VALIDATION AND APPLICATION OF THE PURDIE ASSAY IN THE ANALYSES OF THE MAJOR POLYUNSATURATED FATTY ACIDS

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LIST OF SYMBOLS AND ABBREVIATIONS

Arachidonic acids	AA
Absorbance	Abs
Acceptable intake	AI
Acceptable macronutrients distribution ranges	AMDRs
Alpha-linolenic Acid	ALA
Analysis of Variance	ANOVA
Arachidonic acid-	AA
Association of Official Analytical Chemists	AOAC
Beta-quantification	BQ
Cardiovascular diseases	CVD
Coronary heart disease	CHD
Conjugated linoleic,	CLA
Conjugated linoleic acid	CLA
Cardiovascular heart diseases	CVD
Degrees of freedom	df
Docosahexaenoic,Acid	DHA
Eicosapentaenoic Acid	EPA
Friedewald Formula	FF
Food and Agricultural Product Center	FAPC

High density lipoprotein	HDL
High Density Lipoproteins Cholesterol	HDL-C
Hillcrest Medical Center	HMC
Intermediate Density Lipoproteins	IDL
Long chain fatty acids	LCFA
Low Density Lipoproteins Cholesterol	LDL-C
Linoleic acid	LA
Laboratory Standardization Panel	LSP
Microliter	µL------
Milligram/deciliter	mg/dL
Monosaturated saturated fatty acids	MUFAs
National Cholesterol Education Program	NCEP
Oklahoma State University	OSU
Omega	ω
Phosphate Buffer Solution	PBS
Polyunsaturated Fatty Acids	PUFAs
Reverse Cholesterol Transport	RCT
Recommended daily allowances	RDA
Science Analysis System	SAS
Standard	STD
Total Cholesterol	TC
Technology Business Assessment Group	TBAG

Triglycerides	TG
Very Low Density Lipoproteins	VLDL
World Health Organization	WHO

CHAPTER 1

1.0 INTRODUCTION

Cardiovascular diseases, obesity, diabetes, insulin resistance, cancer and metabolic syndrome, which are closely associated with the accumulation of triglycerides in the body tissues, remain serious causes of human mortality around the world. This is in part due to lack of rapid and sensitive methods to quantify the different types of triglycerides associated with these diseases. Triglycerides are esters delivered from glycerol and three fatty acids. Among the triglycerides, fatty acids have been examined for negative and positive effects on the conditions listed above and many others.[1-3] Fatty acids are carboxylic acids with a long unbranched aliphatic chain with a general formula R-COOH. Fatty acids are divided into 2 major groups: Saturated fatty acids which are fatty acids with single bonds between carbon-carbon atoms and unsaturated fatty acids which include; Monosaturated fatty acids (MUFAs)- (fatty acids with one double bond between carbon-carbon atoms) and Polyunsaturated Fatty Acids (PUFAs)-(fatty acids with more than one double bond between two adjacent).[4] This research was focused on the PUFAs.

1.1 Polyunsaturated Fatty Acids

PUFAs are divided into two groups that include the omega-3 (ω-3) and omega-6 (ω-6) fatty acids. Omega-3 and omega-6 fatty acids are essential fatty acids with a double bond on the third and six-carbon atom of the methyl end, respectively. The major ω-3 and ω-6 PUFAs include: alpha linoleic acid (ALA), Eicosapentaenoic Acid (EPA) and Docosapentaenoic Acid (DHA) while the major ω-6 PUFAs include: Linoleic Acid (LA), Conjugated Linoleic acid (CLA), and Arachidonic Acid (AA) (Figure1.1).

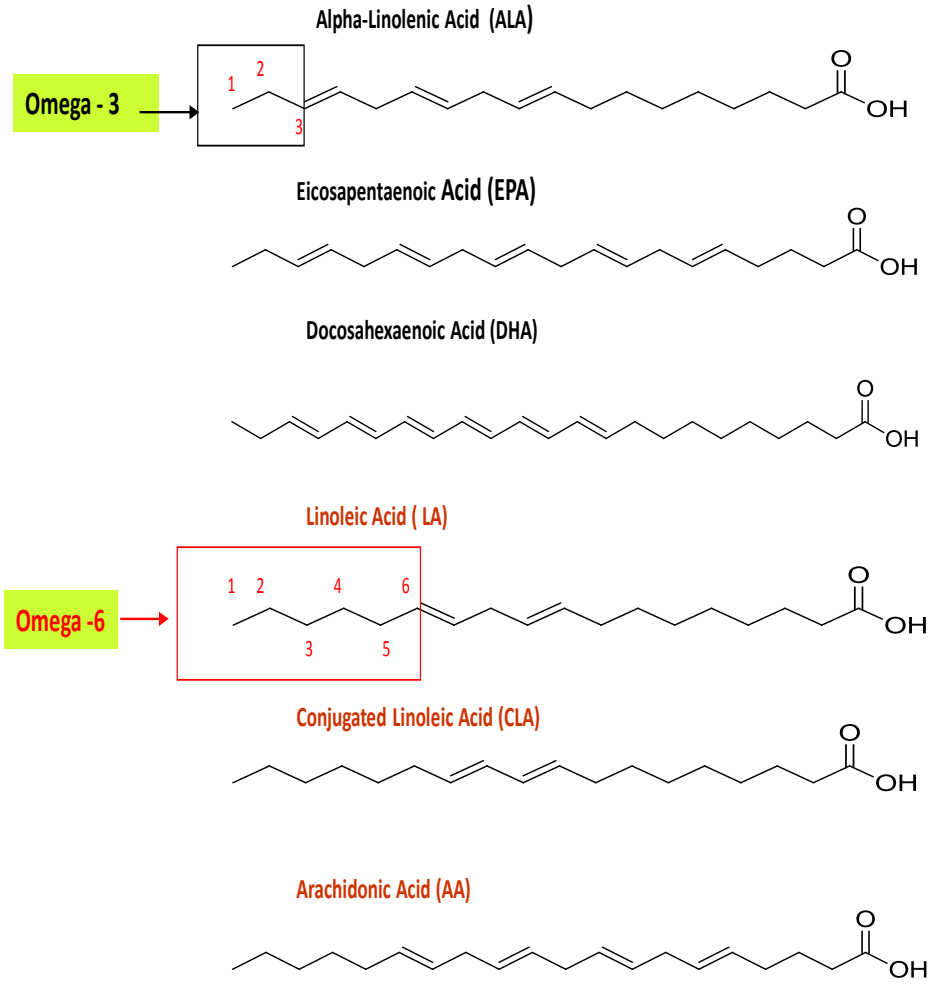


Figure 1.1: The chemical structures of the major PUFAs

PUFAs are found in the vital organs such as the brain, the eye retina and they maintain the body immune system and reduce inflammation. They form vital hormones such as eicosanoid hormone.[5,6] Long chain fatty acids such as linoleic acid and others are considered essential nutrients because they must be consumed in the diet since no *in vivo* path can produce them. The PUFA balance is essential for the maintenance of a healthy

body[7-9] and according to many researchers, PUFA composition of cell membranes is, to a great extent, dependent on dietary intake.[10-13] A ω-6:ω-3 ratio close to one (1) is considered protective against degenerative pathologies. A high ratio is associated with promoting pathogenesis of many diseases such as; cardiovascular diseases, cancer, diabetes, obesity and metabolic syndrome related disease.[14-16] Therefore, appropriate amounts of dietary ω-6 and ω-3 fatty acids need to be considered in making dietary choices and recommendations. However, in general, and mainly in United States of America, the method for reporting fats in foods does not give a substantial description ω-3 and ω -6 PUFAs (Table 1.1).[17]

Table 1.1: A general food label

Nutritional Facts

Serving Size: 1 cup (218g)	
Amount Per Serving	
Calories 1927	Calories from Fat 1927
	% Daily Value*
Total Fat 218 g	**335%**
Saturated Fat 19.84 g	**99%**
Trans Fat	
Cholesterol 0 mg	**0%**
Sodium 0 mg	**0%**
Potassium 0 mg	**0%**
Total Carbohydrate 0 g	**0%**
Dietary Fiber 0 g	**0%**
Sugars 0 g	
Sugar Alcohols 0 g	
Protein 0 g	
Vitamin A 0 IU	0%
Vitamin C 0 mg	0%
Calcium 0 mg	0%
Iron 0 mg	0%

The fats eaten in the diet are important for not only the storage and transportation of energy but also serve as the basis for cellular membranes. These fats are stored as lipoproteins.

1.2 Lipoproteins

Lipoproteins are the colloidal particles that transport cholesterol, insoluble lipids within blood. Lipoprotein classification is based on differences in sizes, floating densities in an ultra centrifugal field or electrophoresis mobilities. Different classes of lipoproteins have different amount of lipids and proteins. These classes includes high density lipoprotein (HDL), Low Density Lipoprotein (LDL), Intermediate Density Lipoprotein (IDL), Very Low Density Lipoprotein (VLDL) and chyromicrons (Figure I.2).[18]

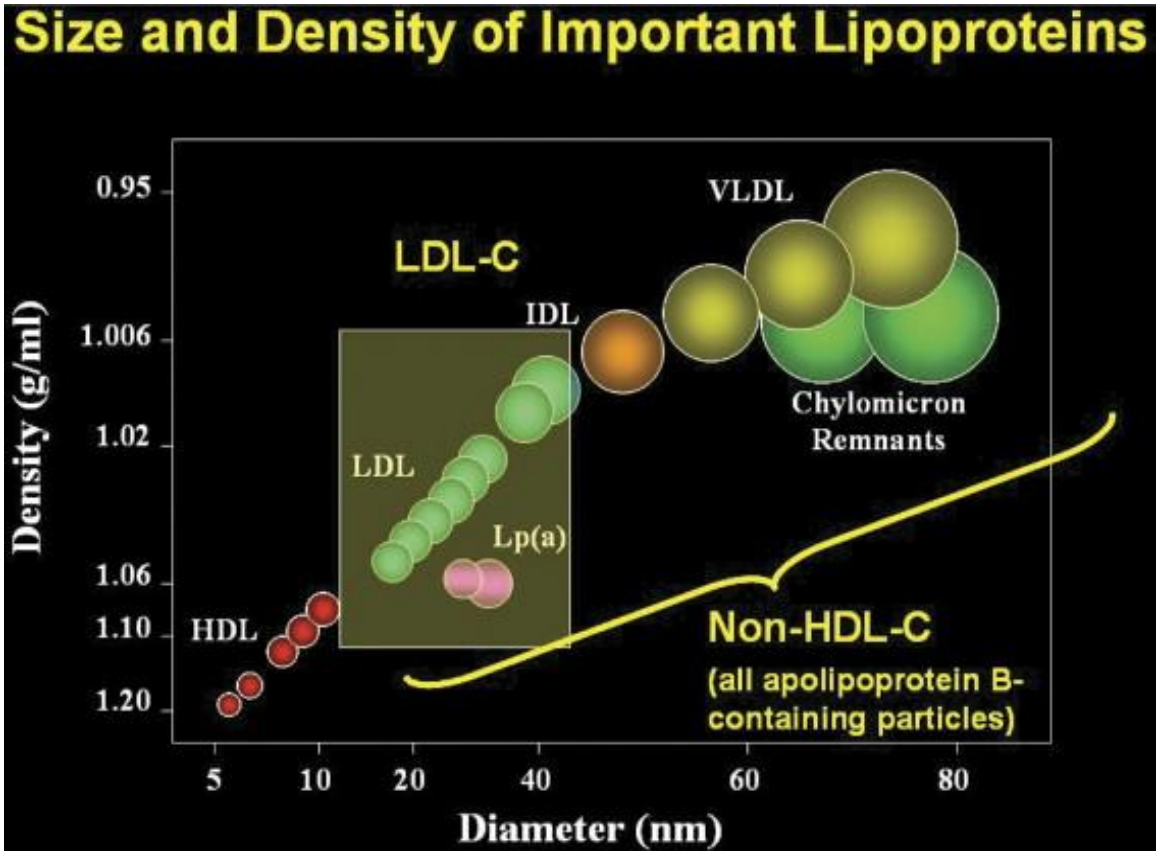


Figure 1.2: Classification of lipoproteins [18]

High-density lipoproteins (HDL) are a fraction of serum lipoproteins characterized by similar molecule density (1.0063-1.21 g/mL) and size (5-17 nm) in diameter. HDL particles have a globular shape and an average percentage by weight composition of 50%

proteins, 30% phospholipids, and 20% cholesterol. The most abundant proteins of HDL is apolipoprotein-A-1, Apo A-11, Apo A-IV, Apo-C, Apo-E and Apo-J are found in lower amounts. Plasma HDL are the vehicles for Reverse Cholesterol Transport (RCT), the mechanism by which peripheral tissue-cholesterol is transferred to the liver for recycling or disposal.[18,19]

The VLDL class of lipoproteins cover a range of particles with a diameter of 280-750 Å, hydrated densities 0.95-1.006 g/mL and floating values form 20-400. The average percentage by weight compositions of VLDL are 55% triglycerides, 20% phospholipids, 15% cholesterol (30% of which is esterified) and 10% proteins. VLDL particles serve to transport endogenous triglycerides from the liver to the peripheral tissues.[18,19]

The LDL includes the range of particles with a density of 0.92-1.2 g/mL.[18,19] In man the principal apolipoprotein of LDL (1.007-1.063 g/mL) is ApoB-100. It has been reported that in other mammalian lipoprotein profiles, ApoA-1 and Apo-E (normally associated with HDL in man) are found in the density range attributed to LDL.[18,20]

The IDL are short-lived lipoproteins derived from VLDL, after hydrolysis of triglycerides of VLDL by the enzyme lipoprotein lipase (LPL). The average percentage by weight composition of IDL is 15% proteins, 7% free cholesterol, 22-26 cholesterol esters, 17% phospholipids, and 35-39 % triglycerides.[18,20]

Chylomicrons are the largest and lightest of the lipoproteins with a diameter of 700-12000 Å and a density of <0.95 g/mL. They are composed mainly of lipids (97.5-99.2%) with average percent by weight composition that are 80-95% triglycerides, 1-3% free cholesterol, 2-4 % esterified cholesterol, 3-6% phospholipids, and 1-2% proteins. They are the principal form of lipoproteins in which absorbed triglycerides are transported from the intestines to the various organs in the body.[18,20]

The level of the stored lipoproteins is measured through a test called lipoprotein profile

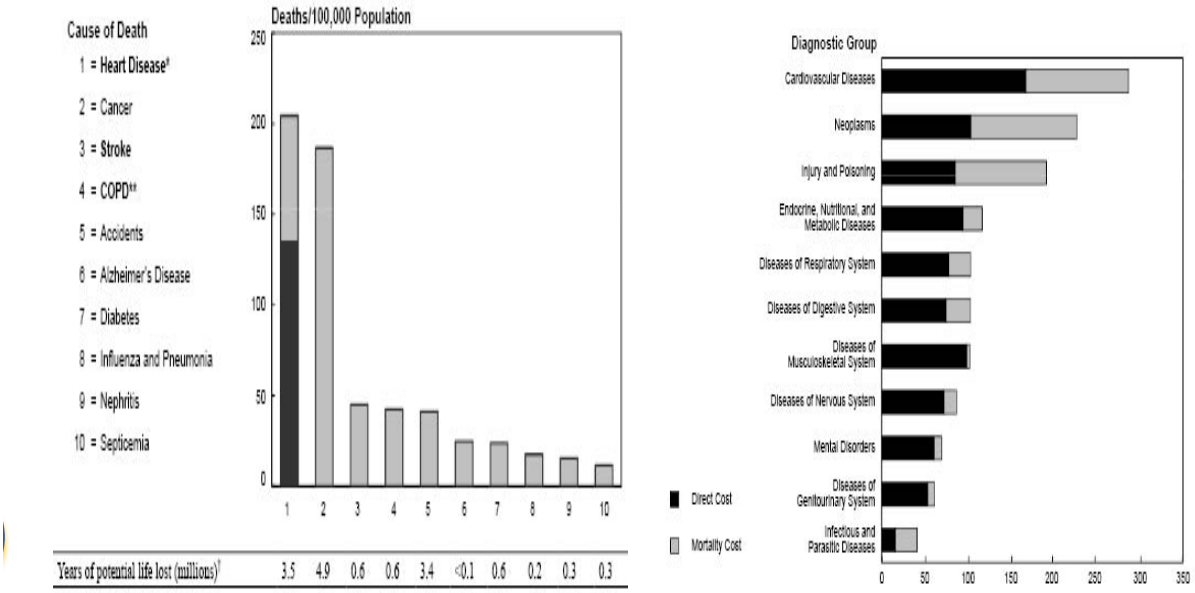
The lipoprotein profile test report includes:

a) Total Cholesterol (TC) - Total cholesterol is a measure of LDL cholesterol, HDL cholesterol, and other lipid components
b) Low density lipoprotein cholesterol LDL, also called "bad" cholesterol)
c) High Density Lipoprotein Cholesterol (HDL-C). HDL cholesterol protects against heart disease by taking the "bad" cholesterol out of the blood and keeping it from building up in the arteries. HDL-C is also called "good" cholesterol.[20]
d) Triglycerides (TG): Fats carried in the blood from the food we eat. Excess calories, alcohol, or sugar in the body are converted into triglycerides and stored in fat cells throughout the body. A full lipid profile report shown by Table 1.2.

Table 1.2: A summary of lipid profile report [21]

Total Cholesterol (mg/dL)	**Total Cholesterol (mg/dL)**
Less than 200	Desirable
200 - 239	Borderline High
240 and above	High
HDL Cholesterol(mg/dL)	**Category**
60 and above	High; Optimal
Less than 40 in men and less than 50 in women	Low
Triglycerides (mg/dL)	**Category**
Less than 150	Normal
150 - 199	Borderline high
200 - 499	High
500 or higher	Very high
LDL Cholesterol (mg/dL)	**Category**
Less than 100	Optimal
100 - 129	Near optimal/above optimal
130 - 159	Borderline high
160 - 189	High
190 and above	Very high

According to Table 1.2, there is no substantial description of the PUFAs and the ratio of ω-6:ω-3. Both the triglycerides and LDL-C are referred to as high risk factors for cardiovascular disease, however, in most cases triglycerides are overlooked and LDL-C is emphasized as the main cause of the death [Figures 1. 3 (a) and (b)].



