POSITIONAL DISTRIBUTION OF FATTY ACIDS IN NATURAL EDIBLE OILS AND STRUCTURED LIPIDS

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FACULTY OF SCIENCE
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ABSTRACT

The main objective of the present study is to investigate the positional fatty acids in natural dietary oils and structured lipids, which has shown relevance to health impact. Quantitative NMR method is selected to analyse the positional fatty acids profiles, instead of using conventional chromatographic techniques. In the present study, long chain ethyl esters were synthesised and subsequently used as starting material in the design of prospective structured lipids to reduce obesity risk. Ethyl behenate was esterified by behenic acid (C22:0) and ethanol under optimised condition. In the effort to monitor the esterification reaction, acid value and glyceride analyses were carried out. Immobilised lipase from the strain of *Rhizomucor miehei* (Lipozyme RM IM, Novozymes^{TM}) was employed in the solvent-free interesterification of ethyl behenate and palm olein (IV= 56) or high-oleic sunflower oil (IV= 92). The newly synthesised structured lipids were subjected to regiospecific analysis by using quantitative ^{13}C NMR (qCNMR) and chromatographic techniques. The structured lipids synthesised using high-oleic sunflower oil (EIE-HOS) contain higher amounts of the desired molecular species 1,3-dibehenoyl-2-oleoylglycerol (BOB) and 1-behenoyl-2,3-dioleoylglycerol (BOO) (76.5%) compared to that from palm olein (EIE-POo) (45.6%). Based on their slip melting point (SMP) (EIE-POo= 39.80℃ ; EIE-HOS= 41.40℃) and solid fat content (SFC), both structured lipids may find potential applications in functional dietary fats, such as cocoa butter equivalent, bakery shortening and *trans*-fat-free margarine. They may also serve as the hard stock for physical blending with other softer cooking oils with the aim of reducing obesogenic effects. A quick and easy analytical method using quantitative ^{13}C NMR (qCNMR) was developed to detect and quantify erucic acid in edible oils and mustard products in this study. An unreported peak at 173.1799 ppm was detected in mustard oil and proposed as *cis*-13 MUFA. The regiospecific analysis method was attainable within 58 minutes without sample

destruction and laborious procedures. Besides, the current method demonstrated high accuracy and low detection limit 0.98% (m/m) in quantitative results. The total content of erucic acid showed an excellent quantitative relation between conventional method (gas chromatography) and quantitative ^{13}C NMR. In this study, selected mustard oil (21.41%) and mustard product C (8.89%) exceeded the permitted maximum levels established for erucic acid (European Union: 5%, United States: 2%). High contents of erucic acid has been reported to cause myocardial lipidosis and heart lesions. More attention should be paid to the tolerable intake of erucic acid because over consumption of erucic acid has no nutritional effect. Thus, quantitative ^{13}C NMR (qCNMR) can be applied to monitor and quantify erucic acid content in a broad range of edible oils and mustard products.

ABSTRAK

Objektif utama kajian ini adalah untuk mengkaji hubungan antara kehadiran atau kedudukan asid lemak dalam triasilgliserol dan kesihatan. Dalam kajian ini, etil ester rantai panjang telah disintesis dan seterusnya, digunakan sebagai bahan permulaan dalam reka bentuk dan sintesis lipid berstruktur yang mungkin dapat mengurangkan risiko obesiti. Etil behenate telah diesterifikasi oleh asid behenik (C22: 0) dan etanol di bawah keadaan optima. Nilai asid dan analisis gliserida telah dijalankan untuk memantau tindak balas esterifikasi. Interesterifikasi etil behenate dan olein sawit (IV= 56) atau minyak bunga matahari yang beroleik tinggi (IV= 92) telah berjaya dijalankan dengan menggunakan lipase terimobilisasi dari *Rhizomucor miehei* (Lipozyme RM IM, Novozymes^{TM}) dalam keadaan tanpa pelarut. Analisisa regiospesifik dengan menggunakan kuantitatif ^{13}C NMR (qCNMR) dan teknik kromatografik telah dijalankan ke atas lipid berstruktur baru tersebut. Hasil Analisa menunjukkan lipid berstruktur berasaskan minyak bunga matahari yang beroleik tinggi (EIE-HOS) mengandungi jumlah spesies molekul dikehendaki *1,3-dibehenoyl-2-oleoylglycerol* (BOB) dan *1-behenoyl-2,3-dioleoylglycerol* (BOO) (76.5%) yang lebih tinggi berbandingkan dengan yang berasaskan olein sawit (EIE-POo) (45.6%). Berdasarkan *slip melting point* (SMP) (EIE-POo= 39.80℃ ; EIE-HOS= 41.40℃) dan *solid fat content* (SFC) masing-masing, kedua-dua lipid berstruktur mempunyai potensi untuk aplikasi produk lemak yang praktikal, seperti mentega koko yang setara, roti lemak sayuran dan marjerin bebas *trans*-asid lemak. Lipid tersebut juga boleh digunakan sebagai lipid pepejal untuk campuran fizikal dengan minyak masak lain lebih lembut dengan tujuan untuk mengurangkan kesan-kesan obesogenik. Satu kaedah analisisa yang cepat dan mudah yang menggunakan kuantitatif ^{13}C NMR (qCNMR) telah dikembangkan untuk mengesan dan menilai kandungan asid erusik dalam minyak makan dan produk-produk *mustard* dalam kajian ini. Puncak 173.1799 ppm tidak

pernah dilaporkan telah dikesan dalam minyak *mustard* dan dicadangkan sebagai *cis*-13 MUFA. Analisisa regiospesifik dilakukan dalam masa 58 minit tanpa kemusnahan sampel dan prosedur yang membebankan. Selain itu, kaedah semasa menunjukkan ketepatan yang tinggi dan had pengesanan yang rendah 0.98% (m/m) dalam keputusan kuantitatif. Jumlah kandungan asid erusik menunjukkan hubungan kuantitatif yang sangat baik antara kaedah konvensional (kromatografi gas) dan NMR ^{13}C kuantitatif. Dalam kajian ini, minyak *mustard* (21.41%) dan produk *mustard* C (8.89%) yang dipilih untuk kajian telah melebihi tahap maksimum asid erusik yang dibenarkan (Kesatuan Eropah: 5%, Amerika Syarikat: 2%). Minyak-minyak dan produk-produk mengandungi asid erusik yang tinggi telah dilaporkan sebagai penyebab miokardium lipidosis dan luka jantung. Pengambilan asid erusik harus terjaga dan dikawal kerana penggunaan asid erusik yang berlebihan tidak berkhasiat. Oleh itu, ^{13}C kuantitatif NMR (qCNMR) boleh digunakan untuk memantau dan mengukur kandungan asid erusik dalam pelbagai minyak makan dan produk *mustard*.

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TABLE OF CONTENTS

CHAPTER TWO: SYNTHESIS OF ETHYL ESTERS

CHAPTER THREE: ENZYMATIC INTERESTERIFICATION, CHARACTERISATION AND PURIFICATION OF STRUCTURED LIPIDS FROM PALM OLEIN AND HIGH-OLEIC SUNFLOWER OIL

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

%	per cent
AR	analytical reagent
BA	behenic acid
BBB	tribehenoylglycerol
BBP	1,2-dibehenoyl-3-palmitoylglycerol
BBS	1,2-dibehenoyl-3-stearoylglycerol
Bhd.	Berhad (limited)
BLB	1,3-dibehenoyl-2-linoleoylglycerol
BLO	1-behenoyl-2-linoleoyl-3-oleoylglycerol
BLP	1-behenoyl-2-linoleoyl-3-palmitoylglycerol
BOB	1,3-dibehenoyl-2-oleoylglycerol
BOO	1-behenoyl-2,3-dioleoylglycerol
BOSt	1-behenoyl-2-oleoyl-3-stearoylglycerol
BSTFA	N,N-Bis(trimethylsilyl)trifluoroacetamide
℃	degree Celsius
^{13}C	carbon-13
CN	carbon number of the three acyl chains
DAG	diacylglycerol
DB	number of double bonds
DHA	docosahexaenoic acid
DNPU	dinitrophenyl urethane
D&S	Dean-Stark
ECN	equivalent carbon number
EIE	enzymatic interesterified
EPA	eicosapentaenoic acid
EtMgBr	ethyl magnesium bromide
FA	Fatty acid
FAC	fatty acid composition
FAME	fatty acid methyl ester
FFA	free fatty acids
GC	gas chromatography
HOS	high-oleic sunflower oil

HPLC	high performance liquid chromatography
Hz	Hertz
i.d.	internal diameter
IM	immobilized
IUPAC	International Union of Pure and Applied Chemistry
IV	iodine value
KOH	potassium hydroxide
LLL	trilinoleoylglycerol
MAG	monoacylglycerol
MUFA	monounsaturated fatty acid
m/v	mass/volume ratio
ND	not detected
NMR	nuclear magnetic resonance
NUFA	number of unsaturated fatty acids
OLL	1,2-dilinoleoyl-3-oleoylglycerol
OOL	1,2-dioleoyl-3-linoleoylglycerol
OOO	trioleoylglycerol
PN	partition number
POo	palm olein
POO	1,2-dioleoyl-3-palmitoylglycerol
POP	1,3-dipalmitoyl-2-oleoylglycerol
POSt	1-palmitoyl-2-oleoyl-3-stearoylglycerol
PPh	phosphoryl phenol
ppm	parts per million
PPP	tripalmitoylglycerol
PUFA	polyunsaturated fatty acid
qCNMR	quantitative ^{13}C NMR
RBD	refined, bleached and deodorised
RM	*Rhizomucor miehei*
RP	reversed phase
rpm	revolutions per minute
SD	standard deviation
Sdn.	Sendirian (private)
SFA	saturated fatty acid
SFC	solid fat content

SLs	structured lipid
SMP	slip melting point
sn	stereospecific-numbering
StOSt	1,3-distearoyl-2-oleoylglycerol
StStSt	tristearoylglycerol
TAG	triacylglycerols
TL	*Thermomyces lanuginose*

CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

1.1.1 Composition of Oils and Fats

Oils and fats mainly consist of triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG) and free fatty acids (FFA) as the major components, and some consist of minor components such as carotenoids, tocopherols, sterols, triterpene alcohols, phospholipids, glycolipids and paraffinic hydrocarbons (Goh *et al.*, 1985). Table 1.1 shows the composition of palm oil. TAG of animal fats usually contains high level content of saturated fatty acids (SFA) which causes animal fats to exhibit relatively high melting points and tend to be solid at room temperature. In contrast, TAG of vegetable or nuts oils contain higher monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) than SFA. Thus, they have lower melting points than animal fats and tends to be liquid at room temperature. Christie (1989) method was applied to determine the complete fatty acid composition (FAC) of TAG. Fatty acid methyl esters were produced and injected into gas chromatography equipped with a flame ionisation detector. Table 1.2 shows the FAC of some edible oils.

Table 1.1: Composition of palm oil

Composition	Percentage (%)
Triacylglycerols	90-98
Diacylglycerols	2-6
Monoacylglycerols	2-5
Free fatty acids	2-3
Minor components	1-2

Refined, bleached and deodorised (RBD) palm olein contains a slightly higher amount of total MUFA (45.2%) than its total SFA (44.2%) due to its major fatty acids (FA) are oleic acid. The most abundant FA in coconut oil is lauric acid (C12:0) because the composition is 49.6%. Coconut oil contain significantly higher amount of total SFA content (90.5%) as compared to RBD palm olein (44.2%), macadamia nut oil (15.8%) and corn oil (13.8%). Oleic acid (C18:1) seems to be the predominant SFA and most abundant MUFA in most oils. Mainly, 45.1%, 57.7% and 30.6% were found in RBD palm olein, macadamia nut oil and corn oil, respectively.

Table 1.2: Fatty acid composition (FAC) of some edible oils

Fatty acid composition (as % methyl esters)	**RBD Palm Olein**	**Coconut Oil**	**Macadamia nut Oil**	**Corn Oil**
8:0	ND	3.7	ND	ND
10:0	ND	5.3	ND	ND
12:0	0.1	49.6	0.1	ND
14:0	0.7	19.1	0.7	ND
16:0	39.2	9.7	8.5	11.5
18:0	3.7	3.1	3.3	1.8
20:0	0.3	ND	2.5	0.5
22:0	0.1	ND	0.7	ND
24:0	0.1	ND	ND	ND
Total SFA	44.2	90.5	15.8	13.8
16:1n7	0.1	ND	21.7	ND
18:1n9	45.1	7.8	57.7	30.6
20:1n9	ND	ND	2.5	0.3
22:1n9	ND	ND	0.2	ND
Total MUFA	45.2	7.8	82.1	30.9
18:2n6	10.5	1.8	1.9	54.4
18:3n3	0.1	ND	0.2	1.0
Total PUFA	10.6	1.8	2.1	55.4

Notes:
SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ND, not detected.

1.1.2 Fatty Acids

Fatty acids (FA) are long hydrocarbon chain carboxylic acids with a functional group (-COOH). In general, FA can be categorised into SFA, MUFA and PUFA. FA fully loaded with hydrogen atoms are known as SFA, while FA with presence of carbon double bonds are known as unsaturated FA. Most natural occurring FA have a chain of an even number of carbon atoms, from C_4 to C_{28}, reflecting the pathway for their biosynthesis from the two-carbon building-block acetyl CoA. Short chain FA consists of hydrocarbon chain with less than six carbons (*e.g.* butyric acid, C_4) while medium chain FA has six to twelve carbons (*e.g.* caprylic (C8:0), capric (C10:0) and lauric (C12:0)) (Bach and Babayan, 1982). Long chain FA have longer hydrocarbon chain (C_{14} to C_{22}) compared to medium chain FA; and FA consists aliphatic chain longer than C_{22} are classified as very long FA. The examples of common FA are depicted in Table 1.3.

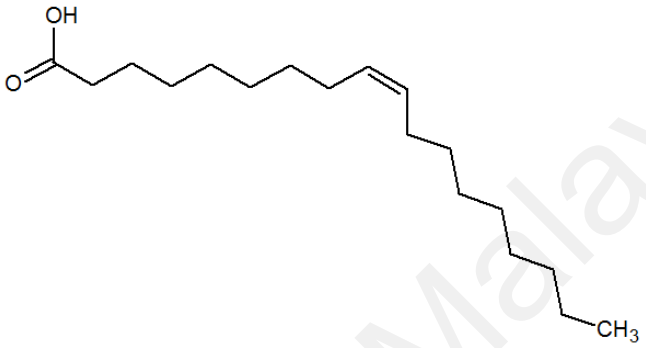
Short chain FA are water soluble and able to be absorbed directly into bloodstream from intestine. They are usually abundant in dairy products for immediate energy needs. Medium chain FA accelerate metabolic conversion instead of being stored as fat, the calories contained in medium chain FA are very efficiently converted into fuel for immediate use by organs and muscles (Tsuji, 2005). However, long chain FA are digested and absorbed by the digestive system to be potentially stored as TAG in adipose tissue if not immediately used as fuel source during physical activity (Dugan and Taylor, 2012). Palmitic acid (C16:0) is the most abundant long chain SFA in animals and plants among all natural occurring SFA.

Table 1.3: Common natural occurring fatty acids

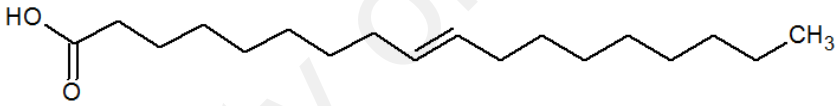
Systematic Name	Trivial Name	Shorthand Name	Structure
Octanoic	Caprylic	8:0	$CH_3(CH_2)_6COOH$
Decanoic	Capric	10:0	$CH_3(CH_2)_8COOH$
Dodecanoic	Lauric	12:0	$CH_3(CH_2)_{10}COOH$
Tetradecanoic	Myristic	14:0	$CH_3(CH_2)_{12}COOH$
Hexadecanoic	Palmitic	16:0	$CH_3(CH_2)_{14}COOH$
Octadecanoic	Stearic	18:0	$CH_3(CH_2)_{16}COOH$
Eicosanoic	Arachidic	20:0	$CH_3(CH_2)_{18}COOH$
Docosanoic	Behenic	22:0	$CH_3(CH_2)_{20}COOH$
Tetracosanoic	Lignoceric	24:0	$CH_3(CH_2)_{22}COOH$
cis-9-tetradecenoic	Myristoleic	14:1n5	$CH_3(CH_2)_3CH=CH(CH_2)_7COOH$
cis-9-hexadecenoic	Palmitoleic	16:1n7	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
cis-9-octadecenoic	Oleic	18:1 n9	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
trans-9-octadecenoic	Elaidic	18:1n9(t)	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
cis-11-octadecenoic	*cis-vaccenic*	18:1n7	$CH_3(CH_2)_5CH=CH(CH_2)_9COOH$
trans-11-octadecenoic	Vaccenic	18:1n7(t)	$CH_3(CH_2)_5CH=CH(CH_2)_9COOH$
cis-13-docosenoic	Erucic	22:1n9	$CH_3(CH_2)_7CH=CH(CH_2)_{11}COOH$
cis-15-tetracosenoic	Nervonic	24:1n9	$CH_3(CH_2)_7CH=CH(CH_2)_{13}COOH$
cis, cis,-9,12-octadecadienoic	Linoleic	18:2n6	$CH_3(CH_2)_3(CH_2CH=CH)_2(CH_2)_7COOH$
cis, cis, *cis*-9,12,15-octadecatrienoic	α-linolenic	18:3n3	$CH_3(CH_2CH=CH)_3(CH_2)_7COOH$
cis, cis, *cis*-6,9,12-octadecatrienoic	γ-linolenic	18:3n6	$CH_3(CH_2)_3(CH_2CH=CH)_3(CH_2)_4COOH$
cis, cis, *cis*, *cis*-5,8,11,14-eicosatetraenoic	Arachidonic	20:4n6	$CH_3(CH_2)_3(CH_2CH=CH)_4(CH_2)_3COOH$
cis, cis, *cis, cis, cis*-5,8,11,14,17-eicosapentaenoic	Eicosapentaenoic (EPA)	20:5n3	$CH_3(CH_2CH=CH)_5(CH_2)_3COOH$
cis, cis, cis, *cis, cis, cis*-4,7,10,13,16,19-docosahexaenoic	Docosahexanenoic (DHA)	22:6n3	$CH_3(CH_2CH=CH)_6(CH_2)_2COOH$

Besides, FA with double bonds can be further categorised in terms of geometric isomerism indicated as *cis*, *trans*-configurations or *Z*, *E*-configurations. A *cis* configuration means that the two hydrogen atoms adjacent to the double bond both on the same side of the chain. The rigidity of the double bond causes the hydrocarbon chain to bend thus restricts the conformational freedom of the FA. In the *cis* configuration, the more double bonds, the less flexibility it has. By contrast, *trans* configuration means the adjacent two hydrogen atoms lie on opposite sides of the chain (Figure 1.1). Hence, the chains bend not much and shape is similar to straight SFA. In

nature, most the unsaturated FA found in human body are *cis* FA, with the exception of retinoic acid (which is present in the eye). Most FA in the *trans* configuration are the result of human processing such as hydrogenation (DeBruyne *et al.*, 2012). In addition, the cell's enzymes breakdown *trans* fats inefficiently, causes *trans* fats accumulate in the body and act as competitive inhibitors to FA metabolizing enzymes.



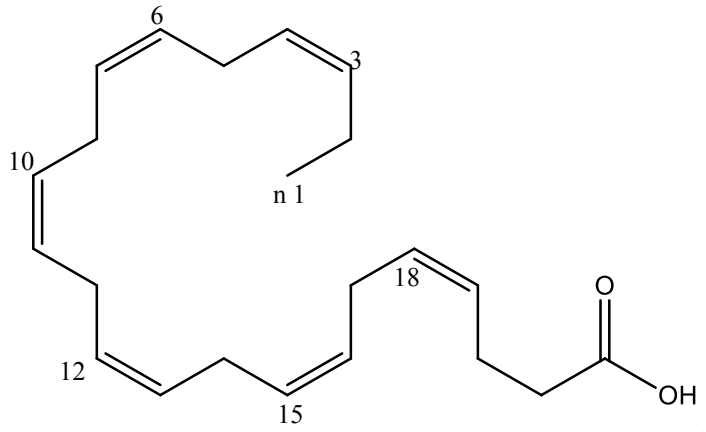
cis-Oleic acid



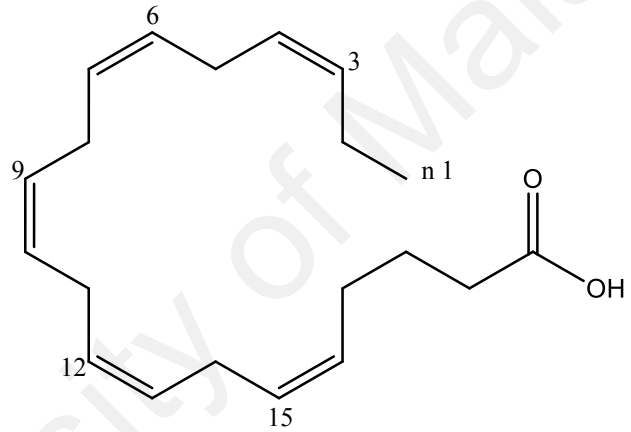
trans-Oleic acid

Figure 1.1: *Cis* and *trans* configuration oleic acid

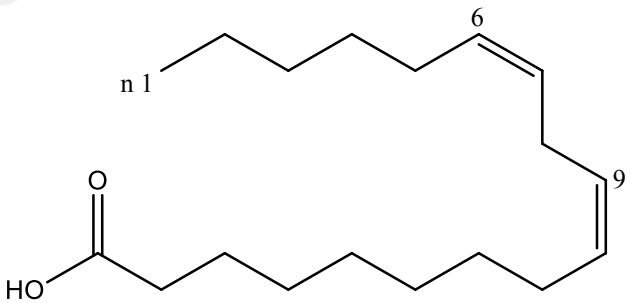
Positions of double bond can state either start from the carbonyl carbon (-COOH) name as alpha (α) or from the methyl end (-CH_3) name as omega (ω / n) of the FA chain (Scorletti and Byrne, 2013). For example, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) which are commonly found in marine oils, are ω3 PUFA as the nearest carbon containing double bond is the third carbon from the methyl end. Linoleic acid is an example of ω6 PUFA as the closest carbon containing double bond is the sixth carbon from the methyl end (Figure 1.2).



