DEVELOPMENT OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR LONG CHAIN FATTY ACIDS DETERMINATION IN ENVRONMENTAL SAMPLE

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(APPLIED ANALYTICAL AND INORGANIC CHEMISTRY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2007

Thesis

Entitled

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was submitted to the Faculty of Graduate Studies, Mahidol University

For the degree of Master of Science

(Applied Analytical and Inorganic Chemistry)

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May 25, 2007

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DEVELOPMENT OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR LONG CHAIN FATTY ACIDS DETERMINATION IN ENVIRONMENTAL SAMPLES.

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ABSTRACT

Fatty acids (FAs) occur widely in natural fats and dietary oil. They are also important as nutrious substances and metabolites in living organisms. This work described the separation and determination of five FAs including lauric acid, myristic acid, palmitic acid, stearic acid and arachidic acid by HPLC with UV detection.

The absorbance of FA was enhanced by pre-column derivatization with phenacyl bromide and naphthacyl bromide to obtain highly chromophoric derivatives. The wavelength at the maximum absorbance of phenacyl ester and naphthacyl ester were 242 and 246 nm, respectively. Various parameters that affected derivatization such as reaction time, reaction temperature and amount of derivatizing agent were studied. The optimum condition of the HPLC system was 93:7 v/v MeOH:H_2O with flow a rate of 1.0 mL/min. Detection limit was in the range of 7 to 42 and 6 to 12 ppb and were obtained from phenacyl ester and naphthacyl ester, respectively. The detection of naphthacyl derivatives was shown to have a higher sensitivity than phenacyl ester. Thus, a naphthacyl derivative is an optimum chromophore for fatty acid analysis.

The method was applied to determine fatty acid profiles in soil and liquid bacterial culture. Liquid-Liquid extraction was employed for lipid removal. The separation of lipid classes were performed using solid phase extraction with a aminnopropyl (NH_2) cartridge. Phospholipid fatty acids (PLFAs) were hydrolysed with potassium hydroxide and the FFAs extracted with hexane. The optimum elution and loading were investigated. Phosphatidylcholine (PC) was spiked in soil and liquid bacterial culture for investigation of recoveries of FAs from PLFAs. This procedure gave a recovery of more than 79% (SD, 1.85 to 7.78 %).

KEY WORDS: FATTY ACIDS / PHENACYL BROMIDE / NAPHTHACYL BROMIDE / BACTERIA / SOIL

84 pp.

การพัฒนาเทคนิคไฮเปอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟี สำหรับหาปริมาณกรดไขมันสายยาวในตัวอย่างสิ่งแวดล้อม (DEVELOPMENT OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR LONG CHAIN FATTY ACIDS DETERMINATION IN ENVIRONMENTAL SAMPLES)

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บทคัดย่อ

กรดไขมันถูกพบมากในไขมันและน้ำมัน ทำหน้าที่สำคัญในการให้พลังงานและเกี่ยวกับการสันดาปในสิ่งมีชีวิต ในงานวิจัยนี้ทำการวิเคราะห์กรดไขมัน 5 ชนิดได้แก่ ลอริค เอซิด, มาย-ริสทิค เอซิด, พาลมิททิค เอซิด, สเตียริค เอซิด และเอราชิดิค เอซิด โดยวิธีตรวจวัดด้วยแสงอุตร้าไวโอเลต.

การเตรียมอนุพันธ์ของกรดไขมันด้วยฟีนาซิล โบรไมด และแนฟธาซิล โบรไมด ทำให้ได้อนุพันธ์ที่มีหมู่ที่ดูดกลืนแสงได้ดี ความยาวคลื่นที่ดูดกลืนแสงได้มากที่สุดของฟีนาซิลและแนฟธาซิล เอสเทอร์คือ 242 และ246 นาโนเมตร สภาวะในการแยกสารอนุพันธ์จะใช้วัฏภาคเคลื่อนที่เป็น 93:7 ของเมทธานอล:น้ำ อัตราการไหล 1.0 มิลลิลิตรต่อนาที อนุพันธ์แนฟธาซิล เอสเทอร์ดูดกลืนแสงได้ดีกว่าฟีนาซิล เอสเทอร์ ดังนั้นอนุพันธ์แนฟธาซิล เอสเทอร์จะถูกนำมาใช้ในการวิเคราะห์กรดไขมัน.

วิธีนี้ถูกนำมาประยุกต์ใช้ในการหาโปรไฟล์ของกรดไขมันในดินและแบคทีเรียที่เพาะเลี้ยง ไขมันจะถูกแยกออกมาด้วยการสกัดและถูกแยกออกเป็นแต่ละประเภทด้วยอะมิโนโพพิล คอลัมน์ ฟอสโฟลิปิด แฟทตี้ เอซิด จะถูกทำให้แตกเป็นกรดไขมันด้วยโพแทสเซียม ไฮดรอกไซด์และสกัดด้วยเฮกเซน นอกจากนั้นศึกษาปริมาตรที่ใช้ชะและความปริมาณที่สามารถรับได้สูงสุดของอะมิโน-โพพิล คอลัมน์ ฟอสฟาติดิลโคลีนถูกเติมลงไปในดินและแบคทีเรียที่เพาะเลี้ยงเพื่อหาร้อยละการได้กลับคืนของกรดไขมันจากฟอสโฟลิปิดพบว่าวิธีที่พัฒนาขึ้นให้ผลมากกว่า 79%

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LIST OF ABBREVATIONS

UV	Ultraviolet
Vis	Visible
MS	Mass Spectrometry
SPE	Solid Phase Extraction
AR	Analytical Reagent
AU	Absorbance Unit
MeOH	Methanol
^{o}C	Temperature in degree of Celsions
i.d..	Inner Diameter
SD	Standard Deviation
mM	Millimeter
µm	Micrometer
nm	Nanometer
µg	Microgram
v/v	Volume by volume
v/v/v	Volume by volume by volume
µg/mL	Microgram per milliliter
µL	Microliter
mL/min	Milliter per minute
min	Minute
Mw	Malecular weight
ppm	Part per million
ppb	Part per billion
M	Molar
g	gram
h	hour
et al.	Et Alli (Latin), and other

LIST OF ABBREVATIONS (CONTS.)

DL	Detection limit
ODS	Octadecylsilane
PBr	Phenacyl bromide
NAPHBr	Naphthacyl bromide
TEA	Triethylamine
viz	Videlicet
FA	Fatty acid
PC	Phosphatidylcholin

CHAPTER 1
INTRODUCTION

Fatty acid profile has been considered as bio-indicator pattern for identification and quantification in microbial samples; including biodiversity, soil quality etc. Fatty acid (FA) consists of carboxylic group containing long chain aliphatic which can be classified as saturated and unsaturated with different carbon numbers. These compounds can be found in various systems such as microorganism, plant tissue, animal tissue, and etc. In our interest, FAs were extracted from culture microorganism and soil to indicate the profile. FAs are part of cellular membrane of all living cells which are mainly consisted of phospholipid fatty acids (PLFAs). These compositions allow us to differentiate between various groups of microorganisms in the samples and also a specific group of microorganism. In addition, the analysis of FAs can be used to observe effect of contamination in soil by comparing between microbial biomass and contaminant loading. The contaminant in soils often decrease microbial activities and also biomass that relates directly with the PLFA profiles.

Analytical technique for determination of these compounds involves with extraction step, separation of lipid classes and following by hydrolysis for releasing FAs. The extraction of lipids and FAs from biological sample has been studied by many researchers. Folch *et al.,* [1957] or Bligh and Dyer [1959] employed a mixture of $CHCl_3$: MeOH (2:1v/v) to remove lipids from the sample. The extracted usually contains neutral lipids and PLFAs fraction, which will be separated into various classes by adsorption chromatographic technique. Mostly used techniques are based on normal-phase chromatography wherein the solutes are retained on the basis of there relative polarity. The stationary phase usually uses in the form of silica [Bitman *et al*., 1983], forisil [Shaikh, 1994], silicic [Sauncles and Horrocks, 1984] or aminopropyl [Kaluzny *et al*., 1985]. After loading the extracted into the column, mixtures of solvent with increasing the polarity were loaded into the column to elute lipids fraction off the bed in order to fractionate lipid classes and polar lipids were

eluted with $CHCl_3$, acetone and MeOH, respectively. The polar fraction containing PLFAs (MeOH fraction) were hydrolyzed by alkaline solution to release FA. Then, FAs can be analysed by various chromatographic techniques such gas chromatography (GC) and high performance liquid chromatography (HPLC). In GC technique, FAs are mostly determined in the forms of fatty acid methyl ester (FAMEs) usin g transesterification reaction with acid/basic catalysts in MeOH. However, FAs do not have chromophore for UV-Vis absorption. They need a derivatization technique for forming FA-derivatives that contain UV-Vis chromophore for HPLC detection or fluorescence. Derivatization reagents such as phenacyl bromide (PBr), naphthacyl bromide (NAPHBr), 2-nitrophenylhydrazine, 9-anthryldiazomethane, bromo methylmethoxy-coumarin are usually used for this propose. In addition, Tsuyama *et al.*, [1992] reported the underivatizaion for FAs analysis.

In this work, we proposed a procedure for the determination of FAs in PLFAs by HPLC using derivatization method for UV detection. PBr and NAPHBr were used as derivatizing agents. This method will apply for analysis lipids from soil and cultural bacteria sample. A mixture of $CHCl_3$: MeOH: buffer (1:2:0.8 v/v/v) was used for lipid removal from the matrix sample. Total lipids in chloroform were fractionated on aminopropyl (NH_2) columns into neutral lipids, free fatty acid (FFAs) and polar lipids with $CHCl_3$/2-propanol, 2% HOAc in ether and MeOH, respectively. Polar lipids fraction containing PLFAs were hydrolyzed with 1.0 M KOH/MeOH and then FFAs were derivatized prior to HPLC analysis using C18 column and 93% MeOH as mobile phase.

CHAPTER 2
OBJECTIVE

The aim of this thesis is to develop a simple liquid chromatographic procedure for determination of FAs; C12:0, C14:0, C16:0, C18:0 and C20:0 in cultured bacteria and soil samples. A high performance liquid chromatography method with UV detector will be developed. This work involves with the study of chromatographic separation and determination of these five FAs in the sample. The sensitivity of the analysis can be improved by using derivatizing agents (PBr and NAPHBr) in the optimum condition. Sample preparation is developed prior to HPLC analysis in order to fractionate PLFAs and eliminate matrix interference. The optimum condition of sample preparation and chromatographic system will be applied for studying FAs profile in the selected samples.

CHAPTER 3
LITERATURE REVIEWS

3.1 Introduction

There are several current procedures used for FA analysis in various sample matrixes. In this chapter, the determination of FAs are summarized. The scope of experimental are mainly focused on bound FAs, which are attached to PLFAs. The analytical method for sample preparation and separation of lipid classed are also reviewed.

3.2 Overview of FA

FA is a long chain carboxylic acid, contains an even number of carbon atoms, from 4 to 36, bonded in the chain. The major roles of FAs are components of more complex membrane lipids and the major components of stored fat in the form of triglyceride (TGs). FAs are predominant in fat and oil by esterified at the three hydroxyl groups of glycerol. Thus, because the FA portions of the TGs make up the larger proportion of the fat molecules, most of the chemical and physical properties result from the effects of the various FAs esterified with glycerol.

The FA is classified as saturated and unsaturated. Saturated FAs are single bond between carbon atoms and unsaturated have one or more double bonds. Saturated FAs have the formula, H $(CH_2)_n$-COOH, where n is number of carbon atoms. FAs are differentiated by their molecular composition. Firstly, differentiating characteristic is the degree of saturation: saturated, monounsaturated or polyunsaturated. Secondly, is chain length, the number of carbon atoms in the FA molecule. Saturated FA of less than eight carbon atoms are liquid at room temperature, whereas those containing more than ten are solid. The presence of double bonds in FAs render the melting point lowers than a saturated FA. The FAs

can be long or branched chain. FAs may occur as free FA or bound FA, which are attached to other molecules, like triglyceride or phospholipids.

FAs are designated by their total number of carbon atoms. Degree of unsaturation is indicated by a number of carbon atoms, followed by the number of sites of unsaturation. The site of unsaturation in a FA is indicated by the symbol Δ and the number of the first carbon of the double bond (e.g. palmitic acid is a 16-carbon FA with no unsaturation and is designated by 16:0 and palmitoleic acid is a 16-carbon FA with one site of unsaturation between carbons 9 and 10, and is designated by $16{:}1^{\Delta 9}$). The prefixes “a”, “I”, “cy”, and “d” refer to anteiso, iso, cyclopropane branching and dicarboxylic FAs, repectively. The hydroxyl FAs are indicated by the prefixes “ω” indicate the position of hydroxyl groups from the aliphatic end of the FAs, and “α” and “β” to indicate the OH groups at positions 2 and 3.[Zelles, 1999]

3.3 Sample preparation

The aim of sample preparation is to produce the sample in a suitable form for introduction into the measuring instrument. FA analysis consist of 3 steps that extraction, separation and determination. In this section will present lipid extraction procedure, in section 3.3.1 will describe separation of lipids to individual class and in section 3.3.2 will discussed analytical technique.

3.3.1 Lipid extraction

Quantitative isolation of lipid from biological sample in free of non-lipid contaminants must be achieved before the analysis is attempted. Lipid samples can be obtained by extraction with various organic solvent. When appreciable the amount of FFA, diacylglycerol, phospholipid and other lipid are detected in extract. Many solvents or solvent combination can be used to extract lipids from the sample. For example, $CHCl_3$ has been widely used in this purpose and also MeOH to obtain polar lipid. Major constitution of solvent extraction is polarity between lipid and solvent usage. So, lipid fraction can be separate with different polarity. From the

literature studying, organic solvent were $CHCl_3$ and MeOH were found that mostly used, as shown bellowing.

Folch and co-worker [1957] developed method for the preparation and purification of brain lipids. These procedure, the lipids were extract by homogenizing the tissue with 2:1 $CHCl_3$-MeOH (v/v) and filtering the homogenate. The filtrate, which contained the tissue lipids and non-lipid substances, was freed from these substances with water or salt solution. The mixture is allow to separate two phase, and then removed the upper phase and removed of it is completed by rinsing with small amount of pure solvent upper phase. The lower phase contains essentially all tissue lipids.

Bligh and Dyer [1959] developed a single operation for the extraction and purification of lipids from biological materials. The wet tissue is homogenized with a mixture of $CHCl_3$ and MeOH in the tissue. The homogenate could then be diluted with water and/or $CHCl_3$ separated into two layers, the $CHCl_3$ layer containing all of the lipids and the methanolic layer containing all the non-lipids. Thus, a purified lipid extract should be obtained when the $CHCl_3$ layer is isolated.

The Bligh and Dyer is probably the most common extraction procedure for lipids. White *et al.,* [1979] modified the Bligh and Dyer extraction procedure for measurement lipid phosphate in sediment. The sediment was suspended in phosphate buffer with anhydrous MeOH and $CHCl_3$. An additional $CHCl_3$ and water were added to the suspension. The proportions of H_2O: MeOH: $CHCl_3$ of 0.8:2:1 (v/v) for single phase extraction and of 0.9:1:1 (v/v) after separation into the second phase were maintained. In addition, Rutters *et al.,* [2002] and Drenovsky *et al.,* [2004] modified Bligh and Dyer procedure for the extraction of lipid in marine sediments and soil.

Hara and Radin [1978] improved method for extracting the lipids from tissue consists of the use of Hexane: 2-propanol, followed by a wash of the extract with aqueous sodium sulfate to remove non-lipid contaminants. This method has advantage over the common usage of $CHCl_3$-MeOH. The solvent are somewhat less toxic, interference in processing by proteolipid protein contamination is avoided, the two phases separate rapidly during the washing step, the solvent density is too enough to permit centrifugation of homogenate as an alternative to filtration.

Bateman and Jenkings [1997] used hepthane: 2-propanol (1:4 v/v) for the extraction of lipid in microbial cultures. Extraction of culture solid that have been pelleted by centrifugation is sufficient for removal of lipid from microbial cultures.

3.3.2 Separation of lipid classes

Solid-phase extraction (SPE) was used to separate various lipids into individual classes. Analytes passing through the SPE column absorb to the absorbent. Absorbed analyte is eluted from the column by changing the eluent. Typical step in SPE include 1) column condition to allow interaction between the solid phase and sample, 2) sample application, 3) column washing to remove substances that interact weakly to the resin. Actually, a wide variety of the chromatography supports are utilized in lipid separation processes.

Lipid extracts from natural sources tend to contain many different classes or groups of compounds. After, a preliminary fractionation has been carried out, into sample lipid, phospholipid or glycolopid groups.

Silica cartridges are also used to purify the non-polar fraction (triglycerides, TG) from other polar compound. Bitman [1983] investigated the lipid composition of breast milk from mother. The total lipid extract was dissolved in hexane and transferred to a silica Sep-Pak cartridge previously washed with $CHCl_3$. The non-polar lipids were eluted by hexane/ethyl ether (1:1). The eluate contained the neutral lipid fraction. The PLFAs were then eluted MeOH and $CHCl_3$: MeOH: H_2O (3:5:2) through the cartridge.

Saunders and Horrocks [1984] developed a new procedure for the separation of arachidonic acid metabolites from neutral lipids and phospholipids. A new sequence was devised for separation of lipid classes on silicic acid columns. The elution sequence was $CHCl_3$ (neutral lipid and FFAs), methyl formate (prostaglandins and cerebrosides), acetone (remaining, glycolipids), and MeOH (phospholipids).

In addition, separation of lipid can be accomplished by using florisil column. Shaikh [1994] prepared forisil column in glass Pasteur pipettes for lipid fractions of myocardial tissue. Lipid extracts were fractionated into PLFAs and neutral lipid fraction by florisil previously dissolved in $CHCl_3$ as slurry. Neutral lipids were first eluted with $CHCl_3$ and PLFAs were then recovered with MeOH.

Kaluzny *et al*., [1985] utilized aminopropyl bonded phase column for separated standard lipid mixtures into individual classes. Lipid mixtures in $CHCl_3$ were applied to the column, under vacuum. All neutral lipids were eluted with $CHCl_3$:2-propanol (2:1 v/v), FA with 2% HOAc in diethyl ether, and all phospholipids with MeOH. Neutral lipids fraction were separated on a new column and fractionated into cholesteryl ester, triglycerides, Cholesterol, diaglycerides and monoglyceride with different organic solvent combination.

Pinkart *et al.,* [1998] developed rapidly separate lipid classes found in microorganism. The method is based on the use of NH_2-SPE column to separate neutral lipids, PHA, polar lipids, TG and sterols.

Giacometti and co-worker [2002] presented the determination of FAs in *sn*-2 position in edible oil TG. 2-MAGs were obtained after lipolysis of the TG fraction by using lipase directed cleavage of FAs at the glycerol 1, 3 carbon and separation of 2-MAG by NH_2-SPE.

Table 3.1 Chemistry of solid phase extraction adsorbent

Sorbent Chemistry	Surface Function
Silica	SiO_2
Silicic acid	$[SiO_x(OH)_{4-2x}]_n$
Florisil	Mg_2SiO_3
Aminopropyl (NH_2)	$Si(CH_2)_3NH_2$

3.4 Analytical procedure of FA

GC and HPLC are commonly used for the quantification of FA. In GC, flame ionization detector (FID) was preferred. In contrast to HPLC, several detections are possible.

3.4.1 Gas Chromatography (GC)

From the very beginning, gas chromatography (GC) has been an important aid in the analysis of FAs in fats and oils and other lipids. Derivatization of FAs for GC analysis is performed to increase the volatility of substances, and improve separation. The methyl derivatives of FAs are most popular because of the broad range of available methylation and transesterification procedure. Esterification of lipids can be carried out with several reagents based on acid-catalysed or base-catalysed reactions.

a) Acid-catalysed transesterification

Common reagents used for acid-catalysed transesterification are methanolic, hydrochloric (HCl) [Meier, 2006] and sulfuric acid (H_2SO_4) [Indarti *et al*., 2005] and boron trifluoride (BF_3) in MeOH [Rule, 1997]. All are suitable for lipid transesterification and also FFA methylation. However, neither acid-catalysed nor BF_3-catalysed reactions proceed at ambient temperature; both types of reaction require heating. BF_3-MeOH methylate FFAs very rapidly but have a limited self life. Methanolic HCl can be carried out by heating the sample in the reagent under refluxing for 2 h.

b) Base-catalysed transesterification

Popular reagent used for base-catalysed transesterification of lipids is sodium methoxide [Jeyashoke, 1998]. In contrast to all acid-catalysed reaction, transesterification with sodium methoxide proceeds at ambient temperature. Therefore, the risk of decomposition of polyunsaturated fatty acids (PUFAs) is lower, and does not require antioxidants such as butylated hydroxytoluene (BHT).

