CHARACTERIZATION OF A NOVEL CHROMOGENIC REACTION FOR ANALYSIS OF UNSATURATED LIPIDS AND APPLICATION TO HUMAN SERUM LIPIDS ANALYSIS

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Thesis Approved:

Thesis Advisor

Dean of Graduate College

DEDICATION

To my parents, Robert and LaVon Studer

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Finally, on a personal note, I am deeply grateful to the Lord, my father, and my family- my parents, LaVon and Robert Studer, and my sister ShaJuan (Swani) Beertema-Studer and her family. I would have not been able to finish without their unconditional love, patience, support and continuous encouragement.

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List of Symbols and Abbreviations

AC	Acetyl Chloride
Apo(a)	Apolipoprotein(a)
ATP	Adult Treatment Panel
BQ	Beta Quantification
CAD	Coronary Artery Disease
CDC	Centers for Disease Control
CFH	Combined Familial Hyperlipidemia
CHD	Coronary Heart Disease
CVD	Coronary Vascular Disease
CM	Chylomicron
DCE	Dichloroethane
DHA	Docosahexaenoic Acid
EPA	Eicosapentaenoic Acid
GC	Gas Chromatography
GP	glycerol-3-phosphate
HDL	High-density Lipoprotein
HDL-C	High-density Lipoprotein Cholesterol
HPLC	High Performance Liquid Chromatography
IDL	Intermediate-density Lipoprotein

IDL-C	Intermediate-density Lipoprotein Cholesterol
L-B	Liebermann-Burchard
LCAT	Lecithin-Cholesterol Acyl Transferase
LDL	Low-density Lipoprotein
LDL-C	Low-density Lipoprotein Cholesterol
Lp(a)	Lipoprotein(a)
LPL	Lipoprotein Lipase
MDA	Malondialdehyde
MLR	Multi-linear Regression
NCEP	National Cholesterol Education Program
NIH	National Institutes of Health
PA	Perchloric Acid
PUFA	Polyunsaturated Fatty Acids
ROS	Reactive Oxygen Species
TBARS	Thiobarbituric Acid Reactive Species
TC	Total Cholesterol
TG	Triglyceride
TLC	Thin Layer Chromatography
VLDL	Very-low-density Lipoprotein
VLDL-C	Very-low-density Lipoprotein Cholesterol

STATEMENT OF PROBLEM

This work focuses on the development of selectivity information and applied methods for the use of a novel chromogenic reaction based on the Chugaev reaction. The emphasis is placed on quantitative comparisons of reactions with various analytes and quantitation of analytes related to the determination of unsaturated lipids in sera.

The use of color forming reactions with organic molecules has been an important part of analytical and medical science. These reactions have allowed the analysis of a variety of compounds found in living systems. Modern colorimetric reactions for the analysis of biological analytes often involve enzymatically-catalyzed reactions. The accuracy of chemical methods, however, often make them the standard methods of analysis by which the enzymatic systems are evaluated.

The reaction used in this study was first disclosed in 1995.[1] It consists of a reaction conducted at ambient temperatures that is known to be chromogenic with a variety of analytes. The reaction was further characterized to be reactive with polyunsaturated fatty acids (PUFA).[2] At that time, the working hypothesis was that the reagent reacted with a variety of unsaturated carbon systems.

The reaction was shown at the time of its disclosure to be quantitative for cholesterol. The ability to quantitate cholesterol is a characteristic of a variety of chemical reactions. The most common chemical reaction for the quantification of cholesterol is the Liebermann-Burchard (L-B) reaction. It was of interest to determine the relationship between the current chromogenic assay and the L-B reaction to understand the differences and benefits of the current assay.

Chapter I addresses the question of what spectral response and what potential this reaction has to selectively quantitate alternative double bond containing compounds. The analytical potential for this reagent in the analysis of various compounds relies on understanding these reaction properties. A variety of analytes was tested to this end primarily in the steroid, terpene, and PUFA categories. Also tested were small unsaturated organic compounds. All of the reactions were conducted at stoichiometric equivalence (where possible) in order to quantitatively compare the spectral response of the analytes. The relationship between the chemical reaction presented with the L-B reaction was considered by conducting chemical reactions with identical analytes using both reagents.

In Chapter II, the use of the chemical assay to simultaneously determine concentrations of analytes in mixtures using multivariate analysis is explored. Synthetic mixtures were prepared as models to allow the development and further refinement of methods to the goal of a routine, reliable assay not requiring separations or other sample pretreatments. Combinations of PUFA and cholesterol mimicking the proportions found in human sera were made and analyzed using multi-linear regression (MLR) in order to test the feasibility of applying multivariate analysis on real samples.

Also considered in Chapter II is the ultimate goal for the chemical assay to be used in the routine clinical analysis of patients' sera. It is believed that the assay's chemical stability, low cost per assay and ability to determine alternative PUFA information can position the reaction as a viable alternative routine assay in the future.

A literature review is presented outlining the current routine clinical analysis methods. Also demonstrated is the dynamic nature of the lipids analysis field and

discussion of some developing analytical methods is made. Finally, a summary of the information in the literature on the importance of PUFA profiles in human disease demonstrates the potential importance of this new assay to the clinical physician. Special emphasis is placed on the quantification of PUFA profiles and the determination of conjugated dienes as an indicator of oxidative stress.

Finally in Chapter II, the use of the chemical reagent in clinical settings was demonstrated when a dyslipidemic patient was monitored over the course of a dietary intervention. This study demonstrated the utility of the reagent in its current (relatively undeveloped) form. A direct comparison between the current standard methods used in routine clinical analysis and the chromogenic reaction was made when the multivariate method developed for the analysis of synthetic mixtures was used for the analysis of the case study sera.

The results presented in this work add to the developing information about this convenient, low cost, robust chemical assay. It adds to the pure and applied scientific knowledge needed to bring the assay to application.

CHAPTER I

CHROMOGENIC REACTIONS OF MODEL COMPOUNDS

One of the oldest and most commonly used color forming reactions is the L-B reaction. The Liebermann reaction was first used in the 19th century,[3] and was later applied to cholesterol by Burchard, thus becoming known as the L-B reaction.[4] The L-B reaction uses sulfuric acid with acetic anhydride in the presence of acetic acid. Another commonly used reaction for the determination of cholesterol is the Zac reaction.[5] Similarly, the Zac reaction takes advantage of the same acetic acid and sulfuric acid solutions, while utilizing ferric ions (Fe^{3+}) in place of the acetic anhydride. The Fe^{3+} is required for the reaction to occur.[6] An alternative chromogenic reaction is the Chugaev reaction. In the Chugaev reaction, the acetic anhydride is replaced by acetyl chloride while the other component of the reaction mixture is zinc chloride. Unlike the L-B reaction, the Chugaev reaction requires heating to facilitate the chromophore production. Studies on the mechanisms of strong acid cholesterol reactions such as the L-B and Zac reactions usually mention the Chugaev reaction. The Chugaev reaction actually seems to be a unique reaction that can, in a modified form, react in selective ways with unique analytes at room temperature.

The mechanism of the strong acid cholesterol reactions has been studied for some time. The area is practically dormant currently as strong evidence has led to mechanisms, which are adequate for the current applications. When strong acids such as perchloric or hexafluorophosphoric acid are added to an organic solution of cholesterol in equivalent stoichiometric amounts a "colorless sterolium salt" forms. If the acid is added in excess

the "colorless sterolium salts" form a purple colored solution of "salts of cholestadiene".[7] It was eventually hypothesized that this acid reaction involves cholesterol undergoing dehydration to form 3,5-cholestadiene and 2,4-cholestadiene that then forms the dimers (1) 3-3'-bis-3,5-cholestadiene and (2) 3,3'-bis-2,4-cholestadiene.[8] Other studies substantiated this hypothesis, and the L-B reaction was believed to also involve this dehydration of cholesterol followed by the formation of dimers.[9, 10] Eventually the dimer hypothesis was proven with the isolation of 3,3'-bis-3,5-cholestadiene.[8] The 2,4 dimer (2) was found in the reaction mixtures of the L-B, Zac and Chugaev reactions.[8, 11, 12] The presence of the dimer(s) does not preclude other significant product formation.

Later research proposed the formation of an extended conjugated system within the ring structure of cholesterol, rather than the dimer, as the primary absorbing product.[13] Much later the L-B reaction product was substantiated as an extended conjugated ring structure by Burke.[6] Burke monitored the production of sulfur dioxide during the L-B reaction to calculate the chromophore structure produced. Dulou also measured sulfur dioxide production in the L-B reaction and found results in agreement with Burke's conclusions.[6, 9, 14]

Along with the controversial nature of the area of strong acid cholesterol color reaction mechanisms, later reports supported the dimer hypothesis. The dimer formation reaction requires the presence of a species to accept the freed hydrogen. In the case of the TCA-HCl (1:1) reaction, the absence of oxygen prevented the dehydration from occurring,[15] therefore preventing the production of the dimer. The reaction does produce 3,5-cholestadiene, cholesteryl chloride, and cholesteryl trichloroacetate. The absorbance spectrum is missing the 560 nm peak that is said to be the dimer.[16] It would be logical to

consider that the dimer would form if a "hydrogen acceptor" were in solution. The authors used oxygen and hydrogen peroxide as the hydrogen acceptor and found the dimer forms with oxygen. The structure hypothesized by Burke.[6] was not isolated in this reaction.[15]

While the mechanism and active product of the strong acid reactions are not fully understood, the activity and analytical utility of these reagents are well known. The L-B reaction is currently the Center for Disease Control (CDC) standard chemical cholesterol determination method. Throughout development of the modern enzymatic cholesterol determination methods, the new systems have been compared to the Abell-Kendall method. The Abell-Kendall method is the CDC standard, which uses transesterification lipid isolation of the cholesterol from human plasma samples and the L-B reaction for quantification.[17] The L-B reaction is also routinely used in modern research laboratory analysis and method development.[18]

Introduction to Model Compounds

The model compounds are composed of three major classes: fatty acids, steroids, and terpenes. Each will be considered as independent groups and finally cross class correlations will be summarized.

Steroids

The steroid compounds are all based on the cholesterol ring template and are structurally unique. The analysis of steroids is a widely used process by industries including agriculture, medicine, forensic, and nutritional sciences.[19-24] Many of the steroids considered are physiologically relevant to mammals.

The nomenclature used in the description of steroids is specialized. It uses a specific carbon numbering system and ring designation to define the structural features. The cholestane skeleton numbering system is as follows: (Figure 1)

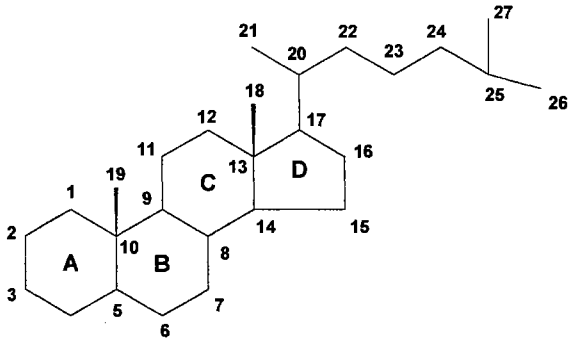

Figure 1. Steroid carbon numbering and ring designations.

There are 26 steroids in this study. They include cholesterol and its derivatives including cholesteryl chloride. Cholesterol (Figure 2(A)(R=H)) has a hydroxide on carbon 3 and an unsaturation at carbon 5 while cholesteryl chloride (Figure 2(A)(Cl on carbon 3)) has a chloride at carbon 3 and the double bond at carbon 5. There were ester derivatives of cholesterol including cholesteryl oleate, cholesteryl nonanoate, cholesteryl myristate, cholesteryl acetate, cholesteryl linolenate, and cholesteryl linoleate. These compounds have an additional carbon chain attached as an ester of the hydroxide on carbon 3 (Figure 2(A)(R=COR))

Other steroids tested are stigmasterol (Figure 2(B)), dihydrocholesterol (Figure 2(C)), 7-dehydrocholesterol (Figure 2(D)), alpha-estradiol (Figure 2(E)), beta-estradiol (Figure 2(E)), ergosterol (Figure 2(F)), stanozolol (Figure 2(G)), norethynadrel (Figure 2(I)), diosgenin (Figure 2(J)), testosterone (Figure 2(K)), 17-alpha-methyl testosterone (Figure 2(L)), 4-cholesten-3-one (Figure 2(M)), 5-cholesten-3-one (Figure 2(N)), 5-

cholesten-3-beta-ol-7-one (Figure 2(O)), progesterone (Figure 2(P)), prednisone (Figure 2(Q)), hydrocortisone (Figure 2(R)) and prednosilone (Figure 2(S)).

Terpenes

The terpenes are polymers of the isoprene unit (CH_2=C(CH_3)-CH=CH_2). They are found mostly in plants. Some of them are important in human physiologically such as vitamin A1. The synthesis of terpenes commonly includes cyclization of the isoprene polymers to form commonly found ring structures. The terpenes, in fact, can be subdivided into those with linear structures and those with cyclic structures. They will be presented in this study as two separate groups. The linear terpenes will also be presented with other small organic model compounds that are not terpenes.

The small organic model compounds are 1,4-pentadiene, trans-1,3-pentadiene and cis-1,3-pentadiene (Figure 3) The linear terpenes are myrcene, squalene and farnesol (Figure 3). Limonene, S-(+)-carvone, alpha-pinene, beta-pinene and camphene comprise the cyclic terpenes (Figure 3)

Polyunsaturated Fatty Acids

Fatty acids in the context of this work are all methyl esters. The fatty acids have varying degree of unsaturation. The natural forms of fatty acid unsaturations are methylene bridged so that the unsaturated fatty acids can be represented as (-CH=CHCH_2-)$_n$ polymers at the double bond containing region. The degree of unsaturation is determined by both dietary and metabolic modification (see Chapter I). The location of the unsaturated region is also physiologically important (see Chapter I).

The nomenclature for lipids has many variations summarized in Table 1.

Table 1. Description of polyunsaturated fatty acid structures.

Name	Total Carbons	Double bonds	Double bond locations	Omega number
Oleate	18	1	9	9
Linoleate	18	2	9,12	6
Conjugated Linoleate	18	2	10,12	6
Linolenate	18	3	9,12,15	3
Arachidonate	20	4	5,8,11,14	6
Eicosapentaenoate	20	5	5,8,11,14,17	3
Docosahexaenoate	22	6	4,7,10,13,16,19	3

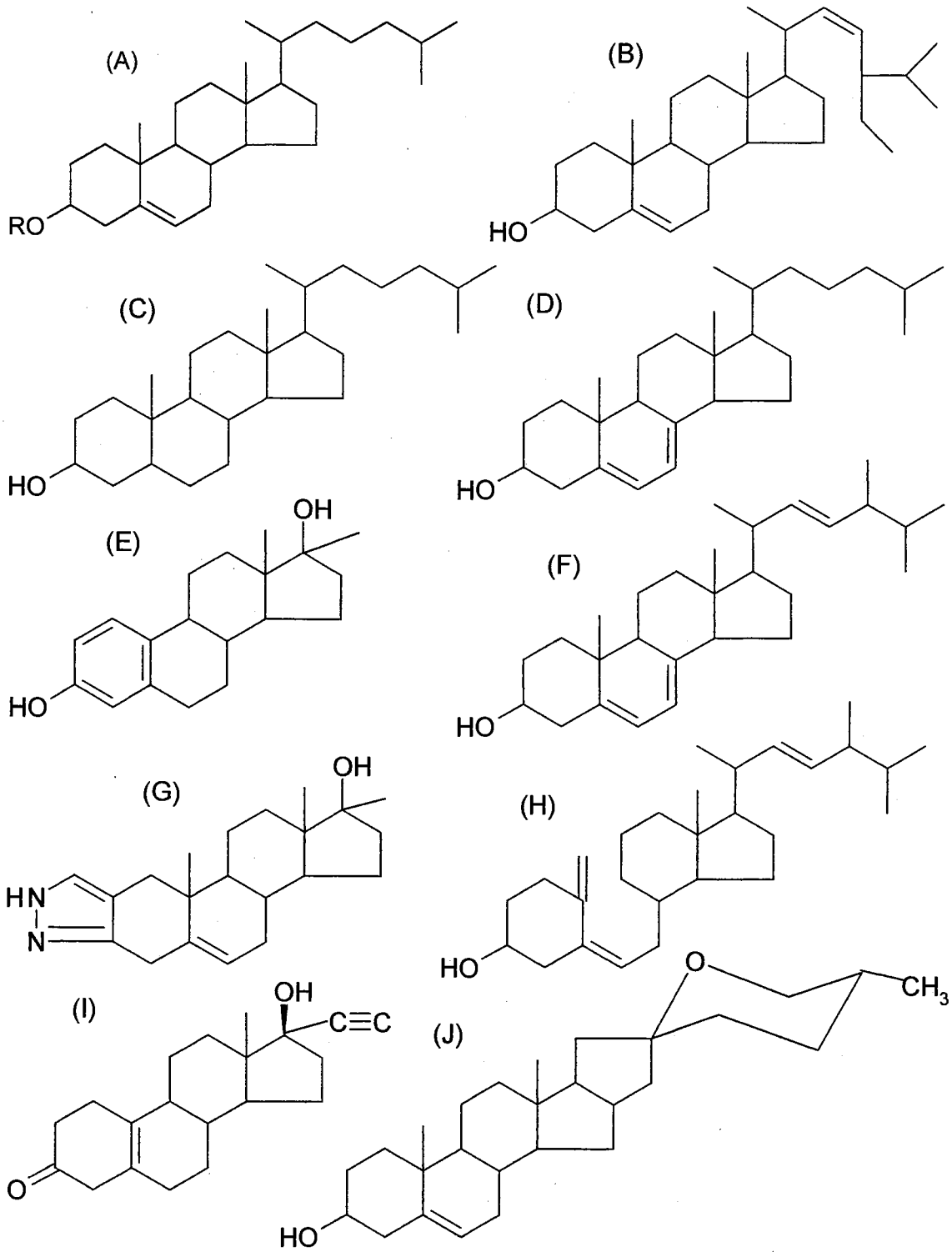

Figure 2. Steroid structures.

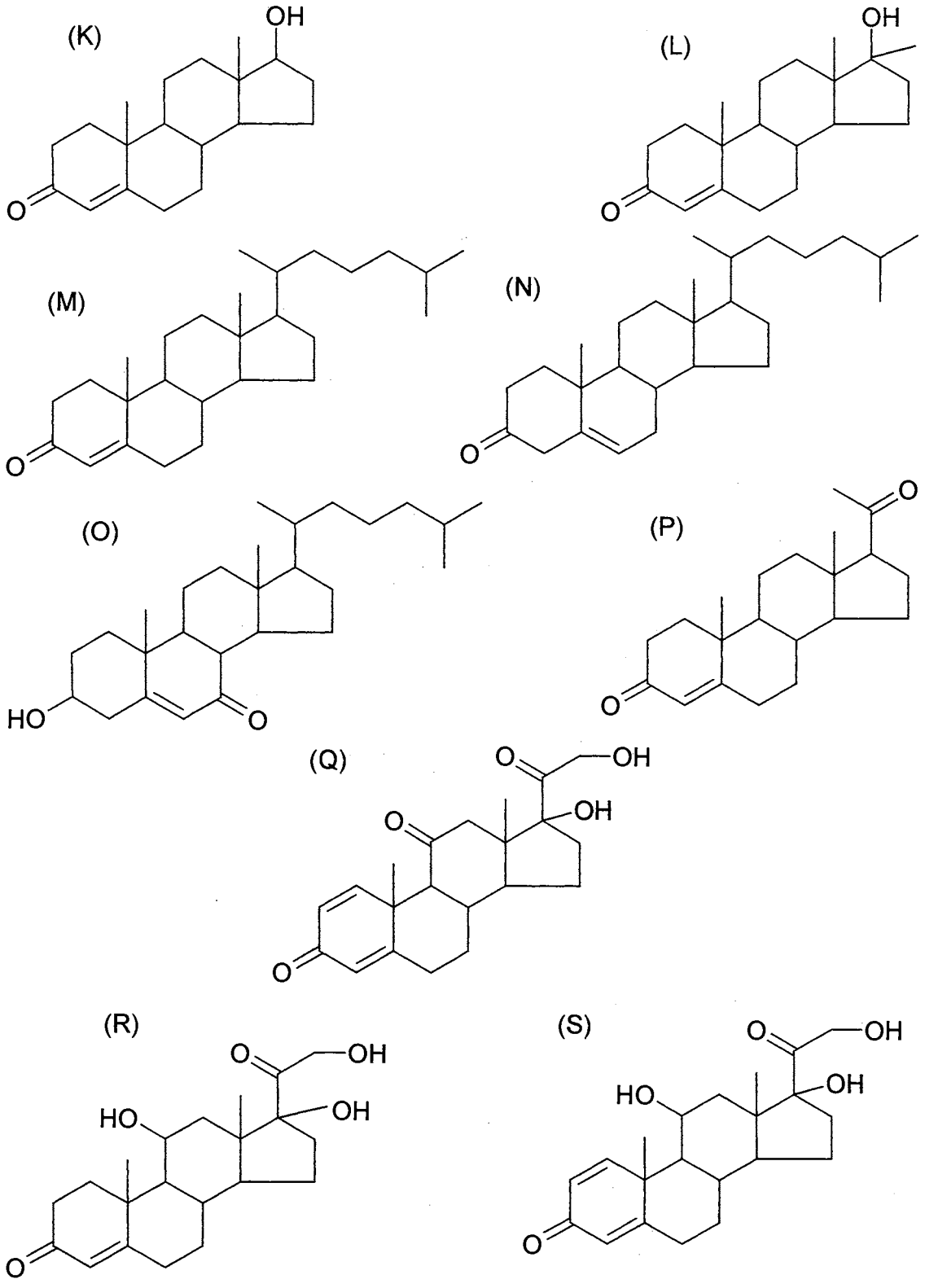

Figure 2. Steroid structures continued.

Experimental Methods

The experimental methods used in this study are based on the reaction of acetyl chloride (AC) with unsaturated compounds and a catalyst. The analytes include steroids, terpenes, fatty acids and small organic compounds. The reaction was discovered during investigation of Chugaev reaction for cholesterol.[25] The original Chugaev method was problematic due to a procedure that involves heating the analytes in AC with $ZnCl_2$. The modified method, presented here, involves the use of an alternative catalyst such as perchloric acid (PA), zinc acetate, or zinc perchlorate.[1] With these alternative catalysts the reaction occurs at ambient laboratory conditions. The reaction is also convenient, as the reaction is initiated by the addition of catalyst and analysis of complex samples such as human serum can be done without pretreatment. The reaction with serum results in the precipitation of protein upon the addition of catalyst. This may occur due to the acetylation of the proteins to less soluble forms. The resulting precipitate is easily removed by centrifugation or filtration. The absence of the requirement for pretreatment is commercially significant as sample analysis times are shortened.

This new reagent has been used for the determination of total cholesterol (TC) by measuring the absorbance at 520 nm. It has also been used for the determination of the high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and very low-density lipoprotein cholesterol (VLDL-C) concentrations by single spectra multivariate analysis.[1] The reactivity of unsaturated fatty acids was demonstrated in the early twenty first century.[2] This information was used in the theoretical recognition of dyslipidemias by spectral comparison.

The reagent is currently composed of 1.0 mL of 98% AC and 40 μL of 70% PA or 0.1 g solid catalyst. The most commonly used solid catalyst is zinc acetate. All reagents were from Sigma-Aldrich. The utilization of 40 μL of perchloric acid introduces 12 μL water into the AC. The reaction of AC with water produces HCl and acetic acid. This reaction occurs in the absence of catalyst. Zinc acetate is a dihydrate salt that is soluble in AC. The total water in 0.1 g of Zinc acetate is 1.6 times the water in 40 μL PA. This is problematic, as the reaction products with water are known to be inhibitory to the reaction. The advantage of zinc acetate is that it is a stable non-hygroscopic salt, which could be pre-weighed for automated high throughput analyses.

An earlier procedure for the acetylation involved the use of dichloroethane (DCE) in the AC as a diluent. The resulting reaction was milder than the reaction with pure AC. DCE, however, does not increase the selectivity or sensitivity of the reagent and it is toxic and difficult to remediate. DCE seems to have the effect of lowering the 520 nm peak. Therefore, the preferred reagent uses pure AC.

Reaction of Pure Compound with Perchloric Acid Catalyst

Analysis is conducted by adding 10 μL of analyte solution to the bottom of a 13 X 100 mm borosilicate disposable test tube. Pure AC (1.0 mL) is added to the analyte. The reaction is initiated when 40 μL PA is added to this mixture. The reaction timer is started immediately after addition of catalyst. The reaction is energetic thus caution should taken to slowly add the PA. The reaction is mixed by hand vortexing for 20 seconds and then either placed in a Teflon 10 mm pathlength cuvette for kinetic determination over 20 minutes or allowed to mature inside the test tube to later be analyzed at the 15-minute

endpoint. Following maturation in the test tube the solution is transferred to the 10 mm pathlength cuvette with a glass Pasteur pipette and full spectral data (350-800 nm) are collected.

Spectrophotometric Determination of Reaction

Absorbance data of the colored products after the reactions were determined on a HP8452a Hewlett Packard spectrophotometer. A 5 second integration time and a 2 nm resolution were used. The blank for each reaction was the reagent (AC) without catalyst. The reagent once mixed with catalyst produces some coloration in the absence of analyte over the 20-minute reaction period. Since the coloration was small, varied, and did not necessarily produce equal amounts with and without analyte, the blank did not include this contribution. For future analytical applications of this reagent, the aforementioned considerations must be addressed in a rigorous way and an appropriate compensating solution implemented.