(a) (b)

Figure 1. 3: (a) Ten Leading Causes of Death: Death Rates, U.S., 2007 [21]
(b) Total Economic Costs of the Leading Diagnostic Groups, U.S., 2007 [21]

The current strategies for reducing the risk of cardiovascular diseases (CVD) involves decreasing the levels of LDL-C but still, despite the intervention aimed at LDL-C, coronary events are the leading cause of death in United States of America (Figure 1.3). In 2010, an estimated 785,000 Americans had a new coronary attack and about 470,000 had a recurrent attack.[21] There is need therefore to shift the focus away from cholesterol as the major risk factor, to the effects of the components of the triglycerides. To address this need, methods using GC, HPLC, GC/MS and TLC have been developed for the analyses of triglycerides. However, these methods have draw backs such as: the methods obtain the total amount of PUFAs instead of the individual PUFAs or the ω-6:ω-3 ratios or/and results are reported as relative percentages of the calculated total peak areas or peak heights.[22] On the other hand, the typical hospital diagnoses report the saturated fatty acids and PUFA data as total triglycerides, ignoring the fact that triglycerides share a

very broad range of different molecular weights, and the LDL-C value is estimated from Friedewald Formula (FF), LDL-C = TC – (HDL-C +TG/5) which is only applicable under specific conditions.[23-25] A method therefore that can quantify the TC, HDL-C, LDL-C and PUFAs without the above-mentioned drawbacks, is paramount. In an attempt to create such a method, the Professor Neil Purdie laboratory at Oklahoma State University developed a test we call the Purdie assay.

1.3 The Purdie assay

1.3.1 Definition of the Purdie Assay

The Purdie assay is a colorimetric test that quantifies 7 analytes (LA, CLA, AA, ALA, EPA, DHA, and cholesterol) in a single step in moles/L without separation or extraction.[26]

1.3.2 The Purdie Assay and the applications of the assay

The assay was developed between Professor Purdie's own research laboratory at Oklahoma State University (1993-2008) and the Groote Schuur Hospital and the University of Cape Town Lipidology group, Department of Internal Medicine (1999-2006), under the direction of Professor A. David Marais.[26] The objective of these joint studies was to better understand the consequences of treatments of patient hyper-dyslipidemias that were independent of age, gender, and race with a primary purpose to offer the test as a source of additional information regarding plasma lipids leading to superior algorithms for the better recognition and more accurate capabilities for the prediction of lipid abnormalities among high risk patients. With a considerable decrease in time and costs, patients can be better served by earlier recognition and be better

informed of their current status and health improvements based upon interventions that include drug therapy, diet, and exercise over the long term. This information will be valuable to the crusade against metabolic syndrome, obesity management, and recognition of disease forms other than only CHD.[26]

1.3.3 History of the Purdie assay

The Purdie Assay originated from the Liebermann-Burchard reaction (1898) for the determination of cholesterol in human subjects that was later modified by Chugaev and Gastev. We made modifications to the original procedure to lessen the high temperature (72 ^{o}C) used and to eliminate the toxic hazards of the experimental conditions that presented personal discomfort and health risks to the users. The original reagent was a 2:1 mixture of 20% w/v $ZnCl_2$ in glacial acetic acid combined with 98% acetyl chloride.[27,28] Progressive refinements of the reagent system led to a final 25:1 mixture of acetyl chloride and 70% perchloric acid (PA), which reacts rapidly under ambient conditions. The assay has been demonstrated to discriminate between the α- and ß-positions at the C-17 carbon of steroids.[29] Later, the work was extended to include terpenes and PUFAs. The next challenge that faced us was how to approach development of a reliable procedure for determining the concentrations for each of the three most prolific ω-3 and three most prolific ω-6 lipids in the forms of esters of LA, EPA, and DHA, ALA, AA, and CLA plus cholesterol and its many esters without involving any form of chemical or physical separations. This was overcome in the Purdie laboratory where a chemometric algorithm were developed and coded.[30]

1.3.4 Principle of the Purdie assay

The assay is based upon a full spectrum visible absorbance detection data using diode array technology, which was a major savings in cost and time over GC/MS analyses. Color induction is done using the single reagent described above. The half-life rates for all six PUFAs are identical to that for cholesterol and its esters and all other allylic groups. In order to assay seven analytes in a single test, multiple wavelengths and multivariate analysis data reduction algorithmic procedures were employed which quantitate all seven individual species. Individually, cholesterol and its esters produce a red color and the PUFAs produce various shades of yellow increasing in intensity with the increasing unsaturation as shown in Figure 1. 4.[31] The visible range extending from 350-550 nm measured at 2 nm intervals leading to 101 data points was chosen.

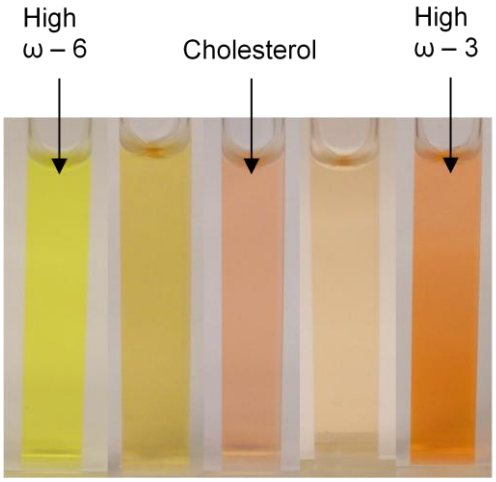


Figure 1.4: Different color shades produced by the Purdie assay[31]

The assay protocol is as described by Figure 1.5

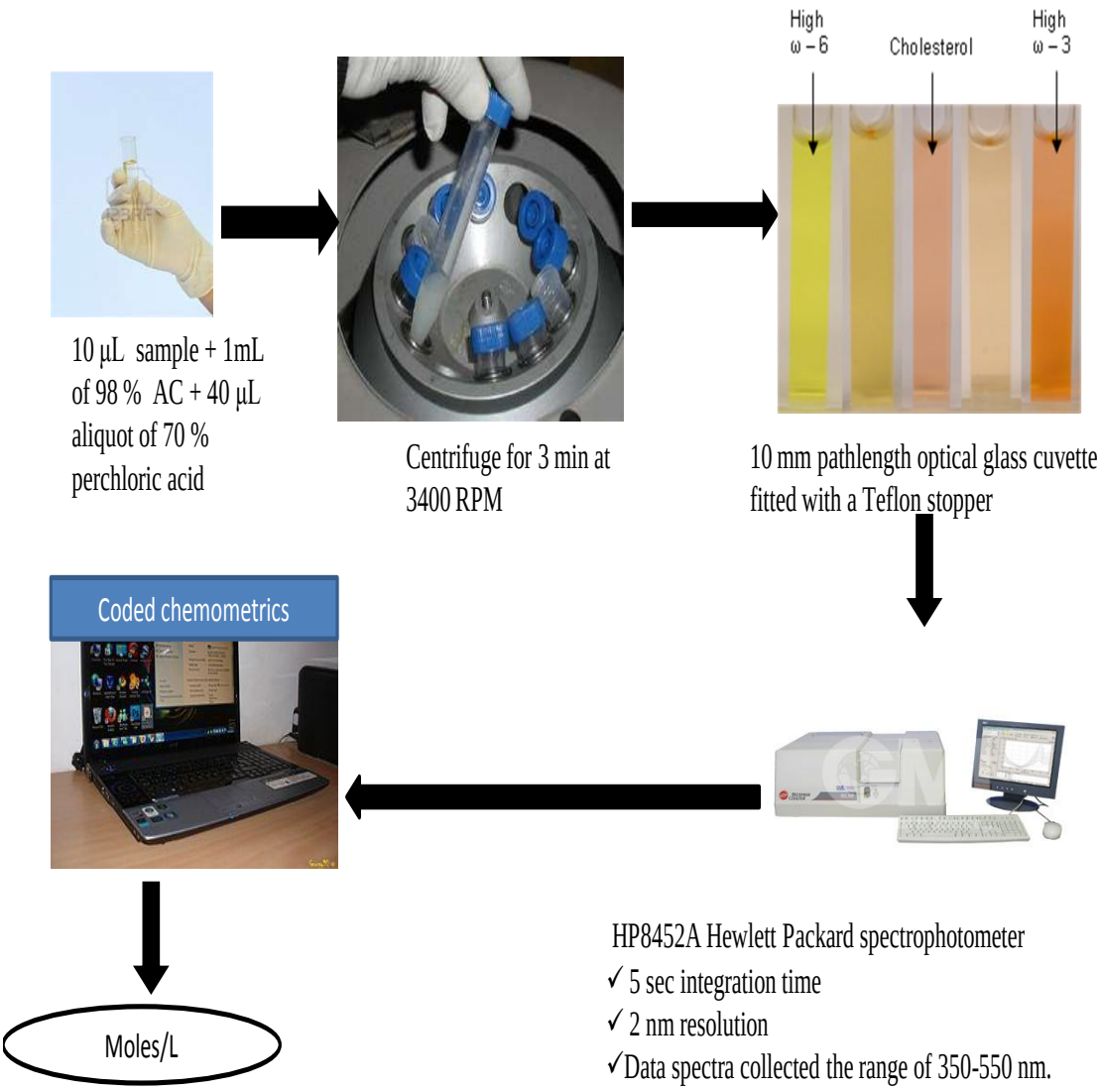


Figure 1.5: The Purdie assay protocol[22, 26]

After developing the assay we were faced with two challenges, which were:

1) Validating the results of this novel spectroscopic protocol against GC/MS analyses, the recognized primary standard.

2) Applying the assay to the quantification of PUFAs.

These two challenges were the basis for this study.

These challenges were addressed by carrying out four projects that are;

1. Quantification of the total ω-6, total ω-3 and ω-6:ω-3 ratio and the validation of the Purdie assay using GC/MS.

2. Comparison of the analyses of total, High Density (HDL) and Low Density (LDL) cholesterol using the Purdie assay and the conventional methods: "A further refinement of the Purdie assay.

3. Quantification of the major ω-3, ω-6 PUFA using the Purdie assay and their ratio in different cholesterol types and the effect of gender and cholesterol types on PUFA levels.

4. Applying the Purdie assay to the labeling of the omega-6 and omega-3 polyunsaturated fatty acid and their ratio among foods.

1.4 Objectives of the study

The objectives of this study included;

1) To determine the sensitivity of a newly developed Purdie assay relative to GC-MS analyses in the quantification of omega-3 (ω-3) and omega-6 (ω-6) polyunsaturated fatty acid (PUFAs) in human serum.

2) To correlate the Purdie assay with the conventional methods.

3) To quantify the levels of ω-3 and ω-6 PUFAs and their ratios in the Total Cholesterol (TC), High Density Lipoproteins Cholesterol (HDL-C) and Low Density Lipoprotein (LDL-C) fractions of human serum using the Purdie assay,

4) To test and quantify the major PUFAs in different types of food.

1. 5 Summary

In summary the first two projects demonstrated the validation of the Purdie Assay, while the last two projects illustrated the application of the Purdie assay in the analyses of the major polyunsaturated fatty Acids (PUFAs). The projects are discussed in chapters 2-5.

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CHAPTER 2

Quantification of Total ω-6, Total ω-3 and ω-6:ω-3 Ratio in Human Serum Using GC/MS

The following chapter has been published in the ***Journal of LCGC North America*** and appears in this thesis with the journal's permission.

2.0 Introduction

The ratio of ω-6:ω-3 as a biomarker for many clinical conditions such as breast cancer, coronary cancer, and cardiac arrest has become an issue of concern to many researchers.[1-4] Omega-3 fatty acids are essential for human development and also most helpful for achieving and sustaining good health.[5] An acceptable balance between the fractions is essential for development of human beings.[6] Simopoulos Artemis[1] and colleagues, for example, suggested that the most important aspect of PUFAs in the prevention of cancer is the PUFA ratio rather than the absolute amount of either. Altogether, many researchers have cited that the ratios in Western diets lie between 10 to 20.[1-4] Epidemiological and experimental research indicates that a ratio around 1/1–2/1 has a protective effect against the development and growth of breast and colon cancers. Short-term biomarker studies in human beings suggest that ω-3 PUFA supplementations at a ratio of 2.5/1 of ω-6 to ω-3 may against colorectal carcinogenesis.[1, 4]

Little information is yet available on the role of ω-6 PUFA relative to ω-3 PUFA on prostate cancer, and the findings are controversial.[1] It has been cited that ω-3 fatty acids compete with ω-6 fatty acids for the cyclooxygenase enzymes, and their products often have the inverse physiologic effect of the ω-6 fatty acid eicosanoid products.[7] This effect therefore calls for use of an analytical method that can detect very low concentrations of total ω-6, total ω-3 and hence more exact ω-6:ω-3 ratio predictions.

It has also been noted over the past 100-150 years that consumption of ω-6 has increased enormously due to the increased intake of vegetable oils from corn; sunflower seeds safflower seeds, cottonseeds, and soybeans.[1, 4] Intake of ω-3 fatty acids has continued to decrease because of a decline in fish consumption and the industrial production of animal feeds rich in grains containing ω-6 fatty acids leading to the production of meat, eggs, and cultured fish rich in ω-6 fatty acids and poor in ω-3 fatty acids.[1, 4,8] In the same way, modern agriculture with the emphasis on production has resulted in decreased ω-3 fatty acid content in many foods, green leafy vegetables, animal meat, eggs and fish.[1,4] This change of feeding behavior has affected the ω-6:ω-3 ratio in the human body. Hence, research on application of ω-6:ω-3 ratios as a clinical biomarker would be of great significance and importance in dietetics and nutrition. The ω-6:ω-3 ratio in the diet is also different based on the population and locations. In the United States, United Kingdom and Northern Europe the ratios range from 15.00:1 to 16.74:[4,8] For Japan the ratio is 4.00:1[4] and in India rural population ratios range from 5:1 to 6.1:1 and from urban populations from 38-50: 1.[4] The concern is how these changes have affected the health of

the people in general and how the ratio of ω-6:ω-3 can be used effectively as a biomarker for diseases.

Other methods that include high performance liquid chromatography (HPLC), gas chromatography (GC), and thin layer chromatography ((TLC)[8,9] have been used for analysis of PUFAs in human serum. The main challenge in these analyses is the fact that the methods obtain the total amount of PUFAs instead of ω-6:ω-3 ratios. At the same time, as happens in the case of GC, when the results are reported as relative percentages of the calculated total peak areas or peak heights, these are prone to change when the number of the integrated peak height or peak area is reduced or increased. This method of expressing data has been shown to be less accurate than absolute concentration data and of lesser meaning in relation to the clinic issue earlier discussed as compared to the ω-6:ω-3 ratios.[9] This study therefore has quantified the total concentrations for the three ω-6 and three ω-3 as well as the ratios.

2.1 Materials and Method

2.1.1 Chemicals

Stable internal standards included the methyl esters of the ω-6 fatty acids (linoleic, conjugated linoleic, and arachidonic), of the ω-3 fatty acids (linolenic, eicosapentaenoic, docosahexaenoic) and tridecanoic acid methyl ester (TDAME), were obtained from Sigma –Aldrich). All the other chemicals and solvents were of high purity and obtained from Sigma -Aldrich and were used without further purification.

2.1.2 Sample

Methyl esters of ω-6 fatty acids (linoleic, conjugated linoleic, arachidonic), ω-3 fatty acids (linolenic, eicosapentaenoic, docosahexaenoic) were examined. All of the standards were 90 to 99 % pure based on gas chromatographic analysis and were all purchased from Sigma-Aldrich. Stock solutions for each of the analytes with maximum total concentrations of 0.02 M were prepared. The stock solutions were used to prepare mixtures analyzed to obtain data for drawing the calibration curve. Serum samples for this work were provided by staff and volunteers at the Hillcrest Medical Center (HMC) in Tulsa, Oklahoma. The anonymous samples from HMC were from volunteers who were already requesting a lipid profile and had given consent. No attempt was made to solicit samples nor was any extensive medical information derived from the samples except for the cholesterol concentration, which was determined by an outside clinical lab using an enzymatic test. Subjects fasted for at least 12 hours prior to the collection of the sample. A venous blood sample was collected into a Vacutainer^{TM} red and grey-capped separation tube. After inversion of the tube five times to mix the blood and the components of the collection tube, the sample was centrifuged at 3400 RPM for 15 minutes. The collection tube contained a clotting activator which takes approximately 30 minutes to activate and allow a floating gel to separate the red blood cells from the serum during the centrifugation step. The serum, which was the top layer in the tube, was then transferred to a 10 mL glass vial with a screw cap. The samples were analyzed using GC/MS and Purdie assay technique.[10-12] GC/MS analyses and the Purdie Assay were completed on the sample within one day of receiving the sample. Samples were stored in a refrigerator at 2-4 °C and were allowed to return to room temperature prior to analyses. HMC samples were drawn from patients with normal to elevated cholesterol levels.

2.2 Preparation of the sample

2.2.1 Preparation of the sample for analysis s with GC/MS

Human blood serum was esterified using the method given by Guy Lepage and

C.Roy.[10,11] The benzene upper phase was drawn off using a 100 µL micropipette and diluted to 500 µL using benzene.[11,12] Analysis was performed using gas chromatography mass spectrometry (GC/MS). Six PUFAs were quantified using tridecanoic acid methyl ester as the internal standard. A 1µL aliquot of the upper benzene phase of the esterified serum was chromatographed as methyl esters on 30-m fused silica column with an internal diameter of 0.320 mm. The column was wall-coated with 0.25 mm of DB-23. Analysis was performed on a Shimadzu (GCMS-QP2010) gas chromatograph. Helium was used as the carrier gas. The injection temperature was held at 250 ^{o}C and the column oven temperature was at 50 ^{o}C. A splitless injection mode was used and the oven temperature program was held for 2.0 minutes at 50 ^{o}C and then raised 180 ^{o}C at

10 ^{o}C/minute. After 5.0 minute hold, the temperature was raised to 240 ^{o}C at a rate of 5.0 ^{o} C/minute and held for 13 minutes. The GC/MS instrument was tuned every day before running the samples. Peaks were identified by the use of pure reference compounds. Six polyunsaturated fatty acids (PUFAs) from C_{18} to C_{22} were identified.

