Part - I

Compositional Studies Of Minor Seed Oils
Chapter - I

Minor Seed Oils

Component fatty acids of natural fats

Lipids are heterogeneous class of compounds whose general solubility in organic solvents and insolubility in water distinguishes them from other cellular constituents such as proteins, carbohydrates and nucleic acids. The acyl lipids are all derivatives of long-chain fatty acids. Over eight hundred fatty acids have been discovered and their number is increasing rapidly. The natural fatty acids usually contain an even number of straight chain carbon atoms with a terminal carboxyl group. The most commonly occurring acids are: palmitic, stearic, oleic, linoleic and linolenic. Besides these usual fatty acids, a large number of functionalized fatty acids have also been discovered as a major and/or minor constituent of seed fats. The composition of seed fats varies between genus to genus, species to species and
family to family because the individual plant species may respond differently to environmental effects such as temperature, light, humidity etc.

Seed fats of the plants belonging to the same botanical family often show similarity in their fatty acid composition. It is also observed that some times specific acids are restricted or preferred to a particular group of plant species. Such as seed fats of Proteaceae family normally contain a large proportion of hexadecenoic acid. Lythraceae and Lauraceae contain short-chain acids. Umbelliferae, Flacoutrtiaceae, Santalaceae and Cruciferae plants contain petroselinic, cyclopentene, acetylenic and erucic acids respectively. Labiatae seed fats produce oils of high percentage of unsaturation and are also reported to contain allenic (laballenic) acid.

An imposing number of new and novel fatty acids have been discovered possessing structural features quite unusual according to our earlier concept. The unusual structural features of recently discovered natural fatty acids are described here. Reviews dealing with structure of unusual fatty acids have been published\textsuperscript{1-2} from time to time.

An unsaturated acid of C\textsubscript{16} chain-length (82.2\%) has been reported by Spencer \textit{et al.}\textsuperscript{3} in the seed oil of \textit{Thunbergia alata}. Recently from author's laboratory \textit{cis}-9-hexadecenoic acid
has been reported in Zanthoxylum alatum, Ochna squarrosa and Ochna artopurpurnia seed oils. Spencer and Kleiman have reported an unusual seed oil, Rourcopsis obliquifoliata which contains preliminary C₆ fatty acids (palmitic 50%; palmitoleic 3.2%). All cis-5,9- and 5,9,12-acids have been reported in the seed oils of Taxus baccata and Larise leptolepis respectively. Plattner and Kleiman reported ω-5 monoene in the seed oil of Grevillea robusta. The first allenic acid, octadeca-5,6-dienoic (laballenic) acid was reported in Labiatae seed oil by Bagby et al. In author's laboratory two seed oils of Labiatae family Leucas cephalotes and Leucas urticifolia have been reported to contain laballenic acid. The seed oil of Diplocyclos palmatus of Cucurbitaceae has been found to contain octadeca-cis-9, trans-11, cis-13-trienoic (puinic) acid to the extent of 38.2%.

An interesting series of acetylenic acids have been found to occur in seed oils of Olacaceae, Compositae, Santalaceae and Simarubaceae families. A couple of unknown fatty acids, 17-octadecen-6-ynoic and 6-eicosynoic acids have been reported by Pearl et al. Smith has reported the presence of crepenynic (octadec-cis-9-ene-12-ynoic) acid in the seed oil of Saussurea candicans.

Among the naturally occurring cyclic fatty acids, apart from the common sterculic and mlavalic acids other acids are:
sterculynic\textsuperscript{17}, 2-hydroxy sterculic\textsuperscript{18} and dihydroxy sterculic acids\textsuperscript{19}. A number of seed oils\textsuperscript{20-29} have been reported from our laboratory to contain sterculic and malvalic acids in varying amounts.

An idea of the complexity and diversity of fatty acids can be obtained from the occurrence of a variety of oxygenated acids in the seed oils. An additional rich source of ricinoleic (12-hydroxy-cis-9-octadecenoic) acid, Hiptage benghalensis seed oil was reported by Siddiqui \textit{et al.}\textsuperscript{30} An isomer of ricinoleic acid, isoricinoleic (9-hydroxy-cis-12-octadecenoic) acid was first reported by Gunstone\textsuperscript{31} in the seed oil of Strophanthus sarmentotus (Apocynaceae). From author's laboratory isoricinoleic acid has been found to be the major component of the fatty acids of \textit{Wrightia tinctoria}, \textit{W. tomentosa}\textsuperscript{32} and \textit{W. coccinea}\textsuperscript{33} seed oils. Recently from author's laboratory two new dihydroxy acids, 9,14-dihydroxyoctadecanoic and 11,13-dihydroxytetraicos-trans-9-enoic acids have been reported in \textit{Peganum harmala}\textsuperscript{34} (Rutaceae) and \textit{Balliospermum axillare}\textsuperscript{35} (Euphorbiaceae) seed oils. Also a new positional isomer of ricinoleic acid, 9-hydroxy-cis-11-octadecenoic acid was reported as a minor constituent of \textit{Plantago major}\textsuperscript{36} (Plantaginaceae) seed oil.