Rate curves were calculated using standard software onboard the spectrophotometer. The data were collected from 350 to 800 nm for each spectral collection. The usual spectral range of importance for the analytes in this study was from 350 to 650 nm. There are 150 data points in the spectra from 350 to 650 nm. Absorbance data were automatically recorded every minute for 20 minutes. Any data between the addition of catalyst and completion of data preparation were lost at the beginning of the reaction. The resulting data were a 20 X 225 element matrix. It was found that the kinetics of the reaction demonstrates significant information about the pure

compounds, and may prove useful for the quantification of the known analytes in mixtures.

Model Compound Calibration Data

Calibration curves were done in duplicate with a minimum of 14 concentration data points per analysis. The calibration data were comprised only of the 15-minute spectra for each of the concentrations on the curve. Molar extinction coefficients were determined for 2-3 of the maxima in the spectra. Average data for each analyte were collected by repeated spectral determination. Using the molar extinction coefficient from the calibration curves, the best fit to the average spectra was made in order to determine the molar extinction coefficient at every wavelength. The resulting graph contained the molar extinction coefficient for the compound determined at the wavelength at which the calibration curve was obtained and extrapolated to all other wavelengths in the spectral range.

Calibration curves were linear from 0.05-0.90 absorbance units with R-squared values in the range of 0.980 or better. The maximum molar absorptivity values for the model compounds analyzed were from 250-9000 Absorbance units per mole. The highest absorptivities were found in the conjugated dienes in linear carbon systems and in cholesterol. Limits of detection were determined for a subset of model compounds and are in the order of 0.01-0.08 mM.

Fatty acids

Analyte stock solutions were either 0.02 M or 0.01 M in spectroscopic grade chloroform (Aldrich) depending on the molar extinction coefficients. For an analysis, 10

μL of the stock analyte is used in the reaction the chosen reagent. A total of 2.0×10^{-7} moles of analyte is all that is needed for each analysis. All of the PUFAs are prone to autoxidation and were stored under argon, at less than 0°C, and kept from light.

Liebermann-Burchard Reagent Preparation

The L-B reagent was made by addition of ice cold 20 mL acetic anhydride and 1.0 mL of ice cold concentration sulfuric acid. The mixture was stirred for 10 minutes on ice. To the cold mixture, 10 mL of acetic acid was added and the mixture was allowed to come to room temperature.

Liebermann-Burchard Reaction

The L-B reaction was conducted by the addition of 50 μL of analyte dissolved in chloroform (0.02 M) to a 13 X 100 mm borosilicate disposable test tube. To the analyte 1.0 mL of L-B reagent was added. The reaction timer was started immediately after the addition of the L-B reagent. The reaction was allowed to occur for 30 minutes. The sample was then transferred to the Teflon sealed 10 mm cuvette using a glass Pasteur pipette. The sample was analyzed by UV/Visible spectrophotometry at 30 minutes into the reaction from 350 to 800 nm. A catalyst is not required. The final color of the reaction is blue-green.

Results and Discussion

Steroids

Steroids with 5-ene-3-β-substituents (cholesteryl esters)

A common physiological derivative of cholesterol involves esterification of a side chain at the 3-beta hydroxide. The reagent is effectively blind to the ester and leads to spectrum practically identical to cholesterol (Figure 3(C)). The cholesteryl esters are important in the hydrophobic transport of cholesterol (Figure 2(A)(R=H)). For all esters that have saturated chains, the spectra were very similar including cholesteryl nonanoate (Figure 3(D)), cholesteryl myristate (Figure 3(E)), and cholesteryl acetate (Figure 3(F)). Cholesteryl oleate (Figure 3(B)) has the monounsaturated oleate fatty acid attached at the 3-beta ester. The spectrum indicates a similar electronic spectrum over all wavelengths below 550 nm. The contribution of oleate at 0.02 M should be very small in the short wavelength range and not affect the region around 520 nm. All of the cholesteryl esters have a maximum absorbance at 360 nm, 420 nm, and 520 nm with the 520 nm peak having the largest absorptivity.

The final cholesteryl ester compounds considered were those with polyunsaturated ester linked side chains. These include cholesteryl linoleate (Figure 4(B)) and cholesteryl linolenate (Figure 4(A)). For these cholesteryl compounds the spectrum is not equal to that for cholesterol (Figure 4(C)). It would be expected that the linoleate and linolenate functional double bonds would react to form a spectrum similar to the methyl esters of linoleate (Figure 4(E)) and linolenate (Figure 4(D)). In fact when the 0.01 M spectra for methyl linoleate and linolenate are subtracted from the cholesteryl linoleate and linolenate respectively, the resulting spectra (Figure 4(insert: G,H)) are very

similar to that of cholesterol (Figure 4(insert)(C)). It is not clear why there is a consistent rise in the 520 nm region and lowering in the shorter wavelengths. The spectra are the same within experimental errors.

One compound with a non-ester substitution at the 3-β position was considered. Cholesteryl chloride (Figure 3(A)) has chloride as an alternative functional group in place of the hydroxide. The chloride at the 3-β position alters the resulting spectrum by increasing the overall absorptivity at all wavelengths and shifting the maximum absorbance from 520 nm to 514 nm.

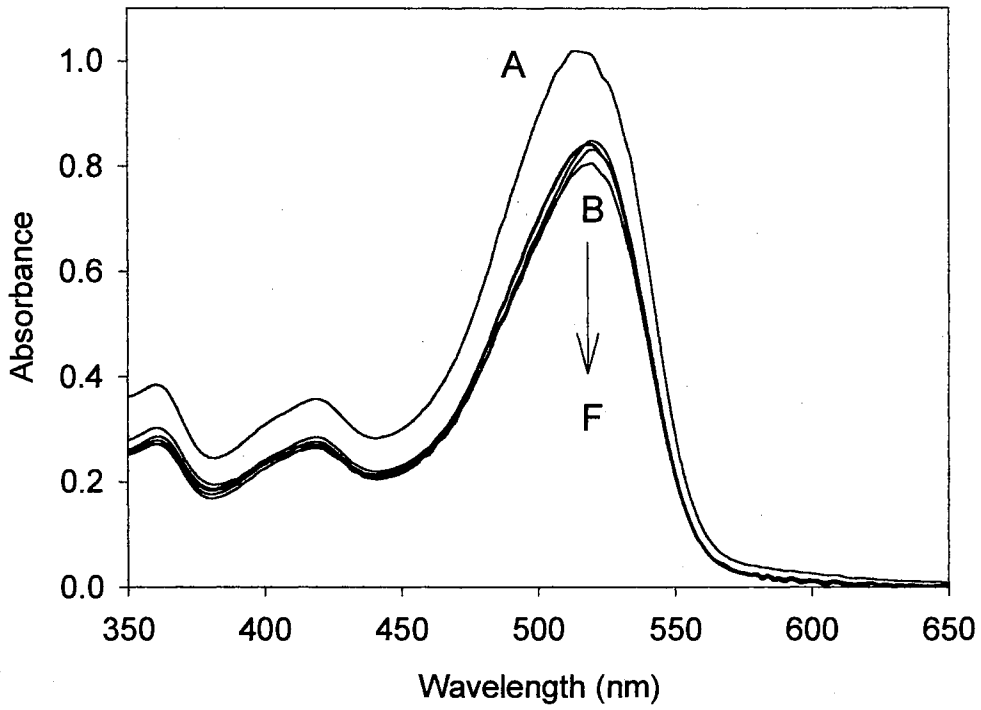

Figure 3. Overlaid spectra of cholesterol derivative including A) cholesteryl chloride and B) cholesteryl oleate C) cholesterol D) cholesteryl nonanoate E) cholesteryl myristate F) cholesteryl acetate.

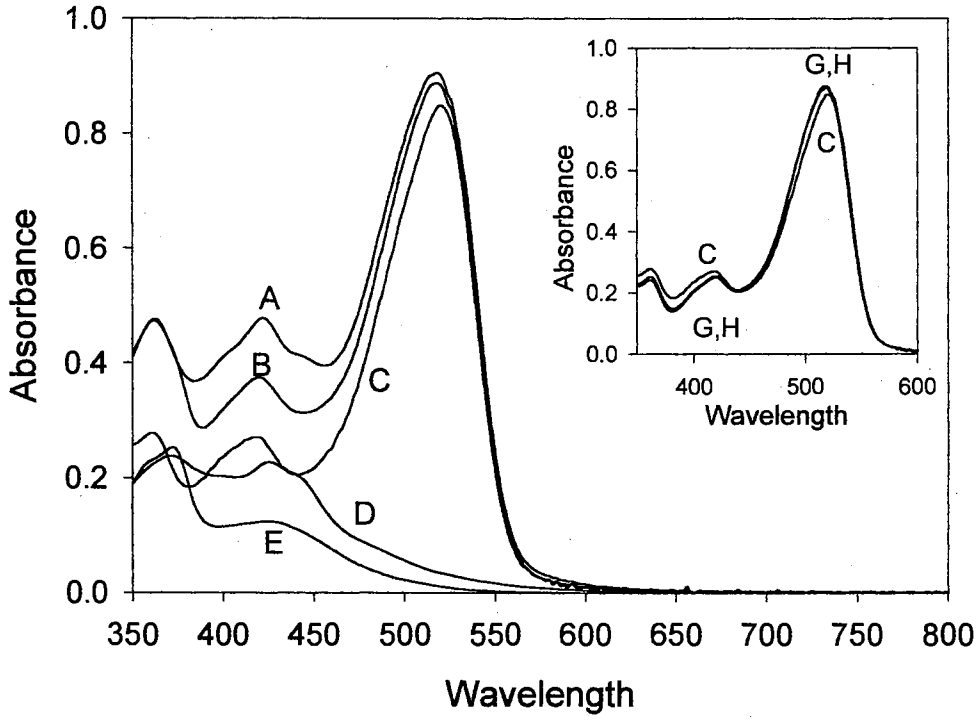

Figure 4. Overlaid spectra demonstrating the additivity of cholesterol and methyl linoleate and methyl linolenate. A) cholesteryl linolenate B) cholesteryl linoleate C) cholesterol D) methyl linolenate E) methyl linoleate all at 0.01M insert G) methyl linoleate subtracted from cholesteryl linoleate H) methyl linolenate subtracted from cholesteryl linolenate and C) cholesterol.

3-β-ol 5-ene compounds

Cholesterol has 3-β-ol and 5-ene functional groups. Three other compounds share these exact functional groups. They are diosgenin (Figure 5(A)), 5-cholesten-3-β-ol-7-one (Figure 5(B)), and stigmasterol (Figure 5(C)). Comparing the resulting spectra and the steroid structure that produced them, the spectrum for diosgenin is significantly different from those for the other cholesterol like compounds.

Diosgenin only varies structurally at the 17th carbon were there is added ring in place of the aliphatic chain of cholesterol (Figure 2(J)) The electronic spectrum of diosgenin has a broad peak with maximum at 420 nm. The spectrum extends from 350 nm out to 650 nm with several underlying maxima present. Differential analysis shows the maxima at 362, 396, 420, 444, 498, and 570 nm. The spectrum is completely different from cholesterol with significant broadening and obvious lack of the 520 nm peak. There is no obvious explanation for this result.

5-cholesten-3-β-ol-7-one is structurally different from cholesterol in the active area (Figure 2(O)). The added ketone is in close proximity to the active unsaturated region on carbon 5. It is not clear how the ketone effects the reaction in terms of the product formed, however, the spectral differences are significant (Figure 5(B)). The spectrum shows a maximum at 444 nm, a large absorbance in the short wavelength past 350 nm and a small absorbance at 526 nm. The conjugated ketone formed by the addition of the ketone to carbon seven has been reviewed in a number of compounds addressed later.

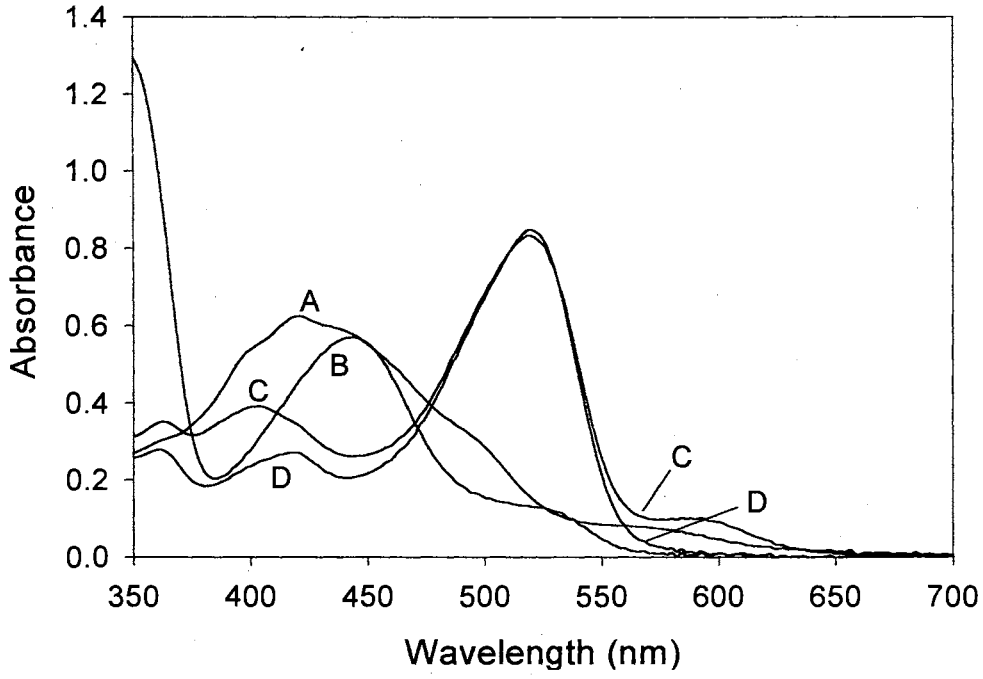

Figure 5. Overlaid spectra A) diosgenin B) 5-cholesten-3-β-ol-7-one C) stigmasterol D) cholesterol.

Stigmasterol only varies from cholesterol at the 17 carbon aliphatic chain (Figure 2(B)). The side chain has an unsaturation at the 19th carbon and an ethyl group at carbon 21. Knowing the double bond is cis for stigmasterol, we can best correlate the double bond to methyl linolelaidate (Figure 15(A)). In both cases, the absorption at 420 nm is increased. Stigmasterol also has another larger absorbance at 404 nm. This larger absorbance makes the 420 nm absorbance a distinguishable shoulder. Furthermore, stigmasterol has a 362 nm peak similar to cholesterol except with a larger absorbance. Stigmasterol has almost exactly the same absorbance at 520 nm as cholesterol at equal molarity. Finally, there is an absorbance at 594 nm that is unique to stigmasterol among the 3-β-5-ene compounds.

Other Steroid Compounds

Another compound tested containing sterol skeletons is dihydrocholesterol. Dihydrocholesterol is composed of exactly the same carbon framework, as cholesterol except it does not have the 5-carbon unsaturation (Figure 2(C)). The reaction of dihydrocholesterol results in no appreciable color formation (Figure 6(F)). Another compound tested also did not undergo chromogenesis with the AC reagent was β-estradiol. Alpha and β-estradiol are both composed of a sterol skeleton with an aromatic benzene-like structure on ring A (Figure 2(E)). No spectral response was seen after reaction with the acetyl reagent (Figure 6(E)).

While β-estradiol gives no coloration upon reaction, α-estradiol forms one of the most intense spectra of the sterols examined (Figure 6(A)). The spectrum includes a

weak broad absorbance around 384 nm with an intense absorbance at 512 nm. There is also a shoulder to the largest peak around 454 nm. The two differ only in the enantiomeric orientation of the 17-hydroxide. The variation of response between the two compounds is profound.

7-dehydrocholesterol has the same structure as cholesterol except there is an additional unsaturation at carbon-7 (Figure 2(D)). This second unsaturation produces a conjugation double bond in ring B of the sterol backbone. The resulting spectrum shows a large absorption at 392 nm and a smaller absorption that seems to split with minima at 486 nm and 508 nm (Figure 6(C)). Ergosterol has the same structure as 7-dehydrocholesterol except it has an unsaturation on carbon-19 and a methyl group off carbon-21 (Figure 2(F)). The unsaturation and hydroxide configuration are exactly equivalent in the first two rings (A and B) of the structures. The double bond on the side chain has a trans conformation. It is therefore similar to methyl oleate. The spectrum for ergosterol is similar to that of 7-dehydrocholesterol except there is an increase in the absorbance and there is an overall broadening of the spectrum (Figure 6(B)). The maximum absorbance is at 388 nm and 486 nm. There is a significant tail beyond 570 nm extending out to 800 nm.

Stanozolol has a unique structure with a nitrogen heterocycle fused to ring A of the sterol skeleton (Figure 2(G)). It has a 17-hydroxyl similar to the estradiols spectroscopically stanozolol has a relatively simple spectrum (Figure 6(D)). It has a single broad peak around 394 nm.

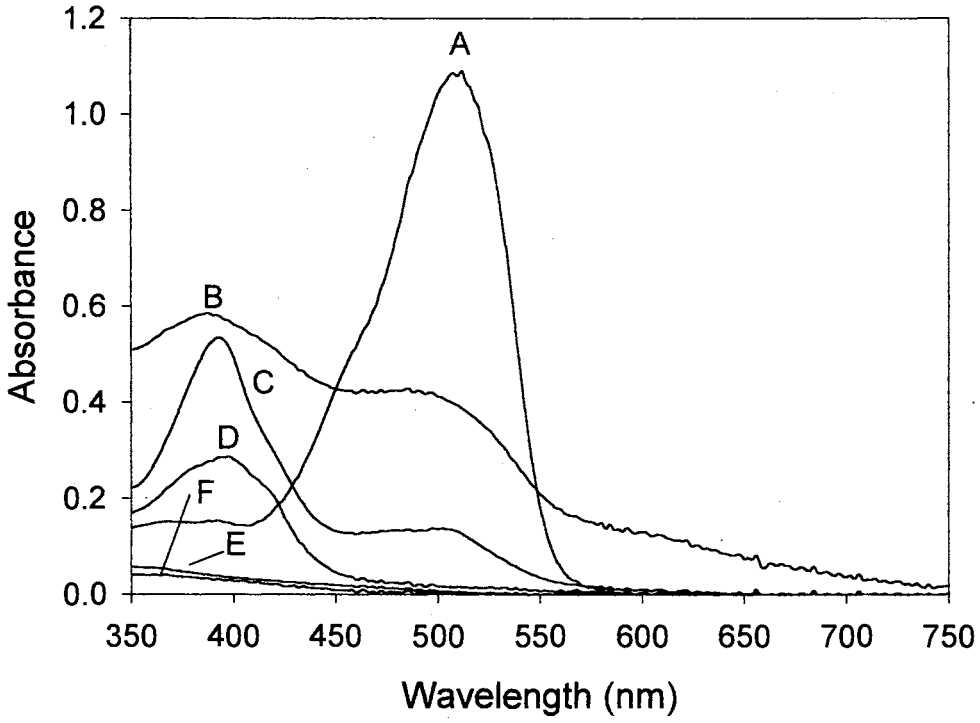

Figure 6. Overlaid spectra of steroid compounds A) α-estradiol B) ergosterol C) 7-dehydrocholesterol D) stanozolol E) β-estradiol F) dihydrocholesterol.

Compounds with Conjugated 3-ones

A number of compounds have the hydroxide at the 3-carbon of cholesterol replaced by a ketone. In a majority of the sterols considered in this study, the ketone is accompanied by an unsaturation at the 4-carbon forming an unsaturated ketone on ring A. Testosterone is a good example of this type of compound. Testosterone is a 4-ene-3-one structure with a 17-hydroxide (Figure 2(K)). The spectral response for testosterone is comprised of a large absorption at 368 nm (Figure 8(C)). The spectrum of testosterone is very simple. Other 4-ene-3-one compounds are more complex in their spectral response. 5-cholesten-3-one is very different structurally from testosterone (Figure 2(N)). The 3-position ketone is no longer conjugated as the unsaturation is at the 5 carbon as is the case with cholesterol. The spectral response includes a maximum absorbance at 372 nm and a smaller absorbance at 526 nm (Figure 7(C)). There is little similarity between 5-cholesten-3-one and testosterone. There are more structural similarities between 4-cholesten-3-one and testosterone (Figure 2(M)). The spectrum of 4-cholesten-3-one includes a maximum at 372 nm and a smaller peak at 526 nm the same as 5-cholesten-3-one (Figure 7(D)). Both 4-cholesten-3-one and 5-cholesten-3-one have the aliphatic group from cholesterol on the carbon-17 position. This difference may contribute to the lack of similar spectra in the 4-cholesten-3-one and testosterone.

Another 4-ene-3-one compound is progesterone. Progesterone is the same sterol structure as testosterone except the 17- position has an α ketone ethyl group in place of testosterone's hydroxide group (Figure 2(P)). Progesterone has a maximum absorbance at 368 nm just like testosterone with almost the same molar extinction coefficient.

Progesterone also has a small broad absorbance in the longer wavelength around 590 nm (Figure 7(B)). The absorption at 590 nm may be due to the 17-ethyl ketone group.

Hydrocortisone is also a 4-ene-3-one containing compound. Hydrocortisone is more highly substituted than the other 4-ene-3-one compounds. It has a hydroxide on ring C and at the 17- position, there is a hydroxide and a ketone ethanol group (Figure 2(R)). The spectral response for this compound involves an absorbance at 366 nm reduced in size from the corresponding absorbance in testosterone (Figure 7(E)). It is common in these reactions that hydroxides react to lower absorbance of unsaturated groups. This is best illustrated by the fact that small alcohols can act to prevent the reaction all together.

Testosterone can be modified so its 17-β-hydroxyl group is bonded to the same carbon as an enantio-specific carbon (Figure 2(L)). This 17-α-methyl forces the hydroxide to be alpha. The response to 17-α-methyl testosterone includes the absorbance at 368 nm with almost exactly the same extinction coefficient, and a number of absorbances not found in testosterone (Figure 8(B)). These 17-α-methyl testosterone specific absorbances include 496, 580, and 620 nm forming a significant absorbance in the longer wavelength range.

3-one compounds

Norethynadrel has a carbon-3 ketone and a double bond between ring A and ring B (Figure 2(I)). Instead, it has a double bond between ring A and B. Further complicating the structure, the 17 position has a hydroxide and ethyne group attached to

it. The spectral response for norethynadrel includes a peak at 384 nm with a shoulder at 426 nm (Figure 8(A)). Norethynadrel also has absorbances at 520 and 584 nm.

Double Conjugated 3 Ketone

Two of the sterol compounds have unsaturations at the 1 and 4 carbons with a ketone at the 3- position. This forms a double unsaturated ketone compound. Prednisone and prednisolone are structurally similar to hydrocortisone except the double bond on carbon-1 for prednisolone and prednisone and the ketone in place of the hydroxide on ring C for prednisone (Figure 2(Q and S)). The absorbance spectra of both prednisone and prednisolone are baseline (Figure 8(D and E)).

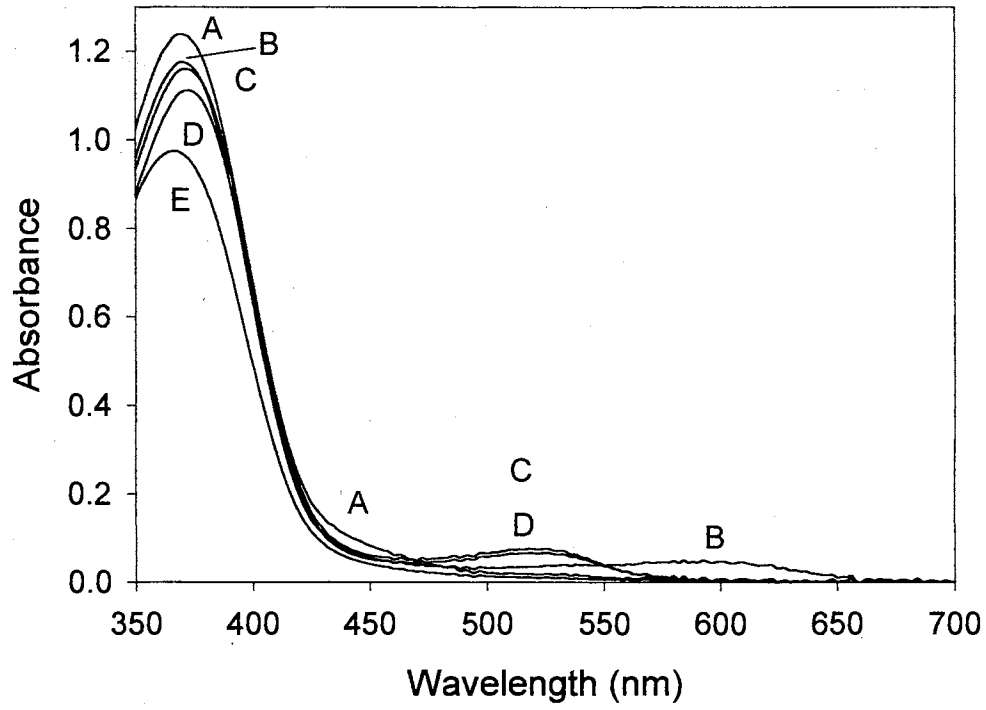

Figure 7. Overlaid spectra A) testosterone B) progesterone C) 5-cholesten-3-one D) 4-cholesten-3-one E) hydrocortisone.

