Targeted Lipidomics: Analytical Strategies for Fatty Amides

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TARGETED LIPIDOMICS:
ANALYTICAL STRATEGIES FOR FATTY AMIDES

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ABSTRACT

TARGETED LIPIDOMICS:
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Dissertation supervised by Mitchell E. Johnson, Ph.D.

There are many significant compounds whose resting levels in biological systems are at nanomolar concentrations or below. In order to more effectively study these compounds work is being done to develop a completely automated system on a microchip. Several steps will be taken to start setting up this system because, at these low concentrations, detection is problematic for many current methods of analysis. First, develop a high performance liquid chromatography with mass spectrometry detection (HPLC/MS) method using atmospheric pressure chemical ionization (APCI) to detect primary fatty acid amides (PFAMs), one of the lipid classes of interest. Additives such as formic acid will be added to the mobile phase in an HPLC/MS method in order to increase the analyte signal and lower detection limits. Second, develop a LC method to replace the solid phase extraction (SPE) method that uses a gradient elution instead of the
step elution currently done. The LC method would allow for analysis with electrospray mass spectrometry (ESI-MS) in order monitor the separation between the “polar” neutral lipid classes. Third, develop a method using capillary electrochromatography (CEC) to separate the fatty amines that have been derivatized for fluorescence detection on a microchip packed with a C18 chromatographic stationary phase.
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Chapter 6

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Chapter 1

Methods of Separating and Detecting “Polar” Neutral Lipids

1.1 Abstract

Lipids are a class of compounds that vary widely in polarity and function. Some lipids form the structural component of living cells, while others are involved with intercellular communication and energy storage. This review will focus on the neutral lipid classes – more specifically the more polar of the neutral lipids (free fatty acids, primary fatty acid amides, monoacylglycerol, diacylglycerol, and N-acylglycines, N-acylethanolamines and N-acylamino acids). These lipid classes are biologically active, but because they are found at trace levels in the cells, the analysis can be tedious and in some cases has not been studied extensively. This review will focus mainly on the analytical techniques that have been developed for separating and analyzing the “polar” neutral lipids – GC/MS, LC/MS, MALDI/MS, SPE and TLC.
1.2 Background and Significance

Lipids vary greatly in structure and functionality among the different lipid classes. One of the few similarities among all the classes of lipids is that they are insoluble in water. Lipidomics is the study of lipids focused on fingerprinting the entire cellular lipidome in order to be able to understand lipid metabolism and lipid-mediated signaling [2]. The goals of lipidomics include quantifying each molecule, identifying the sub-structures of the cell formed by lipids, and determining the mechanisms with which the lipids interact with each other [2]. The motivation of these projects is to be able to gain new insights into health and disease. Preliminary lipid studies of the lipidome have already shown that phosphatidylethanolamine (PtdEtn) is the main storage deposit for arachidonic acid (AA) in resting platelets, that there are different effects from saturated and unsaturated acyl functional groups in cellular metabolism, and that there are different lipid concentrations in the postmortem gray and white matter found in the brain samples from subjects with varying levels of Alzheimer’s disease [2]. The example list of diseases influenced by lipid production includes rheumatoid arthritis, sepsis, asthma, cancer and stroke [3, 4]. Because sample size can be limited and often the lipids are found at trace levels, methods need to be developed to allow for quantitation of the lipids at these low levels. Some work is being done to study the normal levels of a one lipid. For example, while looking for improved clinical and biochemical techniques to quantify the cholesterol levels in human skin fibroblast samples, Liebisch et al. developed an ESI-MS/MS method [5]. The goal of this method was to quantify the free cholesterol and cholesteryl ester levels by derivatizing the lipids with acetyl chloride before infusing into the MS. A few reviews summarize the different experimental approaches to lipidomics
research [4, 6-12]. With current technology, it is impossible to analyze the total lipid profile of a complex tissue sample at one time. One column is not able to separate the various polarities, chain lengths, and varying bond positions completely. Two dimensional or even multiple dimensional separations are being developed to allow for the analysis of more types of lipids in one sample, but this analysis is still limited to a few classes of lipids. A more common approach is to focus on one or two lipid classes to study in detail [13-15].

Lipids can be divided into classes based on polarity, structure or function. A naming system for lipids, described by Fahy et al. [16], has been adopted. This system breaks the lipids down into eight classes based on structure and function: fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterol lipids (ST), prenol lipids (PR), saccharolipids (SL), and polyketides (PK). Using this classification of lipids the “polar” neutral lipids are in the following subclasses: fatty acyls (FA), and glycerolipids (GL), shown in Table 1-1. The free fatty acids (FFAs), the primary fatty acid amides (PFAMs), the N-acyl ethanolamines (NAEs), and the N-acyl amino acids (NAAs) are part of the FA lipid class. The N-acyl glycines (NAGs) are included as part of the NAA subclass. FAs are hydrophobic in nature because of the long carbon chains, which can be saturated or contain double or triple bonds. For these studies (see Chapters 2-6) only the straight chain FAs were included, but lipids in this class can contain rings or branches. The monoacylglycerols (MAGs) and the diacylglycerols (DAGs) are part of the GL lipid class. The GL class contains all the glycerol-containing lipids that can be mono-, di-, or tri-substituted, except for the GPs which form their own class. GLs are used as metabolic fuels and signal molecules.
<table>
<thead>
<tr>
<th>Class Name (abbreviation)</th>
<th>Example Name (subclass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty Acyls (FA)</td>
<td>oleic acid (free fatty acid – FFA)</td>
</tr>
<tr>
<td></td>
<td>lauramidine (primary fatty acid amide - PFAM)</td>
</tr>
<tr>
<td></td>
<td>anandamide (N-acyl ethanolamines - NAE)</td>
</tr>
<tr>
<td></td>
<td>N-arachidonoyl glycine (N-acyl amino acids - NAAs)</td>
</tr>
<tr>
<td>Glycerolipids (GL)</td>
<td>1-monoleoyl-rac-glycerol (monoacylglycerol - MAG)</td>
</tr>
<tr>
<td></td>
<td>1,3-dipalmitoyl-rac-glycerol (diacylglycerol - DAG)</td>
</tr>
</tbody>
</table>

Table 1-1 Example lipids for each subclass of interest grouping them by lipid class.

1.3 Separation and Analysis Methods

When developing analysis methods for a particular class of lipids, the presence of the other classes of lipids needs to be considered. Usually the unwanted lipid classes are removed during sample preparation (i.e. solid phase extraction, thin layer
chromatography or liquid-liquid extraction) [17-20]. If the unwanted lipids are not removed they can interfere with the analysis either by blocking the transfer lines or increasing background noise. Solubility is also an issue as the total mass of lipids is increased.

### 1.3.1 Free Fatty Acids (FFAs)

The free fatty acids are part of the fatty acyl class of lipids. A few reviews have been written summarizing the different instruments that have been used in FFA studies [7, 21-23]. GC/MS analysis is popular for analyzing the FFAs because of their low molecular weight [20, 24-29]. The analysis conditions vary depending on the number of FFAs included and the required detection limits. Several reviews have been published summarizing the FFA GC/MS analysis methods. The analysis parameters for the different GC separation of the longer FFA have been summarized by Rezanka and Votruba [30]. In these cases, the FFA had been derivatized before separation. The method of detection was included but the detection limits were not included. A few books have chapters dedicated to the separation of FFAs. Kramer et al. compared the separations of C18:2 isomers on different columns [31]. This summary includes the extraction method, but does not note how much the percent recovery changed from one method to the next. Tevini and Steinmueller reviewed the separations of saturated FFA and a few with some degrees of unsaturation [32] while Sébédio and Ratnayake reviewed the separations focusing on the trans C18:1 isomers [33]. Christie wrote a review
showing the fragmentation patterns of different fatty acid derivatives [34]. A variety of analysis methods have been developed to focus on either saturated or a series of unsaturated FFAs.

Most of the LC/MS analysis of FFA has been done on derivatized fatty acids [18, 19, 28, 35-40], but separations have been developed for un-derivatized fatty acids [20]. The different types of derivatization agents to enhance sensitivity for UV and fluorescence have been summarized by Smith [41], Rosenfeld [38], and Brondz [42]. When several FFA are present, the analysis can be lengthy. In one example, the total analysis was over two hours long between the derivatization and separation of the fatty acids [18] (See Figure 1-1). The HPLC separations have mostly been done on reverse phase columns (RP-C4 [37], RP-C8 and, RP-C18), but other stationary phases have been used including silver ion columns and monolithic columns [20, 26, 31, 40, 43-46]. The use of RP-C8 and RP-C18 columns has been summarized by Lima et al. [47], but there have been a few methods developed recently [18, 48]. Silver ion columns are usually used for separating a series of FFA with the same chain length but the double bonds in different locations. Additionally the FFA will elute in three groups, FFA with all trans double bonds, FFA with a mixture of cis and trans double bonds, and FFA with all cis double bonds [26, 45]. Dobson et al. have summarized the FFA separations done with silver ion chromatography [49].
Figure 1-1 Chromatogram of standard fatty acid derivatives (corresponding injected amount 35.7 pmol). The fatty acids were derivatized with acridone-9-ethyl-p-toluenesulfonate before separating. Chromatographic conditions: column temperature at 30 °C; excitation wavelength 404 nm, emission wavelength 440 nm; Eclipse XDB-C₈ column (4.6 x 150 mm, 5 mm); flow rate = 1.0 mL/min. 1. formic acid; 2. acetic acid; 3. propionic acid; 4. butyric acid; 5. valeric acid; 6. hexanoic acid; 7. heptonic acid; 8. octoic acid; 9. pelargoic acid; 10. deconoic acid; 11. undecanoic acid; 12. dodecanoic acid; 13. tridecanoic acid; 14. tetradecanoic acid; 15. pentadecanoic acid; 16. hexadecanoic acid; 17. heptadecanoic acid; 18. octadecanoic acid; 19. nonadecanoic acid; A. acridone-9-ethanol; B. actidone; C. reagent peak. Reprinted from reference [18].

The total analysis time varies on the gradient and the number of analytes being studied. For the most part, the published separations only include a few FFA or they do not completely separate between two FFA positional isomers. There are some published chromatographic conditions that have been optimized to separate a partial series of FFA with the same chain length and degree of unsaturation [19, 35, 36, 46, 50-52]. A summary of the different liquid chromatography (LC) mobile phases and detection methods that have been used in FFA analysis have been complied by Rezanka and Votruba [30] and Lima et al. [47].
Because FFA are often found at trace levels (FFA were reported at less than 1% of the total lipids found in green-lipped mussel [53], for example) in the sample matrix, detection limits need to be considered. Detection limits for the LC separations vary depending on the detector being used. For example Mehta et al. found the UV detection limit to be in the nmol range for the six phenacyl bromide FFA derivatives [36], while Czauderna and Kowalczyk found the UV detection limits to be in the pmol range for the dibromacetophenone FFA derivatives [51]. The detection limits for MS depend on the type of source being used. Sanches-Silva et al. report detection limits around 130 pmol for C18:1, C18:2 and C18:3 using atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) for detection [20]. Li et al. found the atmospheric pressure chemical ionization-multidimensional mass spectrometry (APCI-MS/MS) detection limits to be between 12 and 44 fmol for 19 different FFA when monitoring the fragment ion m/z 195.8 SRM mode [18], while Yang et al. found the electrospray ionization mass spectrometry (ESI-MS) detection limits to be between 40 and 160 fmol injected onto the column for derivatized FFA between C10 and C24 when monitoring for the [M-H] peak [37]. Detection of fluorescent derivatives allows for the lowest detection limits. For example, Prados et al. reported detection limits between 4-7 fmol in the LC separation of FFA labeled with 4-N,N-dimethylaminosulfonyl-7-N-(2-aminoethyl)amino-2,1,3-benzoxadiazole (DBD-ED) [54].

In general, the FFAs are separated with either a C8 or C18 LC column (5 μm particles). The column is equilibrated with a high percentage of water (~70%) and then ramped to 100% of either methanol or acetonitrile. Although APCI-MS/MS allows for
detection limits in the tens of femtomoles, derivatization and fluoresce detection allows for detection limits less than 10 fmol.

Capillary electrophoresis (CE) has also been used to separate a series of FFA [55-58]. Gallaher and Johnson showed that a series of derivatized FFA could be separated by CE [56, 57]. The FFA were labeled with a polymethine cyanine near-infrared fluorophore so that laser induced fluorescence could be used for detection [57] (See Figure 1-2). Heinig et al. showed that by using indirect detection the FFA did not need to be derivatized before analysis [55]. This method was compared to HPLC methods where the FFA (even chains C12:0 through C24:0, C17:0, C19:0, C23:0, C18:1\textsuperscript{\alpha}, C18:1\textsuperscript{\text{trans} 9}, and C18:2\textsuperscript{9,12}) were derivatized before analysis. These experiments showed that HPLC method had a better separation, but the CE analysis was completed in a third of the time.

**Figure 1-2** CE separation of C18 saturated (E) and unsaturated (A-D) FFA homologues in methanol/tetraethylammonium chloride with 25 kV applied voltage. A, γ-linolenic (C18:3\textsuperscript{9,12,15}); B, linoleic (C18:2\textsuperscript{9,12}); C, petroselanic (C18:1\textsuperscript{6}); D, oleic (C18:1\textsuperscript{\alpha}); E, stearic (C18:0). Reprinted from reference [56] with permission from the American Chemical Society.
1.3.2 Primary Fatty Acid Amides (PFAMs)

The separations of the PFAMs followed the same patterns as the separations of FFAs under similar conditions. On a reverse phase column, the saturated PFAMs will elute by chain length with the smallest PFAM eluting first. When a double bond is present in the compound, the retention would be approximately the same as a saturated PFAM with two carbons less in the chain. In general, the analysis methods for the PFAMs have not been explored as extensively as the FFA analysis. Therefore, it is common to use the analysis of FFAs as a starting point when developing PFAM analysis methods. They both have a long carbon chain, but different polar head groups. They can be found in the fatty acids and conjugated sub-class of the fatty acyls lipid class. However, the two vary enough in polarity that when separating them by TLC [17, 59] or by normal phase LC (Chapter 5), the two groups separate. The FFA class will elute first from the normal phase LC column because the FFAs have a less polar head group. Like the FFA analysis, GC/MS [17, 59-64] and LC/MS [60, 65-68] (Chapter 4) are the most common methods for further separating the sub-class.

Derivatizing the PFAMs is common before GC analysis. Gee et al. explored several different derivative methods to improve the detection of PFAMs with GC/MS analysis [61, 62]. From these studies, it was determined that derivatizing the PFAMs with bis-trimethylsilyltrifluoroacetamide (BSTFA) in toluene produced the highest signal-to-noise ratios. Using selected ion monitoring (SIM) mode in the ion trap GC/MS, the detection limits were in the low pmol range. Sultan and Johnson further developed this method in order to separate a series of six derivatized C18:1 PFAMs [17] (See Figure 1-3). Unfortunately, further studies show that the derivatizing method is not
efficient at low concentrations. Analysis can be done on underivatized PFAMs. For example, Sultana and Johnson separated a series of PFAMs (even chains C12:0 – C22:0) on a HP-5MS column [17, 59]. This method was limited to separating the PFAMs by chain length and degree of unsaturation. This method cannot separate by bond position, so all of the C18:1 PFAMs co-eluted. The non-polar column does not have enough selectivity. Additionally, using GC/Cl-MS instead of GC/EI-MS with un-derivatized PFAMs improved the analysis so that the detection limits are in the high fmol range (Chapter 4). The GC methods have been summarized in Table 1-2.

Derivatizing the PFAMs before analysis has been done to improve detection limits among positional isomers. The largest series of positional isomers separated was derivatized before injecting onto a polar GC column [17]. Although, the separation of cis/trans isomers can be done without derivatization [64]. The PFAMs can be easily separated by chain length using GC, but the separation of positional isomers cannot be done on non-polar columns.

**Figure 1-3** Separation of derivatized unsaturated C18 amides on a BPX70 column. The relative intensities of m/z 67, 81, 122, 124, 136, and 138 are plotted against retention time. Average linear velocity of He was 30 cm/s. Reprinted from reference [17] with permission from Elsevier.
<table>
<thead>
<tr>
<th>GC Column</th>
<th>Dimensions</th>
<th>Derivative (Reagent)</th>
<th>PFAMs</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP1</td>
<td>25 m x 0.32 mm; 1 μm film</td>
<td>No</td>
<td>C18:0, C18:1&lt;sup&gt;6&lt;/sup&gt;, C22:1&lt;sup&gt;13&lt;/sup&gt;</td>
<td>[69]</td>
</tr>
<tr>
<td>BP20</td>
<td>25 m x 0.22 mm; 0.25 μm film</td>
<td>No</td>
<td>C18:0, C18:1&lt;sup&gt;6&lt;/sup&gt;</td>
<td>[70]</td>
</tr>
<tr>
<td>BPX70</td>
<td>60 m x 0.25 mm; 0.25 μm film</td>
<td>Trimethylsilyl derivatives (Bis-trimethylsilyl trifluoroacetamide)</td>
<td>C18:1&lt;sup&gt;trans&lt;/sup&gt; 6, C18:1&lt;sup&gt;trans&lt;/sup&gt; 9, C18:1&lt;sup&gt;6&lt;/sup&gt;, C18:1&lt;sup&gt;9&lt;/sup&gt;, C18:1&lt;sup&gt;trans&lt;/sup&gt; 11, C18:1&lt;sup&gt;13&lt;/sup&gt;, C18:2&lt;sup&gt;trans&lt;/sup&gt; 9,12, C18:2&lt;sup&gt;8,12&lt;/sup&gt;</td>
<td>[17]</td>
</tr>
<tr>
<td>CP-Sil</td>
<td>10 m x 0.53 mm</td>
<td>No</td>
<td>C18:1&lt;sup&gt;9&lt;/sup&gt;, C20:1&lt;sup&gt;11&lt;/sup&gt;, C22:1&lt;sup&gt;13&lt;/sup&gt;</td>
<td>[71]</td>
</tr>
<tr>
<td>CP-Sil</td>
<td>25 m x 0.32 mm</td>
<td>No</td>
<td>C18:1&lt;sup&gt;9&lt;/sup&gt;, C20:1&lt;sup&gt;11&lt;/sup&gt;, C22:1&lt;sup&gt;13&lt;/sup&gt;</td>
<td>[71]</td>
</tr>
<tr>
<td>CP-Sil 5 CB</td>
<td>25 m x 0.32 mm; 1.2 μm film</td>
<td>No</td>
<td>C18:0, C18:1&lt;sup&gt;9&lt;/sup&gt;, C22:1&lt;sup&gt;13&lt;/sup&gt;</td>
<td>[69]</td>
</tr>
<tr>
<td>Dexil 300 GC</td>
<td>3 m x 2 mm</td>
<td>No</td>
<td>C16:0, C18:0, C18:1&lt;sup&gt;9&lt;/sup&gt;</td>
<td>[63]</td>
</tr>
<tr>
<td>HP 5MS</td>
<td>30 m x 0.25 mm; 1 μm film</td>
<td>No</td>
<td>C16:0, C18:1&lt;sup&gt;9&lt;/sup&gt;, C18:0</td>
<td>[66]</td>
</tr>
<tr>
<td>HP-5MS</td>
<td>30 m x 0.25 mm; 0.25 μm film</td>
<td>No</td>
<td>C12:0 – C20:0 (even), C13:0, C18:1&lt;sup&gt;9&lt;/sup&gt;, C22:1&lt;sup&gt;13&lt;/sup&gt;</td>
<td>[17]</td>
</tr>
<tr>
<td>HT5</td>
<td>12 m x 0.32 mm; 0.1 μm film</td>
<td>No</td>
<td>C18:0, C18:1&lt;sup&gt;9&lt;/sup&gt;, C22:1&lt;sup&gt;13&lt;/sup&gt;</td>
<td>[69]</td>
</tr>
<tr>
<td>Methyl silicon film</td>
<td>15 m x 0.261 mm; 0.25 μm film</td>
<td>Trimethylsilyl derivatives (N-methyl, N-trimethylsilyl trifluoroacetamide)</td>
<td>C16:0, C16:1, C18:1&lt;sup&gt;9&lt;/sup&gt;, C18:1&lt;sup&gt;trans&lt;/sup&gt; 9, C18:2&lt;sup&gt;8,12&lt;/sup&gt;</td>
<td>[72]</td>
</tr>
<tr>
<td>Permabond CW</td>
<td>30 m x 0.2 mm; 0.25 μm film</td>
<td>No</td>
<td>C18:0, C18:1&lt;sup&gt;9&lt;/sup&gt;, C22:1&lt;sup&gt;13&lt;/sup&gt;</td>
<td>[60]</td>
</tr>
<tr>
<td>Simplicity-5</td>
<td>30 m x 0.25 mm; 0.25 μm film</td>
<td>Trimethylsilyl derivatives (N-methyl, N-trimethylsilyl trifluoroacetamide)</td>
<td>C7:0 - C9:0, C12:0 - C18:0 (even)</td>
<td>[73]</td>
</tr>
<tr>
<td>GC Column</td>
<td>Dimensions</td>
<td>Derivative (Reagent)</td>
<td>PFAMs</td>
<td>Reference</td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------</td>
<td>----------------------------------------------------------</td>
<td>--------------------------------------------</td>
<td>-----------</td>
</tr>
</tbody>
</table>
| Simplicity-5 | 30 m x 0.25 mm; 0.25 μm film | Trimethylsilyl derivatives (N-methyl, N-trimethylsilyl trifluoroacetamide) | C12:0 - C20:0 (even)  
C16:1⁹  
C18:1⁹  | [61, 62] |
| Simplicity-5 | 30 m x 0.25 mm; 0.25 μm film | Trimethylsilyl derivatives (N-methyl, N-trimethylsilyl trifluoroacetamide) | C18:1⁹  
C18:1¹¹  
C18:2⁶,¹²  
C18:3⁶,¹²,¹⁵  
C18:1⁸,¹²,OH  | [61, 62] |
| SP-2330    | 30 m x 0.32 mm; 0.2 μm film | No                                                       | C14:0 – C22:0 (even),  
C16:1⁹  
C16:1trans ⁹  
C18:1⁹  
C18:1trans ⁹  
C18:2⁶,¹²  
C18:3⁶,¹²,¹⁵  
C22:1¹³  | [64] |

Table 1-2 Summary of GC separations of PFAMs on both polar and nonpolar columns.

Predominantly the PFAM separations have been done on nonpolar columns (BP1, CP-Sil, Dexsil 300, HP-5MS, HT5, or Simplicity-5) to separate by chain length. When the mixture includes a complex series (i.e. mixture included cis/trans isomers), then a polar column (BPX70 or SP-2330) is needed. If the PFAMs are derivatized before separating, N-methyl, N-trimethylsilyl trifluoroacetamide is used as the derivatizing reagent. The best detection limits are in the tens of femtomoles using EI-MS for detection.

The HPLC separations of PFAMs have mainly been done on C18 reverse phase columns [60, 65, 66, 68, 74, 75] (Chapter 4). Acetonitrile, methanol, and water are common solvents in the reverse phase gradients [60, 65, 66, 68, 75], but dichloromethane and tetrahydrofuran have also been used [74]. A normal phase column has also been used for the separation [67]. In this case, hexane and chloroform were part of the mobile phase [67]. Additives such as formic acid and acetic acid have been added to the mobile
phase to increase the ionization efficiency for APCI-MS detection [66, 67] (Chapter 4). Overall, the methods depended on the number of PFAMs of interest for the study and variation in degree of unsaturation. The LC PFAM separations have mainly been done on C18 reverse phase columns with 4-5 μm particles. The length of the columns was either 150 or 250 mm and the diameter of the columns was either 3.9 or 4.6 mm. The best gradients start at approximately 30 % water and ramp to 100 % organic (methanol or acetonitrile). In order to improve detection limits for APCI-MS methods, formic acid was added to the mobile phase. These separations show that a series of saturated PFAMs can be separated by chain length. The difficulties in separating positional isomers are discussed in Chapter 4. Overall, more studies need to be done to test the limits of the separation on smaller columns (both dimensions and particle size) so that the position of the double bond can be determined by retention time. Knowing the exact structure will be important for profiling the PFAM content of biological tissues.

<table>
<thead>
<tr>
<th>LC Column</th>
<th>Dimensions</th>
<th>Solvents</th>
<th>PFAMs</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waters XTerra RPC18</td>
<td>150 x 3.9 mm; 5 μm particles</td>
<td>acetonitrile and water</td>
<td>C12:0 – C22:0 (even)</td>
<td>[65]</td>
</tr>
<tr>
<td>Alltech Associates Nucleosil 100</td>
<td>250 x 4.6 mm; 5 μm particles</td>
<td>hexane, chloroform and acetic acid</td>
<td>C18:1[^{9}], C12:0 – C12:0 (even), C8:0, and C8:1[^{9}]</td>
<td>[67]</td>
</tr>
<tr>
<td>Waters Nova Pack C18</td>
<td>150 x 3.9 mm; 4 μm particles</td>
<td>methanol and water</td>
<td>C18:1[^{9}] and C22:1[^{13}]</td>
<td>[68]</td>
</tr>
<tr>
<td>Cosmosil 5C18</td>
<td>250 x 4.6 mm; 5 μm particles</td>
<td>methanol, dichloromethane and tetrahydrofuran</td>
<td>C16-C30 (even)</td>
<td>[74]</td>
</tr>
<tr>
<td>Waters C18 Nova Pack</td>
<td>150 x 3.9 mm; 5 μm particles</td>
<td>acetonitrile and methanol</td>
<td>C18:0, C18:1[^{9}] and C22:1[^{13}]</td>
<td>[60]</td>
</tr>
<tr>
<td>Zorbax Eclipse XDB C18</td>
<td>150 x 4.6 mm; 5 μm particles</td>
<td>acetonitrile, methanol, water, and formic acid</td>
<td>C14:0, C16:0, C17:0, C18:1[^{9}], C18:0</td>
<td>[66]</td>
</tr>
<tr>
<td>Zorbax ODS</td>
<td>250 x 4.6 mm</td>
<td>methanol</td>
<td>C1:0, C3:1[^{2}]</td>
<td>[75]</td>
</tr>
<tr>
<td>Waters XTerra RPC18</td>
<td>150 x 3.9 mm; 5 μm particles</td>
<td>methanol, water, and formic acid</td>
<td>C12:0 – C22:0 (even), C18:1, C22:1[^{13}]</td>
<td>Chapter 4</td>
</tr>
</tbody>
</table>
Detection limits are similar to the reported FFA detection limits and vary
depending on the detector and experimental conditions. Carpenter et al. found the UV
detection limits for underivatized C18:0 to be 1 nmol injected [65]. Madl and Mittelbach
found the detection limit of PFAMs to be around 20 fmol when using APCI-MS for
detection [66] (Chapter 4). Single ion monitoring was needed to achieve these detection
limits. Table 1-4 summarizes the MS detection limits for the different methods of
ionization. The detection limits are in the tens of femtomoles for each of the different
MS interfaces: LC/MS, GC/MS, and MALDI-TOF-MS. In cases where positional
isomers are studied, a separation prior to the MS was needed to differentiate between the
PFAM isomers. Because the injection volume for an LC separation is larger than the
injection volume for a GC separation, the sample has to be more concentrated when
injecting on to a GC column versus a LC to get the same mass load detection limit. It is
difficult to compare MALDI-TOF-MS detection limits, because they depend on the
percentage of the spot ablated during analysis and whether the spot was homogeneous.

<table>
<thead>
<tr>
<th>Mass Load</th>
<th>PFAMs</th>
<th>Detector</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 fmol</td>
<td>C16-C30 (even)</td>
<td>APCI-MS</td>
<td>[74]</td>
</tr>
<tr>
<td>20 fmol</td>
<td>C14:0, C16:0, C17:0,</td>
<td>APCI-MS</td>
<td>[66]</td>
</tr>
<tr>
<td></td>
<td>C18:1&lt;sup&gt;9&lt;/sup&gt;, C18:0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>360 pmol</td>
<td>C18:1&lt;sup&gt;9&lt;/sup&gt; and C22:1&lt;sup&gt;13&lt;/sup&gt;</td>
<td>APCI-MS/MS</td>
<td>[68]</td>
</tr>
<tr>
<td>1 – 100 nmol</td>
<td>Derivatized C7:0 – C9:0,</td>
<td>CI-MS</td>
<td>[73]</td>
</tr>
<tr>
<td></td>
<td>C12:0 - C18:0 (even)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17 nmol</td>
<td>C1:0, C3:1&lt;sup&gt;2&lt;/sup&gt;</td>
<td>EI-MS</td>
<td>[75]</td>
</tr>
<tr>
<td>1 nmol</td>
<td>C18:1&lt;sup&gt;9&lt;/sup&gt;</td>
<td>EI-MS</td>
<td>[76]</td>
</tr>
<tr>
<td>Mass Load</td>
<td>PFAMs</td>
<td>Detector</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------------------------------------</td>
<td>----------</td>
<td>-----------</td>
</tr>
<tr>
<td>6 fmol – 90 fmol</td>
<td>Derivatized C16:0, C16:1, C18:1&lt;sup&gt;9&lt;/sup&gt;, C18:1&lt;sup&gt;trans 9&lt;/sup&gt;, C18:2&lt;sup&gt;9,12&lt;/sup&gt;</td>
<td>EI-MS</td>
<td>[72]</td>
</tr>
<tr>
<td>30 – 1500 fmol</td>
<td>C22:1&lt;sup&gt;13&lt;/sup&gt;</td>
<td>EI-MS</td>
<td>[77]</td>
</tr>
<tr>
<td>4 – 40 pmol</td>
<td>Derivatized C18:1&lt;sup&gt;9&lt;/sup&gt;</td>
<td>EI-MS</td>
<td>[78]</td>
</tr>
<tr>
<td>20 - 6000 fmol</td>
<td>C12:0 – C20:0 (even), C13:0, C18:1&lt;sup&gt;9&lt;/sup&gt;, C22:1&lt;sup&gt;13&lt;/sup&gt;</td>
<td>EI-MS</td>
<td>[17]</td>
</tr>
<tr>
<td>0.001 – 10 nmol</td>
<td>Derivatized C12:0 – C20:0 (even), C16:1&lt;sup&gt;9&lt;/sup&gt;, C18:1&lt;sup&gt;9&lt;/sup&gt;, C18:1&lt;sup&gt;11&lt;/sup&gt;, C18:2&lt;sup&gt;9,12&lt;/sup&gt;, C18:3&lt;sup&gt;9,12,15&lt;/sup&gt;, C18:1&lt;sup&gt;9,12-OH&lt;/sup&gt;</td>
<td>EI-MS</td>
<td>[61, 62]</td>
</tr>
<tr>
<td>0.1 – 5 nmol</td>
<td>C18:1&lt;sup&gt;9&lt;/sup&gt;</td>
<td>EI-MS</td>
<td>[76]</td>
</tr>
<tr>
<td>35 nM– 350 mM</td>
<td>C18:1&lt;sup&gt;9&lt;/sup&gt;</td>
<td>ESI MS/MS</td>
<td>[78]</td>
</tr>
<tr>
<td>90 fmol</td>
<td>C18:1&lt;sup&gt;9&lt;/sup&gt;</td>
<td>ESI-MS&lt;sup&gt;3&lt;/sup&gt;</td>
<td>[79]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MALDI-TOF-MS</td>
<td>[80]</td>
</tr>
</tbody>
</table>

**Table 1-4** Summary of MS detection methods for PFAMs. The mass load is either the mass of each PFAM loaded onto the column/plate or the concentration injected into the MS depending on the technique.

1.3.3 N-acylethanolamines (NAEs)

NAEs are defined by a long carbon chain with a head polar group similar to the PFAM head group, but containing two extra carbons and a hydroxyl group. NAEs separations have not been extensively studied, but have been studied more than PFAMs. GC/MS and LC/MS have both been used for analysis NAEs samples.

N-Arachidonylethanolamine (anandamide), an endogenous cannabinoid receptor ligand, is the most commonly studied NAE, so most of the LC methods focus on separating anandamide from the other NAEs in order to purify the sample. Hansen et al. and Yang et al. have summarized results to show that effective detection methods would have to be able to analyze pmol of NAE per gram of tissue to low nmol of NAE per gram of tissue.
depending on the tissue being examined [81, 82]. However, Muccioli and Stella have reported that detection limits may not need to be as low as originally reported [83]. When comparing results, it is important to note the time of obtaining a sample to the analysis time because there can be postmortem changes in endocannabinoid levels [84].

Most of the published NAE separation methods only include a few NAEs. The GC/MS methods have been summarized in Table 1-5. In each case, the NAEs were derivatized prior to injection with one of four derivatization reagents (derivative): pyridine (O-acetyl-NAE), bis-trimethylsilyl trifluoroacetamide (BSTFA) (trimethylsilyl ether), tert-butyldimethylchlorosilane/ imidazole reagent (tert-butyldimethylsilyl) or dimethyl isopropylsilyl imidazole (dimethyl isopropylsilyl ether). When the column dimensions were described, the separation was done on a column 30 m x 0.25 mm. Fontana et al. published the chromatogram including the most NAEs (Figure 1-4). The shortest NAE elutes first. Most of the published separation methods have been developed on a HP-5 MS column (5 % phenyl). These separations include both saturated and unsaturated carbon chain lengths, but none of the published methods have included a series of positional isomers to test the limits of the separation.

Detection limits vary on detector and derivatizing agent. Detection limits for O-acetyl derivatives are between 0.05 and 0.1 nmol when using a flame ionization detector [85], while the detection limit for BSTFA derivatized oleylethanolamide is 0.1 pmol using MS in SIM mode for detection [86]. Detection limits for the dimethyl isopropylsilyl (DMiPSi) ether derivatized anandamide is 10 pg (~30 fmol) using SIM mode [87]. The NAEs have also been derivatized with tert-butyldimethylsilyl (t-BDMS) before GC analysis and the detection limits are approximately 2.9 pmol for anandamide
Because all of the published methods do not include detection limits, the derivatization reagents cannot be directly compared to determine which is the most effective for lowering detection limits.

