A STUDY OF THE TRIGLYCERIDE COMPOSITION OF SOME NATURAL VEGETABLE OILS

being a Thesis presented by

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to the

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in application for

The Degree of Doctor of Philosophy

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Declaration

I hereby declare that this Thesis is based on results of experiments carried out by me, that it is my own composition, and that it has not been presented previously for a higher degree.

The research was carried out in the Chemical Research Laboratories of the United College in the University of St. Andrews under the direction of F. D. Gunstone, D.Sc., F.R.I.C.

F. B. PADLEY

CERTIFICATE

I hereby certify that Mr. Frederick Bolton Padley has spent eleven terms at research work under my supervision, has fulfilled the conditions of Ordinance 16 (St. Andrews) and that he is qualified to submit the accompanying Thesis in application for the degree of Doctor of Philosophy.

F. D. GUNSTONE
Research Supervisor

University Career

I entered the Faculty of Science and Technology, UNIVERSITY of MANCHESTER, in October, 1957. I pursued a recognised course and graduated B.Sc. Tech. with Upper Second Class HONOURS in Chemistry in 1960.

From 1960-1963 I carried out research in the Oils and Fats Division, UNILEVER RESEARCH LABORATORIES, PORT SUNLIGHT, CHESHIRE.

I was admitted as a Research Student in the United College, UNIVERSITY of ST. ANDREWS, in January, 1963 and was awarded a D.S.I.R. studentship which I held until October, 1965.

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F. B. PADLEY

Papers Published Prior to my Research Studentship

The separation of Glycerides by Thin-layer Chromatography on Silica Impregnated with Silver Nitrate.
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Papers Published from my Research Studentship Work

Thin-Layer Chromatography of Lipids - A Review. F. B. Padley, "Thin-layer Chromatography" G. B. Marini-Bettolo (editor) Elsevier Publishing Company, Amsterdam, 1964.

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SUMMARY

A quantitative method of determining the triglycerides in seed oils containing saturated, oleic, linoleic and linolenic acids has been developed. The method relies on our ability to separate triglycerides according to their degree of unsaturation by thin-layer chromatography on silica impregnated with silver nitrate. The separated triglycerides were completely extracted from the adsorbent and a known amount of methyl heptadecanoate added as an internal standard to the extracts prior to the determination of their fatty acid composition by gas-liquid chromatography.

The triglyceride composition of the fractions was calculated from their fatty acid composition assuming that each fraction contained glycerides with a specific number of double bonds. The weight of each fraction was obtained by comparing the amount of methyl heptadecanoate (internal standard) with the amount of normal methyl esters in the fraction.

Five seed oils, soya, wild rose, rubber, candlenut and linseed, were analysed by this method. The results agreed favourably with those calculated by Coleman's procedure from lipolysis results and those calculated according to Gunstone's theory.

The triglycerides of Sapium sebiferum kernel oil were also determined.

A number of seed oils containing linolenic or conjugated unsaturated acids were analysed by the enzyme-hydrolysis technique. The composition of the acids at the 2- position of the triglycerides was thus determined and is discussed in terms of enrichment and selectivity factors.

An attempt was made to detect optical activity in long chain triglycerides by partially hydrolysing them with pancreatic lipase

or Geotrichum candidum microbial lipase. The diglycerides produced were isolated and examined for optical activity.

The triglycerides in maturing sunflower seeds were analysed by thin-layer chromatography and enzyme hydrolysis. The lipids isolated from the maturing seeds were mainly triglyceride, only traces of free fatty acid were detected. An unusual glyceride composition was observed in the oil isolated from seeds in the very early stages of maturation. Reasons for this are discussed. Throughout the remaining period of maturation however the pattern of fatty acid distribution was similar to that found in the mature seeds.

INTRODUCTION

METHODS OF ANALYSING TRIGLYCERIDES, AND THEORIES OF FATTY ACID DISTRIBUTION IN NATURAL FATS

Since Chevreul (1823) and later Berthelöt (1860) made their observations on the chemical composition of natural fats, innumerable attempts to determine their glyceride composition have been made. Many theories have been propounded, only to be superseded by others based on hard won experimental evidence. The analysis of fats has been an especially difficult problem because they contain a large number of triglycerides having similar chemical and physical properties. In the last decade however many new chromatographic techniques have been devised but prior to our work, had not been fully exploited in triglyceride analysis.

In this introduction to Parts I and II of this thesis the various methods of analysing triglyceride mixtures are reviewed, along with the theories concerning fatty acid distribution in natural fats.

A. Fractional Crystallisation, Oxidation and Countercurrent Distribution

A brief mention is made of methods which have relied on one of the principles above, which under the best circumstances have only provided a partial insight to the composition of natural fats.

The fractional crystallisation of fats was widely exploited by Hilditch and co-workers [1,2]. Individual glycerides were not obtained but the composition of a fat was deduced after making certain assumptions about the glycerides in each fraction. In some cases the results are misleading, especially for the more unsaturated oils. More recently an improved procedure using solvents containing silver nitrate was described [3].

The trisaturated content of oils was determined after oxidation (1,4). Kartha (5,6) estimated the tri, di and mono-unsaturated triglycerides by separating the azeleoglycerides

produced on oxidation, but other workers [7,8] found the method unsatisfactory. The mild oxidising agent described by von Rudloff [9] was used by Youngs [10] and gives reliable results. Chromatographic methods of separating oxidised triglycerides are described later.

Dutton and co-workers [11,12,13] separated triglycerides according to molecular weight and degree of unsaturation by counter-current distribution. The method is more efficient than fractional crystallisation, especially for the more unsaturated oils and the improved resolution enables one to make fairly accurate estimations of some of the glycerides present.

Thermal gradient crystallisation, first described by Baker and Williams [14] for separating high polymers, was used to separate triglycerides [15,16,17]. The method is limited by the formation of eutectic mixtures [15], but useful separations were obtained between glycerides whose fatty acids differed sufficiently in chain length or unsaturation.

The possibility of obtaining sharp separations of triglycerides by chromatographic methods has been visualised for many years but only recently has any measure of success been achieved. Coleman [18] and Kaufmann [19] have written earlier reviews on this subject.

The chromatography of triglycerides is conveniently divided into two main sections, adsorption and partition. The various techniques of column, paper, thin-layer and gas-liquid chromatography are therefore dealt with under these two main headings.

B. ADSORPTION CHROMATOGRAPHY

Adsorbents of silica or alumina have been used predominantly in attempts to separate triglycerides. Despite the reported hydrolysis of ester linkages and isomerisation of double bonds [20,23]

by alumina this adsorbent has been used by numerous workers who apparently observed little or no modification of the triglycerides after chromatography. The modification of organic compounds on silica and alumina was discussed by E. Lederer and M. Lederer [24], and Landa and Markovel [25]. Wren [26] in a recent investigation using silica columns concluded that the oxidation of unsaturated compounds, although reported previously [20], did not occur provided that reasonable precautions were taken. It is concluded therefore that the chemical modification of compounds during chromatography depends, not only on the activity of the adsorbent and purity of solvents, but also on chromatographic technique.

Attempts to separate triglycerides by adsorption chromatography on silica and alumina have met with very limited success however. Separation is only achieved when the glycerides differ greatly in molecular weight or contain some unusual acid, e.g. hydroxy or epoxy, which makes the triglyceride more polar. These methods are therefore described only briefly in this introduction.

Vastly improved separations have been obtained on adsorbents impregnated with silver nitrate. Silver ions form π-complexes with olefinic double bonds [27] and numerous workers [28-34] have utilised this effect to separate compounds primarily according to the number of double bonds they contain. The separations are affected by the configuration and position of the double bonds and to a minor extent by chain length.

1. Chromatography on Silica, Alumina, Charcoal and Bleaching Earth

i) Column Chromatography. Chromatographic separations of triglycerides were reported as early as 1938 by Kaufmann [35]. Lower molecular weight triglycerides were the most strongly adsorbed on all the adsorbents above except alumina [36,37] and tristearin

was completely separated from tributyrin on bleaching earth [36]. Attempts to separate linseed [38,39], sardine [40] and soya bean [40,41] oil triglycerides on alumina were unsuccessful but adsorption depended upon the degree of unsaturation. Sahasrabudhe and Chapman [42] found that on silica, adsorption depended upon both the degree of unsaturation and molecular weight of the triglyceride. Holman and co-workers [43,44] completely separated stillingia oil triglycerides into two fractions on silica and alumina. The most polar fraction contained deca-2,4-dienoic acid.

Displacement analysis of triglycerides, first proposed by Claesson [45], was developed by Hamilton and Holman [46,47] who separated some simple long chain triglycerides. Unsaturated glycerides had low adsorbtivities and were inseparable however.

The difficulties encountered in monitoring column eluates have recently been overcome with the development of a simple automatic device described by James et al [48].

ii) Thin-layer Chromatography on Silica. Thin-layer chromatography has in many ways provided the bridge between column and paper chromatography. Thin-layer chromatographic separations are generally much sharper than those obtained by paper chromatography but preparative thin-layer chromatography cannot handle the large amounts of material normally associated with column chromatography. It is in the field of thin-layer chromatography that some of the major developments in triglyceride analysis have taken place.

The separations obtained by adsorption column chromatography are applicable to T.L.C. Adsorption chromatography is of value therefore only when the triglycerides to be separated vary widely in molecular weight or polarity [49]. The long chain triglycerides of Sapium sebiferum were separated from those containing deca- 2,4-dienoic acid on silica [44]. Milk triglycerides [50] were separated into two fractions by chromatography on silica prior to lipolysis, and silica-silver nitrate chromatography. The

triglycerides contained 0,1 or 2 short chain acids and non-random distribution of the fatty acids in the long chain triglycerides was observed.

Mangold and Morris [51] examined numerous seed oils containing epoxy, hydroxy, keto, cyclopropane or acetylenic acids and compared the extracts from healthy and diseased sterculia nuts by silica chromatography.

2. Chromatography on Silica - Silver Nitrate

i) Column Chromatography. de Vries [52,53] first described the separation of glycerides according to their degrees of unsaturation and configuration of the double bonds, on columns of silica impregnated with silver nitrate. Thus tristearin, oleodipalmitin, stearodiolein, and triolein were distinctly separated from one another as was a mixture of elaidodipalmitin and oleodipalmitin. Palm oil was separated into six different fractions with fatty acid compositions corresponding closely to tripalmitin, dipalmitolein, dipalmitolinolein, palmitodiolein, mixtures of palmitolinolein and triolein, and lastly glycerides with four or more double bonds.

Highly unsaturated glycerides have not been separated by column chromatography because of the bad tailing of one glyceride into another; the method has been largely superseded by thin-layer chromatography.

ii) Thin-layer Chromatography. Barrett, Dallas and Padley [34,54] separated glycerides with 0-6 double bonds on silica gel G impregnated with silver nitrate (10-30%). 1- and 2-oleodistearin were also separated and this provides the only instance of the chromatographic separation of positional isomers. Separations dependent on the configuration of the double bonds in the glyceride were obtained by de Vries [55,56] thus oleodistearin and elaidodistearin were resolved. More recently we [57] found that

the two double bonds in lincleic acid formed a stronger complex than the combined effect of two double bonds in two oleic chains; more surprisingly the three double bonds in linolenic acid formed a stronger complex than the four double bonds in two linoleic chains. It was therefore possible to separate glycerides with the same number of double bonds (c.f. ref. 30).

The chromatoplates are best prepared according to the method of Barrett et al [54] who prepared a slurry of silica gel G in aqueous silver nitrate solution. The silica may also be impregnated by spraying a silica chromatoplate with a saturated methanolic solution of silver nitrate [32]. Morris has shown that the concentration of silver nitrate is not critical until quite low concentrations are reached.

Triglyceride mixtures are separated by eluting the chromatoplate with a variety of solvents including a carbon tetrachloride:chloroform:acetic acid mixture (60:40:0.5) containing small quantities of ethyl alcohol (0-3%) depending on the degree of unsaturation of the triglyceride. Mixtures of benzene and ether are also useful developing solvents because the polarity is more easily controlled.

The separated components are detected by spraying with a methanolic solution of dichloro-fluorescein and viewing by U.V. light. The glycerides are also detected by charring at 250° after spraying with phosphoric (50% aqueous) or sulphuric (50% aqueous) acid. A simpler method is to pass the plate (unsprayed) smoothly under a compressed-air/gas, blow torch flame.

The quantitative estimation of the separated components has been achieved in a number of ways.

Barrett et al [54] charred the separated triglycerides on the plate and determined the intensity of the spots using a photodensitometer. Standard glyceride mixtures were run alongside the unknown mixture because the response varied with different glycerides and also with their position on the plate. Results, accurate to ± 2 units per cent, were obtained for mixtures of known composition, and the natural fats, palm oil, cocoa butter, shea butter, and lard were analysed. The method provides results in a relatively short time but is only applicable to chromatograms where fairly sharp separations are obtained. More recently Kaufmann and Mukherjee [58] using two dimensional chromatography charred the separated triglycerides on a non-impregnated portion of the plate [59].

Triglycerides have been separated by preparative-thin-layer chromatography thus allowing amore detailed examination of each fraction [55,57,60].

Triglyceride mixtures (20-200 mg) are readily separated using a larger chromatoplate (20 cm x 40 cm long) and a thicker adsorbent layer (0.3-1 mm). The plate is developed either vertically or horizontally [61] for 2-3 hours and the separated components detected with dichlorofluorescein. The triglycerides may then be extracted from the adsorbent with suitable solvents and their relative amounts determined.

The triglycerides have been extracted with ether, either by shaking [55] or by continuous extraction [62,63]. Wehave found that highly unsaturated triglycerides (trilinolenin) are strongly adsorbed and complete recovery was obtained by extracting the adsorbent six times with a methanol:ether:water (50:50:10) mixture in centrifuge tubes [3,57].

de Vries, Jurriens and Schouten determined the proportion of each triglyceride fraction either by weighing [55] or by estimating

by titration [62,64] the glycerol produced when the triglycerides were saponified. Both methods when applied to synthetic mixtures gave results in good agreement with the known values. Practically complete analyses of palm [63], groundnut and soya bean oils [64] were obtained by quantitative T.L.C. and lipolysis of the isolated fractions [65].

Kaufmann and Wessels [60] chromatographed sunflower seed oil triglycerides, weighed the extracted fractions and determined their fatty acid composition by G.L.C. Each fraction was also characterised by lipolysis and by reverse-phase thin-layer chromatography.

We [3,57,66] have determined the relative amounts of the extracted fractions by adding a known amount of methyl heptadecanoate. The triglycerides were converted to methyl esters and the total methyl esters determined by G.L.C. The amount of each fraction was calculated from the relative proportions of methyl heptadecanoate it contained. The triglyceride composition was calculated from the fatty acid composition of the fraction assuming that the glycerides contained a limited number of double bonds. The compositions of a large number of linoleic and linolenic acid containing seed fats were determined [57,66]. A similar method has recently been described by Blank, Verdino and Privett [67] who also examined the fractions by lipolysis.

Apart from photodensitometry, these quantitative methods depend on the complete recovery of the triglycerides from the adsorbent. Provided that this is accomplished then the best methods of determining the relative proportions of the fractions are either by direct weighing, or incorporation of an internal standard prior to G.L.C. In direct weighing it is imperative that no contamination

or loss of the sample occurs, whereas in the latter procedure all the fatty acid methyl esters must be eluted from the G.L.C. column and detected.

Although silica-silver nitrate chromatography does not differentiate between the saturated acids, in conjunction with the lipolysis technique it permits a very detailed analysis of natural oils.

3. Separation of Modified Glycerides

(i) Column Chromatography. Triglycerides have been separated as their mercuric acetate adducts on silica by Hirayama [68]. Triglycerides with more than four double bonds were strongly adsorbed and could not be eluted quantitatively. The results, obtained for a number of vegetable oils, are given. Youngs [10] oxidised synthetic and natural mixtures of glycerides [9] and separated the resulting azelaoglycerides on silica into two fractions. One fraction contained the trisaturated glycerides and the monoazelaoglycerides (from S_2U)* and the other fraction contained the di- and triazelaoglycerides (from SU_2 and U_3). Each fraction was hydrolysed with pancreatic lipase and from the overall results it was possible to calculate six glyceride classes of the whole fat (S_3, SUS, SSU, USU, U_3). The results for five natural fats are reported. All the unsaturated acids are oxidised to azelaic acid and are therefore indistinguishable. The method is extremely useful for distinguishing the glyceride classes above, especially when the oxidised glycerides are separated by gas-liquid chromatography.

(ii) Thin-layer Chromatography. Prior to the development of silica-silver nitrate chromatography Privett and Blank [69,70] described an ingenious method of separating unsaturated

* Abbreviations, page 100.

triglycerides. The triglycerides were converted to their corresponding ozonides which were then separated according to the number of ozonide groups (i.e. double bonds) in the molecule. Each fraction was then converted, by reduction, to esters of aldehyde fatty acids which were further separated by thin-layer chromatography.

The distribution of the double bonds in the triglycerides of each fraction was deduced from the relative amounts of the different "aldehyde cores". The saturated acids in each fraction were determined by gas-liquid chromatography.

C. PARTITION CHROMATOGRAPHY

1. Reverse-phase Liquid-liquid Partition Chromatography

The underlying principle in liquid-liquid partition chromatography is that a non-polar stationary phase and a polar mobile phase are used. The result is that glycerides are separated primarily according to the number of carbon atoms they contain (carbon number). An olefinic double bond effectively decreases the carbon number by approximately 2- carbon atoms. Many glycerides therefore have similar R_F values or retention times, for example triolein and tripalmitin, and are termed "critical pairs".

Column, paper and thin-layer chromatographic techniques have been described which utilise this principle. The only satisfactory column chromatographic system was described by Hirsch [71,72] who used factice as the stationary phase.

Paper and thin-layer chromatography have been exploited mainly by Kaufmann and co-workers. Excellent glyceride separations have been described, including the separation of "critical pairs"

by multi-development thin-layer chromatography. Only very small amounts of material are normally separated however and certain difficulties in detecting and quantitatively estimating the separated components have been encountered.

The methods should not be underestimated as they could provide one solution to the problem of analysing the more saturated natural fats.

(i) Column Chromatography. Excellent separations of a mixture of simple short and long chain triglycerides were obtained by Hirsch [71,72] using factice (polymerised soybean oil) as a stationary phase and 5% water in acetone as the mobile phase. Chromatography of linseed oil gave eight separate triglyceride peaks, the first being pure trilinolenin (9 double bonds) and the second peak contained linoleodilinolenin (8 double bonds). Subsequent peaks, although more complex, had the equivalent of one double bond less than earlier fractions.

Less effective glyceride separations were described by Black and Hammond [73] using silane treated celite as the support for a two phase mixture of acetone, heptane and water.

The partial separation of nine glyceride components of coconut oil on rubber columns was described by Trowbridge, Herrick and Bauman [74]. Dry columns were introduced in attempts to transfer the separations of layer chromatography to column chromatography [75,76]. Stainer and Bonar [77] prepared a column using dry cellulose powder coated with liquid paraffin as the stationary phase. Some of the main glycerides of cocoa butter were separated by elution with an acetone-methanol mixture.

(ii) Paper Chromatography. The great potentialities of reverse-phase chromatography for triglyceride analysis were

indicated by Mangold, Lamp and Schlenk [78] when they separated trilaurin, trimyristin, tripalmitin and tristearin on silicone oil impregnated paper. Priori [79] separated vegetable oil triglycerides using liquid paraffin as a stationary phase and detected 5% rapeseed oil in olive, sesame and arachis oil.

Many of the advances in paper chromatography of triglycerides have been made by Kaufmann and co-workers [80,86] and the following paragraphs summarise their work.

The paper chromatogram is prepared by impregnating the paper either permanently or temporarily with a non-polar stationary phase. Liquid paraffin and, less frequently, silicone oil are used for permanent impregnation [80] and lower boiling hydrocarbons (undecane, tetradecane) for temporary impregnation [83]. In temporary impregnation the stationary phase is removed after development of the chromatogram to facilitate the detection of unsaturated triglycerides and to assist chemical reactions on the paper. The best separations, however, are obtained using liquid paraffin as the stationary phase.

The chromatogram is developed with a polar solvent generally acetone-acetonitrile (8:2), glacial acetic acid, or acetone-methanol (9:1). Only a small amount (10-100 μg) of the triglyceride mixture is applied to a chromatogram to obtain the most complete separations. Larger amounts (40 mg) may be separated either by ascending development or by circular paper chromatography at the expense of certain triglyceride separations [86]. The separations are considerably improved by developing the same chromatogram a number of times.

After development the unsaturated triglycerides are easily detected with iodine vapour either before or after removal of the

stationary phase. No satisfactory method of detecting saturated glycerides on paraffin impregnated paper has been described. The glycerides can be detected however by removing the stationary phase and either spraying with, or immersing the paper in, an aqueous alcoholic solution of Sudan Black (0.01%) [83]; other dyes suitable for detecting components have also been described [81]. A method which readily detects unsaturated components and to a lesser extent saturated components on liquid paraffin impregnated papers is a combination of α-cyclodextrin and iodine [78].

The interpretation of chromatographic separations is simplified by calculating the paper-chromatographic index, P.C.I.[83] for each glyceride. The P.C.I. is equal to the total number of carbon atoms in the triglyceride fatty acids minus 2 for each double bond in the triglyceride. Triglycerides with the same P.C.I. have approximately the same R_F values. Deviations from this general rule occur when critical pairs of glycerides differ widely in the numbers of double bonds they contain.

The relationship between R_F values and molecular properties has been discussed [83,84,87,101]. Kaufmann and Makus [81] separated twelve simple and mixed-fatty acid triglycerides from tricaprin to tristearin using a number of stationary phases, in particular undecane, with glacial acetic acid as the mobile phase. Seventeen natural fats were also examined using similar chromatographic conditions. Many of the glycerides in natural fats have the same paper chromatographic index and are inseparable, thus only three components were detected in lard, olive oil and cocoa butter whereas butterfat gave ten. Marked differences between soya bean oil triglycerides, before and after interesterification, were observed by Kaufmann and Schnurbusch [80,88]. Kaufmann and Makus [83] separated the mono-acid triglycerides from tricaprylin to tristearin

and also demonstrated the occurrence of critical pairs of triglycerides in chromatography. The separated glycerides of corn, soya bean and cottonseed oils were extracted from the chromatogram, and their fatty acids determine quantitatively by paper chromatography.

Kaufmann and Ahmad [89] discussed the errors incurred in the quantitative determination of fatty acids by paper chromatography, a method by which triglycerides may also be estimated.

Stainer and Bonar [90] separated the mono-unsaturated glycerides of cocoa butter which were then estimated by comparing the areas of the spots.

Natural glycerides were partially resolved by Swartwout and Gross [91] using silica coated, or uncoated, glass paper. The glycerides were characterised by converting them to fatty acid methyl esters and running the chromatogram in the second dimension. The charred components could be estimated by photodensitometry. Cry [92] separated acetoglycerides on silica impregnated paper by adsorption as distinct from reverse-phase chromatography.

Hirayama and co-workers [93,94] have described a number of methods of separating and estimating triglycerides.

Some interesting developments have been made by Vereschchagin and co-workers [95-101]. Apart from estimating the separated triglycerides by photodensitometry [95] they have also extracted the glycerides from the chromatogram and analysed their fatty acid methyl esters by gas-liquid chromatography [96]. In this way they characterised and estimated thirteen glycerides, including stearodiolein (1.5%) and palmitodiolein (2.5%), of cottonseed oil. More recently Vereshchagin and Skvortsova [99,100] combined the separations of reverse-phase with those of silver nitrate

chromatography. Triglycerides were separated using dodecane as the stationary phase and a saturated solution of silver nitrate and dodecane in 97.5-100% methanol as the mobile phase. The fatty acid methyl esters of the separated triglycerides were analysed by gas-liquid chromatography. In this way glyceride separations depend not only on the paper chromatographic index but also on the number and arrangement of double bonds in the glyceride molecule. The following glycerides were separated and characterised: dipalmitolinolein, palmitoleo linolein, stearodilinolein, dioleolinolein, palmitodilinolein, oleodilinolein and trilinolein. All these separations are obtained on silica-silver nitrate [57] except for the separation between palmitodilinolein and stearodilinolein.

(iii) Thin-layer Chromatography. Kaufmann and co-workers[102,107] have made an extensive study in this field and have obtained some remarkable triglyceride separations. The techniques are analogous to those of paper chromatography except that the support is more fragile in thin-layer chromatography.

The best stationary phases described are tetradecane (240°-250° b.pt) [102,107], silicone oil [104] or liquid paraffin [106] supported on Kieselguhr G, alumina, or gypsum. Tetradecane, as in paper chromatography is used as a temporary stationary phase [102]. Aqueous acetic acid or acetone-acetonitrile mixtures are normally used as the mobile phase. If a temporary stationary phase is used the separated components may be detected using iodine vapour, aqueous rhodamine B (0.05%), 2'7'-dichlorofluorescein (0.1% in ethanol) or phosphomolybdic acid [102]. Saturated glycerides are more difficult to detect than unsaturated ones especially if the stationary phase cannot be removed. It was possible to use the chemical reactions and detection procedures of paper chromatography when layers of

gypsum [105] were used to support the stationary phase. For a similar purpose Kaufmann [103] has described a method of silanising the silica layer which was then strong enough to be immersed in reagents. Glycerides could also be detected using a transparency phenomenon.

Numerous critical pairs of triglycerides were separable after hydrogenating or brominating [107] the mixture on the chromoplate.

