5-2007

Reversed-Phase HPLC Determination of Cholesterol in Food Items.

David Christian Essaka
East Tennessee State University

Reversed-Phase HPLC Determination of Cholesterol in Food Items.

A thesis

presented to

the faculty of the Department of Chemistry

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Master of Science in Chemistry

---

by

Essaka David Christian

May 2007

Dr. Chu-Ngi Ho, Chair

Dr. Scott Kirkby

Dr. Ismail Kady

Keywords: Cholesterol, Foods, Lipoproteins, Reverse-Phase Liquid Chromatography.

ABSTRACT

Reverse-Phase HPLC Determination of Cholesterol in Food Items.

By

Essaka David Christian

Cholesterol is a fat-like molecule found among lipids in animal (including human) tissues. It is needed for maintaining good health. However, health issues have been raised because of the strong correlation between high levels of cholesterol in the body and cardiovascular disease. An HPLC method for quantitative determination of cholesterol in foods is presented. This involves a C-18 stationary phase using a 70:30 methanol: 2-propanol mobile phase with an UV detector set at 212 nm. The method showed linearity in the range 5.0 to 100.0 μg/mL and also good reproducibility with relative standard deviation of 4.22%, 2.71%, 4.8%, and 3.7% for the different samples analyzed. The mean recovery of the butter sample was 106.5%. The samples under investigation were common food items such as butter, lard, and two different types of cheese.

DEDICATION

I would like to dedicate this thesis to the people who inspired me throughout my life, those who always believed in me and encouraged me in so many ways to persevere in my efforts.

First, I thank God for all the abilities He blessed me with. I am grateful for the world in which He created me, for the family, the friends, the good and bad moments of life that I have been able to appreciate.

Next, I am grateful and thankful to my parents, Francoise and Josue Essaka, who have always done all that they could to provide me with a good education and all other related things for me to have a better life. Likewise, I appreciate the constant moral support and solicitude from the other members of my family, especially during the difficult periods. Thank you for your love and support.

ACKNOWLEDGMENTS

I would like to express my gratitude to my research advisor Dr. Chu-Ngi Ho for his guidance, patience, and support throughout this project. I am very appreciative to him for all the teachings, which often went beyond the scope of a chemistry course and contributed to my enhancement as an individual.

I thank Dr. Ismail Kady and Dr. Scott Kirkby for their involvement in my thesis committee and teaching; I appreciate the concern and guidance they provided me during my work. Also, I am grateful to Dr. Ray Mohseni for all the help he provided me as far as the handling of all the instruments in the department and Professor Jeffrey Wardeska whose teaching, availability, and suggestions have been valuable resources.

I would also like to thank the faculty and staff of the chemistry department for making my experience at East Tennessee State University a wonderful one.

Last but not least, I would like to express my gratitude to all my Cameroonian friends and family who have been exceptionally supportive and caring throughout.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Some Facts about Cholesterol

Cholesterol is a soft, waxy substance found among the lipids or fats in the bloodstream and in all cell membranes. It is a non-saponifiable lipid essential in maintaining good health, as it is used to form cell membranes, several hormones, vitamin D, and bile acids needed to digest the fats present in our foods *(1)*. Cholesterol is the major sterol in the human body and belongs to the class of molecules called steroids, which are derivatives of the perhydrocyclopentanophenanthrene ring system *(2)*. Its chemical structure is shown in Figure 1.

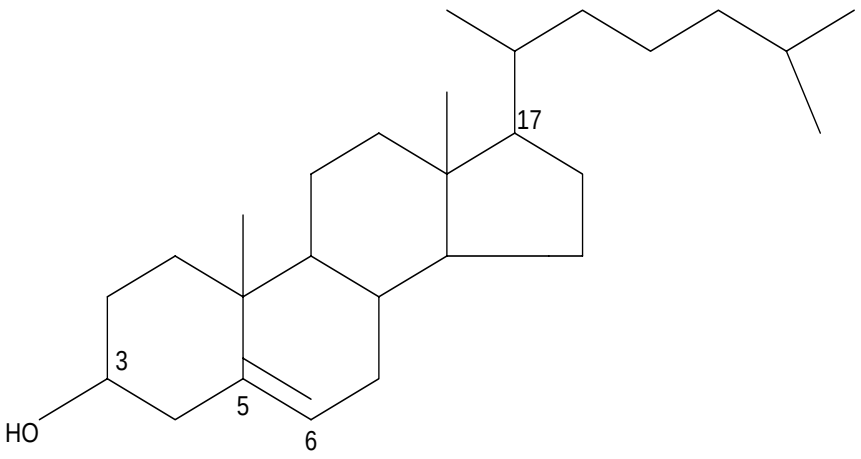


Figure 1. Structure of Cholesterol ($C_{27}H_{46}O$).
Note: The double bond is between carbons 5 and 6, and the hydroxyl group is bonded to carbon 3 of the ring nucleus.

Sterols are steroids that contain a hydroxyl group at C_3 and an aliphatic chain of at least eight carbon atoms attached to C_{17}. Hence, cholesterol has an eight-carbon atom hydrocarbon chain that is numbered 20-27 as a continuation of the steroid nucleus. Some of the cholesterol present in humans is esterified; that is, the hydroxyl group that projects from C_3 is attached to a fatty acid residue with an ester linkage, as shown in Figure 2 *(2)* .

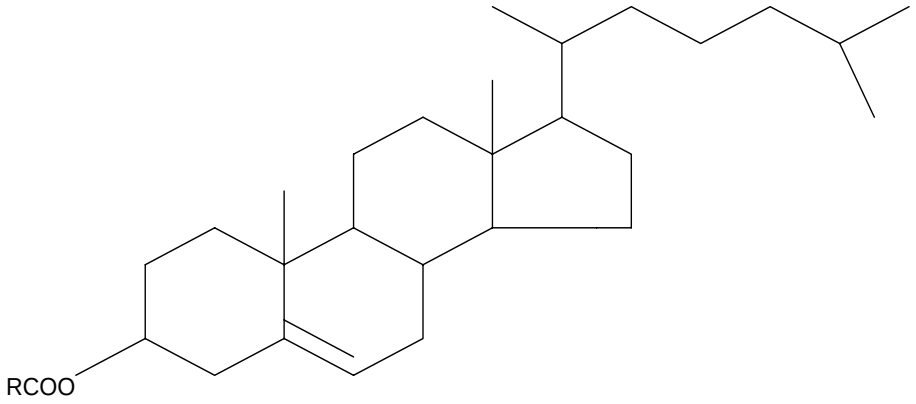


Figure 2. Structure of Cholesteryl Ester.

Cholesterol is synthesized in many types of tissue but particularly in the liver and intestinal walls. It is formed from squalene via lanosterol *(3, 4)*. Cholesterol can thus be produced by most cells in the body. Foods that are derived from animal products are rich in cholesterol. These include eggs, dairy products such as butter, cheese, and cream, as well as most meats. Some of the cholesterol contained in these animal products is in the form of cholesteryl esters, so the ordinary diet contains a mixture of cholesterol and cholesteryl esters *(2)*. Products that are not of animal origin such as vegetables, fruits, and grains do not contain cholesterol.

Cholesterol and other fats cannot dissolve in blood. They have to be transported to and from the cells by special carriers called lipoproteins, which are essentially a combination of lipids and proteins. These are of several kinds, but the ones to focus on are low-density lipoproteins (LDL) and high-density lipoproteins (HDL) *(1)*. Because it contains both hydrophobic and hydrophilic groups, cholesterol serves to help solubilize the nonpolar material that must be transported by the lower density lipoproteins. In addition to serving as a structural component, cholesterol also is one of the lipids that are actually transported by the plasma lipoproteins, predominantly in the form of cholesteryl esters *(2)*. The most important concept to understanding plasma cholesterol transport is that cholesterol does not exist free in the solution. Together with the plasma phospholipids and triglycerides, the other hydrophobic fats, cholesterol enters and leaves the plasma bound to specific proteins, and it is only with these solubilizing

lipoproteins that lipids are transported through the bloodstream *(5)*. Table 1 shows the guidelines developed by the National Cholesterol Education Program (NCEP) of the National Heart, Lung, and Blood Institute, National Institutes of Health (NIH) and the U.S. Department of Health and Human Services *(6)*.

Table 1. Serum Cholesterol and Triglycerides Levels (mg/dL) and risk of heart diseases.

Total Cholesterol	
Less than 200	Desirable
200-239	Borderline high risk
240 and above	High risk
LDL Cholesterol	
Less than 100	Optimal
100-129	Near optimal/above optimal
130-159	Borderline high risk
160-189	High risk
190 and above	Very high risk
HDL Cholesterol	
Above 60-90	Desirable
35-40	High risk
Less than 35	Very high risk
Triglycerides	
Less than 100	Normal
100-149	Borderline high risk
200-399 or above 400	Very high risk

Low-density lipoproteins (LDL) are the major cholesterol carriers in the blood. They transport and supply cholesterol to different parts of the body. They are also referred to as "bad" cholesterol because if too much LDL cholesterol circulates in the blood, it can slowly build-up in the arteries feeding the heart and brain. This can subsequently lead to formation of plaque, a thick, hard deposit that can clog those

arteries, resulting in atherosclerosis. The LDL cholesterol level should be less than 160 mg/dL (or 100 mg/dL for someone already having heart disease) *(1)*.

High-density lipoproteins (HDL), on the other hand, also known as "good" cholesterol, are believed by medical experts to carry cholesterol away from the tissues and back to the liver, its main source of origin in the body, where it is then metabolized and passed out of the body. It is also believed that HDL removes excess cholesterol from plaques and thus slows their growth, helping to reduce the risk of heart attack or stroke. So a high HDL level is a good sign of a healthy body; but a low HDL cholesterol level (less than 40 mg/dL for men and less than 50 mg/dL for women) also indicates a greater risk *(1)*.

Biosynthesis of Cholesterol

Konrad Bloch and associates were the first, in the early 1940s, to reveal that all the carbon atoms of cholesterol are derived from acetate. This finding subsequently led to further investigations and elucidation of the enzymatic complexities of cholesterol biosynthesis *(7)*.

Cholesterol is formed from squalene, a C_{30} hydrocarbon intermediate, via lanosterol, the first sterol to be formed. This synthesis can be viewed as a four-stage process, starting from the two-carbon acetate group of the Acetyl-CoA (acetyl-coenzyme A): the first stage is the synthesis of mevalonate from acetate, followed by mevalonate conversion to two activated isoprenes in a second stage. In the third stage, six activated isoprenes units condense to form squalene, which finally converts to the four-ring steroid nucleus *(4)*.

Two reactions leading to the production of mevalonate from three acetates are involved in the first stage. In the first reaction, catalyzed by hydroxymethylglutaryl-CoA synthase, 3-hydroxy-3-methylglutaryl-CoA is synthesized by condensation of acetyl-CoA and acetoacetyl-CoA; then follows a reduction step catalyzed by hydroxymethylglutaryl-CoA reductase that produces mevalonate *(7)*. This is used for the synthesis of two key isoprenoid precursors of cholesterol; the first two reactions at this second stage are phosphorylations requiring ATP (adenosine triphosphate), followed by a decarboxylation by the enzyme phosphomevalonate decarboxylase to furnish the isoprenoid derivative 3-

isopentenyl pyrophosphate. The enzyme isopentenyl pyrophosphate isomerase then interconverts 3-isopentenyl pyrophosphate and 3,3-dimethylallyl pyrophosphate in an isomerization reaction. Those two derivatives subsequently condense in the third stage to form geranyl pyrophosphate, a C_{10} intermediate that is then transferred to isopentenyl pyrophosphate to synthesize farnesyl pyrophosphate, a C_{15} intermediate. Two farnesyl pyrophosphates then condense to produce squalene in a two-step mechanism: the first step yields an asymmetric C_{30} condensation product that is then reduced and rearranged by squalene synthase, nicotinamide adenine dinucleotide phosphate (NADPH) as co-enzyme, to yield the symmetrical squalene molecule. Squalene is subsequently converted into a tetracyclic steroidal configuration in two reactions: squalene 2,3-epoxide is obtained from the first reaction; it then undergoes cyclization (in a concerted movement of electrons as well as migration of two methyl groups) to yield lanosterol. Cholesterol is finally synthesized from its direct precursors 7-dehydrocholesterol and desmosterol which both come from lanosterol *(7).*

Metabolism of Cholesterol

The liver is the major site of cholesterol synthesis in the body. Other tissues like the intestine, the skin, or the nervous tissues also produce it. An adult can synthesize approximately 800 mg of cholesterol every day. In addition, an individual's diet also provides substantial amounts of cholesterol *(7).* Because there are two "independent" sources for cholesterol, the internal levels of the sterol must be controlled in order to avoid reaching levels giving rise to health issues. Cholesterol synthesis is actually regulated by the dietary cholesterol intake, the caloric intake, certain hormones, and bile acids: that is referred to as "feedback inhibitory" effect *(9).* Michael Brown and Joseph Goldstein elucidated the mechanism whereby cholesterol regulates its own biosynthesis by inhibiting the synthesis of the reductase that converts 3-hydroxy-3-methylglutaryl CoA to mevalonate *(7).*

Cholesterol is needed in all growing animal tissues for membrane synthesis. In addition, a key metabolic function of cholesterol is to serve as the precursor of the following five classes of steroid hormones: progestagens, glucocorticoids, mineralocorticoids, androgens (male hormones), and estrogens (female hormones) *(7).*

Bile salts constitute the major breakdown products of cholesterol; they are polar derivatives of cholesterol synthesized in the liver, stored and concentrated in the gallbladder, and then released in the small intestine. They solubilize dietary lipids, hence promoting their hydrolysis by lipases and facilitating their absorption *(8)*. About 80% of the body's cholesterol is converted by the liver into various bile acids, of which the major ones present in humans are cholic, chenodeoxycholic, deoxycholic, and lithocholic acids. These then conjugate with glycine or taurine to produce bile salts. Glycocholate, for example, which is the main bile salt, is produced by condensation of cholate and glycine *(7)*.

Continuous conversion of cholesterol into bile acids in the liver prevents the body from becoming overloaded with cholesterol, as its excessive accumulation in the tissues is harmful. In normal humans, excess cholesterol is excreted in the feces either in the form of neutral sterols or after conversion to bile acids *(9)*.

Cholesterol and Health Issues

Cholesterol plays an essential role in the normal functioning of many animals including humans, serving in the build-up of the structure of many membranes and as a precursor of steroid hormones and bile acids. However, our diet does not require any cholesterol intake, because all cells can synthesize it from simple precursors. Moreover, high levels of cholesterol in the blood and the occurrence of cardiovascular diseases have been strongly correlated. This is the main reason why nowadays in the media more emphasis is put on the drawbacks of cholesterol, as opposed to its benefits *(4)*.

The transport of cholesterol and other triglyceride fats from their sources of origin (mainly the liver), to the different parts of the body where they are needed is achieved by very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL) and low-density lipoproteins (LDL). This deposition of fats in various places in the body is responsible for the narrowing and hardening of the lumen of a blood vessel. Atherosclerosis, resulting in an obstruction of blood vessels, is a condition linked to high levels of LDL-bound cholesterol leading to heart attacks when coronary arteries become totally blocked. When atherosclerosis affects blood vessels supplying the brain, this results in cerebral vascular disease, eventually leading to a stroke.

