PROFILING OF TRIACYLGLYCEROLS AND IDENTIFICATION OF MARKERS IN EDIBLE FATS AND OILS USING LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

SYED IZMUHAR BIN SYED IDRUS

UNIVERSITI SAINS MALAYSIA
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by

SYED IZMUHAR BIN SYED IDRUS

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TABLE OF CONTENTS

LIST OF APPENDIX

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AC	Alternating Current
ACN	Acetonitrile
Act.	Activation
Act.Q	Activation Q
ADRP	Adipose Differentiation Related Protein / Adipophilin
AF	Ammonium Formate
APCI	Atmospheric Pressure Chemical Ionization
arb	arbitrary units
cAMP	cyclic Adenosine Monophosphate
CI	Chemical Ionization
CID	Collision-induced Dissociation
DAG	Diacylglycerol
DB	Double bond
DNA	Deoxyribonucleic Acid
ECN	Effective carbon number
ESI	Electrospray Ionization
FA	Formic Acid
FAB	Fast Atom Bombardment
FTCIR	Fourier Transform Ion Cyclotron Resonance
FT-OT	Fourier Transform Orbitrap
GC	Gas Chromatography
HETP (H)	Height Equivalent to a Theoretical Plate
HPLC	High Performance Liquid Chromatography
i.d.	Internal Diameter
Iso	Isolation
IT	Ion Trap
IPA	Isopropanol / Propan-2-ol

JAKIM	Jabatan Kemajuan Islam Malaysia
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LIT	Linear Ion Trap
M	Molarity
MALDI	Matrix-assisted Laser Desorption Ionization
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MSn	Number of Mass Spectrometry
MW	Molecular Weight
NIOSH	National Institute for Occupational Safety and Health
N	Normality
NMR	Nuclear Magnetic Resonance
NP	Normal Phase
PI	Photoionization
Q	Quadrupole
RF	Radio Frequency
RP	Reverse Phase
RPM	revolution per minute
sn	stereospecific number
SIMS	Secondary-ion Mass Spectrometry
TAG	Triacylglycerol
TC	Total Carbon
TLC	Thin Layer Chromatography
TOF	Time-of-flight
UHPLC	Ultra High Performance Liquid Chromatography
USFDA	United State Food and Drug Administration
WHO	World Health Organization

LIST OF SYMBOLS

ºC	degree Celsius
%	percentage / percent
^{252}Cf	californium-252
Å	Angstrom
arb	arbitrary unit
C18	octadecyl carbon chain
DA	daltons
kDA	kilodaltons
dH_2O	deionized water
g	gram
g/m^2	gram per meter square
kV	kilovolt
µA	microampere
min	minutes
µl	microliter
ml	milliliter
µm	micrometer
mM	milimolar
mm	millimeter
ms	miliseconds
m/z	mass-to-charge ratio
nmol	nano Mole
nm	nanometer
t_R	retention time
V	volt
v/v	volume per volume

PEMPROFILAN TRIASILGLISEROL DAN PENGENALPASTIAN PENANDA DALAM LEMAK MAKAN DAN MINYAK MENGGUNAKAN KROMATOGRAFI CECAIR SPEKTROMETRI JISIM TANDEM

ABSTRAK

Persijilan dan pengesahan Halal diperlukan di seluruh dunia terutama di Malaysia yang ditadbir oleh badan penguatkuasa seperti Jabatan Kemajuan Agama Islam Malaysia (JAKIM). Untuk mencapai matlamat ini, kaedah ujian benda asing perlu ketat supaya pengenalpastian benda asing dapat dianalisis secara tepat. Analit sasaran untuk analisis yang paling utama adalah Asid Deoksiribonukleik (DNA) dan pengujian yang terlibat dalam analisis DNA seperti Reaksi Berantai Polimerase (PCR). Oleh itu, analit dan teknik sasaran yang baru diperlukan untuk menambah baik analisis ujian semasa dengan menggunakan Fasa-Songsang Kromatografi Cecair Spektrometri Jisim Tandem (UHPLC-MS) yang mensasarkan Triacylglycerols (TAGs). Objektif utama kajian ini adalah untuk membangunkan kaedah yang optimum untuk lajur teras C18 teras dan menghasilkan profil jisim spektrometri TAG dalam lemak dan minyak makanan dengan menggunakan kaedah dan teknik yang dibangunkan dalam mengesan TAG dalam sampel makanan untuk mengenal pasti penanda makanan. Pengiraan perbandingan lajur menunjukkan bahawa lajur teras C18 mempunyai resolusi 2.23, iaitu lebih tinggi dengan resolusi lajur berliang C18 0.91 pada kadar aliran yang sama 400 µl / min. Spesifikasi keseluruhan kecekapan dan kapasiti puncak untuk lajur teras C18 adalah masing-masing 0.72 dan 27.0 yang mana ia tidak terlalu banyak menyimpang daripada lajur berliang C18 sambil mengekalkan resolusi pemisahan yang tinggi. Lajur teras C18 juga memisahkan minyak jagung dengan menjumpai oleic-oleic-linolenic yang tidak terdapat dalam lajur berliang C18. Penanda makanan boleh dikenal pasti berdasarkan keunikan asid

lemak dan peratusan asid lemak. Ghee, yang merupakan campuran lemak haiwan dan minyak sayuran mempunyai 37.9% asid palmitik iaitu komponen tinggi seperti dengan minyak khinzir, 31. 5%. Walaupun minyak sawit mempunyai jumlah asid palmitik tertinggi, minyak kelapa sawit mempunyai asid lemak unik, asid nonadecyclic. Minyak zaitun mempunyai dua asid lemak unik asid gadoleic dan asid achidic, yang kedua-duanya mempunyai 2.8% daripada jumlah komponen asid lemak dalam semua lemak dan minyak yang diuji. Teknik ini memperlihatkan potensi untuk mengenal pasti penanda makanan dan juga sebagai kaedah ujian alternatif.

