PART - II

STUDIES ON NATURAL PRODUCTS (OILS)
CHAPTER - I

SECTION - I

FATTY ACID COMPONENTS OF NATURAL FATS:

A REVIEW:-

Natural fats consist in most cases of triglycerides which are esters of glycerol with three fatty acids. Since these fatty acids comprise some 90% of the weight of any fat, they determine to a large extent the properties of the fats which they constitute. Knowledge of the nature of the constituent fatty acids in fats is therefore of considerable importance.

Accounts of the naturally occurring fatty acids are found in a number of standard text books, notably those of Hilditch and Gunstone. In this section, an attempt will be made to review briefly some points of fatty acid chemistry with special reference to the fatty acids actually found in the latter work.

Generally natural aliphatic mono carboxylic acids with carbon chain lengths of over four are classed as fatty acids. Lower acids have on occasions been found in natural fats, but are exceptional. The vast majority of fatty acids are linear with occasional exceptions, both branched-chain and cyclic.

1. Saturated Fatty acids:

1.1 Commonly occurring: Palmitic (16:0) and stearic (18:0) acids are certainly the most widely occurring
Saturated fatty acids found in nature. Almost all common natural fats are rich in palmitic acid and its useful sources include cotton seed oil (22-28%), palm oil (35-40%), and Chinese vegetable tallow (60-70%). Stearic acid occurs as a major component in only a few fats such as coca butter (35%) and Borneo tallow (40%). In a few seed fats, the so called vegetable tallow, palmitic acid is accompanied by considerable proportions of stearic acid, giving the fat a hard, tallow like consistency. A good example is the seed fat of Shorea robusta (Sal) (palmitic 8%, stearic 35%). Animal tallow are of course rich in both palmitic and stearic acids, particularly so those from Indian animals whether cows, sheep or goats. In the present work, the seed oils of Butea frondosa and Garcinia Xanthochymus were found to contain a high content of palmitic acid, 22.4% and 39.9% respectively.

1.2 Higher saturated acids:

Arachidic (20:0), behenic (22:0) and lignoceric (24:0) acids are major components in only a few rather uncommon seed fats.

Traces of arachidic acid frequently accompany stearic acid in many fats, but larger amounts are rare. Among the best known material containing arachidic acid is the seed fat of Schleichera trijuga.
commonly known in India as Kusum oil, which contains about 20%. Ground nut oil is another very common fat characterised by the presence of a small but definite amount of this acid.

Behenic acid is less common than arachidic acid. Kusum, ground nut and sal fats each contain small amounts. The acid draws its name from the seed of ben (Indian drumstick, Moringa oleifera), though the quantity present in the fat is only about 2-7%. Higher quantities than 7% have not so far been recorded in any seed fat, but in the present investigation this acid was found to the extent of 11% in the seed fats of Butea frondosa belonging to Leguminosae family.

Lignoceric acid is a rare constituent of seed fats. The common groundnut oil contains about 1.5 - 2% of it. A very rich source is the fat of Indian coral nut, Adinanthara pavonina, which carries Ca. 28%, a truly remarkable amount of so high melting a fatty acid (84.2 C). The seed fat of Butea frondosa which was in the present investigation contains 4% of this acid, which must be considered a fairly high proportion.

1.3 Lower saturated acids:

Cow milk fat contains the four carbon butyric acid (10% mol.) with smaller amounts of the caproic, caprylic, capric and lauric acids. Sheep and
Goat milk fats contain these same acids with capric acid in greatest amount (upto 10% mol.). The capric acid is a major component (60%) in the seed fat of the olive tree and the caprylic and capric acids (each 5-10%) accompany the high proportion of lauric acid in coconut oil and to a lesser extent, in palm kernel oil.

Lauric (12:0) and Myristic (14:0) acids occur extensively in the seed fat of the Lauraceae and Myristicaceae respectively. The Indian seed fat pisa (Actinodaphne Lookeri) contains 88% of lauric acid and it is also present in cinnamon oil (80-90%), coconut oil (45-50%) and palm kernel oil (45-55%). Myristic acid is very high in nutmeg butter and is also present in coconut and palm kernel oil (15-18%).

