DEVELOPMENT AND APPLICATION OF A SPECTROPHOTOMETRIC AND CHEMOMETRIC METHOD FOR THE DIRECT DETERMINATION OF LIPIDS IN SERUM AND SYNTHETIC MIXTURES

By

LISA M. REILLY

Bachelor of Science in Chemistry
Mercyhurst College
Erie, Pennsylvania
2000

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 2006

DEVELOPMENT AND APPLICATION OF A

SPECTROPHOTOMETRIC AND

CHEMOMETRIC METHOD FOR

THE DIRECT DETERMINATION

OF LIPIDS IN SERUM

AND SYNTHETIC

MIXTURES

Dissertation Approved:

Neil Purdie

Dissertation Adviser

Allen Apblett

Barry Lavine

Andrew Mort

Mark Rockley

A. Gordon Emslie

Dean of the Graduate College

PREFACE

Polyunsaturated fatty acids (PUFA) have become a focus in both nutritional sciences and medical studies in the evaluation of risk factors for cardiovascular diseases, dyslipidemias, diabetes, obesity, and other diseases. Studies have disclosed that unsaturated fatty acids can offer health benefits which include reducing cardiovascular death rates, lowering blood pressure, and promoting other positive health effects for patients. However, other studies have demonstrated the involvement of unsaturated fatty acids in oxidative stress and the development of atherosclerosis and other diseases. With these known connections of fatty acids to various diseases, the development of a direct, inexpensive, and routine colorimetric assay for the measurement of the concentration of serum lipids would enable routine monitoring to incorporate fully the PUFA as a risk factor for any disease. An accurate colorimetric assay combined with chemometric analysis has been developed that can routinely measure serum and synthetic mixture levels of cholesterol and PUFA simultaneously. This method provides for a more comprehensive unsaturated lipids' panel for serum which may be added to the current cholesterol and lipoprotein panel for inclusion in disease risk assessment models. Although this study is a beginning and further testing will be required on a wide range of human serum samples, it is hoped that the addition of both quantitative and qualitative

data resulting from the assay may not only be applied in routine clinical tests of humans but may be used in other fields such as food analysis.

Since a dissertation is not the single effort of just one person, I would like to take this time to express my gratitude and appreciation to all the people who have helped me reach this milestone. My advisor, Dr. Neil Purdie, was not only my research advisor but also a mentor for the kind of professor whom I hope to become in the future. His commitment to the development of the assay, his ability to teach students at many levels, and his extensive chemical knowledge provide everything a graduate could hope to have in his or her advisor. The support, knowledge, and wonderful research opportunities that Dr. Purdie provided me during my time in his laboratory have made me a research chemist; and I am grateful. Dr. A. David Marias, a collaborator from the University of Cape Town, was my research advisor during a semester working in his clinic on the development of this assay. The time he spent sharing with me the possible medical applications of this procedure and his constant assistance and commitment since my departure to the further development of the assay were invaluable. The members of the University of Cape Town's Lipid Clinic, who assisted on the project, welcomed me not only to their lab, but also to their homes, during my visit there and inspired me both as a chemist and as a person. I hope that one day I will be able to return their kindness. I would like to thank the Director of the Oklahoma State University Wellness Center, Robin Purdie, and the members of the staff of the Wellness Center who have assisted with the development of the assay through the years. Dr. Barry Lavine, a second collaborator, taught me the application of a number of chemometric methods and his assistance helped greatly. Through their informative conversations, Dr. Mark Rockley

and Dr. Lionel Raff provided me with an understanding of wavelength selection and neural networks that proved to be instrumental in this research. I would like to acknowledge two additional members of my committee, Dr. Andrew Mort and Dr. Allen Apblett, for their time and efforts in serving on a doctoral committee. Thanks also to the other faculty members of the Department of Chemistry and Physics and, especially, the staff of the Department of Chemistry for all of their assistance during my time at Oklahoma State University. The much appreciated financial support of the Department of Chemistry and the UCT Lipid Clinic has made my graduate studies at OSU possible.

Prior to attending Oklahoma State University, I had the opportunity to have two great mentors whose encouragement led me to a career in the sciences. Kathleen McCleary, my high school chemistry teacher, combined the learning of chemistry with practical and enjoyable activities. She has been a constant supporter of me during my education ever since, and I am grateful for her inspiration and friendship. Dr. Candee Chambers, my undergraduate advisor, exposed me to the field of physical chemistry during my first summer of college. Those experiences led me to choose chemistry as my major, and, hopefully, now, as a lifelong career.

My friends, who helped keep my life in balance, need to be thanked for their patience and kindness. Tracy Evan, my best friend for over 10 years, has been through it all with me. Her ability to make me laugh, her emails and cards, and all of the other innumerable ways she brought me up when I was down were greatly appreciated. My OSU friends, both inside and outside the department, assisted me by not only being a sounding board but also taught me how to be a better person and citizen of the world. Pauline Aad, Bill Alley, Dany and Maya Doughan, Antoine El Khoury, Michel

Goldstein, Christina Hummel-Meyer, Pedro Lima, Nizam Najd, Tarek Trad, Nevin Uras and Jannah Zubaidi each need to be thanked for their friendship and assistance.

Words cannot really express how grateful I am to my family for their love and constant support. Blessed with two wonderful parents, I would not be where I am today without their encouragement. I only hope that someday they will really understand how much they have done for me and that I will be able to repay them in some way. Thanks to my brother Chad for his sense of humor and his constant reminders about what is truly important in life. Also, I would like to thank my Grandfather Cornelius Reilly and my extended family for their continual thoughts and prayers.

Finally, I would like to dedicate this work to my grandparents who unfortunately passed away: Grandmother Margaret Reilly, Grandmother Hilda Harris; and, especially, my Grandfather Ralph Harris who encouraged me from the beginning to get an education. Grandpa was a very intelligent man and role model who always told me that I could finish my degree.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

TABLE OF SYMBOLS AND ABBREVIATIONS

γ-Linolenic acid, cis,cis,cis-6,9,12-octadecatrienoic acid	GLA
α-Linolenic acid, cis,cis,cis-9,12,15-octadecatrienoic acid	ALA
3-Hydroxy-3-methylglutaryl	HMG
Acylcholesterol acyltransferase	ACAT
Adenosine monophosphate	AMP
Adenosine triphosphate	ATP
Adult Treatment Panel III	ATP III
American Diabetes Association	ADA
American Heart Association	AHA
Apoprotein	apo
Arachidonic acid, cis,cis,cis,cis-5,8,11,14-Eicosatetraenoic acid,	AA
Artifical neural network	ANN
Body mass index	BMI
Cardiovascular disease	CVD
Center for Disease Control	CDC
Circular dichromism	CD
Cis-11,14,17- eicosatrienoic acid	ETA
Cis-7,10,13,16-docosatetraenoic acid methyl ester	DTA
Coenzyme A	CoA
Conjugated linoleic acid, cis-9, trans-11, trans-10, cis-12, trans-9, trans-11, and trans 10, trans-12-octadecadienoic acid	CLA
Coronary artery disease	CAD
Coronary heart disease	CHD
Diet and Reinfarction Trial	DART
Docosahexaenoic acid, cis-4,7,10,13,16,19-docosahexaenoic acid	DHA
Eicosapentaenoic acid, cis-5,8,11,14,17-eicosapentaenoic acid	EPA
European Association for the Study of Diabetes	EASD

Familial combined hyperlipidemia	FCHL
Familial hypercholesterolemia	FH
Fatty acid methyl esters	FAME
Flame ionization	FID
Free Fatty Acids	FFA
Gas chromatography	GC
Gas chromatography – mass spectrometry	GC-MS
Generalized standard addition method	GSAM
High performance liquid chromatography	HPLC
High-density lipoprotein	HDL
High-density lipoprotein cholesterol	HDL-C
Inorganic pyrophosphate	PP_i
Intermediate density lipoproteins	IDL
Lechithin-cholesterol acyltransferase	LCAT
Liebermann-Burchard	L-B
Linoleic acid, cis-9,12-octadecadienoic acid	LA
Lipoprotein (a)	Lp (a)
Liquid-chromatography-mass spectrometry	LC-MS
Long chain polyunsaturated fatty acids	LCPUFA
Low-density lipoprotein	LDL
Low-density lipoprotein cholesterol	LDL-C
Mass spectrometry	MS
Messenger ribonucleic acid	mRNA
Monounsaturated fatty acid	MUFA
Multiple linear regression	MLR
Myocardial infarction	MI
National Cholesterol Education Program	NCEP
National Heart, Lung, and Blood Institute	NHLBI
National Institutions of Health	NIH
Nicotinamide adenine dinucleotide	NADH
Non-esterified fatty acids	NEFA
Non-insulin dependent diabetes mellitus	NIDDM
Nonlinear iterative partial least squares	NIPALS
Non-steroidal anti-inflammatory drugs	NSAID
Omega-3	n-3
Omega-6	n-6
Omega-9	n-9
Partial least squares	PLS
Polyunsaturated fatty acids	PUFA
Principal component	PC
Principal component analysis	PCA
Root mean square error of calibration	RMSEC
Root mean square error of prediction	RMSEP
Root mean square error of validation	RMSEV

Root mean squared error of prediction	RMSEP
Standard addition method	SAM
Thin layer chromatography	TLC
Total cholesterol	TC
Triglyceride(s)	TG
Ultraviolet-visible	UV-VIS
University of Cape Town	UCT
Very low density lipoprotein	VLDL
Very low-density lipoprotein cholesterol	VLDL-C
World Health Organization	WHO

CHAPTER I

INTRODUCTION

Coronary heart disease, hypercholesterolemia, obesity, metabolic syndrome, diabetes, insulin resistance, and cancer are all health conditions that are a top priority for research laboratories and are often discussed in the news in Westernized societies. A broad search is underway to find clinical factors that can be used to detect, predict, diagnose, and monitor these health conditions. One such set of factors are polyunsaturated fatty acids, which have been examined for positive and negative effects on the conditions listed above and many others. Although polyunsaturated fatty acids can be detected by chromatographic methods, a low cost method to quantify polyunsaturated fatty acids in serum samples with little to no separation or sample preparation would enable polyunsaturated fatty acids to be monitored and detected during routine clinical serum analysis. Using a reagent system previously developed by the Purdie laboratory, the calibration and application of a possible routine clinical assay for the detection of polyunsaturated fatty acids was attempted.

Lipids are approximately 25 to 45% of the dietary energy consumed in most developed societies.[1] The fatty acids in the diet are important for not only the storage and transportation of energy but also serve as the basis for cellular membranes, biologically active compounds such as eicosanoids, and nuclear receptor interactions. In particular,

the omega-3 and omega-6 fatty acids, named due to the location of the last double bonds from the end of the carbon chain, have been the focus of research studies for their health effects The long chain fatty acids, such as linoleic acid and others, are considered essential nutrients because they must be consumed in the diet since no *in vivo* path can produce them. Omega-6 fatty acids are found in vegetables, vegetable oils, and meats. Omega-3 fatty acids which are consumed in the human diet through fish or fish products and dietary supplements of fish oil capsules and other forms have become major sources for these nutrients. The trend in the Western diet has been shifted from a low ratio of omega-6:omega-3 fatty acids to a high ratio.[1] The alteration to this ratio and the increased rates of coronary heart disease, diabetes, obesity, and cancer in these societies have raised questions focusing on the possibility of an association between these two observations.[1]

Since the mid-1960s, polyunsaturated fatty acids, especially the longer chain compounds, have been studied for their role in the prevention or onset of obesity, coronary heart disease, and other health conditions. The development of coronary heart disease and atherosclerosis occurs over a period of time and can be the result of other conditions that contribute to their development. Metabolic syndrome is a collection of related conditions that were determined to originate from metabolic changes that can lead to the development of atherosclerotic cardiovascular disease and diabetes. The conditions include high waist circumference and elevated cholesterol, triglyceride, and glucose levels. Elevated free fatty acids are included due to their link to insulin resistance.[2]

Insulin resistance is one prevailing pathological pathway for the development of metabolic syndrome and dyslipidemias.[2-8] An increase in the concentration of fatty acids in serum is a major contributor to the development of insulin resistance. Fatty acids in serum can be from adipose tissue, triglycerides, or the products from lipoprotein lipase.[3] Insulin plays a role in both processes. Once the excess fatty acid reaches insulin sensitive tissues such as muscles, they inhibit the glucose intake on the insulin mediated pathway.[3] In the liver, elevated free fatty acids increase the glucose, triglycerides, and the very low and low density lipoproteins, and decrease the high density lipoproteins released.[3] All of these markers are offered as current measures for the definition of metabolic syndrome, except the reason for the causes of these changes, which are the concentrations of the free fatty acids.

Recent studies have begun to look at the effects of a particular sub-set of fatty acids, polyunsaturated fatty acids (PUFA), and their potential to prevent the onset of insulin resistance and, therefore, the prevention of metabolic syndrome and the other conditions that may develop. Omega-3 fatty acids, particularly fish oil, and to a lesser extent omega-6 fatty acids have been linked to the prevention and the mediation of insulin resistance.[5-8] More studies have associated the dietary intake of fish oil which is primarily composed of omega-3 fatty acids with the prevention of the symptoms of insulin resistance. The omega-3 fatty acids found in fish oil have been used in dietary studies and have lowered triglyceride (TG) and very low density lipoproteins (VLDL) serum levels which are related to both insulin resistance and coronary heart disease risk factors.[5-7] One interesting study completed by Stene and Joner that focused on fish oil intake demonstrated that the risk of the development of type I diabetes mellitus was

lowered through the consumption of cod liver oil during the first year of life.[9] Other studies have demonstrated that fish oil can slow the onset of diabetes through delays in the progression of glucose intolerance.[5-7] All of the beneficial effects of the polyunsaturated fatty acids are related to their anti-inflammatory, antithrombotic, and antiarrhythmic properties.

The relationship of PUFA with coronary heart disease (CHD) has been the subject of numerous research studies. Miettinen et al. revealed that linoleic acid, arachidonic acid, and eicosapentaenoic acids (EPA) were lower in the phospholipids of subjects who sustained a severe cardiac event that was either fatal or non-fatal as compared to subjects who did not suffer a heart problem.[10] In a similar study examining the fatty acid composition of plasma cholesteryl esters and phospholipids in subjects with CHD, Wang et al. discovered that there were higher percentages of stearic acid, dihomo-gamma-linolenic acid and total saturated fatty acids, and lower percentages of arachidonic acid and total polyunsaturated fatty acids.[11] In a review of clinical and epidemiological studies, it was stated that the fatty acids that protect against CHD are linoleic acid, linolenic acid, EPA, and docosahexaenoic acid (DHA).[12] Recent studies offer conflicting results. In two dietary studies involving men who were either recovering from a myocardial infarction (MI) or had stable angina, the experimental intent was to investigate the effect of increasing the intake of fatty acids in fish either through consumption of fish or fish oil supplements. The study revealed mixed results. In one set, the fatty acid increase with the MI patients decreased the mortality of the subjects in the two year follow-up.[13] In the angina set, the patients who ate fish did not show a decrease in mortality; and the patients who consumed fish oil supplements actually had a higher

risk of a cardiac event or sudden death.[13] The discrepancies may be due to the use of capsules versus the consumption of actual fish, variations in conditions, or other possible differences in the mechanisms that need to be examined further.[13]

In an additional examination of the consumption of dietary PUFA, it was demonstrated that the serum linoleic acid and the PUFA concentration were negatively correlated to the overall mortality from cardiovascular events in male subjects.[14] Hooper et al. completed a metanalysis of randomized controlled trials and cohort studies that revealed no clear positive effect on total mortality from cardiovascular events when there was an increase in dietary omega-3 fatty acids.[15] The authors state that while the evidence is not clear, it is not yet appropriate to alter the dietary recommendation of increasing the intake of omega-3; but further research and evaluation should be completed.[15] Even treatment with statins for the lowering of cholesterol to decrease the risk of CHD has an effect on the PUFA in serum. The relative percentages of linoleic acid and alpha-linolenic acid decreased while arachidonic acid and docosahexaenoic acid increased.[16, 17]

In the current regulated methods for the detection of cholesterol and PUFA, each is measured independently of the other. The routine method for the measurement of cholesterol is a simple enzymatic test that is used in every clinical laboratory. The enzymatic test is standardized against the Center for Disease Control (CDC) reference method that is based on the Liebermann-Burchard reagent which reacts directly with cholesterol for the color development.[18] For PUFA analyses in serum, there currently is no standard reference method. The majority of studies use GC (gas chromatography) methods. This method first involves the extraction of the fatty acids using either the

method developed by Foche in the 1950's or an altered method by various authors.[19-26] One such alternative is by a 2:1 methanol:hexane extraction.[16] In a comparison of methods for lipid extraction, the most popular solvent, 2:1 chloroform-methanol (v/v), was only able to extract 90% of the triglycerides which can alter the percentage of PUFA detected in the GC method.[24] The fatty acid samples are then reacted with acetyl chloride and heated for one hour at 100 °C to be converted into methyl esters.[20] The calibrations require the addition of an internal reference, such as methyl docosatrienoate, to the sample.[16] The relative amount of each fatty acid is determined by dividing individual peak areas by the sum of all the fatty acid peaks.[16] The resulting units are neither molarities nor mg/dL, except where GC is combined with mass spectrometry. Alternative methods include combining mass spectrometry with nuclear magnetic resonance. Problems that hinder the technique from becoming a clinical method are the possibility of errors in the extraction, the methylation, the difficulty in obtaining meaningful concentrations, and the labor intensity of the procedures. These limitations strengthen the case for the development of a simple and direct method that does not require separation and reacts directly with the polyunsaturated fatty acids.

The use of colorimetric reactions for clinical detection and quantification has been the key reaction for the development of a number of routine clinical tests. Cholesterol, triglycerides, and free fatty acids all have colorimetric reactions that involve enzymes and eventual color reactions for the quantification of these compounds. As previously mentioned, cholesterol can be directly quantified by the Liebermann-Burchard (L-B) reaction, which is the Center for Disease Control standard reaction for cholesterol. The Purdie reaction which was developed as a modification of the L-B reagents was first

published in 1991 for the quantification of the lipoprotein particles of cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and very low-density lipoprotein (VLDL).[27, 28] After modification and further analysis, the reaction system was determined to react with $–CH_2$-CH=CH- complexes, including steroids, terpenes, and polyunsaturated fatty acids, in addition to cholesterol.[29-33] The products of the reaction have intense absorptions in the visible region from 350 to 550 nm that vary based on the location and number of complexes. The spectra are additive when multiple analytes are present, and have no significant interferences in serum except for lyzed serum samples where red blood cells have entered the sample. [29-33]

The goal of this research project was to develop a simple, direct alternative method for the determination of the polyunsaturated fatty acids in addition to cholesterol in serum and possibly other samples that contain these analytes. Previous attempts to measure these components using the Purdie reagent system have been reported.[31] Initial attempts by the Purdie laboratory to determine the concentrations using multiple linear regressions (MLR) did not yield acceptable results. Using the information of past attempts and having acquired a new understanding of the reaction system and chemometric methods, the intent of this study was to determine the concentration of all seven components believed to be reacting with the assay performed with in serum. Additionally, the spectral patterns from a collection of serum spectra from a collaborating laboratory were analyzed for a possible diagnostic test for dyslipidemias.

CHAPTER II

CHOLESTEROL AND POLYUNSATURATED FATTY ACIDS AND THEIR RELATIONSHIP WITH CARDIOVASCULAR DISEASE

I. Cholesterol

Cholesterol, along with fatty acids, composes the membranes of animal and human cells, is a precursor of bile acids and steroid hormones such as progesterone and testosterone, and is a major component of the nervous system including the brain. The basic structure of cholesterol, pictured in Figure II.1, is a derivative of the perhydrocyclopentanophenanthrene skeleton. Its molecular weight is 387 g/mol.

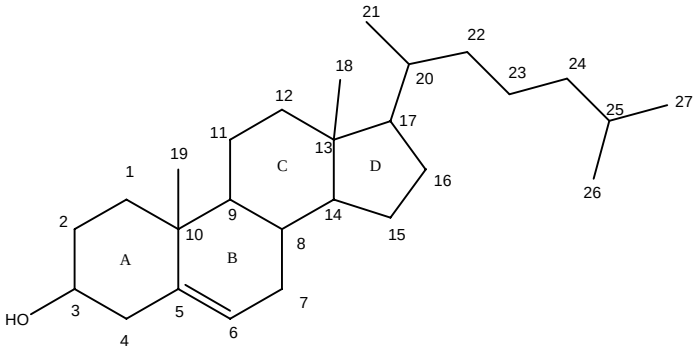


Figure II.1: Structure for cholesterol

Cholesterol can be formed endogenously through the following synthesis: acetate to mevalonate, to isopentyl pyrophosphate, to squalene, and finally to cholesterol.[34] The rate-limiting enzyme of this synthesis is 3-hydroxy-3methylglutaryl-CoA reductase

which produces mevalonate.[18] The synthesis primarily occurs in the liver but can also occur in the intestine.[34] A typical adult on a low cholesterol diet will produce 300 to 1000 mg of cholesterol a day.[18] Formation of cholesterol by the body is the primary source, with diet as a secondary source. It is estimated that the average American diet includes 350 to over 450 mg of cholesterol per day.[18] Meat, eggs, dairy products, and poultry are the major sources of cholesterol.

The reactions of cholesterol occur mainly on the hydroxyl group, at the double bond, or at carbon 7. These reactions result in the formation of bile acids to aid the digestion of lipids, to the synthesis of steroid hormones, or to the formation of Vitamin D.[34] The formation of bile acids is the important elimination step to remove lipids from the body. A fraction of the total cholesterol is in the form of cholesteryl esters resulting from a condensation of the alcohol group with long chain fatty acids. Cholesteryl esters are approximately 70% of the total serum cholesterol and are important in the high lipid carrying capacity of the lipoproteins.[18] The esterification is catalyzed by lecithin-cholesterol acyltransferase (LCAT) in plasma and acylcholesterol acyltransferase (ACAT) inside the cell. LCAT will continue to esterify plasma cholesterol even after blood collection so immediate analysis or sample cooling is necessary if determination of esterified and nonesterified cholesterol is essential.[18]

II. Lipoproteins

Cholesterol, triglycerides, phospholipids, and a group of apoproteins are the major components of lipoproteins. Triglycerides (TG) are molecules with a glycerol backbone that have three fatty acids attached. Phospholipids are molecular complexes with a carbon backbone that can be glycerol, nitrogen containing alcohol, fatty acids, and a

phosphate group. The basic structure of a lipoprotein particle is a core of cholesteryl esters and triglycerides surrounded by a monolayer of phospholipids and free cholesterol, which are more polar to allow the particle to be soluble in aqueous plasma. There are five major categories of lipoproteins based on their composition and physical properties which are summarized in Table II-1.

Table II-1: Physical properties of the major lipoproteins

Composition and Density of Human Lipoproteins[18, 34, 35]					
	Chylomicron	**VLDL**	**IDL**	**LDL**	**HDL**
Density (g/mL)	<0.95	0.95-1.006	1.006-1.019	1.019-1.063	1.063-1.210
Diameter (nm)	75-1,200	30-80	25-35	18-25	5-12
Components (% dry weight)					
Protein	1-2	10	18	25	33
Triglycerol	83	50	31	10	8
Cholesterol and Cholesteryl esters	8	22	29	46	30
Phospholipids	7	18	22	22	29
Apolipoproteins (* denotes major)	A-I*, A-II, B-48*, C-I*,C-II*,C-III*, E	B-100*, C-I*,C-II*,C-III*, E*	B-100*, C-I, C-II, C-III, E*	B-100*	A-I*,A-II*, C-I, C-II, C-III, D, E

Chylomicrons are the initial lipoproteins formed in the intestine and are the largest in size. These particles are mostly composed of lipids which are primarily in the form of triglycerides. The apoproteins are apo A-I, A-II, C-I, C-II, C-III, E, and a fragment of apoB-100, apo B-48. Once formed, the particle is transferred to the lymphatic system for distribution to the rest of the body.[35] In adipose tissue and muscle, lipoprotein lipase catalyzes the hydrolysis of the triglycerides.[35] The remainder of the

chylomicron particle is sent to the liver. Chylomicrons are highest in concentration immediately following a meal, and the half-life for the removal of these particles is four to five minutes with none present in the twelve hour fasting blood samples normally collected in lipid analysis.[18]

The liver generates VLDL which supply the cells with fatty acids and is also the source of HDL. Once the cells release the fatty acids from the VLDL, the lipoprotein forms intermediate density (IDL) and eventually LDL. VLDL particles are released by the liver through capillaries to the extrahepatic tissues.[35] These large, high percentage TG lipoproteins have apo B-100, C-I, C-II, C-III, and E as their apoproteins. Like the chylomicrons, the TG is hydrolyzed by lipoprotein lipase resulting in a reduction in diameter and the removal of the apo C proteins.[18] The new particle is the third classification, IDL resulting from VLDL having a clearance value of 1 to 3 hours.[18]

IDL is converted into LDL by the action of lipoprotein lipase, and its cholesteryl ester composition is increased by cholesteryl ester transfer protein from high density lipoprotein resulting in high cholesterol content. About 60% of total cholesterol is LDL cholesterol.[18] On top of the monolayer, apoprotein B-100 surrounds the outside surface. LDL that is absorbed by cells is degraded in the lysosomes by lysosomal acid lipases. Once released from the lipoprotein, ACAT is activated for the formation of cholesteryl esters which can be stored in the cell. Approximately half of the lipids present in LDL are PUFA.[36] A number of antioxidants including alpha and gamma tocopherol, beta and alpha carotene, lycopene, and others are present to protect the fatty acids from oxidation.[36] LDL particles are able to stay in circulation for about 3 days.[18] LDL cholesterol particles can be classified into subfractions based on size, density,

composition of lipids, susceptibility to oxidation, and affinity for the apoB/E receptor resulting in small, dense LDL and large, less dense LDL.[18]

HDL lipoprotein structure is similar to LDL, but the composition of the proteins and lipids is different. The properties of HDL that are different include the following: an increase in density (1.063 to 1.210 g/mL), the particle size is smaller in diameter, an increase in the percentage of protein, and a decrease in the lipid percentage. The major apolipoproteins that compose HDL are A-I, AII, C-I, C-II, C-II, D, and E. HDL contains 20-30% of the total serum cholesterol.[18] HDL has the major role in the reverse cholesterol transport mechanism by removing cholesterol from tissue and taking it to the liver for removal in bile as bile acid or free cholesterol.[37] There is evidence that HDL also protects LDL from oxidation.[37] HDL_2 and HDL_3 are two subclasses of this lipoprotein which are separated based on density.[18] VLDL can lead to the production of HDL_3 which can have the cholesterol components converted to cholesteryl ester by LCAT to form the larger HDL_2 particle.[18]

A special class of lipoprotein is Lipoprotein(a) (Lp(a)). Lp(a) has a similarity to LDL in lipid composition with the major apolipoprotein being apo B-100.[18] Additionally, Lp(a) contains apolipoprotein(a) which is a carbohydrate-rich protein that is attached to apo B-100 by a disulfide bond.[18] The particles have a density of 1.040-1.130 g/mL and a diameter of 25-30 nm.[18] The major lipids are cholesteryl esters and phospholipids.[18]

III. Coronary Heart Disease (CHD) and Cholesterol

Coronary heart disease (CHD), also called coronary artery disease (CAD) and atherosclerotic heart disease, is a disease that is characterized by the accumulation of plaque on the walls of arteries. Atherosclerosis is a condition where the build up of plaque on the walls of the arteries leads to a narrowing of the path for the blood to flow. If the artery were to become blocked completely, the patient could experience a stroke or myocardial infarction. An increase in LDL levels leads to an increase in the LDL rate of entry into the artery walls and in the adhesion of monocytes circulating in the blood to the endothelial cells.[36 38 39] Inside the intimal region of the artery walls, the LDL is in an environment that does not have sufficient anti-oxidants to protect its components from oxidation. In blood and tissue, there is a high concentration of antioxidants and antioxidant enzymes, such as human serum paraoxonase; but in the walls of the arteries, the LDL is only equipped with the limited concentration of anti-oxidants that are in its complex, such as alpha-tocopherol and beta-carotene. [36 40] If the LDL becomes oxidized, the particles can be absorbed by macrophages and smooth muscle cells to form foam cells. [36 38 39] Growth of the foam cells is caused by the oxidized LDL entering the cells through a scavenger pathway which lacks control. [36 38 39] Stage one of atherogenesis is the development of these foam cells.[39] Stage two is development of the core of the plaque that develops on the artery walls.[41] The final stage called thrombosis eventually leads to the breaking of the artery wall.[41]

The free radicals that attack LDL can modify all of the components present. Oxidized LDL has altered properties ranging from the lack of anti-oxidants that are consumed to a decrease in the fatty acids and total cholesterol.[36 40] After the anti-

oxidants are consumed, the free radicals can modify the PUFAs and cholesterol. The products from the reactions include oxysterols, lipid hydroperoxides, hydroxides, conjugated dienes, and aldehydes.[36 38 42] The products of lipid peroxidation are also damaging to proteins; and, as a result, apolipoprotein B also undergoes modifications.[43] The oxidants can attack the amino acids which results in a break in the peptide chain; or the aldehydes that are generated as part of the lipid oxidation can establish acid side chains to the proteins[38 40] The products of both the lipid oxidation and the protein oxidation are used to monitor both in vitro and in vivo oxidative stress damage to lipoproteins. The generation of oxygen free radicals, the presence of lipid substrates, and the activity of antioxidants are three control factors for the oxidation of LDL.[44]

There is evidence that HDL also protects LDL from oxidation.[37] In human serum experiments, the lipids in HDL are oxidized by peroxyl radicals first and then LDL.[37] It has also been shown that HDL can protect LDL from both copper (Cu^{+2}) and iron (Fe^{3+}) mediated oxidation.[37] Like oxidized LDL, oxidized HDL has different physiological effects. When HDL becomes oxidized, it inhibits the lipoprotein's ability to remove cholesterol from tissue.[45] Oxidized HDL has been observed in plaques from arteries in addition to oxidized LDL, which suggests HDL has the possibility of being both a benefit and a risk factor in the development of atherosclerosis.[45]

IV. Treatment Interventions

The National Cholesterol Education Program (NCEP) supported by the National Institutions of Health (NIH) released a third report on the detection, evaluation, and treatment of high cholesterol in adults (Adult Treatment Panel III, ATP III) in 2001. The report states that elevated LDL cholesterol is the major risk factor for CHD which was

determined by a number of experimental animal tests, laboratory investigations, epidemiology, and genetic forms of hypercholesterolemia.[46] Patients are assessed into three CHD risk categories: highest risk: LDL cholesterol greater than or equal to 130 mg/dL, middle level: LDL levels between 100-129 mg/dL, and lowest: LDL cholesterol less than 100 mg/dL. ATP-III recommends that an LDL concentration below 100 mg/dL lowers a person's risk of developing CHD and suggests treatments to obtain these levels.[46] Elevated LDL cholesterol levels can be a secondary dyslipidemia and conditions such as diabetes, hypothyroidism, chronic liver failure, liver disease, and drug-elevated values must be excluded first.[46] Additional risk factors for CHD are smoking, hypertension, low HDL cholesterol, family history, and age which are also considered in assessing treatment.[46] According to ATP-III, HDL values are inversely connected with the risk of CHD; and patient values should be higher than 60 mg/dL to maintain satisfactory health.[46] Besides the cholesterol in the lipoprotein particles, there are additional biomarkers that are being considered for use in relation to CHD. The ratio of the apoproteins apoB/apoA-1, C-reactive protein, and LDL particle size have shown a direct relationship with CHD.[47-51]

A statin, the most widely used drug treatment for hypercholesterolemia, is a 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase inhibitor which blocks the formation of mevalonate. After being released almost twenty years ago, statins have proven to be effective in the lowering of cholesterol and low density lipoprotein cholesterol (LDL-C) concentrations and contributed to a reduction in the mortality rates of CHD patients.[46] Administering bile acid sequestrants is a second drug option. Bile acid sequestrants reduce serum cholesterol levels by increasing the excretion of the bile

acids from the body. The resulting effect is the liver then must convert more cholesterol into bile acid, reducing the total cholesterol. Fibric acids lower TG and raise HDL levels and, as a result, lower the LDL cholesterol concentration.[46] Nicotinic acid, a form of vitamin B, is a fourth drug option.[46]

Dietary treatments include the increased intake of plant sterols. Plant sterols are similar to cholesterol with only side chain variations, but they are poorly absorbed by the intestine. The daily intake of plant sterols is 200-300 mg.[18] A dose of 5 to 15 g/d of plant sterols can inhibit the absorption of dietary cholesterol and can be used as a treatment for hypercholesterolemia.[18] The ATP-III has recommended a therapeutic diet change which reduces the intake of saturated fats and cholesterol and the inclusion of plant sterols and fiber.[46] Additional lifestyle changes of weight reduction and increased exercise are recommended.[46]

V. Dyslipidemias

Elevated lipoprotein concentrations can be related to genetic or diet induced hyperlipoproteinemias. Lipoprotein disorders are divided into primary or secondary varieties. Sources of secondary hyperlipoproteinemias must be eliminated before considering primary ones. Secondary causes can be divided into five sources: diet, diabetes, other metabolic disorders, drugs, and non-metabolic diseases.[52] Dietary sources include excess saturated fat or calorie intake, weight gain, and eating disorders such as anorexia, in which the body has overproduced lipoproteins to compensate for the lack of nutrients.[52] Drug treatment for cardiovascular disease, endocrine function, and antiretroviral drug therapy can alter the lipid profile.[52] Diabetes, both Types I and II, insulin resistance, hypothyroidism, and other metabolic disorders have raised TG levels,

decreased HDL-C levels, and elevated LDL-C levels; especially when the condition is not controlled.[52] Liver and renal diseases can alter lipoproteins concentrations as well.[52] Each of these five secondary dyslipidemias needs to be treated first before a diagnosis of primary dyslipidemia can be considered.

The clinical primary dyslipidemias are classified into six phenotypes based on their patterns of total cholesterol (TC), LDL, HDL, TG and apolipoprotein levels. Fredrickson *et al.* proposed the classification, and it was adopted by the World Health Organization in 1970.[53] Table II-2 summarizes the types. The Fredrickson Types were commonly used but have been replaced with the genetic lipoprotein disorders described in Table II-3.[53]

Table II-2: Fredrickson classification

Fredrickson Classification of Lipid Disorders[53]				
Type	**Elevated Particles**	**Associated Clinical Disorders**	**Serum Total Cholesterol Concentration**	**Serum, Total Triglycerides Concentration**
I	Chylomicrons	Lipoprotein lipase deficiency, apolipoprotein C-II deficiency	normal	greatly increased
IIa	LDL	Familial hypercholesterolemia, Polygenic hypercholesterolemia, nephrosis hypothyroidism, familial combined hyperlipidemia	greatly increased	normal
IIb	LDL, VLDL	Familial combined hyperlipidemia	greatly increased	increased
III	IDL	Dysbetalipoproteinemia	increased	increased
IV	VLDL	Familial hypertriglyceridemia, familial combined hyperlipidemia, sporadic hypertriglyceridemia, diabetes	normal to increased	greatly increased
V	Chylomicrons, VLDL	Diabetes	increased	greatly increased

Table II-3: Major genetic lipoprotein disorders

Major Genetic Lipoprotein Disorders[53]				
Disorder	**Principal Plasma Abnormality**	**Fredrickson Classification**	**Clinical Features**	**Estimated Frequency**
Heterozygous familial hypercholesterolemia	Increased LDL only	IIa	Tendinous xanthomas, Corneal arcus, Premature CAD, Family history of hypercholesterolemia	0.2% of general population; 5% of MI survivors <60 yr old
Familial defective apolipoprotein B	Increased LDL	IIa	Same as heterozygous familial hypercholesterolemia	Same as heterozygous familial hypercholesterolemia
Familial combined hyperlipidemia	1/3: increased LDL 1/3: increased VLDL 1/3: increased LDL and VLDL	IIa IV IIb	Usually > 30 yr old Often overweight Usually no xanthomas Premature CAD Different generation have different lipoprotein abnormalities	0.5% of general population 15% of MI survivors < 60 yr old
Polygenic hypercholesterolemia	increased LDL	IIa	Premature CAD No xanthomas No family history of hypercholesterolemia	Unknown
Familial hypertriglyceridemia	increased VLDL; TG concentration (200-1000 mg/dL)	IV	Often overweight > 30 yr old Often diabetic Hyperuricemic May or may not have premature CAD Determined by family history and HDL-C	1% of general population 5% of MI survivors <60 yr old
Severe hypertriglyceridemias	increased Chylomicrons and VLDL; (> 1,000 mg/dL)	V	Usually middle-aged Often obese Often hyperuricemic Usually diabetic Risk for recurrent pancreatitis	Unknown
Familial hypoalpha-lipoproteinemia	decreased HDL; <30 mg/dL in males; < 35 mg/dL in females	none	Premature CAD	1% of general population 25-30% of patients with premature CAD
Dysbeta-lipoproteinemia	increased IDL;increased chylomicron remnants; TC: 250-500 mg/dL TG:250-600 mg/dL	III	Yellow palmar creases Palmar xanthomas Tuberoeruptive xanthomas Premature CAD	Uncommon 3% of MI survivors

Type I is a deficiency in lipoprotein lipase activity resulting in the inability to convert chylomicrons into chylomicron remnants. Since chylomicrons are TG rich, the disorder is marked by hypertriglyceridemia. Cholesterol and the remaining lipoproteins are in the normal to low range. Clinical signs of the disorder included eruptive xanthomas when TG levels reach 2000 to 4000 mg/dL and attacks of pancreatitis which present in childhood.[18] The condition is genetic and is extremely rare. The occurrence rate is about one in a million.[18] Due to the lack of the other lipoproteins, especially LDL, Type I patients do not have high occurrences of CHD or atherosclerotic development.[53]

Type II is divided into two sub-classifications, Type IIa and IIb. Type IIa can be heterozygous familial hypercholesterolemia, familial defective apolipoprotein B, familial combined hyperlipidemia, and polygenic hypercholesterolemia.[53] Familial hypercholesterolemia (FH) is an important condition that was studied by Brown and Goldstein to examine LDL receptors in the body.[54] Homozygotes have little to no LDL receptors, and heterozygotes have about half of the normal count. [54] This lack of receptors prevents the liver and cells from normal removal of LDL from serum, resulting in the high levels.[18] IDL is also increased because its entry into the liver is also controlled by LDL receptors allowing for more IDL to be converted into LDL in the plasma.[18] The dyslipidemia appears to result from increased production of VLDL. Homozygotes have an average of 680 mg/dL of cholesterol compared with the heterozygotes having 300 mg/dL.[34] The condition can produce xanthomas, which are fat deposits that are usually found on knees, ankles, hands, and other joints. Type IIa can be familial combined hyperlipidemia (FCHL) with increased levels of TC and LDL-C and normal to low concentrations of HDL.[18] Type IIb has the additional elevated levels of

TG. Type IV has elevated levels of TC and TG.[18] The disorder is genetic occurring in 1 out of 100 people but can be induced by diet and other disorders such as diabetes.[18] Familial defective apolipoprotein B is an inherited abnormality of apoB that interferes with the binding of LDL receptor which leads to premature development of coronary artery disease.[53] Polygenic hypercholesterolemia expresses as an elevated LDL concentration that presents with no family history or physical characteristics like xanthomas.[53]

Dysbetalipoproteinemia Type III, is a genetic disorder characterized by a defect in apoE binding resulting in increases in TC, TG, and apoE concentrations.[55-57] The chylomicrons and VLDL remnants are not efficiently removed resulting in the cholesterol rich lipoproteins that predisposes patients to premature development of artherosclerotic disease[55-57]. Hormonal and environmental factors, diabetes, renal failure and other diseases can bring the onset of dysbetalipoteinemia.[55-57] Physical signs of the conditions include tuboeruptive cutaneous and tendinous xanthomata and palmar crease xanthomas.[55-57] Non-denaturing polyacrylamide gradient gel electrophoresis of the apo B containing particles, the apoB/TC ratio, and genetic confirmation are means to confirm a diagnosis.[55, 56]

Familial hypertriglyceridemia, Type IV, is defined by a moderate increase in TG, a slight increase in TC, and a slight to moderate decrease in HDL.[18] The VLDL particles are larger in size in this condition resulting in the elevated TG levels and decreased lipase activity.[53] This genetic condition occurs in 1% of the population.[53] Familial combined hyperlipidemia can also present as a Fredrickson Type IV where the VLDL is elevated and apo-B production is increased.[53]

Severe hypertriglyceridemia, Type V, is an increase in chylomicrons and VLDL which results in an elevated level of TG as well. It is a combination of dietary fat, lipase activity, and other apoprotein variations.[18] Rarely seen in children, it is presented in adulthood with the development of xanthomas, pancreatitis, and abnormal glucose tolerance with hyperinsulinism.[18] Although genetically inherited, the exact genetic expression is not known. [18]

VI. Fatty Acids

Fatty acids are biological molecules that serve as building blocks for cell membranes and as a source of energy. Their metabolites are also precursors for prostaglandins, thromboxanes, and leukotrienes. Fatty acids are simple carbon chains with a carboxylic acid functional group at one end. Fatty acids can exist as free fatty acids, non-esterified fatty acids (NEFA); or through esterification reactions the fatty acids can be synthesized into phospholipids such as phosphatidylcholine, triglycerides, and cholesteryl esters which can be formed into parts of the lipoprotein partials. Serum lipids are found in lipoproteins, and the fatty acids in these lipoproteins not only reflect dietary intake, but also are closer to metabolic sites of cholesterol regulation. Fatty acids are necessary for biological functions, but the human body cannot produce all of the fatty acids. Therefore, most must be acquired through diet. Due to their involvement in a number of mechanisms and the fact that they must be in the human diet, fatty acids have become the focus of a number of studies attempting to determine their relationship to diseases from cardiovascular disease to mental illnesses.

A. Fatty Acid Structure, Nomenclature, Dietary Sources, and Metabolism

1. Fatty Acid Structure and Nomenclature

Fatty acids are classified based as either saturated or unsaturated. Saturated fatty acids contain all single bonds between carbons in the chain. Unsaturated fatty acids contain one or more double bonds. Molecules with only one double bond are referred to as monounsaturated fatty acid (MUFA). Those with more than one double bond are PUFA. A shorthand notation of the basic structure is (A:B) where A is the number of carbons and B is the number of double bonds. The omega classification of PUFA is the most common reference for these types of molecules and is based on the location of the last double bond from the end of the chain away from terminal methyl group. Omega-9 (n-9), for example, is oleic acid (18:1). Omega-6 (n-6) fatty acids include cis-9,12-octadecadienoic acid, common name, linoleic acid (LA) (18:2); cis,cis,cis-6,9,12-octadecatrienoic acid, common name, γ-linolenic acid (GLA) (18:3); cis,cis,cis,cis-5,8,11,14-Eicosatetraenoic acid, common name, arachidonic acid (AA) (20:4); and conjugated linoleic acid (CLA) (18:2). Conjugated linoleic acid exists as a number of different isomers of which cis-9, trans-11, trans-10, cis-12, trans-9, trans-11, and trans 10, trans-12-octadecadienoic acid are the major forms. The active form of CLA is considered to be cis-9, trans-11.[58] Omega-3 (n-3) fatty acids include cis,cis,cis-9,12,15-octadecatrienoic acid, common name, α-linolenic acid (ALA) (18:3); all cis-5,8,11,14,17-eicosapentaenoic acid (EPA) (20:5), and all cis-4,7,10,13,16,19-docosahexaenoic acid (DHA) (22:6). All structures of these compounds are shown in Figure II-2. The formation of trans fatty acids occurs during the industrial hydrogenation process of

vegetable oils. The unsaturated fats formed have one or more double bonds that have the trans-configuration.