PROFILING OF TRIACYLGLYCEROLS AND IDENTIFICATION OF MARKERS IN EDIBLE FATS AND OILS USING LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

ABSTRACT

Halal certification and authentication has much been sought out globally especially in Malaysia that is govern by regulatory bodies such as Jabatan Kemajuan Agama Islam Malaysia (JAKIM). In order to achieve this, adulterants testing method has to be stringent so the identification of adulterant can be accurately analyzed. Target analyte for analysis has primarily been Deoxyribonucleic Acid (DNA) and testing that involved in DNA analysis such as Polymerase Chain Reaction (PCR). Therefore a new target analyte and techniques are needed to supplement this current testing analysis by using Reverse Phase Ultra High Liquid Chromatography tandem Mass Spectrometry (UHPLC-MS) targeting Triacylglycerols (TAGs). The main objectives of this study are to develop an optimization method for C18 core-shells columns and generate mas spectrometric profiling of TAGs in edible fat and oils and to apply the developed methods and techniques in detecting TAGs in food sample in identifying possible food markers. The column comparison calculation shows that C18 core-shell column has a resolution of 2.23, higher than the C18 porous column resolution of 0.91 at the same flow rate of 400 µl/min. The overall specifications of the C18 core-shell efficiency and peak capacity of 0.72 and 27.0 respectively did not deviate too much from the C18 porous while maintaining the high resolution of separation. The C18 core-shell has also separation corn oils that yield Oleic-Oleic-Linolenic that was not present in the C18 porous column. Food marker can possibly be identified based on the uniqueness of fatty acids and percentage of fatty acids. Ghee, which is a mixture of animal fats and vegetables oils, has 37.9 % of palmitic

acid which is highest component similar to lard oil, 31. 5 %. Although palm oil has the highest palmitic acid in total, palm oil has a unique fatty acid, nonadecyclic acid. Olive oil has two unique fatty acids, gadoleic acid and achidic acid, which both has 2.8 % of the total fatty acid components in all of the fat and oils tested. This technique exhibits its potential as an alternative testing method.

CHAPTER 1

INTRODUCTION

1.1 Food Law and Regulation in Malaysia

Malaysia has in placed Food Law and Regulation that are regulated by the Ministry of Health in order to provide guidelines and regulatory requirement for during food manufacturing. The Food Act of 1983 stated for adulterated food as;

"Adulterated food

13B. (1) No person shall prepare or sell any adulterated food.

(2) For the purposes of and without prejudice to the generality of subsection (1), any food shall be deemed to be adulterated if—

(a) it contains or is mixed or diluted with any substance which diminishes in any manner its nutritive or other beneficial properties as compared with such food in a pure, normal or specified state and in an undeteriorated and sound condition, or which in any other manner operates or may operate to the prejudice or disadvantage of the purchaser or consumer;" (*Laws of Malaysia - Food Act 1983*, 1983)

These legal requirements would be adhering to by the JAKIM for the Halal certification and authentication specifically for Muslim consumer. The structures of these legal requirements are based on the current food analysis and adulterants testing.

1.2 Halal and Adulterant Testing

Jabatan Kemajuan Islam Malaysia introduced the Malaysian Standard on Preparation and Production of Halal Food, MS 1500:2009 to assure the concept of Halal, permissible, is used practically from farm to table. To receive the Halal certificate from JAKIM the manufacturer necessarily ensures preparation, slaughtering, ingredients, cleaning, handling, processing, transportation and distribution is truly comply with Syariah laws, whereby it is given the right to display the Halal logo. Nevertheless, to inspire Malaysian as world Halal Hub, high quality standards of ISO9001 certified, and infrastructure and technologies support for Halal authentication analysis to provide scientific evidence to substantiate any claims regarding the presence or absence of the non-Halal ingredients and/or additives (Sahilah, Laila Liyana, Aravindran, Aminah, & Mohd Khan, 2016).