A branched chain 5-carbon acid occurs in the body fats of the dolphin and porpoise.

Acetic acid is generally considered a fatty acid, but has recently been unequivocally shown to be present in considerable quantity particularly in the seed fats of the family Celastracae.

Triglycerides carrying combined acetic acid are optically active, giving a small but specific rotation of about $-0.57^\circ$. These are only known optically active triglycerides not containing optically active acids such as ricinoleic or chaulmoogric.
2. Unsaturated FATTY ACIDS:

2.1 Commonly occurring: Three 18-carbon, ($18-\omega$) acids oleic, linoleic and linolenic, 18:1, 18:2, 18:3 all occur very widely. All carry the first cis double bond at C-9, the double bonds also cis being at C-12 in linoleic and C-12 and C-15 in linolenic acids. Oleic acid is most common of all fatty acids. It occurs in practically all fats, attaining a very high proportion in many readily available sources such as olive oil (75%), almond oil (75%), ground nut oil (55%), cocoa butter (40%), and animal fats (35-40%). Garcinia xanthochymus seed fat which was analysed in this present investigation was found to be very rich in oleic acid (46%).

Linoleic acid sometimes the major component acid as in safflower (70%) and cotton seed oils (50%).

Caesalpinia bonducella seed oil containing (66%) of linoleic acid was investigated in the present work.

Linolenic acid is the main acid in linseed oil in which it forms over 50%.

2.2 Other monoene fatty acid:

These may be divided into various types.

(a) Palmitoleic and myristoleic acids, 16:1 and 14:1, each with the double bond at C-9 as in oleic acid, occur in fair quantities in fish and ruminant milk fats. In seed fats, they usually accompany the corresponding saturated
acids in very small amounts. One exception of long
standing has been the occurrence of 20% of palmitoleic
acid in a few seed fats of the family Protaceae.
Recent studies would make it appear that the acid is also
found in the seed fats of the sub-family Papilionatace,
family Leguminosae, to the extent of 7-9% (15-17) and
also in seed fat of the family Elaeocarpaceae in the
surprisingly large amount of 15%.18

The present gas liquid chromatographic (GLC)
analysis has shown an exceptionally high amount (11%) of
a 16:1 acid presumably 9-hexadecenoic, in the seed fat of
Garcinia xanthochymus, family Guttiferae. GLC after
hydrogenation supported the structure.

(b) Erucic and Petroselenic acid: Erucic acid, 22:1
with the double bond at C-13, occurs extensively in
rape-mustard and other seed oils of the Cruciferae
family.1 Petroselenic acid, 18:1, the C-6 double bond
isomer of oleic acid, is only found widely in seed fats
of the umbelliferae family.1 No example was studied
in the present investigation.

(c) Cyclopropene fatty acids: Two acids with cyclopropene
rings, the C-10 sterculic acid and 18-C malvalic acid,
occur in natural fats. Seed fats of the family Malvaceae,
of which cotton seed oil is a common example, contains
small amounts (1%) of these acids while some, though not
all, oils of the Sterculia family contains substantial
proportions of sterculic acid. The best known, *Sterculia foetida*, has a 70% content of sterculic acid.

(d) Trans Monoene acids: While large amounts of trans acids are produced during the catalytic hydrogenation of oils and though their presence in small quantities in many animal milk and body fats has been generally accepted, natural trans monoene acids have only recently been isolated from a few seed fats. That of *Thalictrum polycarpum* and several others of this genus, family *Ranunculaceae* carried trans 5-octadecenoic and trans 5-hexadecenoic acids (accompanied by diene and triene acids also carrying a 5-trans first double bond). Similarly 3-trans 18- and 16-carbon monoene acids, accompanied by a 3-trans, 9-cis diene acid were found in *Grindelia oxylypis* oil family *Compositae*. The same monoene and diene acids have recently been found in the seed oils of *Aster alpina* and *Arctium minus*, both of the *Compositae* family.