2.2.2 Calibration Curve

A stock solution of 0.02 M mixture of ω-6 and ω-3 PUFAs and TDA was made using benzene as the solvent. Then 1.5 mL of this the stock solution was diluted in a series of 2:1, 4:1, 8:1, 16:1 stock solutions: benzene and 50 µL of 0.03 g/mL TDA in 4:1 methanol: benzene were added to each diluted solution. Then 1µL of this mixture was

injected into the GC/MS and analyzed in splitless mode for 45 minutes. Each diluted sample was analyzed 4 times (n = 4) at different dilutions. Calibration curves of peak area against conc. in moles per liter were plotted for TDAME, LAME, LNAME, CLAME, AAME, EPAME, and DHAME. Consecutive calibration (n=4) exhibited linearity with R^2 ranging from $R^2 = 0.9414$ and $R^2 = 0.9967$.[13]

2.3 Identification and quantification

The identities of the methyl esters were determined by comparing their relative retention times with those of well-known FAME standard obtained from Sigma-Aldrich. Quantification of FAMEs (peak area response) was accomplished with the help of C13 methyl ester internal standard. The peak area percentages obtained were used for quantification of the individual ω-3 and ω-6 PUFAs in moles per liter and then converted to grams per liter from which the total concentration of the ω-3 and ω-6 were determined and hence the ω-6:ω-3 ratio.[13]

The reproducibility of the method was assessed through analysis of PUFAs prepared from the same blood serum sample the process was repeated in sequence within the same day or of duplicate samples collected simultaneously from the subject and processed and analyzed four times (immediately, and after 1-3 week storage period at -4 ^{o}C in the presence of BHT. Calibration (n = 4) exhibited linearity and reproducibility with CV of 4.24 and $R^2 = 1$.[3]

2.4 Post analytical processing

After data acquisition, customized reports of the splitless run were automatically generated. Certain peaks were selected from many peaks and manually integrated and then the peaks were merged into a single-page final report. The data were used to calculate the concentration of each PUFA in moles per liter. Calculations were based on peak area of the PUFAs of interest, the peak area and concentration of well-known FAMEs that correspond in their retention time with the analyte and the peak area of the internal standard (Equation1). The concentration in moles per liter was converted to concentration in grams per liter and then the total percentage of ω-3 and ω-6 was calculated and ω-6:ω-3 ratio were determined. Table I shows the results obtained from these calculations.[13]

$$\text{LA Conc. (mol/L)} = serum\ LA\ PA \times \frac{[LA]prep\ mix}{prep\ mix\ LAPA} \times \frac{Serum\ StdPA}{prep\ mix\ StdPA} \times dilfactor$$

……Equation 1

Key:

prep mix – Prepared mixture, LA- Linoleic Acid

PA -Peak area, Std –Standard

2.5 Preparation of sample for Spectrophotometry analysis

2.5.1 Serum Samples

A 10 μL sample of serum is added to a 13 x 100 mm borosilicate disposable test tube. One milliliter of 98 % acetyl chloride (Acros) is added to the test tube. A 40 μL aliquot of perchloric acid (70 % ACS reagent grade, GFS) is carefully added down the inside of the test tube and slowly introduced to the acetyl chloride, sample solution. The process must be slow and controlled to prevent excessive splashing that can occur if the solution is added too quickly because of the reaction of acetyl chloride with water in the acid. The reaction starts on first contact with the perchloric acid. The solution is shaken by hand for twenty seconds to allow for the release of the small amount of hydrochloric acid gas from the reaction test tube. The test tube is covered with a Teflon cap and placed into a centrifuge and spun for 3 minutes at 3400 RPM. After centrifugation, precipitated proteins are separated, and the reagent solution is transferred to a 10 mm pathlength optical glass cuvette that is fitted with a Teflon stopper for the remaining time. Absorbance spectra are measured after 15 minutes on an HP8452A Hewlett Packard spectrophotometer. A 5 second integration time and 2 nm resolution were used to collect the spectra over the range of 350-550 nm. The blank for each reaction was pure acetyl chloride. The reagent mixture of acetyl chloride with perchloric acid does produce a slight color at 15 minutes. Due to the possibility of variability and small absorbance value, the combination of acetyl chloride and perchloric acid was not used as a blank.[12, 14] The results obtained were analyzed using chemometrics PLS2 algorithm. [10]

2.6 Results

2.6.1 Quantification of total ω-6, total ω-3 and ω-6/ ω-3 ratios using GC/MS

Quantification of total ω-6, total ω-3 and ω-6:ω-3 ratios using GC/MS was done as described earlier. The results (n = 6) are shown in the Table 2.1 below

Table 2.1: Calculation for total ω-6, total ω-3 and ω-6: ω-3 ratios[15]

Subject's code		***P1***	***P2***	***P3***	***P4***	***P5***	***P6***
LAME	Conc. mol/l	5.41E-03	1.01E-02	5.02E-03	5.14E-03	3.01E-03	2.85E-03
	Conc. g/L	1.59	2.97	1.48	1.51	0.890	0.840
	Total conc. g/L	3.57	6.78	3.72	3.13	2.45	2.01
	% conc.	44.6	43.9	39.7	48.3	36.1	41.9
AAME	Conc. mol/l	1.05E-03	9.32E-04	7.84E-04	1.74E-04	4.56E-04	2.47E-04
	Conc. g/L	0.330	0.296	0.250	0.055	0.150	0.079
	Total conc. g/L	3.57	6.78	3.72	3.13	2.45	2.01
	% conc.	9.36	4.38	6.70	1.77	5.92	3.92
CLAME	Conc. mol/l	1.02E-05	7.35E-04	1.94E-04	3.49E-05	5.17E-04	9.50E-05
	Conc. g/L	0.003	0.220	0.060	0.010	0.150	0.030
	Total conc. g/L	3.57	6.78	3.72	3.13	2.45	2.01
	% conc.	0.080	3.19	1.53	3.13	6.20	1.40
Total ω-6		**54.1**	**51.5**	**47.9**	**50.4**	**48.2**	**45.6**
EPAME	Conc. mol/l	2.23E-03	8.58E-03	4.85E-03	1.85E-03	2.20E-03	4.04E-04

	Conc. g/L	0.700	2.72	1.53	0.590	0.700	0.140
	Total conc. g/L	3.57	6.78	3.72	3.13	2.45	2.01
	% conc.	19.7	40.1	41.2	18.7	28.4	6.90
DHAME	Conc. mol/L	2.73E-03	1.47E-03	1.03E-03	2.77E-03	1.60E-03	2.20E-05
	Conc. g/L	0.940	0.500	0.350	0.950	0.548	0.01
	Total conc. g/L	3.57	6.78	3.72	3.13	2.45	2.01
	% conc.	26.2	7.43	9.47	30.3	22.3	0.320
LNAME	Conc. mol/L	4.46E-06	2.42E-04	1.76E-04	6.50E-05	9.19E-05	2.51E-02
	Conc. g/L	1.00E-03	0.070	0.050	0.020	0.030	7.40
	Total conc. g/L	3.57	6.78	3.72	3.13	2.45	44.6
	% conc.	0.040	1.04	1.38	0.610	1.09	16.6
Total ω-3		**45.5**	**48.5**	**52.1**	**49.8**	**51.8**	**52.1**
ω-6:ω-3 ratio		**1.18**	**1.06**	**0.920**	**1.02**	**0.930**	**0.920**

Table 2.1 shows the conversion of the peak areas obtained from GC/MS analysis into concentration in grams per liter and to percentage concentration. Then ω-6, ω-3 and ω-6:ω-3 ratios were calculated using the total percentages of the PUFAs obtained from GC/MS analysis. The study therefore was able to convert the peak area percentages obtained from GC/MS analysis to concentration percentages that cannot be altered even when the peak areas integrated are increased or decreased. This makes it possible for GC/MS quantitative measurements to be compared with other quantitative measurements

done using other instruments. It was noted that though this is possible, it requires a qualified personnel to do the analysis moreover, the process of analysis and calculations involved in quantification into mM or g/dL is tedious time wasting and cannot be done in one step. This calls for more research on analytical methods that can quantify PUFAs in a single step.[13]

The following chapter has been published in the ***Journal of Validation Technology*** and appears in this thesis with the journal's permission.

2.7 Analysis of spectrophotometry data

The data obtained using the Purdie assay was analyzed using different chemometric techniques. The best chemometric technique was determined by one analyst using results obtained from spectrophotometric analysis and then the data obtained were validated by comparison with GC/MS results obtained by a different analyst. The results of the comparison are in Tables 2.2- 2.5.

The results obtained with the spectrophotometer (PLS2) algorithm were compared with the results obtained by analyzing the same serum sample with GC/MS. Tables 2.2-2.4 shows the comparison for total omega ω-6 and ω-3 and their ratio using GC/MS and spectrophotometry. The ω-6 and ω-3 total concentrations and the ratio of ω-6:ω-3 evaluated with partial least squares (PLS2 chemometric vs. GC/MS were essentially identical.[15]

Table 2.2: Comparison of K-Matrix ridge regression (RR) (350-550 nm) analysis with GC/MS analysis[15]

	ω-6 total Conc. %	ω-6 total Conc. %	ω-3 total Conc. %	ω-3 total Conc. %	ω-6:ω-3	ω-6:ω-3	ω-6:ω-3 % Error
Subject's Code	K-Matrix RR	GC/MS	K-Matrix RR	GC/MS	K-Matrix RR	GC/MS	
P1	42.00	54.05	57.99	45.95	0.7200	1.180	38.98
P2	46.76	51.46	53.24	48.54	0.8800	1.060	16.98
P3	45.10	52.34	54.90	47.66	0.8200	1.100	25.00
P4	42.45	47.93	57.55	52.07	0.7400	0.9200	19.56
P5	45.81	50.42	54.18	49.58	0.8500	1.020	16.67
P6	39.33	48.22	60.67	51.78	0.6500	0.9300	30.11
P7	37.76	47.17	62.24	52.82	0.6100	0.8900	31.47

Table 2.3: Comparison of P-Matrix regression (PM) (350-550 nm) analysis with GC/MS analysis[15]

	ω-6total conc. %	ω-6 total Conc. %	ω-3 total Conc. %	ω-3 total Conc. %	ω-6:ω3	ω-6:ω-3	ω-6:ω-3 % Error
Subject's Code	P-Matrix	GC/MS	P-Matrix	GC/MS	P-Matrix	GC/MS	
P1	46.93	54.05	53.07	45.95	0.88	1.180	25.42
P2	44.86	51.46	55.14	48.54	0.8130	1.060	23.30
P3	49.73	52.34	50.27	47.66	0.9900	1.100	10.00
P4	49.87	47.93	49.87	52.07	50.13	0.9200	7.070
P5	48.77	50.42	51.23	49.58	0.9500	1.020	6.860
P6	49.72	48.22	50.28	51.78	0.9900	0.9300	6.450
P7	49.38	47.17	50.62	52.83	0.9800	0.8900	10.11

Table 2.4: Comparison of Principal Component Regression (PCR) analysis with GC/MS analysis[15]

Subject's Code	ω-6total Conc. % PCR	ω-6 total Conc. % GC/MS	ω-3 total Conc. % PCR	ω-3 total Conc. % GC/MS	ω-6:ω-3 PCR	ω-6:ω-3 GC/MS	ω-6:ω-3 % Error
P1	90.99	54.05	9.010	45.95	10.10	1.180	755.9
P2	74.28	51.46	25.72	48.54	2.890	1.060	172.6
P3	71.64	54.05	9.010	45.95	10.10	1.180	162.7
P4	69.41	47.93	30.59	52.07	2.270	0.9200	146.7
P5	81.43	50.42	18.57	49.58	4.380	1.010	333.6
P6	86.52	48.22	13.48	51.78	6.420	0.9300	590.3
P7	92.97	47.17	7.030	52.78	13.21	0.8900	1384.

Table 2.5: Comparison of Partial least squares (PLS2) (350-550 nm) analysis with GC/MS analysis[15]

Subject's Code	ω-6 total Conc. % PLS2	ω-6 total Conc. % GC/MS	ω-3 total Conc. % PLS2	ω-3 total Conc. % GC/MS	ω-6:ω-3 PLS2	ω-6:ω-3 GC/MS	ω-6:ω-3 % Error
P1	50.12	54.05	49.88	45.95	1.000	1.180	15.25
P2	48.61	51.46	51.39	48.54	0.9500	1.060	11.32
P3	51.42	52.34	48.58	47.66	1.060	1.090	3.640
P4	48.83	47.93	51.17	52.07	0.9500	0.920	3.260
P5	51.59	50.42	48.41	49.58	1.070	1.020	4.710
P6	49.05	48.22	50.95	51.78	0.9600	0.930	3.220
P7	47.86	47.17	52.14	52.83	0.9200	0.890	3.370

2.8 Discussion

From the above results (Tables 2.2-2.5) it was observed that the PLS2 model for human sera yielded comparable results in the total ω-6, total ω-3 and ω-6:ω-3 ratios with GC/MS analyses results with minimum percentage error as compared to the other models. This confirmed the results obtained by the Purdie assay that rated PLS2 as the best when applied to human sera.[15,16]

A scatter plot of PLS2 Vs GC/MS shows a linear relationship. The results of analysis of the data in Table 2.2-2.5 using (SAS (r) proprietary software 9.2 (TS2Mo) PROC GLM at $\alpha = 0.05$, are shown on Table 2.6 (This table has been modified to include, Mean ± STDEV STD, Error Confidence and Confidence Interval).

Table 2.6: Analysis of data from tables 2.2-2.5[15]

	Mean ± STDEV	STD Error	Confidence interval	df	F1,12	P
K-Matrix	0.752 ± 0.102	0.038	0.075	1,12	22.0	0.005
GC/MS	1.01 ± 0.107	0.048	0.079			
P-Matrrix	0.82 ± 0.311	0.118	0.012	1,12	0.980	0.340
GC/MS	1.01 ± 0.107	0.230	0.040			
PCR	7.05 ± 4.17	1.57	3.08	1,12	14.6	0.002
GC/MS	1.17 ± 0.356	0.134	0.263			
PLS2	0.994 ± 0.056	0.021	0.041	1,12	0.320	0.580
GC/MS	0.978 ± 0.057	0.022	0.042			

From the above results (Tables 2.6) it is observed that the PLS2 and P-matrix model to human sera yielded comparable results in the total ω-6, total ω-3 and ω-6 to ω-3 ratios with GC/MS results (Table 2.6). We conclude that there is a significant difference between K-matrix and GC/MS, and between PCR and GC/MS but there is no significant difference between P-marix and GC/MS and between PLS2 and GC/MS. However, the PLS2 model displayed a lower standard error than P-Matrix (Table2.6). This agrees with the conclusion drawn from analysis with spectrophotometry.[15,17] This study therefore suggests that PLS2 model is a better model to use for the quantification of the PUFAs in human serum and therefore validated the best chemometrics.

2.9 Limiting factors influencing fatty acid measurements in GC/MS and the superiority of GC/MS over spectrophotometry analysis.

Long storage periods, resulting from a backlog of samples to be analyzed, can result in changes in the profiles of the polyunsaturated fats, because these are temperature and oxygen sensitive. [3,11] Careful storage under nitrogen gas and at -80 °C will minimize this loss. The throughput for these analyses can be a limiting factor in sample turnover causing analyses in many cases to take months or even years to be completed. At the same time, the sample is processed and heated for a period of 1 hour. This may cause degradation or loss of the sample. Analyses with GC/MS are time consuming, laborious and expensive.[11] On the other hand, it was noted that, the spectrophotometric analysis are only limited to a certain concentration range. If the concentration of the analyte is below a threshold of 10^{-3}, the logarithm (PLS2) gives the concentration of the analyte as zero, whereas, as for GC/MS, the analytes were quantified and values obtained even at very low concentrations. This study demonstrates the superiority of GC/MS over

spectrophometry and suggests that for analytes of very low concentration GC/MS would give better results than a spectrophotometer.

2.10 Advantage of the Purdie assay over conventional methods

The assay quantifies 7 analytes in serum simultaneously in moles per liter, offers speed analysis (no pre-sampling steps are involved and there is no loss of analyte and the duration of an assay is less than an hour from the point of initiation to the final analysis for seven components). Moreover, the anticipated cost of this assay should be less than the current routine tests used for cholesterol and triglycerides.[12,18]

2.11 Conclusions

The greatest achievement of this study is that, the method is applied to the quantification of total ω-6, total ω-3 and ω-6:ω-3 ratios in human serum using GC/MS for the first time, therefore, improving the GC separation and detection an aspect that is of great importance to chromatographers and medical technicians. The study was also used for the validation of Purdie assay.[13-15]

2.12 References

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CHAPTER 3

COMPARISON OF THE ANALYSES TOTAL, HIGH DENSITY (HDL), LOW DENSITY (LDL) - CHOLESTROL USING THE PURDIE ASSAY AND THE CONVENTIONAL METHODS. "A FURTHER REFINEMENT OF THE PURDIE ASSAY"

This work has been submitted to *Journal of Validation Technology*

3.0 Introduction

The National Cholesterol Education Program (NCEP) recommends that cholesterol levels be measured at least once every five years by everyone over the age of 20. The screening test that is usually performed is a blood test called a lipoprotein profile. Experts recommend that men ages 35 and older, and women ages 45 and older, be routinely screened for lipid disorders. The lipoprotein profile includes LDL, HDL ("good" cholesterol), and the triglycerides.[1,2]

Recently there has been significant research based on the analyses of TC, HDL-C, and, on the accuracy of LDL-C measurements, many researchers have cited significant

discrepancies between calculated and direct measured LDL-C concentrations. The two methods currently used to assess LDL-C are FF and beta-quantification (BQ).[3] In clinical practice, serum TC and HDL-C are obtained as mg/dL, while the LDL-C concentrations are most often obtained as a calculated value derived from the FF. [4] This formula is known to inaccurately estimate the levels of LDL-C, especially when serum triglyceride (TG) concentrations are >4.52 mmol/L.[4,6.]

On the other hand, TC and HDL-C are analysed using different assays. An assay that directly and simultaneously quantifies the TC, HDL-C and LDL-C in human serum is essential. This study used the Purdie assay to directly quantify TC, HDL-C and LDL-C in 24 human serum samples in moles per liter with an objective to test any significant difference between the results from the assay and conventional methods used by HMC. The study prostrates, there is no significant difference between the TC and HDL-C results obtained using the two methods but there is a significant difference between the analyses of LDL-C.

3.1 Chemicals and sample used

The chemicals used in this study include: 98 % acetyl chloride (Acros), perchloric acid (70 % ACS reagent grade, GFS), HDL and LDL/VLDL cholesterol Assay Kit (ab65390) from abcam, and 2X LDL/VLDL Phosphate Buffer Solution (PBS). Blood serum was obtained from 24 volunteering patients who had already requested a lipid profile and had

given consent (with serum TG range of 74-558 mg/dL (262 ±141mg/dL) from Hillcrest Medical Center in Tulsa after fasting over night.

3.2 Methods

3.2.1 Analyses of TC, HDL-C by the HMC Clinicians

3.2.1.1 Analyses of TC

Prior to obtaining blood from an antecubital vein, patients assumed a sitting position for 5 minutes, since posture change can alter serum cholesterol concentrations. The blood samples were collected into tubes with heparin anticoagulant and centrifuged to obtain serum after separation from the clot within two hours. The coded sera were analyzed for TG, TC, HDL-C and LDL-C. The LDL-C was calculated using the FF and was stored at -80 ^{o}C and transported to the Purdie laboratory for the analyses of the levels of TC, HDL-C, and LDL-C.