DHA



EPA



Linoleic acid

Figure 1.2: n numbering of DHA, EPA and linoleic acid

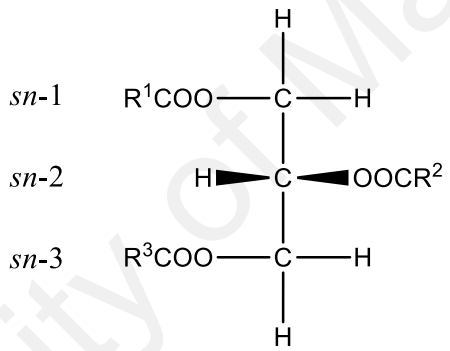
Chemically, MUFA are FA that have only one double bond (C=C) in the hydrocarbon chain. The most common MUFA is oleic acid (C18:1 n9), palmitoleic acid (C16:1 n7) and *cis*-vaccenic (C18:1, n7). In general, MUFA are highly found in edible oils and nuts such as olive oil (75%), camellia (tea seed) oil (90%), macadamia nuts (83%) and almonds (63%). MUFA have a higher melting point than PUFA but a lower melting point than SFA since viscosity and melting temperature increases with decreasing number of double bonds. Some other examples of common MUFA are depicted in Table 1.3.

Polyunsaturated fatty acids (PUFA) are FA that have more than one double bond (C=C) in the hydrocarbon chain. PUFA can be found easily in nuts, seeds, fish, algae, leafy greens, and krill. Some examples of PUFA are linoleic acid (C18:2 n6), EPA (C20:5 n3) and DHA (C22:6 n3). In preliminary research, high dietary intake of n3 PUFA (EPA and DHA) are able to lower the risk of heart attack, reduce 25% risk of additional breast cancer (Patterson *et al.*, 2010), efficient in decreasing low density lipoprotein (LDL) as well as increasing high density lipoprotein (HDL) (Osborn and Akoh, 2002). In addition, DHA is vital for the grey matter structure of the human brain, as well as retinal stimulation and neurotransmission (Bain, 2010). On top of that, n6 PUFA (linoleic acid) may reduce the risk of cardiovascular disease. Some studies have suggested that n6 PUFA should be consumed in a 1:1 ratio to n3 PUFA (Simopoulos, 2006) because the imbalanced ratio can affect how the other is metabolised since n6 and n3 PUFA are both essential FA that are metabolised by the same enzymes.

1.1.3 Triacylglycerols

Triacylglycerols (TAG) are triesters which are formed by combining one mole of glycerol with three moles of FA. TAG are the main constituents of vegetable or nuts

oils and animal fats. In fact, properties of TAG such as melting point depend on the saturation level of FA chains which are attached to the glycerol backbone. Animal fats solidify easily due to the high level of SFA chains. To avoid designation problem in complex TAG, IUPAC recommended stereospecific numbering (*sn*) system. In a Fischer projection, the carbons numbered as 1, 2, and 3 from top to bottom (Figure 1.3). Since the secondary acyl group is shown to the left of the C2 atom, the position distribution of FA in glycerol backbone designated is as *sn*-1, *sn*-2 and *sn*-3 positions. However, the TAG structure will be chiral if the FA at both *sn*-1 and *sn*-3 positions are different.



Notes:
R^1, R^2, R^3 are corresponding to alkyl groups attached to the carbonyl carbons at *sn*-1, *sn*-2 and *sn*-3 positions, respectively.

Figure 1.3: Fisher projection of a triacylglycerol

1.1.4 Esters

Esters are chemical compounds with functional group (-COO) derived from an acid in which an alkoxy (-O) group replaces hydroxyl (-OH) group. Low molecular weight esters are commonly used in the fragrance and flavour industry or as essential oil because of their unique pleasant and fruity aroma. Belsito and his researchers reported 21 esters (*e.g.* butyl stearate, ethyl laurate and methyl hexanoate) which were approved by the Food and Drug Administration (FDA) and 20 esters (*e.g.* allyl cinnamate, isoamyl cinnamate and isopropyl cinnamate) were recognised by the Flavour and

Extract Manufacturers' Association (FEMA, 1965) as flavour ingredients without safety concerns (Belsito *et al.*, 2007). Subsequently, 21 of the esters (*e.g.* triethyl citrate and benzyl cinnamate) were also included in the Council of Europe's list (Council of Europe, 2000) and 19 of the esters (*e.g.* butyl acetoacetate and cinnamyl formate) were evaluated by the International Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2000) as safe food flavours. Other example includes benzyl acetate, a natural compound found in cloves and chamomile, is extensively used as a fragrance ingredient in perfumes, soap, creams, lotions and detergents (VCF, 2010).

Besides used in the food industry, esters have been used as biodiesel to replace petroleum diesel in diesel engines due to environmental concerns and diminishing petroleum reserves (Yong, 2007; Demirbas, 2006). Biodiesel is made by chemically reacting oils or fats with an alcohol producing FA esters. Biodiesel is considered renewable because one of the reactant such as ethanol can be fermented from sugarcane or corn which rich in glucose (Knothe, 2013). The usage of biodiesel has been increasing in the United States after the approval of the Energy Policy Act of 2005. In 2010, fuel suppliers were constrained by the Renewable Transport Fuel Obligation to include 5% biodiesel fuel in all transport fuel sold in the United Kingdom. Both Dagostin and Serres teams have published biodiesel production from transesterification using soybean oil (Dagostin *et al.*, 2015; Serres *et al.*, 2015). Furthermore, other research groups have also reported biodiesel (mixture of ethyl esters) synthesised from transesterification using palm oil (Rocha *et al.*, 2014), canola oil (Oliveira *et al.*, 2011; Ferreira *et al.*, 2015) and sunflower oil (Mesquita *et al.*, 2011; Bessa *et al.*, 2015).

1.1.5 Structured Lipids

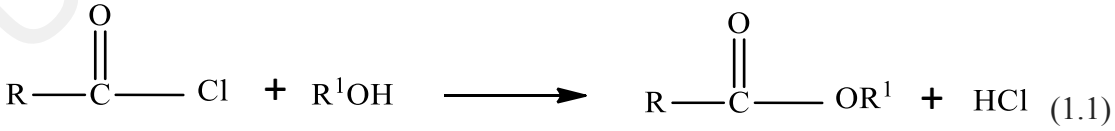
Structured lipids (SLs) are defined as restructured TAG with modified FAC and/or their positional distribution in glycerol molecules by chemical or enzymatical processes. In this definition of SLs, the scope of lipids includes TAG as well as DAG, MAG and glycerophospholipids. It may have synthesised along with the incorporation of FA nutritional aspects, lipid absorption and characteristics of lipases. Incidentally, SLs can offer the most efficient way to deliver the required nutrients and FA for therapeutic purposes (Akoh, 1995).

1.2 Literature Review

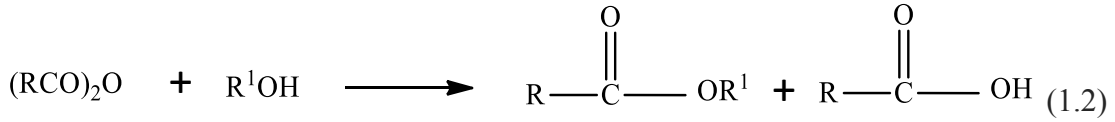
1.2.1 Synthesis of Esters

1.2.1.1 Alcoholysis

Esters are synthesised when alcohols react with acyl chlorides (Equation 1.1) or acid anhydrides (Equation 1.2) (Sano *et al.*, 1999; Dhimitruka and SantaLucia, 2006). Higher yield of esters can be obtained during the reaction between alcohols and acid anhydrides under 0.05-2 mol% of dimethylaminopyridine (Sakakura *et al.*, 2007). However, it is an expensive method and anhydrous condition is required due to reactivity of acyl chlorides and acid anhydrides with water. The reactions are irreversible simplifying work-up.



(1.1)



(1.2)

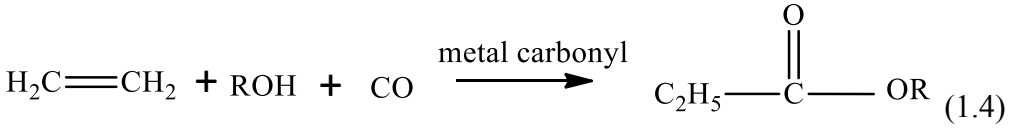
1.2.1.2 Alkylation of Carboxylate Salts

Alkylation of carboxylate salts with alkyl halides can also synthesise esters (Equation 1.3) (Pfeffer and Silbert, 1976). Based on Finkelstein reaction, iodide salt can catalyse the reaction if an alkyl chloride is used. However, some reports concluded that this reaction is more suitable for the preparation of hindered ester, giving high yields and conversions along with not much elimination of by-product (Wagner and Zook, 1953; Mills *et al.*, 1962; Pfeffer *et al.*, 1972; Moore *et al.*, 1979). The reaction was quenched with water, acidified with 10% hydrochloric acid and extracted three times with petroleum ether. The petroleum ether layer was washed four times with 30 mL portions of dilute hydrochloric acid and water followed by sodium thiosulfate solution to remove any liberated iodine. The organic layer was dried and solvent removed by rotary evaporation (Pfeffer and Silbert, 1976).

(1.3)

1.2.1.3 Carbonylation

Esters can also be synthesised by introducing carbon monoxide into organic and inorganic compounds through carbonylation reaction (Buchan *et al.*, 1985). For instance, alkenes react with alcohol in the presence of metal carbonyl catalysts (Equation 1.4) or methanol react with carbon monoxide yields methyl formate when it catalysed by sodium methoxide (Equation 1.5).



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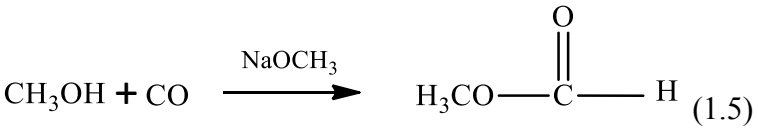
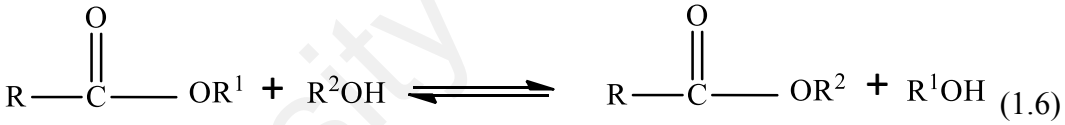


(1.5)

Tsuji *et al.* (1984) discovered a useful method to gain *β*,*γ*-unsaturated ester in the presence of catalysts (palladium-phosphine complexes). Allyl alkyl carbonates react smoothly with carbon monoxide at 50 °C under atmospheric or low pressure (Tsuji *et al.*, 1984). The mixture is cooled, extracted with ether and the extract is washed several times with 20% hydrochloric acid, then with saturated sodium bicarbonate solution and finally with water. After drying and filtering the solution, ether is removed by evaporation, and the crude ester is purified by chromatography, distillation and recrystallization.

1.2.1.4 Transesterification

Transesterification is an organic synthesis involves exchanging the organic group (R^1) of an ester with the organic group (R^2) of an alcohol (Equation 1.6). This reaction is well known and mostly presents as synthesis reaction for biodiesel.



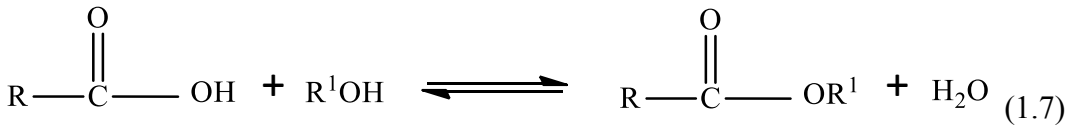
(1.6)

Fatty acid methyl esters (biodiesel) are synthesised from the transesterification reaction of animal fats and vegetable oil with aliphatic alcohols with the aid of an acid or base catalyst (Kurle *et al.*, 2013). Sulphuric acid, aluminium alcoholate and *p*-toluenesulfonic acid was used as acid catalyst, the first being preferred (Rehberg and Fisher, 1944). These catalysts give very high yields in alkyl esters, but the reactions are slow, requiring, typically, temperatures above 100 °C and more than 3 hours to reach complete conversion (Freedman *et al.*, 1984). In contrast, base catalysts (*e.g.* sodium carbonates and potassium carbonates) are less corrosives than the acid catalyst and the base-catalysed of transesterification reaction proceeds faster than the acid-catalysed reaction (Freedman *et al.*, 1984; Freedman *et al.*, 1986). Besides that, transesterification

can be carried out in both heterogeneous and homogeneous catalytic processes. However, the heterogeneous catalytic process is preferred since heterogenous catalyst is easier to be removed and reused, higher activity, and release less pollution into the environment than a homogeneous catalyst (Birla *et al.*, 2012). At the same time, another research also showed esters were prepared by the direct transesterification of carboxylic esters in boiling alcohols catalysed by Scandium Triflate (Remme *et al.*, 2007). The transesterification product was allowed to stand in a separating funnel for glycerol separation. In order to separate glycerol from the ester and removes the residual catalyst, 10 g of pure glycerol was added to the transesterification product and the product was allowed to stand an hour. The glycerol layer will be separated from the ester layer due to the difference in the density between the two phases. Crude esters were washed several times (up to 10) with 50 mL of hot distilled water (50 °C) in a separating funnel until neutral pH and heat at 110 °C (George *et al.*, 2009).

1.2.1.5 Esterification

Esterification of carboxylic acid with alcohol is another prominent method to give esters (Equation 1.7). It is a highly reversible reaction in which the products and reactants are in equilibrium (Ishihara *et al.*, 2002; Srinivas *et al.*, 2003). The presence of concentrated sulphuric acid not only speeds up the reaction but also isolate water because sulphuric acid acts as a catalyst and dehydrating agent in the reaction. Concentrated sulphuric acid can be used for this purpose because it reacts rapidly with water to form a hydrated form of sulphuric acid, effectively removing the water from the reaction mixture.



(1.7)

According to Le Chatelier's principle, the equilibrium can be shifted to favour the formation of products by removing one product from the reaction mixture. For instance, removal of water by either distilling the mixtures of carboxylic acid and alcohol in low boiling azeotropes with toluene by connecting it with a Dean-Stark apparatus, will drive the reaction to the production of esters, and therefore increasing the yield of esters. The esterification product mixed with water was allowed to stand in a separating funnel for separation. Initial washing with water will remove most of the water-soluble species which includes any excess alcohol, carboxylic acid and sulphuric acid. The ester is not very soluble in water so will separate into a separate layer. The ester is less dense than water so the ester layer floats on top of the aqueous layer. After washing there will still be traces of acid left in the organic layer. An aqueous solution of sodium carbonate or sodium hydrogen carbonate can be used to neutralise any remaining acid. The final rinsing with water should remove traces of the water-soluble salt formed.

1.2.2 Synthesis of Structured Lipids

There are a few published reports on tailor made fats in the mid 1950s and early 1960s. The first examination of SLs was from acetic acid and long chain FA sustained growth in rats was published by Mattson (Mattson *et al.*, 1956). Fernandes obtained medium chain rich SLs by transesterification of caprylic acid (C_8) and olive oil (Fernandes *et al.*, 1962). Huang and Akoh (1996) successfully transesterified caprylic acid ethyl ester and triolein by lipase-catalysis. Besides transesterification reaction, interesterification, direct esterification and acidolysis can be employed in the synthesis of SLs too. Direct esterification reaction between alcohol and carboxylic acid enables the synthesis of novel SLs. For example, trielaidin was incorporated directly from the reaction between elaidic acid and glycerol (Adlof and List, 2007). Apart from that, acidolysis demanded

exchange of acyl group between TAG and FA (Xu, 2000). Akoh and Yee (1997) synthesised SLs through enzymatic interesterification between tricaprin (medium chain saturated TAG) and tristearin (long chain saturated TAG). Meanwhile, Yang *et al.* (2001) incorporated stearic acid (long chain SFA) mainly into the *sn*-1 and/or *sn*-3 positions of triacetin (short chain saturated TAG).

1.2.2.1 Interesterification

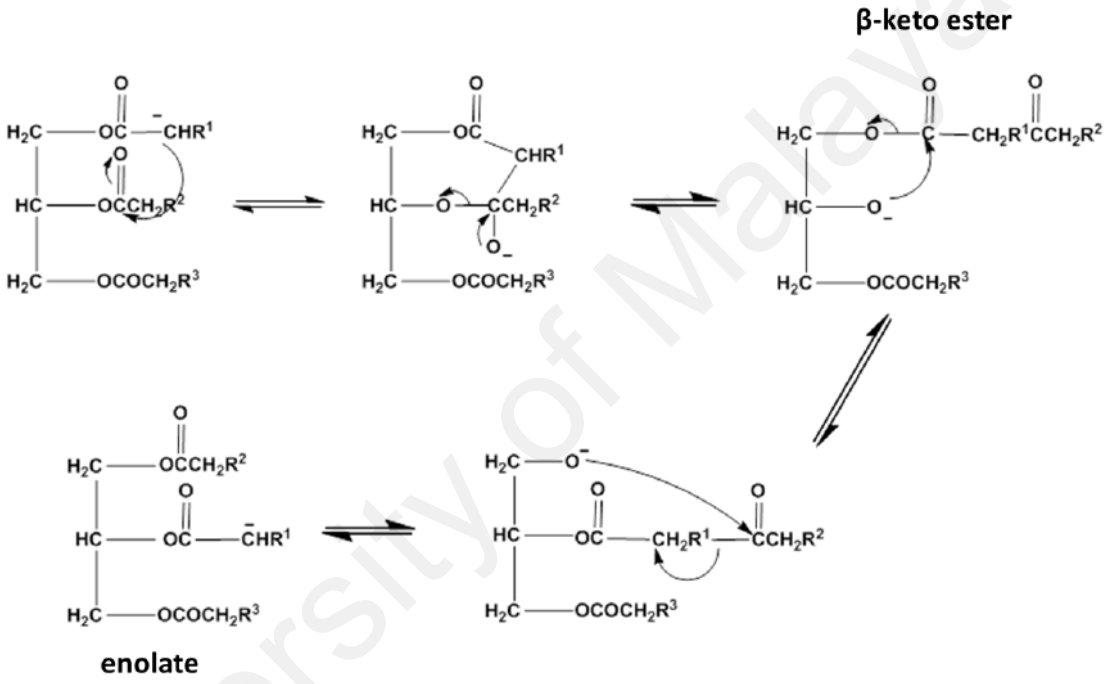
In order to acquire targeted physical properties with low caloric value and low melting point, SLs obtained long chain SFA at the *sn*-1,3 positions prevented the deposition of visceral fat (Kojima *et al.*, 2010). Modification of TAG can be achieved by the incorporation of FA or intramolecular and intermolecular redistribution the positions of FA. SLs can synthesised through interesterification reactions, by chemically or enzymatically.

1.2.2.2 Chemical Interesterification

Chemical interesterification is an important process for oils and fats. It denotes as a random reaction that randomises the acyl chains moieties at the *sn*-1, *sn*-2 and *sn*-3 positions (Silva *et al.*, 2009). Chemical interesterification is classified into two types, namely, random and direct interesterification (Sreenivasan, 1978). In random chemical interesterification, the reaction completely random if the reaction temperature is above the melting point of the mixture. However, the TAG with higher melting point will be crystallised during low temperature, thus, resulted in direct interesterification reaction.

Eckey (1945) introduced chemical interesterification process which can be induced by chemical catalysts such as sodium methanolate or sodium methoxide at low temperature (<100°C). Two types of catalytic mechanisms have been proposed to explain the

chemical interesterification. Batles suggested that the intermediate is a glycerolate in the sodium methanolate-catalysed interesterification while methanolate act as an alcoholate (Batles, 1960). However, another mechanism involving the formation of an enolate ion caused by the *α*-hydrogen of an acyl group being attacked by the sodium methanolate was proposed by Weiss *et al.* (1961) and then, verified by Liu (2004). The increase of *β*-keto ester concentration during interesterification reaction will reduce the occurrence of this mechanism (Figure 1.4).



Notes:
R^1, R^2, R^3 are corresponding to alkyl groups attached to the carbonyl carbons at *sn*-1, *sn*-2 and *sn*-3 positions of triacylglycerol, respectively.

Figure 1.4: Rearrangement of the acyl group *via* Claisen condensation (Liu, 2004)

Chemical interesterification seems to be the most beneficial method low cost and large scale production of SLs. Nonetheless, chemical interesterification leads to a random distribution of FA on the TAG and results in the formation of large variety of TAG species in most cases (Marangoni and Rousseau, 1998).

1.2.2.3 Enzymatic Interesterification

Enzymatic interesterification is a intermolecular catalytic reaction that rearranges FA on glycerol either between one TAG and another TAG (Shin *et al.*, 2010) or between TAG and alkyl esters (Wang *et al.*, 2006) when enzyme is introduced. The interesterification between TAG and alkyl esters will give mixtures of TAG and unreacted alkyl esters. Thus, further purification such a short path distillation to improve both purities and yields are necessary (Shimada *et al.*, 2000).

Commercial lipases are available from microbial, plant, and animal sources. In general, there are two types of lipases, namely, non-specificity (*Candida rugosa*, *Corynebacterium acnes* and *Staphylococcus aureus*) and specific (*Aspergillus* sp., *Mucor* sp. and *Rhizopus* sp.). For instance, Novozym 435 from *Candia antarctica* lipase B is a typical immobilised non-specific lipase and Lipozyme RM IM from *Rhizomucor miehei* is an immobilised 1,3-specific lipase. Non-specific interesterification usually gives a similar product as the chemical interesterification, but regiospecific lipases are commonly used to produce symmetrical products.

Many different types of lipases have been investigated for the enzymatic interesterification of oils and fats. In a study reported by Ibrahim *et al.* (2008), positive synergetic effect was obtained in enzymatic interesterification of palm stearin with coconut oil by applying a dual lipase system (*Pseudomonas flourescens*, *Thermomyces lanuginose* (Lipozyme TL IM), *Rhizomucor miehei* (Lipozyme RM IM) and *Candida antarctica* B). While another similar study compared SLs that been produced using different lipases from Lipozyme TL IM, *Rhizopus* sp. and the mixture of both lipases (Speranza *et al.*, 2016).

The structured lipids (SLs) which resemble human milk fat, are enzymatically interesterified by commercial lipases *Candida cylindracea* (AY30™) and *Mucor circinelloides* (M10™) from lard and soybean oil blends. It was found that *sn*-1,3-specific M10 lipase has greater reduction in the softening point, consistency, and solid fat content compared to AY30 lipase (Silva *et al.*, 2009). Human milk fat was synthesised from tripalmitin and a blended vegetable oil (coconut, safflower, and soybean oils) by *sn*-1,3-specific Lipozyme RM IM (Maduko *et al.*, 2007).

Enzymatic interesterification has advantages over chemical interesterification, owing to the selectivity and regiospecificity of lipases. Benefits such as greater control of the positional distribution of FA on glycerol backbone, and milder reaction conditions, are desirable less adverse effect to the flavour of the product (Rousseau and Marangoni, 1998; Soares *et al.*, 2009). For instance, the enzymatic interesterification results of milk fat and soybean oil blends with *sn*-1,3 specific lipase from *Rhizopus oryzae* immobilised showed the TAG's profile change potentially (Paula *et al.*, 2010). The highest interesterification degree (2.60) was achieved with the medium of 65% of milk fat. Therefore, the feasibility of using *Rhizopus oryzae* immobilised to reduce the milkfat consistency and turning it more spreadable under cool temperature has been demonstrated.