2.3 Other diene fatty acids:

These are of rare occurrence. Rape-mustard oil of *Cruciferae* contains about 0.2% of a 22:2 acid. A few natural conjugated diene acids are known. An unusual one is the 10-carbon conjugated diene acid found in *Stillingia* oil.

2.4 Other triene fatty acids:

Among the conjugated trienes, *θ*-eleostearic acid (18:3, 9 θ, 11 θ, 13 θ) a major component acid of
tung oil (70-80%), is the best known and it gives the oil its special utility in quick and hard-drying surface coatings. Other isomers of α-eleostearic acid have recently come to light, carrying the triene conjugation either as a 9, 11, 13 system (punice, catalpic, licanic and kamlolonic) or as a 8, 10, 12 system (Calendic, jacaric), varying only in their stereo forms. A 5, 9, 12 acid has also been found in *Thalictrum polycarpum* seed fat.

3. Some unusual Fatty Acids:

3.1 Oxygenated Acids: Ricinoleic acid, (12-hydroxyoctadec-9-enoic acid, is the major acid in castor oil (about 90%). It is the most readily available and the most studied natural hydroxy acids. Other hydroxy and epoxy acids are present in the glycerides of certain fungi and some seed oils. Siddiqi et al. have recently reported the occurrence of ricinoleic acid in the seed fat of *Hiptage benghalensis*, family Malpighiaceae, to the extent of 81%.

3.2 Branched Chain and alicyclic acids: Though the great majority of fatty acids are straight chain compounds, there remains a number of interesting acids which carry either a branched chain or a cyclic group. These structural changes lead to a lowering of melting point and in some cases, to the possibility of optical isomerism. The branched acids are found as trace constituents of many animal fats and also are recognized in mycobacteria. The cyclopropene acid, steruclic, occurs widely in seed oils.
or fruit fats of **Malvaceae, Sterculiaceae, Tiliaceae** and **Bombaceae** families accompanied sometimes by another cyclopropene acid, malvalic acid. Chaulmoogra oil and some related seed oils of the family **Flacourtiaeae** contain acids having a cyclopentene group. Hydrocarpic (16-C) and Chaulmoogric (18-C) acids are most common.

No conjugated or any unusual fatty acids were detected in any of the present seed fats either during preliminary study of their UV and IR spectra or in the later GLC studies.
REFERENCES:


11. A.H. Gill and C.M. Tucker, Oil and Fat Ind. 7 (1930) 101.


SECTION - 2

ANALYSIS OF COMPONENT FATTY ACIDS:
A REVIEW:

Despite the great abundance of fats in natural substances and their importance in the process of life, little was known about their component fatty acid composition, until the studies of Wilditch and his colleagues, beginning in 1926, were well advanced. Since then, a number of methods for the quantitative analysis of the fatty acid composition of natural fats have been developed. To-day the use of gas liquid chromatography (GLC) has led to an immense reduction in time and an enormous increase in accuracy while enabling the use of very small amounts of experimental material.

Different techniques applied to fat analysis include the earlier fractional distillation, crystallisation, and urea fractionation, and more modern techniques like counter current distribution, partition chromatography on columns, paper chromatography, thin layer chromatography (TLC), gas liquid chromatography (GLC), ultraviolet spectroscopy, infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (NMR), mass spectrometry, crystallography etc. Though fractional distillation, urea fractionation and crystallisation are classical methods of analysis, to-day these techniques are mainly preparative. These methods are very well known and
details are readily available in the literature. This short review will have a bias towards the fatty acids actually encountered in the present investigation and will emphasise the use of GLC as applied to the analysis of fatty acids with notes on other methods also used in the later experimental work. The analysis of fats has commonly been carried out not with free acids but with their methyl esters.

1. Preparation of methyl esters:

A number of papers have described methods for the preparation of methyl esters from long chain fatty acids or triglycerides. In general these methods use anhydrous methanol containing either an acidic or basic catalyst and involve reaction times varying from 0.5 to 2 hrs. at reflux temperature. The methods using boron-trifluoride-methanol reagent or diazomethane are rapid and give excellent yields and are especially useful in the preparation of small quantities of methyl esters used for GLC.

