1999

Evaluation of columns for lipid separation with the ELSD

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**EVALUATION OF COLUMNS FOR LIPID SEPARATION WITH THE
ELSD**

A Thesis

Presented to

**The Faculty of the Department of Chemistry
San Jose State University**

**In partial Fulfillment
Of the Requirement for the Degree
Master of Science**

By

Chidi Obi

August 1999

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ABSTRACT

EVALUATION OF COLUMNS FOR LIPID SEPARATION WITH THE EVAPORATIVE LIGHT SCATTERING DETECTOR

BY

CHIDI OBI

The high performance liquid chromatography (HPLC) separation of lipids was investigated on monol, diol and C18 columns using mobile phases consisting of acetone and acetonitrile in varying proportions. HPLC coupled to the evaporative light scattering detector (ELSD) was used in evaluating the chromatograms from these three columns.

It was found that the best mobile phase composition for analyzing triglycerides using the C18 column was acetone/acetonitrile (65/35) v/v. A decrease in mobile phase polarity by increasing the acetone content resulted in a decrease in the retention time and resolution of triglycerides. However, for the monol and diol columns the best mobile phase composition was found to be from acetone/acetonitrile (80/20) to acetone (100) v/v depending on the sample. The resolution of triglycerides was very poor in both the monol and diol column analysis. Olive oil, olive oil standard, fish oil, unsalted butter and corn oil were the samples used for this research. Chromatograms obtained from these samples were evaluated.

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Table of contents

Chapter I – INTRODUCTION	1
1. Background	1
(A) Lipids	1
(B) High Performance Liquid Chromatography (HPLC)	3
(C) Columns	6
(D) Detectors	7
(E) Aim of this research	13
Chapter II – EXPERIMENTATION	13
(A) Mobile phase solvents	13
(B) Triglyceride standard	14
(C) Samples	14
(D) Instrumentation	15
(E) Analytical columns	15
(F) Analytical conditions	17
Chapter III – RESULTS AND DISCUSSION	19
(A) Chromatographic analysis	19
(B) Effect of mobile phase polarity on columns	32
(C) Detector performance	38
Chapter IV – CONCLUSION	40
REFERENCES	42
APPENDIX	43

List of Figures

Figure	Page no.
Fig. 1: Structure of a triglyceride	2
Fig. 2A: Structure of a phospholipid	2
Fig. 2B: Structures of a fatty acid	3
Fig. 3: The principle of operation of the ELSD	12
Fig. 4A: Isocratic HPLC analysis. Sample: blank, Column: C18, Mobile Phase: 100% ace	44
Fig. 4B: Isocratic HPLC analysis. Sample: olive oil, Column: C18, Mobile Phase: 100% ace	45
Fig. 5: Isocratic HPLC analysis. Sample: olive oil, Column: C18, Mobile Phase: 90/10 ace/acn	46
Fig. 6: Isocratic HPLC analysis. Sample: olive oil, Column: C18, Mobile Phase: 80/20 ace/acn	47
Fig. 7: Isocratic HPLC analysis. Sample: olive oil, Column: C18, Mobile Phase: 65/35 ace/acn	48
Fig. 8: Isocratic HPLC analysis. Sample: olive oil, Column: C18, Mobile Phase: 50/50 ace/acn	49
Fig. 9: Isocratic HPLC analysis. Sample: olive oil, Column: C18, Mobile Phase: 40/60 ace/acn	50
Fig. 10: Isocratic HPLC analysis. Sample: unsalted butter, Column: C18, Mobile Phase: 100 ace	51
Fig. 11: Isocratic HPLC analysis. Sample: unsalted butter, Column: C18, Mobile Phase: 90/10 ace/acn	52
Fig. 12: Isocratic HPLC analysis. Sample: unsalted butter, Column: C18, Mobile Phase: 80/20 ace/acn	53
Fig. 13: Isocratic HPLC analysis. Sample: unsalted butter, Column: C18, Mobile Phase: 65/35 ace/acn	54
Fig. 14: Isocratic HPLC analysis. Sample: unsalted butter, Column: C18, Mobile Phase: 50/50 ace/acn	55
Fig. 15: Isocratic HPLC analysis. Sample: unsalted butter, Column: C18, Mobile Phase: 40/60 ace/acn	56
Fig. 16: Isocratic HPLC analysis. Sample: fish oil, Column: C18, Mobile Phase: 100% ace	57
Fig. 17: Isocratic HPLC analysis. Sample: fish oil, Column: C18, Mobile Phase: 90/10 ace/acn	58
Fig. 18: Isocratic HPLC analysis. Sample: fish oil, Column: C18, Mobile Phase: 80/20 ace/acn	59
Fig. 19: Isocratic HPLC analysis. Sample: fish oil, Column: C18, Mobile Phase: 65/35 ace/acn	60
Fig. 20: Isocratic HPLC analysis. Sample: fish oil, Column: C18, Mobile Phase: 50/50 ace/acn	61
Fig. 21: Isocratic HPLC analysis. Sample: fish oil, Column: C18, Mobile Phase: 40/60 ace/acn	62
Fig. 22: Isocratic HPLC analysis. Sample: corn oil, Column: C18, Mobile Phase: 100 ace	63
Fig. 23: Isocratic HPLC analysis. Sample: corn oil, Column: C18, Mobile Phase: 90/10 ace/acn	64
Fig. 24: Isocratic HPLC analysis. Sample: corn oil, Column: C18, Mobile Phase: 80-20 ace/acn	65
Fig. 25: Isocratic HPLC analysis. Sample: corn oil, Column: C18, Mobile Phase: 65/35 ace/acn	66
Fig. 26: Isocratic HPLC analysis. Sample: corn oil, Column: C18, Mobile Phase: 50/50 ace/acn	67
Fig. 27: Isocratic HPLC analysis. Sample: corn oil, Column: C18, Mobile Phase: 40/60 ace/acn	68
Fig. 28: Isocratic HPLC analysis. Sample: olive oil std, Column: C18, Mobile Phase: 90/10 ace/acn	69
Fig. 29: Isocratic HPLC analysis. Sample: olive oil std, Column: C18, Mobile Phase: 80/20 ace/acn	70
Fig. 30: Isocratic HPLC analysis. Sample: olive oil std, Column: C18, Mobile Phase: 65/35 ace/acn	71
Fig. 31: Isocratic HPLC analysis. Sample: olive oil, Column: Diol, Mobile Phase: 100% ace/acn	72
Fig. 32: Isocratic HPLC analysis. Sample: olive oil, Column: Diol, Mobile Phase: 90/10 ace/acn	73
Fig. 33: Isocratic HPLC analysis. Sample: olive oil, Column: Diol, Mobile Phase: 80/20 ace/acn	74
Fig. 34: Isocratic HPLC analysis. Sample: olive oil, Column: Diol, Mobile Phase: 65/35 ace/acn	75
Fig. 35: Isocratic HPLC analysis. Sample: unsalted butter, Column: Diol, Mobile Phase: 100% ace	76
Fig. 36: Isocratic HPLC analysis. Sample: unsalted butter, Column: Diol, Mobile Phase: 90/10 ace/acn	77
Fig. 37: Isocratic HPLC analysis. Sample: unsalted butter, Column: Diol, Mobile Phase: 80/20 ace/acn	78
Fig. 38: Isocratic HPLC analysis. Sample: unsalted butter, Column: Diol, Mobile Phase: 65/35 ace/acn	79
Fig. 39: Isocratic HPLC analysis. Sample: fish oil, Column: Diol, Mobile Phase: 100% ace	80
Fig. 40: Isocratic HPLC analysis. Sample: fish oil, Column: Diol, Mobile Phase: 90/10 ace/acn	81
Fig. 41: Isocratic HPLC analysis. Sample: fish oil, Column: Diol, Mobile Phase: 80/20 ace/acn	82
Fig. 42: Isocratic HPLC analysis. Sample: fish oil, Column: Diol, Mobile Phase: 65/35 ace/acn	83
Fig. 43: Isocratic HPLC analysis. Sample: corn oil, Column: Diol, Mobile Phase: 100% ace	84
Fig. 44: Isocratic HPLC analysis. Sample: corn oil, Column: Diol, Mobile Phase: 90/10 ace/acn	85
Fig. 45: Isocratic HPLC analysis. Sample: corn oil, Column: Diol, Mobile Phase: 80/20 ace/acn	86
Fig. 46: Isocratic HPLC analysis. Sample: corn oil, Column: Diol, Mobile Phase: 65/35 ace/acn	87
Fig. 47: Isocratic HPLC analysis. Sample: olive oil std, Column: Diol, Mobile Phase: 100% ace	88