Natural keto or oxo acids are much less common than hydroxy and epoxy acids. \textit{Argemone mexicana} seed oil\textsuperscript{37} was shown
to contain three long-chain (28:0, 30:0, 28:0) oxo acids which might arise from the saturated C\textsubscript{18} and C\textsubscript{20} acids in a chain-extension reaction which maintain the keto group in the first extension cycle:

\[
\begin{array}{ccc}
18:0 & \rightarrow & 3\text{-oxo} \\
20:0 & \rightarrow & 11\text{-oxo} \\
22:0 & \rightarrow & 9\text{-oxo}
\end{array}
\]

(The first natural furanoid fatty acid was isolated from *Exocarpus cuperssiformis*\textsuperscript{38} (Santalaceae). It is a C\textsubscript{18} acid with a 9,12-furanoid system. An antifungal acetylenic furanoid keto ester has been reported from the shoots of *Vicia faba*\textsuperscript{39}. Neutral lipid of *Hevea brasiliensis* latex\textsuperscript{40} has been found to contain a furanoid acid.

The most unusual of all fatty oils is the seed oil of *Dichapetalum toxicarium*\textsuperscript{41}, which was reported to contain \(\omega\)-fluoro-oleic and \(\omega\)-fluoropalmitic acids. Spencer et al.\textsuperscript{42} have identified Lactobicillic and two other related branched olefinic acids in the seed oil of *Byrsocarpus cocuneus*. It also contains large proportion of \(\text{cis-11-octadecenoic acid}\).
Isolation and Characterization of Fatty Acids

Over the last decade advances in the methodology for lipid analysis have been noteworthy. The valuable techniques in the analysis of oils are thin-layer chromatography (TLC), gas-liquid chromatography (GLC), column chromatography, high-performance liquid chromatography (HPLC), urea and thiourea adduct separation, argentation chromatography, counter current distribution, spectroscopic and chemical methods.

Among the chromatographic techniques, TLC is one of the most important analytical tool of the current lipid research. TLC proved successful and widely adaptable in the separation of lipids in contrast to the older chromatographic methods. The use of adsorption, reversed-phase, argentation chromatography etc., was rapidly adapted in the analytical procedures for detection, separation, isolation and characterization of various classes of fatty acids.

In recent years the liquid chromatography has also been used for lipid analysis. The preparative gas-liquid chromatography has successfully been exploited in the isolation of pure fractions from a complex lipid mixture. In the chain of chromatographic techniques HPLC is the latest innovation. The HPLC method is simple, convenient and gives precision and absolute values which are consistent with those from traditional methods.
Many useful spectroscopic techniques like proton nuclear magnetic resonance ($^1$H NMR), $^{13}$C nuclear magnetic resonance ($^{13}$C NMR), liquid chromatography-mass spectrometry (LC-MS) and gas-chromatography-mass spectrometry (GC-MS) offer many advantages for the structure analysis of unknown fatty acids.

Besides the above described recent techniques some classical chemical methods generally used for the analysis of lipids are: catalytic hydrogenation, hydroxylation, oxidative degradation, partial hydrogenation and partial oxidation, Diels-Alder reaction and hydrogen bromide reaction.

During the last few years, there has been a steady progress in the development of new analytical procedures and improvement in the existing methods such as ion-exchange chromatography for the separation of acidic and neutral lipids, vacuum dry column chromatography for speedy separation of fatty mixtures, silver resin chromatography, high performance TLC, alumina plate TLC for the separation of unsaturated acids, silver sulphamate as an argentation and charring reagent, wideline and high resolution NMR, rapid iodine value (IV) determination using mercuric acetate and alkylthiolation of unsaturated fatty acids to determine the position of unsaturation. Halphen and Tortelli-Jaffe tests are used for the presence and colorimetrically assaying of cyclopropenoid fatty acids.
India is a major oilseed producing and oil consuming country. The vegetable oil industry in India today occupies a pivotal position in the mainstream of the country's economic development. The acute scarcity and rising prices of vegetable oils for edible purposes and industrial use have stimulated research in the screening of oil-bearing seeds from wild plants for finding non-traditional sources of vegetable oils. It is now realized that systematic screening of indigenous seed oils may discover oils containing either a high concentration of one of the common natural fatty acids or less common or unknown acid having a structure of industrial interest. These current trends are associated with the phytochemical screening of plant seeds found abundant throughout the world. Another significant role expected to be played by vegetable oils is in solving the energy crisis.

In an attempt to search economically feasible oil yielding species from a broad spectrum of Plant Kingdom, a programme
has been taken up at the author's laboratory for the last several years for the collection and analysis of uncultivated seeds. Recently Osman and Ahmad have published a review of this work. The review has described a few oil-rich species that show sufficient promise as alternative sources of vegetable oils. Till now, the species from rare or less familiar botanical families have received only limited attention. However, some of these little known species seem to provide excellent candidates for the production of desirable seed oils.