1.2.3 Analysis of Positional Distribution of Fatty Acid in Triacylglycerols

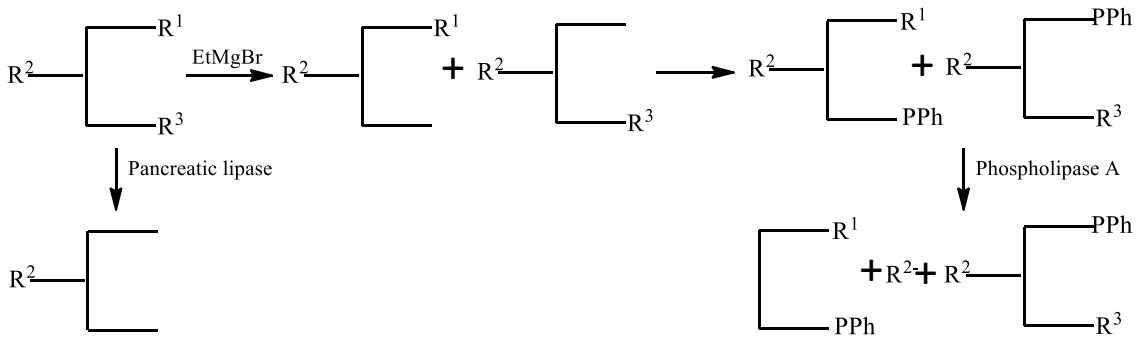
As described earlier, edible oils and fats are majorly composed of mixtures of TAG. In view of nutritional, the positional distribution of FA on glycerol backbone of TAG is crucial. Numerous regiospecific and stereospecific analyses of TAG in oils and fats have developed in past decades. Regiospecific analysis has been widely used to distinguish between the *sn*-1,3 and *sn*-2 positions on TAG, while, stereospecific

analysis referred to the determination of the FA of TAG at *sn*-1, *sn*-2 and *sn*-3 positions of the glycerol.

1.2.3.1 Stereospecific Analysis

Deacylation of TAG to DAG is the first step of stereospecific analysis. Mixtures of 1,2-DAG and 2,3-DAG can be resulted using Grignard reagent, pancreatic lipase, milk lipase or *Geotrichum candidum* lipase. The stereospecific analysis using Grignard reagent was introduced by Brockerhoff in 1965. The Grignard reagent reacts with one of the ester linkages, followed by its hydrolysis to form DAG and tertiary alcohol. Ethyl magnesium bromide is the Grignard preferred reagent in this reaction since it produces a tertiary alcohol that is easily separated from the DAG during chromatographic isolation of the reaction products. Subsequently, DAG can be converted into phospholipids by chemically or enzymatically.

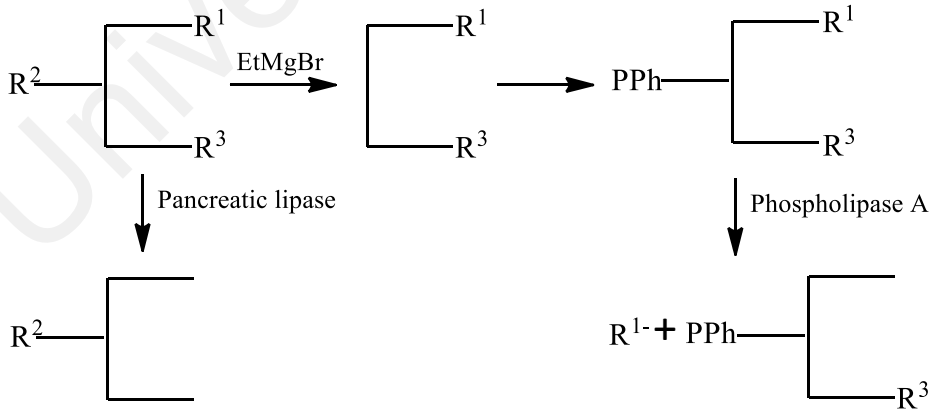
In Brockerhoff's first method, equimolar mixtures of 1,2-DAG and 2,3-DAG can be prepared by reacting the TAG with Grignard reagent (Brockerhoff, 1965). Mixtures DAG are then converted synthetically to phosphatidylphenol by employing phenyl dichlorophosphate and later hydrolysed by phospholipase A (Figure 1.5). The FAC is determined by GC, while prep TLC has isolated lysophosphatide. Additionally, 2-MAG can be determined independently by pancreatic lipase hydrolysis. The main disadvantage of this method results minor errors for the FAC of position 3 since the FAC of position 3 is not determined directly but through calculation.



Notes: PPh = phosphoryl phenol
EtMgBr = Ethyl magnesium bromide

Figure 1.5: Stereospecific analysis of Triacylglycerols by Brockerhoff's first method (1965)

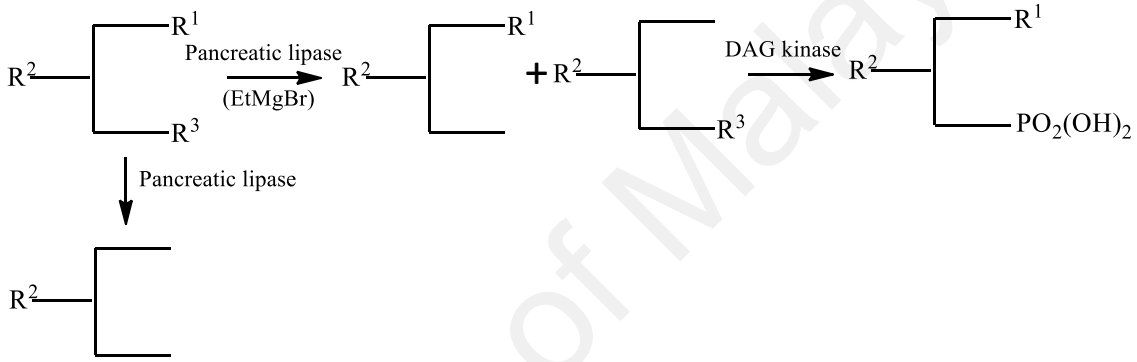
In year 1967, Brockerhoff modified his first method (Figure 1.5). 2-MAG were determined by pancreatic lipase hydrolysis as before, but 1,3-DAG phosphorylated and reacted with phospholipase A after its formation by reacting the TAG with Grignard reagent (Figure 1.6). The FA at *sn*-1 position are cleaved and left a lysophosphatide which contains FA chain at *sn*-3 position. As a result, positional purity of phospholipid can be checked by TLC.



Notes: PPh = phosphoryl phenol
EtMgBr = Ethyl magnesium bromide

Figure 1.6: Stereospecific analysis of Triacylglycerols by Brockerhoff's second method (1967)

Another analytical method using an enzyme from *Escherichia coli* cells has been reported by Lands *et al.* (1966). Mixtures of 1,2-DAG and 2,3-DAG are prepared by reacting the TAG with pancreatic lipase or Grignard reagent. DAG kinase has been employed for the conversion of 1,2-DAG to 1,2-diacyl-3-phosphatidate while 2,3-DAG remained unphosphorylated (Figure 1.7). Then, 2-MAG is determined separately by pancreatic lipase hydrolysis. Only the *sn*-2 position is determined directly, but *sn*-1 and *sn*-3 positions must be calculated.



Notes: EtMgBr = Ethyl magnesium bromide

Figure 1.7: Stereospecific analysis of Triacylglycerols by Lands' procedure (1966)

In comparison, the analysis method presented by Brockerhoff (1965) is more accurate since the FAC of the *sn*-1 position is determined directly. On the contrary, analysis method revealed by Lands *et al.* (1966) show that both *sn*-1 and *sn*-3 positions are determined by calculation only. However, Lands' method can be completed in about 2-3 days because no hydrolysis is needed. Both Brockerhoff methods which include deacylation, phosphorylation, and phospholipase A hydrolysis steps; takes 3-4 days of laborious work for each stereospecific analysis.

Alternative stereospecific analysis that uses simple chemical degradative and derivatization steps, followed by high performance liquid chromatography (HPLC)

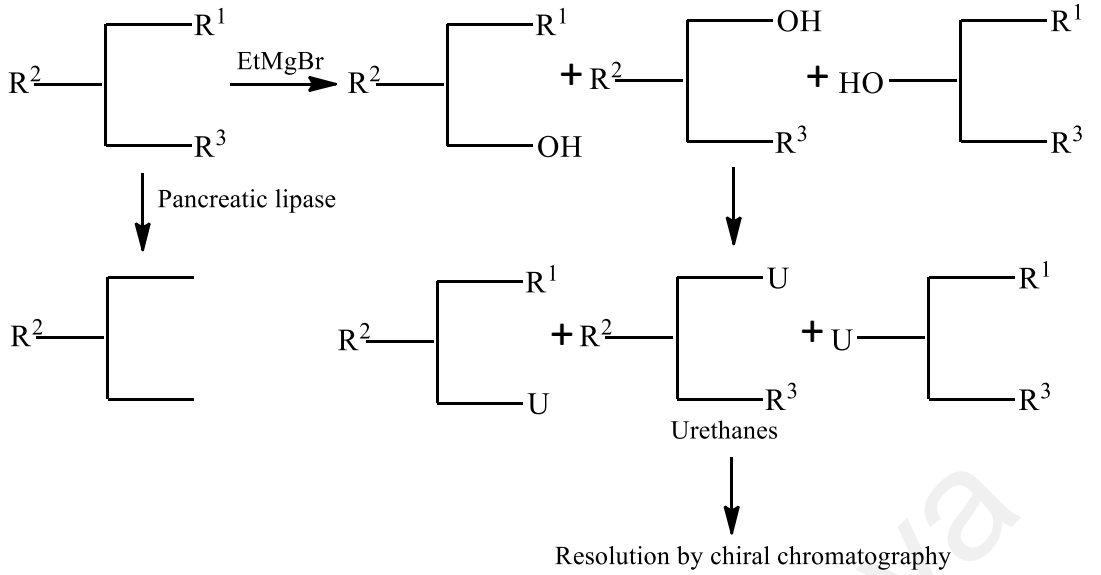
analysis, has been suggested to overcome long analysis time. HPLC analysis of MAG and DAG derivatives can done by normal-phase chromatography, reversed-phase chromatography and chiral-phase chromatography. To achieve good separation of diastereoisomeric derivatives (1,2-DAG, 1,3-DAG and 2,3-DAG), Laakso and Christie (1990) had employed normal-phase chromatography by using silica gel columns, connected in series, and isocratic mobile phase of hexane-isopropanol. The elution orders of DAG formed by single FA are unlikely in the order expected in normal or reverse-phase.

Reversed-phase chromatography has been used much less frequently than adsorption chromatography for stereospecific analysis of TAG. The separations of 3,5-dinitrophenylurethane (DNPU) derivatives from 1,2- and 2,3-DAG have been carried out by Semporè and Bèzard (1991) on an ODS column using an acetonitrile-acetone mobile phase. Furthermore, Angers *et al.* (1998) separated milk fat TAG into fractions of the same partition number (PN) by reverse-phase HPLC before Grignard degradation to analyse the FA distribution. He concluded that the unsaturation degree of the FA and the carbon number (CN) of TAG would influence the *sn* positions within the same PN.

In the chiral-phase chromatographic method, preparation of diastereoisomeric derivatives can be avoided since the stationary phase presents chiral molecules that have chemically bonded to a silica gel supported medium. Itabashi and Takagi (1986) reported that good separation of DNPU derivatives of MAG and DAG can be achieved by using chiral column (*S*)-2-(4-chlorophenyl)isovaleroyl-*sn*-phenylglycine with hexane-dichloroethane-ethanol (40:12:3 v/v/v) mobile phase. The chiral column stationary phase chemically bonded to a silanised aminopropyl silica support, thus, 1-MAG and 3-MAG were well separated (Laakso and Manninen, 1997).

Takagi (1990) and Ando *et al.* (1996) have also converted MAG and DAG prepared from TAG to the DNPU derivatives, followed by resolution by HPLC. The DNPU derivatives contribute to charge-transfer interactions with *pi* electrons on the stationary phase, and thus the separation of enantiomers with C_{18} atoms and double bonds (zero to three) improved. The method can be applied to a complex TAG by decreasing the column temperature and lowering flow-rate. However, this technique is very time-consuming (6 hours or more) and it also fails to resolve a few FA. Another alternative approach which has achieved good separations of enantiomers is by utilising chiral column (R)-(+)-1-(1-naphthyl) ethylamine chemically bonded to a silica gel support and a hexane-dichloro-ethane-ethanol mobile phase. Apart from improving the resolution of the enantiomers, shorter columns and higher flow rate can be employed, thus shortening the analysis time.

The resolution of the DAG urethanes by HPLC on a column of silica gel was proposed by Christie *et al.* (1991). 1,3-DAG urethanes can be eluted early and recovered easily, then followed by 1,2-DAG and 2,3-DAG urethanes (Figure 1.8). The 1,2-DAG and 2,3-DAG urethanes formed are separable in a non-chiral environment as long as the derivatizing agent is a chiral and single enantiomer.



Notes: EtMgBr = Ethyl magnesium bromide

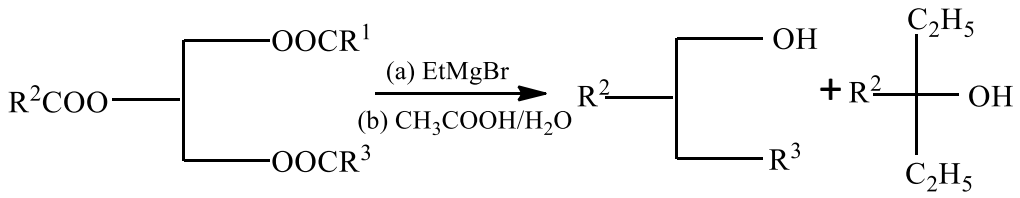
Figure 1.8: Stereospecific analysis of Triacylglycerols and resolution of the diacylglycerol urethanes by HPLC

Stereospecific analysis of TAG can also be carried out using enzymatic hydrolysis to cleave FA from *sn*-1,3 positions of TAG selectively. Another method uses Grignard reagents to hydrolyse TAG which are then derivatised to DAG urethanes partially. These can be separated using chiral GC. However, these methods are very time-consuming, limited to simple mixtures only and large amount of organic solvents. Enzymatic hydrolysis shows some selectivity towards FA of particular chain lengths and degree of unsaturation, as well as a possibility of acyl migration during analysis. Argentation HPLC, using silver ion impregnated column, together with polar solvents, has been shown to separate ABA and AAB type positional isomers, where A and B represent different fatty acyl moieties, but gives no information on the position of FA in an ABC type TAG. In comparison, the Grignard degradation is the most reliable method, but it is not always applicable, and possible isomerisation of glycerol accompanies it.

1.2.3.2 Regiospecific Analysis

Pancreatic lipase was first introduced by Savary and Desnuelle (1955) and by Mattson and Beck (1956). In order to determine FAC of the *sn*-1,3 and *sn*-2 positions through regiospecific analysis, TAG has to be hydrolysed with the enzyme pancreatic lipase in an appropriate buffer. A 2-MAG will be isolated if the FA hydrolysed from the *sn*-1,3 positions. In addition to this, calcium ions and bile salts are helpful for the reaction of TAG to be well dispersed by vigorous shaking, then lipid products are extracted, and the MAG are isolated by TLC for methylation and GC analysis (Luddy *et al*., 1964). 1,3-specific lipase such as *Rhizopus arrhizus* was also employed in the hydrolysis of TAG (Arcos *et al.*, 2000). It resembles pancreatic lipase in many aspects and has an absolute specificity for the primary bonds of glycerolipids. The most important is 1,3-specific lipase because it does not require the calcium ions and bile salts to be effective.

In 1966, Yurkowski and Brockerhoff perfected chemical deacylation with Grignard reagent (Figure 1.9). TAG was partially deacylated to DAG and MAG in regiospecific analysis by using Grignard reagents such as ethyl magnesium bromide (Christie and Moore, 1969; Angers and Arul, 1999), methyl magnesium bromide (Yurkowski and Brockerhoff, 1966) and allyl magnesium bromide (Becker *et al.*, 1993). The regiospecific study which introduced allyl magnesium bromide, obtained the more accurate results and smaller standard derivations, compared to other conventional deacylating agents, such as ethyl magnesium bromide or pancreatic lipase (Becker *et al.*, 1993).



Notes: EtMgBr = Ethyl magnesium bromide

Figure 1.9: Regiospecific analysis of Triacylglycerols: chemical deacylation with Grignard reagent

However, both lipase and Grignard methods have some disadvantages. The listed *sn*-1,3 positions by specific lipases do not provide an accurate analysis of all FA. Either the *sn*-1 and *sn*-3 positions or the *sn*-2 position is determined directly, but other positions must be calculated. Furthermore, acyl migration occurs during hydrolysis may lead to invalid accumulation of 2-MAG in the reaction mixture. Thus, regiospecific analysis of oils containing PUFA mainly cannot be accomplished entirely by this enzymatic method. On the other hand, Grignard method requires close attention because the reaction will be interrupted even by the presence of small amount of moisture.

1.3 Objective of Present Study

The role of dietary oils and fats in human nutrition is one of the most critical areas of concern and investigation in nutritional science. For the purpose of achieving energy needs, intakes of dietary fat must be sufficient to meet daily requirements for essential FA and fats-soluble, namely, vitamins A, D, E, and K to efficiently digest, absorb, and transport together with fats.

According to Oil World Statistic (2015), the world average per capita consumption of all oils and fats was 27 kg in 2014. Appropriate amounts of dietary fats are essential for health as excessive dietary oils and fats intake have been linked to increased risk of obesity and coronary heart disease. The mechanisms by which these link are complex, varied and in many instances not clearly understood. Not all fats may partition for storage with similar efficiency. The degree of risk for other factors may vary according to type and level of FA intakes, percentage of energy from total fat, dietary cholesterol, lipoprotein levels and health status. Literature review on the subject has revealed that FA at different *sn*-position will be subjected to various rates of intestinal absorption. Thus, the FA positional information of oils and fats are essential for nutritional reasons, where saturation and unsaturation FA in positional distribution must be investigated for suitability as diet.

In the present study, investigation of enzymatic, namely ethyl behenate will esterify under optimised condition in current research. The effect of reaction set-up, molar ratio, concentration of catalyst and reaction time also will be studied. In the effort to minimise the use of hazardous substances, 1,3-specific lipase will be employed in the solvent-free interesterification of edible oils and ethyl behenate. The newly synthesised structure

lipids will be subjected to regiospecific analysis and chromatographic analysis of triacylglycerol molecular species.

Apart from the effects of positional distribution of FA on the obesity and coronary heart disease, excessive consumption of erucic acid can also cause heart diseases. Most of the edible oils and mustard products on the market do not mention the content of erucic acid per serving or recommended product consumption on the food label due to lack of quick and easy erucic acid detection methods. In order to avoid excessive consumption of erucic acid, it is very important to ensure the actual amount of erucic acid in edible oils and mustard products from the market. Therefore, a regiospecific analysis for quick and easy detection of erucic acid in edible oils and mustard products will be developed. Both the conventional methods and new regiospecific method for analysis of positional FA will be compared. This new regiospecific method not just enables nutritionist to monitor the intake of erucic acid effectively but it also provides nutritionist for correlating their clinical findings with the positional FA.

In summary, the general objective of the present study is to investigate the positional fatty acids in natural dietary oils and structured lipids, which has shown relevance to health impact. The objective can be achieved through the synthesis of low-calorie SLs enzymatically while long chain ethyl esters used as starting material, analyse the positional FA distribution in TAG using qCNMR, and develop a rapid and direct erucic acid analysis method for edible oils and mustard products.

CHAPTER TWO
SYNTHESIS OF ETHYL ESTERS

2.1 Background

Ethyl esters consists of ester linkage which is derived from a fatty acid (FA) where an ethyl group replaces the hydroxyl group. Ethyl esters can be employed in the synthesis of structured lipids (SLs) through interesterification since esters are produced by natural oils and fats. Enzymatic interesterification between ethyl esters and oils do not require high temperature hence it minimises acyl migration compared to acidolysis (Vikbjerg *et al.*, 2005). In acidolysis reaction, the existence of diacylglycerols (DAG) in oils will cause side-reactions (acyl migration) that leads to the formation of undesirable products (Yang *et al.*, 2005; Xu, 2000). Besides that, side-reaction and by-product acidic fumes, namely, hydrogen chloride may also form in the alcoholysis reaction.

In the synthesis of esters, alcoholysis was found to be expensive and specific anhydrous conditions are required owing to both acyl chlorides and acid anhydrides react with water (Sakakura *et al.*, 2007). There were several reports on the alkylation of carboxylate salts reaction with inactivated halides that results in ineffective, giving poor yields and conversions along with a lot of elimination (Wagner and Zook, 1953; Mills *et al.*, 1962; Pfeffer *et al.*, 1972; Moore *et al.*, 1979). Besides that, transesterification of esters needed either homogeneous or heterogeneous catalysts. The homogeneous catalyst was most frequently employed in transesterification reaction because of its cost effectiveness and high activity (Yan *et al.*, 2010). However, homogeneous catalysts have undesirable reaction with the free fatty acids (FFA) present in the oil to produce soaps (Freedman *et al.*, 1984; Ma *et al.*, 1998). Thus, oils contain high levels of FFA and water are not encouraged to be used in the production of esters. As consequences, it

is tough to develop a suitable catalyst which can employ in the transesterification reaction since FFA present as minor components in oils.

In carbonylation reaction, carboxylic acid esters are obtained by reacting unsaturated hydrocarbons with high carbon monoxide pressure (150 to 700 atm) and alcohol in the presence of metal carbonyl or carbonyl-forming metals at elevated temperature. However, this known method has drawbacks of fluctuating yield and the reaction has to be carried out under highly toxic and flammable carbon monoxide (Wu *et al.*, 2014). In contrast, esterification of carboxylic acid with alcohol in the presence of acid is relatively straightforward. Thus, ethyl esters should be carried out by the esterification instead of alcoholysis, alkylation of carboxylate salts, carbonylation and transesterification.

Short chain alcohols such as methanol and ethanol are the most frequently employed in esterification reaction. Nevertheless, ethanol is selected in the study owing to its high dissolving power for oils and mass transfer limitation between reagents (Zhou *et al.*, 2016). In the perspective of environment-friendly, ethanol is considered renewable as it can be obtained from fermentation of sugarcane (Huang *et al.*, 2015). Even though methanol has advantages in term of low cost and high polarity, methanol must be generated through pressurised catalytic hydrogenation of carbon monoxide.