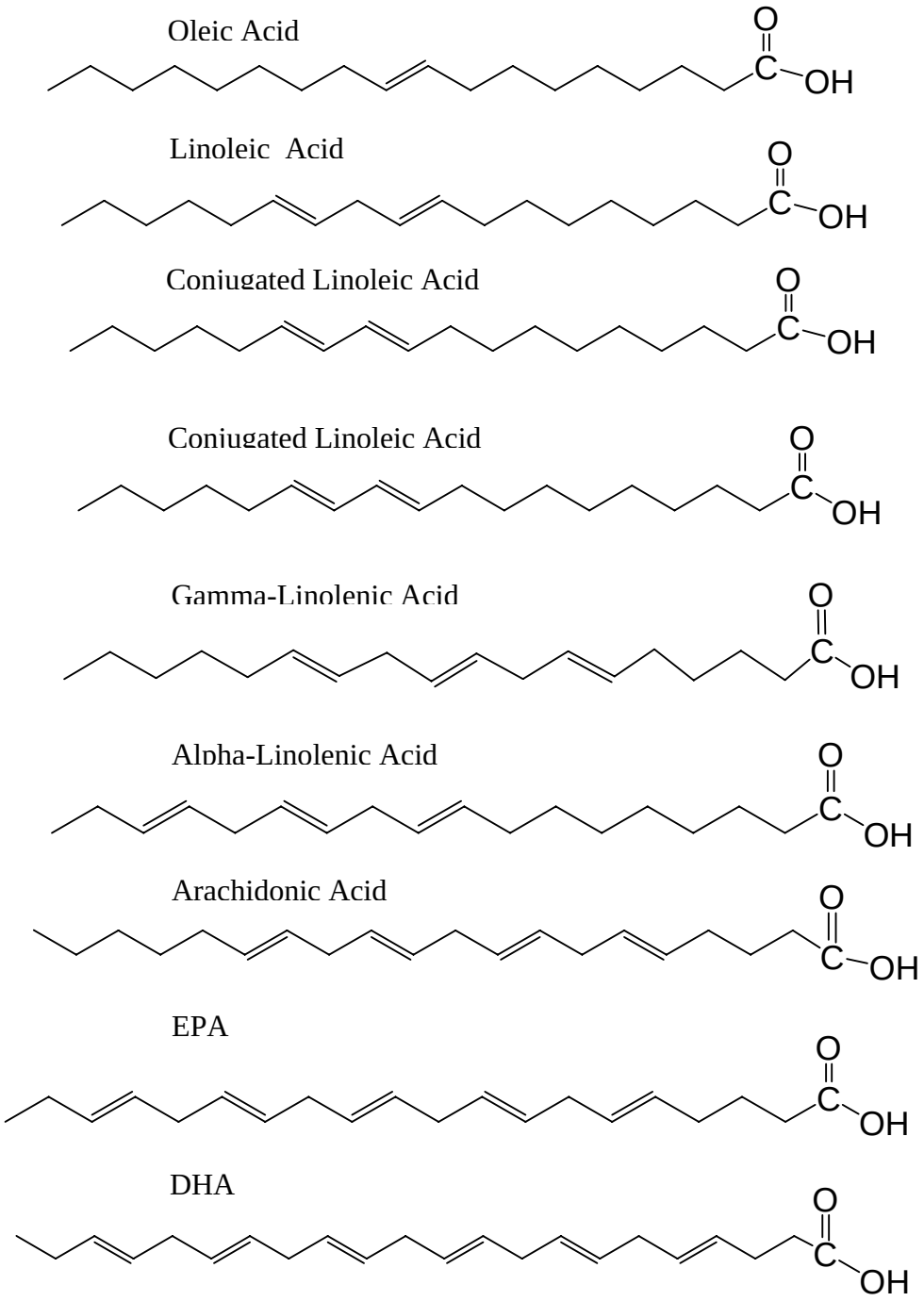


Figure II.2: Structures of MUFA and PUFA

2. Dietary Sources of Fatty Acids

Due to the importance of fatty acids in the formation and properties of cell membranes, they are essential for human and animal development. Saturated fatty acids are primarily consumed in the diet from animal fat in the form of meat and dairy products. Sources of high concentrations of saturated fats include butter, lard, milk, and milk products such and cream and cheese.[59] Plant sources of saturated fat include cottonseed oil, palm oil, and coconut oil.[59] Saturated fatty acids occur naturally in the animal and human body, and all forms do not need to be consumed. The essential fatty acids are LA and ALA which can be metabolized to form additional acids. MUFAs, such as oleic acid, are not considered essential since they are able to be derived through other precursors.[60] The unsaturated fatty acids can be consumed in the form of plant oil, fish oil, and seafood. MUFAs are highest in olive oil, avocados, and nuts such as peanuts and almonds.[59] Omega-6 fatty acids are found in high concentrations in corn, safflower, and sunflower oils.[59] CLA is primarily found in animal sources and processed animal products due to Δ^9-desaturase production of CLA in lactating cows and the exogenous production of CLA by rumen bacterium *Butyrivibrio fibrisolvens*.[58, 61] High amounts of CLA are found in lamb and various forms of beef.[58] Poultry and seafoods are lower in CLA, but turkey is an exception with 2.5 ± 0.04 mg of CLA/g of fat.[58] Processed cheeses and milk products such as buttermilk and condensed milk have amounts ranging from 2.9 to 7.1 mg/g of fat.[58] Plant oils, which are high in LA, have only a small concentration of CLA. ALA, an omega-3 fatty acid, is found in flaxseed, soybean, walnut, and grapeseed oils.[59] The additional omega-3 fatty acids, DHA and EPA, are found in marine animals such as salmon, tuna, flounder, and shellfish.[62] Trans fatty acids, which form as a result

of the hydrogenation of vegetable oils, are primarily found in packaged snack goods, margarines, fast foods, and bakery products. The average consumption of these fatty acids is 4 to 7 % of the total fat in a typical diet in industrialized countries.[63]

The consumption of these fatty acids in humans has changed in the last few decades. Early human beings who consumed wild animals had an estimated 1:1 ratio of omega-6 to omega-3 fatty acids in their diet.[1] The current Western diet is estimated to be between 10 to 20 omega-6 to 1 omega-3.[1, 62] Since the mid to late twentieth century, there has been a shift from animal-based oils to vegetable-based oils in order to lower cholesterol. The modern agricultural business has contributed to the increase in omega-6 fatty acids by changing the feed of livestock.[1] Although it increases production, the high omega-6 content in the grain fed to the animals decreases the omega-3 and lowers the overall PUFA composition when compared to wild animals of the same species.[1] The omega-6 to omega-3 ratio in the diet is also different based on populations and locations. The rates in the United States and the United Kingdom and northern Europe are calculated to be 15.00:1 and 16.74:1, respectively.[1] The ratio in Japan is currently 4.00:1.[1] In India, there exists a difference based on rural (5 to 6.1:1) and urban (38-50:1) populations.[1] The concern is how this change has affected the health of Westernized humans. To examine the effects, the metabolism of the fatty acids must be discussed.

3. Metabolism of Fatty Acids

The essential fatty acids are the PUFA of linoleic acid and alpha-linolenic acid that serve as the beginning compounds in the metabolism of fatty acids. Linoleic acid, the starting compound for the omega-6 (n-6) series, is converted to GLA by Δ^6 desaturase.[60, 64] GLA is expanded into dihomo-γ-linolenic acid through the elongase enzyme. The

latter can be converted into a series of prostaglandins referred to as the 1 series or into AA via Δ^5 desaturase.[60, 64] AA can further metabolize through a series of enzymes of cyclo-oxygenase and lipoxygenase into the 2 series of prostaglandins of TXA_2, PGE_2, and PGI_2 or 4 series leukotrienes of LTB_4, LTC_4, and LTE_4.[60, 64]

The n-6 synthesis path is considered to promote platelet aggregation and is considered to lead to pro-inflammatory molecules.[60, 64] The omega-3 (n-3) synthesis, however, leads to prostanoids and leukotrienes that are considered to be anti-inflammatory.[60, 64] Due to the negative effects, inhibitors for the omega-6 pathway have been developed, including the inhibition of phospholipase A, which is the enzyme that signals the release of AA.[65] ALA, the n-3 synthesis series initial compound, is converted into EPA by Δ^6 desaturase, chain elogation and Δ^5 desaturase.[60, 64] DHA is formed from additional Δ^6 desaturase enzyme. EPA through a series of enzymes, cyclo-oxygenase and lipogenase, is converted in the n-3, anti-inflammatory eicosanoids of the 3 series prostanoids TXA_3, PGE_3, and PGI_3 and 5 series leukotrienes LTB_5, LTC_5-LTE_5.[60, 64] The omega-6 and omega-3 metabolic series is detailed in Figure II-3. It is important to note that humans lack the enzyme to convert LA into ALA.[1] Each synthesis mechanism plays a role in the pathology of a number of conditions including cardiovascular disease which will be discussed in detail.

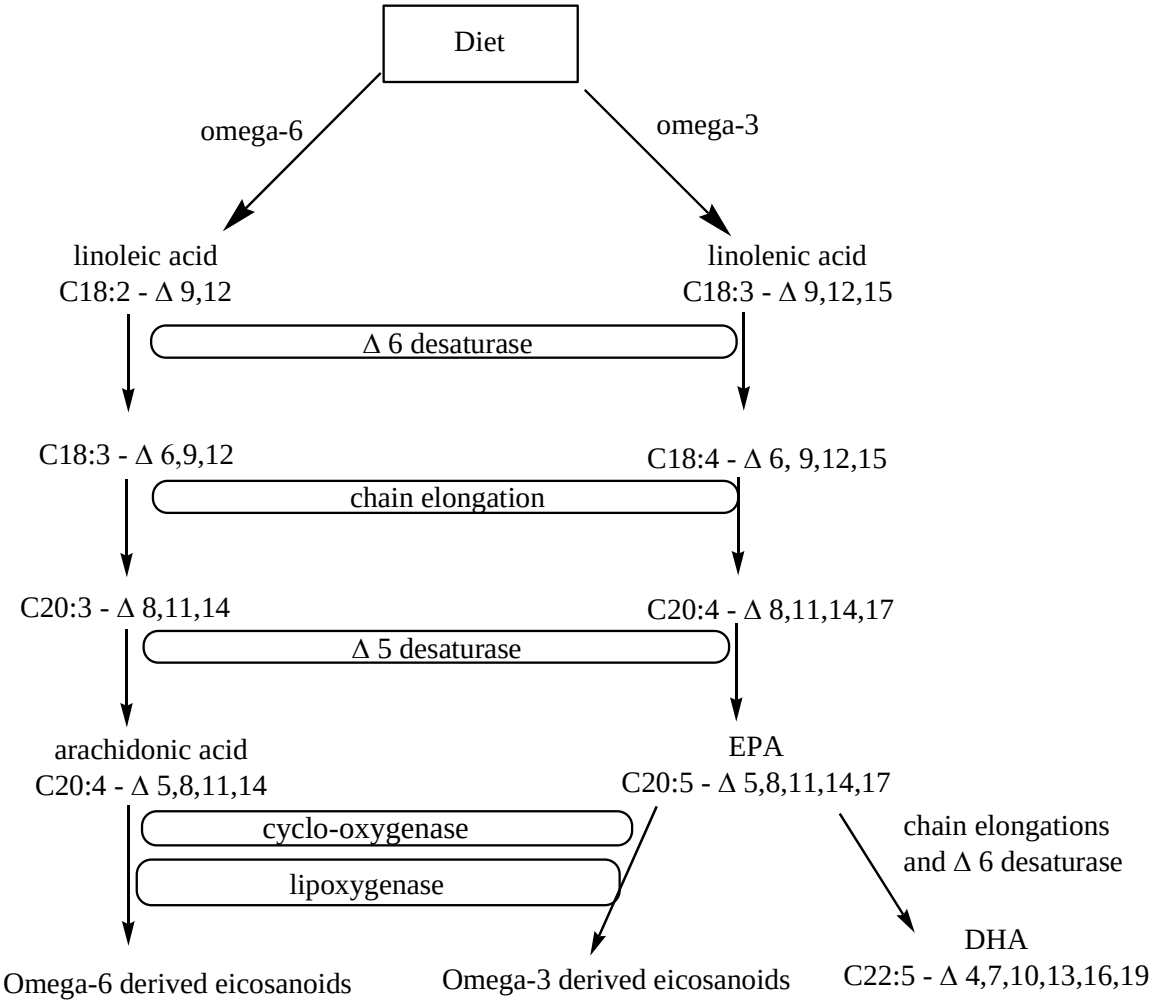


Figure II.3: Metabolic pathways for the essential fatty acids of linolenic acid and α-linolenic acid [64, 66]

Due to the inability to convert LA into ALA, the omega-3 and omega-6 pathways compete for the same set of enzymes, elongase, Δ^6 desaturase, and Δ^5 desaturase, making the two pathways interact with each other for the same resources. The generally accepted preference for the enzymes is omega-3, then omega-6, followed by omega-9.[60] This order raises two important observations. The first is that if cell walls have a high abundance of omega-9 in modern humans, then it is suggestive that there is a deficiency of the omega-3 and omega-6 compounds in the biological system since there should only

be small amounts of the omega-9 compounds based on this order.[60] The rate limiting reaction is the conversion of ALA to EPA using Δ^6 desaturase and the enzyme favors ALA over LA.[67] Typically, however, in the modern diet there is a higher concentration of LA than ALA available.[1, 67] With the increase in concentration, the conversion of LA with Δ^6 desaturase becomes the favored reaction pathway resulting in an increase in the production of AA.[67]

A difference in gender affects the rate of conversion of ALA into EPA and DHA. In a comparison of males and females, the conversion of ALA into EPA and DHA is greater in women, possibly due to the presence of estrogen.[67] The increased rate of conversion may be necessary especially in pregnant and lactating women to meet the needs of the developing fetus or child.[67] The developing fetus during the final weeks of the last trimester requires 15-35 mg/week of DHA for brain development alone.[68] The women's conversion rate may be high to accommodate these needs. In a recent study to test this hypothesis of gender difference, 36 subjects between the ages of 18 and 35 of mixed genders were used to determine the concentration of circulating omega-3 PUFA in plasma with no changes in their diets.[69] The concentration of DHA in plasma phosphatidylcholine, triglycerides, and NEFA was higher in women which is in support of the hypothesis.[69] In both genders, the location of adipose tissue deposits on the body can have an effect. Upper-body adipose tissue deposits release free fatty acid into circulation faster than lower-body deposits and creates a high concentration of fatty acids available for processing.[70] Women release free fatty acids faster than men but have an undetermined mechanism that prevents a higher concentration of free fatty acids or more oxidized fatty acids.[70] Regardless of the gender differences, the overall efficiency rate

for human conversion of ALA into the longer chain fatty acids is lower than in other species.[67] The conversions of ALA to EPA, EPA to the DHA precursor and the precursor to DHA are 0.2%, 0.13 % and 0.05% efficient, respectively.[67] For this reason, in order to increase the amount of DHA and EPA, a dietary supplement of these fatty acids is usually used for metabolic and nutritional studies instead of a supplement of ALA.

A number of dietary and health factors affect the activity of both the Δ^5 and Δ^6 desaturase enzymes. Cholesterol, saturated fats, alcohol, and trans fatty acids are capable of decreasing the activity of these enzymes.[60, 63] Protein deficiency, fasting, a glucose-rich diet, and increasing age have been shown to lower the activity of Δ^6 desaturase enzyme.[60] In order to maintain normal activity, Δ^6 desaturase needs to have the co-factors of pyridoxine, zinc, and magnesium.[60] Insulin, a reduced calorie diet, and a fat free diet can increase the Δ^6 desaturase performance.[60] In a study of mice, conjugated linoleic acid (CLA) was able to increase the activity and the messenger ribonucleic acid (mRNA) expression of Δ^6 desaturase.[71] In the mice that were fed a 1.5% diet of either CLA, LA, or a saturated fatty acid, palmitic acid, the mRNA expression of Δ^6 and Δ^5 desaturase was higher in the mice fed the CLA diet than those receiving the other two.[71] Based on this result, the CLA may have a positive effect on the conversion of LA and ALA into the longer chain fatty acids. Health conditions, such as diabetes mellitus, hypertension, hyperlipidemia, and metabolic syndrome X, lower the activities of Δ^6 and Δ^5 desaturase.[60, 72]

B. Relationship of Fatty Acids to Disease and Health Conditions

1. Coronary Heart Disease

For over thirty years, the relationship of fatty acids in CHD, specifically the long chain PUFA, has been examined in a variety of studies ranging from animal to small nutritional to larger epidemiological studies. The evidence from these studies offers support for the relationship, but some question its effectiveness in all populations and the extent of the benefits gained by altering the omega-3 or omega-6 fatty acids consumption in a diet.

a. Epidemiological and Observational Studies

One of the first studies of dietary fatty acid intake and its effect on plasma lipids was based on an observation that Greenland Eskimos, who have a diet high in marine animals, had a very low occurrence of coronary atherosclerosis or CHD. An initial study of this population showed that they had lower concentrations of cholesterol, triglycerides, and lipoproteins than a control population.[73] Using gas chromatography, Dyerberg *et al*. showed that the esterified fatty acids in the Eskimo group had a lower percent total of linoleic acid and a higher percent total of EPA when compared to the control groups[73] Although the authors state that the difference in esterified fatty acid composition and the lower concentration of the serum lipids suggest a diet like the Greenland Eskimos may be linked to lower CHD risk factors, but it does not offer a definitive answer.[73]

Kromhout *et al.* completed a twenty year study of the men in a fishing town in the Netherlands. Fish consumption, dietary, and health histories were collected in 1960, and then a follow-up of these 852 men was completed in 1980.[74] The mortality rate from

CHD was more than 50% lower in the men who consumed at least 30 grams of fish per day, and an inverse relationship between fish dosage and 20-year mortality from CHD was determined.[74] Ten years after this publication in 1985, a population control study was completed on patients who had one cardiac event; and a control group that contained primarily married men between the ages of 25 and 74 from the Seattle, Washington, area.[75] Both the control and case study patients had blood specimens analyzed, and their spouses were asked to complete a questionnaire on their diets.[75] One fatty fish meal per week was associated with a 50% reduction in the risk of having a primary cardiac event.[75] Using gas chromatographic analysis of the red blood cell membranes, they demonstrated an inverse relationship between the amount of n-3 PUFA and the risk of primary cardiac arrest.[75]

The U.S. Physicians' Health Study is a large observational study involving 22,071 US male physicians who were between the ages of 40 and 84 in 1982 with no incidences of myocardial infarction (MI), stroke, transient ischemic attack, or cancer.[76, 77] The group was randomly assigned to receive aspirin, beta carotene, both drugs, or two placebos. At the start of the study, each subject completed a questionnaire that assessed the factors for cardiovascular disease, diet, and exercise.[76, 77] Follow-up questionnaires were completed twice during the first year and annually thereafter.[76, 77] Analysis of the sudden cardiac deaths, which is death that results within one hour of the onset of symptoms, showed a decreased risk in subjects who had at least one serving of fish per week compared to the subjects who only consumed fish once a month.[77] There was a reduction in overall mortality, but no decrease in risk for total MI, non-sudden cardiac death or total cardiovascular mortality.[77] Baseline serum samples from the subjects who had

experienced sudden cardiac death were analyzed for serum levels of fatty acids using gas chromatography and then compared to control samples.[76] The n-3 baseline concentration was inversely proportional to the risk of sudden death before and after other cardiac risks factors, and other fatty acid groups were adjusted in the model.[76] By comparing the lowest quartile with the highest, there was an 81% lower risk of sudden death.[76]

In a similar study design like the U.S. Physicians' Health Study, the Health Professionals' Follow-Up Study is a cohort study with 45,722 male US health professionals who had no incidences of cardiovascular disease at the baseline in 1986.[78] The dietary intake of n-6 and n-3 PUFA was determined by questionnaires completed by the subjects at intervals over a 14 year period.[78] The risk of sudden death was lowered with a higher intake of EPA and DHA (≥ 250 mg/day) and was independent of the n-6 consumption.[78] EPA and DHA consumption did not have an association with the risk of nonfatal MI or total CHD.[78] Higher ALA intake was inversely associated with risk of nonfatal MI, regardless of high or low n-6 consumption; but, ALA and n-6 did not show any association with sudden death.[78] Men with less than 100 mg/d of EPA and DHA had an interesting outcome. With each 1 g/d of ALA, there was a lowering of the risk of nonfatal MI and total CHD (58%, MI and 47% CHD).[78]

Most of the studies discussed above relied on the dietary intake for analysis of the relationship of the omega-3 fatty acids and CHD. Nonesterified fatty acids (NEFA) or free fatty (FFA) acids are a possible risk marker that can be directly measured in the serum and is free of biases from the subject. In the Paris Prospective Study, a high fasting NEFA was an independent risk factor for sudden cardiac death, but not for MI or overall cardiovascular mortality.[79, 80] In the same study, the concentration of NEFA was

predictive of cancer mortality and was stronger in smoking and/or alcohol-related tumors.[79] In contrast to the Paris study, the Ludwigshafen Risk and Cardiovascular Health Study concluded that the NEFA levels predicted all-cause and cardiovascular mortality in the subjects who had a coronary angiography at the start of the study.[81] These results were independent of other cardio risk factors including BMI, HDL-C, LDL-C and triglyercides.[81] These two studies involved less than 5,000 subjects, and the use of such small population samples limits these results. Additional studies need to be completed to determine if NEFA can be considered for inclusion as a cardio risk factor.

Not all studies have been able to determine an inverse relationship between cardiac events and the intake of n-3 fatty acids. The Health Professionals' Follow-up Study showed no statistically significant relationship between the fish consumption and the risk of sudden death, nonfatal MI, and other CHD related events.[82] The EURAMIC (EURopean multicenter case-control study on Antioxidants, Myocardial Infarction, and breast Cancer) study involves subjects from eight European countries and Israel. Patients with their first MI (n=639) and controls (n=700) had subcutaneous adipose tissue samples collected and the fatty acid content was analyzed using gas chromatography.[83] An inverse relationship between ALA percentage in the adipose tissue and the risk of an MI was evident, but the protective effect of DHA was not demonstrated.[83] In the Seven Countries' Study, an initial positive relationship was seen between fish consumption and 25-year mortality from CHD; but after adjusting for confounding effects of saturated fatty acids, antioxidants such as flavonoids, and smoking, the relationship was no longer statistically significant.[84]

As mentioned in the metabolism of the fatty acids, women are able to convert ALA into EPA and DHA to yield higher concentrations than men. Up to this point, the epidemiological studies have focused on the male sex. The Nurses' Health Study began in 1976 with 121,700 women between the ages of 30 and 55 who were nurses in 11 US states.[85] Dietary information from 84,688 of these subjects who did not have cardiovascular disease or cancer was collected through questionnaires in 1980, 1984, 1986, 1990, and 1994.[85] After adjustments for cofactors including age and smoking, the omega-3 fish consumption was inversely proportional to the risk of CHD death and to a lesser extent CHD and nonfatal MI.[85] In the same study, *trans* fatty acids were analyzed for their risk of CHD. Mozaffarin et al. conducted a review of healthy women involved in the Nurses' Health Study I and II. Based on dietary intake of *trans* fatty acids and markers for systemic inflammation, there was a positive relationship between CLA and the inflammation biomarkers.[63] A consumption related trend of lowering incidences of CHD with higher numbers of fish servings per week was shown in a review of the subjects in the Nurses' Health Study who were diagnosed with type 2 diabetes with no history of CHD at the start of the study.[86] In the twenty-year follow-up, the inverse relationship of lowering risk of CHD with PUFA and a positive relationship of trans fat with CHD was held with all cofactors' adjustments made. Oh et al. showed that younger women and women who are overweight (body mass index (BMI) > 25 kg/m^2) had the most evident inverse relationship between fish consumption and risk of CHD.[86] ALA dietary intake was linked to a reduced risk of sudden cardiac death, but not to nonfatal MI or other CHD cardiac events in the Nurses' Health Study.[87] When comparing the lowest

quintile of ALA consumption and the highest two, those in the highest had a 38 to 40 % reduction in risk of sudden cardiac death.[87]

Differences in the epidemiological studies may be due to variations in definitions of the cardiac end points such as sudden death, differences in the populations studied, and the difficulty of having subjects estimate their servings of fish. Case-control studies have limitations including vulnerability to biases based on the subjects' recall of his or her diet and in the selection process of focusing on one disease. Cohort studies reduce this vulnerability, but they are limited in the classifications set at the baseline of the study, changes to diet in between follow-ups not being collected, and the loss of subjects during the extended study can limit the validity of the results of these studies.

b. Diet Intervention Studies

Diet and Reinfarction Trial (DART) was the first controlled dietary intervention study able to link omega-3 fatty acid and a positive response in the secondary prevention of myocardial infarction (MI). The study focused on 2033 men who had recovered from a MI.[88] The men were randomly selected into groups in which one group was asked to increase their intake of fatty fish to at least two servings per week or were given a fish oil supplement if they could not eat fish.[88] After a two year follow-up, the men advised to eat fish had a 29% ($P<0.05$) decrease in all-cause mortality, but no reduction in a second MI was seen.[88] Also, subjects who consumed about 4 grams of fish per day lowered serum TG by 25 to 30% .[88] Supporting evidence for this initial study was found in the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico Prevenzione (GISSI- Prevenzione) study which was a randomized trial of 11,324 patients who had suffered a MI within three months.[89] The groups consisting of 85% males were placed on

supplements of n-3 PUFA with 850 -882 mg of EPA and DHA daily, 300 mg/day of vitamin E (α-tocopherol), both types, or a placebo for 3.5 years.[89] The relative all-mortality risk decreased by 15% (P=0.023), and a 45% (P=0.01) decrease in sudden cardiac death was seen.[89]

In support of the previous studies, a randomized study of the treatment for the prevention of a second MI was completed on an Asian male population in the Indian Experiment of Infarct Survival. In an 18 hour period after an apparent MI, the patients were randomized into a fish oil (EPA, 1.08 g/day), mustard oil (ALA, 2.9 g/day), or placebo group.[90] After a one-year follow-up, the fish oil group had significant reductions in complications after a MI and in total cardiac deaths when compared to the placebo.[90] The mustard oil group did not have a reduction in total cardiac deaths; however, it did show a reduction in complications seen after a MI such as cardiac arrhythmias and ventricular enlargements.[90] Although this study is small in number, it suggests that a secondary prevention effort using fish oil or mustard oil should be started early after the initial symptoms of an MI. Using the idea that initial treatment for secondary prevention must be started early, a Norwegian study examined the effect of a daily four gram dose of n-3 or corn oil.[91] Each n-3 capsule contained 850-892 mg of EPA and DHA.[91] The comparison between the groups yielded no benefit of the one type of oil over the other in the prevention of another cardiac event which included sudden cardiac death, second MI, or unstable angina for a one to two year period.[91] This study may show that a population which already has a diet high in n-3 fatty acids may not benefit from an increase in these fatty acids after a cardiac episode. Also, the comparison of n-3 fatty acids and the corn oil, which is rich in n-6 and n-9 fatty acids, may not be a controlled study. A control with

an actual no oil placebo would have been a comparison similar to that seen in previous studies. The study did, however, see an increase in HDL cholesterol and a lowering of TG concentrations which show that n-3 fatty acids may have a positive effect on these cardio risk factors.[91] The Norwegian study is not the only one to not see a positive effect. A trial of 3,114 men with angina under the age of 70 was divided into random groups and provided with dietary advice to eat two servings of oily fish per week or to take 3g of oil as a substitute, eat four to five portions of fruits and vegetables per day, eat a combination of both, or advised to eat sensibly.[92] The all-cause mortality reduction seen in other studies was not demonstrated here. The fruit and vegetable group had poor compliance but saw results.[92] The men advised to eat fish saw no improvement and; in fact, the members of the group that took fish oil capsules had a higher risk of cardiac death (11.5% v 9%, P=0.2).[92] The authors state that the lack of evidence to match other studies may be a reflection of the lack of compliance by the subjects or the adjustments for other risk factors being inappropriate.[92]

The measurement of serum lipids and statistics on the cardiac events after n-3 fatty acids supplementation are not the only measures of the effects of these fatty acids on CHD. A coronary angiogram is a specialized X-ray that uses an injected dye to view the blood vessels of the heart. A trial of 223 patients who were clinically proven to have coronary artery disease were randomized into a control group that received a fatty acid tablet with a ratio similar to the European diet and a group that received a capsule with fish oil that was approximately 55% EPA and DHA.[93] Besides the lowering of cardiac events, the patients on the fish oil supplement saw more regression of the coronary artery

disease on the angiogram than the placebo group.[93] The authors also noted an increase compared to the controls in the LDL cholesterol concentration.[93]

c. Omega-6 Fatty Acids

Omega-3 fatty acids have been the focus of the majority of studies completed on the role of fatty acids in CHD, but omega-6 fatty acids have been the center of attention in a few studies. Omega-6 fatty acids are essential; but like fat-soluble vitamins, a level must be maintained that is both biologically necessary and beneficial. The inhabitants of Israel can be considered a test population for the effects of omega-6 fatty acids. The general Israeli diet is higher in LA than the diets in the United States and Europe.[94] Israel has seen an increase in the cases of cardiovascular disease, hypertension, non-insulin dependent diabetes mellitus (NIDDM), and obesity.[94] All of these diseases are linked to hyperinsulinemia and insulin resistance which can be induced by LA.[94] The paradox of omega-6 fatty acids is that they have been shown to reduce the total cholesterol concentration and high-density lipoprotein cholesterol (HDL-C) which reduces the risk of CHD mortality; but at the same time, the measurement of the LDL oxidation susceptibility and risk of acute MI is increased, as seen in the Jerusalem Nutrition Study.[94, 95] The hypothesis for omega-6 actions is that LA and the hydroperoxides of LA may reduce the activity of prostacyclins in the vascular wall and aid in platelet aggregation.[94, 95]

The adipose tissue and platelet concentration of fatty acids was determined by gas chromatography in 226 patients who were having a coronary angiogram due to coronary artery disease (CAD) or valvular heart disease.[96] The linoleic acid percentage of the total fat in each type of sample was positively associated with myocardial score, which is the

measure of the location and severity of the coronary lesions on a scale of 0 (best) to 15 (worst), in men.[96] Platelet EPA in men and platelet DHA in women was inversely associated with the myocardial score.[96] Recently, Laaksonen *et al.* completed a 15 year cohort study on 1551 middle-aged men which included an examination of their dietary intake and the measurement of the serum fatty acid concentration by gas chromatography.[14] When the upper third was compared to the lower third of dietary consumption of LA and ALA, both lowered the risk of mortality by cardiovascular disease to 61 % less likely for LA and 30 to 42% less likely for ALA.[14] The total serum concentration of the esterified PUFA was more strongly associated to the risk of CVD mortality than the measurement of the dietary PUFA intake, and no association of total fat intake was seen with CVD mortality.[14] No association was also found between serum omega-3 fatty acid concentrations and CVD mortality.[14] In a New Zealand 1997 National Nutritional Study, the serum cholesteryl and fatty acid concentrations in cholesteryl esters, phospholipids, and triglycerides were determined.[97] The myristic acid in all three lipid measurements was positively associated with total serum cholesterol, and the cholesteryl ester form of myristic acid was a stronger predictor of total cholesterol than TG or phospholipids.[97] LA inversely correlated with the total serum cholesterol.[97] This is one test that supports the use of fatty acids as a predictor of serum cholesterol; and since cholesterol is one of the primary risk factors of CHD, fatty acids may be an important predictor to include. The role of CLA in CHD is not clear. It has been determined that the role of CLA is isomer dependent and cis-9, trans-11 and trans-10,cis-12 have different effects on lipids, but the complete effect on human health is not

clear.[98] Testing on isolated isomers needs to be completed in order to determine the health advantages or disadvantages.

d. Systematic Reviews

Since a large number of studies have been completed on omega-3 fatty acids, meta-analysis or systematic reviews of the trials have been published. Harris *et al.'s* (1997) analysis reached the following conclusion on omega-3 consumption: total cholesterol is not affected, LDL-C and HDL-C rises slightly (5-10%, 1-3%, respectively), and TG is reduced by 25 to 30 %.[99] ALA did not have the same effect.[99] In a systematic review, the higher risk reduction in CHD mortality is seen in high-risk subjects who consume 40-60 g of fish daily.[100] Little to no risk improvement is seen in already low-risk subjects.[100] Buther et al. completed a meta-analysis of 11 trials completed between 1966 and 1999 and determined that both dietary and supplemental intake of omega-3 fatty acids reduces total CHD mortality, mortality due to MI, and sudden death in patients with CHD.[101] The two most recent systematic reviews of omega-3 studies offer conflicting results. Hooper et al. examined 48 randomized controlled trials and 41 cohort studies that measured effects of omega-3 for 6 months or longer.[15] No effect of omega-3 fatty acids on total mortality, cardiovascular events, or cancer was statistically significant.[15] There was also no separation of studies that looked at primary vs. secondary MI events. Wang el al. examined both primary and secondary studies separately in their meta-analysis. Fourteen randomized control trials examining secondary prevention yielded a strong reduction in all-cause mortality, MI, and cardiac and sudden death.[102] Although weaker, a similar relationship was found in the 25 primary prevention studies and no relationship of ALA to CHD endpoints was seen.[102] A

number of issues plagued these studies including dose variations, differences in the types of fish or supplements consumed, variations in the inclusion criteria, and limited data on ALA and women.

e. Fatty Acids and Simvastatin

Simvastatin, the major form of treatment for hyperlipidemia, can have an effect on the fatty acid metabolism and alter the serum fatty acid composition in subjects. Simvastatins inhibit the 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase, a key enzyme in cholesterol synthesis, which makes it available to act in the fatty acid metabolic synthesis through malonyl-CoA or oxidation in the Krebs cycle by acetyl-CoA.[103] As a result, a number of studies have begun to examine how simvastatin alters the fatty acid composition and how a combination therapy of statins with omega-3 may benefit CHD patients. Two separate studies on hypercholesterolemic patients examined fatty acid composition of serum by gas chromatography from patients who were on a 30 to 40 mg/d dose of statins and were monitored at 12 to 24 weeks after the initial dose. In both cases, the total sum of the PUFA was decreased by 13 and 22%.[16, 103] In the first study, the relative percentages, but not the concentrations of LA and ALA decreased and AA and DHA increased.[16] The second study demonstrated a similar decrease in LA and ALA and an increase in AA, but the study also showed an increase in GLA.[103] The sum of EPA, DHA, and AA had a significant increase.[103] Based on the metabolic pathway, these results suggest that the statins increase the activity of the Δ^5 and Δ^6 desaturases which leads to the production of more AA, EPA, and DHA. Further studies have to be completed to demonstrate if this hypothesis is valid. In an examination of free fatty acid metabolism changes with high-dose simvastatin in type 2 diabetics,

plasma palmitate, a saturated fatty acid, had not changed when compared to the normal controls.[104] No other free fatty acids were monitored. In a study of the combination of atorvastatin with omega-3 supplements, the subjects with the combined treatment had an increase in the HDL-C concentration and a decrease in systolic blood pressure when compared to the controls and subjects with only statin.[105] Omacor, a concentrated omega-3 capsule that contains 44% EPA and 36% DHA, was given to patients with hypertriglyceridemia who were not being controlled by simvastatins. The serum TG decreased by 20-30% and VLDL-C by 30-40% with no effective change in the LDL-C or HDL-C concentrations.[106] The unchanged HDL-C serum concentrations may be due to the fish oil causing a decrease in the production and fractional catabolic rate of HDL apo A-1 and HDL apo A-II resulting in no significant change.[107] Atorvastatin did not have an influence on either rate.[107]

f. Mechanism of Action

The mechanisms of action, particularly the protective effects of the omega-3 fatty acids, are not clear. Fatty acids incorporate into lipoproteins and cell membranes, where they affect membrane characteristics, alter eicosanoid production, increase the relaxation of the blood vessels, and induce dose dependent reductions in platelet aggregation, leukocyte activation, and plasma triglycerides, all of which might reduce the risk of coronary heart diease.[83] The first mechanism and the one that influences other diseases besides CHD, is the anti-inflammatory effect. The omega-6 fatty acids are known to produce eicosanoids that increase platelet aggregation and are pro-inflammatory.[60] With an increase in the omega-3 fatty acids, and given the fact that the common enzymes are preferential to them, the shift of the metabolism would be to the

omega-3 fatty acids which produce anti-inflammatory eicosanoids.[60] A second mechanism is the inhibition of endothelial activation, which is the process of increasing the endothelial expression of leukocyte adhesion molecules in atherogenesis.[108] The greater number of double bonds in n-3 fatty acids showed a greater inhibition of key factors in endothelial activation.[108] The omega-3 function may include a reduction in adhesion molecule expression and platelet-derived growth factor.[109] As seen in the DART trial, the third mechanism of lowering the CVD risk is by the lowering of the TG serum levels.[88] Doses of 3 to 5 grams have been used in the treatment of hypertriglyceridemia.[64] The exact mechanism of this function is unknown, but according to Chan et al., fish oil supplements lowered plasma TG concentrations by decreasing the VLDL apo-B production.[107] The other potential mechanism reduces susceptibility of ventricular arrhythmia, is antithrombogenic and slightly hypotensive, and promotes nitric oxide-induced endothelial relaxation.[60, 109]

g. Dosage and Adverse Effects

The American Heart Association (AHA) recommendation for the consumption of omega-3 fatty acids for the prevention of CHD is dependent on a person's risk for CHD. AHA recommends that adults eat a least two servings of fish per week and/or ALA consumption through plant sources like walnuts, flaxseeds, and soybeans if no history of CHD exists.[109] Patients with CHD are recommended to intake at least 1 gram of EPA and DHA per day either by fish or omega-3 fatty acid capsules.[109] A higher dose of 2 to 4 grams of EPA and DHA is recommended for patients who have hypertriglyceridemia.[109] Most of the adverse affects of omega-3 fatty acids are dose dependent. Omega-3 fatty acids can be an anticoagulant and affect bleeding time, so high

doses of fatty acids should be monitored by a physician.[62, 109] As stated in the omega-3 trials, an increase in LDL-C can occur. Additional side effects can include fishy aftertaste and gastrointestinal problems.[62] With oily fish consumption, one must be concerned with toxins in the fish. Methylmercury, polychlorinated biphenyls, and dioxins can be found in fish such as shark, swordfish, king mackerel, and other large, predatory fish.[62, 109] Some of these toxins may aggravate CHD and are not recommended for women who are pregnant or lactating and young children due to possible effects on development.[62, 109] There are a number of options for other fish that have lower concentrations of these toxins but still have high concentrations of EPA and DHA that can be consumed to limit exposure.

2. Diabetes and Metabolic Syndrome

Metabolic syndrome is a collection of related conditions that were determined to be from metabolic changes that can lead to the development of atherosclerotic cardiovascular disease and diabetes. The idea of metabolic syndrome has been in the literature for years, but in the late nineties, a push to define the condition was started. Organizations such as the World Health Organization (WHO), European Group for the Study of Insulin Resistance, and the NCEP:ATP III all produced definitions of metabolic syndrome. The syndrome definition according to the ATP III, for example, is that a patient who has metabolic syndrome will have three of the following: waist circumference greater than 102 cm in men and 88 cm in women, serum triglycerides greater than or equal to 1.7 mmol/L, blood pressure greater than or equal to 130/85 mmHg, HDL cholesterol less than 1.0 mmol/L in men and 1.3 mmol/L in women, and a serum glucose greater than or equal to 5.6 to 6.1. [2-4] All of the definitions agree on the

basic components, glucose intolerance, obesity, hypertension, and dyslipidemia, but not on the minimum criteria.[2-4] In late 2005, two scientific statements from joint groups related to metabolic syndrome appeared from the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) and the American Heart Association (AHA) and National Heart, Lung, and Blood Institute (NHLBI).[2, 4] The claims made in the ADA/EASD statement focus on the lack of the syndrome existing purely and not having dual patients who are both diabetic and have metabolic syndrome.[4] Kahn et al. stated that the development of the syndrome is unclear, expressed doubt regarding its use as a marker for CVD risk, and questioned how the syndrome was any different than the individual factors that define it.[4] The AHA/NHLBI statement supported the premise that the syndrome exists and set recommendation guidelines for the treatment of the condition.[3] Despite the conflict over the criteria and the existence of the syndrome, the ending conclusion for both statements is that more research needs to be conducted. In the AHA/NHLBI statement, it lists measures that are associated with the syndrome that need to be studied in greater detail; and free fatty acids are included due to their link to insulin resistance.[2]

Insulin resistance is one prevailing pathological path for the development of metabolic syndrome and dyslipidemias.[2-8] An increase in the concentration of fatty acids in serum is a major contributor to the development of insulin resistance. Fatty acids in serum can be from adipose tissue triglycerides or the product from lipoprotein lipase of lipoproteins rich in triglycerides.[3] Insulin plays a role in both processes. Once the excess fatty acid reaches insulin sensitive tissues such as muscles, they inhibit the glucose intake by inhibiting the insulin mediated pathway.[3] In the liver, increased free fatty acids

increased the concentrations of glucose, triglycerides, very low density lipoproteins, and low density lipoproteins and decreased the release of high density lipoproteins.[3] All of these markers are current measures for the definition of metabolic syndrome, except for the causes of these changes which are the concentrations of the free fatty acids. It is interesting to note here that the waist circumference guideline in the defining criteria of metabolic syndrome is the accumulation of visceral fat. As related to the development of insulin resistance, the larger amount of adipose tissue available allows for a great source of triglycerides that can be released as free fatty acids. Garaulet et al. found that central obesity was positively related to the omega-6 PUFA and inversely related to both mono unsaturated fatty acids and polyunsaturated fatty acids.[110]

Recent studies have begun to look at the effects of a particular set of fatty acids, polyunsaturated fatty acids, and their possible ability to prevent the onset of insulin resistance; and, therefore, the prevention of metabolic syndrome and the other conditions that may develop. Omega-3 fatty acids, particularly fish oil, and to a lesser extent omega-6 fatty acids have been linked to the prevention and the mediation of insulin resistance.[5-8] Vessby et al. combined the results of a number of studies that examined the serum lipid fatty acid concentration of patients with conditions related to insulin resistance such as diabetes, obesity, and coronary heart disease.[8] Vessby et al. determined that when compared to controls, the disorders showed a decrease in linoleic acid and an increase in dihomo-gamma linolenic acid.[8] They also indicated an increase in $\Delta 9$ and $\Delta 6$ desaturases and a decrease in Δ^5 desaturases activities which can be monitored through the use of long chain polyunsaturated fatty acid (LCPUFA) concentrations.[8] More studies have associated the dietary intake of fish oil which is

primarily composed of omega-3 fatty acids with the prevention of the symptoms of insulin resistance. EPA and DHA, omega-3 fatty acids found in fish oil, have been used in dietary studies and have lowered triglyceride and VLDL levels in serum which are related to both insulin resistance and coronary heart disease risk factors.[5-7] One interesting study completed by Stene and Joner that focused on fish oil intake demonstrated that it lowered the risk of the development of type I diabetes mellitus through the consumption of cod liver oil during the first year of life.[5, 9] There are also studies that fish oil can delay the onset of diabetes through slowing the progression of the disease through its anti-inflammatory, antithrombotic, and antiarrhythmic properties.

3. Other Diseases

Fatty acids have a number of functions in biological systems that make them essential including controlling membrane characteristics by their structure, the production of eicosanoids, and other functions previously discussed. Their nature leads them to involvement not only in CHD, but also in other diseases related to inflammation. The mechanisms of breast, prostate, and other cancers in *in vitro* studies have been altered favorably, but epidemiological evidence has not come to the same conclusion in all cases.[111] In a review by Terry *et al.*, five of the twelve epidemiologic studies of breast cancer risk showed a significant inverse association with fish or marine fatty acids.[111] Prostate cancer research showed that 5 out of 11 studies in which the n-6 diet was higher and the n-3 intake was lower, found that marine fatty acid reduced the risk of prostate cancer.[111] The possible mechanism of n-3 anticarcinogenic function include the prevention of AA converting to its eicosanoids which are known to be pro-inflammatory, changed cell growth and metabolism by influencing gene expression and signal

transduction, increasing or decreasing free radicals, and altering the metabolism of estrogen.[112, 113] Rheumatoid arthritis, a disease related to inflammation, may be improved through an increase in omega-3 fatty acids in the diet.[65, 114] The dietary intervention trials of omega-3 fatty acids have shown a reduction in the symptoms of rheumatoid arthritis in patients and, in some cases, the subjects are able to stop the use of non-steroidal anti-inflammatory drugs (NSAIDs).[115-117] The omega-3 fatty acid mechanism in this case is to inhibit the production of prostaglandin E2, which is a product of the cyclo-oxygenase (COX) enzyme that promotes inflammation and is also inhibited by NSAIDs.[115-117]

The effects of the omega-6 increase in the modern diet have also been suggested for the rise in mood disorders including depression, bipolar disorder, and postpartum depression.[118] In a review by Parker *et al.*, the increased consumption of fish was associated with a lower incidence of these types of mood disorders in a majority of the studies reported.[118] The use of EPA and DHA for the treatment of major depressive disorder, bipolar disorder, and borderline personality disorder has been completed in eight studies where five showed an improvement in the scoring of the condition or, if on a mood stabilizing drug, a decrease in the side effects of the medication.[118] EPA has also been linked to a decrease in the occurrence of osteoporosis in post-menopausal women by the suppression of osteoclast, which induces bone resorption, and by the inhibition of its precursor's cytokine formation.[119]

CHAPTER III

Review of Cholesterol and Fatty Acid Quantification Methods

I. Measurement of Cholesterol, Triglycerides, and Lipoproteins

A multitude of measurement methods have been used to determine the concentrations of cholesterol, triglycerides, and lipoprotein subfractions since each was linked to disease, especially CHD. The CDC has established both the reference method and simpler, routine methods for each of these measurements. The CDC, the NCEP, and other organizations have established these methods in clinical laboratories and have set guidelines to ensure standardization and reliable measurements at each individual lab.[120-122]

A. Cholesterol

A method developed by Abell *et al.*[123] has become the basis for the CDC reference method for cholesterol.[18] Procedurally, a 0.5 mL sample of serum is treated with 5.0 mL of alcoholic KOH which hydrolyzes the cholesteryl esters.[18] After the cholesterol is extracted with 10 mL of hexane, a portion of the cholesterol solution is dried and exposed to the Liebermann-Burchard[124, 125] reagent. The L-B reagent was first published by Liebermann and then applied to cholesterol by Burchard. The current version of the reagent is acetic acid, sulfuric acid, and acetic anhydride. The reagent

reacts with cholesterol and the absorbance of the product is read at 620 nm.[18] The proposed mechanism by Burke *et al*. starts with the protonation of the –OH group and a loss of water followed by successive protonation resulting in an exceedingly conjugated product which is pictured in Figure III.1.

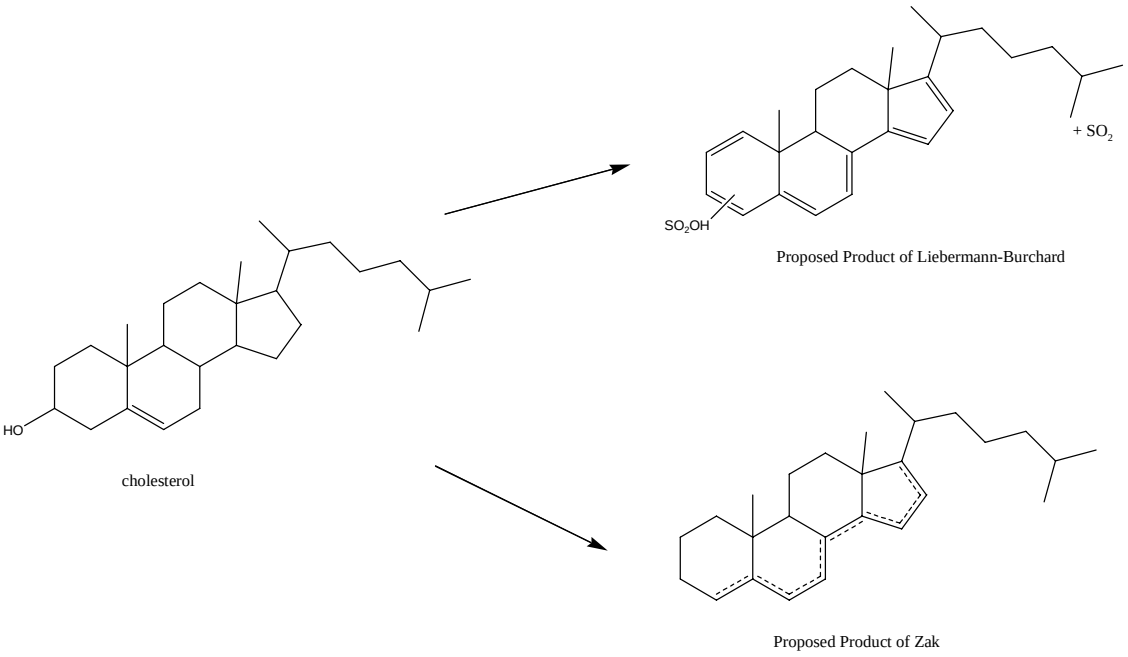


Figure III.1: Proposed products of the Liebermann-Burchard and Zak reagents by Burke et al. [126]

Under strict protocol and the use of pure cholesterol as a standard for calibration, the bias when compared to isotope dilution mass spectrometry is 1.6%.[18] Isotope dilution mass spectrometry is the standard method of detection utilized by the National Institute of Standards and Technology.[18] Cholesterol concentration is expressed as either mg/dL or mmol/L.

