SYMPOSIUM: ANALYSIS OF UNUSUAL AND MINOR CONSTITUENTS. PART I

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Effect of Unusual Acids on Selected Seed Oil Analyses[1]

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Abstract

In the course of chemical compositional studies on seed oils from more than 2600 species of uncultivated plants, a number of new acids were isolated and structurally characterized at our laboratory. Also included in the studies were many fatty acids previously identified but not widely distributed or readily available. Oils containing unusual functional groups often gave unexpected responses to analytical procedures in frequent use. To exemplify the analytical results, data are presented relative to behavior of oils containing oxygenated fatty acids, cyclopropene rings, unusual types and positions of unsaturation, differing chain length, and sundry combinations of these, in the following procedures: Determination of polyunsaturated acids by ultraviolet spectrophotometry; iodine value versus refractive index plot to single out unusual oils; oxirane oxygen by HBr titration; gas-liquid chromatography; infrared, and nuclear magnetic resonance spectroscopy; and lipoxidase assay. Preliminary experimentation on an automatic analytical hydrogenation apparatus indicates good potential, in its aplication to both usual and unusual oils, for a rapid determination of total unsaturation with acceptable accuracy and precision.

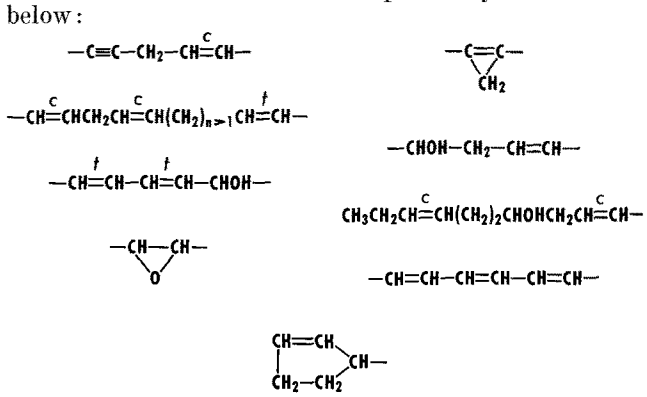
Introduction

ABOUT SEVEN YEARS ago the Department of Agriculture initiated research directed toward the discovery and development of new oilseeds, having chemical composition different (12,16) from that of domestic oilseeds presently available. Since then the presence was demonstrated in seed oils of about a dozen previously unknown fatty acids of novel structure, a number of which comprise a major percentage of their respective seed oil glycerides.

As a result of these researches a variety of new fatty acids, and a number of previously identified acids, but ones which are not widely distributed, readily available, or usually found in common seed oils of commerce were investigated to observe their response to selected analytical procedures used frequently for examining seed oils. Examples selected for this report reflect the dependence of analytical results on the chemical structures and functional groups in the seed oil. In many cases anomalies or deviations from expected answers emphasize limitations of particular methods and reinforce the cautions suggested for their routine application to unknown raw materials. Also described are our preliminary results with the automatic laboratory hydrogenation apparatus of Brown et al. (8) of Purdue University with a number of different types of seed oil samples.

The research summarized draws heavily on the results of M. O. Bagby, F. R. Earle, K. L. Mikolajczak, C. R. Smith, Jr., and their co-workers at the Northern Utilization Research and Development Division.

The structures concerned are primarily those shown below:



[1] Honorable mention, Bond Award, Fall, 1964.
[2] No. Utiliz. Res. and Dev. Div., ARS, USDA.

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Polyunsaturated Acids by Ultraviolet Spectrophotometry

The empirical procedure of alkaline isomerization of oils followed by spectrophotometric determination of conjugated components (1a) is used less frequently for determining the fatty acid composition of glycerides since the advent of gas-liquid chromatography (GLC). Comparison of these two approaches by various investigators (19) has revealed deficiencies of the UV method. Because the UV procedure has been widely used and considered historically to be of fairly general applicability to natural oils containing polyunsaturation, some recent experiences with its use seem worth reviewing.

Oil from *Crepis foetida,* a sunflower family plant similar to the dandelion and native to Turkey, according to UV analysis after 25 min isomerization in 6.6% potassium hydroxide, contains 88% linolenic acid. There was a peak at 236mμ but none at 233mμ; calculation of linoleic acid in the usual fashion (from value at either wavelength) provided a negative value. After 3 min in alkali the absorption was equivalent to 142%of linolenic acid in the original sample (at 268μ). However, GLC analysis (Fig. 1) of methyl esters from the oil revealed no linolenic acid and 28% of linoleic acid. Also present was a peak representing about 60% of a component having a retention position which did not match that of any of the common, naturally occurring, fatty acids. When an iodine value (I.V.) was calculated from the GLC analysis, assuming that the unknown peak was a nonconjugated diene, the value of 150 was in close agreement with the experimentally determined figure of 154. After the structure of the previously unknown *C. foetida* acid was shown to have the methylene-interrupted enyne grouping (27) (Fig. 1) and after the purified acid was found to undergo a facile rearrangement in good yield to a conjugated triene under the influence of alkali, the spectrophotometric behavior of the oil was readily explained. Likewise, I.V.'s become reconciled since acetylenic bonds reportedly (25) absorb only 1 mole of halogen under analytical conditions.

