DEVELOPMENT OF A LASER-BASED INFRARED DETECTOR FOR HIGH PRESSURE LIQUID CHROMATOGRAPHY. THE ANALYSIS OF CHOLESTEROL, CHOLESTERYL ESTERS AND TRIGLYCERIDES IN SERUM.

BY

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Development of an infrared laser detector for liquid chromatography.

Department of Chemistry

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For Michael

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An infrared detector is constructed for use in High Pressure Liquid Chromatography. The detector uses a helium-neon laser which emits at 3.39 microns in the infrared as the source and a commercial lead selenide semiconductor as the detector The detector when used in the flow-injection analysis mode can detect all classes of lipid. fatty acids, triglycerides, phospholipids, cholesterols and cholestryl esters. Chromatography using a normal phase column with separation into the lipid classes of cholesterol, cholestryl esters and triglycerides is performed. A purchased serum sample has been prepared and separation and quantitation of the 3 lipid classes is performed.

DEVELOPPEMENT D'UN DETECTEUR LASER INFRAROUGE POUR CHROMATOGRAPHIE A HAUTE PERFORMANCE; PERMETTANT L'ANALYSE DU CHOLESTEROL, D'ESTERS CHOLESTERYL ET DES TRIGLYCERIDES PRESENT DANS LE SERUM

Un détecteur infrarouge a été construit pour utilisation en chromatographie à haute performance. Un laser hélium-néon qui émet à 3.39 microns dans la région des infrarouges a été utilisé en tant que source et un semiconducteur commercial plomb séléniure en tant que détecteur. Le détecteur utilisé en analyse à injection en continu (flow-injection analysis) peut détecter différentes classes lipidiques, tels que les acides gras, les triglycérides, les phospholipides, les cholestérols et les esters cholestryl. La chromatographie avec séparation des classes lipidiques du cholésterol, des esters cholestryl et des triglycérides sur colonne à phase normale a de même été utilisée Un échantillon de serum commercial a été préparé et la séparation et quantification des 3 classes lipidiques a été réalisées.

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CHAPTER 1

STRUCTURE AND ANALYSIS OF LIPIDS

Lipids encompass a vast number of different types of molecules with such differing structures and functions that the only characteristic that they have in common is that they are not soluble in water. They are, however, soluble in organic solvents such as chloroform, methanol, hexane and benzene to name a few (1).

I. FUNCTIONS (2)

Lipids have numerous biological functions in all cells. Lipids are present in cell membranes, and along with proteins, make up the structure of membranes.

Lipids are important in the storage and transport of metabolic fuel. Lipids, or fats as they are often referred to when speaking of dietary lipids, are important in the diet as energy sources, vitamins (e.g. A,D) and also in their contribution to the palatability of food (3). Fats provide more energy than protein or carbohydrate and are particularly important since the glycogen store (storage mode of carbohydrate in the liver) cannot expand, but the adipose tissue (or storage depot of lipids) can expand enormously. Many years ago it was thought that fats were only important to the diet as an energy source; however, it was soon discovered that a deficiency state could be produced on a fat free diet. It was then determined that the deficiency state could be eliminated by the addition of certain "essential fatty acids", in particular linoleic acid (3).

Some lipids are important as a protective coating on the surfaces of some organisms. Waxes are one type of lipid which provide this water repellant barrier to the environment.

Cell surface components which are important in recognition, species specificity and tissue immunity are other functions of lipids These are not all of the functions of lipids, but merely some of the more important ones.

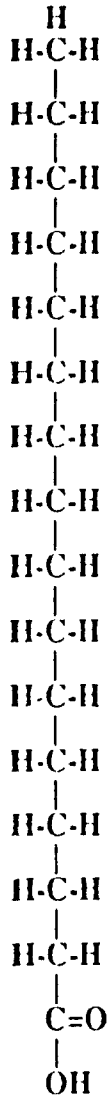
II. CLASSIFICATION

Lipid classification is most often done on the basis of backbone structure; however, the way that this is done can differ slightly from one textbook to the next. Lehninger (2) broadly divides lipids into 2 classes· complex (saponifiable yielding salts of fatty acids (soaps) on alkaline hydrolysis) and simple (nonsaponifiable and therefore they have no fatty acids) They can also be divided into neutral and polar lipids. Regardless of the system of classification, lipids can all be broken down and classified by their functional groups.

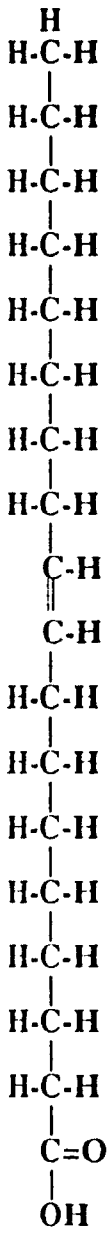
III. SAPONIFIABLE LIPIDS

1. FATTY ACIDS

Fatty acids are the building blocks of saponifiable lipids They are long chain hydrocarbons with a terminal carboxylic acid group. They can be saturated or unsaturated, with the double bond most often being *cis* as opposed to *trans*. The 16-carbon and 18-carbon (Fig. 1) fatty acids predominate and the shorthand notation consists of the number of carbons in the fatty acid followed by a colon followed by the number of double bonds followed by a superscript of the position of the double bonds (see Table 1) (2) All fatty acids are cis unless otherwise indicated Hence, 18.1^{9} (oleic acid) is an 18-carbon fatty acid with one carbon- carbon double bond at the carbon number 9 position. As can be seen from Table 1, most fatty acids do not have conjugated double bonds, usually there is a methylene group between subsequent double bonds.



A)



B)

FIG 1 Some common fatty acids

A) Palmitic acid
B) Oleic acid

TABLE 1 SHORTHAND NOTATION OF SOME COMMON FATTY ACIDS

symbol	structure	common name
16:0	$CH_3(CH_2)_{14}COOH$	palmitic acid
18:0	$CH_3(CH_2)_{16}COOH$	stearic acid
20:0	$CH_3(CH_2)_{18}COOH$	arachidic acid
$18:1^{9}$	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	oleic acid
$18:2^{9,12}$	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$	linoleic acid
$18:3^{9,12,15}$	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$	linolenic acid

Since fatty acids are water insoluble and blood is an aqueous solution, fatty acids in human blood are not free, but are usually bound to albumin, a protein. Therefore, albumin-bound fatty acid (ABFA) is the same as free fatty acid (FFA) which is the same as non-esterified fatty acid (NEFA) (the carboxyl group of the fatty acid can be esterified to a hydrocarbon group) (3).

2. TRIACYLGLYCEROLS

Triacylglycerols have a backbone of glycerol and to each of the hydroxyl groups of glycerol is esterified a fatty acid. Alternate names are neutral fats and triglycerides. The International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB) Commission on Biochemical Nomenclature (4) have recommended the name triacylglycerols as well as mono- and di-acylglycerols for the mono- and di- substituted molecules (Fig. 2). However, triglycerides is the name found throughout the literature and will be used here.

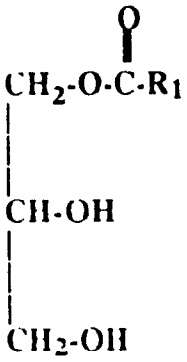
The fatty acids which are esterified to the triglyceride can be all different, all the same, saturated or unsaturated. The triglycerides are the most abundant family of lipids and are the major storage form of lipids in adipose tissue. Triglycerides are insoluble in water and form soaps and glycerol on alkaline hydrolysis.

3. PHOSPHOGLYCERIDES (2)

Phosphoglycerides, also called glycerol phosphatides or phospholipids, have the same glycerol backbone that the triglycerides do. They differ in that one of the primary hydroxyl groups of the glycerol is esterified to a phosphoric acid. The other primary hydroxyl group and the secondary hydroxyl group are esterified to fatty acids in the same manner as in triglycerides (Fig. 3). The X group in Fig. 3 can be any of a number of different groups giving a wide variety of phospholipids (see Table 2).

As can be seen from their structure, phospholipids are amphipathic. The polar end of the molecule is the phosphorus containing portion and the nonpolar portion is the 2 long hydrocarbon chains contributed

A)



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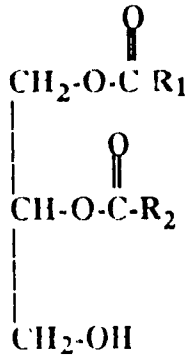

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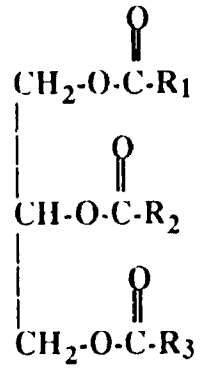


FIG 2 Structures of the acylglycerols

A) 1-monoacylglycerol
B) 1,2-diacylglycerol
C) triacylglycerol

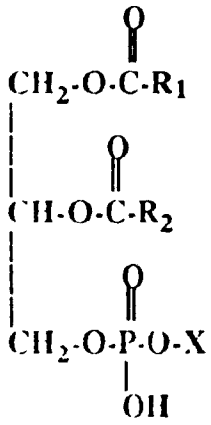


FIG 3 Structure of a phosphoglyceride

TABLE 2 SOME COMMON PHOSPHOGLYCERIDES

X group	phosphoglyceride name
-H	phosphatidic acid
$-CH_2CH_2NH_3^+$	phosphatidylethanolamine
$-CH_2CH_2N^+(CH_3)_3$	phosphatidylcholine (lecithin)
$-CH_2CHCOO^-$ NH_3^+	phosphatidylserine

by the 2 fatty acids. This amphipathic property is important since it means that phospholipids form micelles in water. Micelles are charged aggregates which form with the nonpolar portion of the molecule hidden away from the aqueous environment and the polar end exposed to the aqueous environment. Phospholipids can form similar monolayers (nonpolar tail in air) at air-water interfaces as well as bilayer structures with an internal aqueous component (Fig 4). These bilayers (called liposomes) have been used to study cell membranes since the basic structure of the membrane is believed to be a phospholipid bilayer (although it is certainly more complex than a liposome since there are also proteins in membranes) (2).

4. SPHINGOLIPIDS

Sphingolipids are present in brain and nerve tissue and their name is derived from the presence of sphingosine or a related base (e g. sphinganine) (Fig. 5) All sphingolipids have 3 components: a fatty acid, a sphingosine or related base and a polar head group When the sphingosine base is amide linked to a fatty acid, a ceramide is the new molecule formed (Fig 6) This is the parent structure of all sphingolipids and various polar head groups are attached to the hydroxyl group at the 1-position of the sphingosine base. The most abundant sphingolipid is sphingomyelin and is shown in Fig. 7 with a phosphorylcholine as its polar head group (2)

5. GLYCOSPHINGOLIPIDS (2)

Neutral glycosphingolipids contain 1 or more neutral sugars as the polar head group in the sphingolipid structure If there is only one sugar present (a monosaccharide) the lipid is known as a cerebroside If the monosaccharide is D-galactose, it is a galactocerebroside and if it is D-glucose then it is a glucocerebroside A sulfate esterified to position 3 of D-galactose produces a lipid called a sulfatide.

Acidic glycosphingolipids or gangliosides have a sialic acid (usually N-acetylneuraminic acid, NANA) as one of the residues in the oligosaccharide (2-10 residues) chain. This gives the polar head group a net negative charge at pH 7 0, hence the name "acidic glycosphingolipid".

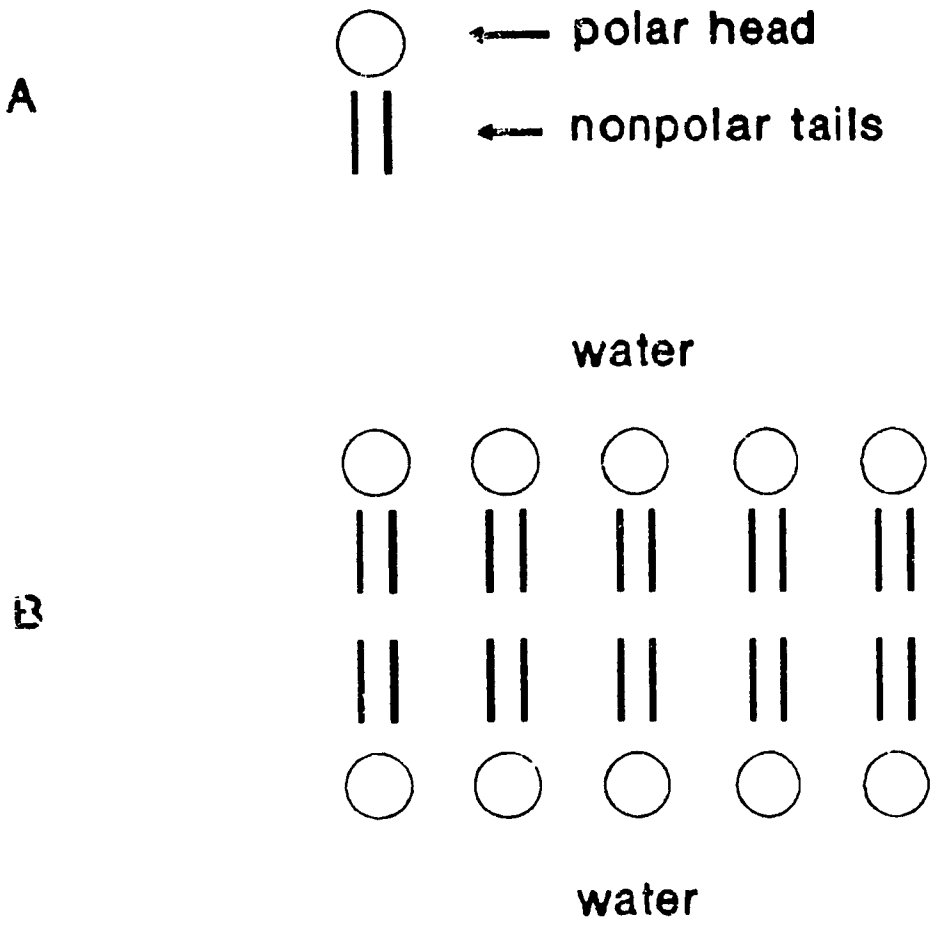
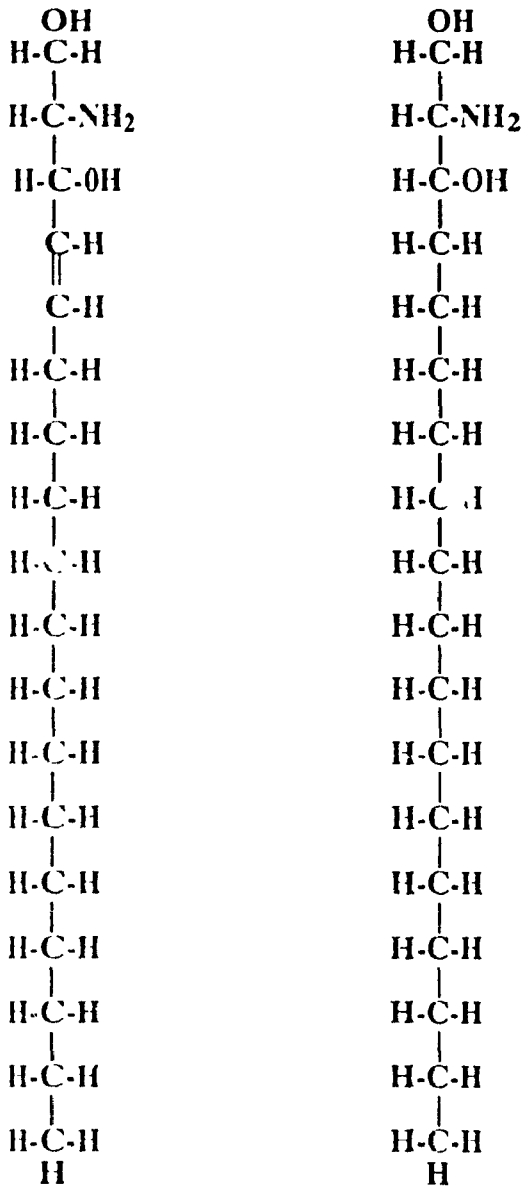


FIG 4 Lipid bilayer in water
A) Phosphoglyceride
B) Lipid bilayer



A) B)

FIG 5 Sphingosine and sphinganine
A) Sphingosine (4-Sphingenine)
B) Dihydrosphingosine (Sphinganine)

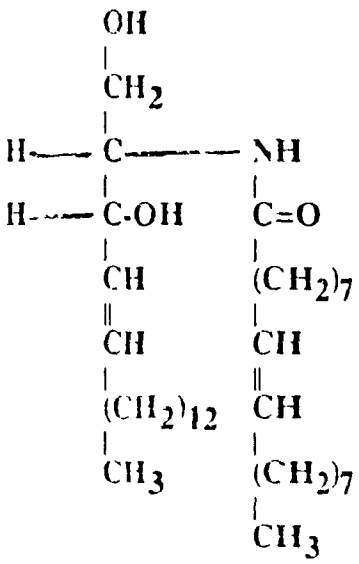


FIG 6 Acramide

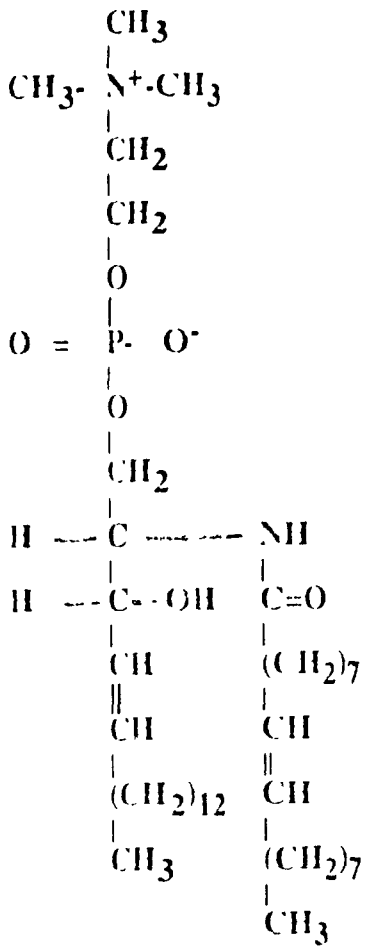


FIG 7 A Sphingomyelin

Glycosphingolipids are present in red blood cells giving blood group specificity and also organ and tissue specificity. They are important in tissue immunity and cell-cell recognition sites; cancer cells have different glycosphingolipids than normal cells.

6. WAXES (2)

Waxes have a fatty acid esterified to a long chain alcohol. They are important in providing a barrier to the environment such as on skin and protective coatings on leaves and fruits. Beeswax and lanolin (wool-fat) are examples.

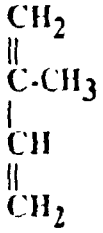
IV. NONSAPONIFIABLE LIPIDS

Up until this point, all the lipids which were discussed were saponifiable, that is, produced soaps on alkaline hydrolysis. Therefore, saponifiable lipids contain fatty acids as building blocks.

The nonsaponifiable lipids contain no fatty acids and hence do not produce soaps on alkaline hydrolysis.

1. TERPENES (2)

Terpenes have a backbone of the 5 carbon group called isoprene (Fig. 8). Terpenes are formed using numerous isoprene units joined in a linear or cyclic fashion. Terpenes are particularly important in plants but also in animals. Squalene (Fig. 9), is a precursor of cholesterol and the carotenoids are also terpenes. β-carotene (Fig. 10) is a precursor of vitamin A which is important in sight. Vitamins E and K are also important terpenes.



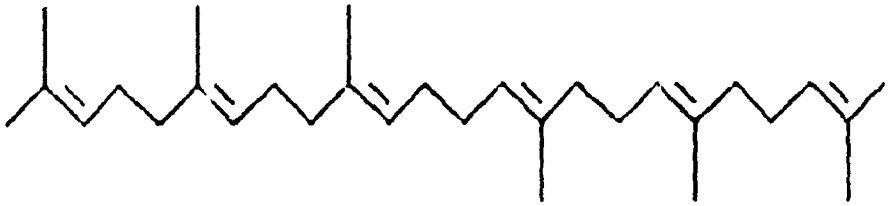
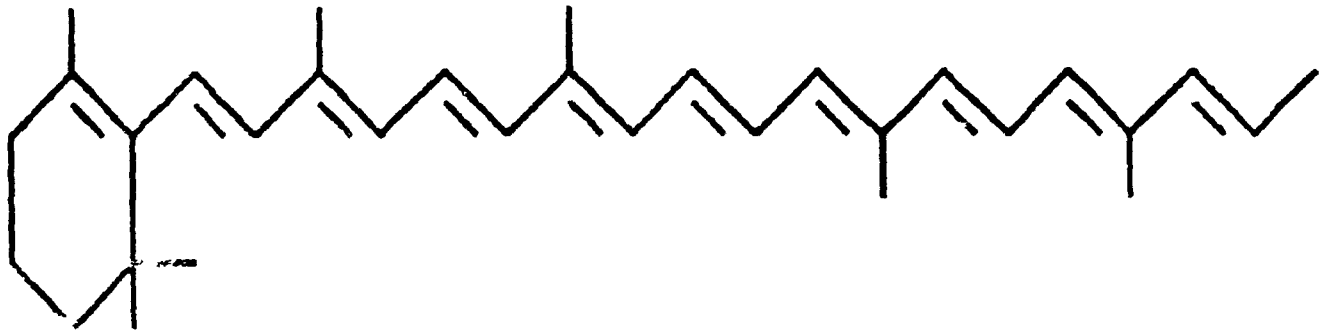
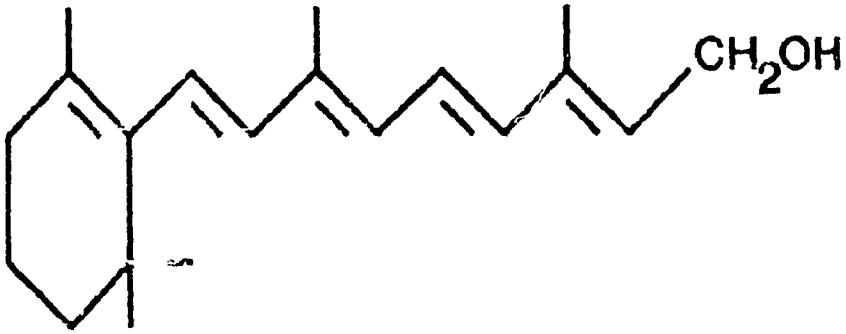


FIG 8 Isoprene unit

FIG 9 Squalene, precursor of cholesterol



A



B

FIG 10 β- arotene and vitamin A_1
A β-carotene
B) Vitamin A_1

2. STEROIDS (2)

Steroids have as their backbone the tetracyclichydrocarbon, perhydrocyclopentanophenanthrene (Fig. 11). Steroids differ in number and position of double bonds, the type, location and number of substituent groups and the configuration (α or β) of the bonds of the substituent groups and the ring structure and also in the ring structure itself (since it has a number of different centers of asymmetry). The most prevalent steroid in animal tissues is cholesterol (Fig. 12).

Cholesterol is the precursor of many other biologically important molecules including: the bile acids which play an important role in the absorption of lipids from the intestine, the male sex hormones, the androgens; the female sex hormones, the estrogens; the hormone progesterone; and also the adrenocortical hormones such as corticosterone and aldosterone.

3. PROSTAGLANDINS (2)

Prostaglandins are fatty acid derivatives which have hormonal and regulatory roles. They are formed by cyclization of the 20 carbon unsaturated fatty acids, such as arachidonic acid (which is derived from linoleic acid, one of the essential fatty acids). Carbons 8 through 12 of the fatty acid form the 5 member ring giving the parent compound, prostanoic acid (Fig. 13). There are a wide variety of prostaglandins with a variety of biological activities which include lowering blood pressure, and induction of smooth muscle contraction.

V. STEREOISOMERISM

The IUPAC-IUB Commission on Biochemical Nomenclature (4) faced a complex problem when trying to agree on a system of distinguishing stereoisomers of lipids. In the literature there are many different systems of designating glycerol alone, all with their respective advantages and disadvantages. The decision was made to use a system of stereospecific numbering The pivotal point of this system states that

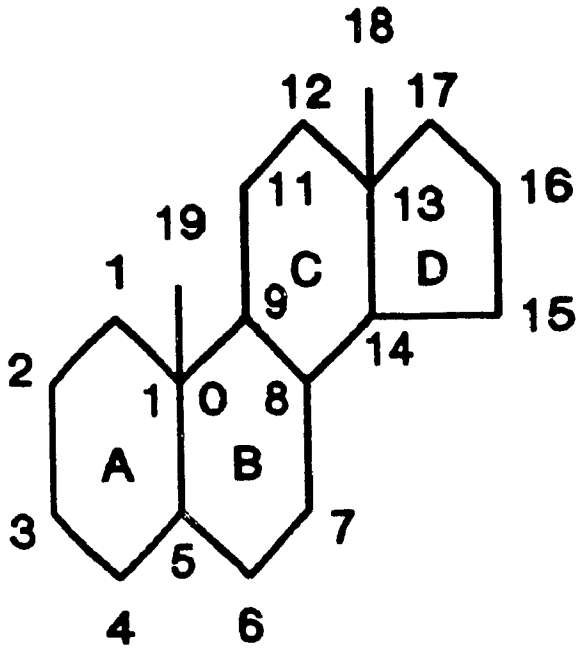


FIG 11 Perhydrocyclopentanophenanthrene structure

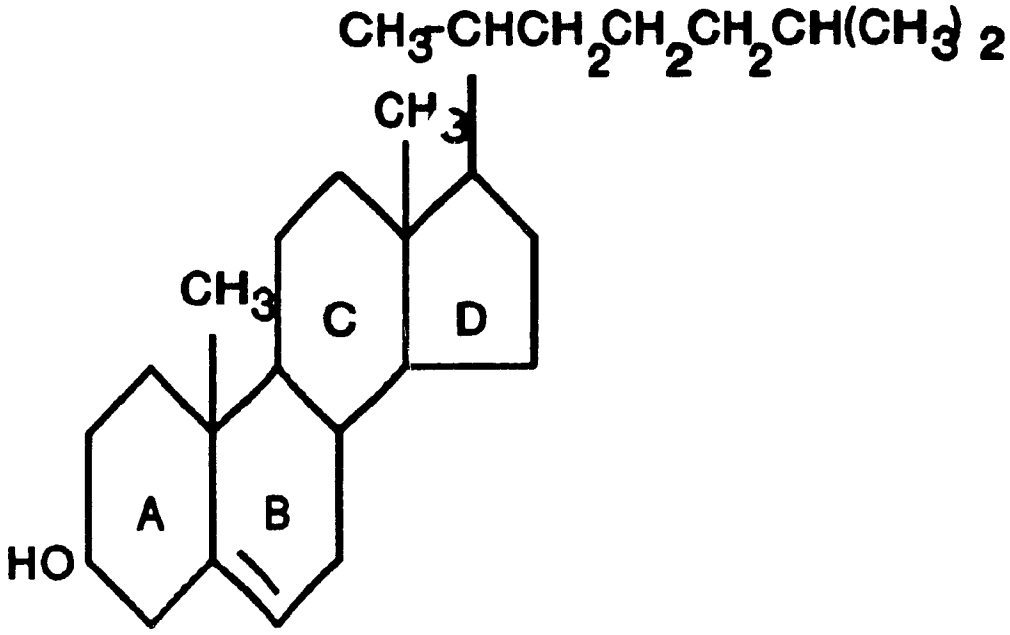


FIG 12 Cholesterol

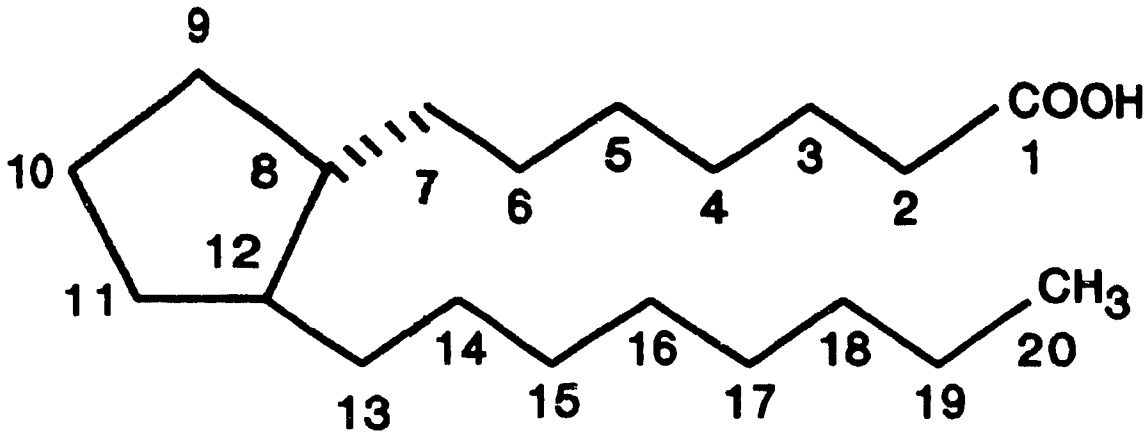


FIG 13 Prostanoic acid

for the secondary hydroxyl of glycerol shown to the left in a Fischer projection, the carbon above the secondary hydroxyl is carbon number 1 and the carbon below is number 3. The prefix *sn* is used before the compound name. Hence glycerol-3-phosphate becomes *sn* glycerol-3- phosphate.

There is one inherent disadvantage to this system and that is that chirality is not shown in the usual manner by a prefix such as R/S or D/L system. However, the fact that carbon number 1 and carbon number 3 lie across a plane of symmetry is enough to show that *sn* glycero-1-phosphoric acid (Fig.14-A) is the optical enantiomer of *sn* glycero-3-phosphoric acid (Fig. 14-B).

VI. LIPIDOSES (3)

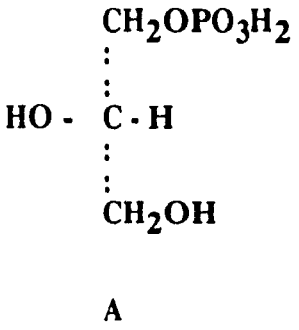
Lipidoses are diseases of an enzyme deficiency. The enzyme, which is missing, causes breakdown of a particular glycosphingolipid and therefore because it is deficient there is accumulation of that particular lipid. Since the lipid very often accumulates in the brain and nervous tissue, there is very often mental retardation and nervous dysfunction (2). Hence, the lipidoses are lipid storage diseases.

1. SPHINGOMYELINOSIS

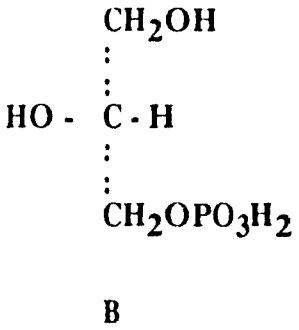
This is also called Niemann-Pick disease and there is deposition of sphingomyelin in every organ and tissue. The missing enzyme is sphingomyelinase.

2. GANGLIOSIDOSES

There are 2 different gangliosidoses, each caused by the accumulation of different gangliosides. Tay-Sachs is the most common with accumulation of the ganglioside in ganglion cells and demyelination. This causes mental dysfunction and impairment of vision. The missing enzyme is a specific N-acetyl galactosidase In neurovisceral gangliosidoses, the ganglioside accumulates because the specific β-galactosidase is absent.



A



B

FIG 14 Enantiomers of glycero-3-phosphoric acid
A) *sn*-glycero-1-phosphoric acid
B) *sn*-glycero-3-phosphoric acid

3. CEREBROSIDOSES

Gaucher's disease is one of the most common lipid storage diseases with accumulation of the cerebroside in the spleen, liver and bone marrow. The missing enzyme is a glucocerebrosidase. Fabry's disease is caused by accumulation of a cerebroside due to the missing specific galactosidase ceramide trihexosidase. Metachromatic leucodystrophy is caused by the accumulation of sulphatide due to absence of sulphatase.

VII. LIPOPROTEINS (5)

Since lipids, by their definition, are not soluble in water, they cannot be transported in blood as such, but form complexes with proteins. These lipoprotein complexes have hydrophobic bonds but few covalent bonds. The complexes are strong enough to resist dissociation during isolation yet allow exchange between the complex itself and body tissues.

All lipids in plasma circulate complexed to protein. Free fatty acids are unesterified fatty acids which are complexed to albumin All other lipids are also complexed to protein and this complex is called a lipoprotein. There are 4 kinds of lipoprotein complexes which are usually separated by either of 2 methods: ultracentrifugation (this method separates lipoproteins by flotation characteristics which are based on their size and density) or electrophoresis. (An electric field is applied to a medium and molecules are separated depending on their mobility and charge A number of different media are used for the separation: paper, starch, etc)

All 4 lipoprotein complexes have a density less than 1 21 g/ml, whereas other plasma proteins (albumin and globulins) have a density of 1.33-1.35 g/ml. The 4 types of lipoproteins can be particles (which scatter light and are large enough to be seen in a light microscope) or soluble lipoprotein complexes (which are soluble in plasma) and can be named by 2 different methods. When classified by their density in

the ultracentrifuge, they are chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL), in order of increasing density. When named by their electrophoretic mobility, they are (in the same order as above) chylomicrons, pre-β (or α_2), β and α lipoproteins (Table 3)

The lipoproteins have various amounts of lipid and protein (as can be seen from Table 3) and have various functions. As the amount of protein increases, the density increases, hence HDL with the largest percentage of protein has the highest density.

1. THE LIPOPROTEIN APOPROTEINS

The proteins which are present in the lipoprotein complexes are called apolipoproteins or simply apoproteins. Fredrickson et al (5) in 1967 described an A and a B protein with the possibility of a C protein. Keyser (6) in 1979 noted the presence of 2 non-identical peptides A-I and A-II, as well as B, C-I, C-II, C-III, D and E. Scanu et al. (7) name the same ones as Keyser in addition to a C-III-0, C-III-1, C-III-2 and an arginine-rich peptide. This situation was made even more complex by the differing nomenclature used by different authors. The classification was done by alphabet (as above), carboxy terminal amino acids or an apo-suffix with the lipoprotein that the protein is associated such as apoVLDL. Certain proteins are associated only with certain lipoproteins (see Table 4) (6).

2. FUNCTIONS AND ORIGINS OF THE LIPOPROTEINS

Chylomicrons carry exogenous fat in the plasma. (Exogeneous fat is fat of dietary origin, whereas endogenous fat is synthesized in the body in the liver.) Chylomicrons appear in the plasma early after fat ingestion. When fat is consumed during a meal, there is dispersion and some digestion by bile salts The glycerols, monoglycerides, diglycerides and free fatty acids are formed into micelles, then become chylomicrons and travel to the venous blood through the lymphatic system Endogenous glycerides which are synthesized in the liver (mostly from carbohydrate) and are the source of free fatty acid, are carried on

TABLE 3 THE TRANSPORT LIPOPROTEINS

	chylomicrons	VLDL	LDL	HDL
electrophoretic mobility	origin	preβ(α_2)	β	α
density (g/ml)	<0.94	0.94-1.006	1.006-1.063	1.063-1.21
protein (%)	1-2	10	25	45-55
triglyceride (%)	80-95	55-65	10	3
phospholipid (%)	3-6	15-20	22	30
free cholesterol (%)	1-3	10	8	3
esterified cholesterol (%)	2-4	5	37	15

TABLE 4 THE APOPROTEINS

lipoprotein class	apoprotein(s) found
chylomicrons	all
VLDL	apo B,C-I,II,III,E
LDL	apo B
HDL	apo A-I,II,C-I,II,III D,E

the pre-β lipoproteins (VLDL). The α (HDL) and β (LDL) lipoproteins between them carry most of the phospholipid and cholesterol in the plasma (see Table 3), fulfilling their function as transport lipoproteins.

VIII. ABNORMAL LIPOPROTEINS (8)

There are some abnormal lipoproteins which have been found, mostly occurring in disease states. A floating β or broad β or a VLDL of β mobility has been seen in uncontrolled diabetes, Tangier's disease and Type III hyperlipoproteinemia (discussed later). The lipoprotein-X (Lp-X), which has phospholipid, cholesterol and VLDL apoproteins, has been seen in obstructive liver disease. HDL_T or α_T lipoprotein has been seen in small amounts in Tangier's disease.

IX. HYPOLIPOPROTEINEMIA

The hypolipoproteinemias are rare and are all due to a decrease or absence of a particular class of lipoprotein.

1. ABETALIPOPROTEINEMIA (9)

Abetalipoproteinemia, as the name suggests, is the absence of β lipoproteins (LDL). There are no chylomicrons produced after fat feeding and the plasma values of cholesterol (20-90 mg/100 ml), phospholipid (95 mg/100 ml) and glyceride (less than 10-20 mg/100 ml) can all be seen to be less than "normal limits" when compared to the normal limits values in Table 5.

2. HYPOBETALIPOPROTEINEMIA (9)

This is a deficiency of the β lipoproteins (LDL) as opposed to an absence as in the previously mentioned disease. There is 10-50% of the normal amount of β lipoprotein. There is a decrease in the plasma cholesterol, phospholipid, glyceride and probably also essential fatty acid. This is a different

TABLE 5 LIPID CONCENTRATION IN BLOOD PLASMA

	mg/100 ml
lipids (total)	400-700
triglycerides	100-250
phospholipids	150-250
cholesterol & esters	150-250
free fatty acids	8-20

disease when compared to that of the abetalipoproteinemia, caused by a different genetic mutation. The hypobetalipoproteinemia can occur secondary to other diseases such as acute infections.

3. FAMILIAL ALPHALIPOPROTEIN DEFICIENCY (9)

This deficiency disease is also known as Tangier's disease. The plasma cholesterol is reduced as is the plasma phospholipid There is a slight elevation of glyceride in the postabsorptive state. There is no a lipoprotein seen on paper electrophoresis but some can be found when immunochemical studies are performed, however it is abnormal. There is gross deposition of cholesterol esters in the body and it is this deposition which causes the clinical features of the disease, such as enlarged tonsils, liver and spleen.

X. HYPERLIPOPROTEINEMIAS (10)

The hyperlipoproteinemias originally were known as hyperlipemias (hypercholesterolemia, hyperglyceridemia, etc.), until the landmark articles by Fredrickson, Levy and Lees in the New England Journal of Medicine in 1967 (5, 9-12) The origins of these diseases can be two-fold. Either primary which is a familial or inherited disease or secondary in which case the hyperlipoproteinemia is due to another disease A secondary hyperlipoproteinemia is generally ignored with the assumption that if one successfully treats the primary disease, the symptoms of the hyperlipoproteinemia will disappear. There are many diseases causing a secondary hyperlipoproteinemia Some examples are hypothyroidism and nephrotic syndrome. The hyperlipoproteinemias are numbered Type I to V starting with the origin on paper electrophoresis Hence, since chylomicrons are generally at the origin, Type I is a chylomicronemia, and so on The diagnosis of the disease is done using a number of tests: usually total lipid concentration (often plasma cholesterol and glyceride as well), serum or plasma turbidity and electrophoresis (Electrophoresis is done more often since it is more convenient and economical than ultracentrifugation which is more expensive and time-consuming.)

1. TYPE I HYPERLIPOPROTEINEMIA (10)

This is a chylomicronemia with an increased concentration of chylomicrons up to 14 hours following the last meal (Normally the chylomicrons are cleared very quickly after a meal and therefore there should be none after fasting.) There is an increased plasma cholesterol and triglycerides with a milky serum with a cream layer on top due to the chylomicrons. This is a rare disease though it has been seen secondary to other diseases such as uncontrolled diabetes, pancreatitis and acute alcoholism

2. TYPE II HYPERLIPOPROTEINEMIA (11)

This very common hyperlipoproteinemia is diagnosed by the presence of clear serum, increase in cholesterol levels, normal or increased triglycerides and an increase in the β migrating lipoproteins (there may be a modest increase in the pre-β lipoproteins) (Beaumont et al. (8) differentiated Type II into an a and b. Type IIa has increased β lipoproteins (LDL) with a normal pre-β (VLDL) and increased cholesterol and normal triglycerides. Type IIb has increased LDL and VLDL with increased cholesterol and triglycerides.) There is deposition of fat in arteries with the involvement of the coronary artery and Type II has been seen sporadically in some patients, presumed to be due to the relatively high fat diet which is an American way of life. The therapy includes reducing weight and dietary cholesterol, using polyunsaturated fats as opposed to saturated and with the use of some drugs which are thought to increase the catabolism of cholesterol or decrease the absorption of cholesterol in the stomach and intestine. Type II is also present secondary to other diseases such as hypothyroidism, obstructive hepatic diseases, myelomas, and hypoproteinemias.

3. TYPE III HYPERLIPOPROTEINEMIA (11)

Type III is a variant of Type II but is clearly a different disease since it is caused by a different mutation. The serum is turbid, the cholesterol and triglyceride are increased, there is an excess of β lipoproteins, however they are at an abnormally low density (floating β or broad β pattern). There is

deposition of lipid in the palms of the hands which is characteristic of this disease. The therapy is similar to Type II: maintain ideal weight, lower cholesterol and eat polyunsaturates. Drugs may be used, however their effectiveness appears uncertain.

4. TYPE IV HYPERLIPOPROTEINEMIA (12)

Along with Type II, Type IV is the most common of the hyperlipoproteinemias. It is diagnosed by the turbid serum, the increase in cholesterol and glycerides and the large increase in pre-β (VLDL) lipoproteins, with no chylomicrons present. The endogenous glycerides are excreted into the liver at such high rates that they cannot be successfully removed. This is accompanied by an increase in body weight, glucose intolerance, and a correlation with coronary artery disease. Type IV is present secondary to insulin dependent diabetes mellitus (youth onset), pancreatitis and alcoholism, etc. Control of the disease is performed by managing body weight and avoiding excess carbohydrate.

5. TYPE V HYPERLIPOPROTEINEMIA (12)

Type V is very rare and is diagnosed by turbid or milky serum (with a cream layer), increased cholesterol and triglycerides Chylomicrons are present with an increase in the pre β lipoproteins This is an exogenous and endogenous hyperlipoproteinemia with abnormal glucose tolerance. There is no evidence for accelerated atherosclerotic heart disease There is some evidence that Type IV and V may be the same disease, homo- and heterozygous, respectively.

6. ATHEROSCLEROSIS (6)

The hyperlipoproteinemias have relatively rare occurrence, however they are important for the information they bring on atherosclerotic heart disease (ASHD) or arteriosclerosis, as it is more commonly known (7) Atherosclerosis is seen as an accumulation of lipids in the arterial wall, in particular of cholesterol esters Some work indicates that the concentration of HDL is inversely proportional to the risk of ASHD or coronary heart disease The size of the body pool of cholesterol increases with a decrease in

plasma HDL. Since HDL (with LDL) carries most of the cholesterol in the body, this was termed a good form of cholesterol by the popular press. There is some evidence that the HDL competes with cholesterol for receptors on the arterial wall and therefore reduces the deposition of cholesterol.

XI. ANALYSIS OF LIPIDS

Lipids have been separated and analyzed by a number of methods. Initially, they were analyzed by wet chemical means and then as analytical techniques developed by thin-layer chromatography (TLC), gas chromatography (GC), and then high pressure liquid chromatography (HPLC).

1. DIRECT METHODS FOR DETERMINATION OF SERUM TRIGLYCERIDES (13)

In order to determine serum triglycerides, the lipids are extracted (using petroleum ether, chloroform or diethyl ether or most commonly using the Folch method: chloroform-methanol 2:1 (v/v)), then the phospholipids are removed (usually using an adsorbent such as Zeolite) and the lipids are saponified using for example, 0.2 - 0.1M ethanolic potassium hydroxide at 60-70 °C for 20-60 minutes. The saponification yields salts of fatty acids and glycerol. The glycerol is most often analyzed. Glycerol can be oxidized by periodic acid to formaldehyde. Two moles of formaldehyde are produced per mole of glycerol. The periodic acid (HIO_4) oxidizes molecules with two adjacent hydroxyl groups or a molecule with a hydroxyl and amino group adjacent. Hence possible interferents include glucose, serine, sphingosine and glycerol phosphate from phospholipids.

The formaldehyde produced by periodic oxidation can then be determined by a number of different reagents. Reaction of chromotropic acid (4,5-dihydroxy-2,7-naphthalenedisulfonic acid) in 9-10M sulfuric acid with formaldehyde at 100°C for 30 minutes produces a violet-pink compound which can be measured at 570 nm.

