THE CHEMICAL ANALYSIS OF LEBANESE OLIVE OIL AND THE INFLUENCE OF OIL ENRICHED DIET ON PLASMA LIPID PROFILE IN RATS

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Requirements For The Degree Of Doctor Of Pharmacy

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ABSTRACT

THE CHEMICAL ANALYSIS OF LEBANESE OLIVE OIL AND THE INFLUENCE OF OIL ENRICHED DIET ON PLASMA LIPID PROFILE IN RATS

By Karen Zarikian

In the second half of this century, emphasis on diet related diseases increased as the major cause of mortality shifted from infections to chronic diseases. Cardiovascular disease (CVD) has been the number one killer in the United States since 1900. According to the American Heart Association 2002 Heart Statistical Update, one death is reported from CVD every 33 seconds. Atherosclerosis is the major underlying cause of coronary heart disease. Diet rich with cholesterol and saturated fat is believed to increase plasma levels of total and low density lipoprotein cholesterol, the primary lipoprotein mediating atherosclerosis. According to the 2001 guidelines of the National Cholesterol Education Program Panel III, dietary treatment must proceed pharmacological therapy in hyperlipidemic patients for a three month period. It has been hypothesized that vegetable oils such as olive oil, canola oil, soybean oil, are major sources of monounsaturated and polyunsaturated fatty acids and that they have more favorable effects on the distribution of cholesterol. Olive Oil is the major fat used in the

Mediterranean diet. Different environmental factors and harvesting techniques influence the chemical composition of olive oil.

The primary objective of this study was to identify the fatty acid, sterol and squalene concentrations and acidity in the Lebanese olive oil prepared by the cold press and automated methods. Another objective was to observe the influence of a ten week olive oil enriched diet on the plasma lipid profile, insulin levels, coagulation time and Apolipoprotein B 100 and B 48 levels in rats.

Olive oil samples were obtained from 11 different Lebanese areas. The identification of the fatty acids, sterols and squalene was performed on gas chromatography mass spectrometry (GCMS) and the acidity was identified by titration. In the second phase of the study, 42 rats were divided into three groups and fed for 10 weeks the following diets: a) normal rat diet, b) diet enriched with olive oil prepared by the automated method and c) diet enriched with oil prepared by the cold press method .

The oil of black olives prepared by the automated method from Beshaale (Northern Lebanon) contained the highest percentage of oleic acid and sterol concentration, whereas the oil from Zrarieh (southern Lebanon) contained the least. On the other hand, the highest squalene concentration was found oil obtained from green olives brought from Beshaale. The most acidic oil was the one obtained from Koura, Kfar Akka (northern Lebanon), prepared by the automated method.

Following ten week low fat verses high fat diet, no significant difference was observed among the various groups in lipid profile, insulin and Apo B 100 & 48 levels. There was a decrease in clotting time in the group fed the automated olive oil compared to the

control and an increase in the clotting time in the group fed cold press olive oil compared to the automated. These findings suggest possible altered fat metabolism in rats. Further studies in humans might yield better conclusive results.

In conclusion, the present study was able to identify the chemical composition of Lebanese olive oil in terms of fatty acids, sterols and squalene content. In addition, the increased acidity in some of the consumed olive oils is of great concern. In order to observe difference in lipid profile, well designed dietary experiments in humans is needed, because the squalene metabolism in rats is different than in humans.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

Apo B : apolipoprotein B

CAD : coronary artery disease

CVD: cardiovascular disease

ELISA: enzyme linked immunosorbent assay

GCMS: gas chromatography mass spectrometry

HDL : high density lipoprotein

IOOC : international olive oil council

LDL : low density lipoprotein

MUFA : monounsaturated fatty acids

OO : olive oil

PUFA : polyunsaturated fatty acids

SFA : saturated fatty acids

VLDL : very low density lipoprotein

TO MY BELOVED PARENTS

Chapter 1

Introduction & Literature Review

In the second half of this century, emphasis on diet related diseases increased as the major cause of mortality shifted from infectious to chronic diseases. Cardiovascular disease (CVD) has been the number one killer in the United States since 1900. According to the American Heart Association 2002 Heart Statistical Update, one death is reported from CVD every 33 seconds. [1]

1.1 Atherosclerosis and coronary artery disease

Atherosclerosis is the major underlying cause of coronary artery disease (CAD). It is a progressive disease that begins in childhood and is manifested in middle – late adulthood. The atherosclerotic lesions arise from transport and retention of plasma low density lipoprotein cholesterol (LDL-C) through the endothelial cell layer. Once in the artery wall, LDL is oxidized by various oxidative products produced locally. These oxidized particles are among the major contributing factors for atherosclerosis development, since they recruit monocytes into artery wall, which once transformed to macrophages accelerate LDL oxidation. Macrophages don't have LDL receptors on their surface. Instead, they have scavenger receptors that recognize only LDL particles that have been chemically modified. One modification occurs when linoleic acid in the lipoprotein particle becomes peroxodized. These macrophages then form foam cells and fatty streaks, the initial lesions of atherosclerosis. These fatty streaks progress to a fibrous plaque, which eventually grow larger and occlude the artery causing myocardial infarction. [2]

1.2 Lipid Transport

The lipoproteins are spherical complexes of lipids and specialized proteins that transport cholesterol, triglycerides and phospholipids.[2] LDL and High Density Lipoprotein (HDL) are among the major lipoproteins in the plasma that are classified according to their density, which is inversely proportional to their lipid content. LDL particles are the most atherogenic, since they are rich in apolipoprotein B and cholesterol. [2,3]

1.2.1 Lipoproteins

Cholesterol and phospholipids are among the plasma lipids that are associated with the membranes of various cells. These molecules are insoluble and can't be easily transported in the aquous environment of the blood. This problem has been solved by stabilizing the lipid particles with a coat of amphiphilic compounds – phospholipids and proteins. The protein moieties are known as lipoproteins and have much more than a stabilizing role. They also confer specificity on the particles, which help them for recognition by receptors. [2,3]

1.2.2 Lipoprotein Structure

Lipoprotein core contains triacylglycerol molecules and more hydrophobic lipids, cholesteryl esters. These hydrophobic molecules are protected from the aquous environment by a coat of more hydrophilic lipids, the phospholipids and free cholesterol. The molecules are arranged with their more hydrophobic portions oriented towards the core of the lipoprotein particles and their hydrophilic regions pointing outwards towards the plasma. The other major components of the lipoprotein particles are the proteins known as apolipoproteins. The particles contain an amphipathic helix, where the

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hydrophobic face interacts with the acyl chain of the phospholipids and the hydrophilic region faces the polar region of phospholipids. The major sites of apolipoprotein biosynthesis are the liver and intestinal mucosa. [3]

Biosynthesis of human apolipoprotein B48 (Apo B48) occurs exclusively in the intestine, whereas apolipoprotein B100 (Apo B100) appears to be of hepatic origin. [4] The two forms of Apo B are the surface components of both triacyglyceride rich lipoprotein (very low density lipoprotein and chylomicrons) and low density lipoprotein . Therefore Apo B serves as a marker of lipoprotein particle and unlike other lipoproteins it does not exchange between different lipoprotein particles.

1.2.3   Lipoprotein Classification

Lipoproteins are classified by their density into four major classes; chylmicrons, very low density lipoprotein (VLDL), low density lipoprotein and high density lipoprotein (HDL). The lipid content is inversely proportional to their density. LDL is the primary carrier of cholesterol for delivery to all tissues, therefore they are the most atherogenic. The exclusive apolipoprotein of LDL is Apo B 100. [2]

1.3   Cardiovascular disease and diet

Cholesterol is an essential component of cell membrane and precursor of steroid hormone, therefore adequate levels have crucial effects on survival. The cholesterol pool in the body is derived from absorption of dietary cholesterol and biosynthesis in the liver. Dietary sources of cholesterol include animal fat and some plants. [5]

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Researchers have been trying since 1908 to elucidate clear links between diet and cardiovascular disease to be able to cure and prevent it. [6] Different dietary factors were found to be correlated with the incidence of coronary artery disease among which the type and amount of fat consumed.

1.4   Lipids in Human Nutrition

Lipids in human diet are derived from storage and structural lipids of plants and animals. Dietary fat consists mainly of triacylglycerols each molecule having three fatty acids esterified to glycerol. The major classes of fatty acids are saturated, monounsaturated and polyunsaturated. [7]

1.4.1   Fatty Acid Classification

The fatty acids can be synthesized in the human body except the two simplest polyunsaturated fatty acids, linoleic and α-linolenic acids which can be formed only in plants. These are referred to as essential fatty acids since they are essential to human life and health. Fatty acids are called saturated when the molecules attain the maximum number of hydrogen atoms such as palmitic and stearic acids. When the fatty acid molecule contains one double bond it is called monounsaturated. The oils of olive and canola are examples of monounsaturated fat (MUF). When the molecule possesses more than one double bond the fatty acid is called polyunsaturated [3],such as linoleic and linolenic acids. Table 1 shows a comparison of the three fatty acid contents in the most commonly consumed oils.

Dietary fat	Saturated Fat	Monounsaturated Fat	Polyunsaturated Fat
Canola oil	7 %	61 %	21%
Corn oil	13 %	29 %	57 %
Safflower oil	10 %	14 %	76 %
Sunflower oil	12 %	16 %	71 %
Olive oil	15 %	75 %	9 %
Soybean oil	15 %	23 %	54 %
Palm oil	51 %	39 %	10 %
Butter fat	68 %	28 %	3 %
Coconut oil	91 %	7 %	2 %

(From Lebanese Association for Nutrition and Food Sciences conference; June 1, 2002)

Table 1 : Fatty Acid Content of Most Commonly Consumed Oils

Carbon Atoms/ Double Bonds	Structure	Common Name
Saturated Fatty Acids		
12:0	$CH_3(CH_2)_{10}CO_2H$	lauric acid
14:0	$CH_3(CH_2)_{12}CO_2H$	myristic acid
16:0	$CH_3(CH_2)_{14}CO_2H$	palmitic acid
18:0	$CH_3(CH_2)_{16}CO_2H$	stearic acid
20:0	$CH_3(CH_2)_{18}CO_2H$	arachidic acid
Unsaturated Fatty Acids		
16:1	$CH_3(CH_2)_5CH{=}CH(CH_2)_7CO_2H$	palmitoleic acid
18:1	$CH_3(CH_2)_7CH{=}CH(CH_2)_7CO_2H$	oleic acid
18:2	$CH_3(CH_2)_4(CH{=}CHCH_2)_2(CH_2)_6CO_2H$	linoleic acid
18:3	$CH_3CH_2(CH{=}CHCH_2)_3(CH_2)_6CO_2H$	linolenic acid
20:4	$CH_3(CH_2)_4(CH{=}CHCH_2)_4(CH_2)_2CO_2H$	arachidonic acid

Figure 1: The Most Abundant Fatty Acids In Fats, Oils And Biological Membranes

1.4.2 Role of Essential Fatty Acids

Linoleic and α-linolenic acids play two major important roles in the body. The first role is stabilizing biological membranes by creating physical properties that are optimal for the transport of substances across the membrane and for the biochemical reactions occurring in the membrane. The second major role is related to their metabolic conversion into oxygenated compounds, the eicosanoids. These molecules are involved in platelet aggregation, kidney function regulation, reactions of immune system, secretion of hormones and constriction or dilation of blood vessels. [7]

1.4.3 Dietary Fat and Lipid Profile

Saturated fat was found to increase total and LDL cholesterol. These findings were confirmed in a study where substitution of saturated fat with monounsaturated fat resulted in 15% reduction in plasma LDL cholesterol concentration.[8] This effect was justified by the fact that saturated fats increase LDL cholesterol concentration by inhibiting LDL receptor mediated catabolism.[9] On the other hand, monounsaturated fats were found to enhance LDL apo B clearance relative to saturated fats, thus resulting in hypocholesterolemic effect.

These findings led to the promotion and a better consumption of vegetable oils rich in unsaturated fatty acids as corn oil, olive oil, sunflower oil, soybean oil, canola oil, substituting for saturated fat like butter and coconut oil. Olive oil has the highest MUFA content among the other vegetable oils which accounts for 75% of its fatty acid composition. Controversy arose whether to use monounsaturated fatty acids (MUFA) or polyunsaturated fatty acids (PUFA) as replacement for saturated fat, since these two fatty

acids have similar effects on lipid profile. Recently some studies documented that serum total cholesterol may decrease to a similar extent when oleic acid, a MUFA or linoleic acid, a PUFA are substituted for SFA.[10]

Other studies showed that, diets rich in MU fat was as effective as a diet rich in PU fat in lowering LDL cholesterol.[11] Another study showed that MUFA decreased LDL oxidative susceptibility and decreased platelet aggregation, thus suggesting a possible protective effect against thrombogenesis. [12]

Overall data indicate that MU fats do not lower LDL or HDL cholesterol relative to saturated fat as much as does polyunsaturated fat.[9] Unsaturated fat, however have greater tendency for oxidation due to their nature. In contrast to polyunsaturated fats, monounsaturated fats have the important advantage of being less susceptible to oxidation. Lipid peroxidation is pronounced in PUFA because more double bonds are present in these fatty acids, which allow easy removal of hydrogen atoms. For every one gram of PUFA, 0.4 units of vitamin E is needed to counteract oxidation.[7]

1.5 Dietary Modifications in Hyperlipidemia

The National Cholesterol Education Program Panel III recommends 12 week dietary therapy for hyperlipidemic patients before considering the use of medications [13]. If the plasma lipid profile wasn't improved, pharmacotherapy along with dietary modifications follow to assist achieving recommended LDL goals. According to these recent guidelines, one diet is recommended for all patients as opposed to the two step diet in the previous guidelines. These modifications in the diet were after the results of a recent study that showed 13% lower triglyceride concentrations in the plasma of patients

consuming monounsaturated fatty acid rich with olive oil and 11% higher concentrations in those on step II diet. In addition, the HDL-C was not changed in monounsaturated fatty acid consumers but was lowered by 4% in patients consuming step II diet. The study concluded that olive oil rich diet decrease CHD risk by 25% compared to step II diet which decrease the risk by 12% .[12]

The step II diet restricts saturated fat daily intake to < 7%, polyunsaturated and monounsaturated to < 10% and cholesterol to < 200 mg/day. The one diet recommended for all hyperlipidemic patients include total fat intake ranging between 25-35% of which < 7% is saturated fat, < 10% polyunsaturated fat and < 20% monounsaturated fat. There was an increase in monounsaturated fat consumption requirements in the new guidelines. The adult daily total fat intake is 60 g/day of which saturated fat intake is 12 g, polyunsaturated fat is 17 g and monounsaturated fat is 31 g. Therefore, dietary modification of fat remains the cornerstone of CHD prevention.

1.6 Mediterranean Diet

The Mediterranean diet is rich with vegetables, fruits, grain and olive oil. In 1993, the International Conference on the mediterranean diet held in Cambridge developed mediterranean diet pyramid where the major source of fat was the monounsaturated fatty acid, oleic acid. Linoleic acid, a polyunsaturated fatty acid is present in smaller quantities. The MU fat content of olive oil not only improves lipid profile, but also reduces platelet aggregation by decreasing activation time of factor VII.[14] In addition, the antioxidant properties of vitamin E present in OO is considered first line of defense against LDL oxidation. Recently, the Seven Countries study revealed longer life

expectancy and low rate of CAD among the Cretan population, regular consumers of olive oil, compared with other populations with different eating habits.[15] Alarcon de la Lastra et al conducted a study in type II diabetes and suggested that diet high in monounsaturated fatty acid content represents a valid option to classic diet rich in carbohydrate as it ameliorates glucose tolerance and exerts a positive effect on lipid profile.[18] Figure 2 shows the Mediterranean Diet Pyramid.

1.7   Olive Oil

The evergreen tree of olive symbolizes peace and life. The origin of olive tree dates back to 5000 years ago in the mediterranean region. Its leaves were regarded as curative when boiled in water and taken as infusion for hypertension and various heart conditions and diuretic. [16]

1.7.1   The International Olive Oil Council

The International Olive Oil Council (IOOC) an intergovernmental organization founded in 1959, is responsible for administering the international agreement on olive oil. The council has over 25 members, including Lebanon since 1973. The international agreement lays down the policy that members should take on the standardization of the market for olive oil and determine the quality of the products. [17]

The Traditional Healthy Mediterranean Diet Pyramid

Daily Beverage Recommendations:

6 Glasses of Water

Wine in moderation

Monthly — MEAT

Weekly — SWEETS, EGGS, POULTRY, FISH

CHEESE & YOGURT

OLIVE OIL

FRUITS, BEANS, LEGUMES & NUTS, VEGETABLES — Daily

BREAD, PASTA, RICE, COUSCOUS, POLENTA, OTHER WHOLE GRAINS & POTATOES

Daily Physical Activity

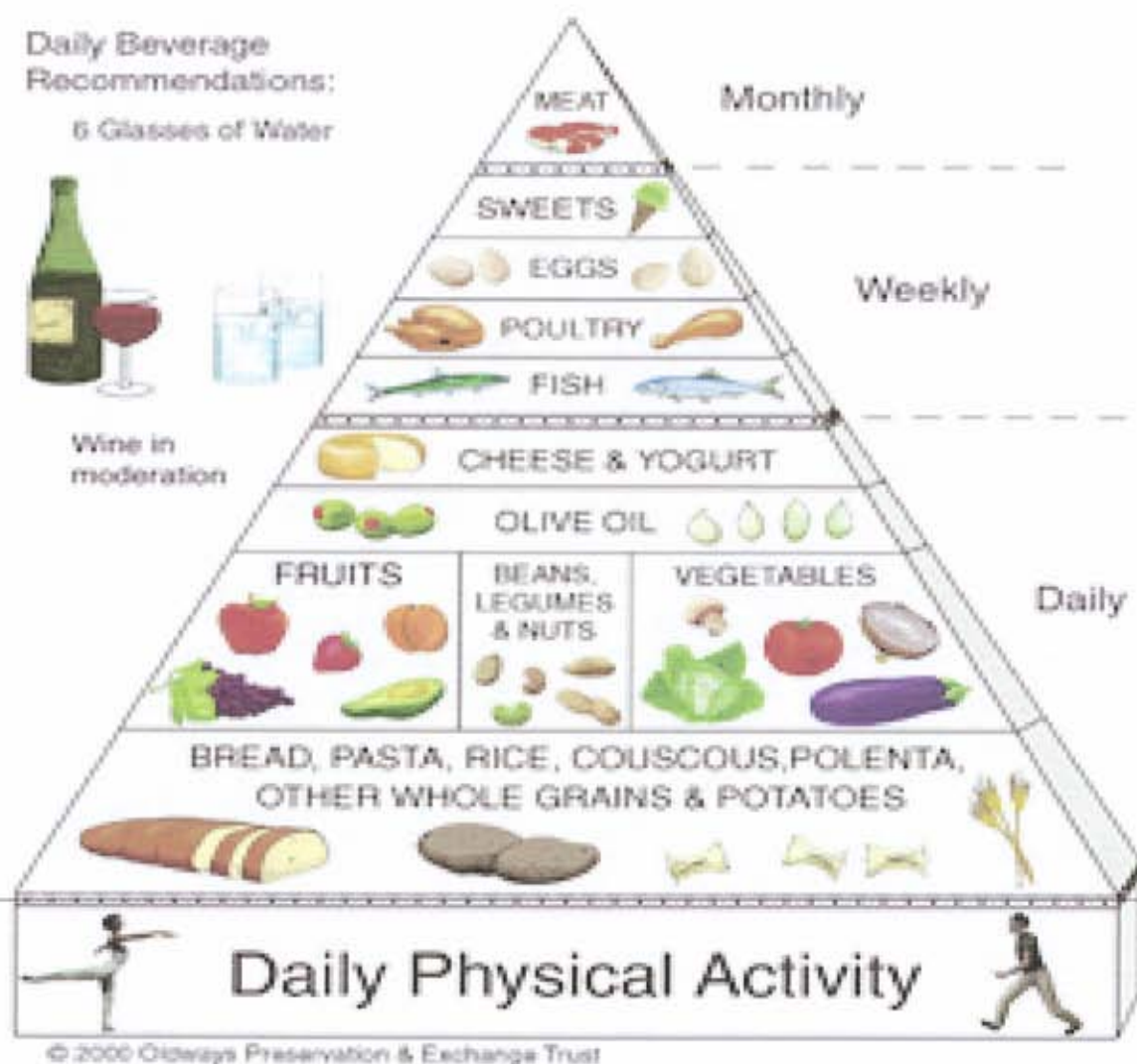© 2000 Oldways Preservation & Exchange Trust

Figure 2: Mediterranean Diet Pyramid

1.7.2   Types of Olive Oil

Olive oil is the oil obtained from the fruit of the olive tree *Olea europaea*. This oil contains four major fatty acids. The major fatty acid is the MUFA oleic acid, to a lesser extent the two SFA palmitic and stearic and the PUFA linoleic (Figure one).The IOOC has developed three definitions for olive oil: Virgin olive oil, Refined olive oil and Olive oil. Virgin oil is obtained from the fruit of olive tree by mechanical or other physical means. The oil is not subjected to heat nor chemicals. This type of oil is subdivided into Extra virgin oil with free acidity of less than1%; Virgin oil with free acidity of less than 2%; ordinary virgin oil with acidity of less than 3.3% and the one with an acidity of more than 3.3% is not fit for consumption.

