AN ALTERNATIVE ROUTINE METHOD TO MEASURE SERUM TOTAL CHOLESTEROL AND ITS DISTRIBUTION AMONG THE MAJOR LIPOPROTEINS

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PREFACE

Serum cholesterol is one of the analytes that is frequently measured in the clinical laboratory. This is because of the established relationship between elevated serum cholesterol level and the risk of coronary heart disease (CHD). Lately, the distribution of cholesterol among the major solubilizing lipoproteins, namely, very low density lipoproteins (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) has also become important in determining CHD risks. Studies have shown that the risk of developing CHD is positively correlated with total cholesterol and LDL and negatively correlated with HDL. These findings led to an increased effort to measure not only total cholesterol but also its distribution among the lipoprotein fractions for early detection and management of CHD. Current routine methods for measuring the lipoprotein fractions involve three enzymatic tests and are not applicable to samples with triglyceride levels > 400 mg/dL. This study offers an alternative routine method for obtaining the lipid profile in a single non-enzymatic test. The applicability of the alternative method to samples that have high triglyceride levels as well as samples with lipid disorders was evaluated.

I would like to thank several people who have helped me throughout my graduate study. I am very much indebted to my research adviser Dr. Neil Purdie, who let me work under his guidance and has since looked out for me. I would also like to thank Dr. Horacio Mottola, Dr. Ziad El

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I would like to dedicate this accomplishment to my family and all my relatives who always inspire me to work harder. My dad would have been very happy if he had seen me finish my doctorate degree. The hard work of my mom to support our family is very much appreciated. My sisters Geraldine and Cecil's friendly advice and the loving arms of my grandmother are very much treasured.

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LIST OF SYMBOLS AND ABBREVIATIONS

CHD = Coronary heart disease

MI = Myocardial infarction

NCEP = National Cholesterol Education Program

LSP = Laboratory Standardization Panel

CDC = Center for Disease Control

VLDL = Very low density lipoprotein

IDL = Intermediate density lipoprotein

LDL = Low density lipoprotein

HDL = High density lipoprotein

TC = Total cholesterol

TG = Triglyceride

VLDL-C = Cholesterol in the very low density lipoprotein particle

IDL-C = Cholesterol in the intermediate density lipoprotein particle

LDL-C = Cholesterol in the low density lipoprotein particle

HDL-C = Cholesterol in the high density lipoprotein particle

$[\alpha]$ = optical activity

ε = molar absorptivity

Lp(a) = lipoprotein a

Lp-X = lipoprotein X

Lp-Y = lipoprotein Y

Lp-E = lipoprotein E

LPL = lipoprotein lipase

LCAT = lecithin cholesterol acetyl transferase

FHS = Framingham Heart Study

LRCF = Lipid Research Clinics Prevalence Mortality Follow-up Study

LRC-CPPT = Lipid Research Clinics Coronary Primary Prevention Trial

MRFIT = Multiple Risk Factor Intervention Trial

ATP = Adult Treatment Panel

PEG = polyethylene glycol

L-B = Liebermann Burchard

FIA = Flow injection analysis

ID/MS = Isotope dilution / mass spectrometry

GC/MS = Gas chromatography / mass spectrometry

HPLC = High performance liquid chromatography

UWC = Oklahoma State University Wellness Center

RBL = Roche Biomedical Laboratories

SMC = Stillwater Medical Center

UCT = University of Cape Town Medical School

CD = circular dichroism spectropolarimeter

MLR = Multiple linear regression

PCA = Principal component analysis

PCR = Principal component regression

PLS = Partial least squares

MVRA = Multivariate regression analysis

SRM = Standard reference material

t_{exp} = result of Student's t-test

t_{table} = theoretical t value from statistical table

FH = Familial hypercholesterolemia

r = correlation coefficients

CV = coefficient of variation

n = sample size

α = significance level

df = degrees of freedom

SD = standard deviation

CHAPTER I

INTRODUCTION

Diseases of the heart and blood vessels are still the leading cause of death in the United States and other industrialized societies. The largest groups of these deaths are associated with myocardial infarction (MI) and coronary heart disease (CHD).[1] Coronary heart disease is a common, life-threatening and disabling disease which can be difficult to treat and very expensive to cure. In the United States alone, about one million Americans suffer MI each year, and more than six million have symptoms of CHD. Illness due to CHD costs over $50 billion annually for care, loss of earnings, and productivity.[2]

Coronary heart disease and myocardial infarction are a consequence of atherosclerosis, a disease of the arteries. Accumulation of lipids in the cytoplasm of arterial cells is an early manifestation of atherosclerosis at the cellular level. Initial deposition of intracellular lipids transported into the vessel wall by lipoprotein plays an important role in the initiation of atherosclerotic lesions.[3,4] Once a lesion has started, lipids, particularly cholesterol, accumulate within the extracellular space of the intima of human arteries. This process thickens the arterial wall and reduces the

lumen of the artery, resulting in reduced blood flow. Reduced blood flow will decrease the supply of oxygen to the tissues and can cause injury or even death to tissues. When this happens in the coronary arteries, the result is a MI or heart attack.

Results from many studies have shown that there are a large number of factors involved in the development of atherosclerosis. The following factors seem to be the most important: hyperlipidemia, genetic factors, hypertension, cigarette smoking, type A personality, elevated blood glucose, obesity, race, age, gender, and lack of exercise.[5] Recently, the effects of serum levels of lipoprotein(a)[6,7] and fibrinogen[8] have been added to the list. The precise way in which these risk factors promote atherosclerosis is not clear.

Among the lipids, cholesterol is the most atherogenic. A large body of evidence[9-12] has shown that an elevated blood cholesterol level is one of the principal risk factors for CHD. In 1985, the United States' National Institute of Health inaugurated the National Cholesterol Education Program (NCEP) with the goal of planning strategies to reduce the prevalence of elevated blood cholesterol in the United States and thereby reduce the incidence of CHD. The NCEP strongly encourages the public to have their serum cholesterol levels examined and be aware of the implications of elevated cholesterol. This recommendation created the need for automated methods for serum cholesterol determinations that can provide results quickly in order to be able to handle the huge numbers of samples that would be analyzed in the various screening programs. Cholesterol screening outside the confines of the conventional laboratory are not at all unusual today. Physicians' offices, pharmacies, shopping malls, worksites, supermarkets, and high traffic public areas are all common venues. Even

home testing of cholesterol levels is now possible. The methods used for cholesterol determination differ in complexity from simple non-instrumental card tests to sophisticated multi-analyte analyzers.

Cholesterol and other lipids play an important role in human metabolism, participating in such diverse functions as the maintenance of cellular integrity, the storage of energy, the provision of metabolic intermediates and the transmission and transduction of signals.[13] In order to be solubilized in the aqueous environment of the blood and be transported through the body, lipids are associated with amphipathic proteins. The complex micellar structures of lipids and proteins are called lipoproteins. Protein moieties of the lipoprotein are known as apo-lipoproteins or apoproteins and serve as co-factors in enzyme and receptor site reactions for synthesis and degradation of lipoprotein complexes.

Three major classes of lipoproteins in the normal fasting state of human serum are usually recognized. These are very low density lipo-proteins (VLDL), low density lipoproteins (LDL) and high density lipo-proteins (HDL). Each of these lipoproteins is heterogeneous in terms of size, lipid composition, and apoprotein composition. Very low density lipoprotein and LDL components transport lipids from the liver to the various cells in the body. Very low density lipoprotein transports mainly triglyceride, while LDL is the main carrier of cholesterol in human blood plasma. Low density lipoprotein taken up by cells lining the arteries are responsible for atherosclerosis. High density lipoprotein fractions on the other hand, facilitate removal of excess cholesterol and other lipids from the blood and body cells by returning them to the liver for degradation to bile acids and ultimate excretion.

In the last two decades, more and more attention has been focused on

the role of the different lipoprotein classes and their relative effects on CHD risks. Studies have shown that the risk of developing CHD is positively correlated with total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C), and negatively correlated with high density lipoprotein cholesterol (HDL-C).[14-18] There has been no direct evidence to implicate the very low density lipoprotein cholesterol (VLDL-C) in CHD risk determinations, but a high triglyceride (TG) level can be a serious problem by itself. However, HDL-C and TG are said to exhibit a strong inverse association.[19] The NCEP concluded that lipoproteins, particularly LDL-C and HDL-C offer more precise information than TC as risk factors for predicting the chances of CHD.

The need for better criteria to characterize CHD risks in patients has led to a rapid increase in the demand for the measurement of lipoprotein classes by clinical laboratories. Assay of TC is now largely relegated to the status of a screening test. Although many methods are currently available for lipoprotein determination, the current routine method used to obtain a lipid profile is based on three independent measurements. Total cholesterol, HDL-C, and TG are measured directly by enzymatic methods. To determine HDL-C, VLDL-C and LDL-C are selectively precipitated with one of the commonly used reagents, such as manganese heparin, dextran sulfate, or magnesium phosphotungstate.[20] The HDL-C remaining in solution is measured by the same enzymatic method used for TC. Based on considerable evidence, the VLDL-C fraction is taken to be equal to one-fifth of the TG value as long as TG is not >400 mg/dL. Low density lipoprotein cholesterol is estimated using the Friedewald formula,[21]

$$\text{LDL-C} = \text{TC} - (\text{HDL-C} + \text{TG}/5) . \qquad \text{(eq. 1)}$$

The LDL-C fraction is the one known with least accuracy because of the propagation of errors in the measurement of TC, HDL-C, and TG, and the empirical nature of the equation.

The Laboratory Standardization Panel (LSP) of the NCEP has emphasized the need to improve upon the precision and accuracy of the measurements of lipoproteins among different clinical laboratories, and has recommended that clinical laboratories should ultimately obtain a precision consistent with coefficients of variation (CV) of $\leq 3\%$ for TC and LDL-C and $\leq 4\%$ for HDL-C.[22-23] In terms of accuracy, LSP recommends a bias of $\leq \pm 3\%$ of the result of the reference method. Despite great improvements in accuracy and precision in the measurement of TC and the lipoprotein classes, there is still a need for reliable methods for the measurement of serum LDL-C[24] and HDL-C[25] that are suitable for routine use in the clinical laboratory.

The goal of this research project was to develop an alternative method to determine the three major lipid fractions directly in a single experiment. Previous attempts[26,27] have been reported to measure these three lipid fractions simultaneously. Results from these studies yielded only a very good correlation between methods for LDL-C. The intent of this study was to improve the correlation for all three major lipid fractions and to surpass the LSP requirements for analytical accuracy and precision. The current routine method has a limitation in estimating LDL-C for hyper-triglyceridemic serum (TG >400 mg/dL). As a further aspiration, the applicability of the alternative method to measuring cholesterol in hyper-triglyceridemic samples was evaluated.

CHAPTER II

THE CHEMISTRY OF CHOLESTEROL AND ITS RELATION TO CORONARY HEART DISEASE

I. Early Chemistry of Cholesterol

Cholesterol was first described towards the end of the eighteenth century by the French chemist de Fourcroy who isolated a crystalline substance from the alcohol-soluble fraction of human gallstones.[28] This substance was also mentioned by Poulletier de la Salle more than twenty years earlier. De Fourcroy considered that his substance was related to cetyl palmitate; but early in the next century, Chevreul showed this not to be so. Chevreul also gave de Fourcroy's substance the name cholesterine. Lecanu detected the presence of cholesterol in the blood of humans while Chevreul and Couerbe had found it in human and animal bile as well as in the brain. It was soon detected as a normal constituent of all animal cells, as well as in several secretions and pathological deposits. For instance, Vogel found it in atheromatous arteries and Muller found it in a type of tumors which he called cholesteatomes.[29]

In 1859, Berthelot showed that cholesterol was an alcohol and

prepared esters of it. Other cholesterol esters like the oleate and palmitate were isolated from serum by Hurthle while the palmitate and stearate were found in normal adrenals by Rosenheim and Tebb in 1909.[29]

Wislecenus and Moldenhauer prepared a dibromide of cholesterol, indicating the existence of a double bond. In 1898, Reinetzer published the correct empirical formula and Windaus in 1919 proposed a tentative structural formula for cholesterol. Advances in x-ray crystallography and synthetic chemistry enabled both Windaus and Wieland and Dane to deduce the correct structural formula for cholesterol in 1932.[29,30]

II. Physical and Chemical Properties of Cholesterol

Cholesterol is the principal sterol of mammalian tissue. In humans, it occurs in particularly high concentrations in the brain, spinal cord and adrenal glands. About 17% of the solid matter of the brain is free cholesterol.[28]

The basic structure of cholesterol is a tetracyclic perhydrocyclopentanophenanthrene skeleton that is typical for steroid molecules. Its molecular weight is 387 g/mole. Fig.1a shows the flat structural form and Fig.1b the conformational representation of the cholesterol molecule.

Substituents are a double bond at carbon atom 5 (C-5), a saturated branched side chain of eight carbon atoms at C-17, and two β–oriented methyl groups at C-10 and C-13. The hydroxyl group at C-3 and the hydrogen atom at C-8 are also β– oriented. Hydrogen atoms at C-3, C-9, C-14, and C-17 are all situated below the plane of the molecule (α-oriented). The A to B, B to C, and C to D ring junctions are all in trans-

configurations. Substituents at C-20 are in the R-configuration.[31]

The 3-β-hydroxyl group is the only polar group in the cholesterol molecule. It is only very slightly soluble in water (solubility is about 0.2 mg / 100 mL water) and quite soluble in organic solvents. It has a melting point of 149.5-150°C.[32]

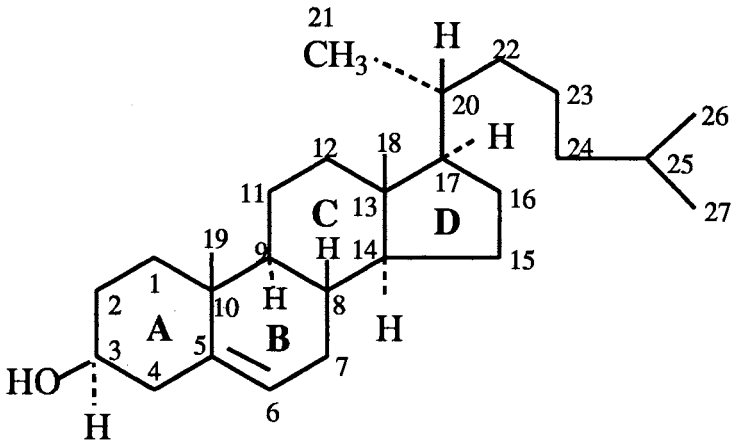

Fig.1a. Flat structural representation of cholesterol.

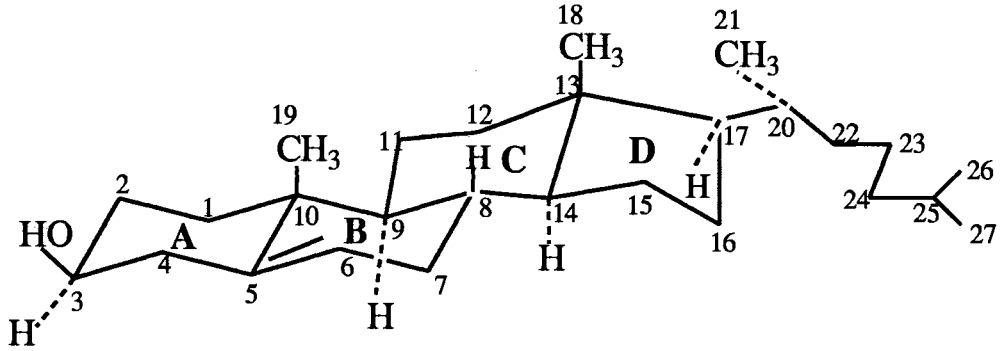

Fig.1b. Conformational representation of cholesterol.

The presence of several asymmetric carbon atoms render solutions of cholesterol optically active and this property is an aid in identifying and

in ascertaining the analytical purity of cholesterol preparations. The optical activity of cholesterol is $[\alpha]_D^{20}$= -31.5° in ether and $[\alpha]_D^{20}$= -39.5° in chloroform.[32] When crystallized from anhydrous organic solvents, cholesterol forms triclinic needles, and when crystallized from 95% alcoholic solution, it separates as monohydrate, rhomb-shaped triclinic plates, which lose water at 70-80°C. The absorption maximum of cholesterol in ethanol occurs at 206 nm ($\varepsilon_{1.0}^{M}$ = 3400).[33]

The chemistry of cholesterol is not particularly complex. Most of the reactions occur at the hydroxyl group, at the double bond or at carbon 7.[29]

A variable fraction of cholesterol is present in the human body as cholesterol esters formed by condensation of the alcohol group with long chain fatty acids that generally contain 16-20 carbon atoms.[33]

The glycoside digitonin, the saponins tigonin and gitonin, and the alkaloid tomatine interact with the 3β-hydroxyl group of cholesterol causing it to be precipitated. The reaction is specific for sterols containing the 3β-hydroxyl group, which provides a basis for procedures designed to measure free cholesterol in biological materials.[33]

The double bond between C-5 and C-6 can be hydrogenated and halogenated. The formation of halide, especially dibromide, has been of great practical use in purifying cholesterol obtained from natural sources. Interactions between cholesterol and sulfuric acid yield intensely colored compounds and this has been the basis of a number of colorimetric determinations.[33]

Cholesterol complexes readily with the salts $CaCl_2 \bullet 2H_2O$ and

$MgCl_2 \cdot 6H_2O$. Complexes contain two cholesterol molecules and one molecule of the salt. It is probable that these salts occur at sites of cholesterol deposition.[31]

Cholesterol readily undergoes auto-oxidation even when stored in crystalline form. The main auto-oxidation products are 7-ketocholesterol, 7α- and 7β- hydroxycholesterols, cholestane-3β, 5α-, 6β-triol, and 25-hydroxycholesterol.[31]

III. Origin and Function of Cholesterol in the Body

Cholesterol present in human and animal tissues has a dual origin. Part originates in the diet and the remainder is synthesized in the body from acetyl CoA, the active form of acetic acid. The quantitative contributions of these two sources are highly variable.[33] The daily dietary intake of cholesterol ranges from 0 to 1.2 g,[34-35] while the total quantity synthesized in the human body averages 1.0 g/day, about 80% of which is produced by the liver.[36-38] Numerous studies [36,39-43] have shown that dietary cholesterol, absorbed from the gastrointestinal tract, can inhibit cholesterol synthesis in the liver, which appears to be the principal mechanism by which the level of cholesterol in the body is controlled.

Cholesterol is the major sterol in man and a component of virtually all cell surfaces and intra-cellular membranes. It is also a substrate for the formation of other essential substances that include: (1) the bile acids, which are synthesized in the liver and function to facilitate the absorption of dietary triglycerides and fat, (2) various steroid hormones (e.g. progesterone, corticosteroids, estrogens), which have widely different physio-

logical properties, and (3) vitamin D_3, the only vitamin normally synthesized in sufficient quantities by the body and therefore not required in the diet.[44]

IV. Transport of Cholesterol and Other Lipids in the Body: The Lipoproteins

To solubilize the hydrophobic cholesterol and other lipids in the aqueous environment of the blood and to transport them throughout the body, lipids are associated with proteins. The lipids and proteins are not covalently attached but are associated by hydrophobic interaction.[45] The macromolecular lipid-protein complexes are referred to as lipoproteins and have characteristic sizes, densities and compositions. Protein moieties of the lipoproteins are known as apoproteins or apolipoproteins. They are responsible for the metabolism of the lipoproteins by interacting with the different enzymes and receptors in the body. Besides lipid transport, lipoproteins also regulate lipid synthesis and metabolism.[46]

Plasma lipoproteins are globular, essentially spherical micelle-like particles, that consist of a nonpolar core of triglycerides and cholesteryl esters sorrounded by an amphipathic coating of protein, phospholipid, and free cholesterol.[45] The several classes of lipoproteins are based upon their differences in sizes, floating densities in an ultracentrifugal field, or electrophoretic mobilities. Each lipoprotein class has a characteristic composition, but the amounts of lipid and protein do not occur in fixed proportions within each class.[47-49] Lipoproteins are, therefore, viewed as a continuous spectrum of molecular aggregates with a changing pattern of

components.[48,50] The different human serum lipoproteins and their properties and composition are summarized in Table 1.

A. Chylomicrons

The term chylomicron was first used by Gage[52] in 1920 to describe the luminous lipid particles observed under the dark field microscope in blood samples after ingestion of fat. Chylomicrons are the largest and lightest of the lipoproteins, with diameters ranging from 700-12000Å and a density less than 0.95 g/mL.[50-51,53-54] They are composed mainly of lipids (97.5-99.2%) with average percent by weight compositions that are 80-95% triglyceride, 1-3% free cholesterol, 2-4% esterified cholesterol, 3-6% phospholipids, and 1-2% protein.[53] On electrophoresis of lipoproteins on paper, agarose gel or polyacrylamide gel, chylomicrons remain at the origin. The major protein components are apo-C and apo-B.[50-51,53-54] Resemblances have been noted between the composition of the protein moiety of the chylomicrons and that of HDL. As such it has been suggested that the HDL are utilized by the intestinal cells for the synthesis of chylomicrons.[55]

Chylomicrons are the principal form of lipoproteins in which the absorbed triglycerides are transported from the intestines to the various organs in the body.[56] They are synthesized in the small intestine in response to the absorption of dietary fat. Most, but not all, of the lipid is of dietary origin. The absorbed lipid is re-esterified within the intestinal epithelial cell and then undergoes further synthetic steps involving phospholipids and apoproteins before secretion from the cell as a chylo-

Table 1. Classification, Properties, and Composition of Human Serum Lipoproteins.[51]

Measurement	Chylomicron	VLDL	IDL	LDL	HDL
Hydrated density,	0.93	0.97	1.003	1.034	1.121
Solvent density for isolation, g/ml	<1.006	<1.006	1.006-1.019	1.019-1.063	1.063-1.21
Molecular weight	(0.4-30) x 10^9	(5-10) x 10^6	(3.9-4.8) x 10^6	2.75 x 10^6	(1.75-3.6) x 10^5
Diameter, nm	>70	25.0-70.0	22.0-24.0	19.6-22.7	4-10
Electrophoretic mobility (paper, agarose)	Origin	Pre-β	Broad β (between β and pre-β)	β	α
Composition, % by weight					
Cholesterol, unesterified	2	5-8	8	13	6
Cholesterol, esterified	5	11-14	22	49	13
Phospholipid	7	20-23	25	27	28
Triglyceride	84	44-60	30	11	3
Protein	2	4-11	15	23	50
Synthesis	Intestine	Liver,intestine	Intravascular	Intravascular	Intestine, liver
Apoproteins, %total					
AI	7.4	Trace	-	-	67
AII	4.2	Trace	-	-	22
B-100	Trace	36.9	50-70	98	Trace
B-48	22.5	Trace	Trace	-	-
CI, CII, CIII	66	49.9	5-10	Trace	5-11
EII, EIII, EIV	-	13.0	10-20	Trace	1-2
D	-	-	-	-	Trace

micron.

Following release from the intestinal mucosal cells into the lymph, nascent chylomicrons enter the systemic circulation via the thoracic duct, and are rapidly metabolized with a plasma half life of 5 to 15 minutes. Transfer of apo A-I and apo A-II to HDL and reciprocal transfers of C and E apoproteins from HDL serve to enhance the hydrolysis of chylomicron-triglycerides by the enzyme lipoprotein lipase (LPL), and provide a source of chylomicron surface components that act as precursors of nascent HDL particles. This process occurs predominantly in the capillary beds of muscle and adipose tissue, and results in the progressive delipidation of the chylomicron particle. Progressive hydrolysis of chylomicron's triglycerides leads to the formation of a smaller chylomicron, the chylomicron remnant.[57]

Plasma drawn from normal human subjects more than 12 hours after ingestion of fat contains few if any chylomicrons.[58] In certain disorders however, the concentration and persistence of chylomicrons may be greatly increased. In a hereditary disease known as Fredrickson type I hyperlipoproteinemia[59] (or hyperchylomicronemia), there is a defect in the activity of the enzyme LPL, which is responsible for the removal from plasma of the triglyceride of both chylomicrons and VLDL. Lack of LPL activity may be due either to its absence or to the lack of the activator enzyme, the protein Apo C-II.[51] In another disorder known as endogenous (carbohydrate induced) lipemia, chylomicrons compete with the increased VLDL for LPL induced triglyceride hydrolysis.

