USING DIRECT SERUM LIPOPROTEIN SUBFRACTION ANALYSIS TO IDENTIFY VARIOUS DYSLIPIDEMIAS

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PREFACE

It is widely known that a high serum cholesterol level predisposes one to a variety of diseases and events including atherosclerosis, coronary artery disease, heart attack, and stroke. It has been estimated that one in two Americans will die from some type of cardiovascular disease. Given the gravity and scope of this problem it is little wonder that countless papers have been published over the last fifty dealing with the effects of dietary fat on serum cholesterol / lipoprotein concentrations and their relation to disease. Yet, in spite of so much attention, a rigorous and thorough understanding of the relationship between the various forms of lipids and proteins and the diseases to which they have been linked has remained elusive. It is the purpose of this research to develop a new method of identifying various blood lipid disorders. In this study, the traditional approach of measuring individual lipoprotein levels is abandoned in favor of a more direct method. Using this method, the evidence presented here suggests that it may be possible to simultaneously identify and measure multiple blood serum components in a single assay. It is shown that the major constituents of the lipoprotein complexes may be identified spectroscopically. The proportions of each component, which vary according to lipoprotein class, are then shown to account for spectral difference among the lipoprotein classes and ultimately the serum as a whole. These spectral differences form the basis for the discrimination of dyslipidemias. It is hoped that this study will form the foundation of future studies to develop more accurate lipid measurement techniques and a better understanding of the risk factors associated with cardiovascular disease.

Any single achievement in one's life is almost always due to the efforts of many people, my time in graduate school has been no exception to this rule. Therefore, I would be remiss if I were to overlook the contribution of the people who helped me get to this point. First of all, I would like to thank my wife, Amber, and my sons, Prescott and Pierce, to whom I dedicate this body of work. It was because of you that I got up each morning and worked hard to get this thing finished. I would also like to thank those

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LIST OF SYMBOLS

AC – acetyl chloride

ACAT – acyl-cholesterol acyltransferase

ADP – adenosine diphosphate

ATP – adenosine triphosphate

CDC – Centers for Disease Control

CVD – coronary vascular disease

CHD – coronary heart disease

CM - chylomicron

cps – counts per second

DHA – docosahexaenoic acid

FA – fatty acid

FAME – fatty acid methyl ester

FC – free cholesterol

GAC – glacial acetic acid

GC – gas chromatography

GP - glycerophosphate

HDL – high-density lipoprotein

HDL-C – cholesterol associated with high-density lipoprotein

[HDL-C] – concentration of cholesterol associated with the high-density lipoprotein

HPLC – high-performance liquid chromatography

HTG - hypertriglyceridemia

IDL – intermediate-density lipoprotein

IDL-C – cholesterol associated with intermediate-density lipoprotein

[IDL-C] – concentration of cholesterol associated with the intermediate-density lipoprotein

IF – interference factor

LAME – linoleic acid methyl ester

LCAT – lecithin-cholesterol acyltransferase

LDL – low-density lipoprotein

LDL-C – cholesterol associated with low-density lipoprotein

[LDL-C] – concentration of cholesterol associated with the low-density lipoprotein

MUFA – monounsaturated fatty acid

NCEP – National Cholesterol Education Program

NIH – National Institues of Health

PA – perchloric acid

PG – prostaglandin

PUFA – polyunsaturated fatty acid

RDA – recommended daily allowance

SDS – synthetic dyslipidemic serum

SFA – saturated fatty acid

SM – synthetic mixture

SRM – standard reference material

SRS – standard reagent system

SS – synthetic serum

TC – total cholesterol

[TC] – total cholesterol concentration

TG - triglyceride

TLC – thin-layer chromatography

VLDL – very-low-density lipoprotein

VLDL-C – cholesterol associated with very-low-density lipoprotein

[VLDL-C] – concentration of cholesterol associated with the very-low-density lipoprotein

CHAPTER I

INTRODUCTION

The term lipid applies to a broad class of compounds that are soluble in organic solvents and nearly insoluble in water. Unlike carbohydrates and proteins, which are defined in terms of their structures, lipids are defined by the physical operation used to isolate them. Not surprisingly, then, lipids include a variety of structural types. Found in all body tissues, lipids, commonly called fats, play an important role in a variety of biological functions. They serve as hormones and hormone precursors, provide energy storage and metabolic fuel, aid in digestion, act as functional and structural cellular components, and form insulation to prevent heat loss. Lipids are generally synthesized in the liver and the intestine and are then transported to distant tissues and organs to execute their various physiological functions. Due to the hydrophobic nature of these fats, transport and delivery via lymph or plasma would be impossible without some form of hydrophilic adaptation. Consequently, lipids are transported throughout the body via micellular structures called lipoproteins.

This study deals with the five major lipoprotein classes: high-density lipoprotein (HDL), low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL), very-low-density lipoprotein (VLDL) and chylomicrons (CM),. (Throughout this text, HDL, LDL, etc. refer to the entire lipoprotein structure, whereas HDL-C, LDL-C, etc. refer only to the cholesterol associated with the respective lipoprotein. (When the acronym is enclosed in brackets, i.e. [HDL], the concentration of the lipoprotein is implied.) Although there are many lipids known to exist in humans, only a limited number are of clinical importance.[1] Clinically important lipids can be broadly divided into five groups based on their chemical structure (see Table 1-0).

Table 1.0. CLASSIFICATION OF CLINICALLY IMPORTANT LIPIDS	
Sterol Derivatives	**Glycerol Esters**
Cholesterol and Cholesterol Esters	Triglycerides
Steroid Hormones	Phosphoglycerides
Vitamin D	**Sphingosine Derivatives**
Bile Acids	Sphingomyelin
Fatty Acids	Glycosphingolipids
Short Chain (2-4 carbon atoms)	**Terpenes**
Medium Chain (6-10 carbon atoms)	Vitamin A
Long Chain (12-26 carbon atoms)	Vitamin E
Prostaglandins	Vitamin K

These lipids, especially cholesterol, triglycerides (TGs), and fatty acids (FAs), have received enormous attention throughout the 20th century. As early as 1910, Windaus[2] reported finding cholesterol in the lesions of arteries. Since that time, many studies have confirmed the accumulation of free and esterified cholesterol in the aorta, coronary arteries and cerebral vessels.[3-8] This thickening of the vascular wall by the localized deposition of lipid and cellular material results in reduced blood flow and weakened arteries. This condition has come to be known as atherosclerosis. Today, it is widely known that a high serum cholesterol level predisposes one to a variety of diseases and events including atherosclerosis, coronary artery disease, heart attack, and stroke.[9-17] While not the sole etiological factor in atherosclerosis, population and epidemiological studies have shown that serum cholesterol levels play a key role in its development.[18-23]

Unlike the consensus on cholesterol, the relationship between high serum TG levels, hypertriglyceridemia (HTG), and atherosclerosis is uncertain.[24] Numerous studies[25-27] seem to indicate a link between HTG and coronary heart disease (CHD), but in 1993 a National Institutes of Health (NIH) Consensus Conference on Triglycerides and CHD[28] failed to establish increased fasting triglyceride concentration as an independent risk factor. However, more recent studies[29-34] suggest that elevated TG

levels are indeed an independent CHD risk factor. The huge body of literature on the triglyceride-disease relationship reflects this ambiguity. Yet, despite so much attention, a complete and thorough understanding of the relationship between the various TGs and the diseases to which they have been linked remains elusive.

A closely related and also controversial subject is that of the relative health effects of saturated vs. unsaturated FA consumption. Over the last few decades, there has been increasing concern expressed about the composition of our dietary fats and oils. The main question being asked was, "What is the desirable ratio of saturated, polyunsaturated, and monounsaturated fatty acids in the diet?"[35] It was generally accepted that monounsaturated fatty acids (MUFAs) lower LDL-C levels and raise HDL-C levels. Polyunsaturated fatty acids (PUFAs) were thought to have an overall cholesterol lowering effect and saturated fatty acids (SFAs) had been associated with increasing the overall cholesterol level. (See Chapter II for a more complete description of saturation in FAs and the various forms of cholesterol.) In light of recent studies, however, these generalizations have broken down and researchers are beginning to examine FAs more specifically. For instance, there have been a number of studies on individual FAs of various saturation levels and their link to diseases such as cancer,[36-52] cirrhosis of the liver,[53-56] and atherosclerosis.[57-67]

Whether or not the risk factors are free cholesterol (FC), TGs, FAs, or some combination thereof remains to be seen; however, one fact is certain, cardiovascular disease (CVD) is the number one cause of death in the United States. It has been estimated that one in two Americans will die from some type of CVD.[68] Given the gravity and scope of this problem it is little wonder that countless papers have been published over the last five decades dealing with the physiological effects of plasma lipid concentrations and their measurement.

Various methods have been used to measure plasma lipids and lipoproteins (see Chapter III - Current Analytical Techniques). The Centers for Disease Control and Prevention (CDC) have developed reference methods for the quantitative analysis of total cholesterol (TC),[69] TGs[70] and HDL-C[71], but not for LDL-C. Because these methods require a high degree of expertise and are time consuming, simpler methods are routinely and widely used. These routine methods involve multi-step enzymatic processes for

both TC and TG quantitation. High-density lipoprotein cholesterol determination is further complicated by precipitation and ultracentrifugation steps. Once the [TC] and [HDL-C] have been determined, the [LDL-C] is approximated using the Friedewald equation. This empirical equation developed by Friedewald et al.[72] is based upon two suppositions. The first is that the total cholesterol concentration is the sum of the [HDL-C], [LDL-C] and [VLDL-C]. This premise does not hold true in all cases, especially in individuals with high concentrations of IDL or certain apolipoproteins. It also does not hold true in cases where CMs are present. The second supposition is that the [VLDL-C] may be approximated by dividing the [TG] by five. This approximation breaks down when the [TG] is greater than 400 mg/dl.

In light of the controversy surrounding the role of lipids in the evolution of atherosclerosis, it is easy to understand the utility and potential of an analytical method capable of directly measuring all lipoprotein fractions in a single step. Even more useful would be a simple one-step method capable of measuring all blood lipids individually. Such a tool would be of enormous utility to a clinical chemist. Unfortunately, it has yet to be invented. However, there are several groups working towards the development of such a tool.[73-78]

Several such studies[79,80] conducted by Purdie and coworkers have demonstrated that the [TC], [LDL-C] and [VLDL-C] can be measured simultaneously in a single, non-enzymatic, spectroscopic assay. In these studies, it was assumed the reagent system reacted with cholesterol and its esters in a serum sample producing one or more chromophores. Once the visible spectrum of the sample was obtained, it was subjected to a multivariate analysis subroutine that had been "trained" or calibrated to discriminate and quantify various lipoprotein profiles. The results of these studies showed strong correlations between experimental and external laboratory values for the [LDL-C], [VLDL-C], and [TC].

This research project may be viewed as a continuation of work done by Purdie et al.[79] on the direct measurement of TC and its distribution among the major serum lipoproteins. The goals of this research project are as follows: 1) refine the reagent system and technique proposed in the previous studies 2) systematically identify which lipid classes chromogenically react with this reagent system and 3) use the information gained from the completion of these two goals to develop a method for identifying various

dyslipidemias. It is further expected that this study will serve as a foundation upon which a method might be developed that could simultaneously identify and quantify multiple serum lipid components.

Chapter II of this thesis provides background information on: the structure and physiological roles of the five clinically important classes of lipids; lipoproteins; and dyslipidemias. Chapter III goes on to outline current lipid analysis techniques. Chapter IV describes the experimental technique used and developed in this study, while Chapter V discusses the results of these experiments. Chapter VI provides a summary of the work done on this project thus far and concluding remarks.

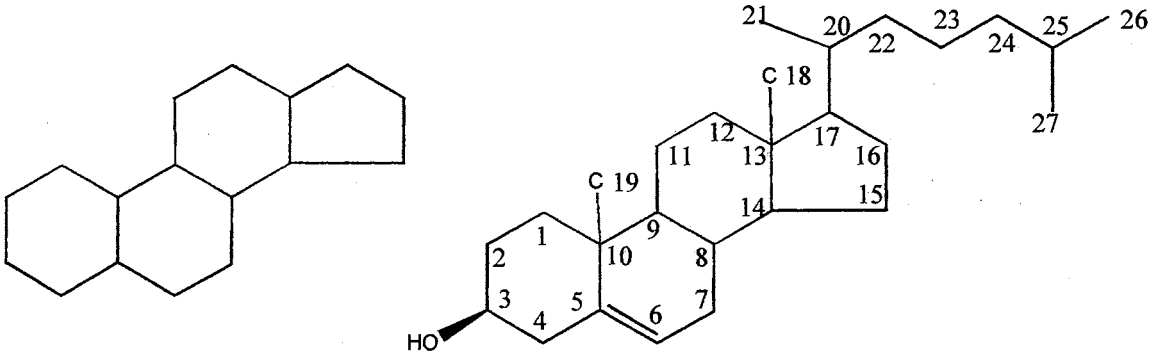
CHAPTER II

BACKGROUND

Structures and Physiology of Lipids

Sterol Derivatives

Cholesterol, like other sterols, is a solid alcohol of high molecular weight (386.64 g/mol) possessing a tetracyclic perhydrocyclopentanophenanthrene (sterane) skeleton. The molecule contains 27 carbon atoms, numbered as shown in Figure 2-0.



sterane skeleton cholesterol

Figure 2-0. Planar representation of the structure of cholesterol.

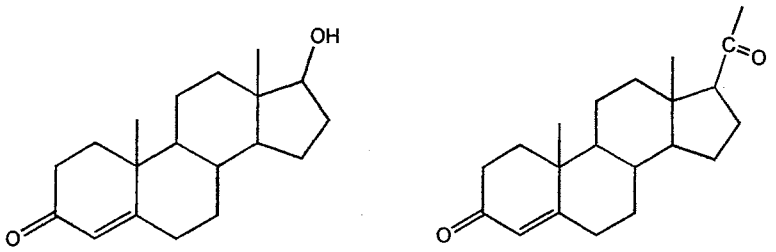
The principle sterol of mammalian tissue, cholesterol is found in high concentrations throughout the nervous and endocrine systems. In fact, about 17% of the solid matter of the brain is free cholesterol.[81] Exclusive to humans and animals, cholesterol is found in virtually all cells and body fluids.

The primary source for cholesterol is the body itself. Almost 90% of the body's total cholesterol is synthesized by the liver and intestines from simpler molecules, particularly acetate.[82] The other source is the diet. Animal products, especially meat, egg yolk, seafood, and whole-fat dairy products provide the bulk of dietary cholesterol.[83] It is estimated that the average American diet contains 300-500 mg of cholesterol per day.[84] Maximum absorption of dietary cholesterol occurs in the small intestine. In addition to animal-based cholesterol, several hundred milligrams of plant sterols, such as beta-sitosterol, are ingested daily but are poorly absorbed. Once synthesized or absorbed, cholesterol is released into circulation for transport by lipoproteins.

Serum cholesterol is found in both free and esterified forms. Practically all cholesterol in the gut is present in the unesterified form, since esterified cholesterol is rapidly hydrolyzed by cholesterol esterases in the pancreatic and small intestinal secretions. Esterification of free cholesterol occurs primarily within the vascular compartment, where cholesteryl esters account for approximately 70% of the TC.[1] This esterification is important as it serves to enhance the lipid-carrying capacity of the lipoproteins. The esterification reaction is catalyzed by the enzymes lecithin-cholesterol acyltransferase (LCAT) in the plasma and acyl-cholesterol acyltransferase (ACAT) intracellularly.

Cholesterol also serves a substrate for the formation of other important sterol derivatives such as steroid hormones, bile acids, and Vitamin D.

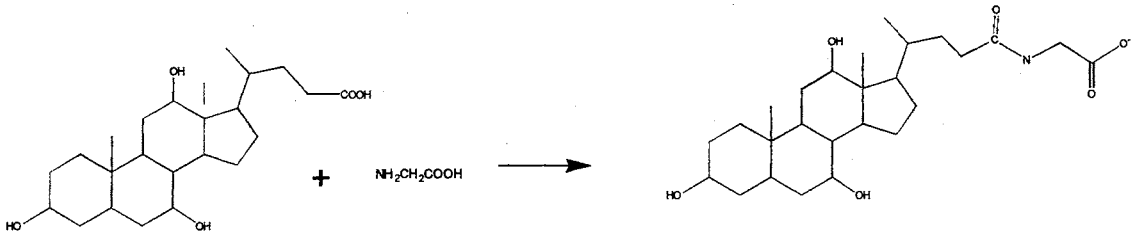
Steroid hormones are synthesized from cholesterol in the adrenal glands or the gonads. Most of these hormones are derived from low-density lipoproteins in circulation, but may be produced de novo.[85] While there are many interesting and physiologically important steroid hormones, this study focuses on only two of the sex hormones, testosterone and progesterone. The structures for these compounds are shown in Figure 2-1.



testosterone progesterone

Figure 2-1. Structures of testosterone and progesterone.

Bile acids are derivatives of cholesterol and are synthesized in the liver. In this process, cholesterol is converted into one of two primary bile acids, cholic or chenodeoxycholic acid. These acids are metabolized into secondary acids and conjugated to an amino acid (glycine or taurine) to yield the conjugated form that is used to facilitate lipid digestion (see Figure 2-2).



cholic acid glycine glycocholic acid

Figure 2-2. Conjugation of cholic and with glycine.

Bile acids are amphipathic, that is, they contain both hydrophobic (lipid soluble) and polar (hydrophilic) regions. The cholesterol portion of a bile acid is hydrophobic and the amino acid conjugate is polar and hydrophilic. Their amphipathic nature enables bile acids to carry out two very important functions. The first is the emulsification of lipid aggregates. Bile acids have a detergent action on particles of dietary fat

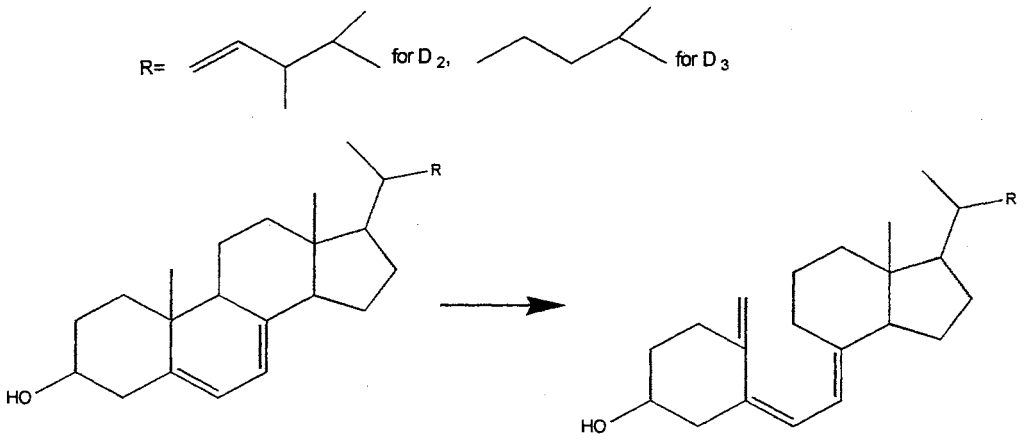
which causes fat globules to break down or be emulsified into minute, microscopic droplets. Emulsification is not digestion per se, but is of importance because it greatly increases the surface area of fat, making it available for digestion by lipases, which cannot access the inside of lipid droplets. The second function is the solubilization and transport of lipids in an aqueous environment. Bile acids are lipid carriers and are able to solubilize many lipids by forming micelles - aggregates of lipids such as FAs, cholesterol and triglycerides - that remain suspended in water. Bile acids are also critical for transport and absorption of the fat-soluble vitamins.

Hepatic synthesis of bile acids is the major mechanism for the breakdown of cholesterol in the body. In humans, roughly 500 mg of cholesterol are converted to bile acids and eliminated in bile every day. This route for elimination of excess cholesterol is particularly predominant in situations of massive cholesterol ingestion.[86]

While large amounts of bile acids are secreted into the intestine every day, only relatively small quantities are lost from the body. This happens because approximately 95% of the bile acids delivered to the small intestine are routed via the portal venous system back to the liver. [87] Hepatocytes extract bile acids very efficiently and little escapes into systemic circulation.

Vitamin D has two forms, ergocalciferol (D_2) and cholecalciferol (D_3). Vitamin D_2 is produced by the ultraviolet irradiation of ergosterol, which occurs in plants, while in humans, vitamin D_3 is produced by the ultraviolet irradiation of 7-dehydrocholesterol in the skin (see Figure 2-3). Each has approximately the same biological activity. The only chemical difference between the two forms is in the side chain.

When ingested, Vitamin D is absorbed in the small intestine and bound directly to chylomicrons. Much of this vitamin hydroxylated at the terminal side chain position in the liver to yield 25-hydroxycholecalciferol, which is the major metabolite of vitamin D in plasma (see Figure 2-4).[88] Vitamin D is generally stable to heat, alkalis, acids and oxidation.[89] It has a wide range of physiological functions, however, the major role it plays deals with bone growth and development.



Ergosterol, (provitamin D_2),

7-Dehydrocholesterol (provitamin D_3)

Vitamins D_2 and D_3

Figure 2-3. Structures of Vitamins D_2, D_3, and their provitamin forms.

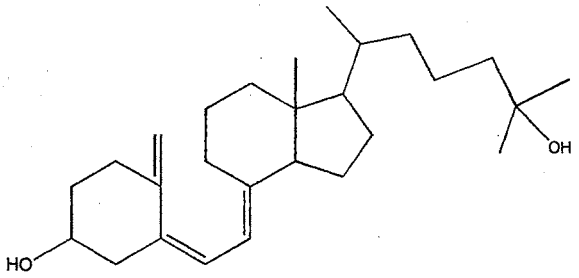


Figure 2-4 Structure of 25-hydroxycholecalciferol.

Fatty Acids

Fatty acids are simply carboxylic acids. They have the simplified formula of RCOOH, where R is an alkyl chain. The most common classification is based on chain length: 2-4 carbon atoms (short chain), 6-10 carbon atoms (medium chain), and 12-26 carbon atoms (long chain). Each class of FA is found in the diet. Once absorbed, however, they are all broken down and reassembled into FAs of the long chain class and all contain even numbers of carbons.

Further classification can be made according to their degree of saturation. Saturated fatty acids (SFAs) contain an alkyl chain without any double bonds between carbon atoms, monounsaturated fatty acids (MUFAs) contain one double bond, and polyunsaturated fatty acids (PUFAs) have more than one double bond in the alkyl chain. A shorthand notation has evolved to describe FAs. For instance, (C_{18}:$3^{9,12,15}$) is used to describe linolenic acid as it has 18 carbons and three double bonds, one at the 9, one at the 12, and one at the number 15 carbon. This notation completely describes the FA and allows one to discriminate between, say linolenic acid (C_{18}:$3^{9,12,15}$) and gamma-linolenic acid (C_{18}:$3^{6,9,12}$) which also has 18 carbons and three double bonds, but in different locations.

This example suggests the value of another common classification of FAs, the ω- classification. This classification groups FAs of varying lengths together based upon the position of their endmost double bond. For instance, a FA whose endmost double bond is six carbons from its methyl end, is an ω-6 FA. If the endmost double bond were three carbons from the methyl end it would be an ω-3 FA. Therefore, linolenic acid is an ω-6 FA and gamma-linolenic is an ω-3 FA. (Note: By convention and for the reader's convenience, common names or shorthand notation will be used throughout this text. A full listing of the compounds referenced in this study along with their various names can be found in Appendix A.)

In mammals, all naturally occurring unsaturated FAs are *cis-* isomers. *Trans-* FAs are a result of catalytic hydrogenation, a process used to harden fats in the manufacture of certain foods. Most fats in the human body are derived from the diet, but, if needed, humans can also synthesize the majority of the FAs required. Linoleic acid (C_{18}:$2^{9,12}$), however, cannot be synthesized by mammals; it is only found in plants. This particular FA is vital for maintenance of health, as well as growth and development, and is termed an *essential fatty acid.* Other nutritionally and physiologically important FAs include stearic acid (C_{18}:0) , oleic acid (C_{18}:1^{9}), linoleic acid (C_{18}:$2^{9,12}$), linolenic acid (C_{18}:$3^{9,12,15}$), gamma-linolenic acid (C_{18}:$3^{6,9,12}$), arachidonic acid (C_{20}:$4^{5,8,11,14}$) , eicosapentaenoic acid (C_{20}:$5^{5,8,11,14,17}$), and docosahexaenoic acid (C_{22}:$6^{4,7,10,13,16,19}$).

The carboxyl group of a FA has a pKa of ~4.8; thus, free FA molecules in both plasma and intracellular fluid exist in an ionized form. Most FAs in the body are present in the form of esters of cholesterol or glycerol.

Prostaglandins are cross-linked derivatives of FAs. The prostaglandins (PGs) are a series of C_{20} unsaturated FAs containing a cyclopentane ring; the parent FA is prostanoic acid (see Figure 2-5). Although their full physiological role is not completely known, these compounds are extremely potent, producing diverse physiological actions at concentrations as little as one microgram per liter. Prostaglandins appear hormone-like in action and are synthesized at the site of action and in almost all tissues. Linoleic acid is a precursor of two of the three 20- carbon FAs that form prostaglandins . The other precursor is linolenic acid. Although sixteen naturally occurring prostaglandins have been discovered, only seven are commonly found throughout the body and are called the primary prostaglandins.

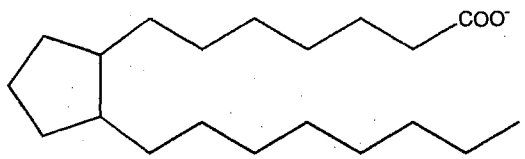
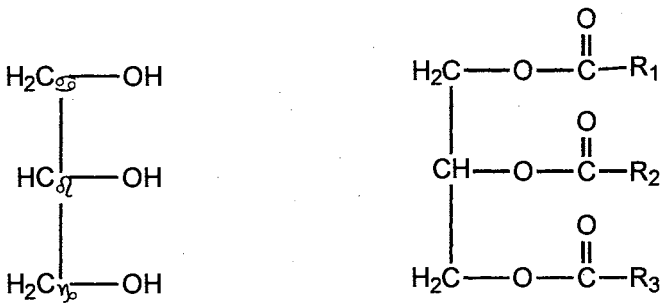


Figure 2-5. Structure of prostaglandins.

Glycerol Esters

Only a small portion of the total lipid fraction consists of long chain carboxylic acids. Most of the carboxylic acids of biological origin are found as esters of glycerol, more specifically, as triacylglycerols. (In some literature triacylglycerols are referred to as triglycerides, or simply as glycerides. In IUPAC nomenclature, because they are esters of glycerol, they should be named as glyceryl trialkanoates, glyceryl trialkenoates, and so on. In this text triglyceride and triacylglycerol are used interchangeably.) Triglycerides are fats and oils of plant or animal origin and include common substances such as peanut oil, soybean oil, corn oil, fish oil, sunflower oil, safflower oil, butter, lard, and tallow. Triglycerides that are liquid at room temperature are commonly called oils; those that are solids are called fats. They can be *simple triglycerides* in which all three acyl groups are the same, or, more commonly, as *mixed triglycerides* in which all three of the acyl groups are different.

Figure 2-6 shows the structure and nomenclature convention for glycerol and glycerol esters. The first carbon on the glycerol is denoted with a subscript alpha, the central carbon with a subscript beta and the final carbon with a subscript gamma. In the glycerol esters , R1, R2, and R3 are FA residues of varying lengths. Depending on the extent of esterification, the products are mono-, di-, or triglycerides, respectively.



Glycerol Glycerol Ester

Figure 2-6. Structures of glycerol and glycerol ester.

The most prevalent dietary glycerol esters are the triglycerides. Constituting 95% of the fat stored in tissue, TGs are the predominant form of glycerol ester found in plasma. Their FA compositions vary considerably but usually include saturated and unsaturated combinations of long-chain FAs. Triglycerides undergo digestion and absorption in the small intestine. After absorption, they are hydrolyzed by lipases and bile acids into glycerol, FAs, and monoglycerides and resynthesized in the intestinal epithelial cells to form lipoprotein components.

Another major class of glycerol esters is the phosphoglycerides or phospholipids. These have FAs (R_1, R_2) esterified on the alpha and beta carbons, respectively, but contain phosphoric acid at the gamma carbon. Usually a group such as a choline, serine, inositol, or ethanolamine is substituted on the phosphoric acid residue (see Figure 2-7). If the substituent group is choline, the compound is referred to as phosphatidylcholine, or more commonly a lecithin. If the substituted group is serine, inositol, or ethanolamine the compound is phosphatidylserine, phosphatidylinositol, or phosphatidylethanolamine, respectively. These three groups of compounds are collectively known as cephalins.

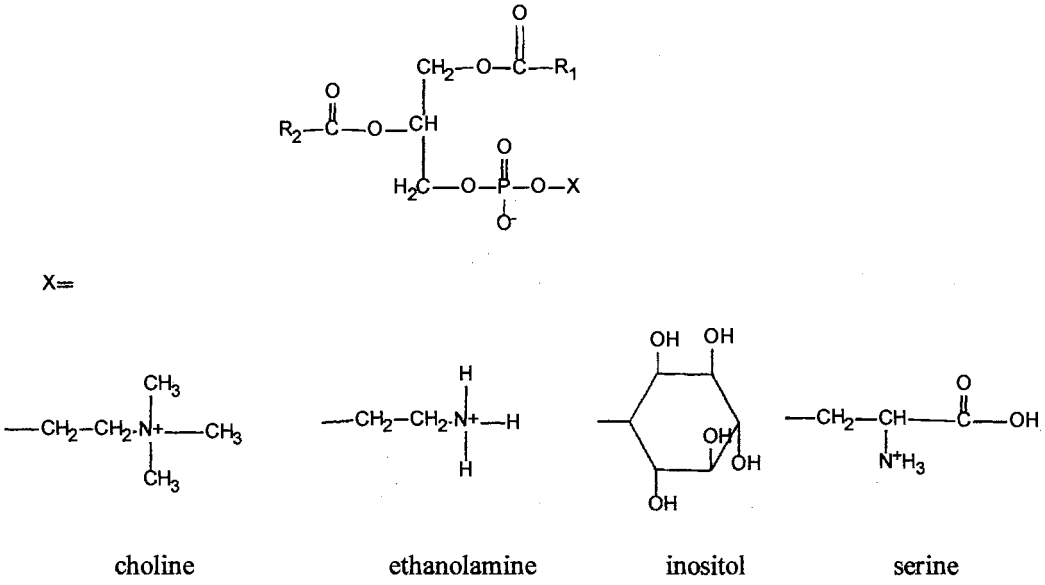
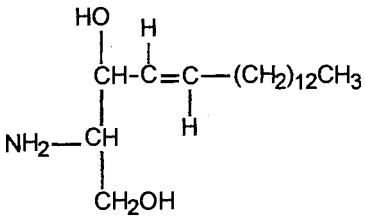


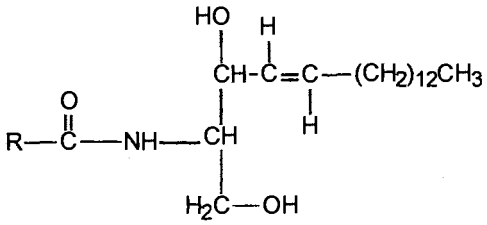
Figure 2-7. Structures of phosphoglycerides and common groups associated with them.

Sphingosine Derivatives

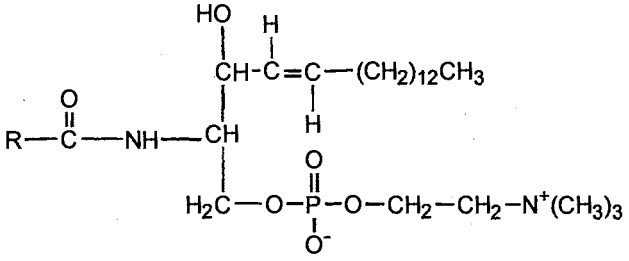
The fourth class of lipids found in humans is derived from the amino alcohol sphingosine. When a FA containing 18 or more carbons binds to the amino group a ceramide is formed. Additional substitutions at the terminal hydroxyl end form sphingomyelin, glycosal ceramides, and other complex sphingolipids (see Figure 2-8).



Sphingosine



Ceramide



Sphingomyelin

Figure 2-8. Sphingolipid structures.

Complex sphingolipids form the major lipids of cell membranes and the central nervous system. While it is well beyond the scope of this text to discuss the origin and function of the many and highly complex sphingosine derivatives, in this study it is useful to look at the major phospholipid distributions in humans[90] (see Table 2-0).

Table 2-0. Phospholipid Distribution (% weight) in Human Lipoproteins			
Species	VLDL	LDL	HDL
Phosphatidylcholine	71.2	70.3	77.3
Sphingomyelin	23.1	25.3	13.2
Phosphatidylethanolamine Phosphatidylserine Phosphatidylinositol	5.7	4.4	6.3

Terpenes

The fifth class of lipids is the terpenes. Terpenes are polymers of isoprene, 2-methyl-1,3-butadiene, and include vitamins A, E and K. Along with vitamin D, these are sometimes called the "fat soluble" vitamins. Vitamins are organic compounds required in trace amounts for health, growth and reproduction. They are natural substances that may be derived from the diet or biologically synthesized. Fat-soluble vitamins are absorbed, transported and stored in a manner similar to other fats and have a much longer retention time in the body than the water-soluble vitamins.

Vitamin A has two natural forms, retinol (A_1) and 3,4-dehydroretinol (A_2) (see Figure 2-9). These compounds are yellowish oils at room temperature and are sensitive to both oxygen and ultraviolet light. Vitamin A_1 predominates, especially in the form of long-chain FA esters, in the liver of mammals and salt-water fish, whereas the less biologically active vitamin A_2 is found in fresh-water fish oils.[91]

Physiological roles of vitamin A occur in growth, reproduction, and maintenance of epithelial tissue and vision. Humans cannot synthesize vitamin A_1, but can derive the aldehyde retinal from provitamin carotenes of plants. Retinal is then reversibly reduced to retinol. Carotenoid compounds such as beta-carotene constitute the main dietary source of what ultimately becomes vitamin A.[91] Like other fats retinol is taken up in the mucosal cells of the small intestine. Free retinol is then esterified and the

esters, in association with chylomicrons, are transported via the lymphatic system to the liver where it is stored.

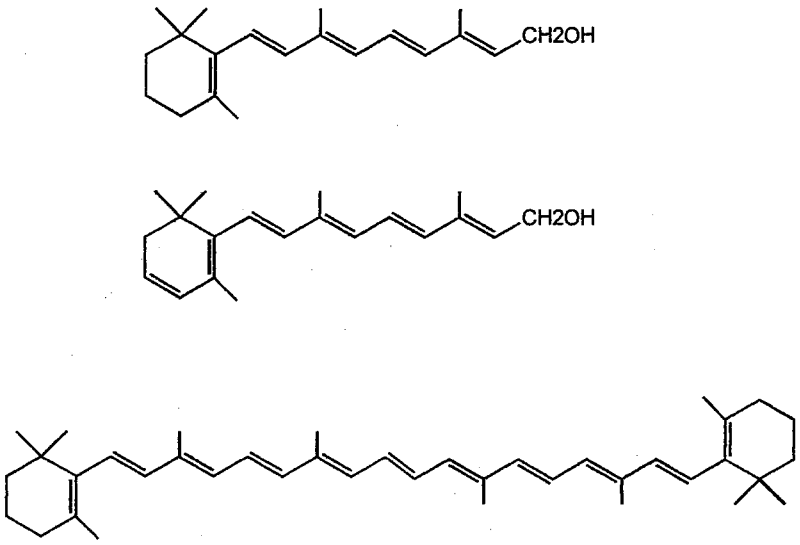


Figure 2-9. Structures of A_1 (top), A_2 (middle) and beta-carotene(bottom).

The most biologically active form of vitamin E is d-alpha-tocopherol (see Figure 2-10). While humans cannot synthesize this vitamin, it is commonly found in plants and especially abundant in vegetable oils.[92] This compound is stable to acid and heat in the absence of oxygen, but labile to oxygen in alkaline solutions and to ultraviolet light.[93] Absorbed in the small intestine in the presence of bile, it is associated with chylomicrons and very-low-density lipoproteins. Vitamin E is stored in most tissues, with the greatest concentration found in adipose tissue. The most well known physiological role for vitamin E is as an antioxidant for unsaturated fatty acyl moieties of lipids within phospholipid membranes.

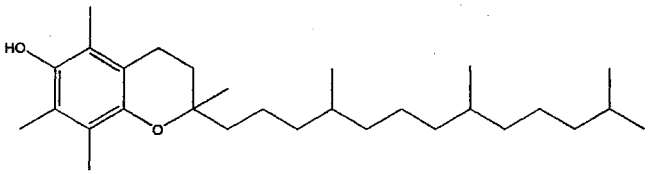


Figure 2-10. Structure of d-alpha-tocopherol.

There are two main compounds in the vitamin K series, phylloquinones (K_1) and menaquinones (K_2). Structures are shown in Figure 2-11. Phylloquinones are synthesized in plants while menaquinones are of bacterial origin and vary in side chain length from 5 (n=1) to 65 (n=13) carbons. These compounds are destroyed by alkaline solutions and reducing agents and are also sensitive to ultraviolet light. No specific recommended daily allowance (RDA) is given for vitamin K since intestinal bacteria in healthy individuals synthesize adequate amounts. However, there is also ample supply in the average diet. Absorption of vitamin K occurs in the small intestine and is facilitated by bile. Vitamins K_1 and K_2 are bound to CMs and transported to the liver for use and storage. Vitamin K plays an integral part in the formation of blood clotting factors.[94]

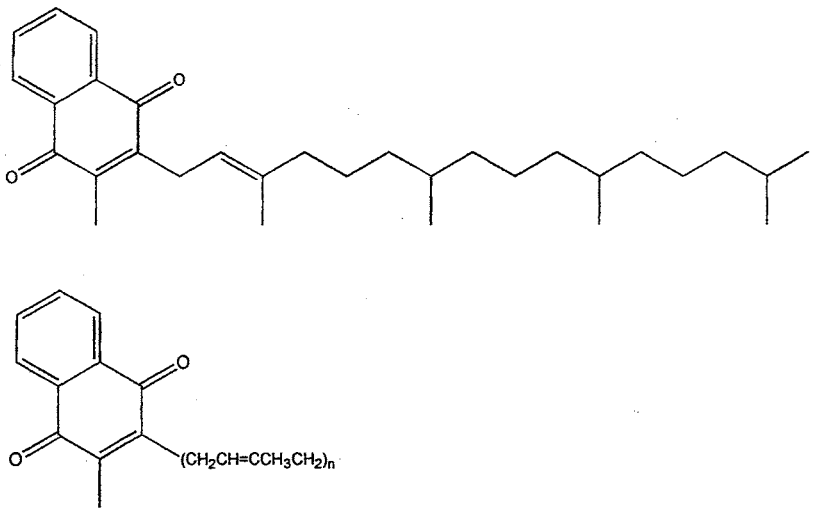


Figure 2-11. Structures of Vitamins K_1 (top) and K_2 type (below).

Lipoproteins

As mentioned in the Introduction, lipoproteins are micellular structures used to transport fats throughout the body. They consist of an outer layer of proteins, called apolipoproteins, and polar lipids and have an inner core of neutral lipids. (For the purposes of this study, emphasis is placed on the lipid fractions and not the apolipoproteins as will be discussed later.) By convention, lipoproteins are divided

into five main ultracentrifugal density classes: HDL, LDL, IDL, VLDL and CM.. Each lipoprotein class has a characteristic composition (see Table 2-1), but it should be noted that this composition is a generalization and the lipid proportions are not rigidly fixed.[95-97] In fact, far from being simple shuttles delivering needed supplies, lipoproteins undergo a series of complex metabolic processes in which changes and exchanges occur continuously in and between the various lipoproteins.[98-100] These processes are referred to as the lipoprotein cascade.[1] For clinical purposes, lipoprotein concentrations have traditionally been expressed in terms of their cholesterol content because they carry virtually all of the cholesterol that circulates in the plasma.. Normal reference lipoprotein levels vary according to age, sex, and race.[101-103] Figure 2-12 shows an electron microscope photomicrograph of HDL, LDL, VLDL and CM particles.

Chylomicrons are the largest in diameter (>70nm) and the least dense (0.93g/ml) class of lipoproteins. They are responsible for the transport of dietary fat. Synthesized in the small intestine, CMs are composed mainly of TGs (80-90%), with about 7% cholesterol and the same percentage of phospholipid. Once secreted into the lymphatic system, they are eventually transported to the thoracic duct and enter the main systemic circulation, where they are the primary means of delivery of TGs to the various tissues.[104] Once in circulation, lipoprotein lipase enzymes hydrolyze the TG component to monoglycerides, glycerol, and free FAs, which are taken up at the cellular level. The remainder of the CM is then known as a CM remnant.[105] The remnants are rapidly removed from circulation by the liver. Subjects in a fasting state have few if any CMs in their plasma.[106]

Very-Low-Density lipoprotein is the other TG rich lipoprotein. Consisting of 50% or more of TG, VLDLs also contain about 20% cholesterol and 20% phospholipid. Slightly more dense (0.97g/ml) than CMs, VLDLs range in size from 25-75nm. Produced in both the liver and the small intestine, they contain the majority of the plasma TG in the fasting state. Like the CM, VLDLs are acted upon by various lipoprotein lipases, depleting them of their TG content. However, unlike CMs whose remnants are destroyed, the remaining portion of the VLDL may be removed via the liver or further catabolized to form short-lived IDLs. These IDLs are more dense (1.003 g/ml) than their VLDL predecessors and

contain approximately equal amounts of TG and cholesterol. The next step in the cascade process is the further lipolysis of the IDL, removing even more TG, to form the LDL.

Low-density lipoproteins range in size from 19.6-22.7 nm with a density range of 1.019-1.063 g/ml. The major lipid constituents by weight are cholesterol (43%), phospholipid (26%) and TG(11%). Low-density lipoproteins are the major cholesterol carrying lipoprotein in the plasma and have a lifetime of about three days.[107] Catabolism of LDL takes place both in the liver and the tissues. Studies have shown that the tissue or receptor mediated pathway is largely responsible for the clearing of the plasma LDL pool.[108] Numerous studies have identified high serum [LDL] as a major risk factor in atherosclerosis.[109-115]

High-density lipoproteins are the smallest in diameter (4-10nm) and the most dense (1.063-1.21 g/ml) of the lipoprotein classes. Secreted from the liver or intestine, nascent HDL particles are disk-shaped. Through the addition of surface components from TG-rich particles, nascent HDL is converted to spherical particles. Free cholesterol from the cell membranes is also transferred to the HDL particle where it is esterified by LCAT. The size and composition of the HDL particle depends strongly on the LCAT activity. High-density lipoproteins contain approximately 20-30% of the total plasma cholesterol. The whole function of HDL is to transport cholesterol back to the liver for reuse or disposal. This mechanism is commonly called the HDL reverse cholesterol transfer pathway. Many studies have shown a negative correlation between high [HDL] and atherosclerotic disease.[116-120] A further subdivision of the HDL class is sometimes used to designate HDL_2 and HDL_3, which vary in the number of cholesterol molecules per unit.[121] However, in this study no such distinction is made.

Table 2-1. Classification, Properties and Composition of Human Serum Lipoproteins					
MEASUREMENT	Chylomicron	VLDL	IDL	LDL	HDL
Hydrated density (g/ml)	0.93	0.97	1.003	1.034	1.121
Solvent density for isolation (g/ml)	<1.006	<1.006	1.006-1.019	1.019-1.063	1.063-1.21
Molecular weight	$(0.4\text{-}30)\times10^{9}$	$(5\text{-}10)\times10^{6}$	$(3.9\text{-}4.8)\times10^{6}$	2.75×10^{6}	$(1.75\text{-}3.6)\times10^{5}$
Diameter, nm	>70.0	25.0-70.0	22.0-24.0	19.6-22.7	4-10
Composition, % by weight					
Cholesterol, unesterified	2	7	8	9	6
Cholesterol, esterified	5	14	22	34	13
Phosphoglycerides	7	21	25	26	28
Triglyceride	84	54	30	11	3
Protein	2	4	15	20	50
Synthesis	Intestine	Liver, Intestine	Intravascular	Intravascular	Intestine

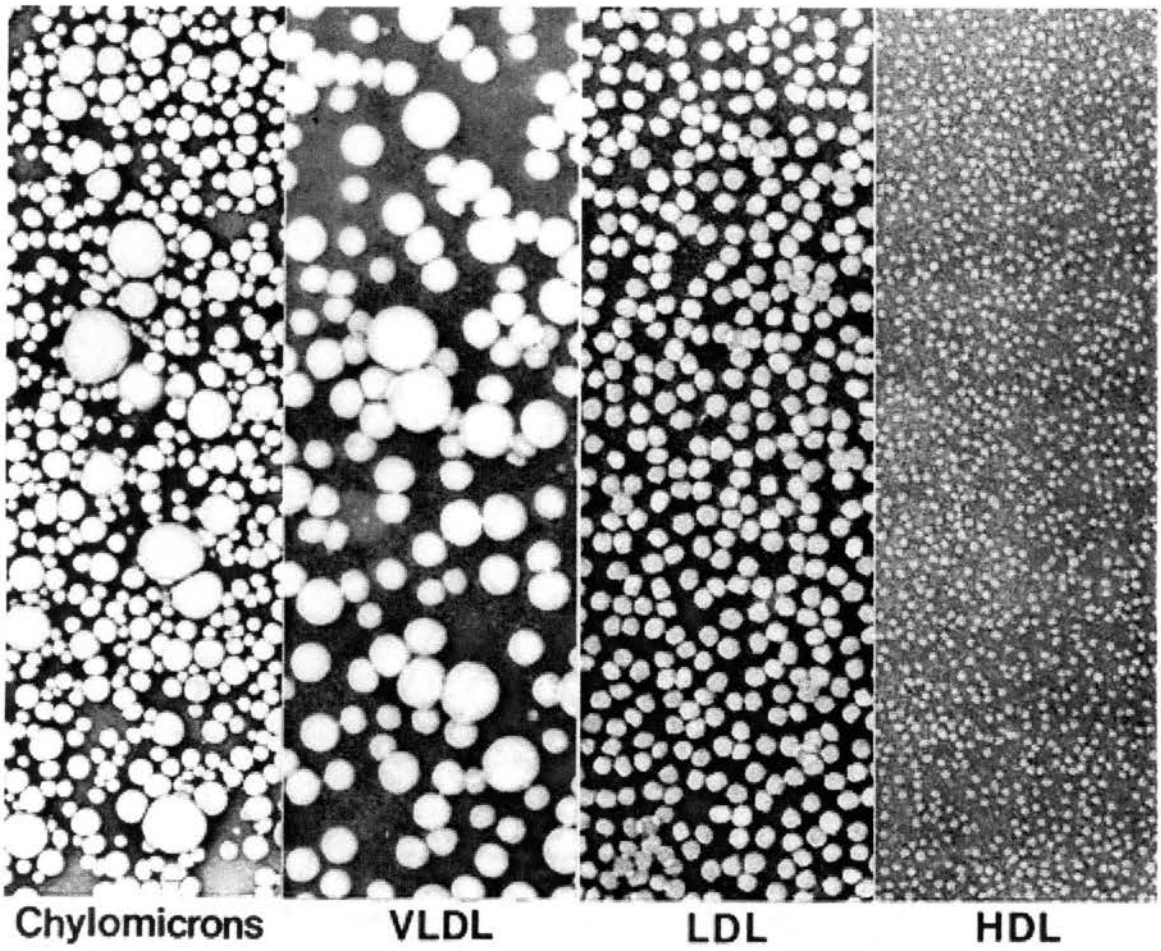


Figure 2-12.

Lipoproteins were separated from blood plasma utilizing the preparative ultracentrifuge. These electron microscopic photomicrographs were prepared by Robert Hamilton, Ph.D. of the Cardiovascular Research Institute, University of California, San Francisco. Magnification for the chylomicrons, (left panel) is 60,000. Magnification for the very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) was 180,000. Pictures were taken after negative staining, and lipoproteins are visualized on a dark background.

Dyslipidemias

Traditionally, hyperlipoproteinemias have been classified as plasma phenotypes, i.e., particular patterns of lipid and lipoprotein levels with strong correlations to clinical disease. Fredrickson and coworkers[122] initially proposed this classification and it was adopted by the World Health Organization in 1970. This classification accounts for the TC level, TG level, HDL, LDL and apolipoprotein levels. This study will consider all of these except the apolipoprotein levels as discussed in Chapter IV. Table 2-2 summarizes the various lipoprotein phenotypes.[123]

Type I disorder is due to a genetic defect.[124] It is characterized by the massive accumulation of CMs in the circulation due to an inability to catabolize dietary fat. This disorder is extremely rare, literally one per million individuals. Triglyceride levels have been reported as high as 10,000mg/dl[125], but more commonly in the range of 2000-4000mg/dl.[124] The concentration of VLDLs is usually normal with [HDL-C] and [LDL-C] concentrations low. Interestingly, patients with this disorder do not appear to be predisposed to atherosclerotic disease.[126,127]

Type II disorders fall under the more general term familial combined hyperlipidemia (FCHL). Individuals with FCHL have either increased [TC] and increased [LDL-C] levels (type IIa) or increased [TC], [LDL-C], and [TG] levels (type IIb). Both of these subtypes have normal to decreased [HDL-C]. About 10 to 15 % of patients with CHD have FCHL.[128] This disorder, also genetic in origin, appears to result from the overproduction of VLDL[129,130], and has a prevalence of 1 in 100 people.[131,132] When elevated, [TG] levels are usually between 200 and 400 mg/dl and LDL levels are about 190 mg/dl, but both may be significantly higher.

Type III dyslipidemia is also of genetic origin. In this disorder, CM and VLDL remnants are not efficiently removed resulting in a build up of [IDL-C].[133] Plasma cholesterol and [TG] levels are also increased, whereas both [LDL-C] and [HDL-C] levels are decreased.[134] Premature atherosclerosis is found in 30 to 50% of individuals with Fredrickson type III hyperlipoproteinemia.[135,136]

Type IV dyslipidemia is characterized by a moderate increase in the [TG], a slight increase in the [TC] and a slight decrease in the [HDL-C] level. The [TG] increase is achieved, not by producing more

VLDL particles, but by producing larger ones with greater TG and cholesterol content. This production of VLDLs with high TG content has been identified as the cause of familial hypertriglyceridemia (FHTG).[137] The exact cause of this disorder is unknown; however, studies indicate it is also probably genetic with an estimated frequency of 1 in 500 people.[138]

Finally, type V hyperlipoproteinemia is a disorder characterized by an increase in both chylomicrons and VLDL, subsequently [TG] is markedly elevated. The etiology of this disease is not known.[1] Little else is known about this disorder except that it does not seem to be risk factor for CHD.

For the sake of completeness is should be noted that another hyperlipidemia, namely hyper-α lipoproteinemia, does exist. In this "disorder", the TC is normal to moderately elevated due to an increase in HDL. According to E.A. Stein in Fundamentals of Clinical Chemistry, "No clinical features have been associated with hyper-α-lipoproteinemia....At present it is merely a biochemical description associated with a less than average cardiovascular disease."

Table 2-2. Classification of Hypercholestermias

Phenotype	Lipoprotein Abnormality (Increase)	Total Cholesterol	Triglyceride	LDL	HDL
Type I	Chylomicrons	normal to moderately elevated	markedly elevated	normal	normal to decreased
Type IIa	LDL	elevated	normal	elevated	normal to decreased
Type IIb	LDL, VLDL	elevated	elevated	elevated	normal to decreased
Type III	IDL	elevated	elevated	normal to decreased	normal to decreased
Type IV	VLDL	normal to slightly elevated	moderately to markedly elevated	normal	normal to decreased
Type V	VLDL, chylomicrons	slightly to moderately elevated	markedly elevated	normal	normal to decreased
Hyper-α-lipoproteinemia	HDL	normal to moderately elevated	normal	normal	elevated

CHAPTER III

CURRENT LIPID ANALYSIS TECHNIQUES

Since the first attempt to measure cholesterol colorimetrically by Liebermann[139] and Burchard[140] in the late nineteenth century, hundreds of analytical techniques have been developed to measure blood lipids, lipoproteins or lipoprotein subfractions. Such techniques have included chemical, enzymatic, and physical methods.[141-183] This proliferation of non-standardized methods led to significant biases between values obtained at different laboratories, in fact, laboratory specific "normal" reference values were not uncommon. The lack of standardization in the fields of lipid measurement and diagnosis standards moved the National Cholesterol Education Program (NCEP) to convene two panels of experts to develop guidelines for reliable lipid and lipoprotein measurements.[71] The NCEP Laboratory Standardization Panel[184] focused on the measurement of total cholesterol, while the NCEP Working Group on Lipoprotein Measurement[185] addressed the measurement of TGs, HDL-C and LDL-C.

Over the last 20 years, the CDC has maintained reference methods for the quantitative analysis of TC, TGs and HDL-C. Because CDC methods were used in several seminal lipid-disease relationship studies and were already accepted as authoritative by the general laboratory and research community, the NCEP panels adopted them as the reference standard in the evaluation of existing or newly developed techniques. It is important to note, however, that this adoption does not require all laboratories to use these methods; it only requires that the method used be capable of giving values equivalent to ones obtained by the CDC methods. The present discussion focuses on CDC reference methods, more routine clinical methods and general examples of the various techniques used in lipid and lipoprotein analysis.

CDC Reference Methods

Total Cholesterol

The reference method for cholesterol is a CDC modified version[69] of the ALBK method[186]. In this method, a 0.5mL aliquot of fasting serum is treated with 5.0mL of ethanolic KOH (0.36M) for 60 minutes at 50°C to hydrolyze the cholesteryl esters. The cholesterol is then extracted from the mixture with 10mL hexane for 15 minutes. An aliquot of the extract is dried in vacuo, and the dry residue is treated for exactly 30 minutes with 3.2mL of the Liebermann-Burchard reagent (a 20:10:1, by volume, mixture of acetic anhydride, glacial acetic acid and sulfuric acid, respectively) to develop the color, which is measured at 620 nm. This method is performed following a strict protocol requiring multiple runs. By convention, cholesterol is reported in terms of milligrams per deciliter, but may also be expressed in terms of molar concentration. Conversion between molar and mass concentration can be achieved using Equation 3-0.

$$\frac{mg}{dL} = \frac{mmol}{L}(38.7) \qquad (3\text{-}0)$$

Triglycerides

Triglycerides, unlike cholesterol, are not unique chemical entities. Triglycerides consist of three fatty acid acyl groups attached to a glycerol backbone through ester linkages. These fatty acyl groups can vary in chain length and degree of unsaturation. For this reason, triglyceride methods usually measure the glycerol backbone expressing the triglyceride concentration in terms of molar concentration. To convert to mass concentration, an assumption is made about the average molecular weight of the triglyceride mixture. Because the major fatty acids are of the C:18 class and have similar molecular weights, the

average TG molecular weight is approximated to be 885 g/mol, the molecular weight of triolein. Equation 3-1 is used to convert mmol/dL to mg/dl.

$$\frac{mg}{dL} = \frac{mmol}{L}(88.5) \qquad (3\text{-}1)$$

In the CDC reference method for TGs[70], the TGs are first extracted from the serum with chloroform to remove water soluble interferents such as glucose and free glycerol. The extract is then treated with silicic acid to remove any phospholipids. Triglycerides in the extract are subjected to alkaline hydrolysis to produce unesterified fatty acids and glycerol. The glycerol is oxidized with a periodate salt to form formic acid and formaldehyde. The formaldehyde is then reacted with chromotropic acid for color development. The absorbance of the chromagen at 570 nm is then measured.

High-Density Lipoprotein Cholesterol

The CDC reference method for [HDL-C] determination[71], sometimes called the beta-quantification method, uses a combination of ultracentrifugation and polyanion precipitation to prepare the HDL containing fraction. Because the term HDL encompasses a number of particles of various cholesterol content, "HDL" is here defined in terms of the method used to isolate the HDL containing fraction. The method is as follows: An accurately measured fasting plasma sample is ultracentrifuged for 18 h at 105,000g. During ultracentrifugation, VLDLs, as well as any chylomicrons present, accumulate as a floating layer at the top of the test tube. The tube is then sliced to remove the VLDL / chylomicron layer and the infranatant is recovered quantitatively. The infranatant now contains IDL, LDL and HDL fractions. Both IDL and LDL contain an apolipoprotein called apo B-100. This apolipoprotein is precipitated by the addition of heparin sulfate (1.3mg/ml) and $MnCl_2$ (0.046mol/L) leaving only HDL in the solution. The precipitate is removed via centrifugation and the [HDL-C] in the supernatant is measured using the CDC reference method for total cholesterol.

Routine Methods

Obviously, CDC reference methods are quite involved, time consuming, and require a high level of technical expertise. These disadvantages effectively prohibit the use of reference methods in a busy clinical environment. The following simplified techniques have been widely adopted for routine clinical use.

Total Cholesterol

Commercially available enzymatic kits have become the standard method for cholesterol determination. The kits combine all of the enzymes and other components into a single photometric reagent. A serum sample of 3 to 10 μL is mixed with the reagent, incubated under controlled conditions and the absorbance is measured, usually at about 500nm. The general reaction scheme is as follows:
In the first step, cholesteryl ester hydrolase converts the cholesterol esters to free cholesterol and free FAs.

$$\text{cholesteryl ester} + H_20 \xrightarrow{\text{cholesteryl ester hydrolase}} \text{cholesterol} + \text{fatty acid} \qquad (3\text{-}2)$$

From there, the 3-OH group of cholesterol is oxidized by cholesterol oxidase to form cholest-4-en-one and hydrogen peroxide.

$$\text{cholesterol} + 0_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholest-4-en-3-one} + H_2O_2 \qquad (3\text{-}3)$$

In the last step, peroxidase catalyzes the reaction of the hydrogen peroxide with phenol and 4-aminoantipyrine to produce quinoneimine dye, which has an absorbance maximum around 550nm.

$$H_20_2 + \text{phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{quinoneimine dye} + H_2O \qquad (3\text{-}4)$$

These methods have several interference factors (IFs) such as bilirubin, ascorbic acid, hemolysis, uric acid and other substances that consume H_2O_2. Studies have shown these affects to be pronounced at

[IF] above 5 mg/dl.[187-189] Further, these methods are not specific to cholesterol because β-hydroxy sterols and plant sterols, such as β-sitosterol, also react.

In practical applications the reagent mixtures vary from manufacturer to manufacturer. Generally the reagent mixture from a particular manufacturer will have been optimized for use with a specific instrument or family of instruments sold by that manufacturer. Therefore, routine lipid measurement methods should be thought of as "measurement systems" composed of reagent, instrument, and calibrating standards. When these measurement systems are automated, measurements usually fall within 1 to 3% of reference values.[82]

Triglycerides

Triglycerides are also measured enzymatically in either plasma or serum. Commercially available single kits combining all the required enzymes, cofactors and buffers are available and widely used. The TG kits are optimized for use with a particular instrument-calibrator system. Several enzymatic methods have been used and are outlined below.

In all of the methods, the first step is the hydrolysis of TGs to glycerol and fatty acids.

$$\text{triglyceride} + 3\,H_2O \xrightarrow{\text{lipase}} \text{glycerol} + 3\ \text{fatty acid} \qquad (3\text{-}5)$$

Glycerol then reacts with adenosine triphosphate (ATP) in a reaction catalyzed by glycerokinase to produce glycerophosphate (GP) and adenosine diphosphate (ADP).

$$\text{glycerol} + \text{ATP} \xrightarrow{\text{glycerokinase}} \text{GP} + \text{ADP} \qquad (3\text{-}6)$$

In most other methods, GP is then oxidized to dihydroxyacetone and H_2O_2 in a glycerophosphate oxidase-catalyzed reaction.

$$\text{GP} + O_2 \xrightarrow[\text{oxidase}]{\text{glycerophosphate}} \text{dihydroxyacetone} + H_2O_2 \qquad (3\text{-}7)$$

Then, just as in reaction (3-4), the H_2O_2 is reacted with phenol and 4-aminoantipyrine to produce quinoneimine dye, whose concentration is then measured.

Alternate methods react the GP produced in reaction (3-6) with NAD to produce NADH which is measured spectroscopically at 340 nm (reaction (3-8)) or reacted with tetrazolium dye to produce formazan where the absorbance can be measured at 500 nm (reaction (3-9)).

$$GP + NAD \xrightarrow{\text{glycerophosphate dehydrogenase}} \text{dihydroxyacetone phosphate} + NADH + H^+ \qquad (3\text{-}8)$$

$$NADH + \text{tetrazolium dye} \xrightarrow{\text{diaphorase}} \text{formazan} + NAD^+ \qquad (3\text{-}9)$$

Still other methods calculate the triglyceride concentration by measuring the amount of ADP produced in reaction (3-6). These methods react the ADP with phosphoenol pyruvate to produce ATP and pyruvate, reaction (3-10). The pyruvate is then reacted with NADH to produce lactate and NAD^+, as described in reaction (3-11). The loss of the NADH is measured photometrically at 340nm.

$$ADP + \text{phosphoenol pyruvate} \xrightarrow{\text{pyruvate kinase}} ATP + \text{pyruvate} \qquad (3\text{-}10)$$

$$\text{pyruvate} + NADH + H^+ \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + NAD^+ \qquad (3\text{-}11)$$

Enzymatic triglyceride methods are specific in that they do not detect glucose or phospholipids. They are linear up to approximately 700 mg/dL, and when automated have coefficients of variation of 2 to 3%.[82]

Because all of the aforementioned methods effectively measure free glycerol derived from triglycerides, each is susceptible to bias from endogenous free glycerol. Glycerol concentrations in fresh serum or plasma samples are usually in the range of 1-10 mg/dL.[82] At this normal level the effects of the free glycerol are simply ignored. However, in cases of hyperglycerolemia, free glycerol levels can be elevated 50 to 100 fold,[190] effectively invalidating routine triglyceride measurements. In such cases, a triglyceride blank is taken and subtracted from the total triglyceride measurement. The triglyceride blank is simply a measure of the free glycerol present in the sample and requires a separate analysis. This can

be achieved enzymatically by reactions (3-6) through (3-11), using a reagent mixture identical to the triglyceride reagent, but lacking the lipase.

High-Density Lipoprotein Cholesterol

High-density lipoprotein cholesterol is most commonly measured in the supernatant of a plasma or serum sample following the precipitation of the apo B-100-containing lipoproteins.[191] As indicated earlier, the apo B containing lipoproteins include VLDL, IDL, LDL, and chylomicrons. Once these lipoproteins are removed, the remaining cholesterol, ostensibly from HDL, is measured using the routine enzymatic methods previously mentioned.

Several precipitating reagent systems that have been used, however, all consist of a polyanion-divalent cation combination.[185] In this approach, the polyanions react with the positively charged groups on the lipoproteins while the cations interact with the negatively charged groups of the lipoprotein immediately forming a heavy precipitate. Precipitation is complete in 10 to 15 minutes at room temperature and the precipitate is sedimented by centrifugation for at least 45,000 g-min. The [HDL-C] is then measured in the supernatant.

While this method seems relatively straightforward, there are some analytical considerations to keep in mind. First, this method assumes the cholesterol in the supernatant is entirely from the HDL lipoprotein; however, this may not always be the case. The idea of being able to precipitate select lipoproteins while keeping others in solution is based upon the premise that there are compositional differences between the lipoprotein classes making some molecules more susceptible to precipitation than others.[192] The lipoprotein cascade mentioned in Chapter II blurs this delineation between the lipoprotein classes making such an assumption tentative at best. Furthermore, the various precipitating reagent systems vary in their ability to precipitate apo B-100-containg lipoproteins producing an inherent bias between precipitation methods.[185,193] Second, in samples with triglyceride-rich lipoproteins incomplete sedimentation can occur. High triglyceride concentrations (>400mg/dL) reduce the density of the lipoprotein-precipitating reagent complex causing some of the complex to remain in the supernatant

producing large error in the [HDL-C] measurement. Third, matrix effects, i.e. interference from the sample itself or from the reagent added, can influence the measurements. For instance, anticoagulants such as EDTA and additives such as citrate and fluoride have large osmotic effects causing water to shift from the cells to the plasma diluting the lipoprotein and producing erroneously low values.[194] Finally, the enzymes used in the final step of this method, in fact all the enzymatic methods outlined in this section, can be a source of error. As with all enzymatic procedures, deterioration of or variation in the source of the enzyme can occur. These effects appear as lot-to-lot and instrument-to-instrument imprecision.

Low-Density Lipoprotein Cholesterol

As mentioned in the introduction, [LDL-C] determination methods are indirect and based on the assumption that the [TC] level may be approximated by equation (3-12).

$$[TC] = [VLDL\text{-}C] + [LDL\text{-}C] + [HDL\text{-}C] \qquad (3\text{-}12)$$

In the most widely used indirect method for [LDL-C] determination, [TC], [TG], and [HDL-C] are measured and the [LDL-C] is calculated using the Friedewald equation:

$$[LDL\text{-}C] = [TC] - [HDL\text{-}C] - [TG] / 5 \qquad (3\text{-}13)$$

Where [TG] / 5 is an estimate of the [VLDL-C] based on an average ratio of TG to cholesterol in VLDL. The justifications for these assumptions are as follows: 1) [IDL-C] accounts for only about 2-3mg/dL of the [TC][82], 2) the 95th percentile for fasting plasma TGs in the United States is <300mg/dL[195], 3) normal fasting plasma samples do not contain chylomicrons and 4) the prevalence of both type III and IV dyslipidemias is only about 1 per 500 people.[190,195]

The assumptions enumerated above break down under the following circumstances. In the case of type III dyslipidemia, chylomicron and VLDL remnants are not efficiently removed resulting in a build up of IDL and decreased HDL and LDL levels. This condition results in the majority of the [TC] coming from the [IDL-C]. In the case of type IV dyslipidemias, the "normal" composition of the VLDL particle is

changed and the factor [TG] / 5 to approximate the [VLDL-C] no longer holds. In samples that have high [TG], >400mg/dL, or that contain chylomicrons (non-fasting samples) the error in the Friedewald equation becomes unacceptably large, i.e. >10%.[196]

In cases where the Friedewald equation may not be successfully applied an alternate method is routinely used. This method[197], based on a procedure used in the Lipids Research Clinic (LRC) Program at the National Institutes of Health (NIH), uses a combination of ultracentrifugation and polyanion precipitation.

In this method, the [TC] is first measured by routine methods. Then a measured aliquot of plasma is ultracentrifuged at 1.006 g/mL (plasma density) at 105,000 x g for 18 hours at 10°C. After centrifugation, the VLDL and any chylomicrons present appear as a floating layer with the infranate containing the HDL, LDL, and IDL. The supernatant is then removed via a tube slicer. The infranate is reconstituted to a known volume and its cholesterol content is measured. The infranate may also be treated to precipitate the apo B-100-containing lipoproteins IDL and LDL and the residual HDL-C is then measured in the clear supernate. Cholesterol concentrations of VLDL and LDL are measured as follows:

$$[\text{VLDL-C}] = [\text{TC}] - [\text{d} > 1.006\ \text{g/mL cholesterol}] \qquad (3\text{-}14)$$

$$[\text{LDL-C}] = [\text{d} > 1.006\ \text{g/mL cholesterol}] - [\text{HDL-C}] \qquad (3\text{-}15)$$

It is clear in both of these equations that approximations are still being made. In equation (3-14) the [VLDL-C] would include any chylomicrons present, necessitating the further analysis of the supernatant. In equation (3-15), the [IDL-C] is altogether ignored. The benefit to this approach is that, barring elevated IDL levels, the [LDL-C] is unaffected by chylomicrons or other TG-rich lipoproteins. Despite these shortcomings and indications that IDL-C may be associated with increased risk of CHD, the NCEP Working Group on LDL-C felt that LDL-C levels should not be corrected for the contribution of other atherogenic lipoproteins; further recommending that research be conducted to establish the individual contributions of IDL-C and LDL-C to CHD risk as reflected in current LDL-C measurements that include the three main lipoprotein classes, HDL, LDL and VLDL.

Other Methods

Chromatography

Chromatography is defined as "...a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary while the other moves in a definite direction."[198] This broad definition encompasses a number of various approaches to lipid analysis including, but not limited to,: thin layer chromatography (TLC), gas chromatography (GC), and high-performance liquid chromatography (HPLC). The principle goal of each of these methods is to separate a mixture into its individual components or analytes. A chromatographic separation requires a sample to be introduced into a flowing stream of gas or liquid (mobile phase) that passes by or through a bed, layer, or column containing the stationary phase. This stationary phase may be particles of a solid, liquid or gel. If liquid, it may be distributed on solid particles that may or may not contribute to the separation process. In general, as the mobile phase carries the sample past the stationary phase, the solutes with lesser affinity for the stationary phase remain in the mobile phase and travel faster and separate from those that have a greater affinity for it. Various separation mechanisms include adsorption chromatography, affinity chromatography, ion-exchange chromatography, partition chromatography and size exclusion chromatography. Once the sample passes by/through the stationary phase, the effluent, i.e. mobile phase with its separated solute zones, then passes through a detector which processes the signal and prints out the information graphically as a series of peaks (a chromatogram). The information represented in the chromatogram can be used to quantify and/or partially identify the solute(s).