Refined olive oil is the oil obtained from virgin olive oil by refining methods as neutralization, bleaching and deodorization. This oil can be used for cooking. Finally the "olive oil" is the oil that consists of a blend of refined olive oil and virgin olive oil fit for consumption.

1.7.3   Factors Influencing Oil Composition

Different environmental factors and harvesting techniques influence the chemical composition of various olive oil samples.[7] Pollution, weather, soil, altitude and preparation methods contribute to the presence of altered chemicals in oils produced at different geographical areas. The further south olives are cultivated, the concentration of saturated fatty acids and linoleic acid of the oil increases, whereas the content in oleic acid diminishes. In addition, the ratio unsaturated/saturated fatty acid rises with the altitude and the ratio of oleic/linoleic increases similarly. The harvesting techniques

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include careful picking of olives to eliminate bruising and therefore prevent oil acidity. The quality of olive oil depends among other variables on the ripeness of olives and on the period of harvest. To obtain good quality oil, olives should be healthy, picked from the tree not from the ground and taken without delay to the oil mill for processing. If the olive is picked from the ground the quality of oil will be poor. [7,18]

The ripeness of olives is important for their harvesting, because the accumulation of fatty acids rises with maturity. Virgin olive oil obtained from overripe olives has the highest yield. Late harvesting, however increases the likely hood of higher free acidity. [7]

The main process leading to the deterioration of lipids are lipolysis and oxidation. [7] In olive oil, the former process usually begins while the oil is in the fruit, whereas the latter is mainly produced during the extraction process and storage. Although lipolysis is important in determining the taste of the oil, it is unlikely to be of any nutritional significance. Oxidation; however, leads to the formation of both unpalatable and toxic compounds and is thus nutritionally undesirable.

As lipids oxidize, they form hydroperoxides, which are susceptible to further oxidation or decomposition to secondary reaction products. These compounds adversely affect flavor, aroma, taste and nutritional value. Olive oil is oxidized when it comes into contact with oxygen, although certain substances in the oil act as antioxidants such as α-tocopherol.

1.7.4 Olive Oil Preparation Methods

There are several methods of OO preparation, the cold press, the automated, cetrifugation and percolation. Each method involves unique techniques that result in oils different in chemical composition and texture.

In order to extract the oil, olives must first be ground in a morter with stones. In the cold press method, olives are crushed and pressed to form the paste. The pressing system has been improved from manual procedures to electrically driven pumps. This paste moves into a cylindrical trough to form a homogenous mass. Then the oil is separated from paste by means of centrifugation. The oil prepared from this method is not subjected to heat nor chemicals. The residue that results from the cold press preparation is treated with heat and water. The oil extracted from the residue is usually of poor quality and higher acidity. This oil is used for soap manufacturing. [7,16]

On the other hand, the automated oil preparation involves the use of modern mills that use stainless steel equipment worked by electricity and computer. The washed olives disappear into a hopper at one end and the oil emerges at the other. This method clears impurities from the pressed oil, through a process known as refining. In the physical process of refining, the oil is put in a drum and heated to $180^{0}C$.

1.8 Significance and Objective

A recent study conducted in Spain evaluated the effect of 25 ml daily olive oil ingestion for one week on LDL resistance to oxidation in 16 healthy volunteers. They concluded that daily intake of 25 ml of olive oil improves resistance to LDL oxidation and lowers plasma LDL levels. [19]

On the other hand, another publication in the Journal of Lipid Research (2000) conducted in Finland, showed that OO rich diet results in higher concentrations of LDL cholesterol.[20] The chemical composition of the oil used consisted of significant amount of

squalene, a triterpene, the precursor of endogenous cholesterol. This chemical is naturally present in the olive oil at concentrations ranging from 3-9 mg/g.

The primary objective of this study was to identify the fatty acid, sterol and squalene concentrations and acidity in the Lebanese olive oil prepared by the cold press and automated methods. Another objective was to observe the influence of a ten week OO enriched diet on the plasma lipid profile, insulin levels and coagulation time in rats.

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Chapter 2

MATERIALS AND METHODS

2.1   Olive Oil Analysis

The study was approved and financed by the university research committee. OO samples were collected from 11 different Lebanese areas. Three from the southern region, three from the north, three from high altitude and one from Bekaa area.

2.1.1   Fatty Acid Analysis and Identification

Each OO sample was extracted using Fatty Acid Methyl Ester extraction procedure. The analysis of only one sample obtained from Monsef was not feasible due to extreme humidity. 10 µl of OO was pipetted into 1ml extraction solution. The mixture was heated for 10 min on 100^0C. After cooling to room temperature, 250 µl of reagent A (Boren Trifluoride) was added. This was heated for 30 min at 100^0C. After cooling the mixture, 500 µl of reagent B (buffer salt) was added. Following vortex, 2 ml reagent C (hexane, isooctane and ether mixture) was added and the solution was centrifuged at $3200min^{-1}$; 20^0C for 5 min. 1 µl of upper layer was injected into gas chromatography mass spectrometry (GCMS)[21] [mass selective detector HP 5973; GC system: HP 6890; column: HP-5MS, 30 m x 250 µm x 0.25 µm thickness; helium: carrier gas , velocity: 41 cm/sec. Pressure   of 99.6 Kpas, flow: 1.2 ml/min.; analysis done on programmed temperature mode from 125 to 250^0C with rate of 16^0C min^{-1}, isothermal for 6 minutes; injector temperature: 265^0C.]

2.1.2 STEROL IDENTIFICATION

Each OO sample was extracted using sterol extraction procedures. In order to quantify the ß-sitosterol content, a standard ß-sitosterol of 100 µg/ml solution was obtained. A 0.5 ml (containing 50 µg ß-sitosterol) of this solution was diluted in 50 mg of butter (butter was chosen because it does not contain sterol nor squalene). 50 µl containing 50 µg internal standard (5-α-cholestane) was added to the standard ß-sitosterol mixture and this solution was extracted using the sterol extraction procedure described below.

Forty micro liter of each oil was pipetted and 40 µl of internal standard (5-α-cholestane) were added to each oil sample prior to extraction. The mixture was vortexed and heated at 75^0C for 10 min and cooled to room temperature. 2 ml of reagent A (buffer salt) was added to the mixture and vortexed, after which 3 ml reagent B (hexane, isooctane and ether) was added. This mixture was centrifuged for 5 min at 3200 min^{-1} at 20^0C. Then 1ml of the upper layer was transferred into a clean tube. This was evaporated to dryness under gentle stream of nitrogen. 50 µl of reagent C (trimethy-silic derivative) was added to the dry test tube. The capped test tube was heated at 75^0C for 30 min. Then 1 µl of the final 50 µl solution was injected into the GCMS. [mass selective detector HP 5973; GC system: HP 6890; column: HP-5MS, 30 m x 250 µm x 0.25 µm thickness; helium: carier gas , velocity: 41 cm/sec. Pressure of 99.6 Kpas, flow: 1.2 ml/min.; analysis done on programmed temperature mode from 125 to 250^0C with rate of 16^0C min^{-1}, isothermal for 6 minutes; injector temperature: 265^0C.]

The chromatogram report included the area ratio and peak ratio of three peaks: squalene, ß-sitosterol and 5-α-cholestane. The concentration of ß-sitosterol in each sample was

calculated from the area ratio of the standard ß-sitosterol / cholestane solution which contained 50 µg ß-sitosterol and the area ratio of each sample which contained an unknown concentration of ß-sitosterol.

2.1.3   SQUALENE IDENTIFICATION

Standard squalene sample (3 ml) of 97% purity was added to 1g butter. 1 g of the internal standard 5-α-cholestane was added to the squalene mixture. This sample was then extracted using the sterol extraction procedure described in section 2.1.2. 1 µl of this sample was injected into the GCMS [mass selective detector HP 5973; GC system: HP 6890; column: HP-5MS, 30m x 250µm x 0.25µm thickness; helium: carrier gas , velocity: 41 cm/sec. Pressure of 99.6 Kpas, flow: 1.2ml/min.; analysis done on programmed temperature mode from 125 to 250^0C with rate of 16^0C min^{-1}, isothermal for 6 minutes; injector temperature: 265^0C.]

The chromatogram report included the area ratio and peak ratio of three peaks: squalene, ß-sitosterol and 5-α-cholestane. The concentration of squalene in each sample was calculated from the area ratio of the standard squalene / cholestane solution which contained 3 µg ß-sitosterol and the area ratio of each sample which contained an unknown concentration of squalene.

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2.1.4 Oil Acidity

The acidity of the 11 OO samples was measured by titration. 28 g of each sample was weighed and transferred into an erlenmeyer flask.[22] Meanwhile, 50 ml 95% ethanol was mixed with 15 drops of indicator phenolphthalein. This solution was neutralized with 0.1N sodium hydroxide and then heated to 40^0C. Each oil sample was mixed with an ethanol solution and titrated with 0.1N sodium hydroxide. The mixture was shaken vigorously until the first permanent pink color appeared. The color persisted for 30 seconds. The volume of the sodium hydroxide was recorded. The percentage acidity was calculated according to the following equation:

$$\frac{\text{ml of alkali x Normality x 28.2}}{\text{Mass of sample (g)}}$$

2.2   Animals and Dietary Facts

Forty two male sprague-dawley rats weighing 150 g were housed in metallic cages in an animal room with twelve hour light and dark cycles. The room was maintained at a temperature of 21^0C. The 42 rats were subdivided into three groups each containing 14 rats. All rats were fed for 10 weeks. The first group the control, were fed starch instead of olive oil, the second group was fed olive oil prepared by the automated method and the third group was fed olive oil prepared by the cold press method. All the animals were on normal rat diet to which 5% w/w olive oil prepared by the two methods was added or an equivalent caloric value from starch 11.25 g was added. The final fat compositions by

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weight were 9.75% and 4.47% respectively. The weight of all the rats was measured at baseline and bi weekly.

2.2.1   PLASMA MEASUEREMENTS

Following 10 weeks, blood samples were drawn after 12 hour fasting for lipid profile, insulin and Apo B analysis. Samples were divided into three categories:

2.2.1.1   BLOOD STUDY

After 12 hours fasting, the animals were anaesthetized using diethyl ether and placed supine on a homeothermic table. Anaesthesia was maintained by the use of a nose cone. A midline abdominal incision was made for about two thirds of the length of the abdomen and the inferior vena cava was exposed by exteriorizing the intestine that was displaced in saline – soaked cotton at 37^0 C.

Using 10 ml syringes containing disodium ethylene diamine tetraacetic acid (Na_2EDTA, 1 mg/ml), about 8 ml of blood were withdrawn from the inferior vena cava. Then the blood was collected in 10 ml tubes and centrifuged at a speed of 2000 g for 20 min at a temperature of 4^0C. Then the plasma was collected and divided into three samples: one sample (V = 2.5 ml) was used to isolate the lipoprotein fractions as described later; whereas the second and third volume of serum were collected in 1.5 ml Eppendorf tubes to be used for insulin and lipid profile analysis.

2.2.1.2   LIPID PROFILE ANALYSIS

Two ml was sent to Saliba Laboratories for total cholesterol, LDL, HDL, triglyceride and coagulation time analysis. The samples were collected into 200 µl sodium citrate. The plasma of 42 rats was analyzed using

2.2.1.3   INSULIN MEASUREMENTS

The plasma insulin level was determined using rat insulin ELISA kit (DRG international, Inc.)   25 µl blood was collected and centrifuged at 14000 min^{-1} , 20^0C for 10 min. The plasma samples were kept at room temperature. A conjugate solution was prepared by diluting 0.6 ml conjugate stock solution through the addition of 6ml conjugate buffer. 25 µl of each sample was dispensed into the appropriate wells of the kit. Then 50 µl of Anti-Insulin-Horse Reddish Peroxide conjugate into all wells. This was incubated for 120 minutes at room temperature on a horizontal shaker set at 700 ± 100 RPM. The plate was then washed by aspiration of the liquid from each well dispensing 350 µl of washing solution into each well and finally aspirating the contents of each well. The last two steps were repeated five times. After the final wash, the plate was inverted and tapped firmly against the absorbent paper. Following the washing step, 200 µl of peroxidase substrate was dispensed into each well within 15 min. The plate was then incubated for 15 min at room temperature away from sunlight. 50 µl of stopping reagent 1M sulfuric acid was dispensed into each well. The absorbances were read at 450 nm within one hour and results calculated.

2.2.1.4 APO LIPOPROTEIN B 100 ANALYSIS

2.2.1.4.1   Isolation of Lipoprotein Fractions

Each 2.5 ml of collected plasma was placed in the bottom of a 10 ml polycarbonate ultracentrifuge tube (Sorvall, Kendro, Laboratory Products) put on ice. To minimize proteolytic degradation, a preservative solution containing aprotonin, 2 mg/l, and phenylmethylsulfonylfluoride (PMSF), 10 mM dissolved in 2-propanol, were added at concentrations of 5 µl/ml plasma and 10µl/ml plasma respectively. Solid NaCl (0.4 g/ml) was added to the plasma samples and vortex mixed in order to increase the density to 1.1 g/ml.

A density gradient must be ensured, therefore three solutions of 1.065, 1.020 and 1.006 g/ml NaCl were prepared, each containing 0.01% (w/v) Na_2EDTA and 0.02% sodium azide (NaN_3) with the PH adjusted to 7.4.

Two ml of each salt solution were then sequentially layered on top of the plasma in the ultracentrifuge tube using the peristaltic pump machine (Watson-Marlow Limited, England). The gradients were then centrifuged for 24 hours at 15^0C to remove lipoprotein fraction Sf > 20 that includes the VLDL fraction.

After centrifugation, the top 0.5 ml of the density gradient (d < 1.006) was removed. This fraction was used for Apo B (Apo B 100 & B 48) analysis.

2.2.1.4.2 Preparation of Samples for Apo B analysis

Isolated samples of apoB-containing lipoproteins were delipidated according to Karpe et al. (1996)[23] in a methanol/diethyl ether solvent system. A volume of 400 μl of the lipoproterin fraction sample was injected into 4 ml ice-cold methanol in a 10 ml glass tube using syringes to ensure efficient delipidation. 4 ml ice-cold diethyl ether was then added to the methanol, followed by centrifugation for 30 minutes at a speed of 4000 g at a temperature of 1^0C. After centrifugation, the solvent was gently removed using a suction device. The protein material was then dissolved in 100 μl of 0.15 M sodium phosphate, 12.5% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) mercaptoethanol, 0.001% (w/v) Bromophenol blue, PH 6.8 at room temperature for about 30 min. This was followed by immediate addition of another 4 ml ice-cold diethyl ether and the sample vortexed, then undergone a second centrifugation for 20 minutes at a speed for 4000 g and at a temperature of 1^0C, after which , the diethyl ether was removed by the same suction device.

2.2.1.4.3 Preparation of Plasma Samples For Total Apo B Quantification

To each volume of plasma, 9 volumes of solution containing 0.15M sodium phosphate, 12.5% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) mercaptoethanol, 0.001% (w/v) bromophenol blue, pH 6.8.

2.2.1.4.4   **Sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)**

Principle

Proteins are amphoteric compounds that carry a net negative charge above their isoelectric point; hence have the ability to migrate towards the anode in an electric field. Separation of proteins by SDS-PAGE electrophoresis is based on coating all proteins with sodium dodecyl sulfate (SDS) so that all proteins have a uniform charge. Separation is therefore dependent on the size of the protein sodium dodecyl sulfate complex. Hence, in SDS-PAGE separations, migration is determined not by intrinsic electric charge of polypeptides but by molecular weight[24].

SDS is an anionic detergent with a long hydrophobic tail. It binds to the hydrophobic side chains of amino acids at a constant ratio of 1.4g of SDS to 1 g of polypeptide, thus conferring a net negative charge to the polypeptide in proportion to its length and to the molecular weight of the protein[25]. Larger proteins also bind to more SDS molecules. This ratio ensures that all SDS-protein complexes have a similar mass: charge ratio; hence, eliminating the intrinsic charge of the protein as a factor affecting migration[26]. Moreover, SDS blocks hydrophobic interactions, and substantially unfolds the protein molecules, minimizing differences in molecular form by eliminating the tertiary and secondary structures[27]. When proteins are treated with 2-mercatoethanol or dithiothreitol (DTT) (in addition to SDS), all disulphide bonds between cysteine residues are broken, producing subunits of proteins[25]. Urea may also be employed to disrupt hydrogen bonds.

In SDS-PAGE, a polyacrylamide porous gel is polymerized from a solution of acrylamide monomers into crosslinking chains, forming a semisolid matrix, where the pore size is similar to the size of the protein molecules[27]. This matrix is then buffered to weakly basic, so most proteins will be anionic and migrate to the anode of the gel when an electric current is applied. The pulling force of the electrical voltage and the opposing force of the gel matrix result in different migration rates fro the proteins. The migration rate is affected by: the electric current, the pore size of the gel and the size of the protein (i.e. smaller proteins move faster in the gel)[24]. In polyacrylamide gels, the effective pore size is inversely related to acrylamide concentration in the polymerization mixture [28.] Following SDS-PAGE separation, the protein bands are visualized directly on the polyacrylamide gel by staining with Coomassie Blue. Usually, 0.2-0.5 µg of any protein can be detected in a sharp band using this dye; and staining is quantitative up to 15-20µg for at least some proteins[28]. Not all proteins have the same chromogenicity with this dye since, different proteins bind Coomassie blue to ditterent extents. Therefore, the molecular weight can be estimated by comparison to standards.

Stock Solutions

- *Sample butter (two – fold concentrated)*: 20 ml glycerol was added to 1.5 g Tris dissolved in 25 ml of distilled water and PH adjusted to 6.75 with 12 M HCl. This was followed by the addition of 10 ml of 2-mercaptoethanol, 4 g SDS and 2 mg of bromophenol blue and diluted with distilled water to a final volume of 100 ml.

