
2. To detect porcine in lard-adulterated tapioca starch and wheat flour pastes using qPCR
3. To extract oil from lard-adulterated wheat biscuits using the Soxhlet method and to determine lard adulteration using chemometrics and random forest-assisted GC-MS

1.5 Overview of the Research

This thesis demonstrated the detection of lard in lard-adulterated starch-based food using two different approaches i.e. firstly, DNA-based detection using real-time polymerase chain reaction (qPCR) and secondly, fatty acid-based detection using gas chromatography-mass spectrometry (GC-MS). The samples used in this research encompassed lard-adulterated samples using tapioca starch and wheat flour as the main ingredients. Due to the plausible formation of the starch-lipid complex during gelatinisation of starch added with lard, starch-hydrolysing enzymes were employed as a pre-treatment step before the CTAB extraction protocol to break down the complex. The efficiency of the first approach using real-time PCR was then determined based on the DNA concentration and the

quantification cycle (Cq) values in real-time PCR. Due to the limited success of DNA-based detection, fatty acid-based detection was also attempted to detect the presence of lard specifically in laboratory-prepared lard-adulterated wheat biscuits using chemometric-assisted GC-MS. In addition, the chemometric analysis of the fatty acid profile data proposed a specific fatty acid compound as a potential biomarker for lard detection in lard-adulterated wheat biscuits. The overall flowchart of the study is depicted in Figure 1.

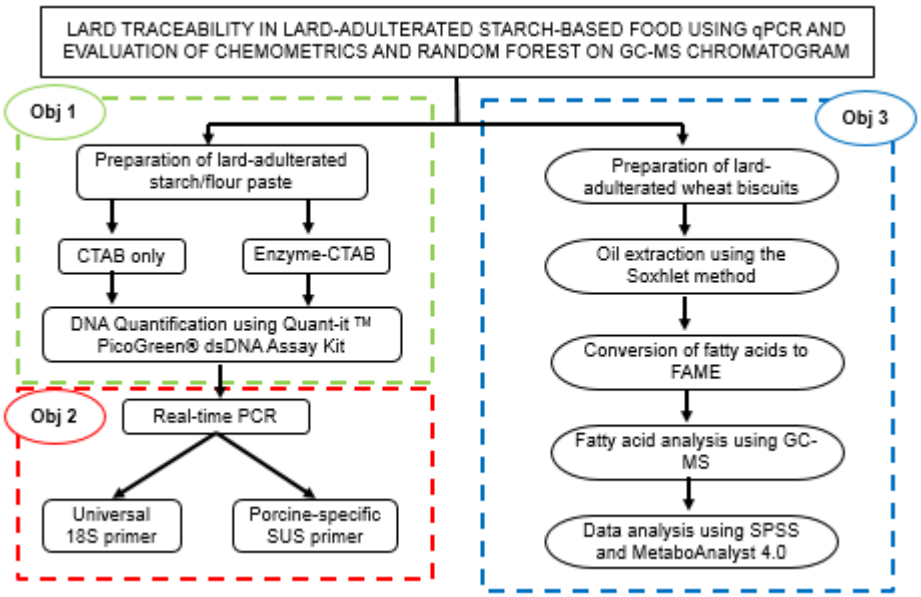

Figure 1: The overview of this research

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