Formaldehyde can be determined by addition of 0.03M phenylhydrazine hydrochloride. After ten minutes 0.005M ferricyanide is added. The solution is chilled for 4-5 minutes at 0°C. Concentrated hydrochloric acid is added and after 10-12 minutes, the absorbance of what is presumed to be 1,5-diphenylformazan can be taken at 540 nm.

Formaldehyde can be reacted with 1M ammonium acetate and 0.01M acetylacetone at a pH of 5.5-6.5 for 40 minutes at 37°C. A yellow compound, 3,5-diacetyl-1,4-dihydrolutidine, can be measured at 412 nm or the fluorescence can be taken.

Reaction of formaldehyde with 3-methylbenzothiozolin-2-one and ferric chloride yields a chromagen which absorbs at 620 nm.

Other methods rely on the reaction of glycerol rather than formaldehyde in the determination of triglycerides. Glycerol in the presence of o-aminophenol, sulfuric acid and arsenic acid (oxidizing agent) at 170°C produces 8-hydroxyquinoline, which when chelated with magnesium fluoresces.

Glycerol can also be determined enzymatically. In the presence of glycerokinase (and magnesium and cysteine in hydrazine buffer, pH 9.4), glycerol and adenosine triphosphate (ATP) yield α-glycerolphosphate (and adenosine diphosphate (ADP)). The α-glycerolphosphate is then converted by glycerolphosphate dehydrogenase to dihydroxyacetone phosphate (DHAP). This enzyme requires NAD^+ (nicotinamide adenine dinucleotide) which is converted to NADH. The change in absorbance at 340 nm before and after addition of α-glycerolphosphate dehydrogenase is proportional to the amount of glycerol which was present.

Another method starts out in a similar way by conversion of glycerol to α-glycerolphosphate by glycerokinase. However, in this method, further reactions take place with the ADP produced by the glycerokinase. The ADP is converted to ATP while phosphoenolpyruvate (PEP) is converted to pyruvate in the presence of pyruvate kinase. The pyruvate is converted to lactate by lactic dehydrogenase in the

presence of NADH (converted to NAD^+). The glycerol is determined by the change in absorbance at 340 or 366 nm. This is specific for glycerol since any possible interferents of the glycerokinase (such as dihydroxyacetone phosphate or glyceraldehyde) are not present in serum.

There are many other methods for the determination of triglycerides in serum and many of these methods have been automated.

2. COLUMN CHROMATOGRAPHY (13)

Before HPLC was widespread, column chromatography was used to separate the lipids. Large-bore columns were used and the solvent was fed by gravity. Silicic acid, lipophilic sephadex, TEAE- (triethylaminoethyl-) and DEAE- (diethylaminoethyl-) cellulose were among the packings used to separate the lipids (13). The different lipids were then separated and collected in subsequent fractions by using a number of different mobile phases to elute each lipid

Neutral lipids could be separated on silicic acid columns. Typically 3-18 mm i d columns could separate 0.5-20 mg of total lipid into major lipid classes such as neutral lipids, phospholipids and glycosphingolipids.

Phospholipids could be separated by ion-exchange chromatography using TEAE-cellulose and DEAE-cellulose. These ion-exchange columns separated ionic lipids into 5 different groups Eluting first were the neutral phospholipids phosphatidylcholine (PC), lysoPC (a phosphoglyceride which has had one of the fatty acids lysed off), and sphingomyelin, then very weakly acidic phospholipids including phosphatidylethanolamine (PE), lysoPE, and N-methyl phospholipid derivatives, weakly acidic free fatty acids; more polar weakly acidic fatty acids including phosphatidylserine (PS) and lysoPS; and strongly acidic compounds such as phosphatidic acid

A typical separation of lipids on TEAE-cellulose starts with application of a 100-200 mg sample in 5-10 ml of chloroform. The elution includes 7 steps: 1. five column volumes of chloroform elutes

sterols, sterol esters, triglycerides, and ceramides; 2. eight column volumes of 9:1 (v/v) chloroform-methanol elutes choline phospholipids (phosphatidylcholine (PC), lysoPC and sphingomyelin); 3. eight column volumes of 2:1 (v/v) chloroform- methanol elutes ceramides polyhexosides; 4. eight column volumes of 2:1 (v/v) chloroform-methanol containing 1% glacial acetic acid elutes phosphatidylethanolamine (PE), lysoPE, free fatty acids and dimethylPE; 5. eight column volumes of glacial acetic acid elutes phosphatidylserine; 6. eight column volumes of methanol removes all excess acetic acid; 7. ten column volumes of 4:1 (v/v) chloroform-methanol made with 0.1M potassium acetate and 20 ml/l 28% aqueous ammonia added followed by six column volumes of methanol elutes acidic phospholipids.

3. THIN-LAYER CHROMATOGRAPHY (13)

In thin-layer chromatography a glass plate is covered with an aqueous slurry of an adsorbent material. The adsorbent could be silica or cellulose, often with the addition of silver nitrate. (The silver ions form a charge-transfer complex with unsaturated lipids, allowing separation of saturated and unsaturated lipids.) The plate is dried and baked and then the sample to be analyzed is spotted onto the bottom of the plate. The lower edge is dipped into the solvent and the solvent rises by capillary action similar to ascending paper chromatography Once the solvent front reaches the top, the plate is dried and the separated lipids can be visualized by spraying with a number of different indicators (2). TLC has been furthered by the development of two-dimensional TLC. One solvent is used and then a second solvent is run at right angles to the first This allows a more complete separation.

Silica gel is most often used for separation of lipids by TLC. The sample is generally in a solution of 19:1 (v/v) chloroform- methanol at a concentration on the order of 25 mg/l.

Neutral lipids can be separated by the developing solvent hexane-diethyl ether-acetic acid 85:15:2 (v/v/v) into cholesterol esters, triglycerides, free fatty acids and cholesterol. To separate phospholipids and glycosphinglipids, a number of different solvents have been used, the first dimension solvent was

chloroform-methanol-aqueous ammonia 65:35:5 (v/v/v) with the second dimension of chloroform-acetone-methanol-acetic acid-water 5:2:1:1:0.5 (by volume) among others. Ceramides and weakly polar lipids have been separated with chloroform-methanol-ammonia 95:5:0 5 (v/v/v) in the first dimension and chloroform-acetone-methanol-acetic acid-water 8:1.5:0.5:0.5:0.5 (by volume).

Detection reagents for visualizing all lipids on TLC plates are numerous. The most common is the char reagent in which the basic ingredient is usually sulfuric acid as the oxidizing agent. Fifty percent sulfuric acid has been used but this does not char saturated lipids uniformly. A 0.6% potassium dichromate solution in 50% sulfuric acid and 37% formaldehyde-98% sulfuric acid 3:97 (v/v) both char all lipids uniformly.

The char reagent is sprayed onto the TLC plate in a fine mist. The plate is baked for 20 minutes at 175-180 °C. The disadvantage of the char reagent is that it is destructive, hence no further analysis can be done with the lipids.

There are several non-destructive sprays for all lipids including water (the least sensitive), 0.001% rhodamine 6G in water and 0.03% 2'7'-dichlorofluorescein in 0.01N sodium hydroxide. In the case of the latter two, the lipids can be visualized under an ultraviolet lamp.

There are also spray reagents for specific lipids, such as ninhydrin for amino phospholipids.

Quantitation of samples after separation by TLC can be done using a number of different reagents. The spots are removed from the TLC plate by scraping with a razor blade or aspiration.

Phosphorus determination after charring is done by oxidizing the sample with a solution of 85% sulfuric acid-72% perchloric acid 2.1 (v/v). The sample is heated at 260°C. for at least 3 hours. The reducing agent 1-amino-2-naphthol-4-sulfonic acid (ANSA is prepared by dissolution of 2.72 gm sodium bisulfite, 0.5 gm sodium sulfate, and 0.1 gm ANSA diluted in a 100-ml volumetric flask) is added to the tube and then a 2 5% solution of ammonium molybdate is added. The color of the phosphomolybdate

solution is developed at 100°C. for 10-15 minutes. The absorbance is read at 820 or 795 nm against a reagent blank.

Glycosphinglipids can be determined with trinitrobenzene sulfonic acid (TNBS). A 1.0-ml aliquot of 14% boron trifluoride in methanol is added and heated at 110°C. for 1.5 hours. The tube is cooled and 1.5 ml water added and then extracted 3 times with 2-ml portions of chloroform. Nitrogen is used to evaporate the solvent and redissolution is done in methanol. A 1-ml aliquot of 4% sodium bicarbonate and 1 ml 0.1% aqueous TNBS are added with heating for 1 hr at 40°C. A 1-ml aliquot of 1N hydrochloric acid in methanol is added with 3 extractions into 2-ml of n-hexane. The solvent is again evaporated with nitrogen and redissolution in 4 ml of 95% ethanol. The absorbance is read at 340 nm.

Cholesterol can be determined with the zinc chloride-acetyl chloride reagent. The spots to be analyzed are scraped into tubes and dried over potassium hydroxide pellets in a dessicator. The sample is then dissolved in 2 ml of chloroform and 1 ml of the zinc chloride-acetic acid reagent (The zinc chloride-acetic acid reagent is prepared by heating 40 g of anhydrous zinc chloride in 150 ml of glacial acetic acid at 80°C for two and a half hours) followed by 1 ml of acetyl chloride. The mixture is warmed at 65°C for 15 minutes and then placed in an ice bath for 15 minutes. The solution is diluted with 5 ml chloroform and the samples are centrifuged to remove the TLC adsorbent. The absorbance of the supernatant is read at 528 nm against a reagent blank.

Free fatty acids, sterol esters and triglycerides are converted to fatty acid methyl esters and determined by addition of 1.5 ml of 14% boron trifluoride in methanol. This solution is heated at 110°C for 5 min The solution is cooled, 1.5 ml of water added and the fatty acid methyl esters are extracted into hexane 3 times using 2 ml each time. The hexane is evaporated with nitrogen and 3 ml of absolute ether, and 0.1 ml of alkaline hydroxylamine is added (3% ethanolic hydroxylamine solution in 95% ethanol). This solution is then evaporated to dryness in a warm water bath at 65°C. One sample is taken to completion at a time The sample is evaporated at reduced pressure to remove the ethanol. A 6-ml ferric

perchlorate aliquot is added and after 30 minutes, the absorbance is read at 530 nm against a blank of 95% ethanol.

Densitometry of the TLC spots can also be done and this technique has the advantage of being simple and fast.

4. GAS CHROMATOGRAPHY (13)

Lipids have been determined by gas chromatography. GC has been particularly useful for the separation of fatty acid esters. Fatty acid methyl esters have been analyzed in concentrations of 10-20 mg/ml in a solution of pentane or hexane.

The determination of fatty acids of neutral lipids can be done in either of 2 ways: after removal of all interfering phospholipid or after saponification of the lipid (with 0.02-0.1M alkali, 60-70 °C, 20-60 minutes) but having omitted the removal of the phospholipid. The phospholipid is often adsorbed onto a suitable adsorbent such as Zeolite. In addition, triglycerides can be determined after conversion into fatty acid methyl esters. Triglycerides cannot be determined directly since they are not volatile (14)

5. INFRARED SPECTROMETRY (13)

Infrared spectrophotometers as well as Fourier Transform Infrared Spectrophotometers (FTIR) have been extensively used to determine lipids. The useful features include the carbon-hydrogen stretching at 3 to 4 μ (3300-2500 cm^{-1}) and the carbonyl (C=O) at 5.85 μ (1710 cm^{-1}).

Lehmann et al. (15) used a Perkin Elmer model 735B infrared spectrophotometer to measure phosphatidylcholine-sphingomyelin ratios. The measurement of phospholipids in amniotic fluid is useful in determining fetal lung maturity. (Phosphatidylcholine is one of the major lung surfactants in the alveolus (the small air sacs in the lungs where oxygen and carbon dioxide exchange with the blood take place) and play a major role in the decrease in surface tension there. Hence a decrease in phospholipids may lead to

respiratory distress syndrome (RDS). The concentration of sphingomyelin is constant and greater than the concentration of phosphatidylcholine until the 26th week of pregnancy and after this time the concentration of phosphatidylcholine increases.) Measuring the phosphatidylcholine-sphingomyelin ratio is often done during premature labor, and if a diagnosis of immature lungs is made, efforts can be taken to stop the labor until the lungs have matured

Lehmann et al.(15) analyzed commercial standards of sphingomyelin and phosphatidylcholine and also a simulated amniotic fluid sample containing various ratios of the two phospholipids. An infrared spectral scan from 4000 to 400 cm^{-1} was taken using 0.1 mm pathlength sodium chloride cells against a reference blank of chloroform. Peaks were observed at 1720 cm^{-1} .

Teramae and Tanaka (16) use a Digilab FTS-15 FTIR spectrophotometer; however, they couple the FTIR to a micro (0.5 mm) LC column. The column is packed with the porous polymer styrene-divinylbenzene. The flow cells (either 0.025 mm pathlength sodium chloride with 0.5 ml volume or 0.2 mm pathlength potassium bromide flow cell with 1.2 ml volume) were placed directly into the sample compartment of the FTIR. Teramae and Tanaka use, among others, samples of diethylphthalate (detected at 1740 cm^{-1}) and p-nitrotoluene (detected at 1520 cm^{-1}). In addition, Taylor (17) coupled an FTIR with a liquid chromatograph for the separation of aliphatic and aromatic hydrocarbons. Combellos et al. (18) coupled a HPLC with a Nicolet FTIR for the detection of aromatics. The FTIR could, therefore, be extended to analyze lipids.

6. LIPOPROTEIN SEPARATION

Lipoproteins have been separated by electrophoresis and the ultracentrifuge as was mentioned in the section on lipoproteins

7. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

As HPLC became popular, it was not long before lipids were being analyzed by this technique. HPLC is a much simpler and faster technique for analysis of mixtures as can be seen when compared with column chromatography (Section XI-2) and the many different solvents used as well as the large volumes required in comparison to HPLC. In addition, HPLC analysis of the separated components is generally done on-line, not the case in column chromatography where fractions are collected and analyzed later, a lengthier procedure.

A number of different types of detectors were used for HPLC analysis of lipids, some more successful than others. Transport ionization detectors were successful in conjunction with gas chromatography and also to some extent in HPLC. In FID (flame ionization detection), there is deposition of a portion of the eluate from the column onto a moving chain, wire or a number of other media After evaporation of the solvent, the solute is transported into a flame of an FID (or argon ionization) (19, 20) There are numerous problems with this method of detection. The size of the detector signal depends on the flow rate, the speed of the moving carrier, the wetting properties of the solvent or affinity of the solute for the carrier surface (20). The detector can be used with gradient elution, however, this may be difficult since some detectors take all of the eluate, while others take only a reproducible portion of the eluate, causing difficulties in quantitation. An additional disadvantage is that the detector is totally destructive These detectors have been available commercially, however, their reliability, reproducibility and quantitative performance are not well established (19). FID detectors have been used to detect skin and blood lipids, cholesterol esters, triglycerides, waxes and brain cerebrosides (19).

Refractive index detectors are considered universal detectors. They monitor the refractive index between the reference mobile phase and the column effluent. Hence all solutes can be detected if the proper mobile phase is used so that there is a difference in refractive index between the solute and solvent They are temperature and flow sensitive and are not considered very useful with gradients because of the

refractive index change (21). Refractive index detectors have been used to detect triglycerides (22) and also cholesterol and cholesterol esters (22).

Ultraviolet-visible photometers and spectrophotometers have a high sensitivity for many solutes that absorb in the ultraviolet or visible region of the spectrum. The sample concentration in the flow cell is ideally determined by Beer's Law. These detectors are useful with gradients and many solvents are available. The problem with this detector is that the solute must have a high extinction coefficient in the ultraviolet or visible region of the spectrum. Lipids generally do not have a very high absorptivity in the ultraviolet or visible region. Some work has been done in the low end of the ultraviolet region of the spectrum (approximately 200 nm) Phosphatidylcholine and sphingomyelin have been detected at 203 nm (24) and also at 206 nm (25), as have triglycerides at 210 nm (26) The lipids that have been detected in this region generally have at least one carbon-carbon double bond.

Lipoproteins have been separated using aqueous gel permeation columns. In particular, the column material TSK gel type PW which is made of microspheres of hydrophilic polymer and the column material TSK gel type SW which is made of a chemically modified silica gel based aqueous support, have been used by a group of Japanese researchers (27, 28) to separate lipoproteins. The solvent used is a Tris-HCl buffer with a pH of 7.4 or a 0.15M sodium chloride solution. The detector used was an absorbance detector at 280 nm. It was noticed in the first paper (27) by these researchers that when using a single column (any one of the following G3000SW, G4000SW, G5000SW, G6000SW) of 600 mm length, 7.5 mm i.d., and separating a mixture of VLDL, LDL, HDL and albumin; separation was incomplete. It was further noted that the G3000 SW column separated the low molecular weight fractions (HDL_2, HDL_3 and albumin) (HDL_2 and HDL_3 are 2 subclasses of the high density lipoproteins with densities of 1.063-1.125 and 1.125-1.210 g/ml, respectively) and the G5000PW or G6000PW columns separated the high molecular weight fractions (VLDL and LDL). Therefore in order to attain a good overall separation of the mixture, 2 or 3 columns were used in series (G5000PW and G3000SW or G5000PW and two G3000SW or G6000PW and G3000SW or G6000PW and two G3000SW) (27). Any of these combinations of columns separated the

mixture into VLDL, LDL, HDL_2 and HDL_3. This was used on normal and pathological blood samples where the subject had been fasting for 12-16 hours. Differences in the chromatograms (i.e. the amounts of each lipoprotein) could be seen between males and females, and normal and pathological blood (e.g. coronary heart disease, cirrhosis, hyperlipidemia).

Since the UV absorbance detector is a very sensitive detector it is the detector of choice if the compound of interest has an absorbance in this region. However, often the absorptivity is too low, hence derivatives are formed to increase the detector response for a particular compound or class of compounds in the sample (21). This has been done for lipids as well. Benzyl, p-nitrobenzyl, 2-naphthacyl esters are useful derivatives for fatty acids, benzyloxime, 2,4-dinitrophenyl-hydrazones for carbonyl-containing steroids and p-nitrobenzoate esters for hydroxyl compounds such as ceramides and other glycolipids (19). These derivatives can be formed before or after the chromatography and very often this derivatization step can be automated

A particular case is the automation of the Nash reaction (d in Section XI-1) where 2,4-pentanedione (acetylacetone) in 2M ammonium acetate reacts with aldehydes (of particular interest is formaldehyde) to give 3,5-diacetyl-2,6-dihydrolutidine. Triglycerides have been separated on an octadecylsilane column with a post-column reaction to produce the 3,5-diacetyl-2,6-dihydrolutidine product with detection at 410 nm (29).

Compton and Purdy (30), in order to improve the reaction, observed that the reaction of ammonia and 2,4-pentanedione produces a stable and easily isolated product 4-amino-3-penten-2- one, called Flural-P. The Flural-P is then used in the continuous flow analysis of formaldehyde This reagent is a very useful one for continuous flow analysis since the reaction product can then be analyzed by absorption (410 nm) or fluorescence (excitation wavelength 410 nm and emission wavelength 510 nm)

Since infrared spectrometry had been performed on lipids, the use of infrared detectors with HPLC seemed logical. The real problem with infrared detectors is the lack of suitable solvents which have

an optical transmission which is sufficiently high to enable detection of eluants from the column (31). The halocarbons have been useful, in particular chloroform and methylene chloride, and depending on the wavelength of interest, methanol, acetonitrile and tetrahydrofuran. The deuterated solvents would be useful if it were not for the cost and the presence of the protio-isomer in small amounts (32). Water and methanol have large absorbances in certain portions of the infrared spectrum, the costly deuterated solvents do not have these large absorbances. Chen and Kou (33) use deuterated water and methanol to separate phospholipids and sphingomyelin in plasma using a commercial infrared detector.

DuPont has an infrared LC detector on the market and this particular instrument has been used for detection of lipids. Payne-Wahl et al. (34) used the DuPont detector to detect methyl palmitate, mono-, di-, and tripalmitin. Parris (31) used the DuPont detector for detection of tributyrin. In addition, the DuPont detector has been used with gradient elution for the determination of triglycerides in citrus leaves (35). Parris and Payne-Wahl both used the variable wavelength infrared detector at 5.72 μ which is the wavelength for detection of the carbonyl group In addition to carbonyl detection, Chen and Kou (33) use the DuPont detector at 6.15 μ for detection of the amide group of sphingomyelin.

A helium-neon laser which emits at 3 39 μ (in the region of the C-H stretching of the CH_2 group) in the infrared has been used as the source in a novel infrared detector (32). This detector produced a linear calibration curve for tripalmitin using trichloroethylene as the solvent.

REFERENCES

1. Christie, W W , Lipid Analysis "Isolation, Separation, Identification and Structural Analysis of Lipids", 2nd edition, Pergammon Press, Oxford, 1982, p 1

2. Lehninger, A L , "Biochemistry", 2nd edition, Worth Publishers, Inc , New York, 1975

3. Gurr, M.I and James, A J , "Lipid Biochemistry an Introduction", 3rd edition, Chapman and Hall, London, 1980, p 152-155.

4. IUPAC-IUB commission on Biochemical Nomenclature of Lipids, Biochem J (1967) *105*, 897

5. Fredrickson, D S , Levy, R I , and Lees, R S , N.Engl J Med (1967) *276*, 34

6. Keyser, J W , "Human Plasma Proteins Their Investigation in Pathological Conditions", John Wiley and Sons, Chicester, 1979, p 98

7. Scanu, A M , Edelstein, C and Keim , P in "The Plasma Proteins Structure, Function and Genetic Control", vol I Putnam, F.W., ed , 2nd edition, Academic Press, New York, 1975, p 326

8. Beaumont, J L , Carlson, L A , Cooper, G R , Fejfar, Z , Fredrickson, D S , and Strasser, T., Bull World Health Organization, (1970) *43*, 891

9. Fredrickson, D S , Levy, R I and Lees, R S , N Engl J Med (1967) *276*, 94

10 Fredrickson, D S , Levy, R I and Lees, R S , N Engl J Med (1967) *276*, 148

11 Fredrickson, D S , Levy, R I and Lees, R S , N Engl J Med (1967) *276*, 215

12. Fredrickson, D S , Levy, R I and Lees, R S , N Engl J Med (1967) *276*, 273

13. Nelson, G J , ed , "Blood Lipids and Lipoproteins Quantitation, Composition and Metabolism", Wiley Interscience, New York, 1972

14. Stolyhwo, A., Colin, H and Guichon, G , Anal Chem (1985) *57*, 1342

15. Lehmann, C , Rosenfeld, M and Parker, M , Anal Lett (1980) *13*, 1303

16. Teramae, N and Tanaka, S , Spec Lett (1980) *13*,117

17. Taylor, L.T , J Chrom Sci (1985) *23*, 26

18. Combellos, C , Bayart, H , Jasso,R , Caude, M , and Rosset, R , J. Chrom. (1983) *259*, 211.

19. Aitzetmuller, K , J Chrom (1975) *113*, 231

20. Aitzetmuller, K , J Chrom Sci (1975) *13*, 454

21 Snyder, L R , and Kirkland, J J , "Introduction to Modern Liquid Chromatography", 2nd edition, John Wiley and Sons, Inc , New York, 1979, p 144

22 Bezard, J A , and Ouedraogo, M A , J Chrom (1980) *196*, 279

23 Perkins, E G , Hendren, D J , Bauer, J E , and El-Hamdy,A-H , Lipids (1981) *16*, 609

24. Jungalwala, F B , Evans, J E , and McCluer, R H , Biochem J (1976) *155*, 55

25 Hax, W M A , and Geurts van Kessel, W S M , J Chrom (1977) *142*, 735

26 Kapoulas,V and Andrekopoulos, N K , J Chrom (1986) *366*, 311

27. Okazaki, M , Ohno, Y , and Hara, i , J Chrom (1980) *221*, 257

28 Ohno, Y., Okazaki, M., and Hara, I , J. Biochem. (1981) *89*, 1675

29. Kondoh, Y., and Takano, S , Anal Chem (1986) *58*,2380

30 Compton, B J., and Purdy, W C , J Liq Chrom (1980) *3*, 1183

31. Parris, N A , J Chrom Sci (1979) *17*, 541

32 Freeman, N.K , Upham, F T , and Windsor, A A , Anal Lett (1973) *6*, 943.

33 Chen, S , and Kou, A Y , J Chrom (1984) *307*, 261.

34 Payne-Wahl, K , Spencer, G F , Plattner, R D , and Butterfield, R.O., J. Chrom. (1981) *209*, 61.

35 Nordby, H.E , J.Am Oil Chem Soc (1984) *61*, 1020

CHAPTER 2

THE LIQUID CHROMATOGRAPHIC DETECTOR

A high pressure liquid chromatographic detector was needed for lipids. As mentioned in the previous chapter, all lipids absorb in the infrared in the 3-4 μ region. It was decided to use a helium-neon laser, which emits in the infrared at 3.39 μ, as the source for the detector. Each component of the detector will be described: the laser, the flow cell, and the detector. The considerations used for the choice of each particular component will also be discussed. A schematic of the detector is shown in Fig. 1 with arrows for the direction of the light paths which are followed.

I. THE LASER

LASER is an acronym for Light Amplification by Stimulated Emission of Radiation. Lasers can be classified by a number of different means, for example by the lasing medium: solid, liquid, gas or semiconductor. The classification can be by the state of the lasing material, that is, neutral atom, ion or molecular laser or it can be classified by the type of laser output produced, that is, continuous wave (CW) or pulsed laser.

1. History

Schawlow and Townes (1) predicted operation of the laser in 1958. In 1960, this prediction came true with the production of the ruby laser by Maiman (2) The ruby material is aluminium oxide (Al_2O_3) doped with chromium(III) ions. The chromium ions produce the laser light The ends are silvered parallel faces irradiated with a high power flash lamp at 5500 angstroms In 1961, Javan et al (3), produced the first gas laser (a helium-neon laser) which lased in the infrared at a number of different wavelengths the strongest of which was at 11530 angstroms. The laser tube was 80 cm long with a 1.5 cm internal diameter,

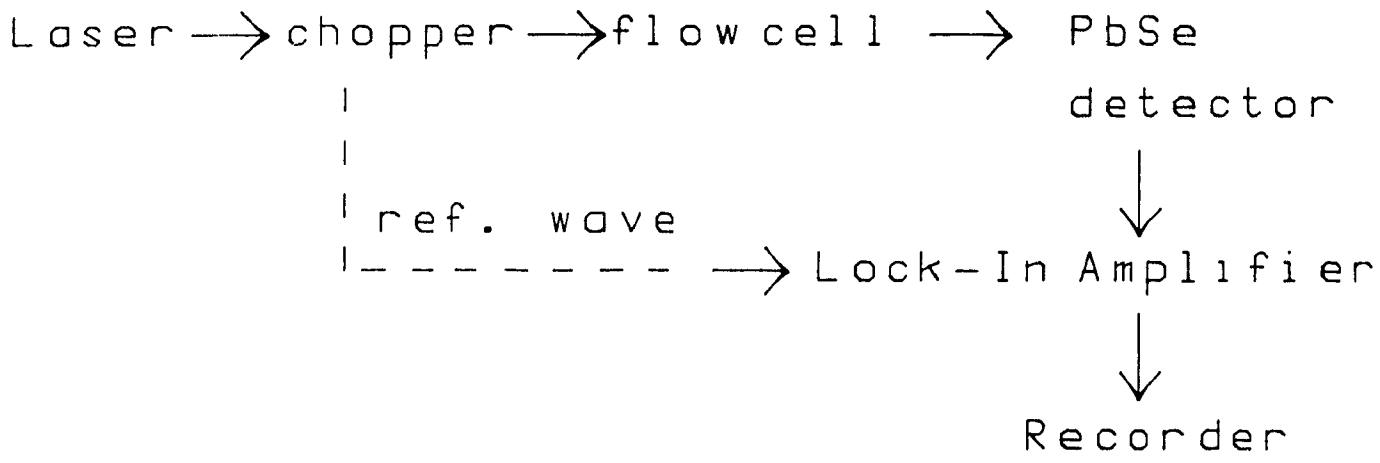


FIG 1 Schematic of HPLC infrared detector

filled with 0.1 mm Hg neon, 1 mm Hg helium and excited with an RF discharge. White and Rigden (4) produced the first red helium-neon laser (6328 angstroms) in 1962.

There are three components which all lasers have in common, though these components can differ widely from laser to laser: a medium that exhibits gain, a system that produces feedback and a pump. In the case of a gas laser, they are a tube filled with the gas that will lase, two mirrors facing each other to produce feedback and the pump is a DC or RF power supply (Fig. 2). Usually one of the mirrors is totally transmitting (M1) and the other is partially transmitting (M2), hence the laser light exits through the partially transmitting mirror (M2).

2. Mechanism of operation

In order to understand a laser one must understand the processes that go on in the atom, molecule or ion. There are many energy levels present in, for example, an atom. Absorption occurs when incoming light of the correct wavelength excites an electron from a lower energy level to that of a higher energy level. The wavelength is equal to hc/E_f-E_i (where h is Planck's constant, c is the speed of light, E_i is the energy initially and E_f the energy after the absorption) (Fig. 3). Spontaneous emission occurs when the electron drops from any of its higher energy levels down to one of the lower energy levels with light being emitted (Fig. 4). Spontaneous emission is a random process and hence yields incoherent radiation. Stimulated emission, which is the process which occurs in a laser, occurs when incoming photons of a precise wavelength and energy collide with excited electrons causing these excited electrons to relax to a lower energy level and at the same time emit a photon which has the same wavelength and energy as that of the incoming photon (Fig. 5). This emitted photon is in phase with the photon that stimulated the event and hence the two photons are coherent (5) All these processes occur in the molecule or ion.

In order to have a laser, one needs stimulated emission and in order to have stimulated emission, most lasers need a population inversion. Normally, in atoms and molecules the lower energy states have a higher population distribution than the higher energy states. Hence, a population inversion, as the words

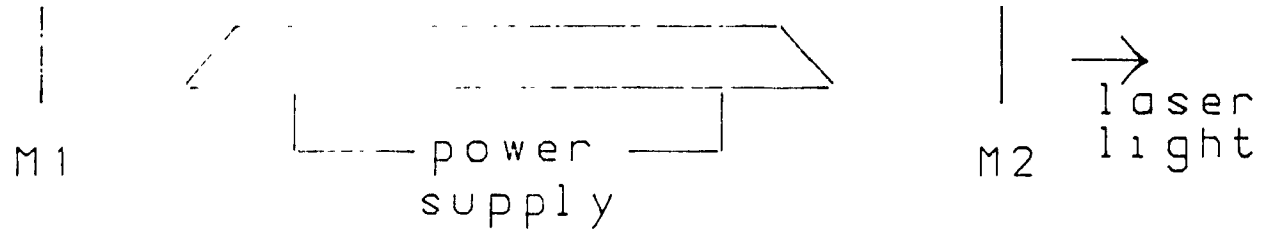


FIG 2 Schematic of a gas laser

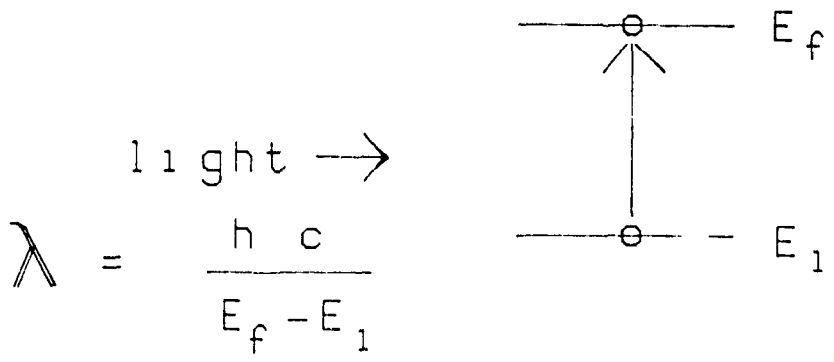


FIG 3 Absorption

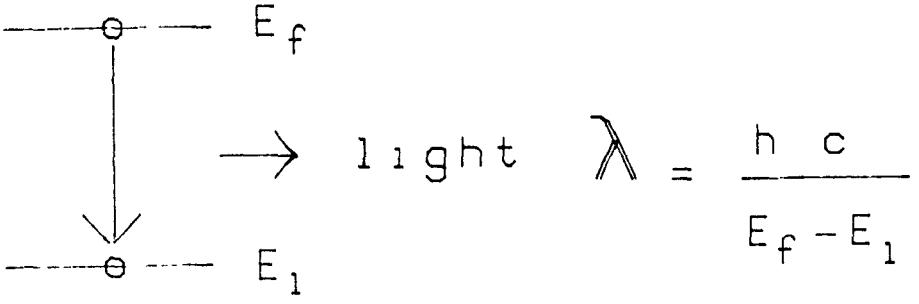


FIG 4 Spontaneous emission

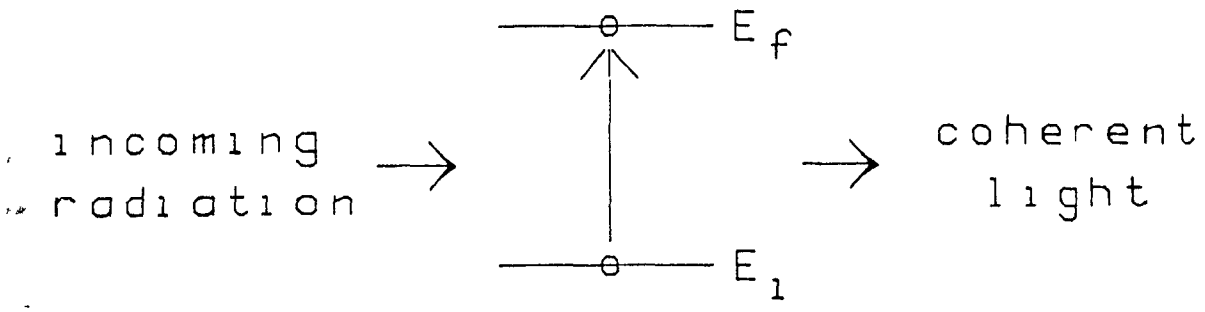


FIG 5 Stimulated emission

imply, is a condition in which for a given pair of energy levels the higher energy state is more populated than the lower energy state.

At equilibrium the Boltzmann distribution applies.

$$N_{upper} = N_{lower} \exp -h\nu/kT \qquad (2.1)$$

where N_{upper}, N_{lower} are the number of atoms in the upper and lower energy states respectively, h is Planck's constant, ν the frequency of light, k Boltzmann constant, T the temperature in °K. Under normal equilibrium conditions and positive temperature, N_{upper} is always less than N_{lower} (the population of the upper energy level is always less than the population of the lower energy level). However, in a laser, a population inversion is required which implies that the population in the upper level is greater than that in the lower level. Under these conditions, solving the Boltzmann distribution gives a negative temperature, non-equilibrium condition (6). The role of the pump or power supply is to produce this non-equilibrium state so that lasing can occur. When a population inversion is produced, incident radiation stimulates the electrons to emit their radiation giving stimulated emission (7). A requirement for the upper level state is that the lifetime be longer than that of the lower level state. Hence the upper level is often a metastable state and consequently lasing rarely occurs with an emission to the ground state since intuitively, one realizes that the ground state would have the largest population distribution and hence it would be very difficult to obtain a population inversion over the ground state (7).

In gas lasers there are a number of different mechanisms by which the population inversion is produced. The two more common mechanisms will be explained.

$$A + e \rightarrow A^* + e \qquad (2.2)$$

The first is direct electron excitation (7), collision of the first kind (8) or excitation by electron impact (9) depending upon which reference is consulted. The excitation is of the atom (A) to a higher level (A^*) upon collision with an energetic electron (e)(equation 2.2). Examples are the noble gas lasers.

$$A^* + B \rightarrow A + B^* \qquad (2.3)$$

The second mechanism is a resonant process where an excited atom (A*)close in energy to an excited state of the lasing atom (B)can transfer its energy to the lasing atom (7). That is, the two atoms each have an energy level which are very close together in energy (equation 2.3). This is the process which occurs in the helium-neon laser where the excited atom is helium and the atom which will become excited and lase is neon.

Lasers can also be categorized as three or four level systems. In the three level laser, the atoms are pumped from the same level as the lower laser level (6). That is, they are excited from a particular level and after stimulated emission drop to the same laser level. The ruby laser is an example of a three level laser (Fig. 6). In the four level laser, none of the laser levels are involved in the pumping process (6) The helium-neon laser is a four level laser.

The pump that is present in a particular laser is either an optical pump or a power supply (DC or RF) The ruby laser and the helium-neon laser are examples of the different types of pumps possible. The ruby laser uses a Xenon flashlamp (often helically wound around the ruby rod) to excite the electrons up to the energy level called $^4F_2$ (Fig. 6). The electron then drops down to the ^2E level and lases back down to the $^4A_2$ where it started (a three level laser). (The optical pump can also be another laser as is the case for dye lasers.) The helium-neon laser uses a power supply, either DC or RF. (The first helium- neon laser produced by Javan et al (3) used an RF discharge.) The electrons of the helium atom are excited up to either the 2^1S or 2^3S level and then through a collision transfer their energy to the $3s_2$ level or $2s_2$ energy levels of neon. As can be seen from Fig. 7, the 2^1S of helium and the $3s_2$ level of Neon are very close in energy as are the 2^3S of helium and the $2s_2$ of neon The laser is then produced by neon electrons dropping to any of a number of levels producing a number of different wavelengths (Fig. 7) (a four level laser) (Direct electron excitation of the neon is also possible and a neon laser without the helium has been produced.)

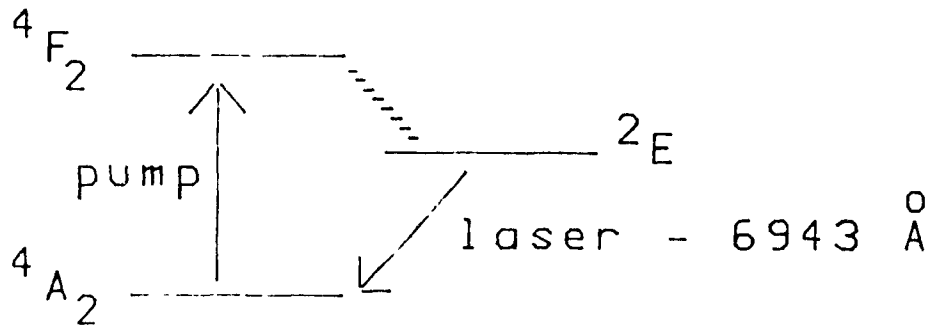
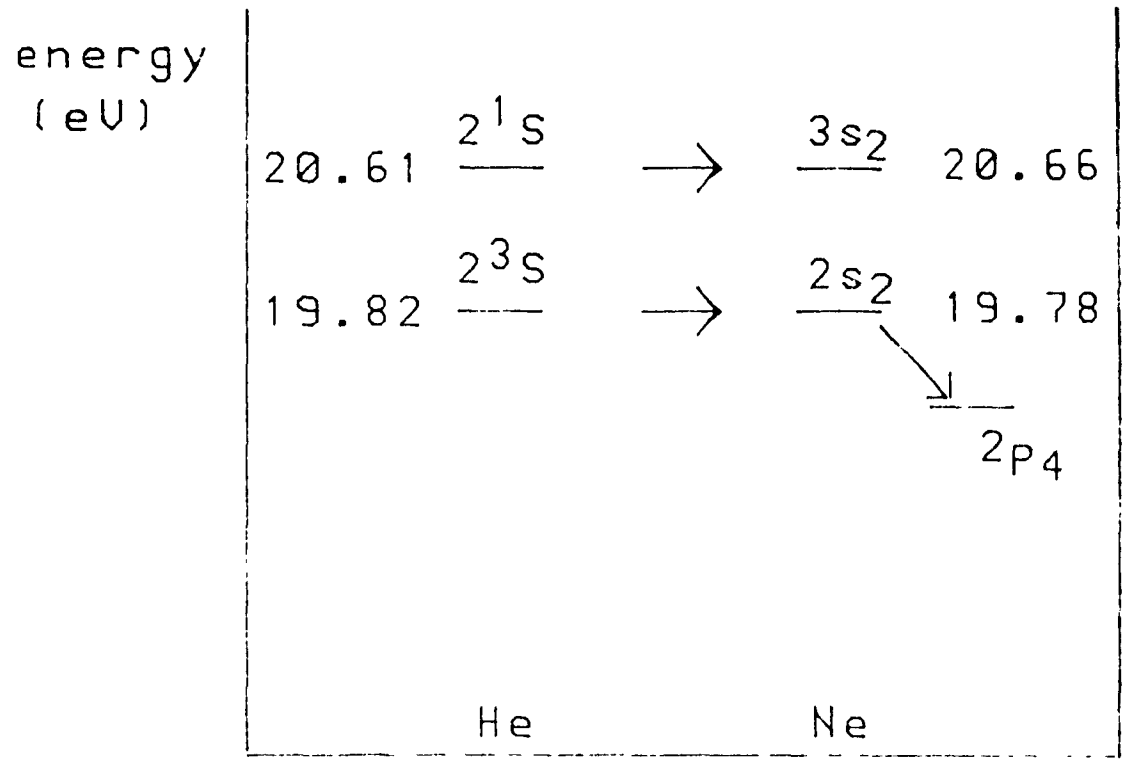


FIG 6 The ruby laser
A three level laser



$3s_2 \rightarrow 3p_4$ 3.39 microns

$3s_2 \rightarrow 2p_4$ 6328 angstroms

$2s_2 \rightarrow 2p_4$ 11523 angstroms

FIG 7 The helium-neon laser
A four level laser
Laser wavelengths in the helium-neon laser

3. Brewster angle (7)

Most lasers, in particular gas lasers, will have external mirrors, that is the mirrors are not attached directly to the laser tube, but are mounted separately on the optical bench. Hence windows are attached to the tube between the mirrors and the gaseous or lasing medium. These windows are generally angled at the Brewster angle. The Brewster angle is the angle of the window to the perpendicular of the laser light or laser tube itself (Fig. 8). The Brewster angle (θ) is calculated from equation 2.4 in with the tan of the

$$\tan \theta = n \qquad (2.4)$$

angle being equal to the refractive index (n) of the window material. The Brewster window polarizes the laser light and more important, the laser light is totally transmitted, hence the losses at the windows are less than the losses at the mirrors

4. Resonator Configuration (7)

A number of different types of mirrors and combinations of mirrors can be used to obtain lasing. The different mirrors and combinations are illustrated in Table 1 and produce various beam characteristics. The more commonly used ones are the hemispherical and long-radius configurations. Javan et al. (3) in the first gas laser used the plane parallel configuration which is also called a Fabry-Perot configuration. This resonator is not often used anymore since it is very sensitive to angular misadjustments and slight deviations from parallelism cause large losses In addition, the diffraction losses in the plane parallel resonator are larger than in any of the other resonators using spherical mirrors (7).

5. Losses

The losses which occur in a laser cavity are due to transmission losses since the mirrors have less than 100% reflectivity and since the mirrors have a finite area. There may be scattering losses due to imperfect Brewster windows The efficiency of operation of most lasers (calculated by using the input of

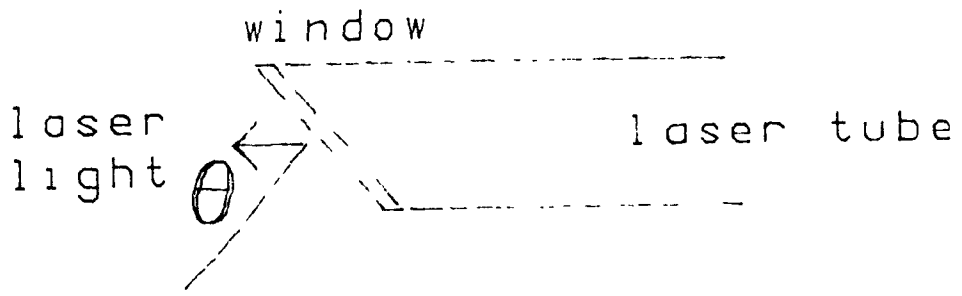


FIG 8 Brewster angle windows

TABLE 1 RESONATOR CONFIGURATIONS

mirror type	radius of curvatures
plane parallel	$b_1, b_2 = \infty$
large radius mirrors	$b_1 >> d$, $b_2 >> d$
confocal	$b_1 = b_2 = d$
spherical	$b_1 = b_2 = d/2$
concave-convex	$b_1 > d$, $b_2 = -(b_1 - d)$
hemispherical	$b_1 = d$, $b_2 = \infty$

b_1, b_2 = radii of curvature of the mirrors

d = distance between mirrors

electrical energy and output power) is on the order of 0.03% (7). The exception is the carbon dioxide laser with attainable efficiencies on the order of 20%.