- *3M Tris buffer (PH 8.8):* 36.6g Tris was dissolved in about 80 ml distilled water and PH adjusted to 8.8 with 12 M HCl. The solution was then diluted with distilled water to a final volume of 100 ml. This solution is stable for several weeks at 4^0 C.

- *0.5 M Tris buffer (PH 6.8):* 6.02 g Tris was dissolved in about 90 ml distilled water and PH adjusted to 6.8 with 12 M HCl. The solution was then diluted with distilled water to a final volume of 100 ml. This solution is stable for several weeks at 4^0 C.

- *Urea/SDS/dithiothreitol solution :* 19.2 g urea, 12.3 mg dithiothreitol and 0.08 g SDS were dissolved in 30 ml of distilled water and the final volume was made up to 40 ml.

- *1.5% ammonium persulfate solution:* 0.15 g of ammonium persulfate was dissolved in 10 ml of distilled water.

- *Electrode buffer (PH 8.3):* 42.6 g glycine and 9.18 g Tris were dissolved in 2 L of distilled water, then 3 g of SDS were added to the Tris/Glycine solution and the final volume made up to 3 L with the addition of 1 L distilled water.

- *Prorogel:* 30 g acrylamide and 0.8 g N,N-bis-methylene acrylamide were dissolved in approximately 70 ml of distilled water; the final volume was adjusted to 100 ml.

- *Water-saturated n-butanol:* 90ml n-butanol + 10ml water.

2.2.1.4.5   SDS-PAGE preparation

Preparation of resolving and stacking gels

Following the method of Hames (1990) according to the original method of Laemmli (1970)[29], linear gradient (5-20%) polyacrylamide gels were prepared to allow an increased protein sieving effects and a better separation of a larger range of protein molecular weights.

A 5% acrylamide resolving gel, a 20% acrylamide resolving gel and a staking gel, to be layered on to p of the resolving gels, were prepared as shown in Table

	Resolving gel		Stacking Gel
	5%	20%	
Sucrose	----	6.75ml	----------
Tris	5.55ml	5.55ml	3.75
Protogel	7.50	30.0	1.85
Urea/SDS/Dithiothreitol	4.50	0.45	0.30
1.5% ammonium persulfate	1.05	1.05	1.15
Water	30.30	4.05	7.50

Table 2: Gel mixtures for 5-20 % gradient gel

Pouring of the Gels

The glass plates of the electrophoresis apparatus (Hoefer SE 600 Amersham Pharmacia Biotech) were assembled using two side spacers and clamps according to the manufacturer's instructions. To make sure that the plates were tightly clamped to prevent leakage, running in water from the gradient former tested it.

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Immediately before pouring the resolving gel, 11.5 µl of tetramethylethylenediamine (TEMED) were added to each of the gels to initiate polymerization. The 20 % gel was placed in the mixing chamber and the 5% gel in the reservoir chamber of a gradient forming apparatus (Coleparmer, USA). The mixing chamber outlet was connected to a peristaltic pump (Watson Marlow 503, U.K.) discharging between the glass plates of the electrophoresis apparatus. As the gels were poured into the chambers, stirring started and the valve outlet of the mixing chamber was opened. Simultaneously, the peristaltic pump and the valve separating the two chambers was opened, forming a 5-20% concentration gradient. After the resolving gel has been poured, it was overlayered with water-saturated butanol and allowed to polymerize. 45 min later, the water-saturated butanol was rinsed off with several washes of distilled water and discarded. The remaining droplets of water were removed with a filter paper. Before overlayering the polymerized resolving gel with the stacking gel, 11.25 µl of TEMED were added to the stacking gel. Immediately after pouring the stacking gel, a 15-well comb was dipped in a previously prepared electrode buffer, shaken dry and quickly inserted in the stacking gel between the glass plates leaving about 5 mm between the resolving and stacking gels at the bottom of each well. The poured stacking gel was allowed to polymerize for 30 min after which the upper and lower electrode chambers were filled with electrode buffer and the comb was carefully removed.

Preparation of Standard Apolipoprotein B100

To quantitate the apoliprotein B bands of rat lipoprotein samples, it was necessary to include in every slab gel, human serum samples previously standardized to their

apolipoprotein B 100. apolipoprotein B 100 derived from LDL was used as a reference protein for standard curve construction. The apo B 100 preparation was derived from LDL that is isolated from fasting plasma samples by the density gradient ultracentrifugation procedure. The LDL subfraction was recovered from a 1 ml portion located 5.5 to 6.5 ml below the to p of the gradient after 16 hour of ultracentrifugation. Fast and careful desalting was achieved by passing the LDL through a PD-10 co,umn (Pharmacia). The total protein content was then determined using the Lowry assay (1951) with addition of SDS (final concentration 1%) to the reagent turbidity. The mean of several determinations was taken as the final protein value. After delipidation of LDL as previously described, the dissolved protein mixture was denatured at 80^0C for 10 minutes. Batches of the delipidated and denatured apo B 100 protein standard can be stored at -80^0C and thawed immediately before use.

Loading Samples

Samples previously prepared by delipidation as described in section 2.2.1.4.1 were dissolved in sample buffer and stored at -20^0C. just prior to use, samples were thawed in preparation for loading. Similarly, the molecular weight markers (Sigma Chemical Co.Ltd) that were previously dissolved in sample buffer according to the supplier's instructions, aliquoted and stored at -20^0C.

Using disposable gel loading tips, 5 µl of molecular weight marker solution was loaded into the first gel lane. 0.2, 0.8 and 3.2 µg of standardized plasma samples were loaded in lanes 2-4. Then, 25 µl of the lipoprotein fractions or 15 µl of plasma under examination were loaded into the remaining lanes. All samples were run in duplicate.

Electrophoresis

After loading the samples, the upper chamber was covered with its lid and the electrodes were properly attached to the power supply (Consort E455, Belgium). The water-cooling system and the power supply were then switched on . Electrophoresis was carried out at 60 mA for two-gels until the bromophenol blue marker reached the resolving gel after which the current was raised to twice its starting value. Four hours later, the power was switched off, the water-cooling system detached and the gel carefully removed for later staining with Coomassie Blue

2.2.1.4.6   Staining and Quantitation of Proteins

The protein staining solution was prepared by dissolving Coomassie Blue R-250 (0.1% w/v) in water: methanol : glacial acetic acid (5:5:2 by volume). Wharman No. 1 filter paper was used to filter the solution so that any insoluble material was removed before use. The protein destaining solution that consisted of 250 ml 30% methanol and 10% glacial acetic acid was then prepared. The two solutions were placed in two containers connected to an automated gel stainer - destainer apparatus (Hoefer processor plus, Amersham Biotech). The stained gel slabs were carefully removed, placed between two transparencies and scanned using a desk top scanner (Scanjet 6100 C, Hewlett Packard). The image saved on the computer diskette, was used for quantitation of the apolipoprotein B bands using 1-D advanced software (Advanced American Biotechnology, 1166E Valencia Dr, #6C, Fullerton CA. 92831).

The concentrations of apo B were determined after constructing the standard curve.

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Chapter 3

RESULTS

3.1 Olive Oil Analysis

Eleven olive oil samples were collected from northern to southern Lebanon. Table 3 gives the details about the collected sample oils.

Sample Number	Geographical area	Preparation method
1	South - Marjayoun	Cold press
2	Bekaa	Cold Press
3	South - Zrarieh	Automated
4	South - Zrarieh	Cold press
5	North- Beshaale – black olives	Cold press
6	North - Beshaale – black olives	Automated
7	North - Beshaale – green olives	Cold Press
8	North - Akar	Cold Press
9	North - Koura	Cold Press
10	North - Koura	Automated
11	Mount Lebanon - Monsef	Cold press

Table 3: Geographical areas of Olive Oil samples and their preparation methods

3.1.1 Fatty acid

The fatty acid content of each of the olive oil was determined as percent content. This percentage of fatty acid in each oil sample was detected using the Hewlet Packard software area percent report. A standard containing the four fatty acids: stearic, oleic, palmitic and linoleic was injected before all the samples. The stearic acid peak appeared at around 8 minutes after the injection, Linoleic acid at 9 min, oleic acid at 9.09 min and palmitc acid at 9.22 min. The chromatograms of the samples are shown in appendix 1.

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Following the standard solution the 11 samples were injected into the GCMS. The percentage of each of the fatty acids is shown in table 4 . The oleic acid ranged from 65.132- 73.840%. The black olives prepared by the automated method contained the highest percentage of oleic acid (73.840%), whereas the oil from Zrarieh contained the least percentage (65.132). Linoleic acid ranged form 7.726-11.790 %. The oil from Koura had the highest percent. Sample 2 had the two final peaks overlapping although a split ratio of up to 110:1 was used for better separation.

Sample No	$C_{16:0}$ Stearic acid	$C_{18:0}$ Palmitic acid	$C_{18:1}$ Oleic acid	$C_{18:2}$ Linoleic acid
Standard	9.126	3.396	82.143	5.335
1	16.591	6.302	67.251	9.856
2	13.728	5.608	80.665	----------
3	16.809	6.606	65.132	11.454
4	17.627	4.847	69.660	7.866
5	11.685	6.965	71.406	9.944
6	11.887	4.857	73.840	9.416
7	10.430	7.688	73.426	8.456
8	15.984	6.246	68.354	9.9416
9	15.447	4.543	70.855	9.155
10	15.221	5.643	67.341	11.790

Table 4: percentage of the four major fatty acids in the 11 olive oil samples

3.1.2 Beta Sitosterol

Analysis of the sterols mainly the β-sitosterol was performed on all olive oil samples. The standard β-sitosterol with the internal standard was injected before all the samples. The area percent report showed 3 peaks: cholestane (6.47 min), cholesterol (10.51 min) and β-sitosterol (14.90 min). The peak height and the area of the three peaks is shown in appendix 2.

Subsequently the 11 samples were injected into the GCMS. The chromatogram showed three peaks: squalene(5.7minutes), cholestane (6.4 minutes) and β-sitosterol(14.9 minutes) . The concentration of β-sitosterol in each sample was calculated from the standard β-sitosterol/cholestane area ratio, which was equal to 0.248. This ratio corresponded to 50 μg of β-sitosterol in 50 mg butter. The concentration of β-sitosterol in the oil samples was determined from the area ratio of each sample relative to the standard area ratio (which corresponds to 50 μg of β-sitosterol). The area ratios of all the samples is shown in appendix 2.

Table 5 shows the concentrations of β-sitosterol in the various oil samples.

Sample No	Sterol Concentration
Standard	0.248
1	2.66
2	1.74
3	2.907
4	2.203
5	3.286
6	2.085
7	2.089
8	2.846
9	2.81
10	2.584
11	3.059

Table 5: sterol concentrations in the 11 oil samples expressed as mg/g

3.1.3 Squalene concentrations

Similarily, the concentration of the squalene was calculated from the standard squalene/cholestane area ratio which was equal to 2.210, where 3 µl of 97% squalene was mixed in 1 g butter. The area ratio of squalene/cholestane of each sample was obtained from the chromatogram report. The weight of squalene was determined relative to the standard. Table 6 shows the concentration of squalene in the various oil samples.

Sample No	Squalene Concentration
Standard	2.210
1	5.08
2	4.862
3	4.299
4	4.230
5	4.509
6	3.838
7	4.4
8	5.298
9	4.433
10	4.28

3.1.4 Oil Acidity

The acidity of the 11 oil samples was detected using the titration procedure described in section 2.1.4 The volume of sodium hydroxide needed to neutralize the oil samples ranged from 2.9-46.1 ml. Table 7 shows the volume of NaOH used, the weight of each oil sample and the calculated acidity expressed as percent oleic acid, since this fatty acid accounts for the largest percent of fatty acids in olive oil.

Sample No	NaOH volume needed to neutralize oil	Weight of oil measured	% Acidity
1	24	28.118	2.4
2	2.9	28.036	0.29
3	9.7	28.051	0.975
4	11.7	28.371	1.16
5	7.3	28.035	0.734
6	25.4	28.030	2.55
7	12.5	28.032	1.25
8	8.9	28.036	0.89
9	38	28.056	3.81
10	46.1	28.016	4.64
11	24.2	20.1	3.4

Table 7: Percent acidity to the 11 olive oil samples

According to the IOOC, olive oils with percent acidity of greater than 3.3 % are not suitable for consumption. There are four oil samples among the ones tested that exceed the 3.3 %. This increased acidity could be attributed to the increased oxidation due to various reasons as air or light exposure or to the harvesting techniques used like picking overripe olives and grinding them with the other olives.

3.2 Plasma Measurements

Following 10 week low fat verses high fat diet among 42 rats which completed the study, blood samples were drawn for lipid profile analysis, coagulation time, insulin level measures and Apo B 100 & 48 levels. The weight of all the rats was measured at baseline and bi weekly. Weights are shown in appendix 3.

3.2.1 Lipid Profile Analysis

The levels of total cholesterol, LDL, HDL and triglyceride levels of the three experimental groups were measured. There was no significant difference among the various measured parameters among the three groups. The measured values and the corresponding statistical analysis are shown in appendix 3.

3.2.2 Coagulation Time

The coagulation time of 12 rats was determined, since 2 samples were coagulated and the test was not feasible. This was attributed to the inadequate citrate amounts during sample collection. The coagulation time was significantly decreased in the automated oil fed group compared to control (p = 0.00016). On the other hand, the coagulation time of the cold press prepared oil fed group was significantly increased compared to the group fed the automated oil (p = 0.017). The values of the coagulation time of the various samples with the corresponding statistical analysis are shown in appendix 3.

3.2.3 Insulin Measurements

Insulin levels were measured using the rat insulin ELISA kit. There was no significant difference in the plasma insulin of the three groups fed different fat diets. The values of the plasma insulin are found in appendix 3.

3.2.4 Apo B 100 & 48 measures

Apo B 100 & 48 are the lipoproteins that reflect the atherogenicity of LDL. There was no significant difference among the three experimental groups. The values of the Apo B 100 & 48 are found in appendix 3.

Chapter 4

Discussion and Conclusion

Following the study conducted in Finland, where olive oil rich in squalene had a negative effect on lipid profile, we attempted to analyze Lebanese olive oil in terms of fatty acid, β-sitosterol, squalene concentrations and percent of oleic acid acidity.

The fatty acid content of the various samples was relatively influenced by the geographical area where the olives were matured. The oleic acid was highest in olive oils obtained from the northern region, unlike the oils from the south. The content of palmitic acid was also influenced by the cultivated area. The highest content was found in the oil obtained from the southern area and the lowest in the high altitudes. These findings confirm the conclusion of the study conducted by Alarcon et al., where the content of saturated fatty acids of the oil increase towards the southern area. In addition, the ratio of unsaturated / saturated fatty acids rises with the latitude.

On the other hand, linoleic and stearic acid content were not consistent with the cultivated environment. Lower linoleic acid content, however, has the advantage of less oxidation.

Sterols are the analytical finger prints that enable olive oil to be identified, since the β-sitosterol accounts for around 93% of oil sterol content. Plant sterols interfere with intestinal cholesterol absorption and have the potential to lower total and LDL cholesterol. Thus, olive oils containing high amounts of sterols, render cholesterol

absorption and might improve lipid profile. The highest β-sitosterol was found in olive oil obtained from black olives cultivated in Beshaale.

Squalene is the precursor of cholesterol. Olive oils containing the least amount of squalene are favored, since this will lower the extent of endogenous cholesterol synthesis. The highest amount of squalene was found in the oil obtained from Beshaale from green olives at 5.29 mg/g. The squalene content in the olive oil used in the Finland study was 3.65mg/g. This finding suggests that further investigation on olive oil diet high in squalene be conducted.

Among the 11 oil samples, 3 samples had acidity greater than the amount specified for consumption. This increased acidity might influence the gastric mucosa negatively and initiate serious health problems.

Rats were chosen for the experiment for the convenience of diet control. It was difficult to find healthy volunteers who would be reliable to complete the rules of the study, specially the control over high fat and low fat diet. The olive oil used in the rat diet was from Koura with squalene concentration of 4.33 mg/g (oil prepared by cold press method) and 4.28 mg/g (oil prepared by automated method). The analyzed lipid profile, insulin and Apo B 100 / 48 levels were changed without statistical significance among the three experimental groups. This could have been attributed to the difference in lipid metabolism in rats compared to humans, specially that the olive oils used in the diet contained 4mg/g squalene.

A study conducted by Tilvis et al[30]. indicated that an increase in dietary squalene load decrease the absorption of squalene but does not affect the absorption of cholesterol in

rats. They concluded that the rat intestine has a marked capacity for absorbing dietary squalene and that the absorbed squalene is converted to bile acids following which they are excreted in the feces. Our results were consistent with these findings, specially that plasma total cholesterol was not changed significantly. The process of squalene metabolism in human system might be somehow different than the one observed in rats.

There was no significant difference in insulin levels between the low fat and high fat diet groups. This could have been attributed to the normal insulin receptor activity, since non of the rates were considered diabetic. Some studies suggest the beneficial role of olive oil on Type II diabetes, therefore further studies on the role of olive oil on diabetic rats is needed.

In conclusion, the olive oil content of Lebanese olive oil was analyzed and facts about the fatty acid, β-sitosterol and squalene concentrations reported. The acidity measures were of great concern. This requires intervention and close regulation of the characteristics of consumption oils. There was no change on lipid profile in olive oil rich diet in rats. These findings, however can't be applied on humans, since there is difference in fat metabolism and squalene fate in these two different species. Further studies evaluating the effects of olive oil rich diet compared to other monounsaturated oils in humans must be conducted to observe the influence on lipid profile.

