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Primary fatty acid amides in mammalian tissues: Isolation and analysis by HPTLC and SPE in conjunction with GC/MS

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This work is dedicated to my loving family

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Tamanna Sultana (Ph.D., Analytical Chemistry)

Primary fatty acid amides in mammalian tissues: Isolation and analysis by HPTLC and SPE in conjunction with GC/MS

Abstract of a Dissertation at Duquesne University

Dissertation supervised by Professor Mitchell E. Johnson

Primary fatty acid amides (PFAM's) are a novel class of bio-active lipids present in mammals in trace level. Selective isolation of PFAM's from lipid extracts is crucial for obtaining them in pure and concentrated form for interference-free instrumental detection and analysis. Synthesis of these commercially unavailable lipids is also important for method development and quantitative analysis by instrumental means. In this study a wide variety of long chain saturated and unsaturated primary fatty acid amides were synthesized and characterized. This list includes the positional and geometrical isomers of naturally abundant PFAM's. Two isotopically enriched PFAM's were also synthesized which are important for establishing the fragmentation patterns of the amides by mass-spectral analysis as well as for use as internal standards in quantitative analysis. The separation of saturated and some unsaturated amides was obtained using a non polar HP-5MS column. A more polar column, BPX70, was employed for the separation of the geometric and positional isomers of the unsaturated amides.

Two methods for the isolation of PFAM's from lipid extracts were developed and validated. First method is a high performance thin layer chromatography (HPTLC),

which was found to be efficient in quick profiling of different lipid classes present in a total lipid extract. Any lipid class of choice can also be further analyzed by scraping it off the HPTLC plates. The second method is a solid-phase extraction (SPE) for selectively extracting the amides in a single fraction by a specific elution solvent from a normal phase column. This fraction can be dried and brought to the desired concentration for further instrumental analysis. Lowest mass of amides to be loaded and recovered by SPE was also optimized. Both HPTLC and SPE amide isolation methods were validated using the total lipids extracted from $N_{18}TG_2$ mouse neuroblastoma cell line. The detection limits of the amides by gas chromatography/mass spectrometry (GC/MS) were found to be in the order of 5-30 pg. PFAM's isolated from rabbit brain and heart tissues by these methods were identified as palmitamide, stearamide and oleamide. Trace amounts of linoleamide and eicosenoamide were also observed in brain tissues.

Table of Abbreviations

2,5-dihydroxybenzoic acid	DHB
2-arachidonoyl glycerol	2-AG
Acyl-CoA:glycine *N*-acyltranferase	ACGNAT
Argentation high performance thin layer chromatography	Ag-HPTLC
Atmospheric pressure chemical ionization	APCI
Bis- Trimethylsilyltrifluoroacetamide	BSTFA
Cannabinoid receptor	CB
Capillary electrophoresis	CE
Chemical ionization	CI
Chlorotrimethylsilane	TMSCl
Cholesterol	Ch
Cholesteryl ester	CE
Cholesteryl palmitate	ChP
Collision induced decomposition	CID
Delta-9-tetrahydrocannabinol	Δ^9-THC
Diacyl glycerol	DAG
Dipalmitin	DP
Discovery aminopropyl phase	DSC-NH_2
Discovery silica phase	DSC-Si
Dulbecco's Modified Eagle's Medium	DMEM
Electron impact	EI
Electrospray ionization	ESI
Fast atomic bombardment	FAB
Fatty acid amido hydrolase	FAAH
Fatty acid methyl ester	FAME
Fetal bovine serum	FBS
Flame ionization detector	FID

Fluorescein isothiocyanate	FITC
Free fatty acids	FA
Gamma-aminobutyric acid	GABA
Gangliosides	G
Gas chromatography/mass spectrometry	GC/MS
High performance liquid chromatography	HPLC
High performance thin-layer chromatography	HPTLC
Kodak scientific imaging software	KODAK/SIS
Laser induced fluorescence	LIF
Liquid chromatography/mass spectrometry	LC/MS
Mass to charge ratio	m/z
Matrix-assisted laser desorption ionization	MALDI
Micellar electrokinetic capillary chromatography	MECC
Monoacyl glycerol	MAG
Monocaproyl glycerol	MCG
Monooleoyl glycerol	MOG
Monopalmitoyl glycerol	MPG
N-acylethanolamine	NAE
N-acylglycine	NAG
N-acylphosphatidylethanolamine	NAPE
Nerve growth factor	NGF
N-methyl-*N*- trimethylsilyltrifluoroacetamide	MSTFA
N-oleoylethanolamine	NOE
N-oleoylglycine	NOG
Normal phase	NP
N-tert-butyldimethylsilane	*N*-*t*-BDMS
Nuclear magnetic resonance	NMR
Octadecylsilane	ODS
Oleamide	OM
Oleic acid	OA

Pentafluorobenzyl	PFB
Peptidylglycine α-amidating monooxygenase	PAM
Phosphatidylcholine	PC
Phosphatidylethanolamine	PE
Polar lipid	PL
Primary fatty acid amides	PFAM's
Reversed phase	RP
Single ion monitoring	SIM
Solid-phase extraction	SPE
Sphingomyelin	Sph
Squalene	Sq
Thin-layer chromatography	TLC
Total ion monitoring	TIM
Triacyl glycerol	TAG
Tricaproin	TC
Trimethyl sulfonium hydroxide	TMSH
Trimethylsilane	TMS
Tristearin	TS
Ultraviolet-visible	UV-vis

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Chapter 1

Review of lipids and fatty acid amide analysis

1.1 Introduction

Lipid analysis is one of the most investigated topics by bio-analytical/bio-chemists due to the diverse structures, nature and the activity of lipids in biological systems. One of the lipid classes, fatty acid amides, is long known for their hormone-like activities. They attracted recent attention when anandamide was identified as an endogenous cannabinoid or endocannabinoid. Anandamide is a twenty carbon fatty acid ethanolamine with four point of unsaturation (*cis,cis,cis,cis*-5,8,11,14 eicosatetraenoyl ethanolamine). It was first identified to be produced in brain and to bind to the cannabinoid receptor (CB1). It was also found to mimic all the activities of delta-9-tetrahydrocannabinol (Δ^9 THC) [1]. It is necessary to point out here that Δ^9 THC is the compound that is responsible for all the pharmacological activity of marijuana and is an exogenous cannabinoid [2, 3]. Cannabinoids are involved in so called retrograde or backward signaling that can change the regular neurotransmission. It has also been found that three specific neurotransmitters glutamate, dopamine and acetylcholine that play key roles in neuronal activities, initiate the synthesis and release of endocannabinoids [see Ref. 4 for review]. Therefore this fatty acid amide class (*N*-acyl-ethanolamides) faced intensive study over the last decade.

The other groups of fatty acid amide, the primary fatty acid amides, have not been found to show any affinity for CB1 receptor but are catabolically related to the

cannabinoid receptor class fatty acid amides. One such primary amide, oleamide, was extracted from the cerebrospinal fluid of sleep-deprived cats and hence was thought to be involved in neurobehavioral activities in brain. Oleamide and other primary amides have therefore been also studied along with the endocannabinoids. These extensive studies include their synthesis, biochemical studies, isolation from tissues and cells etc. and moreover, their characterization by instrumental means.

The instruments that are most often used in lipid analyses includes nuclear magnetic resonance (NMR), high performance liquid chromatography (HPLC), liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS), gas chromatography/mass spectrometry (GC/MS), matrix-assisted laser desorption ionization/time-of-flight (MALDI/TOF). High performance thin layer chromatography/thin layer chromatography (HPTLC/TLC) and solid phase extraction (SPE) are often used for sample preparation. In this chapter the analyses of fatty acid amides by instrumental means, including the techniques used in sample preparation will be reviewed. The main target of this chapter is to recognize the work that has been done and to approach what could further be done in order to isolate and analyze the fatty acid amides free of interaction from other lipid classes. Some basic information about lipids and lipid analyses will also be provided as introduction.

1.2 Lipid definition

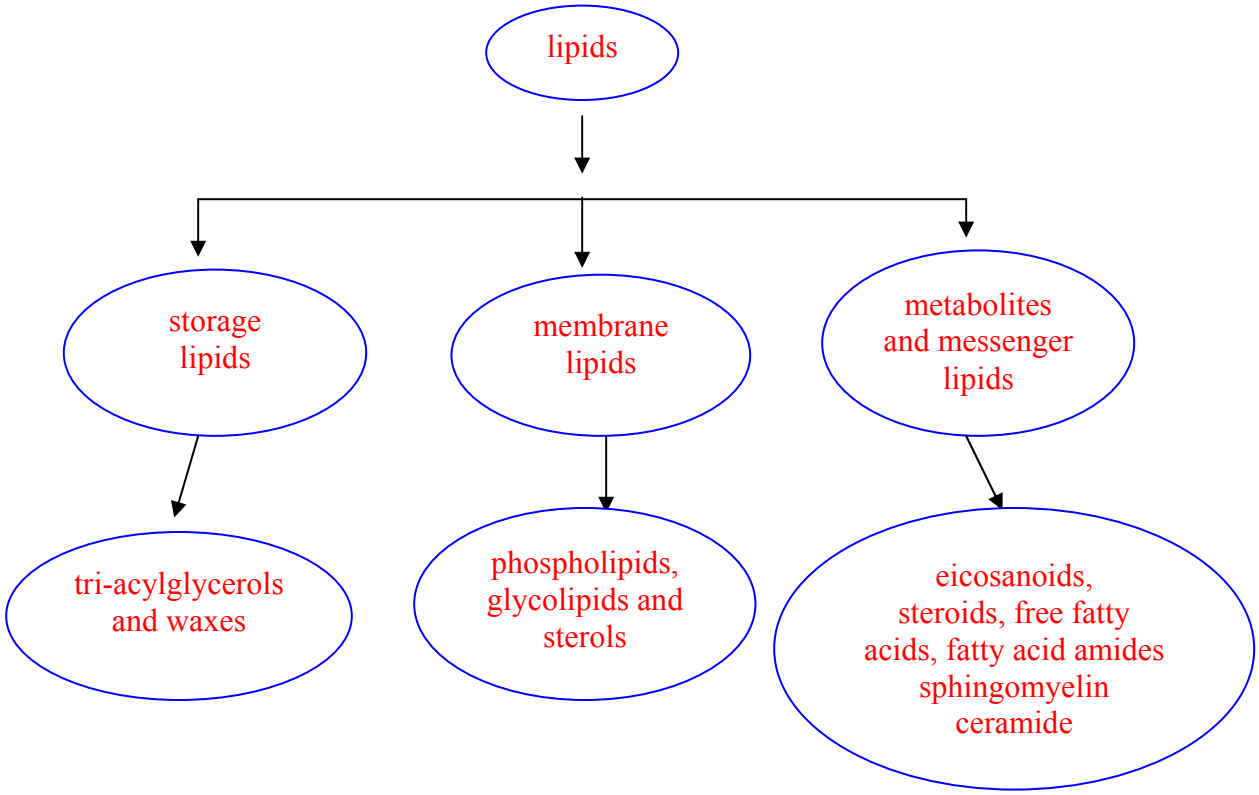
The term lipid refers to the substances that are soluble in organic solvents and are insoluble in water. This is rather a book definition that avoids the fact that many non-lipid substances are also soluble in organic solvents and some lipids show more affinity

for water than organic solvents. Lipids can be defined as the molecules that are synthesized by biological systems and can be extracted from the biological samples by dissolution into organic solvents. The word 'fat or oil' previously used for lipids mainly refers to one class of lipid (triacylglycerol), which is the major stored form of energy in animals and plant seeds [5, 6]. Classification of lipids is described in detail in the section below.

1.3 Lipid classification

These important biochemical molecules range from low molecular weight fatty acids to high molecular weight complex phospho- and glyco-lipids. The two main branches of lipid are storage lipids (which do not contain any polar head groups) and membrane lipids (which contain polar head groups attached to the lipid backbone). Storage lipids are mainly relatively non-polar triacylglycerols containing glycerol backbones esterified with saturated or unsaturated fatty acids. Waxes are another kind of storage lipids, which are mainly the esters of long chain fatty acid with long chain alcohols. They also have water repellent properties and firm consistency. Membrane lipids are called structural lipids because they form the structure of the cell membrane. Membrane lipids can be phospholipids, glycolipids or sterols. Based on the backbone, phospholipids are classified as glycerophospholipids (containing glycerol backbone) or sphingolipids (containing sphingosine backbone). All the phospholipids have a polar head group attached to the glycerol or the sphingosine backbones through a phosphodiester linkage, whereas all the glycolipids have a simple sugar or complex oligosaccharide attached to the sphingoshine backbone. Some membrane lipids have fatty acids attached through

either ether or vinyl ether bonds at the other end of the glycero or phosphoglycero backbone. These are called ether lipids or plasmalogens. Sterols, another class of membrane lipids, contain a steroid nucleus of four fused rings. There are other lipids that are present in much smaller amount and are synthesized as necessary. These are the metabolites and the messenger lipids such as eicosanoids, steroids, free fatty acids and the fatty acid amides. Some of the phospholipids and plasmalogens also serve as metabolites and second messengers. These types of lipids play crucial roles in numerous biological activities. Fatty acids and sterols serve as the precursors for these biologically important classes of lipids [7, 8].



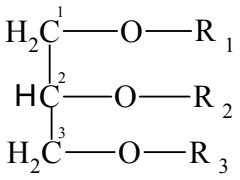
Scheme 1.1: Relational diagram of lipid classes.

Scheme 1.1 gives basic idea about the lipid classes possible even though there are many in between classes discussion of which is beyond the limit of this review.

1.4 Basic structure and biological importance of each lipid class

1.4.1 Storage lipids

1.4.1.1 Triacylglycerols

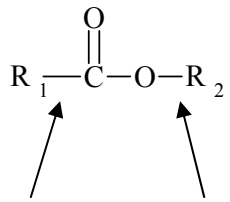


R_1, R_2 and R_3 represent saturated or unsaturated fatty acids

In vertebrates, energy consumption occurs continuously. Triacylglycerols (TAG) serve as the stored form of energy in specialized cells called adipocytes. These lipids serve this purpose best because they occupy less volume per calorie of stored chemical energy than carbohydrate and protein. Adipocytes contain a large amount of triacylglycerols (more than 80% of the mass) that almost fills the cell. 12-14% of normal body weight in male and more than 25% of that in female is adipose tissue or fat. In most eukaryotic cells, TAGs float as oily droplets in the aqueous cytosol, serving as metabolic fuel. TAGs are also stored as oils in the seeds of many plants, proving energy as needed. When fuel is required at a particular body site, the enzyme TAG lipase expressed in adipocytes and seeds catalyzes the hydrolysis of TAG, releasing free fatty acids and sending them to that site to be used as fuel through β-oxidation. Therefore, these fat cells maintain a stable supply of energy by synthesizing TAG from

carbohydrates to be stored and again hydrolyzing them to be used as fuels as necessary [8, 9]. Mono or diacylglycerols (MAG or DAG) are formed when any two or any one of the OH groups on the glycerol backbone respectively remains un-esterified. MAG and DAG have profound biological activities as metabolites and messengers.

1.4.1.2 Waxes



Fatty acid long chain alcohol

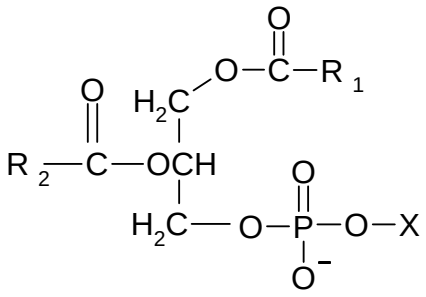
Biological waxes are the esters of long-chain fatty acid with long chain alcohols. The chain length varies from 14 to 36 carbons. These waxes are secreted by different insects, birds and animals and have significant importance for them as well as to humans. The water-repellent property of waxes helps not only to keep the body waterproof but also to keep water from evaporating. It also protects the body against outside attack. Due to its firm consistence property it is extracted from animal and plants, and used in manufacturing industries for various purposes such as in the manufacture of lotions, ointments and polishes [8].

1.4.2 Membrane lipids

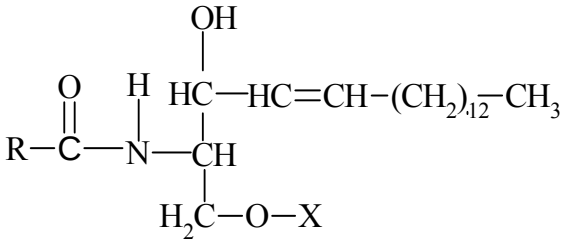
1.4.2.1 Phospholipids and glycolipids

Glycerophospholipids are similar in structure as the TAGs except for an H or a polar head group is attached to the sn-3 position of the glycerol backbone via a

phosphodiester linkage. In sphingolipids, fatty acids are joined by amide linkages rather than ester linkages to the sphingoshine backbone. Phosphosphingolipids have a phosphate group attached between the sphingosine backbone and the polar head group.



Glycerophospholipid



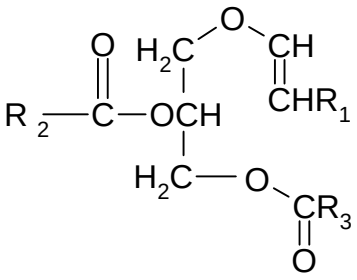
Sphingolipids

X = H or a polar head group

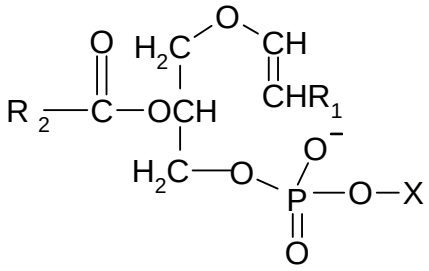
Glycolipids are sphingolipids that lack the phosphate group but have a simple sugar or a complex oligosaccharide attached as the head group. Membrane lipids constitute the 5-10% of the dry mass of most cells and play important roles in the cell structure. They form an impermeable barrier that surrounds cells and cellular compartments. They are mainly amphipathic with a hydrophobic backbone and a hydrophilic polar head group. Sphingolipids play a significant role in cell biology and

cellular functions ranging from intercellular signaling as a second messenger in cellular apoptosis to membrane structural functions [6].

1.4.2.2 Plasmalogens



Non-polar plasmalogen



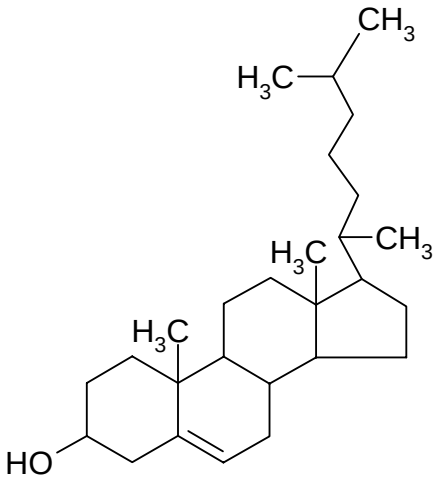
Polar plasmalogen

R_1, R_2 and R_3 here represent long alkyl chains

Plasmalogens are another kind of phospholipids that are also called vinyl-ether lipids and can be non-polar or polar in nature. Non-polar plasmalogens are glycerol esters of two fatty acids with a vinyl ether group in position 1; whereas polar plasmalogens are phospholipids consisting of polar head group (X) in position 3, fatty acid ester in position 2 and vinyl ether bond in position1. Usually the highest proportion of the plasmalogen form is in the phosphatidylethanolamine class with little or no other phospholipids. In phosphatidylcholine, a larger proportion is in the alkyl ether form rather than vinyl ether form, except in heart lipids, where it is reversed. Based on the distribution and properties of plasmalogens in various cell types and changes that occur in plasmalogen metabolism in certain mutant cells, it is suspected that plasmalogens may have a number of other functions in addition to being structural components of cell membranes. They may serve as a store of poly-unsaturated fatty acids (PUFA) that can be released by specific stimulant molecules, may act as intracellular signaling

compounds, may have a role as antioxidants, and are implicated in aging and various degenerative diseases [10].

1.4.2.3 Sterols



Cholesterol

Sterols are another class of membrane lipids that constitute the cell membrane. They also serve as precursors for various biologically active hormones and other signaling molecules. The characteristic sterol structure consists of one five carbon and three six carbon fused rings.

1.4.3 Metabolites and messenger lipids

1.4.3.1 Eicosanoids

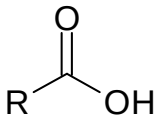
Eicosanoids are derivatives of fatty acid and they act on cells near the point of synthesis. They are all derived from 5,8,11,14-eicosatetraenoic acid ($20:4^{5,8,11,14}$) and therefore are called eicosanoids. The eicosanoids are known to be involved in a variety of activities in cells. Some of the examples are stimulation of muscle contraction,

regulation of blood flow and wake-sleep cycle, induction of inflammation and pain (prostaglandins), formation of blood clots as well as the reduction of blood flow at the site of clot (thromboxanes), induction of muscle contractions in lungs causing asthmatic attack (leukotrienes) [8].

1.4.3.2 Steroids

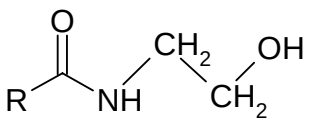
Steroids are derivatives of sterols and they travel through blood stream from the point of synthesis to act on different target tissues. They have very high affinity for their receptors and therefore are potent signaling hormones. They can act against eicosanoids by sending the signal for the reduction of their synthesis thereby acting as anti inflammatory and anti-asthmatic medicine.

1.4.3.3 Free fatty acids

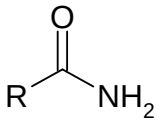


Fatty acids are the structural components of almost all the lipids and serve as body energy through β-oxidation. They also act as second messengers.

1.4.3.4 Fatty acid amides



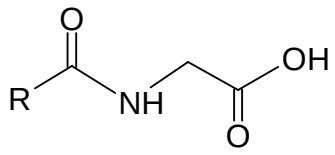
N-acylethanolamine



Primary fatty acid amides

Fatty acid amides belong to an important bio-regulatory group of lipids that act as messenger lipids. *N*-acylethanolamines and primary fatty acid amides are the two most well recognized classes of this group. They are mainly the derivatives of long chain saturated and unsaturated fatty acids. Primary fatty acid amides (PFAM's) can be formed via amidation of the corresponding acid *in vitro* and from *N*-acylglycine *in vivo* [11]. *N*-acylethanolamines can be synthesized by the hydrolysis of *N*-acyl phosphatidylethanolamine (*N*-acyl PE) *in vivo* [12].

1.4.3.5 *N*-acylglycines

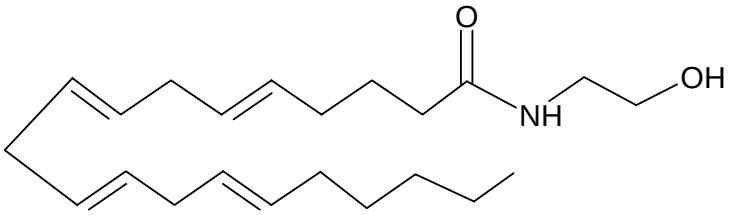


N-acylglycine

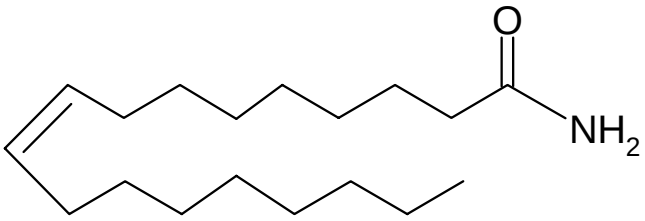
N-acylglycines (NAG) are metabolite lipids that are recently shown to be substrates for PAM (peptidylglycine α-amidating monooxygenase) in PFAM biosynthesis [11, also see Scheme 1.2]. Short chain *N*-acyglycines are produced by the action of acyl-CoA:glycine *N*-acyltranferase (ACGNAT; found in liver and kidney) on acyl-CoA thioesters and glycines. Because acyl-CoA thioesters with acyl chains containing more that 10 carbon atoms has very low V/K values, it is very unlikely that long chain NAG synthesis is catalyzed by ACGNAT. But the identification of long chain NAGs in mammals and insects indicate the presence of an enzyme that catalyzes their synthesis. Very recently Merkler *et al.* [13] have found that $N_{18}TG_2$ cells grown in ^{14}C oleic acid under the conditions known to stimulate PAM expression increases

oleamide production and in presence of a PAM inhibitor produces ^{14}C *N*-oleoylglycine (^{14}C NOG). Their findings therefore strongly support NAG as a substrate for PAM in primary fatty acid amide biosynthesis *in vivo*.

1.5 Biological significance of fatty acid amides (ethanolamines and primary amides)



Arachidonyl ethanolamine (anandamide)



cis-9-octadecenoamide (oleamide)

The biological significance of fatty acid amides have been reviewed in detail in references 4 and 14. Kuehl *et al.* [15] has first shown that *N*-palmitoylethanolamine extracted from soybean, peanut oil and egg yolk is a naturally occurring anti-inflammatory agent. Palmitoylethanolamine was also shown by Ganley *et al.* [16, 17] to have anti- inflammatory and anti- anaphylactic properties and was discovered by Bachur *et al.* [18] as endogenous products of brain, liver and skeletal muscles of fasted

rats and guinea pigs, brain containing the highest amount (they also found the presence of stearoylethanolamine 3-10% of the total palmitoylethanolamine). In 1992, Raphael Mechoulam and his coworkers [1] discovered another endogenous fatty acid amide *N*-arachidonyl ethanolmaine (anandamide) in porcine brain. It was found to bind cannabinoid receptor (CB1), and mimic all the activities of Δ^9 THC. As described earlier, Δ^9 THC was previously found to bind to rat CB1 receptor [2].

Receptors are small proteins found in almost all cell membranes, including neurons. Specific molecules bind to specific receptors resulting in the changes in cells. Cannabinoid receptors are the specific brain membrane receptors that are coupled to the G-proteins. The compounds that have the affinity for these receptors are called cannabinoids or cannabinoid receptor-active. Those cannabinoids which are synthesized endogenously are termed endogenous cannabinoids or endocannabinoids. The two types of cannabinoid receptors discovered are CB1 found in brain and spinal cord and CB2 found in peripheral tissue. CB1 is one of the most abundant G-protein coupled receptors in brain. It is found in high densities in cerebral cortex, hippocampus, hypothalamus, cerebellum, basal ganglia, brainstem, spinal cord, and amygdala [19-21]. It was found by Mackie, Freund, and coworkers [22, 23] that cannabinoid receptors occurred only in very specific position of certain neurons that release the inhibitory neurotransmitter GABA (gamma-aminobutyric acid). The reason it was densely packed on the GABA releasing neurons is that the CB1 receptors can be activated by retrograde (backward) signaling and therefore can reduce or terminate the release of GABA, changing the regular neurotransmission. Even though most of the physiological roles of the cannabinoid (CB1) receptors and hence those of the

cannabinoids are still not known, their presence in the parts of the brain that are responsible for psychoactive power (cerebral cortex), memory impairment (hippocampus), pain reduction and vomiting reflex (brain stem and spinal cord), appetite (hypothalamus), emotional responses (amygdale), etc. associate them with those kinds of activities. CB2 is known to be involved in the immune system. Besides anandamide, four other ethanolamines γ-linolenoylethanolamine, docosatetraenoyl-ethanolamine, docosahexaenoylethanolamine and eicosatrienoylethanolamine were also found to be endocannabinoids [24-27]. 2-arachidonylglycerol (2-AG) has been recently discovered as an endocannabinoid, which binds to both CB1 and CB2 [28, 29]. It was found that three specific neurotransmitters glutamate, dopamine and acetylcholine can induce the synthesis and release of endocannabinoids in specific regions of brain [4]. Because the activation of the CB1 receptors virtually inhibits the secretion of these neurotransmitters neuronal functions can very well be regulated by fatty acid ethanolamines [30].

Between the late 1950's and early 1970's palmitoylethanolamine was identified as an inhibitory agent of inflammation, traumatic shock, and toxic effects of anti-cancer drugs and ethanol [14, 31-39]. Long chain *N*-acylethanolamines were found to affect heart and liver mitochondrial functional parameters. In heart mitochondria they can inhibit the release of Ca^{+2}, which are usually accumulated by the action of some Ca^{+2} releasing agents. This accumulation of Ca^{+2} inside the mitochondria occurs via induction of increased permeability of the inner mitochondrial membrane by these releasing agents [40]. After anandamide was identified as a brain natural product that mimics the activities of marijuana, a number of other activities of this fatty acid amide

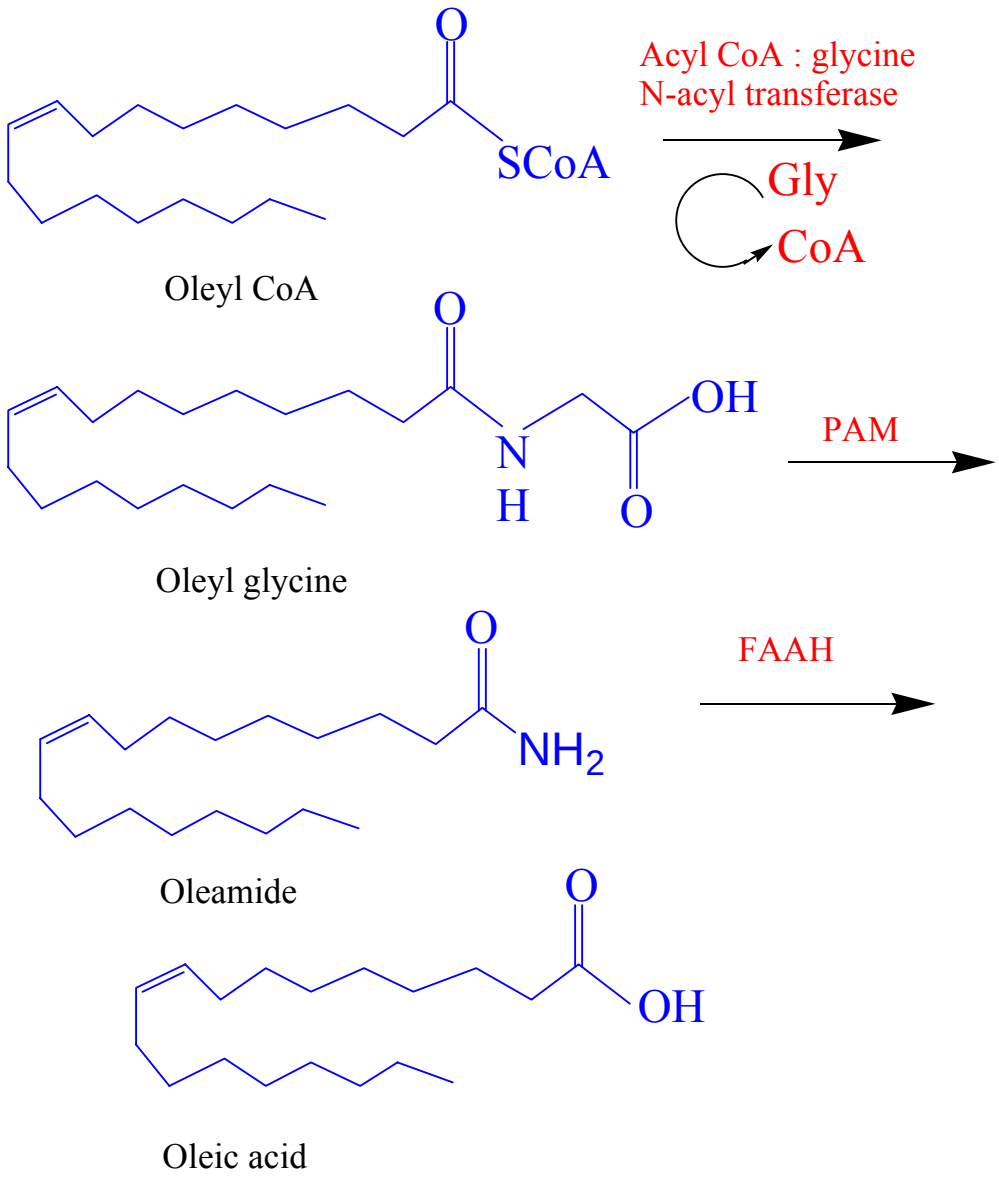
were discovered. Stein *et al.* [41] found that anandamide injection causes rapid increase of bradycardia by its direct action on the blood vessels and subsequent lowering of blood pressure by its inhibition of the secretion of noradrenaline by nerves in the hearts and blood vessels [42-43]. Very recently this so called antinociceptive and possible neuroprotective agent has also been found to have affinity for other targets especially for vanilloid (TRPV1) receptors [reviewed in Ref. 44]. The author pointed that the efficacy of anandamide as a TRPV1 agonist is influenced by several factors such as receptor reserve, phosphorylation, metabolism and uptake, CB1 receptor activation, voltage, temperature, pH and bovine serum albumin. The endocannabinoid system may play a role in the modulation of TRPV1 receptor activation, which might have potential implications in the treatment of inflammatory, respiratory and cardiovascular disorders. Therefore, the diverse effect of anandamide in bio-regulation is still not completely understood.

Biosynthesis of ethanolamines was investigated long before their affinity for CB receptor was discovered. Their biosynthesis is catalyzed by an enzyme transacylase that synthesizes *N*-acylphosphatidylethanolamine (NAPE) from phosphatidylethanol-amines (PE) [45] and by the action of a second phospholipase D type enzyme (phosphodiesterase) that hydrolyzes NAPE to *N*-acylethnolamine (NAE) [12]. The liver, brain, and kidney mitochondria, microsomes and cytosols were found to be the main points of their synthesis even though the synthesis of anandamide is most active in brain cytosol. The enzyme transacylase was first identified by the abnormal accumulation of NAPE and hence NAE in damaged dog-heart mitochondria [46-50] and phosphodiesterase was first characterized in rat heart membrane and was later

found to be present in almost all rat tissues examined with higher amounts present in young rat brains [reviewed in ref 51]. Schmid and co-workers [50, 52] while studying these endogenous cannabinoids first discovered that these compounds are hydrolyzed into free fatty acids and ethanolamines by a specific enzyme. The enzyme that hydrolyzes anandamide to arachidonic acid was named anandamide amidohydrolase and was characterized by a number of scientists. This membrane bound enzyme, now called fatty acid amido hydrolase (FAAH), was also found to catalyze the hydrolysis of a series of *N*-acylethanolamines with similar catalytic activities [53-56].

While the functions and metabolic pathways of NAEs are well established [51, 57-60] very little is known about the biosynthesis and biodegradation pathways of PFAM's. They were first identified in human luteal phase plasma [61] and came to major attention when oleamide was isolated from the cerebrospinal fluid of sleep-deprived cats. In 1995 Cravatt *et al.* [62] separated oleamide from the cerebrospinal fluid of sleep deprived cats and characterized it as a sleep-inducing lipid. Even though no evidence has been found so far that oleamide is also an endocannabinoid, it was found to be catabolically related to the endocannabinoids [reviewed in Ref. 51]. Oleamide is also known to modulate serotonin neurotransmissions [63], increase affinity of GABA for its receptors [64] and inhibit lymphocyte proliferation [65], to prevent gap junction communication in osteoblastic and glial cells [66-68], to increase food intake in rats upon injection [69], to inhibit synovial fluid phospholipase A_2, [70], to possess seizure limiting properties in mice [71, 72], as well as to modulate memory in rat [73]. Of the other amides, erucamide may simulate angiogenesis [74] and regulate fluid imbalance [75]; arachidonamide inhibits human synovial phospholipase

A_2 [70] and inhibits leukotriene biosynthesis [76]; linoleamide increases Ca^{2+} flux [77] and inhibits *erg* current in pituitary cells [78]. Even though the actions of most of the other amides are yet not known, it is almost certain that fatty acid amides have interesting biological activity in mammals.



Scheme1.2: Biosynthetic pathway of PFAM's proposed by Merkler *et al*. [11].

In search for answers to the biosynthesis and degradation pathways of PFAM's, a number of pathways have been proposed. The most recognized pathway involves the production of *N*-acylglycines (NAG) from acyl-CoA and glycine by the enzyme called acyl-CoA: glycine *N*-acyltransferase followed by the PAM (peptidylglycine α-amidating monooxygenase) catalyzed reduction of NAG to primary fatty acid amides [11, also see Scheme1.2]. The amides are then hydrolyzed by the action of FAAH (able to hydrolyze anandamide and a wide variety of fatty acid amides) [reviewed in Ref. 79], which is probably why the concentration of amides found in mammals is very low [80-82]. PAM has also been known to catalyze the oxidation of many neural and endocrine peptide hormones to amides [83, 84]. One model cell line that is used to study oleamide biosynthesis is $N_{18}TG_2$ mouse neuroblastoma cell because of its ability to express PAM. These cells not only express PAM but also show dramatic increase in PAM expression upon cellular differentiation [85]. However, questions were raised about the above pathway when ^{14}C oleamide was found to be produced by $N_{18}TG_2$ cells grown in ^{14}C oleic acid [86]. The authors pointed that because the levels of oleamide were not significantly influenced by stimulation with ionomycin, but were slightly increased by incubation with FAAH inhibitor, $N_{18}TG_2$ cell membranes may contain an enzymatic activity catalyzing the synthesis of oleamide from oleic acid and ammonia. They argued that these data suggest that a reverse FAAH-like enzyme may be responsible for the formation of oleamide in cell-free preparations but not in whole cells. Very recently Merkler *et al*. [13] have found that $N_{18}TG_2$ cells grown in ^{14}C oleic acid under the conditions known to stimulate PAM expression increases oleamide production and under the conditions known to inhibit PAM activity produces ^{14}C *N*-oleoylglycine (^{14}C

NOG). Their findings that oleamide can also be generated from the cells grown in NOG strongly support NOG as an intermediate and PAM as a catalyst in oleamide biosynthesis in vivo.

N-acylethanolamine (NAE) and its biosynthetic precursor *N*-acyl phosphatidylethanolamine (NAPE) can be found in almost all vertebrate tissues, cells, body fluids, invertebrates, and plants [87-91]. It has been found that both NAE and NAPE can accumulate in injured areas [92-97], which suggests that cellular levels of both NAE and NAPE are tightly regulated under physiological conditions. They tend to accumulate highly in response to stress, cellular and tissue degeneration and membrane degradation. The types of NAEs that are observed in these cases are mainly the saturated and monounsaturated NAE, suggesting these amides have profound action in preventing tissue damage and stabilizing membranes [51]. The inhibitory activity of NOE in lipid peroxidation in rat heart mitochondria [98, 99] and the inhibitory activity of NPE (*N*-palmitoylethanolamine) in the glutamate induced excitotoxic death of the cerebellar granule neurons suggest that these cannabinoid receptor inactive amides may prevent or delay the breakdown of the cannabinoids by competing for their degradation by the hydrolyzing enzyme FAAH [100, 101]. It was also claimed that the NAEs may also inhibit or compete for the anandamide transporter by increasing the availability of the endocannabinoid for interaction with the CB1 receptors [102]. These theories might also be true for the primary fatty acid amides. Because oleamide was detected in $N_{18}TG_2$ mouse neuroblastoma cells about 80 times higher than the reported anandamide level in the same cells [86], it is possible that oleamide may be a biological enhancer of anandamide activity. Leggett *et al*. [103] have very recently presented data indicating

oleamide as an agonist for CB1 receptor *in vitro* but *in vivo* data reported so far is conflicting with this finding [104] and therefore is inconclusive.

The synthesis of both of these cannabinoid receptor active and inactive bioregulators *in vitro*, the research on their biochemical and pharmacological properties, and their quantification in numerous biological samples has led us to the understanding of their various activities so far. In this paper our main goal is to review the analysis of fatty acid amides by instrumental means with special attention paid to the sample preparation.

1.6 Instrumental analysis

The techniques used over the years for lipid analysis are mainly gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE) coupled with various types of detectors. Ultraviolet-visible absorption (UV-vis), fluorescence (F), diode-array UV-vis, flame ionization (FID), laser induced fluorescence (LIF) detection, and electron ionization (EI) or chemical ionization (CI) by a mass spectrometer (MS) are the common detection techniques of which the latter is the most widely used for many years. Due to the nonvolatile nature of lipids derivatization is usually required in order to make them volatile and thermally stable to be analyzed by gas chromatography. The other advantages of derivatization are better resolution, improved detector response and peak shape. This situation led researchers to find numerous techniques for lipid derivatizations, most of which works fine, but the formation of artifacts makes the techniques complicated for identifying the target itself. Derivatization in LC is also often required in order to improve (i) detectability, (ii) resolution and selectivity of

analytes, (iii) analyte identity in a complex matrix, (iv) chromatographic behavior as well as (v) stability of analytes under study. One example is that fluorescence tagging is required for improving the detectability of the PFAM's by LC/UV. Even though unlike GC the majority of the compounds can be analyzed by LC without prior derivatization, one advantage of GC over LC is its higher peak capacity due to the availability of the longer column length. In recent years the newly developed ionization techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) have been the choice of lipid chemists due to their ability to form directly gas phase molecular ions for simple as well as complex lipids. CE is another technique that has not been widely applied to the lipid analyses but provides great hope for amide analysis.

1.6.1 Liquid chromatography-mass spectrometry (LC/ESI/APCI/MALDI-MS)

High performance liquid chromatography (HPLC) is a separation technique that has advantages over gas chromatography due to its ability to analyze nonvolatile and thermally fragile molecules. LC can be applied for the separation of a wide range of chemically and biologically significant molecules such as small metabolites to large proteins. The basic HPLC system consists of mobile phase reservoirs, a pump for solvent delivery, an injector, a column and detectors of choice.

The most used HPLC separation techniques are normal-phase (NP) and reversed-phase (RP) HPLC. The adsorption in NP occurs by the interaction of polar functional groups of the solute with discrete sites on the stationary phases. Depending on the strength of these polar interactions, NP chromatography selectively separates

solutes with different number of electronegative atoms or with different functional group. This type of chromatography is therefore suited for class separation. Of the two types of LC stationary phases, silica is used more due to its high sample capacity and less catalytic activity than alumina. Because bare silica can strongly retain some of the very polar compounds, bonded silica stationary phases (diol, cyanopropyl or amino propyl bonded) are used in their separation. Using these less polar phases it is easier to clean the column and the presence of water in mobile phases creates less of a problem in the retention time reproducibility. The drawback of NP chromatography is that separation selectivity cannot be achieved by varying the particle size or shape. Selectivity in this case depends primarily on varying the mobile phases. Reversed-phase chromatography utilizes non polar stationary phase with polar mobile phases, which is reverse of the case of NP chromatography. In RP, retention of solutes occurs through nonspecific hydrophobic interactions with the stationary phases. Therefore the most polar solutes in this case have the least retention time. The advantage of RP over NP is that the individual compounds in a class can be separated in this chromatography. The stationary phase in RP can be bonded silica or different polymeric phases, although bonded silica being the most widely used. Variation in retention and selectivity can be achieved somewhat by using different RP columns, but varying the mobile phase composition provides the greatest selection in separation as in the case of NP [105].

The types of detectors used in conjunction with LC are refractive index, UV-vis, fluorescence, diode-array UV-vis and mass spectrometer (MS). All of them provide spectral data in the form of signal as a function of time whereas the latter provides signal as a function of mass to charge ratio (m/z) as well. The most used LC/MS

ionization involves the ionization of the analyte molecules at atmospheric pressure and the common sources of ionization are electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI). In ESI the analyte in appropriate solution is sprayed into a chamber in the presence of a strong electrostatic force and heated dry N_2 gas at atmospheric pressure and therefore dissociated into gas phase ions. These gas phase ions are attracted through a dielectric capillary entrance and enter into the mass analyzer. In APCI, the sample in appropriate solvent is sprayed through a heated vaporizer at atmospheric pressure resulting in the formation of gas-phase solvent molecules, which in tern are then ionized by electrons discharged from a corona needle. The charges on the solvent molecules are then transferred to the analyte molecules through chemical reactions and the analyte ions thus enter into the mass analyzer through the dielectric capillary entrance. In the relatively new technique APPI, the sample in appropriate solution is converted into gas phase molecules, which are then ionized by photons of narrow range ionization energies generated from a discharge lamp. The advantage of APPI is that the carefully chosen ranges of photon energies allow the formation of as many analyte ions as possible minimizing the ionization of the solvent molecules. The ions formed then enter in the mass analyzer the same way as ESI and APCI [106]. The other most popular ionization technique that creates direct gas phase molecular ions is the matrix-assisted laser desorption ionization (MALDI). MALDI was first introduced by Tanaka *et al*. [107] for which, he was awarded the 2002 Nobel Prize in chemistry. In MALDI, the analyte is co-crystallized with a matrix (usually 1:1000 molar ratio of analyte to matrix), which absorbs in the laser region (337 nm for a N_2 laser, for example). The matrix is ionized

upon bombardment of the mixture with short duration pulses of UV light from a nitrogen laser. The analyte is ionized by energy transfer from the matrix to the analyte rather than by direct laser ionization. The ions produced then are guided to the mass analyzer by applying a high potential electric field.

The most common types of mass analyzers used are ion trap, quadrupole, time-of-flight (TOF) and fourier transform-ion/cyclotron-resonance (FT/ICR or FT/MS). In ion trap mass analyzer the ions entering into the ion trap chamber are trapped by electromagnetic fields and selectively ejected by another field. Ion trap has an advantage of performing multiple stages of mass spectrometry without additional mass analyzers. The simplest and the least expensive mass analyzer is the quadrupole mass analyzer, which consists of four parallel rods arranged in squares. When the analytes enter the quadrupole, the electromagnetic fields generated by applying voltages to these rods detects which mass-to-charge ions can pass through the filter at a given time. TOF mass analyzer consists of a flight tube through which the entering ions are allowed to accelerate by the application of a uniform electromagnetic force. Because the lighter ions travel faster, the mass-to-charge ratios of the ions are determined by the time required by the ions to reach the top of the flight tube and reflect back to the detector. The most expensive mass analyzer, FT/MS, operates in ion trapping mechanism where the ions entering the analyzer are trapped in circular orbits by powerful electrical and magnetic fields. The ions excited by a radio-frequency (RF) electrical field generate a time dependent current, which is converted to orbital frequencies corresponding to the mass-to-charge ratios of the ions by fourier transform. Even though the relatively soft ionization techniques discussed above are able to form gas phase molecular ions and are

more suitable for nonvolatile lipid analysis then the previously used techniques such as EI or CI, they are only capable of generating few fragment ions which are not enough to achieve structural information. The most powerful analytical tool developed in recent years is the collision-induced decomposition (CID) of the molecular ions, which can provide molecular weight information as well as create more fragmentation, allowing the elucidation of structural details [106].

Liquid chromatography has been widely used over the years for the analysis of lipids due to its ability to handle nonvolatile substances, which is a crucial factor in lipid chemistry. The native structures of complex phospholipids can be precisely obtained using MALDI and ESI which was impossible with electron impact MS. On-line HPLC and tandem MS with its capability to separate and identify complex lipids occurring as a mixture in natural products and biological samples is a significant analytical tool for lipidomics. A number of authors have reviewed lipid analysis over the years. These reviews include but are not limited to those by Kim *et al.* [108], Careri *et al.* [109], Murphy *et al.* [110], Volin *et al.* [111], Byrdwell [112, 113], Schiller *et al.* [114], and Griffiths [115]. Our discussion will be in the light of these reviews as well as recent papers, with special attention paid to the fatty acid amides.

1.6.1.1 Liquid chromatography (LC) of lipids

In lipid analysis high-performance liquid chromatography (HPLC) has become a great importance not only for separation of individual lipid classes but also for the sample preparation. Usually after the Folch-pi type of lipid extraction from any tissue/cells, HPLC is employed for further extraction into individual lipid classes. Separation of

each lipid within the classes can also be achieved before mass-spectral analysis. Both the normal phase (NP) and reverse phase (RP) HPLC can be used for the separation of lipids. Phospholipids, for example, are the mostly studied lipid classes by LC. In normal phase HPLC (silica) phospholipids are separated by the polarity of head group, *i.e*., the neutral phospholipid classes elute first and phosphocholine-phospholipids elutes later. Therefore NP is mostly for the separation of lipids by individual classes. Whereas RP can be used to separate individual components within classes provided that the separation of each class was achieved by NP prior to RP. The reason behind that is, in RP, elution is largely depended on the fatty acyl chain rather than on the polar head group resulting in the co-elution of molecules within different classes. For simplicity NP is employed for their separation by classes followed by MS for further identification of each species. One disadvantage of using silica-based column though is the issue with the reproducibility of the retention time if water is present in the solvent system, which can change the affinity of phospholipids for silica. The typical NP mobile phases are hexane/alcohol mixtures whereas typical RP mobile phases are acetonitrile/alcohol mixtures modified depending on the lipids to be separated [reviewed in Ref 116]. For the separation of fatty acids RP is most widely used. Because of the absorptivity of the carboxyl group at low UV region, fluorescent tagging is often required to form derivatives, which fluoresces in the UV region for peak monitoring. Another example is the analysis of eicosanoid which requires concentration in order to increase sensitivity for RPLC analysis. A limitation in their MS analysis is that some isomers produce identical spectra, therefore it is necessary to chromatographically separate all the

isomers before hand. Separation of PFB esters of eicosaniods on a NP column is also useful before MS study [for review see Ref. 117].

1.6.1.2 ESI/APCI/MS

1.6.1.2.1 ESI/APCI/MS of storage lipids

The identification as well as the determination of the acyl position is a crucial factor for the analysis of the lipids. Even though triacylglycerols (TAG) are the most volatile of the complex lipids, derivatization is required for their analysis by GC/MS. Separation and identification is somewhat limited due to the present of numerous different TAG species containing different fatty acyl chain along the sn-1, 2 and 3 position of the glycerol backbone. The advantage of ESI or APCI to form direct gas phase ions has made the analysis of intact TAG species easier. ESI yields $[M+Na]^+$, $[M+NH_4]^+$, $[M+K]^+$ ions for the monoacylglycerols (MAG) diacylglycerols (DAG) or the triacylglycerols [118, 119]. The alkali adduct ion formation is usually facilitated by the addition of those corresponding ions to the electrospray buffers. One problem in these cases appears to be the relation of the absolute abundance of these ions to the total number of double bonds in the acyl chains of these lipid species. The lipid containing saturated acyl chain results in poor yields of the adduct ion, whereas the one containing unsaturated acyl chain result in higher yields. Further CID of these adduct ions provide information about the location of the acyl group as well as the location of the double bonds. For example, Cheng *et al*. [120] revealed the complete structure of the TAG by the high energy CID studies of the NH_4 and the Na adduct ions. APCI of TAG also results in the formation of $[M+H]^+$ ions but poor yield of these ions of the TAG species

with saturated acyl chain remains to be the problem [119]. Silver ion HPLC along with APCI detection was reported for the separation and detection of numerous TAG species in a number of different oil samples [121].

1.6.1.2.2 ESI/APCI/MS for phospholipids

Analysis of glycerophospholipids is somewhat more challenging if the identification of the position of the two acyl chains is required. Even though field desorption-MS was first used [122] to analyze glycerophospholipids, fast atomic bombardment (FAB)-MS was most widely used due to the useful information that could be found from the FAB generated molecular ions [123-131]. The disadvantage of the FAB was the considerable variation in the ion yields for the different classes of the phospholipids, which seemed to be taken care of by the use of ESI-MS [132-135]. In these studies $[M+H]^+$ and [M+ alkali metal]$^+$ ions were reported to be the abundant ions. These parent ions were further fragmented by collision induced decomposition (CID) yielding significant information about the polar head group as well as the diglyceride ions resulting from the neutral loss of the head groups. $[M-H]^-$ ions were also yielded by glycerophospholipids and were successfully used in the analysis of these species in biological samples [136-137]. Analysis of glycerophospholipids as their [M + acetate]$^+$ ions is also possible by using acetate buffers for ESI. Additional ion found to be abundant was $[M-15]^-$, which resulted from the CID of the [M + acetate]$^+$ ions during tandem quadrupole MS [138]. The formation of carboxylate ions from glycerophospholipids by CID in the negative ion ESI mode offers identification of the acyl chains esterified in the sn-1and sn-2 position even though the sn-1 carboxylate ions

predominate [139]. Collisional activation of the negative molecular ions $[M-H]^-$ can also yield ions corresponding to the loss of polar head groups [140].

The sphingolipids also have a polar head group, except ceramides, which lack the polar head group. This group also forms abundant $[M+H]^+$ ions using both ESI and APCI [141, 142]. The CID of this molecular ion yields ions corresponding to the loss of water as well to the cleavage of the amide link. Negative ions are also generated during ESI yielding $[M+Cl]^-$ ions which upon CID form Cl^- ion at m/z 35 [143]. Sphingomyelin also derive $[M+H]^+$ ions as well as $[M-15]^-$ ions specific to phosphocholine lipids. CID of the molecular ions but not the $[M-15]^-$ ions yield the abundant ion for phosphocholine head group at m/z 184 [131]. Formation of $[M+Li]^+$ ion in ESI-tandem mass spectrometer was described by Hsu *et al*.. [144] where CID was found to yield $[M+Li-183]^+$ and $[M+Li-183-18]^+$ ions corresponding to the loss of phosphocholine headgroup and water respectively. Fatty acyl chains and the base could be identified from the less abundant ions, but this analysis is not very promising. As said before, membrane lipids are the most complex lipids and a challenge for the analytical techniques available due to presence of acyl chains as well as varied polar head groups to the glycerol or to the sphingosine backbone. APCI seemed to be more successful in the acyl chain analysis in sphingomyelin than ESI due to the yield of several product ions such as the one $[M-N(CH_3)_3-H_2O+H]^+$ due to the abundant loss of $N(CH_3)_3$ and water as well as the loss of ceramide-specific ions [145, also see Figure 1.1]. APCI and ESI were both used to analyze sphingolipids in brain and human plasma [146, 147]. Rather complex glycosphingolipids containing a carbohydrate linked to the ceramide base instead of a polar head group are more difficult to analyze.

Successful use of ESI, MALDI and FAB were reported for the analysis of these lipids [148-163].

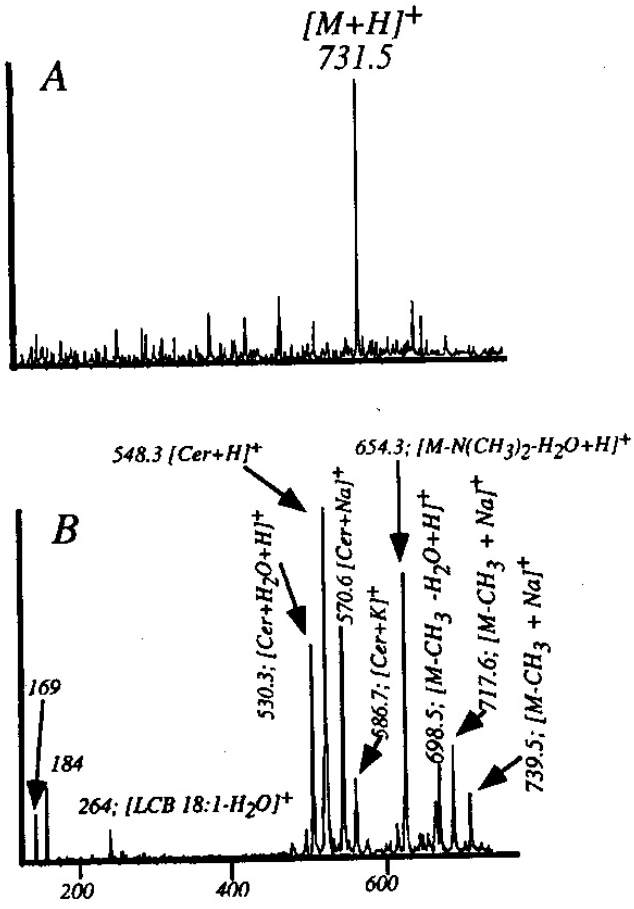


FIGURE 1-1: Positive ions from sphingomyelin (d18:1/18:0) obtained by A) ESI and B) APCI. Reproduced from Ref.110.

