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UNIVERSITY OF NORTHERN COLORADO

Greeley, Colorado

The Graduate School

CHROMATOGRAPHIC ANALYSIS OF FATTY ACIDS USING 9-CHLOROMETHYL-ANTHRACENE AND 2-BROMOMETHYL-ANTHRAQUINONE

A Thesis Submitted in Partial Fulfillment
of the Requirement for the Degree of
Master of Science

Jesus B. Tapia

College of Natural and Health Sciences
Department of Chemistry and Biochemistry

August, 2014

This Thesis by: Jesus B. Tapia

Entitled: *Chromatographic analysis of fatty acids using 9-chloromethyl-anthracene and 2-bromomethyl-anthraquinone*

has been approved as meeting the requirement for the Master of Science in College of Natural and Health Sciences in Department of Chemistry and Biochemistry

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Dean of the Graduate School and International Admissions

ABSTRACT

Tapia, Jesus B. *Chromatographic analysis of fatty acids using 9-chloromethyl-anthracene and 2-bromomethyl-anthraquinone.* Unpublished Master of Science Thesis, University of Northern Colorado, 2014.

Fatty acids are carboxylic acids with long alkane or alkene chains and are important components of living organisms. The presence of the carboxylic acid is analytically very important since it allows for derivatization with fluorescence reagents such as 9-chloromethyl-anthracene (9-CMA) for HPLC analysis. A method for the analysis of fatty acids using 9-CMA and 2-bromomethyl-anthraquinone (MAQ-Br) was developed. The method utilizes a modified protocol previously used for the analysis of short-chain carboxylic acids with 9-CMA. The modified protocol was applied to medium- and long-chain fatty acids for analysis by HPLC with UV-visible and fluorescence detection. After successful derivatization and analysis using 9-CMA, MAQ-Br was used for derivatization with successful results. Six fatty acid standards were derivatized with 9-CMA and utilized to develop a separation method and quantification. With the use of a Zorbax Rx C8 column, good separation was achieved and calibration curves ranging from 1 to 100 pmol with good linearity and R^2 values. The limit of detection was approximately 50 fmol and the maximum limit of quantitation was approximately 100 pmol for the 9-CMA derivatives. After successful separation and quantitation, analyses of fat-free cooking spray and cloprostenol, a prostaglandin, were performed.

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CHAPTER I

INTRODUCTION

Fatty acids are important components of living organisms. Various kinds of fatty acids play important roles in a variety of physiological and biological functions (Fang, et al., 2007). Therefore, analysis of the fatty acid content in biological samples is important. To analyze the fatty acid composition of biological lipids, the complex lipids must be pretreated so that the individual fatty acids are available for chromatographic analysis (Li & Watkins, 2001).

Fatty acids in biological lipids must be hydrolyzed, extracted, and typically converted to their corresponding methyl esters (Li & Watkins, 2001) for gas chromatographic (GC) analysis. A major disadvantage of GC analysis of fatty acids is the use of elevated temperatures that affect thermally-labile compounds such as monounsaturated, polyunsaturated, and hydroxy fatty acids (Sun, You, Song, & Xia, 2011) that can undergo thermal decomposition since the fatty acid alkyl chain is susceptible to oxidation at the double bonds (Frankel, 1984).

To overcome the disadvantage of the high temperature GC analysis, high-performance liquid chromatography (HPLC) can be used. The use of HPLC at room temperature reduces the risk of decomposition of heat-labile compounds (Sun et al.,

2011); however, most fatty acids show neither natural absorption in the visible or ultraviolet (UV) regions, nor do they fluoresce. Therefore, derivatization of fatty acids with labeling reagents has been widely adopted, since HPLC with UV-vis or fluorescence detection also offers higher sensitivity than GC (Guoliang, et al., 2011).

Many different fluorescent labeling reagents have been investigated for the analysis of fatty acids. Some examples (Figure 1) include 2-(2-(anthracen-10-yl)-1*H*-phenanthro[9,10-*d*]imidazol-1-yl)ethyl-4-methylbenzenesulfonate (APIETS) (Sun et al., 2011), 2-(11*H*-benzo[a]carbazol-11-yl)-ethyl-4-methylbenzenesulfonate (BCETS) (Li, et al., 2011), and 2-(2-(anthracen-10-yl)-1*H*-naphtho[2,3-*d*]imidazol-1-yl)ethyl-*p*-toluenesulfonate (ANITS) (Fang, et al., 2007) which were successfully used to analyze fatty acids in different biological samples.

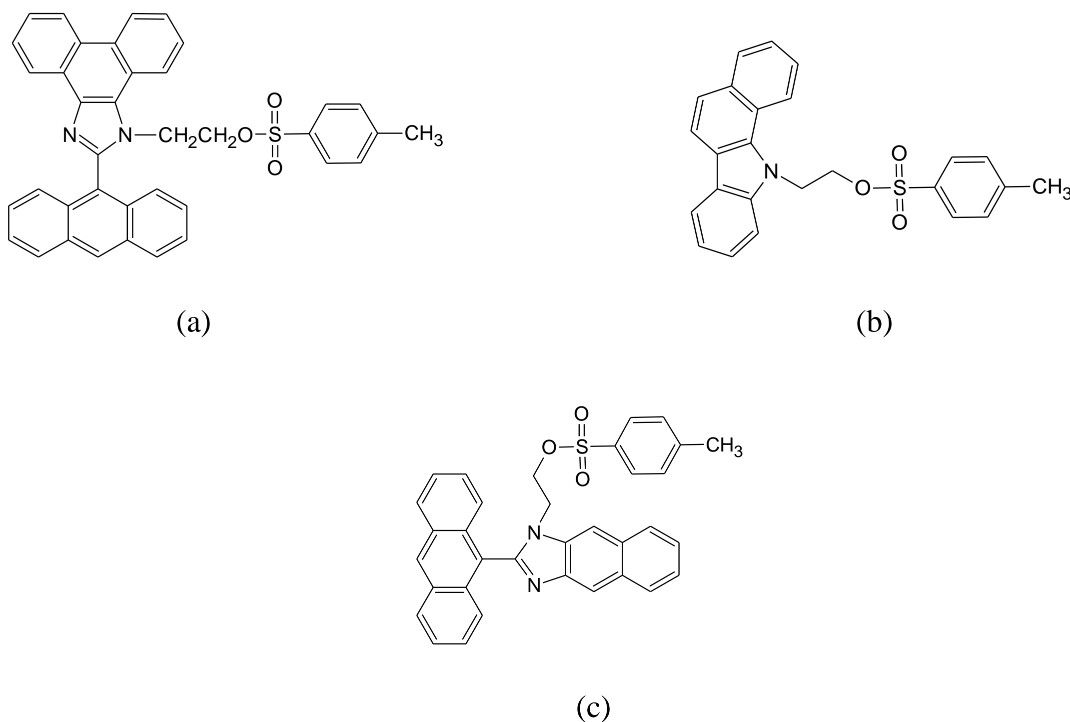


Figure 1. Previously studied fluorescent labeling reagents. (a) 2-(2-(anthracen-10-yl)-1*H*-phenanthro[9,10-*d*]imidazol-1-yl)ethyl-4-methylbenzenesulfonate (APIETS); (b) 2-(11*H*-benzo[a]carbazol-11-yl)-ethyl-4-methylbenzenesulfonate (BCETS); (c) 2-(2-(anthracen-10-yl)-1*H*-naphtho[2,3-*d*]imidazol-1-yl)ethyl-*p*-toluenesulfonate (ANITS).

The aim of this study was to investigate the use of 9-chloromethyl-anthracene, which had been previously used for the analysis of short chain carboxylic acids (Xie, Yu, & Deng, 2012), and 2-bromomethyl-anthraquinone (Figure 2) as labeling reagents for the analysis of fatty acids in biological samples. This study included method validation of the protocol developed by Xie et al. (2012), application of the method to medium chain fatty acids, long chain fatty acids, and prostaglandins, development of a derivatization method with 2-bromomethyl-anthraquinone, and the development of quantitative methods for the analysis of biological samples using both chromophoric tags.

Figure 2. Chromophoric labeling reagents (a) 9-chloromethyl-anthracene (b) 2-bromomethyl-anthraquinone.

CHAPTER II

REVIEW OF LITERATURE

Fatty Acids

Fatty acids are carboxylic acids with long alkane or alkene chains (Figure 3). They are rarely "free" in nature but occur in esterified form as part of more complex molecules, including several classes of lipids (Voet & Voet, 2010).

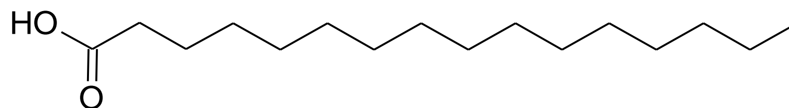
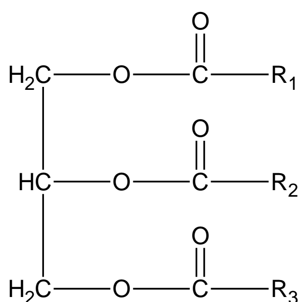


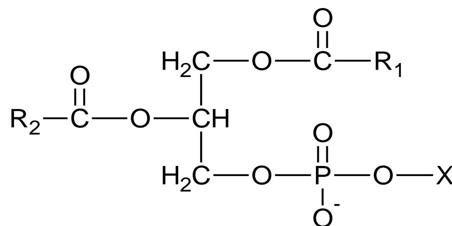
Figure 3. Structure of palmitic acid

Lipids obtained from biological samples have complex structures and often fall into the following classes (Figure 4): triacylglycerols which are fatty acid triesters of glycerol that function as energy reservoirs in animals and plants and are therefore the most abundant class of lipids; acyl phosphoglycerols which consist of *sn*-glycerol-3-phosphate esterified at the C1 and C2 positions to fatty acids with its phosphoryl group esterified to an alcohol, "X," which are the major lipid components of biological membranes; sphingolipids which are also major membrane components are derivatives of the C_{18} amino alcohols sphinosine, dihydrosphinosine, or their C_{16}, C_{17}, C_{19}, and C_{20}

homologs and either an ester of phosphocholine or an acetal of an oligosaccharide; and sterols which are derivatives of cyclopentanoperhydrophenanthrene and precursors of cholesterol, a vital component of cell membranes and a precursor of the steroid hormones and bile salts (Voet & Voet, 2010).



(a)



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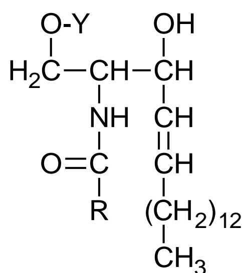

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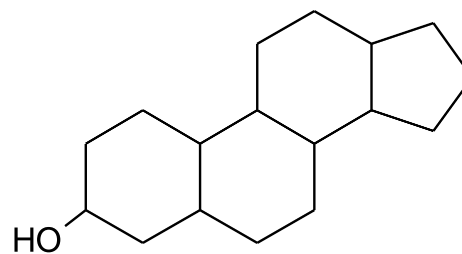


(d)

Figure 4. Major classes of lipids. (a) A triacylglycerol, (b) an acyl phosphoglycerol where X is a phospho alcohol, (c) a sphingolipid where Y is phosphocholine or an oligosaccharide, and (d) a sterol.

Fatty acids can contain 40 or more carbon atoms and can have an odd or even number of carbon atoms in the chain; however, naturally occurring fatty acids typically have an even number of carbons because they are usually biosynthesized by the concatenation of C_2 units (Voet & Voet, 2010). They can also be branched with between one and 20 carbon atoms in a branch. Branched fatty acids may be *iso*-acids or *anteiso*-acids (Figure 5).

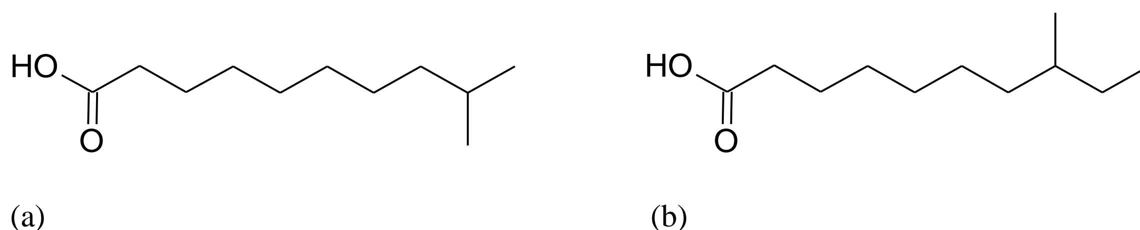


Figure 5. Structures of branched fatty acids. (a) A branched *iso*-acid with a single methyl group attached to carbon 9 and (b) a branched *anteiso*-acid with a single methyl group attached to carbon 8.

Naturally occurring fatty acids may be saturated (Figure 6a), monounsaturated (Figure 6b), non-conjugated polyunsaturated or conjugated polyunsaturated acids (Figure 6c-d). Unsaturated fatty acids have either *cis* or *trans* configurations (Brondz, 2001).

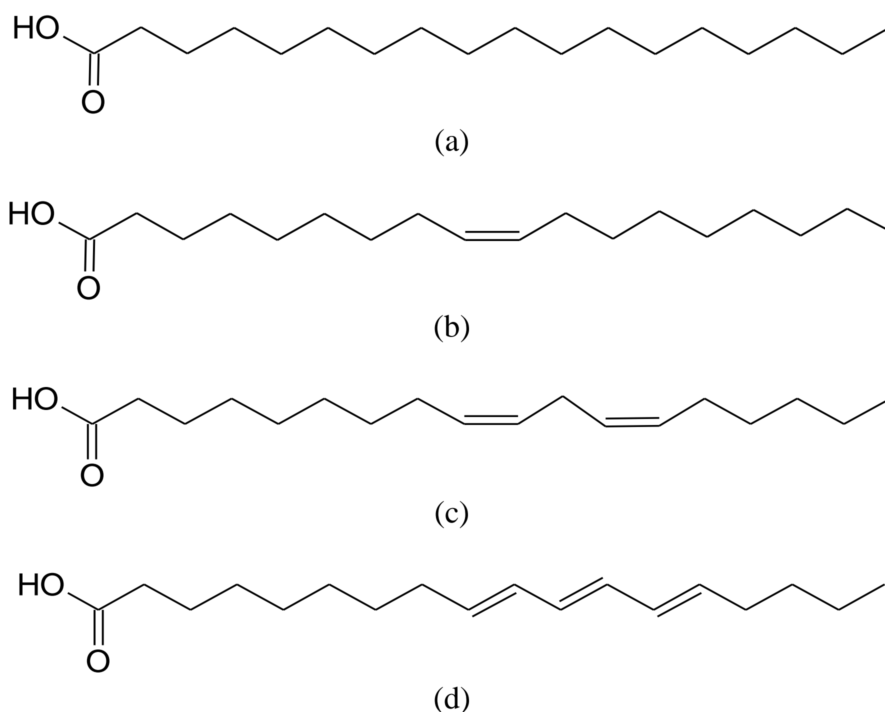


Figure 6. Structures of saturated and unsaturated fatty acids (a) Octadecanoic (stearic) acid, a saturated fatty acid, (b) (9*Z*)-octadecanoic (oleic) acid, a monounsaturated fatty acid, (c) (9*Z*,12*Z*)-9,12-octadecadienoic (linoleic) acid, a polyunsaturated, non-conjugated fatty acid, and (d) (9*E*,11*E*,13*E*)-9,11,13-octadecatrienoic (β-eleostearic) acid, a polyunsaturated, conjugated fatty acid.

A simplified nomenclature for fatty acids specifies the chain length and number of double bonds with location, separated by a colon. The positions of any double bonds are specified by superscript numbers following Δ (delta). For example, the 16-carbon saturated palmitic acid is abbreviated 16:0, and the 18-carbon oleic acid, with one double bond at C-9, is $18{:}1\Delta^{9}$; a 20 carbon fatty acid with one double bond at C-9 and another at C-12 is designated $20{:}2\Delta^{9,12}$ (Nelson & Cox, 2004). Structures and names of some naturally occurring fatty acids are shown in Table 1.