Adulteration in food has been a concern not only decreases the quality of food products but also results in a number of ill effects on health. Authentic testing of food and adulterant detection of various food products is required for value assessment and to assure consumer protection against fraudulent activities. The possibility of food causing health risks imposed by adulterants and the detection methods to detect those adulterants need to be available. Concerns about food safety and regulation have ensured the development of various techniques like physical, biochemical/immunological and molecular techniques, for adulterant detection in food (Nurrulhidayah, Yaakob, Mohammad Aizat, Suhaimi, & Hassan, 2011)

Various methods, based on morphological/anatomical characterization, organoleptic markers (odor, color, texture) and chemical testing, have been developed to authenticate traded food commodity and to check for adulterants (Shaw

et al., 2002). Today three basic strategies can be followed for demonstrating adulteration i.e. By demonstrating the presence of foreign substance or a marker in the commodity By demonstrating that a component is deviated from its normal level and By demonstrating that a profile is unlikely to occur Among these, the first strategy of detection of adulterants by the demonstration of the presence of foreign substances or a marker is considered as the best and simplest (Wilhelmsen, 2004; 2006) (Bansal, Singh, Mangal, Mangal, & Kumar, 2017)

1.3 Problems Statements

The development of current technology enables the product of the food produced to be analysed in terms of its contents accurately and so on, the determination of illegal halal can be done effectively. Several techniques in identifying adulterants such as Enzyme Linked Immunosorbent Assays (ELISA), Radio Immunoassays (RIA), HPLC, FTIR, Electronic Nose coupled with GC-MS and PCR assays have been applied to analysing food, in either processed and unprocessed. For example, the use of instruments such as the Fourier Transform Infrared (FTIR) spectroscopy to detect pig derivatives in meat products (Marikkar, Ghazali, Che Man, Peiris, & Lai, 2005). This led to large capital invested into developing rapid analysis for DNA and protein especially in *Halal* testing (Langley, 2014).

This is due to high possibility of denaturation of target molecules during storage and processing, which leads to time-consuming and labor-intensive process (Kim, Seo, Yum, Jeong, & Yang, 2017). Thus using alternative macromolecules for example lipid as a target analyte for food testing would be useful as complementary

analyte for testing as a whole. Lipid molecules have a higher stability in contrast to DNA and protein; even though lipid has a much large derivative and difficult to isolate the sub-type of lipid needed to analyzed

1.4 Significance of the Study

The findings of the study is to provide a potential alternative methodology in adulterants testing through new technology such in the form of chromatography column using Ultra High Pressure Liquid Chromatography Tandem Mass Spectrometry for lipid analysis and generating TAGs profiles that could possibly identified food markers in edibles oils and fats. The column applied a new technology in the form of high-carbon core-shell as appose to the old technology that uses low-carbon porous shell that would separate and identified TAGs with the highest efficiency possible. This will ensure that food is free of adulterants especially that is legally required not to present and safe for consumption as stated in the JAKIM guideline during food manufacturing process (Mohamad, 2013).

1.5 Objectives

The differentiation of edible lipids such as fats and oils in foods would require a technique that uses TAGs profiles. The focus of the current work is to utilize TAGs mass spectrometric profiling in separating and also identifying markers in edible oils and food products.

The main objectives for this study are:

a) To optimize TAGs in edible fat and oils profiling methodology by using C18 porous and C18 core-shell type liquid chromatography columns
b) To apply the developed method and technique in detecting TAGs in food samples and identify possible markers.

CHAPTER 2

LITERATURE REVIEW

2.1 Adulterants and Food Testing

The food ingredients are defined as ingredients which are listed in descending order by weight on a food label. Food has to be described in a way which is not misleading, the name prescribed by law, and precise description to inform consumers of its true nature and application (JAKIM, 2010). It also may serve as technological function in the manufacture, processing, preparation, treatment, packaging, transport, or storage of the food. It includes any preservatives, coloring substances, flavorings, flavor enhancer, antioxidant and other food conditioners, but shall not include nutrient supplement, an incidental constituent (JAKIM, 2010). For examples, some of food ingredients may either contain pork gelatin, lard, alcohol, and hormones; or food colors (E100-199), preservatives (E200-299), oxidant and antioxidant (E300-399), gelling agent, emulsifiers, anti-caking, stabilizers (E400-499), enzymes, glycerin/glycerol (E422) and flavor enhancers (E600-E699) (Ismail, 2006). The Muslim consumer should be inform of the ingredients of the finished food products that the Halal status is clear (Sahilah et al., 2016).

There are various approaches to detect and quantify the level of adulterants in food products. The first approach is by determining the ratios between some chemical constituents and assuming that these ratios are constant in particular food products. This approach seems to make sense that any addition in any food products will modify or change these ratio values or will highlight an anomaly in its chemical

compositions. There are three ways of analyzing; first is a set number of analyses and the use of chemometrics. Next, is by using analytical methods derived from physical analysis by taking into account the whole samples to show the adulteration effects on the physic-chemical properties. Lastly the third approach is by searching a specific marker in food products, either chemical constituents or morphological components, which proves the presence of adulterants in food products. The analytical methods used for the detection of adulteration of oils and fats including lard are based on the differences in the nature and the composition of the minor and major components of the adulterant and those of the unadulterated oils or fats (Nurrulhidayah et al., 2011; Salahudin, Ramli, Zulkepli, & Razak, 2018).

The advance in food technology had progressed so much and getting more complicated. All sorts of ingredients had been used in foods which are difficult to understand by the consumer, unless they are involved directly in the related field. In addition, the task of halal authentication cannot rely only on expertise from Shariah alone, but also require other related technical fields such as food science and technology, chemistry and veterinary science. Halal authentication cannot rely solely on physical inspection and documentation anymore, but also using the latest high technology analytical instrumentation. In the analytical field, there were many principal techniques that have been successfully applied to detect and identify porcine based ingredients adulteration in food. Che Man & Mirghani (2001) have developed a Fourier-transform infrared (FTIR) spectroscopic method for detecting lard in mixtures of other animal fats, such as chicken, lamb and cow. The results demonstrated that the FTIR could qualitatively differentiate between the pure animal's fats and their blends. They have developed a method for species identification from pork and lard samples and shown to be potentially reliable

techniques for detection of pig meat and fat for halal authentication (Nurrulhidayah et al., 2011).