Thin-layer chromatography, also known as planar chromatography, has been used for the separation of lipid mixtures nearly since its conception by Stahl[199] in 1956.[200-206] In this method, a thin layer (usually 0.2mm) of sorbent material is uniformly spread on a sheet of glass, plastic or aluminum.[207] A small spot of sample is applied to the plate and the plate is then placed into a closed container containing a small volume of the mobile phase. The plate is placed in a vertical position in the tank such

that the mobile phase is below the spot of sample application. The mobile phase then ascends the plate by capillary action and resolves the mixture by differential migration. Reference compounds are chromatographed next to the unknown to aid in identification of the analyte. After the mobile phase reaches a desired height, the plate is removed and allowed to air dry. Further separation is sometimes achieved by developing the plate in a second dimension and/or with a different mobile phase. After drying, the separated components may be located by a variety of procedures. These procedures include ultraviolet illumination, spraying with chromagenic reagents, or oxidation and charring. Thin-layer chromatography is a popular method of lipid analysis for several reasons: 1) good qualitative results can be obtained using a minimum of equipment and expense, 2) analyses are done quickly and many samples can be analyzed simultaneously, 3) the separated classes may be visualized and the chromatograms kept as a permanent record or, if necessary, 4) the appropriate sorbent region may be scraped to recover the analyte for further analysis. The drawbacks of this techniques are that quantification is difficult because the size and density of the spots depend on many variables. Furthermore, just because a reference compound and the analyte of interest migrate at the same rate is not sufficient evidence to identify that compound with any certainty.

Gas chromatography is a process by which a solution is separated into its constituent components by forcing a gaseous mixture of it and the mobile phase, a carrier gas, through a column containing the stationary phase. The basis for this separation is the relative differences in the vapor pressures of the components and their interaction with the stationary phase. Compounds with high vapor pressures elute more quickly than those with low vapor pressures and those having little interaction with the stationary phase will elute faster than those with stronger interactions with the stationary phase.

This method has been used extensively in the analysis of all the major lipid classes.[208-214] In many cases GC analyses are performed on intact molecular species. However, in the case of nonvolatile or thermally unstable substances, i.e. TGs and phospholipids, the high temperatures required for the elution of intact lipids can cause thermal decomposition. Loss of material is dependent on the molecular weight and degree of unsaturation of a given compound.[215] While many fats and oils, especially saturated ones, may be analyzed by this method, the analysis of fish oils (high in PUFAs) has shown the

unsuitability of GC in the analysis of TGs high in PUFA content. Further, loss of steryl fatty acid ester bearing moieties, i.e. cholesteryl arachidonate, also occur.[215]

High-performance liquid chromatography is the analog of gas chromatography. In this technique the stationary phase is held in a metal column and the liquid mobile phase is pumped through an efficient, or tightly packed, column under high pressure. As the column efficiency is inversely related to the particle size of the column packing, high pressure is required to force the liquid through the column. The high-pressure pump can be operated in either isocratic or gradient mode. In isocratic mode, the mobile phase composition remains the same throughout the run. This is the most common mode and is used to separate compounds with similar structures and/ or retention times. When a large number of compounds must be separated or when the range of compound polarities is great, gradient elution is used. In the gradient mode, the composition of the mobile phase is changed, either stepwise or in a continuous fashion, throughout the run to further effect the separation. Once separated, the analytes may be quantitatively measured and sometimes qualitatively identified using a variety of detection techniques including ultraviolet and visible photometry, fluorescence, refractive indexometry or mass spectrometry.

Traditionally, the greatest value of HPLC had lain in the analysis of components which were too involatile or too unstable for the high temperatures required in gas chromatography. It was also used for preparative scale separations for the isolation of particular FAs for structural analysis. Today, however, HPLC is widely used in the analysis of all lipid classes. In recent years, with the advancement of column technology and detector interfaces, especially for mass spectrometers, HPLC has come into its own as the preeminent technique in lipid analysis. It has been proven effective in the analysis of free fatty acids[216-219], fatty acid methyl esters[219,221], monoacylglycerols[222], diacylglycerols[223,224], triacylglycerols[225,226], phospholipids[227-230], cholesterol[231] and cholesterol esters[232].

The main drawbacks to this technique are its high equipment and operational costs, the high maintenance involved, and the high level of technical expertise required. While a full review of HPLC is beyond the scope of this text, the interested reader is directed to recent review articles[233-244] of its current role in lipid analysis.

Electrophoresis

Electrophoresis refers to the migration of charged solutes or particles in a liquid or gel medium under the influence of an electrical field. The rate of migration of the charged species is directly proportional to the net charge of the molecule and inversely proportional to the size of the molecule and viscosity of the electrophoresis medium. Other factors affecting the rate of migration are electric field strength and temperature.

This method was first used to study proteins by Tiselius[245] in 1937. Since that time, electrophoresis has been applied to a wide range of solutes of interest in clinical chemistry including lipoproteins. Because the lipoproteins are zwitterionic, containing both positive and negative charges, macromolecular complexes, the net charge on the lipoprotein is determined by the pH of the solution in which it is found. For instance, in a solution more acidic than the isoelectric point of the solute, the lipoprotein takes on a positive charge and migrates toward the cathode. Conversely, when the solution is more basic than the isoelectric point of the lipoprotein, the lipoprotein is in anionic form and migrates toward the anode. The large disparity in size (see photo #) and the difference in the net charge of the lipoprotein classes are the primary bases of lipoprotein electrophoresis.

Once the lipoprotein fractions have been separated, stains are usually used to visualize and locate the fractions. Results are customarily reported in terms of the percentage of each fraction present or, if the total quantity of lipoprotein is known, in terms of absolute concentration. Quantitation of the dye in the respective zones is accomplished by direct densitometry. Densitometry is an instrumental method in which the electrophoretic strip is moved past an optical measuring system which automatically records and integrates the absorbance peaks of each fraction.

In an approach marketed by Zaxis, Inc. (Hudson, OH), serum samples are separated by gradient gel electrophoresis.[82] In this method, size separation of the lipoproteins is achieved by electrophoresis of the solutes through a polymer which acts as a molecular sieve. As the charged solutes migrate through the polymer network, they become hindered, with large molecules being more hindered than small ones, effecting the separation. The electropherogram is then densitometrically scanned and converted to

equivalent lipoprotein cholesterol concentrations using assumed average cholesterol compositions for the lipoproteins. This technique, however, has not been well received because of resolution problems and differences in staining intensity of the various lipoproteins. Other methodological difficulties and limitations have been well reviewed[186,246] and have prompted the NCEP Working Group on Lipoprotein Measurement to recommend that electrophoretic methods not be used for lipoprotein quantitation.[185]

CHAPTER IV

EXPERIMENTAL METHODS

The present study is based on a color producing reaction first described by Chugaev and Gastev.[247] In this reaction, a mixture of zinc chloride ($ZnCl_2$), glacial acetic acid and acetyl chloride was reacted with cholesterol to produce a colored product. In its original form, the reagent system was a 2:1 mixture (by volume) of 20% $ZnCl_2$ in glacial acetic acid (weight/volume) and acetyl chloride. The experimental procedure in early studies using this reagent mixture was to add a 2mL aliquot of the reagent to a 50μL aliquot of serum sample and incubate the solution for 8 minutes at 67°C for color development. Several absorbance studies[248-251] have used this reagent system to successfully measure total cholesterol levels. The obvious drawback to this formulation is the high incubation temperature required for color production.

In more recent studies by Purdie et al.,[79,80] the original Chugaev reagent system was modified until a mixture was found that was chemically stable and, when reacted with cholesterol, produced color of sufficient intensity under ambient laboratory conditions. These studies have demonstrated that [TC], [HDL-C], [LDL-C], and [VLDL-C] levels can be measured simultaneously in a single, non-enzymatic, spectroscopic assay. In each of these studies, however, it was assumed that the reagent system reacted with only cholesterol and/or its esters in the serum sample. Color variations between the various lipoprotein classes were attributed to "matrix" or solvent-like effects. By the beginning of the present study, the experimental method, i.e. the spectral absorbance data collection method for sera, had evolved into the following general routine. To a 13x100mm glass test tube add 1) 10μl of serum; 2) 1mL of a 3:2 mixture (by volume) of acetyl chloride (AC) and 1,2-dichloroethane (DCE); 3) 40μl of 70% perchloric acid (PA) and start the reaction timer. Centrifuge the mixture for 3 minutes at 3400 rpm then transfer the

supernatant into a glass cuvet. After a 15 minute incubation at room temperature, measure the absorbance spectra from 350nm to 750nm.

This study is a continuation of the work done by Purdie and coworkers in the development of the aforementioned reagent system with the following well defined goals: 1) refine the current reagent system and technique proposed in the previous studies; 2) systematically identify which lipid classes chromogenically react with this reagent system; and 3) use the information gained in the first two goals to develop a method for identifying various serum dyslipidemias.

Reagent Formulation and Refinement

The robustness of the reagent system used in the studies mentioned previously[79,80] was explored by varying the proportion of each component in turn and studying the resultant spectral effects. For this series of experiments, a standard solution of cholesterol, 210mg/dL, was prepared by dissolving 21mg of pure cholesterol in 10mL of spectrophotometric grade chloroform. This standard solution of cholesterol was used for each of the following experiments. The procedure consisted of adding a 10μL aliquot of the standard cholesterol solution to a 13x100mm glass test tube followed by a 1mL aliquot of a 3:2 AC:DCE mixture (by volume) and a 40μL aliquot of PA, in that order. Immediately following the addition of the PA, a reaction timer was started. (It should be noted that this procedure should always be carried out in a fume hood with proper protection as AC and DCE are both strongly irritating to the eyes, respiratory system and skin, and, upon the addition of the PA, a strong evolution of HCl gas occurs.) This solution was immediately transferred to a semi-micro glass cuvet (Starna Cells, Inc., Atascadero, CA.) with a volume capacity of 1.4mL and a path length of 10mm (UV/VIS cell) and incubated for a total of 20 minutes at room temperature. After the 20 minute incubation period, the cell was placed in the cell holder of the spectrophotometer and the absorbance spectrum of the solution was measured from 350-750nm using a Hewlett-Packard 8452A diode-array spectrophotometer (HP 8452A) with an integration time of 1.0 second. The entire procedure was performed thirteen times, each time varying only the AC:DCE

mixture ratio. The following ratios of AC:DCE were examined: pure DCE, 1:4, 1:3, 1:2, 2:3, 1:1, 3:2, 2:1, 3:1, 4:1, 5:1, 10:1, and pure AC. The absorbance spectra were inspected for the number of peaks produced and peak intensity. The decision was made to use pure AC for the remainder of this study (see Chapter V - Results and Discussion).

The effects of varying the amount of PA added to pure AC were compared kinetically. This was accomplished by again preparing a standard solution of cholesterol (220mg/dL) to be used in a series of experiments. The procedure consisted of adding a 10μL aliquot of the standard cholesterol solution to a glass test tube followed by a 1mL aliquot of pure AC and a carefully measured aliquot of PA, in that order. Immediately following the addition of the PA, the solution was transferred to a UV/VIS cell. The absorbance spectrum of the solution was measured from 350-750nm each minute for a total of twenty minutes. Unless specifically stated otherwise, these spectrophotometer settings and procedures are used throughout the remainder of this study. The entire procedure was performed six times, progressively increasing the aliquot of PA from 0μL to 100μL in 20μL increments. Plots of peak heights versus time at wavelengths of 362, 412 and 520nm were then constructed for each run. The amount of PA added was seen to make little to no difference over the range of 20 to 100μL. Unless expressly stated otherwise, the newly refined reagent system, also referred to as the standard reagent, consisting of 1mL of AC immediately followed by a 40μL addition of PA is used for the remainder of this study.

Sample Preparation

In the present context a sample may be either an individual lipid from the various classes outlined in Table 1-0, or a mixture of these lipids, or a human serum. A listing of all individual compounds used in this study is found in Appendix A. Appendix B lists all compounds found to chromogenically react with the newly modified reagent system. Each pure lipid compound, hereafter generically referred to as a standard reference material (SRM), as well as all reagents and solvents were individually purchased from the Sigma-Aldrich Fine Chemical Company, St. Louis, Mo. Upon receipt,

standard materials were stored according to shipping and storage instructions in a standard refrigerator/freezer unit. At the time of analysis, each SRM was dissolved in spectrophotometric grade chloroform to prepare individual solutions with concentrations ranging from about 200-400mg/dL. Mixtures of the SRMs were likewise prepared at various concentrations. (Note: when an aliquot of a "sample" is said to be taken, it should be understood that the sample is dissolved in chloroform, except in the case of serums which are never put into chloroform.) All SRM solutions were analyzed within 24 hours of preparation.

Human serum samples were obtained from the Oklahoma State University Wellness Center (OSUWC). The donors were volunteers who had requested a lipid profile. The subjects were not screened in any way and include individuals in both fasting and postprandial states. Serum collection was done by drawing a venous blood sample from the arm of each subject into two Vacutainer™ serum separation tubes. The tubes were allowed to cool to room temperature. After about 15 minutes, they were centrifuged (Roche Biomedical Laboratories VanGuard 6000 table-top centrifuge) at 5,000 rpm for about five minutes or until the blood cells and plasma had completely separated. One tube was taken to the Oklahoma State University Department of Chemistry for spectral analysis, the other was sent to Regional Medical Laboratory, Inc. (RML) of Stillwater, Oklahoma to be analyzed for [TC], [TG], [HDL-C] and [LDL-C]. Sera for spectral analysis were transferred from the Vacutainer™ tube via a sterile syringe to a glass scintillation vial and stored at 4° C until analyzed.. For each serum sample, both absorbance and fluorescence spectra were taken within 24 hours of collection.

Spectral Acquisition Procedures

Absorbance - Standard Material

A 10μL aliquot of each SRM was prepared and analyzed as previously outlined.

Absorbance - Serums

In the case of a serum sample, a precipitate formed immediately upon the addition the PA. The procedure is exactly the same as above except that a centrifugation step was added. Following the addition of the PA, the solution was centrifuged for 3 minutes at 3400 rpm using a Fisher™ Centrific™ Model 228 tabletop centrifuge. Following centrifugation, the supernatant solution was transferred to a UV/VIS cell and its absorbance spectrum was taken each minute for 17 minutes.

Fluorescence

In a fluorescence emission spectrum, the sample is excited at a single wavelength, e.g. 400nm, and the emission spectrum is measured over a given range, e.g. from 350-650nm. In this study, a series of emission spectra were obtained for each sample by varying the excitation wavelength from 350-650nm in increments of 5nm. The resultant spectra were then collected together to produce a 3-dimensional plot of the entire emission matrix. The excitation spectra were handled in a similar manner with the emission wavelengths being varied from 350-650nm in increments of 5nm.

Kinetics plots of peak heights versus time in the absorbance spectra showed that the 520nm peak associated with cholesterol was 98% developed after 20 minutes; however, the 360nm and 412nm peaks associated with PUFAs in both serum and standard material continued to react for several hours. Each fluorescence matrix scan consisted of sixty individual scans and took approximately one hour to complete. The obvious problem here was that the concentration of the product(s) changed throughout the scan, in

effect deforming the true surface profile of the 3-dimensional emission/ excitation matrix. This problem was addressed in two ways. First, it was found that the addition of a small amount of glacial acetic acid (GAC) to the solution would halt any further reaction for about one hour. This addition of GAC provides one with a "snapshot" of the reaction at twenty minutes that may be studied fluorometrically over a period of one hour. Second, to minimize the time each sample spends in the fluorometer, fresh solutions of each sample were prepared for each emission and excitation scan. The exact procedure is outlined below.

Fluorescence - Standard Material

Two 10μL aliquots of the standard material sample to be analyzed were transferred into separate glass 13x100mm test tubes, hereafter referred to as A and B. The standard reagent system was added to each tube and both solutions were left in the hood to age for 20 minutes. At the end of the twenty minutes, the absorbance spectrum of the solution in A was measured from 350-650nm using the HP8452A spectrophotometer. The solution in B was then diluted with standard reagent (1ml AC:40μL PA) accordingly to get a maximum absorbance between 0.1 and 0.2 absorbance units (A.U.). For every 1ml of AC in B, 50μL of GAC was added and the diluted solution was transferred to a standard glass fluorometric cell with a path length of 10mm and a volume capacity of 3.5mL (Starna Cells, Inc., Atascadero, CA.). Fluorescence emission matrix scans were measured on a SPEX FLUOROLOG-3-Tau-11 spectrofluorometer (Horiba Group, Edisn N.J.). The procedure was repeated exactly for the fluorescence excitation matrix scans. All emission and excitation spectra were measured from 350nm to 650nm in 5nm increments. Integration time was set to 0.1s with slit widths of 3nm for both emission and excitation. The steady-state fluorescence is measured at a right angle to the incident radiation and the signal is divided by the lamp intensity at each wavelength, no other spectral correction were made. Other options chosen from the SPEX software package to enhance the spectra were dark offset, a 2 second pause between each scan, and the masking of first and second order Rayleigh peaks.

Fluorescence - Serums

The procedure for obtaining the emission and excitation matrix spectra of serums is exactly the same as for the standard material except that following the addition of the standard reagent to both A and B, each is centrifuged for three minutes of the twenty minute aging period.. Following centrifugation the supernatant liquid is poured off into clean test tubes again marked A and B respectively and allowed to age for seventeen more minutes. The remainder of the procedure is the same.

CHAPTER V

RESULTS AND DISCUSSION

Reagent Formulation

The robustness of the reagent system used in previous studies[79,80] was explored by varying the proportionality of each component in turn and studying the resultant spectral effects. As outlined in Chapter IV, the AC:DCE ratio in the reagent system was varied over a wide range while holding the proportions of perchloric acid and cholesterol standard constant. Figures 5-1 to 5-13 show the absorbance spectra collected for each of the ratios examined. It was found that all of the AC:DCE ratios produced three distinct absorption bands in the range of 350-650nm. These three features were found at 362, 412 and 520nm in each plot. The ratios of the peak intensities were the major differences in the spectra. Acetyl chloride alone was chosen to be the preferred reagent for two reasons. First, there were no more or fewer peaks obtained with pure AC than for any of the other mixtures and pure AC gave the greatest overall spectral intensity. Second, eliminating the carcinogenic DCE from the mixture simplified the experimental procedure and greatly reduced possible health hazards.

In a subsequent series of experiments, the amounts of pure AC and cholesterol standard in the mixture were held constant and the effects of varying the amount of PA added to the system were studied kinetically. As described in Chapter IV, the amount of PA added varied from 0 to 100μL in 20μL increments. Figures 5-14 to 5-18 plot the time evolutions of the peak heights (at 362, 412 and 520nm from one to twenty minutes) for each increment. It was found that the PA was necessary for color development, but the amount of PA added made little to no difference over the range of 20 to 100μL. A 40μL aliquot was chosen because it produced good overall absorbance intensities at each of the three

wavelengths of interest. It should be noted, however, that 60μL of PA also produced good overall intensities and could just as well have been chosen. For the sake of clarity, it should be noted that the newly refined standard reagent system (SRS) of 1mL AC and 40μL PA is used for the remainder of this study.

The SRS itself was found to be chromogenic and develop a weak absorption spectra (approximately 0.04 A.U.) over a period of twenty minutes. Figure 5-19 plots the absorbance spectra of the SRS at twenty minutes. The SRS was also found to fluoresce slightly. (Throughout this study, it should be understood that absorbance and fluorescence wavelengths of interest ranges from 350-650nm.) Figures 5-20 and 5-21 are contour plots of the emission and the excitation fluorescence spectra, respectively, of the SRS. The largest intensity recorded in each of the SRS fluorescent spectra was on the order of 10^5 counts per second (cps). The Z-axis of all fluorescent plots in this study is the emission intensity in cps. Each contour plot is scaled about the Z-axis to reflect the greatest number of spectral features; this scaling is not uniform from one contour plot to another.)

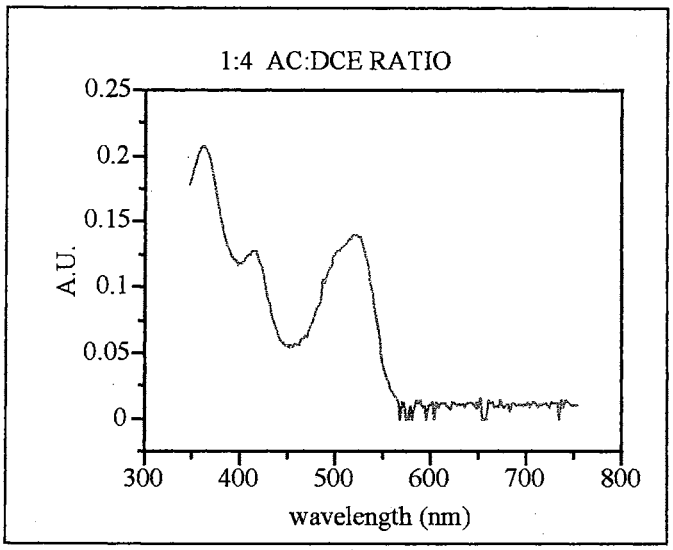


Figure 5-1. Absorption spectrum of the colored products of the reaction of cholesterol and a reagent system consisting of perchloric acid and a 1:4 mixture (by volume) of acetyl chloride and 1,2 dichloroethane after twenty minutes.

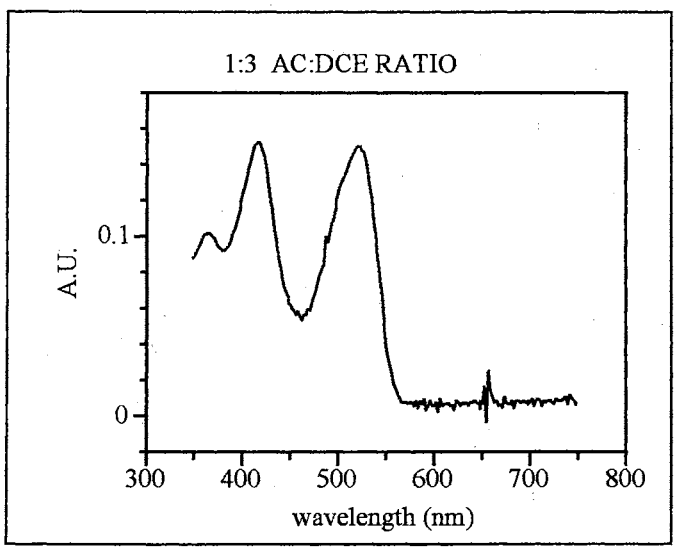


Figure 5-2. Absorption spectrum of the colored products of the reaction of cholesterol and a reagent system consisting of perchloric acid and a 1:3 mixture (by volume) of acetyl chloride and 1,2 dichloroethane after twenty minutes.

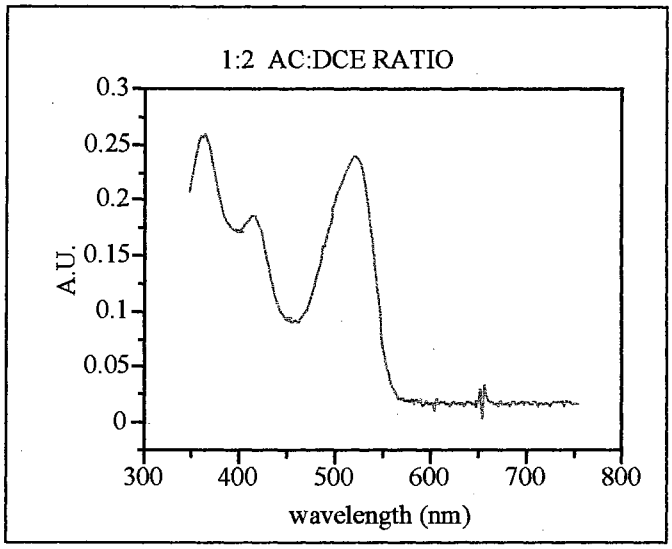


Figure 5-3. Absorption spectrum of the colored products of the reaction of cholesterol and a reagent system consisting of perchloric acid and a 1:2 mixture (by volume) of acetyl chloride and 1,2 dichloroethane after twenty minutes.

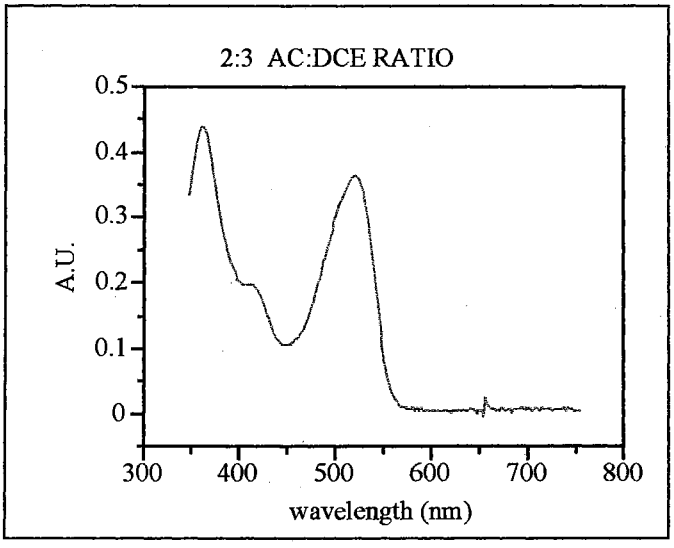


Figure 5-4. Absorption spectrum of the colored products of the reaction of cholesterol and a reagent system consisting of perchloric acid and a 2:3 mixture (by volume) of acetyl chloride and 1,2 dichloroethane after twenty minutes.

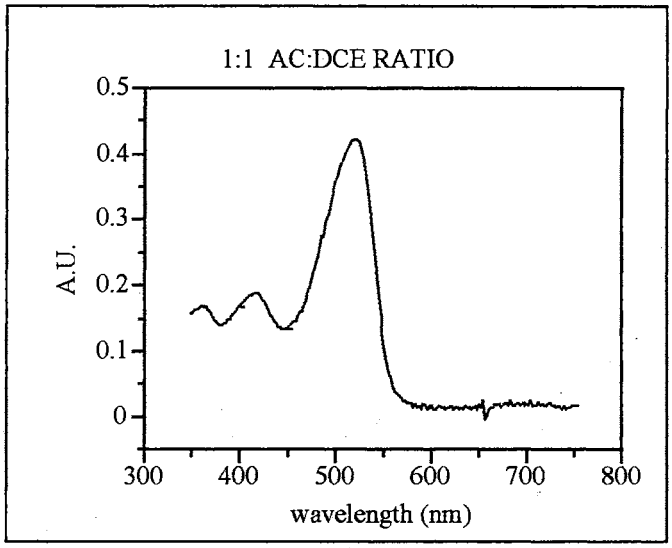


Figure 5-5. Absorption spectrum of the colored products of the reaction of cholesterol and a reagent system consisting of perchloric acid and a 1:1 mixture (by volume) of acetyl chloride and 1,2 dichloroethane after twenty minutes.

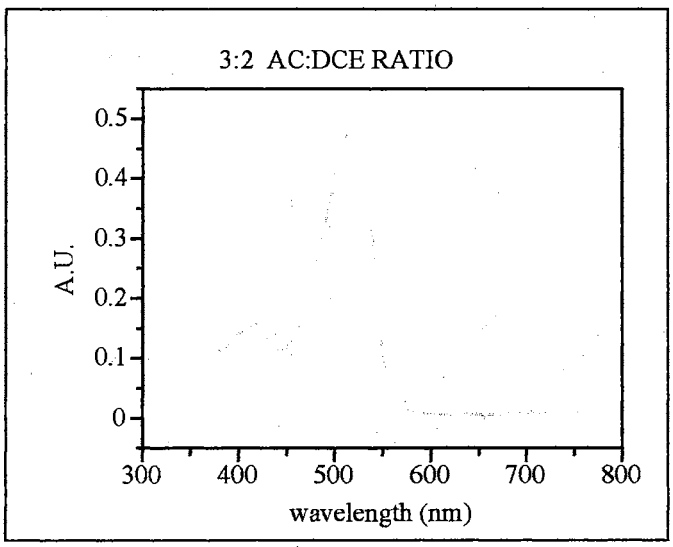


Figure 5-6. Absorption spectrum of the colored products of the reaction of cholesterol and a reagent system consisting of perchloric acid and a 3:2 mixture (by volume) of acetyl chloride and 1,2 dichloroethane after twenty minutes.

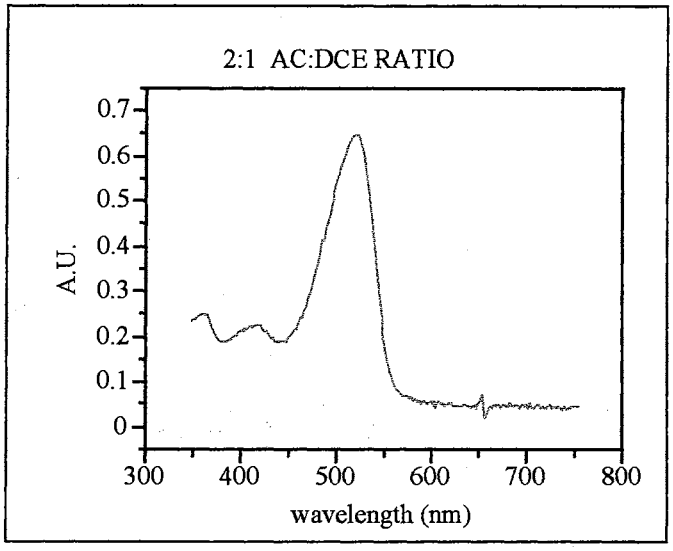


Figure 5-7. Absorption spectrum of the colored products of the reaction of cholesterol and a reagent system consisting of perchloric acid and a 2:1 mixture (by volume) of acetyl chloride and 1,2 dichloroethane after twenty minutes.

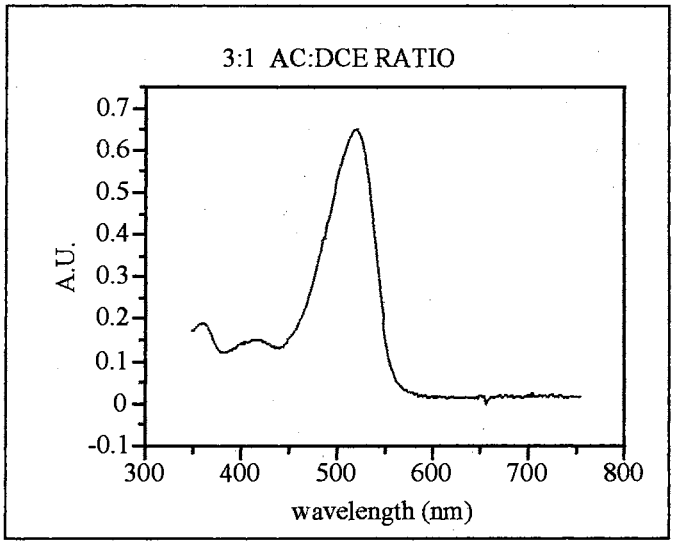


Figure 5-8. Absorption spectrum of the colored products of the reaction of cholesterol and a reagent system consisting of perchloric acid and a 3:1 mixture (by volume) of acetyl chloride and 1,2 dichloroethane after twenty minutes.

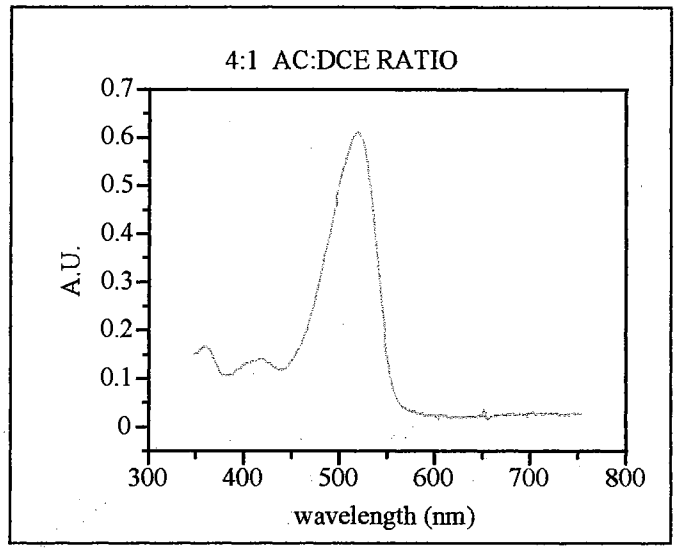


Figure 5-9. Absorption spectrum of the colored products of the reaction of cholesterol and a reagent system consisting of perchloric acid and a 4:1 mixture (by volume) of acetyl chloride and 1,2 dichloroethane after twenty minutes.

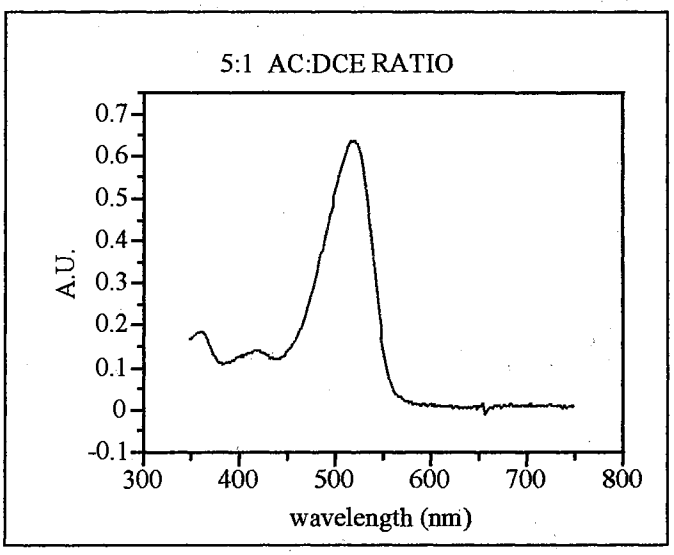


Figure 5-10. Absorption spectrum of the colored products of the reaction of cholesterol and a reagent system consisting of perchloric acid and a 5:1 mixture (by volume) of acetyl chloride and 1,2 dichloroethane after twenty minutes.

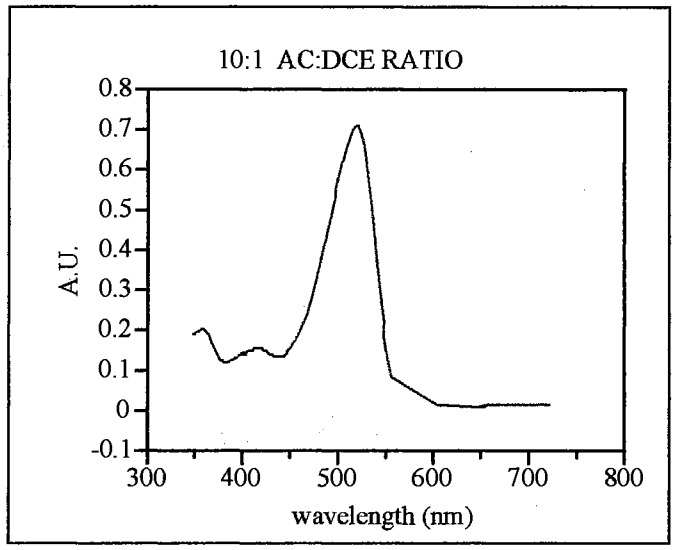


Figure 5-11. Absorption spectrum of the colored products of the reaction of cholesterol and a reagent system consisting of perchloric acid and a 10:1 mixture (by volume) of acetyl chloride and 1,2 dichloroethane after twenty minutes.

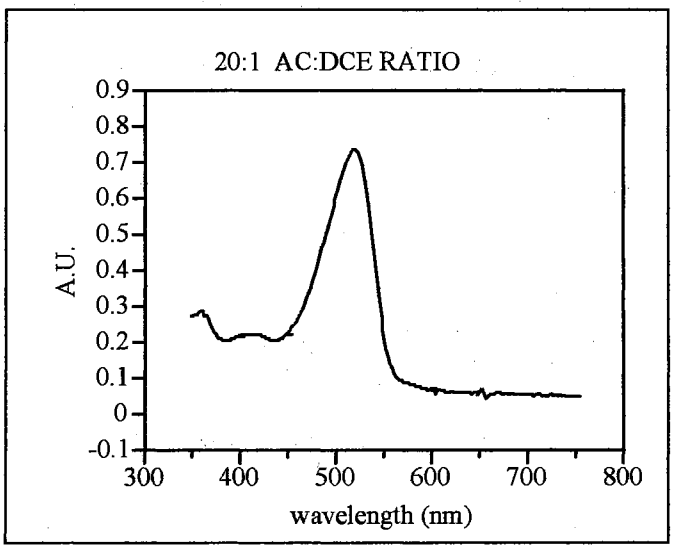


Figure 5-12. Absorption spectrum of the colored products of the reaction of cholesterol and a reagent system consisting of perchloric acid and a 20:1 mixture (by volume) of acetyl chloride and 1,2 dichloroethane after twenty minutes.

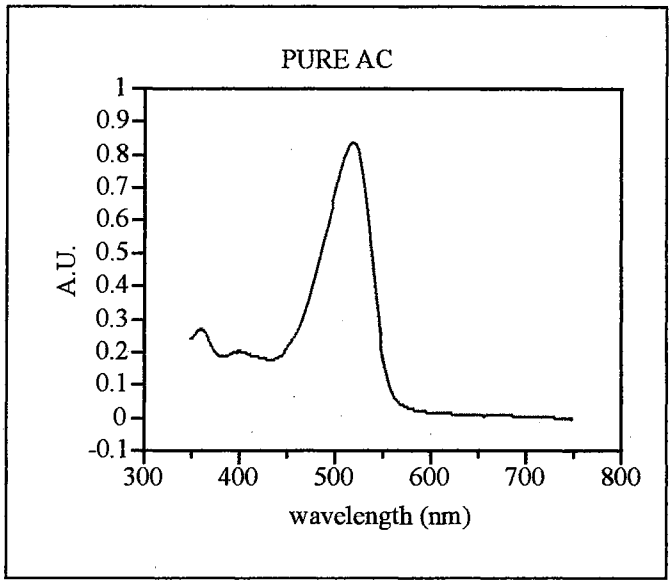


Figure 5-13. Absorption spectrum of the colored products of the reaction of cholesterol and a reagent system consisting of perchloric acid and pure acetyl chloride after twenty minutes.

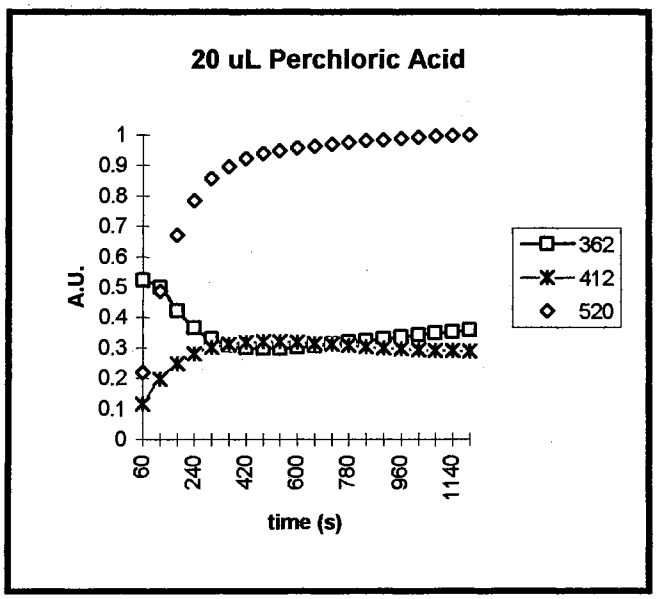


Figure 5-14. Kinetics plot of the absorbance peak height growth at 362, 412 and 520nm for the reaction of cholesterol and a reagent system consisting of 20μL perchloric acid and pure acetyl chloride from one to twenty minutes.

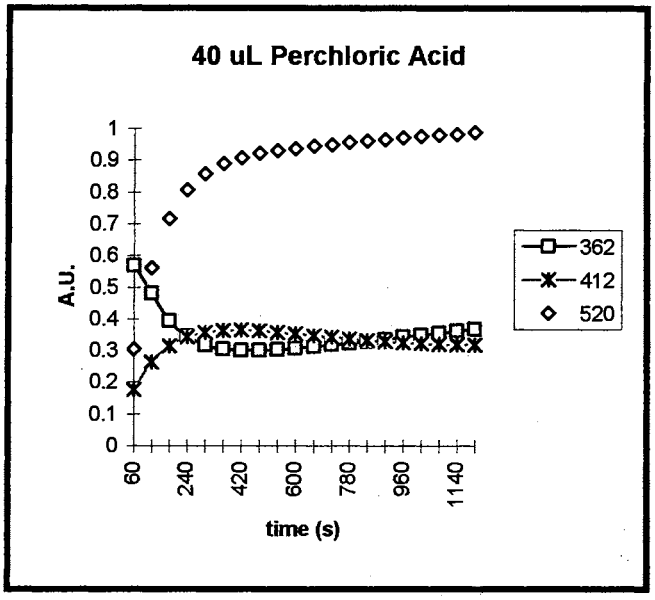


Figure 5-15. Kinetics plot of the absorbance peak height growth at 362, 412 and 520nm for the reaction of cholesterol and a reagent system consisting of 40μL perchloric acid and pure acetyl chloride from one to twenty minutes.

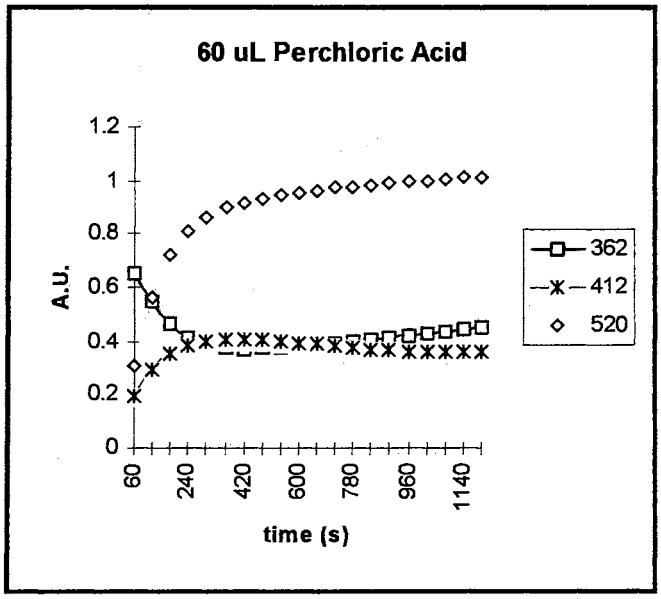


Figure 5-16. Kinetics plot of the absorbance peak height growth at 362, 412 and 520nm for the reaction of cholesterol and a reagent system consisting of 60μL perchloric acid and pure acetyl chloride from one to twenty minutes.

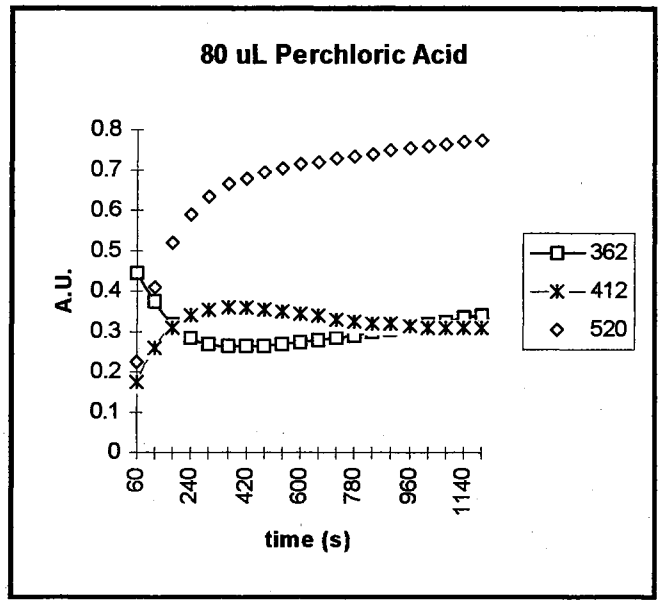


Figure 5-17. Kinetics plot of the absorbance peak height growth at 362, 412 and 520nm for the reaction of cholesterol and a reagent system consisting of 80μL perchloric acid and pure acetyl chloride from one to twenty minutes.

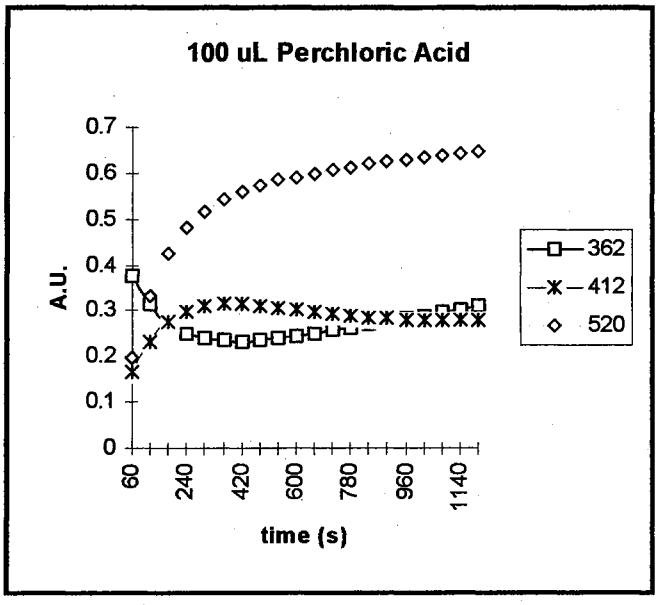


Figure 5-18. Kinetics plot of the absorbance peak height growth at 362, 412 and 520nm for the reaction of cholesterol and a reagent system consisting of 100μL perchloric acid and pure acetyl chloride from one to twenty minutes.

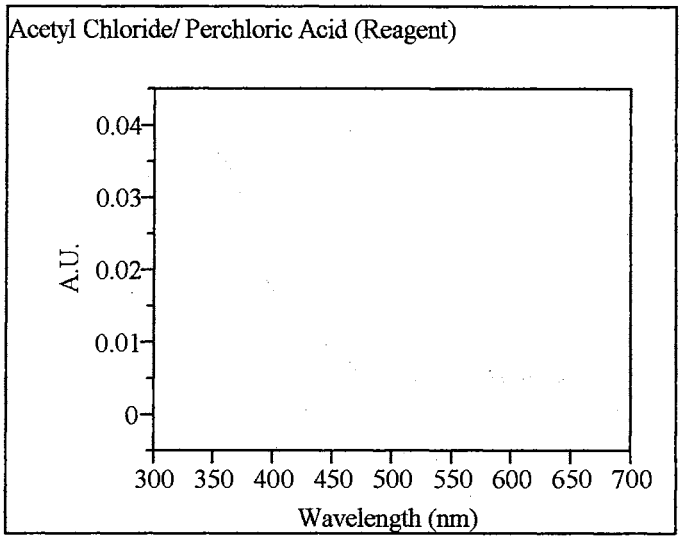


Figure 5-19. Absorption spectrum of the colored products of the reaction of the standard reagent system after 20 minutes.

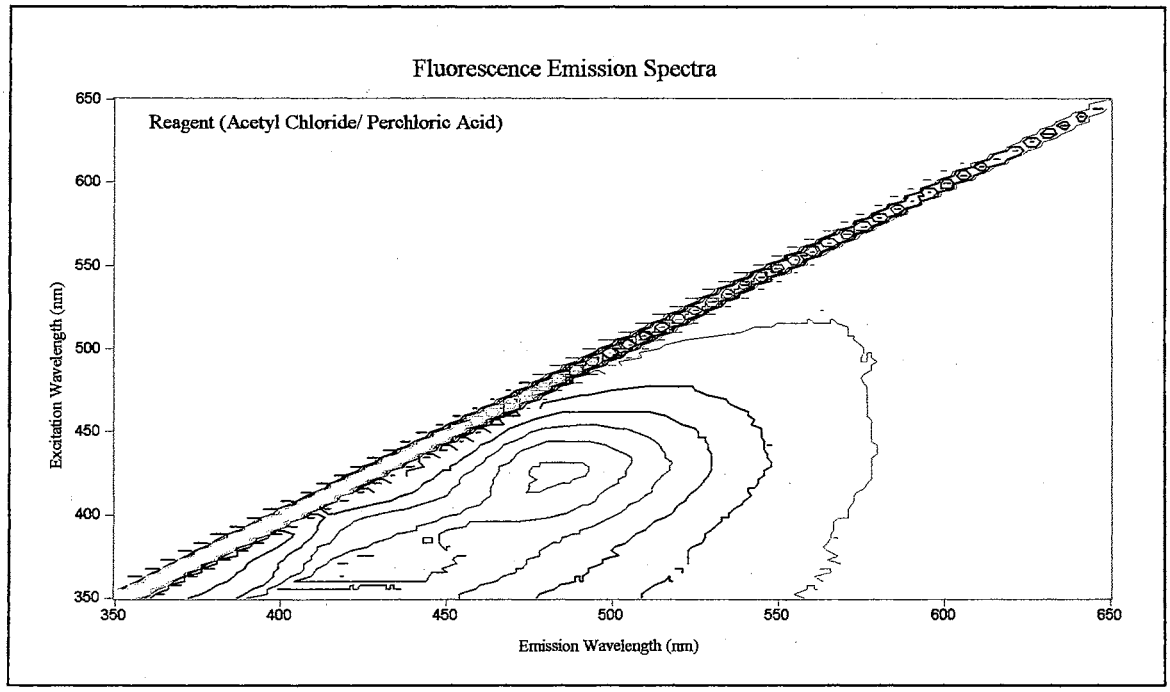


Figure 5-20. Contour plot of the fluorescence emission spectra of the standard reagent system after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

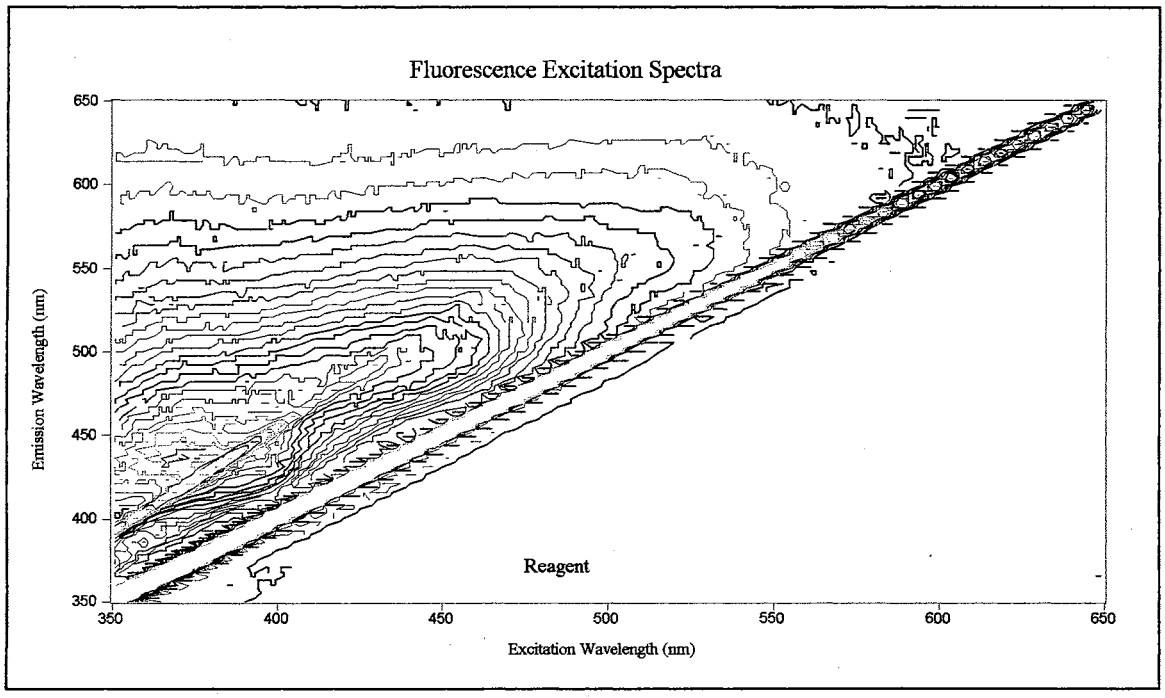


Figure 5-21. Contour plot of the fluorescence excitation spectra of the standard reagent system after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

The second goal of this study was to systematically identify which lipid classes chromogenically react with the SRS. Previous studies using similar reagent systems focused solely on cholesterol or cholesterol esters as the source(s) of the chromophore(s). In a richly diverse biological matrix such as serum it seemed implausible that cholesterol would be the only absorbing species. A more complete understanding of serum spectra taken in this study, previous studies and any future studies requires a rigorous examination of all lipid classes. To this end, representative lipids from the various classes outlined in Table 1-0 were spectroscopically (absorbance and fluorescence spectra) examined. It should be understood that this study was qualitative in nature. The purpose here was simply to identify possibly chromogenic material present in a serum sample and catalog the spectral "patterns" associated with each SRM.

FATTY ACIDS

This study focused on the nutritionally and physiologically important long chain FAs including myristic acid (C_{14}:0), palmitic acid (C_{16}:0), stearic acid (C_{18}:0) , oleic acid (C_{18}:1^{9}), linoleic and linolelaidic acid (C_{18}:$2^{9,12}$), linolenic and γ-linolenic acid (C_{18}:$3^{9,12,15}$), arachidonic acid (C_{20}:$4^{5,8,11,14}$) , eicosapentaenoic acid (C_{20}:$5^{5,8,11,14,17}$), and docosahexaenoic acid (C_{22}:$6^{4,7,10,13,16,19}$). (Prostaglandins, short, and medium chain FAs were not examined.) Each FA was examined in methyl ester form as the esterified form was more chemically stable and less susceptible to oxidation than the free acid. Absorbance, fluorescence emission and excitation spectra were taken for each FAME.

None of the saturated FAMEs chromogenically reacted with the SRS. Oleic acid methyl ester, a MUFA, produced an absorbance spectrum similar to the absorbance spectrum of the SRS, but due to the weak absorbance intensity, it was unclear whether or not a chromophore was being produced by the SRS. Figure 5-23 shows the absorbance spectrum of oleic acid methyl ester after a twenty minute incubation period. The much more sensitive fluoroscopic approach proved that oleic acid could indeed be detected using this technique Figures 5-30 and 5-31 are contour plots of the fluorescence emission and excitation

matrix scans of the oleic acid methyl ester. The remaining fatty acid methyl esters were polyunsaturated and all produced unique absorbance and fluorescence spectra. Figures 5-23 to 5-30 show the absorbance spectra for all of the FAMEs examined. Figure 5-22 is an overlay plot of five absorbance spectra of FAMEs ranging in degree of unsaturation from two to six double bonds. It can be clearly seen that with increasing unsaturation the "tail" of the spectra is red-shifted. Figures 5-31 to 5-45 are contour plots of the fluorescence emission and excitation matrix scans of the PUFAs. The degree of unsaturation proved to be the critical factor influencing the absorbance and fluorescence spectra of the FAMEs. Conformational isomers such as linoleic acid (cis-9,cis-12-octadecadienoic acid) and linolelaidic acid (trans-9,trans-12-octadecadienoic acid) produced the same spectra. Configurational isomers such as linolenic acid (9,12,15-octadecatrienoic acid) and γ-linolenic acid (6,9,12-octadecatrienoic acid) also produced very similar spectral characteristics.

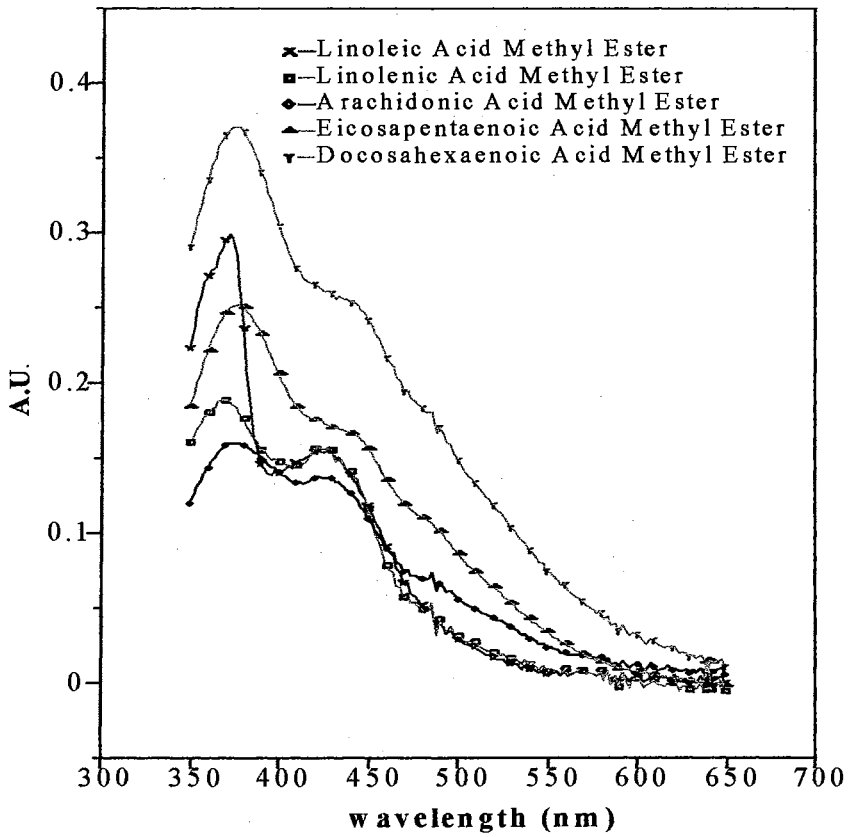


Figure 5-22. Overlay plot of the absorption spectra of PUFA methyl esters after 20 minutes.

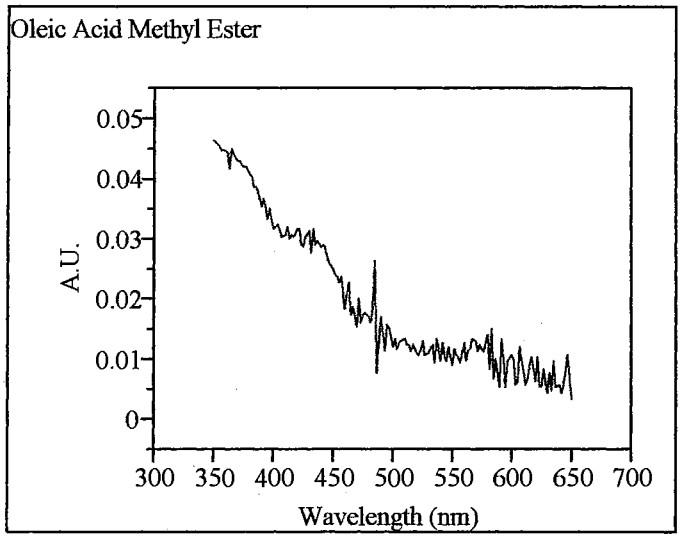


Figure 5-23. Absorption spectrum of the colored products of the reaction of the standard reagent with oleic acid methyl ester after 20 minutes.

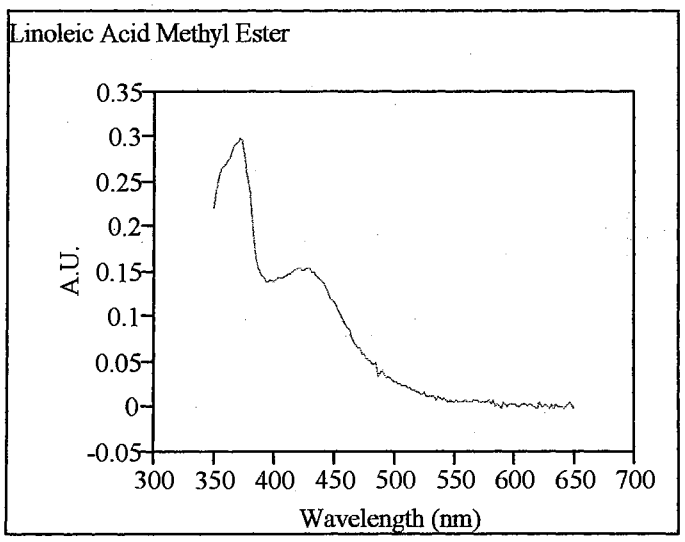


Figure 5-24 Absorption spectrum of the colored products of the reaction of the standard reagent with linoleic acid methyl ester after 20 minutes.

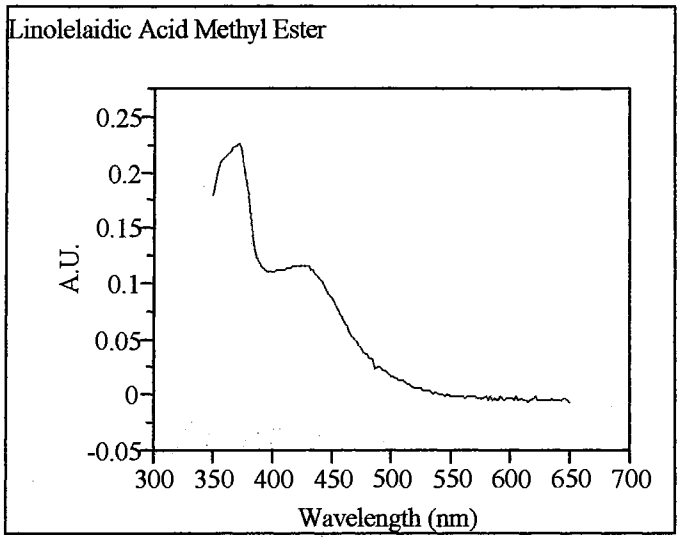


Figure 5-25. Absorption spectrum of the colored products of the reaction of the standard reagent with linolelaidic acid methyl ester after 20 minutes.

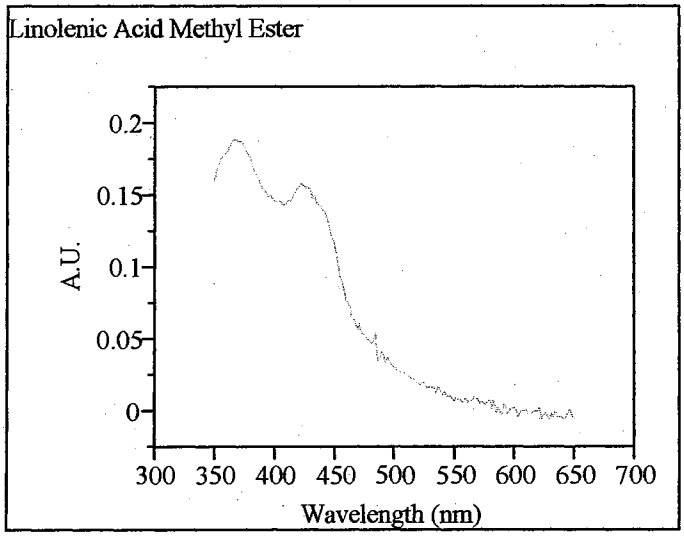


Figure 5-26. Absorption spectrum of the colored products of the reaction of the standard reagent with linolenic acid methyl ester after 20 minutes.

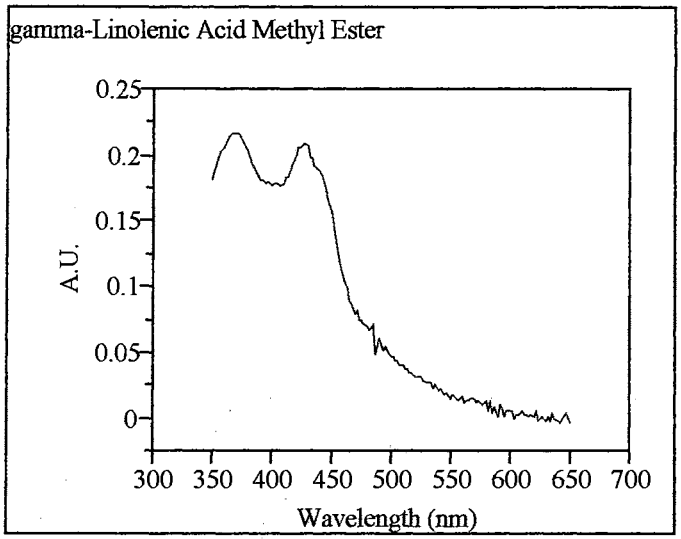


Figure 5-27. Absorption spectrum of the colored products of the reaction of the standard reagent with gamma-linolenic acid methyl ester after 20 minutes.

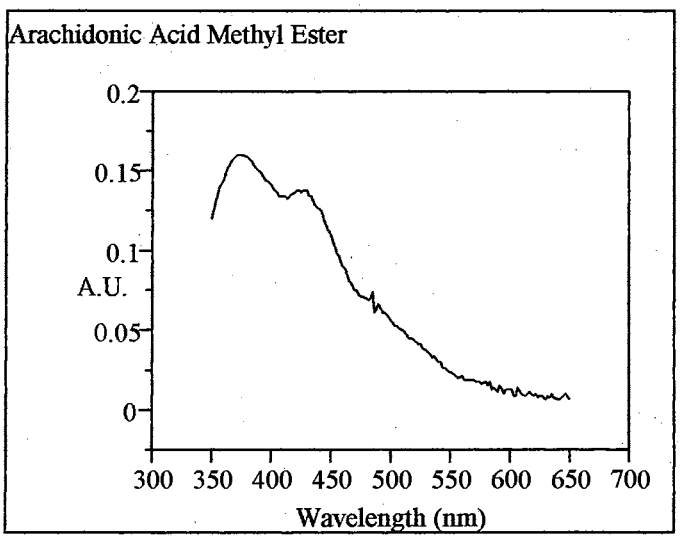


Figure 5-28. Absorption spectrum of the colored products of the reaction of the standard reagent with arachidonic acid methyl ester after 20 minutes.

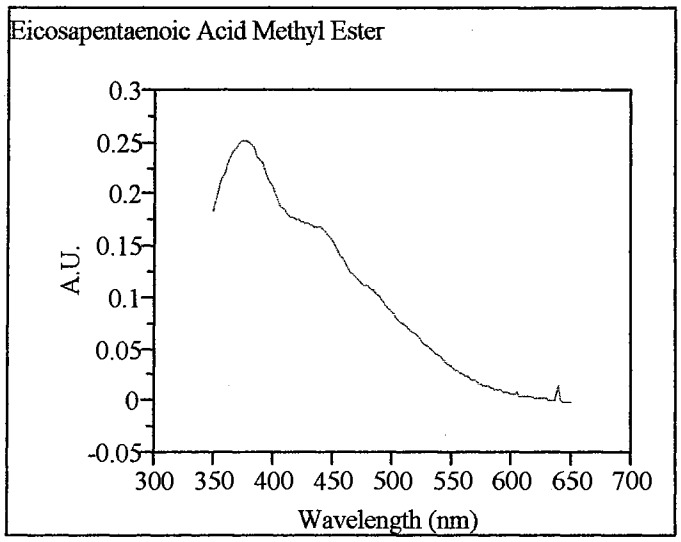


Figure 5-29. Absorption spectrum of the colored products of the reaction of the standard reagent with eicosapentaenoic acid methyl ester after 20 minutes.

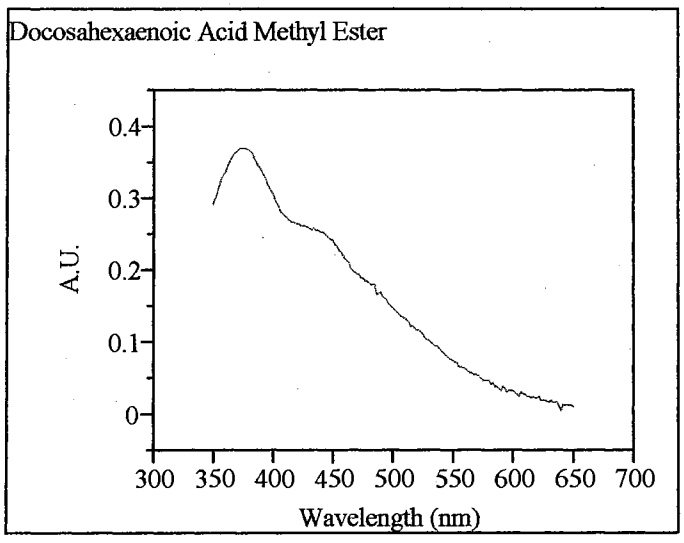


Figure 5-30. Absorption spectrum of the colored products of the reaction of the standard reagent with docosahexaenoic acid methyl ester after 20 minutes.