B. Very low density lipoprotein (VLDL or pre-β)

The VLDL class of lipoproteins covers a wide spectrum of particles, having a diameters from 280-750Å, hydrated densities of 0.95-1.006g/mL and flotation values from 20-400.[47] From their relative mobility on electrophoresis they are sometimes referred to as pre-β lipoproteins.[53] Particle sizes are directly proportional to the TG content and inversely proportional to the phospholipid and protein content.[47] Average percent by weight compositions of VLDL are 55% triglyceride, 20% phospholipid, 15% cholesterol (30% of which is esterified), and 10% protein.[56] The major apoprotein of VLDL is apo B-100 with lesser amounts of apo-C polypeptides and apo-E.[57]

Very low density lipoprotein particles serve to transport endogenous triglycerides from the liver to the peripheral tissues. The liver is the major site of synthesis of VLDL, for which apo B-100 is a constitutive apoprotein but the small intestine will also produce significant amounts of VLDL.[60] Many factors including nutrient intake, plasma concentration of free fatty acids, and levels of insulin and epinephrine in plasma, appear to modulate the hepatic secretion of VLDL, which in turn influences VLDL concentrations in plasma.[57]

Particles of VLDL are metabolized more slowly than chylomicrons. Under normal conditions, the half life of VLDL apo-B is 6 to 12 hours. The metabolisms of VLDL and chylomicrons show many similarities, but one important difference is that the remnant particles, which result from VLDL lipolysis, can be either taken up by the liver via apo E mediated catabolism or subsequently converted to LDL. Metabolism of VLDL to

LDL is the major source of the latter lipoprotein in human plasma.[57]

There are many diseases and metabolic disorders in which moderate to massive increases in VLDL are observed. One such disorder is Fredrickson type IV hyperlipoproteinemia,[59,61] an inherited recessive disease. Increased VLDL production by the liver or reduced VLDL removal are important factors in the development of type IV hyperlipoproteinemias.[51]

C. Intermediate density lipoproteins (IDL)

The IDL's are short-lived lipoproteins derived from VLDL after the hydrolysis of the triglyceride of VLDL by the enzyme LPL.[51] The average percent by weight composition of IDL is 15% protein, 7% free cholesterol, 22-26% cholesteryl esters, 17% phospholipids, and 35-39% triglycerides.[50] It is significant that cholesterol and triglyceride contents of IDL are almost the same. The major apoproteins are apo-B and E.

During the catabolism of one VLDL particle, all constituents, except apo-B, are removed from the particle. Of the original amounts, 7% of apo-C, 20% of triglycerides, 40% of free cholesterol and phospholipids and 60% of esterified cholesterol are recovered with the IDL particle.[50] Each IDL particle however, retains the full complement of apo-B present in the original VLDL particle. Thus, only one IDL is formed from each VLDL particle.[56] Apo-E is responsible for the continued conversion of IDL to LDL for hepatic uptake and degradation.[51]

Disorders, known as Fredrickson type III hyperlipoproteinemia, occur where there is an abnormal accumulation of IDL because hepatic

receptors, which have high affinity for apo-E, do not bind or remove chylomicron remnants and IDL in a normal manner, due to lack of the isoform known as apo-EIV.[51]

D. Low density lipoproteins (LDL)

Low density lipoproteins range in size from 210-250Å, are isolated between densities 1.019-1.063 g/mL, and display β–mobility on electrophoresis.[53] The major lipid constituents by weight are cholesteryl esters (47-55%), unesterified cholesterol (10-11%), phospholipids (28-30%), and triglycerides (8-10%).[47] The major protein content is apo-B, which comprises over 90% of the total LDL protein.[47,50]

Low density lipoprotein is the major cholesterol carrying particle present in plasma. It is normally formed by hepatic delipidation of IDL by hepatic protein lipase. As delipidation occurs, apo-B is unmasked, allowing LDL to bind to specific membrane receptors.[51]

The degradation of LDL occurs in both peripheral tissues and the liver, but the liver is responsible for the catabolism of 70% of LDL in normal human subjects. Catabolism is facilitated by both receptor mediated and non-receptor mediated pathways, but in normal human subjects, the receptor mediated pathway predominates and is responsible for the clearance of up to 75% of the plasma LDL pool.[57] The sequence of events of LDL degradation occur in this order: LDL interacts with high affinity receptor sites and the bound LDL is internalized and subjected to lysosomal degradation that ultimately hydrolyzes the apo-B to amino acids, and the esterified cholesterol is hydrolyzed to free cholesterol which enters the

cytoplasm.[51]

Certain disorders result in either the abnormal absence or abnormal accumulation of LDL. In Fredrickson type II hyperlipoproteinemia (or familial hypercholesterolemia), LDL levels increase significantly and are characterized by a strikingly high incidence of premature atherosclerosis, which tends to cause disease and death at an early age.[51] Patients with familial hyperlipoproteinemia have a defective gene that codes for LDL receptor. The elevated plasma levels of LDL cholesterol cause an increased uptake of LDL by macrophages and smooth muscle cells of arterial wall tissues, resulting in premature atherosclerosis. In the opposite LDL dysfunction, no LDL at all has been found in the plasma of patients with abetalipoproteinemia. The inherited defect in the disease may be the inability to combine lipid with the B protein.[62]

E. High density lipoproteins (HDL)

High density lipoproteins are ultracentrifugally isolated in the density range of 1.063-1.21 g/mL and have an average percent by weight composition of 50% protein, 30% phospholipids, and 20% cholesterol.[50] They are sometimes referred to as α-lipoproteins because they migrate with α-globulins during electrophoresis. There are two subclasses based upon density differences: HDL_2 with densities ranging from 1.063-1.120 g/mL, and HDL_3 which ranges from 1.120-1.210 g/mL.[47] HDL_2 has a mean molecular weight of 360,000 and is composed of 60% lipid and 40% protein, while HDL_3 has a mean molecular weight of 175,000, of which 55% is attributed to the apoprotein. The major proteins are apo A-I and

apo A-II which constitute about 90% of the total protein while apo-C form the minor components.[50]

The major function of HDL is to transport cholesterol from peripheral tissues to the liver for its catabolism and excretion. In addition, HDL plays a major role as a scavenger of lipid and apolipoprotein during the normal catabolism of chylomicrons and VLDL. It is also known to play an important part as a plasma reservoir for apo-CII.[51]

High density lipoproteins are derived from direct secretions by the liver and intestine, transfer from other lipoproteins, and transfer from peripheral tissues.[14] Little is known about the sites of HDL catabolism. The liver and kidney are probably involved.[51]

Several factors have been shown to increase HDL concentrations. Among these are regular exercise, moderate consumption of alcohol, and correction of hypertriglyceridemia.[63-64] A particular genetic disorder, called Tangier's disease, also exists in which HDL particles are entirely absent. The disease is associated with a very low serum cholesterol level and the generalized deposition of cholesterol esters in tissues. These findings led to the concept that HDL has an important function in cholesterol transport.[58]

F. Other lipoproteins

(1) Lipoprotein (a) ("Sinking" pre-β-lipoprotein or Lp(a))

Lipoprotein (a) was discovered in 1963 by Berg[65] and became important because of its antigenic properties. It is found in human plasma

in varying amounts at the density interval of 1.055-1.085 g/mL.[50] Lipoprotein (a) has pre-beta mobility on electrophoresis and is composed of 27% protein, 65% lipid and 8% carbohydrates.[66-67] The protein moiety of Lp(a) lipoprotein was reported to be composed of 65% apoB, 20% of Lp(a) apoprotein, and albumin (less than 15% of total protein). Cholesterol makes up 41.7% and phospholipid 19.2% of the total lipid of Lp(a).[67] Immunochemically, Lp(a) cross reacts with LDL but it differs from LDL in both chemical composition and physical properties.[50]

Increased plasma concentrations of Lp(a) are associated with increased risk of atherosclerosis, but the precise physiologic function and the metabolism of Lp(a) remain unknown.[68] Apparently, even the near absence of Lp(a) from plasma does not cause a deficiency syndrome or any kind of disease.

(2) Lipoproteins occurring in disease states: Lp-X, Lp-Y, and Lp-E

Lipoprotein-X is an abnormal lipoprotein, composed predominantly of free cholesterol and lecithin. It appears in the LDL density range of 1.006-1.063 g/mL from patients with obstructive jaundice,[50] and contains about 65% lecithin, 20-30% unesterified cholesterol, and 5% protein. The protein moiety is composed of albumin (20-40% of total protein) and apo-C.[50] Manzato et al.[69] postulated that flowing back of bile into the blood stream might represent the actual pathway of Lp-X synthesis.

Lipoprotein-Y occurs in patients with cholestasis and lecithin cholesterol acetyl transferase (LCAT) deficiency.[70] It is a triglyceride rich LDL, which showed, in addition to apo-C, the presence of apo-B and

"lipoprotein B", a lipoprotein particle with higher triglyceride and free cholesterol contents than those of normal individuals and an unusually high content of apo-C.[14] The mechanism of Lp-Y synthesis has not been elucidated completely but it may be related to reduced activity of hepatic lipase in liver diseases.

Lipoprotein-E is a lipoprotein which is very rich in apoE. It was first noticed in LCAT deficient plasma by Utermann et al.,[71] and was also reported in patients with liver disease or secondary LCAT deficiency. The mechanism of Lp-E synthesis is not yet known.[14]

V. Studies on the Relationship of Lipoproteins with Coronary Heart Disease

The role of cholesterol and other lipids in the development of atherosclerosis is widely recognized. It is known that the two main processes of atherogenesis involve cell proliferation and blood lipid infiltration.[72] Chemical analysis of atherosclerotic plaques from humans indicate that on the average it is composed of about one half lipid components and one half protein. Most of the cells that accumulate in the atherosclerotic plaque are modified smooth muscle cells. These have undergone changes that have made them less contractile cells in the media of the artery and enable them to divide and synthesize collagen and take up lipid.[73]

The relationship of the various lipids and lipoproteins to CHD and their various manifestation have been the major epidemiological objectives of the Framingham Heart Study (FHS).[18,74-77] It was concluded that

LDL-C is positively correlated, while HDL-C is negatively correlated to CHD risk. The study determined that VLDL-C is also positively related to CHD risk but the relationship tends to disappear when all the lipoprotein fractions are considered simultaneously. Other major study programs in the United States which include the Lipid Research Clinics Prevalence Mortality Follow-up Study (LRCF), the Lipid Research Clinics Coronary Primary Prevention Trial (LRC-CPPT), and the Multiple Risk Factor Intervention Trial (MRFIT) have also made important contributions towards understanding the roles of lipoproteins as CHD risk factors.

The FHS, started in 1949, was an epidemiological study of cardio-vascular disease involving a group of 5209 men and women then aged 30 to 59 years. In 1968, 2815 men and women from the group, ages 49 to 82 years had their lipids and lipoproteins characterized after an overnight fast. The progress of all volunteers had been monitored by means of routine biennial medical examinations where possible, and from morbidity and mortality data provided by hospitals and other sources.[18]

Based on the FHS data, Lavie et al.[78] identified the importance of HDL-C in preventing CHD. The group observed that CHD is rare when HDL-C is high and LDL-C is low. Also, even when LDL-C levels are very high, CHD is fairly uncommon if HDL-C levels are 65 mg/dL or more, and it is rare when HDL-C levels are as high as 85 mg/dL. On the other hand, even when LDL-C levels are very low (≤100 mg/dL), CHD is still common when HDL-C levels are also very low (≤25 mg/dL). Using pooled data from four large prospective epidemiologic studies (FHS, LRCF, LRC-CPPT, and MRFIT), Gordon et al.[79-80] concluded that HDL-C represents a strong independent risk factor for CHD and that a 1 mg/dL increment in HDL-C is associated with a decrease in CHD risk of 2% men and 3% in

women.

Two major hypotheses exist to interpret the role of HDL in restricting the development of CHD.[19] In the first hypothesis HDL is described as a protector against atherosclerosis by its ability to trap excess cholesterol from cellular membrane by esterification and to transfer the esterified cholesterol to the triglyceride rich lipoproteins that are subsequently removed by hepatic receptors. This reverse cholesterol transport from the peripheral cells to the liver prevents the deposition of cholesterol at sites where excessive cholesterol levels produce atherosclerosis. High HDL-C levels will signify a high rate of reverse cholesterol transport. In the second hypothesis, it is proposed that HDL does not interfere directly with cholesterol deposition in the arterial wall, but instead is a measure of the rate of conversion of the TG rich lipoproteins to atherogenic remnants. High HDL-C levels then will indicate an efficient metabolism of TG-rich lipoproteins and a low production rate of atherogenic remnants.

The Lipid Research Clinics Prevalence Study, done during 1972-1976 in 10 collaborating North American centers, is another epidemiological study of lipid and other cardiovascular risk factors.[79] In 1977, a mortality follow-up study (LRCF) was begun by involving all participants in the Prevalence Study who were at least 30 years old at that time.[81] The primary objective of this study was to acquire data on the prevalence of different types of hyperlipoproteinemia in various age and ethnic groups.

The LRC-CPPT study was a multicenter, randomized double blind trial of the efficacy of lowering LDL-C levels in reducing CHD risk in 3806 asymptomatic middle-aged men with primary hypercholesterolemia ($\geq$ 265 mg/dL).[9,82] Findings of the LRC-CPPT confirmed that reducing TC

by lowering LDL-C levels can diminish the incidence of CHD morbidity and mortality in men whose high risk for CHD is a consequence of elevated LDL-C levels. A decrease of 22.3 mg/dL in LDL-C was associated with a 16% to 19% reduction in CHD risk.

Low density lipoprotein is the main carrier of cholesterol from the bloodstream. Elevation of plasma LDL-C therefore results in the deposition of cholesterol in extrahepatic tissues, including the arterial intima, and leads to atherosclerosis. It has been suggested that a more dense fraction of LDL is the one responsible for atherosclerosis, although this proposal is controversial.[83] Dense LDL is associated with a more atherogenic type of lipoprotein profile, with increased levels of TG and apo-B and lower levels of HDL-C and apoprotein-AI. One explanation for the relationship between dense LDL and atherogenesis relates to the increase susceptibility of dense LDL to lipid peroxidation. Evidence showed[84] that oxidized LDL is taken up more rapidly by the arterial cells. Another explanation of the atherogenic property of dense LDL is proposed by Zilversmit[85] and it suggests that the presence of dense LDL may be an indicator of a delay in the post prandial catabolism of potentially atherogenic, TG-rich remnant lipoprotein.

The MRFIT study was a randomized, multicenter clinical trial to test the effect of a multifactor intervention program on the mortality from CHD in 12,866 high risk men aged 35 to 57 years. The subjects were without clinical CHD manifestations but were at high CHD risk (upper 10%-15%) because of a combination of hypertension, cigarette smoking, and elevated plasma cholesterol.[86] Based on the analysis of the MRFIT data, Stamler et al.[87] demonstrated that serum TC levels of about 180 mg/dL and

above are associated with increased risk for middle aged American men, and not just levels that are equal to or greater than 220 to 240 mg/dL.

In 1985, the United States' National Institute of Health inaugurated the National Cholesterol Education Program (NCEP) with the goal of planning strategies to reduce the prevalence of elevated plasma cholesterol in the United States thereby reducing the incidence of CHD. Since then, the NCEP has issued periodic reports developed by its Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel or ATP-I) and its Laboratory Standardization Panel (LSP) on the validity of measurements.[88] The second report (ATP-II) presents the NCEP's updated recommendation for cholesterol management. The report of ATP-II is similar to ATP-I in its outline and fundamental approach to the treatment of high blood cholesterol although it does give more attention to HDL-C as an inverse CHD risk factor.[89] ATP-II still continues to identify LDL-C as the primary target for cholesterol lowering therapy. Dietary therapy remains the initial and principal mode of treatment, and drug therapy is reserved for patients at high risk of CHD.[90]

The guidelines issued by ATP-II for cholesterol management are as follows.[89] All American adults 20 years of age and over should have serum TC and HDL-C measured at least once every 5 years. Further risks classifications are based on answers to a series of CHD related questions. For those without a history of CHD, non-fasting TC and HDL-C measurement is sufficient. If any of the following are observed: (1) TC $\geq$ 240 mg/dL, (2) HDL-C $\leq$ 35 mg/dL or (3) TC between 200 to 239 mg/dL in association with two or more CHD risk factors, a fasting lipoprotein analysis is recommended. Individuals whose LDL-C levels are $\geq$ 160

mg/dL, and those having LDL-C levels between 130 and 159 mg/dL as well as two or more CHD risk factors would be candidates for dietary intervention. Those with existing CHD conditions must submit to fasting lipoprotein analysis, regardless of their blood cholesterol level. Low density lipoprotein cholesterol values greater than 100 mg/dL would be indications for dietary intervention. After an adequate trial of dietary modification, consideration might be given to drug therapy if LDL -C levels remain 30 mg/dL higher than the initial levels.[89]

Additional risk factors identified and expressed in ATP-II are:[89] (1) age; male 45 years and older; female 55 years and older or having premature menopause without estrogen replacement therapy; (2) family history of CHD; (3) cigarette smoking; (4) hypertension (blood pressure of 140/90 mm Hg and higher) or use of antihypertensive drugs; (5) HDL cholesterol level less than 35 mg/dL; and (6) diabetes mellitus.

CHAPTER III

REVIEW OF ANALYTICAL METHODS FOR CHOLESTEROL AND LIPOPROTEIN DETERMINATION

I. Methods to measure serum total cholesterol

A. Colorimetric methods

Interest in the determination of cholesterol originated in the late 19th century, and since then many methods have been developed. A comprehensive review of colorimetric methods for cholesterol determination was published by Zak and Ressler[91] in 1955 and by Tonks[92] in 1967.

The first attempt to measure cholesterol by color determination was made by Liebermann[93] in 1885 and applied by Burchard[94] in 1890. In 1910, Grigaut[95] introduced a procedure for the quantitative estimation of cholesterol based on the Liebermann-Burchard (L-B) reagent. At almost the same time, Windaus[96] employed a microgravimetric analysis for the determination of cholesterol using digitonin as the precipitating agent.

The L-B color reagents consist of a mixture of acetic anhydride and concentrated sulfuric acid, and occasionally glacial acetic acid. The color reaction was studied extensively and a number of modifications appeared which improved upon some of the drawbacks of this reaction. Among these drawbacks are its complexity and sensitivity to many variables, e.g. the composition of the mixture, the amount of water (if any) in the final reaction mixture, the solvent used, the reaction time and temperature, the effect of light, the wavelength at which the color is measured, and the occurrence of interfering substances such as bilirubin and unreacted digitonin. Neither the reagent nor the color of the final product is particularly stable.[92]

A series of modifications involved different extraction procedures. Autenrieth and Funk,[97] for instance, digested blood with a base and then extracted the cholesterol with chloroform or ether before carrying out the L-B reaction. Bloor[98] extracted cholesterol from the blood using an alcohol-ether mixture, evaporated the solvent, re-dissolved the residue in chloroform and ran the L-B reaction in the choloroform solution. In yet another procedure, cholesterol is extracted into an acetone-alcohol mixture and precipitated as the digitonide salt.[99] This is re-dissolved in acetic acid which is used as the solvent for the L-B reaction. In the Abell et al.[100] method, the work-up involves hydrolysis of cholesterol esters with alcoholic potassium hydroxide, extraction of the free cholesterol into petroleum ether, before measuring it by the L-B reaction.

To improve upon the reagent stability, Huang et al.[101] used a mixture consisting of glacial acetic acid, acetic anhydride, and sulfuric acid plus anhydrous sodium sulfate. Huang's procedure could be applied directly

to serum without using prior extractions. Trinder[102] modified the reagent to a mixture of acetyl chloride and sulfuric acid. The color produced in this reaction was said to be much more stable than the colors produced by modifications of the L-B reaction.[92]

Another group of color reagents for cholesterol determination uses p-toluene sulfonic acid (p-tsa) and related reagents. The reagents and the resultant colors are more stable compared to the L-B reaction.[92] A mixture of glacial acetic acid, p-tsa, and acetic anhydride, used by Pearson et. al.[103] in the direct analysis of serum cholesterol, was modified by (a) including sulfuric acid;[104] and (b) substitution of p-tsa with dimethyl-benzene sulfonic acid[105] or sulfosalicylic acid.[106]

Salts of iron (III) in concentrated sulfuric acid were the bases for other alternative color reagents. Zlatkis et al.[107] chose the metal chloride plus acetic acid. Sensitivity, precision and stability were improved using phosphoric acid in dissolving ferric chloride instead of acetic acid;[108] using ferric chloride in a mixture of phosphoric and perchloric acid;[109] using ferric chloride, glacial acetic acid and citric acid;[110] and finally ferric perchlorate, ethyl acetate and concentrated sulfuric acid.[111]

Another color reagent based upon a metal salt substrate was developed in 1910 by Tschugaeff.[112] The reagent is more sensitive than the L-B reagent and has been used by several scientists for cholesterol determination in blood.[112-116] Flourometric detection was also used as an alternative to absorbance detection in the L-B [117] and Tschugaeff reactions.[118]

B. Enzymatic methods

The high selectivity provided by enzymes have also been used to advantage in cholesterol determinations in steps that are preliminary to absorbance detection. Cholesterol dehydrogenase was used by Flegg[119] to oxidize cholesterol to Δ^4- cholestenone and the absorbance was measured at 240 nm. Richmond[120] isolated cholesterol oxidase and applied the purified enzyme to the direct assay of cholesterol in saponified serum. The hydrogen peroxide produced in the reaction of cholesterol oxidase with serum cholesterol was reacted with xylenol orange and quadrivalent titanium and the absorbance of the complex was measured at 550 nm. The method of Allain et al.[121] involved the use of three enzymes to determine total cholesterol in serum. The third enzyme hydrolyzes the cholesterol esters and eliminates the saponification step.

The hydrogen peroxide produced by the action of cholesterol oxidase has been coupled with a number of co-reagents to produce a stable color. Allain et al.[121] used 4-amino-antipyrine and phenol with absorbances measured at 500 nm. Huang et al.[122] chose homovanillic acid in the presence of peroxidase to form a highly fluorescent compound. Papastathopoulos and Rechnitz[123] reacted the hydrogen peroxide with iodide in a molybdenum (VI) - catalyzed indicator reaction and monitored the change in iodide concentration with an ion selective membrane electrode.

Flow injection analysis (FIA) together with enzyme immobilization has been used for cholesterol determinations. Combinations of these two techniques offers several advantages that include selectivity, low cost per

analysis, small sample consumption, and short analysis time. Methods have been proposed for the determination of cholesterol using FIA and immobilized enzyme with amperometric detection[124-127] as well as photometric and fluorometric detection.[128] Petersson et al.[129] and Malavolti et al.[130] developed flow injection systems coupled with luminol-hydrogen peroxide chemiluminescence detection. Use of a fiber optic cholesterol biosensor was reported by Trettnak and Wolfbeis[131] and the detection was based on immobilized cholesterol oxidase and an oxygen-quenched fluorescent reaction. Krug et al.[132] investigated the feasibility of a photometric method using the fiber optic detection approach employing the dye 2-2'-azino-bis-(3-ethyl-benzthia-zoline-6-sulfonic acid) diammonium salt. A cholesterol sensor was also fabricated by Dong et al.[133] based on electrodeposition of palladium and enzyme immobilization on glassy carbon electrode. The hydrogen peroxide produced is monitored by measuring the current of peroxide oxidation electrocatalyzed by dispersed palladium particles.