II. CONSTRUCTION OF THE LASER

1. The Helium-Neon laser

The helium-neon laser used was built at the University of Toronto in the Laser Physics laboratory of Dr. A.D. May. Helium-neon lasers are regularly built there and all design considerations of the tube were decided upon by Dr. May That is, Dr. May was asked to construct a helium-neon laser upon being given the following information: the wavelength of emission (3.39 μ), the radius of curvature of the mirrors (since these had already been purchased) and the power which was thought to be required (1 mW).

The tube, made of medium wall pyrex, was approximately 68.6 cm long with an internal diameter (I.D.) of 0.6 cm. The actual discharge length was approximately 75 cm measuring from electrode to electrode (Fig. 9).

The 1st electrode is 0.0254 cm wall 2024 aluminum with a 5.1 cm outer diameter (O.D.) and 7.6 cm long (Fig 10). The electrode is actually a sheet of aluminum made into a cylinder of the above dimensions. A 0.0254 cm nickel strip connects the aluminum to a 0 32 cm nickel length which is then connected to the power supply. The entire electrode is enclosed in 0.6 cm I.D. pyrex of dimensions 15.5 cm long with a 1 cm O.D. tube connecting the electrode to the laser tube.

The nickel electrode is a 2.5 cm length of nickel tubing with a wall of 0.16 cm. It is connected to an 0.013 cm thick titanium wire which is wrapped on a 4 5 cm length of Chromel-A wire of 25 gauge with a resistance of 2.05 ohms/ft. (Fig. 11) The entire electrode is enclosed in 0 6 cm I.D. pyrex of dimensions 12 cm long and 5 cm in diameter with a 1 cm O.D. tube connection to the laser tube.

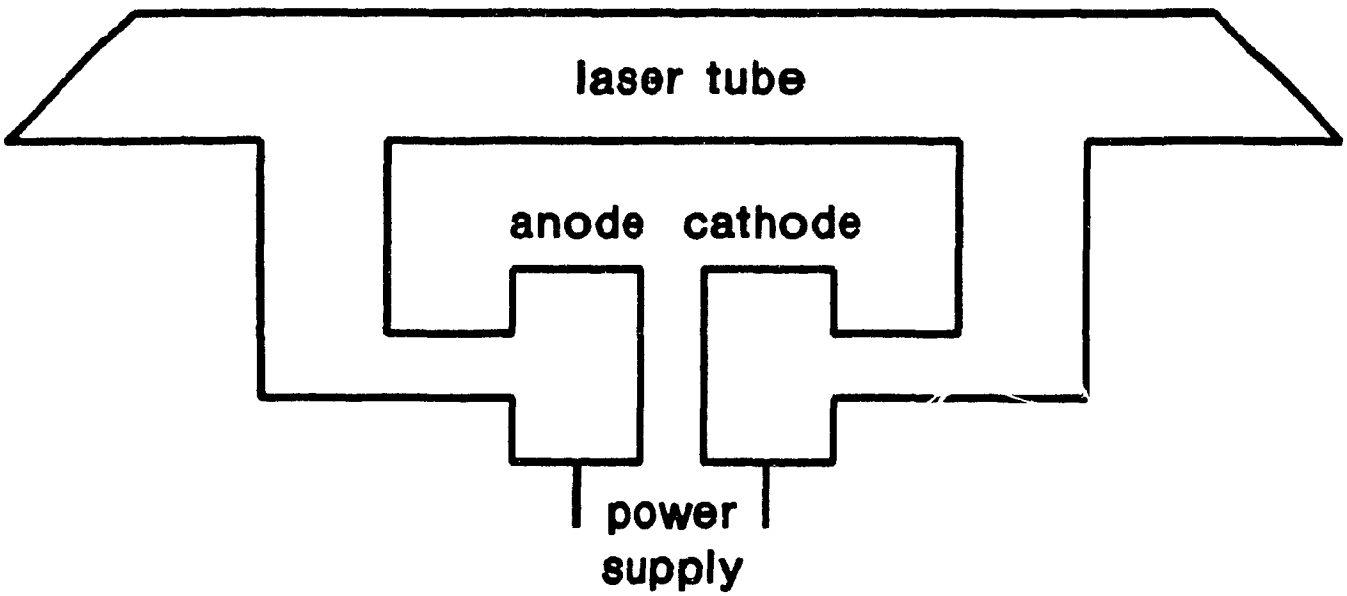


FIG 9 Schematic of the helium-neon laser tube

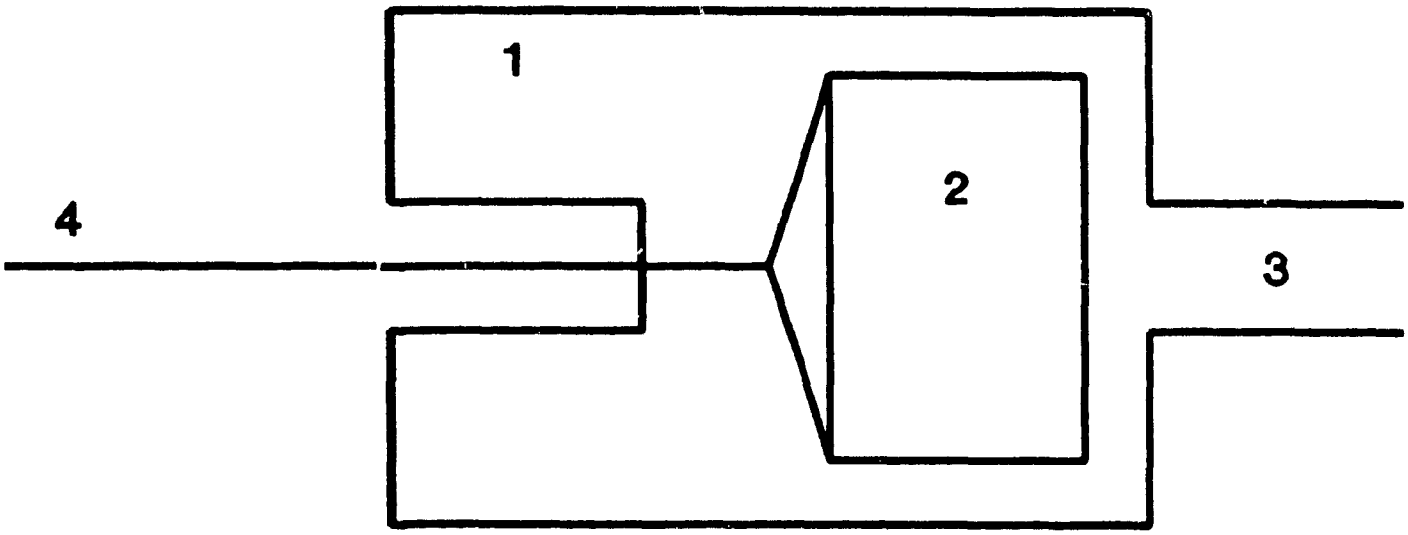


FIG 10 Schematic of aluminum electrode
1. Electrode compartment
2. Aluminum electrode
3. Laser tube connection
4. Connection to power supply

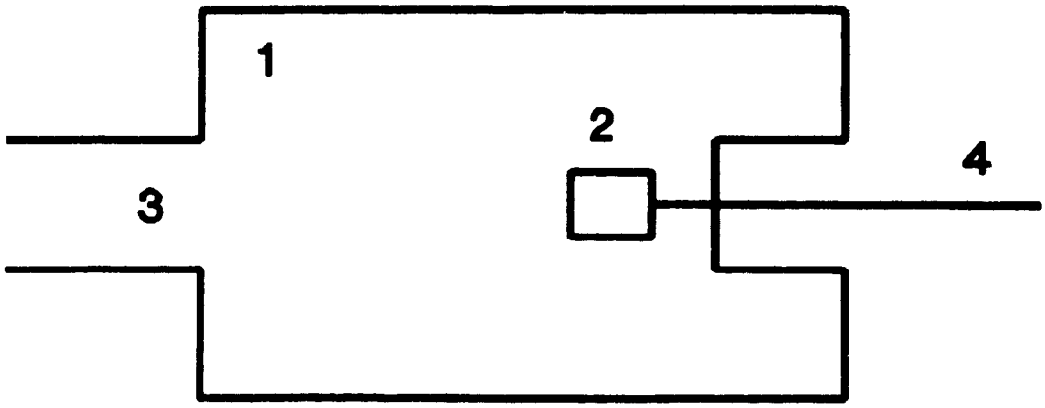


FIG 11 Schematic of nickel electrode
1. Electrode compartment
2. Nickel electrode
3. Laser tube connection
4. Connection to power supply

2. Cleaning the tube

All the glassware used in the laser (the laser tube and the glass electrode compartments) were very carefully cleansed. The glassware is washed with detergent and then rinsed for 5-10 minutes with warm running tap water. It is then soaked for 15-30 minutes in a boiling mixture of sulfuric acid and hydrogen peroxide and then rinsed well with distilled water. The glassware is then dried in the drying oven.

Before placing the electrodes into their respective compartments, they are cleaned separately. The aluminum electrode has been prepared by mechanically polishing it on a lathe and then degreasing it with acetone. The aluminum is etched with sodium hydroxide for approximately 1 minute and then soaked in solution (sulfuric acid:nitric acid:hydrochloric acid 1:1:1 (v/v/v) with a few drops of hydrogen fluoride) for a few minutes until the aluminum is bright. The aluminum cathode is then rinsed and inserted into the pyrex compartment The nickel is electropolished and inserted into the anode compartment. The electrode compartments are then attached by the glassblower to the laser tube.

The Brewster angle windows can then be attached once they have been cleaned. The windows are made of quartz and they are also cleansed in detergent and then rinsed well with distilled water. They are then cleansed in a degreasing bath The windows are hung on metal wires from a large (e.g. 1 litre) beaker into which is placed methanol. The methanol is gently boiled for 30- 60 minutes and the vapor hits the window and condenses. The windows are carefully attached to the laser tube in a clean air (dust-free) environment using an epoxy glue (e.g Torr seal). All of the above cleaning procedures are performed so as to minimize touching the glassware or windows and disposable gloves are used.

3. Filling the tube

The laser tube is then attached to an oil diffusion pump and pumped to 1 torr. A current of 50-100 milliamps (mA) is run through the tube for about 10 minutes to oxidize the aluminum cathode. The pump is then taken down to 10^{-6} torr. Heater tapes are wound around the laser tube, cathode and anode The

temperature is held at about 180°C. and the pressure held constant for at least 24 hours. This outgasses the glass removing any impurities. The tube is then filled with Helium at 0.85 torr and Neon at 0.15 torr. The laser tube can then be disconnected from the diffusion pump by the glassblower.

4. The mirrors

Concave mirrors were chosen because of the greater ease of alignment. The mirrors were 1 inch in diameter, made of the material BK7 with a radius of curvature of 1.47 m. (Perkin-Elmer, Electro-Optics Div., Danbury, CT.). The mirrors were dielectrically coated for operation at 3.39 μ. One mirror was coated for maximum reflection (99.5 ± 0.5%) with the back of the mirror anti-reflection coated. The other mirror is the partial transmitter with a 2% transmission (reflection of 98 ± 0.5%). The 1 inch diameter mirrors are screw mounted in mirror mounts to fit the 6 inch wide optical bench (Figs. 12A,B, and C) constructed in the machine shop at McGill University (the design for the mirror mount originated at the University of Toronto, Laser Physics group). The mirror mounts are useful when aligning the mirrors for lasing since they allow movement in 2 different axes. Movement in each axis is independent of the other. (Essentially one axis moves the mirror right and left and the other axis moves the mirror up and down.)

5. Aligning the laser

The laser tube is mounted on a 0.75 inch thick aluminum optical bench (constructed in the machine shop at McGill University). The mirror mounts are also placed on the optical bench and have been constructed to fit onto it.

The mirrors and laser tube are aligned using a visible helium-neon laser (Spectra-Physics Inc., Mountain View, CA.). Small cutout cardboard circles are placed in the laser mounts where the mirrors normally are placed. These cutouts have a pinhole through them and the red helium-neon laser is aligned so that the beam goes straight through the center of both of the pinholes. The mirror furthest from the laser is then put in the mount. The laser light is reflected back onto the cardboard pinhole and the mirror is adjusted

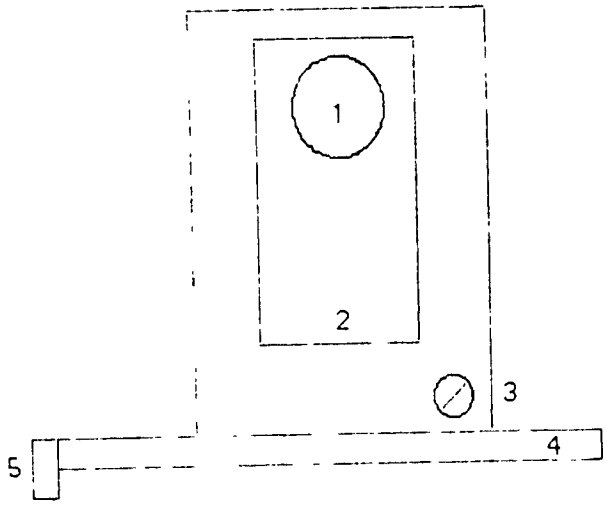


FIG 12A Front view of mirror mount
1. Where the mirror is mounted
2. First axis movement
3. Second axis movement
4. Platform to fit optical bench
5. Holds mount to optical bench

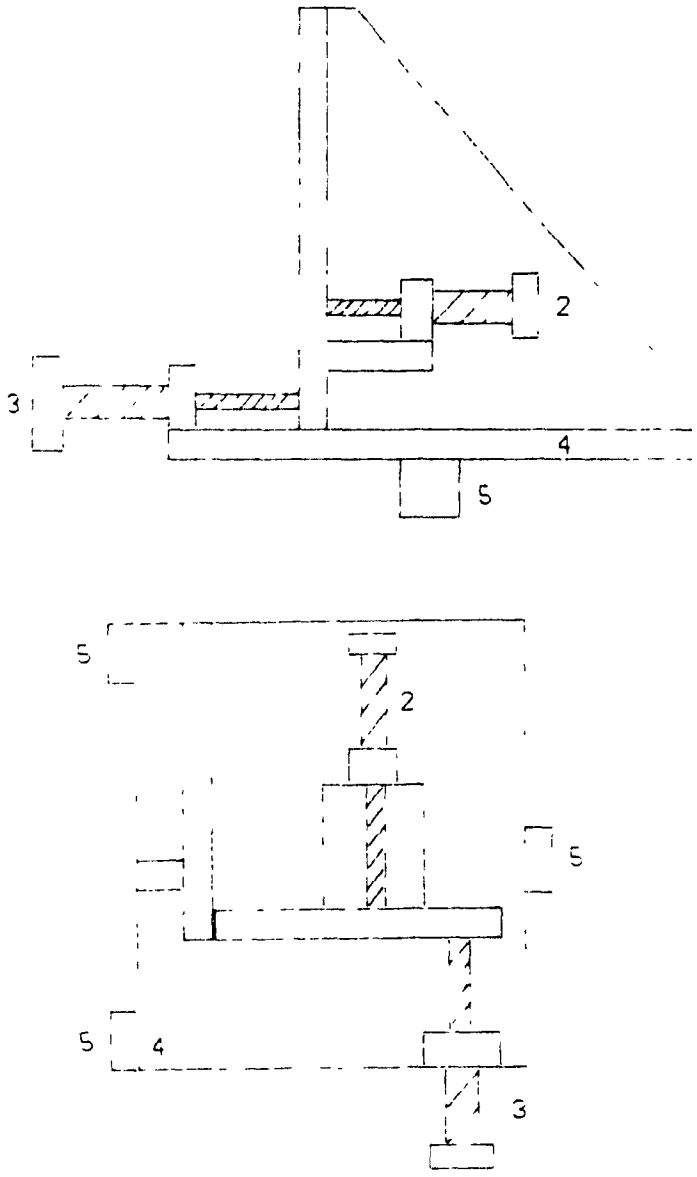


FIG 12B Side view of the mirror mount

FIG 12C Top view of the mirror mount

until the light is reflected back onto the center of the pinhole. The other mirror is then mounted and that mirror is adjusted until the reflected spot is exactly on the output of the laser. The laser tube is then mounted on the optical bench using 2 clamps and adjusted so that the red laser light goes straight through the tube with no reflections in the tube. This is the most difficult step because the laser tube is very narrow.

6. The power supply

The nickel electrode is then connected to a high voltage DC power supply (Beta Electric, New York, N.Y.) through a 50 kiloohm ballast resistor. The aluminum electrode is grounded to the optical bench and the ground of the power supply is also grounded to the optical bench. The power supply is turned on and the discharge started with a tesla coil (Electro-technic Products, Chicago, Ill.). The power supply is adjusted to provide 10-15 mA, with approximately 2500 volts DC. At this point with a properly aligned laser, the laser should be lasing.

III. THE INFRARED REGION OF THE SPECTRUM (10)

The infrared region of the spectrum is broadly divided into three areas: the near infrared from 14,300 to 5000 cm^{-1} (0.7 - 2.0 μ), the infrared from 5000 to 400 cm^{-1} (2.0 - 25 μ), and the far infrared from 400-20 cm^{-1} (25 - 500 μ).

The near infrared region contains the photographic region (14,300 - 7,700 cm^{-1} or 0.7 - 1.3 μ) and the overtone region (7,700 - 5000cm^{-1} or 1.3 - 2.0 μ). The infrared region is the fundamental vibration region and the far infrared is the rotation region.

IV. FLOW CELLS

There are numerous materials which can be used in the construction of infrared flow cells such as sodium chloride, potassium bromide, calcium fluoride, barium fluoride, cesium bromide, silver bromide, Irtan-2, polyethylene, KRS-5, silver chloride. All of these materials transmit light at 3.39 μ, however, some are inappropriate since they are very hygroscopic (sodium chloride and potassium bromide) and fog easily (cesium bromide). Others darken in ultraviolet light (silver chloride). Some are not useful for high pressure work (calcium fluoride is useful at high pressures).

In addition to selection of cell material, pathlength and cell volume should also be considered. The pathlength should be maximized and the cell volume minimized so that maximum peak height is attained while minimum band broadening of peaks is produced during chromatography.

The cell decided upon was one made of Suprasil Quartz (Hellma Cells, Toronto, Ontario). The quartz transmits (80%) at 3.39 μ and is not hygroscopic. The cell has a pathlength of 10 mm and a cell volume of 8 μl and is suitable for high pressure liquid chromatography. The flow cell has an aperture of 1 mm diameter. The flow cell of dimensions 1.27 cm x 1.27 cm x 3.50 cm (the 1 mm aperture is 1.27 cm from the bottom of the flow cell) was placed in a mount which was built in the machine shop at McGill University and fitted onto the optical bench (Figs. 13A, B, C.).

V. INFRARED DETECTORS

Infrared detectors can be broadly divided into two different classes. Imaging detectors are infrared film, the vidicon and the image convertor and as is derived from their name, they provide a total image of the signal. Elemental detectors are point detectors and respond to the average irradiance presented to them. The elemental detectors would have to be scanned sequentially to get the same image provided in the imaging detectors. Hence both types of detectors can ultimately give you the same information, the only

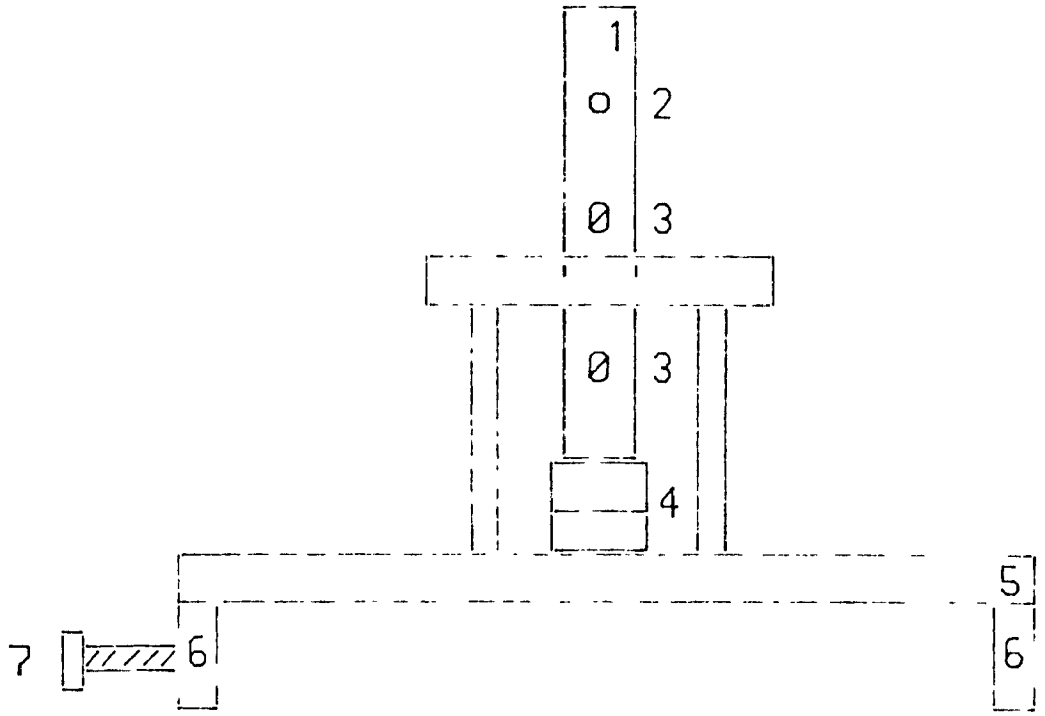


FIG 13A Front view of flow cell mount

1. Holder for flow cell
2. Aperture for laser light
3. Screw
4 Up, down adjustment
5 Platform to fit optical bench
6. Holds mount to optical bench
7. Screw to hold mount to optical bench

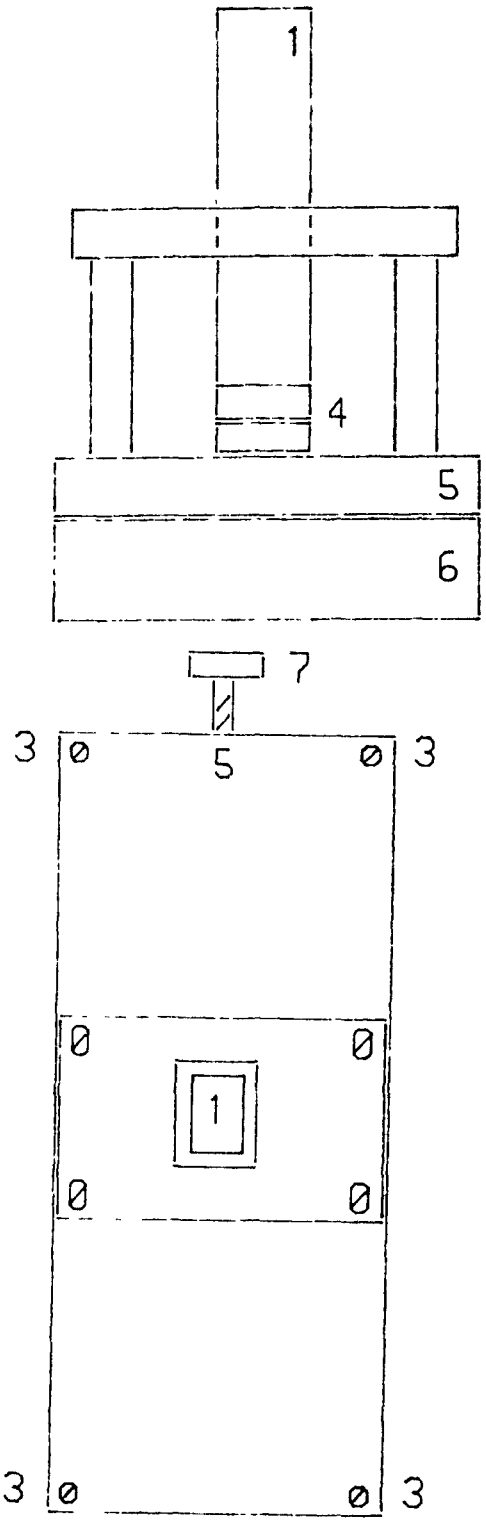


FIG 13B Side view of flow cell mount

FIG 13C Top view of flow cell mount

difference is that the imaging detector gives you the information immediately and the elemental detector must make a number of operations to supply the same data. Only the elemental detectors were considered as possible detectors for the infrared detector since imaging detectors are very expensive and not necessary in a liquid chromatographic detector.

The infrared detectors can be further broken down into 2 groups. thermal detectors (which respond to the heat of the incident radiation causing a change in some physical or electrical property of the detector), and photon or quantum detectors (which respond to the incident photon by an interaction between the photon itself and electron of the detector material). Hence the response of the thermal detector is due to the energy absorbed and the response of a photon detector is due to the number of photons absorbed.

1. Thermal Detectors

The thermal detectors include the thermocouple, thermopile, bolometer, pneumatic or golay detector and the calorimetric detector. These detectors were discovered before photon detectors and respond to all wavelengths. They have time constants on the order of milliseconds.

2. Photon or Quantum Detectors

Photon detectors have the advantage of being one to two orders of magnitude more sensitive than thermal detectors, however, many of them have the added disadvantage that they must be cooled to cryogenic temperatures Time constants are on the order of microseconds since the incident photon and the electrons of the detector interact directly.

If the electron acquires sufficient energy to escape the detector material entirely this is called the **photoelectric** or photoemissive effect If the photon causes an electron to move from a non-conducting state to a conducting state, the result is a **photoconductive** effect Photoconductive detectors are generally semiconductors. Photons can produce an electron-hole pair near a p-n junction and the electric field across

the junction separates the electron-hole pair into two carriers producing a photovoltage or **photovoltaic** effect. Electron-hole pairs produced at the surface of a semiconductor can diffuse deeper into the semiconductor to reestablish electrical neutrality. The electron-hole pair can be separated by a magnetic field to give a voltage called a **photoelectromagnetic** effect. (This technique is rarely used.)

VI. NOMENCLATURE FOR DETECTOR TERMINOLOGY (11)

The responsivity (R) is the detector output per unit input. Since most detectors use an ac or chopped signal, the value used to calculate the responsivity are rms values. Equation 2.5 is the equation used to calculate this value.

$$R = V_s/HA_d \qquad \text{(volt/Watt)} \qquad (2.5)$$

where V_s = rms of the signal voltage, volts.

A_d = area of the detector, cm^2.

H = rms of irradiance on detector, $watt/cm^2$

Responsivity is dependent on a number of conditions including the bias applied to the detector. The response time or time constant is the time it takes for the detector to reach 63% of its final value. Responsivity gives no idea of the minimum amount of radiation which the detector can sense.

The amount of noise that is present in the output of the detector is calculated in the term, Noise equivalent power, NEP, which is the amount of radiant flux necessary to produce a signal equal to the noise of the detector. Measurements are done at higher signal levels since it is difficult to measure a signal when the signal-to-noise ratio is equal to 1 The NEP is then calculated from Equation 2.6.

$$NEP = HA_d/(S/N) = HA_dN/S \qquad \text{(watts)} \qquad (2.6)$$

where N = rms noise voltage

S = rms signal voltage

This equation assumes that the relationship between the input and output is linear.

When comparing detectors, the one with the best responsivity is the one with the highest output for a given input. However, when comparing detection ability, i.e. in terms of minimum radiant flux that is detectable, the detector with the lowest NEP is the best. This has been incorporated into another term, the detectivity, in Equation 2.7.

$$D = 1/NEP \qquad (watts^{-1}) \qquad (2.7)$$

Detectivity is a function of wavelength, temperature, chopping frequency, bias applied, detector area, and bandwidth of the electrical circuit. There is no simple relationship between the detectivity and most of these variables except for the area and the electrical bandwidth. Hence they are simply reported with the proviso that the chopping frequency is selected to be low enough so that there is no limitation by the time constant of the detector and the applied bias is chosen to be the bias that maximizes the detectivity

There is a relationship between the detectivity and the detector area and the electrical bandwidth as shown in Equation 2.8.

$$D^* = D\ (A_d\ D\ f)^{1/2} \quad (cm\ hz^{1/2}/W) \qquad (2.8)$$

The "D-star" or D* term allows one to compare detectors since the area has been normalized to 1 cm^2 and the electrical bandwidth to 1 Hz.

The significance of D* is that it is the signal-to-noise ratio with 1 watt of incident radiation on the detector with a sensitive area of 1 cm^2 and the noise is measured with an electrical bandwidth of 1 Hz

D* is most often seen with two numbers in parentheses, the wavelength of incident light followed by the chopping frequency. It is understood that the electrical bandwidth is 1 Hz since this is present in the definition of D*.

VII. THE LEAD SELENIDE DETECTOR (12, 13)

The lead salt detectors (lead sulfide and lead selenide) are inexpensive to purchase, easy to construct and are versatile in use The detectors are chemically deposited films on an appropriate substrate. Photolithography defines the active area

The film is connected to gold electrodes which minimize the noise at the contacts between lead out gold wires and the film itself. By changing the degree of oxidation, temperature of deposition and the number of coatings in the manufacturing process, one can change the resistance, sensitivity (responsivity), time constant, spectral distribution of response, noise, and signal-to-noise ratio of the detector.

The lead selenide detector can be considered to be a light sensitive variable resistor when connected to a bias power supply The photons induce a change in conductivity and this changes the current through the detector (as described in the section on photoconductive detectors) which can then be amplified and used in the analysis

The lead selenide film is sealed between a coverplate and the substrate by an epoxy cement The coverplate material is often quartz or sapphire depending on the wavelengths to be analyzed.

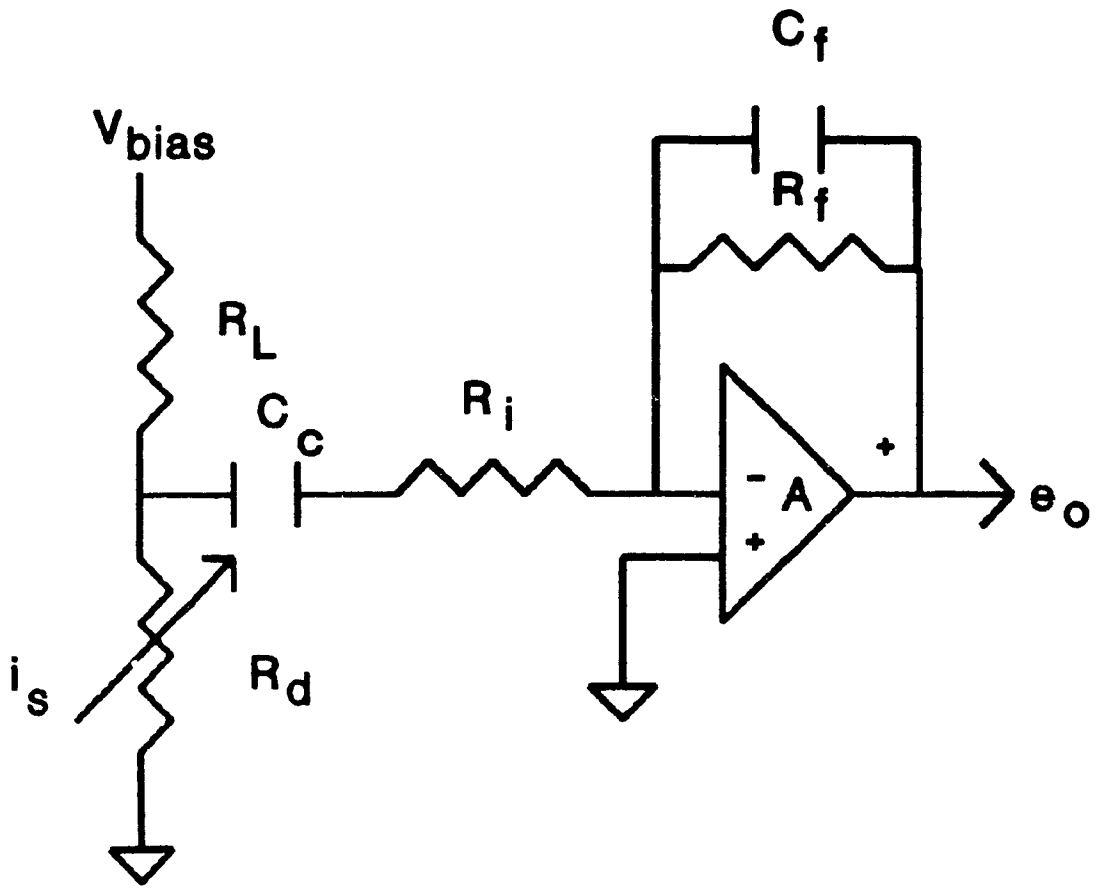
The detector can be cooled, by any of a number of means, since the performance has a negative temperature coefficient. That is, the performance decreases as the temperature increases. Hence this detector is more sensitive at dry ice and liquid nitrogen temperatures.

The lead selenide detector which was purchased was the model 5055 PbSe detector (Infrared Industries, Inc., Orlando, Florida). The detector is inexpensive. The specfications are listed in Table 2. The detector requires a bias voltage or current The simplest case is to match a load resistor to the dark resistance of the detector (Fig 14).

TABLE 2 SPECIFICATIONS OF THE LEAD SELENIDE DETECTOR

D* (500°K, 750 Hz, 1Hz)	2.8×10^8
D* (λ_{pk},750 Hz, 1 Hz)	2.5×10^9
wavelength of maximum response	3.8-4.0 μ
dark resistance	0.5 M ohms
Responsivity (λ_{pk}, 750 Hz)	3000
optimum detector bias	100 V dc

Note: All specifications quoted are from the Infrared Industries catalog and are typical performance characteristics of a 2 X 2 mm PbSe detector.



$$e_o = (i_s R_L \times R_f / R_i)$$

FIG 14 Detector with matched load resistor
R_L - load resistor
R_d - detector
A - amplifier

The detector mount (to fit the 0.375 in diameter detector) (Figs. 15A, B, C) was also built in the machine shop in McGill University to fit the optical bench.

VIII. LOW LIGHT LEVEL MEASUREMENT (14)

Numerous different methods enable one to perform low level light measurements. Some of these techniques are useful for dc signals and some for ac signals. The techniques which will be discussed are photon counting, the electrometer, the lock-in amplifier, and the waveform averager.

1. Photon counting

The photon counting method consists of the photon striking a detector usually a photomultiplier tube, though other detectors could be used. The pulse is amplified and sent to a discriminator, which is set so that it ignores all signals below a certain pre-set limit. In this way, noise is disregarded. The discriminator output is a pulse of a certain amplitude and duration. This output can be fed into a counter and the output can be displayed on a digital meter or converted to an analog signal by a digital-to-analog (D/A) convertor and displayed on a strip-chart recorder. The entire system must be well shielded since photon counting is very sensitive to interference. It is the most sensitive detection system, since it can, in effect, detect one photon.

2. Electrometers

Use of an electrometer or digital voltmeter (DVM) is a very simple means of signal measurement. The output of the detector is connected to the DVM, usually through some sort of current-to-voltage convertor since the output of the detector is current The DVM is a zero frequency or dc measurement technique. Hence it is very susceptible to many different sources of noise. 1/f noise, stray light, etc. The DVM has the advantage of being inexpensive and simple to use.

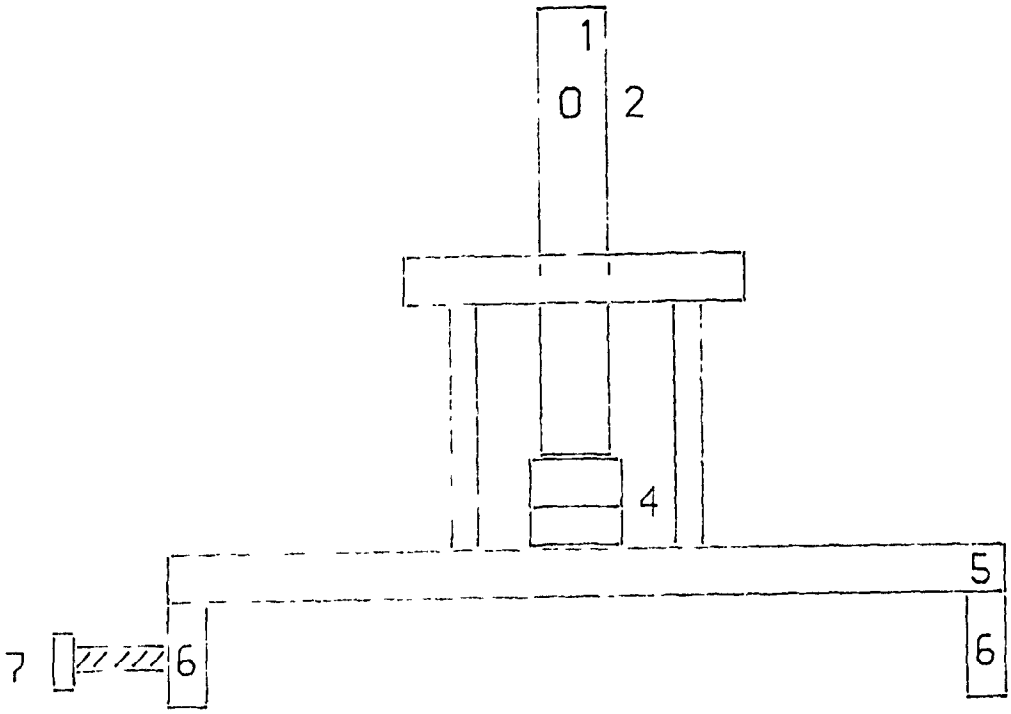


FIG 15A Front view of detector mount
1. Mount for PbSe detector
2. Aperture for laser light
3. Screw
4. Up, down adjustment
5. Platform to fit optical bench
6. Holds mount to optical bench
7. Screw to hold mount to optical bench

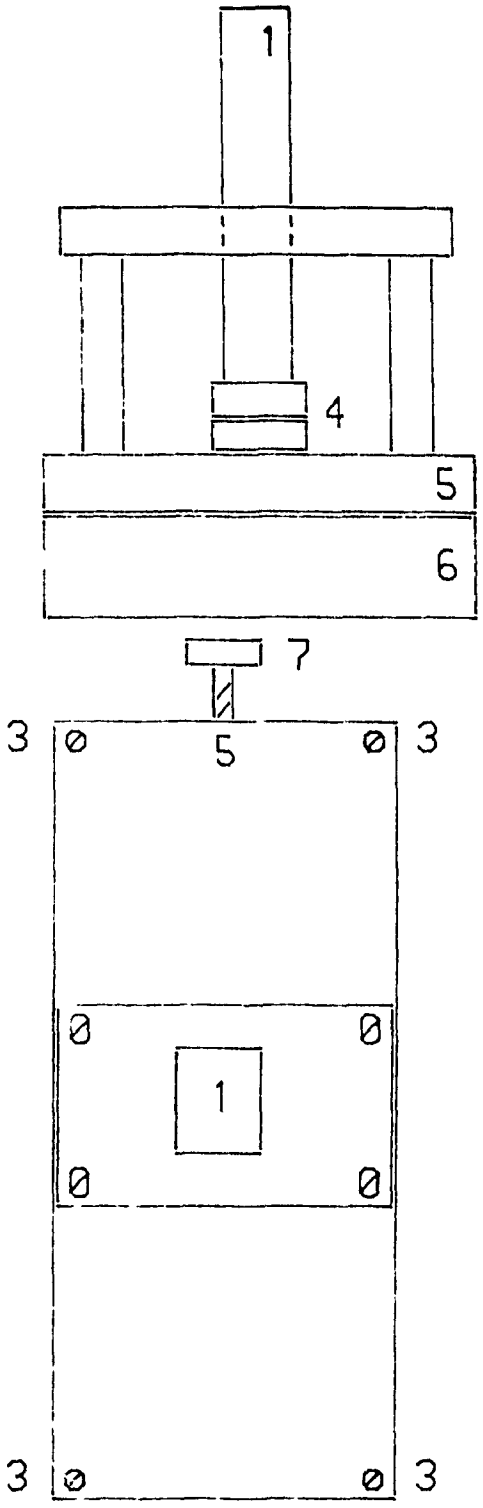


FIG 15B Side view of detector mount

FIG 15C Top view of detector mount

When examining a noise power scan as a function of frequency the noise levels are highest at low frequencies. This is the reason that the DVM, which is a zero frequency system is very susceptible to noise. The problem can be solved, in the case of the DVM, by use of a low pass filter which would filter out some of the low frequency noise. Another alternative is to simply move the signal to another frequency where the noise is reduced and use the next low light level measurement technique, the lock-in amplifier.

3. The lock-in amplifier

The lock-in amplifier is used to detect low level modulated signals. The signal is modulated by a chopping mechanism and hence is moved from a relatively noisy frequency at zero Hz or a dc signal to a less noisy frequency, f_m. The lock-in amplifier or phase sensitive detector requires a reference signal which is the same frequency as the signal frequency and both the reference and sample signal should be in phase. The sample signal is sent in parallel to an inverting and a non-inverting unity-gain amplifier. The inverting and non-inverting outputs are switch-selectable and determined by the polarity of the reference signal. When the reference and sample signal are in phase, the amplifier output will be a maximum. An RC filter smooths the signal and a dc voltage is outputted.

Since the signal and reference may be offset, the output voltage is actually equal to the input voltage multiplied by the phase shift between the reference and sample signal. Harmonic frequencies are eliminated by using tuned amplifiers or heterodyning systems.

The lock-in amplifier needs a chopper or some means of modulating the signal and since the signal is turned on and off some of it is lost. This is more than compensated for by the ability of the lock-in amplifier to recover the signal even when buried in noise.

4. The waveform averager

Waveform averagers are most often used for pulsed signals where the information that is needed is in the shape or length of the light signal or the amplitude of the pulse with a poor duty factor. The

waveform averager has been used for fluorescent decay measurement, optical time domain reflectometry and time resolved spectroscopy.

The input signal is amplified and sent to an RC exponential averager through a switch. An exciting source is usually the trigger and this determines when the switch is closed. Internal delay and gate closing durations are selected so that the amount of time that the switch is closed is short with respect to the time constant of the low pass RC filter. As the switch is opened and closed with each succeeding input, the stored capacitor signal moves closer and closer to the average value of the input signal. The noise is attenuated by the low pass filter. (The duty factor must be taken into consideration when calculating the actual time constant.) A single channel averager has been described but multiple channels are also available.

For all the detection systems mentioned, the electrometer is usually used for continuous light sources, the lock-in amplifier for chopped light sources and the boxcar or waveform averager for pulsed light sources. Photon counting can be used for all of the above-mentioned light sources.

IX. THE CHOPPER

Since a bias voltage is applied to the lead selenide detector, a means of removing this bias voltage must be found. Any of a number of means may be used. It was convenient to use a lock-in amplifier since it was available. The lock-in amplifier requires an ac signal, hence a chopper was constructed to modulate the output of the infrared helium-neon laser. The chopper was built in the machine shop at McGill University and constructed to fit onto the optical bench. The chopper uses a number of inexpensive electrical components including an LED (the source) and a phototransistor (the detector) and the accompanying electronics to power the components and provides a reference wave of the characteristics required for the reference input of the PAR Model 5101 Lock-In Amplifier (Princeton Applied Research, Princeton, New Jersey).

The lock-in amplifier requires a noise-free reference wave against which it compares the input signal (in this case the laser signal). In addition, the reference signal must be coherent with the input laser signal, that is be the same frequency and must have a stable phase relationship with the input laser signal (15). Therefore, a wave generator is not an adequate reference signal since it will never be coherent with the signal wave.

The chopper is run off a variable power transformer (Variac). The chopper includes a mount for a red LED and a phototransistor. The chopper blade simultaneously chops the light from the LED and the laser producing square waves which are coherent. Choppers can be purchased which will accomplish all that this one does, but at a considerably higher cost.