2.2 Lipidomics

Accurate quantifying lipids through lipidomics technology has been a challenge in recent years, but current advancements have made it easier to overcome it. Generally, analysis of lipids starts from the biological sample, extraction, pre-treatment sample and finally mass spectrometric data acquisition. Each of these steps have to be followed with strict and standardize protocol in order to ensure the accuracy of the lipid analysis (Lam, Tian, & Shui, 2017; Triebl, Hartler, Trötzmüller, & Köfeler, 2017). Previous qualitative analysis is now transform to quantitative based on lipidomics advancement that can yield higher accuracy in quantifying lipids. This would benefit fields of study from diseases biomarker to cellular pathways (Gross & Holčapek, 2014; Lam et al., 2017).

2.3 Lipids

Lipid compounds include monoglycerides, diglycerides, triglycerides, phosphatides, sterols, terpenes, fatty alcohols, and fatty acids. Some plants store energy in their seeds as fat in solid form or commonly called oil in its liquid form (Lin & Oliver, 2008; Mu & Høy, 2004). In animals, especially humans, fats from dietary lipid mediates the metabolism and energy levels, which are a more concentrated energy source than carbohydrates (“11th International Symposium on Atherosclerosis,” 1997; “Atherosclerosis X,” 1994).

Dietary fat-soluble compounds, vitamins such as A, D, E, K are a source of antioxidant, which could possibly be used in treatment for diseases that involve disrupting the lipid metabolic enzymes and pathway (Bamba, Lee, Matsubara, & Fukusaki, 2012; Farbstein, Kozak-Blickstein, & Levy, 2010). Lipids such as fatty acids are needed for the production of compounds, which form an essential portion of cell membranes and nerve fibers, and also for the synthesis of certain hormones. Animals can synthesize their own fat from an excess amount of absorbed sugars, but they are limited in their ability to synthesize essential fatty acids (Mu & Porsgaard, 2005). Thus, fatty acids are not just an alternative energy source, but they are vital dietary ingredients more so than carbohydrates (Mensink, 1994).

Lipids in lipidomics are vital components of cellular membranes, lipid-transport and storage vesicles, that can elicit a myriad of bio-chemical functions in a variety of cellular processes (Gross & Holčapek, 2014; Lam et al., 2017).Lipids is one of the four major molecular components of biological organisms, along with proteins, sugars and nucleic acids (Zehethofer & Pinto, 2008). Subsequently, lipids are designated as a group of naturally occurring compounds that have high solubility with organic solvent such as hydrocarbons, chloroform, ethers and alcohols. They include a varied range of compounds, including fatty acids and their derivatives (http://lipidlibrary.aocs.org/Lipids/whatlip/index.htm).

Therefore, the diverse range of compounds inevitably treats cholesterol and plant sterols as a lipid, and could be interpreted to include bile acids, tocopherols and certain other compounds. It also enables the classification of compounds such as gangliosides as lipids. However, it need not include natural substances such as steroidal hormones and some carotenoids or simple terpenes, except in rare circumstances (http://lipidlibrary.aocs.org/Lipids/whatlip/index.htm).

The Figure 2.1 details the classification system for all of the lipids class and subclass. While their definition of a lipid is too broad, it is based on sound scientific principles. The most common lipid classes in nature comprise of fatty acids linked by an ester bond to the trihydric alcohol-glycerol; to alcohols such as cholesterol; by amide bonds to sphingoid bases; or to other amines (Fahy et al., 2005).