High-density lipoproteins (HDL) on the other hand, have a negative correlation with arterial diseases; that is, high levels of HDL are a sign of good health. This is because HDL transport cholesterol and other fats from the body back to the liver where it is recycled.

Several factors can affect the cholesterol levels in the human body. The first factor that we can easily have control over is diet. Foods rich in cholesterol, saturated fats, and trans-fats raise the blood cholesterol levels. Hence, reducing the intake of saturated fats and cholesterol in the diet will have the effect of lowering the blood cholesterol levels. Being overweight is also linked with high cholesterol levels. Losing weight can help lower LDL and total cholesterol levels, as well as raising HDL levels. Exercising on a regular basis not only helps in losing weight but also tends to raise the HDL levels and thus lowers the chances of developing a heart disease *(6)*.

Some other factors cannot be corrected because they are inherent to each individual, but they should still be acknowledged in order to have a better feel of the measures to be taken. One of them is age and gender. Cholesterol levels rise as one gets older. After the age of menopause, women's LDL levels tend to rise even more. Also, heredity determines to some extent the amount of cholesterol one naturally produces *(6)*.

It is equally possible to remedy high blood cholesterol level, the objective being to reduce LDL levels so as to minimize the risk of developing heart diseases or having a heart attack. The Expert Panel on Population Strategies for Blood Cholesterol Reduction released a report in 1990 *(10)*, emphasizing expansion of awareness of high blood cholesterol as a cause of heart disease through mass media channels. Interventions at the population level include diet, exercise, and weight control; these constitute what is called therapeutic lifestyle changes. Another alternative to reducing high blood cholesterol level is drug therapy.

Lipid-lowering therapy is known to be successful in reducing morbidity and mortality in patients with coronary artery disease, even though the effect is more pronounced in high-risk patients with greater risk of coronary artery disease (CAD) than that of low-risk patients. The recommendation from the Adult Treatment Panel III of the US National Cholesterol Education Program (NCEP) *(10)* is that patients with CAD,

diabetes, or global risk of CAD above 20% over 10 years and LDL cholesterol (LDL-C) levels above 130 mg/dL, should receive drug therapy with a goal of reducing LDL-C levels to less than 100 mg/dL. Statins, or 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, are the most effective and well-tolerated lipid-lowering agents *(11)*. Five of them are approved by the US Food and Drug Administration for general use: atorvastatin, achieving up to 55% LDL-C reduction, pravastatin, fluvastatin, lovastatin, and simvastatin. Table 2 shows the amounts of statins required to achieve 30-40 % reduction of LDL-C levels *(12)*.

Table 2. Doses of currently available statins and corresponding LDL-C reductions.

Drug	Dose, mg/dL	LDL reduction, %
Atorvastatin	10	39
Lovastatin	40	31
Pravastatin	40	34
Simvastatin	20-40	35-41
Fluvastatin	40-80	25-35

Bile acid sequestrants are another drug therapy with moderate effectiveness and gastrointestinal side effects. Niacin, the oldest lipid-lowering drug, is still used mainly because it produces 10 to 15% increase of the HDL-C, as well as good reduction of triglycerides. Also, plant stanols that are given as dietary supplements help in decreasing LDL-C in modestly hypercholesterolemic patients *(11)*. Combination therapy is also worth mentioning. Recent findings show that the use of statins with the addition of a second agent such as a bile sequestrant (nicotinic acid, or ezetimibe, a new cholesterol-absorption inhibitor) further reduced LDL-C in some patients *(12)*.

Summary

Heart disease is the number one killer of women and men in the United States of America *(6)*. The reason why there is so much attention on cholesterol is the strong correlation between coronary heart disease and high blood cholesterol levels. Because of

its unique nature as a lipid and a steroid, cholesterol is a substance vital for the normal and healthy functioning of the body. The problem resides in the presence of excessive cholesterol in the blood, especially in the low-density form, as this brings about conditions like hypertension, coronary artery disease, or stroke, when atherosclerotic plaques form in arteries and occlude blood vessels. High blood cholesterol does not present any symptoms, so there is no other way to be aware of a potential risk for developing heart disease than to take a blood test or have a lipoprotein profile.

So the need for developing methods allowing fast, precise, and reliable measurements of the amount of cholesterol present in the blood and in foods is obvious. The design and evaluation of one such method is the purpose of this research project.

CHAPTER 2

METHODS OF CHOLESTEROL ANALYSIS

With all the publicity that has been going on about cholesterol, publicity justified by the strong connection between high blood cholesterol levels and cardiovascular diseases, it is clear that the determination the amount of cholesterol in blood or in foods has become a necessity. Many studies have thus been carried out over the years on this topic, each one trying to achieve a simple, economical, and reliable method of cholesterol determination.

The breakthrough in determination of cholesterol dates back to the late nineteenth century with the development of a practical color reaction first described by Salkowski *(13)*. It was modified by the Nobel Prize winner Windaus *(13)*, who also discovered that digitonin quantitatively precipitates cholesterol and does so relatively easily. The gravimetric method developed by Windaus was the first reference procedure for the determination of cholesterol.

Because of the efforts invested by the scientific community in designing suitable methods of cholesterol analysis, various methods have been developed over the years. It is useful to categorize these methods according to their applications in order to discuss them thoroughly. The literature classifies cholesterol methods into two main groups: direct reactions and extraction methods, which are further subdivided into partial purification of cholesterol with organic solvents and complete isolation of cholesterol *(13)*. In direct reaction methods, reagents are added to the samples without any pre-treatment so that there is no phase separation. On the other hand, extraction methods, as the name implies, involves extraction of the cholesterol from the samples into a suitable solvent followed by a color reaction after the purification. Some of the different methods used to analyze cholesterol are grouped and reviewed according to the analytical techniques used.

Spectrophotometric Methods

Spectrophotometry refers to the measurement of absorption and emission of light by materials. This section reviews some of those methods involving an optical end-point reaction.

Liebermann and Burchard were the pioneers in the colorimetric methods of cholesterol determination, which they established in 1885 *(14)*. The basic procedures later used by others were more or less modified versions of their methodology. Three compounds, acetic anhydride, glacial acetic acid, and sulfuric acid, are required for the preparation of Liebermann-Burchard (L-B) reagent *(14)*. In 1969, Edward Kim and Morris Goldberg *(15)* prepared and used a single stable L-B reagent for serum cholesterol assay. In the process, they first cooled acetic anhydride and concentrated sulfuric acid in ice water. To a 500-mL amber glass bottle, they added 220 mL of cold acetic anhydride and 200 mL of glacial acetic acid (room temperature). After mixing by inversion, 30 mL of cold concentrated acid sulfuric acid was added. The reagent was then ready for usage or storage (for at least six months) at 4°C in the dark *(15)*. In the procedure, the cholesterol standard was prepared by dissolving 100 mg cholesterol in 50 mL glacial acetic acid; 6.0 mL of L-B reagent was pipetted into 19 × 105 mm cuvets labeled "U", "S", and "B" for unknown, standard, and blank respectively. To "U" and "S" cuvets, 0.1 mL of unknown serum and 0.1 mL of cholesterol standard were respectively added then mixed using a vortex mixer. All cuvets were then incubated at 37°C for 18 minutes, after that the tubes were read at 625 nm at regular intervals over 10 minutes against an L-B reagent blank set to 100% transmittance. The concentration of the unknown was calculated using the standard photometric formula

$$C_u = C_s \times A_u/A_s \qquad [1]$$

The above study gave a standard deviation value of ± 5.7, which was deemed acceptable *(15)*.

In 1934, Rudolf Schoenheimer and Warren M. Sperry published a paper *(16)* on the determination of free and total cholesterol in blood and other biological material. Amounts of cholesterol ranging between 0.02 and 0.15 mg could be determined. The procedure involved precipitation of cholesterol with digitonin, and then a color reaction was applied to the precipitate. A modified L-B reaction was employed, which allowed direct comparison of the color given by digitonin with that of cholesterol in the range 610-620 nm using a sensitive photometer. The calculation of the amount of

cholesterol was based on the results of a long series of determinations on known amounts of cholesterol in which it was shown that the specific extinction coefficient is constant over the range studied *(16)*. In terms of accuracy, this method gave values very close to those found in the Windaus procedure.

Enzymatic methods have also been extensively investigated. Charles C. Allain and co-workers, proposed in 1974 a method preferable to older ones because of its specificity, dynamic range, and simplicity *(17)*. No prior treatment of the sample was required. Cholesterol esters are hydrolyzed to free cholesterol by cholesterol ester hydrolase. The free cholesterol produced is oxidized to cholest-4-en-3-one by cholesterol oxidase with the production of hydrogen peroxide. The hydrogen peroxide thus generated is measured by the oxidative coupling of 4-aminoantipyrine and phenol in the presence of peroxidase to yield a chromogen with maximum absorption at 500 nm. The method yielded a coefficient of variation of 0.5% for a cholesterol concentration with a mean value of 384.4 mg/dl and a standard deviation of 1.91%; also a coefficient of variation of 1.0% for a cholesterol concentration with a mean value of 46.9 mg/dL and a standard deviation of 0.48% *(17)*.

The first and second methods above are direct and extraction methods respectively, both non-enzymatic methods, whereas the third is another direct enzymatic method. Extraction methods generally present the disadvantage of being time-consuming, and require considerable manipulative skills and care in the process; also, the L-B color is known to fade on exposure to light. On the other hand, direct methods suffer from the presence of interfering chromogens, hemoglobin, and bilirubin. Another shortcoming is the depression of the final color produced in cases where standards in organic solvents are being used because of water in the serum *(18)*.

Mass Spectrometric Method

An isotope dilution/ mass spectrometric (ID/MS) method for total serum cholesterol was developed independently at the Karolinska Institutet (KI) and the National Bureau of Standards (NBS), now National Institute of Standards and Technology (NIST). The compatibility of both studies was then assessed in a study published in 1982 *(19)*. In the KI procedure, cholesterol-d_4 and purified unlabeled

cholesterol served as labeled internal standard and primary standard respectively. Calibration curves were made from the averages of two single independent molecular-ion ratio measurements on the unlabeled and the labeled cholesterols separately and on four such standard mixtures. Aliquots of the same solution of labeled cholesterol were used for the serum sample analyses. After saponification using alcoholic potassium hydroxide and extraction, ratios of the molecular-ions at m/z 390 and 386 from the two forms of cholesterol were independently measured twice by gas Chromatography-mass spectrometry (GC-MS) *(19).*

On the other hand, the NBS method involved the use of cholesterol-d_7. After saponification, the extracted cholesterol was converted into the trimethylsilyl ether derivative by overnight reaction with 0.5 mL of 6/4/3 by volume of pyridine/hexamethyldisilazane/trimethylchlorosilane. Combined GC-MS with selected ion monitoring at m/z 465 and 458 was used. Each observed ratio for a sample is converted into a cholesterol-d_7 to $-d_0$ weight ratio by a linear interpolation between the ratios observed for two standard mixtures. GC-MS showed that lathosterol interfered in the method at the KI. The NBS results were found to be more precise; but the mean values for the second series of analysis by the KI method lied within ± 1.3% of the NBS values so that they could be used as laboratory reference method values. Still, the ID/MS analyses required rigorous sampling and were time-consuming *(19).*

Electrophoresis Method

The interest in electrophoretic analysis of cholesterol resides in the fact that it allows fractions of cholesterol, namely HDL, LDL, VLDL, and also lipoprotein(a) [Lp(a)], to be quantified, as they play an important role in assessing an individual's risk of cardiovascular disease. Lp(a) was described in 1963 *(20)* as a genetic variant of LDL and has been established as an independent predictor of coronary artery disease, stroke, and retinal occlusion. The study by Nauck and co-workers *(20)* described the rather difficult separation of Lp(a) from the other cholesterol fractions, overcoming the requirement for preparative ultracentrifugation by using agarose gels in which Lp(a) and VLDL are resolved electrophoretically. Cholesterol and triglycerides were determined enzymatically; VLDL, LDL, and HDL were isolated by sequential ultracentrifugation at

less than 1.006 kg/L for VLDL, between 1.019 and 1.063 kg/L for LDL, and between 1.063 and 1.21 kg/L for HDL. Lp(a) was then prepared from the regenerate fluid of a dextran sulfate-based LDL aphaeresis system by sequential centrifugation and gel filtration. Lipoprotein separation followed according to the Lipid Research Clinics Program's protocol *(20)*. Agarose gel lipoprotein electrophoresis and staining was then performed with a rapid electrophoresis system (REP). The new agarose gel contains a buffer with divalent cations and urea, which allows the separation of VLDL and Lp(a). The gel also contains 10.72 g of sodium barbital, 0.28 g of EDTA, 0.40 g of NaN_3, 5.0 g of guanidine-HCl, 850 mmol of urea, and 15 mmol of $MgCl_2$ per liter, with pH adjusted to 8.60. To this mixture, 50.0 g/L sucrose, 10 g/L agarose, and 15 g/L albumin were added. Electrophoresis was carried out at 250 volts for 40 minutes, and cholesterol was enzymatically stained by cholesterol dehydrogenase and nitroblue tetrazolium chloride as dye. The gels were scanned densitometrically at 570 nm with the densitometer of the REP system. Lp(a) was determined with a polyclonal antibody on a Behring nephelometer. This method had excellent agreement with most published immunological methods; the total CVs for LDL-C and HDL-C were < 9.5% throughout, the CVs for VLDL-C and Lp(a)-C were between 7.8% and 23.3% and between 6.8% and 17.7% respectively *(20)*.