Table 1

Structure and Nomenclature of Some Naturally Occurring Fatty Acids

Carbon Skeleton	Structure	Systematic Name	Common Name
12:0	$CH_3(CH_2)_{10}COOH$	*n*-dodecanoic acid	lauric acid
14:0	$CH_3(CH_2)_{12}COOH$	*n*-tetradecanoic acid	myristic acid
16:0	$CH_3(CH_2)_{14}COOH$	*n*-hexadecanoic acid	palmitic acid
18:0	$CH_3(CH_2)_{16}COOH$	*n*-octadecanoic acid	stearic acid
20:0	$CH_3(CH_2)_{18}COOH$	*n*-eicosanoic acid	arachidic acid
24:0	$CH_3(CH_2)_{22}COOH$	*n*-tetracosanoic acid	lignoceric acid
$16{:}1(\Delta^{9})$	$CH_3(CH_2)_5{=}CH(CH_2)_7COOH$	(9*Z*)-hexadecanoic acid	palmitoleic acid
$18{:}1(\Delta^{9})$	$CH_3(CH_2)_7{=}CH(CH_2)_7COOH$	(9*Z*)-octadecanoic acid	oleic acid
$18{:}2(\Delta^{9,12})$	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	(9Z,12*Z*)-octadecadienoic acid	linoleic acid
$18{:}3(\Delta^{9,12,15})$	$CH_3CH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	(9Z,12*Z*,15*Z*)-octadecatrienoic acid	α-linolenic acid
$20{:}4(\Delta^{5,8,11,14})$	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_3COOH$	(5*Z*,8*Z*,11*Z*,14*Z*)-icosatetraenoic acid	arachidonic acid

Note. From (Nelson & Cox, 2004).

Biological Importance of Fatty Acids

Triacylglycerols constitute approximately 90% of the dietary lipid and are the major form of metabolic energy storage in humans (Voet & Voet, 2010). Triacylglycerols consist of glycerol triesters of fatty acids such as palmitic and oleic acids (Figure 7).

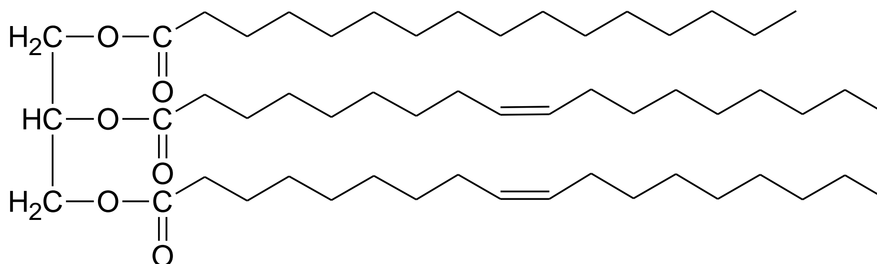


Figure 7. 1-Palmitoyl-2,3-dioleoyl-glycerol, a triacylglycerol composed of palmitic and oleic acids.

Like glucose, fatty acids can be metabolically oxidized to CO_2 and H_2O. Yet, since most carbon atoms of triacylglycerols have lower oxidation states than those of glucose, the oxidative metabolism of fats yields over twice the energy of an equal dry weight of carbohydrate or protein (Voet & Voet, 2010). Before fatty acids can be oxidized, they must be "primed" for reaction in an ATP-dependent acylation reaction to form fatty acyl-CoA and transported across the mitochondrial membrane. Once in the mitochondrial matrix, fatty acids are dismembered through the β-oxidation of the fatty acyl-CoA to produce acetyl-CoA(Voet & Voet, 2010).

Unsaturated Fatty Acid Thermal Decomposition

Unsaturated fatty acids can be subjected to conditions that promote oxidation of their unsaturated components during storage and analysis. The fatty acid alkyl chain is susceptible to oxidation both at double bonds and adjacent allylic carbons. Free-radical

and photooxidation at allylic carbons are responsible for deterioration of unsaturated fatty acids producing hydroperoxides in allylic bonds which may change the position and geometry of the double bonds (Frankel, 1984). The decomposition of lipid hydroperoxides proceeds by homolytic cleavage to form alkoxy radicals which undergo carbon-carbon cleavage to form breakdown products including aldehydes, ketones, alcohols, hydrocarbons, esters, furans, and lactones (Frankel, 1984).

Analysis of Fatty Acids by Gas Chromatography

Fatty acid composition analysis of lipids is usually performed by gas-liquid chromatography (GLC). To analyze the fatty acid composition of food lipids, the complex lipids must be pretreated so that the individual fatty acids are available for chromatographic analysis. For this purpose, fatty acids in complex lipids are converted to their corresponding methyl esters by various derivatization methods to make them more volatile for GLC analysis (Leray, 2014).

Two mechanisms are used to convert the fatty acids in a complex lipid to fatty acid methyl esters (FAMEs): methylation following hydrolysis of the fatty acids from the complex lipids or direct transesterification. The first mechanism involves base hydrolysis (saponification) (Li & Watkins, 2001) or acid-catalyzed hydrolysis (Leray, 2014) in which the ester bond is cleaved between the fatty acid and the glycerol moiety, followed by methylation performed in the presence of an acidic catalyst in methanol. Direct transesterification is usually a one-step reaction involving alkaline or acidic catalysts (Li & Watkins, 2001).

Analysis of FAMEs by GLC has been shown to have very good sensitivity, typically within the µM range. The work performed by Blanco-Gomis, Mangas Alonso,

Margolles Cabrales, & Arias Abrodo (2001) reported limits of detection (LODs) between 0.7 and 2.5 ng/µL, and the work performed by Yang, Feng, Zhao, & Li (2009) reported LODs between 1 and 12 ng/µL. A major disadvantage of GC analysis of fatty acids, however, is the use of elevated temperatures that affects thermally-labile compounds such as monounsaturated, polyunsaturated, and hydroxy fatty acids (Sun et al., 2011) by increasing the chances of oxidation to take place.

High Performance Liquid Chromatography and Fluorescence Detection

Fatty acid composition of lipids can be analyzed using high-performance liquid chromatography (HPLC). Some advantages of using HPLC include better detection limits and the use of a lower temperature during analysis, which is very important for heat-labile fatty acids and volatile (short chain) fatty acids (Leray, 2014). Positional and conformational isomers are more easily separated by HPLC than GLC. Different kinds of detectors may be used, but separations of derivatized fatty acids are usually monitored with a UV-visible or fluorescence detector (Leray, 2014).

The fluorescence detection method is known as one of the most efficient and sensitive systems for fatty acid analysis (Xie et al., 2012). Some derivatization reagents (Figure 8) such as 1-[2-(*p*-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (TSPP) (You, et al., 2007), 6-oxy-(acetyl piperazine) fluorescein (APF) (Du, Zhang, Guo, Deng, & Wang, 2007), 2-(2-(anthracen-10-yl)-1*H*-phenanthro[9,10-*d*]imidazol-1-yl)ethyl-4-methylbenzenesulfonate (APIETS) (Sun et al., 2011), 2-(11*H*-benzo[a]carbazol-11-yl)-ethyl-4-methylbenzenesulfonate (BCETS) (Li, et al., 2011), 2-(2-(anthracen-10-yl)-1*H*-naphtho[2,3-*d*]imidazol-1-yl) ethyl-*p*-toluenesulfonate (ANITS) (Fang, et al., 2007), 4-(5,6-dimethoxy-2-phthalimidinyl)phenylsulfonyl semipiperazide

(DPS-Pz) (Inoue, Ikeno, Ishii, & Tsuruta, 1998), and 1-(9H-carbazol-9-yl) propan-2-yl-methanesulfonate (CPMS) (Zhang, You, Zhou, Li, & Suo, 2012) have been used to make fluorescent derivatives of fatty acids (Figure 9).

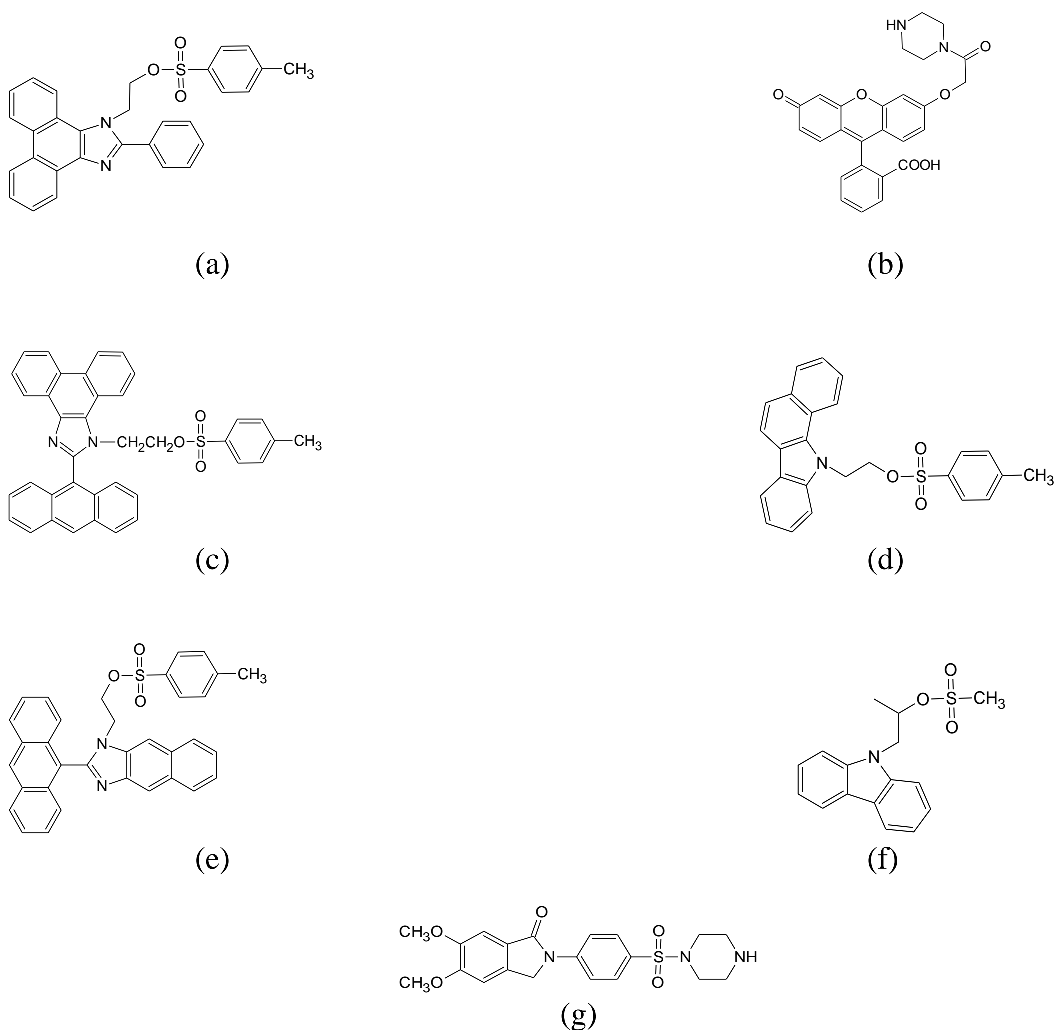


Figure 8. Fluorescent reagents used for fatty acid analysis. (a) 1-[2-(*p*-Toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (TSPP); (b) 6-oxy-(acetyl piperazine) fluorescein (APF); (c) 2-(2-(Anthracen-10-yl)-1*H*-phenanthro[9,10-*d*]imidazol-1-yl)ethyl-4-methylbenzenesulfonate (APIETS); (d) 2-(11*H*-benzo[a]carbazol-11-yl)-ethyl-4-methylbenzenesulfonate (BCETS); (e) 2-(2-(Anthracen-10-yl)-1*H*-naphtho[2,3-*d*]imidazol-1-yl) ethyl-*p*-toluenesulfonate (ANITS); (f) 1-(9H-carbazol-9-yl) propan-2-yl-methanesulfonate (CPMS); (g) 4-(5,6-dimethoxy-2-phthalimidinyl)phenylsulfonyl semipiperazide (DPS-Pz)

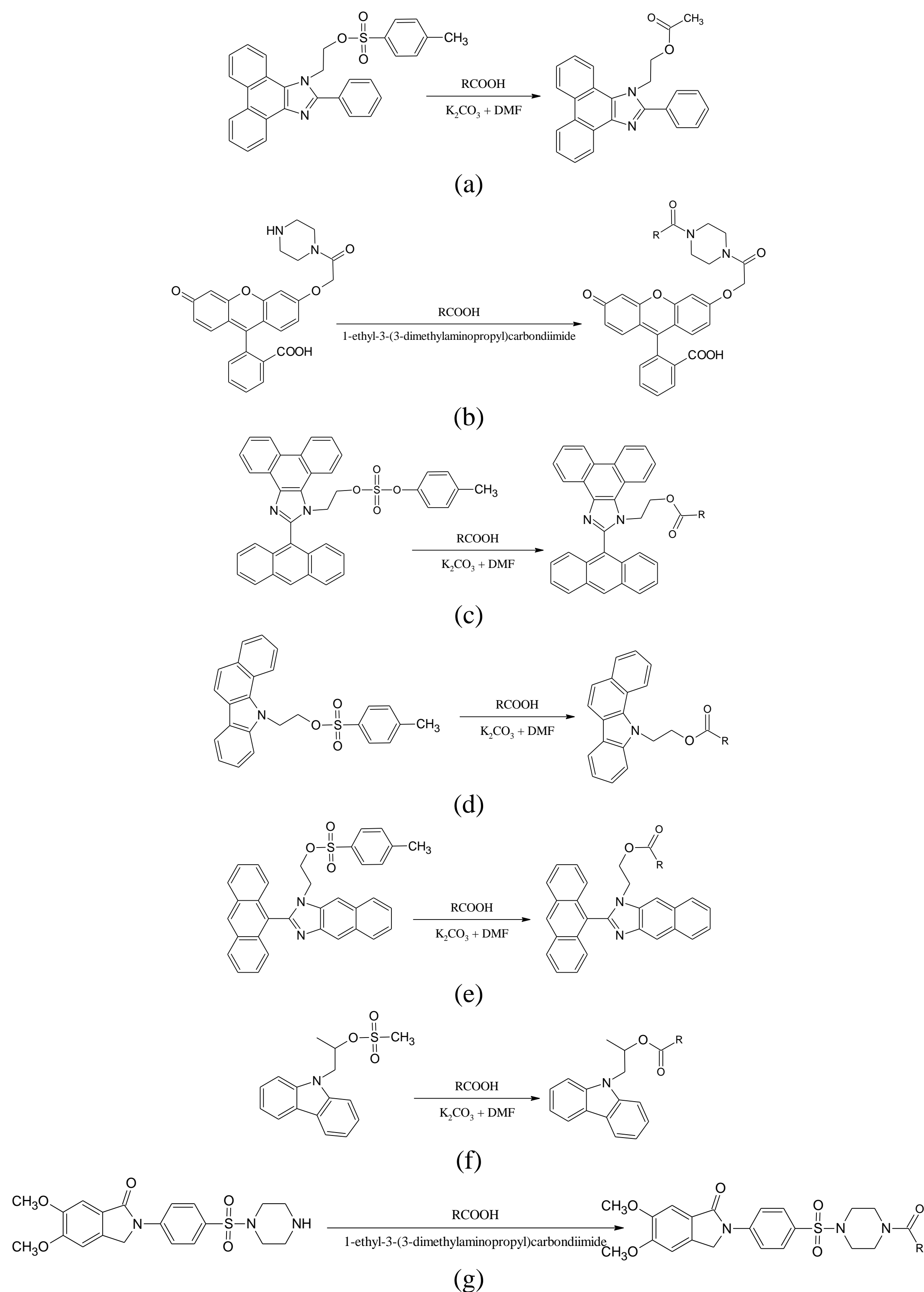


Figure 9. Fatty acid derivatization reactions with different fluorescent reagents. (a) Derivatization with TSPP; (b) derivatization with APF; (c) derivatization with APIETS; (d) derivatization with BCETS; (e) derivatization with ANITS; (f) derivatization with CPMS; (g) derivatization with DPS-Pz.