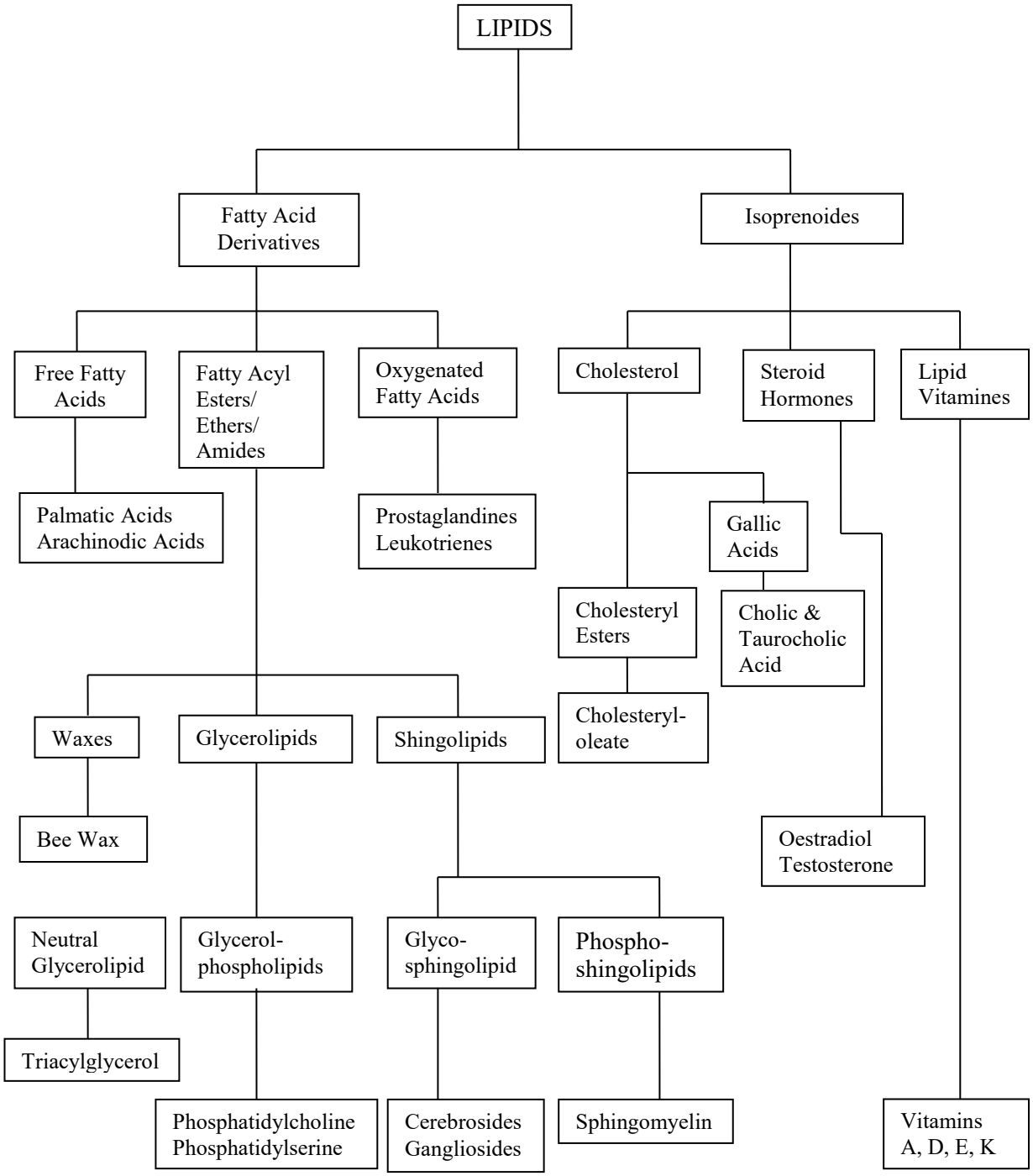

Figure 2.1 Full lipid classes [modified from (Fuchs, Süß, Teuber, Eibisch, & Schiller, 2011)]

In addition, they may contain alkyl moieties other than fatty acids, phosphoric acid, organic bases, carbohydrates and many more components, which can be released by various hydrolytic procedures (Mckee & Mckee, 2003). Definitions of simple lipids are those that during hydrolysis yield two types of primary product per mole; whereas complex lipids yield three or more primary products per mole during hydrolysis. Alternatively, the terms "neutral" and "polar" lipids respectively are used to define these groups, but they are less exact (Mckee & Mckee, 2003).

2.3.1 Fatty Acids

Fatty acids (FA) that can either be in free form or part of complex lipids, have a few of key roles in metabolism that serves as a major metabolic fuel in terms storage and transport of energy, an critical component of all type of membranes, and also as gene regulators. In addition, dietary lipids provide polyunsaturated fatty acids that are precursors to compounds such as the eicosanoids, powerful metabolites. As part of the complex lipids, fatty acids are also important for thermal and electrical insulation, and for protection, mechanically.

Fatty acids are structurally an elongated hydrocarbon chains with a methyl group at one end of the molecule and a carboxyl group at the other end. Based on Figure 2.2, the carbon atom next to the carboxyl group is called α carbon, and the subsequent are β carbon. The letter *n* is commonly used to describe the position of the double bonds (Rustan & Drevon, 2005).

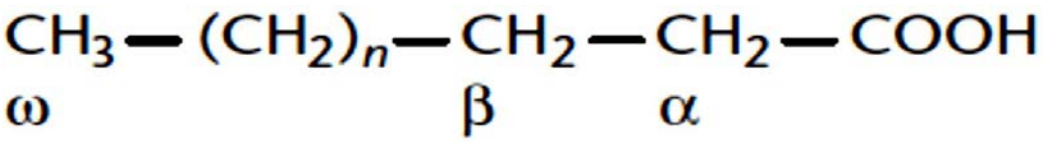

Figure 2.2 A simple fatty acid chain structure [adapted from (Rustan & Drevon, 2005)]

The systematic nomenclature for fatty acids are based on length, degree of saturation, the presence or absence, of the hydrocarbon chain and which possibly include the location of double bonds in context to the carboxyl group (Rustan & Drevon, 2005). The degree of saturation of fatty acids can be saturated and unsaturated, with either mono-, poly- or any other combination for the degree of saturation in a single fatty acid molecule. The most common fatty acids nomenclatures are listed in Table 2.1.

Table 2.1 The common fatty acids nomenclature and characteristics.

