TABLE III

Analysis of Natural Monoglyceride Preparations

	Crude			Refined [a]	
	TLC	Periodate analysis	Mol. dist.	Mol. dist.	Periodate analysis
Safflower	60.8	52.0	16.9	50.2	51.6
Menhaden	58.5	56.0	21.6	53.5	55.0
Lard	60.8	54.5	26.6	46.0	54.5

[a] Silicic acid column or 25% HCl treatment.

washing operation, because values obtained on the refined products agreed well with those obtained by periodate analysis. The crude preparations were refined by treating them with 25% HCl or passing them through a column of silicic acid, using ethyl ether as the solvent for the elution. About 2% of the samples remained on the column in the latter technique, including most of the pigments. The main effect of these treatments appeared to be in the removal of the traces of materials that catalyzed disproportionation.

TLC values for monoglyceride are slightly higher than the periodate oxidation values (α-monoglyceride content) because they represent total monoglyceride content. The difference between the periodate values for monoglyceride content and the TLC values represents the amount of β-monoglyceride in these products. The amount of β-monoglyceride also may be determined directly by TLC analysis after oxidizing the α-monoglycerides with periodic acid (11).

Although molecular distillation may be used for the analysis of mono-, di-, and triglycerides, provided no substances are present which cause disproportionation, TLC has a number of features which make it an ideal technique for the analysis of these compounds. First, because of the relatively large differences in polarity between these compounds, they are readily separated. The method is fast and simple even when it is necessary to perform a preliminary hydrogenation, and it can be carried out on a micro-scale with a high degree of accuracy and precision. It is extremely sensitive and permits the routine analysis of α- and β-monoglycerides and 1,2- and 1,3-diglycerides.

Acknowledgment

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Naturally-Occurring Epoxy Acids. III. Methods for Their Isolation [1,2]

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The adaptation of a wide range of isolation methods to the separation of epoxy components from seed oils has been accomplished. The application of these methods to samples ranging from a few micrograms to 50 g. or more has been considered, and recommendations as to the most suitable methods for specific sample sizes are made. Most of the procedures described are equally suitable for the isolation of hydroxy and other oxy-acids and esters. Some of them, in conjunction with gas-liquid chromatography, provide methods for total analysis of oils containing oxy-acids, which are more accurate and convenient than current analytical methods.

SINCE THE RECOGNITION by Gunstone in 1954 of the first known naturally-occurring epoxy acid (1) several other natural epoxy acids have been described (2–7). The number of known sources of these and possibly other epoxy acids is rapidly growing (8–16), and the possibility that these acids may have some biological importance is now being recognized (1,3,14). Parts I and II of this series delineate procedures for the detection and estimation of epoxy components of biological samples (14,15). This paper describes methods for their isolation.

The isolation of two natural epoxy acids from various oils by countercurrent distribution and/or crystallization has been reported (5,6,10,15). However, in view of the many excellent methods now available for the separation of fatty acids and esters, it seemed probable that these procedures were not the most suitable. This work was undertaken to adapt other methods to the isolation of epoxy acids or esters and to compare their efficiency and convenience for various sample sizes. It should be noted that most of the methods described are only for the isolation of epoxy components as a class and, if a mixture is present, will only partially separate individual epoxy acids or esters in that class. Further modifications will be required to isolate individual components

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[2] Supported by grants from the Hormel Foundation and the National Institutes of Health (Research Grant No. H-3559).

from such a mixture. However most of the natural sources of epoxy acids, thus far investigated, contain only one epoxy component (1,3,5–10), which could be isolated in a pure state by these methods.

Materials and Methods

The mixed esters from *Vernonia anthelmintica* seed oil, which contains more than 70% of *cis*-12:13-epoxyoleic acid (1), were used for evaluating the methods. The acids were obtained by saponifying the oil at room temperature, as described by Hopkins and Chisholm (10), and acidifying the saponifiable portion of the hydrolysate with dilute hydrochloric acid at 0°C. To minimize cleavage of epoxy groups acidification was carried out to pH 5, and the fatty acids were immediately extracted with ether (16). The mixed acids were subsequently esterified with excess diazomethane, and the esters were stored under nitrogen at −30°C. All operations during the preparation of the mixed esters and in the various separation procedures were carried out under nitrogen to minimize oxidation.