A second seed oil which showed an apparent anomaly in the UV procedure for polyunsaturated fatty acids was that from *Thalictrum polycarpum,* an uncultivated perennial herb native to the West Coast of the United States and sometimes called meadow-rue. The value of −35% for the amount of saturated acids in *T. polycarpum* oil (11), calculated from experimental data, obviously involved factors beyond the summation of minor errors and represented gross inapplicability of the procedure. The method failed because two double bonds in a major trienoic fatty acid constituent

$$CH_3(CH_2)_4\overset{c}{C}H{=}CHCH_2\overset{c}{C}H{=}\overset{t}{C}H(CH_2)_2CH{=}CH(CH_2)_3COOH$$

were methylene-interrupted while a third olefinic bond was removed from the other pair by two methylene groups and was isomerized to conjugated form in a negligible amount by alkali under normal conditions (4). Similar instances have been found in our investigations of other natural oils containing isolated unsaturation (3,5).

UV examination of seed oils has of course been routinely used and found to be most helpful in detecting and determining preformed conjugation of various types.

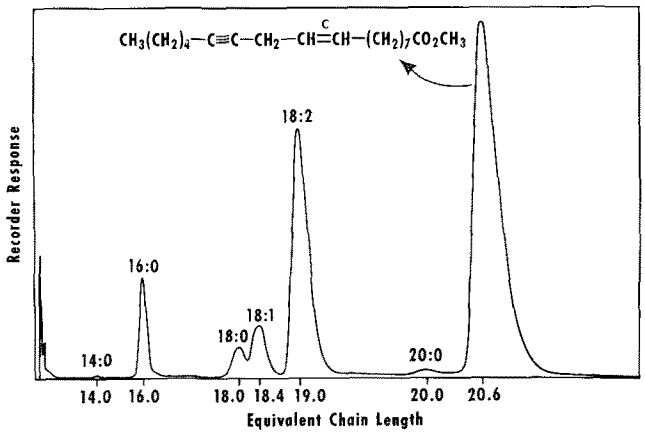


FIG. 1. GLC of *Crepis foetida* methyl esters on 125 x 0.3 cm polyester column (20% LAC-2-R 446 on 60-80 mesh Celite) at 190C.

Iodine Value and Refractive Index

A useful qualitative analytical device, which has permitted the detection of certain unusual lipids, has been the relation between I.V. and refractive index of the oil as compared with a standard regression line (Fig. 2). Constants for oils containing various proportions of only oleic, linoleic, linolenic, and the usual saturated acids fall on a straight line. A group of 70 assorted oils provided a standard line almost identical to that obtained earlier for groups of soybean and linseed oils (11). No significant influence on the oil constants was conferred by variations in constituent fatty acids with respect to position or geometric isomerism of unsaturation, and the presence of cyclopropene or cyclopentene rings caused only slight displacement. In contrast, points farther off the line resulted from presence of conjugated unsaturation, of hydroxyl or epoxide groupings, of isolated acetylenic linkages, or of greater-than-usual quantities of unsaponifiable substances. Carbon chain

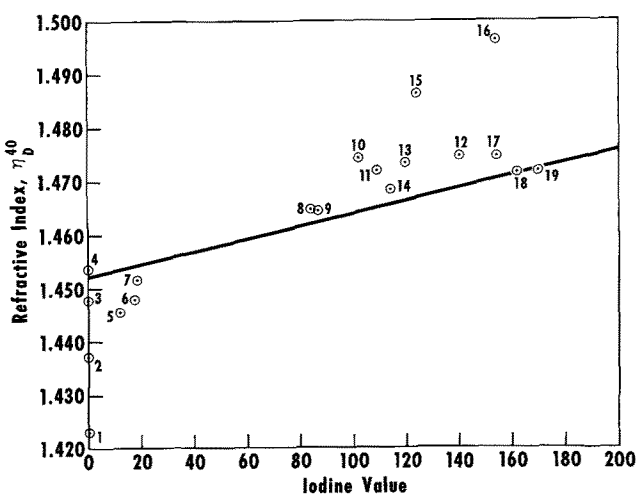


FIG. 2. Iodine value versus refractive index of oils. Numbered points refer to the following triglyceride or crude seed oil samples: (1) Triacetin; (2) Tricaproin; (3) Trilaurin; (4) Tristearin; (5) *Cuphea painteri,* mostly $C_{8:0}$; (6) *Cuphea llavea* mostly $C_{10:0}$; (7) *Cuphea carthagenensis,* mostly $C_{12:0}$; (8) *Sterculia foetida,* cyclopropenes; (9) *Limnanthes douglasii,* mostly C_{20} and C_{22}; (10) *Vernonia anthelmintica,* epoxy; (11) *Lesquerella fendleri,* hydroxy C_{20} monoene; (12) *Lesquerella stonensis,* hydroxyl between olefinic bonds; (13) *Penstemon spectabilis,* high in unsaponifiables; (14) *Flacourtia indica,* cyclopentene; (15) *Dimorphotheca sinuata, alpha*-hydroxy conjugated diene; (16) *Calendula officinalis,* conjugated triene; (17) *Crepis foetida,* nonconjugated enyne; (18) *Symphytum officinale,* 6,9,12-triene; (19) *Thalictrum polycarpum,* isolated *trans.*