Separation Techniques

A technique coupling gas chromatography and mass spectrometry has been mentioned earlier (page 21) in this chapter. In this section, gas and liquid chromatography are the focus of interest. Because of the awareness that high intake of cholesterol in foods is an unfavorable factor in raising plasma cholesterol, it is important to develop methods to assess cholesterol content in various foods. The study by D. J. Fletouris and co-workers *(21)* in 1998 presented a rapid and reliable method for the determination of cholesterol in dairy products by direct saponification and using a capillary column gas chromatography (GC) system equipped with a flame ionization detector (FID). A 0.2 g sample was weighed into a tube to which 5 mL of methanolic KOH was added. After being vortexed for 15 seconds, the lower half of the tube was immersed for 15 minutes in an 80°C bath while vortexing every 5 minutes. After cooling

the tube with tap water, 1 mL of water and 5 mL of hexane were added, and the contents vortexed vigorously for 1 minute then centrifuged for another minute. An aliquot of the top layer was then transferred into the autosampler vial for GC analysis. Injecting 1 μL from standard solutions and plotting the recorded peak area against the corresponding mass of analyte injected yields the calibration curve. The concentration of the cholesterol in the samples is then obtained by using the equation

$$C = M \times V \times 2.5 \qquad [2]$$

where M is the computed mass (in nanograms) of the analyte in the injected extract, and V is the volume dilution factor, if any. The accuracy of the method was assessed by a standard addition procedure. The linearity was acceptable, with an estimate of 98.6% overall recovery. The overall precision was found to be 1.4% *(21).*

Liquid chromatographic methods have been researched as well, with the use of high performance liquid chromatography (HPLC) either with a normal phase or a reverse phase. The article by Michael D. Greenspan and co-workers *(22)* mentioned a combination of a normal phase chromatographic system with spectral detection using a diode array detector in the separation and identification of cholesteryl esters, triglycerides, ubiquinone, α- tocopherol, dolichol, cholesterol, 7-dehydrocholesterol, and retinol. Standard curves were prepared by chromatographing known amounts of triolein, cholesterol, 7-dehydrocholesterol, and retinol. Ultraviolet spectra from 190 to 370 nm were taken every 0.2 second in the analysis of samples. The lipids were first extracted from a dog liver, then the final residue was dissolved in 0.5 ml of chloroform and added to a washed Bond Elut column. The lipids were eluted with 5 mL of diethyl ether. An aliquot of the lipid extract was injected onto the cyanopropyl column and the lipids were eluted isocratically with 0.1% isopropanol in heptane at 1 mL/minute at ambient temperature. This technique is advantageous in that it allows integration of two close chromatographic fractions as various wavelengths can be scanned. However, there is no mention of the possibility of reliably quantitating those lipids *(22).*

Another interesting study is the simultaneous liquid chromatography determination of cholesterol, phytosterols, and tocopherols in foods by H. E. Indyk in

1990 *(23)*. It describes a quality-control sample preparation scheme to overcome the disadvantages of conventional saponification strategies. The HPLC system is coupled in series to a variable-wavelength detector, a fluorescent detector, and a dual-pen chart recorder. The column is a 5-µm $C_{18,}$ and the mobile phase could be either hexane-isopropyl alcohol (99.9 : 0.1, v/v) or 100% methanol. Serial detection was done by UV (210-214 nm) and fluorescence measurements (λ_{ex} = 295 nm; λ_{em} = 330 nm). In the procedure, 0.025 mg/mL of cholesterol and phytosterol working standards were prepared in hexane, 2 µg/mL for α-tocopherol and 0.02 mg/mL for squalene. For the sample, a few grams (from 0.1 to5.0 g depending on the food type) were accurately weighed into a test tube. Ten milliliters of ethanol was added to each sample followed by agitation. Then 2 mL of KOH solution were added and the stoppered tubes were incubated for 8 minutes at 70°C. After cooling, 20.0 mL of hexane-diisopropyl ether (3+1) was added and the tubes were shaken for 5 minutes. Water (30 mL) was then added, and the tubes re-stoppered, inverted 10 times, and centrifuged at 1000 rev. min^{-1} for 10 minutes. For cholesterol determination, 20 µL of the top layer was directly injected. When tocopherols were also required, 10.0 mL of the top layer was evaporated to dryness, the residue was dissolved in 1 mL of either hexane or ethanol, and 20 to 50 µL of the solution was injected *(23)*.

Though the last method gave estimated values that agreed with literatures values and had the advantages of being rapid and simple, as well as selective and sensitive towards tocopherols, the better efficiency of the gas chromatographic systems compared to this method was acknowledged. With the ever-increasing need for determining cholesterol content in various foods, the possibility of HPLC procedures with speed, simplicity, efficiency, and reliability is worth pursuing.

CHAPTER 3

METHODOLOGY OF HPLC

With the continuous improvement of its technical capabilities, high performance liquid chromatography (HPLC) has become one of the most widely used analytical tools today. HPLC involves the separation, identification, and determination of the different components present in a mixture. This process starts with the injection of the sample through an injection port; the sample is then transported in the system by a mobile phase to the column, where interactions between the different components of the mixture and the stationary phase take place. Separation then occurs because different components will have different kinds of interactions (migration rates inside the column) resulting in different elution times of each component. Comparison of the elution times obtained for each component with that of the standard components alone allows for identification of a given component, and quantitation can be achieved with the aid of a calibration curve obtained from injecting different amounts of the standards. This chapter gives a glimpse of all the parameters that have to be taken into account in order to achieve the best results in an analysis.

Types of Chromatography

The use of a stationary phase and a mobile phase has been applied to a number of various chromatographic methods, which can thus be divided into column chromatography and planar chromatography. In column chromatography, the stationary phase is held in a narrow tube and the mobile phase is forced through it by pressure or gravity, whereas in planar chromatography, the stationary phase is supported on a flat plate, and the mobile phase moves through it by capillary action or gravity *(24)*. Also, chromatographic methods are categorized depending on whether the mobile phase is a gas, a liquid, or a supercritical fluid. Clearly, HPLC falls into the column liquid chromatography classification. Table 3 shows the characteristics of some of the chromatographic techniques.

Table 3. Gas and Liquid Chromatographic Techniques.

General Classification	Specific Method	Stationary Phase	Equilibrium Involved
Gas Chromatography mobile phase: gas	Gas liquid	Liquid adsorbed on a solid	Partition between gas and liquid
	Gas-bonded phase	Organic species bonded to a solid surface	Partition between liquid and bonded surface
	Gas-solid	Solid	Adsorption
Liquid Chromatography mobile phase: liquid	Liquid-liquid or partition	Liquid adsorbed on solid	Partition between immiscible liquids
	Liquid-bonded phase	Organic species bonded to a solid surface	Partition between liquid and bonded surface
	Liquid-solid or adsorption	Solid	Adsorption

Furthermore, HPLC could be denoted as normal-phase, or reversed-phase, according to the type of interactions taking place between the stationary phase and the solutes or components in mixtures. Ion-exchange and gel permeation are the other types of interaction in liquid chromatography, but they will not be treated here.

Normal and Reversed-Phase Chromatography

In normal-phase chromatography, the stationary phase is more polar than the mobile phase. The stationary phases are usually made up of inorganic polymers with large surface areas, like hydrated silicon-oxide (silica gel), or aluminum-oxide polymers (alumina) *(25).* In this type of system, the least polar component of a mixture is eluted

first, and increasing the polarity of the mobile phase decreases the elution time of that component *(24).* The term reversed-phase in chromatography comes from the fact that the polarities of the mobile and stationary phases are interchanged as compared with the normal-phase system; that is, the mobile phase is now polar whereas the stationary phase is nonpolar. One type of stationary phase used in reversed-phase chromatography is composed of organic polymers beads; but the most commonly used are nonpolar groups bonded onto silica, such as methyl $–CH_3$, octyl $–C_8H_{17}$, and octadecyl $–C_{18}H_{37}$ *(25).* Here, the most polar component of a mixture will elute first, and increasing the polarity of the mobile phase will increase the elution time of that component *(24).*

Reversed-phase HPLC accounts for more than 70% of the liquid chromatography assays today *(25).* The essential elements constituting the HPLC system are the solvent reservoir where the eluent (mobile phase) is stored. This eluent will transport the analytes to the stationary phase. A pump, usually a reciprocating pump, is used to ensure the flow of the eluent and solute through the system. Then there is an injection port through which the sample is introduced into the system with a syringe. The heart of the system is a column that houses the stationary phase, this is where interactions and thus separation occur. After elution, the analytes pass through a detector, the device that supplies an output signal related to the amount or rate of change of the amount of sample injected; finally a recorder translates the detector's signal into chromatograms. An illustration of a typical HPLC system is given in page 39.

Separation of components in a mixture occurs in the column, according to the molecular differences of the species involved such as their charges, size, mass, polarities, boiling point, chirality, redox potential, acidity constants, and affinity for complexation. A dynamic equilibrium exists between the analytes in the mobile phase and in the stationary phase. Upon changing the conditions of the HPLC experiment (for example changing the mobile phase composition or the pH), that equilibrium is going to shift in one direction or the opposite, because of interactions of analytes with the mobile phase relative to the interactions with the stationary phase. Different analytes will thus elute from the column with different retention times, giving rise to separate peaks in the chromatogram. Several factors help in obtaining the best separation during an analysis. The most important factor is the composition of the mobile phase, as the polarity of the

stationary phase cannot be changed. Some examples of mobile phase compositions include aqueous solutions containing various concentrations of other solvents such as methanol, acetonitrile, or tetrahydrofuran *(24)*.

Detectors Used in HPLC

The detector is the most complex and expensive piece of equipment in the HPLC instrument. Several types of detectors are in use and available commercially. Some of these detectors are ultraviolet-visible absorption, refractive index, fluorescence, heat of adsorption, electrical conductivity, and flame ionization *(26)*. The performance criteria such as sensitivity, detection limit, linearity, repeatability, peak shape and spreading, ease of operation and maintenance, as well as applicability, are to be considered in the choice of any kind of detector.

UV-Visible Absorption Detector. This type of detector is one of the most widely used as it presents the best compromise of all the mentioned performance criteria. It involves absorption of electromagnetic radiation by molecules because of the presence of chromophores such as hydroxyl –OH, carboxyl –COOH, carbonyl –CO, aromatic (benzene ring), or other types of chromophores on the molecules. The ultraviolet-visible range comprises wavelengths between 190 and 600 nm. Molecules are exposed to light coming from a mercury (Hg) or deuterium lamp (in the UV region) or a tungsten halogen source (visible region), and absorption is measured through the transmitted radiation. Monochromators associated with a continuous broad spectrum light source are often used, thus providing a selection of wavelengths and extended applicability: ultraviolet-visible detectors can be used in analysis of proteins, enzymes, and amino and nucleic acids. The sample concentration in the flow cell is linked to the fraction of light absorbed according to Beer's law *(26)*:

$$A = \log (I_o/I) = \varepsilon\, b\, C \qquad [3]$$

with A the absorbance, I_o the incident light intensity, I the transmitted light, ε the molar absorptivity, b the cell path, and C the molar concentration of the sample. Photomultiplier

tubes and photodiode arrays are the most common transducers for measuring absorbance.

Differential Refractive Index Detectors. Refractive index detectors depend on light bending properties of liquids and involve changes in light path as the index of refraction of the eluent changes in the presence of solutes. This type of detector is more universal in the sense that any solute present produces a response *(25)*. In the differential design, light from a pulsed light-emitting diode passes through the cell windows and the sample, is reflected from a mirror, and again passes through the sample and then the window back to a detector. Different angles of refraction cause changes in the amount of light that falls on a photocell. Two photodiodes are used here. The first receives light from the cell with the flowing eluent, while the second receives light from the reference-filled cell. So the detector's response is the amplified difference in light falling on both photocells *(25)*. The major shortcomings for this type of detector are the required temperature control of the cells as well as the impossibility to use gradients (change of mobile phase composition with time).

Flame Ionization Detectors (FID). These detectors have been developed for gas chromatography but can also be used in liquid chromatography. Different designs of FID can achieve the separation of the sample from the solvent in the effluent stream then vaporize or decompose the sample and detect it by that sensitive detector *(26)*.

Optimizing Parameters

The aim of the HPLC experiment is to achieve the best separation possible of the components in a mixture so that each component present produces a well-defined peak or signal with a specific retention time different from others. A good resolution yields sharp narrow peaks in the resulting chromatogram. The choice of the mobile phase is the most critical factor in the separation using reversed-phase HPLC. The mobile phase chosen should be able to dissolve the sample, have a low viscosity, be compatible with the detector, be pure, and be commercially available at a reasonable price *(26)*. Besides the mobile phase, some other factors mentioned in the next few sections affect the separation process of molecules.

Resolution. The resolution in HPLC refers to the degree of separation achieved in an analysis. The resolution between two peaks is given by the equation *(26)*:

$$R = 2\,[t_{R2} - t_{R1}] / (w_2 + w_1) \qquad [4]$$

where t_{R2} and t_{R1} are retention times of retained components, w_1 and w_2 are their respective peak widths in units of time. The resolution depends on the narrowness of the peaks and the distance between the peak maxima; these in turn have to do with the choice of the stationary phase, the mobile phase, temperature, pH, and length of column.

Column Efficiency. The column plays an essential role in achieving a good separation, as the column efficiency determines the peak width. The column efficiency is expressed as follows:

$$H = L / N \qquad [5]$$

where L is the length of the column (in millimeters); N is the number of theoretical plates, which is determined by factors in the column construction such as the quality of the material used in packing the column and how well the packing is achieved. The column efficiency, H, also called the height equivalent to a theoretical plate (HETP), further depends on parameters such as flow rate, particle size, and column diameter. An efficient column produces narrow peaks and thus keeps peak zones from spreading. Three main sources for zone spreading have been identified: multiple paths traveled by solutes, molecular diffusion, and mass transfer. The first is because of the flow velocity varying inside the column and can be minimized by ensuring homogeneous packing of uniformly sized particles in intermediate diameter columns (1-6 mm i.d.) *(26)*. For the second source, the more time the solute spends in the column the more molecular diffusion and thus zone spreading occurs. Operating at a higher flow rate can help minimize this problem. Mass transfer zone spreading on the other hand comes from the dynamic nonequilibrium between the analyte in the mobile phase and the analyte in the stationary phase. This is reduced by the use of thin stationary films in columns *(26)*.

Selectivity Factor. This is also called separation factor. The selectivity factor for two peaks is given by the equation:

$$\alpha = t'_{R2} / t'_{R1} = K_2 / K_1 \qquad [6]$$

where t'_{R1} and t'_{R2} are the adjusted retention times of component 1 and 2 respectively, K_1 and K_2 are distribution coefficients of components 1 and 2, respectively. The distribution coefficient is the ratio of the concentration of analyte in the stationary phase over the concentration of the analyte in the mobile phase.

One single equation summarizes the important factors required to produce a good separation of a mixture:

$$R = \tfrac{1}{4} N^{1/2} [(\alpha-1)/ \alpha] [k'/ (k'+1)] \qquad [7]$$

where the first term N refers to the column efficiency as defined earlier. The second term, $(\alpha-1)/ \alpha$ defines the separation factor. The last term, $k'/(k'+1)$ defines the retention factor, which for any i^{th} component is given by

$$k'_i = t'_{Ri} / t_M \qquad [8]$$

where t'_{Ri} is the adjusted retention time of the i component, and t_M is the time taken by the mobile phase to pass through the column and reach the detector. The N value should be as high as possible to achieve the best separation. One way to do this is by slowing down the flow rate. But, this has the inconvenience of lengthening the analysis time and favoring molecular diffusion. One could also use a longer column, provided that the back pressure does not go too high and still allows stable analysis conditions. The separation factor α, could also be acted upon, in order to improve separation. The improvement is done by increasing the value of α, either by a change in the stationary phase or the mobile phase. Varying the retention factor is definitely the easiest thing to do and, therefore, the one to start with. An increase of k' value will improve separation, as the solute will be retained longer in the column. Typical k' values range from 1 to 5.

Another efficient method to achieve good separation is simply by changing the composition of the mobile phase, or change the pH, or the temperature.

Proposed Research

Chapter 1 pointed out the importance of developing methods in the determination of the analyte of interest, cholesterol, in foods in general, to help minimize the unnecessary daily intake of products with high content in cholesterol and thus reduce the risks of heart diseases. The second chapter reviewed some of the methods that have been developed by scientists to achieve that goal. The merits and disadvantages of the reputed methods have also been discussed.

The literature mentions a great number of methods that simply show that attaining a satisfactory method for analysis is still needed. However, some methods have proven very reliable like the use of Liebermann-Burchard reagent. Different factors are to be taken into account when developing a new analytical method: the ease or simplicity with which the procedure is carried out, the cost of the materials and reagents to be used, as well as their availability. Environmental concerns or the "greenness" should also be addressed in the process. Moreover, the reproducibility, accuracy, precision, and applicability of the method have to be thoroughly assessed in order to validate the method.

The present chapter presented the technique to be used in our attempt to develop and evaluate a new method for the determination of cholesterol in foods. In light of the discussion above, this project will strive to fulfill the following objectives:

1. To establish optimal reversed-phase HPLC conditions for determination and quantitation of cholesterol in various food samples
2. To develop a simple method in the preparation of samples
3. To establish the figures of merits for this HPLC method: linear dynamic range, reproducibility, recovery, and accuracy.
4. To evaluate the applicability of our technique by using various food samples.