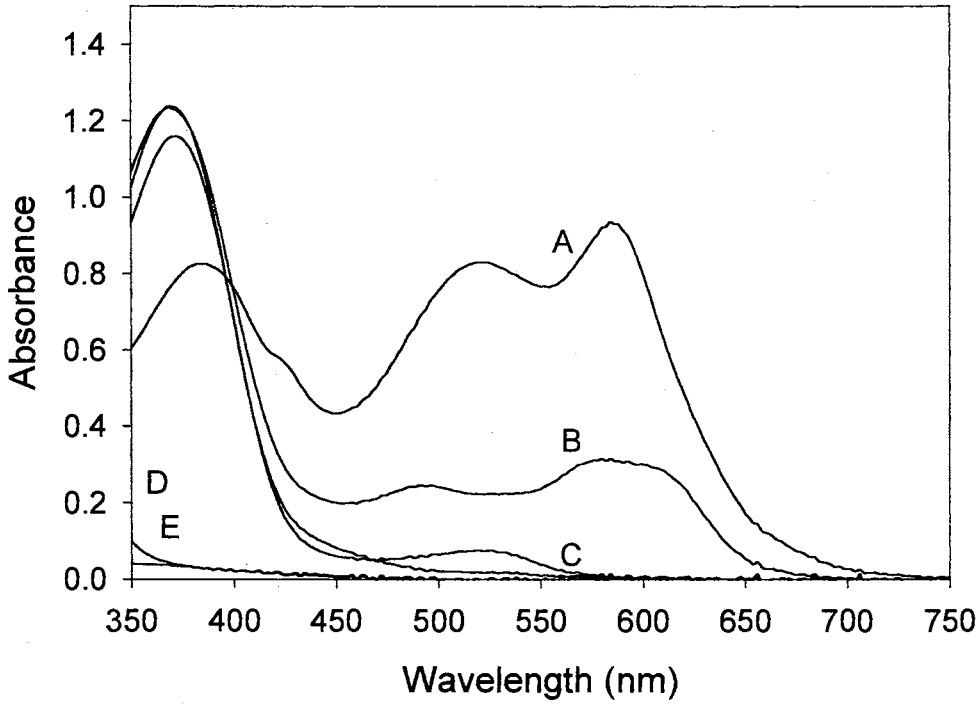

Figure 8. Overlaid spectra A) norethynadrel B) 17-α-methyl testosterone C) testosterone D) prednisone E) prednosilone.

Terpenes

The calibration plots for terpenes are linear between 0.05 to 1.2 mM. The correlation coefficients for the Beers' law plots are for example 0.987 for α-pinene at the 390 nm maxima. The correlations for farnesol are 0.988 at 386 nm, and 0.991 at the 486 nm maxima. The limits of detection are in the order of 1.0×10^{-5} M.

Linear Model Compounds

Pentadienes

Included are non-terpene compounds such as 1,4-pentadiene, trans 1,3-pentadiene and cis 1,3-pentadiene (Figure 9. Trans 1,3-pentadiene, cis 1,3 pentadiene and 1,4-pentadiene all have very similar spectra (Figure 10(E,F,G)). They all have single maxima at 412 nm. All three also have monotonic kinetics leading to the 15 minute maximum. There is a shoulder at 495 nm for all three pentadienes and the appearance of a minimum around 360 nm for the two 1,3-pentadienes may indicate an absorbance below 350 nm.

Trans 1,3-pentadiene is 0.075 absorbance units higher than cis 1,3-pentadiene at 412 nm at the 0.02 M stock concentrations. This difference can be related to the absorbance difference between methyl linoleate and methyl linolelaidate fatty acids. This may indicated that the cis / trans conformation of the 1,3-pentadienes does affect the resulting spectrum. However the conjugation in the double bonds of the two 1,3-pentadienes would be analogous to conjugated methyl linoleate. The conjugation in methyl linoleate leads to a large increase in the resulting absorptivities and large variation in the kinetics of the reaction in the 422 nm region compared to methylene bridged linoleate. Furthermore, the large absorptivity of the product with conjugated methyl

linoleate is not formed in the reaction with a terminal double bond (1,3-pentadiene). Therefore, the difference in the conjugated double bonds between the pentadienes and the conjugated methyl linoleate is the absence of a peripheral saturated carbon. It has been hypothesized in this laboratory that the reaction requires a local methylene (CH_2) (or possibly methyl) group in order to proceed. This hypothesis is supported by the pentadiene data.

The 1,4-pentadiene does have a centralized methylene CH_2 group yet it produces almost identical spectral features as the 1,3-pentadienes. This similarity of spectral responses is not likely due to double bond rearrangement of the 1,4-pentadiene into 1,3-pentadiene configuration. If this type of rearrangement were a feature of this reagent system it would occur in the methyl linoleate and / or conjugated methyl linoleate systems. It is however, possible that the terminal double bonds are susceptible to rearrangement. In the case of three pentadienes, it is illogical to assume that the 1,4-pentadiene with two terminal double bonds should have the same absorbance as the 1,3-pentadienes with only one terminal double bond. Future work should include the analysis of short double bond systems to elucidate the reaction mechanisms and products.

Another point can be made from the reactions with small double bond containing compounds such as the pentadienes. The reaction mechanism hypothesis involving the buildup of a carbon structure from the double bond system is supported in these reaction as there is no possibility of the standard strong acid cholesterol reaction hypothesis involving dehydrogenation to create conjugated unsaturations any larger than already exist on the small molecules. The occurrence of the chromogenic reaction with the

pentadienes is strong evidence toward a mechanism for the reaction that involves an alternative mechanism than the acid reactions currently used in sterol determination.

Linear Terpenes

The most simple terpene is isoprene. The spectral response of isoprene involves a large absorbance at 362 nm (Figure 10(A)). The absorptivity is very small at 0.02 M, and isoprene is structurally similar to 1,3-pentadiene with conjugated double bonds being found on the end of the carbon chain. The difference between isoprene and 1,3-pentadiene is in the presence of a methyl carbon sidechain from the internal carbon of one of the two terminal double bonds rather than off one of the outside double bond carbons as with 1,3-pentadiene. The extremely small absorptivity cannot be explained by the presence of two terminal double bonds as 1,4-pentadiene also contains two terminal double bonds although they are methylene bridged rather than conjugated. The cause of the spectral response of this double bond configuration will only be understood when the mechanism is better understood.

Myrcene, squalene and farnesol are different from the pentadienes and from each other (Figure 9). Myrcene is characterized by a broad maximum at 370 nm with a broad shoulder around 454 nm (Figure 10(D)). Squalene has a maximum at 390 nm and a broad tail across the longer wavelengths (Figure 10(C)). Finally, farnesol has two distinct maxima at 384 and 486 nm (Figure 10(B)).

All of the linear terpenes and the included small-unsaturated compounds have unique spectra and should be discriminable in mixtures. It is important to remember that the equivalent maximum absorbance observed for the two 1,3 and the 1,4-pentadiene

does not preclude spectral determination and isolation of the compounds. The spectra at 415 nm are different while all three spectra are very similar at 494 nm. This is a good indication that the spectra are linearly independent from each other and therefore determinable by a variety of multivariate analysis methods.

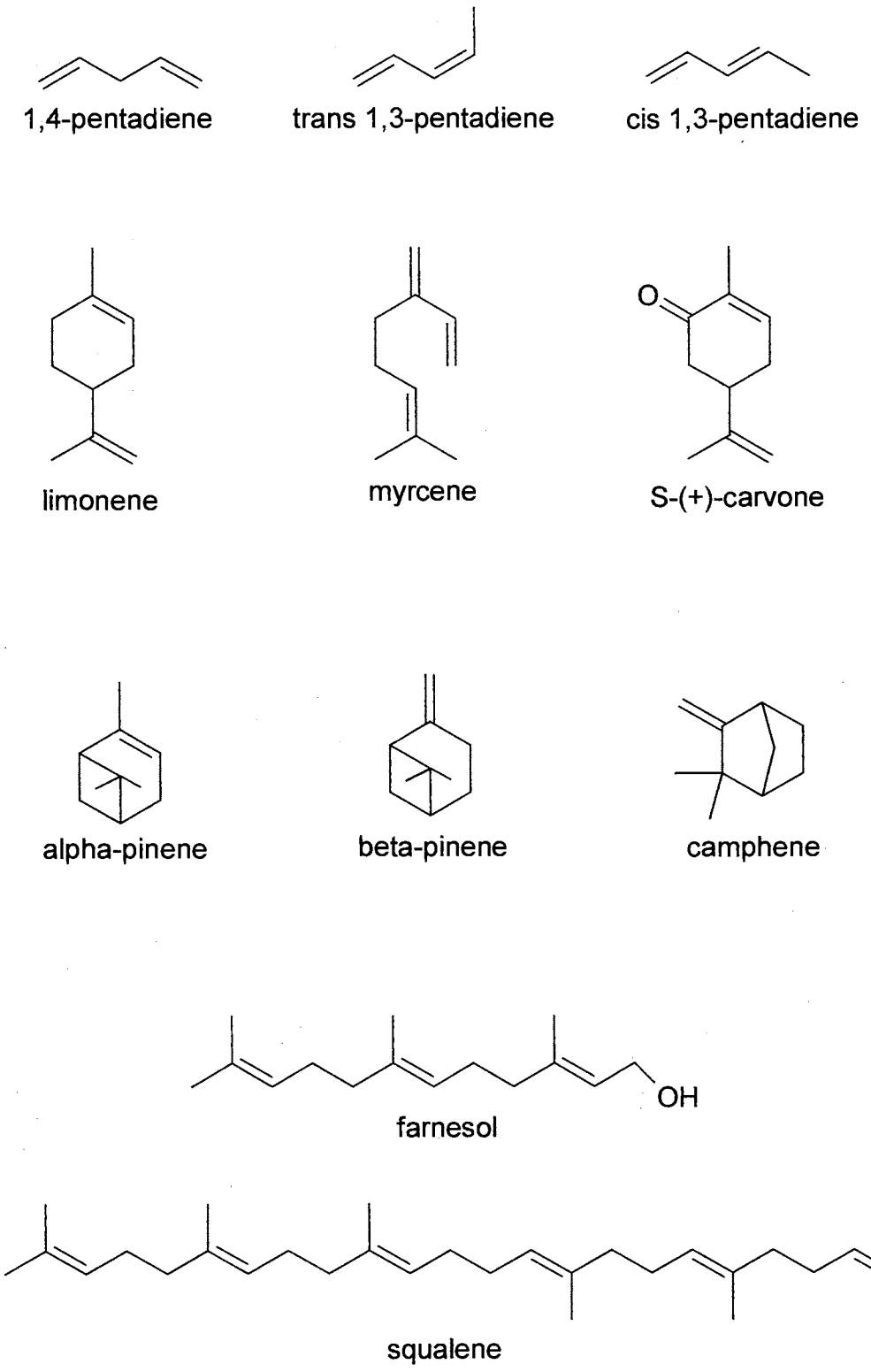

Figure 9. Terpene structures.

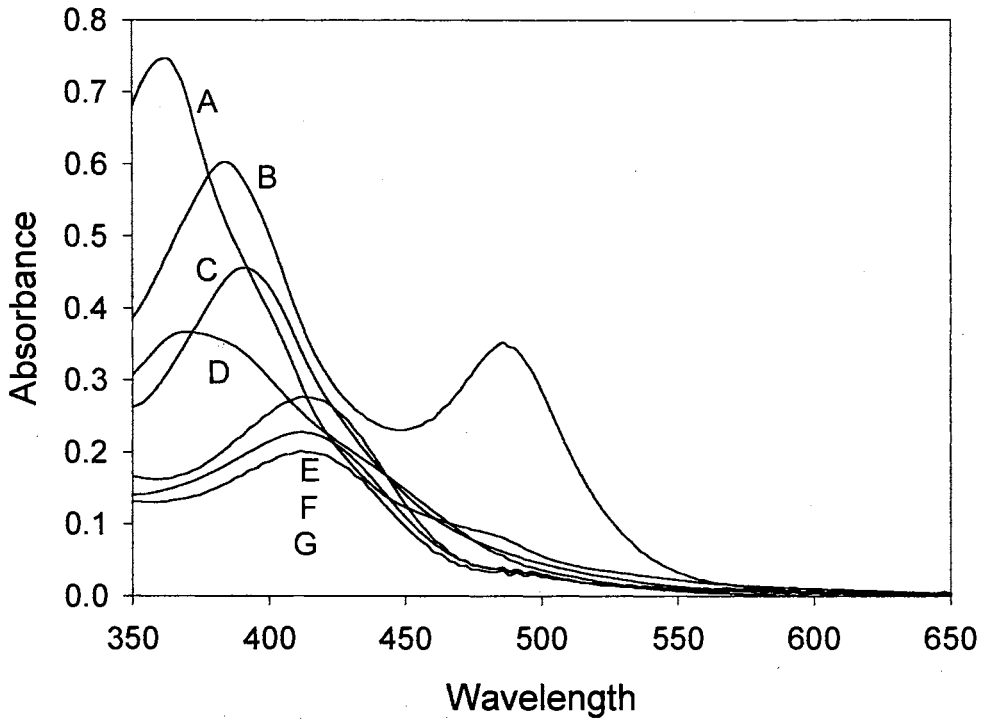

Figure 10. Overlaid spectra of all linear terpenes including A) 10 µl of 0.25M isoprene, B) farnesol, C) squalene, D) myrcene, E) trans-piperylene, F) 1,4-pentadiene, G) cis-piperylene.

Cyclic Model Compounds

The cyclic terpenes are particularly interesting due to the homogeneity in structural motifs. For example limonene, carvone, and α-pinene are all very similar structurally (Figure 9). The spectral response for α-pinene, limonene and β-pinene are almost exactly the same (Figure 9). All three have a large maximum at 388 nm and smaller absorbance at 494 nm (Figure 11(A,B,C)). Limonene and α-pinene are structurally similar, as the double bond for both is endocyclic. Limonene has another double bond on its sidechain. Because the spectra for the limonene is so close to that of α-pinene, the difference of the two spectra would indicate the contribution by limonene's external double bond would be inconsequential. β-pinene is similar to α-pinene except the double bond is exocyclic rather than endocyclic. This structural variation would be expected to give significantly different spectra in the chromogenic reaction. However, the resulting spectrum for β-pinene is very similar to the response for α-pinene.

The structural similarity between limonene and carvone is dramatic. The only difference is the presence of a ketone forming an unsaturated ketone with the endocyclic double bond. The response of carvone is dramatically lower than that of limonene (Figure 11(E)). This indicates that the presence of the ketone prevents the production of the chromophore associated with limonene. There is some coloration formed, however, it is less intense and less sharp than that of limonene.

The side chain of limonene is analogous to the methyl-substituted double bond found in isoprene. It may be that terminal double bonds with a single methyl substitution are structurally inappropriate for the chromogenic reaction with AC.

Camphene has an exocyclic double bond similar to the double bond of β-pinene. The spectral response to camphene is, however, significantly lower than that of β-pinene. Camphene is also slightly shifted from 288 nm to 284 nm and has a broadened base at longer wavelengths (Figure 11(D)). The only differences in the local structures of the two compounds are the substitution on the β carbons and the bicyclic structure.

From the camphene results it seems the reaction only requires a $-CH=CH-CH_2-$ group to occur. The possible combinations of carbons that create this simple structural motif are vast. The reaction is highly influenced by the structural environment in the analyte. Variations as simple as an added ketone or as subtle as the differences in camphene and β-pinene contribute to the spectral uniqueness of practically all of the analytes tested.

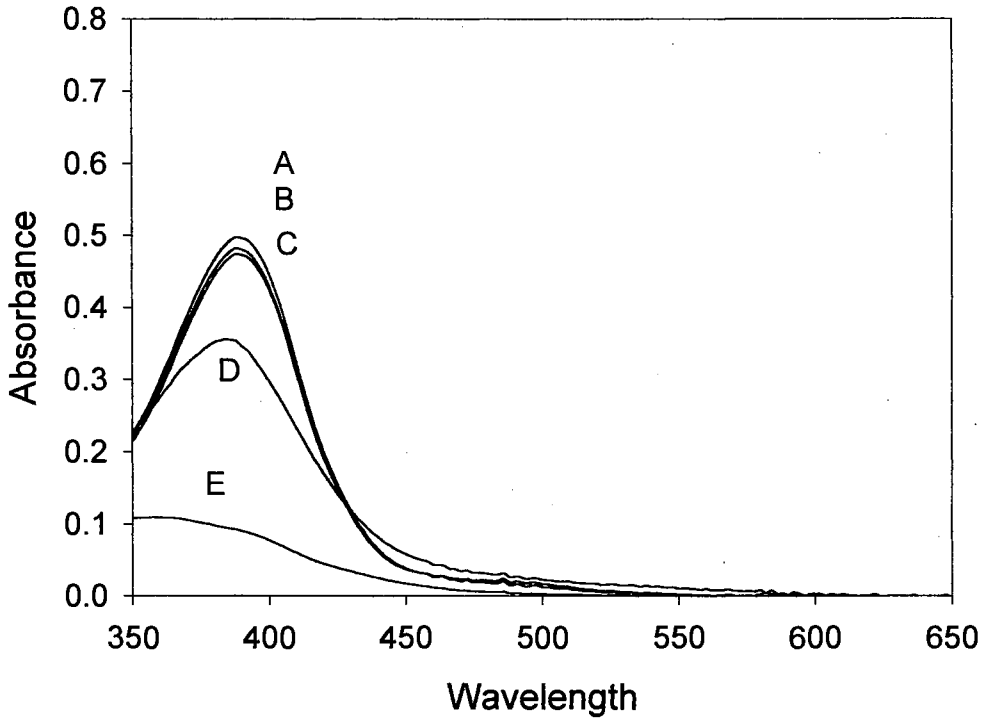

Figure 11. Overlaid spectra of all cyclic terpenes including A) α-pinene, B) limonene, C) β-pinene, D) camphene, E) carvone.

Polyunsaturated Fatty Acids

The reaction of saturated fatty acids gives essentially background absorbance. Oleic acid has a single unsaturation in the middle of the 18-carbon chain. It was previously suspected that single unsaturated acids did not give spectroscopic responses in the chromogenic reactions. It was discovered that a small response does occur with AC and both zinc acetate and PA catalysts. An aliquot containing 5 X 10^{-7} moles of oleic acid gives a small response. Oleic acid as a monounsaturated chain gives a very small absorbance that requires large concentrations to detect (Figure 12(H)). This makes both saturated and monounsaturated species physiologically irrelevant. All other fatty acids were observed at the same concentration (except conjugated linoleic) so that stoichiometric comparisons can be made.

Methyl linoleate has a maximum absorbance at 362 nm. The second absorbance at 422 nm has a relatively lower absorptivity (Figure 12(G)). The result for the trans isomer of methyl linoleate (methyl linolelaidate) indicates a difference in the absorptivity at 422 nm while close correlation exists at the 362 nm region (Figure 12(F)). This variation at 15 minutes seems to indicate the ability to distinguish between the cis and trans linoleic acid isomers in serum (Figure 15(A and B)). Gamma-linolenate and linolenate acids also present unique spectra at 0.02 M stock concentration (Figure 12(D and E)). Conjugated methyl linoleate (Figure 12(A)) is significantly different in intensity from methyl linoleate (Figure 12(G)). It is clear that conjugated methyl linoleate is not proportional to methyl linoleate at 15 minutes (Figure 14(inset)). Even greater differentiation is found when the kinetics of conjugated methyl linoleate and methyl linoleate are compared (Figure 13(Top & Bottom)). The kinetics at 422 nm is

significantly more complex for the conjugated methyl linoleate with a decline in the spectral intensity after seven minutes at 422 nm.

The apparent ability to distinguish cis and trans isomers in linoleate and linolelaidate has significance in the determination of both physiological and foodstuff composition of trans fatty acids. It is currently necessary to do separations for the determination of trans fatty acids found in complex mixtures such as foodstuff or biological samples. The potential for a detection method for cis and trans fatty acids isomers cannot be overstated.

The regioisomer differences observed for methyl linolenate and gamma methyl linolenate are not easily explained. The unsaturations affect the rigidity of the fatty acid chain. It is clear that regioisomers interact with physiological systems in a unique ways (see Chapter I). In the living system, such structure dependence is to be expected. The kinetics of the reaction could be affected by the change in the flexibility of the fatty acid chain depending on the unsaturation location. This phenomenon is of interest and warrants further study.

The kinetic difference between conjugated methyl linoleate and methyl linoleate could allow the determination of the two in a mixture. In binary and more complex mixtures, quantitative analysis of the kinetics of the reaction may allow quantitation of the levels of conjugated methyl linoleate not possible by simple analysis at a standard time. It should be noted that the differences between linoleate and methyl linolelaidate at 15 minutes are not due to dramatic kinetics differences as with conjugated methyl linoleate and methyl linoleate. They are rather do to a small difference in the monotonic

progression in the kinetics at 422 nm resulting in a larger absorbance for that wavelength at the 15 minute endpoint (Figure 15(including inset)).

The primary hypothesis for the reagent's selectivity in PUFAs attributes the difference in spectral response to the number of unsaturations. The ability of the reagent to produce color with fatty acid chains is different from purely acidic reaction such as the L-B reaction which itself does not give a response with PUFAs (Figure 16(B-D)). While the cholesterol molecule gives a good response (Figure 16(A)). Taking into account the current hypothesis for color formation in the case of acid reactions with sterols involve the process of dehydrogenation, it is difficult to see how dehydrogenation is a viable mechanism for the formation of coloration in lipid chains. The isolated double bounds in all of the fatty acids are methylene bridged and for all practical purposes equivalent in terms of their potential to be involved in dehydrogenation reactions. This means the dehydrogenation to form conjugated chromophores would not be as selective as the presented chromogenic reaction. The alternative hypothesis involves a buildup of cyclic structures utilizing the fatty acid as a template (see Chapter I). In this mechanism, unique energy levels within the electron orbitals of the product associated with the spectral absorbances would not be independent of the template. The reaction does not depend on the locations of the double bonds but rather the number of methylene bridged double bonds in a group.

The spectral dependence on the number of unsaturations, the regioisomers and cis/trans confirmations support the hypothesis of a synthetic mechanism. The one anomaly in this deductive trend is the fact that the PUFAs with 5 and 6 unsaturations appear to have almost the same spectra at 15 minutes. Eicosapentaenoate and

docosahexaenoate are both omega-3 fatty acids with no obvious reason for the similarity in their spectra (Figure 12(B and C)). Methyl linolenate contains three unsaturations in the omega-3 positions and forms an independent spectral response. This means the omega-3 unsaturation alone does not cause Eicosapentaenoate and docosahexaenoate to be similar.

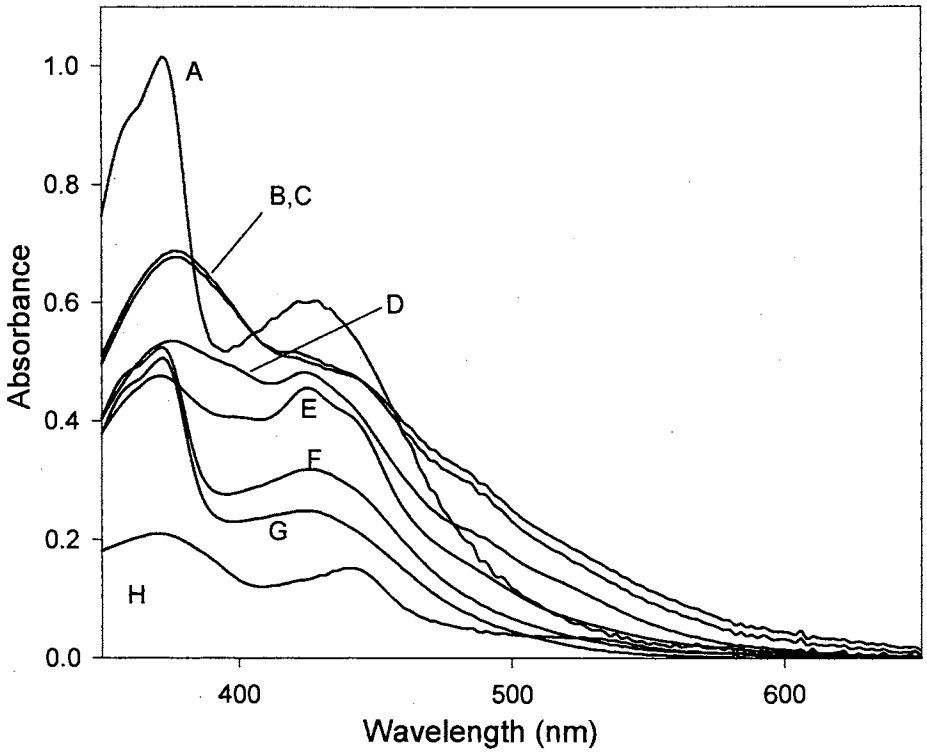

Figure 12. Overlaid polyunsaturated fatty acid spectra for 10 μL of 0.02 M (unless stipulated otherwise) reacted with 1.0 mL acetyl chloride catalyzed by 40 μL of perchloric acid at 15 minutes including; A) methyl conjugated linoleate (0.01M stock) B) methyl eicosapentaenoate C) methyl Docosahexaenoate D) gamma-methyl linolenate E) methyl linolenate F) methyl linolelaidate G) methyl linoleate H) methyl oleate (2.5 times more concentrated).