<table>
<thead>
<tr>
<th>GC Column</th>
<th>Dimensions</th>
<th>Derivative (Reagent)</th>
<th>NAEs</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP-Si8 CB</td>
<td>30 m</td>
<td>Trimethylsilylether (BSTFA)</td>
<td>C16:0, C17:0, C18:1&lt;sup&gt;9&lt;/sup&gt;, C18:0, C20:4&lt;sup&gt;6&lt;/sup&gt;, C20:0</td>
<td>[83]</td>
</tr>
<tr>
<td>OP-15</td>
<td></td>
<td>O-acetyl-NAE (pyridine)</td>
<td>C12:0 – C24:0 (even) C20:4&lt;sup&gt;6&lt;/sup&gt;, C20:1, C18:1, C18:2&lt;sup&gt;9&lt;/sup&gt;</td>
<td>[85]</td>
</tr>
<tr>
<td>HP-5 MS</td>
<td>30 m x 0.25 mm</td>
<td>tert-butylidimethylsilyl (tert-butylidimethylchlorosilane/imidazole reagent)</td>
<td>C18:0, C16:0, C18:1&lt;sup&gt;9&lt;/sup&gt;, C18:1&lt;sup&gt;9&lt;/sup&gt;, C20:4&lt;sup&gt;6&lt;/sup&gt;</td>
<td>[89]</td>
</tr>
<tr>
<td>HP-5 MS</td>
<td>30 m x 0.25 mm</td>
<td>Trimethylsilylether (BSTFA)</td>
<td>C16:0, C18:0, C18:1&lt;sup&gt;9&lt;/sup&gt;, C20:4&lt;sup&gt;6&lt;/sup&gt;</td>
<td>[90]</td>
</tr>
<tr>
<td>DB-17</td>
<td>30 m x 0.25 mm; 0.25 μm thickness</td>
<td>tert-butylidimethylsilyl (tert-butylidimethylchlorosilane/imidazole reagent)</td>
<td>C16:0, C18:0, C18:1&lt;sup&gt;9&lt;/sup&gt;, C18:2&lt;sup&gt;9&lt;/sup&gt;, C20:4&lt;sup&gt;6&lt;/sup&gt;, C22:5&lt;sup&gt;6&lt;/sup&gt;</td>
<td>[91]</td>
</tr>
<tr>
<td>HP-5 MS</td>
<td>30 m</td>
<td>tert-butylidimethylsilyl (tert-butylidimethylchlorosilane/imidazole reagent)</td>
<td>C16:0, C17:0, C18:0, C18:1&lt;sup&gt;9&lt;/sup&gt;, C18:2&lt;sup&gt;9&lt;/sup&gt;, C20:4&lt;sup&gt;6&lt;/sup&gt;</td>
<td>[88]</td>
</tr>
<tr>
<td>HP-5 MS</td>
<td>30 m x 0.25 mm</td>
<td>Trimethylsilylether (BSTFA)</td>
<td>C16:0, C18:1&lt;sup&gt;9&lt;/sup&gt;, C20:4&lt;sup&gt;6&lt;/sup&gt;</td>
<td>[86]</td>
</tr>
<tr>
<td>HP-5 MS</td>
<td>30 m</td>
<td>tert-butylidimethylsilyl (tert-butylidimethylchlorosilane/imidazole reagent)</td>
<td>C16:0, C18:0, C18:1&lt;sup&gt;9&lt;/sup&gt;, C18:1&lt;sup&gt;9&lt;/sup&gt;, C18:2&lt;sup&gt;9&lt;/sup&gt;, C20:4&lt;sup&gt;6&lt;/sup&gt;</td>
<td>[92]</td>
</tr>
<tr>
<td>DB-1</td>
<td>30 m x 0.317 mm; 0.1 μm film</td>
<td>Dimethyl isopropylsilyl ether (dimethyl isopropylsilyl imidazole)</td>
<td>C20:4&lt;sup&gt;6&lt;/sup&gt;, 2-AG</td>
<td>[87]</td>
</tr>
</tbody>
</table>

Table 1-5 Summary of NAE analysis by GC/MS.
Reverse phase [85, 93-98], normal phase [99-101], and silver ion [102] HPLC methods have also been explored to separate and/or purify NAEs (See Table 1-6). The reverse phase method has been developed to include more NAEs in the separation [85, 95]. Fontana et al. have compared a few columns showing that a free fatty acid HP column allows for a better separation between N-γ-linoleoyl-ethanolamine and anandamide than a Nova-Pak column [85]. Williams et al. found that working with a cyano column rather than typical reverse phase columns improved the resolution between the analytes [98]. Additives such as silver acetate [102], sodium acetate [97], ammonium acetate [96, 98] and formic acid [96] have been added, depending on the ionization source, to the mobile phase to improve detection limits. Sugiura et al. published the separation of the most diverse mixture of NAEs [95] (Figure 1-5). The reverse-phase separation included 18 different NAEs, but baseline separation was not reported for all NAEs in the mixture especially ones with the same carbon chain length and degree of un-saturation. Similar problems are also seen in FFA and PFAM separations.
The majority of the NAE separations have been done on reverse phase columns (C18) 4.6 x 250 mm (5 μm particles). Isocratic conditions were used with the mobile phase containing water (less than 35 %) and organic (acetonitrile, methanol, and/or 2-propanol). These conditions separate by chain length. Currently, the published methods have not explored the conditions needed to separate positional isomers. Since anandamide and deuterated anandamide are separated on a UPLC column (1.7 μm particles) future studied should develop methods on column with smaller the particle sizes.

<table>
<thead>
<tr>
<th>LC Column</th>
<th>Dimensions</th>
<th>Solvents</th>
<th>NAEs</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waters Free Fatty Acid</td>
<td>4.6 x 150 mm; 5 μm particles</td>
<td>acetonitrile, tetrahydrofuran and water</td>
<td>C18:3, C18:2, C16:0, C20:4, C18:1, C22:4, C18:0, C20:1</td>
<td>[85]</td>
</tr>
<tr>
<td>Waters Resolve silica</td>
<td>3.9 x 150 mm; 5 μm particles</td>
<td>2-propanol and n-hexane</td>
<td>C16:0, C18:0, C18:1&lt;sup&gt;9&lt;/sup&gt;, C20:4&lt;sup&gt;6&lt;/sup&gt;</td>
<td>[90]</td>
</tr>
<tr>
<td>Shiseido Capcell Pack C18</td>
<td>4.6 x 250 mm</td>
<td>acetonitrile, 2-propanol, and water</td>
<td>C16:0, C18:0, C18:1&lt;sup&gt;7&lt;/sup&gt;, C18:1&lt;sup&gt;9&lt;/sup&gt;, C18:2&lt;sup&gt;6&lt;/sup&gt;, C20:4&lt;sup&gt;6&lt;/sup&gt;, C22:4&lt;sup&gt;6&lt;/sup&gt;, C22:5&lt;sup&gt;6&lt;/sup&gt;</td>
<td>[93]</td>
</tr>
<tr>
<td>HP C18 Hypersil</td>
<td>4.6 x 100 mm; 5 μm particles</td>
<td>methanol and water</td>
<td>C16:0, C18:1&lt;sup&gt;9&lt;/sup&gt;, C20:4&lt;sup&gt;6&lt;/sup&gt;</td>
<td>[94]</td>
</tr>
<tr>
<td>Shiseido Capcell Pack C18</td>
<td>4.6 x 250 mm</td>
<td>acetonitrile, 2-propanol, and water</td>
<td>C14:0, C16:0, C16:1&lt;sup&gt;7&lt;/sup&gt;, C18:0, C18:1&lt;sup&gt;7&lt;/sup&gt;, C18:1&lt;sup&gt;9&lt;/sup&gt;, C18:2&lt;sup&gt;6&lt;/sup&gt;, C20:4&lt;sup&gt;6&lt;/sup&gt;, C22:6&lt;sup&gt;3&lt;/sup&gt;</td>
<td>[95]</td>
</tr>
<tr>
<td>Thermo Electron Hypersil BDS C18</td>
<td>2.1 x 100 mm; 3 μm particles</td>
<td>aqueous silver acetate solution and methanolic silver acetate solution</td>
<td>C16:0, C18:1&lt;sup&gt;9&lt;/sup&gt;, C20:4&lt;sup&gt;6&lt;/sup&gt;</td>
<td>[102]</td>
</tr>
<tr>
<td>Waters Acquity UPLC BEH C18</td>
<td>2.1 x 50 mm; 1.7 μm particles</td>
<td>ammonium acetate, formic acid and acetonitrile</td>
<td>C20:4&lt;sup&gt;6&lt;/sup&gt; and deuterated C20:4&lt;sup&gt;6&lt;/sup&gt;</td>
<td>[96]</td>
</tr>
<tr>
<td>Beckman ultrasphere ODS</td>
<td>4.6 x 250 mm; 5 μm particles</td>
<td>methanol and sodium acetate</td>
<td>C14:0, C16:0, C18:0, C18:1&lt;sup&gt;9&lt;/sup&gt;, C20:4&lt;sup&gt;6&lt;/sup&gt;</td>
<td>[97]</td>
</tr>
<tr>
<td>Agilent Technologies Zorbax SB-CN</td>
<td>2.1 x 50 mm; 5 μm particles</td>
<td>ammonium acetate and methanol</td>
<td>C16:0, C18:1&lt;sup&gt;9&lt;/sup&gt;, C20:1&lt;sup&gt;12&lt;/sup&gt;, C20:4&lt;sup&gt;6&lt;/sup&gt;, C20:5&lt;sup&gt;5&lt;/sup&gt;, C22:6&lt;sup&gt;6&lt;/sup&gt;</td>
<td>[98]</td>
</tr>
<tr>
<td>LC Column</td>
<td>Dimensions</td>
<td>Solvents</td>
<td>NAEs</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------</td>
<td>---------------------</td>
<td>-----------------------------------</td>
<td>-----------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Tosoh TSKgel ODS[103]</td>
<td>4.6 x 150 mm; 5 µm particles</td>
<td>hexane, 2-propanol and methanol</td>
<td>C16:0, C18:1(^{\alpha}), C20:3(^{b}), C20:4(^{b}), C22:4(^{b})</td>
<td>[103]</td>
</tr>
<tr>
<td>Normal phase Whatman</td>
<td>4.6 x 250 mm; 5 µm particles</td>
<td>2-propanol and n-hexane</td>
<td>C12:0, C14:0, C16:0, C18:2, C18:1, C18:0</td>
<td>[101]</td>
</tr>
<tr>
<td>Waters XTerra MS C8</td>
<td>2.1 x 10 mm + 2.1 x 150 mm; 3.5 µm particles</td>
<td>ammonium acetate, formic acid, methanol, acetonitrile, and water</td>
<td>C16:0, C18:0, C18:1, C20:4, C22:4</td>
<td>[104]</td>
</tr>
</tbody>
</table>

Table 1-6 Summary of NAE analysis by LC.

Figure 1-5 Separation of 1-anthroylderivatives of various types of N-acylethanolamines by reverse phase HPLC. Copyright 1996. Reprinted from reference [95] with permission from Blackwell Publishing.

Unless MS is used for detection, the analytes need to be derivatized in order to get trace detection limits. Fontana et al. found UV detection limits to be between 4 nmol and 200 nmol depending on NAE [85]. Qin et al. found the detection limits of derivatized ethanolamines with o-phthaldialdehyde to be 1.0 pmol using a UV detector [97]. Sugiura et al. reported a detection limit of 0.3 pmol for the 1-anthroyl derivative of anandamide.
using a fluorescence detector [95]. The method was further developed by Arai et al. by switching to derivatizing with 4-N-chloroformylmethyl-N-methylamino-7-N,N-dimethylaminosulfonyl-2,1,3-benzoazadiazole (DBD-COCl) improving the detection limits be approximately 10 fmol for anandamide [105]. Schreiber et al. have shown that, when using ESI-MS/MS for the analysis of anandamide and palmitoylthanolamide, the detection limits are between 25 fmol and 100 fmol [102]. In this case, silver acetate was added to the mobile phase and the [M+Ag]^+ ion was monitored. Williams et al. used an APCI source on a triple quadrupole MS to detect approximately 10 pg (~30 fmol) NAEs found in bovine serum albumin (BSA) [98]. Lam et al. reported the best detection limits for anandamide using UPLC-MS/MS [96]. Using an ESI source in positive MRM mode the transition signals were 348.3 to 62.3. The limit of quantification was 0.22 fmol (signal to noise ratio > 10) loaded onto the column, while the detection limit was 0.055 fmol (signal to noise ratio = 3) loaded onto the column.

1.3.4 N-Acyl Amino Acids (NAAs)

NAAs have been postulated as being part of the biological cycle where peptidylglycine α-amidating enzyme (α-AE) will convert the N-acyl glycine (NAG) to the corresponding PFAM [106], but only a few studies have been done to improve the detection of this sub-class of lipids. The NAA sub-class co-elutes with the NAE sub-class when the TLC separation developed by Sultana and Johnson was used [17, 59], but the classes were separated when doing normal phase chromatography (Chapter 6).
The published NAA separations include a wide variety of compounds. Direct comparison between the methods is difficult because of the variation in head groups or only a few NAAs were included in the separation.

The GC separations have been summarized in Table 1-7. These separations were not done on commercially available columns. Costa et al. developed a separation (Figure 1-6) between glycine conjugates using a GC with a CI-MS detector in negative mode [1]. The limits of detection for this method ranged from 0.2 nmol of 2-methylbutyrylglycine per liter of urine to 3 nmol of suberylglycine per liter of urine. In general, published results for NAAs’ separations have not shown the detection limits nor explored the limits of the separation between a series of NAAs. Rinaldo et al. used ammonia chemical ionization to detect NAAs derivitized with diazomethane [107]. Detection limits were not reported for this method, but the lowest calibration point was 0.0167 μg/mL (approximately 10 fmol when 1 μL was injected (in split mode 1:20) onto the column). However, the authors of the published separations have taken different approaches in developing their method.

<table>
<thead>
<tr>
<th>GC Column</th>
<th>Dimensions</th>
<th>Derivative (Reagent)</th>
<th>NAA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP-sil 19</td>
<td>25 m x 0.25 mm; 0.4 μm film</td>
<td>3,5-bis(trifluoromethyl)benzyl ester derivatives (3,5-bis(trifluoromethyl)benzyl bromide)</td>
<td>HG, PPG, SG, BG, IVG</td>
<td>[1]</td>
</tr>
<tr>
<td>Phenyl methylsilicone gum</td>
<td>25 m x 0.2 mm; 0.33 μm film</td>
<td>N-acylated amino acids (diazomethane)</td>
<td>HG, PPG, SG</td>
<td>[107]</td>
</tr>
<tr>
<td>3 % Dexsil 300</td>
<td>180 cm x 3 mm</td>
<td>N-O-bis-(trimethylsilyl)-N-acylglycine (BSTFA)</td>
<td>PG, IVG, BG, MBG, HG, MAG, CG, MCg, TG, 2-HG</td>
<td>[109]</td>
</tr>
</tbody>
</table>
Table 1-7 GC methods for separating derivatized NAAs. Abbreviations: N-palmitoyl-D,L-alanine (C16:1Gly), N-stearoylglycine (C18:0 Gly), N-stearoyl-D,L-alanine (C18:1 Gly), hexanoylglycine (HG), phenylpropionylglycine (PPG), suberylglycine (SG), butyrylglycine (BG), isobutyrylglycine (IVG), propionylglycine (PG), methylbutyrylglycine (MBG), methylacrylglycine (MAG), crotonylglycine (CG), methylcrotonylglycine (MCG), and tiglyglycine (TG).

Figure 1-6 Ion current trace for negative mode CI-MS at m/z (M-BTFMB) from a pool urine spiked with a mixture of glycine conjugates. (A) isobutyrylglycine, (B) butyrylglycine, (C) 2-methylbutyrylglycine, (D) isovalerylglycine, (E) hexanoylglycine, (F) phenylpropionylglycine, and (G) suberylglycine. Reprinted from reference [1] with permission from Elsevier.

A few HPLC separations have been developed with NAAs. The separations have been summarized in Table 1-8. Most of the separations only include a few NAAs and none include a complete series of positional isomers to test the limits of the separation.
All of the published separations, except the ones done on the CSP2 column [110, 111], have been done on reverse phase columns. Carpenter et al. separated seven NAAs (C2-C12, saturated NAAs) using sodium phosphate and acetonitrile as the mobile phase on a C8 reverse phase column [65] (See Figure 1-7). Using UV for detection, the limits of detection were around 1 nmol. The authors also showed that if three C18 analogs (NAA, FFA, and PFAM) are combined in the same separation, the NAA analog will elute first from the C8 column. Goddard and Felsted developed an HPLC method using a reverse phase column to separate five azlactone derivatized NAAs [112]. For this method, water and acetonitrile made up the mobile phase. The detection limits were between 5 and 10 pmol using UV absorbance at 350 nm. Both positive mode [104, 113] and negative mode [114, 115] MS/MS have been used to detect the NAA depending the additives in the mobile phase. The best detection limits reported for positive mode are in the tens of picomoles when the fragment corresponding to the loss of ethanolamine is monitored [104]. The detection limits are two orders of magnitude lower (approximately 100 fmol) when negative mode API is used in MRM mode to monitor 338 and 74 m/z for the detection of N-oleoylglycine [115].

The separations that included the most NAAs in the mixture were done on either a C8 or C18 column (depending on the average chain length of NAAs in the mixture) 4.6 x 250 mm (5 μm particles). The gradients ramped from almost 100 % water to 100 % organic (acetonitrile). The separations were based on chain length and none of the published methods included a series of positional isomers.
<table>
<thead>
<tr>
<th>LC Column</th>
<th>Dimensions</th>
<th>Solvents</th>
<th>NAA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODP50</td>
<td>250 x 10 mm</td>
<td>acetonitrile, water, and triethylamine</td>
<td>N-acyl-L-tyrosines with acyl side chains C8 – C18</td>
<td>[116]</td>
</tr>
<tr>
<td>CSP2</td>
<td>250 x 4.6 mm; 5 μm particles</td>
<td>2-propanol and hexane</td>
<td>Carbon chains C1-C7 with various amino acid head groups</td>
<td>[110, 111]</td>
</tr>
<tr>
<td>Vydac C-18 protein/peptide</td>
<td>250 x 4.6 mm; 5 μm particles</td>
<td>acetonitrile and water</td>
<td>C10:0 Gly, C12:0 Gly, C14:0 Gly, C16:0 Gly</td>
<td>[112]</td>
</tr>
<tr>
<td>Phenomenex Luna C8</td>
<td>250 x 4.6 mm; 5 μm particles</td>
<td>50 mM sodium phosphate and acetonitrile</td>
<td>C2:0 Gly, C3:0 Gly, C4:0 Gly, C6:0 Gly, C8:0 Gly, C10:0 Gly, C12:0 Gly</td>
<td>[65]</td>
</tr>
<tr>
<td>Zorbax Eclipse C18</td>
<td>50 x 2.1 mm</td>
<td>acetonitrile and 10 mM ammonium acetate</td>
<td>N-arachidonyl glycine</td>
<td>[117]</td>
</tr>
<tr>
<td>Phenomenex Luna C8</td>
<td>4.6 x 250 mm; 5 μm particles</td>
<td>acetonitrile, water and trifluoroacetic acid</td>
<td>oleoylglycine</td>
<td>[113]</td>
</tr>
<tr>
<td>In house packed with Microm Bioresources’s Magic C18</td>
<td>9 cm x 7 μm; 3 μm particles</td>
<td>acetonitrile, water and formic acid</td>
<td>C18:0 Gly, C16:0 Gly, C18:1 Gly</td>
<td>[114]</td>
</tr>
<tr>
<td>Waters XTerra MS C8</td>
<td>2.1 x 10 mm + 2.1 x 150 mm; 3.5 μm particles</td>
<td>ammonium acetate, formic acid, methanol, acetonitrile, and water</td>
<td>N-arachidonyl glycine</td>
<td>[104]</td>
</tr>
</tbody>
</table>

**Table 1-8** LC separation conditions for NAA s. Abbreviations: decanoylglycine (C10:0 Gly), lauroylglycine (C12:0 Gly), myristoylglycine (C14:0 Gly), palmitoylglycine (C16:0 Gly), stearoylglycine (C18:0 Gly), oleoylglycine (C18:1 Gly). The number in the other abbreviations refers to the number of carbons in the acyl chain.
Figure 1-7 Separation of short chain N-acylglycines. Peaks are identified according to the number of carbons in the saturated acyl chain. The gradient on the C8 column is shown graphically; the other mobile phase component is 50 mM aqueous phosphate buffer. Reprinted from reference [65] with permission from Elsevier.

1.4 Steps towards 2D/LC

In order to profile different mammalian tissues, it would be useful to have a comprehensive LC method where one dimension separated the neutral lipids by class and the second dimension could be varied to further separate the lipids in the class by chain length, double bond position, and cis/trans orientation. Such a method is currently being developed. Experiments from this lab have shown that a normal phase PVA-Sil column can separate the “polar” neutral lipid classes (data shown in Chapter 5). Further analysis on the PFAMs can be done by collecting the PFAM fraction and loading it onto a C18 reverse phase column to separate by chain length and degree of unsaturation (Chapter 6). This is just one approach to developing a comprehensive method. Other research groups
have taken different approaches and focused on different sub-classes of lipids [10, 101, 105, 118, 119].

Brydwell has started to develop a parallel LC method [10]. For this method, the polar and the non-polar lipids were analyzed separately for two different sample tissues: bovine brain and sand bream filet. The polar lipids were separated using normal phase LC with an ion trap MS for detection. The non-polar lipids, which eluted in the dwell volume were diverted to a reverse phase column to be separated and then detected with a triple quadrupole MS for detection. These two gradients were run in parallel and monitored by two different MS instruments to reduce analysis time. The LC gradients are able to separate the classes of lipids, but this method relies on the MS to separate the lipids further. The drawback of this method is that isobaric lipids cannot be positively identified.

A 2D-LC method, combining a silver ion column and a reverse phase column, has been developed for the characterization of TAGs [120, 121]. The first dimension used a HotSep™ microbore column (150 x 1 mm; 5 μm particles) flushed with a silver nitrate aqueous solution. The second dimension used a Chromolith™ Performa RP-18 guard column (5 x 4.6 mm) and column (100 x 4.6 mm). Using this method, the authors were able to identify 57 different TAGs in donkey milk fat. The total analysis time of this method was 155 min. The authors noted that the concentration injected onto the first dimension is critical. If the concentration is too high, the column could be overloaded, but if the concentration is too low, the TAGs present at trace levels could be diluted to a concentration below the detection limit of the APCI-MS.
2D analysis systems have been developed where fractions are collected from a HPLC separation and then analyzed on the GC/MS. For example, Chapman et al. purified NAEs from seeds using normal phase HPLC, then further analysis was done using a GC/MS after the NAE fraction was derivatized with bis(tri-methylsilyl)trifluoroacetamide [101]. This method did not explore collecting additional fractions to analyze the TAGs also found in the seeds.

Arai et al. developed a method using a phenyl column and an octadecylsilica column to separate derivitized anandamide from other compounds found in rat brain [105]. The sample was loaded onto the phenyl column, where the dervitized anandamide fraction was collected onto a trapping column (an octadecylsilica column). Then, the sample was loaded onto the second column (an octadecylsilica column) for further purification. This method allows for the detection of anandamide down to 10 fmol, but does not track other NAEs though the separation.

Sommer et al. has developed a heart cutting method 2D/LC analysis [118]. The heart cutting approach to 2D/LC focuses on a few fractions where the lipids of interested are expected to elute instead of continuous fraction collection. Fractions were collected from the first dimension, a normal phase column, where the glycerolphosphoethanolamine and the glycerophosphocholine classes were purified. Further separations within the classes were done by analyzing the two fractions separately on a reverse phase column followed by nanospray MS/MS detection by monitoring neutral loss scans in both positive and negative mode.
1.5 Summary

Out of the FA lipid class, the FFAs have been studied the most. Several GC and LC methods have been published and the best detection limits are using LC and fluorescent detection to detect down to 4 fmol. CE separations have been developed to reduce the analysis time. Methods still need to be developed in order to determine bond position. Even though the PFAMs have not been studied as extensively, the PFAMs are similar enough that the FFA separation methods can be easily adapted to analyze a series of PFAMs instead. When using APCI-MS for detection, the detection limit is approximately 20 fmol. Most of the NAEs analysis has been focused on purifying and detecting anandamide. The detection limits on both the GC/MS and LC/MS are usually in the low femtomole range, but one method shows that hundreds of attomole detection is possible with ESI-MS/MS. The NAAs have mostly been studied on the GC/MS. UV detection has mostly been used for LC methods. The detection limits are lower using GC/MS, but the LC detection limits could be improved if MS detection methods were developed.

When using a reverse phase LC column (i.e. C18) or a nonpolar GC column (i.e. 5 % phenyl), the saturated forms of the lipids in the classes described above (FFA, PFAM, NAE, and NAA), elute with increasing chain order. When a double bond is present in the compound, the retention would be approximately the same as a saturated form with two carbons less in the chain. It has been demonstrated that when three C18 analogs (NAA, FFA, and PFAM) are combined in the same separation on a C8 column, the NAA analog will elute first followed by the PFAM analog [65]. The FFA analog eluted last. Therefore, the retention times cannot be compared across class in order to
determine structural information. The GC methods tend to be more complex because the methods require derivatization prior to separations, but more GC methods have been developed to separate a series of positional isomers than LC methods. This is partially the result of the GC methods being studied more than the LC methods.

For each of the lipid classes, femtomole detection is possible. The best detection limits reported are for the NAE lipid class when ESI-MS/MS is used for detection. The head group for the lipid class determines whether positive mode or negative mode MS ionization is more effective for detection. The FFAs and NAAs have the best detection limits in negative mode, while PFAMs and NAEs have the best detection limits in positive mode. Ammonium acetate is commonly added to the mobile phase to improve negative mode detection, while formic acid is used for improving positive mode detection limits. The detection limits between SIM mode on both GC/MS and LC/MS with a single stage quadruple MS are approximately the same when comparing the moles loaded onto the column. However, because the LC methods allow for a larger injection volume, the detection limit for LC/MS is lower when comparing the concentration of the solution injected onto the column. MRM mode MS/MS methods usually have lower detection limits than SIM mode MS methods.

There are a few problems that need to be addressed before the 2D/LC method can be fully developed. When developing the analysis methods for the different lipid classes, pure standards are not commercially available in a variety of carbon chain lengths and varying bond positions. Synthesis methods that convert between the different sub-classes do always produce a pure standard of the final lipid. Out of the lipid classes described, the FFAs are the easiest to commercially obtain. Secondly, the overall goal is to develop
a 2D/LC method, but since extensive research has not been done on each of the sub-
classes, the separations within the sub-class need to be explored before the 2D/LC
method can be fully developed. Because each of the lipids have the long carbon chains, it
is possible that the second method will only need minor modifications to be able to
analyze a different lipid sub-class.
1.6 References


Chapter 2

Microchip Reactions Followed by Capillary Electrochromatography and Laser-Induced Fluorescence for Detecting Fluorescent Fatty Amine Derivatives

2.1 Abstract

Published research on PFAMs has hypothesized that being able to analysis the levels of PFAMs present in biological samples could aid in medical diagnosis. Unfortunately, current analysis methods to do provide the accuracy and detection limits required. One approach to lowering the detection limits of primary fatty acid amide (PFAM) analysis is to convert the PFAM into the corresponding fatty amine and then derivatize the fatty amine with a fluorescent dye. The fatty amines can be derivatized with several different fluorescent dyes including 5-fluorescein isothiocyanate (FITC), a common dye used for laser-induced fluorescence (LIF) detection. The Johnson lab has been exploring several different analytical techniques to improve the detection. Two different techniques are presented in this chapter: (1) show that a microchip platform containing a C18 column can be used to reduce the reaction time between FITC and the
fatty amines and (2) show that a capillary electrochromatography (CEC) method can be used to separate the derivatives. For the reaction experiments, HPLC with fluorescent detection was used to monitor the derivatization reaction. For the second set of experiments, LIF detection monitored the separation of the derivatives. The purpose of these experiments was to explore separation and detection methods for analyzing the PFAMs found at trace levels in biological matrixes.

2.2 Introduction

The use of microchip interfaces has become a popular method for analytical development in recent years. There are many advantages in using the microchip over conventional methods. These include reducing the reagent consumption by a factor of \( \sim 10^4 \), reducing the size of instrumentation, allowing for reactions that are problematic on a larger scale, and reducing total analysis time [1, 2]. These advantages are important when analyzing certain biological molecules, because they are found at nanomolar or lower concentrations in the biological system. Current methods for separating and/or derivatizing are often lengthy and inefficient. Scaling principles applied to microchannels show that the initial sample size, mass or volume, can be significantly reduced over traditional methods. Depending on the design of the microchip and the form of the analytes, a variety of different functions can be performed including: derivatization, purification, polymerase chain reaction (PCR) amplification, and
separation. The analytes and reagents can be loaded and moved around the microchip either by using pressure driven flow or electroosmotic flow.

Although the microchips provide a good platform for manipulating trace amounts of analytes, they are limited by the detection methods. The detectors have to be able to detect the small amounts of analytes loaded onto the microchip. Manufacturers have chip interfaces that introduce the sample into a mass spectrometer; however, laser-induced fluorescence (LIF) can be the best choice if sensitivity is the most important issue because, if the conditions are right, LIF is able to detect single molecules [3-5]. It is important to note that single molecule detection is also dependent on the single molecule’s probability of being in the window of detection.

For this project fatty amines were chosen as the test analytes. Amines are related to the primary fatty acid amides (PFAMs). Common examples of biologically occurring PFAMs are oleamide and erucamide [6, 7]. PFAMs are a class of neutral lipids that can be converted to their corresponding amines via a Hofmann rearrangement [8]. Because of the variation and number of lipids that can be present in a sample, most methods would require three steps in order to isolate each of the PFAMs from a tissue sample or cell. The first step would be a liquid-liquid extraction to collect the neutral lipids from the tissue. The second step would be to purify the lipid class of interest either by solid phase extraction (SPE) or by normal phase liquid chromatography (LC). Details about this step are discussed in Chapter 5. Once the PFAM fraction is purified, they can be converted into their corresponding amines and derivatized with a fluorescent tag such as 5-fluorescein isothiocyanate (FITC) [8].
The derivatized analytes could then be analyzed with capillary electrochromatography (CEC) or capillary electrophoresis (CE). The estimated detection limit for this method when using LIF detection is sub-zmol, significantly lower than any of the other current detection methods [9]. However, if the amines will be derivatized, the reaction efficiency needs to be considered. The rate of reaction is reduced as the concentration of the solution is lowered. In order to be able to work with tens of nanomoles of amines, the sample could be pre-concentrated on a microchip packed with a C18 stationary phase before reacting with the fluorescent tag on the stationary phase. In an attempt to develop a trace analytical method for the detection of fatty amines, the analytes were derivatized on a microchip and analyzed using HPLC with fluorescence detection. The CEC/LIF detection method was developed using a series of purified derivatized amines.

Unlike HPLC, there is not a variety of pumps, detectors, and consumables commercially available for CEC analysis in part because the limited number of research groups currently working with the technique. Although, the technique is becoming more popular, there are still only a few commercially available CEC columns (summarized by Dittman [10]). Currently the columns do not have a wide variety of diameters and stationary phases. Therefore, CEC column needed for this analysis had to be packed by hand. In order to try different stationary phases in the CEC column, the sol-gel reaction would have to be modified to create variations of the CEC column. The advantage of CEC methods is that they are not limited by pressure, allowing for the use of smaller particles in the stationary phase. CEC methods can be completed in a third of the time it takes for a HPLC separation [11].
CEC would be easier to integrate onto a microchip interface. In order to put an HPLC column on a microchip interface, the fittings would have to withstand the pressure from the mobile phase being pushed through the column. Secondly, a simpler design for the microchip would involve the use of a sol-gel for the stationary phase. The sol-gel would hold the stationary phase in place eliminating the need for weirs (see Figure 2-1) that are required to hold the stationary phase in place for an HPLC column.

Figure 2-1 Diagram of the straight design microchip. This microchip design was used for previous experiments. This microchip was packed with Phenomenex’s Luna C18 bulk packing (100 Å pore size, 15 μm diameter) and baked overnight at 115 °C.

Derivatization on a microchip platform and CEC separations are just two of many analytical techniques explored by the Johnson group in order to develop a method that extracts a class of neutral lipids and then further separated the lipids in the class so that each lipid could be positively identified and quantified. The advantage of a microchip platform would be that it could incorporate both the reaction and separation on the same devise. The analytes would be able to be loaded onto the first column to be preconcentrated and reacted with a derivatizing agent. This is demonstrated in
section 2.3. After reacting the analytes could be moved directly onto a second column used for separation. The separation parameters are demonstrated in section 2.4. This design could reduce sample loss by reducing the dead volume zones. Contamination from handling the sample would also be reduced.

2.3 Fluorescence Derivatization on a Microchip

2.3.1 Microchip Background

Carpenter was able to derivatize nanomoles of phenylalkylamines on a 2.1 x 10 mm guard column containing C_{18} as the stationary phase [12]. This procedure improved the detection limit and decreased total analysis time. For these experiments, the samples were analyzed with HPLC for separation and UV-vis for detection. Experiments were done to determine the effectiveness of preconcentration on the phase by pumping a 200 nM solution for 2.5 hours to load 2.5 nmol of each of the phenylalkylamines onto the column. Build up of back pressure prevented the use of lower solution concentrations. Also, an excessive load time makes it impractical with that set-up. It was recommended that a fluorescence detector be used to achieve lower detection limits [2].

There have been several different types of reactions done on microchips, suggesting that the reaction can be scaled down even further. Fluri et al. achieved post column reactions on a microchip following capillary electrophoresis separation [13]. The microchip was used to mix the amino acids with o-phthaldialdehyde. This technique was
monitored with a fluorescence detector which allowed for detection limits of about 2 μM. It has been estimated that 1-3% of the amino acids are derivatized on the column from the time they are mixed to the time they reach the detector, making this an on-column reaction.

Harrison’s group, with the use of fluorescent dyes, has been able to detect sub-pM concentrations of peptides and amino acids using a 300 pL, 200 μm long SPE bed [14]. The high surface area of the octadecylsilane (ODS) beads allows for preconcentration factors as high as 500. Efficient preconcentration is necessary when analyzing many of the biological compounds which are found at nanomolar concentrations in the system. Although beads have been used in various applications to improve reactions because of their high surface area, it is not until recently that they have been integrated into chips with etched channels. The addition of a side channel perpendicular to the main channel was used to control the packing and unpacking the column. This design has been used for selective preconcentration and solid-phase extraction. [15]

Another method of preconcentration on a microchip involves electrokinetics and a silicate membrane [16]. If the right pore size is chosen, the silicate membrane will collect the large molecules while supporting an electrical current. One of the advantages of this type of packing is that it has low flow resistance, allowing flow rates up to 10 μL/min. In this case it was more effective to use lower flow rates when preconcentrating the Phe-Gly-Phe-Gly peptide.
2.3.2 Reagents

Five different fatty amines were used: n-decylamine, 95 % (Sigma, St. Louis, MO), 1-dodecylamine, 98 % (Acros Organics, Morris Plains, NJ), 1-tetradecylamine, 96 % (Aldrich, Milwaukee, WI), 1-hexadecylamine, 99 % (Aldrich), and octadecylamine, 97 % (Aldrich). Fluorescein (laser grade) was from Kodak (Rochester, NY). Sodium bicarbonate (reagent grade), 5-fluorescein isothiocyanate (isomer 1, 90 %) and chlorotrimethylsilane (98 %), were from Acros Organics. Acetonitrile (HPLC grade), methanol (HPLC grade), toluene (ACS grade), sodium phosphate monobasic, sodium phosphate dibasic, hydrochloric acid and sodium hydroxide were from Thermo Fisher Scientific (Pittsburgh, PA). The solutions were filtered with 0.22 μm nylon membrane filters from Whatman (Maidstone, England) before use. The microchip bulk stationary phase, C$_{18}$(2) 100 Å, was from Phenomenex (Torrance, CA). The 100 μm i.d. capillary was from Polymicro Technologies (Phoenix, Az). The nitrogen used for drying was from Air Gas (Pittsburgh, PA). Distilled water was deionized with a Barnstead (Dubuque, IA) Nanopure water system.
2.3.3 Microchips

Chips were manufactured by Dr. Mitchell Johnson at the University of Virginia according to published procedures [17] with a few modifications. The chip substrate was borofloat glass (Nanofilm, Westlake Village, CA, 0.040" thick or Telic, Valencia, CA, 1.1 mm thick) precoated with chromium and AZ 1518 negative photoresist (5300 Å thick). The image for the desired chip features was created by emulsion printing on mylar film (Pixels, Charlottesville, VA) from a postscript file created in AutoCAD LT (Autodesk). The chip features were clear on the photomask. A "sandwich" of clear glass, photomask, and substrate was exposed under UV radiation, transferring the pattern to the photoresist. The resist/substrate was baked at 110 °C for 30-60 min to polymerize the unexposed portion of the resist, and the exposed resist and the chromium under it were removed with solvent. Thus, the underlying glass was exposed only where desired chip features (such as channels) were present in the original film photomask. The exposed glass was etched with hydrofluoric acid: nitric acid: water 50:14:36 for about 45 min to etch channels approximately 90 μm deep and 300 μm wide. The remaining resist and chromium were removed and the plates were washed thoroughly. Top and bottom chips were exposed and etched at the same time. Individual chips were scored and cut, drilled (for inlet and outlet capillary connections), cleaned again, assembled, and baked at 110 °C for 30 min, 550 °C for 1 hr, and finally at 680 °C for 8 hr. The weir structures were created by leaving a gap in the channels in the photomask which was etched only part way through. The isotropic etching process thus created a sloping bottom in the channel with a very small open portion at the top. When bonded to a top
channel, the net result was a channel with a double trapezoidal structure, 300 µm wide in the middle, 120 µm wide at top and bottom, with an elliptical weir that was about 10-15 µm wide at the widest (top to bottom).