Figure	Page no.
Fig. 48: Isocratic HPLC analysis. Sample: olive oil std, Column: Diol, Mobile Phase: 90/10 ace/acn	89
Fig. 49: Isocratic HPLC analysis. Sample: olive oil, Column: Monol, Mobile Phase: 100% ace	90
Fig. 50: Isocratic HPLC analysis. Sample: olive oil, Column: Monol, Mobile Phase: 90/10 ace/10	91
Fig. 51: Isocratic HPLC analysis. Sample: olive oil, Column: Monol, Mobile Phase: 65/35 ace/acn	92
Fig. 52: Isocratic HPLC analysis. Sample: olive oil, Column: Monol, Mobile Phase: 50/50 ace/acn	93
Fig. 53: Isocratic HPLC analysis. Sample: unsalted butter, Column: Monol, Mobile Phase: 100% ace	94
Fig. 54: Isocratic HPLC analysis. Sample: unsalted butter, Column: Monol, Mobile Phase: 90/10 ace/acn	95
Fig. 55: Isocratic HPLC analysis. Sample: unsalted butter, Column: Monol, Mobile Phase: 65/35 ace/acn	96
Fig. 56: Isocratic HPLC analysis. Sample: fish oil, Column: Monol, Mobile Phase: 100% ace	97
Fig. 57: Isocratic HPLC analysis. Sample: fish oil, Column: Monol, Mobile Phase: 90/10 ace/acn	98
Fig. 58: Isocratic HPLC analysis. Sample: fish oil, Column: Monol, Mobile Phase: 65/35 ace/acn	99
Fig. 59: Isocratic HPLC analysis. Sample: fish oil, Column: Monol, Mobile Phase: 50/50 ace/acn	100
Fig. 60: Isocratic HPLC analysis. Sample: corn oil, Column: monol, Mobile Phase: 100% ace	101
Fig. 61: Isocratic HPLC analysis. Sample: corn oil, Column: monol, Mobile Phase: 90/10 ace/acn	102
Fig. 62: Isocratic HPLC analysis. Sample: corn oil, Column: monol, Mobile Phase: 65/35 ace/acn	103
Fig. 63: Isocratic HPLC analysis. Sample: corn oil, Column: monol, Mobile Phase: 50/50 ace/acn	104
Fig. 64: Isocratic HPLC analysis. Sample: olive oil std, Column: Monol, Mobile Phase: 100% ace	105
Fig. 65: Isocratic HPLC analysis. Sample: olive oil std, Column: Monol, Mobile Phase: 90/10 ace/acn	106
Fig. 66: Isocratic HPLC analysis. Sample: olive oil std, Column: Monol, Mobile Phase: 65/35 ace/acn	107

List of Tables

	Page no.
Table 1. Structure of active sites on the various columns	35

Chapter I – INTRODUCTION

1. Background

The analysis and detection of lipids has been a growing and improving field since the advent of the evaporative light scattering detector (ELSD). High performance liquid chromatography (HPLC) coupled to an evaporative light scattering detector has been shown to be an effective tool for lipid analysis and a large number of papers have been published (1).

(A) Lipids

Lipids are extremely important food products and accordingly, much attention has been paid to their analysis. They are distinguished from other biologically produced organic materials by their solubility in ether and similar non-polar organic solvents. Their structures are primarily hydrocarbon-like and they are insoluble in water. The lipids of physiological importance for humans have three main functions namely:

- They serve as structural components of biological membranes
- They provide energy reserves, predominantly in the form of triglycerides
- Both lipids and lipid derivatives serve as vitamins and hormones

There are four classes of lipids that include triglycerides, phospholipids, free fatty acids and sterols. Among these classes of lipids, triglycerides, fatty acids and phospholipids are most often analyzed using HPLC coupled to an ELSD. Triglycerides (Fig. 1) are composed of three fatty acids attached to a “backbone” molecule called glycerol. These compounds can be saturated or unsaturated depending on the composition of the three fatty acids attached to the glycerol. If the fatty acid component of the triglyceride contain

no carbon-carbon double bonds, they are termed saturated; those that contain fatty acids with carbon-carbon double bond are termed unsaturated triglycerides.

Fig. 1 Structure of a triglyceride

Phospholipids (Fig. 2A) like triglycerides are also in human systems. They are consumed daily. Phospholipids are made up of a glycerol “backbone” with two fatty acids attached. Also attached to this backbone are a phosphate and a nitrogen base.

Fig. 2A Structure of a phospholipid

Fatty acids (Fig. 2B) are long-chain hydrocarbon molecules containing a carboxylic acid moiety at one end. They could be saturated, mono-unsaturated or poly-unsaturated depending on the nature of its carbon chain. They fill two major roles in the body:

1. As the component of more complex membrane lipids
2. As the major components of stored fat in the form of triglycerides

Below are two structures of fatty acid, one is saturated (no carbon double bond) and the other is unsaturated (it has a double bond in its structure).

Fig. 2b Structures of a fatty acid

(B) High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography is one mode of chromatography. It is, however, the most widely used analytical separation technique. Chromatographic

processes can be defined as separation involving mass-transfer between stationary and mobile phases. HPLC utilizes a liquid mobile phase to separate the components of a mixture. These components (or analytes) are first dissolved in a solvent and then forced to flow through a chromatographic column under a high pressure. In the column, the mixture is resolved into its components. The degree of resolution is important, and is dependent upon the extent of interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material in the column (2). The interaction of the solute with the mobile and stationary phase can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC possesses a high degree of versatility not found in other chromatographic systems and has the ability to easily separate a wide variety of chemical mixtures.

There are many ways to classify liquid column chromatography. If this classification is based on the nature of the stationary phase and the separation process, three modes can be specified:

- **Adsorption chromatography** – In adsorption chromatography, the stationary phase is an adsorbent (like silica gel or any other silica-based packings) and the separation is based on repeated adsorption-desorption steps.
- **Ion-exchange chromatography** – In this method, the stationary bed has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control the elution time.

- **Size exclusion chromatography** – In this method, the column is filled with material having precisely controlled pore sizes, and the sample is simply screened or filtered according to its solvated molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the pores of the packing particles and elute later.
- In adsorption chromatography, two modes are defined depending on the relative polarity of the two phases: normal and reversed phase chromatography. In normal phase liquid chromatography, the stationary bed is strongly polar in nature (e.g. silica gel), and the mobile phase is non-polar (such as n-hexane or tetrahydrofuran). Polar samples are thus retained on the hydrophilic surface of the column packing longer than less polar materials. In the reversed phase mode, the stationary bed is non-polar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. In this mode, the more non-polar the material is, the longer it will be retained. Eluent polarity plays the dominant role in all types of HPLC. There are two elution types: isocratic and gradient. In the isocratic mode, constant eluent composition is pumped through the column during the entire analysis. In the gradient mode, the eluent composition (and strength) is steadily changed during the run. Both normal and reversed phase HPLC make up almost 90% of all chromatographic applications. The reversed phase mode is the most widely used in lipid analysis and it is the mode used in this research.

(C) Columns

Several types of columns have been used in HPLC analysis of lipids. Most columns used in HPLC are silica based. The most popular bonded phases are C1, C4, C8, and C18. Silica based adsorbents modified with trimethylchlorosilane (C1) and butyldimethylchlorosilane (C4) have a few applications in HPLC, mainly for protein separation or purification (2). These adsorbents show significant polar interactions, although they do not have specific interactions caused by acidic silanols. Octyl (C8) and octadecyl (C18) modified adsorbents are the most popular ones. Almost 80% of all HPLC separations have been developed with these adsorbents. In this research C18 was one of the columns used. Other columns include:

Phenyl – Propylphenylsilane ligands attached to the silica gel show weak dipole induced dipole interactions with polar analytes. Usually, this type of bonded phase is used for group separation of complex mixtures. Amino compounds show some specific interactions with the phenyl-modified adsorbent (2).

CN – A cyano modified surface is very slightly polar. Columns with this phase are useful for fast separations of mixtures consisting of very different components. These mixtures might show a very broad range of retention times on the usual columns. Cyano columns can be used in both normal and reversed phase modes of HPLC. This column has been used in analyzing lipids (3).

NH₂ – The amino phase is a weak anion-exchanger. This type of column is mainly used in the normal phase mode especially for selective retention of aromatic compounds (2).

Diol – Diols are a slightly polar adsorbent for normal and reversed phase separations.

These materials are useful for the separation of complex mixtures of compounds with different polarities, and which usually show a strong retention on unmodified silica. It has been employed in phospholipid separation (4) and was also used in this research.

Monol – Monols are polar adsorbents like diols. In this research, 1-octene-8-ol was used and its structure makes it adaptable to both normal and reversed phase HPLC.

Different kinds of chromatograms are obtained by using different columns. The particle size, length of column and packing material all affect separation (2). A column's performance is also dependent on other factors such as the mobile phase composition, which leads to some columns performing better under certain conditions. In this research, monol, diol, and C18 columns were used and the chromatograms obtained were evaluated.