The chopper system is shown in Figs. 16A and B. A breadboarded circuit which was constructed (Fig. 17) with the help of the Electronics Workshop, was powered by a low noise (± 1 mV ripple) ±15 V power supply (Hammond HPCD 015 001). The electronics power the LED and the phototransistor and consist of a current-to-voltage convertor (since the phototransistor produces a current) followed by an operational amplifier configured as a divider to produce a reference wave of the characteristics suitable to the specifications of the lock-in amplifier. The reference wave must be of particular characteristics depending on the model of lock-in amplifier used. In the case of the PAR model 5101, for the best phase accuracy, a 1 volt sine wave is recommended. However, square waves and triangle waves are all acceptable, the minimum requirement being that the reference wave swing 100 mV with respect to its mean. The square wave is the easiest to produce with a chopper system of this sort. The Zener diode limits the voltage output of the electronics to 1.8 volts.

The mount and motor of the chopper are from an old cooling fan, but the fan blades have been replaced by the chopper blade.

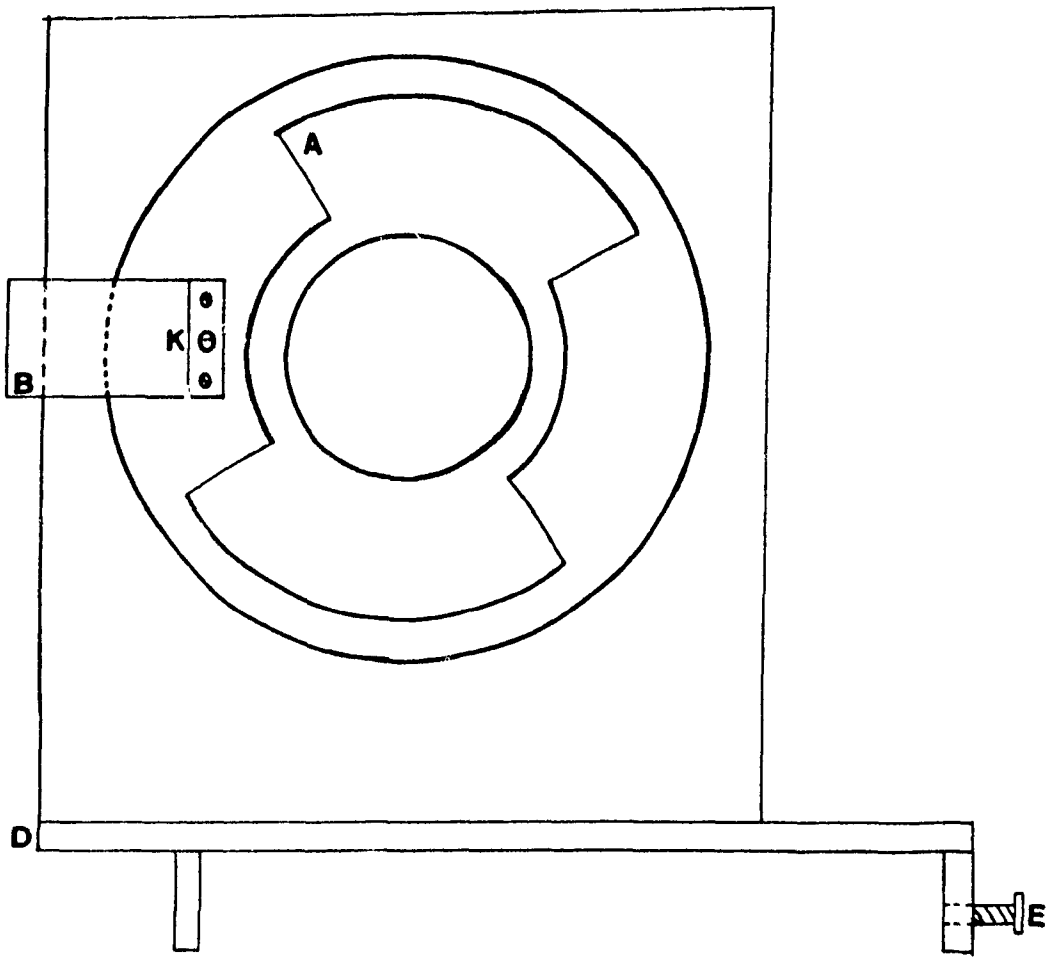


FIG 16A Top view of chopper
A - chopper blade
B - bracket
C - motor and housing
D - base
E - screw
F - rod
G - teflon spacer
H - teflon screws
J - light emitting diode
K - phototransistor

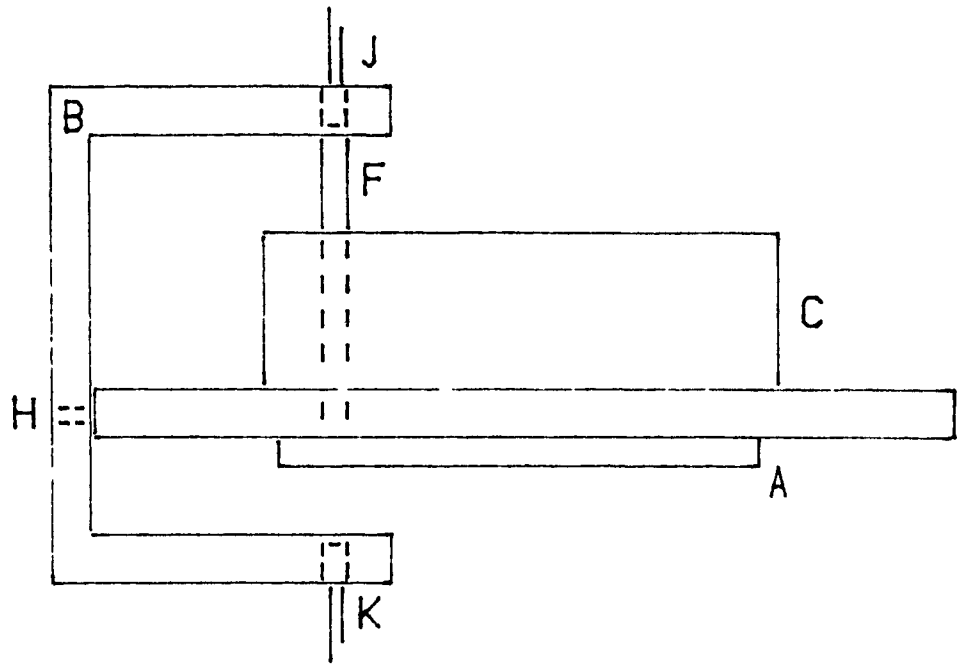


FIG 16B Front view of chopper

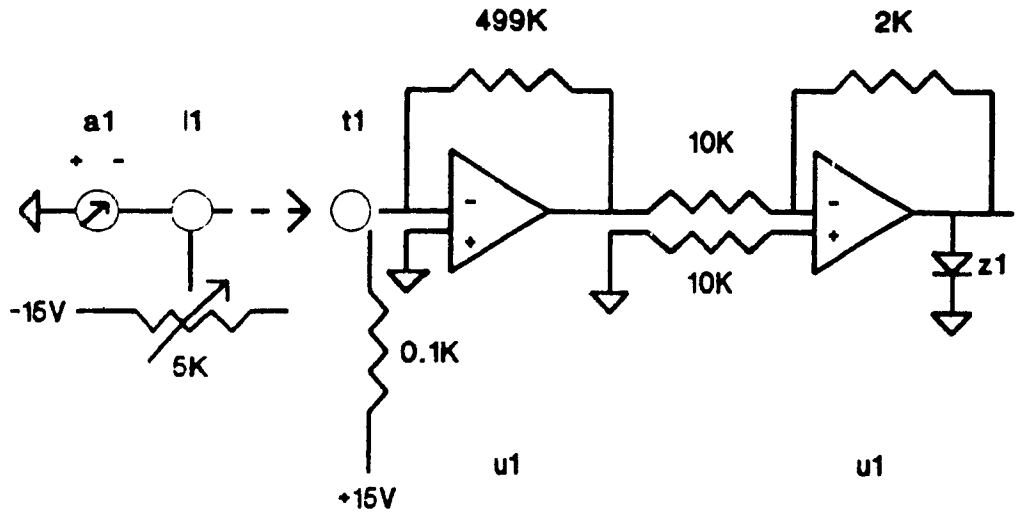


FIG 17 Schematic of electronics to produce reference wave
a1 - ammeter
l1 - light emitting diode (mv 5020)
t1 - phototransistor (til 81)
u1 - i/v convertor (lm 741 cn)
z1 - zener diode (mz2361)

1. Description of Chopper (16)

Figures 16A and B show a schematic drawing of the front and top view, respectively, of the chopper. (A) is the 4.25 in chopper blade itself which is attached to a motor housing (C). There is a bracket (B) which holds the LED (J) and the phototransistor (K). The phototransistor has a rod (F) attached to minimize its detection of exterior light, minimizing the noise. The bracket is attached to the frame of the chopper with Teflon screws (H) and a Teflon spacer separates the bracket from the frame. The Teflon isolates the aluminum chopper from the grounded aluminum optical bench thereby ridding the system of "ground-loops". The base (D) is made of dimensions compatible to the optical bench and the screw (E) merely secures the chopper to the optical bench. The whole chopper is spray painted flat black to minimize the hazardous reflections from the laser.

X. OTHER CHROMATOGRAPHIC INFRARED DETECTORS

The construction of the infrared detector discussed here is similar to that discussed in Freeman et al. (17). An infrared laser of the same wavelength is used and the dimensions (volume and pathlength) and material of the cell are the same. Since the cell was purchased in both cases and there are not many flow cell manufacturers, it is no surprise that the dimensions and material are the same in both cases. Freeman et al. use polytetrafluoroethylene windows to condense the light beam which are not used in the detector described here. The lead selenide detector, chosen for simplicity, ease of use and cost is the same in both detectors. The choice is limited and the only detector with a better D* is indium antimonide (11) with a much higher price and the added complication of cooling. Freeman et al. use glass plates as attenuators in front of the PbSe detector. Attenuators are not necessary here. A Thermocouple amplifier with pre-amplifier is used by Freeman et al. with a homemade chopper (not described) instead of the chopper and lock-in amplifier system used here.

Both tripalmitin and cholestryl palmitate are detected by the Freeman detector, however a calibration curve is only shown for tripalmitin. The only information given about the "separation" is that trichloroethylene is the solvent and that a pellicular silica column is used for cholestryl palmitate. The chromatography of these two compounds has not been studied by Freeman et al.and the chromatography will be studied with the infrared detector described here. A single solvent mobile phase such as trichloroethylene will likely not separate tripalmitin and cholestryl palmitate and they were probably analyzed in a flow-injection mode.

The DuPont infrared detector for liquid chromatography used by Parris (18) and Payne-Wahl et al. (19) for the determination of lipids was produced by Foxboro-Wilks (South Norwalk, CT) for DuPont. DuPont no longer markets it, however Foxboro still does. The infrared detector is a MIRAN 1A-CVF detector with a price of $5900 U.S. The source is a Nichrome wire with a pyroelectric detector which can be scanned from 2.5 to 14.5 μ. It is a single beam instrument which can be used in the absorbance or transmittance mode. It has a response time of 1, 4, 10 or 40 second with a wide variety of cells available depending on the application.

The advantage of the infrared detector described here is cost since constructing it from available parts and the purchase of some new parts were considerably less than $5900 U.S. An additional advantage is in the helium-neon laser since a nichrome wire has an output on the order of μW and the helium-neon laser has an output on the order of mW, an increase of 3 orders of magnitude. In addition this output wavelength of the helium-neon laser is in one narrow band since this is a monochromatic laser. The MIRAN detector can scan wavelengths, however this is not necessary here since we are only interested in one wavelength and one application. The response time of the PbSe detector is faster (microseconds) and this will be useful for very fast peaks. The DuPont detector used by Parris (18) and Payne-Wahl et al. (19) was used at 5 72 μ which is the region of the carbonyl stretch. Therefore, there are different constraints for solvents in both cases. At 5.72 μ, solvents with carbonyl groups would not be useful (large background absorbance) whereas at 3.39 μ solvents with methylene groups (CH_2) are not very useful. It is easier to find

organic solvents (needed to dissolve lipid) with no carbonyl group than to find solvents with no methylene group (see Chapter 4 for discussion of solvent considerations). One would expect the detection at 3.39 μ to be more sensitive since the lipids have many more CH_2 groups than carbonyl groups. However this cannot be proven unless the absorptivity of each group could be determined. These data have not been found in the literature. However, intuitively it seems to be a good assumption since there are many more CH_2 groups than carbonyl groups.

The MIRAN detector has been proven useful for lipids (18,19); the next step is to prove the usefulness of the detector built in this study. In addition, chromatography should be performed since chromatography was not described in the experiment by Freeman et al.

REFERENCES

1. Schawlow, A.L., and Townes, C.H. Phys. Rev. (1958) *112*, 1940.

2. Maiman, T.H. Nature (1960) *187*, 493.

3. Javan, A., Bennett , W.R., Jr., Herriot, D.R., Phys. Rev. Lett.(1961) *6*, 106.

4. White, A.D., and Rigden, J.D., Proc. Inst. Radio Engrs., (1962) *50*, 1697.

5. Skoog, D A., and West, D.M , "Principles of Instrumental Analysis", 2nd edition, Saunders College, Philadelphia, 1980, p. 121.

6. Allen, L ,"Essentials of Lasers", 1st edition, Pergammon Press, Oxford, 1969, p.29.

7. Bloom, A L.,"Gas Lasers", 1st edition, John Wiley & Sons, Inc., New York, 1968, p. 2.

8 Allen, L., and Jones, D G C , "Principles of Gas Lasers", 1st edition, Butterworths & Co., 1967, p. 59-72.

9 Lytle, F E , "Laser Fundamentals", in "Lasers in Chemical Analysis", Hieftje, G.M., Travis, J.C., Lytle, F.E., ed., 1st edition, The Humana Press, Clifton, N.J., 1981, p 21.

10. Meloan, C E , "Elementary Infrared Spectroscopy", 1st edition, The Macmillan Company, New York, 1963, p. 4.

11 Hudson, R D , "Infrared System Engineering", 1st edition, Wiley Interscience, New York, 1969, p. 264

12. Harris, R E , Laser Focus/Electro-Optics, 1983 *Dec* ,87.

13 Harris, R.E , Electro-Optical Systems Design 1977 *March*, 42-44

14. Doherty, H , Lasers & Applications, 1983 *July*, 41.

15. Skoog, D A and West, D M , "Principles of Instrumental Analysis", 2nd edition, Saunders College/Holt, Rinehart and Winston, 1980, p 75

16. Personal communication with Dr D Simkin, McGill University, Montreal, Canada.

17. Freeman, N.K , Upham, F T , and Windsor, A.A , Anal. Lett. (1973) *6*, 943

18 Parris, N A , J. Chrom. Sci (1979) *17*, 541

19 Payne-Wahl, K , Spencer, G F., Plattner, R.D , and Butterfield, R.O., J. Chrom (1981) *61*, 1020

CHAPTER 3

CHARACTERIZATION OF THE INFRARED DETECTOR

I. EMISSION SPECTRUM OF THE HELIUM-NEON LASER

It is useful to be able to record an emission spectrum of the helium-neon laser being used in order to see if more than one laser line is present and to determine the shape of the actual laser line.

To do this a Pye Unicam SP1000 infrared spectrophotometer (Pye Unicam Ltd., Cambridge, England) was used. The infrared source of this spectrophotometer is a nichrome wire. The radiation from the nichrome wire is reflected by two mirrors through a reference and a sample path on its way into the pneumatic detector of the spectrophotometer. The mirror which sends the radiation through the sample path is removed (Fig. 1). This mirror is removed, mirror mount and all, since this will make reassembling the spectrophotometer much simpler since no alignment of the mirror will need to be done.

The laser and the spectrophotometer must then be aligned so that the laser radiation is sent directly into the spectrophotometer. This is not trivial since the laser radiation is not visible. The procedure used was simplified with the help of a second person.

The detector normally used to detect the helium-neon laser, the lead selenide (PbSe) detector, was partially dismantled from its mount (described in Chapter 2) and held in front of the entrance to the spectrophotometer. The laser was then moved around until the laser light was detected on the PbSe detector. The PbSe detector is then removed and an emission spectrum could then be taken of the laser using the infrared spectrophotometer A blank spectrum was done with the laser blocked to ensure that the peaks seen are actually due to the laser (Fig. 2).

As can be seen in Fig. 2, the emission spectrum shows only one emission line at 2950 cm^{-1}(3.39 μ) which is the line expected. As predicted, there are no other peaks present because the laser mirrors were dielectrically coated to produce reflection only at 3.39 μ. (The peak in the vicinity of 1000 cm^{-1} is due to a slit change in the

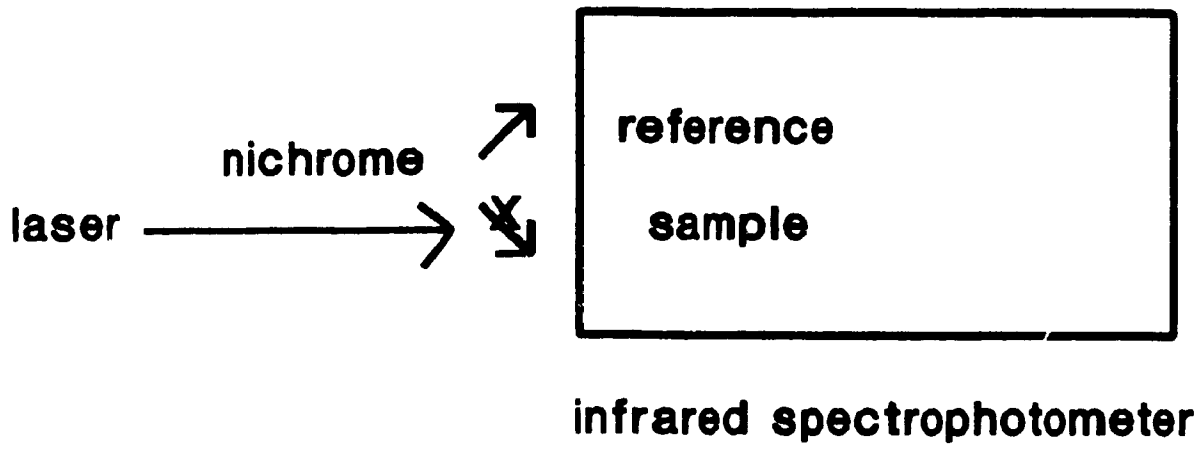


FIG 1 Schematic of laser with infrared spectrophotometer

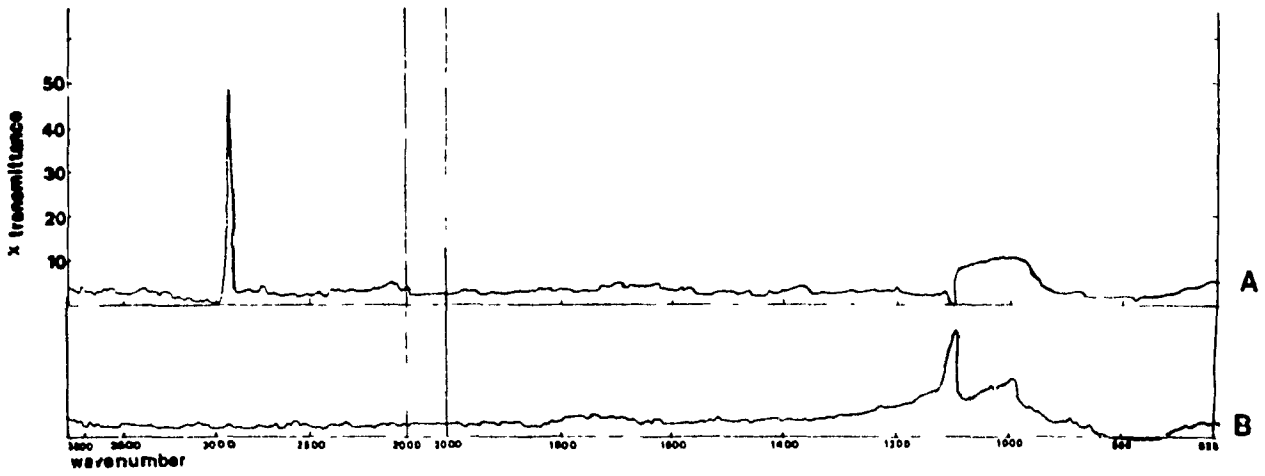


FIG 2 Emission spectra taken with infrared spectrophotometer
A - Laser emission spectrum
B - Blank emission spectrum

spectrophotometer and is present in both the blank and laser spectrum.) The laser line is approximately 20 cm^{-1} wide.

The system, as described, was scanned as a double beam system. That is, the laser line was compared to the reference (nichrome wire) radiation. This causes no problem since all that interests one is which lines were present. The system could probably be altered to run single-beam if the instrument being used were able to perform single beam scans.

II. LASER BEAM DIAMETER

The laser beam diameter can very crudely be determined by taking a piece of cardboard and drilling holes of varying diameter in 1 mm. increments. The hole is placed in front of the detector increasing the hole size until there is no drop in laser power to the detector. Using this method, the diameter of the laser beam was approximately 4 mm.

A more precise method was used with the help of a computer program available from Apollo Lasers, Inc. (1) entitled Laser Beam Characteristics. The following parameters were entered in response to the prompts in the program: radius of curvature of mirrors (R_1,R_2), cavity length L (895 mm), and wavelength of the laser λ (.00339 mm) and distance from the beam waist at which the beam radius is to be calculated Z (842 mm). The beam waist is also calculated by the same program and is midway between the 2 mirrors for a confocal resonator, that is 447 mm and the beam radius at the waist is W_0.

$$W_z^2 = W_0^2 [\ 1 + (\lambda) z / \pi W_0^2]^2 \qquad (3.1)$$

The calculated beam radius, W_Z, is 1.36 mm or a diameter of 2.72 mm not far off the crude manipulation carried out above.

III. DETERMINATION OF OPTIMUM OPERATING POINT OF THE DETECTOR

Since the lead selenide detector requires an external bias to be applied to it (see Chapter 2), it would be advantageous to determine what the optimum bias would be. In Fig. 3, the circuit in which the lead selenide detector is located is shown. The detector is in series with a load resistor. The dc voltage across the load resistor is calculated by the following equation where V_L is the voltage across R_L, the load resistor, V_b is the bias voltage, and R_d is the dark resistance of the detector.

$$V_L = V_b (R_L/R_d + R_L) \qquad (3.2)$$

When light is incident on the detector, a change in the detector conductivity occurs with a change in voltage across the load resistor, that is the signal voltage. Hence a high bias voltage implies a high signal voltage. However, one cannot increase the bias voltage to infinity, since there is only a maximum amount of power which the detector can dissipate. The maximum bias voltage, $V_{b\,max}$ can be calculated by the following equation

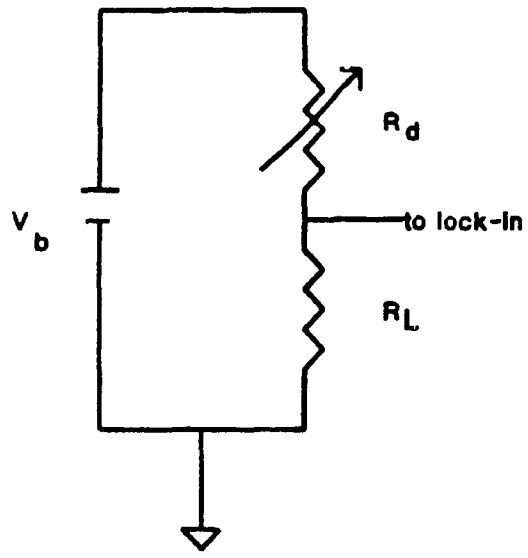
$$V_{b\ max} = [0.1A_d\ (R_d + R_L)^2/R_d]^{1/2} \qquad (3.3)$$

where A_d is the detector area, R_L and R_d as mentionned above.

Hence in the case of the lead selenide detector being used, the maximum bias voltage calculated is 56 volts (or rounded to 60 volts) using an area of 0.04 cm^2 with a dark resistance of 220 k ohms and a load resistor of 200 k ohms.

Since the maximum signal voltage occurs when the load resistor and detector are matched, this condition was chosen. In order to determine the optimum bias voltage, the signal and noise are measured as a function of applied bias. Hudson (2) defines optimum bias as the bias voltage that has the maximum signal-to-noise (S/N) ratio and hence detectivity. The resulting plot is a curve that increases to a maximum and then drops off again, since signal voltage increases linearly; however the noise voltage increases less rapidly except at high bias voltages where it increases more rapidly than the signal.

An experiment was performed to determine the optimum bias. The signal is determined with a digital voltmeter, taking readings at 10 sec. intervals. The noise is determined with the laser light blocked and taking



R_d - detector

R_L - load resistor

V_b - bias voltage

FIG 3 PbSe detector circuit

readings off the DVM at 10 sec. intervals. All other S/N calculations are performed in this manner throughout the chapter. The data are listed in Table 1 and the curve obtained is plotted in Fig. 4.

The optimum voltage under the conditions used is 40 volts. Since the PbSe detector will have this bias voltage applied to it, any changes in light detected will result in a current or voltage (as is the case here, since a resistor has been placed in series). If the bias voltage is 40 volts, it is no simple task to observe changes in light level and therefore changes in voltage, since one would generally use a recorder and most recorders have at best a 10 volt full scale span. Modulating and later demodulating the signal in the end still produces a dc signal but without the very large bias voltage.

IV. REFERENCE WAVE OPTIMIZATION

As previously mentioned in Chapter 2, the lock-in amplifier requires a reference signal to which it compares the input signal and then produces a dc signal which can be read off the panel meter or sent to a recorder. The reference signal must be of particular characteristics depending on the model of lock-in amplifier used. In the case of the Princeton Applied Research (PAR) model 5101, for the best phase accuracy, a 1-volt sine wave is recommended. However, square waves and triangle waves are all acceptable, the minimum requirement being that the reference wave swing 100 mV with respect to its mean (see Chapter 2).

By adjusting the 5K-ohm potentionmeter which powers the LED, one can vary the intensity of the LED and hence the intensity of the reference wave. In Table 2, the 5K-ohm potentionmeter is varied to produce reference waves of 200 mV, 300 mV and 400 mV, peak-to-peak. As can be seen from the data, the 400 mV reference signal would be preferred because of the increased signal-to-noise ratio. The intensity of the LED was not raised higher than this for fear of breakdown of the LED.

TABLE 1 DATA USED IN DETERMINATION OF OPTIMUM BIAS

bias applied (volts)	signal voltage (volts)	noise (mV)	S/N
20	.282	1.60	176
30	.339	1.29	263
40	.408	1.00	408
50	.482	2.03	237
60	.591	2.93	202

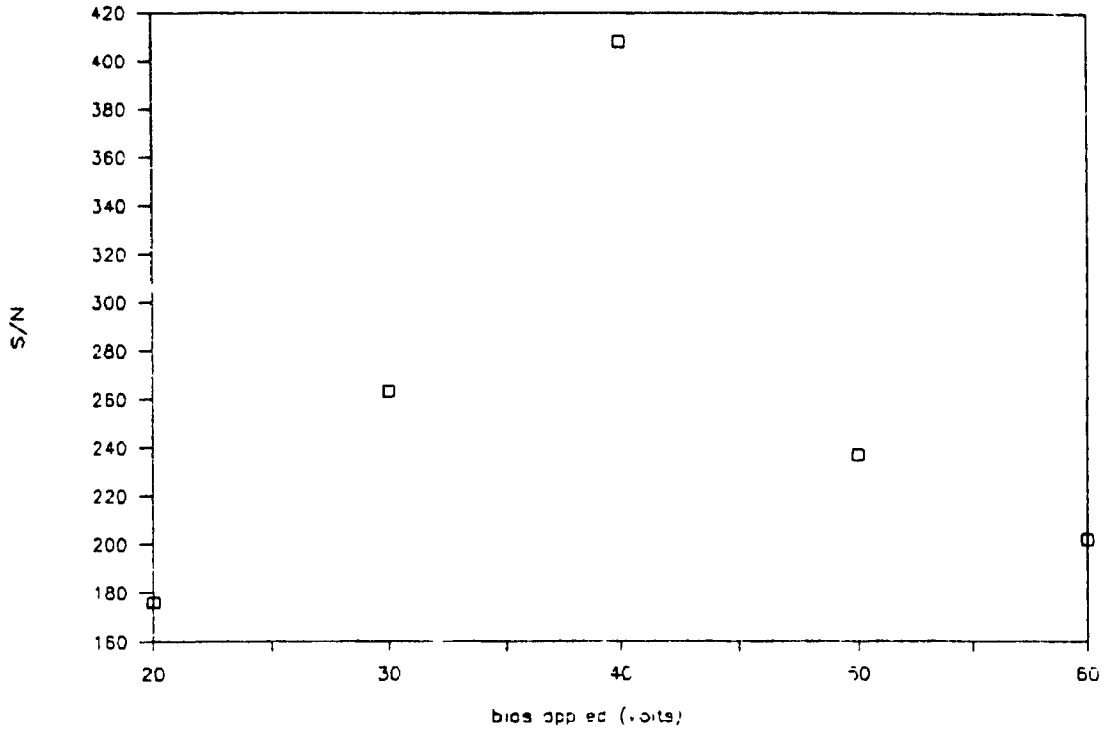


FIG 4 Optimization of PbSe detector

TABLE 2 OPTIMIZATION OF REFERENCE WAVE

reference wave (mV)	S/N
200	74
300	207
400	287

V. DETERMINATION OF THE QUANTUM EFFICIENCY OF THE PBSE DETECTOR

For a background-limited photoconductive detector, such as is used here, (the detector is said to be background limited, if the major source of noise is the photons associated with the background (1)), the maximum threoretical value of the detectivity, D* at a given wavelength is given by the following equation

$$D^* = \lambda \eta^{1/2}/(2hc)\, Q_b^{1/2} \qquad (3.4)$$

where h is Planck's constant, c the speed of light, λ the wavelength impingent on the detector, η the quantum efficiency and Q_b the photon flux.

Substituting for h and c, one gets the following equation

$$D^* = 2.52 \times 10^{18} \lambda\, (\eta^{1/2}/Q_b^{1/2})^2 \qquad (3.5)$$

Rearranging the above equation gives the following equation

$$\eta = [D^*\, Q_b^{1/2}]^2/[2.52 \times 10^{18} \lambda]^2 \qquad (3.6)$$

At 3.39 μ which is the wavelength of the helium-neon laser being used, the background flux (Q_b) is 4 x 10^{14} photons/sec-cm^2 (3) at 23 °C. or 300 °K (since Q_b is temperature dependent) Using a D* of 1.17 x 10^9 cm-Hz$^{1/2}$/W (4), one gets a quantum efficiency of 8 x 10^{-6} or 8 x 10^{-4}%.

The quantum efficiency can be used to determine the precision of the experiment if it were shot noise limited, i.e. limited by the amount of laser light. For a 100 mV laser (from a peak-to-peak oscilloscope reading off of the PbSe detector, a mV reading) and using a dark resistance of 200 K ohms and Ohms law, the current obtained is 5 X 10^{-7} A. Since there are 1.6 X 10^{-19} C/photon, this gives one 3 X 10^{12} photons. The quantum efficiency calculated above can then be multiplied by the number of photons to give 2 5 X 10^7 photons which are actually seen by the detector. The precision which is the inverse of the signal-to-noise or N/S, is calculated by the inverse of the square root of the number of photons which in this case is 0.02% precision if shot noise limited

This is the case without the flow cell in the light path When the flow cell is placed into the light path, the power drops approximately 2 orders of magnitude. This power drop is due to the fact that the cell material absorbs some of the light and also because of the small aperture of the cell (1 mm) which is much smaller that the actual

diameter of the laser beam. The power drop is due to the fact that Suprasil Quartz transmits approximately 80% at 3.39 μ and due to the fact that the cell aperture is 16% of the total laser beam diameter (4 mm to 1 mm). In using these numbers one calculates the power with the flow cell in place to be approximately 10 mV (on oscilloscope). There may be other sources of power drop such as incorrect alignment of flow cell and error associated with reading the mV of power off an oscilloscope display. These errors are difficult to quantitate.

If the laser is now 1 mV from a peak-to-peak oscilloscope reading, the current will be 5×10^{-9} A, which gives 3×10^{10} photons. Multiplying by the quantum efficiency, gives 2.5×10^{5} photons seen by the detector, which gives a precision of 0.2% if shot noise limited. This implies that if the laser is the largest source of noise in the whole infrared detector, the precision would be 0.2%. This is acceptable.

VI. LONG TERM POWER

An examination of the long term laser power can give some insight into the characteristics of the laser. Fig. 5 shows a period of approximately 2 months where the signal of the laser (without the flow cell) is plotted versus time. Day 1 is the first day of the study, Day 71 the last.

As can be seen the power fluctuates from day to day showing what an unstable source the laser is. After Day 13 when the power dropped dramatically, the mirrors were realigned with a much better power output on Day 15. From this point on, the laser power gradually dropped off. This is as is expected since the laser is open to the atmosphere and hence sensitive to dust accumulation on all surfaces. In addition the laser is not in a sealed cavity and therefore prone to vibrations etc. which will tend to slowly decrease the power.

After one to one-and-a-half years continuous use, the laser power drops off irreversibly because of leakage in the laser tube The laser then needs to be refilled.

One would hope to see a much better response from a laser which was not homemade. For comparison purposes, a study of the red helium-neon laser which was used to alignment of the laser mirrors (Spectra-Physics,

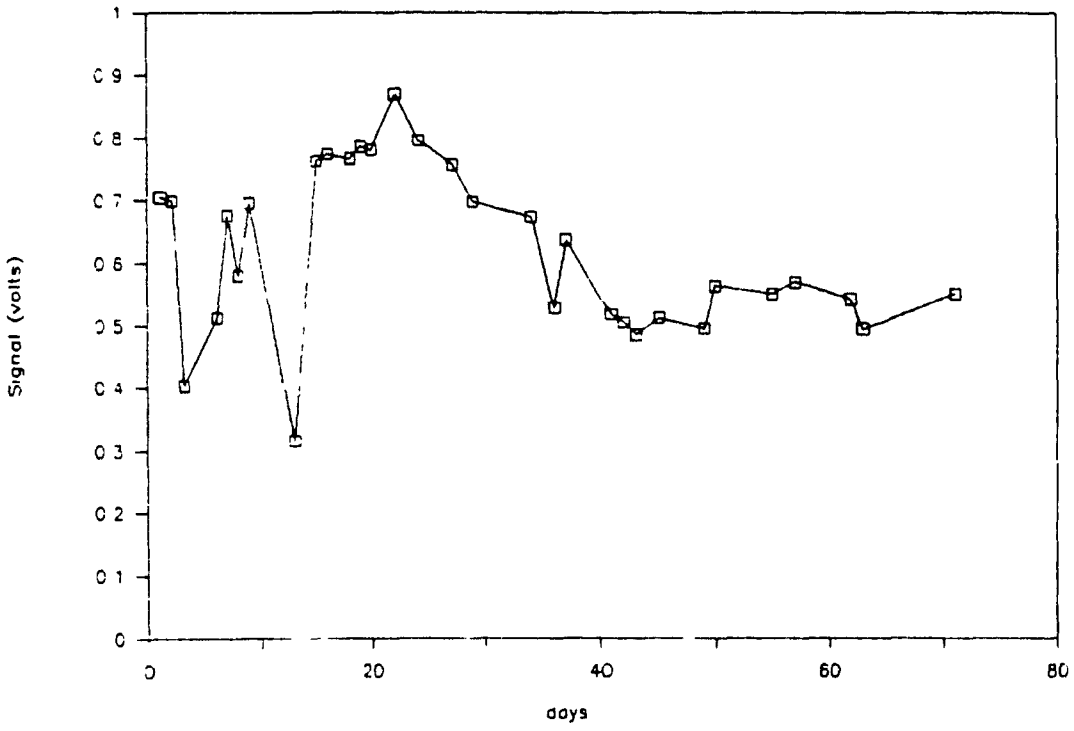


FIG 5 Laser power

Mountain View, CA), we can see that it is not so (Fig. 6). This is perhaps due to the age of the laser, since the red helium-neon laser is quite old and one would hope for better performance from a new laser.

Using a beamsplitter and designing a double beam detector would reduce the effect that these overall fluctuations in the laser and also be of benefit since many solvents useful for chromatography absorb in the infrared. Some thought was given to the idea of double-beaming the instrument, however beam splitters coated for 3.39 μ cost on the order of $1000 U.S. Before such an expensive and time-consuming step was taken, it should be determined whether the infrared detector works as it was designed; that is can lipids be detected.

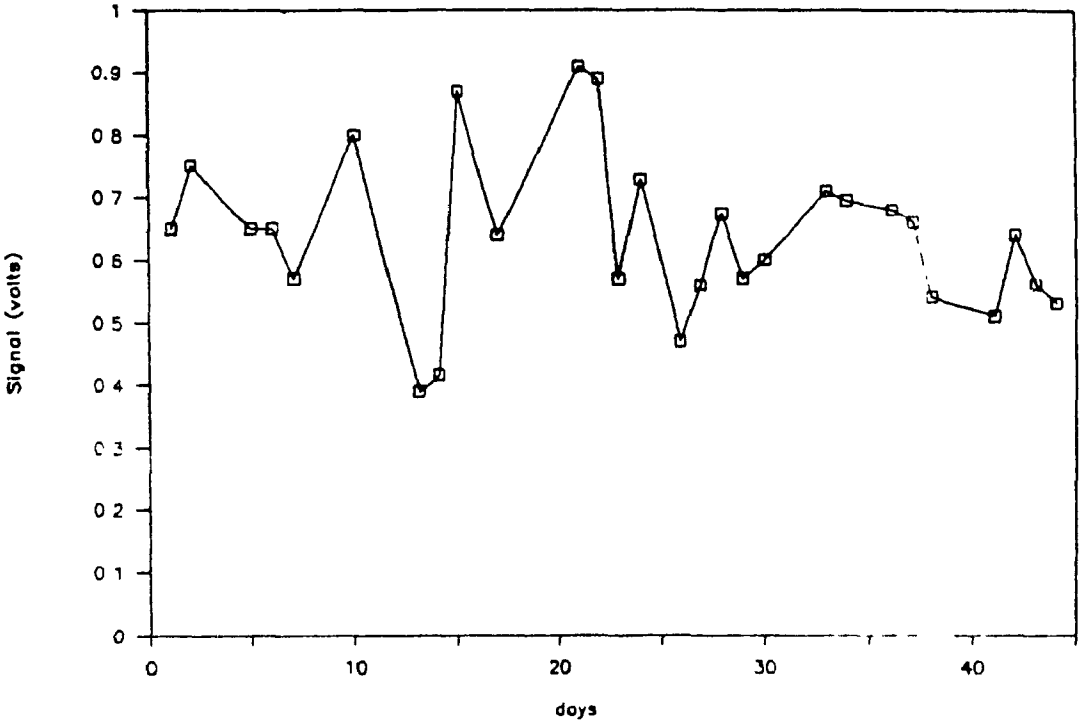


FIG 6 Signal output of red helium-neon laser

REFERENCES

1. Pitlak, R T., "Computer Programs for use in ElectroOptics", Apollo Lasers, Inc., Box 2730, Chatsworth, CA 91311.

2. Hudson, R.D., "Infrared System Engineering", 1st edition, John Wiley & Sons, New York, 1969, p. 349.

3. Communication with Dr B. Horter, Infrared Industries, Orlando, Florida.

4. Model 5055 lead selenide detector specifications, Infrared Industries, Orlando, Florida.

CHAPTER FOUR

FLOW-INJECTION ANALYSIS OF LIPIDS

It can be seen from Chapter 3 that the laser and, in fact, the infrared detector were working. The next step was to place the flow cell in its mount and determine whether lipids can be determined.

I. EXPERIMENTAL

The chromatographic equipment used was a Waters M-6000A chromatographic pump (Waters Associates, Inc., Milford, MA) with a Rheodyne model 7125 sample injector (Rheodyne, Inc., Cotati, CA) with a 500-µl loop (Waters Associates, Inc.). It was decided not to use a column in the initial stages of analyzing lipids.

All lipids were purchased from Sigma Chemical Co., St. Louis, MO.

1. Solvent Considerations

Since lipids, by their definition, are not soluble in water, only organic solvents need be considered as possibilities for use in this system.

Infrared spectra are available from reference books; therefore, one is saved the trouble of determining whether they are transparent in the infrared region of interest (2950 cm^{-1}) (1). Alkanes, by their chemical structure, are not useful. Since we are observing the absorbance of the carbon-hydrogen stretch in methylene groups, alkanes would absorb in this region as well. Normal, branched, and cyclo-alkanes such as n-hexane, isooctane and cyclohexane would not be useful. In addition, ether, p-dioxane, acetic acid, acetone and tetrahydrofuran would be unsuitable. Some of the solvents which were useful were

the halogen substituted alkanes such as chloroform, methylene chloride, methylene bromide, carbon tetrachloride, 1,2,3-trichlorobenzene, bromochloromethane, as well as benzene and carbon disulfide.

In addition to the infrared transparent consideration, there is the problem of whether these solvents are readily available, available at reasonable prices and whether the solvents are available highly purified for High Pressure Liquid Chromatography (HPLC). Some of the less common solvents (in terms of HPLC) are either not available in a highly purified form or are available at enormous cost. (An extreme example is that of deuterated chloroform, which is less infrared absorbing than chloroform (at 3.39 μ); however, it would not be practical to use because of the high cost.) Because of cost and availability considerations, acetonitrile and methanol were considered despite the fact that they exhibit considerable infrared absorbance, though not to such an extent as the alkanes.

The final consideration was that of safety. Information on many hazardous materials is readily available in References 2 and 3. Potential hazards of the possible solvents include flammability of carbon disulfide; methanol can cause blindness, narcosis, headache, nausea, giddiness and loss of consciousness; methylene chloride causes dermatitis; carbon tetrachloride, chloroform and trichloroethylene are all suspected carcinogens. All of the above compounds require the use of gloves, protective clothing and adequate ventilation.

The choice of gloves (4) depends on the compound used. For the following solvents: chloroform, carbon tetrachloride, methylene chloride and trichloroethylene, nitrile or high grade PVC gloves are recommended over natural rubber, neoprene or normal PVC gloves. Nitrile gloves were purchased from Safety Supply, Inc. (Montreal, Que.).

In addition, there is an explosion potential if the following come into contact: 1) acetone and chloroform in the presence of base; 2) carbon disulfide and sodium azide; 3) chloroform or carbon tetrachloride and powdered aluminum or magnesium. Incompatible chemicals can cause violent reactions,

heat production or toxic products if brought into contact. Organic halogen compounds are incompatible with groups IA and IIA metals and aluminum.

In summary, if possible one should substitute a less hazardous chemical. (The classic example is the substitution of toluene or xylene for carcinogenic benzene.) One should adequately protect oneself with protective eyewear, and hand and clothing protection.

In addition, since the vapor of the organic solvents to be used was harmful, solvent reservoirs left open to the atmosphere were not acceptable. Because of the high vapor pressure of these solvents, one could not merely stopper the reservoir. An inline filter was developed which consisted of a 4-5 inch length of plastic tubing. Glass wool, alumina, charcoal, alumina, glass wool was the sequence to fill the plastic tube. This absorbed the vapor. The filters were changed once a day.

Chloroform was chosen as the solvent to begin analysis of lipids. It was infrared transparent and dissolved most lipids. It is relatively toxic though less so than carbon tetrachloride. The chloroform used was distilled in glass, Accusolv grade (Anachemia, Montreal, Que.). The solvent was degassed with helium gas for a minimum of 10 minutes prior to use.

All solutions were prepared by dissolving the lipid in chloroform. Subsequent dilutions were prepared from this solution. All lipids were weighed into volumetric flasks. Lipid solutions were not stored but prepared fresh every morning.

II. FLOW INJECTION ANALYSIS OF LIPIDS

Flow injection analysis of representatives of the four classes of lipids were performed. A flow rate of 1.0 ml/min was used for all experiments in this chapter as well as the subsequent chapters. The flow rate precision according to the specifications of the Waters M-6000A high pressure pump is ± 0.1% with an accuracy of ± 1.0% (or ± 0.005 ml/min). However this was never actually tested and one would expect it to

be less than this since the pump was relatively old. Fatty acids (saturated and unsaturated), triglycerides, cholesterol and esters and phospholipids were detected.