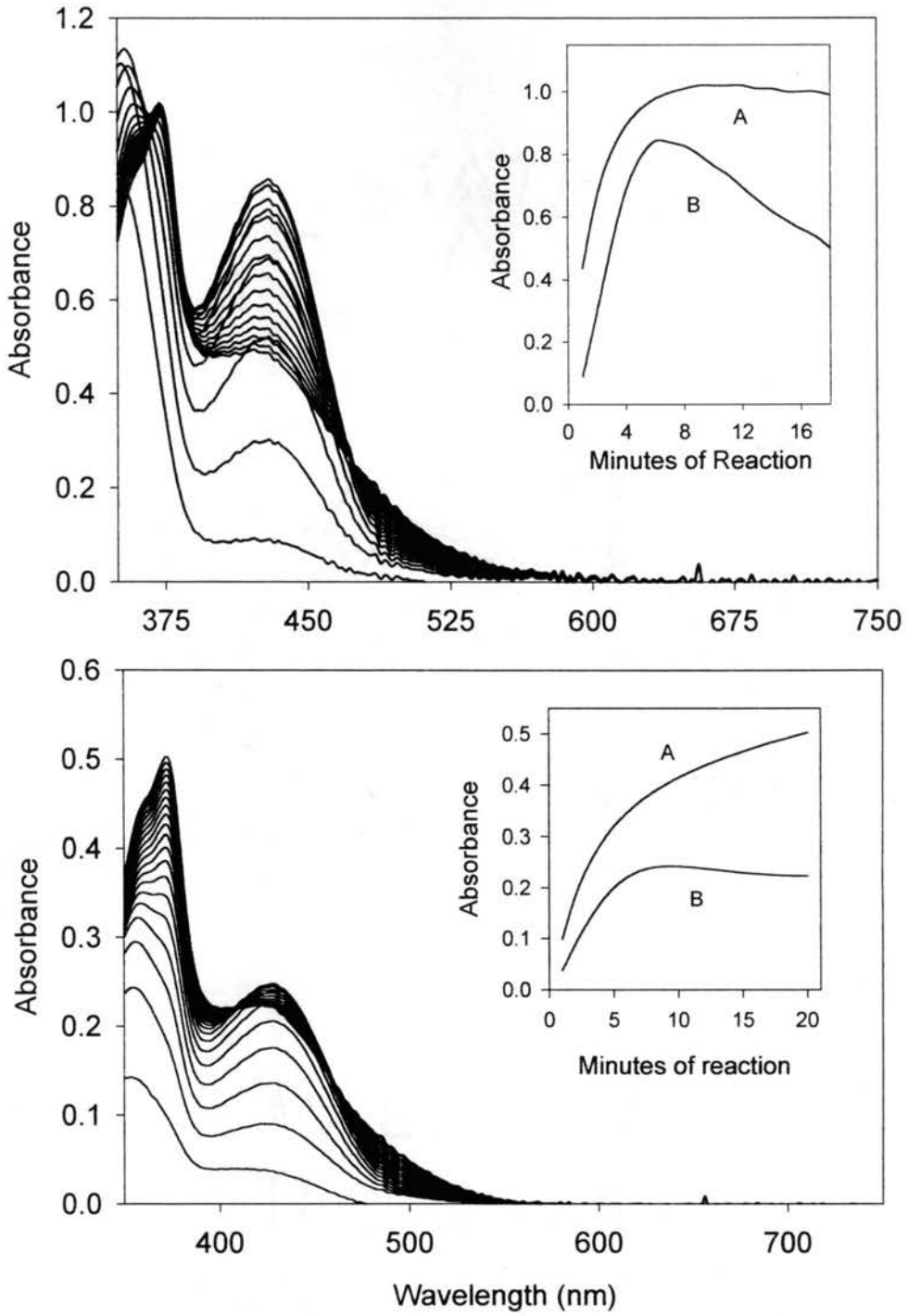

Figure 13. (TOP)) Electronic absorbance spectra of the reaction of 10 μL 0.01 M conjugated methyl linoleate with 1.0 mL acetyl chloride and 40 μL perchloric acid over 20 minutes Inset: Kinetic plots of 372 and 422 nm over reaction time. (BOTTOM)) Electronic absorbance spectra of the reaction of 10 μL 0.02 M methyl linoleate with 1.0 mL acetyl chloride and 40 μL perchloric acid over 20 minutes Inset: Kinetic plots of 372 and 422 nm over reaction time.

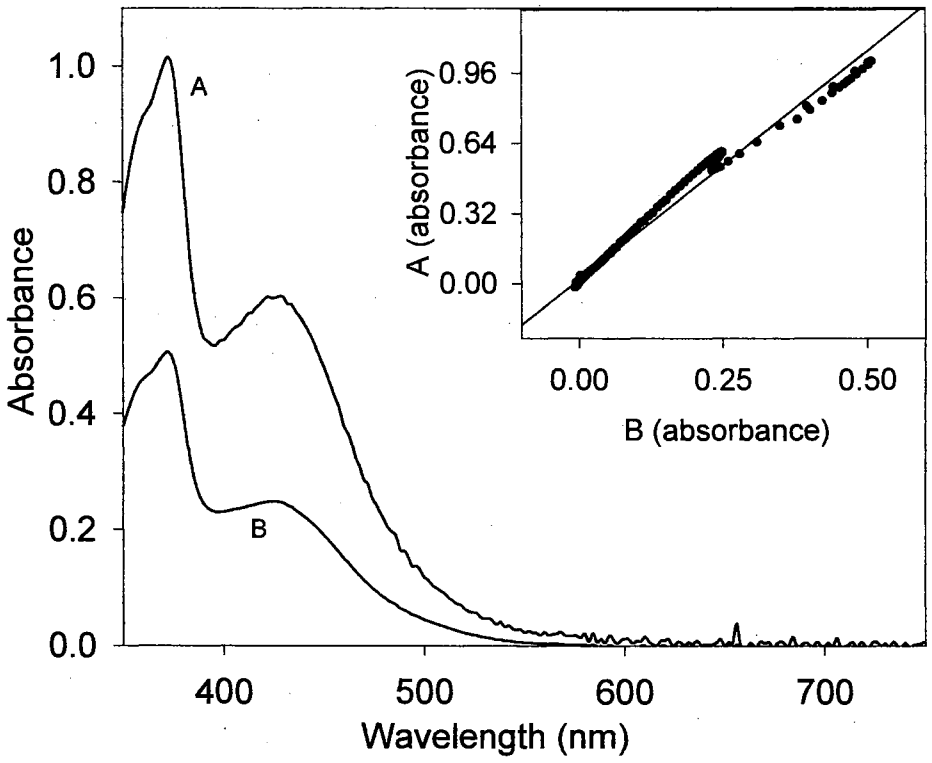

Figure 14. Overlaid A) 10 µl of 0.01 M conjugated methyl linoleate and B) 10 µl of 0.02 M methyl linoleate. Inset cross-correlation plot of the absorbance values of conjugated methyl linoleate versus absorbance values of methyl linoleate.

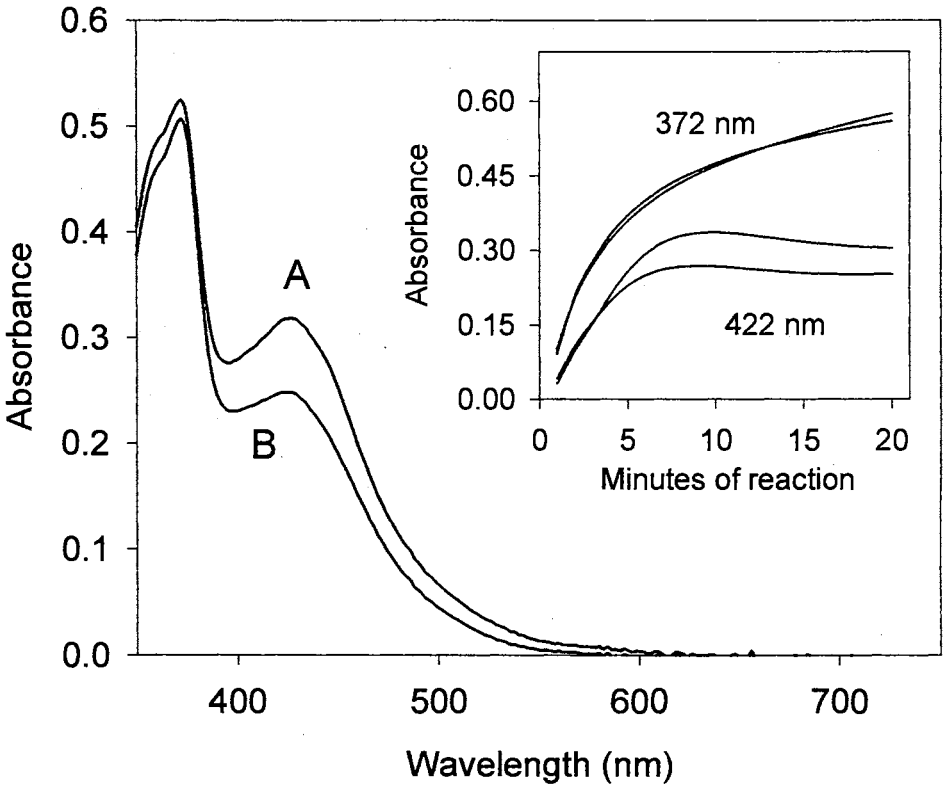

Figure 15. Overlaid spectra of A) linolelaidic acid methyl ester (average of 10 runs) 10 µl 0.02M in 1 mL acetyl chloride with 40 µl perchloric acid and B) linoleic acid methyl ester (average of 9 runs) insert) overlaid kinetics plots for methyl linoleate and methyl linolelaidate at 372 and 422 nm over the 20 minute reaction.

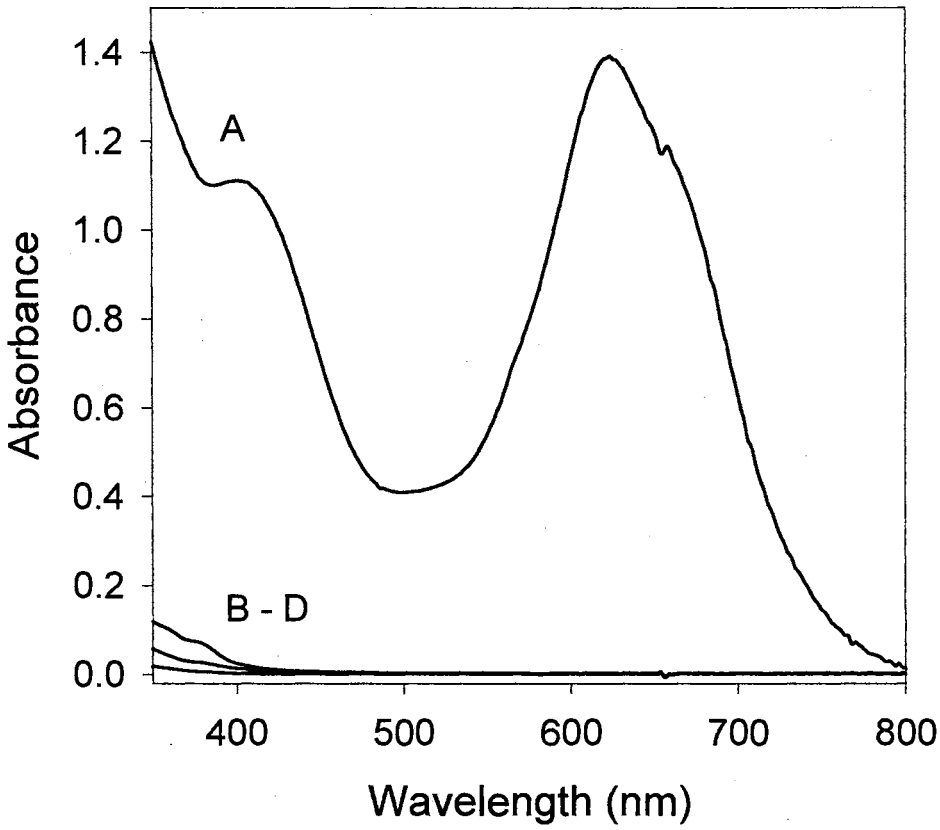

Figure 16. Liebermann-Burchard reaction (1 ml L-B reagent) with A) cholesterol 50 µl of 0.02M B) methyl linolenate 50 µl of 0.02M C) conjugated methyl linoleate 50 µl of 0.02M D) methyl linoleate 50 µl of 0.02M.

Summary

The results presented in Chapter I give strong evidence that proves the selectivity of this reaction in the analysis of unsaturated organic compounds. The reactions on model compounds indicate the selectivity of the reagent for a variety of analytes. The 15 minute spectra of 17 from 26 total steroids (including four saturated cholesteryl esters) can be resolved as unique to the other spectra. Eight of the twelve terpene compounds can be considered unique compared with the other spectra. From the PUFA set of compounds, seven of the eight compounds have unique spectra. The reaction can distinguish among conformational and location isomers. Compounds with differences as small as a single carbon, as in the analysis of 17-alpha-methyl testosterone and testosterone, can be determined.

The results indicate an alternative reaction mode with the selectivity of species that have equivalent unsaturations. The hypothesis before this series of reactions was that the reagent only reacted with double bonds. The current results, especially those of the steroid compounds, raises questions about the influence the unsaturated and heteroatomic structure of an analytes may have on the reaction products and resulting spectra. While the mechanism of the reaction has not been elucidated, the understanding about the nature of the chromogenic reaction has been increased. Both unsaturated and saturated portions of the analyte may contribute to the spectral response of the reaction. This indicates a large increase in the selectivity the reagent has with any given analyte.

The results presented also indicate the additive and quantitative nature of the reagent with a variety of analytes. The compounds considered in Chapter I are potentially important analytes in a variety of analytical applications. The determination

of molar extinction coefficients was done for all of the physiologically relevant PUFA (except methyl oleate). Linearities for all of those PUFA were good with R squared terms above 0.95. Determinations of some of the terpenes indicated limits of detection on the order of $1X10^{-5}$ M and R squared terms around 0.988. Cholesterol has been characterized as linearly related to concentration from the inception of the reagent. Also of importance is the additive nature of the compounds as indicated by the difference spectra of the unsaturated cholesteryl linoleate and cholesteryl linolenate with methyl linoleate and linolenate respectively. This additivity and consequential lack of mutual interference between the analytes is a requirement for the utility of the reagent in the simultaneous determination of mixtures using multivariate analysis methods.

The determination of cholesterol and PUFA is directly related to the analysis of human sera for the determination of disease states. Current clinical methods utilize the determination of cholesterol for the analysis of disease states. The utility of analyzing PUFA is not well developed as a separate field in the current literature, however there are a variety of diseases that result in, or are caused by, a variation in the PUFA profiles of the blood (see introduction Chapter II).

The ability to determine conjugated unsaturated species such as conjugated methyl linoleate is related to the determination of oxidative intermediates and oxidative stress (see introduction Chapter II). If the reagent can be used in the determination of oxidative stress levels, the early detection of a number of oxidative stress related diseases, from atherosclerosis to Alzheimer's disease, may be impacted by the availability of this reagent.

Results from Chapter I indicate the unique nature of the new chromogenic reaction presented. When the results of the standard colorimetric cholesterol reaction, the L-B reaction, is compared to the new chromogenic assay it becomes apparent that the new reaction is different. First, the new reaction creates a larger absorbance with an equal amount of cholesterol indicating an increased molar extinction coefficient resulting in increased sensitivity for cholesterol. The second difference between the two reactions is the inability of the L-B reaction to detect PUFA. This difference may be indicative of a variation in the reaction mechanism. In fact, comparison of the accepted reaction mechanisms for L-B with the results with the new reagent further supports the idea of an alternative reaction mechanism. The dehydration mechanism is not a viable option for compounds such as the unsaturated pentadienes.

CHAPTER II

DETERMINATION OF MIXTURES

Lipoproteins Introduction

Lipoproteins are the carriers of the hydrophobic cholesterol and triglycerides considered in the routine clinical analysis of serum. They are composed of hydrophobic cholesterol esters and triglycerides in the interior of the particle. The more hydrophilic free cholesterol and phospholipids are on the surface of the particle. Also on the surface are apolipoproteins. These charged proteins help make the lipoprotein soluble. They also act in the function and interaction of the lipoprotein within the living system.[26]

Chylomicrons are large lipoproteins that originate in the gut. Chylomicrons are primarily composed of triglycerides (90%). They have four apolipoproteins associated with them including a fragment of the apolipoprotein B-100 called apo B-48 as well as apo A1, C, and E. They are present in large numbers immediately after meals. They are taken up by the liver with the apo E acting in the control mechanism. Chylomicrons are cleared within twelve hours of a meal, and are the reason that fasting is incurred prior to serum sampling.[26]

Very low-density lipoproteins (VLDL) are large lipoproteins originating from the liver. They have apolipoprotein B-100, apo C, and apo E. VLDL is active in the transport of triglycerides to the cells of the body. The VLDL loses triglycerides metabolically, consequently becoming smaller in diameter. During this process, the apolipoprotein C dissociates from the particle. The resulting particle is the intermediate density lipoprotein (IDL).[26]

Fifty percent of the IDL that forms from VLDL is taken up by the liver. Apolipoprotein E facilitates this uptake. The other 50% lose the apolipoprotein E that remained from the VLDL form. Leaving a particle that contains only apolipoprotein B-100 and is high in cholesterol. This new form of IDL is the low-density lipoprotein (LDL) particle.[26]

LDL particles are taken into cells that need cholesterol through endocytosis. This endocytosis is facilitated by the apolipoprotein B-100 (VLDL, IDL, and LDL all have B-100). Roughly sixty seven percent of the TC in the serum is found in LDL particles.[26]

High-density lipoprotein (HDL) particles act in the removal of excess cholesterol from the cardiovascular system. This uptake function is unique to the HDL particle. HDL is formed in the liver, intestine or circulatory system, and contains 20-30% of the total serum cholesterol. The HDL particle contains lecithin-cholesterol acyl transferase (LCAT), which acts to esterify free cholesterol. Apolipoprotein A-1 acts in the cholesterol esterification in conjunction with LCAT. Apo A-1 is the main apolipoprotein in HDL particles. Cholesterol ester transfer protein acts to move esterified cholesterol from HDL to IDL or LDL. The cholesteryl ester is cleared via the uptake mechanisms for these lipoproteins in the liver. Cholesterol is eliminated from the body in the bile as either free cholesterol or bile acids.[26]

Current Lipoproteins Determinations and Diagnostics

The measurement of lipids in sera involves the quantification of TC or total triglycerides (TG) and the determination of the lipoproteins. Currently there are a variety of diagnostic tools for the analysis of lipids and lipoproteins. The current standard method is the enzymatic determination of TC, TG and HDL-C followed by the Friedewald approximation, which calculates the LDL-C. Prior to the enzymatic method, there were chemical methods such as the L-B reaction (see Chapter I) and ultracentrifugation analysis (beta quantification or BQ) of the lipoprotein fractions. These two techniques eventually became the CDC standard methods BQ and Abell-Kendall for cholesterol analysis.

A number of new techniques have become available in the last decade. One of the most important is the direct LDL-C concentration determination method. This *technique* involves the selective precipitation of all lipoproteins except LDL and Lp(a). The LDL and Lp(a) are subsequently measured using the cholesterol enzymatic determination. Other analytical techniques under development for routine use include gradient gel electrophoresis, NMR, and lipoprotein high performance liquid chromatography. These alternative methods do not enjoy the acceptance of the enzymatic Friedewald approximation due to the lack of substantiating studies.

As new analytical tools for lipoprotein analysis are developed, new risk factors for cardiovascular disease need to be addressed. A number of new risk factors are currently in the verification stages. For a risk factor to be considered for routine or semi-routine analyses it must have substantial prospective and retrospective clinical supporting

evidence. The most commonly known new risk factors include trans-fatty acids, apolipoprotein a, and homocysteine. Other future risk factors are fibrinogen, antioxidants, LDL oxidizability, and the subclasses of HDL and LDL lipoproteins.[26]

Standard Lipoprotein Clinical Methods

The current standard methods used for the primary screening of patients uses the enzymatic analysis of TC and TG combined with the Friedewald equation to approximate the LDL-C value. Beyond this initial screening, standard methods for the analysis of dyslipidemias include analysis of lipoproteins. If lipoprotein analysis warrants concern, the primary screening is followed by more specialized tests such as pheno- and genotyping of lipoproteins or quantitative analysis for the presence of chylomicrons in fasting serum. Furthermore, direct determination of the LDL-C can be done using ultracentrifugation or selective precipitation to help verify the dyslipidemic state.

Both plasma and serum measurements are used for the analysis of lipids in the blood. When collecting plasma samples, EDTA treatment for coagulation prevention is preferred to avoid secondary outcomes such as oxidation and bacterial growth. Both cholesterol and triglycerides are 3 % greater in serum than in plasma. HDL-C concentrations are 5-10 % lower in non-fasting serums. This results in an acceptable error on the side of increased risk that is resolvable with further testing.[26] Biological variances can be significant in the routine determination of serum lipids. The (%) biological variances are: TC (6.1), triglycerides (22.6), LDL-C (9.5), and HDL-C (7.4).[27]

The patient should be fasting 10-12 hours prior to the blood draw. The patient should also have avoided alcohol, and the diet of the patient should be normal and steady for 2-3 weeks prior to sampling. In addition, blood work should be avoided during a

period of 2-3 weeks after minor illness. If the patient has undergone a major illness, surgery, or trauma, blood sampling should be avoided for 3 months. The degree of physical activity just prior to taking a venous sample is a variable that has to be controlled. It is recommended the subject be at rest for five minutes prior to the blood draw. Finally, any medication that alters lipid levels should be discontinued, if possible, 3 weeks before samples are taken.[26]

Enzymatic Methods

There are a several variations of the enzymatic methods used to determine the lipids in serum and plasma. The routine method involves enzymatic reactions that lead to products that can then form chromophoric indicators of concentration. An example of this type of diagnostic procedure precipitates the LDL and VLDL lipoproteins with an appropriate agent such as dextran sulfate or phosphotungstic acid. The HDL-C that remains is modified by hydrolyzes of the cholesterol esters using cholesterol esterase. The resulting free cholesterol is converted by cholesterol oxidase to cholest-4-en-3-one and free H_2O_2. The H_2O_2 reacts with precursors, which in turn react with a dye to form the colored product from which quantitative absorbance measurements are made. From the whole serum, the TC is determined separately by the same enzymatic procedure. For TG determination, a separate serum sample is treated with lipase to hydrolyze the ester bonds between the fatty acids and the glycerol molecule. The glycerol is reacted with glycerol kinase to form glycerol-3-phosphate (GP). GP is then reacted with glycerol-3-phosphate oxidase to form dihydroxyacetone phosphate and H_2O_2. The H_2O_2 undergoes a similar reaction as in the determination of cholesterol to give a colorimetric response.

Friedewald Equation

Knowing the TC, HDL-C, and TG concentrations, and from extensive clinical studies presuming the VLDL-C is equal to the 0.2TG the LDL-C is determined according to the Friedewald's approximation:

$$\text{LDL-C} = \text{TC} - 0.2\,\text{TG} - \text{HDL-C} \qquad (1)$$

Unfortunately, the Friedewald's approximation results are never accurate. If the TG is greater than 200 mg/dL, the calculated value of LDL-C compared with a direct measurement is marginal. If the TG is greater than 400 mg/dL, the model fails.[28] If the patient has Type II dyslipidemia or chylomicrons are present the equation is also unreliable.[26] The use of the Friedewald approximation also requires fasting, as the TG levels are uncertain in the presence of chylomicrons. Even if accurate, TG constraints exist. The Friedewald approximation also ignores other potentially significant entities such as Lp(a) lipoprotein, which can lead to inaccurate LDL-C values.[29]

Beta Quantification

The CDC standard method for determining lipoprotein fractions is the BQ procedure. In this procedure, the serum lipoproteins are separated by ultracentrifugation enabling LDL-C to be determined directly. This avoids the problems associated with hypertriglyceridemic patients and the Friedewald approximation. The Friedewald equation is an attempt to approximate the results of direct LDL determination by the BQ. Today direct determination of LDL-C is possible by a variety of isolation methods, however the National Cholesterol Education Program (NCEP) still recommends the

approximation due to an epidemiological study link that demonstrated the importance of LDL-C levels.[30] It is anticipated that eventually the BQ and Friedewald approximation methods will be supplanted by one or more of the direct LDL-C determination methods.

Alternative Lipoprotein Determination Methods

Electrophoresis

Very early in the study of blood lipids physical separations were used to analyze lipoproteins and lipoprotein concentrations. Electrophoresis involves the migration of serum lipoprotein components through a separating media utilizing electromotive force. Electrophoresis of serum components has been used in the analysis of serum components since the beginning of lipoprotein studies. However, the more modern forms of electrophoretic methods have increased their usefulness in lipoprotein analysis. Various separation media can be used for electrophoresis in the analysis of lipoproteins. Common separation media are non-denaturing polyacrylamide and agarose gels. Agarose gel electrophoresis can be used for accurate lipoprotein determinations.[31] Specialized separation media are also used. Immuno-electrophoresis allows selective separation based on specific interaction with the apolipoproteins present and has the potential for direct measurement of apolipoproteins and assays that are more specific.[32, 33] For instance, a modified electrophoresis method allows the determination of glycated lipoproteins as a means for detection of diabetes.[34]

Electrophoresis can be used to directly determine the level of cholesterol of all lipoprotein fractions in sera.[35, 36] Electrophoresis can consequently be used to describe and identify dyslipidemias.[37] Furthermore, it can be used for the determination of

lipoprotein remnants.[38, 39] When electrophoresis of the lipoproteins is done a number of disorders can be elucidated. This is particularly useful for patients with borderline cholesterol levels. If the beta band in the electropherogram is broad it is an indication of Type III dyslipidemia, when a combined hyperlipoproteinemia exists. If the beta and pre-beta bands are resolvable, a patient has Type II-B dyslipidemia, when both cholesterol and triglycerides are at high concentration.[26] If the patient has Type III dyslipidemia the electropherogram can also indicate beta-VLDL, an altered VLDL particle. This is done by first separating the VLDL particles from the other lipoproteins by ultracentrifugation and separating them by electrophoresis. If the VLDL shows beta mobility instead of alpha-2 mobility, they are beta-VLDL. In electrophoresis, any chylomicrons present do not migrate. This fact can also be used to determine chylomicron presence.[26]

Electrophoresis can also give an indication of the charge of the lipoproteins. This is important since the oxidized LDL particles are linked to atherogenesis.[40, 41] The oxidized LDL's surface becomes more electronegative which is detected by increased electrophoretic mobility on the electrophoresis gel.[42, 43] These oxidized LDL particles are termed "LDL-" and they are minimally oxidized with the majority of reactive oxidized species being on the surface of the particle.[44] Electrophoresis, therefore, can be used to assess the level of oxidative stress in a biological system.[45]

Electrophoresis also gives information about lipoprotein particle size. There are variations in LDL size, which are associated with increased risk for atherogenesis.[46, 47] HDL particle size is indicative of the development stage of the lipoprotein and its associated action in cholesterol transport.[48] Electrophoretic methods can determine the

standard subclasses of lipoproteins such as LDL, HDL, VLDL cholesterol without need for precipitation and in an automatable fashion.[49, 50] LDL and HDL subclass deviations are better indicators of CAD than lipoprotein cholesterol levels and this is detectable with electrophoresis almost exclusively.[51-53] LDL subclasses may indicate acute risk of myocardial infarction.[54]

Electrophoresis has many other potential benefits in analysis. For example, standard equipment can be used for the analysis of fatty acids by electrophoresis.[55] The utilization of efficient capillary electrophoresis for the determination of lipoproteins is a developing separation method.[56, 57] Capillary electrophoresis is beneficial in its high throughput potential. Capillary electrophoresis is currently readily automatable and commercially available platforms are available for this type of electrophoresis.