1.6.1.2.3 ESI/APCI/MS of metabolites and messenger lipids

Free acids are the mostly studied lipid class by the highest number of various instrumental techniques. Negative ion ESI of saturated fatty acids yield $[M\text{-}H\text{-}H_2O]^-$

ions whereas unsaturated fatty acid shows additional ions derived from the bond cleavage at the α- and γ-position of the unsaturation [164]. APCI/MS of fatty acids mostly produces M^+ peak but in case of the hydroxy fatty acids, $[M-H_2O+H]^+$ was observed as a base peak. The other peaks observed were the protonated molecular ion peaks, which were prominent if the hydroxy group were located near the carboxylate end [112]. Eicosanoids produce abundant carboxylate anion under ESI. Due to the complexity of the eicosanoids, CID is generally used to reveal further fragmentation information. The detection limit of various eicosanoids using unique CID product ions could be as little 1.6 ng/mL. ESI of steroids yield $[M+H]^+$ ions as well as additional ions corresponding to the loss of one and two water molecules. APCI on the other hand yields only $[M+H]^+$ or $[M+H]^-$ ions. Femtomole level detection was achieved in the analysis of several steroids even though the low yield of the ions is a serious drawback in their analysis. The analysis of steroid conjugates is more successful as the yields of the product ions are higher and further fragment ions from CID can be monitored selectively for quantitation in femtomole level [for review see Ref. 110].

1.6.1.3 MALDI/MS

Even though MALDI is used mainly for the analysis of water-soluble compounds and proteins, the interest in lipid analysis by MALDI is currently increasing. MALDI has been used successfully for the analysis of lipids in various samples including vegetable oils [165, 166, 167-170], crude tissue extracts [171-185], extracts of body fluids and cells [186-196] and it has been well established that quantification with reproducible results can be obtained by MALDI/TOF given adequate sample preparation and

homogeneity of the analyte and matrix. The use of a specific matrix to absorb the laser energy as well as to keep the analyte from cluster formation makes lipid analysis feasible. The use of 2,5-dihydroxybenzoic acid (DHB) is suitable in the lipid analysis because it does not tend to form cluster. Also very low yield of matrix ions are generated which is a plus point for analysis of complex compounds. Sinapinic acid, a common matrix used in MALDI should be avoided in lipid analysis because it tends to undergo polymerization, resulting in the saturation of detector [197, 198]. Because lipid and the matrix are both organic compounds, it is very easy to make a homogenous mixture of analyte and the matrix. In MALDI, a solid sample rather then an analyte in solution is required, which calls for minimal sample preparation. Another main advantage in MALDI is that singly charged ions predominate, therefore less complication is expected. Even though multiply charged molecules are also generated, they show a higher tendency toward neutralization, leaving the stable singly charged ions easily detectable [199]. The extraction of lipid prior to analysis gives better results than applying the sample without extraction. DHB as a matrix requires quick drying of the sample on the MALDI plate after mixing with the matrix in order to obtain small and homogeneous analyte/matrix co-crystals. The ions that are observed in the lipid analysis are mainly $[M+Na]^+$, $[M+K]^+$ and $[M+H]^+$. The first two cations [Na^+ and K^+] are typically found in the lipid extracts and $[M+H]^+$ arises from the addition of TFA [~0.1%] to the matrix. The adduct pattern is strongly influenced by applied laser intensity as well as the ion composition of the applied solvent.

1.6.1.3.1 MALDI of storage lipids

Analysis of diacylglycerols by MALDI-MS was reported using 2,5-dihydroxy benzoic acid (DHB) as a matrix [162] where $[M+Na]^+$ ions appeared to result in better yields. MALDI was able to identify TAG as $[M+Na]^+$ ions in olive and cod liver oil without prior separation using R-cyno-4-hydroxy-cinnamic acid as a matrix [165, 200]. The analysis of vegetable oil samples were possible by MALDI-TOF [166] due to the simplicity of the adduct formation (mostly Na^+ adduct were visible). In MALDI, DAG and TAG show very little fragmentation of the stable $[M+Na]^+$ ion adduct. For DAG, formation of carbenium ions has been observed due to the loss of NaOH [201]. For TAG, loss of the Na salt of a fatty acid residue results in fragment patterns which might be also the case in DAG but is less pronounced due to the interference with the peaks from matrix. The fatty acid composition also has an effect on the fragmentation behavior. For TAG and DAG, the formation of Na^+ adduct is more prominent than the H^+ adduct, which is due to the different tendencies of the Na^+ ion and H^+ ions to bind to the glycerol backbone than to the polar head groups.

1.6.1.3.2 MALDI of membrane lipids

Cholesterol esters behave the same way as TAG and DAG, giving the $[M+Na]^+$ adduct whereas cholesterol gives an $[M+H-H_2O]^+$ ion due to the loss of water and its high affinity for H^+ ion. For phospholipids the intensity of $[M+H]^+$ adduct is typically stronger than the alkali adduct in MALDI spectra. Because the adduct pattern strongly depends on the solvent composition and the applied laser intensity, enhanced signal for alkali adduct can be generated by increasing laser intensity. Sphingolipids can be

analyzed as intact molecules and can yield several peaks due to the heterogeneity in the sphingosine or the acyl chain. One of the most prominent peaks is that derived from the sphingosine residue at m/z 264.0. Analysis of complex glycerophospholipids in human neutrophils by MALDI/TOF with DHB containing 1% TFA as a matrix resulted in the formation of $[M+H]^+$ and $[M + alkali]^+$ ions [197]. MALDI was also used along with FT/ICR [202] for the analysis of these lipids using DHB as a matrix and both $[M+H]^+$ and $[M-H]^-$ ions as well as $[M+ Na]^+$ ions were observed as abundant ions. CID of the negative molecular ions yielded abundant carboxylate ions of which sn-2 carboxylate found to be predominant. Using MALDI it is possible to profile a mixture of sphingolipids and glycolipids by their Na^+ adducts [203]. In order to avoid complication, sample preparation with TLC prior to MALDI is recommended [204].

1.6.1.3.3 MALDI of metabolites and messenger lipids

Free fatty acids can be analyzed by MALDI with low detection limit due to their low molecular weight. Saturated free fatty acids were found to form Na^+ adduct of the sodium salt of the free fatty acid but the presence of unsaturated ones shifted the molecular weight by +14 for all the fatty acids in a mixture [114]. Use of MALDI has not been quite occurred yet in the analysis of eicosanoids or steroids, but can be of great importance. Providing the development of appropriate matrices, application of MALDI with tandem MS can provide more advanced identification of complex eicosanoids.

1.6.2 Gas Chromatography/mass-spectrometry (GC/MS)

Gas chromatography (GC) is a unique and versatile technique used with many types of samples with little or no sample modification. It was developed for the analysis of

volatile compounds and from there it has been expanded for the chemical separation and analyses of gaseous samples, liquid solutions and volatile solids. Typical GC system consists of an oven (which controls the temperature of the column), an injection port and a detector connected to the column. The columns employed usually vary in phase polarity depending on the nature of the compounds to be analyzed. If there is no interaction between the column phase and the analyte no retention occurs, therefore the non polar compounds are analyzed by non polar columns (95-100% dimethyl polysiloxane phase) and polar compounds by polar columns (14-100% polar group substituted dimethyl polysiloxane phase). Column length (l), internal diameter (r) and film thickness (d_f) are the factors that are usually taken into consideration while choosing a capillary column. The regular values for these parameters are r: 0.1 mm to 0.5 mm; l: 30-100 m; and d_f: 0.10 μm to 5.0 μm. It is ideal that the column be as short as possible to keep the analysis time and cost low. The chromatographic resolution and the analysis time depend on the value of r. Usually at constant d_f , the lower the r the higher the resolution and better the separation between critical pairs. If MS is used as a detector, 0.32 is the maximum value of r used. The combination of the values of r and d_f should be so chosen that the value of the column phase ratio is higher for better separation.

A number of different types of detectors are used in conjunction with GC of which the most frequently reported ones for lipid analysis are the flame ionization detector (FID) and the electron impact or chemical ionization mass spectrometry (EI/CI/MS). With FID the analytes are burned in a hydrogen flame and thus are ionized. The negative potential of the jet results in the neutralization of the positive

ions and the electrons are captured by an electrode to produce a signal current. In FID only substances with at least one C-C or C-H bond are detected and as a universal technique FID is not well suited for the trace analysis in complex matrices. FID is also not suitable for the analysis of the highly chlorinated or brominated substances [205]. In the MS mode, two different ionizations electron impact ionization (EI) or chemical ionization (CI) can be used. EI is the most frequently used ionization process in all GC/MS systems. EI involves the interaction between an energy-rich electron beam with the outer electron of the analyte molecule giving rise to the formation of the molecular ion [$M^{+\bullet}$]. The excitation in the rotational and vibrational level of the $M^{+\bullet}$ ion by excess kinetic energy impacted by the electron beam produces further fragmentation. Because the typical ionization potential used in most EI processes is 70 eV, which is almost 5 times higher than the potential required to ionize the organic molecules, sometimes in EI daughter or granddaughter ions are observed instead of the $M^{+\bullet}$ ions. Therefore in EI, determination of molecular weight is sometimes troublesome and the application of CI is necessary. In CI, a reagent gas is used for the ionization of the analyte molecules instead of a beam of electrons. The two step reaction involves the formation of a reagent gas cluster through electron bombardment followed by the reaction between the cluster and the analyte molecule in order to form protonated molecular ion. CI can be used in either positive or negative mode depending on the need of a particular analysis and because it uses considerably less ionization potential, little or no fragmentation of the molecular ion occurs, providing important information about the molecule itself. The data can be monitored by selecting either the total ion mode (TIM) or the selected ion mode [SIM] in most GC/MS systems.

The main disadvantage of gas chromatography in lipid analysis is its incapability of direct analysis of the larger nonvolatile lipid molecules. Even though recently commercialized modified columns are suitable for free fatty acid and amides analysis, most of the lipids needed to be derivatized to a suitable volatile molecule prior to injection. Another disadvantage of GC/MS is the lower sensitivity for trace analysis and the analysis of compounds in complex matrices. Conjunction of GC/MS with TLC and SPE is more preferable for the purification and preconcentration of the analytes of interest.

1.6.2.1 GC/MS of storage lipids

Triacylglycerols (TAG) are probably the mostly studies lipids by GC/MS. In studies, that require the quantification of total fat content in food, transesterifiction under acidic/basic condition is usually done and the fatty acids are analyzed as their methyl ester (FAME). Almost all FAME can be separated on a relatively polar column giving the analyst an idea of available types. EI spectra of TAG usually produces $[M\text{-}R_nCO_2]^+$, $[R_nCO+128]^+$, $[R_nCO+74]^+$ and $[R_nCO]^+$ ions, providing information about carbon chain and the number of double bonds. But the studies that require identifying the location of the acyl chain in glycerol backbone as well as the position of the double bonds in acyl chain are quite challenging. ESI/APCI are mostly employed for such tasks. Separation and detection of MAG is much simpler and can be conveniently carried out by GC/MS due to the presence of only one acyl chain. Analysis and quantification of DAG is also a sensitive technique by GC coupled with negative ion CI. They can be separated and detected as their pentafluorobenzyl (PFB) ester, even

though the derivatization technique results in the migration of the acyl groups into stable 1, 3 positions. The detection limit was found to be 30 fmol of an endogenous DAG species extracted from human basophils [115, 206-210].

1.6.2.2 GC/MS of membrane lipids

Whereas LC/ESI/MS is the most widely used technique for the analysis phospholipids and sphingolipids, use of GC/MS has also been employed for a long period of time for their analysis. Numerous amount of work have been reported in this regard which requires suitable derivatization techniques but none are adequate for the complete structural details. For example, hydrolysis of ceramide followed by GC/MS analysis provides no more information than the identity of the fatty acyl chain and the bases. Ceramides can also be analyzed in intact form as their trimethylsilyl (TMS) or methylated derivatives. The analysis of the methylated glycosphingolipids from human and mouse body organs shows that ceramides separate according to the chain length of the fatty acids but co-elution of some ceramides under each GC peak was observed. Separation in subclasses by TLC or SPE before GC/MS is usually helpful in this type of situation [163, 211-219].

1.6.2.3 GC/MS of metabolites and messenger lipids

Gas chromatographic separation and EI/CI detection can be carried out for the identification of the fatty acids after derivatizing them into a suitable volatile ester, typically the methyl ester [220-223, also see Figure 1-2]. Due to the presence of numerous different species in biological samples this type of analysis is difficult in clarifying the individual origin of the too many fragment ions formed. Optimized

analysis conditions such as sample clean up and select ion monitoring usually improve this situation [224-225].

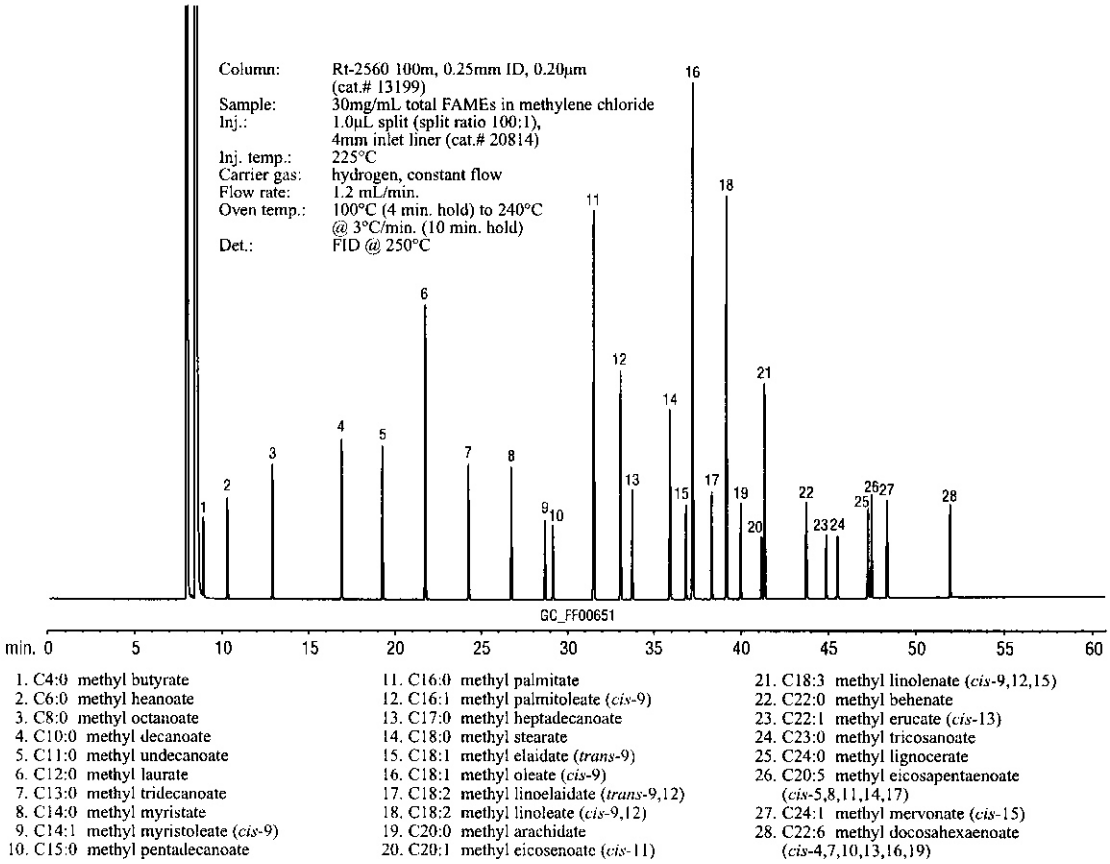


FIGURE 1-2: Separation of C4 to C24 fatty acid methyl esters on restek Rt-2560 column. Reproduced from Restek website (www.restekcorp.com).

One example is the analysis of the arachidonyl containing phospholipids in human histiocytic lymphoma U937 cell line by negative ion CI/MS as their PFB ester [131]. The predominant carboxylate anions were monitored for the assay of the arachidonic acids in this case. Methylation conditions were optimized by Park *et al*.

[226] for the quantitative analysis of the isomers of conjugated linoleic acids (CLA) in various lipid samples. They found that methylations only under different conditions are suitable for different types of CLA and GC was not suitable for the analysis of samples containing low amounts of CLA. Quantitation of eicosanoids by GC/MS requires different derivatization and purification procedures for different eicosaniods, which is complicated and time consuming [227-236]. Use of suitable derivatization agent and careful sample preparation can solve these problems. For example, the analysis of eicosaniods after solid phase extraction (SPE) and derivatization with tert-butyl dimethylsilyl ether (*t*-BDMS) was reported by Tsukamoto *et al*. [237]. Use of one internal standard per eicosanoid and SIM provided the assay of six different eicosanoids from cultured cells. Their method was found to be linear from 10 pg to 100 ng.

1.6.3 CE-LIF/CE-MS

Capillary electrophoresis (CE) is a novel analytical tool which can provide selectivity and higher chromatographic resolution for a wide variety of samples. The high voltage separation and the narrow capillary zone offer fast and efficient separation. It also has an advantage of low sample and solvent consumption over other analytical techniques. Capillary electrophoresis, (CE), was introduced by Jorgenson and Lukacs [238-242] in the early 1980's. CE coupled with various detectors offers the automated analytical equipment, fast analysis times and on-line detection of the separated peaks. The possible CE modes are capillary zone electrophoresis, micellar electrokinetic capillary chromatography, capillary gel electrophoresis, capillary isoelectric focusing, and capillary isotachophoresis with various detection systems such as UV-Vis, fluorescence,

Raman, and laser induced fluorescence (LIF) [243]. The separation based on size and charge differences between analytes is termed capillary zone electrophoresis (CZE) or free solution CE (FSCE); separation of neutral compounds using surfactant micelles is refereed as micellar electrokinetic capillary chromatography (MECC); sieving of solutes through a gel network is termed as capillary gel electrophoresis (GCE); and separation of zwitterionic solutes within a pH gradient is called capillary isoelectric focusing (CIEF). Capillary electrochromatography (CEC) is an associated electrokinetic separation technique which involves applying voltages across capillaries filled with silica gel stationary phases. Separation selectivity in CEC is a combination of both electrophoretic and chromatographic processes. FSCE and MECC are the most frequently used separation techniques in pharmaceutical analysis. GCE and CIEF are of importance for the separation of biomolecules such as DNA and proteins, respectively, and are becoming of increasing importance as development of biotechnology derived drugs is becoming more frequent. So far most of the work done by CE/LIF / CE/MS focused on the analysis of mainly peptides and proteins. A very few papers on lipid analysis by CE have been published most of which focused on the separation of the fatty acids [151, 244-261].

1.6.4 Fatty acid amide analysis

1.6.4.1 Sample preparation

1.6.4.1.1 Lipid extraction

Lipid extraction is the first step of lipid analysis and is the most important task. Folch-Pi and Bligh/Dyer [262-263] extractions are the two types of techniques that are widely

being used and modified. Usually a 20 fold volume of chloroform:methanol (2:1, v/v) is added to the tissue/cell for homogenization. The volume of a tissue sample is computed on the assumption that the specific gravity of tissue is the same as that of water. After homogenization the sample is filtered and 20% water (or aqueous salt solution) is added to the total volume of filtrate. The mixture separates into two phases: the lower phase (chloroform:methanol:water; 86:14:1, v/v/v) containing lipids and the upper phase (chloroform:methanol:water, 3:48:47, v/v/v) containing non lipid contaminants. The upper phase is then discarded and lower phase is dried under N_2 to be dissolved in a suitable solvent [262]. Modifications of this method are allowed providing the required ratio of tissue/cell to solvent is maintained. Modification also depends on the types of lipid classes to be extracted. For example, centrifugation can be used instead of filtration, but 20% methanol needs to be added to the homogenate in order to lower its specific gravity. Therefore, after centrifugation, twice the amount of chloroform must be added to the supernatant in order to have a 2:1 chloroform to methanol ratio. Also the Folch solvent can be replaced by suitable solvents such as isopropanol instead of methanol or dichloromethane instead of chloroform, etc. One disadvantage of the Folch process is that the gangliosides remain entirely soluble in the upper aqueous phase. If gangliosides are to be extracted, dialysis of the upper phase to remove ions and other low molecular weight compounds followed by lyophilisation is done. Fatty acid amides being only moderately polar can be extracted readily with the total lipid using the conventional Folch-Pi extraction.

1.6.4.1.2. Sample clean-up/ derivatization

Before analyzing the sample by the choice of instrument, it is common practice use TLC or SPE to separate target analyte or analytes. The pure and concentrated analyte is then injected into the GC/MS or LC/MS system in order to increase the sensitivity and detactibility (see Section 1.7, Chapter 2 and Chapter 3 for more detail on sample clean-up by TLC and SPE). As discussed above, if the analyte is non volatile, or has absorptivity only in the low UV region, derivatization is often required. Fatty acid amides are one such lipid class that possess both the characteristics and hence need to be derivatized for instrumental analysis. Even though amides can be successfully analyzed in GC polysiloxane columns without derivatization, severe tailing due to the NH_2 group hinders trace analysis of amides. This is especially true when analyzing amides from biological samples, which contain numerous other species. Sample clean up is therefore an important part of amide analysis. HPLC analysis of amides is also troublesome due to the abosoptivity of amide group in low UV region (`210 nm). Fluorescent tagging is required for the formation of fluorophore which fluoresces in the UV region allowing peak monitoring (see Chapter 6 for more information on derivatization for GC analysis).

1.6.4.2 Chromatography methods

Gas chromatography (GC) is the most widely applied technique in the separation of fatty acid amides. As mentioned in the earlier section, volatile derivative formation is usually required for GC analyses. Even though the analysis of underivatized fatty acid amides can be performed in polysiloxane columns, quantitation at trace level is

troublesome due to the severe tailing from the NH_2 group [264]. *N*-acyl ehanolamines were detected in both derivatized (O-acetyl or TMS) and underivatized forms using SE-30 GC and DV-5 columns [1, 18]. Separation of a mixture of low molecular weight fatty acids, PFAM's and NAEs using a chromosorb-101 column was also achieved [265]. Separation of C12-C22 NAEs as their O-acetyl analogs showed that the unsaturated ones eluted right before their saturated analog on OV-1/OP-15 columns [266]. Schmid and co-workers [267, 268-270] reported the analyses of *N*-acyl PE (precursors of NAEs) and NAEs as their *tert*-butyldimethylchlorosilyl (*t*-BDMS) derivatives by a HP-5MS column. The use of a similar column (BP5) for the separation of NAE and their precursors without prior derivatization was also achieved [271]. Primary fatty acid amides were analyzed without derivatization [272-274] using 3% Dexil 300/CP-Sil 5 CB/BP1 columns but only oleamide, stearamide and erucamide were separated. In 1985 Arafat *et al.* reported [61] the extraction of five long chain saturated and unsaturated PFAM's from human luteal phase plasma and their analysis as trimethylsilyl (TMS) derivatives by an HP-5MS column. Separation of TMS derivatives of C2-C20 fatty acid amides (both saturated and unsaturated) was also shown on a Supelco Simplicity-5^{TM} column by Gee *et al.* [275]. They found that the peak intensity was largely dependent on the silylating agent used. Use of BSTFA (*bis*-trimethylsilyltrifluoroacetmide) and MSTFA (*N*-methyl-*N*-trimethylsilyltrifluoro-acetmide) provided the most complete reactions and the derivatized amides with these two reagents produced largest peak areas. They also studied the derivatization of amides by TMSCl (chlorotrimethylsilane) which is usually used as a catalytic

element for selectively derivatizing lipids. No derivatization occurred when TMSCl was used alone. Presence of TMSCl was found to prevent the reaction between MSTFA and amides. The extent of the reaction of amides with BSTFA was found to be reduced in presence of TMSCl. BSTFA was chosen over MSTFA as a derivatizing agent because of the greatest signal-to-noise and signal-to-background ratios in the former case. Toluene was used as a solvent because (i) amides as well as the silylated amides are soluble in toluene, (ii) reaction in toluene and ethyl acetate produced maximal peak areas for the silylated amides, and (iii) reaction in toluene gave rise to higher signal-to-noise and signal-to-background ratios than in ethyl acetate. They also optimized the temperature and time for the derivatization of oleamide in BSTFA-toluene. HP-5MS was used to study the both derivatized and underivatized oleamide which showed that *N*-trimethylsilyl oleamide and *N*-*t*-butyldimethylsilyl oleamide had better detection limit then the oleamide itself [264]. Even though the detection limit improves upon derivatization, one should not discount the possibility of artifact formation. Vosmann *et al.* [276] reported that the derivatization of lipid mixture containing primary fatty acid amides and NAEs with trimethylsulfonium hydroxide [TMSH] led to the formation of *N*- and /or *O*-methylated derivatives which can result in faulty conclusion. It is therefore necessary to use the judgement where derivatization is actually required. Detection limits need to be determined for derivatized and underivatized amides. If the amounts of amides in a sample fall above the quantitation limit for underivatized amides, derivatization should be avoided. If it is absolutely necessary to use

derivatization, the optimum conditions must be used so that as low as possible derivatization agent can be used under mildest conditions.

Liquid chromatography (LC) is the other technique used in fatty acid amide analyses even though it has not been as widely employed as GC. LC is usually used for oxidized, highly branched or relatively polar samples (e.g., phospholipids). One advantage of LC over GC is that the derivatization is not required. But the absorptivity of the -$CONH_2$ group in low UV region (~210 nm) gives rise to poor sensitivity [277]. In order to increase sensitivity, fluorescence tagging is often required with compounds that fluoresce in the UV region. Carpenter *et al.* [277] studied the separation of a series of *N*-acylglycines (NAG) and PFAM's (C2-C12) using a Phenomenex Luna C_8 column. The mobile phases for NAG were 50 mM sodium phosphate (pH 6.0): acetonitrile as gradient and those for PFAM's was water: acetonitrile as gradient followed by isocratic elution. The separation of long chain PFAM's (C12-22) were achieved using a Waters C_{18} column and water: methanol as gradient. They (Carpenter *et al.*) mentioned that a longer run time (over 25 min) for the separation of saturated and unsaturated amides was required. C18 Reversed-phase HPLC was also used to assay PAM activity for the conversion of *N*-acylglycine to the primary fatty acid amides by Carpenter *et al*. They achieved the separation of *N*-oleoylglycine, oleamide and oleic acid on a Phenomenex Luna C18 column using acetonitrile : methanol as a gradient. Carpenter and co-workers has also optimized the conditions for APCI using a C18 column for PFAM's. In order to analyze unsaturated and longer chain PFAM's, ($C \geq 22$) using this technique (for both reversed and normal phase columns), the gradient of mobile phase needs to be

optimized. HPLC separation of PFAM's was also carried out on a Nuclesil-100 column by Jasperse [278] and separation of caprylamide, capramide, lauramide, myristamide, palmitamide, oleamide, stearamide and arachidamide was reported. Reverse phase HPLC using a Nova-pak-C18 column was able to resolve 5 separate NAEs based on the chain length and the degree of unsaturation. The co-eluting NAEs were furthered resolved using a free fatty acid HP column from Waters [266]. The separation of anandamide, *N*-palmitoylethanolamine, and *N*-oleoylethanolamine in biological samples was also achieved on a C18 Hypersil column [279]. Koga *et al.* [280] used TSKgel C18 $80T_M$ / TSKgel silica-60 columns for the determination of *N*-acylethanolamides. Using their method, separation and quantification of anandamide and other NAEs in rat brain and peripheral tissues was possible. Normal phase HPLC using evaporating light scattering detection for the screening of the lipid classes found in food packaging materials established the separation of 12 different lipid classes including erucamide on a LiChrospher Diol column [281]. From the literature review it is clear that the resolution largely depends on the nature and the gradient of the mobile phase used. It is therefore might be possible to separate wide range of co-eluting fatty acid amides with careful selection of mobile phases and their gradient.

The analysis of fatty acid amides by capillary electrophoresis (CE) can be of great importance because the high sensitivity and best resolution is required for these compounds. Separated of free fatty acids by non-aqueous CE with near infrared fluorescence detection was reported [247, 248]. Feng *et al.* [282] were the first to investigate the separation of amidated amino acid by MECC/LIF after selective

fluorescence derivatization. The same group has also shown [283] that CE/LIF can be a very sensitive and efficient method for analyzing the PFAM's. The amides can be converted to corresponding amines through Hofmann rearrangement [284, 285], and the amines are then derivatized with an amine-reactive probe. Fluorescein isothiocyanate (FITC) and fluorescein succinimidyl ester (FSE) were used as the reactive probe but were not found to be useful in methanolic CE due to the lack of charge in methanol. Even though the use of CE/MS for determination of peptides and proteins is common, its application towards lipids analysis has not been reported to date but can be of significant importance.

1.6.4.3 Detection method

Mass spectrometry is the main detection technique usually employed in fatty acid amide analysis with GC and LC. The common ionization techniques used for GC/MS analysis of amides are electron ionization (EI) and chemical ionization (CI). Devane *et al*. [1] had characterized anandamide by GC/MS as both underivatized and TMS- derivatized conditions. Direct exposure CI (isobutene-DCI) gave rise to the protonated molecular $[M+H]^+$ ions (m/z = 348) which under CID produced significant fragments at m/z 287, 62 and 44 due to the formation of $[M+H-C_2H_7NO]^+$, most abundant protonated ethanolamine ions $[HOCH_2CH_2NH_3^+]$ and due to the formation of dehydrated protonated ethanolamine ($[HOCH_2CH_2NH_3-H_2O]^+$) respectively. The TMS derivative of anandamide gave rise to M^+ ion at m/z 419 and $[M-CH_3]^+$ adduct ion at m/z 404. Upon CID of m/z 404, two major fragments were observed due to the formation of $Me_2Si^+OCH_2CH_2NH_2$ (m/z 118)

and $Me_2Si^+OCH_2CH_2NHCOCH=CH_2$ (m/z 172) respectively. The EI mass spectral patterns of *O*-acetyl-NAEs showed [266] the formation of M^+ ions as well as the common ions formed due to McLafferty rearrangement (m/z 85 and m/z 145) and due to gamma cleavage (m/z 98 and 158). Schmid *et al*. [267] reported detection limits for *tert*-butyldimethylchlorosilyl (*t*-BDMS) derivatives for NAEs. Limits for saturated NAEs were found to be 0.1 ng and those for unsaturated NAEs were found to be 1 ng (anandamide) under single ion monitoring mode (SIM, m/z = M-57]. Primary fatty acid amides were also analyzed by GC/FID and GC/MS as slip agents in plastics [272-274]. Detection limits of palmitamide, stearamide, oleamide and erucamide from various polymeric matrices using GC-FID and/or GC/MS were found to be less than 50 ppb [273]. Trimethylsilyl (TMS) derivatives of long chain saturated and unsaturated amides by EI/MS showed characteristic molecular $[M]^+$ ions; $[M-15]^+$ ions due to the loss of $-CH_3$ at the aliphatic end and formation of a ketene; $[M-71]^+$ ions due to the loss of $-C_5H_{11}$ also at the aliphatic end and m/z 59 and m/z 72 ions due to McLafferty rearrangement [61]. Similar patterns were also observed for erucamide and oleamide isolated from blood plasma and bacterial cells respectively [75, 286]. The underivatized amides on the other hand showed characteristics M^+ ions; $[M-17]^+$ ions due to the loss of NH_3 and formation of a ketene; $[M-43]^+$ ions due to the loss of $-C_3H_4$ at the aliphatic end; m/z 59 and m/z 72 ions due to McLafferty rearrangement; fragment ion due to the allylic cleavage between C_7-C_8, followed by rearrangement and the fragment ions due to the C_6-C_7 and C_5-C_6 cleavage of the ketene [86]. BSTFA derivatized fatty acid amide analysis using an ion trap analyzer was carried out by Gee *et al*. [275]. The amides

separation was achieved for ethanamide to eicosanamide in the total ion mode and for the positional isomers of C18 unsaturated amides in the single ion mode. The detection limit was found to be in the order of 1 pmol and quantitation limit was between 1 to 10 pmol. The fragmentation pattern in this case was different from that observed by Arafat *et al.,* which was attributed to the use of a different mass analyzer (Arafat *et al.* used a quadrupole mass analyzer). The mass spec patterns observed in the case of Gee *et al.* included the $[M-71]^+$ ions due to the loss of a pentyl group (unsaturated amides having double bond position closer to the cleavage site C_{13}-C_{14} did not show this peak) as well as the m/z 59 and m/z 72 ions formed by McLafferty rearrangement. No M^+ or $[M-15]^+$ peaks were observed. These patterns were further confirmed using the fragmentation patterns of isotopically labeled amides [287]. Quadrupole mass analyzer was also used for the determination of underivatized, TMS, nitrile, and *N*-*t*-BDMS derivatized oleamide [264]. Mass spectral patterns of derivatized and underivatized oleamide are shown in Figure 1-3.

The common ionization techniques used for LC/MS in fatty acid amide analysis are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). The major ions observed in negative ion ESI/MS for NAPE (precursors of NAEs) was deprotonated $[M-H]^-$ ions which upon CID gave further fragmentation and information for identifying the *N*-acyl group attached to the NAPE. The detection limit was found to be 0.1 ng (100 fmol) per injection [288, 289]. The major ions observed in positive ion ESI/MS of NAEs [279] are $[M+H]^+$ and $[M+Na]^+$. Koga *et al.* [280] have used APCI for the determination of NAEs. The NAEs were detected as $[M+H]^+$ ions and detection limit was found to be about

200 pmol per injection for all the NAEs. The drift voltage was found to have an inverse effect on the intensity of the $[M+H]^+$ ion and a directly propotional effect on the $[M+H-H_2O]^+$ ion. For all the NAEs the maximum intensity of the $[M+H]^+$ ions were found to be maximum at a drift voltage of 20 V with a nebulizer temperature of 200 ºC.

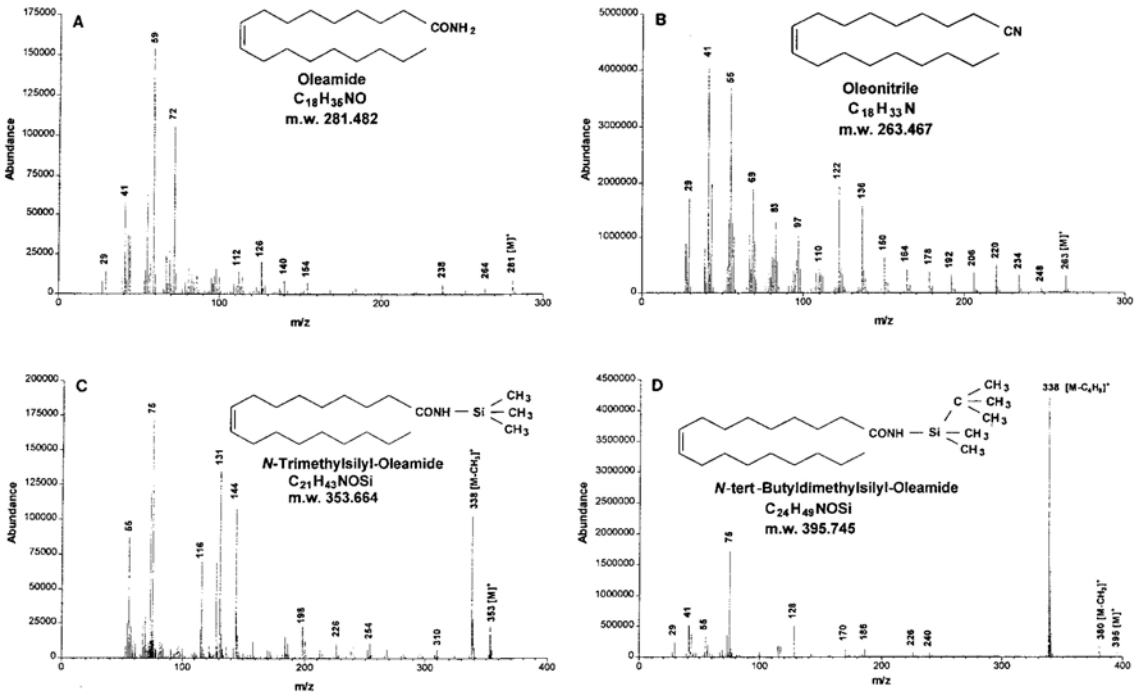


FIGURE 1-3: EI/MS of A) underivatized, B) nitrile derivatized, C) TMS derivatized, and D) *N*-*t*-BDMS derivatized oleamide. Lower levels of M^+ and $[M-17]^+$ ions (m/z 281 and m/z 264 respectively) are observed in A and the improvement of M^+ ions is observed in B upon nitrile derivatization. Predominant fragment peaks are found at lower m/z where the peak interference from biological sample might be substantial but converting oleamide to TMS or *N*-*t*-BDMS ester yields high sensitivities for the m/z 338, which can be used for reliable quantitation. Reproduced from Ref. 264.

Cravatt *et al*. [290] in 1996 published the structural determination of oleamide by mass spectrometry after it was discovered to appear and disappear upon sleep deprivation and resting respectively in cat cerebrospinal fluid. High resolution FAB/MS provided $[M+Na]^+$ ion at m/z 304.2614 for oleamide and tandem ESI/MS produced $[M+H]^+$ parent ions (m/z 282) as well as further fragmentation providing detailed structural information. ESI/MS of oleamide from rat cerebrospinal fluid yielded the $[M+H]^+$ ion which upon CID gave rise to further fragmentation but was found to be too complicated to predict due to the presence of numerous signals even in the blank [264].

Together all these outcome points toward the fact that quite a large number of instrumentation techniques are available for the analysis of fatty acid amides but have their own drawbacks. Sensitivity is a critical issue because sample matrices of interest are mostly biological tissues or cultured cells. Sample preparation for amides prior to the analysis is essential for increased detector lifetime, lower detection limit/higher sensitivity, which usually results in quantitative data with good percent recovery for these types of samples.

1.7 Sample clean-up in lipid analysis

Thin layer chromatography (TLC)/High performance thin layer chromatography (HPTLC) and solid-phase extraction (SPE) are the two main sample preparation techniques frequently used by analytical chemists. Use of TLC and SPE in lipid separation has also been explored. TLC/HPTLC can be used for easy profiling of the lipid classes and the separated samples can be scraped off the plates and analyzed by the

instrument of choice without any interference from the other classes. A number of authors [177, 291-304] have reported such analyses with successful TLC separation of various lipid classes followed by instrumental analyses. Similar sample preparation which is very recently being explored is SPE and the separations of different lipid classes from various types of samples by different SPE columns have been performed and the advantages and disadvantages have been reviewed [305-318]. The most popular SPE columns for the extraction of various lipid classes are the silica and the aminopropyl bonded phase. Due to the highly polar nature of the silica it can adsorb polar lipids and different classes can then be eluted by mobile phase with increasing polarity. The aminopropyl column is more popular then silica because it is a little bit less polar, which is advantageous for the complete recovery of the polar lipids. Another advantage of the aminopropyl column is that it can also be used as an ion exchange column. Details of TLC/HPTLC and SPE studies will be discussed in the introduction sections of the chapters three and four respectively.

1.8 Conclusions

The object of this chapter is to introduce the instrumental analysis done in lipid chemistry in brief with especial attention in fatty acid amide class analysis and to provide an idea of the objective of this thesis. We are interested particularly in primary fatty acid amides (PFAM's) because this fatty acid amide subclass has not been explored as extensively as the *N*-acyl ethanolamides (NAEs). The idea that they possess significant bio-regulatory activities and hormonal behavior as the NAEs besides being catabolically related to them is still to be explored. Careful study is needed to

identify their specific action, which requires sole isolation, identification and quantitation of these species from biological samples. The presence of a huge number of different lipids in the sample matrix makes it difficult to analyze any particular class without interaction from other classes. A method for the isolation of amides before instrumental analysis is therefore very crucial. As pointed out in the earlier sections that a set of standards as well as the use of deuterated amides as internal standard is required for the quantitative analysis of the amides. Before instrumental analysis, amides need to be extracted from the total lipid fractions so that other lipid classes do not interfere with the particular amide analysis conditions. In Chapter 2 of this thesis, we will present the synthesis and purification of the long chain PFAM's including the geometric and positional isomers. Synthesis of deuterated standards will also be discussed. This chapter will also focus on the separation of the amides by a HP-5MS column and its incapability of separating *cis/trans* and positional isomers. The synthesized standards will then be used for the method development of the amide isolation from total lipid extract and amide analysis. Chapter 3 will focus on the total lipid profiling of the $N_{18}TG_2$ mouse neuroblastoma cells by HPTLC followed by the isolation and analysis of cell amides. Chapter 4 will be mainly on the solid-phase extraction of amides from standard lipid mixture followed by method validation with $N_{18}TG_2$ cell lipids. Application of both methods for the analysis of PFAM's in mammalian tissues will be discussed in Chapter 5. Chapter 6 will focus on the separation of the co-eluting amides using argentation HPTLC as well as GC using a BPX-70 column.

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Chapter 2

Small-scale synthesis and characterization of naturally abundant and isotopically enriched primary fatty acid amides

2.1 Abstract

A simple procedure for the synthesis of commercially unavailable primary fatty acid amides (PFAM's) and isotopically labeled PFAM's in small scale has been successfully established. Synthesized amides can be used as standards and the isotopically enriched amides can be used as internal standards for their analysis, especially for the analysis of biologically active amides, in cell and tissue samples by instrumental means. The amides were synthesized from corresponding acids, thionyl chloride and anhydrous ammonia. Deuterated or ^{13}C labeled acids were employed in the synthesis of deuterated or ^{13}C labeled amides respectively. All the precursors used in the synthesis are available commercially. The two step synthesis requires 1 h reflux for the 1st step and ~30 min at around 100 ºC for the 2nd step. The product was highly pure yielding 67-98% depending on the nature of the amide. No other impurities besides a small amount of corresponding acids in some cases were observed. Purity of the products and their structures were confirmed by gas chromatography/mass spectrometric (GC/MS) analysis. ^{1}H NMR was used for the characterization of the geometric isomers (cis/trans) of the unsaturated amides. The products were found to be very stable over a period of three years inside desiccators at room temperature.

2.2 Introduction

It was not until Cravatt *et al.* [1] published report on the biological activity of primary fatty acid amides (PFAM's) that they caught attention as mammalian hormones. PFAM's were mainly used as slip additives in plastic industries before then. Slip additives, added during the formulation of plastics, provide useful surface properties to the plastic by gradually blooming to the surface. These properties include lubrication, prevention of films from sticking together to molds and to each other and reduction of static charge [2]. The primary amides that are used in various types of plastic as slip additives are mainly oleamide, erucamide, palmitamide and stearamide [2-4].

In 1989 Arafat *et al.* [5] had reported the identification of 5 saturated and unsaturated long chain primary fatty acid amides (palmitamide, palmitoleamide, oleamide, elaidamide and linoleamide) in human luteal phase plasma, but their function was not attributed. They also reported a synthetic procedure for the amides. It was shown by Wakamatsu *et al.* [6] in 1990 that erucamide in bovine mesentery is an angiogenic factor. Its angiogenic activity was also shown in regenerating skeletal muscle but erucamide significantly did not show any proliferative activity in endothelium, muscle and connective tissue [6, 7].

In 1995 isolation of oleamide from the cerebrospinal fluid of sleep-deprived cat and induction of physiological sleep in rats upon injection of synthetic oleamide turned attention to the fact that primary fatty acid amides might be an unrecognized class of important bioregulators [1]. Since then primary fatty acid amides, especially oleamide was widely studied and various activities of oleamide as well as other amides have been

identified [6-21]. Bisogno *et al.* [22] have reported the production of oleamide by $N_{18}TG_2$ mouse neuroblastoma cells and pointed out that oleamide might be synthesized *in vivo* from oleic acid. This is contradictory to the fact that oleamide is produced *in vivo* by the catalytic activity of PAM on *N*-oleoylglycine [23].

Recently Merkler *et al.* [24] have found that $N_{18}TG_2$ cells grown in ^{14}C oleic acid under the conditions known to stimulate PAM expression show increased oleamide production and under the conditions known to inhibit PAM activity produces ^{14}C *N*-oleoylglycine (^{14}C NOG). Their findings that oleamide can also be generated from the cells grown in NOG, strongly support NOG as an intermediate and PAM as a catalyst in oleamide biosynthesis *in vivo*. In order to carry out such studies, quantitation is a crucial factor for complete understanding of these bio-regulators. Unfortunately, primary fatty acid amides are not found commercially and they must be synthesized to be used as standards for any quantitation study. The more stable isotopically labeled amides are also required in order to be used as internal standards in such types of studies [5].

Synthesis of primary amides can be done conventionally by the reaction of ammonia with the corresponding acids, anhydrides, esters or acyl halides [25]. The direct reaction of carboxylic acid with ammonia requires harsh conditions which may be suitable for the synthesis of short chain amides with no double bonds but may result in the formation of undesired byproducts in the case of amides with reactive double bonds. Reaction of acid anhydrides with ammonia can also lead to the formation of undesired imides as byproducts [26]. A number of researchers [27-30] reported the coupling reaction between acids and amines for the formation of amides under mild conditions, but no long chain unsaturated or unsaturated primary fatty acid amides were included in

these studies. Lipase-catalyzed synthesis of butyramide and oleamide under mild conditions was studied by Litjens *et al.* [31]. This method is mild and selective but takes about 17 days for the formation of amides. Ammonolysis of triacyl glycerol was also studied by Zoete *et al.* [32] but 72 h was required to yield oleamide by this method.

Very recently, the use of microwaves has become a popular synthesis device for synthetic chemists due to the rapidness of the process. Amide syntheses under different conditions were also studied by several researchers [33-37]. Unfortunately no results for PFAM's were reported. In 1989 Arafat *et al.* [5] reported the synthesis of long chain primary fatty acid amides by the ammonolysis of corresponding fatty acid chloride. They used ammonium hydroxide as a source of ammonia, which introduces water into the system and hence keeps the window open for incomplete conversion and byproduct formation. Anhydrous ammonia in this case can be a good candidate and in fact Philbrook [38] reported the formation of lower aliphatic amides using a similar procedure. In this chapter we have described the synthesis of a wide range of long chain saturated and unsaturated primary fatty acid amides with high purity and higher yield. This range also includes geometric and positional isomers of PFAM's as well as two isotopically enriched PFAM's. After synthesis and purification, amides were characterized by GC/MS and ^{1}H NMR.

2.3 Experimental

2.3.1 Reagents

Dodecanoic (lauric), tetradecanoic (myristic), hexadecanoic (palmitic), octadecanoic (stearic), and docosanoic (behenic) acids were purchased from Acros organics (Morris Plains, NJ); tridecanoic (tridecylic), heptadecanoic (margaric), eicosanoic (arachidic), hexadecanoic-1 ^{13}C (palmitic-1 ^{13}C) acids and thionyl chloride were purchased from Aldrich Chemical Co. (Milwaukee, WI); *cis*-9-tetradecenoic (myristoleic), *cis*-9-hexadecenoic (palmitoleic), *trans,trans*-9,12-octadecadienoic (linoleladic), *trans*-11-octadecenoic (vaccenic), *cis*-6-octadecenoic (petroselenic) and *trans*-9-octadecenoic (elaidic) acids were purchased from ICN Biomedicals Inc. (Aurora, OH); *trans*-6-octadecenoic (petroselaidic), *cis*-13-octadecenoic, *cis*-10-nonadecenoic, *cis*-5-eicosenoic, *cis*-11-eicosenoic, *cis*-13-eicosenoic, *trans*-11-eicosenoic, *cis*-11-14-eicosadienoic, *cis,cis*-9,12-octadecadienoic (linoleic), *cis,cis,cis*-9,12,15 octadecatrienoic (α-linolenic), and *cis,cis,cis*-6,9,12 octadecatrienoic (γ-linolenic) acids were purchased from Sigma Chemical Co. (St. Louis, MO); Heptadecanoic-D33-acid was from CDN Isotopes (Quebec, Canada). Laboratory grade toluene, heptanes, isopropanol, and ethyl acetate were obtained from Fisher Scientific (Fair Lawn, NJ). Anhydrous NH_3 was supplied by Air Products & Chemicals Inc. (Allentown, PA).

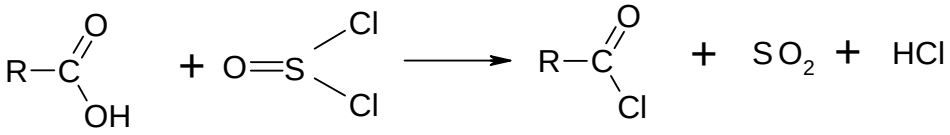
2.3.2 Instrumentation

An Agilent Technologies Network GC/MS system (6890 GC with 5973 mass selective detector and 7683 series injector) was used for the analysis of synthesized amides. The column used was an HP-5MS (0.25 mm internal diameter, 0.25 μm film thickness, 30 m

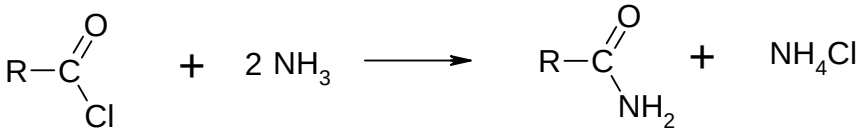
long, Agilent Technologies Inc., Palo Alto, CA) able to analyze fatty acids and amides without prior derivatization. The GC/MS method used for analysis was as follows: starting temperature was 55 ºC, ramped to 150 ºC at 40 ºC per min, held at 150 ºC for 3.62 min, ramped to 300 ºC at 10 ºC per min and finally held at 300 ºC for 2 min. Electron impact ionization (EI) at 70 eV was used and mass range was kept from 40 to 400 m/z for total ion monitoring (TIM) mode. The fragment ions used for the single ion monitoring (SIM) mode were m/z 59, m/z 72, m/z 62, and m/z 76. The temperatures of the injection port and the transfer line were 250 ºC and 280 ºC respectively. Injection volume was 1 μL splitless [39]. A Varian 500 NMR spectrometer was used to record the ^{1}H NMR at 500 MHz of the *cis* and *trans* isomers.

2.3.3 Synthesis procedure

Synthesis of primary fatty acid amides included two steps. The 1st step was the preparation of the acid chlorides from the corresponding acids and the 2nd step was the formation of amides from the acid chlorides. The 1st step was optimized from Arafat *et al.* [5] and the 2nd step was adopted with some modification from Philbrook [38], which was originally for the synthesis of lower aliphatic amides.



Fatty acid Thionyl chloride Acid chloride



Acid chloride PFAM's

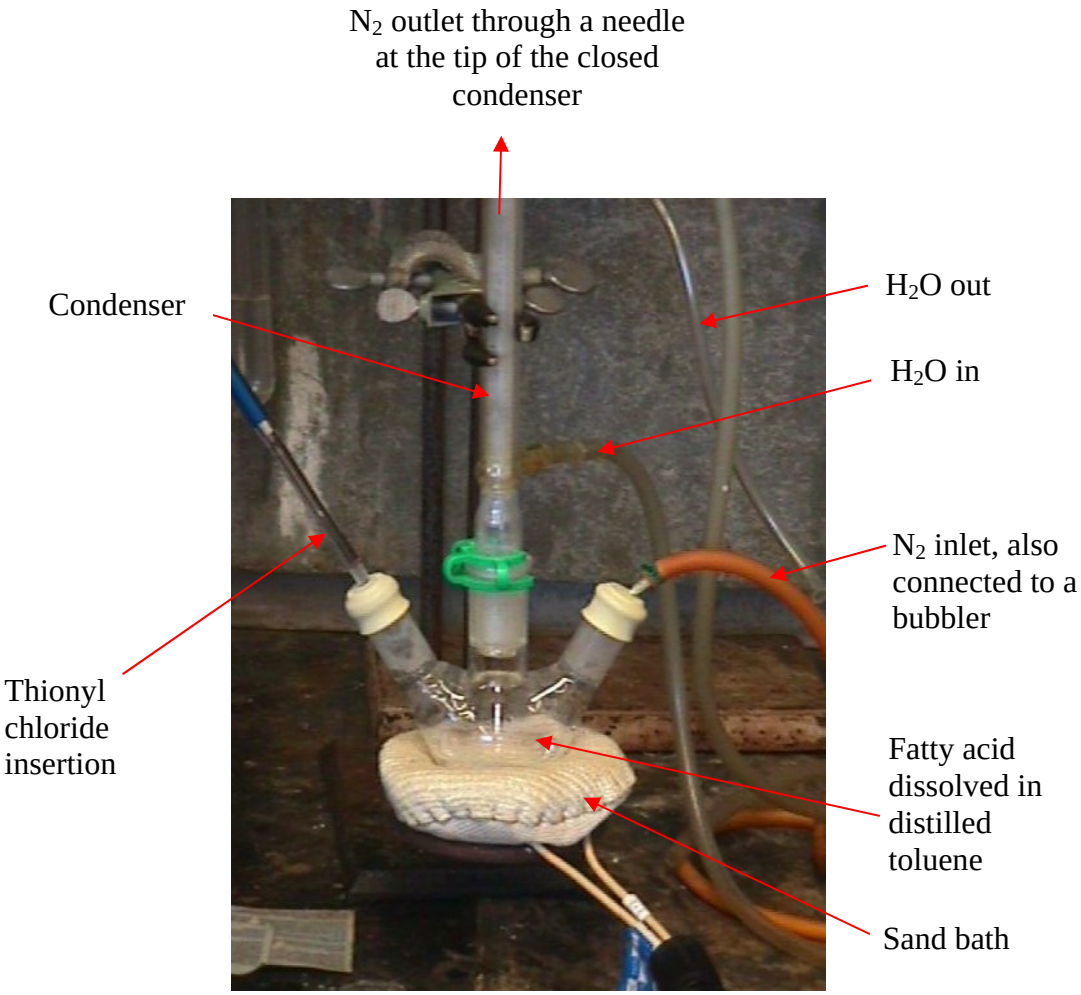


FIGURE 2-1(a): Set-up for PFAM synthesis 1st step.