Reference

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APPENDICES

Appendix one : Chromatograms of the Fatty Acid Methyl Esters

First peak: stearic acid
Second peak : linoleic acid
Third peak: oleic acid
Fourth peak : palmitic acid

```
Operator    : Karen
Acquired    : 17 Jun 2002  12:52    using AcqMethod FAMESTD3
Instrument  :  GC/MS Ins
Sample Name: STD fame
Misc Info   :
Vial Number: 1
```
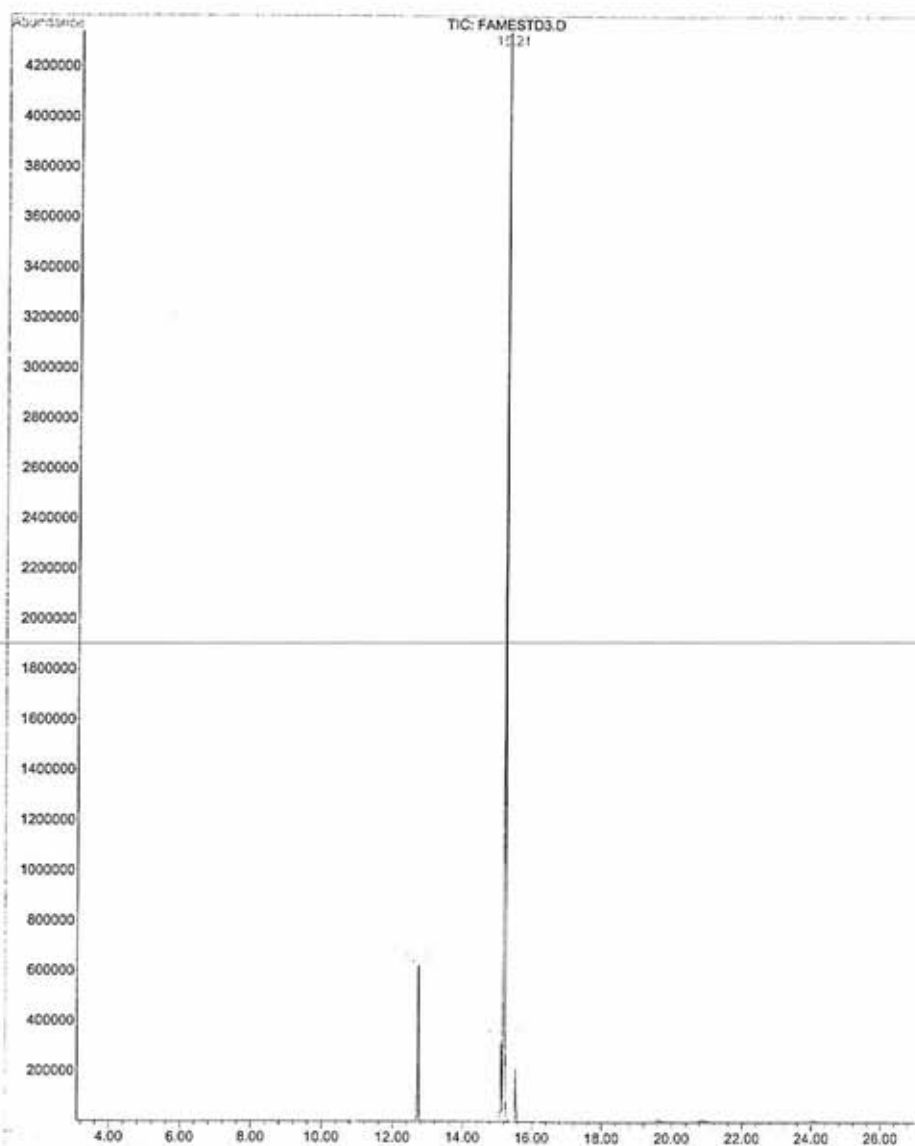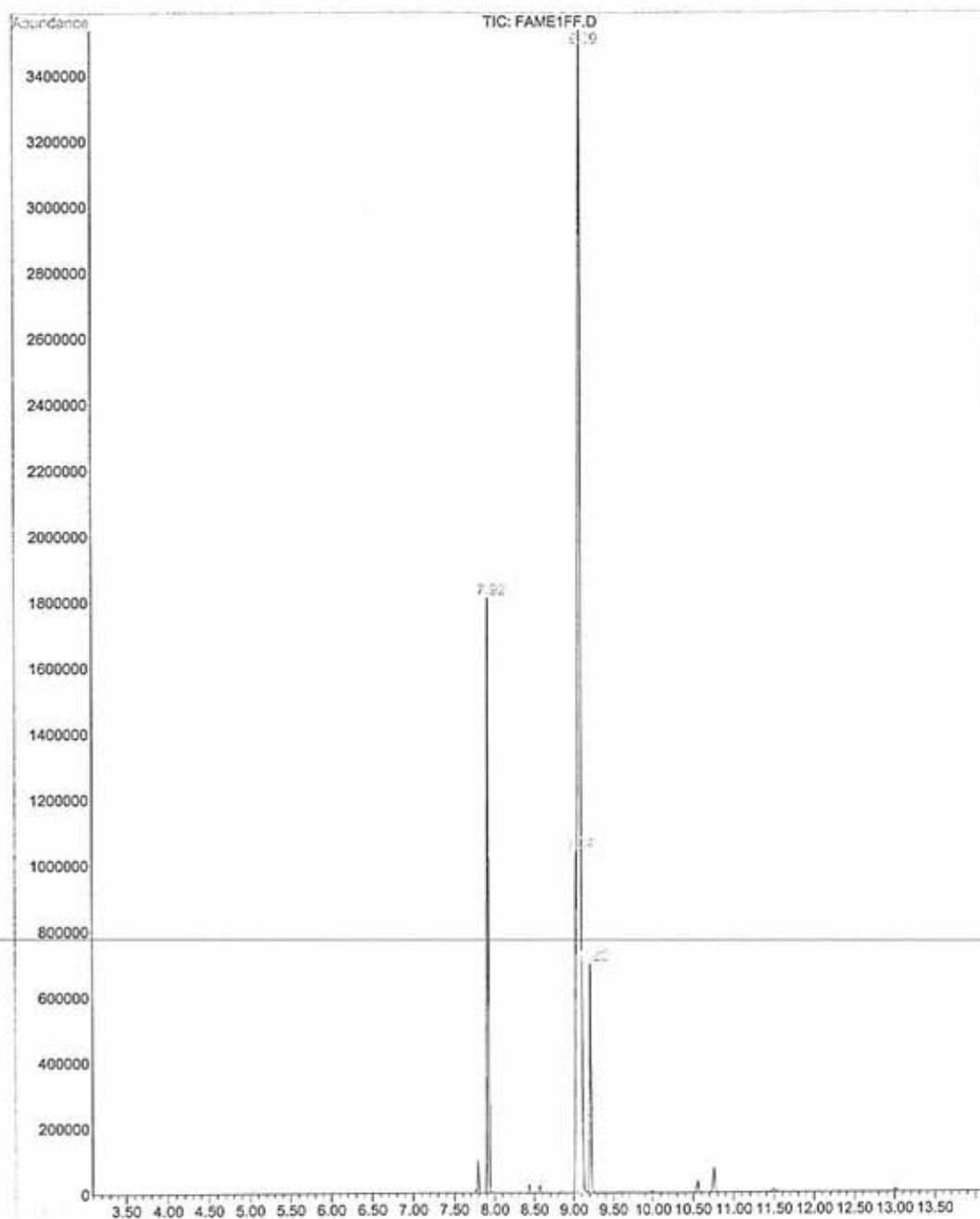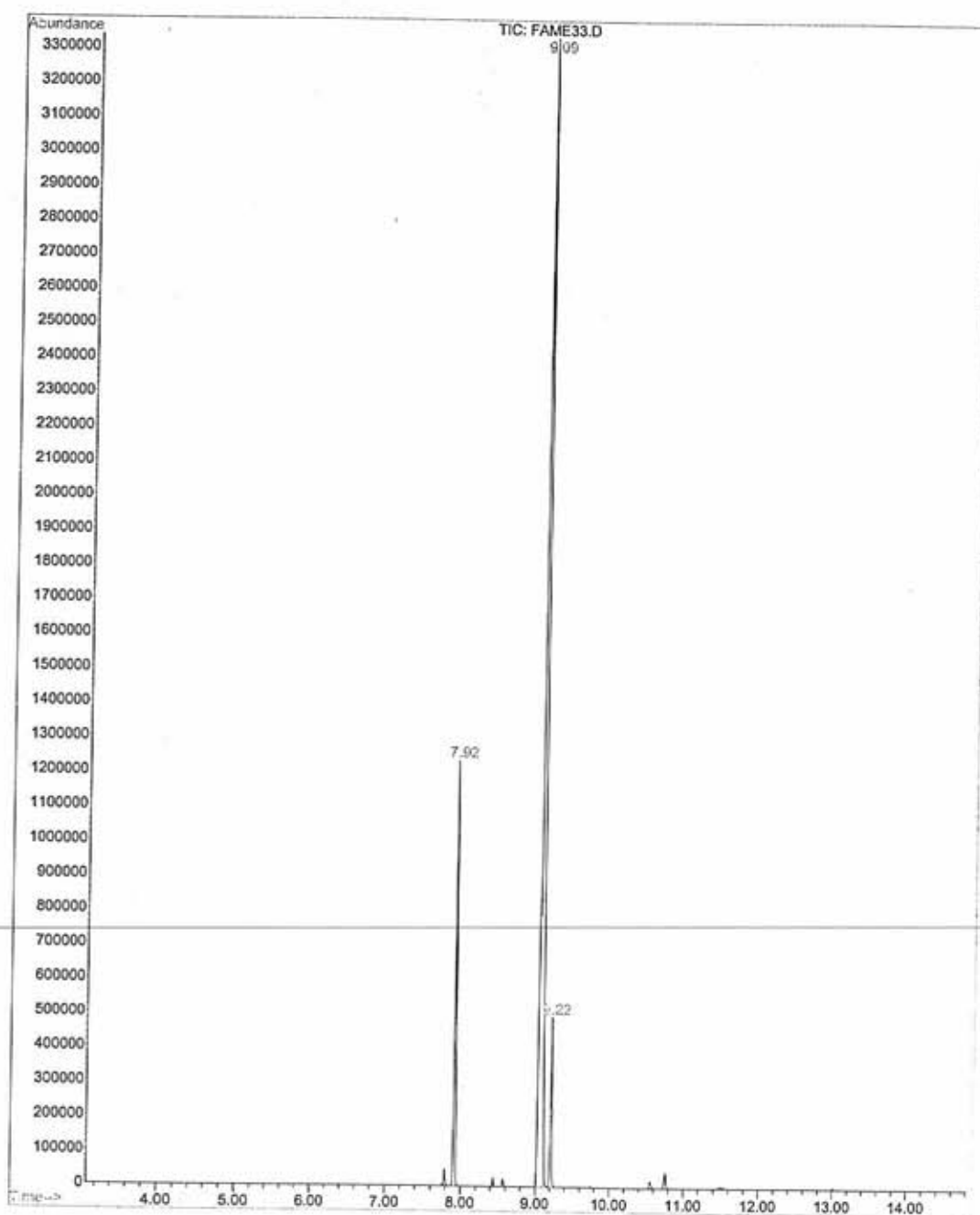

Figure 1.1 : standard FAME

Figure 1.2: Olive oil # 1 (Marjayoun – South)

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File   : C:\HPCHEM\1\DATA\FAME33.D
Operator   : karoon
Acquired   : 2 Jul 2002 13:52   using AcqMethod FAMESP75
Instrument :   GC/MS Ins
Sample Name: oo # 3
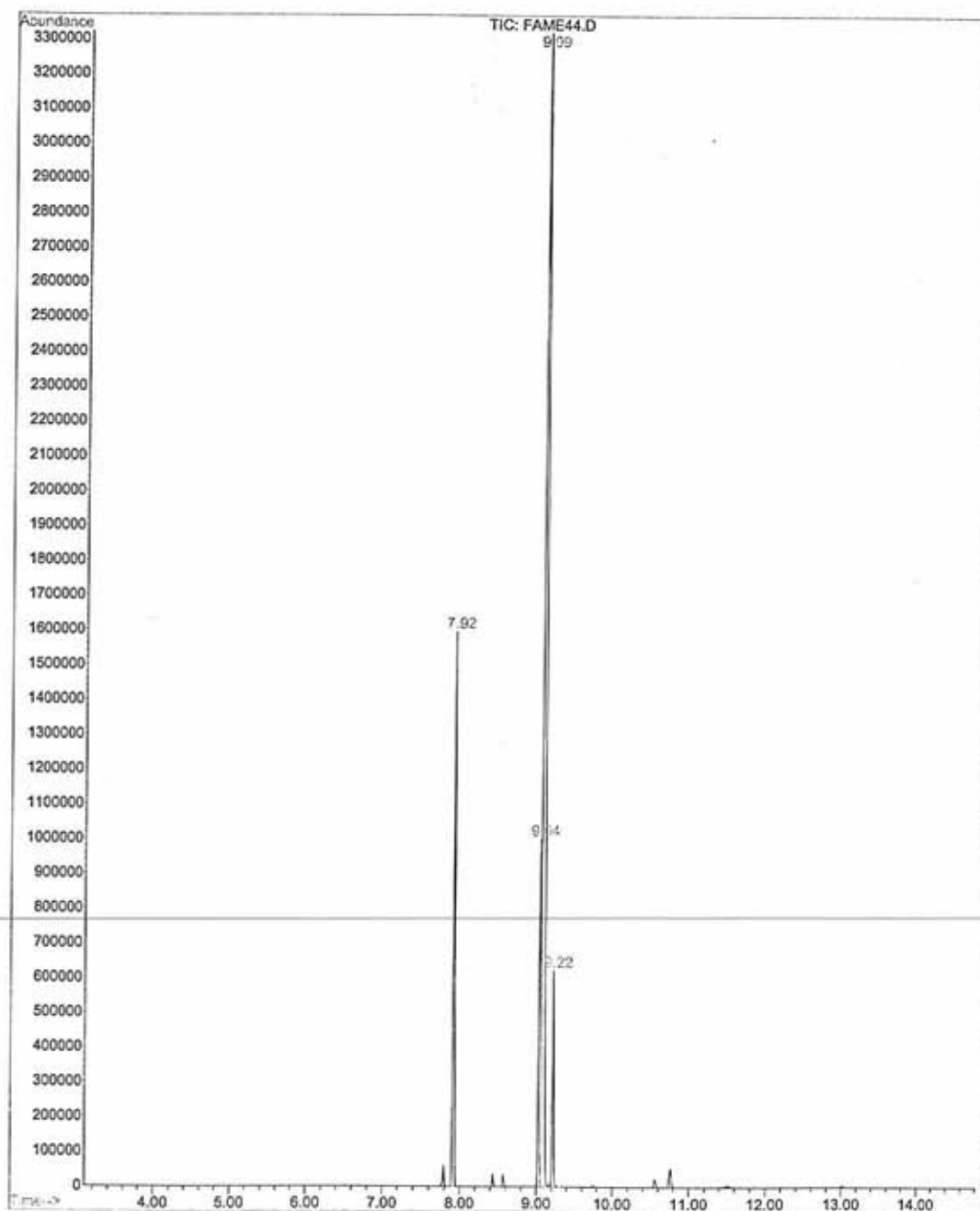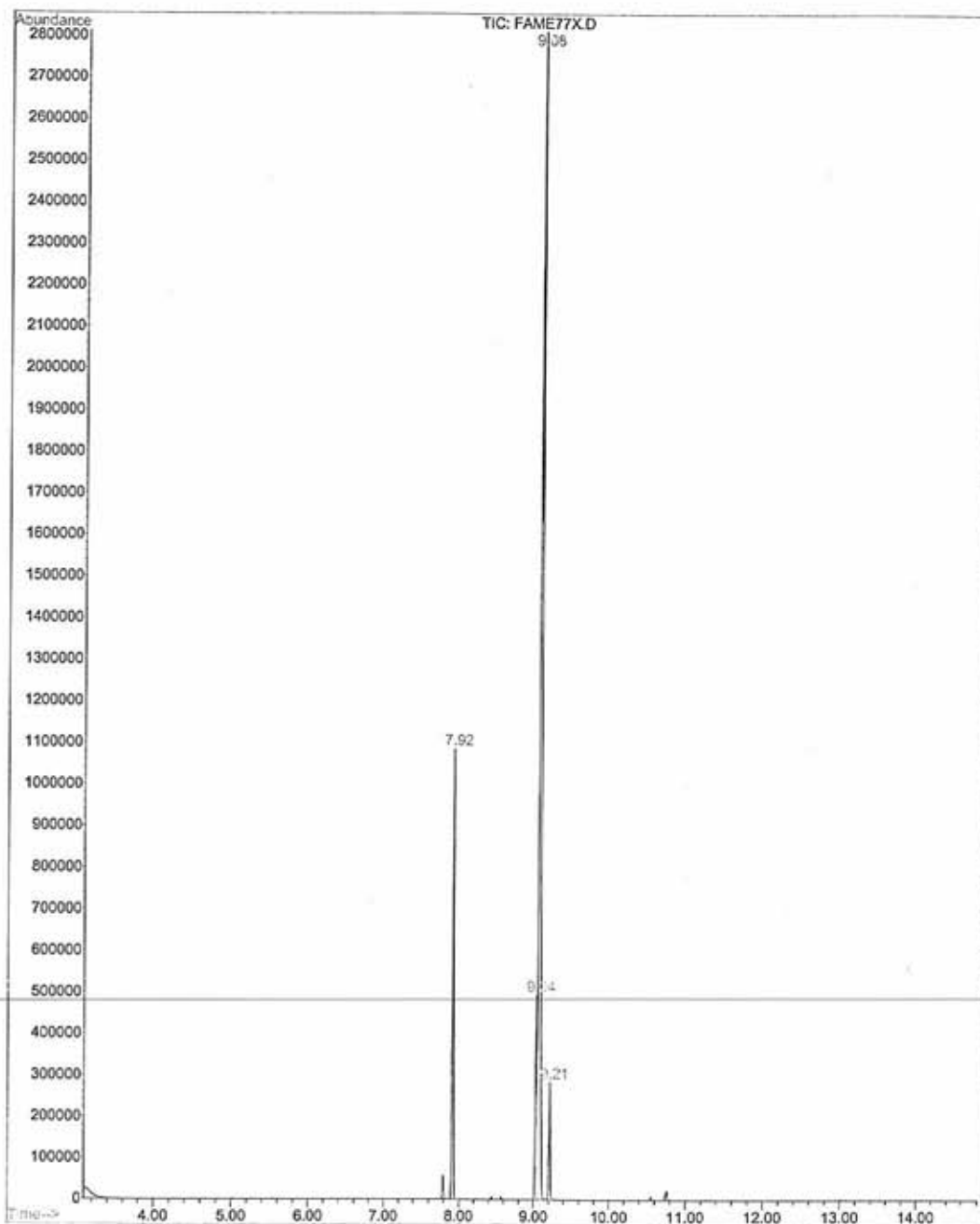Misc Info   :
Vial Number: 1

Figure 1.3 : Olive oil # 2 (Bekaa)

Figure 1.4 : Olive oil # 3 (Zrarieh – South)

Figure 1.5 : Olive oil # 4 (Zrarieh – South)

Figure 1.6 : Olive oil # 5 (Beshaale – Black olives)

Figure 1.7 : Olive oil # 6 (Beshaale – Black olives)

Figure 1.8 : Olive oil # 7 (Beshaale – Green olives)

Figure 1.9 : Olive oil # 8 (Akar – North)

```
File        : C:\HPCHEM\1\DATA\FAME101.D
Operator    : karoon
Acquired    : 4 Jul 2002  10:57      using AcqMethod FAMESP75
Instrument  : GC/MS Ins
Sample Name: OO # 10
Misc Info   :
Vial Number: 1
```
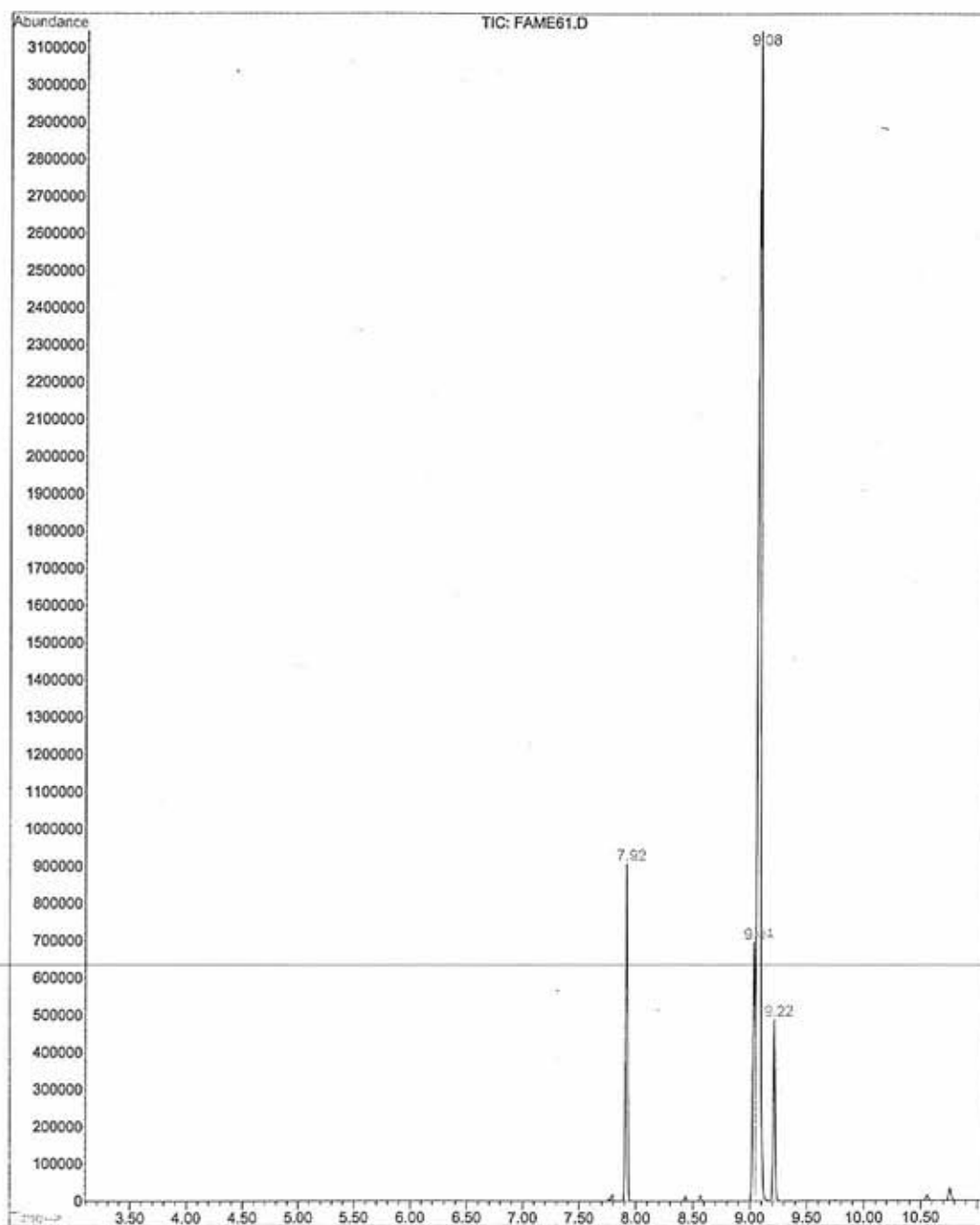