Fractions were monitored almost exclusively by thin-layer chromatography (TLC), using either 5% or 10% diethyl ether in hexane as an eluting solvent (14). Purity of the final product was assessed by the same procedure. A purity of more than 99% of epoxy component was assumed when only an "epoxy" spot was apparent on TLC of a sample of 1 mg. or more. A sample of epoxyoleate, isolated from *Vernonia* esters by adsorption chromatography (*vide infra,* IV [b]) and judged pure in this way, had the following properties:

Analysis

Found, carbon, 73.57%; hydrogen, 11.00%.
Theoretical, carbon, 73.56%; hydrogen, 10.97%.
Oxirane oxygen (17), 5.15%; theoretical, 5.16%.

Refractive index $n_D^{25°} = 1.4589$; dispersion, $n_f - n_c = 0.00896$.

The purity of this ester was further established by GLC, which gave a single symmetrical peak (14).

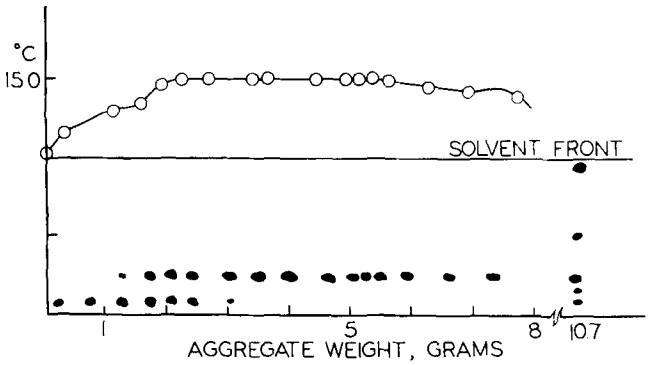

FIG. 1. Temperature/weight curve of fractions obtained by distillation of 10.7 g. of *Vernonia* esters on a whirling band column, along with a reproduction of paper chromatograms of the fractions. Solvent system for paper chromatograms was 50% aqueous acetonitrile.

Results

I. *Distillation. Vernonia* ester samples (10–50 g.) were fractionally distilled through a whirling band column.[3] Figure 1 shows the weight/temperature graph of one such distillation. The fractions in this case were monitored by paper chromatography (14), but a sample of the distilled epoxyoleate was subsequently also judged to be pure by TLC and GLC.

TABLE I

Analyses of *Vernonia* Mixed Esters by Several Methods

	A	B	C	D
14:0	tr.		tr.	tr.
15:0	tr.		tr.	tr.
16:0	2.9		2.5	3.3
17:0	tr.	3.6	tr.	tr.
18:0	1.4		1.1	1.5
20:0	0.2		0.2	0.3
21:0(?)	(?)		0.2	(?)
16:1	tr.		tr.	tr.
18:1	2.0	12.2	2.1	3.2
18:2	9.0		8.6	10.3
18:3	(?)		0.4	0.4
Epoxy 18:1	78.4	78.5	78.6	80.6
Hydroxy etc.	5.9	5.7	6.0	not measured

Columns A and C represent percentage of compositions obtained by separation of nonoxygenated esters, epoxy ester, and hydroxy esters by partition chromatography (IIIc) and adsorption chromatography (IVb), respectively, and subsequent gas-liquid chromatographic analysis of the nonoxygenated esters. Column B represents the weight percentages of the four groups of esters separated by reversed-phase partition chromatography (IIId). Column D is the result obtained by GLC analysis of *Vernonia* total mixed esters.

This distillation was completed in less than 6 hrs., and this short time may be the reason for the absence of a less polar degradation product which was encountered in other, slower distillations. The yield of pure methyl epoxyoleate, comprising fractions 8–16, was 4.2 g., amounting to 39.3% of the original charge of 10.7 g., and 50% of the epoxyoleate in *Vernonia* esters (cf. Table I). Oxirane oxygen values of 5.14, 5.13, 5.15, and 4.92 (theory, 5.16) were obtained for Fractions 8, 12, 15, and 16, respectively, by the method of Swern *et al.* (17).