2.2 Present Study

Prior literature suggested that long chain saturated fatty acid (SFA) at *sn*-1 and *sn*-3 positions are hardly absorbed and easy excreted (Ong and Goh, 2002). Long chain SFA at *sn*-1,3 positions of triacylglycerols (TAG) were found to reduce fat deposition effectively (Gouk *et al.*, 2013b; Gouk *et al.*, 2014). Another research also revealed long

chain SFA at *sn*-1,3 positions of TAG prevents visceral fat deposition which agrees on the relations of SFA excretion to FA chain length directly (Kojima *et al.*, 2010).

For the purpose of obtaining starting material for the synthesis of structured lipids (Chapter 3), ethyl behenate will be synthesised by esterification reaction. Consequently, ethanol and long chain FA, namely, behenic acid (BA) were employed. Since esterification is highly reversible reaction, the percentage yield of the esters can be improved by Le Chatelier's principle. For instance, continuous removal of water can be achieved by connecting the reflux system with a Dean-Stark apparatus, to drive the equilibrium to the product side and increase the production of esters. For the purpose of obtaining high conversion of esters, investigations on parameters such as molar ratio of FA to alcohol, percentage mass of catalyst, reaction duration and reaction set-up were conducted. The current study aims to synthesis high yield ethyl behenate with low acid value through optimised esterification reaction.

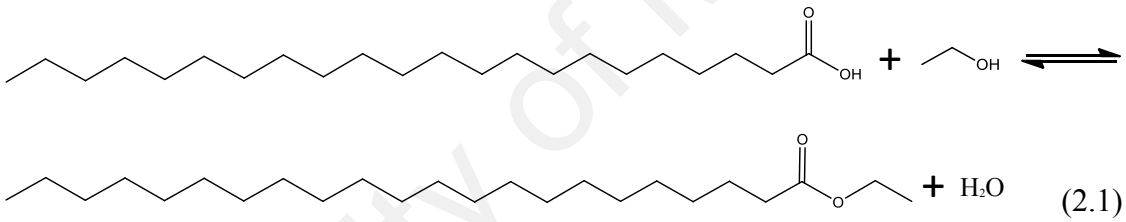
2.3 Experimental

2.3.1 Material

Behenic acid (BA) (minimum 80% purity) was purchased from Tokyo Chemical Industry, Tokyo, Japan. Analytical grade ethanol, isopropanol, concentrated sulphuric acid, hexane and chromatography grade dichloromethane were purchased from Merck, Darmstadt, Germany. Triacontane, potassium hydroxide, phenolphthalein indicator, potassium hydrogen phthalate (KHP), N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA), fatty acid methyl ester (FAME) C_4-C_{24} mix and methyl behenate were purchased from Sigma Chemicals, St. Louis, MO, United States.

2.3.2 Synthesis of Ethyl Behenate

Esterification between 22 g of behenic acid (BA) and 30 mL of ethanol (molar ratio = 1:8) was carried out in a round bottom flask (Equation 2.1). The reaction mixture was refluxed in the presence of 0.50% concentrated sulphuric acid (total mass of reactants) as catalyst. Upon completion of the reaction, 50 mL of hexane was added to the reaction mixture and then transferred into a separating funnel. Hexane layer containing esters was washed with 60°C distilled water to remove the acidic catalyst. The final product can be obtained after the removal of hexane under reduced pressure and successively used as the starting material for the subsequent interesterification without further purification. The preparation of ethyl behenate was done in three replicates to examine the consistency and reproducibility.



(2.1)

2.3.2.1 Molar Ratio

The synthesis of ethyl esters was optimised under few set of parameters (molar ratio, reflux apparatus, concentration of catalyst and reaction time). Acid value and thin layer chromatography were used as monitoring tools for the kinetics study of ethyl behenate. Experiments were carried out by using a condenser connected to Dean-Stark by using 22 g of BA and 38 mL of ethanol (molar ratio = 1:10), refluxed for two hours under the presence of concentrated sulphuric acid (1.00% of total mass of reactants). The conditions remained the same while molar ratios at 1:10, 1:9, 1:8, 1:7 and 1:6 were investigated. Upon completion of the reaction, similar procedures as described in Section 2.3.2 were carried out.

2.3.2.2 Reaction Set-up

A 22 g of behenic acid (BA) and 30 mL of ethanol (molar ratio = 1:8) were mixed well in a round bottom flask, concentrated sulphuric acid (1.00% of total mass of reactants) was added as catalyst. Then, round bottom flask was either connected to a Lie-big condenser, allowed to reflux up to 10 hours. Individual samples were withdrawn at selected intervals (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 hours). Upon completion of the reaction, similar procedures as described in Section 2.3.2 were carried out. The experiment was repeated using a condenser equipped to a Dean-Stark, to investigate the effectiveness of the set-up.

2.3.2.3 Concentration of Catalyst and Reaction Time

Preparation of ethyl esters by reacting 22 g of behenic acid (BA) and 30 mL of ethanol (molar ratio = 1:8) was placed in a round bottom flask which was attached to a condenser connected with a Dean-Stark trap. The reaction mixture was then refluxed in the presence of concentrated sulphuric acid at selected catalyst concentration (0.50, 0.75, 1.00, 1.50 and 2.00% of total mass of reactants). Individual samples were withdrawn at selected intervals (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 hours) for kinetics study. Upon completion of the reaction, similar procedures as described in Section 2.3.2 were carried out.

2.3.3 Determination of Acid Value

Potassium hydrogen phthalate (KHP) was dried in an oven at 120°C for two hours and cooled. A 0.4 g of pre-heated KHP was diluted in 50 mL of distilled water and added with a few drops of phenolphthalein indicator. Potassium hydroxide was then standardised by using the KHP solution to a pink end point. Isopropanol was placed in a conical flask added with a few drops of phenolphthalein indicator. Then, isopropanol was neutralised by potassium hydroxide by dropwise until a faint pink colour was

obtained. Sample (2.5 g) was dissolved in neutralised isopropanol (50 mL) then titrated with the standardised potassium hydroxide to a pink end point. Analyses were carried out in three replicates. The aforedescribed method is in accordance to PORIM test method.

2.3.4 Analysis of Ethyl Behenate

A 20 mg of sample, 0.2 mL internal reference solution (0.02 g of Trioctane in 25 mL dichloromethane) and 1.3 mL of BSTFA solution (16 mL of BSTFA in 25 mL dichloromethane) were pipetted into a 2 mL autosampler vial. The vial was shaken well and then heated for four hours at 70°C - 75°C for silylation process.

A 1 μL of sample was injected into a GC (Shimadzu, GC-2010A series) equipped with a flame ionisation detector and a BPX5 capillary column of 30 m x 0.25 mm internal diameter. An initial temperature of 100°C was held for 1 minute and subsequently increased to 310°C at the rate of 10°C per minute. The oven, injector and the detector ports were set at 100, 245 and 370°C, respectively. The carrier gas was helium with column flow rate of 2.0 mL per minute.

2.4 Results and Discussion

2.4.1 The Effect of Molar Ratio

The synthesis of ethyl behenate was optimised through the kinetics study and monitoring by acid value and thin layer chromatography. The stoichiometry of the esterification reaction requires 1 mole of ethanol and 1 mole of BA to yield 1 mole of ethyl behenate. As the reaction is reversible, it is necessary to use a large excess amount of ethanol in order to shift the equilibrium to the products. A molar ratio of 1:6 (FA: alcohol) is generally used in industrial processes to obtain higher yield of esters (Petchmala *et al.*, 2008). In the present study, the molar ratio of 1:8 achieved the highest

conversion to ethyl behenate as depreciated by the lowest mean acid value 3.27 ± 0.01 mg KOH / g sample demonstrated (Figure 2.1). However, the yields of ethyl behenate for the molar ratio of 1:9 and 1:10 were found to be lower. This result could be attributed to the high water content in the reactants as the high percentage of the analytical grade ethanol was used. Water would react readily with ethyl behenate under subcritical water condition, thus lowering the overall yield.

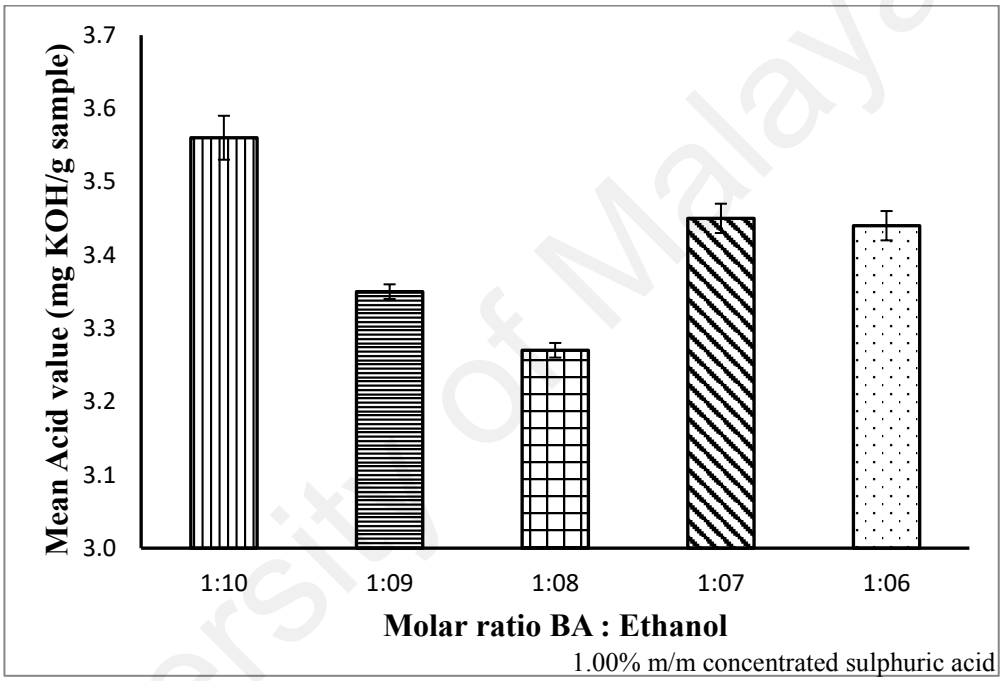


Figure 2.1: Mean acid value of synthesised ethyl behenate, against the molar ratio of behenic acid and ethanol used a condenser connected to Dean-Stark

2.4.2 The Effect of Reaction Set-up

Another important variable affecting the yield of esters is the reaction set-up. When reaction set-up with Dean-Stark was used, lower acid value was obtained along the experiment (Figure 2.2). From these results, it can be seen that set-up with only condenser, without Dean-Stark trap always exhibits higher acid value than that with Dean-Stark set-up for 7 hours of reaction. However, the acid value of both reactions set-up has overlapped after 8 hours. Nevertheless, the lowest acid value still attained by

Dean-Stark set-up at 5 hours. In fact, reactions conducted with Dean-Stark trap exhibits low acid value trend from the beginning of reaction.

The Dean-Stark set-up is suitable for application of foods with low moisture contents and foods containing volatile oils. It able to distilled off with the reaction water in the trap of the Dean-Stark receiver. Besides that, this equipment is relatively cheap, easy to setup and operate. The set-up with Dean-Stark is relatively time saving if compared to the set-up with only condenser, without Dean-Stark trap. The line graph represented condenser connected to a Dean-Stark set-up indicated a steeper gradient at first two hours; this means a better conversion rate is achievable at the early stage of this reaction set-up (Figure 2.2). Thus, condenser attached with Dean-Stark is more suitable become the reaction set-up in the following experiments.

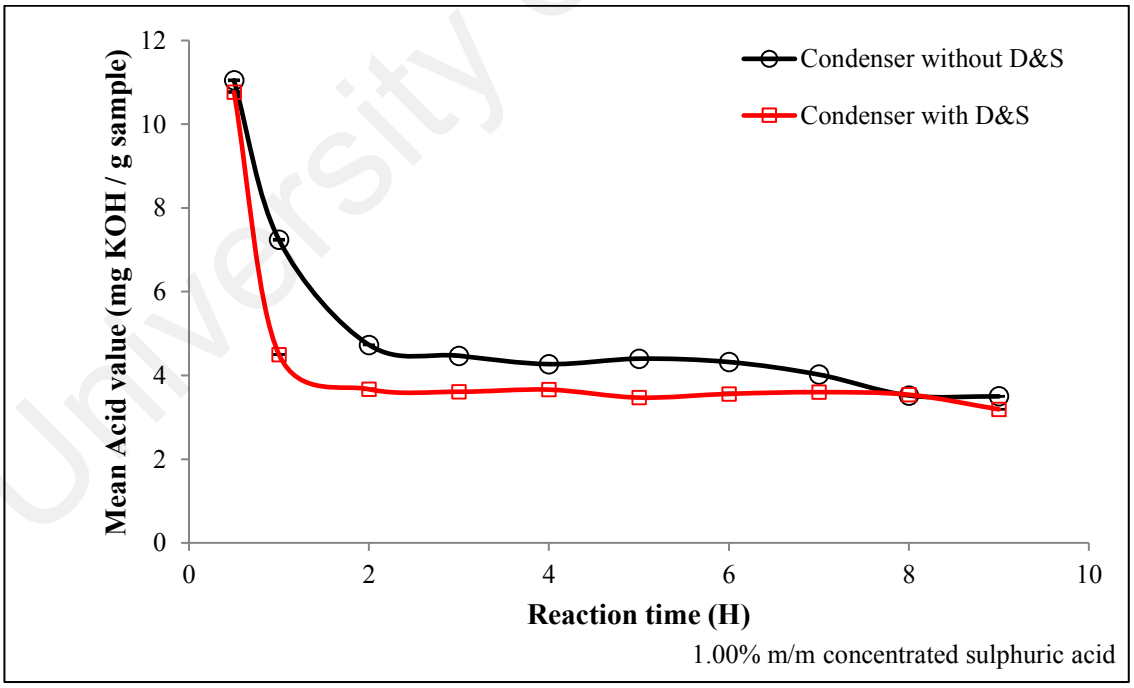


Figure 2.2: Mean acid value of synthesised ethyl behenate, against the reaction time at molar ratio of behenic acid:ethanol (1:8)

2.4.3 The Effect of Concentration of Catalyst, Reaction Time and Unreacted Behenic Acid

Generally, esterification is carried out in the presence of concentrated sulphuric acid as catalyst because of its high acid strength that release hydrogen ions (Lucena *et al.*, 2008). Sulphuric acid was found to be more efficient catalyst for esterification of acrylic acid with ethanol as it induces the maximum conversion of acrylic acid (Jyoti *et al.*,2016). Thus, concentrated sulphuric acid was used as a catalyst to speed up the chemical reaction without involving in the reaction. High concentration represents great amount of molecules in same volume hence more successful collisions may occur. Theoretically, the conversion rate will increase with the reaction time when a higher catalyst concentration was used. In the present study, the lowest mean acid value was achieved by the highest concentration at 2.00% (m/m) (Figure 2.3). Mean acid value drastically reduced within the first hour and then followed by a steady reduction (7.34 to 2.33 mg KOH / g sample). In comparison, the reaction catalyst by 1.50% (m/m) concentrated sulphuric acid also indicated an interesting gradient but relatively higher mean acid value (9.15 mg KOH / g sample) at 0.5 hours.

Subsequently, the reaction catalysed by 0.50% (m/m) concentrated sulphuric acid demonstrated a less steep curve (0.5 hour to 3 hour) as compared to that by 0.75% and 1.00% (m/m). There was a stagnant acid value from 3 to 6 hours and then continued to decrease (Figure 2.4). The highest amount of unreacted BA was attained when catalyst concentration of 0.50% (m/m) was used. It decreased sharply within 4 hours of esterification reaction carried out using the condenser only. The lowest amount of unreacted BA (1.44%) was observed in reaction catalysed by 0.75% (m/m) concentrated sulphuric acid. Similarly, both reactions using 0.75% and 1.00% (m/m) fluctuated from 4 to 6 hours but the mean acid value of concentration 0.75% (m/m) remarkably dropped after 6 hours (Figure 2.3).

A study has indicated that the demand of the high amount of catalyst is useful for the improvement in conversion (Beula and Sai, 2013). In general, as the concentration of catalyst increases the rate of initial reaction also increases, which also accompanied by greater rate of conversion. At the constant molar ratio of 1:8, the influence of increasing the catalyst has a greater effect than increasing the reaction time (Figure 2.3). Unfortunately, both catalyst concentrations at 1.50% (m/m) and 2.00% (m/m) were considered overloaded due to chemical burn and colour stain observed in the products. Concentrated sulphuric acid which higher than 1.00% (m/m) can cause chemical burns *via* hydrolysis and secondary thermal burns through dehydration which is a very serious damage. The reaction was very slow during the first minute as time was required for mixing and dispersion of ethanol into BA, but most of the reactions proceeded very fast thereafter and within the first two hours.

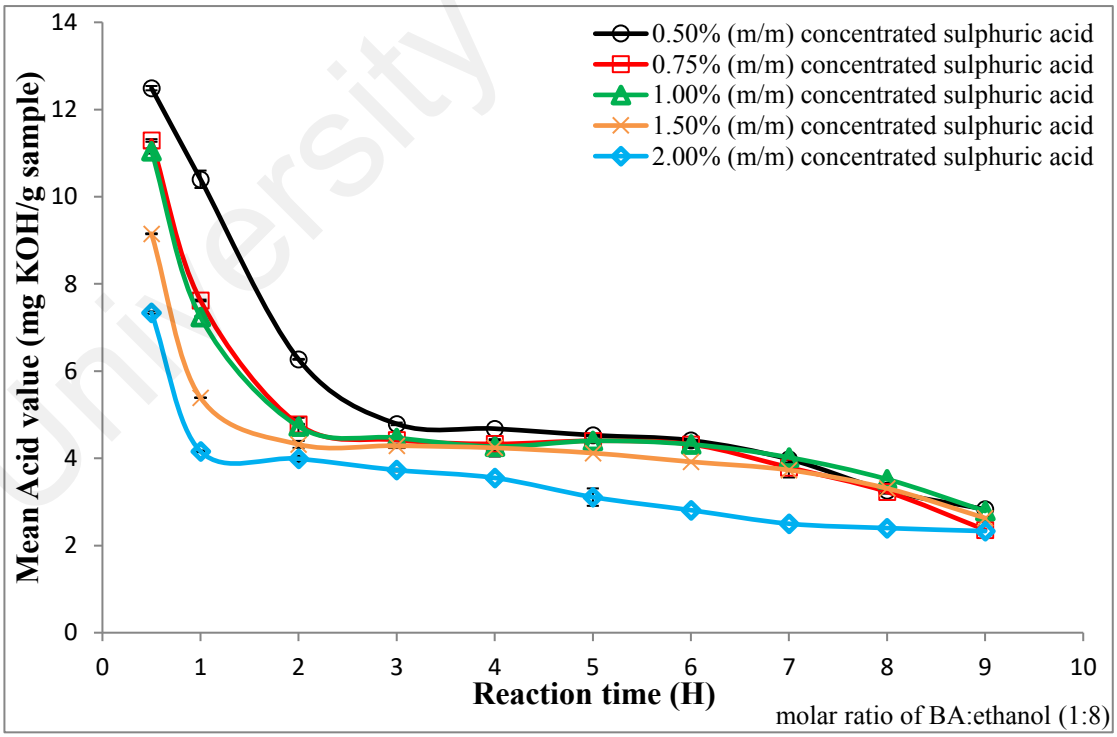


Figure 2.3: Mean acid value of synthesised ethyl behenate, against the reaction time reflux by condenser

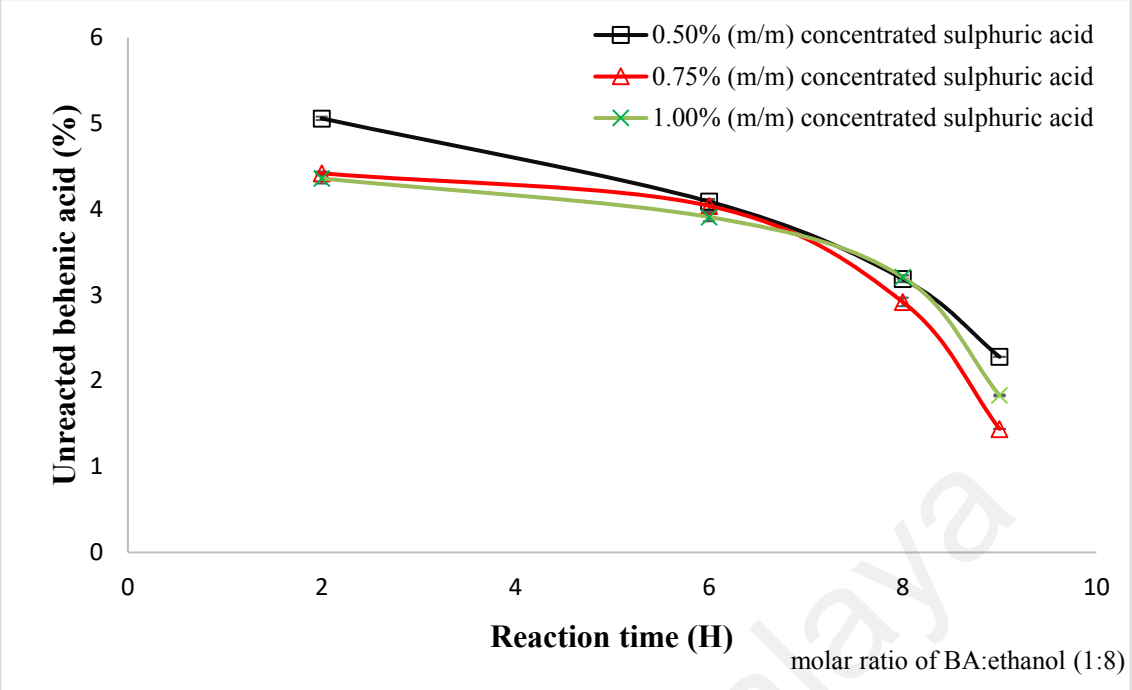


Figure 2.4: Percentage of unreacted behenic acid, against the reaction time reflux by condenser

A kinetic study at a molar ratio of 1:8 and concentrated sulphuric acid at selected concentration (0.50%, 0.75% and 1.00% of total mass of reactants) by using a condenser attached with Dean-Stark was carried out (Figure 2.5). A steep gradient was shown by the curve representing catalyst concentration at 1.00% (m/m) then followed by 0.75% (m/m) for the first two hours. There is no notable change of mean acid values found between the 0.50% (m/m) curve and 1.00% (m/m) curve from 3 to 7 hours. In addition, the means acid values of both curves exhibited inconsistency after 7 hours. However, a relatively lower mean acid value (2.57 mg KOH / g sample) was obtained at 10 hours and catalyst concentration of 0.50% (m/m) when compared to that by 1.00% (m/m). Similarly, the unreacted BA of catalyst concentration at 0.50% (m/m) decreases uniformly from 3 to 10 hours except at 8 hours (Figure 2.6).