Fatty Acids	Chemical Nomenclature	Molecular Weight (MW)	Carbon No.	Double Bond No.
Caproic acid	$C_6H_{12}O_2$	116	6	0
Caprylic acid	$C_8H_{16}O_2$	144	8	0
Capric acid	$C_{10}H_{20}O_2$	172	10	0
Lauric acid	$C_{12}H_{24}O_2$	200	12	0
Myristic acid (M)	$C_{14}H_{28}O_2$	228	14	0
Palmitoleic acid (Po)	$C_{16}H_{30}O_2$	254	16	1
Palmitic acid (P)	$C_{16}H_{32}O_2$	256	16	0
Linolenic acid (Ln)	$C_{18}H_{30}O_2$	278	18	3
Linoleic acid (L)	$C_{18}H_{32}O_2$	280	18	2
Oleic acid (O)	$C_{18}H_{34}O_2$	282	18	1
Stearic acid (S)	$C_{18}H_{36}O_2$	284	18	0
Nonadecylic acid	$C_{19}H_{38}O_2$	298	19	0
Gadoleic acid (G)	$C_{20}H_{38}O_2$	310	20	1
Arachidic acid (A)	$C_{20}H_{40}O_2$	312	20	0

[Adapted from (La Nasa, Ghelardi, Degano, Modugno, & Colombini, 2013)]

2.4 Triacylglycerols (TAGs)

Triacylglycerols (TAGs) are the major components of naturally occurring fats and oils from animal and vegetable sources. Since TAGs are universal in plants and animals, TAGs molecules have adapted to a wide range of environments and functions. TAGs can occur as complex mixtures which are made up of saturated, monounsaturated and polyunsaturated fatty acid chains with a multitude of assortment of chain lengths that will give TAGs its identity (Cai, Short, Syage, Potvin, & Curtis, 2007). Figure 2.3 shows a TAG molecule, which consists of a glycerol backbone acylated with three fatty acids.

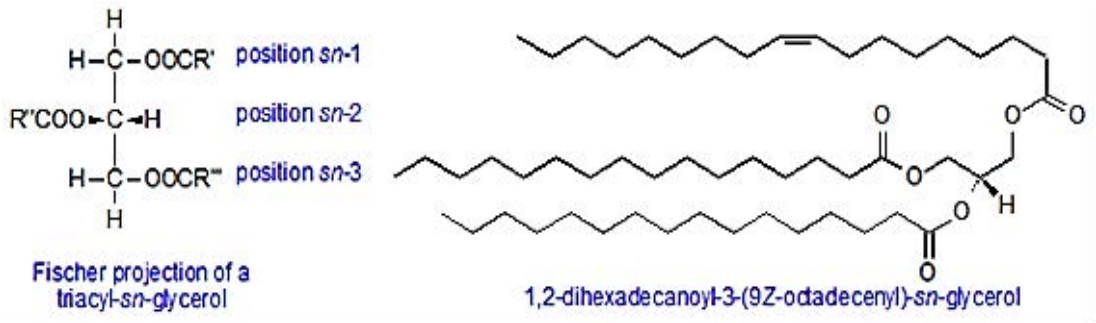

Figure 2.3 *Left*: Fisher's projection of TAG molecule. *Right*: Example of TAG molecule with fatty acid chain attached [adapted from (Fahy et al., 2005)]

2.5 Lipid Extraction Procedures

There are several established methods available for the extraction of lipids from biological samples. The procedures may differ among samples and hence may require modifications, depending on the characteristics of each sample. Table 2.2 illustrates some of the established methods to extract lipids from biological samples. These can also be used in food sample. However, these are basic extraction procedures, therefore extensive examination of sample-by-sample basis is still

required and modifications based on the suitability of the samples are still needed to optimize the extraction of target analyte.

Table 2.2 The established lipid extractions procedures for further lipid analysis.

Solvent system	Usage
Chloroform/methanol (1:1, v/v) "Bligh and Dyer Method"	Lipid extraction in water–rich systems
Chloroform/methanol (2:1, v/v) "Folch Method"	Lipids from animals, plants and bacterial tissues
Butanol saturated in water	Plant lipids, that are entrapped in starch and weak polar lipids
Hexane/isopropanol (3:2, v/v)	Low content of non-lipids (proteins, pigments, small molecules) in the extract because the used solvent mixture is highly polar
Chloroform/isopropanol (7:11, v/v).	Particularly suitable for erythrocytes with a high lipid content
Chloroform/methanol/12N hydrochloric acid (2:4:0.1, v/v/v)	Acidic phospholipids such as phosphatidylserine and particularly phosphoinositides

Compiled from (Bligh & Dyer, 1959; Iverson, Lang, & Cooper, 2001; M. Li, Yang, Bai, & Liu, 2014)

2.5.1 Bligh and Dyer Method

The Bligh and Dyer method is one of the most common and used extraction procedures for lipids. It was developed in the late 1950's by E.G. Bligh and W.J. Dyer (Bligh & Dyer, 1959) as an economical method of extracting the lipids from large volumes of wet tissues, specifically from frozen fish with the minimum volume of solvents. In essence, the endogenous water in the tissue is considered as a ternary component of the extraction system and sufficient chloroform and methanol are to be added in order to obtain a single-phase system for homogenization. After filtering, the residue has to be re-homogenized with fresh chloroform to ensure that the simple lipids are extracted completely. Subsequently, the combined organic layers are added to fresh saline solution that will produce a biphasic (two phase) mixture. A thorough practice is needed in order to make sure that the chloroform layer contains the lipid in question. If applied correctly to wet tissues, this procedure gives good recoveries of the more important lipid classes including TAGs. It is rapid and therefore suited to many routine applications (Bligh & Dyer, 1959).