(D) Detectors

Detectors equipped with a flow through cell were a major breakthrough in the development of modern liquid chromatography. Current liquid chromatographic detectors have a wide dynamic range normally allowing both analytical and preparative scale runs on the same instrument. They have high sensitivities that result in detection of nanograms of material and the advanced models are very flexible, allowing rapid conversion from one mobile phase to another and from one mode to another. Almost all liquid chromatographic detectors are on-stream monitors. The only relatively successful off-line detector is the FTIR spiral disk monitor, which requires sample transfer on a germanium disk followed by scanning in the FTIR instrument (1). HPLC detectors are always used under continuous flow conditions and the sample is always dissolved in the

eluent during detection. In the last decade, there has been significant progress in the development of HPLC/mass spectrometer interfacing systems. A mass spectrometer as an on-line HPLC detector is the most sensitive, selective and the most universal detector. But it is also the most expensive making it unaffordable to many scientists. There are many different types of detectors available, each with its own limitations (5) and the following four are commonly used for lipid analysis:

- ✓ **Refractive index (RI) detector.** This detector cannot normally be used with a gradient because a small change in the refractive index of the mobile phase as a result of composition change will lead to the generation of negative and unwanted peaks that cannot be analyzed. This results in optimum separation not being achieved. Sensitivity is also poor (6).
- ✓ **Ultraviolet (UV) detector.** The absorption region of 200-300 nm (ester bond) is used to detect triglycerides. However, many suitable solvents for lipid chromatographic separation (e.g. acetone) also absorb in this region and so cannot be used (6). Because acetone and acetonitrile are the most widely used mobile phase solvents as well as the most effective in lipid analysis, UV detection is limited by the aforementioned problem.
- ✓ **Transport flame ionization or moving wire detector.** This detector is no longer commercially available. It could be used with any volatile solvent, and hence solvent gradients. However, it lacked sensitivity as the majority of the eluent was not retained for detection (6).

- ✓ **Infrared (IR) detector.** This detector can be used with gradient elution, although some baseline drift occurs in the absorption region used for lipids (5.75 μm , ester carbonyl region) (5).

A fifth type of detector is the evaporative light scattering detector. This device is a new tool that has been used in lipid analysis because of significant advantages over other detectors. This detector was used in this research. The efficiency, sensitivity, and linearity of the evaporative light scattering detector (ELSD) response are correlated to the structural features of the instrument. In particular the nebulization mode and the cell geometry are the most crucial factors. The type of elution can also affect the ELSD performance. The principle of ELSD applies to all solutes having a lower volatility than the mobile phase used in the separation. In fact, the lower temperatures at which the new generation ELSD can operate allow for the quantitation of semi-volatile analytes, although the detector's sensitivity may be lowered.

Figure 3 illustrates the three main processes that occur successively within an evaporative light scattering detector:

1. nebulization of the chromatographic effluent
2. evaporation of the chromatographic effluent
3. light scattering by the residual particles, ideally constituting the analytes

Each process has a well-defined function but also influences, or is influenced by the other processes. All commercial ELS detectors incorporate the above processes, but differ in the methods by which they are performed. An ELSD is susceptible to non-linear responses (7).

- ◆ **Nebulization.** This step transforms a bulk liquid phase into fine droplets from which the solvent can be more easily vaporized; the larger the droplet size, the higher the temperature needed in the evaporation step. The bigger the residual solute particles, the more significant the scattered light intensity will be. The quality and type of the nebulizing gas employed in the system can affect the instrument performance. The choice of the gas is partly influenced by the mobile phase. Nitrogen and air are widely used as nebulizing gases. Carbon dioxide was used in this research because of its high heat capacity. It is also inexpensive and readily available. These nebulizing gases must have a specific degree of purity and must not contain contaminants with molecular dimensions that can generate noise in the system. To avoid the introduction of contaminants, the nebulizing gas is filtered.
- ◆ **Evaporation.** This process takes place as the droplets are carried by the gas flow from the nebulization chamber into a heated drift tube, where the solvent is completely removed to produce particles or droplets of pure solutes. It is important to use as low a temperature as possible for this process to maintain the uniformity of particle size. In addition, low temperature evaporation enhances solute crystallization; the larger the solute particle, the higher the intensity of the light scattering. Furthermore, it has been clearly demonstrated that solid particles of a specific size will scatter light more effectively than liquid particles of the same size (7). At high temperatures, too rigorous solvent vaporization can cause non-uniform particle sizing or inhibit crystal formation thereby adversely affecting the light scattering process (8).

- ♦ **Detection.** The solute particle cloud emerging from the drift tube enters the optical cell, where the solute passes through a light beam. The amount of light scattered by the solute particles is measured using a photomultiplier or photodiode. The incident light beam is produced either by a polychromatic source in the visible range, such as a simple halogen lamp, or by a monochromatic source such as laser emitting diode. The laser source offers no advantage over the polychromatic source. In fact, a laser light beam will be unable to detect a molecule having a chromophore absorbing at the same wavelength as the laser (9).

The ELSD has the following advantages over other detectors used in lipid analysis:

1. It is suitable for narrow bore columns and in effect less solvent is used
2. It has a stable baseline because it is insensitive to changes in the mobile phase composition
3. There is rapid transit of solutes resulting in a small contribution to band broadening
4. It is easy to use, inexpensive, and has good reproducibility
5. It can be used for direct quantification with a wide range of solvents
6. It is applicable to both isocratic and gradient elution conditions

Fig. 3 Principle of operation of ELSD

(E) Aim of this research

This research has two main objectives. The first is finding the best separations for three different columns: monol, diol and C18. This goal is accomplished by varying the proportions of mobile phase composition (acetone and acetonitrile) and keeping all other parameters the same. Four samples (olive oil, unsalted butter, corn oil and fish oil) are used for this study. The information obtained here will give an insight into how lipids behave chromatographically.

The second objective is to evaluate each column for the different samples. Specifically, the goal is to find (from the three columns) the best column in the analysis of lipids.

Chapter II - EXPERIMENTATION

A. Mobile phase solvents

All mobile phase solvents used for high performance liquid chromatography experiments were HPLC grade. The mobile phase solvents used were acetone (99.6%, Fisher Chemical, Springfield, NJ) and acetonitrile (99.9%, Beckman Co., Palo Alto, CA). Both solvents were chosen because of their favorable properties that include purity, detector compatibility, solubility of the sample, low viscosity, chemical inertness and reasonable price. Acetone was also chosen for its high heat capacity. All the samples that were used in this research were diluted in acetone. In lipid analysis, acetone and acetonitrile are the most common mobile phase solvents although tetrahydrofuran, chloroform and dichloromethane have also been used. The mobile phase solvents were degassed with compressed high purity grade helium from Air Products (Allentown, PA).

The degassing step is important to eliminate possible bubbles that might affect detection since the ELSD is very sensitive to small perturbations in eluent composition.

B. Triglyceride Standard

The standard used in this research was an olive oil triglyceride mixture from Sigma Chemical Company (St. Louis, MO). All components are 99% pure. The percent composition of the five components in the standard mixture are: Trilinolein (2%), 1,2-Dilinoeoyl-rac-glycerol (4%), Triolein (60%), 1,2-Dioleoyl-3-palmitoyl-rac-glycerol (30%) and 1,2-Dioleoyl-3-stearoyl-rac-glycerol (4%). The standard sample was prepared to about 8% concentration (wt/vol) in acetone before use. It was stored under argon at less than 0°C.

C. Samples

The samples used in this research were unsalted butter (Challenge butter), corn oil (100% pure Mazola corn oil), olive oil (Safeway extra-virgin olive oil), and fish oil (Alaska deep sea fish oil). They were purchased from a local grocery store. The unsalted butter was prepared for analysis by melting at 40°C in a water bath and filtering hot through a glass fiber to remove possible impurities and/ or contaminants. The resulting liquid was then dissolved to 8% concentration (wt/vol) in acetone. Olive oil, fish oil and corn oil were also prepared to 8% concentration (wt/vol) in acetone. All samples were completely soluble in acetone. Injections of 20 μL of all sample solutions including the olive oil triglyceride standard were made in each analysis.

D. Instrumentation

The HPLC instrumentation includes a Model 8800 ternary liquid chromatographic pump (Spectra Physics, San Jose, CA) which was used to mix and pump the mobile phase through the column. The pump has the capability of both isocratic and gradient elution. An SP4400 Chromjet Integrator (Spectra Physics, San Jose, CA) was used for data analysis. Detection was done with a laser light scattering detector (from A.Stolyhwo, Gdansk, Poland). The samples were injected through a Rheodyne injector (Cotati, CA) with a 20 μ L loop. A 10 μ L syringe (Hamilton Co., Reno, NV) was used for the injections. Carbon dioxide (from Air Products, Allentown, PA) was used as the driving gas. After each sample injection, the syringe was rinsed with acetone. The injection port was also rinsed with acetone after each sample analysis. This procedure is done in order to eliminate or reduce the possibility of sample contamination from previously injected samples.

E. Analytical column

The three analytical columns used were C18 (Spheri-5-RP18) from Spectra Physics, San Jose, CA. The monol and diol (bonded to Vydac silica) columns were prepared by Dr. J. Pesek's group according to a synthetic method that is explained below:

Synthetic process for bonding 1-octene-8-ol and 7-octene-1, 2-diol to Vydac H

The modification is accomplished via silanization of the silica surface using triethoxysilane reagent (TES). The resulting monolayer of Si-H surface linkages is then reacted with the organic moiety through a hydrosilation reaction (10). For the monol

column, 1-octene-8-ol was attached to the silica surface during the hydrosilation process.

For the diol column, 7-octene-1,2-diol was attached during the hydrosilation process.

The reactions can be described as follows:

(I) TES SILANIZATION

(II) HYDROSILATION

For the monol column:

For the diol column:

(7-octene-1,2-diol bonded to Vydac silica)

Catalysts used are:

- hexachloroplatinic acid
- AIBN - Azo-bis(isobutyronitrile) - free radical initiator

These two columns have been characterized and tested for a variety of applications (10,11). In analyzing the various samples used in this work, the three columns were subjected to the same operating conditions. Each column was equilibrated prior to use.