Peak heights were used and absorbance calculations performed. The absorbance was calculated from Equations 1 and 2.

$$T = \frac{E_s - E_{0\%T}}{E_r - E_{0\%T}} \qquad (4.1)$$

$$A = -\log T \qquad (4.2)$$

Equation 1 is used for the calculation of the transmittance (T) where E_s is the voltage due to the sample injection, E_r is the baseline voltage (reference voltage) and $E_{0\%T}$ the voltage when the laser is blocked (zero transmittance). Equation 2 is used for the calculation of Absorbance (A).

In order to minimize the error involved in the injections, it was decided to perform full loop injections. All of the experiments were done in this manner. The model 7125 Rheodyne sample injector is a rotary valve, a six port sample injection valve with injection of the sample through a needle port into the valve shaft. The needle port (in the rotor seal) is used to fill the sample loop (load) and in the inject position the loop is switched into the solvent stream. There are two methods of loading the sample: the complete and partial filling method. The complete loop filling method was used. An excess of sample is injected to insure that the sample loop is completely filled. The actual volume injected is determined by the actual volume of the loop. The highest precision is obtained by this method since there can be no operator error since the injection volume is determined by the sample loop, which does not change.

Processing of data was done by measuring peak heights and determining the absorbance using Equations 4.1 and 4.2. Two injections were made for each concentration and the average absorbance was then plotted vs. concentration to give a calibration curve. However, calibration curves were only linear for a very small portion and then levelled off. The reason for this was the very small volume of the flow cell. Peak heights increased up to a certain point and then levelled off, the peaks becoming wider instead of

higher as concentrations increased. Peak areas normally would be used, however cannot be used here, since this is a transmittance detector. Peak areas can only be easily used for true absorbance detectors, where absorbance is plotted on the recorder.

1. Fatty acids

The fatty acids determined were palmitic acid (Fig. 1), a 16 carbon saturated fatty acid, stearic acid (Fig. 2), an 18 carbon saturated fatty acid, and oleic acid (Fig. 3), an 18 carbon fatty acid with one double bond at carbon 9. (In addition, arachidonic acid was detected though no calibration curve was made.)

2. Triglycerides

The representative triglycerides determined were tristearin (Fig. 4), and triolein (Fig. 5).

3. Phospholipids

Only one phospholipid was analysed (due to the excessive cost of phospholipids). Phosphatidylcholine (PC) was analysed (Fig. 6), one of the more common phospholipids.

As can be seen from the calibration curve of PC, % dilution is the label on the concentration (x) axis. The sample was purchased in a vial. Since most of the lipids which are liquids at room temperature are fairly viscous, it was decided to weigh the sample. However, since the sample was present in a chloroform solution (100 mg/ml), the concentration could not be calculated, because the producer of the PC did not know the density of the PC in chloroform. Further analysis of PC was not done, since at this point the object was only to determine whether phospholipids were detected in the infrared detector and that had been accomplished.

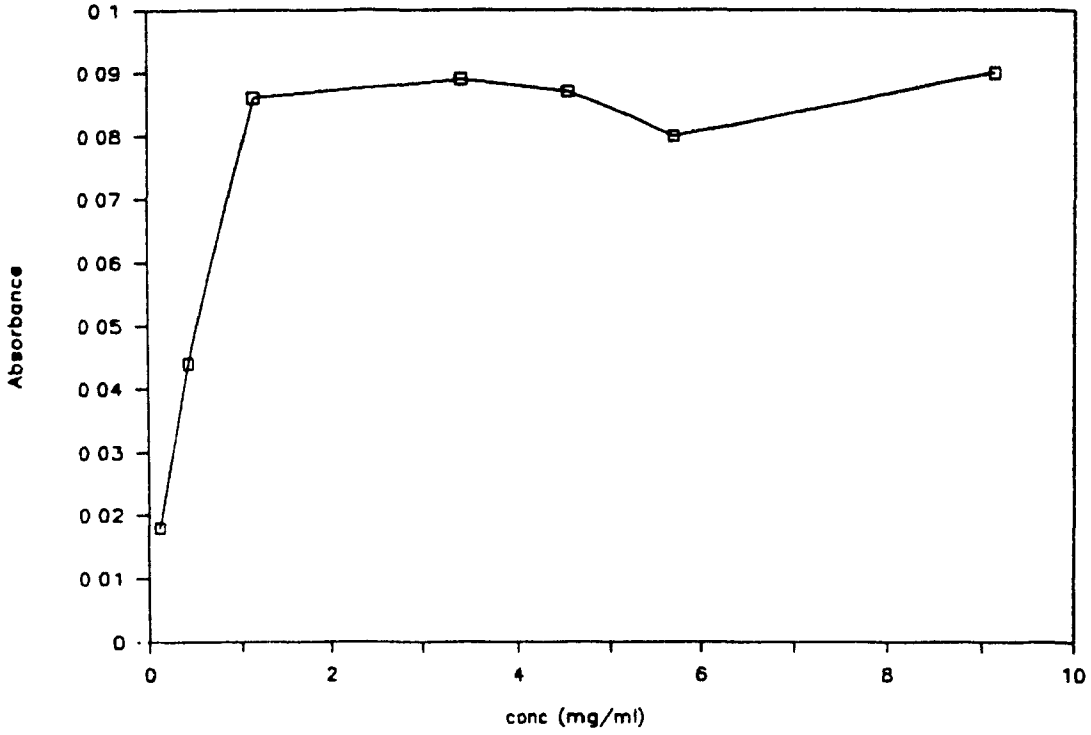


FIG 1 Calibration curve for palmitic acid

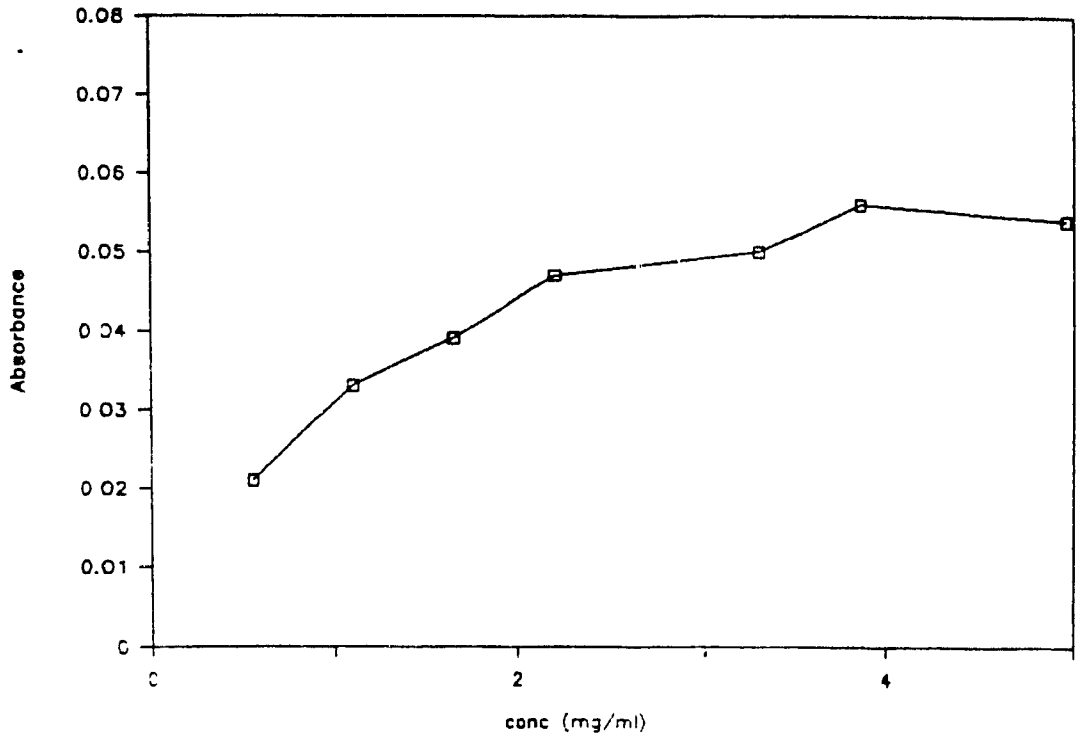


FIG 2 Calibration curve for stearic acid

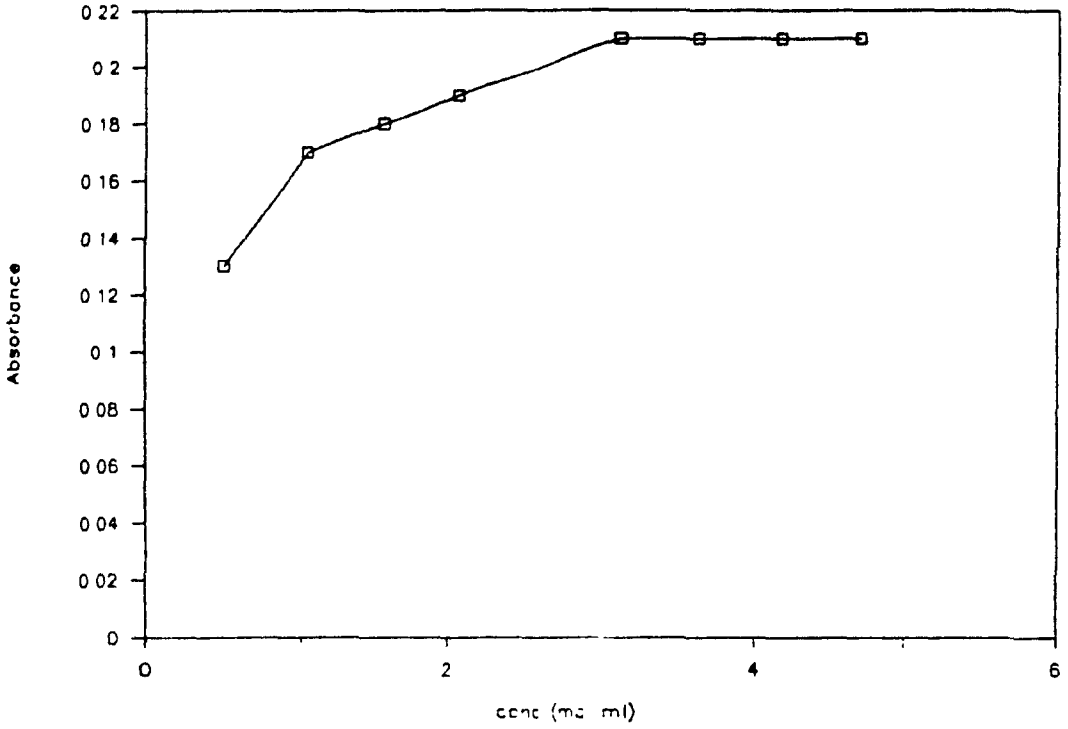


FIG 3 Calibration curve for oleic acid

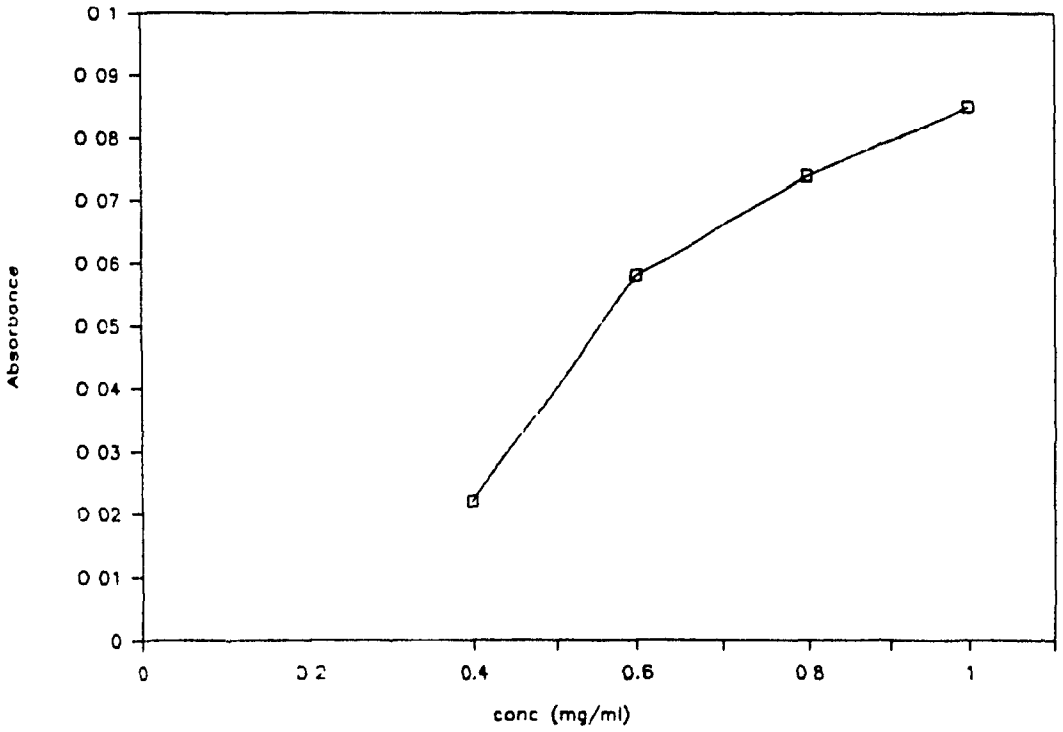


FIG 4 Calibration curve for tristearin

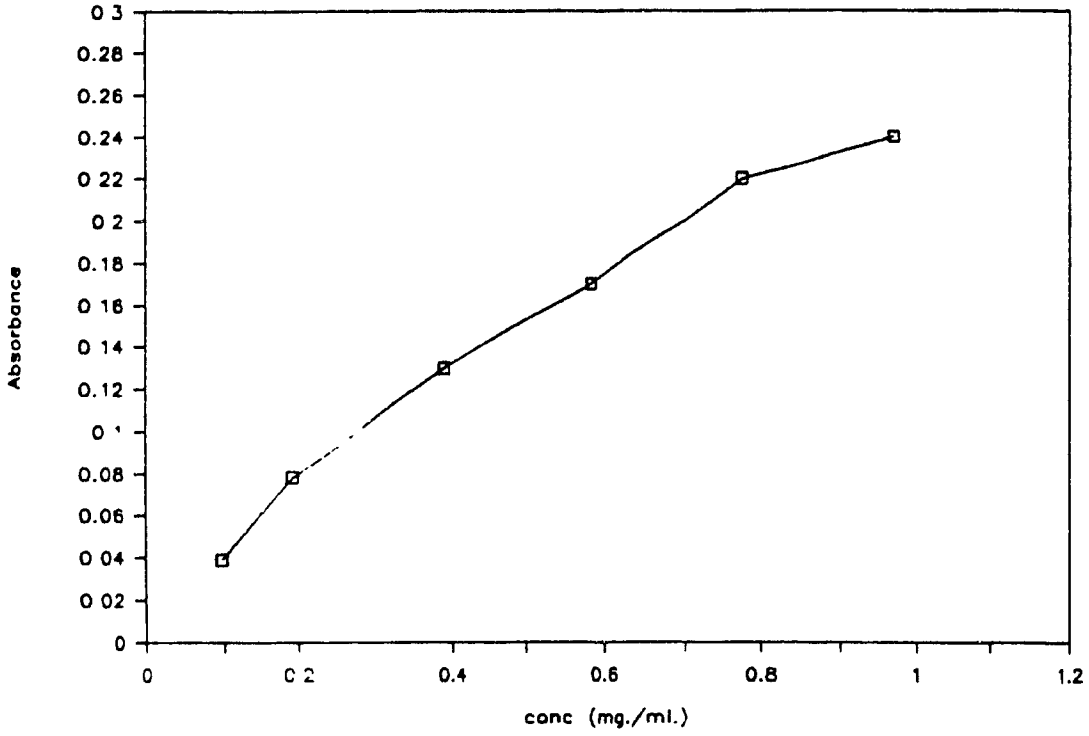


FIG 5 Calibration curve for triolein

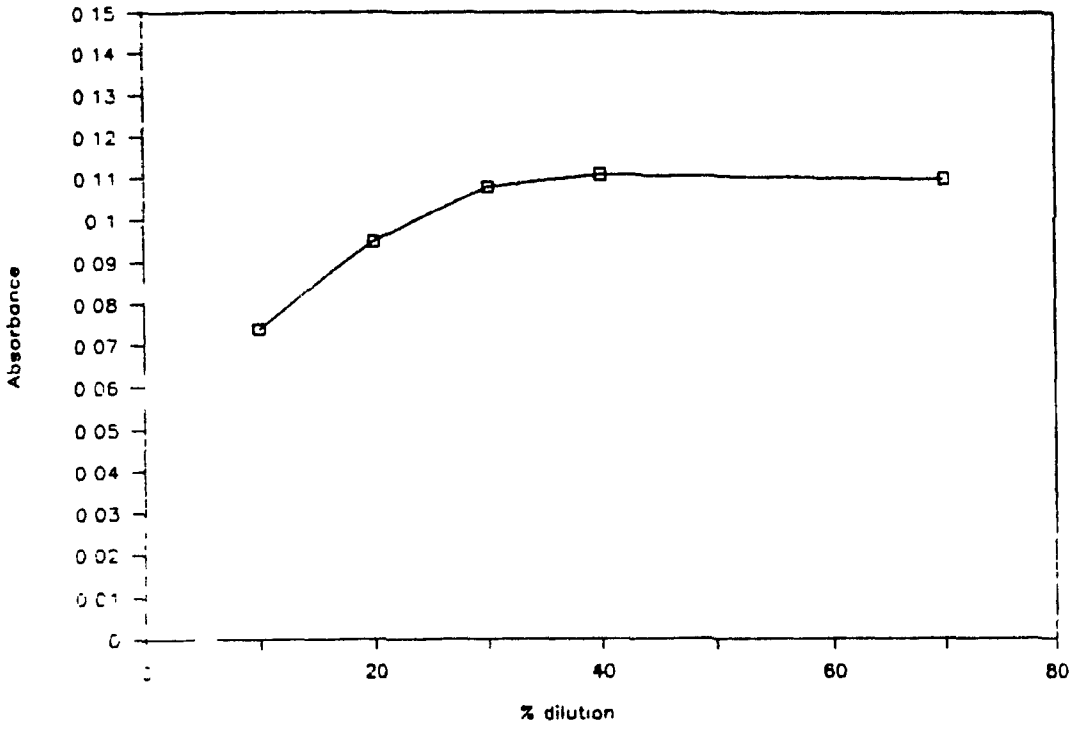


FIG 6 Calibration curve for phosphatidylcholine

4. Cholestryl esters

Cholesterol (Fig. 7) was determined as well as the following cholestryl esters: cholestryl palmitate (Fig. 8), cholestryl stearate (Fig. 9), and cholestryl oleate (Fig. 10).

5. Detection limits

Plots of signal-to-noise versus concentration were performed for representatives of the four classes of the lipids analyzed. The signal was the maximum sample voltage subtracted from the average baseline voltage. The noise was due to the background. that is, 10 readings at 10 second intervals were determined (with solvent flowing) and the standard deviation was determined. The curve was plotted using Lotus 123 and an equation for the best curve was determined using the Omicron PLOTRAX program (Engineering- Science, Inc., Atlanta, Georgia). The detection limit can be obtained from the equation of the curve and calculating the concentration for which the S/N is equal to 2 (by definition). This was done for a representative of each class of lipid: oleic acid (Fig. 11, correlation 0.98), triolein (Fig. 12, correlation 0.99), and cholestryl oleate (Fig. 13, correlation 0.99).

Table 1 shows a table of detection limits for some of the lipids determined. The infrared detector would be able to analyze blood or serum samples for lipids since normal amounts of lipid are well above the detection limit (Chapter 1). More likely is the possibility that the lipid sample would need to be diluted to be in the range of the calibration curve.

III. BEER'S LAW

Beer's law states (Equation 4.3) that the absorbance is proportional to the concentration. Hence for a calibration curve where absorbance versus concentration is plotted, one would ideally get a straight line.

$$A = abc \qquad (4.3)$$

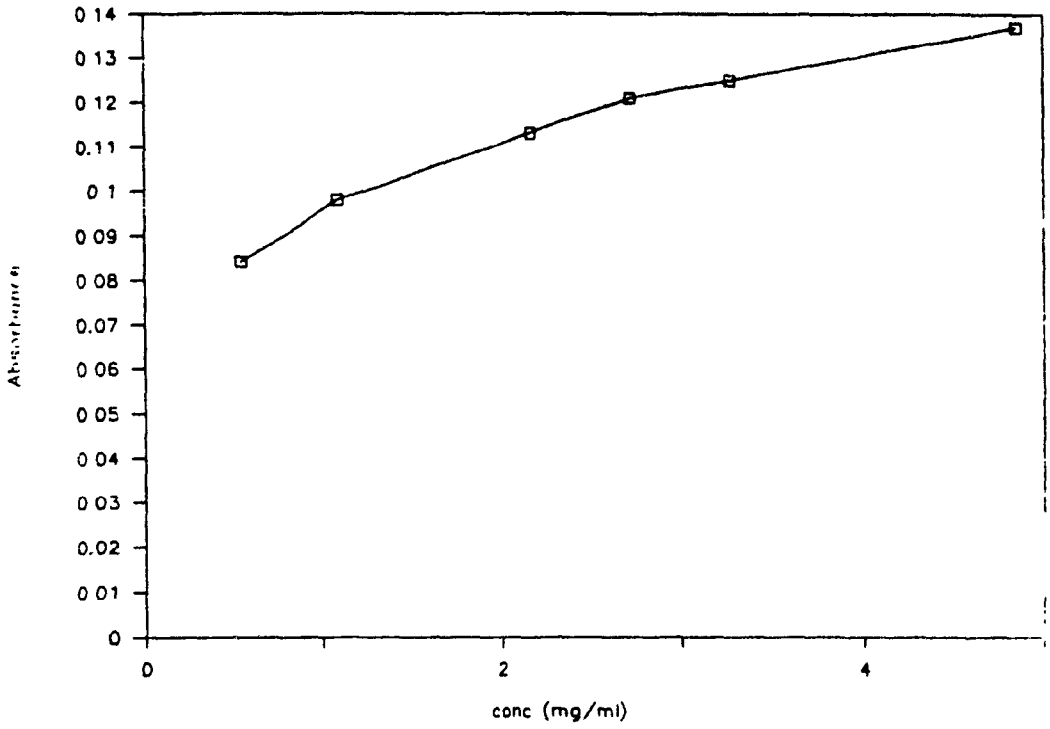


FIG 7 Calibration curve for cholesterol

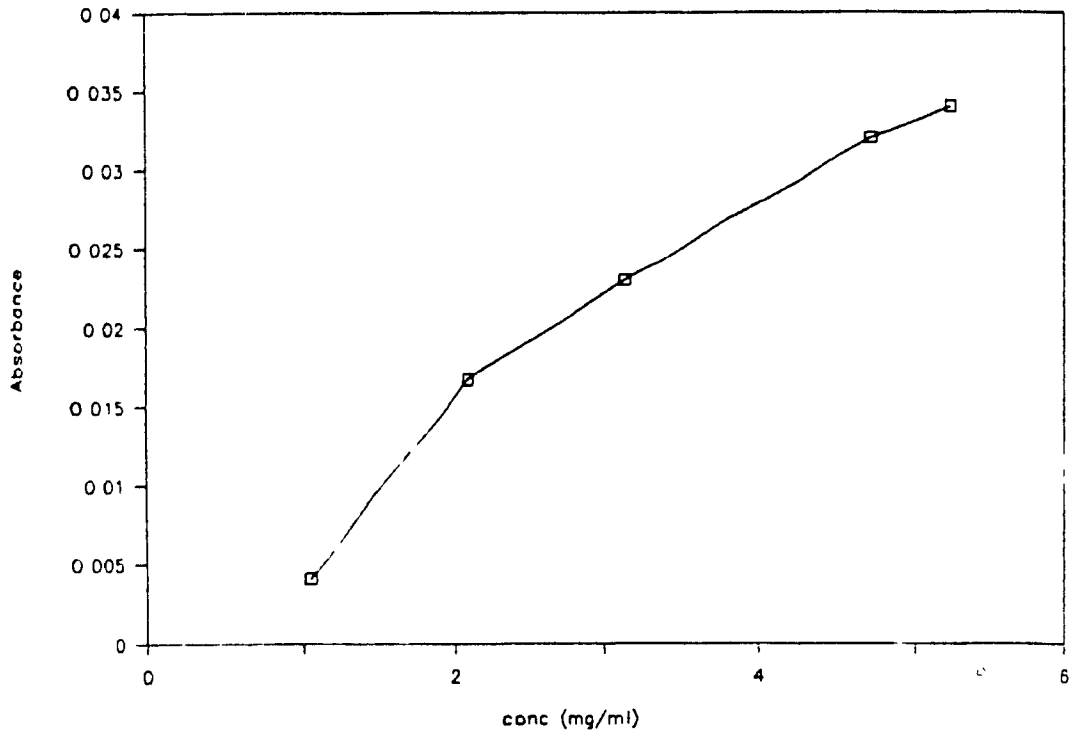


FIG 8 Calibration curve for cholestryl palmitate

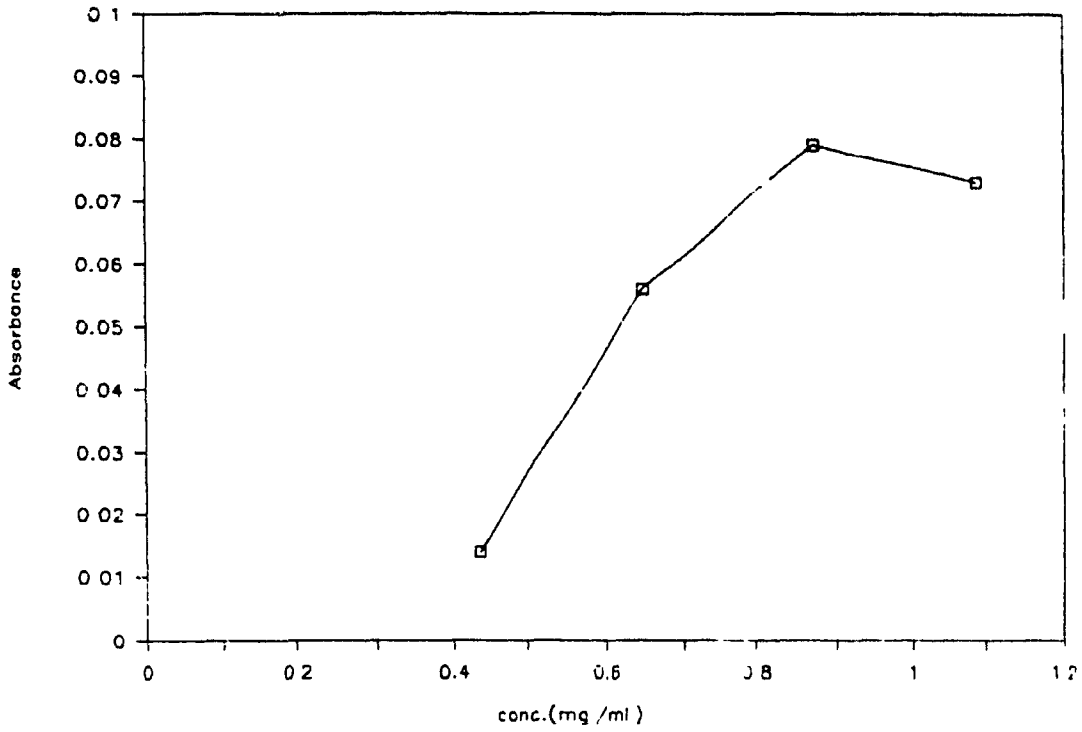


FIG 9 Calibration curve for cholestryl stearate

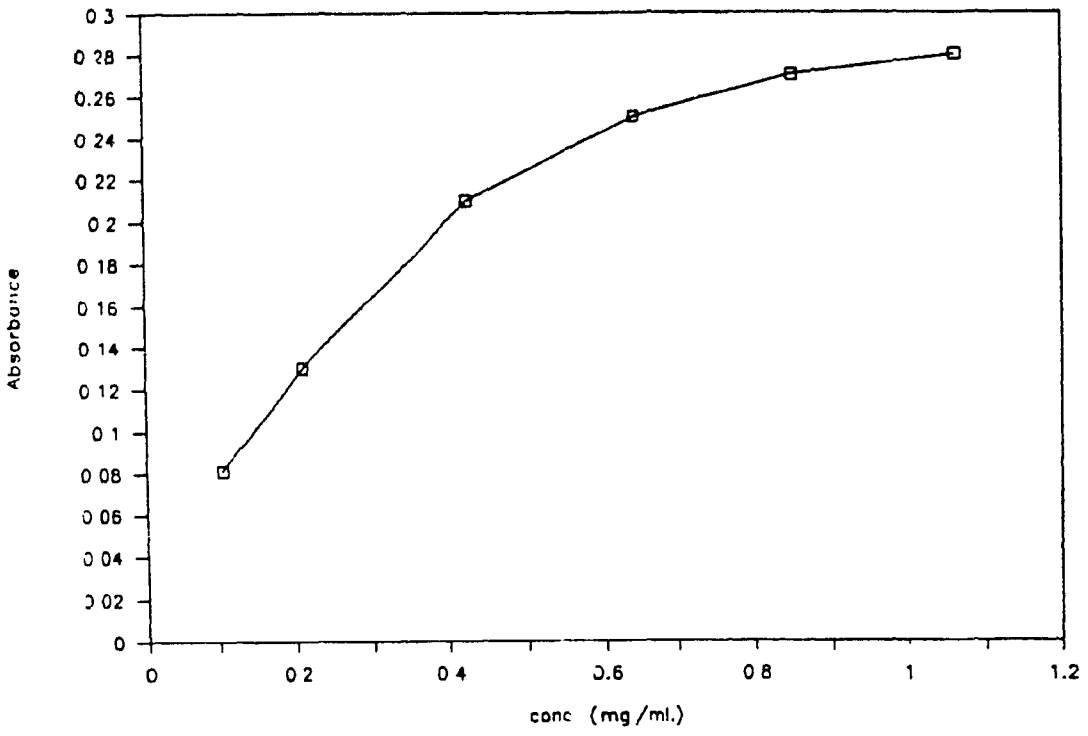


FIG 10 Calibration curve for cholestryl oleate

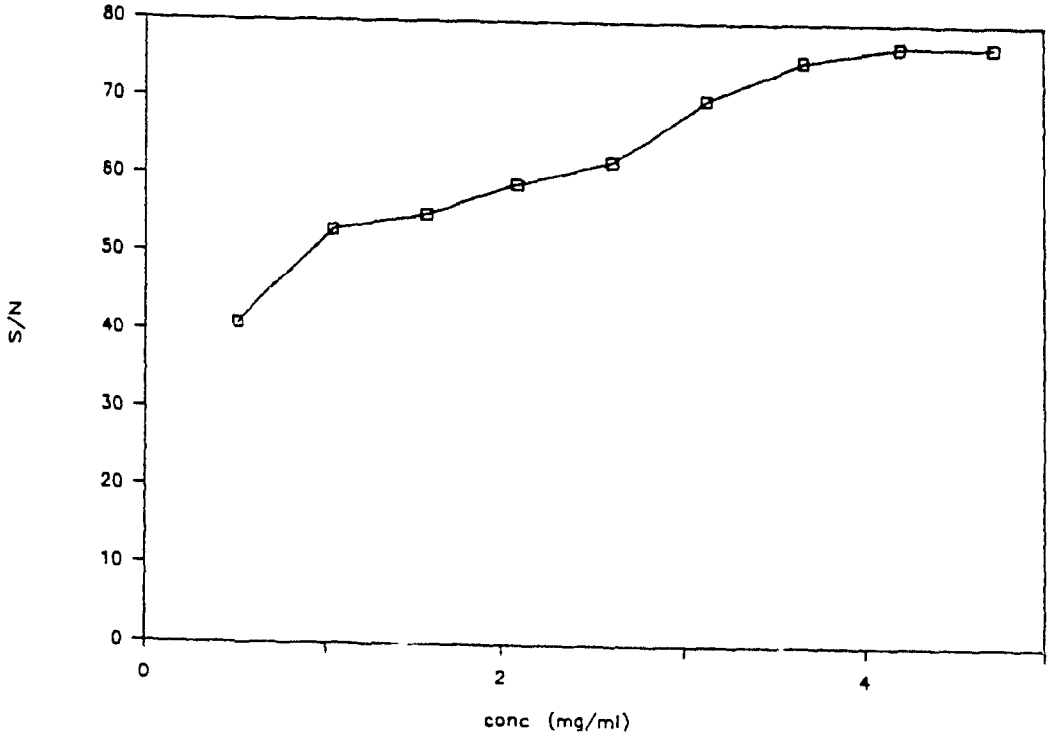


FIG 11 S/N curve for oleic acid

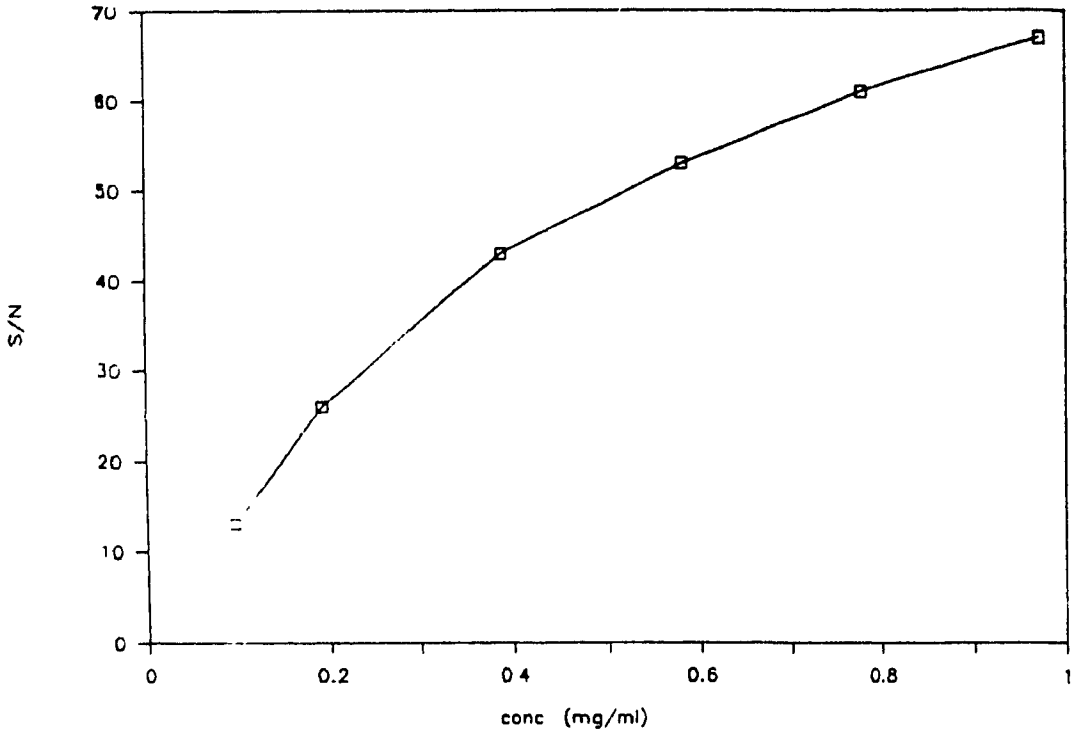


FIG 12 S/N curve for triolein

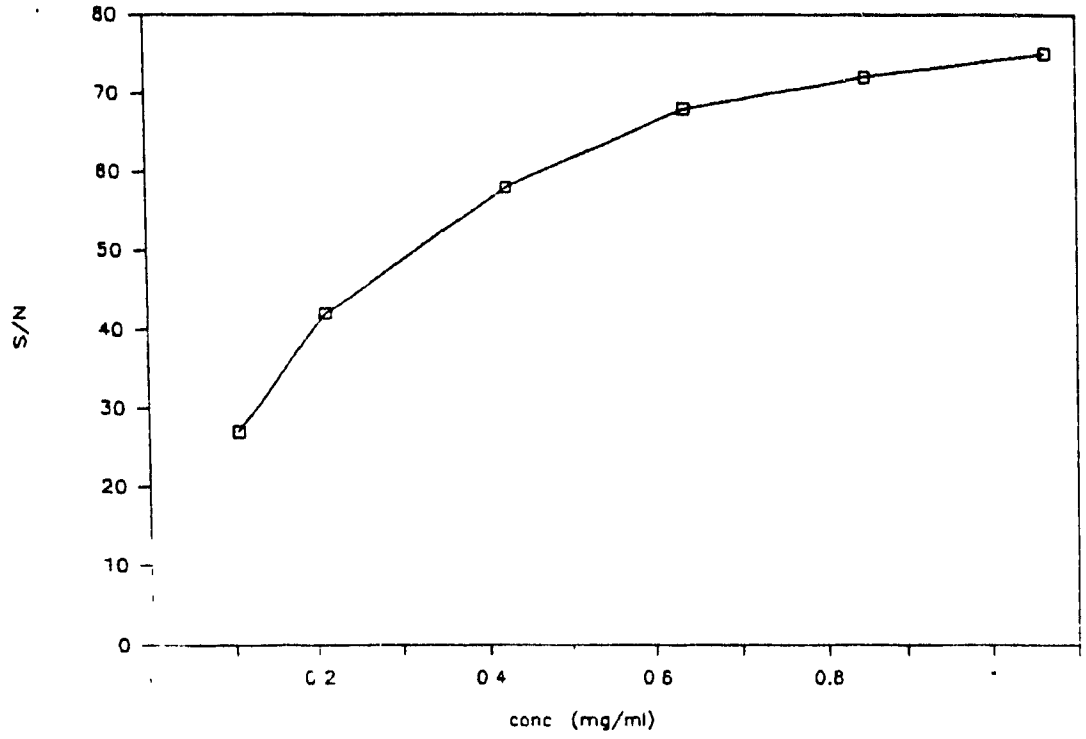


FIG 13 S/N curve for cholestryl oleate

TABLE 1 DETECTION LIMITS OF SOME LIPIDS

	detection limits (mg/ml)
palmitic acid	0.2
oleic acid	0.05
tristearin	0.3
triolein	0.07
cholestryl oleate	0.03

where A is the absorbance (no units), a is the absorptivity or molar absorptivity (l/mol-cm), b is the pathlength in cm, and c is the concentration.

As can be seen in most of the calibrations curves illustrated in this chapter, Beer's law does not apply throughout the concentration range. That is, deviations are observed. This is, in fact, fairly common and is frequently encountered.

The limitation that most often is present is simply due to the limitation in the law itself. Beer's law only applies to dilute solutions (<=0.01M usually) (7). At higher concentrations, there is an interaction between the molecules which are absorbing since the molecules come close enough together so that they can alter the charge distribution of the neighboring species. This can alter their ability to absorb a given wavelength of light. Since this effect will be dependent on concentration, there is a deviation from linearity in the calibration curve. It should be noted that the 0.01M concentration is not absolute, that is exceptions occur particularly with large organic ions or molecules.

There are numerous other reasons for deviations of Beer's law from linearity. The absorptivity is dependent on the refractive index of the solution. Concentration changes may alter the refractive index of the solution. However, a correction factor can be substituted into the Beer's law equation (7). Chemical deviations are due to associations and dissociations (example dimer formation, the dimer would absorb at a different wavelength). This is probably not significant in the case of lipids.

Beer's law is linear for monochromatic radiation only. This would not cause a deviation in this experiment, since in Chapter 3 it was illustrated that the laser was monochromatic. Scattered or stray radiation reaching the detector can cause deviations from linearity of Beer's law (7). The nonlinearity may also be due to a saturation of the PbSe detector. The detector specifications state that the PbSe is sensitive to and may saturate in fluorescent light, if left continuously in fluorescent light. Hence when not in use the detector was stored in the dark. In addition, when turning out the room lights no change in the output was observed. However this was done very crudely, that is the lights were turned off and no change was noticed

in the voltage output of the PbSe nor any significant change in the noise on the recorder tracing. This was never pursued since it was not convenient to work in the dark for extended periods of time.

The infrared detector studied here does not give a linear calibration curve as can be seen from all the calibration curves shown, therefore Beer's law does not apply. Beer's law is the ideal case, however, if one does not have a linear calibration curve, the solution is to plot many more points (than would be needed if the calibration curve were linear). Using many points in a calibration curve would define it exactly and enable one to use the detector for the study of real samples.

REFERENCES

1. Pouchert, C. J., "The Aldrich Library of Infrared Spectra", 3rd edition, Aldrich Chemical Co., Milwaukee, Wisconsin, 1981.

2. Situg, M., "Hazardous and Toxic effects of Industrial chemicals", Noyes Data Corp , Park Ridge, N.J., 1979.

3. Sunshine, I , ed., "CRC handbook of Analytical Toxicology", The Chemical Rubber Co , Cleveland, Ohio, 1969, p 585-670.

4. Bretherick, L , "Hazards in the Chemical Laboratory", 4th edition, The Royal Society of Chemistry, London, England, 1986, p 30

5. Snyder, L R , and Kirkland, J.J. "Introduction to Modern Liquid Chromatography", 2nd edition, John Wiley and Sons,Inc , New York, 1979, p 546-549

6. Christian, G D , "Analytical Chemistry", 4th edition, John Wiley and Sons, Inc , New York, 1986, p 88-91.

7. Skoog, D A., and West, D M , "Principles of Instrumental Analysis", 2nd edition, Saunders College/Holt, Rinehart, and Winston, Philadelphia, 1980, p 153.

CHAPTER 5

HIGH PRESSURE LIQUID CHROMATOGRAPHY OF LIPIDS

It had been determined that all the different classes of lipids could be detected by the laser based infrared detector (Chapter 4). The next step was to insert a column in the chromatographic stream and change from a simple flow-injection system to a chromatographic system.

I. THE STATIONARY PHASE

A number of different methods have been employed to separate lipids. Many different detectors (as is mentioned in Chapter 1) as well as many different columns have been used. Four different types of columns may be considered when chromatography on lipids is to be performed. Gel permeation and ion exchange columns will not be considered since they would not be useful for the separation of lipids. (For the sake of completeness it should be mentioned that cholesterol in human serum lipoproteins was determined by separation of the lipoproteins using aqueous gel permeation columns (TSK Gel type PW and type SW). This is not separation of free lipids as will be done here, but separation of lipoproteins (1).) What remains is normal phase and reverse phase separations. These 2 designations may be considered to be arbitrary since the designations are due to relative polarities of the stationary and mobile phase. Normal phase chromatography is when the stationary phase is more polar than the mobile phase. Reverse phase chromatography is the opposite of normal phase and therefore the stationary phase is less polar than the mobile phase Reverse phase chromatography has been very widely used due to the fact that an aqueous mobile phase is most often used. Since water is a major component of the mobile phase, this keeps the cost of the mobile phase at a minimum.

Since lipids, by definition, are insoluble in water, aqueous reverse phase chromatography is not an option. However, non-aqueous reverse phase chromatography has been performed by Parris (2) using a Zorbax octadecylsilane (ODS) column. Detection of glycerides was done with a commercially available infrared detector set at a wavelength of 5.72 μ.

Separations of lipids have been performed repeatedly by reverse phase columns. HPLC of cholesterol and esters has been done using an ODS 5 μ Supelcosil column (3). Triglycerides have also been separated using reverse phase columns usually with columns based on the ODS system but often produced by different companies. Examples are Zorbax ODS, mentioned above, and μBondapak (4), both ODS columns.

Normal phase columns can be classed according to the functional group on the base material giving weak (example diol), medium (nitrile) and high (amino or aminopropyl) polarity groups. Information from manufacturers reveals that the base material is silica but with little other data available (5). Payne-Wahl et al. (6) use a Partisil PXS 10/25 PAC column to separate classes of methyl ester, mono-, di-, and triglycerides. Infrared detection at 5.72 μ and gradient elution is done. Partisil 10 PAC is a silica based material (called Partisil) with a nitrile functional group.

Since reverse phase columns generally separate within a class of lipids (that is separation of cholesterol and its esters or separation of triglycerides), it was decided to use a normal phase column in order to separate classes of lipids. In the separation of classes of lipids, all the different triglycerides in a sample would elute at the same place on a chromatogram. The column which was purchased was a Partisil 5 PAC (5 μ) 25 cm long, 4.6 mm i.d. (PM Instruments, Inc., Toronto, Ontario).