3.4.2 High performance liquid chromatography (HPLC)

A major difficulty in analysis of FAs by HPLC is related to the absence of chromophores in these molecules to facilitate the detection by UV-Vis absorption or direct fluorescence. For this reason, GC has been used for FA quantification. The use of a capillary column coupled with a flame ionization detector (FID) provides a high resolution for FA analysis. A major advantage of HPLC over GC is the lower temperature used during analysis which reduces the risk of isomerization of unsaturated FAs. In order to increase the sensitivity and selectivity of

detection, a number of UV-absorbing, fluorescent, chemiluminescent and electrochemically active derivatives have been prepared.

a) UV-VIS detection

An ideal UV-Vis derivatizing agent for FAs should form a complex detectable in UV region in higher wavelengths than mobile phase and sensitive enough to be detected in low concentrations. The more commonly used derivatization with 2-bromoacetophenone or PBr enhances sensitivity.

Mehta *et al*., [1998] reported a rapid and sensitive method for separation and quantitation of FFAs in human plasma. Prior to derivatization and analysis, FFAs were extracted from plasma. Two phenacyl ester derivatives, PBr and ρ-bromophenacyl bromide (BrPBr) were investigated in order to achieve optimal separation of individual plasma FFAs. PBr esters of plasma FAs were best resolved using octadecysily column. An isocratic elution method using ACN: H_2O (83:17) at 2 mL/min with UV detection at 242 nm and detection limit approximately 1 nmol.

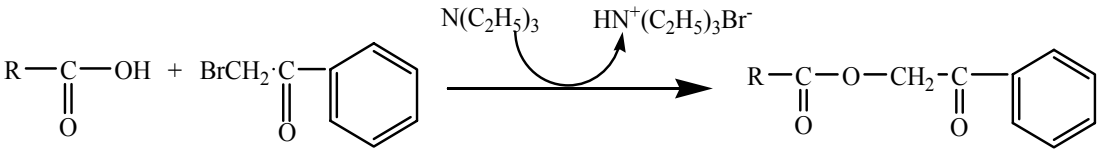

Figure 3.1 FFA derivatization reaction as FA-phenacyl esters.

Siliva and Ferraz [2006] used domestic microwave oven for fat hydrolysis and derivatization with phenacyl chloride (PCl) in fat product. The total reaction time was 6 minute, recoveries were above 94%. Resolution of C14:0 and linolenic acid failed with MeOH: H_2O and the critical pair of C16:0 and C18:1 well resolved. Microwave saponification is delicate to perform especially because of rapid heating and overpressure problem. The detection limit (S/N=3) was 30 ng or 0.25% of standard starting amount of 20 mg/mL.

Naphthacyl esters were studied with the objective of increasing the detection sensitivity. Their molar absorbtivity was reported to be about $3x10^4$ L

mol^{-1} cm^{-1} at 247 nm. Rioux *et al.,* [1999] studied FA metabolism as naphthacyl derivatives by reverse-phase HPLC. The separation of standard mixture of FA-napthacyl esters (25 FAs; C 7:0-C 22: 6) was achieved by a ternary elution gradient of MeOH-ACN-H_2O and monitored by UV absorbance at 246 nm. The linear concentration range was widely, running from 2 ρmoL up to 10 nmol and the limit of detection was found to be about 0.5 ng of FA per injection. The validation of FA analysis was performed by comparing the values obtained with methyl ester by GC and naphthacyl ester by HPLC.

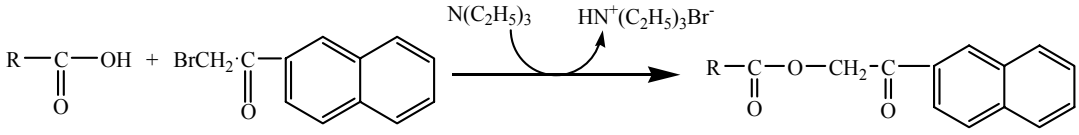

Figure 3.2 FFA derivatization reaction as FA-naphthacyl esters.

Substituted hydrazines have been used to derivatize FAs. The most important of these have been the 2-nitrophenylhydrazines (2-NPH) coupling 1-ethyl-3-(3-dimethylaminopropyl) carbodiimde hydrochloride (1-EDC)/pyridine which provide good sensitivity. Miwa and Yamamoto [1986] developed method for determination of biologically important C10:0–C22:6 FAs as their 2-nitrophenylhydrazides. FAs were separated by reversed-phase HPLC after derivatization with 2-NPH HCl in the presence of 1-EDC. Each FA could be quantitated over the range 2.5-5000 ρmol per injection. For the determination of esterified FAs in fat and oil, the saponified mixture was directly derivatized without extraction. Peris and co-worker [2004] reported that this reaction was carried out using microwave heating for 6 min. The first step in this reaction is the activation of carboxylic acid with EDC, catalysed by pyridine. After the activation, carboxylic hydrazine derivative were obtained in aqueous environment. Method showed good sensitivity, with a detection limit of 15 μmol^{-1}.

In addition, Peter *et al*., [2004] described the determination carboxylic acid after pre-column derivatization with 2-NPH and detected with diode

array detection. The unknown 2-NPH compound can be identified with on-line atmospheric pressure chemical ionization mass spectrometry (APCI-MS) base on the molecular mass or/and the fragmentation of the derivative.

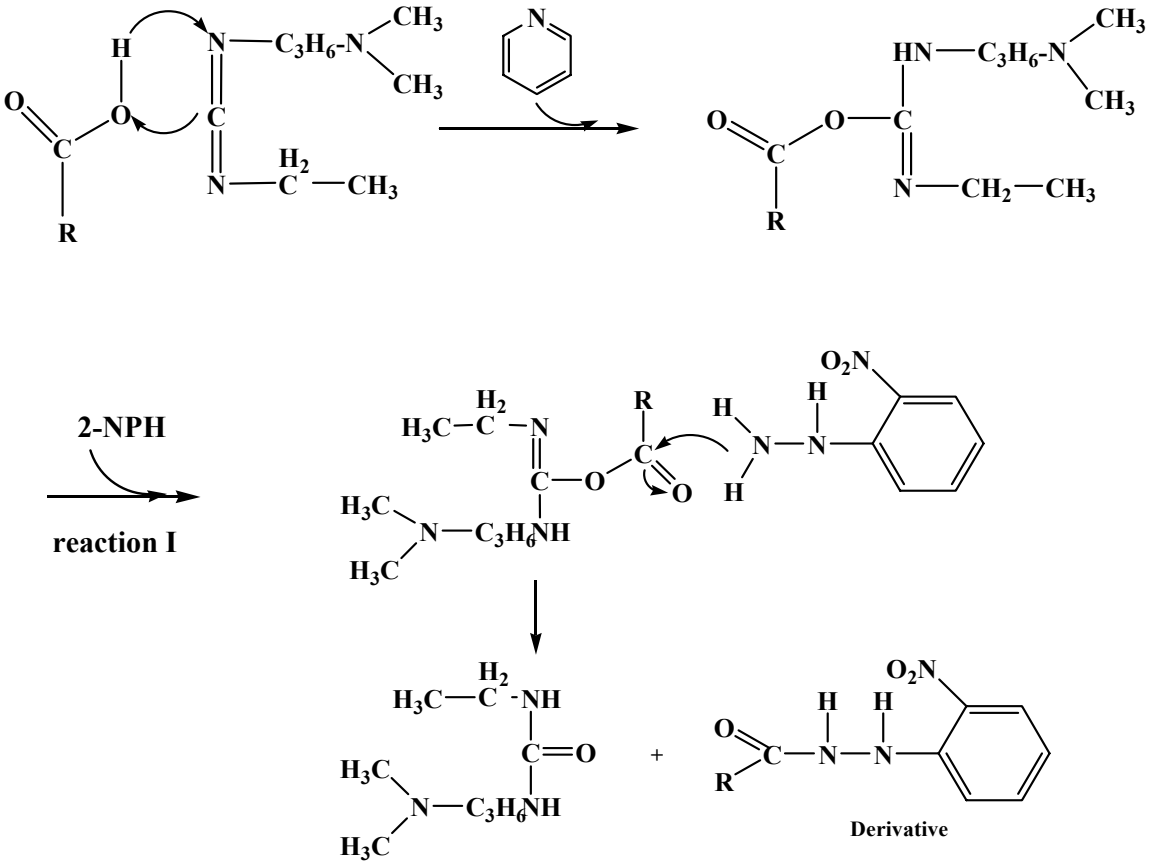

Figure 3.3 Schematic of reaction of carboxylic acid and 2-NPH, using EDC coupling agent by pyridine.

Bravo and co-worker [2004] developed method for and identification quantification of carboxylic FA (CFAs). 2, 4-dinitrophenylhydrazine (2, 4-DNPH), benzyl chloride (BC), and phenylhydrazine (PH) were used for derivatization of different FAs by microwaves oven. After the on-line derivatization, products were separated and quantified by reverse phase column. Microwave irradiation was more efficient for the derivatization than the conventional such as heating in a water bath. Reaction time can decrease almost 10 times.

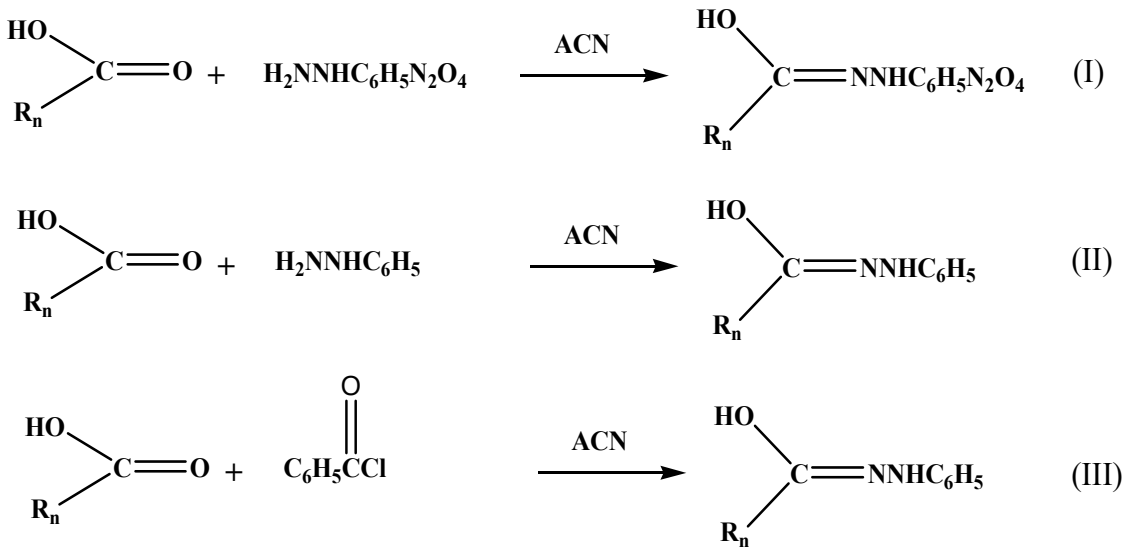

Figure 3.4 Chemical reaction of FA with (I) 2, 4-DNPH, (II) BC and (III) PH.

Nichols and Davies [2002] proposed the use of Atmospheric Pressure Chemical Ionization (APCI) mass spectrometry of FAs as their 2-oxo-phenylethyl esters resulting in a powerful technique in term of detection and allowing simultaneous UV detection. Assignment of chain length and degree of instauration is achieved based on observation of protonated molecular ions. When this is carried out on tandem mass spectrometry, confirmation of chain length and degree of unsaturation is available by observation of the appropriate acyl daughter ions produced by collisional activation of the protonated molecular species. This study reported the FA composition of *S. pealeana*, as determined by concurrent analysis of FA as methyl esters by GC-MS and analysis of 2-oxo-phenylethyl esters by LC-MS. APCI LC-MS analysis proved a superior method for the identification of polyunsaturated FA (PUFA) chain length and degree of unsaturation compared to GC-MS. A PUFA component was detected and identified by LC-MS analysis from the characteristic protonated parent and MS/MS daughter ions. In addition, the capability of PUFA identification afforded by the APCI LC-MS system is clearly demonstrated in the co-elution of C 22:5 and C 17:1.

b) Fluorescence detection

Several fluorogenic tagging reagent have been used for FA analysis resulting in sensitive and selective detection of FA derivatives. The fluorgenic compound should react with FAs rapidly and selectively. The derivative should be stable and have maximal fluorescence in the longer wavelength region to avoid interference by other compounds present in biological samples.

Karages and Rudy [1990] developed a high sensitive method for the separation and quantitation of FA in human serum using 9-anthryldiazomethane (ADAM). The esterification proceeds at room temperature without heat and catalysis. The reaction of FAs with ADAM was allowed to derivatized in the drak at room temperature for 8 hours. Detection limit of FFA serum were less than 10 ρmol/μL. ADAM did not require post-column alkaline hydrolysis to yield fluorescent derivatives. It was unnecessary to remove excess derivatizing agent from the reaction mixture because any excess reagent is eluted within the first 10 minutes of the HPLC run. The limiting factors for using ADAM are the presence of contaminants and decomposition products as well as, its instability.

Prodos and co-worker [1997] described the synthesis of fluorescent dramatization reagent for carboxylic acid, 4-N, N-dimethylaminosulfonyl-7-N-(2-aminoethyl) amino-2, 1, 3-benzoxadiazole (DBD-ED) and investigate its reactivities towards FA and non-steroidal anti-inflammatory drugs (NSAIDs). Its fluorescence properties and separation of these adducts were investigated using LC. The activity of DBD-ED towards carboxylic acids was examined with C20:0 and ibuprofen. The product of adducts at room temperature reached a plateau after 30 min for ibuprofen and *ca*. 1 h for arachidic acid. These adducts were stable for at least 24 h. The detection limits (S/N=3) attained for FAs were 4-7 fmol per injection.

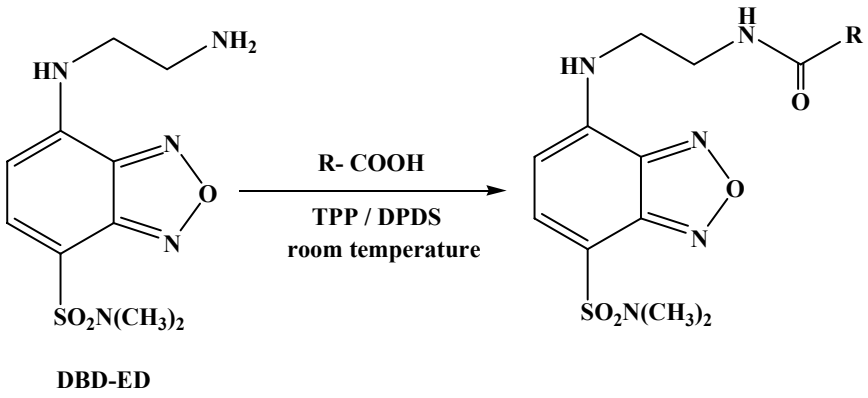

Figure 3.5 Chemical structure of DBD-ED and its reaction product with carboxylic acids.

Wolf and Korf [1990] publish a method for the automated precolumn derivatization of FAs in serum and rat blood. Bromo methylmethoxycoumarin (BrMMC) has been extensively used as labels for derivatization. The suspension of potassium carbonate in crow ether solution was mixed with the sample and subsequently with BrMMC solution. The reagent of FAs with BrMMC and the suspension every FA showed two peaks. The second peak is produced in a competitive reaction with an impurity in the BrMMC.

Santa *et al.,* [1998] described the synthesis of pre-column fluorescent derivatization reagent for carboxylic, *N*-(4-nitro-2, 1, 3-benzoxadiazoyl-7-yl)-*N*-methyl-2-aminoacetohydrazinde (NBD-CO-Hz). NBD-CO-Hz reacted with 2-arylpropionic acids (2-APAs), a group of non-steroidal anti-inflammatory drugs (NSAIDs) in the presence of a condensing agent, EDC and pyridine at room temperature for 2 h to give fluorescent adducts. The detection limits were in fmol range.

You *et al.,* [2007] determinded of long chain FA (LCFAs) (>C20:0) using 1-[2- (*p*-toluenesulfonate)-ethyl]-2-phenylimidazole-[4, 5 - f]-9, 10-phenanthrene (TSPP) as tagging reagent with fluorescence detection and identification with post-column MS have been developed. TSPP could easily and quickly label Lucas at 90^{o}C in the presence of potassium carbonate (K_2CO_3) catalyst in N, N-dimethylformamide (DMF). Eleven free LCFAs from the extracts of

bryophyte plants were sensitively determined. Calculated detection limits from 1.0 pmol injection, at a (S/N=3) were 26.19-76.67 fmol.

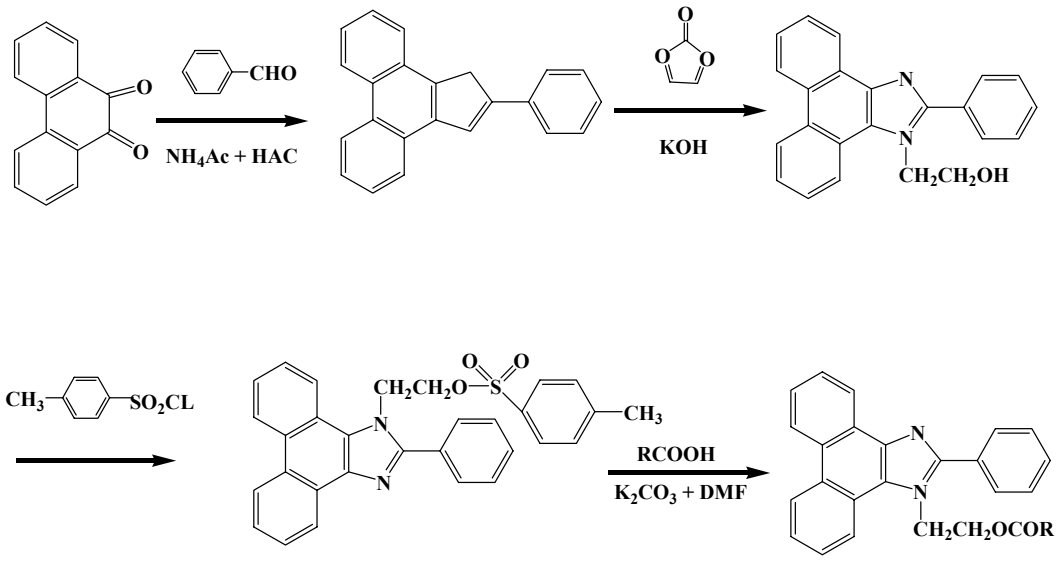

Figure 3.6 The scheme of derivatization procedure for the labeling of long chain FAs. (1): Phenylimidizole-[4, 5, f]-9, 10-phenanthrene; (2): 1-(ethanol)-2-phenylimidazole-[4, 5, f]-9, 10-phenanthrene (EPP); (3): 1-[2-(p-Toluenesulfonate)-ethyl]-2-phenylimidazole-[4, 5, f]-9, 10-phenanthrene (TSPP); (4): Corresponding derivative.

c) Other detection

The C12:0–C18:0 FFA has been separated on reversed-phase ion-pair HPLC method with conductivity detection by Tsuyama *et al.,* [1992]. The separation and determination of underivatized FAs was achieved by the use of an isocratic eluent consisting of MeOH–5 mM tetrabutylammonium (TBA). The limit of detection at a (S/N=3) was *ca* 2 ng, base on the detectability of 1 -µg/mL of margaric acid (2-µL injection). This method had several advantages: no need for derivatization, isocratic elution, good reproducibility, fair high sensitivity and rapidity.

6-[*N*-(3-propionohydrazino) tiered] benzoic [g]-phthalazine-1, 4-(2*H*, 3*H*)-dione (PROB) was developed as an interesting chemiluminescent (CL) reagent by Yoshida *et al.,* [1999]. PROB has a hydrazine group as a reactive

functional group for carboxylic group, and reacts with FAs in the presence of EDC and pyridine at room temperature for 40 min. PROB derivatives of FA were separated by C18 column and detected with post-column CL detection followed by mixing with H_2O_2 and potassium hexacyanoferrate (III) in alkaline medium. The detection limits (S/N=3) for the FAs were 11.8-64.4 fmol on column.

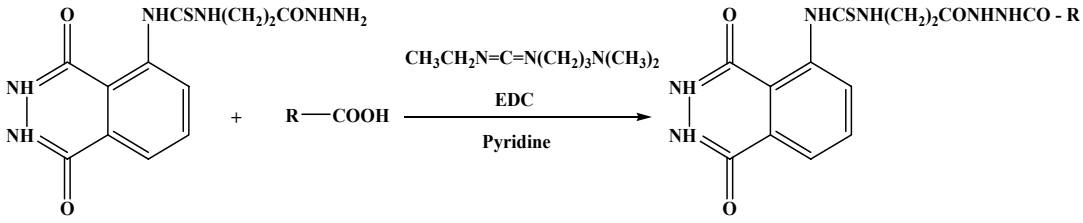

Figure 3.7 Derivatization reaction of PROB

Kawasaki *et al*., [1990] described a novel HPLC method for the detection of individual Fast without the labeling procedure, using the immobilized acyl-CoA synthetase (ACS) and acyl CoA oxidase (ACO) reaction system, coupled with chemiluminescence detection of hydrogen peroxide catalysed by micro-peroxidase (mPOD). The system was used with the enzyme reactor inserted in front of detection unit. Each FA eluted from the HPLC column was mixed with reagent R1 and subjected to enzymatic reaction in the immobilized-ACS-ACO column to form hydrogen peroxide, which was mixed with the chemiluminescence's reagent R2 and the light emitted in the flow cell was detected by the photomultiplier. The reaction conditions for the detection of hydrogen peroxide were carried out by a flow-injection method without the HPLC column. The limit of detection was 5 ρmol.