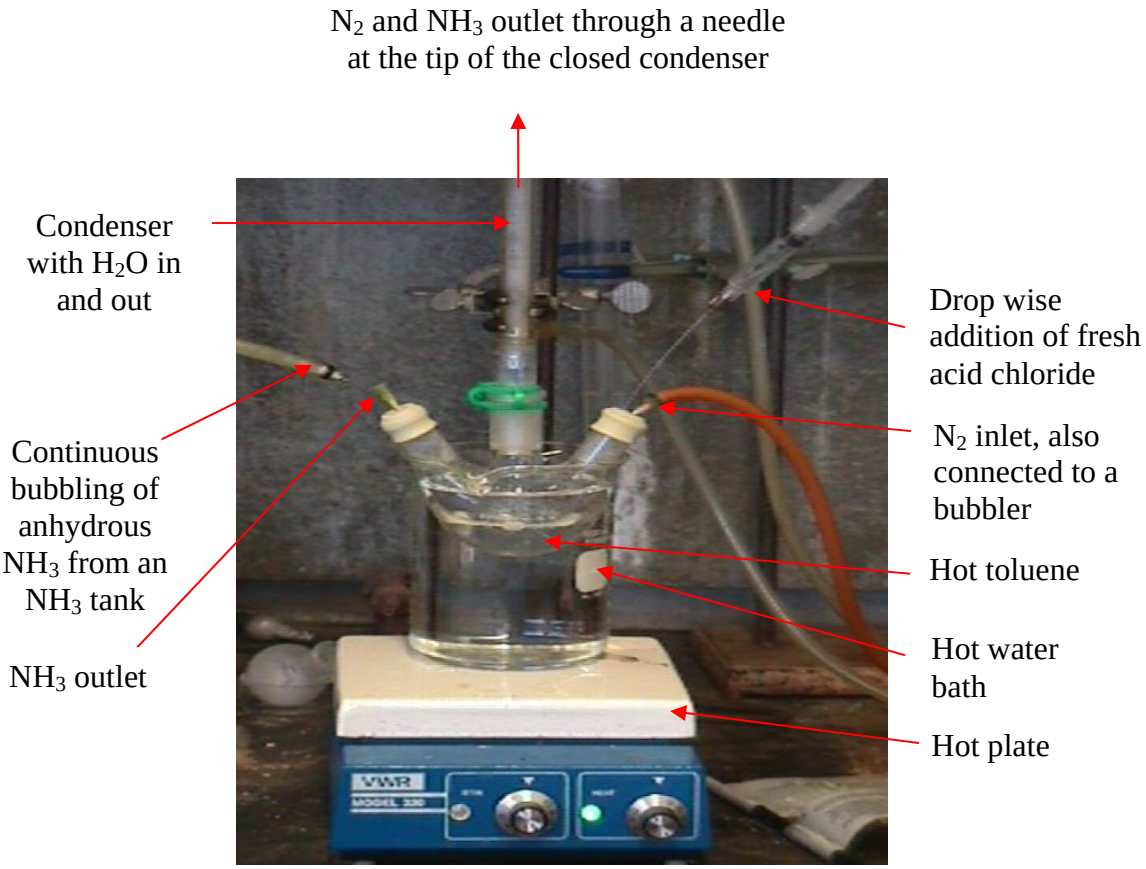


FIGURE 2-1(b): Set-up for PFAM synthesis 2nd step.

In order to carry out the 1st step [Figure 2-1(a)], 10 to 500 mg of acid (depending on the amount available) was dissolved in 5 to 10 mL (depending on the amount of acid used) of toluene (distilled over CaH_2) inside a three-neck round bottom flask, which was placed in a sand bath attached to a temperature regulator. Two side necks were sealed using two Sura Seal White-Rubber Septa and the center neck was attached to a condenser. The top of the condenser was sealed with a Sura Seal White-Rubber Septa letting a N_2 inlet and an outlet. N_2 was allowed to pass for several minutes in order to

achieve an inert environment while the acid was allowed to dissolve in hot toluene. Thionyl chloride was added to the dissolved acid through a glass syringe needle and the mixture was allowed to reflux for an hour. When the reaction mixture was semi-cold it was transferred to a closed, N_2 flushed round bottom flask to be used in the 2^{nd} step.

In order to carry out the 2^{nd} step [Figure 2-1(b)] 5 to 10 mL of toluene was placed through a glass syringe needle in the same three-neck round bottom flask with condenser attached to it. The flask was then placed in a boiling water bath allowing toluene to be warmed. At this point anhydrous ammonia was continuously bubbled into the hot toluene through one side neck and the acid chloride was added drop wise through the other side neck using a glass syringe needle. After the addition of acid chloride was complete, NH_3 was allowed to bubble through the reaction mixture for 20 additional minutes in order to ensure the complete conversion to acid chloride to the corresponding amide. N_2 was kept on during the entire synthesis time.

2.3.4 Purification procedure

2.3.4.1 Saturated primary amides

The reaction mixture was then cooled on an ice bath, filtered and washed three times with hot DDI water under vacuum to remove excess thionyl chloride and NH_4Cl. The sample was then dried inside a desiccator under vacuum. The sample was then run by GC/MS in order to check the purity of the product. In case any other peaks besides the amide peak were observed, the product was purified by column chromatography (procedure described at the end of this section).

2.3.4.2 Unsaturated primary amides

In case of the synthesis of unsaturated fatty acid amides, small amount of acid precursor was available (10-50 mg). Therefore the amount of amide formed stayed dissolved in toluene after synthesis. In this case, the reaction product was filtered while hot and the residue was washed with hot toluene once. The filtrate containing the amide was dried using a rotary evaporator and kept inside a desiccator under vacuum for further drying. The purity of the product was checked by GC/MS and similarly if any other peaks besides amide peak were observed, the product was purified by column chromatography.

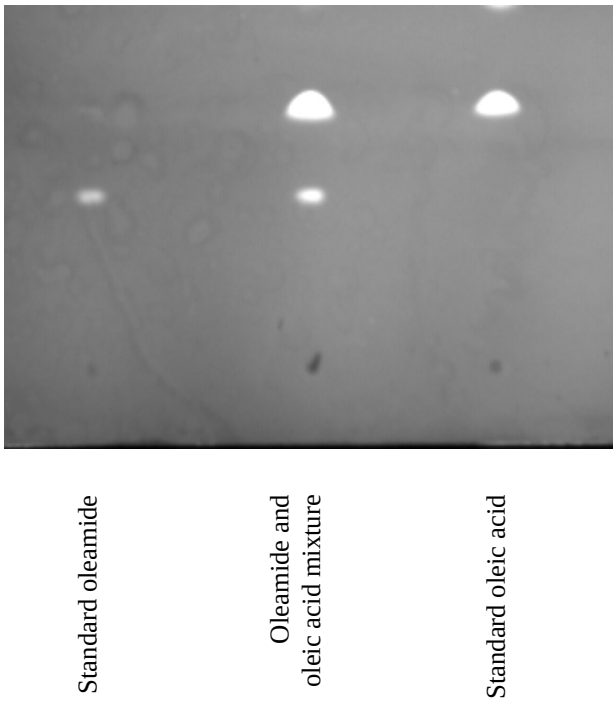


FIGURE 2-2: Separation of oleamide and oleic acid by TLC.

2.3.4.3 Column chromatography method

Only a small amount of acid was observed with amides in some of the final products. Therefore, a thin layer chromatographic (TLC) method (Figure 2-2) was developed for the separation of acids from amides and this method was adapted for the purification of amides by column chromatography. The products found to contain some acids were dissolved in isopropanol and loaded onto a silica column (pre-soaked in 80:20; heptanes: isopropanol). Product was eluted with heptanes: isopropanol: ethyl acetate (80:16:4) and fractions were collected in 10 mL test tubes. Each fraction was identified by comparing the spot on a TLC plate to that of a standard amide and acid. Fatty acid fractions were discarded and the amide fractions were pulled together, dried under rotary evaporator, and stored inside desiccators under vacuum for further drying.

2.4 Results and Discussion

2.4.1 Optimization of synthesis conditions

A total of seven stearamide syntheses were carried out following the procedure described in the above section in order to achieve an optimized conversion of acid to corresponding acid chloride. Optimization parameters are listed in Table 2-I. The effect of reflux time, amount of toluene and the amount of thionyl chloride required for maximum yield were studied. It is shown in Table 2-I that percent yield increases from 50% to 85% with the increases of reflux time from 30 min to 60 min and decreases to 52% upon 90 min reflux. The percent yield increases from 85% to 94% by increasing the amount of $SOCl_2$ from 128 µL (number of moles of $SOCl_2$ required to react with the number of moles of stearic acid in 0.5 g of acid) to 140 µL (10% excess $SOCl_2$) and

remained similar by further increasing the amount to 160 µL (20% excess $SOCl_2$). Decreasing the amount of toluene showed tremendous decrease in yield whereas increase in toluene amount did not have much effect on the percent yield indicating proper dissolution of the acid in toluene is a key to better yield. The optimum conditions were therefore 10 mL of toluene, 1 h. reflux and 10% $SOCl_2$ above the stoichiometric amount. These conditions were followed for the syntheses of all of the other amides. Table 2-II shows the percent yield for 25 amides. Unsaturated amides were found to have lower yields than saturated and isotopically labeled amides, which were expected due to the higher reactivity of unsaturated acids. The amides synthesized by this method were found to be stable over a three year period of time.

TABLE 2-I: Optimization conditions for the 1st step in the synthesis of stearamide (C18:0).

Optimization steps	Amount of stearic acid (g)	Amount of toluene (mL)	Amount of $SOCl_2$ (µL)	Reflux time (min)	Yield (%)
#1	0.5	10	128	30	50
#2	0.5	10	128	60	85
#3	0.5	10	128	90	52
#4	0.5	10	140	60	94
#5	0.5	10	160	60	91
#6	0.5	5	140	60	64
#7	0.5	15	140	60	91

2.4.2 Characterization of the synthesized amides

2.4.2.1 Gas chromatography and mass spectrometry (GC/MS)

Upon drying after synthesis, the amides were dissolved in isopropanol and were run by GC/MS. GC of 16 different amides is shown in Figure 2-3. It can be noticed from the Figure 2-3 that the detection limit for the unsaturated amides is very high compared to the saturated ones and the retention time of the amides increase with the increasing number of carbon in the chain as expected. Unsaturated amides with the same number of carbons elutes in the same region for example all the unsaturated 18 carbon amides elute around 16.65 to 16.75 min under the GC/MS method and column conditions described in the previous section. An $M^{+\cdot}$ ion gas chromatogram of ten 18-carbon (C18) amides is shown in Figure 2-4 where X and Y axes have been shifted for each peak in order to show these co-eluting amides. A more polar column such as BPX70 (70% cyanopropyl) can be employed for the separation of these amides. One disadvantage though is that the derivatization of these amides to their trimethylsilyl (TMS) ester is required for analysis by this column. Results from this study will be discussed in Chapter 6.

TABLE 2-II: Percent product yield for the synthesized amides.

Fatty acid amide	% yield	Scientific symbol *
Lauramide	98	C12:0
Tridecanoamide	68	C13:0
Myristamide	85	C14:0
Myristoleamide	81	$C14:1^{9}$
Palmitamide	97	C16:0
Palmitoleamide	89	$C16:1^{9}$
Palmit-1-^{13}C-amide	95	C16 (^{13}**C**-1):0**
Heptadecanoamide	75	C17:0
Heptadecano-D33-amide	93	C17**D33**:0 **
Stearamide	94	C18:0
Oleamide	Commercially available	$C18:1^{9}$
Elaidamide	98	$C18:1^{trans\ 9}$
Petroselenamide	80	$C18:1^{6}$
Petroselaidamide	90	$C18:1^{trans\ 6}$
vaccenamide	92	$C18:1^{trans\ 11}$
Cis-13-octadecenoamide	90	$C18:1^{13}$
linoleamide	77	$C18:2^{9,12}$
linolelademide	84	$C18:1^{trans\ 9,12}$
α-linolenamide	98	$C18:3^{9,12,15}$
γ-linolenamide	96	$C18:3^{6,9,12}$
Cis-10-nonadecenoamide	89	$C19:1^{10}$
Eicosanoamide	72	C20:0
Cis-5-eicosenoamide	67	$C20:1^{5}$
Cis-11-eicosenoamide	65	$C20:1^{11}$
Trans-11-eicosenoamide	78	$C20:1^{trans\ 11}$
Cis-13-eicosenoamide	90	$C20:2^{13}$
Cis-11,14-eicosadienoamide	75	$C20:2^{11,14}$
Docosanoamide	88	C22:0
Erucamide	Commercially available	$C22:1^{13}$

* Scientific symbols represent the number of carbon atoms in the chain, position and geometrical configuration of the double bond.

** Shows the carbons or hydrogens in red that are isotopically labeled.

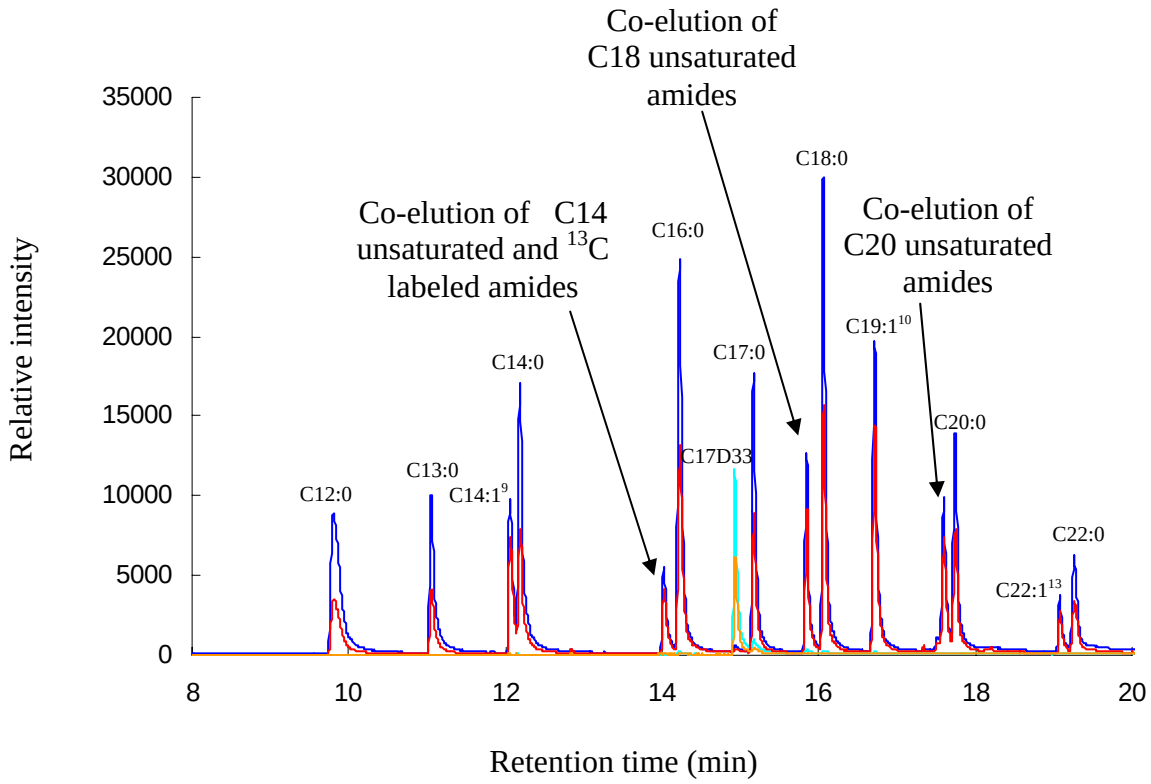
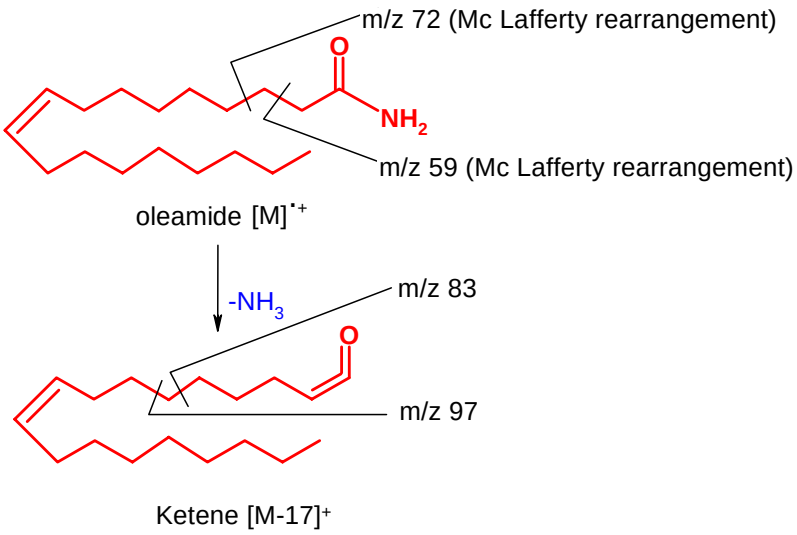


FIGURE 2-3**:** Elution profile for C12-C22 amides on a HP-5MS column. The relative intensities at m/z **59**, **72**, **62** and **76** are plotted against retention time.

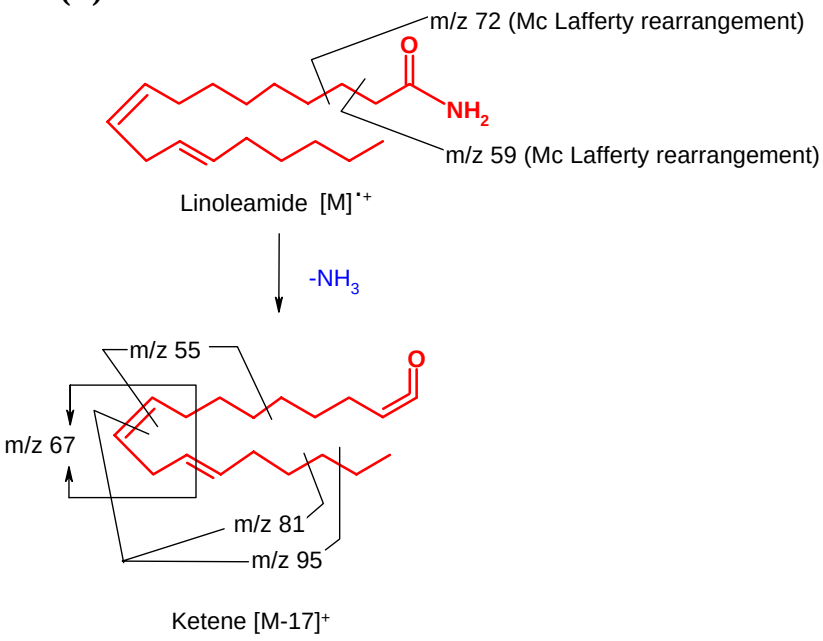
The electron impact ionization MS patterns of four 18-carbon amides are shown in Figure 2-5 and the structures for the common key fragment ions formed have been drawn for illustration (also see Scheme 2.1). All the amides showed similar fragmentation patterns which included the molecular ion peak, $[M]^{+\bullet}$; the peak due to the loss of NH_3 and formation of a ketene, $[M-17]^+$; the peak due to the loss of $-CH_2-CH_2-CH_3$, $[M-43]^+$; the peaks due to the consecutive loss of methylene groups (14 u); and the peaks at m/z 59 and m/z 72 due to the McLafferty rearrangements. The other fragmentation patterns observed were due to the fragment ions from the C6-C7 cleavage

and C5-C6 cleavage of the ketene, for example at m/z 97 and m/z 83 respectively for both oleamide and stearamide [22, 39]. The poly unsaturated fatty acids showed additional fragment ions from cleavages between C5-C6 and C9-10 (i.e., m/z 55); from cleavages between C7-C8 and C12-13 (i.e., m/z 67); from cleavages between C5-C6 and C12-13 (i.e., m/z 95). The ions specific for linoleamide (C18:2 ^{9,12}) and linolenamide (C18:3 ^{9,12,15}) arises from cleavages between C9-C10 and C15-16 and from cleavages between C9-C10 and C16-17 (m/z 81 and m/z 95 respectively for linoleamide and m/z 79 and m/z 93 respectively for linolenamide). These fragmentation patterns show that the double bonds are preserved in the fatty acid amides after their synthesis from corresponding fatty acids. The MS patterns for the isotopically labeled amides were the same as the naturally abundant amides. The peaks at m/z 60 and m/z 73 in case of ^{13}C-1-palmitamide [Figure 2-6(a)] and the peaks at m/z 62 and m/z 76 in case of heptadecano-D33-amide [Figure 2-6(b)] were also due to McLafferty rearrangement. The shift of m/z 59 to m/z 60 for the first one and m/z 62 for the latter are due to one ^{13}C and two deuterium atoms, respectively. The shifts of m/z 72 to m/z 73 and m/z 62 respectively can also be explained similarly (Figure 2-6). Their molecular ion peaks resided at m/z 256 and m/z 302, respectively.

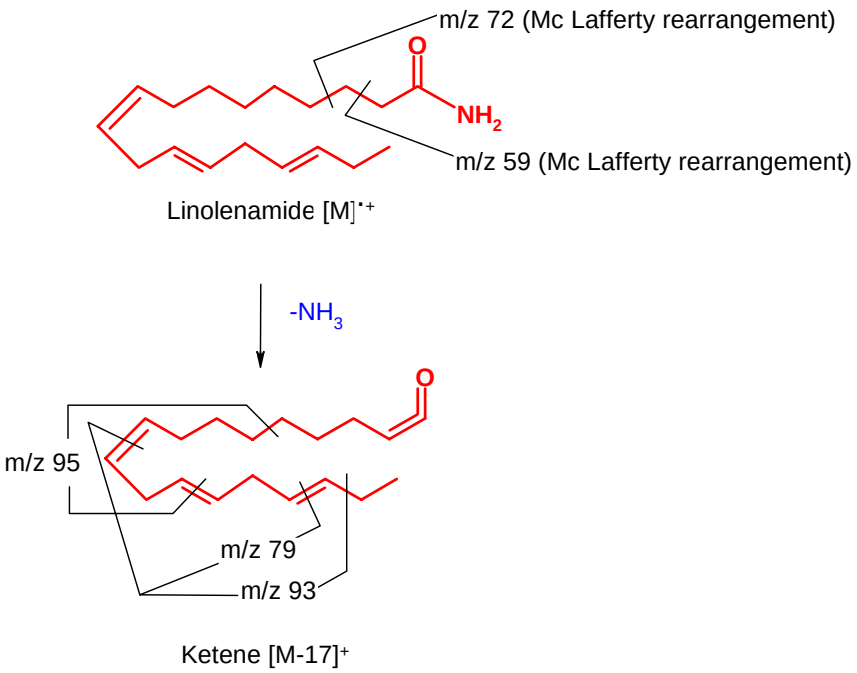
Scheme 2.1(A)



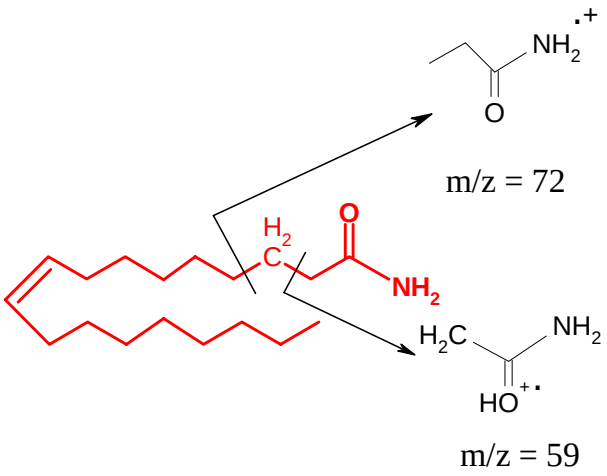
Scheme 2.1(B)



Scheme 2.1(C)



Scheme 2.1(D)



Scheme 2.1: Mass-spectral (quadrupole) fragmentations for A) oleamide (saturated amides shows similar patterns), B) linoleamide (C18:2 [9, 12]) and C) linolenamide (C18:2 [9, 12, 15]). D) McLafferty rearrangement ions common to all amides.

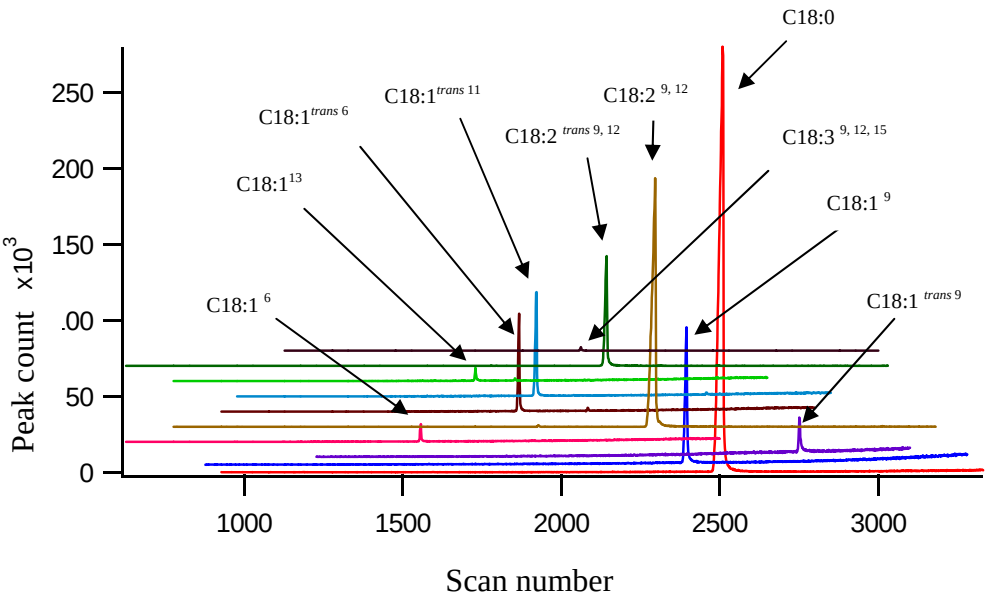
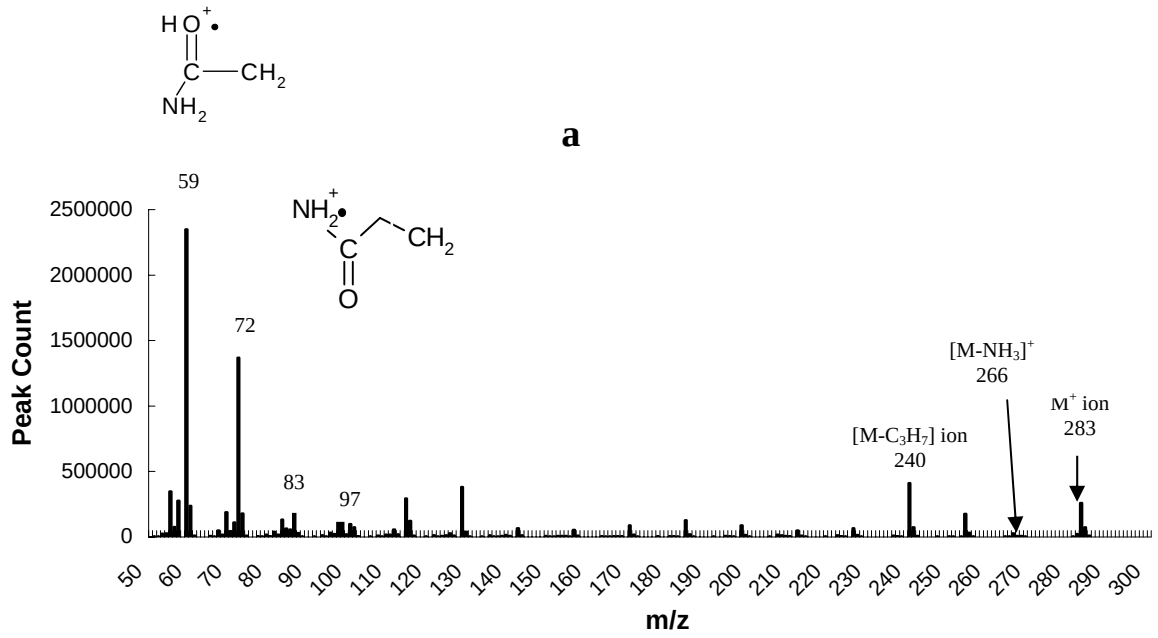
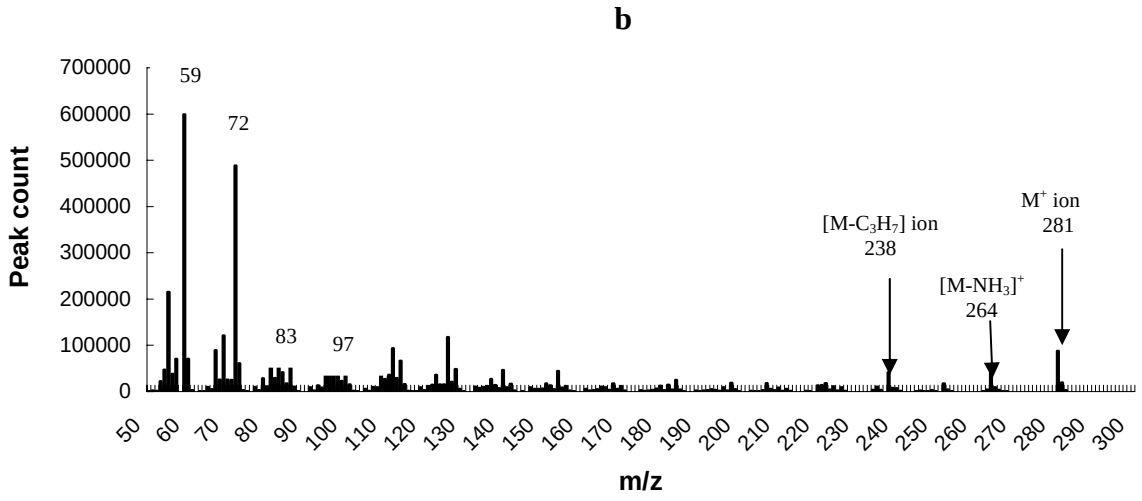


FIGURE 2-4: M^+ ion gas chromatogram of ten 18-carbon (C18) amides. X and Y axes have been shifted for each peak in order to show the co-eluting amides.


a
Peak Count
m/z
59
72
83
97
[M-NH3]+
266
M+ ion
283
[M-C3H7] ion
240


b
Peak count
m/z
59
72
83
97
[M-C3H7] ion
238
[M-NH3]+
264
M+ ion
281

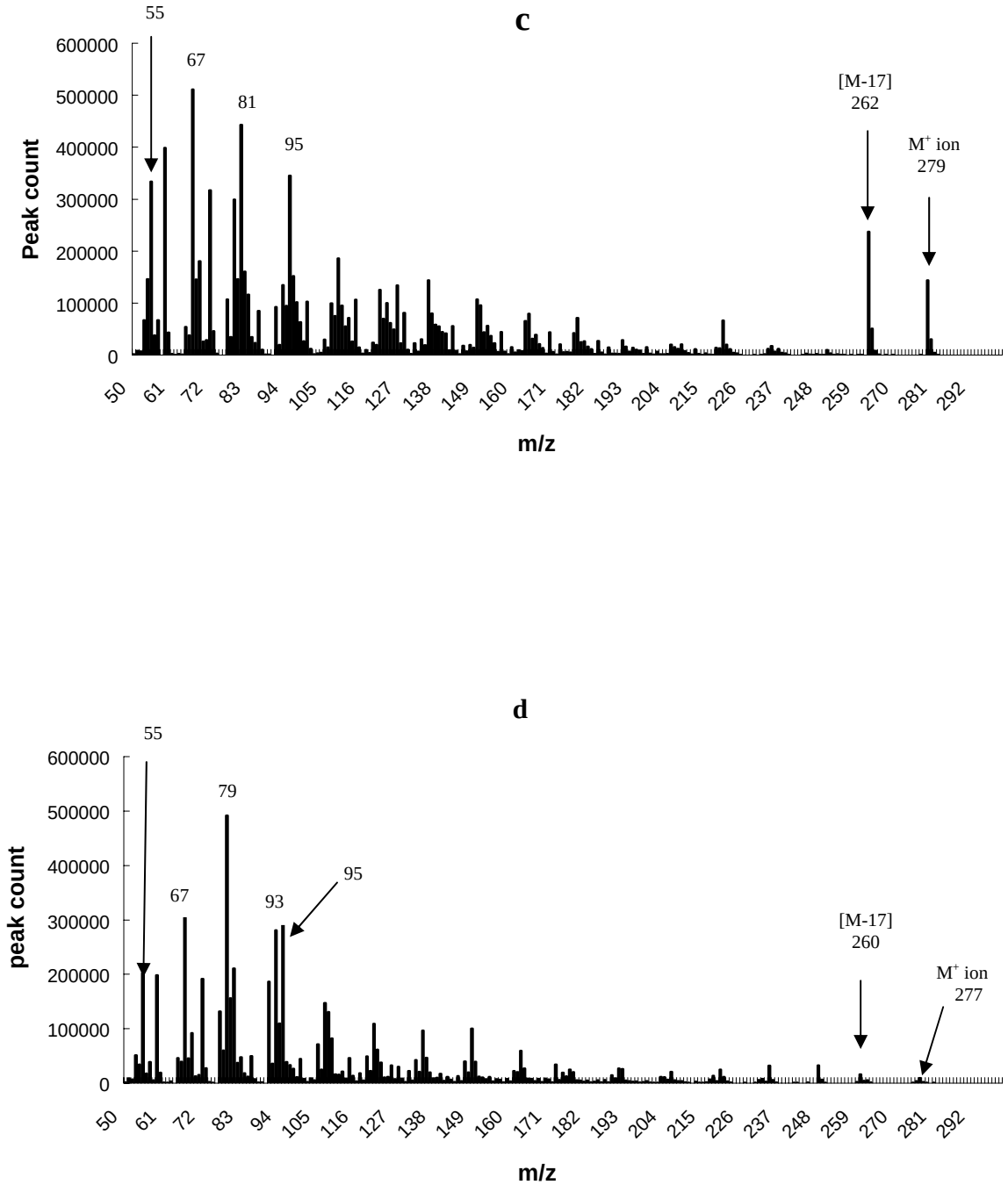


FIGURE 2-5: Mass spectrum patterns of four 18-carbon amides (a) stearamide; C18:0 (b) oleamide; C18:1 (*cis*-9), (c) linoleamide; C18:2 (*cis*,*cis*-9,12) and (d) linolenamide; C18:3 (*cis*, *cis*, *cis*-9,12,15).

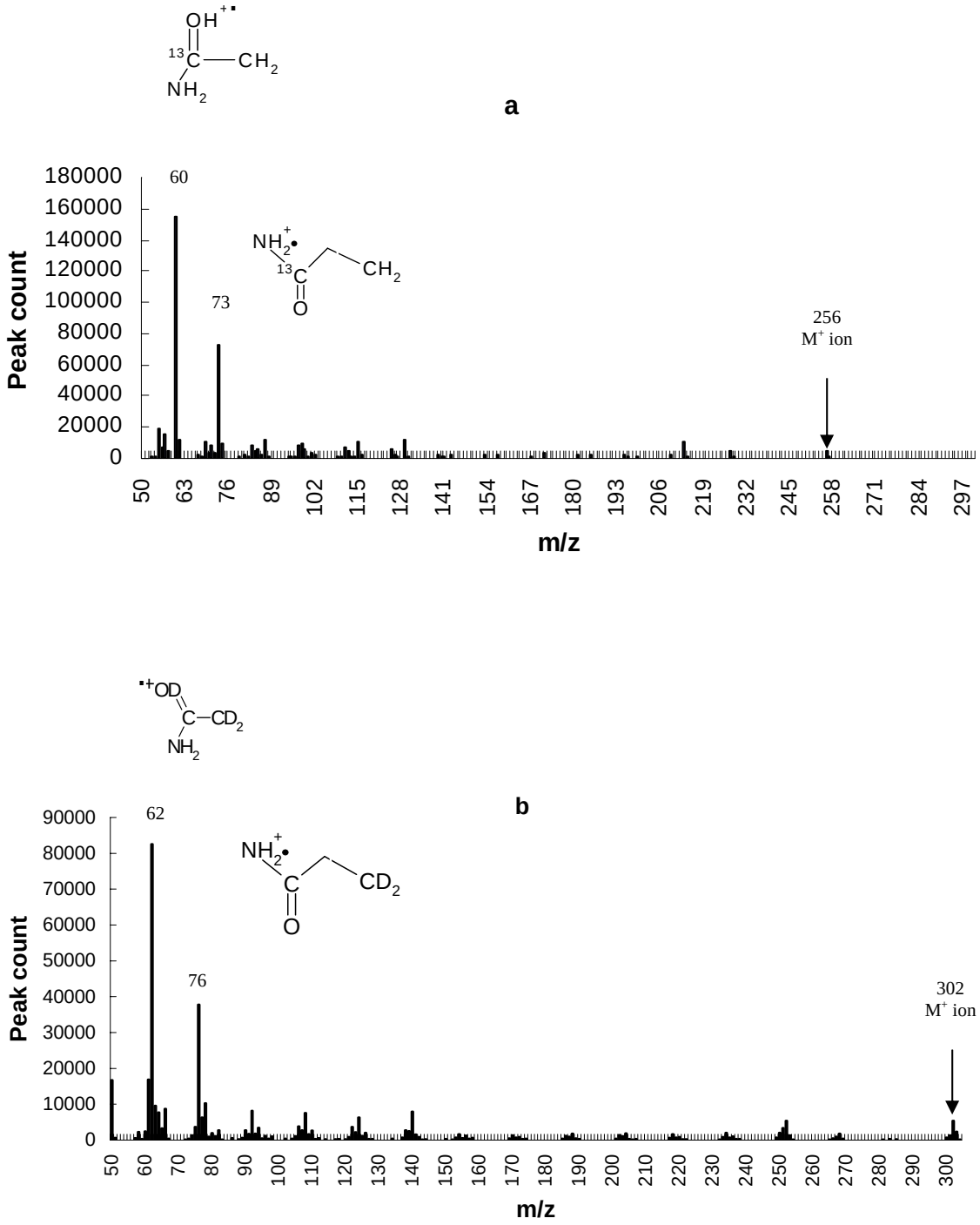


FIGURE 2-6: Mass Spectrometric pattern of (a) ^{13}C-1-palmitamide and (b) C17D33-amide.

2.4.2.2 Nuclear magnetic resonance

^{1}H NMR was used to characterize the *cis* and *trans* isomers of some of the amides. Figure 2-7 shows the NMR spectra of eight C18 amides (including commercially available *cis*-9-octadecenamide, C18:1[9]) using benzene d[6] as a solvent (7.15 ppm). Peaks for the synthesized amides were compared with the predicted values and also the literature values for C18 amides [40, 1]. In all the spectra, peaks due to methyl group (multiplet, 0.78 to 0.95 ppm), broad peaks due to long alkyl methylene protons (multiplet, 1.12 to 1.42 ppm), peaks due to C**H$_2$**-CH$_2$-CO-NH$_2$ (multiplet, 1.42-1.56 ppm), allylic protons (multiplet, 1.58-1.71 ppm), and C**H$_2$**-CO-NH$_2$ (multiplet, 1.86-2.10) and peaks due to alkene protons (multiplet, 5.30-5.50 ppm) were observed. The *cis* isomers showed a slight downfield shift for all the peaks compared to those for the *trans* isomers [Figure 2-7 (a-h)]. As the position of the double bond moves further away from the carbonyl group, slight upfield shift of the peak of the alkene protons was observed [Figures 2-7(a), 2-7(c) and 2-7(f)].

Fig. 2-7 (a) C18:1[6]

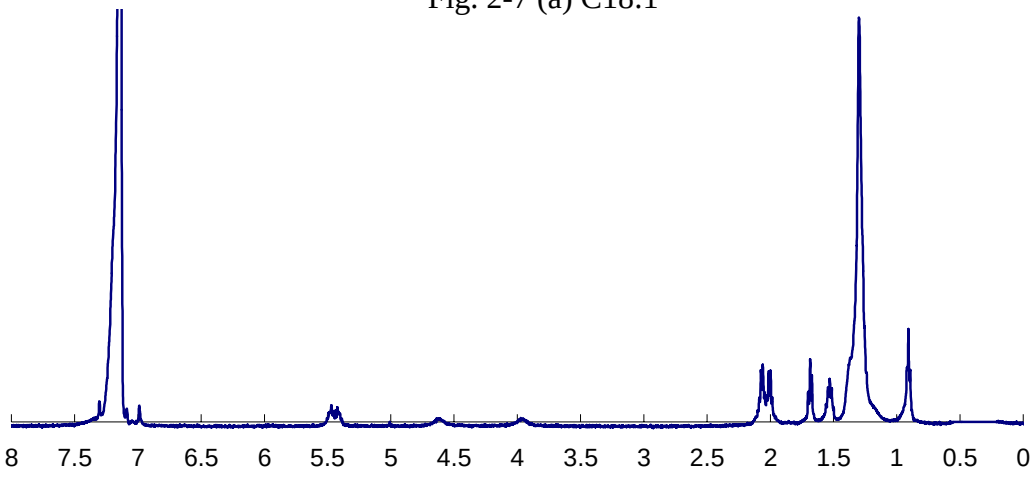


Fig. 2-7 (b) C18:1 *trans*6

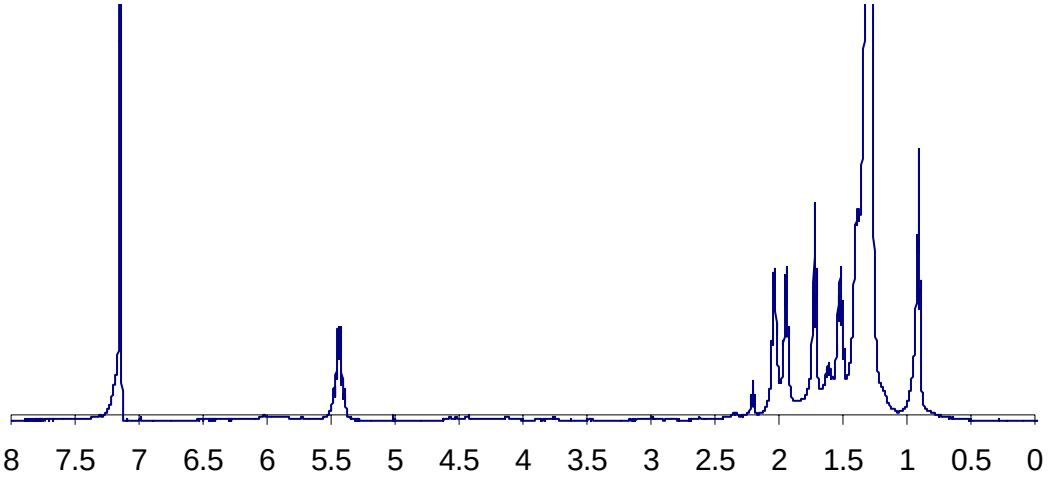


Fig. 2-7 (c) C18:1 ^{9}

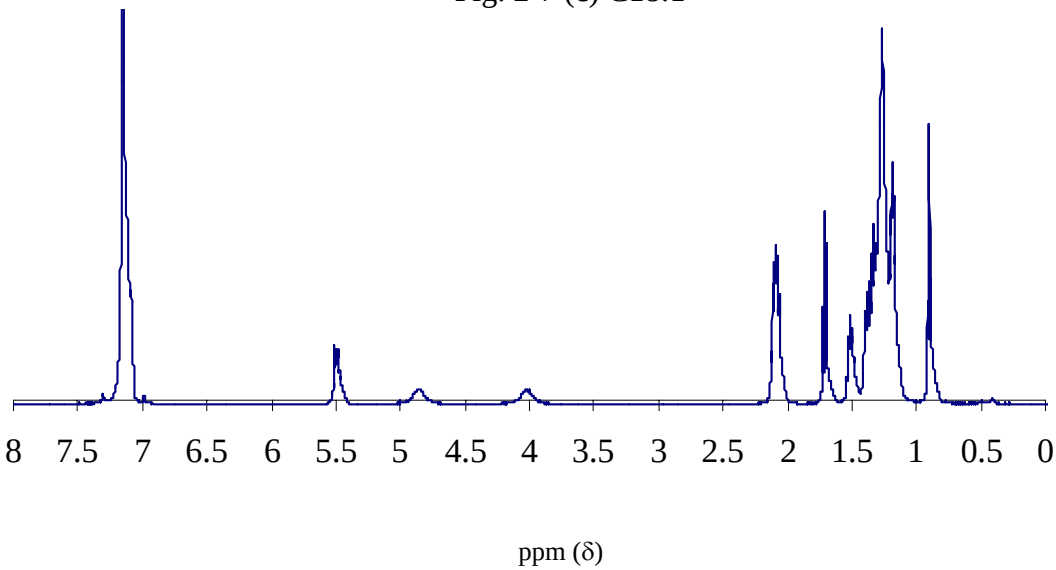


Fig. 2-7 (d) C18:1 *trans*9

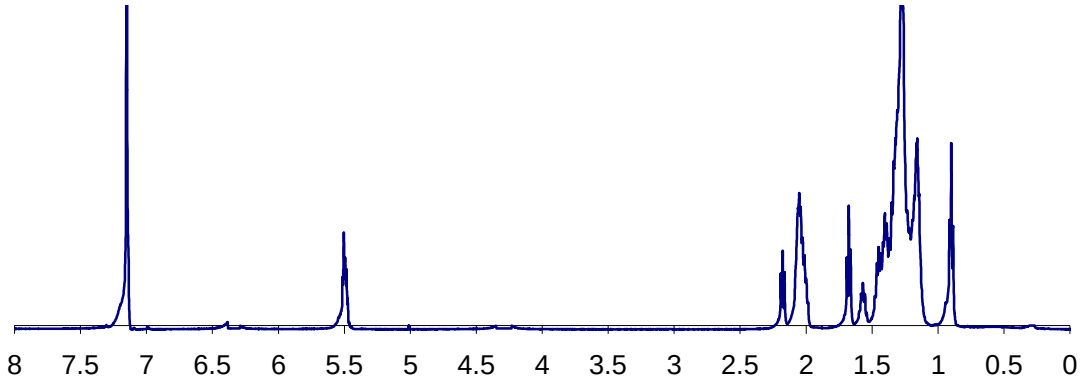


Fig. 2-7 (e) C18:1 *trans*11

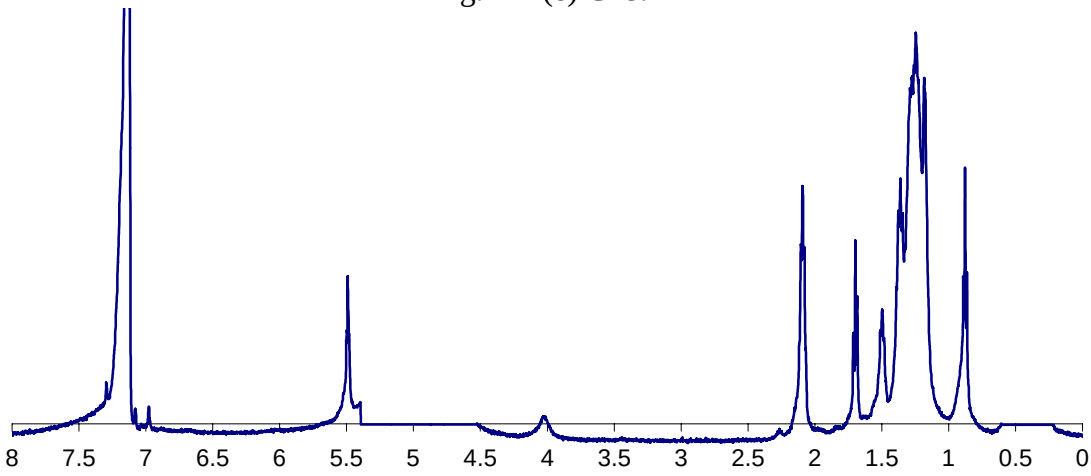


Fig. 2-7 (f) C18:1[13]

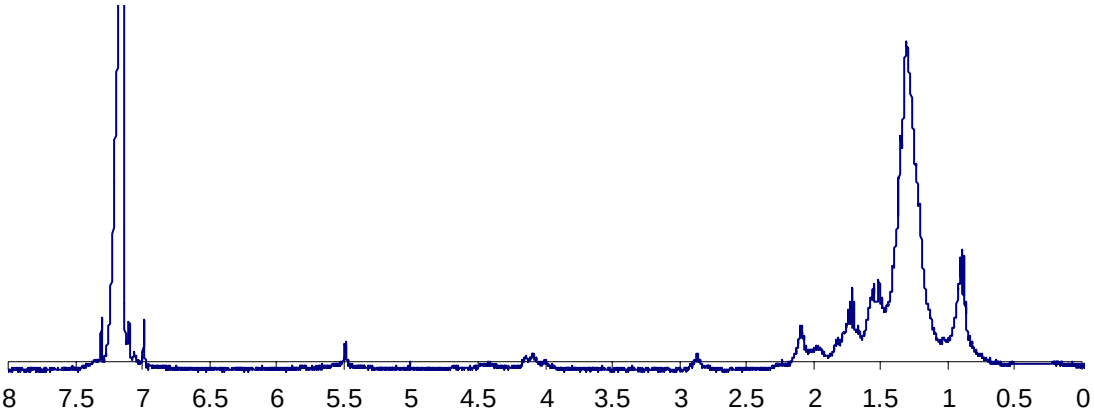


Fig. 2-7 (g) C18:2 [9, 12]

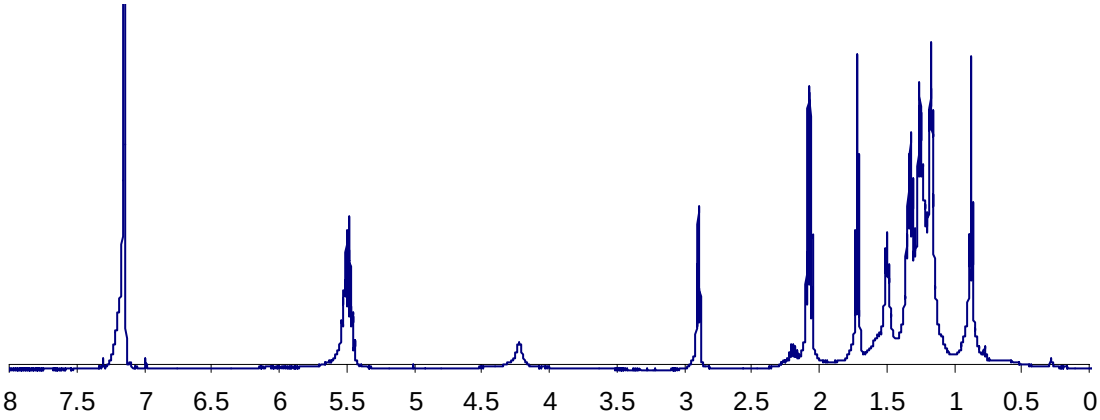


Fig. 2-7 (h) C18:2 *trans* 9, 12

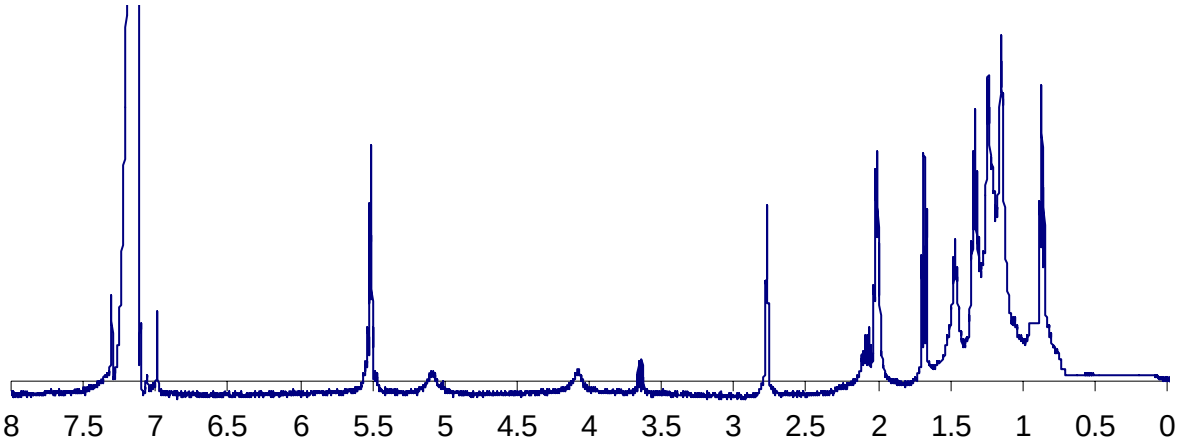


FIGURE 2-7**:** NMR spectra of C18 unsaturated primary fatty acid amides. Please refer to Table 2-II for symbol description.

2.5 Conclusions

In conclusion, we have developed an efficient method for the synthesis of primary fatty acid amides by modifying the simple amide synthesis. Naturally and isotopically abundant PFAM's were synthesized with comparative efficiency. Unsaturated amides as well as their positional and geometric isomers were also synthesized with similar efficiency. Amides synthesized by our method were found to be stable over a long period of time when stored in desiccators at room temperature under N_2. Stearamide was found to be stable over a three year period of time. The *trans* isomers and the isotopically enriched amides were more stable than the rest. They also showed higher

percent yields. Even though some of the unsaturated amides were not separated by HP-5MS column, possible use of BPX70 column for their separation will be discussed in Chapter 6. The synthesized amides were successfully employed in the study of total lipid profiling by HPTLC [41], amide extraction by SPE [42] and also in the separation study of the PFAM's by HPTLC [43], HPLC [44, 45] and GC/MC [manuscript under preparation].

2.6 Acknowledgements

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Chapter 3

Total lipid profiling of $N_{18}TG_2$ mouse neuroblastoma cells by HPTLC and quantification of primary fatty acid amides (PFAM's) by GC/MS

3.1 Abstract

A method for total lipid profiling by HPTLC from a standard lipid mixture was developed. Bioactive primary fatty acid amides (PFAM's), their biosynthetic precursors *N*-acylglycines, as well as the cannabinoid receptor class *N*-acylethanolamine were included in this profiling as lipid classes. The method was validated using the extracted lipids from amide spiked $N_{18}TG_2$ mouse neuroblastoma cells, which have been shown to have catalytic activities necessary to produce PFAM's. Extracted lipids were then quantified by comparing with the calibration curve of standard lipids using Kodak scientific imaging system (SIS). Isolated PFAM spots were scraped out of the HPTLC plates for quantification by GC/MS and percent recoveries from GC/MS analysis were found comparable to the results obtained by SIS. HPTLC is a simple sample clean-up step, which can be useful for analyzing PFAM's also in conjunction with LC/MS, and/or CE.

3.2 Introduction

The analysis of primary fatty acid amides (PFAM's) is rather troublesome due to their low abundance in biological samples and higher detection limits for instrumental analysis. The low volatility of these compounds generally requires the formation of thermally stable derivatives for gas chromatographic/mass spectrometric (GC/MS) analysis [1-10]. The absorption of the $-CONH_2$ group at low UV region (~210 nm) gives rise to poor sensitivity for liquid chromatographic (HPLC) determination [11] even though a number of researchers have reported successful analysis of PFAM's by HPLC [6, 12-17]. Liquid chromatography with tandem mass spectrometry (LC/MS/MS) [18, 19] as well as capillary electrophoresis with laser induced fluorescence (CE/LIF) [20, 21] can be good choices for PFAM analysis but sample clean-up is a crucial factor for any instrumental analysis [22] to increase the detector lifetime as well as the sensitivity. Since PFAM's are usually extracted from cells or tissues along with the other lipids present in the total lipid extract, their complete isolation from the lipid extract can provide interference free instrumental analysis. The use of clean samples will generally result in higher sensitivity and lower detection limit.