F. Analytical conditions

All analyses were done under isocratic conditions. The drift tube temperature was set at 63°C and the detection temperature was set at 60°C. The driving gas (CO₂) pressure was 1.25 kg/cm². The attenuation setting was 64. The maximum pressure for the pump was set at 3000 psi and the mobile phase solvent flow rate was 0.3 mL/min.

0.3 mL/min is the maximum flow rate that is allowed through the detector based on the system configuration. Prior to the first analysis on any given day, the instrument (detector, pump, and integrator) were turned on and allowed to warm up for about one hour. During this time, the mobile phase solvents were degassed and allowed to flow through the column while the carbon dioxide was allowed to flow through the detector. All operating conditions except the injection of the samples were monitored during this warm-up period to allow for proper equilibration and checks for any gas leaks that might come from the CO₂ or helium flow to the instrument. At the end of the equilibration, the detector background signal was set at 0 nA. After verifying that the detector signal was steady, the sample was injected. The various mobile phase compositions used were:

1. 100% acetone
2. 90% acetone / 10% acetonitrile
3. 80% acetone / 20% acetonitrile
4. 65% acetone / 35% acetonitrile
5. 50% acetone / 50% acetonitrile
6. 40% acetone / 60% acetonitrile

All compositions are in vol/vol. The analysis times were about 30 min for all samples.

Chapter III – RESULTS AND DISCUSSION

(A) Chromatographic Analysis

The mode used in the present study is reversed-phase HPLC (RP-HPLC). In this method of analysis, the mobile phase composition is one of the most important parameters. The type of mobile phase used will have a significant effect on the retention. It can promote or suppress ionization of the analyte molecules, and can shield accessible active adsorption centers on the support surface. The main requirement for the mobile phase is that it has to dissolve the analytes at concentration levels suitable for detection. Variation of the eluent composition provides great flexibility in HPLC separations. The mobile phase in RP-HPLC has to be polar and compete for the adsorption sites or the stationary phase with the analyte molecules. In most reversed-phase HPLC, mixtures of water and methanol or acetonitrile are used as the mobile phase. However, in lipid analysis using the ELSD detector, acetone and acetonitrile are the solvents of choice. The best weak solvent for lipid analysis seems to be acetonitrile, while the best strong solvent (based on the samples used in this study) is acetone (12). Water is avoided in lipid analysis with the ELSD for two main reasons: (a) some lipids do not dissolve in water and this will affect their resolution and detection and (b) for proper detection in the ELSD, it is important for all the mobile phase solvents to be completely evaporated prior to light scattering. The vaporization of water requires very high cell and drift tube temperatures which could cause problems in the detector. Also at the high temperatures

required for water vaporization in the detector, some low boiling components such as fatty acids begin to evaporate.

C18 column chromatographic analysis

At the start of each sample analysis, a blank run is done to ensure that there are no factors that may affect separation and/ or detection. An example of a blank run is seen in Figure 4A. From this chromatogram, it is seen that the background signal shows no impurities or noise. Figures 4B-9 show the chromatograms obtained from olive oil analysis. The data shows that the shape and number of peaks obtained by varying the mobile phase composition changes as a function of the ratio between the solvents. The peaks seen in the first few minutes of the chromatograms (in many cases unresolved and broad) in all the samples analyzed using the three columns are triglycerides (TGs). Several small sharp peaks from fatty acids (FAs), phospholipids (PLs), hydroperoxides and other impurities in the sample mixture follow. Any reference to small sharp peaks eluting after the TGs in this discussion refer to solutes other than TGs. Figure 4B shows the chromatogram obtained from olive oil at a mobile phase composition consisting of 100% acetone. This mobile phase composition is the least polar since it contains no acetonitrile. A partially resolved TG peak is seen at the beginning of the separation with several small sharp peaks (from trace components of the mixture) following. The fact that this mobile phase composition is the least polar makes it more suitable for the elution of the non-polar components of the sample being analyzed. There is better resolution of the triglyceride peaks in Figure 5 (with an increase in the polarity of the mobile phase by the addition of acetonitrile) than in Figure 4B. In Figure 5 almost all peaks eluted in less than 10 minutes. A further decrease in the acetone content of the mobile phase (Figure 6)

shows better resolution of the TG peaks than in the previous chromatograms (Figs. 4B and 5) and a greater number of peaks are seen. Most of the major peaks were also eluted in less than 10 minutes. There are also several small peaks (from solutes other than TG) eluting. The best resolved TG peaks are seen in Figure 6 when 80% acetone / 20% acetonitrile (vol/vol) was used as the mobile phase composition. In Figure 7, unlike in Figures 4B, 5, 6, the retention time for the major (TG) peaks exceeded 10 minutes. Also seen in the chromatogram (Fig. 7) are fewer small sharp peaks when compared to Figures 4B and 6. Figures 8 and 9 show broad peaks obtained as the concentration of the acetone is further reduced (50% acetone / 50% acetonitrile and 40% acetone / 60% acetonitrile (vol/vol)). The entire chromatograms obtained show only broad peaks with the loss of the small sharp peaks that were seen in Figures 4B, 5, 6, and 7. There was also an increase in the retention times due to an increase in the polarity of the mobile phase.

From the chromatograms obtained, it is seen that as the concentration of acetone is decreased (Figs. 6 and 7) the peaks become sharper and better resolved. A further decrease in the acetone content by the addition of more acetonitrile in the mobile phase (Fig. 8) lead to broad triglyceride peaks. An increase in the acetone content leads to a decrease in the retention time. The best chromatograms were seen in Figure 6. Figure 7 gave a fairly resolved baseline separation whereas Figure 6 gave good resolution, better and sharper peak shape at shorter retention times. Among all samples analyzed in the present work, olive oil was the only mixture that exhibited good resolution at two different mobile phase compositions. The reduction of the acetone content with an increase in the acetonitrile composition of the mobile phase as seen in Figures 8 and 9

gave very broad peaks and further reduction in the acetone content resulted in a chromatogram where peaks could not be distinguished from the baseline.

The chromatograms obtained in reversed phase HPLC analysis of unsalted butter as seen in Figures 10-15 exhibited similar characteristics as other samples analyzed on the C18 column. Figure 10 shows the chromatogram obtained using 100% acetone as the mobile phase. In this chromatogram, a large unresolved peak is seen at the beginning of the analysis followed by several small peaks. Figure 11 followed the same pattern as Fig. 10 but has a broader main peak with more small peaks eluting. There is slight resolution of the early eluting peaks in Figure 12 with several small peaks following afterwards. Also seen is an increase in the retention time. The chromatogram obtained at a mobile phase composition of 65% acetone / 35% acetonitrile is seen in Figure 13. This condition results in good peak resolution. About 38 well-resolved peaks are seen and unlike in Figures 10, 11, and 12 there are no broad peaks. Figures 14 and 15 show the chromatograms obtained at 50% acetone / 50% acetonitrile and 40% acetone / 60% acetonitrile (vol/vol). From these two chromatograms, it is seen that there is loss of resolution with reduction in the acetone content of the mobile phase. Although several peaks are eluted, they are broad and provide little information about the complex sample. Although butter contains many components, very good resolution of the TG peaks was obtained as seen in Figure 13 by using isocratic conditions and one column. In most analysis done on butter, gradient elution conditions and/ or two columns in series are often used (13). More peaks were seen in the unsalted butter analysis compared to all other samples analyzed and this is expected because butter contains the most components

in the mixture as determined in other analyses. The optimum mobile phase condition for the analysis of TG in unsalted butter is 65% acetone / 35% acetonitrile (Fig. 13).

Figures 16-21 show the chromatograms obtained from fish oil analysis using varying mobile phase compositions. In Figure 16 one broad peak is seen within the first few minutes of the analysis. It is followed by a few small peaks. Figure 17 looks like Figure 16 but a broader initial peak is seen and there are fewer small peaks. Almost all peaks were eluted in about 10 minutes. From Figure 18 it is observed that the previously unresolved broad peak seen in Figures 16 and 17 is slightly resolved in this chromatogram. There is also an increase in the retention time as expected with the reduction of the acetone content in the mobile phase. In Figure 19 about seven fairly well resolved TG peaks are seen and all peaks eluted in less than 15 minutes. The mobile phase composition (65% acetone / 35% acetonitrile) that gave this optimum resolution is consistent for all other samples analyzed. At a mobile phase composition of 50% acetone / 50% acetonitrile (Fig. 20), broad peaks are observed. A further reduction in the acetone content in the mobile phase with an increase in the acetonitrile content (Fig. 21), shows less resolved and broader peaks when compared to Figure 20. From the chromatographic data obtained for the fish oil analysis, it is seen that this sample exhibited the least number of peaks (TGs, PLs, FAs and hydroperoxides) in comparison to the other samples analyzed. It could be that fish oil is the simplest of all samples analyzed in the present study and therefore contained the least number of components in the mixture. The best chromatogram is seen in Figure 19. Figures 16, 17, 18, 20, and 21 show loss of resolution with an increase or decrease in the acetone content of the mobile phase from the optimum composition.