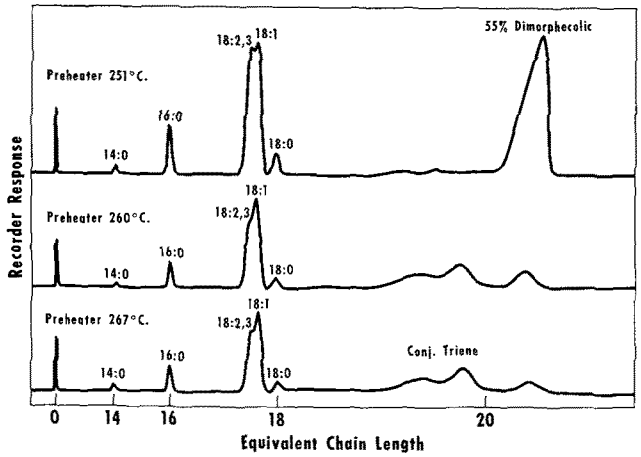


FIG. 3. GLC's of *Dimorphotheca sinuata* methyl esters at three preheater temps. Apiezon L column at 248–250C with 5–7-μl samples.

length affects refractive index, as shown in Figure 2, in that oils or glycerides containing primarily shorter chain acids give points below the line and longer chain acids lead to points above the standard curve. The effect is, however, not large even when the predominant acids in the glycerides vary over the wide range from 12 to 22 carbon atoms.

Although this procedure has been helpful in sorting out oils of unusual composition, quite apparent are its limitations in applicability to selected groupings, and even then only as a rough qualitative empirical guide.

Oxirane Oxygen

The tentative AOCS procedure for determination of oxirane oxygen (1b) by titration at ambient temp with HBr in benzene-glacial acetic acid lacks the specificity required when the analysis is applied to a large variety of seed oils.

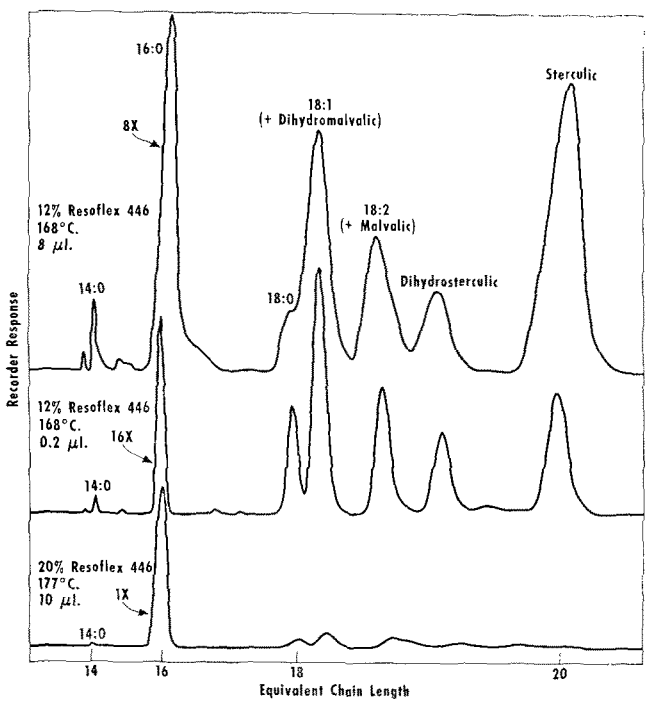
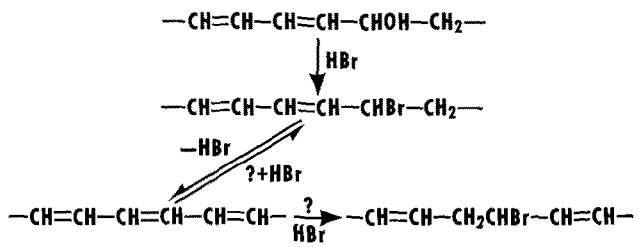


FIG. 4. GLC of *Bombax oleagineum* methyl esters showing effect of liquid phase level and sample size on apparent sterculic acid content. Column, 0.6 x 200 cm; solid support, Celite 545 60-80 mesh, acid washed, base neutralized, and acetone and hexane extracted.