CHAPTER 4

EXPERIMENTAL PROCEDURES, RESULTS, AND DISCUSSION

Experimental Procedures

This chapter describes the experimental procedures involved in the proposed method of cholesterol analysis. These procedures mention the reagents that are used, their source and preparation, the source and preparation of the standards, and sample solutions that are used throughout the analysis. The instrumentation parameters upon which the analysis is based are also evaluated.

Most of the reagents come from the same source; they are used in the preparation of the standards upon which the proposed method is based as well as the samples on which the analysis is carried out. More details are given in later sections on the mixture of those reagents to yield the mobile phase composition to be used throughout.

The standards solutions to be prepared are those upon which this cholesterol analysis method relies; a detailed presentation of their preparation is provided in this chapter. An analysis of various samples is the key to the evaluation of our method, as the reproducibility and recovery studies are carried out on those samples, the preparation of those samples is going to be emphasized.

The conditions for the HPLC experiment used in the project are also given in this chapter. A full description of the HPLC system is provided in the following pages as well as the optimized parameters yielding the best results, such as the composition of the mobile phase, the flow rate, the wavelength of the UV-VIS detector. The ultimate goal is to attain a method offering good precision, accuracy, and applicability.

Reagents

The following reagents are all ACS certified.

1. Deionized Water, produced with one of US Filter Company devices, (Pittsburgh, PA).
2. Methanol (HPLC grade), obtained from Fisher Scientific (Fairlawn, NJ). It is one of the components of the mobile phase, and is also used as solvent in the preparation of a solution of potassium hydroxide.
3. 2-Propanol (HPLC grade) also from Fisher Scientific, serves as the other

component of the mobile phase used in this research project. It is also used as the solvent for all dilutions of samples before analysis in the HPLC system.

4. Hexane (HPLC grade), from Fisher Scientific, is the extraction reagent to be used in the procedure.
5. Potassium Hydroxide crystals used to prepare a methanolic potassium hydroxide solution.
6. Cholesterol 96% pure, from Fisher Scientific, used in the preparation of our standard solutions.
7. Sweet Cream and Salted Butter was obtained from a local Kroger store in Johnson City, TN.
8. Armour Lard (lard and hydrogenated lard) is distributed by ConAgra Foods and found in a local Kroger store as well (Johnson City, TN).
9. Swiss and Kraft Singles (American Pasteurized Prepared Cheeses Product) cheese also obtained from a Kroger store in Johnson City, TN.

Stock Solutions and Reagents Preparations

The following reagents and stock solutions were prepared:

1. Cholesterol stock solution (1.00 mg/mL): 0.0251 grams of 96% pure cholesterol was weighed and placed in a 25-mL volumetric flask and dissolved with 2-propanol after 5 to 10 minutes of sonication.
2. Cholesterol working solution (10 μg/mL): 100 μL of stock cholesterol solution was pipetted into a 10-mL volumetric flask and diluted to the mark with 2-propanol.
3. Mobile phase: 500 mL of methanol and 500 mL of 2-propanol were placed in 2 separate containers; they were both degassed by passing helium gas through them for 15 to 30 minutes before use.

Standard Solutions for Calibration Curve and Linearity Studies

A series of six standards solutions were prepared for the linearity study: 50, 75, 100, 200, 500, and 1000 μL of the stock cholesterol solution were pipetted into six separate 10-mL volumetric flasks; 2-propanol was then added up to the mark. These

dilutions resulted in 5.0, 7.5, 10.0, 20.0, 50.0, and 100.0 μg/mL solutions, respectively.

Preparation of saponification solution

A 0.5 M methanolic potassium hydroxide (KOH) solution is prepared by dissolving 5.6 g of KOH in methanol and diluting to 200 mL with methanol. This solution is prepared fresh weekly.

Preparation of Commercial Samples for Analysis

The sweet cream and salted butter together with the lard samples are those upon which our analysis method was established. The cheese samples (Swiss and Kraft Singles) also served to evaluate the applicability of the method.

Saponification of the different samples was required in order to isolate our compound of interest, cholesterol, from the other lipids present in the commercial samples. This saponification process was a slight modification of the procedure used by Fletouris and co-workers *(21)*. It was carried out as follows:

- About 0.8 g of each sample (butter, lard, cheese) was accurately weighed out into different sample tubes, to which 20.0 mL of 0.5 M methanolic KOH were added
- The tubes were tightly closed and immersed in a 75°C water bath for 45 minutes.
- After heating, the tubes were cooled with tap water, and then 2.0 mL of water followed by 10.0 mL of hexane were added.
- The resulting mixtures were first sonicated then vigorously shaken manually for a couple of minutes; upon standing, the different phases separated and one could take out the upper phase that contained cholesterol for dilution and analysis.

Solutions for the Reproducibility Studies

Prior to dilution, the upper phase withdrawn from the above procedure was gently heated at temperatures around 60-65°C for 10 minutes to evaporate the hexane after the extraction. Hence, out of 10.0 mL of hexane added for extraction only about 5.0 mL of solution was left after evaporation. Specifically, 4.0 mL of solution was left from evaporating the butter sample solution; 400 μL aliquots were then pipetted into 8 different 5-mL volumetric flasks and diluted to the mark with 2-propanol, ready for

analysis in the HPLC system. As for the lard, some white precipitate formed upon heating to evaporate the hexane, so after the evaporation process, the 5.2 mL of solution left was carefully centrifuged before any aliquot was withdrawn. Eight aliquots of 500 μL were then pipetted into separate 5.0 mL volumetric flasks and diluted to the mark with 2-propanol.

Solutions for the Recovery Study

The butter sample was used to evaluate the accuracy of our analysis. About 0.799 g of butter sample was saponified according to the steps mentioned earlier in this chapter. After hexane evaporation, 6.6 mL of solution was left. Nine aliquots of 500 μL each were pipetted into separate 5.0 mL volumetric flasks. The first 3 samples were diluted to the mark with 2-propanol, whereas 100 μL of standard stock cholesterol solution was added into the next 3 flasks, and similarly 200 μL of standard stock cholesterol solution was added into the last 3 flasks. These were also diluted to the mark with 2-propanol.

Preparation of Commercial Samples for Application Study

Swiss and Kraft Singles cheese samples were also included to evaluate the applicability of this method. About 0.793 g and 0.798 g of Swiss cheese and Kraft Singles cheese were saponified following the procedures mentioned in page 37. After evaporation of the hexane, 4.8 mL of the Swiss cheese sample solution was left; 500 μL aliquots were pipetted into 3 separate 5.0 mL volumetric flasks and diluted to the mark with 2-propanol, ready for the HPLC analysis. Similarly, 5.8 mL of the Kraft cheese sample was left after evaporation process, and 500 μL aliquots from this solution were transferred into 3 separate 5.0 mL volumetric flasks as for the Swiss cheese sample, ready for analysis.

Instrumentation

Cholesterol analysis in our research project is based on the popular technique of reversed-phase high performance liquid chromatography (RP-HPLC), a versatile method for separation and analysis of samples from diverse sources. The specific system being used here is a combination of Waters HPLC System and Perkin-Elmer chromatography

pump. A simple representation of the instrumental set-up is displayed in Figure 3.

The HPLC system comprises a reservoir for the mobile phase; our method makes use of two of such containers: one for the methanol and the other for the 2-propanol. A Perkin-Elmer Series 410 pump is used because the original Waters 501 reciprocating pump that is normally connected to the instrument broke down. The injector is a Model 7725 Rheodyne injector with a 20 μL sample loop. Our column is a Betasil 150 mm × 4.6 mm with reversed-phase C-18 packing. For detection, a Model 484 Tunable UV absorbance detector is used. A Model 745B data module records and processes the data.

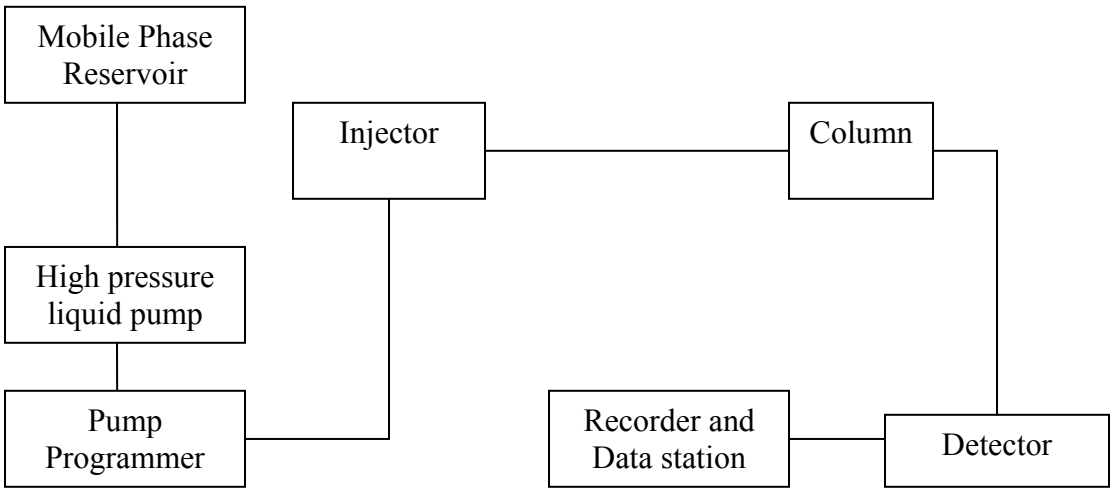


Figure 3. Schematic Diagram of the HPLC Instrumentation.

Experimental Conditions for HPLC Analysis

The analysis is carried out at a constant flow rate of 1.0 mL/ min all throughout, with an average pressure of about 2100 psi. Prior to injection of any sample into the system, the mobile phase is pumped through the column for at least 30 minutes in order to warm up the instrument and achieve equilibration of the column. The mobile phase is a mixture of methanol and 2-propanol in a ratio of 70:30 (methanol: 2-propanol). The pH of the mixture is between 8.0 and 8.5. It is useful to deaerate the mobile phase to avoid the presence of air bubbles. As two containers for each component of the mobile

phase are used, the pump programmer is set in such a way as to pump both solvents at the desired proportion. The different components in our samples are detected by a variable wavelength UV detector set at 212 nm, and the data station displays the recorded data in the form of chromatograms at a chart speed of 1.0 cm/min. This setting is changed to 0.5 cm/min later in the experiment.

Data Analysis

Every sample, from the standards to the commercial samples, was analyzed and injected in triplicate in the HPLC system. The resulting peak areas were saved and processed in Microsoft^{R} Office Excel 2003 software for Windows^{R} XP. Means of the peak areas for each sample were calculated. Standard deviations and relative standard deviations for them were all calculated using Excel. A plot of the calibration curves showing average peak areas as a function of cholesterol concentration was obtained from the standard solutions prepared. The calculated mean values from the various samples were then substituted into the regression line equations obtained in order to get the cholesterol content.

Results and Discussions

The results obtained from our analysis method are presented. Results from the reproducibility, recovery, and applicability studies are presented, and the quality of these results is discussed and evaluated by comparison with other studies mentioned earlier in Chapter 2. It is worth mentioning that this research project started with the use of the Waters HPLC equipment, but the Model 501 pump used then failed, while still investigating our HPLC conditions; that pump has been replaced by the Perkin-Elmer Series 410 pump mentioned in an earlier section of this chapter.

Optimization of the Wavelength of Detection

Cholesterol possesses a polar secondary hydroxyl head group at the C_3 position in the non-polar hydrocarbon chain. This chromophore makes it suitable for absorption of light, a molecular property that is used for its detection. In order to determine the best wavelength of detection, a UV-Vis spectrum of two standard cholesterol solutions at two

different concentrations was taken; the spectrum showed a maximum absorption at 208 nm. The standard solutions were then analyzed in the HPLC at those wavelengths, and also in the range 210 to 216 nm. A slightly higher absorption peak was observed for the wavelength 212 nm as compared with the other wavelengths. This wavelength was then chosen as the optimum one and used throughout the entire analysis. Two similar studies in the literature used the same range of wavelengths. One of them was a study by Indyk H. E. *(23)*, who made use of series detection by UV (210-214 nm) and fluorescent measurements. The other study is by W. Jeffrey Hurst and co-workers *(27)*, who used direct detection at 205 nm.

Optimization of the Mobile Phase

One of the objectives of this project was to develop a method that would be as economical and environmentally friendly as possible. This aspect was considered in the choice of the mobile phase components: methanol and 2-propanol. The most widely used mobile phases in reverse phase HPLC are various mixtures of water and methanol, which make them very attractive when considering their cost and environment concerns. Water is readily available at virtually no cost besides the initial cost to acquire the device producing it. A mixture of water with methanol would do little harm to the environment. Even though methanol is a listed pollutant, its use is still widely accepted because of its polarity as compared to other solvents. However, cholesterol is not soluble in water; so water was not used as one of the components of the mobile phase, and an organic solvent, 2-propanol, was used instead.

Two separate reservoirs were used for the storage of methanol and 2-propanol; this allowed for the possibility to change the composition or ratio of the mixture whenever needed. This control was particularly useful during the preliminary or exploratory studies. Various ratios of methanol to 2-propanol were tried; 60:40, 70:30, 80:20, 90:10, and even a 70:20:10 methanol: 2-propanol: water. The latter was tried to make the mobile phase a little aqueous so as to prevent the materials in our column from being washed away with a too dry mobile phase. That attempt did not work because the resulting mixture would just disturb the equilibrium between the analyte in the mobile phase and the analyte in the stationary phase, yielding unstable and inconsistent

chromatograms. The 60:40 mobile phase produced a good resolution of the analyte's peak with a retention time of around 5.84 minutes with an average back pressure of 2400 psi. The 70:30 mobile phase produced a satisfactory separation of the analyte with a retention time of 7.66 minutes and an average back pressure of 2100 psi. The last set of conditions was better than the precedent 60:40 conditions because the low pressure ensured stable conditions during the entire analysis. With more methanol in the mixture (80:20 and 90:10 mobile phase ratios), the retention time would increase as expected and the analysis time would be longer, which was not desirable. The 70:30 methanol: 2-propanol seemed to be the best composition for our method and was thus adopted.

Both studies mentioned earlier by H. E. Indyk *(23)* and by W. Jeffrey Hurst and co-workers *(27)* used either 100% methanol or (0.1 + 99.9, v/v) isopropyl alcohol-hexane. Their methods resulted in a very long analysis time (around 30 minutes) *(23)* and a lower efficiency in resolution *(27)* respectively, as compared to the present method: the analysis is done in less than 10 minutes and there are no peaks overlapping or interferences on cholesterol signal.

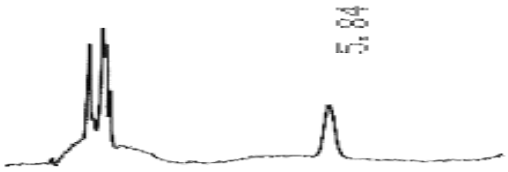
The retention time of cholesterol from the proposed method is observed to be between 7.2 and 7.7 minutes depending on the samples, on the fluctuations in the flow of the mobile phase, but also on how closely the injection buttons are pressed on the HPLC instrument: the injection on the data station has to be pressed at the same time as the sample is injected in the loop. The system was set to obtain each chromatogram in 9 minutes.