Description of the chromatographic conditions used for analysis of fatty acids with TSPP, APF, APIETS, BCETS, ANITS, CPMS and DPS-Pz as the fluorescent tags are shown in Table 2.

Table 2

Conditions for Previously Studied Fluorescent Tags for Fatty Acid Analysis

Fluorescent tag	Column	Detector $\lambda_{ex}/\lambda_{em}$	Mobile phase
TSPP	Reversed-phase Eclipse XDB-C8 (150 × 4.6 mm, 5 µm packing)	260 nm/380 nm	Eluent A: 100% acetonitrile Eluent B: 50% acetonitrile-50% dimethylformamide
APF	Reversed-phase C18 (250 × 4.6 mm, 5 µm packing)	467 nm/512 nm	Eluent A: 100% methanol Eluent B: 100% water
APIETS	Reversed-phase SunFire Prep-C18 (150 × 10 mm, 10 µm packing)	315 nm/435 nm	Eluent A: 30% acetonitrile in water Eluent B: 100% acetonitrile
BCETS	Reversed-phase Eclipse XDB-C8 (150 × 4.6 mm, 5 µm packing)	333 nm/390 nm	Eluent A: 100% water Eluent B: 50% acetonitrile-50% dimethylformamide Eluent C: 100% acetonitrile
ANITS	Reversed-phase Eclipse XDB-C8 (150 × 4.6 mm, 5 µm packing)	250 nm/512 nm	Eluent A: 50% acetonitrile in 30 mM ammonium formate Eluent B: 100% acetonitrile
CPMS	Reversed-phase Hypersil BDS C8 (200 × 4.6 mm, 5 µm packing)	293 nm/360 nm	Eluent A: 5% acetonitrile in water Eluent B: 100% acetonitrile
DPS-Pz	Reversed-phase Symmetry C8 (150 × 3.9 mm, 5 µm packing)	317 nm/380 nm	Eluent A: 100% 10 mM phosphoric acid Eluent B: 100% acetonitrile

9-Chloromethyl-anthracene and 2-bromomethyl-anthraquinone are two other compounds that can be used as chromophoric tags. 9-Chloromethyl-anthracene (Figure 2a) has been used for the analysis of short chain carboxylic acids (Xie et al., 2012) with

detection limits between 0.18 and 2.53 nM; however, it has not been used for the analysis of long chain fatty acids. 2-Bromomethyl-anthraquinone (Figure 2b) has not been reported for its use as a chromophoric tag for the analysis of fatty acids.

Application of Fluorescence Detection to Prostaglandins

Prostaglandins (Figure 10) are cyclopentanoic acids (eicosanoids) derived from arachidonic acid (Garrett & Grisham, 2013). Prostaglandins were discovered in Sweden in the 1930s by Ulf von Euler. The name prostaglandin was given to them because von Euler made his discovery when studying human prostate glands. Prostaglandins, however, are synthesized in the seminal vesicles, and similar substances are synthesized in most animal tissues (male and female) (Garrett & Grisham, 2013).

The analysis of prostaglandins is of importance because they are responsible for certain inflammatory responses, smooth muscle constriction, vasoconstriction, and vasodilation (Garrett & Grisham, 2013). Prostaglandins, are difficult to analyze because they can be present in the human body at extremely low levels. Prostaglandin $E_{2\alpha}$ ($PGE_{2\alpha}$) is present in human serum at levels of less than 10^{-14} M (Garrett & Grisham, 2013); therefore, being able to detect the presence of prostaglandins requires the use of a highly sensitive method of detection.

Current methods of prostaglandins analysis include liquid chromatography with mass spectrometry detection (LC-MS) such as the work performed by Golovko & Murphy (2008) and Taylor, Bruno, Frei, & Traber (2006) and liquid chromatography with UV-vis detection such as the work performed by Hsu, Tsao, Chiou, Hwang, & Hwang (2007). Analysis of prostaglandins can possibly also be performed using HPLC

with fluorescence detection. The presence of a carboxylic acid group (Figure 10); allows for derivatization with 9-chloromethyl-anthracene.

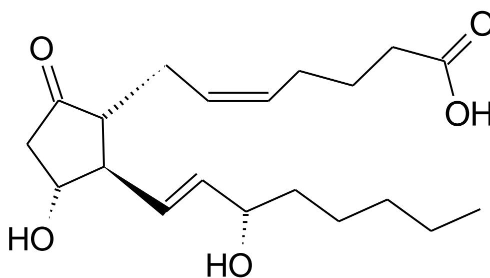


Figure 10. Structure of prostaglandin $E_{2\alpha}$ ($PGE_{2\alpha}$). The presence of the carboxylic acid group allows for derivatization with 9-CMA, making the prostaglandin detectable by fluorescence.

Summary

Fatty acids found in complex lipids can be extracted and derivatized for their analysis by GLC or HPLC, both of which offer sensitive and reliable results. However, due to the possible thermal decomposition of unsaturated fatty acids by analysis with GLC, the development of an HPLC method was studied. The analysis of fatty acids with HPLC was done with the use of fluorescence detection employing 9-chloromethyl-anthracene, or with the use of UV detection employing 2-bromomethyl-anthraquinone as the chromophoric tags.

CHAPTER III

MATERIALS AND METHODS

Materials

Reagents

All reagents employed were analytical grade or higher purity. All solutions were prepared in HPLC-grade acetonitrile (catalog number 300000000) purchased from Pharmco-Aaper. High performance liquid chromatography-grade methanol (339000000) used for HPLC startup and shutdown methods was purchased from Pharmco-Aaper. High performance liquid chromatography-grade water was produced using a Milli-Q® A10 water purification system, herein referred to as HPLC-grade water. The following reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO): triethanolamine (90279), tetrabutylammonium bromide (426288), 9-chloromethyl-anthracene (196517), 2-bromomethyl-anthraquinone (444650), benzoic acid (242381) tridecanoic acid (T0502), hexadecanoic (palmitic) acid (P-5585), octadecanoic (stearic) acid (17536-6), (9*Z*)-octadecanoic (oleic) acid (75090), (9*Z*,12*Z*)-octadecadienoic (linoleic) acid (23392), and (9*Z*,12*Z*,15*Z*)-octadecatrienoic (linolenic) acid (62160). Acetic acid (A38C-212), potassium hydroxide (UN1813) and concentrated hydrochloric acid (A144CSI-212) were purchased from Fisher Scientific, and formic acid was

purchased from Mallinckrodt (2592-05). Cloprostenol was obtained from Dr. Patrick Burns at the University of Northern Colorado School of Biological Sciences.

Instrumentation and Equipment

The following Shimadzu (Tokyo, Japan) HPLC components were used for all HPLC analyzes: LC-10AT liquid chromatograph with a SCL-10A system controller, DGU-14A degasser, SPD-M10A diode array detector, and RF-10AXL fluorescence detector. All chromatograms were generated and analyzed using the CLASS-VP 7.2.1 SP1 software program. An Ohaus Explorer® Pro analytical balance was used for all mass measurements. A Fisher Scientific Accumet® 925 pH meter was used for the pH measurements of sample solutions; a three-point calibration was performed using buffers at pH 4.0 (SB 101-500), 7.0 (SB-107-500) and 10.0 (SB-115-500) obtained from Fisher Scientific. A Symphony VWR oven was used to dry all glassware and to facilitate the derivatization procedure. An IEC Model CL International Clinical Centrifuge was used to centrifuge reaction vials prior to sample injection. An F-2500 Hitachi fluorescence spectrophotometer was used to determine the excitation and emission wavelengths of 9-CMA and an Agilent 8453 diode array spectrophotometer was used to determine the λ_{max} of MAQ-Br.

Methods

Preparation of Derivatization Solutions

Methanolic potassium hydroxide (methanolic-KOH) (1.00 M). For the preparation of 1000-mL, 56.11-g potassium hydroxide (KOH) was added to a 1000-mL volumetric flask, dissolved, and diluted to volume with water.

Hydrochloric acid (HCl) (1.00 M). For the preparation of 1000-mL, 82.5-mL concentrated HCL were measured in a graduated cylinder, added to a 1000-mL volumetric flask and diluted to volume with water.

9-Chloromethyl-anthracene (9-CMA) (1.00 mM). For the preparation of 100-mL, 0.0227-g 9-CMA was added to a 100-mL volumetric flask, dissolved, and diluted to volume with HPLC-grade acetonitrile. The solution was stored at 4 °C until use.

2-Bromomethyl-anthraquinone (MAQ-Br) (1.00 mM). For the preparation of 100-mL, 0.0301-g MAQ-Br was added to a 100-mL volumetric flask, dissolved, and diluted to volume with HPLC-grade acetonitrile. The solution was stored at 4 °C until use.

Tetrabutylammonium bromide (TBAB) (20.0 mM). For the preparation of 100-mL, 0.645-g tetrabutylammonium bromine was added to a 10-mL volumetric flask, dissolved, and diluted to volume with HPLC-grade acetonitrile. The solution was stored at 4 °C until use.

Triethanolamine (TEA) (5% v/v). For the preparation of 100-mL, 5.0 mL triethanolamine was added to a 100-mL volumetric flask and diluted to volume with HPLC-grade acetonitrile. The solution was stored at 4 °C until use.

Preparation of Fatty Acid Standards

Acetonitrile/dimethylformamide (ACN/DMF) 1:1(v/v) solvent. For the preparation of 1000-mL, 500-mL dimethylformamide were added to 500-mL acetonitrile.

Tridecanoic acid standard (1.00 mM). For the preparation of 100-mL, 0.0214-g tridecanoic acid was added to a 100-mL volumetric flask, dissolved, and diluted to

volume with HPLC-grade acetonitrile/dimethylformamide. The solution was stored at 4 °C until use.

Palmitic acid standard (1.00 mM). For the preparation of 100-mL, 0.0256-g tridecanoic acid was added to a 100-mL volumetric flask, dissolved, and diluted to volume with HPLC-grade acetonitrile/dimethylformamide. The solution was stored at 4 °C until use.

Stearic acid standard (1.00 mM). For the preparation of 100-mL, 0.0284-g stearic acid was added to a 100-mL volumetric flask, dissolved and diluted to volume with HPLC-grade acetonitrile/dimethylformamide. The solution was stored at 4 °C until use.

Oleic acid standard (1.00 mM). For the preparation of 100-mL, 0.0282-g oleic acid was added to a 100-mL volumetric flask, dissolved, and diluted to volume with HPLC-grade acetonitrile/dimethylformamide. The solution was stored at 4 °C until use.

Linoleic acid standard (1.00 mM). For the preparation of 100-mL, 0.0280-g linoleic acid was added to a 100-mL volumetric flask, dissolved, and diluted to volume with HPLC-grade acetonitrile/dimethylformamide. The solution was stored at 4 °C until use.

Linolenic acid standard (1.00 mM). For the preparation of 100-mL, 0.0278-g linolenic acid was added to a 100-mL volumetric flask, dissolved, and diluted to volume with HPLC-grade acetonitrile/dimethylformamide. The solution was stored at 4 °C until use.

Combined fatty acid standards stock solution (1.00 mM). For the preparation of 100-mL, the previously stated mass measurements for each individual fatty ac id

standard were added to a 100-mL volumetric flask, dissolved, and diluted to volume with HPLC-grade acetonitrile/dimethylformamide. The solution was stored at 4 °C until use.

Preparation of Cloprostenol Standard

For the preparation of 50-mL, 1.0-mL of the provided 0.5884 mM solution was pipetted to a 50-mL volumetric flask and diluted to volume with HPLC-grade acetonitrile. The solution was stored at 4 °C until use.

Calibration Curve Standards

Eight standards with concentrations of 0.1, 0.3, 0.7, 1.0, 3.0, 5.0, 7.0 and 10.0 µM were prepared by performing the appropriate dilutions using the 1.00 mM combined fatty acid stock solution and diluting with the HPLC-grade acetonitrile/dimethylformamide.

Chromatographic System

Shimadzu HPLC components were used for all HPLC analyses, and a Zorbax HPLC reverse-phase column, Rx-C8 (150 mm × 4.6 mm, 4.6 µm particle size) was used for all routine analyses. The following mobile phase (solvent) configuration was used for all analyses: solvent line A-HPLC-grade water, solvent line B-HPLC-grade acetonitrile, solvent line C-HPLC-grade water, and solvent line D-HPLC-grade methanol.

Saponification and Extraction Procedure

In a 13 × 100 mm screw cap vial, no more than approximately 0.1 g of sample was added followed by the addition of 1.0 mL 1.00 M methanolic-KOH. The vial was sealed using a Teflon lined screw cap and allowed to react at 80 °C for 30 min in the VWR oven. Upon completion of the reaction, the contents of the vial were allowed to cool to room temperature. The contents of the vial were acidified with 1.00 M HCL to

approximately pH 3 (measured with pH paper). The free fatty acids were extracted three times using 5.0 mL of n-hexane for each extraction. The combined n-hexane extracts were dried over anhydrous $MgSO_4$. The n-hexane was evaporated leaving the free fatty acids ready for derivatization.

Derivatization Procedures

Derivatization with 9-chloromethyl-anthracene. In a 10-mL volumetric flask, depending on the desired concentration, the appropriate volume of standard fatty acid was added and diluted to volume with the 1.00 mM 9-CMA solution. The mixed standard fatty acid and 9-CMA were then transferred to a 13 × 100 mm screw cap vial to which 0.5 g K_2CO_3 were added. The vial was sealed with a Teflon lined screw cap and allowed to react in the VWR oven at 85 °C for 35 min with shaking at 5-min intervals. The derivatization reaction is shown in Figure 11. After the reaction was completed, the mixture was allowed to cool to room temperature, and then centrifuged for 5-min in a centrifuge. The supernatant was transferred to a clean 13 × 100 mm vial and stored at -20 °C until analysis.

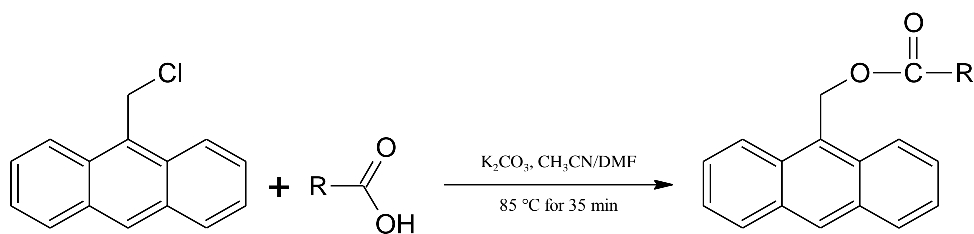


Figure 11. Derivatization reaction with 9-chloromethyl-anthracene.

Derivatization with 2-bromomethyl-anthraquinone. The derivatization of the standard fatty acids was performed with 2-bromomethyl-anthraquinone using the same steps as those for 9-chloromethyl-anthracene (see previous section). The derivatization reaction is shown in Figure 12.

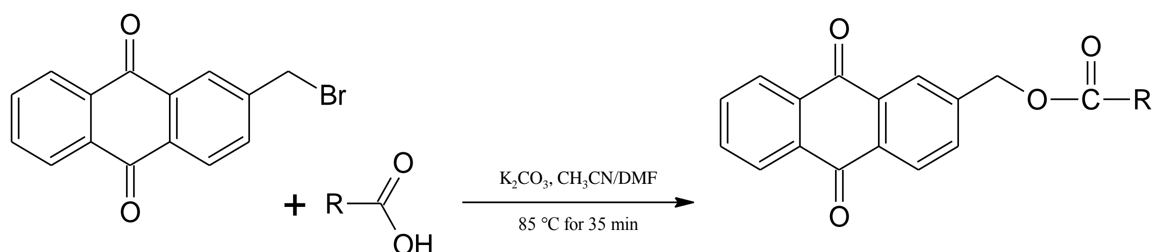


Figure 12. Derivatization reaction with 2-bromomethyl-anthraquinone.

