DEVELOPMENT OF A LIQUID PHASE MICROEXTRACTION TECHNIQUE FOR THE GAS CHROMATOGRAPHIC DETERMINATION OF FATTY ACIDS IN VEGETABLE OILS

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by

GAN HUI SIANG

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LIST OF ABBREVIATIONS

Alpha-Linolenic Acid	**ALA**
Capillary Electrophoresis	**CE**
Capillary Gas Chromatography	**GC**
Cerebro Vascular Disease	**CVD**
Cloud Point Extraction	**CPE**
Capillary Zone Electrophoresis	**CZE**
Dispersive Liquid-liquid Microextraction	**DLLME**
Enrichment Factor	**EF**
Evaporative Light Scattering Detectors	**ELSD**
Electrokinetic Membrane Extraction	**EME**
Fatty Acid	**FA**
Fatty Acid Methyl Ester	**FAME**
Flame Ionization Detector	**FID**
Fluorescence Detector	**FLD**
Free Fatty Acid	**FFA**
Gel Permeation Chromatography	**GPC**
^{1}H-Nuclear-Magnetic Resonance	**^{1}H-NMR**
High Density Lipoprotein	**HDL**
High Performance Liquid Chromatography	**HPLC**
Hollow Fiber- Liquid Phase Microextraction	**HF-LPME**
Homogeneous Liquid–liquid Extraction	**HLLE**
Linoleic Acid	**LA**
Liquid-liquid Extraction	**LLE**

Liquid Phase Microextraction	**LPME**
Long Chain PUFA	**LCPUFA**
Low Density Lipoprotein	**LDL**
Malaysian Palm Oil Board	**MPOB**
Mass Spectroscopy	**MS**
Matrix Solid Phase Extraction	**MSPD**
Monounsaturated Fatty Acids	**MUFA**
Polydimethylsiloxane	**PDMS**
Polyunsaturated Fatty Acids	**PUFA**
Soxhlet Extraction	**SE**
Stir Bar Sorptive Extraction	**SBSE**
Saturated Fatty Acids	**SFA**
Supercritical Fluid Chromatography	**SFC**
Single Drop Microextraction	**SDME**
Support Liquid Membrane	**SLM**
Solid Phase Extraction	**SPE**
Solid Phase Microextraction	**SPME**
Thin Layer Chromatography	**TLC**
World Health Organization	**WHO**

PEMBANGUNAN KAEDAH PENGEKSTRAKAN MIKRO FASA CECAIR UNTUK PENENTUAN KROMATOGRAFI GAS ASID LEMAK DALAM MINYAK SAYURAN

ABSTRAK

Tesis ini difokuskan kepada pembangunan pengekstrakan mikro fasa cecair gentian berongga 2-fasa menggunakan kromatografi gas dengan alat pengesan pengionan nyala untuk penentuan profil asid lemak (laurik, miristik, palmitik, stearik, palmitoleik, oleik, linoleik, linolenik dan arahidik) dalam minyak sayuran. Ester metil asid heptadekanoik diguna sebagai piawaian dalaman. Asid lemak ditransesterifikasi melalui kaedah dua langkah menjadi ester metil masing-masing untuk diekstrak kemudian. Dalam keadaan yang optimum (pelarut pengekstrakan, n-tridekana; masa pengekstrakan, 35 minit; kadar pengacauan, 1700 rpm; suhu, suhu bilik; tanpa penambahan garam). Analit yang diekstrak daripada 10 mL fasa penderma akan melalui 5 μL pelarut organik yang terserap pada liang-liang rongga gentian ke dalam fasa penerima yang terdapat di dalam rongga gentian polipropilene tersebut. Faktor perkayaan 37-115 telah diperolehi. Graf kalibrasi untuk sembilan asid lemak adalah linear di dalam julat 10-5000 μg L^{-1} dengan $r^2 > 0.994$ dan had pengesanan terendah (LOD) (isyarat: kebisingan 3:1) masing-masing ialah 4.73-13.21 ng L^{-1}. Keputusan ini menunjukkan pengekstrakan mikro fasa cecair gentian berongga 2-fasa merupakan alternatif teknik penyediaan sampel yang canggih berbanding dengan teknik penyediaan sampel yang sedia ada terhadap asid lemak. Teknik ini telah berjaya digunakan dalam penentuan FA dalam minyak kelapa sawit (buah, isirung, cair dan minyak masak carotino) dan minyak sayuran lain (kacang

soya, zaitun, kelapa, dedak padi dan labu). Faktor perkayaan yang didapati amat memuaskan dan membuka minat terhadap profil kandungan asid lemak yang minor dalam minyak kelapa sawit dan minyak sayuran yang lain.

DEVELOPMENT OF A LIQUID PHASE MICROEXTRACTION TECHNIQUE FOR THE GAS CHROMATOGRAPHIC DETERMINATION OF FATTY ACIDS IN VEGETABLE OILS

ABSTRACT

This thesis is focused on the development of a two phase hollow fiber liquid-phase microextraction technique, followed by gas-chromatography-flame ionization detection (GC-FID) for the profiling of the fatty acids (FAs) (lauric, myristic, palmitic, stearic, palmitoleic, oleic, linoleic, linolenic and arachidic) in vegetable oils. Heptadecanoic acid methyl ester was used as the internal standard. The FAs were transesterified by a two step method to their corresponding methyl esters prior to the extraction. Extraction parameters such as type of extracting solvent, temperature, extraction time, stirring speed and salt addition were studied and optimized. Recommended conditions were: extraction solvent, n-tridecane; extraction time, 35 min; stirring rate, 1700 rpm; extraction temperature, ambient; without addition of salt. The analytes were extracted from 10 mL donor phase through 5 μL of an organic solvent immobilized in the pores of a porous polypropylene hollow fiber and then into the acceptor phase present inside the hollow fiber. Enrichment factors varying from 37 to 115 were achieved. Calibration curves for the nine FAs were well correlated ($r^2 > 0.994$) within the range of 10–5000 μg L^{-1}. The limit of detection (signal: noise, 3) was 4.73 – 13.21 ng L^{-1}. The results presented show that the hollow fiber liquid-phase microextraction techniques can serve as excellent alternative methods to conventional sample preparation techniques or other microextraction in the analysis of FAs. The method was successfully applied to the profiling of the FAs

in palm oils (crude, olein, kernel, carotino cooking oil) and other vegetable oils (soybean, olive, coconut, rice bran and pumpkin). The encouraging enrichments achieved offer an interesting option for the profiling of the minor and major FAs in palm and other vegetable oils.