Among the structures, other than epoxides, which absorb approximately stoichiometric amounts of HBr are the α-hydroxy conjugated diene grouping, as found in dimorphecolic acid, and the cyclopropenoid system of sterculic and malvalic acids. Studies of the former acid and of synthetic model compounds having the same functional grouping, carried out by Lohmar and co-workers (21) at the Northern Laboratory, showed that initial uptake of HBr during rapid titaration, in which 1 mole of HBr is taken up per mole of compound, was caused by a replacement reaction since essentially all the dienoid absorption is preserved immediately after titration. Subsequently



on standing, HBr is lost as triene is formed from a portion of the diene. The course of the reaction is not fully known, but possibly there is some readdition of HBr as shown above.

One procedure to determine oxirane oxygen in oils containing also cyclopropenoid acids involves utilization of the HBr titration procedure before and after reduction by lithium aluminum hydride (35). This reagent reduces epoxides, but has no effect on cyclopropenes or α-hydroxy conjugated dienes. Difference in HBr absorption before and after reduction is therefore a measure of oxirane oxygen, assuming of course that other unknown HBr-reactive substances are absent. Dimorphecolic acid may, if present, be determined quantitatively by UV spectroscopy. Preliminary evidence suggests that some seed lipids contain unsaponifiable fractions which also react with significant amounts of HBr.

Recently, additional procedures for using halogen acids analytically to estimate epoxides and cyclopropenes when they co-occur have been reported (15,24). One of these (24) involves stoichiometric addition of HCl followed by a quantitative determination of chlorine, with prior removal of epoxides, if present, by a pretreatment procedure. A more elegant method (15), yet simpler, permits the selective titration of epoxides in the presence of cyclopropenes at low temp (3C), whereas both classes of compounds react with HBr at higher temp (55C). Thus, composition of a mixture can be evaluated by an initial titration to a cold end point, and continuation with the same sample to the 55C end point.

Gas-Liquid Chromatography

Though GLC has brought to the oil chemist a more useful and versatile analytical tool than any which preceded it, several examples will suffice to show some limitations of this technique in application to many types of samples. Figure 3 shows the effect of preheater temp on the analysis of an oil containing dimorphecolic acid. At temps near 270C dehydration to conjugated triene is extensive; at 260C almost as much triene is formed, the dimorphecolic ester peak representing only about 5% of the amount present. Near 250C most of the methyl dimorphecolate appears at its own peak position with lesser evidence of dehydration, with about 10% of peak area between equivalent chain length (ECL) 19.0 and 20.0. Ob-

viously, the analysis of *Dimorphotheca* and related oils requires special care in the selection or development of suitable GLC operating conditions, and the possibility of some dehydration makes verification of the results by an independent procedure most desirable.

Complications also arise in GLC analysis of oils containing cyclopropenoid acids. Methyl linoleate and methyl malvalate are either unresolved or incompletely resolved on both nonpolar and polar columns. Such difficulties have been circumvented (38,40) by hydrogenating oils or fatty acid mixtures having cyclopropenes to mixtures containing cyclopropanes and methyl-branched substances. Such hydrogenation and hydrogenolysis products were separable by GLC, and permitted calculations of fatty acid composition. A similar procedure was later adopted by Cornelius and Shone (10). Miwa (28) has reviewed the complexity of such systems particularly when malvalic acid co-occurs with large amounts of sterculic acid.

The GLC analysis of cyclopropenoid acids appears to be greatly affected by operating conditions, interalia by level of liquid phase on the support, size of injected sample, and column temp in the range of 170-220C. The curves in Figure 4 show the effects of three sets of GLC operating conditions on the analysis of methyl esters from seed oil of *Bombax oleagineum*, which is reported to contain about 58% palmitic acid and 27% of cyclopropenoid acids (22% sterculic, 5% malvalic) (10). The bottom curve was obtained under conditions frequently used, employing an instrument with a thermal conductivity detector. No distinct peak for sterculic acid is seen; most of it is apparently retained in the column. However, at a level of 12% instead of 20% of stationary phase, an injection of similar size provided a well-developed peak which represents an amount of sterculic acid comparable to that determined by other methods. In this determination the flame-ionization detector was used.

The center curve shows that injection of smaller samples can also lead to erroneously low results. The relationship between amount of sample added and the calculated percentage of sterculic acid from the resulting chromatogram under selected operating conditions is shown further in Figure 5. With increase in sample size in this series the percentage of sterculic acid became greater until it approached 17-18%. There was probably a holdup of sterculate ester on the column, which percentagewise is less significant with the larger samples. Interchange of flame ionization and thermal conductivity detectors or glass and copper columns showed no significant differences in the results when larger samples were used. The holdup of methyl sterculate in the column is of less significance at 220C even at a sample size of 0.2 μl. However, at this temp the polyester liquid phase bleeds extensively, and except for short durations, temps above 200C were not employed. At temps 200C or less, conditioning of the 20% polyester column by injection of several large doses of this sterculate-containing sample did not "saturate" the column, since no changes could be observed in its behavior.