Kaufmann et al improved their earlier techniques and separated "critical pairs", e.g. triolein from palmitodiolein, by developing the chromatogram two or three times [106]. The separated components were detected with iodine (unsaturated glycerides) or iodine and α-cyclodextrin (saturated and unsaturated glycerides). Soya bean, linseed, maize, palm kernel and groundnut oils were examined by this method. Cocoa butter and cocoa butter substitutes were also distinguished from one another [108].

Michalec, Sulc and Mestan [109] separated synthetic and natural mixtures of triglycerides and Anker and Sonani [110] checked the identity and purity of some natural oils. Kwapniewski [111] described analytical and preparative methods of separating glyceride mixtures.

In no case were these separations as complete as those obtained by Kaufmann's multi-development procedure.

2. Gas-Liquid Chromatography

The analysis of volatile organic mixtures by gas-liquid chromatography is now accomplished with ease and great reproducibility. A number of problems arise when the technique is applied to triglyceride separations however [112,113].

The high temperature required to vaporise the triglycerides makes the choice of a suitable stationary phase difficult, and more important, may cause degradation of the triglycerides. The most stable stationary phase is a silicone gum rubber (S.E. 30) which unfortunately is non-polar. Triglyceride separations have therefore depended primarily on the number of carbon atoms they contain and each chromatographic peak may represent a mixture of triglycerides. The chromatograms are usually temperature programmed to ensure that all the components give fairly sharp and well defined peaks.

Litchfield, Harlow and Reiser [124] have recently described the optimum conditions for the quantitative analysis of triglyceride mixtures. We therefore have an invaluable method of triglyceride analysis which is both quick and precise.

Low molecular weight triglycerides were separated by Huebner[114] who analysed mono- and diglyceride mixtures after acetylation. Fryer, Ormand and Crump [115] were, however, the first to apply the technique to long chain fatty acid triglycerides and although moderate degradation occurred, a 'fingerprint' chromatogram of several edible oils was obtained.

Huebner [112] separated a mixture of simple triglycerides from triacetin to tristearin on a temperature-programmed silicone rubber column. The components were detected with a thermal conductivity detector and it was found that Peak Area $\propto$ Per cent Mole for trihexanoin-tristearin. It was established that the components emerging from the column were undegraded triglycerides [116]. Pelick, Supina and Rose [117] separated triglycerides on silicone gum rubber (S.E. 30) and judged from the peak areas that 90% of the triglycerides were eluted from the column. Triolein and trilinolein were separated by Applied Science workers [118].

Kuksis, McCarthy and Beveridge have improved the chromatographic technique and obtained excellent glyceride separations according to molecular weight from trioctanoin to tristearin [119]. Because the triglycerides are separated according to the number of carbon atoms they contain the technique is best applied to oils containing a large number of fatty acids of different chain lengths, e.g. butter and coconut oils. The triglycerides of oils which consist mainly of palmitic, stearic, oleic and linoleic acids are not separated into many fractions, and all give similar chromatograms. For this reason Kuksis et al have analysed butter oil and coconut oil in greater detail [119-123].

The quantitative analysis of a known simple triglyceride mixture [119,120] gave results within ± 5 units per cent, a discrepancy caused mainly by incomplete vapourisation of the large chain triglycerides and not by degradation. The hot wire detector response gave areas approximately proportional to the weight of the component. Within the limits of accuracy of the method (± 5 units per cent) this relationship held for mixtures of long and short chain triglycerides despite the increased proportion of oxygen in short chain triglycerides. A quantitative estimation of butter fat triglycerides was made [120-123] in which 31 glyceride fractions with C.N^{o} 24-54 were detected. The results were confirmed by analysing molecular distillates of butterfat and also butterfats to which known amounts of saturated and unsaturated triglycerides had been added.

Kuksis et al [123] compared butter and coconut fats with those obtained after interesterification. The results showed that a random triglyceride mixture was not produced, especially in the case of interesterified butterfat which contained more long and

short chain triglycerides than predicted by random distribution.

Gas-liquid chromatography of unknown butterfat samples detected 5-10% of added lard in vegetable fat [122]. The ease of detecting adulterants varied with the type of fat added, thus mixtures of lard and coconut oil could be made which closely matched the gas chromatograms of butter fat.

Degradation of tripalmitin (5%) and tristearin (15%) during chromatography was observed by Jurriens and Kroesen [64] and in quantitative work the results were corrected accordingly.

Very recently Litchfield, Harlow and Reiser [124] determined the optimum conditions for the gas-liquid chromatography of triglycerides. This work represents an important step in triglyceride analysis and for the first time describes methods of obtaining reliable quantitative results.

3. Gas-Liquid Chromatography of Oxidised Triglycerides

Gas-Liquid chromatography of triglycerides using non-polar columns does not distinguish between saturated and unsaturated triglycerides. Youngs and Subbaram [125], and McCarthy and Kuksis [126] oxidised triglyceride mixtures (9), converting the unsaturated glycerides into compounds with smaller carbon numbers (azelaoglycerides). Using a flame ionisation detector Youngs found that for a mixture of triazelain and tripalmitin the peak areas were not proportional to weight per cent. The areas were, however, proportional to the mole percentage after making suitable corrections for the number of carboxyl groups in the molecule. Thus trimethyl azelain has 33 carbon atoms and 6 carboxyl groups and therefore 27 carbon atoms effective for flame ionisation detection. Youngs divided the peak areas by the appropriate "effective carbon numbers"

to give mole percentages. The quantitative analysis of tripalmitin-tristearin mixtures showed a loss of tristearin relative to tripalmitin but this error was negligible when analysing natural fats.

Gas-liquid chromatography of oxidised glycerides in its simplest form does not differentiate between unsaturated acids but does distinguish between individual saturated acids. A large number of vegetable and animal fats have been analysed by this method [127]. The isomeric glycerides of cocoa butter, olive oil, cottonseed oil and soyabean oil were also determined by lipolysis of the fractionated azelaoglycerides [10,125]. The results generally agreed with those predicted by lipolysis with the exceptions of human fat, and bitter gourd seed fat (Momordica charantia) [128]. The latter contained 80% stearodiunsaturated glycerides compared to 45% calculated from lipase hydrolysis data. Possible reasons for this unusual result were discussed.

4. The Consecutive use of Silica-Silver Nitrate and Gas-Liquid Chromatography

Youngs and Subbaram [129] were the first to examine the fractions obtained by silica-silver nitrate chromatography by gas-liquid chromatography. The glycerides were fractionated according to their degree of unsaturation and the composition of each fraction was determined by gas-liquid chromatography of the oxidised glycerides [125]. All the chemically different glycerides (excluding positional isomers) of myristic, palmitic, stearic, oleic, linoleic and linolenic acids may be determined. Lard and cocoa butter were analysed and 24 and 18 glycerides determined respectively.

The triglycerides of Cuphea ilavia seed fat were determined by Litchfield and co-workers [130]. The triglycerides were

separated by preparative thin-layer chromatography on silver-nitrate impregnated silica and the triglycerides of each fraction were determined by gas-liquid chromatography without prior modification. The fat was particularly suited to this analysis because of the high proportion of low molecular weight fatty acids it contained (90% decanoic acid), and 17 components were resolved.

The triglycerides of cocoa butter, sumatra palm oil and lard were analysed by Jurriens and Kroesen [64]. The fatty acid composition and the acids in the 2-position of each fraction separated by silica-silver nitrate thin-layer chromatography were determined. The triglycerides in each fraction were further analysed by gas-liquid chromatography after hydrogenation in order to determine the distribution of the saturated fatty acids. In this way the individual glycerides, including positional isomers, were determined.

A theoretical treatment by McCarthy and Kuksis [131] attempts to correlate all the different chromatographic separations. They suggest a scheme whereby a number of techniques may be used consecutively to give the maximum amount of information.

D. Methods of Determining The Composition of the Acids at the 2- Position

1. Pancreatic Lipase Hydrolysis

The discovery that pig pancreatic lipase only hydrolyses the fatty acids from the 1- and 3- positions of a triglyceride [132-139] has provided us with a standard technique for determining the fatty acid composition at the 2- position. The partial hydrolysis products consist of tri, di and monoglycerides, free fatty acid and glycerol [140]. The removal of the acyl groups from the 1- and 3- positions produces a monoglyceride whose fatty acid composition is therefore the same as that of the 2- position in the

original triglyceride or seed oil(Fig. 1)

This technique was a major advance towards elucidating the triglyceride composition of natural fats and has been reviewed by Desnuelle and Savary [141] and by Coleman [18]. A large number of vegetable seed oils have been analysed [18,142-149] and a common pattern of fatty acid distribution in oils containing saturated, oleic, and linoleic acids has emerged [140,148,150,151]. In practically every oil examined the 2- position is occupied almost exclusively by C_{18} unsaturated acids. On this unusual result have been based a number of theories of fatty acid distribution in natural fats.

Results by Mattsen and Volpenhein [147] showed that fatty acid distribution was not governed by unsaturation alone. Unsaturated C_{20} acids behaved similarly to saturated C_{18} acids. Closer examination of their lipolysis results also indicated a significant difference between the relative distribution of oleic, linoleic and linolenic acids [149,151]. Different acids are therefore distributed in different and usually consistent ways.

Gunstone has defined two terms, the enrichment factor and the selectivity factor [149] which simplify the interpretation of lipolysis results. The enrichment factor is given by the ratio of the molar concentration of an acid in the 2- position to its molar concentration in the triglyceride. Enrichment factors > 1 indicate a preference for the 2- position; $= 1$, random distribution and < 1 a preference for the 1,3- positions. Normally saturated acids are esterified almost exclusively in the 1,3- position and are classified as Category I acids. Variations in the saturated acid content of an oil can cause quite big variations in the enrichment factors of C_{18} unsaturated acids (Category II acids). The selectivity factor was therefore defined as the ratio of the enrichment factor of a Category II acid to the enrichment factor

of the total Category II acids. This enables a more useful comparison to be made between the distributions of Category II acids in different oils.

2. Mass Spectrometry

The technical difficulties encountered by Stenhagen [152] have been overcome by the work of Barber, Merren and Kelly [153]. The spectra of some pure triglycerides were obtained by these workers who were able to distinguish between the two positional isomers of oleo-distearin quite readily. Although the technique is useful for characterising pure triglycerides it is doubtful if it will be of any value for analysing complex triglyceride mixtures. It could be very useful for characterising the triglycerides separated by gas-liquid chromatography however.

E. Theories of Fatty-acid Distribution in Natural Fats

Numerous attempts have been made to correlate the growing mass of experimental data obtained for natural fats [1,140,146,150,154,155]. The theories on fatty acid distribution have necessarily changed as our knowledge increased. A number of papers reviewing this subject have been published [1,18,155].

The theories described prior to lipase hydrolysis will be mentioned only briefly. Some early analytical data for animal fats (1,156) indicated that the fatty acids were randomly distributed but more recent work has disproved this [145]. Hilditch and co-workers [1,157,158] described an Even distribution, or in its quantitative form "Widest" distribution [155] theory:- "if the concentration of an acid in an oil $\leq 33.3\%$ it occurs no more than once in any given triglyceride, $\leq 66.7\%$ no more than twice and $> 66.7\%$ two to three times". There was reasonable agreement between theortical oxidation and fractional crystallisation studies. Kartha's

theory of Restricted random distribution corrected the random distribution theory by inserting the experimentally determined values for trisaturated glycerides into the calculation [159-162]. A number of objections to Kartha's theory have been raised [150,163].

All theolder theories were refuted when it was shown by lipolysis that the 2- position in most natural fats is occupied almost completely by C_{18} unsaturated acids [18,142-149].

A positional theory by Youngs [164] assumed that random esterification took place. To account for the unsaturated composition of the 2- position however he suggested that a rearrangement of the diglyceride to a "preferred form" occurred in his synthetic pathway. The theory was later abandoned because of the poor correlation with experimental results [10].

Two groups of workers, V ander Wal [150] and Coleman and Fulton [140] independently suggested a Positional theory of fatty acid distribution. After determining the fatty acid composition at the 2- position the theory assumes that the remaining acids are distributed randomly at the 1,3- positions. The values for isomeric glycerides calculated on this basis are very different from those given by earlier theories. This theory has become one of the most widely accepted for calculating glyceride compositions of oils.

An idealised form of the positional theory was advanced by Gunstone [155]; in it the 2- position is esterified by oleic, linoleic and linolenic acids only, if sufficient are available. This statement has to be modified as the distribution of more unusual acids is determined. The remaining acids are distributed randomly in the 1,3- positions to obtain values which closely represent those obtained experimentally. The proportions of the

six glyceride classes are calculated as follows:-

Ref. s = % saturated acid in fat

α = % saturated acids in 2- position (0 for $s \leq 66.7\%$)

y = % saturated in 1,3- positions = $\frac{3s-\alpha}{2}$

Then $sss^1 = \alpha y^2$

$ssu = 2\alpha y\,(100-y)$

$sus = (100-\alpha)\,y^2$

$suu = 2(100-\alpha)\,y\,(100-y)$

$usu = \alpha(100-y)^2$

$uuu = (100-\alpha)\,(100-y)^2$

The advantage of Gunstone's theory is that it requires the minimum experimental information. It does not however predict the presence of many glyceride isomers which have been found in relatively small amounts in natural fats.

The disadvantage of both positional theories is that they assume the equivalence of the 1 and 3-positions and therefore make no allowance for the possible preferential acylation of one position by saturated acids say.

More recently Tsuda (165,166) described a theory of <u>ordered distribution</u> in which the 1,2 and 3- positions are considered separately. Ordered distribution is defined as preferential esterification of the triglyceride positions in the order 1,2,3 by the saturated acids. Any remaining positions are filled by the unsaturated acids. The amount of ordered distribution will vary however and in some cases the distribution will be random. This is determined by trial and error in conjunction with experimental results. The theory has the advantage that it allows for all

1. Abbreviations, page 100.

types of distribution. The main criticisms of the theory are that it lacks simplicity and the composition of unknown vegetable oils cannot be predicted.

Since our work began there have been many important advances in the techniques of triglyceride analysis. In this review are included over fifty papers which have been published since January 1963, and give some measure of the great interest this subject has aroused. In addition, thirty-five papers were presented at the T.P. Hilditch Symposium on the Analysis of Natural Fat Triglycerides in April 1965 [167]. To obtain some idea of the field as it was when we commenced our research one should discount practically all the work on silica-silver nitrate chromatography and many of the advances made with gas-liquid chromatography.

Our main object was to study the distribution of saturated, oleic, linoleic and linolenic acids and to a lesser extent conjugated unsaturated acids in vegetable seed oils. We have therefore devised a quantitative method for determining triglycerides, based on chromatographic methods. The composition of the fatty acids esterified at the 2- position of numerous seed oils has also been determined by the enzyme-hydrolysis technique. An attempt to distinguish between the acids at the 1 and 3- positions has also been made. Finally the composition of the triglycerides in maturing sunflower seeds was determined.

PART I

THE QUANTITATIVE ANALYSIS OF TRIGLYCERIDES IN SEED OILS CONTAINING LINOLENIC ACID

A. EXPERIMENTAL

Separation and Estimation of Triglycerides

1. Method in Brief ("Standard Method")

The triglyceride mixture (10-40 mg) in ether was applied as a narrow band to a silica-silver nitrate chromatoplate (20x40 cm^2) approximately two inches from one end. The plate was developed horizontally [61] for 3 hours with a benzene-ether mixture whose composition depended upon the type of glycerides to be separated (triglycerides with 6-9 double bonds, ether; 0-6 double bonds, benzene-ether 9:1).

The separated fractions were detected by spraying with 2',7'-dichlorofluorescein (0.2% methanol) and viewing by ultra-violet light.

The glycerides were removed from the adsorbent by extracting it six times with 7-10 ml portions of ether-methanol-water (5:5:1) in centrifuge tubes (12 cm xl.5 cm id). The mixture was stirred mechanically during each extraction for approximately one minute before centrifugation.

A known amount of a standard solution of methyl heptadecanoate (17:0) was added to the combined extracts of each fraction and the mixture poured into an excess of water and extracted three times with hexane.

Each fraction was isolated and transesterified by boiling with methanol (15 ml) and sodium methoxide (0.5%) for three minutes. The solution was poured into water and the methyl esters isolated after extraction with hexane (3x).

The fatty acid composition of the fractions was determined by gas-liquid chromatography (G.L.C.). The weight of each fraction was calculated by comparing the total peak area of the normal fatty acids to the methyl heptadecanoate peak area. The triglyceride composition of each fraction was calculated from its fatty acid composition.

2. The Method in Detail

(i) Purification of Solvents

All solvents were redistilled before use. Ether was dried and stored over sodium. Dry methanol was prepared by Vogel's procedure (169). Standard hexane (referred to as hexane in this report) was obtained from Carless Capell and Leonard (London). The solvents were checked by G.L.C. to ensure that there were no late running peaks corresponding to the methyl esters in the samples being analysed.

(ii) Preparation of Chromatoplates

A slurry of silica gel G (30 g) in water (62 ml) containing silver nitrate (5 g) was spread on glass plates (20x40 cm^2) according to Stahl (170). The applicator was made of perspex and the trailing edge had a gap of 300μ to the glass plate. The plates were dried for 30 min. in an oven at 100°C, and were ready to use after cooling to room temperature. The plates may be stored in the dark in a clean atmosphere.

(iii) Chromatography of Triglycerides

Using a 40μl Hamilton syringe the triglyceride mixture (10-40 mg) in ether, was applied as a continuous band two inches from one end of the chromatoplate (20x40 cm^2). The chromatogram was developed horizontally as described by Brenner and Niederwieser (61) for 2-3 hours with a benzene-ether mixture. The seed oils we

examined contained triglycerides with 0-9 double bonds which differed greatly in polarity. To obtain reasonable separations between the triglycerides we therefore found it necessary to run two chromatograms; one developed with benzene-ether (9:1) to separate glycerides with 0-6 double bonds, and one with ether to separate glycerides with 6-9 double bonds.

The order of elution of the triglycerides was initially determined according to the number of double bonds they contained and by comparison with mixtures of known composition. The relative R_F values were eventually decided after determining the fatty acid composition of the fractions of numerous seed oils by gas-liquid chromatography.

Some of the fractions were easily characterised because they contained only one or two major components. By correlating the positions of these "pure fractions" we were able to determine the sequence of elution. The triglycerides in order of increasing R_F values, and the fractions which provided the sequence are given in Table 1.

It is apparent that the order does not depend solely upon the number of double bonds in the molecule but also on their position. Thus the complexing power of two oleoyl chains (2 double bonds) is less than that of one linolenoyl chain (3 double bonds).

We have correlated the degree of unsaturation and R_F values of the triglycerides by assigning arbitrary values for the complexing power of each chain:- saturated (0), oleic (1), linoleic (2+a), linolenic (4+4a), where a 0.5. Thus the glyceride 311 has a complexing power of 6+4a which is greater than the value 6+3a for the glyceride 222.

Table 1

Triglyceride Fractions of Seed Oils, identified by G.L.C. and arranged in order of increasing R_F values[1]

Triglyceride	Complexing value	Linseed (Appendix 2)	Candlenut (Appendix 4)	Wild Rose (Appendix 3)	Rubber (Appendix 5)
333	12 + 12a	B1[2]	B1[2]		B1[2]
332	10 + 9a	B2	B2		B2
331	9 + 8a	B3	B3		B3
330	8 + 8a	B3	B3		B3
322	8 + 6a		B4		B4
321	7 + 5a	A3	A2, B5		B5
320	6 + 5a	A4	A3		B5
311	6 + 4a	A5	A4		
222	6 + 3a		A4	A3[2]	
310	5 + 4a		A5	A4	
221	5 + 2a		A6	A4	
300	4 + 4a				A5
220	4 + 2a		A7	A5	A5
211	4 + a		A8	A6	A6,7
210	3 + a		A9	A7	A8,0,10
111	3		A10		A11
200	2 + a				A12
110	2				A13
100	1				A14

1. Abbreviations, p. 100.

2. A and B signify the chromatogram, numerals the particular fractions.

Although we have given the saturated acids zero complexing power the separation of stearoyl from palmitoyl glycerides is sometimes apparent. In the chromatography of rubber seed oil for example palmito-oleo-linolein had a lower R_F value than stearo-oleo-linolein (Appendix 5a, fractions 8-11).

(iv) Detection of Separated Triglycerides

Two basic methods which we have found useful for detecting components on a chromatoplate are described.

(a) 2',7'-Dichlorofluorescein (0.2% in Methanol) (54)

The developed chromatoplate was sprayed with a methanolic solution of dichlorofluorescein (0.2%), dried in a current of nitrogen, and viewed by ultra-violet light. The components appeared as yellow luminous spots or bands against a dark background. The sensitivity of the reagent was reduced in the presence of traces of benzene but 0.01 mg triglyceride could be detected depending on the thickness of the adsorbent.

(b) Charring

An extremely sensitive method of detecting components is to char them on the chromatoplate. Obviously the components are destroyed but this method is useful in providing a visual semi-quantitative method of estimating the glycerides and also in providing a chromatogram which can be easily photographed for record purposes.

The triglycerides were charred in two ways:-

Phosphoric acid (50% aqueous)

The chromatoplate was sprayed with aqueous phosphoric acid (50%) and heated at 250°C for 15 min.

Blowpipe flame

The triglycerides were charred by passing the adsorbent on the plate through the tip of a compressed air-gas blowpipe flame. The air-gas mixture and distance of the plate from the flame were determined by trial and error until the flame did not reduce the silver nitrate but charred the components. The plate was passed through the flame at a speed sufficient to char the triglycerides but not so slowly as to crack the plate. The method has two distinct advantages.

(1) No spray reagents are required for adsorbents impregnated with silver nitrate.

(2) The edges of a preparative chromatogram can be detected leaving the components in the centre unharmed.

(v) Extraction of Triglycerides from Silica-Silver Nitrate

In order to estimate and characterise each separated component it was essential that the triglycerides should be completely extracted from the adsorbent. Because linolenic acid is readily oxidised we first examined methods of extraction using methyl ester or triglyceride (Jatropha curcas seed oil) mixtures containing only saturated, oleic and linoleic acids.

Early attempts to obtain a quantitative recovery of Jatropha curcas seed oil from the silica-silver nitrate adsorbent were unsuccessful. The adsorbent containing the oil was refluxed for three hours with excess methanol containing dry HCl (5%). The mixture was poured into water and extracted with ether (3x). A typical analysis of Jatropha methyl esters obtained in this way appears in Table 2, No. 5 and indicates an incomplete recovery of the unsaturated relative to saturated acids.

Table 2

Extraction of Unsaturated Methyl Esters and Glycerides from Silica Impregnated with Silver Nitrate

	Sample	Adsorbent	Extracting Solvent	Number of Extractions in Centrifuge Tube	Component Acids 16:0	18:0	18:1	18:2	18:3
1	Methyl Esters	-	-	-	65.4	4.1	7.7	22.8	-
2	" "	Silica gel G	Methanol-Benzene (1:1)	2	65.7	4.2	7.9	22.2	-
3	" "	Silica-$AgNO_3$	" " "	2	64.8	4.2	8.3	22.7	-
	Jatropha Curcas								
4	Seed Oil	-	-	-	15.2	6.6	41.7	36.5	-
5	" "	Silica-$AgNO_3$	Boiling methanol during transesterification with MeOH/HCl, 3 hours reflux	-	31.0	12.0	33.0	24.0	-
6	" "	" "	Methanol-Benzene (1:1)	2	17.6	7.4	40.2	34.8	-
7	" "	" "	" " "	5	15.6	4.8	41.2	38.4	-
8	" "	" "	Ether	4	14.9	6.0	39.1	40.0	-
9	" "	" "	Methanol-Ether (1:1)	6	16.2	6.2	41.8	35.8	-
10	" "	" "	Methanol-Ether-Water (5:5:1) followed by Methanol-Ether (1:1)	2 4	15.6	6.1	41.6	36.7	-
11	Linseed Oil	-	-	-	5.6	3.3	16.9	14.4	59.8
12	" "	Silica-$AgNO_3$	Methanol-Ether-Water (5:5:1) followed by Methanol-Ether (1:1)	2 4	7.6	4.4	21.7	16.8	49.5
13	" "	" "	Methanol-Ether-Water (5:5:1) followed by Methanol-Ether (1:1)	3 4	6.6	3.8	18.9	15.7	55.0
14	" "	" "	Methanol-Ether-Water (1:1) followed by Ether	6 1	5.6	3.5	16.9	14.2	59.8

It was thought that poor recovery of the unsaturated acids could have been due to either oxidation or selective retention on the silica-silver nitrate.

It was shown in two ways that oxidation was not occurring.

(1) Jatropha oil was separated into fractions on a silica-silver nitrate chromatoplate by developing with a benzene-ether (9:1) mixture. The plate was dried and redeveloped with the same solvent in a direction at right angles to the first development (2 dimensional chromatography). The separated components were detected by charring. If oxidation of the triglycerides had occurred, then the oxidation products would appear near the base line of the second development. No components corresponding to oxidation products were detected.

(2) A mixture of methyl esters containing saturated, oleic and linoleic acids was chromatographed on an ordinary silica plate and on one impregnated with silver nitrate. A portion of the adsorbent (1 g) from each plate was extracted twice with a methanol-benzene (1:1) mixture (7 mls) in centrifuge tubes. The mixture was mechanically stirred for 1 minute before centrifugation. The combined extracts were poured into water, extracted with ether (3x) and the methyl esters examined by G.L.C.

The analyses (1 and 2, Table 2) show that a uniform extraction of saturated and unsaturated methyl esters was obtained and little or no oxidation occurred either on silica or silver nitrate impregnated adsorbents.

When the same extraction procedure was applied to silver nitrate impregnated silica containing Jatropha oil, a small but

significant loss of linoleic acid was observed (Analysis 6, Table 2). By varying the extracting solvents and increasing the number of extractions, a suitable extraction procedure was finally devised (Analyses 9,10, Table 2).