CHAPTER 1

INTRODUCTION

1.1 Fatty acids

Fatty acids (FAs) are the basic components of most naturally occurring lipids that are found in animals and plants. FAs contain hydrocarbon chains that are bound to a carboxyl functional group at one end and a methyl group at the other end (Figure 1.1).

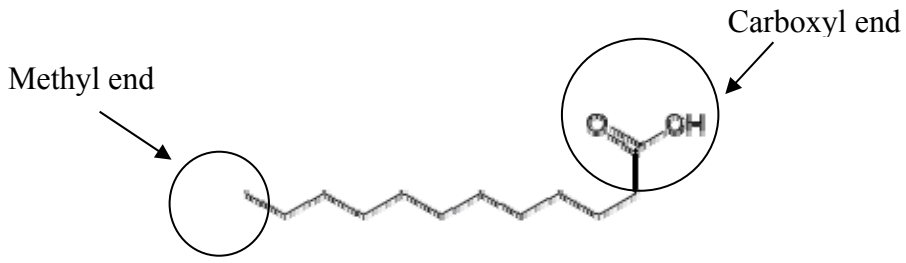


Figure 1.1: The structure of FA

Naturally by occuring FAs commonly have a long hydrocarbon chain (usually unbranched and even numbered), which may be saturated or unsaturated. Saturated fatty acids (SFA) consist of hydrocarbons attached together by single bonds (Figure 1.2)

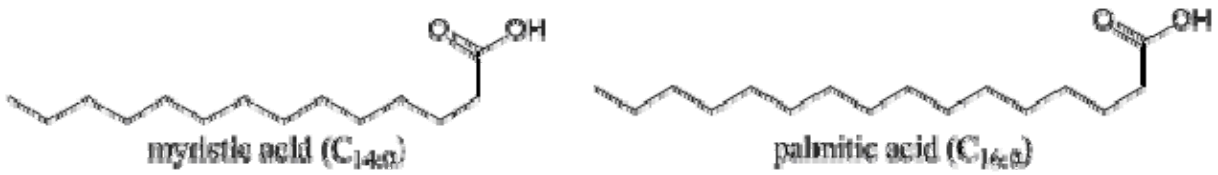


Figure 1.2: Structure of some SFA

The melting point of SFA increase with chain length. The individual SFA has various physical properties as shown in Table 1.1.

Table 1.1: SFA in different oils [1]

Systematic Name	Common Name	No. of Carbon Atoms	Melting Point °C	Typical Fat Source
Ethanoic	Acetic	2	-	-
Butanoic	Butyric	4	-7.9	Butterfat
Hexanoic	Caproic	6	-3.4	Butterfat
Octanoic	Caprylic	8	16.7	Coconut oil
Decanoic	Capric	10	31.6	Coconut oil
Dodecanoic	Lauric	12	44.2	Coconut oil
Tetradecanoic	Myristic	14	54.4	Butterfat, Coconut oil
Hexadecanoic	Palmitic	16	62.9	Most fats and oils
Heptadecanoic	Margaric	17	60.0	Animal fats
Octadecanoic	Stearic	18	69.6	Most fats and oils
Eicosanoic	Arachidic	20	75.4	Peanut oil
Docosanoic	Behenic	22	80.0	Peanut oil

Monounsaturated fatty acids (MUFA) have one double bond (Figure 1.3), and polyunsaturated fatty acids (PUFA) contain more than one double bonds in the chain (Figure 1.4) [2].

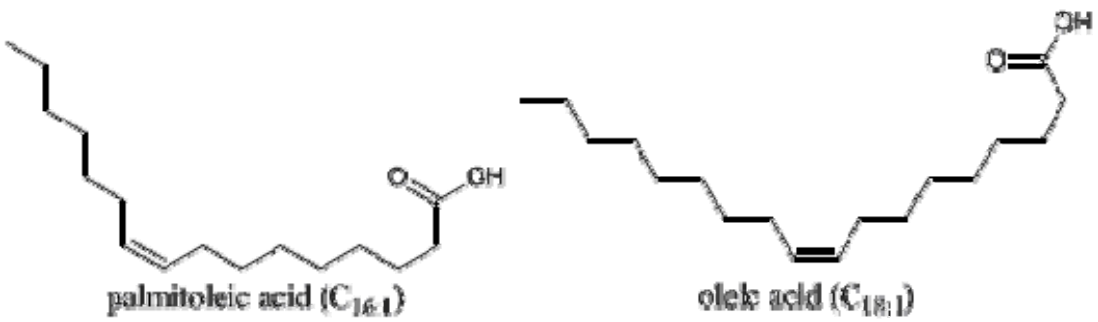


Figure 1.3: Structure of some MUFA

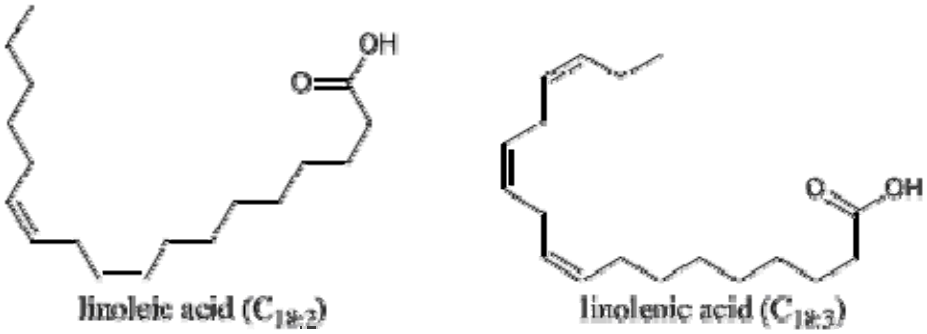


Figure 1.4 Structure of some PUFA

Because of the presence of double bonds, unsaturated fatty acids are more reactive chemically than the saturated fatty acids. The physical properties of some MUFA and PUFA as shown in Table 1.2.