II. *Urea Complex Fractionation.* A sample of approximately 1 g. of *Vernonia* mixed esters and 4 g. of *Vernonia* nonoxygenated esters was dissolved in 100 ml. of methanol and 3-g. portions of urea were added. After each addition the mixture was heated to dissolve the urea and cooled to room temperature. After addition of three portions of urea the first crop of urea complex crystallized and was filtered. A further 3-g. portion of urea was added to the filtrate which was heated, cooled, and filtered again, and this was repeated to give five urea complex fractions. These and the final filtrate were monitored by TLC and analyzed

[3] Mini-cal whirling heliband column, Podbielniak Inc., Division of Dresser Industries, Chicago, Ill.

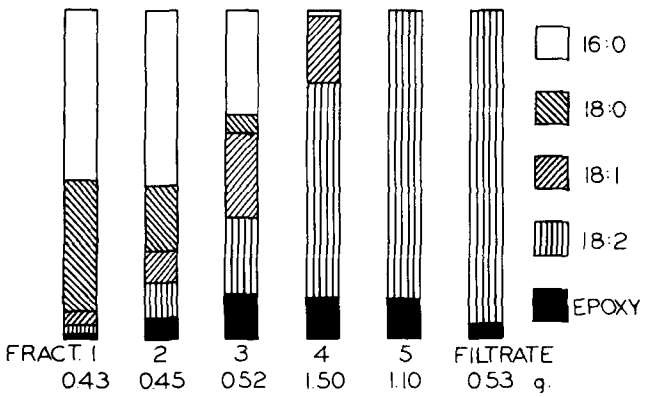

FIG. 2. Weight and composition of esters obtained by fractional crystallization, as urea complexes, of *ca.* 5 g. of esters containing *ca.* 10% of epoxy oleate. Analyses were carried out by GLC on an Apiezon L packed column, having a β-ionization detector. Compositions shown are percentages of total area under the GLC curves.

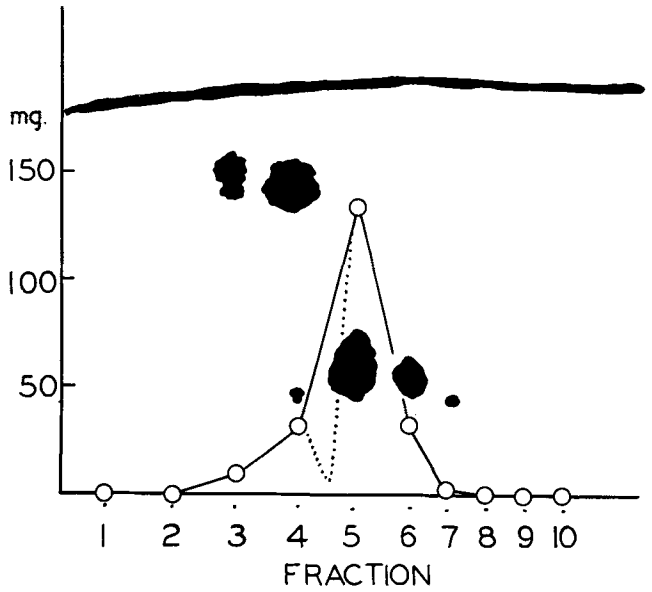

FIG. 3. Weight distribution curve and thin-layer chromatogram of fractions obtained by partition chromatography of 440 mg. of *Vernonia* esters, with acetonitrile as stationary phase and hexane as mobile phase. The chromatogram was developed with 5% ether in hexane.

by GLC. The result is illustrated schematically in Figure 2. Although excellent fractionation of the nonoxygenated esters has been achieved, the hope of concentrating epoxyoleate in the filtrate has not been realized. This ester, in fact, has been shown to have complexing properties roughly equivalent to those of methyl oleate.

III. *Partition.* Several methods involving partition between two phases were studied, and epoxy esters were separated from samples of a few micrograms up to more than 100 g. by such procedures.

a) Gas-liquid chromatography has been used for studies of epoxy esters (4,5,14), and this procedure is suitable for their isolation. The detection system, of course, must be such that components are not destroyed and they may then be isolated by passing the eluent gas through solvent maintained at a low temperature. Under suitable conditions epoxy components were isolated from the column unaltered in any way (5,14).

