not shown individually in Figure 1, but all fall into the bounds of the shaded area.

This initial increase in the oleic acid was not the result of a selective lipolysis for plant lipases have been found to be non-selective (21 and 22). Hydrogenation or dehydrogenation of either the bound or free fatty acids is possible by a fatty acid dehydrogenase system such as has been reported as present in soybeans during germination (4). Although only free long chain acids were reported as precursors for this system, the acceptability of acids bound in triglycerides as precursors should not be neglected. With this last assumption, the accumulation of oleic acid in the residual triglycerides (neutral fat) would not necessitate an active re-esterification process for the incorporation of the newly formed oleic acid into the neutral fat. Such re-esterification would be possible by the lipases as reported by Hilditch (8) or by esterases.

The conversion of other fatty acids into oleic acid as an initial stage in the breakdown of fatty acids appears to be a reversal of that observed in the developing bean. Simmons and Quackenbush (24) concluded from their studies on soybean cuttings that oleic acid was the first formed, and acted as a precursor for the others, which would be possible in the presence of the dehydrogenase system reported by Funicha (4).

With oleic acid as the precursor necessary for further fatty acid breakdown, its later more rapid disappearance during the period of most rapid fat loss could be due to the demand for it exceeding that present in the FFA as a result of the lipase and dehydrogenase systems, and as a result more must be hydrolyzed from the triglycerides. This hydrolysis being non-selective, the other FFA formed must re-esterify and so become prominent in the neutral fat.

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[Received December 15, 1960]

Analysis of the Geometric Isomers of Methyl Linoleate by Gas Chromatography [1, 2]

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Abstract

The four geometric isomers of methyl linoleate have been quantitatively determined by gas chromatogrophy on Apiezon L and DEGS polyester capillary columns. Three peaks were eluted from the Apiezon L column: (a) the 9-*cis*,12-*cis* isomer; (b) the 9-*cis*,12-*trans* isomer; and (c) the 9-*trans*,12-*cis* and 9-*trans*,12-*trans* isomers combined. The DEGS polyester column also resolved three peaks: (a) the 9-*trans*,12-*trans* isomer; (b) the 9-*cis*,12-*cis* and 9-*cis*,12-*trans* isomers combined; and (c) the 9-*trans*,12-*cis* isomer. Since the separation of isomers was different on each column, the content of each of the four isomers could be determined from the combined results. Quantitative results agreed closely with the per cent *trans* bonds as determined by infrared analysis.

Introduction

DURING current investigations on the *cis-trans* isomerization of natural fats, it became necessary to determine the precise fatty acid composition of fats produced by such a process. According to Blekkingh, Janssen, and Keppler (1) and Kass, Nichols, and Burr (2), *cis-trans* isomerization with such catalysts as Se, NO_2, or SO_2 produces only geometric isomerization without involving appreciable movement of the double bond along the carbon chain. However, a small amount of conjugation does occur. In the absence of positional isomers, our problem was to find suitable analyses for the geometric isomers of the three common unsaturated fatty acids: oleic, linoleic, and linolenic. This report describes a procedure for the quantitative determination of the four geometric isomers of methyl linoleate by gas chromatography.

There are four possible geometric isomers of methyl 9,12-linoleate: *cis-cis*, *cis-trans*, *trans-cis*, and *trans-trans*. Very few analytical methods are available for determining the content of these four isomers in a mixture of fatty acids. Infrared analysis at 10.36 microns (3, 4, 5) indicates only the total percent of *trans* bonds present in a mixture. Jackson, Paschke, Tolberg, Boyd, and Wheeler (6) suggested an analytical method based on the different rates of alkali conjugation of the various isomers. Their procedure yielded only approximate results, and their calibration curves were based on only three of the four possible isomers. McGee (7) has described an enzymatic technique for determining the amount of methyl 9-*cis*, 12-*cis* linoleate in a mixture of its geometric isomers. His method is based on the specificity of lipoxidase in

[1] Presented at the fall meeting of the American Oil Chemists' Society, Chicago, Illinois, October 30–November 1, 1961.
[2] Supported in part by a grant from Cargill, Incorporated.

converting only this isomer to a conjugated hydroperoxide, which can than be measured by its ultraviolet absorption at 234 millimicrons. Van Beers, Sparreboom, and Keppler (8) reported an isotopic dilution method for the determination of methyl 9-*cis*,12-*cis* linoleate in the presence of its geometric isomers. Dutton, Scholfield, and Jones (9) have recently reported the separation of methyl linoleate geometric isomers by lengthy countercurrent distribution.