Table 1.2: MUFA and PUFA in different oils [1]

Systematic Name	Common Name	No. of Double Bonds	No. of Carbon Atoms	Melting Point °C	Typical Fat Source
9-Decenoic	Caproleic	1	10	-	Butterfat
9-Dodecenoic	Lauroleic	1	12	-	Butterfat
9-Tetradecenoic	Myristoleic	1	14	18.5	Butterfat
9-Hexadecenoic	Palmitoleic	1	16	-	Some fish oils, beef fat
9-Octadecenoic	Oleic	1	18	16.3	Most fats and oils
9-Octadecenoic	Elaidic	1	18	43.7	Partially hydrogenated oils
11-Octadecenoic	Vaccenic	1	18	44	Butterfat
9,12-Octadecadienoic	Linoleic	2	18	-6.5	Most vegetable oils
9,12,15-Octadecatrienoic	Linolenic	3	18	-12.8	Soybean oil, canola oil
9-Eicosenoic	Gadoleic	4	20	-	Some fish oils
5,8,11,14-Eicosatetraenoic	Arachidonic	4	20	-49.5	Lard
5,8,11,14,17-Eicosapentaenoic	-	5	20	-	Some fish oils
13-Docosenoic	Erucic	1	22	33.4	Rapeseed oil
4,7,10,13,16,19-Docosahexaenoic acid	-	6	22	-	Some fish oils

Fatty acids are also characterized by the size/chain length. Generally, short and medium chain fatty acids have less than 8 and 16 carbons in their backbone, respectively, and comprise of mainly SFA. Long chain fatty acids have more than 16 fatty acids in their backbone, which generally include the bulk of the MUFA and all the PUFA [2]. The term long chain PUFA commonly includes PUFA with 20 or more carbon atoms in their backbone [3]. The diversity of the chain length, degree of unsaturation, geometry and position of double bonds of these FAs determine the characteristic of these lipids and their origins [4]. In this study, ten FAs that was chosen were lauric acid ($C_{12:0}$), myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), arachidic acid ($C_{20:0}$), palmitoleic acid ($C_{16:1}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$) linolenic acid ($C_{18:3}$) and heptadecanoic acid methyl ester an internal standard (IS). The structures of these FAs are shown in Figure 1.5

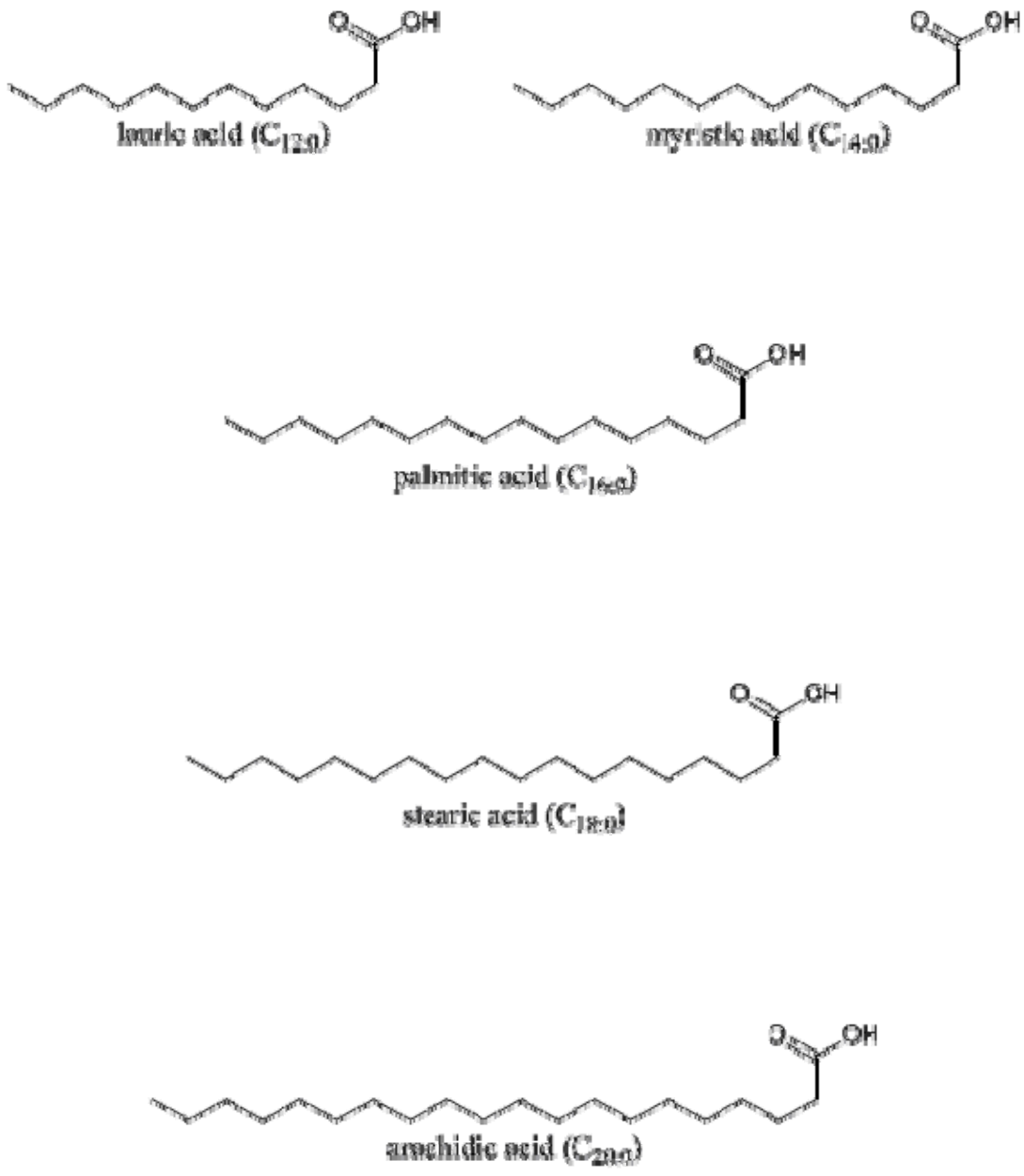
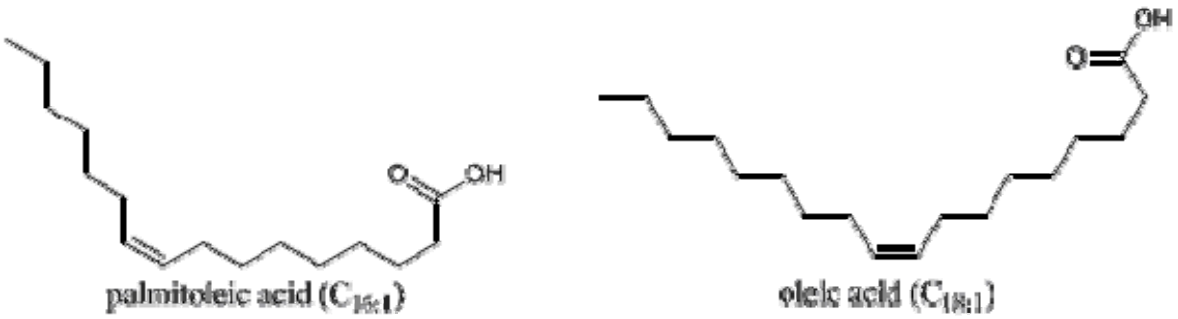
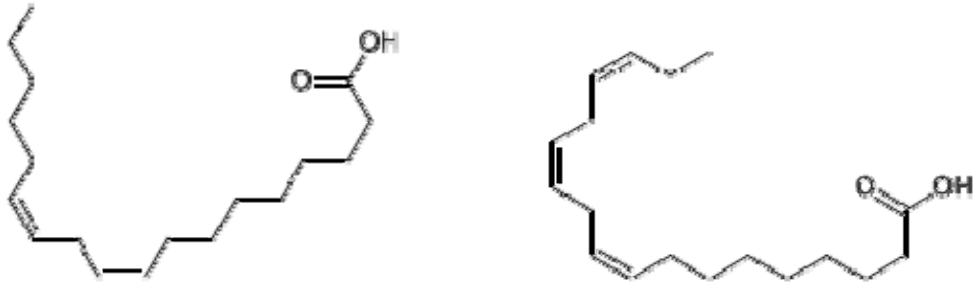