The corn oil chromatographic data are seen in Figures 22-27. When 100% acetone was used as the mobile phase (Fig. 22) an unresolved broad peak is seen at the beginning of the analysis followed by several small peaks. Some of the small peaks eluting after the unresolved triglyceride peak are poorly resolved. The mobile phase composition where acetone is the sole component giving rise to the least polar environment is a favorable condition for the elution of the non-polar components of the sample mixtures. A slight resolution of the formally unresolved TG peak (Fig. 22) shown in Figure 23 is achieved by the addition of acetonitrile in the mobile phase which in effect increases the polarity of the mobile phase. Several small peaks are also eluted. A further decrease in the acetone content of the mobile phase (Fig. 24) shows better resolution of the peaks in comparison to Figs. 22 and 23. Also seen in this chromatogram (Fig. 24) are fewer small peaks and an increase in the retention time. All the major peaks (TGs) eluted in less than 10 minutes. At 65% acetone / 35% acetonitrile (Fig. 25) about thirteen fairly well resolved TG peaks and a few small peaks are observed. There is also an increase in the retention time when compared to Figures 22-24. As the concentration of acetonitrile is further increased in the mobile phase, there is loss of resolution and the number of small peaks eluting within the analysis time decreases (Fig. 26). This is because the solutes become strongly retained in the column with an increase in polarity. A further increase in retention time and loss of resolution is seen in Figure 27 as the acetonitrile content of the mobile is further increased. The optimum chromatogram obtained from this analysis is shown in Figure 25. An increase or decrease in the acetone content after this optimum condition gave fewer and/ or broader peaks.

Chromatograms of the olive oil triglyceride standard (Figures 28-30) were obtained to compare to the separations for the samples purchased from the grocery store. It was also used as a guide in identifying the various peaks eluted. The success of the analysis depends on the ability of the instrument to resolve and detect the various known compounds in the standard mixture. The olive oil standard used in this research contains five components. Good resolution and detection of the five peaks will confirm the effectiveness of the analysis. Figure 28 shows well-resolved triglyceride peaks and a few small peaks. In Figure 29, four peaks (with one of the peaks consisting of two triglycerides) are shown but having a longer retention time when compared to Figure 28. Well-resolved but slightly broader TG peaks are seen in Figure 30. In Fig. 30, all five TG peaks are seen confirming the effectiveness of the separation. Also observed is an increase in the retention time. The optimum mobile phase composition of 65% acetone / 35% acetonitrile used in achieving this separation and was also the most effective in the C18 analyses of oils. The chromatograms obtained from the standard also follow the same pattern (a decrease in retention time with an increase in the acetone content of the mobile phase) as all of the previous samples analyzed. Comparing the data obtained for analyzing olive oil from the grocery store and the olive oil standard under the same analytical conditions (Figs. 5 and 28, 6 and 29, 7 and 30), there are similarities in the peaks detected. In Fig. 29, all the TG peaks eluted in less than ten minutes with the most retained peak (the major TGs) appearing at about five minutes. The same result is seen in Fig 6. In Fig. 5, the first few peaks eluting have symmetrical peak shape as those seen in Fig. 28. In Fig. 7, the peaks (two peaks that were not baseline resolved) seen at about ten minutes exhibited the same symmetrical peak shape as those seen in Fig. 30. A slightly

broad peak barely discernible from the baseline observed at about the seventeenth minute in Fig. 7 is also evident in Fig. 30. The sample from the grocery store however had more peaks which is most likely the result of the presence of compounds produced in the processing of the raw material. The slight differences observed in peak retention times when comparing the olive oil and olive oil standard is most likely due to changes in the column properties and/ or temperature. The aim of the present study is not the identification of the HPLC peaks but a comparison of the chromatograms obtained under various mobile phase compositions in order to determine the best separation conditions.

Diol column chromatographic analysis

The chromatograms obtained from reversed-phase HPLC analysis of olive oil using the diol column are shown in Figures 31-34. Figure 31 with a mobile phase composition of only acetone contains one broad unresolved peak within 10 minutes of the start of the analysis. Also eluted with this broad peak are several small peaks. Fig. 32 which resulted from increased polarity of the mobile phase shows more peaks eluting after the broad triglyceride peak. At a 80% acetone / 20% acetonitrile mobile phase composition (Fig. 33) an unresolved broad peak is seen and almost all small peaks seen at higher acetone concentration are gone. The explanation could be that for olive oil (which is a simple oil), as the polarity of the mobile phase is increased by the decrease in the acetone content of the mobile phase, the polar components of the sample mixture are so strongly retained that their detection is not seen within the 30 minutes duration of the analysis. A further decrease in the acetone content as seen in Figure 34 shows one unresolved peak. It is important to note the dramatic effect of the bonded phase on

separation as seen by comparing Figures 34 and 7 where the only difference is with the column. Whereas Figure 7 shows nicely resolved TG peaks, Figure 34 revealed only a single unresolved TG peak.

The chromatographic data from unsalted butter using the diol column are shown in Figures 35-38. In Figure 35, the chromatogram generally looks like the one shown in Fig. 31 except that the peaks are greater in number and not as well resolved. Unsalted butter being a complex oil is the most difficult to resolve with the diol column. Figure 36 has similar characteristics as Fig. 35 but has more peaks eluting with a reduction in the mobile phase polarity. A further increase in the acetonitrile content of the mobile phase as seen in Fig. 37 resulted in a reduction of the number of small peaks eluting. This reduction in the number peaks with an increase in the mobile phase polarity is a pattern seen in all samples analyzed using the diol column. From Figures 37 and 38, it is seen that more peaks were detected in unsalted butter than olive oil (Figs. 33 and 34) under the same conditions and is in agreement with the fact that butter is more complex and contains more components. It should be noted from Figures 35-38 that the unresolved peak at the beginning of the separation became broader with an increase in the acetone content of the mobile phase. This could be because as the concentration of acetone is increased, the more polar components are eluting with a decrease in the retention time, resulting in poor resolution.

Figures 39-42 show fish oil chromatograms. Fewer peaks are seen in these chromatographic data when compared to other samples analyzed (olive oil, unsalted butter and corn oil). This is the same result obtained for the analysis using the C18 and monol columns. Fish oil as stated earlier is the simplest of all the samples used in the

present study. Fig. 39, which was obtained under the least polar condition of the mobile phase, shows one broad peak and a few small peaks. Fewer peaks are seen with the introduction of acetonitrile in the mobile phase (Fig. 40). A further decrease in the acetone content as seen in Fig. 41 shows only a single broad peak. Figure 42 exhibited the same pattern seen in Fig. 41. This pattern of reduction in the number of peaks with an increase in the polarity of the mobile phase is in agreement with the general behavior seen in all samples analyzed using the diol column.

The corn oil chromatograms are shown in Figs. 43-47. More peaks are seen in Figures 44 and 45. Unlike the chromatograms obtained in the C18 analysis (which showed optimum separation at a specific mobile phase composition of 65% acetone / 35% acetonitrile for all samples analyzed), the diol column analysis show no specific optimum mobile phase composition for any of the samples analyzed. The optimum conditions for the separations vary from sample to sample but generally a greater number of peaks are seen at high acetone concentration. Corn oil, which is also a simple oil, shows fewer peaks than unsalted butter. From corn oil analysis, the least number of peaks is seen in Fig. 43 while the greatest number of peaks is seen in Fig. 45. This corresponds to an optimum mobile phase of 80% acetone / 20% acetonitrile (v/v).

The olive oil triglyceride standard chromatographic data are shown in Figures 47 and 48. Both chromatograms show an unresolved TG peak. From these chromatograms, it is confirmed that the diol column can not be used for the analysis of triglycerides under the conditions used in the present study. The five components that make up the olive oil triglyceride standard were not separated with the diol column. That is unlike the well-resolved peaks seen in the C18 analysis (Fig. 30). From Figs. 47 and 48, it is confirmed

that the broad peaks seen at the beginning of the analysis for all samples analyzed were unresolved triglyceride peaks. The obvious effect of the bonded phase is revealed when comparing Fig. 28 (from the C18 analysis) which shows nicely resolved peaks to Fig. 48 (from the diol column) which shows a single unresolved peak. From the chromatographic data, it is seen that as the acetone concentration is increased, the number of peaks eluting increased. The small peaks eluting at higher concentrations of acetone are from the polar component of the sample mixture. The chromatograms with the most number of peaks (from the diol column analysis) were seen at mobile phase compositions from 80% acetone / 20% acetonitrile to 100% acetone (vol/vol) depending on the sample. Elution is in the same order as with C18 but less resolution is obtained especially for the more complex samples.