In continuation of 'seed oil screening' programme of our laboratory the analysis of eight seed oils of different species was carried out. The petroleum ether extracted oils were first analyzed by chemical, spectral and chromatographic techniques. Conjugation and unusual functions including trans-unsaturation were checked by UV and IR respectively. Absence of unusual groupings was also confirmed by various chromatographic techniques. The oils were then converted into their methyl esters. The esters were analyzed by direct, silver ion and preparative TLC and GLC.

The quantitative estimation of fatty acid components on a gas chromatogram was achieved by comparing retention times with those of the authentic lipid standards. The seeds and oil characteristics are shown in Table-1 along with the GLC analysis of the methyl esters of the component acids.
Erythroxylon monogynum (item 1) of Erythroxylaceae, cultivated in the tea-producing districts of India and Sri Lanka, is grown as an ornamental plant in the gardens of Bombay. Leaves contain alkaloid cocaine, used as antidote to alcohol, opium and tobacco habits. Scanning of the literature revealed no report on fatty acid composition of the species. The fatty acid profile revealed by GLC indicated that the oil had a requisite amount of saturated acids (>68%) for being a cocoa butter substitute, as there is a demand for solid fats for the manufacture of various confectionery products. On the other hand the seeds were also oil-rich (~50%). Thus, E. monogynum deserves agronomic evaluation before it meets any commercial status.

Ipomea obscura (item 2) of Convolvulaceae is cultivated throughout India, Sri Lanka and also in Burma. Leaves are valuable for the application in aphthous affections. The fatty acid composition of the few species of Ipomea have been reported earlier. The determination of polyunsaturated acids in the present study confirms previous reports on the pattern of composition of Ipomea seed oils 18:2>18:1>18:3 but no trace of acids that have less than 16 and more than 18 carbon atoms as in Ipomea dissecta. The oil (item 2) is composed of oleic (24.7%), linoleic (28.7%), linolenic (6.9%) and palmitoleic (1.2%) acids as unsaturated components. Moderate content of palmitic acid (27.7%) is present together with stearic acid (10.7%). Major amount (>50%)
of combined content of oleic-linoleic acids of this oil placed it into the 'semi-drying' group of oils.

Indigofera cordifolia (item 3) of Leguminosae is a well known indigenous seed oil. The flour prepared from the seeds is largely used in Rajputana as a famine food. GLC analysis of methyl esters showed palmitic acid as a major component (32.5%) along with oleic acid (27.1%). The higher content of saturated acids (~55%) is a special feature of this familiar indigenous seed oil. Other unsaturated components (18:2, 10.3; 18:3, 4.8%) were present with small amount of palmitoleic (2.5%) acid.

Citrus sinensis (item 4), Citrus aurantifolia (item 5) and Citrus reticulata (item 6) belong to Rutaceae family. Several forms of Citrus species, varying in size and in the thickness of the rind, are in cultivation in various parts of India. Fruit juice is useful in appetizer, antisepsis, antiscorbutic and in bilious vomiting.

All these seeds were found oil-rich and no report have appeared on the component acids of these seeds except C. reticulata oil (item 6) which has been reanalyzed here. The composition of this oil (item 6) was found similar to the previous investigation except the presence of minor amount of arachidic acid. GLC analysis of these species (items 4, 5 and 6) indicated the high concentration of total unsaturated acids 67-73% and hence suggested that
they may serve as a 'linoleic-rich' drying oil. This property of the oil was found similar to that of other oils from Rutaceae and to cotton seed oil\(^6\). In all these species palmitic acid predominates over stearic acid while palmitoleic acid is poorly present in two species 0.7 (item 4) and 0.4\(^\%\) (item 6).

Artocarpus lakoocha (item 7) of Urticaceae found in Dehra Dun and Eastward in the Sub-Himalaya, is often planted in gardens within the area and road sides. The fruit is largely eaten by the natives of India and its wood yields a yellow dye. The fatty acid composition by GLC analysis indicated that the oil is composed of oleic (10.1\%), linoleic (19.7\%) and a high content of palmitic acid (53.9\%) together with small amount of linolenic acid (2.5\%). An important feature of this species is the high content of total saturated acids (~68\%).