High Performance Liquid Chromatography
Separation and Analysis

The chromatographic system parameters for sample analysis are given in Table 3 and illustrated in Figure 13. The fluorescence detector excitation/emission wavelengths were maintained at 365/410 nm for 9-CMA (Xie et al., 2012) and the UV detector wavelength range was maintained from 250 to 280 nm for MAQ-Br; all other settings were the program defaults.

Table 3

HPLC Mobile Phase Solvent Composition and Pump Parameters for the Assay Method

Time (min)	Module	Action	Value
5.00	Pumps	Solvent B Conc.	82.0%
16.00	Pumps	Solvent B Conc.	82.0%
17.00	Pumps	Solvent B Conc.	80.0%
20.00	RF-10Axl (Det. A)	Zero	
25.00	Pumps	Solvent B Conc.	80.0%
28.00	Pumps	Solvent B Conc.	85.0%
44.00	Pumps	Solvent B Conc.	85.0%
50.00	Pumps	Solvent B Conc.	100.0%
55.00	Pumps	Solvent B Conc.	100.0%
60.00	Pumps	Solvent B Conc.	50.0%
60.00	Controller	Stop	

Note. The pump mobile phase solvent configuration was: solvent line A – HPLC-grade water, solvent line B – HPLC acetonitrile, solvent line C – HPLC-grade water, and solvent line D – HPLC-grade methanol. The initial solvent composition was (A) 50%, (B) 50%, (C) 0%, and (D) 0% at 1.000 mL/min.

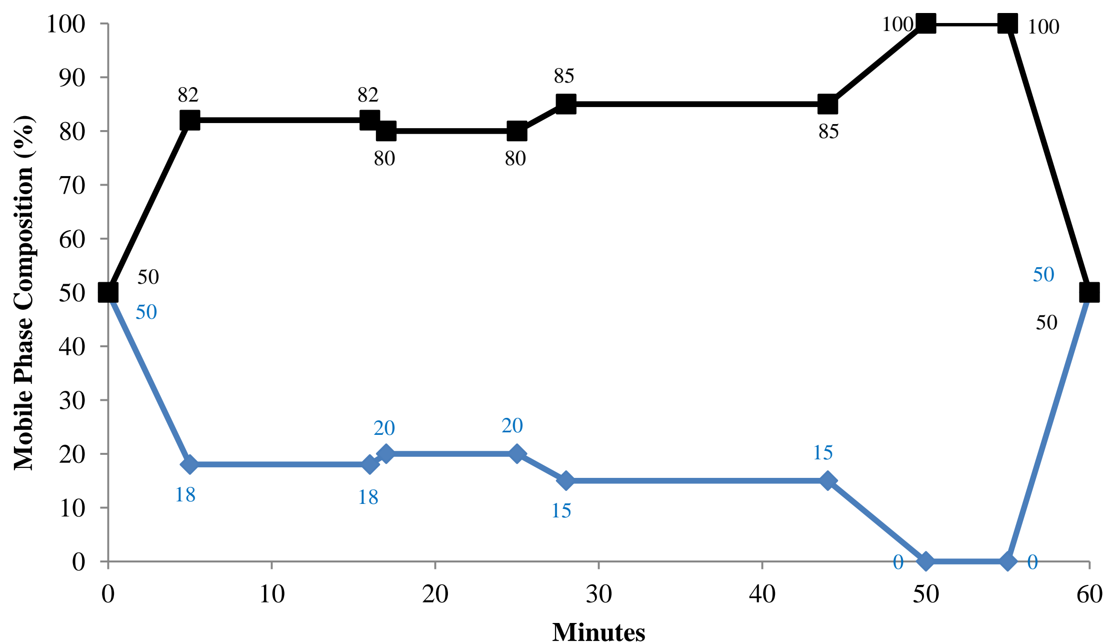


Figure 13. Mobile phase composition profile for the assay. For this method, the initial mobile phase composition and the final mobile phase composition were (A) 50%, (B) 50%, (D) 0%, and (D) 0% flowing at 1.000 mL/min: (♦-HPLC-grade water, ■-HPLC-grade acetonitrile).

CHAPTER IV

RESULTS AND DISCUSSION

The goal of this research was to develop an HPLC-based method suitable for the analysis of fatty acids using 9-chloromethyl-anthracene or 2-bromomethyl-anthraquinone as fluorescence or UV tags, respectively. The fatty acid standards and biological sample were successfully derivatized using both 9-CMA and MAQ-Br.

Derivatization and High Performance Liquid Chromatography Methods Development

The derivatization and separation methods were developed simultaneously. Since data obtained by Xie et al. (2012) suggested a successful method of derivatization, the derivatization and separation methods were attempted simultaneously without major modifications. However, since their methods were not reproducible, a few modifications had to be made in order to obtain successful results.

Derivatization with 9-Chloromethyl-Anthracene (Original Method)

The method developed by Xie et al. (2012) was utilized first for method validation and analysis of fatty acids. The method of derivatization utilized triethanolamine (TEA) as the base, tetrabutylammonium bromide (TBAB) as the phase transfer catalyst, and 9-CMA as the derivatization reagent (Figure 14).

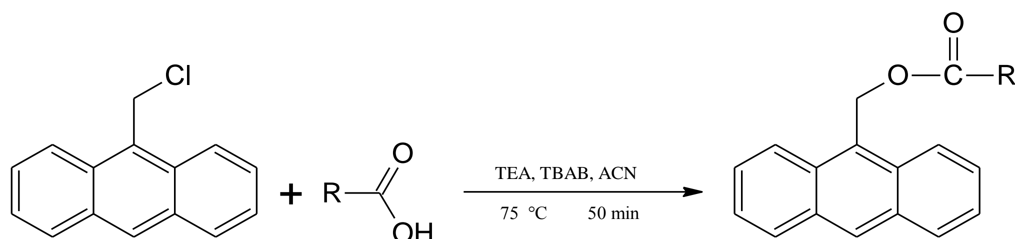


Figure 14. Original derivatization method. Attempts to derivatize carboxylic acid standards were made following this procedure in which TEA, TBAB, and 9-CMA were added and heated at 75 °C for 50 min.

Multiple attempts were made to replicate the derivatization and separation achieved by Xie et al. (2012) using similar standards and separation parameters (Table 4, first protocol). Their analysis was performed using a Heweltt Packard RP-18 column (200 × 4.6 mm, 5 μm packing) with an isocratic mobile phase using 64% acetonitrile and 36% water at 1.0 mL/min. To determine the identity of any present peaks, a reaction "blank" was analyzed (Figure 15). The reaction blank contained everything used for the derivatization reaction except for the short chain acids. The peak at approximately 5 min was determined to be the 9-CMA peak and the peak at approximately 14 min showed what seemed to be successful results; however, the results were not successfully reproduced. After multiple unsuccessful attempts to obtain reproducible data using short chain acids (Figure 16), the same derivatization procedure was used with a palmitic acid standard, and a cooking oil sample due to the high abundance of fatty acids expected to be present. Figures 17 and 18 show the unsuccessful attempts to derivatize palmitic acid and the extracted fatty acids from canola oil, respectively.

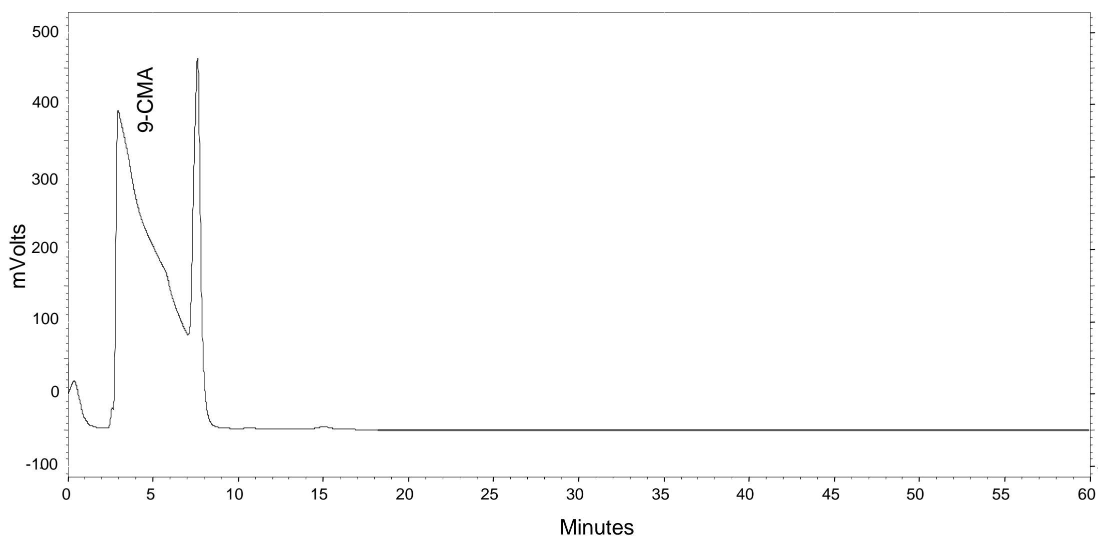


Figure 15. 9-CMA reaction blank.

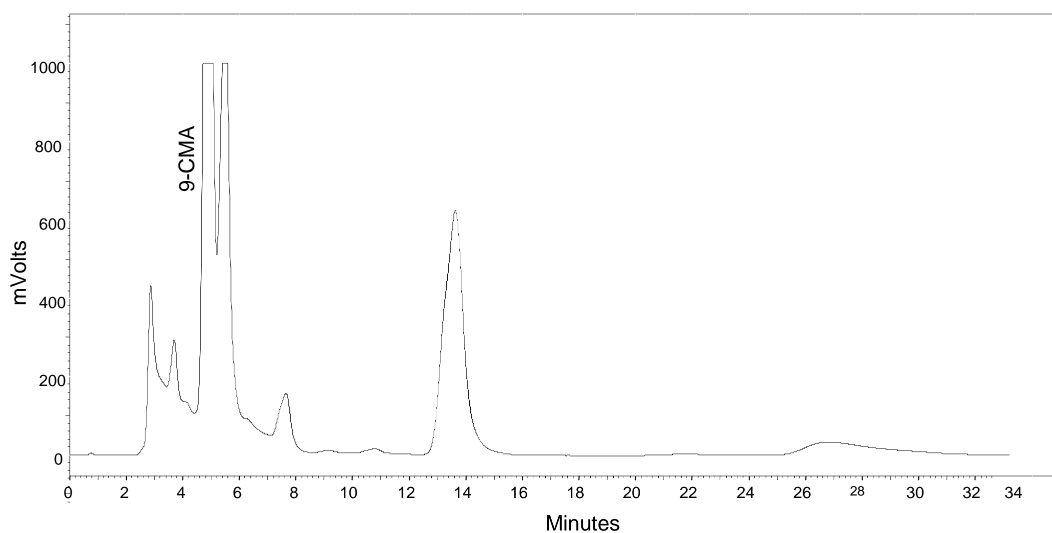


Figure 16. HPLC chromatogram of formic, acetic, and benzoic acid derivatives. The original method developed by Xie et al. (2012) was utilized for method validation/development with unsuccessful results.

The peak at approximately 7.7 min in Figure 17 was believed to be the palmitic acid derivative since no other peaks were present for the total run time. However, after a 9-CMA blank run, it was determined that the peak at approximately 7.7 min was part of the derivatization reagent peaks.

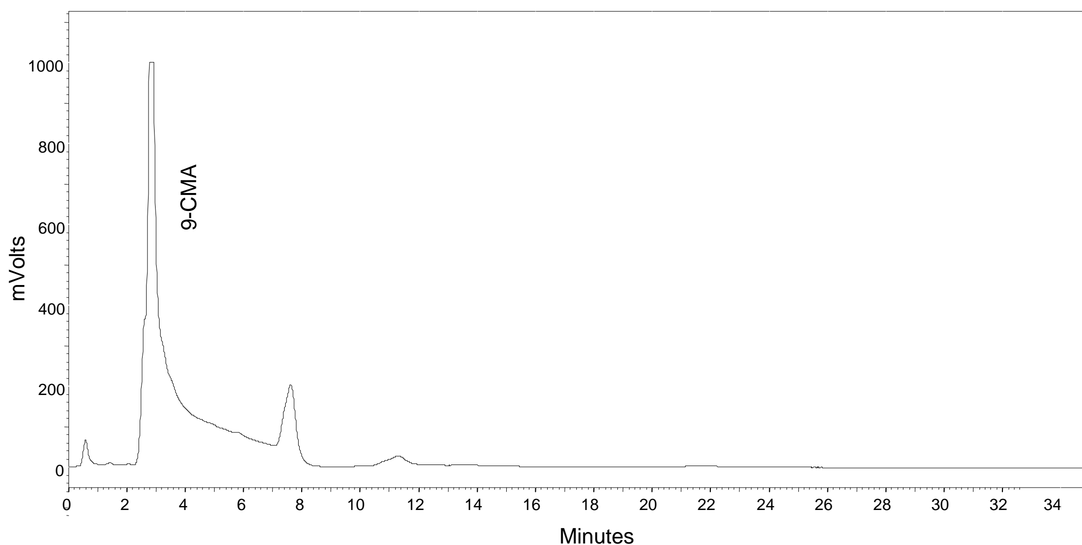


Figure 17. HPLC chromatogram of the palmitic acid derivative. The original method developed by Xie et al. (2012) was utilized in attemts to derivatize and analyze palmitic acid with unsuccessful results.

No peaks were found for the attempted derivatization of the fatty acids extracted from canola oil. After allowing for a total run time of 75 min, the absence of prominent peaks led to the decision to use a different derivatization procedure since there had been no successful results.

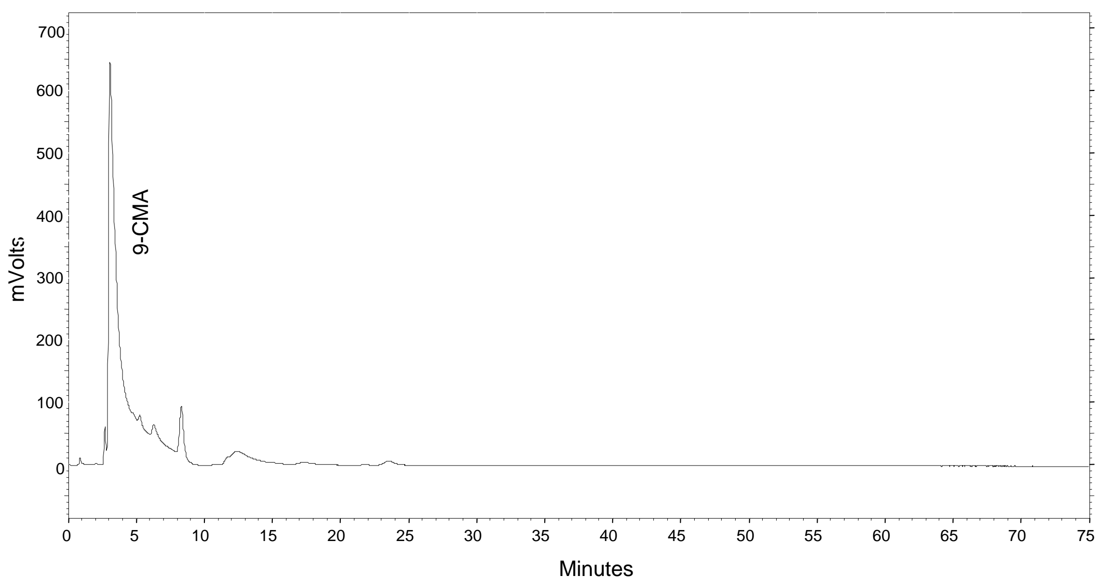


Figure 18. HPLC chromatogram of canola oil fatty acid derivatives. The original method developed by Xie et al. (2012) was utilized to derivatize the fatty acids extracted from canola oil with unsuccessful results.