Before packing the microchip, it was conditioned by pumping about 200 µL each of the following solutions through the microchip: 1 M sodium hydroxide, deionized water, and methanol. A slurry was made of the stationary phase and methanol and this mixture was loaded through the inlet side of the microchip for the straight microchip design. The microchip was packed with Phenomenex’s Luna C₁₈ bulk packing (100 Å pore size, 15 µm diameter). The stationary phase was packed up against the weir by pulling a vacuum on the outlet side, then flushed with methanol and deionized water. In order to hold the stationary phase in place in the straight microchip design, the microchips are baked overnight at 115 °C in a Pro-Set II Stabil Therm Electric Oven (Blue Island, IL). Solutions were pushed through the column using a syringe pump. The straight microchip design is shown in Figure 2-1.

There are a few known problems with the straight design microchip. The reservoirs create a dead volume zone that retains the analytes/reagent instead of loading these onto the microchip. The length of the column varies slightly from one microchip to another because of the packing method. The baking process needed to hold the stationary phase in place degrades the packing. The T-chip design, described in Chapter 5, will eliminate some of these problems, but the T-chip design microchips are more prone to clogging relative to the straight chip design microchips because of the extra weir.
2.3.4 Reaction Set Up

The set-up used for the reaction is shown in Figure 2-2. For this set up the syringe pump (Harvard Compact Infusion Pump model 975) was connected to a series of valves and then the microchip. The first valve was a six port valve (Rheodyne 7000) that switched the flow from loading buffer (95 % phosphate buffer: 5 % method) to elution solvent (100 % methanol). The second valve was an injector valve (Rheodyne 7725) with a 2 μL sample loop for loading the FITC solution and the amine mix. The final valve was a four port valve that was able to divert the flow during the reaction. A fused silica capillary (75 μm i.d.) was used to connect the valves after the injection port while PEEK tubing (0.005 ID) was used to connect the plastic syringes (BD 5 mL) to the first valve. The flow rate was set to 5 μL/min, but the flow varied depending on the condition of the microchip column, because the syringe pump does not have a back pressure regulator. The microchip was placed in a water bath in order to control the reaction temperature. Finger tight fittings were used to keep a tight seal on the inlet and outlet channels to keep the water out of the system.
2.3.5 Silanization

Because the amines, at low concentrations, tend to adsorb to glass, all the collection vials and capillary tubing need to be silanized. First clean vials were soaked in 1 M sodium hydroxide for 20 min. These vials were then rinsed with deionized water and distilled acetone before being dried completely. Each vial was rinsed with toluene and filled with a solution 10% chlorotrimethylsilane: 90% toluene by volume under nitrogen. The next day the solution was removed and the vials were rinsed with toluene before putting in the oven to dry. Before use, the vial was rinsed with methanol to remove any residue. The capillaries were also conditioned in a similar matter. Each solution was pumped at 10 μL/min through the capillary for 15 min: 1 M hydrochloric acid, deionized water, 1 M sodium hydroxide, deionized water, distilled acetone, toluene,
and 10% chlorotrimethylsilane: 90% toluene. The chlorotrimethylsilane: toluene solution was left in the capillary overnight. End caps were put on the capillary to prevent drying. The next day, toluene and then methanol were pumped through the capillary each at 10 µl/min for 15 min. The capillary was then reconnected to the microchip.

2.3.6 Chromatographic Conditions

Separation and analysis were done on a Waters 600 HPLC with a Waters XTerra column (RP18 5µm, 3.9 x 150 mm). A Waters 600 pump equipped with both a Waters 2487 UV-vis absorbance detector (monitoring at 480 nm) and a Waters 470 fluorescence detector (monitoring with excitation 490 nm and emission 520 nm) was used for analysis. The following linear gradient developed by Carpenter was used for analysis of the derivatives: 70% A, 30% B, to 40% A, 60% B over 5 min, hold at 40% A, 60 % B for 4 min before returning to 70% A, 30% B over 5 min, and hold for 2 min to equilibrate for the next injection at 0.8 mL/min where A was 10 mM carbonate buffer adjusted to pH 9.6 and B was acetonitrile [12]. All solutions were filtered with 0.22 µm nylon membrane filter. The injection volume was 5 µL. Millenium software (Waters Corporations) was used for analyzing the chromatograms.
2.3.7 Results and Discussion

The amines and FITC are not soluble in 100 % phosphate buffer, so for these experiments methanol was added to each of the solutions. A 50 μM mix of each of the five different fatty amines was made from the following fatty amines: n-decylamine \((C_{10})\), 1-dodecylamine \((C_{12})\), 1-tetradecylamine \((C_{14})\), 1-hexadecylamine \((C_{16})\), and octadecylamine \((C_{18})\). The solution was made in 35 % methanol: 65 % 10 mM phosphate buffer (pH 7.5). The solution for loading was 150 μM FITC in 5 % methanol: 95 % 10 mM phosphate buffer. These are the lowest percentages of methanol that allowed the analytes to stay in solution. Each of these solutions was filtered before reacting using a 0.22 μm nylon membrane. Figure 2-3 is a diagram showing the three main steps involved in doing the reaction on a microchip.

**Figure 2-3** Block diagram showing the three main steps in doing a reaction on a microchip: load amines, load FITC, and elution followed by HPLC separation and fluorescence detection.
For each reaction, the amines and FITC were first preconcentrated on the stationary phase. An overlap of the analyte and reagent zones would have to occur for the reaction to take place. Then the derivative was eluted off the stationary phase with methanol and injected onto the C18 HPLC column followed by fluorescence detection. The reaction time was optimized by varying the reaction time between 15 min and 60 min finding no significant increase in amount of derivatives formed. Because the chip was in a water bath, the reaction temperature could be controlled. Increasing the temperature of the water bath from 30 °C to 40 °C doubled the amount of derivatives formed, but the packing degraded faster at the elevated temperatures so no further temperature studies were done. Additional changes in the flow rate due to increased temperature were not studied.

The optimized method allowed the column to equilibrate with 95 % phosphate buffer and 5 % methanol for 15 min. The amines were loaded in a 2 μL sample loop and pushed towards the column with 95 % phosphate buffer and 5 % methanol for 9 min. FITC was loaded in the 2 μL sample loop and pushed towards the column with 95 % phosphate buffer and 5 % methanol for 9 min. The flow was stopped to the microchip allowing the reaction to occur for 15 min. During this time the lines going from the first value, through the injection port towards the third valve were flushed with 100 % methanol. After the reaction was done, the derivatives were eluted from the column with 100 % methanol for 15 min. Throughout the entire method, the syringe pump was set to 4.9 μL/min (actual flow rate was around 1 μL/min) and the water bath was set to 30 °C. The eluant was dried down and reconstituted in 30 μL of methanol in order to do three injections on the HPLC with fluorescence detection. The resultant chromatogram is
shown in Figure 2-4. This reaction worked the best when 25 pmol of each amine and 300 pmol of FITC were loaded onto the column. It would take 3-4 hours doing an offline reaction (25 nmol of each amine and 300 nmol of FITC in 3 mL of 65 % methanol and 35 % buffer) with the same 2:1 reaction ratio to get a similar percentage of derivatization. More details about this reaction can be found in Carpenter’s thesis [12].

![Fluorescent chromatogram from reacting either 25 pmol or 15 pmol of each amine with 300 pmol of FITC for 15 min on the microchip column.](image)

**Figure 2-4** Fluorescent chromatogram from reacting either 25 pmol or 15 pmol of each amine with 300 pmol of FITC for 15 min on the microchip column.
2.3.8 Reaction Conclusions

There are several problems limiting this method. Primarily, this method was limited by the fluorescence detector on the HPLC. The lower detection limit was around 5 nM, which limits the mass of the analytes used in the reaction on the microchip. If less than 10 pmol of each amine were loaded onto the microchip, the amount of derivative formed was below the detection limit on the detector. The syringe pump used for loading the solutions onto the chip did not have pressure regulation. As pressure increased, the flow rate going through the microchip decreased causing a variation in the amount of the analytes/reagents that were loaded onto the stationary phase. The baking processes needed to hold the stationary phase in place as well as higher temperature experiments caused the stationary phase to degrade. This caused variation in the efficiency of the reaction occurring from one microchip to another microchip. Overall by reducing the total volume of the solution, the reaction occurred faster. There are still some reproducibility issues stopping this reaction set-up from being used regularly, but using this reaction set-up does derivatize the amines faster than a bench top reaction.

Even though this reaction method does not allow for much variation in the amount of amines loaded onto the column, the percentage of derivatives formed was increased. On a bench top reaction where 50 nmol of each amine was reacted with 500 nmol of FITC approximately 2 % of derivative was formed in 15 min. On the microchip 25 pmol (2,000 fold difference in mass) of each amine was reacted with 300 pmol of FITC approximately 10 % of derivative was formed in 15 min.
Future directions in this project might include switching to laser induced fluorescent (LIF) detection to lower the detection limits. Varying the length of the column on the microchip or varying the percentage of methanol used in the elution buffer might allow for separation between the derivatives allowing for the eluent to go directly into the LIF set-up for analysis. These changes would be dependent on using a pump that can withstand the changes in back pressure caused by switching the mobile phase from phosphate buffer to methanol.

2.4 CEC/LIF Separation and Detection

2.4.1 CEC Background

Capillary electrochromatography (CEC) is a combination between capillary electrophoresis (CE) and HPLC, because the capillary is packed with a stationary phase, but the flow rate is controlled by an applied voltage. The advantage of CEC is high efficiency, high resolution microscale separations with minimal solvent consumption [18]. CEC uses an electric field resulting in a flat flow profile going through the column, while HPLC uses pressure resulting in a parabolic flow profile [18]. (Figure 2-5)
The pressure range on the pump limits the size of the capillary and the flow rate of the mobile phase that can be used for the HPLC separation. The parabolic profile is caused by friction between the solvent and the capillary wall. This friction slows down the molecules moving near the capillary wall causing peak broadening. This effect does not show in electroosmotic flow (EOF) because the flow is driven by the double layer occurring along the walls. The double layer is created by the negatively charged silica groups in the capillary wall and the anions in the buffer solution which are attracted to the capillary walls as well as the cathode. Figure 2-6 shows a representation of the double layers that occur along the capillary wall and also on the stationary phase.

Figure 2-5  Pressure driven flow is parabolic while EOF is flat.
A voltage, usually less than 30 kV [19], across the capillary creates an EOF which moves the mobile phase to either the cathode or the anode depending on the charge of the stationary phase. In this case, the positive charges are attracted to the negative charge from the cathode. This effect cancels the effect of friction and, therefore, allows for a flat profile. The double layer thickness is directly proportional to temperature and inversely proportional to the ionic strength of the mobile phase. Typical double layer thicknesses are between 1 and 10 nm for silica surfaces in aqueous buffers about pH 5 [19]. If the diameter of the capillary or the space between particles is not large enough, the double layers overlap reducing the flow velocity [19]. The EOF is also influenced by the permittivity in vacuum ($\varepsilon_0$), the relative permittivity ($\varepsilon_r$), the zeta potential ($\zeta$), and the solvent viscosity ($\eta$). The EOF can be described by Equation 2-1 [20].

$$\mu_{EOF} = \frac{\varepsilon_0 \varepsilon_r \zeta}{\eta}$$  \hspace{1cm} (2-1)
In theory, CEC is not dependent on particle size, column length, or maximum pressure available [21]. The diameter of the CEC capillary is limited to the same small diameter (100 μm or less) as CE in order to minimize the generation of heat and avoid peak spreading [19]. Increasing temperature affects the resolution of the peaks, often making them broader. Some commercial systems have a cooling system to reduce/prevent the temperature of the capillary from increasing. By multiplying the EOF by the electric field strength (E), as shown in equation 2-2, the linear velocity can be determined.

\[ u_{\text{EOF}} = \mu_{\text{EOF}} E \]  

(2-2)

The same stationary phases used in HPLC columns can be used in CEC columns, but some are unable to create a stable EOF. It is important for the stationary phase to have surface silanol groups to stabilize the EOF [10]. New stationary phases are being developed with larger surface areas and increased silanol activity to help generate a higher EOF, which allows for a better separation [20]. CEC has already been used to separate a variety of analytes including lipids [22, 23], amino acids [24, 25], and proteins [26]. In order to do the separation, there are two major classes of CEC columns from which to choose: open-tubular (OT) columns, and packed columns (PC). In OT columns the stationary phase is bonded to the capillary wall. In PC the capillary is filled completely with a bead-based or monolithic stationary phase. The problem with most of these stationary phases in PC is that they need to be held in place by on-column frits, and the methods used for creating the frits cause a weak spot in the column which causes peak broadening, bubbles, and limit the flow [21, 27]. There is a third class of CEC columns called monolithic columns, where a porous, continuous separation bed is made.
The monolithic packing can be either organic polymer-based or bonded silica-based, but both are chemically bonded to the inner walls of the capillary, eliminating the need for end frits. The advantage of monolithic columns is their pH stability, but the drawback of these columns is that they swell or shrink depending on the solvents used in the mobile phase. By combining silica-based stationary phase with a monolith creates a column that is held in place by the monolithic matrix, but no longer swells or shrinks. This type of monolith is known as a sol-gel [28]. Sol-gels combine organic and inorganic stationary phases allowing for a more selective separation. Figure 2-7 shows diagrams of the different types of columns.

![Diagram showing the view when looking along the side of the three different types of CEC columns: packed column, open tubular and monolithic.](image)

**Figure 2-7** Diagram showing the view when looking along the side of the three different types of CEC columns: packed column, open tubular and monolithic.

Abidi et al. have been able to separate fatty acids found in vegetable oils and plants with PC-CEC followed by analysis with a photodiode array detector system [18]. The samples had to be purified before separation. The authors tried a few different
stationary phases, but were only able to get complete separation with pentafluorophenylsilica (PFPS) packing on a column that was 25 cm long with 75 μm i.d. For the CEC separation 25 mM Tris buffer in a 92:8 mixture of methanol: water was used as the mobile phase. The injections were done at 10 kV for 2 s and the applied voltage was 20 kV to separate. Increasing the percentage of water in the mobile phase increased the resolution of the peaks, but this also increased the run times. The CEC separations resembled the elution patterns from reversed-phase HPLC, using a PFPS packed column, but had better resolution.

Jemere et al. integrated a CEC column onto a glass microchip. The column was packed with ODS coated silica beads [29]. The column length varied between 1 and 5 mm and the packing was held in place by two weirs in the main channel and a polymer seal in the bead introduction channel. By applying a voltage across the microchip, the samples could first be injected and then separated. Only 2 kV was applied during separation. A laser induced confocal epifluorescence detection system with an Ar-ion laser was used for detection. The fluorescent dye used was BODIPY (4,4,-difluoro-1,3,5,7,8-penta methyl-4-bora-3a, 4a-diaza-s-indacene). In these experiments, the detection limit was 10 μM for the two amino acids, arginine and leucine, which is three times lower than the detection limit for CE on a microchip, but still poor relative to typical laser dye experiments.

Dermaux et al. used fused silica columns 50 cm in length, 100 μm i.d., and packed with C\textsubscript{18} particles [22]. Ammonium hydroxide (50 mM) was dissolved in a 57:38:5 mixture of acetonitrile: 2-propanol: n-hexane to make the mobile phase for the CEC runs that separated the eleven triglycerides found in argan oil. The sample (5 mg)
was dissolved in 1 mL of 2-propanol and injected at 10 kV for 3 s. During analysis the voltage was turned up to 30 kV creating a mobile phase velocity of 0.42 mm/s. This method allowed for baseline separation between peaks that normally coeluted in LC.

2.4.2 Reagents

Five different fatty amines: n-decylamine, 95% (Sigma, St. Louis, MO), 1-dodecylamine, 98% (Acros Organics, Morris Plains, NJ), 1-tetradecylamine, 96% (Aldrich, Milwaukee, WI), 1-hexadecylamine, 99% (Aldrich), and octadecylamine, 97% (Aldrich) derivatized with 5-fluorescein isothiocyanate (FITC) and purified by Dr. Carpenter was used. Fluorescein (laser grade) was from Kodak. FITC (isomer 1, 90%), methyltrimethoxysilane (C1-TMOS), and diethylamine were from Acros Organics. Sodium phosphate monobasic, sodium phosphate dibasic, sulfuric acid, sodium hydroxide, ammonium hydroxide, acetic acid and polyethylene glycol 8000 were from Thermo Fisher Scientific (Pittsburgh, PA). Ethyl alcohol 200 proof (Pharmco, Brookefield, CT) was distilled before use in CEC to increase purity. All other solvents were HPLC grade from Thermo Fisher Scientific. All solutions were filtered with 0.22 μm nylon membrane filters from Whatman before use. The 100 μm i.d. capillary was from Polymicro Technologies (Phoenix, Az) and the premium plain glass microscope slides were from Thermo Fisher Scientific. The epoxy was 5 min set from the local hardware store.
2.4.3 Standards

The amine derivative standards were made by Carpenter doing an off-line bench top reaction overnight to form the derivatives [12]. The derivatives were separated and purified using a Waters Prep LC 4000. They were then extracted from the mobile phase, dried down, and then reconstituted in methanol. These samples were used as standards when developing the CEC separation method.

2.4.4 Preparing the Capillary

The C₁ sol-gel used for packing the capillaries was developed by Dr. Braden Giordano (Naval Research Laboratory). In order to prepare the capillary for packing, a 60 cm length of 100 µm i.d. fused silica capillary was flushed separately with 3 mL of 1 M sodium hydroxide, 3 mL of deionized water, and 3 mL of distilled ethanol before drying with nitrogen. To make the gel, 1 mL of the aqueous phase, 558 µL methyltrimethoxysilane (C₁-TMOS), and 390 µL of ethanol were mixed for 2 hrs at room temperature. The aqueous phase was made up of 100 mM acetic acid with 0.2 g/mL polyethylene glycol (8000 molecular weight). Before pumping the mixture into the capillary, 40 µL of 1:1 mixture of diethylamine and acetonitrile was added to induce gelation. Once the capillary was completely filled the ends are capped with septa and the column was baked at 60 °C for 1 hr.
At the desired detection point, a window was created by removing the coating on the capillary, by dripping hot concentrated sulfuric acid over the capillary to dissolve the coating. The window was cleaned with clean methanol and lens cleaning tissue to remove any residue. The window was then centered on a microscope slide and held in place with a drop of epoxy on each side of the window. This mounting method was necessary because the capillary was brittle without its coating, and it allowed the capillary to be clamped down during analysis. The capillary was flushed with distilled ethanol to wet all the packing. This usually took about a day. Then the capillary was flushed with 0.1 mM ammonium hydroxide for about two days to remove the gel precursor used in making the sol-gel. Any remaining gel precursor could affect the double layer and possibly reverse the direction of the flow. The capillary was then flushed with ethanol for several hours before switching to the mobile phase. A low flow rate had to be used because excessive pressure will unpack the capillary. A capillary washer was set-up to flush the capillary column so that the pressure did not exceed 30 psi.

2.4.5 CEC/LIF Set up

The basic CEC set-up is shown in Figure 2-8. The power supply (Spellman 1000R; Plainview, NY, USA) is adjustable so that the number of volts applied across the capillary can be controlled. The ground and voltage probes were put into vials containing the mobile phase. These created the anode and cathode. A capillary containing the sol-
gel connected the anode to the cathode. When a voltage was applied, flow went from the anode to the cathode, because of the negatively charged silica in the stationary phase. The window was aligned with the laser in order to excite the amine derivatives causing a fluorescent emission. In this case the LIF detector was an argon-ion laser (American Laser, Salt Lake City, UT USA), operating at 488.0 nm. The photons were filtered with a filter (for FITC, 525DF40, 525 ± 20 nm; Omega) set at a 90° angle to the laser beam. An avalanche photodiode (APD) (EG&G Optoelectronics, Vaudreuil, Canada), a photon counting detector, used to convert the photons into digital pulses that was measured by a multichannel scaler card. In order to monitor the current through the column, a 100 μΩ resistor was added in series with the circuit and a voltmeter was placed over the resistor. The voltage profile was saved by the computer using the Logger Pro program version 1.0.7 by Vernier Software (Beaverton, OR).
Figure 2-8 Diagram of the CEC set up showing the C1 sol-gel packed capillary connecting the inlet and the outlet vials containing the running buffer. The argon laser was aligned with the capillary to allow for detection of the derivatives. A 100 μΩ resistor was placed between the anode and the ground in series for measurement purposes.

2.4.6 Sample Loading

Electrokinetic injection is done by applying a voltage across the capillary creating an electric field. According to Harris [30], the moles of each ion loaded onto the capillary can be calculated using the following equation:

$$\text{Moles injected} = \mu_{\text{app}} \left( \frac{E \times K_i}{K_s} \right) t \pi r^2 C$$

(2-3)

In this technique, the number of moles injected is dependent on the apparent mobility, described in equation 2-4, the applied electric field (E in V/m), the capillary
radius (r), the sample concentration (C in mol/m$^3$), and the ratio of conductivities between the buffer and sample ($\kappa_b/\kappa_s$), which is one in this case because the sample was dissolved in the running buffer [30]. The apparent mobility (equation 2-4) is affected by the length of the column from injection to the detector ($L_d$), the migration time of a neutral solute added to the sample (t), the applied volts (V) and total length of the column from end to end ($L_t$).

$$\mu_{app} = \frac{L_d}{t \frac{V}{L_t}}$$

(2-4)

The maximum injection volume is dependent on the fraction of allowable peak broadening ($\theta^2$, usually 5%) as shown in equation 2-5 [31].

$$V_{inj} = \frac{\Theta V_{\text{column}}}{\sqrt{N}}$$

(2-5)

The volume of an OT column assumes that the volume is equal to the volume of a cylinder with the same dimensions. The volume of a sol-gel column would be equal to the dead volume in the capillary, which would be less than the calculated value. So a proper injection may need to be smaller than the calculated injection size. The number of theoretical plates is determined using equation 2-6, where $t_r$ is the time of elution and $w_{1/2}$ is the width of that peak at its half height [32].

$$N = \frac{5.55t_r^2}{w_{1/2}^2}$$

(2-6)
2.4.7 Results and Discussion

Before exploring the possibility of using CEC to separate the five different fluorescent derivatized fatty amines several preliminary experiments were done. First an Ohm’s plot was made to show the stability range of the column. As shown in Figure 2-9, the stability range changes depending on the concentration of buffer being used. The results of two different running buffers are shown: 40% methanol in 1 mM phosphate buffer and 40 % methanol in 10 mM phosphate buffer. The pH of the buffer stock solution was 7.5. Increasing the concentration of the ions in solutions, increased the current going through the column, which increased the amount of heat created by the system. The EOF broke down when the double layers overlap or when bubbles formed on the column from the solvent heating. This experiment shows that the conductivity is directly proportional to the concentration of the buffer. In this case, the slope using the 1 mM buffer is 0.324 μA/kV, while the slope using the 10 mM buffer is 2.45 μA/kV.
One method of controlling the separation is varying the applied voltage, so an experiment was done to show the affects of increasing voltage on elution. In this experiment the mobile phase was 40% methanol in 2 mM phosphate buffer. A C1 sol-gel column approximately 30 cm in length was used. All injections were 1 min at 15 kV of 25 nM fluorescein in 40% methanol in 2 mM phosphate buffer. Figure 2-10 shows the results of three different applied voltages: 15 kV, 20 kV, and 25 kV. As the voltage increased, the run times were decreased. The trends were the same as CE done under the same conditions except with an unpacked capillary. The voltage is indirectly proportional to the elution time (see equation 2-4); therefore, the elution time decreases as higher voltages are applied to the capillary. This showed that the highest stable current would shorten the analysis time.
Figure 2-10 25 nM fluorescein injections done for 1 min at 15 kV, but the running voltage was varied: 15 kV, 20 kV, and 25 kV to show the effects on elution time.

In order to test the reproducibility of the injections, three fluorescein injections were then performed on the packed capillary (results shown in Figure 2-11). The capillary was conditioned with 40 % methanol in 2 mM phosphate for an hour before starting and then 30 min in between each injection. Both end vials contained 40 % methanol in 2 mM phosphate during running and the injection vial had 100 nM fluorescein in 40 % methanol in 1 mM phosphate buffer. Injections were 20 sec long at 10 kV and ran at 10 kV. Even though the current increased throughout the runs because of joule heating, the eluting fluorescein peaks from each injection overlapped. Flushing for about 20 min with the buffer solution between injections returned the current to the same starting value each time. For this experiment higher voltages broke down the EOF before the analyte was detected.
Figure 2-11  Three 20 sec injections of 100 nM fluorescein on a 100 μm C1 sol-gel packed capillary ran with 10 kV applied voltage. The left axis shows the LIF detector intensity, while the right axis shows the actual current across a 100 kΩ resistor in series with the capillary.

In order to study the effects of the buffer concentration (i.e. ionic strength) on both the current and the elution time of fluorescein, a series of running buffers were tried: 40 % methanol in 1 mM phosphate buffer, 40 % methanol in 2 mM phosphate buffer, and 40 % methanol in 3 mM phosphate buffer (results shown in Figure 2-12). Each of the injections was done with 25 nM fluorescein in 40 % methanol and 1 mM phosphate buffer for 20 sec at 10 kV. The running voltage was held constant at 10 kV. The capillary was flushed at 5 μL/min with the new running buffer for 30 min before doing the next set of injections. These experiments showed that the running buffer with 2 mM phosphate buffer concentration had the most stable current. Although the 3 mM phosphate buffer concentration was also stable, the lower concentration of phosphate in the running buffer was chosen because of solubility limitations of phosphate in methanol at higher concentrations. It was possible that solubility issues caused the fluorescein in
the 2 mM and the 3 mM runs to have similar retention times, due to 3 mM phosphate not being completely dissolved.

\[ \text{Figure 2-12} \] Three 20 sec injection of 25 nM fluorescein on a 100 μm i.d. x 30 cm C1 sol-gel packed capillary ran with 10 kV applied voltage. The concentration of the phosphate in the running buffer was varied between 1-3 mM in 40 % methanol. The left axis shows the LIF detector intensity, while the right axis shows the actual current across a 100 kΩ resistor in series with the capillary.

A series of injections were then compared where the phosphate buffer was held constant at 2 mM, but the percentage of methanol varied (results shown in Figure 2-13). For each of these solutions, the pH of the phosphate buffer was 7 before adding the methanol. Each of the three injections were 20 sec long at 10 kV with fluorescein (25 nM for 40 % methanol and 100 nM for both 50 % and 60 % methanol) on a 100 μm i.d. x 30 cm C1 sol-gel packed capillary ran with 10 kV applied voltage. The concentration of the phosphate in the running buffer was held constant at 2 mM while the
percentage of methanol was varied from 40-60 %. These injections were run on three separate days. It was expected that increasing the methanol concentration would continually speed up the elution of fluorescein from the column, but this was not the case as 60 % methanol had a longer elution time than 50 % methanol. The 60 % methanol elution could have been slower because of bubble formation on the column or the phosphate buffer not being soluble in the higher concentration of organic solvent. Either way the EOF was not as stable with the higher organic concentration, limiting the solvent elution strength.

Figure 2-13 Three 20 sec injection of fluorescein on a 100 μm i.d. x 30 cm C1 sol-gel packed capillary ran with 10 kV applied voltage. The concentration of the phosphate in the running buffer was held constant at 2 mM while the percentage of methanol was varied from 40-60 %.

A 10 nM each mixture of the five amine derivatives (C10, C12, C14, C16 and C18) in 40 % methanol and 1 mM phosphate buffer was injected on the C1 sol-gel CEC column. The injection was 20 sec long with 10 kV applied during both injection and separation. Results are shown in Figure 2-14. Because the run time had to be set before
injecting, the voltage was only monitored for the first 100 min and the LIF detector run for 110 min. The first peak was assigned to be the FITC, the second peak was assigned to be the C10 derivative, the third peak was assigned to be the C12 derivative, and the fourth peak was assigned to be the C14 derivative while C16 and C18 did not elute. During this experiment, the order of elution was hypothesized and later confirmed during a spiking experiment (Figure 2-15).

![Image of chromatogram](image)

**Figure 2-14** 20 sec injection of 10 nM each mixture of the five amine derivatives on a 100 μm i.d x 30 cm C1 sol-gel packed capillary run with 10 kV applied voltage. The left axis shows the LIF detector intensity, while the right axis shows the actual current across a 100 kΩ resistor in series with the capillary column.

In order to determine the elution order, a series of mixtures were analyzed where one derivatized amine was at a higher concentration than the others in solution. Results are shown in Figure 2-15. For these separations, the mobile phase was a mixture of
50 % methanol and 2 mM phosphate buffer. Each amine derivative was injected at 50 nM except the amine derivative of interest which was added into the mixture at 150 nM. Injections were 10 sec long at 25 kV. Each injection was run right after the previous injection and the current had a flat profile (stable with no joule heating). Although there was some variation in elution times, the elution order was confirmed to be fluorescein, C10, C12, and the C14. Some tailing was occurring on the C14 peak. It was determined that this column was too hydrophobic for C16 and C18 analysis.

![Graph showing the injection of amine derivatives](image)

**Figure 2-15** 10 sec injection at 25 kV of 50 nM of each amine derivatives with the spike concentration at 150 nM on a 100 μm i.d x 30 cm C1 sol-gel packed capillary run with 25 kV applied voltage. Each spike mixture was run twice.
2.4.8 Problems

Most of the time was spent on conditioning the columns. Low flow rates had to be used, because at high pressures the capillaries came unpacked. The ammonium hydroxide wash took the longest. This step was necessary to remove any precursor left after making the sol-gel. The precursor was positively charged, causing the flow to be reversed if any remained on the column. When working with the columns, they needed to be kept as straight as possible. Bending could cause the capillary to break or the packing to separate. Any inconsistency in the packing or capillary causes the voltage to arc in that location, decreasing the stability of the current. If this region was close enough to the end of the capillary, this region was cut off. (Inconsistencies were identified using a microscope.) If this region was close to the capillary window, a new column had to be made. All of the running buffers and the injection solutions had to be degassed before using. Any bubbles in the solution could have been injected onto the column, which caused the current to fluctuate until the bubble was eluted. The bubbles could also have formed when the column over heated from Joule heating especially when using the higher concentration of organic solvents.
2.4.9 CEC/LIF Conclusions

Varying the concentration of methanol and the concentration of the phosphate buffer in the running buffer determines the elution time. The length of the column could have also been varied, but due to the problems encountered trying to keep a stable current going through the column, the length of the column was never varied. By lowering the concentration of phosphate in the mixture, the ionic strength will also be lowered. The lower the ionic strength, the higher the EOF will be. Increasing the EOF, which can also be done by increasing the applied voltage, allows for shorter run times and higher resolution. The power supply can go up to 30 kV, but, depending on the mobile phase, a lower voltage had to be used so that there was a stable current going across the column. By doing an Ohm’s plot, it was determined whether the selected voltage was in the stable range of the current set-up. There will be a lower limit on how much the buffer concentration can be reduced before a current can no longer be sustained across the column. The running buffer was optimized to contain 2 mM phosphate buffer, since experiments show that this concentration produces the most stable current.

Adding methanol to the running buffer was necessary to increase the elution strength of the mobile phase and elute the derivatives off the column. The methanol also needed to be in the loading solution since the amines and the amine derivatives were not soluble in water. The mobile phase cannot be 100 % methanol because phosphate was not soluble in methanol and methanol has a lower boiling point making bubble formation more likely. The running buffer was optimized to contain 50 % methanol since the EOF was not reproducible with the higher concentration. Additionally, the fluorescein
actually took longer to elute with the higher concentration of methanol because of solubility limitations of phosphate in methanol.

Unfortunately, under these conditions, the higher chain amine derivatives (C16 and C18) did not elute from the column. Future work for this column would include optimizing the stationary phase of the column. The sol-gel procedure would need to be examined so that a less hydrophobic stationary phase could be made. Another approach would be to use a buffer salt that is soluble in methanol so that higher concentrations could be used during the analysis.

2.5 Conclusions

Unfortunately, neither one of the phases was able to be completely developed. Both of these analysis methods proved to be very irreproducible in their own way. The microchips often clogged as the stationary phase degraded, making it impossible to optimize the reaction. For the reaction to work on the microchip, the microchip would have to be redesigned so that the stationary phase would not have to be baked into position in order to prevent degradation of the stationary phase. Adding some automation to the solvent/analyte delivery system would deliver the same amount each time and the loading volume would not be dependent on the amount of back pressure. A possible solution would be to use a microchip where the stationary phase is loaded from a side channel off the main separation channel that could be sealed off with epoxy (T-chip design is described in Chapter 5). The CEC separation also proved to be very
irreproducible due to air bubble formation. It often took days to flush the bubbles out of the capillary. The best results show that C12-C16 were able to be separated, but C18 stuck to the stationary phase. In order for this separation to work for longer chain amines, the sol-gel used to pack the CEC column would need to be modified so that it is less hydrophobic. Currently, there are no commercial columns that could be applied to this application, so the columns have to be made in-house. Overall these methods show that reducing the reaction volume allows for a more effective reaction between the dye and analytes and small volumes of analytes can be analyzed using CEC/LIF.

2.6 Acknowledgements

This work is supported by NIH National Institute for Neurological Disorders and Stroke R15NS038443. Dr. Braden Giordano from the Naval Research Laboratory in Washington D.C. supplied the initial C1 sol-gel columns and Dr. Tara Carpenter made the set of standard amine derivatives and helped design the chip based experiments.
2.7 References


Chapter 3

*Exploring Amide Detection Methods: MALDI-MS*

3.1 Abstract

Primary fatty acid amides (PFAMs) are found in biological systems at nanomolar concentrations or below. Currently the standard method for PFAM analysis in this laboratory is a GC/MS method developed by Sultana [1]. Problems with the method include poor reproducibility of the derivation reaction needed to make the compound less polar for a better separation between isomers with different bond positions. In this chapter, the use of time-of-flight mass spectrometry (TOF-MS) with a matrix-assisted laser desorption and ionization (MALDI) source or was explored as a possible alternative method. This analytical technique provides an opportunity to develop a highly selective method to detect the lipids at low concentrations. For this study several different matrices (2,5-dihydroxybenzoic acid, α-cyano-4-hydroxycinnamic acid, caffeic acid, 4-nitroaniline, 2-naphthalene boronic acid, cetrimonium bromide, dithranol, retinoic acid, vitamin D, and sinapic acid) and additives (sodium acetate, lithium acetate, potassium acetate, ammonium acetate and trifluoroacetic acid) were examined to see which
improved the signal of the fatty amides. Optimization experiments were done with four fatty amides: lauramide, oleamide, palmitamide, and stearamide, in order to test each method.

3.2 Introduction

The matrix assisted laser desorption ionization (MALDI) process involves short laser pulses and the matrix is able to absorb the given wavelength [2-6]. When the laser is focused on a spot containing a mixture of the matrix and the analytes, the matrix absorbs the energy from the laser and the surface molecules are then ablated. In the resulting plume of both matrix and analyte molecules, the charge is transferred from the matrix to the analytes, creating the charged analyte ions needed for analysis by mass spectrometry (MS). MALDI is a soft ionization technique; the analytes are more likely to have a single charge or cluster than to fragment. The benefits of using a MALDI source include low detection limits and small sample size.