Portulaca oleracea (item 8, Portulacaceae) grows as a weed and is common in all warm countries. The plant is well known for its medicinal properties in India. The seed oil of Portulacaceae falls in the category of linoleic-linolenic rich oils. GLC analysis indicated a high concentration of total unsaturation (~79\%) and polyunsaturated (~62\%) acids. Seed oils, in which the content of linoleic and/or linolenic acid exceeds 60\%, show promise for use as drying oils. Thus item 8 may serve as a 'linoleic-rich drying' oil.
The main purpose of the present work is to determine by chemical screening what amounts and general classes of fatty acids are contained in minor seed oils from less familiar botanical families (Table-1). Those with suitably high oil content and unique fatty acid composition are of potential practical interest and scheduled for more intensive chemical study.
<table>
<thead>
<tr>
<th>S.N.</th>
<th>Source</th>
<th>Oil Properties</th>
<th>Methyl Esters Composition by GLC as Wt. %</th>
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<tr>
<td></td>
<td></td>
<td>Oil % I.V. a</td>
<td>S.V. b Ref. Index c 16:0 16:1 18:0 18:1 18:2 18:3 Others</td>
</tr>
<tr>
<td>1</td>
<td>Erythroxylon monogynum</td>
<td>50.2 34.5</td>
<td>198.1 1.4752 34.8 - 29.8 29.4 1.4 0.7 14:0(3.7)</td>
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<td>(Erythroxylaceae)</td>
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<tr>
<td>2</td>
<td>Ipomea obscura</td>
<td>5.7 95.3</td>
<td>198.8 1.4850 27.7 1.2 10.7 24.7 28.7 6.9 -</td>
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<td>(Convolvulaceae)</td>
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<td>3.5 63.4</td>
<td>196.5 1.4707 32.5 2.5 9.0 27.1 10.3 4.8 14:0(0.9) 20:0(trace) 22:0(12.5)</td>
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<td>Citrus sinensis</td>
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<td>200.3 1.4745 27.2 0.7 4.9 26.9 35.8 4.4 -</td>
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<td>C. aurentifolia</td>
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<td>C. reticulata</td>
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<td>197.6 1.4853 20.4 0.4 5.1 32.8 38.6 2.3 -</td>
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<td>Artocarpus lakoocha</td>
<td>8.3 55.5</td>
<td>204.3 1.4952 53.9 - 12.2 10.1 19.7 2.5 12:0(0.4) 14:0(1.0)</td>
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<td>(Urticaceae)</td>
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<tr>
<td>8</td>
<td>Portulaca oleracea</td>
<td>17.2 166.8</td>
<td>199.4 1.4865 18.6 - 1.4 17.0 23.7 38.6 20:0(trace) 22:0(0.7)</td>
</tr>
<tr>
<td></td>
<td>(Portulacaceae)</td>
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</tr>
</tbody>
</table>

a- Iodine value; b= Saponification value; c= Refractive index.
Experimental

Materials and Methods

(1) Sources of Oilseeds

The seed samples for the present screening analyses were obtained by staff botanists under contract in various parts of the country or by purchase from commercial seed suppliers.

(2) Extraction of Oil

Cleaned and dried samples of seeds were usually ground in a disintegrator. The powdered seeds were extracted repeatedly with light petroleum ether (40–60 C) in a soxhlet apparatus. The extracted oils were dried over anhydrous sodium sulphate. The solvent was removed in vacuo. The oils were neutralized by passing it (~1 g) in chloroform solution, through a short column of alumina (10 g). The analytical values of oils and seeds were determined according to the AOCS methods. \textsuperscript{69}
(3) **Preparation of Mixed Fatty Acids**

Seed oils were refluxed with ethanolic potassium hydroxide. The unsaponifiable material was removed by ether and the free fatty acids were obtained by acidification of the aqueous layer.

(4) **Methyl Ester**

Esterification was carried out as follows: Samples were refluxed for 1 hr in a large excess of absolute methanol containing 1% sulphuric acid (v/v). In each case, resulting mixtures were diluted to the cloud point with water, chilled in ice bath, and then extracted repeatedly with ether. Combined extracts were dried over anhydrous sodium sulphate and evaporated *in vacuo.*

(5) **Thin Layer Chromatography (TLC)**

Analytical TLC was performed on plates coated with 0.25 mm or 1.0 mm thick layer of silica gel with 20 or 30% ether in hexane as developing solvent. The plates were rendered visual by spraying with a 20% aqueous solution of perchloric acid and heating in an oven (~110 °C) for 10 minutes. Preparative plates of 1.0 mm thickness were sprayed with a 0.2% ethanolic solution of 2',7'-dichlorofluorescein and viewed under UV light. For reversed-phase TLC, the dried coated plates were uniformly impregnated with 5% silicon oil. Acetonitrile-acetic acid-water (70:10:20; v/v/v) were used as developing agent.
(6) **Gas Liquid Chromatography (GLC)**

The quantitative examination of methyl esters were carried out by using a Perkin-Elmer Model 154 vapour fractometer equipped with a thermal conductivity detector, using stainless steel packed column (2mx3mm) coated with diethyleneglycol succinate (DEGS) 15% on chromosorb W (45-60 mesh). The separations were carried out isothermally at 200 °C, chart speed 0.76 m/hr with hydrogen flow of 70 ml/minute.

(7) **Infrared (IR)**

IR spectra were recorded on Perkin-Elmer Model 621 spectrophotometer as a liquid film or as 1% solution in carbon tetrachloride.