The three fats examined in the present work belong to the families *Luguminosae* and *Suttiferae* and contain an appreciable quantity of unsaponifiable matter, which could cause interference in subsequent chromatographic and spectroscopic analysis. Therefore, the classical technique of saponification, extraction of nonsaponifiable matter with ether and subsequent isolation of fatty acids were followed. The fatty acids were then converted to
methyl esters by refluxing with dry methanol containing one per cent sulphuric acid, a well established procedure.

2. Ultra-violet Spectroscopy

Compounds like oleic acid with one double bond absorb ultraviolet light only at wave lengths too low for convenient study and methylene interrupted polyenoic acids, such as linolicic, linolenic or arachidonic all carrying several isolated double bonds, absorb in the same region, but with increased intensity. UV spectroscopy will thus normally provide only negative evidence about the great majority of natural fats which do not contain conjugated unsaturation, but it is invaluable in the study of acids having conjugated unsaturation and of reactions (e.g. alkali isomerization) which yield such acids. In the present investigation the UV spectra of the mixed methyl esters pointed to the absence of conjugated fatty acids in many of these fats.

3. Infrared Spectroscopy

IR spectroscopy is of particular value in the recognition of unusual functional groups and in the study of fatty acids with trans double bonds. It is quantitatively used to estimate the amounts of isolated trans fatty acids present in fats which show a characteristic absorption at 10.36 μ (968 Cm⁻¹). IR spectra have shown the absence of any unusual functional group or trans double bonds in the fatty acids of the fats now studied.
4. Thin Layer Chromatography:

The literature on TLC has been growing enormously and there are several monographs and reviews which give full details of the practical aspects and separation of fatty materials possible by this method. The ensuing discussion is restricted to the fatty acids and their simple methyl esters. The experimental procedure will be described later. Four types of TLC are commonly used in the lipid field, direct TLC, reversed-phase TLC, argentation TLC and separations using other complexing agents such as boric acid TLC.

4.1 Direct TLC: This simple, direct form of TLC does not adequately separate ordinary saturated and unsaturated acids or esters, but has been used to resolve various hydroxy, keto and epoxy acids from one another and from acids not containing such oxygenated functions.

4.2 Reversed-phase TLC: RPTLC separates fatty acids according to chain length and unsaturation. The presence of one double bond in the fatty acid chain has the same effect as reducing the chain length by two carbon atoms, thus oleic moves with palmitic, linoleic with myristic and linolenic with lauric acids. These 'critical pairs' can be resolved by simultaneous hydrogenation or bromination on the plate. Low temperature RPTLC and the use of the solvent system consisting of nitromethane, acetonitrile, acetic acid (75:10:10) also separated these critical pairs.
4.3 Argentation TLC: Separation according to unsaturation is effected on thin layers of silica impregnated with silver nitrate. Olefinic compounds form Pi-complexes of varying stability with silver ions and this allows separation of saturated, mono-, di-, tri- and tetraenoic acids. There are also small differences depending upon the configuration of the double bonds and on their relative positions and minor differences depending on chain length. This procedure has been extensively applied for the detection and isolation of many new acids, present as major or minor components and for the separation of common mixtures such as saturated esters, oleate, linoleate and linolenate.

4.4 Borsic acid TLC: Poly hydroxy esters differing only in the configuration are separated on silica layers impregnated with boric acid.

In the present work, reversed-phase TLC has been used mainly for identifying the presence of saturated components especially higher fatty acids (C_{20}-C_{24}) either before or after hydrogenation. Argentation TLC was also similarly used for the identification of unsaturated components. Direct TLC revealed the absence of any oxygenated fatty acids in the present fats.

5. Gas-Liquid Chromatography:

Gas-liquid chromatography (GLC) has become established as an indispensable tool for research with lipids since its development by Martin and James in 1952.
There are now numerous applications and the literature is extremely large. It provides the most effective method of quantitative analysis of fatty acid mixtures and can give considerable information about acids of unknown structure. The general theory and practice of gas chromatographic methods are extensively treated in many review articles and books. A brief outline will be given of the technique as applied to the analysis of fatty acids encountered in the present work.