Thin layer chromatography (TLC) is the oldest technique used for sample clean up due to its low cost, ease of handling, ability to run multiple samples in parallel and moderate separation times. The separation of almost any compound from its impurities can be achieved by modifying the developing solvent. Solid-phase extraction (SPE) is another sample clean-up technique most recently being used. An advantage of TLC over column chromatography and SPE is that it can provide a quick profiling of all the

compounds present in a sample matrix, giving the analyst an idea of the interfering compounds along with the target analyte. Possible visualization of the TLC plates under UV light makes any method development rather quick and complication free. High performance thin layer chromatography (HPTLC) plates use particle sizes less than ~15 μm, which gives rise to less spot spread and higher resolution in separation than that regular TLC can offer. HPTLC requires improved analytical techniques for spotting the plates for quantification purposes. Samples can be spotted at any desirable amount at constant pressure with minimal spread. The two possible types of TLC development methods are horizontal and vertical development. After sample application TLC plates are placed in a vertical chamber containing developing solvent at the bottom for vertical development. Horizontal development chambers are flat with spaces to fit TLC plates of specific dimensions and have solvent chambers on both sides. This set-up allows TLC development either from one side, which ends at the other side or from both sides, which ends in the middle of the plate. In the latter case, sample can be applied on both sides of the plate increasing overall sample throughput. One disadvantage of horizontal development is that if the surface is not completely horizontal, uniform separation throughout the plate can not be achieved. The separation on TLC plate occurs by adsorption mechanism. The samples applied on the plates are adsorbed on the surface of the silica gel. Because silica gel is very polar, polar compounds are bound more strongly than the non-polar compounds. As the plates are developed by any solvent or mixture of solvents (which are less polar than silica), the most non-polar compounds travel furthest from the point of sample application. TLC plates can be visualized under visible or UV light after development depending on the

absorptivity of the compounds to be analyzed. Colored compounds can be seen with the unaided eye because they absorb light in the visible region but the colorless compounds require the help of UV light for visualization. Some specific compounds such as lipids absorb light in the very low UV region, which are difficult to visualize even in the UV region. Spraying the TLC plates with certain chemicals in order to form fluorophore (which fluoresces in the UV region) is useful in such cases for visualization [23, 24].

The use of TLC in the separation of various lipids from crude lipid extract has now become common practice in lipid analysis (please see Ref. 25 for review). Many of these studies are carried out to quantify lipid class spots on the TLC plate itself by spectrodensitometry [26-35], whereas the remainder were to scrape individual spots and analyze them by the instrument of choice [36-46]. Quantification of various lipid classes on TLC plate largely depends on the visualization technique used. Charring the plates with aqueous ammonium bisulfate, aqueous sulfuric acid, iodine vapors or sulfuric acid-dichromate reagent are the conventional visualization techniques used.

Some researchers reported better sensitivity with cupric acetate charring [29] whereas others [30] reported even better sensitivity when cupric sulfate was used. Cupric acetate was found to react only with the unsaturated species whereas cupric sulfate was found to react with both saturated and unsaturated species [47]. Rhodamine 6G dye, 2, 7-dichlorofluorescein [24, 40] and a lipophilic dye, primuline, were also used by some researchers [41, 48]. The advantage of using such dyes is that they are non-destructive and can be removed from the lipids after scraping the spots for further analysis. It is clear from literature review as well as from personal experience that

optimum and constant spraying, dipping, or charring is required for reproducible results by spectrodensitometry. For instrumental analysis it is ideal not to spray the plates being scraped. Position of the target spot on an untreated plate can be measured by comparing the spots on a treated plate under the same conditions. This practice reduces the sample loss by the sample recovery from the spraying reagent, resulting in the higher percent recovery. The addition of an internal standard (IS) to the sample after scraping is also crucial for quantitative analyses.

Analysis of PFAM's by TLC was reported by a number of researchers. Jasperse *et al.* [13] could separate the PFAM's from the fatty nitriles on a TLC plate but the separation by chain length was achieved only by HPLC. They reported TLC as a quick scanning technique to assess the method development. Bilyk *et al.* [49] studied the separation of free fatty acids, fatty acid amides and fatty acylglycerol on a TLC plate. They described TLC as a rapid means of identifying the components of fatty mixtures with highly reproducible separation even though no data on quantification was available. Kaneshiro *et al.* [50] have used TLC for the detection of amides while studying their bioconversion from oleic acid by *Bacillus megaterium*. They used iodine vapor for the visualization of unsaturated and sulfuric acid charring at 140 ºC for the visualization of saturated amides. Separation of amides was achieved by further analysis with HPLC and GC. TLC was also used for the identification of oleamide produced by *Streptomyces* sp. KK90378, a soil microorganism [51]. After purification by TLC and column chromatography, the structure of oleamide was confirmed using GC/MS and NMR.

In this chapter, we report the inclusion of primary fatty acid amides, *N*-acylethanolamines and *N*-acylglycines as lipid classes in total lipid profiling by high performance thin layer chromatography (HPTLC). The method was validated using the lipids extracted from $N_{18}TG_2$ cell, a model line, that was found to (i) express peptidylglycine α-amidating monooxygenase (PAM), (ii) show increased PAM expression upon differentiation, and (iii) produce both oleamide and anandamide. It is necessary to add here that the biosynthesis of PFAM's from *N*-acylglycines is catalyzed by PAM *in vivo* (please see Section 1.5 of Chapter 1 for more information). The total lipid extract from amide spiked $N_{18}TG_2$ cells was loaded on the HPTLC plates and the lipid classes were profiled [52-53]. Isolated PFAM's spots were scraped off the plates and analyzed by GC/MS free of interference from other lipid classes [54]. Because this method was validated for isolating and analyzing the amides from amide-spiked $N_{18}TG_2$ cells, it is likely that PFAM's as well as NAE and NOG can be isolated and analyzed quantitatively also from any cultured cell type.

3.3 Experimental

3.3.1 Reagents

Oleamide (OM), oleic acid (OA), tristearin (TS), tricaproin (TC), monooleoyl glycerol (MOG), monopalmitoyl glycerol (MPG), monocaproyl glycerol (MCG), dipalmitin (DP), phosphatidylcholine (PC), *N*-oleoylglycine (NOG), *N*-oleyolethanolamine (NOE), sphingomyelin (Sph), gangliosides (G) and squalene (Sq) were purchased from Sigma (St. Louis, MO). Cholesterol (Ch) was purchased from EM Science (Darmstadt, Germany) and cholesteryl palmitate (ChP) from Janssen Chemical (Geel, Belgium).

The solvents used for making solutions and mobile phases were purchased as follows: ACS reagent grade chloroform and methanol from EM Science and EMD (Darmstadt, Germany) respectively; ACS reagent grade hexanes and glacial acetic acid from Fisher Scientific (Fair Lawn, NJ); acetone and diethyl ether from Acros Organics (Geel, Belgium) and Aldrich Chemical Co. (Milwaukee, WI) respectively. Stearamide and palmitamide were synthesized from the corresponding acids according to the published protocol [55] modified in our laboratory.

3.3.2 Cells

$N_{18}TG_2$ cells treated under different conditions were provided by our collaborators Dr. David Merkler and Dr. Kathy Merkler of University of South Florida. The descriptions are as follows: $N_{18}TG_2$ is a mouse neuroblastoma cell line that was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; Braunschweig, Germany). The $N_{18}TG_2$ cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Mediatech Cellgro, Herndon, VA) supplemented with 1% penicillin-streptomycin (Sigma Chemical Co., St. Louis, MO), 10% fetal bovine serum (FBS, Atlanta Biological, Atlanta, GA), and 100 mM 6-thioguanine (Sigma) at 37 °C and 5% CO_2 atmosphere. Several conditions were set up: 1) No Tx: no treatment with differentiation agents, 2) Differentiated (Diff.) Control: Cultures were grown to 60% confluency and then differentiated for two days in low serum (0.5% FBS) DMEM containing 200 ng/mL nerve growth factor (NGF, Boehringer Mannheim, Indianapolis,IN), and 1 mM dibutyryl cAMP (Sigma), 3) Undiff. + oleic acid: undifferentiated cells that were at 60% confluency were treated with DMEM + 200 μM

oleic acid for 2 days and 4) Diff. + oleic/ palmitic / stearic acid: cells at 60% confluency were treated with low serum DMEM + differentiation agents + 200 μM of a particular acid for 2 days. The cells were detached from the tissue culture flask using a cell scraper. The cells were then centrifuged (250 x g) and washed 3 times with Dulbecco's Phosphate Buffered Saline (DPBS). The pellets were flash frozen in a dry ice/methanol bath and stored at -80º C.

3.3.3 HPTLC plates

High performance thin layer chromatography plates (HPTLC) were purchased from Analtech (Newark, DE). These plates were of 10 x 10 cm and with organic binder and no fluorescence indicator incorporated. Primuline dye, (CI 49000; direct yellow 59) used for spraying the HPTLC plates, was obtained from Aldrich.

3.3.4 Systems and instruments

Camag Nanomat and capillary dispenser system, purchased from Camag (Muttenz, Switzerland), was used for easy application of samples on HPTLC plates in the form of spots with minimal spread, precisely positioned and without damage to the layer. 1 μL sample was applied at a time with disposable capillary pipettes. Preval power unit (Precision Valve Corporation, Yonkers, NY) for spraying primuline dye on the HPTLC plates was purchased from a local hardware store. Fluorescent images were obtained using Kodak digital science 440 image station (IS440cF, Perkin Elmer, Boston, MA) and the image analysis was carried out by Kodak 1D scientific image analysis software (Scientific imaging system; SIS, New Haven, CT). An Agilent Technologies Network GC/MS system (6890 GC with 5973 mass selective detector and 7683 series injector)

was used for GC/MS analysis. The column used was an HP-5MS (0.25 mm internal diameter, 0.25 μm film thickness, 30 meter long, Agilent Technologies Inc., Palo Alto, CA) able to analyze fatty acids and amides without prior derivatization. The GC/MS method used for analysis is as follows: Starting temperature was 55 ºC, ramped to 150 ºC at 40 ºC per min, held at 150 ºC for 3.62 min, ramped to 300 ºC at 10 ºC per min and finally held at 300 ºC for 2 min. Electron impact ionization (EI) at 70 eV was used and mass range was kept from 40 to 400 m/z for total ion monitoring (TIM) mode. The fragment ions used for the single ion monitoring (SIM) mode were m/z 59, m/z 72, m/z 62, and m/z 76. The temperatures of the injection port and the transfer line were 250 ºC and 280 ºC respectively. Injection volume was 1 μL splitless.

3.3.5 Method development

12 lipid markers and their mixtures were used for HPTLC method development. These lipids include OM, OA, Ch, ChP, TS, MOG, DP, NOG, NOE, PC, Sph and G. HPTLC mobile phases were made the day before analysis. Solvents were mixed in correct proportions, sonicated for an hour and kept on the bench top with caps on. 2 μL of each standard were applied on HPTLC plates 1 cm from the bottom using the Camag capillary holder. Warm air from a hair dryer was applied continuously for 5 min before sample application in order to activate the silica gel and for 1 more minute after the application in order to dry the spots. For plate development, twin trough chambers for 10 x 10 cm plates from Camag were used. HPTLC separation method was modified from the method used by White, *et al.* [41] in order to separate PFAM's as a lipid class. Firstly, the 10 x 10 cm plates were developed up to 5.5 cm from the bottom in

chloroform: methanol: acetic acid (95:5:1, v/v/v), dried for 5 min with warm air from a hair dryer. Secondly, the dried plates were developed in hexanes: diethyl ether: acetone (60:40:5, v/v/v) up to 8 cm, re-dried. Thirdly and finally, the plates were developed in hexanes: diethyl ether (97:3, v/v) to 9.5 cm. These plates were then dried for 5 min and sprayed with a 0.05 % primuline solution (in 80:20, v/v acetone: water) and re-dried. The dry plates were scanned by Kodak digital Science 440 image station.

3.3.6 Method validation

The developed method was validated using the lipids extracted from $N_{18}TG_2$ cells. Six different $N_{18}TG_2$ cells were used in this study. Cell 1, differentiated (diff.) cells grown in oleic acid; cell 2, diff. control; cell 3, diff. cells grown in stearic acid; cell 4, diff. cells grown in palmitic acid; cell 5, undifferentiated (undiff.) cells grown in oleic acid, and cell 6, No treatment (No Tx) $N_{18}TG_2$ cells. The cells were stored at -80 ºC upon arrival in the laboratory. They were taken out just before the extraction and kept on dry ice until suspending in methanol. Cells 1 and 5 were spiked with oleamide; cells 3 and 4 were spiked with stearamide and palmitamide, respectively, before lipid extraction in order to be able to see the amides on the HPTLC plates and to analyze percent recovery. Spiking was done by suspending the cells with 1 mL amide solutions in methanol (0.947 μmol/mL oleamide, 0.941 μmol/mL stearamide and 1.044 μmol/mL of palmitamide). Lipid extractions from all the cells were carried out according to White *et al.* [41] and Folch-Pi *et al.* [56] with a little modification. The methanol suspension was sonicated for 15 min at room temperature and centrifuged at 4500 rpm for 10 min. The supernatant was separated from the pellet, dried under a stream of N_2 in a warm

water bath at 35-40 ºC. The pellet was re-extracted with 1 mL of 1: 1: 0.1; (v/v/v) chloroform: methanol: water, sonicated for 10 min, vortexed for 2 min and centrifuged for 10 min as above. Supernatant from this step was added to the dried supernatant from the previous step and re-dried the same way. The total extract was then partitioned into lipid and non-lipid portions by adding 1.2 mL of chloroform: methanol (2:1, v/v) and 200 μL of 0.5 M KCl/ 0.08 M H_3PO_4. This partitioned extract was sonicated for 2 min, vortexed for 2 min and centrifuged for 10 min at 4500 rpm. The lower lipid phase was dried under a stream of N_2 in a warm water bath at 35-40 ºC. The dried lipid extract was dissolved in 100 μL of 0.5 mg/mL squalene solution in chloroform: methanol (2:1, v/v). 2 μL of these final lipid solutions (six solutions for six different cells) were applied on HPTLC plates so that the amount of each amide applied on the HPTLC plates is 5.33 μg from the spiked cells.

3.3.6.1 Quantification of lipid classes by KODAK SIS

Because nine different lipid classes were visibly separated in the total lipid extract, calibration curves of nine lipid markers were prepared. All the standard solutions were made with a 0.5 mg/mL squalene (an internal standard) solution in chloroform: methanol (2:1, v/v). Squalene was chosen as an internal standard based on 3 factors: (i) its good resolution from all the lipid classes (it is a non-polar hydrocarbon), (ii) the quantity of squalene applied on the HPTLC plates falls within the range of the amount of lipids applied, and (iii) it can be visualized using primuline dye along with other lipid classes. The sample spots were quantified by comparing their pixel intensities relative

to the internal standard to those of the corresponding standard curves (n = 7 to 10). Kodak 1D scientific imaging software (SIS) was used for this analysis.

3.3.6.2 Quantification of PFAM's by GC/MS

For GC/MS analysis of PFAM's, the spots containing amides were scraped off the HPTLC plates. These plates were not sprayed with primuline and the positions of PFAM's were determined by comparing with plates that were sprayed with primuline. The amides were then treated as follows: (i) amide adsorbed silica-gel was suspended in 2-propanol containing the internal standard (0.8 ng/μL heptadecano-d33-amide) and sonicated for 5 min to dissolve the amide, (ii) the suspension was then centrifuged and the supernatant was collected, (iii) any silica particle left in the supernatant was removed by filtering through 0.2 μm nylon membrane filter (Whatman), and finally (v) the sample was injected into GC. Quantification of cell amides was done by comparing their peak areas relative to the internal standard to those of the corresponding standard curves.

3.4 Results and Discussions

In order to develop a method for total lipid profiling including PFAM's as a lipid class, the method described by White *et al*. [41] was modified. Their method was a multi-step one dimensional TLC development using 4 different solvent systems. In order to follow how each lipid moves after development by each solvent system, six different plates (A-F) were developed. Besides the four solvent systems used by White *et al*., 100 % $CHCl_3$ was included in this method as the first solvent.

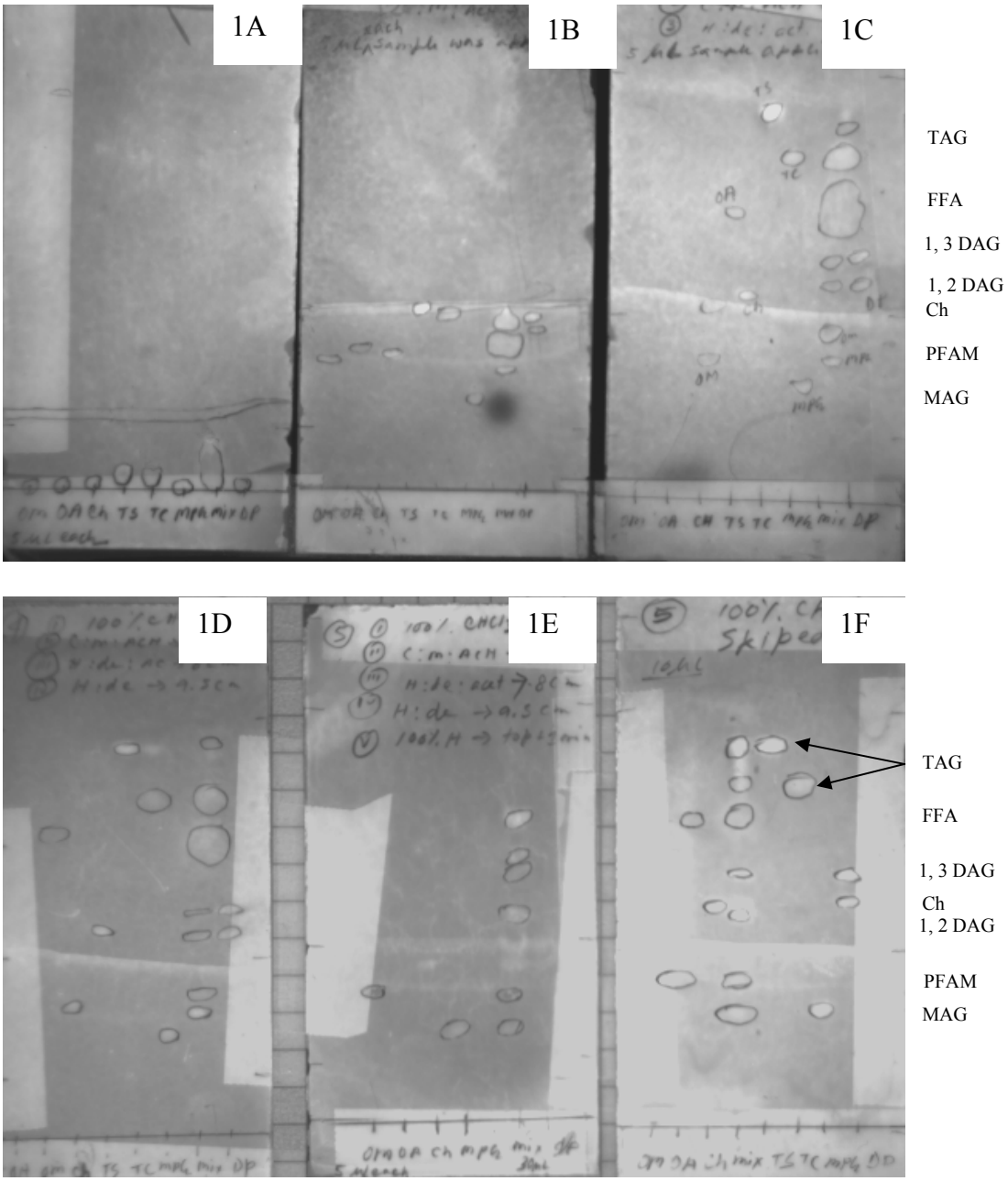


FIGURE 3-1: Step 1 for the method development of the total lipid profiling. The spots on the plates from left to right are OM, OA, Ch, TS, TC, MOG, mixture of lipids and DP except on plate E where the spots are OM, OA, Ch, MPG, mixture and DP. For conditions for each plate, see text.

Therefore the solvent systems used were (i) 100% $CHCl_3$, (ii) $CHCl_3$: MeOH : HOAC (90:10:1; v/v/v), (iii) hexanes : diethyl ether : acetic acid (60:40:5; v/v/v), (iv) hexanes : diethyl ether (97:3; v/v) and (v) 100 % hexanes. The lipids spotted on the plates to start out are OM, OA, Ch, TS, TC, MOG, DP and a mixture of the standard lipids. Plate A was developed in solvent (i) to 2.5 cm; plate B in solvent (i) to 2.5 cm and in solvent (ii) to 4 cm; plate C in solvent (i) to 2.5 cm, in solvent (ii) to 4 cm and in solvent (iii) to 8 cm; plate D in solvent (i) to 2.5 cm, in solvent (ii) to 4 cm, in solvent (iii) to 8 cm and in (iv) to 9.5 cm; plate E in solvent (i) to 2.5 cm, in solvent (ii) to 4 cm, in solvent (iii) to 8 cm, in solvent (iv) to 9.5 cm and in solvent (v) to the top. Plate F was developed as was plate E except solvent (i) was skipped (see Figure 3-1 for A through F TLC images).

It was apparent that the lipids were not moving from the loading position very much with the first solvent. The second solvent was found to be the key solvent that moved the relatively polar lipids, whereas the third solvents carried the relatively non-polar lipids further. The fourth solvent did not seem to have too much effect except for giving better resolution between the lipids. The main point of the use of the fifth solvent was to wash off any non-polar impurities from the plate. The plate F seemed to have better separation then E, therefore the first solvent was skipped for the rest of the study. Figure 3-2 shows the next step taken in the method development. Since the 2^{nd} solvent moves the relatively polar lipids from the position of load, the distance traveled by this solvent was changed (plate C) to observe if any further separation between PFAM and MAG can be achieved. Plate A and B were developed the same way as

plate F and E respectively from Figure 3-1. Plate C was developed as plate F in Figure 3-1 except the distance traveled by the second solvent was 5 cm instead of 4 cm.

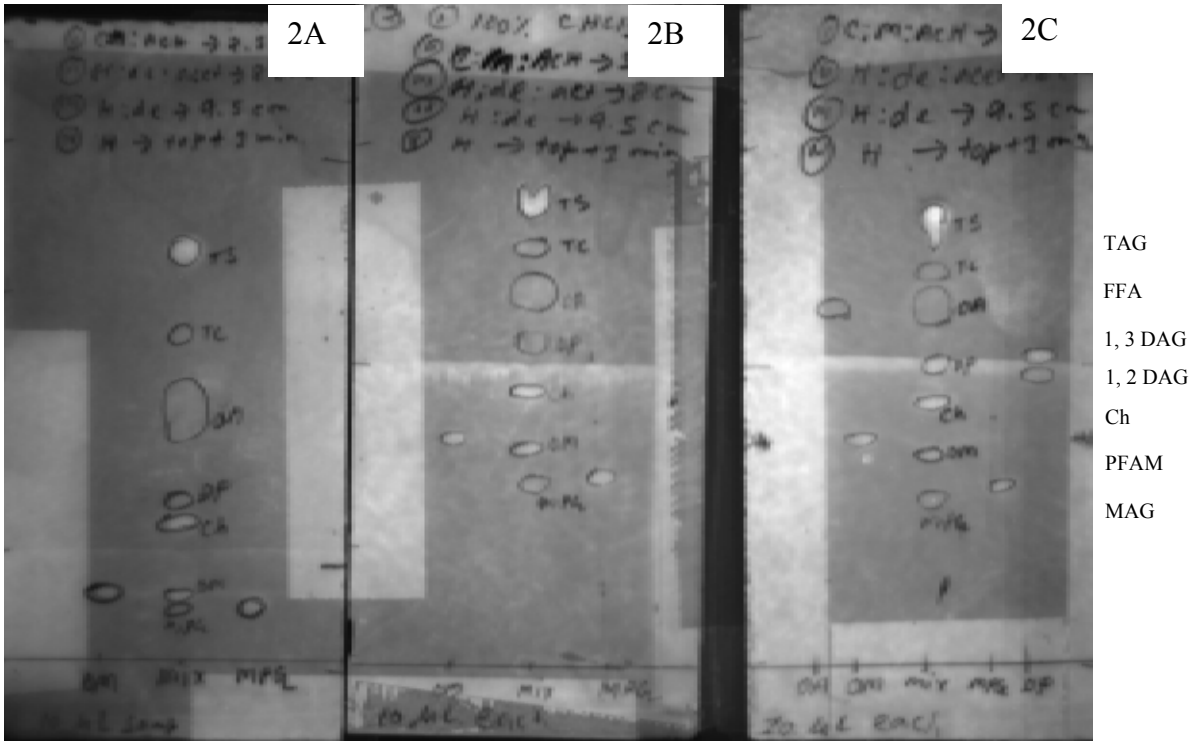


FIGURE 3-2: Step 2 for the method development of the total lipid profiling. The spots on the plates from left to right are OM, mixture of lipids and MOG except on plate C where the spots are OA, OM, mixture, MPG and DP.

As shown in Figure 3-2, a better separation between PFAM and MAG was achieved by the travel of the second solvent to 5 cm. The third step of the study was to see if a different combination of the second solvent provides better resolution between OM and MAG. Figure 3-3 shows that the development of the TLC plate by the second solvent system in a ratio 95:5:1 instead of 90:10:1 indeed gave better separation

between the polar lipids. Introduction of 3 different MAG (monoacyl glycerol) shows that MOG moves a little further than MPG and MCG. The next step therefore was to vary the composition of the second solvent system and the distance traveled by it.

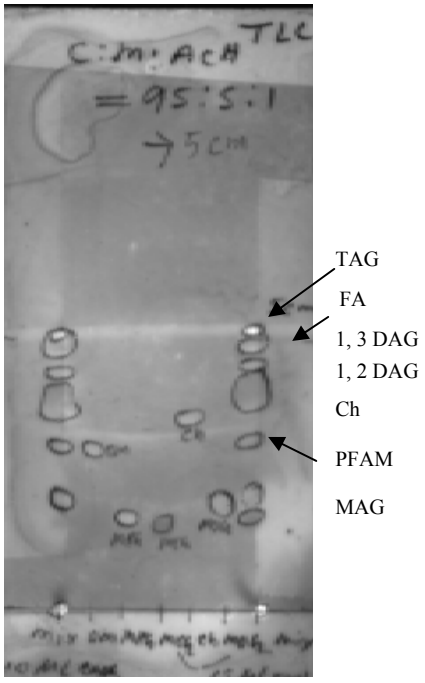
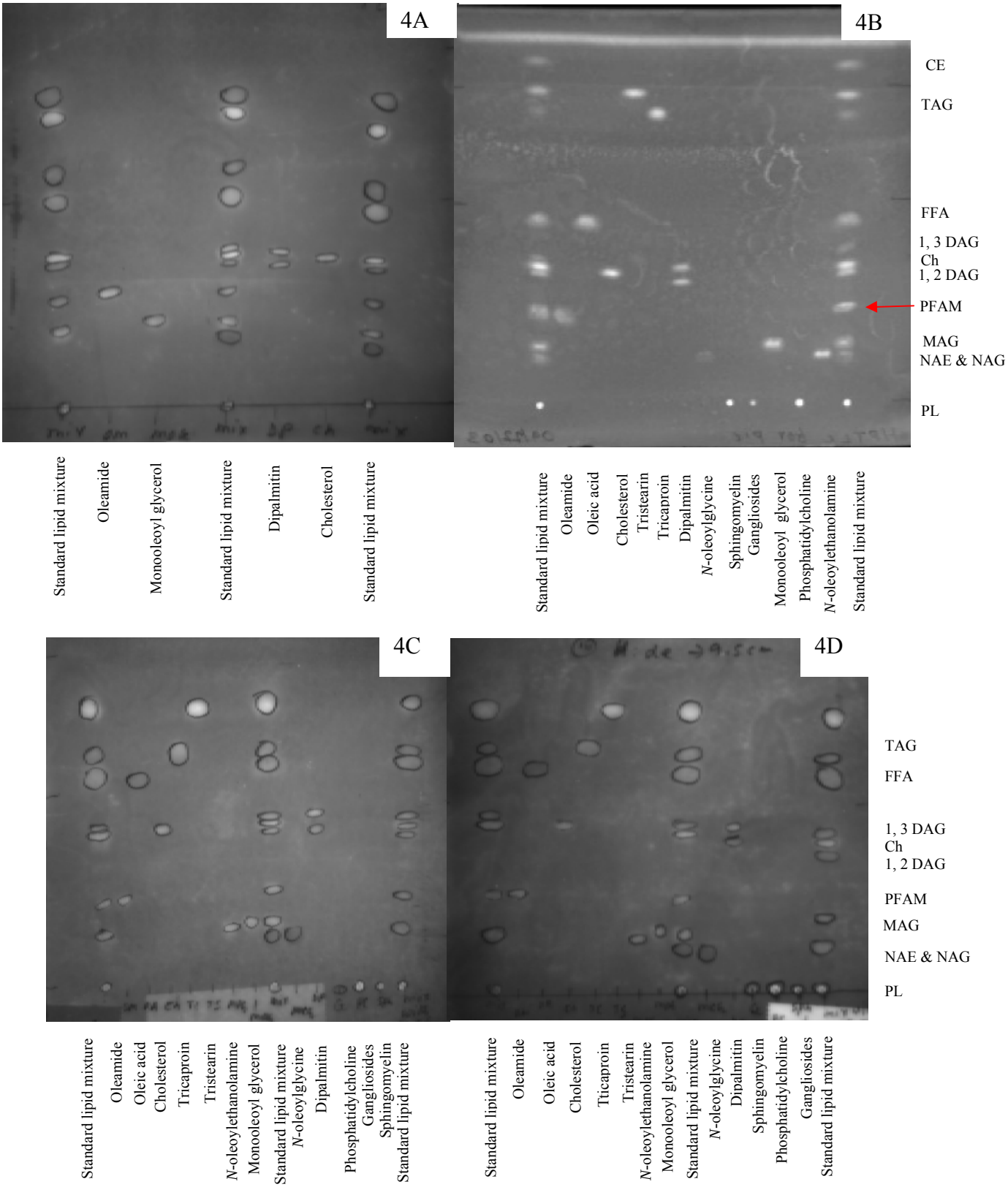


FIGURE 3-3: Step 3 for the method development of the total lipid profiling. The developing solvent for this plate was $CHCl_3$: MeOH : HOAc (95:5:1 ; v/v/v) to 5 cm. The spots on the plate from left to right are mixture of lipids, MPG, MCG, Ch, MOG and mixture of lipids.


4A
Standard lipid mixture
Oleamide
Monooleoyl glycerol
Standard lipid mixture
Dipalmitin
Cholesterol
Standard lipid mixture
4B
CE
TAG
FFA
1, 3 DAG
Ch
1, 2 DAG
PFAM
MAG
NAE & NAG
PL
Standard lipid mixture
Oleamide
Oleic acid
Cholesterol
Tristearin
Tricaproin
Dipalmitin
N-oleoylglycine
Sphingomyelin
Gangliosides
Monooleoyl glycerol
Phosphatidylcholine
N-oleoylethanolamine
Standard lipid mixture
4C
Standard lipid mixture
Oleamide
Oleic acid
Cholesterol
Tricaproin
Tristearin
N-oleoylethanolamine
Monooleoyl glycerol
Standard lipid mixture
N-oleoylglycine
Dipalmitin
Phosphatidylcholine
Gangliosides
Sphingomyelin
Standard lipid mixture
4D
TAG
FFA
1, 3 DAG
Ch
1, 2 DAG
PFAM
MAG
NAE & NAG
PL
Standard lipid mixture
Oleamide
Oleic acid
Cholesterol
Tricaproin
Tristearin
N-oleoylethanolamine
Monooleoyl glycerol
Standard lipid mixture
N-oleoylglycine
Dipalmitin
Sphingomyelin
Phosphatidylcholine
Gangliosides
Standard lipid mixture

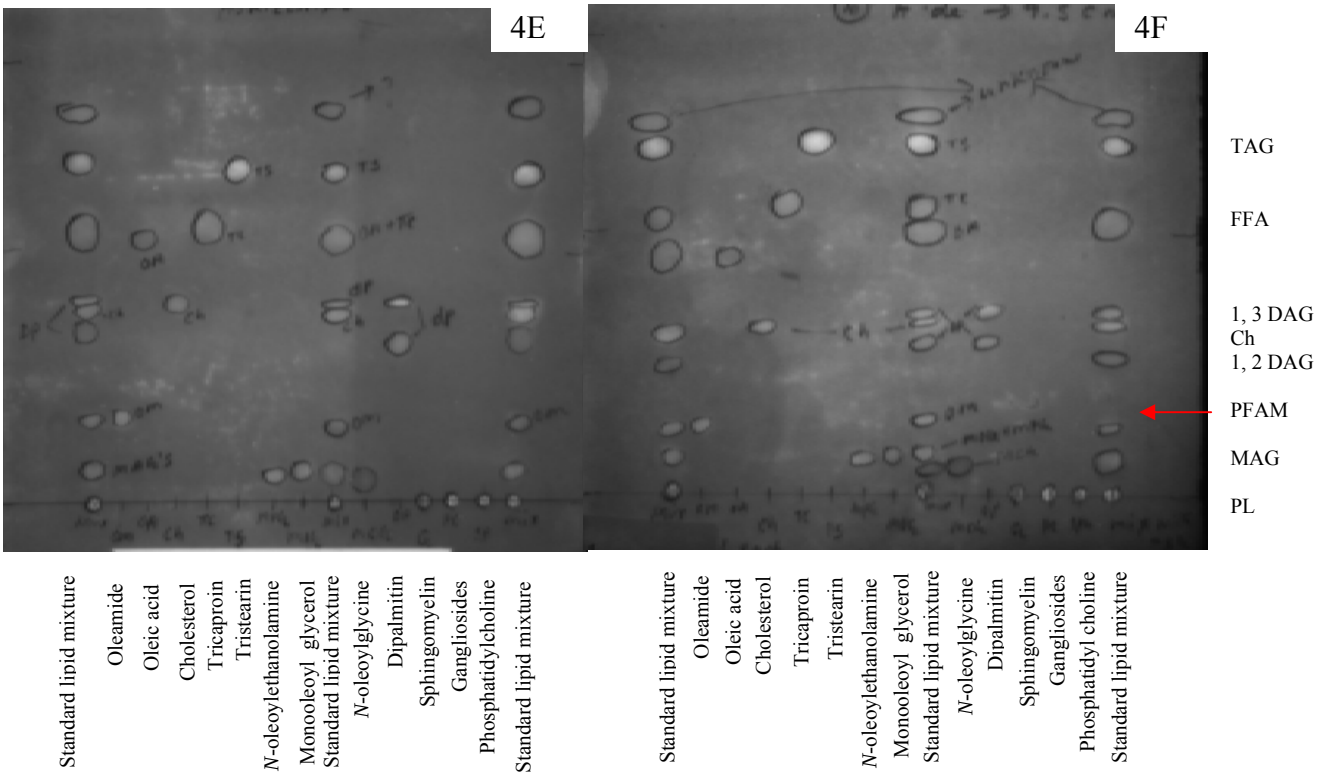
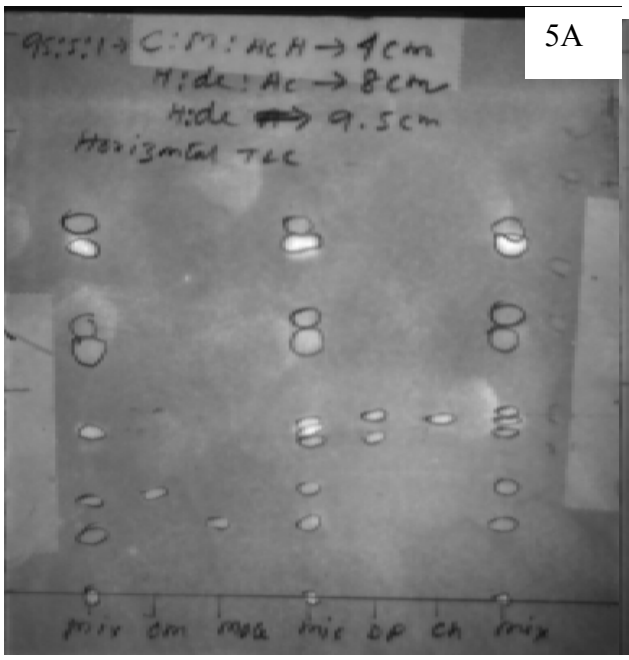
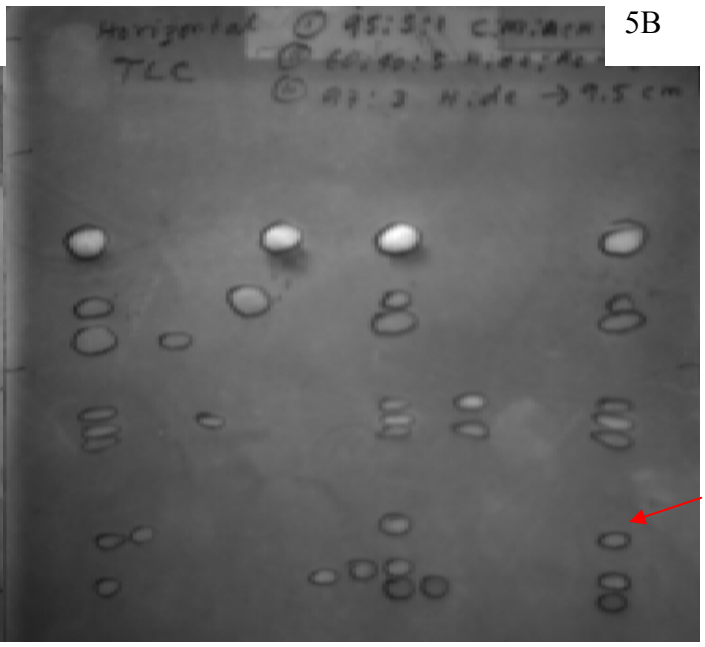


FIGURE 3-4: HPTLC method development step 4. Effect of the composition of the $CHCl_3$: MeOH : HOAc on the total lipid profiling. See text for conditions.

Figure 3-4 shows six different plates A through F with composition of solvent (ii) varying from 94:6:1, 95:5:1, 96:4:1, 97:3:1, 98:2:1 to 98:2:2 respectively. The distance traveled by the solvent was 5 cm. All other conditions of the development were as same as those for plate C of Figure 3-2. Since Figure 3-4 shows that 95:5:1 was giving comparatively better resolution than the other composition, the distance traveled by this solvent was varied. Five different distances were tried with all other conditions the same as the plates in Figure 3-4.

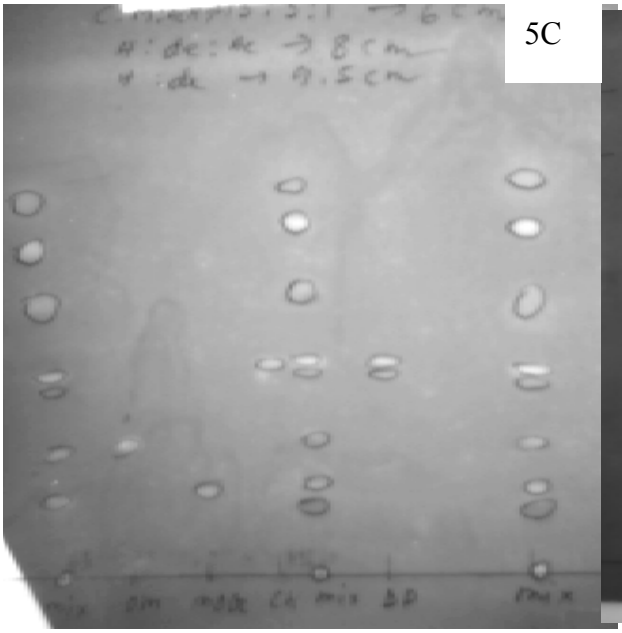


Standard lipid mixture
Oleamide
Monooleoyl glycerol
Standard lipid mixture
Dipalmitin
Cholesterol
Standard lipid mixture

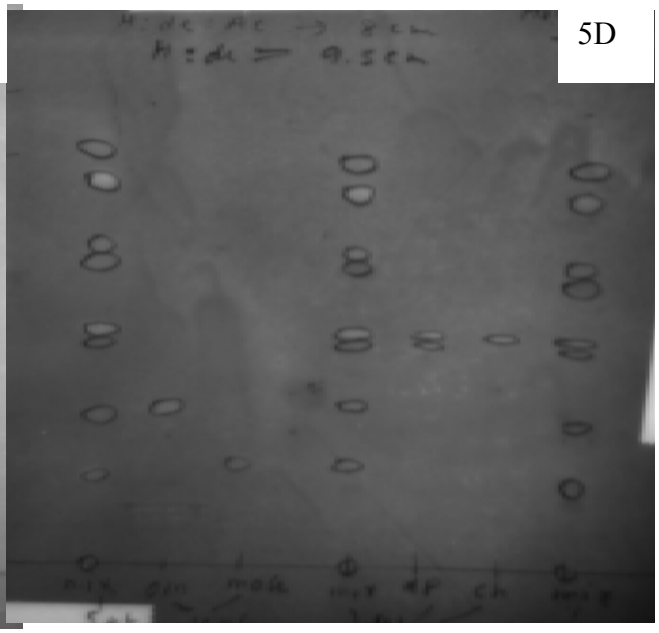


TAG
FFA
1, 3 DAG
Ch
1, 2 DAG
PFAM
MAG
NAE & NAG
PL

Standard lipid mixture
Oleamide
Oleic acid
Cholesterol
Tricaproin
Tristearin
N-oleoylethanolamine
Monooleoyl glycerol
Standard lipid mixture
N-oleoylglycine
Dipalmitin
Sphingomyelin
Phosphatidylcholine
Standard lipid mixture



Standard lipid mixture
Oleamide
Monooleoyl glycerol
Cholesterol
Standard lipid mixture
Dipalmitin
Standard lipid mixture



TAG
FFA
1, 3 DAG
Ch
1, 2 DAG
PFAM
MAG
PL

Standard lipid mixture
Oleamide
Mono-acyl glycerol
Standard lipid mixture
Dipalmitin
Cholesterol
Standard lipid mixture

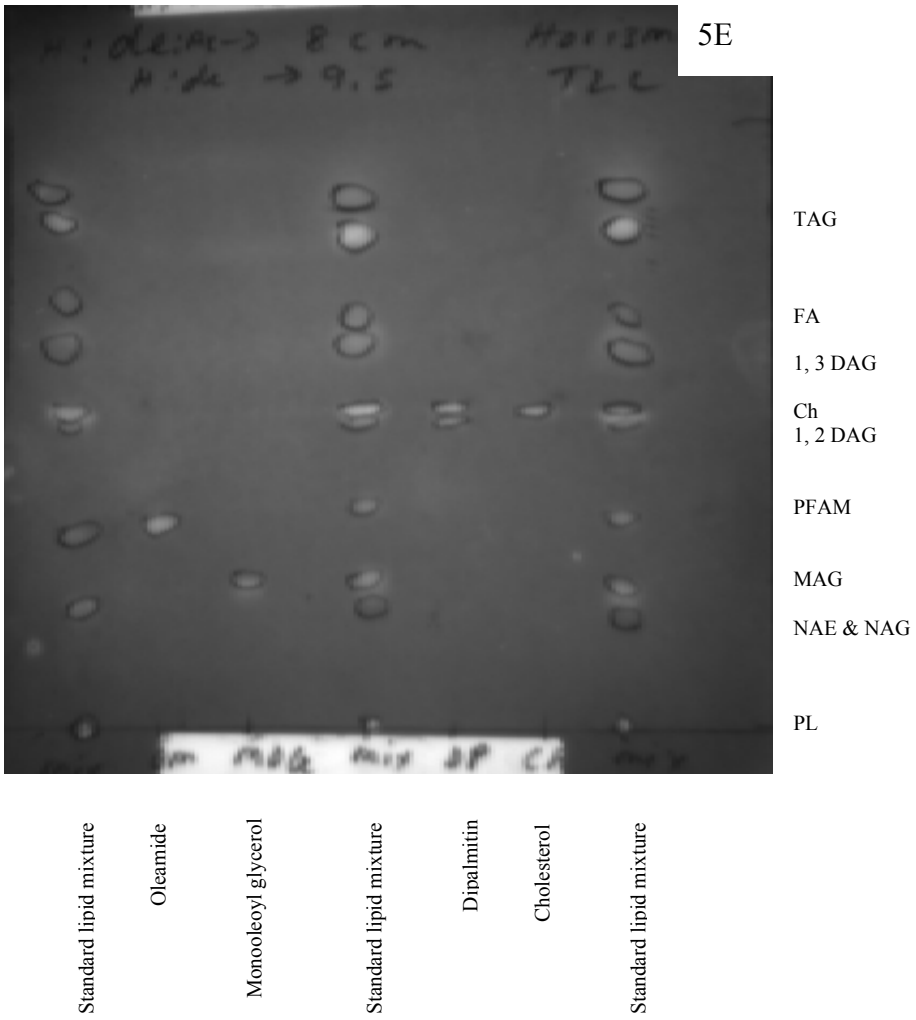


FIGURE 3-5: HPTLC method development step 5. Effect of the distance traveled by $CHCl_3$: MeOH : HOAc (95:5:1; v/v/v) on the total lipid profiling. Distances for plates A-E were 4 cm , 5 cm, 6 cm, 7 cm and 8 cm respectively.

The plates in Figure 3-5 clearly show that 5 cm was the best distance to be used for optimum lipid class separation. The lipids included in the distance study were OM, MOG, DP and Ch movement of which were mostly affected by any change in distance traveled by solvent (ii). Therefore the optimum method was taken as follows:

development in $CHCl_3$:MeOH : HOAc (95:5:1; v/v/v) to 5 cm, in hexane : diethyl ether: acetic acid (60:40:5 ; v/v/v) to 8 cm followed by in hexane : diethyl ether (97:3; v/v) to 9.5 cm.

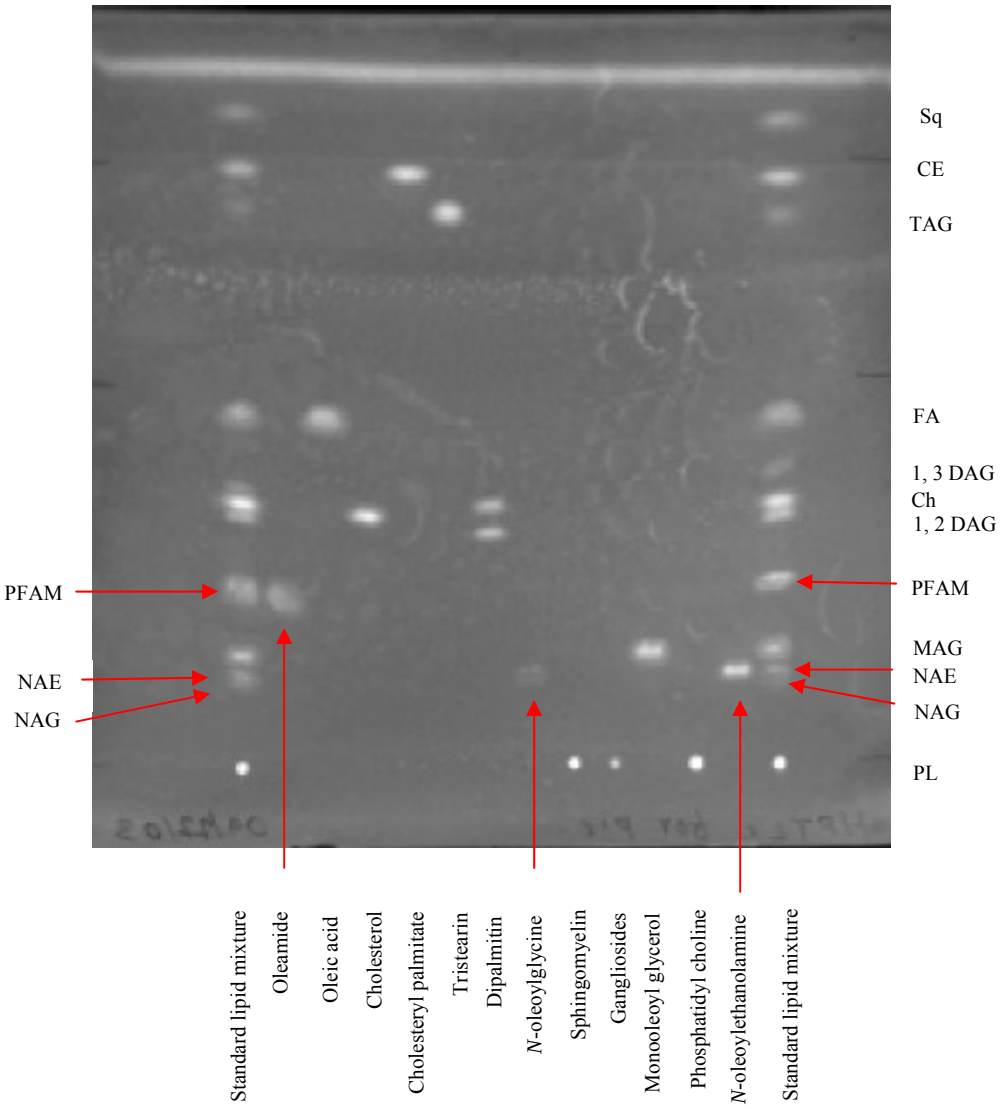


FIGURE 3-6: PFAM's as a lipid class as determined by HPTLC (optimized profiling).

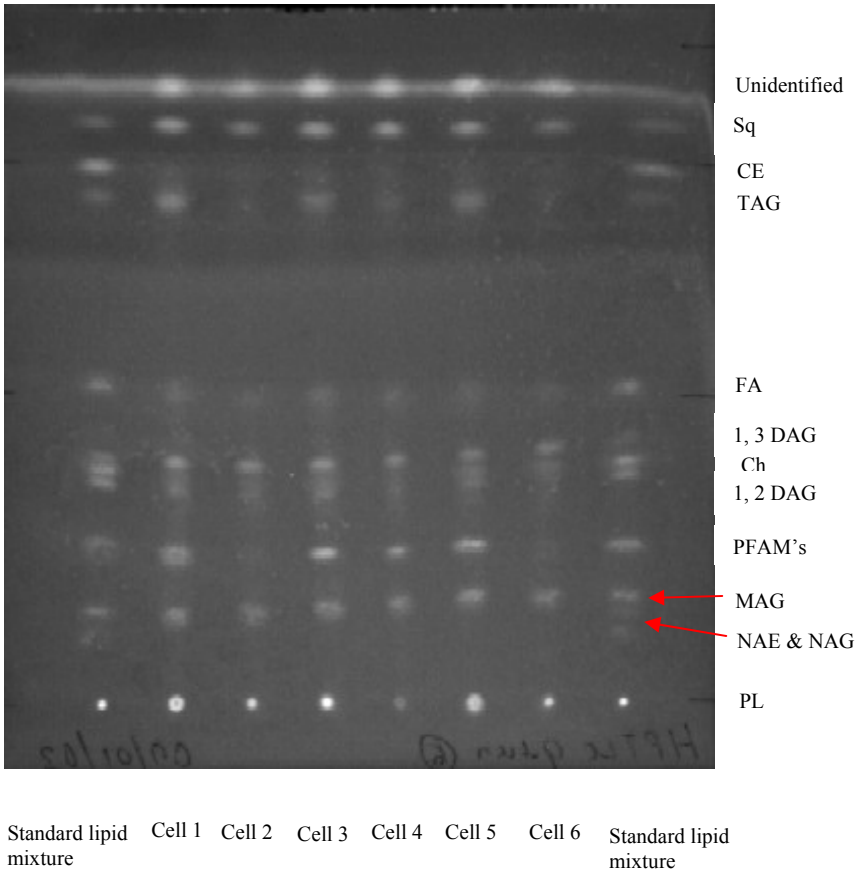


FIGURE 3-7: Total lipid profiling of $N_{18}TG_2$ cell lipids.

In this study 12 lipid markers including PFAM's were successfully included and separated on a TLC/HPTLC plate (Figure 3-7). The lipid classes profiled were phospho and glycolipids (polar lipids; PL), monoacyl glycerol (MAG), primary fatty acid amides (PFAM's), cholesterol (Ch), diacyl glycerol (1,2 and 1,3 DAG), fatty acids (FA), triacyl glycerol (TAG) and cholesteryl ester (CE). *N*-acylglycine and *N*-acylethanolamine (NAG and NAE) were also separated from other lipid classes. Because PL do not migrate from the point of application, the subclasses within this lipid class would have

to be developed separately [41]. Even though, NAE and NAG are not quite separated from each other but separation can be easily improved upon further TLC modification. The spot for NAG and NAE can also be scraped off and separated by LC and/or GC.

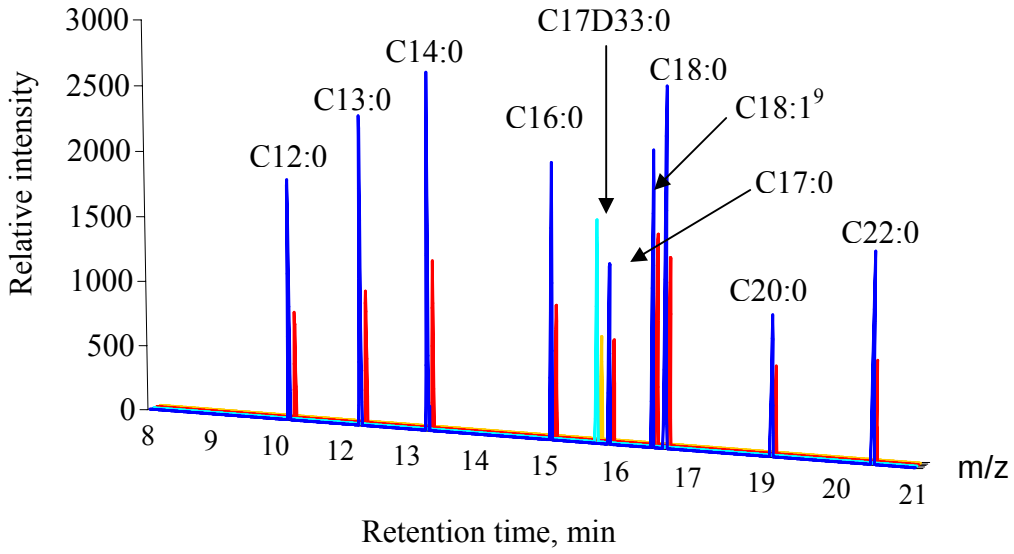


FIGURE 3-8: Elution profile of C12-C22 saturated amides. Relative intensities of m/z are plotted against the retention time. m/z 59 (blue) and m/z 72 (red) are specific to the naturally abundant amides whereas m/z 62 (indigo) and m/z 76 (orange) are specific to the isotopically enriched C17D33 amide used as internal standard (IS).

Our method was equally successful when applied to the separation of lipids extracted from various $N_{18}TG_2$ cells. The resolved lipid classes in these cells shown in Figure 3-8 were MAG, PFAM's, Ch, DAG, FA, TAG and CE. No NAG or NAE were identified in the cells, which may be due to the high detection limits of these lipids. Detection limits for the lipid classes are shown in Table 3-I for both KODAK SIS and

GC/MS. The elution profile for saturated C12 to C22 amides on a HP-5MS column is shown in Figure 3-8. For GC/MS, detection limits for palmitamide, stearamide and

TABLE 3-I: Detection limits (DL) of the lipids for Kodak SIS and GC/MS.