Direct LDL-C Determination

Several direct LDL-C analysis methods currently exist. These methods will allow the analysis of true LDL-C for any patient independent of any dyslipidemic state that may exist. These methods primarily rely on selective precipitation. A number of separation methods have the potential to replace ultracentrifugation including HPLC gel filtration and gradient electrophoresis. Selective precipitations are typically based either on charge neutralization or on antibody binding.[38, 58]

The N-geneous™ LDL-C assay is an example of a direct LDL-C system. It utilizes detergents to selectively solubilize LDL away from the other lipoprotein components. Results have been shown to be very similar to ultracentrifugation[59] and it is not affected by glycemic control. The N-geneous™ system is fully automated and is less

expensive than ultracentrifugation.[60] Direct LDL-C determination methods can be done on people with dyslipidemias, diabetes and are non-fasting with little error.[61-64]

The original studies of cardiovascular risk factors indicated that cholesterol was an independent risk factor. The TC value is enough in cases where the cholesterol concentrations are significantly high or low. A large segment of the patients undergoing cardiovascular screening are borderline patients where TC is misleading in terms of their risk of coronary artery disease (CAD). It is in the intermediate values that the cholesterol lipoprotein distribution is useful in determining the risk for CAD.[65]

If cholesterol is not a suitable risk factor to predict CAD, the lipoproteins are the better predictive risk factors.[65] Specifically, high VLDL-C and LDL-C are both positive risk factors for CAD. High HDL-C concentrations act in a preventive mechanism for CAD.[66, 67] IDL particles are also associated with an increased risk of atherosclerosis[68] It is currently recommended to determine VLDL-C, LDL-C, and HDL-C concentrations before making a diagnosis or prescribing a therapy for the prevention of CAD.[65] The routine determination methods for HDL-C and LDL-C are more time consuming than the determination of TC or TG due to separation steps.[69]

While the lipoprotein fractions are better for borderline patients, for a small number of patients the values for lipoproteins are also not enough to characterize their risk for developing CAD. The subclasses of the lipoproteins are still better as predictive indicators. Lipoprotein subclasses have been characterized in terms of their role in atherogenesis.[70] The LDL particle contribution to atherogenesis largely depends on the "particle size".[54, 71-75] The small LDL particles infer a higher risk of CAD at normal LDL-C concentrations. [65] The associated beneficial effect of HDL does not occur across all of

the HDL subclasses. While the largest HDL particles are protective against atherosclerosis, the two smallest particles are positive risk factors for CAD.[76, 77] The inability of the body to clear chylomicrons is associated with increased risk for coronary heart disease (CHD). The inability to clear chylomicrons causes the presence of larger VLDL lipoprotein particles to appear in fasting serums that correlates with an increased risk of CAD.[78, 79] The lipoprotein subclass profile can demonstrate increased risk for patients with HDL-C and LDL-C values that are considered "normal" by the NCEP definitions.[80] The lipoprotein subclasses are very similar to each other. This results in a 10 time increase in difficulty to speciate them compared to the standard lipoprotein separations.[81]

The determination of VLDL subclasses is not a routine matter and the relationships between the VLDL subclasses and CAD are not well studied. Lipoprotein subclasses can be determined by chemical precipitation, ultracentrifugation, electrophoresis, and chromatography. The analysis of the lipoprotein subfractions requires either a separation or a marker specific for each of the subclasses.[65]

NMR Analysis

NMR can determine the size of lipoprotein particles based on the relative nuclear magnetic resonance shifts.[65] The NMR procedure utilizes the absorbance shift in the methyl group resonance for each different particle size. These different resonances are due to the oriented phospholipids on the surface of the lipoprotein.[82]

One limiting factor of NMR is that the particles are only determined by size and therefore any overlap in the size of the particles, whether of the same or different

lipoprotein classes, are viewed as equivalent. In addition, any interesting chemical variation is lost in NMR analysis. This can be important, for example, because "chylomicron remnants" and VLDL particles are of a similar size. In addition, the apolipoprotein is not distinguished so important variants like Lp(a) derived from LDL are not represented in the data.[65]

Because the NMR method only determines the particles based on their size, errors occur when translating the NMR data to cholesterol concentrations found in the various lipoproteins. The values determined by the standard methods for LDL-C and HDL-C ignore the variation of the concentration of cholesterol in the particles,[83] i.e. only cholesterol in the lipoprotein fraction is determined. The number of LDL particles is determined assuming the LDL particle contains a standard amount of cholesterol. In the case of NMR this source of error in LDL particle number determination is avoided. The actual cholesterol concentrations of LDL and LDL subclasses are determined by analysis of a "bulk lipid signal" from NMR analysis which does not vary based on the transfer of cholesterol from the core of the particle. Therefore, traditional data for the association of lipoproteins to diseases are based on the cholesterol found in the particle. The actual number of lipoproteins is not the standard risk factor. Further, the determination of cholesterol from the total number of lipoproteins is complicated by the same variation of cholesterol that makes the NMR analysis more rigorous.[65]

While the NMR method is limited in a number of important ways, the method does have a few advantages. For example, chylomicrons can be determined with NMR so having a fasting sample is not necessarily needed.[65] NMR also has the potential to do direct determination of conjugated fatty acids in a mixture with pure fatty acids.[84] The

ability of NMR to determine species with specific resonances may prove extremely powerful in the analysis of chemical components of blood. Another limiting factor is that the analysis is based on methyl resonances only and is not specific and readily susceptible to interferences.[65]

Why Lipoproteins are the Current Model for Diseases

Introduction to Dyslipidemias

There are varieties of classification systems for lipid disorders. One system is the Fredrickson classification.[85, 86] Dyslipidemias can be simply classified as hypercholesterolemia, hypertriglyceridemia, high LDL-C, low HDL-C, or some combination of the above. This simplified system ignores the fact that the dyslipidemia can be primary (genetic) or secondary (non-genetic).[26] Some secondary causes of hypertriglyceridemia are obesity, pregnancy, and diabetes mellitus. Some secondary causes of hypercholesterolemia are hypothyroidism, nephrotic syndrome, and cholestasis.[26]

Primary causes of hypercholesterolemia include a single gene mutation in LDL receptors.[87] If this disorder is homozygote the TC levels can rocket to 600-1000 mg/dL. In the heterozygote form TC levels are much lower (300-450 mg/dL). The heterozygote form of the disorder involves a one-half decrease in LDL receptor function.[26] The homozygote form of this disorder can be devastating to the patient who suffers from tendon xanthoma and has atherosclerosis before midlife.[26] The mutations that cause this disorder are well understood.[87].

Hypertriglyceridemia, when taken independently, is a risk factor for CAD. However, when TG concentrations are incorporated with other risk factors its performance as a risk factor falls below acceptable limits.[85, 86] Complex algorithms have been developed to explain the loss of relevance in TG concentration when combined with other factors.[88] A primary (genetic) cause of hypertriglyceridemia is Type IV dyslipidemia. Type IV dyslipidemia is autosomal dominant and results in overproduction of triglycerides in the liver. A characteristic of this dyslipidemia is the presence of larger VLDL particles due to the extra triglycerides available at the liver. There are no biochemical markers for this disorder and there is no evidence to link this disorder to increased risk of CAD. The serum TG levels tend to be less than 600 mg/dL and the LDL-C concentrations tend to be within the normal range.[26] Combined these symptoms make it difficult to diagnose the condition given no specific marker.

TG levels above 1000 mg/dL are associated with chylomicronemia syndrome. Such high levels of TG mean the patient could have pancreatitis.[26] Chylomicronemia can be caused by lowered function of lipoprotein lipase (LPL).[89] This lowered level of enzyme function may be due to lower levels caused by an autosomal recessive disorder, or by lowered level of apolipoprotein C-II that is necessary for LPL to function. LPL deficiency shows up in childhood, and is not linked to early atherosclerosis. Low levels of apolipoprotein C-II is a rare disorder caused by an autosomal recessive gene.[89]

Another cause of chylomicronemia is Type V dyslipidemia. This is especially relevant to the adult onset of chylomicronemia.[26] These cases require the presence of familial hypertriglyceridemia. To this existing disorder some secondary cause (obesity, alcohol, etc) facilitates the chylomicronemia onset. Type V disorder is marked by high

VLDL-C concentrations and the presence of chylomicrons in fasting serums. Type V is genetic in its nature but the details of how it is inherited are not clear.[26]

Combined dyslipidemias occur when both TC and TG levels are above normal. There are two main subcategories of combined dyslipidemia. The first is combined familial hyperlipidemia (CFH); the most common form of combined dyslipidemia.[85, 86, 90, 91] CFH is also called "familial multiple lipoprotein-type hyperlipidemia". This disorder can cause increased concentrations of cholesterol, triglycerides, or both. CFH is autosomal dominant, and causes overproduction of apolipoprotein B-100. Consequently, increased levels of apolipoprotein B-100 are found in VLDL, IDL, and LDL lipoproteins. Patients with CFH have increased risk of atherosclerosis. The diagnosis of this disorder relies on the presence of low HDL-C concentrations and elevated apolipoprotein B-100 levels in the serum.[26] A similar disorder to CFH is hyperapobetalipoproteinemia where there are normal levels of LDL-C and increased levels of apolipoprotein B-100. This disorder is associated with increased risk for CAD.[92]

The second main subcategory of combined dyslipidemias is Type III disorder, also called dysbetalipoproteinemia. In this disorder, the patient is homozygous for the apolipoprotein E-2 gene rather than the normal apolipoprotein E-3. This difference in alleles does not directly cause Type III disorder. One percent of the population is homozygous for apolipoprotein E-2. It requires secondary causes to lead to the onset of Type III disorder. Secondary causes such as obesity or diabetes can lead to the onset of Type III disorder. Apolipoprotein A-2 is not as good at inducing uptake at the liver as the other forms of apolipoproteins E. This results in decreased chylomicron and IDL particle uptake by the liver and their subsequent increased concentration in the circulatory

system.[26] Unique to Type III disorder is the presence of a "broad beta band:" in the electropherogram of the lipoproteins. Due to the high concentration of chylomicrons the analysis of this sample requires direct determination of VLDL-C or LDL-C given the TG concentrations are beyond the working range of the Friedewald approximation. A confirming measure is if the VLDL-C versus TG ratio is greater or equal to 0.30. Type III patients will experience increased atherosclerosis.[93]

In hyperapobetalipoproteinemia, the LDL lipoprotein tends to be the smaller, denser version. These smaller LDL particles are found with many disorders including hypertriglyceridemia, insulin resistance, glucose intolerance, and hypertension. This presence of small dense LDL particles in so many disorders may indicate a pathological relationship between these special LDL lipoproteins and the disorders. The prevalence of this phenomenon has lead to it being called syndrome X by researchers.[94]

The interest in the relationship between serum lipids and their relationship to disease is divided into two fields of study. The first is the study of lipoprotein composition and the second is the study of actual fatty acid composition. Lipoproteinology is currently the model used as a marker for a number of diseases including atherosclerosis, CHD, and susceptibility to stroke. The current standards for lipoprotein cholesterol were reiterated in a report published in 2002 by the NCEP. In the report the standards for lipoprotein cholesterol and serum total values of LDL-C less than 100 mg/dL; TG less than 150 mg/dL; and HDL-C is greater than 40 mg/dL are recommended as the normal range.[28]

The NCEP has issued three Adult Treatment Panels (ATP) since it's inception in 1983. The variation of the standard values is of interest. In the ATPIII (2002) the value

for TG is lower than the value espoused in the ATPII (1993) of 200 mg/dL. ATPII had already lowered the TG value from 400 mg/dL recommended in ATPI (1983). These variations indicate the continued development of the models underlying these values.[28] The need for variation in the cutoff values is made clear by the fact that in 1999 thirty three percent of the patients with CHD had "normal" LDL-C values defined in the ATPII.[95] The ATPII report recommended alternative LDL-C determination if the cholesterol level is normal (<200 mg/dL) but the HDL-C is less than 35 mg/dL; TC is borderline (200-239 mg/dL) and two or more risk factors are present; if TC is greater or equal to 240 mg/dL.[26]

There are many risk factors associated with CAD. One of the risk factors is age. There is an increased risk for CAD in men 45 years and older, and for women 55 years and older. Women with premature menopause without estrogen replacement therapy are considered at greater risk before 55 years-of-age. Another risk factor is a family history of CAD. Smoking is one of the most controllable risk factors. Hypertension and diabetes mellitus are diseases that increase the risk of CAD. If HDL-C is greater or equal to 60 mg/dL this counts as a negative risk factor, i.e. HDL-C is greater or equal to 60 mg/dL helps protect against CAD.[26]

The risk factors currently used may be supplanted or supported by a number of risk factors now being evaluated. These include apolipoprotein concentrations in the blood. For example, higher apolipoprotein B and lower apolipoprotein A-1 concentrations, resulting in a low apo A-1 / apo B ratio, is a marker for CAD. Prospective studies are still necessary for this measure to be accepted as a routine evaluation method.[33, 96] Standardization in measuring apolipoproteins is not complete but

in general, progress is being made. The accurate measurement of apolipoprotein B-100 and apolipoprotein A-1 will be a method in the future.[97] Apolipoprotein B-100 measurement will help diagnose hyperapobetalipoproteinemia that results in atherosclerosis in patients with normal LDL-C concentrations.[26] Apolipoprotein(a) (Apo(a)) is an important apolipoprotein[98-101] found in lipoprotein(a) (Lp(a)). Lp(a) is similar to LDL in lipid composition and apolipoproteins except the addition of Apo(a). Apo(a) is a glycosylated lipoprotein that exists with disulfide bonds linking it to the apolipoprotein B-100 in the surface layer.[26] Apo(a) has high amino acid sequence identity with plasminogen. This fact may prove important for determination of the origin of Apo(a). Apo(a) levels are not readily influenced by external factors. It seems to be under genetic control.[26]

A large number of studies, involving various designs, have shown correlation between LDL-C and atherosclerosis. Some of these studies have shown the effectiveness of treatments that lower LDL-C on CAD.[102, 103]

The Framingham study showed that low HDL-C is a risk factor for CAD.[66] Lowered HDL-C can result from secondary factors including smoking, diabetes, and obesity. A primary (genetic) cause of lowered HDL-C is hypoalphalipoproteinemia.[26] Exceptions to the trend of low HDL-C as beneficial do exist. Individuals with apolipoprotein A-1 mutation have lowered HDL-C concentrations but no increased risk of atherosclerosis.[88] Hyperalphalipoproteinemia produces HDL-C levels greater than 70 mg/dL. These individuals seem to be at lower risk for CAD.[26]

Alternatives to Lipoprotein Analysis

Total Lipids Determination and Human Diagnostics

The lipid profile of the serum has been investigated in a variety of studies utilizing a variety of analytical methods. The most common methods are chromatographic methods: high performance liquid chromatography (HPLC), gas chromatography (GC), and thin layer chromatography (TLC). The results of these studies are largely dependent on the hypothesis being tested. Overall, these studies give an indication of the importance of the serum fatty acid composition in relation to disease states.

How Do Unsaturations and Cholesterol Affect Health

PUFAs are known to affect the lipid composition of the serum. Fish oil, high in PUFAs, lowers levels of VLDL and IDL lipoproteins when taken at 1 g/day.[104] Fish oil has also been used successfully in the treatment of type IV, V, combined and chylomicronemia dyslipidemias.[105,106] Fish oil can counter the results of hypertriglyceridemia that is associated with carbohydrate intake.[107] In the liver of "hyperlipidemic rats" docosahexaenoic fatty acid (DHA) prevents the excessive production of lipids.[108]

Omega-3 fatty acids are essential dietary components related to cancer development and treatment. Cancer is related to diet 20-60% of the time, depending on the type of cancer.[109] Prospective studies have suggested fish supplementation can be protective for women against colorectal cancer.[110] When challenged with carcinogens, eicosapentaenoic acid (EPA) and DHA inhibit carcinogenesis in the colon of rats.[111] Fish

oil gave similar results in carcinogen challenged rat models.[112] HT29 human cancer cells in the nude mouse colon also responded with in a positive way to fish oil treatment.[113] Liver cancer rat models had positive effects for n-3 PUFA and n-6 PUFA on adenoma but not carcinoma. In this study, the PUFA source was plant based and therefore did not contain EPA or DHA.[114] A study has shown positive effects when hepatic carcinomas are treated with EPA and DHA ethyl esters.[115]

The mechanism of action for EPA is that it prevents cell proliferation, and DHA acts to stimulate apoptosis.[116] The effect of n-3 PUFA on breast cancer is less clear. Animal models showed that EPA and DHA decreased the chance of breast cancer but they did not prevent cancer proliferation.[117] A review of data from 1966 to 1994 indicates that n-6 PUFA and saturated fatty acids are associated with increased growth of breast cancer, while n-3 PUFA are slightly inhibitory.[118] Overall, the lack of congruence between the studies is likely due to confounding factors not considered in all studies.[116] n-3 PUFA does prevent metastasis of breast cancer.[119-121] EPA prevented cancer growth and metastasis in rat breast cancer models.[119] Breast cancer growth and lung metastasis is prevented with menhaden fish oil supplementation.[120] Using transplanted tumor models the protective effect on n-3 fatty acids are seen with inhibition of breast cancer proliferation and metastasis.[121] EPA treatment inhibits the metastasis of liver cancer.[122] However, fish oil treatment resulted in promotion of metastasis in comparison to a safflower diet.[123]

In mouse models lung cancer also has increased metastasis with n-3 PUFA supplementation compared with n-6 PUFA or saturated oil diet.[124] In mouse models of

highly metastatic cancer cells EPA and DHA supplementation resulted in lower lung metastasis when compared to linoleic and arachidonic acids.[125]

The beneficial effect of PUFA is associated with increased PUFA peroxidation.[126] The ability of fish oil to prevent mouse carried breast cancer is limited by vitamin E.[127] Vitamin E supplementation increased the likelihood of cancer and the proliferation of the carcinoma.[128] In a study seeking to increase lipid peroxidation and evaluate its effect on human breast cancer cells in mouse hosts, the increase of peroxidation by added iron and a peroxidation inducing drug limited cancer cell growth proportionally.[129] The cytotoxicity of PUFA oxidative products is a significant factor. Hydroperoxides of unsaturated fatty acids are cytotoxic. The ability of DHA to affect cancer cells depends on the cells ability to counter oxidation processes. The "hydroperoxy-DHA" species is a major cytotoxic byproduct of DHA peroxidation.[130] Sarcoma cells were treated with "n-3 PUFA (DHA>ALA>EPA)" and the n-3 PUFA acted to inhibit cell growth. Vitamin E inhibited the toxic effect of these fatty acids while lipid peroxidation was increased with all of the fatty acids in the study.[131] Most leukemia cells are affected by arachidonic acid, EPA, and DHA. The leukemia cells die by both necrosis and apoptosis. EPA causes necrosis rather than apoptosis. The effect of EPA is counteracted by the presence of vitamin E.[132]

Malignant pancreas cells are induced to undergo apoptosis by EPA, DHA and n-6 arachidonic acid in vitro. In these cases apoptosis depended on lipid oxidation.[133] Mitomycin C is an anticancer drug that causes increased oxidation in vivo. EPA has been shown to increase the effectiveness of mitomycin C. The increased unsaturated fatty acids in the tumor allowed for higher toxicity in the tumor cell.[134]. Dietary intake of "pro-

oxidants" facilitated the effectiveness of anthracyclins against breast cancer. Addition of dietary vitamin E inhibited this effect.[135] The use of DHA and EPA also allow the relief of cardiotoxic effects of anthracyclins in rat models.[136] The amount of n-3 PUFA in breast adipose is directly correlated to chemotherapy response. The level of DHA was high in patients who responded well to chemotherapy and low in people who did not respond well to chemotherapy.[137] Anthracyclin effectiveness increased with increased levels of DHA. Lipid peroxides and lipid peroxidation products are therefore associated with the mechanism of drug effectiveness and the use of these factors can assist in future chemotherapy treatment schemes.[138] The use of pro-oxidants combined with fish oil supplementation effectively slowed breast cancer cells in mice.[129]

The increased effectiveness of chemotherapy with n-3 PUFA may not be universally due to peroxidation. Cyclophosphamide has enhanced in vivo performance with menhaden oil due to the effect of the PUFA in modulating liver detoxifying enzyme function and altering liver and carcinoma enzymes that activate cyclophosphamide.[139] The presence of PUFA in cancer cells also assist in the toxic effects of radiation treatment.[140] Treatment of rat hosted cancer cells as treated by hyperthermia is enhanced with addition of ALA and GLA.[141] In the radiation treatment of cancerous astrocytoma cells n-6 GLA and n-3 EPA and DHA all increase tumor toxicity especially GLA.[142] GLA is toxic only to "neoplastic astrocytoma cells" not normal astrocytes making it a good candidate for cotreatment of these cancer cells with radiation therapy.[143]

EPA and DHA are taken into membranes of rat cells without making them more susceptible to peroxidation.[144] n-3 PUFA is taken into several parts of rat liver cells rapidly.[145] The "physiological conditions" in which the PUFA exists is important since

antioxidants can act as prooxidants depending on the partial pressure of oxygen.[146] The levels of n-3 PUFA can be manipulated with diet to give optimum n-3/n-6 PUFA ratios within a few months. This effect can have benefits for several types of cancer especially breast cancer.[147] It is vital that n-3 PUFA effect be considered in terms of n-6 PUFA and antioxidant components. The toxicity of PUFA is higher for cancer cells than normal cells. This is probably due to the lower levels of antioxidants during "malignant transformations".[116]

Lipid Oxidation

Lipids undergo oxidation in the mitochondria in order to be used as energy sources. Lipoxygenases are the enzymes that induce hydrogen removal and dioxygen reaction in a regiospecific manner. The reaction is very similar to lipid autoxidation except it is directed to a specific area of the lipid.[148]

Autoxidation

Unsaturated lipids can undergo autoxidation through peroxidation. The process involves the introduction of both oxygen atoms of diatomic oxygen onto the carbon chain of the lipid in a free radical mechanism.[149] Reactive oxygen species (ROS) can induce a carbon radical in the chain by heterolytically removing hydrogen from the carbon.

$$-CH_2CH=CHCH_2- \rightarrow -CH=CH-\dot{C}H-CH_2- + H^{\bullet} \quad (2)$$

The initial reaction is important because the initiation rate strongly influences the reaction products.[150] Normally unsaturations in lipids are methylene-bridged. However, when the free radical is formed in the initiation of lipid peroxidation the double bonds rearrange to eliminate the methylene group to become conjugated. The free radicals react with dioxygen forming peroxide radicals.[149]

$$-CH_2CH{=}CHCH{=}CH^{\bullet}CH- + O_2 \rightarrow \ldots CH{=}CHCHOO^{\bullet} \qquad (3)$$

The peroxide radical can abstract hydrogen form another carbon chain. The nature of these free radical reactions dictates that large numbers of "initiations" can be achieved by this regeneration of initiator. If the chain has more than one methylene carbon between the double bonds, they will not rearrange into conjugation.[149] Peroxides tend to form on the outside methylene at the two ends of the unsaturated regions. They are less likely to form between double bonds on the methylene bridge carbon.[151]

Transition metals can catalyze the formation of alkylperoxy radicals or alkoxy radicals from existing lipid peroxides. The alkoxy radical can further react to form aldehyde, ketone, and hydroxy containing products. Alkoxy radicals can also breakdown through chain cleavage producing "short chain aldehydes".[149] Linoleate autoxidation can form compounds that are unsaturated aldehydes, such as hept-2-enal, oct-2-enal, and deca-2,4-enal. Linolenate autoxidation can form products such as deca-2,4,7-trienal, hepta-2,4-dienal, prop-2-enal, and pent-2-enal.[152] The unsaturated ketone is ubiquitous in peroxidation breakdown. Malondialdehyde (MDA) is an autoxidation product commonly considered in analytical techniques for oxidation. MDA is produced from a lipid that is

at least triunsaturated. The rearrangement of the middle unsaturation to conjugation with either of the other double bonds is followed by peroxide formation between the double bonds. The peroxide is located closest to the conjugated double bonds leaving a methylene group between the peroxy-carbon and the third unsaturation. In this position, the peroxy radical can form a peroxy bridge with the third unsaturation. This peroxy bridge decomposes into two ketones with the concomitant scission of the bonds beyond the alpha carbons to each ketone. Determination of the extent of oxidative degradation is achieved by monitoring the products of the autoxidation reactions. MDA is a common analyte. As with all free radical reactions the end of the reaction comes with a termination step. This involves the reaction of the reactive species on carbon chains with other free radicals to terminate both reactive species. The overall result is lipid cross-linking through carbon-carbon bonds and peroxides.[149]

Aldehydes can form protein adducts via protein free radical oxidation or Michael Addition of conjugated aldehydes with protein thiol groups. The thiol attacks the carbon of the double bond forming an enol that tautomerizes into an unsaturated aldehyde. These reactions result in unnatural proteins with lowered or lost functionality, making unsaturated aldehyde adducts toxic to cells. The cytoplasmic concentration of total "carbonyls" has been estimated as a means of detecting lipid oxidation. Lipid peroxidation products are hydrophobic. There is, therefore, an increased level of them in the cell membranes. DNA adducts can also be formed with aldehydes such as hex-2-enal and malonyldialdehyde.[149] This leads to abnormal DNA processing and mutagenesis.