Additional color reactions similar to the L-B reaction have been developed and analyzed for their ability to determine the cholesterol concentration in serum samples.

Webster completed a review and comparison of three of these colorimetric reactions that work satisfactorily with cholesteryl esters. The Sperry-Webb reagent has a composition that includes glacial acetic acid, acetic anhydride, and sulfuric acid while the Trider color reagent utilizes dichloroethane, acetyl chloride, and sulfuric acid.[19] The Zak reaction is much similar to the L-B reagent system with Fe^{3+} replacing the acetic anhydride in the acetic acid and sulfuric acid system which produces a colored product.[127] The Zak reagent system was determined to be the best reagent due to its greater sensitivity and stability.[128] Another color reagent adopted by Chugaev and Gastev used a 2:1 mixture of 20% w/v $ZnCl_2$ in glacial acetic acid combined with 98% acetyl chloride.[129] The Chugaev and Gastev reagent was used to measure cholesterol with great accuracy by researchers Hanel and Dam.[130]

The enzymatic method is the primary clinical routine method for the measurement of cholesterol. The reagent system is commercially available in standard manual kits and offers specialized automated instruments that can handle the method. The general procedure is to mix the reagent with 3-10 μL of serum or plasma, incubate for a period of time, and then measure the absorbance.[18] The general series of reactions are as follows:[18]

$$\text{Cholesteryl ester} + H_2O \xrightarrow{\textit{cholesterol ester hydrolase}} \text{cholesterol} + \text{fatty acid} \quad (3.1)$$

$$\text{Cholesterol} + O_2 \xrightarrow{\textit{cholesterol oxidasae}} \text{cholest-4-en-3-one} + H_2O_2 \quad (3.2)$$

$$H_2O_2 + \text{phenol} + \text{4-aminoantipyrin} \xrightarrow{\textit{Peroxidase}} \text{quinoneimine dye} + 2\ H_2O \quad (3.3)$$

Interferences that may include high concentrations of bilirubin, ascorbic acid, compounds consuming H_2O_2, and plant sterols, can occur when the method is utilized because the reaction is not selective to only cholesterol.[18] The reaction is not affected though by hemolysis, uric acid, or bilirubin in concentrations below 5mg/dL.[18] With proper

calibration and laboratory controls, the accuracy is between 1 and 3% with a coefficient of variation of 1.5 to 2.5%.[18] Additional methods such as thin layer chromatography, gas chromatography, mass spectrometry, and near infrared reflectance spectrometry have been developed, but only the standard reference colorimetric method and the routine enzymatic method are used by clinical laboratories.[18]

B. Triglycerides

The reference method for triglycerides first involved the extraction of the compounds from serum or plasma. Preprocessing includes a chloroform extraction that removes the water-soluble compounds of glucose and glycerol that interfere with the test and an application of silicic acid to remove the phospholipids.[18] The isolated triglycerides undergo alkaline hydrolysis with KOH to produce unesterified fatty acids and glycerol.[18] The free glycerol is oxidized to form formaldehyde that can react with chromotropic acid to produce a colored product that is measured at 570 nm. The summary of the reaction is the following.[18]

$$\text{triglycerides} + \text{KOH} \longrightarrow \text{fatty acids} + \text{glycerol} \qquad (3.4)$$

$$\text{glycerol} + \text{periodate} \longrightarrow \text{formic acid} + \text{formaldehyde} \qquad (3.5)$$

$$\text{formaldehyde} + \text{chromotropic acid} \longrightarrow \text{chromogen} \qquad (3.6)$$

Like cholesterol, the measurement is expressed as mg/dL or mmol/L.

The enzymatic method for the routine analysis of triglycerides does not involve an extraction step. Like cholesterol's enzymatic method, the reagent kits and automated instruments are widely available for clinical use. Although multiple options exist for TG determination by enzymes, the first step is to separate the fatty acids from the glycerol

backbone using lipase to catalyze the reaction.[18] The following is the common reaction path.[18]

$$\text{Triglycerides} + 3\ H_2O \xrightarrow{Lipase} \text{glycerol} + 3\ \text{fatty acids} \quad (3.7)$$

$$\text{glycerol} + ATP \xrightarrow{Glycerokinase} \text{glycerophosphate} + ADP \quad (3.8)$$

$$\text{Glycerophosphate} + O_2 \xrightarrow{Glycerophosphate\ oxidase} \text{dihydroacetone} + H_2O_2 \quad (3.9)$$

$$H_2O_2 + \text{phenol} + \text{4-aminoantipyrin} \xrightarrow{Peoxidase} \text{quinoneimine dye} + 2\ H_2O \quad (3.10)$$

Additional options include spectrophotometric measurement of the NADH produced in a reaction of glycerophosphate with nicotinamide adenine dinucleotide (NAD) or measurement of the production of adenosine diphosphate (ADP) in Equation 3.8 by measuring the resulting loss of NADH. Enzymatic TG methods are linear to 700 mg/dL and have a coefficient of variation between 2 and 3 % in automated systems.[18] Correction for endogenous glycerol is needed in cases of hyperglycerolemia, a rare disorder that results from a deficiency of glycerokinase, where the concentration of glycerol in serum is 50 to 100 times greater than the normal range of 1 mg/dL.[18] The concentration of the glycerol is determined by completing reactions 3.5 through 3.7 on the serum which will be the concentration of the endogenous glycerol.[18] This value is then subtracted from the TG values to determine the actual concentration of TG in the sample.[18]

C. Lipoprotein Subfractions

The CDC primary reference method for the determination of lipoprotein fractions is based on ultracentrifugation of the serum sample. One method is sequential differential ultracentrifugation which requires repeated ultracentrifugation steps that raise the solvent density to extract each lipoprotein fraction. Therefore, the densities of the

KBr solutions are changed dependent on the specific lipoprotein subfraction targeted. To float VLDL and chylomicron, the necessary density is 1.006 g/mL; for IDL 1.019 g/mol; for LDL 1.063 g/mL, and for HDL 1.21 g/mL. VLDL, IDL, HDL and LDL sub-fractions can be isolated using the vertical spin density gradient ultracentrifugation that was described by Chung *et al.*[131-133] Based on their method, the plasma will have to be adjusted to a density that is equal to or greater than HDL, which is the densest particle that will be separated (1.21 g/mL), through the use of potassium bromide, KBr. [131-133] The density-adjusted plasma will be layered under NaCl and centrifuged under conditions appropriate for the type of rotor and the sample size used.[131-133] The benefit of the Chung *et al.* method over the standard density gradient method using swingout rotors is that the separation occurs in a short time period which limits the possibility of having native oxidation of the lipoproteins and limits the time the particles are exposed to high g forces and salt concentrations.[131-133] Although effective in separating the fractions, the methods are time consuming and are usually limited to the specialized lipid medical or research clinics.

The CDC reference method for high-density lipoprotein cholesterol is a combination of ultracentrifugation and polyanion precipitation to prepare the HDL for quantification of the cholesterol by the reference method previously discussed. [18] Through ultracentrifugation, VLDL lipoproteins and any chylomicrons are removed from the top layer after 18 h at 105,000 x g.[18] The apo B-100 containing lipoproteins of the remaining IDL, LDL, Lp(a), and HDL in the infranatant are removed by precipitation with heparin sulfate and $MnCl_2$ followed by centrifugation. The remaining HDL particles are measured for cholesterol using the standard reference method. VLDL-C is

equal to the total cholesterol minus the concentration of the total infranatant cholesterol. LDL-C is the total infranatant cholesterol minus HDL-C. These estimations are possible and reasonable in normal subjects due to the concentration of IDL and Lp(a) being 2-3 mg/dL of the TC value.[18] An indirect measurement for the determination of LDL-C is the Friedewald Equation which is

$$[\text{LDL-C}] = [\text{TC}]-[\text{HDL-C}] - [\text{TG}]/5 \qquad (3.11)$$

where the [TG]/5 is based on the average ratio of TG to cholesterol in VLDL particles.[18] This equation is not accurate in cases where TG concentrations are greater than 400 mg/dL which is a problem in lipid clinics who have patients who can have TG levels in the thousands.[18] The only option in these cases is to complete the ultracentrifugation and precipitation procedure described above referred to as the beta-quantification method.

Additional methods for lipoprotein analysis have been developed. Precipitation methods that isolate one or more fractions from the others in the sample have been successful. One such example was described in the beta-quantification method. POLYMEDCO developed a method that complexes the precipitant with magnetic particles which can be removed by magnetic disk instead of a centrifugation step.[18] Chromatographic separation of the lipoproteins either by high performance liquid chromatography (HPLC) or agarose column chromatography has been successful in the separation of VLDL, LDL, HDL_2, and HDL_3. Like ultracentrifugation, the process is time consuming and expensive and limited to specialized laboratories. Immunochemical assays have been developed and are successful in the quantification of the fractions due to the difference in proteins on the particles. The last method used for the separation of lipoprotein particles is gel electrophoresis. The net charge on the surface of the

lipoprotein particle from the amino acid side chains and the phosopholipids and the size of the particles allow for separation. HDL travels the farthest on the gel followed by Lp(a), VLDL, LDL, and chylomicrons. The pattern of the sample on the gel can separate patients into normal, Type III hyperlipoproteinemia, hypertriglyceridemia, and other dyslipidemic states.[55, 56]

II. Polyunsaturated Fatty Acid Methods

For serum PUFA analyses there currently is no standard reference method. The current methods for determination of the PUFA are through thin layer chromatography (TLC), gas chromatography (GC), and high performance chromatography. New methods in mass spectrometry and nuclear magnetic resonance as part of the advancing field of lipidomics will be discussed.

A. Extraction Methods

The first step in the analysis of PUFA is the separation of the fatty acids from the serum or other biological sample. Since serum and other biological samples are complex mixtures, the extraction step is necessary in order to remove proteins, sugars, and other molecules that can interfere with the chromatographic techniques. The extraction methods can be divided into liquid-liquid extractions and solid-phase extraction.

Liquid-liquid extractions are the primary methods used for the separation of the fatty acids. The liquid-liquid extractions will have an organic/hydrophobic solvent which will dissolve the lipids and an aqueous/hydrophilic solvent for the non-lipid components. The solvent chosen is dependent on the form of lipid that is being extracted. Neutral lipids like cholesterol and triglycerides can be extracted using non-polar organic solvents

such as hexane.[134] Phospholipids, an example of a polar, complex lipid, require the use of a polar organic solvent such as methanol or ethanol.[134] The first extraction of the fatty acids from serum using liquid-liquid extraction was developed by Folch *et al.* in the 1950s.[23] Folch *et al.* used 10 mL of chloroform-methanol solution 2:1 (v/v) for every 1 mL of serum or the solution can be adjusted for use with biological tissues.[23] The sample was mixed, centrifuged at 2500 x g for 10 min at 20 °C, and the aqueous phase collected for fatty acid analysis.[23] Additional extraction solvent combinations include hexane, hexane-methanol, hexane-isopropanol, and ether.[24] In a comparison of solvents for lipid extraction, the most popular solvent, 2:1 chloroform-methanol (v/v), was only able to extract 90% of the triglycerides, which can alter the percentage of PUFA detected, and 99% of the cholesterol.[24] Hexane-isopropanol removed 53% of the TG and 94% of the cholesterol, and ether and hexane only were very poor in extracting the TG with only 2% of the original TG recovered.[24] A detergent extraction system first designed by Bordier was also tested. Modest results of 73% TG recovery were demonstrated.[24] An important note to make is that the chloroform-methanol and hexane-isopropanol solvent pairings also precipitated high concentrations of proteins where the ether, detergent system, and hexane only test did not.[24] The key choice for the liquid-liquid extraction is dependent on the fatty acids that need to be extracted. For example, fatty acid methyl esters (FAME) are non polar and can be extracted with non-polar organic solvents such as hexane.[26] A Morrison and Smith procedure is two volume extraction with pentane and one volume of water which obtained a 97-99% recovery of FAMEs.[26]

Solid-phase extraction will use a solid phase and a liquid phase to separate the sample. Phospholipids can be extracted with this method. The solid phase is composed

of silica modified with cyanopropyl-, aminoproply- or dihydroxypropoxypropyl groups which become the absorbent material.[134] Organic solvents dissolve the phospholipids and remove them from the column. In comparison with liquid-liquid extraction, solid-phase is rapid, reliable, and requires a smaller sample size; however, most methods still use liquid-liquid because larger sample sizes are available.

Once extraction has occurred, samples must be watched for degradation by oxygen, light, and solvents. All samples should be stored at -70 °C and stored under nitrogen or argon if samples will not be analyzed for an extended period of time. An anti-oxidant can be added, but care must be taken to choose one that will not interfere with any analysis.

Preparation of the methyl esters of the fatty acids in TG, phospholipids, free fatty acids, and other forms must occur before analysis. Acid-catalyzed transesterification includes methanolic, hydrochloric, and sulfuric acid, and boron trifluoride in methanol as possible reagents.[26] All of these reagents require heating, and the one that is most often used is the boron trifluoride-methanol reagent. The boron reagent though has a limited shelf life, and an overconcentrated solution can cause a loss of PUFA. Additional reagents such as acetyl chloride and aluminum chloride have also been used.[26] Base-catalyzed transesterification is also possible through the use of sodium methoxide and potassium hydroxide in methanol which have an advantage over the acidic catalysts in that the reactions can occur at ambient temperatures and proceed rapidly.[26] The disadvantage is that it does not esterify free fatty acids and the conditions must be anhydrous.[26] These methods methylate non-esterified fatty acids (NEFAs) and transesterify lipid-esterified fatty acids in samples.

A method for the methylation of NEFAs without transesterification of esterified lipids can be completed through the use of a procedure designed by LePage and Roy. To obtain compounds with lower boiling points for use in analysis, the LCPUFA must be converted into methyl esters. The sample is placed in a Teflon-lined capped tube and dissolved/suspended in 4.0 mL of methanol-benzene 4:1 (v/v), prior to which an internal standard of 50 to 300 μg of tridecanoic acid is added for the purpose of quantification.[20] While stirring, 200 μL of acetyl chloride is slowly added over one minute. The reaction tube is heated at 100ºC for one hour and, after cooling, 5.0 mL of 6% K_2CO_3 solution is added to stop the reaction.[20] The tubes must be centrifuged, and the benzene layer is available for use in various analysis techniques.

B. Chromatographic methods

Thin-layer chromatography (TLC), gas chromatography (GC), and high performance liquid chromatography (HPLC) with various detectors such as ultraviolet-visible (UV-VIS), fluorescence, and evaporative light scattering detection have each been applied to the analysis of PUFA. Combination techniques such as liquid-chromatography-mass spectrometry (LC-MS) and gas chromatography – mass spectrometry (GC-MS) have expanded the capabilities of these methods. This expansion has led to advancements in the study of lipidomics.

Thin-layer chromatography (TLC) consists of a plate coated with silica gel or alumina and a mobile phase of an organic solvent such as chloroform, methanol, water, or combinations of these. The fatty acid will migrate on the stationary phase (the plate) while being carried by the mobile phase. The distance traveled is based on the fatty acid's affinity for the mobile phase. Identification of the fatty acid occurs based on the

retardation factor (R_f) which is the ratio of the distance traveled by the analyte to the distance traveled by the solvent. By comparing unknown samples with standards, a fatty acid can be identified. The analyte locations on the plate are visualized through the application of a reagent that makes the spot visible through UV detection or other methods. TLC provides a quick separation of the fatty acids, but the technique does destroy the fatty acids and is not very reproducible. It is best used as a test before running a sample through a more complex chromatographic method.

Gas chromatography is the method most applied to fatty acid analysis. GC is used for the separation of volatile organic compounds. FAME is the form of fatty acid that is easily analyzed with this method. All forms of the fatty acid must be converted to FAME in order to be analyzed with this method. The stationary phase is a capillary, non-polar column with a carrier gas of hydrogen. The use of a non-polar column will separate FAME based on boiling point which will result in the MUFA and PUFA eluting first followed by the saturated components. Quantification of the samples requires peak integrations. Scale calibrations require the addition of an internal reference, such as methyl docosatrienoate, to the sample.[16] The relative amount of each fatty acid is determined by dividing individual peak areas by the sum of all the fatty acid peaks.[16]

GC has become the primary analytical method for the detection of fatty acids so research work using slight variations of the method is extensive. Flame ionization (FID) and mass spectrometry (MS) are the two major forms of detection for GC. The resolving of peaks is comparable in both, but MS has the benefit of allowing for the verification of peak identity by its mass spectrum. Using GC/MS and extraction and esterification or direct transesterification methods described previously, the intraassay CVs ranged

between 2.5 and 13.2% with interassay CVs ranging from 4.6 to 22.9%.[21] After spiking serum samples, the recovery ranged from 76 to 106%.[21] The quantification of the analyte is based on the calibration of the closest standard. The linearity and reproducibility had a coefficient of variation of 9.8% and $r^2 > 0.923$.[21] The concentration range for adults in a study of control subjects is provided in Table III-1.

Table III-1: Concentration ranges of serum polyunsaturated fatty acids

FATTY ACID	RANGE OF CONCENTRATION FOR AN ADULT (> 17 YEARS) [21], μMOL/L
α-Linolenic Acid, C18:3, ω3	50-130
Eicosapentaenoic Acid, C20:5, ω3	14-100
Docosahexanoic Acid, C22:6, ω3	30-250
Docosapentaenoic, C22:5, ω3	20-210
Total ω3 (mM)	0.2-0.5
γ-Linolenic Acid, C18:3, ω6	16-150
Arachidonic Acid, C20:4, ω6	520-1490
Linoleic Acid, C18:2, ω6	2270-3850
Homo-γ-linolenic, C20:3, ω6	50-250
Docosapentaenoic, C22:5, ω6	10-70
Docosatetraenoic, C22:4, ω6	10-80
Total ω6 (mM)	3.0-5.4
Palmitoleic, C16:1, ω7	110-1130
Vaccenic, C18:1,ω7	280-740
7-Hexadecenoic, C16:1, ω9	25-105
Oleic Acid, C18:1, ω9	650-3500
Mead, C20:3,ω9	7-30
Tetracosanoic, C24:1, ω9	60-100
Total polyunsaturated (mM)	3.2-5.8

In a comparison of GC/MS with GC/FID completed on the same serum samples, the GC/FID results indicated a higher concentration of linoleic acid than GC/MS.[21] GC/FID results were expressed as relative percentages of the total fatty acids, which is common in

a number of studies, whereas GC/MS results were expressed as actual concentrations. Langerstedt et al. make the point that the relative concentrations can provide incorrect results in cases where high levels of nonessential fatty acids can falsely lead to a decreased percentage of the essential fatty acids that are being examined.[21] Results and outcomes based on a percentage of the total have to be questioned until absolute concentrations are determined.[21] Numerous additional studies have examined serum fatty acids, either as free fatty acids or as esters, using GC with its various forms of detection.[16, 22, 76, 103, 106, 135-137] With the same sample preparation and GC instrumentation, tissue samples, including adipose tissue, have allowed for the analysis of various fatty acids that are stored in the body.[83, 110]

Although successful, especially, in comparing relative percentage change in fatty acids for clinical studies, GC's disadvantages include the derivatization steps which can alter the structure of the fatty acid or create side-products that can overlap with the analytes needed.[26] Short chain FAME can be eluted quickly and missed.[138] Also, the procedures are quite labor intensive; and it is difficult to obtain meaningful concentrations when using only a limited number of standards. These limitations strengthen the case for the development of a simple and direct method that does not require separation and reacts directly with the polyunsaturated fatty acids.

Where GC uses a gas as its mobile phase, HPLC uses a liquid one and a solid or liquid coated solid in its stationary phase. The analytes are absorbed on the stationary phase to varying degrees resulting in separation based on its affinity to the solid or liquid-coated solid. HPLC is advantageous over GC due to speed, resolution, sensitivity, specificity, sample protection, and its lower temperature operation which reduces the risk

of isomerization of the fatty acids.[134] The method, however, is used for the separation of the lipid class due to overlapping peaks that are improved with the use of the reverse-phase method and different detectors.

Fatty acids do not contain any chromophores that allow them to be directly detected by UV-VIS or fluorescence. As a result, the detection occurs through flame ionization detection (FID), electrochemical detection, or chemiluminscence detection. FID, as in GC, has high sensitivity and is primarily used for the analysis of serum samples without additional sample preparation other than the extraction and methylation of the fatty acids.[138] Electrochemical detection can be done without tagging the fatty acids; however, the addition of the tagging reagent can increase the sensitivity.[138] HPLC with electrochemical detection was used to determine the free fatty acids in plasma with no tagging agent. The resolution of eight examined fatty acids, saturated and unsaturated, was completed in 10 minutes on the column with a resolution only low (0.12) between palmitoleic acid and linoleic acid.[139] The relative standard deviation was less than 1.5 in mixtures of the standards and less than 5% in serum samples spiked with known concentrations of the standard fatty acids.[139] Derivation of the fatty acids is necessary for detection by UV-VIS, fluorescence, and chemiluminscence. A number of agents can be used including benzyl, p-nitrobenzl, phenacyl, p-bromophenacyl and others.[138] HPLC with UV detection was completed for the determination of long chain fatty acids in plasma and lipoproteins. After saponification of the sample, 2-nitrophenylhydrazine was added for the derivatization prior to the extraction of the fatty acids from the sample.[140] The coefficients of variation range between 3 and 31%.[140] Recovery of a standard solution of fatty acids ranged between 95 and 110%.[140] LA is the

most abundant long-chain fatty acid followed by palmitic acid with LDL and HDL compositions of fatty acids appearing to reflect the composition of plasma.[140] It is important to note the derivatization reaction can be affected by the amount of reagent, the reaction temperature, and the incubation time so care must be taken to ensure strong attachment and for the prevention of side chains.[138] Cis- trans- isomers of the fatty acids can be detected through silver ion chromatography with a silver loaded HPLC column.[138] The separation is based on the formation of weak charge transfer complexes between the silver ions and double bonds.[138] HPLC with these various forms of detection have been used for the fatty acid analysis of animal and other human biological samples, products of the oxidation of lipids, foods, plant extracts, and oils.[138, 140-142]

Alternative methods including combining mass spectrometry with GC and LC, LC-MS/MS, multidimensional mass spectrometry, and nuclear magnetic resonance are all advanced techniques that are used to examine fatty acids and their metabolites in the growing field of lipidomics. Through the use of multidimensional mass spectrometry, new classes of phospholipases and new roles for lipids in cerebral function have been discovered.[143] A technique of reverse phase LC-MS/MS surveyed with precursor ion scanning and succeeding product ion scanning was able to increase the detection limits of phospholipid metabolites by 5 to 20 fold allowing for even minor components of studies to be detected.[143]

The last method of fatty acid analysis is the determination of free fatty acids by an enzymatic method. Shimizu *et al.* first developed the enzymatic method in 1979 and colorized it in 1989. It is dependent on the activation of free acids by acyl-CoA synthetase. The reaction of the extracted serum fatty acids involved are the following.[144]

$$\text{FFA + CoA + ATP} \xrightarrow{\textit{acyl-CoA synthetase}} \text{acyl-CoA + AMP + PPi} \quad (3.12)$$

$$\text{AMP + ATP} \xrightarrow{\textit{myokinase}} \text{2ADP} \quad (3.13)$$

$$\text{2 ADP + 2 phosphoenolpyruvate} \xrightarrow{\textit{pyruvate kinase}} \text{2 ATP + 2 pyruvate} \quad (3.14)$$

$$\text{2 pyruvate + 2 NADH} \xrightarrow{\textit{lactate dehydrogenase}} \text{2 lactate + 2 NAD}^{+} \quad (3.15)$$

The reaction was then coupled using acyl-coA oxidase from yeast to react with the acyl-CoA generated by the fatty acids.[144] One product of the reaction, hydrogen peroxide, can be further reacted to produce red quinine dye.[144] A microfluorometric method using these reactions allowed for direct analysis of the serum's free fatty acids with no extraction step which led to improved sensitivity and lower cost.[145] This method of determination of the free fatty acids is widely available in kits from multiple suppliers.

III. Purdie Assay Development

The Purdie Assay was developed based upon a reaction by Chugaev (Tshugaev) and Gastev. The Chugaev and Gastev reaction uses a 2:1 mixture of 20% w/v $ZnCl_2$ in glacial acetic acid combined with 98% acetyl chloride.[129] The Chugaev and Gastev reagent was used to measure cholesterol with great accuracy by Hanel and Dam.[130] The reagent was also used for the determination of dolichol, any number of long-chain unsaturated isoprene groups with a terminal alcohol group, and dolichyl derivatives.[146] The major goal of the original project was the determination of the cholesterol in each of the lipoprotein fractions without a separation step. As mentioned previously, the determination of LDL-C, HLD-C, and the other fractions required either a precipitation of some of the fractions or a sequential ultracentrifugation process. A direct method was attempted when it was determined that the Chugaev color product of cholesterol was active using circular dichroism (CD). Two milliliters of the Chugaev reagent were added

to 50 μL of serum. After 8 min of incubation at 67 °C, the solution was cooled to room temperature and 1 mL of chloroform was added before CD analysis from 625-325 nm. [28, 147, 148] The CD detection exhibited good linearity for the correlations between TC measurements and determinations of both HDL-C and (VLDL+LDL)-C.[28, 147, 148] The separation of VLDL-C and LDL-C was not possible.

Refinement of the reagent system and a switch to visible absorption spectroscopy improved upon the previous results. The modified reagent was changed to a higher concentration of $ZnCl_2$ which contained 980 g of acetyl chloride and 350 g of $ZnCl_2$.[149] The increased concentration heightened the intensity of the signal allowing the sample size to be reduced.[150] The resulting spectra were assumed to be the product of cholesterol and its esters with the reagent. Using a simple algorithm and calibrations, the concentration of each of the subfractions of cholesterol was determined and compared to the standard enzymatic test. The correlation between the spectrophotometric and enzymatic measurements for TC and LDL-C were strong with low coefficients of variances.[149, 150] The correlation for both VLDL-C and HDL-C was not as strong.[149, 150] The correlation between TC and the LDL-C values do validate the spectroscopic method, but further work on including IDL-C in the mathematical model could have improved the results.[150] Two patents resulted from this work.[151, 152]

During this period, the reagent system was also being modified and examined for any possible interference. Zinc chloride, zinc acetate, zinc perchlorate salts, and perchloric acid with acetyl chloride all produce colors of various intensities.[150] Acetic acid was found to inhibit the reaction requiring a longer incubation time to achieve the final color.[150] Hemoglobin was the only protein that caused interference with the

spectrum, and it only occurred at high concentrations.[150] This is important to note because venous blood collection can rupture red blood cells releasing hemoglobin into the serum sample.[150]

Further refinement of the reagents' system led to 1 mL of 98% acetyl chloride, 40 μL of 70% perchloric acid and 10 μL of sample.[29] In the case of serum where precipitation of the proteins occurred, a 3 minute period in a centrifuge at 3400 rpm was required before decanting the solution into a glass cuvette. The spectrum was recorded at 15 min over 350 – 750 nm.[29] Upon examination of additional uses of the Purdie Assay, it was determined that cholesterol and its esters, unsaturated fatty acids, triglycerides, and phospholipids with unsaturated fatty acids attached reacted with the Purdie reagent and produced absorbance and fluorescence spectra.[29] This work showed that the absorbance spectra was not only cholesterol, but also included other compounds in serum that reacted with the reagent. Methyl ester forms of myristic acid (C_{14}:0), palmitic acid (C_{16}:0), stearic acid (C_{18}:0), oleic acid (C_{18}:1^{9}), LA acid, linolelaidic acid (C_{18}:$2^{9,12}$), ALN and GLN (C_{18}:$3^{9.12.15}$), AA, EPA, and DHA were a few tested.[29, 31, 32] Saturated fatty acids did not react with the Purdie reagent system. Oleic acid had a minimal absorbance. Food oils including olive oil, safflower seed oil, and fish oil were also tested.[29, 31, 32] The remaining fatty acids had unique absorbance patterns based on the number of double bonds present. Slight differences between LA and linolelaidic and ALN and GLN existed. Due to this ability, the Purdie assay could serve as a quality control test reagent for standard reference materials and natural oils.[32] Sterols including beta-sitosterol, stigmasterol, and Vitamin D were shown to produce a colored product.[29] Cholesteryl esters were determined to be the sum of the cholesterol spectra and the PUFA that was

attached. Synthetic serum samples and synthetic dyslipidemic samples were prepared to model these conditions and correlations between the synthetic mixtures and human serum samples were positive.[29] Slight variations between the two spectra existed, but the overall fit was good for an initial attempt to mimic dyslipidemic disease states.

Using the 25:1 mixture of acetyl chloride and 70% perchloric acid, the ability for reaction to occur with steroids opens the door for a second quality control assay.[33] The Purdie reagent has selectivity for acylation of the α- over the β- position at the C-17 which would allow for differentiation of anabolic steroids.[33] It was also determined that if multiple reactants are present, the resulting spectrum is the addition of each of the components, assuming no interferences are present.[33] The selectivity of the reaction was completed by an examination of the steroids, PUFA, and terpenes that were able to react with the Purdie assay. It was determined that the reaction is selective to $-CH_2-C=C-$ and can distinguish isomers like conjugated linoleic acid from linoleic acid.[31]

Once it was established that the reagent system was reacting with the cholesterol and the PUFA in the serum, the quantification of these components was attempted using multiple linear regression (MLR) techniques. Using K-matrix methods and the molar extinction coefficients for cholesterol and each PUFA, prepared mixtures were analyzed.[31] Cholesterol, LA and CLA at high concentrations were determined.[31] Large errors were associated with the LNA, AA, EPA and DHA.[31] Multiple reasons are possible for this including experimental error, low signal to noise ratio for the lower concentrations of these four PUFA, and analysis errors related to the algorithm chosen. The analysis was useful in a dietary intervention study on one male, obese subject who was placed on a low fat diet. The sum of the PUFA determined by MLR and the

enzymatic TG concentrations over the 4 month study followed the same qualitative pattern.[31] This suggests that the Purdie assay could offer a qualitative monitoring assay and with further attempts with additional mathematical algorithms, complete determination of all components can turn it into a quantitative assay.

The Purdie assay and its pattern differences seen in samples of serum from dyslipidemia patients were tested in an exploratory clinical trial. Ninety-six subjects with normal , Type I, familial hypercholesterolemia (FH), Type III, and diabetic conditions had their serum samples analyzed with the Purdie assay.[30] Using principal component analysis (PCA) and clustering, pattern recognition techniques allowed for an 81.3% assignment of each spectrum into a group of the same conditions.[30] It is important to note here that some of these patients were already on lipid lowering drugs which can place the spectra into a different group or cluster resulting in a lower fitting accuracy.[30] More information on treated and untreated patients may lead to a higher rate of clustering accuracy.

CHAPTER IV

EXPERIMENTAL METHODS

The determination of the concentration of analytes present in a solution involves multiple steps. The first is the reaction of the sample with the Purdie Assay. Adjustments must be made depending on the sample type which can include serum, a prepared mixture sample that is in chloroform, or a food sample. After obtaining the spectra, the analyses both quantitative and qualitative can be completed. Each method explored for these types of analysis will be explained.

A. Reaction with Purdie Assay

As discussed in Chapter 2, the Purdie Assay has been well developed over the last few years; and, therefore, the methods associated with the assay are well established. The procedures followed for this study are discussed by the sample type and the inclusion or exclusion of water added to the reaction system. The water effect will be discussed in the following chapter.

1. Serum Samples

A 10 μL sample of serum is added to a 13 x 100 mm borosilicate disposable test tube. One milliliter of pure acetyl chloride (Arcos) is added to the test tube. A 40 μL of perchloric acid (70% ACS reagent grade, GFS) is added to the side of the test tube and

slowly introduced to the acetyl chloride/sample solution. The process must be slow and controlled to prevent excessive splashing that can occur if the solution is added too quickly. The reaction time is started as soon as the perchloric acid is added. The solution is mixed by hand for twenty seconds to allow for the release of the small amount of hydrochloric acid gas from the reaction test tube. The test tube is covered with a Teflon cap and placed into a centrifuge for 3 minutes at 3400 RPM. After centrifugation, the precipitated proteins are separated, and the liquid is transferred to a 10 mm pathlength optical glass cuvette with Teflon stopper for the remaining time. Absorbance spectra are taken at 15 minutes using a HP 8452A Hewlett Packard spectrophotometer. A 5 second integration time and a 2 nm resolution were used to collect the spectra over the range of 350-800 nm. The blank for each reaction was pure acetyl chloride. The reagent mixture of acetyl chloride with perchloric acid does produce a slight color at 15 minutes. Due to the possibility of variability and small absorbance value, the combination of acetyl chloride and perchloric acid was not used as the blank. Spectra of the colored species generated from the mixture of the two reagents were obtained and used in the quantitative analysis of the spectra. Additionally, the procedure was altered for the examination of the kinetics of the reaction. After the centrifugation and separation of the sample, the spectrophotometer was set to collect spectra every minute for 20 minutes using the onboard software.

2. Prepared Mixtures, With and Without Water

Throughout the course of the study, it was realized that the water content of the perchloric acid and serum in the reaction solution has an effect on the reaction. Mixtures were prepared from standards of cholesterol and the methyl esters of the fatty acids in a

chloroform solution. Centrifugation was unnecessary since no proteins were added. Data collection remains the same as for serum. The inclusion of water involves the addition of 10 μL of distilled water as the first step, followed by the acetyl chloride, the chloroform mixture sample, and finally the perchloric acid. The remaining steps of the assay remained the same as serum in order to maintain constancy during the 15 minute reaction period. As stated with the serum, collection of the spectra every minute for 20 minutes can be completed using the software of the spectrophotometer for examination of the kinetics.

3. Other Mixtures

New samples that contain the analytes of interest are constantly being considered for use with the Purdie Assay. With each new analyte, consideration must be given to the composition of the sample and any possible interference that might cause alterations in the analysis. In samples such as food oils including sunflower, safflower, and fish that have been analyzed with the Purdie Assay, the procedure was the same as the prepared mixture discussed previously. Procedures with and without water added to the reaction were completed for analysis in attempted quantitative methods. Any sample that does not have water or proteins present can be analyzed using the established prepared sample procedure. If proteins are present, such as in animal blood and milk samples, the procedure will have to include the decanting step that is discussed with the established serum method. The Purdie Assay can be adapted further as more samples are considered as demonstrated with the various mixtures examined to date.

B. Samples

Methyl linoleate, methyl linolenate, methyl arachidonate, methyl eicosapentaenoate, methyl docosahexaenoate, methyl conjugated linoleate, and free cholesterol were all used to prepare mixtures. All of the standards were 90 to 99 % pure based on gas chromatographic analysis and were all purchased from Sigma-Aldrich. Stock solutions for each of the analytes with a maximum total concentration of 0.02 M and 0.04 M were prepared. The stock solutions were used to prepare mixtures to limit the majority spectral response to ranges between 0.2 and 0.9 absorbance units. The mixtures prepared for each quantitative analysis method varied and will be discussed with each of the methods.

Serum samples for this work were provided by staff and volunteers at the Oklahoma State University Wellness Center (OSUWC) and from the Lipidology Laboratory and Clinic at the University of Cape Town Medical School (UCT), South Africa. The anonymous samples from OSUWC were from volunteers who were already requesting a lipid profile and had given consent, Appendix I. No attempt was made to solicit samples nor was any extensive medical information derived from the samples except for the cholesterol concentration which was determined by an outside chemical lab using the enzymatic test. The subjects fasted for at least 12 hours prior to the collection of the sample. A venous blood sample was collected into a Vacutainer™ red and grey capped separation tube. After inversion of the tube five times to mix the blood and the components of the collection tube, the sample was centrifuged at 3400 RPM for 15 minutes. The collection tube contains a clotting activator which takes approximately 30 minutes to activate and a floating gel that separates the red cells from the serum during

the centrifugation step. The serum, which was the top layer in the tube, was then transferred to a 10 mL glass vial with a screw cap. The Purdie Assay was completed on the sample within three days of receiving the sample. Samples were stored in a refrigerator at 2-4 °C. Samples were allowed to return to room temperature prior to analyses. OSUWC samples, about 20 in number, were from patients with normal to elevated cholesterol levels.

UCT samples were collected from patients who were admitted to the Cape Town Clinic under the direction of Professor A. David Marais for the identification and treatment of lipid disorders. All samples were collected and separated by the staff of the clinic using the same containers and procedures as the OSUWC samples. Permission to work with the samples was obtained through the UCT Lipid Clinic, Appendix II. Once separated, a portion of the serum was placed into microcentrifuge tubes instead of the 10 mL glass vials. The remaining portion of the serum was used for other methods in the Lipidology Laboratory. The 619 samples collected were analyzed over a three month period. The cholesterol values and a diagnosis of the lipid disorder, where possible, were determined by the UCT Laboratory.

C. Quantitative and Qualitative Analysis and Multivariate Modeling of the Spectra

The objective in this section was to measure molar concentrations for the seven major serum lipids from a single absorbance spectrum. Separations are not involved. Although a challenging problem, it is to our advantage that indications are that individual spectra are additive. The determination of the concentration of an analyte first involves defining a model or approach that can involve calibration with standard solutions followed by a prediction of the concentrations of each component in the sample. The

defining of the model and subsequent calibration are needed to determine the relationship between the concentration of the analyte and the resulting response, i.e. the spectral absorbance. If the relationship can be clearly defined, then the prediction of unknown samples can occur. The key is to find an approach that best defines the relationship between the concentrations and the absorbance.

In the following sections, four approaches will be described that were used for the calibration and validation of the total multi-component assay. The ultimate verification will be to show the validity of the additivity assumption.

1. Multivariate Approaches

In order to explain the multivariate analysis methods that will be employed, the spectrographic problem must be clearly defined. According to Beer's Law, absorption by a single component at a single wavelength is defined by the following equation:

$$a = \varepsilon d c \qquad (4.1)$$

where a is the absorbance, $\boldsymbol{\varepsilon}$ is the absorption coefficient, d is the cell thickness and $\mathbf{c}$ is the molar concentration of the sample solution. For the same cell thickness for all of the samples, Beer's law can be restated as

$$a = k c \qquad (4.2)$$

where k is the reduced absorption coefficient. If multiple components, m, are in the system, the expression expands to:

$$a_i = k_{i1}(c_1) + k_{i2}(c_2) + \ldots + k_m c_m = \sum_{j=1}^{m} k_{ij} c_j \qquad (4.3)$$

where a_i is the absorbance at wavelength i; k_{ij} is the absorption coefficient of the j component at wavelength i and c_j is the concentration of the jth component. When p

wavelengths from the spectra are used, matrix notation can be used to arrive at the following equation:

$$A = Kc \qquad (4.4)$$

where A is a p x 1 vector that is the resultant spectra, $\boldsymbol{K}$ is a p x m matrix of the normalized molar absorptivities, and c is a m x 1 vector of the concentrations of the m components. By determining the matrix c , the concentrations for all six PUFAs and cholesterol will be obtained.

a. Direct Calibration

The direct method is based on measuring pure component spectra to obtain the molar absorptivities which requires standards of each of the analytes to be available for analysis. There are two key requirements. First, there must not be any interactions between the different components or between components and the solvent.[153] Second, no known matrix constituents can interfere with the resultant spectrum.[153] In the context of the current problem, all of the components are available in pure form, and their molar absorptivities can be obtained to complete the matrix $\boldsymbol{K}$. During initial experiments, no matrix effects or other interactions were encountered. Using this method, the unknown concentration vector can be determined by:

$$c_o = K^T (KK^T)^{-1} a_o \qquad (4.5)$$

where c_o is the vector of the predicted concentrations, $\boldsymbol{K}$ is the matrix of the absorptivities, and a_o is the vector of the sample spectrum.[153] Equation (4.5) applies when the number of wavelengths used in the analysis is greater than the number of components. Relative error can be low as long as the two requirements are true, and multiple wavelengths are used.

To determine the components of the **K** matrix for each of the analytes, molar absorptivities were determined. Multiple linear regressions were completed for every 2 nm wavelengths between 350 nm and 800 nm for each analyte to determine the molar absorptivity for each of the seven analytes of this study. The concentration ranges for the chloroform solution containing the methyl esters of LA, ALA, AA, EPA, and DHA ranged from 0.0025 M to 0.02 M with 10 to 20 concentrations chosen in equal increments over this range for the calibration curve. Cholesterol and CLA had ranges from 0.001 to 0.01 M. The difference in the concentration ranges were required in order to maintain the absorbance falls within the range of 0.2 to 0.9 absorbance units. Molar absorptivities for each analyte with and without water were determined.

b. Indirect and Inverse Calibration

In these procedures, calibration absorptivities are determined using mixtures. The methods will allow for any interactions that exist between components or between components and the solvent to be accounted for in the answer. These methods are divided into two parts; calibration and analysis. To calibrate, a set of spectra for samples of mixtures with known concentrations of the components are measured. K-matrix approach uses a matrix of the known concentrations and a matrix of the respective absorbancies. The matrix **K** can be solved using the general least square solution equation:

$$K = (c^T c)^{-1} c^T A \qquad (4.6)$$

where **c** is the matrix of the known concentrations, **A** is the matrix of the spectra of the samples, and **K** is the matrix solution to the molar absorptivities of the components.[153] The **K** matrix is then used in the analysis step to determine the concentrations of the

components in the unknown sample using the equation:

$$c_o = a_o K^T (KK^T)^{-1} \qquad (4.7)$$

where c_o is the vector of the concentrations of the unknown sample, and a_o is the spectrum of the unknown sample.[153] The advantage of this approach is that the K matrix generated is representative of real absorptivities. Disadvantages are that every component that absorbs must be known, and the method relies on the inversion of two matrices which becomes a problem if the components have similar absorption spectra, in which case, a singular matrix may occur.

The inverse calibration method is based on the model

$$C = AP \qquad (4.8)$$

where **C** is the matrix of the concentrations, **A** is the matrix of the absorbance, and **P** is a matrix of the calibration coefficients.[153] The matrix **P** is determined by

$$P = (A^T A)^{-1} A^T C \qquad (4.9)$$

by using ordinary least squares analysis.[153] Using a sample spectrum, $\boldsymbol{a_o}$, and multiplying it by the generated ***P*** matrix, the unknown concentrations in the sample spectra can be determined. Unlike the matrix **K** in the K-approach which are absorptivities related to the analytes, the components of the P-matrix do not have any physical meaning.[153] P-matrix approach does have the advantage of involving only one matrix inversion.[153]

c. Principal Component Analysis

Principal Component Analysis (PCA) is an unsupervised pattern recognition method which takes high dimensional data and projects it to a lower dimensional space. The method is considered unsupervised because no prior knowledge of the classification is included in the calculation. The goal of PCA is to use the matrix of the original set of

data, **X**, and approximate it by producing two smaller matrices called the score, **T**, and the loadings, **L**. In terms of a matrix equation,

$$X = TL^T \qquad (4.10)$$

where **X** is defined as *n* row of objects and *p* columns of features; **T** is represented by *n* rows with *d* columns of principal components, $\mathbf{L}^T$ is the transpose matrix of L and is defined by *d* columns and *p* rows[153]. To determine the principal components, the nonlinear iterative partial least squares (NIPALS) algorithm was used. NIPALS determines the principal components based on the maximum variance criterion which requires that each component describes the maximum amount of variance that the first did not model with its vector. As a result, the first principal component models the most variance followed by the remainder. Each principal component will be orthogonal to all the others. The number of principal components required for the system being examined was determined by percentage of explained variance, eigenvalue-one criterion, and/or cross-validation. PCA can be completed on the correlation or covariance matrix generated from the data. The correlation matrix is used when the variables have large differences in the variance or the units of measure are different. The data is then standardized first before proceeding with PCA. The principal components can be combined with multiple linear regression to obtain the predicted concentration values in the principal component regression method.[154] This method was examined, but partial least squares regression has advantages. PCA for this study was primarily used for the generation of the principal components which can be used in cluster analysis for pattern recognition and as a possible input for neural networking. All principal component analysis was completed using SAS Institute's statistical software, *JMP*[155].

d. Partial Least Squares (PLS) Regression

Partial Least Squares (PLS) regression methods are widely used in all fields including chemistry. PLS was developed in the late sixties in the field of economics by H. Wold and was later applied to chemical analysis by S. Wold, and H. Martens.[154, 156] The model parameters in PLS have been shown to be more robust to changes in calibration samples than the previous standard methods of multiple linear regression and principal component regression.[154] In previous methods, the concentration matrix was determined by decomposing the **A** and **C** matrix separately. In the case of PLS, the decomposition of the absorbance matrix **A**, which is the **X** matrix in the general theory, and the concentration matrix **C**, which is the **Y** matrix in the general theory**,** are estimated simultaneously.[154] PLS's approach is similar to PCA and is based on the NIPALS algorithm with the exception that both the **X** and **Y** matrices will be decomposed into latent variables instead of just the **X** matrix in PCA.[154] The initial formula is based on the equation

$$Y = XB \qquad (4.11)$$

where **Y** is the concentration matrix, **X** is the absorbance matrix, and **B** is the relationship between the two. The new variables are called X-scores and serve as predictors of both **X** and **Y**.[156] The X-scores that are the elements of matrix **T** are linear combinations of the original variables in matrix **X** with calculated weights **W** for form

$$T = XW \qquad (4.12)$$

which allows **X** to now be defined as

$$X = TP^T + H \qquad (4.13)$$

where **T** are the scores of **X**, the elements of **P** are the loadings, and the errors are represented by the matrix **H**.[156] The X-scores must be determined so that, when multiplied by the loadings, they provide a good summary of X and maintain small residuals in equation 4.13. A corresponding procedure is completed for the **Y** matrix. The Y-scores that are the elements of matrix **U** are linear combinations of the original variable **Y** with calculated weights **Q** for form

$$U = YQ.^{156} \qquad (4.14)$$

which allows **Y** to now be defined as

$$Y = UQ^T + G \qquad (4.15)$$

where **U** are the scores of **C**, the elements of **Q** are the loadings, and the errors are represented by the matrix **G**.[156] The X-scores must also be good predictors of **Y** to yield

$$Y = TQ^T + F \qquad (4.16)$$

which can be written by including Eq. 4.12 to

$$Y = XWQ^T + F = XB + F \qquad (4.17)$$

which is a multiple regression model.[156] The PLS-regression coefficients can be related to the matrix **X** resulting in **B** written as

$$B = W(P^TW)^{-1}Q^T \qquad (4.18)$$

where the relation has been written to directly relate with **X**.[156] The final equation, which is the same as multiple linear regression, can be expressed as

$$Y = XB + E \qquad (4.19)$$

where **Y** is the concentration of the analytes, **X** is the absorbance matrix, **B** is the matrix of the PLS regression coefficients *b*, and **E** is the matrix of the residuals. The advantage

of this method over others is that the directly related decompositions of the two matrices creates an algorithm that is robust to calibration changes.[153] Two types of this approach have been developed. When each analyte is calibrated and predicted one at a time, the method is referred to as PLS1. When two or more components are calibrated and predicted simultaneously, then the PLS2 algorithm must be used. A comparison between both PLS1 and PLS2 was completed for this study. All PLS analysis was completed using *The Unscrambler* software package.[157]

As with any of the methods discussed to this point, the results will only be as good as the data that is supplied to the algorithm. Mean centering of the spectral data is necessary for reduction in the errors and is a common pre-processing step in the use of PLS. The optimum number of principal components for PLS was determined by cross-validation [156]. The cross-validation method will remove one or a group of samples prior to the model being calibrated. The left out points are used to determine the prediction residuals which allow the samples to both determine and test the model. The calibration is repeated again with different samples removed until all of the residuals have been computed for each sample. The root mean square error of calibration (RMSEC) and root mean square error of validation (RMSEV) are calculated for each model. The general equation is

$$RMSE = \sqrt{\frac{\sum_{i=1}^{N} (\hat{y}_i - y_i)^2}{N}} \qquad (4.20)$$

where y and y' are the predicted and actual concentrations and N is the number of samples. The model with the minimum values for RMSEC and RMSEV and the lowest

number of factors is the model with the optimum number of PCs. A plot of the RMSEV against the number of PCs in the model can indicate the appropriate model.