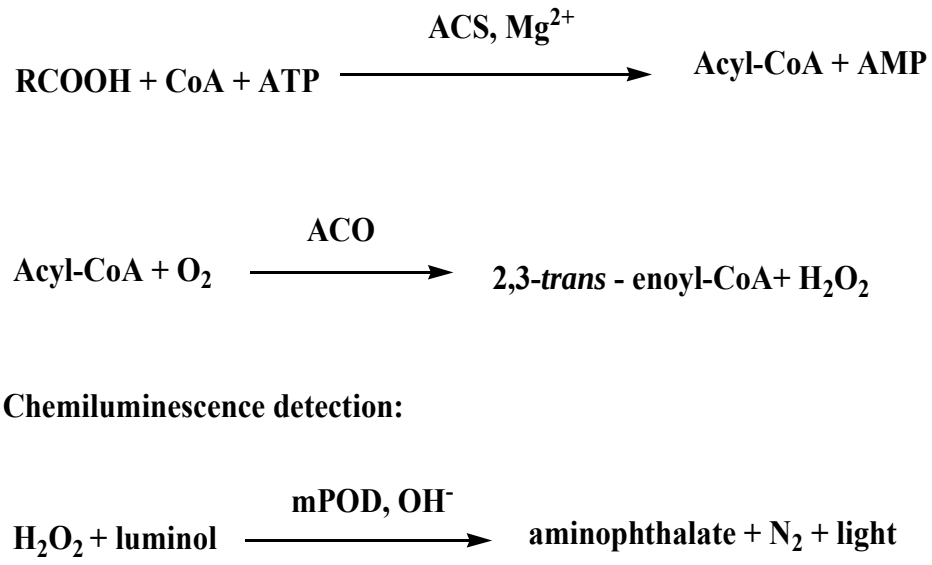

Figure 3.8 Determination of FA using ACS and ACO reaction system

Table 3.2 Summary some literature review on the derivatization of FAs analysis by UV-Vis and FL tagging reagents

Derivatizing reagent	**Catalyst**	**Condition**	**Detecion limit**	**References**
PBr	TEA	Heat 100^{o}C	<1 nmol	Mehta A. *et al.*, 1998
PCl	TEA	Microwave irradiation	30 ng	Silva F. and Ferraz V., 2006
NAPHBr	TEA	Heat 90^{o}C	0.5 ng	Rioux V. *et al.*, 1999
2-NPH	1-EDC/Pyridine	Heat 60^{o}C	-	Miwa H, and Yamamoto M, 1986
2-NPH	1-EDC/Pyridine	Microwave irradiation	15 μmol^{-1}	Peter R. *et al.*, 2004
2, 4-DNPH	-	Microwave irradiation	-	Bravo E. *et al.*, 2004
BC	-		-	
PH	-		-	
ADAM	-	Room temperature	10 pmol	Kargas G. and Rudy T., 1990
DBD-ED	TPP/DPDS	Room temperature	4-7 fmol	Prodos P. *et al.*, 1997
BrMMC	Crown ether	Room temperature	-	Wolf J.H. and Korf J., 1990
NBD-CO-Hz	-	Room temperature	2-4 fmol	Santa T. *et al.*, 1998
TSPP	K_2CO_3	90^{o}C	26.19-76.67 fmol	You J. *et al.*, 2007

CHAPTER 4
MATERIALS AND METHODS

4.1 Introduction

In this work, a reversed-phase high performance liquid chromatography with UV detector using isocratic elution was employed as a method for the identification and determination of C12:0, C14:0, C16:0, C18:0 and C20:0. Instrumentation, reagents, procedures for reagent preparation and sample clean up are described. The chromatographic method was optimized for the separation of fatty acids. Initially, composition of mobile phase was employed in order to improve separation and reduced analytisis time. The FAs were derivatized and optimized for improving the sensitivity using PBr (2-bromo-acetophenone) and NAPHBr (2-bromo-2′-acetonaphthone).

4.2 Instrumentation

The HPLC system used for the chromatographic experiments and other equipment used for preparation of reagents and samples are given in Table 4.1.

Table 4.1 HPLC instrumentation

Instrument	Model	Company
1. Injector	7125	Rheodyne (Cotati, USA)
2. Delivery system	Water 510	Waters (Milford, MA,USA)
3. Column	Hypersil ODS 5 μm (250 x 4.0 mm i.d.)	Hewlett Packard (USA)
4. Detector	Water 484 Tunable Absorbance Detector	Waters (Milford, MA,USA)
5. Data system	Powerchrom 280	eDAQ, Australia

Table 4.2 Equipment and materials for preliminary study and preparation of reagents.

Instrument	Model	Company
1. UV-Visible Spectrophotometer	HP 8483	Hewlett Packard, Germany
2. Analytical balance	CP225D	Sartorius, Germany
3. Ultrasonic bath	ULTRAsonik 280H	Yuciapa CA, USA
4. Vortex mixer	Vortex-Genie G560-E	New York, USA
5. Aspirator	A-3S	Eycla, Tokyo Rikakikai Co.,Ltd , Japan

4.3 Reagents and chemicals

All chemicals and solvents are listed in Table 4.3 as following

Table 4.3 List of reagents, suppliers and reagent grade.

Chemical	Supplier
1. Methanol (HPLC grade)	Fisher Scientific,(Loughboroug,UK)
2. Water (DI grade)	Millipore (Milford, USA)
3. Chloroform (AR grade)	Lab-scan (Bangkok,Thailand)
4. Diethyl ether (AR grade)	Lab-scan (Bangkok,Thailand)
5. Triethylamine (AR grade)	Fluka, (Buchs, Switzerland)
6. Acetic acid (AR grade)	Merck (Darmstadt, Germany)
7. n-Hexane (HPLC grade)	Lab-scan (Bangkok,Thailand)
8. Acetone (HPLC grade)	Fisher Scientific,(Loongberg UK).
9. Hydrochloric acid (AR grade)	Merck (Darmstadt, Germany)
10. Isopropanol (AR grade)	Lab-scan (Bangkok,Thailand)
11. *2*-bromo-2′- acetonaphthone (AR grade)	Aldrich (Wisconsin, USA)
12. *2*-bromo-acetophenone (AR grade)	Fluka, (Buchs, Switzerland)
13. Lauric acid (AR grade)	Fluka, (Buchs, Switzerland)
14. Myristic acid (AR grade)	Fluka, (Buchs, Switzerland)
15. Palmitic acid (AR grade)	BDH Chemical (Poole England)
16. Margaric acid (Standard grade)	Sigma (St.Louis, USA)
17. Stearic acid (AR grade)	Fluka, (Buchs, Switzerland)
18. Arachidic acid (AR grade)	Fluka, (Buchs, Switzerland)
19. L-α-Phosphatidylcholine (Standard grade)	Sigma (St.Louis, USA)
20. Potassium hydroxide (AR grade)	BDH Chemical (Poole England)

4.4 Preparation of solution

4.4.1 Standard solution of FAs

Stock standard solution of 1000 µg/mL of each fatty acid, viz, C12:0, C14:0, C16:0, C18:0 and C20:0 was prepared by dissolving an accurate weight of approximately 10.00 mg of pure compound in acetone and made up to 10.00 ml in a volumetric flask with acetone. These stock solutions were stored in the refrigerator at 4 ^{o}C. The stock solution was diluted to give suitable working solution for the preparation of the calibration standard, chromatographic studies were prepared by aliquot each of the 5 stock standard solutions (1000 µg/mL) in a 10.00 mL volumetric flask and making up to volume with acetone to give concentration of 100µg/mL.

4.4.2 Internal standard solution (1000 µg/mL)

The stock solution of margaric acid (C17:0) was prepared by dissolving 10.00 mg of margaric acid in acetone and made up to 10.00 mL in a volumetric flask with acetone. These solutions were stored in refrigerator at 4^{o}C. The stock solution was diluted to give suitable working solution for the preparation of the calibration standard and for soil sample.

4.4.3 Phosphatidylcholine standard solution (PC) (1000 µg/mL)

Stock solution of 1000 µg/mL of PC was prepared by dissolving 10.00 mg of PC in chloroform and made up to 10.00 mL in a volumetric flask with chloroform. This solution was stored in refrigerator at 4^{o}C.

4.4.4 PBr or (*2*-bromo-acetophenone) solution (20 mg/mL)

Stock solution of 20 mg/mL of PBr was prepared by dissolving 20.00 mg of PBr in acetone and made up to 10.00 mL in a volumetric flask with acetone. This solution was stored in refrigerator at 4^{o}C.

4.4.5 NAPHBr or (*2*-bromo-*2′*- acetonaphthone) solution (20 mg/mL)

Stock solution of 20 mg/mL of NAPHBr was prepared by dissolving 20.00 mg of NAPHBr in acetone and made up to 10.00 mL in a volumetric flask with acetone. This solution was stored in refrigerator at 4^{o}C.

4.4.6 Triethylamine solution (25 mg/mL TEA)

The solution was prepared by adding 345 µL of TEA in acetone and made up to 10.00 mL in a volumetric flask with acetone to give 25 mg/mL.

4.4.7 Acetic acid solution (10 mg/ml HOAc)

The solution was prepared by adding 195 µL of HOAc in acetone and made up to 10.00 mL in a volumetric flask with acetone to give 20 mg/mL.

4.4.8 Mobile phase preparation

The various ratio (v/v) of MeOH to water was prepared. For each new composition of the mobile phase was filtered through a 0.45 µm nylon membrane and degassed in an ultrasonic bath for 30 min before use.

4.4.9 50 mM phosphate buffer pH 7.4

8.709 g K_2HPO_4 was dissolved in 1000mL of H_2O-3A. Adjust pH to 7.4 with 6N hydrochloric acid (HCl). This solution was stored in refrigerator at 4^{o}C

4.4.10 1.0 M KOH /MeOH

56.11 g of KOH was dissolved in 1000 mL of MeOH. This solution was stored in refrigerator at 4^{o}C

4.4.11 2% v/v HOAc in diethyl ether

The solution was prepared by adding 2.00 mL of acetic acid in ether and made up to 100.00 mL in a volumetric flask with ether to give 2 %v/v

4.4.12 $CHCl_3$/2-propanol

A mixture of $CHCl_3$: 2-propanol was prepared in the ratio of 2:1 for homogenization process sample.

4.5 Derivatization procedure

4.5.1 Phenacyl ester derivatives

The derivatization with PBr was performed as follows: Aliquot each of the 5 stocks standard solution FA (1000 µg/mL) were transfered in a screw capped tube. PBr solution (20 mg/mL) and TEA solution (25 mg/mL) were added in to this solution. The screw capped tube was placed in a heater block and heated at 100^{o}C for 15 min. This tube was allowed to cool, added acetic acid solution (10 mg/mL). This tube was heated additional 5 min at 100^{o}C, and allowed to cool. After evaporation under a stream of N_2 and then reconstituted in MeOH. The reconstituted solution was filtered by 0.45 µm nylon membrane before injection.

4.5.2 Napthacyl ester derivatives

The derivatization with NAPHBr was performed as follows: Aliquot each of the 5 stock standard solutions FA (1000 µg/mL) were transfered in a screw capped tube. NAPHBr (20 mg/mL) and TEA solution (25 mg/mL) were added in to this solution. The screw capped tube was placed in a heater block and heated at 90^{o}C for 30 min. This tube was allowed to cool, added HOAc solution (10 mg/mL). This tube was heated additional 15 min at 90^{o}C, and allowed to cool. After evaporation under a stream of N_2 and then reconstituted in MeOH. The reconstituted solution was filtered by 0.45 µm nylon membrane before injection.

4.6 Sample treatment

4.6.1 Liquid-liquid extraction for removal interferences.

4.6.1.1 Liquid-liquid extraction of soil sample.

Liquid-liquid extraction of lipid was carried out in order to decrease matrix interferences. This extraction procedure is the Bligh and Dyer method [1959]. 01The procedure is as follows : an accurate weight 5.00 g of soil sample was shaked for 2 h in extraction mixture containing 5.00 mL of $CHCl_3$, 10.00mL of MeOH and 4.00 mL of phosphate buffer (1:2:0.8). After centrifugation for 30 min at 3000 rpm, 5.00 mL of $CHCl_3$ and 5.00 mL of phosphate buffer were added. Sample was mixed and the phases allowed to separate overnight (~18 h). The $CHCl_3$ layer was transferred in a screw capped tube and dried under N_2 stream.

4.6.1.2 Liquid-liquid extraction of cultured bacteria.

Pipette 1.00 mL of cultural bacteria in media solution and shake for 2 h in extraction mixture containing 1.00 mL of $CHCl_3$, 2.00 mL of MeOH and 0.80 mL of phosphate buffer (1:2:0.8). After centrifugation for 30 min at 3000 rpm, 1.00 mL of $CHCl_3$ and 1.00 mL of phosphate buffer were added. Sample was mixed and the phases allowed to separate overnight (~18 h). The $CHCl_3$ layer was transferred in a screw capped tube and dried under N_2 stream.

4.6.2 Separation of lipid classes

Residue of lipids from Section 4.6.1 were dissolved in $CHCl_3$ for the separation procedures of Kaluzny *et al.,* [1985]. 500 mg Aminopropyl (NH_2) bonded phase SPE columns were prewashed with 5.00 mL of hexane and then 3 times of 0.50 mL of lipid mixture was loaded into the column. Neutral lipids were eluted with 5.00 mL of $CHCl_3$/2-propanal, FAs were eluted with 10.00 mL of 2% HOAc in ether, and polar lipids (PL) were eluted with 5.00 mL of MeOH. The polar fraction was dried under N_2 gas.

4.6.3 Hydrolysis of PL

The residue of PL was dissolved in 1.00 mL of 1 M KOH/MeOH. This solution was placed in a heating block at 80^{o}C for 30 min. Allow to cool at room temperature and then add 5 drop of concentrate HCl. 10 µL of 1000 µL/mg C17:0 and 1.00 mL of hexane were added into the solution to extract FFAs. 0.5 mL of hexane layer was dried under N_2 gas and then excess derivatizing agent was added for preparation of ester compounds.

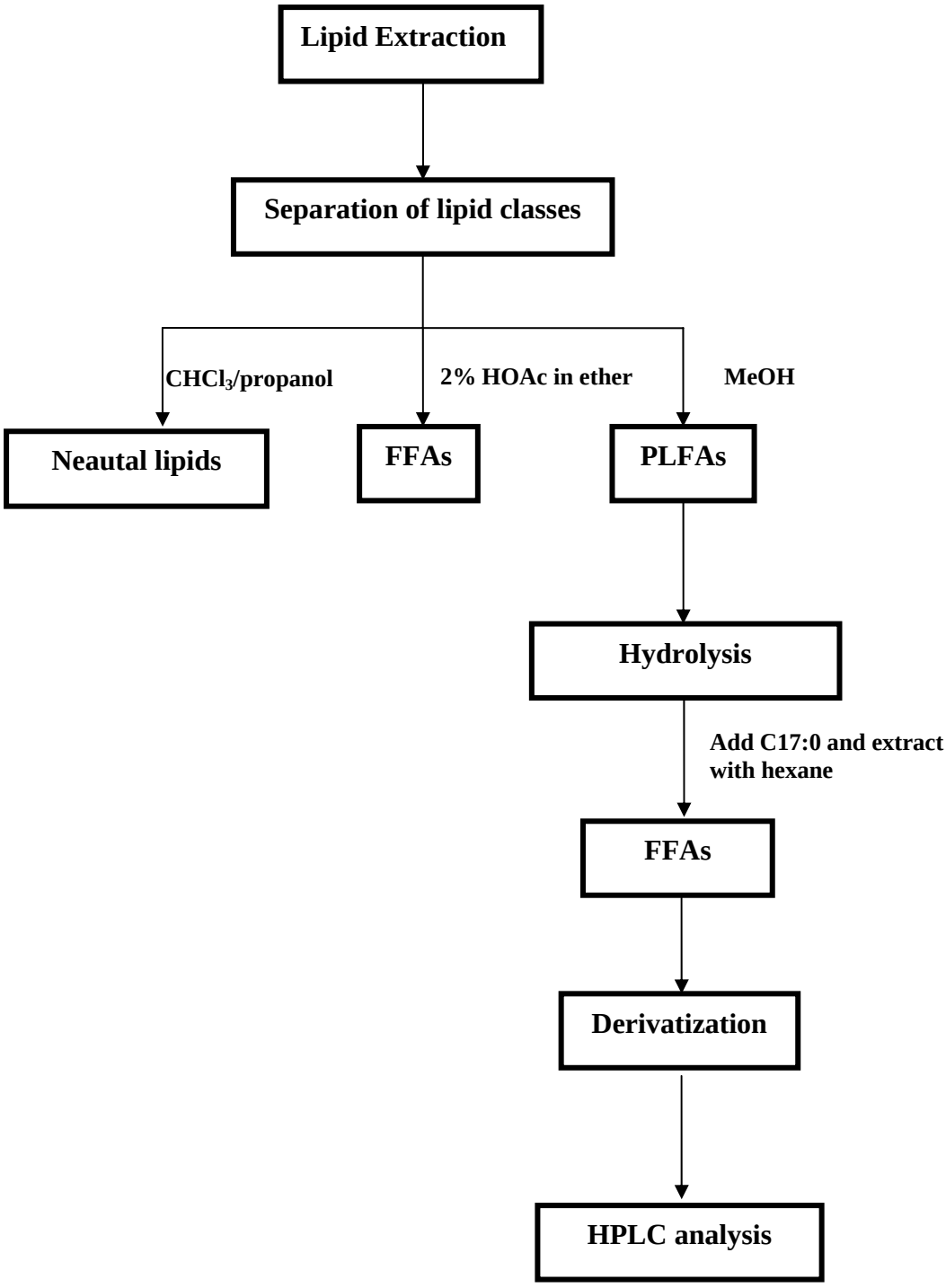

Figure 4.1 The determination of PLFAs ester-linked FAs to in real samples.

4.7 Preparation of calibration graph

4.7.1 Phenacyl ester derivatives

Working standard FAs solution was prepared by 1000 µL of the 5 stock standard solution FAs (1000 µg/mL) transferred in 10.0 mL volumetic flask and making up to volume with acetone. Aliquots of working standard solution (100 µg/mL) were added in a screw capped tube to give different concentration in the range 0.5-50 µg/mL. All solutions contained 5 µL of 1000 µg/mL of C17:0 as the internal standard. FA-phenacyl esters were prepared as described in Section 4.5.1. Each concentration was assayed in triplicate. The various volumes of standard solution, PBr solution (20 mg/mL), TEA solution (25 mg/mL) and HOAc solution (10 mg/mL) are shown in Appendix A

4.7.2 Napthacyl ester derivatives

Working standard FAs solution was prepared by 1000 µL of the 5 stock standard solution FAs (1000 µg/mL) transferred in 10.0 mL volumetic flask and marking up to volume with acetone. Aliquot of working standard solution (100 µg/mL) were added in a screw capped tube to give different concentration in the range 0.1-50 µg/mL. All solution contained 5 µL of 1000 µg/mL of C17:0 as the internal standard. FA-naphthacyl esters were prepared as described in Section 4.5.2. Each concentration was assayed in triplicate. The various volumes of standard solution, NAPHBr solution (20 mg/mL), TEA solution (25 mg/mL) and HOAc solution (10 mg/mL) are shown in Appendix A

4.7.3 Determination of detection limit

The limit of detection is lowest concentration of an analyte that the instrument can reliable detect. Concentration of standard mixture of C12:0, C14:0, C16:0, C18:0 and C20:0 were respectively. 10 µL of 1000 µg/mL of C17:0 was added as internal standard. These samples were derivatized as previously described. The detection limit was determined by injecting 5 times. Standard deviation was then calculated for the data. 3 times of the standard deviation was converted to

concentration and is defined as the detection limit. Details of calculation were shown in Appendix B.

4.8 Recovery study of FAs

4.8.1 Recovery from phosphatidylcholin in soil sample

Soil sample (5.00 g) was spiked with 1000 ug/mL phosphatidylcholin standard solution 50, 100, and 200 µL. These samples were subjected to the extraction, fractionation, hydrolysis and derivatization procedure previously described. The percentage of FAs recovery was calculated by measuring the amount of extracted free FAs from phosphatidylcholin in the spiked sample after sample pretreatment procedure. Triplicates were carried out.