These observations show the large effects of operating variables on attempted GLC analysis of cyclopropenoid fatty esters and suggest caution by investigators who obtain and interpret such data. This is especially so since sterculate apparently does not emerge unchanged from the GLC analysis.

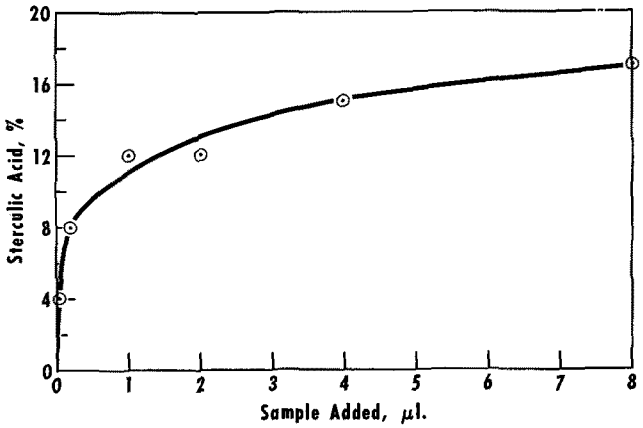


FIG. 5. Variation in sterculic acid content of *Bombax oleagineum* methyl esters according to size of injected sample: 12% Resoflex 446 polyester on 60-80 mesh Celite 545, 0.6 x 200 cm copper column at 168C, flame ionization detector.

In the experiment correlating sterculic acid content with sample size, although the apparent sterculic acid content changed, the percentage of presumed dihydrosterculic acid shown on the chromatogram as the peak just before sterculic acid in Figure 4 remained constant at 4%. This constant value suggests that dihydrosterculic acid is present in the methyl ester preparation from the original oil and is not a conversion product formed in the course of GLC analysis. The sum of dihydrosterculic and sterculic acid is 21% at an injection of 8 μl. This figure is close to the 22% for sterculic acid only in the *Bombax* oil as reported by Cornelius and Shone (10). These workers, however, had determined the sterculic content by GLC analysis of its hydrogenation products, using the assumption that no dihydrosterculic was originally present.

As a final example to illustrate the caution required in interpretation of GLC data, Mikolajczak and Bagby (26) purified a sample of β-eleostearic acid and collected its methyl esters after passage through a GLC polyester column (20% LAC-2-R 446 on 60-80 mesh Celite) at 195C. In addition to the geometric isomerization previously reported by others (30), they found that, although the conjugated triene system was largely preserved intact, extensive bond migration took place. Only 43% of the bonds were in their original position, with 6% of the trienoid system isomerized in position so that it began at C-7, 18.5% at C-8, 25% at C-10, and 7% at C-11.

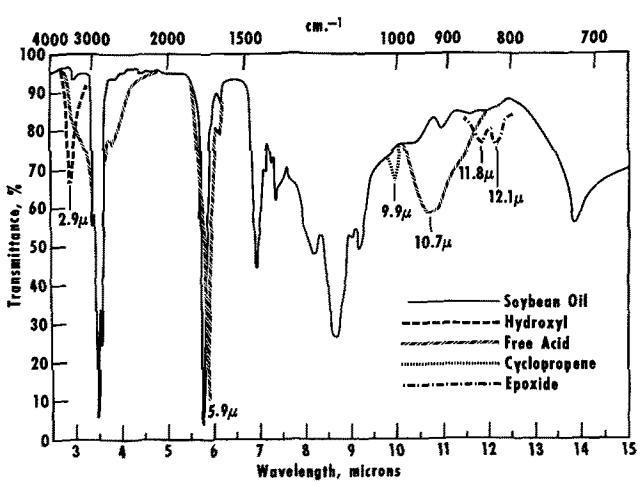


FIG. 6. Expected IR spectral bands in oils having hydroxyl, free acid, cyclopropene, or epoxide groups.

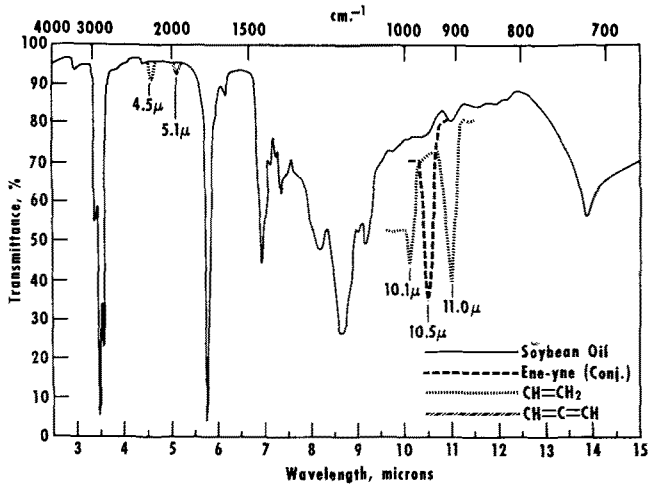


FIG. 7. Expected IR spectral bands in oils having conjugated enyne, terminal olefin, or allene groups.