Monol column chromatographic analysis

The chromatograms obtained from reversed-phase HPLC analysis of olive oil using the monol column are seen in Figures 49-52. Figure 49 which was eluted under a mobile phase composition of only acetone show several well-resolved peaks from the polar components of the sample analyzed. When Fig. 49 is compared to Fig. 31, it is seen that more and better-shaped peaks were eluted when the monol column was used. In Fig. 50 fewer peaks eluted as the polarity of the mobile phase was increased with the addition of acetonitrile to the mobile phase (following the same pattern seen in diol column analysis). With a further decrease in the acetone content of the mobile phase as seen in Figs. 51 and 52, fewer peaks eluted. When an equal amount of both solvents in the mobile phase (50% acetone / 50% acetonitrile) as seen in Figure 52 was used, only

one broad peak was seen and the other peaks were barely discernible from the baseline. These broad unresolved peaks seen in each chromatogram on all samples analyzed using the monol and diol columns are triglyceride peaks. The smaller peaks seen may result from fatty acids, phospholipids, impurities and other non-polar components of the sample. Also eluting are oxidation products (which occur with time as a result of constant opening and closing of the sample bottles and other environmental factors) of the samples being analyzed. These oxidative products were not identified because the goal of the present study was to compare the chromatograms obtained from the samples analyzed using three different columns under the same operational conditions and not the identification of individual peaks.

Shown in Figures 53-55 are chromatograms of unsalted butter. In Fig. 53 better resolution of peaks are seen when compared to Fig. 35 (from the diol column analysis). Figure 54 shows more peaks eluting with the introduction of acetonitrile in the mobile phase. When Fig. 36 (from the diol column analysis) is compared to Fig. 54, the resemblance in the two chromatograms is evident. In Fig. 36, there is clustering of peaks following the elution of a single broad peak. The same pattern is observed in Fig. 54. This chromatographic resemblance is seen in almost all samples studied using the monol and diol columns. Fig. 55 shows a similar pattern as Fig. 54 but with fewer peaks eluting as the polarity of the mobile phase is increased.

For fish oil analysis using the monol column, the chromatograms obtained are shown in Figs. 56-59. Comparing these chromatograms with those obtained from the diol column (Figs. 39-42) similar peak patterns are seen. However a greater number of peaks eluted for all samples analyzed using the monol column. The number of peaks

from the fish oil analysis were fewer than that of the other samples analyzed in agreement with the data obtained from both the diol and C18 column. As expected, with a more polar mobile phase fewer number of peaks are seen as in Figures 58 and 59.

The corn oil chromatograms are seen in Figures 60-63. Comparing these chromatographic data with that from the diol column analysis (Figs. 43-46), similar peak patterns were observed but with more peaks eluting in the monol analysis. When Figures 60-63 are compared to Figures 22-25 (which were obtained from the C18 analysis) the difference was obvious. In Figure 25 a well-resolved triglyceride peak is seen whereas in Figure 45 (from the diol column) and in Fig. 61 (from the monol column) an unresolved triglyceride peak is seen. This was true for all samples analyzed at a mobile phase composition of 65% acetone / 35% acetonitrile.

Figures 64-66 show the chromatograms from the olive oil triglyceride standard. These data also show an unresolved triglyceride peak. Figure 64 in comparison to Fig. 47 shows more peaks in agreement with the trend (elution of a greater number of peaks) observed in comparing the diol and monol column sample analysis. From these chromatographic data, it is seen that as the concentration of the acetone is increased, there is an increase in the number of peaks eluting and a reduction in the retention times. It is also noticed that unlike in the C18 sample analysis, there was no one particular optimum mobile phase composition where the best chromatogram was obtained for all the samples studied in this research. The optimum mobile phase compositions were from 80% acetone / 20% acetonitrile to 100% acetone (vol/vol) depending on the sample. This is also the case with the data from the diol column analysis. The data from the standard sample analysis confirmed that under the conditions tested in this study, the monol

column did not resolve triglyceride peaks. It is important to note that there were variations in the peak width of unresolved TG peaks as the mobile phase composition is varied. This was more evident in the analysis done with the monol and diol columns (Figs. 32, 33, 43, 44, 58 and 59). There were also chromatograms where the unresolved TG peak width were about the same (53, 54, 55 or 56, 57 and 58). These variations suggest that varying mobile phase composition may be a factor in the detector response, which in effect may affect other peaks (PLs, FAs, oxidation products and impurities) eluting. As stated earlier in the thesis, prior to light scattering of the solutes in the detector, the mobile phase solvents are vaporized, however solutes retain some degree of solvation and this may affect detector response. The effect of mobile phase variation on the detector response was not studied in this research and more data are necessary to discuss this point further. The chromatograms in the present study are analyzed based on the peaks seen on the x-axis (time) without consideration for the differences that might result due to change and/or compensation on the y-axis (response).

(B) Effect of mobile phase polarity on columns

Mobile phase polarity has a dramatic effect on the separation of lipids by HPLC as seen from these chromatograms. This is a result of two main factors: (a) competition between the mobile phase and the lipid components for the stationary phase and (b) the increase or decrease in lipid solubility in the mobile phase which can enhance or retard their separation. The polarity effect is chiefly the result of the number of double bonds in the triglyceride, fatty acids or phospholipid molecule, as well as the hydrophobicity due to the number of carbon atoms in the fatty acids making up the lipid. The effect of an

increase in acetone or acetonitrile content is clearly evident from the chromatograms obtained from the various samples analyzed. In C18 column analysis, the chromatograms shown in Figures 7, 13, 19, 25 and 30 were achieved using 65% acetone/35% acetonitrile and the results were satisfactory with some baseline separation. In this reversed-phase analysis, increasing the acetone content decreased not only the separation time but also the resolution, resulting in the clustering of peaks and preventing baseline separation. A further increase in the acetone content impaired resolution as seen in Figures 4B, 10, 16 and 22. A decrease in acetone concentration resulted in band broadening to the extent that detection was impaired as seen in Figures 8,9,14,15,20,21,26 and 27. This band broadening effect was not seen in monol and diol column analysis (Figs.52, 59, and 63). This behavior illustrates the effect of bonded phase content on the partitioning effect of such columns and the retention of triglycerides, fatty acids and phospholipids. The explanation for these broad peaks obtained at low acetone concentration in the C18 column could be attributed to three factors: (1) the sample solution is diluted in a rather poor solvent; (2) the sample is so strongly retained that they are eluted only after a prohibitively long time giving broad peaks and (3) mass transfer effects.

For the monol and diol columns, the chromatograms obtained exhibit similar characteristics. It is important to note that these two columns have minor but significant differences. A greater number of peaks are eluted as the concentration of acetone is increased. The higher the concentration of acetone, the less polar the composition of the mobile phase and the faster the elution of the polar constituents of the sample. At this high concentration of acetone the polar components of the sample such as triglycerides, phospholipids and fatty acids are increasingly eluted. Since both the monol and diol

columns are less hydrophobic, these compounds are eluted slower under these conditions than in the C18 column. The optimum mobile phase composition for the analysis of these lipid samples using the monol and diol columns appeared to be from 80% acetone / 20% acetonitrile to 100% acetone. A decrease in the acetone content of the mobile phase in both the monol and diol columns (Figs. 52, 59 and 63) resulted in fewer peaks eluting. In general, the peaks obtained from the monol column in comparison to the diol column were more numerous, sharper and the retention times were shorter. Looking at Figures 31 and 49, it can be seen that the latter chromatogram contained sharper and a greater number peaks. The explanation could be because the diol moiety has an extra polar (hydroxyl) group attached to its structure and in effect the polar components (specifically TGs, PLs and FAs) may be interacting with the stationary phase to a greater extent thereby increasing their retention times.

As seen from the various chromatograms, the separation of lipids varies with the column type. The nature of the bonded phase plays a very important role in lipid separation. Looking at the structure of the active sites (Table 1), the monol column is unique. It contains an eight-carbon chain with the functional group in the terminal position. The monol exhibits both hydrophobic and hydrophilic properties making it applicable in both normal and reversed phase chromatography. The diol column also has eight-carbon chain but with two hydroxyl groups attached to it making it more polar and in effect more hydrophilic than the monol column. However, the carbon chain content (the hydrophobic portion) makes it also applicable to both reversed and normal phase separation. It is the least hydrophobic of the three columns. The C18 is the most hydrophobic and has the longest carbon chain in its structure. It does not have any polar

group attached thereby making it applicable to only reversed phase chromatography. From the results obtained in this analysis, the reversed phase C18 gave the best overall analysis. The monol column gave better chromatograms when compared to the diol column. This is in agreement with the fact that reversed phase C18 analysis is the most widely used in lipid (especially triglyceride) analysis (12). Under the analytical conditions employed in the present study, the monol and diol columns could be used in the analysis of oxidation products, fatty acids and impurities but not triglycerides. The C18 column however can be used in the analysis of triglycerides, phospholipids, fatty acids, oxidation products and impurities.

Table 1

Structure of active sites on the various columns

<u>Column type</u>	<u>Active site</u>
C18	$-\text{Si}-(\text{CH}_2)_{17}\text{CH}_3$
Diol	$\begin{array}{c} \text{OH} \\ \\ -\text{Si}-(\text{CH}_2)_6-\text{CH}-\text{CH}_2-\text{OH} \end{array}$
Monol	$-\text{Si}-(\text{CH}_2)_6-\text{CH}_2-\text{CH}_2-\text{OH}$

Triglycerides have been analyzed by gas chromatography using polar columns with high thermal stability but less severe conditions however are utilized when they are subjected to HPLC analysis (3). Many types of detectors, such as IR, UV, and flame ionization detector have been tested for the quantification of triglycerides, some showing gradient incompatibility and some displaying non-homogenous detection. ELSD allows the use of a wide range of elution conditions, which vary depending on the nature of the matrix and the fraction or component that are to be quantified. Although the ELSD is a destructive detector, its good sensitivity level allows the insertion of a three way valve just before the detector which makes the splitting of the eluate possible; the various fractions can then be collected and later subjected to GC analysis for the determination of the corresponding fatty acid composition. Both normal and reversed phase modes have been used for TG analysis but reversed phase HPLC is the most widely used.