In HMC laboratories, heparinized blood was centrifuged for 3 minutes to separate the plasma from the cells. A ten micro liters aliquot of heparinized non-hemolyzed plasma was used for the testing. The concentrations of TC were measured using cholesterol/cholesteryl ester detection kit at 520 nm. The concentration of cholesterol was calculate using equation 1

$$\frac{\quad}{\quad} \times \text{Concentration of STD} \quad \text{.....equation 1}$$

3.2.1.2 Analyses of HDL-C

The HDL testing was performed on the Olympus AU640e (New York, USA) analyzer using the Olympus HDL-Cholesterol test (HDL-C) reagent. The absorbance of the blue color complex produced was measured at approximately 600 nm and the HDL-C concentration in the sample was calculated when compared with the absorbency of the HDL Calibrator. LDL-C was estimated using FF equation 2

LDL = TC – (HDL + TG/5)……Equation 2. This calculation is not valid if the Triglyceride is greater than 400 mg/dL.[7]

3.2.2 Analyses of TC, HDL-C and LDL-C using Purdie assay

3.2.2.1 Analyses of TC

No attempt was made to solicit samples nor was any extensive medical information derived from the samples except for the TC, HDL-C, LDL-C and TG levels, which were determined by the HMC clinicians. In the Purdie laboratory, all the blood samples were analyzed for the TC on the same day using the Purdie assay as described by Muriuki *et al.*[8] Ten microliters of samples were reacted in the Purdie assay as described by Muriuki *et al.*[2] The absorbance spectra were measured after 15 minutes on an HP8452A Hewlett Packard spectrophotometer. A 5 second integration time and 2 nm resolution were used to collect the spectra over the range of 350-550 nm (Figure 3.1-3.3). The results obtained were analyzed using a chemometrics algorithm (Table 3.1).[9] The values were converted to mg/dL. The blank for each reaction was pure acetyl chloride[2,8] and each sample was analyzed three times (n = 3).

3.2.2.2 Analyses of HDL-C and LDL-C using Purdie assay

HDL and LDL fractions were separated from each serum sample on the same day after the analyses of TC using the HDL and LDL/VLDL Cholesterol Assay Kit (ab65390) obtained from abcam. Then 100 μL aliquot of 2X Phosphate Buffer Solution (PBS), from abcam, was mixed with 100 μL of serum in a labeled micro-centrifuge tubes. The mixture was incubated at room temperature for 10 minutes, and then centrifuged at 2000 xg (5000 rpm) on a bench-top for 10 minutes. The supernatant (HDL fraction) was transferred into labeled brown sealed air-tight glass vials using a different micropipette for each sample. The precipitates were spun again, and the trace amount of HDL supernatant was carefully removed using a micropipette. The precipitate was then dissolved in 200 μL PBS to obtain LDL/VDL fraction. The solution was transferred into labeled brown sealed–air-tight glass vials using a micropipette. Both HDL and LDL fractions were stored at -80 ^{o}C for analysis. The samples were returned to room temperature before analyses. The analyses of HDL-C and LDL-C were accomplished within three days after separation using the Purdie assay[8]. Each sample was analyzed three times (n = 3). The blank for each reaction was pure acetyl chloride. The spectroscopic results were analyzed using chemometrics algorithm.

3.3 Statistical analysis

Linear regression analysis was used to compare the TC, HDL-C and LDL-C values obtained by the Purdie assay against HMC conventional method in (TG 74-558 mg/dL) samples. SAS software 9.3 (TS1M0) T-tests was used to test the correlation of the two methods. Results were considered significant at $P \leq 0.05$.

3.4 Results

The study consisted of 24 blood samples obtained from patients with the mean age 54.3 ± 14.1 years, (33% female). Each sample was analyzed for the TC, HDL-C and LDL-C, for three times for a total of 648 samples analyzed. The results for each of the three analyses were reproducible, and their average was used to plot Figures 3.1-3.3.

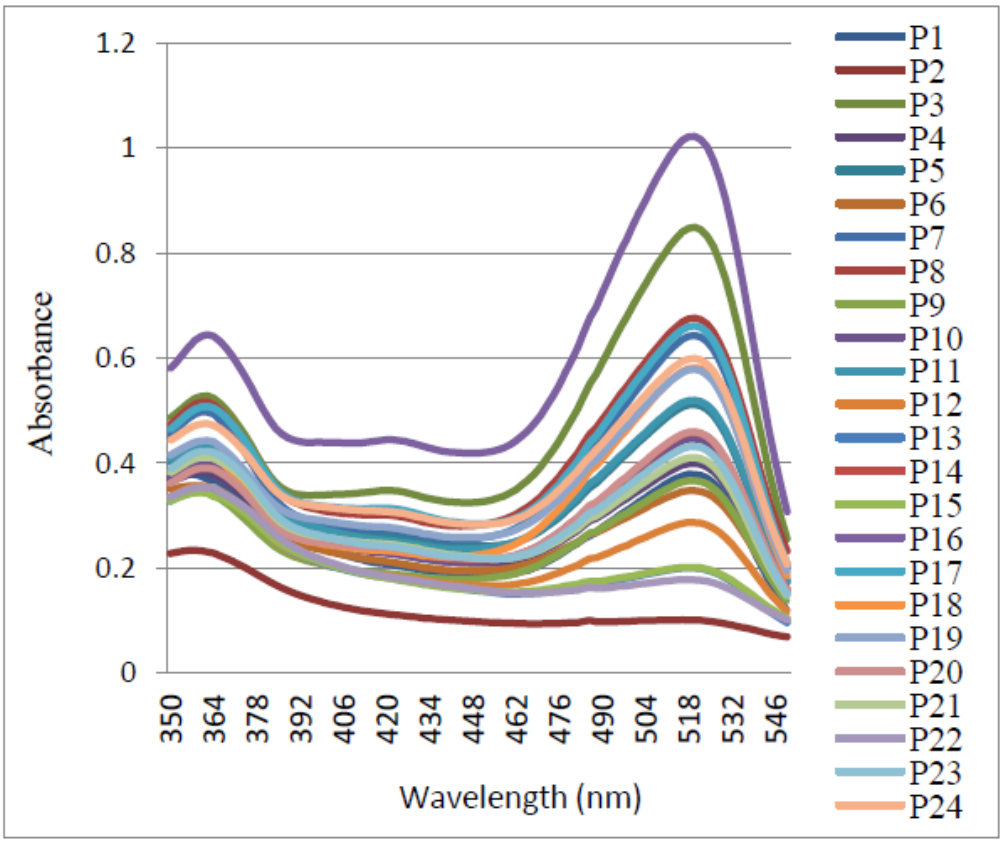

Figure 3.1:Absorbance spectra of TC

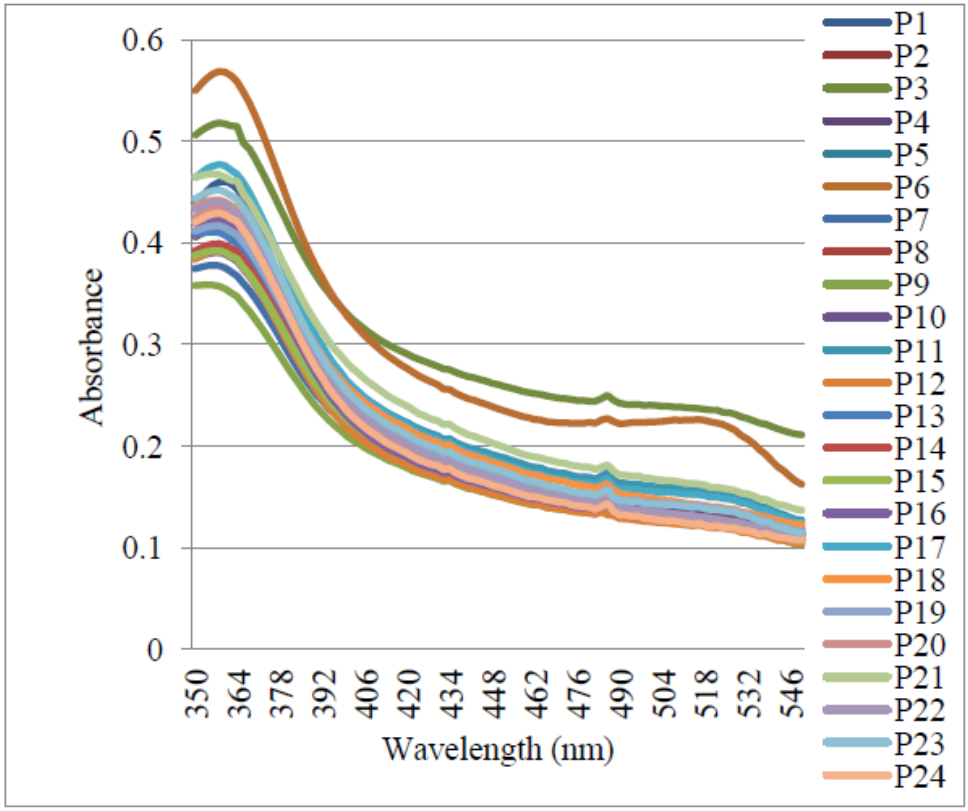

Figure 3.2: Absorbance spectra of HDL-C

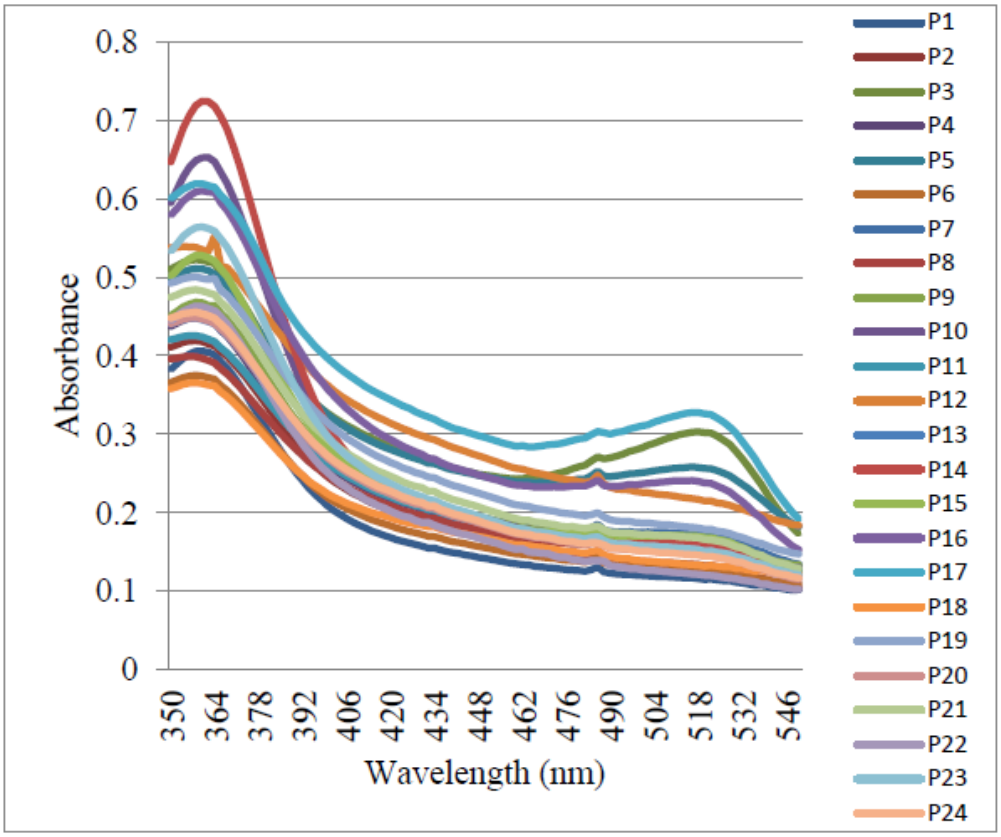

Figure 3.3: Molar absorbance of LDL-C

Linear regression analyses were done for the Purdie assay vs HMC data. As shown in Figure 3.4 a, b and c, the Purdie assay TC and HDL-C data shows a good correlation with HMC data ($R^2 = 0.89$, $R^2 = 0.92$ respectively) but not with LDL-C data ($R^2 = 0.002$).

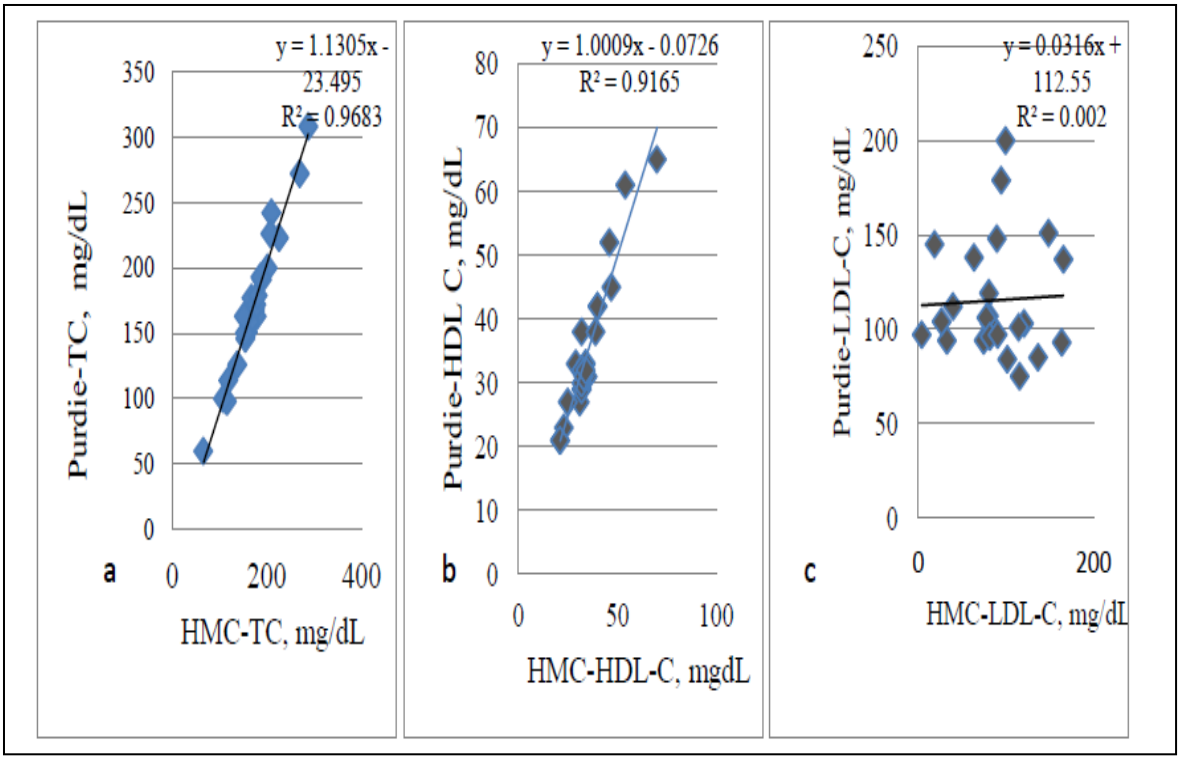

Figure 3.4: Linear regression analysis plot of the Purdie assay Vs HMC analyses of a) TC, b) HDL-C, and c) LDL-C

Table 3.1 shows the mean ± SD mg/dL for the TC, HDL-C and LCL-C obtained by each method for the overall TG (74-558 mg/dL). The mean Purdie assay LDL-C was significantly higher compared with HMC LDL-C, while the mean TC and HDL-C for the two methods did not show any significant difference for the overall TG range (74-558 mg/dL).

Table 3.1: Comparison of TC, HDL-C, and LDL-C determined by the Purdie assay and HMC

Cholesterol	Test	Mean ± SD g/dL	Coeffient of variance %
TC	Purdie assay	172.9 ± 55.6	32.1
	HMC	173.7 ± 48.4	27.9
HDL-C	Purdie assay	35.3 ± 11.3	31.8
	HMC	35.3 ± 10.7	30.5
LDL-C	Purdie assay	115.3± 31.1	27.0
	HMC	88.0 ±43.1	49.0

The correlation of the results obtained by the Purdie assay, and the HMC results are shown in Table 3.2. The TC, and HDL-C concentrations obtained using the Purdie assay were found to be relatively similar to the concentrations obtained by HMC clinicians

(F = 0 .06 df = 1, 46 *P* = 0.81 and F = 0.00 df = 1, 46 *P* = 0.99 respectively), while there was no correlation between LDL-C analyses by the two methods (F = 0.607 df = 1, 46 *P* = 0.02). No consistent trend was observed for discrepancies in excess of 10% in relation to TG concentrations.

Table 3.2: SAS t-test analyses of Purdie assay Vs HMC data

Cholesterol Type	Null hypothesis (Ho)	Alternative hypothesis (H_1)	df	F	P-value	Conclusion
TC	Ho: $\mu_{HMC} = \mu_{Purdie\ assay}$	H_1: $\mu HMC \neq \mu_{Purdie\ assay}$	1, 46	0.06	0.81	Fail to reject Ho
HDL-C	Ho: $\mu_{HMC} = \mu_{Purdie\ assay}$	H_1: $\mu HMC \neq \mu_{Purdie\ assay}$	1, 46	0.00	0.99	Fail to reject Ho
LDL-C	Ho: $\mu_{HMC} = \mu_{Purdie\ assay}$	H_1: $\mu_{HMC} \neq \mu_{Purdie\ assay}$	1,46	6.07	0.02	Reject Ho

*** df =** The number of values in the final calculation of a statistic that are free to vary

3.5 Discussion

LDL cholesterol, whose values are calculated from TC, HDL-C and TG values, is a key factor for pathogenesis of premature cardiovascular diseases (CAD). [10-14] Accurate assessment of TC, HDL-C and TG, therefore, is of great importance in determining the treatment measures for LDL-C. On the other hand, the availability of an accurate and precise method to evaluate LDL-C is of a key importance in clinical assessment of patients at risk of CAD. The current FF method used for the calculation of LDL in human serum is only applicable in fasting serum samples,[7,15] and is unreliable under conditions, such as: a) In presence chylomicrons, b) when plasma TG >400 mg/dL, and c) in patients with Type III hyperlipidemia.[16-19] Furthermore, the Friedewald calculation requires the determination of three different measurements (total cholesterol, triglycerides, and HDL-C), each with its own analytical CV.[7] An assay that quantifies the TC, HDL-C and LDL-C levels independently is paramount.