C. Chromatographic methods

Chromatographic methods have also been adapted to determine cholesterol in human serum. Kritchevsky et al.[134] separated the cholesterol by thin layer chromatography, scraped the cholesterol spot into a test tube, charred it with concentrated sulfuric acid and quantitated by determining the absorbance at 375 nm. Several groups have used gas-liquid chromatography.[135-137] Prior work-up schemes involve saponification, extraction, and sometimes derivatization of cholesterol from the serum. Duncan

et al.[138] prepared the sample according to Abell-Kendall[101] and used reversed phase liquid chromatography with detection at 200 nm. Nomura et al.[139] determined cholesterol by supercritical fluid chromatography on an inert octadecylsilane-silica gel column using supercritical carbon dioxide as mobile phase. The eluent is monitored simultaneously with flame ionization and ultraviolet absorption detector.

D. Mass spectrometry methods

The use of isotope dilution-mass spectrometry (ID/MS) in the determination of cholesterol in serum was first reported by Bjorkhem et al.[140] in 1974. Deuterium-labelled cholesterol ([2,2,3,4 - $^{2}H_4$]-cholesterol) was added to a fixed amount of serum. The ratio of the molecular ions (m/z 386 and 389) measured by GC-MS was used for the determination. Gambert et al.[141] separated cholesterol on a capillary column and used the [3,4 - ^{13}C] isotope of cholesterol because the non-radioactive ^{13}C isotope eliminates radiolysis reactions, exchange processes or isotopic effects. Mass spectrometry was used in the chemical ionization mode leading to simple fragmentation with a greater relative abundance of the high mass ions. Cohen et al.[142] added cholestero-d_7 [cholest-5-en-25,26,26,26,27,27, 27-d_7 -3-ol (3ß)] to the serum and added a derivatization step (trimethyl silyl ether derivative). The intensity ratio of molecular ions at m/z 465 and 458 was followed by GC-MS. Takatsu and Nishi[143] used ID/MS with high performance liquid chromatography instead of GC. The same authors developed a liquid chromatography-atmospheric ionization-MS[144] for serum cholesterol determination.

E. Other methods

A recent approach that uses near infrared reflectance spectrometry detection to measure serum cholesterol was described by Peuchant et al.[145] in 1987. Preliminary spectral calibration is performed using human sera whose cholesterol concentrations are measured by a reference chemical analysis. Calibration makes it possible to select wavelengths that are characteristic of the matrix and its composition. Calibration constants, obtained from regression calculations, are used to quantify serum cholesterol according to a mathematical equation. The determination is performed directly on serum without prior extraction or added reagent. Results are obtained in less than one minute.

II. Methods for serum lipoprotein separation

A. Ultracentrifugation

Differences in the hydrated densities among the lipoproteins make it possible to do fractionation by ultracentrifugation. The Center for Disease Control (CDC) Primary Reference Method for determining lipoprotein classes is based upon separation by ultracentrifugation. The method is time consuming, expensive, and therefore not suitable for large scale use.[146] Its use is restricted to specialized lipid research laboratories. Various types of ultracentrifugation have been employed in lipoprotein analysis.

The separations of lipoprotein classes by sequential differential ultra-centrifugation involves repeated ultracentrifugation (depending on the desired separation) after progressively raising the solvent density. This

method was first reported by Havel et al.[147] and De Lalla and Gofman[148] in the 1950's. Relatively large quantities of plasma can be processed at one time and purification of the lipoproteins from other proteins of density >1.21 g/mL is excellent.[149] However, sequential ultracentrifugation can cause some structural alteration perhaps due in part to long periods of exposure to the high salt concentrations and high **g** forces involved.

The beta-quantification method which has been accepted as a Reference Method,[24] is a combination of ultracentrifugation and precipitation steps. A single ultracentrifugation step is performed, separating the plasma at its own density of 1.006 g/mL. After spinning at 109,000 x **g** for 18 hours, VLDL and chylomicrons float to the top leaving IDL, LDL and HDL in the infranate. The supernatant and infranatant fractions are separated by a tube slicing technique. The cholesterol concentration of plasma, the infranate and the HDL fraction (obtained by chemical precipitation of LDL and IDL in the infranate with heparin-manganese) are measured by the Abell-Kendall method.[100] Very low density cholesterol is calculated as plasma cholesterol minus the total infranatant cholesterol while LDL-C is total infranatant cholesterol minus HDL-C. Additional ultracentrifugation steps are sometimes performed on the infranate to individually separate sub-classes of different densities. Density adjustment to 1.019 g/mL is used to float IDL, adjustment to 1.063 g/mL will float LDL and adjustment to 1.21 g/mL isolates HDL.[24]

Density gradient ultracentrifugation is a different procedure that gained widespread usage in the mid-1970's when Redgrave et al.[150] developed the four step density gradient technique to separate plasma lipoproteins. This technique uses solutions of different densities which are

carefully layered into each tube along with the sample. After ultra-centrifugation to equilibrium, each of the lipoproteins will have migrated into its respective isopycnic density region. The method can separate the lipoprotein classes in a single centrifugation step but incomplete separation may introduce error.[151]

B. Selective precipitation

Precipitation methods were primarily developed in the laboratory of Burstein[152] whose purpose was to isolate lipoproteins from large serum sample volumes or to eliminate LDL-C and VLDL-C from small sample volume in order to determine HDL-C. These methods are fast, simple, and inexpensive which accounts for their popularity.[153] Various precipitating agents have been studied for the precipitation of the serum lipoproteins.

A polyanion such as heparin, either alone or associated with a bivalent cation, selectively precipitates the various lipoprotein classes. At neutral pH and in the presence of a bivalent cation, the ability to precipitate depends upon the relative lipid content. At slightly acid pH, and in the absence of bivalent cation, precipitation is related to the dominant apoprotein.[154] Low density lipoprotein is the major precipitate in the pH dependent procedure and the minor precipitate in the metal ion dependent procedures.

Low density lipoprotein is also precipitated by high molecular weight sulfated polysaccharides at either neutral or slightly alkaline pH. Oncley et al.[155] reported that dextran sulfates with molecular weights over a million, precipitate LDL and VLDL but do not precipitate either chylo-

microns or HDL.

Highly charged anions precipitate LDL and VLDL fractions almost completely. Among these are sodium phosphotungstate ($2Na_2P_2O_5$ $12WO_3,18H_2O$), ammonium paramolybdate ($(NH_4)_6Mo_7O_{24}$, $4H_2O$), and two smaller monomeric bivalent anions, sodium tungstate (Na_2WO_4, $2H_2O$) and sodium molybdate (Na_2MoO_4, $2H_2O$).[154]

One step precipitations of serum lipoproteins can also be accomplished with any of the following reagents: (a) polyphosphates and bivalent cations, (b) tetracyclines and bivalent cations, (c) anionic surfactants and bivalent cations, (d) anionic surfactants and polycations (protamine sulfate), and (e) cationic surfactants and a polyanion (heparin). In each of these associations, the reactants bear opposite charges, and the anion-cation interaction results in the formation of insoluble salts.[154] Non-ionic linear polymers of high molecular weight that are freely soluble in water, such as polyvinylpyrrolidone, polyethelene glycol (PEG) and dextran, have also been used.[154]

Several studies[24,146,153,156-158] have assessed the performance of the different precipitating agents for lipoprotein analysis. Wiebe and Smith[155] compared six precipitating agents used in HDL analysis. They evaluated heparin-$MnCl_2$ at two different concentrations (46 and 92 mmol/L), dextran sulfate-$MgCl_2$ using two different molecular masses of dextran (M_r 50,000 and 500,000), sodium phosphotungstate-$MgCl_2$, and PEG 6000. Their results showed that precipitation with heparin-$MnCl_2$ (92 mmol/L) and PEG gave similar results while dextran sulfate-$MgCl_2$ (M_r 500,000) had the largest proportional and constant bias. All the methods produced

comparable result in the low HDL cholesterol range (25 to 35 mg/dL) but biases were significant at high concentrations. The increased bias in the upper HDL cholesterol range may be due to increased heterogeneity of HDL and the different mechanisms involved in forming the insoluble complexes between lipoproteins and the various precipitating agents.[153] Warnick et al.[156] reported that dextran sulfate (50)-Mg^{+2}, heparin-Mn^{+2} (92 mmol/L) and phosphotungstate-Mg^{+2} give similar results, while heparin-Mn^{+2} (46 mmol/L) and PEG at two different concentrations (75 and 100 g/L final concentration) gave slightly higher values for HDL cholesterol.

C. Electrophoresis

Differences in size and charge among the lipoproteins are the bases for separation by electrophoresis. The net charge on a lipoprotein molecule results from the balance of positive and negative charges on the terminal and side chain amino acid residues, and to a small extent from the phospholipids that are not in the zwitterionic form at electrophoretic pH.[159]

Electrophoretic separation of lipoproteins provides for only qualitative analysis of the particle distribution.[160] It is possible to quantify the bands based on the relative intensities of their stained electrophoretic bands determined by scanning densitometry. However, dye uptake by each lipoprotein is variable and correlates poorly with concentration.[160] Studies have also suggested that many of the electrophoretic methods do not achieve acceptable precision.[161]

Early electrophoretic methods used a liquid phase without supporting

media (free electrophoresis). Although this technique is no longer widely used, it gave valuable information to early investigators. The technique has been abandoned since then and has led to the development of electrophoresis on fixed media. Zone electrophoresis is considered more advantageous since it requires simpler equipment.[162] Media that have been used as supports include starch granules,[163,164] paper,[165] cellulose acetate,[166] agarose,[167,168] and polyacrylamide gel.[169,170]

D. Chromatography

Chromatographic separations of lipoproteins are based on size differences. High performance liquid chromatography (HPLC) has been extensively used in the laboratory of Okazaki[171-175] in the analysis of lipoprotein classes. They developed an HPLC separation method for serum lipoprotein using columns designed for gel permeation chromatography. Quantitation of cholesterol was performed using an enzymatic reaction and absorbances were monitored at 280 nm and 550 nm for protein and cholesterol, respectively. The method successfully separated VLDL, LDL, HDL_2, and HDL_3. It is very simple and gives a direct determination of the fractions in less than 50 minutes without any pretreatment.[171] Other components of the lipoprotein can also be determined with appropriate reagents for selective detection.[174,175] Chromatographic methods are relatively expensive and at present are largely restricted to specialized research laboratories.[160]

Lipoprotein separations have also been made using agarose column chromatography.[176,177] The method is gentle and non-destructive, being

simultaneously preparative and analytical and capable of providing an adequate recovery of lipoproteins after separation. The major drawbacks are long analysis time (24 hours) and the inability to obtain homogeneous lipoprotein fractions.[176] Hydroxyapatite column chromatography[178] has been used for the separation of subfractions 2 and 3 from HDL isolated by preparative ultracentrifugation.

E. Immunochemical methods

Because of differences in the protein moieties among the lipoprotein classes, immunochemical methods have been developed for their quantitation. The ability of proteins to stimulate the production of specific antibodies enabled investigators to identify and quantify proteins in mixtures, even at very low concentrations. Immunochemical methods are more sensitive, selective, reproducible, and potentially more adaptable to automation than non-immunological procedures.[51] Drawbacks are the significant problems associated with producing antisera and in the standardization of lipoprotein assays. Also, immunological cross reactivity makes quantitative immunological analysis of specific lipoprotein fractions in whole plasma difficult .[58]

The introduction of specific antisera for the apoproteins enabled the development of various types of immunoassays leading to the quantification of the protein moieties of VLDL, LDL, and HDL, together with their subclasses. These immunochemical techniques include, radial immuno-diffusion,[179] electroimmunoassay in agar or agarose gel,[179] radio-immunoassay,[180] immunonephelometry,[181] enzyme linked immuno-

assay,[182,183] and fluorescence immunoassay.[184]

III. Proposed alternative method provided by this study

Despite the presence of many analytical methods for cholesterol and lipoprotein determinations, the Laboratory Standardization Panel continues to encourage the development of new methods particularly for LDL-C and HDL-C determinations that is applicable for routine use in the clinical laboratories.[24-25] The method should pass the requirements set by LSP in terms of precision and accuracy.

This study had as a goal the development of a method that measures the three major lipoprotein fractions simultaneously with an acceptable accuracy and precision. Measuring the lipoprotein fractions simultaneously reduces the analysis time and is applicable for routine use. The applicability of the method to measuring cholesterol in hypertriglyceridemic samples was evaluated.

CHAPTER IV

EXPERIMENTAL

A. Sources of Serum Samples

Serum samples for this work were provided by four different laboratories: Oklahoma State University Wellness Center (UWC); Stillwater Medical Center (SMC); Roche Biomedical Laboratories (RBL), Kansas City, Missouri; and University of Cape Town Medical School (UCT), South Africa.

Samples provided by UWC were from volunteers who requested a lipid profile from the Oklahoma State University. No attempt was made to select subjects according to demographic classification, and no demographic data were collected. Subjects were instructed to report to the Wellness Center laboratory having fasted for at least 12 hours. A venous blood sample was drawn from the arm of each subject and placed into two Vacutainer™ red stoppered serum separation tubes. Serum separation tubes have a floating gel to aid in separation of red cells from serum. After letting the serum stand at room temperature for about 30 minutes, it was centrifuged at 5,000 rpm for ten minutes in a table top clinical centrifuge (Roche Biomedical Laboratories VanGuard 6000). One separation tube was taken to the Oklahoma State University Department of Chemistry, for

measurement by the alternative method. Samples were transferred into new 10 mL glass vials with a screw cap. The second separation tube was collected by Roche Biomedical Laboratories personnel for measurement at its Kansas City, Missouri regional laboratory.

Samples from SMC and RBL were exclusively high triglyceride specimens (TG >250 mg/dL). This figure is much lower than the normally accepted cut-off level of 400 mg/dL used for the Friedewald formula.[21] Measurements by SMC and RBL were made in-house using one of the standard routine enzymatic methods. After the serum lipids had been measured, the residuals of the samples were collected and sent to OSU Department of Chemistry.

The majority of the UCT samples were categorized as having lipid disorders (Type III and familial hypercholesterolemia) but a few were normal samples. Splits from these venous samples were shipped overnight to OSU. Lipoprotein fractions were separated in-house by ultracentrifugation, and split portions from these fractions were also shipped to OSU. Cholesterol and TG measurements were made at UCT on the fractions after separation.

The size of the sample pool is currently around 650 samples and is almost equally divided between normal and high triglyceride levels. Some samples were hemolyzed in the collection process and some were creamy in appearance, neither of which produced an observable interference in the method. Samples were not categorized according to gender, race, age, etc. Sample collection and storage conditions before receipt were not standardized, nor was the interval of time that passed between making the tests in the different laboratories. When the samples were received they were kept in the refrigerator maintained at 4°C and were equilibrated to

room temperature for at least 30 minutes before analysis. Serum samples appear to be stable for at least 2-3 months when stored in the refrigerator. Replicate measurements were made in our laboratory to evaluate the imprecisions in the measured spectra that would result from the combined effects of the sampling procedure, and the chemistry of the color reaction.

B. Analytical Methods Used in Independent Laboratories

The laboratories at Roche Biomedical and SMC regularly use one of the current routine commercial methods to measure lipid profiles. The Olympus^{tm} AU560 and Technicon^{tm} Chem-I clinical autoanalyzer are preferred by RBL and SMC, respectively. Three independent measurements are made in order to obtain a lipid profile. Total cholesterol, HDL-C, and TG are measured directly by enzymatic methods.

The enzymatic method for determination of cholesterol is based on the method developed by Allain et al.[121] and uses a color derivatization reaction described by Trinder.[185] The enzymatic reactions involved are as follows:

$$\text{Cholesterol Esters} + H_2O \xrightarrow[\textit{Esterase}]{\textit{Cholesterol}} \text{Cholesterol} + \text{Fatty Acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow[\textit{Oxidase}]{\textit{Cholesterol}} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + \text{4-Aminoantipyrine} + \text{p-Hydroxybenzenesulfonate} \xrightarrow{\textit{Peroxidase}} \text{Quinoneimine Dye} + 4H_2O$$

The quinoneimine dye produced is measured by its absorbance at 500 nm. Although the quinoneimine dye is not structurally related to cholesterol, the absorbance intensity of the colored product is proportional to the amount of cholesterol. For the HDL-C determinatons, the same enzymatic processes were used after VLDL-C and LDL-C were selectively removed by precipitation reaction with a prepared aliquot of dextran sulfate-Mg^{+2}. The VLDL-C fraction is calculated as equal to one-fifth of the TG value as long as TG is not > 400 mg/dL. The routine method for TG determination also involve a four step enzymatic reaction. The LDL-C levels are estimated using the Friedewald formula,[21]

$$\text{LDL-C} = \text{TC} - (\text{HDL-C} + \text{TG}/5). \qquad \text{(eq. 1)}$$

For samples with TG > 400 mg/dL, only TC and sometimes HDL-C results would be reported by the outside laboratories.

Lipid profiles of samples from UCT were determined by sequential ultracentrifugation. Solutions of KBr were added for density adjustments. Adjustment to 1.006 g/mL is used to float VLDL-C and chylomicrons; adjustment to 1.019 g/mL is used to float IDL-C; adjustment to 1.063 g/mL will float LDL-C and adjustment to 1.21 g/mL isolates HDL-C.[24] Cholesterol and TG levels for each fraction were measured by the same enzymatic methods discussed earlier.

C. The Proposed Alternative Method

The method is based upon a color production reaction. The color reagent used in this work has evolved from that first described by

Chugaev and Gastev.[112] In its original form, the reagent was a 2:1 mixture of a solution of 20% w/v $ZnCl_2$ in glacial acetic acid combined with 98% acetyl chloride. The reagent was used succesfully to measure only total cholesterol levels.[113-116] This reagent was also used in trials to measure the cholesterol distribution among the lipoprotein fractions using circular dichroism (CD) spectropolarimeter as the detector.[26] In the CD study, a 2-ml aliquot of the reagent was added to 50 µL of serum. After 8 minutes of incubation at 67°C, the solution was cooled to room temperature and 1 mL of chloroform was added followed by CD measurements from 625-325 nm. Results of the CD study showed that while CD is capable of discriminating HDL-C from the low density fractions, distinction between VLDL-C and LDL-C was not possible.

In a subsequent study,[186] CD was replaced by absorption detection. The $ZnCl_2$ concentration was increased to 35% w/v and the volume ratio of the solvents was inverted so that acetyl chloride was in a 100:1 excess over acetic acid. This modification produced signals of increased intensity and eased some of the problems with protein precipitations when reagent and serum were mixed. Incubation conditions remained the same but, because of the increased sensitivity, the sample size was decreased to 20 µL. Two reagents are kept separate before being added to the serum, which is inconvenient for routine screening work. Experimental conditions still require an 8 minute incubation period at 67°C, which is 14°C higher than the boiling point of acetyl chloride, which is now the solvent in excess.

The reagent that has evolved during this study is a one reagent system. Glacial acetic acid was eliminated altogether and the metal salt was changed to $Zn(ClO_4)_2 \cdot 6H_2O$ (Johnson-Matthey or G. Frederick Smith).

The perchlorate salt dissolves in 98% acetyl chloride (Sigma or Aldrich) directly and is used at a concentration of 0.50 M. Traces of insoluble material, probably ZnO, are conveniently removed by centrifugation for 10 minutes. The reagent is prepared when needed and stored in a tightly sealed amber glass container. A major advantage of these modifications is that color is produced at room temperature which significantly reduces the hostility of the reaction conditions.

The reagent to serum volume ratio was maintained at 100:1. Tests are conveniently done on samples as little as 10-μL, making the method applicable, in principle, to a serum sample as small as a finger-stick. Serum and reagent are added, in that order, to a glass vial and shaken by hand for 15-30 seconds. Proteins precipitated on adding the reagent are removed by centrifugation (Fisher Microcentrifuge Model 59A; speed setting at 9.0) for 3 minutes. Color begins to develop at ambient temperatures immediately after mixing. The supernatant is transferred to a cuvet with a pathlength of 1 cm. Absorbance spectra are measured after 15 minutes from the time of mixing by making five spectral passes from 350-750 nm using a Hewlett-Packard 8452A diode-array spectrophotometer equipped with a temperature controller (25.0 ± 0.1°C). The wavelength range is wider than it was in prior work[186] which facilitates the measurement of lipid profiles, especially for those with high TG levels.

A kinetic study of the color reaction shows it to be 98% complete after a period of 15 minutes, which is the time used for routine measurements. The ultimate color is stable for at least an hour. The function of the Chugaev reagent is not atypical of Friedel-Crafts reagents. Most likely, therefore, the mechanism will involve dehydration and dehydrogenation steps. Friedel-Crafts reagents generally consist of an acid

halide and a Lewis acid catalyst. We found that the zinc chloride, acetate, and perchlorate salts, in combination with acetyl chloride will produce the colored product, as will perchloric acid. Acetic acid, needed to solubilize the zinc chloride for the original Chugaev reagent, appears to retard the reaction rate which accounts for the higher incubation temperature when it is present. Like zinc perchlorate, zinc acetate dissolves directly in acetyl chloride eliminating the need for glacial acetic acid. The perchlorate salt was chosen over the acetate because the latter formed a yellow solution with acetyl chloride after several hours.

From a mechanistic study of the Chugaev reagent,[187] it was proposed that the color is a product of extended conjugation. Similar mechanisms were proposed for the Liebermann-Burchard[188] and Zak[189] reactions. The Chugaev reaction was also run on a number of structurally related steroids.[190] Those that produce color were also found to have antirachitic activity, implying a structural similarity between the product and vitamin D. In other words, the B-ring of the cholesterol template would open at C-10, Figure 2, stimulating dehydrogenation throughout rings C and D.

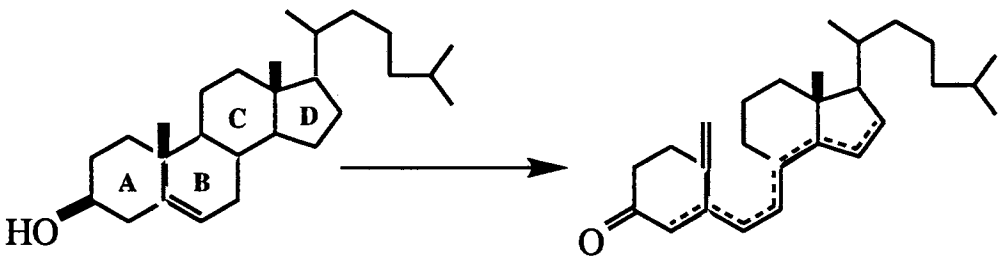

Figure 2: Proposed product of the color reaction.[187]

D. Comparison of Data Analysis Methods

Determining the concentration of an analyte involve two steps: a calibration step and a prediction step. In the calibration step, a mathematical equation is developed that define the relationship between instrument response, A, and the analyte concentrations, C.[191] The calibration equation vary in complexity depending upon the extent of linearity or non-linearity of the correlation. Once the calibration equation has been established, it will be used to predict the concentration of future samples. There are several approaches in obtaining the calibration equation.

Univariate Approach

This approach to calibration typically uses just one A variable (e.g. absorbance at one wavelength) to determine the calibration equation. The calibration equation is obtained by measuring the absorbances of a series of solutions of known concentrations for a standard reference material. In many instances, the entire spectra is obtained but in univariate data analysis only one absorbance (usually the maximum absorbance) will be used. The additional information contained in the spectrum remains unused. The absorbance data are then plotted against concentration to construct a typical Beer's law working curve. Simple linear regression is used to find the statistically best straight line and the corresponding linear calibration equation.