One critical aspect is the wavelength selection. When PLS and other methods were originally introduced, they were considered to be full spectrum methods that would determine the concentrations regardless of the region of spectra used.[158] It has since been proven both experimentally and theoretically that the selection of wavelengths can significantly reduce the prediction errors of the models.[158-163] Wavelengths can be selected using a PLS based method developed by Frenich *et al*.[164] A large coefficient *b* may be an indication of a wavelength that should be included, but it can be a wavelength with a small absolute value and a high variance.[164] In order to use the *b* coefficients, the X variables can be weighted with the inverse of the standard deviation to standardize the X variable space.[164] The new form of the PLS equation is

$$Y = X_{standard} B_w + J \qquad (4.21)$$

where $\mathbf{X_{stand}}$ are the standardized absorbencies, $\mathbf{B_w}$ are the weighted PLS coefficients and **J** is the matrix of the resulting residuals.[164] A large coefficient of the resulting $\mathbf{B_w}$ matrix indicates an important wavelength that should be considered in the wavelength selection process.[164] After selecting a set of wavelengths with high *bw*-coefficients, the PLS model with original data is completed; and the RMSEV for the optimum number of PLS factors is calculated.[164] The process is repeated again with fewer selected wavelengths.[164] The lowest point on a plot of the RMSEV against the number of selected wavelengths will determine the optimum number of wavelengths with high *bw*-coefficients to use for the PLS model. Frenich *et al.* used this method of wavelength selection using PLS1 on two multicomponent mixture problems and were successful in lowering the prediction

error.[164] To determine the wavelength included for each component, they divided the high *bw*-coefficients into five sets based on numerical ranges.[164] Kassim and Simoneit altered this method for PLS2 and used a stepwise elimination method to remove the lowest *bw*-coefficients until the lowest prediction error was found for the optimum model of PLS. A combination of the stepwise elimination method and the numerical criteria were used in this study to examine the effect of wavelength selection on this set of analytes. Wavelength selection should not be applied blindly, and the method used must consider spectrally important regions.[162]

Variations of the PLS method have been developed based on the need for the analysis of various systems. The orthogonal PLS (O-PLS) method was recently developed to handle systematic variations such as baseline, drift, and scatter effects, and impurities that can be present in the spectral matrix.[165, 166] N-way PLS models have been developed to extend PLS to include N variables of objects or samples. The normal PLS is two way with the columns as variables and the rows as samples. In three-way PLS, the matrix becomes a box with a third variable added such as time, change in pH, or another factor. For example, the three-way PLS was used to study the element concentrations of the water and sediment of the rivers Saale and Elbe. Although it was difficult due to the large variability of the system chosen, the three-way PLS was able to improve the prediction of the suspended matter concentrations compared to the two-way PLS model.[167] Although the data collected in this study are not conducive to three-way PLS, it is a method that may be of interest if samples from the same patients are collected over time in any future studies. Comparisons of PLS methods with PCR and artificial neural networks on the same system have been completed, and the artificial neural networks

have been shown to have a lower root mean square error of prediction which demonstrates that multiple methods should be considered when determining quantitative results to select the best method for the current system.[168]

e. Generalized Standard Addition Method (GSAM)

One of the classical methods for the quantitative detection of analytes is an standard addition method (SAM). SAM is based on adding known concentrations of analyte to an unknown sample in order to determine optimally the concentration of the analyte in the original sample. The known concentrations are added to the sample, the response is recorded, and a plot of the response vs. the known added analyte concentrations can determine the unknown concentrations, assuming the system is linear. SAM requires that the chosen response be linear over the entire concentration range. It is zero when no analyte is present, and no other components of the system interfere with the response chosen.[169] If any of these conditions can not be met, then SAM can not be used. In the case of a multicomponent mixture where multiple analytes are of interest, the generalized standard addition method was developed by Saxberg and Kowalski to handle the matrix effects and other drawbacks seen in SAM.[169] GSAM has been successfully applied to spectrophotometry[170], inductively coupled plasma spectrometry[171-173], and anodic stripping voltammetry[174, 175]

For the project at hand, all seven analytes have absorbances that are overlapping in the region of 350 nm to 550 nm. No unique wavelength exists for each of the analytes; therefore, SAM is not a possible option. GSAM, an analysis that simultaneously performs the calibration and prediction using multiple wavelengths, is a possible method.

The lack of a pure blank serum without any of the analytes being studied can be accommodated by GSAM.

In matrix notation, the relationship between the absorbance and the analyte concentrations in GSAM is

$$R = CK = \Delta CK + C_oK = \Delta R + R_o \qquad (4.22)$$

where **R** is the matrix of the measured spectra of the unknown mixture plus the added analyte concentrations at multiple wavelengths, **C** is the concentration matrix with both the added analyte concentration, ΔC, and the unknown analyte concentration, C_o, **K** is the matrix of the absorptivities for each component at each wavelength, **ΔR** is the matrix of the spectra of added analyte, and R_o is the measured spectra of the unknown mixture.[169] Since the addition of the analyte will alter the volume, the corrections for the response **R** in equation 4.19 becomes

$$\Delta Q = \Delta NK \qquad (4.23)$$

where **ΔQ** is matrix of the volume corrected response changes, and **ΔN** is the quantity of the additions in moles, micrograms, or another absolute quantity.[176] In the overdetermined system where more additions than analytes are used, the resulting least squares solution for **K** is

$$K = (N^T \Delta N)^{-1} \Delta N^T \Delta Q \qquad (4.24)$$

assuming that $\mathbf{(N^T \Delta N)^{-1}}$ exists.[176] The final equation that is used to determine the concentration in the unknown analytes is

$$n_o = (KK^T)^{-1} K q_o \qquad (4.25)$$

where n_0 is the vector of the initial concentrations, and q_o is the vector of the volume corrected response for the initial unknown sample.[176] Each addition can be a single

analyte, or it is possible to make multiple additions of analytes at the same time which are referred to as multiple standard additions.[169] The linear model of GSAM as described above is for systems where the components of the **K** matrix are assumed to be constant over the entire range of concentrations. This is the case in the current study; however, GSAM can be changed to include additional terms in the quadratic and cubic forms of the model.[169] A prior two-dimensional method of GSAM to handle both the chromatographic and mass spectrometric data from GC-MS and other combined instruments had already been developed and applied.[177]

The uncertainty of the estimation of K is based on the condition number of the **K** and **ΔN** matrices. The equations have been defined as

$$\frac{\|\delta K\|}{\|K\|} \leq cond(\Delta N)\frac{\|\delta \Delta Q\|}{\|\Delta Q\|} \qquad (4.26)$$

$$\frac{\|\delta_{n_o}\|}{\|n_o\|} \leq cond(K)\left(\frac{\|\delta q_o\|}{\|q_o\|} + \frac{\|\delta K\|}{\|K\|}\right) \qquad (4.27)$$

where || || is the norm of the vector or matrix; cond is the condition number; and δ is the matrix of the small changes between the modeled and actual values of the respective matrices and vectors.[176, 178-180] Equation 4.27 is dependent on having fully selective sensors that do not have any or very low interferences with each other.[176, 178-180] Ideally, the condition number of the **K** matrix would be 1 which would allow for no amplification of error.[176, 178-180] Although it is difficult to define all wavelengths of the current system of this study that do not contain interferences with other analytes, careful wavelength selection can aid in lowering the condition number of **K** and reduce, therefore, the amplification of the error. Although the condition number of the **K** matrix may be high

due to the similarity of the spectra of the six PUFA's, it has been shown that low uncertainties can still be obtained for the analyte concentration as long as there is goodness of fit.[176, 178] Kalivas states that the condition number of **K** is the upper limit of the error.[178] Equation 4.26 is dependent on the experimental design of the additions used in the formation of the **ΔN** matrix. This portion of the error is controlled by the experiment and should be designed to minimize the condition number of the matrix since it is not always possible to minimize the condition number of the **K** matrix due to the analytes being examined.[176, 178-180] The effect of experimental design on this matrix was examined during this study. D-optimal[181] and four component mixture designs[182] were considered and used in the development of the GSAM additions using *The Unscrambler*[157] and the *JMP*[155] software packages. In a study of the volume and matrix effects for GSAM, it was determined that the volume-corrected responses can increase the errors in the determined concentrations resulting in the conclusion that it is best to maintain a constant volume to minimize the variance in the analyte predictions.[183] To reduce this error, a constant volume for the initial sample and each addition was maintained. Each addition for the GSAM study was 10 μL to the mixture plus the reagents' total volume of 1.05 mL. To maintain a constant volume, 10 μL of chloroform was added to the initial unknown sample concentration. GSAM was used for the analysis of both prepared mixtures with four components and serum samples.

f. Artificial Neural Networks (ANN)

ANN have become an important tool in a wide variety of research fields. Inspired by the information processing of neurons found in biological nervous systems, ANN can be trained to find solutions, expose pattern recognitions, and classify data. The basic

method of operation of ANN is defined by the way the individual elements, inputs, neurons, and output are connected and by the strength of the connections which are called weights. The weights are determined by training which follow specified learning rules until the network is able to complete the desired problem. This process is similar to the learning process the biological system undergoes when it is exposed to a new task. New connections or modifications to existing connections are made during this process. Although ANN are not as complex as biological systems, they have been used to solve a number of problems. Productive results have been seen in applications ranging from autopilot enhancements in aerospace, to speech recognition, and even financial models in real estate.[184] ANN have been used for the classification and identification of pharmaceutical and illegal drugs such as heroin using both HPLC and GC data as input.[185, 186] An ANN was developed to aid in the diagnosis of malignant breast nodules from digital mammographic and ultrasonographic images.[187] Besides pattern recognition, ANN have been used for the quantification of mixtures including amino acids, organic dyes, and the oil pollutants in water samples using UV spectra, fluorescence spectra, and combinations of both as input parameters for the ANN.[188-190] Due to the power of ANN, it will be used for a pattern recognition set and for the quantitative determination of the seven components.

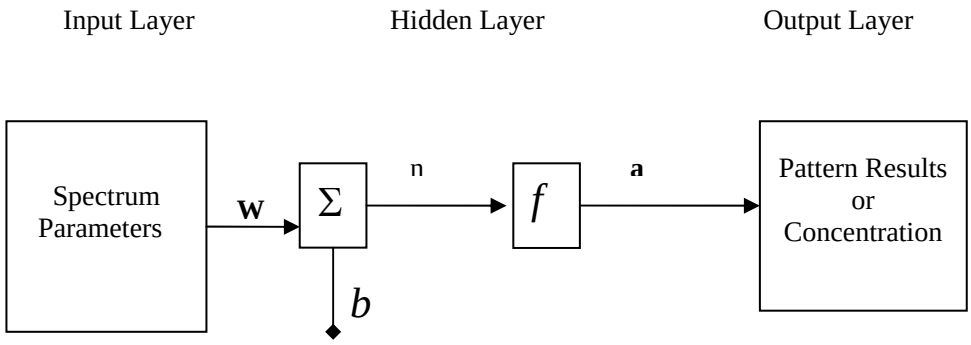


Figure IV.1: Example of a basic neural network design

A basic network design adapted from Hagan *et al.* is shown in Figure IV.1.[184] The network is divided into three layers comprised of input, hidden layers, and output. In the context of the current problem, the input parameters will be numerical descriptors of the spectra. The parameters could have included the absorbance at specified wavelengths for each parameter and characteristics of the spectra including average slopes of the curves and the area under the curves, etc. In the current study, selective wavelengths and principal components were considered. Each of the parameters in the input layer is connected to neurons in the hidden layer. The term hidden is applied due to the fact that inputs and outputs from these layers are not seen in the analysis of the method. The hidden layers can consist of one or more layers. The hidden layer includes a weight matrix (**W**), a summation step (Σ), a bias vector **b**, a transfer function *f*, and an output vector **a**.[184] The number of hidden layers and the number of neurons in each layer is flexible and is determined by examination of the errors in the results. Connections between input layer and the hidden layer are described by the weight matrix **W**. The input parameter matrix, **p**, is multiplied by the weight to form **Wp**, and a bias is added. Each neuron of the hidden layer has a bias b_i, a summation step, a transfer function *f*, and

an output a_i. The bias is summed with the weighted input parameters to form the net input **n**, which is the following equation in matrix form:[184]

$$\mathbf{n} = \mathbf{Wp} + \mathbf{b} \qquad (4.28).$$

The result of the summation step **n** is placed into the transfer function which produces the output vector of the hidden layer,

$$\mathbf{a} = \mathbf{f}\,(\mathbf{Wp} + \mathbf{b}) \qquad (4.29)$$

that leads to the input components of the next layer.[184] The next layer can be an additional hidden layer or can be the final output of the NN. Possible options for the transfer function, **f**, can include both linear and non-linear functions including symmetrical hard limit, log-sigmoid, hyperbolic tangent sigmoid, and other.[184]

The input of the network and its architecture can be defined by the problem and adjusted to improve the fit of the network. The number of inputs used in a network must fully define the problem at hand. The input vector in this study consisted of either selected wavelengths or principal components that were used to describe the spectra for each mixture or serum. The wavelength range from 350 – 550 nm was considered, but the excessive wavelengths proved to increase the prediction errors. The number of hidden layers and number of neurons of each layer define the network architecture which can be altered to find the network that produces the lowest error of prediction. ANN is defined by the number of inputs and the number of neurons in each layer. For example, a 15-30-4 network has 15 input parameters, 30 neurons in the hidden layer, and 4 outputs. Although problems are complex, the goal of ANN is to find a simple network that meets the low error of prediction standards for a clinical assay. If the system has too few or too many neurons or layers, the details of the function or problem are not being described.[184]

A key factor in developing a useful network is the training of the network. Both the weights and the bias are determined during this process. Given a set of data, three groups are randomly formed. Ten percent are the validation set, 10% are placed in the testing set, and 80% are placed in the training set. Each set of data is scaled to have a range that matches the ranges of the transfer functions used in the NN. A number of learning algorithms are available in the software programs. The Levenberg-Marquardt Backprogagation training algorithm was the gradient-based optimization used in this study. The algorithm is designed to minimize the mean square error between the outputs supplied in the training set and the ones generated by the network.[184] To monitor the selection of the number of neurons, a method called "early stopping" was used.[184] The process is used to prevent the over fitting of the training data. The process requires a validation set of input and output from the training set to be monitored during training. The errors produced by the validation set will serve as a control on the process. As training is started, the error will be decreased. The error will reach a low and begin to rise again if overfitting has begun. At that point, the training is completed, and the program is stopped. Monitoring the complexity of the network was completed during the training process through the use of Bayesian regularization. Bayesian regularization and other similar methods add a penalty term to the performance index of the network which is the sum of the squared weights.[184] If this term of the index is too small, the network is overfitted. If the term is too large, important parts of the details of the absorbance/concentration relationship may be missed. The final step is the error analysis of the testing set to determine the accuracy of the NN for prediction. The root mean squared error of prediction (RMSEP), in Equation 4.20 will be used. The prediction set

consisted of prepared mixtures that were not included in the training, validation, or test sets used in the development of the network.

ANN do not have the ability to extrapolate. The range of input parameters used in the training set is the only range that it will be able to analyze accurately. In the case of serum and prepared mixture analysis, the perceptible highest and lowest concentrations of each component *must* be included. In order to get the widest concentration range, possible concentrations from patients with untreated and treated dyslipidemias were included in the training of the NN. For example, a patient with Type I dyslipidemia has an elevated concentration of triglyceride, part of which is PUFA, and a normal level of cholesterol. These vastly different concentrations and spectra had to be considered in the design of the training set. For the four component non-water samples, no experimental design was used. All generated mixtures for that were completed for analysis with other chemometric methods. This resulted in 1500 spectra that were divided into the training, validation, and test sets.

The water added network was prepared differently. The seven component training set was partially designed using full factorial designs with limitations in place to ensure the resulting spectra would remain inside the 0.2 to 1.0 absorbance range across all wavelengths. The resulting matrix of concentrations used to prepare the mixtures is listed in Appendix III. Each of the 130 mixtures was prepared and analyzed at least twice with the Purdie assay. Added to the mixtures were single solutions of each of the seven analytes used to calculate the molar absorptivities of the analytes. A total of 665 spectra were used for the training, validation and testing of the NN. The prediction set contained 16 mixtures that were used for error analysis.

All ANN calculations were completed using the *Neural Network Toolbox*, version 4.0 included as part of the software package *MATLAB R12*.[191] Multiple architectures were attempted and trained since the design must be altered for each data set examined. An example of an ANN architecture used is the three component analysis. We have found that an input of 66 wavelengths in the range of 350 to 480 nm provided the most acceptable network for the determination of the concentrations of three analytes. Through extensive examination of NN architectures, a two-layer network (66-32-3) with 66 inputs, 32 neurons in the hidden layer, and 3 neurons in the output layer was determined to be the best. The transfer functions chosen were the hyperbolic tangent sigmoid function

$$f = \frac{e^{n} - e^{-n}}{e^{n} + e^{-n}} \qquad (4.30)$$

for the hidden layer and

$$f = n \qquad (4.31)$$

for the output layer a linear function.[184] The scaling factor used on the input and output data for use with the range -1 to +1 to match the use of the hyperbolic tangent sigmoid function was

$$A_s = \frac{2(A - A_{\min})}{A_{\max} - A_{\min}} - 1 \qquad (4.32)$$

where A is the absorbance, A_s is the scaled absorbance, and A_{min} and A_{max} are the minimum and maximum absorbance values of the absorbances. Equation 4.32 was applied for each input wavelength individually. Equation 4.32 was also applied to the concentration matrix to scale the input and output data to the range of -1 to 1 to fit the selected transfer function.

2. Pattern Recognition

Using PCA discussed previously, the resulting principal components were plotted versus each other to produce a two and three dimensional representation of the data to determine if any patterns or clustering was occurring. PCA was completed for both prepared mixtures and treated and untreated serum samples. If a pattern was seen, then hierarchical cluster analysis was used to group the data points using the *JMP* software package.[155] In cluster analysis, the process will start with one piece of data and combines groups based on distances from each other in the principal component space.[153] The distances can be determined using a number of different methods including weighted average and Ward's method. Non-hierarchical cluster analysis, which is less frequently used, differs in that it starts with the data divided into groups; and the groups are altered to meet certain conditions such as minimization of the distance and other criteria.[153] The cluster analysis in this study was hierarchical with Ward's method used for the distances.

CHAPTER V

EFFECTS OF WATER ON THE MOLAR SPECTRA

The experimental results for this study have been divided into three parts. The first is the effect of water on the molar absorptivities. Previous studies have published the molar absorptivities of each of the fatty acids, with no water added to the assay method.[29, 31, 32] Since water is approximately 97% of serum, it was tested to see the effect of this amount of water on the reagent system.[192] This simple test revealed significant changes in the molar absorptivities.

As previous work had established, the only major compounds that can react with the reagent system are the PUFA and cholesterol. It should be noted that prior work examined saturated fatty acids, steroids, and other pharmaceutical drugs that contained the –CH=CH-CH_2-reagent selective group. [29-33] Saturated fats did not produce colored products. [29-33] The steroids and other pharmaceutical drugs do react, but their concentration at therapeutic levels in serum would be much too low compared to the PUFA and cholesterol to significantly alter the spectral pattern. [29-33] Therefore, the focus of the analysis is limited to those compounds that will provide the largest contributions to a serum spectrum.

Initially, all calibration attempts with standards of the PUFA and cholesterol were completed with no water added to the reaction as in previous research. After initial

attempts to use the resulting molar absorptivities to fit sample serum spectra, it was determined that the molar absorptivities were too large and had different patterns that, although close, did not fit the serum completely. After considering the reaction system and all possible sources of error, it was determined that the only difference between the serum reactions and the prepared mixture reactions was the additional water and protein found in serum. During the reaction with a serum sample, the proteins are precipitated from the reagent solution and do not alter the spectral pattern. Serum normally has a water to solid percentage ratio of 93:7 where the solids are the proteins and lipids.[192] With the sample size of serum being 10 μL, approximately 9.7 μL of water is added to the reagents when serum is analyzed. The initial test of the water effect was to spike serum. Increments of 5, 10, 20 and 30 μL were added to the reaction. The resulting 15 minute spectrum for each addition is shown in Figure V.1. The general trend is a decrease in absorbance with the region of 380 to 460 nm being affected most. Other spectral regions also diminished in height as more water was added. Although dilution of the mixture is occurring, the changes in the pattern and height are too significant to be fully accounted for by this factor. Follow up tests of adding water to the standards yielded different spectra for each of the analytes.

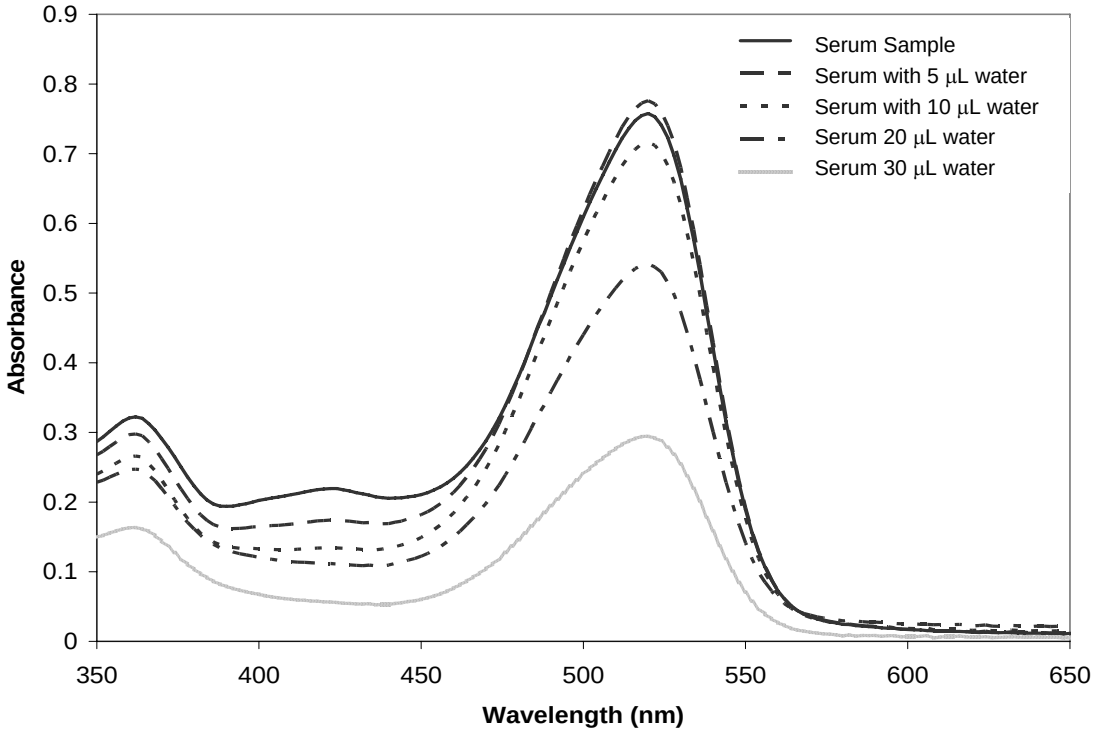


Figure V.1: Overlaid spectra for 10 μL of serum with 5, 10, 20, and 30 μL of water added to the reagent solution prior to reaction.

I. Cholesterol

Cholesterol molar absorbance spectra with and without water have some differences, but only a slight decrease in its maximum peak at 520 nm. A comparison of the molar spectra with and without water is in Figure V.2. Cholesterol is defined by three major peaks occurring at 362 nm, 420 nm, and 520 nm. The 520 nm peak is unique to cholesterol when compared to the other analytes and, as a result, was considered for a single wavelength determination of the cholesterol concentration until a truer measurement of the underlying PUFA was completed. The effect of water on the 520 nm absorbance results is an 8% decrease when 10 μL of water is added. The 362 and the 420 nm peaks have a decrease in absorbance of 33% and 55%, respectively. With only an 8% difference at the 520 nm peak, it offers some explanation as to why cholesterol

calibrations without water differed by less than a 10% error when compared to the enzymatic test. It was not until the PUFA were included in the models that the problem became apparent.

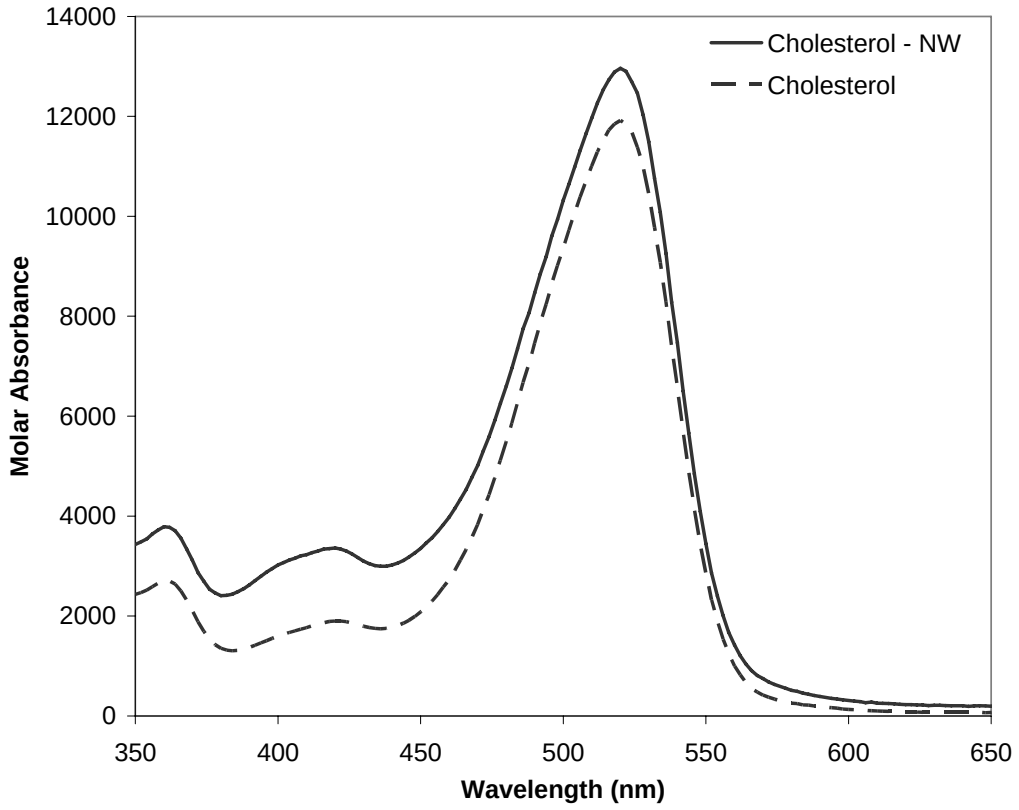


Figure V.2: Overlaid molar absorptivities of cholesterol with and without 10 μL added to the reagent mix. Cholesterol – NW is the cholesterol standard without water in the reagent mixture. Cholesterol is the cholesterol standard with water.

II. Linoleic and Conjugated Linoleic Acid

Spectra for linoleic acid (LA) and conjugated linoleic acid (CLA) originally differed in the magnitudes of molar absorptivities and in spectral patterns. The two maxima in the spectra of LA and CLA both occur at 372 and 426 nm, Figure V.3 and Figure V.4. The percent drop in absorbance at 372 nm for LA was a 55% decrease compared to an 11 % decrease for CLA when water was added. The percent difference at 426 nm, when water was present in the reagent, was a 24% decrease for LA and an

increase of 22% for CLA. The best numerical indicator for the spectral difference between these two isomers is the ratio of the absorbances of the 372 nm peak to the 426 nm peak. Without the presence of water, the ratio was 2.3 for LA and 1.6 for CLA. With water, the ratios are lowered to 1.7 for LA and 1.1 for CLA. This ratio difference between the spectra supports the argument that the concentrations of these compounds in mixtures and serum can be separated. The two factors that influence that statement are the differences in the magnitude of the molar absorbance and the ratio differences between the two peaks demonstrating the pattern differences. The absorbance increase at 428 nm for CLA is very significant to their discrimination since it is the only band to increase in intensity in water.

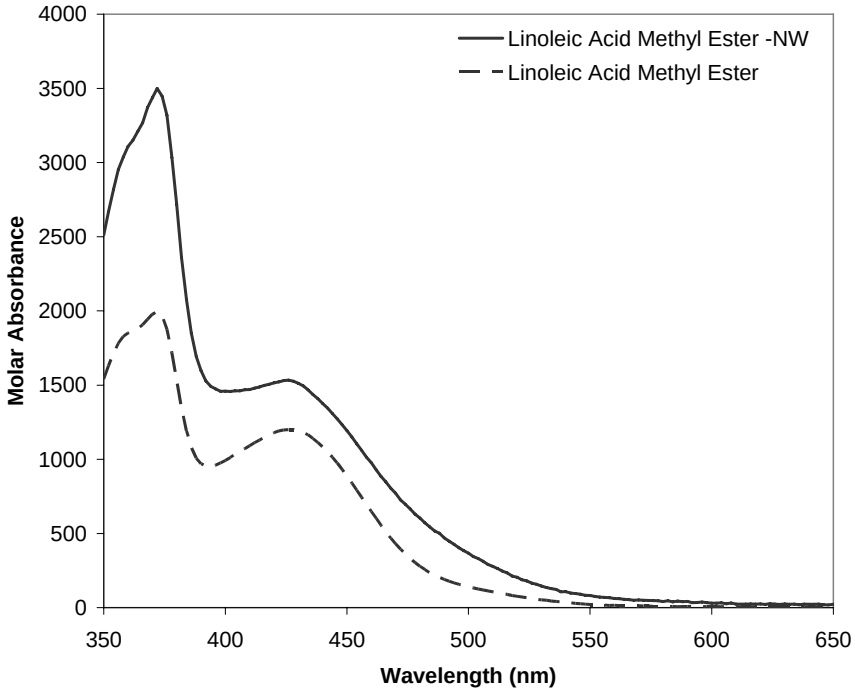


Figure V.3: Overlaid molar absorbance spectra for LA with and without the 10 μL of water. Linoleic Acid Methyl Ester – NW is the PUFA standard without water in the reagent mixture.

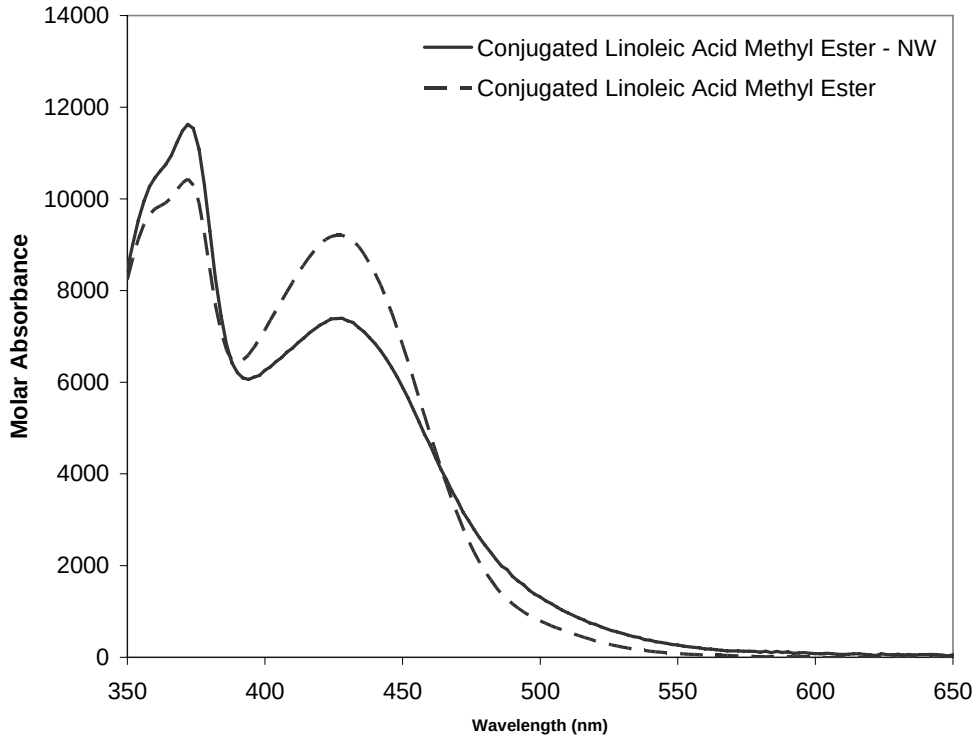


Figure V.4: Overlaid molar absorbance spectra for CLA with and without the 10 μL of water. Conjugated Linoleic Acid Methyl Ester – NW is the PUFA standard without water in the reagent mixture.

CLA and LA were both run in the kinetic mode of the diode –array instrument to investigate the effect of water on the development of the absorbance. Spectra were recorded every minute for a 20 minute time period for each analyte, with and without water. Resulting spectral overlays for a 0.02 M solution of LA are shown in Figure V.5. Figure V.6 displays the corresponding plots for a 0.01 M solution of CLA. Figure V.7 A and B are plots of absorbance versus time in minutes at 372 nm in Figure V.6(A) and 426 nm in Figure V.6(B) for all four kinetic experiments. The significant difference can be seen at the 426 nm peak for the LA solutions. In the non-water reaction, the 426 nm peak reaches an obvious maximum at 11 minutes then begins to decline. In the water reaction, the peak continues to increase in absorbance until the end of the 20 minutes. Only a

small variation exists in the 372 nm peak when comparing the water and non-water sample. The non-water reaction rate is fast during the first six minutes of the reaction compared to the rate for the water sample. This observation suggests that the water hinders the reaction resulting in the decreased absorbance.

The plots in Figure V.7 also demonstrate differences in the kinetics of CLA and LA. Each component has a different rate of reaction, and these differences could possibly be exploited for analyses. The reaction of the standards and the mixtures could be monitored over the reaction time of 3 to 20 minutes to yield more information about the sample to help the analysis. The first three minutes of the reaction, however, are lost during serum analysis due to the required spin time to remove the proteins from solution. Monitoring the reaction over the entire period may be a useful project to consider in the future. The data collected on one sample would be increased from one spectrum to multiple spectra and includes time as a variable. The added information and a proper chemometric method may be able to improve upon the errors in the quantitative results for the determination of the analyte concentrations.

In summary, CLA and LA have a large molar absorptivity difference. The molar absorbance magnitude of CLA is 5.2 times higher than LA in the presence of water. There is also a difference in the pattern that can be determined by examining the ratio of the peaks of 362 nm and 420 nm. The ratio for CLA is 1.7 where LA is 1.1. The differences in magnitude and the spectral patterns allow for the possibility of separation in the deconvoluting methods to determine the concentration of each component.

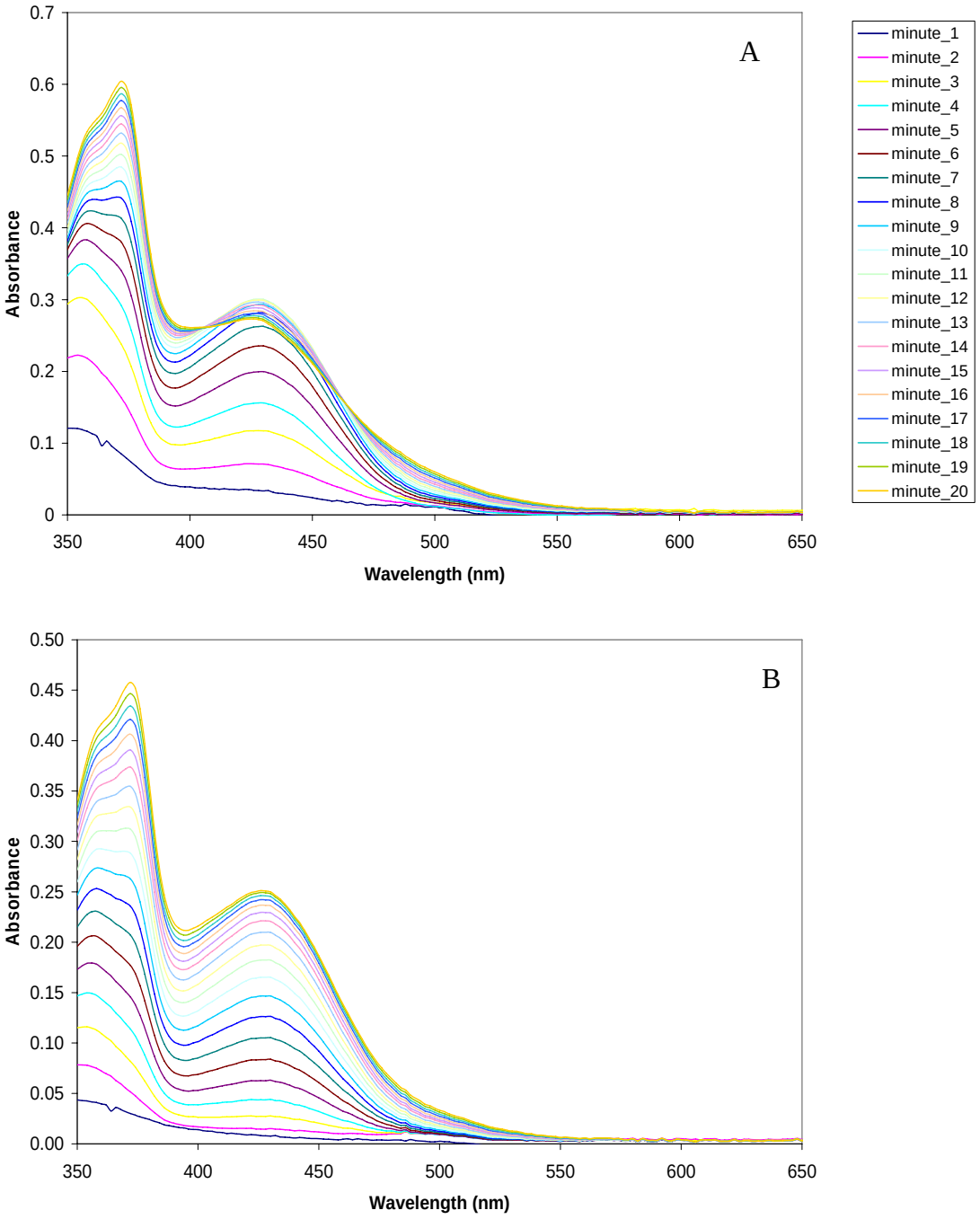


Figure V.5: Spectral ensembles that compare the kinetics of LA (A) absorbance spectra after each minute of the reaction of 10 μL of 0.02 M LA standard in chloroform solution with only the reagents added over a 20 minute time period. (B) Absorbance spectra for each minute of the reaction of 10 μL of 0.02 M LA standard in chloroform solution with 10 μL water added to the reagents of the assay. The legend applies for both figures.

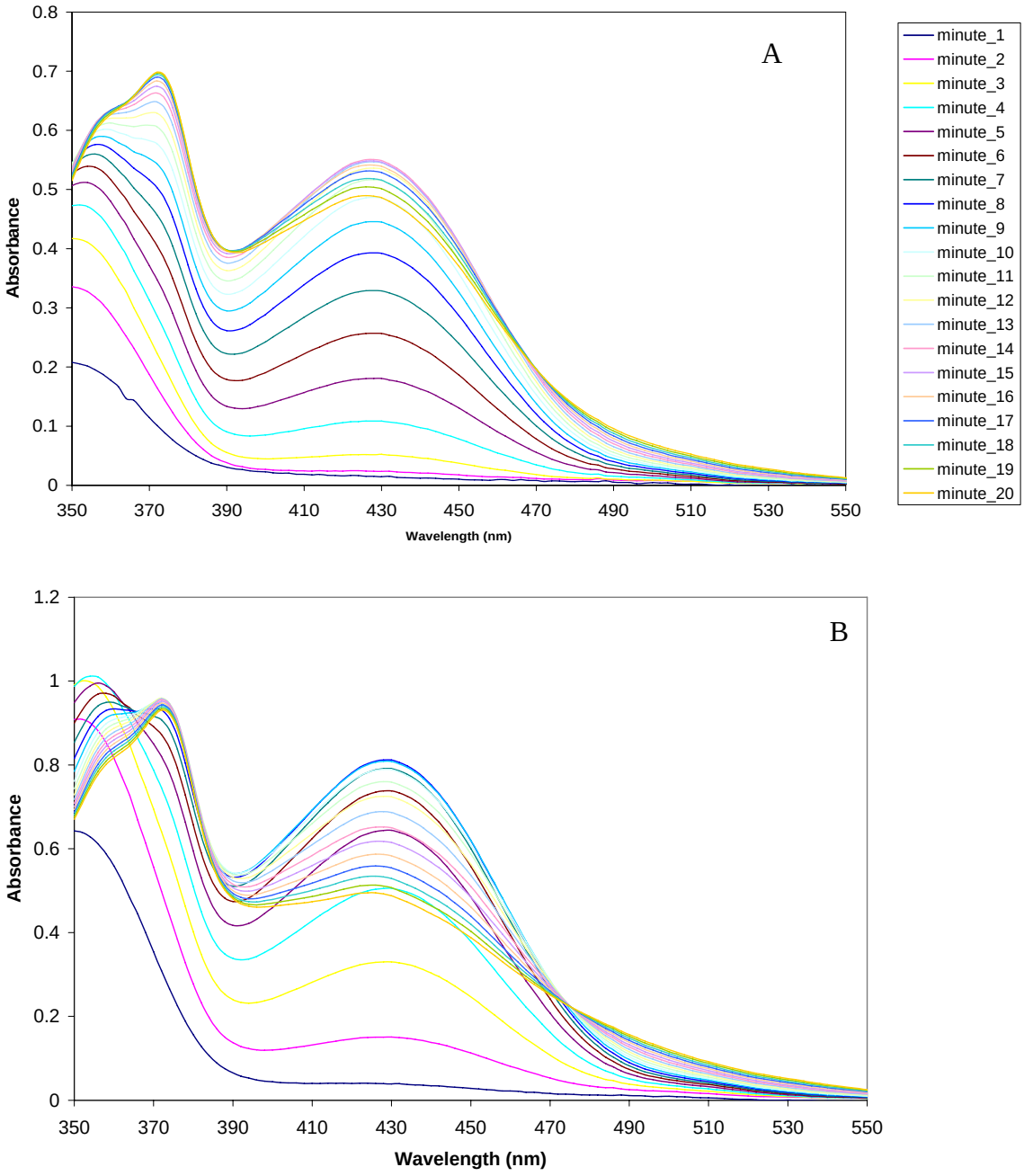


Figure V.6: Spectral ensembles that compare the kinetics of CLA (A) Absorbance spectra for each minute of the reaction of 10 μL of 0.01 M CLA standard in chloroform solution with only the reagents added over a 20 minute time period. (B) Absorbance spectra for each minute of the reaction of 10 μL of 0.01 M CLA standard in chloroform solution with 10 μL water added to the reagents of the assay.