Infrared Spectra

Infrared spectroscopy is used regularly for detecting in oils the presence of less common structures or functional groups. The extensive reviews of O'Connor (32,33) and others provide background for interpreting the useful correlations between specific structures and the spectra that result from them.

Spectra of crude seed oils in the current survey were obtained on each sample from thin liquid films on either silver chloride or sodium chloride plates. The largest proportion of the oils provides a spectrum much like that of soybean oil or other vegetable oils which contain only the most commonly occurring saturated, monoenoic, and polyunsaturated fatty acids and relatively small concentrations of nonglyceridic constituents (Fig. 6). Superimposed in the figure are portions of spectra which represent the type of deviation introduced by the respective functions exemplified. The characteristic hydroxyl band at about 2.9 μ due to O-H stretching and the carbonyl band at 5.9 μ are both also in spectral areas affected when large quantities of free acid are present. Free acid is regarded as present when the bonded OH-stretching vibration causes a broad band at 2.8–4.0 μ. Oftentimes the carbonyl stretching vibration at 5.9 μ is evident due to the presence of a high ratio of free acid. The OH-deformation (out of plane) band at 10.7 μ is additional evidence for the presence of free acid. In a synthetic mixture, 5% of free fatty acid

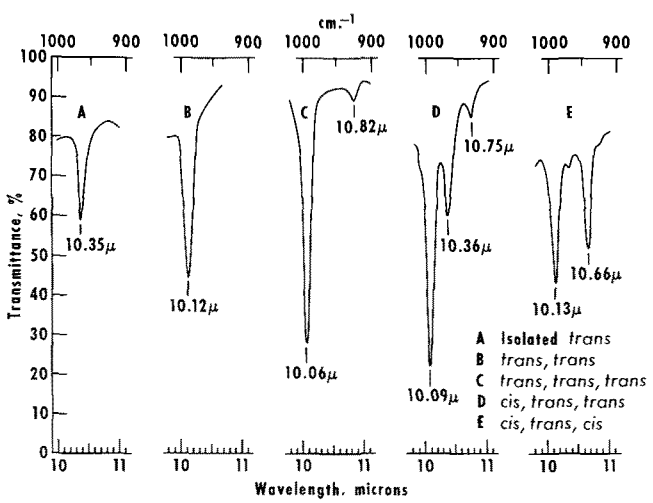


FIG. 8. IR bands in oils containing *trans* unsaturation.

in the glycerides can obscure the presence of up to 20% licanic acid. The respective bands illustrated are those revealed by the presence of substantial quantities of the causative functional groups: i.e., about 50-70% hydroxyl, 60% free acid, 60% epoxy acid, and 14% of cyclopropene in the crude oils. Aditional weaker bands, such as one for the cyclopropenoid grouping at 5.35-5.4 μ, can be demonstrated when purified compounds or fractions are available but are of lesser value in the examination of whole oils. In a similar fashion, Figure 7 shows, also with a soybean oil curve for reference, expected deviations when conjugated enynes, terminal unsaturation, and allenes are present. The latter group has not previously been demonstrated in seed oils, but current research at this Laboratory is providing strong evidence that it is present (6). Isolated disubstituted acetylenic functions, such as the 12,13-bond in crepenync (*cis*-9-octadecen-12-ynoic) acid, do not show characteristic IR bands. IR spectra have been helpful in defining various types of geometric isomerism (Fig. 8) again following literature precedents, and quantitation is useful especially with pure compounds. However, many seed oils, such as those from the family Umbelliferae, have qualitatively different spectra (Fig. 9) and numerous bands not readily assignable to specific functions. Such oils usually contain substantial amounts of unsaponifiable matter, including essential oils.

Proton Magnetic Resonance Spectra

The usefulness of proton magnetic resonance spectra in lipid analysis has been clearly indicated by Storey (39), Hopkins (17), and others. The quantitative nature of the nuclear magnetic resonance (NMR) data, considered with information derived from chemical shifts and coupling effects due to proton environment created by different functional groups, makes it an extremely helpful technique for predicting and confirming structures. For example, Hopkins (17) has shown how it may be profitably applied and interpreted in terms of chemical structures present in the case of cyclopropene and cyclopropane acids, epoxides, esters, ethers, olefins whether conjugated or not, terminal acetylenic or vinyl groups, and branched chains. Storey (39) demonstrated specific application of NMR to the analysis of mixtures of linolenic, linoleic, oleic, and stearic acids, and he indicated the characteristic effect of 15,16-unsaturation in a long-chain fatty acid on its NMR spectrum. NMR spectroscopy has repeatedly been helpful to us (14,27,36) in conjunction with structural problems and analyses of both unusual and usual acids of seed oils.

Figure 10 shows the NMR spectrum of an acid (as its methyl ester) of unusual structure from the seed oil of *Calea urticaefolia*, a Mexican plant of the sunflower family. The acid has been identified as *trans*-3, *cis*-9, *cis*-12-octadecatrienoic acid (3). Also represented on the same figure for comparison are typical bands from spectra of other fatty acids depicting protons analogous to those of the *C. urticaefolia* acid in functionality, but displaced or having a different shape because of proton environment or interactions with other protons.