The univariate approach assumes that the measurement method is specific or selective and that no other matrix constituents influence the

measurement signal. These conditions are not always fulfilled unless the samples are purified and stabilized prior to analysis. Interferences may affect the measurements and make them unsuitable for univariate prediction.[192] For complex mixtures, separation of the desired analyte from the interferences is a pre-requisite for successful calibration and prediction.

Multivariate Approach

Multivariate calibrations, in contrast, combine several different variables (e.g. light absorbance at different wavelengths) in developing the calibration equation. As a consequence interferences can be modelled and/or eliminated and outliers detected.[193] One does not necessarily need the reference materials for calibration. Instead, one reverts to making a broad selection of real samples. The emergence of microcomputer in the laboratory has led to the increased usage of multivariate calibration in data analysis, and it has provided the analytical chemist with the ability to accumulate vast amounts of data in a relatively short period of time.

Multivariate calibration can reduce the need for sample preparation in chemical analysis because various interferences can be accounted for in establishing the calibration equation. Complete separation of the interferences is not a prerequisite compared to the univariate approach. After calibration, one is able to replace cumbersome, slow chemical analysis methods by simple, fast instrumental measurements, provided that the unknown samples belong to the same type of samples used for calibration.[194]

Direct or indirect calibration can be used for multivariate data

analysis. Direct multivariate calibration involves prior measurements of the spectra for pure forms of the individual constituents. In the indirect approach, one foregos the need for the spectra of the pure components. A "representative" set of "reference samples" with measured values for both spectrum and concentration is used for the estimation of the calibration coefficients.[193]

Several multivariate methods are encountered in the literature.[191-196] Only multiple linear regression (MLR), principal component analysis (PCA), principal component regression (PCR), and partial least squares regression (PLS) are discussed in subsequent sections.

Multiple Linear Regression (MLR)

Multiple linear regression is the simplest of the multivariate calibration method. The goal of MLR is to find the linear combinations of the response variable (a_j) in the response matrix **A** such that the model estimates ($\hat{c}_i$) of the values in the concentration matrix **C** in the calibration set, given by,

$$\hat{c}_{i,k} = \sum_{j=1}^{j} a_{ij} \cdot s_{jk} \qquad \text{(eq. 2)}$$

are as close to the known values of **C** as possible.[195] The regression coefficients, s_{jk} are estimated by linear regression with the error term, expressed as

$$\text{Error} = \sum_{k=1}^{k} \sum_{i=1}^{i} (c_{ik} - \hat{c}_{i,k})^2 \qquad \text{(eq. 3)}$$

is kept to a minimum. The general relationship between the concentration matrix **C** and response matrix **A** given by MLR is

$$\mathbf{C} = \mathbf{A}\,\mathbf{S} + \mathbf{E} \qquad \text{(eq. 4)}$$

where **S** is a matrix of regression coefficients and **E** is a matrix of errors associated with the MLR model.

Multiple linear regression is reasonable when dealing with well defined systems (responses are linear, no interfering signals, no analyte-analyte interactions, low noise and no collinearities).[195] All the information in the response matrix **A** is used to establish the mathematical relationship between **A** and **C** regardless of whether or not it is relevant in describing the true relationship.

Principal Component Analysis (PCA) and Principal Component Regression (PCR)

The first step in PCR is PCA. The American Society for Testing and Materials define PCA as " a mathematical procedure for resolving sets of data into orthogonal components whose linear combinations approximate the original data to any desired degree of accuracy. As successive components are calculated, each component accounts for the maximum possible amount of residual variance in the set of data. In spectroscopy, the

data are usually spectra, and the number of components is smaller than or equal to the number of variables or the number of spectra, whichever is less."[196]

Principal component analysis searches for a few uncorrelated linear combinations of the original variables that capture most of the information in the original variables.[197] Geometrically, the first principal component is the line of closest fit to the n observations in the p dimensional variable space. It minimizes the sum of the squared distances of the n observation from the line in the variable space representing the first principal component. The second principal component is a line of closest fit to the residuals from the first principal component.[197] The process goes on until all the variations in the variables are accounted for, but the first few components account for most of the variation.

Algebraically, the first principal component U_1 (also known as scores or factors) for a given measured variables **A**, is a linear combination of the variables in **A** given by

$$U_1 = Y_{11} \cdot A_1 + Y_{12} \cdot A_2 + \ ... + Y_{1p} \cdot A_p = \sum_{i=1}^{p} Y_{1i} \cdot A_i \quad \text{(eq. 5)}$$

such that the variance of the first principal component is maximized. The coefficients Y are referred to as "weights" and are always scaled so the sum of the squared weights is equal to one[198]

$$\sum_{i=1}^{p} (Y_{1i})^2 = 1 . \quad \text{(eq. 6)}$$

Since the variance of U_1 is maximized, the sum of the squared correlations $\sum_{i=1}^{p} (r_{U,Ai})^2$ is also maximized.

The second principal component U_2 involves finding a second weight vector Y_2 such that the variance of

$$U_2 = Y_{21} \cdot A_1 + Y_{12} \cdot A_2 + \ ... + Y_{1p} \cdot A_p = \sum_{i=1}^{p} Y_{2i} \cdot A_i \qquad \text{(eq. 7)}$$

is maximized subject to the constraints that it is uncorrelated with the first principal component and $\sum_{i=1}^{p} (Y_{1i})^2 = 1$.[197] This process can be continued until as many components as variables have been calculated.

The main parameters resulting from a principal component analysis are the matrix of the weighted vector **Y** that is associated with each principal component U and its associated variance λ. The correlations (loadings) of the measured variables with a particular component, can be obtained by multiplying all the elements in the weight vector by the square root of the variance λ of the associated principal component.[197]

The second step of PCR uses MLR to fit the concentration matrix **C** of the calibration samples onto the scores or principal component matrix **U**.[195] This can be represented by

$$\mathbf{U\,S} = \mathbf{C} \qquad \text{(eq. 8)}$$

where **S** is the matrix of regression coefficients obtained by MLR.

For predicting the concentration of unknown solution, one uses the **Y** and **S** matrix. The response of the unknown sample A_{unk} is multiplied by Y to obtain U_{unk} and then U_{unk} is multiplied by S to yield $\hat{c}_{unk}$, the estimate of the concentration of the analytes in the unknown samples.[195]

Partial Least Squares Regression (PLS)

The pioneering work in PLS was done in the late sixties by H. Wold in the field of econometrics. Chemical applications were pioneered by the group of S. Wold and H. Martens in the late seventies.[199] Partial least squares regression is based on H. Wold's general PLS principle in which complicated, multivariate problems are solved by a sequence of simple least-squares regressions.[200] The purpose of using PLS in multivariate calibration is to obtain a good insight and good predictive ability at the same time.

Partial least-squares regression is a modelling procedure that simultaneously estimates the underlying factors in both the response matrix **A** and the concentration matrix **C**.[195] The approach used by PLS is very similar to PCA, the difference being that factors are chosen to describe the variables in **C** as well as in **A**. This is done by using the columns of the **C** matrix to estimate the factors of **A** and at the same time, the columns of **A** are used to estimate the factors for **C**. The resulting models are summarized by

$$\mathbf{A} = \mathbf{U}\,\mathbf{P} + \mathbf{E} \qquad \text{(eq. 9)}$$

and

$$\mathbf{C} = \mathbf{T}\ \mathbf{Q} + \mathbf{F} \qquad \text{(eq. 10)}$$

where the elements of **U** and **T** are called the scores of **A** and **C**, respectively, and the elements **P** and **Q** are called the loadings.[195] The errors associated with modelling **A** and **C** with the PLS model are represented by the terms **E** and **F**. In PLS, the **U** factors are not optimal for estimating the columns of **A** as in PCA, but are rotated so as to simultaneously describe the **C** matrix. The factors for the **A** and **C** matrices have the following relationship

$$\mathbf{T} = \mathbf{B}\ \mathbf{U} + \varepsilon \qquad \text{(eq. 11)}$$

where **B** is the inner relationship between the score matrix **T** and **U**. Partial least-squares regression will compromise the ability of the factors to describe the samples in the individual spaces to increase the correlation of **U** to **T**. It is this compromise that allows for the determination of a factor model that simultaneously describes **A** and **C**.[195]

Two types of approach to PLS calibration have been developed depending on how many of the components of the **C** matrix are considered during calibration. When the calibration and prediction analysis are performed one component at a time, the method is called PLS1. Other concentrations, even if known, are not included in the analysis. Two or more components can be calibrated or analyzed simultaneously by using the PLS2 algorithm.[201]

E. Data Analysis Used in the Study

Since pure reference standard forms for the cholesterol lipid fractions are not available, neither are the absorbance spectra for the individual products of the color reaction. Except for possible competing color reactions with additives included with the samples to enhance the separation processes, the spectra for the fractions separated by ultracentrifugation or precipitation would be considered to be close approximations to reference spectra, Figure 3. Using the fact that the spectra obtained for reactions with standard reference material (SRM) forms of cholesterol and its esters are identical, we have presumed that the same molecular derivatives are produced in the color reaction with serum cholesterol, and that the spectral variations among the cholesterol fractions are due to the different cholesterol compositions and different matrix effects. Matrices in this case are the lipoprotein carriers used to transport cholesterol in serum. A model has to be developed, therefore, that will resolve the spectrum for the whole serum into the contributions from the different parts.

In the original mathematical model,[186] absorbances A(i) were measured at three chosen wavelengths and lipid profiles were calculated by the simultaneous solution of a set of three equations of the form:

$$A(i) = \varepsilon_{VLDL\text{-}C(i)}\cdot[VLDL\text{-}C]\cdot b+\varepsilon_{LDL\text{-}C(i)}\cdot[LDL\text{-}C]\cdot b+\varepsilon_{HDL\text{-}C(i)}\cdot[HDL\text{-}C]\cdot b \quad \text{(eq.12)}$$

where b is the cell pathlength which is equal to 1-cm in all the measurements. Molar absorptivity coefficients ε, were evaluated in a

totally empirical manner and were subject to investigator bias and over-simplifications in the model. One over-simplification, that limited the range of TC over which the model could be applied, was that ε values were presumed to be equal for all three of the lipid fractions at the wavelength of the major maximum (520 nm). There are zero degrees of freedom in this model, making it impossible to use it to predict the composition of every sample with equal precision.

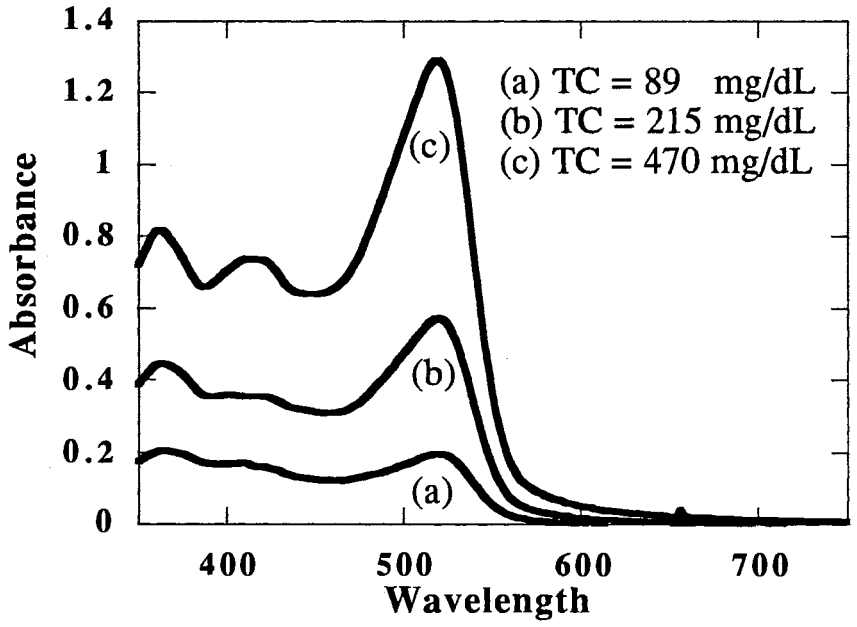

Figure 3. Absorption spectra for the reaction of cholesterol in (a) serum, and the individual fractions: (b) VLDL-C; (c) LDL-C; (d) HDL-C.

To improve upon the mathematical model, more sophisticated multi-variate calibration and prediction analyses were used. The multivariate calibration approach used was PLS2 which is a part of the spectroscopic

analysis software package available in UNSCRAMBLER II (CAMO A/S, Trondheim, Norway). Spectral resolution in the spectrophotometer is 2 nm so a full spectrum data set consists of 200 points, which represent an enormous increase in the number of degrees of freedom compared to the simpler analysis.

As a basis for the multivariate calibration model, a training set was chosen that consisted of 35 samples in which there is a wide distribution in TC, as well as in the various lipid fractions in a conscious effort to approximate the ranges encountered in the entire population. The lipid profiles for these samples had been measured enzymatically at one or other of the external laboratories. As is usual, VLDL was taken to be 0.2xTG and numbers for LDL were calculated using the Friedewald formula. Ranges in values were as broad as we could access (Table 2) using the source laboratories.

Background and weighting corrections were not applied. Other calibration parameters used are shown in Appendix A. The optimum fit to the spectral data for the training set was obtained using four factors. The percent residual variance was about 25% with just the first factor, and less than 0.05% for all four.

As a test to determine if analysis using the full spectrum data set was really necessary, the multivariate regression analysis subroutines were used to identify the optimum wavelengths, i.e. those that are most sensitive to variations of each fraction. Alternative models were tested using reduced data sets that were limited to 100, 30, 14, 6, and 4 wavelengths respectively. Differences in the percent residual variances were minimal from full spectrum through 6 wavelengths, and 4 wavelengths could be used with little loss in the quality of the fit. The primary model used in the

work was based on 6 wavelengths. Measured absorbances for the samples in the training set are given in Table 3.

A justifiable criticism of the computational model is that calibration of the absorbance data to the lipid profiles of the training set is confined by the limitations set by the enzymatic method, namely that VLDL-C is taken to be 0.2xTG, and LDL-C is calculated from the Friedewald formula.[21] Four samples in the training set had values for TG >400 mg/dL, for which HDL-C values had been measured while VLDL-C and LDL-C values calculated. In spite of the risk that the Friedewald formula does not apply to the high TG levels, their inclusion in the training set is crucial so the mathematical model could approximate the entire population. The 6-wavelength calibration model was used to predict lipid profiles for all the sera in the current sample pool. The color reaction was done on high triglyceride samples in precisely the same way, and it was assumed that the same model could be extrapolated to include them.

Table 2. Measured lipid profiles for the training set (mg/dL).[a]

Sample Number	VLDL-C (b)	VLDL-C (c)	LDL-C (b)	LDL-C (c)	HDL-C (b)	HDL-C (c)	TC (b)	TC (c)
1	27	31	128	157	73	53	229	241
2	43	55	91	82	27	28	162	165
3	44	40	187	161	31	55	263	256
4	23	29	96	84	33	38	152	151
5	20	23	110	94	30	46	161	163
6	15	31	101	122	89	51	206	204
7	12	30	132	116	51	41	196	187
8	10	8	116	122	48	44	175	174
9	11	14	121	147	75	52	212	213
10	47	44	159	146	19	40	225	230
11	6	5	127	135	55	49	189	189
12	18	17	125	139	65	49	209	205
13	9	5	92	88	39	44	141	137
14	59	58	162	154	36	43	257	255
15	8	10	145	137	49	53	203	200
16	10	1	136	145	55	60	201	206
17	52	64	221	218	55	57	329	339
18	32	43	106	113	61	42	200	198
19	40	28	173	176	42	60	256	264
20	98	94	152	152	35	30	285	276
21	82	73	147	161	33	37	262	271
22	80	80	177	170	42	41	299	291
23	87	62	87	115	34	39	208	216
24	89	83	115	134	42	31	246	248
25	32	37	132	156	66	44	230	237
26	34	13	98	110	32	42	165	165
27	51	47	222	200	39	60	313	307
28	15	13	114	116	47	45	177	174
29	22	23	111	97	31	34	165	154
30	33	32	157	138	38	50	228	220

Table 2. Continued

Sample Number	VLDL-C (b)	VLDL-C (c)	LDL-C (b)	LDL-C (c)	HDL-C (b)	HDL-C (c)	TC (b)	TC (c)
31	27	47	207	170	42	50	277	267
32	34	44	155	167	78	53	268	264
33	24	23	184	170	49	57	258	250
34	68	64	103	102	29	38	201	204
35	35	22	122	123	35	42	192	187

[a] The time interval between (b) and (c) determinations was variable, sometimes as much as several days.

(b) TC and HDL-C measured enzymatically, VLDL-C calculated as 0.2x TG, and LDL-C calculated from the Friedewald equation. **All measurements were made only once.**

(c) VLDL-C, LDL-C and HDL-C measured spectroscopically, TC calculated as the sum. **Multiple independent measurements were made for all samples.**

Table 3. Absorbance data[a] for the training set.

Sample	A_{362}	A_{388}	A_{420}	A_{456}	A_{520}	A_{546}
1	0.553	0.461	0.442	0.373	0.631	0.250
2	0.459	0.369	0.332	0.271	0.414	0.164
3	0.522	0.432	0.431	0.362	0.650	0.242
4	0.330	0.273	0.251	0.204	0.337	0.126
5	0.319	0.266	0.246	0.200	0.348	0.124
6	0.378	0.312	0.305	0.253	0.467	0.164
7	0.425	0.353	0.335	0.282	0.475	0.185
8	0.349	0.302	0.303	0.255	0.446	0.176
9	0.460	0.384	0.372	0.316	0.555	0.219
10	0.484	0.395	0.403	0.343	0.623	0.233
11	0.337	0.289	0.302	0.254	0.477	0.181
12	0.432	0.362	0.353	0.300	0.529	0.206
13	0.281	0.237	0.215	0.176	0.299	0.116
14	0.568	0.461	0.460	0.387	0.681	0.257
15	0.365	0.308	0.316	0.262	0.489	0.184
16	0.421	0.358	0.335	0.288	0.507	0.199
17	0.821	0.677	0.655	0.566	0.961	0.379
18	0.373	0.303	0.312	0.251	0.464	0.163
19	0.472	0.389	0.411	0.346	0.671	0.243
20	0.762	0.628	0.594	0.505	0.789	0.315
21	0.581	0.463	0.480	0.407	0.747	0.273
22	0.652	0.525	0.536	0.449	0.795	0.297
23	0.488	0.397	0.378	0.314	0.531	0.193
24	0.580	0.468	0.463	0.391	0.670	0.247
25	0.488	0.403	0.415	0.354	0.647	0.244
26	0.351	0.296	0.285	0.238	0.410	0.161
27	0.678	0.561	0.554	0.472	0.826	0.320
28	0.389	0.327	0.304	0.258	0.436	0.173
29	0.365	0.300	0.282	0.236	0.396	0.157
30	0.471	0.390	0.377	0.316	0.550	0.210
31	0.549	0.448	0.459	0.387	0.708	0.265
32	0.538	0.451	0.461	0.384	0.680	0.257
33	0.455	0.376	0.401	0.334	0.642	0.238
34	0.566	0.465	0.412	0.338	0.500	0.200
35	0.403	0.338	0.331	0.280	0.486	0.189

[a] Average absorbance from multiple measurements of each sample.

CHAPTER V

RESULTS AND DISCUSSION

Reagent Modification

The original Chugaev reagent consisted of a 2:1 mixture of a solution of 20% $ZnCl_2$ (w/v) in glacial acetic acid and 98% acetyl chloride.[112] The experimental conditions called for incubation at 67°C for 20 minutes. In an effort to find more convenient experimental conditions, various modifications to the reagent were tried.

The first modification involved changing the relative composition of the reagent mixture. Zinc chloride was increased to 35% (w/v) and the acetyl chloride was increased to be in excess of the acetic acid by 100:1. These modifications increased the intensity of the absorbance signal but the reaction rate was not affected significantly. Incubation at 67°C was still required. The function of glacial acetic acid is as the solvent for the $ZnCl_2$ but it appears to retard the reaction rate. In order to eliminate it, a variety of other salts were evaluated which were dissolved directly into acetyl chloride. Results are summarized in Table 4. The acetate and perchlorate salts of zinc had the best potential as substitutes for $ZnCl_2$. A number of solvents were also evaluated as alternatives to acetyl chloride. Among

these solvents were acetone, chloroform, acetic anhydride, methanol, and dichloromethane. When mixed with zinc perchlorate either the salt did not dissolve or no color was produced when incubated with cholesterol.

Table 4. Metal salts evaluated as alternative to $ZnCl_2$.

Salts	**Solubility in acetyl chloride**	**Color of the solution**	**Gave color when reacted with cholesterol**
$ZnCl_2$	not soluble	-	-
$Zn(ClO_4)_2 \cdot 6H_2O$	soluble	colorless	yes
$Zn(CH_3COO)_2 \cdot 2H_2O$	soluble	turned yellow after about 2 hours	yes
$[CH_3COCH=C(O)-CH_3]_2Zn \cdot xH_2O$	soluble	turned yellow immediately	-
$Zn(SO_4)_2 \cdot H_2O$	soluble	colorless	no
$Zn(NO_3)_2 \cdot 6H_2O$	soluble	turned yellow immediately	-
$ZnCO_3$	not soluble	-	-
$CdCl_2$	not soluble	-	-
$AgNO_3$	not soluble	-	-
$Cd(ClO_4)_2 \cdot 6H_2O$	not soluble	-	-
$Ca(ClO_4)_2 \cdot 6H_2O$	not very soluble	-	-
$Mg(ClO_4)_2$	soluble	colorless	no
$Ba(ClO_4)_2 \cdot 3H_2O$	not very soluble	colorless	yes but very small signal
$NaClO_4$	not soluble	-	-
$KClO_4$	not soluble	-	-

The properties of acetyl chloride were studied extensively by Paul and Sandhu.[202] They found that strongly ionic chlorides are insoluble in acetyl chloride and the development of color when substances are dissolved in acetyl chloride indicates the formation of complexes. Compounds that are capable of acting as Lewis acids are soluble. Acetyl chloride has a dielectric constant of 15.9 at 20°C, low compared to water, which may be a factor responsible for the insolubility of strongly ionic compounds. Solvation plays an important role in the dissolution of Lewis acids and bases in these non-aqueous media.[202]

With zinc perchlorate dissolved directly in acetyl chloride the rate of color production is much faster and signal intensities much higher. One possible explanation is that the perchlorate ion is a stronger base compared to the acetate ion in abstracting hydrogen from the cholesterol molecule making the rate of color formation faster. A kinetic study of the reaction done in our laboratory showed that the reaction is 98% complete in 15 minutes under room temperature conditions. The rate equation is first order in cholesterol and probably first order in zinc perchlorate but because the reagent is present in very large excess compared to cholesterol the order is reduced to pseudo-zero order. Taking the average cholesterol concentration to be 200 mg/dL, the mole ratio $[Zn^{+2}]$: [cholesterol] is approximately 10,000:1.

Color and Spectra of Products

Products of the reactions between the modified Chugaev reagent and standard reference material (SRM) forms of cholesterol and cholesterol esters dissolved in chloroform are pink in color. With serum, the color is a

fluorescent orange, the ultimate shade of orange being a function of the amount of serum cholesterol and its distribution among the lipoprotein fractions. Individual colors for the reactions with the subfractions, separated either by ultrafiltration (Sigma Chemical Co.), or by ultra-centrifugation (Lipids Research Laboratory, Oklahoma Medical Research Foundation) are pink, orange, and yellow for VLDL-C, LDL-C, and HDL-C, respectively. Considering the lipoprotein particles as the local "solvents" for cholesterol in the concurrent reactions, the VLDL-C particles, having the highest proportion of TG and the lowest protein, are the least polar of the lipoproteins and would behave most like chloroform in the test with cholesterol. Colors for the VLDL-C and the SRM of cholesterol are the most alike. The spectral shift from pink to yellow in going from VLDL-C through LDL-C to HDL-C, parallels the relative increase in polarity. Absorbance spectra for the SRM of cholesterol and its esters cannot be used to calibrate the analyses of serum cholesterol because of the environmental effects of the lipoprotein matrices on the electronic absorbance spectra for cholesterol in the various sub-fractions (Figure 3). In contrast, the Liebermann-Burchard reagent, reacted with the lipoprotein subfractions, gives the same spectrum for all three fractions. It cannot, therefore, be used for the discrimination of the lipoprotein fractions.