4.8.2 Recovery from phosphatidylcholin in cultured bacteria

Cultured bacteria in media solution (1.00 mL) were spiked with 1000 ug/mL phosphatidylcholin standard solution 50, 100, and 200 µL. These samples were subjected to the extraction, fractionation, hydrolysis and derivatization procedure previously described. The percentage of FAs recovery was calculated by measuring the amount of extracted free FAs from PC in the spiked sample after sample pretreatment procedure. Triplicates were carried out.

4.9 High Performance Liquid Chromatography Condition

HPLC with isocratic elution was employed to study the separation of FA ester derivatives. Optimum sensitivity for phenacyl and naphthacyl esters detection was obtained on C18 column. The column was first equilibrated with mobile phase for 15-30 min before analysis.

Injector:	Rheodyne 7125 fitted with a 20 µL sample loop.
Injection volume:	20µL
Column:	Hypes ODS 5 µm (250 mm x 4.0 i.d.)
Flow rate:	1.0 mL/min
Detector:	monitoring at 242 nm for phenacyl ester
	monitoring at 246 nm for naphthacyl ester
Data system:	Powerchrom 280

CHAPTER 5
RESULTS AND DISCUSSION

5.1 Introduction

In this chapter, absorption studies, chromatographic studies, sample preparation and sample analysis are presented as shown in the following section. FAs studied in this work were lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0) and arachidic acid (C20:0), respectively. HPLC conditions were optimized for determination of FA naphthacyl and phenacyl ester derivatives on C18 column with UV-Vis detector.

5.2 Absorption Studies

Absorption spectra of FA-derivatives with PBr and NAPHBr were recorded.

5.2.1 Phenacyl ester

PBr reacts with carboxylic group forming ester derivative. Absorption spectra of FA-phenacyl estesrs were studied by preparing the derivatives of FA (C12:0, C14:0, C16:0, C18:0 and C20:0) with PBr (20 mg/mL). PBr was used excess, which was calculated by mole ratio for 100 times more than total FA concentration. The condition of the reaction was described in section 4.5.1. The absorption spectra of each derivative are shown in Figure 5.1. Maximum absorption at wavelength of 200 nm and 245 nm were observed. However, at the wavelength of 245 nm were employed to set the detector of HPLC system.

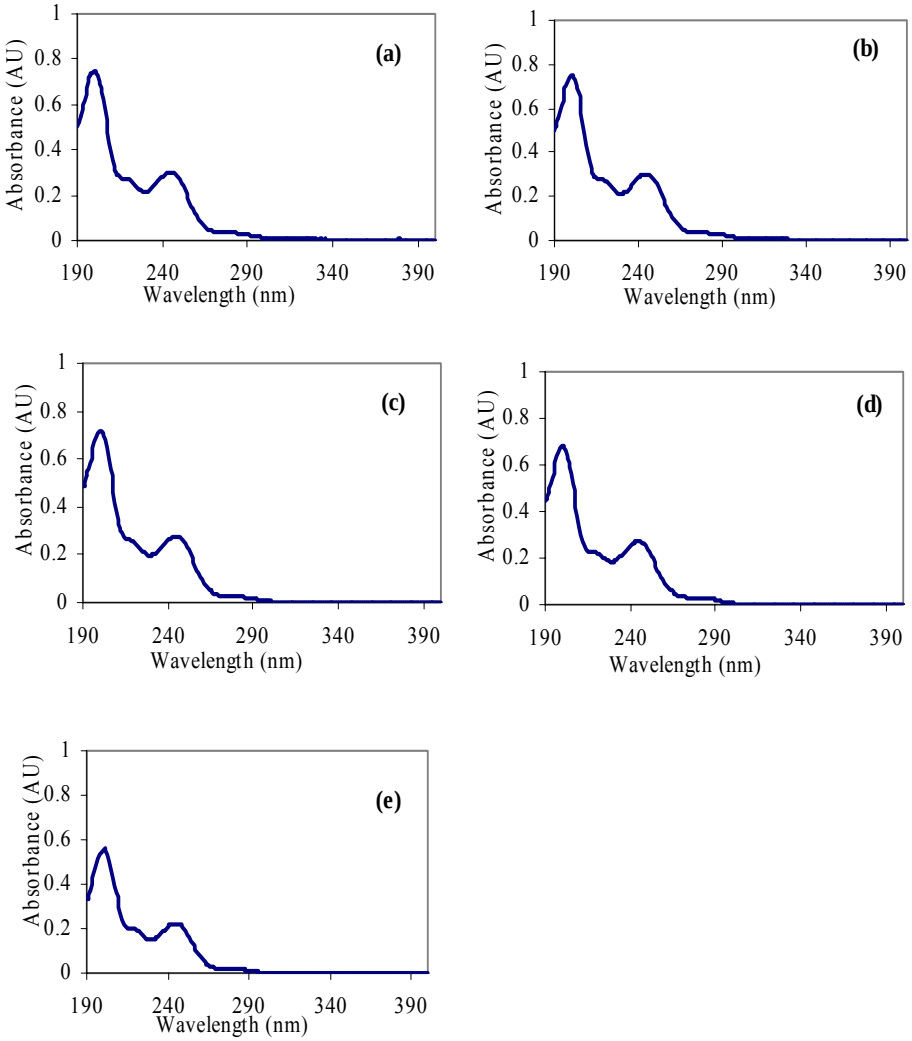

Figure 5.1 Absorption spectra in the range 190–400 nm of FA-phenacyl esters (1 µg/mL); (a) C12:0, (b) C14:0, (c) C16:0, (d) C18:0 and (e) C20:0 in MeOH using UV-Vis spectrophotometer.

From absorption spectrum (Figure 5.1), the maximum region of the spectrum (230-250 nm) was studied by measuring the absorbance with HPLC detector to confirm the maximum wavelength. The results of absorbance reading from the detector are shown in Figure 5.2. The result showed that wavelength at 242 nm was employed for HPLC analysis.

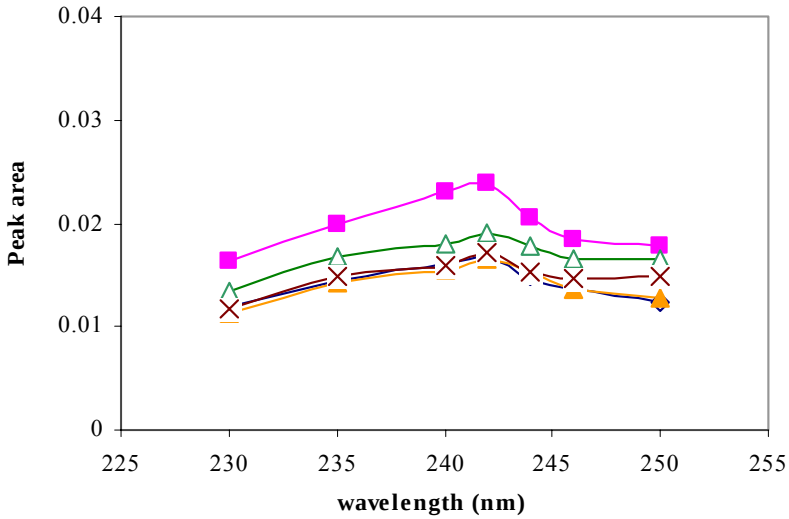

Figure 5.2 Absorption spectrum at the maximum wavelength of FA-phenacyl esters for (◇) C12:0, (■) C14:0, (▲) C16:0, (△) C18:0 and (x) C20:0 using HPLC-UV detector.

5.2.2 Naphthacyl ester

NAPHBr can form ester derivatives with carboxylic group. Absorption spectrum of FA-naphthacyl esters were studied by preparing the derivatives of FAs (C12:0, C14:0, C16:0, C18:0 and C20:0) with NAPHBr (20 mg/mL). Excess amount of NAPHBr was added at 100 times more than total FA concentration. The condition of the reaction was described in section 4.5.2. The absorption spectra of each derivative are shown in Figure 5.3.

Maximum absorption at wavelength 209 nm and 247 nm were obtained. However, the wavelength at 247 nm was selected for the detector of HPLC system.

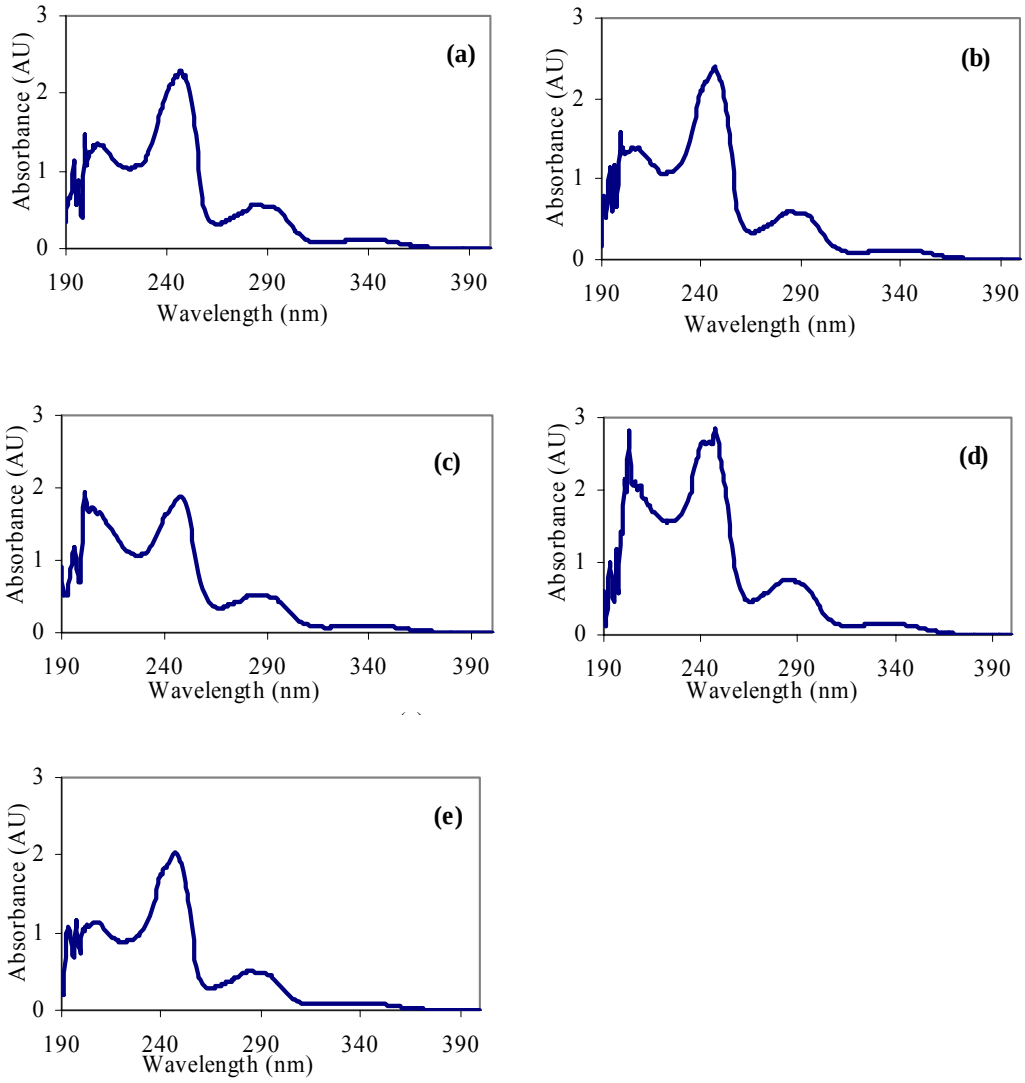

Figure 5.3 Absorption spectra in the range 190–400 nm of FA-naphthacyl esters (1 µg/mL); (a) C12:0, (b) C14:0, (c) C16:0, (d) C18:0 and (e) C20:0 in MeOH by UV-Vis spectrophotometer.

From absorption spectrum (Figure 5.3), the maximum region (230-250 nm) was carried out by measuring absorbance with HPLC detector to confirm the maximum wavelength. Absorbance reading from the detector is shown in Figure 5.4. The result showed that selected wavelength at 246 nm was suitable for HPLC analysis.

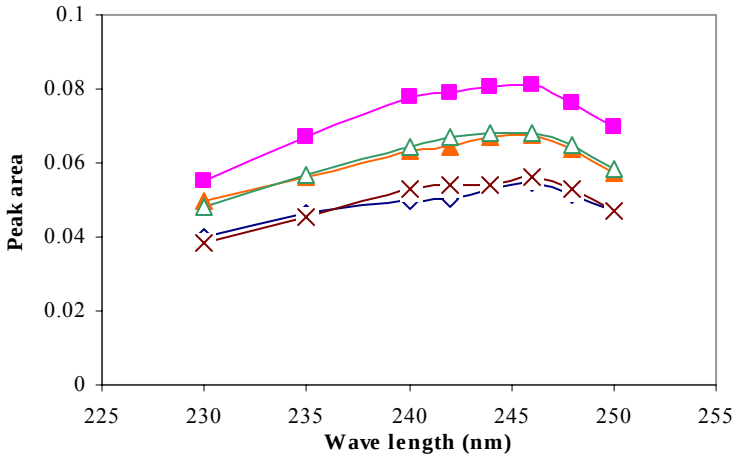

Figure 5.4 Absorption spectrum at the maximum wavelength of FA-naphthacyl esters for (◇) C12:0; (■) C14:0; (▲) C16:0; (△) C18:0 and (x) C20:0 by HPLC-UV detector.

5.3 Chromatographic studies

In this section, FA derivatives were separated on C18 column by varying composition of the mobile phase (MeOH: H_2O). The mobile phase composition was found for the optimum condition. Different wavelength of the detector was set for phenacyl ester (242 nm) and naphthacyl ester (246 nm). Flow rate was set at 1.0 mL/min and injection volume was 20 µL.

5.3.1 FA-phenacyl esters

Standard FAs at the concentration 10 µg/mL was used in this purpose to compare the separation efficiency with the mobile phase composition. Phosphatidylcholin was also investigated as PLFA standard compound because in real sample such as culture bacteria, soil may have many types of these compounds. The compositions of the mobile phase were studied in the range of 90% to 97% of MeOH. The chromatograms are shown in Figure 5.5 and 5.6. Retention time of FA-phenacyl esters retention time increased with the increasing of FA carbon chain length. Retention time for these derivatives decreased when MeOH level in the mobile phase increased. For phosphatidylcholin, (see in Figure 5.6) found that peak of C16:0 and

adjacent peak showed less resolution when MeOH composition was increased. The mobile phase of 93% MeOH gave good resolution and suitable analysis time for FFAs from phosphatidylcholin standard. Therefore, mobile phase with the composition of 93% MeOH was chosen as the optimum composition.

5.3.2 FA-Naphthacyl esters

10 μg/mL standard FAs was used in this work to compare the separation efficiency with the mobile phase composition. In addition, phosphatidylcholin was used to investigate for the separation of C16:0 and C18:0 and some unsaturated FAs of the system. Percentage of MeOH was investigated in the range of 90% to 97%. Results are shown in Figure 5.7 and 5.8. The chromatograms for various mobile phase compositions of FA-naphthacyl estes were separated according to the chain length of FA. Polarity of the mobile phase decreased by adding MeOH, so the retention time of FA-naphthacyl esters was also reduced. Resolution of the analysis decreased when the composition of MeOH increased. Therefore, 93% of MeOH was chosen as the optimum mobile phase, as a compromising between the resolution and the analysis time.

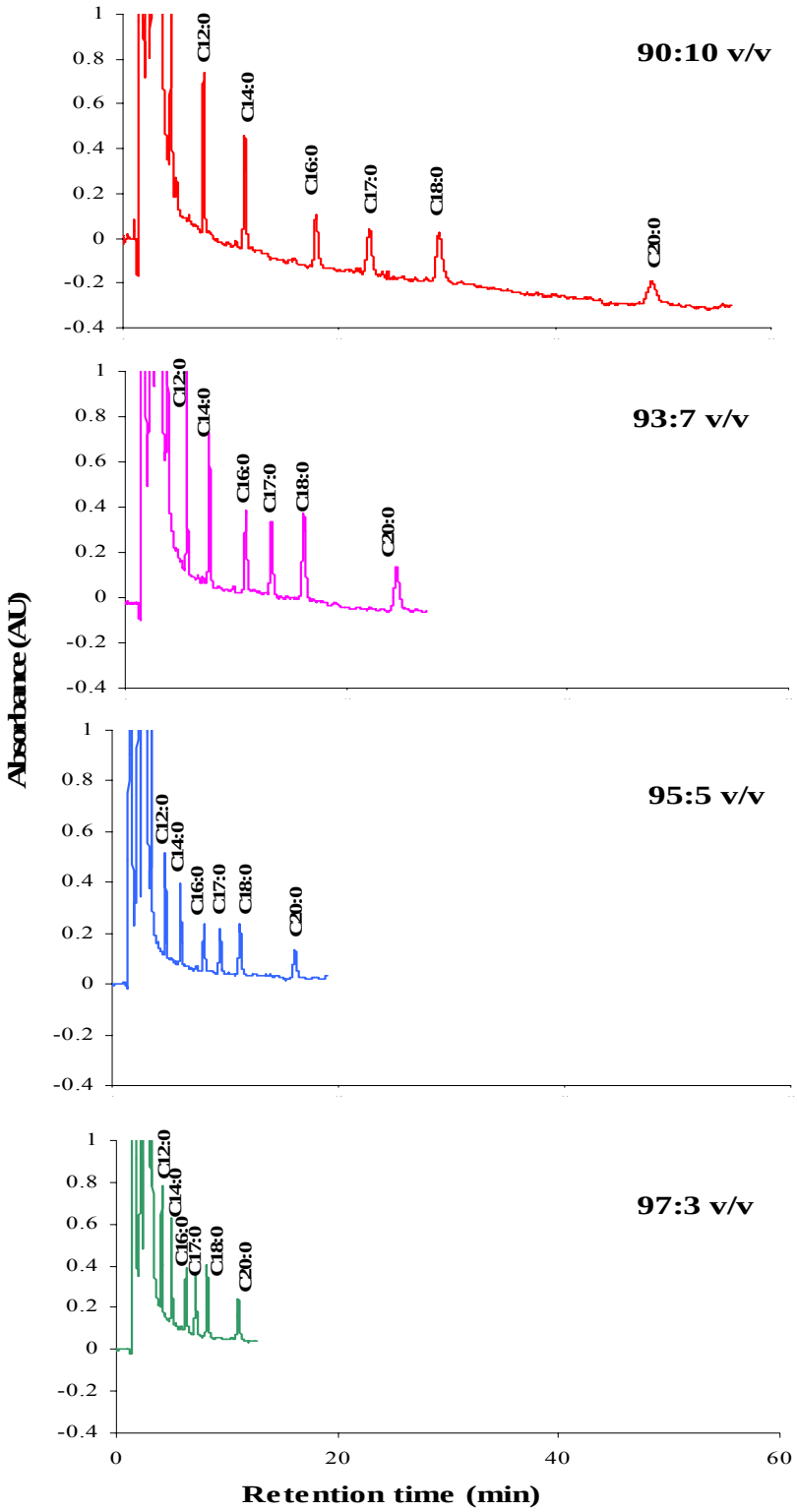

Figure 5.5 Chromatograms of standard FA-phenacyl esters 10.0 µg/mL by varying mobile phase composition of MeOH: H_2O, flow rate 1.0 mL/min, detector set at 242 nm.

