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Naturally occurring epoxy acids: II. detection and measurement of long-chain epoxy acids by near infrared spectrophotometry*†

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SUMMARY

A new method for the detection and estimation of long-chain epoxy acids in seed oils is described. It depends on the measurement of increased absorption at 2.795 μ in the near infrared spectrum caused by chlorohydrins produced from epoxides by treatment with anhydrous ethereal hydrogen chloride. The method is sensitive to approximately 0.2% of epoxy acid in an oil and is specific for epoxides. Hydroxy components of a sample do not interfere since the strongly associated hydroxyl band of chlorohydrins is normally clearly resolved from other OH absorption. The presence of large amounts of vicinally unsaturated hydroxy acids, however, results in large changes in absorption intensity in the 2.8 μ region on HCl treatment and in these cases epoxide concentration cannot be accurately measured but must be estimated. These reactive hydroxy acids, which lead to spurious epoxide values by the conventional methods, lose hydroxyl during the acid treatment, and measurement of the decrease in their absorption at 2.762 μ means that their concentration may be estimated concurrently with that of epoxy components. Other reactive acids, such as cyclopropenoid acids, which result in high epoxide values by the usual methods, do not interfere. Results obtained by this spectrophotometric method are compared, for some oils, with those obtained by the usual chemical methods of epoxide determination.

The increasing evidence of a widespread occurrence of epoxy acids in fats makes desirable the development of more sensitive and more specific methods for their determination.

Currently, the most widely used methods for the estimation of epoxy compounds depend on the uptake of hydrogen halide by the epoxy group. The direct titration of epoxy groups with hydrogen bromide in acetic acid now constitutes a standard procedure (1, 2). This method, however, gives fallacious results with oils containing α,β-unsaturated ketones (3), cyclopropenes (4), or conjugated dieneols (4). The determination, by back titration, of the uptake of hydrogen chloride from anhydrous ether solution (5) or from dioxan solution (3) is less likely to give spurious results, but does so with α,β-unsaturated ketones, such as are found in autoxidation mixtures (3). Both methods seem liable to similar interference by other reactive compounds. Newer methods in which the end point is determined argentometrically (6, 7), or potentiometrically (8), may have the same general disadvantages, although interference by readily hydrolyzable materials and by weak bases is obviated.

However, the basic fault of all these methods is that they measure the uptake of reagent by all components which will react rather than a specific product formed from epoxy groups. The sensitivity of titration methods, too, is such that values for epoxy components present in amounts of less than about 5% are not reliable unless fairly large samples are available.

Other methods for the estimation of epoxy fatty acids which have been tried include polarography (9), which was unsuccessful, and proton magnetic resonance (10), which is a useful tool only when the epoxy component exceeds about 5% of the sample. Colorimetry (11) has found application in the measurement of short-chain epoxides but has not yet been developed for use with long-chain derivatives. Glycol determina-

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tion by periodate oxidation (12), after splitting the epoxy group, offers no advantages over the conventional methods.

The preceding paper (13) describes physical methods, of high sensitivity, for the detection of epoxy acids and esters by paper-, thin-layer-, and gas-liquid chromatography, but quantitative studies by these methods require specialized techniques and may be rather tedious.

Epoxy compounds have infrared absorption in the region of 11 to 13 μ (14) which might be used for their estimation in samples containing high proportions of epoxides, such as epoxy resins (15). The absorption, however, is relatively weak, and in most vegetable oil samples, background absorption in this region is high enough to mask any band due to epoxides present in minor amounts. A near infrared band given by terminal epoxides has been used for the determination of these compounds (16).

The strong hydroxyl absorption in the 2.8 μ region, however, permits the determination of epoxides as hydroxyl derivatives after splitting the epoxy group with an acid. This method has already been used qualitatively as a proof of the presence of a small amount of epoxy acid in *Camelina sativa* seed oil (17).

Two methods described here are based on this principle and measure the products formed from chemical reactions with epoxy groups. They are specific for epoxy groups and can readily detect and measure epoxy components constituting less than 0.5% of a fat. These methods, incidentally, detect and can estimate dimorphecolic acid (9-hydroxy-*trans*-*trans*-octadec-10,12-dienoic acid) or similar compounds in seed oils, since treatment with acid causes dehydroxylation and the reduction in unassociated OH absorption can be measured.