Lipid classes	**DL**		
	SIS (µg)	**GC/MS (pg)**	
PGL	0.08	----	
NAG	0.1	----	
NAE	0.1	----	
MAG	0.36	----	
PFAM's	0.56	C16	25
		C18	10
		$C18^9$	5
Ch	0.05	----	
DAG	0.05	----	
FFA	0.14	----	
TAG	0.09	----	
CE	0.02	----	

oleamide were found to be 25 pg, 10 pg and 5 pg, respectively. The higher detection limit of the amides compared to the other lipid classes on the HPTLC plates is why the spiking was needed in order to be able to see them on the HPTLC plates. In this study HPTLC was proven be an quick and easy method for profiling and quantifying lipids including PFAM's within a specific cell type (see Table 3-II for quantification results)

and the quantification of PFAM's were comparable to that obtained by GC/MS (see Figures 3-9 and 3-10 for standard curves).

GC/MS analysis of the amides scraped off the HPTLC plates is shown in Figure 3-11 which suggests that PFAM's can be isolated and analyzed free of interaction from other lipid classes of any cell or tissue type. Percent recoveries of amides (Table 3-III) obtained by both SIS and GC/MS were found to be comparable within 95 % confidence level except in case of palmitamide. The reason might be attributed to personal or instrumental error for that particular case. Low recovery of stearamide and palmitamide compared to oleamide may be due to the sample loss during the extraction of cells 3 and 4 or due to the sample loss during the isolation of amides from silica particles.

TABLE 3-II: Quantification of lipid classes by KODAK fluorescence imaging system. See Section 3.3.6 for the description of the cells 1-6 used in this study.

	Average mass per cell (ng)								
Cell #	MAG	PFAM's	1,2 DAG	Ch	1,3 DAG	FFA	TAG	CE	PL
1	3.2 ± 0.31	9.8 ± 0.69	0.13 ± 0.02	0.26 ± 0.07	0.25 ± 0.13	0.46 ± 0.15	0.67 ± 0.13	0.17 ± 0.04	2 ± 0.69
2	2.8 ± 0.19	----	0.14 ± 0.02	0.21 ± 0.06	0.23 ± 0.08	0.23 ± 0.05	0.16 ± 0.02	0.04 ± 0.02	0.93 ± 0.32
3	0.45 ± 0.06	0.53 ± 0.21	0.03 ± 0.004	0.03 ± 0.01	0.04 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.03 ± 0.01	0.27 ± 0.10
4	12.4 ± 1.8	10 ± 3.5	0.76 ± 0.17	0.96 ± 0.21	0.76 ± 0.34	2.2 ± 0.35	1.5 ± 0.29	0.32 ± 0.23	----
5	0.29 ± 0.07	0.83 ± 0.19	0.02 ± 0.002	0.02 ± 0.01	0.01 ± 0.004	0.03 ± 0.01	0.06 ± 0.01	0.01 ± 0.002	0.19 ± 0.03
6	0.92 ± 0.16	-----	0.05 ± 0.01	0.06 ± 0.03	0.08 ± 0.04	0.1 ± 0.02	0.07 ± 0.01	0.02 ± 0.01	0.48 ± 0.12

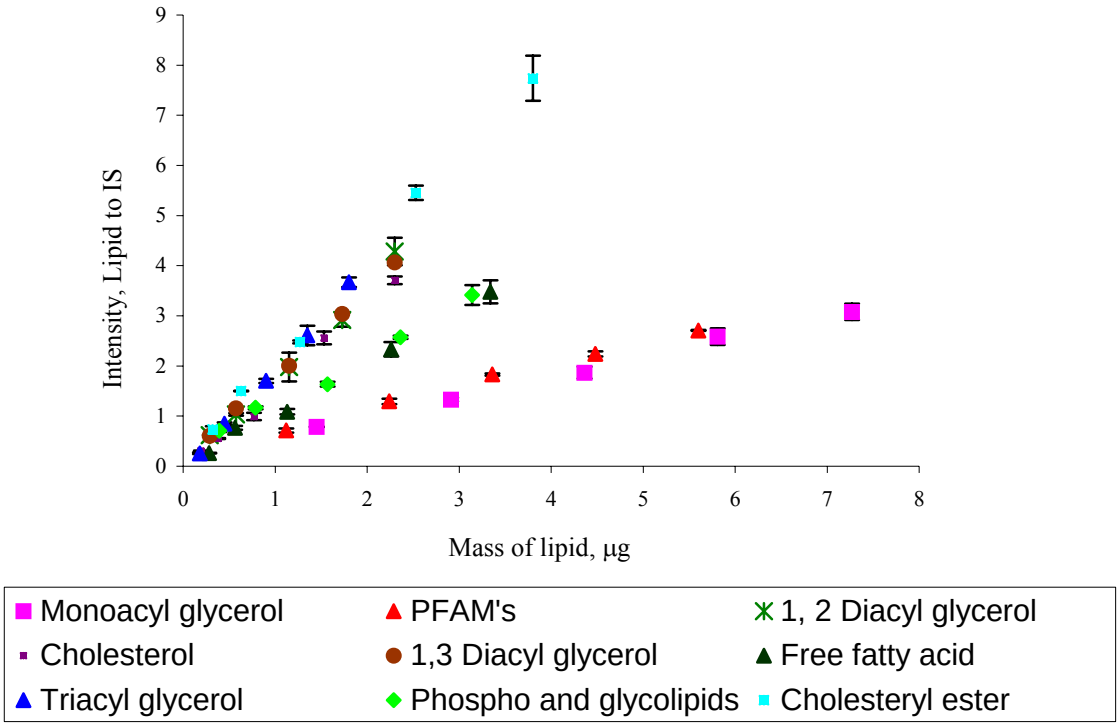


FIGURE 3-9: Standard curves for the quantification of lipid classes on HPTLC. Uncertainties are at 95 % confidence level with n = 6.

Both Table 3-II and 3-III show that no amides were detected in cell 2, differentiated $N_{18}TG_2$ cells and in cell 6, no treatment $N_{18}TG_2$ cells. These cells were not spiked with amides and extracted in the same fashion as the ones spiked with amides. The reason that the amides could not be detected by either HPTLC or GC/MS without a spike was the low cell mass available for this study. Because oleamide is known to be produced in this cell, it might be possible to detect other PFAM's as well, provided a larger sample mass is available.

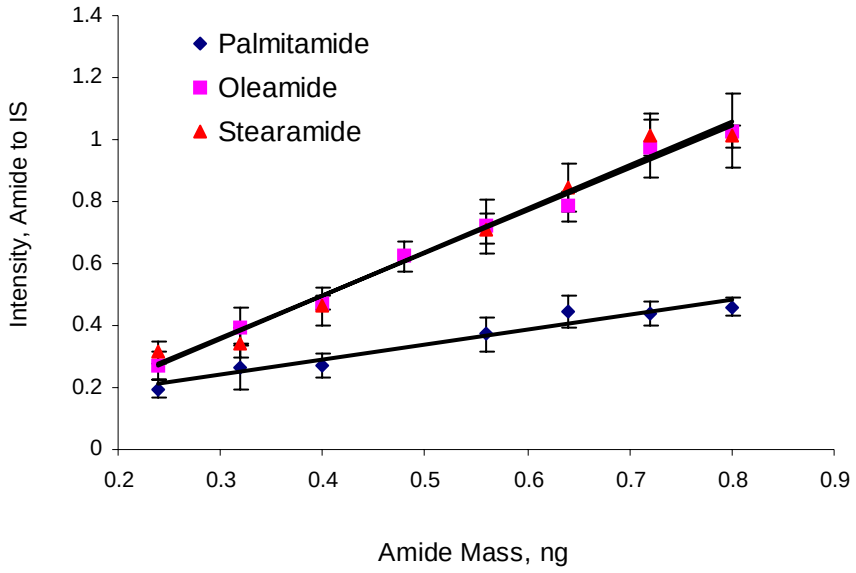
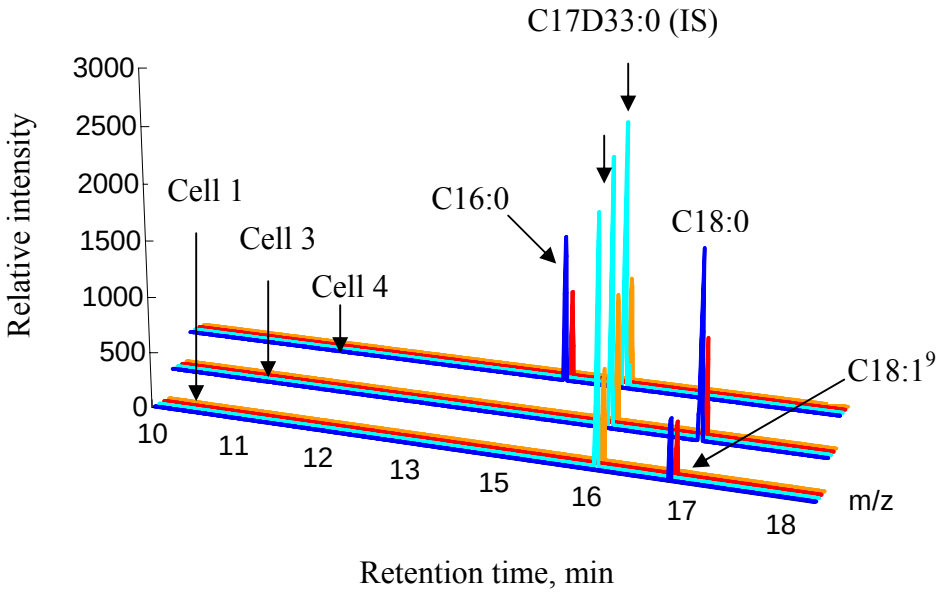


FIGURE 3-10: Standard curves for the quantification PFAM's by GC/MS. Uncertainties are at 95 % confidence level with n = 6.

TABLE 3-III: Percent recoveries of PFAM's from amide spiked $N_{18}TG_2$ cells.

Cell #	Amide spiked	% Recoveries	
		SIS	GC/MS
1	Oleamide	105 ± 8	98 ± 4
2	none	----	----
3	Stearamide	28 ± 12	38 ±1
4	Palmitamide	19 ± 4	36 ± 2
5	Oleamide	80 ± 19	112 ±14
6	none	----	----

Uncertainties are at 95% confidence limit with n = 6 (SIS) and 9 (GC/MS).



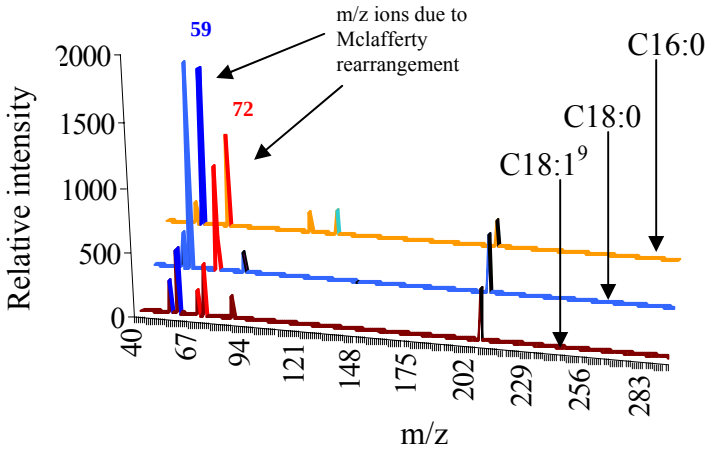


FIGURE 3-11: (a) GC of cell 1 oleamide (C18:1^9), cell 3 stearamide (C18:0) and cell 4 palmitamide (C16:0). Relative intensities of m/z **59**, m/z **72**, m/z **62** and m/z **76** are plotted against the retention time. (b) Mass spectra of oleamide, stearamide and palmitamide recovered from amide-spiked cells. Relative intensities are plotted against m/z values.

3.5 Conclusions

We present a method of isolation of the primary fatty acid amide class, which has recently been recognized for its hormone like activities. After the sample preparation, instrumental analysis of these biologically active hormones can be done free of interaction of other lipids. The method was validated by extracting amides from lipid extracts of $N_{18}TG_2$ cell line, a model for oleamide biosynthesis *in vivo*. Application of the validated method to the lipids extracted from rabbit brain, heart and brain acetone power will be discussed in Chapter 5. Quantification of amides from the biological sample and their percent recovery will be reported. A solid phase extraction method was also developed, which will be detailed in the next chapter. Comparison of the results from HPTLC/GC/MS and SPE/GC/MS of amide isolation can provide strong milestones for these methods.

3.6 Acknowledgements

The author thanks National Institute of Health and National Institute of Neurological Disorders and Stroke for supporting this research (NIH/NINDS R15NS038443), Drs. David and Kathy Merkler of University of South Florida for providing the $N_{18}TG_2$ cells, and Department of Chemistry and Biochemistry at Duquesne University for its instrumental support and other facilities.

3.7 References

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Chapter 4

Solid-phase extraction (SPE) of primary fatty acid amides

4.1 Abstract

Primary fatty acid amides (PFAM's) were isolated from a standard lipid mixture by solid-phase extraction (SPE) and the lowest mass of amide to be loaded and recovered was detected. The method was validated using the lipids extracted from $N_{18}TG_2$ mouse neuroblastoma cells. $N_{18}TG_2$ is a model cell line shown to express PAM (peptidylglycine α-amidating monooxygenase), which is the enzyme for the final step in PFAM biosynthesis. The cells were spiked with seven different amides before lipid extraction. The isolated PFAM's were quantified by GC/MS and percent recoveries were calculated. The detection limit in the single ion monitoring mode (SIM) were found to be 30 pg for amides containing 12 to 14 carbons and 10 pg for amides containing 16 to 22 carbons with the exception of oleamide ($C18:1^9$). Oleamide was found to have a detection limit of 5 pg. Use of SPE for isolating amides from total lipid extract before analyzing them by GC/MS provides clean detection and interference free analysis. SPE sample preparation can thus be useful for analyzing PFAM's also in conjunction with liquid chromatography/mass spectrometry (LC/MS), and/or capillary electrophoresis (CE).

4.2 Introduction

Solid-phase extraction (SPE) implies the adsorption of a target analyte from a mixture onto a suitable solid phase followed by its elution with a chosen solvent. The objective of SPE is to isolate the target analyte in pure concentrated form from a rather complex matrix. The solid phases are so chosen that either the analyte can be selectively adsorbed on the column while the unwanted compounds can be washed off or the impurities are retained while the target analyte is selectively eluted with a mobile solvent of appropriate polarity. The advantages of the SPE over LC includes (i) lower hazardous solvent handing and consumption, therefore least health and safety risk; (ii) capability of handling microgram level samples; (iii) shorter sample preparation time; (iv) increased analyte recovery, as well as (v) less handling and cleaning of glassware.

The variety of solid phases available include silica gel and bonded aminopropyl (normal phase), octadecylsilyl (reversed phase) or the ion exchange media (bonded acidic or basic moieties). The one particular characteristic of silica gel used for SPE is that they have rather larger particle sizes and irregular shapes compared to the traditional HPLC phases, allowing rapid flow of solvent through the sorbent bed. SPE particle sizes generally range form 40-100 μm with 60 Å -70 Å pore diameter. Various bonding techniques and reagents are used by various manufacturers in order to produce monomeric or polymeric bonded phases on the silica surface. Monomeric phase consists of a single bonded silane layer whereas the polymeric phase consists of multiple layers of bonding reagents polymerized on the silica surface. Depending on the nature of the bonding reagent, the SPE phases can be termed as normal (polar) or

reversed (non-polar) phases. The unbonded silica is the most polar phase whereas the octadecylsilyl (ODS) bonded silica by far is the least polar phase. Silica is used for extracting relatively polar compounds followed by their selective elution with mobile phases of increasing polarities, and ODS is mainly used for the extraction of non-polar compounds followed by their elution with mobile phases of decreasing polarities. The disadvantage of using silica or ODS is that very polar or very non-polar compounds respectively can be completely retained by them and therefore become difficult to recover. Application of the moderately polar phases such as aminopropyl bonded phases can be used to avoid such difficulties. Ion exchange phases are also used to selectively extract and elute compounds with cationic or anionic properties. It is a common practice to condition the columns with solvent of similar polarity as the loading solvent before loading the sample, which helps to organize the organic moieties of the bonded silica surface in a regular array, making it more accessible to the analytes [1, 2].

Lipid extraction by SPE has become popular due to the several facts. Firstly, the low concentrations of many of the lipids available in samples can be concentrated by SPE; secondly, any particular class can be selectively extracted and eluted in pure state; and lastly, sensitivity and the limit of detection of the isolated lipid class is improved for instrumental analysis due to the removal of interferents. Normal phase silica columns are the most common types of phases used for lipid extraction even though ODS was one of the first phase to be used for selective isolation of gangliosides from tissue extracts [3]. The reason behind the use of ODS in this case was mainly due to the partition of gangliosides into the Folch upper phase after the Folch extraction of lipids

[4] from tissues. The Folch upper phase is basically polar and passes through the ODS leaving the gangliosides behind, which could thus be selectively eluted by chloroform : MeOH (2:1, v/v). Many other applications for the isolation of various lipid classes as well as purification of total lipid extracts by ODS columns have been described in the literature [5-27].

The use of normal phase SPE is pervasive for the isolation of lipid classes [28-41] due to its polar nature, which offers selective extraction of different lipid classes from various sample sources to be available for further instrumental studies. One example to be mentioned is the separation of *N*-acylethanolamines (NAE: anandamide, an endocannabinoid, belongs to this group) and monoacyl glycerol (MAG: 2-arachidonyl glycerol, a second endocannabinoid, belongs to this group) from mammalian tissue lipid extracts by silica columns [42]. One problem associated with using silica columns is that they tend to show different efficiency from manufacturer to manufacturer or from batch to batch mostly due to variable water retention. This problem can be overcome by drying the phases before use or by using solvents with controlled humidity [43, 44]. The other problem with the silica columns is that the polar lipids (PL) tend to be retained in this rather polar phase.

The use of aminopropyl bonded columns instead of silica columns is favored in lipid analysis because these are comparatively less polar than silica and therefore are less affected by moisture on their capacity for lipid adsorption [45]. Kaluzny *et al* [46] were among the first to use the aminopropyl bonded columns for the separation of seven different lipid classes by piggybacking multiple columns, but unfortunately their method was not found to be reproducible by other laboratories [for review see Ref 1]

including this laboratory. Various modifications of this method have been reported which were better at isolating any particular lipid class [47] or simple lipids [48]. Other uses of aminopropyl bonded phase for the isolation of lipids include but are not limited to the separation of acidic and neutral lipids (NL) from *Escherichia coli* and human spermatozoa lipid extracts [49], separation of NL and polar lipids (PL) from mixed microbial cultures [50], rapid separation of lipid classes in different micro organisms [51], purification of fatty acid ethyl esters from a lipid mixture [52], separation of PL and various NLs from plasma lipid extract [53], separation of plant membrane lipids [54], separation of lipid classes from edible oil and mice tissue samples before their analysis for fatty acid content [55], isolation of chlorinated fatty acids from cell culture medium and fish lipids [56] as well as isolation of serum lipids [57].

Numerous literature reviews are available discussing the principles of SPE, the types of phases available for a particular application, and the application of SPE in lipid analysis [2, 58-61]. The reproducibility of a given SPE method largely depends on the SPE phase, sorbent conditioning, amount of sample loaded, amount of each mobile phase used for elution as well as the flow rate of the mobile phase. Therefore, it is necessary to describe a developed SPE method in detail when publishing in order for others to be able to reproduce the method. It is also not recommended that a vacuum manifold be used for carrying out SPE with non polar solvents. Organic solvents tend to evaporate rapidly under vacuum, which causes partial drying of the stationary phase. For such cases, normal flow by gravity is preferred, but, if required, positive pressure by a syringe can be applied [62].

In the earlier chapters, the biological significance of the primary fatty acid amides (PFAM's) was discussed and the potential problems associated with their analysis were also addressed. It is necessary to point out again that sample clean up such as by HPTLC described in Chapter 3 or by SPE mentioned above before their analysis can provide concentration, higher sensitivity as well as the lower limit of detection. It is important to address that these types of sample preparations not only improve the analysis but also increase the detector lifetime and save money on consumables such as injection liners and septa. In this chapter, a method for the isolation of PFAM's by SPE has been demonstrated [63] and the validation of this method by lipid extracts from $N_{18}TG_2$ cells has been described in detail for ease of reproducibility. The application of this method to the lipids extracted from mammalian tissues will be reported in Chapter 5.

4.3 Experimental

4.3.1 Reagents

Oleamide (OM), oleic acid (OA), tristearin (TS), monooleoyl glycerol (MOG), dipalmitin (DP), phosphatidylcholine (PC), *N*-oleoylglycine (NOG), *N*-oleyolethanolamine (NOE), sphingomyelin (Sph) and bovine brain gangliosides (G) were purchased from Sigma (St. Louis, MO). Cholesterol (Ch) was purchased from EM Science (Darmstadt, Germany) and cholesterol palmitate (ChP) from Janssen Chemical (B-2440 Geel, Belgium). The solvents were purchased as follows: ACS reagent grade hexanes, methanol and glacial acetic acid from Fisher Scientific (Fair Lawn, NJ); acetone from Acros Organics (manufactured in Geel, Belgium); chloroform and diethyl

ether from Aldrich Chemical Co. (Milwaukee, WI). Primary fatty acid amides (except oleamide) were synthesized from the corresponding acids in our laboratory modifying a published protocol [64, also see Chapter 2]. All the free fatty acids were purchased from Sigma unless otherwise mentioned. The undifferentiated $N_{18}TG_2$ cells grown in oleic acid were used, because only this cell type was available in considerable amount to carry out this study. These cells were donated by David and Kathy Merkler of University of South Florida. The cell description is as follows: $N_{18}TG_2$ is a neuroblastoma cell line that was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; Braunschweig, Germany). The $N_{18}TG_2$ cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Mediatech Cellgro, Herndon,VA) supplemented with 1% penicillin-streptomycin (Sigma Chemical Co., St. Louis, MO), 10% fetalbovine serum (FBS, Atlanta Biological, Atlanta, GA), and 100 mM 6-thioguanine (Sigma) at 37 °C and 5% CO_2 atmosphere. Undifferentiated cells that were at 60% confluency were treated with DMEM + 200 μM oleic acid for 2 days and the cells were detached from the tissue culture flask using a cell scraper. The cells were then centrifuged (250 x g) and washed 3 times with Dulbecco's Phosphate Buffered Saline (DPBS). The pellets were flash frozen in a dry ice/methanol bath and stored at -80 ºC.

4.3.2 SPE phases

Discovery DSC-Si and Discovery DSC-NH_2 SPE phases were purchased from Supelco (Bellefonte, PA). Irregularly shaped and acid washed base silica possessed following properties: 50 μm particle size, 70 Å pore diameter, 480 m^2/g specific surface area and

0.9 cm^3/g pore volume. DSC-Si is the unbonded silica phase, which is basically used as a normal phase adsorbent whereas DSC-NH_2 is the aminopropyl bonded silica which can be used as either normal phase or the ion-exchange adsorbent. Polypropylene SPE tubes (empty) of 3 mL volumes with polyethylene frits were also purchased from Supelco. 500 mg of the phases were packed into the tubes between two frits with hand pressure in order to carry out SPE.

4.3.3 HPTLC plates

High performance thin layer chromatography plates (HPTLC) from Analtech (Newark, DE) were used for viewing the lipid contents in different SPE fractions. These plates were of 10 x 10 cm, with organic binder and no fluorescence indicator incorporated. Primuline dye (CI 49000; direct yellow 59) used for spraying the HPTLC plates was obtained from Aldrich Chemical Co. (Milwaukee, WI).

4.3.4 Instrumentation

A Kodak digital Science 440 image station equipped with scientific imaging software (SIS) was used for scanning the HPTLC plates. An Agilent Technologies Network GC/MS system (6890 GC with 5973 mass selective detector and 7683 series injector) was used for GC/MS analysis. The column used was an HP-5MS (0.25 mm internal diameter, 0.25 μm film thickness, 30 m long, Agilent Technologies Inc., Palo Alto, CA) able to analyze fatty acids and amides without prior derivatization. The GC/MS method used for analysis is as follows: Starting temperature was 55 ºC, ramped to 150 ºC at 40 ºC per min, held at 150 ºC for 3.62 min, ramped to 275 ºC at 10 ºC per min and finally held at 275 ºC for 2 min. Electron impact ionization (EI) at 70 eV was used. The

temperatures of the injection port and the transfer line were 250 ºC and 280 ºC, respectively. Mass range was kept from 40 to 400 m/z for total ion monitoring (TIM) and selected masses were 59, 72, 62 and 76 for single ion monitoring (SIM). Injection volume was 1 µL splitless.

4.3.5 Method development

12 lipid markers and their mixtures were used for the SPE method development. These lipids include OM, OA, Ch, ChP, TS, MOG, DP, NOG, NOE, PC, Sph and G. SPE elution solutions were made by mixing them in correct proportions followed by sonication for an hour. To start out the method, 100 µL of each lipid (1 mg/mL) were mixed together, dried under N_2 and re-dissolved in 500 µL hexane. 100 µL of this mixture was loaded into the DSC-Si column so that the total oleamide load onto the SPE tube is 20 µg (total lipid load was 240 µg). The column was completely solvated in hexane before the sample load. After the lipids were adsorbed by the silica phase, 4 mL hexane was used for washing the sample, which was considered as fraction one from DSC-Si column. The lipids were then eluted with 1 mL of each 99:1 hexane: acetic acid, 90:10 hexane: ethyl acetate, 80:20 hexane: ethyl acetate, 70:30 hexane: ethyl acetate, 1.5 mL of 2:1 chloroform: 2-propanol, and finally with 2 mL of methanol. Fraction six was found to contain the amide, monoacyl glycerol, *N*-acylglycine and the *N*-acylethanolamine. This fraction was therefore dried under N_2, re-dissolved in 200 µL of hexane and loaded onto DSC-NH_2 column. The sample was allowed to be adsorbed into the phase and was washed with 2 mL of hexane, which was called fraction one from DSC-NH_2 column. The lipids were then eluted by 2 mL of chloroform, 2 mL of

2% chloroform in 2-propanol, 1 mL of 2% chloroform in 2-propanol, 2 mL of 3% chloroform in 2-propanol and finally with 2 mL of methanol. Fraction three from DSC-NH_2 column was found to contain only amides, which was then dried under N_2 and re-dissolved in 0.25 ng/µL internal standard solution in 2-propanol before injecting into GC/MS. The internal standard used was deuterated heptadecanoamide ($C_{16}D_{33}$-CO-NH_2).

4.3.6 Optimization of amide load

In-order to find out the lowest amount of amide to be loaded and isolated by this method, 9 different oleamide loads were examined. These included 20 µg, 10 µg, 5 µg, 2 µg, 1 µg, 0.5 µg, 0.2 µg, 0.1 µg and 0.02 µg loads. 0.5 µg was found to be the lowest mass loaded and isolated by this method therefore this amount was used for further analysis.

4.3.7 HPTLC method for viewing the lipid contents in each SPE fractions

The fractions from both the columns were applied onto the 10 x 10 cm plates and the plates were developed up to 5.5 cm from the bottom in chloroform: methanol: acetic acid (95:5:1, v/v/v), dried for 5 min with warm air from a hair dryer. The dried plates were re-developed in hexane: diethyl ether: acetone (60:40:5, v/v/v) up to 8 cm and re-dried. The plates were finally developed in hexane: diethyl ether (97:3, v/v) to 9.5 cm. These plates were then dried for 5 min and sprayed with a 0.05% primuline solution (in 80:20, v/v acetone: water) and re-dried. The dry plates were scanned by Kodak image station.

4.3.8 Method validation

4.3.8.1 Lipid extraction

The cells were stored at -80 ºC upon arrival in the laboratory. They were taken out just before the extraction and kept on dry ice until suspending into 4 mL of methanol. 0.1 µg/µL solution of seven amides was made by dissolving 0.001 g of each amide in 10 mL methanol. These amides include lauramide, C12:0; tridecanamide, C13:0; myristamide, C14:0; palmitamide, C16:0; oleamide, C18:1^9; stearamide, C18:0 and eicosanamide, C20:0. 5 µL of this solution was spiked into the methanolic cell suspension so that the amount of each amide spiked into the cell extract was 0.5 µg. Lipid extraction from all the cells were carried out according to White *et al.* [65] and Folch-Pi *et al.* [4] with a little modification. The methanol suspension was sonicated for 15 min at room temperature and centrifuged at 4500 rpm for 10 min. The supernatant was separated from the pellet, dried under a stream of N_2 in a warm water bath at around 35-40 ºC. The pellet was re-extracted with 4 mL of 1: 1: 0.1; (v/v/v) chloroform: methanol: water, sonicated for 10 min, vortexed for 2 min and centrifuged for 10 min as above. Supernatant from this step was added to the dried supernatant from the previous step and re-dried the same way. The total extract was then partitioned into lipid and non-lipid portions by adding 4.8 mL of chloroform: methanol (2:1, v/v) and 800 µL of 0.5 M KCl/ 0.08 M H_3PO_4. This partitioned extract was sonicated for 2 min, vortexed for 2 min and centrifuged for 10 min at 4500 rpm. The lower lipid phase was dried under a stream of N_2 in a warm water bath at around 35-40 ºC. The dried lipid extract was dissolved in 100 µL hexane and loaded into the DSC-Si so that the amount of each amide loaded is 0.5 µg from the spiked cells. Six different

extractions were carried out the same way as described above except no amides were spiked in three extractions (blank).

4.3.8.2 GC/MS analysis of fraction 3 from DSC-NH_2

Fraction three containing amides was dried under N_2 and re-dissolved in 300 µL of 0.25 ng/µL heptadecano-D33-amide solution in 2-propanol. 1 µL of this sample was injected into GC. Quantification of cell amides was done by comparing their peak areas relative to the internal standard to those of the corresponding standard curves.

4.4 Results and Discussion

In order to develop a method for the isolation of primary fatty acid amides from lipid mixture, the SPE method reported by Kaluzny *et al.* [46] for lipid separation was followed to begin with. The method was modified in our own way to isolate amides, which will be discussed in details in this section and has been shown in Figure 4-1 through Figure 4-8.

In an effort to isolate neutral lipids (NL) form polar lipids (PL), the lipids loaded into a DSC-NH_2 column were eluted with $CHCl_3$:2-propanol (2:1), 2% acetic acid in diethyl ether and finally with methanol. TLC of the collected fractions (Figure 4-1) shows that all the neutral lipids in 1st fraction, free fatty acid (FFA) and polar lipids in 3rd fraction with no lipids eluting in 2nd fraction. This result is slightly different from what Kaluzny *et al.* had observed (they found FFA in fraction 2), which is acceptable provided the phase and the solvents were purchased from different vendors. Re-load of the neutral fraction into a separate NH_2 column and their elution with 15% ethyl acetate

in hexane elutes CE and TAG and 2:1 $CHCl_3$: MeOH elutes the rest of the neutral lipids (Figure 4-2).

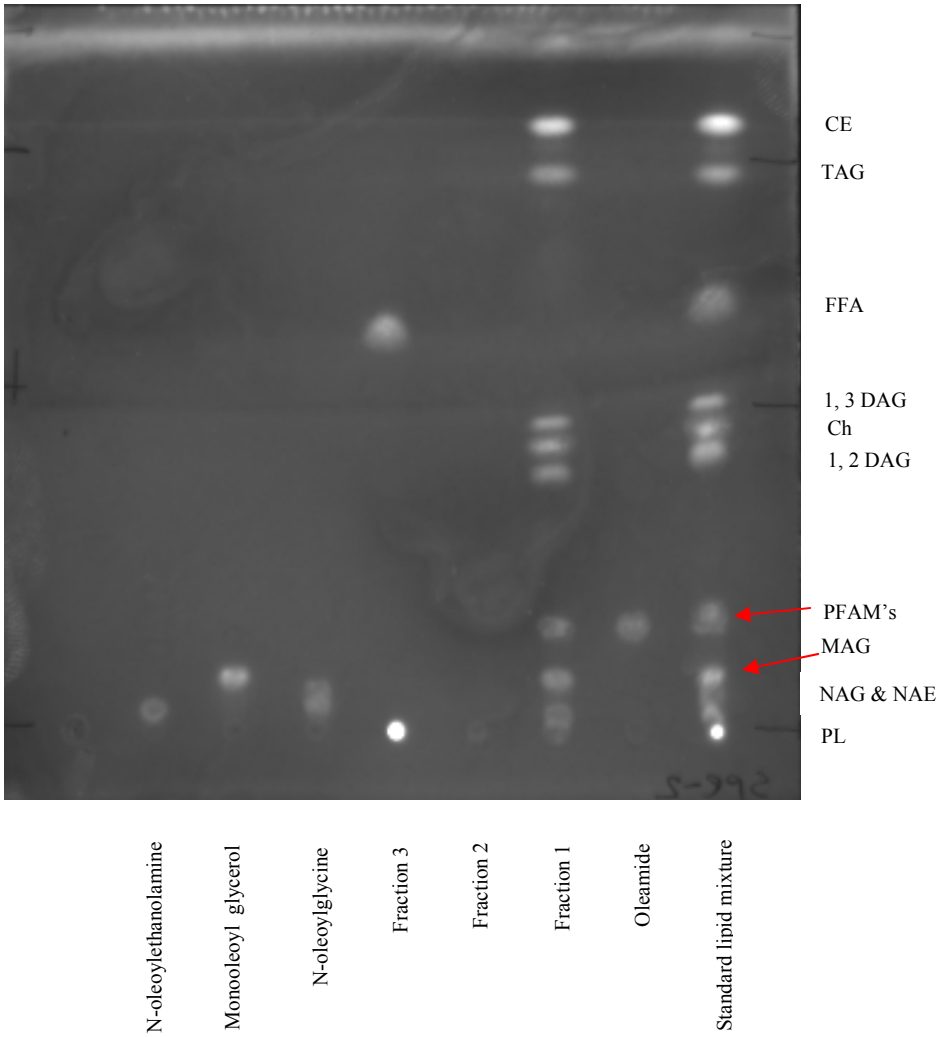


FIGURE 4-1: SPE method development step 1 using a DSC-NH_2 column. Fraction 1: elution by 1 mL of 2:1 $CHCl_3$: 2-propanol; Fraction 2: elution by 1 mL of 2% acetic acid in diethyl ether; Fraction 3: elution by 1 mL of MeOH.

In order to separate amides from the rest of the neutral lipids five separate elution skims were tried with 12% 2-propanol in $CHCl_3$, 3% 2-propanol in $CHCl_3$, 0.5% 2-propanol in $CHCl_3$, 0.2% 2-propanol in $CHCl_3$ and 0.1% 2-propanol in $CHCl_3$ (Figure 4-3) followed by a MeOH wash after each skim. The results show that the first skim eluted all the lipids, second, third, and fourth skims all eluted amides partially. The fifth skim did not elute any amides.

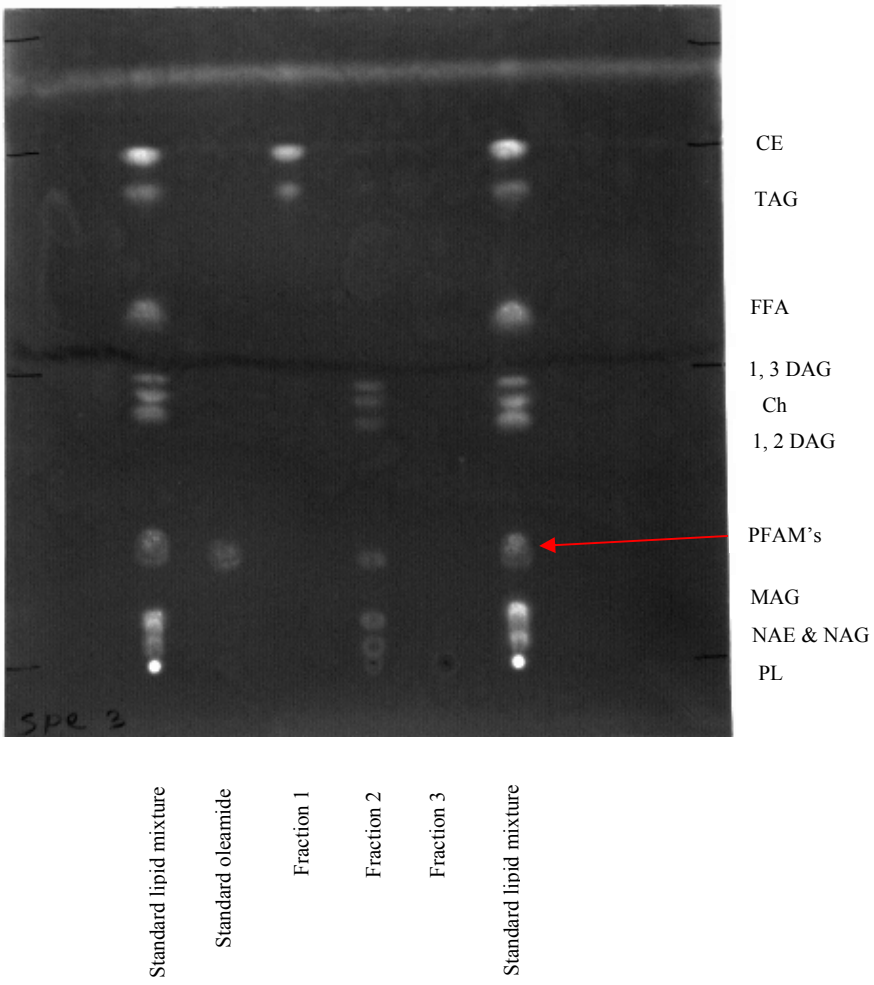
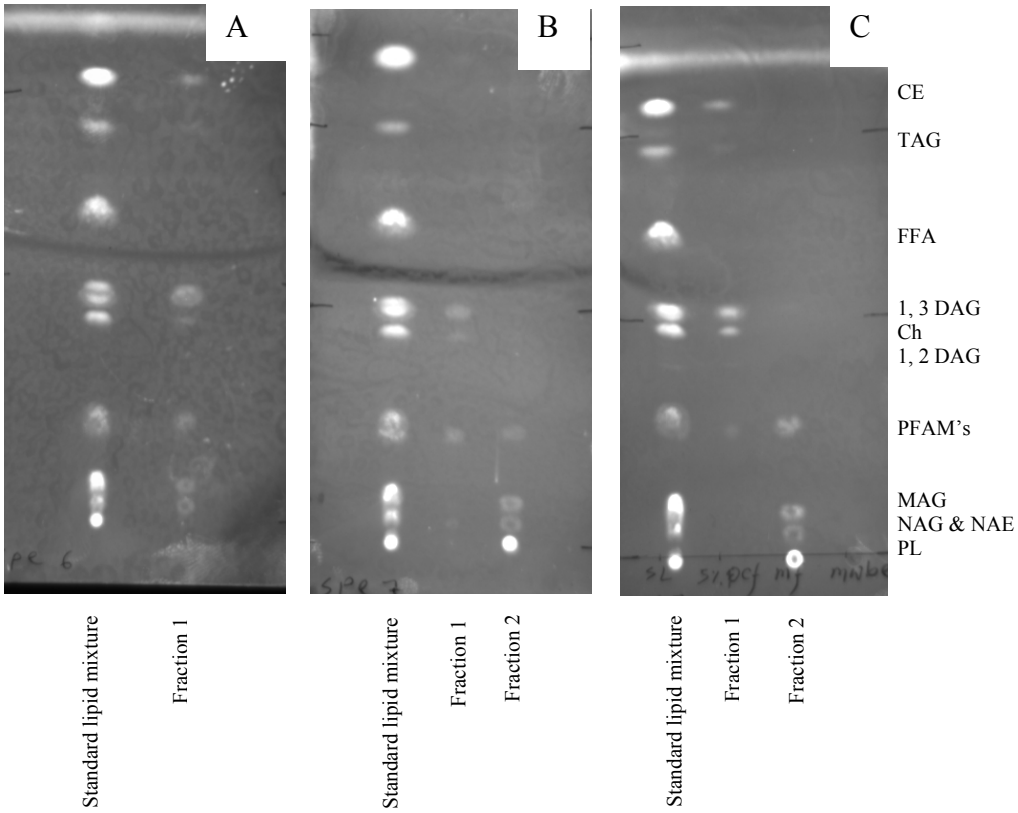


FIGURE 4-2: SPE method development step 2 using a DSC-NH_2 column. Fraction 1: elution by 1 mL of 15% ethyl acetate in hexane; Fraction 2: elution by 1 mL of 2:1 $CHCl_3$: MeOH; Fraction 3: elution by 1 mL of MeOH.

This situation led us to try a different type of phase with which we can separate just the PFAMs, MAG, NOE and NOG out in one fraction and load them into NH_2 column to isolate amides. The use of three different phases to elute NL, PL and FFA is shown in Figure 4-4 which indicates that DSC-Si could be the type of phase we are looking for because it is missing some of the non-polar lipids, which are lost in the $CHCl_3$ wash before elution. Using a DSC-Si column, it was possible to elute all TAG and CE, most FFA and DAG and some Ch by 1 mL of 60:40:5 hexane: diethyl ether: acetone. 1 mL of 95: 5:1 $CHCl_3$: MeOH: acetic acid (HOAc) eluted the rest of the FFA, Ch and some DAG. 2:1 $CHCl_3$: MeOH (1 mL) was found to elute all the amide, MAG, NOE and NOG with a small amount of DAG and Ch. Finally 2 mL MeOH eluted the polar lipids (PL) (see Figure 4-5).



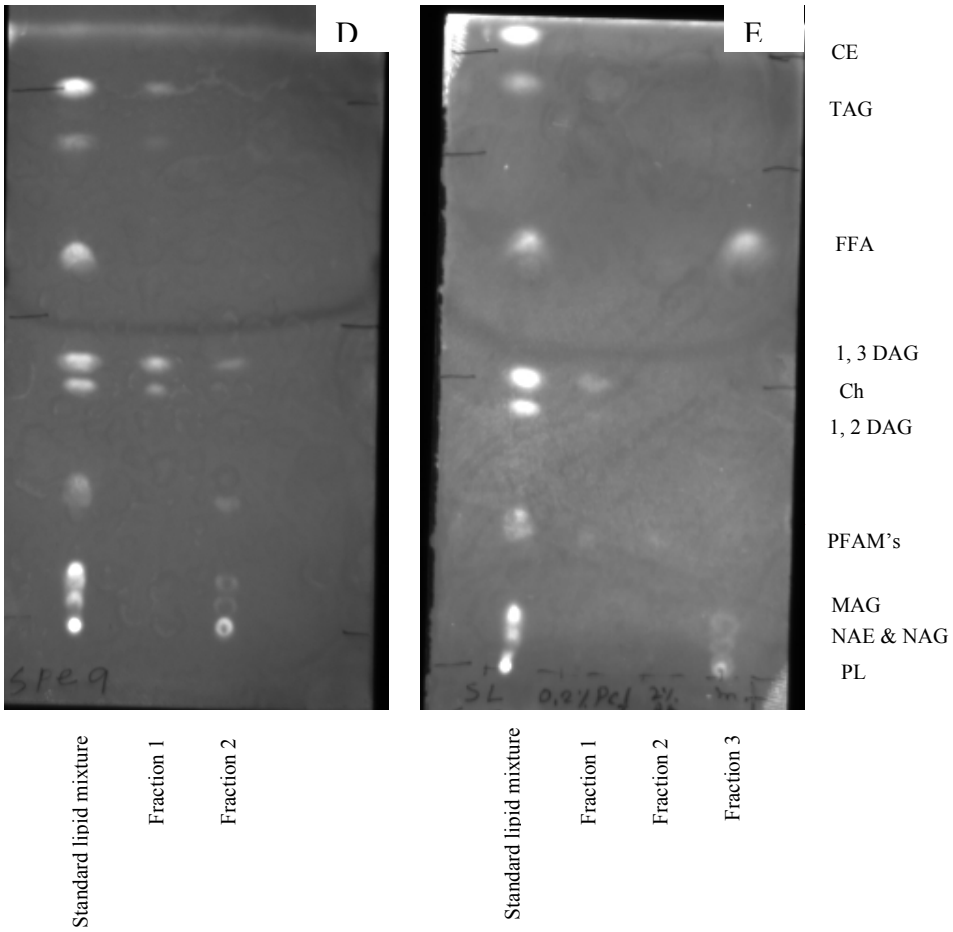


FIGURE 4-3: SPE method development step 3 using DSC-NH_2 columns. A) Fraction 1: elution by 1 mL of 12% 2-Propanol in $CHCl_3$. B) Fraction 1: elution by 1 mL of 3% 2-Propanol in $CHCl_3$; Fraction 2: elution by 1 mL of MeOH. C) Fraction 1: elution by 1 mL of 0.5% 2-Propanol in $CHCl_3$; Fraction 2: elution by 1 mL of MeOH. D) Fraction 1: elution by 1 mL of 0.1% 2-Propanol in $CHCl_3$; Fraction 2: elution by 1 mL of MeOH. E) Fraction 1: elution by 1 mL of 0.2% 2-Propanol in $CHCl_3$; Fraction 2: elution by 1 mL 2% HOAc in diethyl ether; Fraction 3: elution by 1 mL of MeOH.

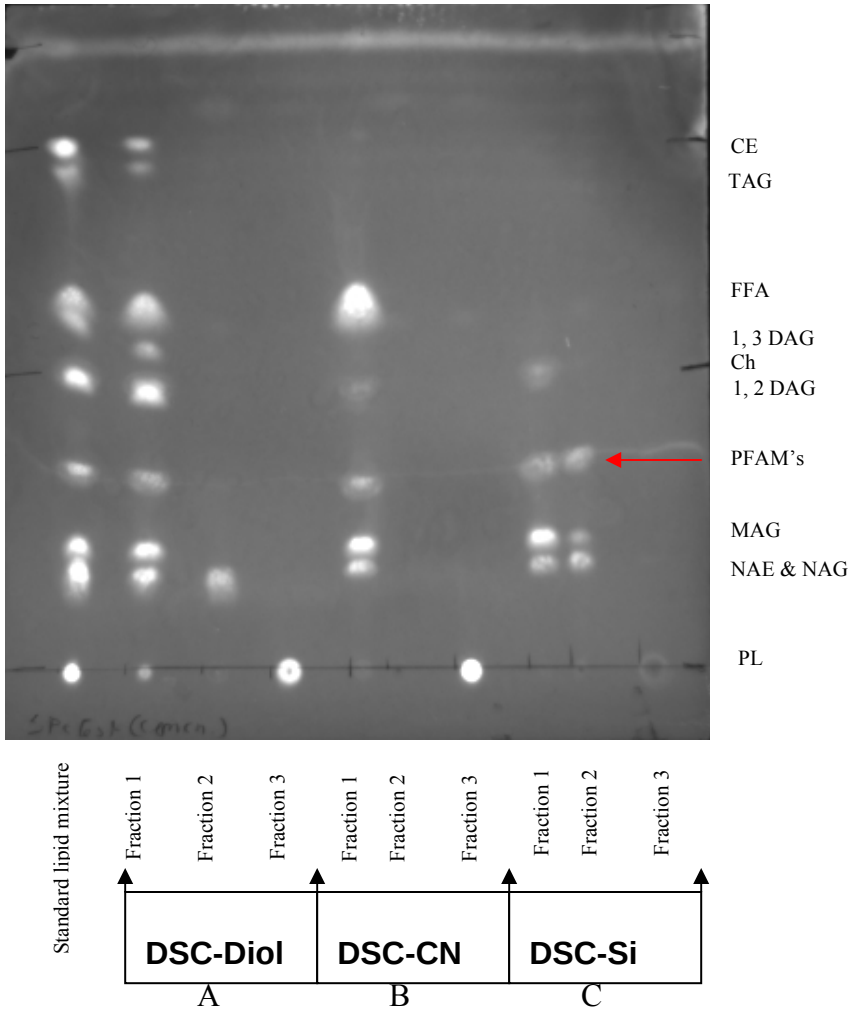


FIGURE 4-4: SPE method development step 4. A) Using DSC-Diol column, B) Using DSC-CN column and C) Using DSC-Si column. Fraction 1: elution by 1 mL of 2:1 $CHCl_3$: 2-propanol; Fraction 2: elution by 1 mL of 2% acetic acid in diethyl ether and Fraction 3: elution by 1 mL of MeOH.

Several attempts in between the 95: 5:1 $CHCl_3$: MeOH: HOAc and the 2:1 $CHCl_3$: MeOH fractions in order to isolate amides in a separate fraction using varying solvent polarity were not very successful. Lipids dissolved in hexane were therefore loaded into a DSC-Si column solvated in hexane and eluted with 1 mL of each hexane, 99:1 hexane: HOAc, 90: 10 hexane: ethyl acetate, 80: 20 hexane: ethyl acetate, 70: 30

hexane: ethyl acetate, 2:1 $CHCl_3$: MeOH and finally, with 1 mL of MeOH. Figure 4-6 showed that hexane wash did not elute any lipids as expected neither did the second elution. The third fraction contained the TAG and CE lipids whereas the next two fractions contained FFA, DAG and Ch.

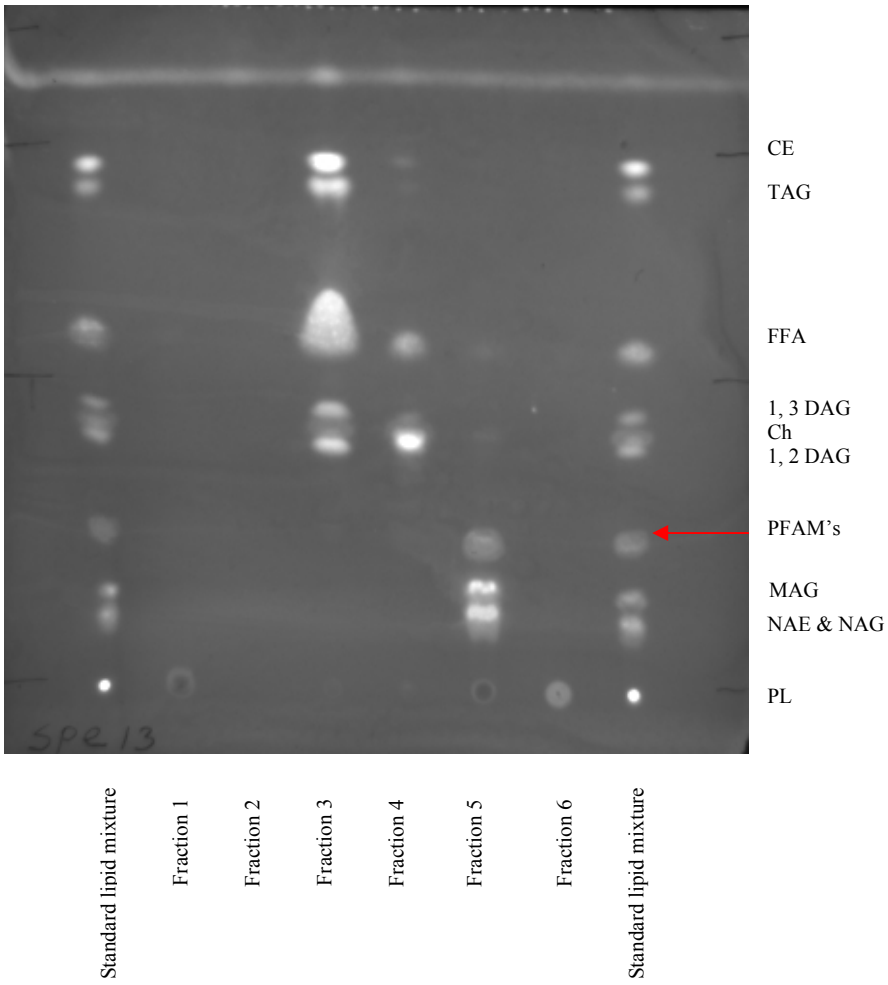


FIGURE 4-5: SPE method development step 5 using DSC-Si column. Fraction 1: MeOH wash of the DSC-Si phase before sample load; Fraction 2: hexane wash after sample load; Fraction 3: elution by 1 mL of 60:40:5 hexane: diethyl ether: acetone; Fraction 4: elution by 1 mL of 95:5:1 $CHCl_3$: MeOH: HOAc; Fraction 5: elution by 1 mL of 2:1 $CHCl_3$: MeOH; Fraction 6: elution by 1 mL of MeOH.

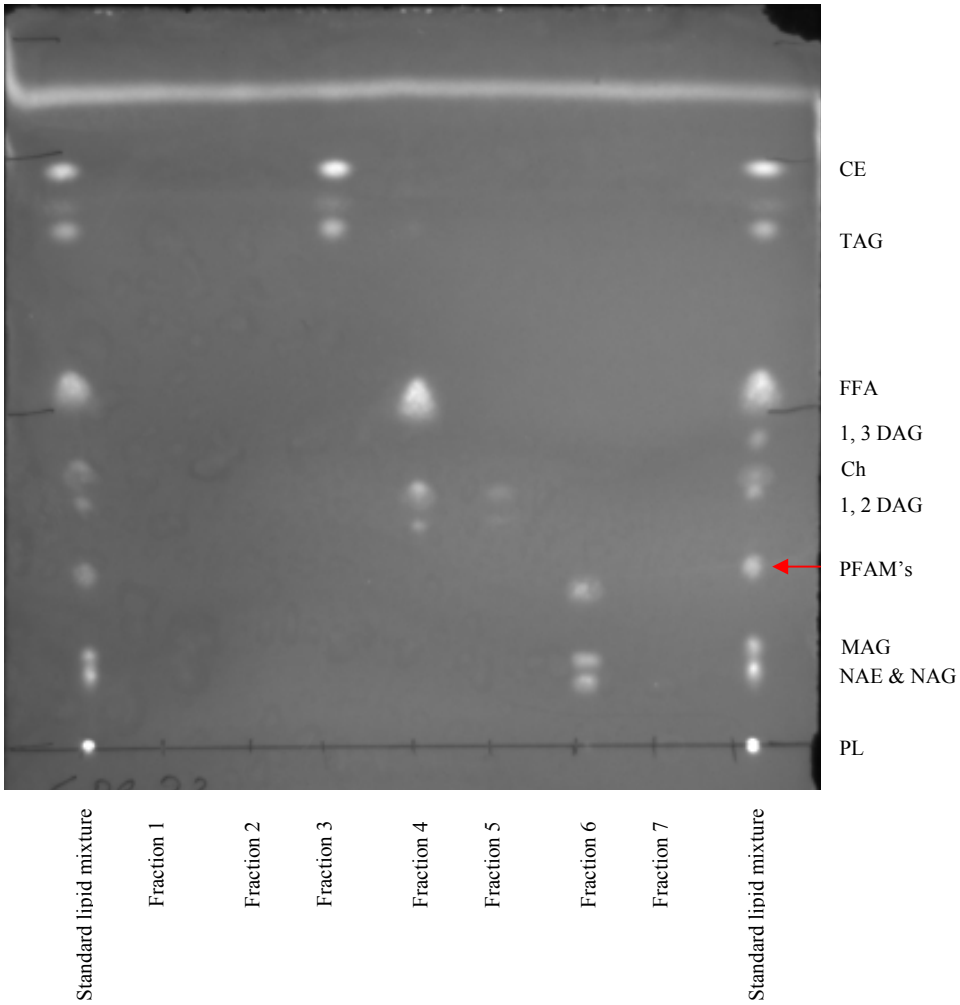


FIGURE 4-6: SPE method development step 6 using DSC-Si column. Fraction 1: 1 mL hexane wash after sample load; Fraction 2: elution by 1 mL of 99:1 hexane: HOAc; Fraction 3: elution by 1 mL of 90:10 hexane: ethyl acetate; Fraction 4: elution by 1 mL of 80:20 hexane: ethyl acetate; Fraction 5: elution by 1 mL of 70:30 hexane: ethyl acetate; Fraction 6: elution by 1 mL of 2:1 $CHCl_3$: MeOH; Fraction 7: elution by 1 mL of MeOH.