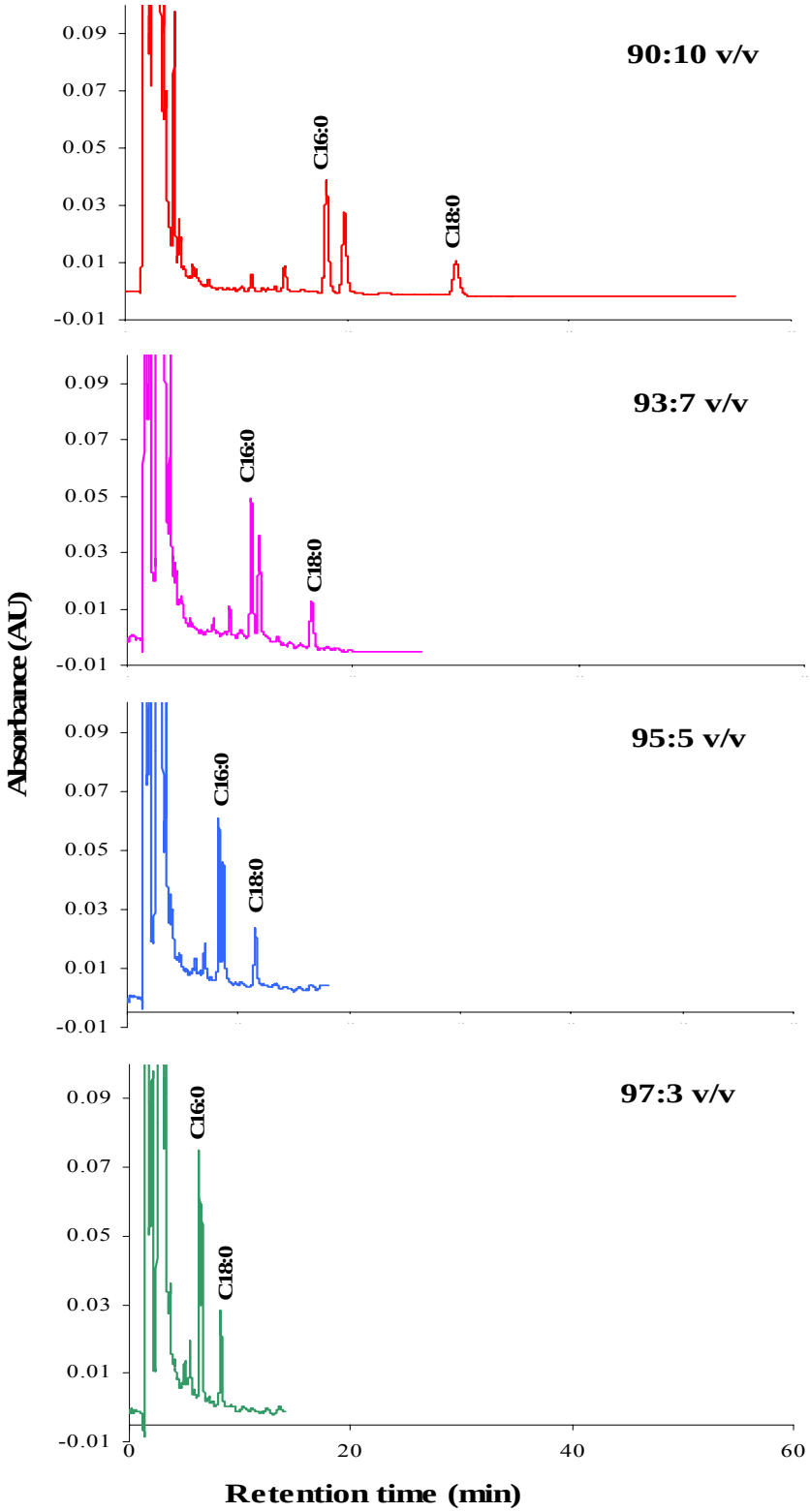

Figure 5.6 Chromatograms of FA-phenacyl esters (prepared from 10.0 µg/mL phosphatidylcholin) by varying mobile phase composition of MeOH: H_2O, flow rate 1.0 mL/min, detector set at 242 nm.

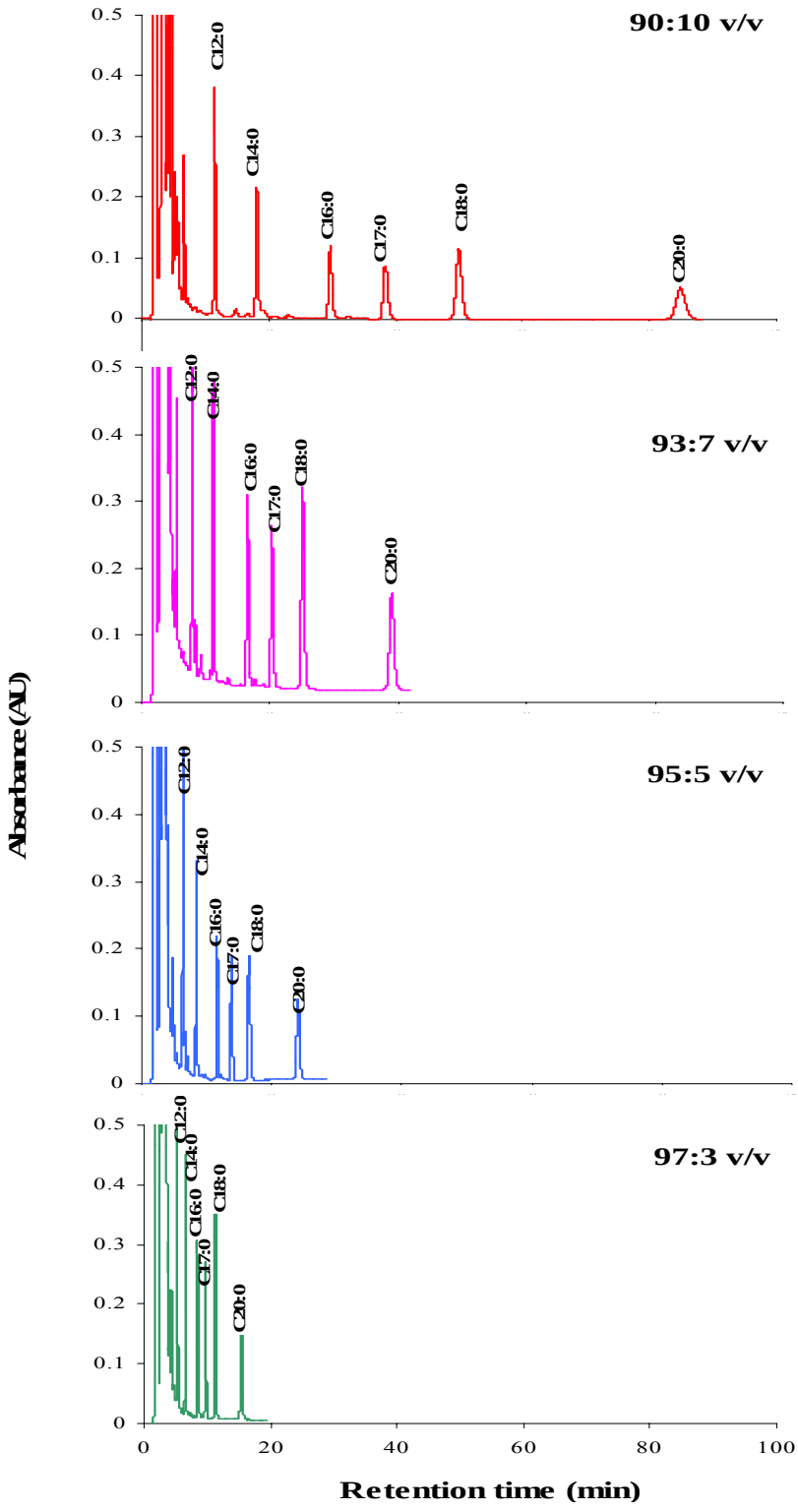

Figure 5.7 Chromatograms standard FA-naphthacyl esters 10.0 µg/mL by varying mobile phase composition of MeOH: H_2O, flow rate 1.0 mL/min, detector set at 246 nm.

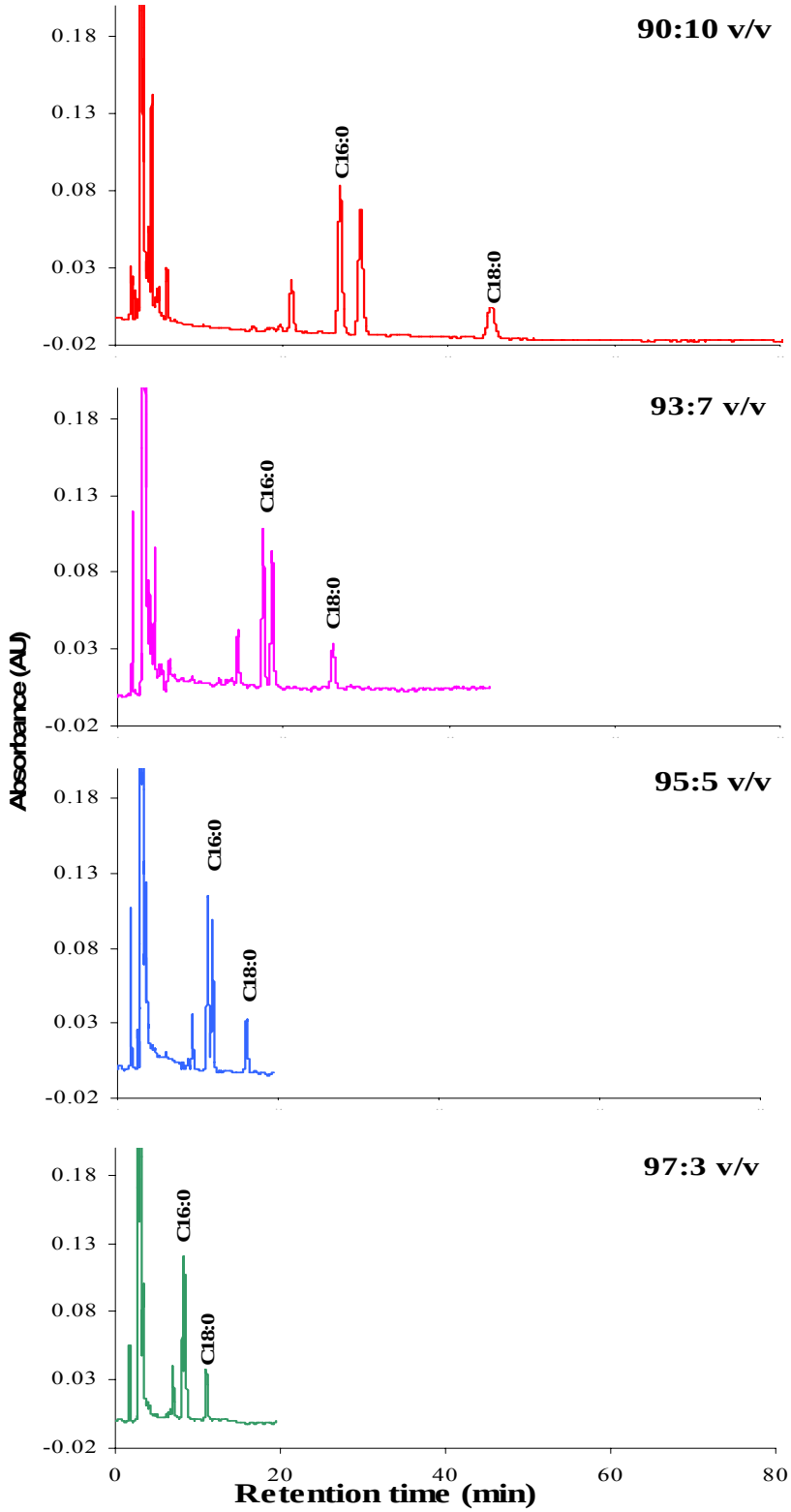

Figure 5.8 Chromatograms standard FA-naphthacyl esters (prepared from 10.0 µg/mL phosphatidylcholin) varying mobile phase composition of MeOH: H_2O, flow rate 1.0 mL/min, detector set at 246nm.

5.4 HPLC conditions

The optimum condition for the analysis of C12:0, C14:0, C16:0, C18:0 and C20:0 as phenacyl ester and naphthacyl ester was 93% of MeOH in H_2O as a mobile phase at the flow rate of 1.0 mL/min. Hypersil ODS C18 column was used. And detector was setted at 242 nm and 246 nm for phenacyl ester and naphthacyl ester, respectively.

Table 5.1 Comparison the retention time between phenacyl and naphthacyl ester.

FAs	Retention time (min) (mean $\pm$ SD)	
	Phenacyl ester	Naphthacyl ester
C12:0	5.19 $\pm$ 0.012	6.52 $\pm$ 0.045
C14:0	7.15 $\pm$ 0.020	9.29 $\pm$ 0.090
C16:0	10.83 $\pm$ 0.055	13.85 $\pm$ 0.200
C17:0	12.68 $\pm$ 0.068	17.12 $\pm$ 0.085
C18:0	15.63 $\pm$ 0.105	21.31 $\pm$ 0.410
C20:0	24.18 $\pm$ 0.216	33.53 $\pm$ 0.926

From Table 5.1, FA-phenacyl esters obtained shorter analysis time than FA-naphthacyl esters. Since, FA-phenacyl esters have interaction with stationary phase less than FA-naphthacyl esters However, increasing the flow rate and/or column temperature may observe shorten analysis time by keeping the column pressure as low as possible.

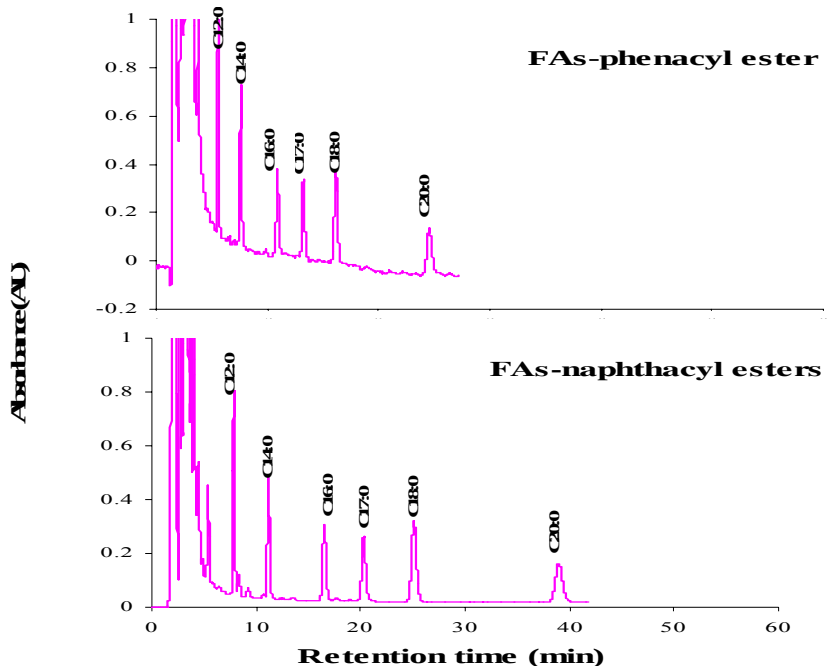

Figure 5.9 Comparison chromatograms of 10 µg/mL FA- phenacyl esters and FA-naphthacyl esters by using 93%MeOH in H_2O, flow rate 1.0 mL/min, detector set at 242 and 246 nm.

FAs contained in real sample are normally composed of saturated and unsaturated FAs, which have the same chain length and different degrees of unsaturation and also different chain lengths and the same degree of unsaturation. These tendencies lead to the occurrence of several pairs of FAs that are difficult to separate. The separation may be considered a criterion of the resolution efficiency of an analytical procedure. It was extremely difficult to resolve C16:0 and C18:1 or C14:0 and C16:1 or C14:0 and C18:3 [Halgunset *et al.*, 1982, Hanis *et al.*, 1988]. In this work, critical pair of FA-naphthacyl esters (like C14:0-C16:1 C16:0-C18:1 and C18:2) were studied by using MeOH-H_2O system. The order of elution and the resolution of a mixture of naphthacyl ester of saturated, monoenoic and polyenoic FAs are given in Figure 5.10. The mobile phase of 93% MeOH gave a good separation of all FA-naphthacyl esters with resolution $\geq$ 1.5 for any two adjacent peaks. As the number of double bond in the hydrocarbon chain increase, the elution

order decrease. This is well illustrated with C18:0, C18:1 and C18:2 all of which contain 18 carbon atoms with 0, 1 and 2 double bonds, respectively.

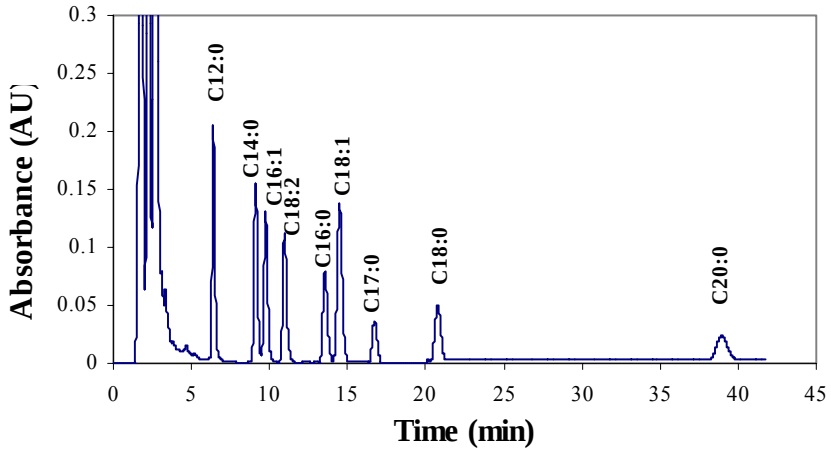

Figure 5.10 Chromatograms of 10 µg/mL mixture standard saturated and unsaturated FA-naphthacyl esters by using 93%MeOH, flow rate 1.0 mL/min, detector set at 246 nm.

5.5 Study of Derivatization

In section 5.2 it was found that FA-phenacyl esters and FA-naphthacyl esters have maximum absorption wavelength at 242 and 246 nm. In this section, factors such as reaction time, reaction temperature and amount of derivatizing agent (PBr, NAPHBr) were studied in order to maximize the absorption. C17:0 was added in the derivatization reaction as internal standard for verification HPLC system.

5.5.1 Phenacyl derivatization

5.5.1.1 Reaction time

The completeness of esterification for FA-phenacyl esters was investigated. The time of deivatization was varied from 5 to 60 min at 100^{o}C of reaction temperature. Results are shown in Figure 5.10. Yield of FA-pencil esters raised rapidly and reached its plateau at 15 min. So, the reaction was completely in 15 min with heating.

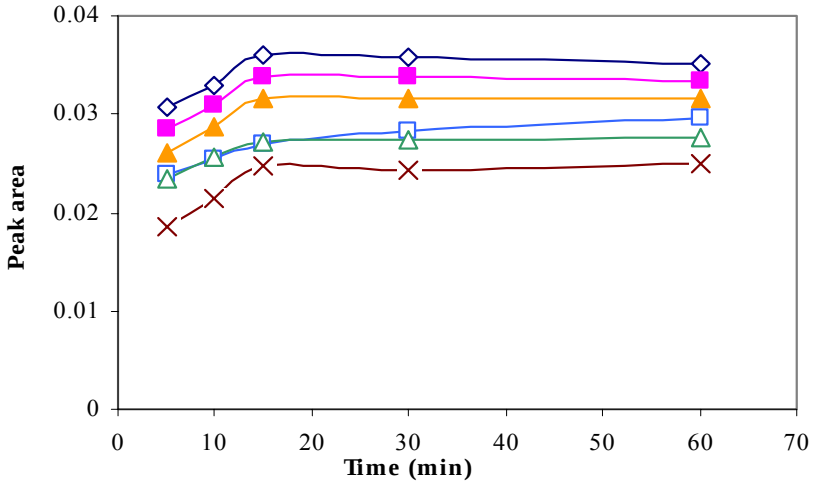

Figure 5.11 Effect of reaction time for FA as phenacyl derivatives; (◇) C12:0, (■) C14:0, (▲) C16:0, (△) C18:0, (x) C20:0 and (□)C17:0 used as internal standard.

5.5.1.2 Reaction temperature

Temperature of derivatization can affect the rate of conversion. Temperature was studied by varying from 50 to 100°C, using 30 min of the reaction time. Results are shown in Figure 5.11. When the temperature increased, the amount of FA-phenacyl derivatives increased. The reaction mixture was significantly enhanced by using heat, when compared FA-phenacyl esters prepared overnight at room temperature [Wood and Lee, 1983]. Heating reduces the time that requires for produce the derivatives. However, higher temperature more than 100°C was not studied. Since, it might be too much heat and evaporated the volatile compounds to boil the solution. The optimum temperature was selected at 100°C for 30 minute as compromising between sensitivity and boiling effect.

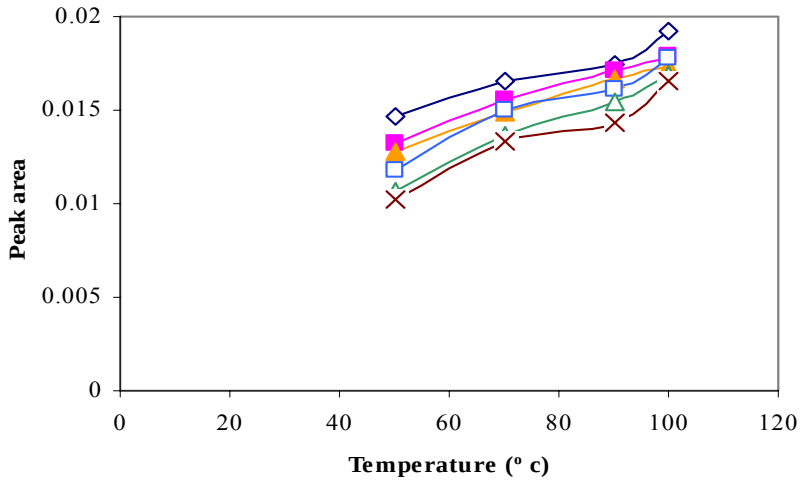

Figure 5.12 Effect of reaction temperature for the FA as phenacyl derivatives ; (◇) C12:0, (■) C14:0, (▲) C16:0, (△) C18:0, (x) C20:0 and (□)C17:0 used as internal standard.