Absorption spectra for the colored products from the reactions of three serum samples with different TC levels are shown in Figure 4. The absorbances at λ_{max} (520 nm) do not obey Beer's Law and the overall shape of the spectra depends upon the distribution of cholesterol among the lipid fractions. In Figure 5, on the other hand, absorption spectra are compared for the colored products of the reactions of cholesterol in three different serum samples which had the same TC but different lipid profiles.

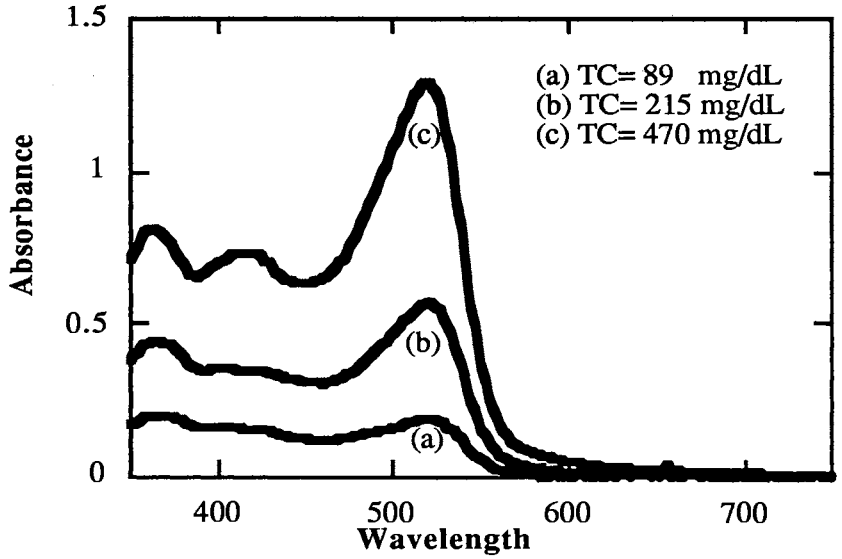

Figure 4. Absorption spectra for the colored product of the reaction of cholesterol with three serum samples with different TC.

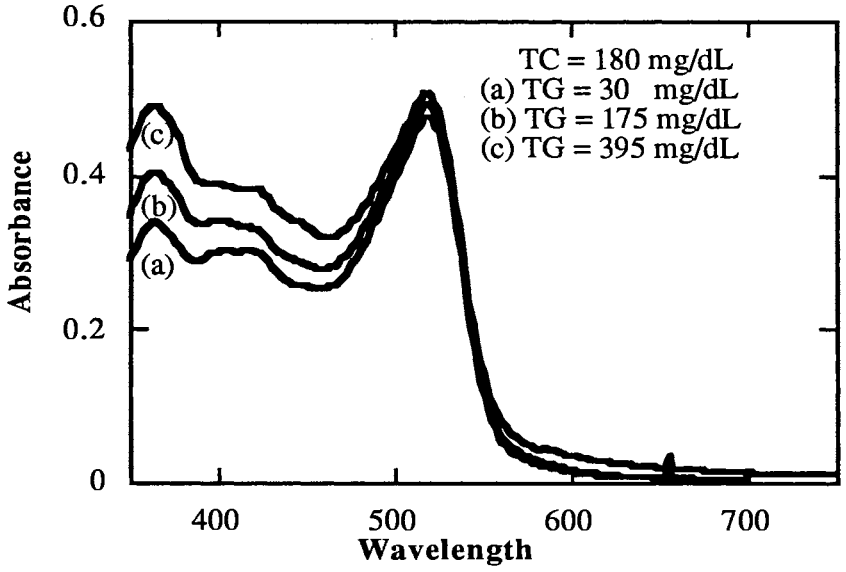

Figure 5. Absorption spectra for the colored product of the reaction with three serum samples with the same TC but different TG levels.

All three have similar absorbance at the 520 nm maximum, representative of the fact that they have the same TC values, 180 mg/dL, as measured enzymatically. This part of the spectrum is dominated by the LDL-C fraction, Figure 3, which, from population averages accounts for as much as 65% of the total serum cholesterol.[52] The critical part of the spectrum for discrimination among LDL-C and other lipid fractions is clearly in the range 360-480 nm. More specifically, absorbances in the 360-430 range are seen to increase as TG increases, Figure 5. The increase in absorbance in the 362 nm absorbance maximum, however, does not correlate linearly with the increase in TG. A linear dependence would only occur if the band was due exclusively to the absorption by VLDL-C, and the approximation was true that VLDL-C = 0.2 x TG at all levels of TG, which it is not. The transition with increasing TG, however is monotonic and smooth which might imply that the mathematical model used to analyze the spectral data will, in fact, be applicable to samples with high TG levels.

Calibration

Since pure forms of the cholesterol lipid fractions are not available, neither are the spectra for the individual products of the color reaction. Therefore, a mathematical model has to be developed that will resolve the whole spectrum into the contribution from the parts.

(a) ***3x3 Matrix Solution:*** In the original work,[186] absorbances A_i, were measured at three chosen wavelength and the lipid profiles were calculated by solving a set of three simultaneous equations of the form:

$$A_{(i)}=\varepsilon_{VLDL\text{-}C(i)}\cdot[VLDL\text{-}C]\cdot b + \varepsilon_{LDL\text{-}C(i)}\cdot[LDL\text{-}C]\cdot b+\varepsilon_{HDL\text{-}C(i)}\cdot[HDL\text{-}C]\cdot b \quad \text{(eq. 12)}$$

where b is the sample pathlength. The nine ε coefficients were evaluated in a totally empirical manner and are subject to investigator bias and over-simplification in the model. The model is also seriously limited by having zero degrees of freedom in the data. Using this method of data analysis, good correlation between methods was obtained only for LDL-C and TC.

(b) ***Multivariate Regression Analysis (MVRA):*** Lipid profile and TC results for the work reported here were determined using MVRA techniques. The use of six data points increased the degrees of freedom.

The training set:

The mathematical model that describes the relationship between absorbance and the concentration of the three major lipoprotein fractions was established by creating a training set or calibration set. The training set consisted of samples that were chosen to have as wide a distribution of TC as well as all the lipid fractions as possible such that it would approximate the distributions in the entire population. This would make the mathematical model predict better the concentration of future samples.

In this study, thirty five samples were chosen for the training set. They included 21 samples from UWC and 4 high TG samples from RBL. Lipid profiles used in the calibration had been measured enzymatically, VLDL-C was taken to be 0.2 x TG, and the numbers for LDL-C were calculated using the Friedewald formula. Ranges in the values of the lipid

fractions were as wide as we could access (Table 2). Using the linear PLS2 algorithm, with six absorbance measurements for each sample, the following calibration equations were obtained

$$[\text{VLDL-C}] = 870.4 \cdot A_{362} - 1702.0 \cdot A_{388} + 415.5 \cdot A_{420} + 1779.0 \cdot A_{456} - 145.81 \cdot A_{520} - 1682.0 \cdot A_{546} \quad \text{(eq. 13)}$$

$$[\text{LDL-C}] = -786.5 \cdot A_{362} + 1569.0 \cdot A_{388} - 17.9 \cdot A_{420} - 1846.0 \cdot A_{456} + 594.6 \cdot A_{520} + 758.0 \cdot A_{546} \quad \text{(eq. 14)}$$

$$[\text{HDL-C}] = -690.5 \cdot A_{362} + 1659.0 \cdot A_{388} + 6.0 \cdot A_{420} - 1935.0 \cdot A_{456} + 509.4 \cdot A_{520} + 271.8 \cdot A_{546} \quad \text{(eq. 15)}$$

and were used to predict the concentrations of the fractions in all of the samples in the training set as well as future samples. Lipid profiles for the members of the training set, predicted using the multivariate analysis, are compared with the results from the independent sources in Table 2.

Between-methods correlation plots and correlation equations for TC and the various fractions of the samples that form the training set are shown in Figures 6 and 7. The y-intercepts are all positive, a result that can be expected when comparing between methods that have quite different experimental uncertainties. The TC correlation is excellent. Because TC is measured by the enzymatic method, but calculated in the spectroscopic method from the sum of the fractions, the validity of the multivariate method is substantiated. Correlations are also very good for VLDL-C and LDL-C. The poorest correlation is seen for HDL-C, but there is a significant improvement over the results from earlier work.[187] Results from paired Student's t-test of the training set showed that correlations

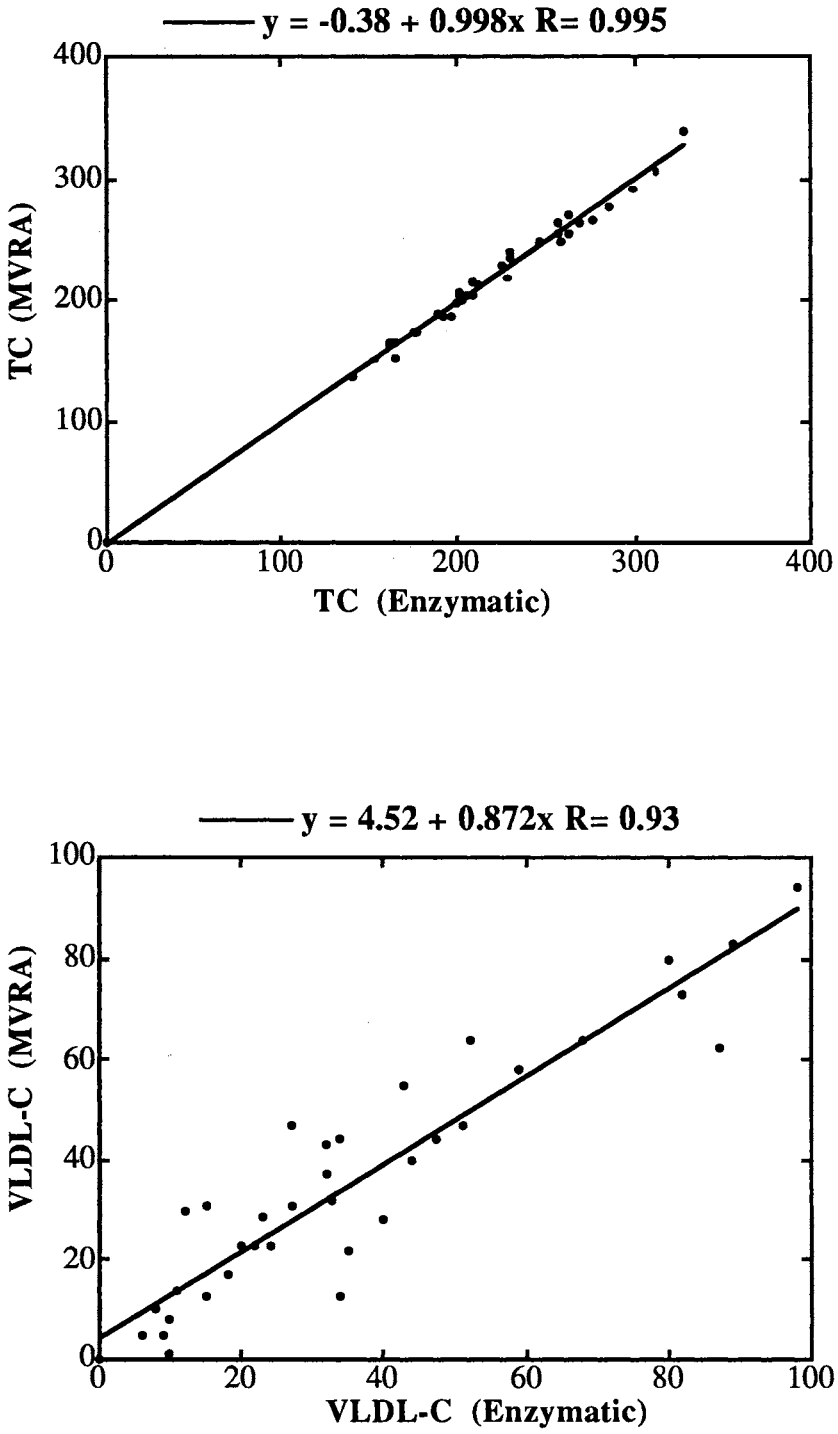

Figure 6. Correlation plots between methods for TC (upper) and VLDL-C (lower) for the training set. Concentrations are in mg/dL.

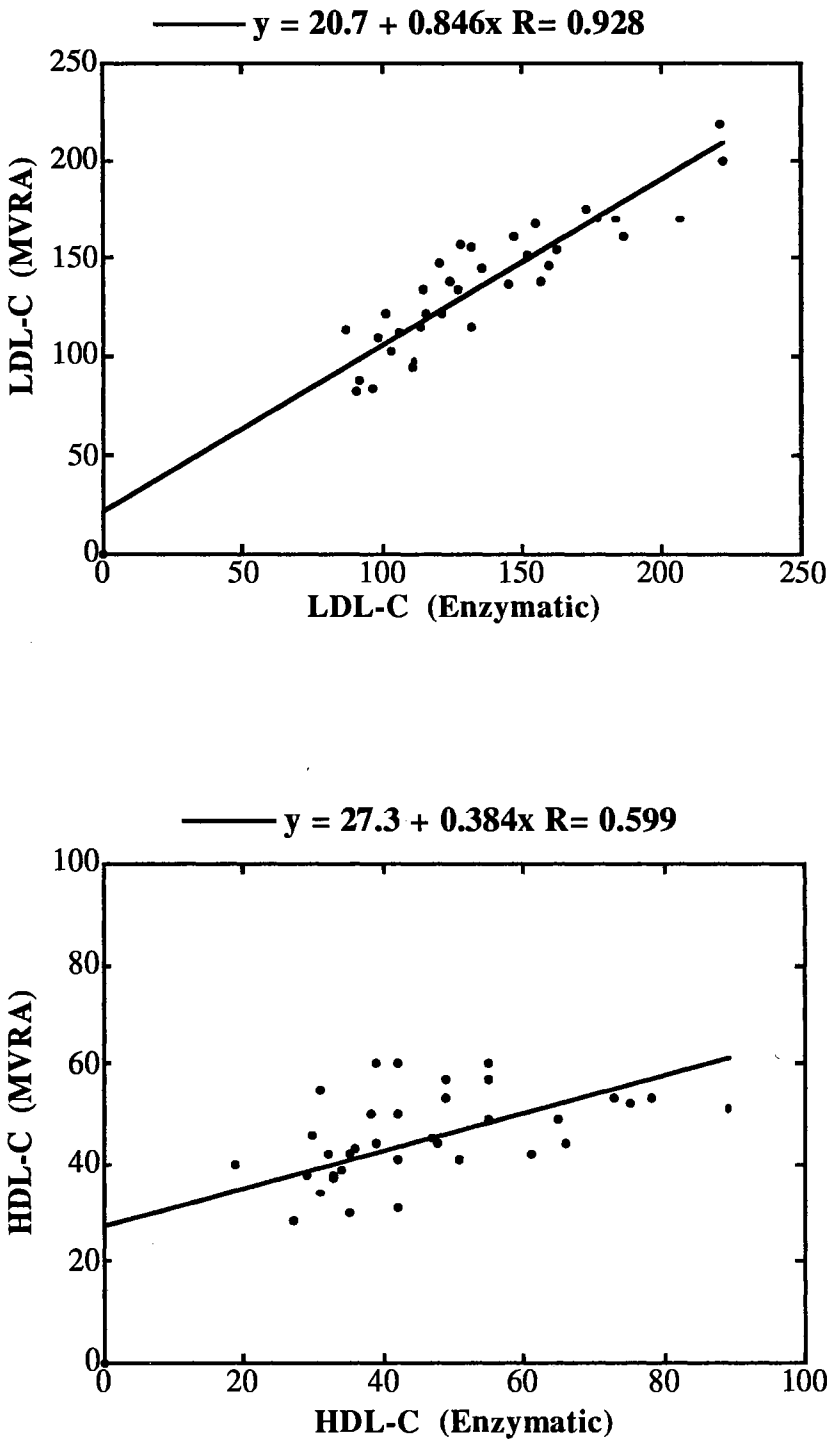

Figure 7. Correlation plots between methods for LDL-C (upper) and HDL-C (lower) for the training set. Concentrations are in mg/dL.

between the enzymatic and the spectroscopic methods do exist, Table 5.

Table 5. Paired student's t-test of samples used in the training set analyzed by the enzymatic and the alternative methods.

(N=35) Fraction	Mean difference	SD difference	t_{exp}
VLDL-C	0.11	9.80	0.066
LDL-C	-0.17	16.23	-0.062
HDL-C	0.20	14.48	0.082
TC	0.94	6.21	0.896
at $\alpha = 0.05$; df = 34	$t_{table} = \pm 2.042$		

Prediction

Calibration equations (eq. 13-15) obtained from data for the training set were subsequently used to predict the concentrations of all the other samples tested. There were several sources of the serum samples as described earlier. The results are discussed independently then collectively in the following sub-sections.

I. OSU Wellness Center (UWC)

The most extensive comparison was made with samples obtained from the UWC (n=304). Some samples were only analyzed by our laboratory, so comparisons are incomplete. Individual results are shown in Appendix B-I. For samples with TG < 400 mg/dL, the VLDL-C fraction

was calculated as TG/5 and LDL-C by the Friedewald formula. For samples with TG > 400 mg/dL, precipitation of HDL-C is usually not done so only TG and TC would be reported by the other laboratory. For some samples, HDL-C values were reported even when TG > 400 mg/dL. The VLDL-C and LDL-C fractions for these samples were estimated using the Friedewald formula although the limit of the formula is exceeded. Very good correlations were obtained for both TC and LDL-C, Figures 8 and 9. The correlation coefficient is much smaller for VLDL-C which can be attributed in part to the empirical way that VLDL-C is calculated in the enzymatic method and the fact that for some samples the upper TG limit was exceeded. The current model indicates that there is no correlation for HDL-C. It is difficult to apportion the relative errors between methods but since VLDL-C and LDL-C are obtained empirically in the enzymatic method, and high TG samples are included in the training set, some of the error is associated with the reference method. Until these have been reduced, the errors in measuring HDL-C by the alternative method cannot be evaluated. The differences between methods appear to be greatest when HDL-C levels are high, > 60 mg/dL, suggesting the absorbance may change non-linearly for this fraction. The only way to resolve the problem is to use ultracentrifugation data as the reference method.

II. Roche Medical Laboratories and Stillwater Medical Center

Samples from these two laboratories were exclusively high TG (>250 mg/dL). These samples were of interest because they represent a

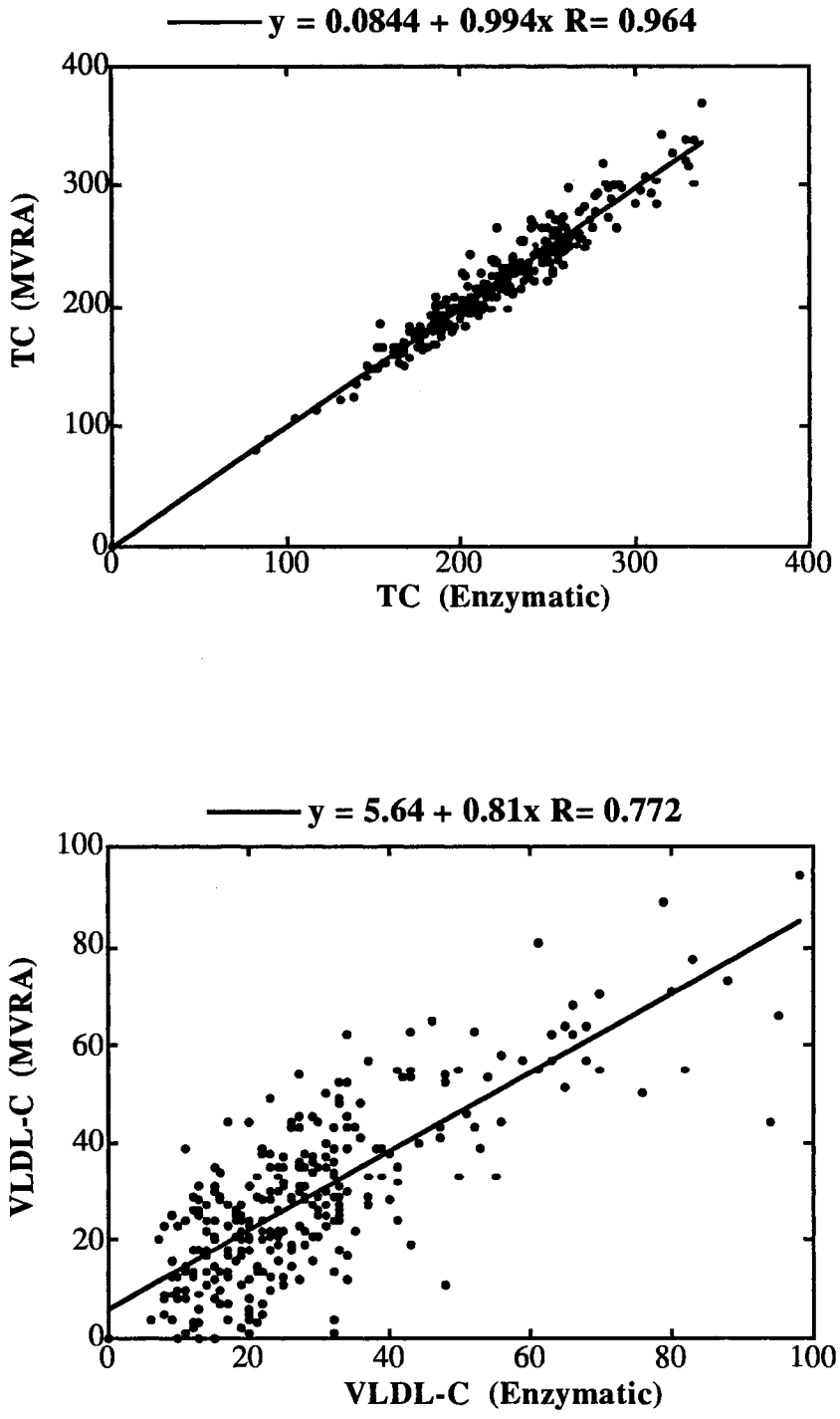

Figure 8. Correlation plots between methods for TC (upper) and VLDL-C (lower) for the OSU Wellness Center samples. Concentrations are in mg/dL.