MATERIALS

Vernonia anthelmintica (purple fleabane) seed oil was hydrolyzed with potassium hydroxide at room temperature and the mixed acids obtained after *careful* acidification. Cleavage of the epoxide group is prevented by acidification at 0° with the theoretical amount of 2 N hydrochloric acid. The methyl esters of these acids were prepared with diazomethane in ether and methyl *cis*-12:13-epoxyoctadec-9-enoate[1] isolated by fractional distillation through a spinning-band column. The product, having 5.14% oxirane oxygen by Swern's method (5) (theory 5.16%), migrated as a single spot on siliconized paper and on thin layers of silicic acid (13). It also gave a single symmetrical peak by gas-liquid chromatographic analysis (13).

cis-9:10-Epoxystearic acid (m.p. 57.5°-58°; reported 59.5°), *trans*-9:10-epoxystearic acid (m.p. 52-54°; reported 55.5°) and *cis,cis*-9:10,12:13-diepoxystearic acid (m.p. 78°-78.5°; reported 79°) were prepared by the methods of Swern *et al.* (18, 19), and their methyl esters were obtained with ethereal diazomethane.

threo-12,13-Dihydroxy oleic acid (m.p. 50°-52°; reported 53°-54°) was derived from methyl epoxyoleate by reaction with acetic acid followed by hydrolysis (20), and its methyl ester prepared with diazomethane. A mixture of methyl *threo*-12,13-chlorohydroxyoleate and methyl *threo*-13,12-chlorohydroxyoleate was prepared from epoxyoleate by reaction with anhydrous ethereal hydrogen chloride (5).

All standard substances were checked for purity by paper- and thin-layer chromatography and, where necessary, purified by adsorption chromatography on silica gel columns.

PROCEDURE

The near infrared spectra of oil samples, as 3.00%, 1.00%, or 0.30% solutions in carbon tetrachloride, were obtained on a Beckman DK-2 Recording Spectrophotometer with fused silica cells of 1 cm path length. The control settings used throughout were scanning time, 5; time constant, 0.1; and sensitivity, 2.0. At this sensitivity the nominal slit width at 2.795 μ was 0.050 mm.

Spectra were recorded of solutions of the same concentration before and after treatment of a sample with anhydrous ethereal hydrogen chloride. This treatment converts any epoxide groups to chlorohydrins and was carried out as follows: A sample of the oil (about 200 mg) was dissolved in anhydrous ether (5 ml) in a glass-stoppered bottle; 0.2 N hydrogen chloride in anhydrous ether solution (20 ml) was added, the bottle stoppered, and allowed to stand for 3 hours. The solution was then diluted with ether, transferred to a separatory funnel, and excess hydrogen chloride removed by washing four times with water. After drying with sodium sulfate, the product was recovered by removal of the solvent under reduced pressure. The untreated oil sample was also carefully dried (Na_2SO_4) in petroleum ether or ether solution, and the sample recovered before its spectrum was recorded.

The absorbance (A) at 2.795 μ was measured from a base line equivalent to the absorption between 1.50 and 1.60 μ, or from a tangent between the minima at

[1] The epoxy ring structure is indicated by a colon, e.g., 12:13-epoxyoctadec-9-enoate, which is synonymous with 12,13-epoxyoctadec-9-enoate.

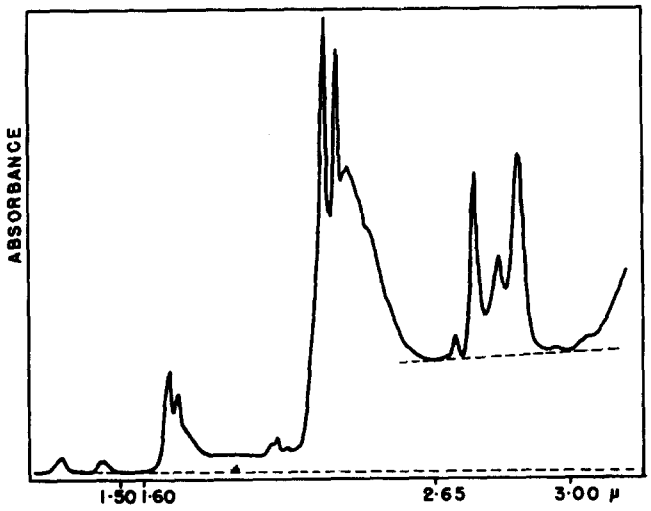

FIG. 1. Portion of near infrared spectrum of *Balanites* seed oil showing the alternative methods of obtaining a base line.