Unfortunately, none of the above methods were suitable for our purposes. Only the procedures of Jackson *et al.* (6) and Dutton *et al.* (9) could determine the content of all four isomers. Results from Jackson's method were too approximate to meet our needs, and we did not have the necessary countercurrent distribution equipment to carry out Dutton's procedure. Since gas chromatography had been used to separate methyl oleate from methyl elaidate (10, 11), we attempted to develop a similar method for the four geometric isomers of methyl linoleate.

Analytical Methods

Gas Chromatography. A Barber-Colman Model 20 gas chromatograph equipped with a capillary column and an argon-ionization detector was used for all gas chromatography analyses. Samples were injected into the flash vaporizer at 275–300C. By means of sample dilution with petroleum ether and a stream-splitting arrangement, approximately 0.001 to 0.010 microliter of methyl esters was placed on the capillary column. The detector was equipped with a radium ionization source and maintained at 205–225C. The ionization voltage applied to the cell electrodes was 1100 volts. A scavenging flow of argon (55 to 65 ml/min) through the detector maintained an effective cell volume of a few microliters.

A 200 ft stainless steel capillary column with an internal diameter of 0.010 in. (0.254 mm) was coated with Apiezon L using the procedure described by Lipsky (10). Analyses with this column were run at 200C with an argon flow of 0.92 ml/min through the column. Under these conditions, methyl stearate had an elution time of about 73 minutes and exhibited a resolution of 37,000 theoretical plates.

A 150 ft stainless steel capillary column with an internal diameter of 0.010 in. (0.254 mm) was coated with diethylene glycol succinate polyester (DEGS) using the same coating procedure mentioned above. Analyses with this column were run at 175C with an argon flow of 0.68 ml/min through the column. Under these conditions, methyl stearate had an elution time of about 38 minutes and exhibited a resolution of 43,000 theoretical plates.

Gas chromatograms were quantitated by the usual triangulation procedure.

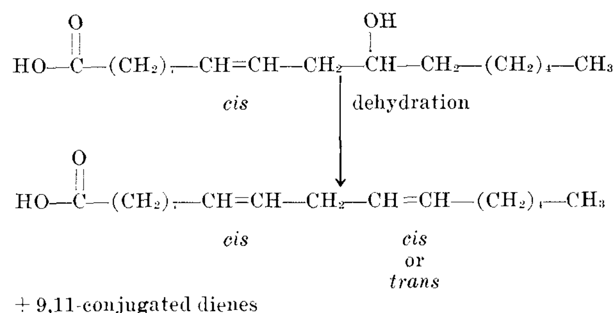
Infrared. Infrared analyses to determine the amount of isolated *trans* bonds were run on Beckman IR-4 and IR-5 infrared spectrophotometers. The AOCS tentative method Cd-14-61 (3) was used with minor modifications. Methyl 9-*trans*,12-*trans* linoleate was used as a primary calibration standard instead of methyl elaidate. Results were then computed in terms of per cent methyl 9-*trans*,12-*trans* linoleate present rather than in terms of methyl elaidate. When gas chromatography showed that a sample contained material other than 9,12-linoleate (such as conjugated dienes), infrared results were corrected to give the per cent *trans* bonds in the 9,12-linoleate fraction alone.

Materials

Methyl 9-*cis*,12-*cis* linoleate was prepared by urea fractionation of safflower oil methyl esters according to the procedure of Keppler, Sparreboom, Stroink, and von Mikusch (12). The final product contained more than 99% methyl octadecadienoate as determined by gas chromatography and had an iodine value of 170.0 (theoretical value: 172.4). It showed no *trans* double bonds by infrared.