The glycerides were quantitatively recovered by extracting the adsorbent six times in centrifuge tubes with ether-methanol (1:1), or using ether-methanol-water (5:5:1) for the first two extractions. The combined effect of mechanical stirring and using aqueous solvents resulted in the adsorbent being broken rapidly into fine particles. The combined extracts were poured into water and extracted with hexane (3x). This extraction procedure was used to isolate triglyceride fractions of Jatropha oil and a quantitative analysis was obtained (Appendix 1). When the same procedure was applied to adsorbents containing linseed oil poor recovery of linolenic acid (18:3) was observed (Analysis 12, Table 2). However by increasing the number of extractions with aqueous solvents (methanol-ether-water, 5:5:1), the silver nitrate was also extracted and a quantitative recovery of all the glycerides was achieved (Analysis 14, Table 2).

Further confirmation that the triglycerides were being completely recovered was obtained by extracting known amounts of candlenut oil from the adsorbent and weighing the products. Recoveries were in the region of 95% weight (92-98%). Poor recovery of unsaturated glycerides or methyl esters is therefore primarily due to poor extraction techniques; oxidation occurred to only a minor extent.

(vi) Preparation of Methyl Esters

The triglyceride fractions in hexane were evaporated in a current of nitrogen on a steam bath. A small amount of acetone was also evaporated from the triglycerides to remove the last traces of water. Methanol 15 ml containing 0.5% sodium methoxide* was added to the triglycerides and the mixture refluxed for three minutes[171].

* Prepared by dissolving 0.5 g clean sodium in 100 ml dry MeoH.

Before proceeding it was ascertained that all the glycerides had dissolved to ensure complete transesterification. This can be assisted by adding 1-2 ml benzene.

After refluxing, the methanolic solution was cooled, poured into water, and the methyl esters extracted three times with hexane. The solvent was evaporated to approximately 0.5 ml and transferred to a tube with a tapered sealed end. The methyl esters were further concentrated by evaporating the solvent to approximately 10μl in a current of nitrogen, and analysed by G.L.C.

(vii) Gas-Liquid Chromatography of Methyl Esters

A Perkin-Elmer 451 fractometer, fitted with a flame ionisation detector was used. The methyl esters were separated on 1 or 2 metre stainless steel columns (o.d. $\frac{1}{4}$") packed with 60-80 mesh firebrick coated with 20% poly (ethylene glycol succinate)* and eluted with nitrogen at 180-190°.

The relative amount of each component was determined by directly comparing the areas under the peaks. Provided that the peaks were symmetrical their areas were estimated by height x width at half height [172,173], otherwise they were determined with a planimeter.

(viii) Calculating the Triglyceride composition of an Oil from the Fatty Acid composition of the fractions

A typical calculation of wild rose seed oil triglycerides will be given to illustrate the general method.

Wild rose seed oil was separated into six fractions by chromatography on silica-silver nitrate. A known amount (1 mg) of methyl heptadecanoate (17:0) was added to each extracted fraction. The fatty acid composition was determined by G.L.C. and the analysis

* Packing material ref. No. P. purchased from Perkin-Elmer

converted to % mol. by calculation. The ratio of the total peak area of the normal fatty acids to the peak area of 17:0 on each gas-liquid chromatogram was also determined.

Then,

$$\frac{\Sigma \text{ Peak Area of normal fatty acids}}{\text{Peak Area of 17:0}} = A$$

but

$$\frac{\Sigma \text{ Peak Area of normal fatty acids}}{\text{Peak Area of 17:0}} = \frac{\text{wt of normal fatty acids}}{\text{wt of 17:0 added}}$$

$\therefore$ wt of normal fatty acids = Ax (wt of 17:0 added).

The relative weights of the fractions were thus determined and converted to per cent mol.

The fatty acid composition and relative amounts of the fractions of wild rose seed oil are given in Table 3. For simplicity the small amount of palmitoleic acid (16:1) which may be present was combined with oleic acid (18:1).

The total composition of the fractions should equal the composition of the original oil. This is a useful comparison to check the recovery and estimation of the fractions. The total fatty acid composition of the fractions was calculated by summing the result of multiplying the fatty acid composition of each fraction by its weight (expressed as per cent mol.).

i.e. Total 16:0 = Σ 16:0 in fraction (% mol.) x Amount of fraction in total oil (% mol.).

The glycerides in each fraction were identified by considering its fatty acid composition and the elution order of the triglycerides. In most cases each fraction contained one or two major triglycerides which were easily recognisable.

Table III

Glyceride Fractions of Wild Rose Seed Oil

Fraction	Component esters (% mol.)				Proportion (%)	Component glycerides (increments % mol.)							Proportion of major glyceride in fraction
	16:0 & 18:0	18:1	18:2	18:3[1]		333	332	331	330	322	321	320	
1	2	1	3	94	6.82	5.53	0.60	0.30	0.39	-	-	-	81%
2	-	2	31	67	17.50	-	16.18	0.99	0.24	0.09	-	-	92
3	11	18	8	63	6.64	-	0.21	3.64	2.10	0.69	-	-	55
4	1	2	61	36	21.06	-	-	1.08	0.66	19.11	0.21	-	91
5	9	20	37	34	15.01	-	-	-	0.36	1.93	8.82	3.90	59
6	9	18	68	5	32.97	-	-	-	-	-	(32.97)		-
					Total	5.53	16.99	6.01	3.75	21.82	(45.90)		

1. Abbreviations page 100.

Having decided which glycerides were in a particular fraction it was a simple matter to determine their relative amounts, provided that the number of triglycerides did not exceed the number of fatty acids (saturated acids in one group). The composition of a typical fraction was calculated as follows

The amount of each glyceride (% mol.) in the fraction was denoted by 3a, 3b, etc. Four simultaneous equations could therefore be written, equating the amount of each fatty acid (% mol.) calculated from the algebraic glyceride composition, to the known amount of fatty acid (% mol.) determined by G.L.C. (see Table 4). The values for a,b,c and d were obtained by solving the simultaneous equations.

In general the total composition of the fractions showed a slight loss (1-2%) of linolenic acid (18:3). The linolenic content was therefore increased by an appropriate amount in fractions where this was considered reasonable. In the majority of cases however no adjustment was necessary.

The glyceride composition of the fractions were thus determine and arranged in Table form (Table 3).

ix. Isolation of Neutral Triglycerides from Vegetable Seeds

Linseeds and soya beans were obtained from J. Bibby and Sons, stillingia seeds (Sapium sebiferum, ex Bombay), rubber seeds (Malaya) and candlenut oil (solvent extracted) from Tropical Products Institute. The wild rose seed was collected locally. The outer shell of the stillingia seed was discarded before extracting the oil from the kernel.

The crushed seeds were thoroughly extracted with light petrol (b.pt 40-60°) in a soxhlet extractor. The extracted oils were examined by T.L.C. on silica gel G, developing the chromatogram with

Table 4

Triglycerides in Fraction 3, Wild Rose Seed Oil

Glycerides	% mol. of fraction	Component fatty acids				Fraction as % mol. of oil
		16:0 + 18:0	18:1	18:2	18:3	
Fraction 3		11.0	18.0	8.0	63.0	6.6
332	3a			0.7	1.4	0.2
331	3b		18.0		36.0	3.6
330	3c	11.0			22.0	2.1
322	3d			7.2	3.6	0.7
Total	100	11.0	18.0	7.9	63.0	

Equating 18:3% mol. $2a + 2b + 2c + d = 63.0$ - - - - - - - - - 1

" 18:2 " $a + 2d = 8.0$ - - - - - - - - - 2

" 18:1 " $b = 18.0$ - - - - - - - - - 3

" 16:0 + 18:0 % mol. $c = 11.0$ - - - - - - - - - 4

a, b, c, and d obtained by solving these simultaneous equations.

light petrol-ether (8:2). The components were detected by charring after spraying with 50% concentrated sulphuric acid. The triglycerides were isolated by eluting the oil (1 g) with benzene (200 ml) from a column of silica (30 g) if appreciable amounts of free fatty acid or partial glycerides were detected in the oil by T.L.C. Otherwise the oil was used directly for analysis.

Iodine values were determined using the procedure described in **British Standard Methods of Analysis of Oils and Fats B.Sc684:1958**

(x) Analysis of seed oil triglycerides

The triglycerides were separated and determined by the method described above ("standard method"). Two chromatograms were developed for each oil, one with ether and one with benzene-ether (9:1) to obtain an analysis of all the glycerides.

All the oils were examined by the lipolysis technique to determine the composition of the acids at the 2- position. The results enabled us to calculate the glyceride composition of the oil according to Coleman [140] and Vander Wal [150]. The experimental technique is described in Part II of this thesis.

(xi) Candlenut oil

In a second analysis, candlenut oil (100 mg) was separated on four silica-silver nitrate chromatoplates (20x40 cm^2) developed with benzene ether (1:1). The separated triglycerides were isolated and similar fractions combined, weighed, and the fatty acid composition determined by G.L.C. The proportion of the glycerides in the oil was calculated from these results and the analysis compared with the one obtained using the "standard method" (Table 5).

Each fraction was further examined by lipolysis in an attempt to determine the proportions of the glyceride isomers. The fractions

generally contained too many components for us to obtain a satisfactory solution however.

(xii) Stillingia oil triglycerides

Stillingia oil was separated into two fractions (containing zero and 1 mol. deca-2,4-dienoic acid) by thin-layer chromatography on silica. The oil (20 mg) was separated on a silica chromatoplate (20x20 cm^2, 300 thick), developed with a light petrol and ether mixture (9:1). The components were detected with dichlorofluorescein and extracted four times in centrifuge tubes with a methanol, ether mixture (1:1). If necessary the separate fractions were re-chromatographed to obtain a pure sample. Larger quantities of oil can be separated by column chromatography [44].

Optical rotations of solutions of the oil and the fractions in chloroform were measured in a 2 ml capacity 1 DM polarimeter tube (Schmidt & Haensch 1926).

Ultra-violet spectra of the triglycerides in methanol were obtained using the Unicam S.P. 700 ultra-violet spectrometer.

The triglycerides of the less polar fraction (saturated, oleic, linoleic andlinolenic glycerides) were separated and determined by the "standard method".

We found difficulty in obtaining reproducible estimations by G.L.C. of the decadienoic acid in the polar fractions. We therefore separated this fraction (0.1 g), on two silica-silver nitrate chromatoplates (20x40 cm^2) developed with benzene, ether (1:1) for 3 hours [61]. After extracting and combining the appropriate triglyceride fractions they were weighed and the decadienoic acid content determined quantitatively by U.V. spectroscopy. The long chain fatty acids were analysed by G.L.C. and the two fatty acid determinations combined. From the fatty acid composition of each fraction we were able to determine its glyceride composition.

Having determined the distribution of the decadienoic acid in the polar fraction we were able to separate and determine the triglycerides by the "standard method". Although G.L.C. gave lower values for the 10:2 content we were able to correct this by making the 10:2 content in each fraction equal to 33% mol. as indicated by our U.V. data.

The results obtained by weighing and by internal standard are given in Table 5.

(xiii) The Composition of Individual Linseeds (Table 9)

The oil from individual linseeds was extracted by crushing the seed in hexane with a pestle and mortar three times. The oil was isolated from the combined extracts and converted to methyl esters by transesterification with methanol and sodium methoxide (0.5%). The composition of the methyl esters was determined by G.L.C.

RESULTS AND DISCUSSION

1. Methods of Analysis

The methods of separating and estimating triglycerides have been reviewed in the introduction. In its present form gas-liquid chromatography is of little value in analysing highly unsaturated oils. Reverse-phase paper chromatography separates glycerides according to chain length and unsaturation and some confusion arises because certain glycerides are inseparable (e.g. triolein, palmito-diolein, dipalmito-olein). The method is probably more suitable for examining the more saturated natural oils. A recently described technique by Vereschagin [99] in which silver nitrate is placed in the developing solvent of a reverse-phase chromatogram enables glycerides to be separated more completely. Saturated acids no

longer form critical pairs with unsaturated ones and the method possibly gives a more complete fractionation of glycerides than silica-silver nitrate.

However thin-layer chromatography on silica-silver nitrate separates triglyceride mixtures into simple fractions whose composition is easy to determine. The method is obviouslyof less value for examining the more saturated oils.

Numerous methods of quantitatively estimating the separated fractions have been reported [55,62-64,66,130]. Photodensitometry[54] can only operate satisfactorily if sharp separations are obtained and is of little use if each detected band contains a number of glycerides. The remaining methods all depend on estimating the glycerides after quantitatively extracting them from the adsorbent.

All the methods at some stage involve a component acid determination of the separated fractions. Some workers have estimated the relative amounts of the fractions by direct weighing[55,60] others have determined the glycerol released on saponification, by titration with periodate [62-64] or by colorimetry using the chromotropic colour reaction [130].

We have devised a new method of estimating the components extracted from the chromatogram. A known amount of methyl heptadecanoate is added as an internal standard to each isolated fraction prior to G.L.C. of the fatty acid methyl esters. The weight of each fraction is calculated by comparing the total peak area of the normal fatty acids to the methyl heptadecanoate peak area. The triglyceride composition is calculated from the fatty acid composition assuming that each fraction contained glycerides with a certain number of double bonds.

Our method therefore provides the most direct route for determining the weight and composition of extracted fractions and has since been used by others [67].

For our analytical results to have any significance we must make some assessment of their accuracy. The two main sources of error occur in the extraction of the glycerides from the adsorbent and in the analysis of the methyl esters by G.L.C.

When known weights of linseed oil were applied to silver nitrate impregnated silica and recovered by the normal extraction procedure 92-98% recoveries were observed. The maximum relative error incurred at this stage will therefore be $\pm 8\%$ assuming that in some cases 100% recovery is observed.

Quantitative gas-liquid chromatography was precise to $\pm 1\%$. This error not only affects the estimation of the relative amounts of the fractions using an internal standard, but also the estimation of the individual glycerides in a fraction.

Thus the relative error in estimating a fraction by adding 20% of an internal standard will be $\pm 6\%$:-

$$\left[\text{Wt of fraction} = \frac{\text{Total Peak Area } (80 \pm 1\%)}{\text{Area of Internal Standard } (20 \pm 1\%)} \times \text{wt of Internal Standard}\right]$$

The relative error in estimating a particular glyceride in a fraction will be $\pm 3\%$ due to inaccuracies in the G.L.C. and an additional relative error of 9% caused by the consistent loss of linolenic acid more than any other acid. The overall relative error is therefore given by [175]:-

$$\sqrt{8^2 + 6^2 + 3^2 + 9^2} = \pm 13\%$$

Although we have not repeated any analyses using the internal standard of estimation, we have analysed two samples twice (Candlenut and Sapium sebiferum seed oils) estimating the fractions in one case by adding an internal standard and in the second by direct weighing (Table 5).* The results agree well and are generally within the limits of precision calculated above.

It is more difficult to assess the effect of "wild errors", e.g. incorrect designation of the glycerides in a fraction, without doing a series of analyses of a sample of known composition. A standard glyceride mixture of the type we have analysed is difficult to obtain; moreover the multiple analysis of a fraction would be very time consuming and probably no more revealing than estimating the accuracy of the method as described above.

A comparison between the composition of the original oil and the total composition of the fractions is a means of confirming the overall completeness of the extraction and correct estimation of the fractions (Table 6). A small loss in the recovery of the more unsaturated acids is generally encountered. We consider this to be primarily due to incomplete extraction of the more unsaturated glycerides and to a secondary extent to oxidation.

Other reported methods of quantitatively determining triglycerides are of the same order of accuracy as ours (54,55,62-64,66,130).

2. The Triglyceride Composition of Linolenic acid containing Seed Oils

The aim of this work was first to devise a method of determining the glycerides in linolenic acid containing oils. The second was to analyse a series of unsaturated oils and to compare the results with the values predicted by Gunstone's theory and those calculated from lipolysis results.

* Tables 5-13 on pages 54-62.

The lipolysis technique is described in detail in the next section. The triglyceride composition determined from these results (Table 7) for linseed, candlenut, wild rose, rubber and soya bean oils is however given in Table 8 and compared with the experimental and theoretical values.

There is a good agreement between our experimental results and those predicted by theory. We have therefore provided experimental confirmation of the theories of fatty acid distribution described by Coleman [140] and Vander Wal [150] (using in part experimental lipolysis results) and the hypothetical form described by Gunstone [155]. This means that the glyceride composition of numerous seed oils can be calculated from their fatty acid composition or more accurately from lipolysis results. The possibility still remains that some oils may deviate from the normal pattern of fatty acid distribution. An example of this has recently been reported by Subbaram, Chakrabarty, Youngs and Craig [128].

In making a final judgement on the comparison between experimental and theoretical triglyceride compositions of natural oils we have considered one other factor.

Numerous workers have reported that in individual seeds there are regional differences in fatty acid composition. Kartha [176] examined numerous seeds of different plants and reported noticeable variations in the iodine value of fat extracted from different parts of the same seed. Similar observations were made by Galoppini and Lotti [177]. Fats used in analysis are generally obtained by extracting a large number of seeds which are unlikely to have identical compositions. To confirm this point we determined the fatty acid composition of six individual linseeds (Table 10). Considerable variation in the oleic and linolenic, but not in the saturated acid content, was noted. Any variation in the linolenic acid content (54-68%) was compensated for by an equivalent increase or decrease

in the oleic acid content (22-11%). Using Gunstone's theory of positional distribution [155] we calculated (i) the glyceride composition of seed A, (ii) the glyceride composition of seed F, (iii) the glyceride composition of an oil having a fatty acid composition intermediate between that of seeds A and F. There was an appreciable difference between (i) and (ii) but not between (iii) and the mean of (i) and (ii) (Table 11).

Very recently Litchfield and Reiser [178] have studied this problem in greater detail. Using a proper mathematical treatment involving integration they calculated the glyceride composition of seeds in which regions differed in fatty acid composition. They concluded that, "when the relative amounts but not the types of fatty acids vary with location, the triglyceride composition can be predicted from the average fatty acid composition. Where different types of fatty acids exist in different regions however, these differences must be taken into account to avoid large errors".

The Individual Oils

The glyceride composition of each oil is now discussed in more detail. The fatty acid and glyceride compositions of the oils are presented in Tables 7, 8, 9, 12, 13. A more general comparison of the oils is made in Table 9 in which the glycerides are classified according to the number of saturated acyl groups and number of double bonds. The experimental data from which the triglyceride composition of the oils was calculated, is presented in the Appendix. A qualitative comparison of the triglycerides in the oils was made on a silica-silver nitrate chromatogram (fig. 1).

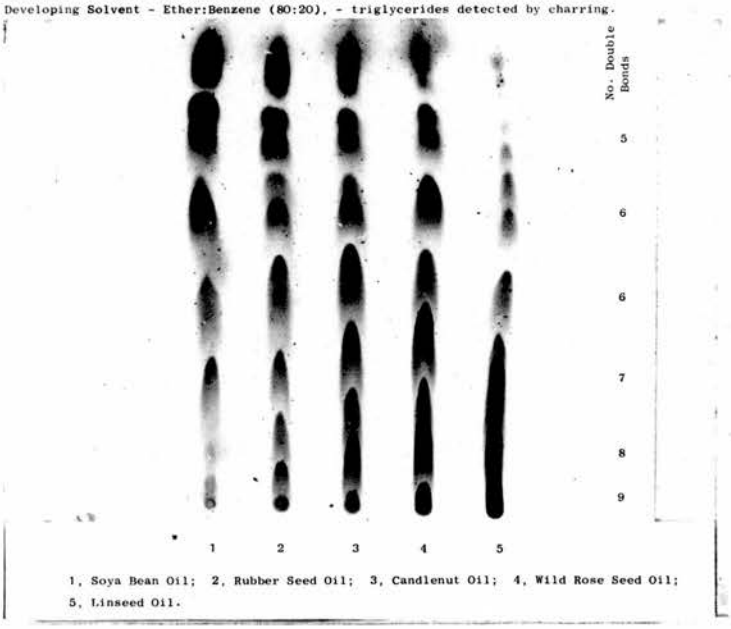
(i) Linseed Oil

Numerous workers have attempted to determine the triglycerides of linseed oil because of its great industrial importance.

SEPARATION OF TRIGLYCERIDES OF SEED OILS CONTAINING LINOLENIC ACID BY THIN-LAYER CHROMATOGRAPHY

Adsorbent - Silica impregnated with 15% Silver Nitrate,

Developing Solvent - Ether:Benzene (80:20), - triglycerides detected by charring.



1, Soya Bean Oil; 2, Rubber Seed Oil; 3, Candlenut Oil; 4, Wild Rose Seed Oil; 5, Linseed Oil.

Fig.1

Crystallisation of the oil or the glyceride mixtures produced on partial or complete hydrogenation met with little success [179,180]. The results of Hilditch and Seavell [181] indicated a preponderance of di- and tri- polyethenoid glycerides. Qualitative data indicating that certain glycerides were present was obtained by fractionally crystallising brominated linseed oil triglycerides [182,183].

Dutton and Canon [11] used a counter-current distribution technique and estimated the proportions of four major components, 333,332,331,322 .

Walker and Mills [38,39] partially fractionated the oil on a column of activated alumina. Repeated fractionations yielded small amounts of linoleodilinolenin , 233 and trilinolenin , 333 . Partial separations on alumina and silica have also been reported by Sahasrabudhe [42].

Reverse-phase chromatography on paper and thin-layer of Kieselguhr was used by Kaufmann and co-workers [81,103,106,107] and others [101,109] to separate linseed oil triglycerides but no quantitative estimations were made.

Hirsch [71] obtained eight different fractions, the first two being 333 and 332, by reverse-phase chromatography on factice.

More recently Youngs [127] analysed the esterified oxidised glycerides by G.L.C., obtaining quantitative results according to the number and type of saturated acids the glycerides contained but not distinguishing between the unsaturated acids.

At the same time as our work was published Vereschagin [99] described a method of determining linseed oil triglycerides using both reverse-phase and silver nitrate, chromatography.

Mattson and Volpenhein [146] determined the composition of the fatty acids at the 2- position by lipolysis.

We obtained probably the first adequate analysis of linseed oil triglycerides (Table 8, Appendix 2). All the possible glycerides except trisaturated were detected and eight were present in amounts greater than or equal to five per cent - 333,332,331,330, 321,320,311,310 .

Trilinolenin (23%) and the dilinoleno-triglycerides, 332,331, 330 (41%) comprised almost two-thirds of the oil.

(ii) Wild Rose Seed Oil (Rosa canina)

There is no previous report on the glyceride composition of this oil and only two publications indicating its fatty acid composition [184,185]. We detected eighteen glycerides (Table 8, Appendix 3), seven being present in amounts greater than or equal to five per cent 333,332,331,322,321,222,221 . The major glycerides were 332 (17%), 322 (21%), 321 (10%), 222 (13%), and 221 (8%).

(iii) Candlenut Oil

Prior to our work the only reports on the composition of Candlenut oil were by Hilditch and co-workers who examined three candlenut oils by low temperature crystallisation [181,186]. This method is now known to be unsatisfactory for highly unsaturated oils.

Because of its fairly uniform fatty acid composition candlenut oil was expected to contain a wide spread of glycerides. In fact we detected nineteen glycerides (Table 8, Appendix 4), eleven being present in amounts greater than or equal to five per cent. The major glycerides were 332 (10%), 322 (13%), 321 (14%), 320 (8%) and 221 (8%).

In a second analysis a larger quantity of candlenut oil (0.1 g) was separated by silica-silver nitrate chromatography and the extracted fractions were weighed. The results compare favourably with those obtained by our normal method (Table 5). These larger fractions were also examined by lipolysis in an attempt to determine the proportions of the triglyceride isomers. The fractions were however too complex for us to obtain an unambiguous solution.

(iv) Rubber Seed Oil (Hevea brasiliensis)

As with candlenut oil, the only previous analysis of rubber seed oil triglycerides was reported by Hilditch and Seavell [181] using low temperature crystallisation.

Our oil contained more saturated acids (21%) than the other linolenic acid containing oils we examined (5-15%). It is interesting to note the chromatographic separation between the palmitic and stearic oleo-linoleotriglycerides (Appendix 5A Plate A, fractions 8-10).

We detected nineteen glycerides (Table 8, Appendix 5), ten being present in amounts greater than or equal to five per cent. The major glycerides were 321 (9%), 320 (8%), 221 (8%) and 210 (11%).

(v) Soya Bean Oil

The commercial importance of soya bean oil is reflected in the numerous reports of its glyceride composition.

The glycerides have been separated by Kaufmann and co-workers using reverse-phase thin-layer and paper-chromatography [81,103,106,107] but no quantitative estimations were made.

Quantitative analyses were reported by Hilditch et al [181] using low temperature crystallisation, and by Scholfield and Hicks [187], using countercurrent distribution.

More recently Youngs [125,127] analysed the esters of oxidised soya bean triglycerides by gas-liquid chromatography. A more complete analysis of the least unsaturated portion of soya bean oil was obtained by Jurriens and Kroesen [64] by estimating the glycerides separated on silica-silver nitrate and also examining them by lipolysis. Results obtained by lipolysis have also been reported [144-146].

Our analysis showed the presence of eighteen glycerides (Table 8, Appendix 6) excluding trisaturated and trilinolenin. Seven glycerides were present in amounts greater than or equal to five per cent - 322,321,222,221,220,211,210 . The major glycerides were 222 (15%), 221 (16%), 220 (13%) and 210 (12%). Our results are compared with other reported analyses in Table 12.