Linear Dynamic Range

The analyte of interest, the cholesterol molecule, was barely detectable at concentrations below 10 μg/mL. For this reason, all the other standard solutions had to be prepared directly from the stock standard (1.00 mg/mL) cholesterol solution (aliquots of 50, 75, 100, 200, 500, and 1000 μL all diluted to the mark in separate 10-mL volumetric flasks with 2-propanol).

Figure 4 shows the chromatograms obtained from the same standard cholesterol solution at different mobile phase ratios.

(A)



(B)

(C)

Figure 4. Chromatograms of the 20.0 µg/mL Cholesterol Standard Solution with Methanol: 2-Propanol 60:40 (A), 70:30 (B), and 90:10 (C). Note: The flow rate is the same throughout, 1.00 mL/min.

A linear dynamic range was established for cholesterol standard solutions of concentrations ranging from 10.0 to 100 µg/mL. A calibration curve within this range was obtained for all subsequent studies carried out such as reproducibility, recovery, and applications studies.

Figure 5 shows chromatograms of the cholesterol standard solutions at three different concentrations.

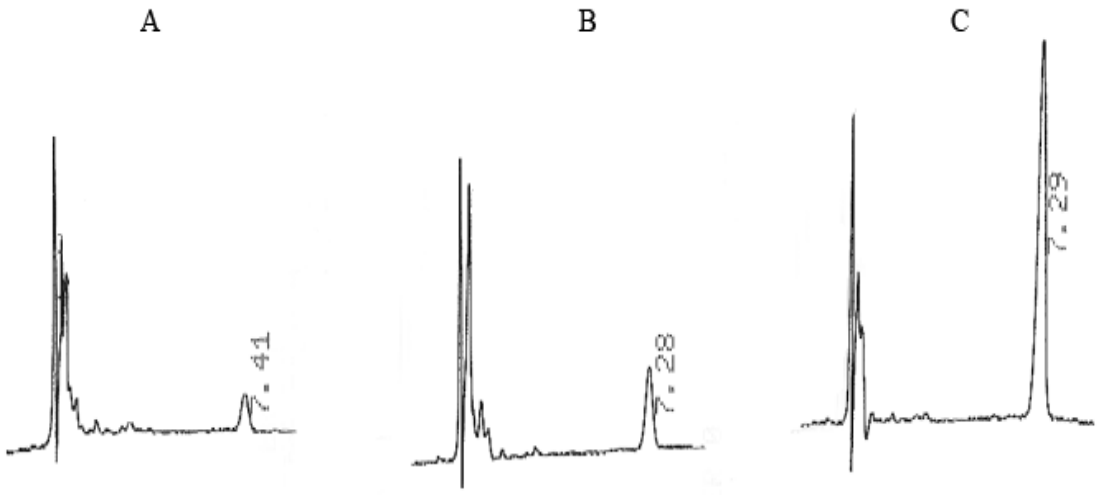


Figure 5. Chromatograms of Cholesterol Standard Solutions with Varying Concentrations.
Note: The mobile phase is methanol: 2-propanol 70:30 flowing at 1.00 mL/min. Concentrations increase from 10 µg/mL for A), 20 µg/mL for B), to 100 µg/mL for C).

Four different concentrations of standard solutions were used in this dynamic range study: 10, 20, 50, and 100 µg/mL. Each solution was injected into the HPLC system in triplicate; the average peak areas for each concentration were plotted against the concentration range. The regression line was observed to follow the equation Y = 4854.3X + 10705 with a correlation coefficient of 0.9959. The results are displayed in Table 4.

Table 4. Average Peak Areas of Cholesterol Standard Solutions for Reproducibility Study of the Butter Samples.
Note: The mobile phase was 70:30 methanol: 2-propanol, at a flow rate of 1.0 mL/min, and the detection wavelength set at 212 nm. Each reported area was the average of triplicate injections, and each relative standard deviation was obtained within each concentration of standard solution.

Cholesterol Conc. (μg/mL)	Average Peak Area	Relative Standard Deviation
10	49050	11.90 %
20	124875	20.90 %
50	244441	3.98 %
100	498227	0.52 %

The observed large values of the relative standard deviation for the first and second cholesterol standards solutions can be explained as resulting from a statistical analysis of four values (four peak areas) instead of three like the last two samples. Indeed, when the 10 and 20 μg/mL solutions were injected, they yielded peak areas with some inconsistencies in the areas of the peaks. So, an extra injection was carried out in order to minimize the effect of these irregularities on the entire analysis. As this experiment was the first to be done before any other on the same day, one could attribute these observed irregularities to the non-equilibrium still existing in the column, even after the instrument has been running for half an hour. The overall correlation coefficient value of 0.9959 did confirm that the obtained calibration curve was linear. In subsequent analysis, however, more time was allowed for the column to equilibrate before any analysis was started, and such large variations were avoided. The plot of the data obtained is shown in Figure 6.

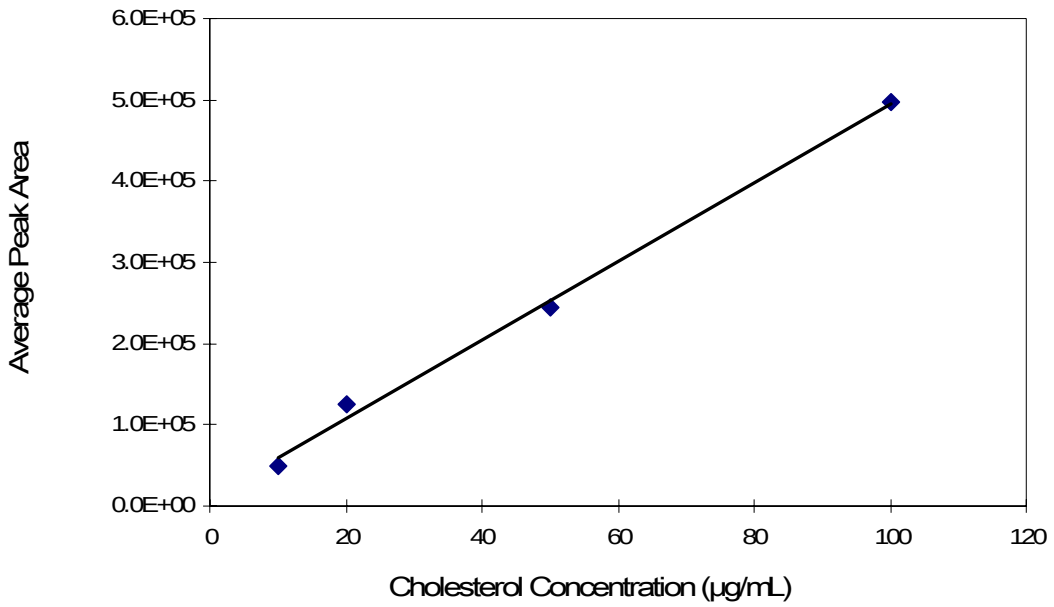


Figure 6. Calibration Curve Using 10, 20, 50, and 100 µg/mL Cholesterol Standards.
Note: The line follows the equation Y = 4854.3X + 10705 with a correlation coefficient value of 0.9959.

Reproducibility Studies

After working with standard solutions of cholesterol to establish the dynamic range of the analysis, the proposed method should be assessed by using real samples. The next step of our project would then be to check whether the method is at all reproducible. The butter and lard samples obtained from local stores in Johnson City, TN were thus chosen to carry out the reproducibility study.

As mentioned earlier, a calibration curve was obtained for every study, in order to determine by extrapolation the amount of cholesterol present in the samples. From the amount of cholesterol written on the label of each sample, it was possible to estimate the expected amount to be found. That estimate was just to ensure that the dynamic range studied would be wide enough to include the amounts of cholesterol present in the samples to be analyzed, or, in other words, to make sure that the amount of cholesterol in the samples studied would fall within the limits of our calibration curve, and that was found to be the case.

Reproducibility Study Using the Proposed Method on the Butter Sample

The butter samples were analyzed on the same day and right after the dynamic range study was done so that there was no need to obtain an extra calibration curve for the reproducibility study of butter. Eight butter samples were prepared according to the procedures outlined earlier in this chapter. Each sample was injected in triplicates for a total of 24 injections. The mean of peak areas from each sample was obtained; those means were plugged into the regression line equation of the calibration curve in order to get the corresponding cholesterol concentrations in μg/mL. Once these concentrations were found, further calculations were performed to get the actual amounts of cholesterol in each sample per gram of butter so as to match the units (mg/g) on the label of the butter package. These calculations were required in order to make a direct comparison between the values obtained from our method and the values reported by the Food companies on their products. The regression line equation used was $Y = 4854.3X + 10705$. And the calculations to get the mg/g of butter were performed as follows:

- The concentration of cholesterol in μg/mL was multiplied by 5.0 mL, which was the final dilution volume before injection. This operation would give the amount of cholesterol in μg.
- The amount obtained above is then divided by the volume pipetted into the 5.0-mL volumetric flask: in this case, 400 μL or 0.40 mL.
- The result from the above is multiplied by the volume of solution left after evaporation of hexane, 4.0 mL in this case.
- The resulting number is the quantity of cholesterol present in the initial amount of butter weighed, so it was divided by the mass of butter to obtain the desired cholesterol quantity per gram of butter.

Figure 7 shows the appearance of the butter samples chromatograms.

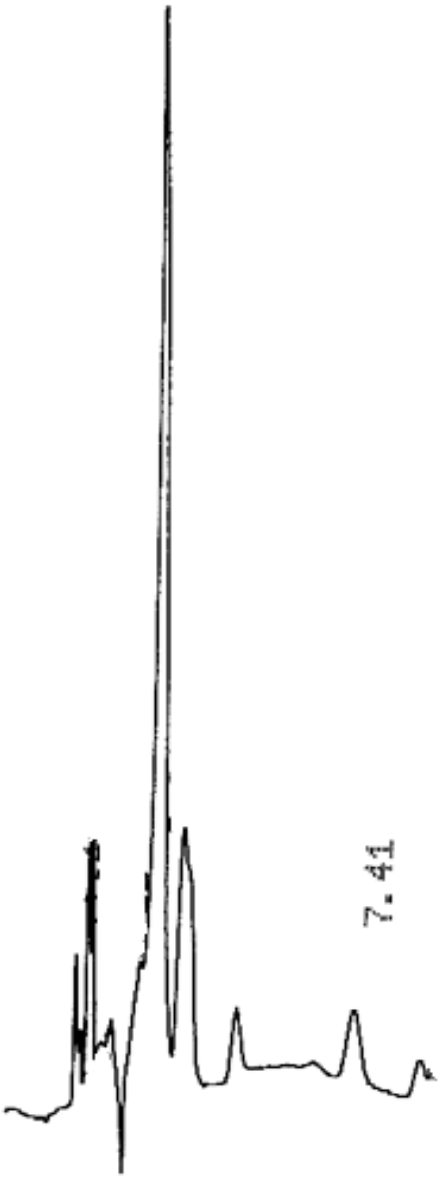


Figure 7. Chromatogram of a Sweet Cream and Salted Butter Sample. Note: The mobile phase was methanol: 2-propanol, flowing at 1.0 mL/min with a detection wavelength set at 212 nm. The analyte's peak (cholesterol) is centered at 7.41 minutes.

The results obtained are presented on Table 5. An evaluation of our method was then possible by comparing the average amount of cholesterol found from our data analysis to the amount reported by the manufacturing company on the label of their product.

Table 5. Results of the Reproducibility Study of the Sweet Cream and Salted Butter Samples.
Note: The mobile phase was 70:30 methanol: 2-propanol, at the flow rate of 1.0 mL/min. The concentrations of cholesterol are expressed in mg/g of butter.

Sample	Cholesterol Concentration (mg/g of butter)
1	1.07
2	1.2
3	1.1
4	1.09
5	1.09
6	1.04
7	1.11
8	1.11
Mean	1.10
Relative Standard Deviation	4.22 %
Amount of Cholesterol Reported on Label	2.14

The label on the butter package reported 30 mg of cholesterol per serving, with one serving being equal to 14 grams of butter; this makes an amount of 30 mg/ 14 g or 2.14 mg of cholesterol per gram of butter. Thus, the percent difference between the determined value and the reported value could be calculated to be about $[(2.14 – 1.1)/2.14] \times 100 = 48.6\%$. The latter number suggested either an unsatisfactory level of efficiency in the proposed method or some inaccuracy in the reported amount of cholesterol found on the label. It should be noted that almost all commercial butter from

different brands and companies have identical labeled amounts of cholesterol on their packages.

Reproducibility Study Using the Proposed Method on the Lard Sample

The lard samples were analyzed on a different day from the butter. A new calibration curve with good linearity (correlation coefficient of 0.9986) had to be established for this set of experiments to be meaningful. This was achieved using the following cholesterol standard solutions: 5.0, 7.5, 10.0, and 20.0 μg/mL. Again, each of the standards was injected in triplicates; the means of the peak areas were plotted against the concentration, the regression line was obtained together with the correlation coefficient to be used in getting the cholesterol concentration in the lard. All experimental procedures and data analysis were just the same as those used with the butter sample. The data for the calibration curve are presented in Table 6. It is notable that the overall relative standard deviations of the triplicate injections are now lower than the ones shown in table 5.

Table 6. Average Peak Areas of Cholesterol Standard Solutions for the Reproducibility Study of the Lard Samples.
Note: The mobile phase was 70:30 methanol: 2-propanol, at a flow rate of 1.0 mL/min, and the detection wavelength set at 212 nm. Each reported area was the average of triplicate injections, and each relative standard deviation was obtained within each concentration of standard solution.

Cholesterol Conc. (μg/mL)	Average Peak Area	Relative Standard Deviation
5.0	24709	9.45 %
7.5	34318	2.65 %
10.0	48906	1.07 %
20.0	95479	1.74 %

The regression line was found to be Y = 4767.8X + 195.6, with a correlation coefficient of 0.9986 showing the linearity of the calibration. This line is plotted in Figure 8.

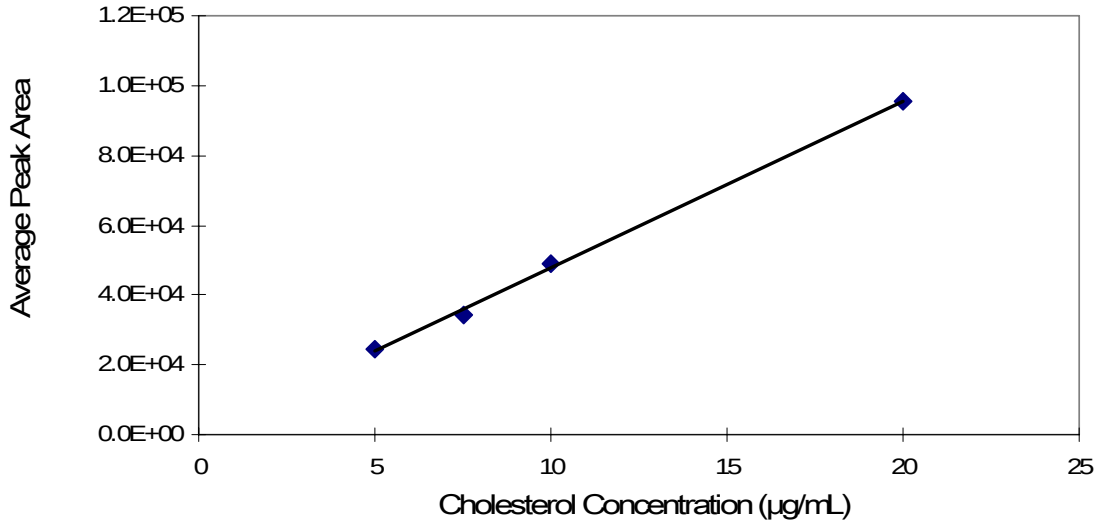


Figure 8. Calibration Curve using 5.0, 7.5, 10.0, and 20.0 µg/mL Cholesterol Standards.
Note: The mobile phase was 70:30 methanol: 2-propanol, at a flow rate of 1.0 mL/min, and the detection wavelength set at 212 nm. The regression line equation was Y = 4767.8X + 195.6, with a correlation coefficient of 0.9986.