The basic equipment for the method consists of a column containing a stationary phase held on an inert support and connected to a source of moving vapour phase and a detector capable of detecting the separated material. The substance for separation is placed on the column, driven through the column by the carrier gas and the materials are separated depending upon their differing interactions with the stationary phase. The separated materials are characterised by measuring some chemical or physical property. A number of commercial instruments are now available for gas liquid chromatography.

The size of samples required is small and usually varies from 0.5 to 10 μg. Micrometer syringes are used to introduce the sample. The supporting materials for the columns are Kieselguhr celite or ground firebricks. The stationary liquid phases are often polar or nonpolar materials. Capillary columns have also been used and give better separation because of the extreme length of the column and the very small quantity of material required.
The carrier gases used in GLC are commonly argon, helium, hydrogen and nitrogen. The detectors most frequently used are the thermal conductivity detector, \( \beta \)-ray argon ionization cell, hydrogen flame ionization detector and gas density balance and these largely decide the choice of the gas phase.

In the present investigation GLC analysis was carried out on an F & M Model 1609 gas liquid chromatograph having both thermal conductivity and hydrogen flame ionization detectors. The fatty acids were analysed as their methyl esters, since the latter are more volatile and do not show the high degree of association that the parent acids do. Two main types of stationary phase, non-polar and polar, were used.

5.1 Separation on non-polar columns: The non-polar phases such as Apiezon greases or silicones tend to resolve mixtures of fatty esters on the basis of chain length only irrespective of degree of unsaturation, a tendency which is further used to advantage by employing a short column of 1-2 ft. in length. Silicones are comparatively temperature stable and can be used without danger of decomposition or volatilisation up to 300°C or more. The support also plays a part, fire brick tending to give a high degree of peak resolution than celite. A silicone (SE 30) supported on cromosorb W (40-60 mesh) was used in this work.

5.2 Separation on polar columns: The polar phases are mainly polyesters derived from dihydric alcohols such as ethylene
glycol, diethylene glycol, or butanediol and dibasic acids such as succinic, glutaric, adipic, suberic, phthalic or iso-phthalic. These polyesters on suitable supports provide the right degree of polarity and viscosity for good resolution of fatty esters. Diethylene glycol succinate or DEGS supported on chromosorb W (45-60 mesh) was used throughout this work. The polyesters are thermally less stable than the non-polar phases and can generally be used at about 200°C. It separates the fatty esters both by chain length and unsaturation giving clearly resolved peaks for 16:0, 16:1, 18:0, 18:1, 18:2, 18:3 in rather large proportion. In such cases, either the silicone run is consulted to establish independently the presence of 20:0 or the material is fully hydrogenated and re-run either on silicone GLC, polyester GLC or reversed phase TLC to determine if any 20:0 acid is present and its proportion.

In polyester GLC, the longer the emergence time and the lower the temperature, the wider and flatter the peaks. Hence esters of 20:0 and beyond, especially when these are present in small amounts as is often the case, give peaks whose areas are impossible to determine with accuracy. There is also a limit to which column temperatures can be raised without severe "bleeding" of the polyester from the column in the flowing gas stream. In these instances, rapid silicone GLC of higher temperatures geared to these esters enables satisfactory estimation.

5.3 Expanded GLC: This technique was used to estimate minor components, generally higher saturated and unsaturated esters. After the regular GLC run (first chroma-
(togram), the experiment is repeated with a larger sample size and at a higher sensitivity of the apparatus, taking care to see that one of the well defined peaks of the first chromatogram remains within the chart as a reference peak. An increase in the area of the peaks of the minor components occurs and can be estimated. Their true percentage in the mixture can then be determined by reference to the relative areas of the reference peak in the first and second chromatograms. The minor peaks can also be well estimated at a higher chart speed, by using a larger sample size and by increasing the sensitivity of the apparatus, during the run just before the emergence of the minor peaks. This technique is applicable to both silicone and polyester columns either with the original or fully hydrogenated esters.