II. THE MOBILE PHASE

Since the column is the same Partisil column as in Ref. 6, the first attempt was to try the same mobile phase. Payne-Wahl et al. (6) use a gradient from hexane-chloroform (60:65) to hexane-chloroform-acetonitrile (25:65:35). The latter solvent was attempted without any success because of the large infrared absorbance (at 3.39 μ) of the solvent. Infrared detection at 5.72 μ was the means employed by Payne-Wahl et al. for detection of lipids which is the carbonyl absorption, hence this is a logical choice for a mobile phase since none of the solvents have carbonyl groups. However at 3.39 μ, hexane, in particular has a very large absorption due to the C-H stretch in the methylene group. (Acetonitrile also has some absorbance at 3.39 μ) Therefore another solvent system had to be used with this column for detection at 3.39 μ.

Freeman et al. (7) use trichloroethylene as a solvent for the detection of tripalmitin at 3.39 μ. Therefore, trichloroethylene can be considered, however when attempted in the chromatographic setup, no separation of lipids was produced (that is all lipids eluted at the void volume). Mixtures of chloroform-trichloroethylene and also dichloromethane-trichloroethylene enabled one to detect the lipid but did not give adequate separation. It was decided to attempt a 3 solvent system and after trial and error, a mobile phase of the mixture chloroform-trichloroethylene-dichloromethane (1:1:0.05) (v/v/v) was used with separation of triglycerides, cholesterol and cholestryl esters (Fig. 1).

III. THE SEPARATION

Figure 1 shows a typical separation of cholestryl stearate, tristearin, and cholesterol (in order of appearance from the time of injection). (The y-axis is 1V full scale recorder setting and 0.5 cm/min and the recorder tracing is to scale.) A calculation of the resolution, R_s (a quantitative measure of the relative separation) using equation 1 is performed for tristearin and cholestryl stearate,

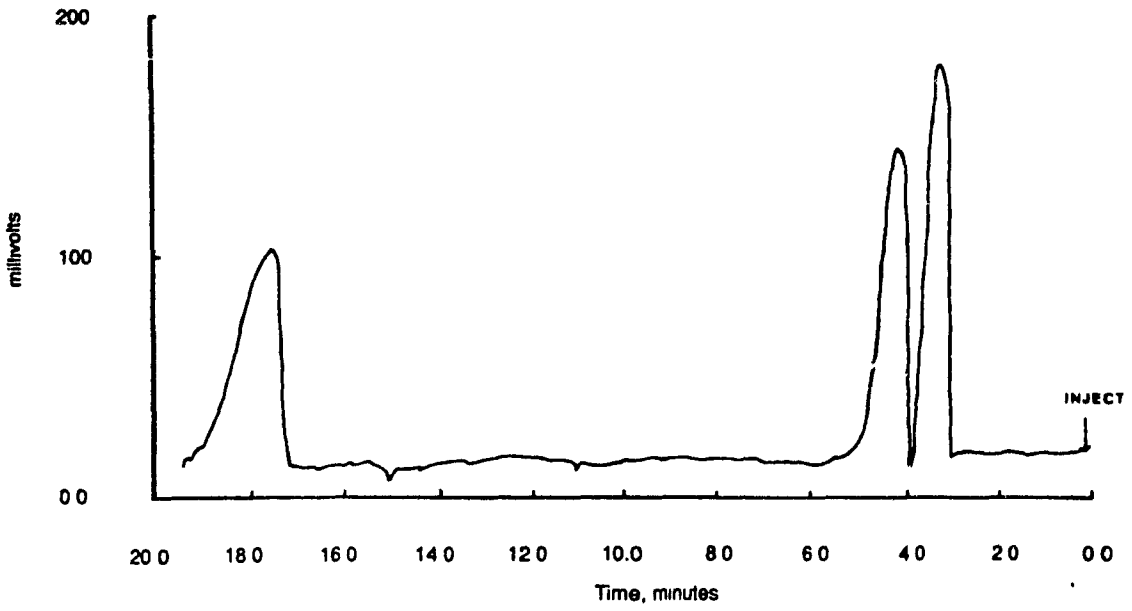


FIG 1 Sample chromatogram: separation of cholestryl stearate, tristearin and cholesterol

$$R_s = \frac{t_2 - t_1}{1/2\ (t_{w1} - t_{w2})} \qquad (5.1)$$

where t_2 is the retention time of the second band (tristearin), t_1 is the retention time of the first band (cholestryl stearate), and t_{w1} and t_{w2} the width of the bands. From Fig. 1, the retention times of cholestryl stearate (3.05 minutes) and tristearin (4.05 minutes) and peak width (1.0 and 1.05 minutes respectively) can be used to determine an R_s of 0.91, which implies a separation of 95-98% of cholestryl stearate from tristearin.

IV. CHROMATOGRAPHY OF LIPIDS

All experimental conditions were the same as those mentioned in Chapter 4 except for the inclusion of the column. All solutions and solvents were prepared as in Chapter 4. The recorder speed was 0.5 cm/min.

Figure 1 shows the separation of a mixture of cholestryl stearate, tristearin and cholesterol. Calibration curves for these three compounds were made for cholestryl stearate, tristearin, and cholesterol (see Appendix B). Similar separations were performed and plotted for a mixture of cholestryl oleate, triolein, and cholesterol (see Appendix B) and also for a mixture of cholestryl palmitate, tripalmitin, and cholesterol (see Appendix B). In each case Absorbances were determined as in Chapter 4 and in all cases full loop (500-μl) injections were performed. In each case a cholesterol calibration curve was made; however, rather than display all of them only one is included.

In all cases, the chromatography was the same for cholestryl esters and for the triglycerides. That is cholestryl stearate, cholestryl palmitate and cholestryl oleate all eluted with the same retention time and tristearin, tripalmitin and triolein all eluted at the same place. In addition, cholesterol in each case also had the same retention time for each separation. These particular cholestryl esters and triglycerides were used

because of their availability and also because they would make up a major portion of the lipids found in serum.

The coelution of cholestryl esters and also of triglycerides can be shown by preparing a solution of cholestryl esters (a mixture of cholestryl palmitate, cholestryl stearate, and cholestryl oleate) and upon chromatography, the mixture gave one peak at exactly the same retention time as a single cholestryl ester. In addition, a similar solution of triglycerides only was chromatographed. The solution was made of tripalmitin, tristearin and triolein. This solution also gave one peak and at the same retention time as any one triglyceride alone.

Another mixture was chromatographed. The solution was made up of cholestryl esters (a mixture of cholestryl palmitate, stearate and oleate) and triglyceride (a mixture of tripalmitin, tristearin and triolein). As was expected there was one peak only for cholestryl esters and one peak only for the triglyceride at exactly the expected retention times.

In summary it has been shown that the triglycerides all elute with the same retention time on the chromatogram and mixtures perform the same way that single components do. This has also been shown for cholestryl esters.

V. SENSITIVITY STUDIES

In this determination, the absorption of the methylene groups in a hydrocarbon chain was being done, one would expect that as the length of the hydrocarbon chain is increased there would be an increase in the sensitivity of the detector to that compound. In applying this concept to the situation at hand, as the length of the hydrocarbon chain attached to the lipid backbone is increased, an increase in the sensitivity of the detector to that lipid is expected. One would expect to see this for cholestryl esters as well as triglycerides.

The sensitivity of the detector is an abstract term. In reality, when Beer's law is used, the absorptivity can be used as a measure of sensitivity. When reported in this manner, higher absorptivities imply greater sensitivity of the detector to this compound in comparison to another compound with a lower absorptivity.

In order to calculate the absorptivity, a plot of absorbance (A) versus concentration (c) is made. A linear regression is performed on the data and from the slope of the line and the pathlength one can calculate the absorptivity from Beer's law (A = abc).

Table 1 shows the data for cholesterol, cholestryl esters and triglycerides. The absorptivity has been calculated in terms of molar concentration since that is the value most often reported in the literature. Concentrations have been illustrated in terms of mg/ml and conversion is done using the molecular weight of each lipid.

As can be seen from Table 1 for both the cholesterol-cholestryl ester series and the triglyceride series, the absorptivity increases with hydrocarbon chain length. In the case of lipids with double bonds, it is expected that the sensitivity would drop off since the carbon-hydrogen absorption is shifted when there is a double bond present. (An olefinic hydrogen (C=C-H) stretching is observed at a slightly higher frequency at 3.25-3.35 μ (3100-3000 cm^{-1}) (9).) Also as can be seen from Table 1, the absorptivities of the triglycerides with the same chain length are higher than those of the corresponding cholestryl esters. This is expected since the triglycerides would have more methylene groups than the cholestryl esters. Appendix B shows the calibration curves used for the calculation of the absorptivities in Table 1. The linear regression line used in the determination of the absorptivities is plotted.

It would be of interest to compare the absorptivities calculated for this infrared detector to any values present in the literature. Unfortunately these data do not seem to be available. Some data were found in Nelson (9) in the form of infrared spectra and from these spectra absorptivities were calculated using Beer's law. Table 2 shows the values calculated.

TABLE 1 ABSORPTIVITIES OF THE LIPIDS

	absorptivity (l/mol-cm)	chain legnth: #double bonds
cholesterol	101	0:0
cholestryl laurate	172	12:0
cholestryl myristate	214	14:0
cholestryl palmitate	226	16:0
cholestryl stearate	278	18:0
cholestryl oleate	280	18:1
cholestryl linoleate	197	18:2
cholestryl linolenate	170	18:3
trilaurin	192	12:0
trimyristin	225	14:0
tripalmitin	355	16:0
tristearin	514	18:0
triolein	465	18:1
trilinolein	441	18:2
trilinolenin	255	18:3

TABLE 2 LITERATURE VALUES OF ABSORPTIVITIES

	solvent	absorptivity (l/mol-cm)
cholesterol	CCl_4	530
cholestryl palmitate	CCl_4	938
tripalmitin	CCl_4	1920
cholestryl oleate	CS_2	1120

The data in Table 2 show higher absorptivities in all cases than found for the chromatographic detector. However in all cases the solvent used was different so no meaningful comparison can be made. Carbon tetrachloride and carbon disulfide will have a smaller absorbance than the mobile phase used here. Also different solvents will have different interactions with the compound of interest. Carbon tetrachloride and carbon disulfide could be used (though it isn't known if a separation would occur); however they weren't because of the safety considerations discussed in Chapter 4.

VI. DETECTION LIMITS

A calculation of the detection limits for each of the lipids used in the sensitivity study was done. The detection limit is calculated for signal-to-noise equal to 2 (by definition). The equation of a plot of the signal-to-noise ratio versus concentration is used. Appendix C shows the curves plotted (S/N vs. concentration and Table 3 the detection limits of the various lipids.

As was mentioned before, the detection limits for lipids are well below the concentrations needed for determination of lipids in blood.

VII. CONCLUSIONS

Only three classes of lipids were able to be determined using the chromatographic system described That is, cholesterol, triglycerides and cholestryl esters were determined. Fatty acids and phospholipids were not able to be determined using the present column and mobile phase. These compounds either appeared in the void volume or did not have sufficient absorbance in this particular mobile phase It was decided not to pursue the separation of these compounds since they are not normally determined in serum in hospital laboratories. The next step is to use a serum sample and determine the lipids in this sample using this system.

TABLE 3 DETECTION LIMITS OF THE LIPIDS

	mg/ml
cholesterol	0.1
cholestryl laurate	0.03
cholestryl myristate	0.02
cholestryl palmitate	0.02
cholestryl stearate	0.02
cholestryl oleate	0.01
cholestryl linoleate	0.1
cholestryl linolenate	0.1
trilaurin	0.02
trimyristin	0.02
tripalmitin	0.02
tristearin	0.02
triolein	0.03
trilinolein	0.01
trilinolenin	0.02

REFERENCES

1. Okazaki, M., Ohno, Y , and Hara,I. J. Biochem.(1981) *89*, 879.

2 Parris, N A., J Chrom (1978) *149*, 615.

3. Perkins, E G , Hendren, D.J , Bauer, J E , and El-Hamdy, A-H., Lipids (1981) *16*, 609.

4. Bezard, J A , and Ouedraogo, M A , J Chrom (1981) *196*, 279.

5. Horvath, C , ed , "High Performance Liquid Chromatography", vol I, Academic Press, New York, 1980, p 88

6 Payne-Wahl, K , Spencer, G F , Plattner, R D , and Butterfield, R O , J Chrom. (1981) *209*, 61.

7. Freeman, N K , Upham, F T , and Windsor, A A , Anal Lett (1973) *6*, 943.

8. Snyder, L R , and Kirkland, J.J , "Introduction to Modern Liquid Chromatography", 2nd edition, John Wiley and Sons, Inc , 1979

9 Nelson, G J , ed , "Blood Lipids and Lipoproteins· Quantitation, Composition and Metabolism", Wiley Interscience, New York, 1972, chapter 4

CHAPTER SIX

SERUM ANALYSIS

Blood is made up of cells (red blood cells, white blood cells and platelets) which are suspended in a fluid called plasma. Plasma is a mixture of inorganic and organic materials dissolved in water. Generally, an anticoagulant such as oxalate, citrate, ethylenediaminetetraacetic acid (EDTA) or heparin is added to the blood specimen for preparation of plasma. However, if no anticoagulant is added to the blood collected, and the sample is allowed to clot, the fluid separated is serum. Serum, therefore, lacks the protein fibrinogen, since the fibrinogen is transformed to fibrin in the formation of the clot (3-6% of the total plasma protein is fibrinogen). Approximately 93% of the plasma or serum is water. The remaining 7% are the solutes, mostly proteins (1).

I. THE SERUM SAMPLES

A serum sample, Lipid-Unitrol (Bio-Merieux, Syntex, Kanata, Ont.), was purchased. Lipid-Unitrol is a lipid control which has been assayed for use in the determination of cholesterol, total lipids, phospholipids and triglycerides. It is a bovine, elevated lipoprotein control serum and contains free cholesterol and esterified cholesterol in the normal range of human serum. Triglycerides are present at an elevated level. The sample is purchased as a powder (lyophilized) in a vial and is reconstituted with 3 ml of distilled water and is to be treated as a serum sample The lipids are dissolved with a swirling motion until all are dissolved. The serum sample is stable for 4 days at 2-8 °C.

A triglyceride control is also analyzed It is produced by the Societe quebecoise de biochemie clinique (Emitrol-1, normal triglyceride, and Emitrol-2, elevated triglyceride) and is used in the interlab

quality control program. It is a liquid bovine control and is treated in the same manner as serum. It was obtained at the Royal Victoria Hospital (Montreal, Quebec).

A third sample is analyzed. The Lipid-Trol sample, which is manufactured by the American Dade Co. (Canlab, Pointe Claire, Que), is also a lyophilized product. It is prepared from human blood with the addition of animal plasma lipoproteins to elevate the various lipid fractions. The sample is reconstituted with the addition of 3 ml of distilled water and is to be treated as a patient serum. The Lipid-Trol sample is found to have elevated triglycerides.

II. SAMPLE PREPARATION

Upon reconstitution of the serum sample (the same procedure is followed for the triglyceride control except there is no reconstitution), the sample is centrifuged for approximately 30 minutes in a bench top centrifuge (International Equipment Company, Boston, Mass.). Typically a 1 , 2-, or 3-ml pipetted aliquot is then removed and the lipids are extracted according to the method described in Nelson (2). The lipid extracted using 2:1 chloroform-methanol (v/v). (This solution also precipitates the protein present.) Twenty- five volumes of solvent are used per ml of serum. The exact procedure for a 1-ml aliquot is to deliver 8 ml of methanol into a beaker The serum is then added dropwise. Half the chloroform is then added (that is, 8 ml) and the solution is stirred for 5 minutes. The remaining 8 ml of chloroform is added and the solution left to stand for a further 5 minutes. The solution is then filtered using a Whatman No. 41 filter paper The above procedure is done in ice in order to decrease breakdown of the lipids. Initially the filtrate was collected in a volumetric flask and diluted to the mark with 2:1 chloroform-methanol. Separation was possible, however quantitation was not possible due to the large background absorbance of methanol

A number of other methods have been used for the extraction of lipids in plasma and serum samples. The Sperry and Brand method (3) described by Nelson (2) is certainly the most popular. However,

other solvent mixtures have been used. Examples are acetone (4), absolute alcohol and ethyl ether (1:1) (5), boiling alcohol-ether mix (3:1) (6), and acetone-absolute ethanol (1:1) (7). All of these solvents were injected onto the column, however, all had large absorbances. Therefore these were unsuitable.

Since some of the authors had used vacuum or steam evaporation, it was decided to rotary evaporate at room temperature in order to not cause lipid loss. The filtrate is collected in a 250 ml round bottom flask and the solution rotary evaporated at room temperature until there is no solvent remaining. The product is then redissolved in the chromatographic solvent.

III. THE SEPARATION

The serum sample can then be injected onto the column. The concentration of the lipids are determined by the standard addition method.

In the standard addition method, one measures a signal for the sample and then after addition of a known amount of standard to the sample, the signal is measured again This should be done at least twice in order to assure linearity. A calibration curve is prepared and an extrapolation to the horizontal axis is done which gives the concentration of the unknown. This method assumes a linear calibration curve and a good blank. This method also compensates for variations caused by physical and chemical interferences in the sample since the standard is subjected to the same matrix as the sample (8).

A number of aliquots of the serum sample are transferred to volumetric flasks One is diluted to volume (generally 5 ml) and in the others a known aliquot of a lipid standard is added. The absorbance is measured after separation on the chromatographic column A calibration curve is prepared and the unknown concentration in the serum sample can be determined by extrapolation of the line to the concentration axis. All the standard solutions are prepared in the same manner as previously described All

Sigma standards are listed as 99% pure and this purity is taken into account in the concentration calculations. The entire extraction procedure with rotary evaporation takes approximately 1-1 1/2 hrs.

IV. RESULTS

The Lipid-Unitrol sample was treated as described above. Cholesterol, cholestryl esters and triglycerides were separated on the chromatographic column as expected from analysis of purchased lipids. The standard addition method was applied and calculation of the serum concentration was done. Cholesterol concentration was calculated to be 2 99 g/l (Fig. 1) after taking into account the fact that the extraction method yield was 95% The correct cholesterol concentration listed in the Lipid-Unitrol specifications is 2 77-3 25 g/l Hence the analysis yields results which are correct for cholesterol

However, after similar calculations for triglycerides (using tripalmitin as the standard in the standard addition method), the results were consistently too low when compared to the results listed in the specifications.

Upon consideration, it was realized that the triglyceride present in serum is not one triglyceride alone but made up of a mixture of many triglycerides If the concentration of triglyceride was .002 molar (which is the approximate molar concentration of triglyceride in Lipid-Unitrol), then knowing the absorptivity (Chapter 5) and the pathlength, one can calculate the absorbance of the solution using Beer's law. If the triglyceride is assumed to be only tripalmitin, the absorbance would be 0.71. However, we know that the triglyceride in serum is not only tripalmitin. If we assume that the triglyceride was a 50-50 mixture of tripalmitin and triolein, the absorbance is calculated to be 0.824, an increase in absorbance of approximately 16% Therefore the actual fatty acid composition of the triglycerides present in serum is important in this determination. That is, we cannot simplify and use any one triglyceride as a standard. This argument would also apply to cholestryl esters.

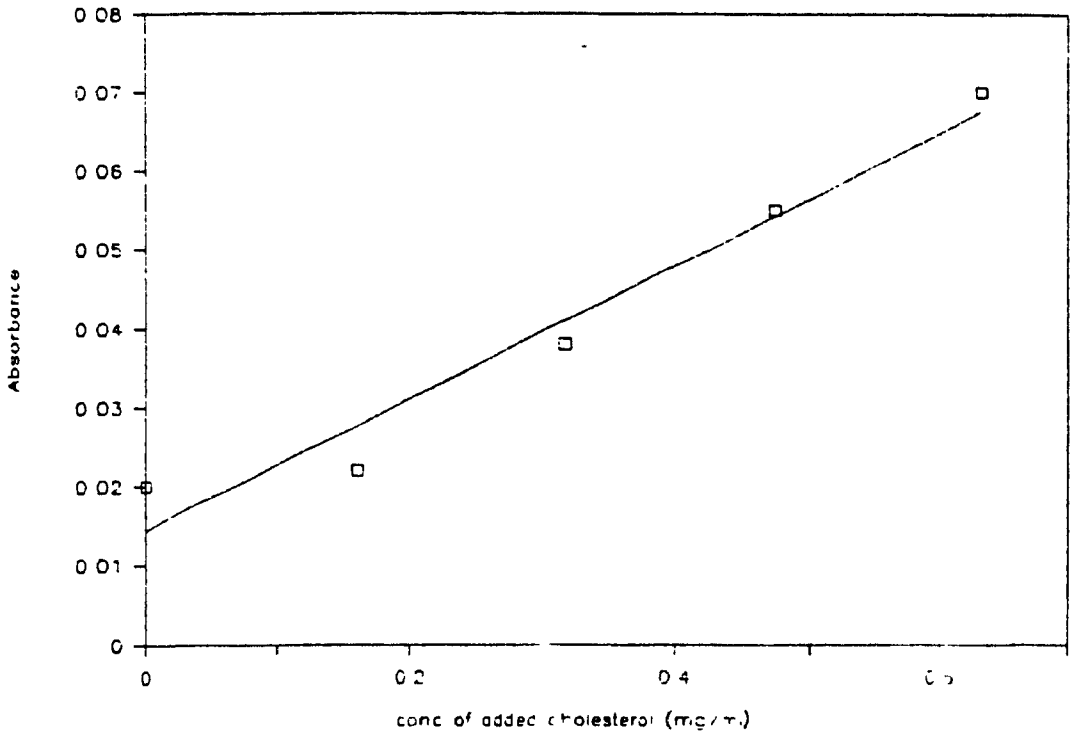


FIG 1 Calibration curve for cholesterol in Lipid-Unitrol

This conclusion was confirmed upon a review of the literature. Numerous studies have been performed in order to determine the fatty acid composition of cholestryl esters, triglycerides, phospholipids and free fatty acids (9,10). It is believed that the fatty acid composition of the various lipids vary due to diet as well as medical health conditions such as heart disease and the hyperlipidemias. The fatty acids present in plasma vary depending on whether the diet is high in saturated or unsaturated fats (11) and therefore it could be assumed that the fatty acid composition of the other lipids will vary to some extent depending on diet as well. To further complicate matters, there appears to be some difference in fatty acid composition of the lipids as well as overall lipid levels depending on age (9).

All this notwithstanding, an approximation of the mixture of triglycerides and cholestryl esters present in serum was made to be used in the standard addition. A mixture of the 6 major triglycerides was used (despite the fact that there are many more than that present in serum, though in very small proportion): 7.5% trimyristin, 31.3% tripalmitin, 5.3% tristearin, 32.7% triolein, 6.1% trilinolein, 17.1% trilinolenin. The 6 major cholestryl esters were also used in the following proportions: 10.4% myristate, 7.8% palmitate, 6.2% stearate, 21.5% oleate, 49.0% linoleate, and 5.2% linolenate.

Using these mixtures, triglyceride was calculated to be 2.38 g/l (Fig. 2) within the range quoted for Lipid-Unitrol (2.34-2.86 g/l) Cholestryl esters were calculated, in spite of the fact that no values are given other than the information that cholestryl esters are within the normal range for human serum. Cholestryl esters were evaluated to be 3.47 g/l (Fig. 3). The cholestryl ester values given in Schrade et al. (9) for healthy persons was 2.0-2.4 g/l and in Guidry (12) the range was 2.13-2.36 g/l.

The Royal Victoria triglyceride control is analyzed and the Emitrol-1 (normal triglyceride level) was determined to be 1.06 g/l (Fig. 4). The correct range for this sample was 0.9-1.01 g/l as determined by the hospital. The Emitrol-2 (elevated triglyceride level) sample is determined to be 2.07 g/l (Fig. 5). The hospital has determined it to be 1.78-1.93 g/l.

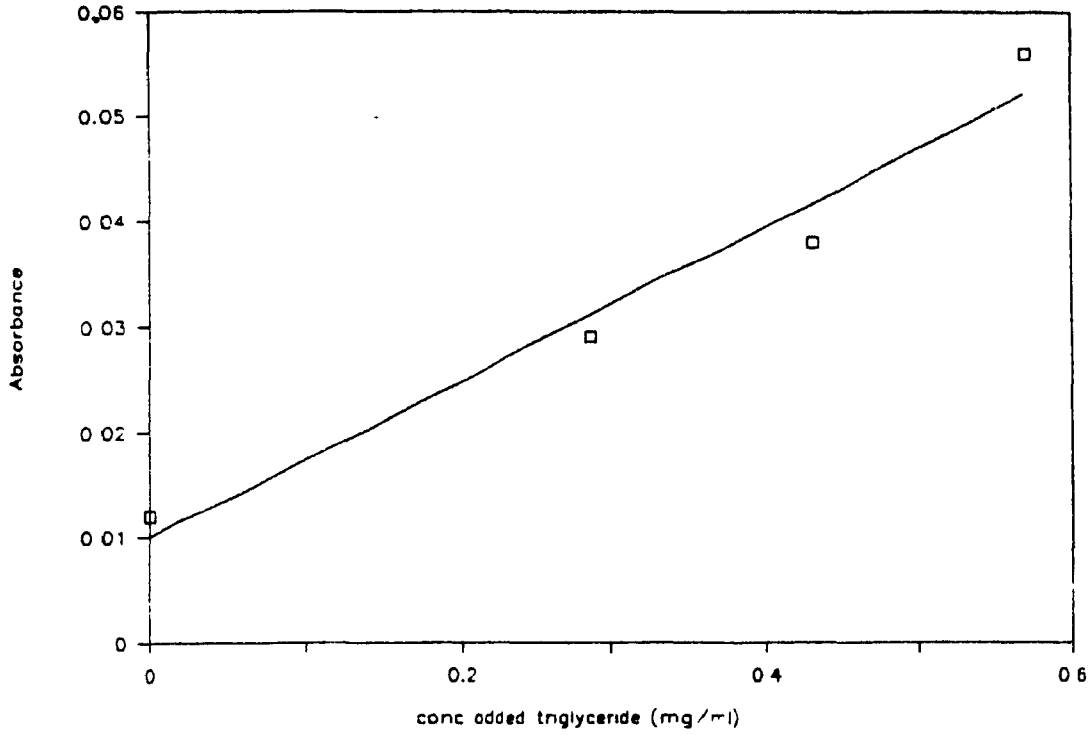


FIG 2 Calibration curve for triglycerides in Lipid-Unitrol

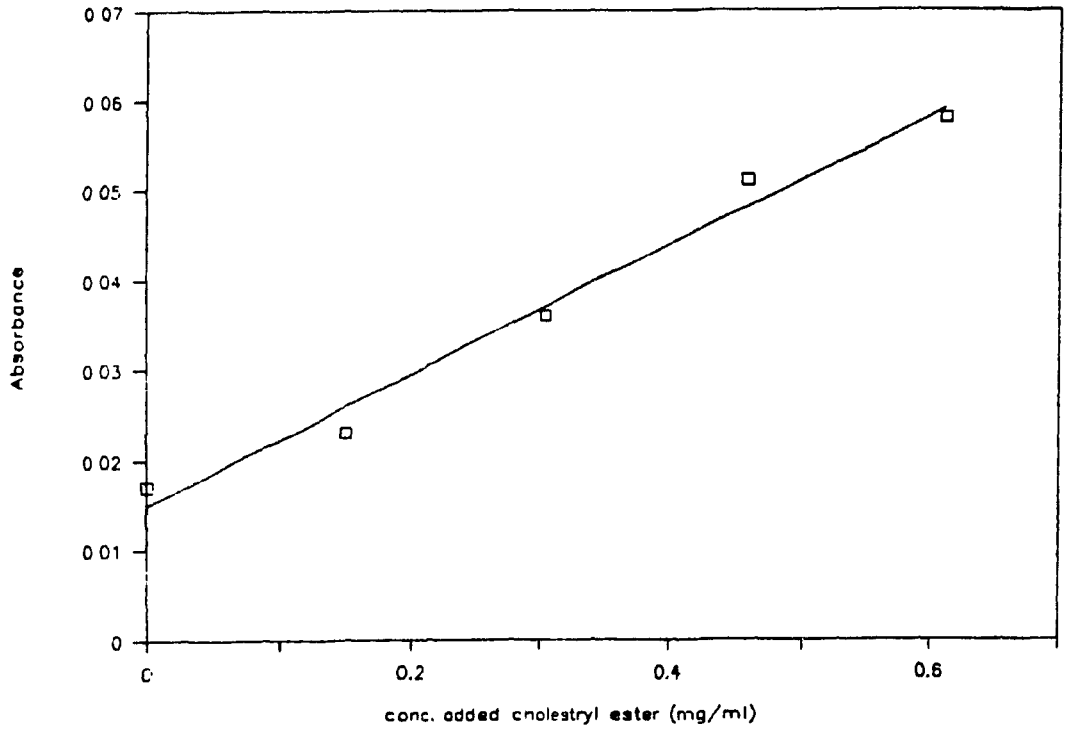


FIG 3 Calibration curve for cholestryl esters in Lipid-Unitrol

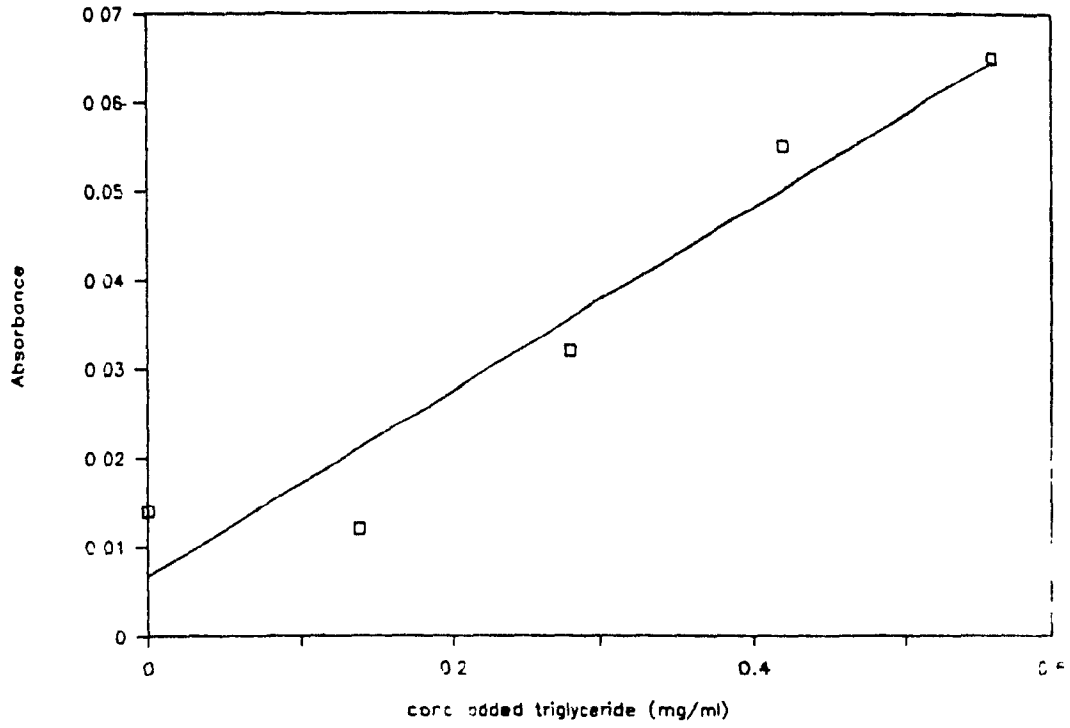


FIG 4 Calibration curve for normal triglyceride control

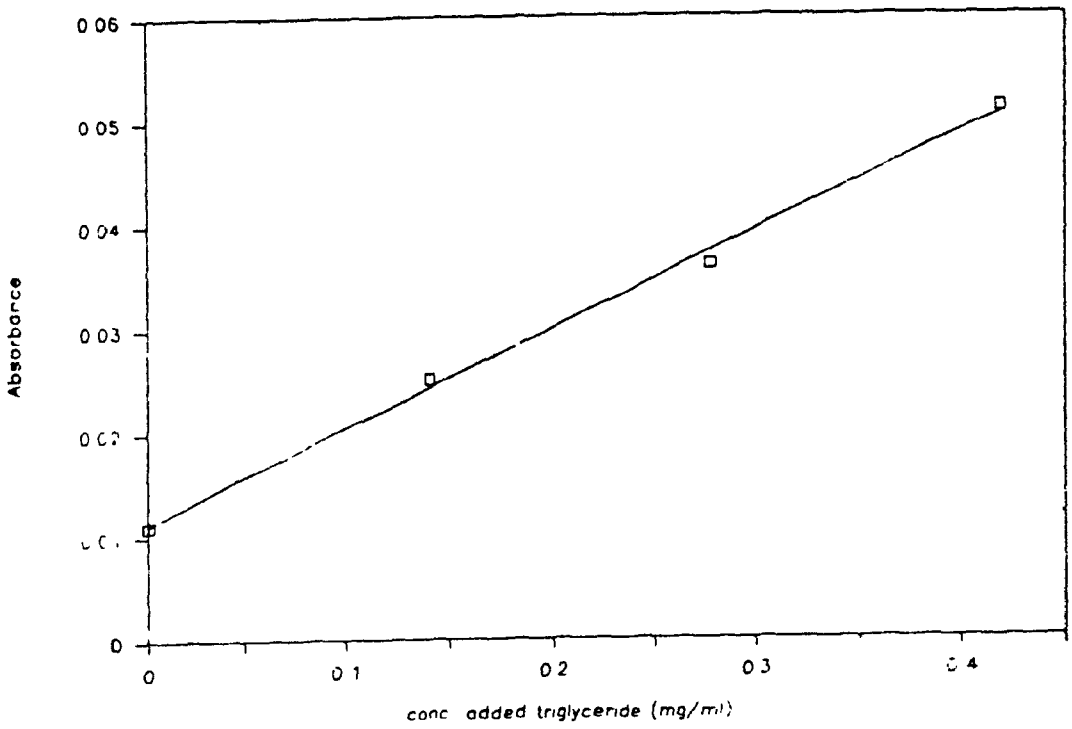


FIG 5 Calibration curve for elevated triglyceride control

The Emitrol-1 and -2 samples are analyzed by individual laboratories and then the results are sent to the Societe quebecoise de biochemie clinique and the results analyzed throughout the province. The average values for the triglyceride controls for over 80 hospitals are compiled. Hence the hospital results are only correct for their individual system. The correct range determined for Emitrol-1 was 0.89 -1.09 g/l and for Emitrol-2 was 1.75 to 1.97 g/l. Hence there is a difference between the hospital range and the overall range. Serum samples were analyzed for cholesterol and triglycerides by a minimum of 2 different methods and as many as 4 different methods.

A third sample, the Dade serum sample Lipid-Trol, was analyzed. The triglyceride was determined to be 3.25 g/l (Fig. 6) which is in the correct range listed in the specifications. The extra analyses of triglyceride in the control and the Dade sample was done since it was the triglyceride (and also the cholestryl ester) which had caused some problems initially. Further analyses were done for confirmation.

V. COEFFICIENT OF DETERMINATION (1)

In order to compare different sets of data or methods, one uses the coefficient of determination (also called the coefficient of variation). The coefficient of variation is the standard deviation divided by the mean times 100. This allows one to compare the results of two different experiments or methods since means cannot be compared directly because all experiments and instruments have different standard deviations Means and standard deviations cannot be compared directly since they may be expressed differently. Table 1 (expanded from the product literature) shows the coefficient of variation for the different methods used to analyze the serum samples The specifications provided by the companies that produced the serum samples included the means and the standard deviations so that the coefficient of

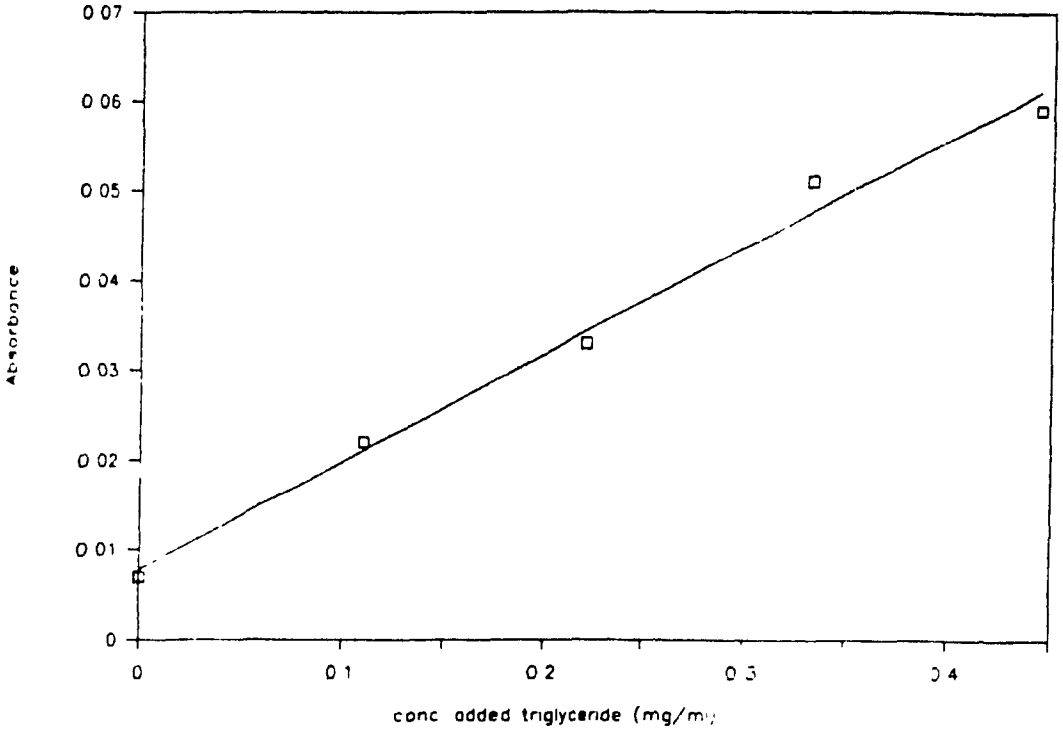


FIG 6 Calibration curve for triglycerides in Lipid-Trol

TABLE 1 COEFFICIENTS OF DETERMINATION FOR TRIGLYCERIDES IN THE SERUM SAMPLES

	mean (g/l)	standard deviation	coefficient of determination
Lipid-Unitrol			
1. Hantzsch	2.23	0.14	6.28
2. enzyme-UV	2.46	0.13	5.28
3. enzyme-PAP	2.60	0.13	5.00
Lipid-Trol	(mg/dl)		
1. colorimetric	291	6.0	2.06
2. enzymatic (Abbott)	322	8.1	2.52
3. Boehringer-Mann	286	9.8	3.43
4. DuPont	286	6.2	2.17

variation can be determined and then compared to that for the infrared detector used here. All of the methods used by Syntex and American Dade Co. are absorption determinations (colorimetric or ultraviolet absorption reactions). The Hantzsch reaction involves the extraction of the lipids followed by hydrolysis of the triglyceride to formaldehyde using sodium metaperiodate. Reaction of the formaldehyde with ammonium acetate and acetylacetone (Chapter 1) is followed by determination at 412 nm. The enzyme-UV and enzyme-PAP method used by Syntex in the determination of triglyceride in Lipid-Unitrol is not described, however it is likely similar to the enzymatic method described by Tietz (1). This reaction involves the alkaline hydrolysis of triglyceride using ethanolic potassium hydroxide. A solution of enzyme, NADH and ATP is added with the absorbance measured at 340 nm. The colorimetric method used by Dade is the same Hantsch reaction used by Syntex. The Abbott and Boehringer-Mannheim systems are simply described as enzymatic quantitative determinations of triglyceride (very likely a variation on the enzymatic determination described above or one of the many described in Chapter 1). The DuPont system is described as an enzymatic procedure with the formation of NADH measured at 340 nm. Hospital laboratories use automated systems similar to those described here depending upon which instrument is actually used.

The coefficient of variation is 12.6% for the infrared detector. Six determinations of the same sample are done on the same day. The coefficient of variation on a day-to-day basis is determined to be 18.2%. It is expected that there is less precision on a day-to-day basis.

The 12.6% determined for this detector is 2-6 times worse than the methods quoted in the specifications. This is expected since these are well known tried and true techniques.

VI. CONCLUSION

The serum samples are analyzed using the HPLC infrared detector. The triglyceride and cholesterol values are within the range quoted as correct. Any differences are due to the differences in standard deviations and technique which is the reason all the purchased serum samples give a number of different "correct" values using a number of different techniques.

The infrared detector is not as sensitive as any of the well-known commercial HPLC detectors. Despite this it has the advantage that it can detect all classes of lipids. (One would expect the DuPont or Foxboro LC detector to be able to detect all classes of lipid since it is also an infrared detector.)

This detector also has the advantage of being non-destructive, which all of the automated methods (usually involving alkaline hydrolysis or periodic oxidation) are not This is important since the original conception of the detector was to build a detector which could detect all classes of lipids and then if necessary these classes could be shunted to a second column and separation within the class could be done using a reverse phase column.

Future work would include double-beaming the instrument to compensate for the laser wander and the absorption of the mobile phase. (A commercial infrared helium-neon laser would also be purchased since commercial lasers have longer lifetimes and hopefully more stable output.) This would certainly improve the coefficient of variation.

In addition, a chromatographic system would be developed so that all classes of lipid could be separated including the fatty acid and phospholipids This could involve the use of a gradient Gradient elution liquid chromatography using an infrared detector has been done by Parris (13) The infrared detector has a clear advantage over the refractive index detector since gradient elution is not easily done with refractive index detectors.

Also a recent survey of the product literature involved the discovery of a flow cell constructed of Infrasil Quartz (Hellma Cells, Toronto, Ontario) which is expected to give less absorption of light at 3.39 μ. It has the same dimensions as the Suprasil Quartz cell used; however, it should absorb less of the laser radiation before the laser light reaches the chromatographic effluent.

REFERENCES

1. Tietz, N.W., ed., "Fundamentals of Clinical Chemistry",2nd edition, W. B. Saunders Co., Philadelphia, PA, 1976, p. 298

2. Nelson, G J., ed., "Blood Lipids and Lipoproteins·Quantitation, Composition and Metabolism", Wiley Interscience,New York, 1972

3. Sperry, W.M , and Brand, F C , J Biol Chem ,(1955) *213*, 69

4. Gorter, E , and Grendal, F , J Exp Med (1925) *41*, 439

5. Folch, J , and van Slyke, D D , J Biol Chem (1939) *129*, 539

6. Christiansen, H N , J Biol Chem (1939) *129*, 531

7. Sperry, W M , and Webb, M , J Biol Chem (1950) *187*, 97.

8 Skoog, D A , and West, D M , "Principles of Instrumental Analysis", 2nd edition, Saunders College/Holt, Rinehart and Winston, Philadelphia, PA, 1980, p 325

9 Schrade, W , Biegler, R , and Bohle, E , J Atheroscler Res (1961) *1*, 47

10. Nikkari, T , Salo,M , Maatela, J , and Aromaa, A , Atherosclerosis, (1983) *49*, 139

11. Fasman, G D , "CRC Handbook of Biochemistry and Molecular Biology Lipids, Carbohydrates and Steroids", 3rd edition, The Chemical Rubber Co , 1975

12 Guidry, M A , and Mendez, J , Circulation (1965) *32*, 106

13 Parris, N A , J Chrom Sci , (1979) *17*, 541.

APPENDIX A. LASER SAFETY (1)

The first step in laser safety is to establish into which class the laser falls. This is determined by the wavelength, power output and length of pulse (for pulsed lasers). In the case of continuous wave (CW) lasers such as very common 6328 angstrom helium-neon laser a Class I laser has a power output of less than or equal to 1 microwatt; a Class II laser has power greater than or equal to Class I but less than or equal to 1 milliwatt; Class III laser's power is greater than that of Class II, but has power of less than or equal to 0 5 watts; and a Class IV laser's power is greater then 0.5 watts. Depending upon the class of the laser (any laser safety book will explain how to classify a laser), an appropriate sign is placed on the door to the laser area Class I lasers are considered to be low-power lasers and require only a CAUTION sign with the proviso "Do not stare directly into the beam". Class II lasers (only visible lasers can be Class II) are considered medium-power lasers and also require a CAUTION sign with the proviso: "Do not stare into beam Never view through optical instruments." Class III lasers are medium-pow. . lasers and require a DANGER sign with the proviso "Avoid direct exposure to beam " Class IV lasers ar high-power lasers requiring a DANGER sign and the proviso: "Avoid eye or skin exposure to direct or scattered radiation."