In search for an assay that can quantify the levels of TC, HDL-C and LDL-C independently, the Purdie laboratory (Oklahoma State University) developed the Purdie assay.[1] The comparison of the performance of the Purdie assay with the conventional method used at HMC shows that, in 99.9% of the samples, serum LDL-C obtained by FF calculation was underestimated as compared to the Purdie assay data. This is in agreement with results obtained by many researchers who reported that calculated LDL-C underestimates the LDL-C values.[6,20,21] Deviations from >10% occurred in 92 % of the samples. The percentage error increased with increase in TG concentration however the

increase was discrete. This is in agreement with many researchers who found that significant relative error emerged with increased TG concentrations.[6,20,21] These discrepancies between HMC and Purdie assay measurement may be due to significant underlying lipid abnormalities existing in the subjects whose health conditions were not revealed and considered in this study. Attempts to use the alternative multipliers to improve the reliability of the Friedewald equation, as compared to Purdie assay values, gave only 15 % better LDL-C values. This is in agreement with the 1992 status report[17] that reports that, the use of alternative triglyceride multipliers gives only marginally better LDL-C values.

3.6 Conclusions

This study adds an important aspect to the analyses of TC, HDL-C and LDL-C in that it describes a single assay for the analyses of TC, HDL-C and LDL-C as compared to other methods which require different assays for the analyses of TC and HDL-C. This study also reinforces the earlier recommendations that direct measurement of TC, HDL-C and LDL-C should be adopted as the clinical standard to measure LDL-C.[16,17] This research further supports other researchers that suggested that, direct HDL-C measurements are equivalent to those based on precipitation, as recommended by the National Cholesterol Education Program **(**NCEP).[16,7,22] We strongly feel that because the Purdie assay analyses of the levels of TC, and HDL-C compare with the conventional method used at HMC, thus most probably the levels of LDL-C obtained by this assay are also accurate. This is evident because the disadvantage of underestimating LDL cholesterol (LDL-C) levels is eliminated by the Purdie assay analyses because LDC-C values are not calculated from TC, HDL-C and TG) but obtained independently in moles per liters. At the same time,

the assay quantifies TC, HDL-C and LDL-C in all the samples even with TG values exceeding 400 mg/dL without diluting. More information about the Purdie assay can be found in Muriuki M et, al; *Journal of Validation Technology, 2011*. The results of the assay were not compared with BQ analyses

3.7 References

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CHAPTER 4

QUANTIFICATION OF THE MAJOR ω-3, ω-6 PUFAS USING THE PURDIE ASSAY AND THEIR RATIO IN DIFFERENT CHOLESTEROL TYPES AND THE EFFECT OF GENDER AND CHOLESTEROL TYPES ON PUFAS LEVELS

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4.0. Introduction

Cardiovascular diseases are the world's largest killers, claiming 17.1 million lives a year[1]. In the United States of America, heart diseases are the leading cause of death and are a major cause of disability. The most common heart disease in the United States is coronary heart disease (CHD). In 2010, an estimated 785,000 Americans had a new coronary attack, and about 470,000 had a recurrent attack [1]. Many researchers have cited that the chance of developing CHD can be reduced by taking steps to prevent and control factors that put people at greater risk[1,4]. This calls for a more elaborate method for diagnosing, predicting and reporting the CHD risk factors such as TC and the PUFAs in TC, HDL-C and LDL-C.

The current methods used in the measurement of TC include colorimetric, enzymatic, chromatographic, and near infrared reflectance spectrometry detection methods[3,6] while those used to measure serum lipids/TGs include ultracentrifugation, selective precipitation, electrophoresis, and immunochemical methods. These methods fail to report the quantitative values of the individual major PUFAs in TC, HDL-C, and LDL-C. Further, serum LDL-C concentrations in clinical practice are most often obtained as a calculated value derived from the Friedewald Formula (FF). This formula is known to inaccurately estimate the levels of LDL-C especially when serum triglyceride (TG) concentrations are >4.52 mmol/L and might also be inaccurate among specific patient groups at these lower TG concentrations.[8,10] A method therefore, that can effectively quantify the serum PUFAs concentrations whether greater, equals or less than 400 mg/dL is essential. On the other hand, a paradigm shifts that might change the focus away from cholesterol lipofraction as the major risk factors, to the effects of the components of the triglycerides (TGs) is important. The "real" numbers for the TG levels are reported in mg/dL and percentages.[5] It is evident that, TGs biochemically break down into long chain saturated fatty acids (LCFAs) and polyunsaturated fatty acids (PUFAs). Of these, the latter are considered as a potential biomarker for deriving quantitative health risk prediction models[5,6] and are divided into 2 groups: ω-3 and ω-6.[6] These are divided into the ω-3, (alpa-linolenic (ALA), eicosapentaenoic (EPA) and docosahexaenoic, (DHA) and ω-6 (alpha linoleic (ALA), conjugated linoleic (CLA), and arachidonic (AA) acids sub-types that are considered "good" and "bad" respectively for human health maintenance. In search for a new and early diagnostic biomarker, it is important to assess the presence of elevated levels of ω-6 fatty acids perhaps even before considering serum cholesterol levels or both. The concern is that although

cholesterol and total TGs are both routinely reported in terms of mg/dL, in clinical laboratories, only cholesterol can be converted into the proper millimolar units.

Cholesterol and PUFA molecules share the same allylic -(CH=CH-CH_2) - functional group that is susceptible to oxidative stress; and the group is absent in both saturated long chain fatty acids (LCFA) and apolipoproteins.[5] If that functional group, present in the B-ring of the cholesterol molecule, is the origin of its role as a CHD risk factor, most probably the PUFAs share a higher role as a CHD risk factor than cholesterol.

According to the National Cholesterol Education Program (NCEP), decreasing the levels of LDL-C is the primary strategy for reducing the risk of cardiovascular disease. However, despite the intervention aimed at LDL-C, 60% to 70% of coronary events still occur.[11] This strongly suggests that factors other than LDL-C might be more important in determining the risk of cardiovascular diseases. Thus, we contend that inclusion of other factors such as the levels of ω-3 and ω-6 PUFAs in TC, HDL-C and non-HDL-C fractions and the ω-6:ω-3 in the patient's diagnoses might give a more and elaborate meaning. This study uses Purdie assay to simultaneously quantify the ω-3, ω-6 PUFAs in TC, HDL-C, non-HDL-C fractions in moles per liter and the ratio of ω-6:ω-3 PUFAs in TC, HDL-C, and non-HDL-C fractions. The method meets the required precision and accuracy as required by Laboratory Standardization Panel (LSP).[12] The serum samples for this study were provided by Hillcrest Medical Center (HMC) Tulsa, Oklahoma.

4.1 Samples, subjects, chemicals and method used

4.1.1. Samples and Subjects

The study analyzed human serum samples obtained from 35 subjects aged between 23 and 99 years (54.8 ± 15.8 years).

4.1.2 Chemicals used

The chemicals used in this study were, 98 % acetyl chloride (Acros) perchloric acid (70 % ACS reagent grade, GFS)

HDL and LDL/VDL cholesterol Assay Kit (ab65390) from abcam, 2X LDL/VDL precipitation buffer.

4.1.3 Methods

4.1.3.1. Analysis of TC, HDL-C and LDL-C ω-3, ω-6 in human serum

Blood serum was obtained from volunteering patients from Hillcrest hospital Tulsa after fasting over night. The anonymous samples from HMC were from volunteers who had already requested a lipid profile and had given consent. No attempt was made to solicit samples neither was any extensive medical information derived from the samples except for the TC, HDL-C, and TG levels, which the HMC clinicians determined. Prior to obtaining blood from an antecubital vein, patients assumed a sitting position for 5 minutes, since posture change can alter the serum

cholesterol concentrations. The blood samples were collected into tubes without anticoagulant and centrifuged to obtain serum after separation from the clot within two hours. The sera was stored at -80 ^{o}C and transported to Purdie laboratory where all the blood samples were analyzed for the levels of the ω-3 and ω-6 PUFAs in the TC using the Purdie assay and the HDL and LDL-C fractions were separated from each serum sample on the same day using HDL and LDL/VDL cholesterol Assay Kit HDL and LDL/VLDL Cholesterol Assay Kit (ab65390) obtained from abcam and stored at -80 ^{o}C. During these analyses, a 1mL aliquot acetyl chloride was added into a 13 x 100 borosilicate disposable test tube containing exactly 10 µL of serum sample. To the acetyl chloride-sample mixture, a 40 µL aliquot of perchloric acid (70% ACS reagent grade, GFS) was carefully added. The mixture was shaken for twenty seconds and the test tube covered with a Teflon cap and placed into a centrifuge and spun for 3 minutes at 3400 RPM. After centrifugation, the reagent solution was transferred to a 10 mm path length optical glass cuvette and fitted with a Teflon stopper and the precipitate discarded.[5] Absorbance spectra were measured after 15 minutes of color development using a HP8452A Hewlett Packard spectrophotometer (Rockville Maryland). A 5 second integration time was used to collect the spectral data over the range of 350-550 nm measured at 2 nm intervals. The ω-3 and the ω-6 PUFAs in the separated HDL-C and LDL-C fractions (referred to as non-HDL-C in this study) samples were analyzed within 3 days using the Purdie assay as described previously.[5] The samples were allowed to return to room temperature before analysis. Each sample was analyzed three times (n = 3). The levels of LNA, CLA, AA, ALA, EPA, and DHA in mol/ L were determined from the molar absorbance spectra using chemometric algorithm PLS1 as described

by Dumancas *et al*., 2010.[12] The values were converted to mg/dL and the total ω-3, ω-6 and the ratio of the ω-6:ω-3 PUFAs were calculated which resulted into 12 variables.

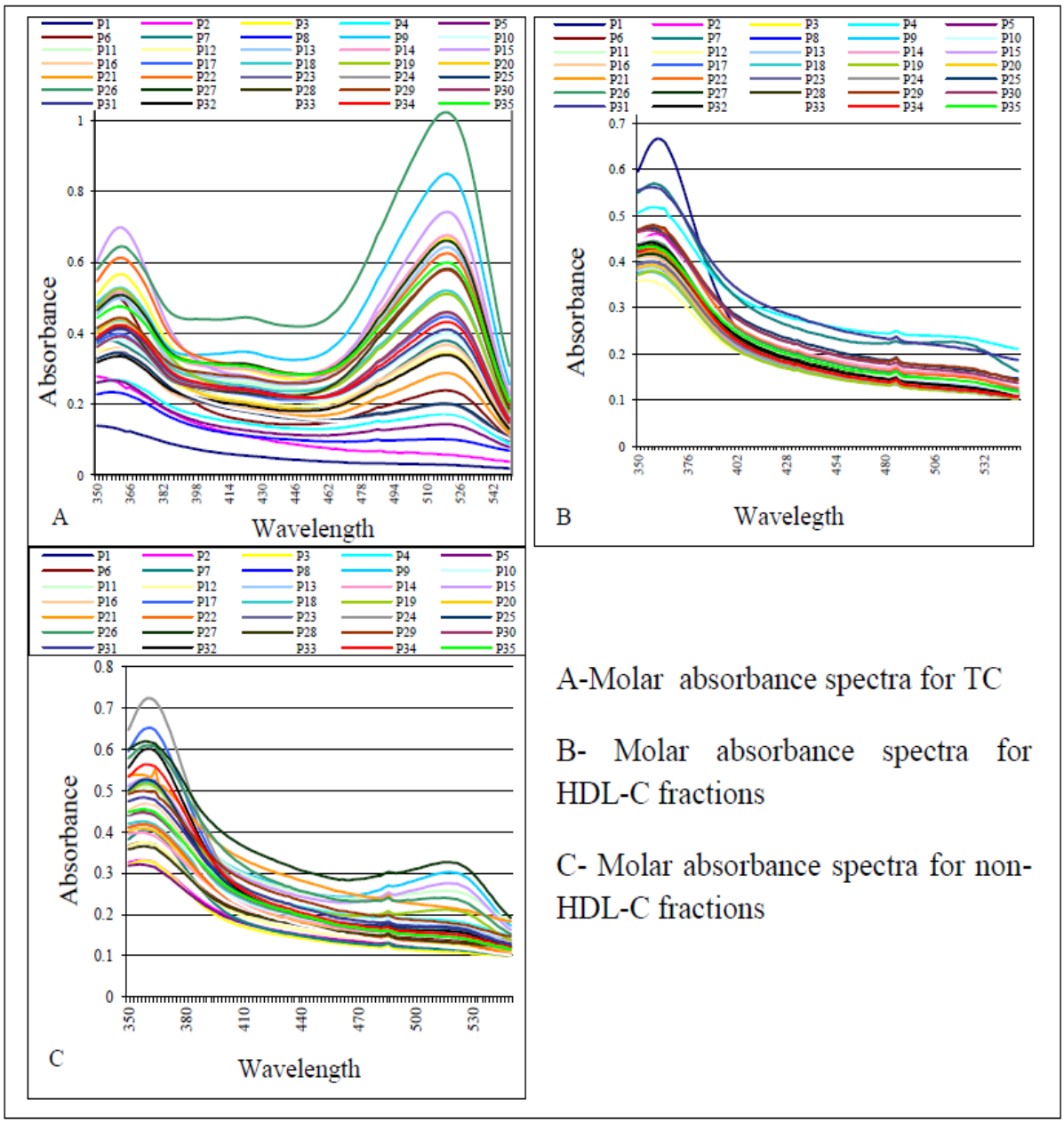
4.2 Statistics

The data were analyzed using SAS software 9.2 (TS2Mo) and SPSS statistical packages (Version 17.0 SPSS Inc. Chicago, USA).[13] Principal Component Analysis (PCA) was used to elucidate the relationship between the different types of PUFAs in TC, HDL-C and non-HDL-C. PCA is a mathematical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of uncorrelated variables called principal components. Each principal component is a linear combination of the original cholesterol variables, with each successive principal component accounting for a smaller percent of the variation in the original data set.[14] Two-way Analysis of Variance (ANOVA) was used to test for the effect of gender and cholesterol type on the levels of the ω-3, ω-6 PUFAs and the ratio of ω-6:ω-3 PUFAs in TC, HDL-C and non-HDL-C. Age was treated as a covariate to control its effects on the levels of PUFAs. Significant means were separated by Tukey's Honestly Significant Differences test. Results were considered significant at $P \leq 0.05$.

4.3 Results

4.3.1 Spectrophotometric spectra for PUFAs in TC, HDL-C and non-HDL-C.

Figures 4.1 A, B, and C shows the absorbance spectra of TC, HDL-C and non-HDL-C, respectively



Figures 4.1 a, b and c shows the molar absorbance spectra of TC, HDL-C and non-HDL-C respectively[15]

Analyses from the molar absorption spectra using chemometric alogarithm are shown in Table 4.1

Table 4.1: PUFAs in different cholesterol types[15]

			Total PUFAs			TC			HDL-C			Non-HDL-C		
Subject's Code	G	A	TC	HDL-C	Non-HDL-C	Total ω-3	Total ω-6	ω-6:ω-3	Total ω-3	Total ω-6	ω-6:ω-3	Total ω-3	Total ω-6	ω-6:ω-3
P1	50	M	2.60	16.2	9.75	1.17	1.43	1.23	7.48	8.70	1.16	5.05	4.67	0.930
P2	48	F	5.20	8.98	5.13	2.34	2.87	1.23	4.51	4.47	0.990	2.78	2.36	0.850
P3	49	F	8.42	6.91	5.58	4.53	3.88	0.860	3.76	3.15	0.840	3.01	2.56	0.850
P4	99	F	3.85	6.97	6.59	2.27	1.58	0.700	4.22	2.75	0.650	3.57	3.03	0.850
P5	65	F	4.72	7.34	4.95	2.70	2.01	0.750	3.99	3.35	0.840	2.75	2.21	0.800
P6	86	F	12.8	8.33	5.78	6.79	6.02	0.890	4.57	3.76	0.820	3.21	2.57	0.800
P7	23	F	5.26	9.33	7.89	3.04	2.22	0.730	4.97	4.36	0.880	3.95	3.94	1.00
P8	61	M	3.91	6.57	7.10	2.12	1.79	0.840	3.51	3.06	0.870	3.75	3.35	0.890
P9	70	F	4.84	6.01	7.39	3.11	1.74	0.560	3.29	2.73	0.830	4.21	3.18	0.750
P10	55	F	4.84	7.17	7.53	3.06	1.79	0.580	3.75	3.42	0.910	4.05	3.49	0.860
P11	57	F	5.04	6.13	7.09	3.09	1.95	0.630	3.35	2.78	0.830	4.10	2.99	0.730
P12	65	M	4.53	5.77	6.26	2.97	1.55	0.520	3.17	2.60	0.820	3.39	2.87	0.850
P13	33	F	7.06	6.23	7.14	4.06	3.00	0.740	3.35	2.88	0.860	3.80	3.34	0.880
P14	86	M	6.65	6.87	6.06	4.05	2.60	0.640	3.80	3.06	0.810	3.31	2.74	0.830
P15	64	F	13.4	6.94	8.24	6.44	6.91	1.07	3.63	3.31	0.910	4.55	3.69	0.830

P16	53	M	5.26	6.67	7.88	3.33	1.94	0.580	3.61	3.06	0.850	4.15	3.73	0.900
P17	56	M	5.23	6.72	14.2	3.22	2.01	0.630	3.53	3.19	0.900	6.76	7.40	1.10
P18	55	M	5.73	8.26	6.51	3.53	2.20	0.620	4.20	4.07	0.970	3.55	2.97	0.840
P19	33	M	9.28	6.59	8.72	5.00	4.29	0.860	3.49	3.10	0.890	4.53	4.19	0.930
P20	67	F	7.83	6.56	6.72	4.11	3.73	0.910	3.50	3.06	0.870	3.66	3.06	0.840
P21	72	M	5.46	7.78	7.38	3.02	2.44	0.810	4.02	3.77	0.940	4.30	3.07	0.710
P22	43	F	9.53	8.41	7.10	4.99	4.54	0.910	4.38	4.03	0.920	3.75	3.35	0.890
P23	46	M	5.10	6.88	7.11	2.63	2.47	0.940	3.66	3.22	0.880	3.81	3.30	0.860
P24	75	M	5.49	8.27	16.8	3.12	2.36	0.760	4.18	4.08	0.980	7.85	9.00	1.14
P25	65	M	4.97	7.18	9.97	2.68	2.29	0.860	4.02	3.16	0.780	5.06	4.91	0.970
P26	45	F	5.65	7.30	10.1	3.63	2.01	0.550	3.93	3.37	0.860	5.12	4.96	0.970
P27	55	M	5.63	6.76	8.64	3.32	2.31	0.700	3.61	3.15	0.870	4.77	3.87	0.810
P28	57	F	5.81	7.84	5.65	3.54	2.27	0.640	4.10	3.74	0.910	3.29	2.36	0.720
P29	45	M	4.92	7.77	7.50	3.23	1.67	0.520	4.23	3.54	0.840	4.16	3.35	0.810
P30	52	F	4.58	7.31	7.25	2.82	1.76	0.620	4.02	3.29	0.820	3.86	3.39	0.880
P31	47	M	4.98	8.19	7.78	2.87	2.11	0.730	4.66	3.53	0.760	4.02	3.76	0.940
P32	57	F	4.27	7.97	12.7	2.70	1.57	0.580	4.07	3.91	0.960	6.17	6.50	1.05
P33	49	M	5.43	7.99	8.92	2.88	2.55	0.880	4.21	3.77	0.900	4.41	4.51	1.02
P34	48	M	5.61	7.42	10.9	3.22	2.38	0.740	4.09	3.32	0.810	5.43	5.52	1.02
P35	33	M	4.76	7.11	7.46	3.07	1.69	0.550	3.79	3.32	0.880	4.01	3.45	0.860

G, Gender; A, Age; Units Mg/dL× 10^2

4.3.2 Correlation and test of significance

Three principal components explained 89% of the total variance based on the 12 variables that were measured Table 2. The first Principle Component (PC) was a linear combination with high loads on HDL-C fraction PUFAs such as, the total ω-3, ω-6 and the ratios of ω-6:ω-3 PUFAs separating patients with high density cholesterol PUFAs from those with low density cholesterol PUFAs. In addition, PC 1 described a strong gradient for patients with a high ratio of ω-6:ω-3 PUFAs in TC. PC 2 was a linear combination with high loads for total non-HDL-C PUFAs, the ω-3 and ω-6 PUFAs in non-HDL-C and the ratio of ω-6:ω-3 PUFAs in non-HDLC fraction describing a gradient for low density cholesterol or non-HDL-C fraction PUFAs. Finally, PC 3 was a linear combination with high loads for the total PUFAs in TC, the ω-3 and ω-6 PUFAs in TC separating patients with high values for total cholesterol PUFAs (Table 4.2).