Effects of unsaturation at the 3,4-position in the *C. urticaefolia* acid merit particular attention. In Figure 10 the doublet (C) at 7.04 τ, resulting from protons on the carbon *alpha* to both the carboxyl group and an olefinic carbon, represents two hydrogen atoms that would usually be on the curve as a triplet

at 7.78 τ (H) since they are affected only by the carboxyl group in compounds such as stearate, oleate, and linoleate (39). Olefinic protons on carbons 3 and 4 are apparently in a sufficiently different environment from those in oleate, linoleate, and linolenate that a multiplet (A) at 4.63 τ replaces the triplet (J) at 4.72 τ usually associated with these olefinic protons. The methylene protons of the 1,4-pentadiene structure in the *C. urticaefolia* acid, linoleate, or linolenate cause a triplet at 7.25 τ (D). The methylene of a 1,4-pentaenyne structure present in a naturally occurring acetylenic acid, *cis*-9-octadecen-12-ynoic (crepenynic) acid (27) indicated a slight deshielding by causing a rather diffuse doublet (K) at 7.16 τ. The doublet (E) at 7.95 τ is the chemical shift band of the methylene protons *alpha* to the unsaturation, and the band (F) at 8.68 τ is due to the shielded methylene protons. Shielded terminal methyl protons show a triplet (G) at 9.10 τ and a broad valley at about 8.9 τ between the chemical shifts for shielded methylenes and shielded methyls. However, when a compound has a methyl *beta* to unsaturation as in methyl linolenate (39) and methyl densipolate (26), the chemical shift shows a slight deshielding effect. The triplet (L) at 9.05 τ is better defined, and it has been possible to use the low-field peak as a quantitative measure of *beta*-methyl protons in mixtures of acids (14,39). Integration of the NMR curve for the *C. urticaefolia* acid provides the numbers and types of protons consistent with the structure represented in Figure 10.

Lipoxidase Procedure

While the lipoxidase procedure developed by MacGee (22) for determining the presence of *cis, cis* methylene-interrupted unsaturation is not applied to all oil samples surveyed analytically, it has been profitably used on numerous occasions in our Laboratory on acids having an unusual structure. Applications have included both demonstration of the absence (5,36) of the *cis,cis* methylene-interrupted unsaturation and its presence (3,4) in trienoic acids containing, in addition, isolated *trans* unsaturation.

Degree of Unsaturation by Hydrogenation

Vegetable oil chemists have always been interested in determining the degree of unsaturation of their raw materials because of its importance in catagorizing their drying properties, in assessing extent of commercial hydrogenation, and in assessing numerous other oil characteristics. A variety of quantitative procedures has been suggested for this determination, including use of (most frequently) halogens, ozone (23), hypochlorous acid (9,31), hydrogen, and various physical methods. The reviews of Allen (2) and of Bolley (7) include a more detailed discussion of procedures, and describe some of their advantages and limitations. The use of quantitative hydrogenation is commonly reported to give good results for total unsaturation which are more reliable than usual halogen addition methods, particularly on oils having structures such as conjugated unsaturation. However, most laboratory hydrogenation methods are limited for widespread usage because of the complexity of their apparatus and procedures, lack of reproducibility in different laboratories, or time and care required in the determination (2,7). Since some of these objections seemed to be of less consequence in the hydrogenator proposed by Brown et al. (8), this apparatus was tested on a number of oils. The results are briefly reviewed in this report, leaving detailed description of the experiments for a subsequent paper (29).

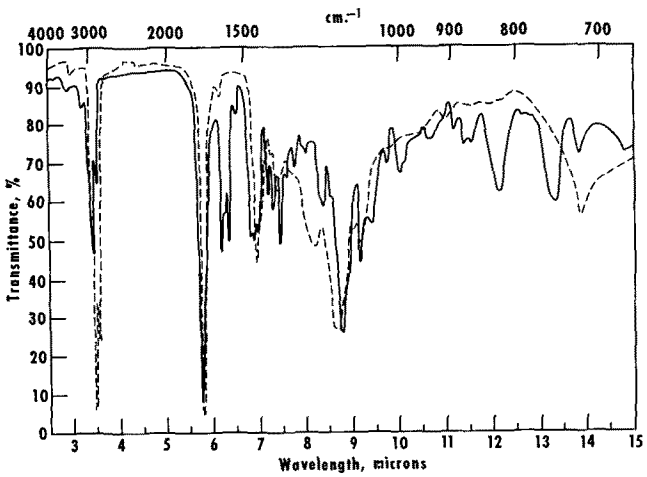
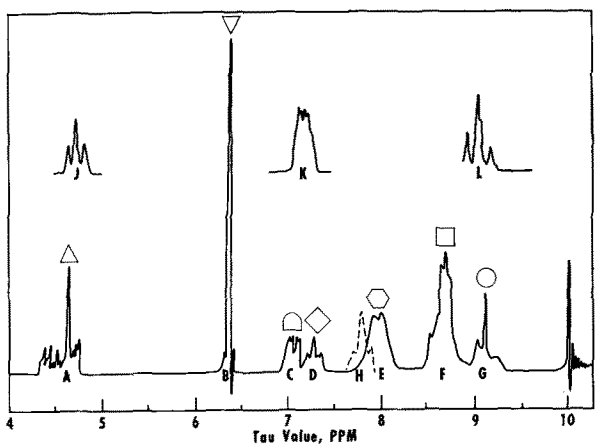
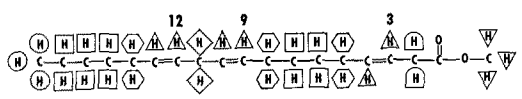