In lipid peroxidation reactions of serum samples, linoleic acid is the predominant fatty acid and therefore is the predominant form of oxidized fatty acid in the serum.

Analysis of the oxidation of LDL has demonstrated the importance of the oxidized form of linoleic acid in oxLDL formation.[153]

Current Determination of Lipid Autoxidation and Oxidative Stress

Currently, the methods for the determination of lipid peroxidation in foodstuffs and medical applications are well developed, and include chemical, immunochemical, and separation methods for the determination of the extent of oxidation of lipids in given situations. UV/Visible, fluorescence and chemiluminescence are all used for analysis of autoxidation products.[149] Headspace GC is also a commonly used technique.[154].

A variety of methods are used and preferred in the determination of autoxidation of foodstuffs. Peroxide value, hexanal and MDA concentrations are among the most common.[155] Thiobarbituric acid reactive species (TBARS), lipid peroxide, fluorescence and volatiles (including hexanal) are also used.[149] Monitoring the consumption of oxygen is useful for foodstuff but is of little use for autoxidation determinations in vivo.[156] Physiological determinations of TBARS is currently the most commonly used method.[149]

As was described earlier, the formation of free radical sites in unsaturated lipids leads to conjugated double bonds. These double bonds absorb light in the UV region at 234 nm. Detection at 234 nm can be used to determine conjugated dienes in a variety of samples. Interference by biological compounds that also absorb in the 234 nm region has seriously limited the use of UV detection. Extraction methods have been used to alleviate this problem.[149] With the advent of second derivative spectroscopic techniques the elimination of the extraction procedures is possible. Methylene bridged dienes absorb at 210 nm, trans-trans conjugated dienes absorb at 234 nm, cis-trans conjugated dienes absorb at 244 nm and ketone dienes absorb at 280 nm.[156]

Determination of peroxides is another procedure commonly used for the determination of oxidation. In foodstuffs the procedure involves the dissolution of the sample into a chloroform / acetic acid mixture. This solution is reacted with potassium iodide. The peroxide acts to form iodine that is reduced with a suitable agent by titration to form iodide. This redox titration is monitored with starch as the indicator.[149] The endpoint of the titration occurs when the dark blue starch complex disappears. The iodine determination can also be monitored with spectrophotometric methods.[157] Enzymatic detection of peroxides with horseradish peroxidase type II is also possible.[158] Peroxides can also be determined with chemiluminescence.[159-161] In one study the link between toxicity of the cancer drug doxorubicin and lipid peroxidation was determined using a chemiluminescent method.[162] Chemiluminescence methods use luminol in reaction with hydroperoxides.[149] Fluorescence is achieved using fluorescent indicators like Diphenyl-1-pyrenylphosphine.[33, 163]

A major class of lipid peroxidation products is carbonyl compounds. Reviews have been written on aldehyde containing products.[164] Determination of carbonyl containing compounds is commonly done by derivatizing the carbonyl with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazones. These 2,4-dinitrophenylhydrazones are detected with UV/Vis spectrophotometry.[149] This system has been used in spectrophotometric detection in HPLC separations of 2,4-dinitrophenylhydrazone aldehyde derivatives.[165] Currently an immunochemical method is being used to detect carbonyl species where the antibody binds directly to the 2,4-dinitrophenylhydrazones. This allows the detection of the carbonyl species within the

context of a living cell.[166] Fluorescence determinations are achieved by forming decahydroacridine derivatives[164, 167] or by other derivatizations.[168]

MDA concentrations can be determined but they are not indicative of lipid peroxidation levels in vivo.[164] A very small amount of MDA can be produced from the diunsaturated linoleic acid.[149] MDA is not the most common peroxidation product.

Summary

This review summarizes current routine clinical methods. It presents the importance placed on lipoprotein determination, and emphasizes the current and developing methods for lipoprotein isolation and quantification. It reviews why determinations of TC and lipoprotein fractions have been traditionally used for the determination of lipids in the sera. The association of these values to a variety of lipids disease states (dyslipidemias) is also discussed.

. The methods of lipoprotein analysis are largely dependent on such techniques as centrifugation, electrophoresis, or selective precipitations. These isolation methods have made the analysis of lipoproteins accessible for the last few decades. The utility of lipoprotein data has been demonstrated in cross-sectional clinical studies to predict risk of CAD. Methods under development, including direct determinations of LDL-C, are dependent on similar clinical studies to prove the reliability of the method for the determination of risk. In fact, every new method, including the one presented in this work, requires such clinical verification.

Methods to separate and analyze individual lipid components have been available for several decades. Lipid components in this sense means cholesterol, its esters, polyunsaturated fatty acids in a transesterified state, etc. The analysis of these lipid species is a tool currently used for the analysis of diseases with associated variations of blood lipids. These analyses have depended primarily on isolation of the species by separation methods. The use of lipid profiles for the prediction, determination or monitoring of disease states is not currently an organized field of scientific study.

That the direct determination of lipids by means of the chemical assay described in this work can be used for the determination of disease states is supported by the literature. The ability of the present method to simultaneously determine the lipid components of sera is unprecedented. The assay can determine mono- and polyunsaturated fatty acid compositions as well as having a superior detection limit over the L-B reaction for the determination of TC. Cholesterol is still considered the overriding determinant of cardiovascular risk. High TC is predictive of risk for CAD regardless of the lipoproteins concentrations. Combined with the ability to determine levels of the PUFA, which are known to be associated with variations of lipoprotein ratios and subsequently cardiovascular risk, the chromogenic assay has the possiblity to revolutionize routine clinical analyses.

Beyond the known analytes of cholesterol and PUFA, there are other beneficial analytes potentially determinable with the reagent. Spectral responses of the reaction have demonstrated positional and enantio-specificity potentially allowing determination of cis vs trans and conjugated double bond isomers. The presence of conjugated double bonds is indicative of oxidative stress, and oxidative stress is associated with a variety of diseases in humans from atherosclerosis to diabetes. With all of the disease implications of the known analytes detected by this assay when combined with the known capability of the assay to cluster diabetics from a set of 100 normal and dyslipidemic patients (unpublished results) further emphasizes the potential of the assay as an alternative routine clinical assay.

Mixture Determination

Introduction

In order to determine the components of a mixture from a spectrum of the mixture a multivariate analysis method must be used. The simplest of the multivariate analysis methods is MLR. In MLR, the components of the mixture and the molar extinction coefficients of all components must be known. In the application of the chromogenic reaction for analysis of serums, the components absorbing the spectra are known. Once calibration data is determined for the full spectrum of each component, MLR should produce an accurate result for the concentration of each component.

Studying synthetic mixtures is the first approach to the development of a real calibration system for the analysis of serum spectra can be developed. This study uses mixtures of lipids at concentration percentages of each component corresponding to the amounts found in actual serum samples. MLR analysis of the synthetic mixtures is then used to determine the feasibility of such a calibration system for real samples.

Multiple Linear Regression

MLR is the most basic of the multivariate analysis methods. The technique involves solving linear equations to determine the concentrations of the components that compose a mixture. The technique is accurate and resilient if all components of the mixture are known and appropriate calibration data are available for each component. In multiple linear regression, the dependent variable is related to a group of independent variables. For systems obeying Beer's law:

$$A = abc \qquad (4)$$

Where A is the absorbance, a is the molar extinction coefficient, b is the pathlength and c is the concentration of the absorbing species. The dependent variable is the absorbance (A) at any wavelength. The absorbance (A) of a mixture is dependent on the sum of absorbance contributions from all of the components at that wavelength:

$$A = a_1 \times c_1 + a_2 \times c_2 + ...a_x + c_x + e \qquad (5)$$

Here b is a consistent pathlength of 10 mm. The above calculation can be done at all wavelengths by solving the simultaneous linear equations, and is easily achieved using matrix algebra. In matrix form equation 2 becomes:

$$A = Xc \qquad (6)$$

Where A is the spectral response from the mixture at all wavelengths, X is the molar absorptivity matrix for all components of the mixture at all wavelengths (also known as the K matrix) and c is equal to the molar concentration vector.

To determine the concentration for each component given the spectra A, solve equation (6) algebraically:

$$c = X^{-1}A \qquad (7)$$

In matrix algebra, the inverse of a non-square matrix does not equal to the algebraic inverse. It is necessary to calculate a "pseudo-inverse" instead. The pseudo-inverse is equal to:

$$X^{-1} = (X'X)^{-1}X' \qquad (8)$$

Where X' is the transpose of the matrix X. Substituting (5) into (4), we get the solvable equation:

$$c = (X'X)^{-1}X'A \qquad (9)$$

In spectroscopic determinations, equation (9) is adequate if the components are present in large enough quantities to contribute to the resulting spectrum. In the analysis of PUFA at concentrations found in "normal" sera, some of the components are present in exceedingly small amounts. When equation (6) was used in the analysis the resulting concentration vector contained values less than zero. The Matlab function lsqnonneg does multiple linear regression analysis while avoiding errors caused by fitting a curve with data that is not significantly represented in the spectra. Lsqnonneg works by doing the minimization of the residual between the actual and calculated spectrum defined as;

$$norm(C \times X - d) \qquad (10)$$

Where C is the K matrix, d is the spectrum that is being fitted, and X is the concentration result of the lsqnonneg calculation. C times X creates a theoretical spectrum representing the concentrations determined by MLR.

For lsqnonneg the values in X are required to be greater than zero. The residuals between the calculated spectra and the actual spectra were evaluated as;

$$Y = norm(C \times lsqnonneg(C,d) - d) \qquad (11)$$

Where C is the K matrix, d is the spectrum that is being fitted, and Y is the residual between the two.

Negative outcomes influence the results for components that are in larger amounts and are adequately represented in the spectrum being analyzed. Lsqnonneg interprets negative results as "out of range" and automatically removes the absorptivity data for those components from the calculation and recalculates without these data, thus relying entirely on the remaining components of the K matrix. The resulting concentration matrix has a zero in place of any negative values. The resulting solutions for the remaining components give a poorer fit to the calculated curve but relieve errors associated by the use of negative concentration values.

The K matrix must contain linearly independent vectors. If the K matrix does not contain linearly independent vectors separation of the individual components would not be possible. In this work, the K matrix was determined to have the rank equal to the number of matrix columns (7), which indicates that the columns of the K matrix adequately represent unique components.

Methods

Mixture Preparations

Mixtures of the seven most common lipid components, methyl linoleate, methyl linolenate, methyl arachidonate, methyl eicosapentaenoate, methyl docosahexaenoate, methyl conjugated linoleate, and free cholesterol were created by approximating the percentages of the PUFAs present in what were considered to be "normal" sera. Stock solutions for each PUFA, based on these percentages, were made with a maximum total PUFA concentration equal to 0.02 M. The total concentration of 0.02 M was chosen so that the spectral response in the lower wavelengths would be between 0.2 and 0.9 absorbance units. The concentration of cholesterol used was also approximated to that found in a "normal" serum. Stock solutions were first made by dissolving the analyte in chloroform. The chloroform in the mixture was then evaporated under a stream of nitrogen. The resulting oily samples were then mixed with 1.0 mL pure AC and the reaction was initiated by 40 μL PA. Absorbance data were measured at 15 minutes into the reaction.

Ratios of PUFA in "normal" sera were calculated using references that summarize human population fatty acid profiles data.[44, 169, 170] Percentages were determined by dividing the concentration of the individual PUFA by the total PUFA concentration. Only PUFA were considered, saturated and monounsaturated fatty acids were not incorporated into the data.

Reaction of Mixtures with Perchloric Acid Catalyst

Analysis was conducted by adding 2, 5 or 10 μL of each proportional analyte stock solution to a 13 X 100 mm borosilicate disposable test tube. The chloroform was removed with a stream of nitrogen. Pure AC (1.0 mL) was added to the analyte mixture. The reaction was initiated by the addition of 40 μL PA. The reaction timer was started immediately after addition of catalyst. The reaction was energetic thus caution was taken to avoid bumping. The reaction was mixed by hand vortexing for 10 seconds and then either placed in the cuvette for kinetic determination over 20 minutes or develop in the tube to later be analyzed at the 15-minute endpoint. Following maturation in the test tube the solution was transferred to the cuvette with a glass Pasteur pipette and full spectral data (350-800 nm) were collected.

Script Calculation Description

The determination of the mixtures was done by automating the calculation through a matlab m-file script. The script is in Appendix A. The script analyzes the spectra using data at wavelengths from 350 to 650 nm to calculate all seven of the known components especially the two most prevalent components (cholesterol and methyl linoleate). It then calculates the theoretical spectra of both cholesterol and linoleate based on the calculated concentrations and the molar extinction coefficients for all wavelengths. Difference spectra are calculated by subtracting the theoretical cholesterol and linoleate spectra from the original spectra. The difference spectra are then determined for all remaining components in a second iteration utilizing a K matrix that excludes data for cholesterol or linoleate. By doing the spectral difference and a second

calculation, it was anticipated the amount of information determinable from any given spectra would be increased.

Results and Discussion

The chemical assay has been demonstrated to have potential use in the analysis of lipids in sera. Experimental investigation into the use of the chemical assay for the analysis of blood lipids was done utilizing mixtures containing seven blood lipid components at physiologically correct concentrations. The results of multi-linear regression of these mixtures representing serum concentrations of PUFA and cholesterol are encouraging. The power of quantitative analysis of sera depends on the ability to quantify all PUFA independent of the concentrations found in sera. The more highly unsaturated PUFA such as EPA and DHA are found at the lowest concentrations and are of the most importance physiologically. The ability to quantitate all PUFA real time could prove a useful routine assay containing information relevant to modern diseases. This notion is supported by the ability to cluster out diabetics from a group of 100 subjects including dyslipidemic patients.

The samples were run independently and the results were independent for each of the samples. Each sample was recorded at 15 minutes into the reaction over the range of 350-800 nm. The samples were determined by MLR analysis using a matlab script as presented in Appendix A. The results of the MLR analysis included the residual for each calculated spectrum. The actual concentrations and MLR determined concentrations of the stock solutions are presented in Table 3. Observing this table, it is clear that the MLR analysis is not ideal for the determination of all PUFA in the mixtures. Indeed, there is a recurring theme throughout the mixture sets that the DHA and EPA concentrations are not accessible to the MLR calculation. From Table 2, the stock concentrations of DHA, EPA and methyl linolenate are all four times lower than methyl arachidonate and 15

times lower than methyl linoleate. It is likely that the inconsistencies in the determination of these PUFA are due to the low concentrations at which they are found. This result does not eliminate the possibility of utilizing this method for the full determination of PUFA in sera. Alternative methods including increasing the concentration of sera in the reaction in order to get better resolution in the low wavelengths or analysis methods which take advantage of the information contained in the kinetics plots of the chromogenic reactions may prove MLR useful for the determination of real samples.

Table 2. Polyunsaturated fatty acids concentrations.

Fatty acid	% PUFA	stock conc
leic	75	0.015 M
lenic	3.4	0.001 M
arach	17	0.004 M
DHA	2.8	0.001 M
EPA	1.4	0.001 M
	total =	0.022 M

The results also indicate that arachidonate is not as accurate as the linoleate and cholesterol values. This is likely due to the three times smaller concentration. This lower concentration does not allow adequate resolution of the concentrations. The "sum" values on the last two columns of the table are the sum of all PUFA determined by MLR analysis and the sum of the actual expected concentrations. It is clear from these values that the nonnegative MLR is detecting and quantitating the majority of the PUFA species present but is not able to separate the values into discrete concentrations.

Some of the mixtures included conjugated methyl linoleate. The resulting concentrations are sometimes determinable but at the lowest concentrations, MLR returns a zero result. Conjugated methyl linoleate concentrations were lower than 5×10^{-7} M in

samples 6,7,13, and 14, and were not determined by MLR analysis. Overall MLR determinations worked well for methyl linoleate, cholesterol and conjugated methyl linoleate concentrations. Conjugated methyl linoleate was more readily determinable at relatively high concentrations.

Table 3. Results including actual and MLR determined concentrations for all seven components (M)

	leic-actual	leic-MLR	lenic-actual	lenic-MLR	arach-actual	arach-MLR	DHA-actual	DHA-MLR
1	2.9E-05	1.5E-05	9.6E-06	2.1E-05	3.8E-05	6.1E-05	9.6E-06	0
2	7.2E-05	4.6E-05	9.6E-06	6.1E-05	3.8E-05	4.6E-05	9.6E-06	0
3	1.4E-04	8.6E-05	1.9E-06	1.3E-04	3.8E-05	6.9E-07	9.6E-06	0
4	1.4E-04	9.4E-05	4.8E-06	1.3E-04	3.8E-05	0	9.6E-06	0
5	1.4E-04	9.9E-05	9.6E-06	1.0E-04	7.7E-06	0	9.6E-06	0
6	1.4E-04	9.2E-05	9.6E-06	1.4E-04	3.8E-05	0	1.9E-06	0
7	1.4E-04	9.8E-05	9.6E-06	1.4E-04	3.8E-05	0	4.8E-06	0
8	1.4E-04	9.6E-05	9.6E-06	1.5E-04	3.8E-05	0	9.6E-06	0
9	1.4E-04	8.6E-05	9.6E-06	1.6E-04	3.8E-05	0	9.6E-06	0
10	1.4E-04	9.5E-05	9.6E-06	1.6E-04	3.8E-05	0	9.6E-06	0
11	1.4E-04	9.1E-05	4.8E-06	1.1E-04	3.8E-05	0	4.8E-06	0
12	0	0	1.9E-06	0	3.8E-05	6.2E-05	4.8E-06	0
13	0	0	4.8E-06	0	7.7E-06	3.1E-05	9.5E-06	0
14	1.4E-04	7.4E-05	0	9.4E-05	1.9E-05	0	1.9E-06	0
15	7.2E-05	5.0E-05	9.5E-06	5.1E-05	0	0	4.8E-06	0
16	2.9E-05	2.0E-05	4.8E-06	4.2E-05	3.8E-05	3.6E-05	0	0
17	0	0	1.9E-06	0	1.9E-05	3.9E-05	9.5E-06	0
18	7.2E-05	4.5E-05	9.5E-06	9.3E-05	3.8E-05	2.6E-05	1.9E-06	0
19	2.9E-05	2.2E-05	9.5E-06	4.1E-05	3.8E-05	6.0E-05	4.8E-06	0
20	1.4E-04	6.6E-05	4.8E-06	9.0E-05	7.7E-06	0	9.5E-06	0
21	1.4E-04	0	9.6E-06	2.5E-04	3.8E-05	0	9.6E-06	0
22	1.4E-04	1.05E-04	9.6E-06	1.5E-04	3.8E-05	0	9.6E-06	0
23	1.4E-04	1.46E-04	9.6E-06	8.3E-05	3.8E-05	3.4E-05	9.6E-06	0
24	7.2E-05	0	4.8E-06	3.4E-04	1.9E-05	0	4.8E-06	0
25	7.2E-05	0	4.8E-06	2.5E-04	1.9E-05	0	4.8E-06	0
26	7.2E-05	5.0E-05	4.8E-06	1.1E-04	1.9E-05	0	4.8E-06	0
27	7.2E-05	5.5E-05	4.8E-06	7.5E-05	1.9E-05	0	4.8E-06	0
28	7.2E-05	4.7E-05	4.8E-06	9.3E-05	1.9E-05	0	4.8E-06	0

Table 3. Results including actual and MLR determined concentrations for all seven components (M) (cont.)

	EPA-actual	EPA-MLR	chol-actual	chol-MLR	conj-actual	conj-MLR	sum-actual	sum-MLR
1	9.6E-06	0	7.2E-05	7.4E-05	0	0	9.6E-05	9.6E-05
2	9.6E-06	0	7.2E-05	7.6E-05	0	0	1.4E-04	1.5E-04
3	9.6E-06	0	7.2E-05	7.7E-05	0	0	2.0E-04	2.2E-04
4	9.6E-06	0	7.2E-05	7.8E-05	0	0	2.1E-04	2.2E-04
5	9.6E-06	0	7.2E-05	7.4E-05	0	0	1.8E-04	2.0E-04
6	9.6E-06	0	7.2E-05	7.2E-05	0	0	2.0E-04	2.3E-04
7	9.6E-06	0	7.2E-05	6.8E-05	0	0	2.1E-04	2.4E-04
8	1.9E-06	0	7.2E-05	7.3E-05	0	0	2.0E-04	2.5E-04
9	4.8E-06	0	7.2E-05	6.6E-05	0	0	2.1E-04	2.5E-04
10	9.6E-06	0	7.2E-05	6.7E-05	0	0	2.1E-04	2.6E-04
11	1.9E-06	0	7.2E-05	6.9E-05	0	0	1.9E-04	2.0E-04
12	9.5E-06	0	7.2E-05	7.1E-05	0	0	5.4E-05	6.2E-05
13	4.8E-06	0	7.2E-05	7.4E-05	0	0	2.7E-05	3.1E-05
14	9.5E-06	0	7.2E-05	6.8E-05	0	4.1E-06	1.7E-04	1.7E-04
15	1.9E-06	0	7.2E-05	7.6E-05	0	0	8.8E-05	1.0E-04
16	4.8E-06	0	7.2E-05	7.2E-05	0	0	7.6E-05	9.9E-05
17	0	0	7.2E-05	7.2E-05	0	0	3.1E-05	3.9E-05
18	9.5E-06	0	7.2E-05	7.3E-05	0	0	1.3E-04	1.6E-04
19	9.5E-06	0	7.2E-05	7.5E-05	0	0	9.1E-05	1.2E-04
20	4.8E-06	0	7.2E-05	7.6E-05	0	1.4E-05	1.7E-04	1.7E-04
21	9.6E-06	0	7.2E-05	6.1E-05	4.8E-05	5.1E-05	2.6E-04	3.0E-04
22	9.6E-06	0	7.2E-05	7.4E-05	4.8E-05	0	2.6E-04	2.5E-04
23	9.6E-06	0	7.2E-05	7.2E-05	1.9E-05	0	2.3E-04	2.6E-04
24	4.8E-06	0	1.4E-04	1.2E-04	9.6E-05	6.0E-05	2.0E-04	4.0E-04
25	4.8E-06	0	1.4E-04	1.3E-04	4.8E-05	3.6E-05	1.5E-04	2.9E-04
26	4.8E-06	0	1.4E-04	1.4E-04	4.8E-05	0	1.5E-04	1.6E-04
27	4.8E-06	0	1.4E-04	1.5E-04	9.6E-05	0	2.0E-04	1.3E-04
28	4.8E-06	0	1.4E-04	1.4E-04	1.9E-05	0	1.3E-04	1.4E-04

Analysis of errors was used to discriminate any obviously erroneous data in the original pool. It was decided that any spectra, which had a 520 nm absorbance of 0.02 absorbance unit below the expected value for the actual concentration, would be removed as erroneous.

The MLR analysis concentrations are used to create a "theoretical" spectrum that is used in the optimization of the calculation. Outside the MLR calculation this spectra, when compared to the original empirical spectra gives some idea of how well the MLR model fits the spectrum. Mixture 3 was analyzed to determine the MLR spectrum and the difference between the empirical spectrum and the MLR spectrum (Figure 17(including insert)). The differences are minimized in the lsqnonneg calculation process.

There seems to be a consistent shift in the 520 nm maximum during the calculation of the MLR spectrum. It is not clear why this occurs in the MLR calculation but not in the empirical spectra. The determination of the absorbance spectrum for pure cholesterol using the K matrix from the MLR analysis does not lead to the 520 nm shift. However, upon addition of other components into the concentration vector the shift occurs.

During the processing of the mixtures, the diluted stocks containing the approximation to physiological amounts of each component were run. They were done with 10 μL of the each one in the normal AC / PA reaction. The results give an indication as to the approximate absorbances occurring due to each concentration at the maximum quantities they were used. They are presented in (Figure 18). The low concentrations of methyl linolenate, DHA, and EPA create low absorbance spectra with increasing associated errors. The lowest concentration of methyl conjugated linoleate

give a similar absorbance as the highly unsaturated fatty acids. This gives some insight into the inability to resolve the lower methyl conjugated linoleate concentrations by MLR analysis.

The results of the MLR analysis of mixtures are not as good as expected. It was hoped that the analysis could recover reliable data for all PUFA species including those at lower concentrations. The determination using a pseudo-inverse K-matrix gave negative values this is attributed to errors in determining the concentrations. This is potentially due to low signal to noise at the levels the more highly unsaturated PUFA occur. It also may be due to errors associated with Matlab numerical determinations used for the analysis.

Matlab uses numerical methods to determine the results for matrix calculations. These types of calculations result in unavoidable errors. If the program uses iterative routines to calculate values, the error accumulates. This type of error is only avoidable if full control of the calculation process is available to the programmer. Matlab does not allow that level of control. If from equation (7) the K-matrix and the concentration vector are known, an absorbance spectrum is determinable. This "theoretical spectra" can then be used in MLR with the same K-matrix to achieve the concentration vector back again. There should be no variation between the theoretical and determined concentrations. When this type of calculation is used for the determination of errors associated with the calculations, the calculation using the standard pseudo-inverse has a small associated error for concentration values down to 1×10^{-14} M. The pseudo-inverse calculation also gives negative responses, however, leading to erroneous results. The preferred lsqnonneg script, for the determination of concentration without the errors due

to negatives, results in a similar error as the standard calculation down to a concentration of about 4 X 10^{-7} M. At and below this concentration the mathematical approach breaks down. The resulting values are not representative of the "theoretical" concentration originally put into the calculation. This result may be due to cumulative errors associated with the iterative calculation. These errors may be circumvented by increasing the concentration in the actual analysis to give high absorbance values in the lower wavelengths. It may also be overcome by more rigorous programming control of errors.