(8) **Ultraviolet (UV)**

UV spectra of oils were recorded on DK-2 Ultraviolet spectrophotometer in methanol.
Epoxy Fatty Acid in Kigelia pinnata Seed Oil

Seed Oil Containing Unusual Fatty Acid

A number of novel fatty acids containing unusual functionalities have been isolated and characterized from the seed oils of various plant species. The structure of these unusual acids of plant origin have been comprehensively reviewed by Smith, Hopkins and Paltenden. In addition to the more common saturated, monoenoic and C18 polyunsaturated fatty acids, plant lipids contain more complex acids which include conjugated, acetylenic and ethylenic of both cis and trans configuration, allenic, cyclopropane, cyclopropene, cyclopentane, cyclopentene, furanoid, epoxy, hydroxy and oxo acids. The sundry combination of these functional groups may be found in a single fatty acid.

Natural fatty acids which absorb approximately stoichiometric amounts of hydrogen bromide are the acids having conjugated dienol, epoxy and cyclopropene groupings. Among the three natu-
rally occurring HBr-reacting acids, epoxy and cyclopropene fatty acids have recently attracted attention of lipid chemists.

Epoxy acids have been reported in the seed oils of more than 60 species in 12 plant families. Krewson\textsuperscript{73} has reviewed epoxy seed oils. Earle\textsuperscript{74} has supplemented Krewson's review and provided a comprehensive list of seeds in which epoxy oils have been found. The structure of vernolic (cis-12,13-epoxyoctadec-cis-9-enoic) acid, first acid of this class, isolated from \textit{Vernonia anthelmintica} seed oil, was elucidated by Gunstone\textsuperscript{75}. Several epoxy fatty acids have been isolated from the seed oils\textsuperscript{70} including coronaric (cis-9,10-epoxyoctadec-cis-12-enoic) acid\textsuperscript{74} and 9,10-epoxyoctadeca-trans-3, cis-12-dienoic acid\textsuperscript{76}. Vernolic acid has also been found in seed oils of \textit{Hibiscus cannabinus}\textsuperscript{77} and \textit{Cephalaria syriaca}\textsuperscript{78}. As a variant vernolic acid group, \textit{Crepis conyzaefolia} (Compositae), reported by Spencer\textsuperscript{79}, contains vernolic acid and two previously unknown acids, \textit{cis}-12,13-epoxyoctadeca-trans-6,\textit{cis}-9-dienoic (14\%) and \textit{cis}-12,13-epoxyoctadeca-cis-6,\textit{cis}-9-dienoic (2\%) acids.

A C\textsubscript{20} homologue of vernolic acid, named alchornic (cis-14,15-epoxy-cis-11-eicosenoic) acid has been isolated from \textit{Alchornia cordifolia} (Euphorbiaceae) seed oil by Kleiman et al.\textsuperscript{80} Conacher and Gunstone\textsuperscript{81} characterized a new epoxy acid, \textit{cis}-9,10-epoxyoctadec-12-ynoic acid as a minor component of \textit{Helichrysum}
bracteatum (Compositae) seed oil, along with the previously identified coronaric acid. Ulchenko et al.\textsuperscript{82} analyzed the seed oil of Onopordium acanthium and reported the presence of mono (epoxy)octadecenoyl diacyltriglycerides which consisted of 63.1\% \( \alpha \)- and 36.9\% \( \beta \)-epoxytriglycerides.

A new epoxy acid, \textit{cis}-3,4-epoxy-\textit{cis}-11-octadecenoic acid has been isolated from the seed oil of \textit{Vernonia roxburghii}\textsuperscript{83} along with vernolic acid in the author's laboratory. Also \textit{Vernonia volkemelifolia}\textsuperscript{84} has been found to be a rich source of vernolic acid. \textit{Mucuna prurita}\textsuperscript{85} (Leguminosae) was also found to contain a previously unidentified, \textit{cis}-12,13-epoxy-\textit{trans}-9-octadecenoic acid along with vernolic acid.

Recently the seed oils of \textit{Mucuna pruriens}\textsuperscript{86}, \textit{Hibiscus mutabilis}\textsuperscript{28}, \textit{Abelmoschus moschatus}\textsuperscript{29}, \textit{Sonchus oleraceus}\textsuperscript{87}, \textit{Cosmos sulphureus}\textsuperscript{88} and few species of \textit{Acacia} have also been found to contain vernolic acid in varying amounts.

These epoxy acids may be regarded as derivatives of oleic, linoleic and linolenic acids, in which one of the usually present double bond is epoxidized through metabolism. Seed oils rich in epoxy acids are of potential interest as replacement for synthetic epoxy compounds used as stabilizers for plastic materials\textsuperscript{89} and also as starting material for the preparation of
other derivatives. Swern et al. have listed epoxy compounds showing carcinogenic activity.