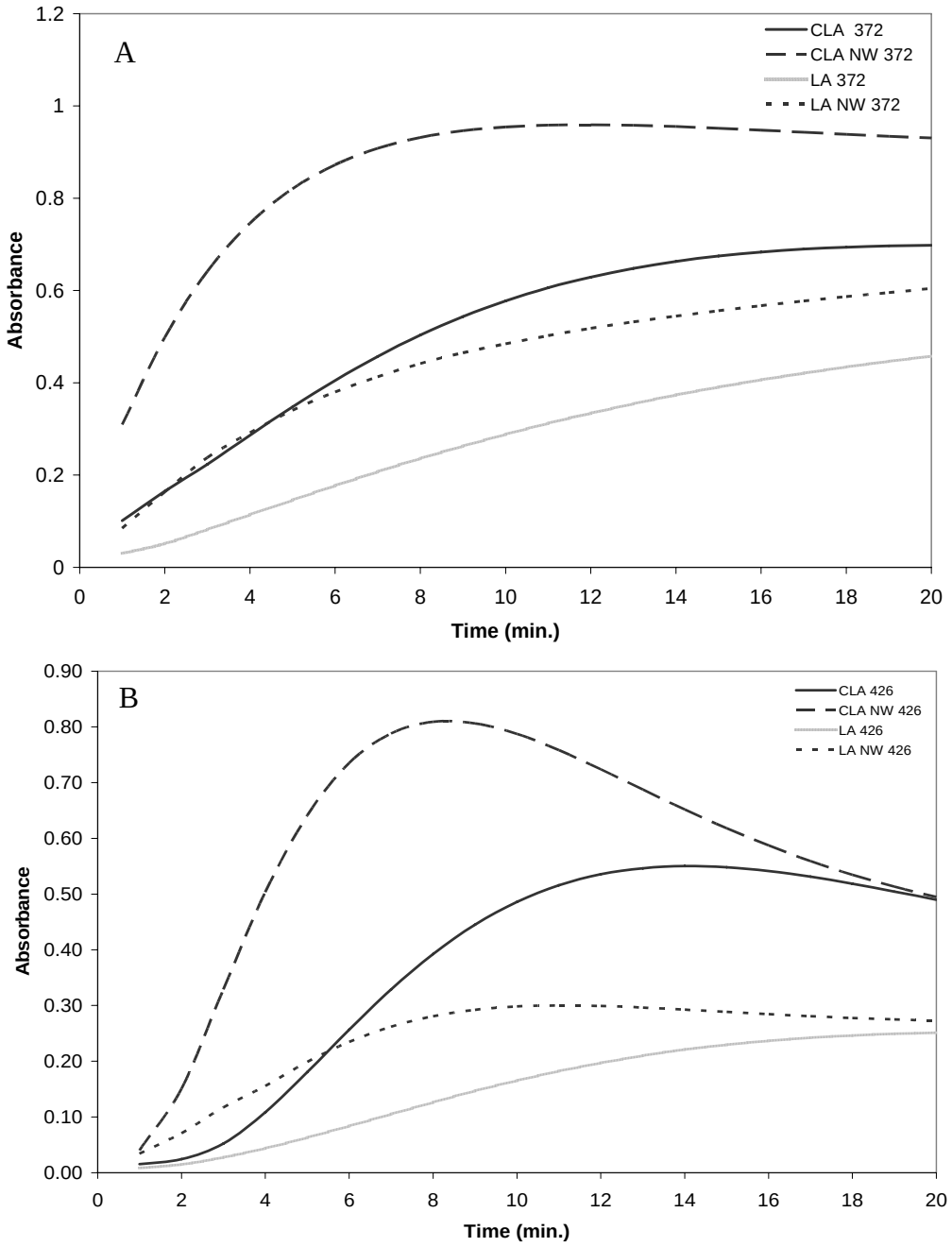
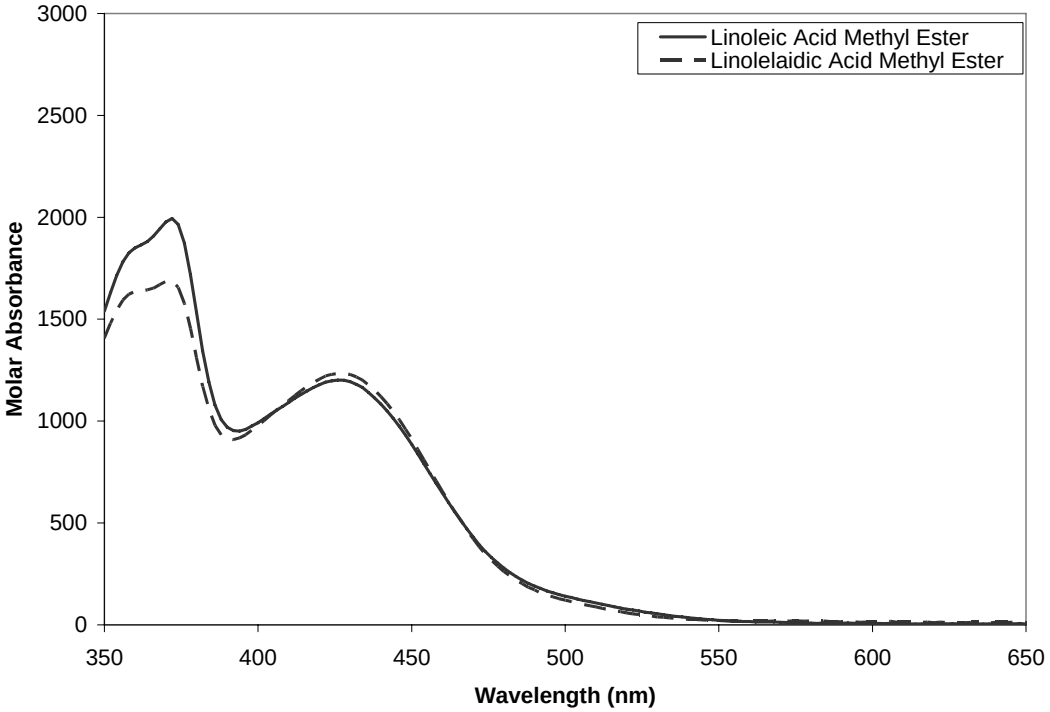
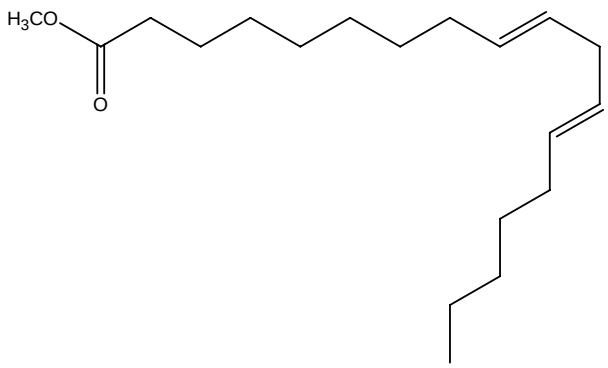


Figure V.7: (A) Absorbance v. time for the peak at 372 nm for the reagents with and without water for CLA and LA. NW means no water was added to the reaction. (B) Absorbance v. time for the peak at 426 nm for the reagents with and without water for CLA and LA.

The two double bonds in LA allow for an investigation of the assay's ability to discriminate between all cis- and all-trans by virtue of a spectral difference. The trans, trans isomer also called linolelaidic acid can be compared to the cis, cis LA isomer, linoleic acid. Standard forms of the methyl esters of LA and linolelaidic acids were obtained and the molar absorptivities of each, with water present, were determined using the methods already described. Their molar absorptivities are compared in Figure V.8 that also includes the structures of each of the analytes. The spectra show strong similarities in the magnitude and overall pattern. The percent difference at the 372 nm peak is 17% with LA being higher than linolelaidic acid. The 426 nm peak is reversed with the linolelaidic acid absorbance being 2% higher than the LA molar absorbance. The ratio between the 372nm: 426 nm molar absorbance is 1.7 for LA and 1.4 for linolelaidic acid which might be within the error limits. The similarities in the molar absorptivity and the ratio of the two major peaks lead to the conclusion that the assay is unable to discriminate between the cis,cis and trans, trans isomers of LA. Tests of this conclusion will have to be reexamined as part of the development of the chemometric methods discussed in the next section of this chapter.



Linolelaidic Acid Methyl Ester
Methyl trans,trans-9,12-octadecadienoate



Linoleic acid methyl ester
Methyl cis,cis-9,12-octadecadienoate

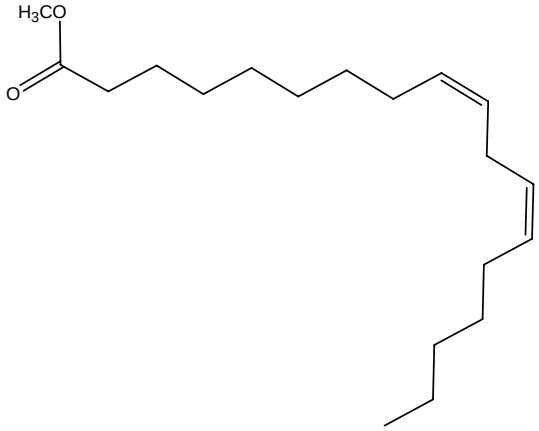


Figure V.8: Molar absorbance spectra for LA and linolelaidic acid methyl ester which were completed with water included in the reaction. The chemical structures for each are displayed below the figure.

III. α- and γ- Linolenic Acid

ALA and GLA are two structural isomers of the PUFA that contain three double bonds in their structure. ALA, an omega-3 fatty acid with double bonds on the 9, 12 and 15 carbons, was examined with and without water added to the reaction. The spectral difference is shown in the graph of the molar absorptivities in Figure V.9. The ratio of the two maximum peaks, 372 nm to 426 nm, is 1.0 without water and 0.97 with water. The percentage of the difference between the molar absorptivities is 56% for 372 and 51% for 426 nm with the water spectra being higher in both cases.

The GLA is an omega-6 fatty acid with double bonds on the 6, 9, and 12 carbons. Because of cost constraints, it was only examined with the addition of water. A second fatty acid methyl ester with three double bonds in a longer carbon chain was examined as an alternative under the same conditions, cis-11,14,17- eicosatrienoic acid methyl ester that has a 20 carbon chain with three double bonds located in it. ALA and GLA have an 18 long carbon chain. Prior to this part of the study, it was unclear if the Purdie assay could discriminate among a series of PUFA with the same number of double bonds, e.g. GLA, ALA, and cis-11,14,17- eicosatrienoic acid. The comparison of the molar spectra for the similar fatty acids is shown in Figure V.10. Chemical structures for each are shown in Figure V.11. There is a difference of only 5% between ALA and cis-11,14,17-eicosatrienoic acid (ETA) at each of the major peaks. ETA has a maximum peak ratio of 0.97 which is the same as the ratio of the peaks in ALA with water. The longer carbon chain does not appear to alter the molar spectrum. Based on this result, all of the PUFA containing three bonds will be expected to produce a similar molar absorptivity. GLA has a different peak ratio of 0.90 and a percent difference of 0.85% for 372 nm and 8.7%

for 426 nm when compared to ALA. With the difference in the peak ratios, a trial of chemometric methods will have to be completed to determine if this difference in pattern is sufficient to lead to a separation of the concentration of GLA from ALA in complex mixtures. With the highly overlapping molar spectra and only small variations between all three spectra, the conclusion that the color development and resulting spectral pattern are dependent on the number of double bonds present can be supported.

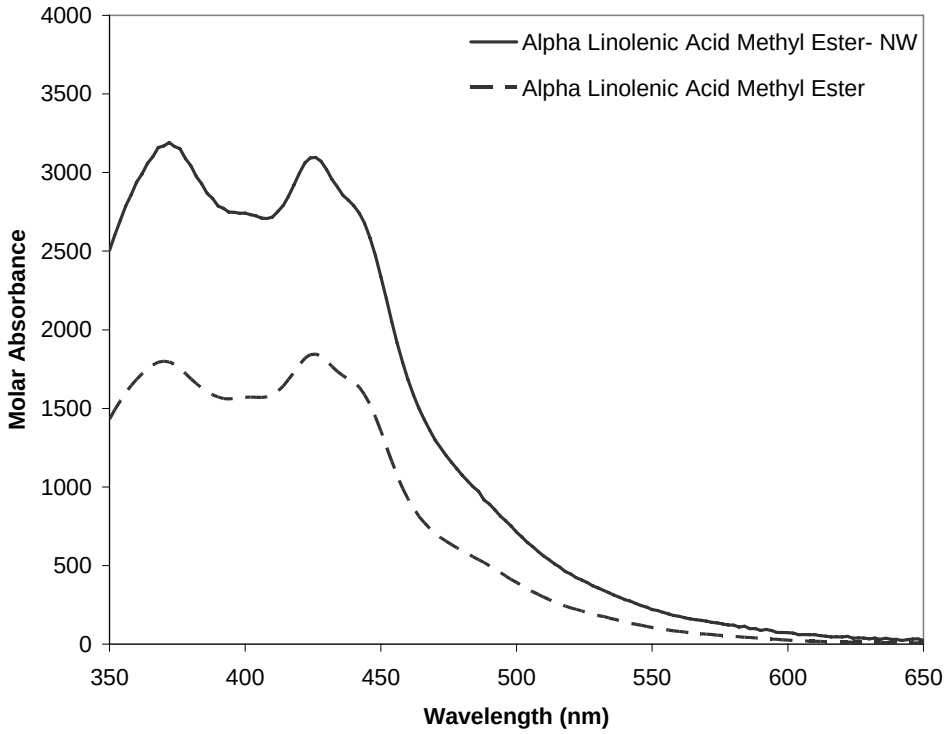


Figure V.9: Comparison of ALA methyl ester under the changed reaction conditions where 10 μL of water was added.

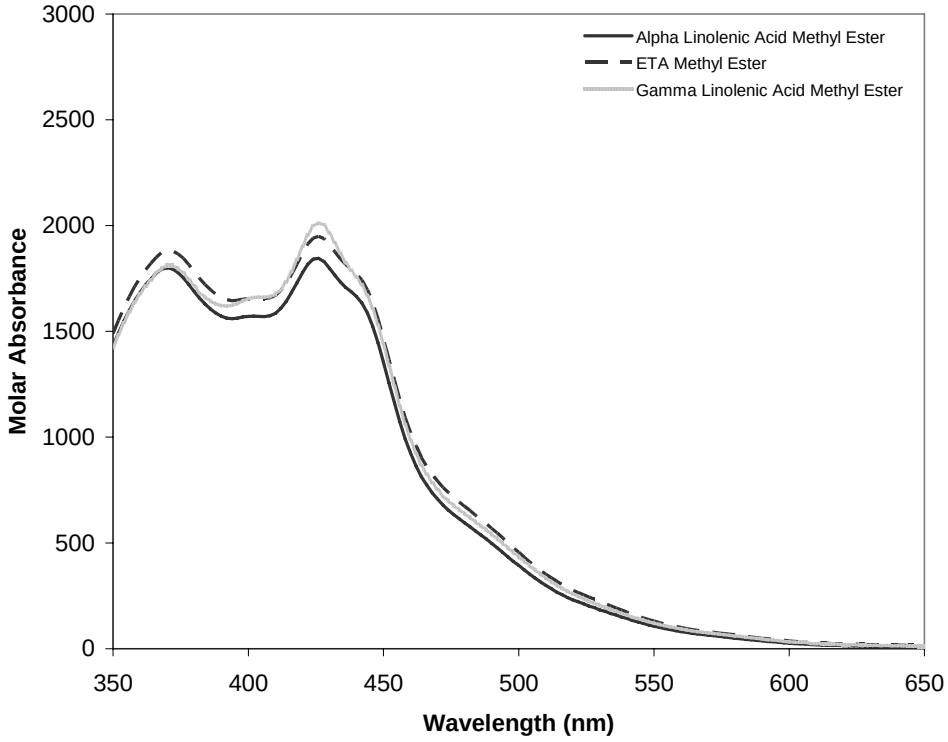
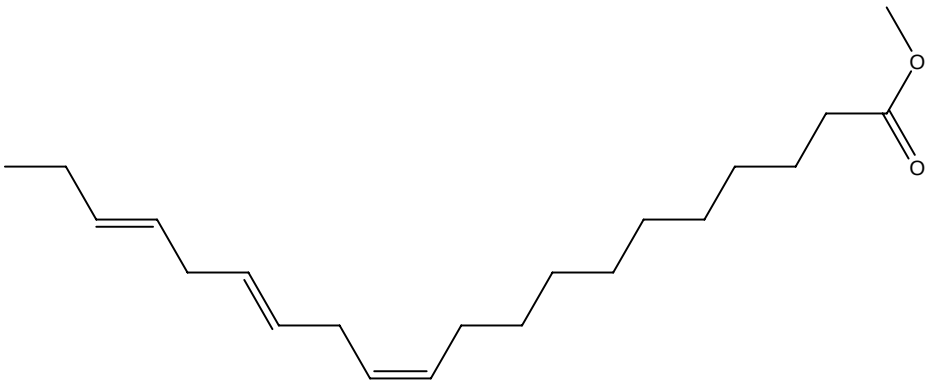
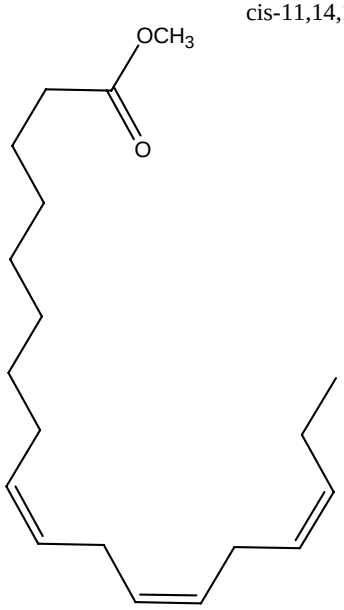


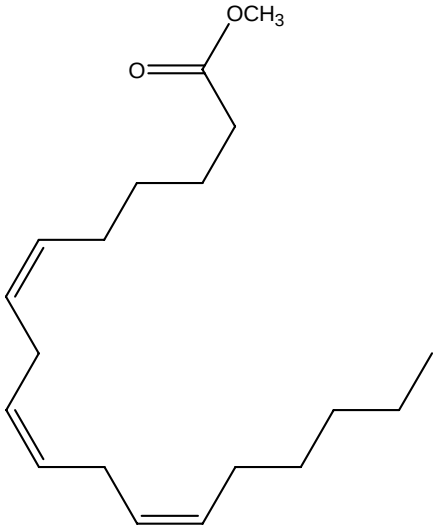
Figure V.10: Molar absorbance spectrum for the fatty acids with three double bonds in the carbon chain. The similarity in the spectra results from the Purdie reagent producing spectra that are the same for the same number of double bonds present in the compound.



cis-11,14,17-eicosatrienoic acid methyl ester



alpha linolenic acid methyl ester



gamma linolenic acid methyl ester

Figure V.11: Chemical structures for each of the PUFA containing three double bonds.

IV. Arachidonic Acid

Additional support for the Purdie reagent producing spectra that are determined by the number of double bonds and not the compound nor the length of the carbon chain is provided by examining AA and cis-7,10,13,16-docosatetraenoic acid methyl ester (DTA). Each of these PUFA contains four double bonds. First, AA was examined under the water and no water reaction conditions, and the molar absorbance spectrum for both is in Figure V.12. AA has two major peaks at 376 nm and 424 nm. The percent

differences between the spectra are 41% for 376 nm and 50% for 424 nm, with the water reaction being lower. The ratio between the 376 nm and 424 nm peak remained the same, so water did not have an effect on the spectral pattern. It just reduced the absorbance.

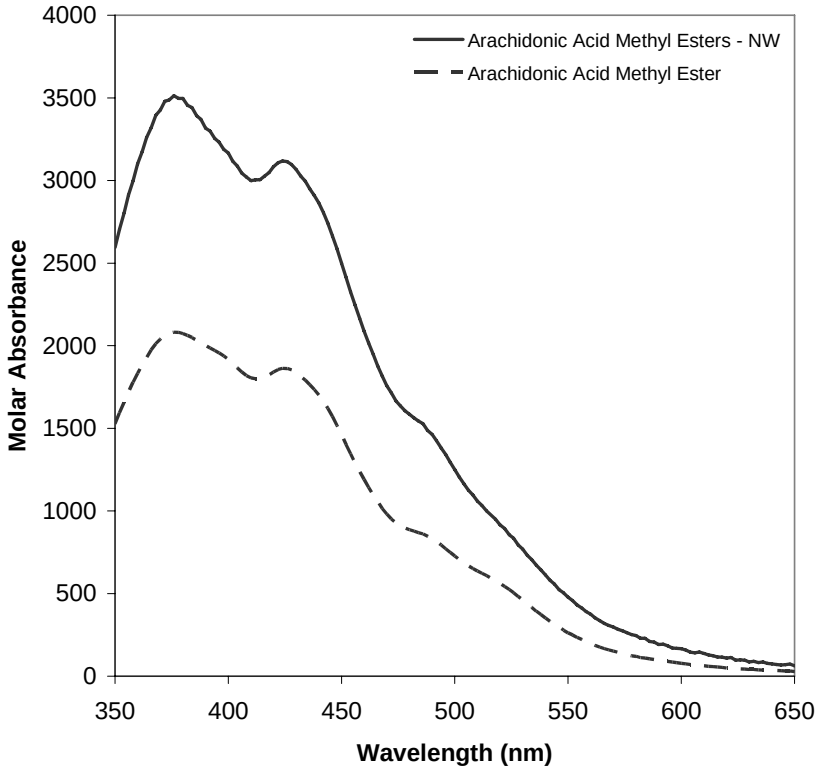


Figure V.12: Comparison of AA methyl ester under the changed reaction conditions where 10 μL of water was added and no water (NW) added.

The molar spectra comparison of AA with DTA with water is shown in Figure V.13 with the chemical structures included in Figure V.14. The resulting spectra show similar patterns in the region of 350 to 460, and then the DTA has an elevated absorbance through 650 nm. It is unclear if this is a true depiction of the molar spectra for DTA or if it is the result of an error that occurred the day that the data was collected. Limited supply of this standard has inhibited reproduction of the experiments. The presence of

the change from 460 to 650 nm is a concern, especially, at the 520 nm region since it is the major absorbance peak for cholesterol. The similarities in the 350 to 460 nm region, where all of the other PUFA absorb, are almost a duplicate of each other. Based on these regional differences and the results from the PUFA with three double bonds, the statement that the Purdie assay will produce a unique spectral response that is dependent on the number of double bonds present is supported.

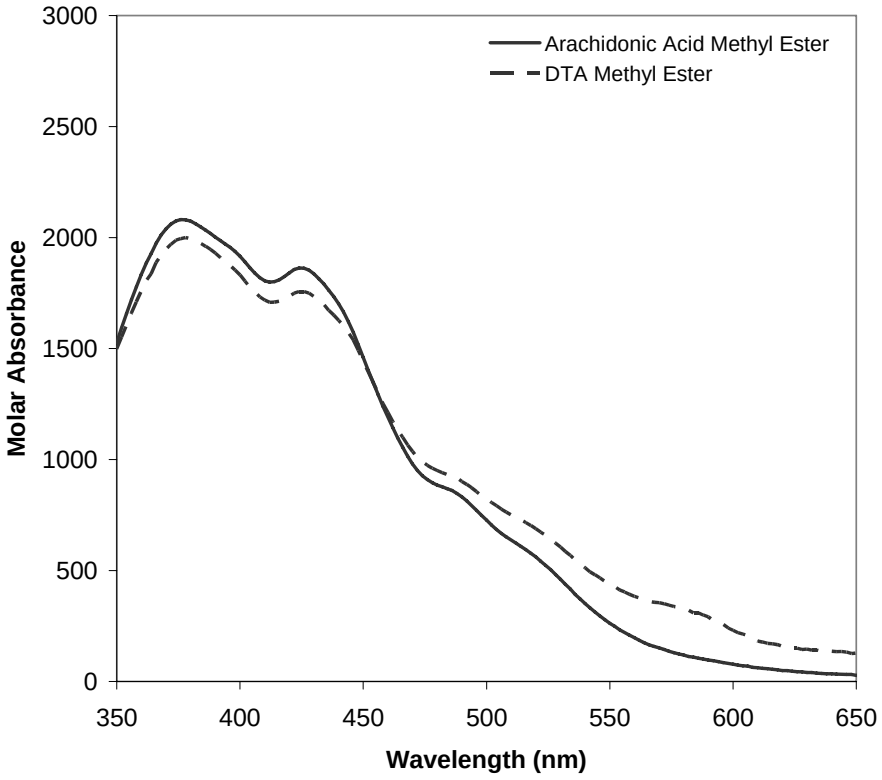


Figure V.13: Molar absorbance spectrums for the fatty acids with four double bonds in the carbon chain. The similarity in the spectra results from the Purdie reagent producing spectra that are identical to those with the same number of double bonds present in the compound.

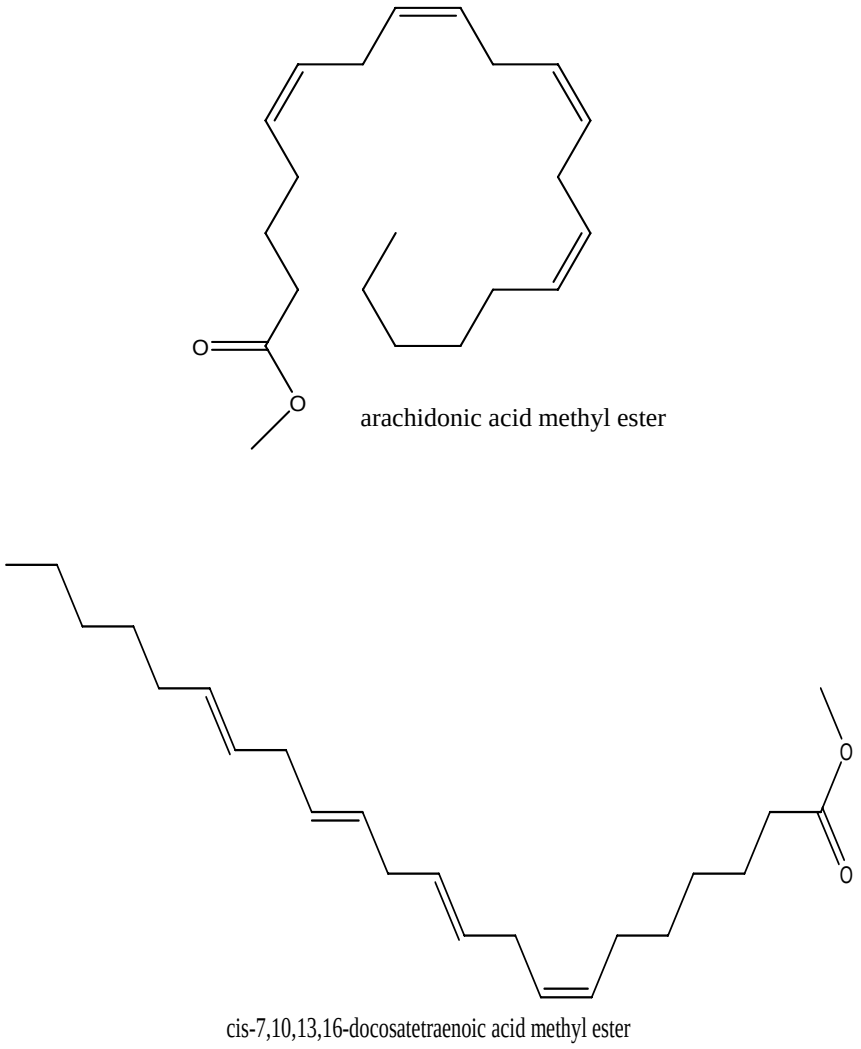


Figure V.14: Chemical structures for AA and cis-7,10,13,16-docosatentraenoic acid methyl ester

V. EPA and DHA

The final two PUFA that are included in this study were EPA and DHA, Figure V.15, reveals the effect of water on the reaction of the Purdie reagent with EPA, and Figure V.16 contains the molar spectrum from the reactions with DHA. The chemical structures for each PUFA are in Figure V.17. EPA, with five double bonds, and DHA, with six double bonds, have a major absorbance peak at 376 nm and a shoulder before a steep descent at 444 nm. EPA had a percent molar absorbance difference of 36% and

43% for each wavelength when water was added to the reaction system. DHA had a 60% decrease in percent difference in molar absorbance at 376 nm and 67% at 444 nm. Both EPA and DHA have a ratio of 376 nm absorbance: 444 nm absorbance of 1.4 without water and 1.6 with water. As previously reported1-3 and confirmed with the water added reaction, EPA and DHA have similar patterns and only differ in the magnitude of the molar absorptivity. A plot of the molar absorptivities of each component versus the other is provided in Figure V.18. The plot is linear with no looping in the pattern which suggests that the patterns are the same. Chemometric methods will have to be tested to determine if separation of the individual concentrations can be achieved. If it is not possible, the combination of all of the spectra may be necessary. Since they are both omega-3 fatty acids, the combination will not be as disconcerting as combining any of the other analytes.

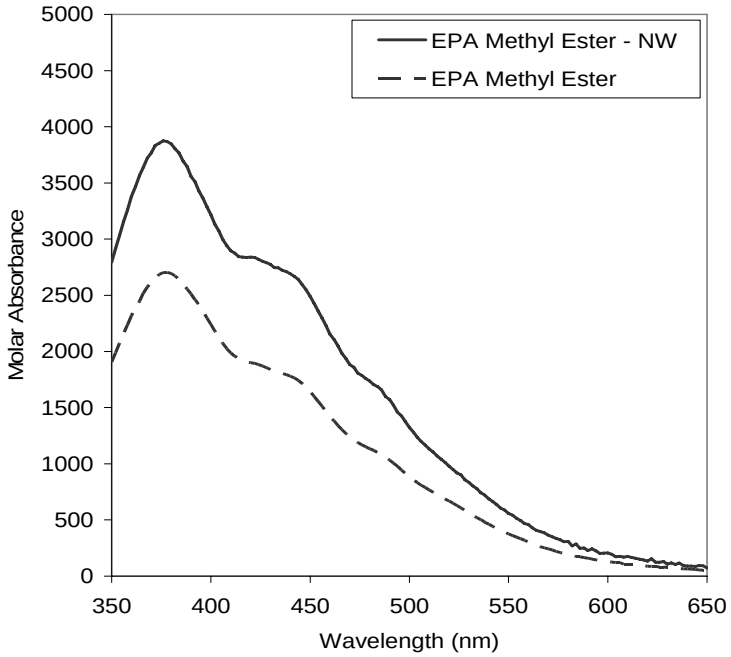


Figure V.15: Molar absorbance spectra for EPA methyl ester with and without (NW) 10 μL of water added to the reaction

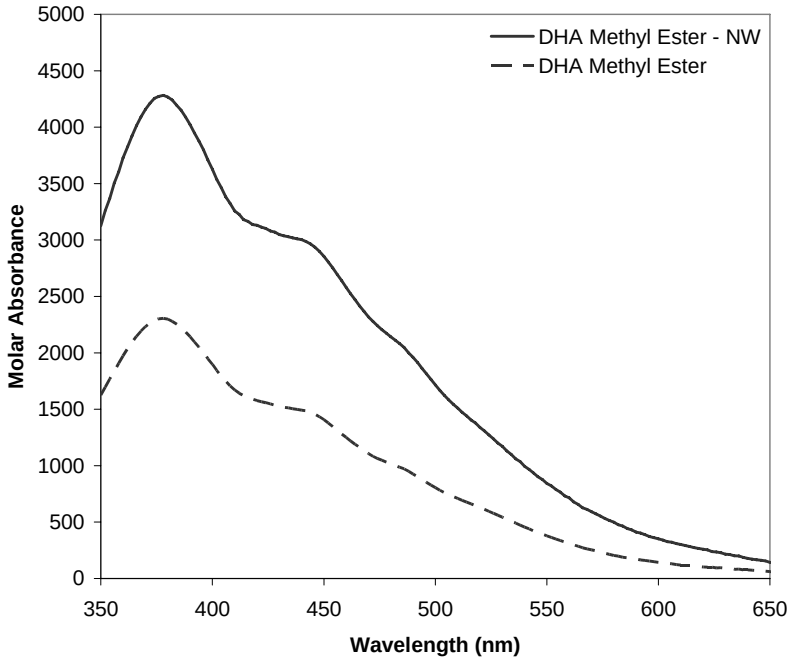
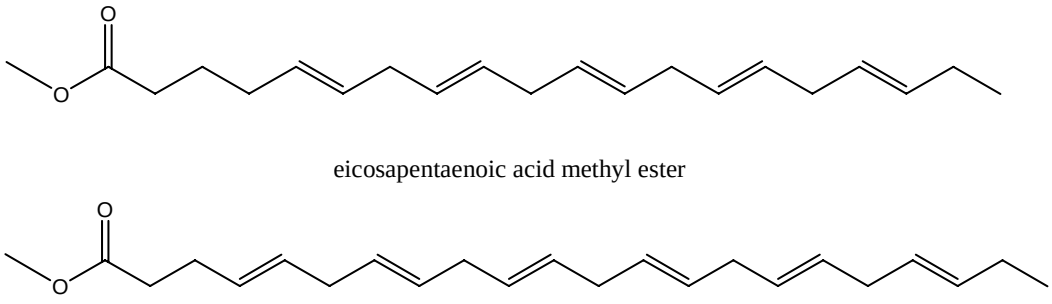


Figure V.16: Molar absorbance spectra for DHA methyl ester with and without (NW) 10 μL of water added to the reaction



eicosapentaenoic acid methyl ester

docosahexaenoic acid methyl ester

Figure V.17: Chemical structures for EPA and DHA methyl esters

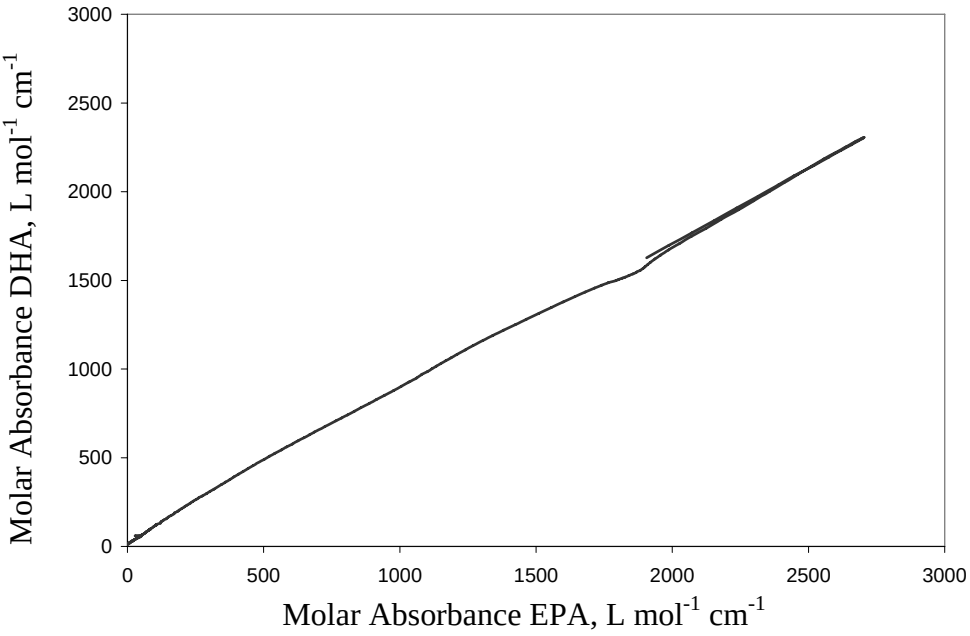


Figure V.18: Molar absorbance of DHA v. molar absorbance of EPA. The equation of the line is y = 0.839x + 30.8 with an R^2=0.999.

In summary, the addition of water to the reaction to simulate its possible influence on serum analyzed with the Purdie Assay produced a significant effect on the magnitude of the molar absorptions. All seven major analytes had a reduction in the molar absorptivity values, with the exception of CLA at 428nm, and experienced changes to their spectral pattern. Through examination of the kinetics of two of the analytes, LA and CLA, it was determined that water slowed the rate of the reaction which resulted in a lower absorbance for the 15 minute spectra. Through the early stage analysis of analytes with the same number of double bonds as part of the chain, the spectral patterns appear to be nearly identical in pattern and absorbance. This results in the conclusion that the Purdie reagent produces a unique spectral pattern for each PUFA with the same number of double bonds in the chain. The exception to this statement is CLA, the conjugated isomer of linoleic acid. CLA has a molar absorbance 5.2 times that of LA with a

difference in the ratio of its major absorbance peaks. As the number of double bonds in the carbon chain increases to five and six, the spectral pattern becomes essentially identical except for an increase in its magnitude. Spectra for EPA and DHA illustrate this problem. It is an issue that will have to be addressed in the quantitative analysis.

CHAPTER VI

CHEMOMETRIC METHODS APPLIED TO ASSAY

Multiple attempts to simultaneously determine the concentration of the seven major analytes in serum by using prepared mixtures as the model system is the second part of the results' section. Results will be examined both with and without the water addition using a variety of methods including PLS, ANN, and GSAM. The final section is focused on the possibility of clustering being applied as a method to determine disease states based on different spectral patterns. Prepared mixtures and a limited set of data generated on serum samples from the Cape Town laboratory will also be discussed. The quantitative and qualitative analyses together describe the basic abilities of the Purdie assay.

I. Description of Data

As seen in the examinations of the molar absorbance spectra, the data for quantitative analysis are divided between two sets. A third set of data from the UCT Lipid lab was used for the qualitative analysis to be discussed in section III of this chapter. The mixture analysis combined with the calibrations for experiments without water resulted in a set of 1471 spectra. After initial analysis, outliers determined by examining influence and residual variance plots were removed to reduce the number to 1410. This set of data was divided into training, prediction, and test sets as needed for

analysis with the multiple multivariate methods. Figure VI.1 consists of the minimum and maximum spectra for this set of samples. Table VI-1 contains the concentration ranges for each of the four components examined with the non-water reagent system. The minimum concentrations are the non-zero minimums. The units of μM are used to represent the concentration in the assay reaction.

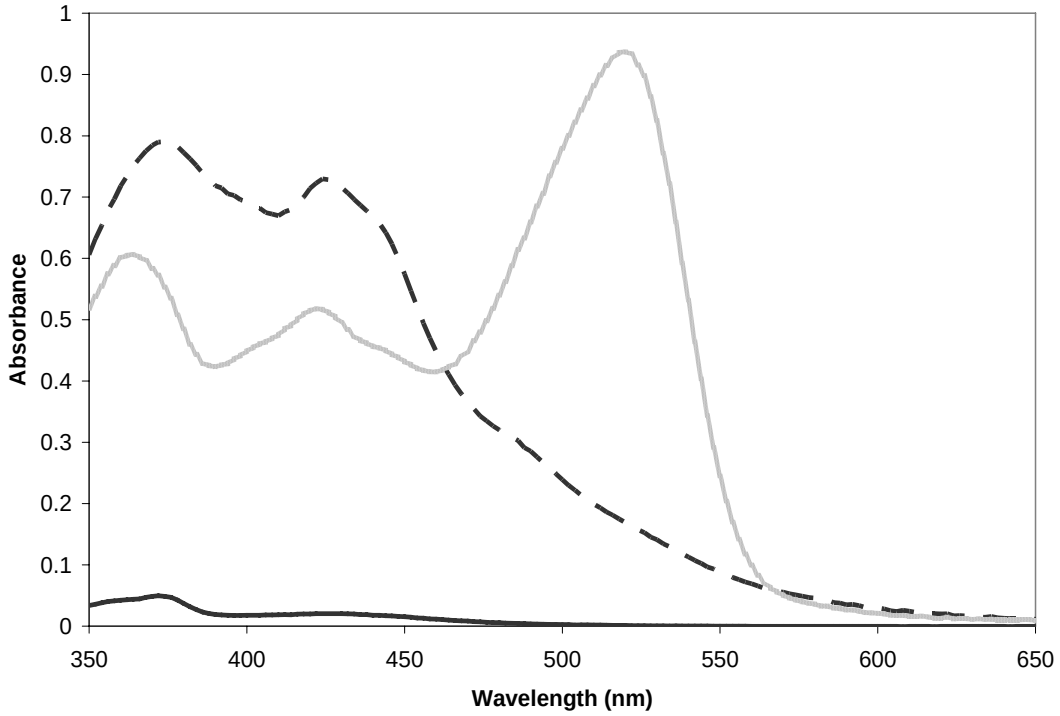


Figure VI.1: Spectra representing the minimum and maximum spectra for the data set completed without water. The minimum spectrum is for a low concentration of LA. The dashed line spectrum is a mixture of ALA and AA. The light gray spectrum is a mixture of cholesterol, LA, and ALA.

Table VI-1: Concentration range of data set without water

	Cholesterol	Linoleic Acid	α-Linolenic Acid	Arachidonic Acid
Min. Concentration (μM)	9.41	15.4	15.9	19.0
Max. Concentration (μM)	151	196	201	201
Average (μM)	80.1	106	109	110

The second data set was for water samples. This set was designed using full factor design with limits in place to maintain the total absorbance under 1.0 absorbance units. The molar concentrations of the solution used are given in Appendix III. Table VI.2 lists the minimum and maximum concentrations in μM. The spectra in Figure VI.2 are the minimum and maximum spectra of the entire data set of 667 mixture spectra. Eight outliers were removed, and the remaining spectra were divided as needed for each method. A set of prediction spectra were completed independently and were used to determine the root mean standard error of prediction (RMSEP) for each of the methods. The concentrations of these mixtures are within the ranges in Table VI-2.

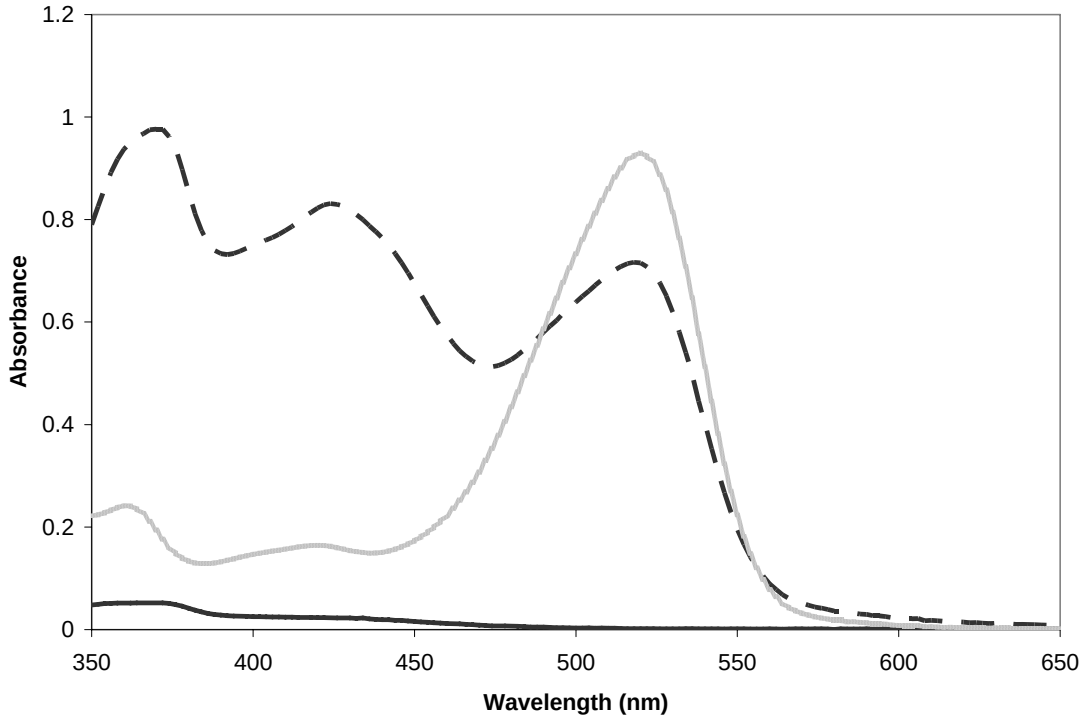


Figure VI.2: Spectra show the limits of the minimum and maximum spectra for the data set completed with water added. The minimum spectrum is for a low concentration of LA only. The dashed line spectrum is a mixture of cholesterol, ALA, AA, EPA, DHA, and CLA. The light gray spectrum is a high concentration sample of cholesterol only.

Table VI-2: Concentration range of the mixture spectra completed with water.

	Cholesterol	Linoleic Acid	α-Linolenic Acid	Arachidonic Acid	EPA	DHA	Conjugated
Min. Concentration (μM)	10.8	19.7	19.4	17.8	18.2	20.8	9.87
Max. Concentration (μM)	77.8	197	204	214	182	214	99.2
Average (μM)	44.3	109	111	116	100	117	54.5

II. Multivariate Approaches

A. Direct and Indirect Calibration Methods

The standard method of analysis for multivariate systems has been the multiple linear regression analysis. In cases where there are more input responses to describe the spectra than analyte concentrations, the system is overdetermined and least squares analysis must be used. As described in the methods section, the basic equation is $\mathbf{C}=(\mathbf{K}^T\mathbf{K})^{-1}\mathbf{K}^T\mathbf{A}$, where **C** is the concentrations of the unknown solution, **K** is the matrix of the molar extinction coefficients, and **A** is the absorbencies for the spectra of the unknown solution. The key is defining the matrix **K**.

In the first method, the K matrix was defined using molar extinction coefficients that were determined by calibrating each standard with multiple dilutions to find the calibration lines (slope and intercept) for each wavelength of the spectra. This method is a direct calibration method. Figure VI.3 is an example calibration line for LA at 372 nm. The equation of the line had a slope of 3500 with an intercept of –0.0174 and an R^2= 0.994. This process was repeated for each of the analytes, and the resulting K matrix was formed and then used to examine prepared mixtures. Full spectral molar absorptivities

used for this analysis are summarized in Figure VI.4. DHA and EPA were combined as one in this set of calibrations.