Figure 1.5 Chemical structures of the FAs studied



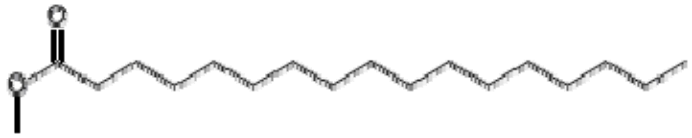
palmitoleic acid ($C_{16:1}$)

oleic acid ($C_{18:1}$)



linoleic acid ($C_{18:2}$)

linolenic acid ($C_{18:3}$)



methyl heptadecanoate (IS)

.

Figure 1.5 Chemical structures of the FAs studied (continue)

1.1.1 Fatty acid nomenclature

Fatty acids can be named in many ways. They are often referred to by their trivial names, but are more commonly identified by their systematic nomenclature according to the International Union of Pure and Applied Chemistry (IUPAC). According to IUPAC recommendations, fatty acids are named by numbering the first, and further the next double bonds, counted from the carboxyl end. However, in nutritional and biological context the double bonds may be more commonly designated from the methyl end. It may be an easier way of numbering unsaturated fatty acids by only defining the location of the first double bond, since they are generally separated by methylene groups (CH_2). The first double bond is given by the term n minus (n-), or omega minus (ω-). Both numeric systems initiate the numeric names by numbering the total carbon atoms next to the number of double bonds. Thus, linoleic acid is referred to as 18:2n-6 (18:2 ω-6) by n-(ω-) system (Figure 1.6).

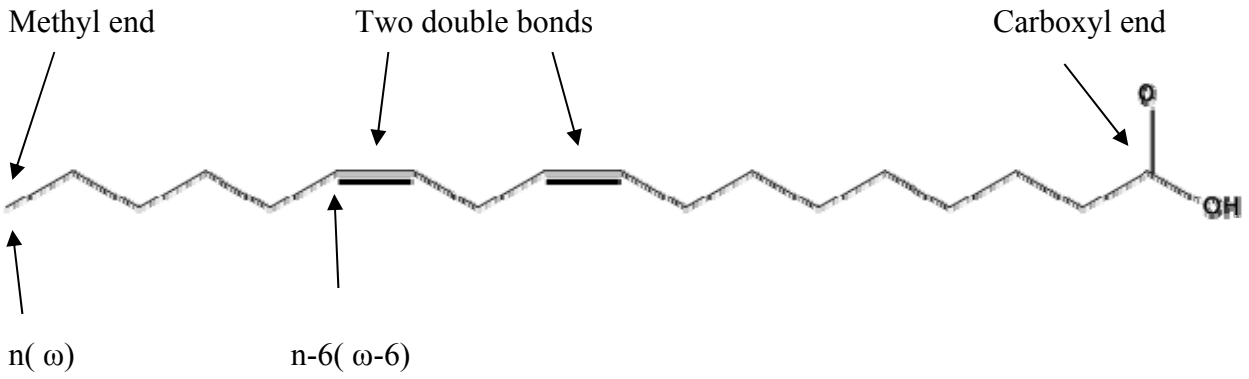


Figure1.6: The structure and the n-(ω-) system nomenclature of linoleic acid. The first double bond is designated from the methyl end [5].

Some common fatty acids are listed in Table 1.3. SFA, such as palmitic acid (16:0), are written only with total carbons relative to zero, since they do not contain any double bonds. Typically, the MUFA have a double bond located at the n-7 or n-9 position, whereas PUFA containing 18 or more carbons in their backbone are restricted to having their first double bond only at n-3, n-6 or n-9 position [2].

Table 1.3: Fatty acid nomenclature [3]

Trivial name	**Abbreviation**	**Numeric name** n- system
Palmitic acid	PA	16:.0
Stearic acid	SA	18:.0
Oleic acid	OA	18:1n-9
Linoleic acid	LA	18:2n-6
ω-Linolenic acid	ALA	18:3n-3
Dihomo ω-linolenic acid	DGLA	20:3n-6
Arachidonic acid	AA	20:4n-6
Eicosapentaenoic acid	EPA	20:5n-3
Docosahexaenoic acid	DHA	22:6n-3

1.1.2 Essential FAs

Essential FAs are important to humans. Vegetable oils are the main sources of essential FAs. The term ‘essential’ implies that they must be supplied in the diet because they are required by the human body and cannot be endogenously synthesized. The human body can produce all but two of the FAs it needs, i.e., linoleic acid (LA) and alpha-linolenic acid (ALA). The essential FAs are widely distributed in plant and animal oils (e.g., fish oils). Although the body to some extent can convert ALA into these longer-chain omega-3 fatty acids, the omega-3 fatty acids found in marine oils help to fulfill the requirement of essential FA (and have been shown to have wholesome properties of their own). Since they cannot be synthesized in the body from other substrates, they must therefore be supplied in food.