Modified protocol of Bligh and Dyer is a much common practice nowadays (Iverson et al., 2001) to suit the target sample and to match the sensitivity of the system used such as the one used in this study.

2.6 Non-Mass Spectrometric Based Lipidomics

This section discusses briefly lipidomics analysis that is based non-mass spectrometric technique. The non-mass spectrometric analysis for lipidomics includes Fourier transform infrared (FTIR), differential scanning calorimetry (DSC), and Raman spectroscopy technique. Spectroscopy technologies in lipid analysis are infrequently used due to its limitation, such as its relatively low sensitivity to molecule mixture compared with MS. This occur due to the small chemical shift range, which produces packed spectra when acquired as a one-dimensional spectrum (M. Li et al., 2014).

FTIR spectroscopy offers analysis of whole sample with no modification. This technique coupled with chemometrics techniques of multivariate calibration of the partial least square was used in lipodomics. This can be used for the determination of lipid in mixed oils sample (a. Rohman, Triyana, Sismindari, & Erwanto, 2012).

DSC is a non-perturbing method for studying the thermotropic phase performance of hydrated lipid dispersions and lipid biological membranes. However, data acquisition and analytical protocol modification is needed due to the downside of the lipid thermotropic diverse phase behavior (Lewis, Mannock, & Mcelhane). Another type of spectroscopy, the Raman spectroscopy, uses a quantitative chemical material in a real-time and nondestructive technique without needing any exogenous modification to the samples (M. Li et al., 2014).

2.7 Mass Spectrometric Based Lipidomics

This study uses fully mass-spectrometric based analysis to profile TAGs from samples. Analytical methods have been developed for the analysis of lipids that includes the commonly chromatography tandem with mass spectrometry (L. Li et al., 2014). Chromatography is scientifically defined as the separation of two components in a molecular mixture based on different distribution between two non-miscible stationary phases, either solid or liquid through a mobile phase (Coskun, 2016; Kenndler, 2004).

Although there are many other types of chromatography, the techniques that most frequently used in the study of lipid separation are Thin-Layer Chromatography (TLC), Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC). The suitable technique will depend on various parameters such as polarity, complexity, duration, volatility and the amount of sample together with the type of solvents to be used (Shan, Jaffe, Li, & Davis, 2008) in addition to the molecular weight (MW) (, stoichiometry, and binding affinity (McKee & McKee, 2003).

The separation compounds on a carrier gas, which usually is nitrogen gas, is in actual fact, the characterization of a gas chromatography technique. The detection is often done through of mass spectrometry in tandem (GC/MS). Even though this technique has been established in fatty acid analysis and quantifying the fatty acids compositions of lipids, the analyte must be highly volatile so that the compounds can be analyzed where, derivatization, changing of the analyte structure is needed (Fuchs. et. al, 2010). Liquid chromatography (LC) will be the focus of this study moving forward.

2.8 Liquid Chromatography

Chromatographic separations in LC with the exception of size-exclusion chromatography functions through interactions developed between the functional groups of solute molecules, solvent molecules and the stationary phase (Romero-González, Frenich, & Vidal, 2010). The interactions in LC are hydrogen bonding, van der Waals forces and electrostatic forces. The modes of LC are classified according to the nature of these interactions. The mode of chromatography best suited for a particular separation depends upon molecular mass, polarity and ionic characters of a solute (Mansoor, 2002). Subsequent sections will discuss the method used and the columns technology comparison between C18 porous and C18 core-shell.

2.8.1 HPLC/Ultra High Performance Liquid Chromatography (UHPLC)

High-performance liquid chromatography is a separation technique on which a matrix is subjected under high pressure by elution with different solvents. The main benefit of this technique is that high qualities of separations are attainable. This is also applicable on a preparative scale unlike TLC. The coupling of HPLC with MS is well-recognized and thoroughly tested on. One of its shortcomings involve more time-consuming and expensive run time than TLC together with the detection of saturated lipids possessing some difficulty. In addition, post-column derivatization can be somewhat challenging. Conversely, HPLC routine method of lipid isolation in many laboratories has been well-established (Fuchs et. al, 2010).

Reversed-phase high-performance liquid chromatography (RP-HPLC) involves the separation of molecules based on hydrophobicity. The separation is determined on the hydrophobic binding of the molecule in the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase; in this instance that would be the column particle. The mixture is initially applied to the particle in the presence of an aqueous buffer, which then the molecules are eventually eluted out by the addition of organic solvent to the mobile phase (Cajka & Fiehn, 2014).

Elution can proceed by one of two conditions; by isocratic where the concentration of organic solvent is constant throughout the run or by gradient elution whereby the concentration of organic solvent increased or decreased over a period of time. The molecules are, therefore, eluted in order of increasing molecular hydrophobicity (Aguilar, 2004). This study used the RP-HPLC technique for TAG analysis.

2.8.2 Liquid Chromatography C18 Columns

Different modes of liquid chromatography have evolved for the analyses of a variety of compounds in diverse types of matrices. The modes of liquid chromatography include: normal-phase liquid chromatography (NPLC), reversed-phase liquid chromatography (RPLC), ion-exchange liquid chromatography (IELC) and size-exclusion chromatography (SELC) (Coskun, 2016). Selection of a liquid chromatographic mode for a particular analysis may also requires selection of a column, if used, (stationary phase) and solvents for the mobile phase. Any mode that is selected for development of methodology, it is important that certain criteria for

method validation are fulfilled (Mansoor, 2002). The study involves LC that emphasizes the different LC columns and solvent mixtures used.