Methyl 9-*trans*,12-*trans* linoleate was prepared by isomerizing methyl 9-*cis*,12-*cis* linoleate with selenium, and recrystallizing the fatty acid from methanol as described by Kass and Burr (13). The final product showed no impurities as determined by gas chromatography. Infrared absorption at 10.36 μ was nearly twice that of methyl elaidate. The melting point of the acid derived by saponification and acidulation of the ester was 27.8–28.4C. (28–29C according to Kass and Burr).

A mixture of methyl 9-*cis*,12-*cis* linoleate and methyl 9-*cis*,12-*trans* linoleate in the absence of the other two geometric isomers was prepared by methanolysis of dehydrated castor oil (Baker Castung 103 G-H). Dehydration of the ricinoleic acid in castor oil can form both 9,11-conjugated and 9,12-unconjugated dienes (14). The double bond formed by dehydration can assume either a *cis* or *trans* configuration.



Since the original 9-double bond in ricinoleic acid had a *cis* configuration, the 9,12-octadecadienoates produced by dehydration were the *cis-cis* and *cis-trans* isomers (6). No *trans-cis* or *trans-trans* isomers were present in the dehydration product. As will be shown later, this mixture can be used for the identification of the methyl 9-*cis*,12-*trans* linoleate peak in a gas chromatogram.

A mixture of methyl 9-*trans*,12-*cis* linoleate and methyl 9-*trans*,12-*trans* linoleate in the absence of the other two geometric isomers was prepared by dehydrating methyl ricinelaidate. Methyl ricinelaidate was prepared from castor oil by the method of McCutcheon et al. (15) (m.p. found: 28.0–29.5C; m.p. reported: 28.9–29.8C). The methyl ricinelaidate was then dehydrated at 240C under nitrogen with 2% $NaHSO_4$ for 17 minutes with magnetic stirring. Water was added and the product extracted with Skelly F. The Skelly F solution was washed repeatedly with water, dried over Na_2SO_4, filtered, and the solvent evaporated. Since the original 9-double bond in the methyl ricinelaidate had a *trans* configuration, the methyl 9,12-octadecadienoates produced by dehydration were the *trans-cis* and *trans-trans* isomers. As will be shown later, this mixture can be used for identification of the methyl 9-*trans*,12-*cis* linoleate peak in a gas chromatogram.

Experimental

To determine if it was possible to analyze the geometric isomers of methyl linoleate by gas chromatog-

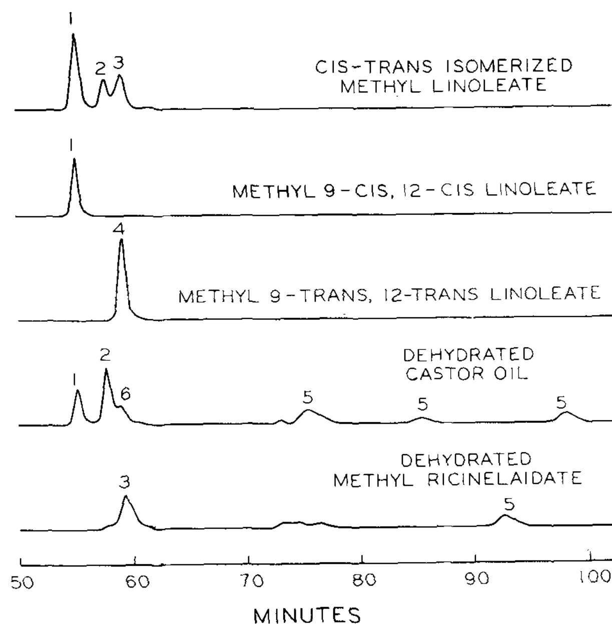


FIG. 1. Separation of the geometric isomers of methyl linoleate on a 200 ft. capillary column coated with Apiezon L showing procedure for identifying peaks: (1) methyl 9-*cis*, 12-*cis* linoleate; (2) methyl 9-*cis*,12-*trans* linoleate; (3) methyl 9-*trans*,12-*cis* linoleate and methyl 9-*trans*,12-*trans* linoleate combined; (4) methyl 9-*trans*,12-*trans* linoleate; (5) conjugated methyl octadecadienoates; and (6) methyl oleate.

raphy, a preliminary experiment was run to determine if the two most dissimilar isomers (methyl 9-*cis*, 12-*cis* linoleate and methyl 9-*trans*,12-*trans* linoleate) could be separated. A partial separation was obtained on a 6 ft x 3.5 mm ID column packed with 60/80 mesh Chromosorb W coated with 25% Apiezon L. With this indication that some separation was possible, it was decided to continue the study using high resolution capillary columns.