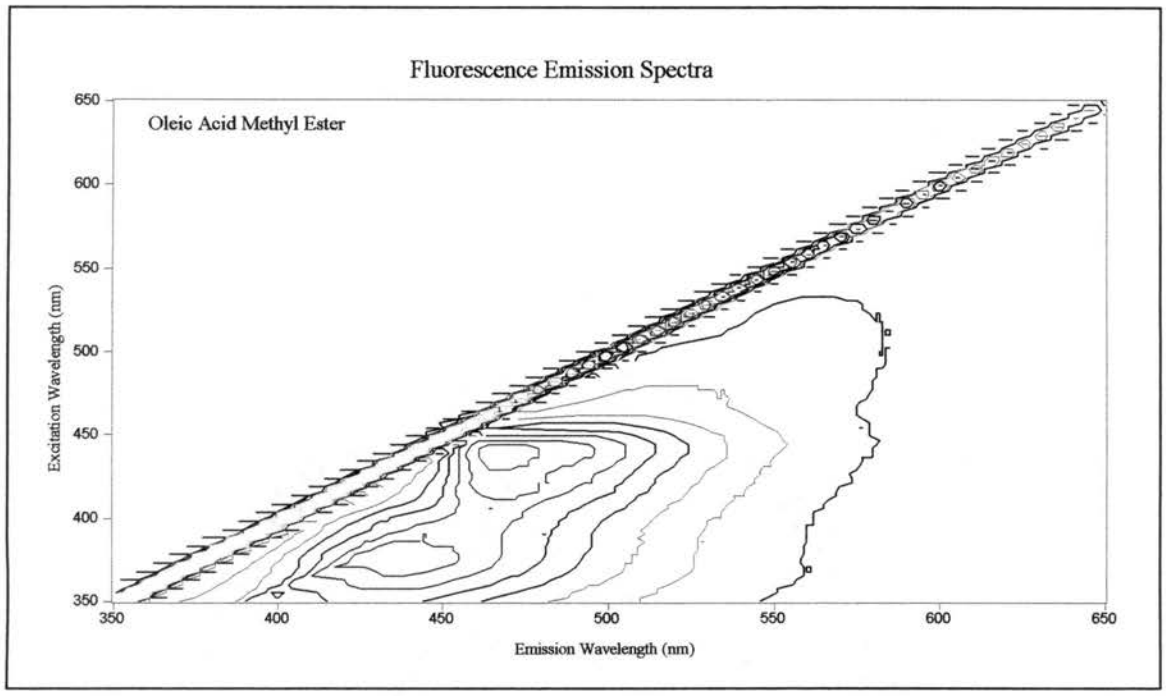


Figure 5-31. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with oleic acid methyl ester after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

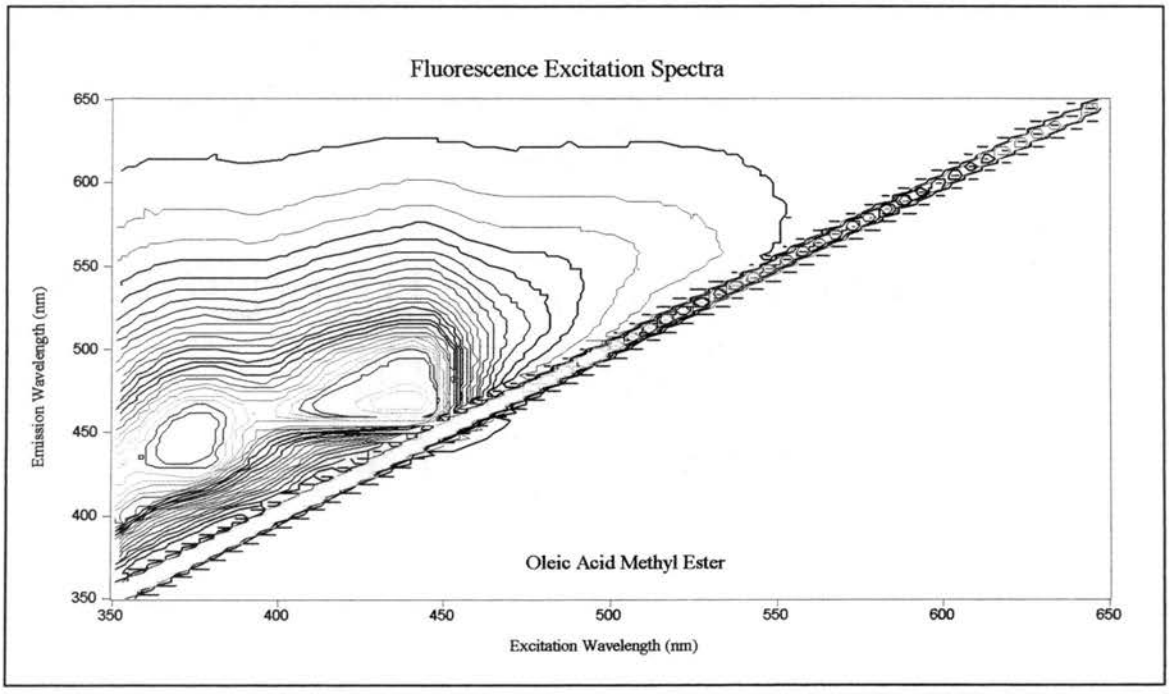


Figure 5-32. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with oleic acid methyl ester after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

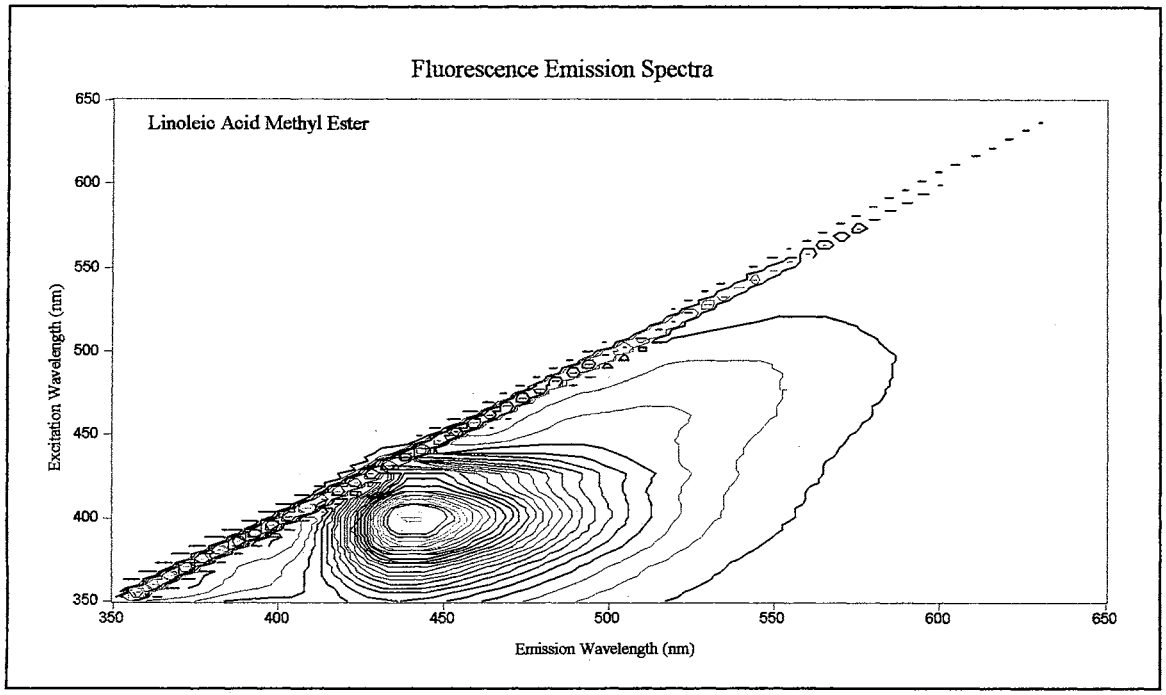


Figure 5-33. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with linoleic acid methyl ester after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

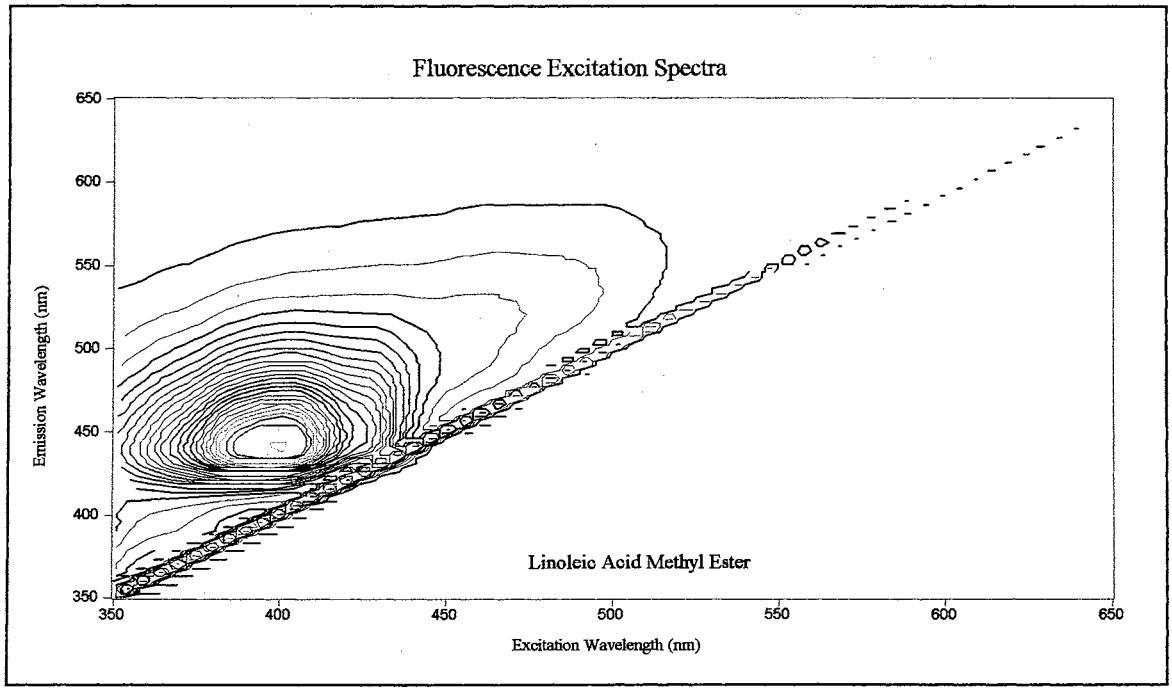


Figure 5-34. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with linoleic acid methyl ester after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

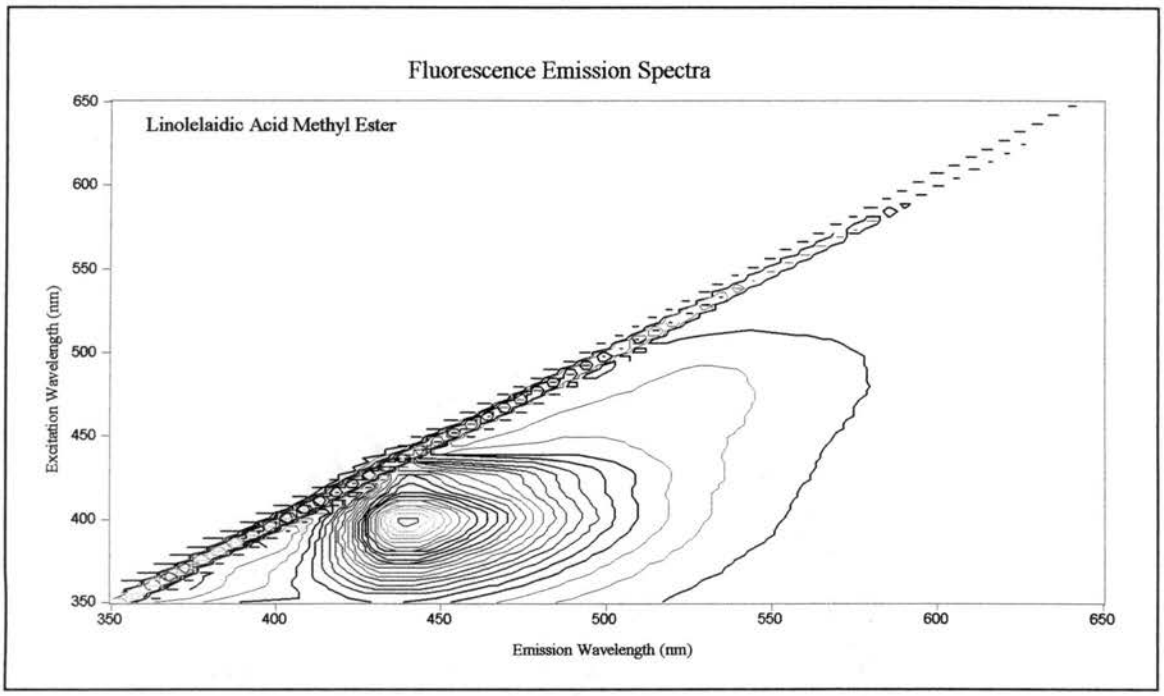


Figure 5-35. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with linolelaidic acid methyl ester after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

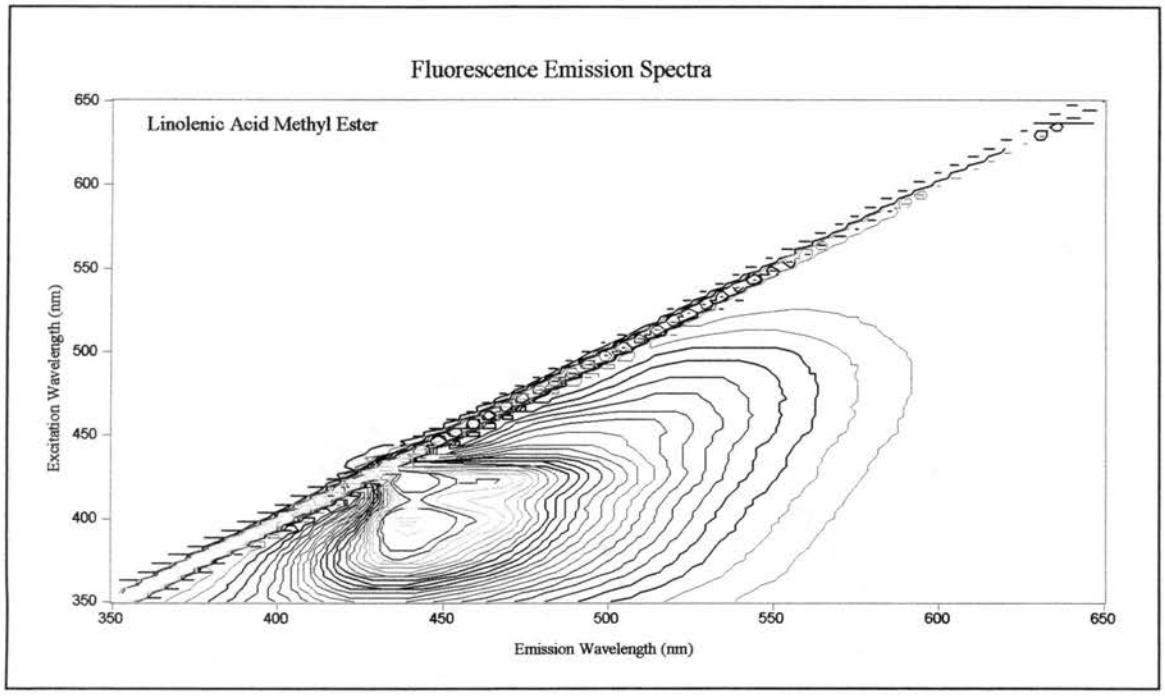


Figure 5-36. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with linolenic acid methyl ester after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

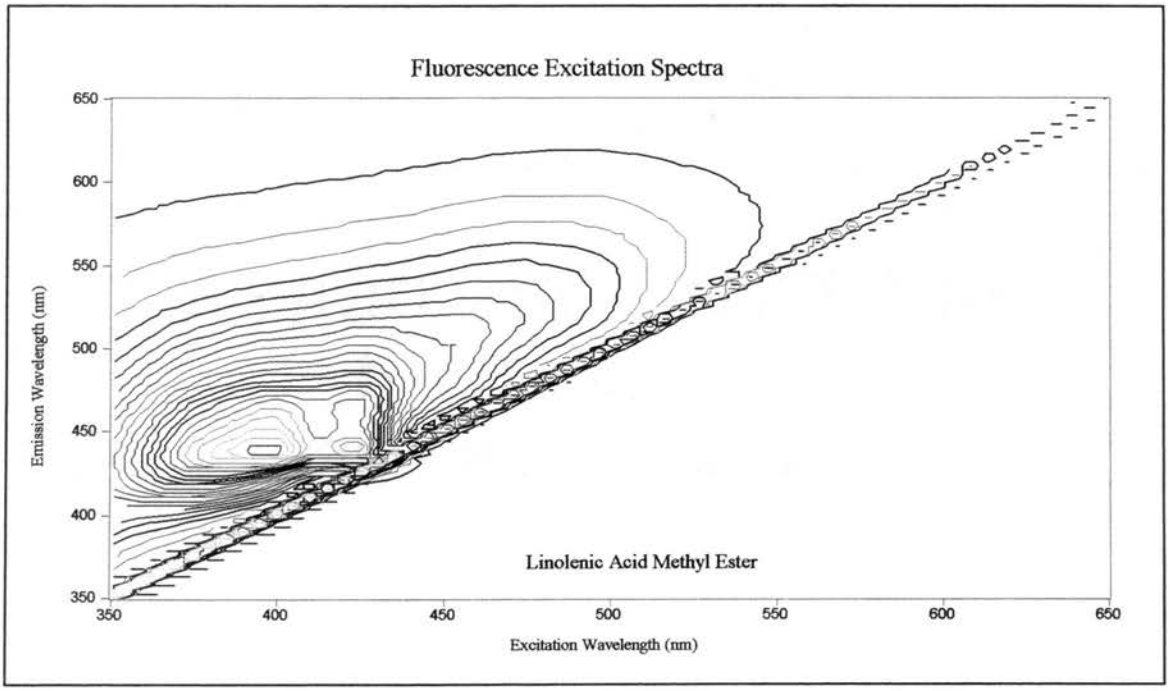


Figure 5-37. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with linolenic acid methyl ester after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

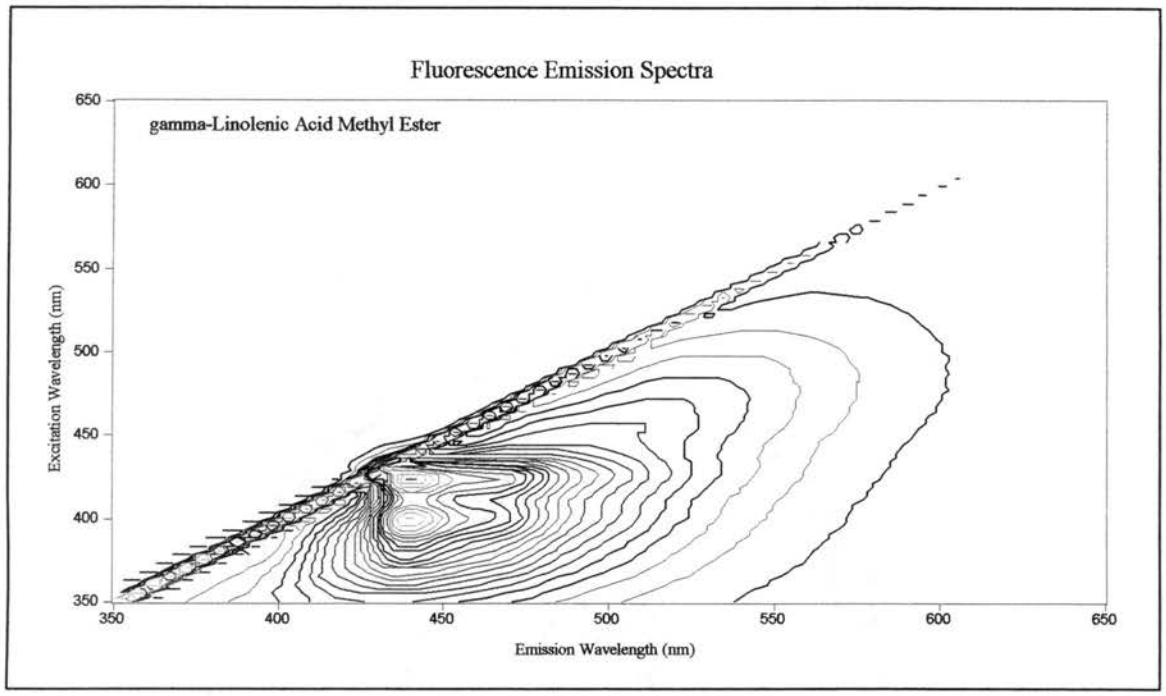


Figure 5-38. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with gamma-linolenic acid methyl ester after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

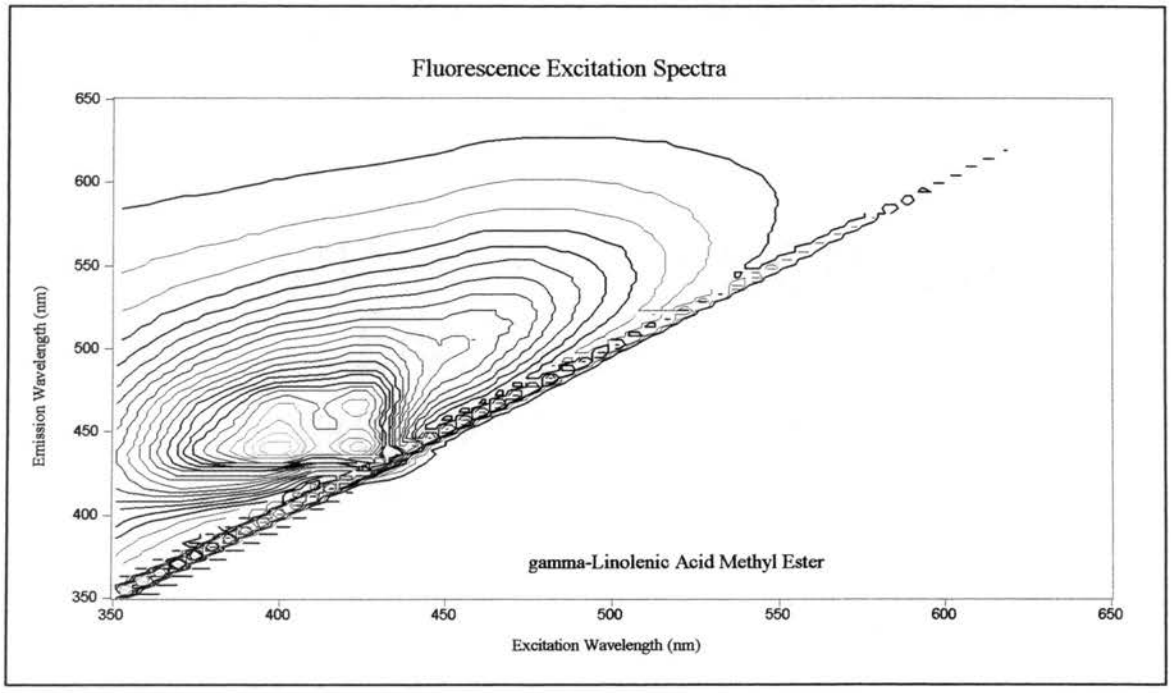


Figure 5-39. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with gamma-linolenic acid methyl ester after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

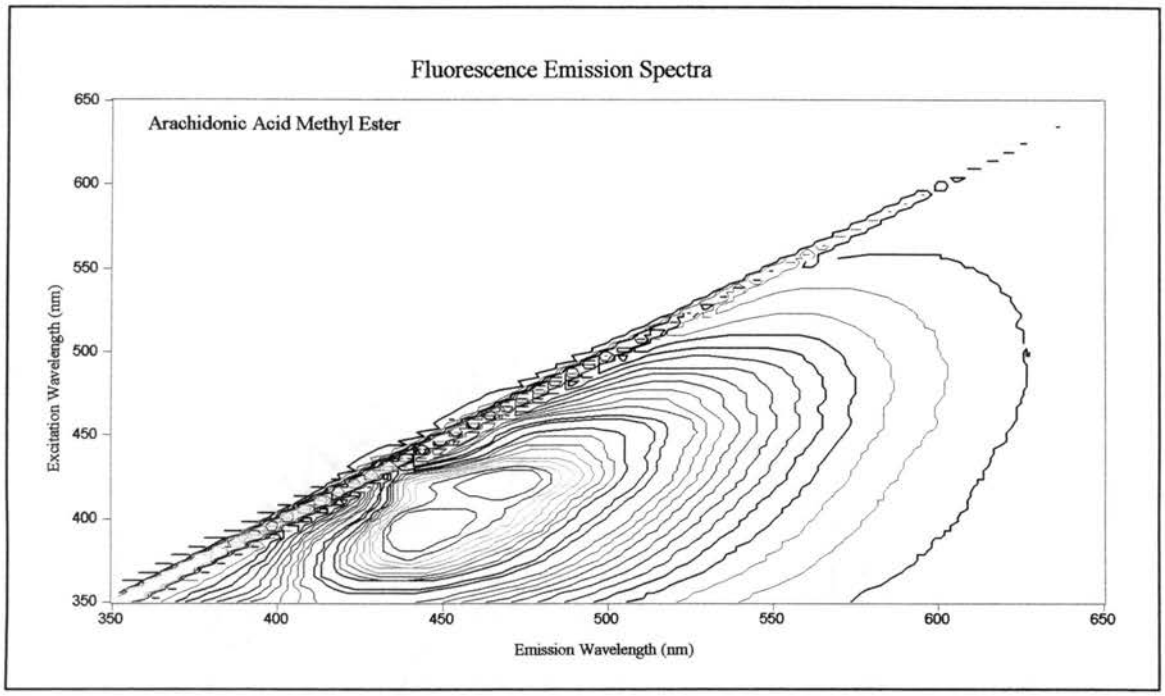


Figure 5-40. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with arachidonic acid methyl ester after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

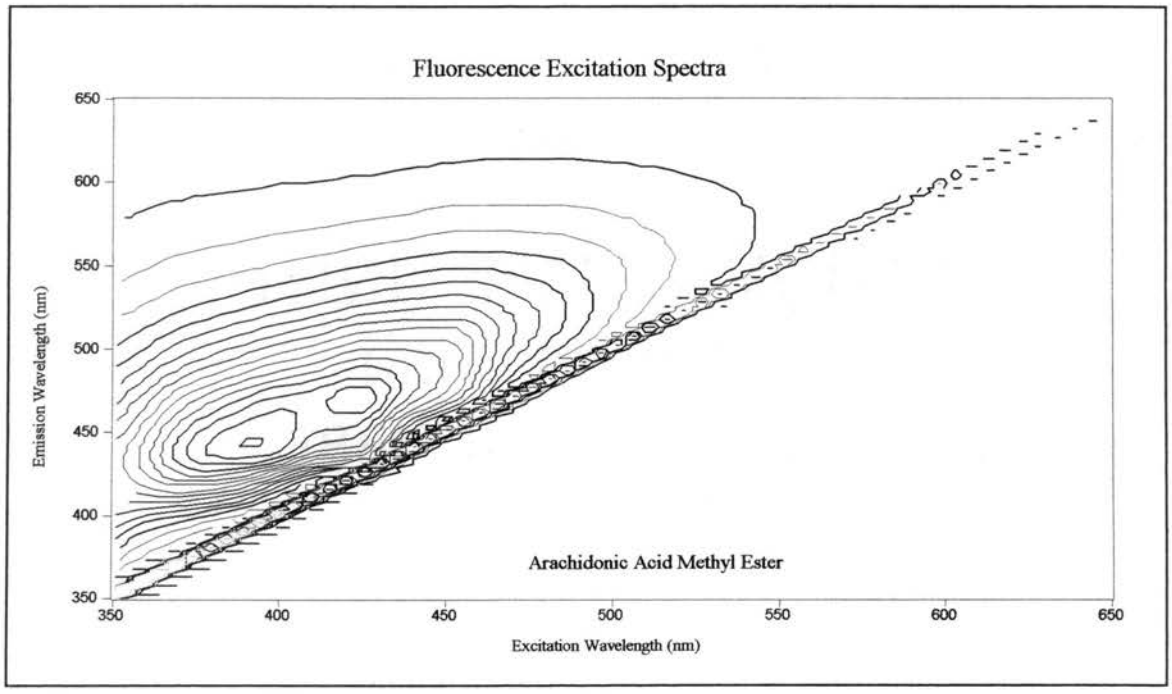


Figure 5-41. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with arachidonic acid methyl ester after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

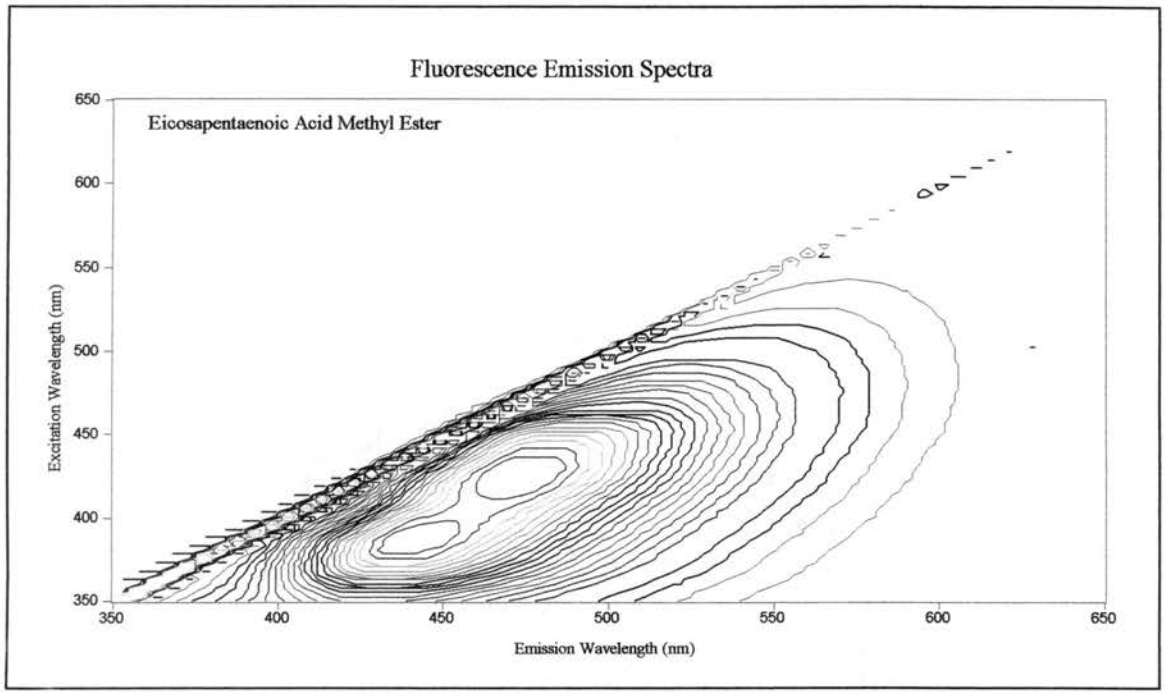


Figure 5-42. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with eicosapentaenoic acid methyl ester after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

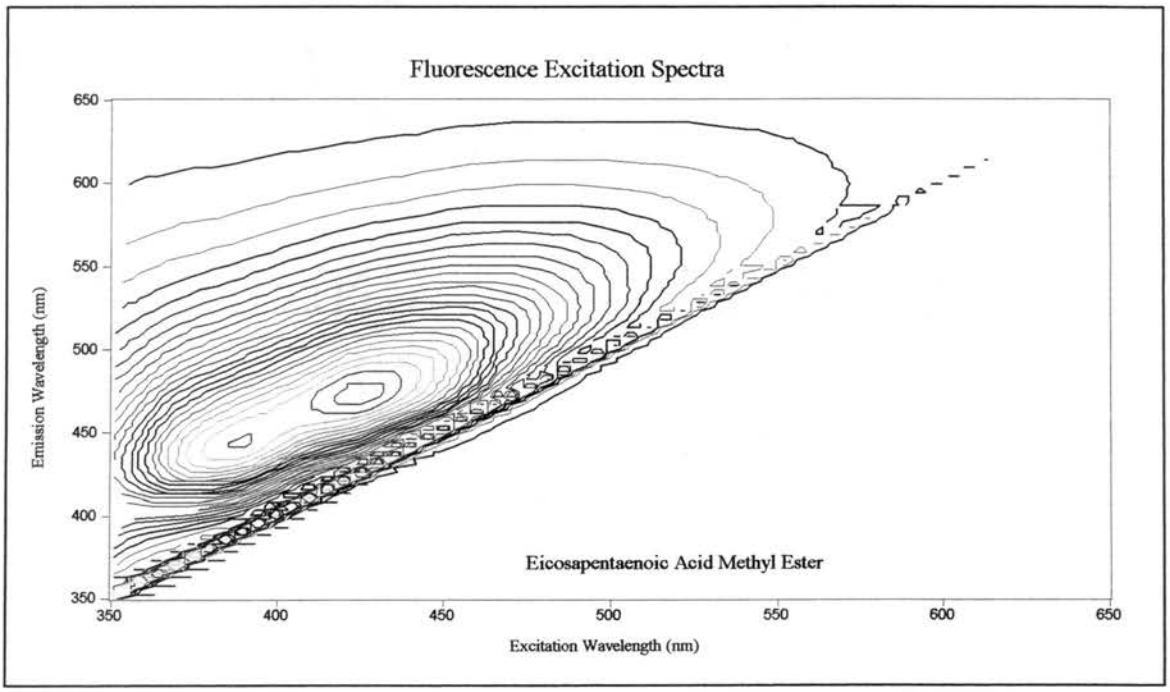


Figure 5-43. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with eicosapentaenoic acid methyl ester after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

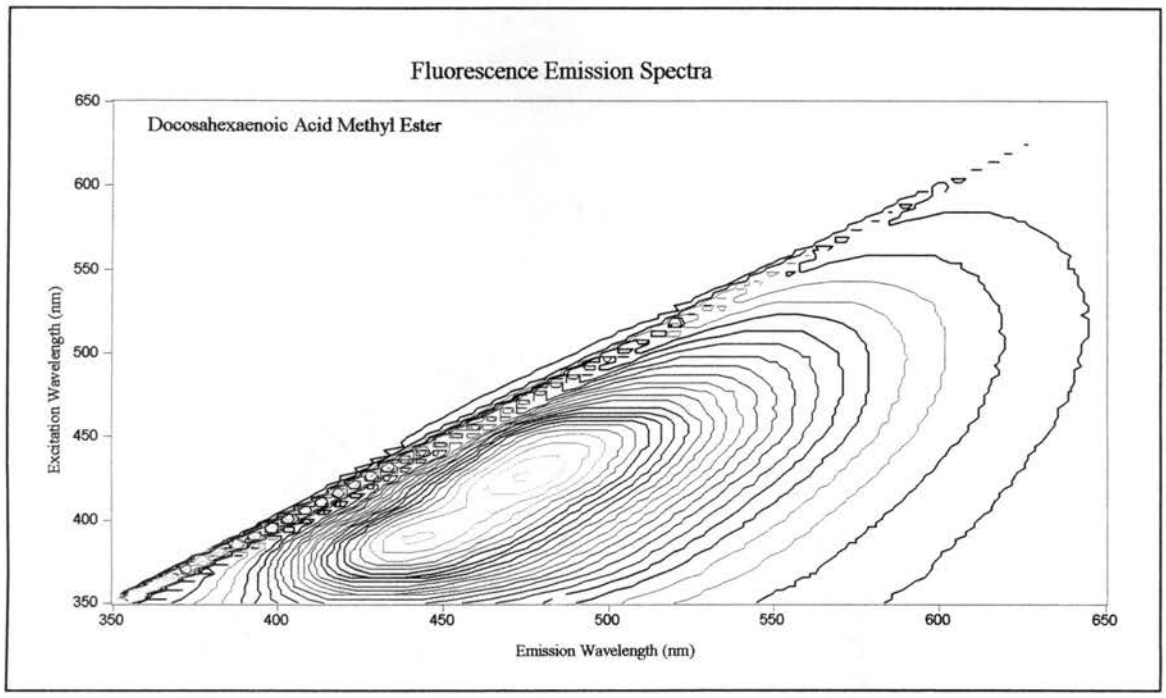


Figure 5-44. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with docosahexaenoic acid methyl ester after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

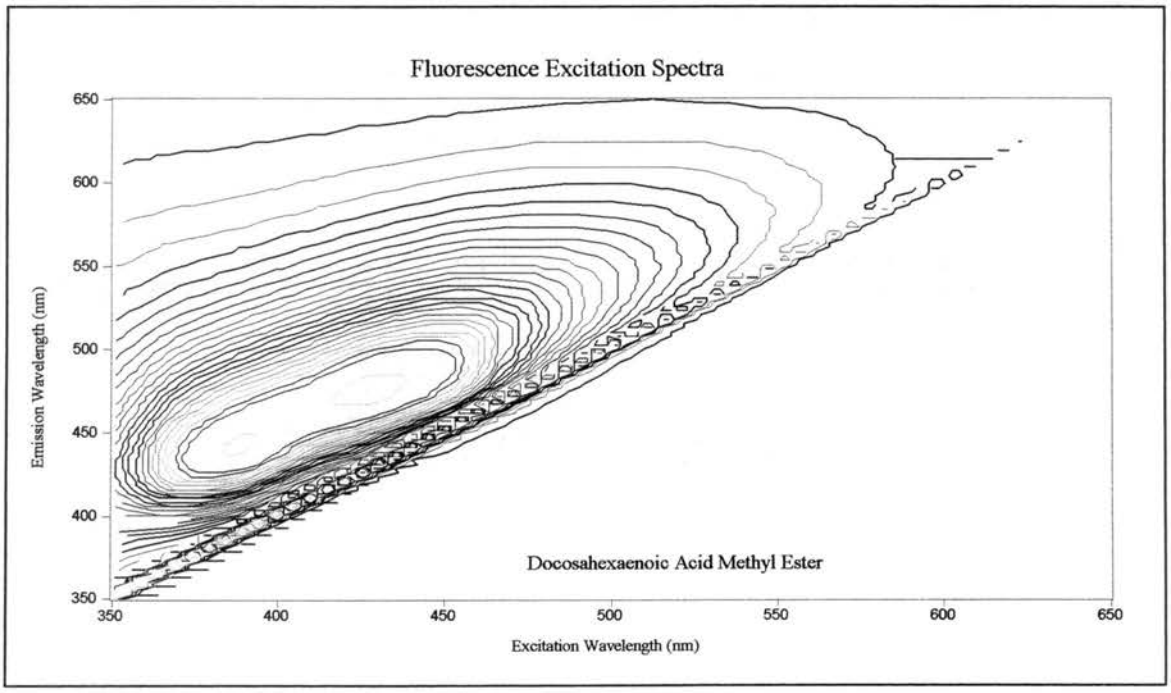


Figure 5-45. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with docosahexaenoic acid methyl ester after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

STEROL DERIVATIVES

Clinically important sterol derivatives include cholesterol and its fatty acid esters, steroid hormones, Vitamin D and bile acids. Sterol derivatives all contain a tetracyclic perhydrocyclopentanophenanthrene (sterane) skeleton (Figure 5-46). In this study, fifteen clinically important sterol derivatives were examined with various substituent groups on and modifications to the sterane skeleton. These compounds are listed and their structures are depicted in Figures 5-48 and 5-49.

Not all steroid derivatives react chromogenically with the SRS or produce fluorophores. In fact, only those steroid derivatives with a double bond in the Δ-5 position (Figure 5-47) develop color and fluoresce. Cholesterol, cholesterol esters, beta-sitosterol and stigmasterol all contained a double bond in the Δ-5 position and all produced similar absorbance and fluorescence spectra. Figures 5-50 to 5-60 show the absorbance spectra of each chromogenic SRM. Cholic acid and 5-alpha-cholestane-3-one contain no double bonds in the sterane portion or the substituents and produced neither absorbance nor fluorescence spectra. Similarly, when a double bond is present in the ring structure in a position other than the Δ-5 carbon, such as in the sex hormones testosterone and progesterone, no absorbance or fluorescence spectra are produced. This evidence suggests that the SRS has a regio/structural specificity and would chromogenically react with any sterol derivative with a double bond in the Δ-5 position. Further, the cyclic nature of the sterane skeleton is not be required for color development as can be seen in the case of cholecalciferol (vitamin D_3). In this instance, the cyclic nature of the sterane skeleton is broken between the 9^{th} and 10^{th} carbons (see Figure 5-48). Cholecalciferol does, however, produce absorbance and fluorescence emission and excitation spectra (see Figure 5-53 and Figures 5-67 and 5-68, respectively).

Cholesterol esters examined in this study were divided between saturated and unsaturated FA forms. When reacted with the SRS, all of the saturated cholesterol esters produce an absorbance spectra identical in form and spectral characteristics to the free cholesterol absorbance spectrum (see Figures 5-4 to 5-6). This finding was not surprising, since none of the saturated FAs themselves produce absorbance or fluorescence spectra. When examined fluoroscopically, however, all of the saturated cholesterol esters produce spectra similar to free cholesterol with an additional peak on the excitation contour plots around

360nm, 425nm (see Figures 5-70,72,74) The emission contour plots for the saturated cholesterol esters (Figures 5-69,71,73) are identical in form to the free cholesterol emission contour plot (Figure 5-62).

The unsaturated cholesterol esters all exhibited marked increases in the 362 and 412nm peaks of the absorbance spectra (Figures 5-57 to 5-60). These increased peak heights are explained by the added PUFA contributions to the absorbance spectra, an assumption that was reinforced upon examination of the fluorescence spectra. Figures 5-75 to 5-82 are contour plots of the fluorescence emission and excitation matrix scans of the unsaturated cholesterol esters. In all of these plots, peaks corresponding to peaks in the unsaturated FAME contour plots are found. For instance, taking the linoleic acid methyl ester (LAME) and the cholesteryl linoleate as examples, one can easily see the single peak in the emission spectra of the LAME (Figure 5-33) is also found in the cholesteryl linoleate emission spectra (Figure 5-77). Analogous results were found for all unsaturated FAMEs and cholesterol esters containing the corresponding fatty acid moieties.

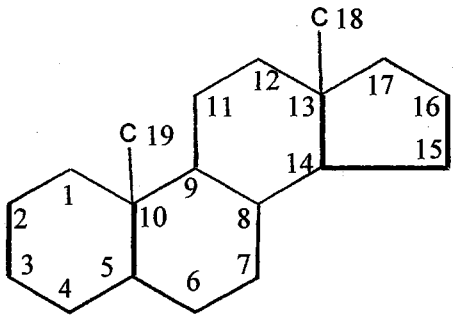


Figure 5-46. Schematic of a tetracyclic perhydrocyclopentanophenanthrene (sterane) skeleton.

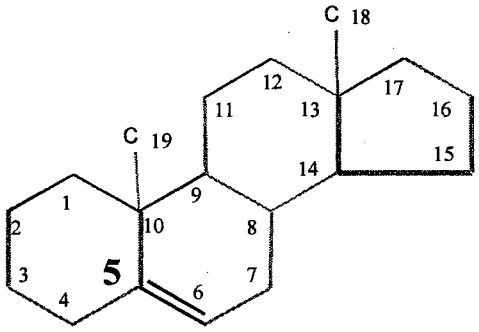
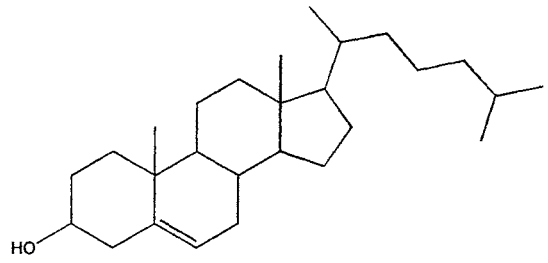
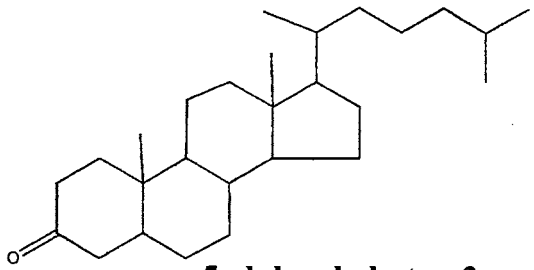


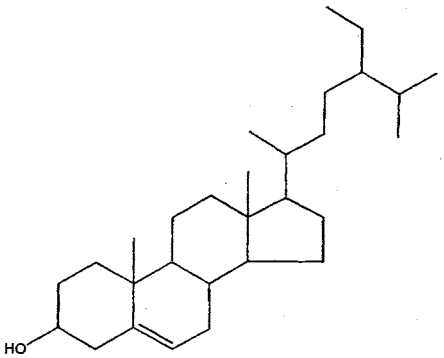
Figure 5-47. Schematic of a tetracyclic perhydrocyclopentanophenanthrene (sterane) skeleton with the Δ-5 double bond highlighted.



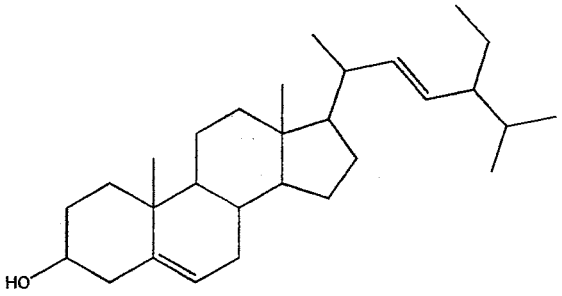
cholesterol



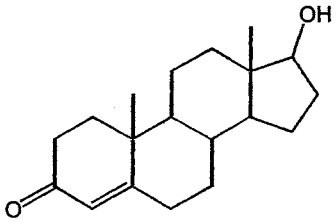
5-alpha-cholestan-3-one



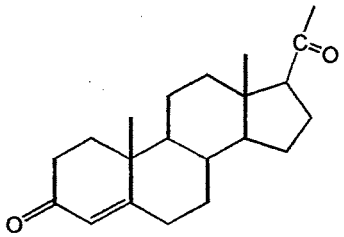
beta-sitosterol



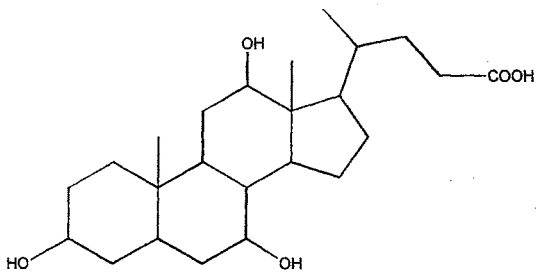
stigmasterol



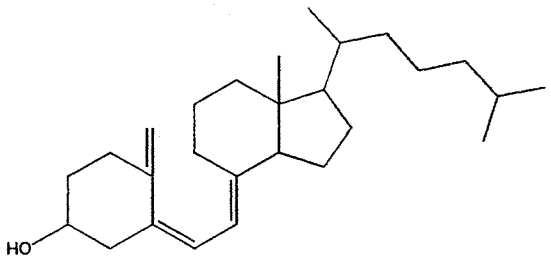
testosterone



progesterone

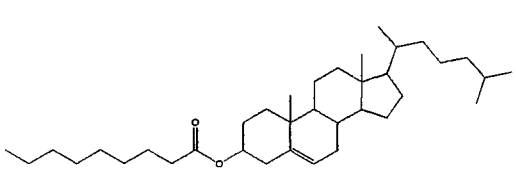


cholic acid

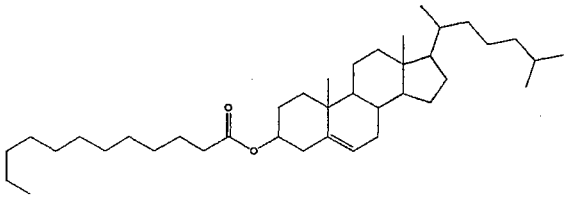


vitamin D_3

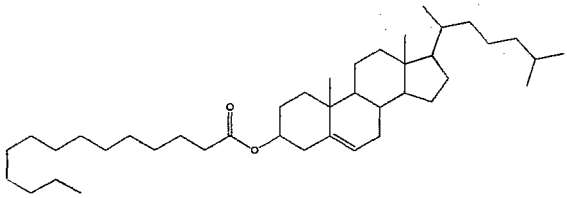
Figure 5-48. Structures of the sterol derivatives used in this study.



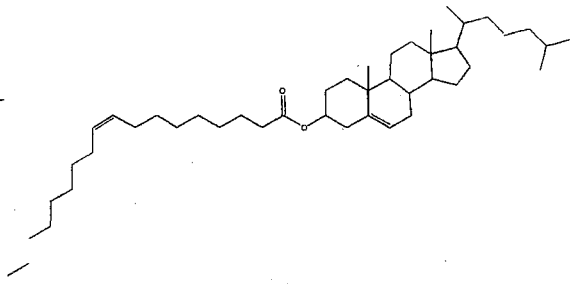
cholesteryl pelargonate



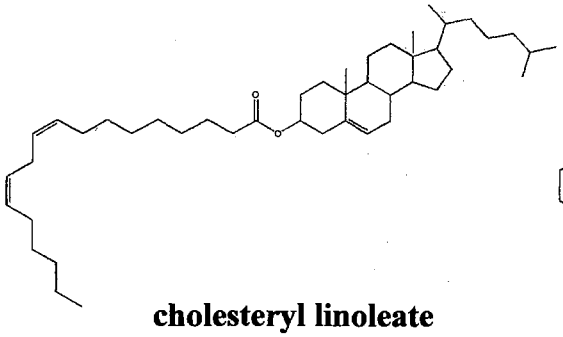
cholesteryl laurate



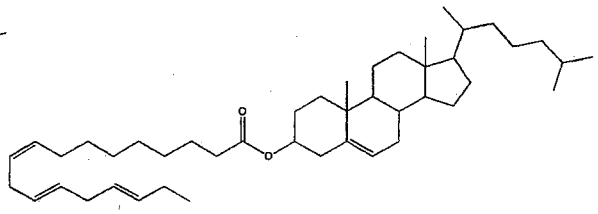
cholesteryl myristate



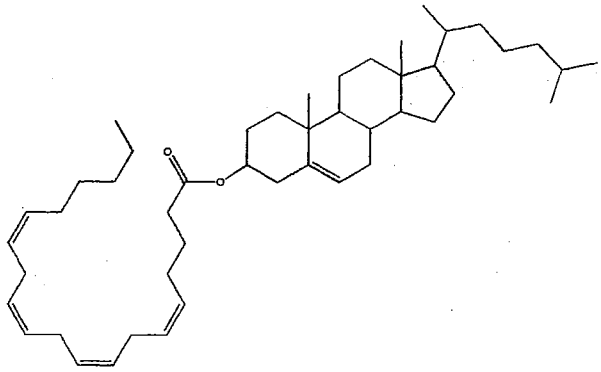
cholesteryl oleate



cholesteryl linoleate



cholesteryl linolenate



cholesteryl arachidonate

Figure 5-49. Structures of the sterol derivatives used in this study.

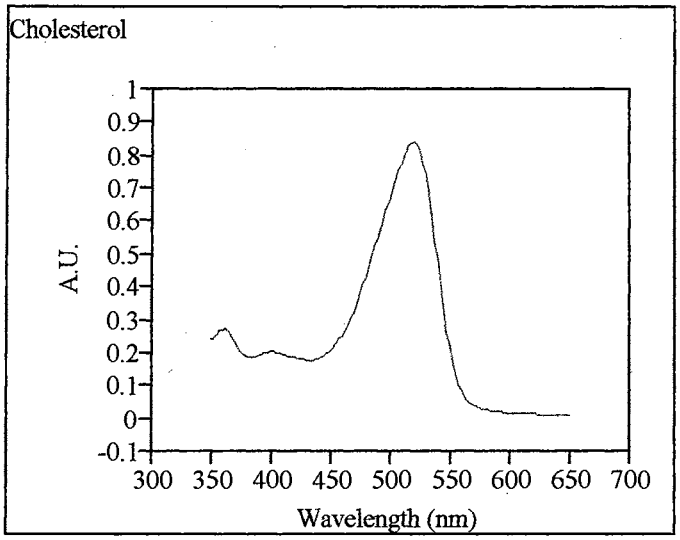


Figure 5-50 . Absorption spectrum of the colored products of the reaction of the standard reagent with cholesterol after 20 minutes.

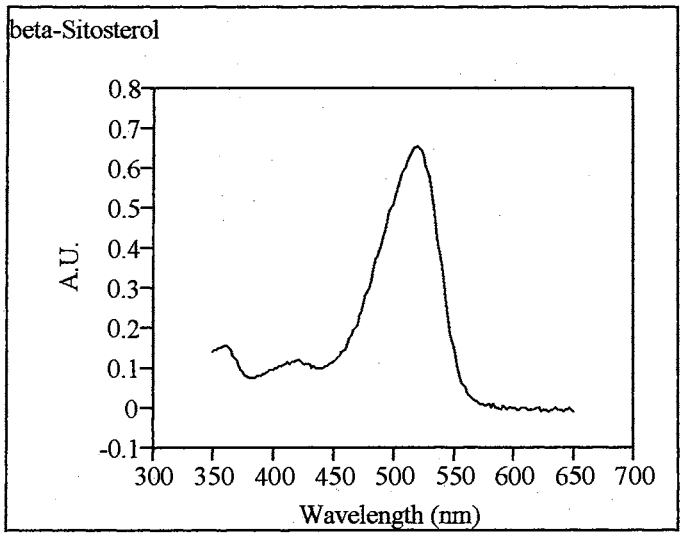


Figure 5-51. Absorption spectrum of the colored products of the reaction of the standard reagent with beta-sitosterol after 20 minutes.

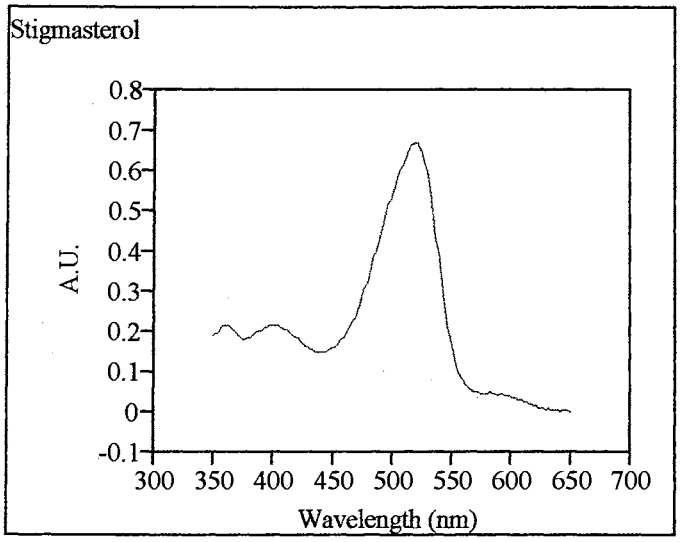


Figure 5-52. Absorption spectrum of the colored products of the reaction of the standard reagent with stigmasterol after 20 minutes.

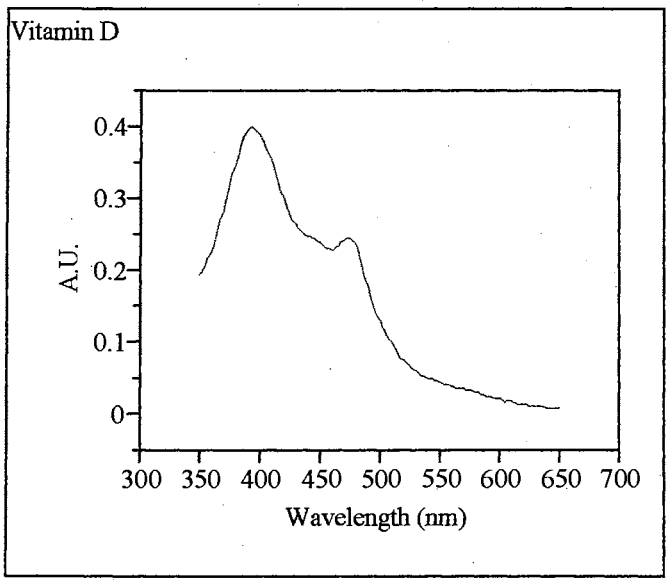


Figure 5-53. Absorption spectrum of the colored products of the reaction of the standard reagent with vitamin D_3 after 20 minutes.

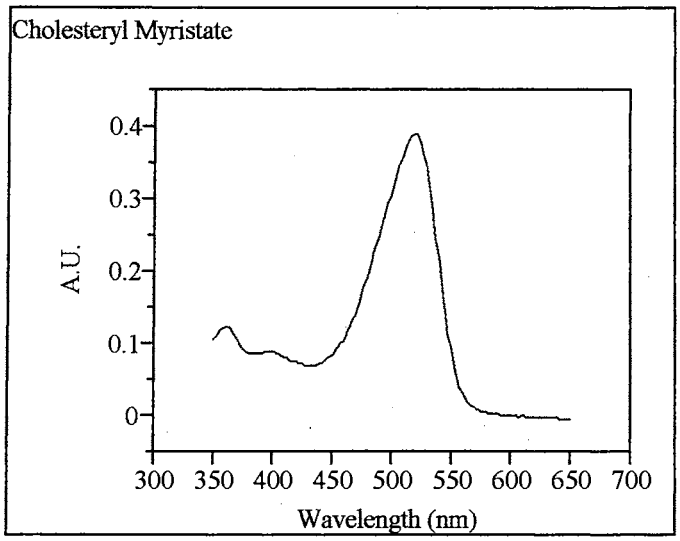


Figure 5-54. Absorption spectrum of the colored products of the reaction of the standard reagent with cholesteryl myristate after 20 minutes.

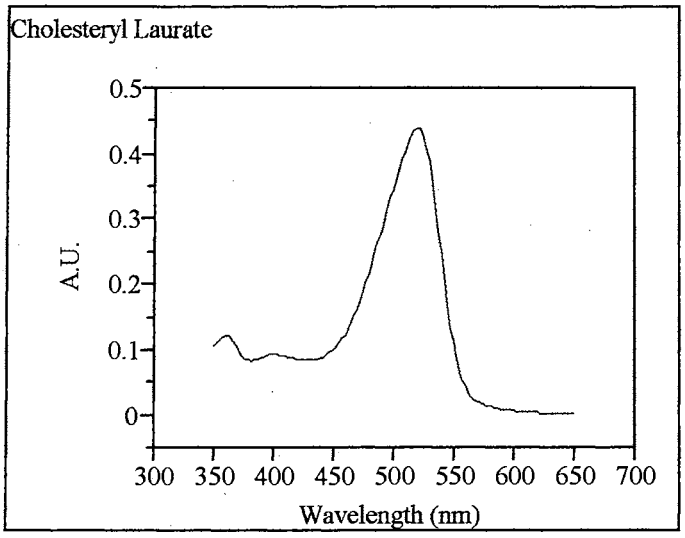


Figure 5-55. Absorption spectrum of the colored products of the reaction of the standard reagent with cholesteryl laurate after 20 minutes.

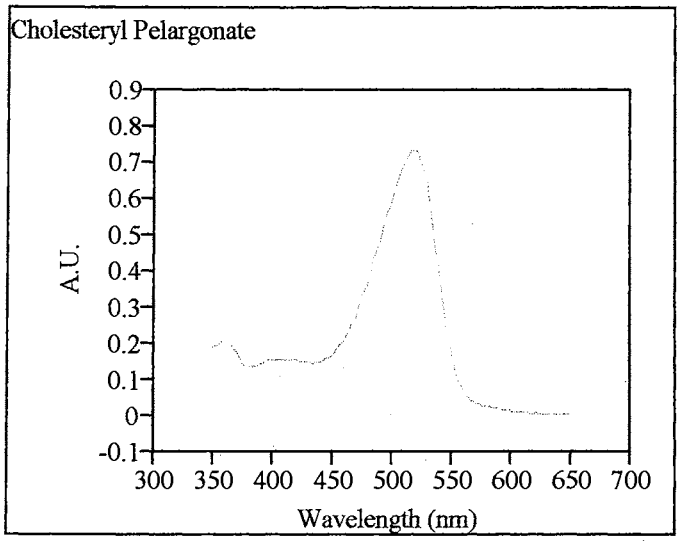


Figure 5-56. Absorption spectrum of the colored products of the reaction of the standard reagent with cholesteryl pelargonate after 20 minutes.

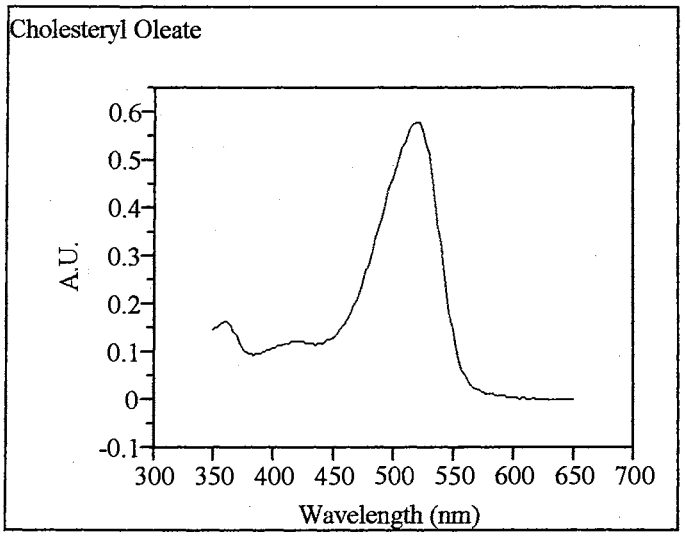


Figure 5-57. Absorption spectrum of the colored products of the reaction of the standard reagent with cholesteryl oleate after 20 minutes.

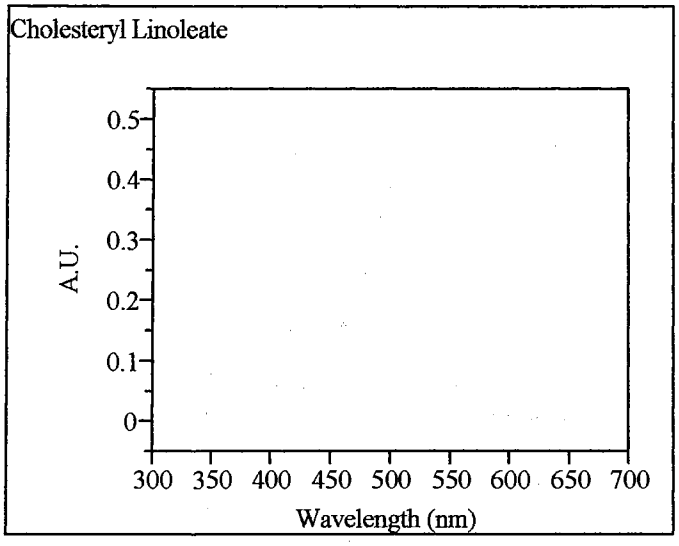


Figure 5-58. Absorption spectrum of the colored products of the reaction of the standard reagent with cholesteryl linoleate after 20 minutes.

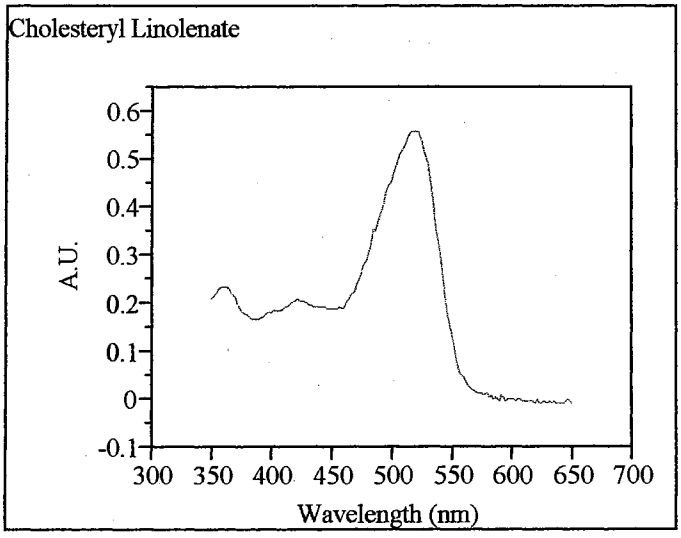


Figure 5-59. Absorption spectrum of the colored products of the reaction of the standard reagent with cholesteryl linolenate after 20 minutes.

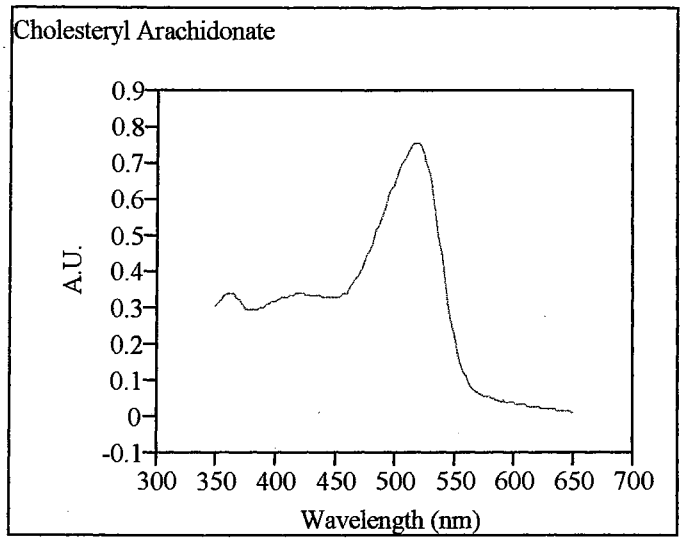


Figure 5-60. Absorption spectrum of the colored products of the reaction of the standard reagent with cholesteryl arachidonate after 20 minutes.