In contrast, there is no notable change of unreacted BA (3.29% to 3.15%) found at catalyst concentration 1.00% (m/m) from 3 to 10 hours (Figure 2.6). Mean acid value was decreasing from 3 to 8 hours and exhibited a consistency from 9 hours onwards when 0.75% (m/m) of catalyst was used. The mean acid value reactions curve used by 0.75% (m/m) catalyst decreases regardless of the reaction set-up. In the present study, the unreacted BA catalysed by concentration 0.75% (m/m) has reduced slowly (2.70% to 2.61%) from 3 to 6 hours and then followed by extreme change after 6 hours of reaction (Figure 2.6). Consequently, reaction catalysed by 0.75% (m/m) concentration and set-up with Dean-Stark has obtained the lowest acid value (1.31 mg KOH / g sample) and lowest amount of BA (1.16%). However, the catalyst concentration and mean acid value are not proportion directly, but it adds to extra cost. It is necessary to remove the catalyst from the reaction medium at the end of the reaction. Besides time-consuming, larger amount of water has to spend on washing when bigger amount of catalyst was used in order to remove the catalyst fully. Therefore, concentrated sulphuric acid should be used appropriately due to its unintended consequences.

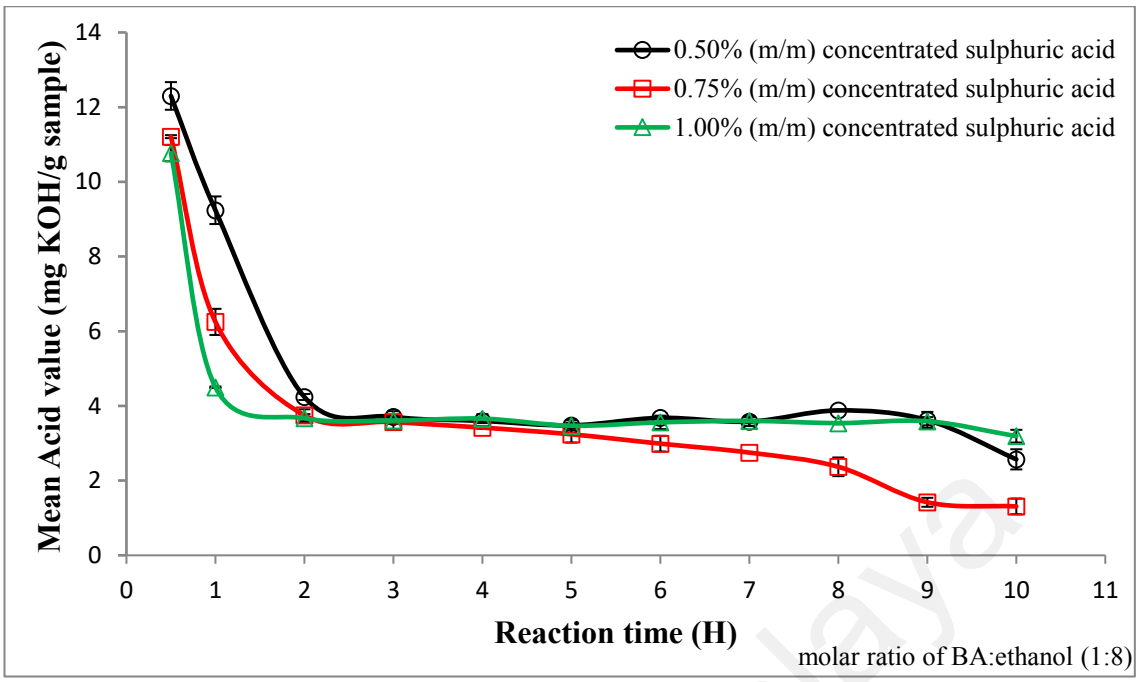


Figure 2.5: Mean acid value of synthesised ethyl behenate, against the reaction time reflux by condenser attached with Dean-Stark

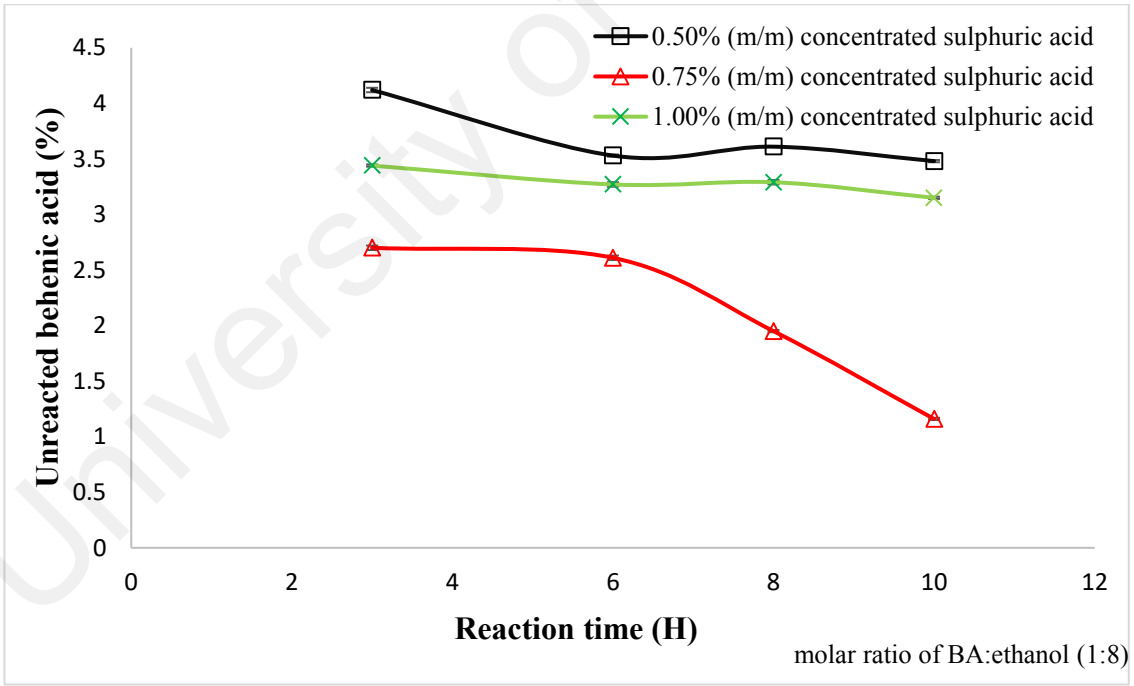


Figure 2.6: Percentage of unreacted BA, against the reaction time reflux by condenser attached with Dean-Stark

In summary, ethyl behenate with the lowest mean acid value (1.42±0.11 mg KOH / g sample) can be attained under optimised parameters at a molar ratio of 1:8, 0.75% (m/m) of concentrated sulphuric acid and with the attachment of Dean-Stark for 9 hours. The unique structure of Dean-Stark allows the chemical equilibrium reactions to be shifted to the products side since continual removal of co-product (water) can be achieved. Meanwhile, an appropriate concentration of catalyst can lead to highest conversion rate without extra cost or samples damage. Besides that, purification of the product can be carried out easier since catalyst have to remove fully.

CHAPTER THREE
ENZYMATIC INTERESTERIFICATION, CHARACTERISATION AND PURIFICATION OF STRUCTURED LIPIDS FROM PALM OLEIN AND HIGH-OLEIC SUNFLOWER OIL

3.1 Background

The worldwide prevalence of obesity increased more than doubled between the year 1980 and 2014. Overweight and obesity have become a major medical and public health problem over the past decades. According to World Health Organization (2016), there are 600 million obese and 1.9 billion overweight adults in the world. Overweight and obesity are linked to more deaths than underweight since it is a major risk factor for cardiovascular diseases, diabetes, musculoskeletal disorders and cancers. Besides that, lack of physical activity and excess intake of foods high in fat lead to energy imbalance of calories and subsequently caused obesity and overweight. In fact, obesity can be prevented if there is sufficient consumption of fruit and vegetable, regular physical activity and limited energy intake from total fats and sugars (World Health Organization, 2016).

Structured lipids (SLs) provide several benefits to food manufacturers such as the production of cocoa butter equivalent, human milk fat substitutes, low-calorie SLs, modified fish oil products and margarine. Cocoa butter is an important material to make chocolate, where its triacylglycerols (TAG) contains 3 main fatty acids (FA), namely, stearic and/or palmitic acid at the *sn*-1,3 positions and oleic acid at *sn*-2 position. For example, a cocoa butter equivalent was synthesised through enzymatic interesterification of palm oil mid fraction with stearic acid using Novo lipase Lipozyme™ (Undurraga *et al.*, 2001).

Among the structured lipids, human milk fat substitute is the most popular lipid used to replace human milk fat. Unlike the TAG in natural oils, the TAG in human milk fat substitutes contain palmitic acid at *sn*-2 position while oleic acid at the *sn*-1,3 positions. A few studies have reported on the production of human milk fat substitutes by a wide variety of lipases. One of the productions of human milk fat substitutes was by introducing lipase *Candida* sp. 99-125 into blends of lard and oleic acid, meanwhile *β*-cyclodextrin was used to improve the product yield (Zhang *et al.*, 2016). In addition, another study used *Rhizopus oryzae* lipase as a feasible biocatalyst to carry out acidolysis of tripalmitin and oleic acid to produce human milk fat substitutes (Tecelão *et al.*, 2012).

In the perspective of dietary, oils and fats always provide higher calorie (9 kcal/g) compared to the carbohydrate and protein (4 kcal/g). Thus, consumption of oils and fats has the directly proportional to obesity and overweight. As a consequence, synthesis of low-calorie SLs was targeted. SLs synthesised from long and short chain FA are proposed as low-calorie fats and are used as confectionery fats (Taek *et al.*, 2001). Short chain TAG provides lower calories than long chains TAG, but TAG contains long chain FA and unsaturated FA at *sn*-1,3 and *sn*-2 positions respectively, are considered less likely to cause weight gain than short chain TAG due to low absorbability. A research group observed that the absorption rate of stearic acid increased when stearic acid dominates the *sn*-2 position of TAG (Mattson *et al.*, 1979). Thus, absorption of FA is dependent on its positional distribution in the glycerol backbone.

A few fats substitute were approved and used as a replacement for fats and oils in the early 1990s. Structured fat such as SALATRIM™ and Caprenin™ are both designed to enable weight loss. OLEAN^{TM}, Caprenin^{TM} and SALATRIM^{TM} offer similar functions,

stability, physical, and chemical characteristics as regular fat but provide lower calories. OLEAN™ is a synthetic fat produced by Procter & Gamble through esterification between FA and a sucrose molecule with eight hydroxyl groups. As a result, it is a molecule bonded to 6-8 FA, which is hard to be digested and absorbed and finally excreted through the intestinal tract intact (Mattson and Volpenhein, 1972; Daher *et al.*, 1996). However, its side effects of gastrointestinal distress caused diarrhoea, loose stools and inhibit the absorption of some vitamins and other nutrients (Aggarwal *et al.*, 1993). OLEAN™ is only approved as fat substitute for savoury snacks by Food and Drug Administration (Kessler, 1996; Code of Federal Regulations, 1996).

Furthermore, another synthesised SLs, SALATRIM™, contained long chain stearic acid (C18:0) and some short chain FA such as acetic (C2:0), propionic (C3:0) or butyric (C4:0) acids (Klemann *et al.*, 1994; Finley *et al.*, 1994) was accepted as a lower-calorie fat replacer by 2003 Novel Food Regulation (EC) No 258/97 of the European Parliament. In addition, SALATRIM™ is able to reduce its absorption in the small intestine thus caused greater amounts of fat in the gastrointestinal tract, which causes sequently decreases appetite and energy intake (Sørensen *et al.*, 2008).

Caprenin™, primarily contains caprylic (C8:0), capric (C10:0), and behenic (C22:0) acids, were substituted into soft candies and confectionery coatings as a low-calorie fat (Webb and Sanders, 1991). Additionally, significant reductions in high-density-lipoprotein cholesterol, high-density-lipoprotein 2 cholesterol and high-density-lipoprotein 3 cholesterol increment of total cholesterol/high-density-lipoprotein cholesterol ratio since no changes for total cholesterol, low-density-lipoprotein cholesterol and serum TAG were noted in hypercholesterolemic men fed with Caprenin™ (Wardlaw *et al.*, 1995). Nevertheless, Caprenin™ was withdrawn from the

market because of its adverse effects on blood lipids and lipoprotein metabolism (Auerbach *et al.*, 1998; Nestel *et al.*, 1998).

3.2 Present Study

Prior literature disclosed that long chain saturated fatty acids (SFA) at *sn*-1,3 positions of TAG reduced fat deposition effectively (Gouk *et al.*, 2013b; Gouk *et al.*, 2014). Moreover, Kanjilal *et al.* reported low-calorie value and lipid deposition obtained while rats and rabbits are fed with SLs from lipase catalysed interesterification of ethyl behenate with sunflower and soybean oils (Kanjilal *et al.*, 1999; Kanjilal *et al.*, 2013). Besides that, Kojima *et al.* also reported that the SFA excretion is related to FA chain length since long chain SFA at *sn*-1,3 positions of TAG prevents visceral fat deposition (Kojima *et al.*, 2010). Hence, SLs obtained from long chain saturated behenic acid are perceived as a potential substrate in the design of low-calorie SLs through restricted intestinal absorption after hydrolysis by 1,3-specific pancreatic lipase. Due to the implication of antioxidants and nutrients of synthesised SLs, palm olein (IV= 56) (POo) and high-oleic sunflower oil (IV= 92) (HOS) were selected as the materials in the present study.

For the purpose of reducing chemical consumption and environmental problems, biocatalysts are often used to catalyse reactions related to modification of oils and fats in the past few decades. Lipases from the strain of *Rhizomucor miehei* (Lipozyme RM IM, Novozymes^{TM}) will be employed in the current work since it performed well as 1,3-specific catalysts in the interesterification reactions (Han *et al.*, 2011). In addition, milder reaction conditions, as well as fewer side reactions were the additional advantages of Lipozyme RM IM (Foresti and Ferreira, 2010). In contrast to the random stereospecific distribution of behenic acids in Caprenin^{TM}, the aimed SLs in the present

study are designed to contain high amount of behenic acid at the *sn*-1,3 positions of its TAG, while the *sn*-2 position is conserved with high unsaturation for favourable nutritional attributes in terms of blood lipid profile and cholesterol levels in humans.

3.3 Experimental

3.3.1 Material

Ethyl behenate was synthesised as the method mentioned in Section 2.3.2. Refined, bleached and deodorised HOS and POo were provided by Intercontinental Specialty Fats Sdn. Bhd., Selangor, Malaysia. Immobilised lipase from the strain of *Rhizomucor miehei* (Lipozyme RM IM, Novozymes^{TM}) was purchased from Novozymes, Denmark. AR grade of toluene, hexane, anhydrous methanol, glacial acetic acid, high performance liquid chromatography (HPLC) grade acetonitrile, dichloromethane, sodium methoxide and deuterated dichloromethane were purchased from Merck, Darmstadt, Germany. Standard TAG (purity ≥ 99%), namely, tripalmitoylglycerol (PPP), trioleoylglycerol (OOO), 1,3-dipalmitoyl-2-oleoylglycerol (POP), tristearoylglycerol (StStSt) and tribehenoylglycerol (BBB) were obtained from Sigma Chemicals, St. Louis, MO, United States.

3.3.2 Synthesis of Structured Lipids by Enzymatic Interesterification between Palm Olein and High-Oleic Sunflower Oil and Ethyl Behenate

Palm olein (IV= 56) (POo) (5 g) was mixed with Ethyl behenate (6.43 g) (molar ratio = 3:1) in a 100 mL Schott bottle with screw cap. The substrates were preheated at 65°C to ensure their homogeneities in a molten state. Thereafter, 10% immobilised lipase from Lipozyme RM IM (total mass of substrates) was loaded in the reaction mixture. Solvent-free interesterification was performed in an orbital shaking water bath (Model SV1422, Memmert, Germany) at 150 rpm and 65°C. Individual samples were withdrawn at selected intervals (0.5, 1, 2, 3, 5, 10, 15, 20, 24 hours), filtered and

subjected to further characterisation after removal of catalyst. The described procedures were repeated using 5 g of HOS (IV= 92) mixed with 6.25 g of ethyl behenate at a molar ratio of 1:3.

3.3.3 Purification of Synthesised Structured Lipids

The interesterified products were subjected to chromatographic separation using silica gel G column (50 g, Kieselgel 60-200 mesh, Merck Art.No 7731, Darmstadt, Germany). A solvent system of 1% ethyl acetate in hexane was used to elute unreacted ethyl behenate, while 5% ethyl acetate for the elution of newly synthesised structured TAG. The remaining free fatty acids and other partial acylglycerols remained undeveloped under the above-mentioned solvent systems.

3.3.4 Characterisation of Synthesised Structured Lipids

3.3.4.1 High Performance Liquid Chromatography

Triacylglycerols (TAG) composition was determined by reversed-phase HPLC on a Waters chromatographic system (Waters SFO, Milford, USA) equipped with an evaporative light scattering detector (Model 2424, Waters, USA). The separation of the molecular species was affected on a Purospher STAR RP-18 end capped column (250 mm x 4.6 mm) of 5-µm particle size (Merck, Darmstadt, Germany). Sample injection was executed by a Sample Manager Waters 2757 injector with 20 µL loops (Waters, USA). The detector drifts tube temperature set at 60°C. Nitrogen gas was used as a nebulizing gas at the pressure of 40 psi.

Sample (8 mg) was dissolved in 10 mL of HPLC grade dichloromethane and subsequently vortexed for 1 minute. Prior to sample injection, the samples were passed through a filter with pore size of 0.2 µm (Millipore, Bedford, USA). The mobile phase consisted of an isocratic elution of acetonitrile/dichloromethane solvent system (40:60, by volume) for 25 minutes and a 1 mL/minute flow rate. The data acquisition and

processing were executed by MassLynx version 4.1 (Waters, USA). The order of elution of TAG molecular species was based on the corresponding equivalent carbon number (ECN). Peak identification was further affirmed on the plot of the logarithm of the retention volume of TAG relative to trioleoylglycerol versus the number of double bonds (Stolyhwo *et al.*, 1985).

3.3.4.2 Gas Chromatography Fatty Acid Composition

Gas chromatography (GC) was used to determine fatty acid composition (FAC). Fatty acid methyl esters were produced by sodium methoxide-catalysed transesterification (Christie, 1989). Methyl ester sample (1 µL) was injected into the GC (GC-2010A series, Shimadzu) equipped with a flame ionisation detector and a BPX70, 30 m x 0.32 mm x 0.25 µm capillary column. An initial temperature of 140°C was held isothermally for 2 minutes, and then increased to 220°C at a rate of 8°C per minute. The column was held at the final temperature for 5 minutes. The oven, injector and the detector ports were set at 140°C, 240°C and 260°C, respectively. Helium was the carrier gas at a flow rate of 1.10 mL/minute operated at a 50:1 split ratio.

3.3.4.3 ^{13}C Nuclear Magnetic Resonance Analysis

Regiospecific analyses of SLs were optimised from previous literature (Gouk *et al.*, 2012). ^{13}C NMR measurements were performed using a JEOL ECA-400 MHz NMR spectrometer operating at 9.4 T. Manual shimming was employed. A spectral width of 1500 Hz at where the acyl chain carbonyl carbons resonate, 8192 data points and 90° pulse excitation were applied. Experimental temperature set at 303.15 K whereas total repetition time of 27.4 s was chosen. Total scans per analysis were 128 times. Deconvolution used as the integration method to separate the overlapped peak and calculate its area.

3.3.4.4 Slip Melting Point

Slip melting points (SMP) of the selected SLs analysed by AOCS Slip Melting Point Cc 3-25. About 1 g of melted sample was placed in a capillary tube to a height of 1 cm. The tube was cooled overnight at 4°C and then immersed in a beaker half-full of distilled water. The water level in the beaker was 2 cm above the upper edge of sample. The initial bath temperature was adjusted to 10°C below the expected SMP of the sample. The water bath was agitated with a magnetic stirrer and heated applying a temperature gradient of 0.5°C/min. The temperature at which the sample in each tube started to melt and become apparent was observed and recorded as its SMP.

3.3.4.5 Solid Fat Content

Solid fat content (SFC) in selected SLs was measured at 5, 10, 20, 30, 35, 40 and 45°C with a low resolution NMR (Minispec MQ20, Bruker) using IUPAC Solid Fat Content 2.150(a) test method. All samples were tempered prior to the measurement of SFC to remove all crystallisation and melting history. SLs were melted at 80°C and held at 60°C for 20 minutes, then cooled at 0°C for 90 minutes. Subsequently, kept overnight at 26.5°C. The samples were stabilised for 35 minutes at each measuring temperature before measuring.

3.4 Results and Discussion

3.4.1 Identification of Chromatographic Peaks

The equivalent carbon number (ECN) is employed to confirm the order of HPLC elution of various TAG molecular species (Perona and Ruiz-Gutierrez, 2003) and illustrated in Equation 3.1:

$$ECN = CN - 2 \times DB - 0.2 \times NUFA \quad (3.1)$$

Where CN is the total number of carbons in acyl chains, DB is the total number of double bonds and NUFA is the number of unsaturated FA in the TAG molecule.

Nonetheless, the ECN values of TAG are not the sole method to assign chromatographic peak. Relative retention volumes express the retention volume of the other peaks relative to a standard and compare the ratio corresponds to known TAG species with the standard. The OOO was used as the reference molecular species and the detailed chromatographic peak identification is achieved by the approach of logarithms of retention volumes (Stolyhwo *et al.*, 1985; Fabien *et al.*, 1993). For example, the logarithm of relative retention volume of PPP can be determined as Equation 3.2.

$$\text{logarithm of relative retention volume of PPP} = \log_{10}\left(\frac{\text{retention volume of PPP}}{\text{retention volume of OOO}}\right) \quad (3.2)$$

Figure 3.1 shows the plot of logarithm of the relative retention volumes against the number of double bonds in the TAG molecule. The chromatographic data were derived from the analysis of POo, HOS and the synthesised SLs by employing the standard saturated TAG, PPP, StStSt and BBB. In Figure 3.1, each plot was joined by two points representing different homogenous TAG while heterogeneous TAG will fall on the plot in between the two points. Those plots had divided the segment into four. For example, 1,3-dibehenoyl-2-oleoylglycerol (BOB) and 1-behenoyl-2,3-dioleoylglycerol (BOO)

were lies on the plot which connected by two points BBB and OOO. In addition, 1-behenoyl-2-oleoyl-3-stearoylglycerol (BOSt) was located in the middle center of the triangle formed by BBB, OOO and StStSt, as well as apart from both BOB and 1,3-distearoyl-2-oleoylglycerol (StOSt) in same distance (Figure 3.1).