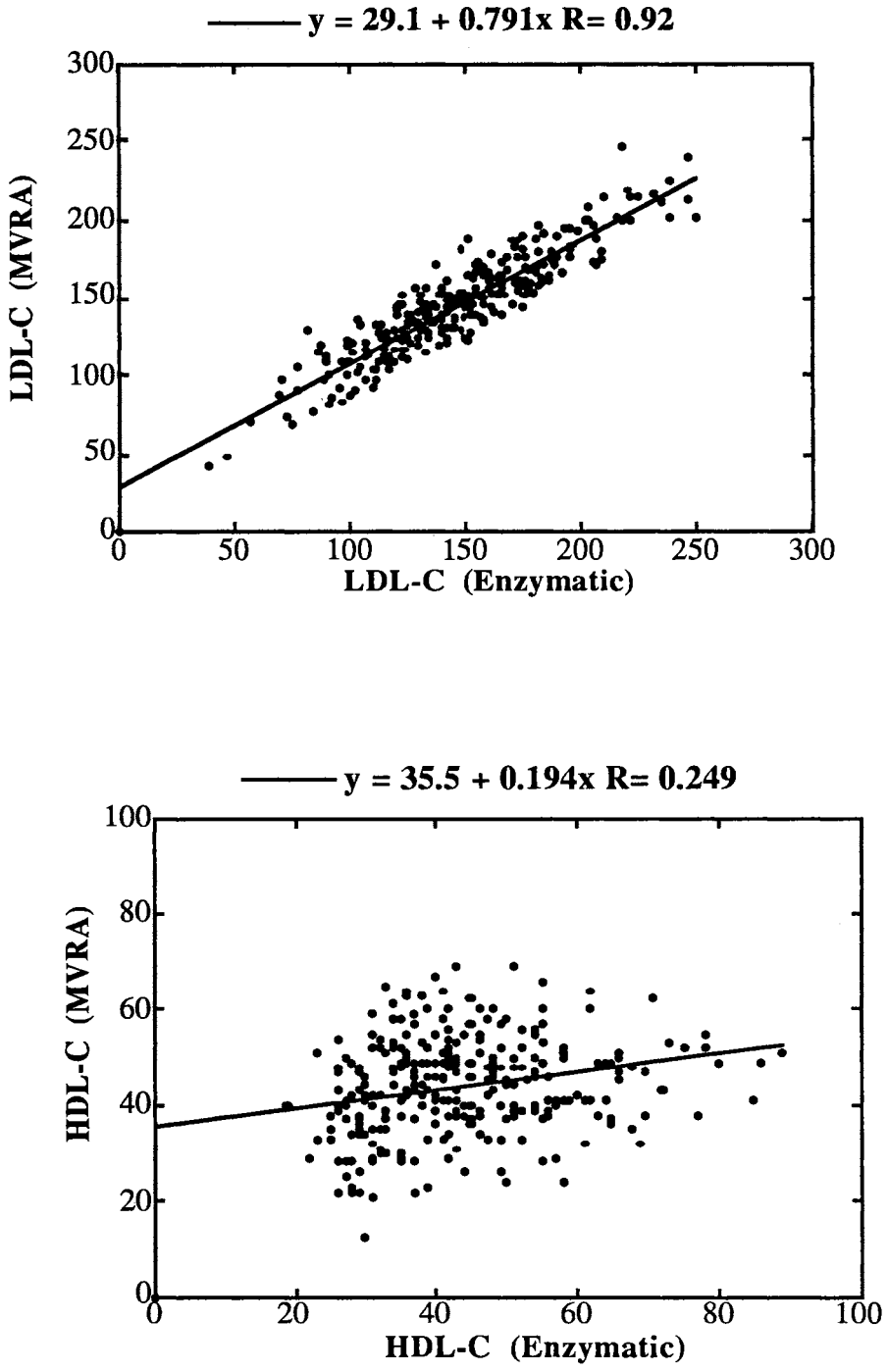

Figure 9. Correlation plots between methods for LDL-C (upper) and HDL-C (lower) for the OSU Wellness Center samples. Concentrations are in mg/dL.

population with any of a number of lipid disorders. Having a routine method for measuring their lipid fractions would be helpful in the early detection and management of the disorders. Current routine methods fail for these samples. Ultracentrifugation is the only way to obtain the information.

Absorbances in the 360-430 nm range increase dramatically with increasing TG (Figure 5). The increase in the maximum absorbance at 362 nm is non-linear with the amount of TG.

Comparisons of the lipid profiles of samples from RBL (n=53) and SMC (n=251) obtained with the enzymatic method and the alternative method are shown in Appendices B-II and III. Correlation plots of TC between the enzymatic and the alternative methods for these samples are shown in Figure 10. The correlations are very good. The VLDL-C obtained by MVRA is generally smaller in comparison to the value obtained by TG/5 for high TG samples. This is consistent to the limitations set by the Friedewald formula. Comparisons between methods for the lipid fractions for the majority of these high TG samples cannot be made. However, HDL-C of some of these high TG samples were reported by the other laboratories (n=21 for RBL and n=4 for SMC) and the values obtained by MVRA were comparable to those obtained for UWC. The LDL-C for these samples obtained by MVRA are greater than those approximated from the Friedewald formula. One possible explanation for the higher LDL-C values is that VLDL-C is underestimated making the LDL-C higher. The obvious failure of the calibration model to predict the lipid profiles of these abnormal samples, even when TC values correlate, suggests that there is an essential factor missing from the model which would redistribute the cholesterol over the fractions.

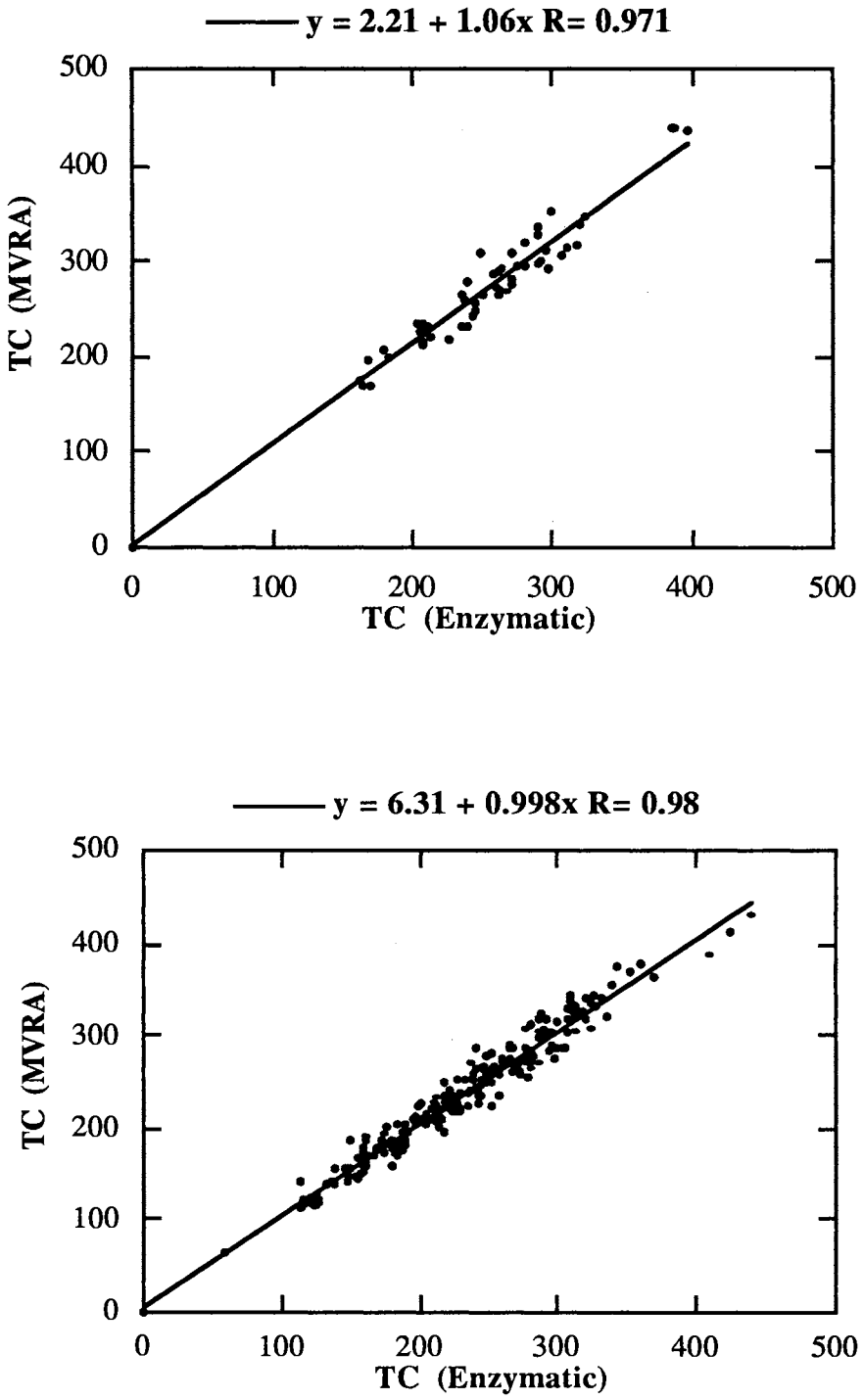

Figure 10. Correlation plots between methods for TC of samples from Roche Biomedical Laboratory(upper) and Stillwater Medical Center (lower). Concentrations are in mg/dL.

III. University of Cape Town Medical School (UCT)

The UCT lipids laboratory is involved with research into lipid dysfunctionalities, particularly Type III lipid disorder and familial hypercholesterolemia (FH). Individuals with FH have significantly high LDL-C levels and are at a very high risk for atherosclerosis. In Type III lipid disorders, the level of IDL-C is increased indicating a defect in the metabolism of the lipoproteins. Detection and management of these disorders requires ultracentrifugation which is both labor and financially intensive. Our collaboration is to evaluate the spectrophotometric method as a potential alternative. Samples from UCT were separated using the preparative ultracentrifuge and lipid profiles obtained by this method can also provide a check on the validity of the current model which is based on the routine enzymatic method.

There are no obvious differences in the spectra for reactions with the plasma of normal, Type III, and FH individuals. Comparison of the lipid profiles of samples from UCT are shown in the Appendix B-IV. Considering the possible effects of lipid dysfunctionalities on both the separation and photometric technologies, correlation plots between the methods are very good for TC and LDL-C for both normal and Type III samples, Figure 11. Once again, the HDL-C correlation is not good (Figure 12). For VLDL-C, there is a good correlation between the two methods if comparing normal samples (Figure 13). The current model is predicting the VLDL-C concentration lower than the one obtained by ultra-centrifugation method for Type III individuals (Figure 13), as it did for high TG samples in the RBL subset.

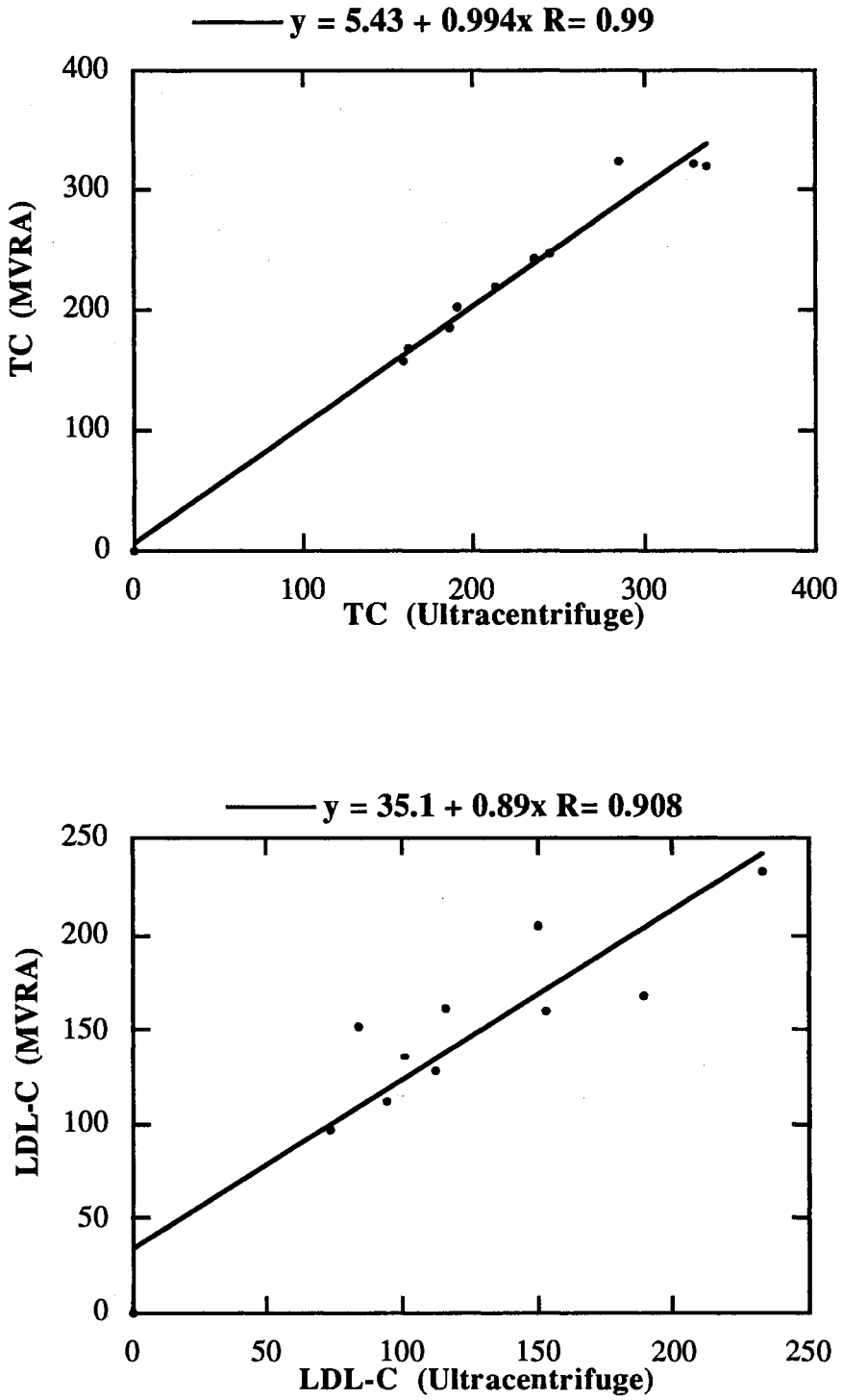

Figure 11. Correlation plots between methods for TC (upper) and LDL-C (lower) for all the samples from the University of Cape Town. Concentrations are in mg/dL.

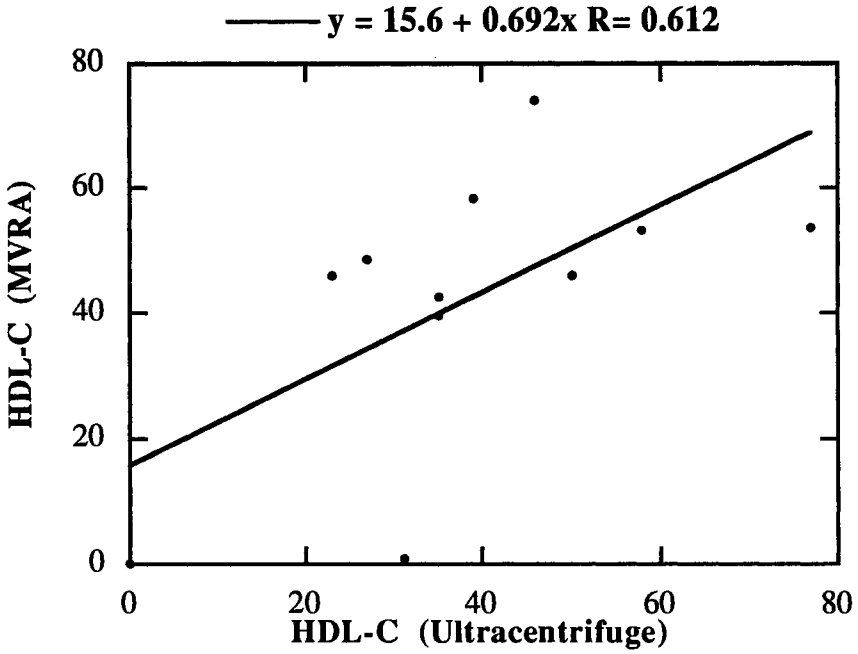

Figure 12. Correlation plot between methods for HDL-C for all the samples from University of Cape Town. Concentrations are in mg/dL.

A significant factor in lipid dysfunctionalities that is not measured in the routine enzymatic screening is the IDL-C fraction. This fraction is described to be VLDL-C like in structure but with a cholesterol loading similar to LDL-C. The IDL-C levels are high in Type III and low in normal and FH cases. A calibration model that includes the IDL-C fraction would be desirable to evaluate the abnormal samples and would probably help improve the correlations among the other lipid fractions particularly HDL-C. An obvious difference can be seen on the spectra of type III and FH or normal individual (Figure14) using the first fraction (fraction A)

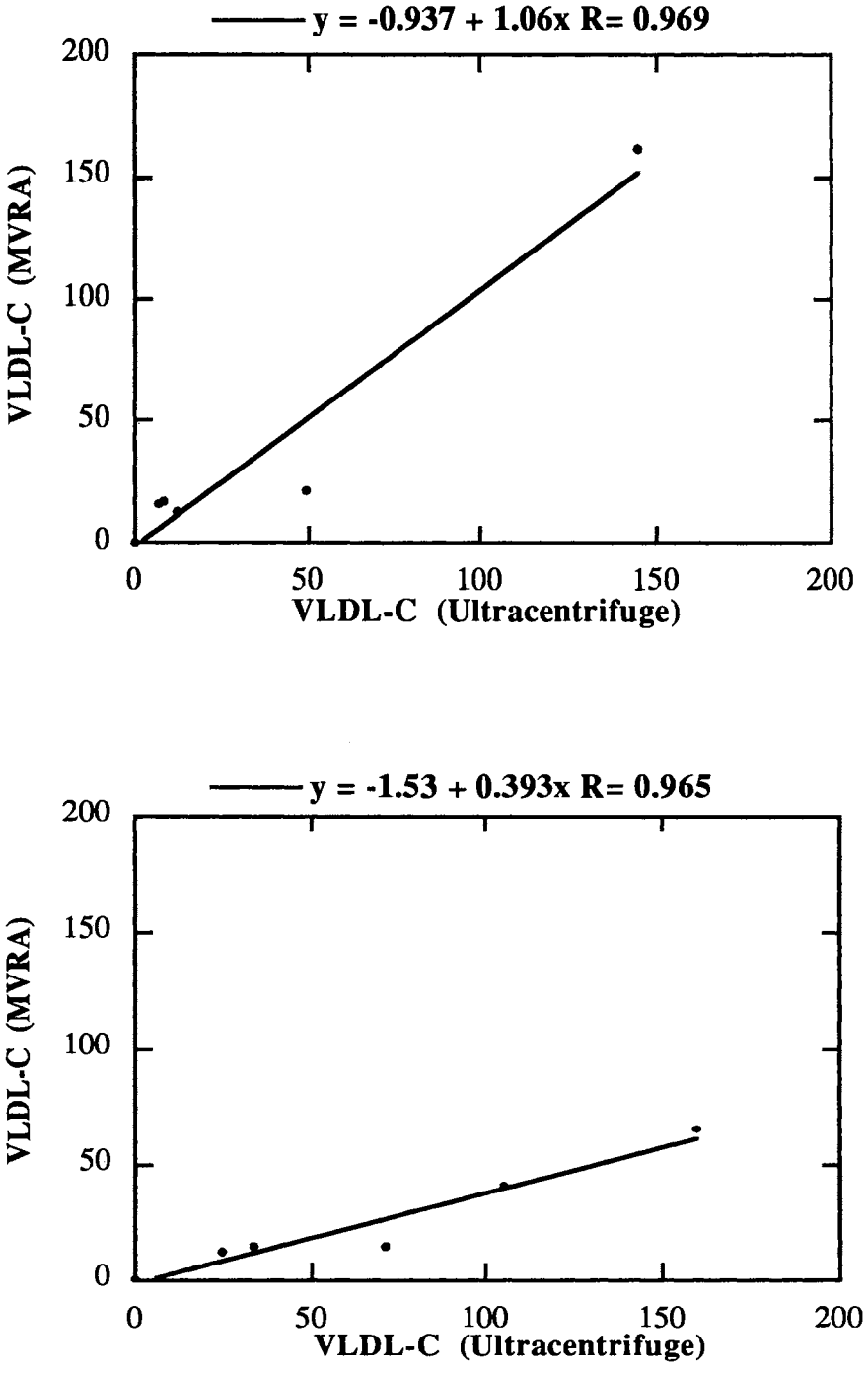

Figure 13. Correlation plots between methods for VLDL-C for the University of Cape Town samples with no lipid disorder (upper) and with known Type III lipid disorder (lower). Concentrations are in mg/dL.

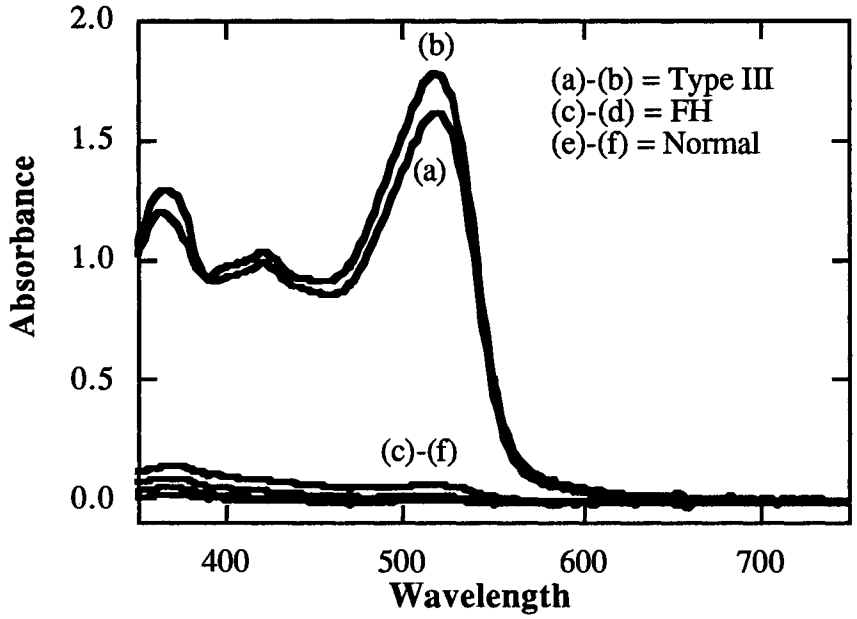

Figure 14. Absorbance spectra of fraction A (obtained by ultracentrifugation) of individuals that are normal or with Type III and FH lipid disorder.

after ultracentrifugation. It is hard to distinguish between the spectra of FH and normal individuals (Figure 15). The A fraction was obtained using a density=1.006 g/mL cushion to float the A layer which is mostly VLDL-C. The spectra of fraction A of Type III individuals show a large absorbance at 520 nm compared to the absorbance at 362 nm. The spectra for fraction A for FH and normal individuals are similar to each other having a very low intensity and the absorbance at 362 nm is greater than the absorbance at 520 nm. More samples having a confirmed Type III and FH conditions need to be analyzed to check if the spectra of the A fraction really correlates with a lipid disorder.

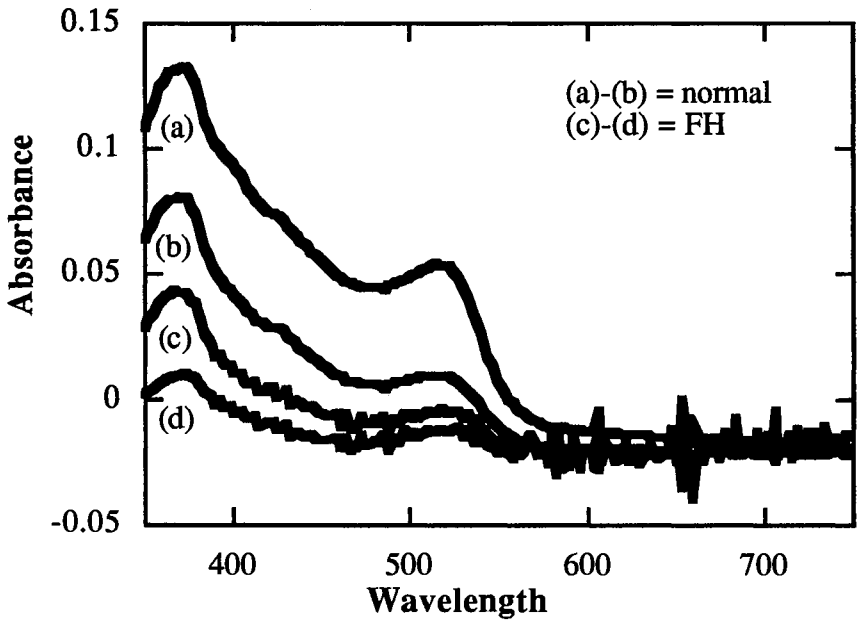

Figure 15. Absorbance spectra of fraction A (obtained by ultracentrifugation) of individuals that are normal and with FH lipid disorder.