Table 4.2: Rotated Component Matrix[a] [15]

PUFAs	Component		
	1	2	3
TC total PUFAs	-0.061	-0.086	0.989
Total PUFAS HDL-C fraction	0.966	0.071	-0.147
Non -HDL-C fraction total PUFAs	0.064	0.988	-0.077
TC ω-3	-0.209	-0.072	0.934
HDL-C ω-3	0.919	0.029	-0.161
Non-HDL-C ω-3	0.052	0.966	-0.073
TC ω- 6	0.074	-0.096	0.989
HDL-C ω-6	0.975	0.099	-0.134
Non-HDL-C ω-6	0.069	0.992	-0.078
TC ω-6:ω-3	0.697	-0.103	0.411
HDL-C ω-6:ω-3	0.754	0.234	0.017
Non-HDL-C ω-6:ω-3	0.124	0.861	-0.074

Extraction Method: Principal Component Analysis.

Rotation Method: Varimax with Kaiser Normalization.

a. Rotation converged in 4 iterations.

The ratio of the ω-6:ω-3 PUFAs was significantly influenced by the type of cholesterol ($F = 10.84$, df = 2, 99, $P = < 0.001$) (Table 4.3) but not by gender or an interaction between gender and type of cholesterol (Figure 4.5). The ratios of ω-6:ω-3 PUFAs in TC fraction were significantly lower than that of HDL-C and non-HDL-C fractions (Figure 4.5). Conversely, a

significant two-way interaction between gender and cholesterol type was observed for different types of PUFAs in TC, HDL-C and non-HDL-C fractions (Table 4.3). The total PUFAs, the ω-3, and the ω-6 PUFAs in TC fraction were significantly higher in females than in males while the non-HDL-C total PUFAs, the ω-3 and the ω-6 PUFAs, were significantly higher in males than in females. Total PUFAs in the HDL-C fraction, the ω-3 PUFAs and the ω-6 PUFAs were not significantly influenced by gender (Table 4.3 and Figures 4.2-4.5).[15]

Table 4.3: Two-way ANOVA results for the effect of gender and cholesterol type on the levels of the PUFAs in TC, HDL-C and non-HDL-C fractions[15]

PUFAs *in TC,HDL-C and non-HDL-C fractions*	**Variables**	**df**	**F**	**P**
Total PUFAs	Cholesterol type	2,99	8.845	0.000
	Gender	1,99	0.220	0.640
	Cholesterol type × gender	2,99	4.206	0.018
ω-3	Cholesterol type	2,99	6.996	0.001
	Gender	1,99	0.088	0.767
	Cholesterol type × gender	2,99	4.291	0.016
ω-6	Cholesterol type	2,99	9.914	0.000
	Gender	1,99	0.348	0.557
	Cholesterol type × gender	2,99	3.899	0.023
ω-6:ω-3 ratio	Cholesterol type	2,99	10.84	0.000
of PUFAs	Gender	1,99	0.579	0.449
	Cholesterol type × gender	2,99	0.674	0.512

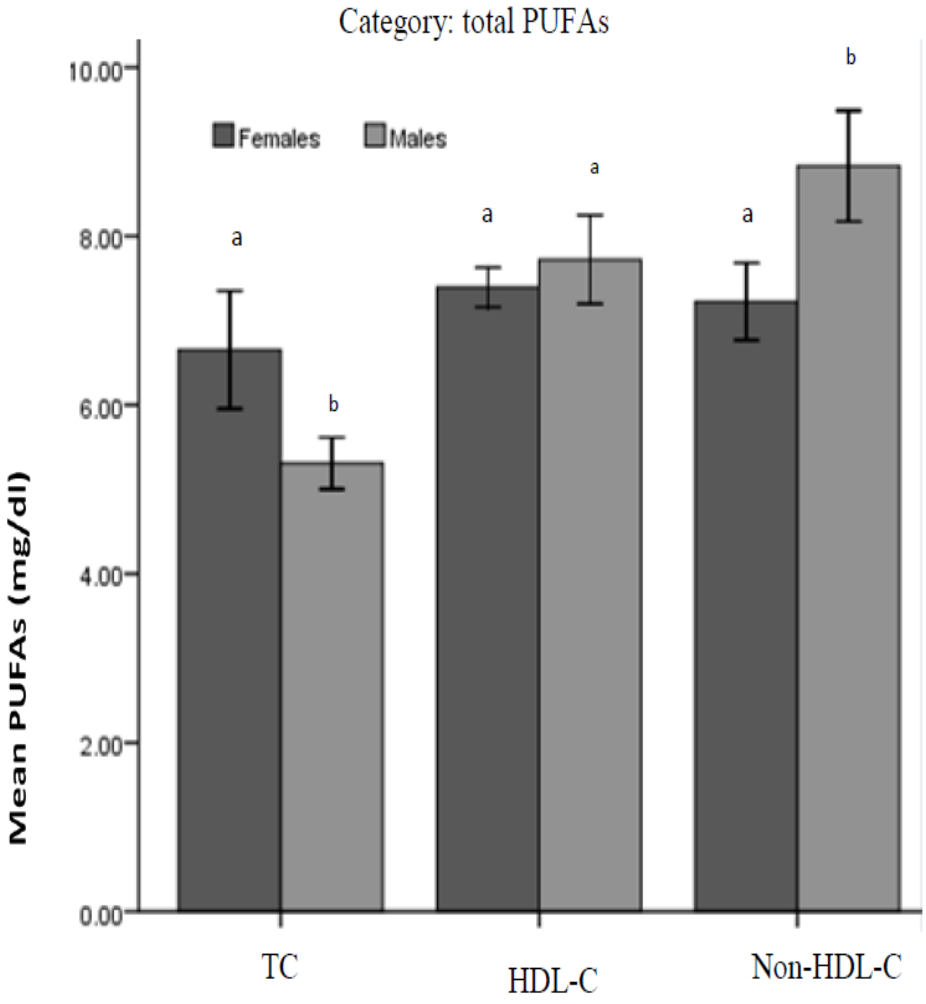


Figure 4.2: Effect of gender on the total PUFAs in TC, HDL-C and non-HDL-C fractions[15]

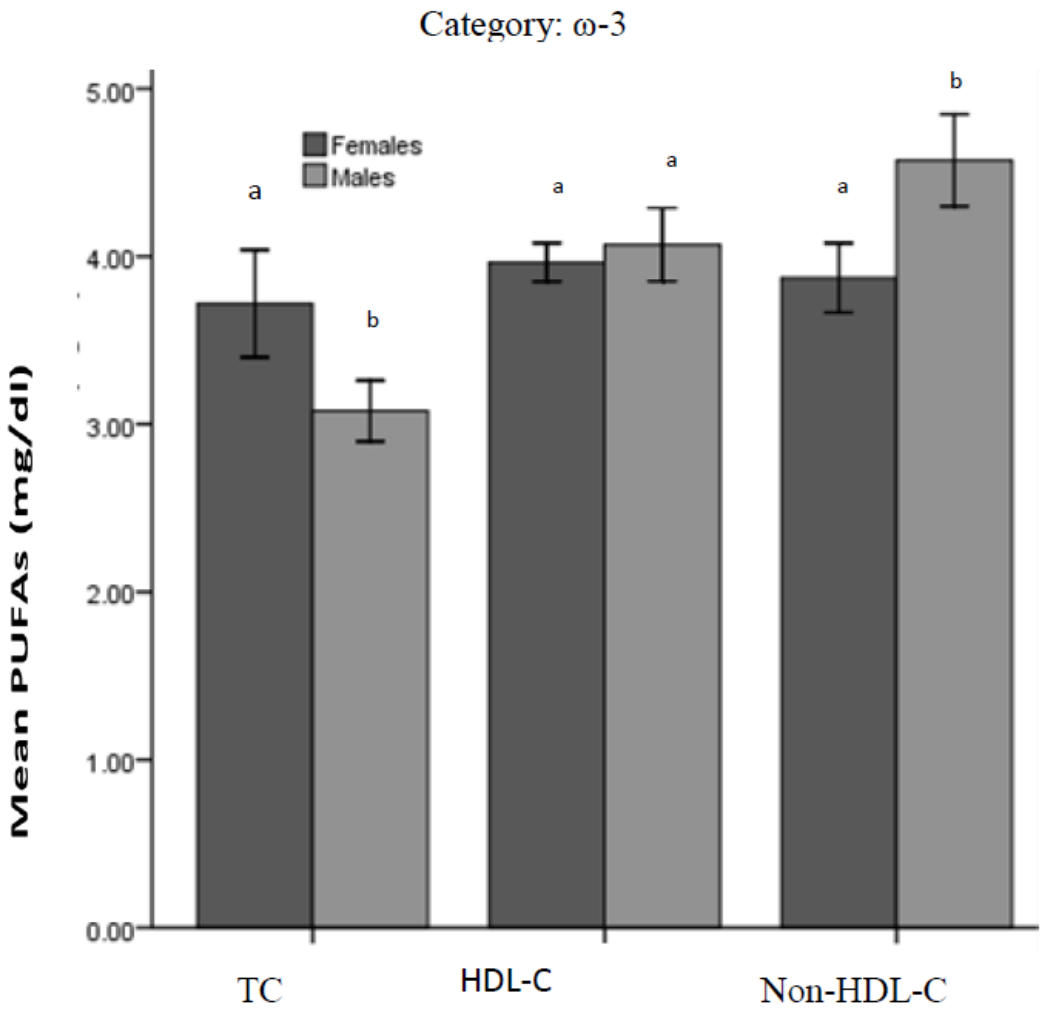


Figure 4.3: Effect of gender on the levels of ω-3 PUFAs in TC, HDL-C and non-HDL-C fractions[15]

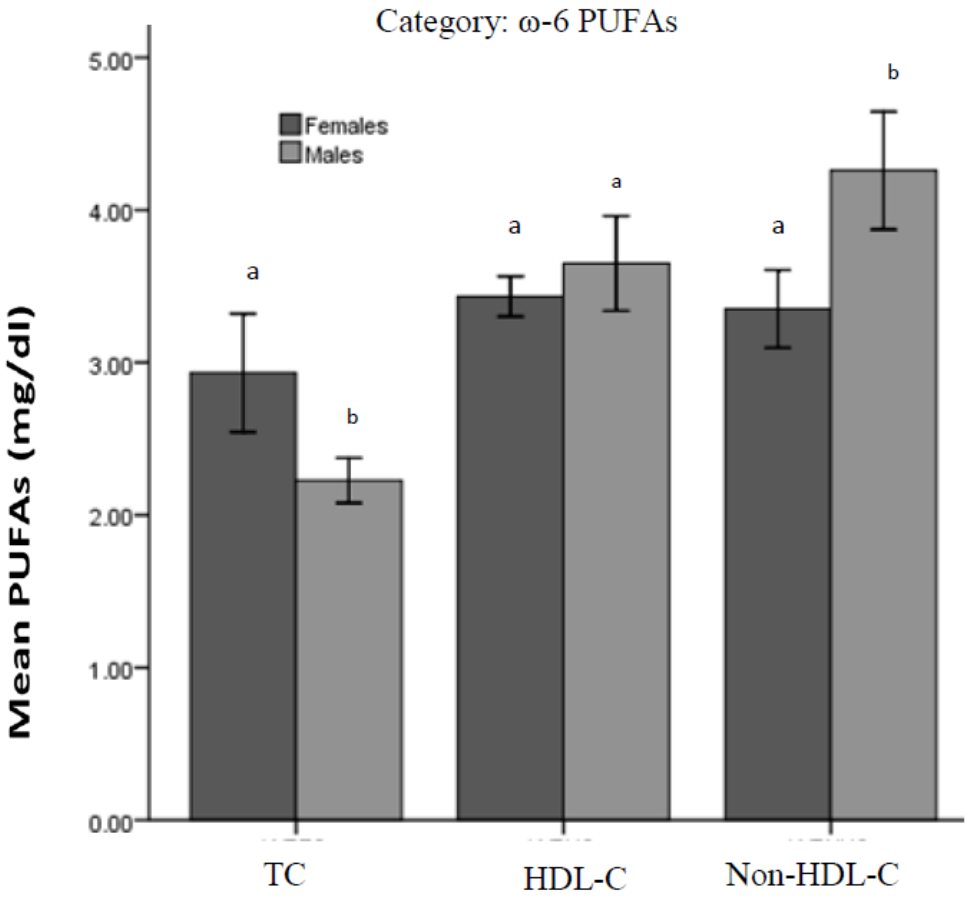


Figure 4.4: Effect of gender on the levels of ω-6 PUFAs in TC, HDL-C and non-HDL-C fractions[15]

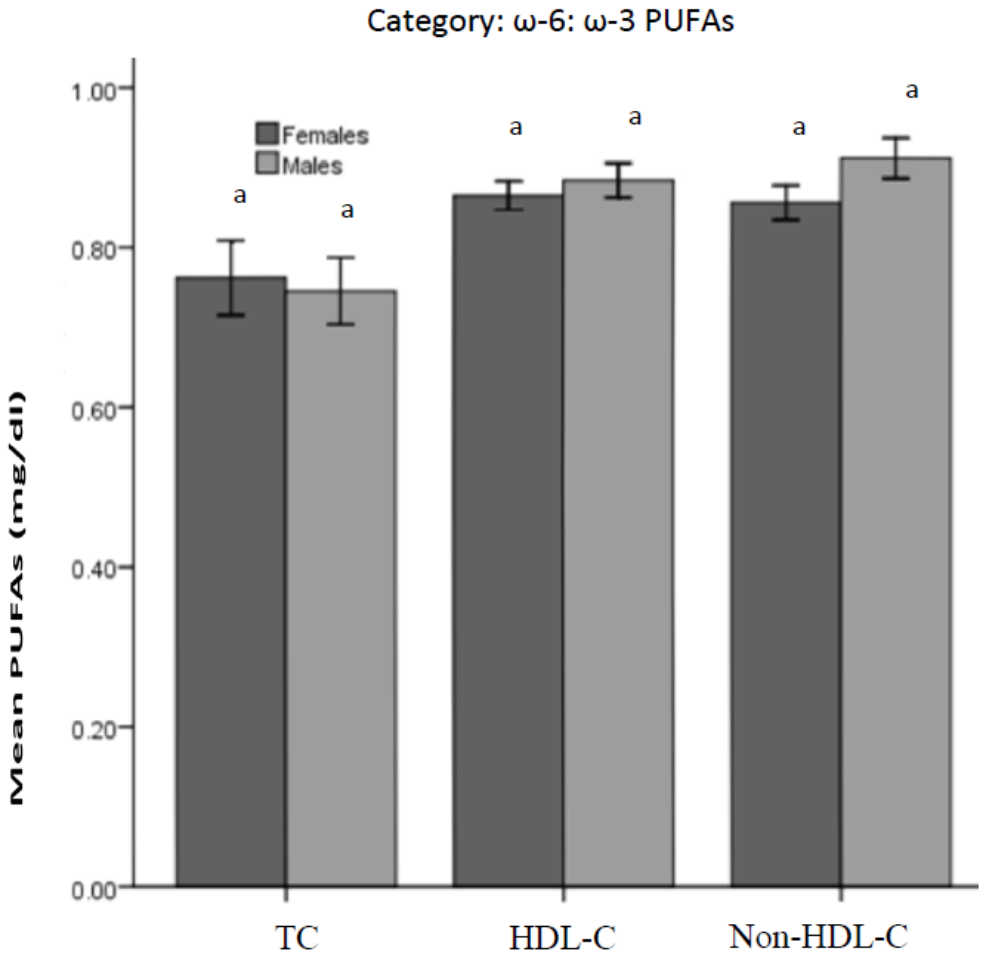


Figure 4.5: Effect of gender on the ratio of ω-6:ω-3 PUFAs in TC, HDL-C and non-HDL-C fractions[15]

4.4 Discussion

4.4.1Interaction PUFAs and the type of cholesterol

Our study demonstrated that the levels of TC, HDL-C, and non-HDL-C fractions in human serum could accurately predict the values ω-3, ω-6 PUFAs as well as the ω-6:ω-3 ratios of

PUFAS and/or the ratio of ω-6, ω-3 PUFAs in human serum can be used to predict the levels of HDL-C, non-HDL-C fractions and TC levels. We suggest that the use the ratio of ω-6:ω-3 PUFAs would be a more elaborate method of reporting the CHD risk factors as compared to the present conventional method of estimating LDL-C levels and ignoring TGs values if 400 mg/dL. At the same time, this work implicates that during the patient's diagnoses, estimating the levels of the PUFAs in TC is a good predictor for the levels of PUFAs in HDL-C fractions but not non-HDL-C fraction (Table 4.2).

4.4.2 Interaction of PUFAs with gender

The gender related difference in the levels of total PUFAs in TC (Figure 4.2) might be related to differences in sex hormone status that result to differential hormonal regulation of PUFAs metabolism. Clinical data supports this hypothesis since estrogens, not androgens, have been shown to increase the concentrations of AA and DHA in the blood lipids of patients treated with sex hormone steroids.[14,17] Several mechanisms which include the partitioning of ALA between β-oxidation, adipose tissue storage and ALA conversion into DHA have been proposed to explain gender related differences in PUFA status.[16,17] Women are generally characterized by higher body fat percentage than men.[17,18] The results showed that age did not have a significant effect on the levels total PUFAs $F = 0.048$, $df = 1, 98$, $P = 0.827$.