Also important is that the MLR calculation is accounting for the background correction associated with the reagent itself. At 15 minutes, the maximum absorbance of the background is 0.02 absorbance units at 350 nm. This is approximately a five percent error for an absorbance of 0.4 absorbance units. The error diminishes for PUFAs as the wavelengths increase.

The error, between the MLR determined concentrations and the theoretical concentrations, also includes experimental errors. These errors are significant because the volumes used were small and many measurements were made. The samples were also made to maximize variation in the components not to assure accuracy through repeated spectral measurements.

Despite difficulties in the calculation of the concentrations found in the spectroscopic analysis of mixtures, the utility of the assay is proven. The method in its first generation form can determine cholesterol and linoleic acid at their natural concentrations as well as conjugated linoleic acid at elevated levels. These data combined represent a new era of rapid PUFA determinations that has the potential to contribute meaningful information to diagnostic determinations made today.

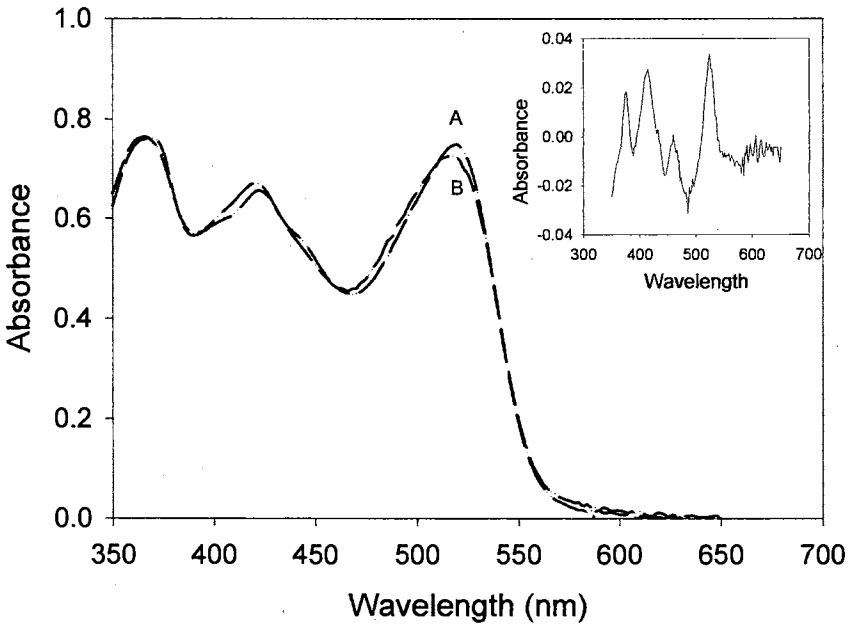

Figure 17. Mix 3 empirical spectrum (A) with overlaid MLR determined spectrum (B). insert: Difference between empirical spectrum minus MLR determined spectrum.

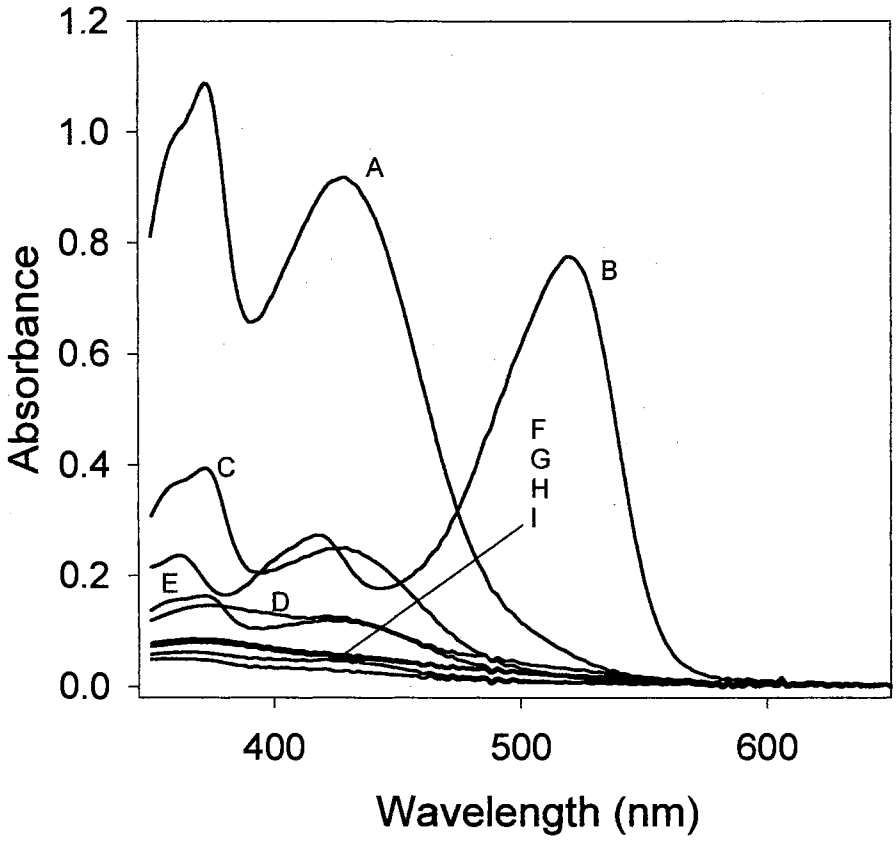

Figure 18: Overlaid spectra of all seven components at the concentrations on table 4. All were created by using 10 µl chloroform sample plus 1.0 ml acetyl chloride catalyzed with perchloric acid. Including: equivalence of 10 µl of A) 0.01 M methyl conjugated linoleate B) 0.0075 M cholesterol C) 0.015 M methyl linoleate D) 0.00034 M methyl arachidonate E) 0.001 M methyl conjugated linoleate F) 0.00028 M methyl EPA G) 0.00078 M methyl DHA H) 0.00068 M methyl linolenate I) 0.0001 M methyl conjugated linoleate

Dietary Intervention Study

Introduction

The diet of a 28-year-old obese Caucasian male volunteer was modified from a high fat diet to a low fat diet. Serum lipids were monitored with regularity over a 4-month period. The dietary intake of the subject was monitored and analyzed for lipid content. The serum lipid modification was also monitored using the novel chromogenic reaction discussed earlier, which is quantitative for cholesterol and PUFAs. The weight of the subject was also monitored.

The expected result of a low-fat dietary intervention is lowered lipids including cholesterol and TG concentrations. This was observed and an attempt was made to also monitor the PUFA content of the serum. The reactions were performed using a modified reagent that was a mixture of 3/2 AC / DCE, plus PA as catalyst. This decision was made in order to make comparisons between this study and a South African study in which diabetics were found to be separated from 100 "normal" and dyslipidemic patients in 2-D and 3-D clustering analysis.

The subject self-monitored his diet for fats, cholesterol, and calories following recommendations from the nutritionist at Oklahoma State University Seretean Wellness Center. Subscribing to a diet that preferred PUFAs over saturated fatty acids; the subject consumed more fish and chicken instead of beef. Simple sugars were also avoided as a means of maintaining low caloric intake. The diet of the subject was recorded in a daily journal, which included food type and portion size. The diet was then analyzed using an Internet database. The subject did not undertake an exercise program for the duration of the trial.

The hypothesis being tested was the ability of the assay to be used in the tracking patient responses to intervention studies. Such studies are particularly well suited to this assay as the cost per analysis is low. Furthermore, quantification of the serum components is not entirely necessary during the study, as the differences in the spectra during the study are more than adequate to determine the effectiveness of the treatment. As described in the Chapter I and in previous research, the assay is linearly dependent on the serum lipid components: cholesterol, linoleic acid and conjugated linoleic acid. The assay can potentially be modified to increase the sensitivity to include data for the PUFA found at lower concentrations. Eventually the quantitative determination of all PUFA, including conjugated dienes will allow the reaction to supplement or supplant currently used methods in routine lipid analysis of human sera.

Methods

Serum Sampling

Samples were drawn at the Oklahoma State University Wellness Center and analyzed at the Regional Medical Laboratory. The samples were taken as venous draws from the arm into two Vacutainer™ red-stoppered serum separation tubes. These tubes use a density gel to assist in separation of the serum from the red blood cells. The samples were allowed to stand at room temperature for 30 min to allow clotting factors to solidify. The vacuum tube was then centrifuged at 5,000 rpm for 15 min to allow separation in a tabletop centrifuge (Fisher Centrific model 228). One of the tubes was submitted to the Regional Medical Laboratory and the other was utilized for the chemical analysis in the Oklahoma State University Department of Chemistry. The samples for chemical analysis were stored in 10 mL vials with screw caps.

Reaction of Serum with Perchloric Acid Catalyst

Analyses were done by adding the usual 10 μL of analyte (serum) to the bottom of a 13 X 100 mm borosilicate disposable test tube. Acylating agent, pure AC/DCE (1.0 mL), was added to the analyte. The reaction was initiated on careful addition of 40 μL PA, and the reaction timer started immediately. Precautions and mixing by vortexing were done as before. Protein precipitants were removed by centrifugation for 3 minutes at 3400 rpm. The sample was separated from the precipitate pellet and either placed in the cuvette for kinetic determination or allowed to develop inside a separate test tube until the 15-minute endpoint was reached. Full spectral data (350-800 nm) were collected as before.

A minimum of three samples of each serum sample was run. The full spectral data (350 – 800 nm) were stored for all reaction times between five and 20 min. Data before 5 min were not available due to the centrifugation process.

It is important to note that the data were taken with a modified reagent. The reagent contained 60% AC to 40% DCE. This reagent was used so the subject's data could be compared to previously collected cross-sectional clinical data.

Clinical Laboratory Results

The clinical laboratory used standard enzymatic reaction and Friedewald approximations (see Chapter III introduction) to determine TC, TG, HDL-C and LDL-C. The clinical laboratory also returned results for trace analytes such as sodium, calcium and liver enzymes (see results and discussion).

MLR Analysis Methods

Analysis of the serum samples included the use of the MLR method described earlier in this chapter. The cholesterol, linoleic acid, and docosahexaenoic acid concentrations for the subject over the trial was determined from the chromogenic reaction spectra using the MLR method. The modified script file for the analysis of serum samples is presented in the Appendix B.

Results and Discussion

Over the course of the study, the TC was progressively lowered from 282 mg/dL to a minimum of 172 mg/dL (Figure 19(B)). The LDL-C concentration was also lowered from 186 mg/dL to a minimum of 85 mg/dL (Figure 19(C)). The HDL-C concentration was lowered slightly during the study from 47 mg/dL to a minimum of 33 mg/dL (Figure 19(D)). TG values were lowered from 392 mg/dL to 155 mg/dL at the lowest point (Figure 20(A)). The subject's weight was lowered from 285 lbs. to 252 lbs. at the lowest point

The alteration of the LDL-C concentration to the HDL-C concentration is of interest in that the lowered LDL-C and/or elevated HDL-C concentration is indicative of diminishing risk of atherosclerosis and heart disease. In this dietary intervention study the LDL-C/HDL-C concentration ratio as determined by the standard clinical assay decreased over the duration of the study from 4.2 to a minimum of 2.2 (Figure 21).

Other serum values measured included glucose, BUN, creatinine, sodium, potassium, chloride, bicarbonate, anion gap, uric acid, calcium, phosphorus, protein total, albumin, bilirubin total, iron, ALK PHOS, LD. None of these analytes altered significantly during the dietary trial.

The liver enzymes aspartate aminotransferase (AST (SGOT)), alanine aminotransferase (ALT (SGPT)), and gamma-glutamyltransferase (GGT) were also determined during the dietary trial and were all above normal values initially. Elevated liver enzymes are a sign of liver damage. There are a number of causes for elevated liver enzyme levels. The use of cholesterol lowering statin drugs can cause increase in free liver enzymes. The subject was not using such medication. Infectious hepatitis can cause

increased liver enzyme levels. The possibility of infectious hepatitis was eliminated by testing for the presence of such viruses. The possible diagnosis from the subject's personal physician was either fatty liver or liver cancer. The recommended diagnosis verification method was liver biopsy. Fatty liver as the alternative diagnosis would be evident in increased fat cells in the liver biopsy. The subject decided to postpone liver biopsy and tracked the liver enzymes as the diet was changed. The values for AST (SGOT) were lowered over the trial from 86 to a minimum of 19. ALT (SGPT) went from 171 to 29. The levels of GGT dropped from 100 to a minimum of 31. All three liver enzymes dropped into the normal range over the course of the study. These results indicated that nonalcoholic fatty liver was the cause of the elevated liver enzymes and that the dietary regimen the subject was using acted as an effective treatment for that disorder. Interestingly, the process of liver biopsy, while routine, is an invasive technique with risks associated with such techniques. The possibility of an alternative noninvasive method of diagnosis was not recommended as an option.

The value of the absorbance at 520 nm was adjusted by multiplying by a constant so the average of the adjusted 520 nm values is equal to the average values of the cholesterol concentration determined by the standard clinical assay in mg/dL. The value of the constant is 437. The clinically measured value of cholesterol overlays the adjusted value of the 520 nm peak for the subject over the time of the study (Figure 19(A)). This indicates there is a close relationship between the cholesterol value and the 520 nm value, and is expected given the understanding that the majority of the 520 nm peak comes from the reaction with cholesterol.

The 362 nm absorbance value was adjusted by multiplication by a constant (908). The result is that the average of the adjusted 362 nm absorbance is equal to the average TG values determined by the standard clinical assay in mg/dL. The adjusted 362 nm values overlay the values of the TG concentration determined by the standard clinical assay (Figure 20(A and B)). Particularly interesting is the upturn of both the clinical TG values and the adjusted 362 nm absorbances. This indicates there is a real increase near the end of the study.

The enzymatic method for the determination of TG only detects the glycerol portion of the triacylglycerol molecule. Concentrations of TG are not equivalent to a particular species of fatty acid. Since there are saturated, monounsaturated and PUFA attached to the glycerol. The fact that the TG concentrations mirror the adjusted 362 nm values is representative of the ubiquitous presence of the PUFA in serum components.

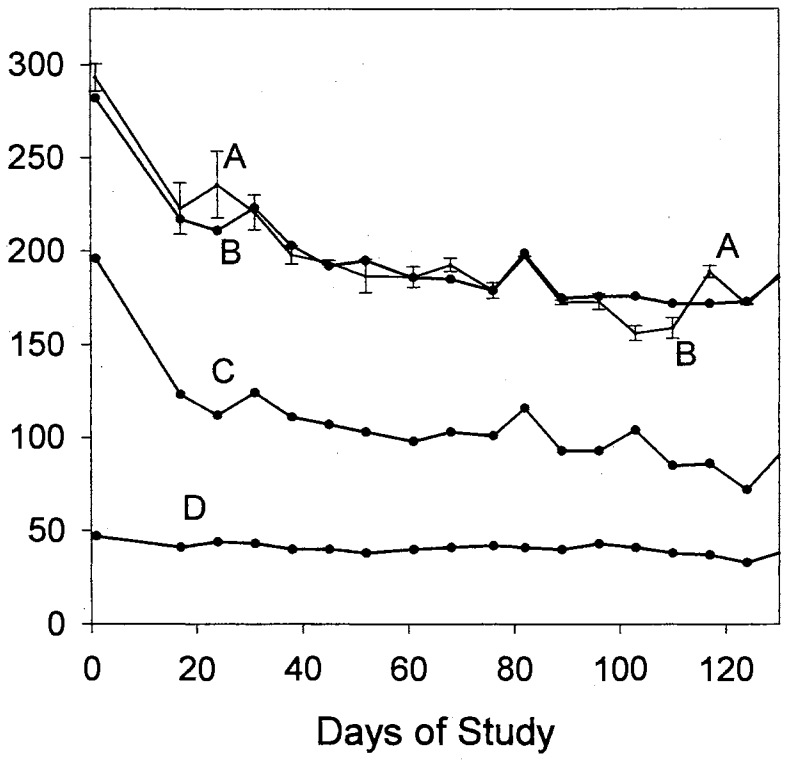

Figure 19. Overlaid graph of all clinical data with adjusted absorbances from chemical assay A) 520 nm absorbance adjusted by a constant (437) so the average of the absorbances over the trial is equal to the concentrations of cholesterol in mg/dL by standard clinical assay B) cholesterol in mg/dL by standard clinical assay C) LDL cholesterol concentration determined by standard clinical assay D) HDL cholesterol concentration determined by standard clinical assay.

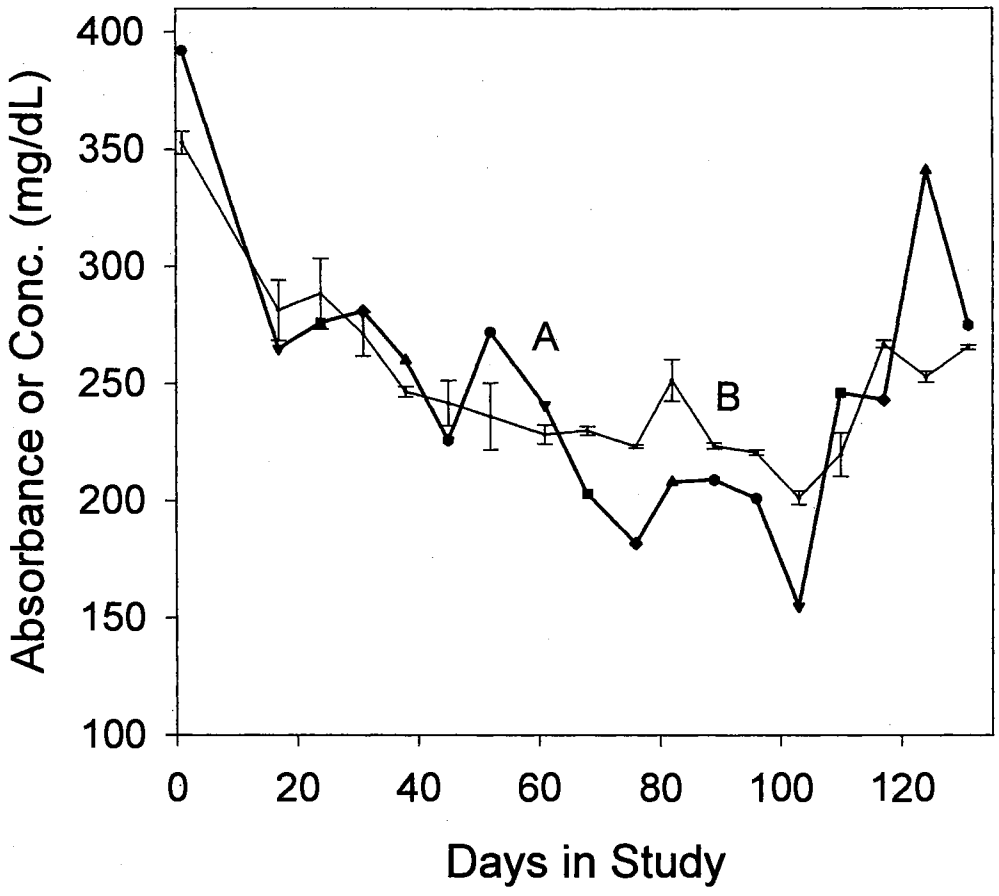

Figure 20. Overlaid graph of A) triglyceride concentration mg/dL B) absorbance at 362 nm adjusted by a constant (908) so that the average adjusted 362 nm absorbance value was equal to the average triglyceride concentration in mg/dL.

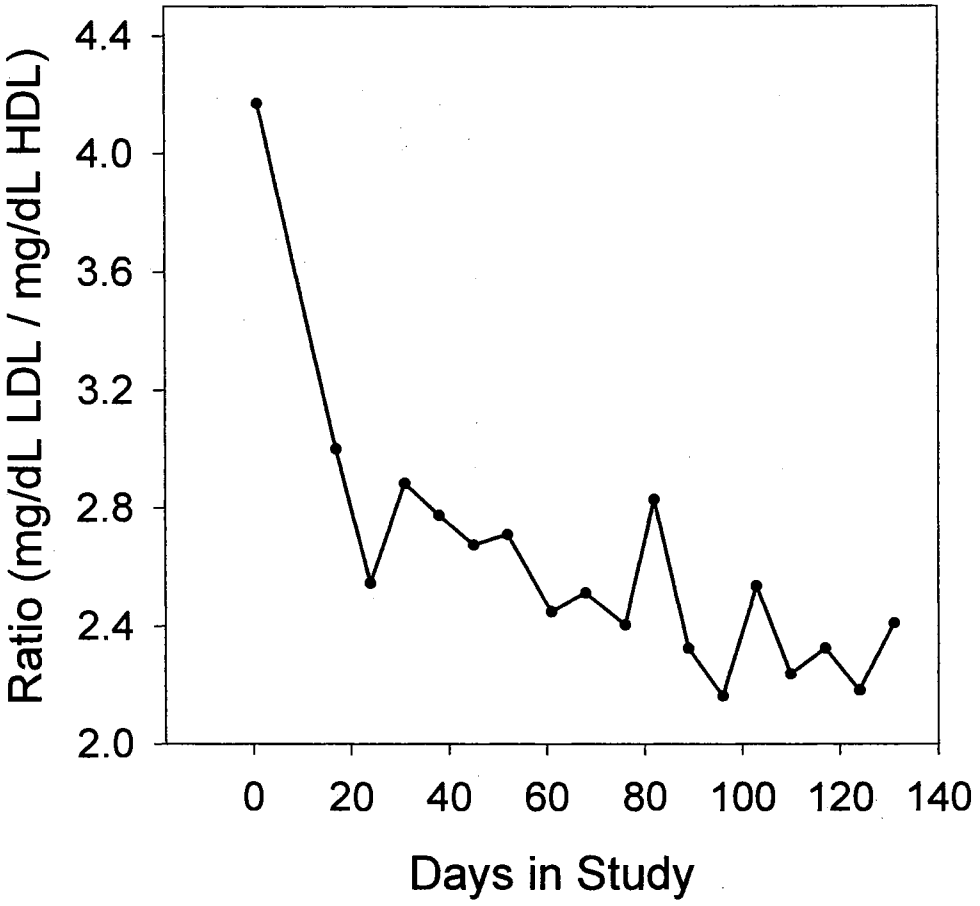

Figure 21. Graph demonstrating decrease in the LDL/HDL cholesterol concentration ratio, determined by the standard clinical method, for the duration of the intervention trial. Lower LDL/HDL ratio is associated with a decrease in the risk of atherosclerosis and heart disease.

MLR results

The method developed for the determinations of lipid components of mixtures presented earlier in this thesis was applied to the determination of lipids in real sera. The reagent used for the determination of the sera is modified from pure AC to 60% AC 40% DCE. The results for the determination of sera using the calibration data from the mixture section are therefore systematically shifted. However, the comparison between the values as they change in this intervention study should are independent of this systematic error.

The relationship between the TC determined by the enzymatic clinical laboratory method and the value by MLR are linearly correlated (Figure 22). The clinical value was converted to molarity in the assay solution by standard conversions. The linear cross-correlation plot of the two sets of values against each other has a slope of 0.7824 and R squared of 0.9249. The y-intercept of this plot is $1X10^{-5}$.

The TC values determined by the standard clinical assay and the MLR analysis of the chromogenic assay data were plotted versus the days of the study. The correlation between the clinical data and the chemical assay data is good. Both the clinical data (Figure 23(B)) and the MLR data (Figure 23(A)) decrease early in the intervention coming to a plateau around 100 days into the study. Both data sets indicate a slight increase in cholesterol towards the end of the study.

Linoleic acid was also determined in the MLR analysis of the chromogenic chemical assay spectra. Interestingly linoleic acid was progressively lowered during the study. However, the ratio between the linoleic acid and cholesterol determined by MLR

analysis seems to be constant for the duration of the study until the end where the ratio increases significantly (Figure 24). This increase occurs in the region of the study at which TG determined by the standard clinical assay increases, which suggests that the increase in the standard clinical assay later in the study is associated with a variation of fatty acid composition while the initial lowering of the lipid levels is not.

There is a correlation between TG and the values for the PUFAs found by MLR. In the plot of the overlaid values for TG determined by the standard clinical assay and the sum of the concentrations of linoleic acid, DHA (possibly highly unsaturated PUFA), and conjugated linoleate versus the days of the study indicates the relationship. Both the clinical TG value (Figure 25(A)) and the value of the MLR determined combined concentrations of the PUFA (Figure 25(B)) decrease at the beginning of the study, reach a minimum around 100 days and increase at the end of the study.

When the TG values determined by the standard clinical assay and the sum of the concentrations of linoleic acid, DHA (possibly highly unsaturated PUFA), and conjugated double bond are plotted versus the TG concentrations in mg/dL, a linear correlation is seen (Figure 26). The MLR determined PUFAs values were multiplied by a constant to bring the average of all the values into the range of the average of all of the TG concentrations. The value needed to normalize the two sets of data was $4.25X10^6$. The result of least squares linear regression was a slope of 0.5088 with R squared of 0.7087 and y intercept of 124.28. The very high y-intercept is a consequence of the variability in the molecular weight of the "average" PUFA in triglyceride structures. The residuals of the MLR calculations are presented on (Figures 23 and 24).