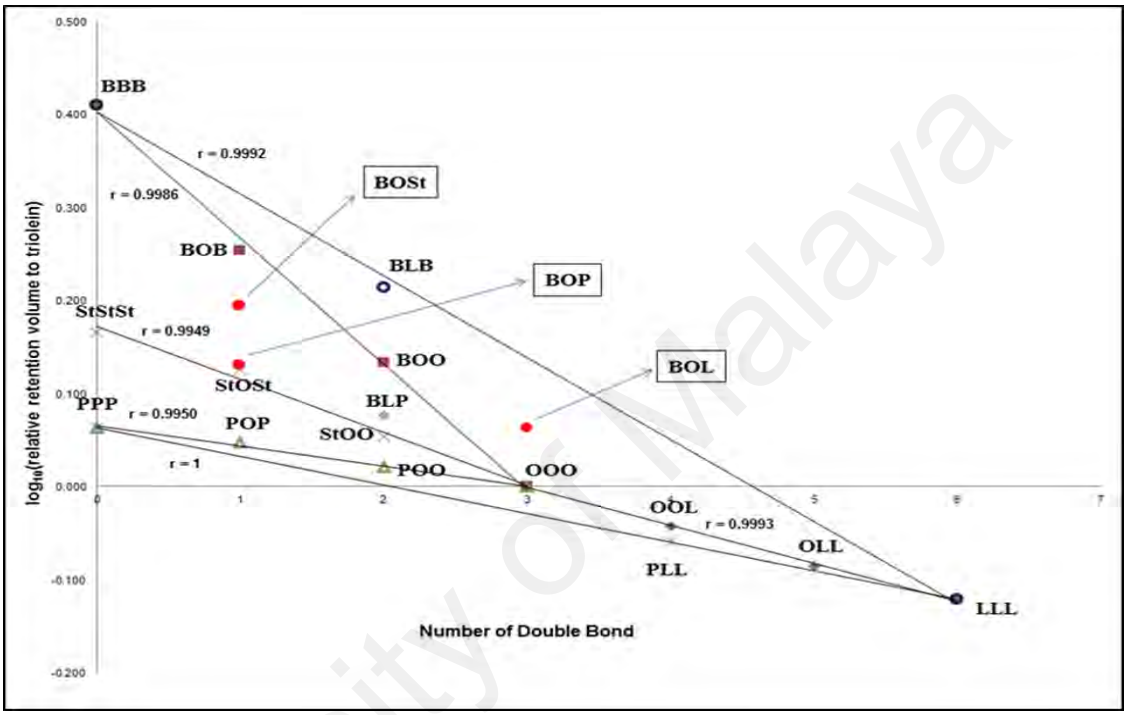


Figure 3.1: Graph of logarithm of the retention volumes of triacylglycerols, relative to trioleoylglycerol (OOO), against the total number of double bonds in the fatty acid acyl chains

The logarithm of relative retention volume and ECN values both are directly proportional while number of double bond was fixed as a constant. In addition, the TAG molecular species as well as eluted prior to OOO displayed negative logarithm values under the x-axis (Figure 3.1). The HPLC experiments was robust and intact throughout all analyses due to the exceptional correlation coefficients obtained (r=0.9949-1.0000). More important, this diagram allows the identification of an unknown chromatographic peak with known relative retention volume. Such as an unknown peak (logarithms of relative retention volume = 0.1365) which obtained two number of double bond was

plotted in Figure 3.1, the plot fell on the linear line of BBB and OOO. Hence, the peak was identified as BOO.

3.4.2 Triacylglycerols Composition

Triacylglycerols (TAG) composition of SLs produced from POo and HOS is presented in Table 3.1. Both BOO and BOB were accumulated during the reaction. There were 27.2% of BOO and 18.4% of BOB detected in enzymatic interesterified palm olein (EIE-POo) (Table 3.1). In another hand, there were 33.0% and 21.4% decrements of POo and OOO contents under half an hour of catalytic action, respectively. Interesterification reaction of HOS produced higher yield of BOO (46.2%) and BOB (30.3%) as compared to interesterification reaction of POo. The OOO content in HOS was reduced from 90.1% to 8.3% within 2 hours of reaction. In the presence of Lipozyme RM IM which act as 1,3-specific enzymes, behenic acid was incorporated into *sn*-1 and/or *sn*-3 positions. Hence, both major targeted species, BOB and BOO were produced in interesterification of ethyl behenate with POo or HOS. However, the major targeted species (BOO and BOB) produced from HOS was generally higher than the targeted species produced from POo. This is because 90.1% of major TAG in HOS is OOO. In fact, it promising yield was achieved in the present synthesis of structured TAG.

Table 3.1: Triacylglycerol compositions of POo, HOS and interesterification products catalysed by Lipozyme RM IM

ECN	Molecular species	Composition of total triacylglycerols (mol%)			
		POo	EIE-POo	HOS	EIE-HOS
41.4	LLL	2.2 ± 0.1	nd	0.5 ± 0.1	nd
43.4	OLL	0.9 ± 0.0	nd	0.6 ± 0.1	nd
43.6	PLL	0.7 ± 0.0	nd	nd	nd
45.4	OOL	7.5 ± 0.1	3.3 ± 0.0	1.9 ± 0.1	2.0 ± 0.1
45.6	POL	12.1 ± 0.1	2.8 ± 0.0	nd	nd
47.4	OOO	31.7 ± 0.2	10.3 ± 0.0	90.1 ± 0.3	8.3 ± 0.1
47.6	POO	42.8 ± 0.2	9.8 ± 0.0	4.6 ± 0.2	2.4 ± 0.2
47.8	POP	2.2 ± 0.1	3.8 ± 0.0	2.0 ± 0.0	nd
49.6	StOO	nd	nd	0.3 ± 0.0	nd
51.6	BLO	nd	4.3 ± 0.0	nd	4.2 ± 0.3
51.8	BLP	nd	8.5 ± 0.0	nd	1.2 ± 0.2
53.6	BOO	nd	27.2 ± 0.0	nd	46.2 ± 0.2
53.8	BOP	nd	2.6 ± 0.0	nd	1.6 ± 0.0
55.8	BOSt	nd	3.9 ± 0.0	nd	1.2 ± 0.1
57.8	BLB	nd	2.7 ± 0.0	nd	2.5 ± 0.0
59.8	BOB	nd	18.4 ± 0.0	nd	30.3 ± 0.1
60.0	BBP	nd	2.3 ± 0.0	nd	nd
62.0	BBSt	nd	nd	nd	nd
66.0	BBB	nd	nd	nd	nd

Notes: ECN, equivalent carbon number; nd, not detected; P, palmitic; St, stearic; O, oleic; L, linoleic; B, behenic.

Beside BOO and BOB, BLP (8.5%) was the next highest yield of the targeted TAG composition by interesterification reaction of POo. Most probably those TAG are contributed by the acyl migration of palmitic acid and linoleic acid in POo. In contrast, BLO (4.2%) was detected as third targeted TAG composition in enzymatic interesterified high-oleic sunflower oil (EIE-HOS). Moreover, species containing linoleic acid, oleic acid and palmitic acid (LLL, OLL and PLL) diminished in the interesterification of EIE-POo. Meanwhile, species containing linoleic acid and oleic acid (LLL and OLL) fully converted into another species through the interesterification of EIE-HOS.

The HPLC profiles of the EIE-POo and EIE-HOS are presented in Figure 3.2. HPLC profiles of EIE-POo exibits smaller amount of undesired TAG species than EIE-HOS. In fact, EIE-HOS (87.3%) displayed higher yield of targeted TAG species (BLO, BLP, BOO, BOP, BOSt, BLB and BOB) compared to EIE-POo (67.6%).

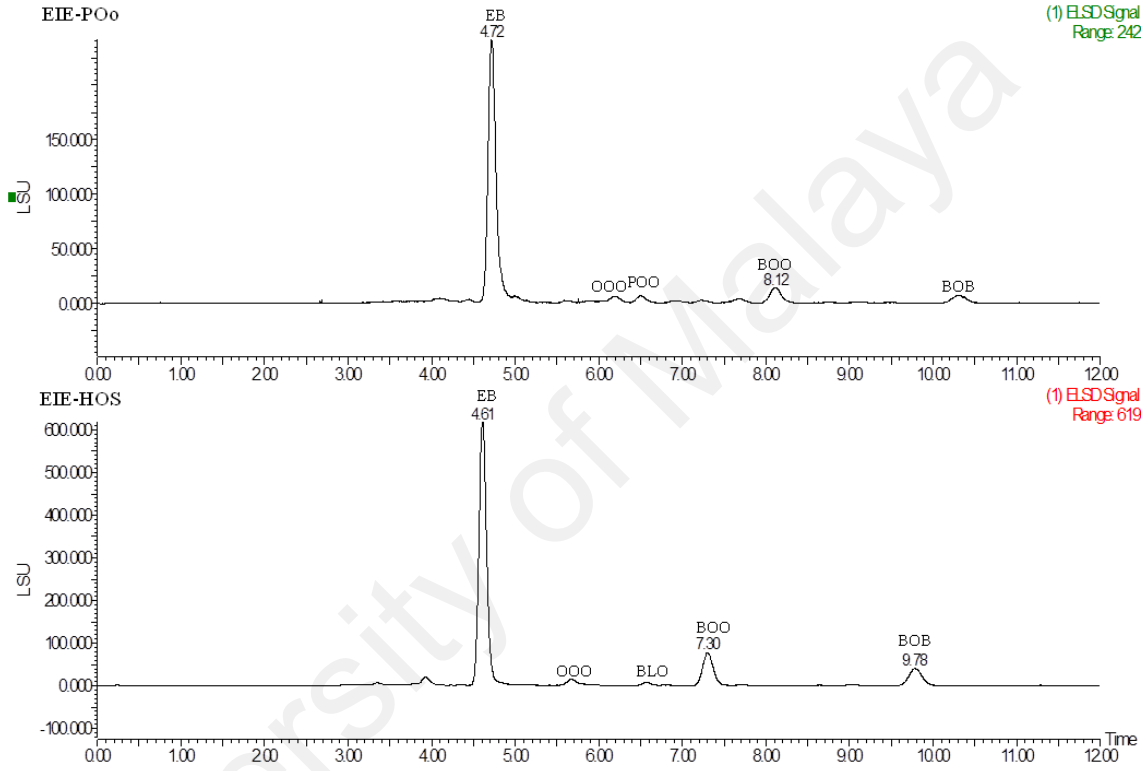


Figure 3.2: High performance liquid chromatography profiles of the interesterified structured lipids EIE-POo and EIE-HOS

3.4.3 Positional Composition

Positional FAC of EIE-POo, EIE-HOS, purified EIE-POo and purified EIE-HOS are presented in Table 3.2. Total SFA content of products was higher than the starting materials, POo and HOS, while the total MUFA compositions depreciated. SFA compositions at the *sn*-1,3 positions of TAG in EIE-POo increased from 71.0 mol% to 82.0 mol% (Table 3.2) by the catalytic action of Lipozyme RM IM. Yet, 64.0 mol% of SFA were obtained at the *sn*-1,3 positions of EIE-HOS (increment of 52.4 mol%).

However, SFA compositions at the *sn*-1,3 positions of TAG in EIE-HOS was relatively lower than those in EIE-POo.

Table 3.2: Positional fatty acid compositions of POo, HOS, purified EIE-POo, purified EIE-HOS and interesterification products catalysed by Lipozyme RM IM

Sample	Composition (mol%) *			
	sn-position	Saturated	Monounsaturated	Polyunsaturated
POo	1,3	71.0 ± 0.4	26.1 ± 0.3	2.9 ± 0.3
	2	7.4 ± 0.9	74.3 ± 0.9	18.3 ± 0.2
	1,2,3	50.3 ± 0.4	41.8 ± 0.5	7.9 ± 0.2
EIE-POo	1,3	82.0 ± 0.3	14.8 ± 1.1	3.2 ± 0.7
	2	13.5 ± 1.2	66.3 ± 0.7	20.2 ± 1.8
	1,2,3	58.8 ± 0.3	32.3 ± 0.9	8.9 ± 1.3
Purified EIE-POo	1,3	63.8 ± 0.2	30.6 ± 1.1	5.6 ± 1.2
	2	3.3 ± 0.8	81.7 ± 0.7	15.0 ± 1.5
	1,2,3	43.5 ± 0.4	47.8 ± 0.9	8.7 ± 1.4
HOS	1,3	11.6 ± 0.3	76.5 ± 0.2	11.9 ± 0.5
	2	nd	86.5 ± 0.4	13.5 ± 0.7
	1,2,3	7.8 ± 0.3	79.8 ± 0.3	12.4 ± 0.7
EIE-HOS	1,3	64.0 ± 1.1	31.7 ± 0.8	4.3 ± 0.3
	2	5.3 ± 0.4	81.8 ± 1.3	12.9 ± 0.1
	1,2,3	44.3 ± 0.4	48.6 ± 0.1	7.2 ± 0.3
Purified EIE-HOS	1,3	71.0 ± 1.1	24.4 ± 1.1	4.7 ± 0.5
	2	4.3 ± 0.3	85.2 ± 0.7	10.6 ± 0.4
	1,2,3	47.7 ± 0.8	45.6 ± 0.3	6.7 ± 0.5

Notes: IV, iodine value; POo, palm olein IV 56; nd, not detected.

* ^{13}C NMR results are mean of three replicates ± standard deviation.

Within 0.5 hour of interesterification reaction of POo with ethyl behenate, increment of saturation level at the *sn*-2 position was 6.1 mol% (7.4 mol% to 13.5 mol%). In the 2 hours of interesterification reaction EIE-HOS, the SFA content at the *sn*-2 position of SLs increased from undetectable in HOS to 5.3 mol%. The accumulation of SFA content at the *sn*-2 position of EIE-HOS was significantly lower than those produced from EIE-POo.

At the *sn*-2 position, EIE-HOS's SFA content of 5.3 mol% decreased to 4.3 mol% (Table 3.2) after purification. The saturation level of EIE-HOS at *sn*-2 position is relatively lower than EIE-POo (13.5 mol%). However, the SFA content of EIE-POo at the *sn*-2 position drastically dropped to 3.3 mol% after purification. Due to the nature and characteristic of POo, EIE-POo (82.0 mol%) showed relatively greater amount of SFA at the *sn*-1,3 positions of TAG than in EIE-HOS. Nevertheless, purified EIE-HOS (71.0 mol%) in Table 3.2 exibits greater amount of SFA at the *sn*-1,3 positions compared with the purified EIE-POo (63.8 mol%). This is in-line with one of the essential criteria for prospective low-calorie SLs.

Fatty acid composition (FAC) at the *sn*-positions of SLs is related to time since both of them were under compilation and involved the evolution of 1,3-specific interesterification reaction. This might caused by the acyl migration of *sn*-2 to *sn*-1, 3 positions. Due to higher stability of secondary hydroxyl group in TAG, minorities of the acyl chain at the *sn*-2 position of TAG was cleavaged and migrated to the *sn*-1,3 positions and then followed by the incorporation of behenic acid into *sn*-2 position in the interesterification reaction. Thus, the undesirable acyl migration was the major cause for variation in FAC at the *sn*-2 position.

3.4.4 Fatty Acid Composition

The FAC of POo, EIE-POo, HOS and EIE-HOS are listed in Table 3.3. The most abundant FA in POo was oleic acid but changed to behenic acid (36.7%) in EIE-POo after the interesterification reaction by Lipozyme RM IM.

Table 3.3: Fatty acid compositions of starting POo, EIE-POo, HOS and EIE-HOS interesterification by Lipozyme RM IM

Acyl chain	Composition of total fatty acids (mol%) [a]			
	POo	EIE-POo	HOS	EIE-HOS
12:0	0.3 ± 0.0	0.1 ± 0.0	nd	nd
14:0	1.0 ± 0.0	0.5 ± 0.0	nd	0.1 ± 0.0
16:0	39.9. ± 0.2	20.0 ± 0.1	3.6 ± 0.1	2.9 ± 0.1
18:0	4.0 ± 0.0	2.6 ± 0.0	2.9 ± 0.0	1.6 ± 0.0
20:0	0.3 ± 0.0	3.6 ± 0.0	0.3 ± 0.0	3.0 ± 0.1
22:0	0.1 ± 0.0	36.7 ± 0.3	1.2 ± 0.0	35.5 ± 0.2
24:0	0.1 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.8 ± 0.0
Total saturated fatty acids	45.7 ± 0.2	63.9 ± 0.2	8.3 ± 0.1	44.0 ± 0.2
16:1	0.2 ± 0.0	0.1 ± 0.0	nd	0.1 ± 0.0
18:1	43.3 ± 0.2	30.6 ± 0.3	83.2 ± 0.1	50.6 ± 0.4
20:1	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
22:1	nd	0.1 ± 0.0	nd	0.1 ± 0.0
Total monounsaturated fatty acids	43.7 ± 0.2	31.9 ± 0.3	83.5 ± 0.1	51.0 ± 0.4
18:2	10.4 ± 0.0	5.0 ± 0.1	8.2 ± 0.0	4.8 ± 0.2
18:3	0.2 ± 0.0	nd	nd	nd
20:2	nd	0.1 ± 0.0	nd	0.1 ± 0.0
22:2	nd	0.1 ± 0.0	nd	0.1 ± 0.0
Total polyunsaturated fatty acids	10.6 ± 0.0	5.2 ± 0.1	8.2 ± 0.0	5.0 ± 0.2

Notes: POo, palm olein IV 56; EIE-POo, enzymatic interesterified palm olein; HOS, high-oleic sunflower oil; EIE-HOS, enzymatic interesterified high-oleic sunflower oil; nd, not detected.

[a] FAC results are mean of three replicates ± standard deviation.

The most abundant FA (oleic acid) in EIE-HOS remained the same as in HOS since HOS is rich in oleic acid (83.2%) naturally. The content of behenic acid obtained from EIE-HOS was 35.5%, which is comparable to that of EIE-POo. However, the incorporation of behenic acid was proven by the increase in total saturation, especially in EIE-HOS (8.3% to 44.0%) and EIE-POo (45.7% to 63.9%). In general, EIE-POo was mainly consisted of palmitic (20.0%), oleic (30.6%) and behenic acids (36.7%), whereas two major FA behenic (35.5%) and oleic (50.6%) acids were found in EIE-HOS.

3.4.5 Slip Melting Point

The SMP of purified EIE-POo and purified EIE-HOS were obtained at 39.80°C and 41.40°C, respectively. The SMP of purified EIE-HOS is higher than purified EIE-POo by 1.60°C due to EIE-HOS consists higher content of total BOO and BOB (76.4%) whereas EIE-POo only contains 45.5%. Purified EIE-POo is preferred in term of lower SMP due to less waxy sensations and near to body temperature (37°C).

3.4.6 Solid Fat Content

Solid fat content (SFC) determines the physical properties (such as spreadability, mouth feel, firmness and stability) of SLs. The SFC against temperature curves of purified EIE-POo and EIE-HOS were both determined by IUPAC 2.150(a) method (Figure 3.3).

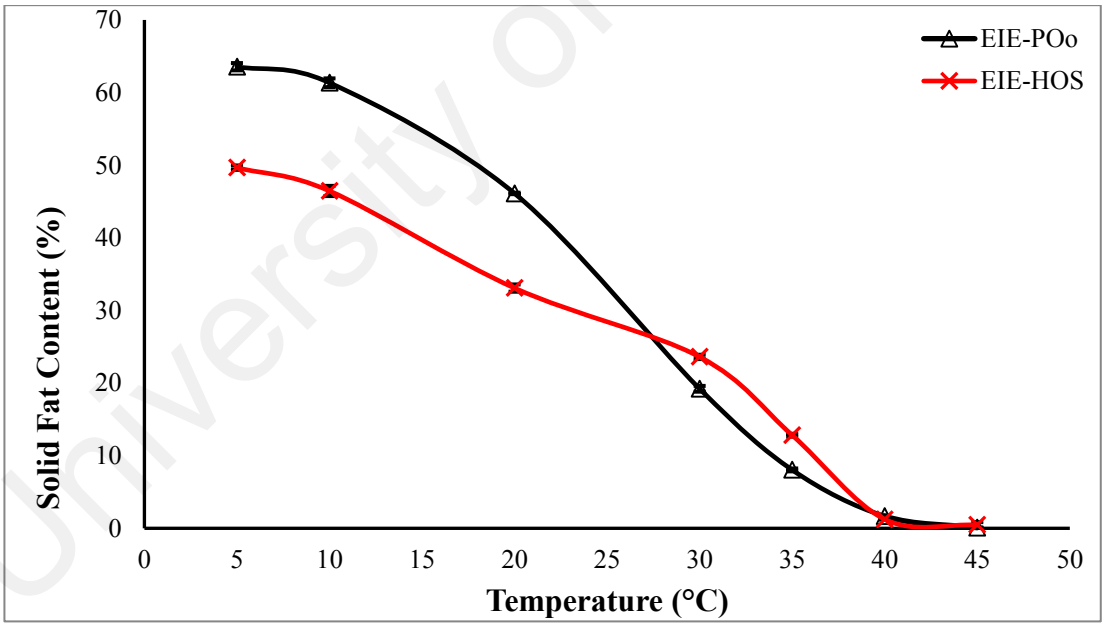


Figure 3.3: Solid fat content curve of purified structured lipids (EIE-POo and EIE-HOS) depending on temperature

There was an increase in density when SLs crystallised and decreased when SLs melted since the density of solid lipids always higher than those of their liquid states (Hui and Sherkat, 2005). At 10℃ , both SLs contain SFC greater than 32%, hardly exhibit

spreadability at the refrigerator temperature (Arifin *et al.*, 2011; Criado *et al.*, 2008). With the exception of temperature between 27.5 and 39.0℃ , the SFC of EIE-POo was higher than the SFC of EIE-HOS. Then, at the temperature between 5 and 27.5°C, EIE-POo may obtain greater hardness compared with EIE-HOS due to the greater SFC. In addition, EIE-POo displayed a notable difference in SFC between 20 and 33°C. Therefore, EIE-POo may offer more intensive cooling sensation and flavour release in our mouth. At the range of 33 to 38°C, both samples have relatively high SFC (more than 3.5%) which may cause waxy sensations (Lida and Ali, 1998; Criado *et al.*, 2007; Torbica *et al.*, 2006). The SFC of a SL is required to fall into the range between 15 and 25% at the usage temperature in order to avoid an excess of liquid and oiliness sensation in baking shortening. For confectionery, EIE-POo may provide better mouth feel and less absorption of FA by intestinal mucosa because of the high contents of targeted species (BOO and BOB) and a good SFC range between 20 and 33°C.