It can be seen that as the laser's power output increases, the precautions to be taken become increasingly stringent, for example, for the Class IV laser, eye and skin exposure is dangerous even after diffuse scattering of the light. The American National Standards Institute (ANSI) Z 136.1 (1980) (2) states: eyewear SHOULD (the word should is to be understood as advisory) be used for Class III lasers and protective eyewear SHALL (the word shall is understood to mean mandatory) be used for Class IV lasers. Particular caution should be taken with invisible lasers.

The necessary safety considerations can be broken down into three different categories: personnel, engineering, and electrical.

1. PERSONNEL SAFETY

Personnel safety involves ensuring that the laser user and all other laboratory workers are also safe. Personal safety concerns eye, skin, and inhalation precautions. Eye protection can take the form of wearing glasses or goggles that offer adequate protection for the type of laser being used, i.e., the wavelength of output and the power output. Laser goggles should be labeled for the wavelength and the laser for which they are to be used and should not be used for any other wavelength or laser. Many people erroneously think that using any pair of laser goggles is better than using nothing at all In most cases indiscriminate use of goggles affords no protection since goggles generally have a very narrow band of protection and offer no protection at all at wavelengths other than those for which they were designed A real danger results when a person wearing the wrong pair of goggles thinks he or she is adequately protected and then looks at the beam.

Eyewear should be inspected periodically for cracking, and discoloration. The frame should be inspected for mechanical integrity and the eyewear should be examined for light leaks that would permit hazardous intrabeam viewing. (Intrabeam viewing is the condition resulting from loss of integrity whereby the eye is exposed to all or part of a laser beam)

Particular care should be taken when using a laser to align optics. For this task, eyewear is usually not practical since the laser beam must be seen. It is usually recommended that laser work be done in a well-lit room so that pupil size is its smallest; therefore, if a beam does hit the eye, there is minimal chance for damage. Since alignment of optics is often done in the dark, this task requires particular care.

Few accidents have occurred as a result of eye exposure to a laser, but most of the exposures that did take place did so because personnel were not wearing protective eyewear Of seven accident cases surveyed (3), six victims were not wearing eyewear and one was, but it had slipped The importance of protective eyewear cannot be overstressed. Glasses or goggles must fit properly and comfortably so that they will do the job for which they were designed. (Prescription safety glasses or goggles are available.

Laser workers requiring corrective lenses should purchase these, or make sure that regular goggles fit comfortably over prescription glasses.)

The American National Standards Institute (ANSI) and Air Force Occupational Safety and Health (AFOSH), as do most of the safety manuals and books, suggest an eye examination before starting laser work, at periodic intervals during work, and upon completion of laser work. AFOSH (4) suggests taking an ocular history, a best corrected distant visual acuity (including cycloplegic refraction, if indicated), an Amsler grid examination, and funduscopic and slit lamp examinations. ANSI has slightly different requirements, but ANSI and AFOSH tend to agree on most points. Most ophthalmalogists include most of the points listed above in any thorough eye examination, so there should be no difficulty getting a proper examination.

In addition to eye precautions, skin protection must be used for high-power lasers and some ultraviolet lasers. Care should be taken if the laser worker is using prescription medication that causes sensitivity to light, since the worker may be especially susceptible to eye or skin damage. Also, the worker's medical and ocular history should be examined for anything that would cause abnormal sensitivity to laser light.

Some lasers produce noxious products; thus ventilation must be adequate. Also, very large capacitors on high voltage power supplies can produce a very loud noise upon discharge, necessitating the use of ear protection.

With some solid-state lasers, such as a ruby laser, the method of producing the population inversion to obtain lasing is a flashlamp, which can produce a very bright flash that can be dangerous. In such cases the laser goggles may not be useful since the flashlamp is usually at a different wavelength than the laser output

One last precaution is to remove all jewelry - watches, rings, etc. - to eliminate any chance of hazardous reflections. (It is also advised that workers not wear any metal when working with high voltages, which is invariably the case with lasers.) It is a good idea to make a habit of removing all jewelry in the morning before starting work and then putting it on again before leaving for the day.

To guarantee everyone's safety, other precautions that should be taken include the installation of curtains, protective housing and warning lights. Warning lights are advised for all lasers and these should be turned on when the laser is on. Red lights are standard and can be connected to the power supply so that the warning lights come on when the power supply is turned on. Most safety manuals suggest using curtains on windows to prevent radiation from escaping. The CRC Handbook of Laboratory Safety (5) suggest heavy black felt drapes as a possible material for the curtains. As long as the curtains are fireproof and opaque, they will be effective in containing radiation. Depending on the wavelength of laser output, other materials may work, such as plastic garbage bags, which are convenient and cheap and are useful, particularly if the laser is only being used for a short period of time.

The laser should not be at eye level for personnel who are sitting or standing, and therefore should be higher than six feet or lower than three feet to minimize the risk of eye exposure.

Specular scattering (scattering off mirror-like surfaces) can be dangerous, so it is advisable to paint all metal surfaces flat black to reduce dangerous reflections. In addition, there can be hazardous reflections off the paint on the walls depending on the wavelength of laser output (6)

A protective housing made of a material that will absorb the laser output is useful since it reduces the danger to the user as well as to other workers. Permanent protective housing will reduce the class of the laser because of the reduction in power output.

2. ENGINEERING SAFETY

Engineering controls can include protective housing and warning lights, safety interlocks and locked doors, and fire extinguishers. Most laser laboratories should be kept locked so that no one can wander in and be inadvertently exposed to laser light. Locking up is a good idea if there is more than one person working in the laboratory. However, anyone working alone faces a problem. Either the door is locked, keeping everyone out for their safety, or the doors are left unlocked so that the person working alone can call for help, or at least not be locked in if help is needed.

Safety interlocks (keys etc.) are suggested so that no unauthorized personnel can turn on the laser. Most commercial lasers usually have some sort of safety interlock. Since some high-power lasers can, because of their high energy, cause ignition, a fire extinguisher should be handy, and all flammable objects should be removed from the beam's path. In chemistry laboratories in particular, all flammable chemicals should be removed.

3. ELECTRICAL SAFETY

Electrical safety is the last category of laser safety. Safety precautions in this category include making sure that the laser user is properly insulated through use of an insulating mat (since all floors are considered conductive), and making sure one's hands are dry and not perspiring (using insulating or lineman's gloves). The electric company branch in most towns would have information on where to purchase these gloves. Best's Safety Directory (7) has a great deal of useful information on where safety products can be purchased

As mentioned previously, no jewelry or metal should be worn when working with the laser. As in all high voltage situations, the "one-hand" rule applies: that is, use one hand only and make sure the other hand is not touching anything that is conductive which could make a complete current path through you;

putting one hand in a pocket is a good idea. An electrical shock causes the muscles to contract. Using the palm to grab something may cause an electric shock that causes the hand to clench, and if the power is high enough, one cannot let go. Using the back of the hand reduces the possibility of being left clenching a live wire.

If someone is found unconscious near a laser, assume the worst and call for help immediately. Always check to make sure that the person is disconnected from the power supply; if he or she is not, make sure the power supply is OFF. Once the person has been disconnected from the high voltage, begin standard first aid procedures. Check for breathing and start artificial respiration if necessary. Then check for a pulse, and, if necessary, begin cardio-pulmonary rescusitation (CPR). Someone in the laboratory should be trained in first aid and in CPR, since cardiac arrest is the most drastic consequence of high voltage accidents. In addition, every worker should know the number to call for help, both within and outside of the institution. It is important to neither work alone with a laser and high voltages, nor to work when overly fatigued, since this is when accidents are most likely to occur.

Several accidents have occurred involving laser workers. Of those accidents involving eye damage, all victims have recovered with either some or no permanent damage. High voltage has permanent effects - it can kill.

4. SUMMARY

Lasers should be treated with respect since they can, when carelessly used, be very dangerous. Safety is important, but it need not be taken to extremes to be effective.

The safety steps taken into consideration with the use of the infrared helium-neon described above were: determination of the class of the laser. The laser was a class III laser and hence the appropriate DANGER sign was placed on the doors leading into the room where the laser would be used. Laser safety

goggles appropriate for the particular wavelength and expected power output of the laser where purchased (Optikon Corp., Waterloo, Ontario). An eye examination was performed by an ophthmalogist. Curtains in a fireproof and opaque material were purchased (Draperies Suzanne Fournel Enr., St. Vincent de Paul, Quebec). Warning lights were placed over the 2 doors leading into the room. Everything placed on the optical bench was painted flat black (if this was feasible). The 2 doors to the room were left unlocked and a fire extinguisher was placed in the immediate vicinity of the laser. An insulating rubber mat for the floor was purchased (Union Electric, Montreal, Quebec) as were lineman's gloves (Safety Supply Co., Montreal, Quebec). The optical bench was grounded and one of my collegues learned cardio-pulmonary rescusitation (CPR) I made sure never to work alone in the lab.

REFERENCES:

1 Stokl, C , Amer Lab , (1984) *July*, 67

2 ANSI Z 136 1 "American National Standard for the Safe Use of Lasers", American National Standards Institute, Inc , 1430 Broadway, New York, N.Y , 10018, 1980, p 13

3 Boldrey, E E , National Safety News, (1981) *Oct* , 31

4 AFOSH Std 161-10, "Health Hazards Control for Laser Radiation", Dept of the Air Force, Washington, D C , 20330, 1980

5 Steere, N V , ed , "CRC Handbook of Laboratory Safety", The Chemical Rubber Co , Cleveland, 1971, p 381

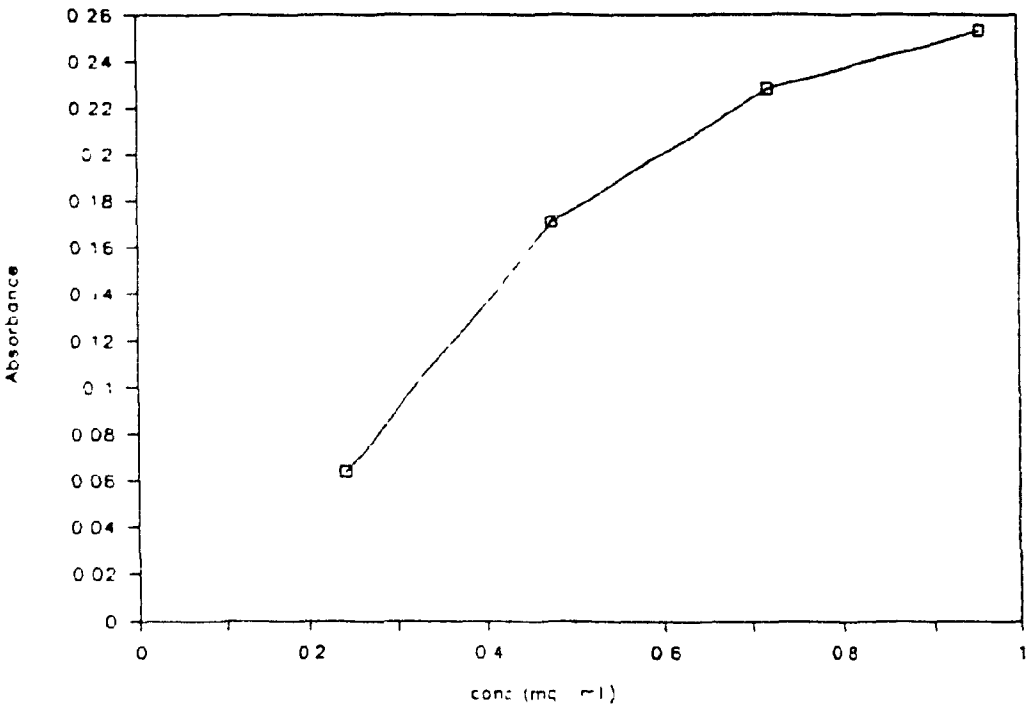
6 Ullrich, O A , and Evans, R M , Battelle, Columbus Labs , Columbus, Ohio 43201, 1981

7 Best's Safety Directory, 17th edition, A M Best Co , Inc , Oldwick, N J , 1977

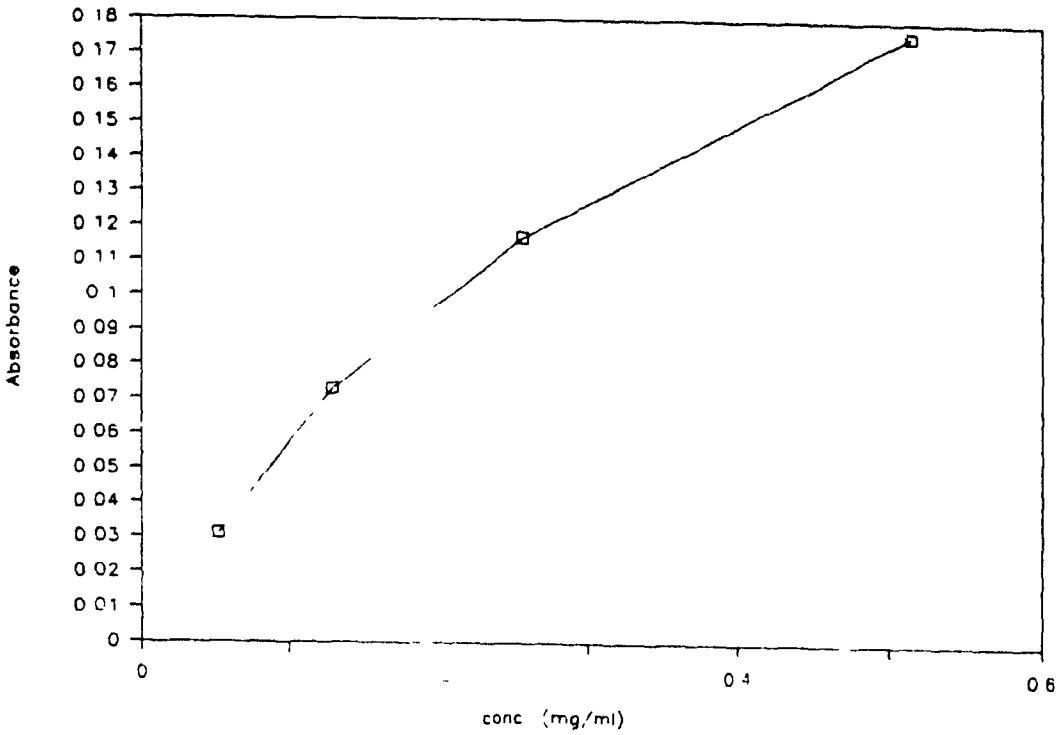
APPENDIX B

The calibration curves (Absorbance vs. concentration) for all the lipids which were used for the calculation of absorptivities in Table 1 in Chapter 5.

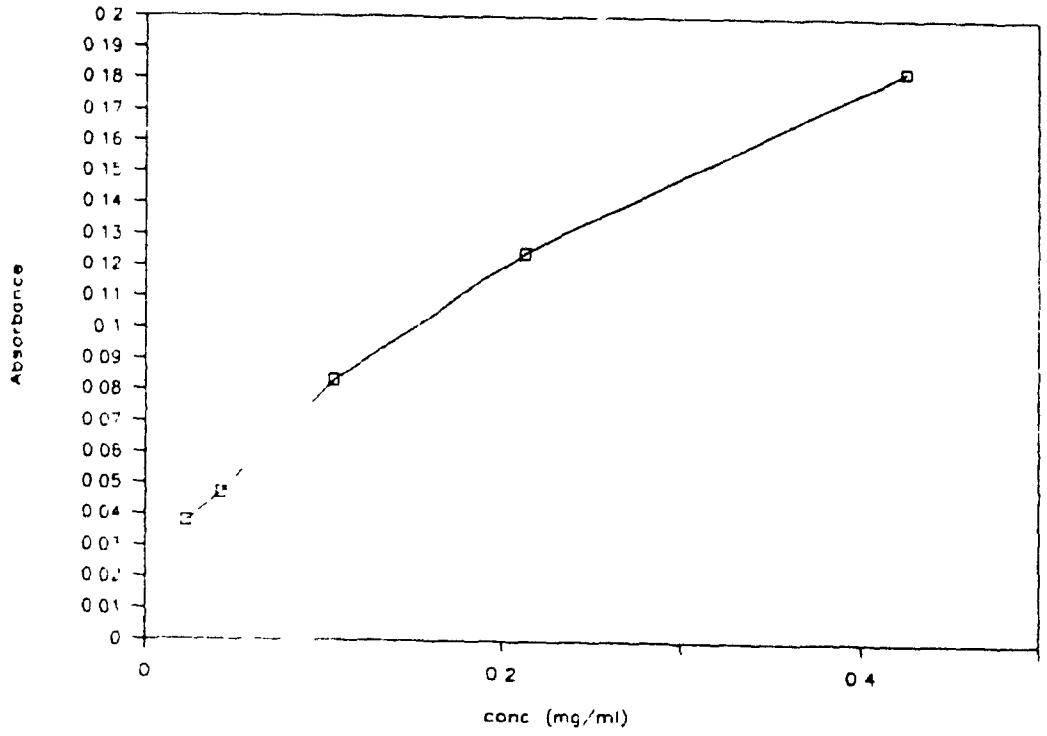
	correlation coefficient	intercept (mg/ml)	absorptivity l/mol-cm
cholesterol	0.920	.0230	101±20.9
cholestryl laurate	0.967	.0277	172±22.3
cholestryl myristate	0.980	.0371	214±17.8
cholestryl palmitate	0.959	.0517	226±33.0
cholestryl stearate	0.965	.0342	278±30.7
cholestryl oleate	0.998	.0392	280±17.3
cholestryl linoleate	0.981	.0375	197±15.9
cholestryl linolenate	0.980	.0979	170±12.3
trilaurin	0.961	.0306	192±22.4
trimyristin	0.985	.0801	225±16.1
tripalmitin	0.989	.0381	355±18.6
tristearin	0.997	.0350	514±16.4
triolein	0.970	.0267	465±47.1
trilinolein	0.983	.0481	441±29.2
trilinolenin	0.974	.0435	255±17.0



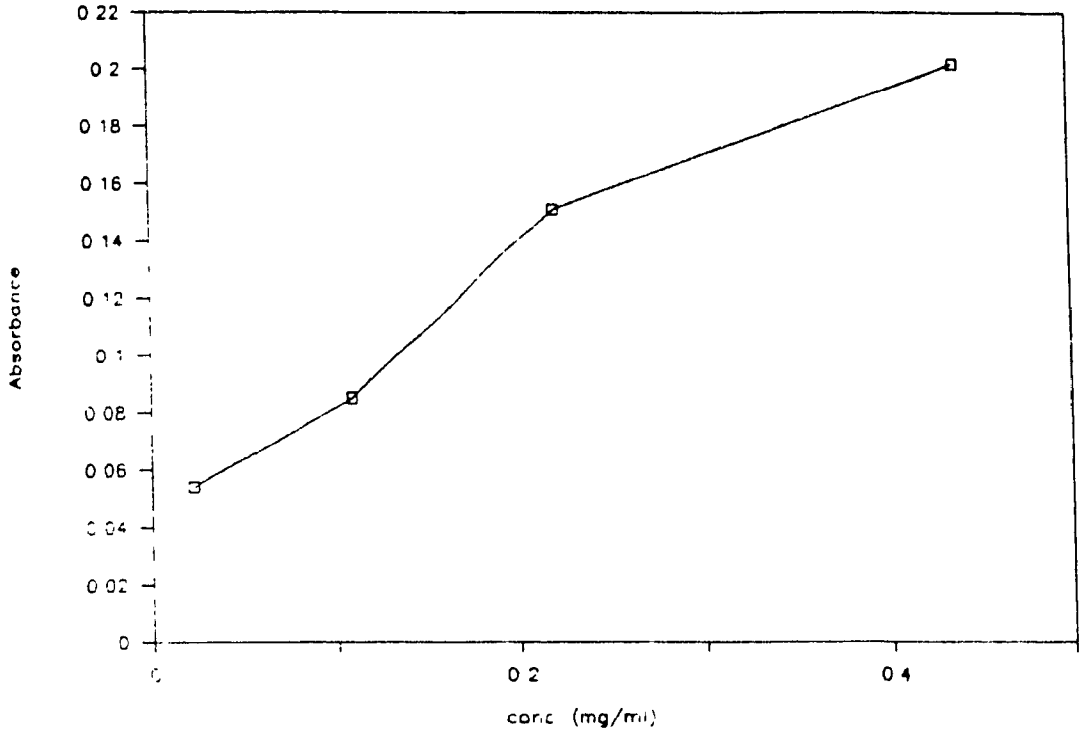
Calibration curve for cholesterol



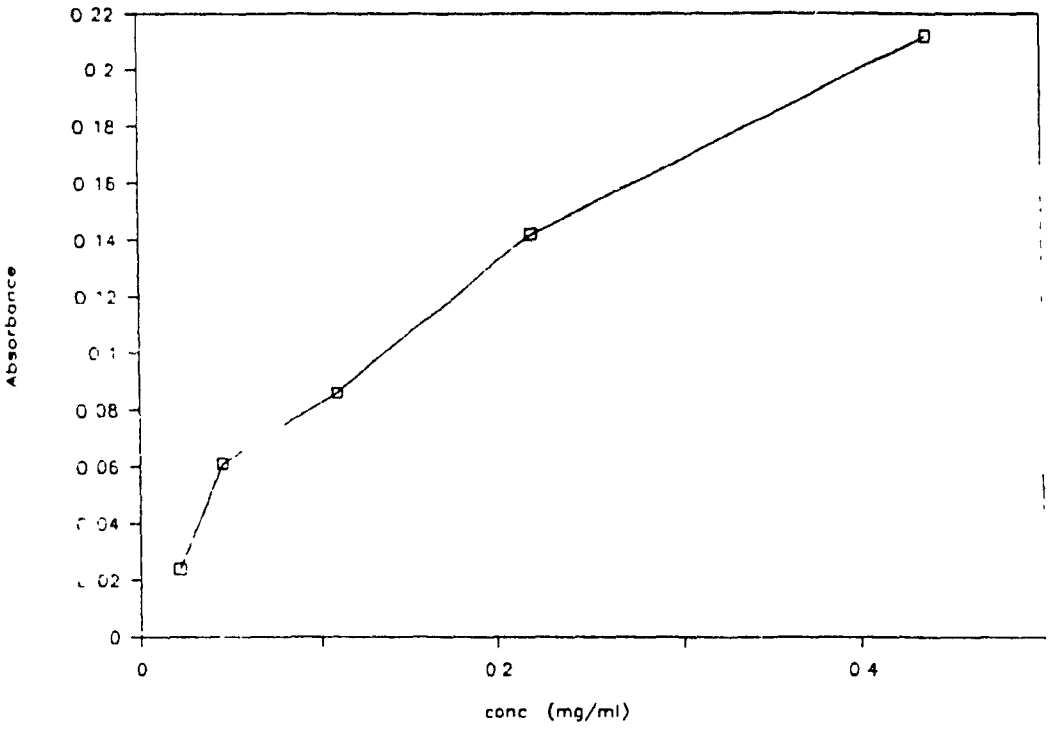
Calibration curve for cholestryl laurate



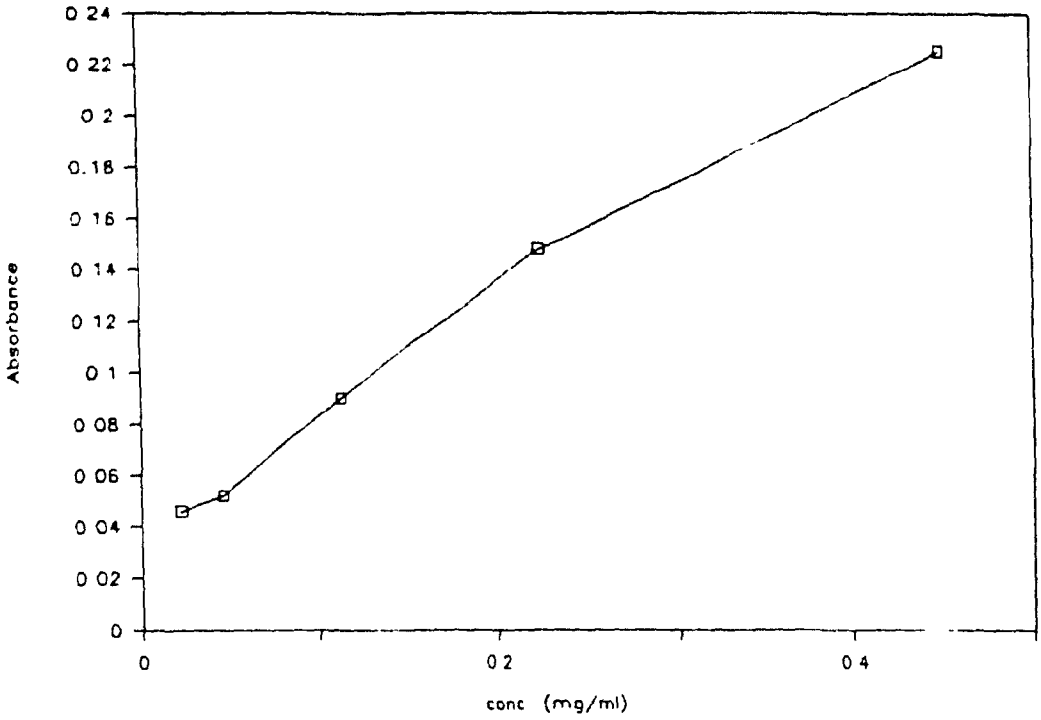
Calibration curve for cholestryl myristate



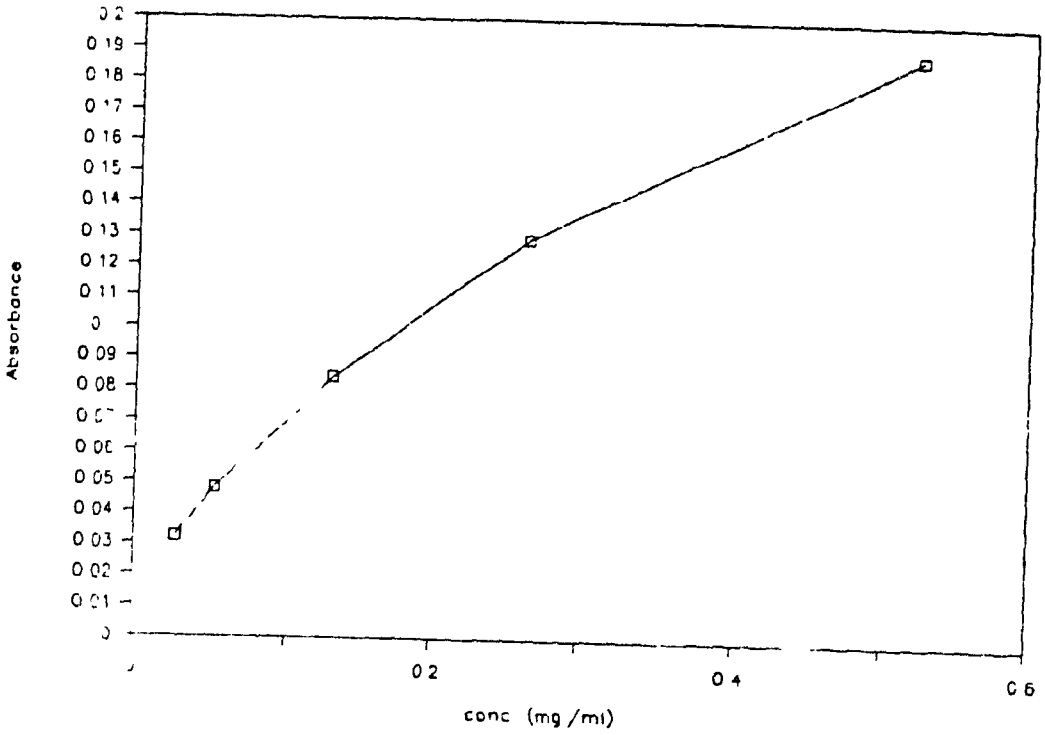
Calibration curve for cholestryl palmitate



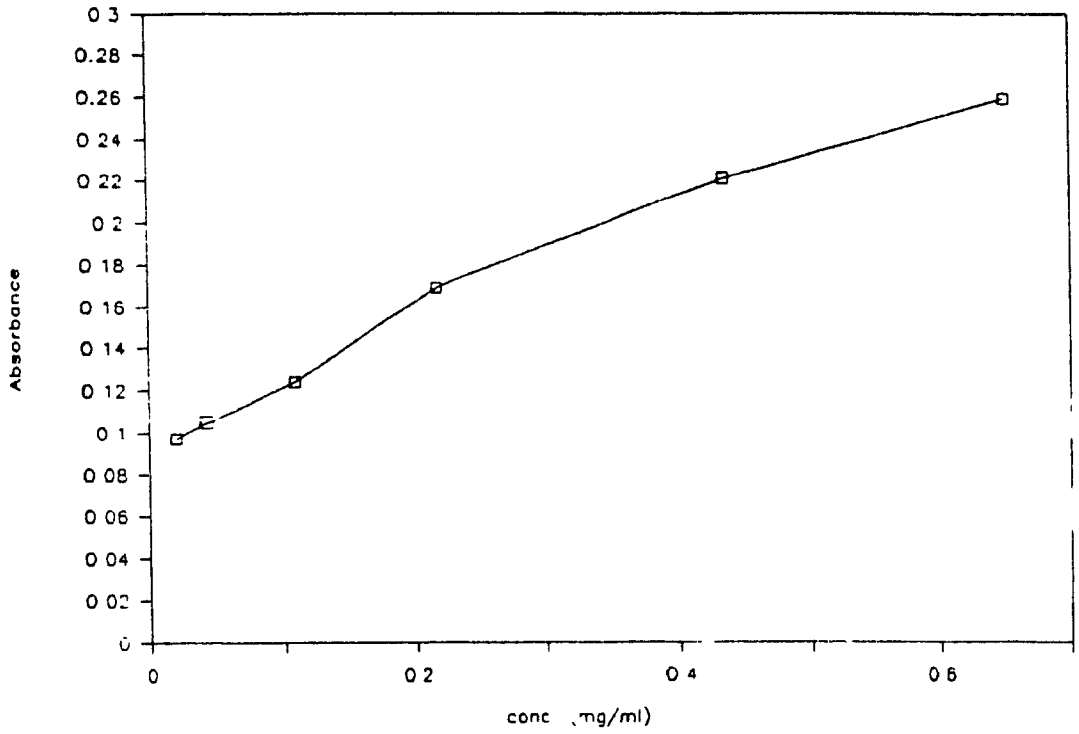
Calibration curve for cholestryl stearate



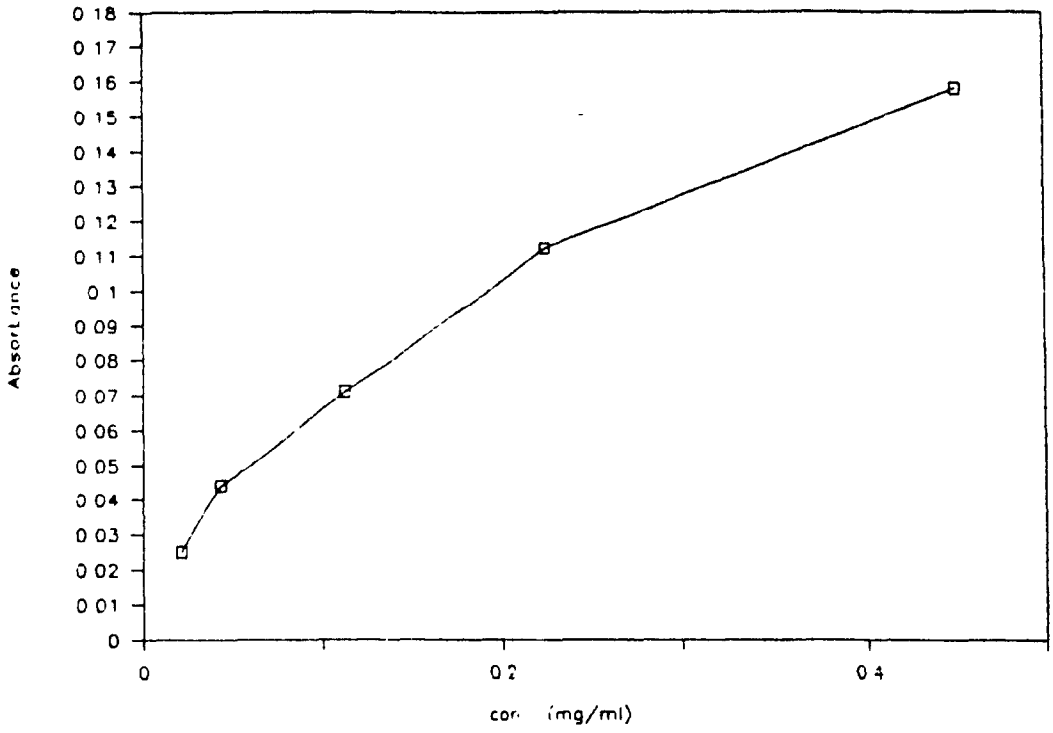
Calibration curve for cholestryl oleate



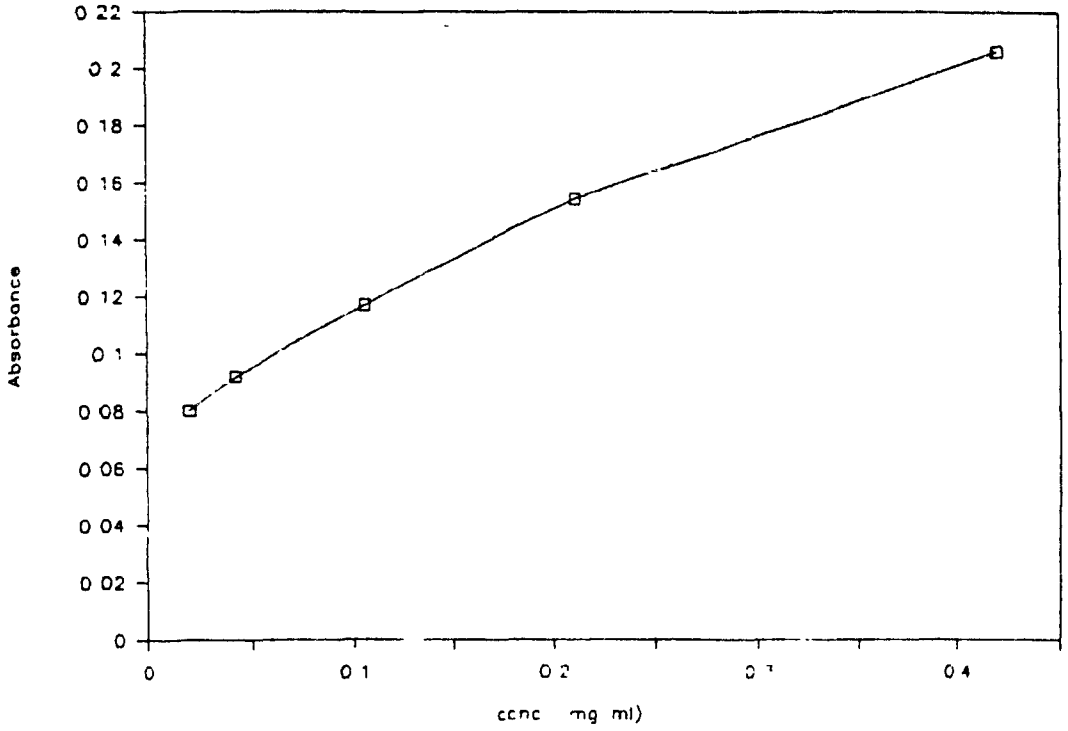
Calibration curve for cholestryl linoleate



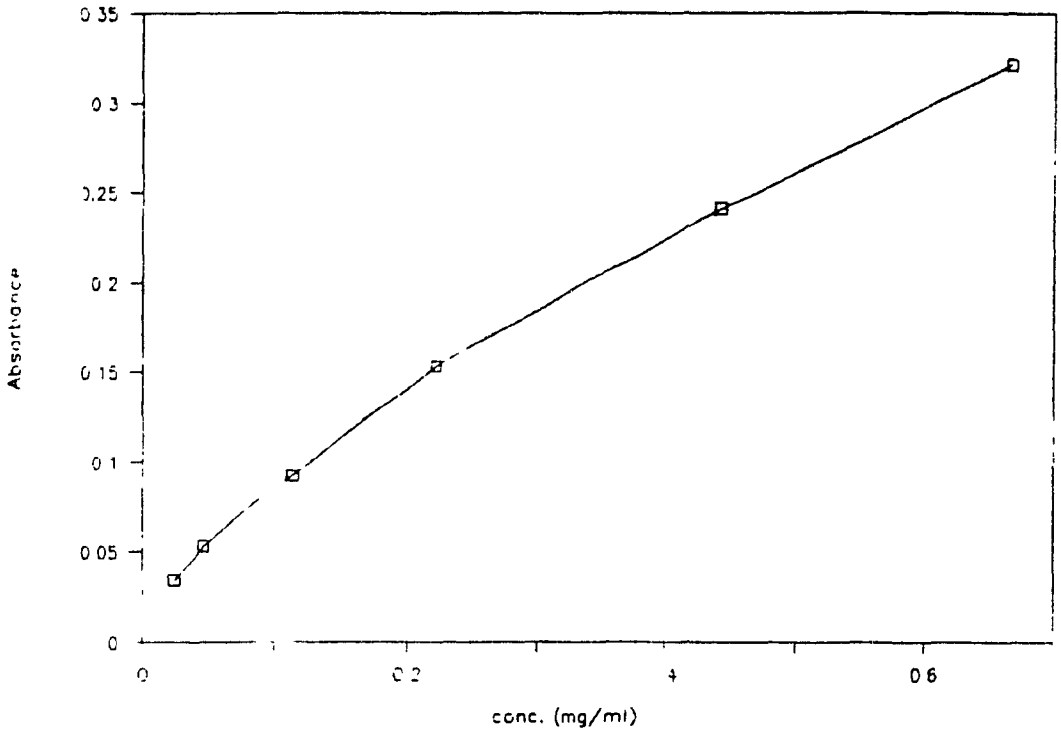
Calibration curve for cholestryl linolenate