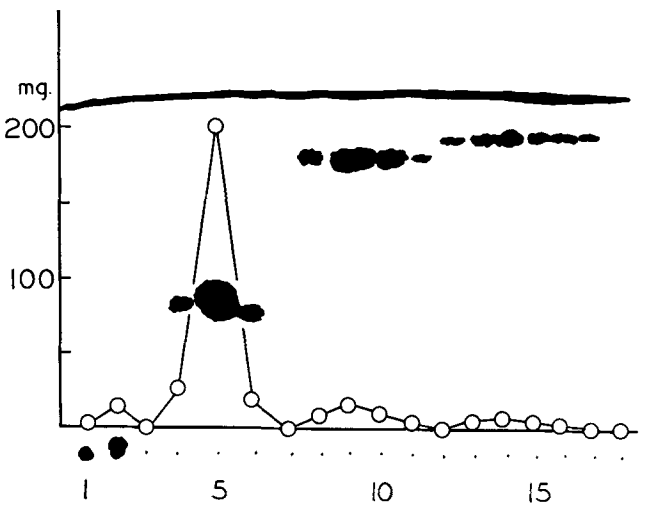

FIG. 4. Weight distribution curve and thin-layer chromatogram of fractions obtained by reversed-phase partition chromatography of 317 mg. of *Vernonia* esters with iso-octane as stationary phase and acetonitrile as mobile phase. Chromatogram was developed with 5% ether in hexane.

b) Paper chromatography may be similarly used when only small samples are available. Using methods already described (13), epoxy acids or esters were readily separated from other components and, after locating the spots with iodine vapor, these were cut out and the epoxy components were eluted from the paper with solvent.

c) Partition chromatography was carried out on a 27 x 1.5 cm. column of 60–80 mesh, acid- and alkali-washed Celite (14 g.) with acetonitrile as the stationary phase. Hexane, equilibrated with acetonitrile, was passed through the column until phase equilibrium was reached, and a sample of *Vernonia* esters (440 mg.) was added and eluted with the same solvent at a flow rate of 2 ml./minute. Fractions of 10 ml. each were collected, monitored by TLC, and weighed. Figure 3 shows the weight curve of the fractions from this separation superimposed on a reproduction of the thin-layer chromatogram showing their composition. Pure epoxyoleate (337 mg., 76.6% of the total sample) was obtained from Fractions 5–9, and the small amount of epoxyoleate in Fraction 4, estimated by TLC to be 8 mg., raises the proportion of methyl epoxyoleate in the original mixed esters to 78.4%. Unoxygenated esters amounted to 15.7%, and material not recovered from the column (26 mg., 5.9%) consisted of more polar hydroxy esters (*vide infra,* III [d] and IV [b]). Gas-liquid chromatographic analysis of Fractions 3 and 4 permitted a complete analysis of the mixed esters to be effected (Table I).

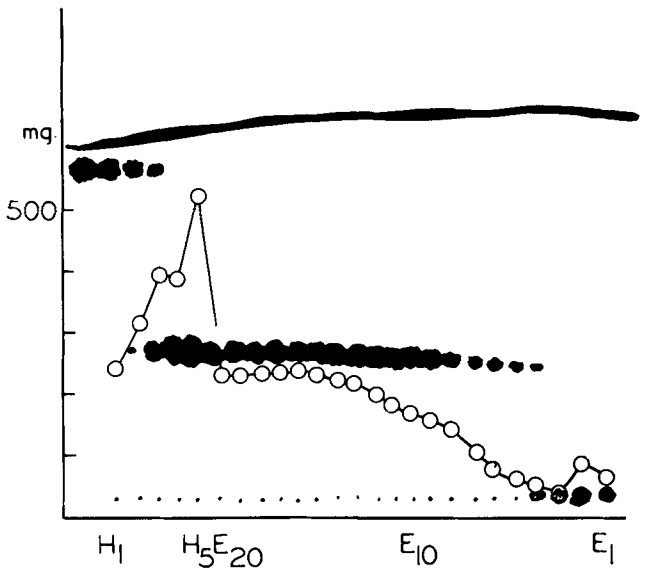

FIG. 5. Weight distribution curve and thin-layer chromatogram of fractions obtained by countercurrent distribution, in a series of five separatory funnels, of 5 g. of *Vernonia* esters between hexane and 80% ethanol. Chromatogram was developed with 10% ether in hexane.

d) Reversed phase partition chromatography was carried out on a 30 x 1.5 cm. column of 60–80 mesh Celite (15 g.), which had been made hydrophobic by exposure to the vapors of dichlorodimethylsilane. *iso*-Octane was the stationary phase, and acetonitrile, equilibrated with *iso*-octane, was passed through the column until phase equilibrium was reached. *Vernonia* esters (317 mg.) were added and eluted with equilibrated acetonitrile at a flow rate of 2 ml./minute.