2.65 and 3.00 μ, as shown in Figure 1. The former mode of measurement is preferred since its base line, unlike the tangential base line, is unaffected by large changes of absorption in the 2.8 μ region. If solutions of the same concentration are used, the increase in absorbance (A_t-A_u) at 2.795 μ for each pair is obtained by difference and the absorptivity[2] due to chlorohydrin calculated. If different concentrations are used, the absorptivities of the original and treated samples at 2.795 μ must each be calculated and the difference obtained. The concentration of epoxy components is then calculated, as percentage of epoxyoleic acid, or as percentage of oxirane oxygen, from a calibration curve of known mixtures or by relation to the absorptivity, similarly measured, of the pure chlorohydrins:

$$\text{Epoxy content (as per cent epoxyoleic acid)} = \frac{a_t - a_u}{0.175}$$

where a_t = absorptivity at 2.795 μ of treated oil
a_u = absorptivity at 2.795 μ of untreated oil
and 0.175 = absorptivity of pure chlorohydroxy oleic acid, calculated from absorptivity of the methyl ester.

[2] The terms "absorption," "absorbance," and "absorptivity" are used according to the Joint Committee on Nomenclature in Applied Spectroscopy, *Anal. Chem.* **24**: 1349, 1952. That is, "absorption" describes the phenomenon, "absorbance" is a measure of the phenomenon without units, and "absorptivity" is a specific measurement referring to the ratio of the absorbance to the product of concentration and optical path length.

The experimental error, including errors in weighing and in measurement, is estimated to be 0.2% epoxyoleic acid for a single determination on samples containing relatively small amounts of epoxy components. The sensitivity is considered to be less than 0.2% if multiple determinations are carried out. When high proportions of epoxy acids are present in an oil, the values obtained by the procedure described above are likely to be low since introduction of a large number of chlorine groups during HCl treatment increases the average molecular weight. Thus the general background absorptivity of the untreated oil sample is higher than that of the treated sample. This can be corrected in one of two ways:

(*a*) If the uncorrected epoxy content by the above procedure is c%, as epoxyoleic acid, the absorptivity of untreated sample a_u and the molecular weights of epoxyoleic acid and chlorohydroxy oleic acid are 296.5 and 334, respectively, the following relationship holds:

$$\text{Corrected } a_u = \frac{100\, a_u}{100 - c + \frac{234}{296.5}c} = \frac{100\, a_u}{100 + 0.113c}$$

The difference between the absorptivity of the HCl treated oil at 2.795 μ and this corrected background is used to obtain the true epoxide content of the oil.

(*b*) Alternatively, the high background absorption in the untreated oil may be reduced by the ratio of the CH_2 band heights at 2.31 μ of treated to untreated oil and the difference in absorbance in the two samples calculated from this value. This method equates the number of CH_2 groups and hence the number of molecules so that a direct comparison is possible.

Assuming very accurate quantification in making up solutions of the HCl-reacted samples, the second correction method is preferable, and great accuracy in making up solutions of the unreacted oils is then unnecessary. Only in samples containing high proportions of epoxy components or having high background absorbance near 2.8 μ is such correction necessary since, in general, the difference is small. *Vernonia* oil gave an uncorrected value of 59.4%, which was raised to 59.9% by the first correction method, and to 60.2% by the second. The corresponding results for *Cephalocroton* oil were 54.2%, 54.7%, and 54.8%.

Some experiments were also carried out where epoxide groups were converted not to chlorohydrins but to 1,2-diols. This was effected by boiling the sample under reflux with glacial acetic acid for 2 hours. The acetylated oil was recovered, hydrolyzed, and the mixed acids esterified. Increase in absorption at 2.792 μ is due to the associated hydroxyl frequency of *threo*-1,2-diols produced from epoxides by this treatment.

RESULTS

Mixtures of known concentrations of pure methyl *cis*-12:13-epoxyoleate in olive oil (epoxy content 0.0%) were analyzed by these methods and calibration curves of A_t-A_u against epoxide content were drawn. Both gave straight lines through the origin (Fig. 2). From the slopes of these lines, the molar absorptivities of the pure diol and of the pure mixed chlorohydrins were calculated to be 71.4 and 58.5, respectively. These values are almost identical with those obtained from curves of the pure components (Fig. 3).