5.4 Column temperatures: The column can be held during GLC at a fixed temperature (isothermal) or the temperature can gradually be raised during the course of the run at any desired rate (temperature programming). The latter enables each ester to be eluted at its appropriate temperature and avoids having to strike a mean for the whole run. The area response is not altered; only the emergence time is shortened and hence the peaks are narrower and more symmetrical. On the other hand, making a series of GLC runs in succession is much easier when the column is held at a steady temperature.

5.5 Detection: Two detection systems are used in the present work; thermal conductivity detector was used for determining the component fatty acid composition of the three fats and the flame ionization detector for the
gyceride studies. The principle involved in the thermal conductivity detector is the alteration in electrical conductivity of a hot wire (a part of the wheatstone bridge system) caused by the presence of the vapour of the fatty ester in the surrounding gas stream. This change in the conductivity is amplified and communicated to a recorder in the form of a signal. The flame ionization detector has the advantages of great sensitivity, implying that very small quantities of material are sufficient and nonresponse to water, which allows aqueous solutions of materials to be resolved. The organic substances ionize at the flame temperature and electrons are captured by a gauze above the flame, the alteration in current thus produced being sufficient to send signals to the recorder after amplification.

5.6 Peak identification: The identification of peaks obtained from an unknown mixture is carried out by a comparison of retention volumes (or retention times so long as flow rate is constant) with those of reference esters, preferably an at least two columns of different nature. This permits the identification of known compounds and can give considerable information about unknown structures.

When the logarithm of the retention time for a series of homologous compounds is plotted against the number of carbon atoms, a straight line is obtained, the slope of which depends on the operating conditions (stationary phase, temperature etc.) Closely related homologous series, such as n-saturated and monoenoic
esters, given lines which are parallel or almost so, esters of iso-acids or dienoic acids give; other lines.

5.7 Carrier gas: Hydrogen gas is used as the carrier gas with thermal conductivity detector in the present work, because of ready availability, cheapness and convenience. Other possible carrier gases are argon and nitrogen.

Nitrogen is generally used as the carrier gas with the flame ionisation detection system. Flame-ionization detector requires the use of three gas streams, hydrogen for the hydrogen flame, air for the burning of the flame and nitrogen as the carrier gas.

5.8 Quantitative analysis: Quantitative measurements using thermal conductivity detectors, flame ionisation detectors or other differential type detectors depend upon the determination of the recorded peak area or peak height and the relationship of these quantities to the amount or concentration of solute in sample. Peak height measurements are usually recommended when small peaks must be measured or when the band width is narrow as in the case of early peaks.

5.8.1 Methods of peak area measurements: Area measurements are commonly carried out by one of the following techniques.

(a) Planimetry: This method is slow but is one of the most accurate. (Peak areas are readily reproducible to \( \pm 0.1 \text{ Cm}^2 \)). Accuracy decreases as peak area decreases. Some authors have recommended the use of fast recorder
chart speed to increase the peak area of small peaks and thus decrease the relative error in the measurement.

(b) Triangulation: This is done by drawing the base line of the peak and true tangents at the outward curve of the peak boundary, taking the precautions of using fine, hard pencil points and measurement of length of the base and height of the triangle with engine turned finely graduated rulers. This method has been used throughout this work.

(c) Integration by weighing: The weight of the unit area of the chart paper is determined. Peak area may be calculated by cutting out the peak and weighing the paper. The accuracy of this method depends upon the care exercised in cutting and on the constancy of thickness and moisture content of the chart paper. This method is destructive and now outdated.

(d) Electromechanical integrators: A number of electronic integrating device have been designed for direct attachment to recorders so that area integration may be performed simultaneously with the recording of the chromatogram.

From the total peak areas, each one can be calculated as a percentage of the total which will give the weight percentages of different components of the mixture. In this work the weight percentages of methyl esters were determined and converted to weight percentage as acids and thence to molar percentages as acids.
REFERENCES:


SECTION 3

GLYCERIDE STRUCTURE OF SOME NATURAL FATS
A REVIEW

Only the relevant theories and methods will be briefly reviewed, since a number of monographs and reviews are available which give a comprehensive account of the theories of glyceride structure of fats.