Combined data from the four laboratories

Some statistics on the raw data of the combined results from the four different laboratories are shown in Table 6. The result of the Student's t-test comparing the alternative method and the method used by the external laboratories are shown in Table 7 while the correlation coefficients are shown in Table 8.

The correlation coefficients in Table 8 show that a linear correlation exists between the alternative method and the method used by the external

Table 6. Statistics on the lipid profiles obtained by two methods of the samples from the four laboratories.

Statistic	VLDL-C		LDL-C		HDL-C		TC	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
minimum	6	-8	39	42	19	1	58	63
maximum	98	107	250	246	89	74	439	439
number of points	469	469	327	327	327	327	611	611
mean	43	37	146	147	43	43	228	231
median	35	35	145	146	41	43	226	228
population standard deviation(SD)	24.3	21.8	39.0	32.0	13.0	11.1	53.6	56.2

(a) Method used by external laboratories.

(b) Alternative method using MVRA.

Table 7. Results of Student's t-test using all the data from the four external laboratories.

Statistic	VLDL-C	LDL-C	HDL-C	TC
minimum difference	-37	-70	-32	-57
maximum difference	81	49	44	31
number of points	469	327	327	611
mean difference	5.5	-0.86	-0.05	-3.5
S.D difference	15.7	17.8	14.7	14.0
t_{exp} (calculated)	7.59	-0.87	-0.06	6.18
$t_{critical}$ at α= 0.05	1.96	1.96	1.96	1.96

Table 8. Correlation coefficients (r).

Alternative method VLDL-C	vs. External laboratories VLDL-C (n=469)	0.770
Alternative method LDL-C	vs. External laboratories LDL-C (n=327)	0.893
Alternative method HDL-C	vs. External laboratories HDL-C (n=327)	0.285
Alternative method TC	vs. External laboratories TC (n=611)	0.969

Critical value of r (α= 0.05) = 0.195

laboratories. The weakest of the correlations is for HDL-C. The model is unable to predict high concentrations of HDL-C suggesting that the response might not be linear at high HDL-C concentrations. Also, it would appear that a model that is based on ultracentrifugation or beta-quantification of the lipoprotein fractions with the IDL-C fraction accounted for would be desirable for proper calibration and prediction. Although a correlation exists between the two methods on TC and the lipid fractions, results of the t-test show a significant difference in TC and VLDL-C. This is posible if there were a constant determinate error in one method making the differences significant even though a good correlation between the methods exist. The estimation of VLDL-C by TG/5 may be a source of error contributing to the significant difference in VLDL-C between these two methods which subsequently affect the TC. Another source of the discrepancy might relate to the fact that in the alternative method the test is done in duplicate but in the external laboratories tests are done only once.

Precision Studies

To assess the precision of the alternative method, analyses were done several times for one serum. Three serum samples were chosen that have different values of their total cholesterol. Table 9 summarizes the results of the precision studies while the lipid profiles obtained for each analysis is

Table 9. Results of the precision studies.

	VLDL-C (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	TC (mg/dL)
Sample 1				
(a) Enzymatic	32	159	42	234
(b) Spectroscopic n=10	25	165	47	238
SD	2.6	4.1	1.9	3.1
%CV	10.2	2.5	4.0	1.3
Sample 2				
(a) Enzymatic	7	47	37	89
(b) Spectroscopic n=6	19	49	23	91
SD	3.4	3.2	1.9	1.9
%CV	17.9	6.7	8.0	2.0
Sample 3				
(a) Enzymatic	20	104	33	157
(b) Spectroscopic n=10	8	107	40	155
SD	4.5	4.1	1.4	3.1
%CV	58.4	3.8	3.5	2.0

shown in Appendix C. Since outside laboratories make one measurement for each serum, comparisons of the precisions cannot be made.

Results obtained from the precision studies showed that the alternative method passes the requirements set by LSP for TC. The requirement of the LSP for CV of ≤ 3% for LDL-C and ≤ 4% for HDL-C is not completely fulfilled particularly at low levels of this lipid fractions.

Interference Studies

To test the effects of other endogenous substances on the alternative method, weighed amount of various solid substances were added to 1 ml aliquots taken from two pooled samples prepared by mixing serum from nine individuals. Spectra and data were analyzed the usual way. Results are shown in Table 10. Initial amounts added to the serum (column 4) were equal to the highest test levels encountered in practice. It should be understood that these amounts are in excess of the "normal" high values (column 2).

Table 10. Results of the interference studies.

Compound added	High "normal" concen-tration (mg/dL)	High test levels (mg/dL)	Amount added to serum (mg/dL)	VLDL-C (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	TC (mg /dL)
serum 1	-	-	-	18	110	46	174
urea	38	500	500	16	109	42	167
creatinine	1.5	30	30	15	108	44	166
fructose	7.5	30	30	18	108	43	169
citrate	3.0	30	30	21	103	40	164
d-glucose	110	1200	1200	22	107	39	168

Table 10 (continued). Results of the interference studies.

Compound added	High "normal" concen-tration (mg/dL)	High test levels (mg/dL)	Amount added to serum (mg/dL)	VLDL-C (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	TC (mg /dL)
serum 1	-	-	-	18	110	46	174
hemoglobin	2.5	500	500	34	118	53	205
			250	36	92	33	161
			125	34	90	42	167
			62.5	32	94	44	170
			31.25	24	105	48	177
serum 2	-	-	-	22	98	40	160
albumin	5000	6000	6000	32	85	33	150
			3000	29	86	34	149
			1500	24	94	37	155
			750	18	100	40	158
γ–globulin	5000	6000	6000	21	94	38	153
			3000	29	85	35	149
			1500	29	85	35	149
			750	28	97	38	163

Results of the interference studies showed that only hemoglobin, albumin and globulin have an effect on the measurement if they are present in very high concentrations. Quantities of the interferences were progressively decreased to determine the treshold level for the interference. These levels are also shown in Table 10. The interference that is of primary concern is hemoglobin because very often in the collection of venous blood some rupture of the red cells will occur. The interference treshold is 15 times greater than the "normal" high serum value.

Linearity Tests

A linear Beer's Law dependence for TC is observed when the SRM of cholesterol is dissolved in chloroform, an isotropic solvent. Correlation tests made by spiking serum with SRM were non-linear because of changing matrix effects. A test was made by mixing two serums which had different TC and different lipoprotein levels in varying proportions and analyzing the spectra the usual way. Results are given in Table 11 and correlation plots are shown in Figure 16.

Table 11. Results of the linearity tests.

Serum 1	Serum 2	VLDL-C	LDL-C	HDL-C	TC
100%	-	21	93	37	151
75%	25%	17	109	38	164
50%	50%	22	131	40	193
25%	75%	20	151	48	219
-	100%	13	165	50	228

Results of the linearity tests showed that the alternative method is sensitive to the increase in concentration in LDL-C, HDL-C and TC. The response was linear over the concentration ranges that were studied. The limits to the ranges need to be evaluated in further work. The majority of the normo-lipid serum measured by the external laboratories are covered by the ranges expressed for TC, LDL-C, and HDL-C in Table 11, which further substantites the linearity correlations between methods for LDL-C and TC, Figures 8-11.

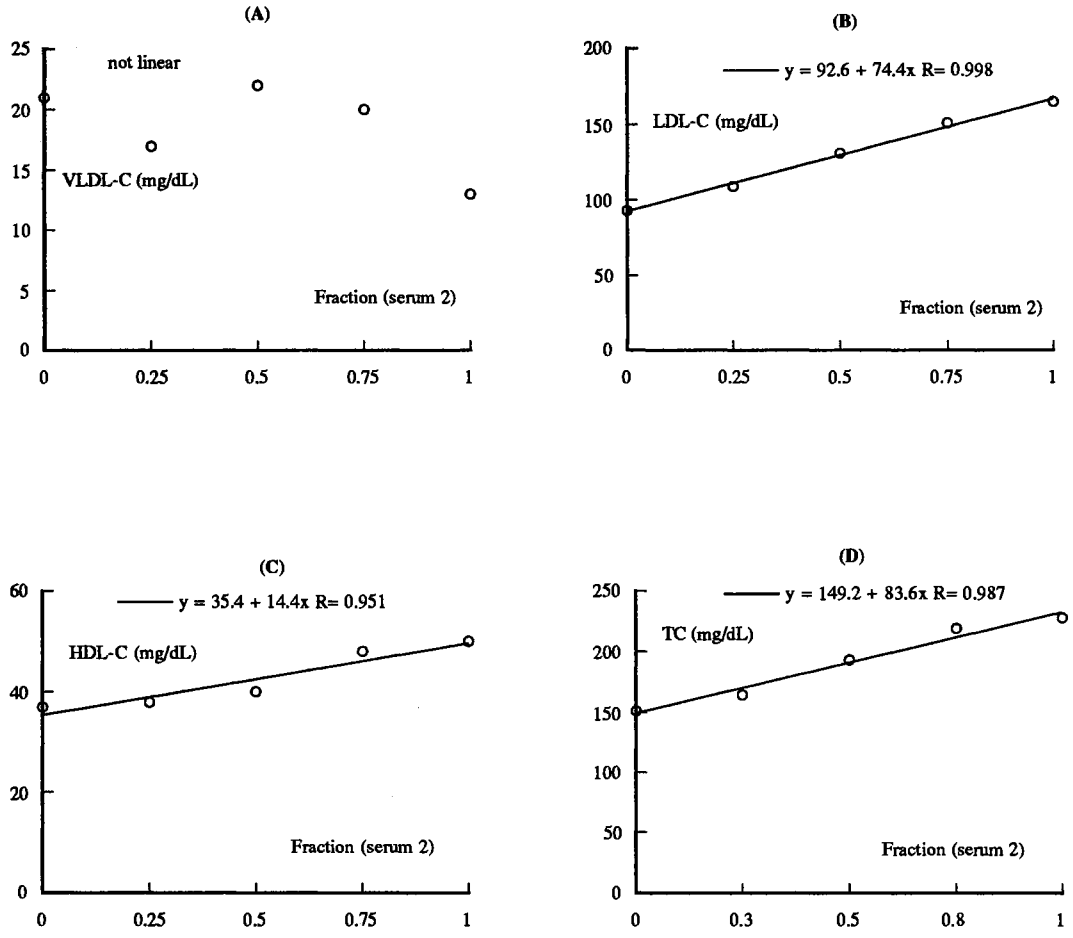

Figure 16. Plots of the concentration of the lipoprotein obtained by the alternative method against fraction of Serum 2 added to Serum 1: (A) VLDL-C, (B) LDL-C, (C) HDL-C, and (D) TC.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The most significant result that has emerged from this study is that, the amount of cholesterol in the major lipoprotein fractions has been measured routinely and directly without resorting to any fractionation step(s). The good correlation between TC values, measured by distinctly different procedures, validates both the physical interpretation of the spectrum for whole serum, and the mathematical model that is used to fit the data. Correlations between the alternative method and current routine methods for all the fractions and TC were better than reported in previous studies.[26-27,186] Also, the experimental conditions are much simpler than the previous studies using a one-reagent system with the reaction occurring at room temperature. The precision of the alternative method for TC is very good and passes the requirement of the Laboratory Standardization Panel.

In the process a method has been developed for the direct determination of LDL-C which is independent of empirical approximations such as the Friedewald equation. Reducing the number of steps needed for profiling from three to one, and eliminating an intermediate selective precipitation that separates low from high density fractions, should have

the effect of improving upon the overall accuracy in the measurements for all fractions.

A third advantage offered by the alternative method is that lipid distributions for high TG samples can be measured. These data might now be considered in assigning risk factors for CHD. Quite often the evidence for failure of the Friedewald approximation in high TG samples are negative values for LDL-C and HDL-C. This will happen because VLDL-C is larger than 0.2xTG in these cases.

Preliminary studies have shown that a spectral difference can be seen in the A fraction of Type III individuals. More samples with confirmed lipid disorders need to be tested to see if the alternative method can provide an easy way to detect the various lipid disorders.

One critique of the method is that it is based on a lipid profile provided by a certified test laboratory but not a primary resource laboratory. The next development step is to prepare a training set using lipid profile data that were obtained using primary reference methods developed and recommended by the Center for Disease Control such as ultracentrifugation data. A training set based on ultracentrifugation which also includes the IDL-C fraction in the training set is the recommended next step. With these as the basis, there should be a significant improvement in the correlation and slope for all the lipid fractions.

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Appendix A

Calibration Parameters Used

Model Center:	Origin	
Default weights:	1/ Standard Deviation	
Outlier Detections:	On	Limit: 3.0
Leverage Detection:	On	Limit: 0.9
Calibration Method:	Partial Least Squares 2	
Validation Method:	Cross Validation	
Add Start Noise?:	Yes	
Segment Selection:	Random	
Number of Segments:	35	

Appendix B

Raw Data

I. Lipid profiles of samples from OSU Wellness Center.

Sam-ple	TG	VLD L-C (a)	VLD L-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
1	280	56	58	101	108	28	42	185	208
2	55	11	39	133	140	62	64	206	243
3	130	26	19	143	161	50	58	219	238
4	95	19	11	129	134	38	42	186	187
5	354	70	55	182	189	28	49	280	293
6	70	14	24	97	109	57	29	168	162
7	140	28	28	69	88	50	24	147	140
8	150	30	25	223	214	38	63	291	302
9	110	22	5	130	140	35	48	187	193
10	60	12	2	147	153	42	52	201	207
11	105	21	3	173	186	40	67	234	256
12	65	13	3	90	109	43	39	146	151
13	135	27	12	196	182	26	54	249	248
14	120	24	30	120	108	37	28	181	166
15	275	55	33	86	115	29	34	170	182
16	552		105		118		8	234	231
17	215	43	19	177	175	40	55	260	249
18	195	39	39	118	112	31	29	188	180
19	40	8	23	85	76	39	23	132	122
20	280	56	44	141	143	38	40	235	227
21	250	50	33	148	152	41	41	239	226
22	115	23	18	248	240	51	69	322	327
23	85	17	23	73	74	49	26	139	123

Sam-ple	TG	VLD L-C (a)	VLD L-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
24	165	33	26	217	201	50	58	300	285
25	160	32	36	132	157	66	45	230	238
26	50	10	8	117	122	48	43	175	173
27	440		91		133		18	241	242
28	55	11	14	126	146	75	52	212	212
29	1710		276		41		-61	236	256
30	524		113		133		14	253	260
31	235	47	43	159	146	19	40	225	229
32	30	6	4	128	135	55	49	189	188
33	170	34	12	99	109	32	42	165	163
34	85	17	23	177	152	43	49	237	224
35	135	27	31	129	156	73	53	229	240
36	255	51	46	223	199	39	60	313	305
37	215	43	55	92	82	27	28	162	165
38	105	21	15	137	134	39	52	197	201
39	75	15	12	115	116	47	45	177	173
40	250	50	55	123	111	25	35	198	201
41	60	12	29	133	115	51	40	196	184
42	175	35	22	122	123	35	41	192	186
43	110	22	22	112	97	31	35	165	154
44	135	27	30	182	168	34	53	243	251
45	90	18	16	126	139	65	49	209	204
46	295	59	57	162	154	36	43	257	254
47	45	9	4	93	87	39	43	141	134
48	165	33	31	157	137	38	49	228	217
49	40	8	9	146	137	49	52	203	198
50	135	27	45	208	170	42	50	277	265
51	205	41	35	173	163	36	55	250	253
52	165	33	24	152	150	46	54	231	228
53	65	13	6	133	146	71	62	217	214
54	488	98	94	152	151	35	29	285	274
55	50	10	0	136	145	55	60	201	205
56	260	52	63	222	219	55	57	329	339
57	115	23	13	189	173	41	58	253	244
58	65	13	18	135	139	56	46	204	203

Sam-ple	TG	VLD L-C (a)	VLD L-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
59	125	25	31	166	165	58	51	249	247
60	305	61	55	127	139	41	33	229	227
61	135	27	36	160	136	37	43	224	215
62	170	34	30	191	175	32	54	257	259
63	115	23	22	204	200	45	62	272	284
64	210	42	53	160	166	38	53	240	272
65	170	34	43	156	166	78	52	268	261
66	130	26	29	113	109	37	46	176	184
67	75	15	15	131	130	42	48	188	193
68	95	19	22	153	144	46	51	218	217
69			22		116		43		181
70	160	32	43	107	112	61	41	200	196
71	160	32	34	220	199	41	64	293	297
72	120	24	22	185	170	49	57	258	249
73	100	20	12	155	146	47	58	222	216
74	200	40	38	163	158	48	49	251	245
75	325	65	64	122	152	66	47	253	263
76	130	26	15	165	154	34	58	225	227
77	200	40	28	174	176	42	60	256	264
78	185	37	29	135	125	45	57	217	211
79	2995		243		77		-23	263	297
80	120	28	35	153	123	45	40	226	198
81	220	44	40	188	161	31	55	263	256
82	115	23	28	96	83	33	37	152	148
83	115	23	21	187	164	46	60	256	245
84	100	20	23	111	93	30	46	161	162
85	75	15	30	102	121	89	51	206	202
86			22		142		52		216
87	465		97		118		25	246	240
88	340	68	64	104	102	29	38	201	204
89	75	15	8	134	137	54	55	203	200
90	80	16	14	211	174	45	62	272	250
91	155	31	37	178	144	26	40	235	221
92	155	31	35	203	173	36	49	270	257
93	90	18	24	118	103	35	30	171	157

Sam-ple	TG	VLD L-C (a)	VLD L-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
94	75	15	35	183	158	58	50	256	243
95	75	15	24	144	143	64	49	223	216
96	50	10	23	103	91	43	39	156	153
97	85	17	18	179	155	42	54	238	227
98	160	32	33	184	160	36	43	252	236
99	205	41	32	156	156	36	42	233	230
100	205	41	24	166	172	54	50	261	246
101	115	23	30	138	120	33	35	194	185
102	150	30	25	193	166	37	44	260	235
103	75	15	20	116	114	45	37	176	171
104	135	27	31	148	147	50	37	225	215
105	215	43	63	167	172	65	37	275	272
106	50	10	13	145	144	47	48	202	205
107	476		86		201		34	329	321
108	80	16	29	154	163	80	49	250	241
109	95	19	27	127	136	62	41	208	204
110	315	63	62	117	109	28	28	208	199
111	90	18	21	190	170	48	47	256	238
112	55	11	24	112	103	57	40	180	167
113	55	11	15	138	144	66	50	215	209
114	80	16	34	162	143	42	49	220	226
115	140	28	31	240	201	36	64	304	296
116	160	32	34	111	104	27	40	170	178
117	60	12	26	89	98	64	41	165	165
118	125	25	37	167	157	49	48	241	242
119	180	36	48	178	152	35	48	249	248
120	110	22	38	180	160	52	52	254	250
121	65	13	31	153	128	43	50	209	209
122	45	9	25	140	128	57	41	206	194
123	100	20	31	141	131	40	41	201	203
124	150	30	35	160	150	47	42	237	227
125	130	26	28	169	152	41	49	236	229
126	315	63	57	164	151	30	41	257	249
127	110	22	39	126	134	70	38	218	211
128	115	23	35	143	124	27	41	193	200

Sam-ple	TG	VLD L-C (a)	VLD L-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
129	215	43	53	135	127	30	34	208	214
130	45	9	9	154	144	53	45	216	198
131	85	17	14	143	121	29	43	189	178
132	95	19	20	158	134	41	45	218	199
133	195	39	33	251	201	23	51	313	285
134	125	25	37	210	179	55	49	290	265
135	190	38	39	175	161	34	44	247	244
136	95	19	24	179	157	43	41	241	222
137	80	16	28	163	140	51	44	230	212
138	105	21	14	121	142	68	35	210	191
139	155	31	27	183	170	37	43	251	240
140	160	32	29	184	163	30	44	246	236
141	205	41	55	154	143	42	29	237	227
142	125	25	35	181	166	50	45	256	246
143	70	14	14	130	124	40	41	184	179
144	145	29	36	168	139	29	37	226	212
145	165	33	52	172	145	49	33	254	230
146	235	47	41	166	164	38	44	251	249
147	120	24	35	151	153	60	42	235	230
148	240	48	52	157	150	42	34	247	236
149	110	22	28	117	112	40	36	179	176
150	85	17	44	150	147	56	41	223	232
151	155	31	25	133	156	37	47	201	228
152	100	20	8	122	145	43	47	185	200
153	135	27	54	126	125	58	24	211	203
154	330	66	68	163	177	39	33	268	278
155	240	48	54	140	131	22	29	210	214
156	100	20	44	162	161	65	36	247	241
157	130	26	44	178	169	54	40	258	253
158	120	24	19	123	116	31	28	178	163
159	165	33	27	189	179	45	46	267	252
160	402	80	71	104	133	28	23	212	227
161	90	18	26	116	112	55	37	189	175
162	70	14	18	75	68	29	26	118	112
163	180	36	41	150	137	29	37	215	215

Sam-ple	TG	VLDL-C (a)	VLD L-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
164	170	34	39	108	98	26	28	168	165
165	170	34	45	166	161	43	38	243	244
166	140	28	38	190	173	39	46	257	257
167	150	30	44	174	155	35	38	239	237
168	969		135		89		-8	214	216
169	65	13	26	176	158	54	46	243	230
170	80	16	34	162	157	56	39	234	230
171	185	37	57	149	130	33	30	219	217
172	155	31	50	152	146	46	34	229	230
173	55	11	10	130	119	40	36	181	165
174	115	23	38	196	181	32	31	251	250
175	265	53	39	132	142	28	22	213	203
176	230	46	65	233	215	51	38	330	318
177	175	35	43	196	176	32	35	263	254
178	85	17	27	247	212	43	69	307	308
179	185	37	33	101	88	26	43	164	164
180	130	26	43	200	193	52	56	278	292
181	415	83	77	152	164	25	33	260	274
182	350	70	107	154	145	31	21	255	273
183	170	34	52	125	110	44	38	203	200
184	1146		183		126		-15	309	294
185	150	30	26	194	195	33	51	257	272
186	45	9	16	90	111	63	38	162	165
187	115	23	49	134	142	69	32	226	223
188	135	27	35	147	146	40	39	214	220
189	521		97		155		16	241	268
190	80	16	10	183	196	48	60	247	266
191	120	24	33	121	144	59	41	204	218
192	125	25	32	183	178	41	46	249	256
193	145	29	45	205	208	54	47	288	300
194	100	20	24	197	195	35	58	252	277
195	90	18	25	139	152	45	49	202	226
196	350	70	70	128	134	32	30	230	234
197	140	28	35	177	181	35	49	240	265
198	105	21	29	198	184	31	52	250	265