From this line equation, the cholesterol concentrations in µg/mL were found in the eight different lard samples injected in triplicates. The calculations as outlined previously were also performed for the lard to obtain the actual amount of cholesterol in mg/g of lard, except that the volumes here were slightly different from those used with the butter. Indeed, after evaporation of hexane here, 5.2 mL of solution were left. Three aliquots of 500 µL each were the diluted to 5.0 mL with 2-propanol. The data obtained are presented in Table 7.

The low values of the relative standard deviations show that the method has good precision as observed values agree with each other. The present procedure determined an average amount of 0.45 mg of cholesterol per gram of lard in the sample analyzed. The amount reported on the label of the lard package is 10 mg per serving size, with one serving being equal to 13 g of lard; so the reported value is about 10 mg/13g of lard or

0.77 mg of cholesterol per gram of lard. Comparing our value with the label's reveals a percent difference of $[(0.77 – 0.45)/0.77] \times 100 = 41.56$ %.

Table 7. Results of the Reproducibility Study of the Lard Samples.
Note: The mobile phase was 70:30 methanol: 2-propanol, at the flow rate of 1.0 mL/min. The concentrations of cholesterol are expressed in mg/g of lard.

Sample	Cholesterol Concentration (mg/g of lard)
1	0.48
2	0.45
3	0.44
4	0.45
5	0.46
6	0.45
7	0.44
8	0.45
Mean	0.45
Relative Standard Deviation	2.71 %
Amount of Cholesterol Reported on Label	0.77

A chromatogram of the lard sample peak at retention time 7.42 min is shown in Figure 9.

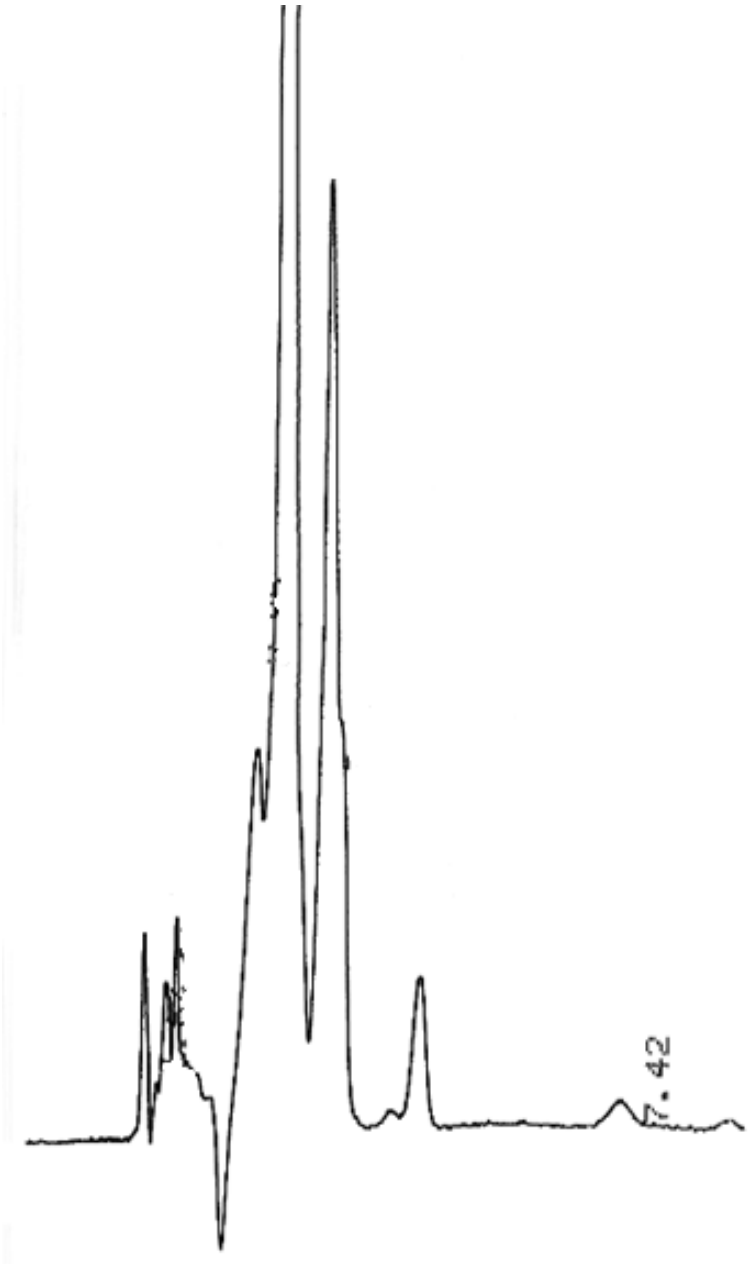


Figure 9. Chromatogram of a Lard Sample.
Note: The mobile phase is 70:30 methanol: 2-propanol, flowing at a rate of 1.0 mL/min, with the detection wavelength set at 212 nm. The peak of interest is centered at 7.42 minutes.

Recovery Study

Another step forward in the evaluation of our method of analysis was the assessment of its accuracy by carrying out the recovery study. This study was done on a different day, and a new calibration curve with correlation coefficient of 0.9998 was required in order to determine the concentration of cholesterol found. The following concentrations of standard solutions were used for the calibration curve: 7.5, 10.0, 20.0, 50.0, and 80.0 μg/mL. The data obtained are summarized in Table 8, and the calibration curve is plotted in Figure 10.

Table 8. Average Peak Areas of Standard Cholesterol Solutions for the Recovery Study.
Note: The mobile phase was 70:30 methanol: 2-propanol, at a flow rate of 1.0 mL/min, and the detection wavelength set at 212 nm. Each reported area was the average of triplicate injections, and each relative standard deviation was obtained within each concentration of standard solution.

Cholesterol Conc. (μg/mL)	Average Area Peaks	Relative Standard Deviation
7.5	33454	3.43 %
10.0	46242	7.47 %
20.0	98089	2.50 %
50.0	237462	1.15 %
80.0	386629	1.50 %

Consistency and precision in the measurements were still observed, as statistically proven by the low relative standard deviation numbers.

The butter was used for this study again. Nine aliquots of butter sample were prepared as described earlier. The first three aliquots did not have any added cholesterol from the standard stock solution, whereas the next three had 100 μL of the 1.00 mg/mL cholesterol standard added, and the last three had 200 μL of the same standard added. Each sample was injected in triplicates. The addition of 100 μL from the 1.00 mg/mL

stock cholesterol standard was synonymous to adding 100 µg, before the final dilution to 5.0 mL with 2-propanol. So 100 µg and 200 µg were respectively added.

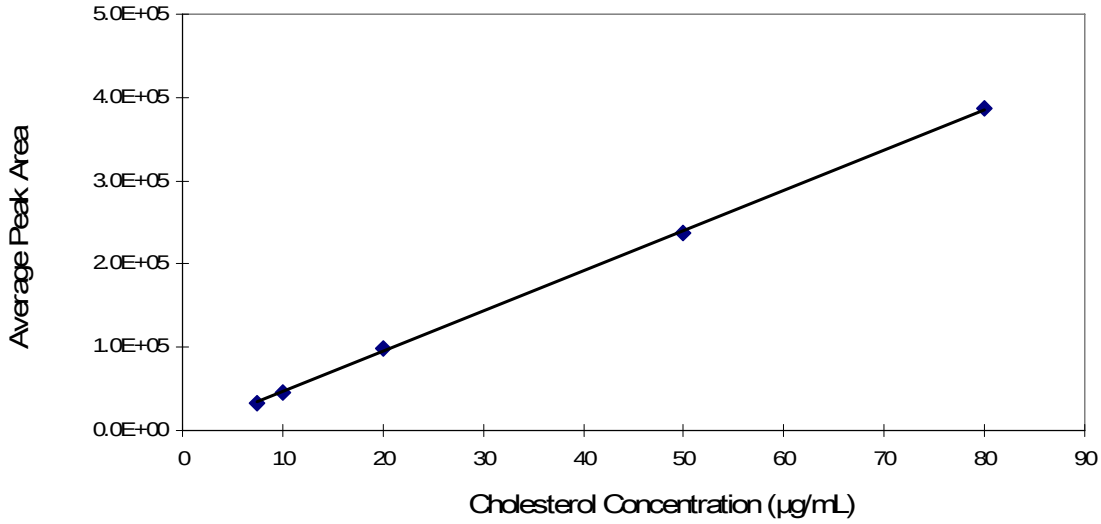


Figure 10. Calibration Curve using 7.5, 10.0, 20.0, 50.0 and 80.0 µg/mL Cholesterol Standards.
Note: The mobile phase was 70:30 methanol: 2-propanol, at a flow rate of 1.0 mL/min, and the detection wavelength set at 212 nm. The regression line equation was Y = 4841.5X - 1816, with a correlation coefficient of 0.9998.

The amounts of cholesterol added in the butter samples were calculated as follows: 100 µL × 1.0 mg/mL = 100 µg. This was then diluted to 5.0 mL with 2-propanol in volumetric flasks ready for the HPLC analysis. After this dilution, the added concentration of cholesterol would be 100 µg / 5.0 mL = 20.0 µg/mL. Likewise, the added concentration of cholesterol in the second set of butter samples would be twice as much, that is 40.0 µg/mL. Figure 11 shows the chromatograms from the butter sample with 100 µL standard added and the one with 200 µL standard added.

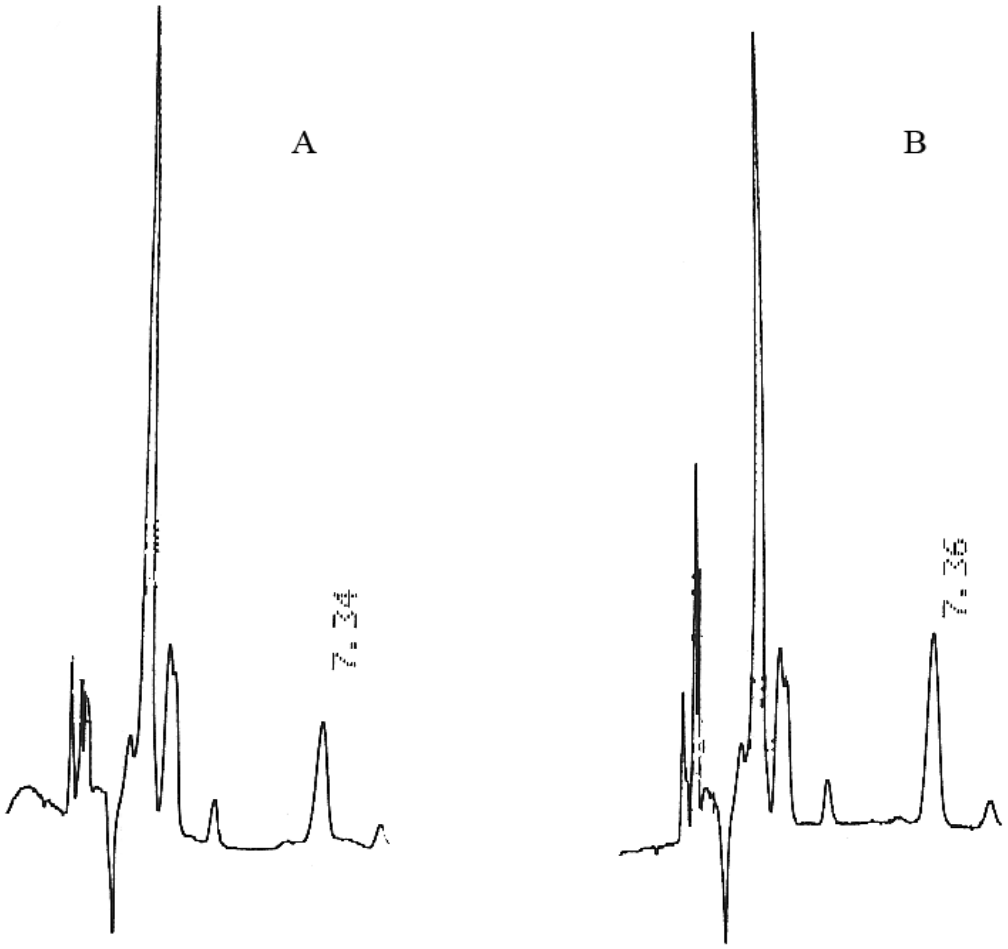


Figure 11. Chromatograms of Sweet Cream and Salted Butter Samples with Added Cholesterol Standard Solutions.
A) Sample + 100 µL standard added. B) Sample + 200 µL standard added.
Note: The mobile phase is 70:30 methanol: 2-propanol, flowing at a rate of 1.0 mL/min, with the detection wavelength set at 212 nm. The peaks of interest are centered at 7.34 and 7.36 minutes respectively.

The data obtained for this study are presented in Table 9. The average peak areas were obtained within each concentration, and the relative standard deviations were also calculated. The percent recovery is given by the following equation:

% Recovery = [Mean peak area of (analyte + standard added) – Mean peak area of Analyte] / Mean peak area of standard added to the analyte [9]

Table 9. Recovery Study Results for the Analysis of Butter Samples.
Note: The mobile phase was 70:30 methanol: 2-propanol, at a flow rate of 1.0 mL/min, and the detection wavelength set at 212 nm. The average retention time of the analyte's peak was 7.35 minutes.

Samples	µg of Standard stock cholesterol solution added	% Recovery (RSD %)
1	100	102.1 (1.05 %)
2	200	111.0 (0.53 %)

The percent recovery yielded values that are acceptable, and the conclusion is that the proposed method is reliably accurate. Indeed, ideal values for the percent recovery should be around 100 %, meaning that the matrix in which the analyte is present has no or negligible effect on its absorptive properties, thus allowing the analyte to be effectively determined without much interference. Moreover, the low relative standard deviation within each sample suggests a good deal of consistency and precision of the method. Having enough confidence in the accuracy of this method, the next step in its establishment is the application study.

Application of the Proposed HPLC Method to Other Commercial Samples

At this point, the precision, the consistency, and the accuracy of the proposed method have been investigated and all proved to be acceptable based on relative standard deviations below 5% and a mean percent recovery of 106.5%. The objective of the next set of experiments is to establish the possibility of applying this same method to other types of samples and to get reliable results as well. Two different types of cheese are chosen for this purpose: Swiss cheese and Kraft Singles cheese.

Swiss and Kraft Singles cheeses were prepared as mentioned before. Three samples of each type of cheese were ready for the analysis, and triplicate injections were made. Another calibration curve was required here, as this experiment was done separate from all the others. Standard cholesterol solutions with concentrations 5.0, 7.5, 10.0, and 20.0 µg/mL were used to establish the linear dynamic range. Each one was injected in triplicates. A plot of the data obtained is shown in Figure 12. The regression line was found to follow the equation: $Y = 4782.7X - 893.97$, and displayed a correlation coefficient of 0.9900.