1. Concept of fatty acid distribution in triglycerides.

1.1 Even distribution:

The rule of even distribution as stated by Hilditch leads to heterogeneity in any glyceride molecule and homogeneity in the glyceride mixture i.e. the fat.

1.2 Random distribution:

The glyceride types possible by this rule can be calculated from the binomial expansion of \( (S + U)^3 \), where \( S \) and \( U \) are saturated and unsaturated acids in mole per cent.

1.3 Restricted random distribution:

Kartha found even and random distributions inadequate and put forward the restricted-random distribution (RRD) theory on the basis of glyceride compositions of natural fats determined by his acetone-acetic acid permanganate oxidation procedure. This
rule states that variation from the proportions of the glyceride types required by random distribution occurs when there is restriction in the formation of GS. Kartha has developed a rule based on this concept for calculation of the glyceride types from the molar proportions of S and GS.

1.4 Specific distribution:

The results obtained from lipolysis of natural fats have shown the predominant occurrence of S at the primary position of the glycerides. Vander wal developed the 1-3-random, 2-random concept on the basis of this observation. The two assumptions involved in this hypothesis are (i) that the fatty acids attached to the 1-, 2-, and 3-positions are distributed therein at random, regardless of their proportions and (ii) that the 1- and 3-positions are occupied by identical proportions of all fatty acids. Van der wal and Coleman and Fulton have independently derived a set of calculations to determine the glyceride composition of fats from lipolysis data.

Based on Hilditch's suggestion, Gunstone postulated that the secondary hydroxyl is preferentially acylated by $C_{18}$-U and the 1-, and 3-positions are then esterified by the remaining acids including any $C_{18}$-U not required at the secondary hydroxyl. Within these limits the distribution at each position is statistical.

Mattson and Volpenhein, from study of
a large number of fats, concluded that the 1- and 3-positions are preferentially acylated, though not at random, by S and fatty acids with more than 18 carbon atoms, and then all the unoccupied positions are acylated by the remaining acids consisting mostly of $\text{C}_{18}^-\text{U}$ which are distributed at random.

Coleman\(^{26}\) introduced a factor in Gunstone's calculations for glyceride types to account for the S found in the 2-position, in the case of those fats containing less than two thirds of S.

The RRD theory was extended by Kartha\(^{9}\) to include two more rules A and B. Rule A is applied when $\alpha, \beta$ enzyme mechanism acts alone and rule B is applied when it acts along with fatty acid specificity. Of the $\alpha$- and $\beta$- lipases assumed to be present in ripening seeds, the former promotes reversible esterification of the 2-position, with only higher acids. According to this theory\(^{9,27}\) specific esterification takes in the order $1 \rightarrow 2 \rightarrow 3$-position. The glyceride composition calculated on this basis\(^{27}\) agree with Kartha's experimental data.

Savary and Desnuelle\(^{28}\) proposed that $\alpha$-glycerophosphate is esterified with $\text{C}_{18}^-\text{U}$ at the 2-position by a specific enzyme. A second enzyme with no fatty acid specificity esterifies the 3-position. In the synthesis of triglycerides, the 1-position is acylated after the phosphoryl group is split off by the second enzyme or similar enzyme with no fatty acid specificity.
Kartha has put forward an explanation for the nonconformity of certain fats, which he terms the high order composite fats, to the RRD rule. According to him the composition of the fatty acid pool and consequently the glyceride mixture may vary from one cell to another. So the pooled fat from different cells may or may not conform to the RRD depending on the variations in glyceride composition from cell to cell. The degree of composition which expresses the degree of conformity of a fat to this distribution is calculated from the observed and calculated values of either GS2 U GSU2 or GU2.