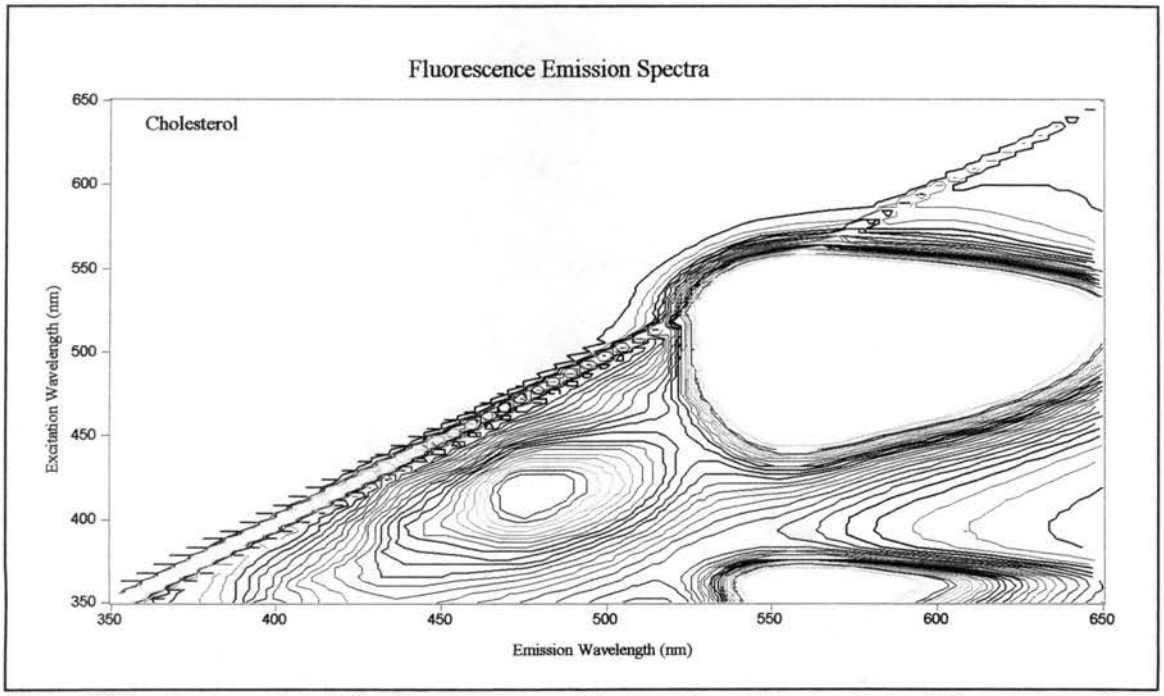


Figure 5-61. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with cholesterol after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

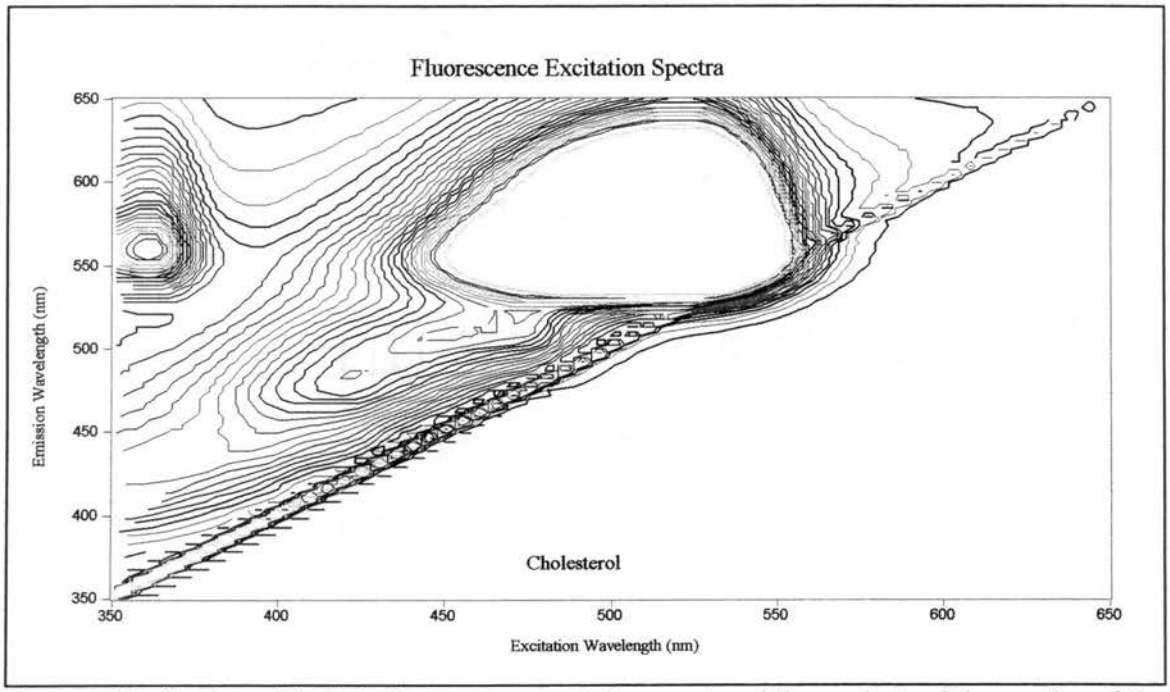


Figure 5-62. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with cholesterol after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

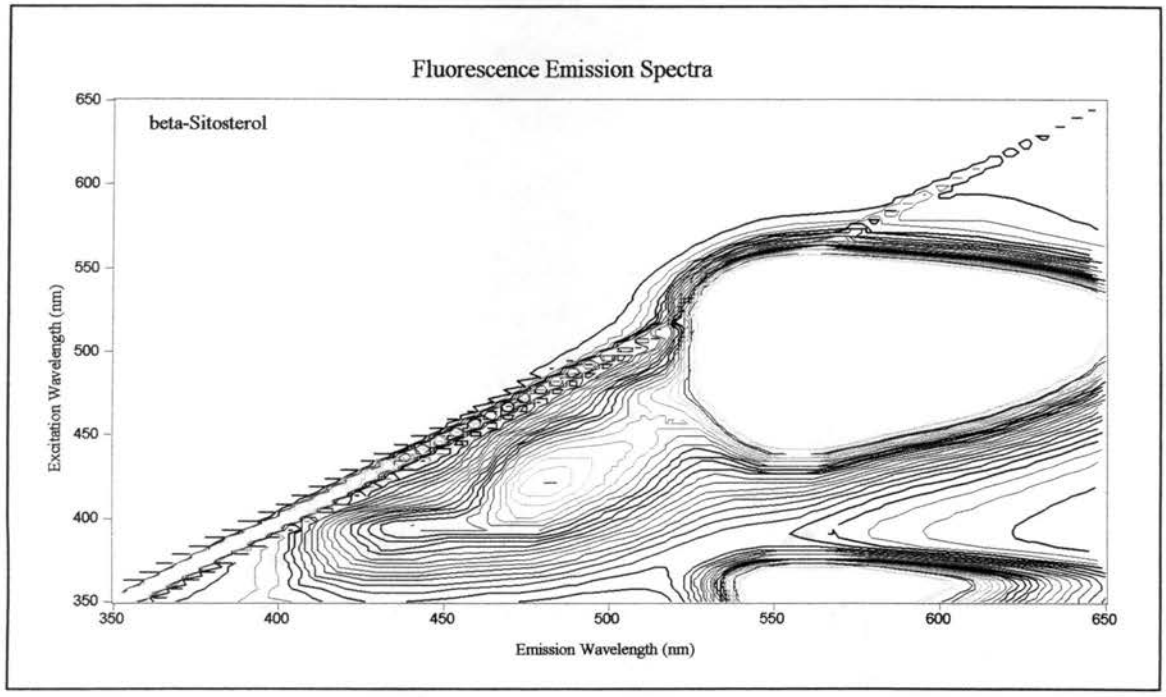


Figure 5-63. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with beta-sitosterol after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

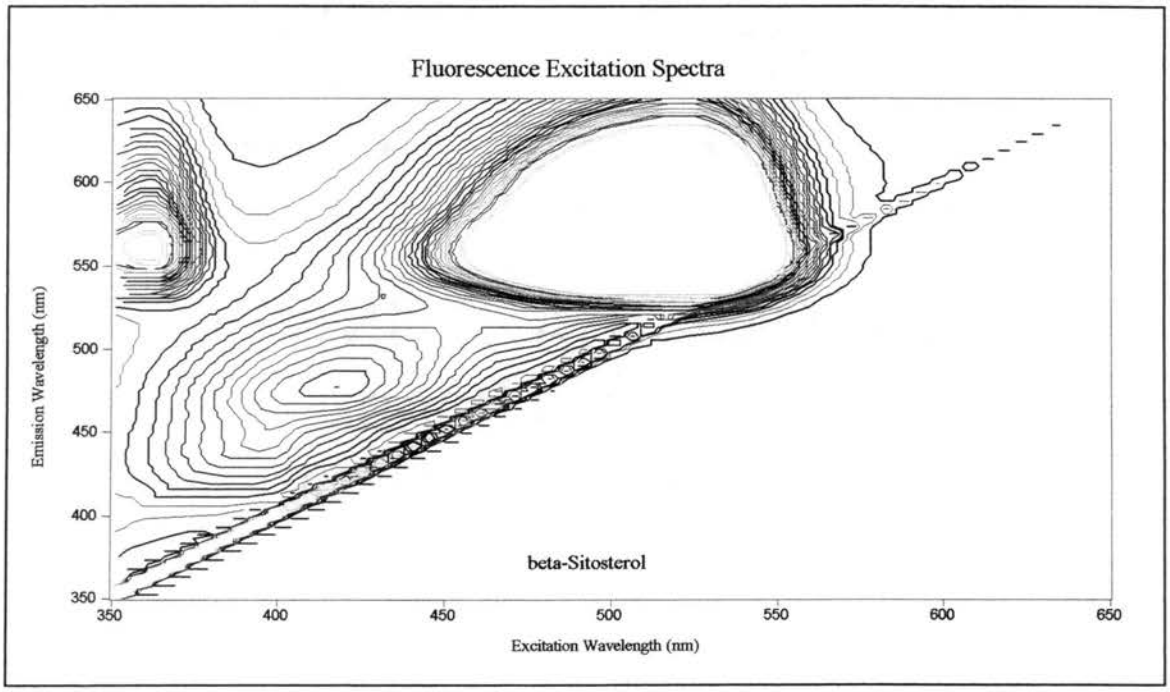


Figure 5-64. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with beta-sitosterol after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

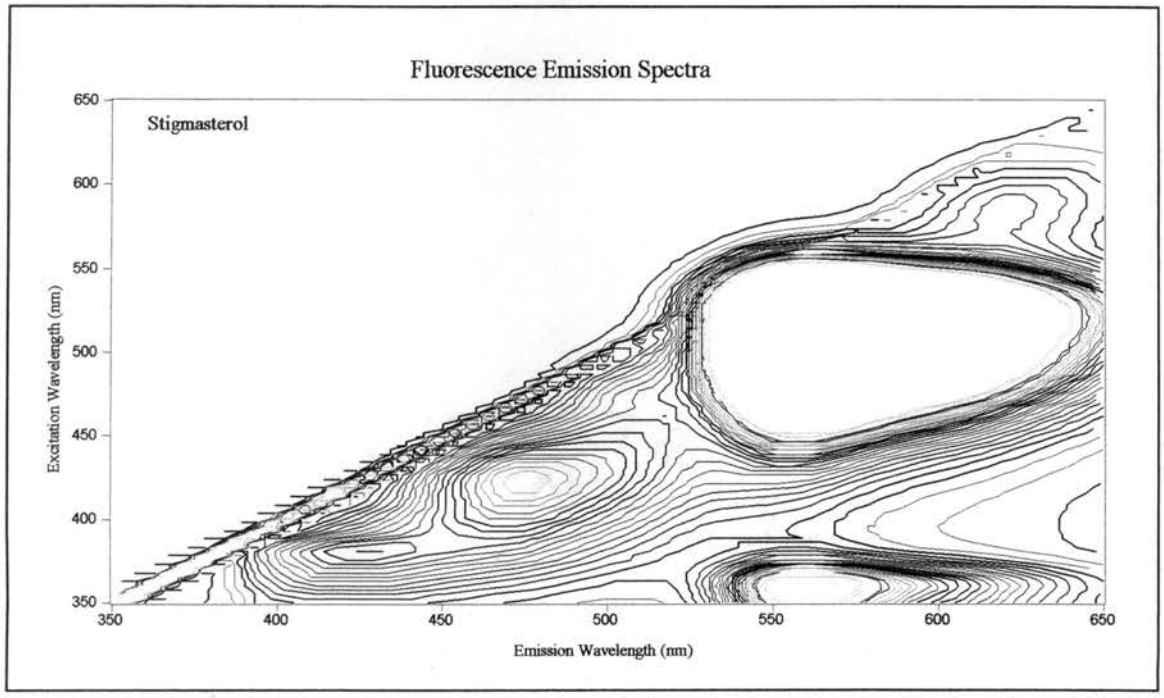


Figure 5-65. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with stigmasterol after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

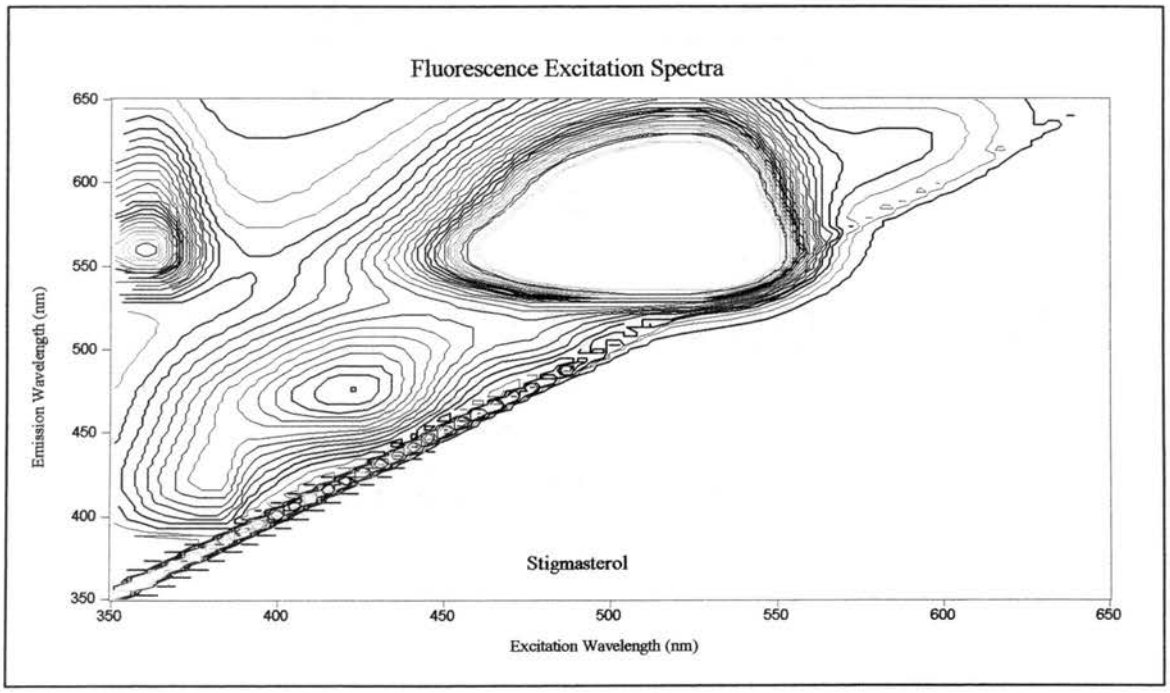


Figure 5-66. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with stigmasterol after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

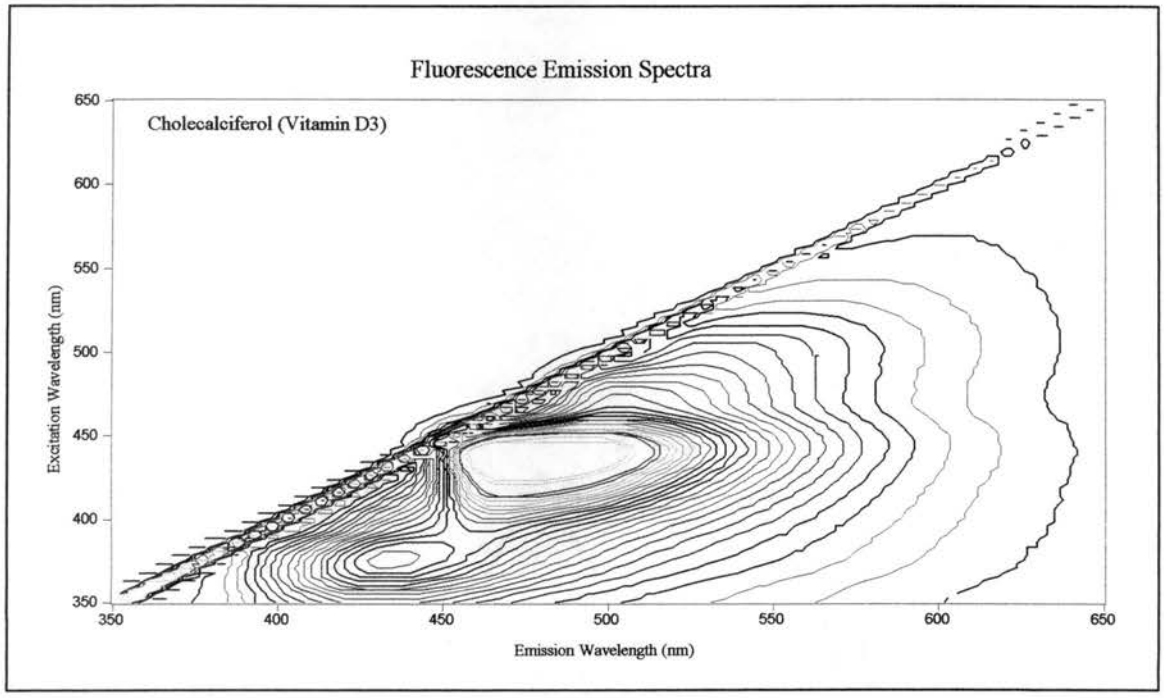


Figure 5-67. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with cholecalciferol (vitamin D_3) after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

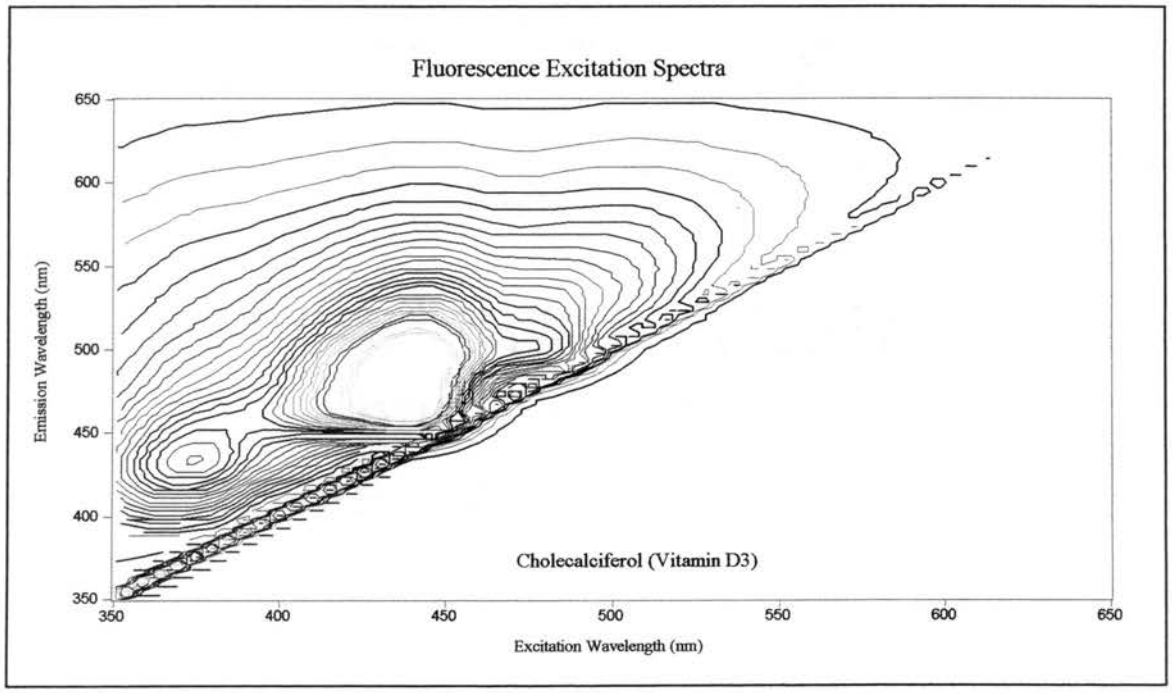


Figure 5-68. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with cholecalciferol (vitamin D_3) after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

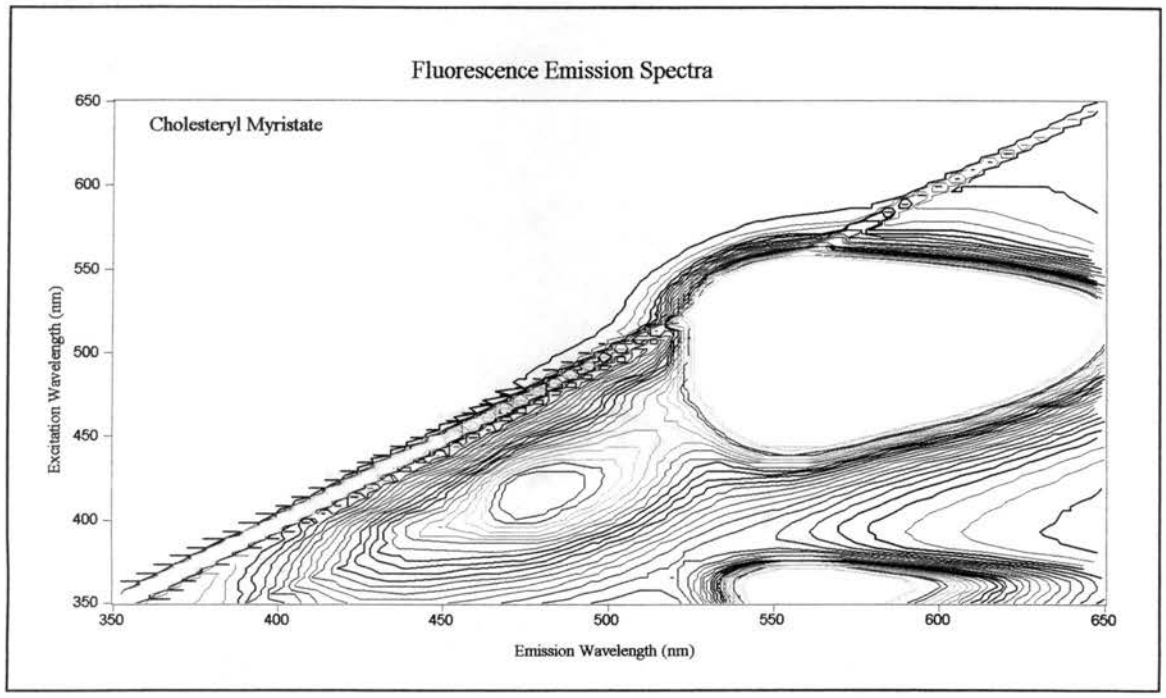


Figure 5-69. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with cholesteryl myristate after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

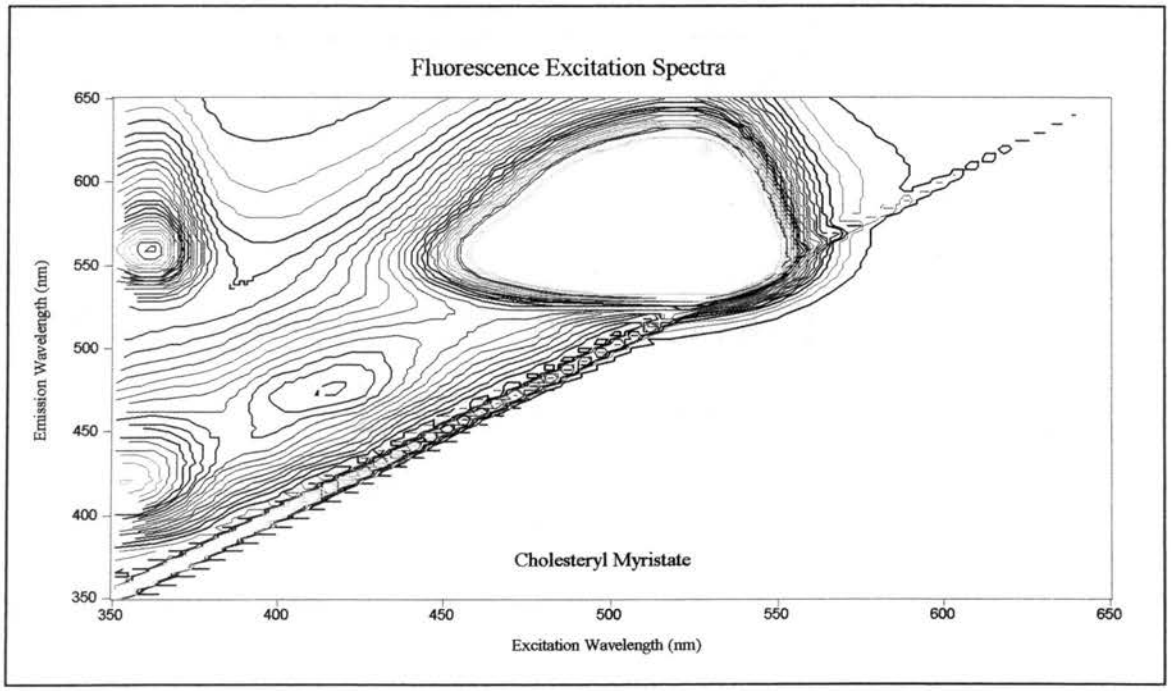


Figure 5-70. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with cholesteryl myristate after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

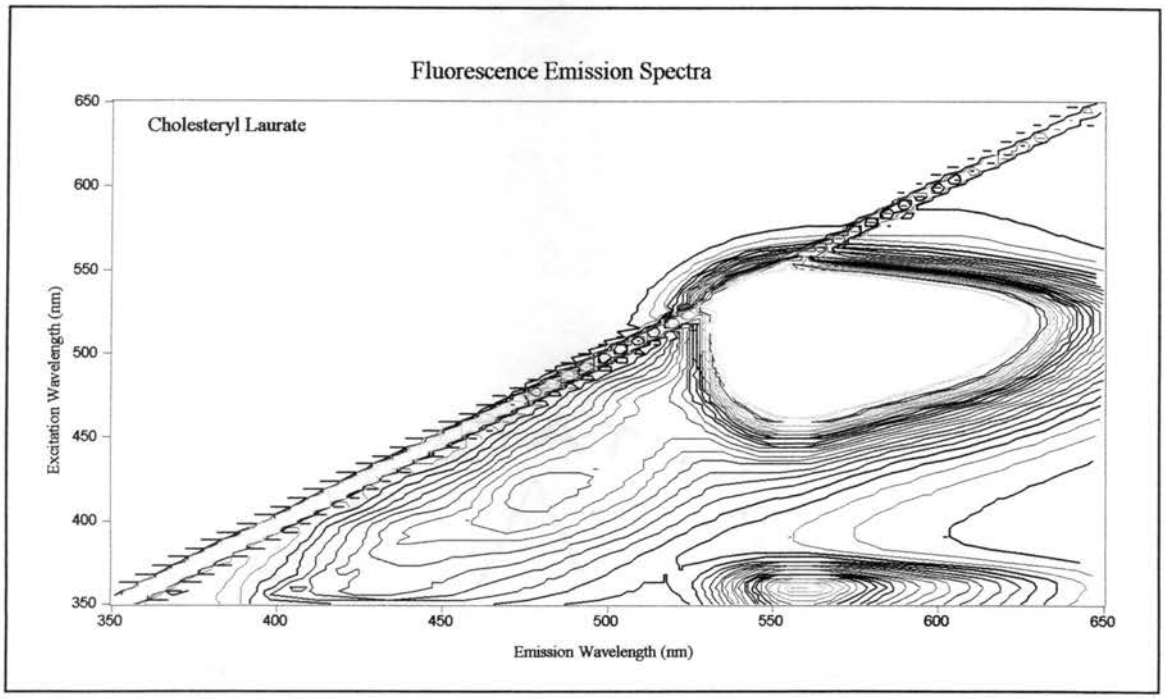


Figure 5-71. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with cholesteryl laurate after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

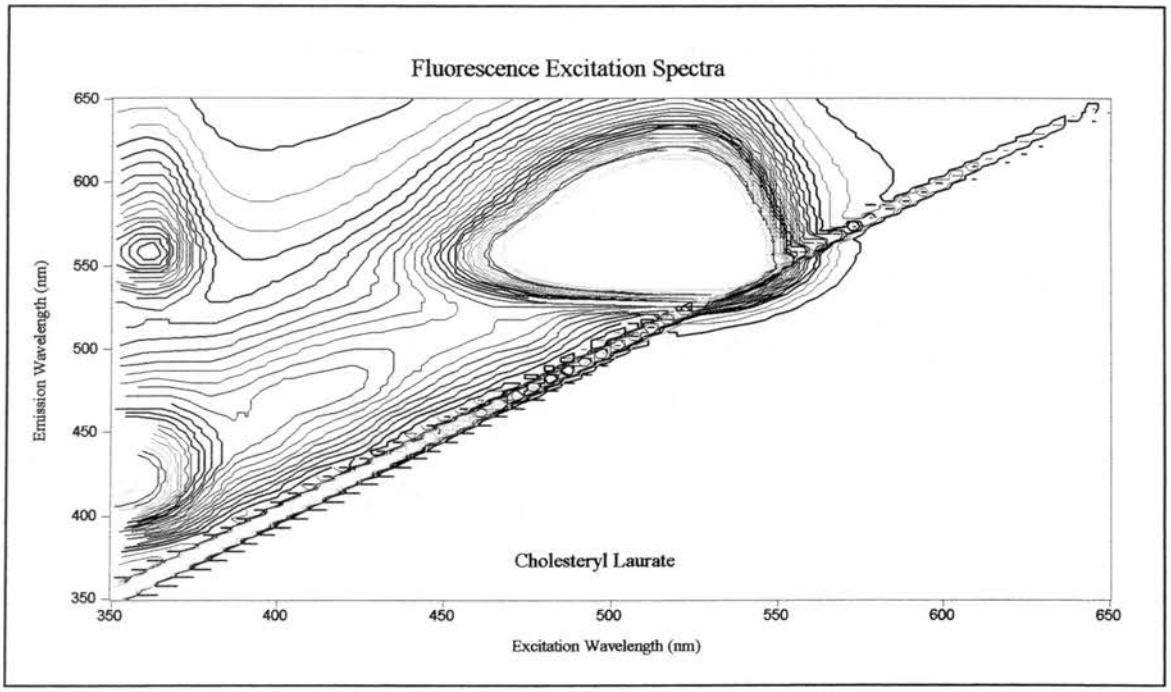


Figure 5-72. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with cholesteryl laurate after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

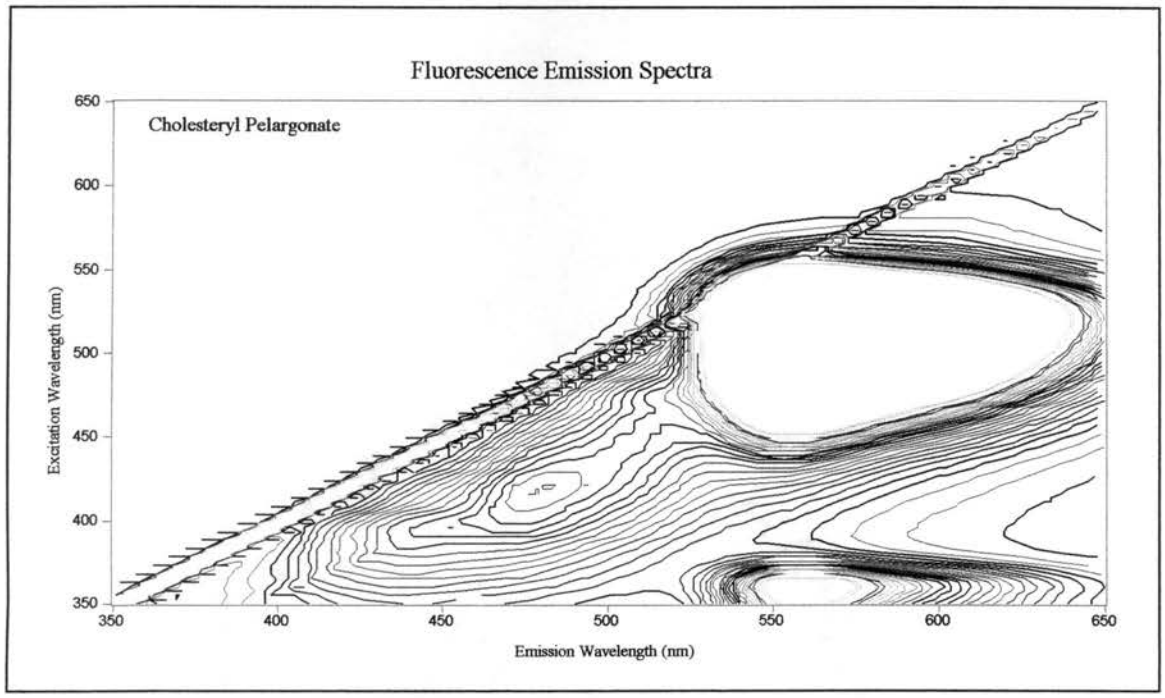


Figure 5-73. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with cholesteryl pelargonate after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

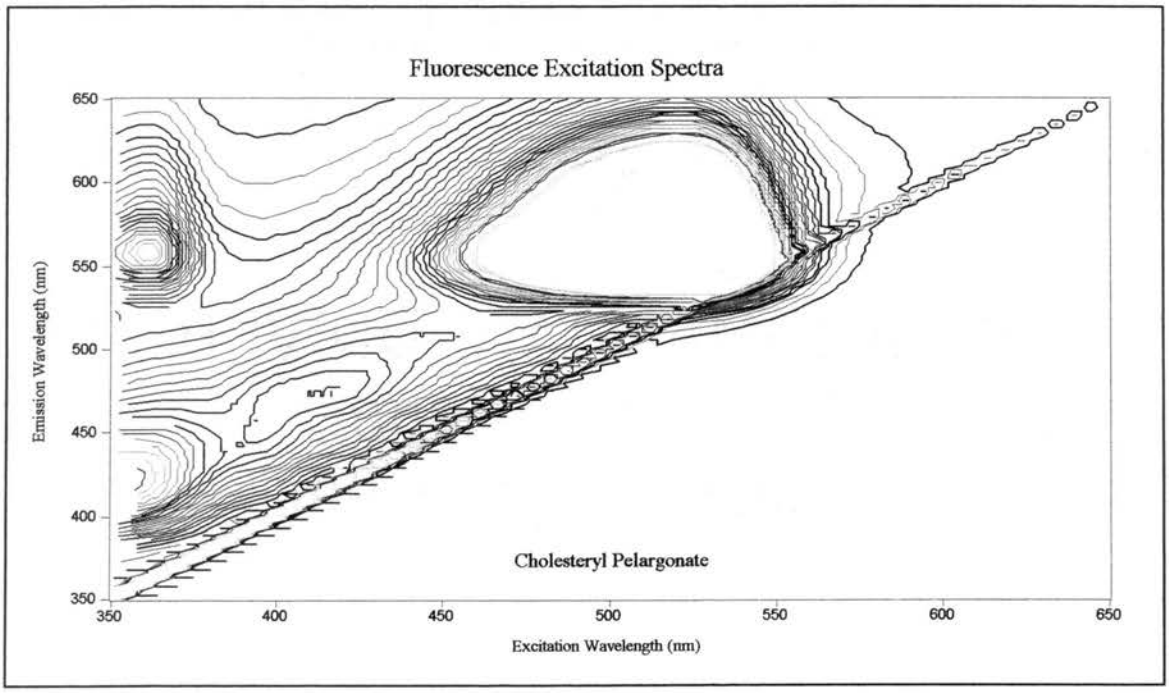


Figure 5-74. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with cholesteryl pelargonate after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

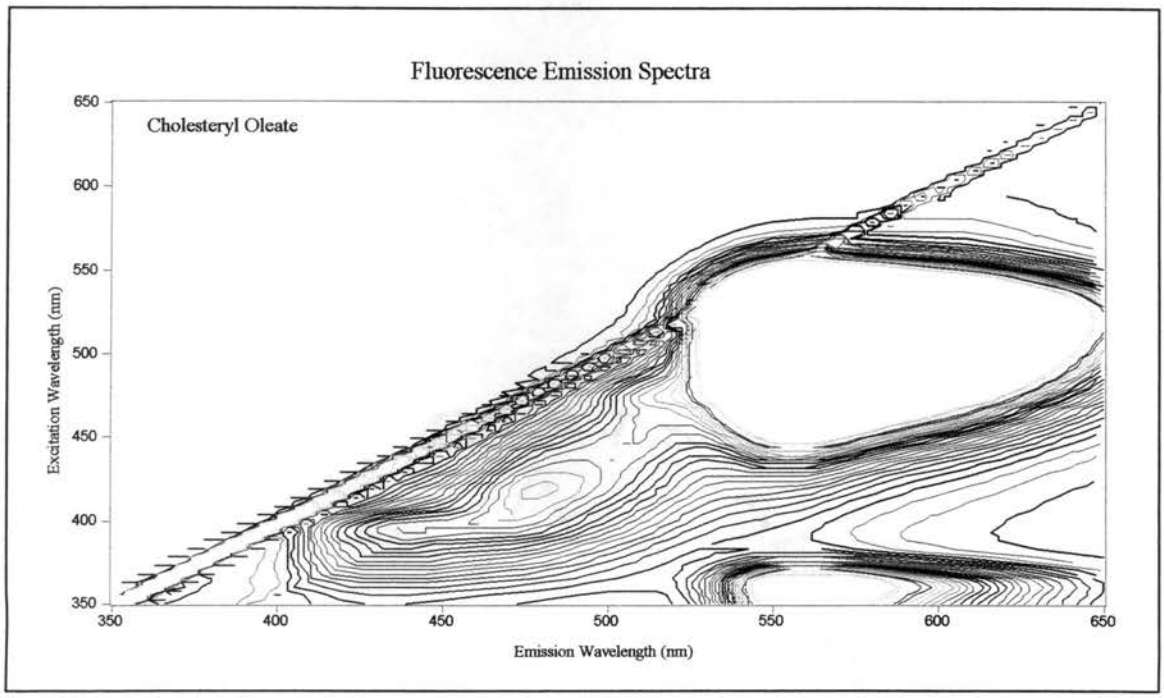


Figure 5-75. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with cholesteryl oleate after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

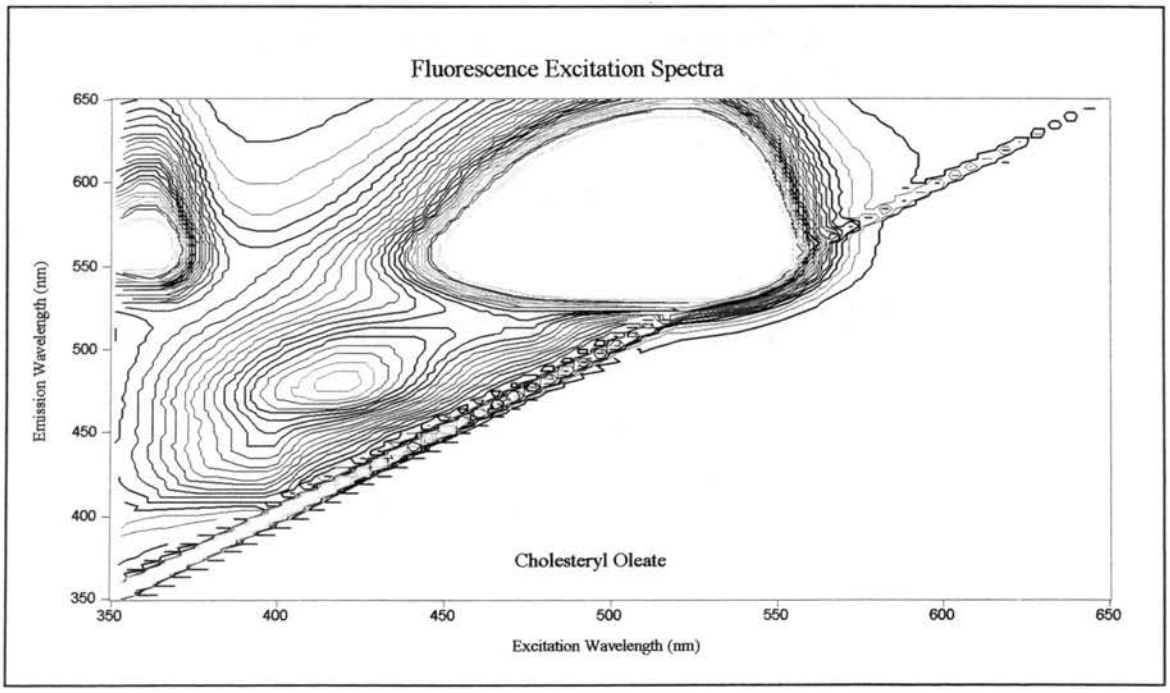


Figure 5-76. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with cholesteryl oleate after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

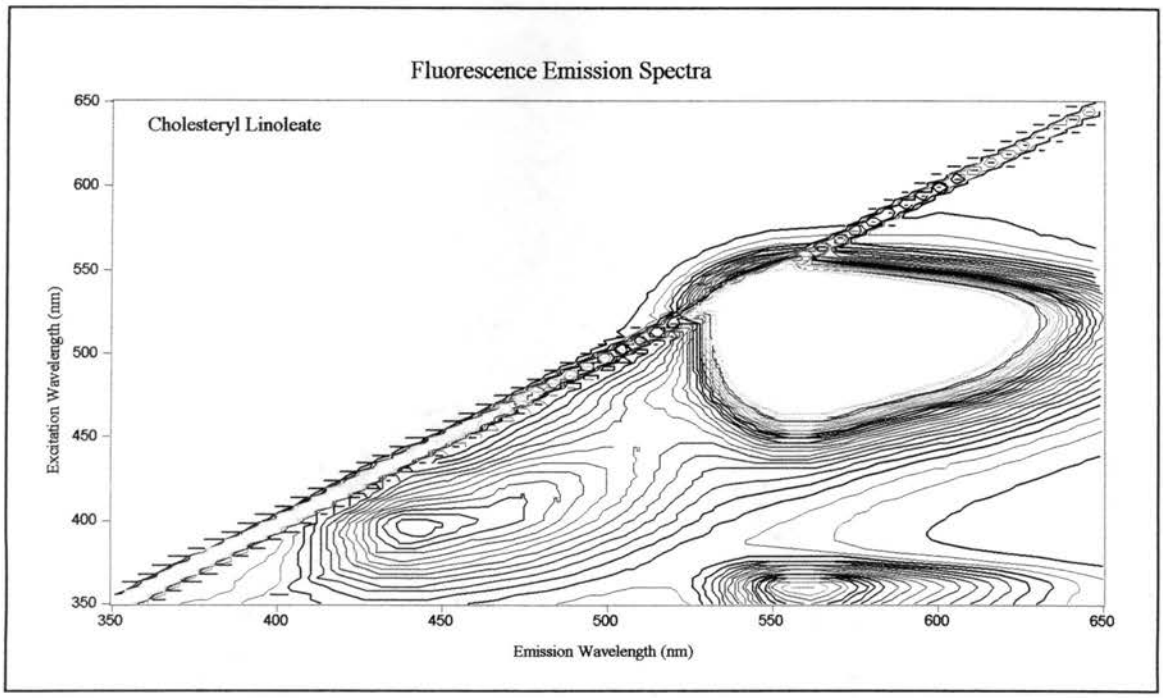


Figure 5-77. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with cholesteryl linoleate after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

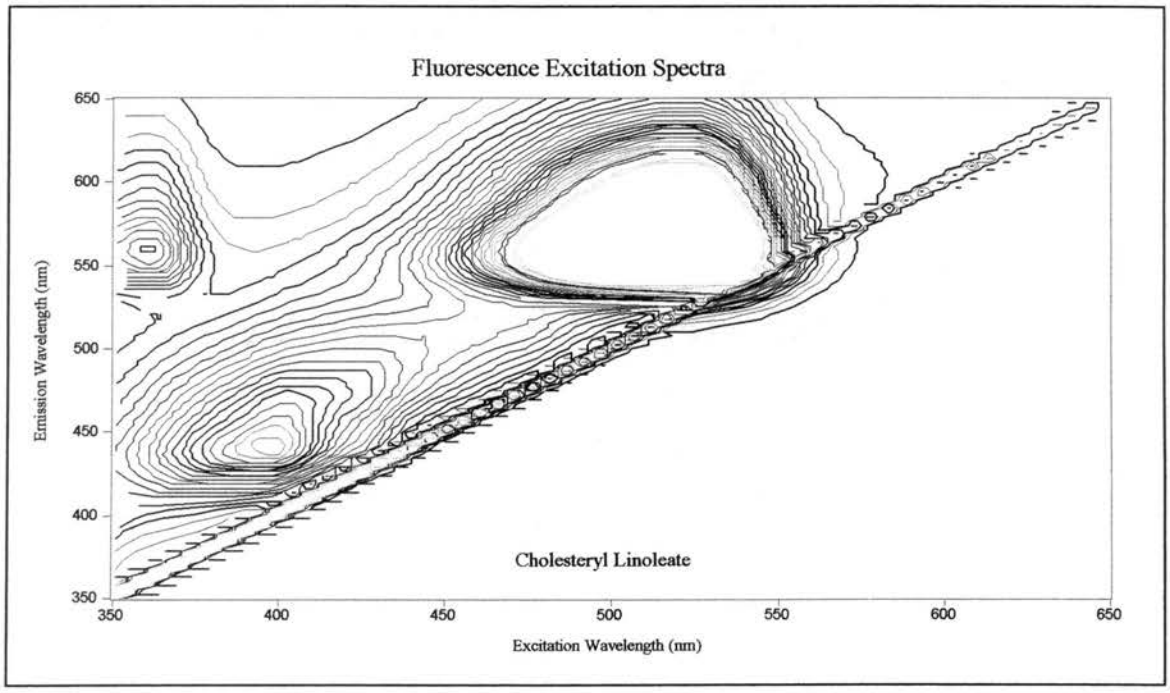


Figure 5-78. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with cholesteryl linoleate after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

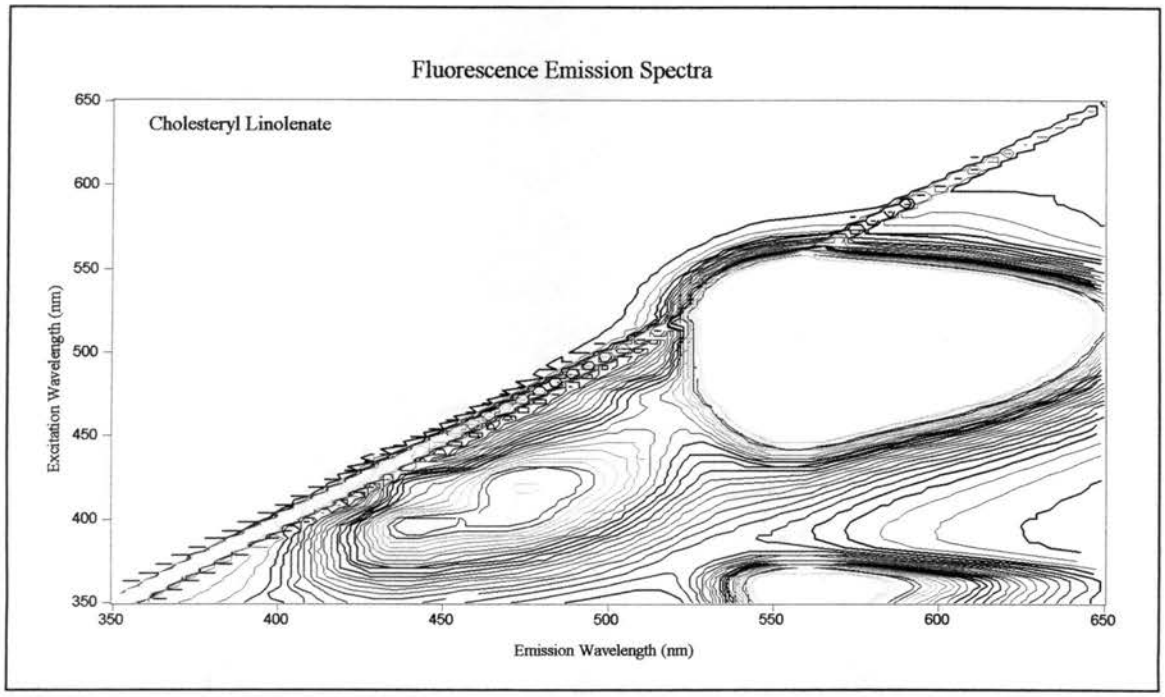


Figure 5-79. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with cholesteryl linolenate after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

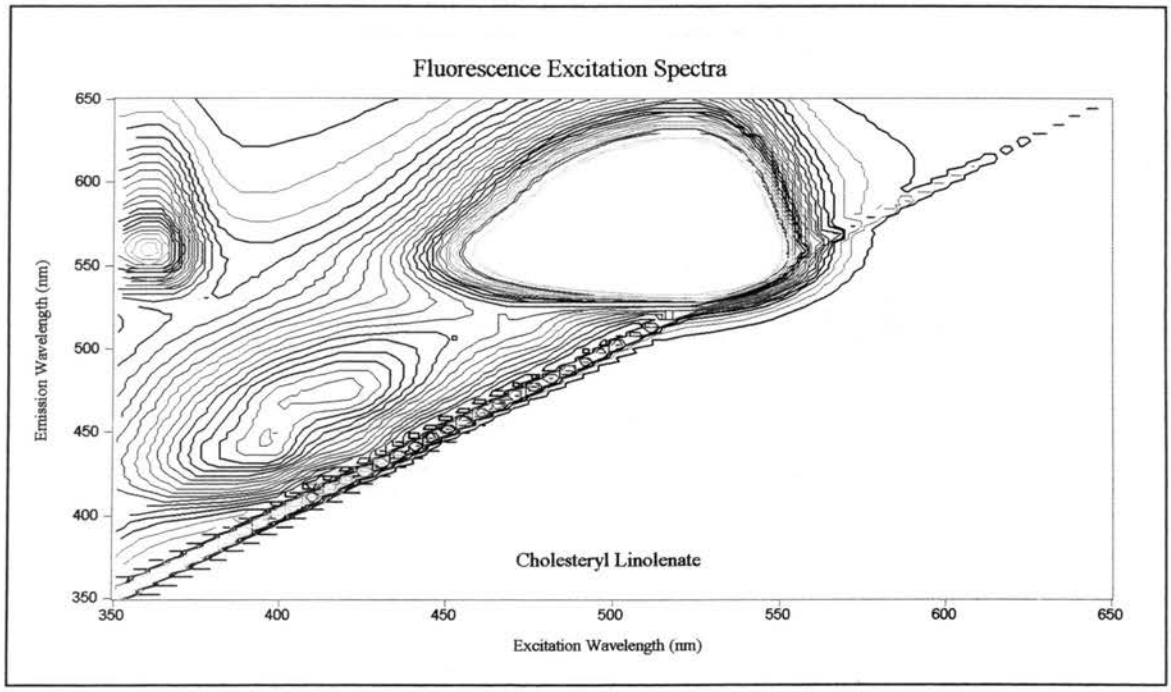


Figure 5-80. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with cholesteryl linolenate after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

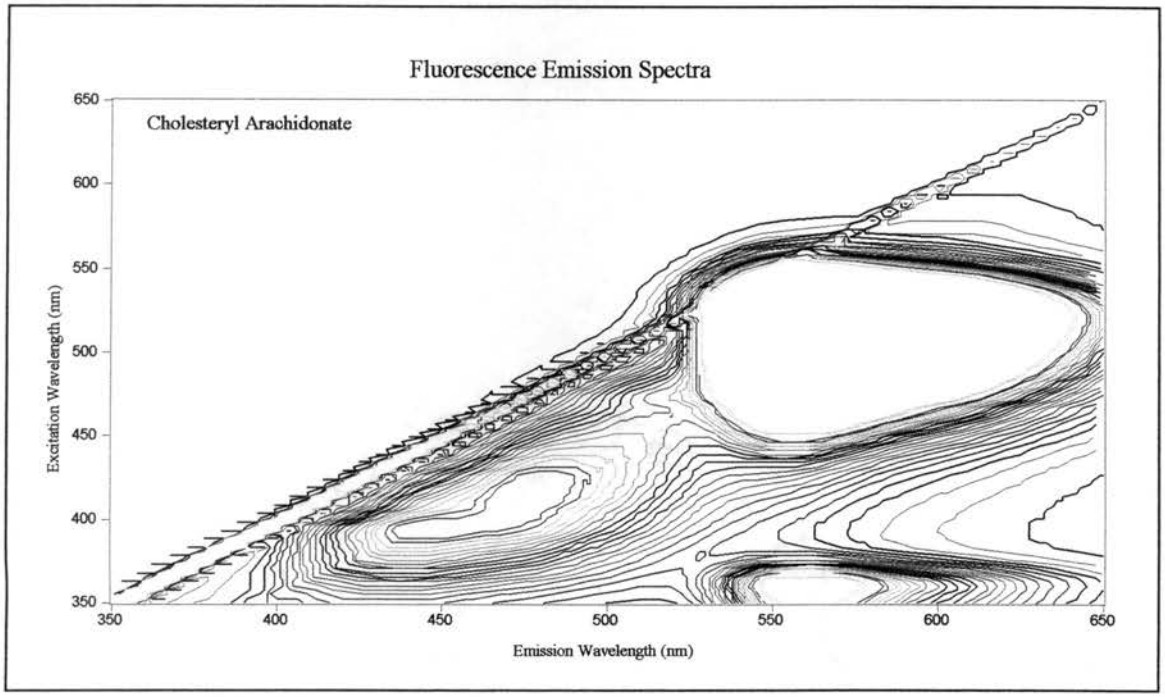


Figure 5-81. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with cholesteryl arachidonate after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

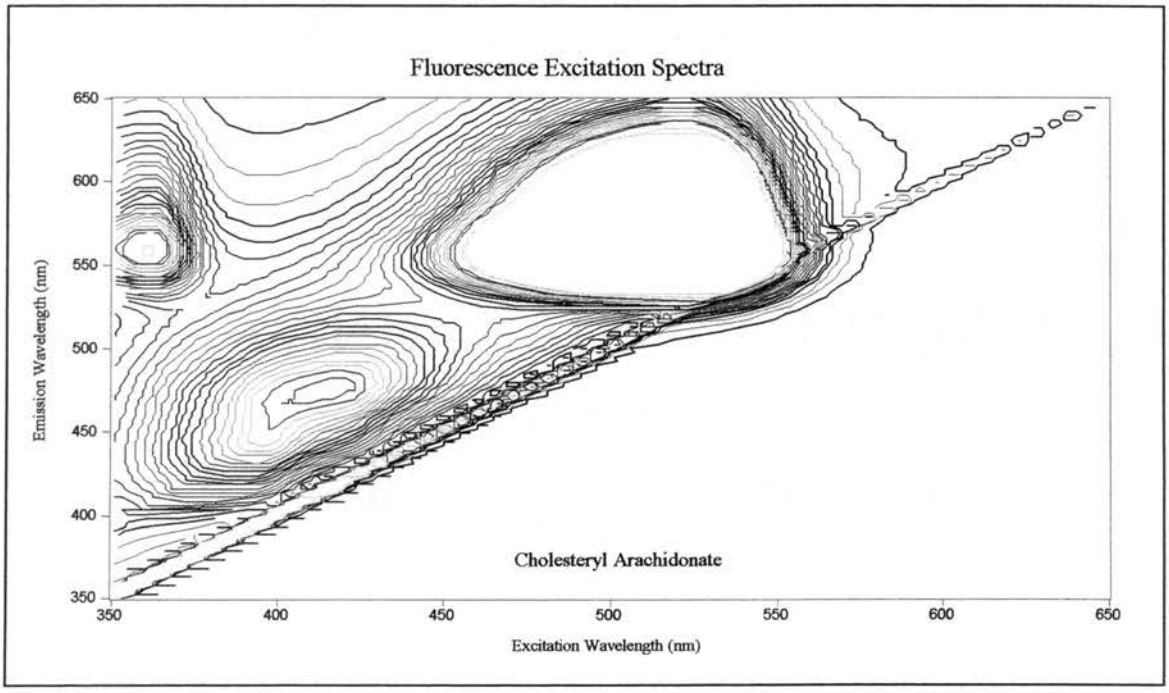


Figure 5-82. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with cholesteryl arachidonate after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

GLYCEROL ESTERS

The most prevalent dietary glycerol esters are the triglycerides. Constituting 95% of the fat stored in tissue, TGs are the predominant form of glycerol ester found in plasma. Their FA compositions vary considerably but usually include saturated and unsaturated combinations of long-chain FAs. In this study, simple triglycerides, both saturated and unsaturated were studied. These simple triglycerides included tristearin, triolein, trilinolein and trilinolenin. Much like the FAMEs, when the saturated triglyceride, tristearin is reacted with the SRS no absorbance or fluorescence spectra develops. The unsaturated FAs, however, all produce absorbance and fluorescence spectra that exhibit the same spectral features as the FAMEs from which they were composed. In other words, the spectra (absorbance and fluorescence) for the TG trilinolein is, in essence, the spectra of the LAME. This behavior held for all of the unsaturated TG SRMs and even held for mixtures of TGs (oils). Each oil examined, whether fish, olive, sunflower seed, soybean or safflower seed oil produced a spectrum that strongly correlated with the spectrum of the predominant unsaturated FA found in each mixture. For example, the collective spectra (absorbance, fluorescence emission and excitation) for fish oil, which is high in docosahexaenoic acid (DHA), correlated strongly with the spectrum of the FAME DHA. Similar correspondences are seen between olive oil, high in oleic acid, and the oleic acid methyl ester as well as between sunflower seed, safflower seed and soybean oils and LAME, see Figures 5-83 to 5-90 for the absorbance spectra and Figures 5-91 to 5-100 for the contour plots of the fluorescence emission and excitation matrix spectra for each of the triglycerides and mixtures of triglycerides examined.

Another major class of glycerol esters is the phosphoglycerides or phospholipids. These have FAs esterified on the alpha and beta carbons but contain a phosphoric acid ester at the gamma carbon. Just as in the case of the triglycerides, phospholipids containing only saturated FA moieties develop no spectra, while phospholipids containing unsaturated FA moieties produce spectra that correlate strongly with the spectra of the FA of which they are composed. Absorbance spectra of the various phospholipids examined in this study containing unsaturated FA moieties are plotted in Figures 5-101 to 5-108. The corresponding fluorescence emission and excitation contour plots are found in Figures 5-109 to 5-120.

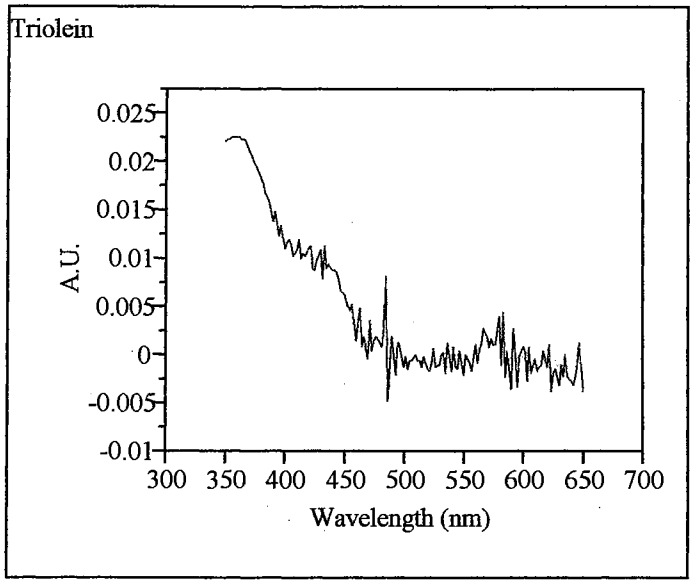


Figure 5-83. Absorption spectrum of the colored products of the reaction of the standard reagent with triolein after 20 minutes.

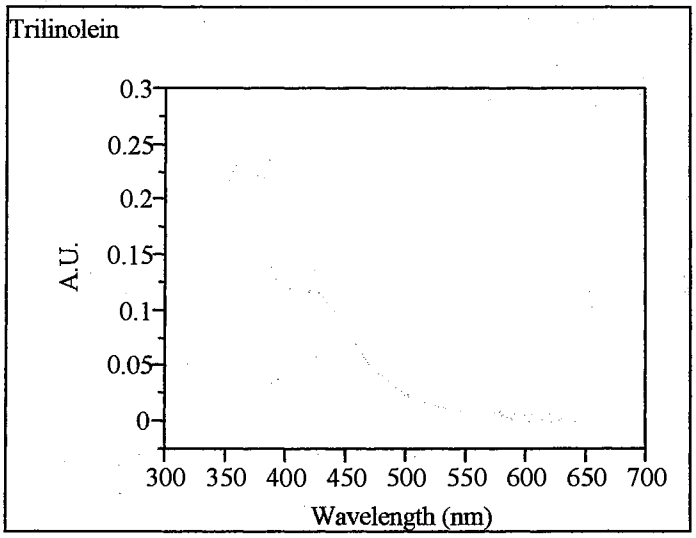


Figure 5-84. Absorption spectrum of the colored products of the reaction of the standard reagent with trilinolein after 20 minutes.

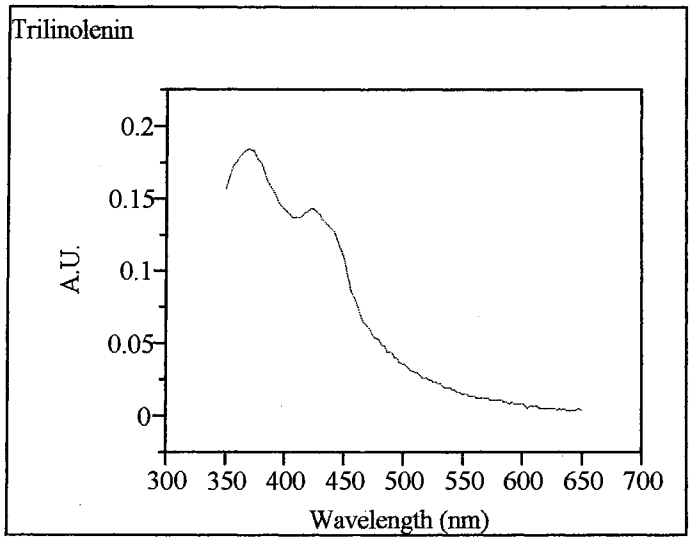


Figure 5-85. Absorption spectrum of the colored products of the reaction of the standard reagent with trilinolenin after 20 minutes.

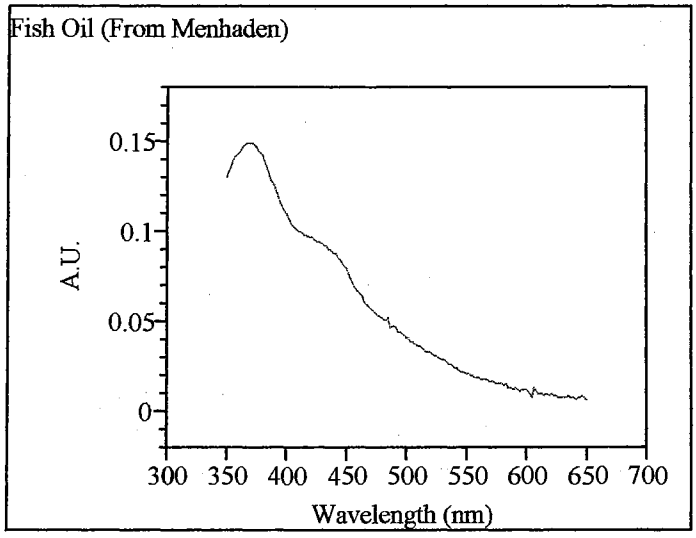


Figure 5-86. Absorption spectrum of the colored products of the reaction of the standard reagent with fish oil (from menhaden) after 20 minutes.

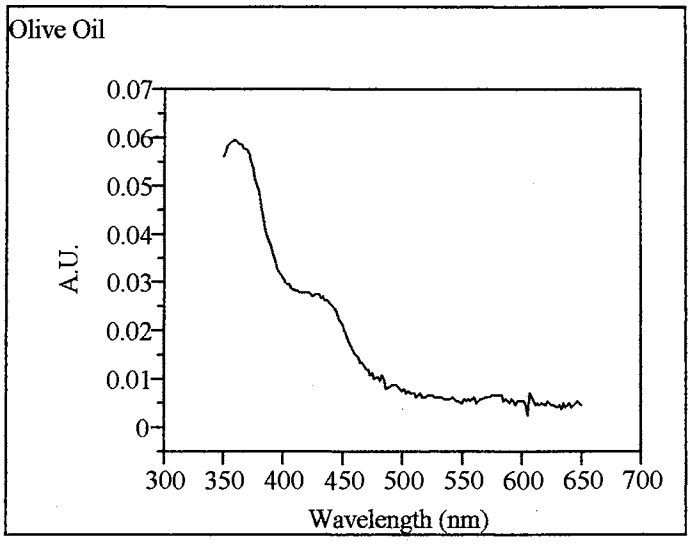


Figure 5-87. Absorption spectrum of the colored products of the reaction of the standard reagent with olive oil after 20 minutes.

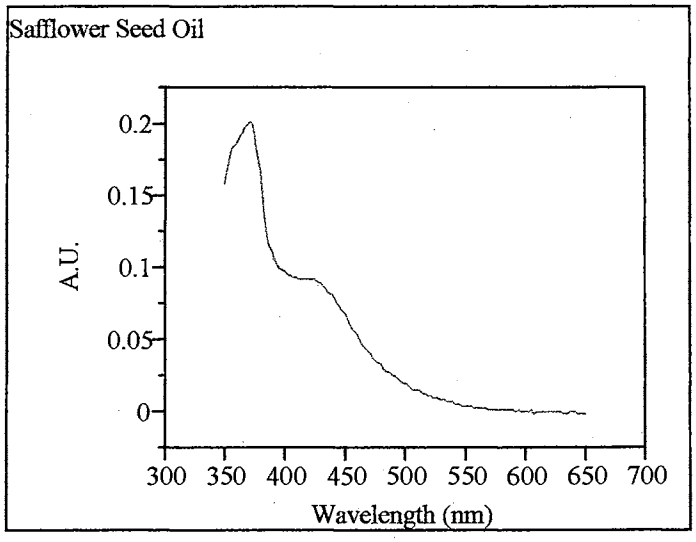


Figure 5-88. Absorption spectrum of the colored products of the reaction of the standard reagent with safflower seed oil after 20 minutes.

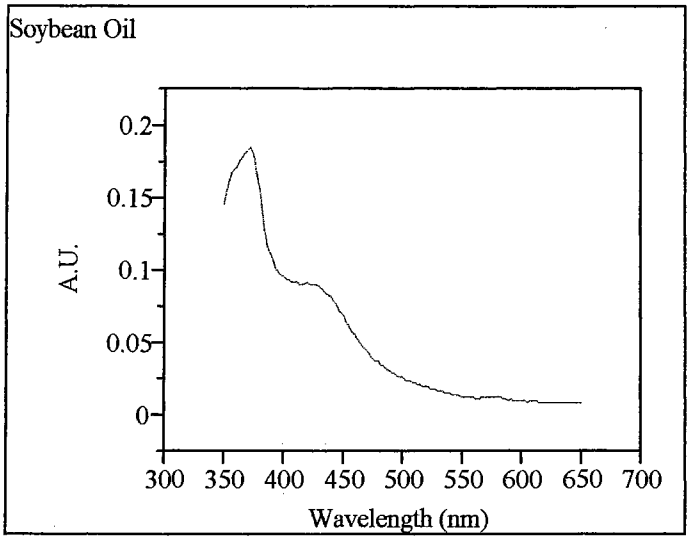


Figure 5-89. Absorption spectrum of the colored products of the reaction of the standard reagent with soybean oil after 20 minutes.

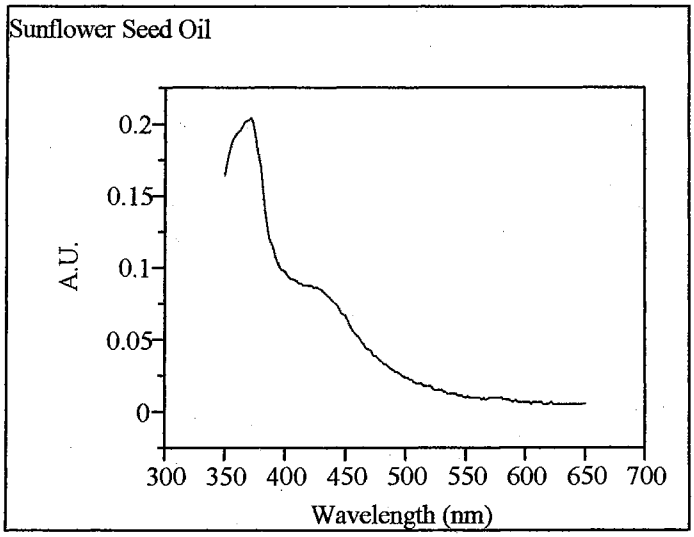


Figure 5-90. Absorption spectrum of the colored products of the reaction of the standard reagent with sunflower seed oil after 20 minutes.