The results are shown in Figure 4. Fractions 1 and 2 contained hydroxy esters (18 mg., 5.7%) and Fractions 4–6 were pure epoxyoleate (249 mg., 78.5%). The nonoxygenated esters were apparently separated into unsaturated esters (Fractions 8–11) and saturated esters (Fractions 13–16) containing 12.0% and 3.8% of the total mixed esters. These values agree well with those found by GLC analysis of other samples (cf. Table I).

e) Countercurrent distribution. A sample of *Vernonia* esters (5.0 g.) was dissolved in 100 ml. of hexane equilibrated with the polar solvent in the first of a series of five 250-ml. separatory funnels. To each of the other four funnels were added 100 ml. of equilibrated hexane. Portions of 100 ml. each of a polar solvent, equilibrated with hexane, were then passed through the series and equilibrated with the nonpolar phase at each step.

The hexane/80% ethanol partition shown in Figure 5 resulted in separation of 3.46 g. of pure epoxyoleate (69.2% of the sample or 87.6% of the epoxyoleate present) in Fractions E5–E20 and Fraction H5. A further five portions of polar phase passed through the system would probably have resulted in almost complete separation of the entire epoxy component.

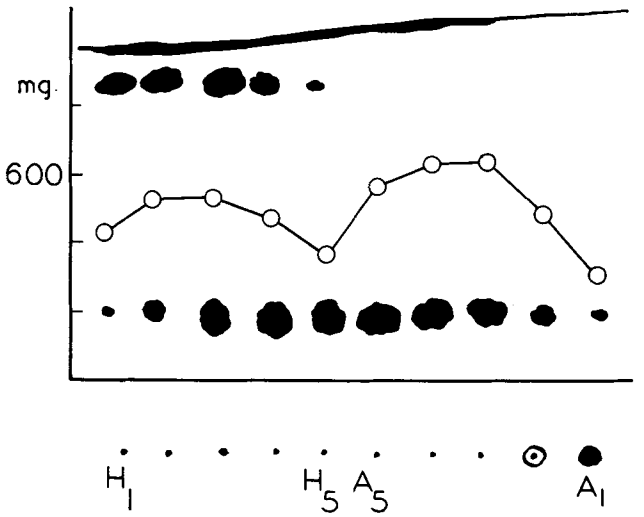

FIG. 6. Weight distribution curve and thin-layer chromatogram of fractions obtained by countercurrent distribution, in a series of five separatory funnels, of 5 g. of *Vernonia* esters between hexane and acetonitrile. Chromatogram was developed with 10% ether in hexane.

Figure 6 shows that partition with hexane/acetonitrile resulted in separation of only 47% of the available epoxyoleate, in the pure state, in Fractions A3–A5, and the first traces of nonoxygenated esters had already advanced to Fraction H5. The nonoxygenated esters therefore were too soluble in the polar phase of this system. The addition of 10% or 20% of water to the acetonitrile would probably result in a better separation.

IV. *Adsorption.* a) Thin-layer chromatography on silicic acid has been shown to give excellent separation of nonoxygenated, epoxy, and hydroxy acids or esters from each other and also some degree of subfractionation within these classes (14,18). Up to 50 mg. were chromatographed on a single plate by spotting as many as 25 1- to 2-mg. samples as closely as possible along one edge of the plate. The separated components were subsequently scraped from the plate and eluted from the adsorbent.