Mammals lack the ability to introduce double bonds in fatty acids beyond carbons 9 and 10. Hence LA acid and ALA acid are essential fatty acids for humans. Essential FAs is important to help human in raising the High Density Lipoprotein (HDL), the so-called good cholesterol. Besides that, human body needs essential FAs to manufacture and repair cell membranes, enabling the cells to obtain optimum nutrition and expel harmful waste products. A primary function of essential FAs is the production of prostaglandins, which regulate important body functions such as heart rate, blood pressure, blood clotting, fertility, conception, and play a role in immune function by regulating inflammation and encouraging the body to fight inflection. Essential FAs also are important in supporting the cardiovascular, reproductive, immune and nervous systems.

1.2 Vegetable oil

Vegetable fats and oils are lipid materials that are derived from plants. It is mainly constituted by triacylglycerol (95 - 98%) and complex mixtures of minor compounds (2- 5%) of a wide range of chemical nature. A triglyceride is formed from one molecule of glycerol and three fatty acids (Figure 1.7).

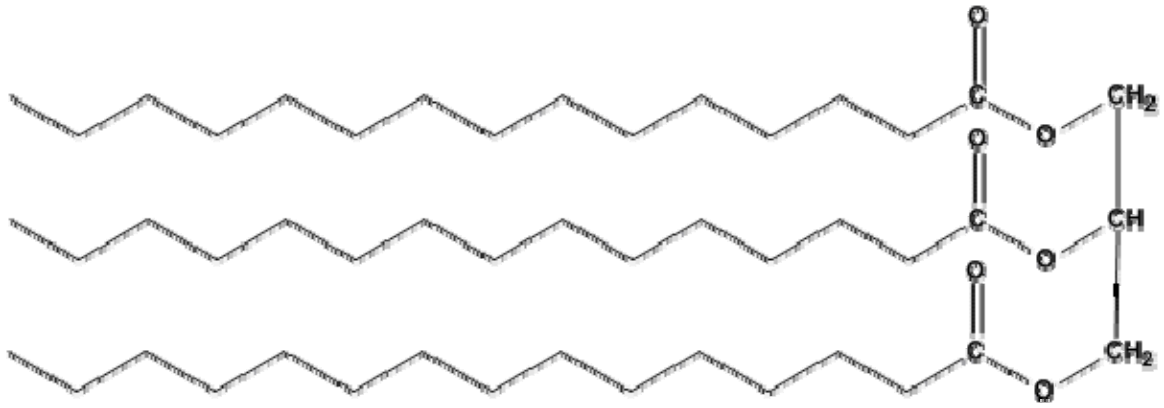


Figure 1.7 Structure of triglyceride

The minor components include mono- and diglycerides, free fatty acids, phosphatides (or phospholipids), sterols, protein fragments, various resinous and mucilaginous materials and oxidative products. FAs occurring in vegetable oils are classified according to their degree of saturation. This FA chains may contain one or more double bonds at specific positions (unsaturated and polyunsaturated), or they may be fully saturated. The physical and chemical properties of a fat depends on the composition of the fatty acid mixture. Physically, oils are liquid at room temperature and fats are solid. This is due to the fact that oil sources contain a higher proportion of unsaturated acids and are often liquids at room temperature due to hydrogen

bonding. The following triglyceride vegetable oils account for almost all worldwide production (by volume) (Table 1.4).

Table 1.4: Total world consumption of major vegetable oils in 2007/2008 [6]

Oil source	World consumption (million tons)	Notes
Palm	41.31	The most widely produced tropical oil. Also used to make biofuel.
Soybean	37.54	Accounts for about half of worldwide edible oil production.
Rapeseed	18.24	One of the most widely used cooking oils, Canola is a (trade marked) variety (cultivar) of rapeseed.
Sunflower seed	9.91	A common cooking oil, also used to make biodiesel.
Peanut	4.82	Mild-flavored cooking oil.
Cottonseed	4.99	A major food oil, often used in industrial food processing.
Palm kernel	4.85	From the seed of the African palm tree
Coconut	3.48	Used in soaps and cooking
Olive	2.84	Used in cooking, cosmetics, soaps and as a fuel for traditional oil lamps

The properties of oils are very much dependent on the FAs profile, which provide information on chain length, percent SFA, MUFA and PUFA. Based on these information, the recommended usage of each oil can be proposed. Profile determination of unsaturated FAs is useful in health care management (e.g., towards the prevention of diseases [7-12]). Oils that's that are highly saturated (e.g. coconut and palm oil) are known as deep fry oils, as they resist oxidation and extreme heat (180 – 200 ºC). Unsaturated FAs can degrade easily to form toxic compounds when heated, leading to atherosclerosis, inflammatory joints and birth defects when consumed. Oleic

acid ($C_{18:1}$) is one of the most abundant monounsaturated FAs in oils; it was shown to be effective in reducing coronary heart diseases mainly via LDL-cholesterol reduction polyunsaturated FAs such as linoleic acid ($C_{18:2}$) or commonly known as omega-6 (ω−6) and linolenic acid ($C_{18:3}$) (commonly known as omega-3 (ω−3)) have long been recognised as essential FAs for normal growth and good health. As mentioned earlier, these FAs cannot be produced by the human organs and thus must be obtained from the daily diet. However, it is important to maintain the appropriate ratio of ω6/ω3 in the diet because an increase in this ratio will lead to rapid mortalities from cancers and allergies [11]. Consuming appropriate amounts of ω3 (1-2 g per day) can lead to beneficial effects such as superior cardiovascular condition, protection from heart attack and stroke. It was also reported that there is a strong link between linoleic acid intake and reduction in incidence of prostate, breast and colorectal cancers [13].

1.2.1 Palm oil

Palm oil has been used in food preparation for over 5,000 years and it is one of the 17 major oils and fats produced and traded in the world today [14]. Currently, most of the world's production of palm oil comes from South-East Asia, in particular Malaysia and Indonesia. Malaysia is the second largest producer of palm oil after Indonesia [15].

The approximate concentration of FA in palm oil, palm kernel oil and RBD palm olein is summarised in Table 1.5.

Table 1.5: Fatty acid constituents (%) of palm oil, palm kernel oil and RBD palm olein [16].