(vi) Stillingia Oil (Sapium sebiferum kernel oil)

Stillingia oil contains deca-2,4-dienoic acid (≈10%) and other unusual acids [188-192] in addition to saturated, oleic, linoleic and linolenic acids.

Crossley and Hilditch [193] examined the oil and concluded that the decadienoic acid occurred only once in any glyceride molecule in which it was present and that it occupied the 2- position. Hirayama [194] separated the glycerides by counter-current distribution obtaining eleven fractions, four of which contained one mole of decadienoic acid per mole triglyceride. Huang et al [43] noted that the oil had a high optical activity. More recently this fact was investigated by Maier and Holman [44] who separated the more polar triglycerides containing the decadienoic acid from the normal long chain triglycerides by chromatography on silica. They confirmed that the 10:2 acid occurred only once in the triglyceride molecule and further concluded that the optical activity exhibited

by the oil was due to an optically active mono-decadienoic acid triglyceride. This was best explained if the decadienoic acid was esterified at the 1- position. The same workers [192] however reported later that the optical activity was due to an acid previously undetected in the oil, 8-hydroxy-5, 6-octadienoic acid. The acid occurred in amounts equivalent to decadienoic acid and was esterified at the 1- position. The free hydroxyl group of the acid was esterified by the decadienoic acid. Holman was unable to detect the hydroxy-allenic acid by G.L.C. possibly because degradation occurred during chromatography.

Our investigation showed that Stillingia oil had a specific rotation $[\alpha]_D^{20} = -8.7^\circ$ and adsorbed in the ultra-violet spectrum at 266 mμ,

$$E_{1\ cm}^{1\%} = 77.$$

Hilditch [190] gives $E_{1\ cm}^{1\%}$ 10:2 methyl ester = 1317 and Crombie, 1209 [195]. Using a mean value of 1263 the proportion of decadienoic acid in Stillingia oil was calculated to be 6.1% (wt).

The polar (26% wt.) were separated from the less polar (70.5%) triglycerides by thin-layer or column chromatography on silica. A very polar fraction (3.5%) was not examined. The fatty acid and triglyceride compositions of the oil and the fractions is given in Table 13. Comparing the two fractions it is interesting to note that the decadienoic acid replaces linolenic acid in the polar fraction almost exclusively.

The least polar triglycerides contained saturated, oleic, linoleic and linolenic acids, were not optically active and did not adsorb at 266 mμ. Nineteen glycerides were detected by chromatography on silica-silver nitrate and six - 333, 332, 331, 330, 322, 320, occurred in amounts greater than 5% of Stillingia oil. The major glycerides were 333 (10.7% Stillingia oil) and 332 (15%) (Table 13, Appendix 7).

The polar fraction represented 25.6% wt stillingia oil calculated from the dienoic acid content of the oil, a value which compares favourably with that found experimentally by chromatography (26%). This fraction contained deca-2,4-dienoic acid, saturated, oleic, linoleic and linolenic acids and some minor components. It hada specific rotation $[\alpha]_D^{20^\circ} = -34.2^\circ$ and adsorbed at 244 mμ, $E_{1\ cm}^{1\%} = 295$, values which correlate well with those of the total oil.

The separated glycerides of the polar fraction were weighed and examined by G.L.C. and ultra violet (U.V.) spectrometry. U.V. spectrometry showed that each fraction contained just below 33% mol. decadienoic acid (lower values were obtained by G.L.C.), thus confirming previous reports (44).

The glycerides in each fraction were determined in the usual way and are given to two significant figures. The major glycerides were 33D* (5.9% of stillingia oil) and 32D (7.2%) (Table 13, Appendix 7).

The glycerides of the polar fraction were also estimated by the standard method (Appendix 7) adding an internal standard and assuming the dienoic acid content to be 33% mol. The two sets of results compare favourably (Table 5). Like Holman we were unable to detect the hydroxy-allenic acid by G.L.C. but this does not affect the relative proportions of the triglycerides which we determined.

* 33D-- Glyceride containing 2 linolenoyl and 1 decadienoyl acyl groups.

Table 5

Component Glycerides of Candlenut oil and the "Polar Fraction" of Sapium sebiferum determined by T.L.C. and estimating the fractions by the addition of an internal standard (A) and by direct weighing (B).

Glyceride[1]	Candlenut		Glyceride	Sapium sebiferum (polar fraction)	
	A	B		A	B
333	2	3	33D	19.1	19.4
332	10	11	32D	23.5	23.8
331	6	8	31D	11.4	11.6
330	3	4	30D	11.2	9.5
322	13	11	22D	10.4	10.9
321	14	13	21D	8.7	9.3
320	8	6	20D	9.6	10.1
311	5	5	11D	1.5	3.5
310	4	6	10D	4.6	1.9
300	-	-			
222	6	7			
221	8	7			
220	6	5			
211	5	4			
210					
200					
111	10	10			
110					
100					

1. Abbreviations, page 100.

Table 6

Total composition of the separated Glyceride Fractions compared with the Component Esters (% mol.) of the Whole Oil[1]

Oil	No. of fractions	16:0	18:0	16:1	18:1	18:2	18:3
Linseed							
Plate A	10	6.9	3.8	0.6	17.3	14.0	57.4
Plate B	6	6.5	3.3	0.5	16.4	14.4	58.9
Triglyceride		6.1	3.2	0.1	16.6	14.2	59.8
Wild Rose							
Plate A	10	4.0	1.4	0.5	10.8	47.7	35.6
Plate B	6	4.0	1.4	0.4	10.7	46.8	36.7
Triglyceride		3.7	0.9	0.3	10.3	49.1	35.7
Candlenut							
Plate A	14	7.1	3.4	-	21.9	38.8	28.8
Plate B	8	7.2	3.1	-	22.0	38.7	29.0
Triglyceride		6.5	3.2	-	22.0	37.6	30.7
Rubber							
Plate A	14	9.9	10.5	-	26.6	34.5	18.5
Plate B	7	9.5	8.1	-	26.6	36.9	18.9
Triglyceride		10.7	10.6	-	25.0	32.7	21.0
Soya							
Plate A	8	12.2	3.5	0.5	24.2	50.9	8.7
Plate B	7	12.4	3.8	0.8	23.3	51.1	8.6
Triglyceride		12.0	3.6	0.6	23.7	51.4	8.7
Stillingia Oil							
(i) Non-Polar Fraction		6.2	1.8	0.3	11.1	26.3	54.3
Plate A	10	6.6	2.4	0,4	11.2	26.4	53.0
Plate B	6	6.6	2.6		11.4	25.8	53.6
(ii) Polar Fraction (33% 10:2)		5.8	1.7	0.2	8.9	22.0	28.1
Plate (33% 10:2)		5.6	1.6	0.3	9.7	21.8	27.7

1. Abbreviations, page 100.

Table 7

Component Acids Esterified at the 2- Position compared with the Composition of the Seed Oil (% mol.)

	16:0[1]	18:0	16:1	18:1	18:2	18:3
Linseed	6.1	3.2	0.1	16.6	14.2	59.8
2-Monoglyceride	0.7	-	0.2	18.8	20.6	59.7
Wild Rose	3.7	0.9	0.3	10.3	49.1	35.7
2-Monoglyceride	0.3	-	0.3	11.0	56.3	32.1
Candlenut	6.5	3.2	-	22.0	37.6	30.7
2-Monoglyceride	0.9	0.5	0.5	26.7	51.3	20.1
Rubber	10.7	10.6	-	25.0	32.7	21.0
2-Monoglyceride	1.2	0.6	-	23.7	48.6	25.9
Soya	12.0	3.6	0.6	23.7	51.4	8.7
2-Monoglyceride	1.8	0.6	0.2	23.2	66.2	8.0
Stillingia (non-polar)	6.2	1.8	0.3	11.1	26.3	54.3
Monoglyceride	1.6	-	-	14.9	39.2	44.3

1. Abbreviations, page 100.

Table 8

Component Glycerides (% mol.) of Linseed, Wild Rose Seed, Candlenut and Soya bean oils determined by T.L.C. (A) by Lipolysis (B) and by calculation from the Component Acids

Glyceride[1]	Linseed			Wild Rose			Candlenut			Rubber			Soya		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
333	23	22	22	5	5	5	2	3	3	1	1	1	-	-	-
332	14	15	15	17	19	19	10	11	11	3	4	4	1	1	1
331	15	18	18	6	4	4	6	6	6	3	3	3	-	1	1
330	12	10	10	4	2	2	3	2	3	3	3	3	1	-	-
322	3	3	4	21	25	26	13	13	13	6	6	6	7	7	7
321	5	8	8	10	11	12	14	15	15	9	10	10	5	6	6
320	5	5	5	4	5	4	8	7	7	8	10	10	4	4	5
311	5	5	5	1	1	1	5	5	4	5	4	4	2	2	2
310	7	6	6	1	1	1	4	4	4	7	7	7	3	2	2
300	1	1	1	-	-	-	-	-	1	3	3	3	2	1	1
222	1	-	-	13	12	12	6	5	5	7	3	3	15	13	13
221	2	1	1	8	7	8	8	9	9	8	8	8	16	19	19
220	-	1	1	4	4	3	6	5	4	6	7	7	13	13	13
211	1	1	1	2	2	2	5	5	5	6	6	6	8	9	9
210	3	2	1	2	2	1	5	5	5	11	11	11	12	12	12
200	-	-	-	1	-	-	1	1	1	4	5	5	4	4	3
111	1	1	1	1	-	-	1	1	1	3	2	2	2	1	1
110	2	1	1	-	-	-	2	2	2	4	4	4	5	3	3
100	-	-	-	-	-	-	1	1	1	3	3	3	-	2	2

1. Abbreviations, page 100.

Table 9

Glyceride categories[1] (% mol.) of Linseed, Wild Rose Seed, Candlenut, Soya bean and Rubber Seed Oils

	Linseed	Wild Rose	Candlenut	Rubber Seed	Soya Bean
S_2U[2]	1(2,2)[1]	1(0,0)	2(2,2)	10(11,11)	6(4,5)
SU_2	29(24,24)	15(14,11)	28(24,25)	39(42,42)	38(35,36)
U_3	70(74,74)	84(86,89)	70(74,73)	51(47,47)	56(61,59)

Glycerides (% mol.) Classified according to Number of Double Bonds

No. of Double Bonds					
1	-	-	1	3	-
2	2	1	3	8	9
3	5	3	6	17	16
4	8	7	15	19	24
5	12	13	21	21	22
6	18	27	23	19	21
7	18	27	19	9	7
8	14	17	10	3	1
9	23	5	2	1	

1. Experimental values given. Values determined by lipolysis and Gunstone's theory respectively are given in brackets.

2. Abbreviations, page 100.

Table 10

Component acids (% wt) of the oil in six individual Linseeds and of oil from a large number of Seeds[1]

Seed	16:0[1]	18:0	18:1[2]	18:2	18:3
A	6	3	11	12	68
B	7	3	12	11	67
C	6	4	12	12	66
D	7	3	12	15	63
E	6	4	20	14	56
F	6	4	22	14	54
Composite oil	6	3	17	14	60

1. Abbreviations, page 100.

2. Includes 16:1 (0.2-0.5%).

Table 11

The Triglyceride Composition (Gunstone) of two Linseeds A and F and a Seed (A+F) with the average fatty acid composition of Seeds A and F

Glyceride[1]	Seed A(i)	Seed F(ii)	Av(i)+(ii)	Seed(A+F)(iii)
333	31.2	15.6	23.4	22.3
332	16.6	12.0	14.3	14.5
331	15.0	19.2	17.1	18.4
330	13.1	9.2	11.1	10.9
322	3.0	3.0	3.0	3.0
321	5.3	9.9	7.6	8.0
320	4.5	5.0	4.8	4.8
311	2.4	8.0	5.2	5.0
310	4.2	7.6	5.9	5.9
300	1.4	1.4	1.4	1.3
222	0.2	0.3	0.25	0.2
221	0.4	1.2	0.8	0.9
220	0.4	0.6	0.5	0.5
211	0.6	2.0	1.3	1.1
210	0.8	1.4	1.1	1.2
200	0.2	0.3	0.25	0.3
111	0.1	1.1	0.6	0.5
110	0.4	1.6	1.0	0.8
100	0.2	0.6	0.4	0.4

1. Abbreviations, page 100.

Table 12

Component Acids and Glycerides (% mol.) of Soybean Oil

	component acids[1]				major component glycerides								
Ref.[2]	Sat.	18:1	18:2	18:3[a]	232	321	322	221	220	211	210[b]	U_3	U_2S
1	19	22	49	10	0	-		52		-	-	42	58
2	20	22	49	9	0	-		48	-	-	-	43	55
3	14	30	49	7	14	9	5	25	-	-	-	-	-
4	17	26	50	7	-	-	-	-	-	-	-	56	38
5	14	23	55	8	-	-	-	19	15	9	10	62	29
present work	16	24	51	9	15	5	7	16	13	8	12	56	38

1. Abbreviations, page 100.

2. Ref.1 Hilditch T.P., Meara & Holmberg, J. Am. Oil Chemists' Soc., 24, (1947), 321.
" 2 Hilditch T.P., A. J. Seavell J. Oil Col. Chem. Assn., 33, (1950), 24.
" 3 Scholfield, C.R., M. A. Hicks, J. Am. Oil Chemists' Soc., 34, (1957), 77.
" 4 Youngs C.G., M. R. Subbaram, J. Am. Oil Chemists' Soc., 41, (1964), 218.
" 5 Jurreus G., Kroesen A.C.J., J. Am. Oil Chemists' Soc., 42, (1965), 9.

Table 13

Stillingia Oil:- Component Acids and

Component Acids (% mol.)	10:2[2]	16:0	1
Whole oil	9.5	6.5	
Non-polar Fraction (70% mol.)	-	6.2	
Polar Fraction (30%)	33.3	5.8	

Component Glycerides (% mol.)

	Non-polar Fraction		
333	11.6	222	1.8
332	15.4	221	1.4
331	8.0	220	1.4
330	6.3	211	1.0
322	6.7	210	0.7
321	4.6	200	0.3
320	6.0	111	TR.
311	1.8	110	0.3
310	2.1	100	0.3
300	0.3	000	-

Glyceride Categories (% mol.)

S_2U - 1	9 double bonds	-	12
SU_2 - 24	8 " "		21
U_3 - 75	7 " "		22
	6 " "		19

1. See "Abbreviations", p. 100.
2. Determined by U.V.
3. D signifies dienoic acid.

PART II

THE POSITIONAL DISTRIBUTION OF LINOLENIC OR CONJUGATED UNSATURATED FATTY ACIDS IN SEED OILS

A. EXPERIMENTAL

1. Isolation of Neutral Oils. The linolenic acid containing seed oils (linseed, candlenut, wild rose, rubber seed and soya bean) were extracted from the crushed seeds with light petrol (b.pt 40-60°) in a soxhlet extractor.

The crushed seeds containing the conjugated unsaturated acids (Calendula officinalis, Catalpa ovata, Catalpa bignonoides, Catalpa speciosa, Jacaranda mimosifolia, Momordica balsamina, Momordica charantia, Tung oil, Centranthus ruber, Parinarium laurinum, Impatiens glanduligera and Santalum acuminatum) were extracted six times with cold hexane in a pestle and mortar. The solutions were filtered and the solvent evaporated in a stream of nitrogen. The percentage oil extracted from the seeds is given in Table 4.

The extracted oils were examined by T.L.C. on silica gel G, developed with light petrol-ether (3:2) and the components detected with iodine or by charring. If appreciable amounts of partial glycerides and free fatty acid were present the neutral triglycerides were isolated by chromatography on silica [174]. This was unnecessary in the majority of cases.

The linolenic acid containing oils were stored at 0° under nitrogen prior to use but oils containing conjugated unsaturated acids were extracted on the same day that they were required for analysis.

2. Lipolysis procedure. The lipolysis conditions were similar to those described by Desnuelle [141] and Fulton and Coleman [140]. The fat

(0.5-1 g), 1.2M ammonium chloride-ammonia buffer (pH 8.5, 20 ml), calcium chloride solution (2 ml of 22% $CaCl_2$, $6H_2O$) and bile salt solution (0.1 ml of 25% solution) were placed in a jacketed vessel and maintained at 40°. Pancreatic lipase (100 mg) was added and the mixture stirred vigorously for 10 minutes during which time the pH of the solution was maintained at 8.5 by addition of ammonia solution (0.88 s.g.).

The lipolysis mixture was then poured directly into N.HCl (200 ml) and extracted three times with ether. The combined extracts were washed with water until neutral.

One of two methods was then used to separate the lipolysis products.

3. Separation and Analysis of Lipolysis Products

(i) Free fatty acid was removed from the etherial solution by passing it down a column of Amberlite IRA 400 (OH)* resin (30 g,) and eluting the column with a further 50 ml ether. The eluate was washed with water and the glycerides isolated. The mono-, di- and triglycerides were separated by the method of Quinlin and Weiser (174). Normally only the monoglyceride fraction was required for analysis, in which case the silica column was first eluted with 250 ml benzene-ether (10%) to remove tri- and diglycerides and then with ether (200 ml) to obtain the monoglyceride.

The free fatty acids from the lipolysis if required were eluted from the resin column with a mixture of 270 ml methanol-ether-hydrochloric acid (100:150:20 v.v.). The eluant was neutralised with sodium carbonate, evaporated and the free fatty acids isolated.

* Generated from IRA 400 (Cl) resin by eluting with 400 ml N.NaOH and washing with water until neutral.

(ii) The lipolysis products (≈ 30 mg) were separated by T.L.C. on silica (300 μ thick) developed with chloroform-acetone-0.880 ammonia (80:20:1 v.v.). The free fatty acid remained on the base line as ammonium salts, the monoglyceride had an Rf value of 0.3 and the di- and triglycerides moved close to the solvent front. The separated components were detected with dichlorofluorescein and extracted from the silica six times in centrifuge tubes with 10 ml portions of solvent. The glycerides were extracted with peroxide-free ether and the free fatty acids with ether-methanol - N.HCl (5:5:1). If the weights of the individual fractions were to be estimated a known amount of methyl heptadecanoate was added as an internal standard to the etherial extracts. The products were then isolated and converted to methyl esters either by transesterification with methanol and sodium methoxide [171] or by esterification using BF_3-MeOH [196].

The analytical conditions used for determining the composition of the fatty acid methyl esters have been described previously. Conjugated unsaturated fatty acid methyl esters were also separated on a diethyleneglycol succinate column (Im. Stainless steel column, $\frac{1}{4}$" o.d. packed with 20% D.E.G.S. on Gas Chrom. Z[1]) at 180°C with nitrogen (90 ml) as the carrier gas.

4. Ultra-violet Spectroscopy (U.V.). The proportion of conjugated trienoic acids in some of the seed oils was also determined by quantitative U.V. spectroscopy. Spectra of methanolic solutions of the oils were read on the Unicam S.P. 700 spectrometer. An average value [197-199] of 1820 was used for the $E^{1\%}_{1cm}$ of α-eleostearic acid in methanol.

5. Characterisation of Linoleic acid in Calendula officinalis

An acid in Calenduala officinalis seed oil, with a similar Rf value to linoleic acid on gas-liquid (P.E.G.S.) and silica-silver

1. Obtained from APPLIED SCIENCE Lab. English agents PYE, Cambridge.

nitrate chromatograms was isolated by preparative thin-layer chromatography on silica-silver nitrate.

The mixed methyl esters (0.1 g) were separated on four chromatoplates (0.3 mm thick layer of silica-silver nitrate 30g:5g:60 ml water) developed with benzene. After detecting the separated components with dichlorofluorescein the band corresponding to linoleic acid was extracted six times with ether in centrifuge tubes.

The pure product (26 mg) was oxidised using von Rudloff's procedure [9] as modified by Craig and Tulloch [200]. The acids produced by the oxidation were isolated, converted to methyl esters using BF_3-MeOH reagent [196] and characterised by comparison with suitable standard compounds on G.L.C.

B. RESULTS AND DISCUSSION

1. General Observations

The composition of the fatty acids at the 2- position of natural oils is readily determined by examining the products of enzyme hydrolysis. This technique has been widely applied [142-149] in the examination of seed oils containing mainly palmitic, stearic, oleic, linoleic and linolenic acids. For the method to be of any analytical value however it is essential that when mixed fatty acid triglycerides are hydrolysed, the different fatty acid chains are removed at similar rates from the 1, 3- positions. This ensures that no acid or group of acids is concentrated in the monoglyceride. If an acid is hydrolysed more rapidly than other acids in the mixture, then the monoglyceride composition will probably show a misleading enrichment of the least hydrolysed acids.

It has been shown [201-204] that the esters of short chain fatty acids (4-10 carbon atoms) are hydrolysed faster than long

chain fatty acids (12-18 C), the latter having similar hydrolysis rates. Significant results are therefore obtained when oils containing only common long chain fatty acids (12:0-18:0, 16:1, 18:1, 18:2, 18:3) are examined by the enzyme-hydrolysis technique.

The action of pancreatic lipase on unusual acids should be determined however before analysing oils in which they occur. Jensen et al [205] and Clement et al [206] for example, found no difference between the hydrolysis rates of elaidic and oleic acids. Acids with double bonds near the α position are hydrolysed at a slower rate than normal long chain acids however [207].

In our work we have analysed two groups of seed oils, the first containing linolenic acid as a major component, and the second, conjugated unsaturated fatty acids.

Before proceeding with our analyses of conjugated unsaturated oils we ensured that the conjugated trienoic acids were not behaving abnormally during lipolysis. The proportions and composition of the enzyme-hydrolysis products of Centranthus ruber seed oil were determined by preparative-T.L.C. and G.L.C. of the fractions containing an internal standard (Table 1). These results may be compared with those obtained for a seed oil (candlenut) containing the more common fatty acids. The monoglyceride fraction of Centranthus ruber contains only 1% triene compared to 52% in the original oil. The free fatty acid and di-, triglyceride compositions however indicate that the lipolysis of the conjugated acid has proceeded normally. The composition of the di- and triglyceride fraction compares favourably with that of the original oil and proves that the triene is being hydrolysed at a similar rate to the other long chain acids. This is confirmed by the relative amounts of saturated acids to triene in the free fatty acid and in the oil. Similar results were obtained for Catalpa ovata (9^{t}, 11^{t}, 13^{c}) and Jacaranda mimosifolia (8^{c}, 10^{t}, 12^{c}) seed oils (Table 1b).

Table 1a

The Fatty Acid composition of the Lipolysis Products of Candlenut, Centranthus ruber and Santalum acuminatum Seed Oils

(i) Lipolysis Products of Candlenut Oil

Component	% Mol. Fatty Acids	Component acids[1] (% Mol.) 16:0	18:0	18:1	18:2	18:3
Triglyceride	29.5	5.4	3.9	20.6	36.1	34.0
Diglyceride	20.6	6.2	4.4	21.3	39.5	28.6
Free Fatty Acid	36.9	7.3	4.0	18.5	34.2	36.0
Monoglyceride	13.0	0.9	0.5	27.2	51.3	20.1
Total Fatty Acids		5.7	3.6	20.8	38.1	31.8
Original Oil		6.0	3.2	22.5	37.7	30.6

(ii) Lipolysis Products of Centranthus ruber Seed Oil

Component	% Mol. Fatty Acids	Component acids (% Mol.) 16:0	18:0	18:1	18:2	18:3	conj. 18:3
Di- & Triglyceride	50.3	3.9	3.2	3.8	39.3	1.8	48.0
Free Fatty Acid	36.5	7.1	5.6	5.5	13.8	3.2	64.8
Monoglyceride	13.2	0.6	-	6.4	90.8	1.2	1.0
Total Composition		4.6	3.7	4.8	36.8	2.2	47.9
Original Oil		4.0	2.8	4.0	36.0	1.3	52.1

(iii) Lipolysis Products of Santalum acuminatum

	Component acids (% Mol.) 16:0	18:0	18:1	a	b	c	d	conj. C.18 enyne	e
Di- & Triglyceride	7.6	5.0	14.5	10.6	55.6	1.9	2.6	2.2	-
Free Fatty Acid	4.4	1.9	37.8	-	7.8	4.0	1.3	42.8	-
Monoglyceride	2.6	1.8	37.0	-	20.6	6.6	Tr	31.4	-
Original Oil	1.1	1.2	27.7	-	14.2	7.8	Tr	30.5	17.5
Carbon Nos. on D.E.G.S.			18.6	19.0	19.4	20.3	21.0	23.0	24.6

1. Abbreviations, page 100.

Table 1b

Fatty Acid Composition of the Lipolysis Products of Catalpa ovata and Jacaranda mimosifolia

C.ovata (9^{t}, 11^{t}, 13^{c})

Component	% Mol. F. Acid	Component Acids[1] 16:0	18:0	18:1	18:2	18:3	a[2]	conj. 18:3.
Di- & Triglyceride	25.0	3.9	3.1	12.0	45.2	1.6	1.3	32.9
Free Fatty Acid	46.0	6.6	4.5	7.4	29.7	1.3	1.9	48.6
Monoglyceride	29.0	0.2	-	16.3	66.6	2.0	1.5	13.4
Original oil		3.0	1.5	9.9	39.9	2.7	2.4	40.6

J.mimosifolia (8^{c}, 10^{t}, 12^{c})

Component	% Mol. F. Acid	Component Acids 16:0	18:0	18:1	18:2	18:3	conj. 18:3
Di- & Triglyceride	5.0	21.0	9.1	10.5	25.8	-	33.5
Free Fatty Acid	64.0	6.4	8.4	11.6	27.7	3.6	42.3
Monoglyceride	31.0	0.5	-	17.2	76.8	-	5.5
Original oil		4.6	3.9	12.2	38.3	2.5	38.5

1. Abbreviations, page 100.

2. C. No. 21.0 on D.E.G.S.

The importance of examining the lipolysis products of unusual seed oils was further underlined however when Santalum acuminatum seed oil was analysed (Table 1a). The oil contains a conjugated octadecenynoic acid [208,209] and an unidentified acid (C.No. 24.6 on D.E.G.S.) detected by us by G.L.C. This acid was absent from the monoglyceride, but the composition of the remaining products indicated that the acid was not being recovered and the analysis was void.