Apiezon L Column. On a 200 ft capillary column coated with Apiezon L, a mixture of the four geometric isomers (Se isomerized methyl 9-*cis*,12-*cis* linoleate) was resolved into three peaks. Figure 1 shows a typical chromatogram. The locations of the *cis-cis* and *trans-trans* isomers were determined by comparison with the relative retention times of pure methyl 9-*cis*,12-*cis* linoleate and methyl 9-*trans*,12-*trans* linoleate (Figure 1). The location of the *cis-trans* isomer was determined by the "mixed chromatogram" technique. A mixture of the *cis-cis* and *cis-trans* isomers was prepared from dehydrated castor oil as described above. A chromatogram of this material (Figure 1) indicated that the 9,11-conjugated octadecadienoic esters present were eluted considerably later than any of the 9,12-octadecadienoic esters. The separation was so great (the conjugated esters were eluted after methyl stearate) that there was no possibility of overlapping of the two groups. The dehydrated castor oil methyl esters showed three peaks occurring where the geometric isomers of methyl linoleate should elute. Two of these peaks were identified as methyl 9-*cis*,12-*cis* linoleate and methyl oleate. The remaining peak (constituting 29.7% of the dehydrated castor oil esters) had the same relative retention time as the middle peak, and was presumed to be methyl 9-*cis*,12-*trans* linoleate.

The location of the *trans-cis* isomer was also determined by the "mixed chromatogram" technique. A mixture of the *trans-cis* and *trans-trans* linoleate isomers was prepared from dehydrated methyl ricinelaidate as described above. As expected, a chromatogram of this material (Figure 1) again showed considerable separation of the conjugated and non-conjugated esters, so that there was no possibility of the two groups overlapping. The dehydrated methyl ricinelaidate showed one peak occurring where the geometric isomers of methyl linoleate should elute. This peak had the same retention time as methyl 9-*trans*,12-*trans* linoleate. It was concluded that both the *trans-cis* and *trans-trans* isomers were combined in one peak.

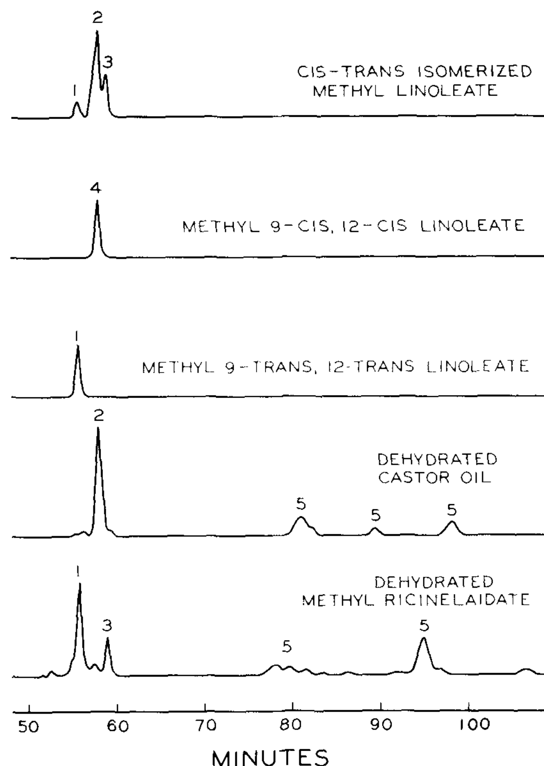


FIG. 2. Separation of the geometric isomers of methyl linoleate on a 150 ft. capillary column coated with DEGS polyester showing procedure for identifying peaks: (1) methyl 9-*trans*,12-*trans* linoleate; (2) methyl 9-*cis*,12-*cis* linoleate and methyl 9-*cis*,12-*trans* linoleate combined; (3) methyl 9-*trans*, 12-*cis* linoleate; (4) methyl 9-*cis*,12-*cis* linoleate; and (5) conjugated methyl octadecadienoates.