According to Pasch and Schrepp, there are three main goals when preparing a MALDI sample: the matrix and sample mix needs to form crystals, the matrix needs to absorb the laser radiation in the MALDI source, and the analyte needs to be ionized [2]. One of the hardest steps is creating homogeneous, uniform crystals. There are several different methods to apply the sample and matrix to the plate: dried droplet procedure, spin coating, formation of thin films, air-spray deposition, electrospray deposition, and solventless [2, 7, 8]. The dried droplet procedure is the most common where a solution
of the matrix is mixed with the sample solution [2, 7, 8]. Approximately 1-5 µL of the final solution is applied to the MALDI plate and allowed to dry. Spin coating requires the samples to be loaded into stainless steel probe tips and then rotated at 300 rpm [2, 9]. The small crystals could then be crushed by applying pressure with a glass slide. Experiments showed a homogeneous mixture of the matrix and analyte. Thin films have been applied by first applying the matrix and then a layer of the analytes [2]. Spraying the mix solution on the MALDI plate with an air-spray brush increases the shot-to-shot reproducibility [2]. This method eliminates some of the inhomogeneous crystals formed with a drop drying. Electrospray deposition uses a set-up similar to the electrospray MS source [2, 7, 8]. This method provides a homogeneous layer, but it is more complicated and could produce fragmentation depending on the analyte. It is estimated that approximately 1-2 µL of the solution is applied to a 2.5 cm diameter circle. The final method is similar to KBr pellets where the matrix and analyte powders are finely ground and mixed together [2, 10]. This method eliminates any inconsistencies due to solvent evaporation.

Graphite has been added to liquid matrices for MALDI analysis [11]. In these experiments, the liquid matrix (glycerol) was diluted in methanol and mixed with graphite particulates. The slurry was applied to the sample holder and the methanol was allowed to dry. If the mixture was able to spread before drying, it created a thin layer of graphite/liquid matrix. The analytes were spotted on top of the graphite/liquid matrix. The problem with this technique is that it could contaminate the source. The advantage of this technique is that the graphite acts as an energy transfer medium and allowed for fmol detection of medium range molecular weight compounds. A separate sample
preparation method called desorption/ionization on porous silicon mass spectrometry has also been used [12]. In this case the analytes are trapped in the porous silicon and released when the laser is aimed at a spot. The advantage of this technique is that small molecules can be analyzed without interferences from the matrix. The disadvantage is that the surface will degrade over time and contaminants from the air can also be trapped onto the surface.

Relatively few MALDI experiments have been done on small molecules. This is mostly due to the baseline noise at low mass-to-charge ratios when vacuum MALDI sources are used. There is less baseline noise when atmospheric pressure MALDI sources are used, but the matrix signal can still interfere. Although MALDI is commonly used in proteomics, experiments have been done to analyze the different lipid classes. For example phospholipids [13], diacylglycerols [14, 15], cholesterol [16] and fatty acids [17] have been detected using a MALDI-MS. Ayorinde et al. developed a MALDI method to qualitatively determine the composition of fatty acids in vegetable oils [17]. This method used meso-tetrakis(pentafluorophenyl)porphyrin (F20TPP) as the matrix and sodium acetate was added as the dopant. The vegetable oil was saponified before mixing with the matrix and dopant solutions. The final mixture was made in methanol for spotting on the MALDI plate. Each of the acids were detected as the \([\text{RCOONa} + \text{Na}]^+\) peak. For this study there was approximately 10 nmol of each acid in one spot.

Additives have been included in the MALDI spot in order to improve the spectrum either by increasing the analyte signal or by reducing the matrix signal. Hanton et al. added alkali cations to the analyte solution [18]. This study found that the analytes were more likely to fragment rather than separate from the metal cation. Guo et al. have
shown that additives such as CTAB can be added at low concentrations to the sample spot in order to suppress the matrix signal [19]. This technique was used to analyze small molecules such as cyclodextrin using alpha-cyano-4-hydroxycinnamic acid (CHCA) as the matrix.

A list of common matrices including common solvents that have been used for MALDI-TOF analysis has been compiled elsewhere [2, 20]. Table 3-1 shows the matrices examined for the PFAM analysis.

<table>
<thead>
<tr>
<th>Name</th>
<th>Molecular Formula/Molecular Mass</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,4-dihydroxycinnamic acid (caffeic acid)</td>
<td>C_9H_8O_4 180.16 amu</td>
<td><img src="image1.png" alt="Structure" /></td>
</tr>
<tr>
<td>Alpha-cyano-4-hydroxycinnamic acid (CHCA)</td>
<td>C_{10}H_6O_3N 189.17 amu</td>
<td><img src="image2.png" alt="Structure" /></td>
</tr>
<tr>
<td>Cetyltrimethyl ammonium bromide (CTAB)</td>
<td>C_{19}H_{42}BrN 364.34 amu</td>
<td><img src="image3.png" alt="Structure" /></td>
</tr>
<tr>
<td>2,5-dihydroxybenzoic acid (DHB)</td>
<td>C_7H_6O_4 154.12 amu</td>
<td><img src="image4.png" alt="Structure" /></td>
</tr>
<tr>
<td>Name</td>
<td>Molecular Formula/ Molecular Mass</td>
<td>Structure</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>----------------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>dithranol</td>
<td>C\textsubscript{14}H\textsubscript{10}O\textsubscript{3} 226.23 amu</td>
<td><img src="image" alt="Dithranol Structure" /></td>
</tr>
<tr>
<td>4-nitroaniline</td>
<td>C\textsubscript{6}H\textsubscript{6}O\textsubscript{2}N\textsubscript{2} 138.12 amu</td>
<td><img src="image" alt="4-Nitroaniline Structure" /></td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>C\textsubscript{11}H\textsubscript{12}O\textsubscript{5} 224.21 amu</td>
<td><img src="image" alt="Sinapic Acid Structure" /></td>
</tr>
<tr>
<td>all-trans-retionic acid</td>
<td>C\textsubscript{20}H\textsubscript{28}O\textsubscript{2} 300.44 amu</td>
<td><img src="image" alt="All-Trans-Retionic Acid Structure" /></td>
</tr>
<tr>
<td>(Vitamin A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cholecalciferol</td>
<td>C\textsubscript{27}H\textsubscript{44}O 384.64 amu</td>
<td><img src="image" alt="Cholecalciferol Structure" /></td>
</tr>
<tr>
<td>(Vitamin D3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Naphthaleneboronic acid</td>
<td>C\textsubscript{10}H\textsubscript{7}B(OH)\textsubscript{2} 171.99 amu</td>
<td><img src="image" alt="2-Naphthaleneboronic Acid Structure" /></td>
</tr>
</tbody>
</table>

**Table 3-1** Structures of matrices analyzed in this study for optimizing PFAM (C12:0, C16:0, C18:0 and C18:1\textsuperscript{9}) detection by MALDI-TOF.
MALDI MS is known for being a trace analysis technique, and a few published experiments have shown that MALDI MS can be used for quantitative analysis. Benard et al. were able to quantify the amount of diacylglycerols (DAGs) by carefully controlling the experimental conditions [14]. The analyte/matrix solutions were dissolved in ethylacetate. It was found that ethylacetate produced a more homogeneous mixture between the compounds (DHB and DAGs) than methanol. Secondly, because alkali metal ions commonly found in chemicals and solvents are not readily soluble in ethylacetate, the ratio between the sodium adduct and the potassium adduct could be kept at an approximate constant. One of the DAGs was used as an internal standard to reduce the differences in intensities from run to run. By setting the laser power to obtain the optimal signal to noise ratio, it was possible to quantify the DAGs in the picomolar to nanomolar range. This method is less sensitive than GC/MS or LC/MS methods, but the sample preparation is less extensive and total analysis time is significantly less.

The two main goals for this project were to test the detection limits for PFAMs with TOF-MS using an AP-MALDI source and to determine whether a method can be developed in order to accurately quantitative the trace levels of PFAMs found in a sample. Several different compounds were evaluated to determine which matrix would increase the analyte signal with minimal baseline interference.
3.3 Reagents

2-Naphthaleneboronic acid, 2,5-dihydroxybenzoic acid (DHB), 3,4-dihydroxycinnamic acid (caffeic Acid, predominantly trans isomer, 99+ % pure), all-trans-retionionic acid (Vitamin A acid, 97 % pure), cetyltrimethyl ammonium bromide (CTAB) and Vitamin D3 crystalline (99+ % pure) were from Acros Organics (Morris Plains, NJ). Dithranol (97+ % pure) and α-cyano-4-hydroxycinnamic acid (CHCA, 97 % pure) were from Aldrich (Milwaukee, WI). 4-nitroaniline was from Kodak (Rochester, NY). Sinapic acid (98 % pure) and oleic acid were from Sigma (St. Louis, MO). The methanol (HPLC grade for solutions and reagent grade for cleaning) and the trifluoroacetic acid (TFA) were from Thermo Fisher Scientific (Pittsburgh, PA). Car wax (paste form by Turtle Wax Inc.) was from the local hardware store and was used to condition the MALDI plates. Lauric acid and stearic acid were from Thermo Fisher Scientific. Palmitic acid was from ICN Biomedicals Inc. (Aurora, OH). The primary fatty acid amide (PFAM) standards [lauramide (C12:0), palmitamide (C16:0), stearamide (C18:0), and oleamide (C18:1\(^\circ\))] were synthesized in house from their corresponding fatty acids with a greater than 95% purity as verified by GC/MS [21].

3.4 Instrument Set Up

For these experiments, an Agilent Technologies (Santa Clara, CA) time-of-flight mass spectrometer was used with an AP/MALDI ion source interface. The instrument was calibrated and checked using the ESI source before switching to the MALDI source.
For these experiments, the fragmenter was set to 300 V, the skimmer was set to 60 V, the Oct RFV was set to 300 V, and the nitrogen gas temperature was 325 °C at 5.0 L/min unless otherwise noted. The intensity of the signal was increased when the laser was focused on a crystal. In order to increase crystal formation when drying, the plates were waxed, which prevented the sample from spreading over the MALDI plate. Figure 3-1 shows the difference in spot sizes between a treated MALDI plate and an untreated MALDI plate. Figure 3-2 shows the differences in mass spectra. There is some additional noise from the presence of the wax, but none of the signal from the wax overlaps with the m/z for the analytes. The signal of the 714 m/z peak is stronger on the waxed MALDI plate vs the untreated MALDI plate. For all the experiments, the waxed MALDI plates were used. The wax was applied as needed when the spots started to spread before drying.

**Figure 3-1** 0.5 µL spots of DHB. On the left where the plate has been waxed, the spots remained condensed and crystals start to form. On the right where the plate has no treatment, the spot spreads out of the block and no crystals form. The results are better when the laser can be aimed at crystals.
Figure 3-2 Comparison of mass spectra between DHB when spotted on a waxed MALDI plate (top) and DHB when spotted on an untreated MALDI plate (bottom).

3.5 Results and Discussions

3.5.1 UV-Vis of Matrix

In order to determine possible matrixes, a series of compounds were examined on the UV-visible spectrophotometer (Cary 1E from Varian, Palo Alto, CA) from 200 to 500 nm to determine the absorbance at 337 nm (Figure 3-3). If the compounds do not absorb at 337 nm from the nitrogen laser on the MALDI source, the compound will not be effective at lifting the analytes off the plate and into the MS source. For these experiments, the compounds were dissolved in methanol at 10 μM. Eight different compounds were analyzed: caffeic acid, CHCA, DHB, CTAB, nitroaniline, dithranol, retinoic acid, and vitamin D3.
3.5.2 Choosing a Matrix

In order to determine the optimal conditions for detecting the primary fatty acid amides (PFAMs), four sample PFAMs (lauramide (C12:0), palmitamide (C16:0), oleamide (C18:1\(^9\)), and stearamide (C18:0)) were compared by varying which matrix was used, the sample to matrix ratio, the analysis time, and the attenuation of the laser. The sample to matrix ratio was run using the ten different matrixes: DHB, CHCA, caffeic
acid, 4- nitroaniline, 2-naphthalene boronic acid, CTAB, dithranol, retinoic acid, Vitamin D, and sinapic acid. Unless otherwise noted, the sample spot size was 0.5 μL and the spectrum was collected for 1 min.

2,5-dihydroxybenzoic acid (DHB, MW = 154.12) contains a benzene ring with two alcohol groups and a carboxylic acid group. The attenuation of the laser was set to nine when using DHB as the matrix. Figure 3-4 shows the profile of the DHB matrix with no analytes. Figure 3-5 shows the signal of four PFAMs (C12:0, C16:0, C18:1⁹, and C18:0) using DHB as the matrix. The MALDI plate was sonicated in reagent grade methanol and allowed to dry before spotting. In order to determine the best spotting conditions, a series of solutions was made from a 0.5 M solution of DHB in methanol and a 10 mM total mix of the four PFAMs: C12:0, C16:0, C18:1⁹, and C18:0 where the ratio of the moles of DHB to the moles of total amides was varied (Figure 3-6). The errors were high for some of the mixtures due to poor signal-to-noise ratio. It was determined that a ratio near 1000:1 would be the best when working with the DHB matrix. However, the overall concentration may need to be increased so that the signal is significantly higher than the baseline. The results from this experiment were difficult to reproduce due to the degradation of the laser, eventually making it impossible to use DHB as the matrix.
Figure 3-4 MALDI profile of the DHB matrix.

Figure 3-5 MALDI spectrum of the PFAM mixture (C12:0, C16:0, C18:1\(^9\), and C18:0) using DHB as the matrix. Approximately 20 pmol of each PFAM and 500 nmol of DHB was spotted.
Figure 3-6  Plot of the log of the average intensity of the analyte peak divided by the mole of analyte in the sample vs the log of the ratio of moles of DHB to moles of amides.

Alpha-cyano-4-hydroxycinnamic acid (CHCA, MW = 189.17) is a cinnamic acid derivative. The attenuation of the laser was set to 7 when using CHCA as the matrix. The MALDI plate was sonicated in reagent grade methanol and allowed to dry before spotting. Figure 3-7 shows the profile of the matrix. Figure 3-8 shows the signal of four PFAMs (C12:0, C16:0, C18:1\(^9\), and C18:0) using CHCA as the matrix. In order to determine the best spotting conditions, a series of solutions was made from a 0.2 M solution of CHCA in methanol and a 10 mM total mix of the four PFAMS where the ratio of the moles of CHCA to the moles of total amides was varied (Figure 3-9). The concentration of the CHCA was limited by the solubility of CHCA in methanol. Again, the ratio 1000:1 showed the best signal with limited interference from the background.
Figure 3-7  MALDI profile of the CHCA matrix.

Figure 3-8  MALDI spectrum of the PFAM mixture (C12:0, C16:0, C18:1^9, and C18:0) using DHB as the matrix. Approximately 20 pmol of each PFAM and 100 nmol of CHCA was spotted.
**Figure 3-9** Plot of the log of the average intensity of the analyte peak divided by the mole of analyte in the sample vs the log of the ratio of moles of CHCA to moles of amides.

3,4-dihydroxycinnamic acid (caffeic acid, MW = 180.16) is a phenolic compound similar to cinnamic acid but contains more hydroxyl groups. The attenuation of the laser was set to 7 when using caffeic acid as the matrix. Figure 3-10 shows the profile of the matrix. The MALDI plate was sonicated in reagent grade methanol and allowed to dry before spotting. In order to determine the best spotting conditions, a series of solutions was made from a 0.2 M solution of caffeic acid in methanol and a 10 mM total mix of the four PFAMS where the molar ratio of caffeic acid to total amides was varied. After analyzing all the samples, it was determined that caffeic acid would not work as a matrix for analyzing PFAMs because there was no signal for the PFAMs in any of the spectra.
4-Nitroaniline (MW = 138.13) is a benzene ring with a nitro group and an amino group in the *para* position. The attenuation of the laser was set to 7 when using 4-nitroaniline as the matrix. The MALDI plate was sonicated in reagent grade methanol and allowed to dry before spotting. Figure 3-11 shows the profile of the matrix. Figure 3-12 shows that even when the spot contains a mixture of PFAMs, the matrix signal was predominate. In order to determine the best spotting conditions a series of solutions was made from a 0.2 M solution of 4-nitroaniline in methanol and a 10 mM total mix of the four PFAMS where the ratio of the moles of 4-nitroaniline to the moles of total amides was varied (Figure 3-13). This data had to be carefully analyzed by scanning the data list to find the m/z corresponding to the PFAMs. Overall the results from CHCA were better than the results from the 4-nitroaniline.
Figure 3-11 MALDI profile of the 4-nitroaniline matrix.

Figure 3-12 100 – 300 m/z MALDI profile of the 4-nitroaniline matrix. Although the spot contained a mixture of PFAMs, the matrix signal is predominant making PFAM analysis difficult.
**Figure 3-13** Plot of the log of the average intensity of the analyte peak divided by the mole of analyte in the sample vs the log of the ratio of moles of 4-nitroaniline to moles of amides.

Cetyltrimethyl ammonium bromide (CTAB, MW = 364.45) has a long carbon chain and a bromide ion. The attenuation of the laser was set to 12 when using CTAB as the matrix. The MALDI plate was sonicated in reagent grade methanol and allowed to dry before spotting. In order to determine the best spotting conditions, a series of solutions was made from a 0.5 M solution of CTAB in methanol and a 10 mM total mix of the four PFAMS where the ratio of the moles of CTAB to the moles of total amides was varied. Figure 3-14 shows the profile of the matrix. After analyzing all the samples, it was determined that CTAB would not work as a matrix for analyzing PFAMs since there was not enough signal to positively identify the PFAMs in any of the spectra. Guo et al. have shown that CTAB actually suppresses the matrix signal when added at low concentrations to the matrix:analyte mixture allowing for better detection of the analyte, but does not work as the matrix alone [19].
Dithranol (MW = 226.2), unlike the other compounds chosen to be studied as a possible matrix, contains two benzene rings. The attenuation of the laser was set to 9 when using dithranol as the matrix. The MALDI plate was sonicated in reagent grade methanol and allowed to dry before spotting. Figure 3-15 shows the profile of the matrix. Figure 3-16 shows the signal of four PFAMs (C12:0, C16:0, C18:1\textsuperscript{9}, and C18:0) using dithranol as the matrix. Because of background noise only C18:1\textsuperscript{9} and C18:0 can be seen on the graph. In order to determine the best spotting conditions, a series of solutions was made from a 0.2 M solution of dithranol in choloroform and a 10 mM total mix of the four PFAMs where the ratio of the moles of dithranol to the moles of total amides was varied (Figure 3-17). Dithranol is not soluble in methanol. Because chloroform had to be used as the solvent, a glass syringe was used to spot the MALDI plate so that smaller spots could be applied to the plate to prevent the spot from spreading while drying. The
main problem with using dithranol as the matrix is that there is a matrix peak with almost
the same m/z ratio as the C18:0 analyte signal. The two signals could be differentiated
by looking at the data table for an exact mass. Although the analytes had reasonable
signal intensities, the signal from the matrix was significantly higher making it difficult to
detect the analyte.

![Figure 3-15](image_url) MALDI profile of the dithranol matrix.

![Figure 3-16](image_url) MADLI spectrum of the PFAM mixture (C12:0, C16:0, C18:1<sup>9</sup>, and C18:0) using
dithranol as the matrix. Approximately 450 pmol of each PFAM and 45 nmol of dithranol was
spotted.
Figure 3-17  Plot of the log of the average intensity of the analyte peak divided by the mole of analyte in the sample vs the log of the ratio of moles of dithranol to moles of amides.

All-trans-retinoic acid (MW = 300.44) is also known as vitamin A. The attenuation of the laser was set to 10 when using vitamin A as the matrix. The MALDI plate was sonicated in reagent grade methanol and allowed to dry before spotting. Figure 3-18 shows the profile of the matrix. Figure 3-19 shows the signal of four PFAMs (C12:0, C16:0, C18:19, and C18:0) using vitamin A as the matrix. Because of the high background noise only C16:0 and C18:0 can be identified on the spectrum. In order to determine the best spotting conditions, a series of solutions was made from a 0.5 M solution of vitamin A in 1:1 methanol:chloroform and a 10 mM total mix of the four PFAMs where the ratio of the moles of vitamin A to the moles of total amides was varied (Figure 3-20). Vitamin A was not soluble in 100% methanol. Because chloroform had to be added, a glass syringe was used to spot the MALDI plate so that smaller spots could be applied to the plate to prevent the spot to spread while drying. Although there are not
any overlapping peaks between the analyte and the matrix, the spectrum for vitamin A was noisy. For this matrix, there does not seem to be much difference in the analyte signal no matter which ratio was used for the analysis. This matrix would be better if the software allowed for background subtraction.

![Figure 3-18](image1.png)

**Figure 3-18** MALDI profile of the vitamin A matrix.

![Figure 3-19](image2.png)

**Figure 3-19** MALDI spectrum of the PFAM mixture (C12:0, C16:0, C18:1, and C18:0) using vitamin A as the matrix. Approximately 100 pmol of each PFAM and 20 nmol of vitamin A was spotted.
Figure 3-20  Plot of the log of the average intensity of the analyte peak divided by the mole of analyte in the sample vs the log of the ratio of moles of vitamin A to moles of amides.

Vitamin D3 (D3, MW = 384.64) was also examined as a possible matrix. The attenuation of the laser was set to 10 when using vitamin D3 as the matrix. The MALDI plate was sonicated in reagent grade methanol and allowed to dry before spotting. Figure 3-21 shows the profile of the matrix. This matrix was tested by mixing a 10:1 solution of 0.2 M vitamin D3 in methanol and 10 mM total mix of the four PFAMS. It was determined that vitamin D3 would not work as a matrix, because the attenuation was on the minimum setting and very little signal was obtained.
Sinapic acid (MW = 224.21) is commonly used for negative mode ionization. The attenuation of the laser was set to 7 when using sinapic acid as the matrix. The MALDI plate was sonicated in reagent grade methanol and allowed to dry before spotting. Figure 3-22 shows the profile of the matrix. This matrix was tested by mixing a 50:1 ratio of moles made from a solution of 0.2 M sinapic acid in methanol (containing 0.1 % by volume TFA) and 10 mM total mix of the four PFAMs. It was determined that sinapic acid would not work as a matrix, because the sensitivity for detecting PFAMs was much better in the positive mode.
2-Naphthalene boronic acid (MW = 171.99) was examined as a possible matrix. The attenuation of the laser was set to 10 when using 2-naphthalene boronic acid as the matrix. The MALDI plate was sonicated in reagent grade methanol and allowed to dry before spotting. This matrix was tested by mixing a 100:1 molar ratio made from a solution of 0.1 M 2-naphthalene boronic acid in methanol and 10 mM total mix of the four PFAMS. Figure 18 shows the profile of the matrix. It was determined that 2-naphthalene boronic acid would not work as a matrix, because the PFAMs were not ionized using this matrix. Additionally, the matrix signal by itself was not very strong.

Overall the two best matrices were DHB and CHCA. The major problem was that the instrument was never designed to run DHB so over time, as the laser degrades from use, the laser no longer has enough energy to ablate the matrix. A few additional studies were done with DHB until the laser degraded. CHCA ended up to be the best matrix, but its solubility properties in methanol limited the solution parameters. The
other matrixes either had too much noise in the baseline in order to be able to detect the
PFAMs or did not absorb the 337 nm wavelength effectively enough.

3.5.3 Attenuation Study

Initially the attenuation was adjusted by aiming the laser at a spot of matrix and
adjusting the laser attenuation for the highest intensity of the matrix spots. Once it was
determined that DHB and CHCA were the best matrixes, a series of runs were done
where the spectrum for the mixture of the four PFAMs was collected for each attenuation
setting. DHB was varied from 6 – 10 (below 6 no signal was obtained) and CHCA was
varied from 2 – 10. For these runs, the run time was 1 min and the spectrum was
collected from 100 to 400 m/z. Figure 3-23 shows the data from the average of three
spots for both DHB and CHCA. Each spot was a 100:1 molar ratio of matrix (40 μM
stock solution) to PFAM (10 mM total mixture of stock solutions). From this study it
was determined that for DHB the attenuation should be set to 10 and for CHCA it should
be set to 4, lower than previous runs. It is important to note that these results will change
as the laser degrades, but when the optimization is checked it is better to examine the
analyte’s signal rather than the matrix signal.
3.5.4 Determining Analysis Time

Initially all of the matrixes were compared by analyzing the integrated spectrum collected over 1 min. In order to determine the best run time for collecting the data, a series of runs were done with CHCA and DHB as the matrix and the PFAM concentration (100:1 ratio with 10 mM total PFAMs in methanol) was held constant so that just the length of analysis was varied (0.5, 1.0, 3.0, 5.0, and 10.0 min (Figures 3-24 and 3-25). Each of the data points had to be multiplied by the time, in seconds, of analysis. This was necessary since the software automatically divides by the time, but this experiment was designed to look at the total signal collected from one spot.

Figure 3-23  Plot of the intensity of the analyte peak vs the attenuation setting on the laser.

Each of the data points were the average from three spots.
Figure 3-24  Plot of the intensity of the analyte peak vs the time in minutes of the analysis time for DHB. Each of the data points were multiplied by the time in seconds of analysis.

Figure 3-25  Plot of the intensity of the analyte peak vs the time in minutes of the analysis time for CHCA. Each of the data points were multiplied by the time in seconds of analysis.
Without the corrections, the DHB runs look the same and for CHCA the shorter the run time the better the signal; however, once the data has been corrected for the length of analysis (data shown above), 3 min run times look the best for both matrixes. There might be slight improvement over 3 min for CHCA if the run time was extended to 10 min, but not significantly enough to justify the longer analysis time. By plotting the signal to noise ratio for DHB (Figure 3-26) and CHCA (Figure 3-27), the results show that a 3 min run time was required for an optimal signal.

**Figure 3-26** Plot of the signal to noise ratio vs the run time using DHB as the matrix. The greatest signal to noise ratio was with a three min run time for each of the PFAMs analyzed.
Figure 3-27 Plot of the signal to noise ratio vs the run time using CHCA as the matrix. The greatest signal to noise ratio with the least amount of error was with a three min run time for each of the PFAMs analyzed.

3.5.5 Optimizing Additives

Adding salts or acids to the matrix and analyte mixture can increase the signal strength of the analytes. Ayorinde et al. used sodium acetate as a dopant in order to detect fatty acid methyl esters on the MALDI-TOF [17]. For this study, a series of salts (sodium acetate, lithium acetate, potassium acetate, and ammonium acetate) and TFA were added to the spots to see if any improvement in the signal was made.

In order to test the different salt additives, the matrix to analyte ratio was 1000:1 in methanol so that each spot contained 420 nmol DHB, 100 pmol each PFAM with 20 nmol of salt. These runs were compared to spots where methanol was added in place
of the salt solution. The spots were 0.5 μL and the run time was 1 min long.

Unfortunately, in this case, the salt suppressed the signal. The best [M + salt]$^+$ signal peak had the same intensity as the [M+H]$^+$ signal peak in the same spectrum (Figure 3-28). Except for C18:1$^\omega$, adding salt to the spot decreased even the combined signal of the [M+salt]$^+$ signal and the [M+H]$^+$ signal for each PFAM.

![Figure 3-28](image)

**Figure 3-28** Plot of the intensity of the analyte peak for each salt examined with the four PFAMs. Each signal is the average of three trials were the [M+H]$^+$ and the [M+salt]$^+$ signal were combined. The blank run only contained [M+H]$^+$ peaks because methanol was added in place of a salt solution. Four PFAMs were in each spot with the DHB matrix at a ratio of 1000:1.

Another approach to increasing the analyte signal was adding trifluoroacetic acid (TFA) to the spot. In order to test the acid as an additive two different concentrations were examined: 0.1 % and 0.5 % by volume. For this experiment the matrix to analyte ratio was 100:1 in methanol made with either 0.2 M DHB or 0.2 M CHCA and a 10 mM mixture of four PFAMs. These runs were compared to spots where methanol was added in place of the TFA. The spots were 0.5 μL and the run time was 3 min long. When analyzing the DHB spots the attenuation was set to 10, but turned down to 4 when
analyzing the CHCA spots. Adding TFA at 0.1 % either does not affect the PFAM signal or slightly improves the signal, however adding more TFA decreases the signal (Figure 3-29).

**Figure 3-29** Plot of the intensity of the analyte peak for each concentration of TFA examined with the four PFAMs for each matrix. Each signal is the average of three trials. Four PFAMs were in each spot with the DHB or CHCA matrix at a ratio of 100:1.

### 3.5.6 Detection Limits

After the parameters were optimized a series of solutions were made where the concentration of the PFAM was decreased. Before optimizing the MS parameters and the spotting conditions the detection limit was approximately 15 pmol when CHCA was used.
as the matrix. The final detection limit experiment was run over two days (Figure 3-30). The amount of matrix, CHCA, in each spot was held constant while the concentration of PFAMs was diluted before mixing with the CHCA solution, therefore the ratio of matrix to analyte varied with each set of spots. Using the optimized parameters, the detection limit for C12:0 was approximately 2 pmol, while the detection limit for C16:0, C18:0 and C18:1\(^9\) was approximately 200 fmol. It is important to note that these detection limits are dependent on the condition of the nitrogen laser.

![Graph showing detection limits](image)

**Figure 3-30** Detection limits of four PFAMs using CHCA for the matrix. The matrix to analyte ratios were 500:1, 5000:1, and 50,000:1.
3.5.7 Problems

One of the problems with the MALDI detection method is that the nitrogen laser continually degrades. Initially, samples were able to be analyzed using DHB as the matrix, but over time, as the laser degraded the laser was no longer powerful enough to create the plume of ions with the DHB compound. Unfortunately, this MALDI source was optimized to use CHCA as the matrix. It is generally accepted that analysis done by MALDI MS is only qualitative and not quantitative because only a portion of the sample spotted on the MALDI plate is ablated during analysis and the spot may not be homogeneous. However, throughout these experiments the intensity of the m/z peak was used to compare the results when optimizing the parameters. Further studies would have to be done in order to determine how much of the sample is actually analyzed.

3.6 Conclusions

A series of matrixes (DHB, CHCA, caffeic acid, 4- nitroaniline, 2-naphthalene boronic acid, CTAB, dithranol, retinoic acid, Vitamin D, and sinapic acid) were examined in attempt to develop a MALDI MS method to analyze PFAM samples. After a series of experiments comparing the analysis of C12:0, C16:0, C18:0 and C18:1\textsuperscript{9} using the different matrixes, it was determined that DHB and CHCA were the best suited for analysis. After exploring MALDI-TOF-MS for the detection of PFAMs as an alternative to the current GC/MS method [1, 21], it was determined that this ionization source was not sensitive enough to allow for trace detection. On average, the detection limits were in
the pmol range, so this project was ended while other PFAM detection methods (Chapter 4) were explored. Additionally, quantization statistics would have to be worked out to determine the accuracy of the method.
3.7 References


Chapter 4

Developing a LC/APCI-MS Separating and Detection Method for PFAMs Analysis

4.1 Abstract

There are many significant lipids whose resting levels in biological systems are at nanomolar concentrations or below. After evaluating several different analytical techniques including time-of-flight mass spectrometry (TOF-MS) with a matrix-assisted laser desorption and ionization (MALDI) source (Chapter 3) and reverse phase liquid chromatography with the use of single quadrupole mass spectrometry with either an electrospray source (ESI) or and atmospheric pressure chemical ionization source (APCI), it was determined that APCI allowed for the lowest detection limits. This chapter describes the details of developing the method in order to detect trace levels of primary fatty acid amides (PFAMs). Optimization experiments were done with four fatty amides: lauramide (C12:0), oleamide (C18:1\textsuperscript{\textcircled{\textdegree}}), palmitamide (C16:0), and stearamide (C18:0), before expanding the method to include all even saturated PFAMs between
lauramide (C12:0) and behenamide (C22:0), plus some PFAMs with one degree of unsaturation: C18:1 and C22:1. The limitations of the method will be discussed.

4.2 Introduction

Primary fatty acid amides (PFAMs), neutral, single chain, bioactive lipids, are part of a class of lipid signaling molecules [1-4]. It has been hypothesized that monitoring the concentration of specific primary fatty acid amides will allow for diagnosis of ocular surface disease [5, 6] and affective disorders such as depression [5]. In some diseases, the ratio of amide to its corresponding fatty acid may be more important than the quantity of amides present when diagnosing certain diseases [6]. Small changes in the carbon chain length change how the amide affects the system; specifically oleamide (C18:1\textsuperscript{9}) has been found to influence sleep [5, 7, 8], erucamide (C22:1\textsuperscript{13}) repairs the circulatory system [9, 10], and linoleamide (C18:2\textsuperscript{9,12}) effects Ca\textsuperscript{2+} signaling [11]. C18:1\textsuperscript{9} is the most commonly studied PFAM and has also been found to influence the memory processes [12], to decrease body temperature [13] and locomotor activity [7], suppress pain [14] and to regulate the voltage-gated Na\textsuperscript{+} channel [15, 16] and the GABA\textsuperscript{A} receptor [7, 12, 16]. The symptoms observed with increasing the dosage of amides vary with the type of unsaturation in the same chain length [3, 8]. For example, trans-9,10-octadecenoamide did not induce sleep for as long as cis-9,10-octadecenoamide at the same dosage [8]. Currently, the pathway involving the biological synthesis of PFAMs is unknown. It is hypothesized that the biosynthetic pathway for oleamide either
involves one of the following proteins: peptidylglycine alpha-amidating monooxygenase (PAM) [17, 18], cytochrome c [19] or fatty acid amide hydrolase (FAAH) [17, 20]. While FAAH is usually expected to degrade the PFAM, Bisogno et al. has shown that FAAH can also synthesize PFAM when the concentration of ammonium ion and the corresponding FFA concentration is high [21].

In order to study the biological pathways involving PFAMs, and to quantitate novel PFAMs in biological samples, analytical methods need to be improved to accurately determine the structure of the PFAMs at femtomole levels or below. Although there are methods available to study a few common PFAMs, the published methods do not include the complete series needed to quantify all the PFAMs found in biological samples. Madl and Mittelbach developed a liquid chromatography separation method followed by atmospheric pressure chemical ionization mass spectrometry for detection (LC/APCI–MS). The lower detection limits for long chain amides around 20 fmol [22]. Only C14:0, C16:0, C18:1⁹ and C18:0 were included in the separation protocol with an internal standard. Sultana achieved similar results (lower detection limit between 50 and 150 fmol depending on amide) using gas chromatography mass spectrometry (GC/MS) with an HP5-MS column (Agilent Technologies) for analysis [23]. This GC method was not able to separate the positional and geometric isomers. Baseline separation of C18:1⁹, elaidamide (C18:1trans⁹), petroselenamide (C18:1⁶), petroselaidamide (C18:1trans⁶), vaccenamide (C18:1trans¹¹), octadecenoamide (C18:1¹³), arachidamide (C20:0), behenamide (C22:0), and erucamide (C22:1¹³) was achieved using a highly polar column (BPX-70, SGE) allowing for the determination of the placement of the double bonds, but the samples needed to be derivatized first. The reaction between the amides and
N, N-bis-trimethylsilyltrifluoroacetamide (N, N-BSTFA) has a low percent yield and often forms other products, increasing the detection limit of the method and causing problems with quantitation. Other GC/MS methods have been developed to analyze C18:1\(^9\) in biological samples [24, 25] and plastic materials [26, 27]. These methods only examined the samples for a few specific PFAMs for qualitative analysis.

### 4.3 Summary of the GC/MS Method

While the LC/MS method was being developed, the samples were run on both the GC/MS and the LC/MS to compare detection limits, total analysis time, and separation limitations. The method was initially developed by Sultana [23, 28] and then adapted by Dent (Duquesne University).

Sultana’s method was transferred to a Varian GC/MS/MS system (CP-3800 GC with Saturn 2000 mass selective detector and CP-8400 autosampler) by Dent. The column was a Varian VF5-MS (0.25 mm internal diameter, 0.25 \(\mu\)m film thickness, 30 m long). The GC method used for analysis was as follows: starting temperature was 55 °C, ramped to 150 °C at 40 °C/min, held at 150 °C for 3.62 min, and finally ramped to 275 °C at 10 °C/min and held for 6.5 min. Helium was used as a carrier gas with a flow of 1 mL/min. The mass range was 50 to 350 m/z in chemical ionization (CI) mode, with methanol as the CI gas. The temperature of the injection port was 250 °C with an injection volume of 1 \(\mu\)L unsplit. The sample was diluted in 2-propanol to avoid methylation that occurs in the injection port when the sample was diluted in methanol.
4.4 LC/APCI-MS Method

This project was initially started after Sommer et al. demonstrated that femtomole detection of PFAMs was possible using APCI-MS [29]. In addition a previous graduate student in the Johnson lab, Tara Carpenter, started developing a reverse phase LC gradient to separate a series of PFAMs [30, 31]. These two ideas were combined to develop a reverse phase LC method using a Waters X-Terra MS C<sub>18</sub> (5 μm, 3.9 x 20 mm) column for the separation and an APCI-MS probe on the Waters ZMD MS for detection. Detection limits and reproducibility were examined.