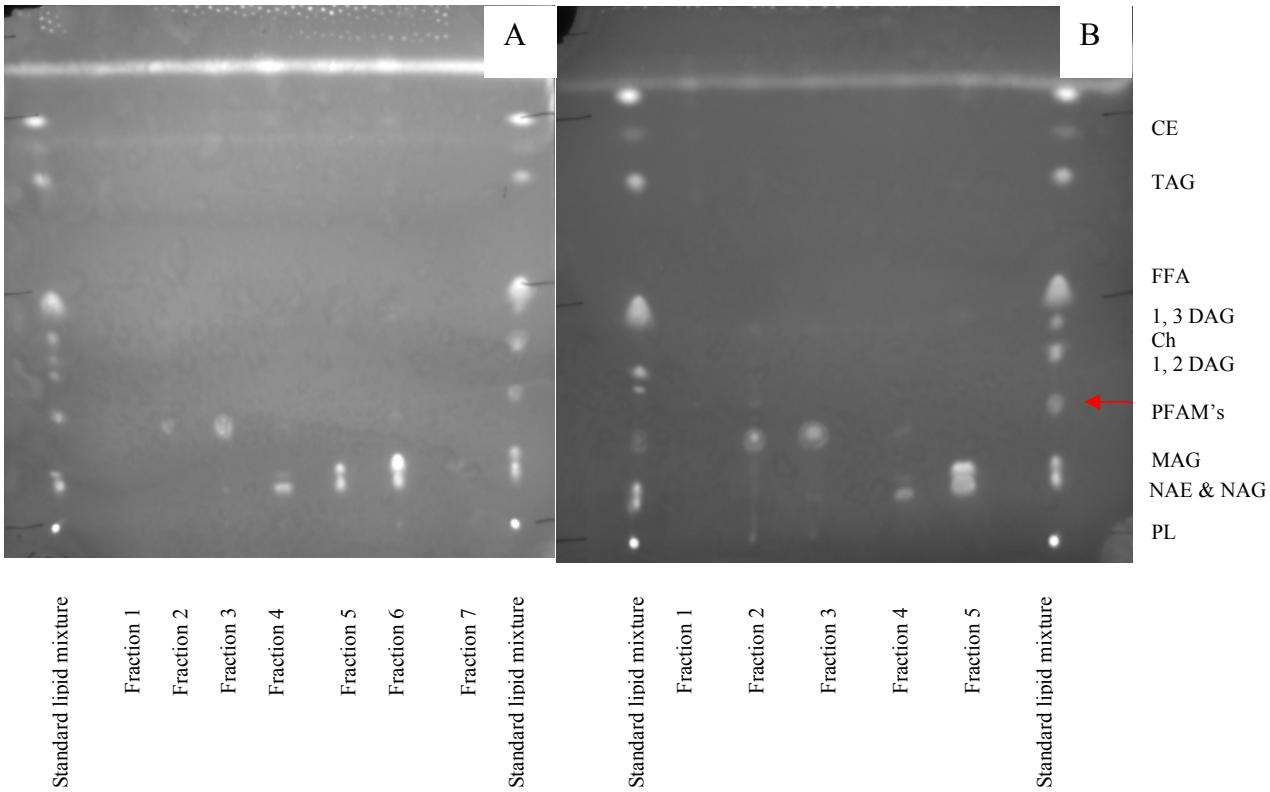


FIGURE 4-7: SPE method development step 7 using DSC-NH_2 columns. A) Fraction 1: 1 mL $CHCl_3$ wash after sample load; Fraction 2: elution by first 1 mL of 2% 2-propanol in $CHCl_3$; Fraction 3: elution by second 1 mL 2% 2-propanol in $CHCl_3$; Fraction 4: elution by third 1 mL 2% 2-propanol in $CHCl_3$; Fraction 5: elution by first 1 mL of 3% 2-propanol in $CHCl_3$; Fraction 6: elution by second 1 mL 3% 2-propanol in $CHCl_3$; Fraction 7: elution by third 1 mL 3% 2-propanol in $CHCl_3$. B) Fraction 1: 1 mL $CHCl_3$ wash after sample load; Fraction 2: elution by first 1 mL of 3% 2-propanol in $CHCl_3$; Fraction 3: elution by second 1 mL 3% 2-propanol in $CHCl_3$; Fraction 4: elution by first 1 mL 4% 2-propanol in $CHCl_3$; Fraction 5: elution by second 1 mL of 4% 2-propanol in $CHCl_3$.

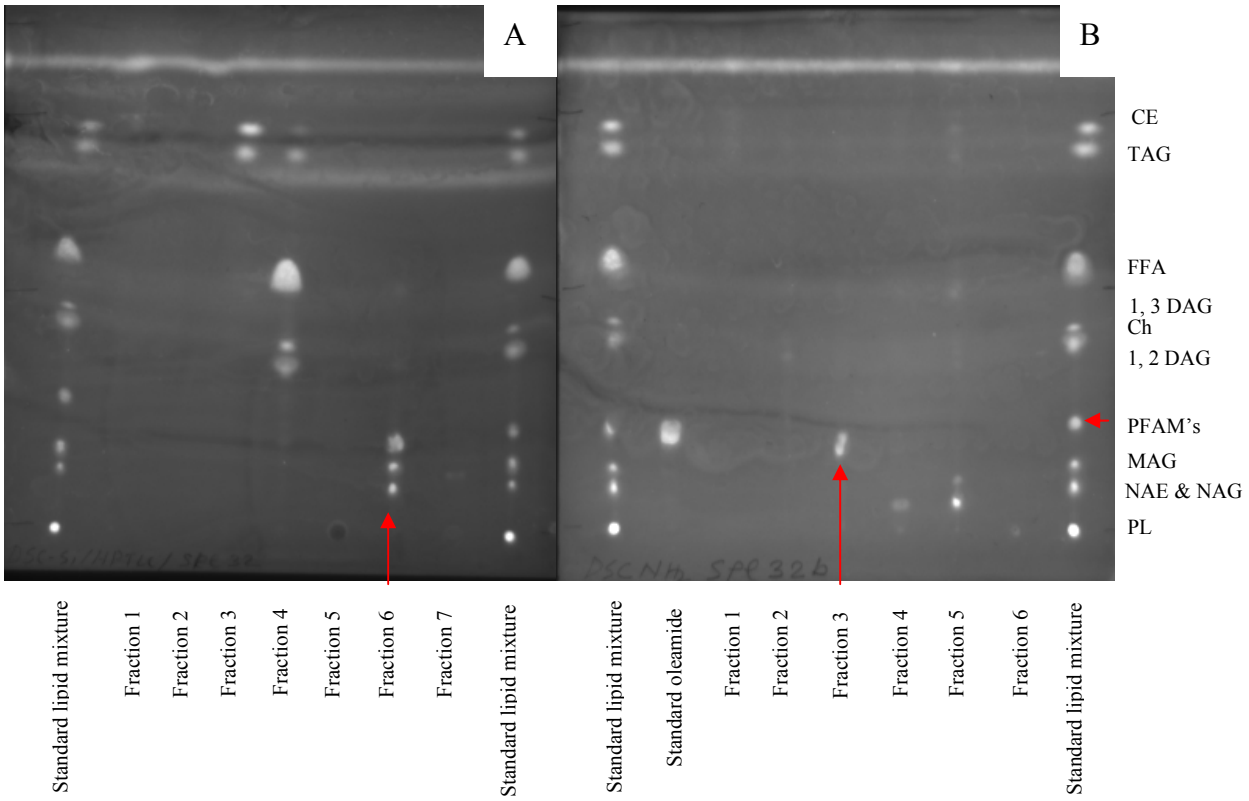


FIGURE 4-8: Optimized SPE method for the isolation of amides by A) DSC-Si and B) DSC-NH_2 columns. A) Fraction 1: 4 mL hexane wash after sample load; Fraction 2: elution by 1 mL of 99:1 hexane: HOAc; Fraction 3: elution by 1 mL of 90:10 hexane: ethyl acetate; Fraction 4: elution by 1 mL of 80:20 hexane: ethyl acetate; Fraction 5: elution by 1 mL of 70:30 hexane: ethyl acetate; Fraction 6: elution by 1.5 mL of 2:1 $CHCl_3$: 2-propanol; Fraction 7: elution by 2 mL of MeOH. B) Fraction 1: 2 mL hexane wash after sample load; Fraction 2: elution by 2 mL $CHCl_3$; Fraction 3: elution by 2 mL of 2% 2-propanol in $CHCl_3$; Fraction 4: elution by 1 mL 2% 2-propanol in $CHCl_3$; Fraction 5: elution by 1 mL 3% 2-propanol in $CHCl_3$; Fraction 6: elution by 2 mL of MeOH.

The sixth fraction contained the desired lipids amide, MAG, NAE and NOG. Because DSC-NH_2 column was already tried for the separation of amide in a different fraction, fraction 6 was dried under N_2, re-dissolved in hexane and loaded into DSC-NH_2 column to carry out further study. This column was therefore eluted with different proportions of 2-propanol in $CHCl_3$. Figure 4-7 shows that the solution that isolated amides from MAG, NOE and NOG was 2 mL of 2% 2-propanol.

Depending on the other tests performed, the final SPE method for the isolation of primary fatty acid amides (Figure 4-8) was taken as the method described in the experimental section. This method was optimized for the lowest mass of amides that could be loaded and isolated successfully from a lipid mixture. The amide loads tried were 20 µg, 10 µg, 5 µg, 2 µg, 1 µg, 0.5 µg, 0.2 µg, 0.1 µg and 0.02 µg while keeping the mass of other lipids constant. Figure 4-9 shows the percent recovery of some of the amide loads, which indicates that the lowest possible mass to be loaded and isolated by this method (500 mg packing in a 3 mL tube) is 0.5 µg. The low percent recovery below this mass was due to the scattering of amides in different fractions, which could be improved using a smaller sorbent mass. This 0.5 µg amount was therefore used for the method validation using $N_{18}TG_2$ cell lipids. The isolated amides from the total lipid extract were separated and detected by GC/MS, providing a nice and clean detection (Figure 4-10 and Figure 4-11). The large peak that comes around 19 min in Figure 4-11 is due to erucamide ($C22:1^{13}$) which was eluted from the polypropelene SPE tubes used for the solid phase packing.

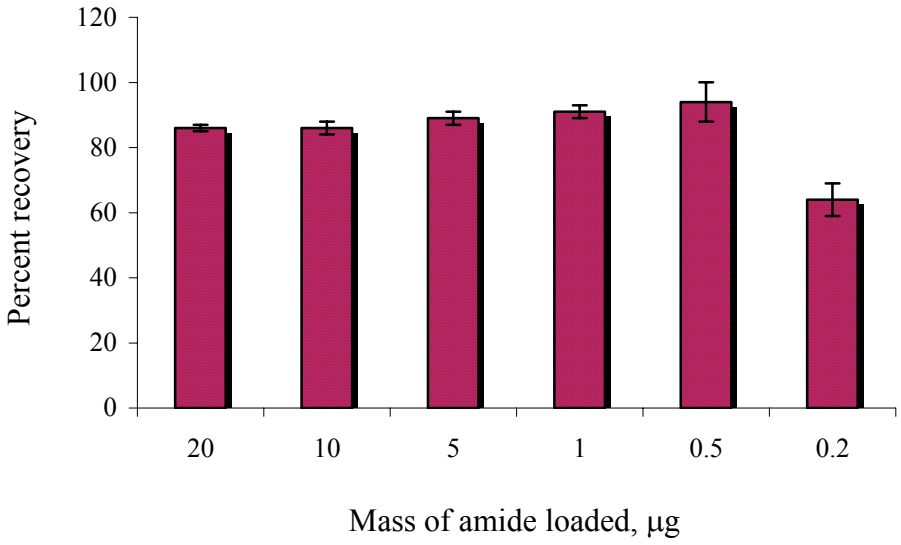


FIGURE 4-9: Percent recoveries of the decreasing mass of amides by SPE. Uncertainities are at 95% confidence limit (n = 9).

As mentioned in Chapter 2 that erucamide, oleamide ($C18:1^9$), palmitamide (C16:0) and stearamide (C18:0) are used as slip additives in plastics. Therefore, in this study, a parallel blank was run for each sample. Trace amounts of eicosanoamide (C20:0), stearamide and oleamide were also detected in blank. The quantitation of the amides was carried out by subtracting blank data from sample data. The percent recoveries of the seven different amides are displayed as bar graph in Figure 4-12. The results indicate that the shorter chain amides are more difficult to recover and/or quantify in trace level. The reason might be the low extractability of the relatively polar short chain amides by the aminopropyl phase. The detection limits for the amides show (Table 4-I) that the shorter chain amides have higher detection limit than that of the

longer chain ones. The instrument response (Figure 4-13) was found linear over a range of around 48 fmol (~5 pg) to 6000 fmol (2500 pg) of amides.

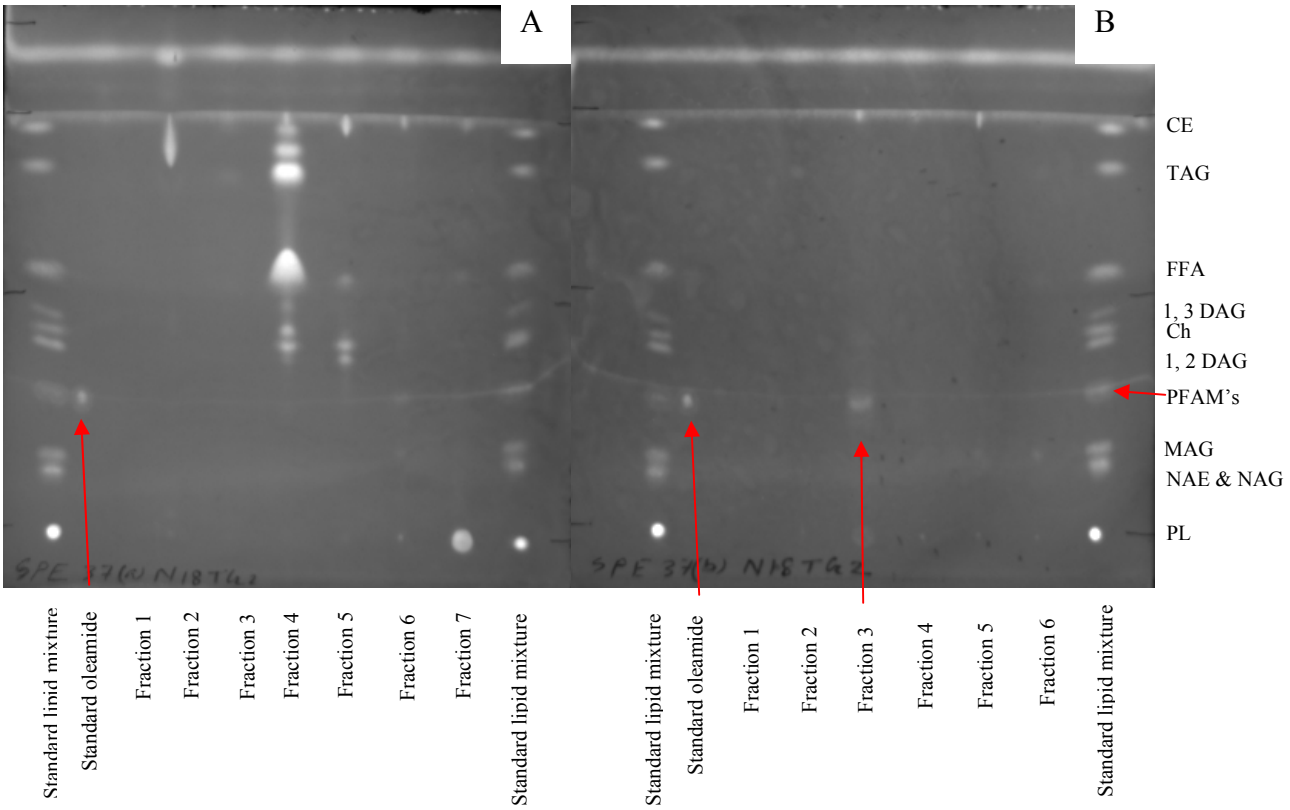


FIGURE 4-10: SPE fractions of the lipids extracted from amide spiked $N_{18}TG_2$ cells A) DSC-Si column and B) DSC-NH_2 column.

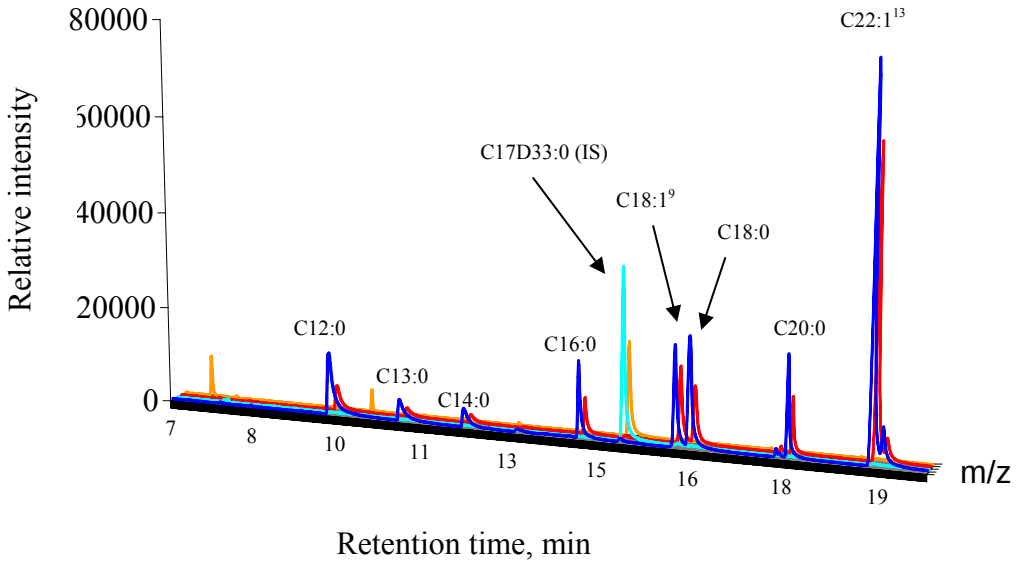


FIGURE 4-11: Separation of PFAM's isolated from amide spiked $N_{18}TG_2$ cells. The relative intensities at m/z **59**, **72**, **62** and **76** are plotted against retention time.

TABLE 4-I: Detection limits (DL) of different amides in single ion monitoring mode (SIM).

Amides	DL, pg
C12:0	30
C13:0	30
C14:0	30
C16:0	10
C17:0	10
$C18:1^9$	5
C18:0	10
C20:0	10
C22:0	10

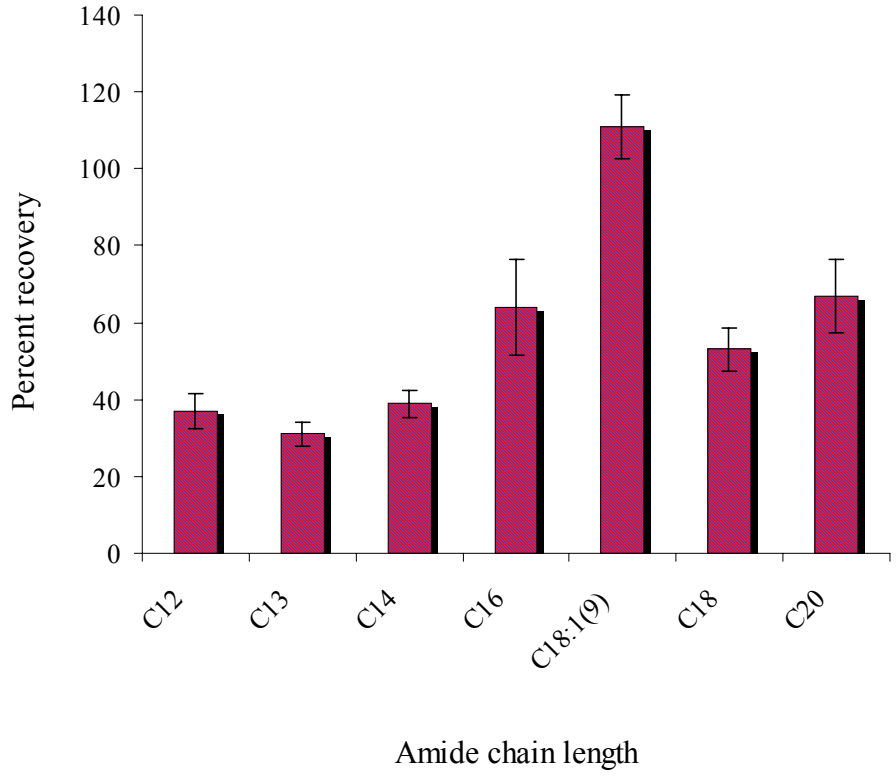


FIGURE 4-12: Percent recoveries of PFAM's from amide spiked $N_{18}TG_2$ cells. Uncertainities are at 95% confidence level (n=9).

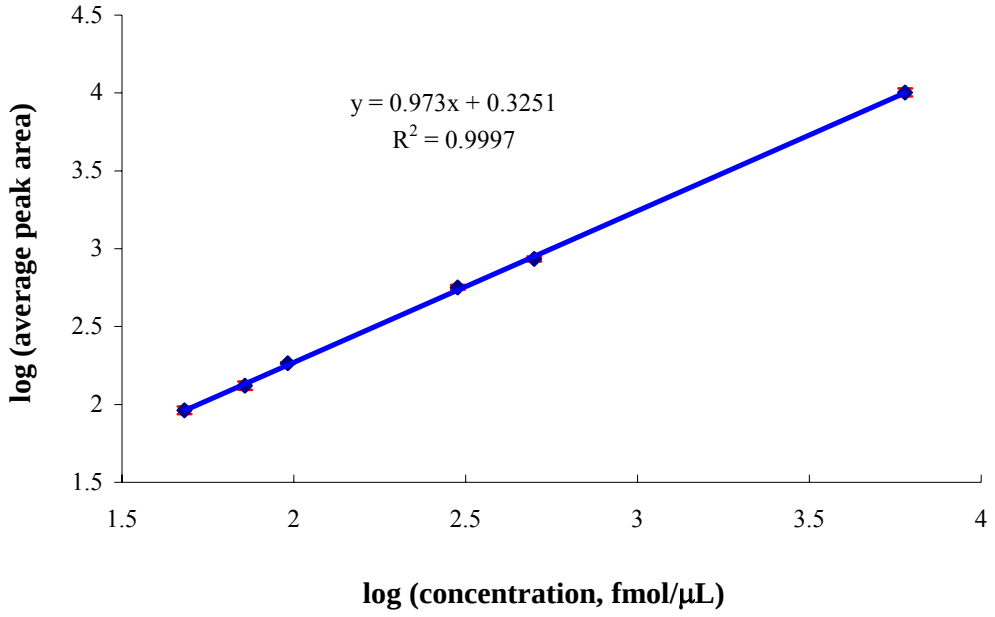


FIGURE 4-13: Mass spectrometer response in terms of the concentration of oleamide (48 femto mol/µL to 6000 femto mol/µL). Uncertainties are at 95% confidence level (n=3).

4.5 Conclusions

This chapter included the discussion of the isolation of the primary fatty acid amides by solid-phase extraction and their quantification by GC/MS. As mentioned earlier, SPE is a simple sample clean up/preparation procedure useful in analyzing compounds, which are not abundant, have higher detection limits and/or are difficult to be quantified in presence of other interfering compounds. The method was validated using the lipids extracted from $N_{18}TG_2$ cells and percent recoveries of seven different amides were successfully calculated. Application of this method for the isolation of amides from

mammalian tissues will be discussed in the following chapter with emphasis on quantification. Comparison of the results obtained from this method to that of the HPTLC/GC/MS method can provide strong milestones for these methods.

4.6 Acknowledgements

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Chapter 5

Distribution of primary fatty acid amides in mammalian tissues

5.1 Abstract

Primary fatty acid amides (PFAM's) were isolated and quantified from the lipid extracts of frozen and powdered rabbit tissues. These biologically active hormones are found in mammals in a very low concentration and their detection limits are relatively higher. High performance thin layer chromatography (HPTLC) and solid-phase extraction (SPE) were used for their isolation prior to the analysis by GC/MS. HPTLC or SPE provided the complete separation of amides from total lipid extract and allowed their concentration for interference free and quantitative detection. These types of sample preparation techniques can also be useful for analyzing PFAM's in conjunction with liquid chromatography/mass spectrometry (LC/MS), matrix-assisted laser desorption ionization (MALDI)/MS, and/or capillary electrophoresis (CE).

5.2 Introduction

Fatty acid amides comprise one of the important classes of bioactive lipids found in mammals. *N*-acylethanolamines (NAE) and primary fatty acid amides (PFAM's) are two important families of this class [1]. Members of both of these families consisting of long chain saturated and unsaturated fatty acids are considered as bioregulators due to their hormone like activities [2]. They became recognized as a signaling class of lipid, when anandamide (*N*-arachidonylethanolamine) was identified in 1992 as a natural product that binds to the cannabinoid receptors in the brain [3]. It was found to mimic all the activities of delta-9-tetrahydrocannabinol (Δ-9-THC) and was thought to be a potent neuromodulator besides its other hormonal activities. Within three years researchers discovered that oleamide, a primary fatty acid amide, is synthesized in the cerebrospinal fluid of sleep deprived cats [4]. Since then the PFAM's were studied widely in various biochemical and pathological ways. They were first identified in human luteal phase plasma [5] even though neither their function nor their bio-synthetic pathway was recognized at them time.

Various reported hormonal-activities of oleamide and the controversy about its biosynthetic pathways were discussed in Section 1.5 of Chapter 1. Quantification of PFAM's by instrumental analysis is problematic due to their presence in biological samples in low concentration as well as due to the interference from other lipids present in lipid extracts of the sample of interest. The peak suppression during trace analysis of PFAM's due to the tailing of $-NH_2$ group gives rise to higher detection limits, which seems to be more prominent in the presence of other non-volatile lipids. Because of this

reason, PFAM's are usually derivatized to stable, amide deactivated compounds for higher sensitivity before GC/MS analysis. These problems can be solved by isolating the PFAM's from interfering lipids followed by diluting the isolate to a preferred concentration. Therefore it is possible to analyze the PFAM's quantitatively from biological samples of interest by the instrument of choice without any interaction from other lipid classes.

Sample preparation by HPTLC and SPE for the study of lipids was discussed in Chapters 3 and 4. Analysis of brain and plasma lipids after clean up by HPTLC or SPE are the long known techniques used by numerous researchers. Mascala *et al.* [6] have separated neutral and acidic lipids from rat and human brain using a DEAE-Sephadex column and then further separated into individual lipid classes by applying them into separate HPTLC plates. They used two solvent systems for the development of the plates. The quantification of the lipid classes was obtained using a scanning densitometer equipped with a Shimadzu CR1A data processor. They reported that the use of an internal standard minimized the variation from plate to plate. The use of cupric acetate for charring was found to be more sensitive than the conventional sulfuric acid-dichromate reagent. Entezami *et al.* [7] on the other hand reported similar analysis of brain lipids by single solvent system HPTLC and found that cupric sulfate charring was more sensitive than cupric acetate charring.

Separation of cholesterol, *N*-acyl glycerol and *N*-butyl palmitamide was achieved by Bilyk *et al.* [8] on a TLC plate with 2 solvent systems and aqueous sulfuric acid charring. They described TLC as a rapid means of identifying the components of fatty mixtures with highly reproducible separation but neither any quantification data

were published, nor the percent recovery results were mentioned. Alvarez *et al.* [9] reported the quantitation of amniotic fluid lipids by HPTLC and reflectance spectrodensitometry with percent recoveries ranging from 82-95%. The detector response was found to be linear over a range of 20 ng/mL (detection limit) to 2 µg/mL. TLC was also employed for the separation of the polar lipids from rabbit tissues by Baldoni *et al.* [10], but the method was used for only qualitative study. Dreyfus *et al.* [11] reported the quantitative analysis of lipid classes extracted from pig and rat tissues by HPTLC and found the percent recoveries around 90-97% for different lipid classes. It is clear from literature review that HPTLC/TLC is an ideal technique for quick profiling of lipid classes present in a sample of interest. With proper developing solvent, it is possible to isolate the target lipid class in higher recoveries from other classes. The isolated lipid can be brought to any desired concentration for further instrumental analysis.

SPE is the most recently used technique for the isolation of a specific lipid class from the crude lipid extract. Two different SPE phases usually employed by researchers, namely, un-bonded silica phases and aminopropyl bonded silica. So far the latter phase has become more popular in lipid analysis due to its comparatively lower polarity than silica and hence less capability of retaining polar lipids. Kaluzny *et al.* [12] had first reported the use of such column for the separation of seven lipid classes from bovine adipose tissue extract with percent recoveries ranging from around 96-101%. They reported that about 10 mg of lipid can be loaded on a 500 mg column. According to literature both types of columns were successfully used in lipid analysis by numerous authors [13-17].

A very few studies were actually done for the isolation of PFAM's from crude lipid extract by HPTLC or SPE. In 1994, before oleamide was identified as a sleep inducing agent, Kaneshiro *et al.* [18] reported the isolation of PFAM's from *Bacillus Megaterium* cell extracts using silica column chromatography (similar to SPE) followed by HPLC and GC analysis for their separation and identification. TLC was used as a primary means of identification of the presence of amides in those cells by comparing with the standard amides. Cravat *et al.* [4] in 1995 isolated a compound from the cerebrospinal fluid (CSF) of sleep-deprived cat and identified it as *cis*-9-octadecenoamide (oleamide), which fueled the later PFAM studies. Bisogno *et al.* [19, 20] had reported the existence of both oleamide and anandamide in $N_{18}TG_2$ mouce neuroblastoma cells, human breast cancer cells and rat adrenal pheochromocytoma cells. They employed a series of sample preparation steps such as TLC, SPE and column chromatography for quantitative determination of these amides. Hanuš *et al.* [21] had also determined the presence of oleamide in rat plasma and CSF quantitatively by GC/MS. They reported the difficulties of analyzing oleamide by electrospray MS without prior chromatography due to the presence many compounds in body fluids. In their case oleamide was analyzed by GC/MS after derivatization in order to increase the peak sensitivity and therefore to lower the detection limit. Oleamide was also identified to be produced in a soil microorganism by Ho Jeong *et al.* [22]. They successfully used preparative TLC before GC/MS in order to obtain oleamide in pure form allowing contaminant free analysis. These types of studies indicate that it is crucial to isolate the target lipid class from other lipid classes present in the complex lipid matrix for interference free analysis [23].

Together all these data suggest that sample preparation before instrumental separation and detection is the key to obtain better and reliable quantitation. It is necessary to point out again that sample clean up such as by HPTLC and/or by SPE mentioned above before their analysis can provide concentration, higher sensitivity as well as the lower limit of detection. This type of interference free analysis results not only in lower peak suppression lowering the detection limit for PFAM's but also provides longer detector lifetime and save money on consumables such as injection liners and septa. The addition of internal standards (IS) to the sample before and after isolation is also crucial for quantitative instrumental analysis. In this study, we report the isolation of PFAM's from rabbit tissues using both HPTLC and SPE followed by quantitative analysis by GC/MS [24]. Rabbit brain and whole heart tissues are known to express PAM, the enzyme for the last step of PFAM biosynthesis (see Chapter 1 for more information). Because heart atrium but not ventricle expresses PAM, atrium removed heart were used in this study as control. The rabbit tissues were chosen for the application of HPTLC and SPE sample preparation methods because of their low cost and commercial availability at the time of this study.

5.3 Experimental

5.3.1 Reagents

ACS reagent grade hexanes, ethyl acetate, methanol, 2-propanol and glacial acetic acid were purchased from Fisher Scientific (Fair Lawn, NJ); acetone, palmitic acid and stearic acid were purchased from Acros Organics (Morris plains, NJ); chloroform and

diethyl ether, heptadecanoic acid and eicosanoic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI); heptadecaoic-D33-acid was purchased from CDN Isotopes (Quebec, Canada). Palmitamide, stearamide, heptadecanoamide, deuterated heptadecanoamide and eicosanoamide were synthesized from the corresponding acids in our laboratory modifying a published protocol [25, also see Chapter 2]. Oleamide (OM) and erucamide were purchased from Sigma (St. Louis, MO).

5.3.2 Tissues

Rabbit whole heart (atrium removed), rabbit whole brain (stripped) and rabbit brain acetone powder were purchased from Pel-Freez Biologicals (Rogers, AR).

5.3.3 HPTLC plates

High performance thin layer chromatography plates (HPTLC) from Analtech (Newark, DE) were used for viewing the lipid contents in different SPE fractions. These plates were of 10 x 10 cm, with organic binder and no fluorescence indicator incorporated. Primuline dye (CI 49000; direct yellow 59) used for spraying the HPTLC plates was obtained from Aldrich Chemical Co. (Milwaukee, WI).

5.3.4 SPE phases

Discovery DSC-Si and Discovery DSC-NH_2 SPE phases were purchased from Supelco (Bellefonte, PA). Irregularly shaped and acid washed base silica possessed following properties: 50 μm particle size, 70 Å pore diameter, 480 m^2/g specific surface area and 0.9 cm^3/g pore volume. DSC-Si is the base silica phase which is basically used as a normal phase adsorbent whereas DSC-NH_2 is the aminopropyl bonded silica which can

be used as either normal phase or the ion-exchange adsorbent. SPE tubes (empty) of 3 mL volumes with polyethylene frits were also purchased from Supelco. 500 mg of the phases were packed into the tubes between two frits applying hand pressure in order to carry out SPE.

5.3.5 Instruments

A Kodak digital Science 440 image station equipped with scientific imaging software (SIS) was used for scanning the HPTLC plates. An Agilent Technologies Network GC/MS system (6890 GC with 5973 mass selective detector and 7683 series injector) was used for GC/MS analysis. The column used was an HP-5MS (0.25 mm internal diameter, 0.25 μm film thickness, 30 m long, Agilent Technologies Inc., Palo Alto, CA) able to analyze fatty acid amides without prior derivatization. The GC/MS method used for analysis is as follows: Starting temperature was 55 ºC, ramped to 150 ºC at 40 ºC per min, held at 150 ºC for 3.62 min, ramped to 275 ºC at 10 ºC per min and finally held at 275 ºC for 2 min. Electron impact ionization (EI) at 70 eV was used and the temperatures of the injection port and the transfer line were 250 ºC and 280 ºC, respectively. Mass range was kept from 40 to 400 m/z for total ion monitoring (TIM) and selected masses were 59, 72, 62 and 76 for single ion monitoring (SIM). Injection volume was 1 μL splitless.

5.3.6 Lipid extraction from rabbit brain and heart tissues

The samples were stored at -80 ºC upon arrival. Right before lipid extraction, samples were taken out of the freezer and placed at dry ice temperature. They were then cut into pieces and weighed before extracting lipids. The lipids were extracted from the tissues

(26, 27) as follows: The weighed tissues/acetone powder were suspended into 20 times of its volume of chloroform: methanol (2:1; v/v) and completely homogenized using a glass-to-glass homogenizer (Kontes Glass Co., Vineland, NJ). The tissue homogenate was then sonicated for 10 min at 1500 g. Before sonication the specific gravity of the homogenate was lowered using 0.2 times of its volume (20%) of methanol. The supernatant was separated from the pellets and chloroform was added to the supernatant in order to adjust the proportion of chloroform: methanol to 2:1 (for example if the total volume of the homogenate was 20 mL, 4 mL methanol was added before sonication and 8 mL of chloroform was added to the supernatant after sonication). To the total amount of supernatant, 0.2 times of its volume of aqueous 0.88% KCl was added (for example if the total volume of the supernatant was 20 mL, 4 mL of aqueous 0.88% KCl was added). The sample was then vortexed for 2 min and allowed to sit for separation into two phases. The upper phase mostly contains proteins and non-lipid contaminants and the lower phase contains the lipid extract. The upper phase was discarded and the lower lipid phase was completely dried under a stream of N_2. The dried lipid extract was dissolved in 4 mL hexane to be loaded onto either HPTLC plates or into the SPE columns. Three separate extractions and three blanks were carried out for this study. An internal standard, heptadecanoamide (C17), was spiked into the tissue suspension as well as into the blanks before homogenization in order to calculate percent recovery. The glass-to-glass homogenizer was silanized using 10% TMS in toluene before use in order to minimize the amide adsorption on the silica surface.

5.3.7 Quantitation of amides from rabbit brain and heart tissues by HPTLC

5.3.7.1 Isolation of amides from TLC plates

30 µL of extracted lipids were applied onto HPTLC plates and the plates were developed as follows: firstly up to 5.5 cm from the bottom in chloroform: methanol: acetic acid (95:5:1, v/v/v), dried for 5 min with warm air from a hair dryer; secondly, the dried plates were developed in hexane: diethyl ether: acetone (60:40:5, v/v/v) up to 8 cm, re-dried and finally, the plates were developed in hexane: diethyl ether (97:3, v/v) to 9.5 cm. Two separate plates were developed at the same time, one of which was sprayed with primuline for visualization. Amide spots adsorbed in silica were scraped off the plate not sprayed with primuline by comparing to the spot position on the sprayed one.

5.3.7.2 Analysis of scraped amides by GC/MS

The scraped silica containing amides was suspended in isopropanol containing the deuterated internal standard, sonicated for 10 min for the transfer of amide to isopropanol from silica. The samples were filtered through 0.2 µm filter paper and 1 µL of the sample was injected into GC/MS.

5.3.8 Quantitation of amides in rabbit brain and heart tissues by SPE

5.3.8.1 Isolation of amides from lipid extract

SPE elution solutions were made by mixing them in correct proportions followed by sonication for an hour. The DSC-Si column was completely solvated in hexane before the sample load. After the lipids were loaded, 4 mL hexane was used for washing the sample, which was considered as fraction one from DSC-Si column. The loads for

brain, heart, and brain acetone powder were 50 μL, 100 μL and 200 μL, respectively. The lipids were then eluted with 1 mL of each 99:1 hexane: acetic acid, 90:10 hexane: ethyl acetate, 80:20 hexane: ethyl acetate, and 70:30 hexane: ethyl acetate, 1.5 mL of 2:1 chloroform: methanol, and finally with 2 mL of methanol. Fraction six was found to contain the amides, monoacyl glycines, *N*-acylglycines and the *N*-acylethanolamines. This fraction was therefore dried under N_2, re-dissolved in 200 μL of hexane and loaded onto DSC-NH_2 column. The sample was adsorbed onto the phase and then washed with 2 mL of hexane, which was called fraction one from DSC-NH_2 column. The lipids were then eluted by 2 mL of chloroform, 2 mL of 2% chloroform in 2-propanol, 1 mL of 2% chloroform in 2-propanol, 2 mL of 3% chloroform in 2-propanol and finally with 2 mL of methanol. Fraction three from DSC-NH_2 column was found to contain only amides.

5.3.8.2 GC/MS analysis of fraction 3 from DSC-NH_2

Fraction three containing amides was dried under N_2 and re-dissolved in 300 μL of 0.25 ng/μL heptadecano-d33-amide solution in 2-propanol. 1 μL of this sample was injected into GC. Quantification of amides was done by comparing their peak areas relative to the internal standard to those of the corresponding standard curves.

5.3.9 HPTLC method for viewing the lipid contents in each SPE fraction

The fractions from both the columns were applied onto the 10 x 10 cm plates and the plates were developed up to 5.5 cm from the bottom in chloroform: methanol: acetic acid (95:5:1, v/v/v), dried for 5 min with warm air from a hair dryer. The dried plates were re-developed in hexane: diethyl ether: acetone (60:40:5, v/v/v) up to 8 cm and re-

dried. The plates were finally developed in hexane: diethyl ether (97:3, v/v) to 9.5 cm. These plates were then dried for 5 min and sprayed with a 0.05 % primuline solution (in 80:20, v/v acetone: water) and re-dried. The dry plates were then scanned by Kodak scientific imaging system.

5.4 Results and Discussion

The total lipid profiling of the rabbit tissues shows the separation of 9 different lipid classes with higher amount of phospholipids and cholesterol present in brain tissues whereas higher amount of triacyl glycerol and fatty acids present in heart tissues (Figure 5-1 and Figure 5-2). *N*-acylglycine (NAG) and *N*-acylethanolamine (NAE), monoacyl glycerol (MAG), primary fatty acid amides (PFAM's), diacyl glycerol (DAG), free fatty acids (FA), triacyl glycerol and cholesteryl esters (CE) were the other classes recognized by comparing to the standard lipids. PFAM's were detectable on a TLC plate (Figure 5-1) upon overloading the plate whereas optimum loading provided nice separation between the classes (Figure 5-2) with PFAM's beyond the detection limit by TLC.

After scraping off amide spots and analysis by GC/MS, three different amides were identified, which as shown in Figure 5-3 and Figure 5-4 includes palmitamide, stearamide, and oleamide. The amides were identified by comparing their retention times and mass spectral patterns with those of the standard amides. The other amides observed in these figures are heptadecanoamide and deuterated heptadecanoamide used as internal standards added to the samples before and after extraction, respectively. The

presence of erucamide was also observed due to their elution from polypropylene vials, which was confirmed by running blanks under the same conditions as the samples.

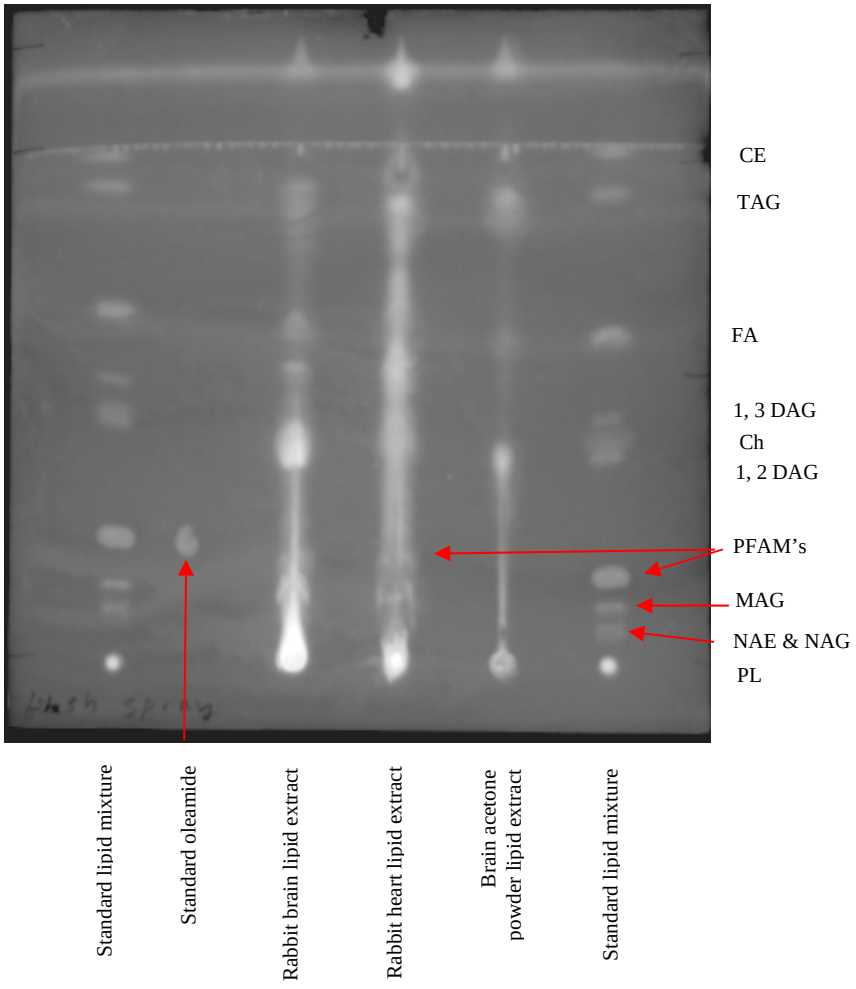

FIGURE 5-1: Total lipid profiling of rabbit tissues (overloading the HPTLC plate).

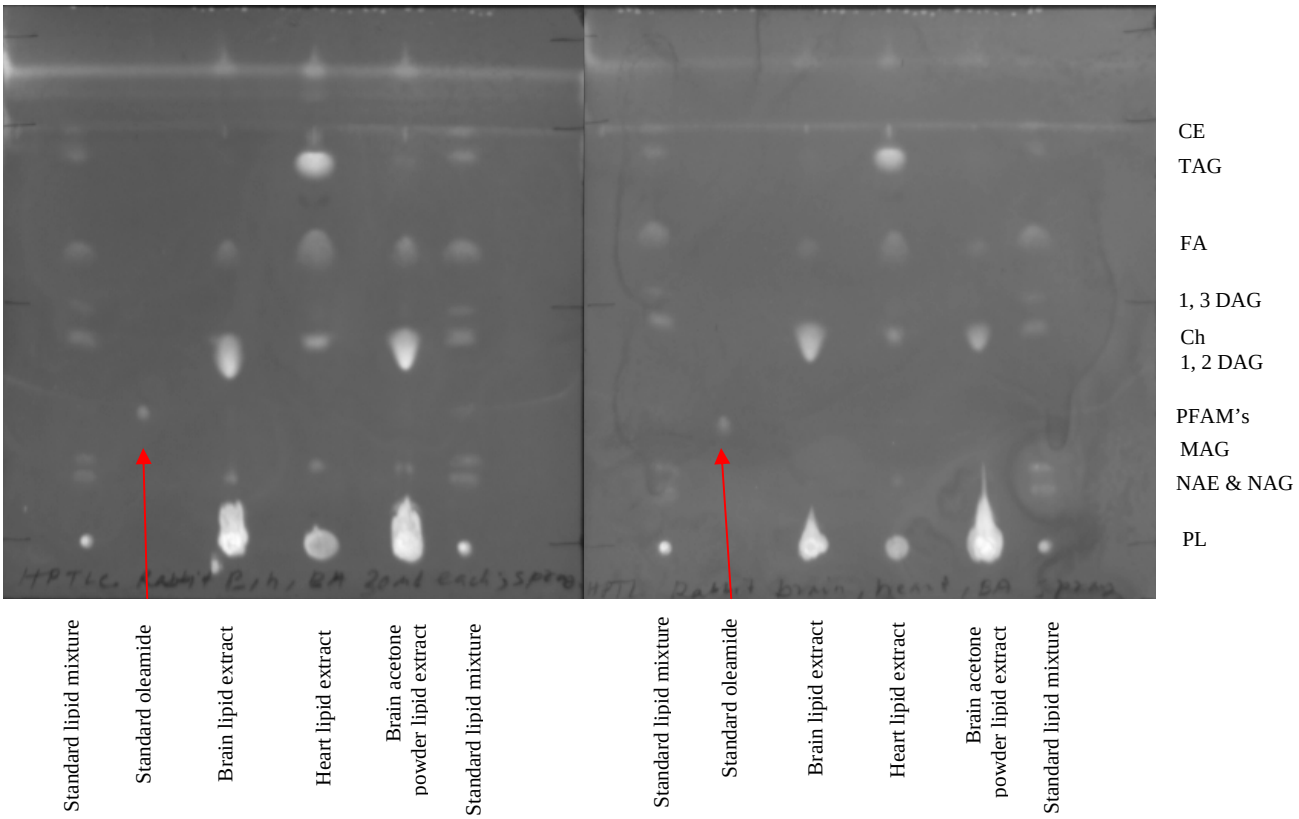

FIGURE 5-2: Total lipid profiling of rabbit tissues (two sets of optimized lipid load on the HPTLC plate).

The HPTLC of the SPE fractions of the rabbit brain and heart tissues are shown in Figure 5-5 and Figure 5-6. The analysis of fraction three from the DSC-NH_2 column by GC/MS (Figure 5-7 and Figure 5-8) confirmed the presence of palmitamide, stearamide and oleamide. Again heptadecanoamide (C17) and deuterated heptadecanoamide (C17D33) were used as internal standards added to the samples before and after extraction, respectively. The presence of erucamide as well as eicosanoamide was also observed, which largely eluted from SPE polypropylene vials.

For most of the cases of GC/MS analysis reported here, the GC method was edited so that the runs end before the erucamide elution in order to increase the sensitivity of the other peaks.

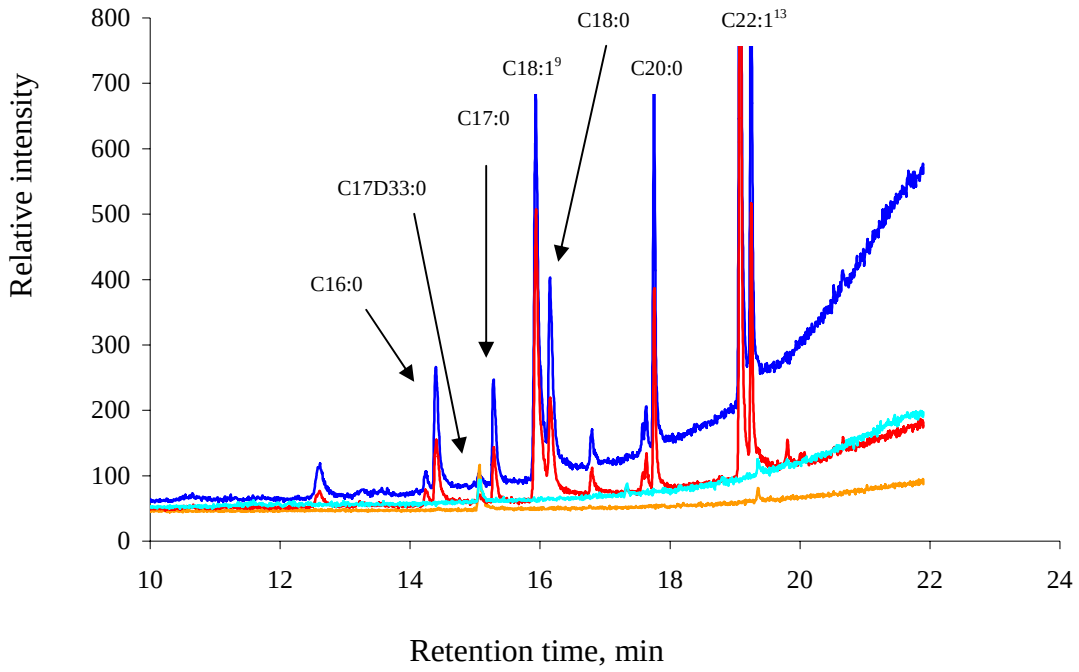

FIGURE 5-3: GC of rabbit brain amides isolated by HPTLC. The relative intensities at m/z **59**, **72**, **62** and **76** (see Chapter 2 for fragmentation patterns) are plotted against retention time.

The ions monitored under SIM were m/z 59 and m/z 72 for the naturally abundant amides as well as m/z 62 and m/z 76 for the isotopically enriched amides (Figures 5-3, 5-4, 5-7, 5-8, 5-9 and 5-10). These ions correspond to the fragmentations due to McLafferty rearrangement (see Chapter 2 for description). It is noticeable that

the relative intensities of the amides isolated by SPE (Figures 5-7 and 5-8) are higher than that isolated by HPTLC (Figures 5-3 and 5-4). The reason is the larger sample capacity of SPE. The optimized amount of the lipid extract that could be loaded on the HPTLC plate was only around 1-30 μL (total mass unknown for the rabbit tissue lipid load).

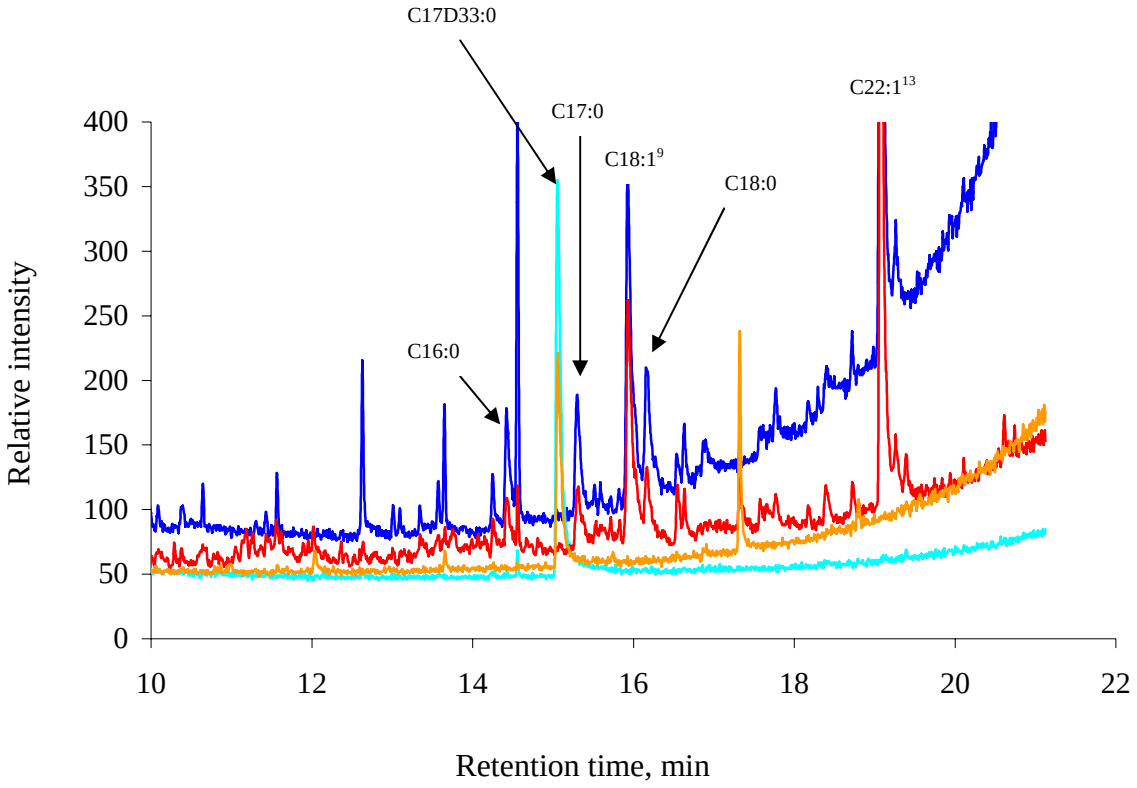

FIGURE 5-4: GC of rabbit heart amides isolated by HPTLC. The relative intensities at m/z **59**, **72**, **62** and **76** (see Chapter 2 for fragmentation patterns) are plotted against retention time.