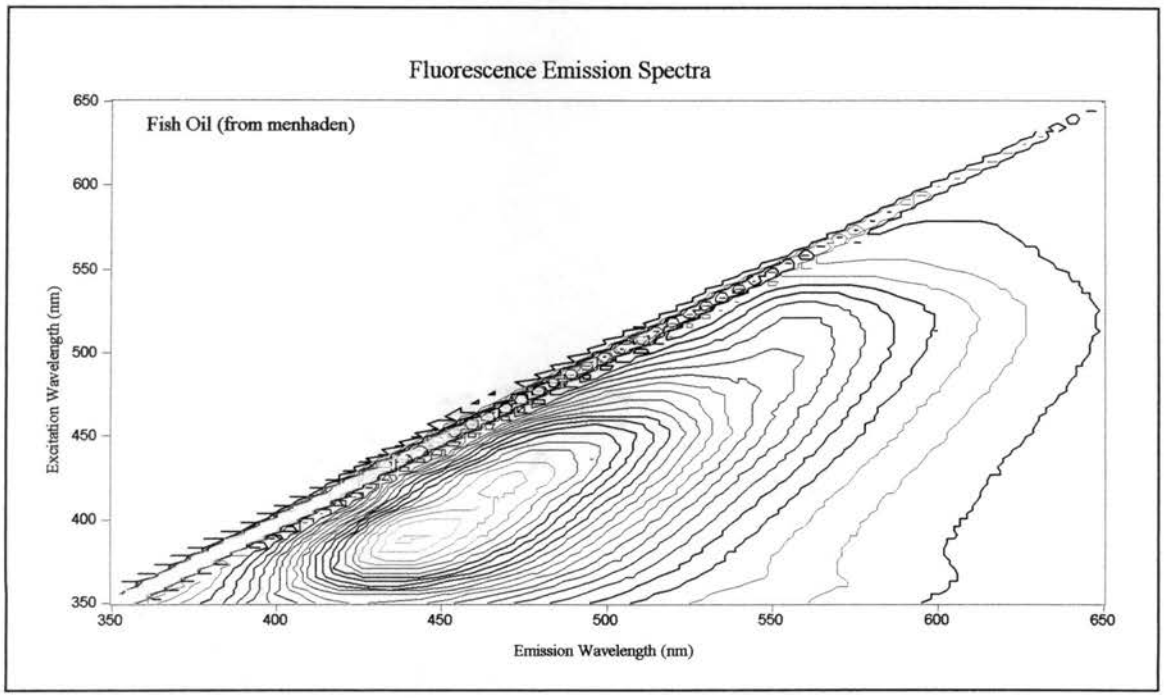


Figure 5-91. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with fish oil after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

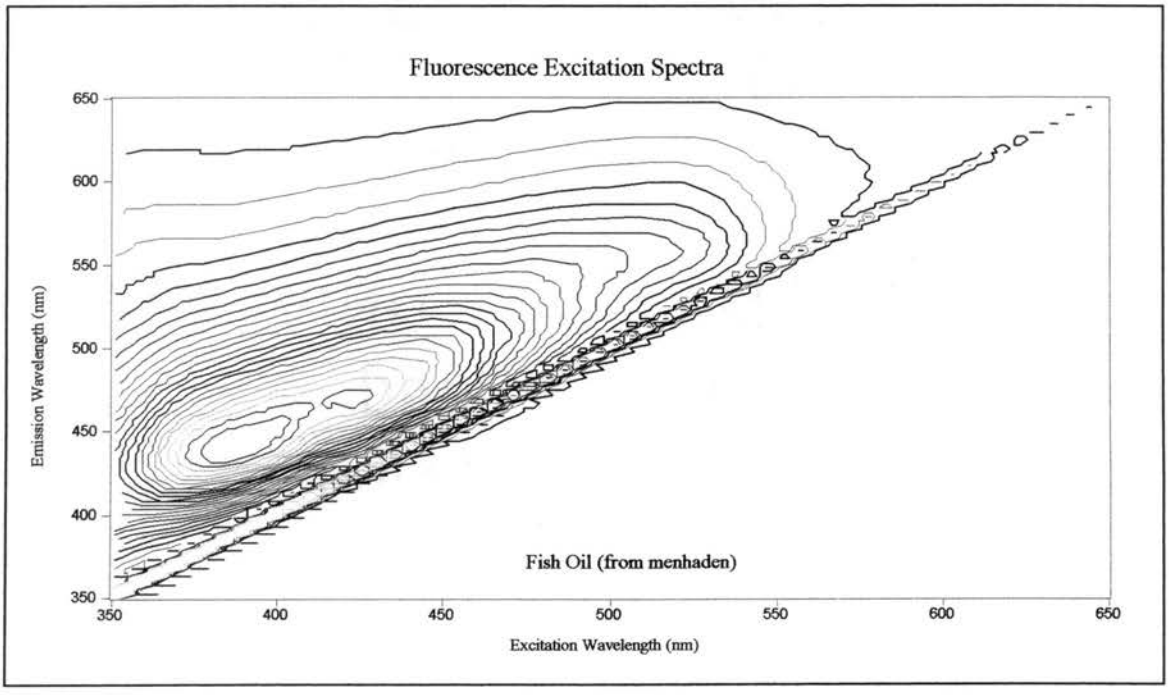


Figure 5-92. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with fish oil after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

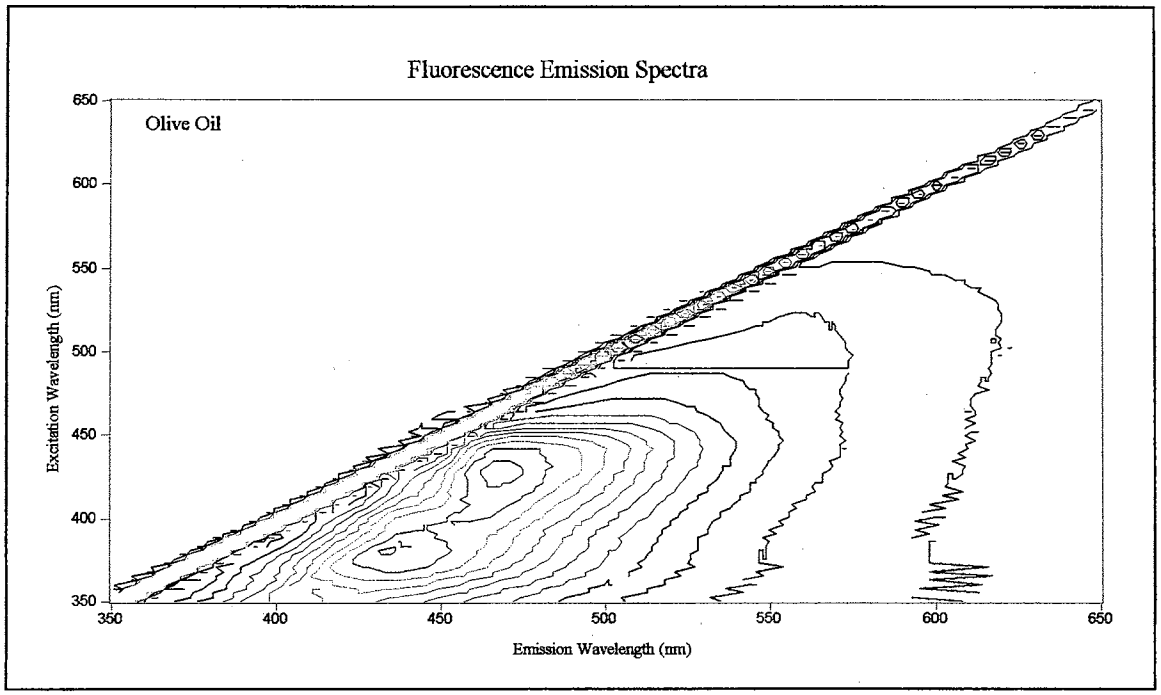


Figure 5-93. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with olive oil after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

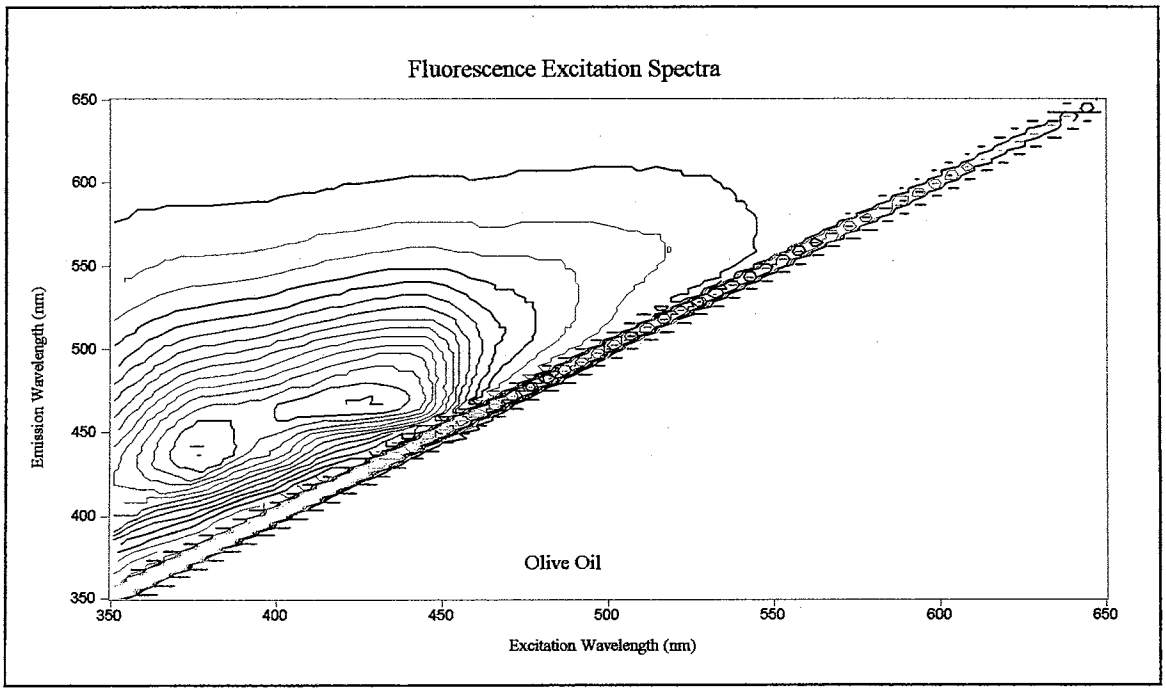


Figure 5-94. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with olive oil after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

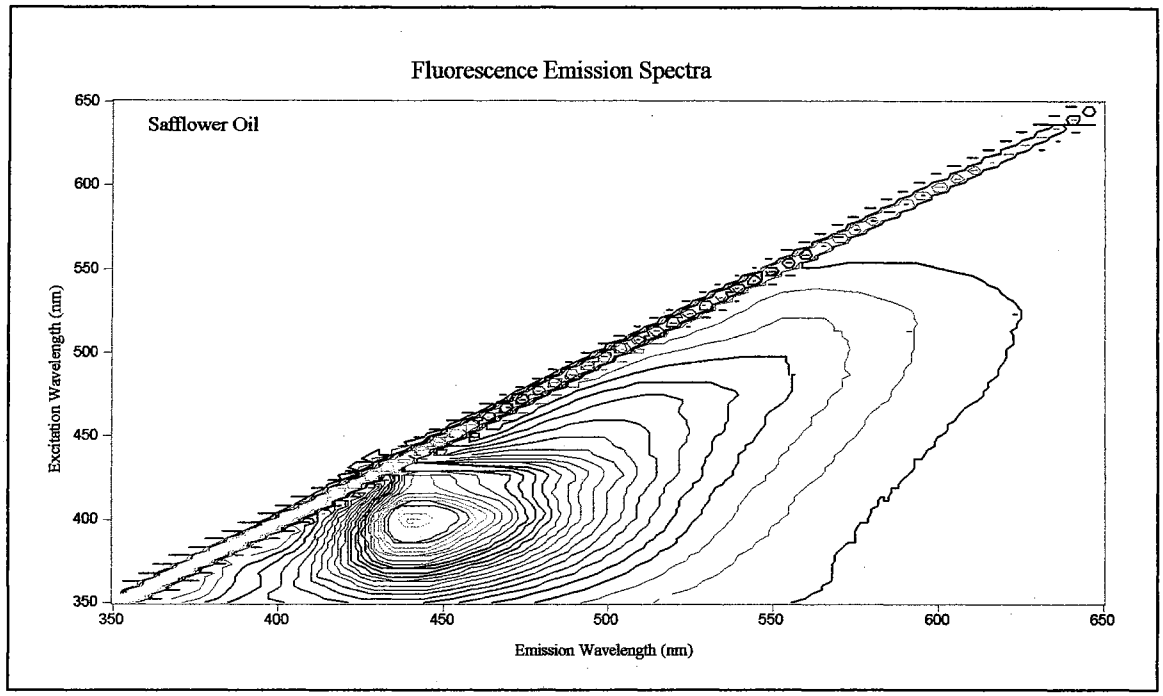


Figure 5-95. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with safflower oil after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

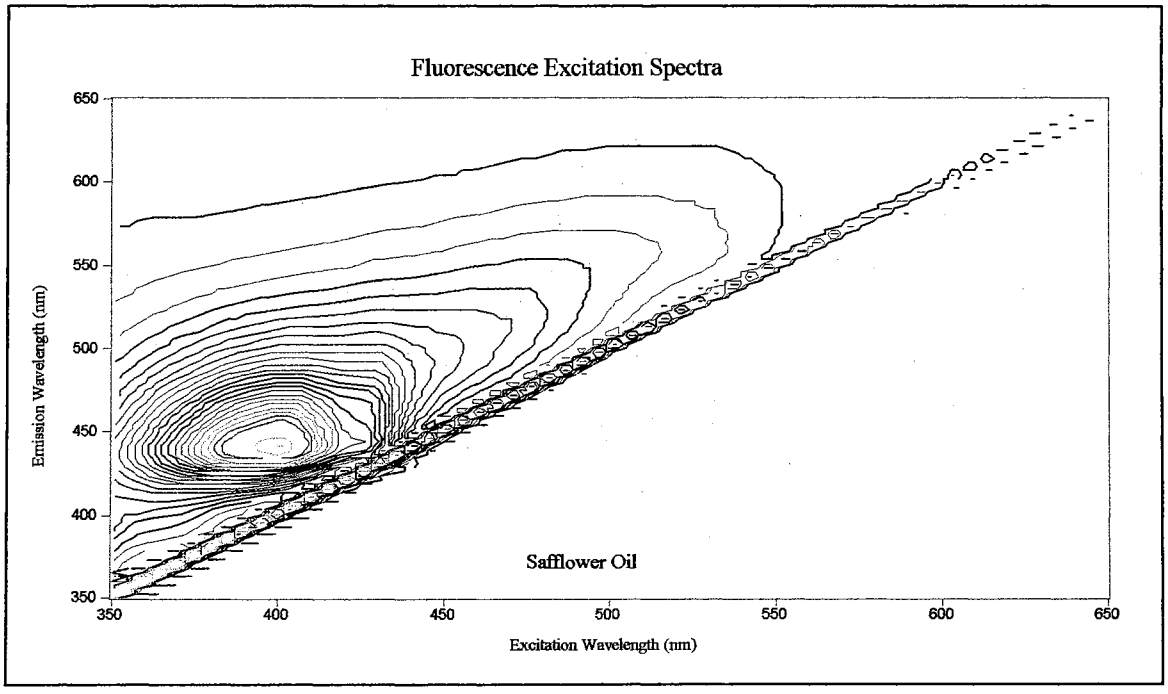


Figure 5-96. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with safflower oil after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

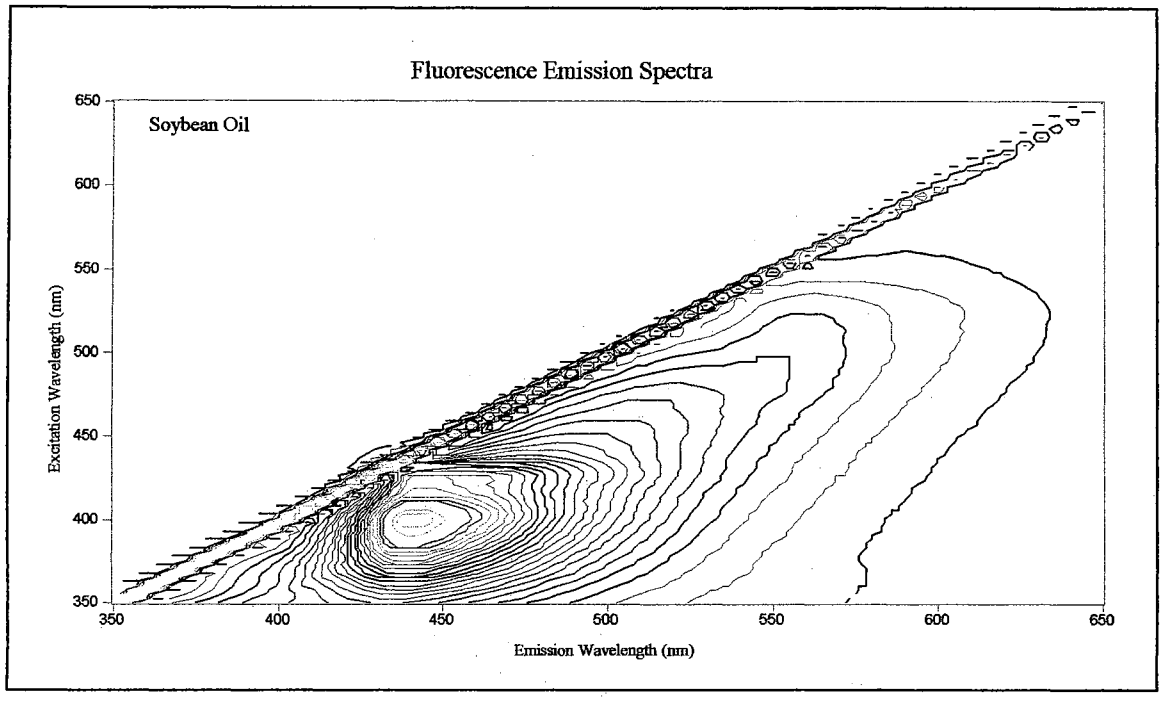


Figure 5-97. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with soybean oil after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

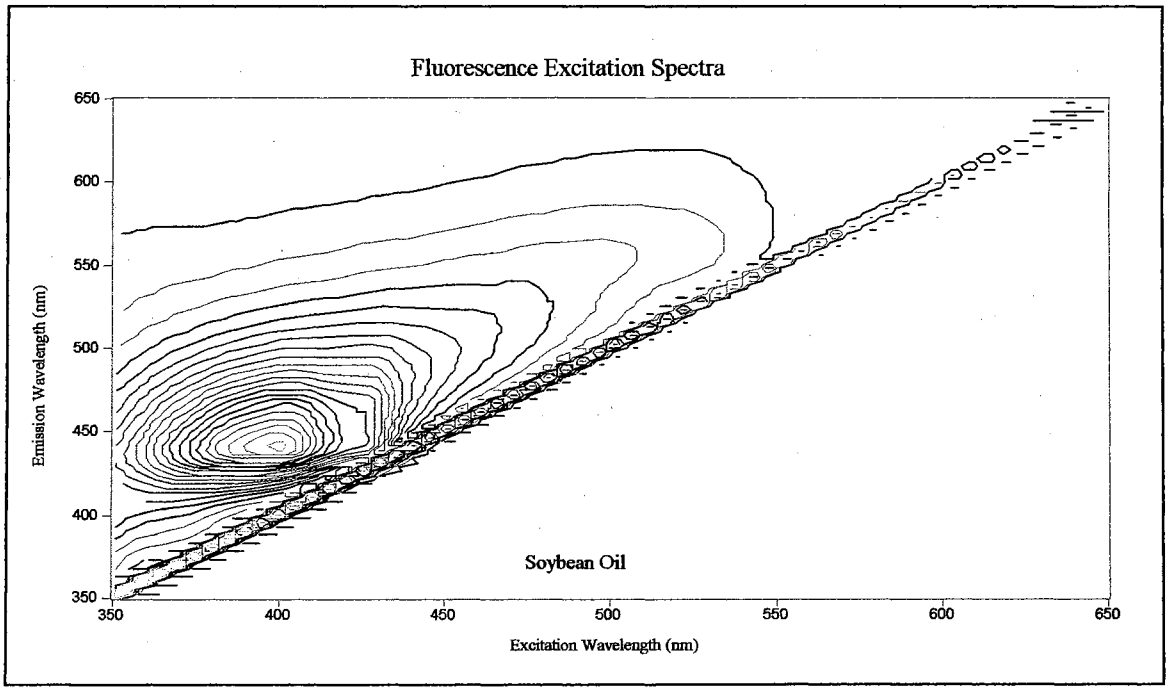


Figure 5-98. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with soybean oil after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

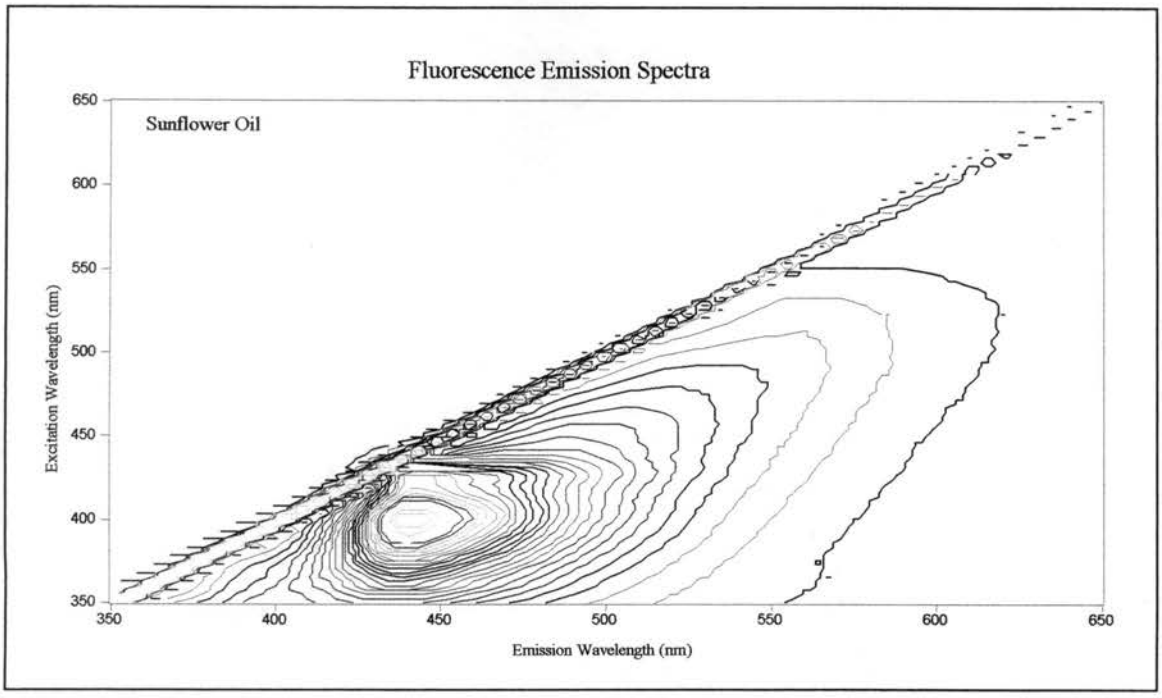


Figure 5-99. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with sunflower oil after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

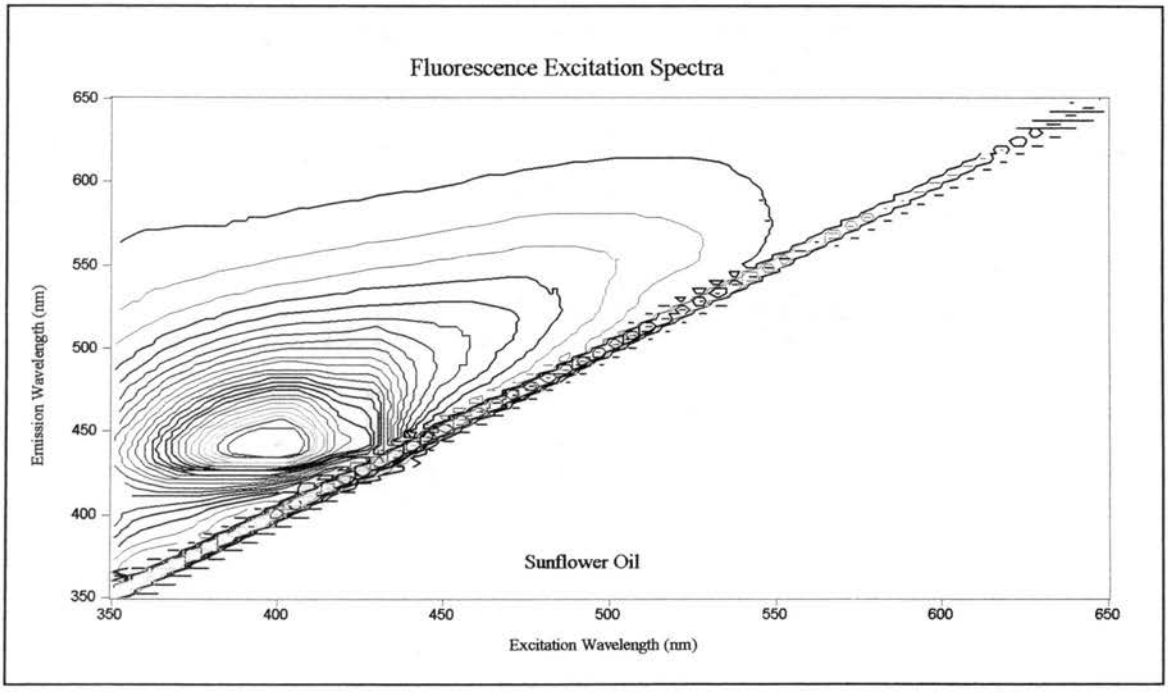


Figure 5-100. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with sunflower oil after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

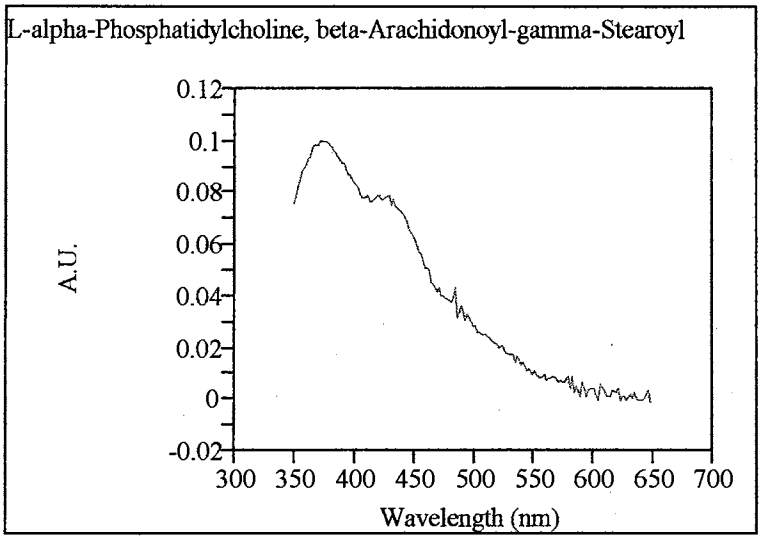


Figure 5-101. Absorption spectrum of the colored products of the reaction of the standard reagent with L-alpha-phosphatidylcholine, beta-arachidonoyl-gamma-stearoyl after 20 minutes.

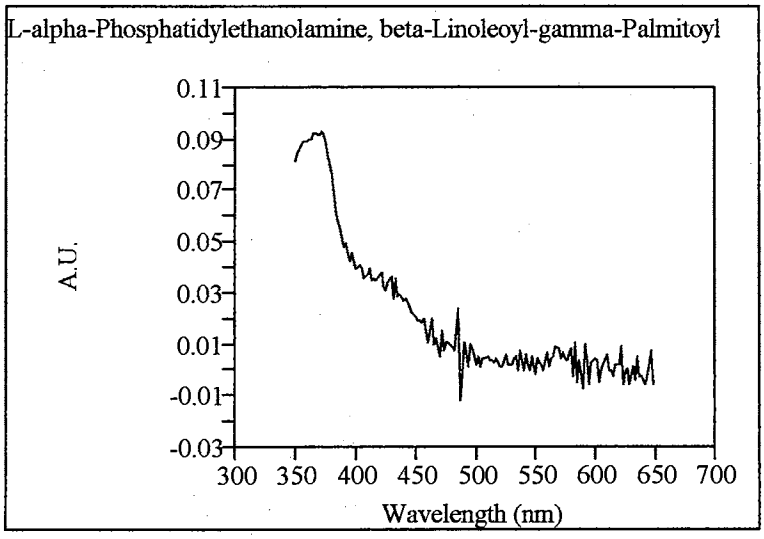


Figure 5-102. Absorption spectrum of the colored products of the reaction of the standard reagent with L-alpha-phosphatidylethanolamine, beta-linoleoyl-gamma-palmitoyl after 20 minutes.

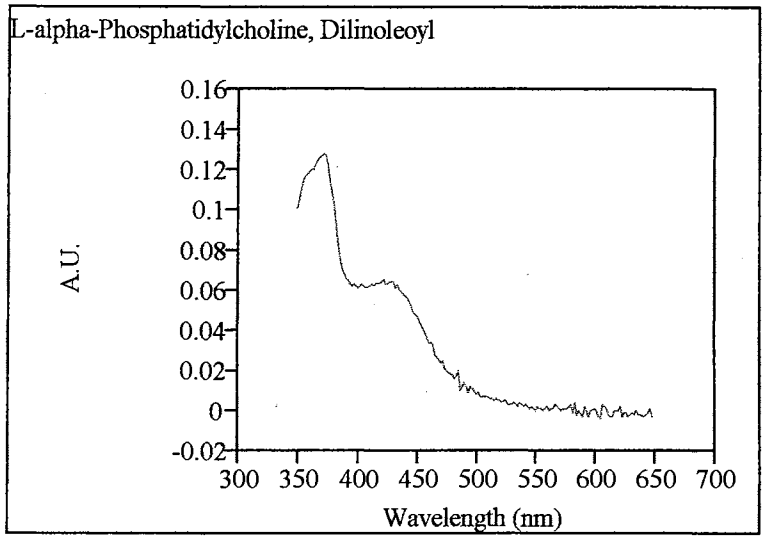


Figure 5-103. Absorption spectrum of the colored products of the reaction of the standard reagent with L-alpha-phosphatidylcholine, dilinoleoyl after 20 minutes.

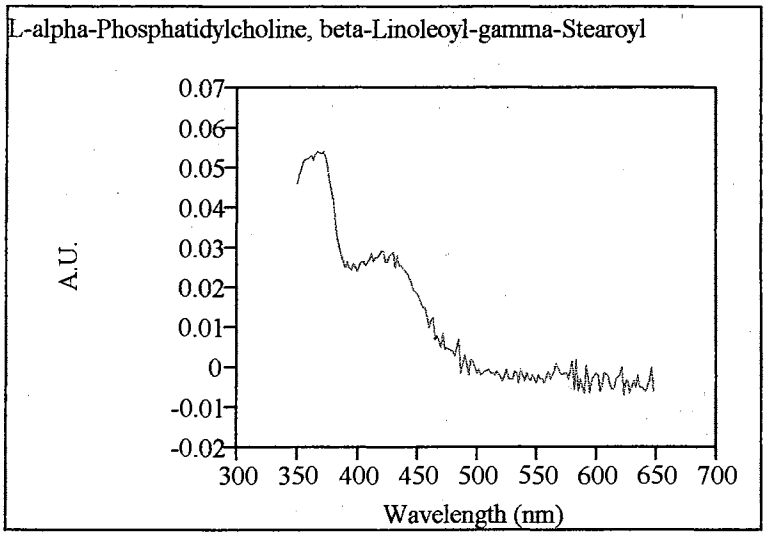


Figure 5-104. Absorption spectrum of the colored products of the reaction of the standard reagent with L-alpha-phosphatidylcholine, beta-linoleoyl-gamma-stearoyl after 20 minutes.

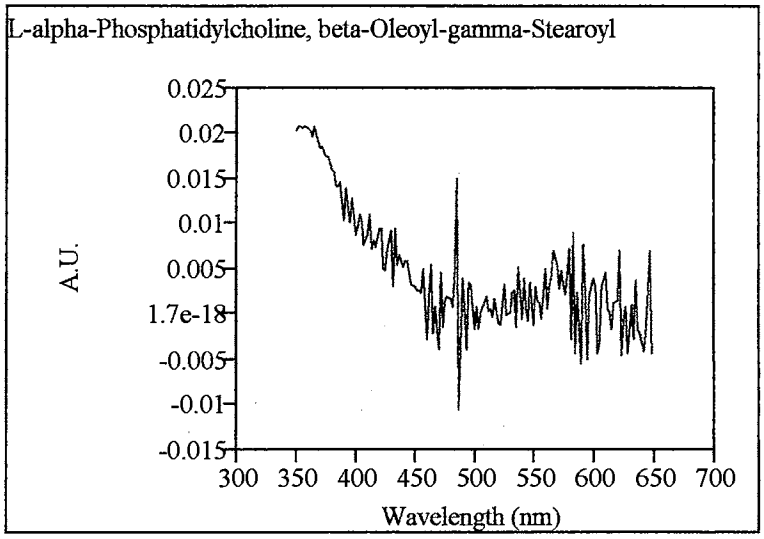


Figure 5-105. Absorption spectrum of the colored products of the reaction of the standard reagent with L-alpha-phosphatidylcholine, beta-oleoyl-gamma-stearoyl after 20 minutes.

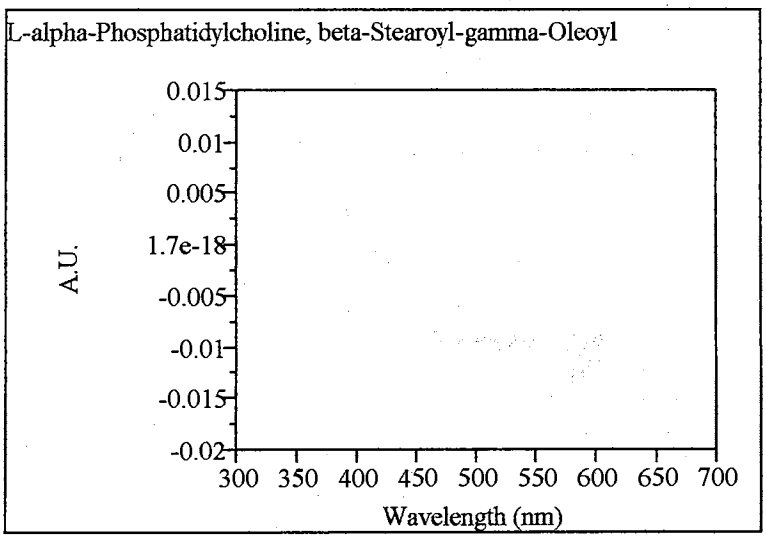


Figure 5-106. Absorption spectrum of the colored products of the reaction of the standard reagent with L-alpha-phosphatidylcholine, beta-stearoyl-gamma-oleoyl after 20 minutes.

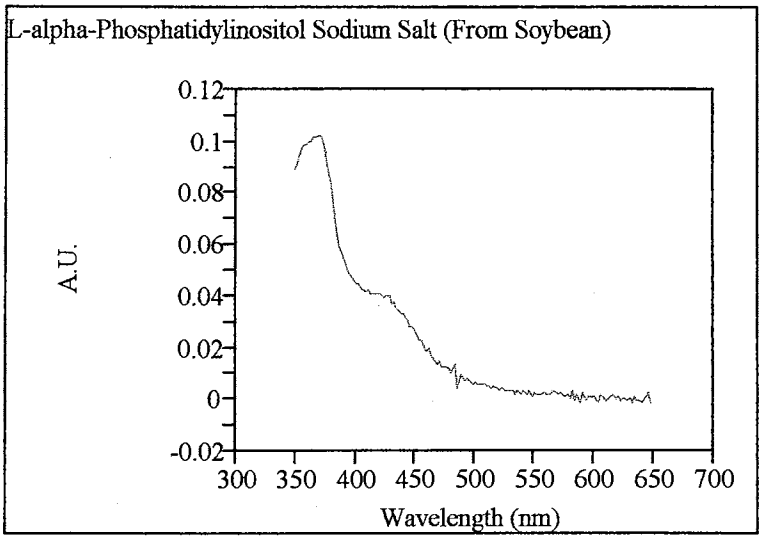


Figure 5-107. Absorption spectrum of the colored products of the reaction of the standard reagent with L-alpha-phosphatidylinositol sodium salt (from soybean) after 20 minutes.

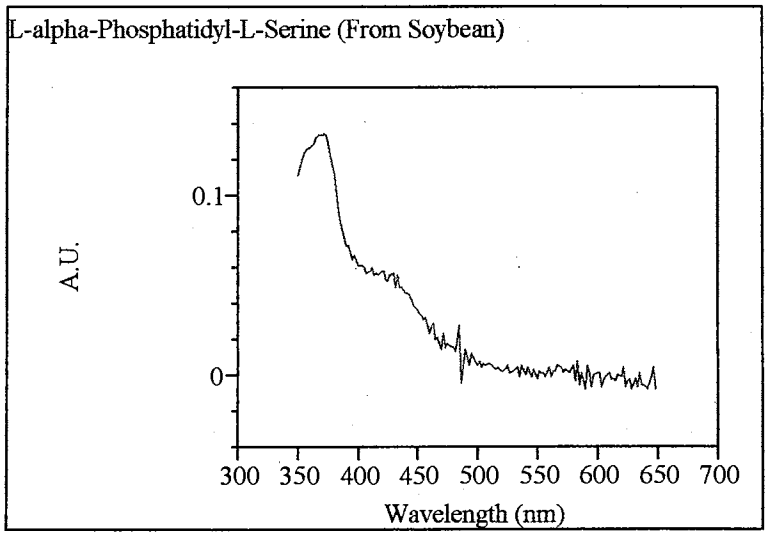


Figure 5-108. Absorption spectrum of the colored products of the reaction of the standard reagent with L-alpha-phosphatidyl-L-serine (from soybean) after 20 minutes.

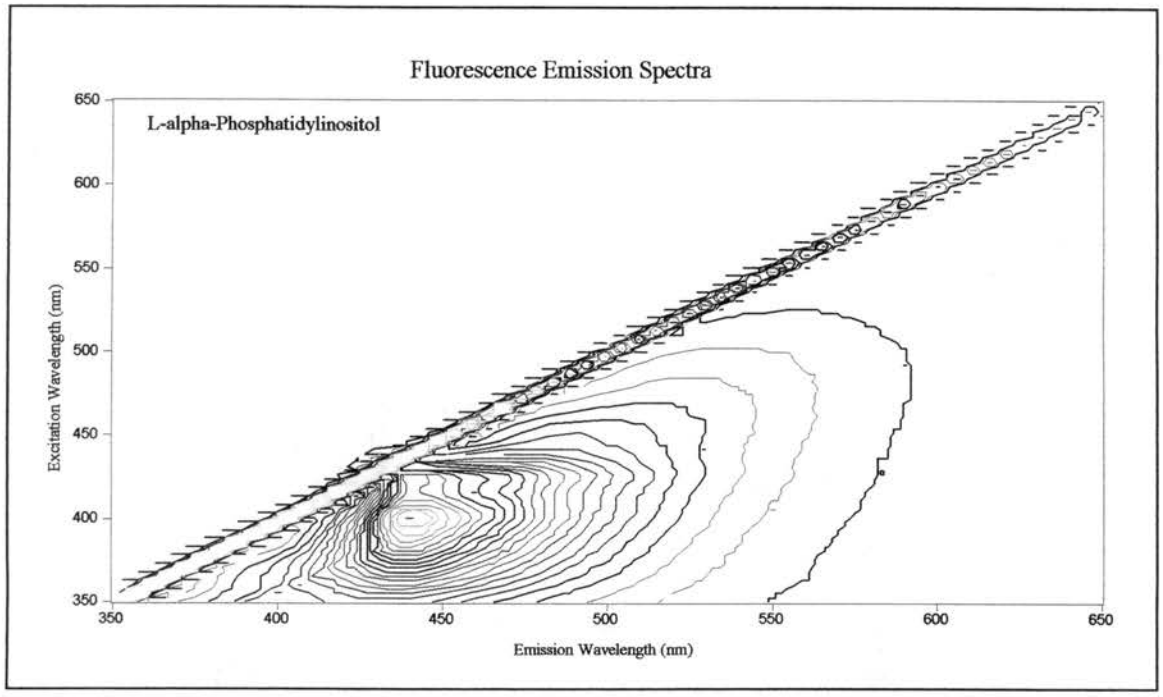


Figure 5-109. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with L-alpha-phosphatidylcholine after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

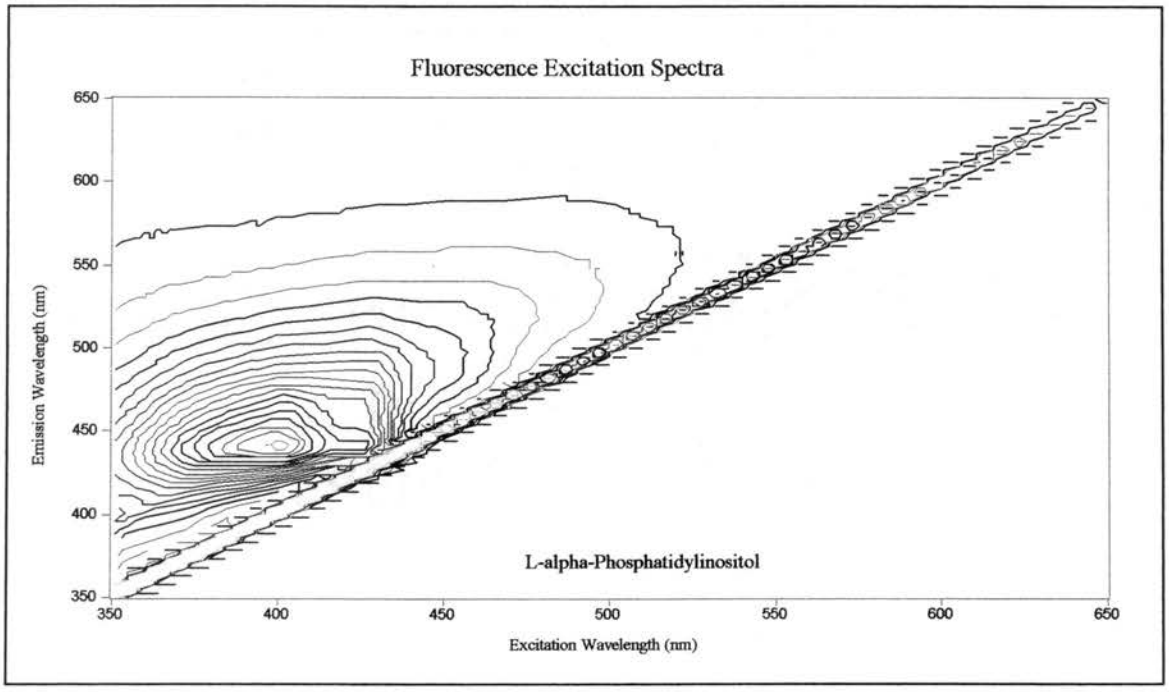


Figure 5-110. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with L-alpha-phosphatidylcholine after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

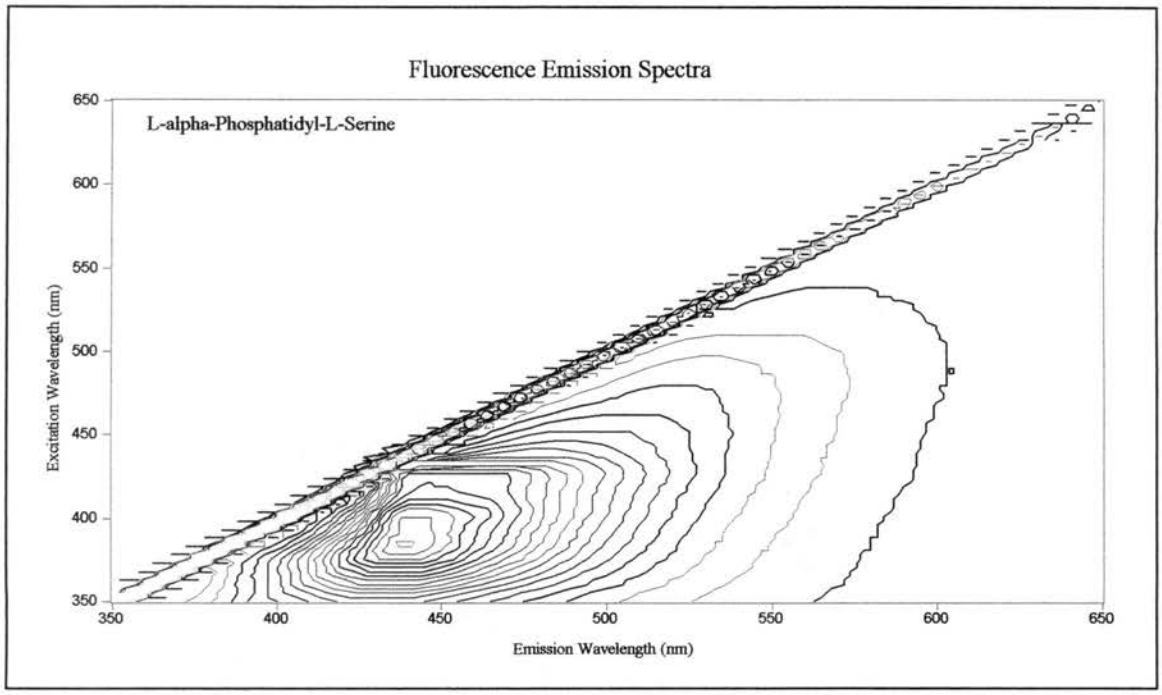


Figure 5-111. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with L-alpha-phosphatidyl-L-serine after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

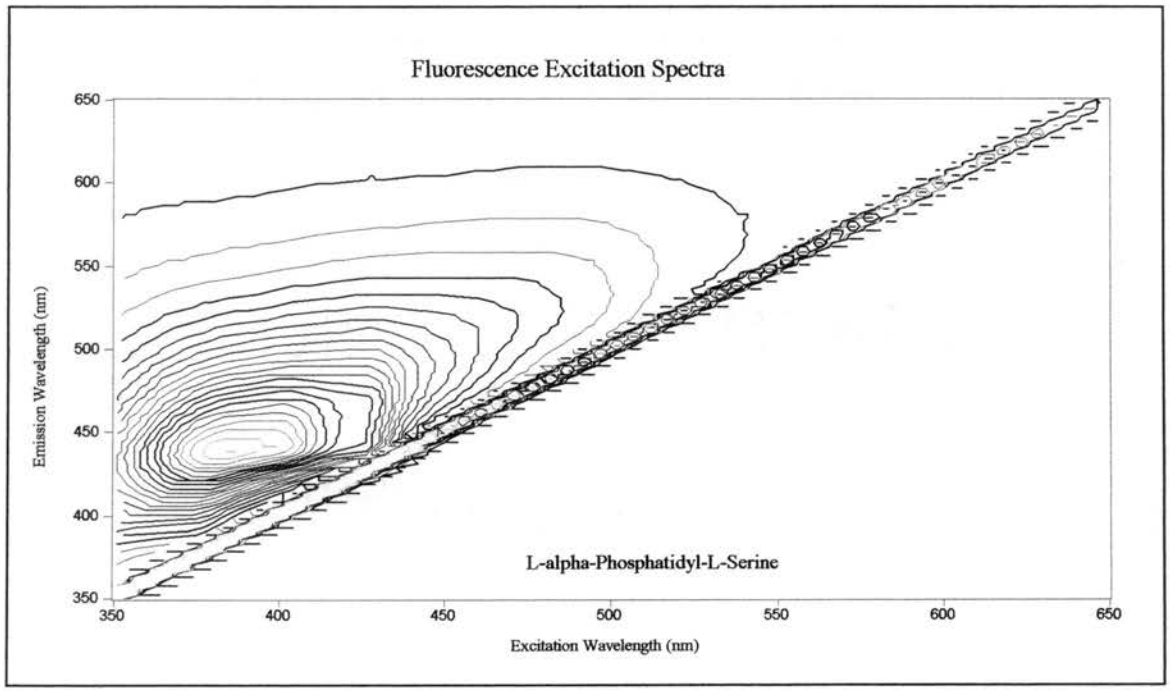


Figure 5-112. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with L-alpha-phosphatidyl-L-serine after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

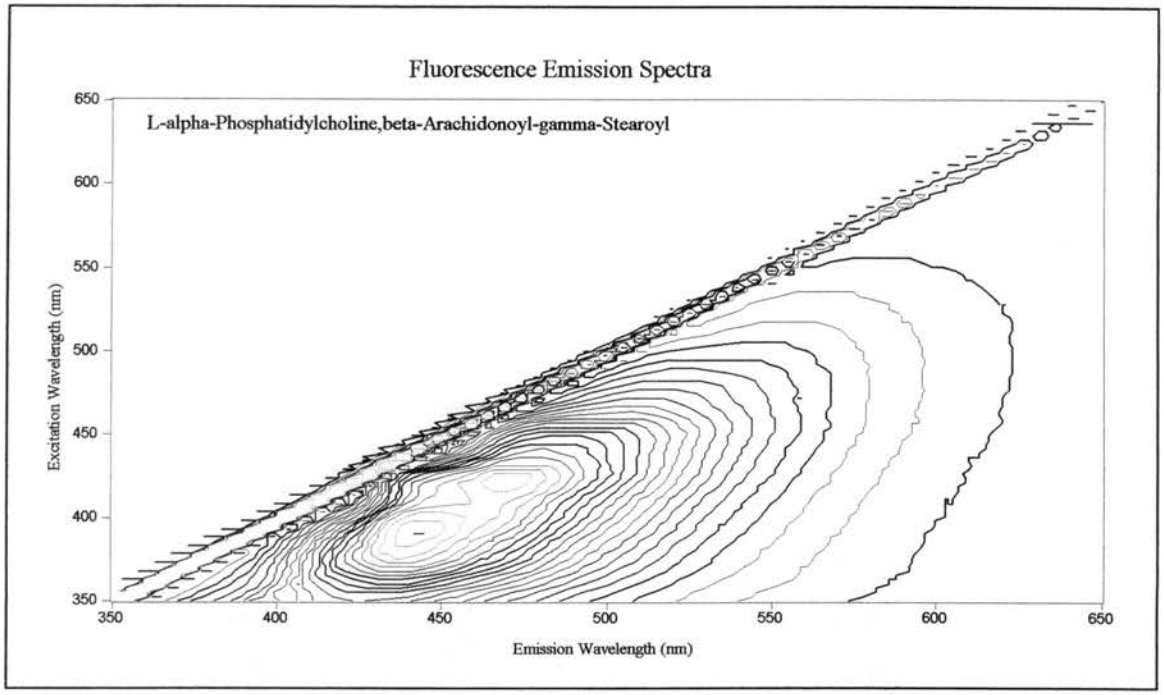


Figure 5-113. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with L-alpha-phosphatidylcholine, beta-arachidonoyl-gamma-stearoyl after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

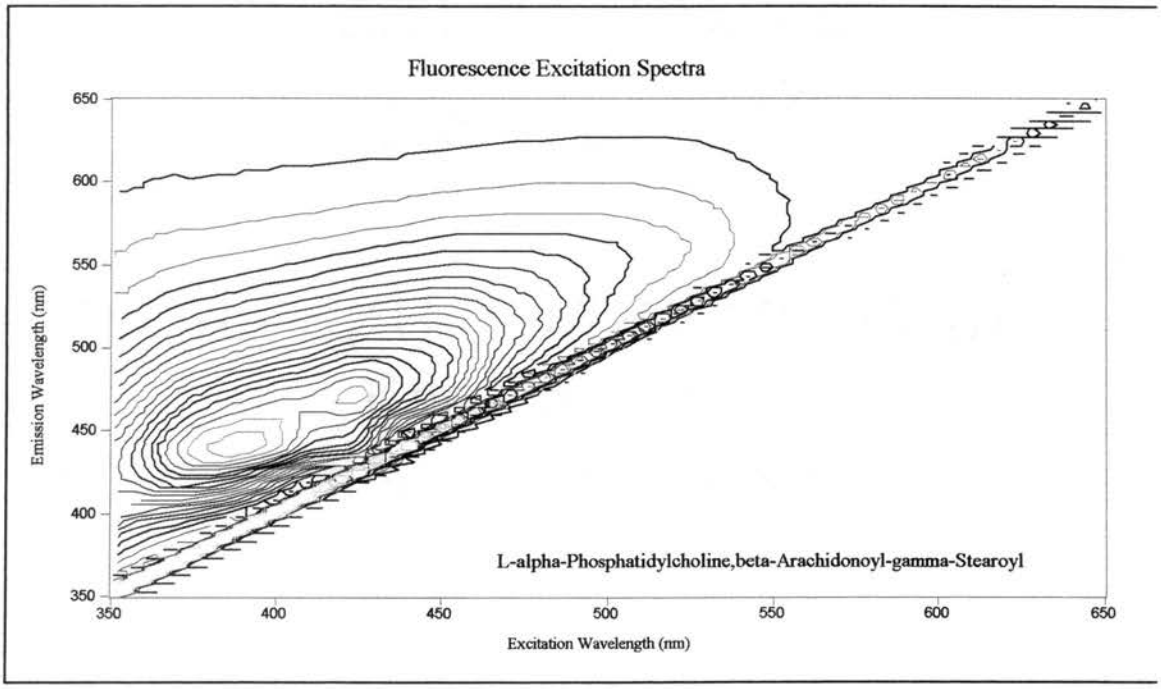


Figure 5-114. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with L-alpha-phosphatidylcholine, beta-arachidonoyl-gamma-stearoyl after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

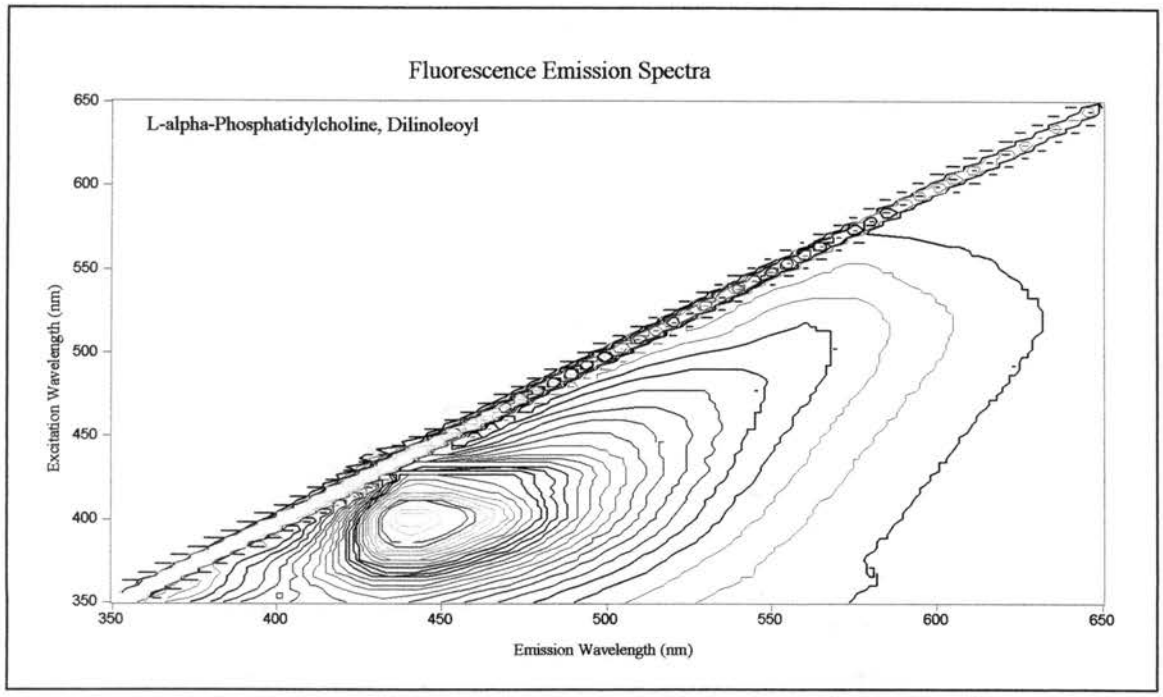


Figure 5-115. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with L-alpha-phosphatidylcholine, dilinoleoyl after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

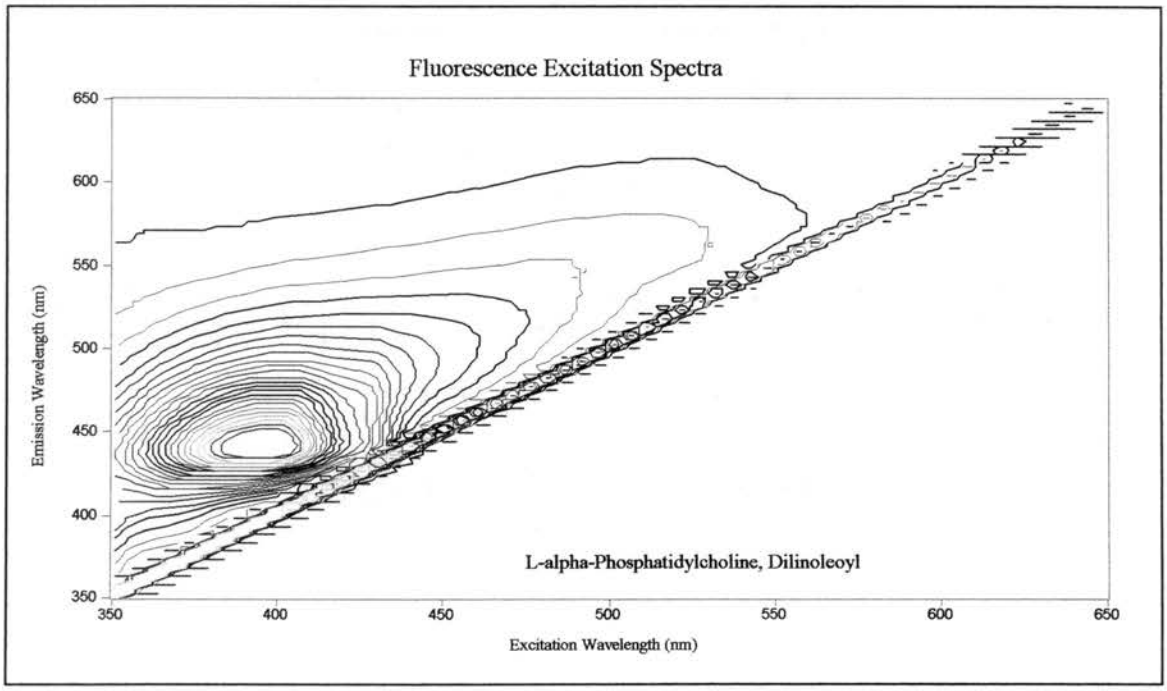


Figure 5-116. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with L-alpha-phosphatidylcholine, dilinoleoyl after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

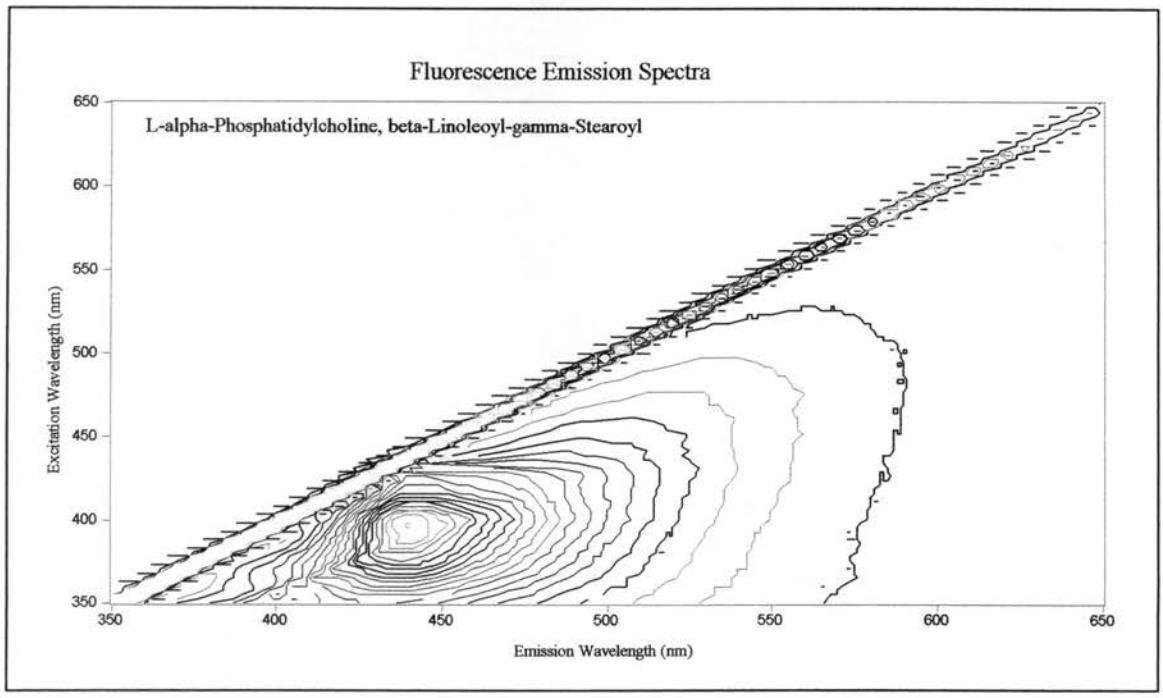


Figure 5-117. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with L-alpha-phosphatidylcholine, beta-linoleoyl-gamma-stearoyl after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

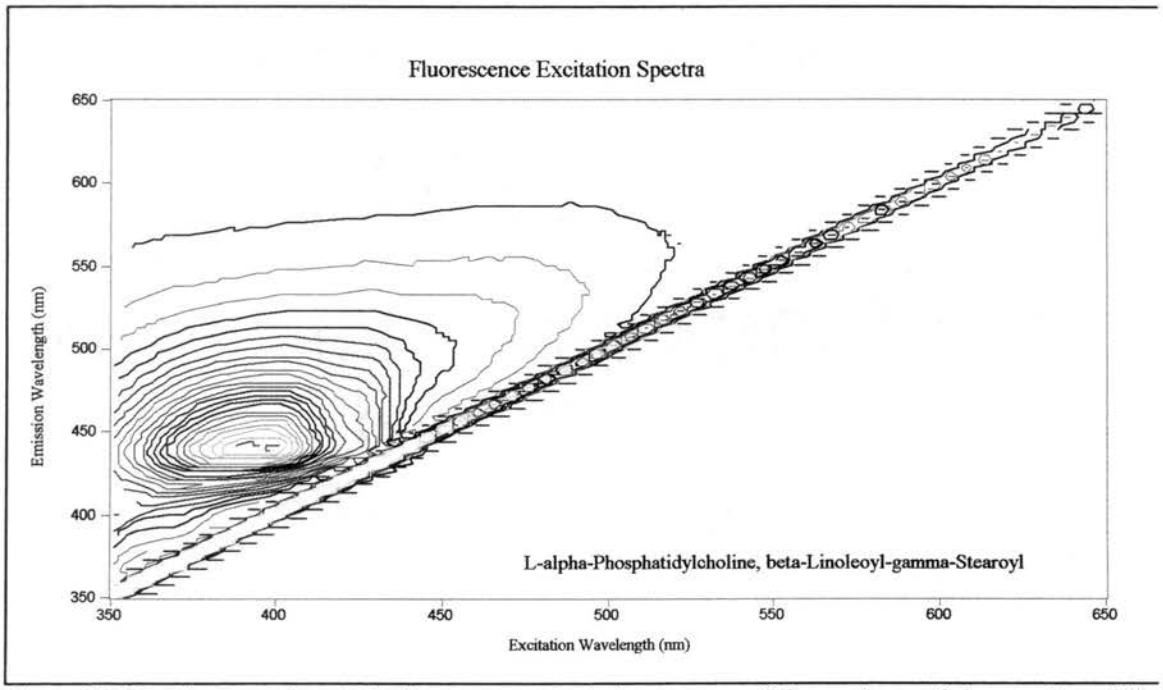


Figure 5-118. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with L-alpha-phosphatidylcholine, beta-linoleoyl-gamma-stearoyl after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

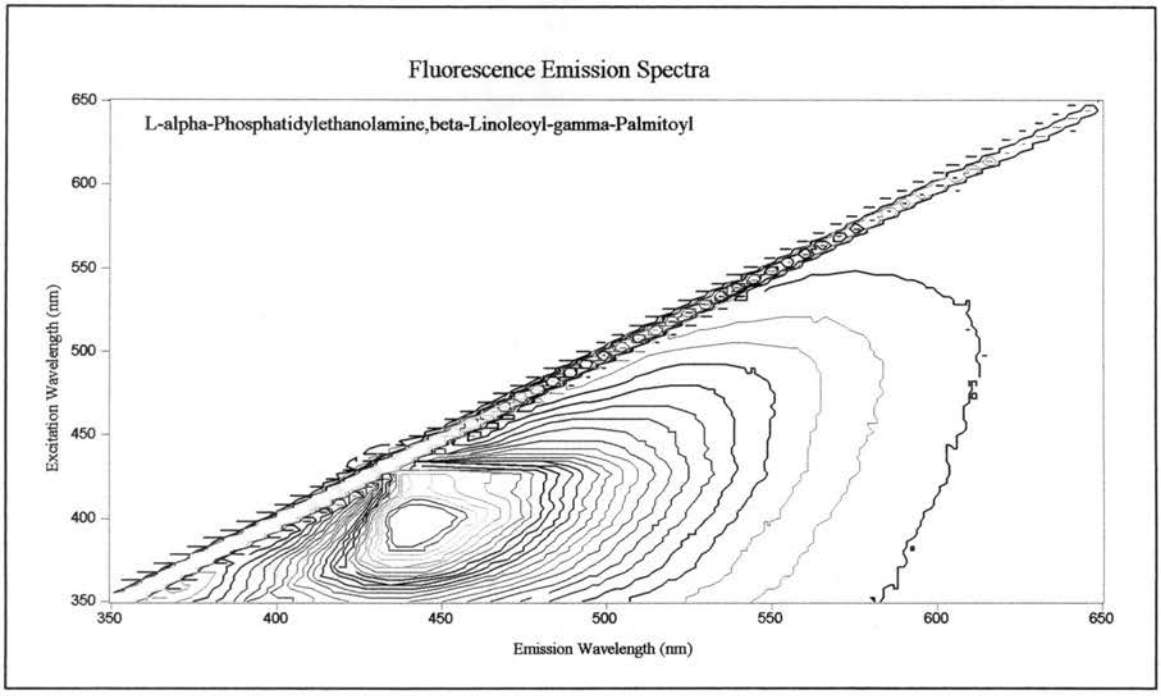


Figure 5-119. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with L-alpha-phosphatidylcholine, beta-linoleoyl-gamma-palmitoyl after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

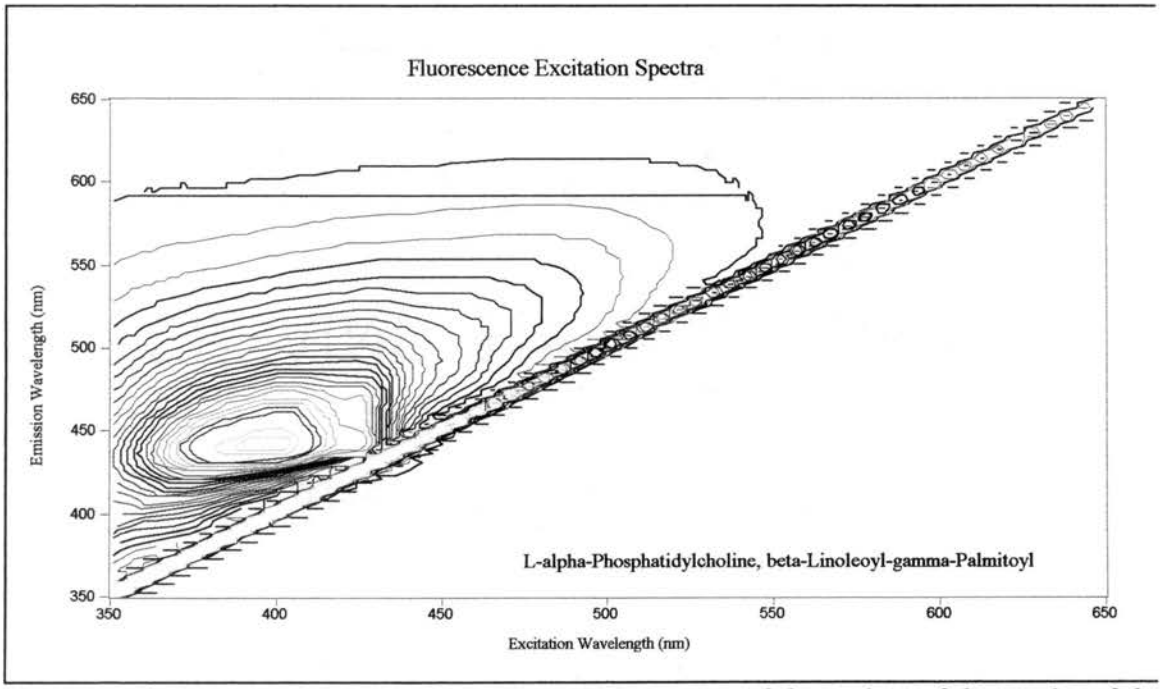


Figure 5-120. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with L-alpha-phosphatidylcholine, beta-linoleoyl-gamma-palmitoyl after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

SPHINGOSINE DERIVATIVES

Sphingosine and sphingomyelin were both examined and found to produce no absorbance or fluorescence spectra when reacted with the SRS. No other sphingosine derivatives were studied.

TERPENES

The terpenes examined included vitamins A (retinol), E (α-tocopherol) and K_1. Of these three, only vitamins A and K produced color when reacted with the SRS and none of the products fluoresced. Of the two color reactions, only the products of retinol were stable when irradiated with light. Figure 5-121 shows the absorbance spectra of retinol. It should be noted that the concentration of retinol used was approximately 200mg/dL. In serum, the concentration of vitamin A would be in the nano-gram range. This effectively eliminates the application of the current technique as a serum assay for vitamin A.

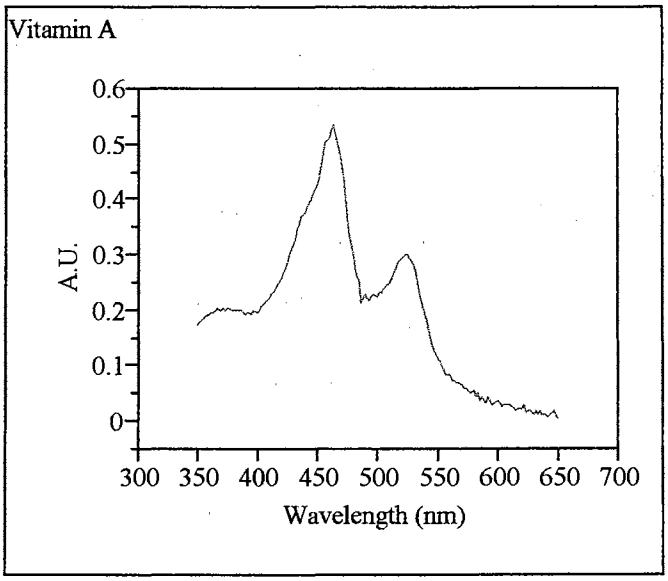


Figure 5-121 . Absorption spectrum of the colored products of the reaction of the standard reagent with vitamin A after 20 minutes.

At this point it is useful to review the purpose of this study and the points covered thus far. The ultimate purpose here was to develop a method capable of identifying and discriminating between blood lipid disorders, dyslipidemias, using a single spectroscopic assay of serum. It has been shown that the major components of serum, i.e. cholesterol and its esters, unsaturated fatty acids and triglycerides/ phospholipids with unsaturated fatty acid moieties, all react chromogenically with the SRS to produce unique absorbance and fluorescence spectra. This information will now be used to develop the third and final goal of this study.

The last goal of this study was much more involved than the previous two goals. The ultimate objective here was to identify a dyslipidemic serum based on its absorbance spectrum. Because of the complexity of lipoproteins and their constantly changing compositions, it is apparent that is a nontrivial exercise.

Traditionally, diagnosing a dyslipidemia has meant quantifying the concentrations of the various lipoprotein classes. This study breaks with that tradition and focuses not on lipoprotein concentrations but on the proportions of the lipid constituents making up the lipoproteins. For example, as Table 2-1 shows, chylomicrons and LDLs are made up of the same fundamental components but in different proportions. These different proportions give rise to the different physical properties i.e. density, size, electrophoretic mobility, etc. of the lipoprotein. It is these physical differences that have traditionally served as the bases for separation, subsequent quantification and ultimately the diagnosis of a particular dyslipidemia. However, if one were able to make a spectral discrimination between a chylomicron and a HDL based solely on composition, a Type I dyslipidemia (elevated chylomicron levels) could be easily detected, that is the spectrum as a whole should have characteristics similar to the spectrum of a chylomicron. This idea of spectral identification of a dyslipidemia based solely on the compositional differences of the lipoprotein forms the crux of this reasearch.

To this point in the present work, it has been shown that cholesterol, cholesterol esters, long chain unsaturated fatty acids and triglycerides and phospholipids containing unsaturated fatty acids will all chromogenically react with the SRS. Reviewing Table 2-1, one can see the major non-protein constituents of the lipoproteins are free cholesterol, esterified cholesterol, triglycerides and phospholipids.

(Serum proteins were found to precipitate out of solution upon the addition of the PA.) Leveraging the knowledge gained in the previous two goals of this study, it seems reasonable to predict, for instance, that chylomicrons would have a different spectral pattern than HDLs. To further explore this idea of spectral discrimination based solely on the compositional differences of the lipoproteins, "synthetic lipoproteins" or mixtures that simulate lipoproteins in that they reflect the average composition of the various human serum lipoproteins were created and analyzed. Figures 5-122 to 5-126 show the absorbance spectra for each of the synthetic lipoproteins. The corresponding fluorescence emission and excitation contour plots are found in Figures 5-127 to 5-136.

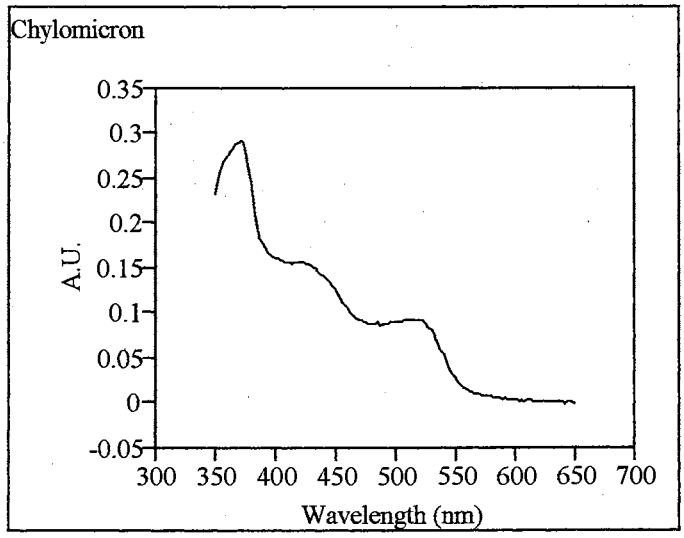


Figure 5-122. Absorption spectrum of the colored products of the reaction of the standard reagent with a synthetic chylomicron mixture after 20 minutes.