Detection and Isolation of Epoxy Fatty Acid

The general analytical procedure find unexpected response by the seed oils containing HBr-reacting fatty acids. This complexity inherent in the analysis of these fatty acids has created problems in their detection, isolation and structure determination. When the presence of oxygenated acids in seed lipid is suspected, the intact lipids should be examined as fully as possible by non-destructive techniques. Various procedures now available are so refined that the identity of products can satisfactorily be established by these methods. TLC is the best technique to detect epoxy fatty acids. Due to greater polarity of oxygenated function, a direct TLC gives spot of lower $R_f$ (retention time) indicating the presence of epoxy acid.

Qualitatively, the compounds with epoxy group are revealed on the TLC plate by picric acid test, whereas the quantitative determination is being made by HBr-titration at 3 °C. The results are expressed as hydrogen bromide equivalent (HBE) and calculated as epoxoyleic acid. Reversed-phase TLC and argentation TLC are also used to separate various isomers of epoxy fatty acids. Erythro and threo isomers of vicinal
dihydroxy acids, and a number of epoxy acids are resolved using silica gel impregnated with silver nitrate or boric acid.

Due to the importance of epoxy compounds, more sensitive methods have been used for their isolation and structure determination. IR, NMR and mass spectral techniques are very much helpful in ascertaining the structure of epoxy acids present in an oil.

The IR spectra of epoxy esters show two characteristic bands for epoxy function at 840 and 826 cm$^{-1}$. Methyl cis and trans epoxyoctadecanoates may also be discerned from each other by the discreet absorption bands at 892 (trans) and 833 cm$^{-1}$ (cis), respectively. NMR is also informative to characterize an epoxy acid, the epoxy protons give a signal around $\delta$ 2.7 (cis-epoxide) or 2.45 (trans-epoxide), though these values change slightly when epoxy group is near to the ester or $\alpha$-methyl group. The epoxy group exerts a weak deshielding influence on the CH$_3$- and the CH$_2$COOMe signals when the epoxy function approaches these groups.

Mass spectrometry is also an excellent tool for the structure determination of long-chain epoxy acids. In the mass spectrum of saturated epoxy esters very intense fragmentation occurs at the site $\alpha$ to the functional groups to give structure-revealing fragments. In addition trans-annular fragmentation also
takes place with concomitant hydrogen transfer to give the prominent ions. Gunstone et al.\textsuperscript{94} have examined the mass spectra of about thirty one isomers of methyl epoxyoctadecanoates.

GLC has been used as a powerful tool for the detection and estimation of epoxy fatty acids. The GLC behaviour of the \textit{cis} and \textit{trans} epoxy esters is summarized by Gunstone \textit{et al.}\textsuperscript{94} The mass spectrometry coupled with gas chromatography (GC-MS) is also a useful technique to study epoxy esters.
Kigelia pinnata Seed Oil: A Moderate Source of Vernolic Acid

The seed oils containing high concentration of epoxy acid appear to be of potential use in plastic formulations, protective coatings and other industrial products. An attempt of searching new epoxy oil-rich species, the present work reveals the presence of vernolic acid (~22%) in the triglycerides of K. pinnata seeds oil. The species, a naturalized variety of K. africana (Benth) is the first member of the family Bignoniaceae, to contain this HBr-active acid.

Oil was extracted from the ground seeds of K. pinnata with petroleum ether (40-60 C). The analytical values of the oil and seeds were determined according to the procedures recommended by the AOCS methods\textsuperscript{69} and the data are summarized in Table-3.

Oil had no absorbance in UV, thus indicating no conjugation in the oil. The IR spectrum of the oil showed moderate
absorption at 840 and 815 cm\(^{-1}\) indicating the presence of cis-epoxy grouping. Analysis of the oil by TLC revealed two spots for oxygenated and non-oxygenated glycerides. The spot at lower \(R_f\) gave a positive picric acid TLC test\(^{91}\) indicating the presence of epoxy function. The oil was transesterified by using 0.5N sodium methoxide in anhydrous methanol. Separation of oxygenated and non-oxygenated methyl esters was accomplished by preparative TLC using hexane-ether as developing solvent.

Fraction I contained a mixture of non-oxygenated esters. Argentation TLC of this fraction revealed spots corresponding to saturates, monoene and diene. IR and UV analysis gave no indication of trans-unsaturation or conjugation respectively. GLC analysis showed the presence of the methyl esters of myristic, palmitic, stearic, oleic and linoleic acids(Table-2).

Fraction II gave a positive colour reaction for epoxy compound with picric acid. Its IR spectrum indicated an isolated cis-double bond\(^{28}\) with absorption at 3015 and 720 cm\(^{-1}\) and a cis-epoxide with intense bands 840 and 815 cm\(^{-1}\). Its NMR spectrum showed general features which resembled the spectra of fatty methyl ester except the diagnostic signals at \(\delta\) 2.3 m

\[
(4H, \text{-CH-CH-CH-CH-(CH}_2)_n\text{-CH}_2\text{-COOCH}_3), 2.8 m (2H, \text{cis-CH-CH-})
\]

and 5.4 m (2H, \text{-CH=CH-}). These IR and NMR spectra were consistent with those of methyl vernolate isolated from \textit{V. anthelmintica} seed.
This suggested that fraction II may be methyl cis-12,13-epoxy-cis-9-octadecenoate.