4.4.1 Reagents

Methanol used for the LC/MS analysis was HPLC grade from either Thermo Fisher Scientific (Pittsburgh, PA) or J.T. Baker (Phillipsburgh, NJ). Distilled water was deionized with a Barnstead (Dubuque, IA) Nanopure water system. Both the methanol and the water were filtered with 0.22 μm Nylon membrane filters from either Millipore (Billerica, MA) or Whatman International (Maidstone, England) prior to use. The mobile phase for the HPLC analysis contained formic acid (99% pure) from Acros Organics (Morris Plains NJ) in both the methanol and water. These solutions were made up fresh each day and sonicated in a 2210 Branson Ultrasonic cleaner (Danbury, CT) for 20 min to mix and degas. Heptadecanoic-D33-Acid was from CDN Isotopes (Quebec, Canada). Lauric acid, myristic acid, heptadecanoic acid and stearic acid were from Thermo Fisher Scientific. Oleic acid, tridecanoic acid and behenic acid were from Sigma (St. Louis,
MO) and arachidic acid was from Acros. All the other fatty acids were from ICN Biomedicals Inc. (Aurora, OH). The primary fatty acid amide (PFAM) standards [C12:0, tridecanoamide (C13:0), myristamide (C14:0), C16:0, heptadecanoamide (C17:0), heptadecano-D33-amide (C17D33:0), C18:0, C18:1\(^{9}\), C18:1\(^{trans\,9}\), C18:1\(^{6}\), C18:1\(^{trans\,6}\), C18:1\(^{trans\,11}\), C18:1\(^{13}\), C20:0, C22:0, and C22:1\(^{13}\)] were synthesized in house from their corresponding fatty acids with a greater than 95% purity as verified by GC/MS [28]. Toluene (HPLC grade) and chlorotrimethylsilane (98%) (TMS) used for silanization of the glassware were from Acros Organics.

### 4.4.2 Instrument

The HPLC/MS system was a Waters 2695 separations module with a Waters 2487 dual wavelength detector operated at 210 nm to monitor the gradient and a Waters ZMD MS (Milford, MA) with an APCI probe in positive mode to monitor the separation. Data was analyzed using Waters MassLynx 4.1 software. Single ion mode was used to monitor the mass to charge ratios corresponding to the [M+H]\(^{+}\) peak for the PFAMs of interest. Each channel monitored one or two mass to charge ratios for the time during which the corresponding peaks were expected to elute to minimize background noise. When calculating the peak area for an analyte, the corresponding mass to charge ratio chromatogram was extracted before integrating. For the HPLC separation, a Waters XTerra MS C\(_{18}\) (5 μm, 3.9 x 150 mm) column with a Waters XTerra MS C\(_{18}\) (5 μm, 3.9 x 20 mm) guard column and an inline filter were used. The solvents were sonicated...
in a 2210 Branson Ultrasonic cleaner (Danbury, CT) for 20 min to degas before running; during the run the in-line degasser was used.

4.4.3 Silanization Procedure

In order to silanize the vials, clean vials were soaked in 1 M NaOH for 30 min before rinsing with ultrapure water and drying completely. The vials were flushed with argon to remove any moist air before filling with 10 % TMS in toluene. The vials were capped after flushing the head space with argon and allowed to sit overnight, then rinsed with toluene. The vials were stored in the drying oven (60 °C) until needed. Prior to using, the vials were rinsed with methanol and dried completely. It was necessary to re-silanized the vials after each use.

4.4.4 MS Optimization

It was necessary to determine which MS conditions produced the best ionization of the PFAMs using the APCI probe on the Waters ZMD. A series of experiments were run to test how much the temperature of the probe, the gas flows, the applied voltages, and the magnification of the detector signal influenced the signal response. Unless otherwise noted, the following conditions were used for optimization experiments. A 20 μL injection of a 0.1 mM each mixture of C12:0, C18:1<sup>9</sup>, and C18:0 was loaded onto the column. More PFAMs were added once the MS parameters had been optimized in
order to determine the limits of the separation. Unless otherwise noted, a 25 min gradient was used where the column was equilibrated in 90:10 methanol:water before injection. After injection, the gradient was ramped linearly to 95:5 methanol:water over 10 min before returning to the initial conditions (2 min ramp). The mobile phase was held at the initial conditions for 13 min before doing the next injection. Formic acid (0.1 %) was in both the methanol and the water. The flow rate was held constant at 1 mL/min. The starting APCI-MS parameters were as follows: corona 3.0 kV, sample cone 25 V, extraction cone 2 V, RF lens 0 V, source temperature 130 °C, desolvation temperature 530 °C, desolvation gas flow 600 L/hr, and the cone gas flow 100 L/hr. The nitrogen gas source was from a high pressure liquid nitrogen dewar. Once a parameter had been optimized, the setting was used for subsequent experiments optimizing the other MS parameters. For these experiments, the three mass to charge ratios corresponding to the PFAMs being analyzed were monitored throughout the analysis in one channel.

In order to optimize the temperature setting for the APCI heater, a series of injections were done where all the parameters were held constant except for the temperature of the APCI heater, which was varied from 450 to 650 °C. Three injections were done at each temperature setting. By comparing at the peak areas, it was determined that 500 °C was the best setting for the APCI heater; however, after several experiments, the APCI heater was turned down to 450 °C to lengthen the life of the heater. For the remaining optimization experiments, 500 °C was used as the setting for the APCI heater. Results from varying the heater temperature are shown in Figure 4-1.
Next the nitrogen flow for the cone gas was optimized. For this experiment, all of the APCI-MS parameters were held constant except for the cone gas setting which was varied from 100 °C to 250 °C. Two injections of 670 pmol each PFAM were done at each gas flow setting. The results are shown in Figure 4-2. The cone gas was optimized at 150 L/hr in this experiment, but, as the method was developed, the gas flow was eventually turned down to 50 L/hr to reduce the background noise in the separation experiments. For the remaining optimization experiments, 150 L/hr was used as the setting for the cone gas.
Figure 4-2 Resulting peak areas from injecting 670 pmol of each PFAM (C12:0, C18:1\textsuperscript{9}, and C18:0) on the XTerra MS C\textsubscript{18} column while varying the cone gas flow setting. Results are the average of two injections with the standard deviation.

In order to optimize the setting for the desolvation gas flow, all the parameters were held constant except the setting for the desolvation gas flow. The flow rate was varied between 300 and 600 L/hr. Two injections of 670 pmol each PFAM were done at each gas flow setting. The results are shown in Figure 4-3. It was determined that the desolvation gas was optimized at 400 L/hr.
Once the gas flows and temperature settings were optimized, the corona voltage was optimized. Three injections of 670 pmol each PFAM were done at each voltage setting. The corona voltage was varied between 2.8 and 3.2 kV, while the rest of the MS parameters were held constant. The results are shown in Figure 4-4. It was determined that the corona voltage was optimized at 3 kV.
Figure 4-4 Resulting peak areas from injecting 670 pmol of each PFAM (C12:0, C18:1<sup>9</sup>, and C18:0) on the XTerra MS C<sub>18</sub> column while varying the corona pin voltage setting. Results are the average of three injections with the standard deviation.

The cone voltage was optimized by varying the setting from 15 to 35 V, while the other MS parameters were held constant. PFAMs (670 pmol each) was injected in triplicate at each voltage setting. The results are shown in Figure 4-5. This experiment shows that the optimal cone voltage setting was 25 V.

Figure 4-5 Resulting peak areas from injecting 670 pmol of each PFAM (C12:0, C18:1<sup>9</sup>, and C18:0) on the XTerra MS C<sub>18</sub> column while varying the cone voltage setting. Results are the average of three injections with the standard deviation.
The optimized MS parameters are as follows: corona 3.0 kV, sample cone 25 V, extraction cone 2 V, RF lens 0 V, source temperature 130 °C, desolvation temperature 450 °C, desolvation gas flow 400 L/hr, and the cone gas flow 150 L/hr. These parameters needed to be adjusted each time maintenance was done on the MS, but the parameters were optimized by infusing a mixture of the three PFAMs (C12:0, C18:1\textsuperscript{9}, and C18:0) in 75:25 methanol:water with 0.3 % formic acid and adjusting the parameters to get the highest signal on the tune page.

The final parameter to optimize was the magnification of the detector. Unlike the other optimization experiments, this parameter was optimized after the LC separation was developed. At this point in developing the method, it was determined it would be better to analyze the slope of a short calibration curve in order to determine the best setting for the magnification of the detector. This experimental set up also ensured that the background noise did not increase causing the detection limit to increase. A 25 min gradient was used where the column was equilibrated in 75:25 methanol:water before injection. After injection, the gradient was ramped linearly to 95:5 methanol:water over 30 min before returning to the initial conditions. The mobile phase was held at the initial conditions for 10 min before doing the next injection. Formic acid (0.3 %) was in both the methanol and the water. The flow rate was held constant at 1 mL/min and a 20 μL injection was done. The APCI-MS parameters were as follows: corona 3.5 kV, sample cone 30 V, extraction cone 3 V, RF lens 0.1 V, source temperature 130 °C, desolvation temperature 450 °C, desolvation gas flow 450 L/hr, and the cone gas flow 50 L/hr. Two injections of each of the three concentrations (2 pmol, 20 pmol, and 200 pmol) were done at each magnification setting (varied between 550 and 800). Data from the 600, 650,
700, and 800 settings is shown in Figure 4-6. For these experiments, the mixture included C12:0, C14:0, C16:0, C17:0, C18:0, C18:1\textsuperscript{9}, C22:0, and C22:1\textsuperscript{13}. C17D33:0 was used as an internal standard so the concentration was held constant in each of the mixtures making the slope zero. It was determined that 700 was the best magnification setting. The slopes of the signal to noise ratio were also plotted, which showed that 800 would be the best setting; however doing a detection limit test showed that 700 was the best detector magnification setting.

![Graph](image)

**Figure 4-6** Resulting slopes from duplicate injections of three different concentrations of the PFAM mixture on the XTerra MS C\textsubscript{18} column at four different magnification setting. Results are shown comparing the slope of the line from the peak areas and when the detector magnification is set to 600, 650, 700, and 800.
4.4.5 Separation Optimization

In addition to optimizing the gradient between methanol and water to improve the separation between the PFAMs, the concentration of formic acid in the mobile phase had to be optimized. The concentration of formic acid had to be examined to determine what range improved the ionization of the PFAMs in the APCI-MS source. Also, experiments were done to see if it was necessary to silanize the glass vials used for injection and which solvents could be used for dilution the samples. Unless otherwise noted, the optimized MS parameters were used.

The initial gradient was 25 min long and the column was equilibrated in 90:10 methanol:water before injection (20 μL). This gradient was a shortened version of the gradient developed by Carpenter et al. [30]. After injection the gradient was ramped linearly to 95:5 methanol:water over 10 min before returning to the initial conditions. The mobile phase was held at the initial conditions for 13 min before doing the next injection. Formic acid (0.1 %) was in both the methanol and the water. The flow rate was held constant at 1 mL/min. However, as more PFAMs were added to the mixture, the gradient ramp had to be slowed down and more water added to the initial conditions. The gradient ended up being 45 min long and the column was equilibrated with 75:25 methanol:water before injection. After injection, the gradient was ramped linearly to 95:5 methanol:water over 30 min before returning to the initial conditions (5 min ramp). The mobile phase was held at the initial conditions for 10 min before doing the next injection. 0.1 % formic acid was in both the methanol and the water. This gradient separated all of the completely saturated PFAMS with even chain lengths varying from C12:0 to C22:0 plus the odd chain lengths C13:0 and C17D33:0. The resulting
chromatogram from injecting 200 pmol each of eight PFAMs is shown in Figure 4-7. This method also partially separated a series of PFAMs with the same chain length and the same degree of unsaturation. The experiments done to optimize the analysis are discussed in detail below.

**Figure 4-7** Chromatogram showing the separation of eight different PFAMs (200 pmol of each was injected) before the gradient was optimized. The percentage of methanol is plotted on the right axis with the dotted line.

A simple test was done in order to determine whether silanization of the glass vials was necessary. For this experiment, a mixture (20 pmol of each PFAM) of six different C18:1 PFAMs were used. There was not a complete separation between the different PFAMs so C18:1<sup>9</sup>, C18:1<sup>trans</sup><sup>11</sup>, and C18:1<sup>13</sup> co-eluted in the first peak, C18:1<sup>trans</sup><sup>9</sup> and C18:1<sup>6</sup> co-eluted in the second peak, and C18:1<sup>trans</sup><sup>6</sup> eluted in the third peak. Six injections were done from three different silanized vials and three different non-silanized vials. Results (Figure 4-8) showed that using silanized vials slightly
improved the peak areas. Statistically at the 95 % confidence level there is no difference between the two measurements; however, because an increase in peak areas was noticed when the same concentration was prepared in a silanized vial as opposed to a non-treated vial, silanized vials were used for the experiments.

![Figure 4-8](image)

**Figure 4-8** Average results from injecting (20 pmol of each PFAM) of six different C18:1 PFAMs were used. There was not a complete separation between the different PFAMs so C18:1 \(^9\), C18:1 \(^{\text{trans}}\) \(^{11}\), and C18:1 \(^{13}\) co-eluted in the first peak, C18:1 \(^{\text{trans}}\) \(^{9}\) and C18:1 \(^{6}\) co-eluted in the second peak, and C18:1 \(^{\text{trans}}\) \(^{6}\) eluted in the third peak. Two different mixtures were made: one using a silanized glass vial and the other using a non silanized glass vial. The error bars are the standard deviation of the three injections.

In order to optimize the formic acid concentration, two injections of the PFAM mixture were done at three different concentrations (2 pmol, 20 pmol, and 200 pmol) for four different concentrations of formic acid in the mobile phase (0, 0.1, 0.3 and 0.5 %). For these experiments, ten PFAMs (C12:0, C14:0, C16:0, C17:0, C18:0, C18:1 \(^9\), C18:1 \(^{\text{trans}}\) \(^9\), C18:1 \(^{\text{trans}}\) \(^6\), C22:0, and C22:1 \(^{13}\)) and the internal standard (C17D33:0) were monitored. The results shown in Figure 4-9 show that 0.3 % formic acid in the mobile
phase gave the best ionization of the PFAMs. It is important that the mobile phase be prepared daily to prevent the formic acid from degrading [32].

Figure 4-9 Average results from injecting a series of PFAMs (C12:0, C14:0, C16:0, C17:0, C18:0, C18:1\textsuperscript{trans} 6, C18:1\textsuperscript{trans} 9, C18:1\textsuperscript{9}, C22:0, and C22:1\textsuperscript{13} from left to right in each group) at three different mixture concentrations (200 pmol, 20 pmol, and 2 pmol injected) at each concentration of formic acid (0 %, 0.1 %, 0.3 % and 0.5 %) in the mobile phase.

Another examined parameter was the solvent that the sample was dissolved in for injection onto the column. Initially, all the samples were dissolved in either 100 % methanol or 1:1 methanol:chloroform (stock solution). However, as the method was developed and the results were compared to GC/MS data, it was convenient to dissolve the sample in 2-propanol so that the same solution could be run on both instruments. Since only 20 μL of the solution was injected onto the column, neither the peak areas nor the retention times varied among any of these solvents. This data is not shown.
A separation with ten PFAMs was done, but the entire spectrum between 250 m/z and 350 m/z was monitored during the run. The MAP function was used in MassLynx 4.1 to plot all the data (Figure 4-10). From this 2D graph a pattern can be seen in the elution pattern. This type of plot may be useful in indentifying additional PFAMs in biological samples; however, further studies would have to be done to determine whether the additional spots would be impurities or more PFAMs. The MAP plot also shows the major impurities contributing to the total ion count background.

![Figure 4-10 MAP plot of the separation between nine PFAMs (C16:0, C17:0, C18:0, C18:1\textsuperscript{trans 6}, C18:1\textsuperscript{trans 9}, C18:1\textsuperscript{9}, C20:0, C22:0, and C22:1\textsuperscript{13}) where the MS scanned from 250 m/z to 350 m/z. The arrows mark the signals from impurities.](image)

### 4.4.6 C18:1 Separation Optimization

After the RP-LC conditions were optimized, further studies were done in order to test the limits of the separation and determine whether it would be possible to completely separate a series of C18:1 PFAMs. The gradient described above was able to separate
between a double bond in the same location, but in the cis versus the trans orientation. However, in a mammalian tissue sample, it is more likely that the double bond would be found in a different location still in the cis orientation as opposed to the trans orientation in the same location [33]. Longer gradients, increasing the temperature, longer columns, and additional solvents were varied to improve the separation. The elution order of the C18:1 series is shown in Figure 4-11. In order to show the separation between the C18:1 PFAMs and positively identify the different PFAMs, each PFAM was injected separately (20 pmol each).

**Figure 4-11** LC/APCI-MS separation and detection of a series of C18:1 compounds with the double bond in different positions. 20 pmol of each amide was injected separately in 20 μL of methanol. Only m/z 282 was monitored. The chromatograms are overlaid to show elution order. Peak 1: C18:1\textsuperscript{\text{trans} 11}, peak 2: C18:1\textsuperscript{9}, peak 3: C18:1\textsuperscript{13}, peak 4: C18:1\textsuperscript{\text{trans} 9}, peak 5: C18:1\textsuperscript{6}, and peak 6: C18:1\textsuperscript{\text{trans} 6}. 
Although the peaks were not completely separated, there was some difference in retention times for each of the C18:1 PFAMs. The data shown above and data from a gradient where the ramp was 60 min long were loaded into the DryLab Program version 2.05 from LC Resources Inc. (Walnut Creek, CA). Unfortunately the gradients predicted by the program to improve the separation were unstable and were not able to be copied using the LC/MS. From this study, it was determined that a better separation would be needed in order to be able to use a computational approach to optimizing the gradient.

Longer gradients were formed by increasing the initial percentage of water in the mobile phase, reducing the slope of the gradient, or adding a hold at the initial conditions before starting the gradient. Figure 4-12 shows two examples of what happened to the separation as the slope of the gradient was reduced. For each of the runs, 2 pmol each mixture of PFAMs (C18:1\textsuperscript{trans} \text{11}, C18:1\textsuperscript{9}, C18:1\textsuperscript{13}, C18:1\textsuperscript{trans} \text{9}, C18:1\textsuperscript{6}, C18:1\textsuperscript{trans} \text{6}) was injected. Gradient A was 75 min long and the column was equilibrated with 75:25 methanol:water before injection. After a 30 min hold at the initial conditions, the gradient was ramped linearly to 85:15 methanol:water over 30 min before returning to the initial conditions. The mobile phase was held at the initial conditions for 10 min before doing the next injection. Gradient B was 75 min long and the column was equilibrated with 75:25 methanol:water before injection. After a 30 min hold at the initial conditions, the gradient was ramped linearly to 90:10 methanol:water over 30 min before returning to the initial conditions. The mobile phase was held at the initial conditions for 10 min before doing the next injection. For both of these gradients, the mobile phase contained 0.1 % formic acid and the injections were done at room temperature. After several
different approaches to reducing the slope of the gradient, it was determined that a
gradual ramp would not separate the series. The separation between the PFAMs was
slightly improved because a shoulder formed on the first peak, but the overall analysis
was significantly lengthened.

Figure 4-12  LC/APCI-MS separation and detection of a series of C18:1 positional isomers.
2 pmol of each amide was injected as a mixture and only m/z 282 was monitored. Two
chromatograms using two different gradients are overlaid to show the effects of slowing down the
gradient. The inlay depicts the differences between the two gradients.

Another approach to improving the separation between the C18:1 series of
PFAMs was to observe how temperature affected the separation. Increasing the
temperature changes the viscosity of the mobile phase allowing for faster flow rates and
faster mobile phase-stationary phase exchange, but for every 10 °C increase in
temperature the retention factor is reduced by 2 to 3 [34]. Figure 4-13 shows a series of
injections done with the same gradient only the column temperature was varied between 30 °C and 40 °C. 10 pmol of each C18:1\textsuperscript{trans 11}, C18:1\textsuperscript{9}, C18:1\textsuperscript{13}, C18:1\textsuperscript{trans 9}, C18:1\textsuperscript{6}, C18:1\textsuperscript{trans 6} was injected as a mixture onto the column. As expected, increasing the temperature did improve peak height so that the peak shape was sharper and the width decreased. Unfortunately, the resolution between the analytes decreased because the elution time decreased as the temperature increased. The resolution between the three peaks varied slightly, but a trend was not noticeable. By plotting the plate heights versus the temperature and the plate number versus the temperature (Figure 4-14), it was determined that the plate height increased and the plate number decreased as the temperature increased. Also, since no shoulders were formed on the peaks, this approach to optimizing the separation was not continued. The data is not shown, but increasing the temperature of the longer gradients did not improve the separation either.

![Figure 4-13](image)

**Figure 4-13** LC/APCI-MS separation and detection of a series of C18:1 compounds with the double bond in different positions. 10 pmol of each amide was injected as a mixture and only m/z 282 was monitored. Four chromatograms are shown where the same gradient was used, but the column temperature was varied. Peak 1 is the co-elution of C18:1\textsuperscript{trans 11}, C18:1\textsuperscript{9}, and C18:1\textsuperscript{13}, peak 2 is the co-elution of C18:1\textsuperscript{trans 9}, and C18:1\textsuperscript{6}, and peak 3 is C18:1\textsuperscript{trans 6}.
Figure 4-14 The trends in plate height (left) and plate number (right) as the temperature was increased. Peak 1 is the co-elution of C18:1$^{\text{trans}}_{11}$, C18:1$^{9}$, and C18:1$^{13}$, peak 2 is the co-elution of C18:1$^{\text{trans}}_{9}$, and C18:1$^{6}$, and peak 3 is C18:1$^{\text{trans}}_{6}$.

Plate number was also increased by connecting two Waters X Terra MS C$_{18}$ (5 μm, 3.9 x 150 mm) columns together still using the Waters X Terra MS C$_{18}$ (5 μm, 3.9 x 20 mm) guard column and an inline filter. It is common to use either a longer column [35] or two columns in a series [36, 37], when more complex separations are desired. For these experiments, the gradient was ramped from 75:25 methanol:water to 80:20 methanol:water over 60 min before returning to initial conditions. The flow rate was 0.7 mL/min, which was limited by the upper pressure limits of the LC system and the columns. Results are shown in Figure 4-15. Connecting two columns in a series improved the separation slightly, as a shoulder could be seen on the first peak, but the C18:1 series compounds still were not completely separated. Based on retention times from when the PFAMs were injected separately, the shoulder was suspected to be C18:1$^{\text{trans}}_{11}$. Very little improvement was made, while the analysis time tripled for the C18:1 PFAMs. Although the PFAMs were retained on the column longer to allow for increased resolution, the extra band broadening from the slower flow rate limited the
resolution. This approach might have improved the separation more if the packing allowed for higher flow rates.

![Figure 4-15](image)

**Figure 4-15** Mixture of 6 C18:1 amides separated using 2 C18 XTerra columns connected in a series. Peak 1 is the co-elution of C18:1\textsuperscript{trans 11} (suspected shoulder peak), C18:1\textsuperscript{9}, and C18:1\textsuperscript{13}, peak 2 is the co-elution of C18:1\textsuperscript{trans 9}, and C18:1\textsuperscript{6}, and peak 3 is C18:1\textsuperscript{trans 6}.

The final approach to improving the separation between the C18:1 series was to add 2-propanol to the mobile phase. 2-propanol was either added at a constant percentage to the mobile phase or slowly added into mobile phase up to 10 %. From these experiments, the best gradient was when the column was equilibrated in 73:25:2 methanol:water:2-propanol. After injection, the initial conditions were held for 30 min before ramping to 83:15:2 over 30 min. The column was returned to initial conditions for 10 min before the next injection. Figure 4-16 shows the resulting chromatogram when 20 pmol of each: C18:1\textsuperscript{trans 11}, C18:1\textsuperscript{9}, C18:1\textsuperscript{13}, C18:1\textsuperscript{trans 9}, C18:1\textsuperscript{6}, C18:1\textsuperscript{trans 6} were injected as a mixture onto the column. Again, the shoulder was expected to be C18:1\textsuperscript{trans 11} based on the elution order from previous experiments.
**Figure 4-16** Mixture of six C18:1 amides separated using two C18 XTerra columns connected in a series. Peak 1 is the co-elution of C18:1\textsuperscript{trans 11} (suspected shoulder peak), C18:1\textsuperscript{9}, and C18:1\textsuperscript{13}, peak 2 is the co-elution of C18:1\textsuperscript{trans 9}, and C18:1\textsuperscript{6}, and peak 3 is C18:1\textsuperscript{trans 6}.

### 4.4.7 Detection Limits

Throughout the optimization procedure the detection limit was monitored. The best detection limits for C16:0, C18:1 and C18:0 were 10 fmol of each, but the detection limits for the other PFAMs in the mixture were higher: C12:0 (1 pmol), C13:0 (100 fmol), C14:0 (200 fmol), and C17:0 (200 fmol). Unfortunately, these detection limits were dependent on the condition of the instrument so the detection limits were usually in the low pmol range. From running these experiments, it was hypothesized that the cleanness of the cones and corona pin, the vacuum seal, and the gas flow affected the ionization of the PFAMs. It was also important for the formic acid to be added to the mobile phase daily to prevent the formic acid from degrading.
4.4.8 Problems

One of the biggest problems with the set up was that the nitrogen came from a liquid nitrogen dewar. Depending on how much liquid nitrogen was left in the tank, the gas flow varied throughout the day. Figure 4-17 shows how the response factor (slope of the line when the area of the analyte signal divided by the area of the standard signal is plotted versus the concentration of the analyte divided by the concentration of the standard) changed throughout 28 injections. The first eight injections were done on one day with a nitrogen dewar that was almost empty. The last twenty injections were done on another day with a nitrogen dewar that had just been filled and delivered. Each injection was done with 16 μM of each PFAM (C16:0, C17:0, C17D33:0, C18:0, C18:1\textsuperscript{9}, and C20:0). Because the concentration of both the analyte and the internal standard was the same, the response factor is just the ratio of the peak area of the sample compared to the peak area in the internal standard. It is important to note that the plotted gas flows are the values at the start of the run and the standard deviation was calculated separately for each day. Overlaying this data shows that the gas flow does influence the response factors, but the starting values of the gas flow saved in the software cannot be used to distinguish a good run from a bad run. The percent error in the response factors after the nitrogen dewar was changed was varied between 4-10 % depending on the PFAM.
Figure 4-17 Plot of the response factors (left axis) from five different PFAMs over the course of 28 injections. The starting gas flows are plotted in the right axis. Each PFAM was added to the mixture so that 320 pmol of each was injected onto the column. C17D33:0 was used as the internal standard at the same concentration. The first eight injections used a different nitrogen dewar than the rest of the injections.

In order to test the influence of the gas flow on the response factors, a nitrogen tank was set up to provide the gas flows. Each PFAM (C12:0, C18:1<sup>9</sup>, and C18:0) was added to the mixture so that 50 pmol of each was injected onto the column. 10 pmol of C17D33:0 was used as the internal standard. The first three and the last three injections used a nitrogen dewar while the middle injections used a nitrogen tank. See Figure 4-18. The standard deviation was calculated separately for each type of nitrogen used. This shows that a steady gas flow will improve the reproducibility of the method. The percent
error of the response factors using the nitrogen tank was varied between 2-3 % depending on the PFAM.

![Graph showing response factors and injection number](image)

*Figure 4-18* Plot of the response factors from five different PFAMs over the course of 12 injections. Each PFAM was added to the mixture so that 50 pmol of each was injected onto the column. 10 pmol of C17D33:0 was used as the internal standard. The first three and the last three injections used a nitrogen dewar while the middle injections used a nitrogen tank.

In order to determine whether the ionization efficiency was varying throughout the separation, isocratic conditions, where most of the PFAMs co-eluted, was run multiple times to determine the error in the response factor. For this experiment the mobile phase was 95:5 methanol:water with 0.3 % formic acid. The chromatogram is shown in Figure 4-19. The response factors from 18 consecutive injections are shown in Figure 4-20.
**Figure 4-19** LC/APCI-MS separation and detection of eight PFAMs. 1000 pmol of each amide was injected as a mixture. All of the m/z ratios corresponding to the PFAMs were monitored in one channel. Isocratic elution with 95:5 methanol:water with 0.3 % formic acid.

**Figure 4-20** Plot of the response factors (left axis) from seven different PFAMs over the course of 18 injections. The starting gas flows are plotted on the right axis. Each PFAM was added to the mixture so that 1 nmol of each was injected onto the column. C17D33:0 was used as the internal standard.
Initially starting this project, some interference was expected from contaminants, especially C18:0, C18:1\textsuperscript{9}, C22:1\textsuperscript{13} because they are used as slip additives in plastics [26, 27, 38, 39]. However, as the method was developed it was determined that the solvents contained other contaminants that interfered with the analysis. These compounds had the same mass to charge ratio and similar polarity as the PFAMs on the LC/APCI-MS, but a different fragmentation pattern on the GC/MS. Distilling the solvents used for sample preparation eliminated most of the interference.

Using this method, there is a partial separation between the C18:1 series, but this method alone cannot tell the difference between bond position and orientation. Unfortunately, the best methods of detecting the PFAMs does not allow for the use of capillary columns (see section 4.5.3) for the separation method. The pressure limits require the use of flow rates through the column that are two to three times lower than the required flow rates for APCI-MS analysis.

A five point calibration curve over three orders of magnitude (10 pmol – 4 nmol) using this method and triplicate injections was analyzed. C17D33:0 was used as the internal standard. Although the data could be fit to a line, the error in each response factor was still approximately 20 % when standards were used to check the accuracy of the curve.
4.4.9 Conclusions

The final method for the LC/APCI-MS method was done at room temperature with the PFAMs dissolved in 2-propanol so that analysis could be done on both instruments (LC/APCI-MS - 20 μL injected and CG/MS – 1 μL injected). The final gradient was 45 min long and the column was equilibrated with 75:25 methanol:water before injection. After injection, the gradient was ramped linearly to 95:5 methanol:water over 30 min and then held at 95:5 for 5 min before returning to the initial conditions. The mobile phase was held at the initial conditions for 5 min before doing the next injection. 0.3 % formic acid was in both the methanol and the water. The resulting chromatogram when 300 pmol of each PFAM is injected is shown in Figure 4-21. The MS parameters are as follows: corona 3.0 kV, sample cone 25 V, extraction cone 2 V, RF lens 0 V, source temperature 130 °C, desolvation temperature 450 °C, desolvation gas flow 400 L/hr, and the cone gas flow 150 L/hr. These are average values for each of the parameters since they needed to be adjusted each time maintenance was done on the MS. Channel one was used to monitor for C12:0 and C14:0 by scanning the mass to charge ratios 200 and 228 starting at 5.0 after injection to 13.5 min. Channel two was used to monitor for C16:0 by scanning the mass to charge ratio 256 from 16.0 to 17.0 min. Channel three was used to monitor for the C18:1 isomers by scanning the mass to charge ratio 282 from 17.0 to 22.0 min. Channel four was used to monitor for the internal standards C17D33:0 and C17:0 by scanning the mass to charge ratios 270 and 303 from 16.0 to 23.0 min. Channel five was used to monitor for the C18:0 by scanning the mass to charge ratio 284 from 21.0 to 25.0 min. Channel six was used to monitor for C20:0 by scanning the mass to charge ratio 312 from 24.5 to
29.0 min. Channel seven was used to monitor for the C22:1 isomers by scanning the mass to charge ratio 338 from 26.0 to 33.0 min. Channel eight was used to monitor for C22:0 by scanning the mass to charge ratio 340 from 29.0 to 38.0 min.

Figure 4-21 LC/APCI MS of 12 amides. 300 pmol of each amide in 20 μL of 1:1 chloroform:methanol was injected. The channels were overlaid to show the separation. (1) C12:0 and (2) C14:0 are detected on channel 1 by monitoring m/z 200 and m/z 228. Channel 2: (3) C16:0 - m/z 256. Channel 3: (4) C18:1\textsuperscript{9}, (5) C18:1\textsuperscript{trans 9}, (6) C18:1\textsuperscript{trans 6} - m/z 282. Channel 4: (7) C17D33:0 and (8) C17:0 - m/z 303 and m/z 270. Channel 5: (9) C18:0 - m/z 284. Channel 6: (10) C20:0 – m/z 312. Channel 7: (11) C22:1\textsuperscript{13} – m/z 338.
Figure 4-22 Retention time of the saturated PFAMs under gradient elution.

4.5 LC/ESI-MS Method

An attempt to separate a series of PFAMs with the same degree of unsaturation but the double bond in different positions was done with a 0.3 μm particle size reverse phase column. The smaller particle size should allow for a higher separation power. Because of the high back flow pressure, low flow rates and ESI-MS were used to separate and detect the PFAMs. Ammonium acetate was added to the mobile phase to increase the ionization efficiency when using ESI-MS for detection. Comparisons were made to the APCI-MS detection method by using a 3.9 μm i.d. reverse phase column and ESI-MS detection.
4.5.1 Reagents

Methanol used for the LC/MS analysis was HPLC grade from Thermo Fisher Scientific (Pittsburgh, PA). Distilled water was deionized with a Barnstead (Dubuque, IA) Nanopure water system. All of the mobile phase solvents were filtered with 0.22 μm Nylon membrane filters from Whatman International (Maidstone, England) and sonicated in a 2210 Branson Ultrasonic cleaner (Danbury, CT) for 20 min prior to use. The ammonium acetate added to the mobile phase was from Thermo Fisher Scientific. Heptadecanoic-D33-Acid was from CDN Isotopes (Quebec, Canada). Lauric acid, myristic acid, heptadecanoic acid and stearic acid were from Thermo Fisher Scientific. Oleic acid and behenic acid were from Sigma (St. Louis, MO) and arachidic acid was from Acros. All the other fatty acids were from ICN Biomedicals Inc. (Aurora, OH). The primary fatty acid amide (PFAM) standards [C12:0, (C14:0, C16:0, C17:0, C17D33:0, C18:0, C18:1\textsuperscript{9}, C18:1\textsuperscript{trans 9}, C18:1\textsuperscript{trans 6}, C22:0, and C22:1\textsuperscript{13}] were synthesized in house from their corresponding fatty acids with a greater than 95% purity as verified by GC/MS [28].

4.5.2 Instrument Set-up for LC/ESI-MS

The HPLC/MS system was a Waters 2695 separations module with a Waters 2487 dual wavelength detector operated at 210 nm to monitor the gradient and a Waters ZMD MS (Milford, MA) with an ESI probe in positive mode to monitor the separation. Data was analyzed using Waters MassLynx 4.1 software. Single ion mode was used to
monitor the mass to charge ratios corresponding to the \([M+H]^+\) peak for the PFAMs of interest; the MS program was set up to monitor several mass to charge ratios in one channel.

For the HPLC separation, either a Waters XTerra MS C\(_{18}\) (5 μm, 3.9 x 150 mm) column with a Waters XTerra MS C\(_{18}\) (5 μm, 3.9 x 20 mm) guard column and an inline filter or a C18 Acclaim column from Dionex (3 μm, 1 x 150 mm) with an inline filter was used. During the run the in-line degasser was used. All separations were done at room temperature (about 28 °C).