The analysis of phospholipids (PLs) is of great importance in the food chemistry and cosmetics fields, and also from biological and metabolic standpoints. PLs are high molecular weight polar compounds that do not present a characteristics absorption profile. When injected into a gas chromatograph, they decompose into diglycerides and under controlled conditions, they give rise to a reproducible series of decomposition products that hamper their study in natural matrices. Regarding HPLC, different detectors have been tested but better qualitative and quantitative results have been obtained with the ELSD (3). Both normal and reversed phase methods have been proposed as analytical alternatives for the determination of phospholipids.

Lipids can easily undergo oxidation. This reaction can occur *in vivo*, in pure lipid systems and lipid containing foods following either chemical or enzymatic pathways. In some cases, oxidation leads to the formation of some compounds whose toxicity has been asserted or is still to be confirmed (3). In the first phase of the chemical oxidation process, hydroperoxides are formed which are commonly known as primary oxidation products. These compounds are unstable and can decompose into a series of secondary products that vary with respect to the chemical functional groups and to the molecular dimensions. Owing to the wide range of products generated from a single molecule, the analytical evaluation of the oxidation products is a difficult task, hence good resolving power is required in addition to a high sensitivity level (3). Considering these facts, GC has often been chosen for the quantification of lipid oxidation products; this choice is supported by the elevated sensitivity level of GC and the possibility of confirming the compound identity by coupling the GC instrument with a mass spectrometer. GC however is not suitable for the study of hydroperoxides since these compounds degrade in the injection port. HPLC coupled to a selective detector and an ELSD instead allows the study of both complex matrices and hydroperoxides. It would therefore be advisable to perform the quantitative analysis of the oxidation products with ELSD since it can provide a measurement of the single compounds and the whole oxidation process.

In the present work, isocratic elution was chosen because of the need for a rapid routine method for the analysis of triglycerides even though gradient elution gives a better separation of triglycerides, phospholipids, oxidation products and fatty acids in complex oils such as butter. In this research, it was possible to compare a large number of experimental conditions using different stationary phases.

(C) Detector performance

Several factors affect the ELSD sensitivity and they include:

Gas quality: CO₂ is used in this study for reasons that have been stated earlier. It is recommended that gasses from all sources be filtered through a sub-micron particle filter before entering the ELSD. This will eliminate a possible source of background noise.

Solvent quality: HPLC grade solvents are preferred. It is important to make sure that these solvents do not have particles in them. The particle content of the solvents is important because such impurities will contribute significantly to background noise.

Column contributions: It is known that certain types of column, and also columns of the same type from different manufacturers, may exhibit varying degrees of hydrolytic instability under certain solvent conditions. Such instability is manifested by non-volatile sub-micron particles of packing material appearing in the effluent: these particles will be detected by the ELSD and contribute to the noise level.

Contributions from each of the above factors should be determined sequentially and periodically, or whenever baseline deterioration occurs. These contributions can be obtained in detector millivolt readouts or by plotting the output signal on a suitable recording device. Fig. 4A is an example of a blank run that shows no background noise or interference. The geometry of the nebulizer is also a critical factor of the detector efficiency. The flow rate of the driving gas should be kept low, in order to increase the solute particle dimensions and thus the sensitivity of the detector. The detector temperature should be adjusted to maximize the signal to noise ratio. If the temperature is too low, the mobile phase is not fully evaporated resulting in a baseline drift. On the

other hand, at higher temperatures, some low boiling components such as free fatty acids begin to evaporate. The main drawback of the ELSD is that it detects the non-volatile compounds under working conditions, usually those with lower volatility than the mobile phase. This is not an important drawback because a large portion of analytes are less volatile than commonly used mobile phases.

Chapter IV- CONCLUSION

Three columns (C18, monol and diol) have been evaluated from the chromatograms obtained by varying the mobile phase composition. This section will summarize the findings and suggest additional work that may be considered in the future.

The data from the research shows clearly that decreasing the solvent polarity by the addition of more acetone resulted in a decrease in the elution time of lipids. This was also due to the combination of both solubility and partitioning effects. Decreasing the solvent polarity by increasing the acetone content also results in a reduction in the retention times and an increase in the elution of the polar components (such as TGs, PLs, FAs and oxidation products) of the sample mixture. The optimum mobile phase composition was different for the various columns. From the results obtained, a mobile phase of 65% acetone/35% acetonitrile (vol/vol) would seem the most appropriate in analyzing mixtures of triglycerides using the C18 column. For the monol and diol columns the optimum mobile phase compositions were from 80% acetone / 20% acetonitrile to 100% acetone and these stationary phases are more applicable to phospholipid, fatty acids and oxidation products analysis.

Based on the overall data, the C18 column had the best performance, followed by ~~the~~ monol column and finally the diol column. The monol and diol columns however did not resolve the triglyceride peaks. This result agrees with the fact that most lipid separations are done with C18 in the reversed phase mode.

Future work should include the use of the gradient elution mode in analyzing these samples to improve the separation. It is known that for complex oil separations,

gradient elution generally gives better results (12). Also, the identification of the various peaks obtained will help determine the similarities and differences between the make up of the various samples. It is known that these samples contain some of the same triglycerides.

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APPENDIX (Figures)

Fig. 4A : Isocratic HPLC analysis. *Sample:* Blank, *Column:* Spheri-5-RP-18, *Mobile Phase:* 100% Acetone, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 4B : Isocratic HPLC analysis. *Sample:* Olive Oil, *Column:* Spheri-5-RP-18, *Mobile Phase:* 100% Acetone, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 5 : Isocratic HPLC analysis. *Sample:* Olive Oil, *Column:* Spheri-5-RP-18, *Mobile Phase:* 90% Acetone/10% Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 6 : Isocratic HPLC analysis. ***Sample:*** Olive Oil, ***Column:*** Spheri-5-RP-18, ***Mobile Phase:*** 80% Acetone/20% Acetonitrile, ***Flow Rate:*** 0.3ml/min, ***Attenuation:*** 64

Fig. 7 : Isocratic HPLC analysis. ***Sample:*** Olive oil, ***Column:*** Spheri-5-RP-18, ***Mobile Phase:*** 65% Acetone/35% Acetonitrile, ***Flow Rate:*** 0.3ml/min, ***Attenuation:*** 64

Fig. 8 : Isocratic HPLC analysis. ***Sample:*** Olive oil ***Column:*** Spheri-5-RP-18, ***Mobile Phase:*** 50% Acetone/50% Acetonitrile, ***Flow Rate:*** 0.3ml/min, ***Attenuation:*** 64

Fig. 9 : Isocratic HPLC analysis. **Sample:** Olive Oil, **Column:** Spheri-5-RP-18, **Mobile Phase:** 40% Acetone/60% Acetonitrile, **Flow Rate:** 0.3ml/min, **Attenuation:** 64

Fig. 10 : Isocratic HPLC analysis. ***Sample:*** Unsalted butter, ***Column:*** Spheri-5-RP-18, ***Mobile Phase:*** 100% Acetone, ***Flow Rate:*** 0.3ml/min, ***Attenuation:*** 64

Fig. 11 : Isocratic HPLC analysis. ***Sample:*** Unsalted butter, ***Column:*** Spheri-5-RP-18, ***Mobile Phase:*** 90% Acetone/10% Acetonitrile, ***Flow Rate:*** 0.3ml/min, ***Attenuation:*** 64

Fig. 12 : Isocratic HPLC analysis. **Sample:** Unsalted butter, **Column:** Spheri-5-RP-18, **Mobile Phase:** 80% Acetone/20% Acetonitrile, **Flow Rate:** 0.3ml/min, **Attenuation:** 64

Fig. 13 : Isocratic HPLC analysis. *Sample*: Unsalted butter, *Column*: Spheri-5-RP-18, *Mobile Phase*: 65% Acetone/35% Acetonitrile, *Flow Rate*: 0.3ml/min, *Attenuation*: 64

Fig. 14 : Isocratic HPLC analysis. **Sample:** Unsalted butter, **Column:** Spheri-5-RP-18, **Mobile Phase:** 50% Acetone/50% Acetonitrile, **Flow Rate:** 0.3ml/min, **Attenuation:** 64

Fig. 15 : Isocratic HPLC analysis. ***Sample:*** Unsalted butter, ***Column:*** Spheri-5-RP-18, ***Mobile Phase:*** 40% Acetone/60% Acetone, ***Flow Rate:*** 0.3ml/min, ***Attenuation:*** 64