Linolenic acid containing oils

Six oils, including the non-polar fraction of Sapium sebiferum, with different proportions of linolenic acid (9-60%) were examined. In order to obtain a more complete picture of the distribution of saturated, oleic, linoleic and in particular linolenic acids some results published by other workers are also included (Table 2). The component acids of the triglyceride and of the 2- position (Table 3) together with the selectivity factors of the unsaturated acids are given (Table 2).-See page 21 for definition.

As expected from previous results, the saturated acids occur only to a minor extent in the 2- position. The selectivity factors of the unsaturated acids are distinctly different and a general trend is noted.

The selectivity factor for linolenic acid is very consistent ($0.8 \pm .1$ in eleven out of thirteen cases) and lower than that of the combined unsaturated acids. Linoleic acid on the other hand has a consistently high selectivity factor ($1.1 \pm .1$ in eight cases). Oleic acid is more erratic in its behaviour but tends to have an average selectivity factor approaching 1.0. These results compare favourably with those reported by Mattson and Volpenhein [147] for seed oils which also contain C_{20} unsaturated acids.

Table 2

Selectivity Factors of Acids in Linolenic Acid containing Seed Oils

Ref.	Seed Oil	Component Acids[6]				Selectivity Factors		
		Sat[d]	18:1	18:2	18:3	18:1	18:2	18:3
1	Wild rose	4	11	49	36	1.0	1.1	0.9
2	Wheat flour	8	27	58	7	1.2	1.1	0.8
1	Stillingia (m.p.)	8	11	26	55	1.2	1.4	0.8
1	Linseed	9	17	14	60	1.0	1.3	0.9
2	Linseed	10	22	15	52	1.1	1.4	0.8
1	Candlenut	10	22	37	31	1.1	1.3	0.6
2	Walnut	11	15	61	12	1.0	1.0	0.9
3	Soya	13	27	53	8	1.4	0.9	0.7
2	Soya	16	25	51	8	0.7	1.1	0.9
1	Soya	16	24	51	9	0.8	1.1	0.8
2	Wheat germ	20	18	55	7	0.8	1.1	0.7
5	Rubber	21	25	33	21	0.8	1.2	1.0
4	Morning glory	35	15	42	8	1.0	1.0	0.8

1. Gunstone, F.D., Padley, F.B., J. Am. Oil Chemists' Soc., 42, (1965).
2. Mattson, F.H., Volpenhein, R.A., J. Lipid Res., 4, 392 (1963).
3. Mattson, F.H., Lutton, E.S., J. Biol. Chem., 233, 868, (1958).
4. Mattson, F.H., Volpenhein, R.A., J. Biol. Chem., 236, 1891, (1961).
5. This Thesis.
6. Abbreviations, page 100.

Table 3

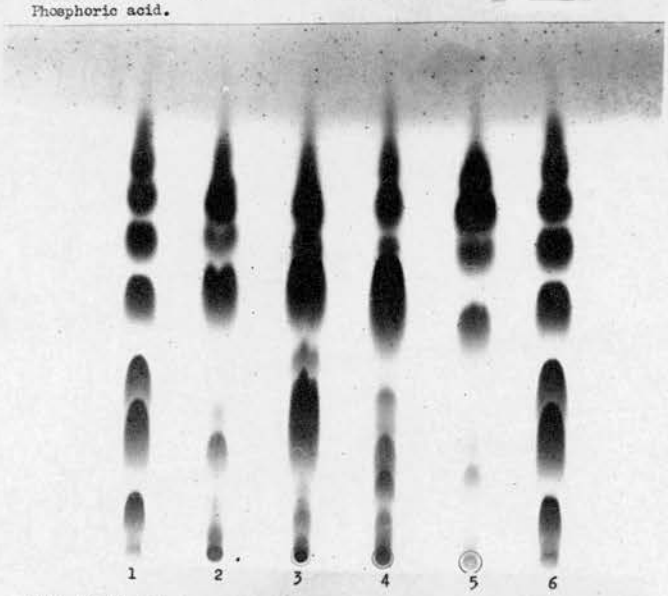
Lipolysis results for Seed oils containing Linolenic Acid

Seed oil	Component Acids[1] (% Mol.)					
	16:0	18:0	16:1	18:1	18:2	18:3
Linseed						
Triglyceride	6.1	3.2	0.1	16.6	14.2	59.8
Monoglyceride	0.7	-	0.2	18.8	20.6	59.7
Wild Rose						
Triglyceride	3.7	0.9	0.3	10.3	49.1	35.7
Monoglyceride	0.3	-	0.3	11.6	56.3	32.1
Candlenut						
Triglyceride	6.5	3.2	Tr	22.0	37.6	30.7
Monoglyceride	0.9	0.5	0.5	26.7	51.3	20.1
Rubber						
Triglyceride	10.7	10.6	-	25.0	32.7	21.0
Monoglyceride	1.2	0.6	-	23.7	48.6	25.9
Soya						
Triglyceride	12.0	3.6	0.6	23.7	51.4	8.7
Monoglyceride	1.8	0.6	0.2	23.2	66.2	8.0
Stillingia (non-polar)						
Triglyceride	6.2	1.8	0.3	11.1	26.3	54.3
Monoglyceride	1.6	-	-	14.9	39.2	44.3

1. Abbreviations, page 100.

Thin Layer chromatography on Silica impregnated with Silver Nitrate, of Seed oils containing conjugated Trienoic Acids.

Chromatograph developed with Benzene/Ether (20%), components detected by charring at 250°C after spraying with 50% aqueous Phosphoric acid.



Name of oil. L to R. 1, Jatropha curcas., 2. Tung oil., 3, Catalpa bignonoides., 4, Calendula officinalis., 5, Momordica balsamina., 6, Jatropha curcas.

Fig.1

3. Oils containing Conjugated Unsaturated Acids

(i) Analytical Methods

Thin-Layer Chromatography. We have not been primarily concerned with the triglyceride composition of the oils we examined but the chromatograms of a number of oils on silica-silver nitrate (T.L.C.) are shown in Fig. 1. The separations are not as effective as for non-conjugated unsaturated compounds because of the lower complexing power of conjugated double bonds, conjugated dienes and trienes complexing only to a slightly greater extent than the double bond in oleic acid. The R_f values of the triglycerides are compared with those of Jatropha curcas triglycerides (Appendix 1).

It is well known that conjugated unsaturated acids polymerise and oxidise very readily. Each stage of our experimental procedure was therefore checked to see if any conjugated trienoic acids were lost. No loss of conjugated acids was observed when Calendula officinalis triglycerides were mixed with buffer solution (NH_3-NH_4Cl. pH 8.5) at 40° for 15 minutes. A small loss (5%) was observed when the triglycerides were eluted from a silica column with benzene, or extracted from a silica chromatoplate with ether.

Gas-Liquid Chromatography. The accuracy of G.L.C. for estimating conjugated acids was also determined. Morris et al (210) observed that conjugated trienoates undergo cis-trans isomerisation during gas-liquid chromatography and concluded that this occurred mainly at injection because of the high temperature of the flash heater. Mikolajczak and Bagby (211) collected α-eleostearic acid after chromatography and found only 81% triene, which was a mixture of isomers produced by double bond migration.

We prepared our methyl esters by transesterification with sodium methoxide-methanol and obtained similar chromatograms to Morris when we used a diethyleneglycolsuccinate stationary phase

at 180° (flash heater 210°). Approximately 10% isomerisation to β-eleostearate (all trans) occurred when methyl α-eleostearate was analysed under these conditions. The proportion of conjugated trienoic acid in a number of oils was determined both by G.L.C. and U.V. spectroscopy. The values compared favourably (Table 6) and G.L.C. was therefore normally used to determine the total fatty acid composition of the seed oils and their lipolysis products. The carbon numbers of the fatty acid methyl esters on a polyester stationary phase (diethyleneglycolsuccinate) are given in Table 5. During our work we noticed that little or no isomerisation occurred in methyl esters obtained from the seed oils of C.ovata, C.speciosa, or M.balsamina. Even when high column (200°) and flash heater (240°) temperatures were used no isomerisation or degradation of C.ovata methyl esters was observed. Two peaks corresponding to conjugated trienes (ctt and ttt) consistently appeared in other oils, however, especially C.ruber (9c, 11t, 13t) in which 30-40% of the triene was the all-trans isomer. The possibility that the all-trans isomer might be present in the original oil was therefore examined (references describing characterisation of conjugated acids given in Table 4).

The infra-red spectra for M.charantia, C.ruber and isomerised tung oil were therefore compared in the 10.43 (cis) and 10.06 (trans) regions (212, 213). The ratio $\frac{\log T \text{ (trans)}}{\log T \text{ (cis)}}$ was determined from spectra of solutions of the oils in carbon tetrachloride. The values obtained were 4.8, 6.3 and 14.8 for M.charantia, C.ruber and isomerised tung oil respectively. These results further indicated that the conjugated triene in C.ruber was a mixture of the ctt and ttt isomers. For additional confirmation we decided to compare the nuclear magnetic resonance (N.M.R.) spectra of some of the oils.

Nuclear Magnetic Resonance

The only previous reported spectrum of an oil containing conjugated trienes was given by Hopkins [214,215] for tung oil at low resolution.

The N.M.R. spectra of seven oils containing different conjugated trienoic acids are given in Fig. 2. No attempt will be made to interpret the 'splitting' of each main signal; the spectra were obtained principally for comparison. The signals obtained for conjugated double bonds are in the region 4.1-4.2 p.p.m. In this region the spectra are identical for three oils, i.e. M.charantia (9c, 11^{t}, 13^{t}). C.speciosa (9^{t}, 11^{t}, 13c) and C.officinalis (8c, 10^{t}, 12^{t}), with the same double bond configuration but different positions. The spectra for oils containing conjugated acids with different configurations are distinctly different however, and the main signals are at different positions i.e. isomerised tung oil (9^{t}, 11^{t}, 13^{t}) - 4.09 p.p.m.; M.charantia (9c, 11^{t}, 13^{t}) - 3.82 p.p.m.; M.balsamina (9c, 11^{t}, 13c) - 3.71 p.p.m.

The spectrum for C.ruber is different from the other spectra in the 3.7-4.1 p.p.m. region which indicates that either the configuration of the double bonds is different from those examined, or the oil contains more than one conjugated trienoic acid. Infra-red spectroscopy and G.L.C. evidence favours the latter possibility and the oil probably contains a mixture of α- and β-eleostearic acids. This might arise from the extraction of seeds which have deteriorated during storage but a visual examination indicated that the seeds were sound; on the other hand it could be the first instance of different conjugated trienoic acids occurring together in the same seed. Additional confirmation is required but unfortunately a fresh sample of C.ruber seeds was not readily available.

Fig.2a

N.M.R. SPECTRA OF OILS CONTAINING CONJUGATED TRIENOIC ACIDS

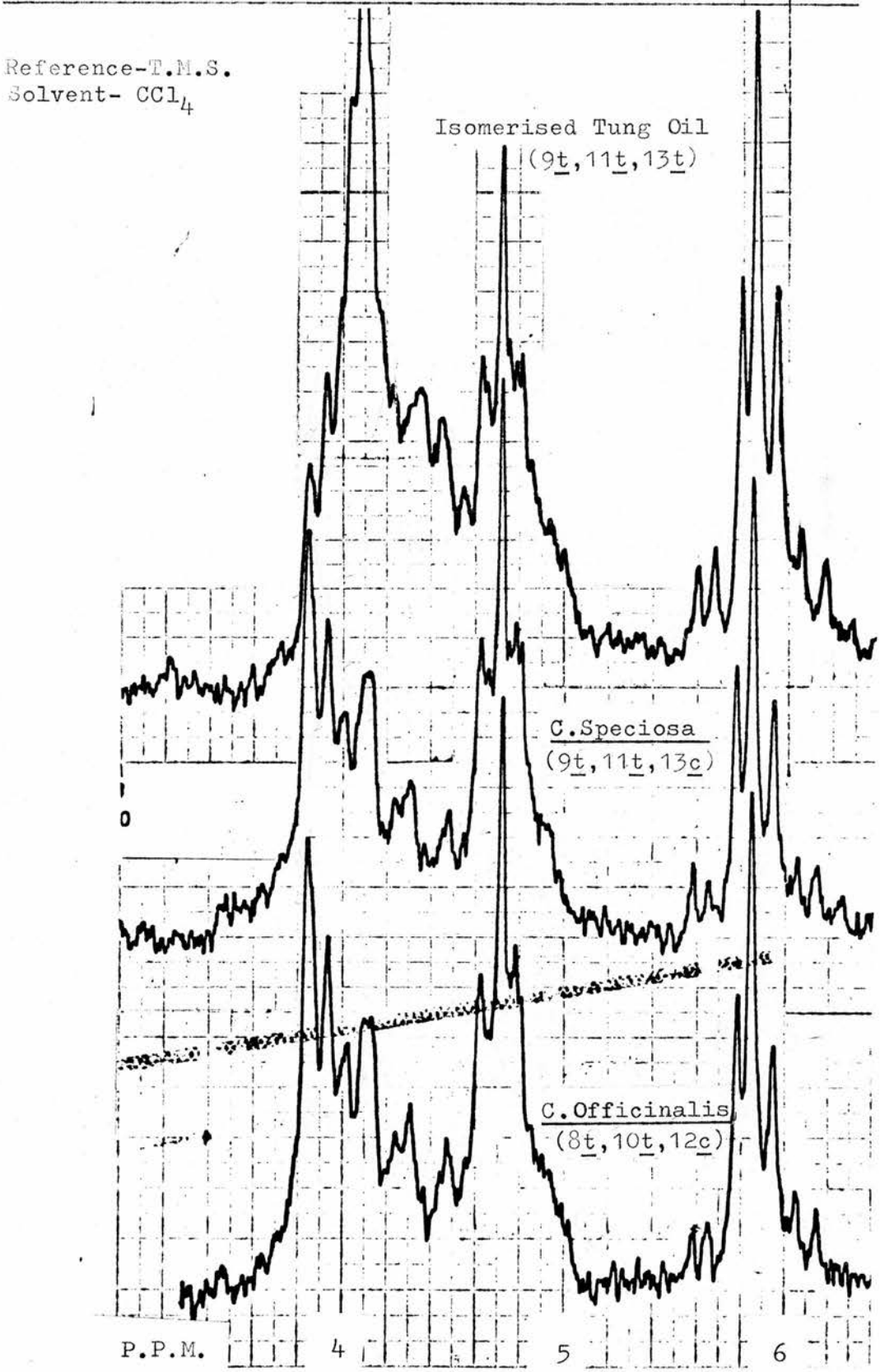
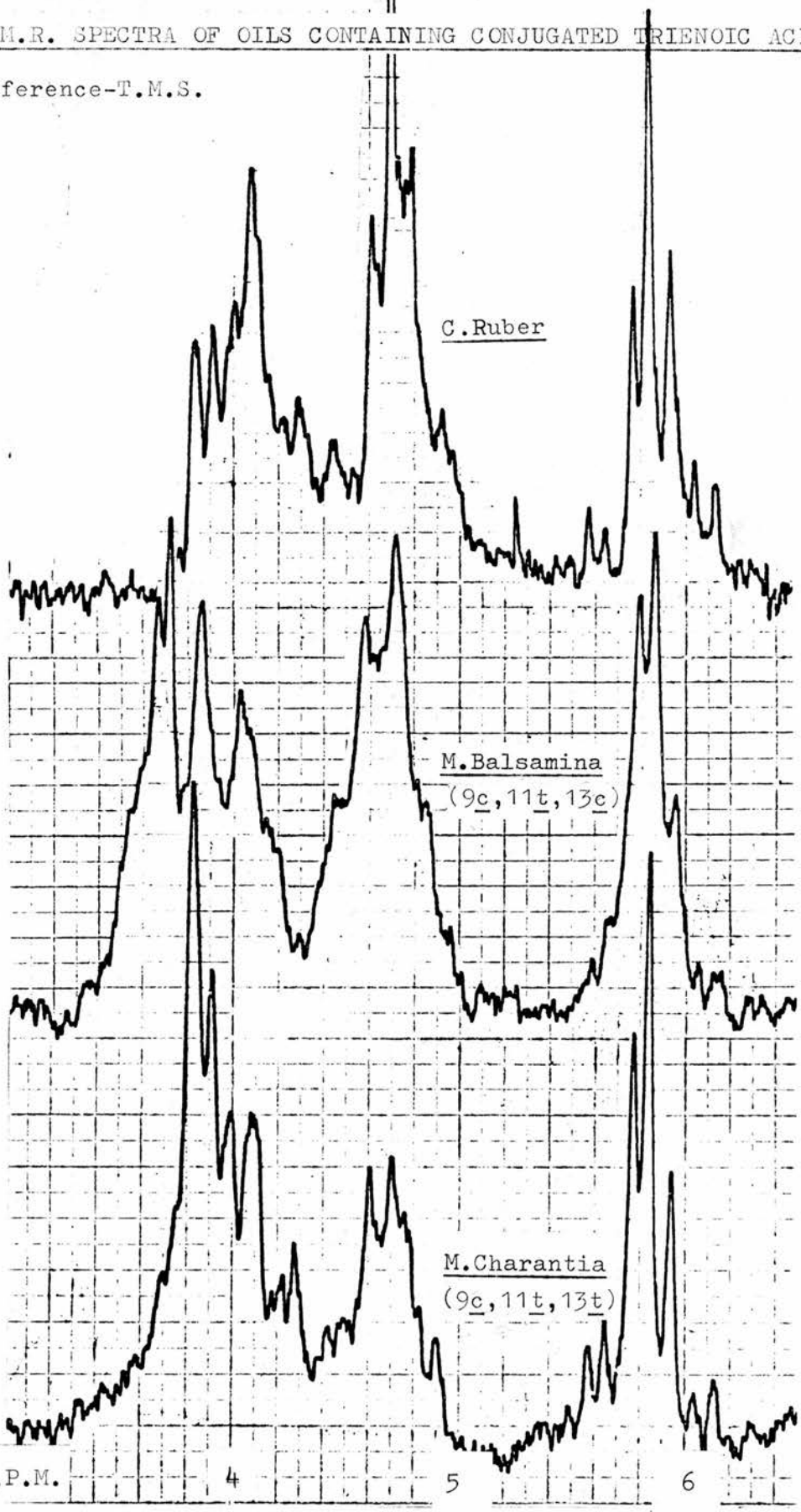


Fig.2b

N.M.R. SPECTRA OF OILS CONTAINING CONJUGATED TRIENOIC ACIDS.

Reference-T.M.S.



These initial results suggest that N.M.R., particularly at higher resolution, may be used to determine the configuration of double bonds in conjugated trienoic acids. Reference spectra of all the configurational isomers (6) are obviously required however before a definite conclusion can be drawn.

(ii) Lipolysis Results

Nine oils containing major amounts of conjugated trienoic acids and two oils containing conjugated tetra-, tri- and dienoic acids were examined. The validity of these analyses is discussed in the first section. The position and configuration of the double bonds in the trienoic acids were determined previously, mainly by Hopkins and Chisholm (Table 4).

Only one previous analysis of this type of oil had been reported prior to our work. Youngs [128] analysed Momordica charantia seed oil, and made the unusual discovery that the experimentally determined triglyceride composition did not agree with that calculated from lipolysis results. This indicated that the fatty-acid distribution in the 1- and 3- positions was not random.

The composition of the fatty acids in the 2- position of the seed oils we examined is given in Table 6 and the enrichment and selectivity factors in Table 7. In the cases where the trienes have very low enrichment factors they have been classified as Category I acids [155] (normally saturated acids) when calculating the selectivity factors.

The oils which contain a conjugated trienoic acid as their major component show remarkable differences in their patterns of fatty acid distribution (c.F. enrichment factors and selectivity factors).

Table 4

Seed Oils containing Conjugated Unsaturated Fatty Acids

Family	Species	Oil in seeds (% wt)	Conjugated acid	Reference
Euphorbiaceæ	Aleurites montana (Tung oil)	-[1]	9c, 11t, 13t	1, 2, 3, 4
Valerianaceæ	Centranthus ruber	19	9c, 11t, 13t	5
Compositæ	Calendula officinalis	14	8t, 10t, 12c	6, 7
Cucurbitaceæ	Momordica charantia	22, 25	9c, 11t, 13t	5
"	Momordica balsamina	21	9c, 11t, 13c	5
Bignoniaceæ	Catalpa ovata	14	9t, 11t, 13c	8
	Catalpa speciosa	19	9t, 11t, 13c	5
	Catalpa bignonoides	21	9t, 11t, 13c	10
	Jacaranda mimosifolia	22	8c, 10t, 12c	9

1. Obtained as the oil.

1. Paschke, R.F., Tolberg, W., Wheeler, D.H., J. Am. Oil Chemists' Soc., 1953, 30, p.97.
2. Bickford, W.G., Dupre, E.F., Mack, C.H., O'Connor, R.T., J. Am. Oil Chemists' Soc., 30,
3. Ahlers, N.H., Brett, R.A., McTaggart, N.G., J. Appl. Chem., 3, (1953), 433. (1953), 376.
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8. Hopkins, C.Y., Chisholm, M.J., J. Chem. Soc., 1962, 573.
9. Hopkins, C.Y., Chisholm, M.J., J. Org. Chem., 27, (1962), 3137/ref. 10. Private communication.

Table 5

Carbon Numbers of some Non-Conjugated and Conjugated Unsaturated Fatty Acid Methyl Esters on Diethyleneglycol succinate (D.E.G.S.)

Analytical Conditions:- 1 metre stainless steel column (¼" o.d.)
Packing:- Gas Chrom. Z (Applied Science Lab.) coated with 20% D.E.G.S.
Column temp. 180°. Flash heater 210°.
Flow Rate. 90 ml N_2/Min.
Flame Ionisation Detector.

Type	C_{18} Acids Position and Configuration of Double bonds	Carbon No.[1]
Oleic	9c	18.6
Linoleic	9c, 12c	19.4
Linolenic	9c, 12c, 15c	20.6
Diene (conj.)	-	22.1
Triene (conj.)		
Punicic acid	9c, 11t, 13c	23.1
Analogue of Punicic acid	8c, 10t, 12c	23.1
α-eleostearic	9c, 11t, 13t	23.3
Catalpic acid	9t, 11t, 13c	23.4
Analogue of Catalpic acid	8t, 10t, 12c	23.4
	8t, 10t, 12t	23.8
β-eleostearic	9t, 11t, 13t	23.8
Tetraene (conj.)	9, 11, 13, 15	25.8 & 26.3

1. Results within ± 0.1.

Calendula officinalis (8_t, 10_t, 12_c)								
Triglyceride	3.0	-	4.3	28.8	-	-	63.9	-
Monoglyceride	0.7	-	4.6	14.4	-	-	80.3	-
Momordica balsamina (9_c, 11_t, 13_c)								
Triglyceride	9.6	5.3	7.4	9.7	-	-	68.0	-
Monoglyceride	1.0	-	6.0	20.7	-	-	72.3	-
Jacaranda Mimosifolia (8_c, 10_t, 12_c)								
Triglyceride	4.6	3.9	12.2	38.3	2.5	-	38.5	-
Monoglyceride	0.5	-	17.2	76.8	-	-	5.5	-
Parinarium laurinum[5] (9, 11, 13, 15)								
Triglyceride	3.4	3.9	1.9	1.8	-	17.8	30.3	40.9
Monoglyceride	1.5	0.9	2.6	3.5	-	28.2	32.8	30.5
Impatiens glanduligera[5,6]								
Triglyceride	8.8	11.8	14.7	29.8	-	10.3	6.6	17.4
Monoglyceride	3.0	9.4	16.5	43.1	-	10.8	5.4	11.0

1. Abbreviations, page 100
2. Proportion of conj. 18:3 determined by U.V. in brackets.
3. Position and configuration of conjugated double bonds.
4. Triglyceride contained 2.4% and monoglyceride 1.5% of component, C.No. 21.0.
5. Configuration of double bonds unknown.
6. Triglyceride contained 0.6% and monoglyceride 0.8% 16:1.