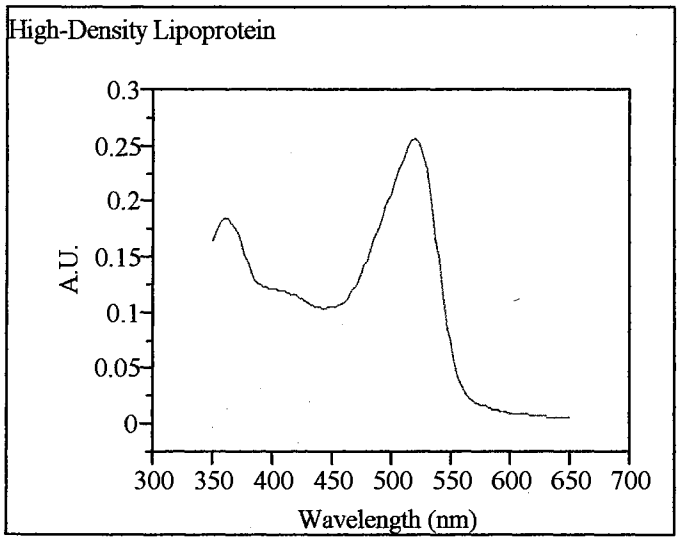


Figure 5-123. Absorption spectrum of the colored products of the reaction of the standard reagent with a synthetic high-density lipoprotein mixture after 20 minutes.

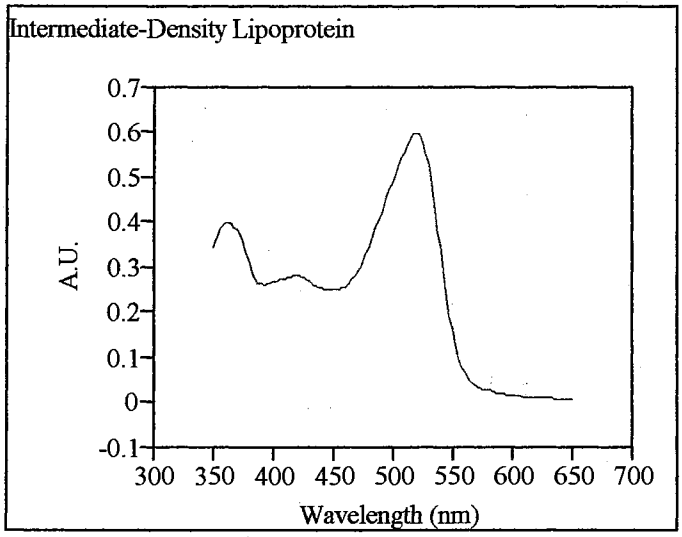


Figure 5-124. Absorption spectrum of the colored products of the reaction of the standard reagent with a synthetic intermediate-density lipoprotein mixture after 20 minutes.

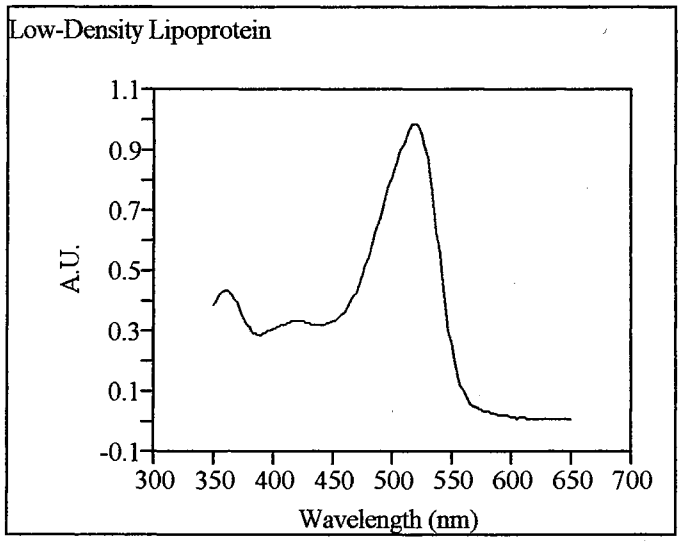


Figure 5-125. Absorption spectrum of the colored products of the reaction of the standard reagent with a synthetic low-density lipoprotein mixture after 20 minutes.

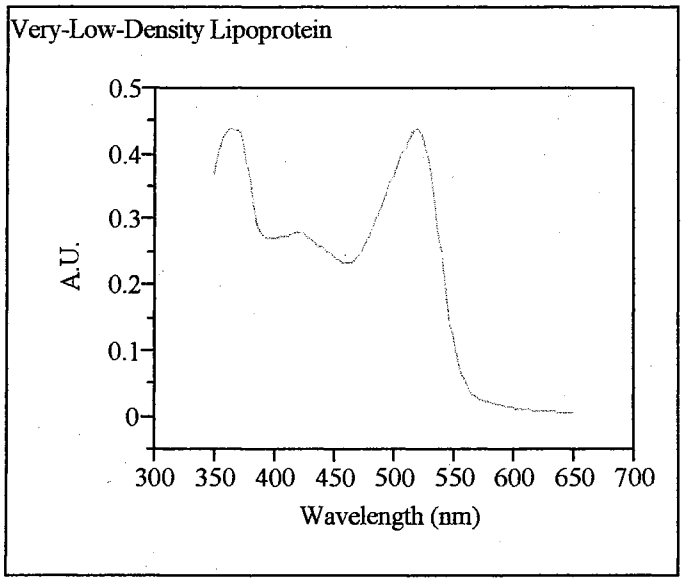


Figure 5-126. Absorption spectrum of the colored products of the reaction of the standard reagent with a synthetic very-low-density lipoprotein mixture after 20 minutes.

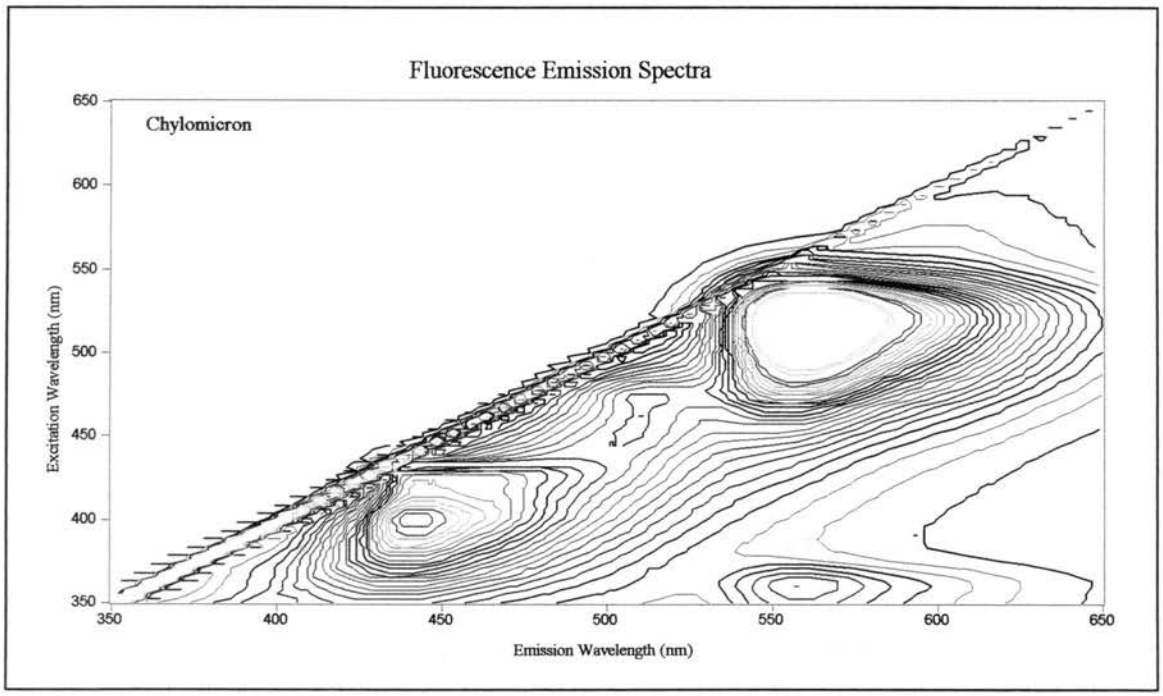


Figure 5-127. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with a synthetic chylomicron mixture after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

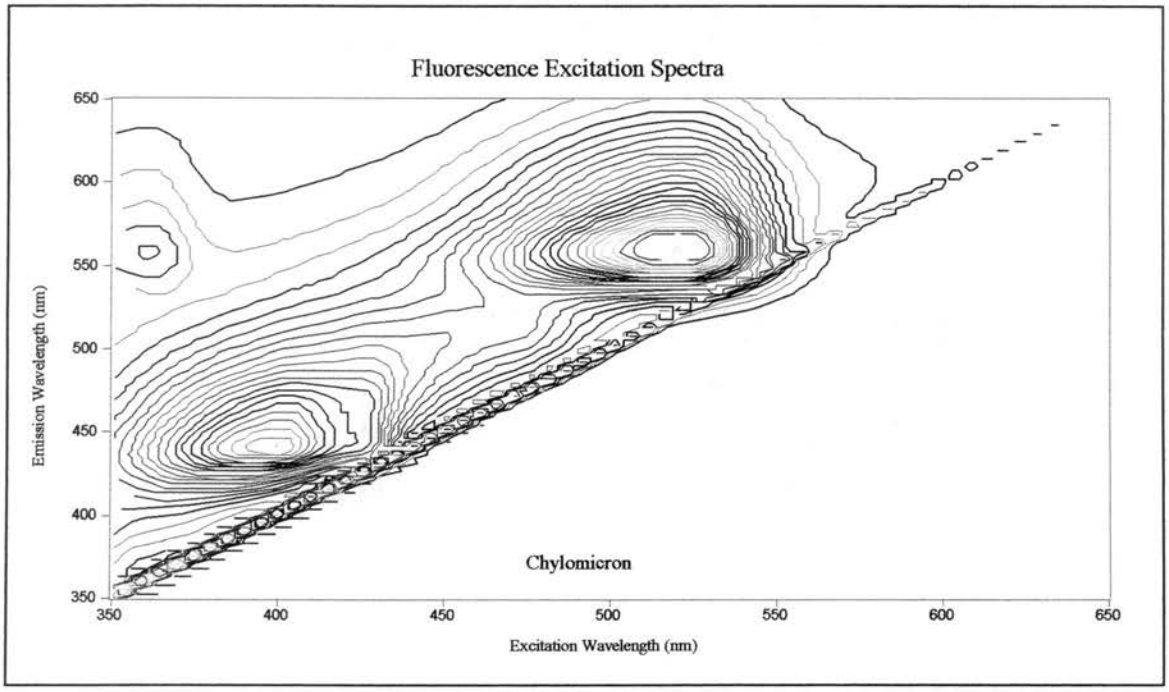


Figure 5-128. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with a synthetic chylomicron mixture after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

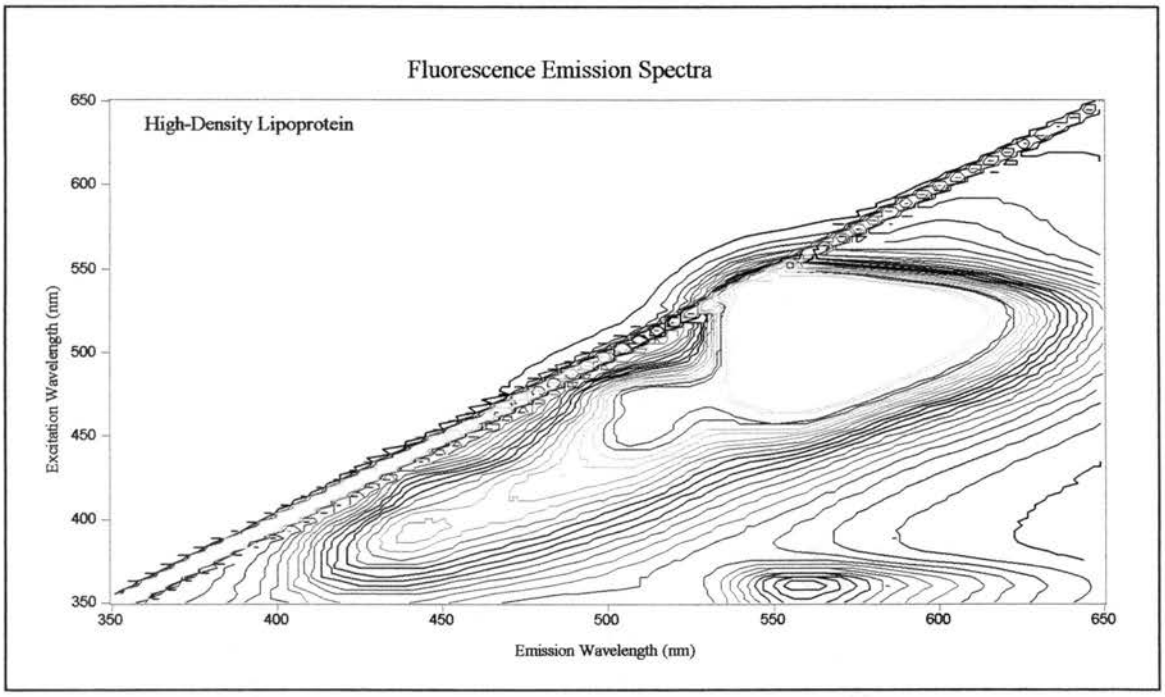


Figure 5-129. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with a synthetic high-density lipoprotein mixture after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

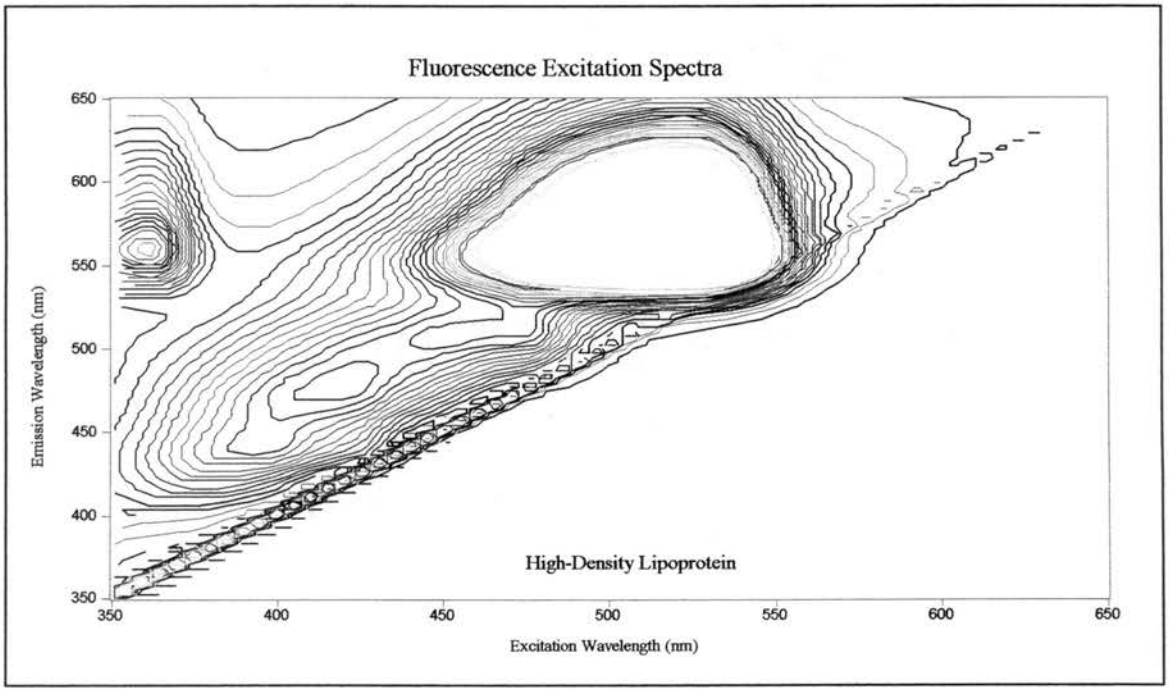


Figure 5-130. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with a synthetic high-density lipoprotein mixture after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

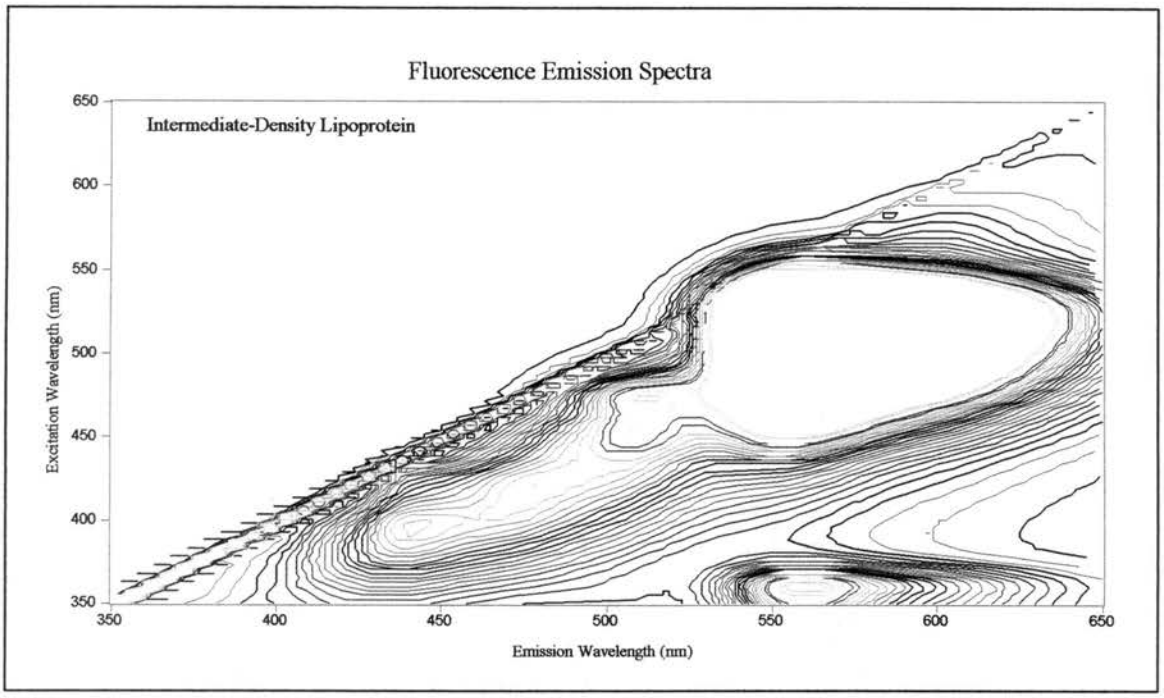


Figure 5-131. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with a synthetic intermediate-density lipoprotein mixture after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

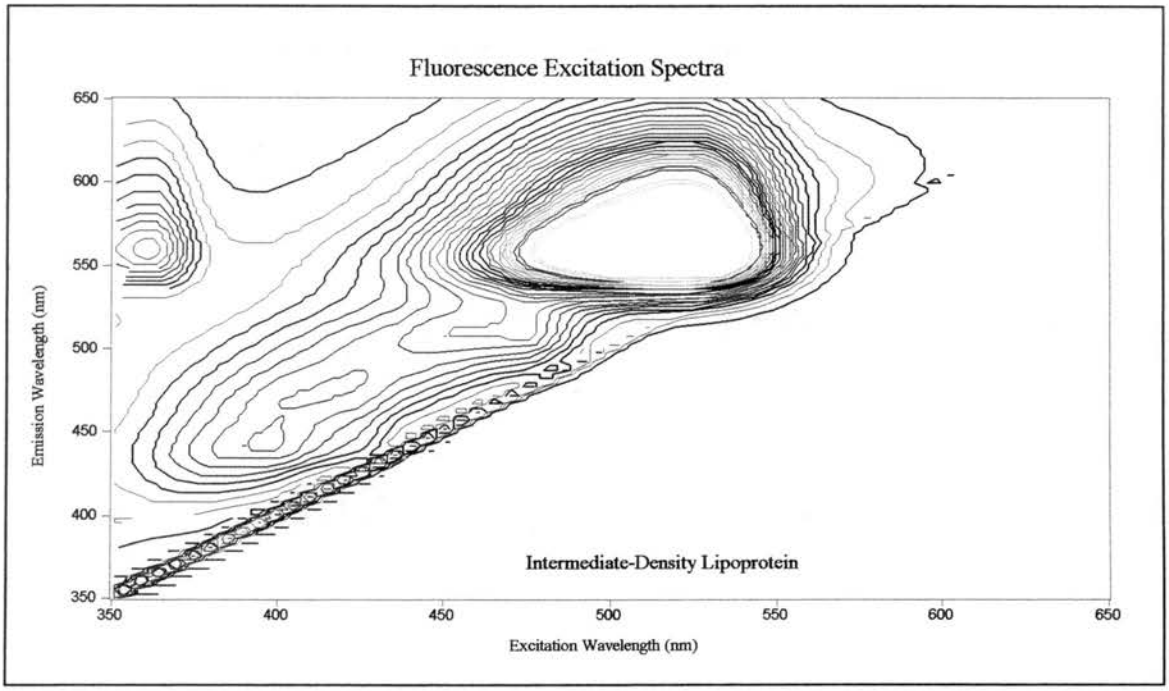


Figure 5-132. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with a synthetic intermediate-density lipoprotein mixture after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

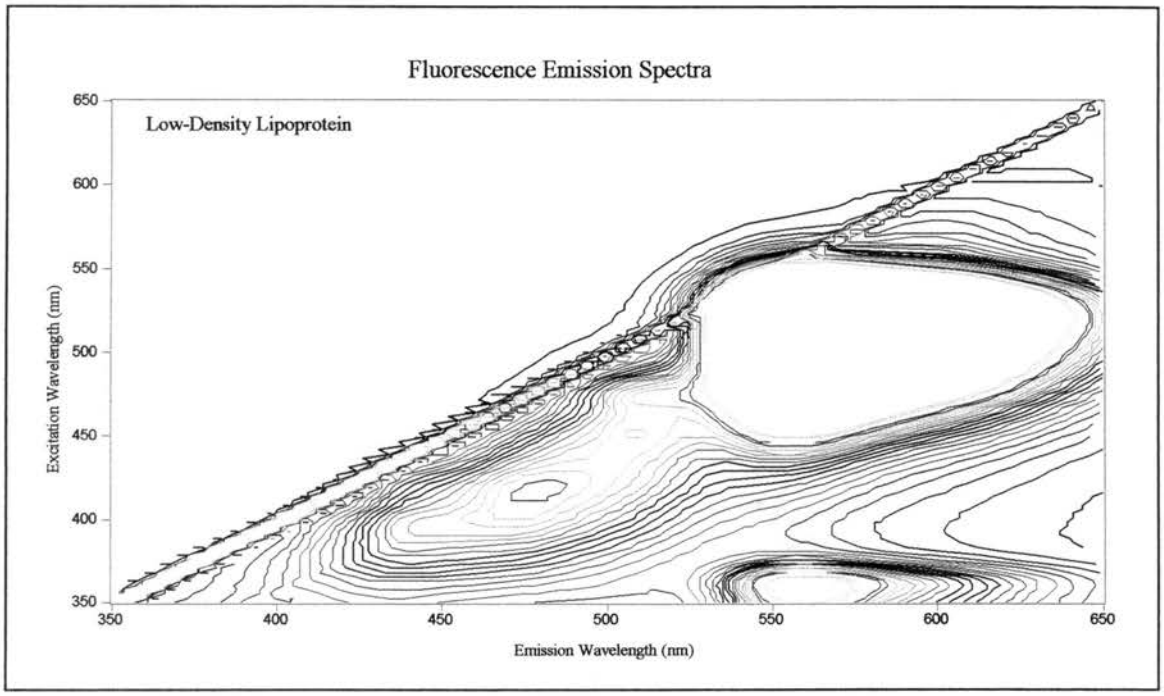


Figure 5-133. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with a synthetic low-density lipoprotein mixture after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

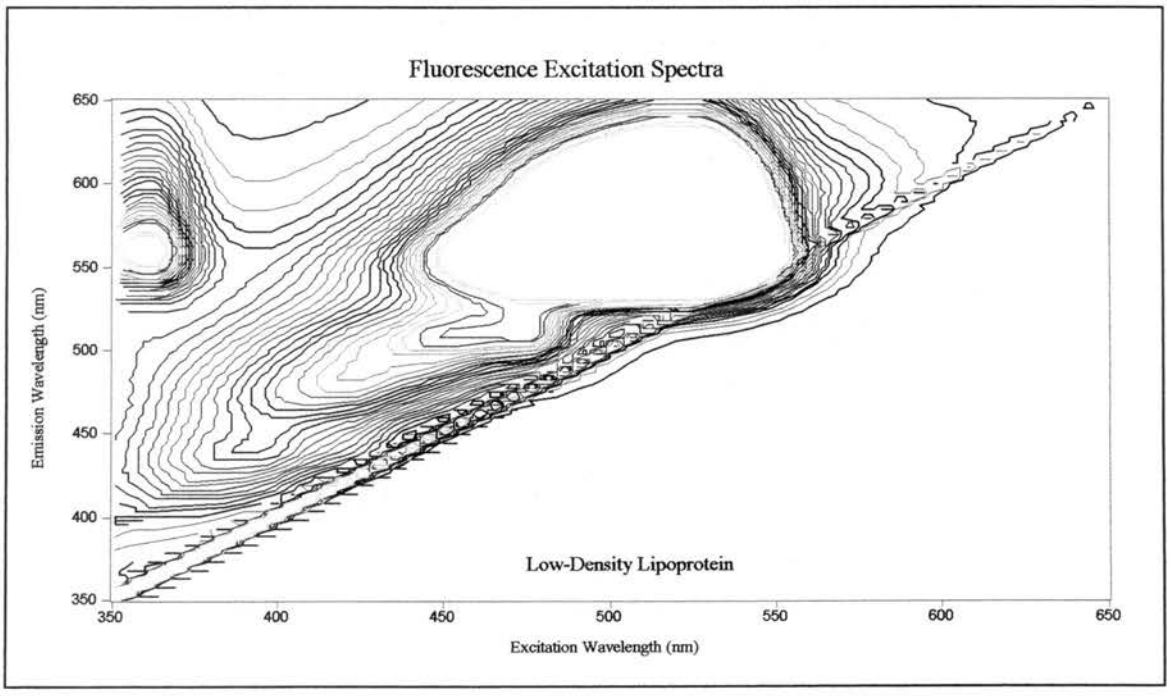


Figure 5-134. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with a synthetic low-density lipoprotein mixture after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

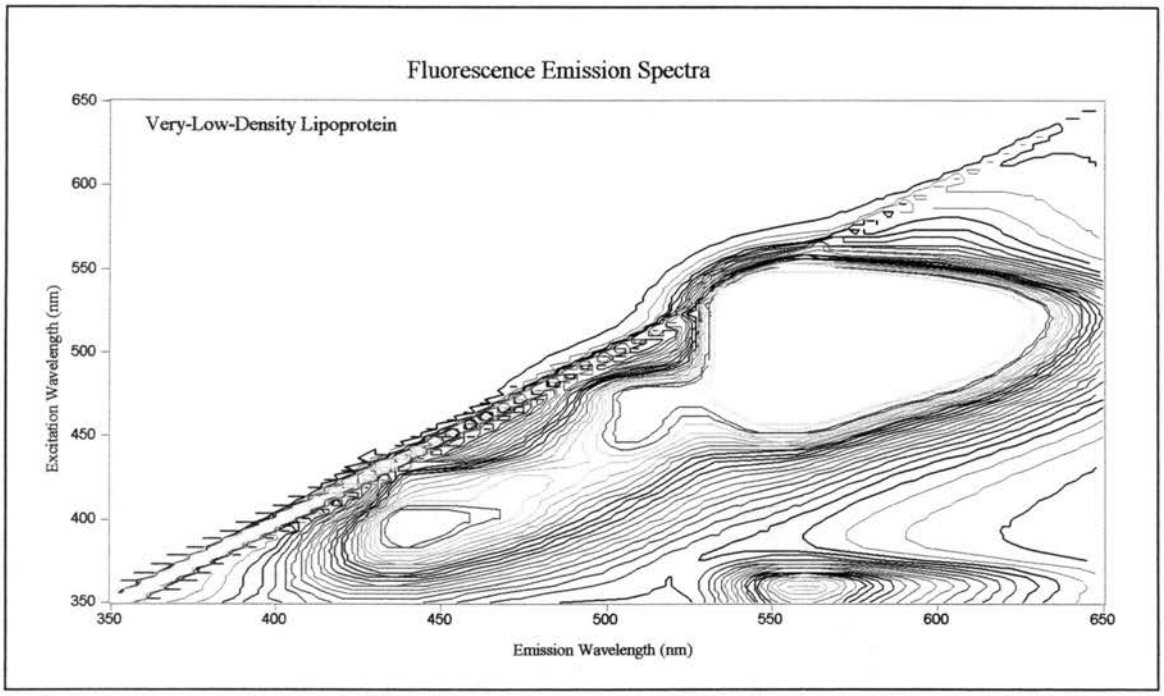


Figure 5-135. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with a synthetic very-low-density lipoprotein mixture after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

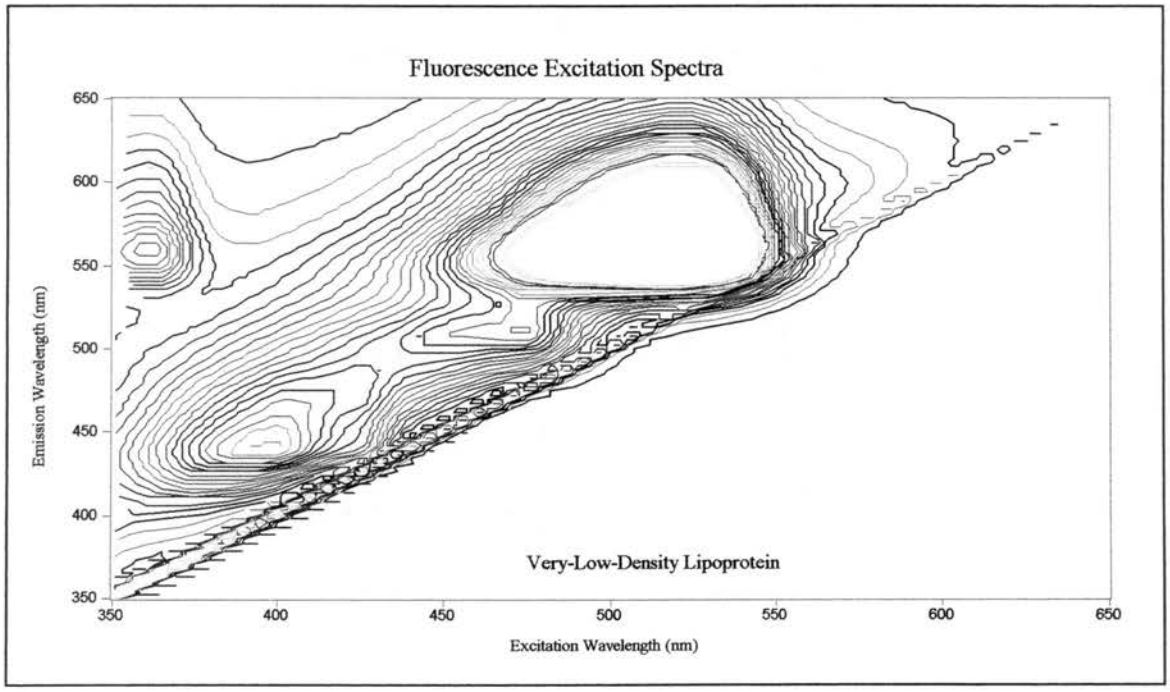


Figure 5-136. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with a synthetic very-low-density lipoprotein mixture after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

One objective of the present work was to determine whether or not the spectra of serum samples treated with the reagent could be represented by a linear combination of the spectra of appropriate combinations of the reagent generated spectra of standard lipoproteins. To validate the idea of making synthetic lipoproteins, six human sera were analyzed using the current technique and again by an independent laboratory where [TC], [TG], [HDL-C] and [LDL-C] were measured using routine laboratory methods. Synthetic serum mixtures (SS01-SS06) reflecting the data reported by the independent lab were then created and analyzed using the current technique. These "synthetic sera" absorbance spectra were qualitatively compared to the true sera absorbance spectra to see how well they reproduced the spectral characteristics of the real serum samples. (This validation method was used because the more direct approach of comparing the actual lipoprotein fractions to the synthetic lipoprotein mixtures required ultracentrifugation which is extremely time and labor intensive and would have required equipment and procedures unavailable to this project.

To understand how the synthetic sera were made, Serum 01 is used as an example. In this serum, RML analyses reported the following values; [TC]=211mg/dL, [TG]=129mg/dL, [HDL-C]=80mg/dL and [LDL-C]=105mg/dL. A total volume for all the synthetic serum mixtures was first chosen to be 10mL, simply for convenience of calculation. The [TC] was assumed to be the sum of the [HDL-C], [LDL-C] and [VLDL-C] fractions. Therefore [VLDL-C]=[TC]-[LDL-C]-[HDL-C], or 26mg/dL. According to Table 2-1, in HDL the mass ratio of unesterified cholesterol to esterified cholesterol to PL is 6:13:28. If the [HDL-C] = 80mg/dL, it follows that the unesterified cholesterol portion is on average 25.3mg/dL, the average esterified cholesterol portion must be 54.7mg/dL and PL must be present at an average concentration of 117.89mg/dL. To make a 10mL solution with an [HDL-C] of 80mg/dL, 2.53mg of unesterified cholesterol, 5.47mg of esterified cholesterol and 11.79g of PL were added to 10mL of chloroform. This procedure was repeated for the VLDL and LDL fractions. The [TG] was simply calibrated for the total volume of the mixture, in this case 12.9mg of TG was added to 10mL of chloroform. In the end, a total of 5.59mg of unesterified cholesterol, 15.51mg of esterified cholesterol, 20.74mg of PL and 12.9mg of TG were added to 10mL of chloroform to make synthetic Serum 01.

Because RML reports data as only the [TC] with no distinction made between the free and esterified forms, a series of three synthetic mixtures was made for each of the human sera analyzed. The first of the series approximated the [TC] by using only free cholesterol. The second approximated the [TC] using a free cholesterol / saturated cholesterol ester (cholesteryl myristate) combination. The third series approximated the [TC] using a free cholesterol / unsaturated cholesterol ester (cholesteryl linoleate) combination. Other standards used in the preparation of these mixtures are phosphatidylcholine (Type XVI-E: from fresh egg) for the PL and soybean oil for the TG. (Soybean oil was chosen for its relatively high ratio of linolenic acid to linoleic acid.) Table 5-1 shows the RML test results for each of the true sera. Tables 5-2 to 5-7 show the corresponding amounts of each standard added to 10mL of chloroform to create these synthetic serum mixtures. Figures 5-137 to 5-142 are overlay plots of each human serum and the respective synthetic serum series. Figures 5-143 to 5-166 are contour plots of each serum/ synthetic serum series. In the case of each serum, the agreement between peak heights and placement in the absorbance overlay plots seemed rather remarkable considering the richly diverse nature of the biological matrix.

It should be understood that there is and should be some variation between the actual human sera and the synthetic series in both the absorbance and the fluorescence plots. After all, the ratio of esterified to unesterified cholesterol in each serum was unknown. Another unknown was the fatty acid composition of the PL, TG and cholesterol esters in each serum sample. Given all of the unknowns inherent in these biological samples, the current technique when applied to synthetic mixtures yielded spectra which were very similar to the absorbance and fluorescence spectra of human sera with equivalent TG, TC and PL concentrations.

Table 5-1. RML Lipid Analysis Results for Serums 01-06						
Serum #	1	2	3	4	5	6
[TC] (mg/dL) RML	211	243	222	213	140	217
[HDL-C] (mg/dL) RML	80	34	32	77	28	41
[LDL-C] (mg/dL) RML	105	190	168	114	75	123
[TG] (mg/dL) RML	129	93	112	112	187	265
[VLDL-C] (mg/dL) (calculated)	26	19	22	22	37	53

Table 5-2. Synthetic Serum 01 (SS01) Composition				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	2.53	5.47	11.79	-
LDL	2.20	8.30	6.34	-
VLDL	0.87	1.73	2.6	-
TOTAL	5.6	15.50	20.73	12.9

Table 5-3. Synthetic Serum 02 (SS02) Composition				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	1.07	2.33	5.01	-
LDL	3.98	15.02	11.49	-
VLDL	0.63	1.27	1.9	-
TOTAL	5.68	18.62	18.40	9.3

Table 5-4. Synthetic Serum 03 (SS03) Composition				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	1.01	2.19	4.71	-
LDL	3.52	13.28	10.16	-
VLDL	0.73	1.47	2.2	-
TOTAL	5.26	16.94	17.07	11.2

Table 5-5. Synthetic Serum 04 (SS04) Composition				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	2.43	5.27	11.35	-
LDL	2.39	9.01	6.89	-
VLDL	0.73	1.47	2.2	-
TOTAL	5.55	15.75	20.44	11.2

Table 5-6. Synthetic Serum 05 (SS05) Composition				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	0.88	1.92	4.13	-
LDL	1.57	5.93	4.53	-
VLDL	1.23	2.47	3.7	-
TOTAL	3.68	10.32	12.36	18.7

Table 5-7. Synthetic Serum 06 (SS06) Composition				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	1.29	2.81	6.04	-
LDL	2.57	9.72	7.44	-
VLDL	1.77	3.53	5.3	-
TOTAL	5.63	16.06	18.78	26.5

SERUM 01- SYNTHETIC SERUM 01 SERIES ABSORBANCE SPECTRA

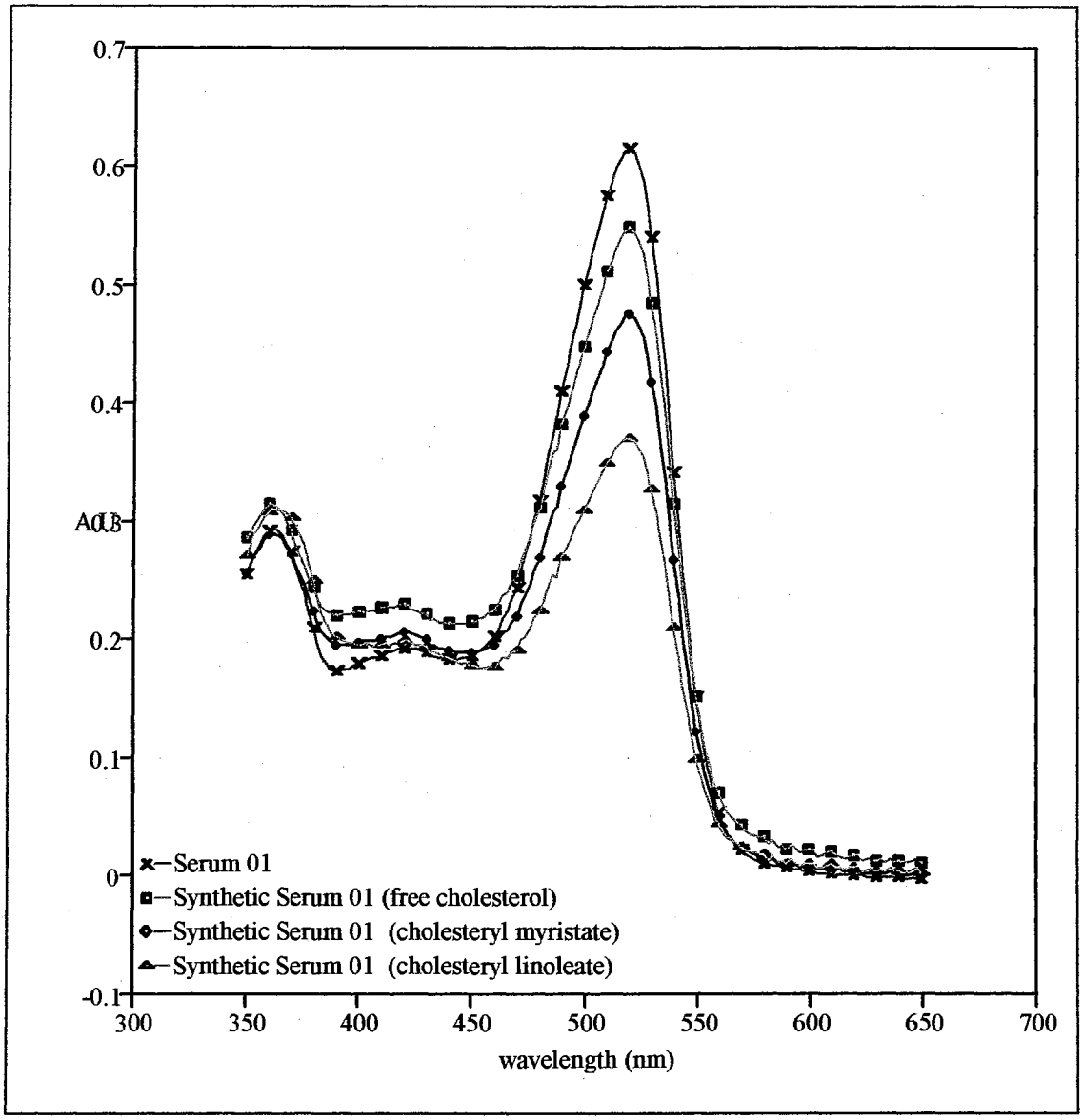


Figure 5-137. Overlay plot of the absorption spectra of the colored products of the reactions of the standard reagent with Serum 01 and the synthetic serum 01 series after 20 minutes.

SERUM 02- SYNTHETIC SERUM 02 SERIES ABSORBANCE SPECTRA

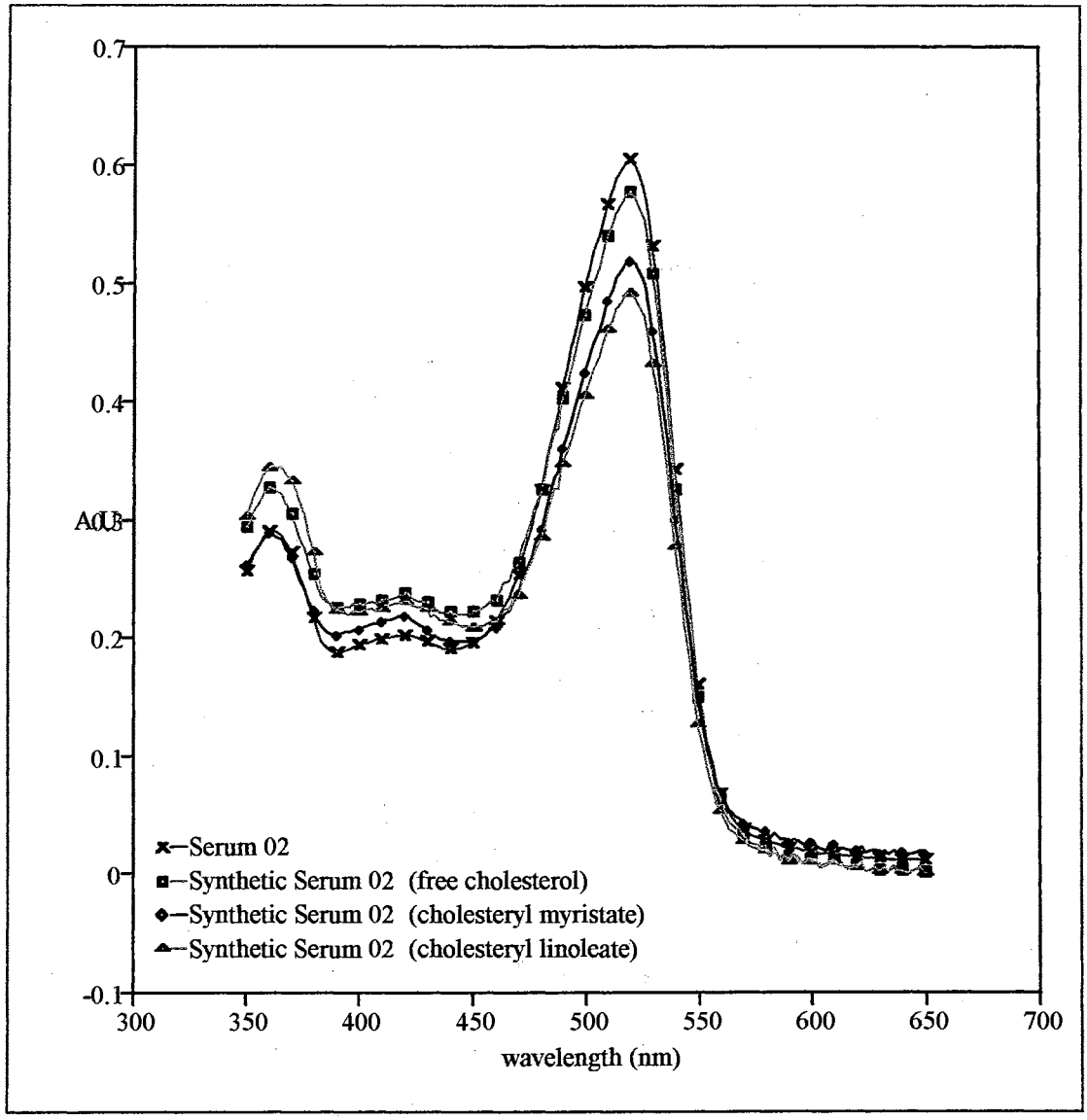


Figure 5-138. Overlay plot of the absorption spectra of the colored products of the reactions of the standard reagent with Serum 02 and the synthetic serum 02 series after 20 minutes.

SERUM 03- SYNTHETIC SERUM 03 SERIES ABSORBANCE SPECTRA

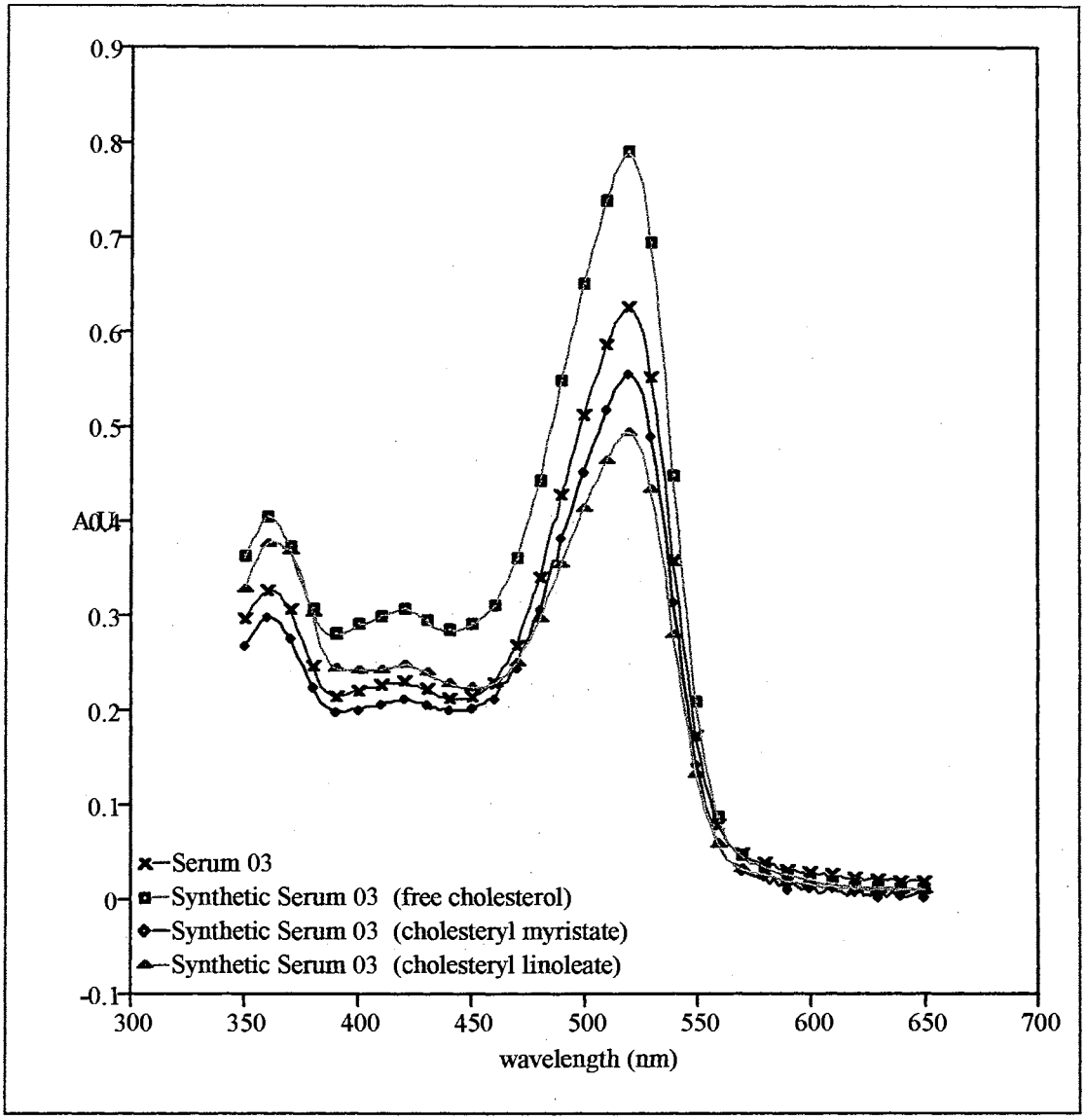


Figure 5-139. Overlay plot of the absorption spectra of the colored products of the reactions of the standard reagent with Serum 03 and the synthetic serum 03 series after 20 minutes.

SERUM 04- SYNTHETIC SERUM 04 SERIES ABSORBANCE SPECTRA

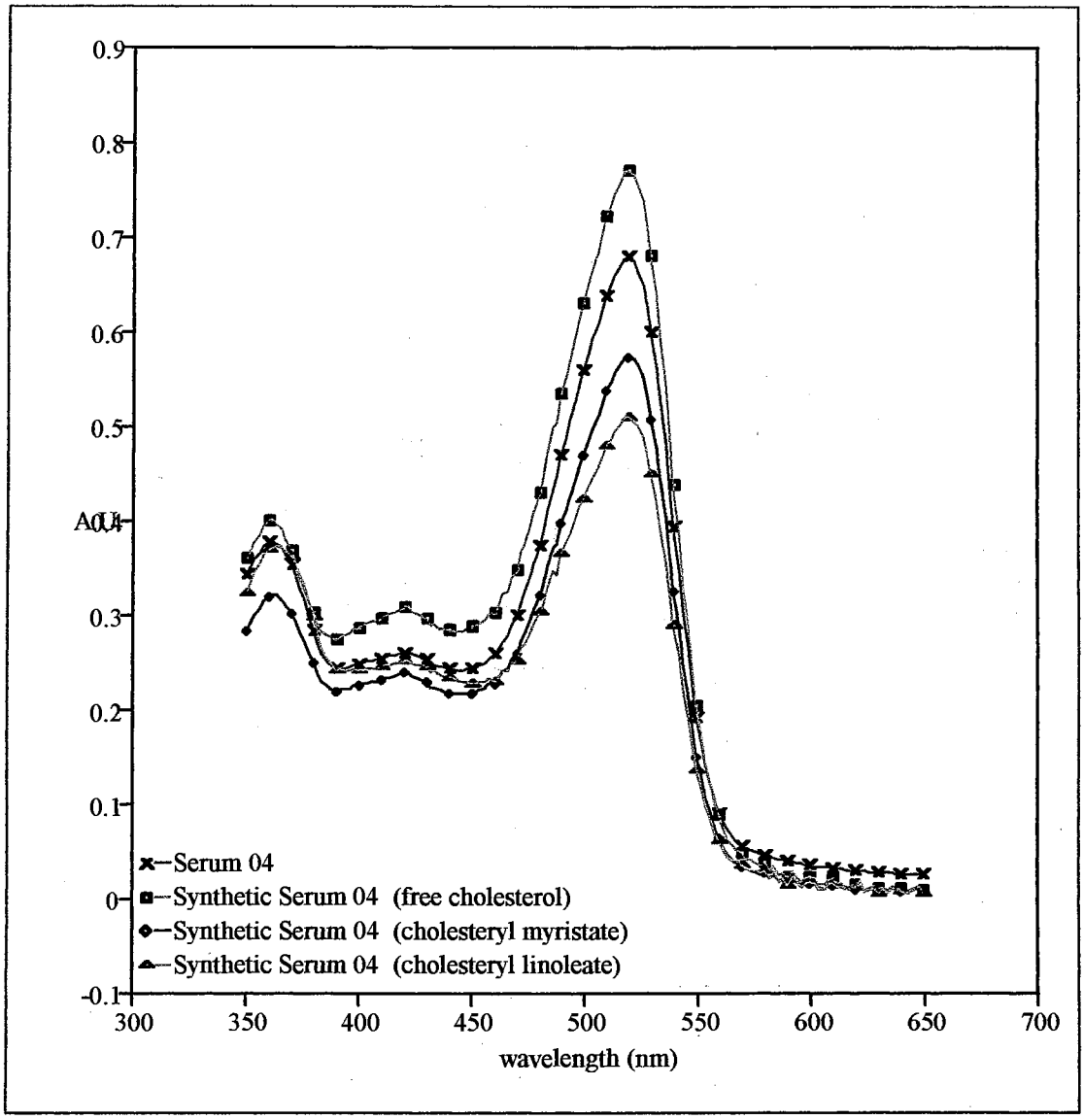


Figure 5-140. Overlay plot of the absorption spectra of the colored products of the reactions of the standard reagent with Serum 04 and the synthetic serum 04 series after 20 minutes.

SERUM 05- SYNTHETIC SERUM 05 SERIES ABSORBANCE SPECTRA

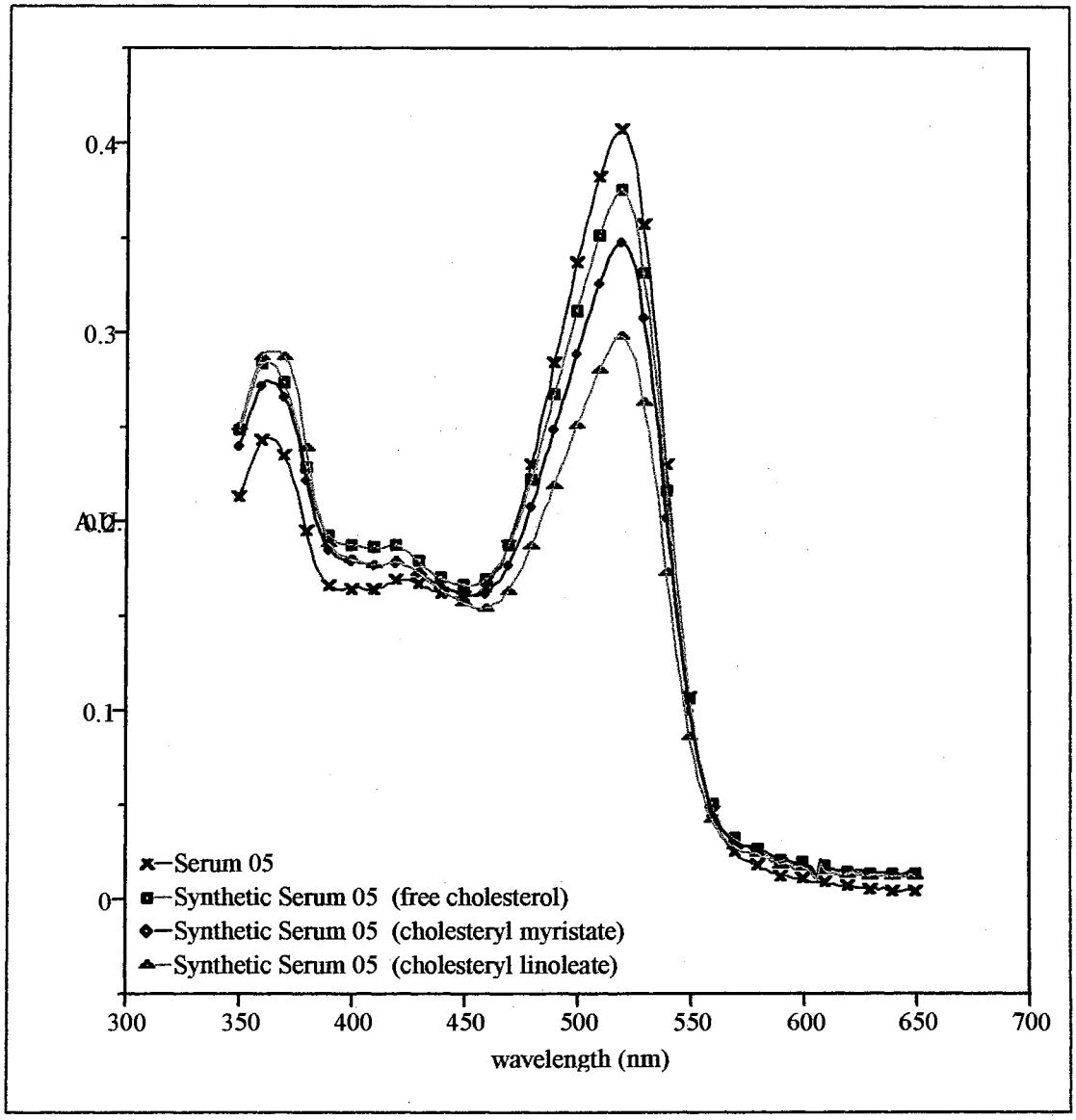


Figure 5-141. Overlay plot of the absorption spectra of the colored products of the reactions of the standard reagent with Serum 05 and the synthetic serum 05 series after 20 minutes.

SERUM 06- SYNTHETIC SERUM 06 SERIES ABSORBANCE SPECTRA

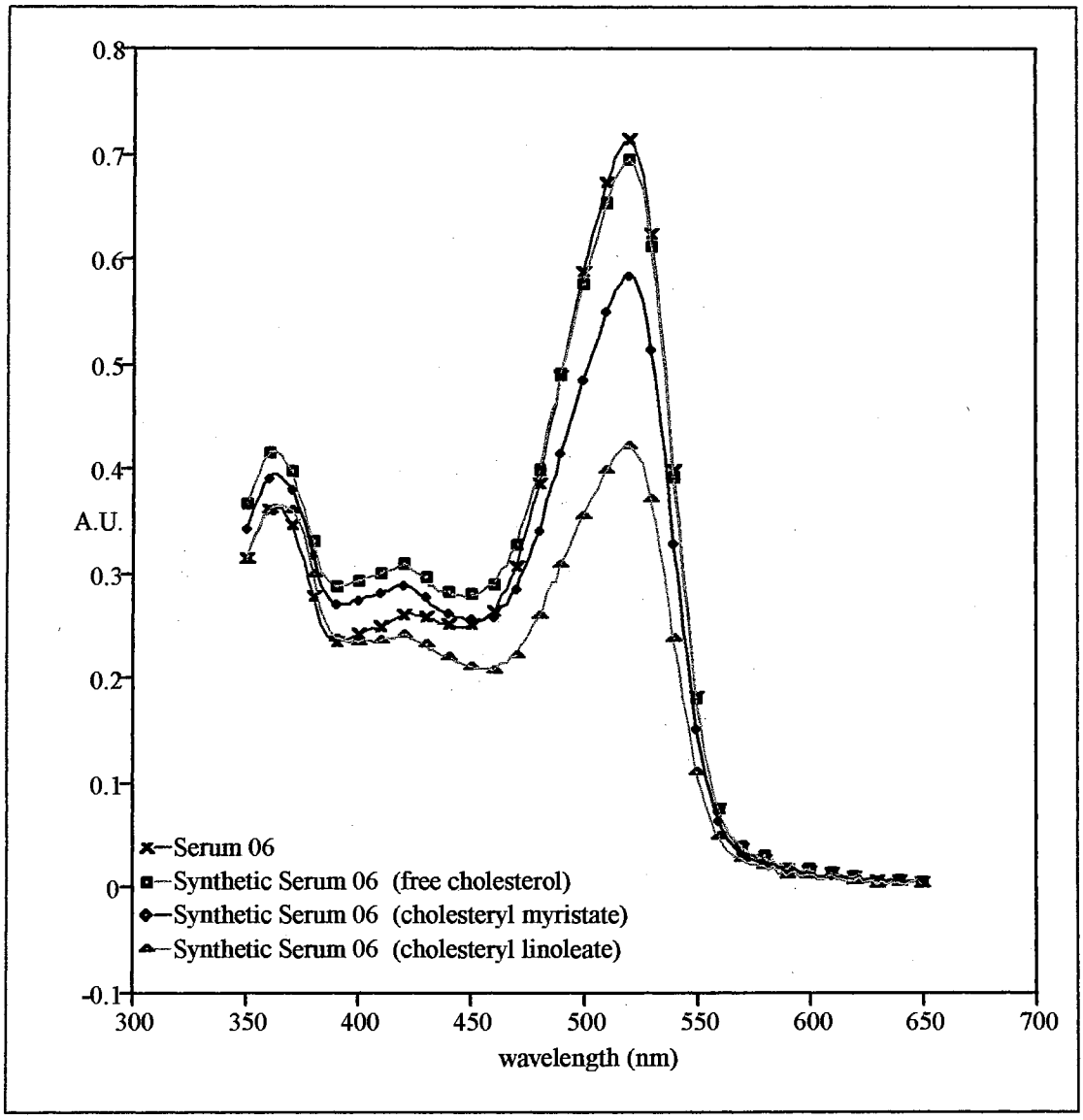


Figure 5-142. Overlay plot of the absorption spectra of the colored products of the reactions of the standard reagent with Serum 06 and the synthetic serum 06 series after 20 minutes.