On the other hand, the amount that could be loaded on SPE was in the order of 1-300 μL (total mass unknown for the rabbit tissue lipid load). The loads for brain,

heart, and brain acetone powder were 50 μL, 100 μL and 200 μL, respectively, onto SPE columns. In order to optimize the amount of load, different loads of the same sample were carried out by SPE and the fraction were visualized by HPTLC until optimal resolution was obtained. The reason only 50 μL load was sufficient for brain compared to 200 μL for brain acetone powder is that (i) amount of brain tissue used was higher than brain acetone powder in our case and (ii) brain contained much higher lipid amount than brain acetone powder (see Table 5-I and 5-II). The later could be due to the lipid loss that might occur during drying the tissues under acetone [28].

Table 5-I and 5-II shows the quantification of amides by both HPTLC/GC/MS and SPE/GC/MS. The percent recoveries of the internal standard (C17) spiked before the lipid extraction was found to be comparable at 95% confidence limit (n=9) for SPE, although this was not the case for HPTLC analysis. Not only the percent recovery of the spiked standard was uncomparable at 95% confidence limit but also the amount of amides found in tissue sample was randomly different from that obtained by SPE. Lower recovery by HPTLC was observed in most cases compared to that by SPE except in some case where larger values were obtained. The larger values could be resulted from possible contamination from TLC developing solvents (or due to peroxide formation) whereas lower values could be the result of sample loss during separation of amides from silica after scraping the spots. Therefore, it is clear from the results that HPTLC is valuable for quick profiling of the total lipids and SPE is more reliable for quantification. Even though it was assumed that no PFAM's would be found from atrium-removed heart, palmitamide, stearamide and oleamide were detected in heart as in brain. This suggests that PFAM's are probably carried into the ventricle by blood.

TABLE 5-I: Quantitation of PFAM's in rabbit tissues by HPTLC/GC/MS.

	Amount of amides, µg/g of rabbit tissue			**Percent recovery of spiked standard**
	C16	**C18**	**C18:1^{9}**	**C17**
Brain	7.2 ± 0.8	3.5 ± 0.2	28 ± 2	85 ± 3
Heart	1.6 ± 0.2	2.0 ± 0.1	4.5 ± 0.4	82 ± 4
Brain-acetone powder	3.6 ± 0.5	1.8 ± 0.1	5.8 ± 0.1	91 ± 3
Blank	----	0.005 ± 0.001	0.0136 ± 0.0001	98 ± 6

Uncertainties are at 95% CL with n = 9.

TABLE 5-II: Quantitation of PFAM's in rabbit tissues by SPE/GC/MS.

	Amount of amides, µg/g of rabbit tissue			**Percent recovery of spiked standard**
	C16	**C18**	**C18:1^{9}**	**C17**
Brain	3.2 ± 0.2	4.4 ± 0.2	23 ± 1	104 ± 9
Heart	1.9 ± 0.1	1.4 ± 0.1	7.5 ± 0.5	92 ± 5
Brain-acetone powder	2.7 ± 0.4	1.7 ± 0.1	15.7 ± 0.8	95 ± 9
Blank	----	0.007 ± 0.001	0.031 ± 0.002	102 ± 7

Uncertainties are at 95% CL with n = 9.

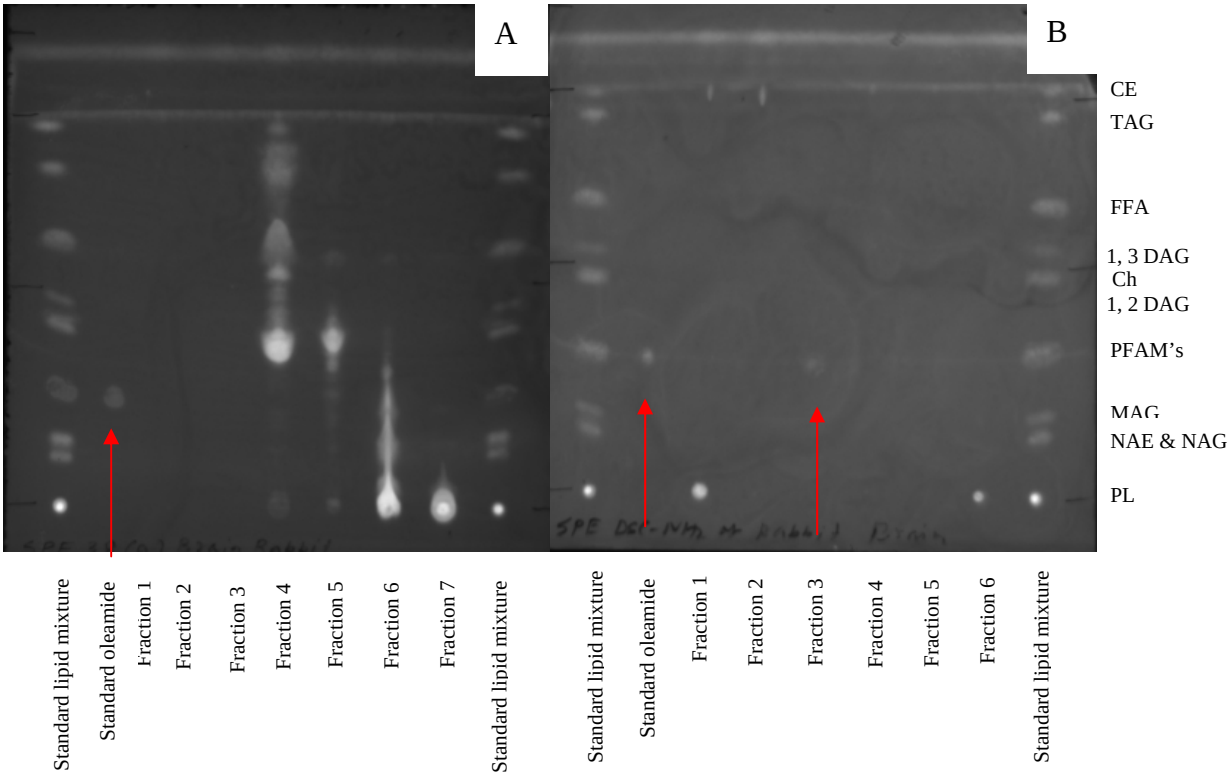

FIGURE 5-5: SPE fraction from rabbit brain lipid load onto A) DSC-Si and B) DSC-NH_2 columns. **Note**: SPE method for the isolation of amides by A) DSC-Si and B) DSC-NH_2 columns. A) Fraction 1: 4 mL hexane wash after sample load; Fraction 2: elution by 1 mL of 99:1 hexane: HOAc; Fraction 3: elution by 1 mL of 90:10 hexane: ethyl acetate; Fraction 4: elution by 1 mL of 80:20 hexane: ethyl acetate; Fraction 5: elution by 1 mL of 70:30 hexane: ethyl acetate; Fraction 6: elution by 1.5 mL of 2:1 $CHCl_3$: 2-propanol; Fraction 7: elution by 2 mL of MeOH. B) Fraction 1: 2 mL hexane wash after sample load; Fraction 2: elution by 2 mL $CHCl_3$; Fraction 3: elution by 2 mL of 2% 2-propanol in $CHCl_3$; Fraction 4: elution by 1 mL 2% 2-propanol in $CHCl_3$; Fraction 5: elution by 1 mL 3% 2-propanol in $CHCl_3$; Fraction 6: elution by 2 mL of MeOH.

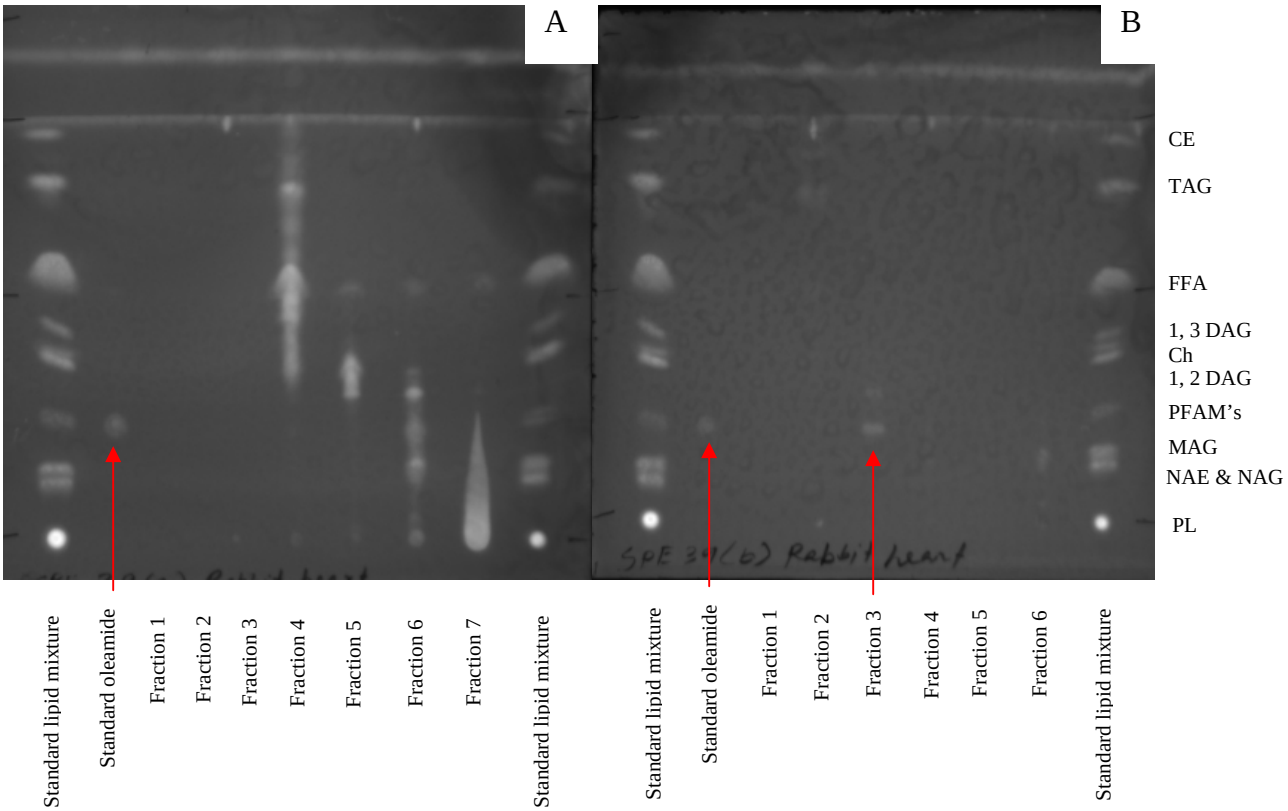

FIGURE 5-6: SPE fraction from rabbit heart lipid load onto A) DSC-Si and B) DSC-NH_2 columns. (Please see the NOTE under Figure 5-5 for clarification).

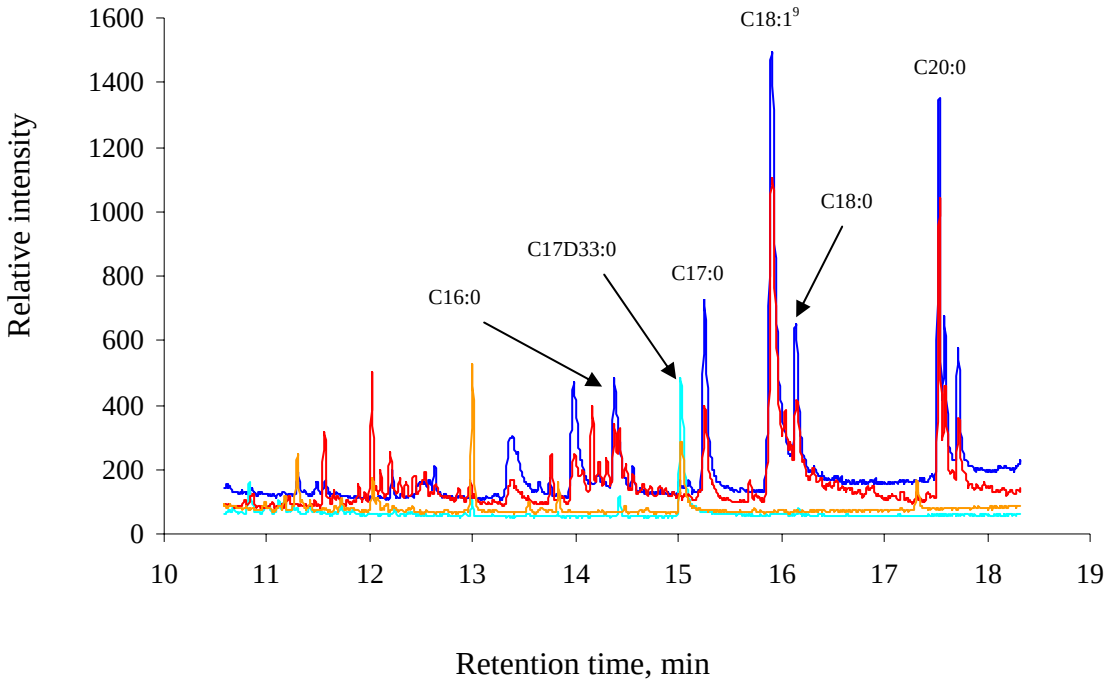

FIGURE 5-7: GC of rabbit brain amides isolated by SPE. The relative intensities at m/z **59**, **72**, **62** and **76** (see Chapter 2 for fragmentation patterns) are plotted against retention time.

The amount of palmitamide and oleamide isolated from human luteal phase plasma [5] were in the order of 3.9 μg/mL and 31.7 μg/mL, respectively, and the amount of oleamide extracted from $N_{18}TG_2$ cells [20] was 0.0155 μg (55.0 ± 9.5 pmol; mean ± SD, n=2)/10^7 cells. Hanuš *et al*. [21] reported the amount of oleamide analyzed as TMS ester to be 9.9 ± 1.0 ng/mL in rat plasma and 44 ± 3.0 ng/mL in rat CSF. They [21] concluded that the higher microgram amount of PFAM's extracted from human plasma was probably due to the contamination coming from the use of plastic

containers. It is to be mentioned that the PFAM's are used as slip additives in plastics in order to improve their surface properties [29-32].

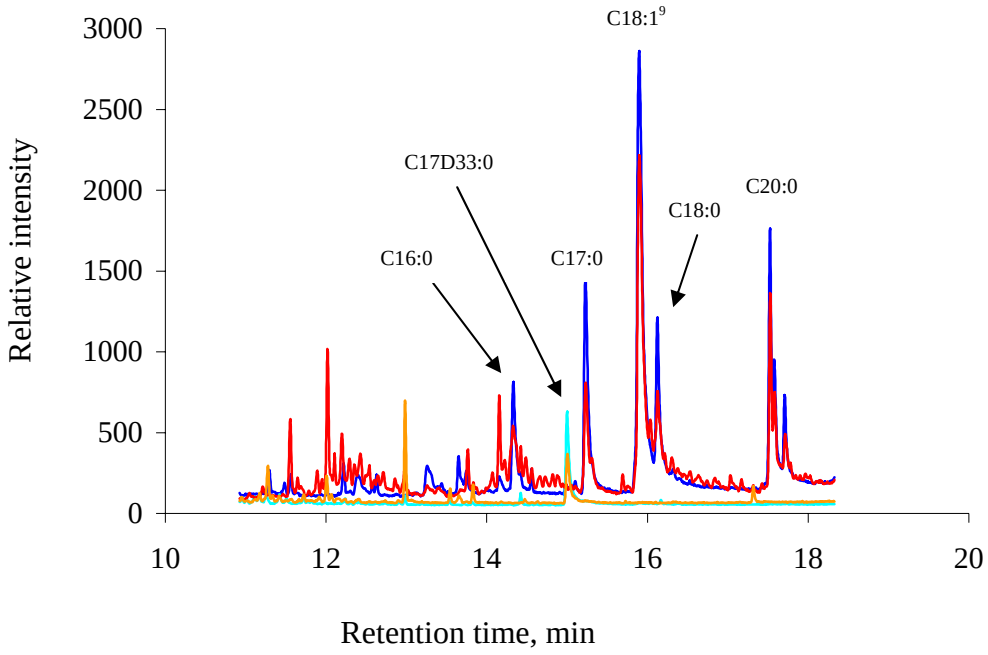

FIGURE 5-8: GC of rabbit heart amides isolated by SPE. The relative intensities at m/z **59**, **72**, **62** and **76** (see Chapter 2 for fragmentation patterns) are plotted against retention time.

The amount of palmitamide (3.2 ± 0.2 μg/g) and oleamide (23 ± 1 μg/g) found in brain by SPE in this study is in accordance with the amounts of those reported by Arafat *et al.* [5]. The comparison was done by assuming that the specific gravity of tissue is the same as the specific gravity of water, therefore the weight of 1 mL of tissue can be taken as 1 g. Even though polypropelyne vials and tubes were used for lipid extraction and SPE purposes in this study, a parallel blank as described above was run

in this case and was subtracted from the sample in order to compensate for any contamination (Figure 5-9). The standard curves for PFAM's were found to be linear in the range of 5 pg/µL to 2.5 ng/ µL at 95% confidence limit (Figure 5-10).

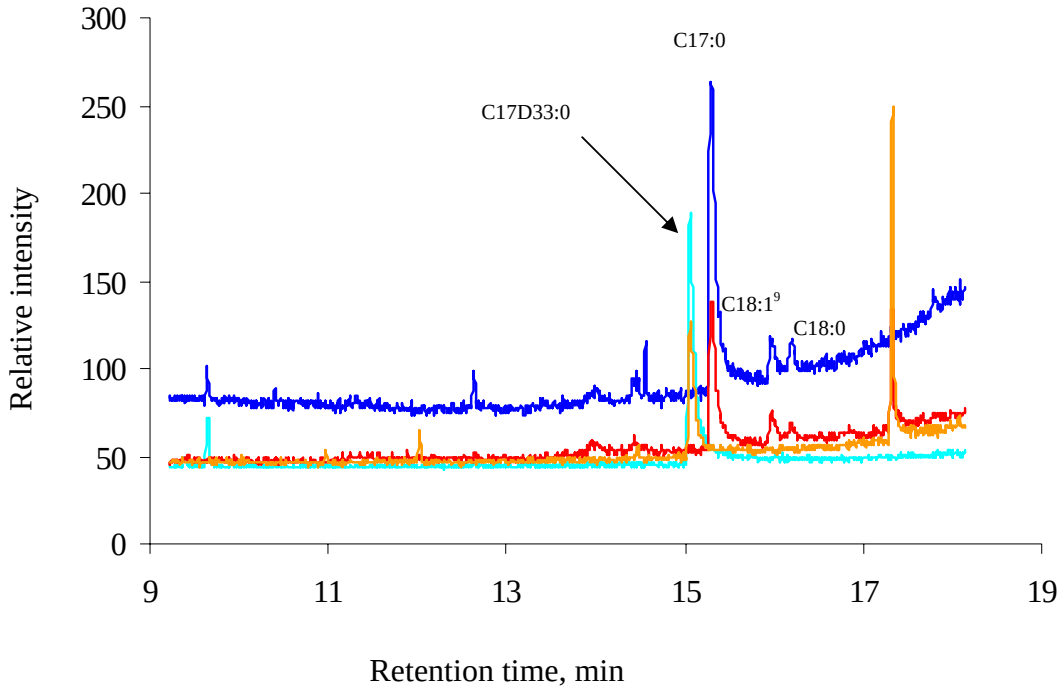

FIGURE 5-9: GC of blank of the lipid extraction process. The relative intensities at m/z **59**, **72**, **62** and **76** (see Chapter 2 for fragmentation patterns) are plotted against retention time.

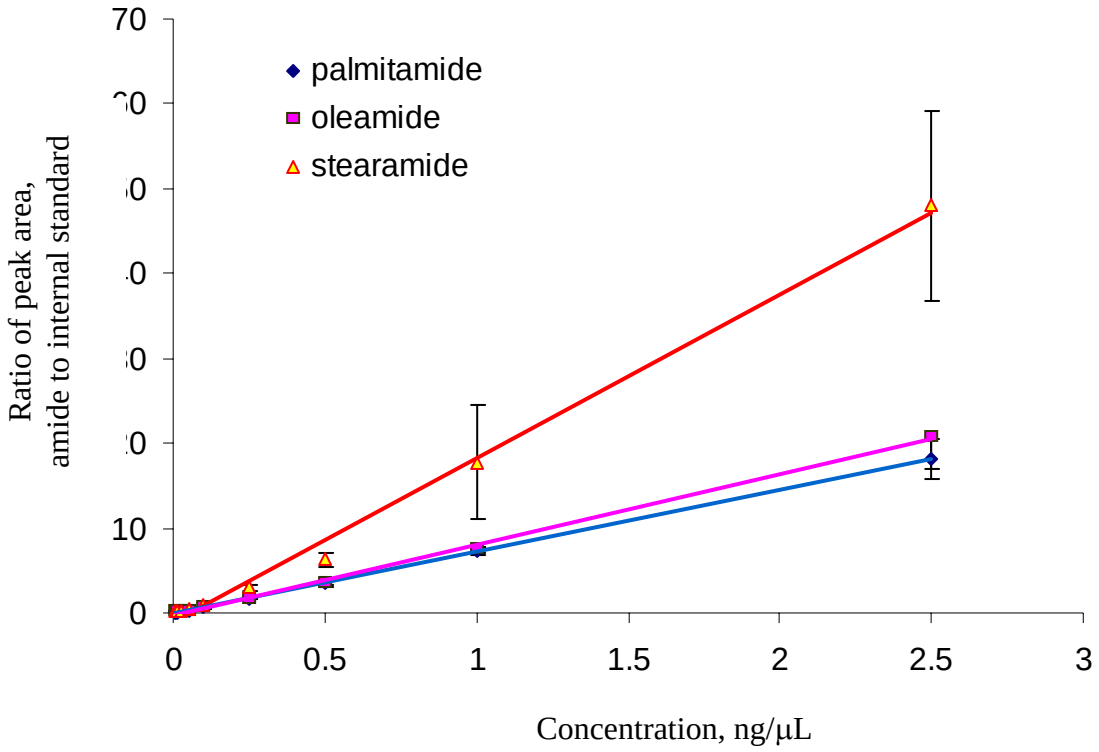

FIGURE 5-10: Standard curves for PFAM's. Uncertainties are at 95% confidence limit (n=3).

One other point to address from the results that the oleamide peak observed in GC could also be the result of the co-elution of different C18 unsaturated amides, which were not separated on a HP-5MS column (Figure 5-11). The fact that, Arafat *et al.* [5] also found palmitoleamide ($C16:1^{9}$; 4.4 µg/mL), elaidamide ($C18:1^{trans9}$; 3.7 µg/mL) and linoleamide ($C18:2^{9,\ 12}$; 2.2 µg/mL) besides palmitamide and oleamide in human plasma, even strengthen this point. In our case, this assumption is still to be proven using a relatively polar column capable of separating unsaturated fatty acids and amides, which will be discussed in Chapter 6.

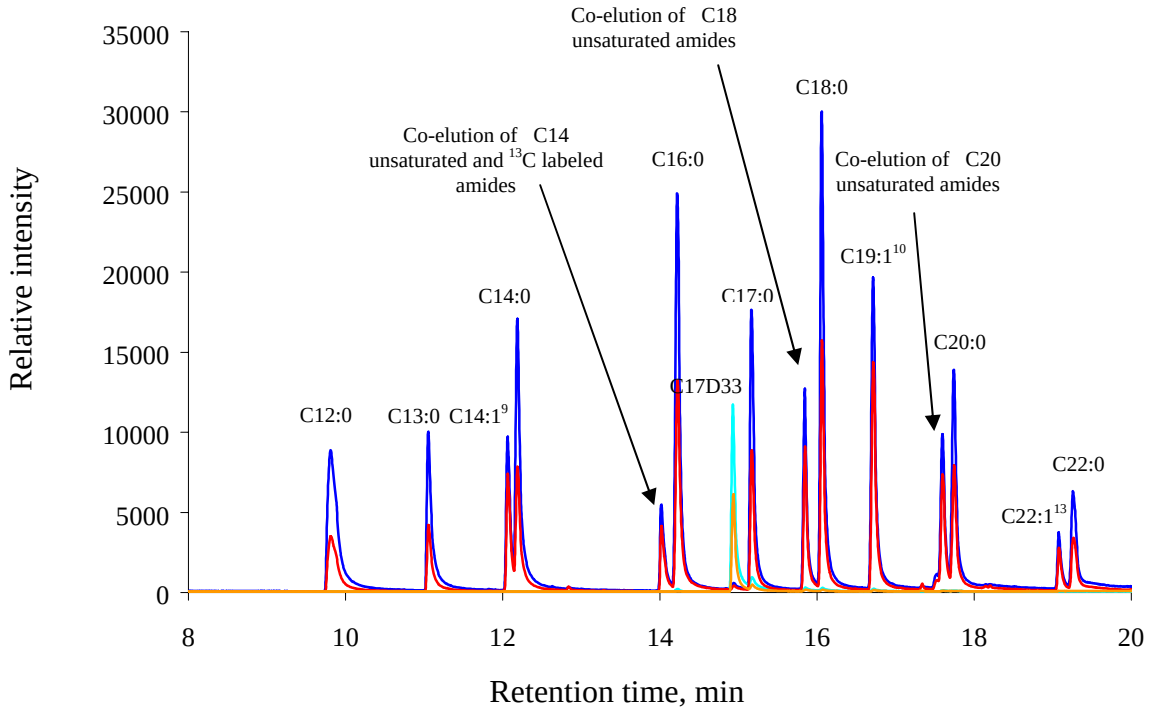

FIGURE 5-11: Elution profile for standard C12-C22 amides on a HP-5MS column. The relative intensities at m/z **59**, **72**, **62** and **76** (see Chapter 2 for fragmentation patterns) are plotted against retention time.

5.5 Conclusions

The application of the HPTLC and SPE sample preparation methods for the isolation of the primary fatty acid amides from tissue lipid extracts prior to their analysis by GC/MS has been established. These sample preparation techniques have been proven to be useful for obtaining clean amide samples free from interfering components. The clean detection techniques gave rise to higher sensitivity and lower detection limits for the amides. Concentration of the samples was also achieved providing the quantitation of

these trace lipid amides in mammalian tissues. The HPTLC method has been proven to be efficient for quick profiling of the total lipid extract whereas the SPE method was found to be efficient in reliable quantification at 95% confidence limit. The amount of palmitamide and oleamide was found in the same order of the amount of those observed by Arafat in human plasma. Nearly 100 % recovery of the spiked internal standard by SPE makes the method reliable for quantitative recovery of the amides from any cell or tissue type. Next chapter will emphasis on the use of a very polar BPX70 column for the separation of any unsaturated amides if co-eluting with the oleamide peak.

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Chapter 6

Separation of unsaturated fatty acid amides by argentation-HPTLC and BPX70 column

6.1 Abstract

Unsaturated primary fatty acid amides were separated by silver-ion high performance thin layer chromatography (argentation-HPTLC) as well as by a BPX70 GC column. The separated amides were all eighteen carbon analogs of *cis*-9-octadecenoamide (oleamide). The separation was achieved in terms of number of double bonds by argentation-HPTLC whereas clear resolutions between geometric and positional isomers were obtained using the BPX70 column. Synthesized standard PFAM's were used for this separation analysis and PFAM's isolated from rabbit brain and heart tissues by solid phase extraction (SPE) were used for the application. The goal of this method was to separate and identify individual peaks if co-eluting with the oleamide peak. Separation of unsaturated amides with same number of carbon cannot be achieved by a less polar column such as HP-5MS. Separation of rabbit brain and heart amides shows that oleamide is the only major C18 unsaturated amides isolated from heart tissues. Trace amounts of linoleamide ($C18:2^{9,12}$) and eicosenoamide ($C20:1^{13}$) were also identified in brain besides oleamide.

6.2 Introduction

Primary fatty acid amides (PFAM's) are one of the most recently emerging classes of bio-active compounds. Long chain amides (C12-C22), isolated from biological samples and assigned different hormonal activity so far, are oleamide (*cis*-9-octadecenoamide), erucamide (*cis*-13-docosenoamide), anandamide (*cis*,*cis*,*cis*,*cis*-5,8,11,14 eicosatetraenoamide), and linoleamide (*cis*,*cis*-9,12 octadecadienoamide) [1-17]. Numerous different PFAM's can be synthesized from commercially available free fatty acids (FFA) *in vitro* and no assumption can be made yet how many different PFAM's are synthesized *in vivo*. For a list of fatty acids refer to Table 6-1 [18]. The next step of the isolation of PFAM's from tissues and samples is their identification followed by quantification. In order to identify each and every single amide present in a sample, they need to be completely separated from each other. Normal phase liquid chromatography (TLC/HPLC) and gas chromatography are the most widely used separation techniques for fatty acids, their derivatives and PFAM's separations [19-48]. Separation of the *cis* and *trans* isomers of FFA as their methyl esters has been achieved using mainly argentation HPTLC/HPLC and highly polar GC columns. Solid-phase extraction (SPE) and supercritical fluid chromatography in Ag-ion mode can also be used [49, 50]. Numerous publications and application notes from various laboratories as well as from different manufacturers are available, which discuss theories and techniques behind these separations [19-48].

TABLE 6-1: Systematic names and selected properties of some of the more important fatty acids of five or more carbon atoms [18].

Common name	Systematic name	Mol. form.	M_r	t_m/°C	S
	Saturated				
Valeric acid	Pentanoic acid	$C_5H_{10}O_2$	102.13	-34	2.5
Isovaleric acid	3-Methylbutanoic acid	$C_5H_{10}O_2$	102.13	-29.3	4.3
Caproic acid	Hexanoic acid	$C_6H_{12}O_2$	116.16	-3	0.967
Enanthic acid	Heptanoic acid	$C_7H_{14}O_2$	130.19	-7.5	0.24
Caprylic acid	Octanoic acid	$C_8H_{16}O_2$	144.21	16.3	0.080
Pelargonic acid	Nonanoic acid	$C_9H_{18}O_2$	158.24	12.3	0.0284
Capric acid	Decanoic acid	$C_{10}H_{20}O_2$	172.27	31.9	0.015
Lauric acid	Dodecanoic acid	$C_{12}H_{24}O_2$	200.32	43.2	0.0055
Tridecylic acid	Tridecanoic acid	$C_{13}H_{26}O_2$	214.35	41.5	0.0033
Myristic acid	Tetradecanoic acid	$C_{14}H_{28}O_2$	228.38	53.9	0.0020
Pentadecylic acid	Pentadecanoic acid	$C_{15}H_{30}O_2$	242.40	52.3	0.0012
Palmitic acid	Hexadecanoic acid	$C_{16}H_{32}O_2$	256.43	63.1	0.00072
Margaric acid	Heptadecanoic acid	$C_{17}H_{34}O_2$	270.46	61.3	0.00042
Stearic acid	Octadecanoic acid	$C_{18}H_{36}O_2$	284.48	69.6	0.00029
Arachidic acid	Eicosanoic acid	$C_{20}H_{40}O_2$	312.54	76.5	
Phytanic acid	3,7,11,15-Tetramethylhexadecanoic acid	$C_{20}H_{40}O_2$	312.54	-65	
Behenic acid	Docosanoic acid	$C_{22}H_{44}O_2$	340.59	81.5	
Lignoceric acid	Tetracosanoic acid	$C_{24}H_{48}O_2$	368.64	87.5	
Cerotic acid	Hexacosanoic acid	$C_{26}H_{52}O_2$	396.70	88.5	
Montanic acid	Octacosanoic acid	$C_{28}H_{56}O_2$	424.75	90.9	
	Monounsaturated				
Caproleic acid	9-Decenoic acid	$C_{10}H_{18}O_2$	170.25	26.5	
Palmitoleic acid	*cis*-9-Hexadecenoic acid	$C_{16}H_{30}O_2$	254.41	-0.1	
Oleic acid	*cis*-9-Octadecenoic acid	$C_{18}H_{34}O_2$	282.47	13.4	
Elaidic acid	*trans*-9-Octadecenoic acid	$C_{18}H_{34}O_2$	282.47	45	
Vaccenic acid	*trans*-11-Octadecenoic acid	$C_{18}H_{34}O_2$	282.47	44	
Erucic acid	*cis*-13-Docosenoic acid	$C_{22}H_{42}O_2$	338.57	34.7	
Brassidic acid	*trans*-13-Docosenoic acid	$C_{22}H_{42}O_2$	338.57	61.9	
Nervonic acid	*cis*-15-Tetracosenoic acid	$C_{24}H_{46}O_2$	366.63	43	
	Diunsaturated				
Linoleic acid	*cis,cis*-9,12-Octadecadienoic acid	$C_{18}H_{32}O_2$	280.45	-12	
	Triunsaturated				
cis-Eleostearic acid	*trans,cis,trans*-9,11,13-Octadecatrienoic acid	$C_{18}H_{30}O_2$	278.44	49	
trans-Eleostearic acid	*trans,trans,trans*-9,11,13-Octadecatrienoic acid	$C_{18}H_{30}O_2$	278.44	71.5	
Linolenic acid	*cis,cis,cis*-9,12,15-Octadecatrienoic acid	$C_{18}H_{30}O_2$	278.44	-11	
	Tetraunsaturated				
Arachidonic acid	5,8,11,14-Eicosatetraenoic acid, (all-*trans*)	$C_{20}H_{32}O_2$	304.47	-49.5	

M_r: molecular weight, t_m: melting point in °C,

S: aqueous solubility at 20 °C in units of grams of solute per solute per 100 g of water.

Silver ion chromatography depends on the formation of weak, reversible, charge-transfer complexes of Ag^+ with unsaturated organic molecules. The mechanism for this type of reaction involves 1) the formation of a sigma bond between the occupied bonding π_{2p} electrons of the double bond and free 5s and 5p orbitals of Ag^+ as well as 2) the formation of a weaker π accepter backbone between the antibonding π^*_{2p} electrons of the double bond and the occupied 4d orbitals of the Ag^+ [51]. The FFA derivatives such as unsaturated FAMEs can be separated on Ag impregnated silica phase based on the number of double bonds present, geometric configuration of the double bonds (*cis/trans*) as well as the position of the double bond. Stronger retention is obtained for the higher degree of unsaturation and for the larger the separation between the double bond (i.e., polyunsaturated fatty acids will retain stronger than diunsaturated ones and methylene interrupted unsaturated fatty acids will retain stronger than conjugated ones). As the double bond position moves away from the carbonyl group of the fatty acids, longer retention is observed. The *trans* isomers are found to retain less strongly than the *cis* isomers [for review see Ref. 52].

Successful separation by silver ion chromatography such as Ag-HPTLC depends on the optimum impregnation of stationary phase with silver. A "dynamic impregnation" technique proposed by Aitzetmueller *et al*. is generally used which includes the development of the TLC plates in 10-20% $AgNO_3$ in acetonitrile [23]. In this process, as the development progresses, Ag gradually travels upwards on the plates with a decreasing concentration. Better separation is usually achieved with such a gradient in silver content on the stationary phase. Even though the earlier works show that 10 to 30% $AgNO_3$ solution are necessary for better resolution [53, 54], recent work

indicates that retention and resolution are not affected at all by impregnating plates with more than 5% $AgNO_3$ solution [55, 57]. Moreover, excellent separation was achieved by developing the plates in 0.5% methanolic $AgNO_3$ [52, 58, 59]. The solvents used for the separation of lipids by Ag-TLC are mainly non polar solvents such as benzene, toluene, chloroform, dichloromethane, diethyl ether, etc. Mixtures of different solvents are also employed depending on the unsaturation of the lipids to be separated. Usually a larger excess of aliphatic hydrocarbons are employed with varying amounts of polar solvents in the mixture [for a review see Ref. 23]. Because the separation profile of FFA/FAME is similar to that of PFAM's as in other chromatographic techniques, Ag-HPTLC could be successfully employed in their separation. A given recipe for FAME might not work for PFAM's in which case method development will be necessary.

Separation and detection of fatty acids by gas chromatography is a long known and probably the most used technique. The analysis of fatty acids in different lipids (such as in triacylglycerols) is done by transesterification of the fatty acids into their methyl esters followed by GC/MS analysis. Earlier work mostly shows the separation in terms of chain length or at best in terms of unsaturation, whereas the recent analyses are concentrated on separation in terms of configuration and position of the unsaturation [35-48]. The later was possible due to the development of polar columns that are capable of separating the positional and geometric isomers of FAMEs. These columns are routinely used in various laboratories for FFA analysis in food, vegetable and fish oil, dairy products, plastics, paints and varnishes, geochemical supplies, biological samples and many others. As mentioned above, PFAM's tend to show similar chromatographic behavior as FFA/FAME. The separation of derivatized PFAM's in

terms of the number of carbon has been achieved using non polar columns [35-37]. In some cases, separation by the number and position of unsaturation was achieved (38, 39) but separation in terms of geometrical and most positional isomers have yet to be accomplished. Use of a polar column for the separation of PFAM's was also described but only a few unsaturated ones were separated, which probably due to the poor column performance [43]. In recent years the development of polar columns has reached tremendous goals of achieving more stable and lower bleed profile. Temperatures as high as 260 °C can be employed, even with the most polar phases (such as cyanopropyl incorporated dimethyl polysiloxane) without breakage of the phase. The bonded phase feature (cross linking) allows such a column to be rinsed to remove contaminants. Employing such a column could result the desired PFAM separation with higher sensitivity and lower detection limit, provided PFAM's are proper derivatized prior to the analysis [see Section 6.4.2 for more discussion on polar columns and derivatization techniques].

In this study, we have employed argentation HPTLC (Ag-HPTLC] [60] and a polar column (BPX70; 70% cyanopropyl dimethyl polysilphenylenesiloxane) to explore the separation of unsaturated PFAM's. The BPX70 column was also used in the separation of amides isolated from rabbit brain and heart tissues. Different pros and cons of the techniques used have been mentioned and areas for further development are discussed in the following sections.

6.3 Experimental

6.3.1 Reagents

PFAM's were synthesized from the corresponding acids (see Chapter 2) unless otherwise mentioned. The PFAM's used in this study were octadecanoamide (stearamide; C18:0), *cis*-9-odecenoamide (oleamide; C18:1^{9}), *trans*-9-octadecenoamide (elaidamide; C18:1^{trans9}), *cis*,*cis*-9,12-octadecadienoamide (linoleamide; C18:$2^{9,12}$), *trans*,*trans*-9,12-octadecadienoamide (linoleladamide; C18:$1^{trans9,12}$), *cis*,*cis*,*cis*-9,12,15-octadecatrienoamide (α-linolenamide; C18:$3^{9,12,15}$), *cis*,*cis*,*cis*-6,9,12-octadecatrieno-amide (γ-linolenamide; C18:$3^{6,9,12}$), *trans*-11-octadecenoamide (vaccenamide; C18:$1^{trans11}$), *cis*-6-octadecenoamide (petroselenamide; 18:1^{6}), *trans*-6-octadecenoamide (petroselaidamide; C18:1^{trans6}), *cis*-13-octadecenoamide (C18:$1^{13)}$, *cis*-5-eicosenoamide (C20:1^{5}), *cis*-11-eicosenoamide (C20:1^{11}), *trans*-11-eicosenoamide (C20:$1^{trans11}$), and *cis*-13-eicosenoamide (C20:1^{13}). The corresponding acids were purchased from Sigma Chemical Co. (St. Louis, MO). Oleamide is commercially available and was purchased also from Sigma. All chemicals and solvents used were of ACS reagent grade. Chloroform and methanol were obtained from EM Science and EMD (Darmstadt, Germany) respectively; acetonitrile, ethyl acetate, hexanes, isopropanol, glacial acetic acid and sodium thiosulfate were all purchased from Fisher Scientific (Fair Lawn, NJ); acetone was from Acros Organics (Geel, Belgium); diethyl ether and silver nitrate were from Aldrich Chemical Co. (Milwaukee, WI).

6.3.2 Tissues

Rabbit whole heart (atrium removed), rabbit whole brain (stripped), and rabbit brain acetone powder were purchased from Pel-Freez Biologicals (Rogers, AR).

6.3.3 HPTLC plates

High performance thin layer chromatography plates (HPTLC) from Analtech (Newark, DE) were of 10 x 10 cm, with organic binder and no fluorescence indicator incorporated. Primuline dye (CI 49000; direct yellow 59) used for spraying the HPTLC plates was obtained from Aldrich Chemical Co. (Milwaukee, WI). Preval^{TM} power unit (Precision Valve Corporation, Yonkers, NY) for spraying primuline dye on the HPTLC plates was purchased from a local hardware store.

6.3.4 SPE phases

Discovery DSC-Si and Discovery DSC-NH_2 SPE phases were purchased from Supelco (Bellefonte, PA). Irregularly shaped and acid washed base silica possessed following properties: 50 μm particle size, 70 Å pore diameter, 480 m^2/g specific surface area and 0.9 cm^3/g pore volume. DSC-Si is the base silica phase which is basically used as a normal phase adsorbent whereas DSC-NH_2 is the aminopropyl bonded silica which can be used as either normal phase or the ion-exchange adsorbent. SPE tubes (empty) of 3 mL volumes with polyethylene frits were also purchased from Supelco. 500 mg of the phases were packed into the tubes between two frits applying hand pressure in order to carry out SPE.

6.3.5 Instrumentation

Camag Nanomat and capillary dispenser system, purchased from Camag (Muttenz, Switzerland), was used for easy application of samples on Ag incorporated HPTLC plates. 1 μL sample was applied at a time with disposable capillary pipettes. Fluorescent images were obtained using Kodak digital science 440 image station (IS440cF, Perkin Elmer, Boston, MA). An Agilent Technologies Network GC/MS system (6890 GC with 5973 mass selective detector and 7683 series injector) was used for the analysis of derivatized amides. The column used was a polar BPX70 column (0.25 mm internal diameter, 0.25 μm film thickness, 60 m long, SGE incorporate, USA). The composition of the column is 70% cyanopropyl polysilphenylenesiloxane. The GC/MS method used for analysis was as follows: starting temperature was 60 ºC, ramped to 170 ºC at 50 ºC per min with 5 min hold time, ramped to 200 ºC at 4 ºC per min with 5 min hold time and finally ramped to 225 ºC at 50 ºC per min with 4.5 min hold time. The total run time was 24.70 min [61]. Electron impact ionization (EI) at 70 eV was used and mass range was kept from 40 to 400 m/z. The m/z values 67, 81, 122, 124, 136 and 138 were used for single ion monitoring (SIM). The temperatures of the injection port and the transfer line were 250 ºC and 280 ºC respectively. Injection volume was 1 μL splitless

6.3.6 Separation of PFAM's by Ag-HPTLC

6.3.6.1 Preparation of HPTLC plates

HPTLC plates were allowed to develop in either a 10% $AgNO_3$ solution in acetonitrile or a 0.5% $AgNO_3$ solution in methanol to the top in a vertical development chamber.

The silver actually travels up to the half of the plate with its concentration gradually decreasing. The Ag impregnated plates were dried at 100 ºC for 5 min.

6.3.6.2 HPTLC method for plate development

Solvents were mixed in correct proportions, sonicated for an hour and kept on the bench top with caps on. 5 µL of each PFAM standard (1 µg/µL) were applied on HPTLC plates 1 cm from the bottom using the Camag capillary holder. Warm air from a hair dryer was continuously applied during sample application in order to dry the spots. For plate development, horizontal chambers for 10 x 10 cm plates from Camag were used. HPTLC separation method is as follows: the 10 x 10 cm plates were developed twice up to 10 cm in hexane: diethyl ether: acetone (40:35:15, v/v/v). The plates were developed in the same direction as Ag was developed and were dried in between runs.

6.3.6.3 HPTLC plate visualization

The developed plates were then dried at 100 ºC for 5 min. Ag was removed by dipping the plates into a saturated solution of sodium thiosulfate followed by dipping into double distilled water. The plates were dried at 100 ºC for 5 min and sprayed with a 0.05 % primuline solution (in 80:20, v/v acetone: water) and re-dried. The dry plates were scanned by Kodak digital Science 440 image station for visualization.

6.3.7 Separation of PFAM's by BPX70 column

6.3.7.1 Derivatization of amides

50 µL of each PFAM standards (1 µg/µL) were placed in separate vials and completely dried under N_2. The dried amides were then placed inside a glove bag and 50 µL *N*, *N*-

bis-trimethylsilyltrifluoroacetamide (*N*, *N*-BSTFA) was added to each amide. The reaction mixture was then heated at 95 ºC for 15 min and allowed to cool. After the products were cold, 300 μL acetonitrile was added to each vial and 1 μL of each of these solutions were injected into GC/MS.

6.3.7.2 Identification of amides

Individual amides were run by GC/MS in order to identify their retention time and mass spectral patterns. Once this goal was established, a mixture of derivatized amides was injected into the GC/MS and the elution profile was observed. Different amides, in a mixture, were therefore identified by comparing their retention times and MS patterns with those of the corresponding individual amide.

6.3.8 Separation of rabbit brain and heart PFAM's by BPX70 column

6.3.8.1 Extraction of lipids from rabbit brain and heart

The samples were stored at -80 ºC upon arrival. Right before lipid extraction, samples were taken out of the freezer and placed at dry ice temperature. They were then cut into pieces and weighed before extracting lipids. The lipids were extracted from the tissues (62, 63) as follows: The weighed tissues/acetone powder were suspended into 20 times of its volume of chloroform: methanol (2:1; v/v) and completely homogenized using a glass-to-glass homogenizer (Kontes Glass Co., Vineland, NJ). The tissue homogenate was then sonicated for 10 min at 1500 g. Before sonication the specific gravity of the homogenate was lowered using 0.2 times of its volume (20%) of methanol. The supernatant was separated from the pellets and chloroform was added to the supernatant in order to adjust the proportion of chloroform: methanol to 2:1 (for example if the total

volume of the homogenate was 20 mL, 4 mL methanol was added before sonication and 8 mL of chloroform was added to the supernatant after sonication). To the total amount of supernatant, 0.2 times of its volume of aqueous 0.88% KCl was added (for example if the total volume of the supernatant was 20 mL, 4 mL of aqueous 0.88% KCl was added). The sample was then vortexed for 2 min and allowed to sit for separation into two phases. The upper phase mostly contains proteins and non-lipid contaminants and the lower phase contains the lipid extract. The upper phase was therefore discarded and the lower lipid phase was completely dried under a stream of N_2. The dried lipid extract was dissolved in 4 mL hexane to be loaded onto SPE columns. Blanks (no tissues) were prepared following the same procedure as samples. The glass-to-glass homogenizer was silanized using 10% TMS in toluene before use in order to minimize the amide adsorption on the silica surface.

6.3.8.2 Extraction of amides from total lipid extract by SPE

SPE elution solutions were made by mixing them in correct proportions followed by sonication for an hour. The DSC-Si column was completely solvated in hexane before the sample load. After the lipids were loaded, 4 mL hexane was used for washing the sample, which was considered as fraction one from DSC-Si column. The loads for heart, and brain acetone powder were 100 µL of the lipid extract in each case. The lipids were then eluted with 1 mL of each 99:1 hexane: acetic acid, 90:10 hexane: ethyl acetate, 80:20 hexane: ethyl acetate, and 70:30 hexane: ethyl acetate, 1.5 mL of 2:1 chloroform: 2-propanol, and finally with 2 mL of methanol. Fraction six containing the amides, monoacyl glycines, *N*-acylglycines and the *N*-acylethanolamines was dried

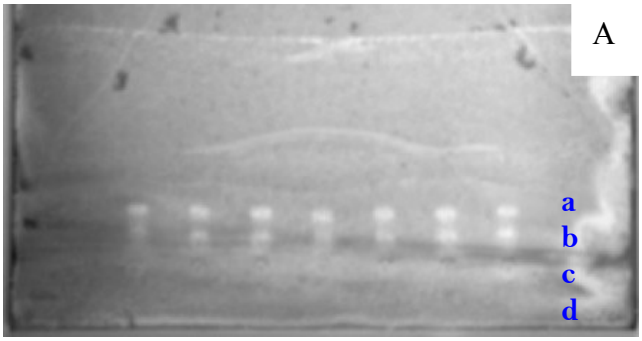
under N_2, re-dissolved in 200 μL of hexane and loaded onto DSC-NH_2 column. The sample was adsorbed onto the phase and then washed with 2 mL of hexane, which was called fraction one from DSC-NH_2 column. The lipids were then eluted by 2 mL of chloroform, 2 mL of 2% chloroform in 2-propanol, 1 mL of 2% chloroform in 2-propanol, 2 mL of 3% chloroform in 2-propanol and finally with 2 mL of methanol. Fraction three from DSC-NH_2 column containing amides was dried under N_2 and derivatized with BSTFA as described above before injecting 1 μL sample into GC/MS.

6.4 Results and Discussion

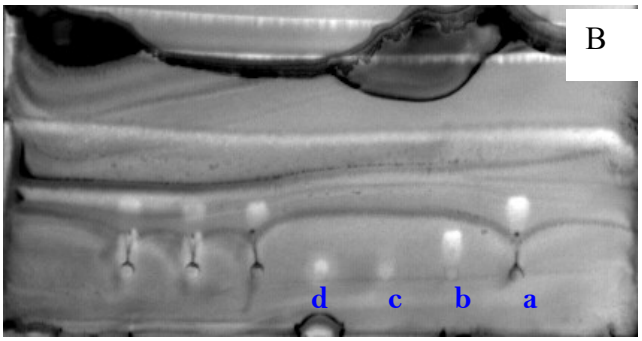
6.4.1 Separation of PFAM's by argentation-HPTLC

In order to develop a method for the separation of primary fatty acid amides by Ag-HPTLC, free fatty acid (FFA) were tried to begin with. The reason behind this is that fatty acid methyl esters are the mostly studied lipid class by Ag-HPTLC (see Section 6.2). The goal was to optimize a method for the separation of FFA and include amides into the method. To start out the method, HPTLC plates were developed in 10% solution of $AgNO_3$ in acetonitrile. Four C18 acids were loaded onto the plates and were developed in various solvents (such as hexane, diethyl ether, methanol, tetrahydrofuran etc) in order to follow how the acids move. These acids were stearic acid (no double bond), oleic acid (one double bond), linoleic acid (two double bonds) and linolenic acid (three double bonds). Unfortunately, FFAs were not found to be separated by a single solvent system. It was clear from the literature review that the fatty acid methyl esters are well separated by developing with a mixture of solvents containing a large portion of nonpolar one. Different solvent systems were therefore tried which included

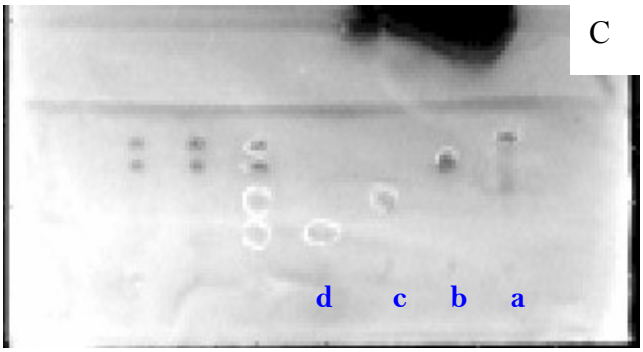
hexane:diethyl ether (97:3), hexane:diethyl ether:acetone (60:40:5) and hexane:diethyl ether:acetone (40:30:15). FFAs were found to be separated by developing the plates in the latter solvent up to 10 cm of the plates (Figure 6-1).



Acid	R_f Value
a: Stearic (18:0)	0.21
b: Oleic ($18:1^{9}$)	0.14
c: Linoleic ($18:2^{9,12}$)	Undetectable
d: Linolenic ($18:3^{9,12,15}$)	Undetectable



Acid	R_f Value
a: Stearic (18:0)	0.31
b: Oleic ($18:1^{9}$)	0.15
c: Linoleic ($18:2^{9,12}$)	~Origin
d: Linolenic ($18:3^{9,12,15}$)	~Origin



Acid	R_f Value
a: Stearic (18:0)	0.55
b: Oleic ($18:1^{9}$)	0.41
c: Linoleic ($18:2^{9,12}$)	0.32
d: Linolenic ($18:3^{9,12,15}$)	0.14

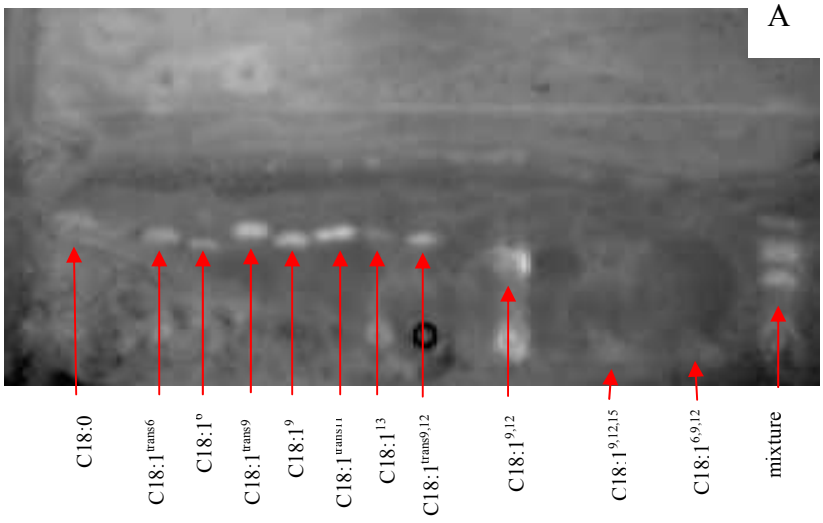
FIGURE 6-1: HPTLC Plate developed A) in hexane: ether: acetone (60:40:5 v/v/v) up to 10 cm, B) twice in hexane: ether: acetone (60:40:5 v/v/v) to the top and C) in hexane: ether: acetone (40:35:15 v/v/v) up to 10 cm.

It is clear from the picture that they are separated based on their number of double bonds, with the most unsaturated one being strongly retained by Ag. These plates were visualized under UV light (254 nm) by spraying primuline after reducing the Ag on the plates (see Section 6.3.6.3).

Similar solvent systems were therefore tried for the separation of ten different C18 PFAM's. Figure 6-2(A) shows that a slight separation of amides occurs using the same solvent system and under the same conditions as those used for the separation of acids. An improved separation was observed by developing the plates in same solvent system twice upto 7 cm [Figure 6-2 (B)]. The amides with higher number of unsaturation were more strongly retained by Ag. For example, linolenamide ($C18:3^{9,12,15}$) with 3 sites of unsaturation did not move from the origin at all. Due to the comparatively higher polarity of the amides than acids, the resolution between the amides was not satisfactory. Even though a slight separation between *cis* and *trans* isomers (*cis* being strongly retained) was observed when separately run, a mixture was not separated. A slight increase in retention strength was also observed for the amides with double bonds closer to the amide functional group (i.e., $C18:1^{6}$ was strongly retained than $C18:1^{9}$, which was again strongly retained than $C18:1^{13}$). These plates were also Ag impregnated by dipping them into 10% $AgNO_3$ solution in acetonitrile. One problem that was encountered during the above procedure was the presence of higher percentage of Ag on the plate. Even though the Ag was being reduced, the dipping procedure in some cases tended to wash the acids off too. Also the reduced portion of the Ag quenched the primuline fluorescence making the visualization difficult. It was therefore necessary to use a lower percent of $AgNO_3$ solution for the

plate impregnation. Literature review indicates that $AgNO_3$ contents higher than 5% in the layer have no effect on retention or resolution, more over 0.5% methanolic $AgNO_3$ solution was found to show excellent resolution for fatty acid methyl esters and triacyl glycerols [see Section 6.2].