Table 6

Lipolysis Results for Seed oils Containing Conjugated dienoic, trienoic and tetraenoic acids

Seed oil	Component Acids[1] (% Mol.)							
	16:0	18:0	18:1	18:2	18:3	conj. 18:2	conj.[2] 18:3	conj. 18:4
Tung oil (9c, 11t, 13t)[3]								
Triglyceride	3.1	2.1	11.2	14.6	-	-	69.0 (70.5)	-
Monoglyceride	0.3	-	8.8	30.5	-	-	60.4	-
Momordica charantia (9c, 11t, 13t)								
(i) Triglyceride	1.8	18.1	8.8	9.9	-	-	61.2 (59.5)	-
Monoglyceride	1.4	3.2	4.5	14.2	-	-	76.7	-
(ii) Triglyceride	1.2	20.9	8.2	6.6	0.5	-	62.6	-
Monoglyceride	0.1	0.6	2.6	8.9	-	-	87.8	-
Centranthus ruber (9c, 11t, 13t)								
Triglyceride	4.0	2.8	4.0	36.0	1.3	-	52.1 (47.5)	-
Monoglyceride	0.6	-	6.4	90.8	1.2	-	1.0	-
Catalpa ovata[4] (9t, 11t, 13c)								
Triglyceride	3.0	1.5	9.9	39.9	2.7	-	40.6 (40.2)	-
Monoglyceride	0.2	-	16.3	66.6	2.0	-	13.4	-
Catalpa bignonoides (9t, 11t, 13c)								
Triglyceride	2.3	1.5	9.6	45.1	-	-	41.5 (40.1)	-
Monoglyceride	0.4	-	14.8	76.2	-	-	8.6	-
Catalpa speciosa (9t, 11t, 13c)								
Triglyceride	4.6	2.0	7.8	37.9	3.2	-	44.5	-
Monoglyceride	1.8	0.8	12.4	70.2	2.8	-	12.0	-

Table 7

Enrichment and Selectivity Factors of Acids in Seed Oils

Seed oil	Component Acids[1] (% Mol.)						
	Sat[d.]	18:1	18:2	18:3	conj. 18:2	conj. 18:3	conj. 18:4
Tung oil (9c, 11t, 13t)	5.2	11.2	14.6	-	-	69.0	-
M.charantia (9c, 11t, 13t)							
(i)	19.9	8.8	9.9	-	-	61.2	-
(ii)	22.1	8.2	6.6	0.5	-	62.6	-
C.ruber (9c, 11t, 13t)	6.8	4.0	36.0	1.3	-	52.1	-
C.ovata[3] (9t, 11t, 13c)	4.5	9.9	39.9	2.7	-	40.6	-
C.bignoncides (9t, 11t, 13c)	3.8	9.6	45.1	-	-	41.5	-
C.speciosa (9t, 11t, 13c)	6.6	7.8	37.9	3.2	-	44.5	-
C.officinalis (8t, 10t, 12c)	3.0	4.3	28.8	-	-	63.9	-
M.balsamina (9c, 11t, 13c)	14.9	7.4	9.7	-	-	68.0	-
J.Mimosifolia (8c, 10t, 12c)	8.5	12.2	38.3	2.5	-	38.5	-
P.laurinum[4] (9, 11, 13, 15)	7.3	1.9	1.8	-	17.8	30.3	40.9
I.glanduligera[4] (9, 11, 13, 15)	20.6	14.7	29.8	-	10.3	6.6	17.4

1. Abbreviations, page 100
2. Considered as Category 1 acids.
3. See Table 6, footnote 4.
4. See Table 6, footnote 5 and 6.

ontaining Conjugated dienoic, trienoic and tetraenoic Acids

Enrichment Factors						Selectivity Factors				
Sat[d.]	18:1	18:2	conj. 18:2	conj. 18:3	conj. 18:4	18:1	18:2	conj. 18:2	conj. 18:3	conj. 18:4
0.1	0.8	2.1	-	0.9	-	0.8	2.0	-	0.8	-
0.2	0.5	1.4	-	1.3	-	0.4	1.2	-	1.1	-
0.1	0.3	1.4	-	1.4	-	0.2	1.1	-	1.1	-
0.1	1.6	2.5	-	< 0.1	-	1.0	1.5	-	-[2]	-
0.1	1.6	1.7	-	0.3	-	1.0	1.0	-	-[2]	-
0.1	1.5	1.7	-	0.2	-	0.9	1.0	-	-[2]	-
0.3	1.6	1.9	-	0.3	-	1.0	1.1	-	-[2]	-
0.2	1.1	0.5	-	1.3	-	1.1	0.5	-	1.2	-
0.1	0.8	2.1	-	1.1	-	0.7	1.8	-	0.9	-
0.1	1.4	2.0	-	0.1	-	0.7	1.0	-	-[2]	-
0.3	1.4	1.9	1.6	1.1	0.7	1.3	1.8	1.5	1.0	0.7
0.6	1.1	1.4	1.0	0.8	0.6	1.1	1.3	0.9	0.7	0.6

Even the same conjugated acid does not behave in a consistent manner, e.g. α-eleostearic acid (9c, 11^t, 13^t) in tung oil, Momordica charantia and Centranthus ruber seed oils. Catalpic acid (9^t, 11^t, 13c) behaved consistently in the three oils we examined, the acid occurring mainly in the 1,3- positions but its analogue (8^t, 10^t, 12c) in Calendula officinalis concentrated in the 2- position. Punicic acid (9c, 11^t, 13c) also behaved differently to the (8c, 10^t, 12c) isomer. No common pattern emerges if we classify the acids according to the position of the double bonds. Although we have analysed insufficient oils to decide if the fatty acid distribution depends on the Family it is interesting to note that the four oils of the Bignoniaceae have similar distributions as do the two oils of the Cucurbitaceae (Table 4). The seed oils readily fall into two groups if they are classsified according to the proportion of conjugated triene they contain. The triene is only enriched at the 2- position of oils containing $>52\%$ of the acid. The significance of this result is not apparent however.

Oleic and linoleic oils do not show the consistent pattern of distribution (c.F. selectivity factors) observed in the linoleic and linolenic acid containing oils. In Calendula officinalis for example the selectivity factor of linoleic acid is 0.5 (normally 1.1). Because of this unusual result we isolated the acid by preparative T.L.C. on silica-silver nitrate and confirmed the position and configuration of the double bonds by oxidation and infra-red techniques respectively.

Two oils, Parinarium laurinum [216,218] and Impatiens glanduligera [219], containing conjugated dienes, trienes and tetraenes (9, 11, 13, 15 - octadecatetraenoic acid-parinaric) were analysed. The configuration of the double bonds in parinaric acid is unknown and the dienes and trienes have not been characterised.

The enrichment factors of the saturated acids were higher than normal (0.3 and 0.6 respectively) as were those for oleic and linoleic acid. The enrichment factors of the conjugated acids varied but were in the order diene > triene > tetraene. In Parinarium laurinum there were only minor amounts of non-conjugated and saturated acids and therefore diene and triene concentrated more in the 2- position than in Impatiens glanduligera. In both oils parinaric acid showed a slight preference for the 1, 3- positions.

PART III

CHARACTERISING OPTICALLY ACTIVE TRIGLYCERIDES

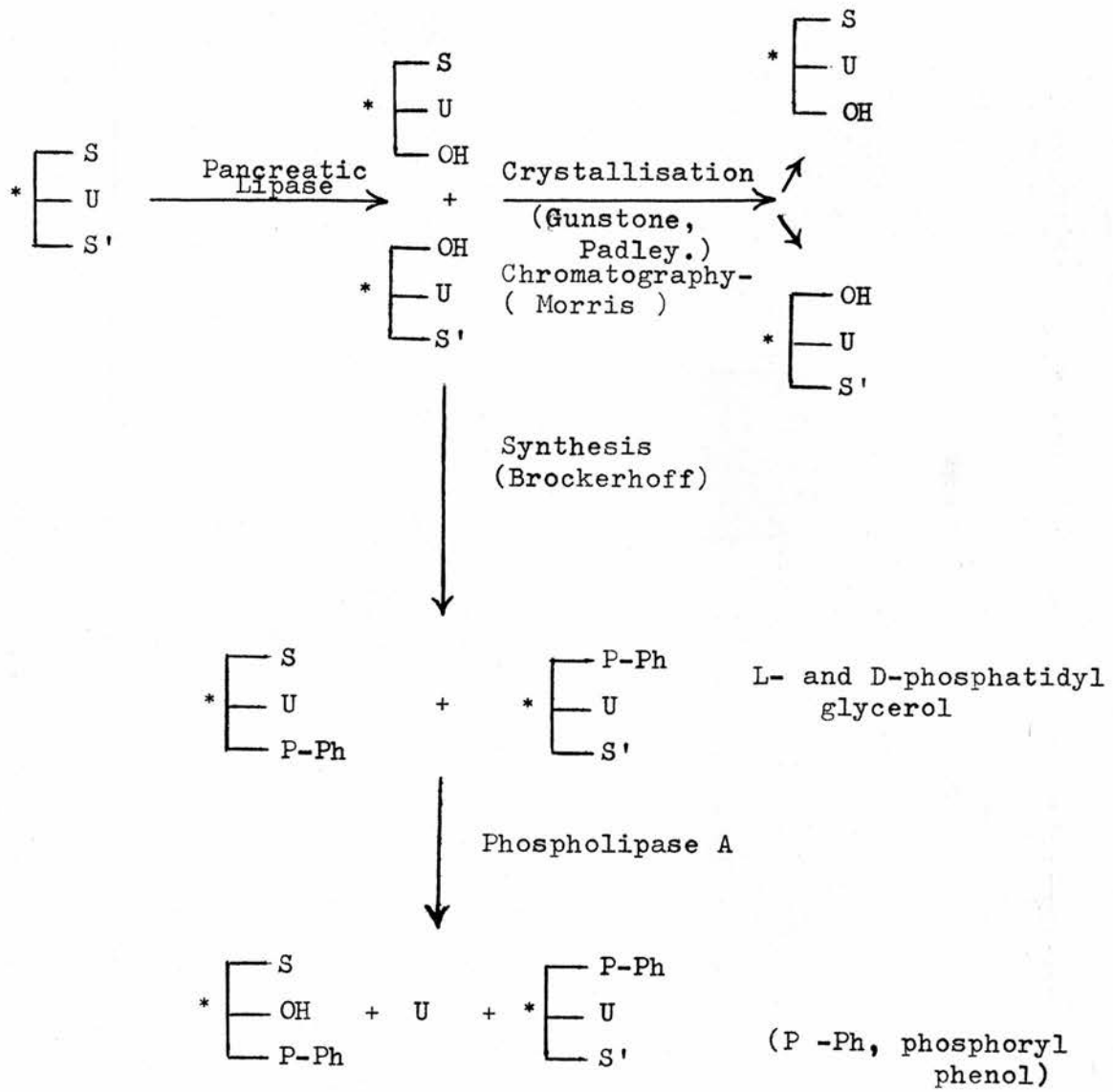
A. INTRODUCTION

The compositions of the acids at the 1 and 3- positions in a triglyceride have not been studied in any detail because until recently no simple techniques were available. The important feature about the glycerol molecule in this case is that the carbon atom at position 2 is asymmetric if the groups at the 1 and 3- positions are different. It was shown by Baer and Fischer [220,221] that the optical rotations of long chain triglyceride enantiomorphs are so low as to be undetectable. Following this observation they pointed out that although natural triglycerides do not exhibit rotatory power they are not necessarily racemates. The Fischer convention [222] of describing enantiomorphs will be used in this work.

Schlenk [223] described two physical methods which distinguish between separate triglyceride enantiomorphs and their racemic mixture. When mechanical pressure is applied to crystals without centres of symmetry, in the direction of structural polarity, they acquire an electric charge. This phenomenon is known as the piezoelectric effect [224] and is exhibited by all crystals of one enantiomorph. The effect is unlikely to occur in crystals of racemates.

The second method relies on X-ray crystallography. The X-ray diagrams of separate enantiomorphs are identical but entirely different from that of the racemic mixture.

More recently two methods relying on enzymic hydrolysis were described (Fig1). Brockerhoff [225] isolated the diglycerides produced when an optically active triglyceride was partially hydrolysed with



METHODS OF CHARACTERISING OPTICALLY ACTIVE TRIGLYCERIDES

Fig. 1.

pancreatic lipase. The diglyceride was mainly a mixture of the 1, 2- diacyl and 2, 3- diacyl isomers. These were converted to the L- and D- phosphatides and hydrolysed with phospholipase A from snake venom [226,227]. Only the acids at the 2- position of the L- phosphatide are attacked by this lipase and a lyso-phosphatide was isolated whose fatty acid composition was the same as the 1- position of the original triglyceride. The composition of the acids at the 2- position were determined by pancreatic lipolysis, and that of the 3- position by difference.

We have investigated two procedures which might detect optical activity in a triglyceride. The methods depend on our ability to modify the triglyceride to give compounds which have measurable rotations, (see **figure** 1).

If a triglyceride enantiomorph (e.g. L-α-palmitodiolein) is partially hydrolysed with pancreatic lipase, the diglyceride produced will be a mixture of L-α-palmitoyl-β-oleoylglycerol, $[\alpha]_D^{25^\circ}$ + 2.8° and D-$\alpha'\beta$-dioleoylglycerol $[\alpha]_D^{25^\circ}$ - 2.8°. These glycerides differ in their degree of unsaturation and may be separated either by crystallisation or chromatography on silica-silver nitrate (fig. 1). For the method to be successful the lipase must be non-stereospecific (228) i.e. must hydrolyse the 1 and 3 positions at equal rates, and racemisation must not occur during lipolysis. This procedure has also been suggested recently by Morris [229,230] but no published results are available.

Our second procedure relies on the specificity of a microbial lipase. Alford, Pierce and Suggs [231] recently examined the properties of some microbial lipases. It was found that Geotrichum candidum lipase preferentially hydrolysed unsaturated acids from mixed fatty acid triglycerides, e.g. palmitodiolein. We therefore considered that if this lipase partially hydrolysed L-α-palmitodiolein

we might obtain a monoglyceride consisting mainly of L-α-monopalmitin which has a measurable rotation $[\alpha]_D^{20}$ - 4.4°.

B. EXPERIMENTAL

1. L-α-Palmito-β,α'-diolein. The procedure described by Baer and Fischer (221,232-235) was used to convert D-mannitol to D-acetone glycerol (232) from which was prepared L-α-monopalmitin (233). This was acylated with oleoyl chloride to give L-α-Palmito-β,α'-diolein (235). The acids used in the synthesis were >99% pure[1] and were converted to acid chlorides by reaction with thionyl chloride (236).

D-acetone glycerol $[\alpha]_D^{20°}$ 13.6° lit. 13.6-13.9° (232)

L-α-monopalmitin m.pt. 70-71° lit. 71-72° (232)
$[\alpha]_D^{20°}$ - 4.4°, 10% solution in pyridine
lit. -4.37 (232)

L-α-palmito-β,α'-diolein Pure by T.L.C.[2]
Fatty acid composition 34% mol. 16:0, 66% mol. 18:1
Fatty acid composition of 2- position:- 96% mol. 18:1
Optical rotation zero (0.5 g in 2 ml $CHCl_3$, 1dM tube)

2. Monosaturated Triglycerides of Jatropha curcas seed oil

J.curcas seed oil (20 g) was fractionally crystallised from a mixture of acetone (400 ml) and methanol saturated with silver nitrate (1000 ml) (151). The mono-unsaturated (S_2U) glycerides were filtered from the solution at -10°. The monosaturated glycerides (SU_2) obtained at -60° (6.8 g) were recrystallised from the same solvent mixture (500 ml) to remove the triunsaturated (U_3) glyceride impurities. Yield 65% theory; Saturated acids 32% mol. Unsaturated acids 68%; Pure by T.L.C. on silica silver nitrate.

3. Lipolysis procedure. The triglycerides (3-5 g) were hydrolysed for 5-10 minutes at 40° with pancreatic lipase (0.5 g) in

1. 16:0 purchased from B.D.H. 18:1 obtained by fractional crystallisation of olive oil fatty acids from acetone.

2. Abbreviations, page 100.

NH_3/NH_4Cl buffer (100 ml 1.2M solution pH 8.0) containing calcium chloride (5 ml of 22% solution) and bile salts (0.1 ml of 25% solution sodium taurocholate). The mixture was stirred vigorously throughout the lipolysis and the pH maintained at 8.0 by adding 0.88 ammonia. The lipolysis products were poured directly into ice-cold N.HCl and extracted with ether (3x). The combined extracts were washed with water until neutral.

4. Isolation of diglycerides from lipolysis products

The ether solution of the lipolysis products was eluted rapidly down a column of I.R.A. 400 (OH) ion exchange resin (30 g, 1.9 cm. i.d.) to remove free fatty acid. The ether eluate was washed with water prior to isolating the mixture of partial glycerides. Traces of water were removed by evaporation with acetone.

The partial glycerides (1.5-3.0 g) were separated on a silica column (100 g dry silica + 5% water, 3.0 cm., i.d.). Triglycerides were eluted with 600 ml benzene and diglycerides with 600 ml benzene containing 10% ether. If required the monoglycerides were eluted with ether.

The mono- and diunsaturated diglyceride fraction ($\approx$ 0.5 g) was fractionally crystallised from acetone (1% solution). The monosaturated diglycerides (80-90% pure) were filtered at -60°C, the diunsaturated fraction of similar purity remained in the mother liquor.

The diglycerides were isolated and characterised by T.L.C. and G.L.C. Prior to polarimetry they were decolourised by eluting a 20% solution of the glyceride in chloroform through a short column of charcoal (2 g in filter tube, porosity 4, 1 cm. i.d.).

5. Optical rotation measurements. The rotations of chloroform solutions of the diglycerides were measured, unless stated otherwise, by the staff at Unilever Research Laboratory[1] using a Perkin-Elmer 141 polarimeter. An automatic instrument with an accuracy of $\pm$ .001°.

1. Unilever Research Laboratory, The Frythe, Welwyn, Herts.

In some earlier work rotations were measured on a Schmidt & Haensch polarimeter (1926) using a low capacity (2 ml) 1dM. centre filled polarimeter tube.

Hydrolysis using the Microbial lipase from Geotrichum candidum

The conditions for the lipolysis were similar to those described by Alford, Pierce and Suggs [231] but on a larger scale.

The triglyceride or fat (1 g) was placed in a jacketed vessel maintained at 35°C containing phosphate buffer solution (20 ml of 0.25 M solution, pH 6.0) and calcium chloride (1 ml, 22% solution). Geotrichum candidum lipase (0.1 g) was added and the mixture was stirred vigorously for 45-60 minutes. Throughout the lipolysis the pH was maintained at 6.0 by adding N. sodium hydroxide. The lipolysis products were isolated and analysed by the method described for pig pancreatic lipase.

RESULTS AND DISCUSSION

Analysis of Diglycerides obtained by Hydrolysis with Pancreatic Lipase

The optical rotations of the diglyceride fractions obtained by the lipolysis of L-α-palmito-β.α'-diolein (L-POO) and the monosaturated (SUU) fraction of J.curcas oil are given in Table 1. The observed rotations are compared with the expected rotations (in brackets) which were calculated assuming that no isomerisation or racemisation occurred and allowing for the presence of another diglyceride with the opposite rotation, e.g. 23, 5, and 20% * S, U, OH in * U, U, OH from the lipolysis of POO.

The rotations of the diglycerides from L-POO indicate that racemisation occurs to a large extent. In two cases the dioleoglycerides (U, U, OH) gave rotations of approximately half the expected values but in one case the rotation was practically zero.

Table 1

Optical Rotations of Diglycerides isolated from the Lipolysis products of Natural and Synthetic Triglycerides

Triglyceride	Diunsaturated diglyceride (* U,U,OH)			Monosaturated diglyceride (*S,U,OH)		
	Purity[1] %	g. in 2ml $CHCl_3$	Rotation[2,3]	Purity[1] %	g. in 2ml $CHCl_3$	Rotation[2,3]
L-POO[4]	77	0.31	-.12 (-.21)	89	0.2	0.00 (+.20)
L-POO	95	0.11	-.002(-.13)	91	0.11	+.003 (+.11)
L-POO	80	0.035	-.013(-.026)	-	-	-
J. curcas[5] (SUU)						
(i)	70	0.23	-.08 ($\pm$0.12)	94	0.17	0.00 ($\pm$.19)
(ii)	84	0.32	-.059($\pm$.273)	92	0.50	-.056 ($\pm$.53)

1. Diglyceride contains impurity of either S, U, OH or U, U, OH diglyceride respectively.
2. Rotations measured on Schmidt and Haensch polarimeter given to two decimal places. Those measured on the Perkin-Elmer 141 polarimeter are given to three decimal places.
3. The rotations of chloroform solutions (column 2) of the diglycerides in 1dM polarimeter tubes are given. These are compared with the rotations (in brackets) which were calculated assuming that the diglycerides were optically active ($[\alpha]_{20}^{D}o = \pm 2.5^{o}$) and allowing for the presence of diglycerides with an opposite rotation, e.g. *U, U, OH in *S, U, OH.
4. Zero rotation, Perkin-Elmer 141 (18% solution in $CHCl_3$, 1dM tube).
5. Rotation of +0.002, Perkin-Elmer 141 (36% solution in $CHCl_3$, 1dM tube).

The monosaturated diglyceride (S, U, OH) also had zero and an extremely low (+ 0.003) rotation in the two cases in which it was isolated in a fairly pure form (c.a. 90%). Racemisation could have occurred in two possible ways. Isomerisation of the 1, 2- diglyceride to the 1, 3- diglyceride, and thence to the 2, 3- diglyceride enantiomorph is one possibility. Thin-layer chromatography of the diglycerides on silica detected only small amounts of 1, 3- diglycerides (≤ 10%), but because diglycerides readily isomerise [237] some racemisation will occur by the above route. Coleman and Fulton [140] have in fact found evidence which suggests that a fraction of pork pancreas is capable of catalysing the isomerisation of diglycerides.

The second possibility is that resynthesis and exchange of acyl groups occurred during lipolysis. Borgström [135] found that labelled free palmitic acid was incorporated in the glycerides of corn oil during lipolysis in the absence of calcium ions. The presence of calcium ions reduced the amount of resynthesis but 5-10% of the activity was found in the glycerides. This interchange of fatty acids which occurs at the 1, 3- positions explains, in part, the observations we have made. It does not explain the difference between the rotations of the dioleo- and palmito-oleo-diglycerides, unless palmitic acid is re-esterified more rapidly than oleic acid; Borgström found no evidence for this however.

An additional problem arises with the results obtained for J.curcas oil because the rotations of the mono- and di-unsaturated glycerides are in the same direction. They should in fact be in opposite directions [238]. These anomalous results may have been caused by asymmetric resynthesis but this seems unlikely [228] following Tattries observation that the lipase hydrolyses glycerides non-stereospecifically. The concentration in the diglycerides of an optically active component present in the original oil is another possibility but the oil was optically inactive.

We must conclude from these results however that because racemisation occurs, this method of detecting optical activity in natural glycerides, in its present form, is unreliable.

The Hydrolysis of Triglycerides by Geotrichum candidum Microbial Lipase

Jatropha curcas seed oil and L-α-palmitodiolein were hydrolysed with Geotrichum candidum lipase and the fatty acid composition of the lipolysis products was determined (Table 2). As reported by Alford et al [231] we have found that the unsaturated acids are hydrolysed more rapidly than the saturated acids (compare F.F.A. with triglyceride composition). The hydrolysis was not as specific for the unsaturated acids as previously reported however. Saturated and unsaturated acids were hydrolysed at different rates with the result that the saturated acids were concentrated in the glyceride fraction. The monoglycerides isolated after the hydrolysis of J.curcas and L-POO triglycerides contained appreciable amounts of unsaturated acids and had zero optical rotation in both cases. This method is therefore unsuitable for detecting optical activity in triglycerides.

From our results it therefore appears unlikely that a reliable method for detecting optical activity in triglycerides will develop from examining the lipolysis products produced either by the action of pancreatic lipase or G.candidum lipase.

The physical methods described by Schlenk seem to be more promising, especially as we now have the chromatographic methods for separating and isolating individual glycerides. Even at this early stage it is interesting to note that both Schlenk [223] and Brocherhoff [225] found racemic triglycerides in the fats they examined - cocoa butter and corn oil respectively.

Table 2

The Lipolysis Products of Jatropha curcas seed oil and L-Palmitodiolein using Geotrichum candidum lipase

J. Curcas (50% hydrolysis)	Component Acids[1] (% mol.)				
	16:0	18:0	16:1	18:1	18:2
Triglyceride after lipolysis	18.9	9.6	2.9	33.7	34.9
Diglyceride	21.6	11.4	3.3	32.2	31.5
Monoglyceride	23.2	15.7	2.2	28.9	30.0
Free Fatty Acid	6.8	TR	3.8	44.1	45.3
Original oil	17.3	3.9	3.2	39.3	36.3

L-Palmitodiolein (40% hydrolysis)

	Component Acids (% mol.)	
	16:0	18:1
Triglyceride	34.0	66.0
Diglyceride	41.1	58.9
Monoglyceride	59.6	40.4
Free Fatty Acid	15.4	84.6

1. Abbreviations, page 100.

PART IV

THE TRIGLYCERIDES IN MATURING SUNFLOWER SEEDS

INTRODUCTION

Our present knowledge about triglyceride biosynthesis in plants is very limited. Some routes whereby fatty acids are synthesised have been discovered [239,240] and a pathway for triglyceride synthesis in animals described [241,242]. However the way in which plants synthesise triglycerides has not been determined.

Kartha [246] detected no acetyl value in the fat extracted from maturing coconuts. He suggested that the triglyceride precursor was adsorbed on the enzyme and was not released until the triglyceride synthesis was complete.

Numerous workers [243-250] have analysed maturing seeds in attempts to obtain some clue as to the method of triglyceride synthesis. At that time it was only possible to determine the total fatty acid composition of the triglycerides.

We have been particularly interested in the distribution of fatty acids in natural triglycerides. Our method of determining triglycerides which has been previously described enables us for the first time to determine the triglyceride composition of maturing seeds. There are a number of ways in which the triglycerides produced in maturing seeds could eventually give the product found in the mature seed:-

1. Individual glycerides are synthesised at different stages of maturation but their total composition at maturity has the typical pattern of fatty acid distribution found in mature seeds.

2. The fatty acids of the triglycerides are distributed differently in the immature and mature seeds. As the seed reaches maturity the fatty acids rearrange to produce the triglyceride mixture of the mature seed.

3. The fatty acids in the immature and mature seeds are distributed in the same way.

We therefore undertook the investigation to determine the fatty acid distribution in triglycerides of maturing sunflower seeds.

The sunflower was chosen for this experiment because it grows readily in the north-eastern climate and the seeds contain a reasonable proportion of triglycerides at maturity.