Figure 1.10: Olive oil # 9 (Koura)
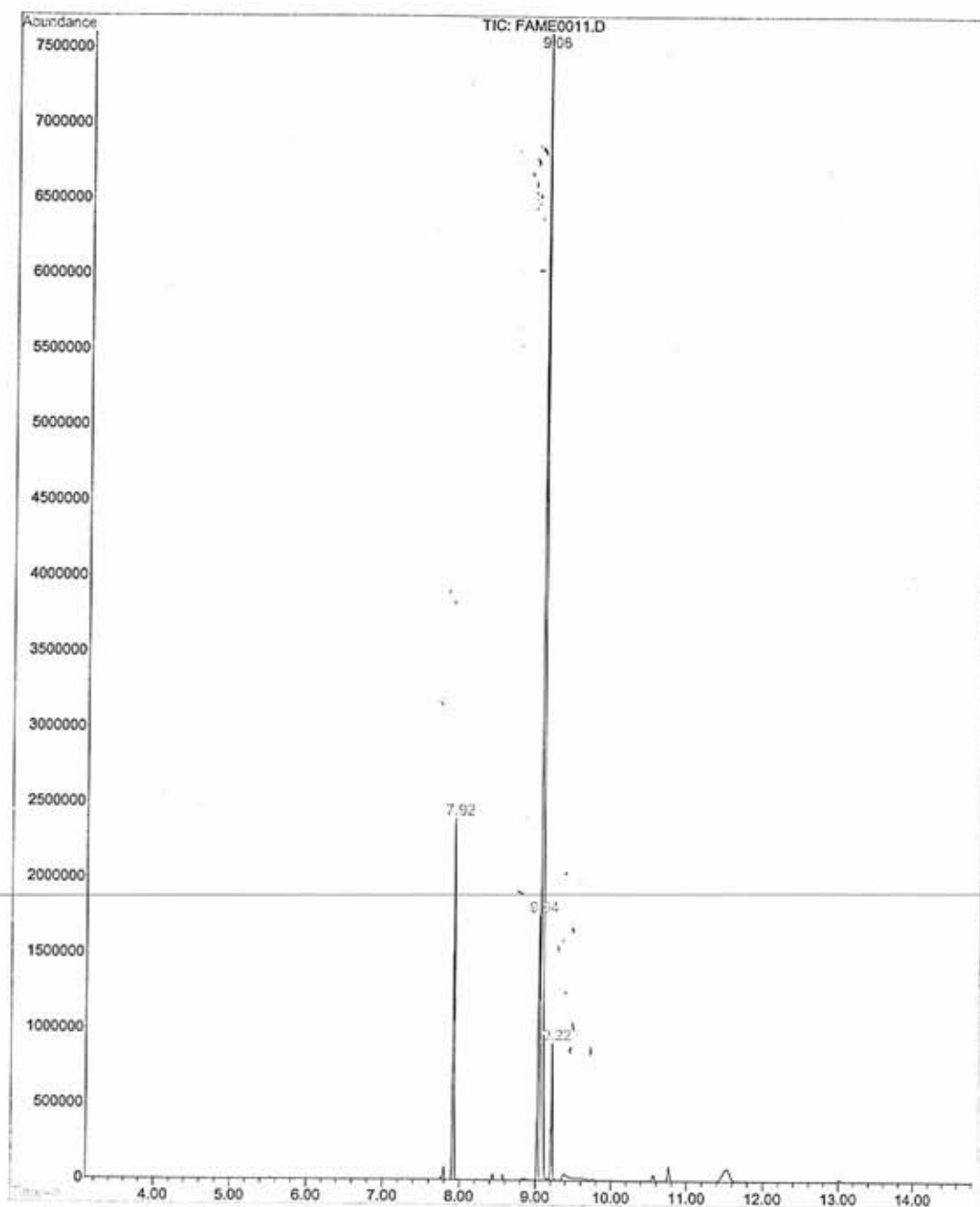
Figure 1.11 : Olive oil # 10 (Koura)

Appendix two: Chromatograms of ß-sitosterol and squalene
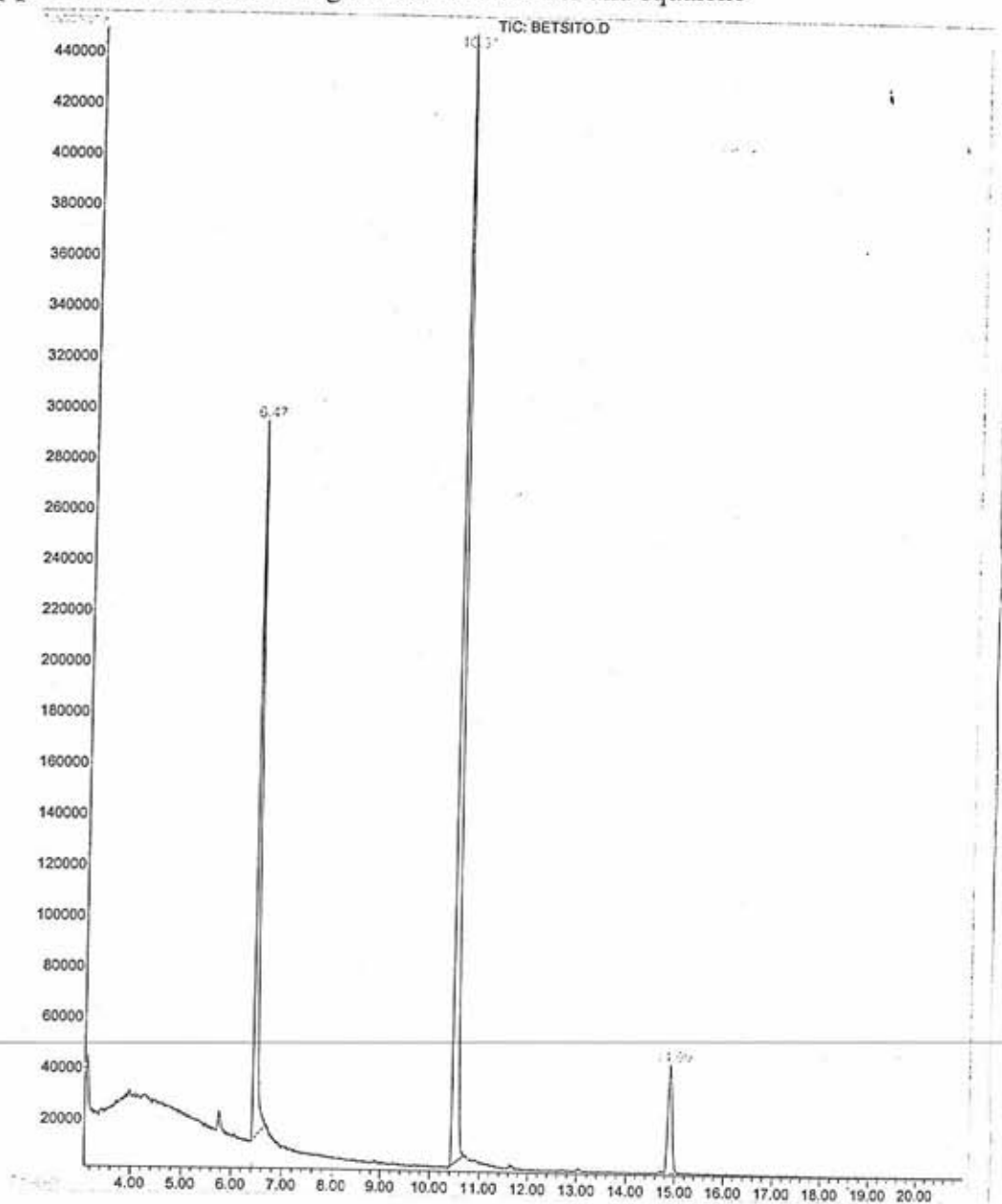
Peak #	Identification	R.T. time	Peak Height	Corr. Area
1	ß-Sitosterol	6.468	280423	10208231
2	Cholesterol	10.515	444671	21840593
3	5-α-Cholestane	14.903	42473	2536884

Figure 2.1: Standard Sterol

File   : C:\HPCHEM\1\DATA\SQUAL.D
Operator   : karen
Acquired   : 17 Jul 2002   15:04   using AcqMethod STEROL
Instrument :   GC/MS Ins
Sample Name: squalene dt
Misc Info   :
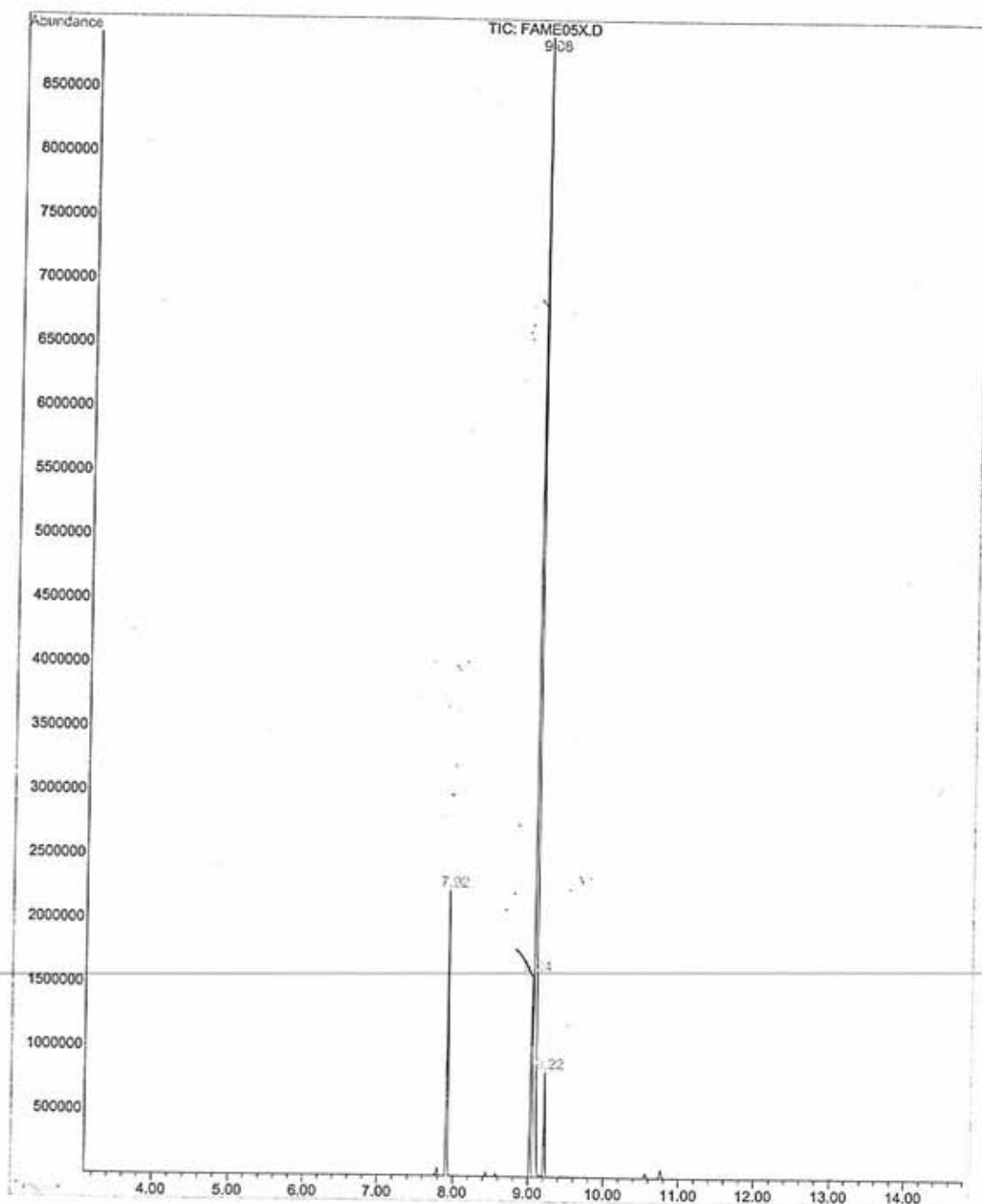Vial Number: 1
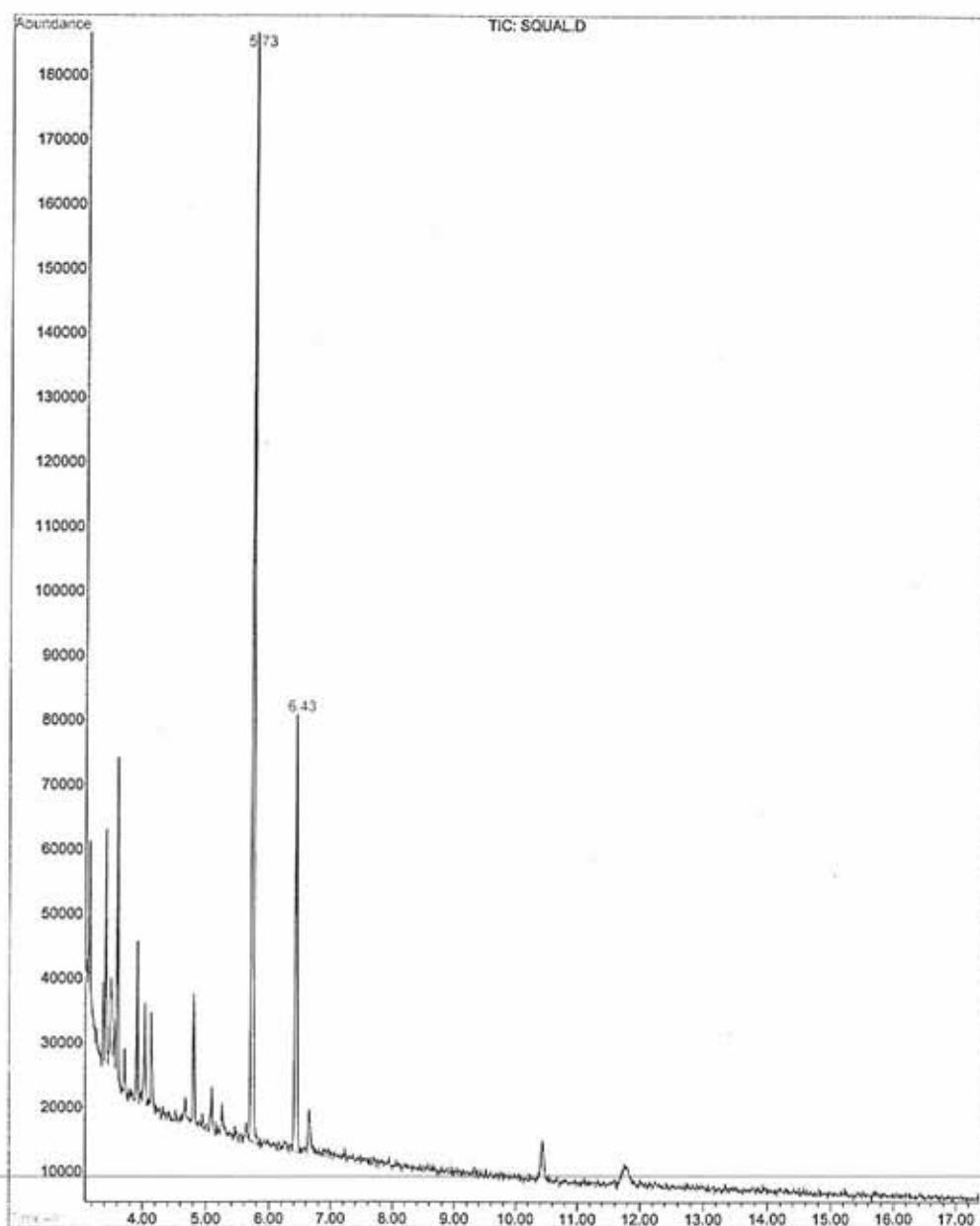
Peak #	Identification	R.T. time	Peak Height	Corr. Area
1	Squalene	5.762	944198	28908220
2	ß-Sitosterol	6.462	239129	7713750
3	5-α-Cholestane	14.903	81171	5098007