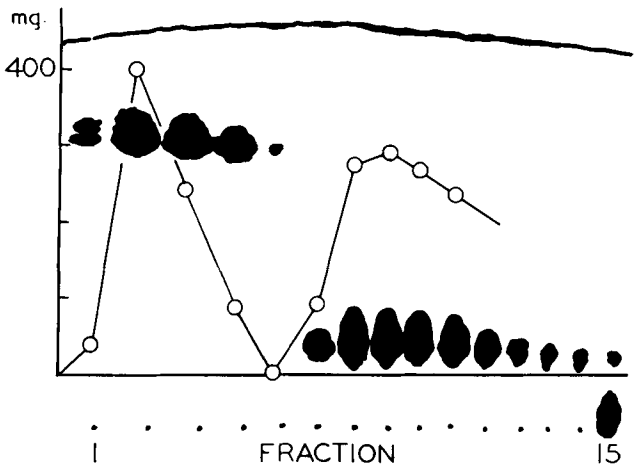

FIG. 7. Weight distribution curve and thin-layer chromatogram of fractions obtained by adsorption chromatography of 5 g. of *Vernonia* esters on silica gel. Fractions 1–10 were 50 ml. each of 3% ether in hexane; Fractions 11, 12, and 13 were 750, 500, and 400 ml., respectively, of the same solvent; Fraction 14 was 300 ml. of 5% ether in hexane and Fraction 15 was 150 ml. of pure ether. The chromatogram was developed with 5% ether in hexane.

b) Column chromatography on silica gel or silicic acid has been used extensively in this laboratory for the isolation of epoxy and hydroxy esters from the mixed esters of seed oils in amounts ranging from a few milligrams to 25 g. by using columns of a suitable size.

As an example, the separation of epoxyoleate from a 5.0-g. sample of *Vernonia* esters will be described. The column used contained 75 g. of silica gel (60–200

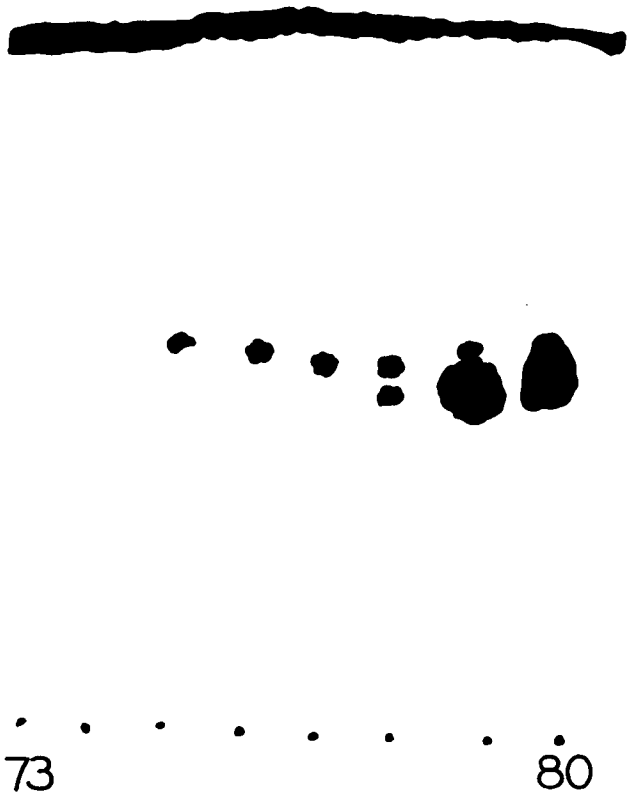

FIG. 8. Thin-layer chromatogram of fractions obtained by adsorption chromatography of 500 mg. of *Vernonia* esters on silicic acid in a Hagdahl segmented column, showing the separation of a trace amount of probable epoxylinoleate immediately before the first fractions containing epoxyoleate. Chromatogram was developed with 10% ether in hexane.

mesh)[4] and was 90 cm. x 1.25 cm. The column was packed with dry adsorbent, and 3% diethyl ether in hexane was run through it until all air bubbles were removed. The sample was applied quantitatively to the top of the column, elution was commenced with 3% ether in hexane, and 10 fractions of 50 ml. each were collected. These were monitored by TLC, worked up separately and weighed; the results are illustrated in Figure 7. Fractions 11, 12, and 13 were 750, 500, and 400 ml., respectively, of the 3% ether in hexane solvent, and the epoxy ester concentration in the eluent was very low by Fraction 13. This was followed by 300 ml. of 5% ether in hexane (Fraction 14), which eluted almost all of the remaining epoxyoleate on the column, and finally by 150 ml. of pure ether (Fraction 15), which eluted the small amount remaining along with all the more highly oxygenated esters. Total recovery from the column was 4.99 g. The composition of the *Vernonia* esters was found to be: 15.4% of nonoxygenated esters (Fractions 1–5), 78.6% of epoxyoleate (Fractions 6–14), and 6.0% of hydroxy esters (Fraction 15). Analysis of the combined nonoxygenated ester fractions by GLC gave the results shown in Table I and permitted the total analysis of the *Vernonia* mixed esters to be achieved.