5.5.1.3 Effect of PBr.

Amount of PBr reagent on derivatization of FA as phenacyl esters was studied. A fixed concentration of standard FAs (1.0, 10.0 ug/mL) solution and variable volume of 20 mg/mL PBr were prepared. The volume of PBr varied from 5 to 300 µL corresponding to PB: FA mole ratio of *ca*. 25 to 150, respectively. The results (see Table 5.2) showed that, when the mole ratio of PBr: FAs increased. The absorbance of FAs increased too. Maximum and constant peak areas could be attained with added amounts of PBr 100 times higher than total FAs. Excess PBr usage showed no significantly different with the detection response. Therefore, the amount of PBr concentration was employed as a compromising between the sensitivity and reagent consumption.

Table 5.2 The effect of amount of PBr on peak area of FA compounds

Mole of total FAs	Volume of PBr(µL)	Mole of PBr	Mole ratio PBr: FAs	Peak area (V·s)				
				C12:0	C14:0	C16:0	C18:0	C20:0
1µg of each	5	5×10^{-7}	25	0.0189	0.0195	0.0143	0.0093	0.0104
FFAs or	10	1×10^{-6}	50	0.0210	0.0203	0.0173	0.0128	0.0138
2.0×10^{-8}	20	2×10^{-6}	100	0.0207	0.0203	0.0171	0.0167	0.0200
mole	30	3×10^{-6}	150	0.0203	0.0204	0.0171	0.0166	0.0206
10 µg of	1	1×10^{-6}	5	0.0310	0.0258	0.0130	0.0113	0.0085
each FFAs	50	5×10^{-6}	25	0.1267	0.1090	0.0688	0.0681	0.0516
or	100	1×10^{-5}	50	0.2114	0.1910	0.1327	0.1441	0.1195
2.0×10^{-7}	200	2×10^{-5}	100	0.2472	0.2486	0.1733	0.1991	0.1684
mole	300	3×10^{-5}	150	0.2479	0.2516	0.1732	0.1996	0.1686

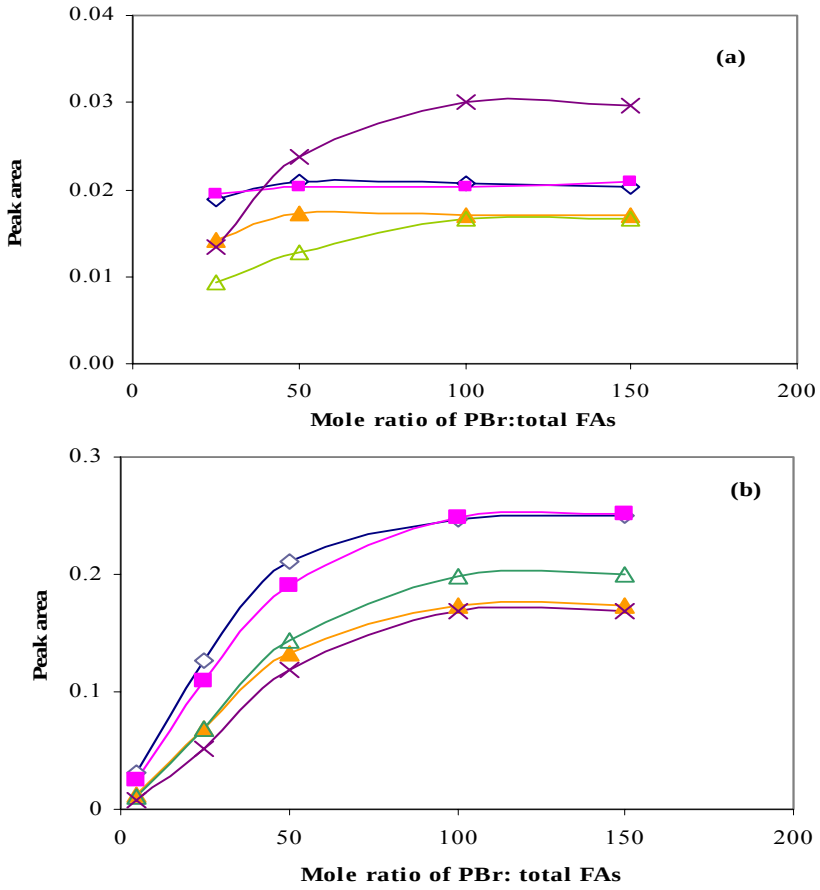

Figure 5.13 Effect of PBr on derivatization; (a) 1µg of each FAs, (b) 10 µg of each FAs.

5.5.2 Naphthacyl derivatization

5.5.2.1 Reaction time

An appropriate time in the derivatization for FAs-naphthacyl esters were studied. The time of deivatization was varied from 5 to 60 min at a fixing temperature of 90°C. Result in Figure 5.13 indicated that conversion of FAs increased with a longer time. Reaction time of 30 min at 90°C was chosen. For 30 min of reaction, the maximum derivatization is accomplished at this time for naphthacyl derivatization.

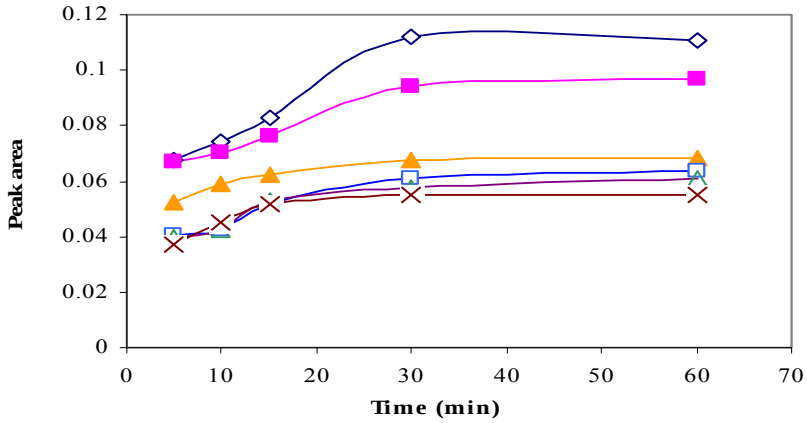

Figure 5.14 Effect of reaction time for the FA as naphthacyl derivatives; (◇) C12:0, (■) C14:0, (▲) C16:0, (△) C18:0, (x) C20:0 and (□)C17:0 used as internal standard.

5.5.2.2 Reaction temperature

In order to increase reaction rate, the reaction temperature of FA-naphthacyl esters was employed using a heating block by varying the temperature from 50 to 100°C for 30 min. Effects of temperature on sensitivity are presented in Figure 5.14. Result showed that the conversion of FA-naphtnhacyl esters was accelerated with higher temperature. The optimum temperature was selected at 90°C for 30 min for the preparation of FA-naphthacyl esters.

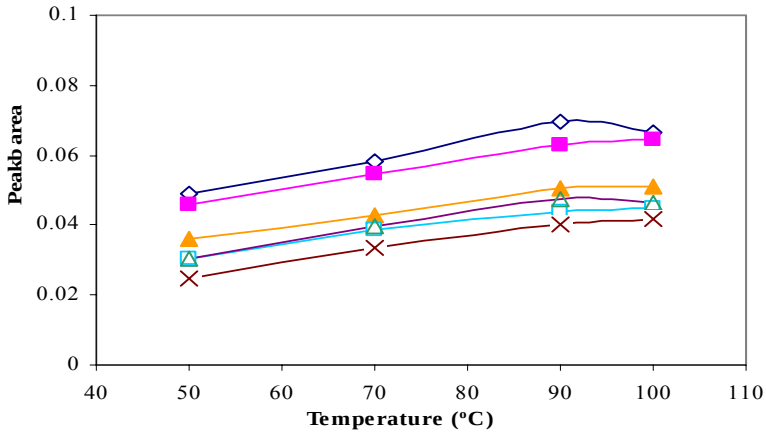

Figure 5.15 Effect of reaction temperature for the FA as naphthacyl derivatives; (◇) C12:0, (■) C14:0, (▲) C16:0, (△) C18:0, (X) C20:0 and (□)C17:0 used as internal standard.

5.5.2.3 Effect of NAPHBr

Amount of NAPHBr reagent on derivatization of FA-naphthacyl esters was studied. A fixed concentration of standard FAs (1.0, 10.0 ug/mL) solution and variable volume of 20 mg/mL NAPHBr were prepared. The volume of NAPHBr varied from 1 to 300 μL corresponding to NAPHBr: FA mole ratio of *ca*. 4 to 120, respectively. The results (see Table 5.3) showed that, the increasing in mole ratio of NAPHBr: FAs resulted increasing in sensitivity of the FA derivatives. In order to improve absorbance, the deivatizing reagent was added in excess. If species were added to the overall reaction, the reaction will favor the side opposing the addition of the species. This excess of NAPHBr should be sufficient to react completely FAs as napthacyl ester. Therefore, the mole ratio between NAPHBr: FAs of *ca*.80 to 1 was selected as the optimum condition.

Table 5.3 The effect of amount of NAPHBr on peak area of FA compounds

Mole of total FAs	Vol. of NAPHBr	Mole of NAPHBr	Mole ratio NAPHBr: FAs	Peak area (V·s)				
				C12:0	C14:0	C16:0	C18:0	C20:0
1µg of each FAs or 2.0×10^{-8} mole	1	8×10^{-8}	4	0.0444	0.0470	0.0327	0.0184	0.0186
	5	4×10^{-7}	20	0.0763	0.0700	0.0504	0.0466	0.0313
	10	8×10^{-7}	40	0.0796	0.0725	0.0528	0.0464	0.0358
	20	1.6×10^{-6}	80	0.0803	0.0751	0.0567	0.0470	0.0359
	30	2.4×10^{-6}	120	0.0809	0.0751	0.0561	0.0467	0.0358
10 µg of each FAs or 2.0×10^{-7} mole	10	8×10^{-7}	4	0.1091	0.0734	0.0708	0.0432	0.0325
	50	4×10^{-6}	20	0.4304	0.3659	0.2694	0.1898	0.2216
	100	8×10^{-6}	40	0.6826	0.5906	0.4654	0.5005	0.4444
	200	1.6×10^{-5}	80	0.7975	0.7343	0.5959	0.6275	0.5503
	300	2.4×10^{-5}	120	0.7969	0.7465	0.5981	0.6250	0.5527

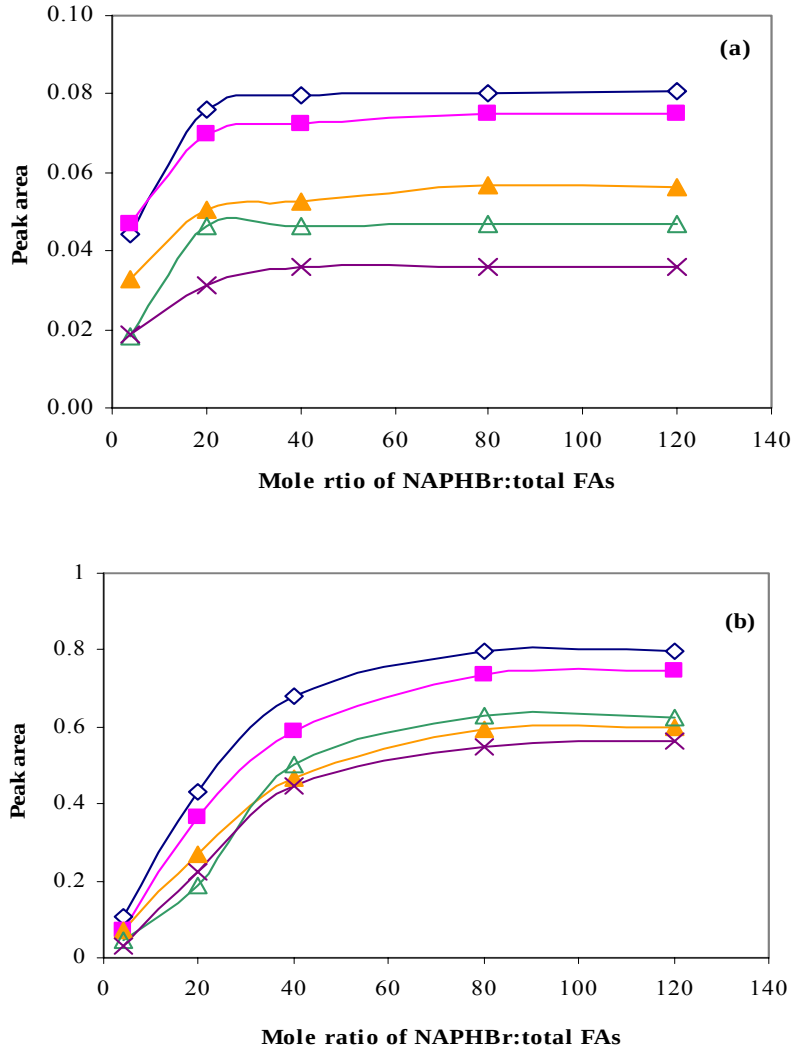

Figure 5.16 Effect of NAPHBr on derivatization ; (a) 1µg of each FAs, (b) 10 µg of each FAs.

5.6 Method performance

5.6.1 FA as phenacyl ester

Analytical data and linear equation of FA as phenacyl ester derivatives at the concentration range of 0.5, 1.0, 10.0, 20.0 and 50.0 µg/mL under the optimum condition are shown in Table 5.4.

Table 5.4 Comparison between external standard and internal standard calibration and detection limit using peak area from standard mixture of phenacyl esters.

FAs	Conc.range (μg/mL)	External standard	Internal standard	DL (ng/mL)
C12:0	0.5 -50	y = 0.0717x + 0.0006 R^2 = 1.0	y = 0.2376x - 0.1316 R^2 = 0.9989	6.4
C14:0	0.5-50.0	y = 0.0701x + 0.0047 R^2 = 0.9998	y = 0.2325x - 0.1177 R^2 = 0.9993	11.4
C16:0	0.5-50.0	y = 0.0524x +0.0167 R^2 = 0.9996	y = 0.174x +0.0624 R^2 = 0.9992	35.6
C18:0	0.5-50.0	y = 0.0829x - 0.0117 R^2 = 1.0	y = 0.2749x - 0.1872 R^2 = 0.9986	36.1
C20:0	0.5-50.0	y = 0.0627x - 0.0111 R^2 = 1.0	y = 0.2077x - 0.1473 R^2 = 0.9987	41.7

The results of the quantitative investigation are shown in Figure 5.17, which is plotted concentrations against peak area of each FA-phenacyl esters and peak ratio between internal standard (C17:0) and each FA-phenacyl esters standard. The plot gave linearity in a range from 0.5-50.0 μg/mL. This result indicates that the derivatization method can be used to quantify FAs in the linearity range that is given.

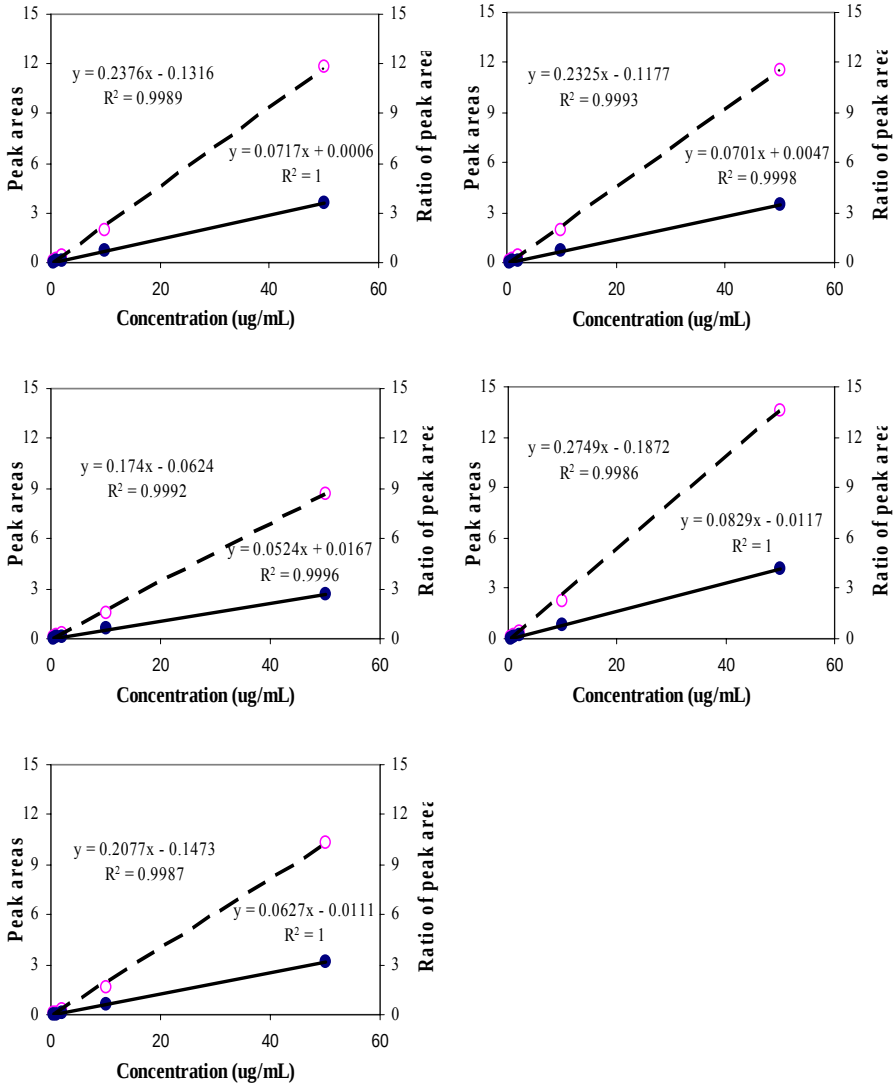

Figure 5.17 External (●) and internal curve (○) for the quantitation of FA as phenacyl derivatives; (a) C12:0, (b) C14:0, (c) C16:0, (d) C18:0, (e) C20:0 separated using the Hypersil ODS column.

5.6.2 FA as naphthacyl derivatives

The calibration graph for FA as naphthacyl derivatives including C12:0, C14:0, C16:0, C18:0 and C20:0 from 0.1, 1.0, 5.0, 10.0 and 50.0 µg/mL under the optimum condition is shown in Table 5.5.

Table 5.5 Comparison between external standard and internal standard calibration and detection limit using peak area from standard mixture of naphthacyl esters.

FAs	Conc. range (μg/mL)	External standard	Internal standard	DL (ng/mL)
C12:0	0.1-50.0	y = 0.2002x + 0.1322 R^2 = 0.9975	y = 0.1438x + 0.1823 R^2 = 0.9924	5.5
C14:0	0.1-50.0	y = 0.2098x + 0.093 R^2 = 0.9989	y = 0.1507x + 0.1479 R^2 = 0.9955	6.2
C16:0	0.1-50.0	y = 0.1704x + 0.0719 R^2 = 0.9996	y = 0.1223x + 0.1195 R^2 = 0.9976	6.8
C18:0	0.1-50.0	y = 0.2506x + 0.081 R^2 = 0.9994	y = 0.1805x + 0.1507 R^2 = 0.9969	9.3
C20:0	0.1-50.0	y = 0.1957x + 0.0162 R^2 = 0.9999	y = 0.1413x + 0.077 R^2 = 0.9985	12.2

The results of the quantitative investigation are shown in Figure 5.18, which are plotted the concentration against peak area of each FA-naphthacyl esters and peak area of internal standard (C17:0) and each FA-naphthacyl esters standard. This result indicates that the derivatization method can be used to quantify FAs in a given linearity range of 0.1-50.0 μg/mL.