**4.5.3 Microbore Column Experiments**

When using the larger XTerra column (5 μm, 3.9 x 150 mm), it was determined that a simple gradient would not completely separate a series of PFAMs with the same carbon chain length, but varying the position of the double bond (data shown in section 4.4.5). One attempt to improve the separation was to use a column with a smaller particle size (C18 Acclaim column from Dionex with 3 μm particles), but because of pressure limitations the flow rate had to be reduced to 8 μL/min; therefore, APCI-MS could not be used to analyze these samples. Initially, isocratic runs were done in order to test the system. Figure 4-23 shows the separation between C12:0, C18:1\(^9\), and C18:0 when the mobile phase was 85 % methanol and 15 % water. A 2 μL injection was done. ESI-MS settings were as follows: capillary 3 kV, sample cone 10 V, extraction cone 5 V, RF lens
0.1 V, source temperature 80 °C, desolvation temperature 100 °C, desolvation gas flow 150 L/hr, and the cone gas flow 50 L/hr.

Unfortunately, running in isocratic mode did not separate the C18:1 series. Several gradients were tried in order to improve the separation, but, because the runs were not reproducible from run to run, it was difficult to monitor trends in the separation as the mobile phase was varied. Ammonium acetate was added into the mobile phase at varying concentrations between 1 and 5 mM, but again the trends were not reproducible. After several more experiments, it was determined that either the injection volume affected the chromatogram or the mass load was too high for the column. The Van Deemter curve was not studied, but it was possible that the flow rate was below the optimal range for the column. The detection limit for this set-up was approximately 60 pmol of PFAM injected onto the column. In conclusion, although this path should have been explored further, the parameters were limited by the instrument capabilities so further studies were not done.
4.5.4 Separation and Detection Limits

In order to determine whether or not further development of the PFAM separation on the smaller column should be continued, the detection limits using ESI MS as the detector were examined. These experiments also allowed for a direct comparison between the ESI-MS source and APCI-MS source. For this HPLC separation, a Waters XTerra MS C_{18} (5 μm, 3.9 x 150 mm) column with a Waters XTerra MS C_{18} (5 μm, 3.9 x 20 mm) guard column and an inline filter were used. During the run the in-line degasser was used. Before injecting, the column was equilibrated in 75:25 methanol:water. After injection, the gradient was ramped linearly to 95:5 methanol:water over 30 min before returning to the initial conditions. The mobile phase was held at the initial conditions for 10 min before doing the next injection. The concentration of ammonium acetate in the mobile phase was varied. The flow rate was held constant at 1 mL/min, but the flow was split so only part of the eluent was directed into the MS. ESI-MS settings were as follows: capillary 4.5 kV, sample cone 40 V, extraction cone 5 V, RF lens 0.2 V, source temperature 100 °C, desolvation temperature 100 °C, desolvation gas flow 640 L/hr, and the cone gas flow 50 L/hr. These values varied slightly every time maintenance was done on the MS.

In order to optimize the concentration of ammonium acetate in the mobile phase, a series of injections were done with 200 pmol of each PFAM except the internal standard C17D33:0 where only 20 pmol was injected at the varying concentrations of ammonium acetate (2.5, 5, 7.5, 10, and 15 mM) in the mobile phase. For these experiments, the flow was split so that 430 μL/min was directed into the MS. Figure 4-24 shows the resulting peak areas as the concentration of ammonium acetate
was changed for each PFAM. This experiment concludes that 10 mM ammonium acetate should be added to the mobile phase to improve the ionization efficiency of the PFAM when using ESI.

![Figure 4-24](image)

**Figure 4-24** Resulting peak areas from injecting 200 pmol of each PFAM (except C17D33:0 which was 20 pmol) on the XTerra MS C\textsubscript{18} (5 μm) column with varying concentrations of ammonium acetate in the mobile phase.

After the signal had been optimized, the detection limit was determined using 10 mM ammonium acetate in the mobile phase. The gradient and the column were the same as above but this time only 80 μL/min was directed into the MS. Because the results were going to be compared to results from other experiments, silanized glassware was used to prevent sample loss. A series of injections were done, varying the amount of each PFAM loaded from 5 pmol to 30 pmol. It was determined that the detection limit was approximately 10 pmol, approximately 2-3 orders of magnitude higher than for APCI-MS (data shown in section 4.4.6).
In order to determine whether the results were reproducible from run to run, several injections of a mixture of C16:0, C17:0, C18:0, C18:1\textsuperscript{9}, C20:0, C22:0 and C22:1\textsuperscript{13} (1 nmol each) and C17D33:0 (500 pmol). Results are shown in Figure 4-25. Depending on the PFAM, the error (relative standard deviation) in the response factor varied from 3-7 % which was approximately the same error in the response factors when APCI-MS was used for detection.

Figure 4-25 – Plot of the response factors from seven different PFAMs over the course of 23 injections. Each PFAM was added to the mixture so that 1 nmol of each was injected onto the column. C17D33:0 was used as the internal standard.
4.5.5 Conclusions

The major problem with working with packing material with a smaller particle size was that the back pressure increase limited the working range of the flow rate. The lower flow rates increased the elution times, but did not increase the resolution. The loading volume was also limited. Unfortunately, the Waters 2695 was not capable of doing reproducible injections at 2-5 μL, so the separation on the C18 Acclaim column from Dionex was not fully explored. All of the analysis done at the lower flow rate had to be done using ESI-MS as the detector because the APCI probe was not capable of handling the lower flow rates. At the same time, the detection limits using ESI-MS as the detector were tested. After optimizing the concentration of ammonium acetate in the mobile phase, it was determined that ESI would not be as sensitive as APCI-MS for detecting the PFAMs.

4.6 LC/APCI-MS Quantitation

Because of the amount of error in the calculations when the full mixture of the PFAMs was analyzed as a standard mixture or as a series of spikes, another gradient was developed for four of the saturated PFAMs and the internal standards. The shorter gradient allowed for a three point calibration curve and the sample to be run in one day. Because the total analysis time was reduced, the instrumental variations during the analysis were also reduced. The results reduced the relative standard deviation in peak areas from run to run using the same concentration.
4.6.1 Reagents

Methanol was HPLC grade from either Thermo Fisher Scientific or J.T. Baker (Phillipsburg, NJ). Distilled water was deionized with a Barnstead (Dubuque, IA) Nanopure water system. Both the methanol and the water were filtered with 0.22 μm Nylon membrane filters from Whatman International (Maidstone, England) prior to use on the HPLC. The mobile phase for the HPLC analysis contained formic acid (99% pure) from Acros Organics (Morris Plains NJ) in both the methanol and water. These solutions were made up fresh each day and sonicated in a 2210 Branson Ultrasonic cleaner (Danbury, CT) for 20 min to mix and degas. Heptadecanoic-D33-Acid was from CDN Isotopes (Quebec, Canada). Heptadecanoic acid and stearic acid were from Thermo Fisher Scientific. Oleic acid, and behenic acid were from Sigma (St. Louis, MO) and arachidic acid was from Acros. All the other fatty acids were from ICN Biomedicals Inc. (Aurora, OH). The primary fatty acid amide (PFAM) standards [C16:0, C17:0, C17D33:0, C18:0, C20:0, C22:0, and C22:1\(^{13}\)] were synthesized in house from their corresponding fatty acids with a greater than 95% purity as verified by GC/MS [28]. Toluene (HPLC grade) and chlorotrimethylsilane (98%) (TMS) were from Acros Organics.

4.6.2 Experimental Parameters

The HPLC/MS system was a Waters 2695 separations module with a Waters 2487 dual wavelength detector operated at 210 nm to monitor the gradient and a Waters ZMD
MS (Milford, MA) with an APCI probe in positive mode to monitor the separation. Data was analyzed using Waters MassLynx 4.1 software. Single ion mode was used to monitor the mass to charge ratios corresponding to the [M+H]^+ peak for the PFAMs of interest. Each channel monitored one mass to charge ratios corresponding to the [M+H]^+ signal for each PFAM in the mixture. For the HPLC separation, a Waters XTerra MS C_{18} (5 μm, 3.9 x 150 mm) column with a Waters XTerra MS C_{18} (5 μm, 3.9 x 20 mm) guard column and an inline filter were used. The column was equilibrated in 80:20 methanol:water with 0.3 % formic acid. After injection, the gradient was ramped linearly to 100 % methanol with 0.3 % formic acid over 10 min. The mobile phase was then returned to the initial conditions (5 min ramp) and flushed for 5 min before doing the next injection. All separations were done at room temperature (about 28 °C) with both gradients. APCI-MS settings were as follows: corona 3 kV, sample cone 30 V, extraction cone 5 V, RF lens 0.1 V, source temperature 130 °C, APCI probe temperature 450 °C, desolvation gas flow 450 L/hr, and the cone gas flow 50 L/hr. These values varied slightly every time maintenance was done on the MS.

4.6.3 Results and Discussion

From previous experiments, it was known that there was less error in the response factors when a shorter gradient was used. A gradient was then developed to separate only a few of the saturated PFAMs and the internal standards. The shorter gradient separated C16:0, C17D33:0, C17:0, C18:0, C20:0, and C22:0 with a 20 min LC method. The
mixture contained 1.2 nmol of each PFAM except C17D33:0 where only 320 pmol was added to the mixture.

![Graph showing separation of four saturated PFAMs and internal standards](image)

**Figure 4-26** – Separation of four saturated PFAMs (1.2 nmol each) and the internal standards.

A three point (2 nmol, 1.2 nmol, and 600 pmol) calibration curve was run. Three injections were done of each solution and averaged together. Three separate calibration curves were run on separate days. When using C17D33:0 as an internal standard to calculate the response factors, the calibration curve had approximately 36 % error. The error was calculated by calculating the concentration of several standard mixtures in which the concentration was known. The calculated concentrations were then compared to the actual concentration values. When the C17D33:0 signals were ignored and a basic linear calibration curve was used the error was reduced to approximately 1 %. An example set of data is shown in Table 4-1.
### Basic Calibration Method

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Basic Calibration Method</th>
<th>Response Factor Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C16:0</td>
<td>C17:0</td>
</tr>
<tr>
<td>100 μM</td>
<td>101%</td>
<td>101%</td>
</tr>
<tr>
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<tr>
<td>Average</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 4-1: The percent recovery of the standard mix at three different concentrations. Each of the values reported are the average of three injections. Two methods of calculations were used: basic calibration method and the response factor method (see Appendix A).

#### 4.6.4 Conclusions

The shorter gradient method reduced the instruments error. The shorter gradient was 20 min long and the column was equilibrated with 80:20 methanol:water before injection. After injection, the gradient was ramped linearly to 100% methanol over 10 min before returning to the initial conditions. 0.3% formic acid was in both the methanol and the water. The error in the basic linear calibration curve was reduced to approximately 1%.

### 4.7 Conclusions

After exploring several different alternative PFAMs detection methods (MALDI-MS (Chapter 3), LC/ESI-MS, and LC/APCI-MS) it was determined that LC/APCI-MS was the best alternative to analyzing the PFAMs with GC/MS. The
Analysis time was slightly longer for the LC method (45 min) than the GC method (25 min plus cooling), but the detection limits were roughly the same when comparing the number of moles loaded onto the column (meaning that the concentration detection limits in the analyte solution were better by about an order of magnitude for LC/APCI-MS, given the larger injection volume of the LC).

The advantage of using LC/MS over GC/MS was that derivatization was not needed, which can improve detection limits and reduce total analysis time. A GC/MS method similar to the method developed by Sultana and Johnson [23] or Gee, Groen and Johnson [40, 41] was used as the standard (comparison) method when developing the LC/MS method. The main modification was to use chemical ionization and an ion trap MS rather than derivatization, electron ionization, and quadrupole MS, as this method was simpler and avoided derivatization. Because of the complexity of the lipids found in a typical tissue sample, detailed analysis of a specific class requires some form of sample preparation prior to introduction to the final analytical instrument. Sultana and Johnson showed that amides could be isolated from a Folch-Pi lipid extract by a two-stage solid phase extraction (SPE) method, provided some care is exercised in the choice of solvents [23]. Because C22:1\textsuperscript{13} and C18:1\textsuperscript{9} are commonly used as slip additives in plastic, considerable care had to be taken to minimize the blank contribution of these compounds. Experiments showing the analysis of PFAMs in bovine omentum are discussed in Chapter 6.

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4.8 References


Chapter 5

Separations of the “Polar” Neutral Lipid Classes

5.1 Abstract

The biological role of primary fatty acid amides (PFAMs), a class of neutral lipids, is not fully understood. It is suspected that being able to monitor the concentration of PFAMs will allow for the diagnosis of ocular surface disease and affective disorders. A Folch-Pi extraction method removes the neutral lipids from the biological tissue sample. In order to be able to detect and monitor the PFAMs in biological systems, further purification is necessary. A solid phase extraction (SPE) method has been developed to isolate the PFAM fraction from the lipid extract, but the method is not automated and is sometimes irreproducible. In this chapter, microchips platforms are used in attempts to scale down the SPE method, while normal phase liquid chromatography (NP-LC) columns are used to automate the separation. The eluent from the NP-LC can be directed into the mass spectrometer with an electrospray source for monitoring.
5.2 Background

Lipidomics is the science of fingerprinting the entire cellular lipidome in order to enable, among other things, studies of lipid metabolism and lipid-mediated signaling [1]. In reality it is impossible, with current analysis techniques, to do a complete, comprehensive analysis of the total lipids found in a complex tissue. One common approach is to focus on one or a few classes of lipids with similar polarities. Once the lipids have been separated by class, further separations or simply direct infusion onto the MS [1, 2] are done to analyze the lipids within the class.

Initial studies of the neutral lipid classes in this lab started with the development of a TLC analysis [3]. This analysis method included the following lipid classes as standards: cholesterol esters (CE), triacylglycerols (TAG), free fatty acids (FFA), diacylglycerols (DAG), cholesterol (Ch), primary fatty acid amides (PFAMs), monoacylglycerols (MAG), N-acylglycines (NAG), and N-acyl ethanolamines (NAE). Polar lipids in the mixture did not move from the baseline. The lipid classes were scraped off the thin layer chromatography (TLC) plate for further analysis [4]. In order to improve sample capacity, the solid phase extraction (SPE) method was developed [3]. The SPE method to purify the PFAM fraction involved a silica based column and an amine based column. There were some problems with reproducing this method. The grade of solvent mattered and the volume of other neutral lipids present influenced the results. The method protocol had to be further modified so that plastic consumables were not used, in order to reduce contamination. Because of the number of different lipid classes, it is impossible to completely study all the lipids in a complex sample at one time. For this study, only the “polar” neutral lipids were studied, with a focus on the
PFAMs, which are part of the fatty acyls group. An extensive list of lipids can be found at www.lipidmaps.org.

<table>
<thead>
<tr>
<th>Class Name (abbreviation)</th>
<th>Example Name (subclass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty Acyls (FA)</td>
<td>Oleic Acid (Free Fatty Acids - FFAs)</td>
</tr>
<tr>
<td></td>
<td>Lauramide (Primary Fatty Acid Amide - PFAM)</td>
</tr>
<tr>
<td></td>
<td>Anandamide (N-acyl Ethanolamines - NAE)</td>
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<td>N-arachidonoyl glycine (N-acyl Amino Acids - NAAs)</td>
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<td></td>
<td>1,2-Dipalmitoyl-rac-glycerol (Diacylglycerol - DAG)</td>
</tr>
<tr>
<td></td>
<td>Tristearin (Triacylglycerol – TAG)</td>
</tr>
</tbody>
</table>

*Table 5-1* Example lipids for each subclass of interest grouping them by lipid class.
Due to problems with reproducibility and contamination, and lengthy analysis time with SPE, an automated method is preferred for the purification step of the PFAMs and other neutral lipid analysis. Normal phase columns have been used to separate a few classes of lipids [5-10], but reverse phase columns have also been used [11, 12]. Carpenter et al. showed that if three C18 analogs - NAG, FFA, and PFAM - are combined in the same separation, the NAG analog will elute first from a C8 column [11]. A similar separation of the three C18 analogs was done by Merkler et al. [13]. In this case a Phenomenex Luna C8(2) column was used. These reverse phase liquid chromatography (RP-LC) methods did not explore whether the lipid classes would overlap when more lipids were included in the separation, but our experience with RP separation of lipids (Chapter 4) leads us to believe that a single RP column does not have the resolving power or peak capacity to separate a large set of lipids across several classes. Another reverse phase method has been developed by Perona and Ruiz-Gutierrez to separate the MAG, DAG, and TAG lipid classes [10]. The gradient varied between acetone and acetonitrile. This gradient partially separates the different lipids within the class, but baseline separation was not achieved between the individual lipids – only the classes.

A normal phase separation was used by Chapman et al. to purify the NAEs from the TAGs also found in the seed extracts [7]. In this method, the NAE fraction was collected, derivatized, and analyzed by GC/MS to identify each of the NAEs present in the sample. Sommer et al. developed normal phase gradients to separate a variety of polar and nonpolar lipid classes [6]. One gradient used methyl t-butyl ether, methanol, 2-propanol, ammonium acetate and water to separate several polar lipid classes (DAG,
FFA, MAG, cerebroside, glycerolphosphoethanolamine, glycerophosphoinositol, glycerophosphocholine, gangliosides, sphingomyelin and monoacylglycerophosphocholine). Baseline separation was not achieved between each lipid class. Another gradient was developed using methyl t-butyl ether, n-heptane, acetic acid, 2-propanol, ammonium acetate, and methanol to separate the nonpolar lipid classes (cholesteryl ester, fatty acid methyl ester, triacylglycerol, DAG, free cholesterol, and MAG). In both cases, the eluent was split and directed into the ESI-MS in order to monitor the separation. Another separation using a similar column was developed by Christie to separate a series of polar lipid classes [14]. The mobile phases were slightly different than other normal phase gradients reported; iso-hexane, methyl-tert-butyl ether, 2-propanol, chloroform, acetic acid, water, and triethylamine were included in the solvent mixes. This separation spanned phospholipids through sterol esters.

5.3 Standards and Reagents

The methanol, methyl t-butyl ether, and 2-propanol used for the LC/MS analysis were HPLC grade from J.T. Baker (Phillipsburgh, NJ). The chloroform was Optima grade from Thermo Fisher Scientific (Pittsburgh, PA). Toluene (HPLC grade) was from Acros Organics. The n-heptane was from Alfa Aesar (Ward Hill, MA). Distilled water was deionized with a Barnstead (Dubuque, IA) Nanopure water system. The post column feed for MS detection was 10 mM ammonium acetate (Thermo Fisher Scientific) in 60:40 methanol:2-propanol. All of the mobile phases were filtered with 0.22 μm Nylon
membrane filters from Whatman International (Maidstone, England) and sonicated for 20 min in a 2210 Branson Ultrasonic cleaner (Danbury, CT) prior to use. The acetic acid was from Aldrich (St. Louis, MO). The acetyl chloride was from Mallinckrodt (Paris, KY). The sodium hydroxide and potassium carbonate was from Thermo Fisher Scientific. Heptadecanoic-D33-acid was from CDN Isotopes (Quebec, Canada). Lauric acid, myristic acid, heptadecanoic acid and stearic acid were from Thermo Fisher Scientific. Oleic acid and behenic acid were from Sigma (St. Louis, MO) and arachidic acid was from Acros. All the other fatty acids were from ICN Biomedicals Inc. (Aurora, OH). The primary fatty acid amide (PFAM) standards [lauramide (C12:0), myristamide (C14:0), palmitamide (C16:0), heptadecanoamide (C17:0), heptadecano-D33-amide (C17D33:0), stearamide (C18:0), oleamide (C18:1\(^9\)), arachidamide (C20:0), behenamide (C22:0), and erucamide (C22:1\(^{13}\))] were synthesized in house from their corresponding fatty acids with a greater than 95% purity as verified by GC/MS [4]. 1-Monopalmitoyl-rac-glycerol (MAG) and tristearin (TAG) were from Sigma (St. Louis, MO) 99 % purity and 1,2-dipalmitoyl-rac-glycerol (DAG) was from MP Biomedicals (Solon, Ohio). The stearoyl ethanolamide (NAE) and N-oleoylglycine (NAG) were from the Cayman Chemical Company (Ann Arbor, MI). The lipids were dissolved in 1:1 chloroform: methanol. The spherical silica gel packing was from Sorbent Technologies (Atlanta, GA). The epoxy was 5 min set from the local hardware store.
5.4 Oleamide Extraction on a Microchip

5.4.1 Set up

Two different attempts were made to scale down the SPE method to fit on a microchip. One attempt focused on the recovery of C18:1\(^9\), while the other determined whether the column was long enough for the classes of lipids to be separated. In both cases, the fractions were collected and analyzed by GC/MS.

The set-up used for the C18:1\(^9\) recovery (Figure 5-1) was simpler than the set-ups used for the reaction experiments described in Chapter 2. Two of the valves used for controlling the flow were removed leaving only the injection valve. For this set up the syringe pump (Harvard Compact Infusion Pump model 975) was connected to an injection valve (Rheodyne 7725). The valve contained a 2 μL sample loop for loading the lipid mixture. A piece of fused silica capillary (75 μm i.d. x 36 cm, Polymicro Technologies L.C.C., Phoenix, AZ) was fitted to the inlet side of the microchip with PEEK fittings and a plastic holder. A piece of capillary was also connected to the outlet side of the microchip and went into the collection vial. The flow rate was set to 20 μL/min, but the flow varied depending on the condition of the microchip column, because the syringe pump does not have a back pressure regulator. The actual flow rate due to back pressure was estimated to be 1 μL/min. Fused silica capillaries (75 μm i.d.) were used to connect each of parts, except PEEK tubing (0.005 ID) was used to connect the syringe (SGE glass 5 mL) to the valve. It is important to use glass syringes instead of plastic syringes because there are amides on the surface of the plastic syringes from the manufacturing process. These amides would have been extracted by the solvents needed for this extraction and would be detected when analyzing. Also, the plastic syringes are
not compatible with chloroform used in the mobile phase. By comparing eluant volumes, it was obvious that the flow rate varied throughout the extraction.

**Figure 5-1** Diagram of the oleamide extraction on a microchip set up.

Before packing the microchip, the channel was conditioned by pumping about 20 μL each of the following solutions through the microchip: 1 M sodium hydroxide, deionized water, and methanol. The microchip was packed with Sorbent Technologies’ Spherical Silica Gel bulk packing (100 Å pore size, 20 μm diameter). A slurry was made with the stationary phase and methanol and was loaded through the inlet side of the microchip. The stationary phase was packed up against the weir by pulling a vacuum on the outlet side, then flushed with methanol and deionized water. In order to hold the stationary phase in place in the straight microchip design, the microchips were baked overnight at 115 °C in a Pro-Set II Stabil Therm Electric Oven (Blue Island, IL). The straight microchip design is shown in Figure 5-2.

Methanol was pushed through by hand reverse flow in order to check that the silica packing was going to stay in place and wash out any debris away from the weir in
the straight chip design. Then the microchip was put in the holder and flushed with hexane to prepare for the extraction. It was estimated that the microchip holds no more than 0.2 μg of packing.

![Diagram of the straight design microchip. This microchip design was used for previous experiments. This microchip was packed with Phenomenex’s Luna C18 bulk packing (100 Å pore size, 15 μm diameter) and baked overnight at 115 °C.](image)

**Figure 5-2** Diagram of the straight design microchip. This microchip design was used for previous experiments. This microchip was packed with Phenomenex’s Luna C\textsubscript{18} bulk packing (100 Å pore size, 15 μm diameter) and baked overnight at 115 °C.

### 5.4.2 Separation Conditions

A few experiments have been done to show that C18:1\textsuperscript{9} can be retained on a microchip packed with silica and then selectively eluted off with a series of solvent washes containing different ratios of chloroform, methanol, acetic acid, and 2-propanol. From the estimated amount of packing on the microchip (0.2 μg) and the upper load limit on the solid phase extraction tubes [4], it was determined that 1 ng of C18:1\textsuperscript{9} should be loaded onto the microchip. The other lipids were not used because it was assumed at the
time that the GC/MS cannot detect them without derivatization and the higher mass load for the microchip was below the detection limit on the HPTLC plates.

For this experiment, the microchip column was flushed with chloroform for 30 min, collecting the last 15 min as a blank. Then 2 μL of 1 μg/μL C18:1\(^9\) in chloroform was loaded onto the column while chloroform was flushed through the system for 15 min. A larger mass load was used to ensure detection by the GC/MS. In order to elute the C18:1\(^9\) from the column, three elution steps were done for 15 min each: 95:5:1 chloroform: methanol: acetic acid, 2:1 chloroform: methanol, and 100 % methanol. The collection vial was changed at each step when the mobile phase was changed. The fractions were reconstituted in 30 μL of internal standard, 0.25 ng/μL deuterated heptadecanoamide (C17D33:0), and dissolved in 2-propanol.

5.4.3 GC/MS Conditions

An Agilent Technologies Network GC/MS system (6890 GC with 5973 mass selective detector and 7683 series injector) was used for the analysis. In the C18:1\(^9\) recovery experiments, the following GC/MS conditions were used: the column was an HP-5MS (0.25 mm internal diameter, 0.25 μm film thickness, 30 m long, Agilent Technologies). The GC/MS method used for analysis was as follows: starting temperature was 55 °C, ramped to 150 °C at 40 °C per min, held at 150 °C for 3.62 min, ramped to 300 °C at 10 °C per min and finally held at 300 °C for 2 min. Electron impact ionization (EI) at 70 eV was used and mass range was kept from 40 to 400 m/z. Four
ions: 59, 62, 72, and 76, were monitored in order to detect the internal standard and the C18:1$^9$. The temperatures of the injection port and the transfer line were 250 °C and 280 °C respectively. Injection volume was 1 μL splitless. This method was developed by Sultana [4].

5.4.4 Results and Discussion

Figure 5-3 shows the GC/MS chromatograms from the last four fractions. Results from the C18:1$^9$ recovery experiments showed some C18:1$^9$ coming off during loading, so either the mass load was too great for the microchip column or the flow rate was too high. Most of the C18:1$^9$ came off during the 95:5:1 chloroform: methanol: acetic acid fraction, but small amounts came off in the following washes. The final wash, 100% methanol, caused column bleed. These experiments showed that a more sensitive method is needed for the analysis of the fractions, so that the mass load can be reduced. Derivatizing the lipids before analysis will lower the detection limits on the GC/MS [4]. Also, switching to the T-chip design (next section) would increase the mass load because a portion of the packing would not be degraded in the baking process. The column length on the T-chip design microchip was more controlled because the length was always the distance between the two weirs. The column length on the straight design microchip depends on how much packing stays in the channel, when the packing was pulled tightly against the weir.
Figure 5-3 (A) Fraction collected from loading 2 μL of C18:1⁹ in chloroform. (B) Fraction collected from elution with 95:5:1 chloroform: methanol: acetic acid. (C) Fraction collected from elution with 2:1 chloroform: methanol. (D) Fraction collected from elution with methanol. The arrows mark the elution times for C18:1⁹ and C17D33:0.

5.5 Lipid Separation on a Microchip

5.5.1 Set up

Before packing the microchip used for the lipid extraction, it was conditioned by pumping about 20 μL each of the following solutions through the microchip: 1 M sodium hydroxide, deionized water, and methanol. The slurry was made of the stationary
phase and methanol and was loaded through the side arm of the t-chip design. The microchip was packed with Sorbent Technologies’ Spherical Silica Gel bulk packing (100 Å pore size, 20 μm diameter). The stationary phase was packed up against the weirs by pulling a vacuum on the outlet sides, then flushed with methanol and deionized water. In order to hold the stationary phase in place in the T-chip design, epoxy was added to the side arm. After the epoxy dried, the microchip was put in the holder and flushed with hexane to prepare for the extraction. It was estimated that the microchip holds no more than 0.2 μg of packing.

**Figure 5-4** Diagram of the T-chip design microchip. This microchip design was used for SPE experiments. This microchip was packed with Sorbent’s spherical silica gel (100 Å, 20 μm).

The set-up used for the lipid extraction (Figure 5-5) was simpler than the set-ups used for the C18:1\(^9\) extractions. All of the valves used for controlling the load were removed because of contamination problems. For this set up, the syringe pump (Harvard Apparatus Pump II) was connected to the microchip with polyethylene tubing (0.38 ID) fitted over the needle (25 G) and a piece of fused silica capillary (75 μm i.d.) that was
fitted to the inlet side of the microchip with PEEK fittings and a plastic holder. A piece of capillary was also connected to the outlet side of the microchip and going into the collection vial. A syringe pump was used to pump the solvents through the column. It is important to use glass syringes instead of plastic syringes because there are amides on the surface of the plastic syringes from the manufacturing process. These amides would have been extracted by the solvents needed for this extraction and would be detected when analyzing. Also, the plastic syringes were not compatible with chloroform in the mobile phase. Even though the syringe pump was set to 20 μL/min, the actual flow rate due to back pressure was estimated to be 1 μL/min. After comparing eluent volumes, it was obvious that the flow rate varied throughout the extraction.

![Diagram](image)

**Figure 5-5** Diagram of the lipid extraction on a chip set up.

### 5.5.2 Separation Conditions

In order to be able to work with the other lipid classes, the samples were derivatized before running on the GC/MS. The upper mass load for the microchip was
below the detection limit on the HPTLC plates. For these experiments, a 0.1 mM mixture of both tristearin (TAG) and myristic acid (FFA) in methanol was used.

The microchip column was flushed with hexane for 30 min, collecting the entire fraction as the blank. Then 5 μL of the lipid mixture in methanol was loaded onto the column while hexane was flushed through the system for 10 min. In order to elute the lipids from the column, three elution steps were done for 20 min each: 90:10 hexane: ethyl acetate, 80:20 hexane: ethyl acetate, and 100 % methanol. The collection vial was changed at each step when the mobile phase was changed. The fractions were dried down and stored in the freezer until transterification and analysis on the GC/MS.

5.5.3 Transterification

Before analysis on the GC/MS the lipids were derivatized. The methods from Lepage [15] and Masood et al. [16] were adapted for small volumes by Mr. Timothy Fahrenholz (Duquesne University). The fractions were dried down and then resuspended in 20 μL of toluene and 80 μL of 0.05 mg/mL butylate hydroxytoluene (BHT) in methanol in a 1.5 mL vial. The solution was cooled on ice before 20 μL of cold acetyl chloride was dripped into the vial. The vial was capped off (in this case two septa were used to ensure a good seal) to prevent evaporation. The vial was heated at 100 °C for three hours, with mixing every 30 min. During the reaction the vials were only placed a quarter of the way into the heating block slots to allow a reflux to occur. After the solution cooled, 250 μL of 6 % potassium carbonate and 300 μL of toluene were added.
The solution was centrifuged at 2.0 x 1000 rpm for 2 min to separate. The top layer was removed and saved for analysis, while 300 μL of toluene was added to the bottom layer. The bottom layer was centrifuged again, and the new top layer was added to the aliquot removed after the first centrifuge step. The volume of the combined top layers was diluted to 1 mL with toluene for analysis. This solution was stored in the refrigerator until the GC/MS analysis could be performed.

5.5.4 GC/MS Conditions

An Agilent Technologies Network GC/MS system (6890 GC with 5973 mass selective detector and 7683 series injector) was used for the analysis. For the lipid separation experiment the following conditions were used for the analysis. The column was a Phenomenex BPX 70 (0.25 mm internal diameter, 0.25 μm film thickness, 60 cm long, SGE). The GC/MS method used for analysis was as follows: starting temperature was 80 °C, after a 5 min hold ramped at 10 °C/min to 220 °C, and finally held at 220 °C for 5 min. Injection volume was 1 μL spiltless. The mass range was 20 to 800 m/z. The temperature of the injection port was 200 °C.
5.5.5 Results and Discussion

Figure 5-6 shows a good run from separating TAG and FFA. Based on flow rate calculations, it was estimated that 600 pmol of each lipid was loaded onto the microchip. The signal in the blank fraction could be a combination of contamination and/or carry over from lipids left in the chip set up from the last experiment. FFA showed promise of being retained on the microchip. TAG was found in every fraction. When looking the percent recoveries (see Table 5-2), all of them are low, putting the concentration of the methyl esters below the calibration curve done with standards on the GC/MS. The percent recovery was tested by reacting an aliquot of sample that did not go through the microchip. Fahrenholz (Duquesne University) was getting approximately 70 % recovery from the transterification process. However, these results show that the percent recovery was less than 1 %. Further experiments could not determine the source of error.

![Graph showing peak area for tristearin and myristic acid](image)

**Figure 5-6** 600 pmol each of tristearin (TAG) and myristic acid (FFA) loaded onto the microchip and then eluted off in three fractions. Each fraction was converted into corresponding methyl esters and then analyzed on the GC/MS. Each bar is an average of three injections on the GC/MS.
Table 5-2  Percent recoveries based on loading 600 pmol of both tristearin (TAG) and myristic acid (FFA). All values are an average of three injections on the GC/MS. The un-separated mix was a separate trial and did not go through the microchip.

<table>
<thead>
<tr>
<th></th>
<th>Tristearin</th>
<th>Myristic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank Avg</td>
<td>0.13%</td>
<td>1.37%</td>
</tr>
<tr>
<td>Load Avg</td>
<td>0.14%</td>
<td>0.89%</td>
</tr>
<tr>
<td>90:10 Avg</td>
<td>0.23%</td>
<td>0.71%</td>
</tr>
<tr>
<td>80:20 Avg</td>
<td>0.16%</td>
<td>1.80%</td>
</tr>
<tr>
<td>MeOH Avg</td>
<td>0.14%</td>
<td>1.69%</td>
</tr>
<tr>
<td>Unseparated Mix</td>
<td>0.19%</td>
<td>0.79%</td>
</tr>
</tbody>
</table>

5.6 Normal Phase Liquid Chromatography Purification Method

5.6.1 Normal Phase LC (small column)/ESI MS

A normal phase LC method was developed using gradient elution so that each lipid class would be in a different fraction. Markers for each lipid class were monitored with ESI MS while the gradient was being optimized. The first gradient developed used a 1 mm i.d. NP column.

5.6.1.1 Experimental Conditions

In order to automate the SPE extraction method, a normal phase liquid chromatography (NP-LC) method was followed by fraction collection. The HPLC system was a Dionex (Bannockburn, IL) UltiMate 3000 LC pump with manual injection
(2 μL sample loop) and a Foxy Jr. fraction collector (Teledyne ISCO, Lincoln, NE). For the separation, a YMC polyvinyl alcohol coated silica (PVA-Sil) (1.0 x 150 mm, 5 μm particles) column with an inline filter was used. The solvents were sonicated for 20 min to degas and mix before use; during the run, the in-line degasser was used. The mobile phase was a mixture of solvent A (0.5% methyl t-butyl ether in n-heptane) and solvent B (0.02 % acetic acid and 10 % 2-propanol in methyl t-butyl ether). Before injecting, the column was equilibrated in 85 % solvent A and 15 % solvent B. The initial conditions were held for 15 min before ramping to 45 % solvent A and 55 % solvent B over 5 min. The final conditions were held for 10 min to wash the column before returning to the initial conditions. The column was flushed for 15 min at the initial conditions before the next injection. The flow rate throughout the method was 100 μL/min.

When monitoring the separation was required, the eluent was directed into a Waters ZMD MS (Milford, MA) with an electrospray (ESI) probe in positive mode. In order to increase the ionization efficiency, a post column feed (10 mM ammonium acetate in 60:40 2-propanol: methanol) was used at a flow rate of 50 μL/min. Single ion mode was used to monitor the mass to charge ratios corresponding to the ion with the strongest signal for each lipid marker for each class and each lipid was monitored in a separate channel. Channel 1 was set to 353 m/z for the monoacylglycerols (MAG) [M+Na]^+ peak, channel 2 was set to 256 m/z for the primary fatty acid amides (PFAM – C16:0 was used as the standard) [M+H]^+ peak, channel 3 was set to 592 m/z for the diacylglycerols (DAG) [M+Na]^+ peak, and channel 4 was set to 350 m/z for the n-aceyl ethanolamine (NAE) [M+Na]^+ peak. In order to set the MS parameters, a mixture of 5 μL of 2 mM DAG, 5 μL of 2 mM MAG, 100 μL of 1 mM PFAM, and 100 μL of 1 mM NAE
was dried down and then reconstituted in 500 μL of Solvent A and 250 μL of the post column feed. The concentrations were different for each lipid class because the ionization efficiency of each class was different. The mixture was infused into the MS at 50 μL/min. The parameters MS were set as follows: the capillary was 3.50 kV, the cone voltage was 40 V, the extractor was 5 V, the RF lens was 0.10 V, the source block temperature was set to 110 °C, the desolvation temperature was set of 450 °C, the cone gas was 50 L/hr and the desolvation gas was 150 L/hr. These parameters varied over time as maintenance was done on the instrument.