The concept a porous shell coating a solid particles within a packing column was first proposed in the late 1960s, but the production of core-shell particles was too expensive and bulky to produce but which was overcome in the late 20th century (Guiochon & Gritti, 2011). The characteristic structure of core-shell particles active in chromatography encompasses of two different parts. One is an inner solid core with a diameter of 0.9 - 3.7 μm. The core of the column can either be inorganic compound or most likely an organic material. The second is a shell formed by several layers of silica covering around the core. The nucleus and the shell together creates a narrow particle size distribution that of a spherical particle that can be acutely adjusted to be 1.3 - 5.0 μm in total diameter, namely the core-shell particles (González-ruiz, Olives, & Martín, 2015).

An important property such as porosity, exerts a prominent impact on the chromatographic performance (Fekete, Olah, & Fekete, 2012). The resolution of a chromatogram can be improved with a thinner porous surface of a core-shell particles based on the eluting strength of the mobile phase reducing to compensate for the smaller retention factor yielded by the drop in the surface area of the particle (Horvath, Gritti, Fairchild, & Guiochon, 2010). The porosities of superficially and fully porous particles have been contrast (Gritti, Cavazzini, Marchetti, & Guiochon, 2007) and concluded the core-shell particles total porosity is lower than that of the totally porous traditional silica, due number of total pores per silica particle was reduce in the solid core. However, the external porosity of the columns possessing core-shell particles has a higher value than those obtained for the columns packed with traditional silica particles. During selection for suitable core-shell particles for a

particular separation, pore diameter and the molecular weight of the analytes parameters must be taken into account (González-ruiz et al., 2015).

Based on the core-shell column, improving chromatographic parameters can be summarize on five accounts. Firstly, as column permeability is increased, the higher the flow rates can be achieved at lower back pressures. Secondly, the high homogeneity and roughness of the particles obtained with this process improves the packing process of the column, avoiding the band broadening. Next, the solid nuclei reduce the longitudinal diffusion. Fourth, the existence of a reduced porous region in the particles reduces the band broadening and diffusion processes and, lastly, the higher thermal conductivity associated with the solid silica nucleus improves heat dissipation thus reducing the existence of radial temperature gradients that contribute to band broadening (González-ruiz et al., 2015).

2.9 Mass Spectrometry Components

Mass spectrometry based approaches have dominated the developed and applied analytical strategies for the analysis of complex biological lipid mixtures. The approaches used for lipid profiling can be categorised as either targeted or untargeted. In targeted workflows, a common strategy is to focus on known lipid molecules, specifically selected for MS analysis, by coupling LC separation (Gethings et al., n.d.). Liquid chromatography-mass spectrometry is a widely used method to quantify small molecules in complex biological matrix due to its high sensitivity, specificity and throughput, making it an ideal method to quantify the levels of TAG species in biological samples. However, mass spectrometry has been demonstrated to possess stronger identification ability, higher sensitivity, higher

specificity, and more simplicity. Thus, it is a very useful tool for analysis of TAG composition (Shan et al., 2008).

Mass spectrometry measurements are performed with charged particles because it is easy to manipulate experimentally the motion and direction of ions. By applying electric and magnetic forces, the energy and velocity of ionic species can be controlled, and both help in their separation and detection. In contrast, neutral gas-phase species move randomly and aimlessly. Their separation by gravitational force is highly impractical, and if attempted at all, then it might require an extremely long flight path, perhaps miles long (Munson & Field, 1966).

The Figure 2.4 shows the steps in mass spectrometry analysis. The first step is ionization that converts analyte molecules or atoms into gas-phase ionic species. This step requires the removal or addition of an electron or proton(s). The excess energy transferred during an ionization event may break the molecule into characteristic fragments. The next step is the separation and mass analysis of the molecular ions and their charged fragments on the basis of their mass-to-charge ratio (*m/z*). Finally, the ion current of these mass-separated ions is measured, amplified, and displayed in the form of a mass spectrum data (Dass, 2007).

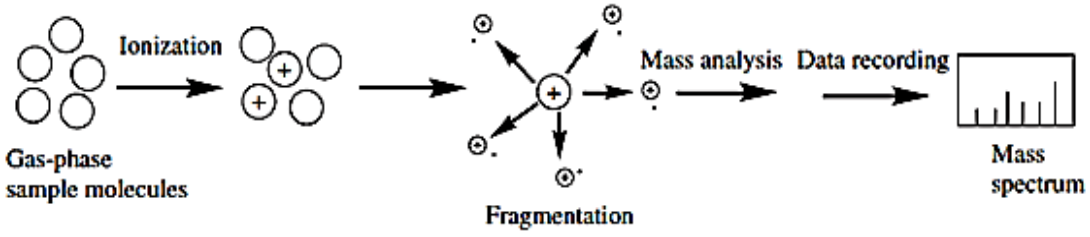

Figure 2.4 Basic concept of mass spectrometry analysis (Adapted from C. Dass, Principles and Practice of Biological Mass Spectrometry, Wiley-Interscience, 2001).