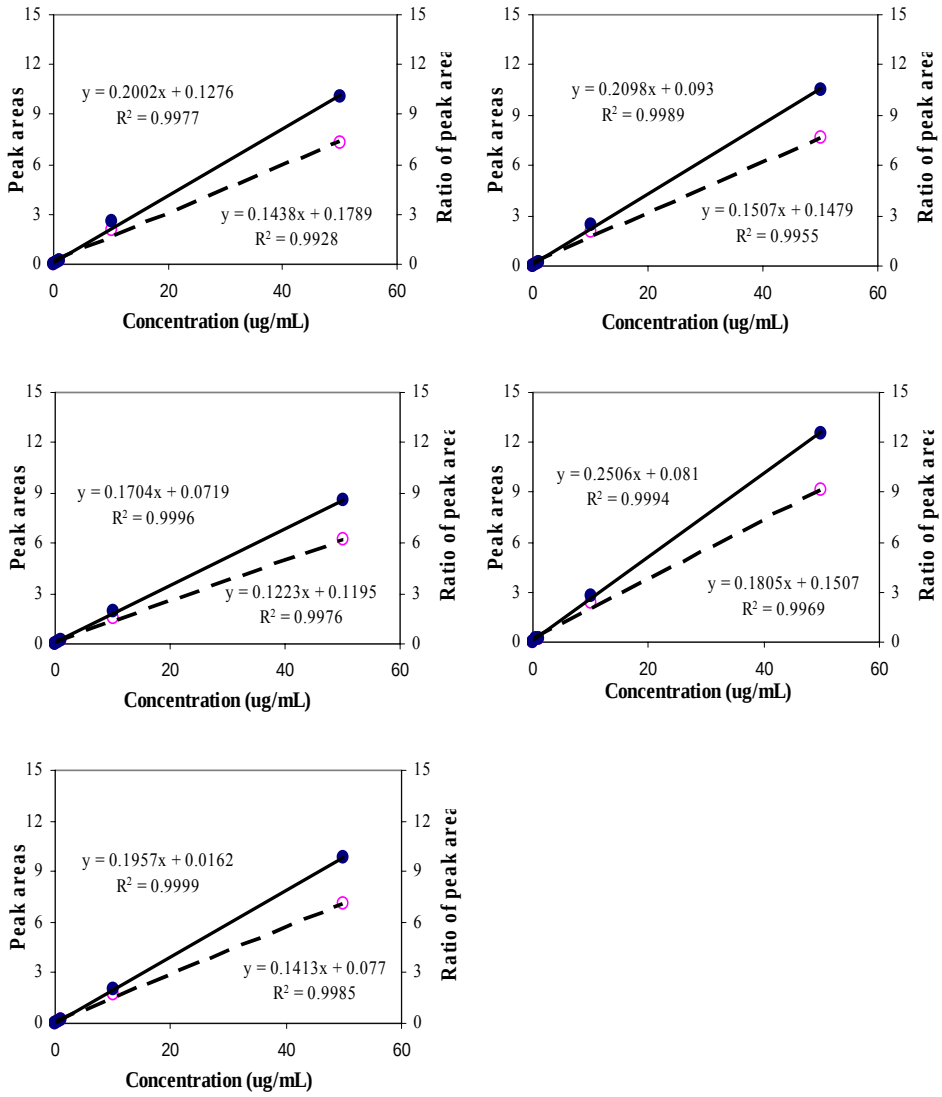

Figure 5.18 External (●) and internal curve (○) for the quantitation of FA as naphthacyl derivatives; (a) C12:0, (b) C14:0, (c) C16:0, (d) C18:0, (e) C20:0 separated using the Hypersil ODS column.

The chromophoric reagents for forming FAs are effectively to improve the sensitivity of the analysis. The detection limits were in ng/mL level. In addition, FAMEs analysis using GC-FID was developed in our research group. Comparison the detection of limits for FAs derivatives between HPLC-UV and GC-FID are shown in Table 5.6. Detection of naphthacyl derivatives was shown to be highly sensitive. So, this chromophoric agent is very useful for the analysis of FA in very low concentrations, enhance with various detection system.

Table 5.6 Comparison detection limits of FA ester derivatives with various derivatizing agent between HPLC-UV and GC-FID.

FAs	Detection limit (ng/mL)			
	HPLC-UV		GC-FID	
	PBr	NAPHBr	5%H_2SO_4	14%BF_3
C12:0	6.4	5.5	270	240
C14:0	11.4	6.2	230	230
C16:0	35.6	6.8	210	200
C18:	36.1	9.3	190	180
C20:0	41.7	12.2	240	210

5.7 Sample preparation

Sample preparation of FAs consists of three steps. The first step is liquid-liquid extraction in order to remove non-lipid from the sample. The method was optimized from Bligh and Dyer procedure [1959].The second step is to separate lipid classes using solid-phase-extraction (SPE). In this step, we focus on the optimization condition for separation between FFAs and PLFAs. Final step is the hydrolysis of PLFAs to release FFAs.

5.7.1 Comparison between Silica and NH_2 bonded phase SPE column.

Packing material were Silica and NH_2 which packed in a column. The column was conditioned with 5.00 mL of hexane. Solution of 1000 µg/mL standard phosphatidylcholin (100 µL) was loaded into the column. Eluent step was carried out with different solvents for lipid fractionation. The volume of eluent was fixed at 10.00 mL and phosphatidylcholin was eluted with MeOH. Results are shown in Table 5.6. Recovery greater 80% was obtained with the used of NH_2 column. Polarity of NH_2 bonded phase is similar to phosphatidylcholin and comparable elution efficiency with MeOH. Therefore, the separation of lipid classes was performed by using NH_2 column.

Table 5.7 Comparison elution recoveries of FAs in phosphatidylcholin standard obtained from NH_2 and silica columns

Ester compound of	%Recovery (mean± SD)	
	NH_2 column	Silica column
C16:0	88.96 ± 3.76	47.56 ± 5.48
C18:0	86.06 ± 3.69	43.62 ± 6.36

5.7.2 Study of overloading column

The effect of sample loading was studied in the range of 20 to 2000 µL using 1000 µg/mL phosphatidylcholin. Our studying was performed in this range of sample loading volume to investigate the performance of the NH_2 column. The results are shown in Table 5.7 and Figure 5.18. It could be seen that there was no overloading of the analyte on the NH_2 column up to 2000 µL the volume. However, greater volume more than 2000 µL was not investigated in this work. This concentration was considered to be large enough to detect in the environmental sample.

Table 5.8 Analytical data obtained from the study of overloading column

Volume of PC* (µL)	Peak Area (V·s)		Regression line correlation coeff.
	C16:0	C18:0	
20	1.024	0.945	
50	1.945	1.312	y = 0.2847x + 0.0077
100	2.920	1.810	R^2 = 0.9985
200	5.549	2.933	
500	12.583	5.702	y = 0.1298x + 0.3563
1000	29.239	13.542	R^2 = 0.997
2000	56.987	26.483	

PC = Phosphatidylcholin

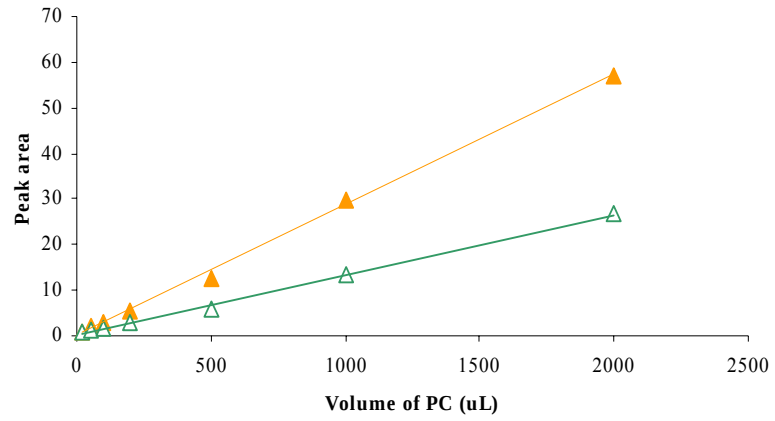

Figure 5.19 Calibration curve from the study of the overloading column

5.7.3 Separation between FFAs and phosphatidylcholin

Using NH_2 column was investigated for the efficiency of fractionation between FFAs and phosphatidylcholin. A mixture of C16:0, C18:0 (5.0, 10.0 and 20.0µg/mL) and phosphatidylcholin (10.0, 25.0 and 50.0 µg/mL) was loaded into the column. Eluent fraction was performed in collecting 1.0 mL for each fraction. FFAs fraction, 2% HOAc in ether was used to elute FFAs. Phosphatidylcholin fraction, MeOH was used to elute phosphatidylcholin. Then, elution profile from the 1st fraction to the 20th fraction was constructed by comparing with the concentration values. Results are in Figure 5.20.

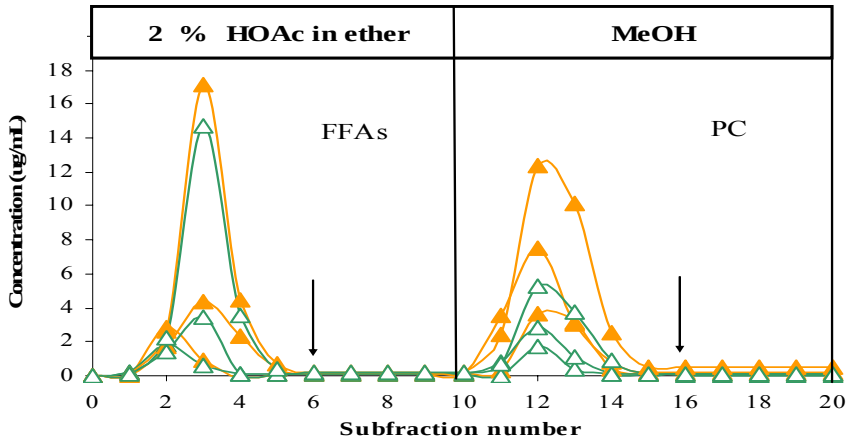

Figure 5.20 The elution volume of 2% HOAc in ether for elution at 5, 10 and 20 µg/mL (▲) C16:0, (△) C18:0 and the elution volume of MeOH for elution (▲) C16:0, (△) C18:0 from 10, 25 and 50 µg/mL phosphatidylcholin.

These compounds at difference concentration were eluted with the same volumes. So, volumes of eluent did not depend on the amount of sample loading. It could be seen that the optimum elution volume were 6.00 mL for each fractionated lipid classes.

5.7.4 Recovery study

Recovery of the method was investigated on two types of samples (soil and cultured bacteria). The recovery and precision of FFAs from phosphatidylcholin are shown in Table 5.8. The method provided satisfactory recoveries for both types of samples (soil: 79.0-89.5 %, cultured bacteria 78.9-84.1 %). The optimizing technique is successfully used in environmental sample. Especially, PLFAs fraction is the most useful for studying bacterial communities.

Table 5.9 Percent recovery of FFAs from phosphatidylcholin in sample

Sample	Volume of PC	%Recovery (mean± SD)	
		C16:0	C18:0
Soil	50 µL	80.1 ± 2.2	79.0 ± 5.2
	100 µL	89.5 ± 4. 8	84.3 ± 7.8
	200 µL	84.7 ± 3. 6	87.1 ± 3.3
Cultured bacteria	50 µL	84.0 ± 4.1	81.9 ± 5.9
	100 µL	79.1 ± 2.8	78.9 ± 5.0
	200 µL	79.7 ± 1.9	84.1 ± 3.5

This method involving several steps, it may be loss of the analytes. The extraction step with hexane can be corrected by using an internal standard (C17:0). Results are presented in Table 5.9.

Table 5.10 Percent recovery of C17:0 in the extraction of hexane

sample	C17:0 concentration (Mean±SD)/µg/mL		Recovery (%)
	Added	Found	
Soil	5.00	4.57 + 0.08	91.34
Cultured bacteria	5.00	4.72 + 0.06	94.32

5.8 Study of FAs profile

The total FAs ester linked to PLFAs a useful as indicators of total microbial biomass and the community structure in environmental sample. The shift in the FA profiles can be detected the changes in community composition. The method has been used for evaluation microbial community shift in different soil type. In this experiment, FAs profile in soil and sediment sample sampling from Phayathai and Salaya campus were compared. These samples showed similar pattern as shown in Figure 5.21.

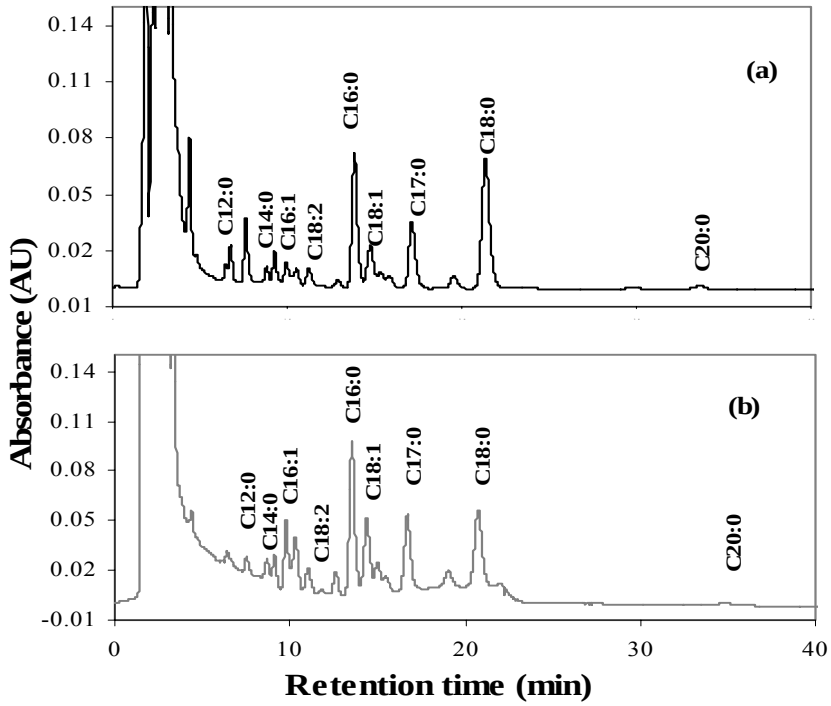

Figure 5.21 FAs profiles of (a) soil at Mahidol Phayathai, and (b) sediment at Mahidol Salaya derivatized with NAPHBr and C17:0 used as internal standard

No significant shifts were observed in the composition of the FA profiles in both samples. However, at different conditions such as temperature, pH or heavy metal may be involved the changes in the microbial community composition.

The information of FAs profile in cultural bacteria was studied. From Figure 5.22, non-coliform showed different peaks (t_R = 11.6, 13.4 and 20.4) when compared with coliform. Predomainantly, C16:0 and C18:0 were found in higher content in these samples. But straight chain saturated FAs in bacteria and other organism could not use to confirm the organism. The comparisons of FAs profile should not consider only these FAs at high concentration. The FAs present in small amounts must be analyzed. In this study, C18:2 (linoleic acid) were found in less amount which as biomarker for fungi.

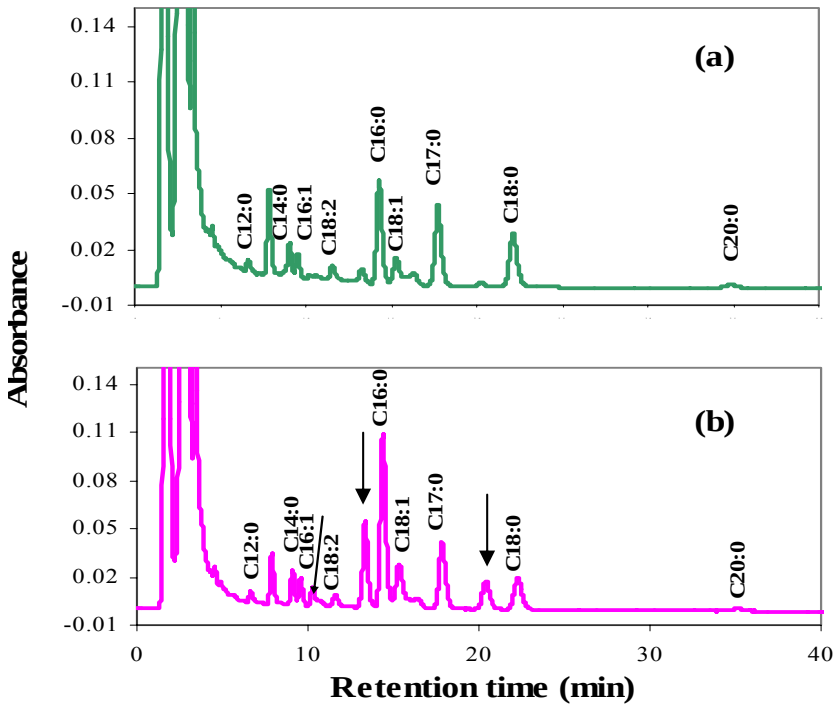

Figure 5.22 FAs profiles of (a) non- choliform, and (b) choliform when derivatized with NAPHBr and C17:0 used as internal standard

Table 5.11 Percentage of saturated FA-naphthayl esters from various samples.

FAs	Respond factor	% saturated FAs composition			
		Soil	Sediment	Non-coliform	Coliform
C12:0	1.44	7.73	6.58	1.71	4.28
C14:0	1.37	4.67	4.39	4.75	6.25
C16:0	1.24	28.47	38.06	40.42	44.54
C18:0	1.01	31.60	44.11	8.75	1.80
C20:0	0.86	1.33	1.45	0.90	2.08

From the chromatogram of real sample, elution with MeOH-H_2O mobile phase only could not resolve some adjacent peaks of C14:0, C16:1 and some unidentified peaks. To optimize chromatographic resolution, the effect of mobile phase composition could be improved. Glajch and Kirkland [1983] optimized mobile phase selectivity, with the addition of a proton donor solvent (such as ACN) to a proton acceptor solvent (such as MeOH) leads to improved the resolution primarily due to hydrogen bonding and dipole-dipole interaction. Whereas, in ACN the number of double bonds seems to be more important, in MeOH the chain length seems to be more important. Utilization of the different properties of these two solvent offer some possibility for the separation of FAs. For example, the increasing in retention times with decreasing ACN concentration is greatest for longer chain FAs. Conversely, as the solvent strength increases, the retention time for shorter chain length or higher degree of unsaturation will decrease.

5.9 Confirmation of FAs with MS detection

Mass spectrometry was used to support structure information of FA-naphthacyl esters. The characterization by retention time (comparison with those standards) and/or UV spectrum is not sufficient. The structure of FA-naphthacyl esters were confirmed by considering the molecular mass or/and fragmentation of derivative. The result shown in Table 5.9 and mass spectrum see in Appendix C.

Table 5.12 Molecular mass data of FA-naphthacyl esters fragmentation

Ester	$[M+1]^+$	$[NaphCH(OH)CHOH]^+$	$[NapCO]^+$
Laurate	369	187	155
Myristate	397	187	155
Palmitate	425	187	155
Stearate	453	187	155
Arachidate	481	187	155

In all ester, the base peak was found at m/z=155 which was assigned to $[NapCO]^+$. In addition, all ester showed rather prominent ion at m/z =187 which may be attributed to the protonated species $[NaphCH(OH)CHOH]^+$.

5.10 Suggestion of Future Work

The method is effective for the determination of FAs in microorganism. Future works should be carried out on other FAs which are present s biomarker of specific group and indicators of microbial stress. Available nutrient status, pH, heavy metal and organic carbon of the soil are also used for monitoring the response of stress condition on the soil environmental. However, they are not sensitive enough to predict early change in the soil status. On the other hand, the soil microbial properties respond much faster to disturbance and perturbation. The induction of certain changes in FAs components can be studied by measuring ratio of *trans* to *cis*–monoenoic unsaturated FAs, proportion of cyclopropyl FAs specific sensitivity to heavy metal and biodiversity assessment [Kaur *et al*., 2005]. The response PLFAs is Fgure 5.23

At present, bio-diesel is an alternative energy from vegetable oil or animal fats for using in diesel oil substitution. The method development also can be used to apply for determining the FAs of oils or fats (triglyceride) in bio-diesel.

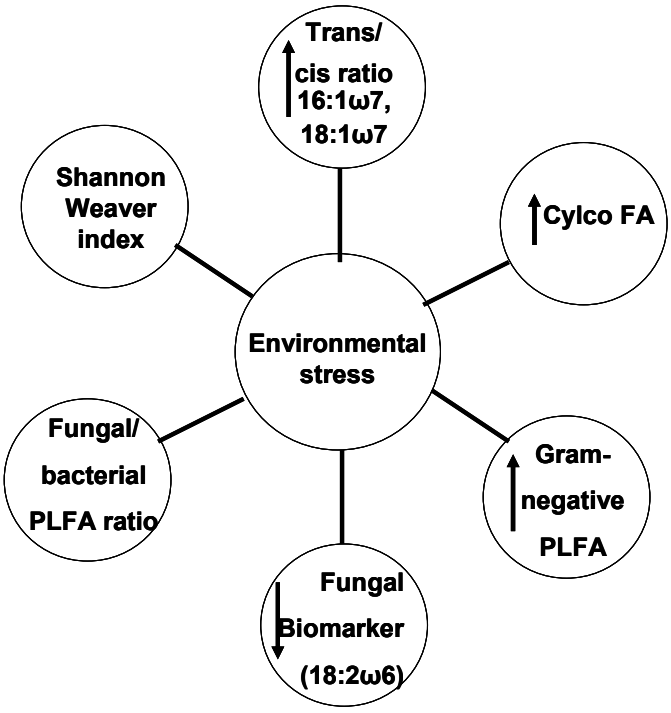

Figure 5.23 Response of PLFA biomarkers to environment stress.

CHAPTER 6
CONCLUSION

FAs have a potential to be biomarker for environmental studies because these compounds contain in living organisms. The profile of FAs provides information for FAs content and dependent and/or independent factors. Content and number of FAs are mainly obtained from chromatographic techniques. Since, saturated FAs do not absorb UV-Vis light, they can not be measured directly with the HPLC instrument. The derivatization methods were optimized and HPLC conditions were examined for determining FAs (C12:0, C14:0, C16:0, C18:0 and C20:0) and then the developed procedure was applied to measure FAs profile in bacterial culture and soil samples.