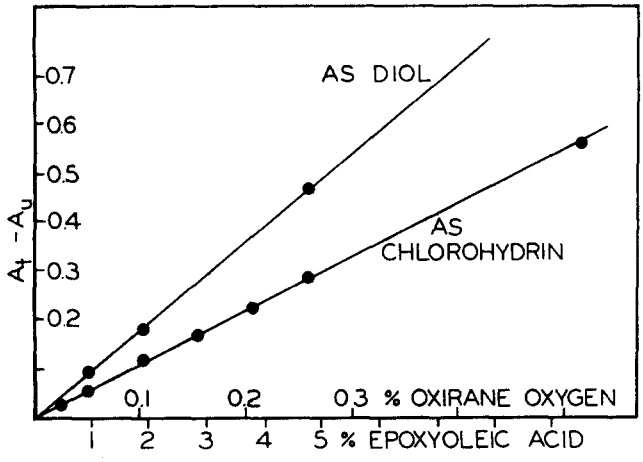

FIG. 2. Relationship of absorbance of treated sample corrected for background absorbance of untreated sample (A_t-A_u) to epoxy content of standard samples after treatment with HCl or acetic acid. Absorbance of diols measured as 4.00% solutions at 2.792 μ and of chlorohydrins as 3.00% solutions at 2.795 μ.

Molar absorptivity of the chlorohydrins from pure *cis*-9:10-epoxystearate (57.4) was the same as for those from epoxyoleate and the maximum occurred at the same wave length. The molar absorptivity of the chlorohydrins from *cis,cis*-9:10,12:13-diepoxystearate (116.0) was twice that of the monoepoxy derivatives, again at 2.795 μ. However, the *erythro*-chlorohydrins, produced by the same treatment from *trans*-9:10-epoxystearate, gave a rather different spectrum with an associated hydroxyl band at 2.793 μ of molar absorptivity 37.4 and an unassociated hydroxyl band at 2.758 μ of molar absorptivity 15.5.

The concentrations of the standard mixtures in carbon tetrachloride were 3.00% for determinations as chlorohydrins and 4.00% for the diols. If unknown samples are studied in the same concentration, any increase in absorbance at 2.795 or 2.792 μ, after hydrochloric or acetic acid treatment, respectively, may be interpolated directly on the appropriate calibration line to obtain the epoxide concentration either as per cent epoxyoleic acid or as per cent oxirane oxygen. This was the method used to obtain the values reported in Table 1.

Estimation of epoxides via the diols is, for several reasons, unsatisfactory. Free acids must be removed from the oil since they would otherwise be measured in the natural sample but not in the esters resulting from treatment. Their removal, however, may mean the loss of some epoxy material present as free acids. Furthermore, free acids are not the only constituents that will give hydroxyl absorption in the natural oil but will be removed during the hydrolysis. Mono- and diglycerides, which also have free hydroxyl groups, will be lost. These facts mean that for many oils a decrease in OH absorption will follow treatment to convert epoxy glycerides to dihydroxy esters, and small amounts of epoxy components may be overlooked because of this decrease. Although it is simple to separate triglycerides from mono- and diglycerides by silicic-acid chromatography, the separation of these components may also mean loss of some epoxy constituents.

With the chlorohydrin method the mono- and di-

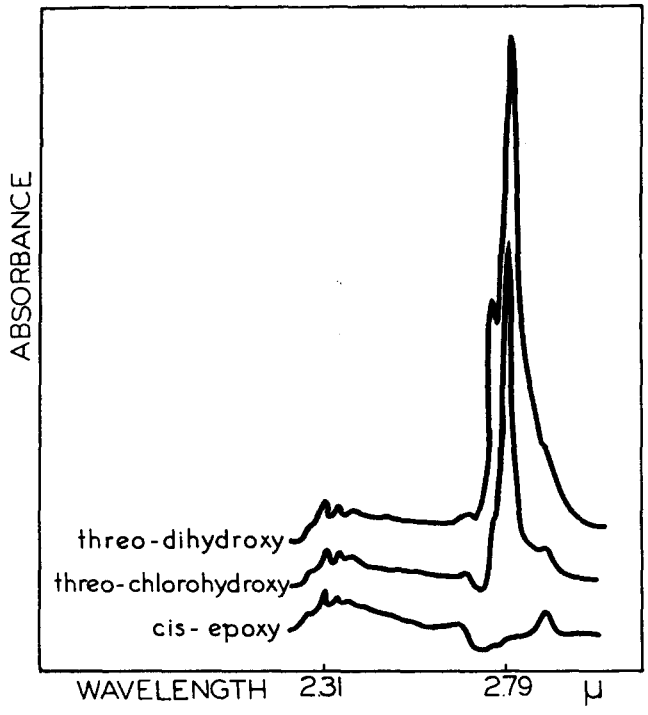

FIG. 3. Portions of near infrared spectra of methyl *cis*-12:13-epoxyoleate, methyl *threo*-12,13- and -13,12-chlorohydroxyoleate mixture and *threo*-12,13-dihydroxyoleate as 0.30% solutions in carbon tetrachloride. Molar absorptivity at 2.795 μ of chlorohydroxyoleate, calculated from this curve, is 58.5 and molar absorptivity at 2.792 μ of dihydroxyoleate is 70.1.