Derivatization with 9-Chloromethyl-Anthracene (Modified Method)

The method developed by Sun et al. (2011) was utilized in combination with the method developed by Xie et al. (2012) using K_2CO_3 as the base, no phase transfer catalyst, and 9-CMA as the derivatization reagent (Figure 19).

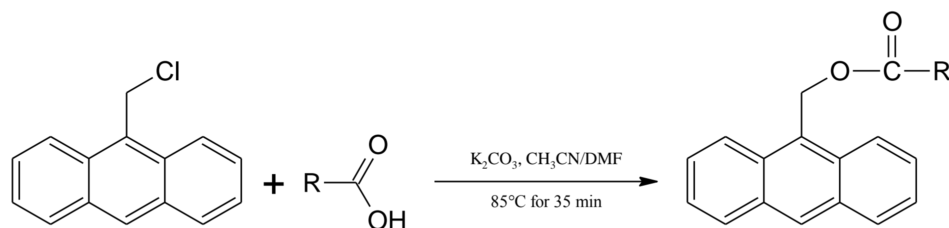


Figure 19. Modified derivatization method.

Palmitic acid was the first fatty acid standard utilized to test the modified method. In this procedure, K_2CO_3 was added in combination with 9-CMA and the mixture was heated at 85 °C for 35 min with shaking in 5 min intervals. Analysis of the products of the reaction with an isocratic mobile phase composed of 80% acetonitrile and 20% water gave the chromatogram in Figure 20 in which the peak at approximately 12 min is the palmitic acid derivative. The analysis was allowed to run for 60 min to verify that there were no other peaks. To verify that the peak at approximately 12 min was the palmitic acid derivative, a reaction containing all the reagents for the derivatization step, except for palmitic acid was performed. After confirming that the peak was the palmitic acid derivative, triplicate runs were performed to verify reproducibility.

Further analysis of the method was performed by derivatizing stearic acid and analyzing it (Figure 21) to verify that the method could be applied to a different fatty acid. The stearic acid derivative was eluted at approximately 18.5 min, but the analysis was allowed to run for 60 min to verify that there were no other peaks present.

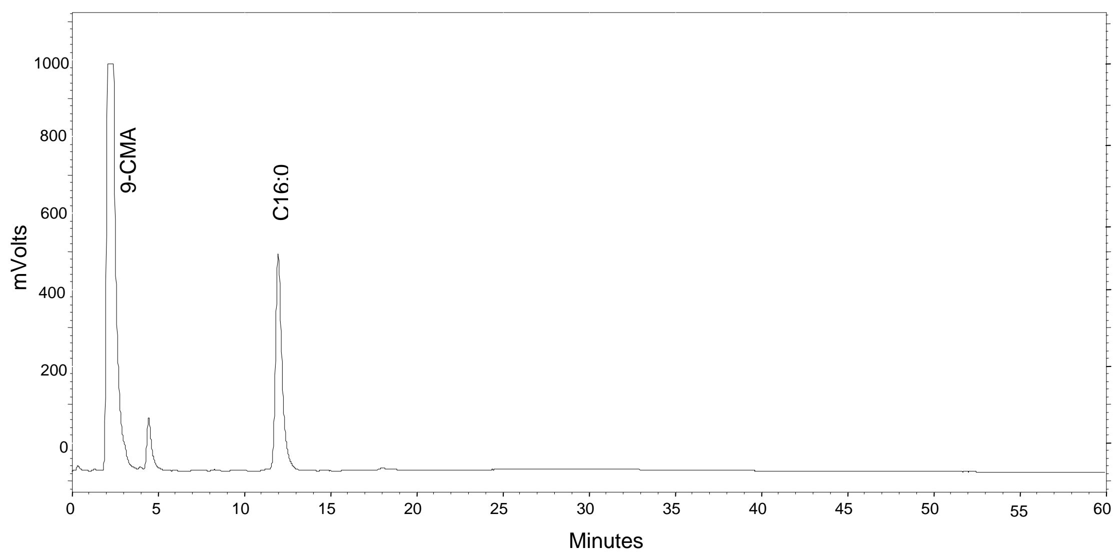


Figure 20. HPLC chromatogram of the palmitic acid derivative using the modified method.

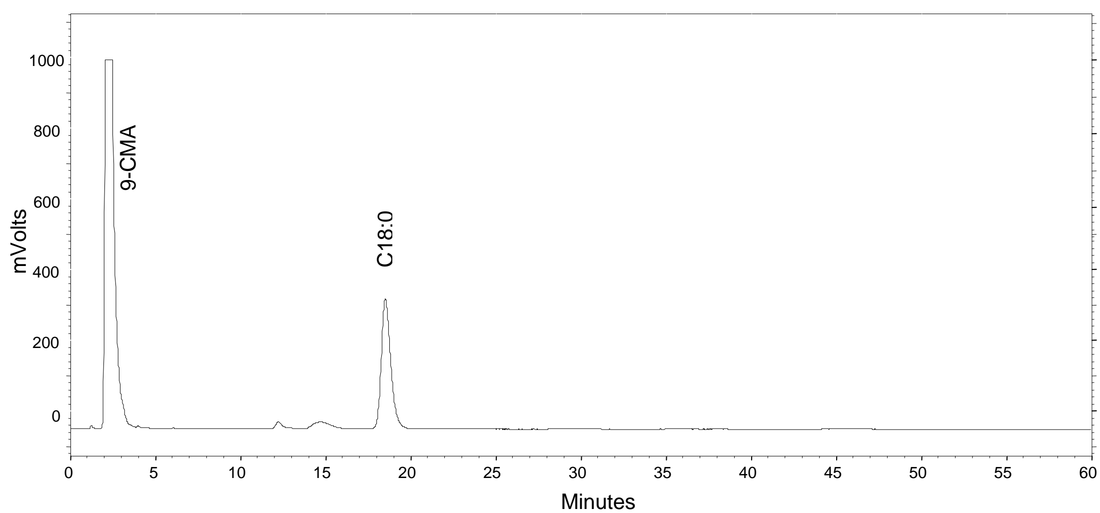


Figure 21. HPLC chromatogram of the stearic acid derivative using the modified method.

To further validate the derivatization method, all six fatty acid standards were derivatized and analyzed in order to optimize the separation (Figure 22). Using a mobile phase composition of 90% acetonitrile and 10 % water, and analyzing individually derivatized fatty acids, each peak was identified. The elution for the derivatives was approximately 8.2 min for tridecanoic acid derivative, 8.6 min for linolenic acid derivative, 11.4 min linoleic acid derivative, 15.0 min for palmitic acid derivative, 16.1 min for oleic acid derivative and 23.8 min for stearic acid derivative.

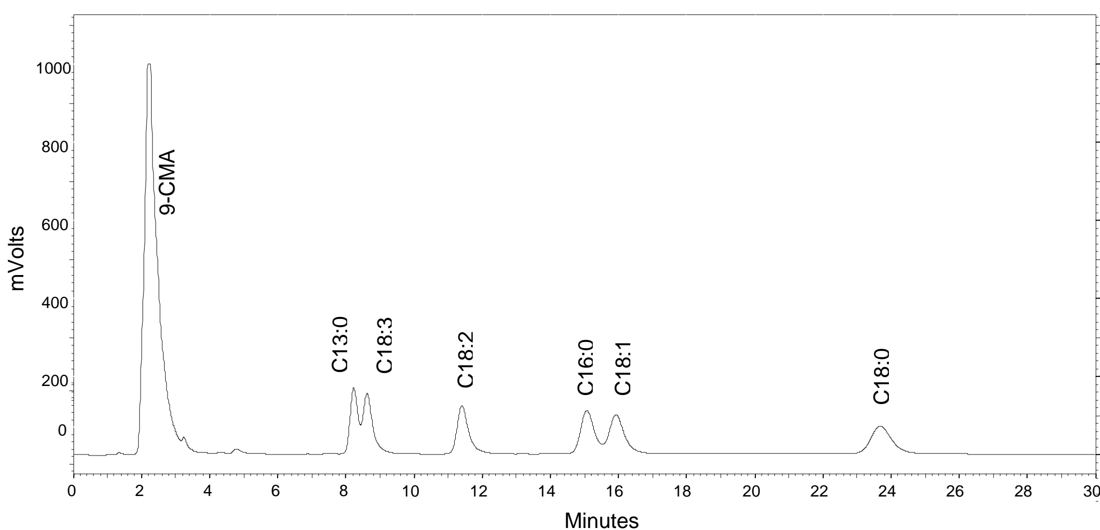


Figure 22. HPLC chromatogram of six fatty acid derivatives.

Separation of 9-Chloromethyl-
Anthracene Derivatives

The development of the separation method with HPLC started by using the 9-CMA derivatized tridecanoic, palmitic, stearic, oleic, linoleic and linolenic acids. A literature search offered several protocols, including and most importantly, the protocols used by Xie et al. (2012) and Sun et al. (2011). This provided a starting point for experimentation with the available column types, mobile phase compositions, mobile phase conditions, and flow rates (Table 4).

Table 4

Experimental Chromatographic Protocols

Column type	Mobile phase composition	Isocratic or gradient	Flow rate (mL/min)	Comments	Ref.
TosoHaas ODS-120T (C18) 100 mm x 4.6 mm particle size 5 µm	64 % acetonitrile, 36 % water	Isocratic and gradient	1.00	Run times of up to 1.5 hr	1
TosoHaas ODS-120T (C18) 150 mm x 2.0 mm particle size 5 µm	80% acetonitrile, 20% water	Isocratic and gradient	0.5	Run times of up to 1.5 hr	1
TosoHaas ODS-80TM (C18) 100 mm x 4.6 mm particle size 5 µm	80% acetonitrile, 20% water	Isocratic	1.00	Run times of up to 1.5 hr	1
TosoHaas CN-80TS 150 mm x 4.6 mm particle size 5 µm	30% acetonitrile, 70% water	Isocratic and gradient	1.00	No separation of peaks achieved. All peaks eluted in less than 10 min.	1 2
Zorbax Rx(C8) 150 mm x 4.6 mm particle size 5 µm	50% acetonitrile, 50% water	Gradient	1.00	Successful separation, good resolution	2
Supelco C8 150 mm x 4.6 mm particle size 5 µm	50% acetonitrile, 50% water	Gradient	1.00	Successful separation, good resolution	2

Note. Protocols were used to determine the optimal column type, mobile phase, and solvent combinations for the separation of the fatty acid standards.
1. Modification of the method of Xie et al. (2012).
2. Modification of the method of Sun et al. (2011).

The first protocol used a TosoHaas ODS-120T (100 × 4.6 mm, particle size 5 µm) column, an isocratic mobile phase of 64% acetonitrile and 36% water at a flow rate of 1.0 mL/min. The excessively long run times led to the use of gradient elution in attempts to reduce retention times to less than 60 min. Changing to a gradient elution reduced the analysis time to less than 60 min. However, this column was not used because the elution time for tridecanoic acid was very long with a retention time of approximately 35 min as

shown in Figure 23. In order to elute in a shorter period of time, the percentage of acetonitrile had to be increased drastically, which in turn led to peaks overlapping.

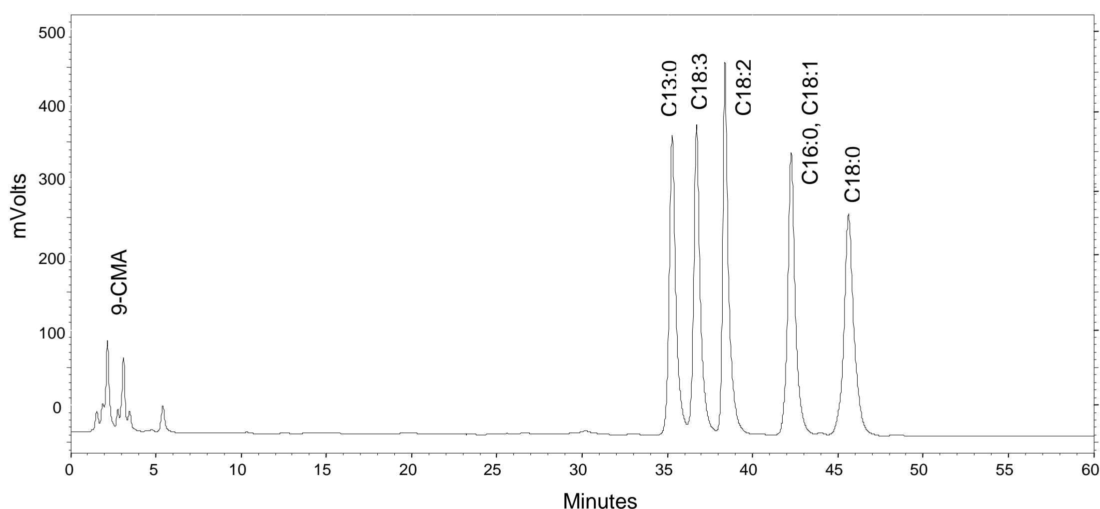


Figure 23. HPLC chromatogram of six fatty acid derivatives using the first protocol. This chromatogram was obtained using gradient elution with a TosoHaas ODS-120T (100 × 4.6 mm, particle size 5 μm) column. The peak at 35 min is the tridecanoic acid derivative.

The second protocol used a TosoHaas ODS-120T (150 × 2.0 mm, particle size 5 μm) column, an isocratic mobile phase of 64% acetonitrile and 36% water at a flow rate of 1.0 mL/min. The relatively long run time led to the use of gradient elution in an attempt to reduce retention times to less than 60 min. As seen in Figure 24, the use of a gradient in which the percentage of acetonitrile and the relatively high flow rate led to a total run time of less than 30 min but with some serious overlap. The peak at approximately 15 min is both the tridecanoic and linolenic acid derivatives, and the peaks centered at approximately 23 min are the palmitic and oleic acid derivatives. This was verified by analyzing each of the individual fatty acid derivatives.

At this point, the use of a TosoHaas ODS-120T (150 × 2.0 mm, particle size 5 μm) column seemed like a good option if an acceptable separation protocol could be

developed. Before the decision was made to use the TosoHaas ODS-120T (150 × 2.0 mm, particle size 5 μm) column, a third, fourth, fifth and sixth protocol were tested.

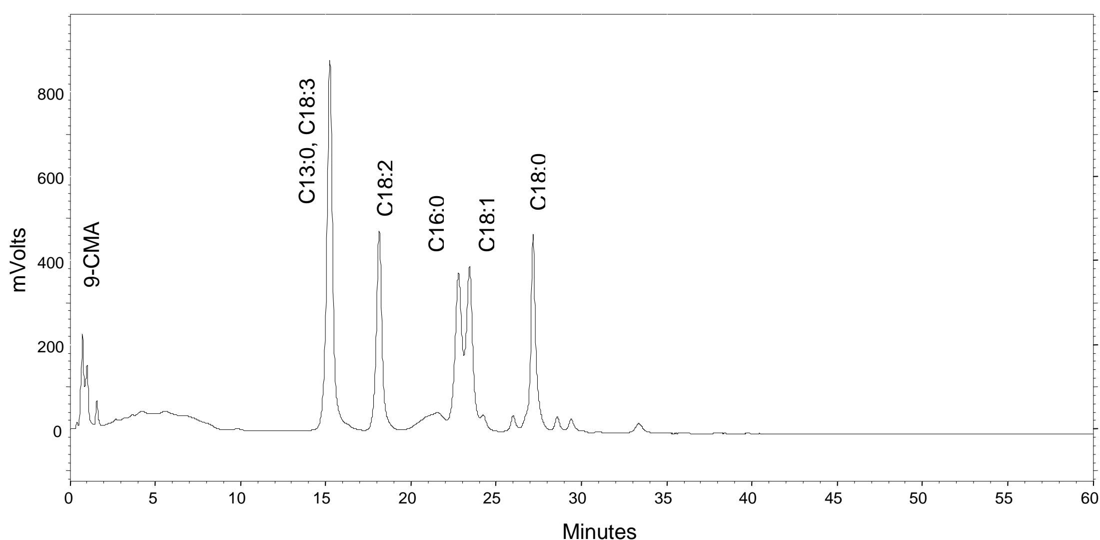


Figure 24. HPLC chromatogram of six fatty acid derivatives using the second protocol. This chromatogram was obtained using gradient elution with a TosoHaas ODS-120T (150 × 2.0 mm, particle size 5 μm) column.