Fatty Acid	Chain Length	Palm Oil	Palm Kernel Oil	RBD Palm Olein
Caproic	$C_{6:0}$	-	0.3	-
Caprylic	$C_{8:0}$	-	4.3	-
Capric	$C_{10:0}$	-	3.7	-
Lauric	$C_{12:0}$	0.3	50.1	-
Myristic	$C_{14:0}$	1.2	15.4	0.89
Palmitic	$C_{16:0}$	44.3	7.3	41.54
Stearic	$C_{18:0}$	4.3	1.8	3.51
Oleic	$C_{18:1}$	39.3	14.5	43.63
Linoleic	$C_{18:2}$	10	2.4	10.43
Others	$C_{18:3}$, $C_{20:0}$ etc.	0.6	0.2	-

RBD: refined, bleached, deodorized

It is obvious from the table that the dominant FA in palm oil, palm kernel oil and palm olein is palmitic, lauric and oleic, respectively. For palm oil, the compositions of saturated and unsaturated fatty acids are almost the same. So palm oil is neither saturated nor unsaturated and interestingly this 50:50 composition occurrs naturally. Currently, biotechnological techniques are being explored towards producing palm oil with higher iodine number (IV) and higher monounsaturated FA [17]. Through gene modification, the saturated palmitate ($C_{16:0}$) can be

converted to monounsaturated oleate ($C_{18:1}$) [16]. Such attempts will open up new possibilities for the oil as useful industrial feedstock as well as in the liquid oil sector.

Palm oil is consumed in the fresh state and/or at various levels of oxidation. Feeding experiments in various animal species and humans have highlighted the beneficial role of fresh palm oil to health. These benefits include reduction in the risk of arterial thrombosis and atherosclerosis inhibition of cholesterol biosynthesis and platelet aggregation, and reduction in blood pressure [18]. However, on being used in the oxidized state possesses potential dangers to the physiological and biochemical functions of the body. Oxidized palm oil induces an adverse effect on plasma lipid profile, free fatty acids, phospholipids and cerebrosides. Additionally, oxidized palm oil induces reproductive toxicity and organ toxicity particularly of the kidneys, lungs, liver and heart [17].

Available evidence suggests that at least part of the oxidized oil impact on health is due to generation of toxicants due to oxidation. The reduction of the dietary level of oxidized oil and/ or the level of oxidation may reduce the health risk [18]. A study by a group of researchers in China comparing palm, soybean, peanut oils and lard showed that palm oil actually increased the levels of good cholesterol and reduced the levels of bad cholesterol in the blood [19]. This group also found that in normal and hypercholesterolemic subjects, the use of palm oil in the diet should be safe and will not increase the risk of cerebro vascular disease (CVD). Toxicological and pharmacological studies show that supplementation with palm tocotrienols up to 2,500 milligrams per day per kilogram of body weight does not produce any significant side effects.

1.3 FAME preparation

Vegetable oil consist of triglyceride molecule that comprises one molecule of glycerol and three ester bonds that linked the FAs molecule [20]. In general, FAME is used as a volatile derivative for the determination of FA. The preparations of which FAMEs are explained in the following sections are performed by transesterification and two-step method (saponification and esterification).

1.3.1 Transesterification

For lipids, fats and oils, often a transesterification procedure involving the direct conversion of FAs to alkyl esters (particularly methyl esters) by alcohol in the presence of a catalyst is often carried out (Figure 1.8).

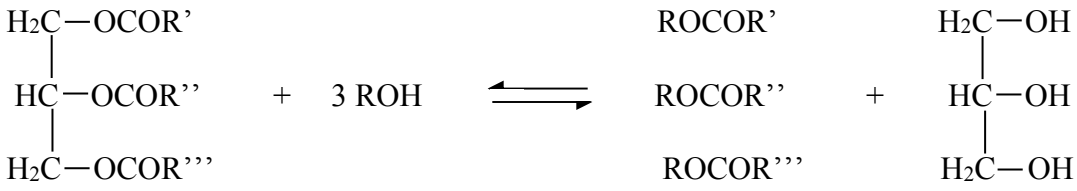


Figure 1.8 General equation for a transesterification reaction.

Transesterification between triglyceride and alcohol is commonly known as alcoholysis and if methanol is used, then it is known as methanolysis. Methanolysis of oil, together with a suitable catalyst, produces FAME and glycerol. At the end of the reaction, the latter settles down as

bottom layer. It is important to note that the main purpose of transesterification is to lower the viscosity and to increase the volatility of the oil.

The conversion of triglyceride to simple esters reduces the molecular weight of oil to one-third of its original value. Together with that, it also reduces the viscosity of oil by a factor of about 8, thus increases the volatility [21].

The overall transesterification process is normally a sequence of three consecutive steps, which are reversible reactions as shown in Figure 1.9. From triglycerides, the first step is the formation of diglycerides, followed by the conversion of diglycerides to monoglycerides and finally from the monoglycerides, glycerol is obtained.

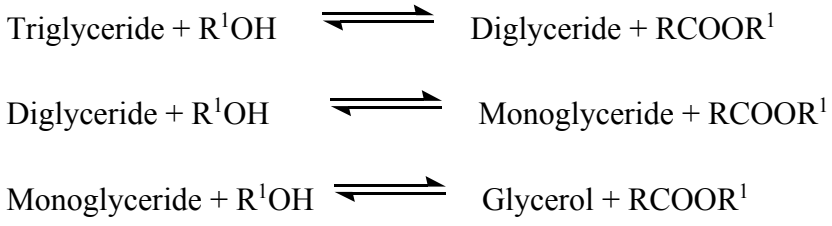


Figure 1.9 Step-wise transesterification reaction of triglycerides [21]

In all the three reactions, one methyl ester molecule is produced at each step. Since the reaction is reversible, an excess of alcohol is usually more appropriate to accelerate the forward reaction, although stoichiometric relation between oil and alcohol is 1:3. In industries, excess alcohol has always been used to accelerate the forward reaction. One of the most important variables affecting methyl esters yield are the molar ratio of alcohol to oil and the reaction temperature [22]. A catalyst is usually used to improve the reaction rate and yield. The common catalysis are

alkaline (NaOH, KOH, $NaOCH_3$, Na_2CO_3 and K_2CO_3) or acidic catalyst (HCl, H_2SO_4, BF_3, H_3PO_4). However, the transesterification reaction using acid catalyst are very slow and requiring long time. By using the base catalysts, the reaction is faster than the acid catalyzed reaction. Furthermore, the base catalyst are less corrosive than acid catalyst. The problem of using base-catalyst transesterification with triglyceride is the formation of soap. The soap prevent the separation of the glycerine and FAME fraction. The reaction also will affects the neutralization of the base catalyst that are no longer available to catalyze the transesterification and give less FAME product. The two-step method was applied in the vegetable oils to resolve this problem.