TABLE 1. EPOXY CONTENT OF SEED OILS BY NEAR INFRARED SPECTROPHOTOMETRY OF THEIR CHLOROHYDRINS

Plant Family	Species (Common Name)	As Epoxy-oleic Acid	Oxirane Oxygen
		per cent	*per cent*
Apocynaceae	*Strophanthus sarmentosus*	0.0	0.00
Bombacaceae	*Ceiba acuminata*	0.0	0.00
Compositae	*Carthannus tinctorius* (safflower)	0.0	0.00
	Cosmos bipinnatus (cosmos)	3.2	0.17
	Helianthus annus (sunflower)	1.9	0.10
	Helianthus var. Improved Dakota	0.4	0.02
	Helianthus var. Giant Grey Stripe	0.5	0.03
	Helianthus var. Double Dwarf Sungold	0.7	0.04
	Chrysanthemum (Ann Merry mixture)	1.2	0.06
	Heliopsis scabra zinnaeflora	0.2	0.01
	Heliopsis pitcheriana	1.0	0.05
	Artemisia absinthium (wormwood)	14.9*	0.81*
	Cynara cardunculus (Argentine thistle)	1.1	0.06
	Dimorphotheca aurantiaca (Cape marigold)	0.6*	0.03*
	Vernonia anthelmintica (purple fleabane)	60.2	3.23
Cruciferae	*Brassica campestris* (rape)	0.0	0.00
Cucurbitaceae	*Apodenthria undulata*	0.7	0.04
Euphorbiaceae	*Cephalocroton cordofanus*	54.8	2.94
	Sapium sebiferum (stillingia)	0.0	0.00
Gramineae	*Zea Mays* (corn)	0.0	0.00
Leguminosae	*Calliandra eriophylla* (fairy dusters)	5.9*	0.32*
Linaceae	*Linum usitatissimum* (linseed)	0.0	0.00
Malvaceae	*Hibiscus cannabinus* (kenaph)	1.8	0.10
Oleaceae	*Olea europea sativa* (olive)	0.0	0.00
Punicaceae	*Punicum granatum* (pomegranate)	0.0	0.00
Sterculiaceae	*Brachychiton acerifolius*	0.2	0.01
Umbelliferae	*Petroselinum sativum* (parsley)	0.0	0.00
Zygophyllaceae	*Balanites aegyptica* (lalob)	3.5	0.19

* These values were estimated because the presence of conjugated diene hydroxy acid made direct measurement impossible.

glycerides, free acids, etc., remain intact. Only epoxy and a few other groups react with hydrogen chloride to any extent, and only epoxides generate new hydroxyl groups on such treatment. Moreover, the hydroxyl group of a chlorohydrin is strongly hydrogen-bonded to the chlorine and the resultant peak at 2.795 μ is generally well separated from normal hydroxyl absorption near 2.76 μ, which does not interfere.

Some oils were studied by the diol method, and the results obtained (Table 2) are in fairly good agreement with those resulting from conversion to chlorohydrins. For reasons stated above, however, the diol method was abandoned in favor of the chlorohydrin method, which was used for most of this work.

DISCUSSION

It was found that the spectra of methyl oleate, of methyl linoleate, and of nine seed oils were unchanged by the action of hydrogen chloride. Thus, increased hydroxyl absorption does not result from reaction with HCl unless epoxy compounds are present.

The difference between the spectra of *threo-* and *erythro-*chlorohydrins, produced by HCl treatment of *cis-* and *trans-*epoxides, respectively, means that the method of determination, as outlined here, is limited to mixtures containing *cis-*epoxides. Since no *trans-*epoxy acid has as yet been shown to occur naturally, the utility of the method for studies of natural oils or epoxidized natural oils is not affected by this limitation. Autoxidation mixtures, however, may contain both *cis-* and *trans-*epoxides and the differences in the spectra of the chlorohydrins from these must be borne in mind in any quantitative determination on such mixtures.