HPTLC plates were therefore developed in 0.5% methanolic $AgNO_3$ solution. After 10 different C18 amides were loaded onto the HPTLC plates, the plates were developed in the same solvent as before [see Figure 6-3(a)]. Developing the plates twice in hexane:ether:acetone (40:35;15; v/v/v) did not improve the resolution except moving them collectively away from the origin [Figure 6-3(B)]. The washing steps were found to be easier in this case with no problem in getting rid off Ag. The detection of the amides improved to a much higher degree due to the presence of lower amount of Ag. One problem observed with this lower percentage of Ag was though the lesser degree of resolution between the amides than that observed with higher silver content [see Figures 6-2(A) and 6-3(A)]. Therefore it will be necessary to optimize the silver content on the plate for the optimum resolution as well as for optimum detection of the primary fatty acid amides.



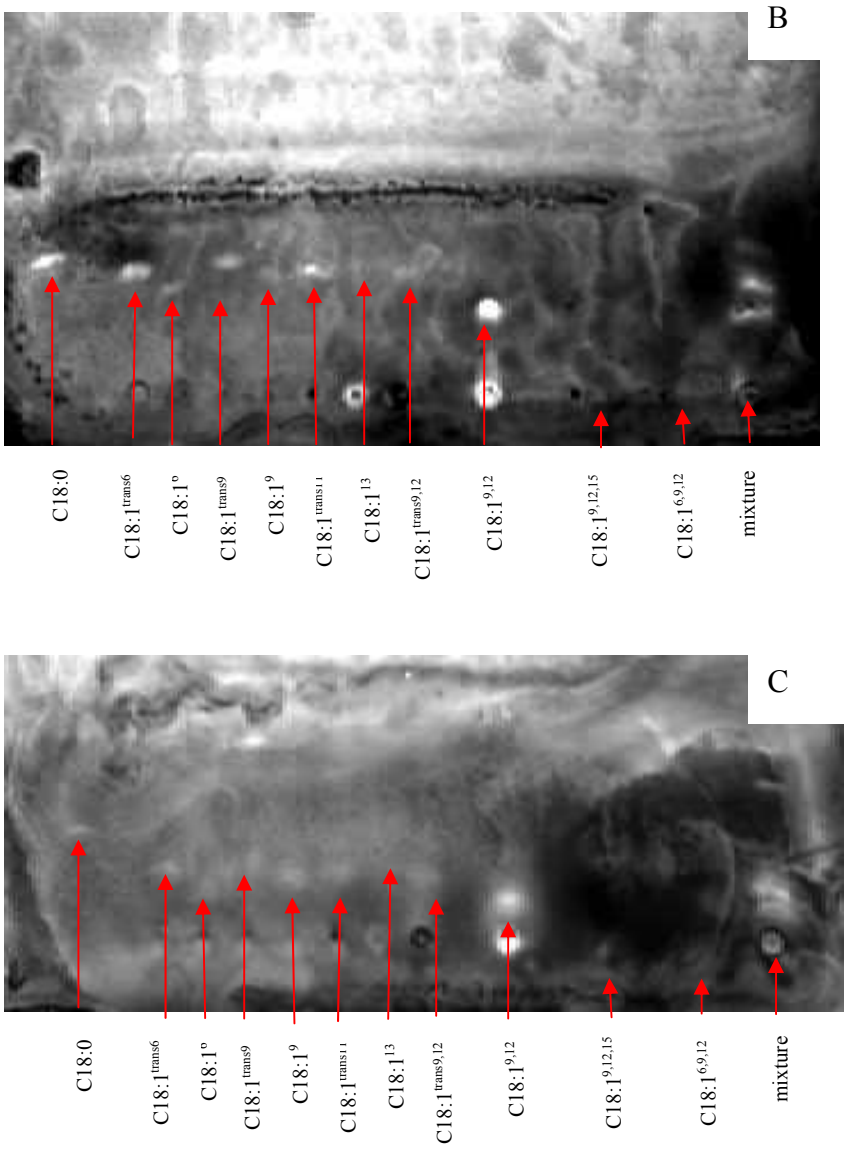


FIGURE 6-2: Separation of C18 unsaturated primary fatty acid amides by Argentation-HPTLC. The plates were impregnated by dipping them in 10% $AgNO_3$ in acetonitrile. Plate development A) in hexane: ether: acetone (40:35:15; v/v/v) up to 10 cm, B) twice in hexane: ether: acetone (40:35:15; v/v/v) to 7 cm, and C) in chloroform: methanol: acetic acid (95:5:1; v/v/v) to 5 cm, in hexane: ether: acetone (60:40:05; v/v/v) to 7 cm and finally in hexane: ether (97:3; v/v) up to 10 cm.

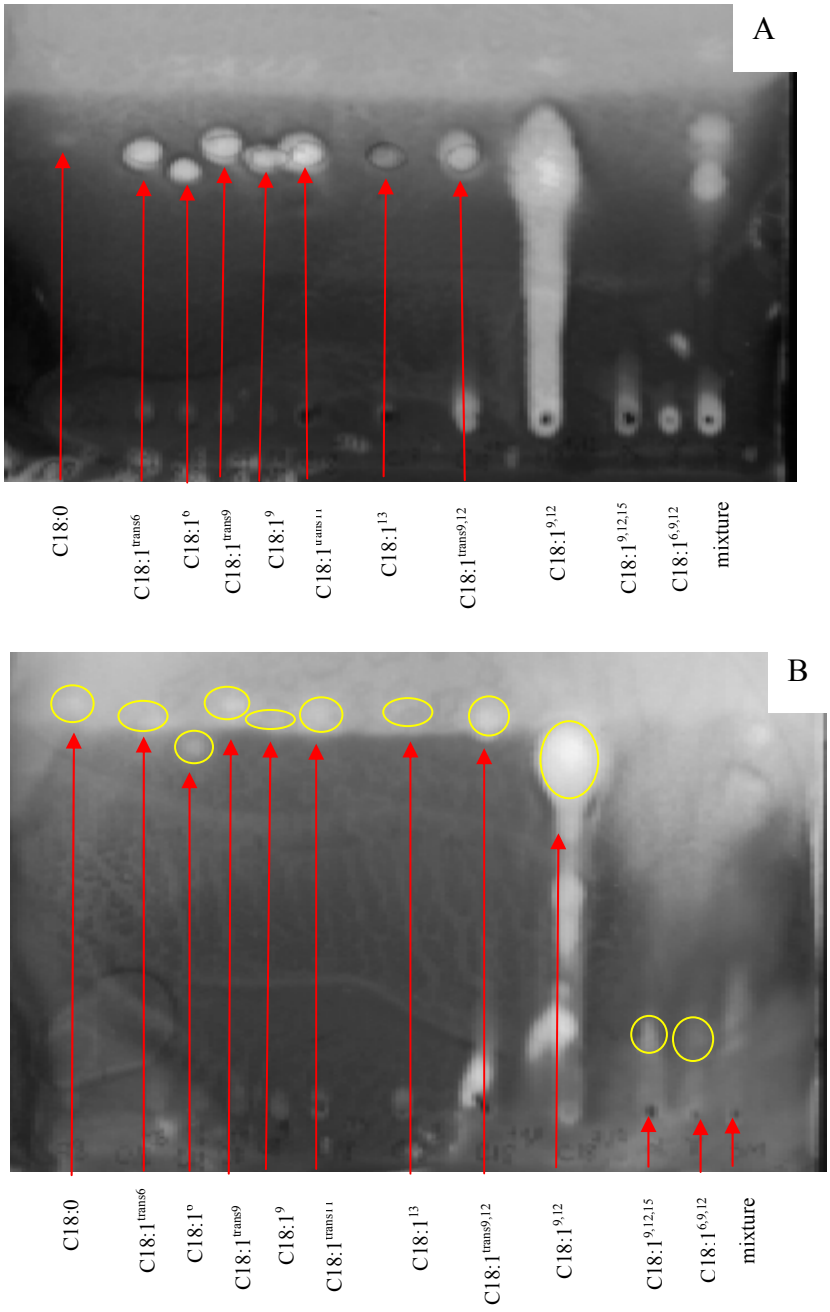


FIGURE 6-3: Separation of C18 unsaturated primary fatty acid amides by Argentation-HPTLC. The plates were impregnated by dipping them in 0.5% $AgNO_3$ in methanol. Plate development A) in hexane: ether: acetone (40:35:15; v/v/v) up to 10 cm, B) twice in hexane: ether: acetone (40:35:15; v/v/v) up to 10 cm.

6.4.2 Separation of PFAM's by BPX70 column

6.4.2.1 Some insight into polar columns

It was discussed in previous chapters that unsaturated PFAM's with the same number of carbons can not be separated by a non polar column like HP-5MS (see Figure 6-4). This was not unexpected because the amides are relatively polar and there is a tight margin between their polarities. Because BPX70 is a highly polar column, it can be utilized for their separation. There are a number of columns available from different manufacturers which have similar polarities as BPX70, but none of them have ever been used in conjunction with a mass spectrometer. The reason behind this is their high bleed profile, which can saturate the detector. These are mainly the highly polar columns with low temperature limit and mostly being used with FID. There are numerous literature/application notes available about these columns being successfully used in the separation of long chain geometric and positional isomers of fatty acid methyl esters (FAME) in very short time. But it is important to point out that most of these separations were carried out with H_2 as carrier gas and FID as a detector. Both of these conditions are not used in majority of the separation laboratories. A very few cases of He use as a carrier gas were mentioned where the separations were rather sloppy and required longer time. BPX70 is the only polar column which has been found to be used with a MS [61] for the separation of FAME. This column was therefore used in this study for the separation of derivatized PFAM's. One crucial requirement for the analysis of PFAM's by the polar column is that they must be derivatized prior to the analysis in order to lower the retention time as well as to increase sensitivity. It is necessary to add here that analysis of non-volatile compounds by GC/MS requires prior

derivatization, but FFA and PFAM's can be analyzed by some non-polar columns (such as HP-5MS) with high sensitivity without derivatization. Derivatization is often required in trace analysis in order to increase the sensitivity. At low concentration, underivatized acids and amides tend to show lower sensitivity due to the tailing by their polar functional groups even on a non-polar column (HP-5MS is only 5% polar). This tailing problem is severe with polar columns and results in very poor sensitivity even when analyzing them at higher concentration. The underivatized compounds can be detected at higher concentration but the resolution between peaks is unacceptable. Therefore in order to analyze the primary fatty acid amides by polar columns, the NH_2 group needs to be deactivated through derivatization

6.4.2.2 Derivatization of PFAM's by BSTFA and analysis by GC/MS

A number of different techniques are available in the literature for the derivatization of the amides (39, 64). The most commonly used one is the derivatization by *N*, *N*-BSTFA. The protocol was optimized in this laboratory and can be successfully used for the derivatization of fatty acids and amides. In this study the PFAM's were derivatized with *N*, *N*-BSTFA at 95 ºC for 5 min and cooled at room temperature before acetonitrile was added. The derivatized amides were then run by GC/MS and individual mass spectral patterns were observed.

Derivatizing with BSTFA involves the replacement of one hydrogen from the amide by trimethylsilyl group forming trimethylsilyl-amide (TMS-derivative). While analyzing the mass spectra of the amides derivatized according to the protocol described above, it was observed that instead of TMS derivatives, nitrile derivatives were formed.

The reason might be the use of acetonitrile as a solvent which quickly converted TMS derivatives to nitrile derivatives in gas phase on-column. Figure 6-5 shows the mass fragmentation patterns of nine C18 amides.

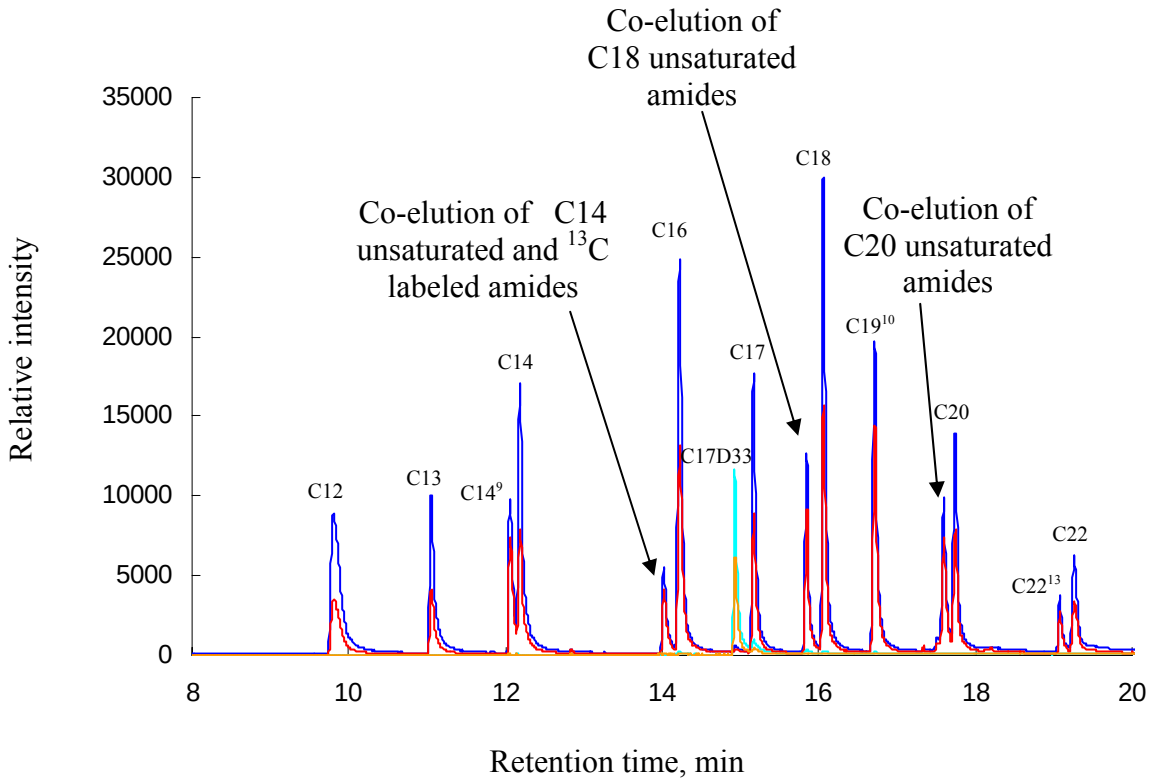


FIGURE 6-4**:** Elution profile for C12-C22 amides on a HP-5MS column. The relative intensities at m/z **59**, **72**, **62** and **76** are plotted against retention time. Refer to Chapter 2 for the description of the fragment ions.

Fig 6-5(a)

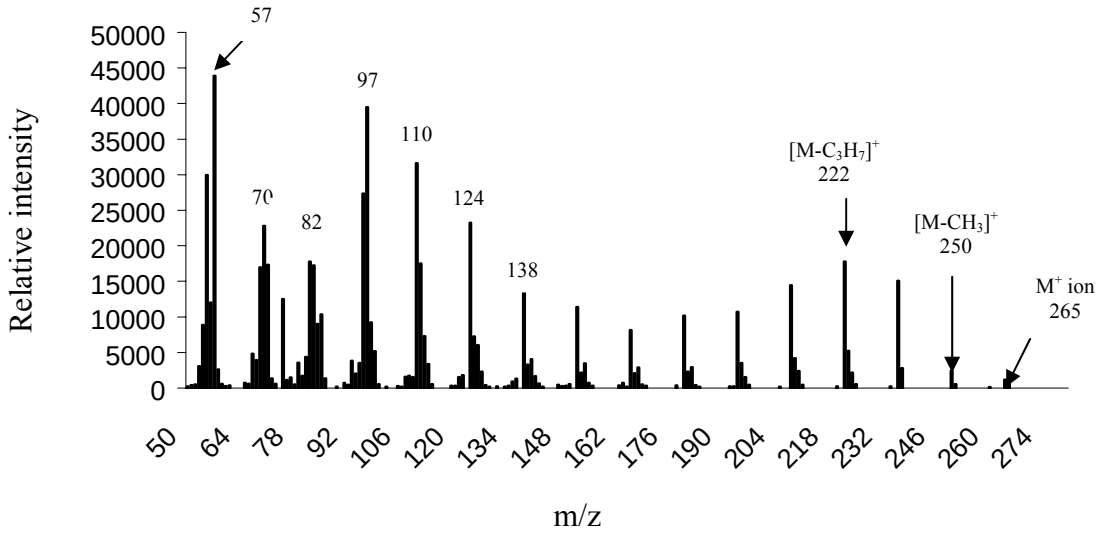


Fig 6-5(b)

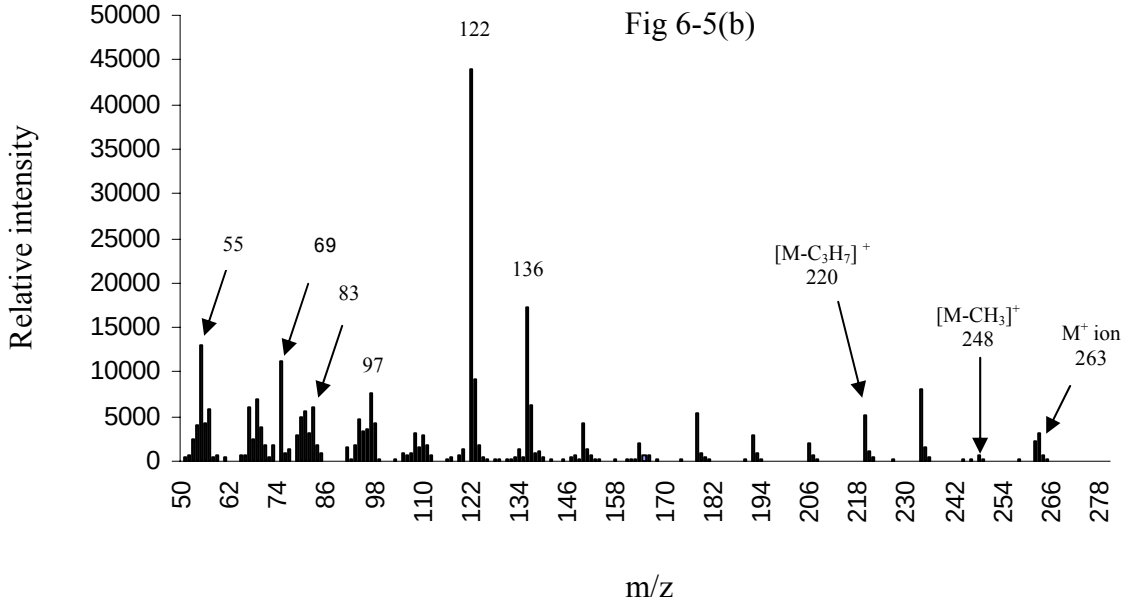


Fig 6-5(c)

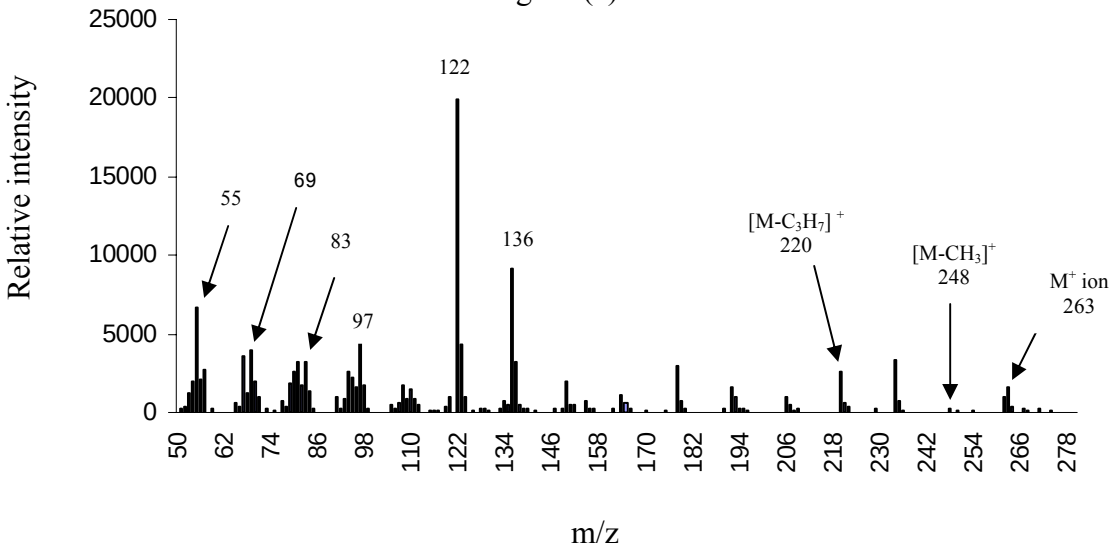


Fig 6-5(d)

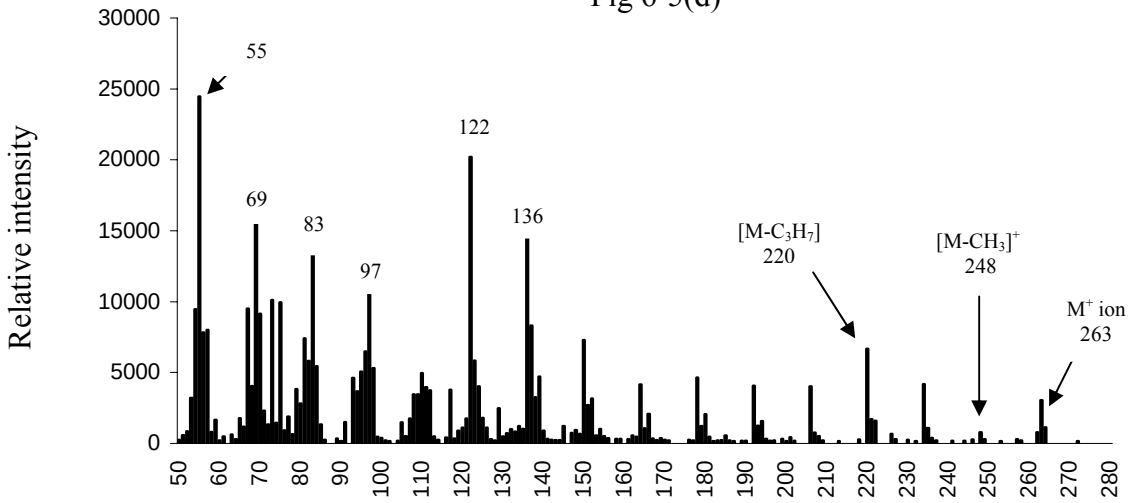


Fig 6-5(e)

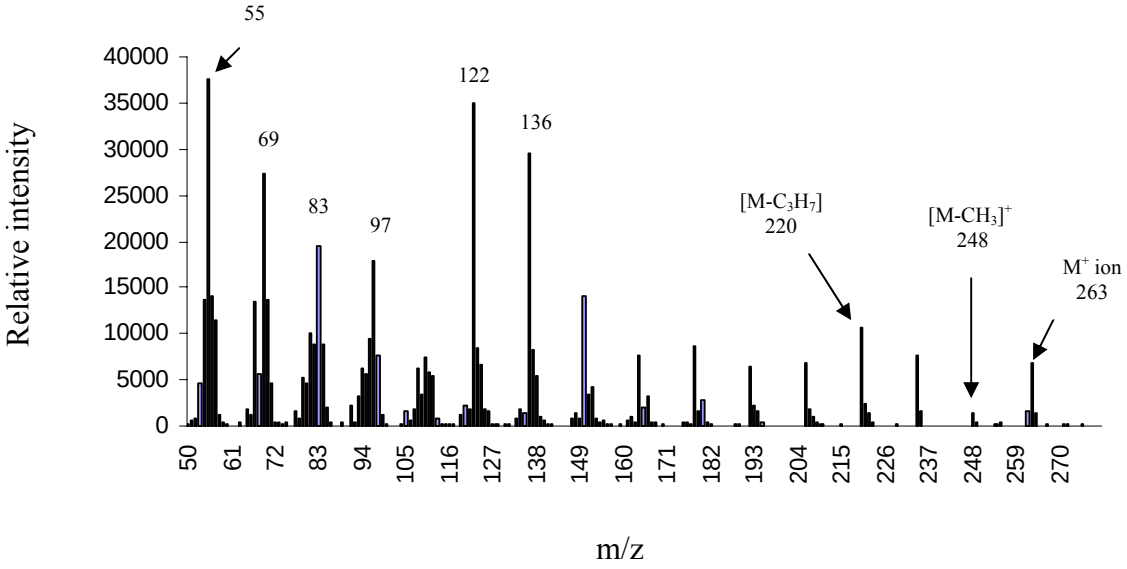


Fig 6-5(f)

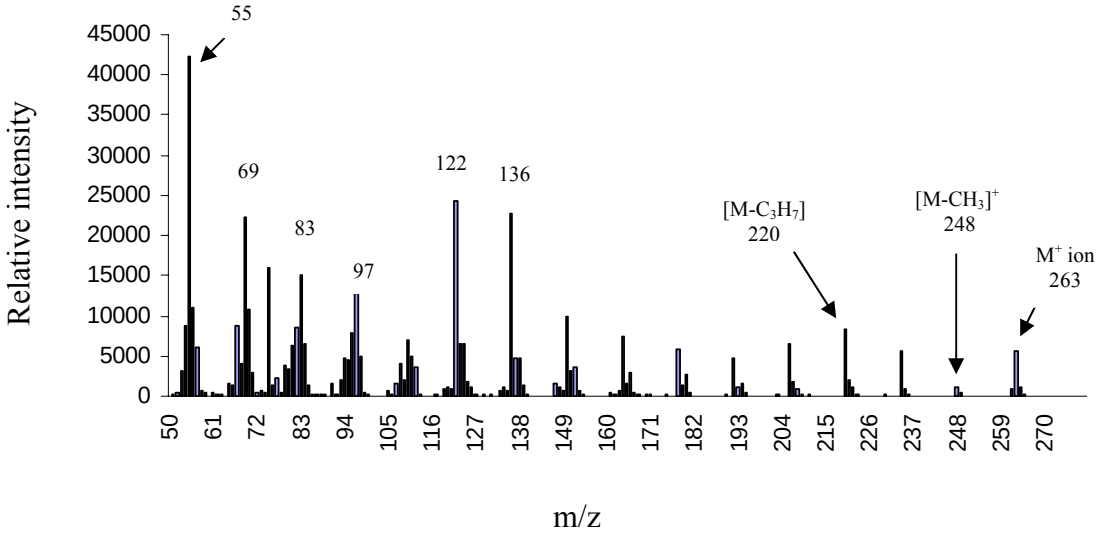


Fig 6-5(g)

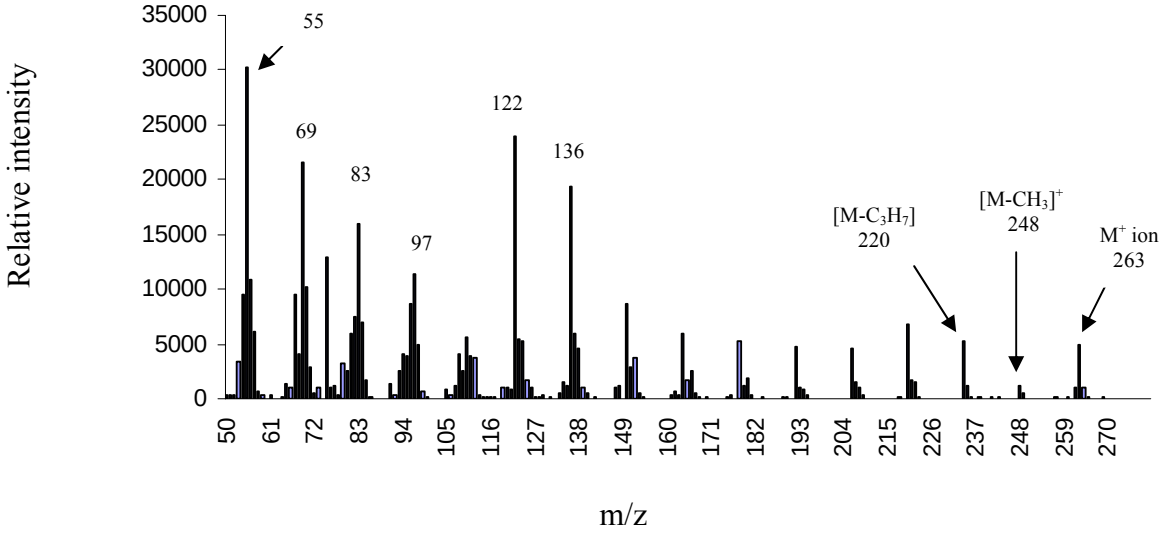


Fig 6-5(h)

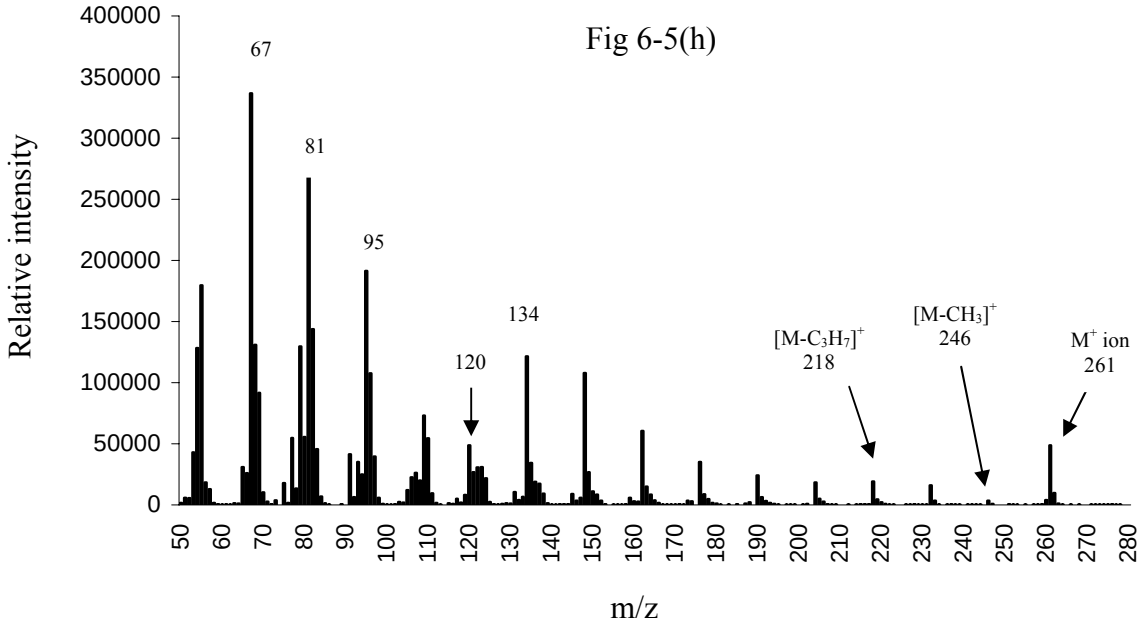


Fig 6-5(i)

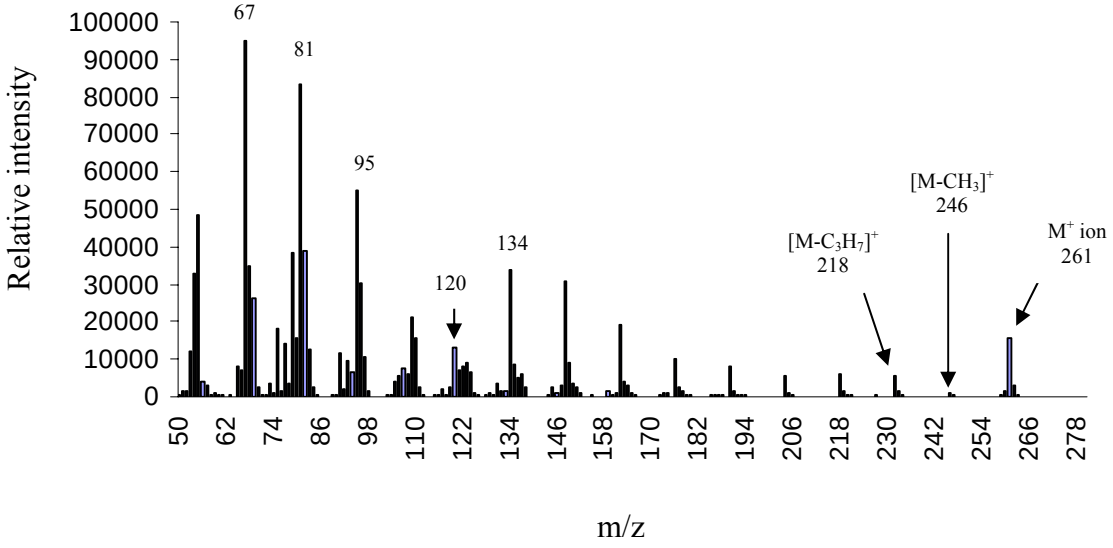


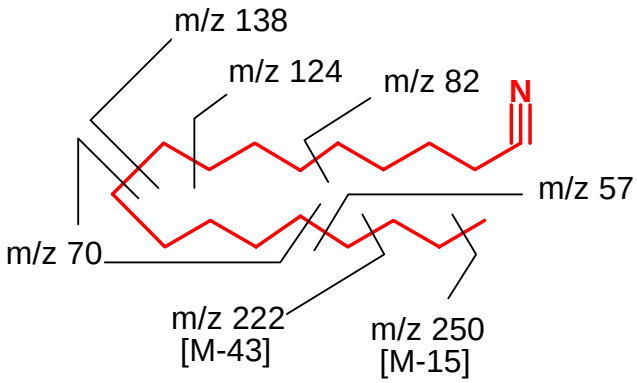
FIGURE 6-5: Mass spectrum patterns of nitrile derivatives of C18 amides (a) stearamide; C18:0, (b) petroselenamide; C18:1^{6}, (c) petroselaidamide; C18:1^{trans6} , (d) oleamide; C18:1^{9} , (e) elaidamide; C18:1^{trans9}, (f) Vaccenamide; C18:1 ^{trans11}, (g) *cis*-13 octadecenamide; C18:1^{13}, (h) linoleamide; C18:1^{9,12}, and (i) linoleladamide; C18:1^{trans9,12} . See scheme 6.1 for illustration of the patterns.

The structures for the common key fragment ions formed have been drawn for illustration in Scheme 6-1. All the amides (both saturated and unsaturated) showed similar fragmentation patterns which included the molecular ion peaks ($[M]^{+\cdot}$); the peaks due to the loss of -CH_3, ($[M-15]^+$); the peaks due to the loss of -CH_2-CH_2-CH_3, $[M-43]^+$ ion; and the peaks due to the consecutive loss of methylene groups (14 u).

Stearamide which is a saturated amide showed peaks at m/z 124 and m/z 138 [Figure 6-5(a)] due to the formation of $(C_8H_{14}N)^+$ and $(C_9H_{16}N)^+$ ions from C8-C9 and C9-C10 cleavages respectively. Stearamide also showed peaks at m/z 57 (base peak), m/z 70, and m/z 82 due the formation of $(C_4H_9)^+$ $(C_5H_{10})^+$ and $(C_5H_8N)^+$ ions

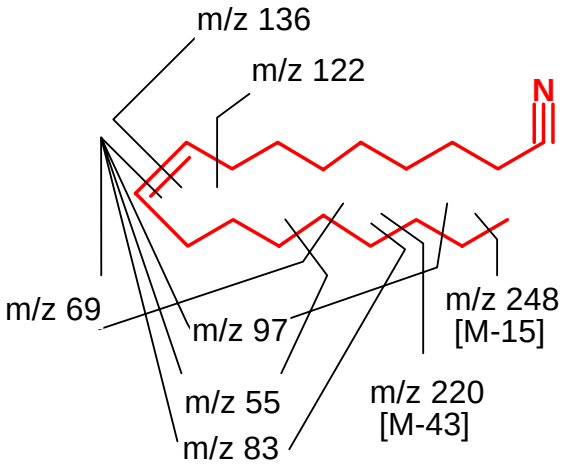
respectively [see Scheme 6-1(A)]. All the mono unsaturated amides showed characteristic peaks at m/z 122 and m/z 136 due to the $(C_8H_{12}N)^+$ and $(C_9H_{14}N)^+$ fragment ions. The other intense fragmentation patterns observed at m/z 55, m/z 69, m/z 83 and m/z 97 were due to the cleavage of the nitrile [see Scheme 6-1(B)]. For petroselenamide ($C18:1^6$) and petroselaidamide ($C18:1^{trans\ 6}$) with a double bond at sixth carbon, the base peak was observed at m/z 122. The intensities of the peaks at m/z 55, m/z 69, m/z 83 and m/z 97 were much lower than those observed for other monounsaturated amides [see Figure 6-5(b)-(c)]. This is most probably due to the position of the double bond, which was included in the most frequently formed peak at m/z 122 [see Scheme 6-1(C)]. The diunsaturated PFAM's did not show any fragment ions at m/z 69, m/z 83 and m/z 97, instead intense peaks at m/z 55, m/z 67, m/z 81, m/z 95 were observed [see Figure 6-5 (h)-(i)] due to the presence of an additional double bond. The other characteristic peaks resided at m/z 120 and m/z 134 due to the formation of $(C_8H_{10}N)^+$ and $(C_9H_{12}N)^+$ ions, respectively [see Scheme 6-1(D)]. These fragmentation pattern shows that the double bonds are preserved in the fatty acid amides after their synthesis from corresponding fatty acids.

Scheme 6-1(A)



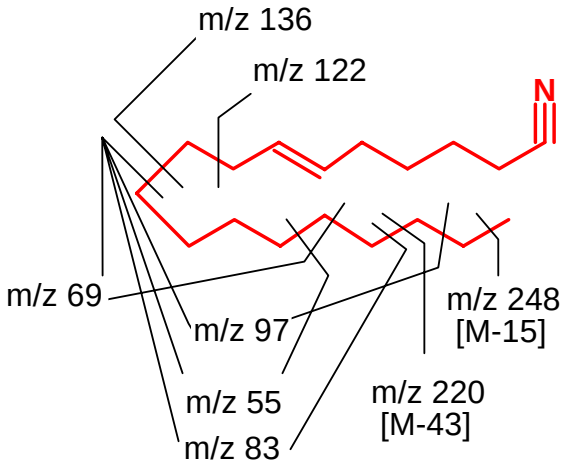
Nitrile derivatized stearamide, m/z 265, [M]

Scheme 6-1(B)



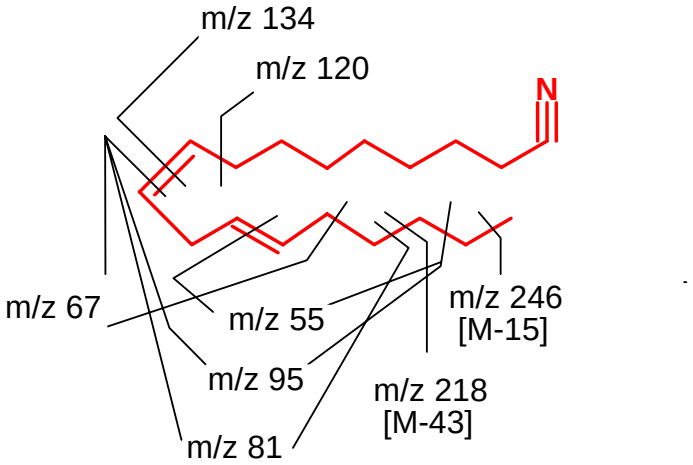
Nitrile derivatized oleamide, m/z 263, [M]

Scheme 6-1(C)



Nitrile derivatized petroselaidamide, m/z 263, [M]

Scheme 6-1(D)



Nitrile derivatized linoleamide, m/z 261, [M]

Scheme 6-1 Mass-spectral (quadrupole) fragmentations for A) stearamide; C18:0, B) oleamide C18:1^{9} (all the unsaturated amides shows similar patterns), C) petroselaidamide C18:1^{trans6}, and D) linoleamide; C18:2 ^{9,12}.

6.4.2.3 Separation of derivatized standard PFAM's

Once the identity and the retention time of the each derivatized amides was confirmed, a mixture of amides was run by GC/MS. Before the analysis, argon (a non- retained gas) was injected into the column and the average linear velocity was calculated to be 27 cm/sec for He carrier gas. Because the amides had high capacity factor, k′, at 27 cm/sec gas velocity, optimization needed to be done. In order to optimize the separation and lower the elution time, separation was carried out at 30 cm/sec, 35 cm/sec, and 40 cm/sec, average He gas linear velocities. At the latter velocity, elution profile was found to be shorter, which is better for the higher carbon amides. It was found that the amides from C20 to C22 elute near the maximum temperature limit of the column. They are therefore difficult to recognize and often co-elute with column bleed peaks. But the resolution between the C18 amides at this velocity was not satisfactory. A slightly better separation was observed using the velocity at 35 cm/sec than that using the velocity at 40 cm/sec, whereas 30 cm/sec provided the best resolution and elution profile among the four velocities tried.

Separation of nine C18 amides is shown in Figure 6-6 indicating excellent resolution between positional and geometric isomers, which was not achieved on a HP-5MS column. The *trans* isomers eluted at least a min before the *cis* isomers. The amides with double bond closer to the carbonyl carbon eluted earlier than those containing double bonds further away from the carbonyl carbon. Even though most of the monounsaturated amides showed nice separation from the unsaturated ones, C18:1^{13} was found to co-elute with C18:$2^{9,12}$ most probably due to the close position of their double bonds. Because the fragments for C18:1^{13} (m/z 122 and m/z 136) are different

from those for C18:2^{9,12} (m/z 67, m/z 87, m/z 120, m/z 134), it will be possible to separate them by monitoring those ions only (see Figure 6-6).

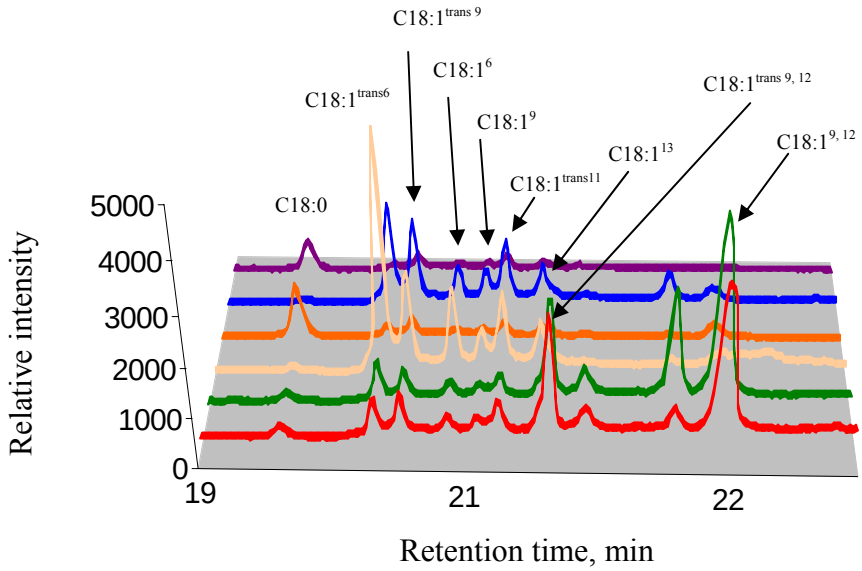


FIGURE 6-6: Separation of derivatized unsaturated C18 amides on a BPX70 column. The relative intensities of m/z 67, m/z 81, m/z 122, m/z 124, m/z 136, and m/z 138 are plotted against retention time (see Figure 6.5 for fragmentation patterns). Average linear velocity of He was 30 cm/sec. Co-elution of C18:1^{13} and C18:2 $^{trans\ 9,\ 12}$ occurs in this separation but still can be separately identified due to the m/z 122, and m/z 134 specific for C18:1^{13} which are not formed by C18:2 $^{trans\ 9,\ 12}$ (m/z 67 and m/z 81 are specific for linoleladmide).

The separation between four C20 amides was also achieved. Table 6-II shows their retention times in two different linear velocities. The retention times indicate that the elution profile for C20 amides follows similar trend as C18 amides in terms of geometric and positional isomers. The *trans* amides elute before the *cis* amides and as

the position of the double bond moves further away from the carbonyl carbon, the retention time increases. The problem with these higher molecular weight amides is that they elute near the maximum temperature of the column and are often accompanied column bleed peaks. They were not included in this study because base line resolution was not achieved for them. It is important to add that this separation indicates not only the position of the double bonds but also the stereochemistry of the bonds was preserved after the synthesis of the amides from their corresponding acids.

TABLE 6-II: Retention time of four C20 unsaturated amides at different average linear velocity of He carrier gas.

Amides	Retention time, min at different He gas average linear velocity	
	30 cm/sec	40 cm/sec
$C20{:}1^{5}$	22.97	20.84
$C20{:}1^{trans\ 11}$	23.37	22.12
$C20{:}1^{11}$	23.81	22.47
$C20{:}1^{13}$	23.92	22.59

6.4.2.4 Separation of PFAM's from rabbit brain and heart

The analysis of the derivatized amides isolated from rabbit brain and heart shows that oleamide is the only major unsaturated fatty acid amide present in those tissues (see Figure 6-7). Trace amounts of linolemide and *cis*-13- eicosenoamide were also observed besides oleamide, stearamide and palmitamide in brain tissues [Figure 6-7(a)]. Heart tissues contained only stearamide and palmitamide besides oleamide [Figure 6-7(b)].

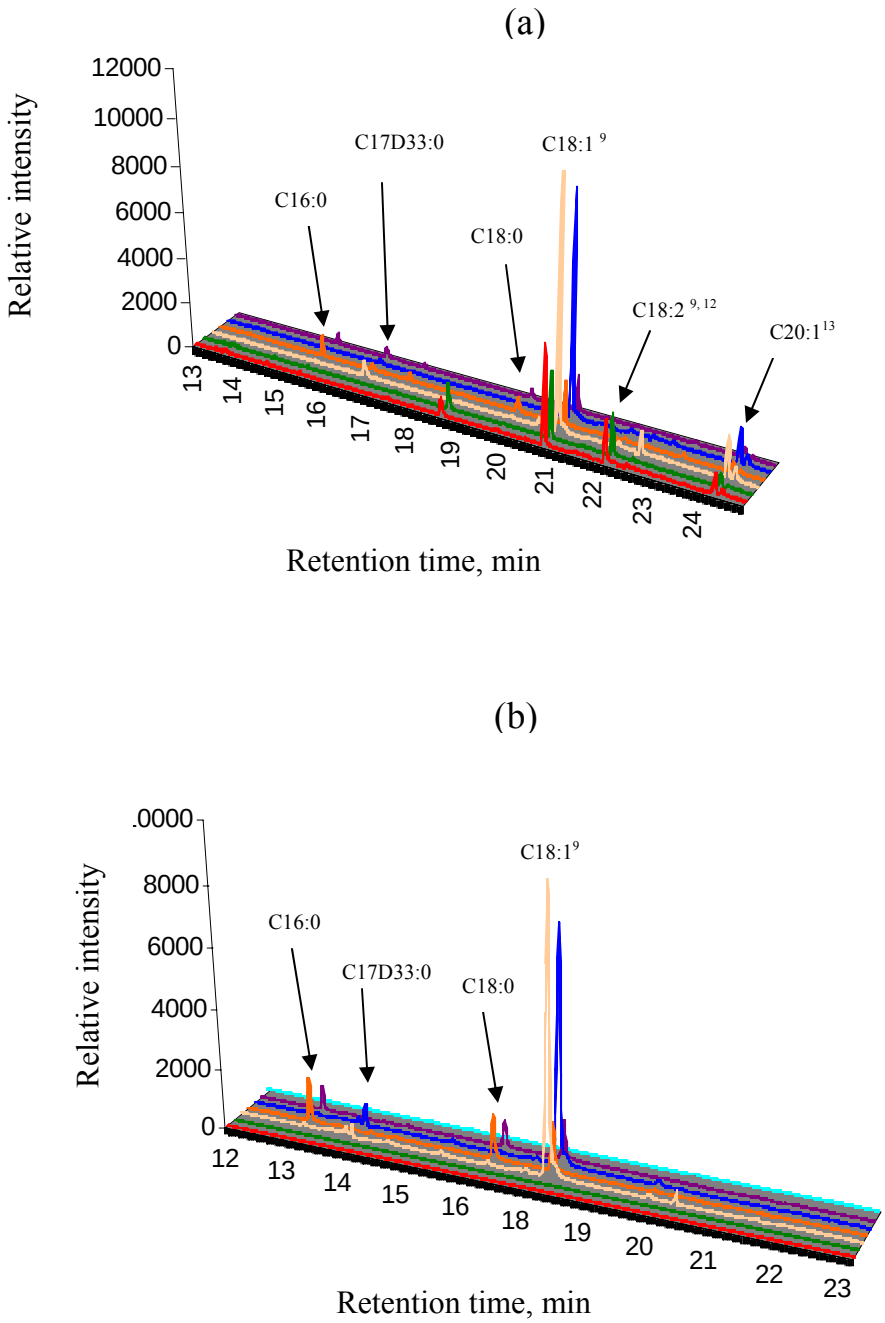


FIGURE 6-7: Separation of derivatized unsaturated C18 amides from a) rabbit brain acetone powder and b) rabbit heart on a BPX70 column. The relative intensities of m/z 67, m/z 81, m/z 122, m/z 124, m/z 136, and 138 are plotted against retention time. (see Figure 6.5 for fragmentation patterns). Average linear velocity of He was 30 cm/sec for

(a) and 40 cm/sec for (b). Peaks in figure (a) therefore eluted around 1.5 min later than those in (b).

Beacuse fatty acid amides are used as slip additives in plastics and can contaminate the sample upon the use of such containers, a method blank was run and subtracted from each sample spectrum. This method therefore could be an excellent way of identifying the types of amides present in different parts of mammalian tissues. It was basically a qualitative study to identify the unsaturated amides that are co-eluting with the oleamide peak. The quantitation is the next step that needs to be done. Also the tissues that were used in this study are available in larger quantities in which case detection is not an issue, but mapping the amides in different parts of tissue will be critical. For example, concentration of amides in different brain parts such as in hypothalamus, neocortex, basal ganglia, hippocampus, amygdale, cerebellum, brain stem and spinal cord could be in the order of picograms. Sensitivity will definitely be an issue for amide identification and quantitation in these types of samples using a polar column. One solution might be to find a better derivatizing agent for producing more stable derivatized amides for better sensitivity as well as lower detection limit.

6.5 Conclusions

In conclusion, we have developed an efficient method for the separation of primary fatty acid amides by BPX70 column. Unsaturated C18 amides were separated with excellent resolution in terms of the number, position and stereochemistry of the double bonds. In this study, the C20-C22 unsaturated amides were not employed due to the incapability of the high bleed, polar BPX70 column to provide baseline resolution for

these amides. An argentation-HPTLC method was also studied for the separation of C18 unsaturated amides. A nice resolution between the saturated and the unsaturated amides were observed in this case. A satisfactory resolution between the geometric and the positional isomers could not be achieved. As discussed in the results and discussion section, optimization of the amount of silver might help improve these types of separation. Even though the amides isolated from rabbit tissues were successfully separated by the BPX70 column, quantification still needs to be carried out for these tissues as well as other tissues known to express PAM. As discussed in earlier chapters, PAM is the catalyst for the final step of oleamide biosynthesis. Tissues known not to express PAM (such as kidney) will also be analyzed and will be a good control for future studies.

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6.7 References

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Appendices

Appendix-A: Publications

1. Khan, Shahed U. M. and **Sultana, Tamanna**, "Photoresponse of n-TiO_2 thin film and nanowire electrodes", *Solar Energy Materials and Solar Cells*, 2003, 76(2), 211-221.
2. **Sultana, Tamanna** and Johnson, Mitchell E. "Analysis of fatty acid amide lipid class by instrumental means", 2005 (review in preparation)
3. **Sultana, Tamanna** and Johnson, Mitchell E. "Small-scale synthesis and characterization of naturally abundant and isotopically enriched primary fatty acid amides", 2005 (manuscript under preparation)
4. **Sultana, Tamanna**; Stamolis, Christina and Johnson, Mitchell E. "Total lipid profiling of $N_{18}TG_2$ mouse neuroblastoma cells by HPTLC and quantification of primary fatty acid amides (PFAM's) by GC/ MS", 2005 (manuscript under preparation)
5. **Sultana, Tamanna** and Johnson, Mitchell E. "Solid phase extraction of primary fatty acid amides: Method development by standard lipids and method validation by $N_{18}TG_2$ cell lipids", 2005 (manuscript under preparation)
6. **Sultana, Tamanna** and Johnson, Mitchell E. "Distribution of fatty acid amides in mammalian tissue", 2005 (manuscript under preparation)
7. **Sultana, Tamanna**; DiCicco, Leigh A.; Shank, Elise; Zatorski, Jennifer; Koulter, Corey and Johnson, Mitchell E. "Separation of unsaturated fatty acid amides by argentation HPTLC and GC/MS", 2005 (manuscript under preparation)

Appendix-B: Poster Presentations

1. **Sultana, Tamanna** and Johnson, Mitchell E. "Isolation of primary fatty acid amides by solid phase extraction: An approach towards method development", *Abstracts of Papers, 228th ACS National Meeting*, Philadelphia, PA, United States, August, 22-26, 2004, ANYL-113.
2. Leigh Anne DiCicco; **Sultana, Tamanna** and Johnson, Mitchell E. "Separation of fatty acids and amides using argentation thin-layer chromatography", *Abstracts of Papers, 35th Central Regional Meeting of the American Chemical Society*, Pittsburgh, PA, United States, October 19-22, 2003, Poster # 287.
3. **Sultana, Tamanna** and Johnson, Mitchell E. "Total lipid analysis of $N_{18}TG_2$ cells by HPTLC", *Abstracts of Papers, 226th ACS National Meeting*, New York, NY, United States, September 7-11, 2003, ANYL-059.

Appendix-C: Oral Presentations

1. **Sultana, Tamanna** and Johnson, Mitchell E. “Solid phase extraction of trace lipid amides from biological samples”, *PittCon-2005*, Orlando, FL, United States, February 27 - March 04, 2005. Paper # 1510-4.
2. **Sultana, Tamanna**, Ferrenc, Chris; Landers, James P. and Johnson, Mitchell E. “Selective extraction of lipid classes using packed beds on microfluidic chips”, *PittCon-2005*, Orlando, FL, United States. February 27 – March 04, 2005. Paper # 1820-9.
3. **Sultana, Tamanna** and Johnson, Mitchell E. “Total lipid analysis of various $N_{18}TG_2$ cells by HPTLC and GC/MS”, *PittCon-2004*, Chicago, IL, United States, March 07-12, 2004. Paper # 6200-800.
4. **Sultana, Tamanna** and Johnson, Mitchell E. “Method development of total lipid analysis by high performance thin layer chromatography (HPTLC)”, *PittCon-2003*, Orlando, FL, United States, March 09-14, 2003. Paper # 2630-6.
5. **Sultana, Tamanna** and Johnson, Mitchell E. “Separation of fatty acid amides by gas chromatography coupled with mass spectrometry (GC-MS)”, *FACSS-2002: The 29th Annul Conference*, RI, United States, October 13-17, 2002. Paper # 628.
6. **Sultana, Tamanna** and Khan, Shahed U. M. “Photoelectrochemical splitting of water on nanocrystalline n-TiO_2 thin film and quantum wire electrodes”, *Proceedings: Electrochemical Society* (2001), 2001-19 (Quantum Confinement VI: Nanostructured Materials and Devices), 9-19.