Figure 2.2: Standard Squalene

File   : C:\HPCHEM\1\DATA\STEROL01.D
Operator   : karoon
Acquired   : 5 Jul 2002   9:37   using AcqMethod STEROL
Instrument : GC/MS Ins
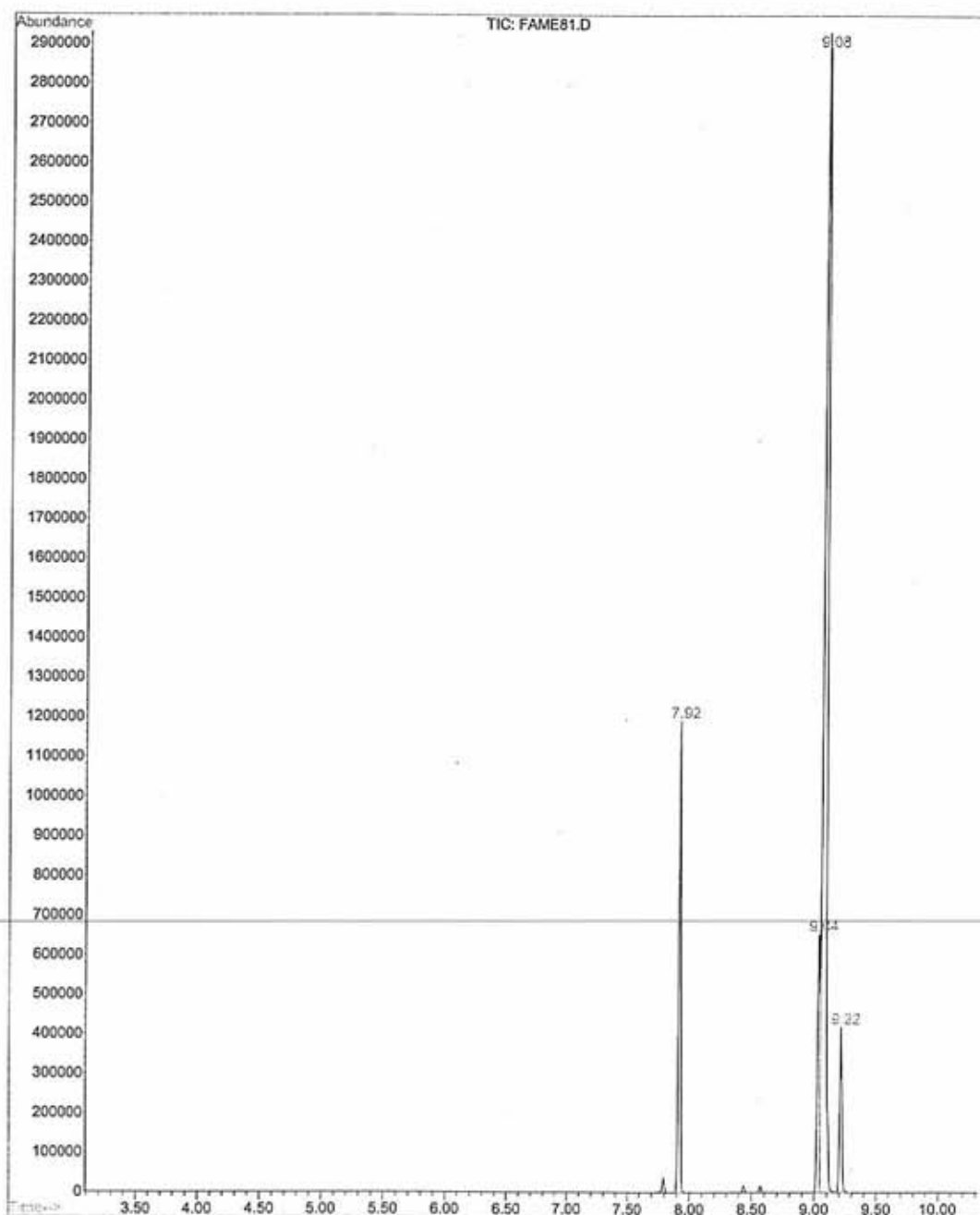Sample Name: OO # 1 sterol
Misc Info   :
Vial Number: 1

Peak #	Identification	R.T. time	Peak Height	Corr. Area
1	Squalene	5.762	944198	28908220
2	Beta-Sitosterol	6.462	239129	7713750
3	5-α-Cholestane	14.903	81171	5098007

Figure 2.3: Olive oil # 1 (Marjayoun – South)

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File   : C:\HPCHEM\1\DATA\STEROL3.D
Operator   : karoon
Acquired   : 5 Jul 2002 10:57   using AcqMethod STEROL
Instrument : GC/MS Ins
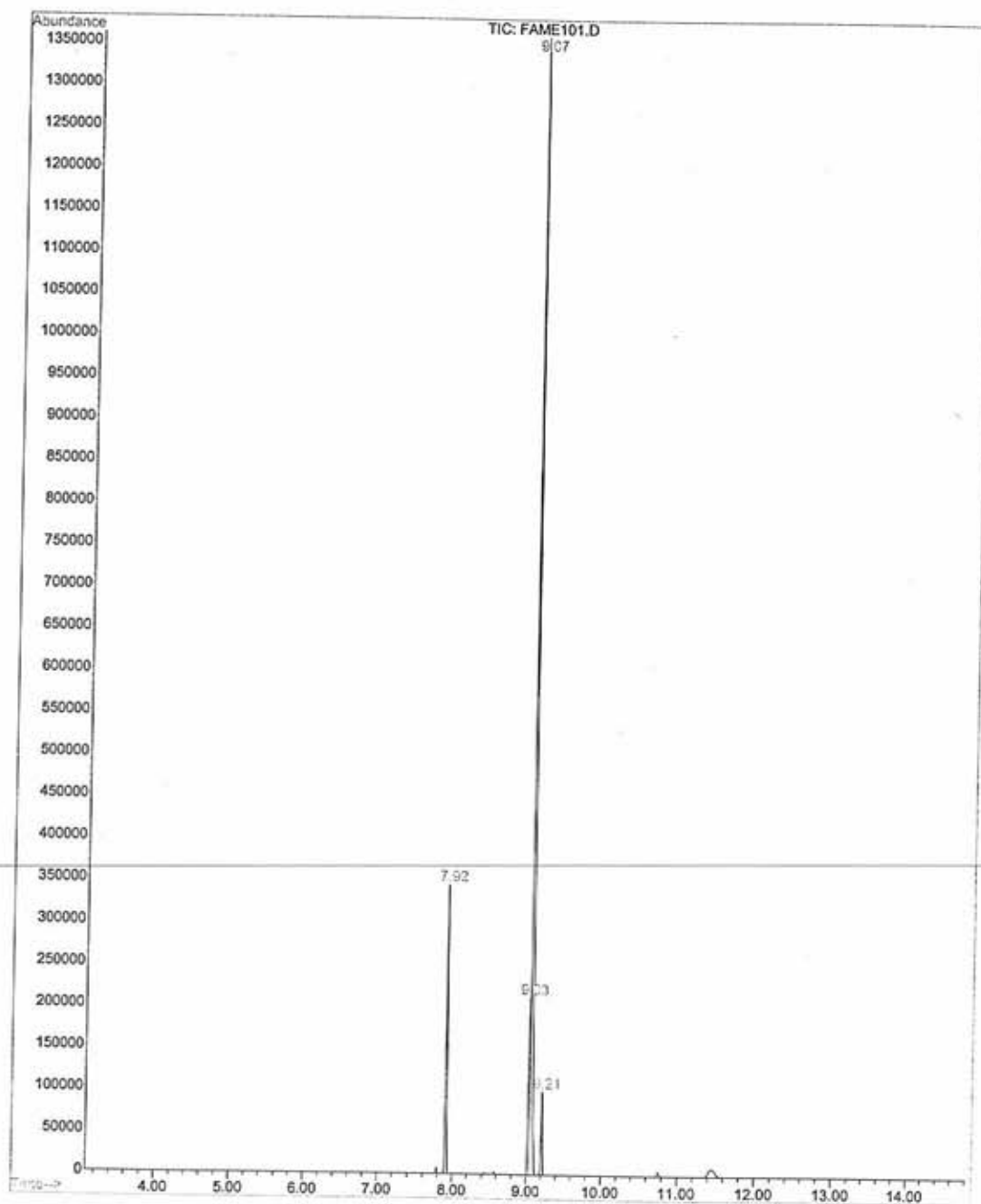Sample Name: OO # 3 sterol
Misc Info :
Vial Number: 1
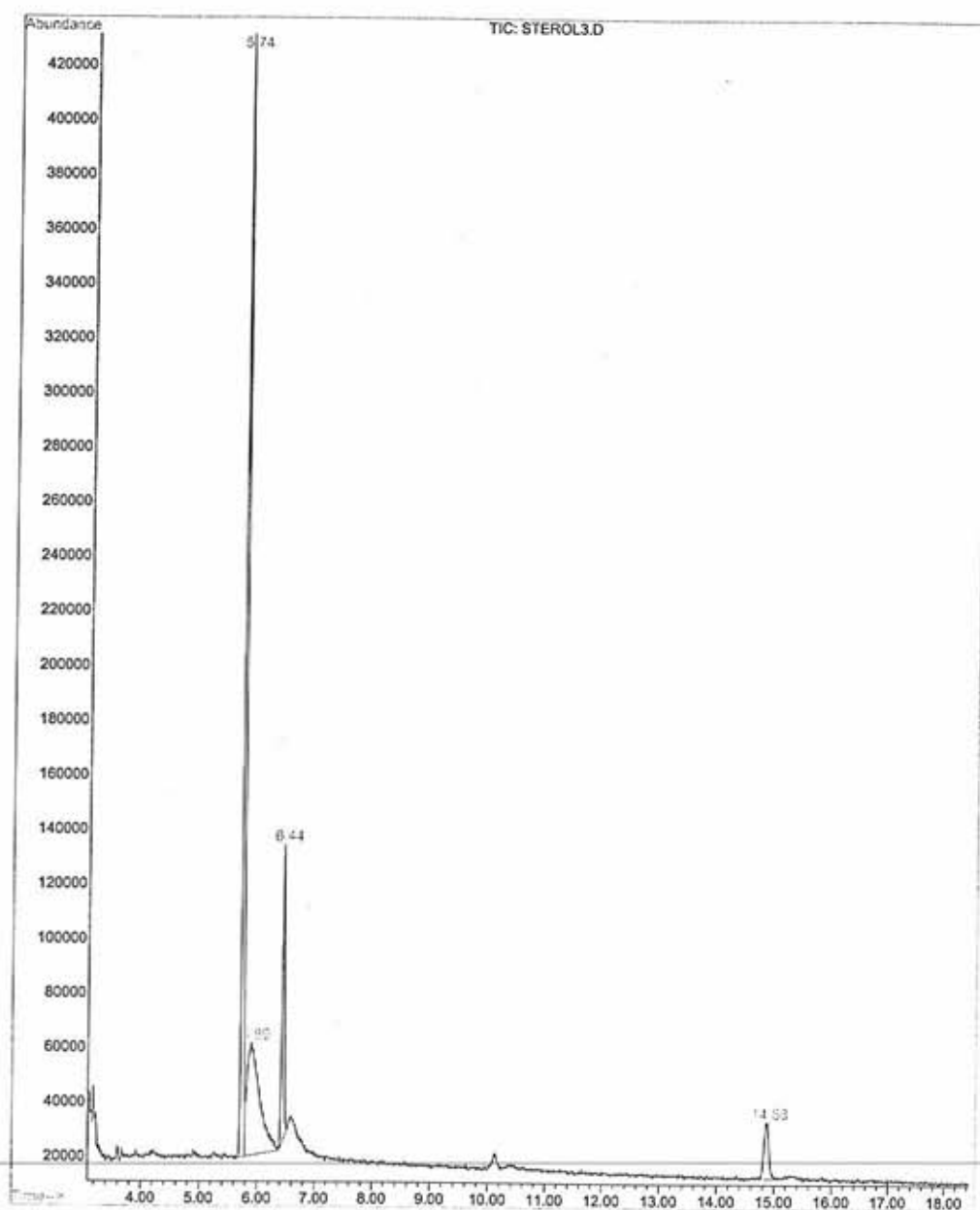
Peak #	Identification	R.T. time	Peak Height	Corr. Area
1	Squalene	5.740	408645	10415165
3	ß-Sitosterol	6.446	107588	2907346
4	5-α-Cholestane	14.881	20686	1259041

Figure 2.4 : Olive oil # 2 (Bekaa)

File   : C:\HPCHEM\1\DATA\STEROL4.D
Operator : karoon
Acquired : 5 Jul 2002 11:49   using AcqMethod STEROL
Instrument : GC/MS Ins
Sample Name: OO # 4 sterol
Misc Info :
Vial Number: 1

TIC: STEROL4.D

Peak #	Identification	R.T. time	Peak Height	Corr. Area
1	Squalene	5.745	680055	20799224
2	ß-Sitosterol	6.451	192129	6567383
3	5-α-Cholestane	14.891	71060	4735147

Figure 2.5 : Olive oil # 3 (Zrarieh – South)
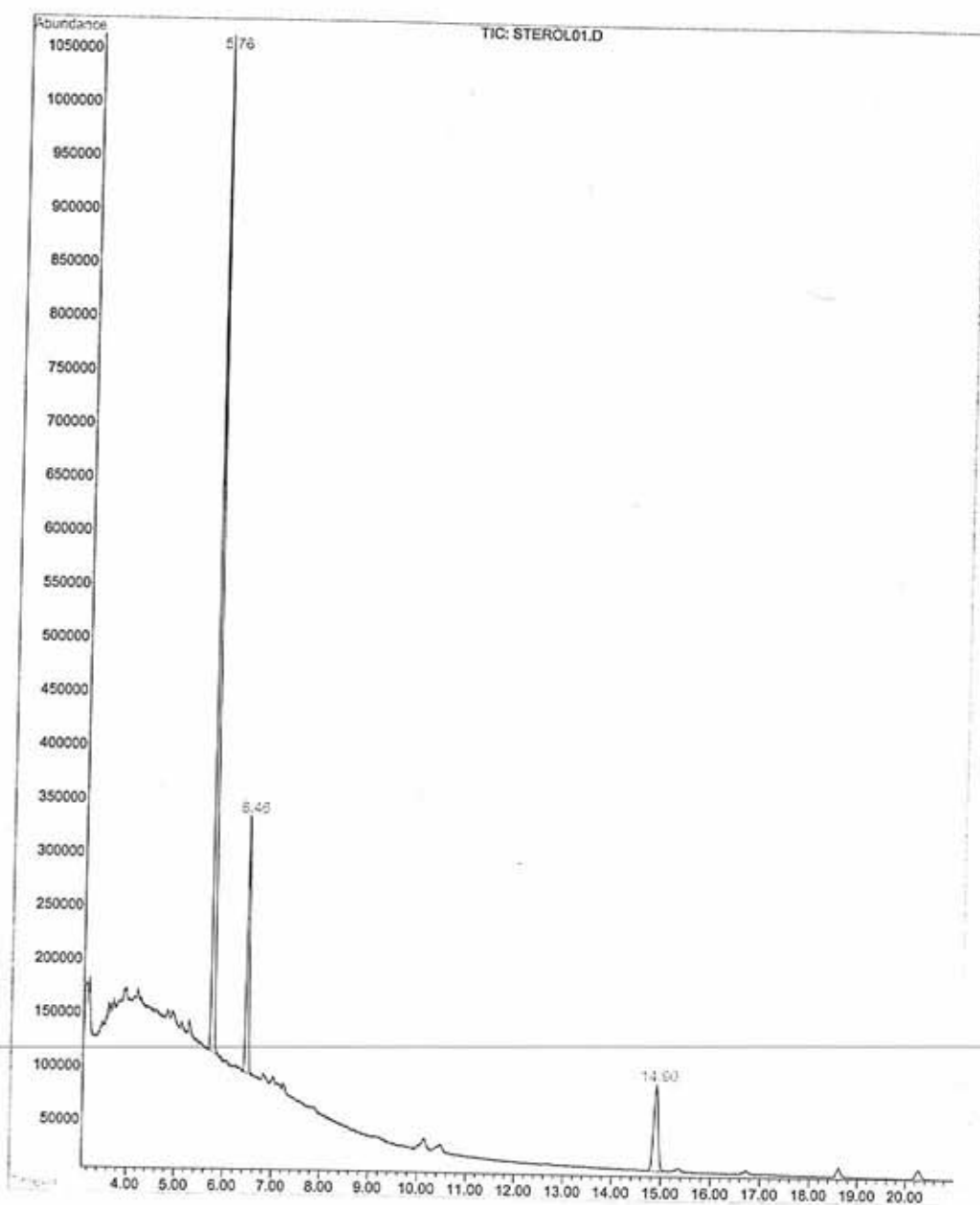
File   : C:\HPCHEM\1\DATA\STERO7.D
Operator : karoon
Acquired : 8 Jul 2002 11:21   using AcqMethod STEROL
Instrument : GC/MS Ins
Sample Name: OO # 7
Misc Info :
Vial Number: 1
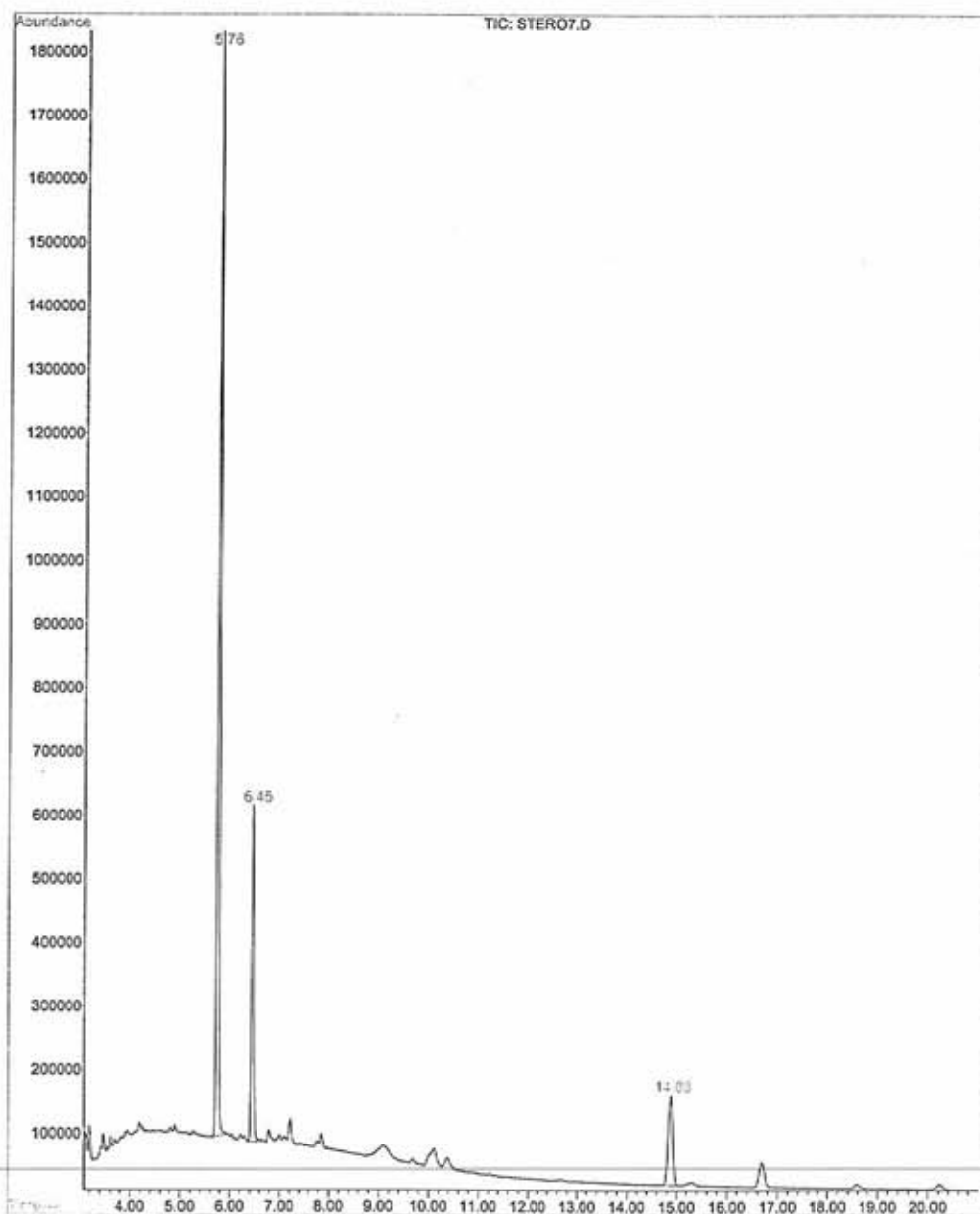
Peak #	Identification	R.T. time	Peak Height	Corr. Area
1	Squalene	5.765	1726538	50188829
2	ß-Sitosterol	6.454	526790	16106181
3	5-α-Cholestane	14.877	141078	8800882

Figure 2.6 : Olive oil # 4 (Zrarieh – South)