The third protocol used a TosoHaas ODS-80TM (100 × 4.6 mm, particle size 5 μm) column, an isocratic mobile phase of 64% acetonitrile and 36% water at a flow rate of 1.0 mL/min. The relatively long run times led to the use of gradient elution in an attempt to reduce retention times to less than 60 min. The use of gradient elution improved the retention times by having all peaks eluted in less than 60 min. However, this column was not used because the elution time for tridecanoic acid was very long with a retention time of approximately 33 min as seen in Figure 25. The peak at approximately 33.5 min is both tridecanoic and linolenic acids, and the peak at approximately 40 min is both oleic and palmitic acid derivatives. The identity of each peak was determined by analyzing each individual fatty acid derivative. In order to elute the peaks in a shorter period of time, the percentage of acetonitrile had to be increased drastically, which in turn led to serious peak overlap.

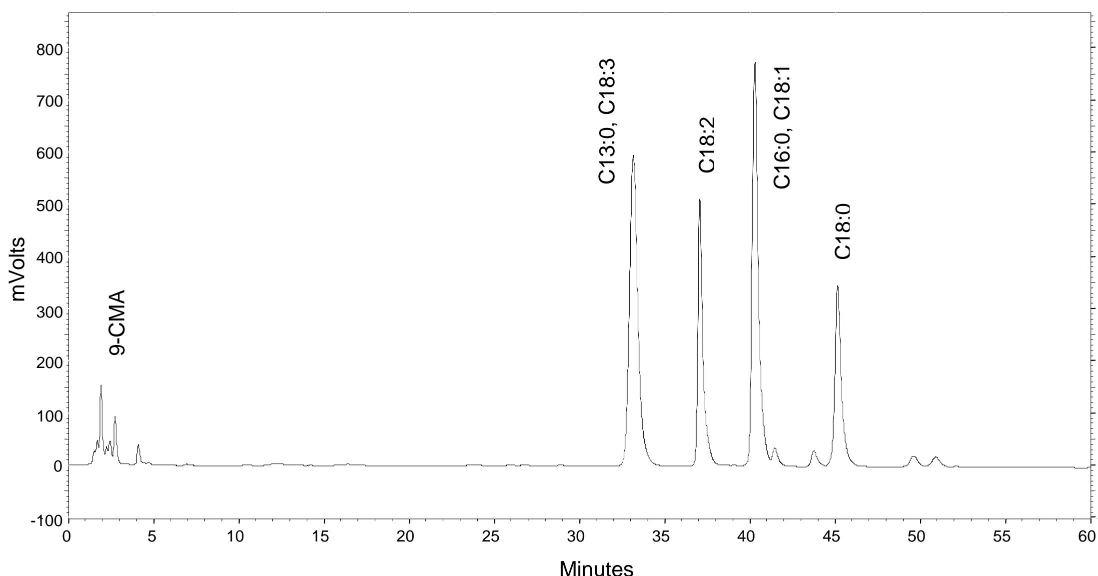


Figure 25. HPLC chromatogram of six fatty acid derivatives using the third protocol. This chromatogram was obtained using gradient elution using a TosoHaas ODS-80TM (100 × 4.6 mm, particle size 5 μm) column.

The fourth protocol used a TosoHaas CN-80TS (150 × 4.6 mm, particle size 5 μm) column, an isocratic mobile phase of 30% acetonitrile and 70% water at a flow rate of 1.0 mL/min. The use of this column led to very short retention times with excessive overlap (Figure 26). The use of a column with a cyano group was not recommended by the literature to separate fatty acid derivatives; however, its separation abilities were still studied. The use of this column did not produce useful results. Separation of the fatty acid derivatives was not achieved with the solvent composition used in all other protocols.

The fifth protocol used a Zorbax Rx C8 (150 × 4.6 mm, particle size 5 μm) column, a gradient mobile phase starting at 50% acetonitrile and 50% water at a flow rate of 1.0 mL/min as described in "HPLC separation and analysis" under Materials and Methods. This protocol was a variation of the method developed by Sun et al. (2011) that showed the first successful separation of all six derivatized fatty acids (Figure 27).

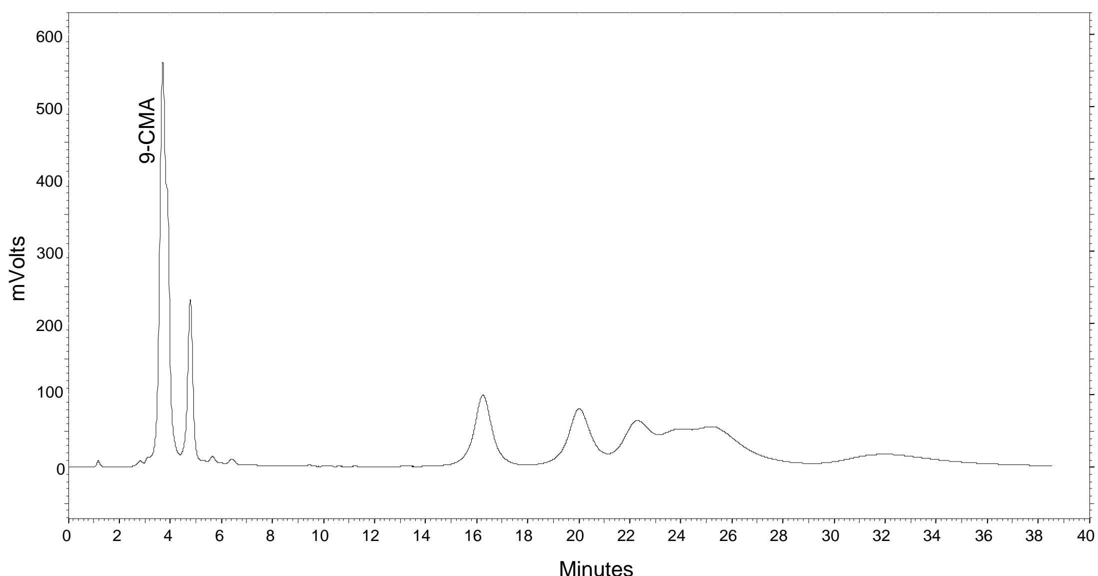


Figure 26. HPLC chromatogram of six fatty acid derivatives using the fourth protocol. This chromatogram was obtained using gradient elution using a TosoHaas CN-80TS (150 × 4.6 mm, particle diameter 5 µm) column.

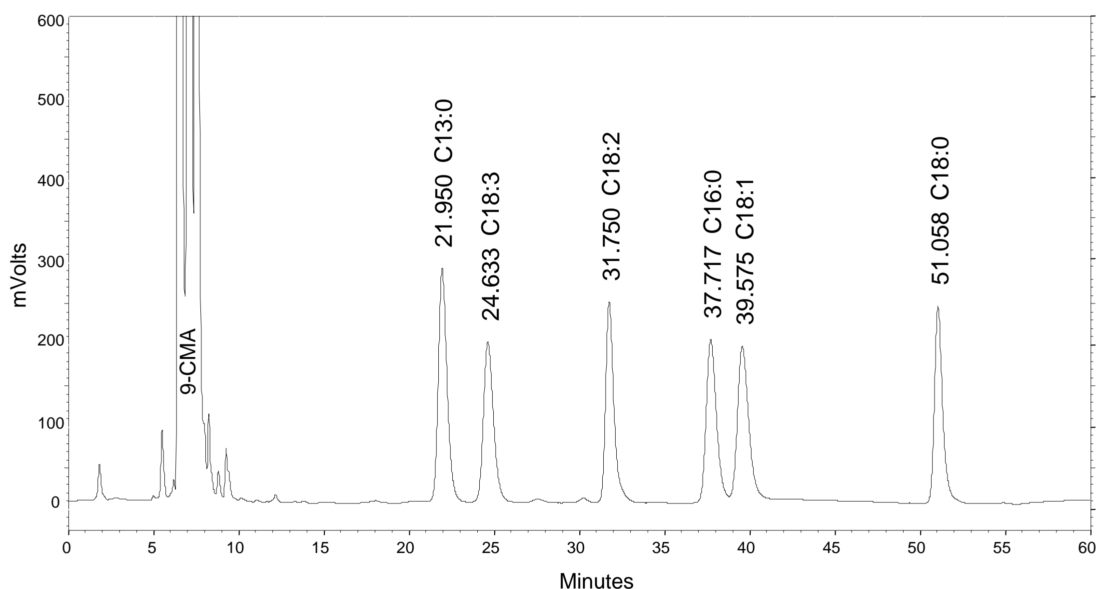


Figure 27. HPLC chromatogram of six fatty acid derivatives using the modified method. Successful separation of the six fatty acid standard derivatives was achieved using the fifth protocol described in Table 4 and in Materials and Methods.

The sixth protocol used a Supelco C8 (150 × 4.6 mm ID, particle size 5 µm) column, a gradient mobile phase starting at 50% acetonitrile and 50% water at a flow rate

of 1.0 mL/min. This protocol is identical to the fifth protocol with the exception of the column. The purpose of this protocol was to test the reproducibility of the HPLC method using a different column with the ultimate purpose of having a backup column (Figure 28).

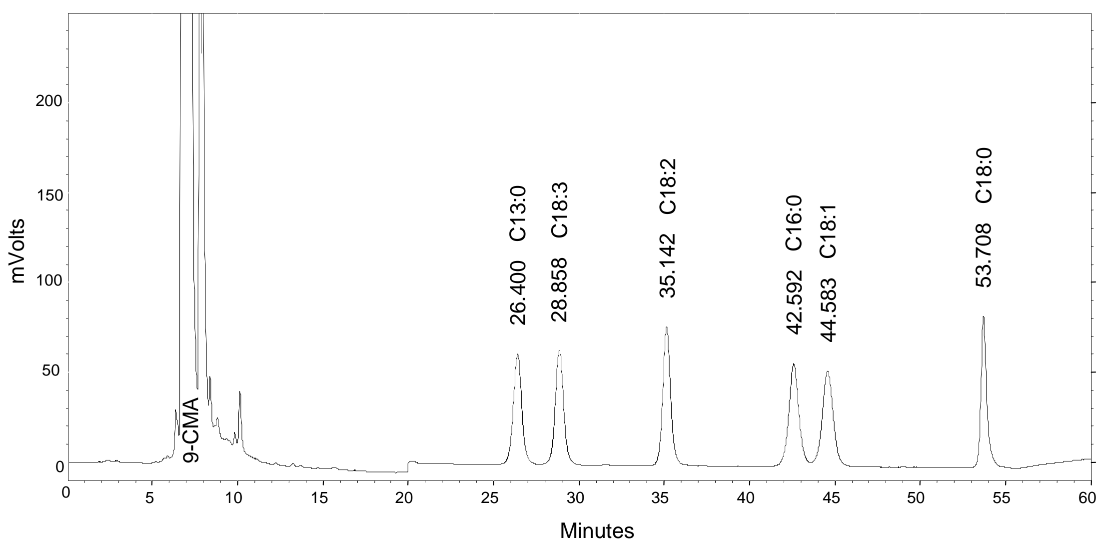


Figure 28. HPLC chromatogram of six fatty acid standard derivatives using the sixth protocol.

Derivatization with 2-Bromomethyl-Anthraquinone (Modified Method)

The method developed by Sun et al. (2011) was utilized to derivatize fatty acid standards in the presence of K_2CO_3 as the base at 85 °C for 35 min with shaking in 5 min intervals. Derivatization of each fatty acid was successful as shown in Figure 29. However, the method was not suitable for analysis with fluorescence detection since 2-bromomethyl-anthraquinone does not fluoresce.

The chromatogram in Figure 29 was obtained using the HPLC conditions described in "HPLC separation and analysis" under Materials and Methods, using the photodiode array detector (PDA) with a range from 250 to 280 nm wavelength detection. Figure 29 contains the six expected peaks that belong to each fatty acid derivative which

confirms the success of the method using 2-bromomethyl-anthraquinone. No further analysis was performed using 2-bromomethyl-anthraquinone.

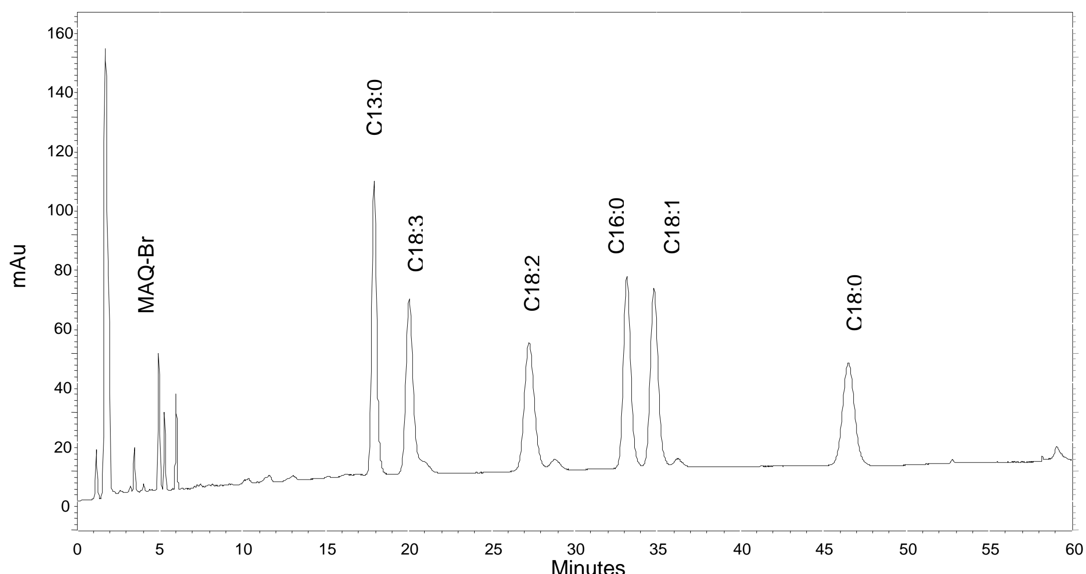


Figure 29. HPLC chromatogram of 2-bromomethyl-anthraquinone fatty acid derivatives. The same elution order as that of the 9-CMA fatty acid derivatives was observed with slight reduction in run time.

Quantitative Analysis

Following the development of the fifth protocol (Table 4) and successful separation of the six fatty acid was achieved, calibration curves were developed to quantify the fatty acid derivatives. Two sets of calibration curves with triple replicates were obtained to test reproducibility, precision, and accuracy. Determination of the limit of detection (LOD) and the limit of quantitation (LOQ) were performed following the determination of the calibration curves.

Calibration Curves

The calibration curves were developed using eight point calibrations for all fatty acid standard derivatives except for stearic acid for which only seven were used due to upper LOQ limitations. As shown in Figure 30, excellent linearity and coefficient of

determination were achieved after plotting the average of the triplicate determinations (Table 5).

Table 5

Standard Calibration Curve Data for Tridecanoic Acid Derivatives

pmol	Trial 1	Trial 2	Trial 3	FR*	St Dev
1	279299	276558	263603	273153.3	8383.61
3	868245	854486	848274	857001.7	10220.4
7	2007304	1988057	2002271	1999211	9981.78
10	2869951	2861212	2837192	2856118	16963.1
30	8433345	8380126	8287211	8366894	73960.1
50	13717989	13552758	13533707	13601485	101344
70	19464788	19210300	18934834	19203307	265046
100	27089375	26999249	26902464	26997029	93475.3

Note. Data collected for tridecanoic acid derivatives varying from 1 to 100 pmol on column. Fluorescence data were obtained by chromatographic analysis described in Materials and Methods. These data are represented graphically in Figure 30.
* FR is the fluorescence response.