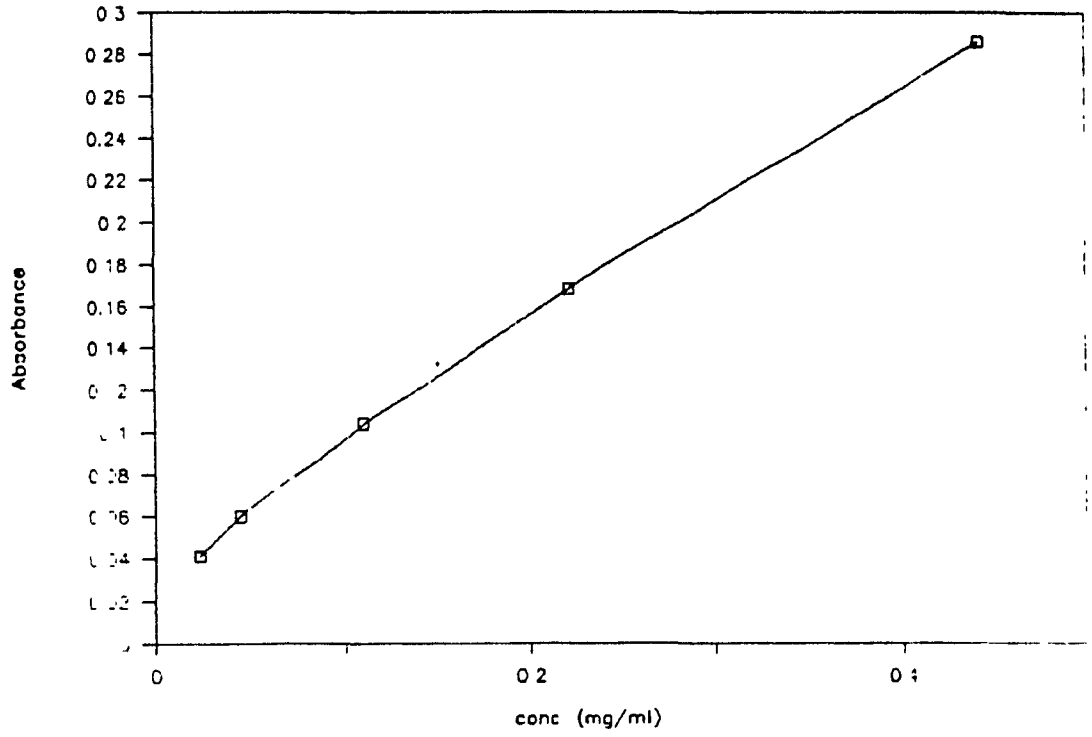
Calibration curve for trilaurin



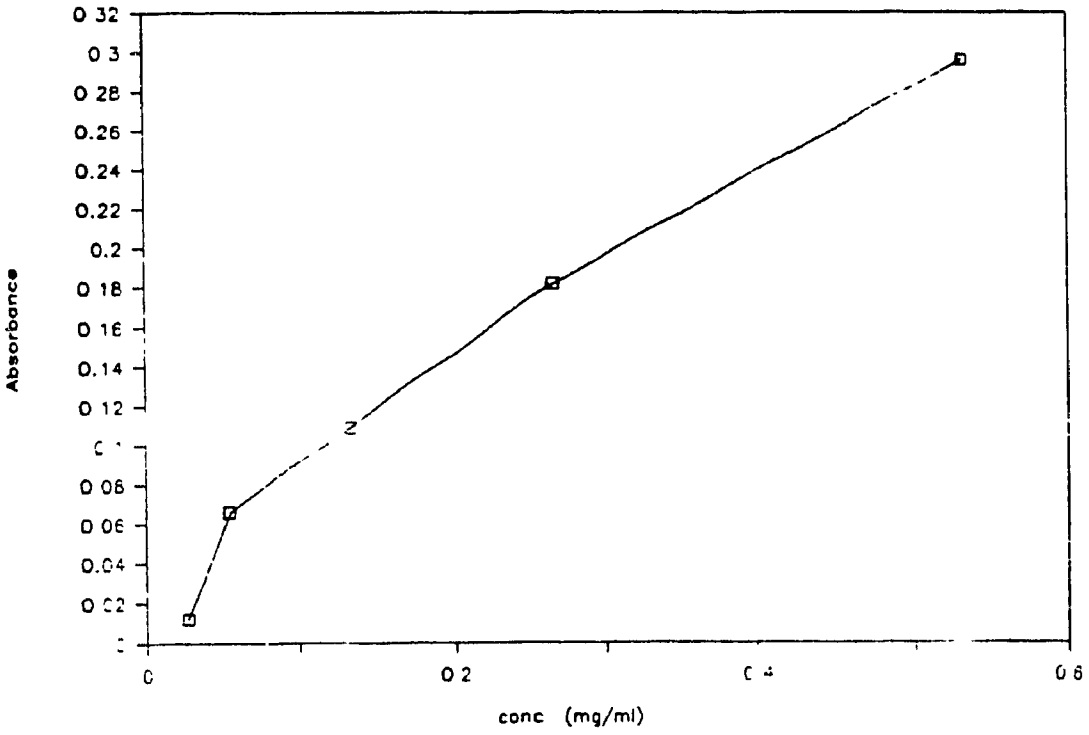
Calibration curve for trimyristin



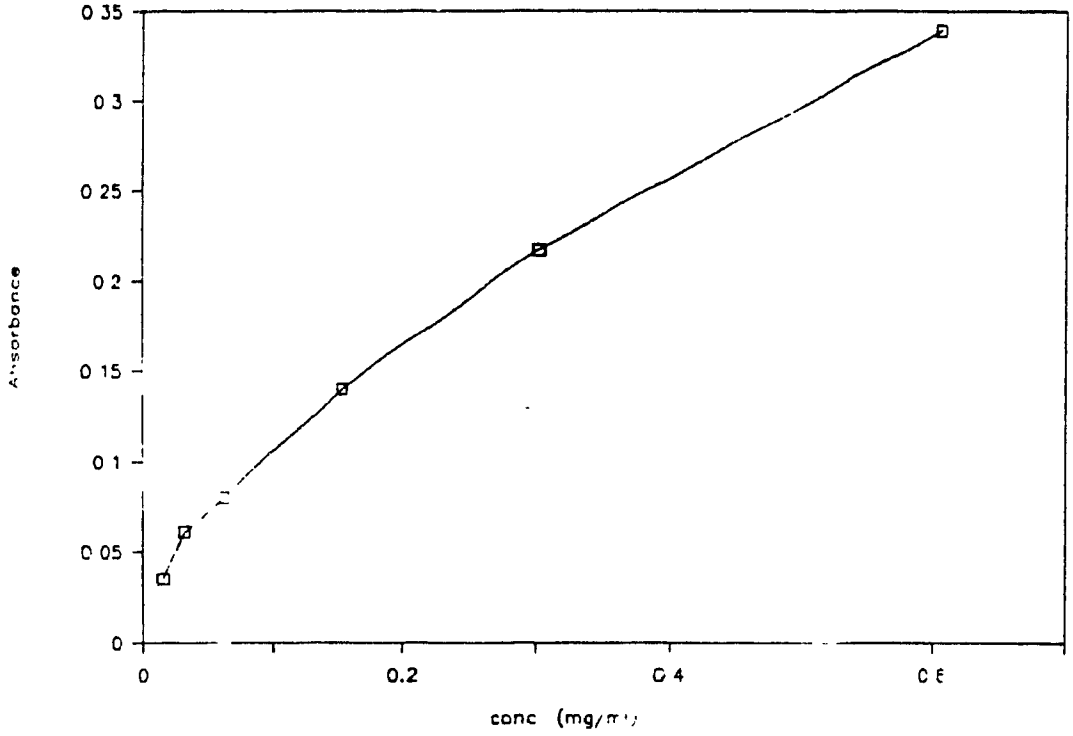
Calibration curve for tripalmitin



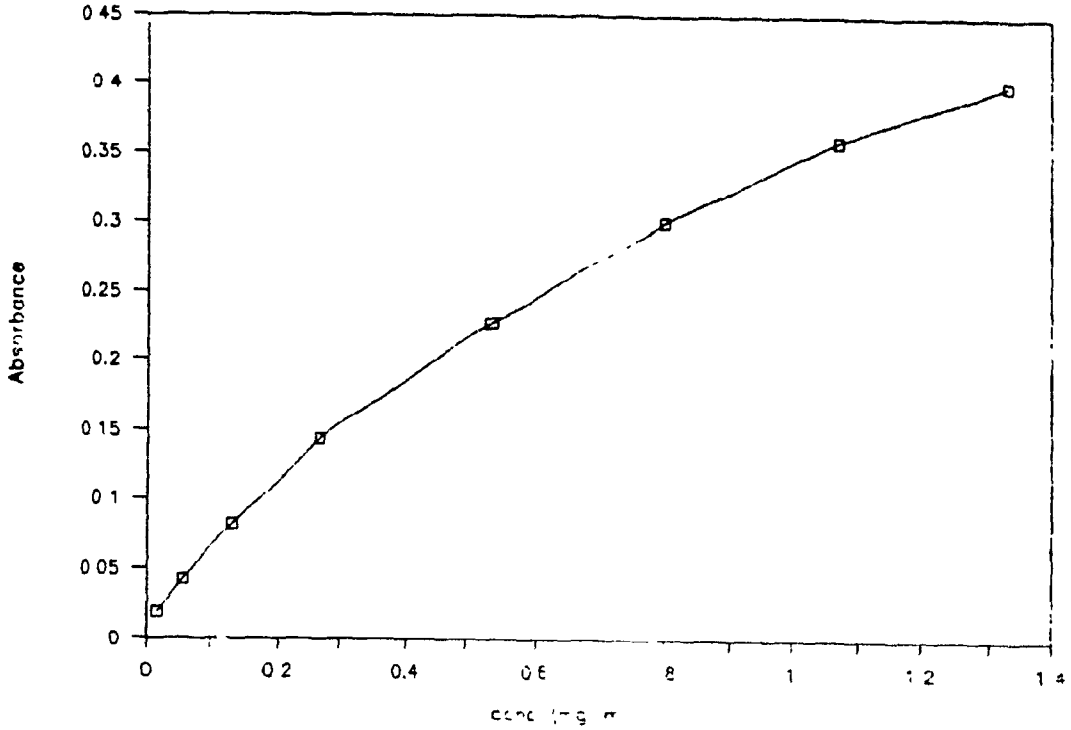
Calibration curve for tristearin



Calibration curve for triolein



Calibration curve for trilinolein

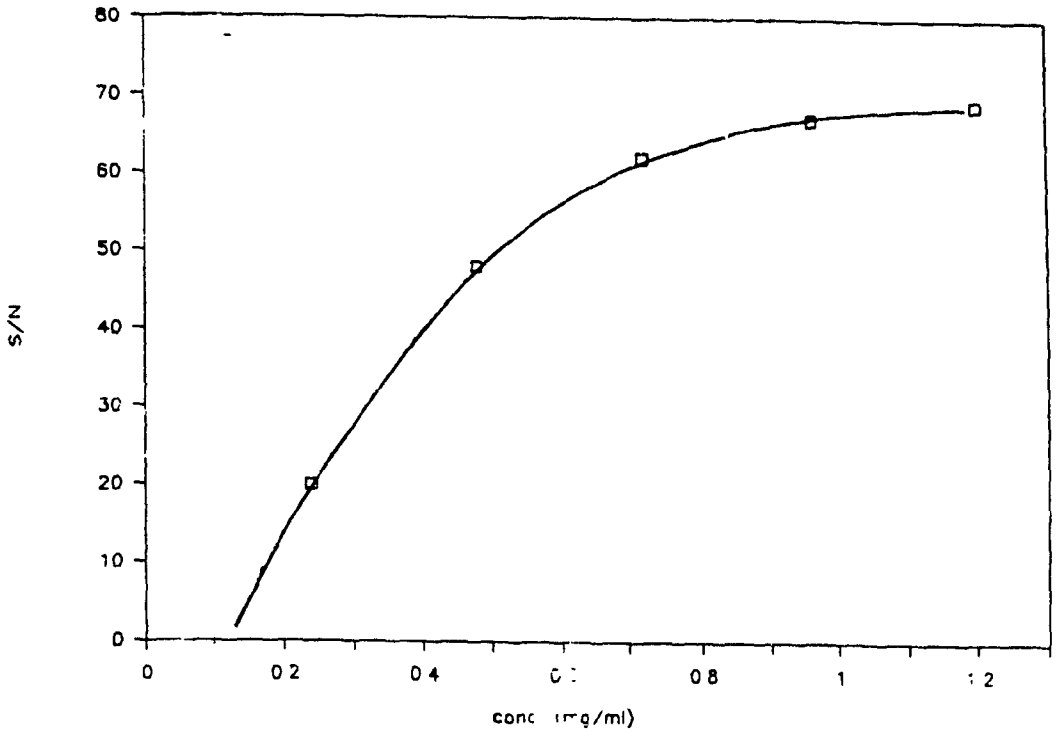


Calibration curve for trilinolenin

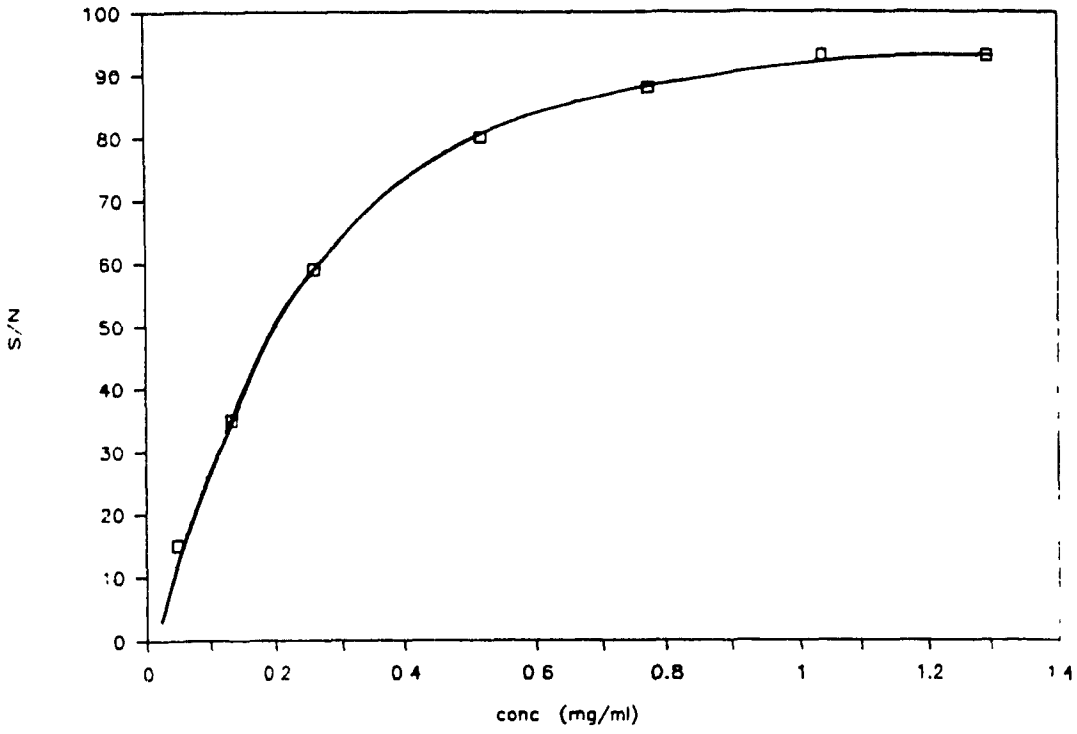
APPENDIX C

Included are the signal-to-noise versus concentration curves for all the lipids which are used in the determination of the detection limits given in Table 2 in Chapter 5.

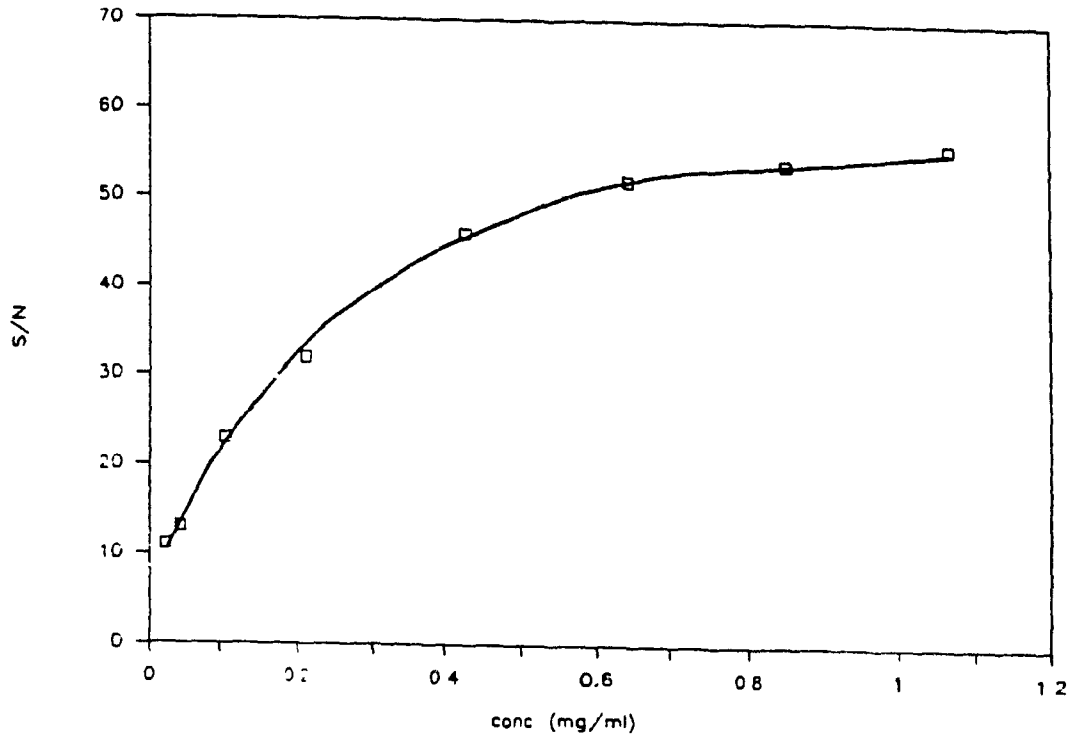
	correlation coefficient
cholesterol	0.98
cholestryl laurate	0.99
cholestryl myristate	0.99
cholestryl palmitate	0.99
cholestryl stearate	0.99
cholestryl oleate	0.99
cholestryl linoleate	0.99
cholestryl linolenate	0.99
trilaurin	0.99
trimyristin	0.99
tripalmitin	0.99
tristearin	0.99
triolein	0.99
trilinolein	0.99
trilinolenin	0.96



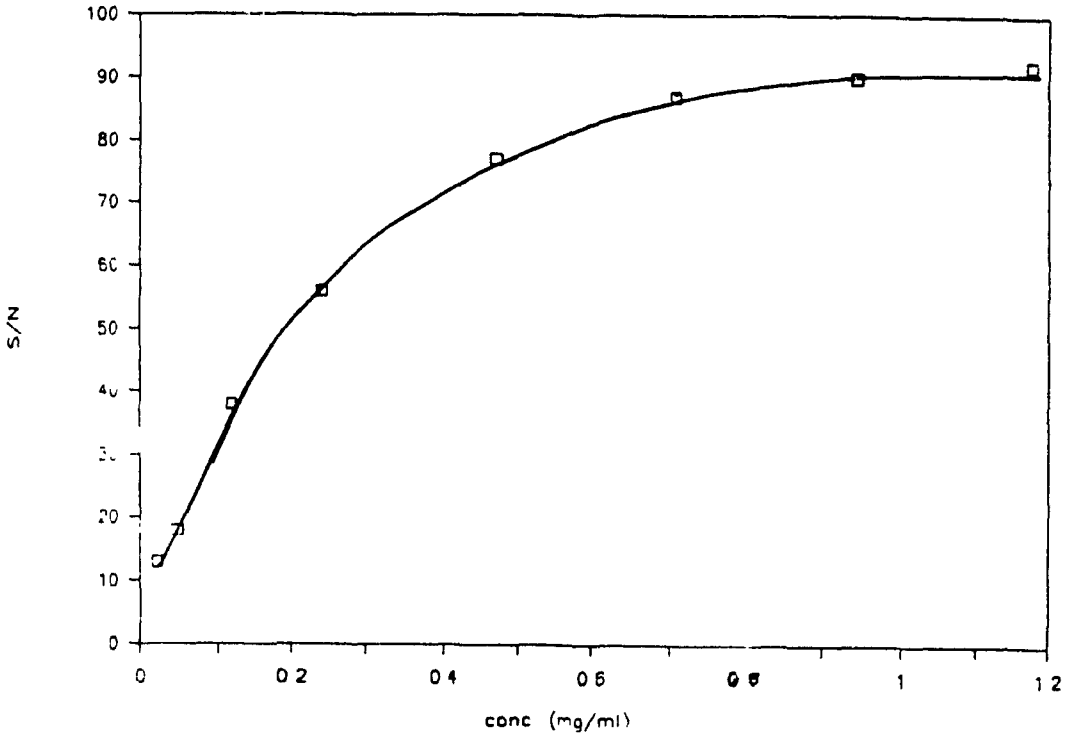
S/N curve for cholesterol



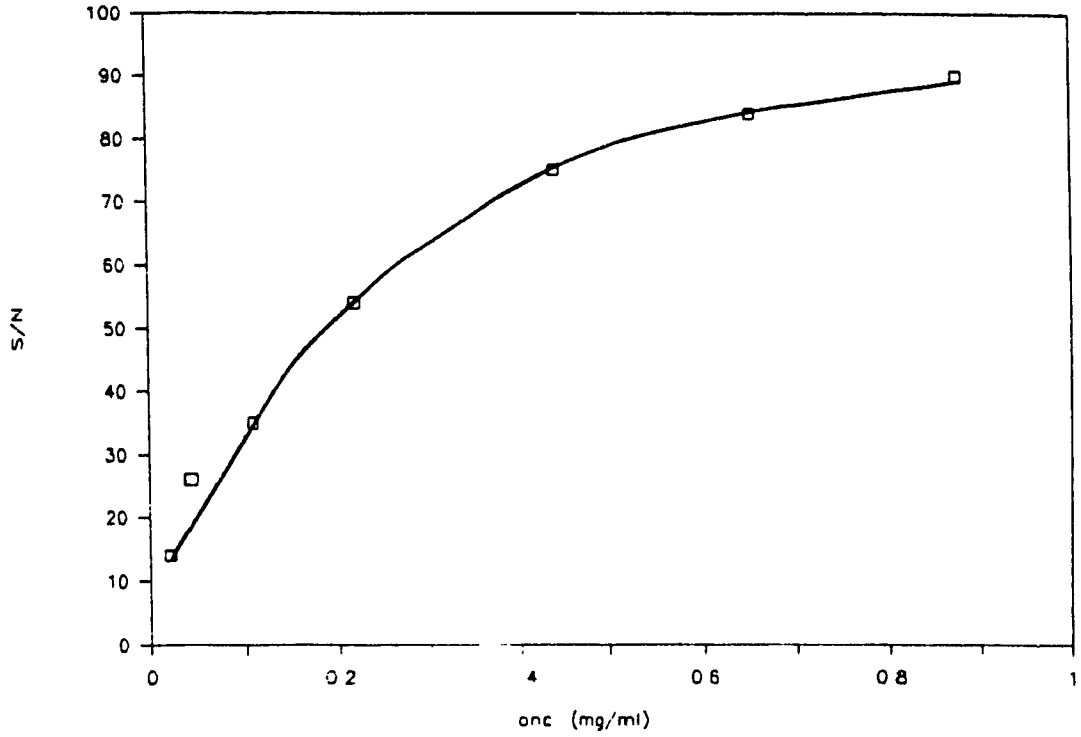
S/N curve for cholestryl laurate



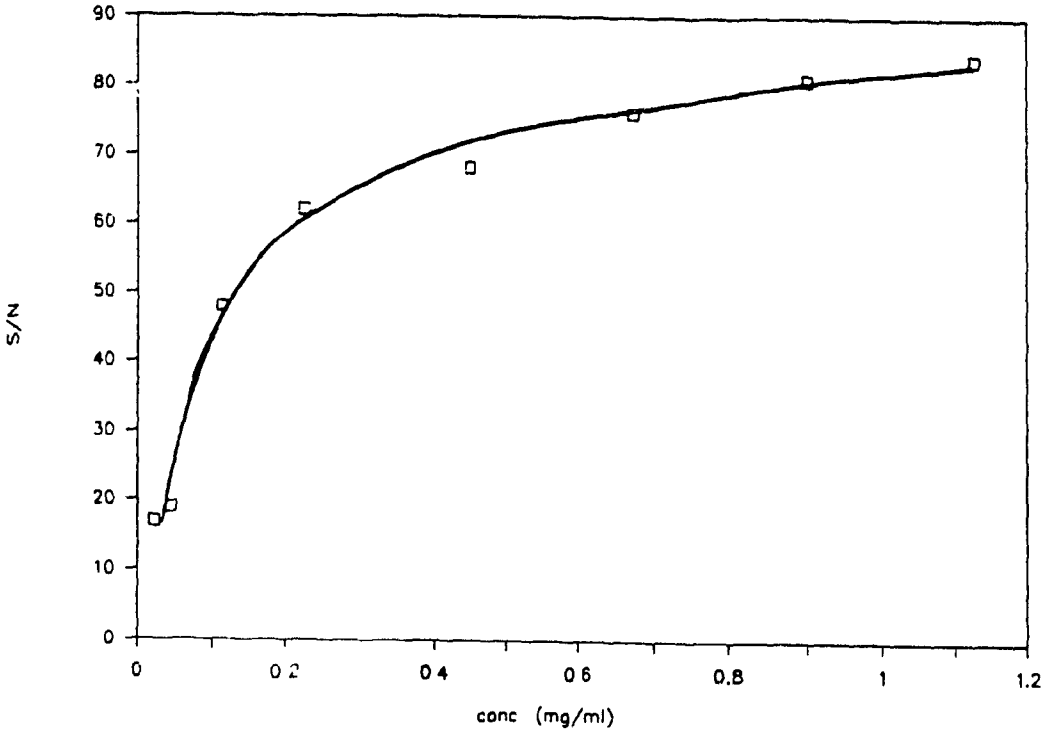
S/N curve for cholestryl myristate



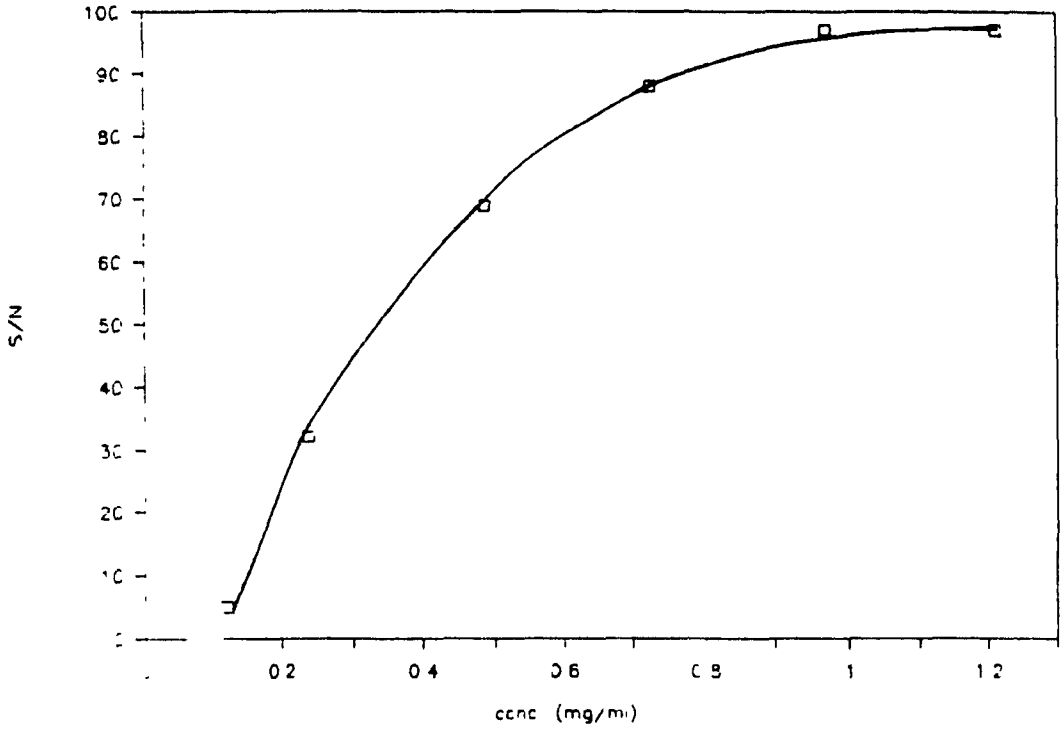
S/N curve for cholestryl palmitate



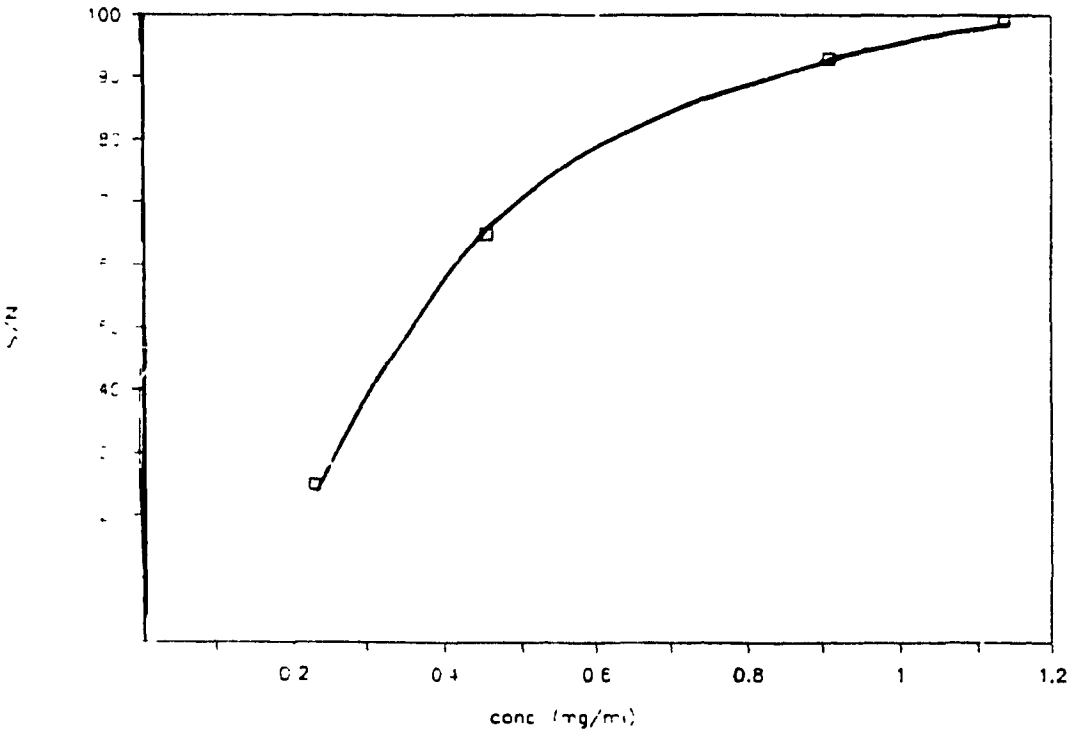
S/N curve for cholestryl stearate



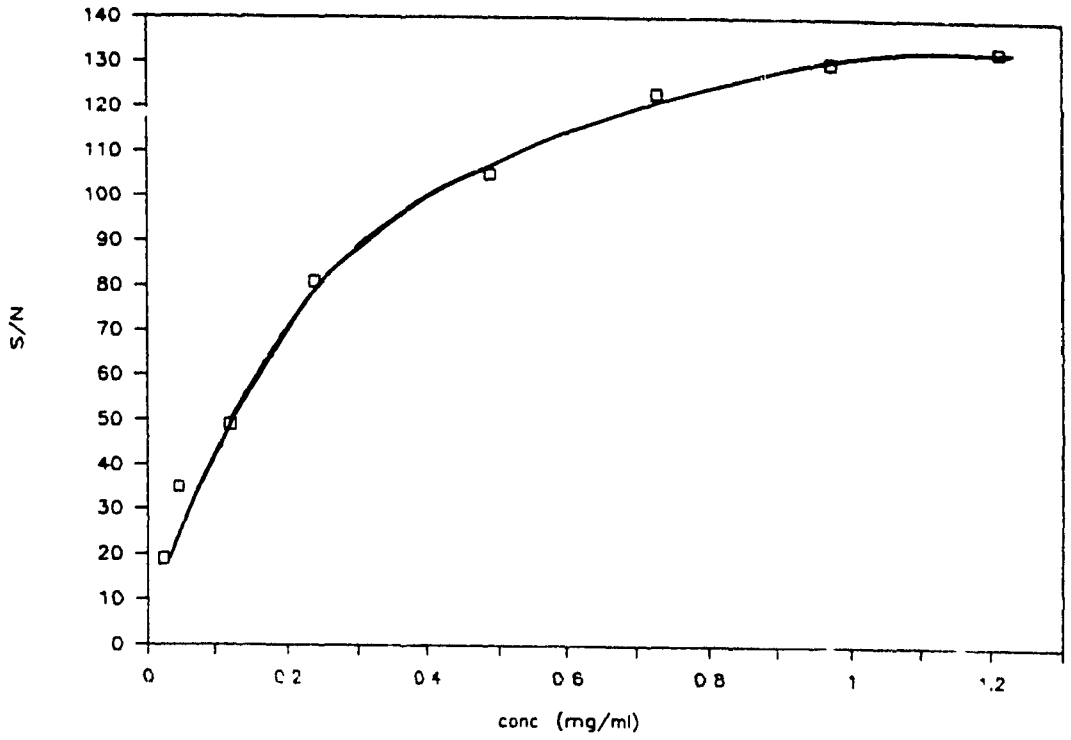
S/N curve for cholestryl oleate



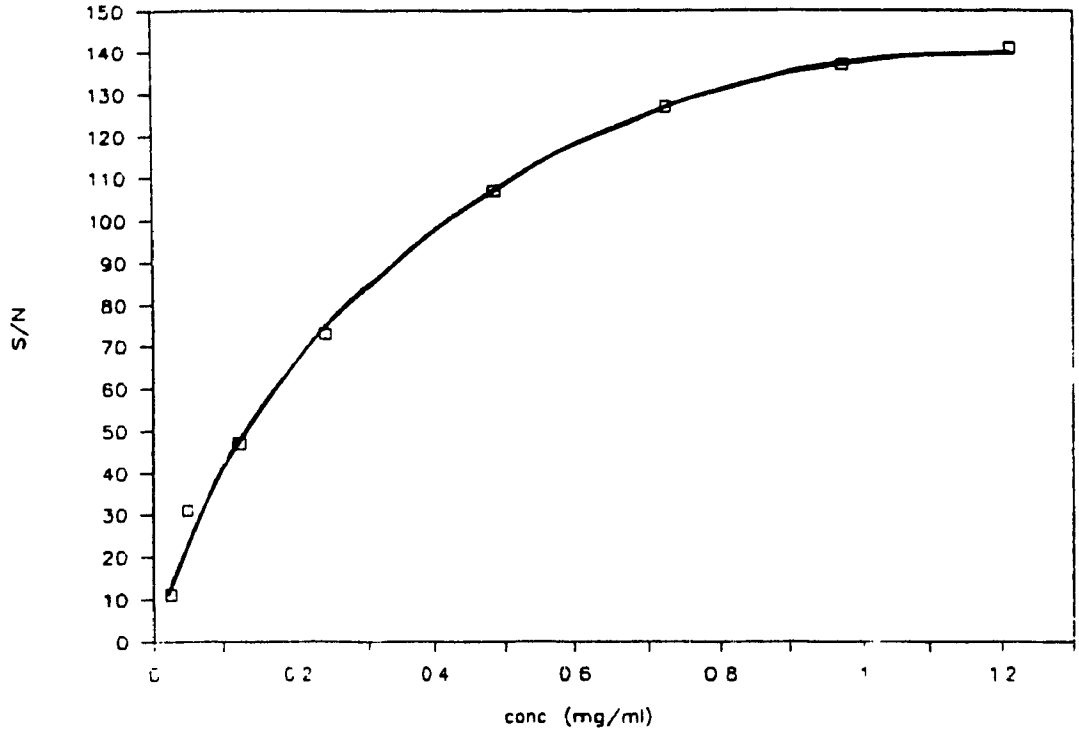
S/N curve for cholestryl linoleate



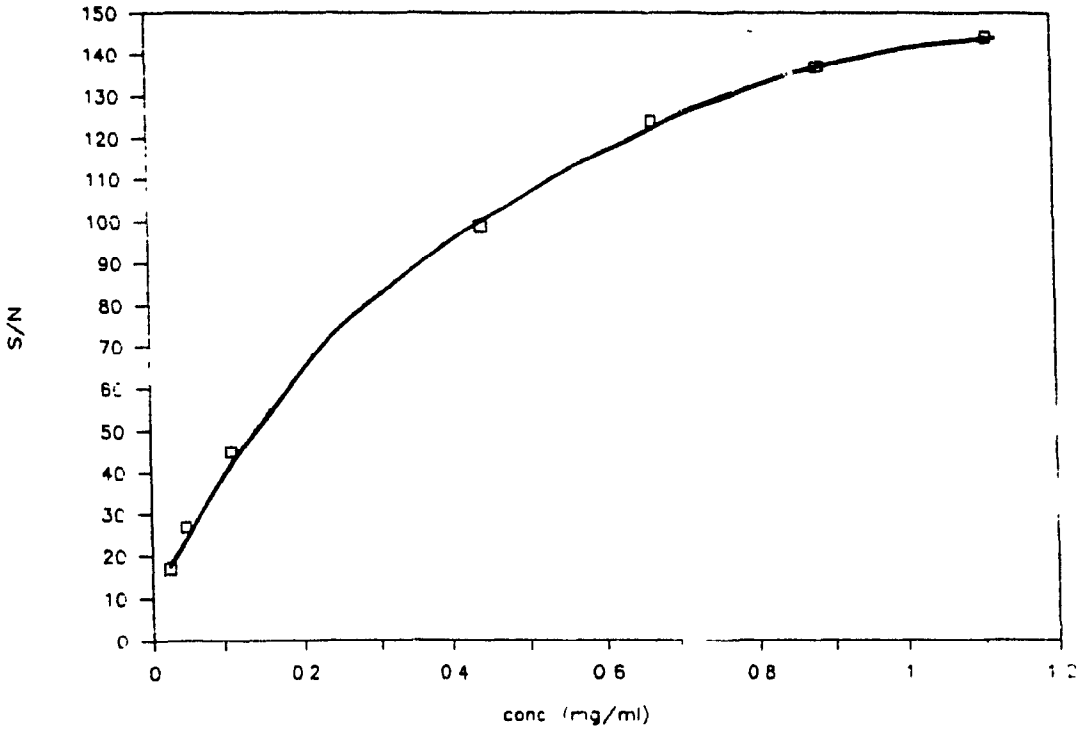
S/N curve for cholestryl linolenate



S/N curve for trilaurin



S/N curve for trimyristin



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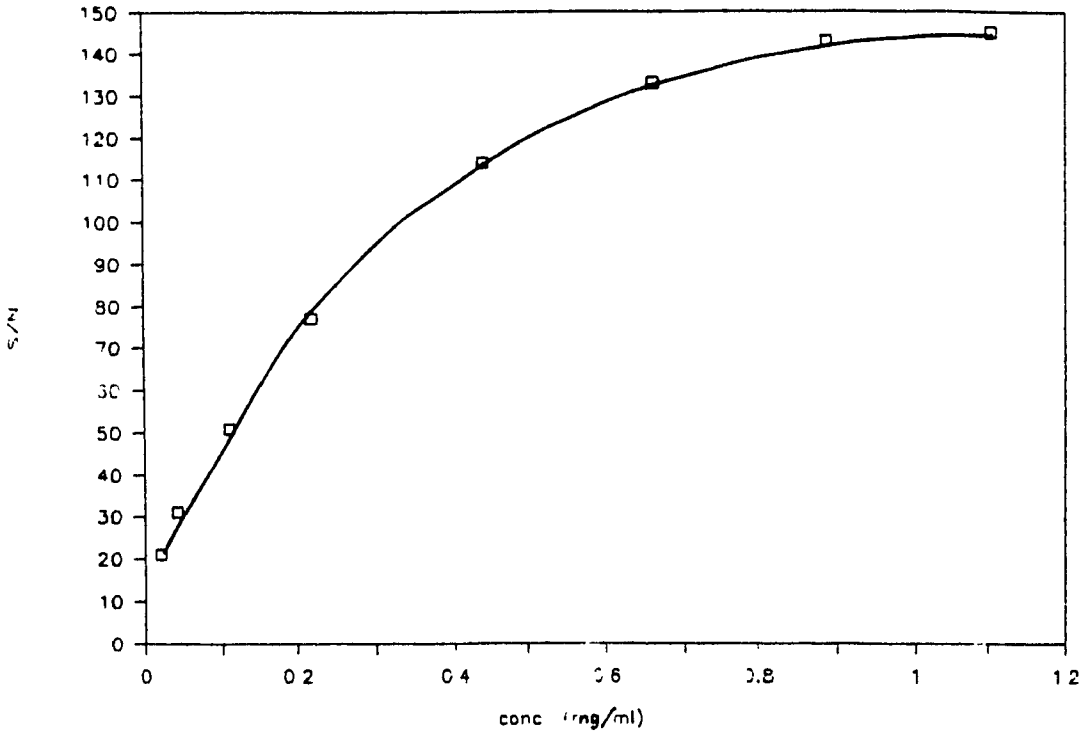
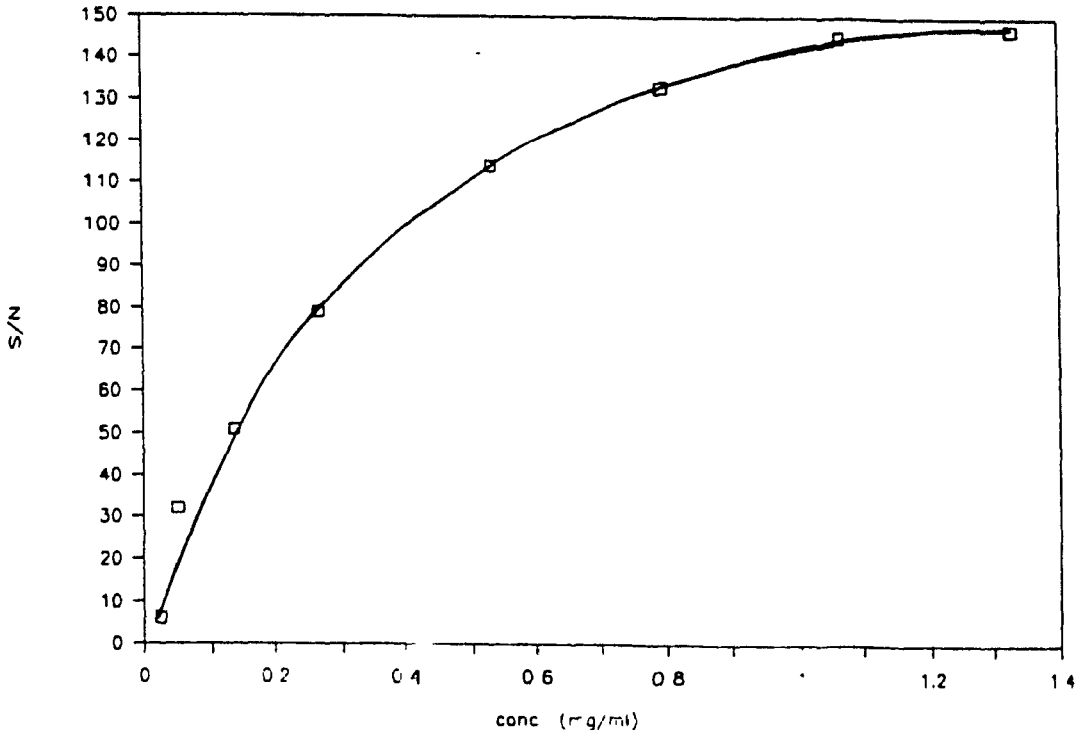
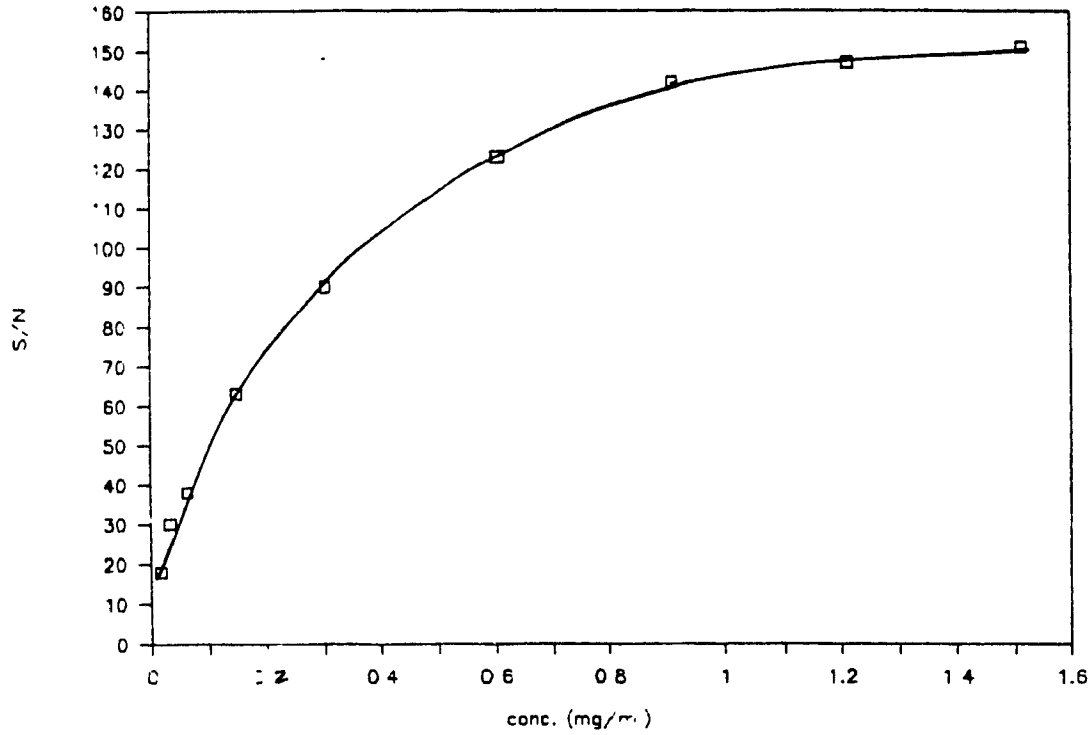


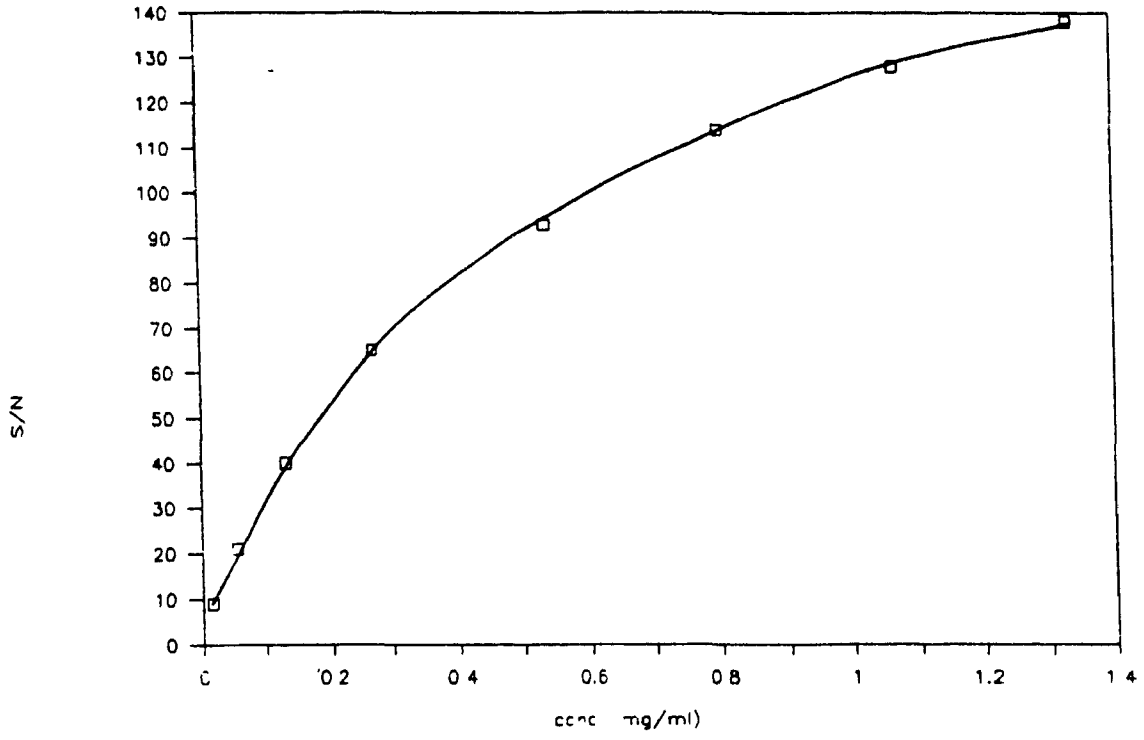
S/N curve for tristearin



S/N curve for triolein



S/N curve for trilinolein



S/N curve for trilinolenin

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. The construction of an infrared detector for High Pressure Liquid Chromatography based on a previously published design.

2. The evaluation and characterization of this detector.

3. The demonstration of the detector as a means of detecting all classes of lipid in the flow-injection analysis mode.

4. The use of the detector in High Pressure Liquid Chromatography for detection of the three most commonly analyzed for classes of lipid: cholestryl esters, triglycerides and cholesterol.

5. The use of a normal phase High Pressure Liquid Chromatography column for the separation of 3 classes of lipids.

6. The use of normal phase chromatography for the determination of the absorptivity and detection limits for cholesterol, cholestryl esters and triglycerides.