Comparison of values obtained by this method and by the methods of Durbetaki and of Swern (Table 2) shows that the last two methods give values which are higher than ours in many instances. The more reactive hydrogen bromide reagent of Durbetaki, in most cases, gives values showing the greatest difference from ours. Many oils are known to contain acids which interfere in conventional epoxide determinations (5). *Dimorphotheca* oil contains a high proportion of 9-hydroxy-*trans*-*trans*-10,12-octadecadienoic acid (21),

TABLE 2. COMPARISON OF RESULTS OBTAINED BY VARIOUS METHODS OF EPOXIDE DETERMINATION

Oil	Epoxide Content as Per Cent Epoxyoleic Acid			
	I.R. (Chloro-hydrins)	I.R. (Diols)	Dur-betaki*	Swern
Vernonia	60.2			63.1
Cephalocroton	54.8			57.2
Cynara	1.1	1.0	1.5	
Apodenthria	0.7			1.2
Stillingia	0.0			0.3
Hibiscus	1.8		4.8	2.9
Ceiba	0.0		1.7	2.0
Brachychiton	0.2		11.0	6.0
Cosmos	3.2	3.2	6.6	7.2
Helianthus	1.9		3.1	9.5
Artemisia	14.9†	15.0†	23.0	20.1
Dimorphotheca	0.6†	1.5†	52.0	30.7
Balanites	3.5		7.9	5.5
Calliandra	5.9†	7.6†	11.0	8.3
Heliopsis pitcheriana	1.0			2.1
Chrysanthemum	1.2			3.1

* Determinations using this method, on the same seed oils, were carried out by F. R. Earle's group at the U.S. Department of Agriculture, Northern Regional Laboratory, Peoria, Ill.

† These values were estimated.

and we have shown (22) that isomers of this acid are the cause of high epoxide values given by the oils *Artemisia, Cosmos* and *Helianthus* (family Compositae), *Calliandra* (Leguminosae), and *Balanites* (Zygophyllaceae). This particular type of reactive acid, containing a vicinally unsaturated hydroxyl structure, loses hydroxyl on treatment with hydrogen halides in organic solvents. The decreased intensity of the unassociated hydroxyl band at 2.762 μ after such treatment enables the concentration of such acids to be estimated concurrently with any epoxy components by the chlorohydrin method here described (22).

When both epoxy and reactive hydroxy acids were present together in an oil and one or the other was in high concentration ($>$ 10%), the large changes in absorption in the 2.8 μ region after HCl treatment made a direct measurement of their concentrations impossible. In these cases concentrations were estimated, making allowances for the interfering bands. The patterns of the interfering bands were constructed, with reference to the absorption curves of other oils showing similar absorption at 2.83 and 2.88 μ. The amounts of absorption due to reactive hydroxy acid in the original oil sample and caused by chlorohydrins in the acid treated sample were then estimated. Although these estimations were carried out on this rather arbitrary basis, the values so obtained for epoxy acid content and for diene hydroxy acid content were in good agreement with the values obtained by other methods (22). The ease of qualitative detection of one such acid in the presence of the other is in no way impaired, since their hydroxyl absorption bands are clearly resolved. In addition to the above six oils, where we have verified the presence of these acids by chromatographic and spectrophotometric studies (22), measurements indicate that *Heliopsis pitcheriana* and the mixed *Chrysanthemum* sample (both of the Compositae) contain a vicinally unsaturated hydroxy acid in a concentration of 0.8% and 0.6%, respectively.

Members of the families Sterculiaceae, Malvaceae, and Bombacaceae contain cyclopropene acids (23, 24, 25) which are reactive toward hydrogen halides. Acids of this type are probably responsible for all the apparent epoxide value of the *Ceiba* oil and for most of it in the *Brachychiton* and *Hibiscus* oils, as determined by the Durbetaki method.

These results demonstrate that the method for the detection and estimation of epoxy components by near infrared spectrophotometry of the chlorohydrins has much greater specificity and sensitivity than the methods in general use. Much smaller samples are required and the procedure is simple and rapid.

We are grateful for the co-operation of F. R. Earle, of the U.S. Department of Agriculture, Northern Regional Laboratory, Peoria, Illinois, in providing us with samples of many of the oils studied and in making available to us the results of epoxide determinations by the Durbetaki method on these oils. We also wish to thank S. B. Penick and Company, New York, for their gift of *Vernonia anthelmintica* seeds. The technical assistance of G. C. Miller is gratefully acknowledged.

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