Further verification of the assigned structure was obtained by chemical methods. The epoxy ester on acetylation yielded the corresponding diol acid. This dihydroxy acid melting at 54-55 °C was characterized as threo-12,13-dihydroxyoctadecenoic acid (II, Scheme) (lit.75 m.p.54-55 °C) by comparison with an authentic sample of threo-12,13-dihydroxyoctadecenoic acid prepared from V. anthelmintica seed oil. Hydrogenation of the unsaturated dihydroxy acid (II) yielded threo-12,13-dihydroxyoctadecenoic acid (III), m.p.96-97 °C (lit.75 m.p.96-97 °C). Its identity was further confirmed by mixed m.p. with an authentic sample prepared from V. anthelmintica seed oil. Cleavage of unsaturated diol (II) by periodate-permanganate gave hexanoic (IV) and azelaic (V) acids identified by GLC analysis. Hence the cleavage points were at C_9-C_10 and C_12-C_13 linkage. The oxidative fission of saturated diol (III) gave hexanoic (IV) and dodecanedioic (VI) acids, showing the hydroxyl group at C_12-C_13 position. The configuration of unsaturated and saturated diols (II and III) were confirmed by comparing the TLC behaviour with standard threo and erythro diols on boric acid TLC. The configuration of diols (II and III) were found to be threo.

The chromatographic, spectroscopic and chemical degradation data collectively identified the dihydroxy ester derived
from the epoxy acid of fraction (II) as methyl threo-12,13-
dihydroxyoctadec-cis-9-enoate (IIb). These results demonstrate
conclusively that the original epoxy acid is vernolic (cis-12,13-
epoxyoctadec-cis-9-enoic) acid (Ia).

GLC analysis of the total methyl esters of oil indicated that these esters contained 22.3% methyl vernolate, along with esters of common acids in the amounts given in Table-2. The percentage of epoxy acid obtained by GLC also agrees with the amount determined by the HBr titration of oil.

Oil from the immature seeds of *K. pinnata* when subjected to HBr-titration showed only 2-3% epoxy content. On the other hand the matured seeds yielded an oil whose HBr-titration indicated a higher percentage of epoxy acid (~22%). The increase in the epoxy content may be attributed to the role of enzyme system in the biosynthesis of oxygenated acid during the ripening period of the seeds.
Scheme

\[
\begin{align*}
\text{CH}_3(\text{CH}_2)_4-\text{CH}-\text{CHCH}_2-\text{CH}=&\text{H}(\text{CH}_2)_7\text{COOCCH}_3 \\
\text{(I)}
\end{align*}
\]

1. CH₃COOH
2. KOH
3. H⁺/MeOH

\[
\begin{align*}
\text{CH}_3(\text{CH}_2)_4-\text{CH}-\text{CHCH}_2-\text{CH}=&\text{H}(\text{CH}_2)_7\text{COOH} \\
\text{(II)}
\end{align*}
\]

MnO₄⁻/IO₄⁻

\[
\begin{align*}
\text{CH}_3(\text{CH}_2)_4\text{COOH} + \text{HOOC}(\text{CH}_2)_7\text{COOH} \\
\text{(IV)} & \text{(V)}
\end{align*}
\]

H₂, Pd/C

\[
\begin{align*}
\text{CH}_3(\text{CH}_2)_4-\text{CH}=&\text{CH}(\text{CH}_2)_7\text{COOH} \\
\text{(III)}
\end{align*}
\]

MnO₄⁻/IO₄⁻

\[
\begin{align*}
\text{CH}_3(\text{CH}_2)_4\text{COOH} + \text{HOOC}(\text{CH}_2)_{10}\text{COOH} \\
\text{(IV)} & \text{(VI)}
\end{align*}
\]
Experimental

(i) General Methods

Melting points were observed on a Kofler apparatus and are uncorrected. Spectroscopic and chromatographic analyses of the oil and methyl esters were performed in the same way as described in the preceding section, except the boric acid TLC. The dihydroxy ester was analyzed on silica gel G plates impregnated with boric acid and developed with hexane-ether-acetic acid (60:40:1; v/v/v). The spots were visualized by charring the plates at 110°C after they had been sprayed with 20% aqueous solution of perchloric acid. The preparative TLC was performed on (20x20 cm) plates coated with 1.0 mm thick silica gel G and were sprayed with 2',7'-dichlorofluorescein and bands were visualized under UV light. Methyl esters were prepared by transesterification with sodium methoxide (0.5N). The uncorrected weight percentages of the non-oxygenated fatty acids were calculated from GLC analysis (Table-2).
(ii) **Seed and Oil Characteristics**

Preliminary analyses of the seeds and its oil were determined by the standard AOCS procedures (Table-3).