The derivatization processes were performed successfully by using PBr and NAPHBr. These derivatizing agents were utilized for preparation of FAs esters. The derivatives of these esters were shown to be very sensitive with the response to UV-Vis detector. The optimum condition found in this study is as follows: derivatizing agents 100 and 80 times more than total FAs (as calculated by mole ratio of Derivatizing:total FAs) and the reaction time of 15 min at 100^{o}C for phenacyl ester and 30 min at 90^{o}C for naphthacyl ester. The separation condition was performed by using Hypersil ODS C18 column and the MeOH: H_2O mobile phase with the ratio of 93:7, flow rate 1.0 mL/min. The derivatives of phenacyl esters showed less retentions time than naphthacyl esters. The concentration range of the analysis was in the range of 0.5-50 µg/mL and 0.1-50 µg/mL for phenacyl ester and naphthacyl ester, respectively and limit of detections were in the level of ppb. Further, we later used NAPHBr for studying condition of sample preparation for lipid classification and real sample analysis.

Sample preparation step consists of extraction, lipid classification and determination. In extraction step, a mixture of $CHCl_3$: MeOH: buffer was used as extractant and total lipids were obtained in $CHCl_3$ layer. The second step, total lipids

were loaded into the conditioned NH_2 column to separate neutral lipids, FFAs and PLFAs from the sample. The elution step from the SPE column was carefully investigated by using elution solvents beginning order of the elution with $CHCl_3$/2-propanol for neutral lipid, 2% HOAc in ether for FFAs and MeOH for PLFAs. The PLFAs were only studied by releasing FAs with alkaline hydrolysis and then extracted FAs with organic solvent. The obtained fraction was further derivatized and determined the FAs profile. Recovery study was performed by spiking standard PC into the samples and high recovery values (>79%) were obtained.

The optimized condition of sample preparation, derivatization and determination of FAs with HPLC system with UV-Vis detection was successfully employed for the cultured bacterial and soil samples. The profile of FAs obtained from PLFAs can be applied for studying contaminant effects in environment.

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APPENDIX

APPENDIX A

PREPARATION OF CALIBRATION GRAPH

The various volumes of working standard solution (100µg/ml), PBr solution (20 mg/mL), TEA solution (25 mg/mL) and HOAc solution (10 mg/mL) are shown in below.

FAs (µL)	PBr (µL)	TEA (µL)	HOAc (µL)
5	10	10	10
10	20	20	20
20	40	40	40
100	200	200	200
500	1000	1000	1000

The various volumes of working standard solution (100µg/mL), NAPHBr solution (20 mg/mL), TEA solution (25 mg/mL) and HOAc solution (10 mg/mL) are shown in below.

FAs (µL)	NAPHBr (µL)	TEA (µL)	HOAc (µL)
5	10	10	10
10	20	20	20
20	40	40	40
100	200	200	200
500	1000	1000	1000

APPENDIX B

DETERMINATION OF INSTRUMENTAL DETECTION LIMIT

The limit of detection is the lowest concentration of an analyte that an analytical process can reliably detect. In this study the method for calculating limit of detection was by injection standard solution at low concentration 5 times. The signal value 3 times of standard deviation was converted to the concentration to give the detection limit.

The calculation for FA-phenacyl esters are shown as follow:

FA-phenacyl esters	Conc. (µg/mL)	Peak height	Average	SD (n=5)	LOD (ng/mL)
Laurate	0.03	0.00029 0.00034 0.00035 0.00032 0.00033	0.00033	$2.3x10^{-5}$	6.4
Myristate	0.05	0.00037 0.00039 0.00033 0.00034 0.00033	0.00035	$2.68x10^{-5}$	11.4
Palmitate	0.1	0.00029 0.00033 0.00037 0.00028 0.00035	0.00032	$3.85*10^{-5}$	35.6

FA-phenacyl esters	Conc. (µg/mL)	Peak height	Average	SD (n=5)	LOD (ng/mL)
Stearate	0.15	0.00038 0.00039 0.00039 0.00036 0.00032	0.00037	$2.95x10^{-5}$	36.1
Arachidate	0.2	0.00034 0.00033 0.00030 0.00030 0.00029	0.00031	$2.17x10^{-5}$	41.7

Peak height 0.00033 was equalvalent to Laurate phenacyl 0.03 µg/mL

Peak height $3x2.3x10^{-5}$ was equalvalent to Laurate phenacyl ($3x2.3x10^{-5}$) x0.03/0.00033 = 6.4 ng/mL

The calculation for FA-naphthacyl esters are shown as follow:

FA-naphthacyl esters	Conc. (µg/mL)	Peak height	Average	SD (n=5)	LOD (ng/mL)
Laurate	0.015	0.00030 0.00030 0.00033 0.00030 0.00029	0.00030	1.52×10^{-5}	5.5
Myristate	0.015	0.00021 0.00024 0.00028 0.00025 0.00030	0.00026	3.51×10^{-5}	6.27
Palmitate	0.025	0.00026 0.00025 0.00024 0.00023 0.00029	0.00025	2.3×10^{-5}	6.8
Stearate	0.05	0.00019 0.00021 0.00022 0.00022 0.00022	0.00021	1.3×10^{-5}	9.3
Arachidate	0.1	0.00031 0.00034 0.00031 0.00032 0.00031	0.00032	1.3×10^{-5}	12.2

Peak height 0.00030 was equalvalent to Laurate naphthacyl 0.015 µg/mL.

Peak height $3x1.52x10^{-5}$ was equalvalent to Laurate naphthacyl

$(3x1.52x10^{-5}) \times 0.015/0.00030 = 5.5$ ng/mL

APPENDIX C

MASS SPECTRA OF NAPHTHACYL ESTER

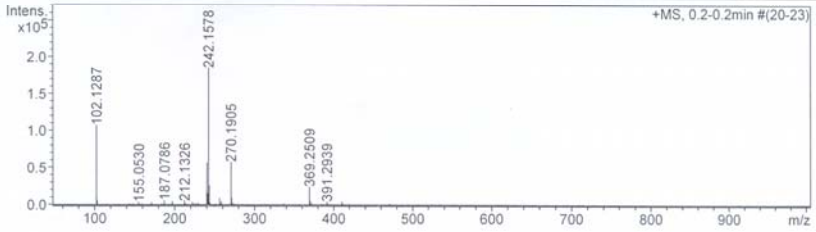
Mass Spectrum List Report

Analysis Info

Analysis Name	Lauric acid_21_01_3077.d	Acquisition Date	2/21/2007 9:57:12 AM
Method	suttiporn new.m	Operator	Administrator
Sample Name	Lauric acid Lauric acid	Instrument	micrOTOF 72

Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Corrector Fill	44 V
Scan Range	n/a	Capillary Exit	90.0 V	Set Pulsar Pull	383 V
Scan Begin	50 m/z	Hexapole RF	60.0 V	Set Pulsar Push	383 V
Scan End	1000 m/z	Skimmer 1	30.0 V	Set Reflector	1300 V
		Hexapole 1	30.0 V	Set Flight Tube	9000 V
				Set Detector TOF	2090 V



#	m/z	Res.	S/N	I	FWHM
1	102.1287	4940	17107.1	106867	0.0207
2	103.1323	5321	1053.7	6607	0.0194
3	155.0530	6404	360.3	3196	0.0242
4	171.0836	6305	218.1	2775	0.0271
5	187.0786	6349	336.8	5575	0.0295
6	197.0986	6446	245.8	4660	0.0306
7	212.1326	2997	209.7	4732	0.0708
8	222.1314	6895	154.0	3383	0.0322
9	226.1263	6688	83.8	1791	0.0338
10	229.0894	7137	103.2	2161	0.0321
11	240.1425	6530	2929.4	56321	0.0368
12	241.1478	6568	797.9	15223	0.0367
13	242.1578	6408	9766.1	184797	0.0378
14	243.1616	6759	1367.3	25669	0.0360
15	244.1650	7143	109.7	2047	0.0342
16	256.1383	6915	524.1	8819	0.0370
17	257.1415	6828	86.3	1442	0.0377
18	258.1527	7498	282.8	4674	0.0344
19	270.1905	6950	3907.1	57471	0.0389
20	271.1940	7388	643.7	9373	0.0367
21	272.1801	4637	112.7	1627	0.0587
22	274.1496	7345	92.9	1313	0.0373
23	337.1300	8006	115.2	1403	0.0421
24	369.2509	8349	2157.3	24091	0.0442
25	370.2549	8202	444.3	4948	0.0451
26	372.2059	7627	170.9	1893	0.0488
27	391.2939	7736	402.0	4160	0.0506
28	410.2226	7408	415.2	4002	0.0554
29	411.2255	7575	117.7	1133	0.0543
30	470.3396	7791	154.1	1118	0.0604

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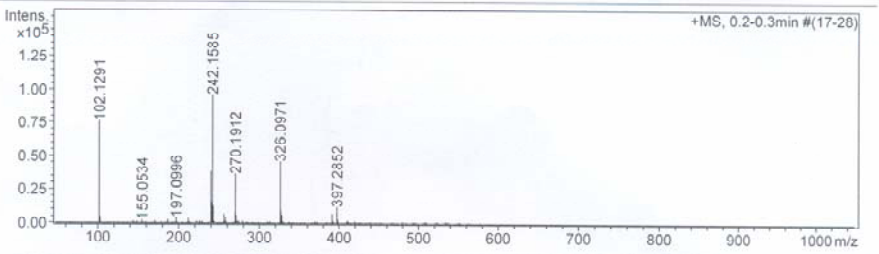
Mass Spectrum List Report

Analysis Info

Analysis Name	Myristic acid_15_01_3076.d	Acquisition Date	2/21/2007 9:51:23 AM
Method	suttiporn new.m	Operator	Administrator
Sample Name	Myristic acid	Instrument	micrOTOF 72
	Myristic acid		

Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Corrector Fill	44 V
Scan Range	n/a	Capillary Exit	90.0 V	Set Pulsar Pull	383 V
Scan Begin	50 m/z	Hexapole RF	60.0 V	Set Pulsar Push	383 V
Scan End	1000 m/z	Skimmer 1	30.0 V	Set Reflector	1300 V
		Hexapole 1	30.0 V	Set Flight Tube	9000 V
				Set Detector TOF	2090 V



#	m/z	Res.	S/N	I	FWHM
1	102.1291	5003	16661.3	75855	0.0204
2	103.1327	5354	1036.0	4744	0.0193
3	144.0608	6008	250.7	1388	0.0240
4	155.0534	6503	327.8	2217	0.0238
5	171.0845	6075	165.7	1540	0.0282
6	187.0791	6091	223.9	2642	0.0307
7	197.0996	6503	288.8	3861	0.0303
8	212.1207	3348	217.7	3426	0.0634
9	222.1315	6674	87.5	1395	0.0333
10	226.1277	6465	91.3	1447	0.0350
11	240.1435	6851	2495.1	38668	0.0351
12	241.1492	6952	996.9	15429	0.0347
13	242.1585	6660	6223.8	96166	0.0364
14	243.1624	6620	807.1	12455	0.0367
15	256.1390	6850	449.2	6802	0.0374
16	258.1534	7481	275.8	4165	0.0345
17	270.1912	6909	2467.7	36572	0.0391
18	271.1949	6999	400.0	5922	0.0387
19	272.1789	4900	98.2	1453	0.0555
20	274.1499	7384	100.6	1485	0.0371
21	279.1658	7291	108.9	1594	0.0383
22	326.0971	7804	3371.8	45849	0.0418
23	327.0985	7776	681.2	9250	0.0421
24	328.0938	7836	449.5	6096	0.0419
25	391.2951	8274	562.5	7173	0.0473
26	392.2998	7607	124.6	1592	0.0516
27	397.2852	8092	925.3	11747	0.0491
28	398.2895	8343	224.2	2848	0.0477
29	410.2242	7380	165.5	2086	0.0556
30	419.3288	8032	109.2	1332	0.0522

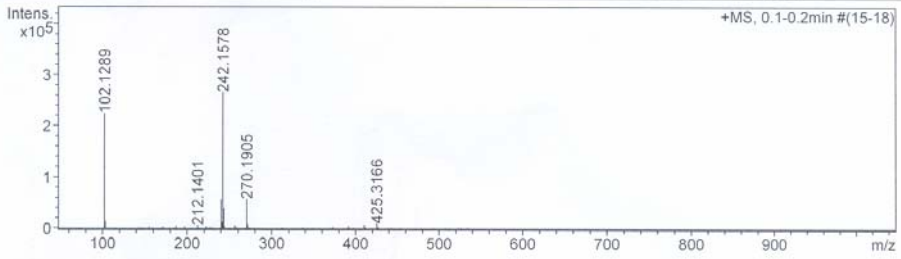
Mass Spectrum List Report

Analysis Info

Analysis Name	Palmitic acid_22_01_3078.d	Acquisition Date	2/21/2007 10:03:02 AM
Method	suttiporn new.m	Operator	Administrator
Sample Name	Palmitic acid Palmitic acid	Instrument	micrOTOF 72

Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Corrector Fill	44 V
Scan Range	n/a	Capillary Exit	90.0 V	Set Pulsar Pull	383 V
Scan Begin	50 m/z	Hexapole RF	60.0 V	Set Pulsar Push	383 V
Scan End	1000 m/z	Skimmer 1	30.0 V	Set Reflector	1300 V
		Hexapole 1	30.0 V	Set Flight Tube	9000 V
				Set Detector TOF	2090 V



#	m/z	Res.	S/N	I	FWHM
1	102.1289	4819	30213.6	223878	0.0212
2	103.1325	5307	1857.9	13773	0.0194
3	155.0529	6260	255.6	2354	0.0248
4	171.0837	6481	174.8	2299	0.0264
5	187.0786	6516	246.4	4207	0.0287
6	197.0988	6429	164.7	3218	0.0307
7	212.1401	3662	271.6	6306	0.0579
8	213.1426	3208	39.0	920	0.0664
9	222.1309	7039	139.9	3132	0.0316
10	226.1264	6516	65.6	1424	0.0347
11	229.0895	6859	79.1	1672	0.0334
12	240.1426	6406	2990.8	56998	0.0375
13	241.1474	6624	659.9	12458	0.0364
14	242.1578	6220	14212.4	265599	0.0389
15	243.1616	6768	2129.1	39402	0.0359
16	244.1646	7034	156.1	2864	0.0347
17	256.1391	7019	336.1	5425	0.0365
18	258.1526	7477	133.7	2111	0.0345
19	270.1905	7011	4251.0	57654	0.0385
20	271.1941	7395	660.9	8844	0.0367
21	272.1833	4659	96.3	1273	0.0584
22	312.2027	8065	129.4	1273	0.0387
23	337.1300	7998	163.6	1379	0.0422
24	372.2053	7949	326.9	2938	0.0468
25	391.2939	7719	381.5	3773	0.0507
26	392.2970	7582	92.7	924	0.0517
27	410.2225	7689	623.1	6719	0.0534
28	411.2261	7628	172.5	1872	0.0539
29	425.3166	8757	963.9	9936	0.0486
30	426.3207	7678	221.1	2269	0.0555

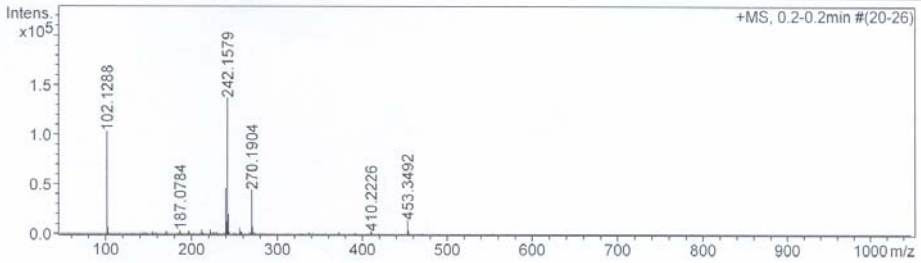
Mass Spectrum List Report

Analysis Info

Analysis Name	Stearic acid_25_01_3081.d	Acquisition Date	2/21/2007 10:20:32 AM
Method	suttiporn new.m	Operator	Administrator
Sample Name	Stearic acid Stearic acid	Instrument	micrOTOF 72

Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Corrector Fill	44 V
Scan Range	n/a	Capillary Exit	90.0 V	Set Pulsar Pull	383 V
Scan Begin	50 m/z	Hexapole RF	60.0 V	Set Pulsar Push	383 V
Scan End	1000 m/z	Skimmer 1	30.0 V	Set Reflector	1300 V
		Hexapole 1	30.0 V	Set Flight Tube	9000 V
				Set Detector TOF	2090 V



#	m/z	Res.	S/N	I	FWHM
1	102.1288	5094	22920.0	103244	0.0200
2	103.1324	5368	1403.3	6335	0.0192
3	144.0610	6164	163.5	790	0.0234
4	155.0530	6195	334.2	2040	0.0250
5	171.0837	6456	272.8	2421	0.0265
6	187.0784	6616	307.7	3580	0.0283
7	197.0987	6204	233.9	3126	0.0318
8	212.1329	3092	235.1	3753	0.0686
9	222.1310	7187	305.6	4720	0.0309
10	226.1262	6704	82.3	1234	0.0337
11	229.0894	7043	101.5	1486	0.0325
12	240.1427	6657	3468.8	46187	0.0361
13	241.1478	6913	926.0	12222	0.0349
14	242.1579	6250	10495.3	137250	0.0387
15	243.1612	6779	1508.3	19548	0.0359
16	244.1645	7010	124.1	1596	0.0348
17	256.1388	7325	502.4	5745	0.0350
18	257.1417	6534	80.3	911	0.0394
19	258.1529	7455	204.1	2287	0.0346
20	270.1904	7263	4522.6	44220	0.0372
21	271.1936	7556	734.8	7099	0.0359
22	272.1841	4649	108.3	1035	0.0586
23	274.1502	7062	86.2	804	0.0388
24	337.1304	7862	217.0	1141	0.0429
25	372.2057	8342	284.0	1462	0.0446
26	391.2959	7697	147.5	818	0.0508
27	410.2226	7579	513.1	3039	0.0541
28	411.2259	7778	161.0	959	0.0529
29	453.3492	8233	2346.6	14346	0.0551
30	454.3520	7705	625.0	3825	0.0590

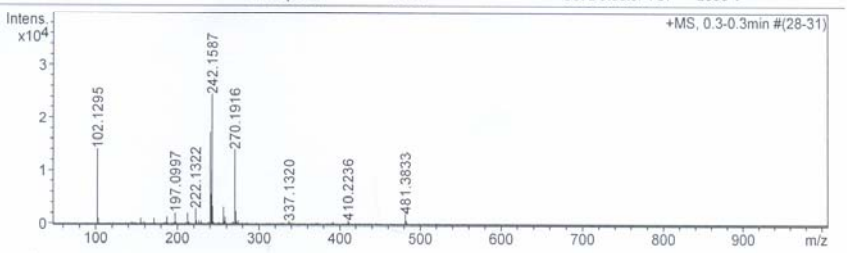
Mass Spectrum List Report

Analysis Info

Analysis Name	Arachidic acid_28_01_3084.d	Acquisition Date	2/21/2007 10:37:58 AM
Method	suttiporn new.m	Operator	Administrator
Sample Name	Arachidic acid Arachidic acid	Instrument	micrOTOF 72

Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Corrector Fill	44 V
Scan Range	n/a	Capillary Exit	90.0 V	Set Pulsar Pull	383 V
Scan Begin	50 m/z	Hexapole RF	60.0 V	Set Pulsar Push	383 V
Scan End	1000 m/z	Skimmer 1	30.0 V	Set Reflector	1300 V
		Hexapole 1	30.0 V	Set Flight Tube	9000 V
				Set Detector TOF	2090 V



#	m/z	Res.	S/N	I	FWHM
1	102.0957	5482	1734.1	3550	0.0186
2	102.1295	5477	6868.8	14064	0.0186
3	103.1326	5383	486.2	1002	0.0192
4	155.0542	6147	334.6	1039	0.0252
5	171.0850	6646	260.9	1070	0.0257
6	187.0796	6636	264.8	1349	0.0282
7	197.0997	6221	359.8	2056	0.0317
8	212.1157	5239	304.0	2021	0.0405
9	213.1155	5394	55.6	373	0.0395
10	222.1322	7194	439.7	2858	0.0309
11	223.1360	6830	86.4	559	0.0327
12	226.1280	6786	97.4	619	0.0333
13	229.0902	6885	89.4	558	0.0333
14	240.1440	6852	2946.0	17159	0.0350
15	241.1491	7000	960.2	5557	0.0344
16	242.1587	6820	4246.0	24410	0.0355
17	243.1621	6632	572.1	3268	0.0367
18	256.1396	7269	605.1	3162	0.0352
19	257.1412	6412	95.0	493	0.0401
20	258.1539	7157	261.4	1346	0.0361
21	268.1765	7574	88.4	423	0.0354
22	270.1916	7519	2979.2	13999	0.0359
23	271.1952	7283	505.1	2355	0.0372
24	272.1824	4645	97.6	452	0.0586
25	274.1508	7039	137.0	624	0.0389
26	337.1320	8253	193.8	538	0.0409
27	391.2981	8715	144.8	393	0.0449
28	410.2236	7324	258.8	728	0.0560
29	481.3833	7648	754.2	1953	0.0629
30	482.3881	7633	240.1	619	0.0632

BIOGRAPHY

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