EXPERIMENTAL

Isolation and Extraction of the Seeds

Maturing seeds were removed from a single sunflower head over a period of 90 days after flowering commenced (August 25th, 1964). At each sampling the seeds (30-10) were removed from three parts of the flower head, equidistant apart and on an outer circumference four seeds in depth. We thus obtained a representative sample of seeds at similar stages of maturity. The fat was immediately extracted by grinding the seeds three times in a pestle and mortar with hexane at room temperature.

Analysis of the Seed oils

The fatty acid composition of each sample was determined by gas-liquid chromatography of the methyl esters. These were prepared from the triglycerides by transesterification with methanol and sodium methoxide [171]. The chromatographic conditions were as previously described in Part I.

The triglycerides were also examined qualitatively by thin-layer chromatography on silica gel G and on silica gel G impregnated

with silver nitrate (slurry of 30 g silica gel G with 65 ml water containing 5 g $AgNO_3$).

The chromatograms on silica gel G were eluted with a light petroleum-diethyl ether mixture (7:3) and the lipids were detected by charring at 250°C after spraying with 50% aqueous sulphuric acid.

The silver nitrate impregnated plates were eluted with a benzene-diethyl ether mixture (7:3) and the components were detected by charring at 250°C after spraying with 50% aqueous phosphoric acid.

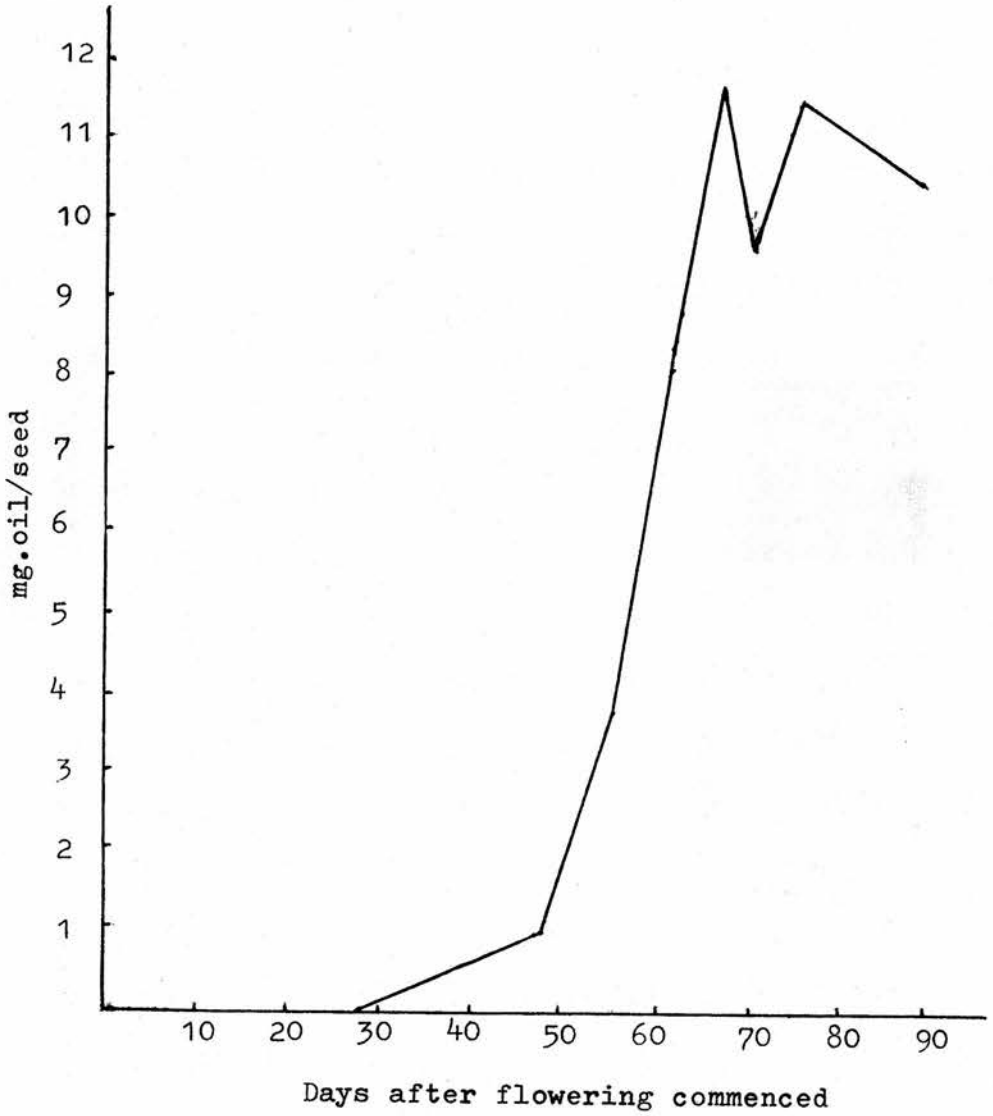
The triglycerides of three samples (38, 55 and 90 days after flowering) were also determined quantitatively by silica-silver nitrate chromatography using the procedure described in Part I. The composition of the acids at the 2- position were determined by enzyme hydrolysis.

RESULTS AND DISCUSSION

By taking seeds from a single sunflower head we had the greatest chance of obtaining seeds at similar stages of maturation [151]. The disadvantage was that only small amounts (19-100 mg) of fat were obtained for analysis. It is a reflection on the sensitivity of the analytical methods that the following comprehensive information was obtained.

The rate of biosynthesis of triglycerides in the maturing seeds is shown graphically in fig. 1 which was derived from the results in Table I.

The average weight of fat extracted from each seed indicates that the triglycerides were synthesised over a relatively short period (3 weeks). After triglyceride synthesis had stopped the seeds continued to lose weight, probably through dehydration. These findings are in agreement with the results of previous



PRODUCTION OF OIL IN THE MATURING SUNFLOWER SEED.

fig.1

Table I

Weight of oil extracted from Maturing Sunflower Seeds

Days after flowering commenced	No. of Seeds	mg oil/seed	% oil/seed
28	35	trace	trace
38	29	0.6	0.9
48	15	1.0	1.2
55	14	3.8	5.3
62	10	8.1	12.9
66	10	11.4	20.0
70	9	9.5	16.8
76	10	11.3	24.4
90	10	10.3	24.6

Table II

Fatty Acid composition of Sunflower Seed Oils during Maturation (wt %)

Days after flowering commenced	Component acids[1] (% wt)					
	16:0	18:0	18:1	18:2	a[2]	b[3]
28	17.0	8.5	18.7	10.1	26.0	19.7
38	7.5	6.8	26.8	58.9		
48	4.8	2.0	16.9	76.3		
55	5.2	1.9	16.1	76.8		
62	5.4	2.0	14.6	78.0		
66	5.4	2.1	14.0	78.6		
70	8.4	1.9	13.2	76.4		
76	6.4	1.9	13.9	77.8		
90	6.7	3.1	15.5	74.7		

1. Abbreviations, page 100.
2. Carbon Number 19.8 on Poly(ethyleneglycol)succinate.
3. Carbon Number 20.2 on " " "

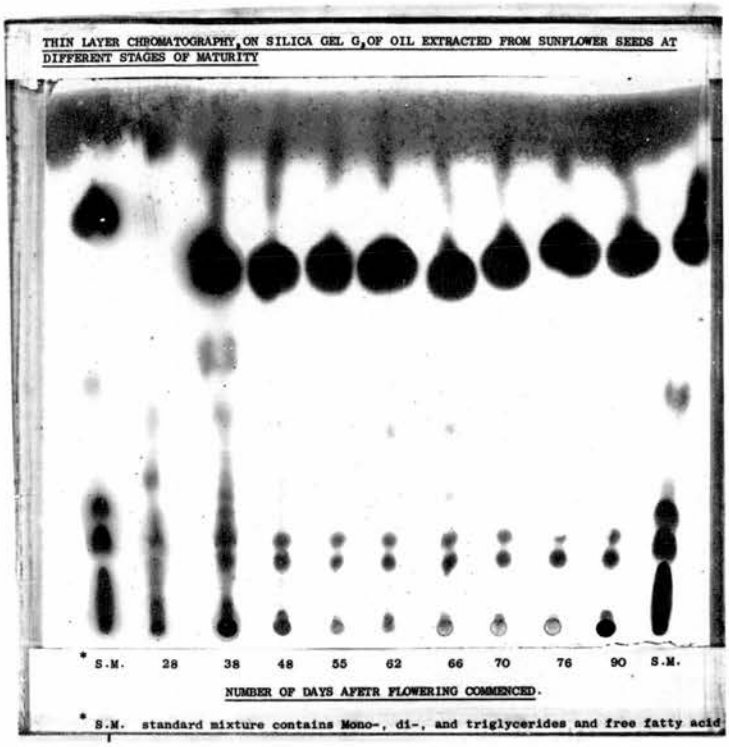
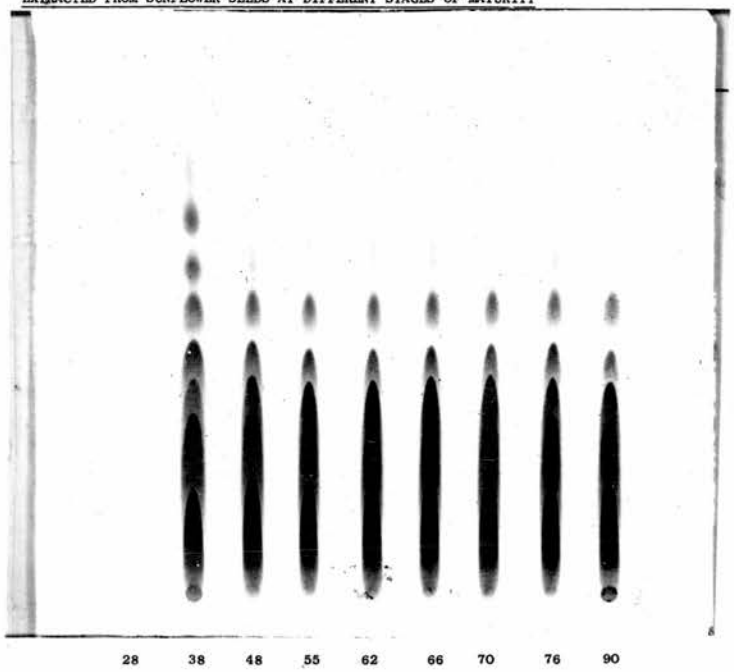

THIN LAYER CHROMATOGRAPHY ON SILICA GEL G, OF OIL EXTRACTED FROM SUNFLOWER SEEDS AT DIFFERENT STAGES OF MATURITY
* S.M.
28
38
48
55
62
66
70
76
90
S.M.
NUMBER OF DAYS AFETR FLOWERING COMMENCED.
* S.M. standard mixture contains Mono-, di-, and triglycerides and free fatty acid

Fig.2

THIN LAYER CHROMATOGRAPHY, ON SILICA GEL G IMPREGNATED WITH SILVER NITRATE, OF OIL EXTRACTED FROM SUNFLOWER SEEDS AT DIFFERENT STAGES OF MATURITY



NUMBER OF DAYS AFTER FLOWERING COMMENCED.

Fig.3

workers (250,252,253).

The qualitative composition of the lipids in the samples was obtained by thin-layer chromatography on silica gel G (fig. 2). Sample 28, isolated during the initial stages of triglyceride biosynthesis, was mainly partial glycerides with only a trace of triglyceride. The later samples were predominantly triglycerides with traces of partial glycerides and other lipids. No free fatty acid was detected.

The fatty acid compositions of the samples are given in Table II.

Samples isolated during the 55-90 day period of maturation had very similar fatty acid compositions, characterised by a high linoleic acid content ($\lesssim$ 75%) and a low proportion of saturated acids ($\lesssim$ 8%).

Sample 38 contained less linoleic acid and more oleic and saturated acids relative to the fats of more mature seeds.

The total fatty acid composition of Sample 28 showed a high saturated acid and very low linoleic acid content, relative to later samples. Two unidentified peaks (Carbon No. 19·8 and 20·2 on polyester) were also detected in the methyl esters from the sample. We were unable to characterise them because insufficient material was available.

During the period therefore when practically all the triglyceride in the sunflower seed was synthesised, the fatty acid composition was constant.

The component glycerides of all the samples were compared qualitatively (fig. 3) and those of 38, 55 and 90 day samples determined quantitatively, by silica-silver nitrate chromatography. The triglycerides of these latter samples were also determined by lipolysis and calculated according to Gunstone's theory. The results are compared in Table III.

Table III

Triglycerides of 38, 55 and 90 day Samples determined by T.L.C.[1], Lipolysis[2] and Theory[3]

Tri-glyceride	38 DAY			55 DAY			90 DAY		
	T.L.C.	Lipolysis	Theory	T.L.C.	Lipolysis	Theory	T.L.C.	Lipolysis	Theory
222[4]	21	20	20	41	44	44	37	41	41
221	24	27	27	27	28	28	26	26	26
220	20	16	16	19	14	14	21	18	18
211	5	12	12	6	6	6	6	5	5
210	11	15	15	4	6	6	8	7	7
200	2	3	3	2	1	1	1	2	2
111	3	2	2	-	-	-	-	-	-
110	9	3	3	1	1	1	1	1	1
100	5	2	2	-	-	-	-	-	-
000	-	-	-						

1. For experimental results see Appendix, p
2. According to Coleman (140).
3. According to Gunstone (155).
4. Abbreviations, page 100.

The similarity of the component triglycerides in the 55-90 day samples, shown qualitatively by thin-layer chromatography (fig. 3), was confirmed by their quantitative analysis. The quantitative results compared favourably with those obtained by lipolysis and predicted by theory (Table III).

The quantitative results of the 38 day sample however differed from those derived by lipolysis or by theory. A possible explanation for this would be that the sample is a mixture of fats of widely different composition.

We observed a wide change in the fatty acid composition of the sample early in maturation (samples 28, 38, 48) and this would be reflected in a rapid change in triglyceride composition. The triglycerides of sample 38 may therefore be obtained from:-

a) Seeds (30) of very different fatty acid composition. The triglycerides from each individual seed may conform to the normal distribution pattern but when combined would produce a fat of unusual composition.

b) Different segments of the same seed. Kartha [176] recently showed that the oil content and iodine value varied from one site to another in a single seed. These differences are probably extremely large in the early stages of maturation and triglycerides from different parts of the same seed would give a fat of unusual composition. The amount of fat in an immature sunflower seed is inadequate for analytical purposes however. It would be of interest to examine some larger individual seeds, e.g. almond, in their early stages of maturation.

c) Seeds at different stages of maturity having widely different fatty acid and triglyceride compositions.

Litchfield [178] has recently discussed the effect of combining natural fats from different parts of the same seed. Provided

Table IV

Lipolysis results for 38, 55 and 90 day Samples

Fatty Acid (mole %)	38 DAY		55 DAY		90 DAY	
	Triglyceride F.A.	Monoglyceride F.A.	Tri. F.A.	Mono. F.A.	Tri. F.A.	Mono. F.A.
16:0	8.1	1.9	5.6	-	7.3	.4
18:0	6.7	1.9	1.9	-	3.0	.7
18:1	26.5	32.0	15.9	13.0	15.3	14.9
18:2	58.7	64.2	76.6	87.0	74.4	84.0

that the relative amounts, but not the fatty acid types, vary with location then the glyceride composition of the total fat can be calculated from the average fatty acid composition.

The unusual triglyceride mixture of Fraction 38 could therefore be obtained only if different parts of the seeds, or individiual seeds, contained different acids. This is most easily explained by assuming the absence of linoleic acid in certain locations as suggested by the relatively low linoleic acid content in Fraction 28.

The composition of the acids esterified at the 2- position of the triglycerides was determined by lipolysis . The results for 38, 55 and 90 day seeds (Table IV) show that the saturated acids are excluded from the 2- position whereas linoleic acid is enriched. Oleic acid is randomly distributed in fractions 55 and 90 whereas in sample 38 it is enriched in the 2- position. Sample 38 therefore has an unusual fatty acid composition at the 2- position as well as an unusual triglyceride composition.

We therefore conclude that the fatty acid distribution in triglycerides during maturation, follows the same pattern that is found in the triglycerides of mature seeds. Only in the very early stages of maturation was any unusual distribution noticed and possible reasons for this are discussed.

When this work was completed two publications by Hirayama and co-workers (254,255) described the development of triglycerides in maturing soya beans and the seed of Sapium sebiferum. The triglycerides were estimated by photodensitometry of the reverse-phase chromatograms and the composition of the acids at the 2- position was determined by pancreatic lipolysis. From their results they also concluded that the specific distribution of the fatty acids occurs during the early stages of seed maturation.

ABBREVIATIONS

G.L.C. - Gas-Liquid Chromatography.

T.L.C. - Thin-Layer Chromatography.

N.M.R. - Nuclear Magnetic Resonance Spectroscopy.

Abbreviations for Acids

S - Saturated.

U - Unsaturated.

16:0, 16:1, 18:0, 18:1, etc. - First number gives the chain length, the second number the number of double bonds, e.g. 18:0 - stearic acid, 16:1 - palmitoleic acid.

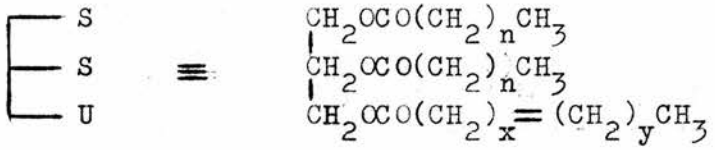
Abbreviations for Triglycerides

S_2U, SU_2, etc. - S and U represent saturated and unsaturated acyl groups respectively. S_2U is a triglyceride containing two saturated and one unsaturated acids. The position of the groups and the degree of unsaturation are not specified.

SUS - A mono-unsaturated triglyceride with the unsaturated acid esterified at the 2- position.

SUU - A di-unsaturated triglyceride with the saturated acid at the 1 or 3- positions.

310, etc. - Each number represents the number of double bonds in one of the acyl groups of the triglyceride, e.g. 310 - Linoleno-oleo-saturated triglyceride. The positions of the acids in in the triglyceride are not specified. (0 includes all saturated acids, 1, all the acids with one double bond).



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APPENDIX 1

Glyceride Analysis of Jatropha Curcas Seed Oil[1]

Fraction	Component Acids[2] (% Mol.) 16:0	18:0	16:1	18:1	18:2	Amount % Mol.	Component Glycerides (% Mol.) 222	221	220	211	210	111	200	110	100	000
1	2.7	0.5	1.1	3.1	92.6	6.6	5.2	.8	.6							
2	0.9	-	1.5	31.5	66.1	12.6		12.1	.3	.2						
3	23.0	6.7	1.0	4.2	65.1	11.6		0.7	10.3	.6						
4	1.2	0.6	2.3	60.5	35.4	13.9			.6	13.1	.2					
5	23.6	7.6	2.6	32.1	34.1	18.8				1.2	17.6					
6	3.0	0.2	3.2	91.4	2.2	8.2					.5	7.6	.1			
7	37.1	10.3	2.0	24.8	25.9	9.6					2.0	1.4	5.5	.7		
8	22.3	9.8	2.9	65.0	-	12.8								12.8		
9	44.8	19.8	2.5	32.9	-	5.6								.4	5.2	
10	37.8	12.3	14.8	35.1	-	0.3								.15	.15	-
Total							5.2	13.6	11.8	15.1	20.3	9.0	5.6	14.0	5.4	-
Original Oil						Whole Nos.	5	14	12	15	20	9	6	14	5	-
						Theory	5	15	10	17	22	6	6	13	6	-

1. Triglycerides separated by "Standard Method". Chromatogram developed with Benzene-Ether (10%).
2. Abbreviations, page

APPENDIX 2a

Glyceride Fractions of Linseed Oil (Plate A[a])

Fraction	Fatty Acid Composition (% Mol.) 16:0	18:0	16:1	18:1	18:2	18:3	Amount % Mol.	333	332	331	330
1	4.0	2.3	0.1	8.0	8.6	77.0	63.3	←	63.3		→
2	2.9	1.6	0.4	5.6	55.8	33.6	3.2	-	-	-	-
3	3.3	0.5	0.7	31.0	32.2	32.3	6.5	-	-	-	-
4	20.3	9.9	1.0	4.9	32.7	31.2	4.6	-	-	-	-
5	3.5	1.1	0.9	54.3	11.8	28.4	6.3	-	-	-	-
6	16.2	10.3	0.8	34.2	13.1	25.4	6.6	-	-	-	-
7	18.2	11.1	1.7	32.3	25.6	11.1	3.7	-	-	-	-
8	17.7	11.1	2.3	54.3	11.4	3.2	5.2	-	-	-	-
9	33.9	11.3	27.0	23.8	4.0	-	0.4	-	-	-	-
10	29.7	11.8	16.5	33.0	9.0	-	0.2	-	-	-	-
Total	6.9	3.8	0.6	17.3	14.0	57.4		←	63.3		→
Original oil	6.1	3.2	0.1	16.6	14.2	59.8					

a. Plate developed with Ether-light petrol (1:1).

Component Glycerides (% Mol. of Total Oil)														
322	321	320	311	222	310	221	300	220	211	210	111	200	110	100
2.2	0.6	0.4												
-	5.8	0.7												
-	-	4.2	0.4											
-	-	-	4.5	0.2	1.0	0.6								
-	-	-	0.2	-	5.0	1.4								
-	-	-	-	-	0.9	-	0.3	0.4	0.7	1.4				
-	-	-	-	-	-	-	0.5	-	-	1.8	1.2		1.7	
-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.2	0.1
-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.2	
2.2	6.4	5.3	5.1	0.2	6.9	2.0	0.8	0.4	0.7	3.2	1.2	0.2	2.1	0.1

APPENDIX 2b

Glyceride Fractions of Linseed Oil

Fraction	Fatty Acid Composition (% Mol.) 16:0	18:0	16:1	18:1	18:2	18:3	Amount % Mol.	333	332	331	330
1	0.5	0.2	0.2	0.6	0.7	97.8	24.3	22.7	0.5	0.6	0.5
2	0.8	0.3	0.3	2.2	30.0	66.4	14.2	-	12.7	1.0	0.5
3	9.1	5.3	0.3	17.1	2.4	65.8	24.9	-	0.4	13.0	10.8
4	4.0	2.8	0.4	6.0	47.7	39.1	5.2	-	-	0.4	0.5
5	10.3	3.7	1.1	20.6	32.8	31.5	10.4	-	-	-	-
6	12.9	6.5	1.0	43.6	16.6	19.4	21.0	-			
Total	6.5	3.3	0.5	16.4	14.4	58.9	Total	22.7	13.6	15.0	12.3
Original oil	6.1	3.2	0.1	16.6	14.2	59.8					
Plate A Analysis								← 71.9 →			
Combined Analysis of Plates A + B								22.5	13.5	15.0	12.0

a. Plate developed with Ether.

(Plate B[a])

Component Glycerides (% Mol. of Total Oil)														
322	321	320	311	222	310	221	300	220	211	210	111	200	110	100
-														
-														
0.7														
3.1	0.6	0.6												
-	4.8	3.7	0.7	0.5	0.7									
						←	21.0						→	
3.8	5.4	4.3	0.7	0.5	0.7	←	21.0					→		
→		5.3	5.1	0.2	6.9	2.0	0.8	0.4	0.7	3.2	1.2	0.2	2.1	0.1
3.5	5.5	5.0	5.0	0.5	7.0	2.0	1.0	0.5	0.5	3.0	1.0	0.5	2.0	TR.