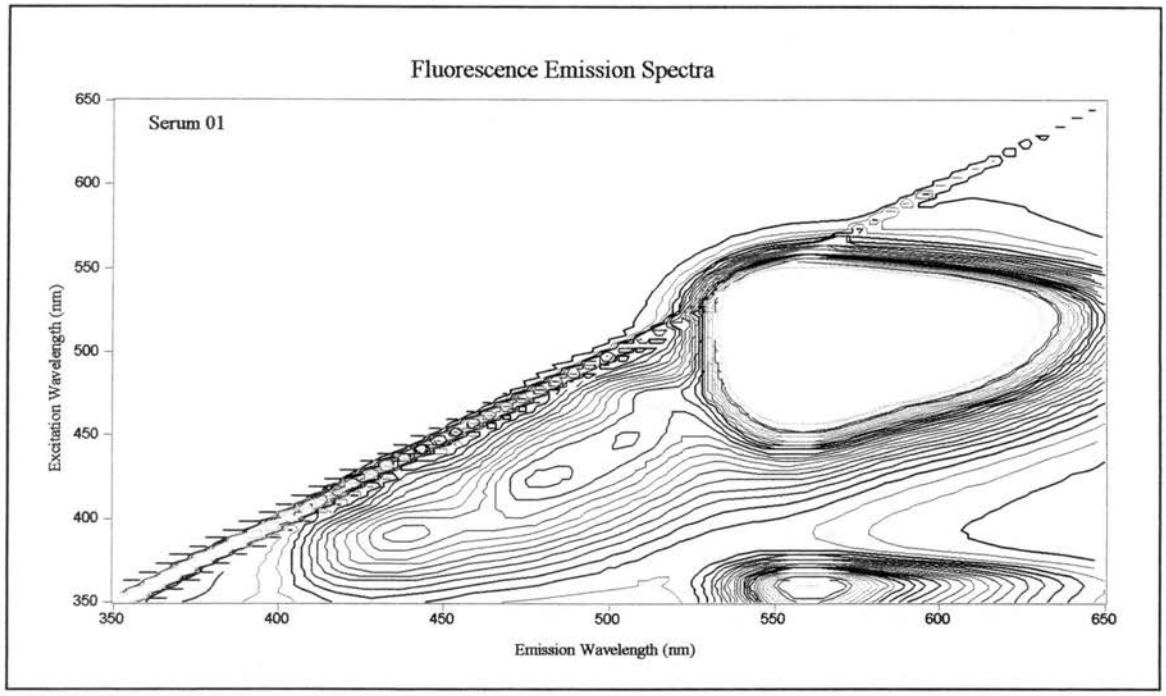


Figure 5-143. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with serum 01 after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

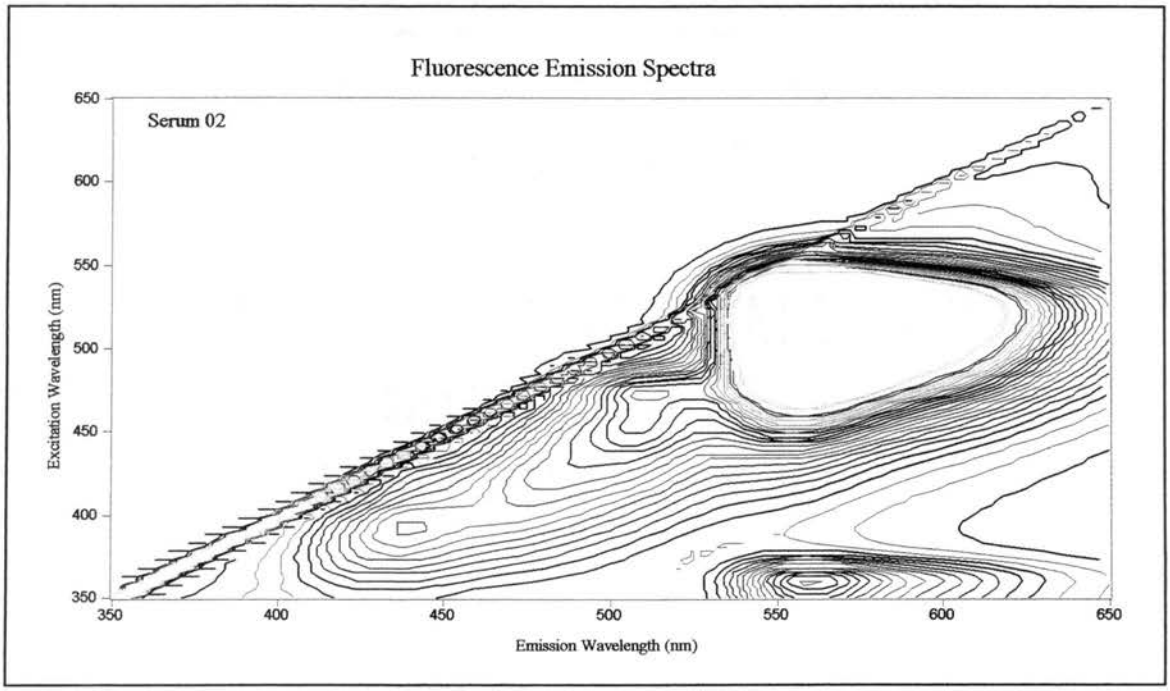


Figure 5-144. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with serum 02 after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

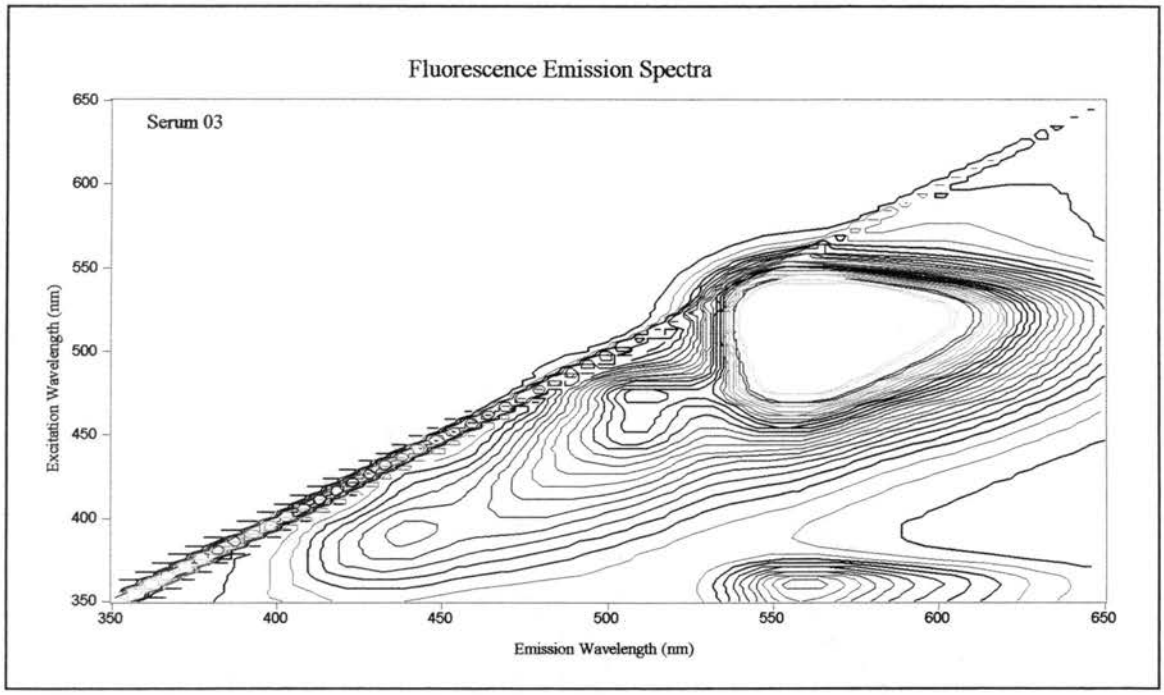


Figure 5-145. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with serum 03 after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

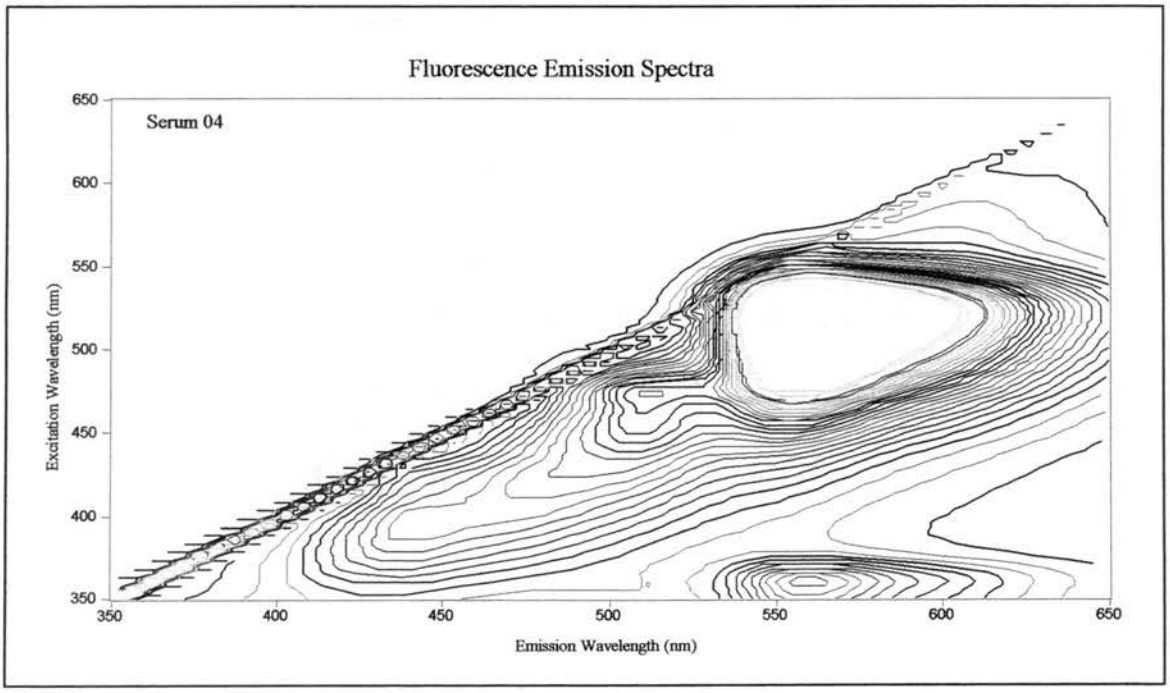


Figure 5-146. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with serum 04 after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

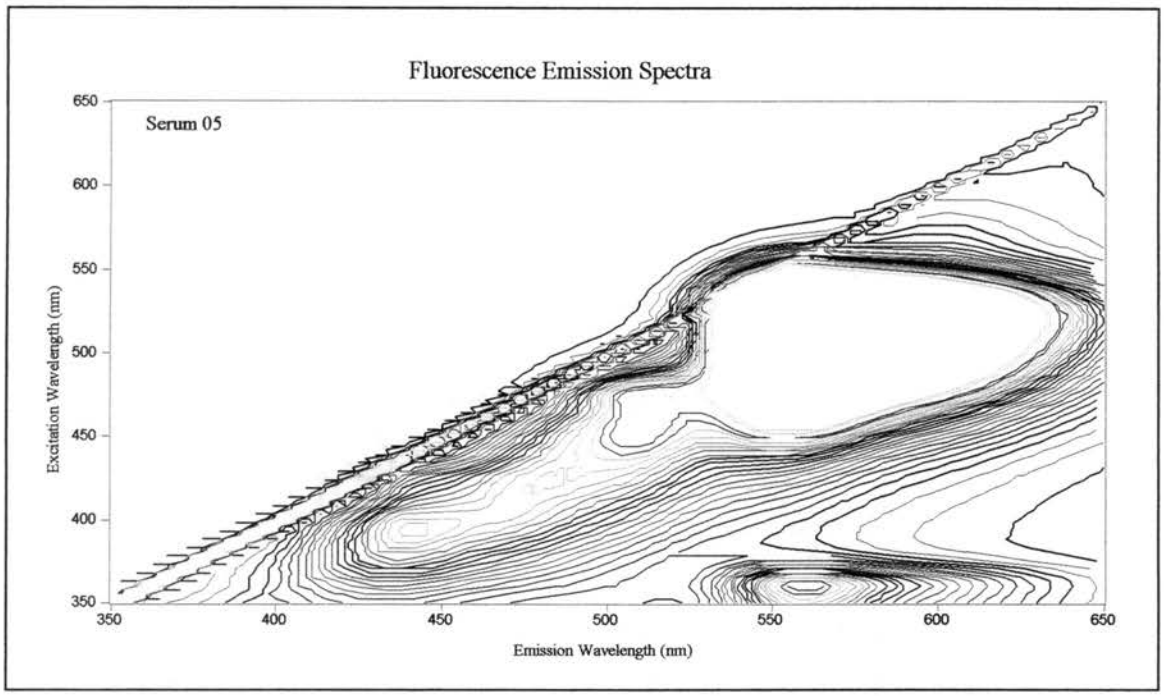


Figure 5-147. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with serum 05 after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

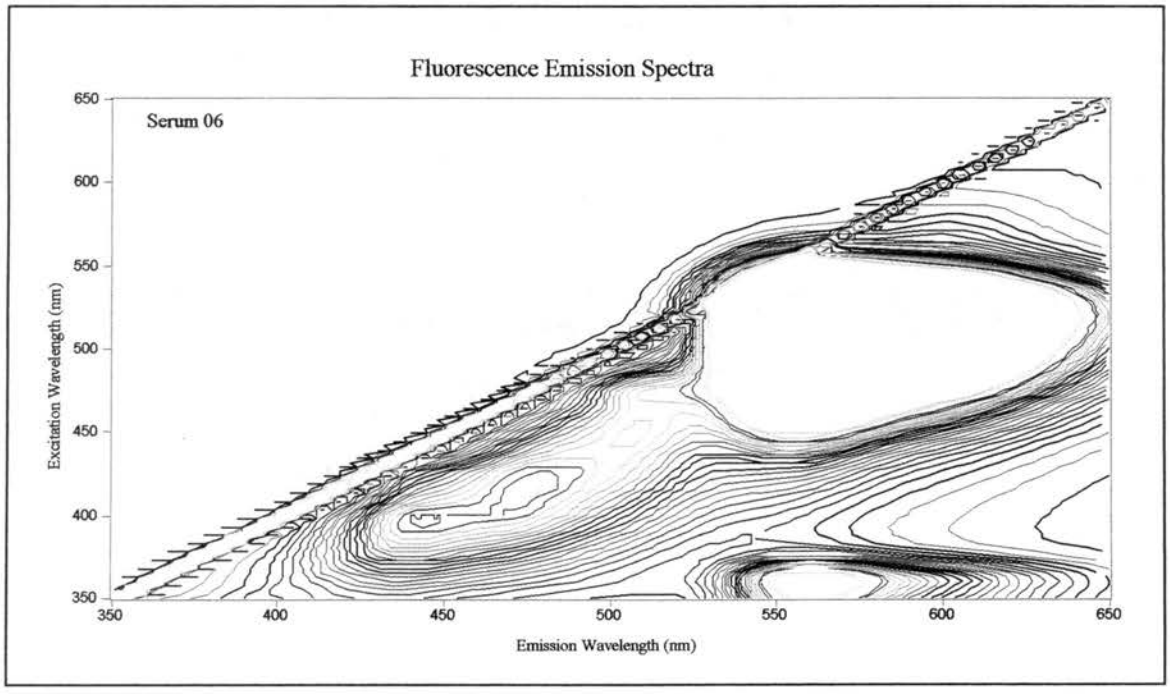


Figure 5-148. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with serum 06 after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

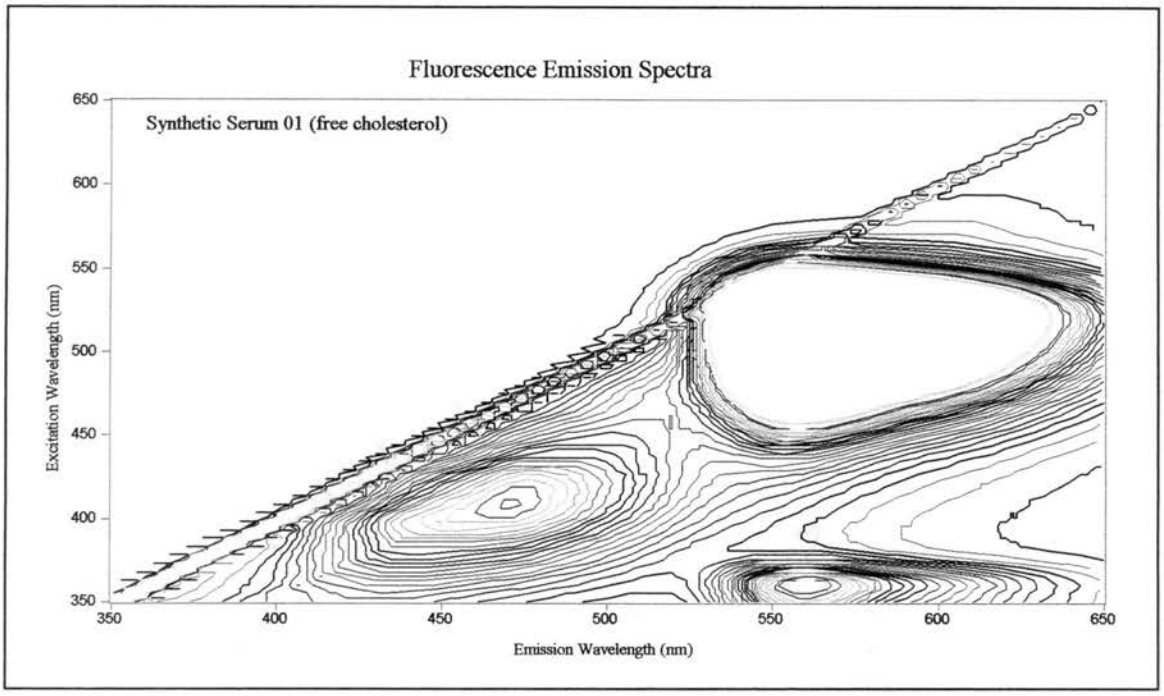


Figure 5-149. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with synthetic serum 01 (free cholesterol) after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

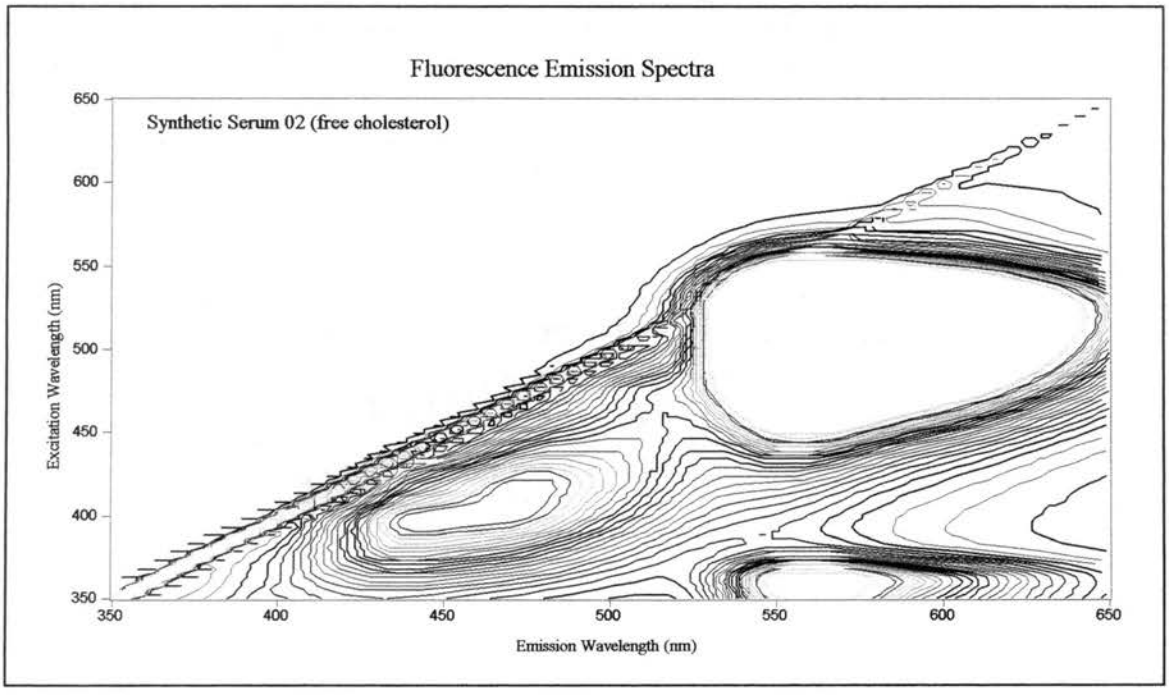


Figure 5-150. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with synthetic serum 02 (free cholesterol) after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

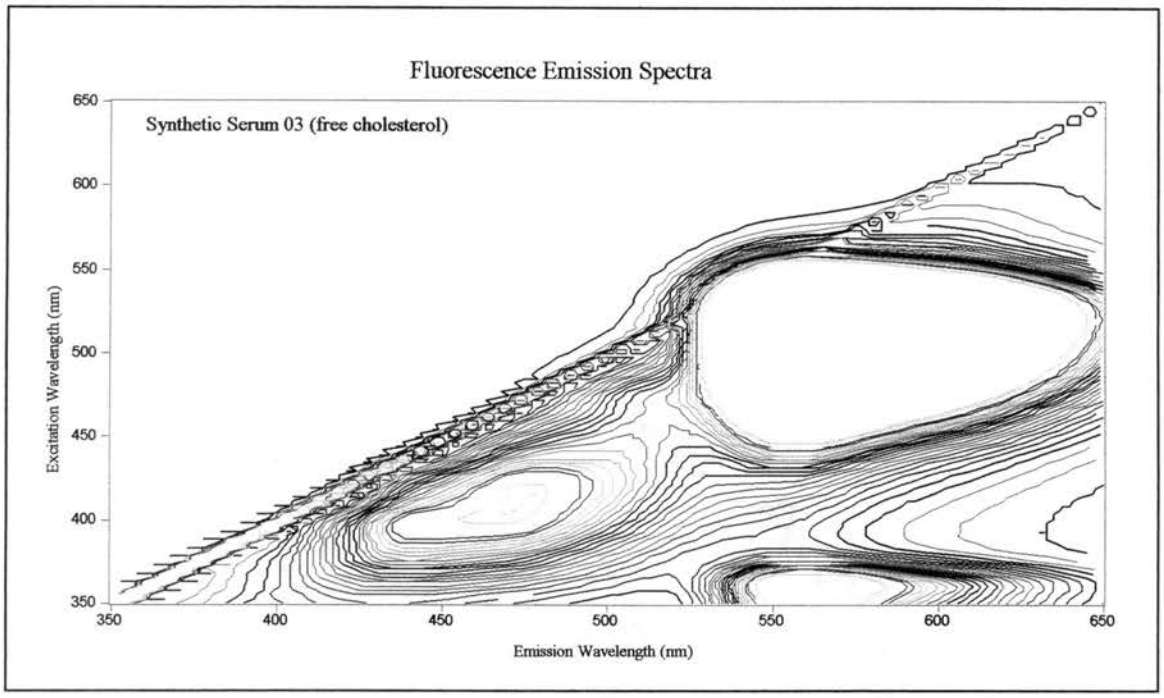


Figure 5-151. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with synthetic serum 03 (free cholesterol) after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

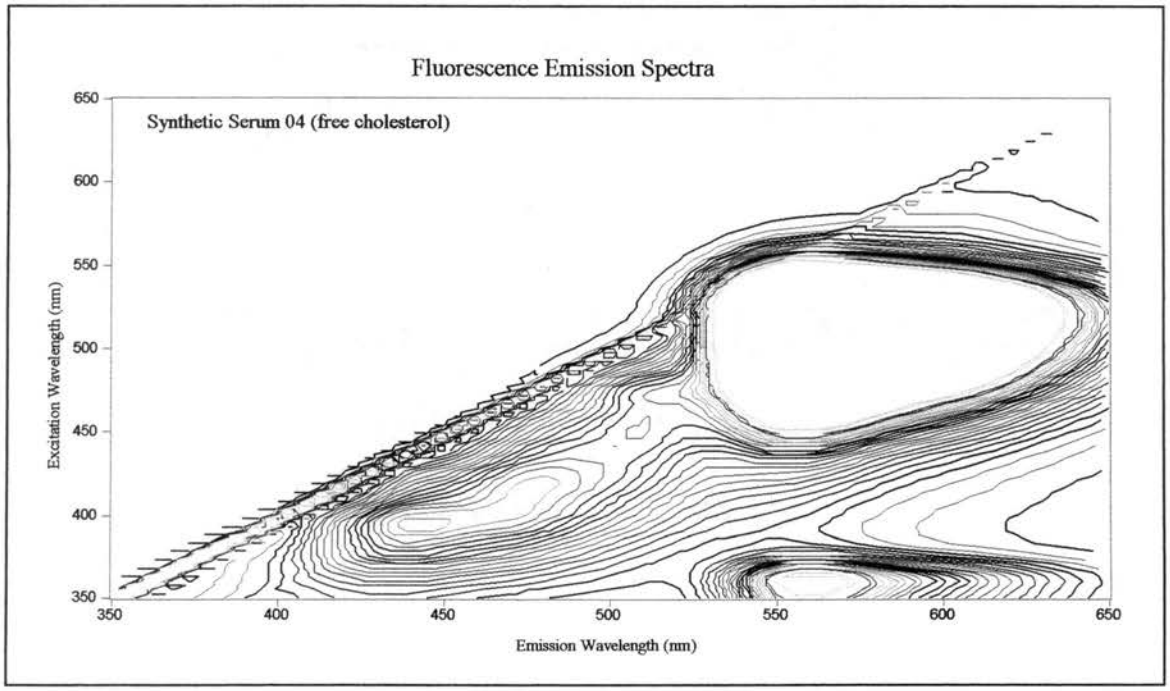


Figure 5-152. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with synthetic serum 04 (free cholesterol) after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

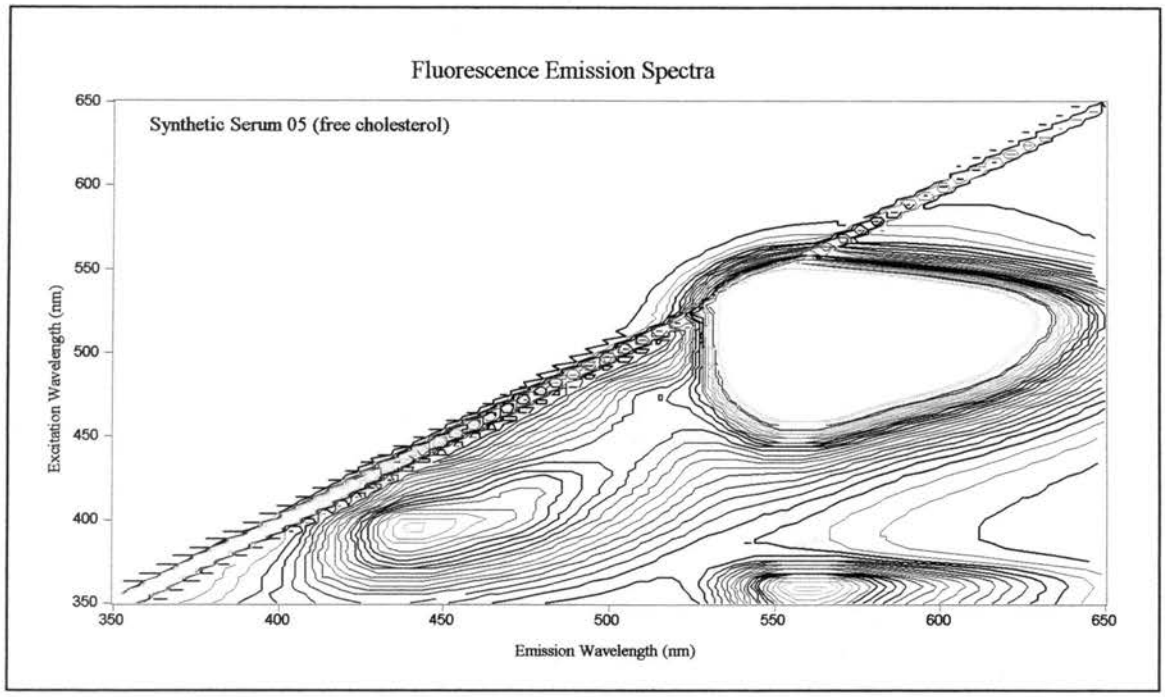


Figure 5-153. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with synthetic serum 05 (free cholesterol) after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

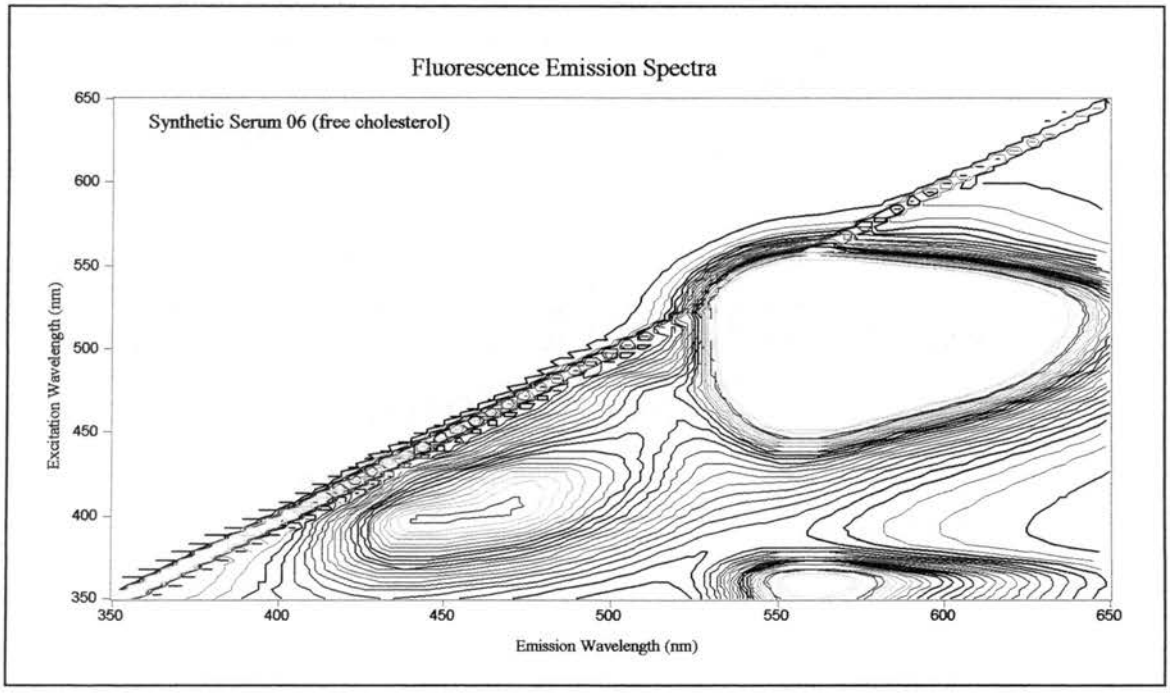


Figure 5-154. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with synthetic serum 06 (free cholesterol) after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

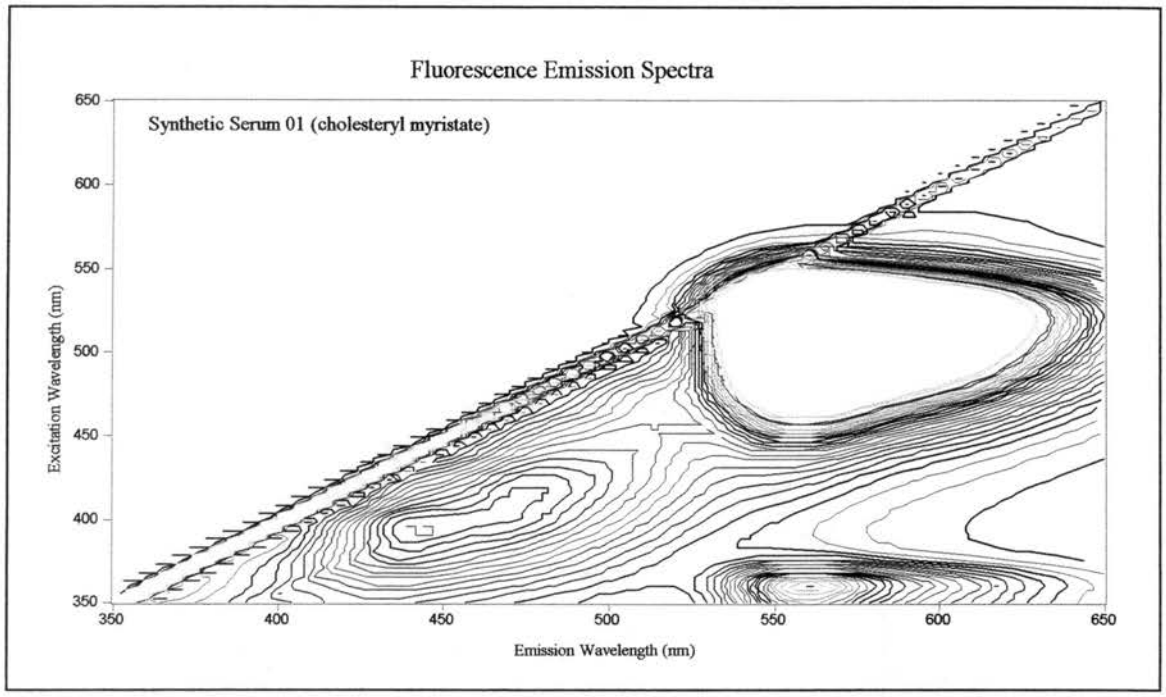


Figure 5-155. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with synthetic serum 01 (cholesteryl myristate) after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

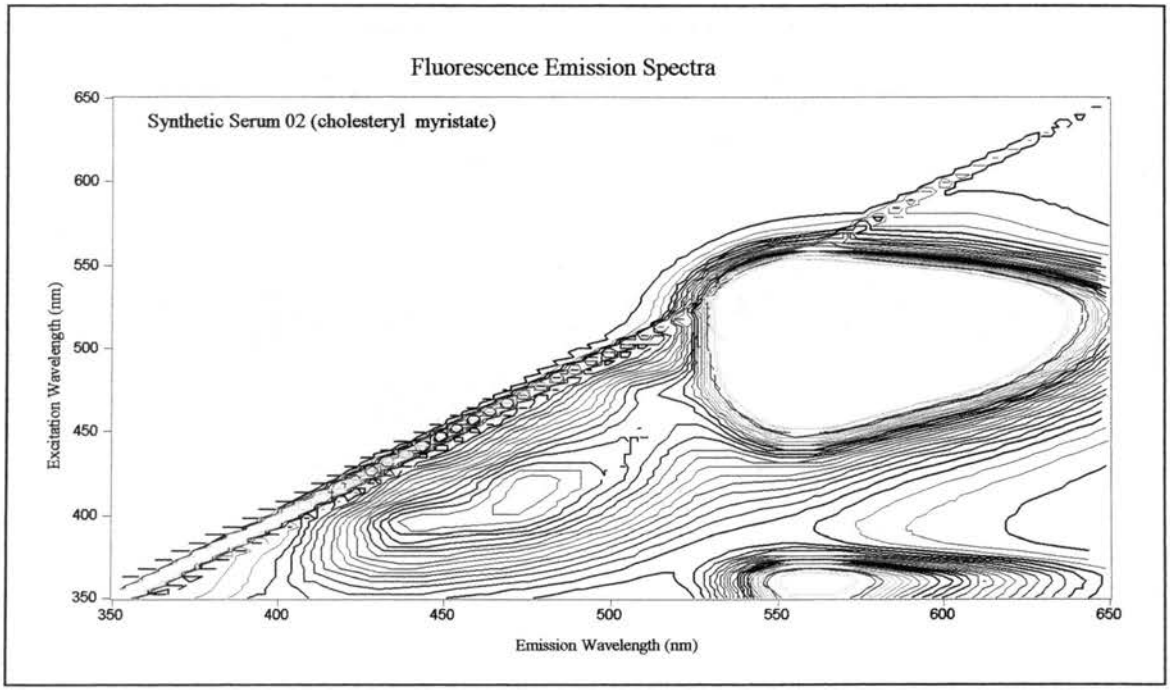


Figure 5-156. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with synthetic serum 02 (cholesteryl myristate) after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

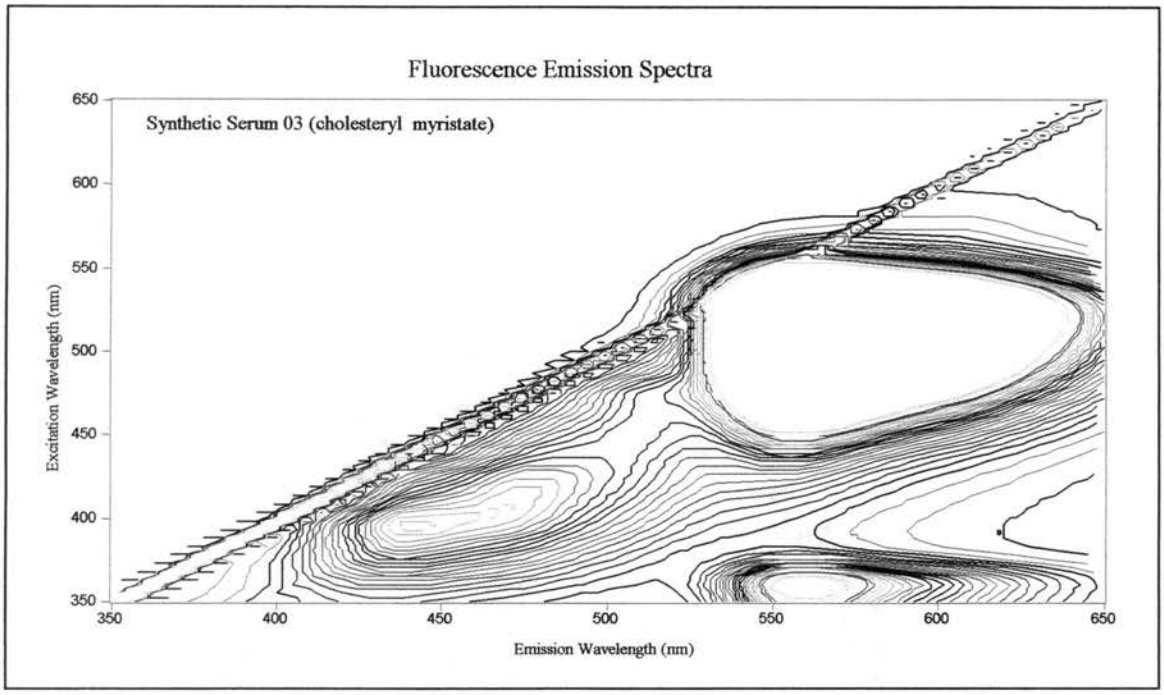


Figure 5-157. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with synthetic serum 03 (cholesteryl myristate) after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

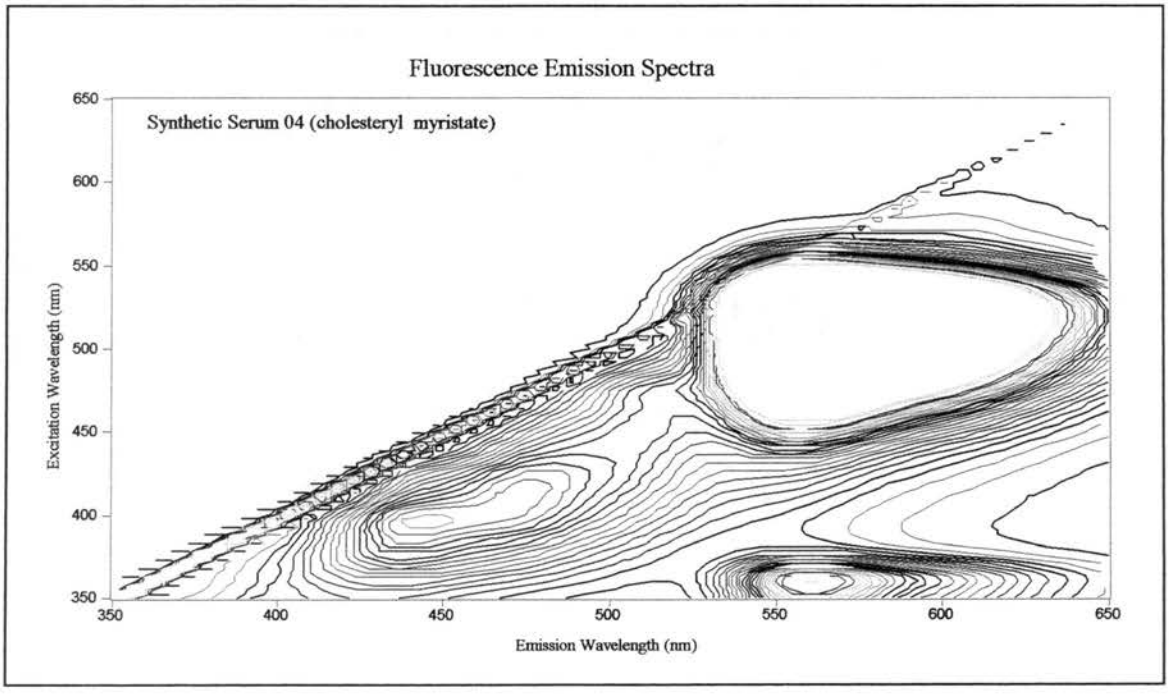


Figure 5-158. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with synthetic serum 04 (cholesteryl myristate) after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

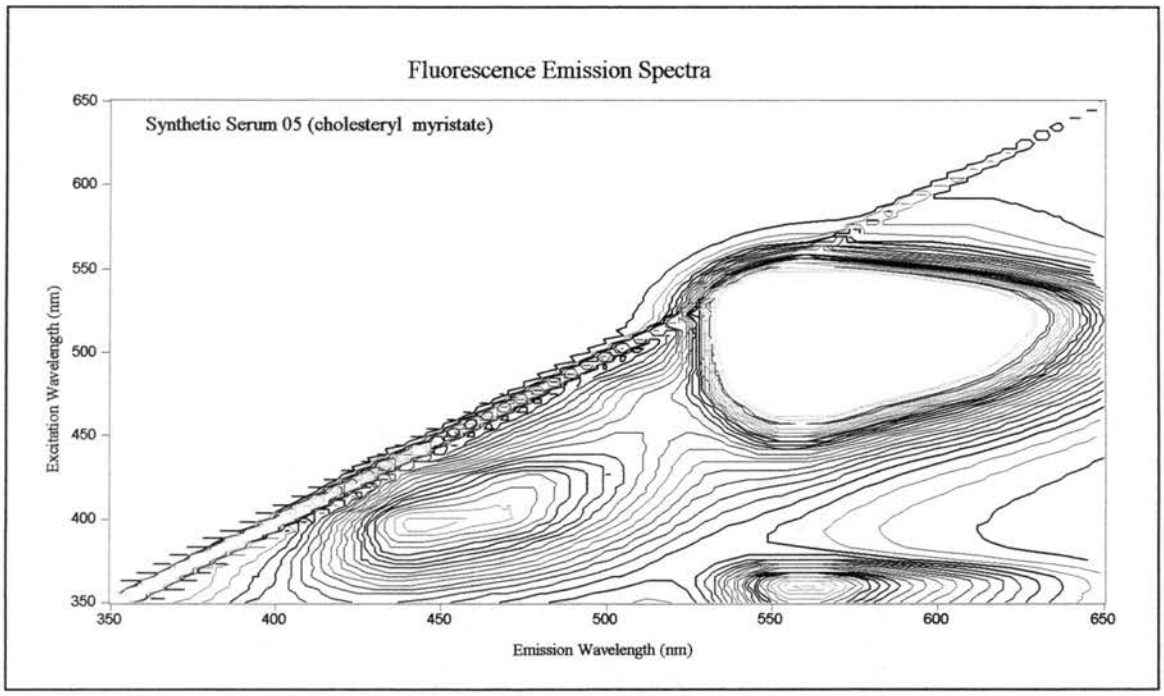


Figure 5-159. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with synthetic serum 05 (cholesteryl myristate) after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

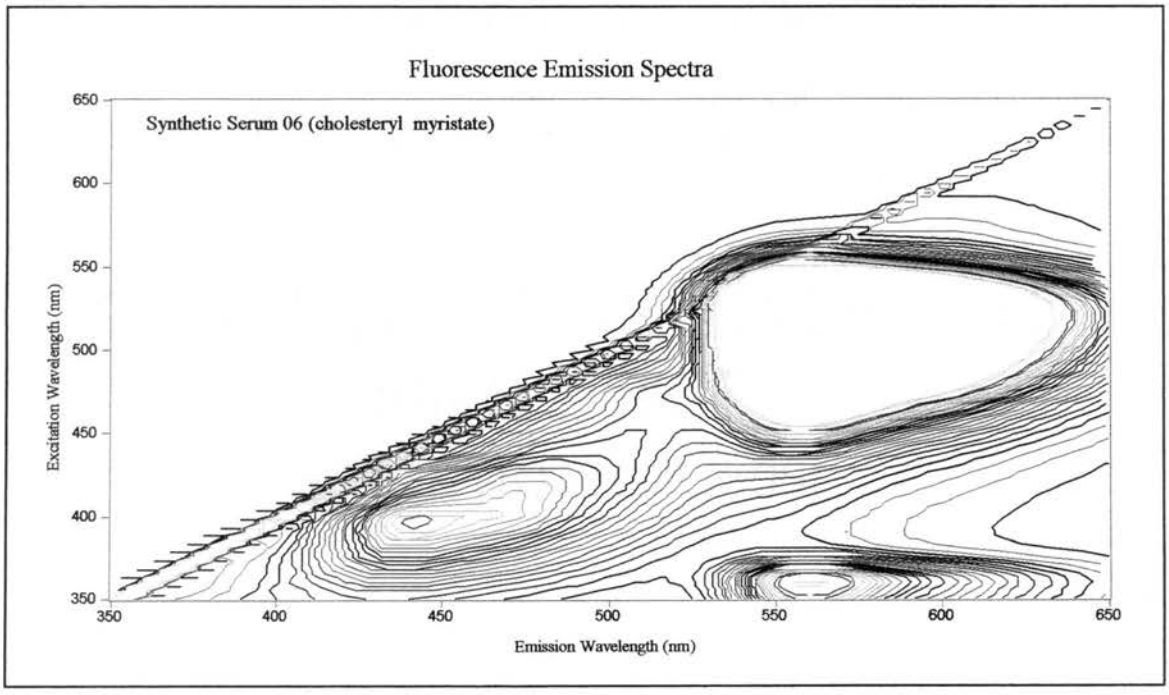


Figure 5-160. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with synthetic serum 06 (cholesteryl myristate) after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

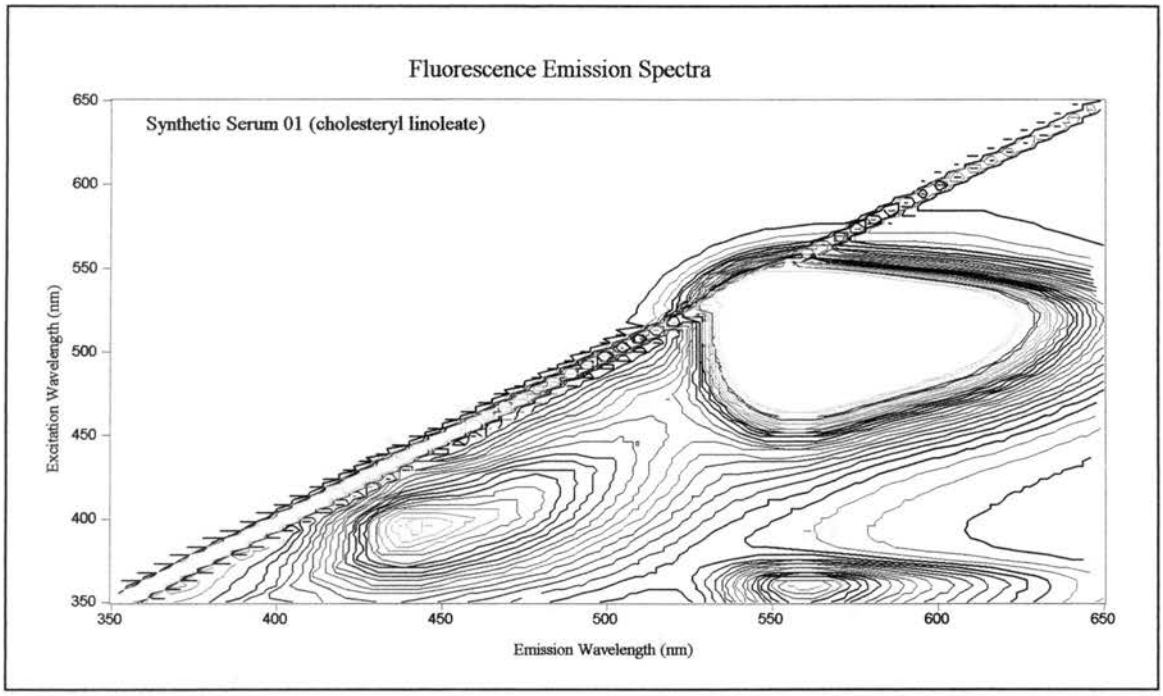


Figure 5-161. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with synthetic serum 01 (cholesteryl linoleate) after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

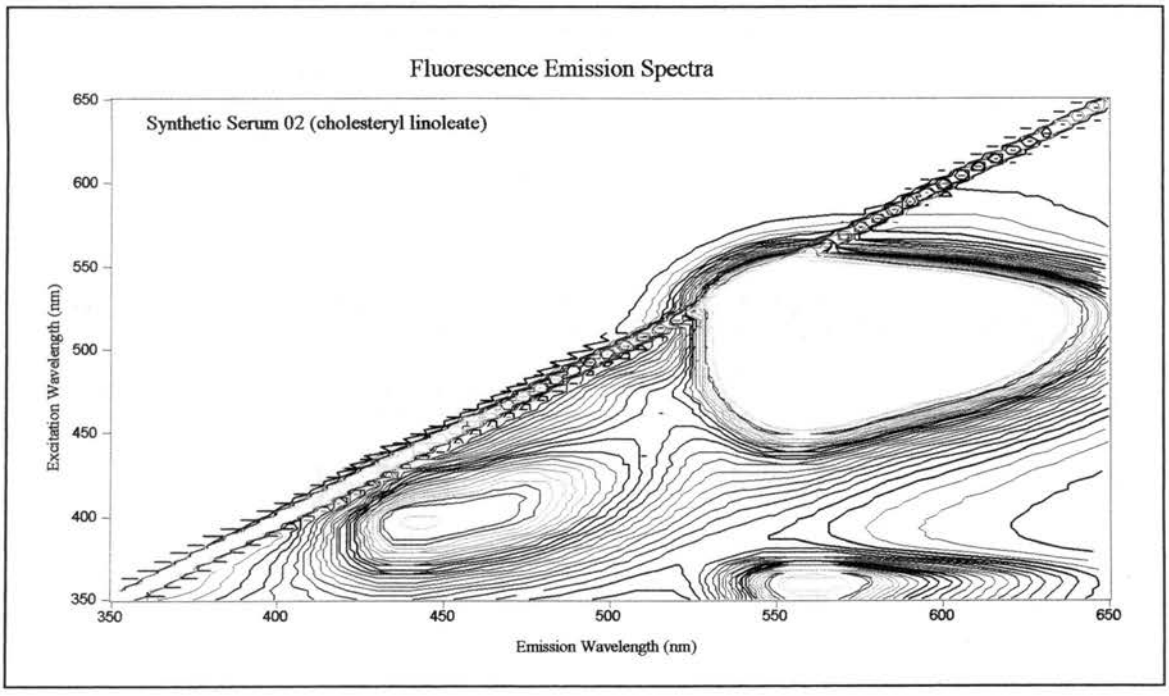


Figure 5-162. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with synthetic serum 02 (cholesteryl linoleate) after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

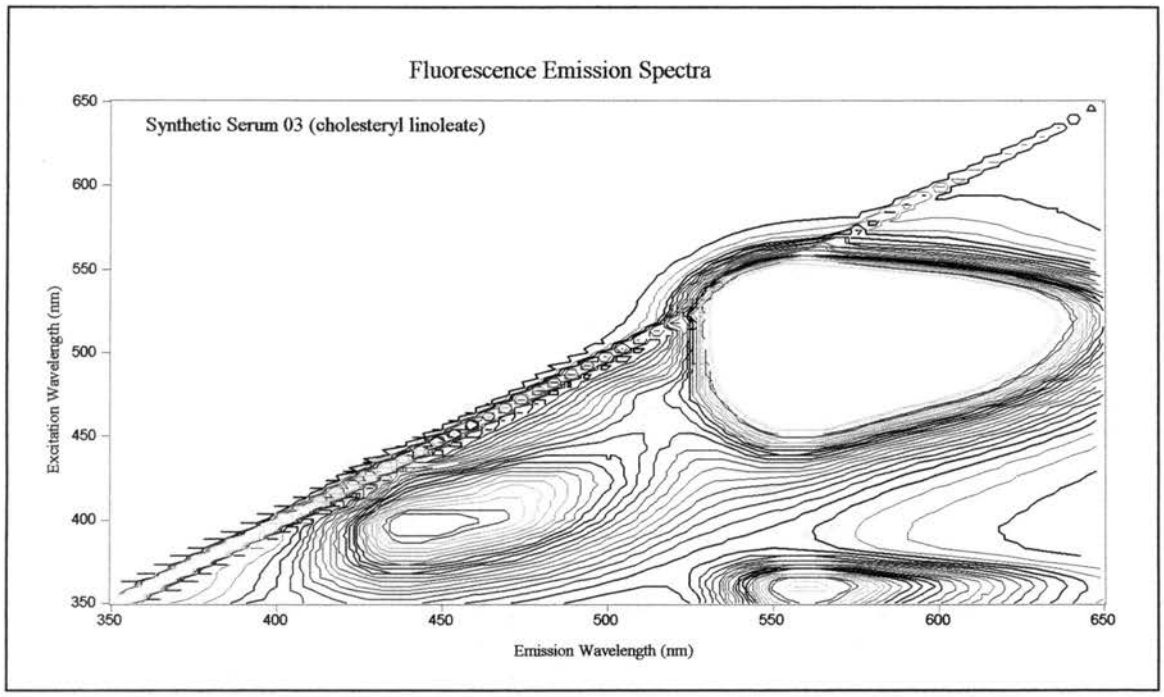


Figure 5-163. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with synthetic serum 03 (cholesteryl linoleate) after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

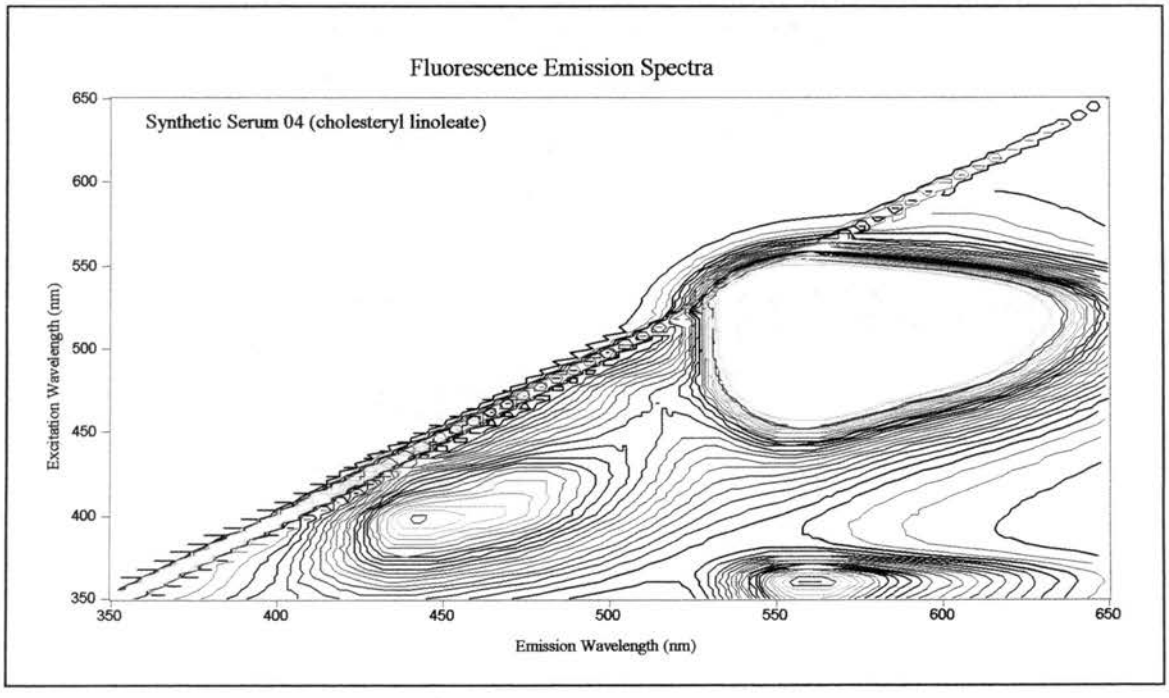


Figure 5-164. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with synthetic serum 04 (cholesteryl linoleate) after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

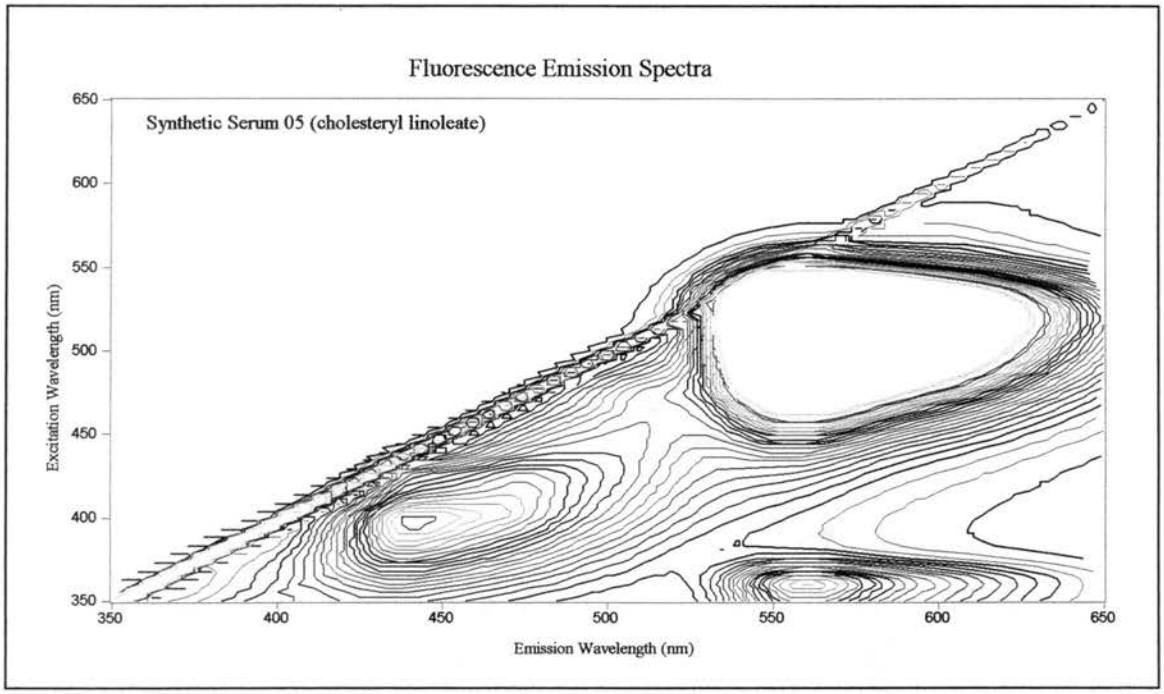


Figure 5-165. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with synthetic serum 05 (cholesteryl linoleate) after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

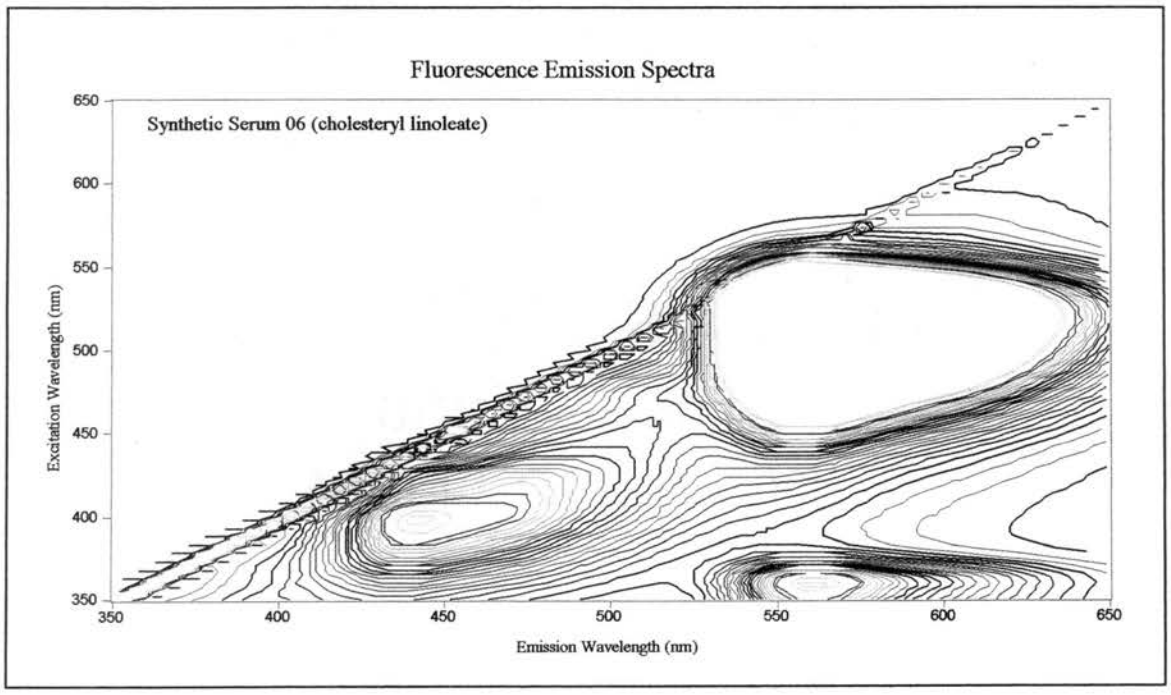


Figure 5-166. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with synthetic serum 06 (cholesteryl linoleate) after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

Having shown that the synthetic sera spectra do reproduce the spectral characteristics of the true serum spectra in a qualitative way, the assumption was made that the synthetic mixtures are, at least component-wise, good approximations of actual blood. The assumption is critical to the next step in this study. The next step was the application of the current technique to the serum samples of diagnosed untreated dyslipidemic patients. However, patients with the coveted dyslipidemias of interest were not available in the local area at the time of the study and are generally quite rare throughout the United States. Therefore, synthetic mixtures representing the various dyslipidemias were made up and analyzed. Table 5-8 outlines the total lipid composition of each of the synthetic dyslipidemic serums (SDSs), while Tables 5-9 to 5-15 break down lipid composition by lipoprotein class in each SDS sample. Figure 5-167 is an overlay plot of the absorbance spectra of each SDS and Figures 5-168 to 5-181 are the respective contour plots of the fluorescence emission and excitation spectra. Figure 5-167 clearly shows unique spectral patterns for most of the SDSs. The exceptions were Types IIB and VI which show a very similar pattern.

Table 5-8. Total Lipid Composition of Synthetic Dyslipidemic Serums (SDSs)							
Dyslipidemia #	I	IIA	IIB	III	IV	V	VI
[TC] (mg/dL)	351.31	250.91	353.40	430.32	296.77	412.81	297.22
[HDL-C] (mg/dL)	25.75	24.80	23.34	21.44	24.23	29.01	193.47
[LDL-C] (mg/dL)	38.84	190.86	193.69	32.36	91.41	91.18	59.88
[IDL-C] (mg/dL)	4.07	7.83	7.37	338.60	7.65	7.63	0
[VLDL-C] (mg/dL)	45.53	27.42	128.99	37.92	173.47	106.87	43.86
[Chylomicron-C] (mg/dL)	237.12	0	0	0	0	178.12	0
[TG] (mg/dL)	2980.67	131.07	392.30	447.78	718.54	2447.75	158.66
[PL] (mg/dL)	347.47	185.90	286.65	371.26	240.22	389.23	365.19

Table 5-9. Lipid Composition by Lipoprotein Class in Synthetic Dyslipidemic Serum I (SDS-I)				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	0.81	1.76	3.79	0.41
LDL	0.81	3.07	2.35	0.99
IDL	0.11	0.30	0.34	0.41
VLDL	1.52	3.04	4.55	11.71
Chylomicron	6.77	16.93	23.71	284.55
TOTAL	10.02	25.10	34.74	298.07

Table 5-10. Lipid Composition by Lipoprotein Class in Synthetic Dyslipidemic Serum IIA (SDS-IIA)				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	0.78	1.70	3.65	0.39
LDL	3.99	15.09	11.54	4.88
IDL	0.21	0.57	0.65	0.78
VLDL	0.91	1.83	2.74	7.05
Chylomicron	0	0	0	0
TOTAL	5.89	19.19	18.58	13.10

Table 5-11. Lipid Composition by Lipoprotein Class in Synthetic Dyslipidemic Serum IIB (SDS-IIB)				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	0.74	1.60	3.44	0.37
LDL	4.05	15.31	11.71	4.95
IDL	0.20	0.54	0.61	0.74
VLDL	4.30	8.60	12.9	33.17
Chylomicron	0	0	0	0
TOTAL	9.29	26.05	28.66	39.23

Table 5-12. Lipid Composition by Lipoprotein Class in Synthetic Dyslipidemic Serum III (SDS-III)				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	0.68	1.47	3.16	0.34
LDL	0.68	2.55	1.96	0.83
IDL	9.03	24.83	28.22	33.86
VLDL	1.26	2.53	3.79	9.75
Chylomicron	0	0	0	0
TOTAL	11.65	31.38	37.13	44.78

Table 5-13. Lipid Composition by Lipoprotein Class in Synthetic Dyslipidemic Serum IV (SDS-IV)				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	0.77	1.66	3.57	0.38
LDL	1.91	7.23	5.53	2.34
IDL	0.20	0.56	0.64	0.76
VLDL	5.10	12.24	14.29	68.37
Chylomicron	0	0	0	0
TOTAL	7.98	21.69	24.03	71.85

Table 5-14. Lipid Composition by Lipoprotein Class in Synthetic Dyslipidemic Serum V (SDS-V)				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	0.92	1.98	4.27	0.46
LDL	1.91	7.21	5.51	2.33
IDL	0.20	0.56	0.64	0.76
VLDL	3.56	7.12	10.68	27.48
Chylomicron	5.09	12.72	17.81	213.74
TOTAL	11.68	29.59	38.91	244.77

Table 5-15. Lipid Composition by Lipoprotein Class in Synthetic Dyslipidemic Serum VI (SDS-VI)				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	6.11	13.24	28.51	3.05
LDL	1.25	4.73	3.62	1.53
IDL	0	0	0	0
VLDL	1.46	2.92	4.39	11.28
Chylomicron	0	0	0	0
TOTAL	8.82	20.89	36.52	15.86

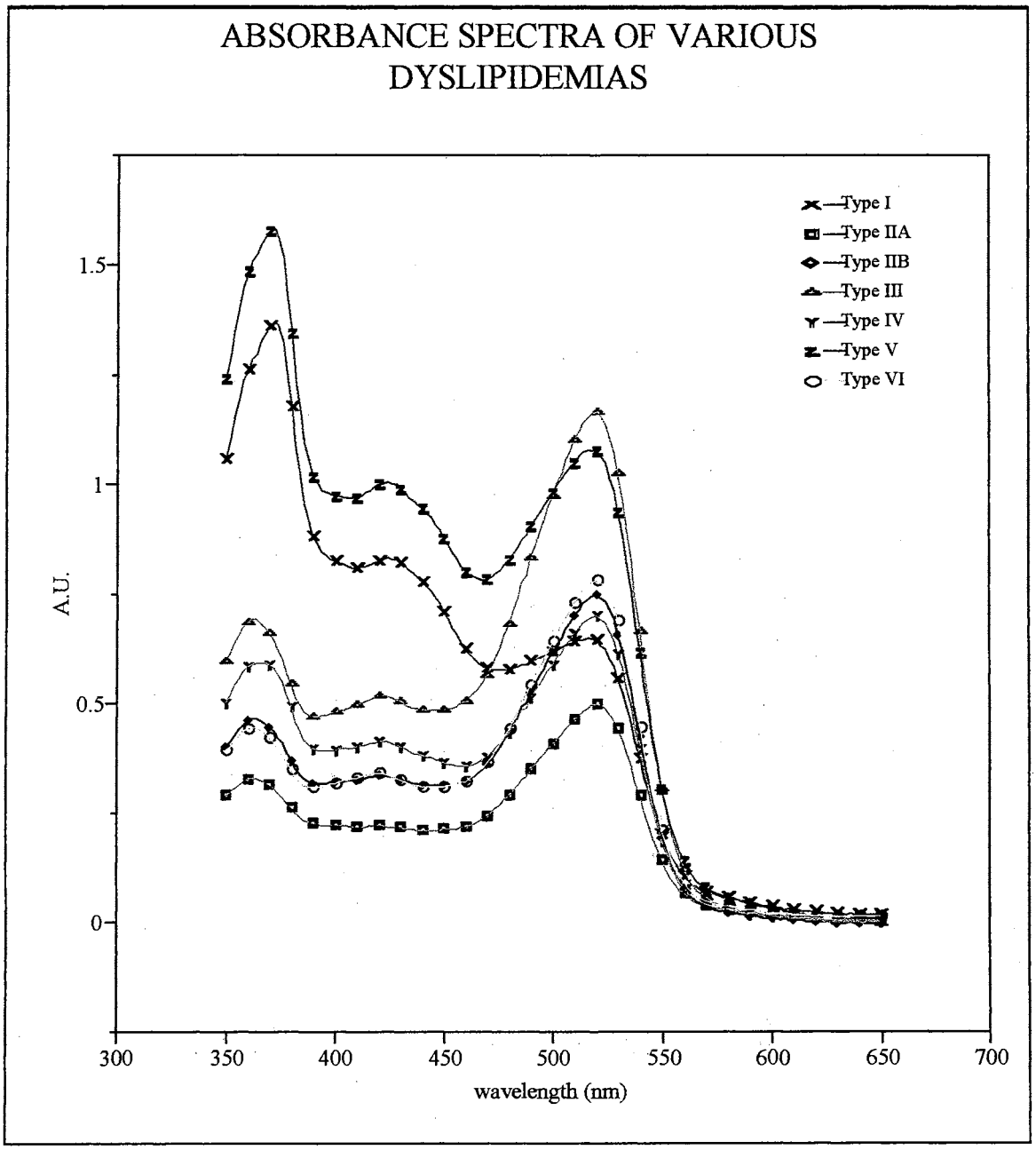


Figure 5-167. Overlay plot of the absorption spectra of the various synthetic dyslipidemias after 20 minutes of reaction with the SRS.