When a complete analysis of the PFAM lipid class was required, the eluant was directed to the fraction collector. Based on retention times of the lipid classes when the flow was directed into the MS, the eluant was collected from 15 min to 24 min as one fraction. The sample was dried down and then reconstituted into 100 μL of 16 μM C17D33:0 in 2-propanol for further analysis with the RP-LC separation followed by atmospheric pressure chemical ionization (APCI-MS) for detection (Results shown in Chapter 6).

5.6.1.2 Results and Discussion

The development of the normal phase LC separation was based on the separation described by Sommer et al. [6]. In order to monitor the separation, the eluant was directed into the MS with an ESI source. Data was collected from several different gradients separating the same lipid mixture; then the separation was optimized using the
DryLab Program version 2.05 from LC Resources Inc. (Walnut Creek, CA). The optimized gradient is described in the experimental conditions section 5.6.1.1. The resulting chromatogram is shown in Figure 5-7. The test lipid mixture contained 560 pmol of each PFAM, 200 pmol of MAG, 200 pmol of DAG and 5 nmol of NAE. A higher concentration of the PFAM class was needed to keep the four lipid class’ signals on the same scale because of the differences in the ionization efficiency between the classes. Switching to a larger column would increase the sample capacity and allow for more concentrated fractions (after drying down and reconstituting) to be loaded onto the reverse phase column.

![Chromatogram](image)

**Figure 5-7** Optimized separation between four neutral lipid classes on the 1 x 150 mm normal phase column. Each lipid class was monitored on a separate channel on the MS. Channel 1 was set to 353 m/z for the MAG (1-monopalmitoyl-rac-glycerol) [M+Na]^+ peak, channel 2 was set to 270 m/z for the PFAM (C17:0) [M+H]^+ peak, channel 3 was set to 592 m/z for the DAG (1,2-dipalmitoyl-rac-glycerol) [M-Na]^+ peak, and channel 4 was set to 350 m/z for the NAE (stearoyl ethanolamide) [M+Na]^+ peak. The lipid mix injected contained 560 pmol of PFAM, 200 pmol of MAG, 200 pmol of DAG and 5 nmol of NAE.
The mass load tolerance of the column was estimated by running several dilutions of the lipid mix. The peak areas from the chromatograms were plotted against the concentration of the lipids to determine the upper limit of the linear response range. The mass load was approximately 8 nmol of PFAM and approximately 600 pmol each of MAG and DAG. The mass load of each individual lipid class loaded onto the column was not examined, so the PFAM load might be affected by the amount of other classes of lipids present in the mixture.

In order to make sure that all of the PFAMs expected in the omentum tissue would co-elute on the normal phase column, a mixture of seven PFAMs: C16:0, C17:0, C18:0, C18:1\textsuperscript{9}, C20:0, C22:0 and C22:1\textsuperscript{13} (560 pmol each) was injected onto the normal phase column. The separation conditions were the same as for the full lipid separation, except the MS method was changed to monitor the mass-to-charge ratio corresponding to the [M+H]\textsuperscript{+} signal for each of the PFAMs in the mixture. The results are shown in Figure 5-8. These results show that all the PFAMs roughly co-eluted and the small difference in the retention times were in the opposite order from the reverse phase separation (the longer chain PFAMs eluted first).

\textsuperscript{A} Unpublished manuscript – Kroniser, K.M. and M.E. Johnson, Liquid Chromatography Mass Spectrometry Analysis of Primary Fatty Acid Amides in Bovine Omentum.
**Figure 5-8** The normal phase LC separation of a mixture of seven different PFAMs to make sure there was no separation occurring within the class. The experimental conditions were the same as the lipid separation except the m/z corresponding to the [M+H]^+ signal for each PFAM were monitored. The mixture contained 560 pmol of each PFAM.

### 5.6.2 Normal Phase LC (big column)/APCI MS

After developing the gradient on a 1 mm i.d. NP column, it was determined that a larger sample capacity would be needed for the 2D/LC analysis method. The gradient from the 1 mm NP column was scaled for the 4.6 mm i.d. NP column. Because of the higher flow rate going through the 4.6 mm i.d. column, the lipid markers were monitored with APCI-MS.
5.6.2.1 Experimental Conditions

In order to increase the sample load capacity of the NP-LC method a larger column (YMC PVA-Sil 4.6 x 250 mm column (5 μm particles) with a guard column and an inline filter) was used. The HPLC system was a Dionex UltiMate 3000 LC pump with manual injection (20 μL sample loop). For the separation, the solvents were sonicated for 20 min to degas and mix before use; during the run, the in-line degasser was used. The mobile phase was a mixture of solvent A (0.5% methyl t-butyl ether in n-heptane) and solvent B (0.02 % acetic acid and 10 % 2-propanol in methyl t-butyl ether). Before injecting, the column was equilibrated in 85 % solvent A and 15 % solvent B. The initial conditions were held for 15 min before ramping to 45 % solvent A and 55 % solvent B over 5 min. The final conditions were held for 10 min to wash the column before returning to the initial conditions. The column was flushed for 5 min at the initial conditions before the next injection. The flow rate throughout the method was 1 mL/min with the post column feed (10 mM ammonium acetate in 60:40 methanol:2-propanol) added at 0.5 mL/min after the column.

Because of the higher flow rates being used in the LC separation, the eluant was monitored using the Waters ZMD MS with an APCI probe in positive mode. Single ion mode was used to monitor the mass to charge ratios corresponding to the lipid marker for each class and each lipid was monitored in a separate channel. Channel 1 was set to 353 m/z for the monoacylglycerols (MAG) [M+Na]⁺ peak, channel 2 was set to 270 m/z for the primary fatty acid amides (PFAM) [M+H]⁺ peak, channel 3 was set to 592 m/z for the diacylglycerols (DAG) [M+Na]⁺ peak, and channel 4 was set to 350 m/z for the
n-acyl ethanolamine (NAE) [M+Na]^+ peak. In order to set the MS parameters, a mixture of 5 μL of 2 mM DAG, 5 μL of 2 mM MAG, 100 μL of 1 mM PFAM, and 100 μL of 1 mM NAE was dried down the reconstituted in 500 μL of Solvent A and 250 μL of the post column feed. The concentrations were different for each lipid class because the ionization efficiency of each class was different. The mixture was infused into the MS at 150 μL/min. The parameters MS were set as follows: the capillary was 3.00 kV, the cone voltage was 30 V, the extractor was 5 V, the RF lens was 0.10 V, the source block temperature was set to 130 °C, the desolvation temperature was set of 450 °C, the cone gas was 50 L/hr and the desolvation gas was 450 L/hr. These parameters varied over time as maintenance was done on the instrument.

5.6.2.2 Results and Discussion

Initially, a mixture of lipids was infused into the MS to determine the best additive to increase the ionization. Although formic acid works the best for improving the signal in the reverse phase separation of the PFAMs (Chapter 4), formic acid was not soluble in hexane. The same solution used for the post column feed for the 1 mm column and ESI detection was used at a higher flow rate in order to be able to detect some of the neutral lipid classes. The gradient is described in the experimental conditions section 5.6.2.1. The resulting chromatogram is shown in Figure 5-9. The test lipid mixture contained 15 nmol of PFAM, 30 nmol of MAG, 30 nmol of DAG and 15 nmol of NAE. Only MAG and NAE were detected under these conditions. After running these
experiments, it was determined that APCI would not work for monitoring the separation. The APCI probe allowed for directing the entire eluent into the MS, but the conditions could not be optimized to detect all the lipid classes in the mixture. The lipid classes ionized better when methanol was used as the mobile phase as opposed to the n-heptane needed for this normal phase separation.

**Figure 5-9** The normal phase LC separation of a mixture of four different lipid classes. Each lipid class was monitored on a separate channel on the MS. Channel 1 was set to 353 m/z for the MAG (1-monopalmitoyl-rac-glycerol) [M+Na]^+ peak, channel 2 was set to 270 m/z for the PFAM (C17:0) [M+H]^+ peak, channel 3 was set to 592 m/z for the DAG (1,2-dipalmitoyl-rac-glycerol) [M-Na]^+ peak, and channel 4 was set to 350 m/z for the NAE (stearoyl ethanolamide) [M+Na]^+ peak. The lipid mix injected contained 15 nmol of PFAM, 30 nmol of MAG, 30 nmol of DAG and 15 nmol of NAE, but only the MAG and NAE classes were detected.
5.6.3 Normal Phase LC (big column)/ESI MS

After developing the gradient on a 1 mm i.d. NP column, it was determined that a larger sample capacity would be needed for the 2D/LC analysis method. Because of poor sensitivity when monitoring the lipid markers with APCI-MS, the eluent was split and the lipid markers were monitored with ESI-MS. The gradient was then optimized on the 4.6 mm i.d. NP column.

5.6.3.1 Experimental Conditions

In order to increase the mass load for the separation, a larger column (YMC PVA-Sil 4.6 x 250 mm column with 5 μm particles) was used for the NP-LC method. Before the column there was an inline filter followed by a YMC PVA-Sil 4.0 x 20 mm guard column. The HPLC system was a Dionex UltiMate 3000 LC pump with manual injection (20 μL or 200 μL sample loop) and a Foxy Jr. fraction collector. The solvents were sonicated for 20 min to degas and mix before use; during the run, the in-line degasser was used. The mobile phase was a mixture of solvent A (0.5% methyl t-butyl ether in n-heptane) and solvent B (0.02 % acetic acid and 10 % 2-propanol in methyl t-butyl ether). When using gradient #1, the column was equilibrated before injection in 60 % solvent A and 40 % solvent B. After the injection, the gradient was ramped to 50 % solvent A and 50 % solvent B over 20 min and held for 5 min before returning to the initial conditions. The column was flushed for 5 min at the initial conditions before
the next injection. When using gradient #2, the column was equilibrated before injection in 95 % solvent A and 5 % solvent B. After the injection, the gradient was ramped to 50 % solvent A and 50 % solvent B over 40 min before returning to the initial conditions. The column was flushed for 15 min at the initial conditions before the next injection. The flow rate throughout both gradients was 1 mL/min and the separations were done at room temperature.

When monitoring the separation was required, the eluent was directed into a Waters ZMD MS with an ESI probe switching between positive mode and negative mode. The eluent was split after the column so that the flow rate into the MS was approximately 200 μL/min. In order to increase the ionization efficiency, a post column feed (10 mM ammonium acetate in 60:40 methanol:2-propanol) was added to the split portion of the eluent at a flow rate of 150 μL/min. Single ion mode was used to monitor the mass to charge ratios corresponding to the lipid marker for each class and each lipid was monitored in a separate channel. Channel 1 was set to 353 m/z for the monoacylglycerol (MAG) [M+Na]^+ peak, channel 2 was set to 270 m/z for the primary fatty acid amide (PFAM) [M+H]^+ peak, channel 3 was set to 592 m/z for the diacylglycerol (DAG) [M+Na]^+ peak, channel 4 was set to 350 m/z for the n-aceyl ethanolamine (NAE) [M+Na]^+ peak, channel 5 was set to 281 m/z for the fatty acid (FFA) [M-H]^− peak, channel 6 was set to 338 m/z for the n-aceyl glycine (NAG) [M-H]^− peak, and channel 7 was set to 914 m/z for the triacylglycerol (TAG) [M+Na]^+ peak. In order to set the MS parameters, a mixture of 600 μL of 2 mM TAG, 150 μL of 2 mM NAG, 75 μL of 0.1 mM FFA, 75 μL of 2 mM DAG, 150 μL of 2 mM MAG, 150 μL of 1 mM PFAM, and 150 μL of 1 mM NAE was dried down the reconstituted in 1 mL of
Solvent A and 500 μL of the post column feed. The concentrations were different for each lipid class because the ionization efficiency of each class was different. The mixture was infused into the MS at 50 μL/min. Two set of parameters were used: one for positive mode and one for negative mode detection. For positive mode detection, the capillary was 4.00 kV, the cone voltage was 70 V, the extractor was 5 V, and the RF lens was 0.10 V. For negative mode detection, the capillary was 4.00 kV, the cone voltage was 45 V, the extractor was 1 V, and the RF lens was 0.10 V. The following parameters were the same for both modes of detection: the source block temperature was set to 110 °C, the desolvation temperature was set of 450 °C, the cone gas was 50 L/hr and the desolvation gas was 200 L/hr. These parameters varied over time as maintenance was done on the instrument.

When analysis of the PFAM lipid class was required, the entire eluent was directed to the fraction collector. Based on retention times of the lipid classes when the flow was directed into the MS, the eluent from 31 min to 35 min was collected as one fraction. Periodically the separation had to be monitored in order to adjust for any variation in retention times. The time the fraction was collected was then adjusted accordingly. The fraction was dried down and then reconstituted into 100 μL of 16 μM C17D33:0 in 2-propanol for further analysis with the RP-LC/APCI-MS (Results shown in Chapter 6).
5.6.3.2 Results and Discussion

The development of the 4.6 mm i.d. NP-LC separation was based on the optimized gradient from the 1 x 150 mm normal phase column. In order to monitor the separation, the eluent was split before being mixed with the post column feed and then directed into the MS with an ESI probe. Data was collected from several different gradients separating the same lipid mixture; then the separation was optimized using the DryLab Program. The optimized gradient is described in the experimental conditions section 5.6.3.1 as gradient #1. The resulting chromatogram shown in Figure 5-10 used gradient #1 and only monitored for the lipid classes detected in positive mode. This lipid mixture contained 15 nmol of each PFAM, 30 nmol of MAG, 30 nmol of DAG, and 15 nmol of NAE. Using this gradient, the four lipid classes in the mix separated and eluted in less than 15 min. When the NAG and FA lipid markers were added to the mix they co-eluted with the MAG and DAG lipid markers respectively. It was suspected that the DAG standard was actually a mixture of isomers.
Figure 5-10 Optimized separation between four neutral lipid classes on the 4.6 x 250 mm normal phase column using gradient #1. Each lipid class was monitored on a separate channel on the MS. The lipid mix contained 15 nmol of PFAM, 30 nmol of MAG, 30 nmol of DAG, and 15 nmol of NAE. A 20 μL sample loop was used.

The separation had to be further optimized to be able to separate all the lipid classes. The optimized gradient is described in the experimental conditions section 5.6.3.1 as gradient #2. This gradient was able to separate seven different lipid class markers: TAG, FA, DAG, NAG, MAG, PFAM, and NAE. For this separation, (Figure 5-11) the lipid mixture contained: 120 nmol TAG, 750 nmol of FA, 15 nmol of DAG, 30 nmol of NAG, 30 nmol of MAG, 15 nmol of PFAM, and 15 nmol of NAE.
Figure 5-11 Optimized separation between seven neutral lipid classes on the 4.6 x 250 mm normal phase column using gradient #2. Each lipid class was monitored on a separate channel on the MS. The lipid mix contained 120 nmol TAG, 750 nmol of FA, 15 nmol of DAG, 30 nmol of NAG, 30 nmol of MAG, 15 nmol of PFAM, and 15 nmol of NAE. A 20 μL sample loop was used.

After the separation was optimized, the injection volume was varied while the mass load was held constant in order to determine whether the injection solvent (80:20 heptane:2-propanol) had any affect on the separation. Figure 5-12 shows the results from a 200 μL injection of the same lipid mixture. When the injection volume was increased, the DAG lipid marker eluted earlier and the peaks for the MAG, NAG, PFAM, and NAE lipid markers broadened. If separation is needed between the FA and DAG lipid classes, the injection volume has to be less than 50 μL.
Figure 5-12 Separation between seven neutral lipid classes on the 4.6 x 250 mm normal phase column using gradient #2. Each lipid class was monitored on a separate channel on the MS. The lipid mix contained 120 nmol TAG, 750 nmol of FA, 15 nmol of DAG, 30 nmol of NAG, 30 nmol of MAG, 15 nmol of PFAM, and 15 nmol of NAE. A 200 μL sample loop was used.

The reproducibility of the separation was tested, by injecting the same mass load five times at three different injection volumes (20, 100 and 200 μL). The variation in response times for the PFAM (C17:0) was less than 1.5 %. Because of the change in peak shape, the injection volume had to be same when comparing results. The larger injection volume (200 μL) was chosen so that the mass load could maximize for the 2D/LC experiments (Chapter 6).

In order to make sure that all of the PFAMs expected in the omentum tissue A would co-elute on the 4.6 mm i.d. NP column using gradient #1, a mixture of eight

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PFAMs: C16:0, C17:0, C17D33:0, C18:0, C18:1^9, C20:0, C22:0 and C22:1^{13} (15 nmol each) was injected (20 μL injection) onto the 4.6 mm i.d. NP column. The detection conditions were the same as for the lipid separation, except the MS method was changed to monitor the mass-to-charge ratio corresponding to each of the PFAMs in the mixture. The resulting chromatogram from using gradient #1 is shown in Figure 5-13. These results show that all the PFAMs co-eluted in a 2.5 min window (the longer chain PFAMs eluted first). The separation was more defined using the 4.6 mm i.d. column versus the 1 mm i.d. column.

![Figure 5-13](image_url)

**Figure 5-13** The normal phase LC separation using gradient #1 of a mixture of eight different PFAMs to make sure there was no separation occurring within the class. The experimental conditions were the same as the lipid separation except the m/z corresponding to the [M+H]^+ signal for each PFAM were monitored. The mixture contained 15 nmol of each PFAM in a 20 μL injection volume.
In order to make sure that all of the PFAMs expected in the omentum tissue would co-elute on the 4.6 mm i.d. NP column using gradient #2, a mixture of eight PFAMs: C16:0, C17:0, C17D33:0, C18:0, C18:1\textsuperscript{9}, C20:0, C22:0 and C22:1\textsuperscript{13} (50 nmol each) was injected (200 μL injection) onto the 4.6 mm normal phase column. The detection conditions were the same as for the lipid separation, except the MS method was changed to monitor the mass-to-charge ratio corresponding to each of the PFAMs in the mixture. The resulting chromatogram from using gradient #2 is shown in Figure 5-14. These results show that all the PFAMs co-eluted in a 7 min window (the longer chain PFAMs eluted first). Using the second gradient and the larger injection volume the peaks were broader, with less resolution between peaks.

![Chromatogram](Figure 5-14)

**Figure 5-14** The normal phase LC separation using Gradient #2 of a mixture of eight different PFAMs to make sure there was no separation occurring within the class. The experimental conditions were the same as the lipid separation except the m/z corresponding to the [M+H]\textsuperscript{+} signal for each PFAM were monitored. The mixture injected contained 50 nmol of each PFAM in a 200 μL injection volume.


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5.7 Conclusions

The initial attempts to scale down SPE purification method were done with columns on a glass microchip. The experiments showed some promise that the method would work, but reproducibility was an issue. Optimizing the method on the microchip was problematic because of the packing degrading and clogs so the same column could not be used throughout the entire experiment. After several different attempts, it was determined that the current microchip design did not contain enough stationary phase particles to be able to effectively retain and then selectively elute off the lipid classes. Redesigning the microchip to include a longer column and less dead volume zones would increase the reproducibility of these methods. Secondly, replacing the syringe pump with a pump with back pressure correction would keep the flow constant from one extraction to the next.

Replacing the SPE purification method with a NP gradient followed by fraction collection improves the reproducibility of the method. Because the gradient can be monitored when problems occur, this eliminates time spent on problem solving due to day-to-day variation in retention time. The NP separation can be monitored using ESI-MS, unlike the SPE method where only fractions can be analyzed. If complete recovery of the sample is not required, the separation could be monitored in real time while collecting fractions. Secondly, the same column was used for each extraction, rather than the two freshly hand packed columns needed to complete the SPE method. One of the limitations of the NP method is that because the separation has been analyzed using ESI-MS, the elution pattern was only known for the lipid classes that ionize. Further, the method was only set up to monitor for one example in each lipid class with
the exception of the PFAMs; therefore, the band width of the entire class was unknown. By following the trends of chromatography, the PFAM fraction was assumed to only have PFAMs. Each separation by itself it shorter using the NP column as opposed to the SPE method. However, multiple SPE tubes can be run at the same time, so if more than four separations were done, the SPE method was shorter.
5.8 References


Chapter 6

Two Dimensional Liquid Chromatography Separations and Mass Spectrometry Analysis of Primary Fatty Acid Amides

6.1 Abstract

In order to be able to study the biological role of primary fatty acid amides (PFAMs), a class of neutral lipids, an off-line two-dimensional liquid chromatography method was developed. It is suspected that being able to monitor the concentration of PFAMs will allow for the diagnosis certain diseases and disorders. In order to be able to detect and monitor the PFAMs in biological systems, several different analytical methods need to be combined in order to be able to completely analyze the complex raw tissue extract for a specific class of lipids. For this method, the Folch-Pi extraction method was used to remove the neutral lipids from the omentum tissue. Two normal phase liquid chromatography methods were developed to separate the neutral lipids by class. This part of the method could be monitored with electrospray mass spectrometry or the PFAM fraction could be collected for further separation. The purified PFAM fraction was
separated by chain length and degree of saturation using reverse phase liquid chromatography. The final analysis was done with atmospheric pressure chemical ionization mass spectrometry. Results include separations done with a standard mix and separations done with PFAMs spiked into bovine omentum tissue samples.

6.2 Introduction

The number of research articles published on the pharmacology and discovery of PFAMs increased substantially following discovery of oleamide and other amides in the cerebrospinal fluid of sleep deprived cats [1]. GC/MS studies of human plasma in luteal phase women found five PFAMs [2]. Hamberger and Stenhagen detected erucamide in body organs from pig (lung, kidney, liver, and brain) and rat (lung, liver, spleen, brain, and intestine) [3]. More recently, oleamide, myristamide (C14:0), palmitamide (C16:0), stearamide (C18:0), and erucamide (C22:1) were found in human meibomian gland secretions [4]. In this work bovine omentum has been chosen as the model tissue for testing the analysis method. Omentum has been used to increase blood flow to the brain in Alzheimer patients [5] and treat progressive ischemia [6]. Wakamatsu et al. extracted erucamide from bovine mesentery and showed that several different long chain amides can induce angiogenesis [7]. Another study has found gangliosides in omentum tissue [8]. Preliminary experiments in this laboratory have shown that a few PFAMs were present in omentum. Furthermore, the work by Wakamatsu et al. has not been repeated,
and a more definitive measure of PFAMs present in omentum, particularly erucamide, is needed.

GC/MS methods have been developed to analyze oleamide in biological samples [9, 10] and plastic materials [11, 12]. These methods only examined the samples for a few specific PFAMs for qualitative analysis. Hanus et al. reported finding 156 pmol of C18:1\text{\textsuperscript{9}} per milliliter of cerebrospinal fluid and 35 pmol of C18:1\text{\textsuperscript{9}} per milliliter of plasma from male Sprague-Dawley rats [10]. Arafat et al. reported finding 15 nmol/mL C16:0, 17 nmol/mL C16:1\text{\textsuperscript{9}}, 113 nmol/mL C18:1\text{\textsuperscript{9}}, 13 nmol/mL C18:1\text{\textsuperscript{trans}9}, and 8 nmol/mL C18:2\text{\textsuperscript{9,12}} in human plasma [2]. HPLC with UV detection or HPLC with refractive index (RI) detection can be done, but the amides being analyzed need to be fully separated by chromatography to be positively identified [13, 14]. The detection limits are about 100 μM for UV detection [14] and about 3 mM for RI [13], and so are inadequate for typical amide concentrations in biological tissue or fluid.

6.3 Standards and Reagents

All solvents used for SPE were HPLC grade solvents (except chloroform which was Optima grade) from Thermo Fisher Scientific (Pittsburgh, PA). All the SPE solvents except the chloroform needed to be distilled before using to remove impurities. The methanol, methyl t-butyl ether, and 2-propanol used for the LC/MS analysis were HPLC grade from J.T. Baker (Phillipsburgh, NJ). Distilled water was deionized with a Barnstead (Dubuque, IA) Nanopure water system. All mobile phase solvents were
filtered with 0.22 μm Nylon membrane filters from Whatman International (Maidstone, England) prior to use. The mobile phase for the reverse phase HPLC analysis contained 0.3 % formic acid (99% pure) from Acros Organics (Morris Plains NJ) in both the methanol and water. The post column feed for the ESI-MS detection method was 10 mM ammonium acetate (Thermo Fisher Scientific) in 60:40 2-propanol: methanol. These solutions were sonicated for 20 min to mix and degas. Heptadecanoic-D33-acid was from CDN Isotopes (Quebec, Canada). Heptadecanoic acid and stearic acid were from Thermo Fisher Scientific. Oleic acid and behenic acid were from Sigma (St. Louis, MO) and arachidic acid was from Acros. All the other fatty acids were from ICN Biomedicals Inc. (Aurora, OH). The primary fatty acid amide (PFAM) standards [palmitamide (C16:0), heptadecanoamide (C17:0), heptadecano-D33-amide (C17D33:0), stearamide (C18:0), oleamide (C18:1 9), arachidamide (C20:0), behenamide (C22:0), and erucamide (C22:1 13)] were synthesized in house from their corresponding fatty acids with a greater than 95% purity as verified by GC/MS [15]. Stock solutions for the PFAMs were 1 mM in 1:1 chloroform: methanol. The small amount of chloroform in the injection sample did not affect the retention time or peak shape. 1-Monopalmitoyl-rac-glycerol (MAG) was from Sigma 99 % purity and 1,2-dipalmitoyl-rac-glycerol (DAG) was from MP Biomedicals (Solon, Ohio). The bovine omentum sample came from a local abattoir and was stored at -20 °C until analysis. Toluene (HPLC grade) and chlorotrimethylsilane (98%) (TMS) were from Acros Organics. The SPE packing materials – Discovery-Si and Discovery-NH2 - were from Supelco (Bellefonte, PA). The argon and nitrogen gases were from Airgas (Radnor, PA). The desiccant (magnesium sulfate anhydrous) was technical grade from Aldrich.
6.4 Sample Preparation

6.4.1 Silanization Method

Glass vials used for fraction collection were silanized before use, because the analysis was done at low concentrations and lipids tend to stick to glass. Clean vials were soaked in 1 M NaOH for 30 min before rinsing with ultrapure water and drying completely. The vials were flushed with argon to remove any moist air before filling with 10 % TMS in toluene. The vials were capped after flushing the head space with argon and allowed to sit overnight, then rinsed with toluene. Prior to using, the vials were rinsed with methanol and dried completely. The vials were silanized before each use. Plastic vials, centrifuge tubes, and SPE tubes cannot be used because the slip additives added to the plastic are often C18:0, C18:1\(^9\), and C22:1\(^{13}\) [11, 12, 16, 17].

6.4.2 Lipid Extraction Method

The lipids were extracted using a sample preparation method previously developed in this laboratory for extracting lipids from N\(_{18}\)TG\(_2\) cells, then modified for tissue samples [15, 18]. The solvents used in this method were distilled first because they contained impurities with the same mass-to-charge ratio as the PFAMs in the LC/MS analysis. Blanks were run to check for any additional contamination. In order to improve reproducibility between samples, several grams of bovine omentum tissue was blended together. Portions (0.5 – 1 g depending on the experiment) were homogenized in a volume of 2:1 chloroform: methanol 20 times the weight of the tissue sample. After
adding C17:0 (IS1) as an internal standard, the sample was transferred to a silanized 50 mL glass centrifuge tube. Methanol (1/5 of the total volume) was used to rinse the homogenizer and then added to the sample. The sample was centrifuged with an Avanti J-20 XP Centrifuge from Beckman Coulter and a JS 5.3 rotor (30 min at 1500 g) and the liquid was decanted into a clean centrifuge tube. Additional chloroform was added to the sample to make the solvent ratio 2:1 chloroform: methanol. A volume of 0.88 % aqueous KCl equal to 1/5 the total volume of the sample was added. The sample was agitated before freezing the sample in order to separate the sample into two phases. The bottom phase was dried down after the top phase had been removed and completely dried down by blowing nitrogen over the sample and then pulling a vacuum. The sample was reconstituted in 1 mL of 80:20 n-heptane: 2-propanol. Sonication was needed to completely dissolve the sample.

6.5 Solid Phase Extraction Method

The extraction was followed by SPE to separate out the PFAM fraction using the method previously described [18]. After washing the column (3 mL silanized glass SPE tube containing 0.5 g of unbonded silica (Discovery-Si from Supelco) in-between two frits), a series of solvents was passed through the column, where the sixth fraction was saved and dried down. In order to further separate the PFAMs from the other neutral lipids (monoacylglycerols, N-acylglycines, and N-acylethanolamines) in the fraction, the sample was loaded onto a second column (3 mL silanized glass SPE tube containing 0.5 g
of amino bonded phase (Discovery-NH$_2$ from Supleco) packing in-between two frits). Again a series of solvents were passed through the column and the third fraction was saved and dried down.

The analysis was done by GC/MS, LC/MS, or both. The PFAM fraction was reconstituted into 150 μL of 16 μM C17D33:0 (instrumental internal standard, IS2) in 2-propanol for further analysis. The LC/MS method development was described in Chapter 4. Detection limits for all the PFAMs (C16:0 – C22:0) were in the tens of femtomoles making the detection limit for the LC/MS analysis method one to two orders of magnitude lower than the detection limit for the GC/MS analysis method when comparing the amount of moles loaded onto the column. The GC/EI-MS method was developed by Sultana and Johnson [18] and the GC/Cl-MS method was developed by Dent (Duquesne University). With the GC/EI-MS method, smaller chain amides (12 to 14 carbons) had a detection limit of 30 pg (about 150 fmol), larger chain amides (16 to 22 carbons) had a detection limit of 10 pg (about 40 fmol), and oleamide (C18:1$^\delta$) had a detection limit of 5 pg (about 20 fmol). Other lipids in the standard lipid mixture could be detected when each fraction from the SPE was loaded onto a HPTLC plate [15]. It was determined that 0.5 μg of oleamide and 20 μg of the other lipids can be separated using 500 mg of silica packing in the SPE purification method.
6.6 Separation Conditions

6.6.1 Normal Phase LC/ESI MS Conditions

In order to automate the SPE extraction method, a normal phase liquid chromatography (NP-LC) method was followed by fraction collection. The HPLC system was a Dionex (Bannockburn, IL) UltiMate 3000 LC pump with manual injection (2 or 200 μL sample loop) and a Foxy Jr. fraction collector (Teledyne ISCO, Lincoln, NE). The solvents were sonicated for 20 min to degas and mix before use; during the run, the in-line degasser was used. The mobile phase was a mixture of solvent A (0.5% methyl t-butyl ether in n-heptane) and solvent B (0.02% acetic acid and 10% 2-propanol in methyl t-butyl ether).

For the small scale normal phase purification method, a YMC PVA-Sil (1.0 x 150 mm) column with an inline filter was used. Before injecting 2 μL of the lipid extract, the column was equilibrated in 85% solvent A and 15% solvent B. The initial conditions were held for 15 min before ramping to 45% solvent A and 55% solvent B over 5 min. The final conditions were held for 10 min to wash the column before returning to the initial conditions. The column was flushed for 15 min at the initial conditions before the next injection. The flow rate throughout the method was 100 μL/min.

When monitoring the 1 mm i.d. NP separation, the eluent was directed into a Waters ZMD MS (Milford, MA) with an ESI probe in positive mode. In order to increase the ionization efficiency, a post column feed (10 mM ammonium acetate in 60:40 2-propanol: methanol) was used at a flow rate of 50 μL/min for the 1 mm column. Single ion mode was used to monitor the mass to charge ratios corresponding to the lipid
marker for each class and each lipid was monitored in a separate channel. Channel 1 was set to 353 m/z for the monoacylglycerols (MAG) [M+Na]$^+$ peak, channel 2 was set to 270 m/z for the primary fatty acid amides (PFAM) [M+H]$^+$ peak, and channel 3 was set to 592 m/z for the diacylglycerols (DAG) [M+Na]$^+$ peak. When analysis of the PFAM lipid class was required, the eluent was directed to the fraction collector. Based on retention times of the lipid classes when the flow was directed into the MS, the eluent from 15 min to 24 min was collected as one fraction. This fraction was dried down and then reconstituted into 100 μL of 16 μM C17D33:0 in 2-propanol for further analysis with the RP-LC/APCI-MS method.

For the larger scale normal phase purification method, a YMC PVA-Sil (4.6 x 250 mm) column was used for the separation of the lipid classes. A 200 μL sample loop was used to load the lipid extract. Before injecting, the column was equilibrated in 95 % solvent A and 5 % solvent B. After the injection, the gradient was ramped to 50 % solvent A and 50 % solvent B over 40 min before returning to the initial conditions. The column was flushed for 15 min at the initial conditions before the next injection. The flow rate throughout the method was 1 mL/min and the entire separation was done at room temperature.

When monitoring the 4.6 mm i.d. NP separation, the eluent was directed into the MS with an ESI probe in positive mode. The eluant was split after the column so that the flow rate into the MS was approximately 200 μL/min. In order to increase the ionization efficiency, a post column feed (10 mM ammonium acetate in 60:40 methanol:2-propanol) was added to the split portion of the eluent at a flow rate of 150 μL/min. Single ion mode was used to monitor the mass to charge ratios corresponding to the lipid marker for each
class and each lipid was monitored in a separate channel. Channel 1 was set to 353 m/z for the monoacylglycerol (MAG) [M+Na]+ peak, channel 2 was set to 270 m/z for the primary fatty acid amide (PFAM) [M+H]+ peak, channel 3 was set to 592 m/z for the diacylglycerol (DAG) [M+Na]+ peak, channel 4 was set to 350 m/z for the n-acyl ethanolamine (NAE) [M+Na]+ peak, channel 5 was set to 281 m/z for the fatty acid (FFA) [M-H]- peak, channel 6 was set to 338 m/z for the n-acyl glycine (NAG) [M-H]- peak, and channel 7 was set to 914 m/z for the triacylglycerol (TAG) [M+Na]+ peak. When analysis of the PFAM lipid class was required, the entire eluent was directed to the fraction collector. Based on retention times of the lipid classes when the flow was directed into the MS, the eluent from the PFAM peak was collected as one fraction. The retention time of the PFAM peak shifted over time, so the separation had to be periodically monitored and the collection times corrected accordingly. This fraction was dried down and then reconstituted into 150 μL of 16 μM C17D33:0 in methanol for further analysis with the RP-LC/APCI-MS. The development and limitations of these methods have been described in Chapter 5.

6.6.2 Reverse Phase LC/APCI-MS Conditions

The HPLC/MS system was a Waters 2695 separations module with a Waters 2487 dual wavelength detector operated at 210 nm to monitor the gradient and a Waters ZMD MS (Milford, MA) with an APCI probe in positive mode to monitor the separation. Data was analyzed using Waters MassLynx 4.1 software. Single ion mode was used to
monitor the mass to charge ratios for the [M+H]^+ signal corresponding to the PFAMs of interest. Each channel monitored one or two mass to charge ratios for the time the corresponding peaks were expected to minimize background noise. For the qualitative RP-HPLC separation, a Water’s X Terra MS C18 (5 μm, 3.9 x 150 mm) column with a Water’s X Terra MS C18 (5 μm, 3.9 x 20 mm) guard column and an inline filter were used. The solvents were sonicated for 20 min to degas before running; during the run the in-line degasser was used. Two different gradients were developed in Chapter 4. Gradient #1 was qualitative and was used to determine which PFAMs present in the sample, while gradient #2 was shorter to reduce the uncertainty in the calculated values.