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File   : C:\HPCHEM\1\DATA\STEROL6.D
Operator : karoon
Acquired : 8 Jul 2002 10:51   using AcqMethod STEROL
Instrument :   GC/MS Ins
Sample Name: OO # 6
Misc Info :
Vial Number: 1
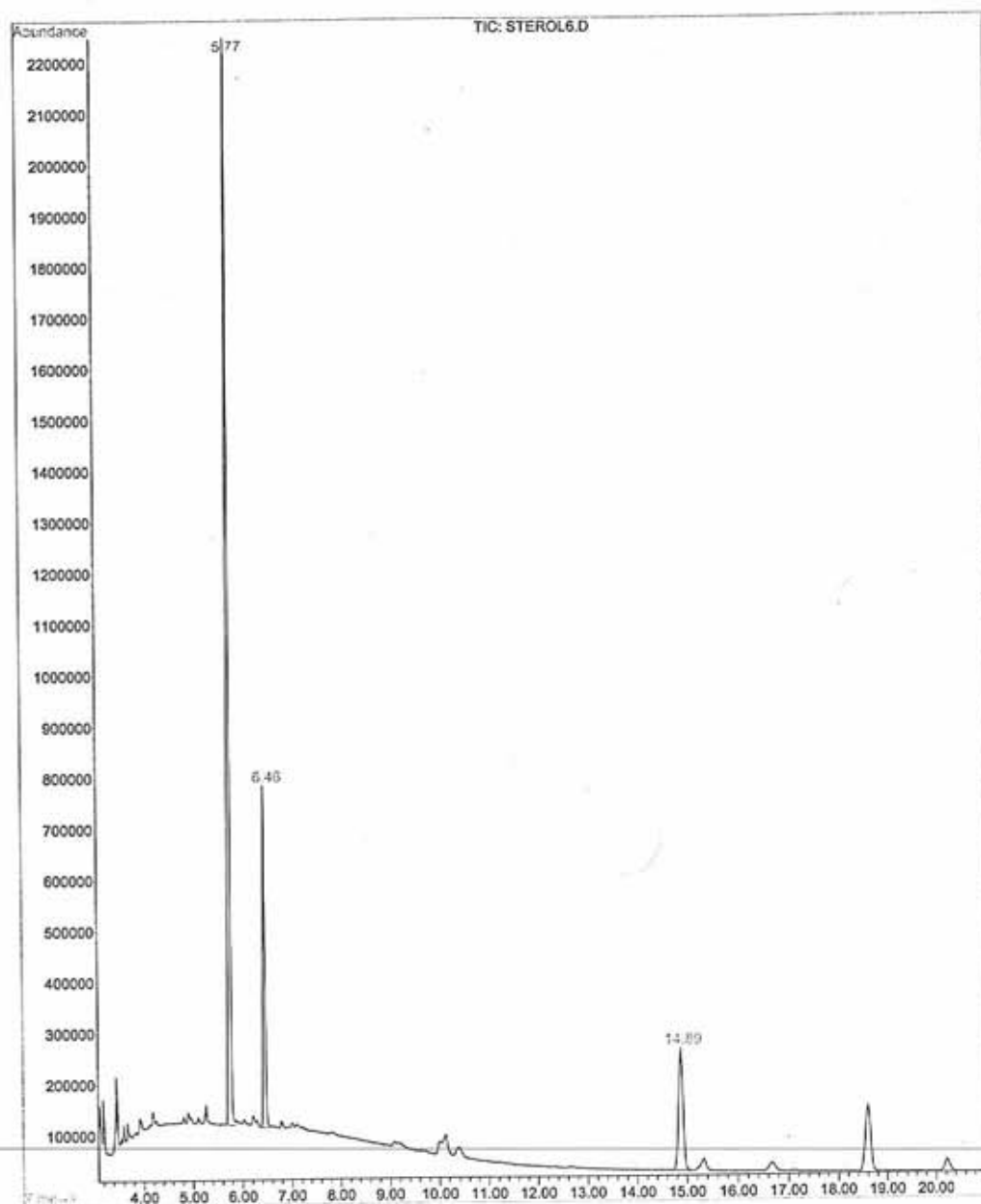
Peak #	Identification	R.T. time	Peak Height	Corr. Area
1	Squalene	5.770	2123865	59903945
2	ß-Sitosterol	6.453	661571	18061010
3	5-α-Cholestane	14.888	239004	14720788

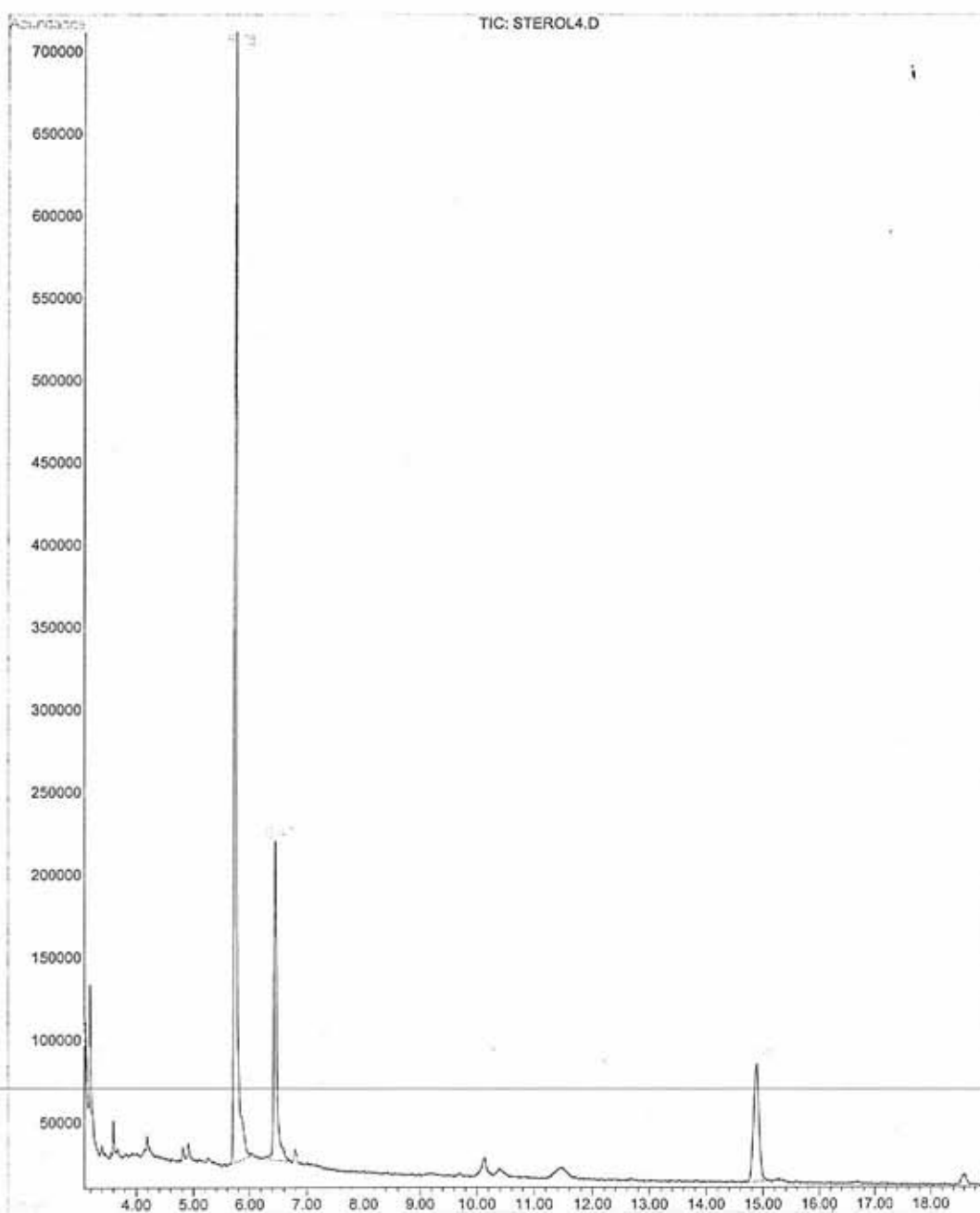Figure 2.7 : Olive oil # 5 (Beshaale – Black olives)

File   : C:\HPCHEM\1\DATA\STEROL5.D
Operator : karoon
Acquired : 8 Jul 2002 10:08   using AcqMethod STEROL
Instrument : GC/MS Ins
Sample Name: OO # 5
Misc Info :
Vial Number: 1

Peak #	Identification	R.T. time	Peak Height	Corr. Area
1	Squalene	5.754	1455374	42030314
2	ß-Sitosterol	6.449	471822	14858837
3	5-α-Cholestane	14.878	124992	7686547

Figure 2.8 : Olive oil # 6 (Beshaale – Black olives)

File   : C:\HPCHEM\1\DATA\STEROL9.D
Operator   : karoon
Acquired   : 9 Jul 2002 11:12   using AcqMethod STEROL
Instrument :   GC/MS Ins
Sample Name: OO #9
Misc Info   :
Vial Number: 1

Peak #	Identification	R.T. time	Peak Height	Corr. Area
1	Squalene	5.757	1887194	51781508
2	ß-Sitosterol	6.445	569003	15975640
3	5-α-Cholestane	14.863	133879	8278769

Figure 2.9 : Olive oil # 7 (Beshaale – Green olives)
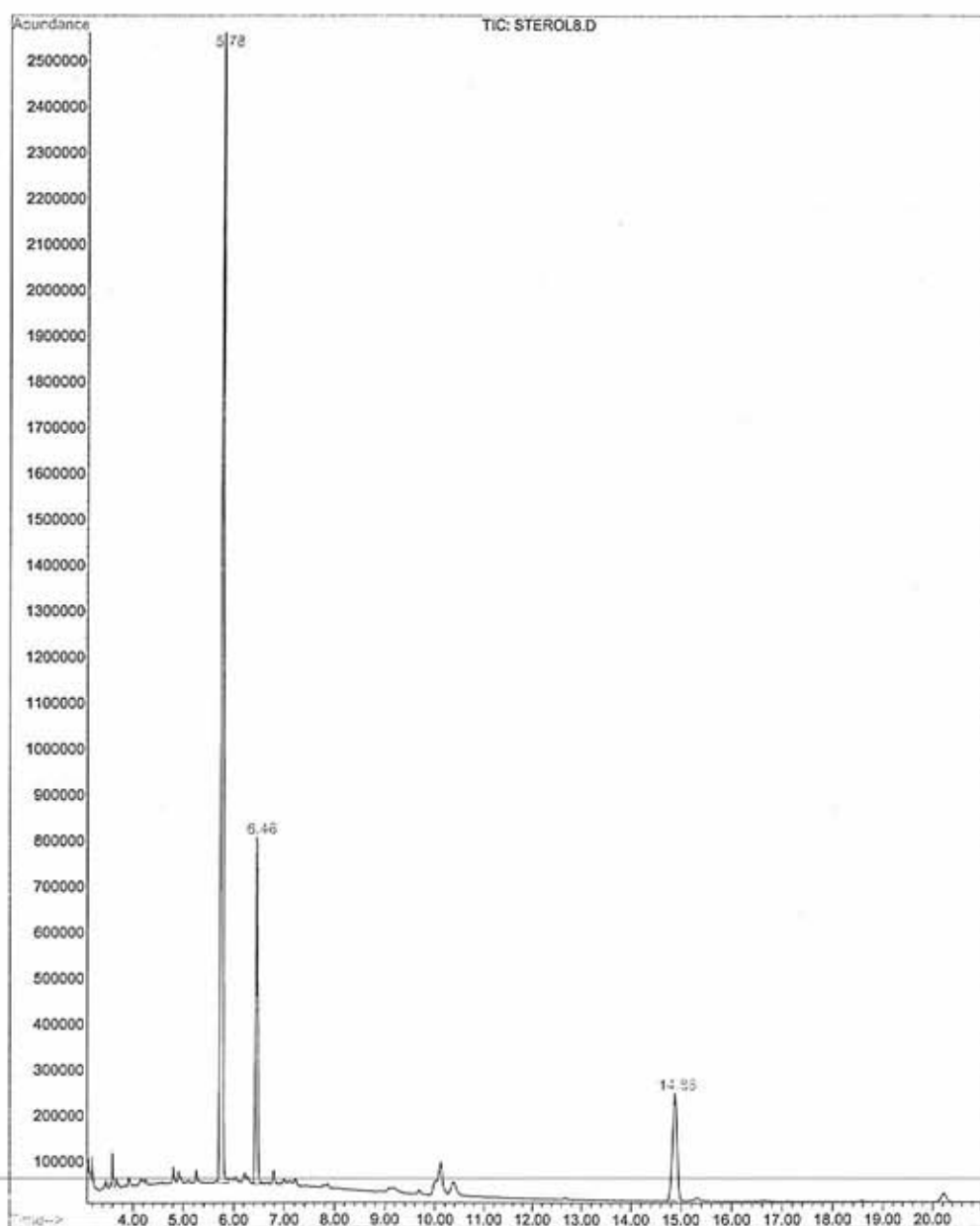
Peak #	Identification	R.T. time	Peak Height	Corr. Area
1	Squalene	5.774	2504086	79452296
2	ß-Sitosterol	6.458	751617	20357039
3	5-α-Cholestane	14.886	234259	14373923

Figure 2.10 : Olive oil # 8 (Akar – North)
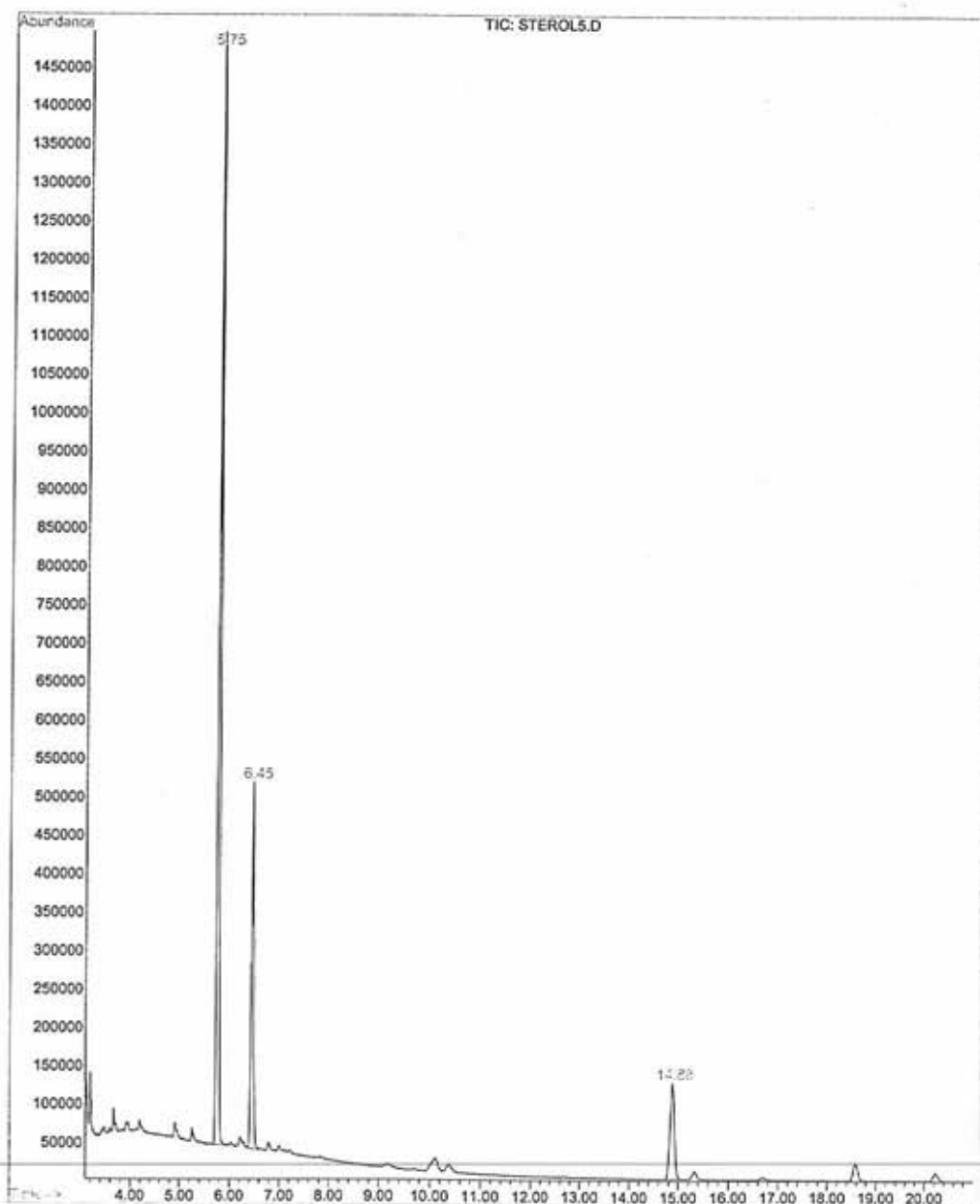
File   : C:\HPCHEM\1\DATA\STEROL10.D
Operator   : karoon
Acquired   : 9 Jul 2002 11:38   using AcqMethod STEROL
Instrument : GC/MS Ins
Sample Name: OO # 10
Misc Info   :
Vial Number: 1