An even wider separation of the three classes of components of a 500-mg. sample of *Vernonia* esters was achieved by using a Hagdahl segmented column (19,20). The column had a total capacity of 160 ml., was packed with 70 g. of silicic acid (100–200 mesh, Mallinckrodt) and operated at a flow rate of *ca.* 40 ml. per hour. The sample was eluted with 10% diethyl ether in petroleum ether, and 5 ml. fractions were collected by an automatic fraction-collector. The separation was monitored by differential refractometry and by TLC of individual fractions. Nonoxygenated esters emerged in Fractions 10–35 and epoxyoleate in Fractions 78–153. None of the hydroxy esters had begun to appear by Fraction 200 when the chromatogram was discontinued. The percentage of nonoxygenated and epoxy esters separated from the sample was the same as reported above from the conventional column. Of particular interest however was the appearance of a very small amount (1 mg., or 0.2%) of a slightly less polar ester than epoxyoleate in Fractions 75–79, overlapping epoxyoleate in the last two of these fractions (Figure 8). This component is probably 15:16-epoxylinoleate, which is likely to migrate to this position on TLC (14), and its separation in such a trace amount is a tribute to the efficiency of the Hagdahl column.

By using an 80 cm. x 30 cm. column containing 400 g. of silica gel, 25-g. and 3.0-g. samples of *Vernonia* esters were chromatographed satisfactorily, and more than 80% of the available epoxyoleate was isolated in a pure state, using an abbreviated elution pattern to finish the separation in 8 hrs. If suitable column dimensions (*i.e.*, length/diameter >20/1) are retained, there seems no reason why epoxy components from samples of 100 g. or more may not be readily isolated.

c) Batch adsorption has been found to be very suitable and convenient for separation of epoxy and hydroxy compounds from nonoxygenated materials with sample sizes of 1–2 g. For example, 0.95 g. of *Vernonia* esters was dissolved in 100 ml. of 3% ether in petroleum ether (b.p. 37°–45°), and 20 g. of silicic acid (100–200 mesh, Mallinckrodt) were added. The mixture was stirred and filtered, and the silicic acid was washed with 100 ml. of the same solvent. The filtered silicic acid was similarly extracted and washed three times, using 5%, 10%, and 25% ether in petroleum ether, in turn. These four fractions were monitored by TLC and weighed after solvent was removed. They contained 8.8%, 28.6%, 34.2%, and 22.9% of the sample, respectively. Fraction I was almost entirely nonoxygenated esters, 2 was a mixture predominantly epoxy oleate, and 3 and 4 were pure epoxy oleate. The 5.5% remaining on the silicic acid consisted of hydroxy esters. This procedure is sometimes more convenient than column chromatography for large samples and was used with 25 g. of *Vernonia* esters to give a similar separation to that reported above.

[4] W.R. Grace and Company, Davison Chemical Division, Baltimore, Md.

Discussion

Distillation is not recommended as an isolation procedure because of the tendency of epoxy esters, particularly unsaturated epoxy esters, to decompose.

For samples containing a relatively low proportion of epoxy components it is convenient first to concentrate the epoxy material by solvent-solvent partition or batch adsorption. These procedures are simple, take little time, and give approximately equivalent results. The former is more suitable for concentration of free acids and, for this reason, may be preferred. For final isolation of pure epoxy esters the choice of method depends largely on the desired sample size.

When only microgram or milligram quantities are available, gas-liquid, paper, or thin-layer chromatography are used. Paper-chromatography is suitable for isolation of epoxy acids or esters as a class but cannot, with present solvent systems, differentiate between components within that class (14) and is thus limited in its usefulness. Both thin-layer chromatography and gas-liquid chromatography can effect separation of some epoxy esters from others. Used in conjunction these two methods can isolate the esters of all known naturally-occurring epoxy acids (14). Thin-layer chromatography is recommended however as the first method to be used in isolating epoxy esters from small samples, and GLC should be used only where necessary since, under some conditions, it causes degradation of epoxy components.

For samples of 50 mg. to a few grams either adsorption, partition, or reversed-phase partition column chromatography are suitable. These methods give rather equivalent results in separating epoxy esters as a class. If isolation of the epoxy components as acids is desired, then the partition or reversed-phase partition chromatographic methods are more suitable than adsorption.