Equivalent chain length values of the various isomers on an Apiezon L capillary column are shown in Table I.

DEGS Polyester Column. On a 150 ft capillary column coated with DEGS Polyester, a mixture of the four geometric isomers was also resolved into three peaks. A typical chromatogram is shown in Figure 2. The DEGS column also eluted the conjugated 9,11-linoleates much later than the 9,12-linoleates (Figure 2) so that peaks could be identified using the same techniques described above for Apiezon L column chromatograms. The dehydrated castor oil and dehydrated methyl ricinoleate showed several processing artifacts eluting near the 9,12-linoleate peaks. These artifact peaks were relatively small and

TABLE I
Equivalent Chain Length Values (16) of the Geometric Isomers of Methyl Linoleate

Compound	Stationary phase	
	Apiezon L 200C	DEGS polyester 175C
Methyl 9-*cis*,12-*cis* linoleate	17.48	19.46
Methyl 9-*cis*,12-*trans* linoleate	17.59	19.46
Methyl 9-*trans*,12-*cis* linoleate	17.64	19.55
Methyl 9-*trans*,12-*trans* linoleate	17.64	19.35
Methyl stearate	18.00	18.00

TABLE II
Composition of Mixtures of the Geometric Isomers of Methyl Linoleate

Sample [b]	Isomers present				% *trans* bonds [a]	
	% *cis-cis*	% *cis-trans*	% *trans-cis*	% *trans-trans*	By gas chromatography	By infrared
A	55.9	21.4	18.2	4.5	24.3	25.5
B	41.2	26.5	23.2	9.1	34.0	33.5
C	18.2	27.9	24.6	29.3	55.6	57.0
D	3.3	8.8	11.6	76.3	86.5	89.3

[a] Calculated as % methyl 9-*trans*,12-*trans* linoleate.
[b] Samples were prepared as follows: (A) Methyl 9-*cis*,12-*cis* linoleate was heated under nitrogen with 0.3% Se at 195C for 15 minutes. (B) Methyl 9-*cis*,12-*cis* linoleate was heated under nitrogen with 0.4% Se at 195C for 30 minutes. (C) Linseed oil was heated under nitrogen with 1.0% Se at 200C for 4 hours. The isomerized oil was converted to methyl esters by the usual H_2SO_4 catalyzed methanolysis procedure and distilled. The methyl octadecadienoate fraction was then isolated by preparative gas chromatography. (D) Methyl 9-*cis*,12-*cis* linoleate was heated under nitrogen with 1.0% Se for 6 hours. After distillation, the corresponding fatty acids were prepared by saponification and acidulation. These fatty acids were then recrystallized once from methanol at −20C to enrich the *trans-trans* isomer content. The product was then esterified with diazomethane.

therefore did not interfere with the identification of the much larger peaks of the 9,12-linoleate isomers.

Equivalent chain length values of the various isomers on a DEGS polyester column are shown in Table I.

Quantitative Results. Neither the DEGS nor the Apiezon L column separated all four of the geometric isomers of methyl linolate. Only three peaks were eluted on either column, which meant that two isomers were always combined in one peak. Fortunately, the two isomers which could not be separated on the Apiezon L column (*trans-cis* and *trans-trans*) were eluted as individual peaks on the DEGS column. And the two isomers which could not be separated on the DEGS column (*cis-cis* and *cis-trans*) were eluted as individual peaks on the Apiezon L column. Thus, it was possible to run a mixture on both columns and with the combined results determine the amounts of each of the four isomers present.

This quantitative technique was applied to four typical samples of selenium-isomerized methyl linoleate. Results are shown in Table II. To check the validity of these quantitative results, the per cent isolated *trans* bonds in each sample was calculated from the gas chromatography data. This value was compared with the per cent isolated *trans* bonds determined from infrared absorption at 10.36 microns. Values determined by both methods show close agreement (see Table II).