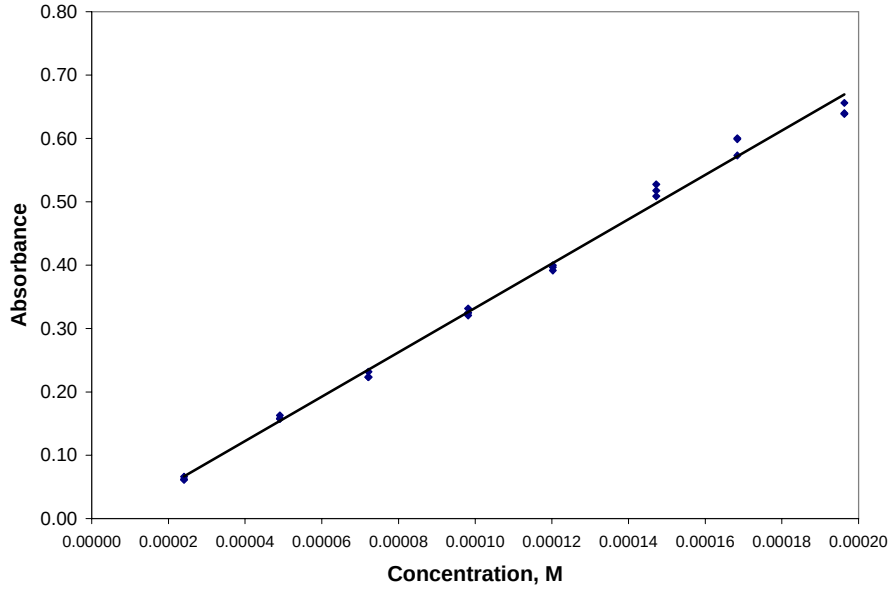


Figure VI.3: Absorbance v. concentration for LA at 362 nm. This is an example of the calibration lines determined for each wavelength examined for each component.

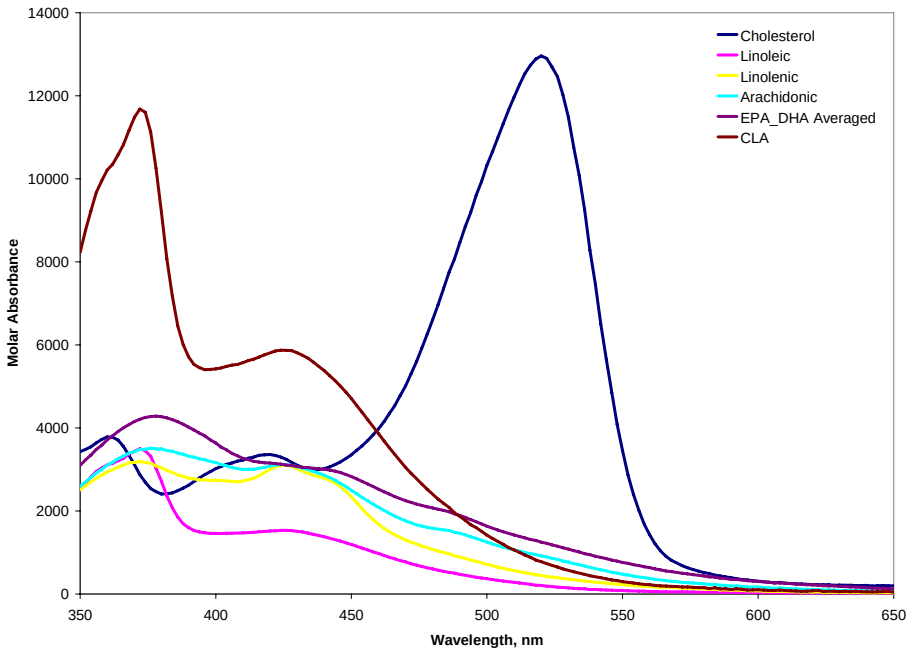


Figure VI.4: Molar absorbance spectra resulting from the calibration of the standard solutions

The resulting concentrations for prepared mixtures did not result in low percent errors. In attempts where all six analytes and 101 wavelength variables formed in the **K** matrix, the resulting concentration matrix could not determine which components were zero. Since the solutions had known components, the K matrix was altered to include only the molar absorptivities of the analytes in the mixture. For example, if the solution contained only cholesterol and LA, then the K matrix was reduced to data for only two analytes. Four representative solutions are displayed in Table VI-3.

Table VI-3: Resulting concentrations from the component dependent K matrix results with percent errors

Mixture 1			
	Experimental	Matrix	Percent Error
	Concentration (μM)	Concentration (μM)	(%)
Cholesterol	64.4	48.3	-2.15
Linoleic	65.4	56.2	-9.59
Linolenic	64.1	70.4	5.28
Mixture 2			
	Experimental	Matrix	Percent Error
	Concentration (μM)	Concentration (μM)	(%)
Cholesterol	80.5	79.6	-1.11
Linoleic	32.7	24.7	-24.7
Linolenic	48.1	42.1	-12.5
Arachidonic	31.6	41.5	31.4
Mixture 12			
	Experimental	Matrix	Percent Error
	Concentration (μM)	Concentration (μM)	(%)
Cholesterol	32.2	36.2	12.3
Linoleic	32.7	30.4	-7.23
Linolenic	32.1	35.4	10.3
Arachidonic	31.6	28.0	-11.4
EPA/DHA	75.1	98.8	31.5
Mixture 14			
	Experimental	Matrix	Percent Error
	Concentration (μM)	Concentration (μM)	(%)
Cholesterol	48.3	48.3	0.08
Linoleic	49.1	56.2	14.6
Linolenic	48.1	70.4	46.3
Conjugated Linoleic	49.6	51.3	3.26

This attempt is obviously not a suitable solution to the problem of finding a method for the quantification of the unknown spectra resulting from the Purdie assay. The first problem is that in an unknown solution, the components are uncertain; therefore, the **K** matrix cannot be defined. The other problem is that while the percent errors are low for the three component mixtures of cholesterol, linoleic, and linolenic, the percent error also increased as the number of analytes increased. It is interesting to note that cholesterol is low in all of the mixtures attempted. Cholesterol has the one unique absorbance peak at 520 nm. All components contribute to the total absorbencies in the range 350 to 480 nm. As the number of the PUFA variables in this region is increased, the error increases. An alternative method had to be found that can analyze overlapping peaks. Figure VI.5 is mixture 1 comparing the results of the least squares solution, the experimental results, and the addition of the known concentrations times each of the molar absorptivities. There is an obvious difference in the experimental spectra and the matrix and addition spectrum. Either there is an error in the calibration, or the matrix method is not best method to solve the problem. Additional attempts at calibrations with water have been completed, but a similar problem with the percent error increasing with the number of analytes was observed.

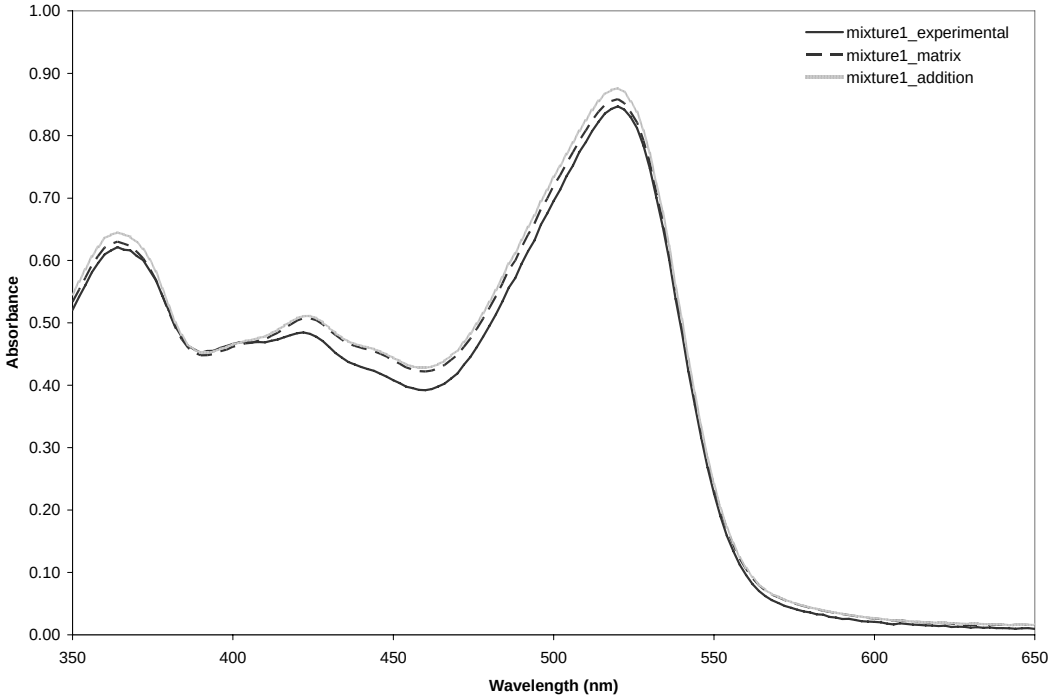


Figure VI.5: Comparison of the least squares solution with the experimental and simple addition spectrum

A second method was attempted that used both the classical least squares method and a non-negative least squares algorithm to solve the over determined system. Using all 101 wavelengths from 350 to 550 nm, the RMSEP for twenty of the mixtures were analyzed using both methods. Table VI-4 compares the two results. The non-negative least square solution approach reduced the RMSEP for four of the PUFA and increased RMSEP for cholesterol and ALA. The non-negative solution was able to predict more of the cases where the components were not present in the mixture compared to the direct calibration. The RMSEP in both cases are too large to lead to any useful prediction model using the directly calibrated K matrix. The ratio of the RMSEP to the mean concentration of each analyte expressed as a percentage reaffirms this statement. Ideally,

the percentage would be under 10%, which is not the case in any of the analytes determined in this calculation.

Table VI-4: Direct calibration results comparing classical least squares to a non-negative least squares solution

	Direct Calibration (RMSEP, μM)	Ratio RMSEP:Mean Concentration, %	Direct Calibration (non-negative) (RMSEP, μM)	Ratio RMSEP:Mean Concentration, %
Cholesterol	2.02	3.90	43.7	84.6
Linoleic Acid	115	226	51.4	101
Linolenic Acid	29.1	59.7	62.1	128
Arachidonic Acid	56.0	130	41.1	95.5
EPA/DHA	29.0	44.2	18.7	28.4
Conjugated	68.8	133	37.7	72.0

The indirect K-matrix and P-matrix calibrations' methods were done on the same set of data as the direct calibration method. The dilution mixtures used in the calibrations of the non-water samples were used to determine both the **K** matrix and **P** matrix in the respective methods. The resulting columns in the **K** matrix from the K-matrix method are the molar absorptive spectra for each of the analytes. DHA and EPA were determined separately by the method. The resulting spectra from using all of the wavelengths are in Figure VI.6.

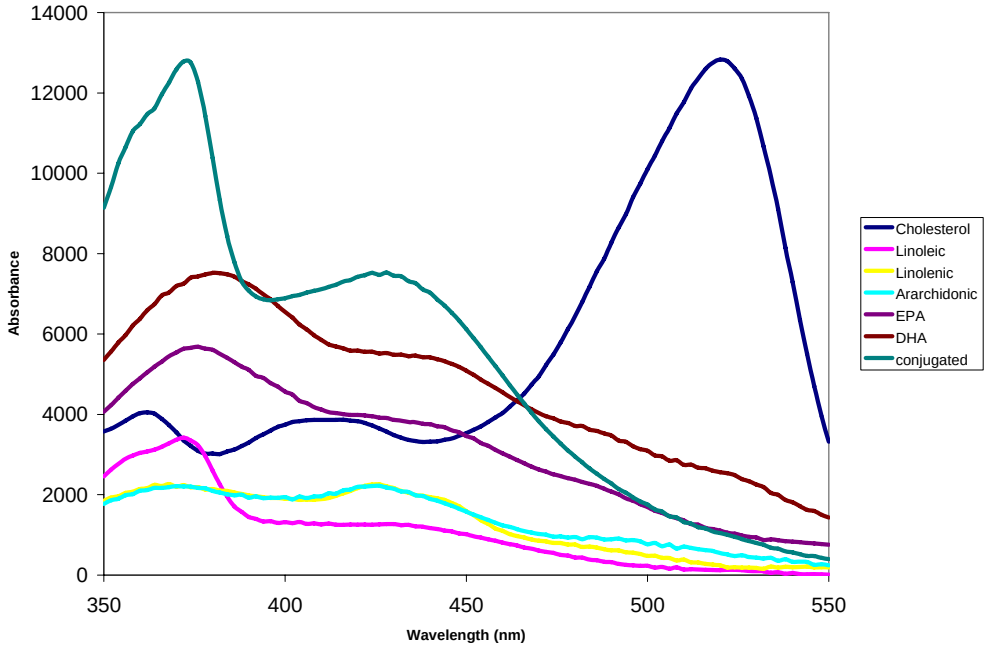


Figure VI.6: Molar absorptivity spectra determined by the K matrix indirect calibration method

The resulting matrix was then used to determine the concentration of the same twenty analytes used in the direct calibration method. The P-matrix indirect calibration was also completed, but one of the drawbacks of the method is that the **P** matrix has no physical meaning. The components are not the molar absorptivities of the analytes. The RMSEP and the relative error compared to the mean of the concentrations for the K-matrix are in Table VI-5. The P-Matrix result had a ratio of RMSEP to mean concentration over a thousand percent. This is the result of poorly designing the concentration matrix. It will be repeated with a designed matrix to obtain the comparison between both methods.

Table VI-5: Indirect calibration results comparing K-matrix method

	K-matrix, RMSEP (μM)	RMSEP:Mean Concentraion, %
Cholesterol	18.7	36.1
Linoleic Acid	36.2	70.9
Linolenic Acid	9.91	20.3
Arachidonic Acid	18.1	42.1
EPA	39.5	88.8
DHA	36.8	71.3
Conjugated	24.0	45.7

Neither method is able to determine the concentrations. The K-matrix approach has provided the best determination of all of the components except for EPA and DHA. The P-matrix approach cannot even approach an answer that is close to the known concentrations of the mixtures. The direct and indirection calibrations methods cannot handle the highly overlapping spectra that are present. The last attempt to improve the error of prediction is to introduce different strategies for wavelength selection and mixture design on the results.

Mixture design for the water samples, as stated previously, was a limited full factorial design to maintain a proper absorbance. Both the direct and indirect methods were completed on the spectra. The **K** matrix for the direct calibration method was composed of the molar spectra for each analyte discussed in Chapter V. The resulting molar spectra from the K-matrix approach using only the mixtures from the full factorial design are shown in Figure VI.7.

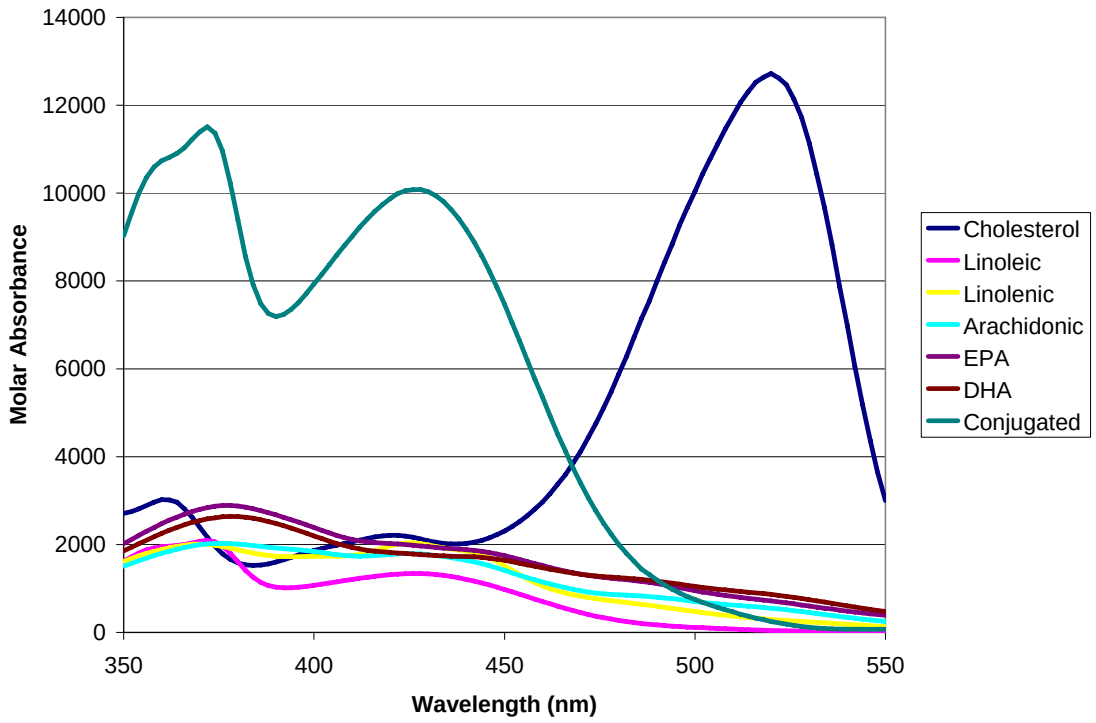


Figure VI.7: Molar absorptivities from the components of the K matrix from the water samples

Overall the spectral patterns compared with the spectra from the calibrations, except for a magnitude shift for all spectra. An average increase in the molar absorption is 11%. The RMSEP for all four methods are in Table VI-6.

Table VI-6: Comparison of direct and indirect calibration methods utilizing a full factorial design for the mixtures

		P Matrix, 101 variables		K Matrix, 101 variables	
	Mean Conc. (μM)	RMEP (μM)	RMSEP: Mean, %	RMEP (μM)	RMSEP: Mean, %
Cholesterol	22.7	4.67	20.6	1.41	6.16
Linoleic Acid	44.3	6.32	14.3	46.1	104
Linolenic Acid	39.1	1.96	5.03	16.9	43.2
Arachidonic Acid	39.0	6.83	17.5	46.5	123
EPA	26.8	15.5	58.0	30.6	114
DHA	38.7	10.6	27.5	21.3	55.1
Conjugated Linoleic Acid	22.9	1.84	8.04	7.59	33.2

		Direct Calibration, Classical Least Squares		Direct Calibration, Non-negative	
	Mean Conc. (μM)	RMEP (μM)	RMSEP: Mean, %	RMEP (μM)	RMSEP: Mean, %
Cholesterol	22.7	4.15	18.2	4.38	19.3
Linoleic Acid	44.3	28.7	64.7	28.7	64.8
Linolenic Acid	39.1	16.0	40.9	9.37	24.0
Arachidonic Acid	39.0	23.1	59.2	11.3	29.0
EPA	26.8	43.3	162	92.5	346
DHA	38.7	25.8	66.8	82.2	213
Conjugated Linoleic Acid	22.9	4.48	19.6	3.49	15.3

The results are a significant improvement over the findings from the previous attempt using spectra that were not produced from a mixture design. Water added to the reagent system is a variation, as well, since it increased the difference in a number of the analyte's spectra. Despite the improvement, the errors are still too significant to use these methods for analysis of serum or other systems. A part of this difference may be the result of the K-matrix approach requiring two matrix inversions. Since the spectra of the analytes are similar, the inversion may result in an almost singular matrix which cannot be used for the final determination of the concentrations.[153] One solution to improve on the K matrix method is to reduce the number of wavelengths used in the calculation.

Wavelength selection can be used to reduce the effects of overlapping spectra. The difficulty in this system is including all of the PUFA. Each of the PUFA absorb in the same region. Table VI-7 displays the six groups of wavelengths chosen to examine the effects. The combination of 75 and 21 were determined from criteria examining the standardization of PLS2 results. The procedure will be discussed in the next section on PLS methods.

Table VI-7: Selected wavelengths chosen to examine the effects of wavelength selection on the determination of the concentrations of analytes

Number of Wavelengths	Selected Wavelengths
101	350 – 550 nm, every 2 nm
75	350, 352, 354,356,358,360,362,364,366,368, 380, 382,384,386,388,390,392,394,398,400, 402,414,416,418,420,424,426,428,430,432 434,436,438,440,442,444,446,448,450,452 454,456,458,460,462,464,466,468,470 472,474,480,482,496,500,502,504,506,508,514 516,518,520,522,524,526,528,530,532,534 536,538,540,542,544
69	350-480, 518, 520, 522
66	350-480
33	360-378, 388-394, 400, 412, 418, 420, 422, 242, 426, 428, 430, 440, 442, 444, 480, 482, 484, 518, 520, 522
21	350, 360,362,366,380,382,390,392,426,440, 444,446,462,464,470,496,528,530,534,536,538

Each of the wavelength groups was attempted using all of the components, cholesterol and the six PUFA. A table comparing the number of variables with the RMSEP and the ratio of REMSEP to the mean concentration is included in Table VI-8.

Table VI-8: Effect of five wavelength selection groups on K Matrix results

		K Matrix, 75		K Matrix, 69		K Matrix, 66	
	Mean Conc (μM)	RMSEP (μM)	RMSEP: Mean, %	RMSEP (μM)	RMSEP: Mean, %	RMSEP (μM)	RMSEP: Mean, %
Chol	22.7	1.40	6.18	1.18	5.20	1.23	5.41
LA	44.3	50.9	246	47.0	106	47.8	108
ALA	39.1	17.0	65.4	6.87	17.6	6.67	17.1
AA	39.0	49.0	278	39.0	99.9	40.3	103
EPA	26.8	30.7	574	34.6	129	24.4	91.0
DHA	38.7	21.7	93.3	33.1	85.6	22.5	58.2
CLA	22.9	8.43	69.0	8.99	39.3	9.12	39.8

		K Matrix, 33		Kmatrix, 21	
	Mean Conc (μM)	RMSEP (μM)	RMSEP: Mean, %	RMSEP (μM)	RMSEP: Mean, %
Chol	22.7	2.93	12.9	2.14	9.43
LA	44.3	9.16	20.7	69.5	157
ALA	39.1	24.5	62.6	2.80	7.17
AA	39.0	76.4	196	63.6	163
EPA	26.8	216	809	85.5	319
DHA	38.7	177	457	61.3	158
CLA	22.9	2.63	11.5	13.2	57.7

Little to no improvement was seen with any of the wavelength groupings. The highest number of variables included in the matrix resulted in similar RMSEP as was seen for the use of all 101 variables. As the number of variables decrease, the RMSEP and the ratio increased significantly for all the analytes, including cholesterol. Cholesterol is the one analyte that should be able to be determined because of the unique 520 nm peak. If cholesterol is not determined within a reasonable error, then the method, in general, will not be a possibility for the quantification of this assay. Using all 101 wavelengths for the K matrix method is the best approach based on these results.

Given the fact that EPA and DHA are so similar and LA and CLA have overlapping spectral patterns with different magnitudes, an attempt was made to examine the K matrix method with only cholesterol, LA, ALA, and AA. Using only the mixtures with these components from the designed mixture matrix, the resulting molar absorptivity spectra (Figure VI.8) place the focus on the similarities in the ALA and AA spectra. In Table VI-9, the RMSEP has been reduced compared to the seven component K matrix results, but the similarity in the ALA and AA spectra result in a high RMSEP for these two compounds. Having a more robust method than indirect or direct calibration that is capable of handling the severely overlapping spectra is required.

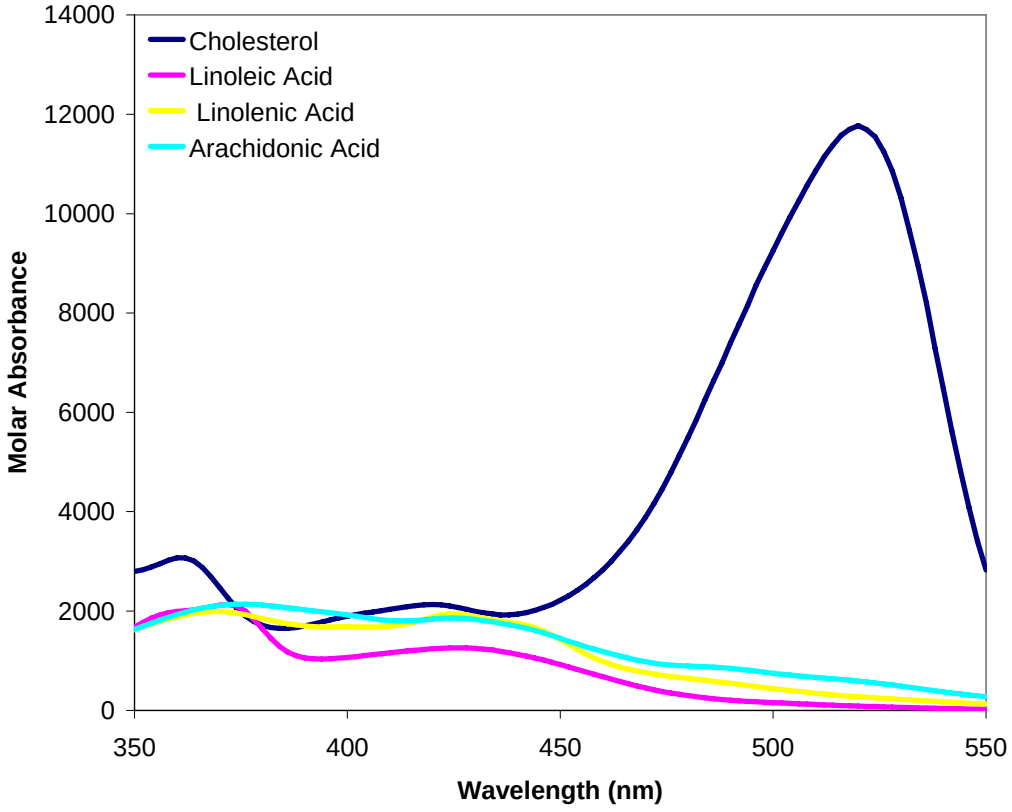


Figure VI.8: Molar absorptivity spectra that are the components of the K matrix from the K matrix approach using four components

Table VI-9: Errors on prediction for K matrix approach using only four components

	Mean Concentration (μM)	RMSEP (μM)	RMSEP:Mean, %
Cholesterol	26.2	5.57	21.2
Linoleic	53.1	24.2	45.5
Linolenic	63.3	47.9	75.7
Arachidonic	65.4	51.2	78.2

B. Partial Least Squares (PLS) Approach

Multivariate calibration methods, PLS1 and PLS2, were applied to the spectral data from both the water and non-water data sets to examine different aspects of the problem. As with the previous methods, the RMSEP was determined for each analyte.

The non-water set was used to examine the differences in PLS1 and PLS2 on the prediction errors. The water samples were used to examine the effect of wavelength selection and the number of components on the calibration of the PLS model.

The RMSEP for PLS1 and PLS2 calibrations for four components are presented in Table VI-10. PLS2 is only slightly better than PLS1 for each analyte. This may be the case because the interference of the compounds in the spectra is taken into account in PLS2 and not in PLS1. PLS1 determines the concentration of one analyte at a time; whereas, in the PLS2 all of the concentrations are determined simultaneously. The higher RMSEP are obtained for LA, ALA, and AA while cholesterol is low. LA, ALA, and AA have absorbance peaks that completely overlap; whereas, cholesterol has regions that do not, which causes the higher RMSEP in the overlapping analytes.

Table VI-10: Comparison of the RMSEP for PLS1 and PLS2

PLS1	Mean Conc. (μM)	RMSEP (μM)	RMSEP:mean conc., %
Cholesterol	62.5	6.15	9.85
LA	88.6	64.0	72.2
ALA	93.5	61.3	65.6
AA	122	34.4	28.2

PLS2	Mean Conc. (μM)	RMSEP (μM)	RMSEP:mean conc., %
Cholesterol	62.5	4.02	6.43
LA	88.6	63.7	71.9
ALA	93.5	61.7	66.0
AA	122	33.0	27.1

Expanding PLS to include all seven components was done using PLS2 on the set of water samples. The error comparisons of the two models are presented in TableVI-11. The combination of EPA and DHA reduced the error for these components and

demonstrated a slight decrease with the other components. Combining DHA and EPA into one component helps to reduce the error and may be considered for use, but the RMSEP will need to be reduced considerably.

Table VI-11: Change in errors seen when DHA and EPA are combined for PLS2 analysis

PLS2 with 7 analytes				
	Percent Error	Maximum Percent Error	RMSEP (μM)	Ratio, %
Cholesterol	9.18	14.6	1.82	7.21
Linoleic	15.4	38.3	7.95	18.0
Linolenic	6.82	18.7	2.71	7.06
Arachidonic	12.0	35.3	6.66	17.2
EPA	59.7	94.3	18.2	64.7
DHA	6.89	55.4	10.6	45.7
Conjugated	6.89	15.3	18.2	9.11
PLS2 with 6 components				
	Percent Error	Maximum Percent Error	RMSEP (μM)	Ratio, %
Cholesterol	9.44	17.4	1.87	7.41
Linoleic	14.6	37.8	8.03	18.2
Linolenic	5.66	18.3	2.35	6.12
Arachidonic	10.4	27.4	6.57	17.0
EPA+DHA	17.9	43.5	12.0	25.9
Conjugated	7.64	18.6	2.10	17.0

A cross-validation method was used to determine the number of PLS1 and PLS2 components to use in the final model. This method performs the calibration on the data set minus one sample and predicts the concentration of the sample that was not included. Each of the samples is left out during the algorithm. Their concentrations are predicted and then compared to the actual concentrations. The RMSEC was determined for various numbers of components to calculate the optimum number to include in the model. An example of the RMSEC for various numbers of components for the PLS2 calibrations with the water samples is located in Figure VI.9. The minimum RMSEC for the PLS2 model pictured is 10PC using all 101 wavelengths. For the PLS1 models, cholesterol

required 2 PCs, LA required 5 PCs, and ALA and AA required 10 PCs. The RMSEC for the PLS2 for the non-water samples required 9 PCs. All plots show a rapid decrease in the RMSEC for CLA and cholesterol. The remaining fatty acids require more components to reduce the RMSEC. The difference in behavior is due to the similarities of the spectra of the PUFA.

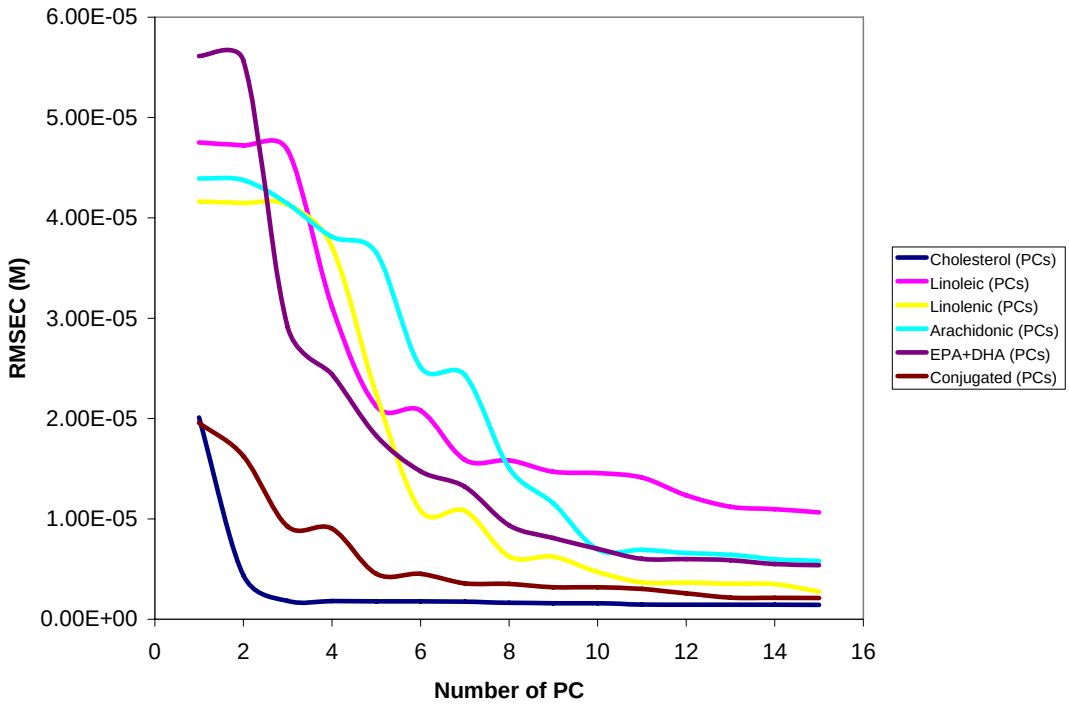


Figure VI.9: RMSEC as a function of the number of PLS2 components for each of the analytes with DHA and EPA combined.

Wavelength selection can be determined by examining the *bw*-coefficients from a PLS model using standardized data. The *bw*-coefficients that are negative are variables that have a high influence from the other analytes. The PLS2 model with the water samples was used with the standardized model due to similar RMSEP seen when

comparing PLS1 with PLS2. The *bw*-coefficients for each of the analytes using 10 PC which had the lowest root mean square error of calibration (RMSEC) are in Figure VI.10.

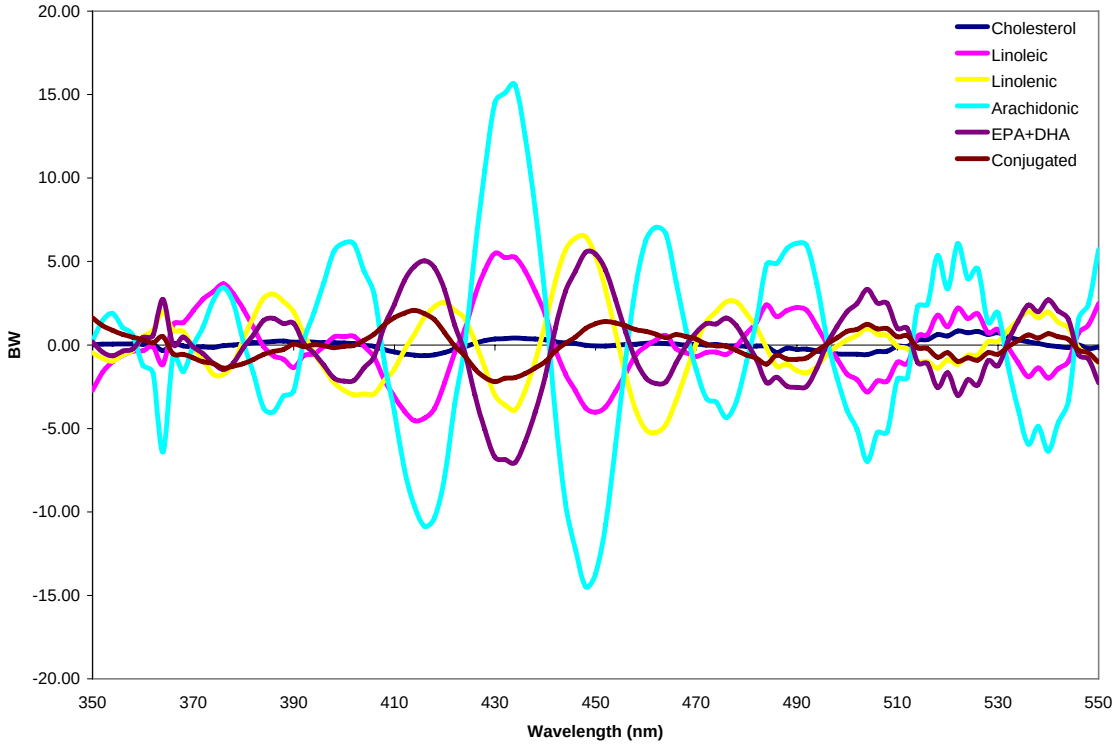


Figure VI.10: *bw* – coefficients from a PLS2 analysis for each of the analytes

Two groups of wavelengths were determined based on the following criteria. A 75 variable collection included wavelengths that had three of the six components with positive *bw*-coefficients. A 21 variable collection consisted of wavelengths that had four of the six components with positive bw coefficients. A 33 variable collection of wavelengths that included the maximum wavelengths for each analyte with some added on either side of the peak maxima in case of shifts in the peaks. The last two wavelength groups studied were the wavelengths in the region of 350-480 nm with and without the 520 nm peak of cholesterol included. Each wavelength grouping was analyzed with

PLS2 using the mean centered data and cross validation method for the determination of the number of PCs used for each model. Each model was then used to predict the concentrations of each analyte. The RMSEP versus the number of variables are reported in Figure VI.11.

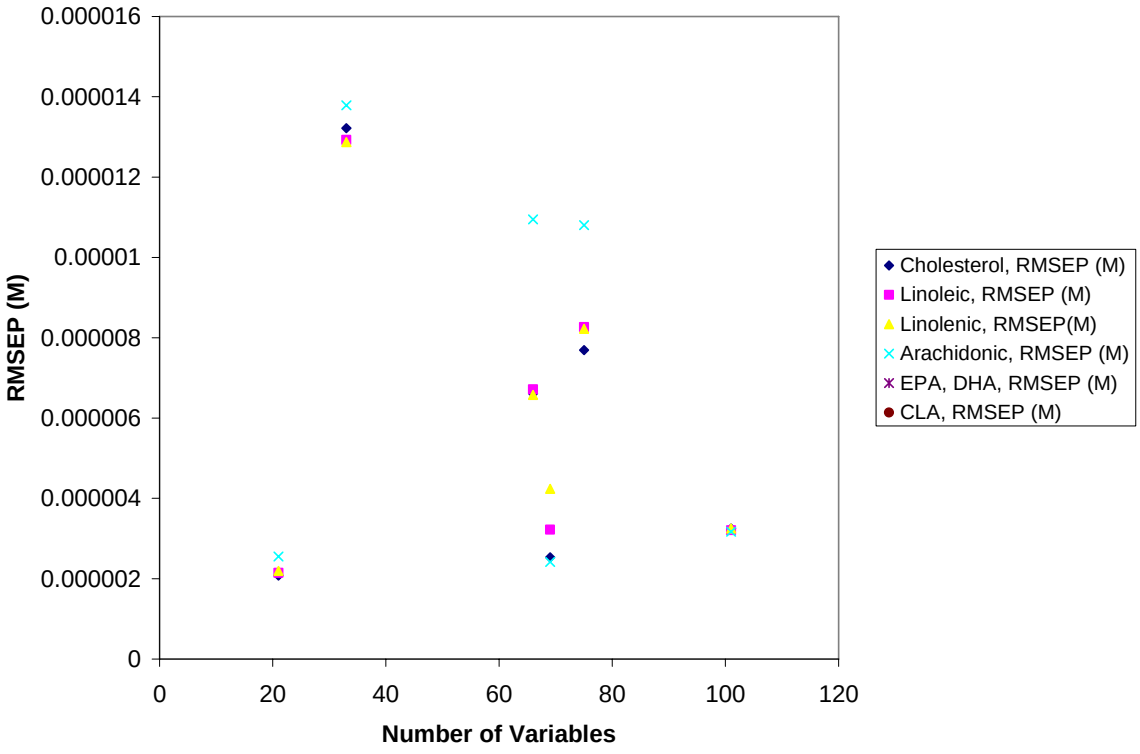


Figure VI.11: RMSEP compared for each analyte at various numbers of wavelength variables

The 21 wavelengths chosen had the lowest RMSEP for all of the analytes. The PLS method is dependent on having the most wavelengths with the least influence from other analytes. The selected regions from 350 to 480 nm and the 33 variables selected based on maximum peaks of the molar spectra produced the highest RMSEP.

Table VI-12: The error analysis of the results on the prediction set from the water analysis and wavelength selection using 21 wavelengths

	Percent Error, %	Maximum Percent Error, %	Mean Concentration, μM	RMSEP, μM	RMSEP:Mean Concentration, %
Cholesterol	7.26	12.0	25.2	3.17	12.6
Linoleic	21.1	46.4	44.2	10.8	24.4
Linolenic	4.12	10.9	38.4	2.42	6.30
Arachidonic	31.1	54.4	38.6	11.0	28.3
EPA+DHA	26.9	63.7	46.5	13.8	29.7
Conjugated	6.30	13.7	22.8	2.55	11.2

Error analysis for the PLS2 model with 21 variables still has a high ratio of RMSEP to the mean concentration for each of the analytes which are shown in Table VI-12. All variables need to be reduced to below 10%.

C. GSAM Approach

The reaction system, unlike other analytical systems, does not have a blank that can be used in the analysis. Serum is a complex system and a serum without any cholesterol and all of the fatty acid removed can not be obtained. One chemometric method that can accommodate for this deficiency and, possibly, improve the error analysis is GSAM. GSAM is based on adding multiple unknown amounts of analytes to the mixture to determine the concentration of the initial mixture. The error associated with this method is based on the matrix of the addition and the resulting matrix of molar absoptivities. Due to the overlapping spectra of the analytes, there is a limited opportunity to lower the error in that matrix. The concentration of the addition can be controlled by mixture design. Extreme vertices and D-optimal design were used to define the mixture matrix.

Extreme vertices design used the matrix of mixtures in Table VI-13 for the additions to the prepared mixtures. The concentrations listed are the concentrations of the prepared mixtures prior to dilution. Mixtures with and without water additions were prepared. Table VI-14 displays a comparison between the two methods. The condition number for the concentration matrix was 7.09, and the condition number of the K matrix was 82.1 for all 101 wavelengths. When GSAM was attempted with wavelengths from 350 to 450 nm, the condition number of the K matrix was 40.3. GSAM theory determines that in cases of interferences, which are the overlapping spectra in this case, the condition number of the K matrix is the upper bound of the uncertainties which are amplified under these conditions.[178] The ideal condition number of the K matrix is 1. Although the K matrix from both of the selected wavelength regions is high, Kalivas has shown that the prediction capability of GSAM may not be altered, which is seen in this set of data.[178]

The samples with water had the lowest RMSEP when compared with the direct and indirect calibration methods. The variation seen in the spectra for the water samples may aid in improving the error. The other difference between the two sets of data is the small improvements on the method that occurred during the experimental time. As the operator gained more experience with the method, the manual components of the reaction were made more uniform. Slight variation in the addition of the perchloric acid to the acetyl chloride and the vigor of the hand shaking will each have an effect on the sample. If an instrument is developed for the assay, these will be eliminated.

Figure VI.12 provides an example of a prepared mixture with each addition. The spectrum that is the lowest is the original sample. Figure VI.13 is a comparison of the

GSAM predicted spectra with the experimental value for one of the samples completed with water. It is clear that the fit of the two spectra is an overlay, except in the high cholesterol peak. One error that cannot be eliminated is related to the GSAM recommendation that the additions be at least double the amount in the initial sample. If completed, the absorbance will be over the limit of 1.0 absorbance for serum. The prepared mixtures were designed to match the concentration seen in a normal serum sample. The cholesterol in the water samples is close to having a ratio of RMSEP to the mean concentration of 10%, which suggests the method and design of addition can have a low occurrence of error. More tests on concentrations that are higher in cholesterol and new designs of the additions to fit future samples will have to be examined.

Table VI-13: GSAM addition mixtures for examining four components. An extreme vertices design was applied for the additions to the prepared mixtures.

Added Mixture	Cholesterol (M)	LA (M)	ALA (M)	AA (M)
1	0.004	0	0	0
2	0	0.02	0	0
3	0	0	0.02	0
4	0	0	0	0.02
5	0.002	0.01	0	0
6	0.002	0	0.01	0
7	0.002	0	0	0.01
8	0	0.01	0.01	0
9	0	0.01	0	0.01
10	0	0	0.01	0.01
11	0.001	0.0075	0.0075	0
12	0.001	0.0075	0	0.0075
13	0.001	0	0.0075	0.0075
14	0	0.075	0.0075	0.0075
15	0.001	0.005	0.005	0.0075
16	0.002	0.01	0.005	0.0075

Table VI-14: GSAM combined results for samples with and without water

Samples without water (n=8)			
	Mean Conc. (μM)	RMSEP (μM)	RMSEP:mean conc., %
Cholesterol	51.8	11.4	22.0
LA	54.9	11.5	21.0
ALA	14.2	24.1	170
AA	13.2	22.9	174
Samples with water (n=10)			
	Mean Conc. (μM)	RMSEP (μM)	RMSEP:mean conc., %
Cholesterol	51.4	6.29	12.3
LA	53.8	6.04	11.2
ALA	14.2	8.27	58.2
AA	17.6	2.65	15.0

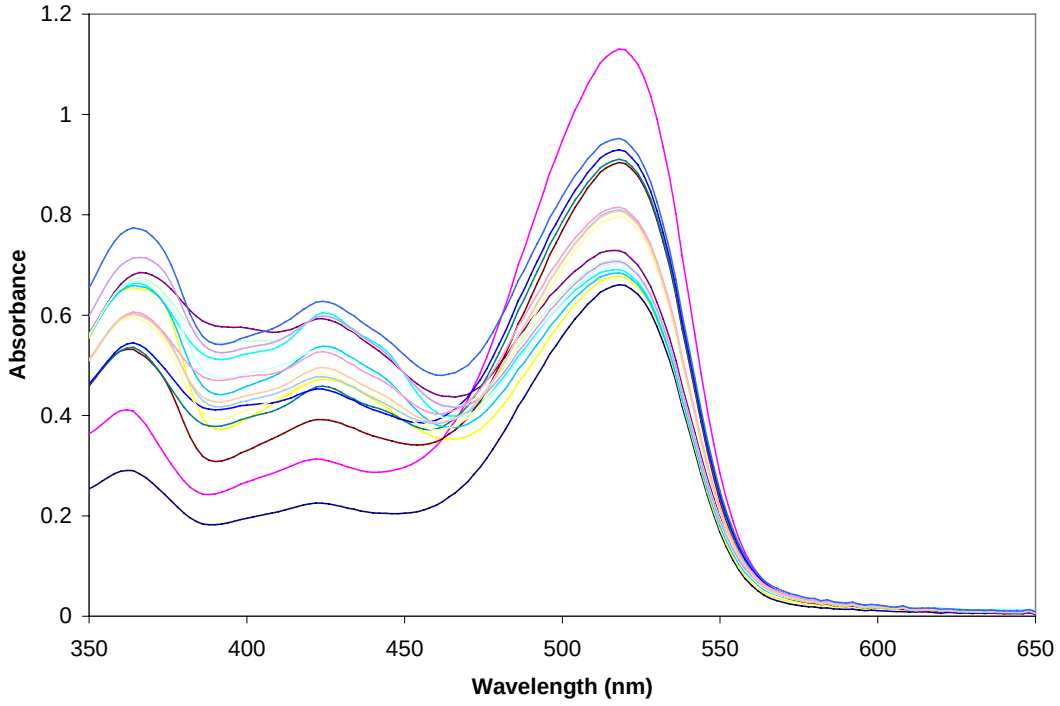


Figure VI.12: Prepared mixtures with the additions added for the GSAM approach for one example

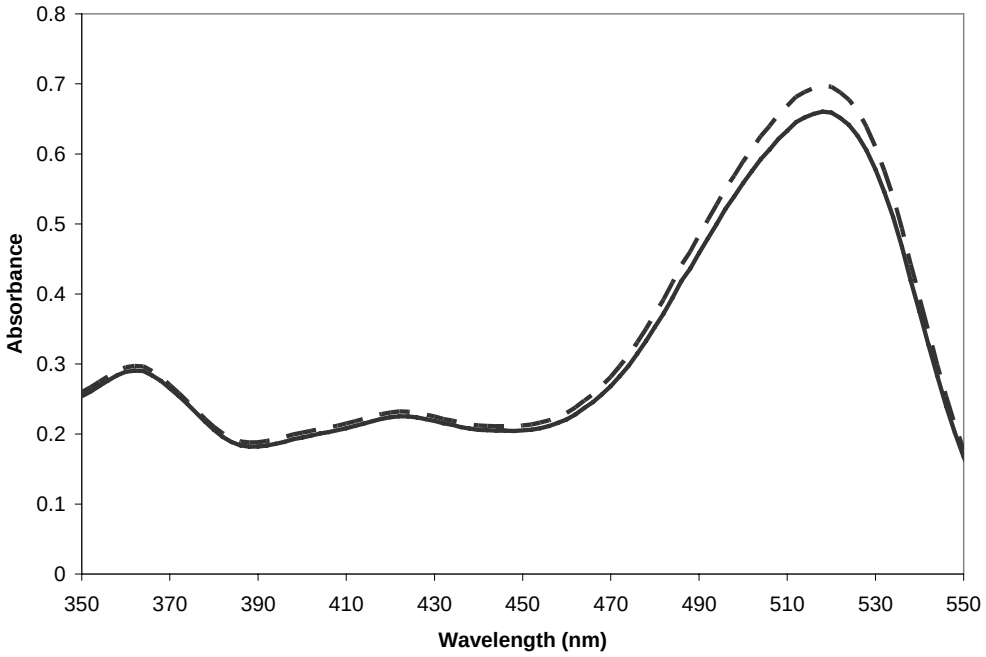


Figure VI.13: GSAM predicted spectra with the experimental spectra. The dashed line is the GSAM predicted spectra. The solid line is the experimental spectra for the prepared mixture.