1.3.2 Two-step method of FAME preparation

FAMEs can be prepared by the two-step method which consist of acid-base and base-acid method. The main purpose we used the two-step method to preparation of FAME is to solve the problems of using acid and base catalyst. This two-step method involved saponification and esterification which can be employed for all the vegetable oils.

Saponificaton reaction was the process to converted triglyceride become soap. After that the soap will convert become FAME by esterification. The general mechanism involves two steps shown in Figure 1.10. First, acids of lipids are esterified by sodium hydroxide, creating glycerol and the salt of the fatty acids. This liberates the fatty acids and is called saponification. Second, transesterification occurs when methanol reacts with the crude soap producing fatty acid methyl esters through esterification [23, 24].

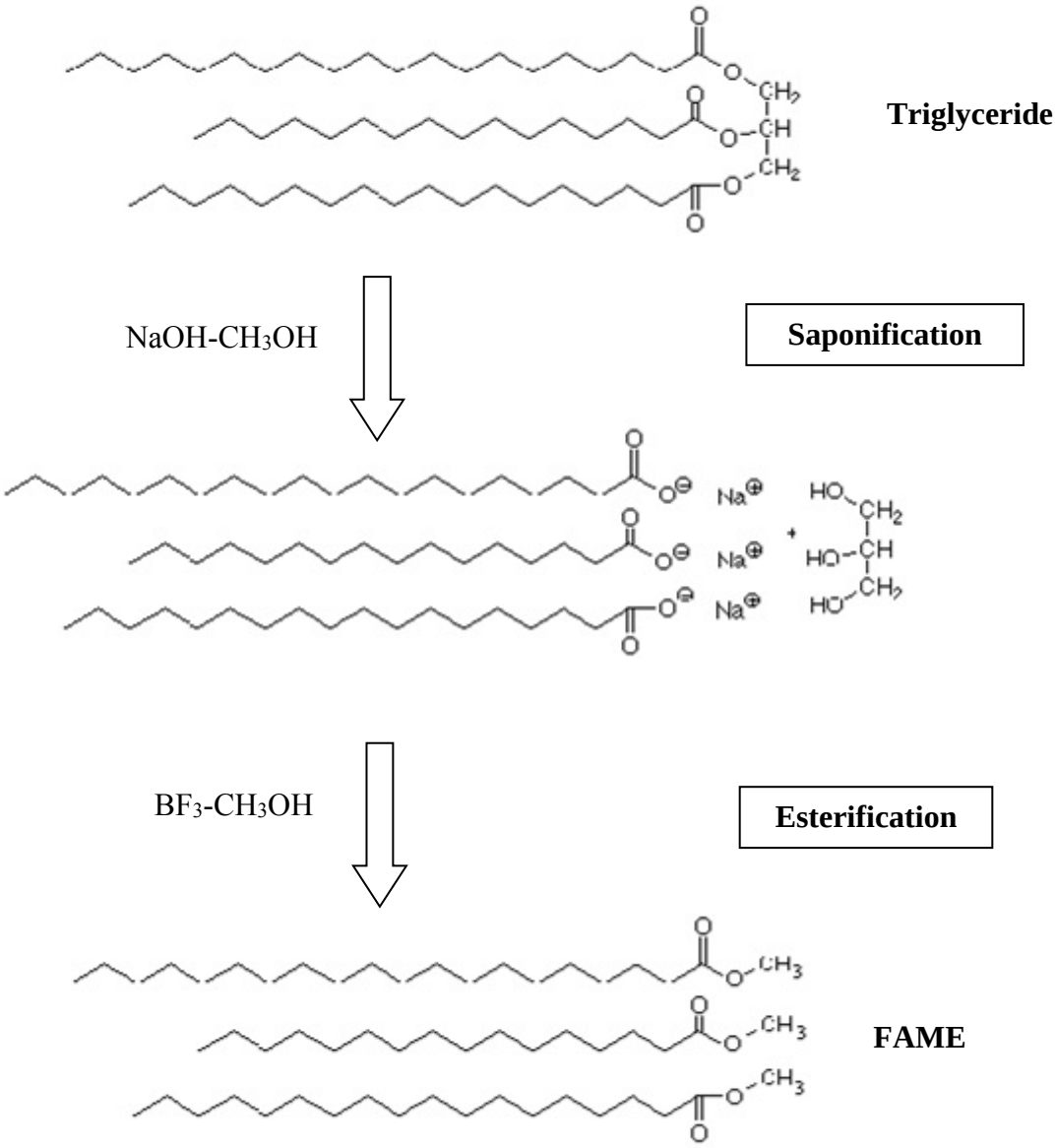


Figure 1.10: Methylation and saponification of triacylglycerides under specific conditions [25].

The method currently use at the American Oil Chemists Society (AOCS) and the Malaysian Palm Oil Board (MPOB) to transesterification vegetable oil become FAME base on this principle according to this method, strong base NaOH under boiling state and in presence of the strong catalyst BF_3 in CH_3OH was need to convert the triglyceride in vegetable oil to FAME. Esterified fatty acids or FAME are more volatile and vaporise easier on the column, which is a criteria for quantification by gas liquid chromatography (GLC).

1.4 Analytical methods for the determination of FAs

Various analytical methods have been developed for analyzing mixtures of FAMEs from the transesterification reaction.

1.4.1 Gas chromatography (GC)

Generally, GC has been the most widely used method for the analysis of FAMEs because of its good resolution, sensitivity and precision [26]. Flame ionization detection (FID) is the most common GC detector for analyzing hydrocarbon compounds. FID has good sensitivity and has a wide linear response [27, 28].

GC-MS (mass spectroscopy) is another alternative method to GC-FID for the analysis FAME due to the selective and sensitive MS detection. It can be used for the analysis of geometric and positional isomers of FAs from the diagnostic fragmentation of saturated and unsaturated FAs [29, 30].