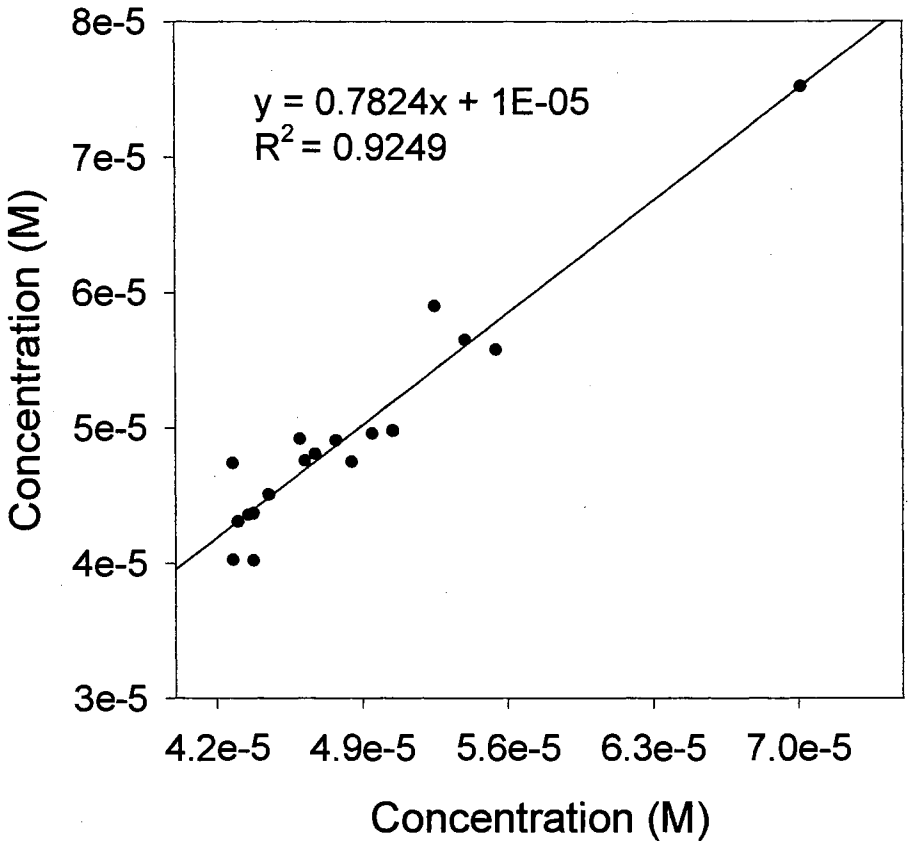

Figure 22. Cross-correlation plot demonstrating linear relationship between cholesterol concentrations determined by MLR analysis of the chemical assay spectra and the cholesterol concentration determined by the standard clinical assay. The linear regression of the cross-correlation plot describes the best fit line as having a slope of 0.7824 with R squared of 0.9249 and a y intercept of $1X10^{-5}$.

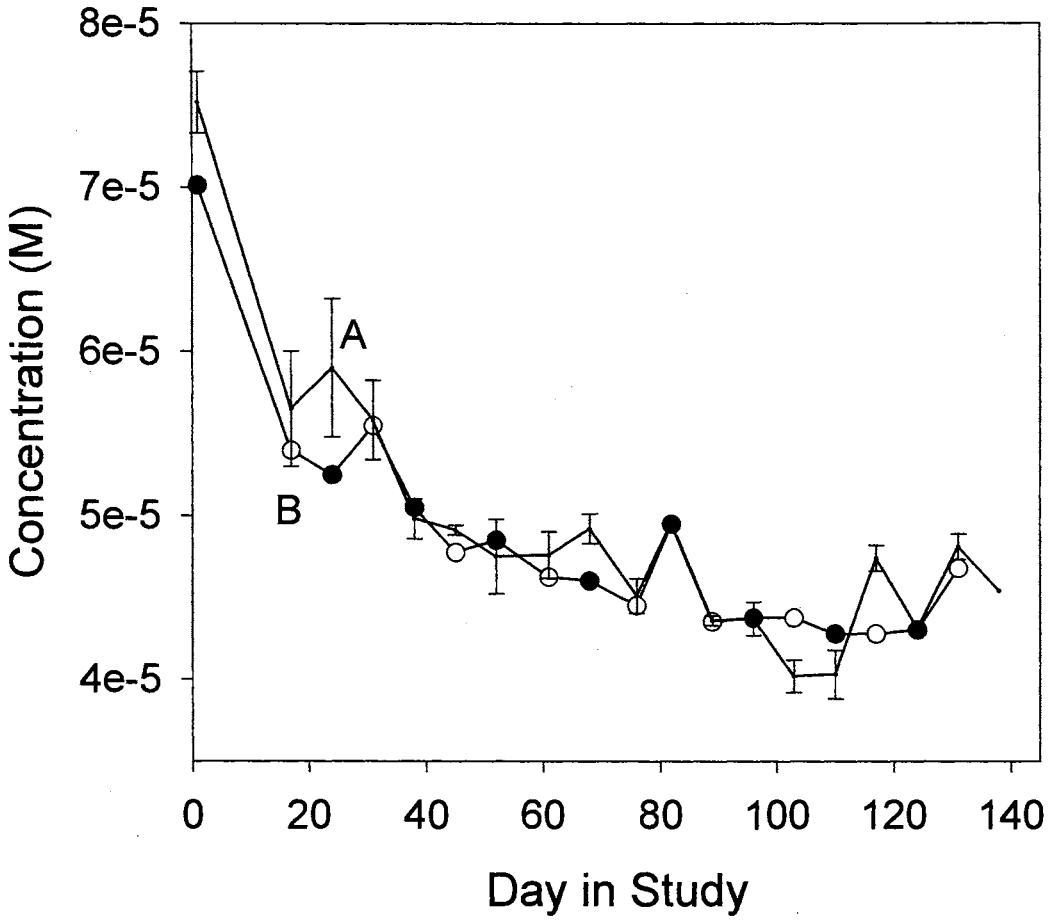

Figure 23. Overlaid graph A) cholesterol concentration determined by the MLR analysis of the chemical assay spectra and B) the cholesterol concentration determined by the standard clinical assay. This demonstrates the close relationship between the determination of cholesterol by MLR analysis of the chemical assay and the determination of cholesterol by the standard clinical assay.

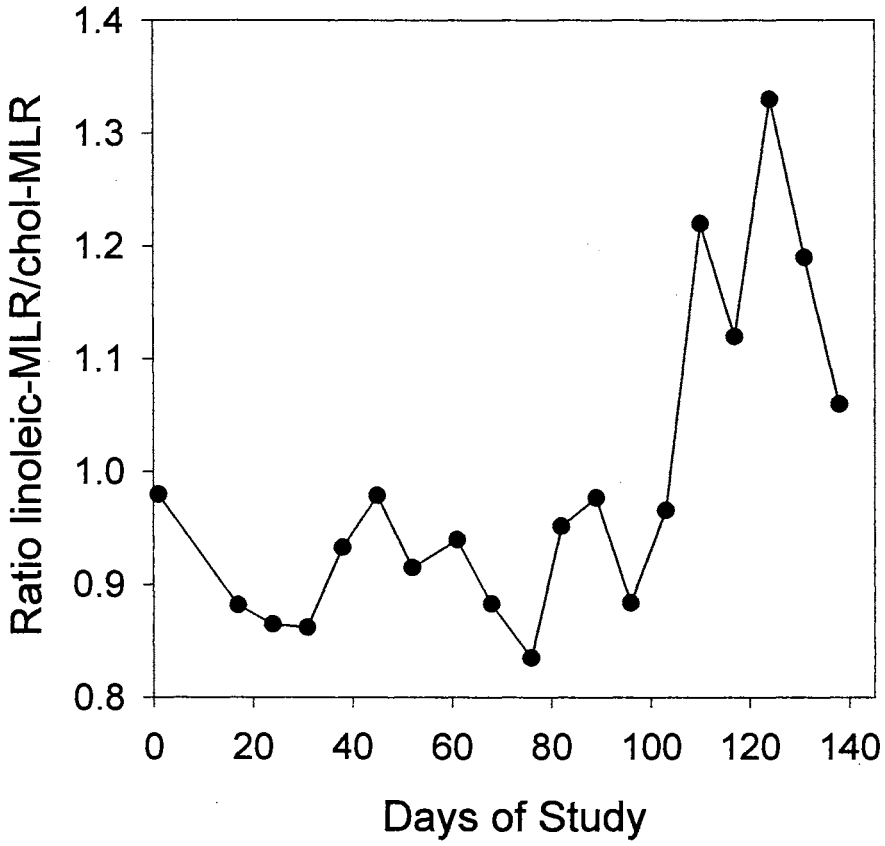

Figure 24. Graph demonstrating the steady nature of the ratio of linoleic acid determined by MLR analysis of the chemical assay spectra and the cholesterol concentration determined by MLR analysis of the chemical assay spectra. The upturn of the ratio between linoleic acid and cholesterol at the end of the trial is particularly interesting.

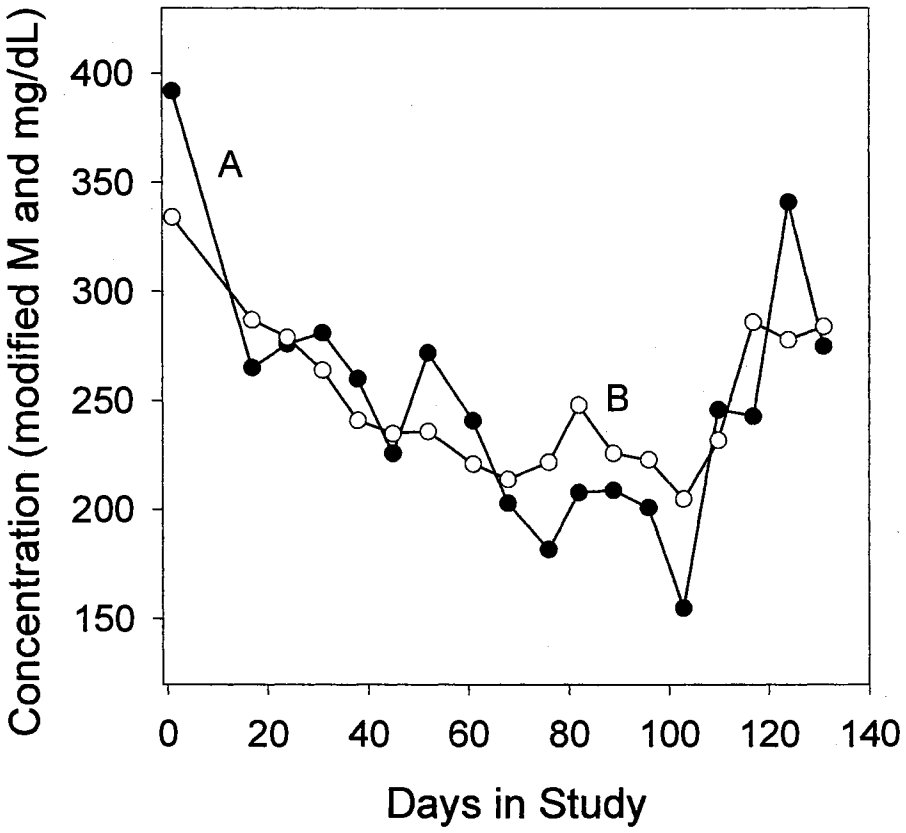

Figure 25. Overlaid graph of A) triglyceride concentrations determined by the standard clinical assay and B) the sum of concentrations of linoleic acid, DHA, and conjugated diene concentrations in molarity determined by MLR analysis of the chemical assay spectra adjusted by multiplication of integer ($4.25X10^6$) to make the average of the concentration of the summed PUFA equal to the average of the triglyceride values determined by the standard clinical assay in mg/dL.

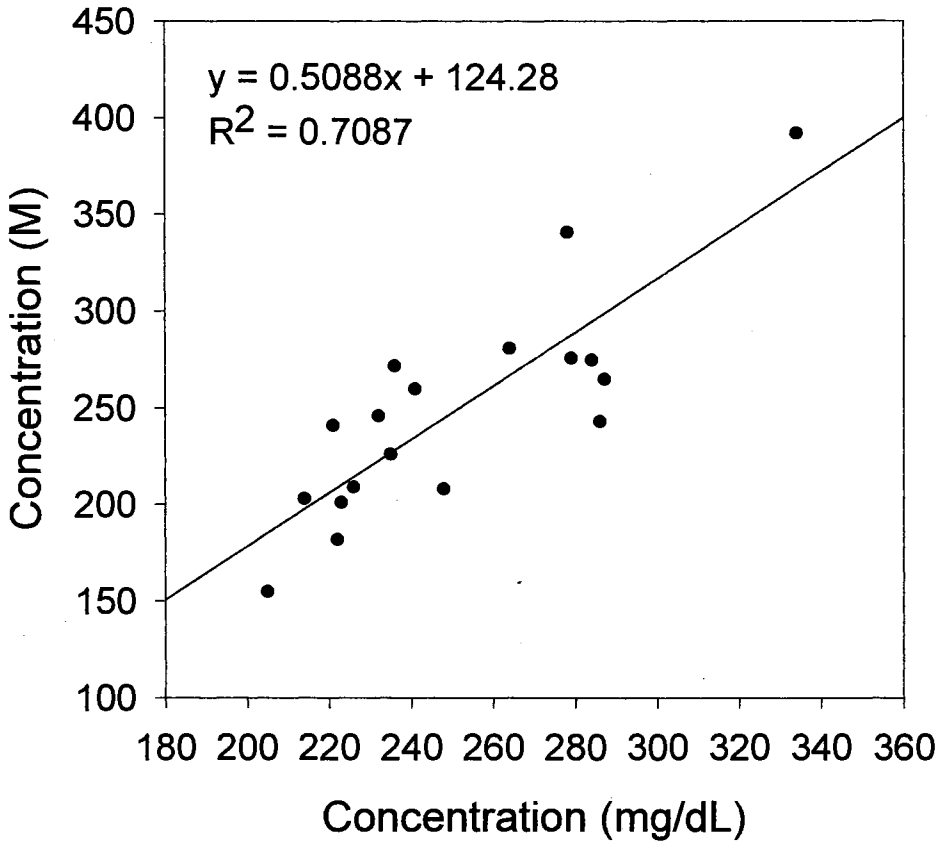

Figure 26. Cross-correlation plot of the adjusted value of the sums of linoleic acid, DHA, and conjugated diene determined by the MLR analysis of the chemical assay spectra versus the) triglyceride concentrations determined by the standard clinical assay in mg/dL

Summary

The determination of lipids in blood is routinely used for the analysis of dyslipidemias and risk for CAD, the leading cause of death in the United States. Results presented in Chapter II give a clear indication of the utility of the reagent for (1) the quantitative determination of the major lipids in concentrations consistent with those found in sera; (2) the ability of the data available from the chromogenic reaction to correlate and consequently model the data determined by routine methods currently being used and (3) the potential utility of quantitative determination of lipids in the sera for routine monitoring of patients and the diagnosis of disease states.

The ability to determine the concentration of major lipid components; cholesterol, linoleic acid and arachidonic acid from mixtures that have PUFA concentration ratios similar to sera is the first step toward an analytical method for the determination of all PUFA in sera by multivariate analysis. Good correlations are currently available with this first generation data analysis method. Future work will extend the boundaries to include all PUFAs and the resultant lipid profiles will have important applications in screening patients for emerging disease risks.

The observed consistency in the correlation between the values for the PUFAs, represented by the 362 nm absorbance values with the standard clinically measured TG concentrations, was an expected result given the ubiquitous nature of the PUFA species throughout the chemical forms in which they occur in the blood. While the determination of "triglyceride" values is not possible with the PUFA concentration data available from the chromogenic reaction, the ability to monitor a patient's fatty acid variation in a longitudinal manner is demonstrated. Furthermore, there is strong evidence in the

literature that the concentration values of individual PUFA species are likely more important for determination of disease states than the collective value as represented by TG.

The ability to quantitatively determine cholesterol, linoleic, arachidonic acid concentrations is indicative of the power the chromogenic reaction has in the analysis of lipids and the subsequent determination of disease states. Even in the preliminary methods presented here the quantitative determination of TC by MLR and the qualitative demonstration of acquiring information about fatty acids in the triglyceride form only available by enzymatic methods routinely used today for analysis is demonstrated. In its present state, the assay could be used for the monitoring of patients and the determination of relative change in PUFA and TC.

The techniques used for the demonstration of the chromogenic reaction's utility took advantage of the unique spectra present in six of the seven components. The ability of the reagent combined with a multivariate analysis method to be perfected in the future will bring a new world of information to the physician's hands in minutes.

CHAPTER III

CONCLUSIONS

Chapter I shows the unique selectivity and quantitative nature of the chromogenic reaction. The chromogenic reaction of AC with unsaturated compounds catalyzed by Lewis or Bronsted acids is shown useful for the analysis of a variety of structural moieties. The emphasis of this thesis is on the determination of lipid components of sera. The reaction has potential in a variety of alternative applications. The ability of the reagent to not only be selective to for the unsaturated portion of the analyte but also the saturated portion supports the idea of analyzing more complex chemical structures.

Broader applications are supported by the evidence presented in Chapter I where the reagent was used to produce chromogenic responses from a broad variety of compounds. Stoichiometric comparisons were made in order to understand the contributions each unique structural region contributes to the spectrum. The results indicate a highly selective reaction for both unsaturated and saturated structures. Molar extinction coefficients were determined by calibration curve analysis for some of the compounds. Linearity and limits of detection for these compounds confirmed the quantitative nature of the reaction. It was found that the spectral response of the various compounds to the reagent was related to the structural components of the compounds and that the reagent also appeared to be selective to positional and stereo-variations. It was also demonstrated that the spectra for individual components in a mixture are strictly additive and spectra from different chromophores in the same molecule are additive.

In analytical applications, the reaction could serve to detect the presence of contaminating unsaturated compounds that produce their own chromogenic response to the reagent. Accordingly, in quantitative assays, the lack of a spectral response for a given pure compound may be indicative of non-chromogenic contaminants. Alternatively, the reagent could be used for quantitative determination of mixtures using multivariate analysis. In complex natural mixtures of PUFA for instance, the determination of the components would allow monitoring and management of the components. In areas such as plant oil processing or biodiesel production, this simple, cheap and automatable method of analysis could increase profitability and quality control in the manufacture process. Finally, the reaction has demonstrated selectivity for unsaturated structural moieties, which could be used for the analysis of unknown chemical structures. This could be useful in the combinatorial drug development where a particular moiety is expected, and the reaction is used for quality control of libraries. It could also be useful if a random structure development process is used to form a library and the chromogenic reaction is used to either screen for known useful or toxic moieties.

A review of the current methods used in lipids analysis was presented. The current paradigm was considered and developing methods were described to demonstrate the progressive nature of the field of lipoprotein analysis as the best methods are still being sought. The review also demonstrates the utility of fatty acid analysis. The relationships between PUFA and a variety of diseases are considered and the determination and use of PUFA values is described. The review also discussed the importance of being able to determine conjugated diene species in the analysis of oxidative stress related diseases. Overall the review describes the imperfect routine

methods currently used and develops an argument for the importance of a fast, economical alternative.

The components of human blood lipids were among the compounds studied in the analysis of model compounds in Chapter I. In Chapter II, combinations of the blood lipids in ratios representing human serum were prepared and determined by MLR. Results for accurately analyzing the major lipids independently were generally good. The ability to determine the minor components was not as good as expected, and is partly due to experimental and calculation errors and partly due to the low concentrations of the minor serum lipid components. In the future analyses can be improved upon by using calculation methods that control the cumulative errors associated with the calculation, or possibly by increasing the concentration of the serum analyte in order to gain better information about the minor components in the sera. Alternatively, multivariate analysis methods capable of taking advantage of the absorbance data collected at 1 minute intervals throughout the reaction may increase both the selectivity and sensitivity. The analysis in its current form resulted in good data for the concentrations of cholesterol, linoleic acid, and elevated levels of conjugated linoleic acid.

The application to a real patient was of interest as prior research shows an ability to cluster diabetics from a group of 100 patients, which included other dyslipidemics. In Chapter II, the utilization of the assay to monitor the response of a patient to dietary intervention is demonstrated. A 29-year-old obese Caucasian male was monitored as his diet was changed resulting in significant lipids lowering and lipoprotein concentration ratio alterations. Results indicated that while the LDL-C/HDL-C ratio was not detected in the chemical assay, the amounts of linoleic acid and TC were quantitated using the

MLR method developed on synthetic mixtures. Also, a linear correlation was shown to exist between the TG concentration in mg/dL and the linoleic acid concentration adjusted to mg/dL; a relationship that may be quantitative within biological variability. There was no evidence for the presence of conjugated linoleic acid in the sera. Future work may prove that quantitation of conjugated linoleic acid at serum concentrations is possible. This is significant in the study of in vivo oxidative stress.

The chemical assay discovered in our laboratory is very flexible in its application. It shows selectivity to a variety of chemical moieties such as positional and enantiomeric isomers, which makes it useful for the analysis of unsaturated systems in quantitative analysis and quality control applications. It is novel; its closest rival, the L-B reaction, is significantly different in its reactive center e.g. L-B does not react with PUFA. Quantitation of blood lipids was conducted in synthetic mixtures and in real sera in order to gauge the usefulness of the assay for sera analysis. Quantitative determinations of cholesterol, linoleic acid, and elevated levels of conjugated linoleic acid were possible in this first generation analysis. Simple manipulations can increase the detection limits by at least three times and improved MLR analysis programming should allow the assay to quantitatively determine all blood unsaturated components including conjugated dienes. The assay is inexpensive to conduct and is manageable in terms of chemical hazards. Overall, it has been demonstrated that the unique selectivity and quantitative ability of this reaction has the potential to revolutionize serum lipid analysis.

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APPENDIX A
LSQNONNEG SCRIPT FOR MIXTURES

```
load kmat450.txt
load kmat650.txt
load lin650.txt
load chol650.txt

for N=1:42
name=['s' int2str(N) '.txt'];
load(name);
end
clear N
clear name

for N=1:42
name=['s' int2str(N)];
tn650=eval(name);
tn450=tn650(1:51);
conc650 = lsqnonneg(kmat650,tn650);
mlr=kmat650*conc650
diffmlr=tn650-mlr
X = conc650(6);
cholspec650= X*chol650;
X = conc650(1);
linspec650 = X*lin650;
totalspec = cholspec650 + linspec650;
minuscholandlin = tn650 - totalspec;
resid650 = norm(kmat650*conc650-tn650);
X=minuscholandlin(1:51);
conc450 = lsqnonneg(kmat450,X);
resid450 = norm(kmat450*conc450-tn450);
temp=['resid450s' ,int2str(N), ' = resid450'];
eval(temp);
temp=['resid650s' ,int2str(N), ' = resid650'];
eval(temp);
temp=['minuscholandlin650s' ,int2str(N), ' = minuscholandlin'];
eval(temp);
temp=['conc650s' ,int2str(N), ' = conc650'];
eval(temp);
temp=['conc450s' ,int2str(N), ' = conc450'];
eval(temp);
temp=['cholspec650s' ,int2str(N), ' = cholspec650'];
eval(temp);
temp=['linspec650s' ,int2str(N), ' = linspec650'];
eval(temp);
temp=['cholandlins' ,int2str(N), ' = totalspec'];
eval(temp);
temp=['mlr650s' ,int2str(N), ' = mlr'];
eval(temp);
temp=['diffmlr650s' ,int2str(N), ' = diffmlr'];
eval(temp);
clear tn650
clear tn450
end
```

```
clear I

res450cmpld=zeros(42,1);
for N=1:42
x=['resid450s' int2str(N)];
res450cmpld(N)=eval(x);
end

clear x
clear N

res650cmpld=zeros(42,1);
for N=1:42
x=['resid650s' int2str(N)];
res650cmpld(N)=eval(x);
end

clear x
clear N
clear I

for I=1:42
X=['clear resid450s' int2str(I)];
eval(X);
end

clear I

for I=1:42
X=['clear resid650s' int2str(I)];
eval(X);
end

clear x
clear N

clear conc650
clear conc450
clear cholspec650
clear linspec650
clear totalspec
clear X
clear I
clear N
clear chol650
clear kmat450
clear kmat650
clear lin650
clear minuscholandlin
clear name
clear temp
clear resid450
clear resid650
```

APPENDIX B
LSQNONNEG SCRIPT FOR SERUMS

```
load kmat450.txt
load kmat650.txt
load lin650.txt
load chol650.txt

for N=1:19
name=['s' int2str(N) '.txt'];
load(name);
end

clear N
clear name

for N=1:19
name=['s' int2str(N)];
conc650 = lsqnonneg(kmat650,eval(name));
X = conc650(6);
cholspec650= X*chol650;
X = conc650(1);
linspec650 = X*lin650;
totalspec = cholspec650 + linspec650;
minuscholandlin = eval(name) -totalspec;
X=minuscholandlin(1:51);
conc450 = lsqnonneg(kmat450,X);
temp=['minuscholandlin650s' ,int2str(N), ' = minuscholandlin'];
eval(temp);
temp=['conc650s' ,int2str(N), ' = conc650'];
eval(temp);
temp=['conc450s' ,int2str(N), ' = conc450'];
eval(temp);
temp=['cholspec650s' ,int2str(N), ' = cholspec650'];
eval(temp);
temp=['linspec650s' ,int2str(N), ' = linspec650'];
eval(temp);
temp=['cholandlins' ,int2str(N), ' = totalspec'];
eval(temp);
end

for I=1:14
X=['clear s' int2str(I)];
eval(X);
end

clear conc650
clear conc450
clear cholspec650
clear linspec650
clear totalspec
clear X
clear I
clear N
clear chol650
clear kmat450
```

```
clear kmat650
clear lin650
clear minuscholandlin
clear name
clear temp
```

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_____

VITA 2

Joe B. Studer

Candidate for the Degree of Doctor of Philosophy

Thesis : CHARACTERIZATION OF A NOVEL CHROMOGENIC REACTION FOR ANALYSIS OF UNSATURATED LIPIDS AND APPLICATION TO HUMAN SERUM LIPIDS ANALYSIS

Major Field: Chemistry

Biographical:

Education: Graduated from Broken Bow High School, Broken Bow, Oklahoma in May 1991; received Bachelor of Science Degrees in Chemistry and Biology from Southeastern Oklahoma State University, Durant Oklahoma in May 1996; completed the requirements for the Doctor of Philosophy Degree at Oklahoma State University in May 2003.

Professional Experience: Research Technician Southeastern Oklahoma State University, 1995–1997 ;Graduate Teaching Assistant, Oklahoma State University, 1997-2002

Professional Memberships: Alpha Chi Sigma; American Chemical Society; Phi Lambda Epsilon