An additional mixture design matrix was completed using D-optimal design. The ratios of the concentrations are in Table VI-15. The condition number of the concentrations prepared is 3.38, which is lower than the extreme vertices trial. The lower condition number results in a lower error according to the theory of GSAM. The results for the matrix, Table VI-16, are an improvement over the RMSEP seen in the extreme vertices trial. Mixture design is important in GSAM. It can reduce the errors in the prediction of the concentrations, as demonstrated, and aid in reducing the number of mixtures that must be added to fully describe the analytes.

Table VI-15: D-optimal design for the additions used in a GSAM attempt. The condition number is 3.3834 for the concentration matrix based on the prepared samples.

	Cholesterol (M)	LA (M)	ALA (M)	AA (M)
Addition 1	0.25	0.25	0.25	0
Addition 2	0.25	0	0	0.25
Addition 3	0.25	0.25	0	0
Addition 4	0.25	0	0	0
Addition 5	0	0	0	0.25
Addition 6	0	0	0.25	0
Addition 7	0.125	0.125	0.125	0.125
Addition 8	0	0.25	0	0
Addition 9	0	0.25	0	0.25
Addition 10	0.25	0	0.25	0
Addition 11	0	0	0	0
Addition 12	0	0.25	0.25	0
Addition 13	0.125	0.125	0.125	0.125
Addition 14	0.125	0.125	0.125	0.125

Table VI-16: GSAM results using D-Optimal design on three prepared mixtures

	Mean Conc., (μM)	RMSEP, (μM)	RMSEP: Mean Conc, %
Cholesterol	36.6	1.67	4.57
LA	85.9	8.51	9.91
ALA	96.5	22.8	23.6
AA	63.5	22.0	34.6

The GSAM approach for four components was attempted with serum samples. The concentration results for the cholesterol and the three PUFA are listed in Table VI-17 with examples of the predicted and experimental spectra in Figure VI.14. The cholesterol for the serum was determined with the standard enzymatic method. Unfortunately, the PUFA could not be determined for comparison. The RMSEP for the cholesterol is 10.7 mg/dL. The ratio of the RMSEP to the mean concentration of cholesterol is 5.83%. Determining the values with all four components while keeping the cholesterol error low is a starting point for the analysis of serum. GSAM requires that all analytes and

interference must be fully described in the addition used. Since serum contains a number of other analytes, such as EPA, DHA and CLA, that were not included, the values of the PUFA may be inaccurate. Due to the time consuming process of analyzing serum using GSAM and the number of additions needed to describe all seven analytes, GSAM is not appropriate for a clinical laboratory that needs to examine hundreds of samples in a week. If the process can be automated, it may be considered.

Table VI-17: GSAM four component analysis of serum samples. RMSEP for the cholesterol is 10.6451 mg/dL. The ratio of the RMSEP to the mean concentration of cholesterol is 5.83%.

	Cholesterol, GSAM pred. (mg/dL)	Cholesterol, Enzymatic (mg/dL)	Percent Error %
Sample 1	204	187	-8.88
Sample 2	207	188	-10.3
Sample 3	188	189	0.54
Sample 4	193	189	-2.28
Sample 5	174	163	-6.78
Sample 6	175	173	-1.13
Sample 7	188	189	0.33

	LA (μg/mL)	ALA (μg/mL)	AA (μg/mL)
Sample 1	291	1250	53.3
Sample 2	619	794	81.4
Sample 3	943	370	621
Sample 4	458	867	199
Sample 5	504	697	84.2
Sample 6	907	178	581
Sample 7	403	745	81.0

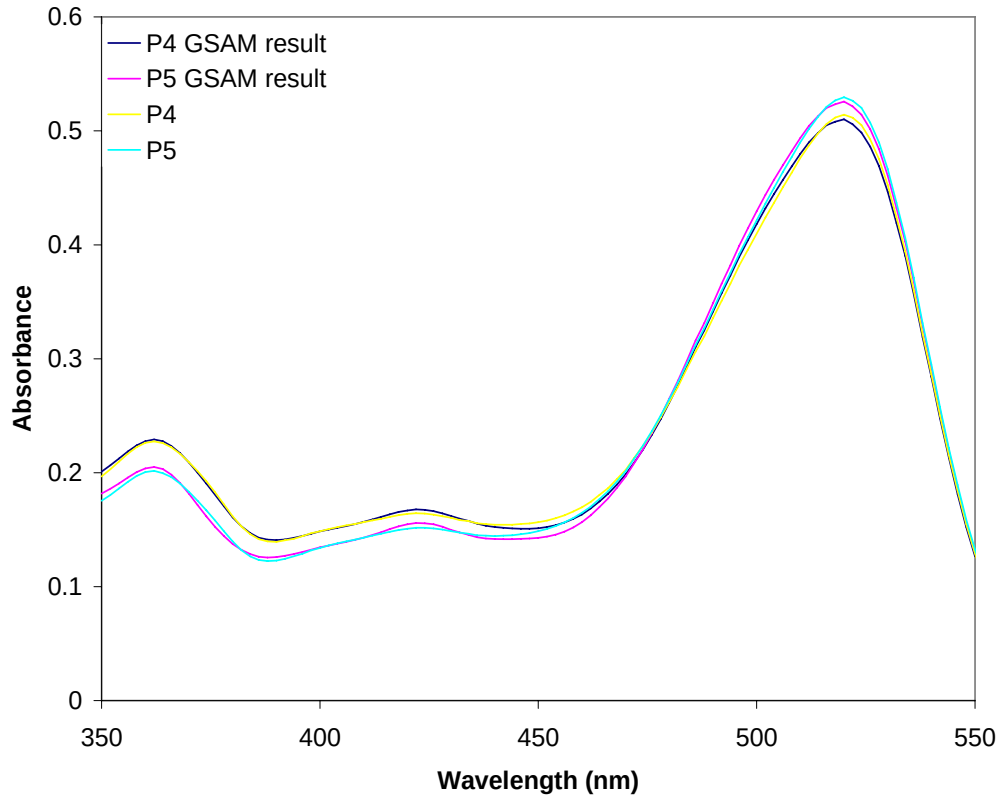


Figure VI.14: Comparison of the GSAM predicted spectra with the experimental spectra for two of the seven samples examined.

D. Neural Networks

The data sets without water have been analyzed using ANN with three and four components. The expansion to the additional analytes is in progress. A collection of 1410 spectra was used for the training. The data set was randomly divided into a training set (80%), a validation set (10%), and a test set (10%). The architecture of the network changes with each analyte and must be determined by examining the RMSEP resulting from each network attempted. The transfer function for the first hidden layer was chosen to be a sigmoid function with the output transfer function being linear. These two functions have been used previously in determining the concentrations in other spectrometric studies.[188, 189, 193] The number of neurons in the output layer equals the

number of concentrations that is being determined by the network. The number of neurons in the first hidden layer is the variable that must be determined. A network is trained using a number of neurons, and then the RMSEP is determined for that network for all of the components. A plot of the RMSEP compared to the number of neurons in the hidden layer for the three component analysis is in Figure VI.15. The number of neurons for each analyte was the lowest for 32 neurons in the layer with the wavelengths of 350 – 480 nm resulting in 66 inputs for the network. A plot of the ratio of the RMSEP to the mean concentration of the predicted set is in Figure VI.16. The reduction in the RMSEP corresponds with a reduction in this ratio and in the average percent error for the prediction set. Any fewer than 15 nodes results in an increase in the RMSEP, and increasing the number of neurons over 40 results in an increase in the RMSEP suggesting overfitting. The balance between not defining and overfitting the system is needed and is accomplished with the use of 32 neurons in the layer. The overall network architecture was 66-32-3. The error analysis is listed in Table VI-18 for this network. The required number of epochs ranged from 76 to 223 for the training of each of the networks. Limiting the number of inputs to 25 selected wavelengths based on absorbance did not demonstrate a significant reduction in the RMSEP, which is shown in Table VI-19.

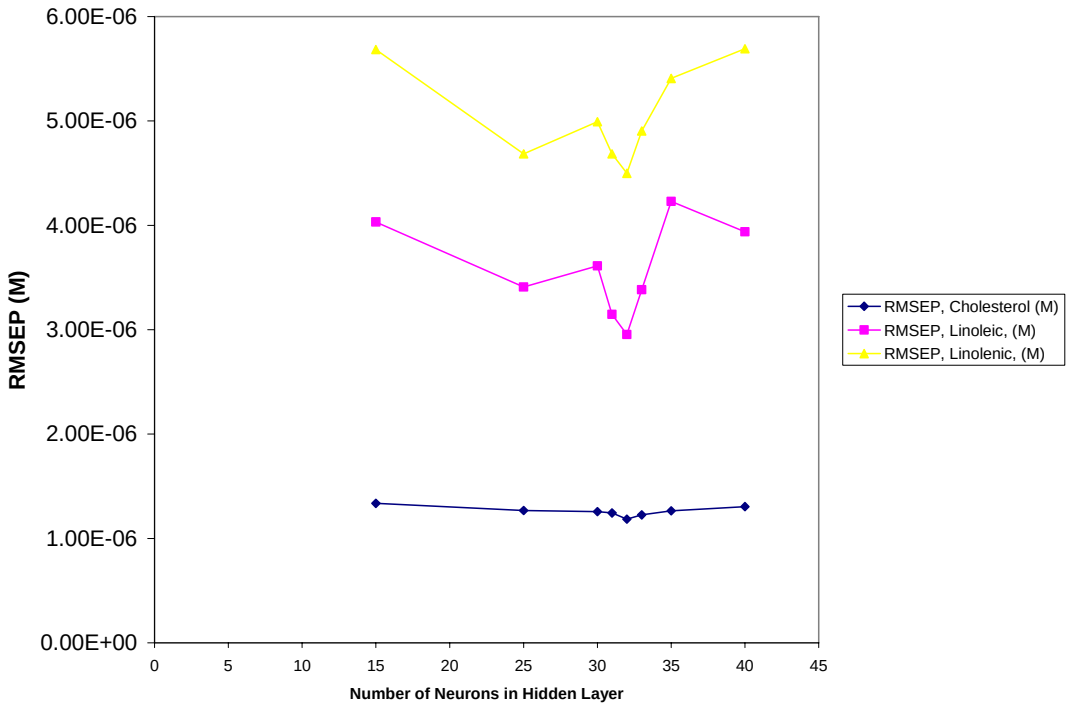


Figure VI.15: RMSEP versus the number of nodes in the hidden layer for the development of the three analyte network

Table VI-18: RMSEP, mean concentration, and the ratio expressed as a percentage for the three component network.

	RMSEP, (μM)	Mean Concentration, (μM)	Ratio RMSEP:Mean Conc., %
Cholesterol	1.19	49.2	8.44
LA	2.95	105	8.03
ALA	4.50	110	7.36

Table VI-19: Prediction error analysis for networks using 25 selected wavelengths

Number of Neurons	RMSEP, Cholesterol (μM)	Ratio, Cholesterol	RMSEP, Linoleic, (μM)	Ratio, Linoleic	RMSEP, Linolenic, (μM)	Ratio, Linolenic
20	2.63	4.06	2.99	3.24	2.86	6.49
32	3.14	4.85	3.04	3.29	4.44	10.1
50	2.17	3.35	4.92	5.34	2.86	6.47
100	6.45	9.95	6.10	6.61	3.66	8.28

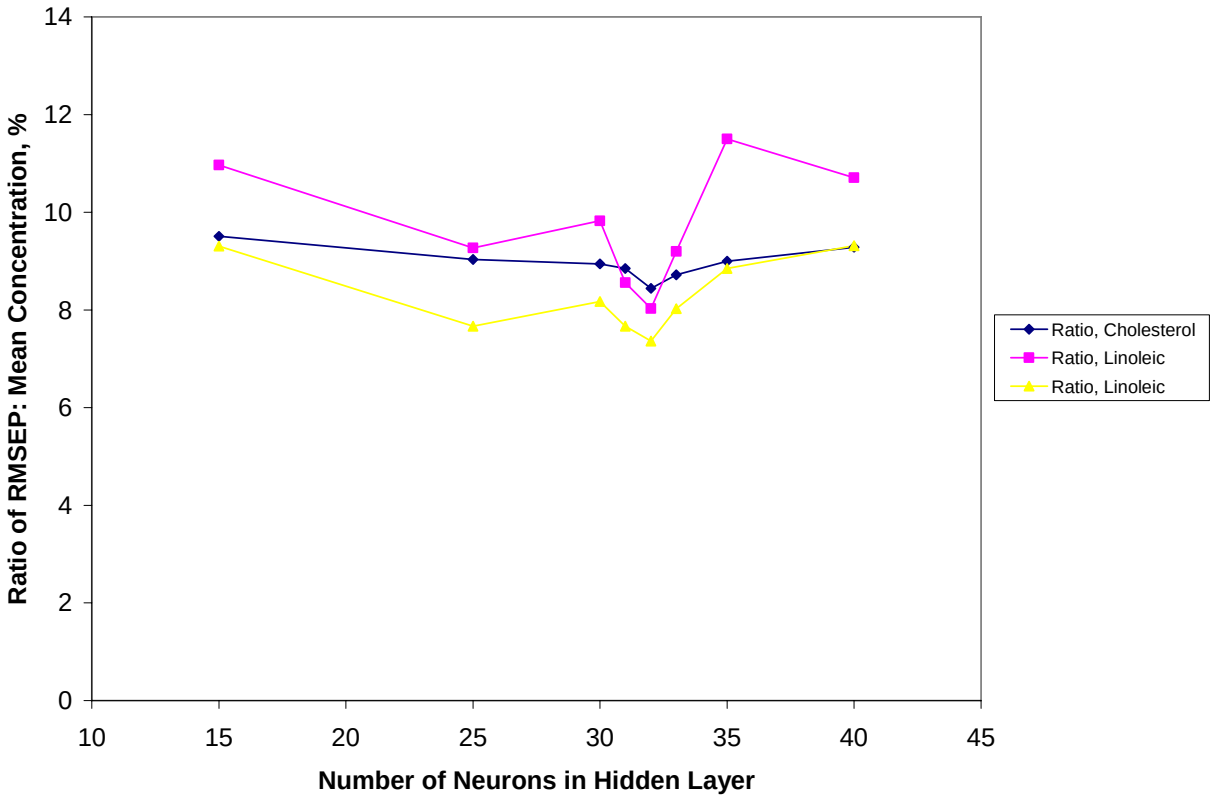


Figure VI.16: Ratio of the RMSEP to the mean concentration versus the number of neurons in the hidden layer

A number of methods and attempts to improve each with wavelength selection, mixture design, and alterations to the reagent procedure have been presented. To summarize the results, a final comparison between GSAM, PLS, and ANN with four component samples completed with no water are compared. The ANN architecture for the four component network was 66-40-4 as determined by examining the RMSEP. The mixtures determined for each method were included in the predictions of all three methods. The combined results are in Table VI-20.

Table VI-20: Summary table for four component analysis with GSAM, PLS2, and ANN

GSAM			
	Mean Conc. (μM)	RMSEP (μM)	RMSEP:mean conc., %
Cholesterol	62.2	4.82	7.75
LA	69.8	5.63	8.06
ALA	31.3	6.45	20.6
AA	28.0	2.34	8.37
PLS2			
	Mean Conc. (μM)	RMSEP (μM)	RMSEP:mean conc., %
Cholesterol	62.2	5.17	8.31
LA	69.8	6.89	9.86
ALA	31.3	1.65	5.29
AA	28.0	5.99	21.4
ANN			
	Mean Conc. (μM)	RMSEP (μM)	RMSEP:mean conc., %
Cholesterol	62.2	3.34	5.37
LA	69.8	3.79	5.43
ALA	31.3	2.57	8.23
AA	28.0	3.45	12.4

Based on the water samples, the ANN approach has the lowest RMSEP for all of the samples. Mixture design and wavelength selections each contributed to the reduction of the errors seen with GSAM and PLS2 methods. ANN has a lower RMSEP. The high RMSEP for ALA and AA in GSAM and PLS2, respectively, may be due to the similarities in the spectra for the two analytes. The error is reduced or at least distributed among both analytes in the case of ANN. The ANN networks expanding to all seven components and with water added to the reaction are in the process of being trained. Once completed, the network will be used to analyze serum since the added water will simulate the same conditions of the serum reactions.

E. Pattern Recognition

Corresponding spectral patterns of the samples can be used for pattern recognition analysis. The water sample selections that were created using full factorial design and the prediction set were analyzed using hierarchical cluster analysis with Ward sampling on the principal components. The resulting plot of PC 2 versus PC 1 and the resulting clusters with 0.95 confidence density ellipses are present in Figure VI.17.

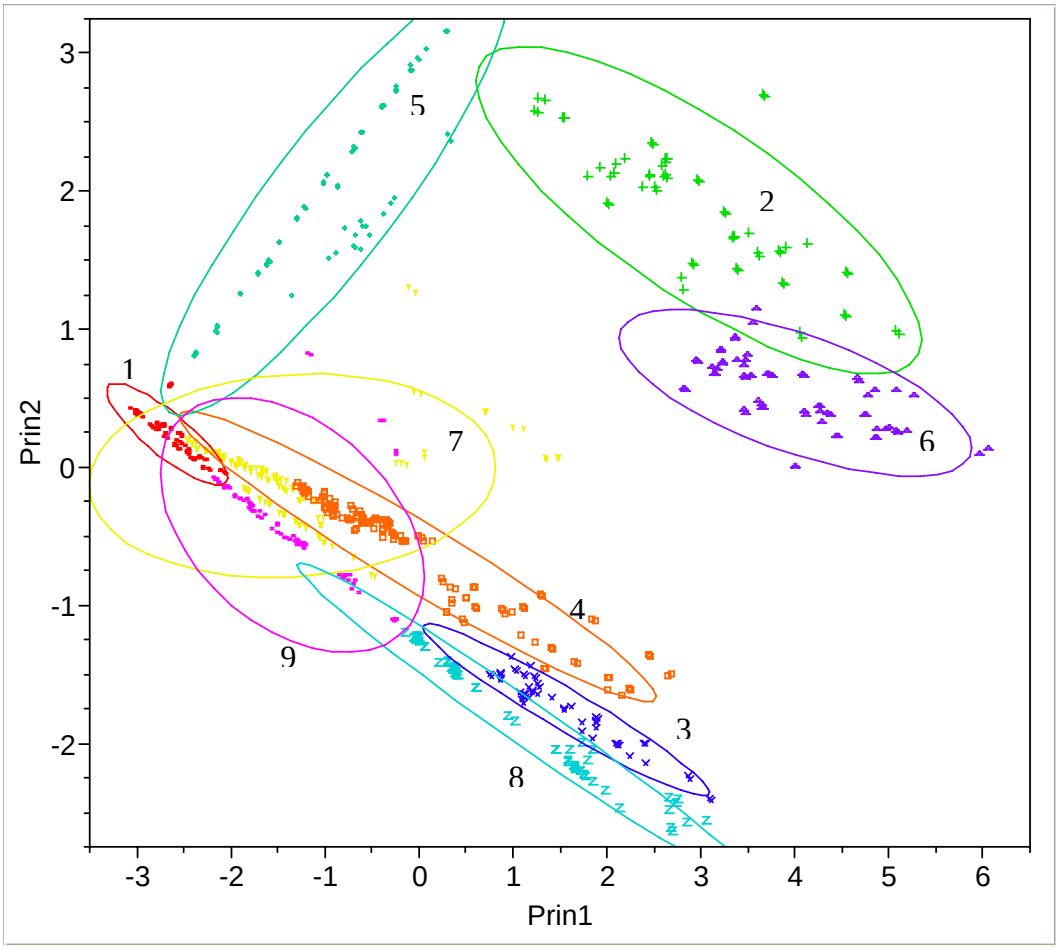


Figure VI.17: Principal component 1 v. principal component 2 for the prepared mixtures that were completed with water. Nine clusters were determined.

The clusters divide the samples into the respective analytes and mixtures. Cluster 1 is the blank with no analytes. Some of the very low concentrations of linoleic acid are included. Clusters 2, 3, and 6 are mixtures of all the components. Cluster 4 and 7 are composed of AA, EPA, and DHA. The lack of separation of these components is the result of the similarities of the spectra for each of these components, especially at lower concentrations. Cluster 7 also contains the ALA samples. They are not divided into any other cluster. Cluster 5 is composed of all the cholesterol samples. The final two clusters, 8 and 9, are the CLA with the division being the changes in concentrations. The separation suggests that the pattern can be used to examine the patterns generated from different mixtures.

A set of data collected by the UCT on samples from patients was analyzed using similar methods. After the cluster analysis, the collaborators in Cape Town determined the clusters are the result of the dyslipidemic state of the patients.

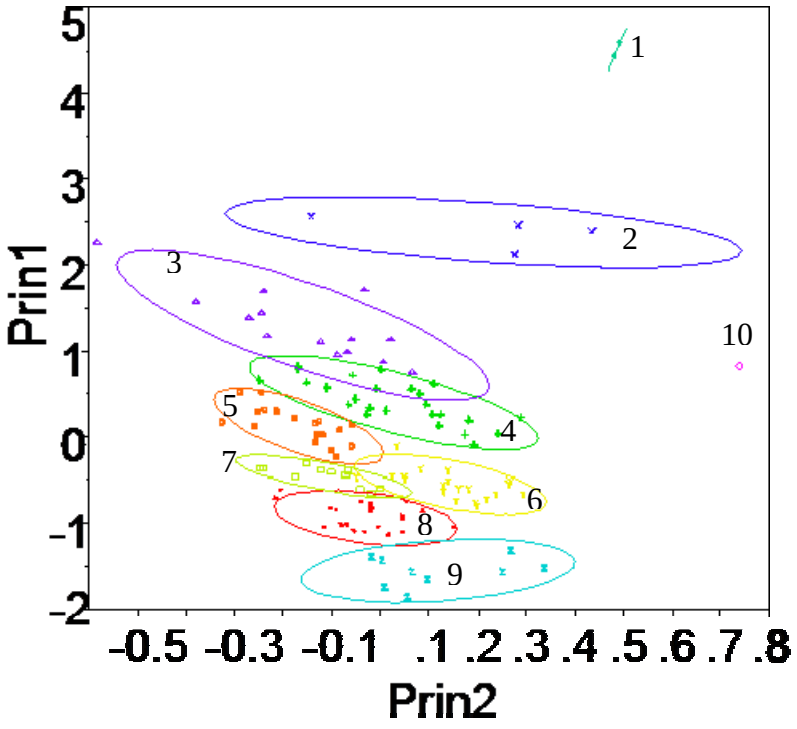


Figure VI.18: PC1 v. PC2 of the untreated serum samples.

The general trend that is seen in Figure VI.18, to this point, is that from top to bottom one goes from the Type V (top) through to the Type IIs in clusters 3,4, and 5 and end with control patients being distributed in 8 and 9. Cluster 1 contains the Type V patient pattern. Cluster 2 is Type III and Type IV (2 of each). Separation will improve with an increase in the sample size of these types and with the inclusion of the fatty acid profiles. Cluster 3 is a Type IIa pattern with a majority considered to be FH (8/14) and FCH (4/14). Cluster 4 is a combination of Type IIa, IIb with controls with cholesterol values over 220 mg/dL. Cluster 5 is a combination of IIa, IIb and some patients on various stages of statin treatments. Cluster 6 is a combination of controls with cholesterol over 200 mg/dL. Cluster 7 is a cluster of samples with cholesterol between 193 and 240 mg/dL, statin treated patients, controls, diet induced hypercholesterolemia,

and people under altered diet treatment for hypercholesterolemia. Cluster 8 and 9 are clusters of controls and some statin treatment patients. The one sample in cluster 10 is a sample of a type V patient in cluster 1 that was run with half the required volume of serum due to the extremely high absorbance of the entire spectra. Although the collaborators were able to complete an initial analysis of the clusters, all of the following groups are subject to change as the fatty acid profile is increased and as more information about the medical history is gathered. Further collection of data and patients' histories including diet, treatments, and other health conditions will have to be collected to determine if this pattern recognition can be used as a screening tool for dyslipidemias in a clinical setting.

CHAPTER VII

CONCLUSION

The goal of this research project was to develop a simple, direct alternative method for the determination of the polyunsaturated fatty acids in addition to cholesterol in serum and, possibly, other samples that contain these analytes. Initial attempts by the Purdie laboratory to determine the concentrations were improved through the use of chemometric techniques and additional knowledge about the reaction conditions. This study was able to determine the concentration of four of the seven components believed to be reacting with the assay in serum. Additionally, the spectral patterns from a collection of serum spectra from a collaborating laboratory demonstrated a possible diagnostic test for dyslipidemias which offers support for an expanded study.

PUFA are a focus in both nutritional sciences and medical studies in the evaluation of risk factors for cardiovascular diseases, dyslipidemias, metabolic syndrome, and other diseases. The studies have disclosed that unsaturated fatty acids can offer health benefits which include reducing cardiovascular death rates, lowering current cardio risk factors such as LDL cholesterol, and promoting positive effects for patients suffering from cancer, arthritis, and mental disorders . With potential connections to these diseases, analytic methods have been developed to determine the concentrations of the PUFA in serum. The current common method of detection is GC with FID or MS

detection, which includes a step of extraction and transformation of the PUFA to methyl esters to complete the detection. To improve on the analytic method of detection for PUFA for quick and easy use in medical and nutritional research, a colorimetric reaction developed in the Purdie laboratory was utilized to develop a quantitative spectrophotometric assay.

The addition of water to the reaction to simulate a possible effect with serum analyzed with the Purdie Assay had a significant effect on the magnitude of the molar absorptions. All seven major analytes had a reduction in the molar absorptivity values and experienced changes to their spectral pattern. Through examination of the kinetics of two of the analytes, LA and CLA, it was determined that water slowed the rate of the reaction which resulted in a lower absorbance for the 15 minute spectra. Through the analysis of analytes with the same number of double bonds as part of the chain, the spectral patterns are nearly identical in pattern and absorbance. This results in the conclusion that the Purdie reagent produces a unique spectral pattern for each PUFA with the same number of double bonds in the chain. The exception to this statement is CLA, the conjugated isomer of linoleic acid. CLA has a molar absorbance 5.2 times that of LA with a difference in the ratio of its major absorbance peaks. As the number of double bonds in the carbon chain increase to five and six, the spectral pattern becomes identical with an increase in its magnitude as seen with EPA and DHA.

A number of multivariate analysis methods were examined for the determination of the concentrations of individual components of prepared mixtures and, ultimately, serum based on the spectra resulting from the Purdie assay. Direct and indirect calibration methods had low percentage errors for binary, tertiary, and quaternary

mixtures containing cholesterol, LA, ALA, and AA. As CLA and the similar components of EPA and DHA were added, the percentage error rose. Despite attempts to improve the analysis through wavelength selection, mixture design, and the addition of water to the assay, the methods were not able to handle the separation of all seven components. PLS, GSAM, and ANN were used to analyze the same set of prepared mixtures of cholesterol, LA, ALA, and AA. The ANN outperformed the other methods with a ratio of RMSEP to the mean concentrations of 5.37 to 12.4% for the four components without the presence of water. GSAM was utilized to examine serum and was able to determine cholesterol with a 1-10 % error compared to the enzymatic test results completed at an independent laboratory. A new training set with the presence of water in the reaction is currently being established, and the resulting spectra will be used to analyze ANN determination of serum samples. Since water has such a major effect on the spectra, in order for ANN to possibly determine serum, the entire training set of spectra must match conditions.

The final multivariate analysis technique to examine the qualitative patterns of the spectra was completed using PCA with cluster analysis. A cluster diagram created from PCA data derived from untreated patients' serum spectra had nine clusters which suggest separation based on dyslipidemias. Prepared mixtures also yielded separation based on the components present and the concentration ranges of each of the components. Both cluster analyses suggest that the spectrum itself can offer a benefit as a possible screening tool for dylipidemias; and, if combined with the quantitative data for PUFA and other cardiac risk factors, may be functional in risk prediction models. Further studies with an

in depth analysis of medical histories must be completed before any final conclusion about the clustering can be established.

The Purdie assay can quantitatively determine the concentration of cholesterol, LA, ALA, and AA in prepared mixtures through the use of multivariate analysis methods with ANN yielding the lowest percentage errors and coefficient of variation. The addition of the three remaining PUFA and expanding the ANN to include the effects of water for all components is a continuing project. Once completed, the assay will be a useful clinical assay for a wide variety of research projects including further studies on the clustering and possible pattern recognition methods for the screening of dyslipidemias.

REFERENCES

1. Simpoulos, A. P., Importance of the Ratio of Omega-6/Omega-3 Essential Fatty Acids: Evolutionary Aspects. In *Omega6/Omega-3 Essential Fatty Acid Ratio: The Scientific Evidence*, Simopulos, A. P.; Cleland, L. G., Eds. Karger: Basel, 2003; Vol. 92, pp 1-22.
2. Grundy, S. M.; Cleeman, J. I.; Daniels, S. R.; Donato, K. A.; Eckel, R. H.; Franklin, B. A.; Gordon, D. J.; Krauss, R. M.; Savage, P. J.; Smith, S. C., Jr.; Spertus, J. A.; Costa, F., Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation* **2005,** 112, (17), 2735-52.
3. Eckel, R. H.; Grundy, S. M.; Zimmet, P. Z., The metabolic syndrome. *Lancet* **2005,** 365, (9468), 1415.
4. Kahn, R.; Buse, J.; Ferrannini, E.; Stern, M., The metabolic syndrome: time for a critical appraisal: joint statement from the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetes Care* **2005,** 28, (9), 2289-304.
5. Lombardo, Y. B.; Chicco, A. G., Effects of dietary polyunsaturated n-3 fatty acids on dyslipidemia and insulin resistance in rodents and humans. A review. *J Nutr Biochem* **2006,** 17, (1), 1-13.
6. Riccardi, G.; Giacco, R.; Rivellese, A. A., Dietary fat, insulin sensitivity and the metabolic syndrome. *Clin Nutr* **2004,** 23, (4), 447-56.
7. Rustan, A. C.; Nenseter, M. S.; Drevon, C. A., Omega-3 and omega-6 fatty acids in the insulin resistance syndrome. Lipid and lipoprotein metabolism and atherosclerosis. *Ann N Y Acad Sci* **1997,** 827, 310-26.
8. Vessby, B.; Gustafsson, I. B.; Tengblad, S.; Boberg, M.; Andersson, A., Desaturation and elongation of Fatty acids and insulin action. *Ann N Y Acad Sci* **2002,** 967, 183-95.
9. Stene, L. C.; Joner, G., Use of cod liver oil during the first year of life is associated with lower risk of childhood-onset type 1 diabetes: a large, population-based, case-control study. *Am J Clin Nutr* **2003,** 78, (6), 1128-34.
10. Miettinen, T. A.; Naukkarinen, V.; Huttunen, J. K.; Mattila, S.; Kumlin, T., Fatty-acid composition of serum lipids predicts myocardial infarction. *Br Med J (Clin Res Ed)* **1982,** 285, (6347), 993-6.
11. Wang, L.; Folsom, A. R.; Eckfeldt, J. H., Plasma fatty acid composition and incidence of coronary heart disease in middle aged adults: the Atherosclerosis Risk in Communities (ARIC) Study. *Nutr Metab Cardiovasc Dis* **2003,** 13, (5), 256-66.
12. Wijendran, V.; Hayes, K. C., Dietary n-6 and n-3 fatty acid balance and cardiovascular health. *Annu Rev Nutr* **2004,** 24, 597-615.
13. Burr, M. L.; Dunstan, F. D.; George, C. H., Is fish oil good or bad for heart

disease? Two trials with apparently conflicting results. *J Membr Biol* **2005,** 206, (2), 155-63.
14. Laaksonen, D. E.; Nyyssonen, K.; Niskanen, L.; Rissanen, T. H.; Salonen, J. T., Prediction of cardiovascular mortality in middle-aged men by dietary and serum linoleic and polyunsaturated fatty acids. *Arch Intern Med* **2005,** 165, (2), 193-9.
15. Hooper, L.; Thompson, R. L.; Harrison, R. A.; Summerbell, C. D.; Ness, A. R.; Moore, H. J.; Worthington, H. V.; Durrington, P. N.; Higgins, J. P.; Capps, N. E.; Riemersma, R. A.; Ebrahim, S. B.; Davey Smith, G., Risks and benefits of omega 3 fats for mortality, cardiovascular disease, and cancer: systematic review. *Bmj* **2006,** 332, (7544), 752-60.
16. Harris, J. I.; Hibbeln, J. R.; Mackey, R. H.; Muldoon, M. F., Statin treatment alters serum n-3 and n-6 fatty acids in hypercholesterolemic patients. *Prostaglandins Leukot Essent Fatty Acids* **2004,** 71, (4), 263-9.
17. Rise, P.; Ghezzi, S.; Priori, I.; Galli, C., Differential modulation by simvastatin of the metabolic pathways in the n-9, n-6 and n-3 fatty acid series, in human monocytic and hepatocytic cell lines. *Biochem Pharmacol* **2005,** 69, (7), 1095-100.
18. Rifai, N.; Bachorik, P. S.; Albers, J. J., Lipids, Lipoproteins, and Apolipoproteins. In *Tietz Textbook of Clinical Chemistry*, 3rd ed.; Burtis, C. A.; Ashwood, E. R., Eds. W.B. Sauders Company: Philadelphia, 1999; pp 809-861.
19. Sperry, W. M.; Brand, F. C., The determination of total lipides in blood serum. *J Biol Chem* **1955,** 213, (1), 69-76.
20. Lepage, G.; Roy, C. C., Specific methylation of plasma nonesterified fatty acids in a one-step reaction. *J Lipid Res* **1988,** 29, (2), 227-35.
21. Lagerstedt, S. A.; Hinrichs, D. R.; Batt, S. M.; Magera, M. J.; Rinaldo, P.; McConnell, J. P., Quantitative determination of plasma c8-c26 total fatty acids for the biochemical diagnosis of nutritional and metabolic disorders. *Mol Genet Metab* **2001,** 73, (1), 38-45.
22. Husek, P.; Simek, P.; Tvrzicka, E., Simple and rapid procedure for the determination of individual free fatty acids in serum. *Analytica Chimica Acta* **2002,** 465, 433-439.
23. Folch, J.; Lees, M.; Sloane Stanley, G. H., A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **1957,** 226, (1), 497-509.
24. Ferraz, T. P.; Fiuza, M. C.; Dos Santos, M. L.; Pontes De Carvalho, L.; Soares, N. M., Comparison of six methods for the extraction of lipids from serum in terms of effectiveness and protein preservation. *J Biochem Biophys Methods* **2004,** 58, (3), 187-93.
25. Eder, K.; Reichlmayr-Lais, A. M.; Kirchgessner, M., Studies on the methanolysis of small amounts of purified phospholipids for gas chromatographic analysis of fatty acid methyl esters. *J Chromatogr* **1992,** 607, (1), 55-67.
26. Eder, K., Gas chromatographic analysis of fatty acid methyl esters. *J Chromatogr B Biomed Appl* **1995,** 671, (1-2), 113-31.
27. Purdie, N. Direct cholesterol anal. by spectrophotometry. 94-US7836 9502826, 19940713., 1995.
28. Purdie, N.; Murphy, L. H.; Purdie, R. B., Direct measure of the low-density fractions of serum cholesterol. *Anal Chem* **1991,** 63, (24), 2947-51.
29. Krouse, J. Using Direst Serum Lipoprotein Subfraction Analysis to Identify

Various Dyslipidemias. Oklahoma State University, Stillwater, OK, 2001.
30. Purdie, N.; Krouse, J.; Studer, J.; Marais, A. D. Direct Serum Lipids Assay for Evaluation of Disease States. 6,737,275, May 18, 2004.
31. Studer, J. Characterization of a Novel Chromogenic Reaction for Analysis of Unsaturated Lipids and Application to Human Serum Lipids Analysis. Oklahoma State University, Stillwater, OK, 2003.
32. Studer, J.; Purdie, N.; Krouse, J., Exploitation of Friedel-Crafts acylation as a color derivatizing reaction for terpene and lipids analyses. *Appl Spectrosc* **2003,** 57, (7), 784-90.
33. Studer, J.; Purdie, N.; Krouse, J. A., Friedel-Crafts acylation as a quality control assay for steroids. *Appl Spectrosc* **2003,** 57, (7), 791-6.
34. Stryer, L., *Biochemistry*. 4th ed.; W.H. Freeman and Company: New York, 1995.
35. Zubay, G. L., *Biochemistry*. 4th ed.; McGraw-Hill: Boston, 1998.
36. Esterbauer, H.; Gebicki, J.; Puhl, H.; Jurgens, G., The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radical Biology and Medicine* **1992,** 13, (4), 341-390.
37. Garner, B.; Witting, P. K.; Waldeck, A. R.; Christison, J. K.; Raftery, M.; Stocker, R., Oxidation of high density lipoproteins. I. Formation of methionine sulfoxide in apolipoproteins AI and AII is an early event that accompanies lipid peroxidation and can be enhanced by alpha-tocopherol. *Journal of biological chemistry* **1998,** 273, (11), 6080-7.
38. Liu, R. H., Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *Am J Clin Nutr* **2003,** 78, (3 Suppl), 517S-520S.
39. Berliner, J., Introduction. Lipid oxidation products and atherosclerosis. *Vascul Pharmacol* **2002,** 38, (4), 187-91.
40. Aviram, M.; Fuhrman, B., Effects of Flavonoids on the Oxidation of Low-Density Lipoprotein and Atherosclerosis. In *Flavonoids in Health and Disease*, 2nd ed.; Rice-Evans, C.; Packer, L., Eds. Marcel Dekker, Inc.: New York, 2003; pp 165-203.
41. Navab, M.; Ananthramaiah, G. M.; Reddy, S. T.; Van Lenten, B. J.; Ansell, B. J.; Fonarow, G. C.; Vahabzadeh, K.; Hama, S.; Hough, G.; Kamranpour, N.; Berliner, J. A.; Lusis, A. J.; Fogelman, A. M., The oxidation hypothesis of atherogenesis: the role of oxidized phospholipids and HDL. *J Lipid Res* **2004,** 45, (6), 993-1007.
42. Bohm, B., *Introduction to Flavonoids*. Harwood Academic Publishers: Australia, 1998; Vol. 2, p 365-395.
43. Mates, J. M.; Perez-Gomez, C.; De Castro, I. N., Antioxidant enzymes and human diseases. *Clinical Biochemistry* **1999,** 32, (8), 595-603.
44. Miller, E. R., III; Appel, L. J.; Risby, T. H., Effect of dietary patterns on measures of lipid peroxidation: results from a randomized clinical trial. *Circulation* **1998,** 98, (22), 2390-2395.
45. Schnell, J. W.; Anderson, R. A.; Stegner, J. E.; Schindler, S. P.; Weinberg, R. B., Effects of a high polyunsaturated fat diet and vitamin E supplementation on high-density lipoprotein oxidation in humans. *Atherosclerosis* **2001,** 159, (2), 459-66.
46. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *Jama* **2001,** 285, (19), 2486-97.

47. Jenkins, D. J.; Kendall, C. W.; Marchie, A.; Faulkner, D. A.; Wong, J. M.; de Souza, R.; Emam, A.; Parker, T. L.; Vidgen, E.; Lapsley, K. G.; Trautwein, E. A.; Josse, R. G.; Leiter, L. A.; Connelly, P. W., Effects of a dietary portfolio of cholesterol-lowering foods vs lovastatin on serum lipids and C-reactive protein. *Jama* **2003,** 290, (4), 502-10.
48. Tsimikas, S.; Willerson, J. T.; Ridker, P. M., C-reactive protein and other emerging blood biomarkers to optimize risk stratification of vulnerable patients. *J Am Coll Cardiol* **2006,** 47, (8 Suppl), C19-31.
49. Rizzo, M.; Berneis, K., Low-density lipoprotein size and cardiovascular risk assessment. *Qjm* **2006,** 99, (1), 1-14.
50. Rizzo, M.; Berneis, K., The clinical relevance of low-density-lipoproteins size modulation by statins. *Cardiovasc Drugs Ther* **2006,** 20, (3), 205-17.
51. Walldius, G.; Jungner, I., The apoB/apoA-I ratio: a strong, new risk factor for cardiovascular disease and a target for lipid-lowering therapy--a review of the evidence. *J Intern Med* **2006,** 259, (5), 493-519.
52. Maritz, F., Secondary Dyslipidaemia. *Continuing Medical Education* **2003,** 21, (7), 365-369.
53. AACE medical guidelines for clinical practice for the diagnosis and treatment of dyslipidemia and prevention of atherogenesis. *Endocr Pract* **2000,** 6, (2), 162-213.
54. Brown, M. S.; Goldstein, J. L., How LDL receptors influence cholesterol and atherosclerosis. *Sci Am* **1984,** 251, (5), 58-66.
55. Blom, D. J.; Byrnes, P.; Jones, S.; Marais, A. D., Non-denaturing polyacrylamide gradient gel electrophoresis for the diagnosis of dysbetalipoproteinemia. *J Lipid Res* **2003,** 44, (1), 212-7.
56. Blom, D. J.; O'Neill, F. H.; Marais, A. D., Screening for dysbetalipoproteinemia by plasma cholesterol and apolipoprotein B concentrations. *Clin Chem* **2005,** 51, (5), 904-7.
57. de Villiers, W. J.; van der Westhuyzen, D. R.; Coetzee, G. A.; Henderson, H. E.; Marais, A. D., The apolipoprotein E2 (Arg145Cys) mutation causes autosomal dominant type III hyperlipoproteinemia with incomplete penetrance. *Arterioscler Thromb Vasc Biol* **1997,** 17, (5), 865-72.
58. Chin, S. F.; Liu, W.; Storkson, J. M.; Ha, Y. L.; Pariza, M. W., Dietary Sources of Conjugated Dienoic Iomers of Linoleic Acid, a Newly Recognized Class of Anticarcinogens. *Journal of Food Composition and Analysis* **1992,** 5, 185-197.
59. O'Brien, R. D., *Fats and oils: formulating and processing for applications*. 2nd ed.; CRC Press LLC: Boca Raton, 2004.
60. Das, U. N., Essential fatty acids: biochemistry, physiology and pathology. *Biotechnol J* **2006,** 1, (4), 420-39.
61. Griinari, J. M.; Corl, B. A.; Lacy, S. H.; Chouinard, P. Y.; Nurmela, K. V.; Bauman, D. E., Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by Delta(9)-desaturase. *J Nutr* **2000,** 130, (9), 2285-91.
62. Covington, M. B., Omega-3 fatty acids. *American family physician* **2004,** 70, (1), 133-40.
63. Mozaffarian, D.; Pischon, T.; Hankinson, S. E.; Rifai, N.; Joshipura, K.; Willett, W. C.; Rimm, E. B., Dietary intake of trans fatty acids and systemic inflammation in women. *Am J Clin Nutr* **2004,** 79, (4), 606-12.