For separation of epoxy components from samples of 5 g. or more, adsorption chromatography on a column of appropriate size is recommended. A suitable elution pattern may be readily worked out to save time and solvents and yet retain almost complete separation of epoxy components. Liquid-liquid partition can also be used for such large samples but is more tedious and gives less complete separation.

Of the methods described, column chromatography by adsorption, partition, and reversed-phase partition, together with GLC analysis of the nonoxygenated ester fraction, are most suitable for quantitative determinations. GLC analysis of a total mixed ester sample containing epoxy components does not give an accurate quantitative determination of its compo-

sition. As noted before (14) epoxy esters give a lower response than nonoxygenated esters when a β-ionization detector is used. In addition, any dihydroxy esters, such as occur in the esters from *Vernonia* oil, will not be measured. The effect of both these factors is to give high values for the nonoxygenated esters (cf. Table I).

Most of the methods described for the isolation of epoxy acids or esters are equally, or more suitable for the isolation of hydroxy or other oxygenated derivatives. Thus a wide range of methods is available for the isolation of epoxy and other oxygenated acids and esters, and some of the methods, in conjunction with gas-liquid chromatography, may be used to give more accurate analyses of the mixed esters of oils containing these derivatives than are possible with older analytical methods.

Acknowledgment

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Antimicrobial Activity of Some Ricinoleic and Oleic Acid Derivatives

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Ricinoleic and oleic acid derivatives were screened for their antimicrobial activity, under optimum growing-conditions, against several species of bacteria, yeasts, and molds. Several ricinoleic acid derivatives and petroselinic (iso-oleic) acid exhibited considerable activity; in fact, their activity against some micro-organisms was comparable to sorbic and 10-undecenoic acid, known antimicrobial agents, as indicated by this test.

THE ANTIMICROBIAL ACTIVITY of various types of fatty acids or fatty acid derivatives has been studied by a number of investigators, using different techniques and organisms. According to Kiesel (10), the antimycotic action of saturated fatty acids increases as the number of carbon atoms in the chain increases up to eleven, and the branched-chain fatty acids are less active than straight-chain fatty acids of equal molecular weights. Tetsumoto (20,21,22) observed that the unsaturated fatty acids are more antimycotic than the corresponding saturated fatty acids, that the normal fatty acids are more active than the isomeric acids, and that the activity is due to the undissociated molecule, not the anion. The fungistatic activity of an organic acid varies with the pH of the test medium; the activity is roughly proportional to the concentration of the nonionized molecules (8). Cowles (4) found the bactericidal action of fatty acids to be greater at low pH values and the long-chain fatty acids to be more active than the short-chain fatty acids. Keeney *et al.* (9) stated that the long-chain fatty acids are more fungicidal than the short-chain fatty acids. Wyss *et al.* (24) found that the antimycotic action of fatty acids increases with chain length up to 12 carbon atoms, that the unsaturated fatty acids are slightly more active than the corresponding saturated fatty acids, and that fatty acids possessing an odd number of carbon atoms are no more active than the even-numbered homologs. The results obtained by Spoehr *et al.* (18) indicated that fatty acids with 16 or fewer carbon atoms exhibit antibacterial activity; that oleic, elaidic, linoleic, β-eleostearic, and β-licanic acids lacked antibacterial power but acquired it on photo-oxidation; that stearic acid lacked antibacterial power even after photo-oxidation; and that glycerides lacked antibacterial power even though the respective fatty acids were antibacterial. McGowan *et al.* (15) suggested that the fungistatic activity of substituted, unsaturated fatty acids is associated with the tendency of the substituents to withdraw electrons from the ethylenic bond. Stedman (19) found that propionic, undecylenic, and caprylic acids exhibit superior antimicrobial action at acidic pH's. Melnick *et al.* (16). reported that α,β-unsaturated fatty acids, such as sorbic acid, are normal transitory metabolites in the oxidation of saturated fatty acids by molds; however high initial concentrations can inhibit the dehydrogenase enzyme system in molds. Inhibition of this important enzyme system is held responsible for the fungistatic or fungicidal activity of sorbic acid. The bacteriostatic action of unsaturated fatty acids was observed by Kodicek (11) to increase as the number of *cis* ethylenic bonds increases. It was noted also

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