Discussion

The separation of the geometric isomers of methyl linoleate on an Apiezon L capillary column is not unexpected, since other workers have demonstrated the separation of methyl oleate and methyl elaidate on similar columns (10, 11). The increased resolution of capillary over packed columns contributes greatly in making an adequate separation, since methyl linoleate has four possible geometric isomers. As with oleate-elaidate separation, *trans* isomers are eluted after their corresponding *cis* isomers.

The separation of the *cis-trans* and *trans-cis* isomers into two separate peaks is surprising, since most previous workers have considered these two compounds practically identical. However, examination of the physical configurations of these two isomers (Figure 3) may give some clue as to why they can be separated. If the carbonyl oxygen on a molecular model of the *cis-trans* molecule is oriented upward, the carbon chain bends *downward* between the 9th and 10th carbon atoms. If the carbonyl oxygen on a molecular model of the *trans-cis* molecule is oriented

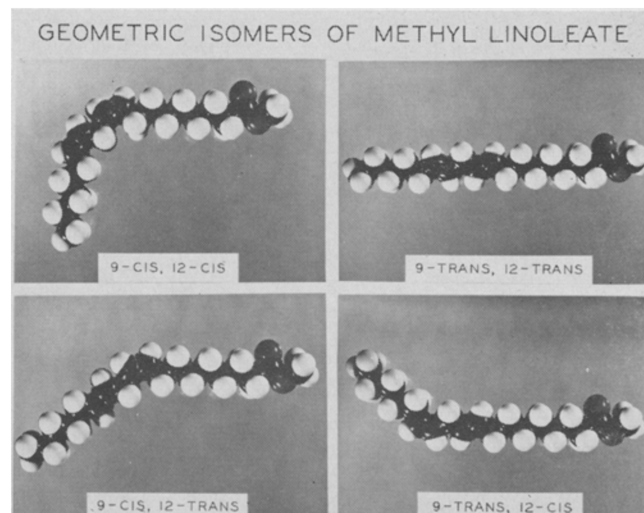


FIG. 3. Molecular models of the four geometric isomers of methyl 9,12-linoleate.

upward, the carbon chain bends *upward* between the 12th and 13th carbon atoms. These configurational differences probably contribute to differences in the vapor pressures and adsorption characteristics of these two isomers, thus aiding their separation by gas chromatography.

The separation of geometric isomers on a DEGS polyester column comes as a surprise, since polyester columns are generally believed to be incapable of distinguishing between *cis* and *trans* double bonds. Evidently, it is just a matter of enough theoretical plates to make the separation. The order of elution of the isomers on a polyester column is puzzling, since there is no discernible pattern. The *cis-cis* and *trans-trans* isomers do not occupy the outside peaks as with the Apiezon L column. At first it was thought that the peaks had been wrongly identified, but repeated rechecking showed them to be correctly labeled. We can offer no explanation for this unusual order of elution.

Future improvements in the resolution of capillary columns may eventually make possible the separation of all four isomers on one column. At the present time, however, we are unable to obtain such a separation with our equipment.

It should be noted than on an Apiezon L column, several other common fatty acid esters have elution times identical with the geometric isomers of methyl linoleate. Methyl 9-*cis*, 12-*cis*, 15-*cis* linolenate has the same retention time as methyl 9-*cis*, 12-*cis* linoleate. The geometric isomers of methyl linolenate are eluted at the same time as the *cis-trans*, *trans-cis*, and *trans-trans* linoleate isomers. The elution time of methyl oleate is almost identical with methyl 9-*trans*, 12-*trans* linoleate, and the two substances usually appear as one misshapen peak. When analyzing the isomer content of the linoleate portion of a *cis-trans* isomerized natural fat, these interfering substances will prevent proper analysis. To overcome this difficulty, a pure linoleate fraction can be prepared by preparative gas chromatography on a packed polyester column. 97–98% pure linoleate material thus produced can be analyzed on the Apiezon L capillary column with no difficulty.

Similar interference problems are not encountered with the DEGS polyester column.

It should be emphasized that the analytical procedure described here is only applicable to the *geometric* isomers of methyl linoleate, such as produced

by the well-known elaidinization catalysts NO_2, SO_2, Se, Te, etc. Catalysts producing extensive positional isomers (hydrogenation catalysts such as Ni, Pd, Pt, etc.) will yield a more complex mixture of geometric *and* positional isomers, and cannot be analyzed by this method.