64. Din, J. N.; Newby, D. E.; Flapan, A. D., Omega 3 fatty acids and cardiovascular disease--fishing for a natural treatment. *Bmj* **2004,** 328, (7430), 30-5.
65. Heller, A.; Koch, T.; Schmeck, J.; van Ackern, K., Lipid mediators in inflammatory disorders. *Drugs* **1998,** 55, (4), 487-96.
66. Daviglus, M. L.; Stamler, J.; Orencia, A. J.; Dyer, A. R.; Liu, K.; Greenland, P.; Walsh, M. K.; Morris, D.; Shekelle, R. B., Fish consumption and the 30-year risk of fatal myocardial infarction. *The New England journal of medicine* **1997,** 336, (15), 1046-53.
67. Burdge, G. C.; Calder, P. C., Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. *Reprod Nutr Dev* **2005,** 45, (5), 581-97.
68. Lauritzen, L.; Hansen, H. S.; Jorgensen, M. H.; Michaelsen, K. F., The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina. *Prog Lipid Res* **2001,** 40, (1-2), 1-94.
69. Bakewell, L.; Burdge, G. C.; Calder, P. C., Polyunsaturated fatty acid concentrations in young men and women consuming their habitual diets. *Br J Nutr* **2006,** 96, (1), 93-9.
70. Koutsari, C.; Jensen, M. D., Thematic review series: patient-oriented research. Free fatty acid metabolism in human obesity. *J Lipid Res* **2006,** 47, (8), 1643-50.
71. Takahashi, Y.; Kushiro, M.; Shinohara, K.; Ide, T., Activity and mRNA levels of enzymes involved in hepatic fatty acid synthesis and oxidation in mice fed conjugated linoleic acid. *Biochim Biophys Acta* **2003,** 1631, (3), 265-73.
72. Das, U. N., Pathophysiology of metabolic syndrome X and its links to the perinatal period. *Nutrition* **2005,** 21, (6), 762-73.
73. Dyerberg, J.; Bang, H. O.; Hjorne, N., Fatty acid composition of the plasma lipids in Greenland Eskimos. *The American journal of clinical nutrition* **1975,** 28, (9), 958-66.
74. Kromhout, D.; Bosschieter, E. B.; de Lezenne Coulander, C., The inverse relation between fish consumption and 20-year mortality from coronary heart disease. *The New England journal of medicine* **1985,** 312, (19), 1205-9.
75. Siscovick, D. S.; Raghunathan, T. E.; King, I.; Weinmann, S.; Wicklund, K. G.; Albright, J.; Bovbjerg, V.; Arbogast, P.; Smith, H.; Kushi, L. H.; et al., Dietary intake and cell membrane levels of long-chain n-3 polyunsaturated fatty acids and the risk of primary cardiac arrest. *Jama* **1995,** 274, (17), 1363-7.
76. Albert, C. M.; Campos, H.; Stampfer, M. J.; Ridker, P. M.; Manson, J. E.; Willett, W. C.; Ma, J., Blood levels of long-chain n-3 fatty acids and the risk of sudden death. *New England Journal of Medicine* **2002,** 346, (15), 1113-1118.
77. Albert, C. M.; Hennekens, C. H.; O'Donnell, C. J.; Ajani, U. A.; Carey, V. J.; Willett, W. C.; Ruskin, J. N.; Manson, J. E., Fish consumption and risk of sudden cardiac death. *JAMA : the journal of the American Medical Association* **1998,** 279, (1), 23-8.
78. Mozaffarian, D.; Ascherio, A.; Hu, F. B.; Stampfer, M. J.; Willett, W. C.; Siscovick, D. S.; Rimm, E. B., Interplay between different polyunsaturated fatty acids and risk of coronary heart disease in men. *Circulation* **2005,** 111, (2), 157-64.
79. Charles, M. A.; Fontbonne, A.; Thibult, N.; Claude, J. R.; Warnet, J. M.; Rosselin, G.; Ducimetiere, P.; Eschwege, E., High plasma nonesterified fatty acids are predictive of cancer mortality but not of coronary heart disease mortality: results from the Paris Prospective Study. *Am J Epidemiol* **2001,** 153, (3), 292-8.
80. Jouven, X.; Charles, M. A.; Desnos, M.; Ducimetiere, P., Circulating nonesterified fatty acid level as a predictive risk factor for sudden death in the population.

Circulation **2001,** 104, (7), 756-61.
81. Pilz, S.; Scharnagl, H.; Tiran, B.; Seelhorst, U.; Wellnitz, B.; Boehm, B. O.; Schaefer, J. R.; Marz, W., Free fatty acids are independently associated with all-cause and cardiovascular mortality in subjects with coronary artery disease. *J Clin Endocrinol Metab* **2006,** 91, (7), 2542-7.
82. Ascherio, A.; Rimm, E. B.; Stampfer, M. J.; Giovannucci, E. L.; Willett, W. C., Dietary intake of marine n-3 fatty acids, fish intake, and the risk of coronary disease among men. *The New England journal of medicine* **1995,** 332, (15), 977-82.
83. Guallar, E.; Aro, A.; Jimenez, F. J.; Martin-Moreno, J. M.; Salminen, I.; van't Veer, P.; Kardinaal, A. F.; Gomez-Aracena, J.; Martin, B. C.; Kohlmeier, L.; Kark, J. D.; Mazaev, V. P.; Ringstad, J.; Guillen, J.; Riemersma, R. A.; Huttunen, J. K.; Thamm, M.; Kok, F. J., Omega-3 fatty acids in adipose tissue and risk of myocardial infarction: the EURAMIC study. *Arterioscler Thromb Vasc Biol* **1999,** 19, (4), 1111-8.
84. Kromhout, D.; Bloemberg, B. P.; Feskens, E. J.; Hertog, M. G.; Menotti, A.; Blackburn, H., Alcohol, fish, fibre and antioxidant vitamins intake do not explain population differences in coronary heart disease mortality. *Int J Epidemiol* **1996,** 25, (4), 753-9.
85. Hu, F. B.; Bronner, L.; Willett, W. C.; Stampfer, M. J.; Rexrode, K. M.; Albert, C. M.; Hunter, D.; Manson, J. E., Fish and omega-3 fatty acid intake and risk of coronary heart disease in women. *JAMA, the Journal of the American Medical Association* **2002,** 287, (14), 1815-1821.
86. Hu, F. B.; Cho, E.; Rexrode, K. M.; Albert, C. M.; Manson, J. E., Fish and long-chain omega-3 fatty acid intake and risk of coronary heart disease and total mortality in diabetic women. *Circulation* **2003,** 107, (14), 1852-7.
87. Albert, C. M.; Oh, K.; Whang, W.; Manson, J. E.; Chae, C. U.; Stampfer, M. J.; Willett, W. C.; Hu, F. B., Dietary alpha-linolenic acid intake and risk of sudden cardiac death and coronary heart disease. *Circulation* **2005,** 112, (21), 3232-8.
88. Burr, M. L.; Fehily, A. M.; Gilbert, J. F.; Rogers, S.; Holliday, R. M.; Sweetnam, P. M.; Elwood, P. C.; Deadman, N. M., Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). *Lancet* **1989,** 2, (8666), 757-61.
89. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. *Lancet* **1999,** 354, (9177), 447-55.
90. Singh, R. B.; Niaz, M. A.; Sharma, J. P.; Kumar, R.; Rastogi, V.; Moshiri, M., Randomized, double-blind, placebo-controlled trial of fish oil and mustard oil in patients with suspected acute myocardial infarction: the Indian experiment of infarct survival--4. *Cardiovascular drugs and therapy / sponsored by the International Society of Cardiovascular Pharmacotherapy* **1997,** 11, (3), 485-91.
91. Nilsen, D. W. T.; Albrektsen, G.; Landmark, K.; Moen, S.; Aarsland, T.; Woie, L., Effects of a high-dose concentrate of n-3 fatty acids or corn oil introduced early after an acute myocardial infarction on serum triacylglycerol and HDL cholesterol. *American Journal of Clinical Nutrition* **2001,** 74, (1), 50-56.
92. Burr, M. L.; Ashfield-Watt, P. A. L.; Dunstan, F. D. J.; Fehily, A. M.; Breay, P.; Ashton, T.; Zotos, P. C.; Haboubi, N. A. A.; Elwood, P. C., Lack of benefit of dietary advice to men with angina: results of a controlled trial. *European journal of clinical*

nutrition **2003,** 57, (2), 193-200.
93. von Schacky, C.; Angerer, P.; Kothny, W.; Theisen, K.; Mudra, H., The effect of dietary omega-3 fatty acids on coronary atherosclerosis. A randomized, double-blind, placebo-controlled trial. *Ann Intern Med* **1999,** 130, (7), 554-62.
94. Berry, E. M., Are diets high in omega-6 polyunsaturated fatty acids unhealthy? *European Heart Journal Supplements* **2001,** 3, (Supplement D), D37-D41.
95. Berry, E. M., Who's afraid of n-6 polyunsaturated fatty acids? Methodological considerations for assessing whether they are harmful. *Nutr Metab Cardiovasc Dis* **2001,** 11, (3), 181-8.
96. Hodgson, J. M.; Wahlqvist, M. L.; Boxall, J. A.; Balazs, N. D., Can linoleic acid contribute to coronary artery disease? *Am J Clin Nutr* **1993,** 58, (2), 228-34.
97. Crowe, F. L.; Skeaff, C. M.; Green, T. J.; Gray, A. R., Serum fatty acids as biomarkers of fat intake predict serum cholesterol concentrations in a population-based survey of New Zealand adolescents and adults. *Am J Clin Nutr* **2006,** 83, (4), 887-94.
98. Tricon, S.; Yaqoob, P., Conjugated linoleic acid and human health: a critical evaluation of the evidence. *Current Opinion in Clinical Nutrition and Metabolic Care* **2006,** 9, (2), 105-110.
99. Harris, W. S., n-3 fatty acids and serum lipoproteins: human studies. *Am J Clin Nutr* **1997,** 65, (5 Suppl), 1645S-1654S.
100. Marckmann, P.; Gronbaek, M., Fish consumption and coronary heart disease mortality. A systematic review of prospective cohort studies. *European journal of clinical nutrition* **1999,** 53, (8), 585-90.
101. Bucher, H. C.; Hengstler, P.; Schindler, C.; Meier, G., N-3 polyunsaturated fatty acids in coronary heart disease: a meta-analysis of randomized controlled trials. *Am J Med* **2002,** 112, (4), 298-304.
102. Wang, C.; Harris, W. S.; Chung, M.; Lichtenstein, A. H.; Balk, E. M.; Kupelnick, B.; Jordan, H. S.; Lau, J., n-3 Fatty acids from fish or fish-oil supplements, but not alpha-linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review. *Am J Clin Nutr* **2006,** 84, (1), 5-17.
103. Jula, A.; Marniemi, J.; Ronnemaa, T.; Virtanen, A.; Huupponen, R., Effects of diet and simvastatin on fatty acid composition in hypercholesterolemic men: a randomized controlled trial. *Arterioscler Thromb Vasc Biol* **2005,** 25, (9), 1952-9.
104. Isley, W. L.; Harris, W. S.; Miles, J. M., The effect of high-dose simvastatin on free fatty acid metabolism in patients with type 2 diabetes mellitus. *Metabolism* **2006,** 55, (6), 758-62.
105. Nordoy, A.; Hansen, J. B.; Brox, J.; Svensson, B., Effects of atorvastatin and omega-3 fatty acids on LDL subfractions and postprandial hyperlipemia in patients with combined hyperlipemia. *Nutr Metab Cardiovasc Dis* **2001,** 11, (1), 7-16.
106. Durrington, P. N.; Bhatnagar, D.; Mackness, M. I.; Morgan, J.; Julier, K.; Khan, M. A.; France, M., An omega-3 polyunsaturated fatty acid concentrate administered for one year decreased triglycerides in simvastatin treated patients with coronary heart disease and persisting hypertriglyceridaemia. *Heart* **2001,** 85, (5), 544-8.
107. Chan, D. C.; Watts, G. F.; Mori, T. A.; Barrett, P. H.; Redgrave, T. G.; Beilin, L. J., Randomized controlled trial of the effect of n-3 fatty acid supplementation on the metabolism of apolipoprotein B-100 and chylomicron remnants in men with visceral obesity. *Am J Clin Nutr* **2003,** 77, (2), 300-7.

108. De Caterina, R.; Liao, J. K.; Libby, P., Fatty acid modulation of endothelial activation. *Am J Clin Nutr* **2000,** 71, (1 Suppl), 213S-23S.
109. Kris-Etherton, P. M.; Harris, W. S.; Appel, L. J., Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Arterioscler Thromb Vasc Biol* **2003,** 23, (2), e20-30.
110. Garaulet, M.; Perez-Llamas, F.; Perez-Ayala, M.; Martinez, P.; de Medina, F. S.; Tebar, F. J.; Zamora, S., Site-specific differences in the fatty acid composition of abdominal adipose tissue in an obese population from a Mediterranean area: relation with dietary fatty acids, plasma lipid profile, serum insulin, and central obesity. *Am J Clin Nutr* **2001,** 74, (5), 585-91.
111. Terry, P. D.; Rohan, T. E.; Wolk, A., Intakes of fish and marine fatty acids and the risks of cancers of the breast and prostate and of other hormone-related cancers: a review of the epidemiologic evidence. *Am J Clin Nutr* **2003,** 77, (3), 532-43.
112. Awad, A. B.; Young, A. L.; Fink, C. S., The effect of unsaturated fatty acids on membrane composition and signal transduction in HT-29 human colon cancer cells. *Cancer Lett* **1996,** 108, (1), 25-33.
113. Larsson, S. C.; Kumlin, M.; Ingelman-Sundberg, M.; Wolk, A., Dietary long-chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms. *Am J Clin Nutr* **2004,** 79, (6), 935-45.
114. Lee, S.; Gura, K. M.; Kim, S.; Arsenault, D. A.; Bistrian, B. R.; Puder, M., Current clinical applications of omega-6 and omega-3 fatty acids. *Nutr Clin Pract* **2006,** 21, (4), 323-41.
115. Cleland, L. G.; James, M. J.; Proudman, S. M., The role of fish oils in the treatment of rheumatoid arthritis. *Drugs* **2003,** 63, (9), 845-53.
116. Cleland, L. G.; James, M. J.; Proudman, S. M., Fish oil: what the prescriber needs to know. *Arthritis Res Ther* **2005,** 8, (1), 202.
117. Stamp, L. K.; James, M. J.; Cleland, L. G., Diet and rheumatoid arthritis: a review of the literature. *Semin Arthritis Rheum* **2005,** 35, (2), 77-94.
118. Parker, G.; Gibson, N. A.; Brotchie, H.; Heruc, G.; Rees, A. M.; Hadzi-Pavlovic, D., Omega-3 fatty acids and mood disorders. *Am J Psychiatry* **2006,** 163, (6), 969-78.
119. Terano, T., Effect of ω3 Polyunsaturated Fatty Acid Ingestion on Bone Metabolism and Osteoporosis. In *Fatty Acids and Lipids - New Findings*, Hamazaki, T.; Okuyama, H., Eds. Karger: Basel, 2001; Vol. 88, pp 141-147.
120. Caudill, S. P.; Cooper, G. R.; Smith, S. J.; Myers, G. L., Assessment of current National Cholesterol Education Program guidelines for total cholesterol triglyceride, HDL-cholesterol, and LDL-cholesterol measurements. *Clin Chem* **1998,** 44, (8 Pt 1), 1650-8.
121. Cooper, G. R.; Smith, S. J.; Duncan, I. W.; Mather, A.; Fellows, W. D.; Foley, T.; Frantz, I. D., Jr.; Gill, J. B.; Grooms, T. A.; Hynie, I.; et al., Interlaboratory testing of the transferability of a candidate reference method for total cholesterol in serum. *Clin Chem* **1986,** 32, (6), 921-9.
122. Ellerbe, P.; Myers, G. L.; Cooper, G. R.; Hertz, H. S.; Sniegoski, L. T.; Welch, M. J.; White, E. t., A comparison of results for cholesterol in human serum obtained by the Reference Method and by the Definitive Method of the National Reference System for cholesterol. *Clin Chem* **1990,** 36, (2), 370-5.
123. Abell, L. L.; Levy, B. B.; Brodie, B. B.; Kendall, F. E., A simplified method for

the estimation of total cholesterol in serum and demonstration of its specificity. *Journal of Biological Chemistry* **1952,** 195, 357-66.
124. Burchard, H., Beitraege zur Kenntnis des Cholesterins. *Chem. Zentralbl* **1890,** 61, 25.
125. Liebermann, N. C., Uber das Oxychinoterpen. *Berichte der Deutschen Chemischen Gesellschaft* **1885,** 18, 1803.
126. Burke, R. W.; Diamondstone, B. I.; Velapoldi, R. A.; Menis, O., Mechanisms of the Liebermann-Burchard and Zak color reactions for cholesterol. *Clinical chemistry* **1974,** 20, (7), 794-81.
127. Zlatkis, A.; Zak, B.; Boyle, A. J., A new method for the direct determination of serum cholesterol. *J Lab Clin Med* **1953,** 41, (3), 486-92.
128. Webster, D., The Direct Estimation of Esterified Cholesterol with a Number of Commonly Used Cholesterol Colour Reagents. *Clin Chim Acta* **1963,** 8, 731-43.
129. Tshugaev, L.; Gastev, A., Cholesterol. I. Application of the Xanthogenic Reaction. *Berichte der Deutschen Chemischen Gesellschaft* **1910,** 42, 4631-4.
130. Hanel, H. K.; Dam, H., Determination of small amounts of total cholesterol by the Tschugaeff reaction with a note on the determination of lathosterol. *Acta Chemica Scandinavica* **1955,** 9, 677-82.
131. Chung, B. H.; Segrest, J. P.; Ray, M. J.; Brunzell, J. D.; Hokanson, J. E.; Krauss, R. M.; Beaudrie, K.; Cone, J. T., Single vertical spin density gradient ultracentrifugation. *Methods in enzymology* **1986,** 128, 181-209.
132. Chung, B. H.; Segrest, J. P.; Cone, J. T.; Pfau, J.; Geer, J. C.; Duncan, L. A., High resolution plasma lipoprotein cholesterol profiles by a rapid, high volume semi-automated method. *Journal of lipid research* **1981,** 22, (6), 1003-14.
133. Chung, B. H.; Wilkinson, T.; Geer, J. C.; Segrest, J. P., Preparative and quantitative isolation of plasma lipoproteins: rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. *Journal of lipid research* **1980,** 21, (3), 284-91.
134. Peterson, B. L.; Cummings, B. S., A review of chromatographic methods for the assessment of phospholipids in biological samples. *Biomed Chromatogr* **2006,** 20, (3), 227-43.
135. Blaha, V.; Solichova, D.; Cernohorsky, D.; Bratova, M.; Vyroubal, P.; Zadak, Z., Bioanalysis of PUFA metabolism and lipid peroxidation in coronary atherosclerosis. *J Pharm Biomed Anal* **2000,** 22, (3), 563-72.
136. Chan, D. C.; Watts, G. F.; Nguyen, M. N.; Barrett, P. H., Factorial study of the effect of n-3 fatty acid supplementation and atorvastatin on the kinetics of HDL apolipoproteins A-I and A-II in men with abdominal obesity. *Am J Clin Nutr* **2006,** 84, (1), 37-43.
137. Soderberg, B. L.; Sicinska, E. T.; Blodget, E.; Cluette-Brown, J. E.; Suter, P. M.; Schuppisser, T.; Vetter, W.; Laposata, M., Preanalytical variables affecting the quantification of fatty acid ethyl esters in plasma and serum samples. *Clin Chem* **1999,** 45, (12), 2183-90.
138. Lima, E. S.; Abdalla, D. S. P., High-performance liquid chromatography of fatty acids in biological samples. *Analytica Chimica Acta* **2002,** 465, (1-2), 81-91.
139. Kotani, A.; Fuse, T.; Kusu, F., Determination of plasma free fatty acids by high-performance liquid chromatography with electrochemical detection. *Anal Biochem* **2000,** 284, (1), 65-9.

140. Bailey, A. L.; Southon, S., Determination of total long-chain fatty acids in human plasma and lipoproteins, before and during copper-stimulated oxidation, by high-performance liquid chromatography. *Anal Chem* **1998,** 70, (2), 415-9.
141. Sanches-Silva, A.; Rodriguez-Bernaldo de Quiros, A.; Lopez-Hernandez, J.; Paseiro-Losada, P., Comparison between high-performance liquid chromatography and gas chromatography methods for fatty acid identification and quantification in potato crisps. *J Chromatogr A* **2004,** 1032, (1-2), 7-15.
142. Browne, R. W.; Armstrong, D., HPLC analysis of lipid-derived polyunsaturated fatty acid peroxidation products in oxidatively modified human plasma. *Clin Chem* **2000,** 46, (6 Pt 1), 829-36.
143. Gross, R. W.; Jenkins, C. M.; Yang, J.; Mancuso, D. J.; Han, X., Functional lipidomics: the roles of specialized lipids and lipid-protein interactions in modulating neuronal function. *Prostaglandins Other Lipid Mediat* **2005,** 77, (1-4), 52-64.
144. Shimizu, S.; Tani, Y.; Yamada, H.; Tabata, M.; Murachi, T., Enzymatic determination of serum-free fatty acids: a colorimetric method. *Anal Biochem* **1980,** 107, (1), 193-8.
145. Miles, J.; Glasscock, R.; Aikens, J.; Gerich, J.; Haymond, M., A microfluorometric method for the determination of free fatty acids in plasma. *J Lipid Res* **1983,** 24, (1), 96-9.
146. Hilderson, H. E.; Steen, L.; De Wolf, M.; Lagrou, A.; Hilderson, H. J.; Van Dessel, G.; Dierick, W., Spectrophotometric determination of dolichol and dolichyl derivatives using the Chugaev color reaction. *Anal Biochem* **1984,** 141, (1), 116-20.
147. Murphy, L. H. Direct Determination of Cholesterol Lipid Distributions Using Circular Dichroism. Oklahoma State University, Stillwater, OK 1991.
148. Talley, M. B. A Comparison of Methods for the Determination of Low Density Lipoprotein Cholesterol. Oklahoma State University, Stillwater, OK, 1992.
149. Purdie, N.; Lucas, E. A.; Talley, M. B., Direct Mesure of Total Cholesterol and its Distribution Among Major Serum Lipoproteins. *Clinical Chemistry* **1992,** 38, (9), 1645-1646.
150. Lucas, E. A. An Alternative Rountine Method to Measure Serum Total Cholesterol and its Distribution Among the Major Lipoproteins. Oklahoma State University, Stillwater, OK, 1995.
151. Purdie, N. Direct Cholesterol Assay Reagent. Nov. 23, 1999.
152. Purdie, N. Direct Cholesterol Assay. Jan.14, 1997.
153. Otto, M., *Chemometrics: statistics and computer application in analytical chemistry*. Wiley-VCH: Weiheim; New York; Chichester; Brisbane; Singapore; Toronto, 1999.
154. Geladi, P.; Kowalski, B. R., Partial least-squares regression: a tutorial. *Analytica Chimica Acta* **1986,** 185, 1-17.
155. SAS *JMP Statistical Discovery Software*, 4.0; SAS Institute, Inc.: Cary, 2000.
156. Wold, S.; Sjostrom, M.; Eriksson, L., PLS-regression: a basic tool of chemometrics. *Chemometrics and Intelligent Laboratory Systems* **2001,** 58, (2), 109-130.
157. CAMO Technologies, I. *The Unscrambler*, Camo Technologies, Inc.: Oslo, Norway, 1986-2005.
158. Goicoechea, H. C.; Olivieri, A. C., Wavelength selection for multivariate calibration using a genetic algorithm: A novel initialization strategy. *Journal of Chemical*

Information and Computer Sciences **2002,** 42, (5), 1146-1153.
159. Abdollahi, H.; Bagheri, L., Simultaneous spectrophotometric determination of Vitamin K3 and 1,4-naphthoquinone after cloud point extraction by using genetic algorithm based wavelength selection-partial least squares regression. *Analytica Chimica Acta* **2004,** 514, (2), 211-218.
160. Meinke, M.; Gersonde, I.; Friebel, M.; Helfmann, J.; Mueller, G., Chemometric determination of blood parameters using visible-near-infrared spectra. *Applied Spectroscopy* **2005,** 59, (6), 826-835.
161. Nadler, B.; Coifman, R. R., The prediction error in CLS and PLS: The importance of feature selection prior to multivariate calibration. *Journal of Chemometrics* **2005,** 19, (2), 107-118.
162. Spiegelman, C. H.; McShane, M. J.; Cote, G. L.; Goetz, M. J.; Motamedi, M.; Yue, Q. L., Theoretical Justification of Wavelength Selection in PLS Calibration: Development of a New Algorithm. *Analytical Chemistry* **1998,** 70, (1), 35-44.
163. Xu, L.; Schechter, I., Wavelength selection for simultaneous spectroscopic analysis. Experimental and theoretical study. *Analytical Chemistry* **1996,** 68, (14), 2392-2400.
164. Frenich, A. G.; Jouan-Rimbaud, D.; Massart, D. L.; Kuttatharmmakul, S.; Martinez Galera, M.; Marinez Vidal, J. L., Wavelength selection method for multicomponent spectrophotometric determinations using partial least squares. *Analyst (Cambridge, United Kingdom)* **1995,** 120, (12), 2787-92.
165. Trygg, J., O2-PLS for qualitative and quantitative analysis in multivariate calibration. *Journal of Chemometrics* **2002,** 16, (6), 283-293.
166. Trygg, J.; Wold, S., Orthogonal projections to latent structures (O-PLS). *Journal of Chemometrics* **2002,** 16, (3), 119-128.
167. Einax, J. W.; Aulinger, A.; Von Tumpling, W.; Prange, A., Quantitative description of element concentrations in longitudinal river profiles by multiway PLS models. *Fresenius' Journal of Analytical Chemistry* **1999,** 363, (7), 655-661.
168. Estienne, F.; Massart, D. L., Multivariate calibration with Raman data using fast principal component regression and partial least squares methods. *Analytica Chimica Acta* **2001,** 450, (1-2), 123-129.
169. Saxberg, B. E. H.; Kowalski, B. R., Generalized standard addition method. *Analytical Chemistry* **1979,** 51, (7), 1031-8.
170. Vandeginste, B.; Klaessens, J.; Kateman, G., Interactive calibration by a recursive generalized standard addition method. *Analytica Chimica Acta* **1983,** 150, (1), 71-86.
171. Al-Ammar, A. S.; Hamid, H. A.; Rashid, B. H., Elimination of interferences and effects from drift in working parameters in inductively coupled plasma atomic emission spectrometry by using a combination of the generalized standard addition method and the generalized internal reference method. *Spectrochimica Acta, Part B: Atomic Spectroscopy* **1990,** 45B, (4-5), 359-66.
172. Kalivas, J. H.; Kowalski, B. R., Generalized standard addition method for multicomponent instrument characterization and elimination of interferences in inductively coupled plasma spectroscopy. *Analytical Chemistry* **1981,** 53, (14), 2207-12.
173. Zagatto, E. A. G.; Jacintho, A. O.; Krug, F. J.; Reis, B. F.; Bruns, R. E.; Araujo, M. C. U., Flow injection systems with inductively-coupled argon plasma atomic emission spectrometry. Part 2. The generalized standard addition method. *Analytica Chimica Acta*

1983, 145, 169-78.
174. Franke, J. P.; De Zeeuw, R. A.; Hakkert, R., Evaluation and optimization of the standard addition method for absorption spectrometry and anodic stripping voltammetry. *Analytical Chemistry* **1978,** 50, (9), 1374-80.
175. Gerlach, R. W.; Kowalski, B. R., The generalized standard addition method: intermetallic interferences in anodic stripping voltammetry. *Analytica Chimica Acta* **1982,** 134, 119-27.
176. Jochum, C.; Jochum, P.; Kowalski, B. R., Error propagation and optimal performance in multicomponent analysis. *Analytical Chemistry* **1981,** 53, (1), 85-92.
177. Xie, Y.; Wang, J.; Liang, Y.; Ge, K.; Yu, R., Background bilinearization by the use of generalized standard addition method on two-dimensional data. *Analytica Chimica Acta* **1993,** 281, (1), 207-18.
178. Kalivas, J. H., Precision and stability in the generalized standard addition method. *Analytical Chemistry* **1983,** 55, (3), 565-7.
179. Moran, M. G.; Kowalski, B. R., Effect of random experimental error on the generalized standard addition method. *Analytical Chemistry* **1984,** 56, (3), 562-9.
180. Sharaf, M. A.; Illman, D., L.; Kowalski, B. R., *Chemometrics*. John Wiley & Sons, Inc.: New York, 1986; Vol. 82.
181. Aguiar, P. F. d.; Bourguignon, B.; Khots, M. S.; Massart, D. L.; Phan-Than-Luu, R., D-optimal designs. *Chemometrics and Intelligent Laboratory Systems* **1995,** 30, (2), 199-210.
182. Anderson, M. J.; Whitcomb, P. J., *DOE Simplified: Practical Tools for Effective Experimentation*. Productivity, Inc.: Portland, OR, 2000.
183. Kalivas, J. H., Evaluation of volume and matrix effects for the generalized standard addition method. *Talanta* **1987,** 34, (11), 899-903.
184. Hagan, M. T.; Demuth, H. B.; Beale, M. H., *Neural Network Design*. PNS Publishing: Boston, MA, 1996.
185. Esseiva, P.; Anglada, F.; Dujourdy, L.; Taroni, F.; Margot, P.; Pasquier, E. D.; Dawson, M.; Roux, C.; Doble, P., Chemical profiling and classification of illicit heroin by principal component analysis, calculation of inter sample correlation and artificial neural networks. *Talanta* **2005,** 67, (2), 360-367.
186. Welsh, W. J.; Lin, W.; Tersigni, S. H.; Collantes, E.; Duta, R.; Carey, M. S.; Zielinski, W. L.; Brower, J.; Spencer, J. A.; Layloff, T. P., Pharmaceutical Fingerprinting: Evaluation of Neural Networks and Chemometric Techniques for Distinguishing among Same-Product Manufacturers. *Analytical Chemistry* **1996,** 68, (19), 3473-3482.
187. Chen, D. R.; Chang, R. F.; Huang, Y. L., Computer-aided diagnosis applied to US of solid breast nodules by using neural networks. *Radiology* **1999,** 213, (2), 407-12.
188. Ganadu, M. L.; Lubinu, G.; Tilocca, A.; Amendolia, S. R., Spectroscopic identification and quantitative analysis of binary mixtures using artificial neural networks. *Talanta* **1997,** 44, (10), 1901-1909.
189. He, L. M.; Kear-Padilla, L. L.; Lieberman, S. H.; Andrews, J. M., Rapid in situ determination of total oil concentration in water using ultraviolet fluorescence and light scattering coupled with artificial neural networks. *Analytica Chimica Acta* **2003,** 478, (2), 245-258.
190. Yin, C.; Shen, Y.; Liu, S.; Yin, Q.; Guo, W.; Pan, Z., Simultaneous quantitative

UV spectrophotometric determination of multicomponents of amino acids using linear neural network. *Comput Chem* **2001,** 25, (3), 239-43.
191. Mathworks, T. *Neural Network Toolbox for Matlab,* 4.0; The Mathworks: Natick, MA, 2000.
192. Zak, B.; Artiss, J. D., Decreased water: increased solids: distorted serum concentrations. *Microchemical Journal* **2002,** 72, (3), 235-240.
193. Long, J. R.; Gregoriou, V. G.; Gemperline, P. J., Spectroscopic calibration and quantitation using artificial neural networks. *Analytical Chemistry* **1990,** 62, (17), 1791-7.

APPENDICES

APPENDIX I

Copy of Oklahoma State University Wellness Center blood testing consent form.

OSU WELLNESS CENTER
INFORMED CONSENT FORM

BLOOD TESTING

Explanation of Test

The blood test you are about to undergo is part of the Oklahoma State University Wellness Program. The test includes selected blood variables analyzed from the fingerstick method or from a venous sample.

It will be determined, prior to testing, that this test is appropriate and safe for you. All testing will be conducted by trained personnel and procedures will be explained to your satisfaction at the outset.

Possible Risks

The potential risks associated with the venipuncture/fingerstick are: (1) Venipuncture/fingerstick may cause some pain or discomfort. The exact amount, if any will be dependent upon individual preconceptions and pain threshold levels. (2) Possible hematoma (bruising) at the venipuncture/fingerstick site following the procedure. The occurrence or non-occurrence will be dependent upon bleeding/coagulation times and adherence to instructions pertaining to holding a cotton ball against the venipuncture/fingerstick site, with pressure, for five minutes following extraction of the needle or following the fingerstick. (3) Slight risk of infection. Any break in the integrity of the skin is associated with a small degree of infection risk. However, if directions are followed the risk is very small.

Consent by Subject

Information, which is obtained in the health screening, will be treated as privileged and confidential. IF USED FOR STATISTICAL RESEARCH PURPOSES, INDIVIDUAL IDENTITIES WILL NOT BE RELEASED. IF RESULTS FROM THE RESEARCH ARE PUBLISHED, NO INDIVIDUALS WILL BE NAMED IN THE ANALYSIS OF THE DATA. DATA will be statistically analyzed in an aggregate manner. The Wellness Center professional/medical staff RESERVE THE RIGHT TO contact you if your results are outside normal REFERENCE limits for recommendations and educational opportunities. In addition, if indicated, a small amount of the blood drawn may be used for research in alternative cholesterol testing.

I have read the foregoing, I understand it, and any questions which may have occurred to me have been answered to my satisfaction.

Date_____

Subject Signature_____

APPENDIX II

Letter of Permission from the University of Cape Town Lipidology Clinic.

UNIVERSITY OF CAPE TOWN

Dr A D Marais, Professor
Internal Medicine, Lipidology
Cape Heart Centre, 5th Floor, Chris Barnard Building,
University of Cape Town Health Science Faculty,
Observatory 7925 South Africa

Tel: 27-21-404 2265
Fax: 27-21-406-6396
Email: DMARAIS@CAPEHEART.UCT.AC.ZA

DATE: 15th November 2006
REF: Phillips/Staff/REILLYL

TO WHOM IT MAY CONCERN

MS L M REILLY
Department of Chemistry
Oklahoma State University
107 Physical Science
Stillwater, OK 74078
United States of America

Tel: (405) 744 5948
Fax No: (405) 744 6007
Email: lisa.reilly@okstate.edu

Dear Sir/Madam

Ms Reilly is finishing her dissertation in the Department of Chemistry at the Oklahoma State University. She has requested a statement about the work done in Cape Town.

Her work formed part of a collaboration between Professor Neil Purdie in the Department of Chemistry at Oklahoma State University and ourselves in Cape Town. She visited our Department to set up a chemical analysis and thereafter analysed anonymous samples from patients with dyslipidaemia who had consented to research investigation. Whilst much of the initial work was done in the laboratory in South Africa, further analysis was done after she had left. Her visit was from 4th February 2004 to 4th May 2004.

Yours sincerely

Professor A D Marais
Head
Lipid Laboratory

APPENDIX III

Table of 130 mixtures from a full factorial design with limitation to maintain an absorbance between 0.2 and 1.0 absorbance units.

	Cholesterol (M)	Linoleic (M)	Linolenic (M)	Arachidonic (M)	EPA (M)	DHA (M)	Conjugated (M)
Mixture1	0	0	0	0	0	0	0
Mixture2	0.008	0	0	0	0	0	0
Mixture3	0	0.02	0	0	0	0	0
Mixture4	0.008	0.02	0	0	0	0	0
Mixture5	0	0	0.02	0	0	0	0
Mixture6	0.008	0	0.02	0	0	0	0
Mixture7	0	0.02	0.02	0	0	0	0
Mixture8	0.008	0.02	0.02	0	0	0	0
Mixture9	0	0	0	0.02	0	0	0
Mixture10	0.007	0	0	0.01	0	0	0
Mixture11	0	0.02	0	0.02	0	0	0
Mixture12	0.007	0.01	0	0.01	0	0	0
Mixture13	0	0	0.02	0.02	0	0	0
Mixture14	0.007	0	0.01	0.01	0	0	0
Mixture15	0	0.01	0.01	0.01	0	0	0
Mixture16	0.007	0.01	0.01	0.01	0	0	0
Mixture17	0	0	0	0	0.02	0	0
Mixture18	0.007	0	0	0	0.01	0	0
Mixture19	0	0.02	0	0	0.02	0	0
Mixture20	0.007	0.01	0	0	0.01	0	0
Mixture21	0	0	0.02	0	0.02	0	0
Mixture22	0.007	0	0.01	0	0.01	0	0
Mixture23	0	0.01	0.01	0	0.01	0	0
Mixture24	0.007	0.01	0.01	0	0.01	0	0
Mixture25	0	0	0	0.02	0.02	0	0
Mixture26	0.007	0	0	0.01	0.01	0	0
Mixture27	0	0.01	0	0.01	0.01	0	0
Mixture28	0.007	0.01	0	0.01	0.01	0	0
Mixture29	0	0	0.01	0.01	0.01	0	0
Mixture30	0.007	0	0.01	0.01	0.01	0	0
Mixture31	0	0.01	0.01	0.01	0.01	0	0
Mixture32	0.007	0.01	0.01	0.01	0.01	0	0
Mixture33	0	0	0	0	0	0.01	0
Mixture34	0.007	0	0	0	0	0.01	0
Mixture35	0	0.02	0	0	0	0.02	0
Mixture36	0.007	0.01	0	0	0	0.01	0
Mixture37	0	0	0.01	0	0	0.01	0
Mixture38	0.007	0	0.01	0	0	0.01	0
Mixture39	0	0.01	0.01	0	0	0.01	0
Mixture40	0.007	0.01	0.01	0	0	0.01	0

	Cholesterol (M)	Linoleic (M)	Linolenic (M)	Arachidonic (M)	EPA (M)	DHA (M)	Conjugated (M)
Mixture41	0	0	0	0.02	0	0.02	0
Mixture42	0.007	0	0	0.01	0	0.01	0
Mixture43	0	0.01	0	0.01	0	0.01	0
Mixture44	0.007	0.01	0	0.01	0	0.01	0
Mixture45	0	0	0.01	0.01	0	0.01	0
Mixture46	0.007	0	0.01	0.01	0	0.01	0
Mixture47	0	0.01	0.01	0.01	0	0.01	0
Mixture48	0.007	0.01	0.01	0.01	0	0.01	0
Mixture49	0	0	0	0	0.02	0.02	0
Mixture50	0.007	0	0	0	0.01	0.01	0
Mixture51	0	0.01	0	0	0.01	0.01	0
Mixture52	0.007	0.01	0	0	0.01	0.01	0
Mixture53	0	0	0.01	0	0.01	0.01	0
Mixture54	0.007	0	0.01	0	0.01	0.01	0
Mixture55	0	0.01	0.01	0	0.01	0.01	0
Mixture56	0.007	0.01	0.01	0	0.01	0.01	0
Mixture57	0	0	0	0.01	0.01	0.01	0
Mixture58	0.007	0	0	0.01	0.01	0.01	0
Mixture59	0	0.01	0	0.01	0.01	0.01	0
Mixture60	0.007	0.01	0	0.01	0.01	0.01	0
Mixture61	0	0	0.01	0.01	0.01	0.01	0
Mixture62	0.007	0	0.01	0.01	0.01	0.01	0
Mixture63	0	0.005	0.005	0.005	0.005	0.005	0
Mixture64	0.006	0.005	0.005	0.005	0.005	0.005	0
Mixture65	0	0	0	0	0	0	0.008
Mixture66	0.007	0	0	0	0	0	0.006
Mixture67	0	0.01	0	0	0	0	0.006
Mixture68	0.007	0.01	0	0	0	0	0.006
Mixture69	0	0	0.01	0	0	0	0.006
Mixture70	0.007	0	0.01	0	0	0	0.006
Mixture71	0	0.01	0.01	0	0	0	0.006
Mixture72	0.006	0.005	0.005	0	0	0	0.004
Mixture73	0	0	0	0.01	0	0	0.006
Mixture74	0.007	0	0	0.01	0	0	0.006
Mixture75	0	0.01	0	0.01	0	0	0.006
Mixture76	0.006	0.005	0	0.005	0	0	0.004
Mixture77	0	0	0.01	0.01	0	0	0.006
Mixture78	0.006	0	0.005	0.005	0	0	0.004
Mixture79	0	0.005	0.005	0.005	0	0	0.004
Mixture80	0.006	0.005	0.005	0.005	0	0	0.004
Mixture81	0	0	0	0	0.01	0	0.006
Mixture82	0.007	0	0	0	0.01	0	0.006
Mixture83	0	0.005	0	0	0.005	0	0.004
Mixture84	0.006	0.005	0	0	0.005	0	0.004
Mixture85	0	0	0.005	0	0.005	0	0.004
Mixture86	0.006	0	0.005	0	0.005	0	0.004
Mixture87	0	0.005	0.005	0	0.005	0	0.004

	Cholesterol (M)	Linoleic (M)	Linolenic (M)	Arachidonic (M)	EPA (M)	DHA (M)	Conjugated (M)
Mixture88	0.006	0.005	0.005	0	0.005	0	0.004
Mixture89	0	0	0	0.005	0.005	0	0.004
Mixture90	0.006	0	0	0.005	0.005	0	0.004
Mixture91	0	0.005	0	0.005	0.005	0	0.004
Mixture92	0.006	0.005	0	0.005	0.005	0	0.004
Mixture93	0	0	0.005	0.005	0.005	0	0.004
Mixture94	0.006	0	0.005	0.005	0.005	0	0.004
Mixture95	0	0.005	0.005	0.005	0.005	0	0.004
Mixture96	0.006	0.005	0.005	0.005	0.005	0	0.004
Mixture97	0	0	0	0	0	0.01	0.006
Mixture98	0.007	0	0	0	0	0.01	0.006
Mixture99	0	0.01	0	0	0	0.01	0.006
Mixture100	0	0.005	0	0	0	0.005	0.004
Mixture101	0.006	0.005	0	0	0	0.005	0.004
Mixture102	0.006	0	0.005	0	0	0.005	0.004
Mixture103	0	0.005	0.005	0	0	0.005	0.004
Mixture104	0.006	0.005	0.005	0	0	0.005	0.004
Mixture105	0	0	0	0.01	0	0.01	0.006
Mixture106	0.006	0	0	0.005	0	0.005	0.004
Mixture107	0	0.005	0	0.005	0	0.005	0.004
Mixture108	0.006	0.005	0	0.005	0	0.005	0.004
Mixture109	0	0	0.005	0.005	0	0.005	0.004
Mixture110	0.006	0	0.005	0.005	0	0.005	0.004
Mixture111	0	0.005	0.005	0.005	0	0.005	0.004
Mixture112	0.006	0.005	0.005	0.005	0	0.005	0.004
Mixture113	0	0	0	0	0.005	0.005	0.004
Mixture114	0.006	0	0	0	0.005	0.005	0.004
Mixture115	0	0.005	0	0	0.005	0.005	0.004
Mixture116	0.006	0.005	0	0	0.005	0.005	0.004
Mixture117	0	0	0.005	0	0.005	0.005	0.004
Mixture118	0.006	0	0.005	0	0.005	0.005	0.004
Mixture119	0	0.005	0.005	0	0.005	0.005	0.004
Mixture120	0.006	0.005	0.005	0	0.005	0.005	0.004
Mixture121	0	0	0	0.005	0.005	0.005	0.004
Mixture122	0.006	0	0	0.005	0.005	0.005	0.004
Mixture123	0	0.005	0	0.005	0.005	0.005	0.004
Mixture124	0.006	0.005	0	0.005	0.005	0.005	0.004
Mixture125	0	0	0.005	0.005	0.005	0.005	0.004
Mixture126	0.006	0	0.005	0.005	0.005	0.005	0.004
Mixture127	0	0.005	0.005	0.005	0.005	0.005	0.004
Mixture128	0.006	0.005	0.005	0.005	0.005	0.005	0.004
Mixture129	0.003	0.0025	0.0025	0.0025	0.0025	0.0025	0.002
Mixture130	0.003	0.0025	0.0025	0.0025	0.0025	0.0025	0.002

VITA

Lisa Marie Reilly

Candidate for the Degree of

Doctor of Philosophy

Thesis: DEVELOPMENT AND APPLICATION OF A SPECTROPHOTOMETRIC AND CHEMOMETRIC METHOD FOR THE DIRECT DETERMINATION OF LIPIDS IN SERUM AND SYNTHETIC MIXTURES

Major Field: Chemistry

Biographical:

Personal Data: The daughter of Thomas and Judith Reilly

Education: Graduate from Cranberry Junior/Senior High School, Seneca, Pennsylvania, May 1996. B.S. in Chemistry, Mercyhurst College, Erie, Pennsylvania, May 2000. Completed requirements for the Doctor of Philosophy in Chemistry at Oklahoma State University in December 2006.

Experience: Instructor, Chemistry in Civilization, CHEM 1014, Oklahoma State University, Fall 2004, 2005, and 2006, Graduate Teaching Assistant, Fall 2000 – Summer 2006. Undergraduate research assistant, Los Alamos National Laboratory, Summer 1999, Undergraduate research assistant, National Science Foundation Research Experience for Undergraduates, University of Pittsburgh, Summer 1998, Undergraduate research assistant, Mercyhurst College, Summer 1997.

Professional Memberships: American Chemical Society, Phi Lambda Upsilon National Chemistry Honors Society, Phi Eta Sigma Freshman Honors Society

Name: Lisa Marie Reilly Date of Degree: December 2006

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: DEVELOPMENT AND APPLICATION OF A SPECTROPHOTOMETRIC AND CHEMOMETRIC METHOD FOR THE DIRECT DETERMINATION OF LIPIDS IN SERUM AND SYNTHETIC MIXTURES

Pages in Study: 181 Candidate for the Degree of Doctor of Philosophy

Major Field: Chemistry

Scope and Method of Study: Polyunsaturated fatty acids (PUFA) have become a focus in both nutritional sciences and medical studies in the evaluation of risk factors for cardiovascular diseases, dyslipidemias, diabetes, obesity, and other diseases. Studies have disclosed that unsaturated fatty acids can offer health benefits which include reducing cardiovascular death rates, lowering blood pressure, and promoting positive health effects for patients with other diseases. However, other studies have demonstrated the involvement of unsaturated fatty acids in the development of atherosclerosis, metabolic syndrome, and other diseases. With the potential connections of fatty acids to various diseases, the development of a direct, inexpensive, and routine colorimetric assay for the measurement of the concentration of serum lipids would enable a PUFA to be incorporated fully as a risk factor for any disease.

Findings and Conclusions: Our research group has focused on the use of a patented reagent developed by Dr. Purdie that is selective to –C-C=C- groups and results in a unique ultra-violet/visible spectrum for cholesterol and the PUFA. The resulting spectrum from a serum or mixture sample is a composite of all seven components. Multivariate analysis methods that have been applied to the de-convolution of the spectral data are: principal component analysis (PCA); partial least squares (PLS); simple standard addition (SAM), generalized standard addition (GSAM); and artificial neural networks (ANN). The comparative results for the same, prepared samples comprised of individual standards up to seven component mixtures using ANN, GSAM, and PLS chemometric algorithms had root mean square error of prediction on the order of 1-20% indicative of a rugged assay procedure. Overall, the ANN results are superior to the other two computational methods. The combination of the Purdie assay and chemometric methods has created a simple, direct method for the determination of cholesterol and PUFA concentrations. Further studies, with examination of the method applied to serum, will conclusively demonstrate the full capabilities of this assay.

ADVISER'S APPROVAL: Dr. Neil Purdie