APPENDIX 3a

Glyceride Fractions of Wild dog rose

Fraction	Fatty Acid Composition (% Mol.)[2] 16:0	18:0	16:1	18:1	18:2	18:3
1	1.2	0.5	0.1	3.7	25.5	69.0
2	2.8	0.7	0.3	8.9	52.4	34.9
3	3.1	1.5	0.2	6.0	79.6	9.6
4	3.1	0.7	0.9	31.7	59.3	4.3
5	21.7	7.8	-	1.8	68.7	
6	2.7	-	1.6	59.4	36.3	
7	21.8	7.4	3.1	35.0	32.2	
8	28.6	10.2	4.2	31.9	28.1	
9	9.5	4.3	7.5	70.7	8.0	
10	41.8	17.9	13.3	27.0		
Total	4.0	1.4	0.5	10.8	47.7	35.6
Original oil	3.7	0.9	0.3	10.3	49.1	35.7

Amount % Mol.	333	332	331	330
34.0	←	34.0		→
28.9	-	-	-	1.4
17.5	-	-	-	-
8.7	-	-	-	-
4.6	-	-	-	-
2.3	-	-	-	-
1.8	-	-	-	-
0.9	-	-	-	-
0.9	-	-	-	-
0.4	-	-	-	-
Total	←	34.0	→	1.4

1. Plate developed with Benzene-Ether(9:1).
2. Abbreviations page 100.

Seed Oil (Plate A)[1]

Component Glycerides (as % Mol. of Total Oil)														
322	321	320	311	222	310	221	300	220	211	210	111	200	110	100
17.8	8.0	1.7												
-	1.9	2.4	0.7	12.5										
-	-	-	0.1	0.3	1.0	7.3								
-	-	-	-	-	-	0.3	-	4.3						
-	-	-	-	-	-	-	-	0.2	2.1					
-	-	-	-	-	-	-	-	-	0.2	1.6				
-	-	-	-	-	-	-	-	-	-	0.3	0.2	0.4		
-	-	-	-	-	-	-	-	-	-	-	0.7	0.2		
-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.3
17.8	9.9	4.1	0.8	12.8	1.0	7.6	-	4.5	2.3	1.9	0.9	0.6	0.1	0.3

APPENDIX 3b

Glyceride Fractions of Wild

Fraction	Fatty Acid Composition (% Mol.)[2] 16:0	18:0	16:1	18:1	18:2	18:3	% Mol. Fraction	333	332	331	330	322
1	1.9	-	-	1.5	2.9	93.7	6.8	5.5	0.6	0.3	0.4	
2	0.4	-	-	1.9	31.1	66.6	17.5		16.3	1.0	0.2	
3	6.7	3.7	-	18.2	7.9	63.5	6.6		0.3	3.6	2.1	0.6
4	0.9	0.2	0.4	1.6	60.8	36.1	21.1			1.3	0.6	19.2
5	7.1	2.4	0.4	19.1	36.8	34.1	15.0				0.4	1.9
6	6.4	2.2	0.8	17.6	67.9	5.1	33.0					
Total Fatty Acids	4.0	1.4	0.4	10.7	46.8	36.7	Total	5.5	17.2	6.2	3.7	21.7
Original oil	3.7	0.9	0.3	10.3	49.1	35.7						
Plate A Analysis							← 53.2					
Combined Analysis of Plates A + B								5.5	17.0	6.1	3.7	21.5

1. Plate developed with Ether
2. Abbreviations page 100

Rose Seed Oil:- (Plate B)[1]

Glyceride Composition of Fractions (% Mol. of Total Oil)

321	320	311	222	310	221	300	220	211	210	111	200	110	100
8.8	3.9 ←			33.0 →									
8.8	3.9 ←			33.0 →									
9.9	4.1	0.8	12.8	1.0	7.6	-	4.5	2.3	1.9	0.9	0.6	0.1	0.3
9.8	4.0	0.8	12.6	1.0	7.5	-	4.4	2.3	1.9	0.9	0.6	0.1	0.3

Oil (Plate A[a])

Component Glycerides (as % Mole. Total Oil)												
320	311	222	310	221	300	220	211	210	111	200	110	100
2.0												
6.5	0.3	1.8										
	4.1	4.3	0.6									
			3.6	1.0								
				7.5	0.1	0.5						
						4.9	0.2					
							5.0					
							0.3	4.6				
								0.2	1.3	0.1		
								0.3		0.6		
											1.6	
												0.7
												0.3
8.5	4.4	6.1	4.2	8.5	0.1	5.4	5.5	5.1	1.3	0.7	1.6	1.0

APPENDIX 4a

Glyceride Fractions of Candlenut

Fraction	Fatty Acid Composition (% Mol.) 16:0	18:0	18:1	18:2	18:3	Amount % Mol.	333	332	331	330	322	321
1	1.7	0.9	7.0	35.7	54.7	35.3	←		35.3		→	
2	3.9	0.7	29.0	34.6	31.8	14.3						12.3
3	14.7	9.6	43.0	45.7	25.7	8.6						
4	2.1	-	32.4	48.2	17.3	9.0						
5	17.2	8.8	34.6	14.0	25.4	4.6						
6	3.1	1.1	31.0	63.8	1.0	8.1						
7	21.9	10.9	2.4	64.8	-	5.1						
8	1.1	-	63.4	35.5	-	5.0						
9	20.9	10.3	35.6	33.2	-	4.9						
10	5.0	2.3	87.2	5.5	-	1.6						
11	37.4	17.5	12.8	32.4	-	0.9						
12	24.5	9.1	64.0	2.4	-	1.6						
13	37.8	22.4	32.6	7.2	-	0.7						
14	33.0	20.9	37.5	8.6	-	0.3						
Total	7.1	3.4	21.9	38.8	28.8	Total	←		35.3		→	12.3
Original oil	6.5	3.2	22.0	37.6	30.7							

a. Plate developed with Benzene-Ether (9:1).

APPENDIX 4b

Glyceride Fractions of Candlenut

Fraction	Fatty Acid Composition (% Mol.) 16:0	18:0	18:1	18:2	18:3	Amount % Mol.	333	332	331	330	322	321
1	3.0	-	3.4	9.6	90.0	2.8	1.6	0.8	0.2	0.2		
2	0.6	-	2.6	30.5	66.3	10.2		9.2	0.8	0.2		
3	7.9	4.2	21.2	2.4	64.3	8.2		0.3	5.0	2.9		
4	0.8	-	2.1	64.2	32.9	14.4					13.1	0.9
5	8.6	4.0	21.2	32.8	33.5	20.7						13.0
6	3.7	-	57.8	8.3	30.2	5.6						
7	9.3	5.1	15.1	57.9	12.6	9.9						
8	11.9	5.1	37.3	45.7	-	28.2						
Total	7.2	3.1	22.0	38.7	29.0	Total	1.6	10.3	6.0	3.3	13.1	13.9
Original oil	6.5	3.2	22.0	37.6	30.7							
						Plate A Analysis ←					60.5	→
						Combined Analysis of Plates A + B	1.6	10.2	6.0	3.3	13.0	13.8

a. Plate developed with Ether.

Oil (Plate B[a])

Component Glycerides (% Mol. of Total Oil)												
320	311	222	310	221	300	220	211	210	111	200	110	100
0.4												
7.7												
0.1	4.6	0.4	0.5									
		5.7	4.2									
			←				28.2		→			
8.2	4.6	6.1	4.7	←			28.2		→			
→		6.1	4.2	8.5	0.1	5.4	5.5	5.1	1.3	0.7	1.6	1.0
8.2	4.6	6.0	4.2	8.4	0.1	5.4	5.5	5.1	1.3	0.7	1.6	1.0

APPENDIX 4c

The Triglycerides of Candlenut Oil, separated by

	Fatty Acid Composition[2] (% Mol.)						Amount[3]					
Fraction	16:0	18:0	16:1	18:1	18:2	18:3	% Mol.	333	332	331	330	322
1	2.2	0.4	0.3	3.4	8.9	84.8	5.1	2.8	1.4	0.6	0.3	
2	1.3	0.5	0.3	6.4	27.5	64.0	14.3		9.4	2.9	0.8	1.2
3	6.5	3.9	0.4	19.3	10.8	59.1	9.5			4.4	3.0	0.9
4	1.0	0.9	1.0	7.5	58.2	31.4	12.9					9.0
5	3.9	1.0	0.4	29.1	34.9	30.7	12.7					
6	8.3	4.4	0.3	20.0	47.4	19.6	15.4					
7	7.9	3.3	0.3	32.5	46.7	9.2	11.0					
8	11.8	4.9	0.4	33.6	49.3	-	8.9					
9	21.3	9.0	0.7	47.0	22.0	-	10.2					
Total	6.9	3.1	0.5	22.0	36.4	31.1	Total	2.8	10.8	7.9	4.1	11.1
Original oil	6.3	2.6	0.2	20.3	39.3	31.3						

1. Plate developed with Ether.
2. Abbreviations, page 100.
3. Determined by direct weighing.

T.L.C.[1] and Weighed prior to G.L.C.

Component Glycerides[2] (% Mol. of Total Oil)													
321	320	311	222	310	221	300	220	211	210	111	200	110	100
1.2													
3.2	0.7												
8.4	1.9	1.4	1.0										
	5.6	3.7	4.1		2.0								
				3.0	6.8		0.7	0.5					
						TR.	4.5	4.2		0.2			
										←		10.2	→
12.8	8.2	5.1	5.1	3.0	8.8	TR.	5.2	4.7	←		10.4		→

APPENDIX 5a

Glyceride Fractions of Rubber Seed

Fraction	Fatty Acid Composition (% Mol.)[2] 16:0	18:0	18:1	18:2	18:3	Amount % Mol.	333	332	331	330	322	321
1	3.0	2.7	8.7	33.4	52.6	16.8	← 16.8				→	
2	3.3	1.8	28.2	36.1	30.7	12.5						8.6
3	8.5	7.5	12.4	52.1	19.5	12.9						
4	6.8	5.7	33.7	38.1	15.7	18.0						
5	19.6	23.1	-	49.6	7.7	8.0						
6	4.9	9.2	50.3	29.8	5.8	2.9						
7	1.6	-	64.3	34.1	-	4.1						
8	29.9	4.0	35.6	34.4	-	0.8						
9	24.0	8.6	34.6	32.8	-	4.1						
10	12.1	19.7	35.9	32.3	-	6.4						
11	11.1	4.1	76.7	8.1	-	3.3						
12	30.4	36.6	-	33.0	-	3.5						
13	18.5	16.6	65.4	-	-	4.3						
14	32.2	29.6	38.2	-	-	2.4						
Total	9.9	10.5	26.6	34.5	18.5	Total	← 16.8				→	8.6
Original oil	10.7	10.6	25.0	32.7	21.0							

1. Plate developed with Benzene-Ether(9:1)
2. Abbreviations page 100

Oil (Plate A)[1]

Component Glycerides (% Mol. of Total Oil)												
320	311	222	310	221	300	220	211	210	111	200	110	100
1.9	1.0	1.0										
6.0	2.3	4.6										
	1.7	1.5	6.8	8.0								
					2.0	6.0						
					0.5	0.2	2.2					
						0.1	3.9	0.1				
								0.8				
								4.0	0.1			
								6.1	0.3			
								0.1	2.5	0.7		
										3.5		
											4.1	0.2
											0.3	2.1
7.9	5.0	7.1	6.8	8.0	2.5	6.3	6.1	11.1	2.6	4.2	4.4	2.3

APPENDIX 5b

Glyceride Fractions of Rubber Seed Oil

Fraction	Fatty Acid Composition (% Mol.)[2] 16:0	18:0	18:1	18:2	18:3	Amount % Mol.	333	332	331	330	322	321
1	4.1	-	2.0	3.0	90.9	1.1	1.0	0.1	0.1	0.1		
2	2.3	-	3.4	28.8	65.5	4.0		3.3	0.4	0.3		
3	7.9	6.2	19.5	4.0	62.4	4.8			2.6	1.9	0.3	
4	1.5	-	2.7	61.2	34.6	6.3				0.3	5.5	0.5
5	7.6	5.5	18.8	31.0	34.1	17.0						6.7
6	8.9	7.6	27.1	37.2	19.2	18.3						
7	12.4	11.3	35.4	39.6	1.3	48.3						
Total	9.5	8.1	26.6	36.9	18.9	Total	1.0	3.4	3.1	2.6	5.8	7.2
Original oil	10.7	10.6	25.0	32.7	21.0							
						Plate A Analysis	←			16.8	→	8.6
						Combined Analysis of Plates A + B	1.0	3.5	3.0	2.7	5.8	8.7

1. Plate developed with Ether
2. Abbreviations page 100

(Plate B)[1]

Component Glycerides (% Mol. of Total Oil)

320	311	222	310	221	300	220	211	210	111	200	110	100
10.3												
	⟵ 18.3 ⟶											
	⟵ 48.3 ⟶											
10.3	⟵ 66.6 ⟶											
7.9	5.0	7.1	6.8	8.0	2.5	6.3	6.1	11.1	2.9	4.2	4.4	2.3
8.0	5.0	7.2	6.9	8.1	2.5	6.4	6.2	11.2	2.9	4.2	4.4	2.3

APPENDIX 6a

Glyceride Fractions of Soya Bean

Fraction	Fatty Acid Composition (% Mol.) 16:0	18:0	16:1	18:1	18:2	18:3	Amount % Mol.	333	332	331	330	322
1	4.1	1.1	0.2	8.8	64.7	21.1	37.7	←				37.7
2	4.8	0.9	1.0	31.7	58.8	2.8	16.8					
3	25.7	7.8	-	1.8	63.0	1.7	14.2					
4	2.1	0.5	0.9	64.8	31.7	-	8.2					
5	23.3	8.0	1.0	34.0	33.5	0.2	11.0					
6	28.4	9.4	1.5	38.5	21.7	0.5	6.5					
7	30.1	10.2	4.0	51.0	4.7	-	5.6					
Total	12.4	0.8	3.8	23.3	51.1	8.6	Total	←				37.7
Original oil	12.0	0.6	3.6	23.7	51.4	8.7						

1. Plate developed with Benzene-Ether(9:1)

Oil (Plate A)[1]

Component Glycerides (% Mol. of Total Oil)											
321	320	311	222	310	221	300	220	211	210	111	200	110	100
→													
				1.4	14.9	0.5							
					0.8	0.7	12.7						
								8.2					
									10.3				
									1.2	2.2	3.1		
											0.8	4.4	0.4
→				1.4	15.7	1.2	12.7	8.2	11.5	2.2	3.9	4.4	0.4

APPENDIX 6b

Glyceride Fractions of Soya Bean Oil

Fraction	Fatty Acid Composition (% Mol.)[2] 16:0	18:0	16:1	18:1	18:2	18:3	Amount % Mol.	333	332	331	330	322
1	16.6	3.8	-	11.7	27.8	40.1	0.3	-	0.1	0.1	0.1	
2	2.8	3.1	0.9	3.7	31.0	58.5	1.6	-	1.2	0.2	0.2	
3	2.0	1.3	0.3	3.3	57.1	36.0	8.5	-	-	-	0.7	6.7
4	11.1	3.4	0.6	17.3	36.4	31.2	9.7	-	-	-	-	-
5	4.0	1.0	0.2	9.8	77.8	7.2	18.4	-	-	-	-	-
6	13.8	3.4	0.4	26.4	55.5	0.5	40.6	-	-	-	-	-
7	21.2	6.8	0.6	46.2	24.6	0.6	20.6					
8	29.7	13.0	5.0	33.3	13.3	5.7	0.3	-	-	-	-	-
Total	12.2	3.5	0.5	24.2	50.9	8.7	Total	-	1.3	0.3	1.0	6.7
Original oil	12.0	3.6	0.6	23.7	51.4	8.7						
							Plate A Analysis	<----------				39.1
							Combined Analysis of Plates A + B	-	1.3	0.3	1.0	6.7

1. Plate developed with Ether.

2. Abbreviations page 100.

(Plate B)[1]

Component Glycerides (% Mol. of Total Oil)													
321	320	311	222	310	221	300	220	211	210	111	200	110	100
0.9	0.2												
4.5	4.2	0.4	0.6										
-	-	1.2	14.1	2.8	0.3								
-	-	-	-	0.5	16.7	0.5	12.7	8.9	11.5	2.2	3.8	4.4	
-	-	-	-	-	-	-	-	-	-	-	0.1	-	0.2
5.4	4.4	1.6	14.7	3.3	17.0	0.5	12.7	8.9	11.5	2.2	3.9	4.4	0.2
→					15.7	1.2	12.7	8.2	11.5	2.2	3.9	4.4	0.4
5.4	4.4	1.6	14.8	3.3	15.8	1.2	12.8	8.2	11.6	2.2	3.9	4.4	0.4

APPENDIX 7a

Glyceride Fractions of Sabium sebiferum

I. Glycerides which do not contain

Fraction	Fatty Acid Composition (% Mol.) 16:0	18:0	16:1	18:1	18:2	18:3	Amount % Mol.	333	332	331	330	322
1	1.1	0.2	0.1	5.4	15.9	77.3	45.7		45.7			
2	7.0	2.5	0.2	7.9	33.3	49.1	23.3			4.4	6.6	11.1
3	11.1	3.8	0.4	17.9	38.0	28.8	18.8	-	-	-	-	-
4	13.8	4.7	0.6	31.4	31.3	18.2	5.2	-	-	-	-	-
5	24.9	9.7	0.9	2.1	56.5	5.9	2.6	-	-	-	-	-
6	3.7	1.0	1.5	56.1	33.2	4.5	1.2	-	-	-	-	-
7	21.8	8.0	4.1	31.5	31.7	2.9	1.5					
8	25.2	12.4	9.8	28.4	24.2	-	0.7					
9	27.2	9.5	7.8	55.5	-	-	0.3					
10	55.1	8.5	6.0	30.4	-	-	0.7					
Total	6.6	0.4	2.4	11.2	26.4	53.0	Total ←		67.8			→
Kernel oil	6.2	0.3	1.8	11.1	26.3	54.3						

a. Plate developed with Benzene-Ether (8:2).

b. Fraction represents 70 of Stillingia Oil.

Kernel Oil

C_{10}-dienoic acid (Plate A[a])[b]

Component Glycerides (as % Mol. of Total Oil)													
321	320	311	222	310	221	300	220	211	210	111	200	110	100
1.2													
5.5	8.4	2.4	2.5										
-	-	-	0.2	2.8	2.2								
-	-	-	-	0.3	-	0.2	2.1						
-	-	-	-	0.1				1.1					
						0.1		0.3	1.1				
									0.2	0.2	0.3		
												0.3	
													0.7
→ 6.7	8.4	2.4	2.7	3.2	2.2	0.3	2.1	1.4	1.3	0.2	0.3	0.3	0.7

APPENDIX 7b

Fraction	Fatty Acid Composition (% Mol.) 16:0	18:0	18:1	18:2	18:3	Amount % Mol.	Glyceride Fractions of I. Glycerides which do not contain 333	332	331	330	322	321
1	0.6	-	0.4	0.7	98.3	17.2	16.3	0.4	0.2	0.3		
2	-	-	0.8	32.1	67.1	22.1		21.6	0.5	-		
3	11.2	4.0	18.4	1.2	65.2	19.2			10.4	8.6	0.2	
4	0.9	-	0.9	64.0	34.2	10.1			0.3	0.3	9.5	
5	12.5	3.8	18.0	33.6	32.1	13.6						7.1
6	14.4	7.3	27.9	40.4	10.0	17.8						
Total	6.6	2.6	11.4	25.8	53.6	Total	16.3	22.0	11.4	9.2	9.7	←
Kernel oil	6.2	1.8	11.4	26.3	54.3	Plate A	←			67.8	→	6.7
		Glycerides of Non Polar Fraction					16.5	22.0	11.5	9.0	9.5	6.5
		Glycerides as % Mol. Stillingia Oil					11.6	15.4	8.0	6.3	6.7	4.6

a. Plate developed with Ether.

b. Fraction represents 70%of Stillingia Oil.

Stillingia Oil

2, 4-decadienoic acid (Plate B[a])[b]

Component Glycerides (% Mol. of Non-Polar Fraction)												
320	311	222	310	221	300	220	211	210	111	200	110	100
6.5 ← 17.8 →												
31.4 →												
8.4	2.4	2.7	3.2	2.2	0.3	2.1	1.4	1.3	0.2	0.3	0.3	0.7
8.5	2.5	2.5	3.0	2.0	0.5	2.0	1.5	1.0	TR	0.5	0.5	0.5
6.0	1.8	1.8	2.1	1.4	0.3	1.4	1.0	0.7	TR	0.3	0.3	0.3

Glyceride Fractions of

II. B. Mono-2,4-decadienoyl

Fraction	Fatty Acid Composition (% Mol.)[d] 10:2	16:0	18:0	16:1	18:1	18:2	18:3	Amount % Mol.	33D	32D	31D
1	33.3	0.7	-	0.2	0.4	1.9	63.5	21.1	19.1	1.2	0.4
2	33.3	1.1	0.1	-	2.0	30.5	33.0	24.7		22.3	1.5
3	33.3	2.8	1.5	0.8	26.1	8.2	28.4	12.5			9.5
4	33.3	21.7	7.0	1.0	1.0	7.5	28.5	9.7			
5	33.3	2.4	0.7	0.7	3.7	59.2		10.6			
6	33.3	4.5	1.0	1.6	27.7	31.8		8.5			
7	33.3	23.0	6.8	1.1	10.1	26.7		9.0			
8	33.3	25.3	7.1	3.3	32.0	-		3.9			
Total	33.3	6.5	2.0	0.7	8.8	21.1	27.6	Total	19.1	23.5	11.4
Original Composition		5.0	2.0	0.2	8.8	21.7	29.0				
								Glycerides as % Mol. Stillingia Oil	5.7	7.0	3.4

a. Plate developed with Benzene-Ether (1:1).
b. Fraction represents 30% Stillingia Oil.
c. Separated fractions determined using an internal standard.
d. Calculated from G.L.C. analyses assuming 10:2 and minor components = 33.3% Mol.
e. D signifies dienoic acid.

Stillingia Oil

Triglyceride Fraction [a.b.c.]

Component Glycerides (as % Mol. diene Fraction)[e]					
30D	22D	21D	20D	11D	10D
0.4					
0.9					
1.5	1.5				
8.4	0.7	0.6			
	8.2	1.4	1.0		
		6.7	1.4	0.4	
			7.2	1.1	0.7
					3.9
11.2	10.4	8.7	9.6	1.5	4.6
3.4	3.1	2.6	2.9	4.5	1.4

APPENDIX 7d

Glyceride Fractions of

II. A. Mono-2,4-decadienoyl Triglyceride

Fraction	Fatty Acid Composition (% Mol.)[d]							$E^{1\%}_{1\ cm.}$	Wt.	% Mol.
	10:2	16:0	18:0	16:1	18:1	18:2	18:3			
1	33.3	0.4	-	-	0.3	1.2	64.8	303	16.4	20.0
2	33.3	0.9	-	-	1.7	30.5	33.5	295	21.0	25.6
3	33.3	3.4	0.5	0.3	25.9	6.9	29.7	312	10.4	12.7
4	33.3	10.0	3.2	0.5	2.3	37.7	13.0	315	15.1	18.4
5	33.3	13.1	4.1	0.5	23.4	25.6	-	248	19.1	23.3
Total		5.6	1.6	0.3	9.7	21.8	27.7			
Original Composition		5.8	1.7	0.2	8.9	22.0	28.1			

Glycerides as % Mol. Stillingia Oil

a. Plate developed with Benzene-Ether 1:1.

b. Fraction represents 30% of Stillingia Oil.

c. Separated fractions determined by weighing.

d. Calculated from G.L.C. analyses and assuming 10:2 and minor components ≡ 33.3% Mol.

e. D signifies dienoic acid.

Stillingia Oil

Fraction - "Polar Fraction" [a.b.c.]

Component Glycerides (as % Mol. of diene Fraction)[e]

33D	32D	31D	30D	22D	21D	20D	11D	10D
19.4	0.4	0.1	0.1					
	23.4	1.5	0.7					
		10.0	1.5	1.2				
			7.2	9.7	1.5			
					7.8	10.1	3.5	1.9
19.4	23.8	11.6	9.5	10.9	9.3	10.1	3.5	1.9
5.8	7.1	3.5	2.9	3.3	2.8	3.0	1.1	0.5

APPENDIX 8a

Quantitative Analysis of Triglycerides of Sample 38 by T.L.C.

Fatty Acid Composition of Fractions (Mole %)

Fraction	Mole %	16:0	18:0	18:1	18:2
1	25.3	1.7	1.4	2.9	94.0
2	24.5	2.2	2.2	30.9	64.7
3	15.4	20.2	10.9	4.3	64.6
4	5.5	6.4	-	57.7	35.9
5	8.5	17.8	14.0	36.3	31.9
6	5.7	21.9	8.5	56.0	13.6
7	8.9	18.8	14.6	58.2	8.4
8	6.2	24.4	35.1	40.5	-
Composition of Total Fractions	-	10.3	7.8	26.1	55.8
Composition of Original Oil	-	8.1	6.7	26.5	58.7

Triglyceride Composition of Fractions as % Mole of Total Fat

Fraction	222	221	211	111	220	210	110	200	100
1	20.7	2.2			2.4				
2		21.5			3.0				
3			1.0		14.4				
4			4.5		0.4	0.6			
5				0.4		8.1			
6				0.5		2.3	2.9		
7				2.2			4.4	2.3	
8							1.4		4.8
Total	20.7	23.7	5.5	3.1	20.2	11.0	8.7	2.3	4.8

APPENDIX 8b

Quantitative Analysis of Triglycerides of Sample 55 by T.L.C.

Fatty Acid Composition of Fractions (Mole %)

Fraction	Mole %	16:0	18:0	18:1	18:2
1	8.0	2.4	-	2.2	95.4
2	34.7	0.4	-	0.6	99.0
3	27.0	3.1	-	32.4	64.5
4	17.2	19.3	10.4	6.1	64.2
5	5.8	3.3	1.2	60.8	34.7
6	4.4	27.5	4.6	33.2	34.7
7	2.9	34.9	16.7	29.9	18.5
Composition of Total Fractions	-	6.9	2.6	16.0	74.5
Composition of Original Oil	-	5.6	1.9	15.9	76.6

Triglyceride Composition of Fractions as % Mole of Total Fat

Fraction	222	221	211	111	220	210	110	200	100
1	6.9	0.5			0.6				
2	33.7	0.6			0.4				
3		24.7			2.3				
4		0.6	1.3		15.3				
5			5.1		0.2	0.5			
6				0.2	0.3	3.9			
7							1.3	1.6	-
Total	40.6	26.4	6.4	0.2	19.1	4.4	1.3	1.6	-

APPENDIX 8c

Quantitative Analysis of Triglycerides of Sample 90 by T.L.C.

Fatty Acid Composition of Fractions (Mole %)

Fraction	Mole %	16:0	18:0	18:1	18:2
1	2.0	3.2	-	3.0	93.8
2	36.5	0.4	-	0.9	98.7
3	26.0	1.6	-	33.4	65.0
4	19.7	20.0	12.4	4.6	63.0
5	5.6	4.6	2.0	55.9	37.5
6	6.5	21.0	9.9	35.2	33.9
7	2.6	24.8	13.5	32.7	29.0
8	1.1	29.1	15.2	55.7	-
Composition of Combined Fractions		7.3	3.7	16.8	72.2
Composition of Original Oil		7.3	3.0	15.3	74.4

Triglyceride Composition of Fractions as % Mole of Total Fat

Fraction	222	221	211	111	220	210	110	200	100
1	1.6	0.2			0.2				
2	35.1	1.0			0.4				
3		24.8			1.2				
4			1.2		18.5				
5			4.5		0.7	0.4			
6			0.5			6.0			
7				0.3		1.6		0.7	
8							0.8		0.3
Total	36.7	26.0	6.2	0.3	21.0	8.0	0.8	0.7	0.3