In conclusion, SLs containing high amount of BOO and BOB have successfully synthesised through lipase catalysed interesterification reactions in the present study. EIE-HOS yielded higher amount of BOO and BOB (76.5%) and a much lower saturation (5.3 mol%) at *sn*-2 position as compared to EIE-POo. Owing to their physical properties at room temperature, EIE-HOS offers higher desirable rheological properties to bakery, confectionery or as a base for *trans*-fat-free products. Moreover, they may serve as the hard stock for physical blending with other softer cooking oils with the aim of reducing obesogenic effects of the latter. Since EIE-HOS contains higher content of BOB (30.3%) than EIE-POo; hence, EIE-HOS is suitable use as the cocoa butter equivalent for chocolate and confectionery coatings. Nevertheless, purified EIE-HOS (41.40℃) obtained relatively higher SMP while compared to purified EIE-POo. Therefore, purified EIE-POo is less waxy sensations because it near to body

temperature (37°C). In other words, both EIE-HOS and EIE-POo still display the fat properties which may lower risk towards excessive fat deposition. Besides, EIE-HOS may be more effective to prevent obesity in confectionery field due to its higher yield of BOO and BOB.

CHAPTER FOUR
DETERMINATION OF ERUCIC ACID IN EDIBLE OILS AND MUSTARD BY USING QUANTITATIVE CARBON-13 NUCLEAR MAGNETIC RESONANCE

4.1 Background

Erucic acid (C22:1 13*c*, *n*-9) is a prominent naturally occurring *cis*-13 monounsaturated fatty acids (MUFA) present in rapeseed, cramble and mustard seed oils (Figure 4.1), especially in members of the *Brassica* genus (Gunstone and Harwood, 2007). Erucic acid has also been found in some marine animal oils. Mustard oil is commonly used as cooking oil in India, Bangladesh, Pakistan and Nepal, while the Europeans, Americans and Asians prefer to consume mustard as sauces, seasonings and salad dressings due to its pungent, strong cabbage and hot nutty taste. However, high content of erucic acid has been reported to cause myocardial lipidosis and heart lesions (Sauer and Kramer, 1983; Zhang *et al.*, 1991; Kramer *et al.*, 1990; Watkins *et al.*, 1995).

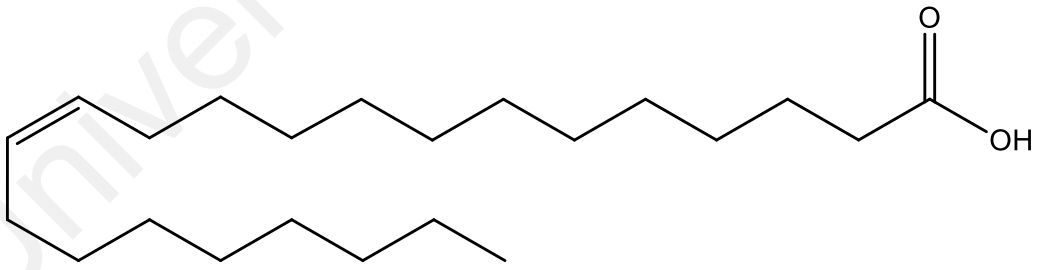


Figure 4.1: Erucic acid

Experimental studies have demonstrated an association between dietary erucic acid and myocardial lipidosis in some species. Myocardial lipidosis is reported to reduce the contractile force of heart muscle (Food Standards Australia New Zealand, 2003). The effect can explain the occurrence of myocardial lipidosis that erucic acid is poorly

oxidised by the mitochondrial *β*-oxidation system, which then led to its accumulation in the heart and liver (Sauer and Kramer, 1983; Zhang *et al.*, 1991). Studies have also demonstrated an association between dietary erucic acid and heart lesions in rats.

Roine *et al.* (1960) were the first to report the toxic effects of rapeseed oil. The rats which fed rapeseed oils at up to 70% of the calorie content of their diet were reported to have developed myocarditis. Weanling rats have been reported to accumulate fat in the heart muscle after one day of feeding high amounts of rapeseed oil (Abdellatif and Vles, 1970). The amount of fat in the heart muscle of these rats was sometimes found to exceed the standard values by four times. The fat droplets are mainly triacylglycerols (TAG) containing a large proportion of erucic acid (Houtsmuller *et al.*, 1970). The fat accumulation decreases over a long time and finally disappears even with continued feeding of rapeseed oil. The fat accumulation is reported to disappear even more quickly if erucic acid is removed from the diet (Kitts, 1996).

In response to the reported potential health concerns in human, food grade rapeseed oil is being regulated in the Europe, Australia, New Zealand and the United States. The Commission of the European Communities (1980) specified a maximum 5% of erucic acid; while Food Standards Australia New Zealand (2003) has set a provisional tolerable daily intake of 7.5 mg of erucic acid per kg of body weight. In a more recent development, food grade rapeseed oil also has been regulated not to exceed 2% of erucic acid by the United States Department of Health and Human Service (2010).

Conventional ^{13}C Nuclear magnetic resonance (NMR) spectroscopy allows identifying the carbon atoms in an organic molecule. The ^{13}C isotope of carbon (natural abundance = 1.1%) can be detected by ^{13}C NMR since main carbon ^{12}C isotope is not detectable by

NMR due to zero net spin. Therefore, only few ^{13}C nuclei present resonate in the magnetic field, and the intensities of the signals are not typically proportional to the number of equivalent ^{13}C atoms. A new quantitative ^{13}C NMR (qCNMR) regiospecific analysis is developed to overcome these drawbacks since monoacylglycerols (MAG), diacylglycerols (DAG), and TAG have characteristic chemical shifts for the three glycerol carbons, C1 and C2 carbons in each acyl chain (Gunstone, 1991; Gunstone, 1994).

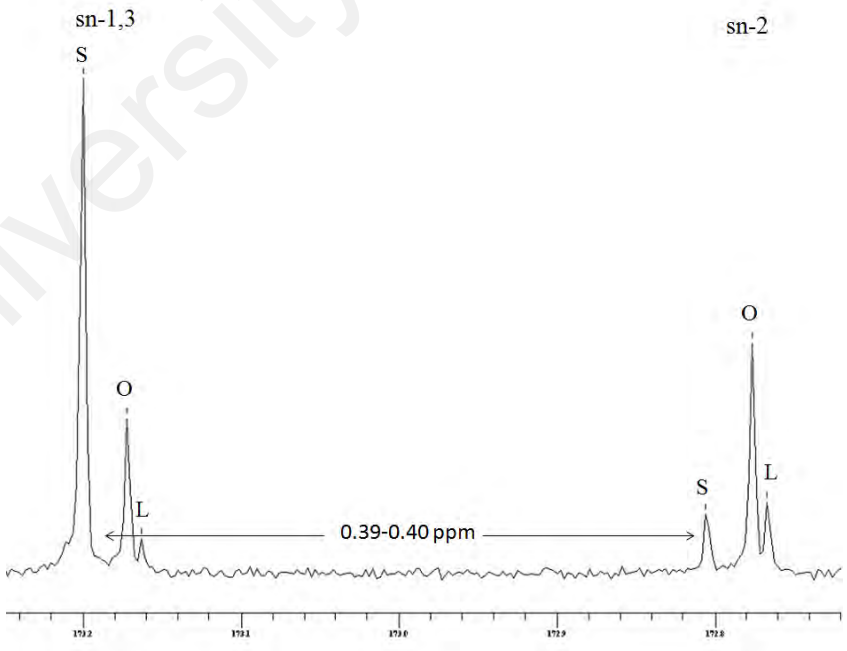
Although sufficient time is required for the nuclei to relax between repeat scans, time needed can be reduced by engaging appropriate pulse sequences. Gouk *et al.* (2012) have reported that accurate regiospecific data can be obtained rapidly using qCNMR. The reported method successfully narrow down the systematic error to 1.0 mol% and less than one hour of analytical time is needed. As discussed in Section 1.2.3, conventional method that consists of chemical hydrolysis and high performance liquid chromatography (HPLC) is very time consuming and usually would take at least 3 days even to a skilled individual.

4.2 Present Study

The qCNMR regiospecific method was developed to analyse the TAG, DAG and MAG to provide the distributions of FA in the *sn*-1,3 and *sn*-2 positions of oils and fats (Ng, 1985) without a risk of positional cross contamination from acyl migration (Diehl *et al.*, 1995). It appears to be especially useful for fish oils which not easily analysed by other means. The high-resolution ^{13}C NMR spectra have utilised the differences (α/β splittings) in the chemical shifts of the carbon atoms in *sn*-1,3 and *sn*-2 positions. The C13 resonances can be categorised into carbonyl (172–174 ppm), unsaturated (124–134 ppm), glycerol (60–70 ppm) and aliphatic (10–35 ppm). However, only the carbonyl

(Ng, 1983) and olefinic (Ng, 1984; Wollenberg, 1990) regions have reported for the regiospecific analysis.

Esters are more shielded (δ 150-180 ppm) as compared to aldehyde and ketone (δ 190-220 ppm). The carbonyl carbons of fatty acids (FA) of palm oil appear as two sets of resonances, the high frequency set (δ 173.2 ppm) includes the acyl chains esterified at the *sn*-1,3 positions of TAG, whereas the low frequency set (δ 172.7 ppm) includes acyl chains esterified at the *sn*-2 position (Figure 4.2). Chemical shifts for the FA at *sn*-1,3 positions are shifted consistently by 0.39-0.40 ppm at the higher frequencies than those of the corresponding FA attached at the *sn*-2 position. This shift difference was explained by their different γ-gauche interaction (Howarth *et al.*, 1995). Besides, the saturated fatty acids (SFA), MUFA and polyunsaturated fatty acids (PUFA) appear from higher to lower frequency in that order within each set of signals (Ng, 1983; Wollenberg, 1990).



Notes: S, saturated fatty acid acyl chain; O, oleic acid acyl chain (MUFA); L, polyunsaturated fatty acid acyl chain (PUFA).

Figure 4.2: ^{13}C NMR spectrum of acyl chain carbonyl resonances of palm oil

The carbonyl carbon resonances resolved by the number of unsaturation centres. The separation between vaccenyl (*cis*-11-monoene) and PUFA chain (0.02 ppm) are slightly wider than the separation between oleyl (*cis*-9-monoene) and PUFA chain (0.01 ppm). Therefore, vaccenyl is at the order of higher frequency than oleyl. Signals from PUFA, linoleyl and linolenyl chains are indistinguishable in both sets of resonances due to the identical position of first double bond occurred at the *γ*-position from the carbonyl carbons and the carbon further from the carbonyl carbon (Bergana and Lee, 1996). However, qCNMR regiospecific method will overcome the shortcomings as mentioned earlier in the conventional method (Section 1.2.3.2). Thus, the goal of present study is to develop a regiospecific analysis for quick and easy detection of erucic acid in edible oils and mustard products.

4.3 Experimental

4.3.1 Material

Refined mustard oil, coconut oil, corn oil, grape seed oil, hemp seed oil, linseed oil, macadamia nut oil, peanut oil, pumpkin seed oil, cold pressed rapeseed oil, rice bran oil, sesame oil, soybean oil, walnut oil, canola oil, mustard sauce H, mustard sauce M and mustard powder C were purchased from local supermarkets. Refined bleach and deodorised (RBD) palm olein, RBD palm oil and RBD palm kernel oil were supplied by Bintulu Edible Oils Sdn. Bhd. Standard Trierucin (>98%) supplied by TCI (Tokyo, Japan). Deuterated chloroform, *n*-hexane (HPLC and AR grades), concentrated sulphuric acid, AR grade toluene, anhydrous methanol, glacial acetic acid, and sodium methoxide were purchased from Merck, Darmstadt, Germany. Sodium Chloride and sodium bicarbonate were purchased from Systerm, Shah Alam, Selangor. Fatty acid methyl ester (FAME) C_4-C_{24} mix was purchased from Sigma Chemicals, St. Louis, MO, United States.

4.3.2 Cold Extraction of Mustard Products

Mustard sauces and mustard powder were cold extracted twice with *n*-hexane in a separating funnel at 3:40 (m/v) and 11:300 (m/v) sample/solvent ratios, respectively. After being shaken for 2 minutes, the hexane extract was transferred into a round bottom flask, and the solvent was evaporated under reduced pressure at 40°C. Sample C obtained the highest yield of extraction (30.89%), followed by M (1.73%) and H (1.18%).

4.3.3 Preparation of Oil Mixtures, Synthetic Mixtures, Extracted Mustard Products and Oil Samples

Mixtures of mustard oil and canola oil were prepared in mass ratios of 5:95, 10:90, 20:80, 30:70, 40:60 and 50:50 (m/m) and then 200 mg of the mixtures was dissolved in 0.5 mL of deuterated chloroform to produce a 2:5 (m/v) sample/solvent ratio. Synthetic mixtures were prepared by mixing standard trierucin and canola oil in concentrations of 0.060, 0.068, 0.088, 0.103, 0.115, 0.120, 0.176 and 0.223 (mol dm^{-3}) and diluted with 0.5 mL of deuterated chloroform.

All oil samples and mustard extracts were dissolved in deuterated chloroform to produce a 2:5 (m/v) sample/solvent ratio. All samples were degassed by purging with nitrogen and ultrasonicated for 2 minutes to ensure the removal of paramagnetic dissolved oxygen. The volume of sample used fixed in 0.5 mL for the best resolution of resultant spectrum.

4.3.4 ^{13}C NMR Regiospecific Analysis

Regiospecific analyses of oils and mustard extracts were optimised from our previous study (Gouk *et al.*, 2013a). All ^{13}C NMR spectra were recorded using JEOL ECA-400

MHz NMR spectrometer at a frequency of 100.40 MHz fitted with a 5-mm-i.d. dual probe. The proton decoupled spectra were acquired using inverse gated heterodecoupling pulse sequence. A spectral width of 1500 Hz at where the acyl chain carbonyl carbons resonate, 8192 data points and 90° pulse width were employed. The inversion recovery pulse sequence was used to measure ^{13}C spin-lattice relaxation times by setting the tau values from 15.0 to 0.5 s and the pulse values from 9.0 to 30.0 μs. Experimental temperature was set at 25°C while 5.5 s of acquisition time and 22.0 s of relaxation delay applied. The total repetition delay was 27.4 s to achieve 99.9% recovery of z-magnetization. Hence, 29.0 s pulse delay was selected. Free induction decay was processed in the exponential window. Deconvolution was done by a mixture coefficient ratio of 1:1 between Lorentzian and Gaussian functions and further optimised by a nonlinear least-square procedure, as established and reported previously (Gouk *et al.*, 2012). All spectra acquired in 128 and 4 dummy scans. Standard JEOL ALICE processing software was used.

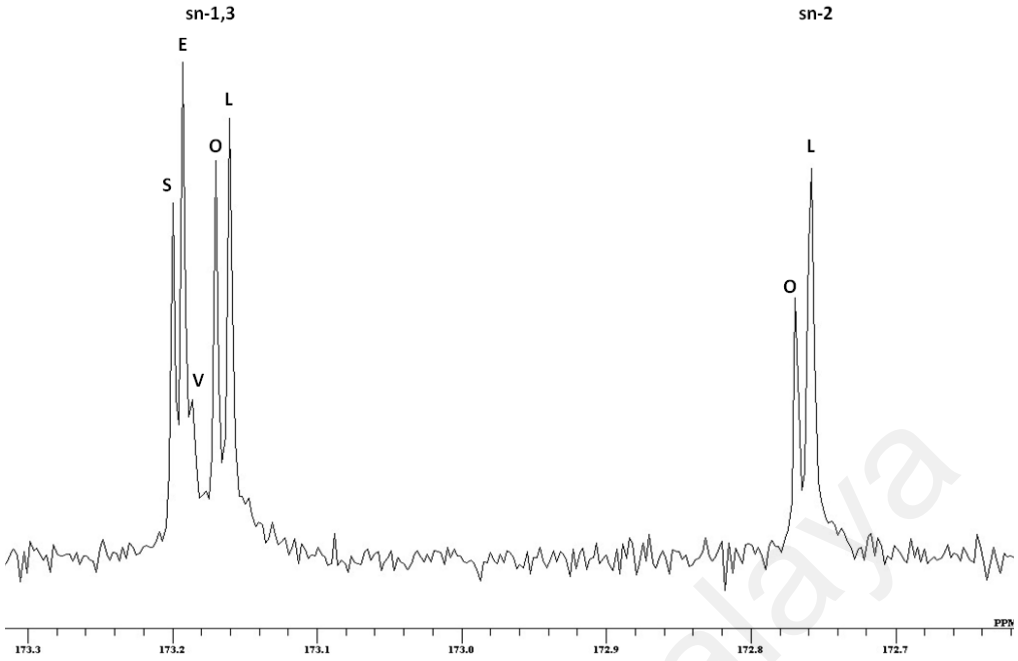
4.3.5 Transesterification and Analysis of Fatty Acid Composition

Fatty acid methyl ester (FAME) of edible oils and mustard extracts produced by sodium methoxide-catalysed transesterification and acid-catalysed transesterification, respectively (Christie, 1989). Methyl ester sample (1 μL) was injected into the gas chromatography (GC) (GC-2010A series, Shimadzu) equipped with a flame ionisation detector and a BPX70, 30 m x 0.32 mm x 0.25 μm capillary column. An initial temperature of 140°C was held isothermally for 2 minutes, and then increased to 220°C at a rate of 8°C per minute. The column was maintained at the final temperature for 5 minutes. The oven, injector and the detector ports were set at 140°C, 240°C and 260°C, respectively. Helium was the carrier gas at a flow rate of 1.10 mL/minute operated at a 50:1 split ratio.

4.4 Results and Discussion

4.4.1 Detection of Erucic Acid in Mustard Oil and Limit of Detection

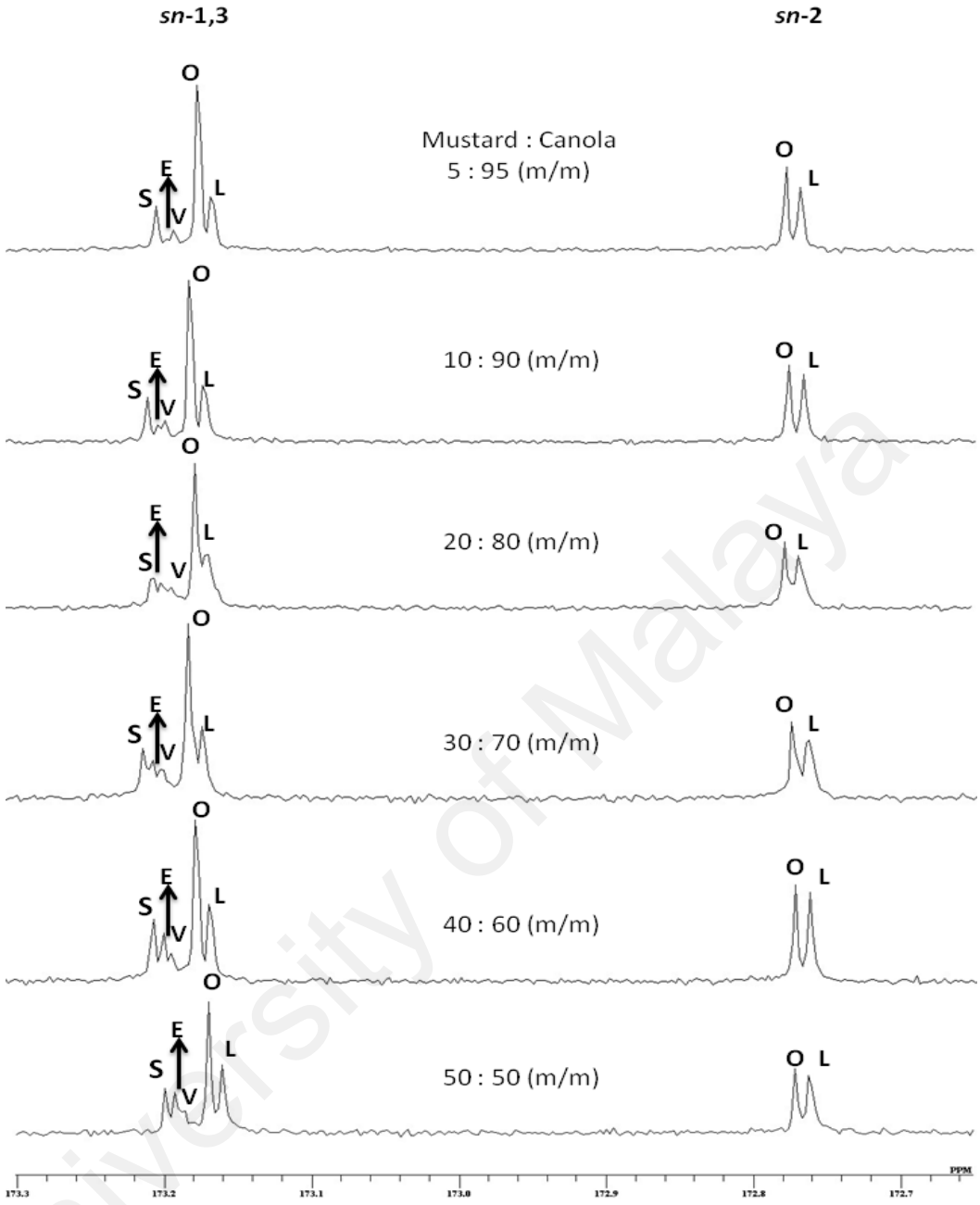
Mustard oil was selected as the test oil because it contains 24 to 40% of erucic acid (Gunstone and Harwood, 2007). The carbonyl (172-174 ppm) carbon nuclei resonance in mustard oil was acquired under the frequency of 100.40 MHz (Figure 4.3). Apart from those reported previously in the carbonyl carbon region (Ng, 1985; Bergana and Lee, 1996; Vlahov, 1998; Gouk *et al.*, 2012), an additional peak recorded at 173.1799 ppm which has not been reported before. This resonance is prepared to be attributed to the presence of *cis*-13 MUFA in mustard oil. The resonance of *cis*-13 MUFA was shifted by 0.0069 ppm at a higher frequency than the *cis*-11 MUFA owing to the distance-dependent electric field effect of the double bond. Besides, it was at a 0.0069 ppm lower frequency about those for saturated species. The effect as mentioned above was prominent as the first double bond occurred further from the carbonyl carbon in *cis*-13 MUFA, resulting in a relatively higher frequency compared with *cis*-11 MUFA resonance.



Notes: S, saturated fatty acid acyl chain; E, *cis*-13 monounsaturated; V, *cis*-11 monounsaturated; O, *cis*-9 monounsaturated; L, polyunsaturated fatty acid acyl chain.

Figure 4.3: ^{13}C NMR carbonyl of triacylglycerols in mustard oil

In order to examine the current methodology, mustard oil mixed with canola oil (good source of *cis*-11 MUFA) in different of mass ratios. A significant and sharp peak corresponded to the *cis*-13 MUFA at *sn*-1,3 positions region was observed in the 50% (m/m) spectrum. The gradual decrease in the mass ratio of mustard oil was accompanied by the evolution of erucic acid peak (Figure 4.4). Hence, the peak appeared at 173.1799 ppm can be confirmed as erucic acid.