It was noted that, the levels of ω-3 PUFAs in TC and mainly DHA were higher in female than in males (Figure 4.3). This is in agreement with the study carried by Childs. *et al*., 2008; which reported that females subjects had higher levels of ω-3 PUFAs in TC than men an aspect attributed to research findings that show that women have a higher capacity to synthesize ω-3 DHA than men.[18-20] A study carried by Audrey *et al.*, 2010,[20] using rat models, showed similar results.[21] At the same time, there was a large variation in the levels of ω-3 PUFAs within the females. This may be attributed to the hormonal differences between individual women, which is influenced by factors such as the degree of stress, type of food eaten, emotions and the menstrual cycle, and age. The males on the other hand, had higher levels of ω-3 PUFAs in non-HDL-C fraction. It was noted that men had higher levels of ALA in non-HDL-C fraction than the females. This supports the suggestion that women could have a greater capacity for ALA conversion into DHA, but the underlying mechanisms are unknown[21-23]. The results showed that age did not have a significant effect on the levels ω-3 PUFAs in different cholesterol types ($F = 0.000$, $df = 1, 98$, $P = 0.997$).

This study suggests that the high levels of non-HDL-C ω-6 PUFAs in the males may be one of the unexplained causes of the male's high CVD risk. The United States Department of Human Services (US DHHS) 1999-2007 report showed that approximately 237.5 males per 100,000 die each year from heart disease as compared to 153.8 female deaths.[24] The results showed that age did not have a significant effect on the levels of ω-6 PUFAs in the different cholesterol types

(F = 0.153, df = 1, 98, *P* = 0.697. The lack of significant interaction between ratio of ω-6:ω-3 PUFAs in TC, HDL-C and non-HDL-C fractions and gender (Figure 4.5) further supports our suggestion that the ω-6:ω-3 ratio of PUFAs could be used as a biomarker for CHD, and other metabolic syndrome related disease regardless of the gender. The results showed that age did not have a significant effect on the ratio of ω-6:ω-3 PUFAs in different cholesterol types (F = 1.855, df = 1, 98, *P* = 0.176).

4.5 Conclusions

The major limitation in this study is the volunteer sample size that limits the external validity, however, despite the limitation; we believe this study adds important information to our current understanding of PUFAs contents in TC, HDL-C and non-HDL-C fractions. In conclusion, this study demonstrates the importance of including the levels of the major PUFAs in TC, HDL-C and non-HDL-C fractions in the patients' diagnoses. For men, more focus should be based on the ratios of PUFAs in the non-HDL-C fractions, which would result to minimized CVD risks. For the female more focus should be on the levels of ω-6 in TC. On the other hand, the dietary guidelines may need to recommend higher intake of preformed ω-3 PUFAs for men than for women to achieve the same tissue levels. This information would be of great importance when considering risk assessment and drug therapy for CHD and the choices of diet for both men and women. Moreover, the study demonstrates that measuring the level of PUFAs in TC can be used as predictor for good or bad cholesterol. This study also introduces an assay we call the Purdie assay that analyzes PUFAs in human serum.

4.6 References

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CHAPTER 5

APPLYING THE PURDIE ASSAY TO THE LABELLING OF OMEGE-6, OMEGA-3 POLYUNSATURATED FATTY ACIDS AND THEIR RATIOS IN FOODS

The following chapter has been accepted for publication in the ***Journal of Food Science and Engineering*** and appears in this thesis with the journal's permission.

5.0 Introduction

The current nutrition recommendations are directed to preventing degenerative pathologies such as cardiovascular diseases (CVD) and cancer[1-4]. The nutrition contents of diets have a profound influence on a number of vital physiological pathways. Furthermore, a strong link exists between dietary trends and a number of common diseases and metabolic disorders such as cancer, diabetes, atherosclerosis, and obesity. Nutrition organizations recommend keeping Trans- fatty acids as low as possible[5-7]. The recommendation for total fat intake should be 25-35% of total daily calories with most fats being monounsaturated and polyunsaturated fatty acids (MUFA and PUFA, respectively), such as fish, nuts, and vegetable oils[1-7].

Over the last 100 years, there has been an increase in the consumption of the ω-6 PUFAs and a decrease of ω-3 PUFAs that are, alpha linolenic acid (ALA), decosahexanoic acid (DHA),

and eicosapentaenoic acid (EPA) in western countries[8-10]. The ω-3 fatty acids are essential for human development and also helpful for achieving and sustaining good health.[5-7] Linoleic acid is the major ω-6 fatty acid, and ALA is the major ω-3 fatty acid. In the body, LA is metabolized to AA, and ALA is metabolized to EPA and DHA.[11] Today this ratio of ω-6 to ω-3 PUFAs in Western diets is about 10 to 1:20 to 25 to 1, indicating that Western diets are deficient in ω-3 fatty acids compared with the diet on which humans evolved and their genetic patterns were established.[10,11] The ω-3 and ω-6 essential fatty acids (EFA) are not interconvertible in the human body and are important components of practically all cell membranes.[12] The ω-6 and ω-3 fatty acids influence eicosanoid metabolism, gene expression, and intercellular cell-to-cell communication. The PUFA composition of cell membranes is, to a great extent, dependent on dietary intake. Therefore, appropriate amounts of dietary ω-6 and ω-3 fatty acids need to be considered in making dietary recommendations and choices. These two classes of PUFA should be distinguished because they are metabolically and functionally distinct and have opposing physiological functions; their balance is important for homeostasis and normal development.[13,14] Studies with primates and human newborns indicate that DHA is essential for the normal functional development of the retina and brain, particularly in premature infants. A balanced ω-6:ω-3 ratio in the diet is essential for normal growth and development and should lead to decreases in cardiovascular disease and other chronic diseases and improve mental health.[15-17]

The dietary fatty acids are derived from both animals and plants. In general, animals are high in saturated fatty acids. Vegetables oils tend to be higher in polyunsaturated fatty

acids and are therefore liquids at room temperature.[10,14] The ω-3 PUFAs are found in some vegetables oils, fish and seafood while the source for the ω- 6 PUFAs includes nuts, most cooking oils, and beef.[14] As micronutrients, fats are not assigned recommended daily allowances (RDA). Fatty acids have acceptable intake (AI) levels and acceptable macronutrients distribution ranges (AMDRs) instead of RDAs. The AI for ω-3 is 1.6g/d (men) and 1.1 g/d (women) while AMDR is 0.6% to 1.2% of total energy as the Food and Nutrition Board recommends.[7] In France, the recommended allowance for ω-3 is 500 mg/d for men and 400 mg/d women.[10,12] The World Health Organization (WHO) recommends 1-2 servings of fish per week, which corresponds to 200-500mg of EPA and DHA.[18] Epidemiological and experimental studies indicate that an ω-3:ω-6 ratio around 1/1–2/1 has a protective effect against the development and progression of breast cancer and colon cancers.[12-14] From these studies it is evident that a balance between the ω-3:ω-6 fractions is essential for health and prevention of many chronic diseases.[12] Therefore we contend that the ω-3 and ω-6 PUFA levels be included on food labels and should also include the ratio of ω-6:ω-3 to label food products with an ultimate goal of improving consumers' food choices for improving health and preventing development of chronic diseases. This study describes a method that we had developed for quantifying ω-3 and ω-6 in several food products.

5.1 Chemicals and foods used

Reagents used were, 98 % acetyl chloride (Acros perchloric acid (70 % ACS reagent grade, GFS), meat, milk, nuts, and pizzas.

5.2 Sample preparation and experimental methods

5.2.1 Samples

Oklahoma State University (OSU) Food and Agricultural Product Center (FAPC) supplied the meat products for this study. The nuts and milk were purchased from local grocery stores (Wal-Mart and Braums; Stillwater, Oklahoma). The OSU dining services provided pizzas.

5.2.2 Preparation of the samples

5.2.2.1 Beef, pizza and nuts samples

Precut test samples, were transferred into a food processor (Bench top model, 110/120V 60 Hz, 1 hp, 7.5 A, 1725 rpm, fan-cooled motor, 4 qt bowl: Model R4Y (robot Coupe, USA, Inc., Jackson, MS, USA) bowl and blended for approximately three minutes. Pizza and cheese samples were also cut into small pieces and prepared similar to the meat samples. All food samples were prepared based upon the principles of Association of Official Analytical Chemists (AOAC) official methods 985.18.[19]

The nuts were first sprayed with distilled water to wash off salt and then dried under the light lamp for a period of 15-25 minutes until the water content was below 10%. The samples were then ground into a fine paste using mortar and pestle then stored in brown sealed-airtight vials.

The milk samples were analyzed as purchased, and no dilution or pre-processing was done.

5.2.2.2 Analysis of the ω-3, ω-6 PUFAs and ω-6: ω-3 ratios of PUFAS

A 2 mL aliquot acetyl chloride was added to a 13 x 100 borosilicate disposable test tube containing exactly 25 mg of solid sample or 20 µL of liquid sample of the food products. To the acetyl chloride-sample mixture, an 80 µL aliquot of perchloric acid (70 % ACS reagent grade, GFS) was added. The mixture was shaken for twenty seconds and the test tube covered with a Teflon cap and placed into a centrifuge and spun for 3 minutes at 3400 RPM. After centrifugation, the reagent solution was transferred to a 10 mm pathlength optical glass cuvette and fitted with a Teflon stopper and the precipitate discarded.[20,21] The foods displayed different color shades depending on the amount of the ω-3 or ω-6 PUFAs in the samples (Figure1). Absorbance spectra were measured after 15 minutes of color development using a HP8452A Hewlett Packard spectrophotometer (Rockville Maryland). A 5 second integration time was used to collect the spectral data over the range of 350-550 nm measured at 2 nm intervals. The molar absorbance spectra are shown in (Figures 2A, 3A, 4A). The blank for each reaction was pure acetyl chloride. Due to the possibility of variability and small absorbance value, the combination of acetyl chloride and perchloric acid was not used as a blank.[21] The levels of LA, CLA, AA, ALA, EPA, and DHA in mole/L were determined from the molar absorptivities using the chemometric PLS1 algorithm.[21] The molar values were converted to mg/100 g units and

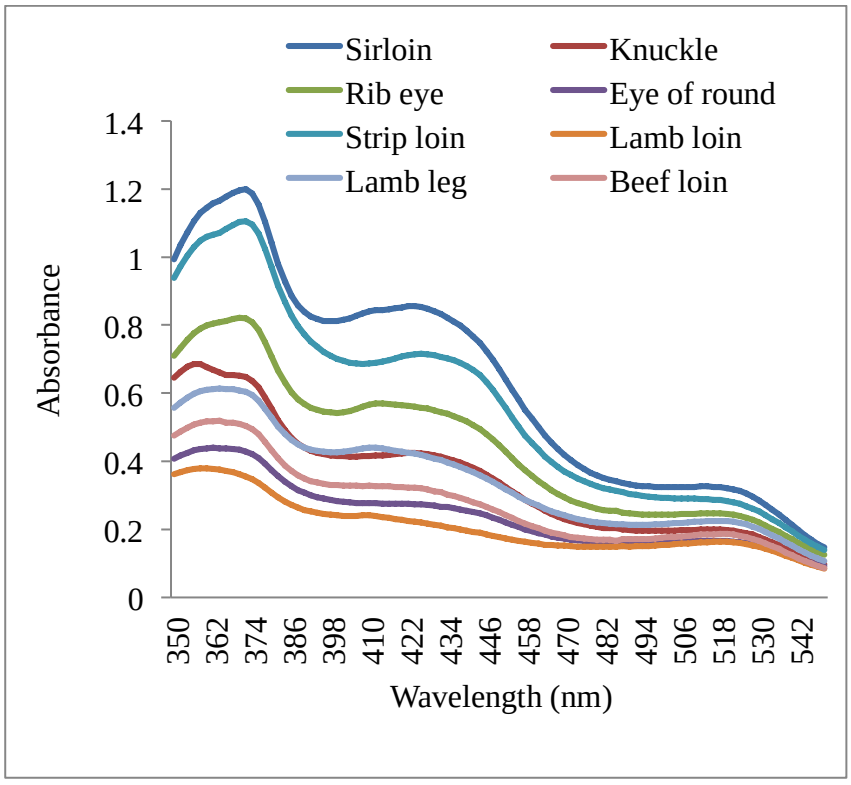
the total ω-3, ω-6, and the PUFAs ratios (ω-6:ω-3) were calculated. Analyses for each sample were done in triplicate.

5.3. Results

A total of 28 samples were analyzed in triplicate. The molar absorbance spectra and the ratio of ω-6:ω-3 PUFAs are shown in Figures 2-5.

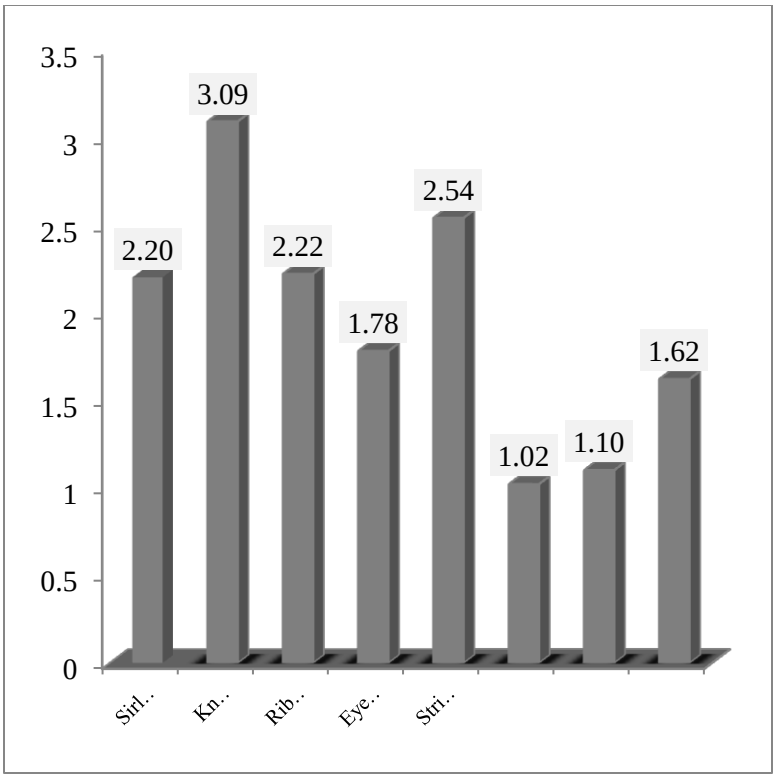
5.3.1 Meat products:

Eight types of meat (2 from lamb and 5 from cattle) were analyzed for the levels of ω-3 and ω-6 PUFAs and the ratios of ω-6:ω-3 PUFAs (Figure 5.1). According to this study, beef knuckle had the highest ratio of ω-6:ω-3 PUFAs (3.09). The ratio decreased in the order: knuckle >sirloin loin >rib eye >sirloin >eye of round >lamb leg >lamb loin >beef loin (Figure 5.2).



!

Figure 5.1 Absorbance spectra of meat brands



5.2 The ω-6: ω-3 ratios of the tested meat products

5.3.2 Pizza products:

Five types of pizza were analyzed (Figure 5.3). The ratio of ω-6:ω- 3 PUFAs for the pizza products range from 1.72-4.01 with pork sausage and vegetable pizza products displaying the highest and the lowest ω-6:ω-3 ratio of 4.01 and 1.72, respectively (Figure 5.4).

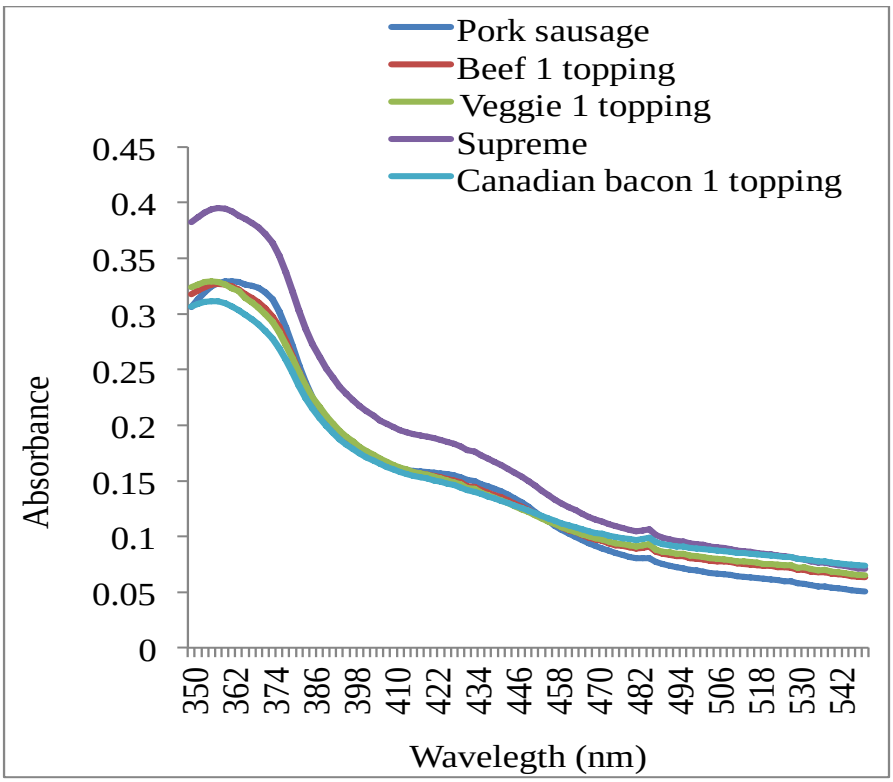


Figure 5.3: Absorbance spectra of pizza products

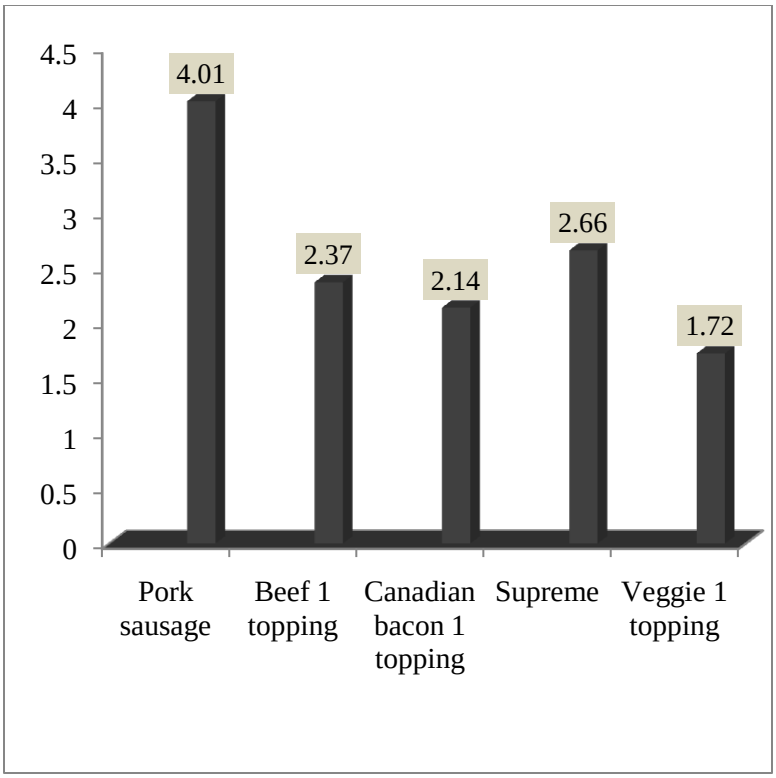


Figure 5.4: The ω-6:ω-3 ratios, of tested pizza products

5.3.3 Nuts

Eight types of nuts were analyzed (Figure 5.5). The nuts displayed a ratio of ω-6:ω-3 PUFAs ranging from 0.73-1.12 with macadamia nuts displaying the lowest ratio (0.73) and pecans had the highest ratio of ω-6:ω-3 PUFAs (Figure 5.6)