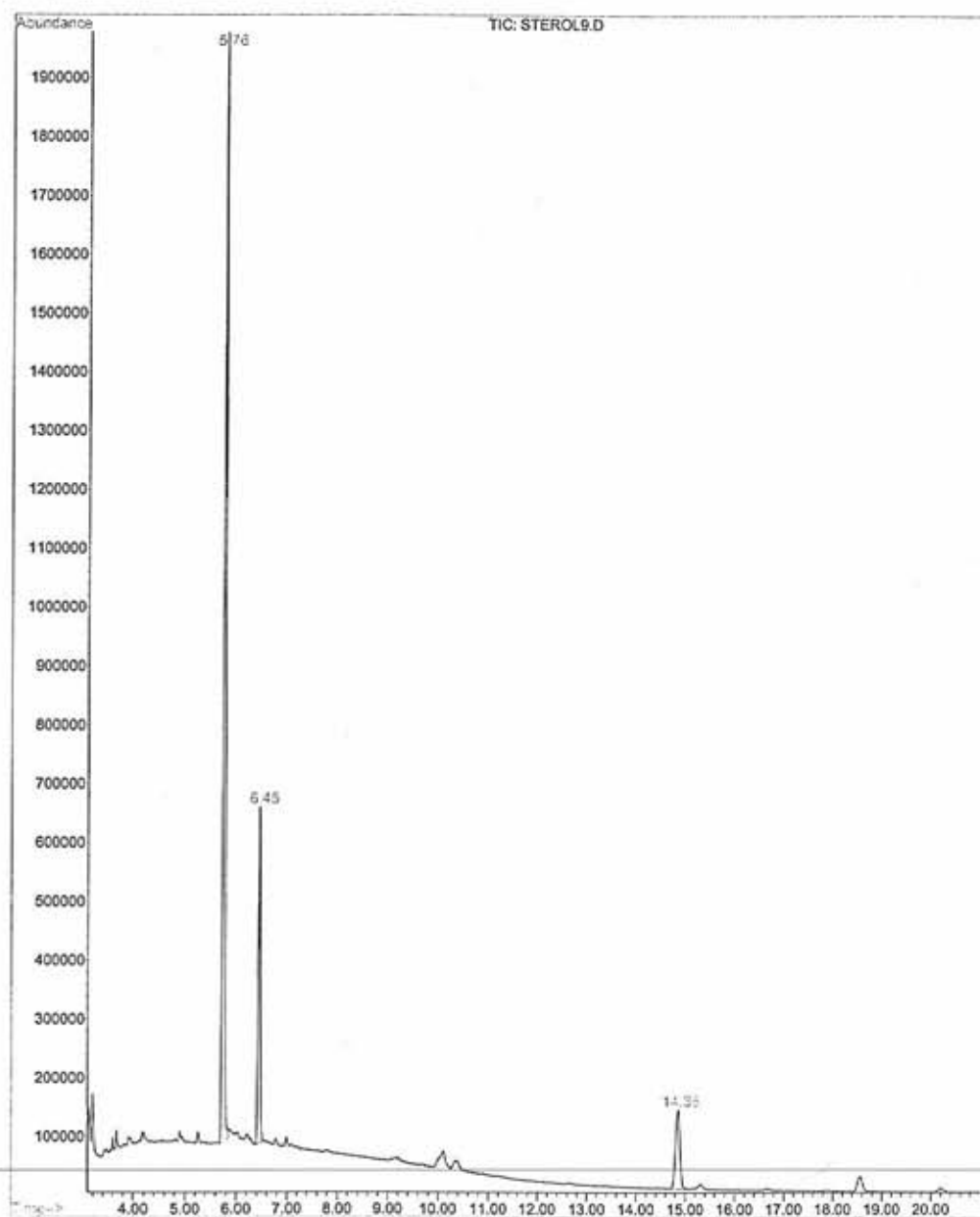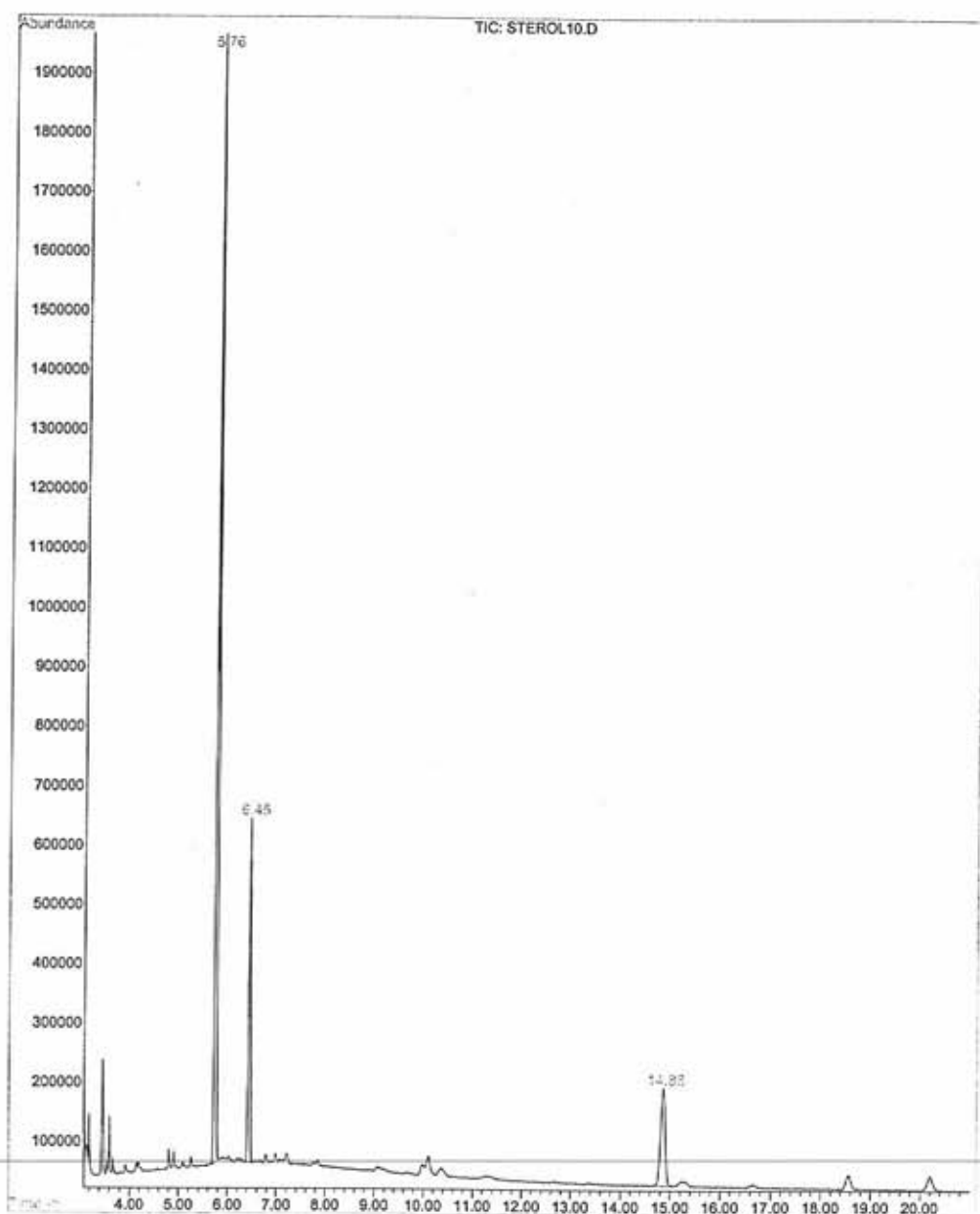| Peak #	Identification	R.T. time	Peak Height	Corr. Area
1	Squalene	5.756	1886894	48106504
2	ß-Sitosterol	6.445	572588	14736027
3	5-α-Cholestane	14.862	163826	10272004

Figure 2.11: Olive oil # 9 (Koura)

File   : C:\HPCHEM\1\DATA\STEROL11.D
Operator   : karoon
Acquired   : 9 Jul 2002 12:13   using AcqMethod STEROL
Instrument :   GC/MS Ins
Sample Name: OO # 11
Misc Info :
Vial Number: 1
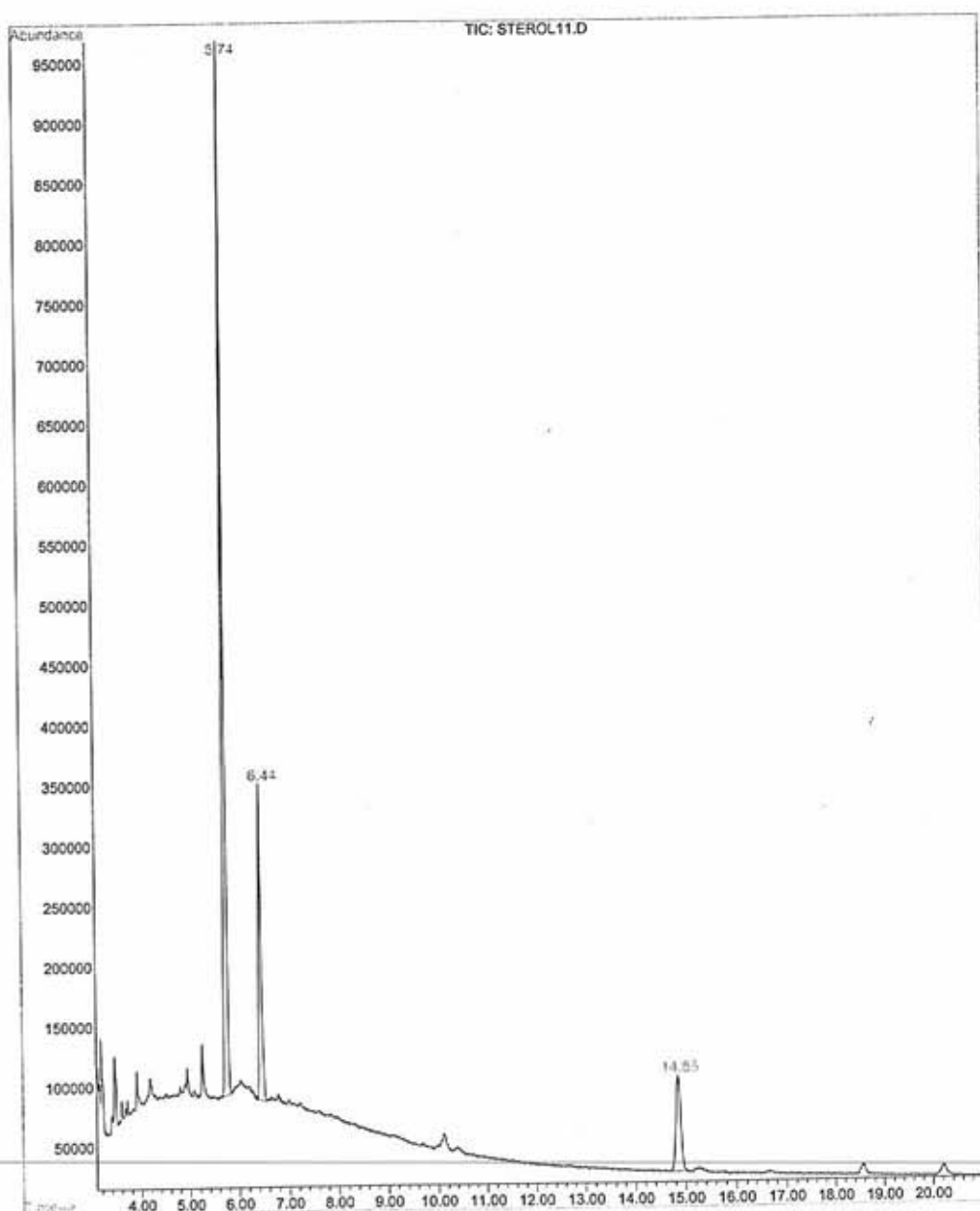
Peak #	Identification	R.T. time	Peak Height	Corr. Area
1	Squalene	5.740	877038	24227371
2	ß-Sitosterol	6.440	261479	8309411
3	5-α-Cholestane	14.846	79705	5332089

Figure 2.12 : Olive oil # 10 (Koura)

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Operator   : karen
Acquired   : 17 Jul 2002 10:54   using AcqMethod STEROL
Instrument :   GC/MS Ins
Sample Name: OO # 12
Misc Info   :
Vial Number: 1
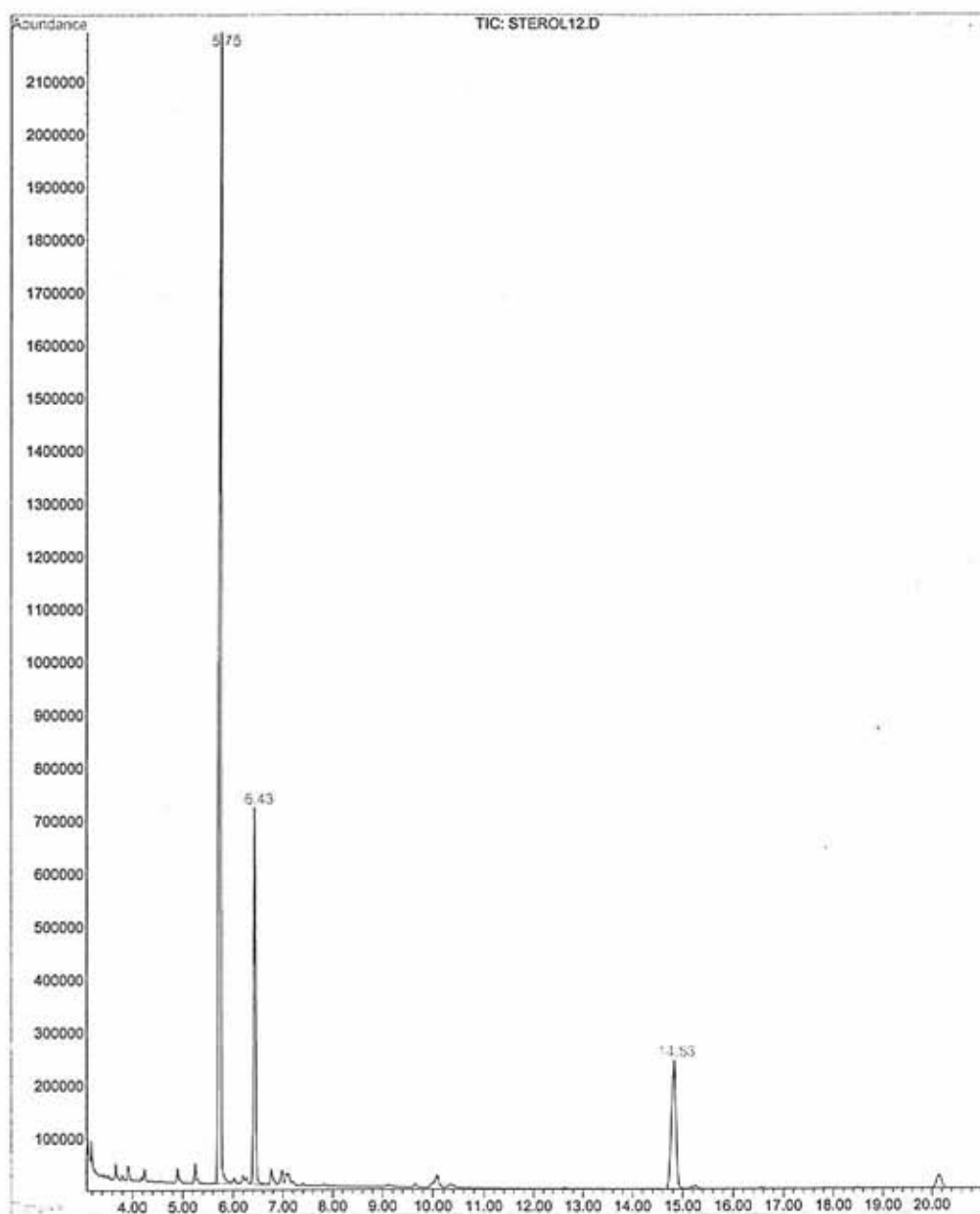
Peak #	Identification	R.T. time	Peak Height	Corr. Area
1	Squalene	5.751	2164761	62935367
2	ß-Sitosterol	6.434	718183	19944611
3	5-α-Cholestane	14.828	243783	15134499

Figure 2.13: Olive oil # 11 (Monsef)

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Appendix three

Lipid profile of the three experimental groups

Weight Measurements: each group contains 7 rats. Weight measured as a group

Group No	Baseline	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8	WK 9	WK 10
1(Control)	150g	2745	2998	3266	3580	3825	4135	4327	4750
2(Automated)	150g	2710	2930	3160	3448	3673	3920	4174	4361
3(cold press)	150g	2558	2735	2947	3232	3410	3753	4060	4215

Total Cholesterol (mg/dl)

NUMBER	CONTROL	AUTOMATED	COLD PRESS
1	48	52	48
2	65	59	56
3	43	58	51
4	54	48	52
5	49	60	47
6	62	57	50
7	45	63	49
8	38	57	53
9	42	61	51
10	54	51	59
11	45	51	58
12	50	55	50
13	66	52	62
14	51	47	46
AVERAGE	50.8571429	55.07142857	52.28571429
STD	8.59209962	4.968582613	4.778615368
T-TEST	C VS A	C VS P	A VS P
	0.12421293	0.591292929	0.142593178

Triglyceride (mg/dl)

NUMBER		CONTROL	AUTOMATED	COLD PRESS
	1	51	27	30
	2	27	34	47
	3	49	42	26
	4	23	40	19
	5	31	56	28
	6	25	41	38
	7	32	34	16
	8	42	33	29
	9	40	35	77
	10	38	47	47
	11	24	32	51
	12	60	34	42
	13	39	43	49
	14	32	29	36
AVERAGE		36.6428571	37.64285714	38.21428571
STD		11.0982129	7.732122237	15.75377157
T TEST		C VS A	C VS P	A VS P
		0.78425567	0.762706527	0.90396609

HDL (mg/dl)

NUMBER		CONTROL	AUTOMATED	COLD PRESS
	1	25	24	22
	2	32	24	27
	3	21	28	23
	4	27	22	29
	5	26	29	21
	6	34	32	31
	7	23	36	23
	8	16	17	13
	9	20	16	19
	10	19	17	17
	11	17	11	18
	12	20	18	16
	13	21	17	19
	14	17	19	16
AVERAGE		22.7142857	22.14285714	21
STD		5.49725206	7.047718047	5.233031185
T TEST		C VS A	C VS P	A VS P
		0.81281833	0.405752954	0.630239534

LDL (mg/dl)

NUMBER	CONTROL	AUTOMATED	COLD PRESS
1	13	23	20
2	28	28	20
3	12	22	23
4	22	18	19
5	17	20	20
6	23	17	11
7	16	20	23
8	14	33	34
9	13	38	16
10	28	24	32
11	23	33	29
12	18	30	25
13	37	26	33
14	28	22	23
AVERAGE	20.8571429	25.28571429	23.42857143
STD	7.44060732	6.305413687	6.653099749
T TEST	C VS A	C VS P	A VS P
	0.10125857	0.101258572	0.455222247

Chol / HDL ratio

NUMBER	CONTROL	AUTOMATED	COLD PRESS
1	1.92	2.16	2.18
2	2.03	2.45	2.07
3	2.04	2.07	2.21
4	2	2.18	1.79
5	1.88	2.06	2.23
6	1.82	1.78	1.61
7	1.95	1.75	2.13
8	2.38	3.24	3.98
9	2.05	3.72	2.65
10	2.89	2.98	3.37
11	2.61	4.42	3.16
12	2.5	2.99	3.06
13	3.16	2.98	3.24
14	3	2.47	2.92
AVERAGE	2.30214286	2.660714286	2.614285714
STD	0.45462036	0.771735948	0.68710893
T TEST	C VS A	C VS P	A VS P
	0.14620243	0.168187555	0.867787914

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Coagulation Time

NUMBER	CONTROL	AUTOMATED	COLD PRESS
1	16.5	14.2	14.3
2	16.2	14.4	13.9
3	16.6	14.3	14.5
4	14.9	14.7	14.6
5	15.8	15.5	14.2
6	14.4	14.3	15.1
7	15.9	13.5	17.1
8	18.3	15	15.6
9	14.5	14.9	16.6
10	17.1	15.1	15.3
11	16.1	14.9	16.4
12	15.8	13.7	N
13	15.4	13.9	15.4
14	15.6	N	16.2
AVERAGE	15.9357143	14.49230769	15.3230769
STD	1.02777365	0.588021454	1.01583615
T TEST	C VS A	C VS P	A VS P
	0.00016281	0.132217694	0.017498602

N : no coagulation, excluded from results

Insulin levels

NUMBER	CONTROL	AUTOMATED	COLD PRESS
1		0.078	0.072
2	0.133	0.125	0.075
3	0.089	0.104	0.066
4	0.059	0.069	0.237
5	0.233	0.18	0.117
6	0.192	0.081	0.059
7	0.063	0.092	0.088
8	0.117	0.09	0.087
9	0.105	0.055	0.082
10	0.065	0.083	0.073
11	0.199	0.085	0.052
12	0.072	0.105	0.099
13	0.05	0.082	0.132
14	0.096	0.085	0.075
AVERAGE	0.11330769	0.093857143	0.093857143
STD	0.05968722	0.029866001	0.046488803
T TEST	C VS A	C VS P	A VS P
	0.28909868	0.351889833	1

Apo B 48 (mg/dl): Each value is the average of two plasma values

NUMBER	CONTROL	AUTOMATED	COLD PRESS
1	10.5	9.41	8.45
2	9.23	10.25	9.76
3	9.51	9.21	9.86
4	7.6	10.65	9.95
5	9.25	8.45	10.2
6	9.94	9.25	10.9
7	n	9.46	10.7
AVERAGE	9.33833333	9.525714286	9.974285714
STD	0.97798603	0.723322361	0.797660269
T-TEST	C VS A	C VS P	A VS P
	0.69903908	0.222577127	0.291997653

Apo B 100

NUMBER	CONTROL	AUTOMATED	COLD PRESS
1	30	30.5	25.3
2	25.4	35	26.7
3	37.8	33.5	35.1
4	29.4	25.6	26.7
5	31.4	34.9	37
6	29.6	30	30.9
7	n		
AVERAGE	30.6	31.58333333	30.28333333
STD	4.05758549	3.628452379	4.882792917
T-TEST	C VS A	C VS P	A VS P
	0.66754506	0.90517807	0.612063291

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