Notes: S, saturated fatty acid acyl chain; E, *cis*-13 monounsaturated; V, *cis*-11 monounsaturated; O, *cis*-9 monounsaturated; L, polyunsaturated fatty acid acyl chain.

Figure 4.4: ^{13}C NMR carbonyl spectrum of oil mixtures made up by mustard oil and canola in various mass ratios

Acceptable coefficient of determination (R^2 = 0.9977) can be obtained while the concentration of trierucin was linearly correlated to the total composition of erucic acid (Figure 4.5). Therefore, this excellent relation further validated high prediction and replication of the current established method. Detection limit as low as 0.98% (m/m)

was then determined from the regression line of the graph (Fonollosa *et al.*, 2014; Kaiser, 1970).

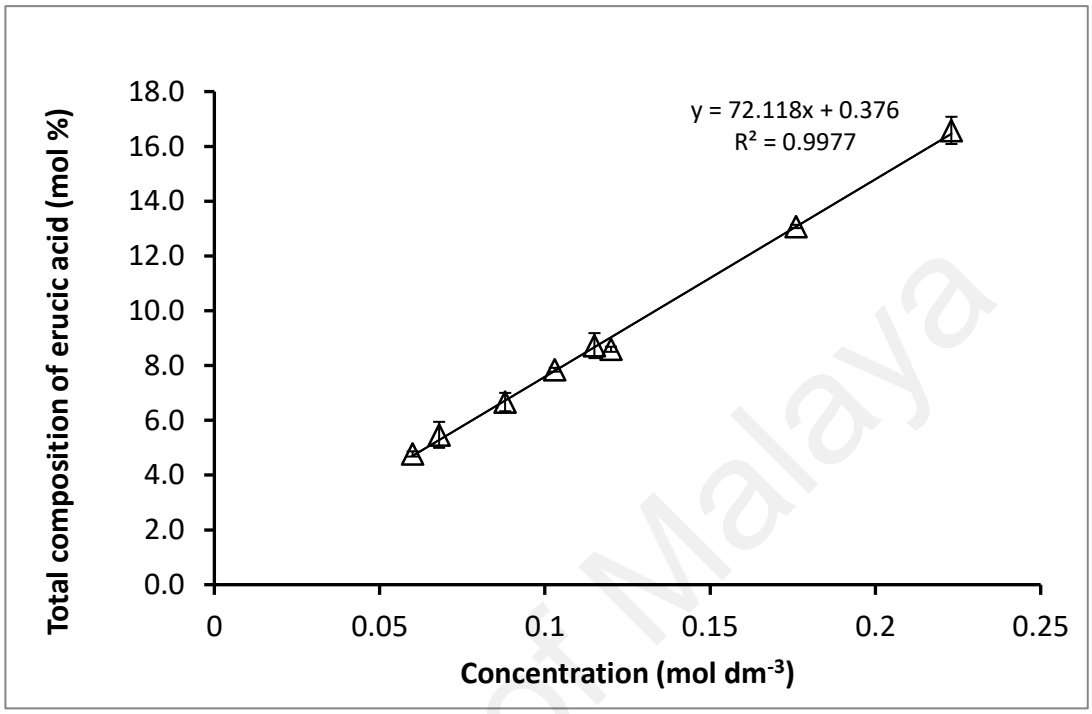


Figure 4.5: Variation of the composition of erucic acid in different mass ratios of standard trierucin and canola. Values are means with their standard errors represented by vertical bars

4.4.2 Conventional Analytical Method Versus Quantitative ^{13}C NMR

The SFA, *cis*-11 MUFA and *cis*-13 MUFA occurred exclusively at the *sn*-1,3 positions. It is noteworthy that *cis*-9 and *cis*-11 structural isomers can be quantified separately. The composition of erucic acid at the *sn*-1,3 positions was 26.6 mol%. With no more than 0.7 mol% of standard errors, all NMR data obtained in high repeatability (Table 4.1). Furthermore, both fatty acid composition (FAC) results determined by NMR and conventional method through methylation and then chromatographic analysis (Christie, 1989) are comparable (Table 4.1).

Table 4.1: Positional distribution of fatty acids in mustard oil obtained by ^{13}C NMR and gas chromatography

Carbon resonance	Acyl chain	Composition (mol%)[a]			Total FAC[b] (mass %)
		sn-1,3	*sn*-2	Total	
Carbonyl carbon	SFA	17.7 ± 0.2	nd	12.4 ± 0.2	12.6 ± 0.1
	cis-13 MUFA	26.6 ± 0.6	nd	18.5 ± 0.6	18.4 ± 0.1
	cis-11 MUFA	6.7 ± 0.1	nd	4.8 ± 0.2	31.5 ± 0.1
	cis-9 MUFA	21.4 ± 0.1	34.7 ± 0.4	25.6 ± 0.1	
	PUFA	27.6 ± 0.7	65.3 ± 0.4	38.7 ± 0.5	37.5 ± 0.1

Notes: FAC, fatty acid composition; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; nd, not detected.
[a] ^{13}C NMR results are mean of three replicates ± standard deviation.
[b] Analysis of total FAC was performed by gas chromatography in mass %.

Previous studies employed saponification, acidification, methylation, thin layer chromatography, ozonisation and GC (Meijboom and Jongenotter, 1980; Mortuza *et al.*, 2006) or gas-liquid chromatography (Harvey and Downey, 1964; Sarwar *et al.*, 2014) to determine erucic acid content. Otherwise, another research reported that it is necessary to add an internal standard during transesterification for quantification of the FA exclusively. The mixture was treated for 1.5 hours at 80°C, followed by addition of 1.5 mL of 1% sulphuric acid in methanol. Both 1 mL of saturated sodium chloride solution and 1 mL of demineralised water were added in advance before the extraction. For the purpose of determining the response factor, the second internal standard needs to be added to the organic layer solution before subjected to GC coupled with the electron ionisation mass spectrometry (Wendlinger *et al.*, 2014). This shows the conventional methods are indeed laborious and time consuming.

Most of the conventional analytical methods are highly chemicals and reagents demanding. Meanwhile, the methods often lead to sample destruction as samples require methylation, laborious procedures and lengthy sample preparation (more than 2 hours). On the contrary, the qCNMR merely takes 1 hour for sample preparation and analysis. As a non-destructive method, original samples can be recovered from the

analysis without chemical derivatization. Therefore, there is a low chance for sample loss or sample contamination.

4.4.3 Erucic Acid Content in Edible Oils and Mustard Products

According to Oil World Statistic (2015), consumption of oils and fats has increased 33.6 million tonnes in the past six years from 162 million tonnes in 2008 to 195.6 million tonnes in 2014. In 2014, per capita consumption of oil and fats was 58 kg in the United States, 61 kg in the European Union, 16 kg in the India and 26 kg in the China, respectively. The world average per capita consumption of all oils and fats was 27 kg or 74.0 g a day in 2014. Thus, it is important to ensure good quality oil is in the market.

The currently established method was applied to determine the content of erucic acid in selected edible oils and mustard products. Generally, no erucic acid was detected in canola oil, coconut oil, corn oil, grape seed oil, hemp seed oil, linseed oil, macadamia nut oil, peanut oil, pumpkin seed oil, rapeseed oil, rice bran oil, sesame oil, soybean oil, walnut oil, RBD palm olein, RBD palm oil and RBD palm kernel oil in the present study (Table 4.2). In fact, ^{13}C NMR regiospecific data of grape seed oil, hemp seed oil, linseed oil, macadamia nut oil, pumpkin seed oil, RBD palm oil and RBD palm kernel oil have not published before (Table 4.2). These new regiospecific data may provide more insight into the role of essential human nutrition.

Table 4.2: Positional distribution of fatty acids in selected edible oils obtained by quantitative ^{13}C NMR

Sample	Acyl chain	Composition (mol%)[a]		
		***sn*-1,3**	***sn*-2**	**Total**
Canola oil	SFA	12.6 ± 0.2	nd	8.3 ± 0.6
	cis-13 MUFA	nd	nd	nd
	cis-11 MUFA	7.6 ± 0.7	nd	5.0 ± 0.6
	cis-9 MUFA	59.0 ± 1.0	51.8 ± 0.9	56.4 ± 0.7
	PUFA	20.8 ± 0.7	48.2 ± 0.9	30.3 ± 0.4
Coconut oil	SFA	90.4 ± 0.4	93.5 ± 0.8	91.4 ± 0.1
	cis-13 MUFA	nd	nd	nd
	cis-11 MUFA	nd	nd	nd
	cis-9 MUFA	9.7 ± 0.4	6.5 ± 0.8	8.7 ± 0.1
	PUFA	nd	nd	nd
Corn oil	SFA	21.9 ± 0.6	nd	14.6 ± 0.1
	cis-13 MUFA	nd	nd	nd
	cis-11 MUFA	nd	nd	nd
	cis-9 MUFA	30.9 ± 0.1	30.2 ± 0.6	30.7 ± 0.1
	PUFA	47.2 ± 0.5	69.8 ± 0.6	54.7 ± 0.2
Peanut oil	SFA	24.1 ± 0.1	nd	16.3 ± 0.1
	cis-13 MUFA	nd	nd	nd
	cis-11 MUFA	5.0 ± 0.3	nd	3.4 ± 0.2
	cis-9 MUFA	59.3 ± 0.9	68.1 ± 1.0	62.1 ± 1.0
	PUFA	11.6 ± 0.8	31.9 ± 1.0	18.2 ± 1.0
RBD Palm olein	SFA	63.0 ± 0.7	9.3 ± 0.0	44.7 ± 0.1
	cis-13 MUFA	nd	nd	nd
	cis-11 MUFA	nd	nd	nd
	cis-9 MUFA	30.6 ± 0.9	72.8 ± 0.3	45.0 ± 0.4
	PUFA	6.4 ± 0.3	17.9 ± 0.4	10.3 ± 0.4
Rice bran oil	SFA	35.3 ± 0.1	2.6 ± 0.2	24.2 ± 0.3
	cis-13 MUFA	nd	nd	nd
	cis-11 MUFA	1.9 ± 0.0	nd	1.3 ± 0.2
	cis-9 MUFA	38.9 ± 0.0	45.1 ± 1.0	40.9 ± 0.1
	PUFA	23.4 ± 0.1	52.3 ± 1.0	33.6 ± 0.4

Table 4.2, continued

Sample	Acyl chain	Composition (mol%)[a]		
		***sn*-1,3**	***sn*-2**	**Total**
Soybean oil	SFA	22.9 ± 0.1	nd	15.4 ± 0.5
	cis-13 MUFA	nd	nd	nd
	cis-11 MUFA	3.0 ± 0.1	nd	2.1 ± 0.1
	cis-9 MUFA	22.8 ± 0.1	22.0 ± 0.2	22.6 ± 0.2
	PUFA	51.2 ± 0.0	78.1 ± 0.2	60.1 ± 0.5
Walnut oil	SFA	14.6 ± 0.8	nd	9.6 ± 0.6
	cis-13 MUFA	nd	nd	nd
	cis-11 MUFA	nd	nd	nd
	cis-9 MUFA	13.5 ± 0.2	15.7 ± 0.7	14.3 ± 0.1
	PUFA	71.9 ± 1.0	84.3 ± 0.7	76.1 ± 0.5
Sesame oil	SFA	24.7 ± 0.5	nd	17.0 ± 0.0
	cis-13 MUFA	nd	nd	nd
	cis-11 MUFA	2.8 ± 0.2	nd	1.9 ± 0.2
	cis-9 MUFA	37.5 ± 0.2	49.7 ± 0.5	41.3 ± 0.2
	PUFA	35.0 ± 0.5	50.3 ± 0.5	39.8 ± 0.0
Rapeseed oil (cold pressed)	SFA	11.4 ± 0.2	nd	8.0 ± 0.2
	cis-13 MUFA	nd	nd	nd
	cis-11 MUFA	11.4 ± 0.6	nd	7.9 ± 0.5
	cis-9 MUFA	57.6 ± 0.2	53.8 ± 0.3	56.5 ± 0.0
	PUFA	19.6 ± 0.6	46.2 ± 0.3	27.6 ± 0.6
Grape seed oil	SFA	17.5 ± 0.2	nd	11.7 ± 0.1
	cis-13 MUFA	nd	nd	nd
	cis-11 MUFA	2.1 ± 0.8	nd	1.4 ± 0.6
	cis-9 MUFA	14.3 ± 0.8	23.0 ± 0.2	17.2 ± 1.0
	PUFA	66.1 ± 1.0	77.0 ± 0.2	69.7 ± 0.8
Hemp seed oil	SFA	15.7 ± 0.1	nd	10.5 ± 0.1
	cis-13 MUFA	nd	nd	nd
	cis-11 MUFA	nd	nd	nd
	cis-9 MUFA	10.9 ± 0.3	12.5 ± 0.1	11.4 ± 0.2
	PUFA	73.4 ± 0.4	87.5 ± 0.1	78.1 ± 0.1
Linseed oil	SFA	14.8 ± 0.1	nd	9.7 ± 0.1
	cis-13 MUFA	nd	nd	nd
	cis-11 MUFA	nd	nd	nd
	cis-9 MUFA	18.1 ± 0.7	21.8 ± 0.3	19.5 ± 0.7
	PUFA	67.1 ± 0.8	78.2 ± 0.3	70.9 ± 0.6

Table 4.2, continued

Sample	Acyl chain	Composition (mol%)[a]		
		***sn*-1,3**	***sn*-2**	**Total**
Macadamia oil	SFA	24.4 ± 0.2	nd	16.8 ± 0.2
	cis-13 MUFA	nd	nd	nd
	cis-11 MUFA	12.0 ± 0.3	nd	8.3 ± 0.2
	cis-9 MUFA	63.6 ± 0.4	100.0 ± 0.0	74.9 ± 0.4
	PUFA	nd	nd	nd
Pumpkin seed oil	SFA	26.6 ± 0.2	nd	17.5 ± 0.1
	cis-13 MUFA	nd	nd	nd
	cis-11 MUFA	2.2 ± 0.6	nd	1.4 ± 0.4
	cis-9 MUFA	41.3 ± 0.6	35.5 ± 0.3	39.4 ± 0.2
	PUFA	29.9 ± 0.3	64.5 ± 0.3	41.7 ± 0.1
RBD Palm kernel oil	SFA	81.6 ± 0.2	78.3 ± 0.3	80.4 ± 0.2
	cis-13 MUFA	nd	nd	nd
	cis-11 MUFA	nd	nd	nd
	cis-9 MUFA	16.4 ± 0.9	18.1 ± 0.4	17.0 ± 0.4
	PUFA	2.0 ± 0.7	3.7 ± 0.7	2.6 ± 0.2
RBD Palm oil	SFA	64.5 ± 1.0	17.8 ± 0.5	48.7 ± 0.6
	cis-13 MUFA	nd	nd	nd
	cis-11 MUFA	nd	nd	nd
	cis-9 MUFA	31.6 ± 0.9	60.9 ± 0.0	41.5 ± 0.3
	PUFA	3.9 ± 0.1	21.3 ± 0.6	9.8 ± 0.3

Notes: FAC, fatty acid composition; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; nd, not detected.
[a] ^{13}C NMR results are mean of three replicates ± standard deviation.

Mustard oil (18.50 mol%, 21.41 mass%, Table 4.1, Table 4.4) has exceeded the maximum levels of erucic acid specified by the aforementioned regulations. The tolerable daily intake of erucic acid for human exposure would be 7.5 mg per kg body weight (Food Standards Australia New Zealand, 2003). Hence, approximately not more than 500 mg of erucic acid should be taken by a 67 kg adult per day. From calculation, only 2.3 g of mustard oil (214.1 mg erucic acid per g sample) used in the present study is suggested to be consumed per day, which is less than one teaspoon (1 teaspoon = 4.2 g). This permitted amount of mustard oil is extremely low in relation to world average per capita consumption per day (74.0 g). It is easy to exceed the maximum consumption level of erucic acid; especially the Indians and Pakistanis. Coincidentally, the World

Health Review (2014) disclosed that coronary heart disease is the primary cause of death in both India (13.70%) and Pakistan (9.87%).

On the other hand, M exhibited the highest amount of erucic acid (33.1 mol%, 35.94 mass%), followed by H (32.2 mol%, 36.97 mass%) and C (25.5 mol%, 28.85 mass%), residing only at *sn*-1,3 positions (Table 4.3, Table 4.4). No erucic acid was detected at the *sn*-2 position of all mustard products. Erucic acid is the most abundant FA in M and H at *sn*-1,2,3 positions with less than 1.0 mol% standard errors. Meanwhile, PUFA (linoleic acid, α-linolenic acid and eicosadienoic acid) is the highest FA determined in C at *sn*-1,2,3 positions. Nevertheless, all mustard extracts have exceeded the maximum levels of erucic acid specified by the aforementioned regulations.

Sample C obtained the highest content of erucic acid per g sample (88.9 mg) when compared to both M (6.4 mg) and H (4.2 mg) that below the maximum levels of erucic acid. Therefore, the permitted amount consumption of C was significantly lower (5.6 g) than M (78.1 g) and H (119.0 g). Overall, a person should consume not more than 11 sachets (44.8 mg of erucic acid per sachet) of M or 17 sachets (29.4 mg of erucic acid per sachet) of H mustard sauce daily as per Food Standards Australia New Zealand (2003).

Table 4.3: Positional distribution of fatty acids in selected mustard products obtained by quantitative ^{13}C NMR

Sample	Acyl chain	Composition (mol%)[a]		
		***sn*-1,3**	***sn*-2**	**Total**
H	SFA	10.6 ± 0.3	nd	7.3 ± 0.3
	cis-13 MUFA	46.9 ± 0.9	nd	32.2 ± 0.0
	cis-11 MUFA	17.3 ± 0.0	nd	11.9 ± 0.3
	cis-9 MUFA	17.9 ± 0.1	49.7 ± 0.6	27.8 ± 0.1
	PUFA	7.4 ± 0.4	50.3 ± 0.6	20.8 ± 0.4
M	SFA	10.9 ± 0.7	nd	7.4 ± 0.5
	cis-13 MUFA	48.6 ± 0.2	nd	33.1 ± 0.1
	cis-11 MUFA	16.8 ± 0.2	nd	11.4 ± 0.1
	cis-9 MUFA	18.0 ± 0.2	47.6 ± 0.6	27.4 ± 0.4
	PUFA	5.7 ± 0.1	52.5 ± 0.6	20.6 ± 0.6
C	SFA	9.8 ± 0.0	nd	6.7 ± 0.0
	cis-13 MUFA	37.0 ± 0.7	nd	25.5 ± 0.5
	cis-11 MUFA	17.1 ± 0.2	nd	11.8 ± 0.1
	cis-9 MUFA	22.1 ± 0.1	37.2 ± 0.6	26.8 ± 0.3
	PUFA	13.9 ± 0.5	62.8 ± 0.6	29.1 ± 0.6

Notes: FAC, fatty acid composition; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunSFAs; nd, not detected.

[a] ^{13}C NMR results are mean of three replicates ± standard deviation.

Table 4.4: The absolute amount and content of erucic acid in the mustard oil and mustard products obtained by quantitative ^{13}C NMR

Samples	Erucic acid content (mol%) [a]	Erucic acid content (mass%) [b]	Absolute amount of erucic acid by weight (%)[c]	Content of erucic acid (mg / g sample)
Mustard oil	18.50	21.41	21.41	214.10
H	32.20	35.94	0.42	4.20
M	33.10	36.97	0.64	6.40
C	25.50	28.85	8.89	88.90

[a] Quantitative ^{13}C NMR regiospecific analysis results (Table 4.1 and Table 4.3).

[b] $\frac{Mass\ of\ erucic\ acid}{Average\ mass\ of\ acids} \times 100$

[c] Extraction yield of mustard products (H: 1.18%; M:1.73%; C:30.89%)

In summary, qCNMR can be applied to monitor and quantify erucic acid content in a broad range of edible oils and mustard products. A quick and direct method has been established without any laborious chemical derivatization before the analysis. As a result of low detection limit (0.98%), erucic acid level below the permitted level (European Union: 5%, United States: 2%) can also be determined by the optimised method. The present findings also indicate that mustard oil and mustard products represent a relevant source for human intake of erucic acid. More attention should be paid to the tolerable intake of erucic acid because over consumption of erucic acid has no nutritional effect.

As discussed in Chapter Two, ethyl behenate with the lowest mean acid value (1.42±0.11 mg KOH / g sample) can be attained under optimised parameters at a molar ratio of 1:8, 0.75% (m/m) of concentrated sulphuric acid and with the attachment of Dean-Stark for 9 hours. In Chapter Three, the ethyl behenate obtained previously has been used as a starting material react with high-oleic sunflower oil through lipase catalysed interesterification reactions. SLs containing high amount of BOO and BOB have successfully synthesised and postulated to have desirable rheological properties for bakery, confectionery or as a base for *trans*-fat-free products with the aim of reducing obesogenic effects. While as presented in Chapter Four, their positional fatty acids profiles can be analysed by quick and direct quantitative NMR method. Same quantitative NMR method can be applied to monitor and quantify erucic acid content in a broad range of edible oils and mustard products as well. Thus, the positional fatty acids in natural dietary oils and structured lipids have been successfully studied using non-conventional method, namely, quantitative NMR method which serves as a quick and non-destructive analytical method for the profile of positional fatty acids.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Refereed Journal Paper

Kok, W. M., Mainal, A., Chuah, C. H., & Cheng, S. F. (2017). Content of erucic acid in edible oils and mustard by quantitative ^{13}C NMR. *European Journal of Lipid Science and Technology*, DOI: 10.1002/ejlt.201700230.

Cheng, S. F., Kok, W. M., & Chuah, C. H. (2017). Enzymatic synthesis of structured lipids with behenic acid at the *sn*-1,3 positions of triacylglycerols. *Food Science and Biotechnology*, DOI: 10.1007/s10068-017-0271-3.

Proceedings

Kok, W. M., Cheng, S. F., & Chuah, C. H. (2014). Synthesis of long chain saturated structured lipids *via* enzymatic interesterification using *Rhizomucor miehei* (Lipozyme RM IM). Poster presented at 5th UM-NUS-CU Trilateral Mini Symposium and Scientific Meeting 2014, 11 February 2014 to 12 February 2014, Kuala Lumpur, Malaysia.

Kok, W. M., Cheng, S. F., & Chuah, C. H. (2015). Long chain saturated structured lipids: synthesis and characterisation. Poster presented at 13th Euro Fed Lipid Congress: Fats, Oils and Lipids, 27 September 2015 to 30 September 2015, Florence, Italy.

Kok, W. M., Cheng, S. F., & Chuah, C. H. (2016). Long chain saturated structured lipids: synthesis and characterisation. Poster presented at Oils and Fats International Congress 2016, 19 October 2016 to 21 October 2016, Kuala Lumpur, Malaysia. (Awarded 3rd Prize)