Before injecting using gradient #1, the column was equilibrated in 75:25 methanol:water with 0.3 % formic acid. After injection, the gradient was ramped linearly to 95:5 methanol:water with 0.3 % formic acid over 30 min with a 5 min hold at the final conditions before returning to the initial conditions. The mobile phase was held at the initial conditions for 5 min before doing the next injection. The flow rate was held constant at 1 mL/min. All separations were done at room temperature (about 28 °C) with both gradients. APCI-MS settings were as follows: corona 3 kV, sample cone 30 V, extraction cone 2 V, RF lens 0.2 V, source temperature 130 °C, APCI probe temperature 450 °C, desolvation gas flow 450 L/hr, and the cone gas flow 50 L/hr. These values varied slightly every time maintenance was done on the MS. The development and limitations of the method have been described in Chapter 4. Channel one was used to monitor for C12:0 and C14:0 by scanning the mass to charge ratios 200 and 228 starting at 5.0 after injection to 13.5 min. Channel two was used to monitor for C16:0 by scanning the mass to charge ratio 256 from 16.0 to 17.0 min. Channel three was used to
monitor for the C18:1 isomers by scanning the mass to charge ratio 282 from 17.0 to 22.0 min. Channel four was used to monitor for the internal standards C17D33:0 and C17:0 by scanning the mass to charge ratios 270 and 303 from 16.0 to 23.0 min. Channel five was used to monitor for the C18:0 by scanning the mass to charge ratio 284 from 21.0 to 25.0 min. Channel six was used to monitor for C20:0 by scanning the mass to charge ratio 312 from 24.5 to 29.0 min. Channel seven was used to monitor for the C22:1 isomers by scanning the mass to charge ratio 338 from 26.0 to 33.0 min. Channel eight was used to monitor for C22:0 by scanning the mass to charge ratio 340 from 29.0 to 38.0 min.

Before injecting using gradient #2, the same column was equilibrated in 80:20 methanol:water with 0.3 % formic acid. After injection, the gradient was ramped linearly to 100 % methanol with 0.3 % formic acid over 10 min. The mobile phase was then returned to the initial conditions and flushed for 5 min before doing the next injection. All separations were done at room temperature (about 28 °C) with both gradients. APCI-MS settings were as follows: corona 3 kV, sample cone 30 V, extraction cone 2 V, RF lens 0.2 V, source temperature 130 °C, APCI probe temperature 450 °C, desolvation gas flow 450 L/hr, and the cone gas flow 50 L/hr. These values varied slightly every time maintenance was done on the MS. The development of the method has been discussed in Chapter 4. For this method, each mass to charge ratio corresponding to the PFAMs in the mixture was monitored in a separate channel for the duration of the run.
6.6.3 GC/MS Conditions

A Varian GC/MS/MS system (CP-3800 GC with Saturn 2000 mass selective detector and CP-8400 autosampler) was used for the analysis of PFAMs. The column was a Varian VF5-MS (0.25 mm internal diameter, 0.25 μm film thickness, 30 m long). The GC/MS method used for analysis was as follows: starting temperature was 55 °C, ramped to 150 °C at 40 °C/min, held at 150 °C for 3.62 min, and finally ramped to 275 °C at 10 °C/min. Helium was used as a carrier gas with a flow of 1 mL/min. The mass range was 50 to 350 m/z in chemical ionization (CI) mode, with methanol as the CI gas. The temperature of the injection port was 250 °C with an injection volume of 1 μL unsplit. The sample was reconstituted in 2-propanol to avoid methylation that occurs in the injection port when the sample is diluted in methanol.

6.7 Results and Discussions

Initial experiments were done by purifying the PFAMs from the bovine omentum lipid extract using the SPE method. In this case, approximately 1 g of bovine omentum tissue was used for the extraction and 100 μL of the 1 mL lipid extract was loaded onto the first SPE column. Gradient #1 was used for the RP-LC analysis. For these experiments the samples were analyzed by both the LC/MS and the GC/CI-MS. The results are shown in Table 6-1. The GC/CI-MS analysis was done by Dent. The amounts in the table were first corrected for any changes in the instrument from run to run using the response of C17D33:0 and then for dilutions during the sample preparation. Next the
amounts were corrected for percent extracted using the percent recovery of C17:0. Sultana and Johnson showed that although the percent recovery of each PFAM from sample is not exactly the same, the percent recoveries are similar [18]. The amounts from three separate injections on the instrument were averaged together before subtracting any PFAMs found in the blank samples. Finally, the numbers were divided by the starting mass of the wet tissue before averaging the results of three separate extractions. C18:0 and C18:1\(^9\) have the largest error because of possible contamination from any plastic used during the sample preparation. Because the detection limit was higher on the GC/MS, the LC/MS method detected more types of PFAMs. Unfortunately, further studies showed that these numbers were irreproducible.

<table>
<thead>
<tr>
<th>PFAM</th>
<th>LC/MS Method (nmol/g)</th>
<th>GC/MS Method (nmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitamide (C16:0)</td>
<td>2.47 ± 1.24</td>
<td>1.41 ± 0.10</td>
</tr>
<tr>
<td>Stearamide (C18:0)</td>
<td>2.23 ± 1.32</td>
<td>2.09 ± 1.19</td>
</tr>
<tr>
<td>*Oleamide (C18:1(^9))</td>
<td>20.05 ± 2.76</td>
<td>11.43 ± 1.71</td>
</tr>
<tr>
<td>*Arachidamide (C20:0)</td>
<td>0.86 ± 0.82</td>
<td>--</td>
</tr>
<tr>
<td>*Behenamide (C22:0)</td>
<td>1.91 ± 0.22</td>
<td>--</td>
</tr>
<tr>
<td>Eruccamide (C22:1(^13))</td>
<td>118.79 ± 139.15</td>
<td>111.47 ± 22.83</td>
</tr>
</tbody>
</table>

Table 6-1  Values are reported at nmol of PFAM per gram of wet tissue and are the average of three separate extractions/analysis. Values were corrected using both internal standards before the blank was subtracted to get the final values. The percent recovery for the LC/MS method was 30% while the percent recovery for the GC/MS method was 32%. The error is the 95% confidence level. The asterisk denotes which reported PFAMs values were statically different between the two methods.

An exact comparison cannot be made between this data and previously published data. Hanus et al. reported C18:1\(^9\) concentrations in male Sprague-Dawley rats at
156 pmol/mL in cerebrospinal fluid and 35 pmol/mL of plasma [10], which is significantly lower than the amount found in the bovine omentum tissue. Arafat et al. reported finding 15 nmol/mL of C16:0 and 113 nmol/mL of C18:1\textsuperscript{9} in human plasma [2]. These values are higher than what was found in the bovine omentum, but C18:1\textsuperscript{9} is more concentrated than C16:0. However, the rough orders of magnitude are similar.

Wakamatsu et al. qualitatively studied omentum tissue but only reported finding C22:1\textsuperscript{13}, which was found at the highest concentration in this study [7]. That result alone is intriguing, given the (scant) evidence for erucamide as an angiogenic agent, and suggests that further study of the role of PFAMs in angiogenesis is warranted.

Validation experiments were done by spiking known amounts of the PFAM standards into the bovine omentum tissue before extraction. A set of spikes were done by adding 2 times, 3 times, and 4 times (see table 6-2) the amount of PFAMs found in the tissue sample. For each of these samples, 100 μL of the lipid extract was loaded onto the SPE column. The RP-LC/APCI-MS results (using gradient #1) are summarized in table 6-3. Because the recovered values did not consistently increase as the concentration of the PFAM increased, it was hypothesized that the concentration of each PFAM was not homogeneous throughout the entire bovine omentum tissue. At times, the blank tissue had more PFAMs than the spiked sample. In future experiments, the error between the lipid extracts with the same spike concentration was reduced by blending several grams of bovine omentum tissue together before the lipids were extracted from 1 g portions.
Table 6-2 The amount (nmols) added of each PFAM to the tissue before extraction.

<table>
<thead>
<tr>
<th>PFAM</th>
<th>C16:0</th>
<th>C18:0</th>
<th>C18:1*</th>
<th>C20:0</th>
<th>C22:1*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X spike</td>
<td>4</td>
<td>4</td>
<td>40</td>
<td>4</td>
<td>200</td>
</tr>
<tr>
<td>3X spike</td>
<td>6</td>
<td>6</td>
<td>60</td>
<td>6</td>
<td>300</td>
</tr>
<tr>
<td>4X spike</td>
<td>8</td>
<td>8</td>
<td>80</td>
<td>8</td>
<td>400</td>
</tr>
</tbody>
</table>

After the NP-LC purification method (Chapter 5) and the RP-LC method (Chapter 4) were developed, the two methods were combined. Initially, standards were used to test the method. By calculating the percent recovery through the method with standards, the error in the method could be calculated. To determine the recovery of the fraction collection, 2 μL of the PFAM mixture (560 pmol each) was injected onto the 1 mm i.d. NP-LC column. Three fractions were saved as they eluted from the column: 10 min to 15 min to check for any PFAM loss, 15 min to 24 min to collect the PFAMs, and 24 min to 30 min to check for any PFAM loss. The eluent was dried down and then reconstituted in 2-propanol which contained 16 μM of the internal standard (C17D33:0) for analysis with the gradient #1 RP-LC method followed by APCI-MS for detection. Results are shown in Table 6-4. This experiment showed that the recovery in the 15 to 24 min fraction of all the PFAMs was close to 100% for each PFAM except C22:0. It is suspected that contamination caused the error because the peak shape was different from calibration runs.

Table 6-3 The amount (nmol/g of wet tissue) recovered from the extraction after subtracting the amount found in the blank.

<table>
<thead>
<tr>
<th>PFAM</th>
<th>C16:0</th>
<th>C18:0</th>
<th>C18:1*</th>
<th>C20:0</th>
<th>C22:1*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X spike</td>
<td>7.56</td>
<td>20.58</td>
<td>41.06</td>
<td>3.30</td>
<td>127.60</td>
</tr>
<tr>
<td>3X spike</td>
<td>2.34</td>
<td>-3.10</td>
<td>16.90</td>
<td>0.67</td>
<td>15.57</td>
</tr>
<tr>
<td>4X spike</td>
<td>8.27</td>
<td>22.87</td>
<td>46.22</td>
<td>4.29</td>
<td>84.78</td>
</tr>
</tbody>
</table>
Table 6-4 Percent recoveries of each PFAM collected from the 1 mm i.d. NP column. C17:0 was used to correct for the variation between extractions, while C17D33:0 was used to correct for the variation in instrument response.

<table>
<thead>
<tr>
<th>PFAM</th>
<th>Percent Recovered</th>
<th>Standard Deviation</th>
<th>95 % Confidence Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0</td>
<td>82 %</td>
<td>7 %</td>
<td>17 %</td>
</tr>
<tr>
<td>C18:0</td>
<td>95 %</td>
<td>6 %</td>
<td>15 %</td>
</tr>
<tr>
<td>C18:1&lt;sup&gt;9&lt;/sup&gt;</td>
<td>86 %</td>
<td>3 %</td>
<td>7 %</td>
</tr>
<tr>
<td>C20:0</td>
<td>93 %</td>
<td>10 %</td>
<td>24 %</td>
</tr>
<tr>
<td>C22:0</td>
<td>217 %</td>
<td>24 %</td>
<td>61 %</td>
</tr>
<tr>
<td>C22:1&lt;sup&gt;13&lt;/sup&gt;</td>
<td>94 %</td>
<td>37 %</td>
<td>91 %</td>
</tr>
</tbody>
</table>

The NP-LC method using the 4.6 mm i.d. column was also validated using standards. Because of the higher flow rates, 1.5 mL fractions were collected from 32 min to 42.5 min. For this experiment 30 nmol of each PFAM (C16:0, C17:0, C18:0, C18:1<sup>9</sup>, C20:0, C22:0, and C22:1<sup>13</sup>) was loaded as a mixture in 80:20 n-hetane:2-propanol onto the 4.6 mm i.d. NP column. Each fraction was collected in a silanized glass vial, dried down, and reconstituted in 100 μL of 16 μM C17D33:0. Each fraction was injected once onto the RP-LC column using gradient #1 and analyzed with APCI-MS. The mixture was injected three separate times onto the NP-LC column. The peak areas were corrected using the C17D33:0 as the internal standard and the results were reported as a percentage of the total signal. There was some shift in the signal between trials, but all of the PFAMs eluted between fractions 3-6 corresponding to 35-41 min in the gradient. Figure 6-1 is a reconstructed chromatogram from the three trials averaged together showing the elution from the 4.6 mm i.d. NP column. The reconstructed chromatogram
agrees with the elution order when the eluent was monitored in real time with the ESI-MS (Figure 5-14).

![Graph](image_url)

**Figure 6-1** The data from the three trials were averaged together and replotted as one "chromatogram" showing the elution from the 4.6 mm i.d. NP-LC column.

Using the RP-LC gradient #1 alone there was over 20 % error in the back calculations when trying to quantify the results. After propagating the error and adding in the experimental error from sample loss, the final numbers were insignificant compared to the error values. The error was a combination of run-to-run variation on the instruments, variation in extraction efficiency from one sample to the next, variations in tissue samples, and experimental error from the sample preparation procedure. In order to reduce the error caused by the APCI-MS, a shorter gradient (described as the gradient #2 in the RP-LC/APCI-MS conditions section) was developed where only six PFAMs were monitored by the MS. This method allowed for a three point calibration
curve and the sample injections to be run in triplicate on the same day. In order to test this method, 20 nmol of each PFAM (C16:0, C17:0, C18:0, C20:0, and C22:0) were injected (200 μL injection) onto the normal phase column. The PFAMs were collected as two fractions (34 – 38 min and 38 – 42 min) which were combined during drying down. The sample was then reconstituted in 150 μL of 16 μM C17D33:0 in methanol before running the RP-LC/APCI-MS in triplicate using gradient #2. Although the second internal standard (C17D33:0) was added before analysis, the error from the calibration curve was smaller when the peak areas were not corrected with the response factors. The samples did need to be corrected with the first internal standard (C17:0) to correct for any sample loss during the purification separation. Results are shown in Figure 6-2 and Table 6-5. The data shows that at the 95 % confidence level, the experimental data agrees with the actual quantities added to the mixture. See Appendix A for calculation procedures, except the values do not have to be corrected for dilutions since a lipid extraction was not done.
Figure 6-2: Percent recovery of each individual PFAM (C16:0, C18:0, C20:0, and C22:0) when collected off of the NP-LC column and then further separated with the RP-LC column using gradient #2. 20 nmol of each PFAM including C17:0 was loaded onto the NP column twice. Error bars are the 95 % confidence limit. The asterisks show when the data was statistically different from 100 %.

<table>
<thead>
<tr>
<th>PFAM</th>
<th>C16:0</th>
<th>C18:0</th>
<th>C20:0</th>
<th>C22:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>93 %</td>
<td>94 %</td>
<td>84 %</td>
<td>78 %</td>
</tr>
<tr>
<td>Sample 2</td>
<td>111 %</td>
<td>108 %</td>
<td>85 %</td>
<td>37 %</td>
</tr>
<tr>
<td>Sample 1 error</td>
<td>12 %</td>
<td>11 %</td>
<td>12 %</td>
<td>33 %</td>
</tr>
<tr>
<td>Sample 2 error</td>
<td>5 %</td>
<td>3 %</td>
<td>3 %</td>
<td>11 %</td>
</tr>
<tr>
<td>Sample 1 95 % C.L.</td>
<td>29 %</td>
<td>28 %</td>
<td>29 %</td>
<td>82 %</td>
</tr>
<tr>
<td>Sample 2 95 % C.L.</td>
<td>11 %</td>
<td>8 %</td>
<td>8 %</td>
<td>28 %</td>
</tr>
</tbody>
</table>

Table 6-5: Percent recovery of each individual PFAM (C16:0, C18:0, C20:0, and C22:0) when collected off of the NP-LC column and then further separated with the RP-LC column using gradient #2. 20 nmol of each PFAM including C17:0 was loaded onto the NP column twice. The values were calculated using a basic calibration curve.

The method on the 1 mm i.d. NP-LC column was verified by analyzing the PFAM content in omentum tissue. The lipid extract was reconstituted in 5 mL of 80:20 n-heptane:2-propanol instead of 1 mL and then 2 μL of the sample was injected onto the
normal phase column. The PFAM fraction was collected and reconstituted in 50 µL of 2-propanol containing 16 µM of the internal standard (C17D33:0) before injecting onto the RP-LC column using gradient #1. Five PFAMs (C18:1, C18:0, C20:0, C22:0 and C22:1) plus the internal standards (C17D33:0 and C17:0), which were added during sample preparation, were identified in the sample. (Figure 6-3) The same PFAMs, except for C16:0, were identified using both the SPE column\textsuperscript{A} and the 1 mm i.d. NP column.

\textbf{Figure 6-3} The sample was purified by loading 2 µL of the lipid extract (reconstituted in 5 mL) onto the normal phase column. The PFAM fraction was collected and reconstituted in 50 µL of 2-propanol before injecting onto the RP-LC column. Five PFAMs (C18:1, C18:0, C20:0, C22:0 and C22:1) plus the internal standards (C17D33:0 and C17:0), which were added during sample preparation, were identified in the sample. Except for C16:0 the same PFAMs were identified using both of the sample preparation methods.

\textsuperscript{A} Unpublished manuscript – Kroniser, K.M. and M.E. Johnson, \textit{Liquid Chromatography Mass Spectrometry Analysis of Primary Fatty Acid Amides in Bovine Omentum}. 

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The method on the 4.6 mm i.d. NP-LC column was verified by analyzing the PFAM content in omentum tissue and then 200 μL of the sample was injected onto the NP column. The PFAM fraction was collected and reconstituted in 100 μL of 2-propanol containing 16 μM of C17D33:0 before injecting onto the RP-LC column using gradient #1. Two PFAMs (C22:0 and C22:1) plus the internal standards (C17D33:0 and C17:0), which were added during sample preparation, were identified in the sample (Figure 6-4). The peak at 30 min was an impurity. The concentration of PFAMs varied greatly from trial to trial, even when the recovery of the internal standard was the same. This led to the hypothesis that the PFAM content was not consistent over the entire bovine omentum sample; therefore, this data cannot be directly compared to the results from the SPE method.

![Graph showing the separation of PFAMs and impurity](image)

**Figure 6-4** The sample was purified by loading 200 μL of the lipid extract onto the normal phase column. The PFAM fraction was collected and reconstituted in 100 μL of 2-propanol before injecting onto the RP-LC column. Two PFAMs (C22:0 and C22:1) plus the internal standards (C17D33:0 and C17:0), which were added during sample preparation, were identified in the sample.
A lipid extraction was done after spiking 30 nmol of C16:0, C17:0, C18:0, C20:0, and C22:0 into the 1 g bovine omentum tissue samples. In this case, the blank was an unspiked sample of tissue. Some solvent remained in the samples after dry-down but before reconstituting in 1 mL because the only method of drying down was blowing nitrogen over the sample. The final lipid extract sample (200 μL) was loaded onto the NP-LC column. Two fractions were collected from the NP-LC column and combined during dry down before reconstituting in 150 μL of 16 μM C17D33:0 in methanol. 20 μL of the solution was injected onto the RP-LC column using gradient method #2 for analysis. The results from two extractions injected separately on the NP-LC column and then run in triplicate on the RP-LC column are summarized in Figure 6-5 and Table 6-6. See Appendix A for calculation procedures. These numbers were better than previous experiments, but the error was still significant. A series of experiments where the mass load on the normal phase column was varied by loaded the same spiked lipid extract at different volumes (100, 150, 175, and 200 μL) onto the column was done. No trends were noticed.
**Figure 6-5** Percent recovery of each individual PFAM (C16:0, C18:0, C20:0, and C22:0) when collected off of the NP-LC column and then further separated with the RP-LC column using gradient #2. 30 nmol of each PFAM including C17:0 was spiked into the omentum tissue. Two injections were done on the NP column and three injections were done on the RP column. Error bars are the 95% confidence limit. The asterisks show when the data was statistically different from 100%.

<table>
<thead>
<tr>
<th>PFAM</th>
<th>C16:0</th>
<th>C18:0</th>
<th>C20:0</th>
<th>C22:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction 1</td>
<td>36%</td>
<td>45%</td>
<td>107%</td>
<td>13%</td>
</tr>
<tr>
<td>Extraction 2</td>
<td>132%</td>
<td>127%</td>
<td>187%</td>
<td>64%</td>
</tr>
<tr>
<td>Extraction 1 error</td>
<td>31%</td>
<td>52%</td>
<td>141%</td>
<td>74%</td>
</tr>
<tr>
<td>Extraction 2 error</td>
<td>7%</td>
<td>7%</td>
<td>9%</td>
<td>7%</td>
</tr>
<tr>
<td>Extraction 1 95 % C.L.</td>
<td>78%</td>
<td>130%</td>
<td>350%</td>
<td>183%</td>
</tr>
<tr>
<td>Extraction 2 95 % C.L.</td>
<td>18%</td>
<td>18%</td>
<td>22%</td>
<td>16%</td>
</tr>
</tbody>
</table>

**Table 6-6** Percent recovery of 30 nmol of each PFAM spike from two extractions loaded on to the NP-LC column and analyzed with gradient #2 on the RP-LC column and APCI-MS. The values were calculated using a basic calibration curve.
In an attempt to determine whether incomplete dry-down of the extract was a source of variability, two new methods were tested for post-extraction processing. One method was to put the centrifuge tubes containing the lipid extract into a vacuum desiccator for about an hour with the pump running in order to completely dry the samples. The other method was to add magnesium sulfate in excess to the lipid extract after removing the top layer. The sample was centrifuged at 1500 g for 30 min to separate out the magnesium sulfate. The liquid was poured through a filter into a clean centrifuge tube and dried down under nitrogen. The samples still needed to be dried under vacuum in order to remove all the solvents.

In order to test both of these dry down methods, 60 nmol of each PFAM was spiked into approximately 0.5 g of bovine omentum tissue before extraction. In these cases, the blank was an unspiked sample of tissue. The samples were reconstituted into 2 mL of 80:20 heptane:2-propanol. 200 μL of the final lipid extract sample was loaded onto the NP-LC column. Two fractions were collected from the NP-LC column and combined during dry down before reconstituting in 150 μL of 16 μM C17D33:0 in methanol. The PFAM solution (20 μL) was injected onto the RP-LC column using gradient method #2 for analysis. The results from two extractions injected separately on the NP-LC column and then run in triplicate on the RP-LC column are summarized in Figure 6-6 and Table 6-7 for the vacuum dry down and Figure 6-7 and Table 6-8 for the desiccant/vacuum dry down. See Appendix A for calculation procedures using the basic calibration method. The percent recovery using the magnesium sulfate as a desiccant did not significantly reduce the dry down time, but the percent recoveries are noticeably lower. The student t test shows that there was less uncertainty in the calculated values.
shown in table 6-7 when the lipid extract mass was completely dried before reconstituting as opposed to some solvent remaining in the lipid extract (data shown in table 6-6). Overall, the percent recoveries were higher when the sample was dried under vacuum after drying under nitrogen. Because the remaining solvent would not have been the same for each sample, the solvent could have affected the concentration of the PFAMs and/or the retention times on the NP-LC column.

![Graph showing percent recovery of 60 nmol of each PFAM spike from three extractions loaded on to the NP-LC column and analyzed with gradient #2 on the RP-LC column and APCI-MS. The samples were dried down using by blowing nitrogen over the sample and then pulling a vacuum. Two injections were done on the NP column and three injections were done on the RP column. Error bars are the 95% confidence limit. The asterisks show when the data was statistically different from 100%.]

**Figure 6-6** Percent recovery of 60 nmol of each PFAM spike from three extractions loaded on to the NP-LC column and analyzed with gradient #2 on the RP-LC column and APCI-MS. The samples were dried down using by blowing nitrogen over the sample and then pulling a vacuum. Two injections were done on the NP column and three injections were done on the RP column. Error bars are the 95% confidence limit. The asterisks show when the data was statistically different from 100%.
Table 6-7 Percent recovery of 60 nmol of each PFAM spike from three extractions loaded on to the NP-LC column and analyzed with gradient #2 on the RP-LC column and APCI-MS. The samples were dried down using by blowing nitrogen over the sample and then pulling a vacuum. The values were calculated using a basic calibration curve.

<table>
<thead>
<tr>
<th>PFAM</th>
<th>C16:0</th>
<th>C18:0</th>
<th>C20:0</th>
<th>C22:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction 1</td>
<td>81%</td>
<td>102%</td>
<td>59%</td>
<td>76%</td>
</tr>
<tr>
<td>Extraction 2</td>
<td>95%</td>
<td>122%</td>
<td>68%</td>
<td>76%</td>
</tr>
<tr>
<td>Extraction 3</td>
<td>85%</td>
<td>101%</td>
<td>44%</td>
<td>59%</td>
</tr>
<tr>
<td>Extraction 1 error</td>
<td>15%</td>
<td>18%</td>
<td>10%</td>
<td>29%</td>
</tr>
<tr>
<td>Extraction 2 error</td>
<td>20%</td>
<td>23%</td>
<td>23%</td>
<td>8%</td>
</tr>
<tr>
<td>Extraction 3 error</td>
<td>10%</td>
<td>17%</td>
<td>19%</td>
<td>8%</td>
</tr>
<tr>
<td>Extraction 1 95 % C.L.</td>
<td>38%</td>
<td>45%</td>
<td>26%</td>
<td>73%</td>
</tr>
<tr>
<td>Extraction 2 95 % C.L.</td>
<td>51%</td>
<td>57%</td>
<td>58%</td>
<td>20%</td>
</tr>
<tr>
<td>Extraction 3 95 % C.L.</td>
<td>25%</td>
<td>42%</td>
<td>48%</td>
<td>20%</td>
</tr>
</tbody>
</table>

Figure 6-7 Percent recovery of 60 nmol of each PFAM spike from three extractions loaded on to the NP-LC column and analyzed with gradient #2 on the RP-LC column and APCI-MS. The samples were dried down using desiccant and then a vacuum. Two injections were done on the NP column and three injections were done on the RP column. Error bars are the 95 % confidence limit. The asterisks show when the data was statistically different from 100 %.
<table>
<thead>
<tr>
<th>PFAM</th>
<th>C16:0</th>
<th>C18:0</th>
<th>C20:0</th>
<th>C22:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction 1</td>
<td>68%</td>
<td>18%</td>
<td>54%</td>
<td>40%</td>
</tr>
<tr>
<td>Extraction 2</td>
<td>67%</td>
<td>33%</td>
<td>144%</td>
<td>78%</td>
</tr>
<tr>
<td>Extraction 3</td>
<td>60%</td>
<td>55%</td>
<td>174%</td>
<td>91%</td>
</tr>
<tr>
<td>Extraction 1 error</td>
<td>17%</td>
<td>11%</td>
<td>16%</td>
<td>11%</td>
</tr>
<tr>
<td>Extraction 2 error</td>
<td>15%</td>
<td>8%</td>
<td>25%</td>
<td>21%</td>
</tr>
<tr>
<td>Extraction 3 error</td>
<td>6%</td>
<td>7%</td>
<td>18%</td>
<td>11%</td>
</tr>
<tr>
<td>Extraction 1 95 % C.L.</td>
<td>42%</td>
<td>27%</td>
<td>39%</td>
<td>28%</td>
</tr>
<tr>
<td>Extraction 2 95 % C.L.</td>
<td>37%</td>
<td>20%</td>
<td>63%</td>
<td>51%</td>
</tr>
<tr>
<td>Extraction 3 95 % C.L.</td>
<td>15%</td>
<td>17%</td>
<td>44%</td>
<td>28%</td>
</tr>
</tbody>
</table>

Table 6-8  Percent recovery of 60 nmol of each PFAM spike from three extractions loaded on to the NP-LC column and analyzed with gradient #2 on the RP-LC column and APCI-MS. The samples were dried down after a desiccant was used to remove the water from the sample using by blowing nitrogen over the sample and then pulling a vacuum. The values were calculated using a basic calibration curve.

### 6.8 Conclusions

The PFAMs were extracted from the tissue sample with the other neutral lipids using Sultana’s method [15, 18]. After the sample is reconstituted, it could be loaded onto either SPE silica based column and then an amine based column or a normal phase LC column. In both cases, the PFAM fraction was collected, dried down, and reconstituted before loading a portion onto the RP-LC column.

Replacing the SPE purification method with a NP gradient followed by fraction collection improves the reproducibility of the method. Because the gradient can be measured when problems occur, time spent on problem solving due to day-to-day variation in retention time is eliminated. The NP-LC separation can be monitored using
ESI-MS, unlike the SPE method where only fractions can be analyzed (either by TLC or by MS). Secondly, the same column was used for each extraction, rather than the two freshly hand packed columns needed to complete the SPE method, reducing run to run variations. One of the limitations of the NP-LC method was that because the separation has been analyzed using ESI-MS, the elution pattern was only known for the lipid classes that ionize. Furthermore, the method is only set up to monitor for one example in each lipid class with the exception of the PFAMs. By following the trends of chromatography, the PFAM fraction was assumed to only have PFAMs and maybe some MAGs. When using TLC to monitor the separation, all of the lipids in a given class are detected, but the method does not differentiate between contamination and lipids.

There were several sources of error including experimenter and instrument. All of the glassware had to be silanized, so any piece of glassware not fully silanized would have contributed to sample loss during sample preparation. All of the samples were homogenized by hand. Two sources of experimenter error would be if the tissue was not fully homogenized or if one of the solvent volumes were incorrectly measured. The first internal standard (C17:0) should have corrected for any of these mistakes during sample preparation. From running replicates of the standard mix solutions, it was determined that the instrument response was not the same from one injection to the next. Initially, a second internal standard (C17D33:0) was added to the extract before injection. However, further experiments showed that using a shorter gradient and a basic calibration curve (i.e. without the second internal standard) was actually more reproducible than the longer gradient and the response factor calculation method. The concentration of PFAMs varied greatly from one bovine omentum tissue sample to the next. Because the percent
recovery of the internal standard was similar, it was hypothesized that the concentration of PFAMs was not homogenous throughout the entire omentum making the tissue sample the greater source of error. In order to reduce variations between samples within the same experiment, several grams of tissue were blended together before homogenizing one gram portions separately.

Two different gradients were developed. The gradient #1 was 45 min long while gradient #2 was 20 min long. Gradient #1 was used for identify which PFAMs were present in the tissue sample. This gradient separated all of the even saturated PFAMs from C12:0 through C22:0 and partially separated a series of PFAMs with one degree of unsaturation and the same carbon chain length. Unfortunately, this method varied over 20 % from one injection to the next. The instrument error was reduced to less than 5 % by using the gradient #2. Gradient #2 did not separate the PFAMs with one degree of unsaturation and the same carbon chain length, but could be used to quantitate a few saturated PFAMs spiked into the tissue sample. Additionally, two different calculation methods were compared: basic calibration method and response factor method. The response factor method was set up to correct for instrument variations, but the results were more accurate when a short calibration curve was used with the basic calibration method. The drawback to this method was that the PFAM concentration in the tissue had to be estimated before setting up the calibration curve. Gradient #1 was used for qualitative results and gradient #2 was used for quantitative results.

Figure 6-8 shows a schematic breakdown of the method. Starting from the end of the method, it has been determined that there was approximately 5 % error between injections of standard mixtures on the RP-LC/APCI-MS using gradient #2. When the
PFAM fraction was loaded onto the NP-LC column, collected, and then analyzed by RP-LC/APCI-MS using gradient #2, the error increased to approximately 15 % except for C22:0. By comparing the variation between the percent recovery of the internal standard (C17:0) between samples versus the absolute percent recovery of the spiked PFAMs, it was determined that most of the error was from the variation in the PFAM concentration in the bovine omentum tissue. For example, looking at the data depicted in Figure 6-6, the error (when comparing sample to sample variation) in the C17:0 recovery was 6 % and the error for the other PFAMs except for C22:0 was less than 10 %. Therefore only approximations can be given for the PFAM concentrations in the bovine omentum tissue using this method.

Figure 6-8 Schematic breakdown of the offline 2D-LC method for the analysis of PFAMs in bovine omentum tissue.
Although these experiments show that an off line 2D-LC method has been
developed, further modifications need to be made to this method in order to develop a
comprehensive 2D-LC method for quantitative lipid analysis. The biggest problem is the
length of the RP-LC gradient. Ideally, the NP-LC separation should be slow enough so
that three to four fractions of each peak can be injected onto the RP-LC column. With
the current NP-LC method, the PFAMs elute in approximately 2.5 min, which would
only allow for one injection onto the RP-LC column. If the total fraction (250 μL) was
directly loaded onto the RP-LC column, the additional solvent would affect the
separation. The RP-LC method was developed with a 20 μL injection volume.
Additionally, the off line 2D-LC method focused on PFAM analysis; therefore only the
PFAM lipid class was collected from the NP-LC column and further separated by RP-LC.
Future experiments would evaluate the effectiveness of separating the lipids in the other
“polar” neutral lipid classes by RP-LC. The RP-LC gradient may need to change as the
polarity of the lipid eluting from the NP-LC column changes.

Erin Divitio will do the method development needed to turn this off line 2D-LC
method into a comprehensive 2D-LC method. Divitio has already started developing a
shorter gradient using a 6460 Agilent Technologies (Santa Clara, CA) rapid resolution
LC and triple quadrupole MS. There are several advantages of using this instrument over
the Waters 2695 LC pump and ZMD MS. The LC pumps have a higher pressure limit;
therefore RP columns with smaller particle sizes and/or gradients with faster flow rates
can be used for the separation of the PFAMs by chain length and degree of unsaturation.
Preliminary experiments show that a partial series of the C18:1 isomers can be separated
using a RP column (2.1 x 50 mm; 1.8 μm particles) and isocratic conditions (80:20
methanol:water with 0.3 % formic acid) in under 10 min. In theory, using a triple quadrupole MS instead of the single quadrupole should reduce the background noise allowing for lower detection limits. The method for the single quadrupole MS monitors for the \([M+H]^+\) mass to charge signal corresponding to the PFAMs of interest. The triple quadrupole method can be set up to scan for the \([M+H]^+\) mass to charge signal, and then further fragment the ion. The fragmentation pattern obtained from the MS/MS would identify if each peak was a PFAM or an impurity.

Future work might include evaluating NP capillary columns for the first dimension. This method would be needed if the RP method cannot be shortened so that several injections can be done on the RP column for every peak eluting from the NP column. By switching to a longer column with a smaller i.d., the NP separation will be lengthened. The sample loop collecting the fractions for the RP column could then be reduced and the number of samples loaded onto the RP column could be increased. Depending on the separation parameters, this should allow for longer analysis times in the second dimension compared to using the 4.6 mm i.d. NP column. It is important that the NP and RP gradients be modified at the same time so that optimizing one dimension does not interfere with the separation in the other dimension.

Currently, this method can be used to screen tissues for PFAMs. The carbon chain length and degree of unsaturation can be determined, but the bond position cannot be positively identified. An approximation of the quantity of each PFAM can be made. However, the amount of uncertainty in these values will be a problem when comparing the quantity of PFAMs in various tissues. Since only a few different tissues have been screened for PFAMs [15], several different tissues will be screened for PFAMs before
focusing on determining the concentration of PFAMs. Knowing where the PFAMs are stored and synthesized in vivo will allowed for more focused analytical methods to be developed for the quantification of the PFAM content.

The second phase of experiments will involve developing the method, so that other lipid classes can be monitored. This will be important for when studying the metabolic pathways. Although, some studies have been done to determine the biological pathway, having a method that is able to monitor all the lipid classes involved at the same time will help confirm the proposed cycles.

In conclusion the current method is good for screening biological tissues for PFAMs. After it has been determined which biological tissues contain the most PFAMs, the comprehensive 2D/LC method can be developed to focus on the lipids present in these tissues. The ultimate goal would be to have a method to separate and detect all the different variations and isomers of the neutral lipids; however, it is only necessary to optimize the separation for the neutral lipids that are naturally found in the tissues of interest.
6.9 References