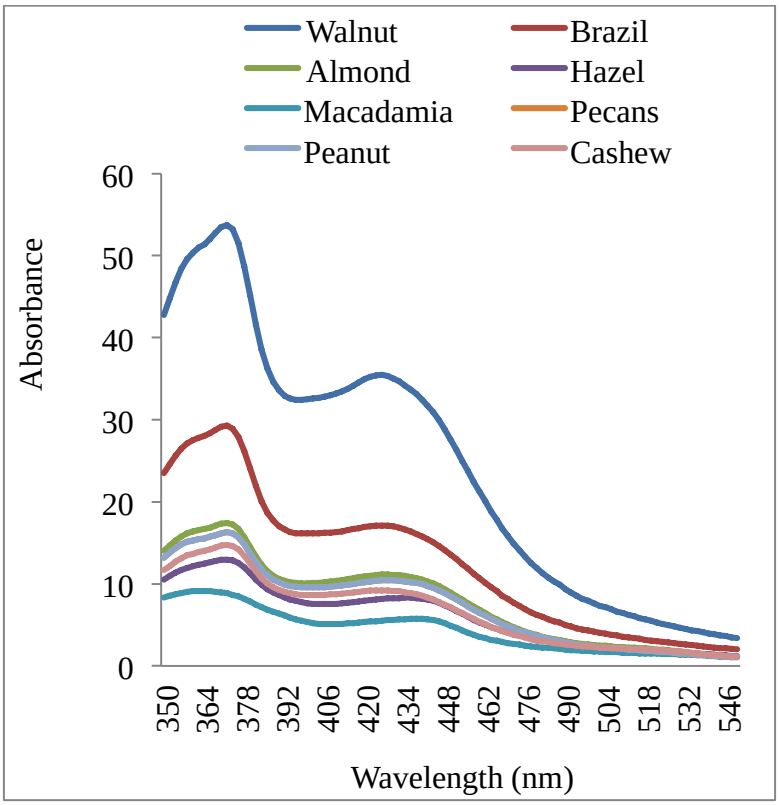


Figure 5.5: Absorbance spectra of nuts tested

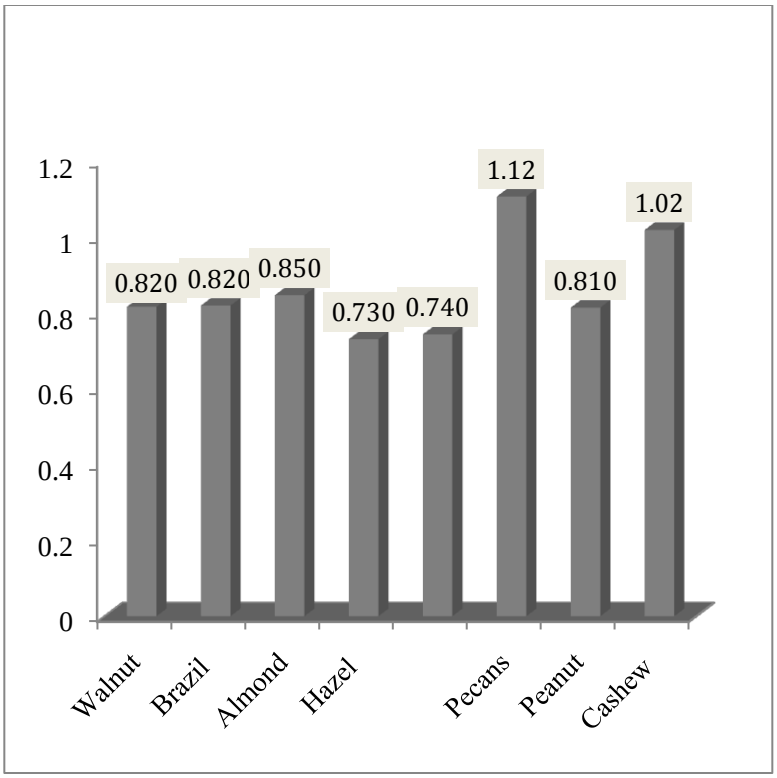


Figure 5.6: The ω-6: ω-3 ratio of the nuts tested

5.3.4 Milk

Seven brands of milk were analyzed in this study (Figure 5.7). The ratio of ω-6:ω-3 PUFAs is between 2.42-3.18 (Figure 5.8). Goat's milk had the lowest ratio of ω-6:ω-3 PUFAs (2.30). The different brands of milk were generally noted to have low levels of ω-3 PUFAs.

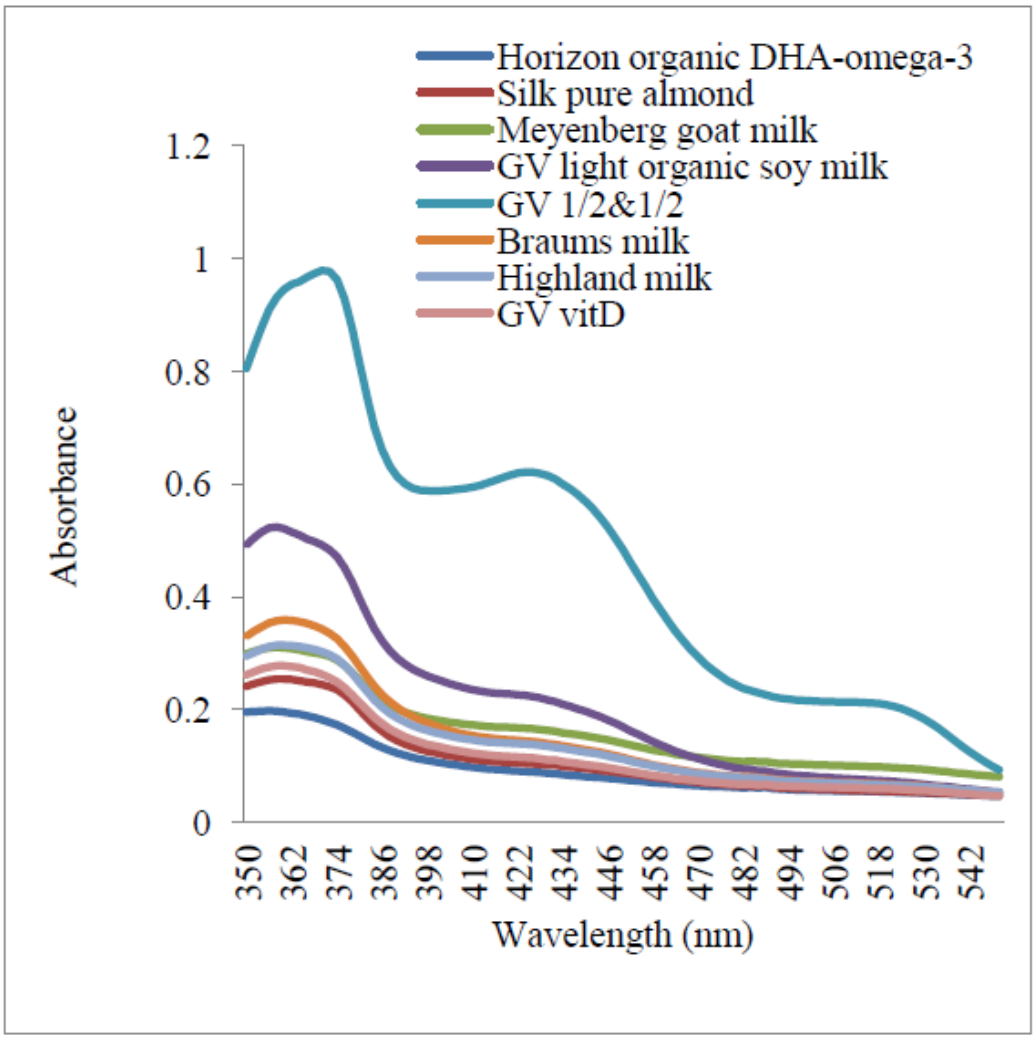


Figure 5.7: Absorbance spectra of the tested milk products

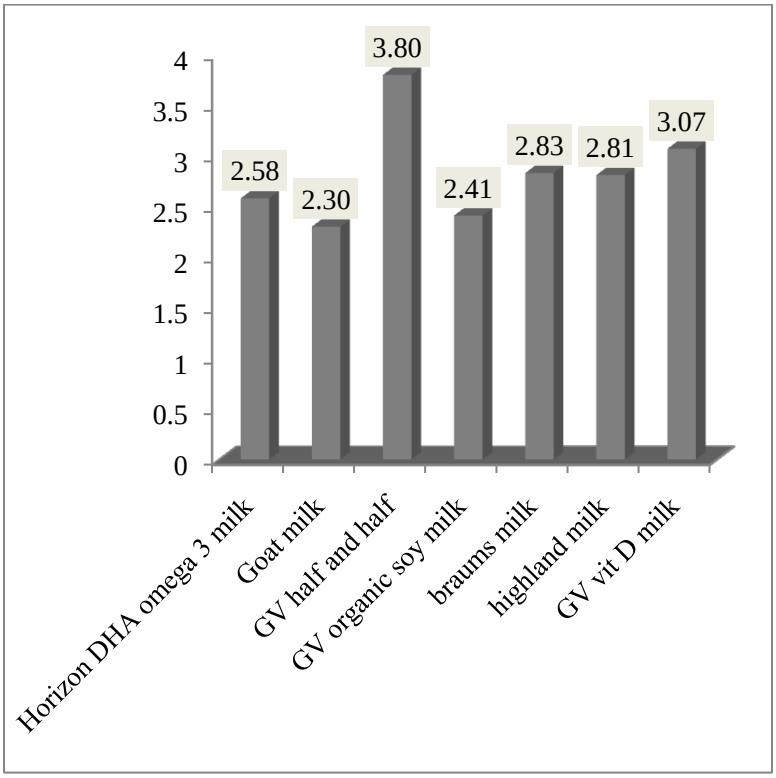


Figure 5.8: The ω-6: ω-3 ratios of tested milk products

5.4 Discussion

5.4.1 Meat

According to many researchers an ω-3:ω-6 ratio around 1/1–2/1 has a protective effect against the development and progression of breast cancer and colon cancers.[12-14] Our findings therefore suggest that beef loin or lamb loin (ω-3:ω-6 ratio 1.02 and 1.10, respectively) would be better choice of meat with respect to the levels of PUFAs. Linoleic acid was noted as the main component of ω-6 PUFAs in meat while AA and CLA were below the detection limit. The US food database report does not differentiate the different PUFAs in meat.[22] This study therefore improves the identification of PUFAs, an aspect that is of importance in food labeling.

5.4.2 Pizza

It was noted that the ω-6:ω-3 ratio of vegetable pizza is 1.72. The main PUFA in vegetable pizza is ω-6. From the pizza products, vegetable pizza is preferably a better choice in terms of ω -6 and ω-3 PUFAs while pork sausage would be the least preferred choice. So far no attempt has been made to label the ω-6:ω-3 on pizza or other foods. Some restaurants that have attempted to label the health values of food only display the caloric content. This suggests that labeling the ratio of ω-6:ω-3 PUFAs in precooked or cooked food followed by awareness campaign would lead to the consumers making the right food choices therefore improving health which could lead to prevention of chronic diseases.

5.4.3 Nuts

Both walnuts and Brazil nuts have a 0.82 ratio ω-6:ω-3 of PUFAs however it was noted that in a given weight, walnuts, have 3 times more ω-3 fatty PUFAs than Brazil nuts. The only fact that makes walnut a better choice is that a smaller amount of walnuts is needed to achieve the adequate intake (AI) of ω-3 and ω-6 PUFAs level as compared to Brazil nuts. Walnuts are therefore more economical than Brazil nuts and all the other nuts analyzed in this study. On the other hand, pecans and cashew nuts had higher levels of ω-6 PUFAs than the ω-3 PUFAs (ω-6:ω-3 ratio 1.11 and 1.02 respectively). The knowledge of the ratio of ω-6:ω-3 in nuts can be utilized by food industries to improve breakfast cereals and snack's nutritive value by designing a nutritious highly palatable product with a specific or a cocktail mixture of nuts that account for the AI of ω-3 and ω-6 PUFAs levels resulting in a superior diet with the same or even improved taste.

5.4.4 Milk

This study suggests that the different types of milk can be fortified to improve the ω-3 levels or new formulas for cattle feeds could be used that would improve the ω-3 levels in milk. The US food nutrient database has no information for EPA and DHA neither CLA, ALA, LA concentrations on dairy products.[22]

5.5 Conclusions

This study adds an important aspect in labeling nutrient on the dairy products[14] (Table 5.1).

Table: 5.1: The proposed improved labeling of foods by including the levels of the major PUFAs

Nutrition Facts	
Size serving: 1 cup (218g)	
Amount per serving	
Calories 1927	Calories from Fat 1927
	% Daily Value
Total Fats 218g	**335%**
Saturated Fat 19.84 g	99%
Major polyunsaturated fats	
Omega -3 (Good Fats)	
18:3 (ALA)	
20:5 (EPA)	
22:6 (DHA)	
Omega-6 (bad Fats)	
18:2 (LA)	
18:2 (CLA)	
20:4 (AA)	
Omega-6: 0mega-3 ratio	
A ratio of 1:1-1:2 is healthy for you	
Trans fat	

In summary, the results of this study demonstrate that it is possible to assess food quality based on the ratio of ω-6:ω-3 ratio. Our method can assist and direct customers to make good food choices. Additionally, this information can also be used to develop and formulate functional foods that are capable of reducing chronic diseases as well as improve the nutrition value of already existing foods and food supplements formulas. The study also describes a novel assay that is economical and fast to quantify PUFAs in foods and might be useful for creating a data bank for the major PUFAs in fresh foods, dairy products, meats, nuts, breakfast cereals and other food products.

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CHAPTER 6

GENERAL CONCLUSIONS AND RECCOMMEDATIONS

6.0 General conclusions

The strong correspondence of the Purdie assay, a non-enzymatic assay, with the Liebermann-Burchard reaction is very convincing evidence that this assay can also qualify as being the primary standard method not only for cholesterol but also for all other substances with allylic groups plus any corresponding functional groups with a triple bond.

The Purdie assay is an inexpensive, sensitive and accurate test that measures the concentrations of the 7 most abundant serum lipids in mole/L and has similar sensitivity with that of GC/MS. It can effectively be used to improve the current methods of reporting PUFAs during lipid profile testing in serum and in foods

6.2 Recommendations

More research on the relationship of PUFAs in TC, HDL-C and non-HDL-C fractions is necessary.

APPPENDICES

APPENDIX 1

Copy of Oklahoma State University Wellness Center blood testing consent form

OSU WELLNESS CENTER
INFORMED CONSENT FORM

BLOOD TESTING

Explanation of Test

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

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

Possible Risks

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

Consent by Subject

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

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

Date_____

Subject Signature_____

VITA

Mary Waruguru Kimani

Candidate for the Degree of

Doctor of Philosophy

Thesis: VALIDATION AND THE APPLICATION OF THE PURDIE ASSAY IN THE ANALYSES OF THE MAJOR FATTY ACIDS

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Name: Mary Waruguru Muriuki Kimani Date of Degree: July, 2012*

Institution: Oklahoma State University Stillwater, Oklahoma

Title of Study: VALIDATION AND APPLICATION OF THE PURDIE ASSAY IN THE ANALYSES OF THE MAJOR FATTY ACIDS

Pages in Study: 94 Candidate for the Degree of Doctor of Philosophy

Major Field: Chemistry

Scope and Method of Study:
Cardiovascular diseases resulting from the accumulation of triglycerides in the heart blood vessels result in human mortality around the world. This is, in part, due to lack of rapid and sensitive methods to quantify the different types of triglycerides associated with these diseases. To address this research gap, the objectives of this study were:

1) To determine the sensitivity of a newly developed Purdie assay relative to GC-MS in the quantification of omega-3 (ω-3) and omega-6 (ω-6) polyunsaturated fatty acids (PUFAs) in human serum.
2) To correlate the Purdie assay with other conventional methods.
3) To quantify the levels of ω-3 and ω-6 PUFAs and their ratios in the Total Cholesterol (TC), High Density Lipoproteins Cholesterol (HDL-C) and Low Density Lipoprotein (LDL-C) fractions of human serum using the Purdie assay,
4) To test and quantify the major PUFAs in different types of food.

This work contributed in part to quantifying 6 PUFAs and their ratios in human serum and foods. Blood serum specimens, obtained from Hillcrest Medical Center (HMC) were analyzed on the same day using GC/MS vs. the Purdie assay. Results obtained from the two methods were compared using the SAS t-test and resulted in a good agreement between the total ω-3 and total ω-6 PUFA levels and for the ω-6:ω-3 ratios. In an attempt to refine the Purdie assay, 24 human serum samples were analyzed for TC, HDL-C and LDL-C using the Purdie assay. The data were correlated with the results obtained by HMC clinical staff at $\alpha = 0.05$. Results showed no significant difference for TC and HDL-C data.

Findings and Conclusions:
Our findings suggest that Purdie assay is an accurate and sensitive assay that can be used to quantify PUFAs in human serum as well as in food products. These properties, along with its relatively low cost, make it a useful assay in identification of the risks posed by different types of foods as well as early detection of triglyceride-related cardiovascular diseases.

ADVISER'S APPROVAL: Dr. Neil Purdie