Fig. 16 : Isocratic HPLC analysis. *Sample:* Fish Oil, *Column:* Spheri-5-RP-18, *Mobile Phase:* 100% Acetone, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 17 : Isocratic HPLC analysis. *Sample:* Fish Oil, *Column:* Spheri-5-RP-18, *Mobile Phase:* 90% Acetone/10% Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 18 : Isocratic HPLC analysis. *Sample:* Fish Oil, *Column:* Spheri-5-RP-18, *Mobile Phase:* 80% Acetone/20% Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 19 : Isocratic HPLC analysis. *Sample:* Fish Oil, *Column:* Spheri-5-RP-18, *Mobile Phase:* 65% Acetone/35% Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 20 : Isocratic HPLC analysis. **Sample:** Fish oil, **Column:** Spheri-5-RP-18, **Mobile Phase:** 50% Acetone/50% Acetonitrile, **Flow Rate:** 0.3ml/min, **Attenuation:** 64

Fig. 21 : Isocratic HPLC analysis. *Sample:* Fish Oil, *Column:* Spheri-5-RP-18, *Mobile Phase:* 40% Acetone/60% Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 22 : Isocratic HPLC analysis. *Sample:* Corn Oil, *Column:* Spheri-5-RP-18, *Mobile Phase:* 100% Acetone, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 23 : Isocratic HPLC analysis. *Sample:* Corn Oil, *Column:* Spheri-5-RP-18, *Mobile Phase:* 90% Acetone/10% Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 24 : Isocratic HPLC analysis. *Sample:* Corn Oil, *Column:* Spheri-5-RP-18, *Mobile Phase:* 80% Acetone/20% Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 25: Isocratic HPLC analysis. *Sample:* Corn Oil, *Column:* Spheri-5-RP-18, *Mobile Phase:* 65% Acetone/35% Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 26 : Isocratic HPLC analysis. ***Sample:*** Corn Oil, ***Column:*** Spheri-5-RP-18, ***Mobile Phase:*** 50% Acetone/50% Acetonitrile, ***Flow Rate:*** 0.3ml/min, ***Attenuation:*** 64

Fig. 27 : Isocratic HPLC analysis. *Sample*: Corn Oil, *Column*: Spheri-5-RP-18, *Mobile Phase*: 40% Acetone/60% Acetonitrile, *Flow Rate*: 0.3ml/min, *Attenuation*: 64

Fig. 28 : Isocratic HPLC analysis. **Sample:** Olive Oil Std, **Column:** Spheri-5-RP-18, **Mobile Phase:** 90% Acetone/10% Acetonitrile, **Flow Rate:** 0.3ml/min, **Attenuation:** 64

Fig. 29 : Isocratic HPLC analysis. *Sample*: Olive Oil Std, *Column*: Spheri-5-RP-18, *Mobile Phase*: 80% Acetone/20% Acetonitrile, *Flow Rate*: 0.3ml/min, *Attenuation*: 64

Fig. 30 : Isocratic HPLC analysis. **Sample:** Olive Oil Std, **Column:** Spheri-5-RP-18, **Mobile Phase:** 65% Acetone/35% Acetonitrile, **Flow Rate:** 0.3ml/min, **Attenuation:** 64

Fig. 31 : Isocratic HPLC analysis. *Sample:* Olive oil, *Column:* Diol, *Mobile Phase:* 100% Acetone, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 32 : Isocratic HPLC analysis. *Sample:* Olive oil, *Column:* Diol, *Mobile Phase:* 90% Acetone/10% Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 33 : Isocratic HPLC analysis. *Sample:* Olive oil, *Column:* Diol, *Mobile Phase:* 80% Acetone/20% Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 34 : Isocratic HPLC analysis. *Sample*: Olive oil, *Column*: Diol, *Mobile Phase*: 65% Acetone/35% Acetonitrile, *Flow Rate*: 0.3ml/min, *Attenuation*: 64

Fig. 35 : Isocratic HPLC analysis. *Sample:* Unsalted butter, *Column:* Diol, *Mobile Phase:* 100% Acetone, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 36 : Isocratic HPLC analysis. ***Sample:*** Unsalted butter, ***Column:*** Diol, ***Mobile Phase:*** 90% Acetone/10% Acetonitrile, ***Flow Rate:*** 0.3ml/min, ***Attenuation:*** 64

Fig. 37 : Isocratic HPLC analysis. ***Sample:*** Unsalted butter, ***Column:*** Diol, ***Mobile Phase:*** 80% Acetone/20% Acetonitrile, ***Flow Rate:*** 0.3ml/min, ***Attenuation:*** 64

Fig. 38 : Isocratic HPLC analysis. *Sample:* Unsalted butter, *Column:* Diol, *Mobile Phase:* 65% Acetone/35% Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 39 : Isocratic HPLC analysis. *Sample*: Fish oil, *Column*: Diol, *Mobile Phase*: 100% Acetone, *Flow Rate*: 0.3ml/min, *Attenuation*: 64

Fig. 40 : Isocratic HPLC analysis. *Sample:* Fish oil, *Column:* Diol, *Mobile Phase:* 90%Acetone/10%Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 41 : Isocratic HPLC analysis. *Sample*: Fish oil, *Column*: Diol, *Mobile Phase*: 80% Acetone/20%Acetonitrile, *Flow Rate*: 0.3ml/min, *Attenuation*: 64

Fig. 42 : Isocratic HPLC analysis. *Sample:* Fish oil, *Column:* Diol, *Mobile Phase:* 65% Acetone/35%Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 43 : Isocratic HPLC analysis. *Sample:* Corn oil, *Column:* Diol, *Mobile Phase:* 100% Acetone, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 44 : Isocratic HPLC analysis. *Sample*: Corn oil, *Column*: Diol, *Mobile Phase*: 90% Acetone/10%Acetonitrile, *Flow Rate*: 0.3ml/min, *Attenuation*: 64

Fig. 45 : Isocratic HPLC analysis. *Sample:* Corn oil, *Column:* Diol, *Mobile Phase:* 80% Acetone/20%Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 46 : Isocratic HPLC analysis. *Sample:* Corn oil, *Column:* Diol, *Mobile Phase:* 65% Acetone/35%Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 47 : Isocratic HPLC analysis. *Sample:* Olive oil std, *Column:* Diol, *Mobile Phase:* 100% Acetone, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 48 : Isocratic HPLC analysis. *Sample:* Olive oil std, *Column:* Diol, *Mobile Phase:* 90% Acetone/10%Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 49 : Isocratic HPLC analysis. *Sample:* Olive oil, *Column:* Monol, *Mobile Phase:* 100% Acetone, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 50 : Isocratic HPLC analysis. *Sample:* Olive oil, *Column:* Monol, *Mobile Phase:* 90% Acetone/10%Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 51 : Isocratic HPLC analysis. *Sample:* Olive oil, *Column:* Monol, *Mobile Phase:* 65% Acetone/35%Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 52 : Isocratic HPLC analysis. *Sample:* Olive oil, *Column:* Monol, *Mobile Phase:* 50% Acetone/50%Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 53: Isocratic HPLC analysis. *Sample:* Unsalted butter, *Column:* Monol, *Mobile Phase:* 100% Acetone, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 54 : Isocratic HPLC analysis. *Sample*: Unsalted butter, *Column*: Monol, *Mobile Phase*: 90% Acetone/10%Acetonitrile, *Flow Rate*: 0.3ml/min, *Attenuation*: 64

Fig. 55 : Isocratic HPLC analysis. *Sample:* Unsalted butter, *Column:* Monol, *Mobile Phase:* 65% Acetone/35%Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 56 : Isocratic HPLC analysis. *Sample:* Fish oil, *Column:* Monol, *Mobile Phase:* 100% Acetone, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 57 : Isocratic HPLC analysis. *Sample:* Fish oil, *Column:* Monol, *Mobile Phase:* 90% Acetone/10%Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 58 : Isocratic HPLC analysis. *Sample:* Fish oil, *Column:* Monol, *Mobile Phase:* 65% Acetone/35%Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 59 : Isocratic HPLC analysis. *Sample:* Fish oil, *Column:* Monol, *Mobile Phase:* 50% Acetone/50%Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 60 : Isocratic HPLC analysis. *Sample:* Corn oil, *Column:* Monol, *Mobile Phase:* 100% Acetone, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 61 : Isocratic HPLC analysis. *Sample:* Corn oil, *Column:* Monol, *Mobile Phase:* 90% Acetone/10%Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 62 : Isocratic HPLC analysis. *Sample:* Corn oil, *Column:* Monol, *Mobile Phase:* 65% Acetone/35%Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 63 : Isocratic HPLC analysis. *Sample:* Corn oil, *Column:* Monol, *Mobile Phase:* 50% Acetone/50%Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 64 : Isocratic HPLC analysis. *Sample:* Olive oil std, *Column:* Monol, *Mobile Phase:* 100% Acetone, *Flow Rate:* 0.3ml/min, *Attenuation:* 64

Fig. 65 : Isocratic HPLC analysis. ***Sample:*** Olive oil std, ***Column:*** Monol, ***Mobile Phase:*** 90% Acetone/10%Acetonitrile, ***Flow Rate:*** 0.3ml/min, ***Attenuation:*** 64

Fig. 66 : Isocratic HPLC analysis. *Sample:* Olive oil std, *Column:* Monol, *Mobile Phase:* 65% Acetone/35%Acetonitrile, *Flow Rate:* 0.3ml/min, *Attenuation:* 64