Sam-ple	TG	VLDL-C (a)	VLD L-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
199	85	17	23	191	190	43	53	251	266
200	145	29	34	211	214	44	55	284	303
201	700		133		176		11	283	320
202	55	11	15	162	166	46	55	219	236
203	135	27	43	117	123	45	36	189	202
204			43		138		37		218
205	305	61	81	130	147	29	37	220	265
206	442	88	73	218	246	32	52	338	371
207	170	34	62	240	225	42	56	316	343
208	155	31	40	156	139	26	39	213	218
209	60	12	18	114	114	42	38	168	170
210	70	14	27	100	100	47	33	161	160
211	165	33	49	138	171	85	41	256	261
212	95	19	18	114	125	44	40	177	183
213	330	66	62	99	119	27	25	192	206
214	155	31	40	217	201	37	57	285	298
215	100	20	20	114	132	58	41	192	193
216	65	13	9	160	169	54	50	227	228
217	395	79	89	226	214	29	36	334	339
218	120	24	26	140	157	54	56	218	239
219	70	14	17	123	129	48	46	185	192
220	105	21	33	106	121	56	38	183	192
221	65	13	28	142	151	68	48	223	227
222	85	17	13	136	134	41	51	194	198
223	155	31	30	152	148	34	47	217	225
224	160	32	24	160	165	42	48	234	237
225	65	13	25	148	147	52	48	213	220
226	160	32	39	150	124	25	38	207	201
227	165	33	48	188	174	37	49	258	271
228	120	24	21	115	111	28	34	167	166
229	75	15	21	154	139	36	43	205	203
230	40	8	5	125	133	50	44	183	182
231	100	20	6	186	192	62	60	268	258
232	115	23	10	108	117	44	38	175	165
233	65	13	0	146	146	43	47	202	193

Sam-ple	TG	VLDL-C (a)	VLD L-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
234	120	24	16	173	160	29	48	226	224
235	100	20	8	104	106	33	39	157	153
236	115	23	28	159	146	31	40	213	214
237	100	20	21	151	145	47	42	218	208
238	412	82	55	145	150	29	35	256	240
239	165	33	18	112	127	46	38	191	183
240	70	14	22	173	182	86	49	273	253
241	60	12	13	120	116	54	39	186	168
242	100	20	12	140	139	43	49	203	200
243	145	29	21	142	143	40	49	211	213
244	40	8	8	91	101	52	39	151	148
245	85	17	17	236	211	34	61	287	289
246	50	10	8	148	151	63	49	221	208
247	150	30	21	140	154	51	44	221	219
248	140	28	19	197	181	35	52	260	252
249	125	25	22	78	106	61	32	164	160
250	55	11	1	146	135	40	46	197	182
251	75	15	8	146	146	51	48	212	202
252	110	22	14	163	163	42	51	227	228
253	110	22	20	131	152	72	43	225	215
254	80	16	7	143	140	39	49	198	196
255	135	27	23	134	139	47	42	208	204
256	110	22	22	157	172	66	51	245	245
257	125	25	13	208	188	31	59	264	260
258	49	10	10		114		43	167	167
259	100	20	18	123	116	30	41	173	175
260	160	32	14	179	168	27	50	238	232
261			29		167		49		245
262	70	14	11	127	122	48	50	189	183
263			5		113		52		170
264			11		164		64		239
265	100	20	4	111	132	55	66	186	202
266	470	94	44	204	208	36	51	334	303
267	145	29	16	167	160	54	46	250	222
268	45	9	13	145	131	58	52	212	196

Sam-ple	TG	VLDL-C (a)	VLDL-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
269	260	52	43	181	154	26	47	259	244
270	140	28	29	70	98	54	40	152	167
271	155	31	23	145	131	29	43	205	197
272	340	68	57	157	151	27	37	252	245
273	75	15	0	207	197	33	65	255	262
274	145	29	27	178	161	29	47	236	235
275	270	54	53	140	136	31	32	225	221
276	110	22	24	132	130	43	43	197	197
277	165	33	25	149	149	31	42	213	216
278			10		108		38		156
279			2		143		48		193
280			16		116		35		167
281			7		202		56		265
282			3		152		45		200
283			0		142		44		186
284			0		120		36		156
285	275	55	33	142	148	32	30	229	211
286	85	17	7	141	152	51	50	209	209
287			4		160		56		220
288			3		108		44		155
289			8		129		42		179
290	95	19	17	99	123	77	38	195	178
291	215	43	19	137	154	35	41	215	214
292			9		120		39		168
293			6		112		41		159
294			7		139		48		194
295	125	25	11	155	139	30	42	210	192
296	95	19	2	156	170	78	55	253	227
297			17		128		55		200
298			16		110		50		176
299	160	32	1	153	150	26	48	211	199
300			0		132		46		178
301			101		94		5		200
302			24		141		45		210
303			7		141		45		193

Sam-ple	TG	VLDL-C (a)	VLD L-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
304	145	29	37	102	115	23	33	154	185
305	35	7	20	45	48	37	22	89	90
306	110	22	7	169	176	36	50	227	233
307	325	65	51	99	120	26	22	190	193
308	85	17	4	148	180	55	53	220	237
309	165	33	29	121	139	42	41	196	209
310	110	22	7	88	120	46	39	156	166
311	240	48	11	176	190	37	59	261	260
312	100	20	4	205	199	36	63	261	266
313	100	20	1	83	130	70	47	173	178
314	110	22	14	150	143	34	52	206	209
315	105	21	15	121	124	40	42	182	181
316	60	12	3	116	128	52	48	180	179
317	380	76	50	153	189	49	40	278	279
318	60	12	14	40	42	29	22	81	78
319	100	20	5	130	132	34	48	184	185
320	150	30	29	108	113	37	37	175	179
321	95	19	25	96	93	52	33	167	151
322	140	28	22	120	129	51	39	199	190
323	170	34	17	126	134	43	31	203	182
324	130	26	18	114	124	44	26	184	168
325	475	95	66	103	136	30	12	228	214
326	160	32	4	172	186	45	46	249	236
327	55	11	8	58	70	35	28	104	106
328	75	15	31	78	91	55	28	148	150
329	185	37	27	170	167	38	49	245	243

(a) Lipid profile reported by the external laboratory.
(b) Lipid profile obtained by the alternative method.

II. Lipid profiles of samples from Roche Biomedical Laboratory.

Sam-ple	TG	VLD L-C (a)	VLD L-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
1	842		69		116		29	208	214
2	406		81		148		39	268	268
3	511		99		104		14	227	219
4	518		102		194		43	321	339
5	416		85		69		16	169	170
6	631		132		155		20	308	307
7	709		137		188		26	300	351
8	433		74		153		37	251	264
9	544		109		107		14	235	230
10	435	87	63	87	115	34	39	208	217
11	1110		185		229		22	396	436
12	537		121		139		23	271	283
13	1305		202		119		-11	272	310
14	443	89	83	115	134	42	31	246	248
15	409		83		194		49	291	326
16	459		86		71		18	163	175
17	436		106		95		19	214	220
18	632		113		137		27	272	277
19	610		103		122		18	244	243
20	411	82	73	147	161	33	37	262	271
21	562		83		149		32	262	264
22	419	84	79	123	140	38	38	245	257
23	474		111		170		33	311	314
24	411	82	95	183	180	53	41	318	316
25	391	78	79	126	121	36	32	240	232
26	398	80	80	177	171	42	41	299	292
27	416	83	93	122	143	30	28	235	264
28	429	86	78	89	126	30	22	205	226
29	412	82	71	175	187	33	39	290	297
30	420	84	80	51	77	29	15	164	172
31	413	83	96	101	118	27	17	211	231
32	459	92	131	196	200	36	14	324	345
33	1239		226		57		-49	204	234
34	895		151		100		-16	207	235

Sam-ple	TG	VLD L-C (a)	VLD L-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
35	681		133		74		-9	183	198
36	3280		504		109		-174	387	439
37	515		79		184		32	281	295
38	585		149		130		-4	260	275
39	537	107	111	129	163	27	17	263	291
40	436	87	110	68	95	25	3	180	208
41	440	88	94	60	120	59	17	207	231
42	558	112	96	125	168	28	27	265	291
43	432	86	67	138	193	51	34	275	294
44	957		137		196		3	291	336
45	681		104		147		7	238	258
46	1413		266		88		-66	259	288
47	759		128		112		-5	204	235
48	481	96	89	176	188	20	22	292	299
49	401	80	49	120	190	39	41	239	280
50	466	93	74	178	203	25	35	296	312
51	842		141		163		3	250	307
52	692		151		157		10	281	318
53	587		126		76		-6	168	196

(a) Lipid profile reported by the external laboratory.
(b) Lipid profile obtained by the alternative method.

III. Lipid profiles of samples from Stillwater Medical Center.

Sample	TG	VLDL-C (a)	VLDL-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
1	1110		168		37		-16	172	189
2	910		102		112		9	198	223
3	1450		257		12		-68	213	201
4	420		62		131		27	217	220
5	990		146		122		0	272	268
6	1350		197		148		-15		330
7	325	65	34		199		77		310
8	330	66	51		149		54		254
9	358	72	64		59		25		148
10	388	78	66		138		44		248
11	472		67		116		31		214
12	306	61	55	117	150	40	44	218	249
13	202	40	12		97		32	147	141
14	253	51	16		214		73	308	303
15	268	54	24		144		48	212	216
16	1710		182		219		10	425	411
17	297	59	21		209		57	300	287
18	322	64	32		137		34	203	203
19	327	65	40		105		25	184	170
20	364	73	51		76		15	154	142
21	366	73	86		98		13	219	197
22	417		60		143		34	258	237
23	641		63		178		34	298	275
24	1542		208		186		-4	410	390
25	666		58		201		48	325	307
26	557		2		90		35	125	127
27	543		74		164		28	260	266
28	539		70		70		7	157	147
29	436		71		126		28	229	225
30	376	75	91	184	168	44	27	303	286
31	630		62		98		20	189	180
32	493		52		185		44	281	281
33	420		48		151		39	236	238
34	373	75	34		188		49	260	271

Sam-ple	TG	VLD L-C (a)	VLD L-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
35	273	55	17		186		55	252	258
36	325	65	37		111		29	172	177
37	337	67	46		152		36	212	234
38	349	70	17		158		52	218	227
39	355	71	41		165		41	250	247
40	398	80	43		227		55	316	325
41	400		33		173		45	248	251
42	402		23		161		48	223	232
43	405		44		131		36	209	211
44	429		78		142		23	237	243
45	443		67		239		51	339	357
46	264	53	28		109		39	158	176
47	278	56	56		179		41	260	276
48	280	56	46		95		21	159	162
49	284	57	50		138		29	213	217
50	293	59	19		76		27	115	122
51	306	61	37		112		31	185	180
52	343	69	44		155		47	245	246
53	316	63	46		188		50	281	284
54	322	64	50		170		43	258	263
55	590		67		193		45	297	305
56	267	53	47		162		43	245	252
57	332	66	60		130		37	209	227
58	461		83		163		29	267	275
59	476		81		131		25	259	237
60	261	52	41		124		36	215	201
61	295	59	48		96		27	174	171
62	311	62	36		171		53	274	260
63	315	63	57		133		35	229	225
64	320	63	33		111		38	184	182
65	325	65	13		74		33	119	120
66	327	65	29		214		64	314	307
67	358	72	51		112		32	191	195
68	379	76	56		144		38	231	238
69	382	76	54		206		57	320	317

Sam-ple	TG	VLD L-C (a)	VLD L-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
70	399	80	71		155		36	270	262
71	420		61		116		29	211	206
72	412		91		153		30	281	274
73	443		68		173		38	284	279
74	443		46		151		48	243	245
75	451		48		133		36	225	217
76	525		68		172		43	295	283
77	577		78		160		35	271	273
78	766		166		36		-28	182	174
79	255	51	37		133		41	214	211
80	254	51	27		112		36	184	175
81	265	53	54		108		31	191	193
82	1540		181		42		-36	188	187
83	310	62	71		120		20	215	211
84	286	57	56		158		48	252	262
85	267	53	31		87		43	161	161
86	280	56	37		88		25	156	150
87	283	57	53	112	135	53	32	222	220
88	299	60	49		174		48	278	271
89	314	63	42	183	181	32	52	278	275
90	332	66	37		172		49	273	258
91	336	67	50		192		49	297	291
92	341	68	61		142		34	246	237
93	344	69	38		98		33	163	169
94	345	69	39		157		42	244	238
95	368	74	51		175		44	286	270
96	294	59	7		75		35	115	117
97	376	75	66		160		36	247	262
98	302	60	49		126		37	215	212
99	297	59	38		133		43	204	214
100	404		51		168		47	260	266
101	409		43		159		49	246	251
102	640		89		50		12	158	151
103	634		83		128		36	252	247
104	552		95		110		21	243	226

Sam-ple	TG	VLD L-C (a)	VLD L-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
105	422		74		69		16	159	159
106	430		81		145		39	282	265
107	678		99		116		5	231	220
108	374	75	67		98		19	187	185
109	350	70	46		108		25	183	179
110	1209		135		5		-23	122	117
111	780		98		81		8	172	187
112	617		102		59		-10	147	151
113	485		87		103		14	197	204
114	466		82		149		25	258	256
115	460		68		47		12	125	127
116	402		61		153		39	228	253
117	283	57	69		190		46	286	305
118	290	58	49		94		31	168	174
119	259	52	39		96		32	154	167
120	687		123		107		9	235	239
121	600		87		96		15	189	198
122	551		77		119		21	230	217
123	511		85		120		7	211	212
124	432		90		106		12	200	208
125	389	78	63		67		10	136	140
126	364	73	59		140		30	223	229
127	346	69	70		225		45	321	340
128	339	68	62		125		22	195	209
129	329	66	58		215		50	289	323
130	326	65	60		95		30	149	185
131	304	61	54		117		29	176	200
132	839		125		151		-4	264	272
133	557		72		172		22	268	266
134	484		42		163		30	243	235
135	455		58		111		5	187	174
136	406		43		215		29	306	287
137	395	79	68		86		6	180	160
138	370	74	58		177		26	268	261
139	370	74	65		135		15	227	215

Sam-ple	TG	VLD L-C (a)	VLD L-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
140	368	74	63		217		42	336	322
141	362	72	66		135		28	226	229
142	357	71	56		210		53	292	319
143	328	66	70		98		15	177	183
144	327	65	51		111		25	179	187
145	302	60	60		98		19	168	177
146	276	55	30		99		28	146	157
147	266	53	48		55		12	125	115
148	253	51	62		312		56	439	430
149	632		134		143		-8	254	269
150	444		54		145		28	222	227
151	272	54	33		23		6	58	62
152	775		90		102		6	187	198
153	636		110		184		15	291	309
154	551		97		185		21	295	303
155	431		95		172		22	266	289
156	426		82		122		10	197	214
157	387	77	82		168		19	248	269
158	387	77	68		145		21	217	234
159	368	74	67		206		34	278	307
160	309	62	61		86		9	138	156
161	624		80		164		25	265	269
162	419		49		201		31	253	281
163	413		71		212		35	308	318
164	392	78	64		166		23	250	253
165	388	78	61		103		16	171	180
166	359	72	65		125		18	208	208
167	383	77	67		214		43	319	324
168	354	71	58		163		31	234	252
169			190		75		-60		205
170	648		79		135		8	236	222
171	490		96		170		19	267	285
172	429		58		79		8	153	145
173	398	80	63		136		24	253	223
174	365	73	61		164		30	279	255

Sam-ple	TG	VLD L-C (a)	VLD L-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
175	398	80	68		122		18	204	208
176	338	68	62		84		13	160	159
177	670		93		205		33	329	331
178	610		115		194		20	307	329
179	604		112		110		0	220	222
180	539		87		138		18	241	243
181	496		39		68		11	120	118
182	458		88		38		-13	114	113
183	391	78	76		159		23	250	258
184	363	73	66		142		27	225	235
185	318	64	71		224		44	333	339
186	338	68	79		184		34	287	297
187	278	56	75		188		37	295	300
188	360	72	67		146		24	222	237
189	355	71	86		167		26	250	279
190	329	66	49		179		43	237	271
191	297	59	75		222		41	310	338
192	309	62	62		207		44	282	313
193	322	64	50		113		29	161	192
194	436		77		160		29	244	266
195	407		62		150		28	222	240
196	510		84		248		43	344	375
197	350	70	63		120		22	183	205
198	353	71	46		151		34	221	231
199	521		86		89		10	171	185
200	279	56	41		102		25	166	168
201	327	65	32		114		32	170	178
202	404		30		114		34	180	178
203	527		76		202		37	300	315
204	532		65		116		21	189	202
205	758		135		107		-4	219	238
206	610		82		164		39	241	285
207	585		78		174		33	268	285
208	570		72		128		22	212	222
209	556		55		166		37	240	258

Sam-ple	TG	VLD L-C (a)	VLD L-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
210	492		43		209		54	290	306
211	492		87		144		21	240	252
212	455		72		115		22	217	209
213	394	79	62		169		32	242	263
214	366	73	-8		211		69	238	272
215	258	52	41		235		55	310	331
216	269	54	53		107		22	171	182
217	271	54	35		111		39	160	185
218	537		57		54		9	119	120
219	421		43		73		25	114	141
220	917		122		18		-15	120	125
221	375	75	49		85		21	150	155
222	318	64	40		240		63	310	343
223	337	67	45		96		33	159	174
224	845		172		151		-1	314	322
225	702		88		39		-4	118	123
226	575		84		211		33	318	328
227	467		38		102		26	159	166
228	401		84		120		22	200	226
229	389	78	49		215		54	287	318
230	368	74	71		125		23	208	219
231	349	70	27		186		51	260	264
232	295	59	72		214		46	314	332
233	291	58	46		112		33	173	191
234	742		112		21		-11	127	122
235	674		94		242		32	353	368
236	484		67		117		22	197	206
237	398	80	58		217		51	312	326
238	380	76	42		172		44	250	258
239	342	68	32		210		57	289	299
240	262	52	35		105		33	159	173
241	250	50	25		72		23	126	120
242	375	75	31		82		24	137	137
243	413		55		81		19	147	155
244	769		185		189		-10	370	364

Sam-ple	TG	VLD L-C (a)	VLD L-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
245	449		68		104		24	190	196
246	440		38		236		59	324	333
247	415		46		205		51	294	302
248	364	73	35		197		55	277	287
249	329	64	11		253		78	326	342
250	302	60	19		282		77	361	378
251	250	50	33		74		30	133	137

(a) Lipid profile reported by the external laboratory.
(b) Lipid profile obtained by the alternative method.

IV. Lipid profiles of samples from University of Cape Town.

Sam-ple	TG	VLD L-C (a)	VLD L-C (b)	LDL-C (a)	LDL-C (b)	HDL-C (a)	HDL-C (b)	TC (a)	TC (b)
1	142	33	14	94	111	35	43	162	168
2	212	71	14	84	151	58	53	213	218
3	389	105	40	116	161	23	46	244	247
4	912	145	162	153	160	31	1	329	323
5	204	24	12	112	127	50	46	186	185
6	460	159	65	150	205	27	49	336	319
7	186	49	21	74	97	35	40	158	158
8	80	12	13	101	136	77	54	190	203
9	230	7	16	233	233	46	74	286	323
10	88	8	17	189	168	39	58	236	243

(a) Lipid profile reported by the external laboratory.
(b) Lipid profile obtained by the alternative method.

Appendix C

Lipid Profiles Obtained for the Precision Studies

A. Sample 1

	VLDL-C mg/dL	LDL-C mg/dL	HDL-C mg/dL	TC mg/dL
(a) Enzymatic	32	160	42	234
(b) Spectroscopic				
1	25	170	48	243
2	28	167	43	238
3	28	165	45	238
4	22	169	49	240
5	26	163	47	236
6	30	157	46	233
7	23	169	48	240
8	25	161	47	233
9	24	164	49	237
10	23	167	48	238
mean	25	165	47	238
S.D.	2.6	4.1	1.9	3.1
%C. V.	10.2	2.5	4.0	1.3

B. Sample 2

	VLDL-C mg/dL	LDL-C mg/dL	HDL-C mg/dL	TC mg/dL
(a) Enzymatic	7	45	37	89
(b) Spectroscopic				
1	19	49	24	92
2	18	50	25	93
3	14	53	25	92
4	18	51	24	93
5	23	45	21	89
6	23	45	21	89
mean	19	49	23	91

S.D.	3.4	3.2	1.9	1.9
%C.V.	17.9	6.7	8.0	2.0

C. Sample 3

	VLDL-C mg/dL	**LDL-C mg/dL**	**HDL-C mg/dL**	**TC mg/dL**
(a) Enzymatic	20	104	33	157
(b) Spectroscopic				
1	5	112	41	158
2	8	105	39	152
3	8	106	41	155
4	6	110	41	157
5	1	112	42	155
6	17	101	37	155
7	4	108	40	152
8	12	102	41	155
9	6	105	41	152
10	10	112	40	162
mean	8	107	40	155
S.D.	4.5	4.1	1.4	3.1
%C.V.	58.4	3.8	3.5	2.0

Appendix D

Institutional Approval for Handling Human Subjects and Biohazards

OKLAHOMA STATE UNIVERSITY
INSTITUTIONAL REVIEW BOARD
HUMAN SUBJECTS REVIEW

Date: 10-25-94 **IRB#:** AS-93-001C

Proposal Title: SERUM CHOLESTEROL MEASUREMENTS IN HYPERLIPOPROTEINEMIAS

Principal Investigator(s): Neil Purdie, Edralin Lucas

Reviewed and Processed as: Continuation

Approval Status Recommended by Reviewer(s): Approved

APPROVAL STATUS SUBJECT TO REVIEW BY FULL INSTITUTIONAL REVIEW BOARD AT NEXT MEETING.
APPROVAL STATUS PERIOD VALID FOR ONE CALENDAR YEAR AFTER WHICH A CONTINUATION OR RENEWAL REQUEST IS REQUIRED TO BE SUBMITTED FOR BOARD APPROVAL.
ANY MODIFICATIONS TO APPROVED PROJECT MUST ALSO BE SUBMITTED FOR APPROVAL.

Comments, Modifications/Conditions for Approval or Reasons for Deferral or Disapproval are as follows:

Continuation extends through 11/01/95.

Signature: _____ Date: October 26, 1994

Chair of Institutional Review Board

Oklahoma State University

DEPARTMENT OF MICROBIOLOGY & MOLECULAR GENETICS
COLLEGE OF ARTS AND SCIENCES

306 LIFE SCIENCE EAST
STILLWATER, OKLAHOMA 74078-0289
405-744-6243
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2 November, 1992

Dr. Neil Purdie
Professor
Department of Chemistry
Oklahoma State University
CAMPUS

RE: Institutional Approval for Biohazards Handling
"Serum Cholesterol Measurements in Hyperlipoproteinemias"

Dear Professor Purdie:

This is to inform you that your research project as proposed in your letter of 19 October, 1992 to me has been registered with the Biosafety Officer. This letter constitutes approval to conduct the research in accordance with the OSHA Regulations (1992), US Department of Health and Human Services Regulations (1987), and Centers for Disease Control Regulations (1987) for the safe handling of blood with special attention to blood-borne pathogens.

Sincerely,

Robert V. Miller
OSU Biosafety Officer

VITA

Edralin Aguinaldo Lucas

Candidate for the Degree of

Doctor of Philosophy

Thesis: AN ALTERNATIVE ROUTINE METHOD TO MEASURE SERUM TOTAL CHOLESTEROL AND ITS DISTRIBUTION AMONG THE MAJOR LIPOPROTEINS

Major Field: Chemistry

Biographical:

Personal Data: Born in Manila, Philippines, on January 22, 1965, the daughter of Virgilio and Aida Aguinaldo.

Education: Graduated from Philippine Science High School, Quezon City, Philippines, March 1981. Bachelor of Science in Chemistry, University of Santo Tomas, Manila, Philippines, March, 1986. Completed requirements for the Doctor of Philosophy in Chemistry at Oklahoma State University in May 1995.

Professional Experience: Graduate Teaching and Research Assistant, Department of Chemistry, Oklahoma State University, August 1989 to present. Assistant Researcher, Analytical Research Department, United Laboratories, Inc., Manila, Philippines, April 1987 to July 1989. Research Trainee, Food and Nutrition Research Institute, Manila, Philippines, April 1986 to March 1987.

Professional Membership: American Chemical Society
Phi Lambda Upsilon Honor Society