(iii) **Picric Acid TLC**

Picric acid TLC was carried out using silica gel G plates (2.5 x 8.5 cm). The developing system was petroleum ether-ether-acetic acid (75:24:1; v/v/v). The plates were then sprayed thoroughly with 0.5N picric acid in 95% ethanol which were saturated with the vapour of ether-ethanol-acetic acid (80:20:1; v/v/v) for 30 minutes, and exposed to ammonia fumes for 1-2 minutes. The orange spot on a yellow background on the TLC plate indicated the presence of epoxy group.

**Hydrogen Bromide Titration**

The procedure of Harris et al. was followed to titrate a weighed amount of oil with 0.1N hydrogen bromide using crystal violet as an indicator at 3°C to a bluish green end point, that persist for 30 seconds. The percentage of oxirane oxygen/epoxide was calculated by the equation:

\[
\% \text{ of Oxirane Oxygen} = \frac{V \times N \times 1.60}{Wt \text{ of the Sample}}
\]

where \( N \) = Normality of HBr.
\( V \) = Volume of HBr solution consumed in titration.
Table 2

Fatty acid components of *K. pinnata* seed oil (Wt %) by GLC

<table>
<thead>
<tr>
<th>Component</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic</td>
<td>0.4</td>
</tr>
<tr>
<td>Palmitic</td>
<td>25.4</td>
</tr>
<tr>
<td>Stearic</td>
<td>0.9</td>
</tr>
<tr>
<td>Oleic</td>
<td>8.9</td>
</tr>
<tr>
<td>Linoleic</td>
<td>42.0</td>
</tr>
<tr>
<td>12,13-epoxyoleic</td>
<td>22.3</td>
</tr>
<tr>
<td>Analytical data on <em>Kigelia pinnata</em> Seeds and Oil</td>
<td></td>
</tr>
<tr>
<td>-------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>Oil content of seeds, %</strong></td>
<td>8.7</td>
</tr>
<tr>
<td><strong>Unsaponifiable content, %</strong></td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Moisture, %</strong></td>
<td>6.4</td>
</tr>
<tr>
<td><strong>Iodine value (Wijs)</strong></td>
<td>102.0</td>
</tr>
<tr>
<td><strong>Saponification value</strong></td>
<td>205.0</td>
</tr>
<tr>
<td><strong>Refractive index, ( n_D^{30} )</strong></td>
<td>1.4854</td>
</tr>
<tr>
<td><strong>Oxirane oxygen, %</strong></td>
<td>0.51</td>
</tr>
<tr>
<td><strong>HBr Equiv. (as % epoxyoleic)</strong></td>
<td>21.65</td>
</tr>
</tbody>
</table>
Isolation and Characterization of Epoxy Acid

The methyl esters of *K. pinnata* and *V. anthelmintica* seed oils were prepared by transesterification with sodium methoxide (0.5N, 12 ml/g of oil). The total methyl ester (5.0 g) of *K. pinnata* was separated into non-oxygenated fraction I (4.0 g) and oxygenated fraction II (100 mg) by preparative TLC.

**Oxygenated Fraction**

IR(Neat): 1740 (C=O) and 840 (cis-epoxide); NMR(CHCl₃): 0.9 t (3H), 1.31 br, s (chain-CH₂), 2.3 m (4H), 2.8 m (2H), 3.6 s (3H) and 5.4 (2H).

**Acetolysis of Fraction II**

Fraction II (40 mg) was refluxed with glacial acetic acid (1.5 ml) for 5 hr. The mixture was saponified and acidified then diluted with water and extracted repeatedly with ether. After removal of solvent and successful crystallization from petroleum ether-ether (2:1; v/v) yielded a solid, unsaturated dihydroxy acid (II) melting at 54-55°C. The unsaturated dihydroxy derivative of vernolic acid was obtained by direct acetolysis of *V. anthelmintica* oil as described above.
Hydrogenation of II

A 200 mg portion of II was hydrogenated in the presence of 10% Pd-C. The usual work up yielded 12,13-dihydroxyoctadecanoic acid (III) melting at 96-97°C. No depression in melting point was observed on admixture with an authentic sample of saturated dihydroxy obtained from vernolic acid.

Permanganate-Periodate Oxidation of II and III

A 200 mg portion of II and III and of potassium carbonate were dissolved in t-butyl alcohol (40 ml). To this mixture, was added a solution of sodium meta periodate (180 mg) and potassium permanganate (2 ml of 0.057 M) in 40 ml of water. The reaction mixture was stirred at ambient temperature for 24 hr, then reduced with sodium metabisulphite, acidified with HCl and extracted with ether. Ether extracts after drying and evaporation yielded a semi-solid mixture which was analyzed by GLC after esterification with ethereal diazomethane. The identity of the cleavage products were made comparing their retention times with those of authentic samples. The dihydroxy monoenoic acid (II) yielded hexanoic (IV) and azelaic (V) acids whereas the dihydroxyoctadecanoic acid (III) gave hexanoic (IV) and dodecanedioic (VI) acids.