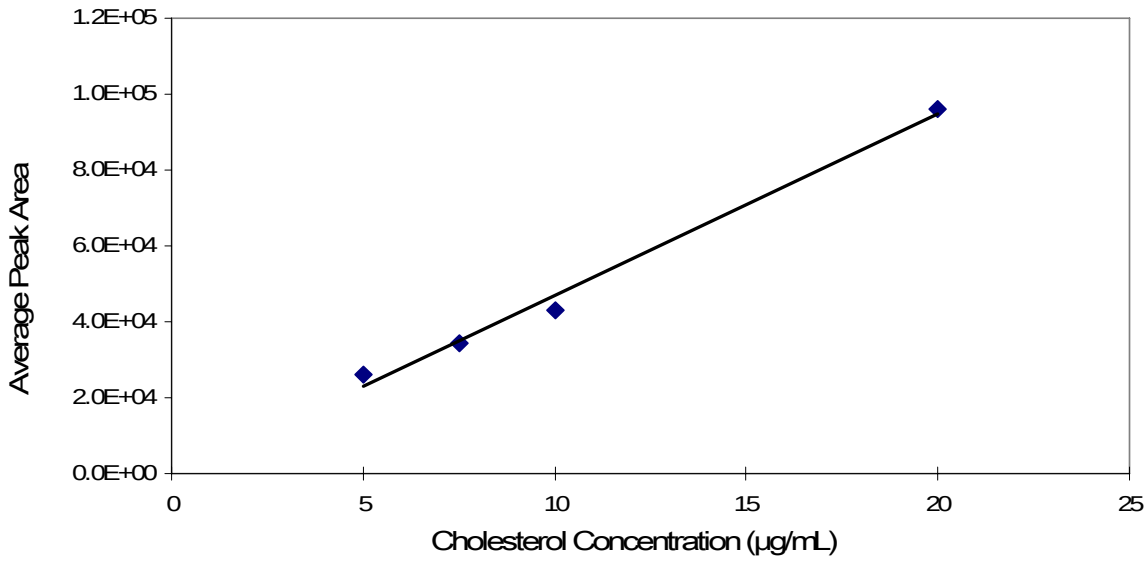


Figure 12. Calibration Curve Using 5.0, 7.5, 10.0, and 20.0 µg/mL Cholesterol Standards.
Note: The mobile phase was 70:30 methanol: 2-propanol, at a flow rate of 1.0 mL/min, and the detection wavelength set at 212 nm. The regression line equation was Y = 4782.7X – 893.97, with a correlation coefficient of 0.9900.

The data obtained from the chromatograms (average peak areas) were used to determine the cholesterol concentrations in µg/mL with the help of the regression line equation; these concentrations were then converted back to the amount of cholesterol present in each sample per serving size, conversions achieved by the calculations steps outlined as done for the butter and the lard samples. All three Swiss Cheese samples were analyzed (with each one being injected in triplicate); but only 2 of the 3 Kraft Singles Cheese were analyzed because the HPLC was running out of paper and mobile phase. However, the data were considered useful. The chromatograms obtained from the Swiss and the Kraft Singles Cheese are shown respectively in Figure 13.

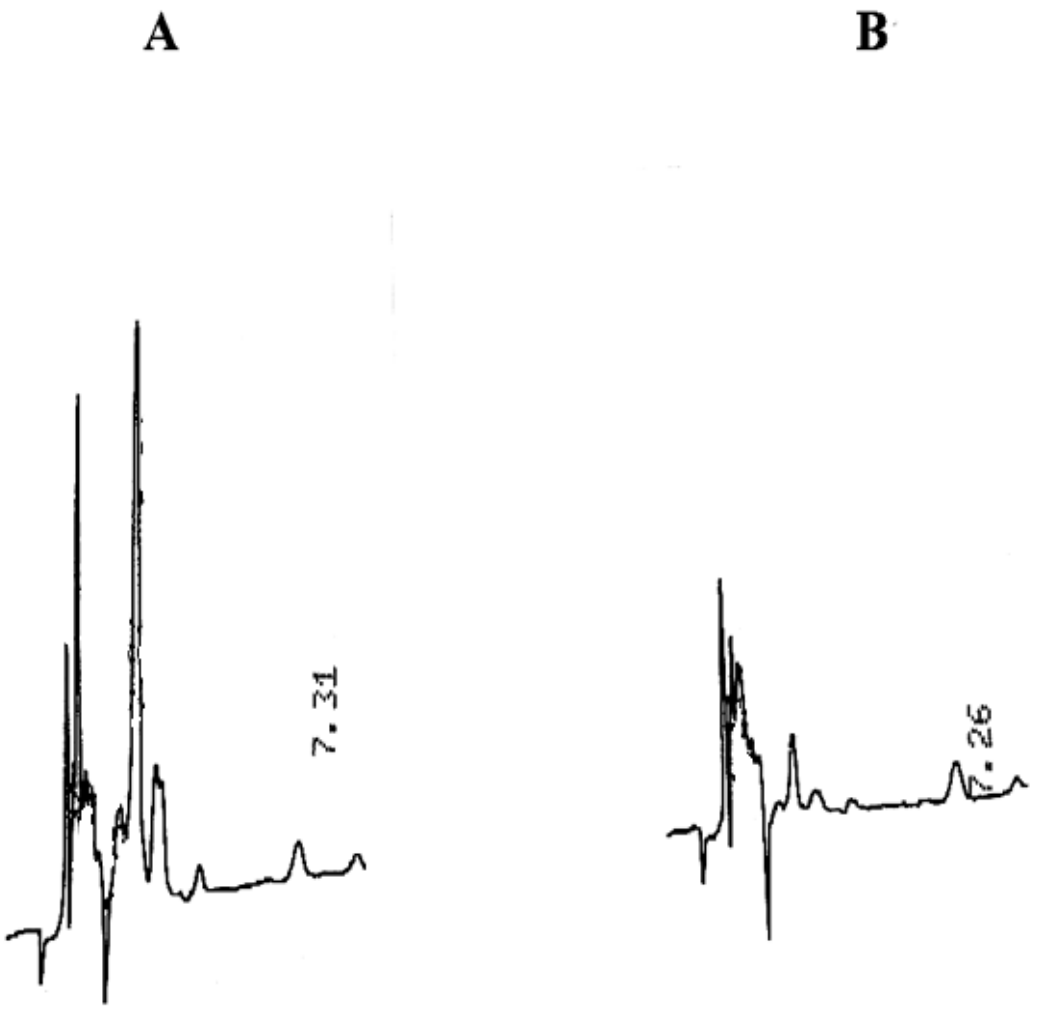


Figure 13. Chromatograms of Swiss Cheese and Kraft Singles Cheese Samples.
A) Swiss Cheese. B) Kraft Singles Cheese.
Note: The mobile phase is 70:30 methanol: 2-propanol, flowing at a rate of 1.0 mL/min, with the detection wavelength set at 212 nm. The peaks of interest are centered at 7.31 and 7.26 minutes for Swiss and Kraft cheeses respectively.

Means and relative standard deviations were calculated. The resulting data are presented in Table 10. The overall average of cholesterol concentration was determined, and a direct comparison with the amounts of cholesterol reported by the food manufacturers is also possible.

Table 10. Amount of Cholesterol Determined in Cheese Samples.
Note: The mobile phase was 70:30 methanol: 2-propanol, at a flow rate of 1.0 mL/min, and the detection wavelength set at 212 nm. The retention time of the analyte peaks were 7.31 and 7.26 minutes. Cholesterol amounts are expressed in mg/g of cheese.

Sample	Amount of Cholesterol Determined (mg/g) (R.S.D)	Amount of Cholesterol Reported on the Label (mg/g)
Swiss Cheese	0.59 (4.8%)	0.92
Kraft Singles	0.61 (3.68%)	0.95

As one can appreciate, the low relative standard deviations obtained for the samples above show how good, or at least acceptable, precision and consistency in the proposed method of analysis are. It is also noticeable that the amounts of cholesterol reported in both types of cheese are just as close together as are the amounts determined by our method. However, there is a difference of about 36% between the reported amounts and those determined. This observation brings back the question on the efficiency of this method in determining cholesterol relative to the accuracy of the amounts reported on the labels by the manufacturers. As mentioned before, the labels for most manufacturers on their dairy products give almost identical values.

One way to investigate whether the present method is indeed giving reliable results is to carry out an independent established method for determination of cholesterol and compare the results obtained from it with the results of the proposed HPLC method. The Liebermann-Burchard reaction is one such method that has proven reliable and precise since the nineteenth century *(15)*.

Modified Liebermann-Burchard Method

To determine the effectiveness of the proposed HPLC procedure, the method using modified Liebermann-Burchard (L-B) reagent was performed. The L-B reaction was the color reaction used to evaluate the total cholesterol contained in each butter sample. It is a complex reagent in 22:20:3 acetic anhydride, glacial acetic acid, and

concentrated sulfuric acid medium. Variables such as the concentration of the reagents, the duration of the reaction, its temperature, the wavelength at which the color is measured, and the form in which the cholesterol is present either (esterified or free cholesterol) affect the reaction and must, therefore, be controlled while the reaction is taking place .

The standard cholesterol solutions for constructing the calibration curve were prepared from cholesterol working solution (1.00 mg/mL) made in methylene chloride. The standard solutions were prepared with 50 µL, 75 µL, 100 µL, 150 µL, and 200 µL from the standard cholesterol pipetted into five respective 10-mL volumetric flasks. In each reaction vial sufficient amount of methylene chloride was added to make the total volume to be 2.00 mL. Five milliliters of L-B reagent were then added, and the mixture was incubated for 45 minutes in the water bath at 37 – 40 ^{0}C for the blue-green color production. As a result, each vial contained a total volume of 7.0 mL, making the concentrations 7.14, 10.71, 14.28, 21.43, 28.57 µg/mL respectively. Absorbances were measured using the spectrophotometer at 620 nm and the data obtained are tabulated in Table 11 on the next page. The results showed a linear calibration curve following the equation Y = 0.0056X -0.0078 with a correlation coefficient of 0.9886. The butter samples were prepared the same way as they were for the HPLC analysis (weighing, saponification, extraction of cholesterol with hexane, then evaporation of hexane), in the procedures outlined in page 46. Approximately 0.804 g of butter was weighed. After evaporation of hexane, 6.5 mL of solution was left, from which 2 aliquots of 500 µL each were pipetted into separate vials, diluted to 2.00 mL with methylene chloride, before the addition of 5.00 mL of L-B reagent followed by 45 minutes incubation at 37-40 ^{0}C. The absorbance was read in triplicates for each sample, and the relative standard deviation was calculated. The amount of cholesterol found is given in Table 11 on the next page, with the standard deviation of the butter samples written in parentheses. Figure 14 shows the calibration curve used in determining cholesterol concentration in the butter samples.

Table 11. Cholesterol analysis using the Modified Liebermann-Burchard Reagent.

Sample	Cholesterol Concentration (µg/mL)	Absorbance (AU)
Standard 1	7.14	0.033
Standard 2	10.71	0.051
Standard 3	14.28	0.070
Standard 4	21.43	0.122
Standard 5	28.57	0.149
Butter sample 1	11.57 (0.46 % RSD)	0.057
Butter sample 2	11.21 (0.24 % RSD)	0.055

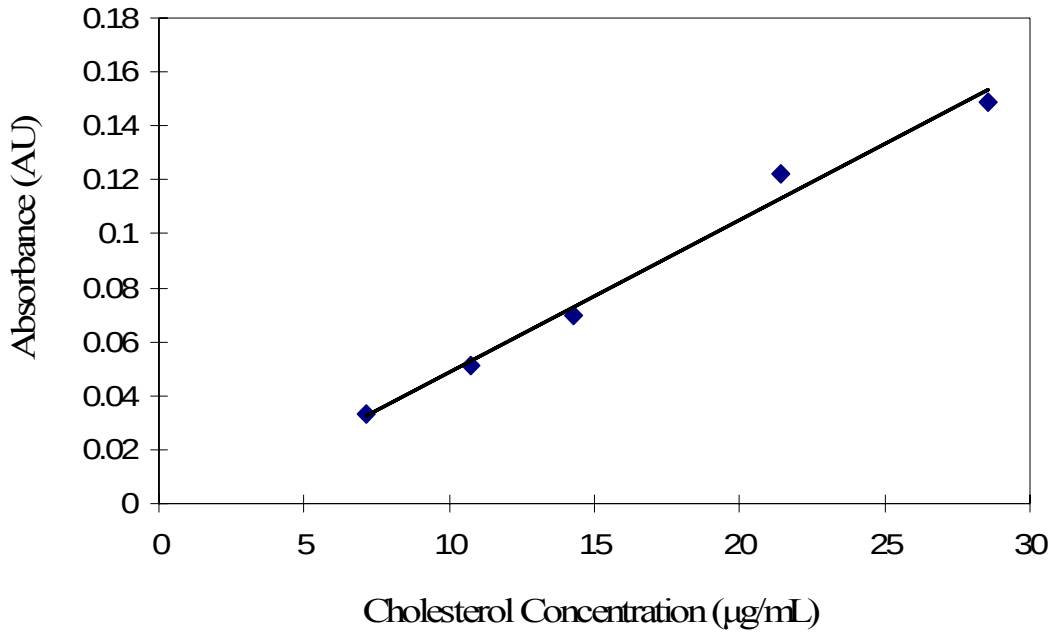


Figure 14. Plot of Absorbance (AU) vs. Cholesterol Concentration (µg/mL) for the modified Liebermann-Burchard method.
Note: The regression line follows the equation Y = 0.0057X – 0.0078 with a correlation coefficient of 0.9886.

The modified Liebermann-Burchard reagent employed in this experiment has been extensively used over the years as a reference method for the determination of cholesterol in serum. Cholesterol amount in μg/mL was found from the calibration curve shown in the previous page. This concentration was then converted into mg/g of butter using the calculations steps outlined in page 46. The obtained results gave an average cholesterol amount of 1.29 mg/g of butter. In light of the present numbers, it could be concluded that while the proposed method needed to be refined in order to avoid any loss of cholesterol in the sample preparation, it provided results that were reliable enough for the analysis to be valid. Moreover, the HPLC method and the L-B method appeared to give results closer in agreement with each other better than with the labeled amount.

The local stores from which the samples were bought did not have any detail concerning the analytical method used to get the amount on their labels because these are given by their suppliers and are based on the general recognition by the United States Department of Agriculture (USDA) that butter contains 31 mg of cholesterol per serving *(36)*. Our butter sample displayed 30 mg of cholesterol for a 14 g serving size. The above mentioned generalization of labels should account for the observed discrepancy between the amount of cholesterol detected with the proposed method and the amount shown on the labels.

CHAPTER 5

CONCLUSIONS

The proposed HPLC method for determination of cholesterol in food items has been thoroughly investigated and evaluated. This chapter gives a brief account of the various steps involved in the process, with a summary and significance of the results obtained. Some ideas about how to improve the present method are also suggested for future investigations.