Scholfield et al. have calculated the composition and structure of unsaturated glycerides of vegetable oils directly from the fatty acids composition of the oil by applying the following three simple rules in sequence. Saturated fatty acids and those with chain length greater than 18 carbon atoms are first distributed equally and randomly on the 1-, and 3-position of the glycerol moiety, oleic and linoleic acids are treated equally, or as a unit and distributed equally and randomly on all three glyceride positions with any excess from the 1- and 3-positions being added to the 2-position, and all remaining positions are filled by linoleic acid. They have found remarkably good agreement between calculated and experimentally determined fatty acid distributions for many vegetable oils whose compositions are reported in the literature.
2. Methods of determination of glyceride composition:

Low temperature crystallisation and counter current distribution methods are tedious.
Permanganate oxidation procedures are not quantitative. Periodate-permanganate oxidation and stereospecific enzymatic methods need to be examined further before they can be considered entirely reliable.
The use of GLC is restricted by the low volatility of glycerides. The lipolysis technique was found favourable for several reasons. It is operationally simple and provide considerable information and also gives data on positionally isomeric glycerides.

Pancreatic lipase hydrolysis method:

That pancreatic lipase is specific in splitting the fatty acids esterified at the outer positions of glycerol was first shown by Mattson and Beck and soon confirmed by others. Tattrie et al. observed that the enzyme does not differentiate between the 1- and 3-positions. The only major drawback is the migration tendency of the liberated 2-monoglycerides.

The lipolysis method was first employed by Mattson and Beck for the investigation of lard and interesterified fat and later for a large number of fats by others.

Lipolysis is usually performed at 37-40° by thorough mixing of lipase and emulsified substrate in a medium containing bile salt, CaCl₂ and buffer (pH-C₃, 8.0).
Several versions of this procedure are available with change either in buffer or bile salt or sample size. In a second method, the buffer is replaced by an electrolyte such as sodium or ammonium chloride solution and the pH is maintained during lipolysis by continuous addition of alkali.

In either of the methods, the time required to attain 50-60% hydrolysis varies from 20-30 mins, depending upon the activity of the lipase and the amount of substrate used. The hydrolysis is stopped by addition of mineral acid. The products are extracted with a solvent and made free of mineral acids.

Analysis of either the 2-monoglycerides or of the liberated fatty acids may in theory be used for calculation of the glyceride composition. However, Mattson and Volpenhein and Coleman have shown that the composition of the liberated fatty acids varies with the extent of lipolysis. This is probably because of acyl migration from the secondary to the primary positions and consequent cleavage and also because of the possible presence of non-specific lipase impurities in commercial lipase which cleaves fatty acids from the 2-position. Their experiments also showed that the composition of the 2-monoglycerides is independent of the extent of lipolysis.

Liberated 2-monoglycerides are isolated in two operations, viz., ion exchange chromatography to remove free fatty acids followed by silicic acid.
or Florisil or liquid column chromatography. They can be isolated in one operation by preparative silica gel TLC.

Luddy et al. developed a semimicro technique whereby hydrolysis can be done in 3-4 mins. on only 50 mg. of fat sample. Preparative TLC was employed to isolate the monoglycerides. The short duration of lipolysis reduces considerable acyl migration.

Tallent et al. simplified the lipolysis procedure especially for fats containing unusual labile acids by methylating the free fatty acids with diazomethane and silylating the partial glycerides in the lipolysis products. The entire mixture was then analysed by programmed temperature GLC. The use of bis (trimethylsilyl) acetamide simplified the procedure by eliminating the esterification step.

Though the enzymatic technique has been applied to a large number of fats, its suitability for fats containing short chain fatty acids is still uncertain; because of the great ease with which short-chain acids migrate from the 2- to the 1-position. It may be noted however that only the lipolysis technique can give a direct estimate of all the six glyceride types, GS3, GSSU, GSUS, GSUU, GUSU and GU3.

In the present investigation, 500 mg of pure natural triglyceride samples were hydrolysed at pH 8 and 37°, using a purified pork pancreatic lipase preparation.
with the addition of Ca$^{++}$ ions and bile salts.

Hydrolysis time varied between 10' and 12 minutes only; about 15 per cent splitting was sought so as to practically eliminate the complication of acyl migration. To ensure further accuracy only the isolated 2-monoglycerides were analysed.
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