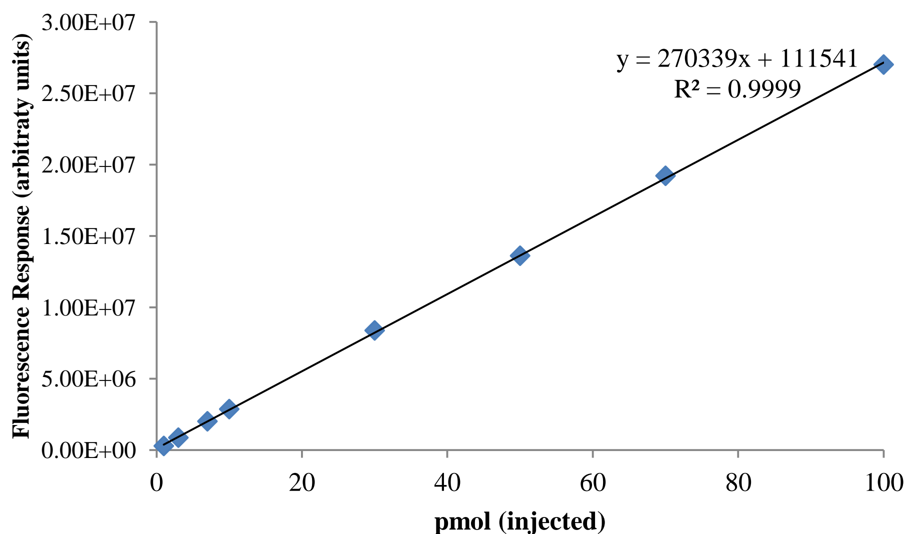


Figure 30. Standard calibration curve of tridecanoic acid derivatives. The curve displays the mean fluorescence responses verses the standards of 1, 3, 7, 10, 30, 50, 70, and 100 pmoles of tridecanoic acid derivatives injected onto the column.

To test reproducibility and accuracy of each calibration curve, two mixed fatty acid standard stock solutions were prepared and analyzed. Data was collected three times

for each data point (injected pmols); the fluorescence response for all standards, mean fluorescence responses, and standard deviations are shown in the Appendix.

Limit of Detection and Limit of Quantitation

The limit of detection (LOD) was determined by diluting a solution starting at a 1.00 mM concentration until the HPLC chromatogram was obtained in which the LOD was determined to be approximately 50 fmol on column (Figure 31).

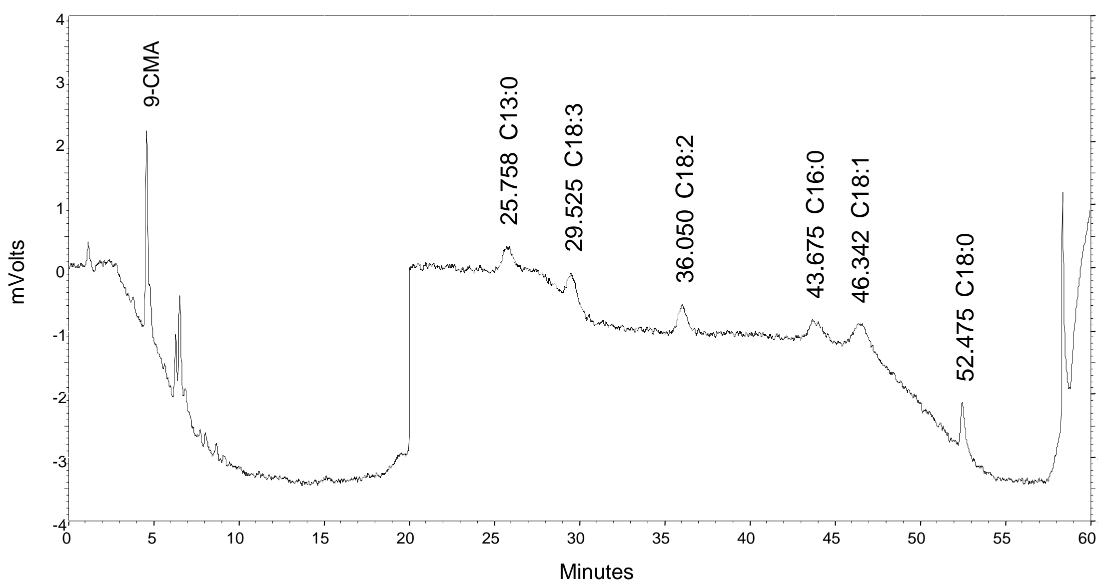


Figure 31. HPLC chromatogram of limit of detection. All six fatty acid derivatives show at least a 3 to 1 signal to noise ratio to give a limit of detection of approximately 50 fmol.

The upper limit of quantitation (LOQ) was determined by the limitations of the fluorescence detector on the instrument itself. Any values above 1000 mVolts were not detectable by the instrument and therefore not quantified. The highest concentration allowable under the developed separation conditions that included all six standards was 70 pmol (injected onto the column) since at 100 pmol (on column) the stearic acid peak was no longer quantifiable (Figure 32).

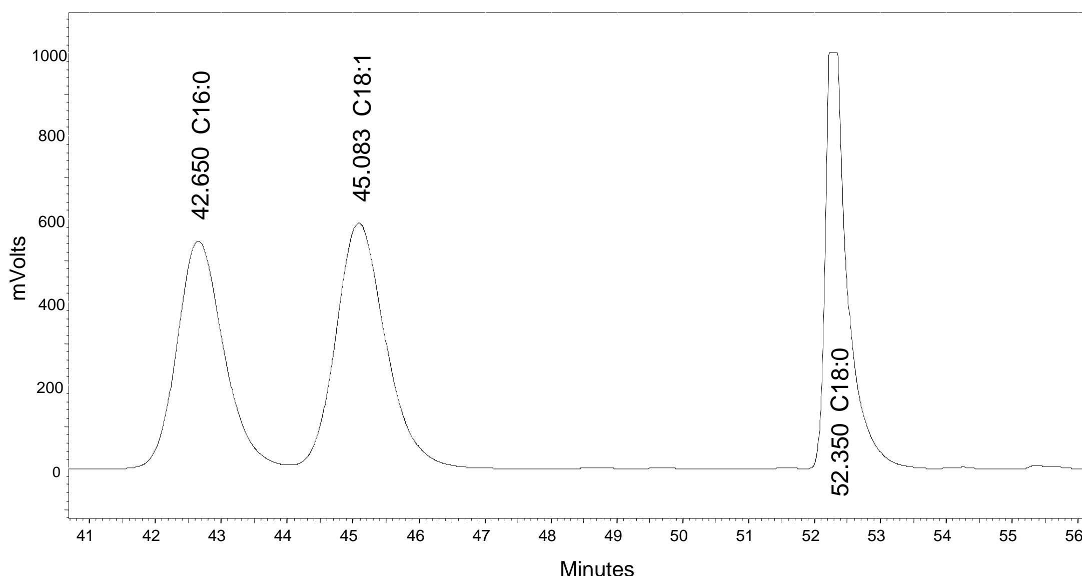
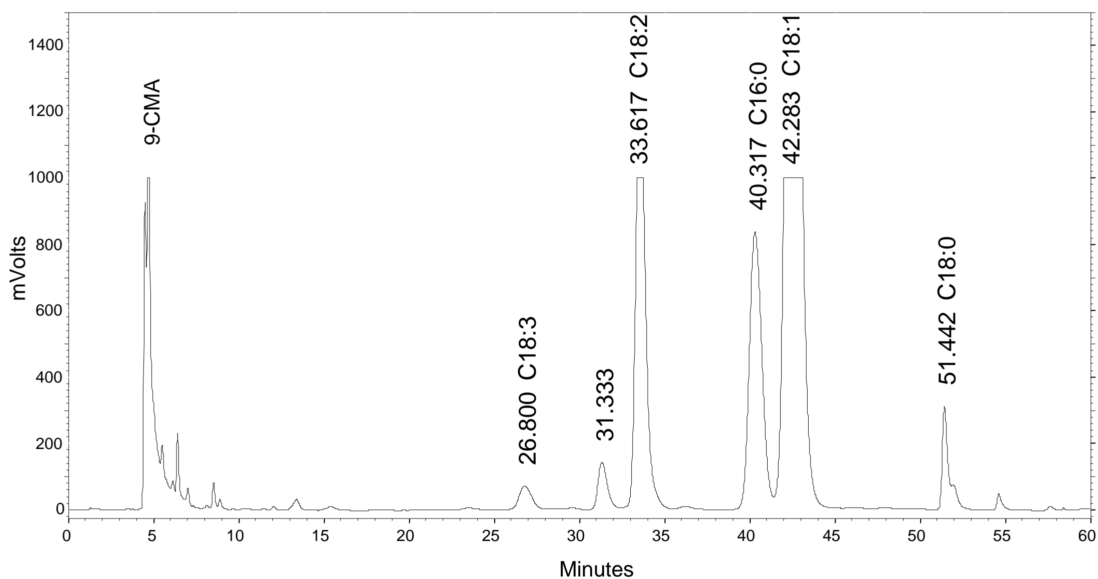


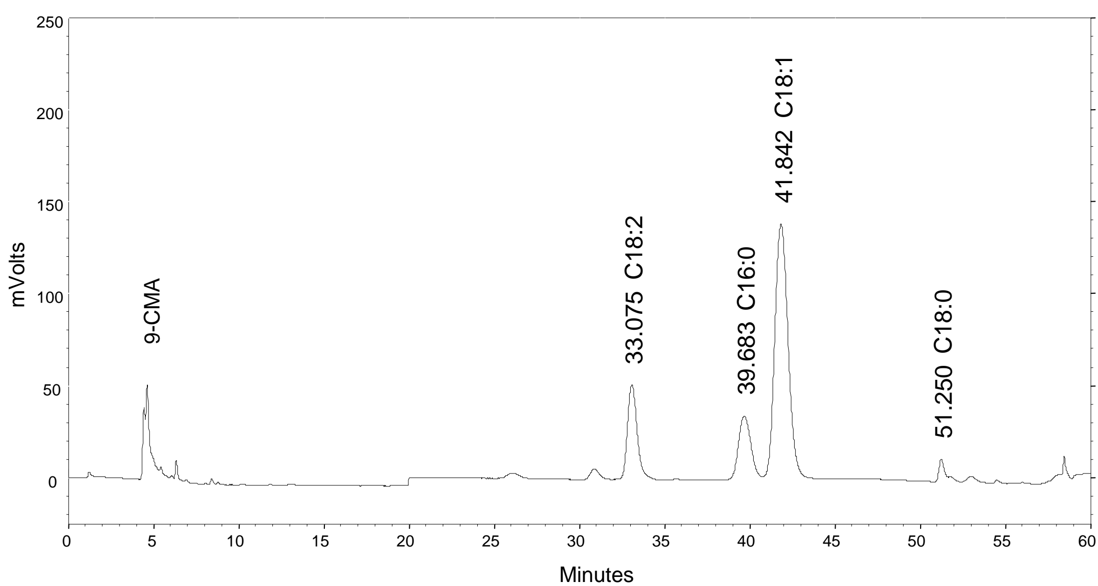
Figure 32. HPLC chromatogram showing the limit of quantitation. The peak at 52.35 minutes (stearic acid derivative) shows the limit of quantitation for the fatty acid standard derivatives.

Analysis of Fat-Free Cooking Spray

To determine whether the method was applicable to fatty acids other than the fatty acid standards, olive oil based fat-free cooking spray was analyzed. Approximately 8 mg of cooking spray (Great Value, Walmart) were treated as described in “Extraction procedure” in Materials and Methods to obtain the fatty acids present in the sample. Following the extraction, the fatty acids were derivatized with 9-CMA as described in “Derivatization with 9-chloromethyl-anthracene” in Materials and Methods prior to HPLC analysis. After derivatization was complete, the fatty acid derivatives were analyzed as described in “HPLC separation and analysis” in Materials and Methods (Figure 33).



(a)



(b)

Figure 33. HPLC chromatogram of fatty acid derivatives from fat-free cooking spray. (a) First analysis of fatty acid content. (b) Analysis of a 1 to 25 dilution of the fat-free cooking spray derivatives to have all peaks fall within detector range.

Analysis of Cloprostenol

Following the successful analysis of fat-free cooking spray, the cloprostenol standard was derivatized as described in “Derivatization with 9-chloromethyl-anthracene” in Materials and Methods prior to HPLC analysis. Figure 34 shows a chromatogram with preliminary results showing successful derivatization and analysis of cloprostenol using 9-CMA. The peak ranging from approximately 4.4 to 7 min is the excess 9-CMA peak (determined after analyzing a reaction “blank”) and the peak at approximately 9.5 min is the cloprostenol derivative since it was the most prominent peak to appear after analysis of the reaction “blank”.

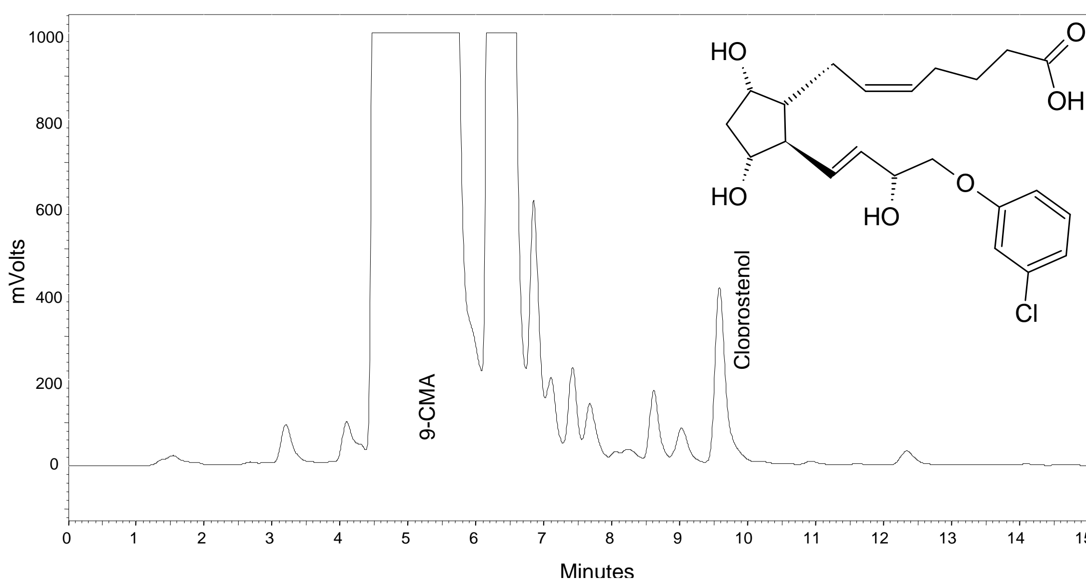


Figure 34. HPLC chromatogram of cloprostenol derivative. The peak at approximately 9.5 min is the cloprostenol derivative.

CHAPTER V

CONCLUSIONS

Fatty acids are carboxylic acids with long alkane or alkene chains and are important components of living organisms. The presence of the carboxylic acid is analytically very important since it allows for methylation or ethylation for GC analysis, or derivatization with fluorescence reagents such as 9-chloromethyl-anthracene (9-CMA) for HPLC analysis. Xie et al. (2012) developed a derivatization method that involved the use of 9-CMA to analyze short-chain acids with successful results.

The goal of this research project was to replicate the results obtained by Xie et al. (2012) and, after successful replication, apply the method to derivatize and analyze medium- and long-chain fatty acids. After multiple attempts to replicate the method developed by Xie et al. (2012) without successful results, a new method was developed in combination with the method developed by Sun et al. (2011).

The method developed was successfully applied to six fatty acid standards, a fat-free cooking spray, and cloprostenol, an analog of prostaglandin $E_{2\alpha}$ ($PGE_{2\alpha}$). The method using 9-CMA is capable of separation and detection of low quantities of the derivatives.