First, a set of optimal conditions for the HPLC analysis was required. The mobile phase was a combination of methanol and 2-propanol in the ratio 70 to 30, flowing at the constant rate of 1.00 mL/min throughout. This ratio allowed the completion of a sample analysis in nine minutes, relatively shorter analysis time as compared to other methods found in the literature. The cholesterol molecule had an average retention time of 7.4 minutes throughout the entire analysis, with very slight variations depending on the samples handled. The pressure also remained constant with an average of 2100 psi for every analysis. The detection wavelength was set at 212 nm, as that particular wavelength produced the highest absorbance signals, allowing efficient detection of the analyte even at the lower concentration ends. The choice of this set of conditions essentially resulted from a couple of desired goals in our method: the stability of the measurements when working at 2100 psi as compared to 2400 psi as discussed in previous chapter (page 41), and a short time of analysis. The time of analysis was not too fast and allowed good resolution of peaks; it was not too slow either, and, as a result, a stable and fast set of HPLC conditions was established for the analysis. Concerning the environmental considerations, methanol is commonly used in HPLC methods of analysis because it does little harm to the environment. Methanol and 2-propanol are also reasonably affordable.

Second, a procedure for the preparation of samples was investigated. At the very early stages of this project, enzymatic reactions were carried out in the various samples to hydrolyze the cholesteryl esters present to free cholesterol. This method of preparation of samples was soon abandoned as the cholesteryl esterase, the enzyme used for the hydrolysis reaction, introduced interferences and did not increase the amount of free cholesterol detected. The hydrolysis method by saponification of the various samples

was then tried and proved to be the better alternative. A totally innovative method for preparing the samples was not achieved in this work; instead, a slight modification of the sample preparation procedure from the work by D. J. Fletouris and co-workers *(21)* was used. The modification consisted of altering the amounts of substances used for the purpose of our analysis: larger samples size, larger volumes of reagents used, and longer saponification process. However, this method remained as simple as the original one, involving not more than 3 main steps: the weighing of samples and saponification, the hexane evaporation process, and the HPLC analysis (as opposed to gas chromatography analysis used in the reference mentioned above). At this point, we had established a simple and rapid set of conditions for the analysis by the proposed method.

Next, the linearity of the study was evaluated. The method showed a fairly wide dynamic range from 5.0 to 100.0 μg/mL of the standard cholesterol solution, consistent range in which the samples could be analyzed with the certainty to reliably account for the amount of cholesterol present. All subsequent calibration curves were established within that range. The reproducibility studies would then tell us how satisfactory the method was. The butter and lard samples were used for these reproducibility studies. They were satisfactory in terms of consistency and precision: 4.22% and 2.71% relative standard deviations were found for the butter and lard respectively, and mean cholesterol amounts of 1.10 mg/g of butter and 0.45 mg/g of lard. However, these amounts were less than the amounts reported on the labels by the respective manufacturing companies.

The accuracy of the method was evaluated through the recovery study. This study was done in order to investigate the effect of the medium (mostly hexane) in which the analyte was found on its separation and absorptive properties. No major or interfering effect was found as confirmed by the percent recovery of 102.1% and 111.02% respectively, for both samples with 100 μL and 200 μL of standard added. The low relative standard deviations found within each sample type (samples with 100 μL of added standard and samples with 200 μL of standard added) also further confirmed the consistency and precision of this study. The accuracy was then deemed reliable with respect to the figures obtained.

The proposed method would not be useful if it could not be applied to samples other than those used to establish it. So the last step in this project was to apply this

method in determination of cholesterol contents of different samples. Two different types of cheese were chosen for this purpose: Swiss and Kraft Singles cheeses. A good deal of precision was achieved within each sample type, with relative standard deviations of 4.8% and 3.68% for the Swiss and Kraft Singles cheese respectively. The amounts of cholesterol determined were 0.59 mg/g of Swiss cheese and 0.61 mg/g of Kraft Singles cheese. Once again, there was a discrepancy between the amounts of cholesterol determined by the present method and the amounts reported on the labels of those samples by their respective manufacturers.

The discrepancy observed between the amounts of cholesterol found in the various samples by this method of analysis and the amounts reported on the labels of each sample could be explained as follows: some loss of cholesterol might have occurred in the procedures used in preparing the samples. Indeed, because of the small sizes of the samples used, any slight loss of samples during their measurements and transfers into reacting flasks would make an appreciable percentage difference in the final amount of cholesterol detected. Moreover, the saponification process might not have reached completion because of slight fluctuations in the temperature of the water bath. However, these errors are inherent to every analysis and should contribute only partly to the unexpected discrepancy. To really resolve this discrepancy issue, one needs to use greater number of samples. The determinations of cholesterol in this project were done on portions of the butter or lard in the same package product, or on different parts of the same piece of cheese, rather than using different packages of samples. Another method is to combine a large amount of sample and make a bigger volume of the dissolved sample to have an "averaging" effect. Then smaller aliquots of it are used for analysis. One could also find another saponification procedure and compare the results from it with the current ones.

A modified Liebermann-Burchard method, which is a reference method in the determination of cholesterol, was used to compare the amount of cholesterol found with that obtained via the HPLC method. The agreement of these values with a 16% difference showed that the proposed method was indeed reliable.

The present method presents the advantages of simplicity, because of the small number of steps involved and rapidity as the time for the HPLC analysis is less than 9

minutes once the samples are ready, as compared to 20-30 minutes with the method by H. E. Indyk *(23)* for example. The use of a mixture of methanol: 2-propanol is another advantage in the sense that those two solvents are preferred to some others like tetrahydrofuran or acetonitrile which are also less polar, thus not suitable for our purpose.

For future investigations, and because of the simplicity of this method as compared to the International Dairy Federation method *(21)* for example, the steps to work on are obvious to identify: first, the procedures involved in weighing the samples could be improved to avoid any sample loss. Second, the saponification process conditions could be made more vigorous and allowed to go beyond the 45 minutes that the present method used. It would also be interesting to investigate the effect of the pH of the mobile phase in the analysis. Cholesterol is not easily ionizable, so the effect of the pH should not be much; but achieving a control of the pH of the mobile phase might help to obtain an even faster analysis than what is achieved now. Any future work on this project would essentially be in the refining of the method.

In light of the results and discussions mentioned in Chapter 4, the proposed reversed-phase HPLC method of determination of cholesterol in foods was established. This method was proven rapid enough for a reliable HPLC analysis as well as being reproducible, precise, and accurate as attested by the satisfactory figures of merits mentioned above (pages 61-62). Its possible application to other sample types might be generalized to quantitative determination of cholesterol in diverse food samples. However, the recovery of the total cholesterol present in the samples must be efficient enough (which was not the case in this work) to ensure that the amount present is indeed completely accounted for.

REFERENCES

1. "*Learn and Live.*" American Heart Association, Inc. 2006 http://www.americanheart.org/presenter.jhtml?identifier=4488. 11/28/2006.
2. Montgomery, R.; Dryer, R.L.; Conway, T.W.; Spector, A.A. *Biochemistry, a case-oriented approach*, 3rd Ed.; The C.V. Mosby Company, 1980; pp 439-463.
3. Christie C.C.; *Sterols and sterol esters: Structure, Occurrence, and Biochemistry Analysis.* Lipid Analysis, 3rd Ed.; 2003, pp 1-7.
4. Nelson, D.L.; Cox, M.M. *Lehninger Principle of biochemistry* 3rd Ed.; Worth Publishers, 2000; pp 799-814.
5. Levy, I.R. "Cholesterol, Lipoproteins, Apoproteins, and Heart Disease: Present Status and Future Prospects." *Clin. Chem.* 1981, *27*, 653-662.
6. *"High Blood Cholesterol; What you need to know"* U.S. Department of Health and Human Services, National Institutes of Health, National Heart, Lung, and Blood Institute. May 2001. Publication No. 01-3290. http://www.nhlbi.nih.gov/health/public/heart/chol/wyntk.htm. 11/28/2006.
7. Armstrong, F.B. *Biochemistry*, 3rd Ed. Oxford University Press, Inc. 1989; pp 370-383.
8. Stryer, L. *Biochemistry.* W. H. Freeman and Company, San Francisco: 1975; pp 487-493.
9. Montgomery, R.; Conway, T.W.; Spector A.A.; Chappell D. *Biochemistry, a case-oriented approach*, 6th Ed. The Mosby Year-Book, Inc. 1996; pp 332-349.
10. Pearson, T.A. "The Epidemiologic basis for Population-wide Cholesterol Reduction in the Primary Prevention of Coronary Artery Disease" *Am. J. Cardiol*. 2004, *94* (suppl.):4F-8F.
11. Stein, E.A., "Managing Dyslipidemia in the High-Risk Patient" *Am. J. Cardiol*. 2002, *89* (suppl.):50C-57C.
12. Chamsi-Pasha, H. "Cholesterol: How low should we go?" *Saudi Med J*. 2005, vol. *26* (1):11-18.
13. Zak, B., "Cholesterol Methodologies: A Review" *Clin. Chem*. 1977, *23/7*, 1201-1214.

14. Burke, R.W.; Diamondstone, B.I.; Velapoldi, R.A.; Menis, O. “Mechanisms of the Liebermann-Burchard and Zak Color Reactions for Cholesterol” *Clin. Chem*. 1974, *20/7*, 794-801.
15. Kim, E.; Goldberg, M. “Serum Cholesterol Assay Using a Stable Liebermann-Burchard Reagent.” *Clin. Chem*. 1969, *15*, 1171-1179.
16. Schoenheimer, R.; Sperry, W.M. “A Micromethod for the determination of free and combined cholesterol.” *Journal of Biological Chemistry*. 1934, pp. 745-760.
17. Allain, C.C.; Poon, L.S.; Chan, C.S.G.; Richmond, W.; Fu, P.C. “Enzymatic Determination of Total Serum Cholesterol.” *Clin. Chem.* 1974, *20/4*, 470-475.
18. Jamieson, A. “A Method for the Rapid Determination of Serum Total Cholesterol using a Modification of the Pearson Reaction” *Clin. Chim. Acta*, 1964, *10*, 530-535.
19. Schaffer, R.; Sniegoski, L.T.; Welch, M.J.; White, E.V.; Cohen, A.; Hetrz, H.S.; Mandel, J.; Paule, R.C.; Svensson, L.; Bjorkhem, I.; Blomstrand, R. “Comparison of Two Isotope Dilution/Mass Spectrometric Methods for Determination of Total Serum Cholesterol.” *Clin. Chem*. 1982, *28/1*, 5-8.
20. Nauck, M.; Winkler, K.; März, W.; Wieland, H. “Quantitative Determination of High-, Low-, and Very Low-Density Lipoproteins and Lipoprotein(a) by Agarose Gel Electrophoresis and Enzymatic Cholesterol Staining.” *Clin. Chem*. 1995, *41/12*, 1761-1767.
21. Fletouris, D.J.; Botsoglou, N.A.; Psomas, I.E.; Mantis, A.I. “Rapid Determination of Cholesterol in Milk and Milk Products by Direct Saponification and Capillary Gas Chromatography.” *J. Dairy Sci.* 1998, *81*, 2833-2840.
22. Greenspan, M.D.; Lo, C.L.; Hanf, D.P.; Yudkovitz, J.B. “Separation and Identification of triglycerides, cholesterol esters, cholesterols, 7-dehydrocholesterol, dolichol, ubiquinone, α-tocopherol, and retinol by high performance liquid chromatography with a diode array detector” *Journal of Lipids Research* 1988, vol. *29*.
23. Indyk, H.E. “Simultaneous Liquid Chromatography Determination of Cholesterol, Phytosterols and Tocopherols in foods.” *Analyst*, December 1990, vol. 115
24. Skoog, West, Holler. “Fundamentals of Analytical Chemistry” 7th Ed.; Saunders College Publishing, 1997, pp. 660-717.

25. Rubinson, K.A.; Rubinson, J.F.; "Contemporary Instrumental Analysis" 1st Ed.; 2000, pp 629-658.
26. Hadden, N.; Baumann, F.; MacDonald, F.; Munk, M.; Stevenson, R.; Gere, D.; Zamaroni, F.; Majors, R. *Basic Liquid Chromatography*. 1st Ed.; Varian Aerograph, 1971, pp. 2.2; 6.1- 6.21.
27. Hurst, W. J.; Aleo, Michael D.; Martin, Robert A. Jr. "Nonaqueous Reverse Phase Liquid Chromatographic Analysis for Cholesterol in Milk Chocolate." *J. Assoc. Off. Anal. Chem.* 1984, vol. *67*, No 4; pp. 698-700.
28. Kautsky, Marie P. *Steroid Analysis by HPLC: Recent Applications.* Chromatographic Science Series, Vol. 16; Marcel Dekker, Inc. New York and Basel. 1981; pp 65-67.
29. Osato, S.; Heki, M. "On the Micro Determination of Lipids in Tissues." *The Journal of Biological Chemistry.* 1929, pp. 541-557.
30. Carroll, R. M.; Rudel, L. L. "Evaluation of a High-Performance Liquid Chromatography Method for Isolation and Quantitation of Cholesterol and Cholesteryl Esters." *Journal of Lipids Research*. 1981, vol. 22; pp. 359-363.
31. Huang, H. S.; Kuan, S. S.; Guilbault, G. G. "Amperometric Determination of Total Cholesterol in Serum, with Use of Immobilized Cholesterol Ester Hydrolase and Cholesterol Oxidase." *Clin. Chem.* 1977, *23/4*; 671-676.
32. Wybenga, D. R.; Pileggi, V. J.; Dirstine, P. H.; Di Giorgio, J. "Direct Manual Determination of Serum Total Cholesterol with a Single Stable Reagent." *Clin. Chem*. 1970, *16*, No 12, 980-984.
33. Katan, M. B.; Van der Har, F.; Kromhout, D.; Schouten, F. J. M. "Standardization of Serum Cholesterol Assays by Use of Serum Calibrators and Direct Addition of Liebermann-Burchard Reagent." *Clin. Chem*. 1982, *28*, No 4; pp. 683-686.
34. Morris, K.; Sherma, J.; Fried, B. "Determination of Cholesterol in Egg Yolk by High Performance TLC with Densitometry." *Journal of Liquid Chromatography*. 1987, *10(7)*, 1277-1290.
35. Pearson, S.; Stern, S.; McGavack, T. H. "A Rapid, Accurate Method for the Determination of Total Cholesterol in Serum." *Analytical Chemistry*. Vol. 25, pp. 813-814.

36. http://www.ams.usda.gov/howtobuy/butter.pdf . 12/20/2006.

VITA

ESSAKA DAVID CHRISTIAN

Personal Data:	Date of Birth: March 24, 1981. Place of Birth: Geneva, Switzerland. Marital Status: Single.
Education:	University of Buea, Cameroon; Chemistry, B. S. 2002. East Tennessee State University (ETSU), Johnson City, Tennessee, USA; Chemistry, M.S. 2007.
Professional Experience:	Graduate Teaching Assistant, ETSU, Johnson City, TN, USA; 2004-2006. Chemistry Tutor, ETSU, 2005-2006. Assistant Camp/Conference Manager, ETSU Housing, Johnson City, TN; 05/2006-08/2006. Pollster, Louis Harris, Orleans (France); 01/2004-07/2004.
Professional Skills:	Fluent in English, French, and Duala. Computer literate.
Extra-curricular:	Executive Member of the International Student Organization, ETSU, Johnson City, TN. Practice of group sports (soccer, basketball) Volunteer at “Rebuild Northwest Florida” on a Mission Trip to Pensacola, FL.