The GC analysis of FAs (C2-C8 and C16-C18) [31] in olive mill waters gave a detection limit of 0.9-5 mg L^{-1}. The analysis of FAs (C6-C18) [32] in cider with gave a limit of 0.7-3.6 mg L^{-1} by using GC-FID compare with determination FAs (C8-C22) [33] in waste water with the detection limit 8-16 ng L^{-1} by using GC-MS have been reported. The comparative study of GC-FID and GC-MS methods to determination of FA has been report in sunflower oil [34], oil from the mophane caterpillar [35], and margarine [36]. The GC-MS has higher sensitivity compared to other common GC detectors.

However, GCMS is more expansive compared to GC-FID. Thus, it is interesting to develop new GC-FID techniques for the determination of FA.

1.4.2 High performance liquid chromatography (HPLC)

HPLC is also a suitable technique for the analysis of fats and oils because of its speed, sensitivity and reproducibility. The major advantage of HPLC over GC is the lower temperatures required and this will reduce the risk of isomerization of double bonds. Furthermore, fractions can be collected for further analysis. Apart from that, HPLC is considered more flexible as the retention characteristics can be easily modified by varying the composition of the mobile phase.

The common HPLC detectors used for detecting FA derivatives are the ultraviolet-visible spectrometer (UV-Vis) and fluorescence detector (FLD). Thus, derivatization procedures is required to “tag” the chromophore or fluorophore to the analyte, rendering them to be detected using UV-vis or FLD [37-40].

The derivatization procedure is important to increase the method sensitivity. However, the derivatization procedure takes time and sometimes incomplete or unstable reaction with the derivatization compound; or unselective labeling that leads to interfering by-product. Some derivatization reagents are expensive and unstable. There is thus, a strong emphasis lately on developing alternative methods that do not require derivatizations [37-40].

HPLC methods for the determination of underivatized FAs involving detectors such as mass spectrometer [41], chemiluminesce [42], electrochemical [4] and evaporative light scattering detectors (ELSD) [43] have been reported. Recently our group also report that determination of underivatized long chain fatty acids using capacitively coupled contactless conductivity detection [44].

1.4.3 Other analytical methods

Other analytical methods for the determination of FAs include supercritical fluid chromatography (SFC), capillary zone electrophoresis (CZE), gel permeation chromatography (GPC), ^{1}H-Nuclear-Magnetic Resonance (^{1}H-NMR) and thin layer chromatography (TLC). SFC is a hybrid between GC and HPLC, especially in terms of the ability to use both capillary GC and packed HPLC columns and FID and UV detectors. FAMES and FFA have been separated by SFC on capillary [45], and packed columns, in both reversed-phase [46] and normal-phase [47] modes. CZE has been used to obtain partial fatty acid profiles of butter and palm oil [48, 49]. It is particularly useful for the rapid determination of the short-chain FAs in such samples. GPC was used for the simultaneous determination of transesterification reaction products such as ethyl esters, mono-, di- and triglycerides and glycerol. The common detector of GPC for detecting

compounds is the refractive index detector [50, 51]. The determination of the yield of transesterification reaction or percentage of methyl esters can be quantified from ^{1}H-NMR spectrum. The yield was investigated from the signal of the methylene and methoxy protons [52]. Although TLC is still applied very extensively for preliminary separation and estimations of the FA present, the instrumental methods are generally used to obtain the quantitative profiles.

1.5 Sample preparation in chemical analysis

Sample preparation step is to isolate and concentrate analytes of interest from interfering sample components, and to convert the analytes to a form that is compatible with the instrument for the final analysis. This step is probably the most important due of three major reasons. Firstly, it involves the possible loss of target compounds and the unintentional introduction of contaminants. Secondly, there is the question of whether the preparation can provide clean sample for chromatographic analysis (selectivity). And finally, the sample preparation must be effective to pre concentrate the analytes which can be measured by the method chosen, i.e. high sensitivity. Good sample preparation methods should have the following features. They should

i. Consume low quantities of organic solvents, to reduce exposure to toxic compounds, and also produce less waste.
ii. Be easily operated and automated, and compatible with various instruments.
iii. Allow large sample throughput.
iv. Have high selectivity and be less affected by matrices.
v. Be economical and be time-efficient.

Generally, conventional liquid-liquid extraction (LLE) is used as sample preparation.

1.5.1 Liquid-liquid extraction (LLE)

LLE is the most common method in sample preparation. Prior to the analytical determination, the analytes need to be isolated from the sample. This is used in the official methods such as those published by the US Environmental Protection Agency (US EPA). LLE is also recommended by regulatory bodies, e.g., the American Oil Chemists Society (AOCS) and the Malaysian Palm Oil Board (MPOB). LLE is a separation process that takes advantage of the relative solubility of solutes in immiscible solvents. The solute dissolves more readily and becomes more concentrated in the solvent in which it has a higher solubility. A partial separation occurs when a number of solutes have different relative solubility in the two solvents used.

During the LLE procedure, the solution containing the analyte (A) and an immiscible solvent is manually or mechanically shaken and allowed to separate.

The advantages of LLE are the availability of pure solvents and the use of low-cost apparatus. However, major problems of the LLE technique are the gross consumption of organic solvents, lack of selectivity, time consuming, labour intensive, and the extra evaporation step required prior to analysis to remove the excess solvent. This can lead to contamination problems and possible loss of analytes [53-55].

1.5.2 Soxhlet Extraction (SE)

The SE created by Franz von Soxhlet in 1879. This is a popular sample preparation technique for the continous extraction of analytes from a solid sample. This technique has been used for the analysis of food such as for the extraction of lipid from wheat grains [56] and pharmaceutical sample [57]. This technique, although exhaustive, is not selective and further clean-up such as

solid phase extraction is necessary. However, SE is still widely found in laboratories and form a standard procedure for many solid-liquid extractions [58].

1.6 More recent sample preparation techniques

More recent techniques have been introduced and they are characterized by [59]:

- The ability to use smaller initial sample sizes.
- Greater selectivity in extraction.
- Potential for automation or for on-line methods, reducing manual operations, errors and time required.
- More environmentally friendly with less waste and the use of significantly small volumes or sometimes no organic solvents.

Driven by these purpose, advances in sample preparation have resulted in a number of techniques such as the solid phase extraction (SPE) and matrix solid phase extraction (MSPD). More recently, microextraction techniques such as the solid phase microextraction (SPME), stir bar sorptive extraction (SBSE) and liquid phase microextraction approaches have been used.