Calibration curves, limit of detection (LOD) at 50 fmol and upper limit of quantitation (LOQ) at 100 pmol were obtained for each of the fatty acid derivatives as

part of the quantitative analysis. Good linearity and coefficients of determination greater than or equal to 0.9999 were obtained within the range used for quantitation.

Recommendations

Based on the results of this study, the following recommendations are suggested for further investigation of the HPLC determination with fluorescence detection of fatty acid derivatives.

1. To limit errors introduced by extraction of fatty acids and derivatization, an internal standard should be used. Tridecanoic acid would be most appropriate since it did not interfere with the fat-free cooking spray that was analyzed.
2. To limit errors during quantification of extracted fatty acids the percent recovery should be calculated. This can be performed by adding a known concentration of the internal standard to the sample to be analyzed and comparing it to the fluorescence response of the internal standard alone.
3. To have uninterrupted shaking/stirring during the derivatization reaction, the use of a water bath on a magnetic stir plate is recommended. This will ensure constant mixing without changing the temperature which occurs when removing the reaction vial from the oven every 5 min.
4. To limit errors during HPLC separation and quantitation, the use of a column oven would be most ideal. Analysis of fatty acid derivatives produced chromatograms with slightly different retention times probably due to laboratory ambient temperature variations. Retention time variations can lead to different fluorescence responses for the same standard which could produce unreliable standard curves.

5. To limit errors introduced in between the hydrolysis-extraction and derivatization steps, a method that reduces the number of reaction steps should be developed. Being able to derivatize the fatty acids in the same reaction vial in which the hydrolysis is performed would minimize the possibility of losing free fatty acids prior to derivatization.
6. The choice of the HPLC column for this method was based on columns available at the start of the method development. Improved retention times, resolution and sensitivity could possibly be achieved by using an octyl column with different specifications. An octyl column with a smaller internal diameter, smaller particle size, or both, should provide those improvements. These recommendations are presented based on the theory behind chromatographic separations.

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APPENDIX

CHROMATOGRAPHIC DATA

Table 6

Standard Calibration Curve Data for Tridecanoic Acid Derivative (duplicate)

pmol	Trial 1	Trial 2	Trial 3	FR	St Dev
1	262728	271936	264327	266330.3	4920.043
3	861699	872135	850781	861538.3	10677.91
7	2042227	2072337	1965364	2026643	55163.02
10	2916132	2888361	2800745	2868413	60224.51
30	8679027	8679743	8392850	8583873	165431.4
50	14386157	14394718	13763700	14181525	361872.4
70	19927354	20070416	19151304	19716358	494551.5
100	27953506	28614237	27308828	27958857	652721

Note. Data collected for tridecanoic acid derivatives varying from 1 to 100 pmol for a second fatty acid standards stock solution.

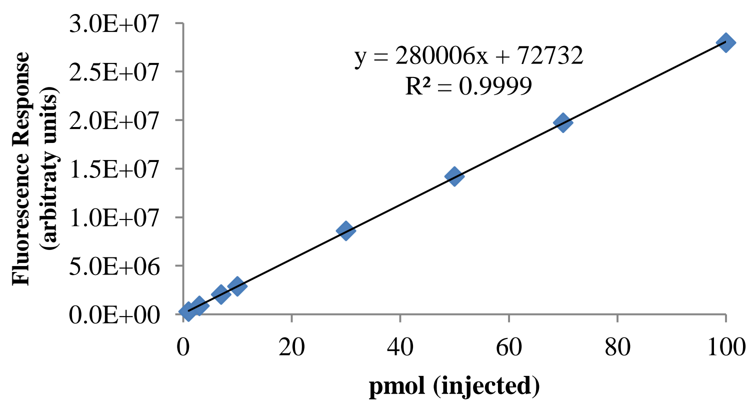

Figure 35. Standard calibration curve of tridecanoic acid derivatives (duplicate).

Table 7

Standard Calibration Curve Data for Linolenic Acid Derivatives

pmol	Trial 1	Trial 2	Trial 3	FR	St Dev
1	342509	339757	344321	342195.7	2298.08
3	941011	935401	954232	943548	9668.45
7	2206840	2262870	2112815	2194175	75825
10	3144959	3163374	3057037	3121790	56828.6
30	9329345	9363870	9080445	9257887	154636
50	15465023	15516906	14834638	15272189	379817
70	21450422	21629982	20599198	21226534	550657
100	30056853	30804253	29351116	30070741	726668

Note. Data collected for linolenic acid derivatives varying from 1 to 100 pmol.

Table 8

Standard Calibration Curve Data for Linolenic Acid Derivatives (duplicate)

pmol	Trial 1	Trial 2	Trial 3	FR	St Dev
1	270857	221426	262694	251659	26498.8
3	903865	888223	881016	891034.7	11681.1
7	2134972	2119109	2123703	2125928	8162.21
10	3017263	2976862	2955669	2983265	31292.2
30	8795740	8737588	8659039	8730789	68603.6
50	14302310	14143918	14197760	14214663	80537.5
70	19878860	20107087	19738664	19908204	185956
100	28215162	28109813	27981264	28102080	117141

Note. Data collected for linolenic acid derivatives varying from 1 to 100 pmol for a second fatty acid standards stock solution.

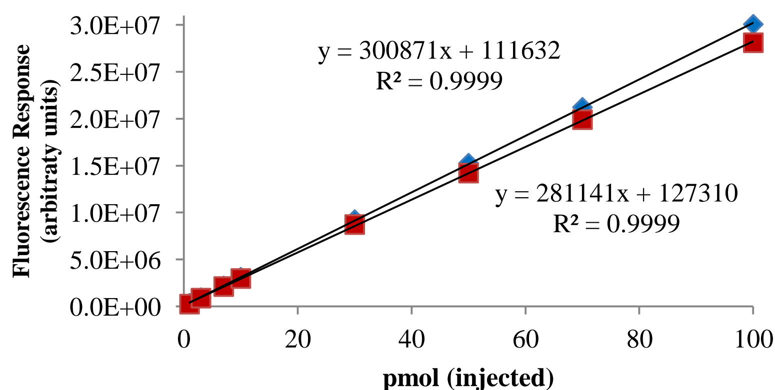

Figure 36. Standard calibration curves for linolenic acid derivatives. Both sets of data are plotted to compare the resulting calibration curves. ♦ Data from Table 7, ■ data from Table 8.

Table 9

Standard Calibration Curve Data for Linoleic Acid Derivatives

pmol	Trial 1	Trial 2	Trial 3	FR	St Dev
1	288836	299989	305848	298224.3	8642.2
3	936352	944295	916157	932268	14506.8
7	2203742	2216136	2137876	2185918	42064.6
10	3164511	3152196	3041588	3119432	67695.2
30	9409971	9387779	9096957	9298236	174665
50	15602055	15568035	14860268	15343453	418796
70	21617595	21668462	20676477	21320845	558618
100	30325637	30969130	29485267	30260011	744105

Note. Data collected for linoleic acid derivatives varying from 1 to 100 pmol.

Table 10

Standard Calibration Curve Data for Linoleic Acid Derivatives (Duplicate)

pmol	Trial 1	Trial 2	Trial 3	FR	St Dev
1	312602	298501	299894	303665.7	7770.37
3	952039	953395	942542	949325.3	5913.53
7	2223179	2200300	2205470	2209650	11998.5
10	3161951	3146481	3143270	3150567	9988.42
30	9310639	9228134	9142073	9226949	84289.3
50	15112776	14928255	14967732	15002921	97163.3
70	20943408	20923220	20939651	20935426	10736.6
100	29705730	29618239	29558751	29627573	73932.8

Note. Data collected for linoleic acid derivatives varying from 1 to 100 pmol for a second fatty acid standards stock solution.

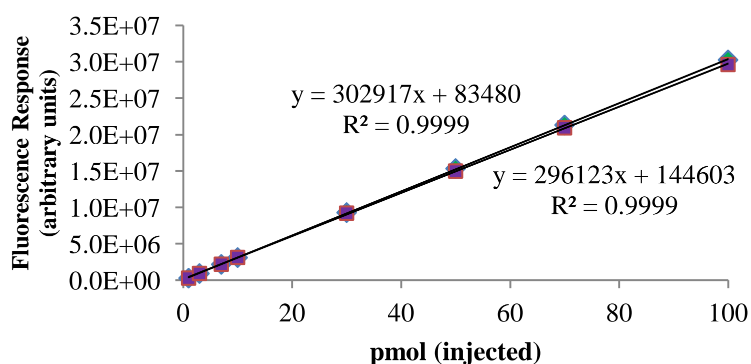

Figure 37. Standard calibration curves for linoleic acid derivatives. Both sets of data are plotted to compare the resulting calibration curves. ♦ Data from Table 9, ■ data from Table 10.

Table 11

Standard Calibration Curve Data for Oleic Acid Derivatives

pmol	Trial 1	Trial 2	Trial 3	FR	St Dev
1	224359	232033	224732	227041.3	4326.93
3	814046	795844	771321	793737	21440.3
7	1903444	1911001	1834786	1883077	41991.6
10	2750311	2739528	2627841	2705893	67810
30	8215171	8204406	7970954	8130177	137996
50	13610379	13638050	13056051	13434827	328321
70	18883459	19030691	18185239	18699796	451659
100	26578640	27176124	25929531	26561432	623475

Note. Data collected for oleic acid derivatives varying from 1 to 100 pmol.

Table 12

Standard Calibration Curve Data for Oleic Acid Derivatives (Duplicate)

pmol	Trial 1	Trial 2	Trial 3	FR	St Dev
1	231934	235113	234587	233878	1703.97
3	816140	810148	794268	806852	11302.4
7	1923421	1878971	1890963	1897785	22996.9
10	2728267	2713196	2719476	2720313	7570.28
30	8069208	8036845	7955993	8020682	58312.4
50	13166034	13016347	13065754	13082712	76270.7
70	18308183	18252905	18208875	18256654	49760.1
100	26011426	26006666	25841077	25953056	97006.1

Note. Data collected for oleic acid derivatives varying from 1 to 100 pmol for a second fatty acid standards stock solution.

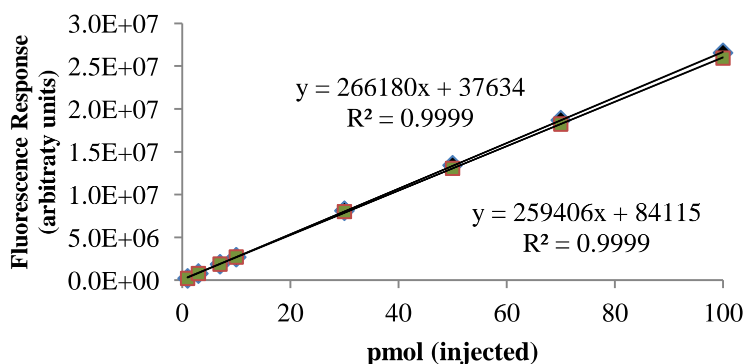

Figure 38. Standard calibration curves for oleic acid derivatives. Both sets of data are plotted to compare the resulting calibration curves. ♦ Data from Table 11, ■ data from Table 12.

Table 13

Standard Calibration Curve Data for Palmitic Acid Derivatives

pmol	Trial 1	Trial 2	Trial 3	FR	St Dev
1	334366	325555	321237	327052.7	6691.41
3	986895	951427	924218	954180	31429.1
7	2249926	2226731	2147792	2208150	53542.4
10	3212062	3197230	3082044	3163779	71171.7
30	9524993	9498607	9221835	9415145	167930
50	15747859	15729462	15052954	15510092	396000
70	21927925	21907551	20969950	21601809	547300
100	30890929	31256127	29835823	30660960	737550

Note. Data collected for palmitic acid derivatives varying from 1 to 100 pmol.

Table 14

Standard Calibration Curve Data for Palmitic Acid Derivatives (Duplicate)

pmol	Trial 1	Trial 2	Trial 3	FR	St Dev
1	302025	301207	306269	303167	2717.37
3	905434	888202	875190	889608.7	15171
7	2037872	1995684	2009914	2014490	21463
10	2921042	2885631	2886394	2897689	20227.9
30	8556562	8421846	8347866	8442091	105811
50	13754660	13619466	13661550	13678559	69183.3
70	19113991	19053660	18996056	19054569	58972.8
100	27103919	27273641	26995853	27124471	140030

Note. Data collected for palmitic acid derivatives varying from 1 to 100 pmol for a second fatty acid standards stock solution.

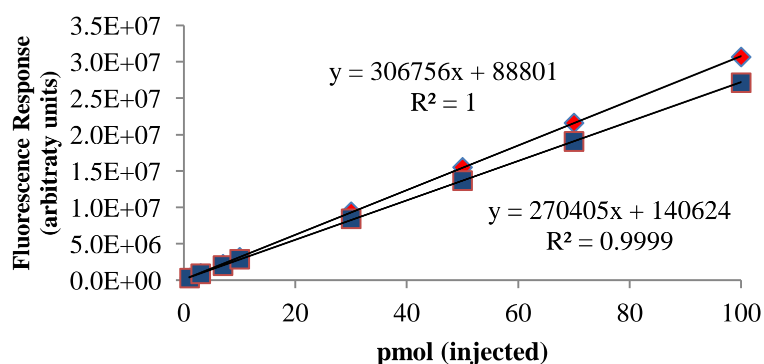

Figure 39. Standard calibration curves for palmitic acid derivatives. Both sets of data are plotted to compare the resulting calibration curves. ♦ Data from Table 13, ■ data from Table 14.

Table 15

Standard Calibration Curve Data for Stearic Acid Derivatives

pmol	Trial 1	Trial 2	Trial 3	FR	St Dev
1	259775	267364	259095	262078	4590.42
3	779631	779291	756227	771716.3	13415.2
7	1789164	1795916	1736626	1773902	32458
10	2547259	2559052	2474105	2526805	46019.2
30	7566114	7606179	7338721	7503671	144249
50	12531060	12598129	12131842	12420344	252090
70	17435721	17657457	16923659	17338946	376350

Note. Data collected for stearic acid derivatives varying from 1 to 100 pmol.

Table 16

Standard Calibration Curve Data for Stearic Acid Derivatives (Duplicate)

	Trial 1	Trial 2	Trial 3	FR	St Dev
1	268856	263578	257077	263170.3	5900.07
3	786121	786560	778455	783712	4557.98
7	1819997	1793999	1809070	1807689	13053.9
10	2575230	2553734	2541602	2556855	17029.9
30	7532577	7428912	7379299	7446929	78211.3
50	12206628	12085665	12138008	12143434	60663.7
70	16934356	16833876	16833759	16867330	58046

Note. Data collected for stearic acid derivatives varying from 1 to 100 pmol for a second fatty acid standards stock solution.

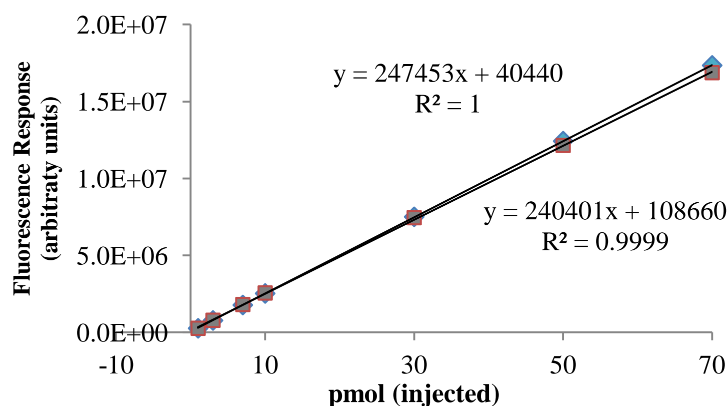

Figure 40. Standard calibration curves for stearic acid derivatives. Both sets of data are plotted to compare the resulting calibration curves. ♦ Data from Table 15, ■ data from Table 16.