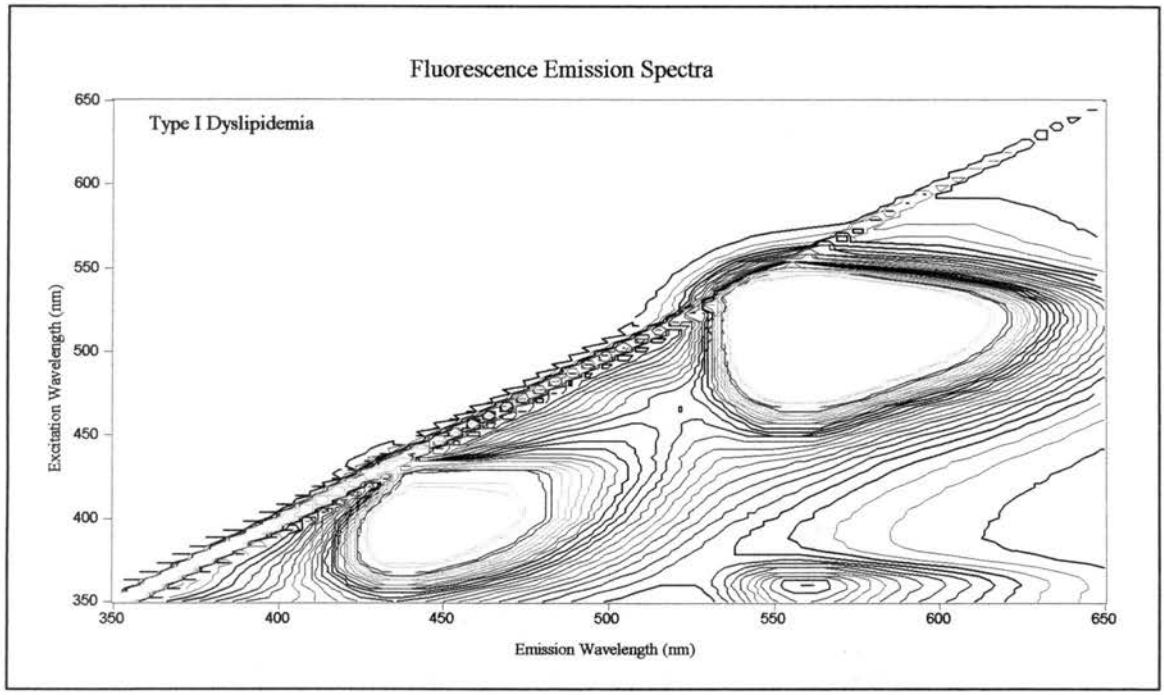


Figure 5-168. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with a synthetic Type I dyslipidemia mixture after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

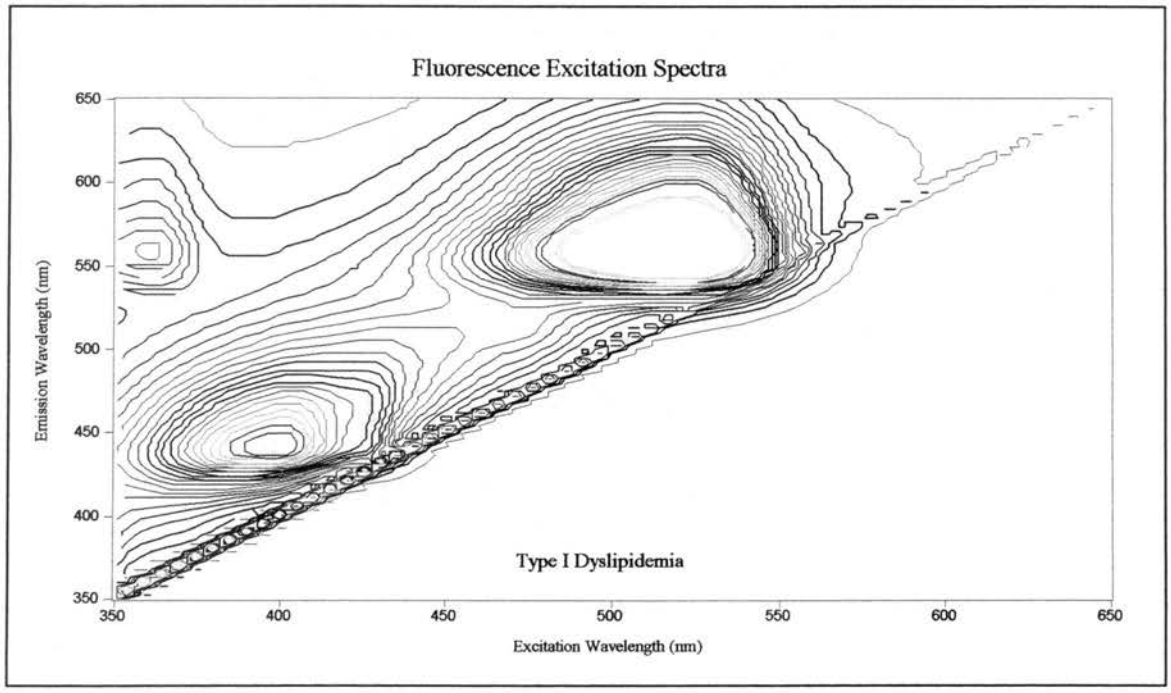


Figure 5-169. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with a synthetic Type I dyslipidemia mixture after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

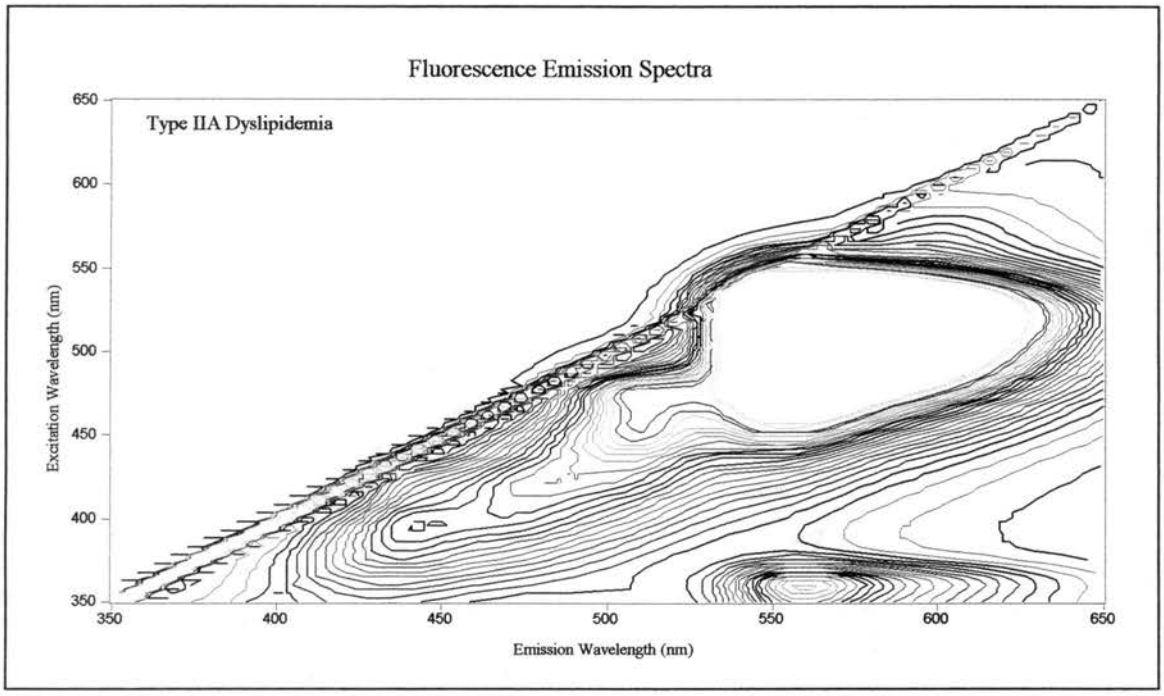


Figure 5-170. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with a synthetic Type IIA dyslipidemia mixture after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

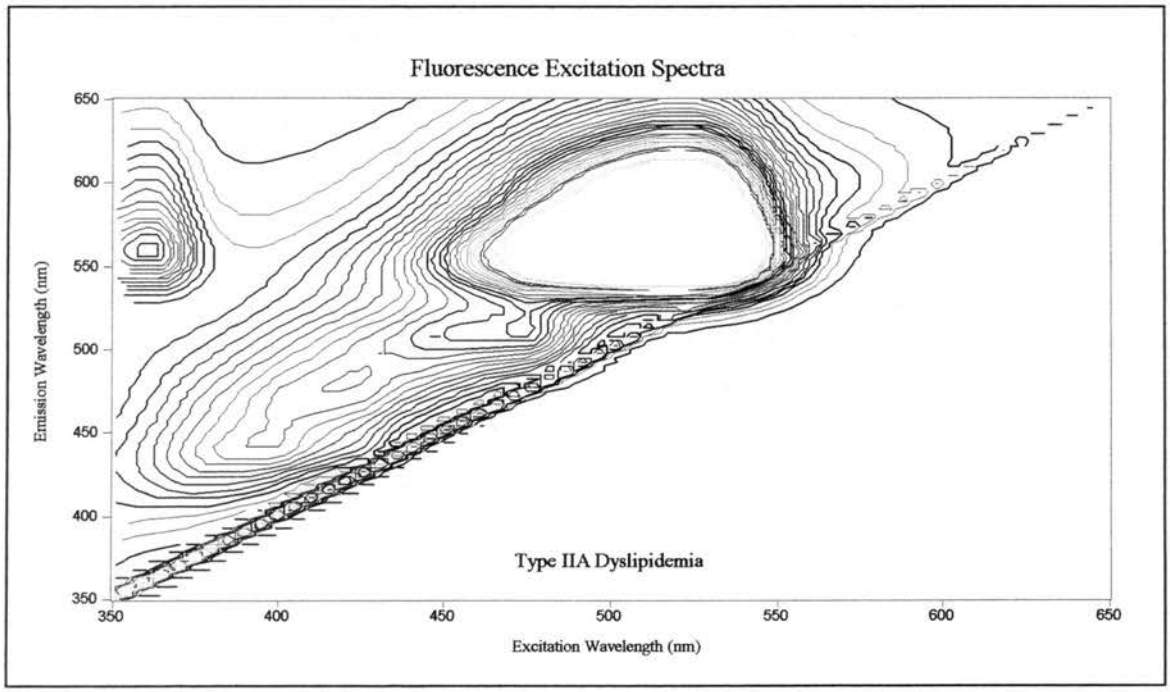


Figure 5-171. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with a synthetic Type IIA dyslipidemia mixture after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

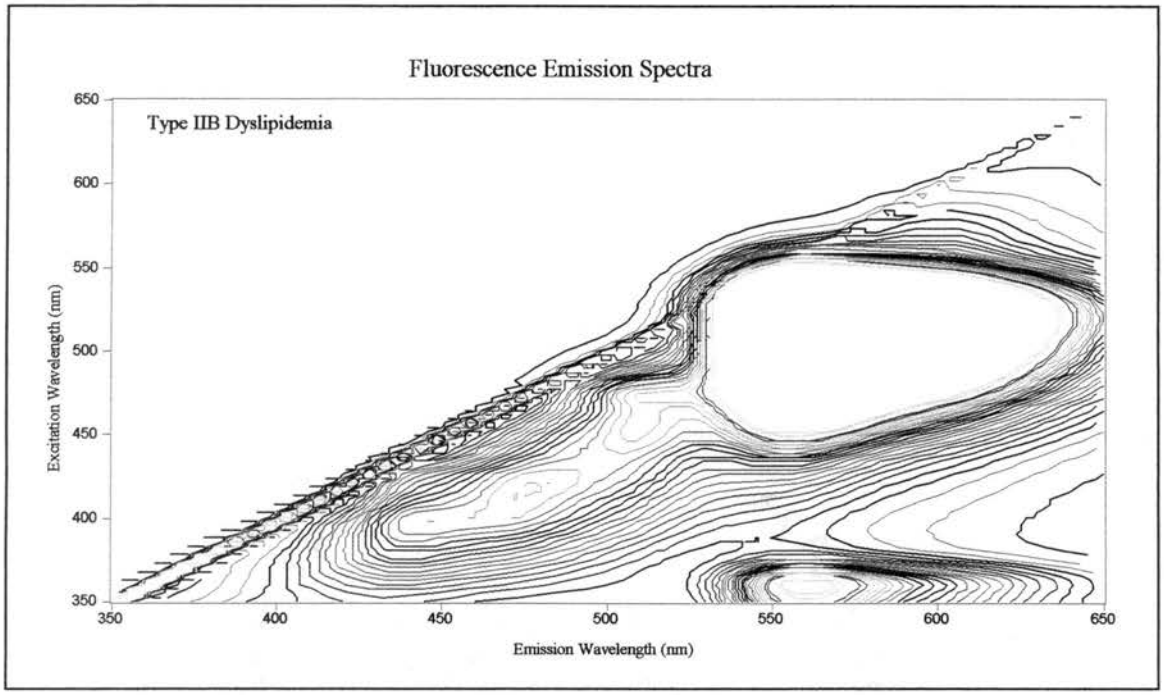


Figure 5-172. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with a synthetic Type IIB dyslipidemia mixture after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

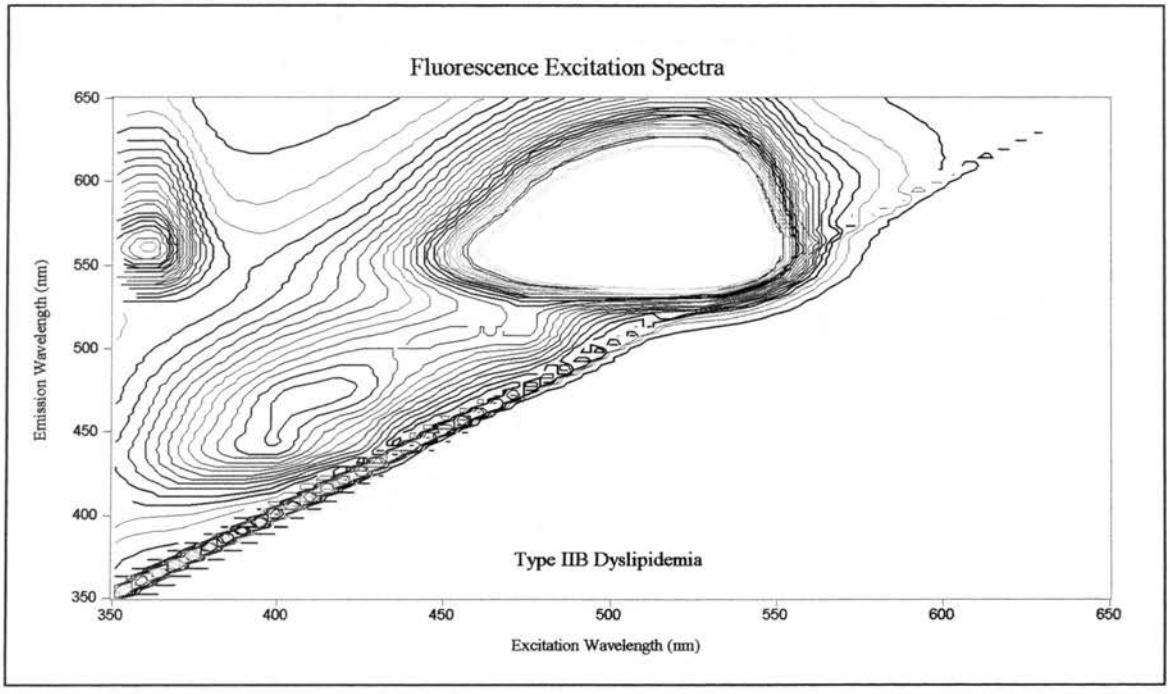


Figure 5-173. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with a synthetic Type IIB dyslipidemia mixture after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

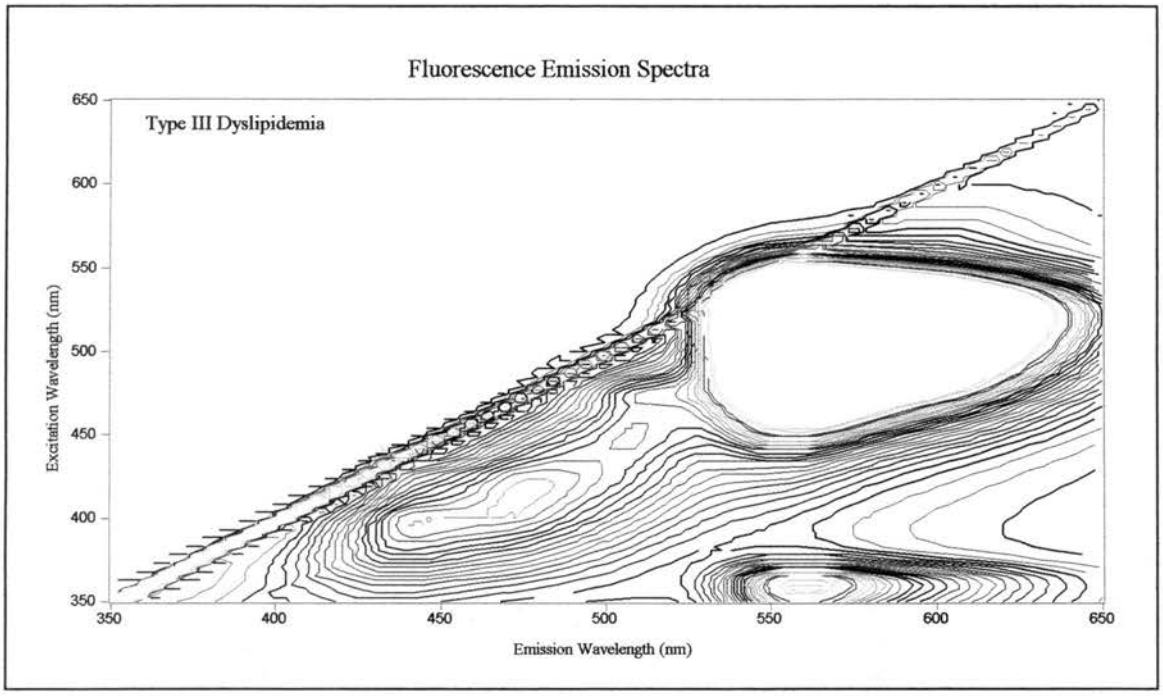


Figure 5-174. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with a synthetic Type III dyslipidemia mixture after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

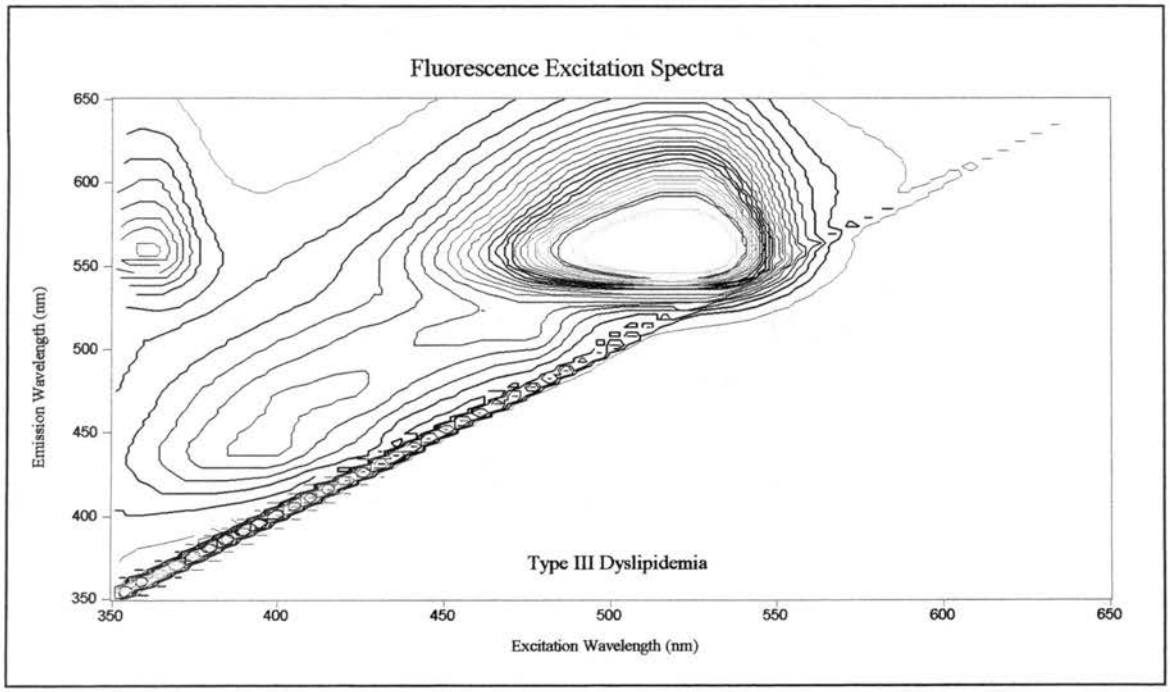


Figure 5-175. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with a synthetic Type III dyslipidemia mixture after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

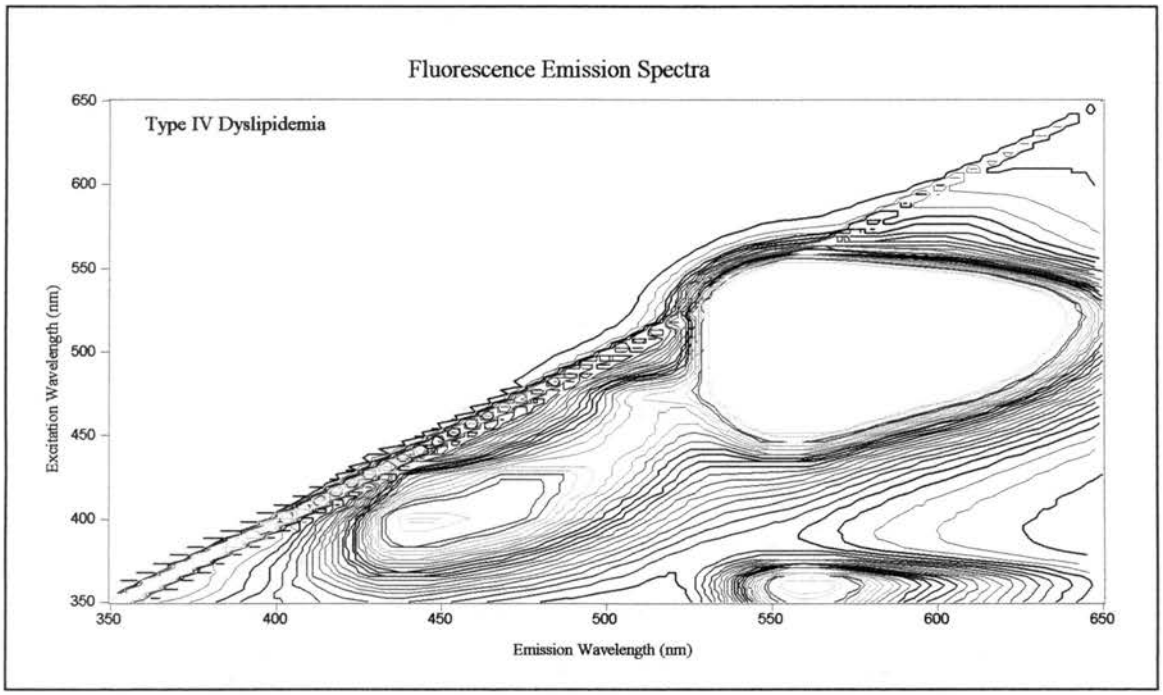


Figure 5-176. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with a synthetic Type IV dyslipidemia mixture after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

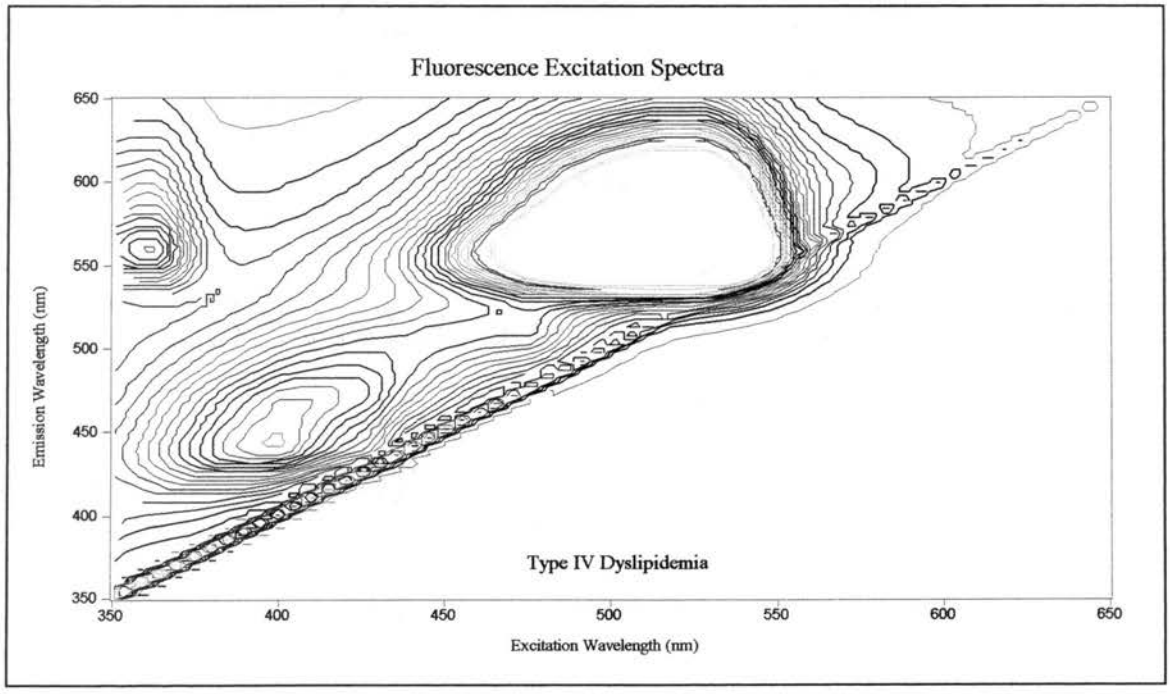


Figure 5-177. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with a synthetic Type IV dyslipidemia mixture after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

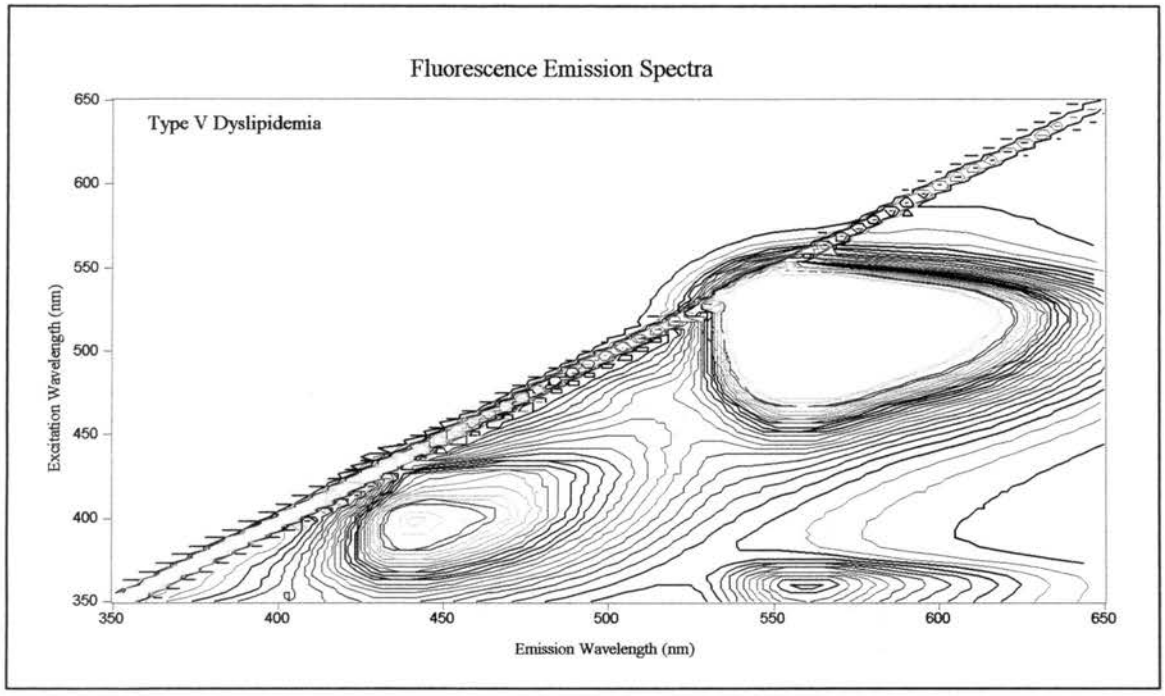


Figure 5-178. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with a synthetic Type V dyslipidemia mixture after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

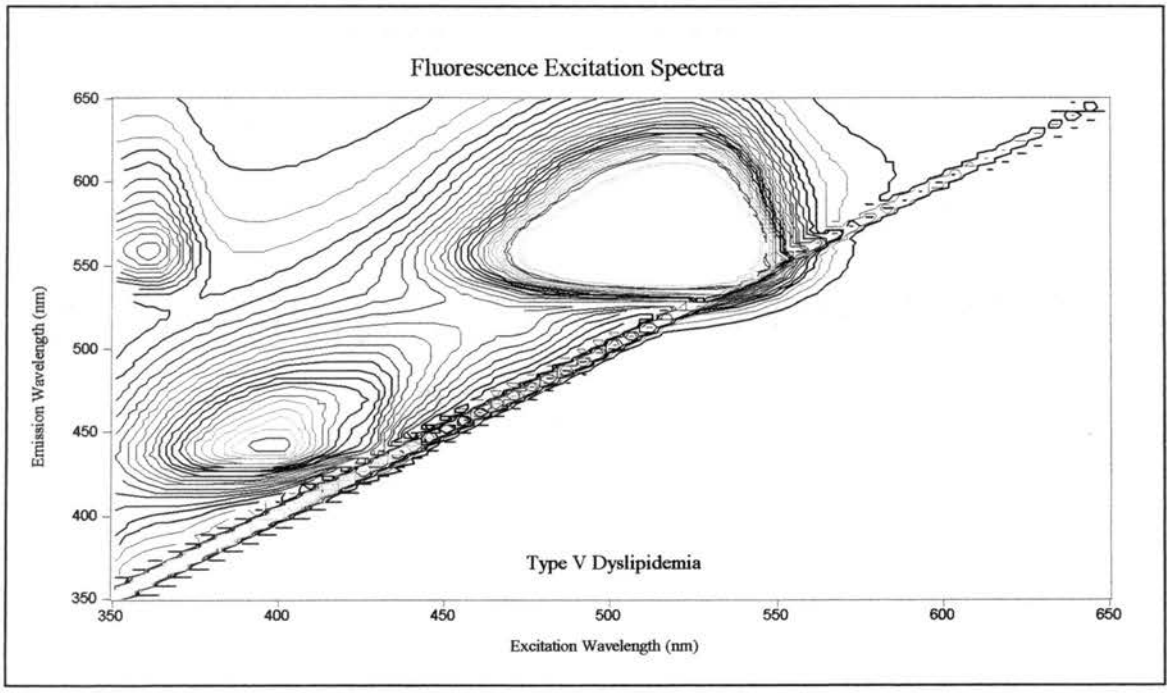


Figure 5-179. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with a synthetic Type V dyslipidemia mixture after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

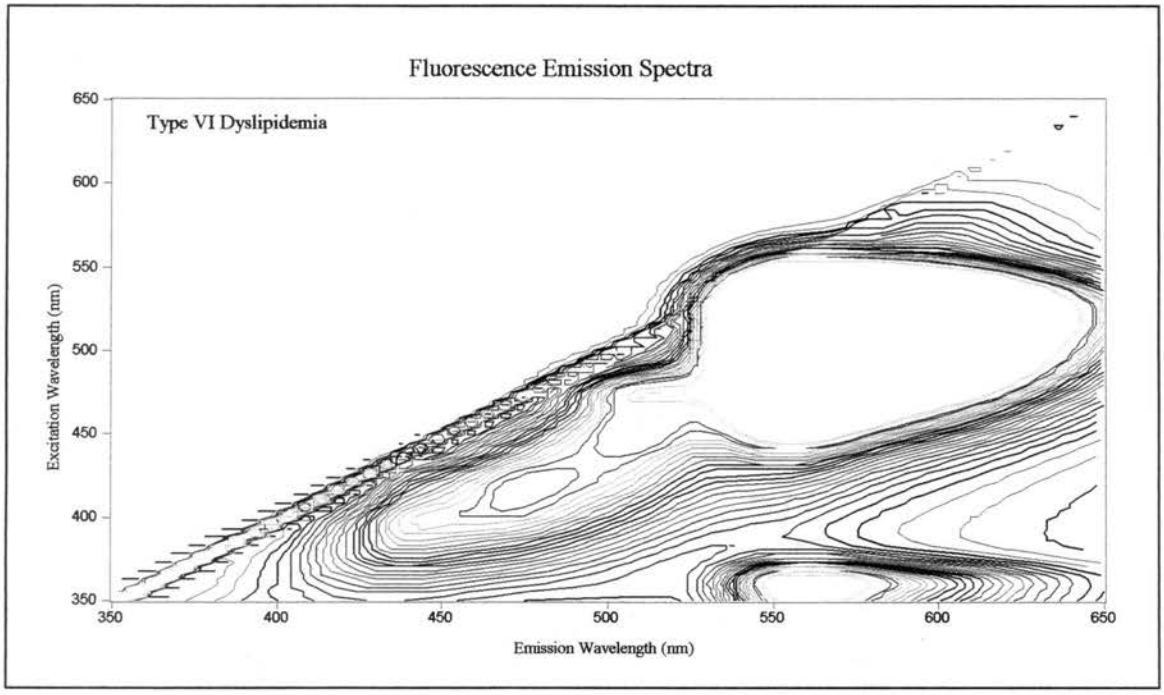


Figure 5-180. Contour plot of the fluorescence emission spectra of the products of the reaction of the standard reagent with a synthetic Type VI dyslipidemia mixture after 20 minutes. Excitation wavelengths range from 350-650nm in 5nm increments.

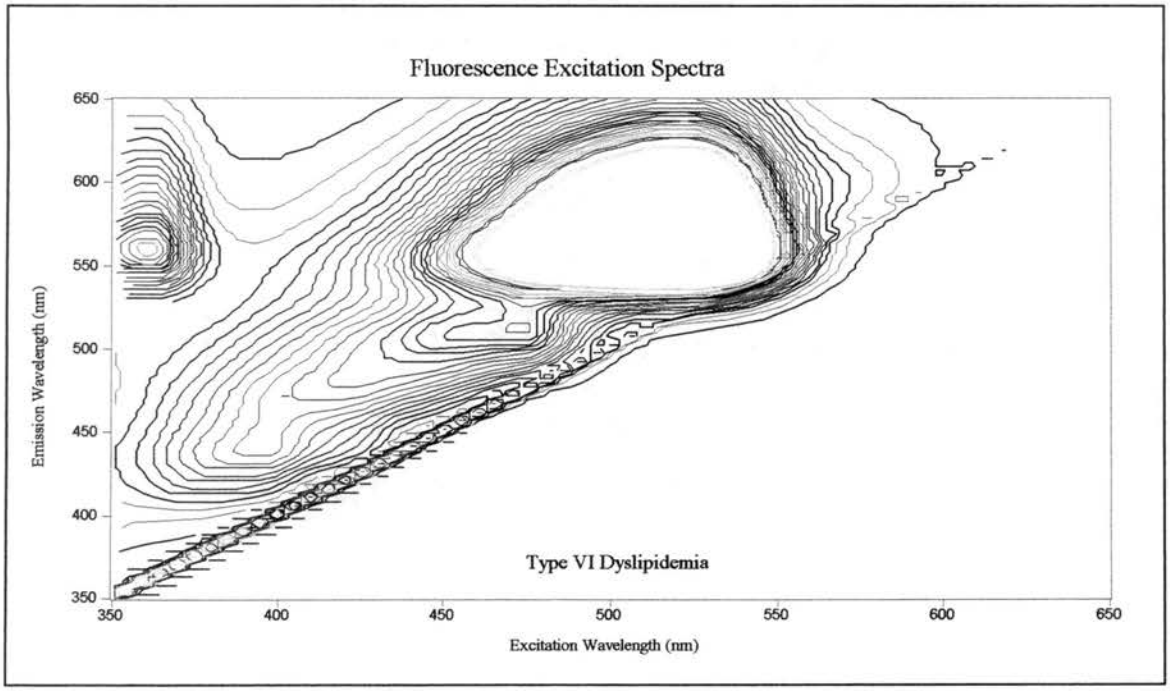


Figure 5-181. Contour plot of the fluorescence excitation spectra of the products of the reaction of the standard reagent with a synthetic Type VI dyslipidemia mixture after 20 minutes. Emission wavelengths range from 350-650nm in 5nm increments.

Collectively, the spectra of the SDSs may be thought of as a library. Any future spectra can be compared to this library and correlational assessments can be made to determine if a disease state exists. In the larger context of practical application, this library would include spectra representing healthy individuals with a wide range of blood lipid levels as well as individuals with varying degrees of each disease state. To illustrate the utility and effectiveness of this approach, sera analyzed previously (Serum 01-06) and series of synthetic mixtures (SM01-SM06) were made up to represent both healthy and dyslipidemic subjects. (Because normal lipid concentrations vary demographically, a "normal" serum in this study is considered to be that of a 29 year old white male with a [TC] between 140 and 220 mg/dL, a [HDL-C] from 30 to 70mg/dL, a [LDL-C] from 60 to 175mg/dL, a [PL] from 125 to 275mg/dL and a [TG] from 44 to 185mg/dL.[82] The synthetic mixtures were made up to reflect the following states of health: SM01 - Type I dyslipidemia; SM02 - Type IIa dyslipidemia; SM03 - Type IIb dyslipidemia; SM04 - Type III dyslipidemia; SM05 - Type IV dyslipidemia; SM06 - Type V dyslipidemia; SM07 - normal (high end); SM08 - normal (low end). Table 5-16 outlines the total lipid composition of each SM. Tables 5-17 to 5-24 list the lipid composition by lipoprotein class for each SM. Figures 5-182 to 5-189 plot the absorbance spectra of the SM series. Spectral data obtained from the sera and the SM series were correlated with spectra from the library of dyslipidemic spectra to determine which, if any, disease states could be predicted. Correlations were done using the JMP® Statistical Discovery Software program on a Windows™ based personal computer. In the JMP® program, the Analyze → Multivariate options were chosen to create cross-correlation plots between every possible pair of spectra and to determine the degree of linearity between the pair. The multivariate option routine uses the Pearson-product moment method to measure the strength of the linear relationship between two variables. For the response variables X and Y, a Pearson-product correlation coefficient, r, is computed as shown in Equation 5-1. If a perfectly linear relationship exists between the two variables, the correlation is 1 or -1, depending on whether the variables are positively or negatively related. If there is no linear relationship, r approaches zero. In using this approach, the assumption was made that if a perfectly linear relationship exists between two spectra then those two spectra contain the same proportions of, though not necessarily the same quantity of, each chromogenic lipid class. Furthermore, knowing each lipoprotein differs in its lipid composition, it is

assumed that the absorbance spectrum reflects the proportions of the lipoproteins and hence the overall state of health of the individual.

$$r = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum (y - \bar{y})^2} \sqrt{\sum (x - \bar{x})^2}} \qquad \text{Eq. 5-1}$$

Figure 5-190 is a cross-correlation matrix plot of the synthetic mixtures SM01, SM02 and SM06 and dyslipidemia Types I, IIA and V. In this matrix, the absorbance units at a given wavelength in the spectrum of each SM are one of the variables, say X, while the absorbance units at corresponding wavelengths in the spectrum of SDSs are the other variable, Y. (Ellipsoids of the density estimation fitting the bivariate distribution of points are shown to emphasize the degree of linearity.) It is easily seen that the strongest correlations or the most linear relationships exist between SM01 and SDS-I (r=0.9991); SM02 and SDS-IIA (r=0.9992); and SM06 and SDS-V (r=0.9986). Comparing the composition of SM01 in Table 5-16 with the composition of SDS-I in Table 5-8, it can be seen that the [TC], [TG], and [PL] are all greater in SM01 (85,>1000, and 89mg/dL, respectively), however each mixture still falls into the category of a Type I dyslipidemia. Similar comparisons can be made for the other two pairs of spectra. In each case, the overall compositions were vastly different, yet the absorbance spectra for each pair was strongly correlated because each pair fell into the same dyslipidemia category.

Figure 5-191 is another cross-correlation matrix plot. Here the absorbance spectra of the synthetic mixtures SM01, SM02 and SM06 and dyslipidemia Types I, IIA and V are plotted against each other. In Figure 5-191, it was shown that the strongest correlations exist between SM03 and SDS-IIB (r=0.9941); SM04 and SDS-IIB (r=0.9954); and SM05 and SDS-IV (r=0.9851). The pairs (SM03, SDS-IIB) and (SM05, SDS-IV) correctly predict the synthetic mixtures to be dyslipidemic sera Types IIB and IV, respectively. However, in the case of the (SM04, SDS-IIB) pair, an incorrect correlation to disease state was made. The synthetic mixture SM04 was made up in proportions reflecting a Type III dyslipidemia, but the strongest correlation was made with the Type IIB SDS. It should be noted however that strong correlations were also made to SDS Types III and IV.

Figure 5-192 is a cross-correlation plot matrix of "normal" sera and two synthetic mixtures. The two synthetic mixtures, SM07 and SM08, were made up to reflect the highest and the lowest blood lipid levels considered to be normal. There are several remarkable features in this matrix. First, it was shown that all the normal sera had very strong correlations (0.9876 or better) with each other despite widely varying lipid concentrations. Second, as can be seen in Table 5-1, RML reported values of lipid levels in Serums 01-06 were all closer to the high end of the normal range than the low end. For each of the sera, the correlation coefficient matrix clearly shows stronger correlations with the high-end SM07 mixture. Serum 05 had the lowest reported lipid values in almost every category and, of the six sera, clearly showed the strongest correlation to the low-end SM08 mixture. These patterns suggest that the current method may indeed be useful in identifying and discriminating between dyslipidemic disease states. What is more, these patterns indicate that it may be possible to follow the progress of an individual from one state of health to another. Overall it was clearly shown that several types of dyslipidemias may indeed be identified using this technique of spectral analysis.

Table 5-16. Total Lipid Composition of Synthetic Mixtures 01-08 (SM01-08)								
Synthetic Mixture #	01	02	03	04	05	06	07	08
[TC] (mg/dL)	435.27	320.64	442.79	545.07	319.79	509.86	297.45	138.71
[HDL-C] (mg/dL)	25.13	23.34	21.74	15.29	26.29	31.74	70.19	30.99
[LDL-C] (mg/dL)	26.54	264.13	246.00	43.26	72.12	136.83	175.29	60.50
[IDL-C] (mg/dL)	3.97	7.37	6.86	452.72	7.55	7.16	3.82	17.17
[VLDL-C] (mg/dL)	55.56	25.80	168.19	33.80	213.84	125.30	48.15	30.04
[Chylomicron-C] (mg/dL)	324.07	0	0	0	0	208.83	0	0
[TG] (mg/dL)	4046.47	144.96	505.72	553.12	872.91	2875.34	183.57	114.80
[PL] (mg/dL)	436.02	226.04	354.69	459.76	264.74	469.61	260.76	126.61

Table 5-17. Lipid Composition by Lipoprotein Class in Synthetic Mixture 01 (SM01)				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	0.79	1.72	3.70	0.40
LDL	0.56	2.10	1.60	0.68
IDL	0.11	0.29	0.33	0.4
VLDL	1.85	3.70	5.56	14.29
Chylomicron	9.26	23.15	32.41	388.89
TOTAL	12.57	30.96	46.58	404.66

Table 5-18. Lipid Composition by Lipoprotein Class in Synthetic Mixture 02 (SM02)				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	0.74	1.60	3.44	0.37
LDL	5.53	20.88	15.97	6.76
IDL	0.20	0.54	0.61	0.74
VLDL	0.86	1.72	2.58	6.63
Chylomicron	0	0	0	0
TOTAL	7.33	24.74	22.60	14.50

Table 5-19. Lipid Composition by Lipoprotein Class in Synthetic Mixture 03 (SM03)				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	0.69	1.49	3.20	0.34
LDL	5.15	19.45	14.87	6.29
IDL	0.18	0.50	0.57	0.69
VLDL	5.61	11.21	16.82	43.25
Chylomicron	0	0	0	0
TOTAL	11.63	32.65	35.46	50.57

Table 5-20. Lipid Composition by Lipoprotein Class in Synthetic Mixture 04 (SM04)				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	0.48	1.05	2.25	0.24
LDL	0.91	3.42	2.62	1.11
IDL	12.07	33.20	37.73	45.27
VLDL	1.13	2.25	3.38	8.69
Chylomicron	0	0	0	0
TOTAL	14.59	39.92	45.98	55.31

Table 5-21. Lipid Composition by Lipoprotein Class in Synthetic Mixture 05 (SM05)				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	0.83	1.80	3.87	0.42
LDL	1.51	5.70	4.36	1.84
IDL	0.20	0.55	0.63	0.75
VLDL	6.29	15.09	17.61	84.28
Chylomicron	0	0	0	0
TOTAL	8.83	23.14	26.47	87.29

Table 5-22. Lipid Composition by Lipoprotein Class in Synthetic Mixture 06 (SM06)				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	1.00	2.17	4.68	0.50
LDL	2.86	10.82	8.27	3.50
IDL	0.19	0.53	0.60	0.72
VLDL	4.18	8.35	12.53	32.22
Chylomicron	5.97	14.92	20.88	250.60
TOTAL	14.20	36.79	46.96	287.54

Table 5-23. Lipid Composition by Lipoprotein Class in Synthetic Mixture 07 (SM07)				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	2.22	4.80	10.34	1.11
LDL	3.67	13.86	10.60	4.48
IDL	0.10	0.28	0.32	0.38
VLDL	1.61	3.21	4.82	12.38
Chylomicron	0	0	0	0
TOTAL	7.6	22.15	26.08	18.35

Table 5-24. Lipid Composition by Lipoprotein Class in Synthetic Mixture 08 (SM08)				
Standard Added	unesterified cholesterol (mg)	esterified cholesterol (mg)	PL (mg)	TG (mg)
HDL	0.98	2.12	4.57	0.49
LDL	1.27	4.78	3.66	1.55
IDL	0.46	1.26	1.43	1.72
VLDL	1.00	2.00	3.00	7.73
Chylomicron	0	0	0	0
TOTAL	3.71	10.16	12.66	11.49

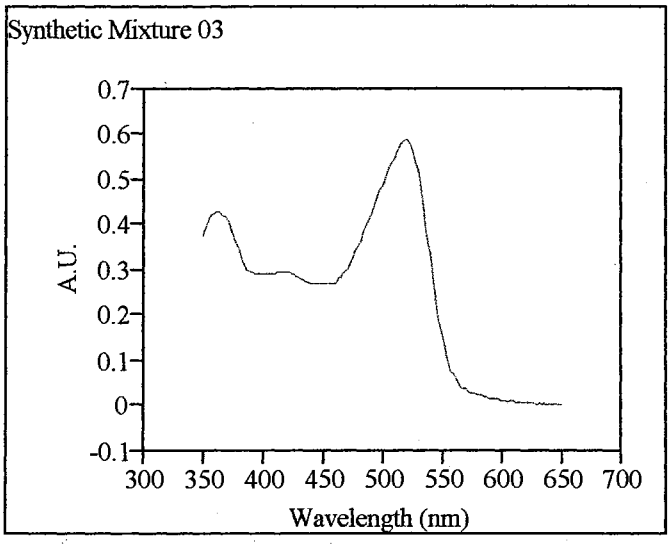


Figure 5-184. Absorption spectrum of the colored products of the reaction of the standard reagent with synthetic mixture 03 after 20 minutes.

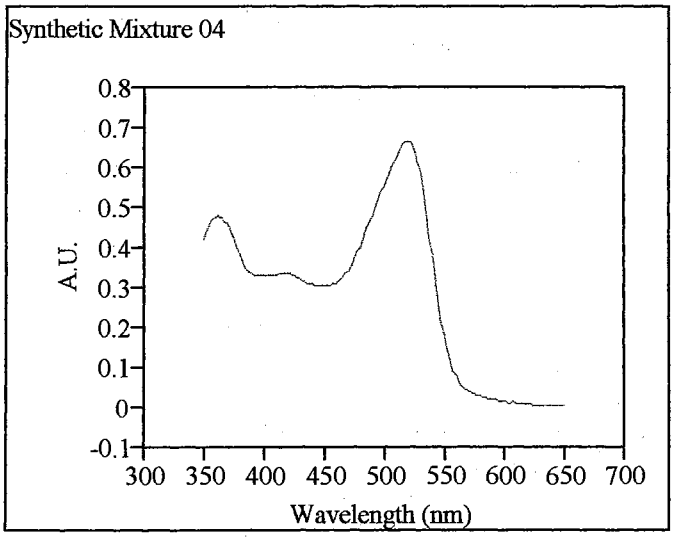


Figure 5-185. Absorption spectrum of the colored products of the reaction of the standard reagent with synthetic mixture 04 after 20 minutes.

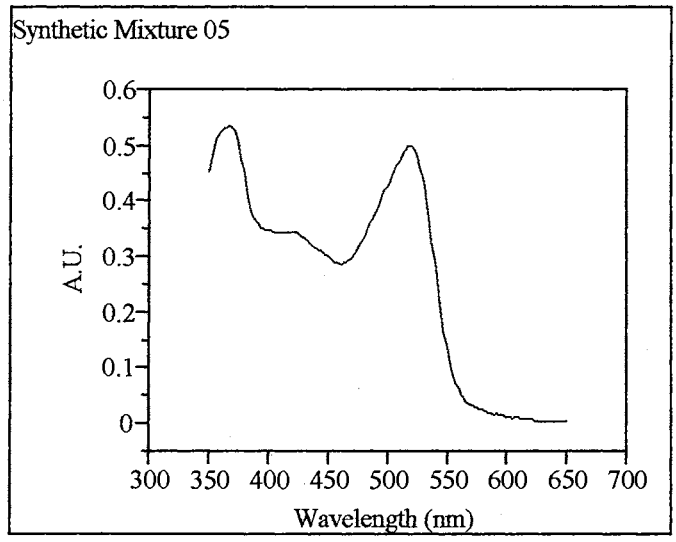


Figure 5-186. Absorption spectrum of the colored products of the reaction of the standard reagent with synthetic mixture 05 after 20 minutes.

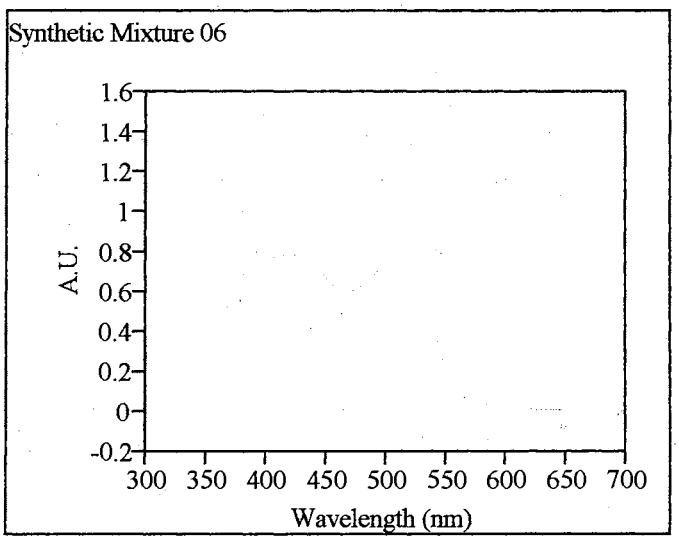


Figure 5-187. Absorption spectrum of the colored products of the reaction of the standard reagent with synthetic mixture 06 after 20 minutes.

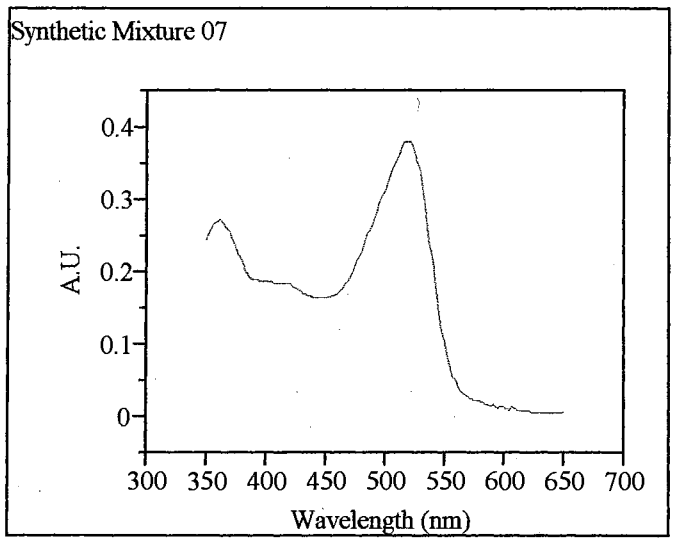


Figure 5-188. Absorption spectrum of the colored products of the reaction of the standard reagent with synthetic mixture 07 after 20 minutes.

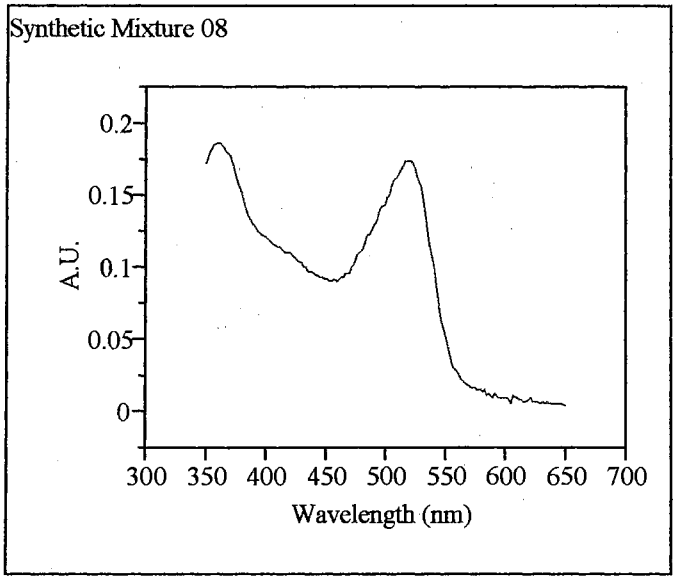


Figure 5-189. Absorption spectrum of the colored products of the reaction of the standard reagent with synthetic mixture 08 after 20 minutes.

	SDS-I	SDS-IIA	SDS-V	SDS-VI	SM01	SM02	SM06
SDS-I	1.0000	0.6986	0.9770	0.6418	0.9991	0.7154	0.9861
SDS-IIA	0.6986	1.0000	0.8343	0.9961	0.6720	0.9992	0.8077
SDS-V	0.9770	0.8343	1.0000	0.7902	0.9680	0.8466	0.9986
SDS-VI	0.6418	0.9961	0.7902	1.0000	0.6125	0.9934	0.7600
SM01	0.9991	0.6720	0.9680	0.6125	1.0000	0.6896	0.9792
SM02	0.7154	0.9992	0.8466	0.9934	0.6896	1.0000	0.8215
SM06	0.9861	0.8077	0.9986	0.7600	0.9792	0.8215	1.0000

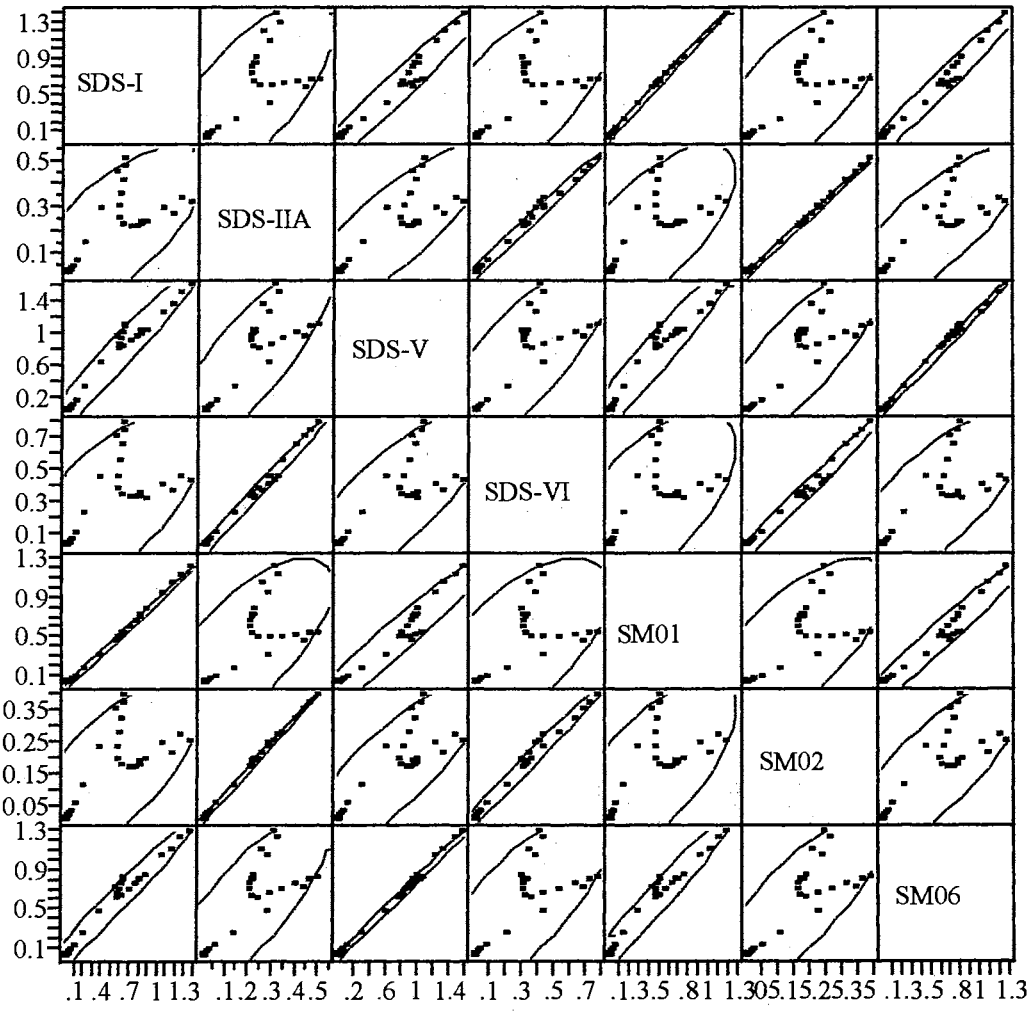


Figure 5-190. Cross-correlation plot matrix of synthetic mixtures SM01, SM02 and SM06 and synthetic dyslipidemia Types I, IIA and V. Above the plot matrix are the Pearson-product correlation coefficients for each plot.

	SDS-IIB	SDS-III	SDS-IV	SM03	SM04	SM05
SDS-IIB	1.0000	0.9993	0.9748	0.9941	0.9954	0.9228
SDS-III	0.9993	1.0000	0.9670	0.9897	0.9915	0.9094
SDS-IV	0.9748	0.9670	1.0000	0.9927	0.9912	0.9851
SM03	0.9941	0.9897	0.9927	1.0000	0.9999	0.9587
SM04	0.9954	0.9915	0.9912	0.9999	1.0000	0.9551
SM05	0.9228	0.9094	0.9851	0.9587	0.9551	1.0000

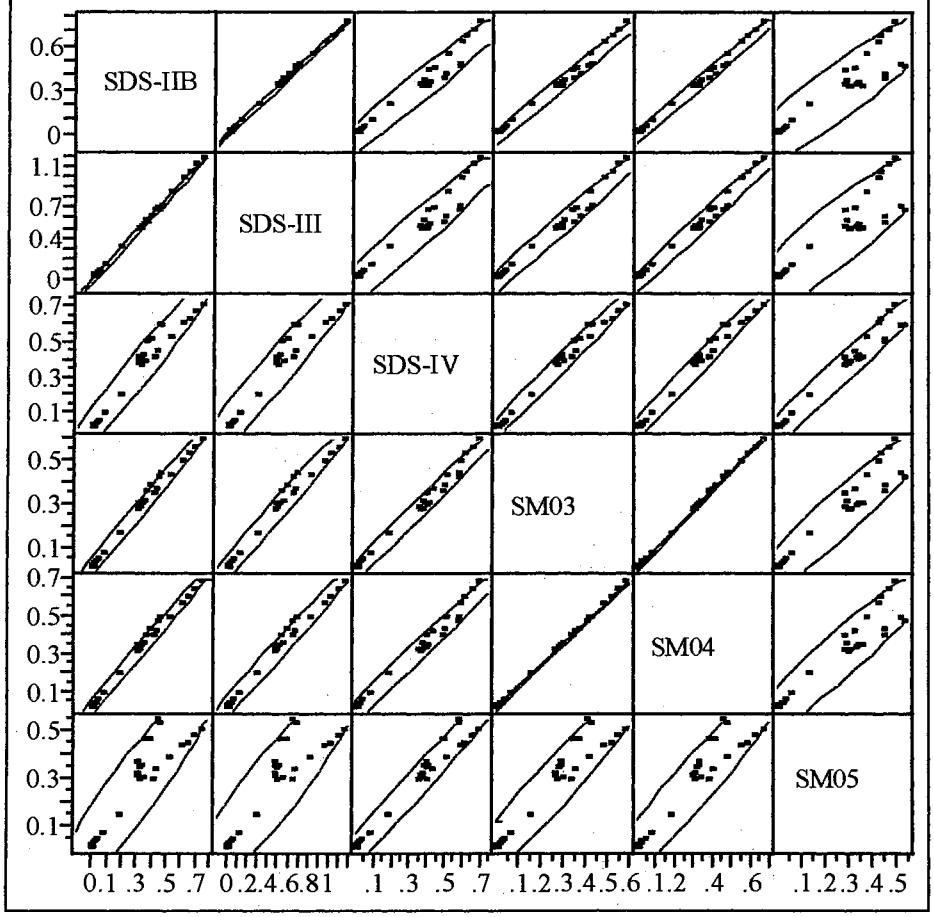


Figure 5-191. Cross-correlation plot matrix of synthetic mixtures SM03, SM04 and SM05 and synthetic dyslipidemia Types IIB, III and IV. Above the plot matrix are the Pearson-product correlation coefficients for each plot.

	Serum 06	Serum 05	Serum 04	Serum 03	Serum 02	Serum 01	SM07	SM08
Serum 06	1.0000	0.9946	0.9992	0.9995	0.9984	0.9982	0.9753	0.8646
Serum 05	0.9946	1.0000	0.9959	0.9932	0.9878	0.9876	0.9928	0.9115
Serum 04	0.9992	0.9959	1.0000	0.9995	0.9974	0.9975	0.9796	0.8766
Serum 03	0.9995	0.9932	0.9995	1.0000	0.9990	0.9990	0.9738	0.8625
Serum 02	0.9984	0.9878	0.9974	0.9990	1.0000	0.9999	0.9633	0.8402
Serum 01	0.9982	0.9876	0.9975	0.9990	0.9999	1.0000	0.9631	0.8409
SM07	0.9753	0.9928	0.9796	0.9738	0.9633	0.9631	1.0000	0.9532
SM08	0.8646	0.9115	0.8766	0.8625	0.8402	0.8409	0.9532	1.0000

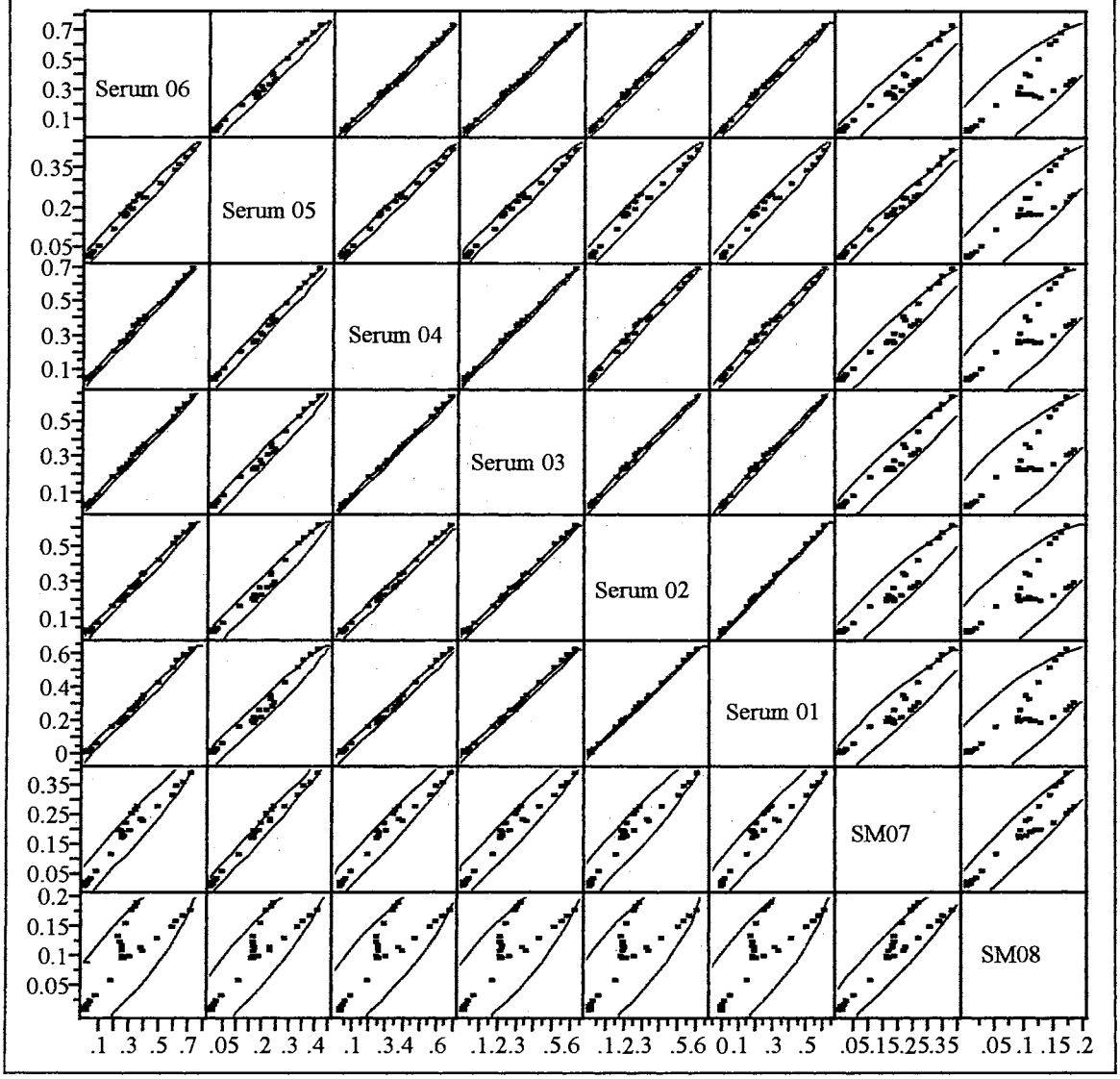


Figure 5-192. Cross-correlation plot matrix of synthetic mixtures SM07, SM08 and Serums 01-06. Above the plot matrix are the Pearson-product correlation coefficients for each plot.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The purpose of this research was to develop a new method of identifying various blood lipid disorders (dyslipidemias). Traditionally, dyslipidemias have been identified by quantifying individual serum lipoprotein levels using a combination of enzymatic, precipitation and ultracentrifugation techniques. These routine methods have a number of inherent flaws rendering them useless in a variety of situations. The present study abandons the traditional approach in favor of a more revolutionary direct chemical method. Three primary goals were identified: 1) refine a previously proposed reagent system and technique to identify as many lipid classes as possible; 2) systematically identify which lipid classes chromogenically react with this reagent system; and 3) use the information gained in the first two goals to develop a method for identifying various serum dyslipidemias.

The evidence presented here suggests that it may be possible to simultaneously identify and measure multiple blood serum components in a single assay. It was demonstrated that the major lipid components of serum, i.e. cholesterol and its esters, unsaturated fatty acids and triglycerides/ phospholipids with unsaturated fatty acid moieties, all react chromogenically with the SRS to produce unique absorbance and fluorescence spectra. It was further shown that using data from the current technique to analyze linear combinations of these lipids, it is possible, to a good degree, to reproduce the spectral characteristics of true human sera. This discovery was then leveraged against the fact that dyslipidemic patients were not available to provide the needed serum samples; therefore, synthetic dyslipidemic samples were created and analyzed. Seven synthetic dyslipidemic samples were prepared and analyzed to create a "library" of spectra with which other synthetic mixtures and true sera could be correlated.

Strong correlations were found to exist between true serum spectral data and data for synthetic mixtures made up to reflect the overall lipid composition of the true serums thus proving the validity of this approach. Other remarkable correlations were found between the spectra obtained by analyzing real sera. Serum samples were all taken from healthy individuals and, despite widely varying lipid concentrations, showed a high degree of linearity between them. This strong correlation suggests that it is the ratio of lipids, not the concentrations of the individual blood components that are responsible for the overall spectral pattern. The idea is further reinforced by a close examination of the synthetic mixtures. Synthetic mixtures representing serums of dyslipidemic patients were correlated with the library of spectra taken from other dyslipidemic mixtures. Again, strong correlations were found between mixtures of the same dyslipidemia phenotype, but having radically different lipid concentration levels. These preliminary observations suggest that the method may indeed be useful in the identification and diagnosis of various disease states, however, the robustness and efficacy of this technique can only be proved in clinical trials. It is anticipated that this study will form the foundation for future studies to develop more accurate lipid measurement techniques and a better understanding of the risk factors associated with cardiovascular disease.

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APPENDIX A

STANDARD REFERENCE MATERIALS USED IN THIS STUDY

Common Name(s)	IUPAC Nomenclature
25-hydroxycholecalciferol	9,10-Secocholesta-5,7,10(19)-triene-3b,25-diol
5-alpha-cholestan-3-one	5-alpha-cholestan-3-one
7-dehydrocholesterol	Cholesta-5,7-dien-3b-ol
arachidic acid	eicosanoic acid
arachidonic acid methyl acid	methyl arachidonate
behenic acid	docosanoic acid
beta-sitosterol	22,23-dihydrostimasterol
chenodeoxycholic acid	3a,7a-Dihydroxy-5b-cholan-24-oic acid
cholecalciferol (vitamin D3)	9,10-Secocholesta-5,7,10(19)-trien-3b-ol
cholesterol	Cholest-5-en-3beta-ol
cholesteryl laurate	5-cholesten-3beta-ol 3-dodecanoate
cholesteryl linoleate	5-cholesten-3beta-ol 3-linoleate
cholesteryl linolenate	5-cholesten-3beta-ol 3-linolenate
cholesteryl myristate	5-cholesten-3beta-ol 3-tetradecanoate
cholesteryl oleate	5-cholesten-3beta-ol 3-oleate
cholesteryl pelargonate	5-cholesten-3beta-ol 3-nonanoate
cholic acid	3a,7a,12a-Trihydroxy-5b-cholan-24-oic acid
docosahexaenoic acid methyl ester	cis-4,7,13,16,19-docosahexaenoic acid methyl ester
eicosapentaenoic acid methyl ester	cis-5,8,11,14,17-eicosapentaenoic acid methyl ester
ergocalciferol, vitamin D2	9,10-Secoergosta-5,7,10(19),22-tetra-en-3b-ol
ergosterol	Ergosta-5,7,22-trien-3b-ol
fish oil (from menhaden)	
gamma-linolenic acid methyl acid	6,9,12-octadecatrienoic acid methyl ester
L-alpha-phosphatidyl-L-serine (from soybean)	
L-alpha-phosphatidylcholine (type XVI-E from fresh egg yolk)	
L-alpha-phosphatidylcholine, dilinoleoyl	
L-alpha-phosphatidylcholine, distearoyl	
L-alpha-phosphatidylcholine,beta-arachidonoyl-gamma-stearoyl	
L-alpha-phosphatidylcholine,beta-linoleoyl-gamma-stearoyl	
L-alpha-phosphatidylcholine,beta-oleoyl-gamma-stearoyl	
L-alpha-phosphatidylcholine,beta-stearoyl-gamma-oleoyl	
L-alpha-phosphatidylethanolamine,beta-linoleoyl-gamma-palmitoyl	
L-alpha-phosphatidylinositol sodium salt	

(from soybean)	
lauric acid	Dodecanoic acid
lignoceric acid	tetracosanoic acid
linoleic acid	9,12-Octadecadienoic acid
linoleic acid methyl ester	methyl linoleate
linolelaidic acid methyl ester	methyl linolelaidate
linolenic acid	9,12,15-octadecatrienoic acid
linolenic acid methyl ester	methyl linolenate
myristic acid	Tetradecanoic acid
oleic acid	9-Octadecenoic acid
oleic acid methyl ester	methyl oleate
olive oil	
palmitic acid	Hexadecanoic
palmitic acid methyl ester	methyl palmitate
palmitoleic acid	9-Hexadecenoic acid
progesterone	Pregn-4-ene-3,20-dione
safflower seed oil	
sphingomyelin	
stearic acid	Octadecanoic acid
stearic acid methyl ester	methyl stearate
stigmasterol	3beta-hydroxy-24-ethyl-5,22-cholestadiene
sunflower seed oil	
testosterone	17b-Hydroxyandrost-4-en-3-one
trilinolein	
trilinolenin	
triolein	1,2,3-Tri[cis-9-octadecenoyl]glycerol
vitamin A	retinoic acid
vitamin E	(+)-alpha-tocopherol
vitamin K1	2-methyl-3phytyl-1,4-naptho-quinone

APPENDIX B

STANDARD REFERENCE MATERIALS FOUND TO BE CHROMOGENIC

Common Name(s)	IUPAC Nomenclature
25-hydroxycholecalciferol	9,10-Secocholesta-5,7,10(19)-triene-3b,25-diol
7-dehydrocholesterol	Cholesta-5,7-dien-3b-ol
arachidonic acid methyl acid	methyl arachidonate
beta-sitosterol	22,23-dihydrostimasterol
cholecalciferol (vitamin D3)	9,10-Secocholesta-5,7,10(19)-trien-3b-ol
cholesterol	Cholest-5-en-3beta-ol
cholesteryl laurate	5-cholesten-3beta-ol 3-dodecanoate
cholesteryl linoleate	5-cholesten-3beta-ol 3-linoleate
cholesteryl linolenate	5-cholesten-3beta-ol 3-linolenate
cholesteryl myristate	5-cholesten-3beta-ol 3-tetradecanoate
cholesteryl oleate	5-cholesten-3beta-ol 3-oleate
cholesteryl pelargonate	5-cholesten-3beta-ol 3-nonanoate
docosahexaenoic acid methyl ester	cis-4,7,13,16,19-docosahexaenoic acid methyl ester
eicosapentaenoic acid methyl ester	cis-5,8,11,14,17-eicosapentaenoic acid methyl ester
fish oil (from menhaden)	
gamma-linolenic acid methyl acid	6,9,12-octadecatrienoic acid methyl ester
L-alpha-phosphatidyl-L-serine (from soybean)	
L-alpha-phosphatidylcholine (type XVI-E from fresh egg yolk)	
L-alpha-phosphatidylcholine, dilinoleoyl	
L-alpha-phosphatidylcholine,beta-arachidonoyl-gamma-stearoyl	
L-alpha-phosphatidylcholine,beta-linoleoyl-gamma-stearoyl	
L-alpha-phosphatidylcholine,beta-oleoyl-gamma-stearoyl	
L-alpha-phosphatidylcholine,beta-stearoyl-gamma-oleoyl	
L-alpha-phosphatidylethanolamine,beta-linoleoyl-gamma-palmitoyl	
L-alpha-phosphatidylinositol sodium salt (from soybean)	
linoleic acid	9,12-Octadecadienoic acid
linoleic acid methyl ester	methyl linoleate
linolelaidic acid methyl ester	methyl linolelaidate
linolenic acid	9,12,15-octadecatrienoic acid
linolenic acid methyl ester	methyl linolenate
oleic acid	9-Octadecenoic acid
oleic acid methyl ester	methyl oleate

olive oil	
safflower seed oil	
stigmasterol	3beta-hydroxy-24-ethyl-5,22-cholestadiene
sunflower seed oil	
trilinolein	
trilinolenin	
triolein	1,2,3-Tri[cis-9-octadecenoyl]glycerol
vitamin A	retinoic acid
vitamin K1	2-methyl-3phytyl-1,4-naptho-quinone

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_____

8

VITA

Justin Ashley Krouse

Candidate for the Degree of

Doctor of Philosophy

Thesis: USING DIRECT SERUM LIPOPROTEIN SUBFRACTION ANALYSIS TO IDENTIFY VARIOUS DYSLIPIDEMIAS

Major Field: Chemistry

Biographical:

Personal Data: Born in Shreveport, Louisiana, on February 8, 1972.

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