Acknowledgment

We wish to thank Mrs. J. E. Lord for her help in preparing some of the materials used in the investigation and Mr. J. Q. Walker of the Barber-Colman Company for making the capillary columns used in the study. We are also indebted to the Baker Castor Oil Company for supplying the dehydrated castor oil used.

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[Received December 22, 1961]

Vernonia anthelmintica (L.) Willd. Trivernolin, 1,3-Divernolin and Vernolic (Epoxyoleic) Acid from the Seed Oil[1]

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Abstract

The epoxy fatty acid components isolated from the seed oil of *V. anthelmintica*, Indian ironweed, where the seed had been allowed to undergo lipolysis after grinding, were trivernolin, 1,3-divernolin, and vernolic acid. By inactivation of the hydrolytic enzyme system present in the seed, oil containing more than 50% trivernolin may be obtained. This species has potentialities as a replacement crop for those now in surplus; its seed contains 20 to 26% of an oil rich in epoxyoleic (vernolic) acid combined as glycerides amounting to 70 to 75%.

Introduction

RECENT INTEREST in *Vernonia anthelmintica* (L.) Willd. centers upon the unique character of its seed oil giving the plant potentiality as a new oilseed crop which might replace those now in surplus. This species of *Vernonia*, native to India, has been difficult to grow for seed production, flowering in early fall in many locations only to be frost-killed before reaching seed maturity. Improved prospects for successful cultivation of the plant in the U.S. came in 1960: a new introduction from India produced seed in Nebraska, North Carolina, and Texas. Adaptation studies are in progress on a wider scale at a number of locations. This paper deals with the extraction of the oil from the seed and the isolation and identification of its constituents: trivernolin, 1,3-divernolin, and vernolic acid. These are required in quantity for developmental studies of their utility in plastic formulations and in the preparation of chemical derivatives.

The literature on *V. anthelmintica*, commonly called purple fleabane or Indian ironweed, is voluminous. The plant was well-known to the early Greeks and Theophrastus described it about 300 B.C. (1–3). Before Linnaeus' time more than a dozen Latin synonyms were used for its designation and in a preliminary search more than 70 vernacular terms have been encountered, mostly references to Indian tribal names. In India, *V. anthelmintica* is often confused with *Psoralia cordylifolia* L. (4). In 18th century literature it is described by Linnaeus (5), having received its present name in 1804, given to it by Karl Ludwig Willdenow (6). He was the first to place it in the genus *Vernonia*, named for 17th century English botanist, William Vernon. This genus is one of the largest in the family Compositae, with an estimated 400–600 species (7,8). There are many references to the medicinal (9–16) and early ones to the insecticidal (1,3) properties of *V. anthelmintica*. It is claimed to be anthelmintic, purgative, good for asthmatic and kidney disorders, hiccough, and inflammatory swellings and skin diseases, especially leucoderma and psoriasis. A comprehensive report on the earlier literature, rich in folk-lore, is in preparation. The following is a review of late 19th and 20th century contributions to the chemistry of this plant:

Lindley and Moore (8) mention the production of oil by pressing the seed. Dymock *et al.* (9) extracted the oil from the seed and claimed the presence of an alkaloid in an alcoholic extract which they named *Vernonine;* they also reported that the seed contained 7.7% ash, a figure in close agreement with 7.2% obtained here. Kesava-Menon (10) was among the first to record information on the properties of the seed oil and Bhaduri (17) stated that the seed contained a glycoside which he named *shomerjin*. Chopra *et al.* (11,12) found no alkaloid present in the seed but reported the presence of a bitter principle, presumably accountable for the medicinal properties. Vidyarthi (18) first announced the presence in the seed oil of a new acid which he named *vernolic* but he considered it to be a hydroxy-acid isomeric with ricinoleic. He also reported on the sterols found in the unsaponifiable material. The details of these studies on fatty acid composition appeared in other publications (19,20), Majumdar (13) worked on

[1] Presented at the A.O.C.S. meeting in St. Louis, 1961.
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