FIG. 9. IR spectrum typical of many Umbelliferae (species shown *Angelica ampla*) oils (solid line), with soybean oil (broken line) shown for comparison.

The apparatus (Fig. 11) consists of a flask for the in situ preparation of catalyst and generation of hydrogen, connected to a refillable buret containing standardized borohydride solution and to a mercury manometer. Between the buret tip and the flask is a mercury seal. Samples are introduced into the closed system via a hypodermic needle through a rubber septum. Following catalyst preparation and equilibration of the system, the apparatus is ready for automatic use. Solvent in the flask contains excess acetic acid and the free space in the system contains hydrogen gas. Reactions were carried out in diglyme [bis (2-methoxyethyl) ether]-isopropanol as





Symbol	Structure	PPM τ	No. Protons
○	ω-Methyl protons	9.10	3
□	Insulated methylene protons	8.68	10
⬡	α-Olefinic methylene protons	7.95	6
◇	Di-α-olefinic methylene protons	7.25	2
⌂	α-Olefinic-α-carboxyl methylene protons	7.04	2
▽	Methoxy protons	6.36	3
△	Olefinic protons	4.63	6

FIG. 10. NMR spectral data on the methyl *trans*-3,*cis*-9,*cis*-12-octadecatrienoate from *C. urticaefolia* oil, with bands of functional groups from other acids shown for comparison with its curve (see text).

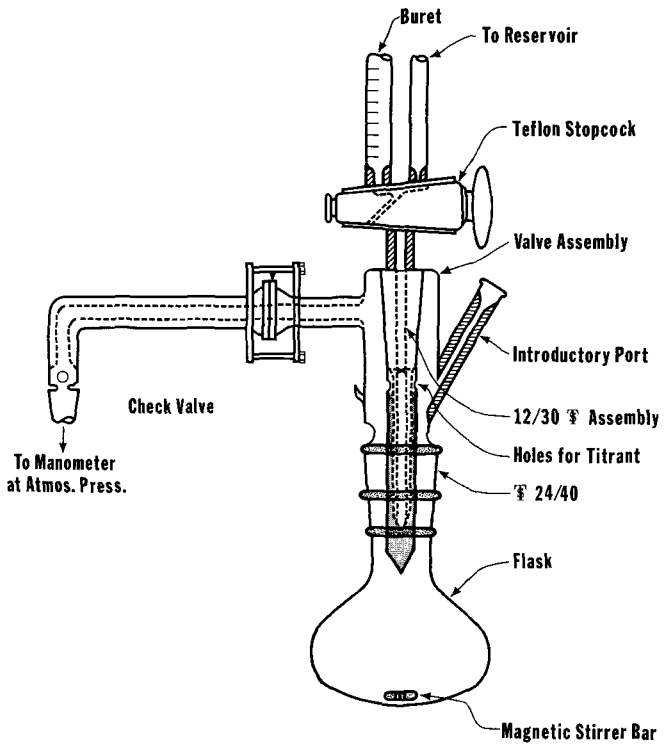


FIG. 11. The hydrogenation apparatus of Brown et al. (8).

solvent in accordance with a suggestion by Dr. H. C. Brown of Purdue University. The standard borohydride 0.05M titrant solution was in diglyme-isopropanol (1:9 v/v).

A given amt of oil (100 μl, weighed to 0.1 mg) is injected, which immediately begins to absorb hydrogen. The decrease in gas pressure creates an imbalance at the mercury seal that results in a flow of sodium borohydride reagent into the flask. The borohydride reacts with acetic acid to release hydrogen and the mercury seal is restored until the hydrogen pressure within the flask drops again. This process is automatically repeated until the sample no longer absorbs hydrogen. Straight-forward calculations based on the amt of standard sodium borohydride solution drawn in from the buret, with suitable correction factors for the volumes of sample and titrant introduced into the flask, enable determination of the "hydrogen iodine value" (iodine equivalent of the hydrogen absorption, expressed in proper units) of the sample. Borohydride solutions were standardized by injection of a known amt of pure octene-1.

Before application of the Brown procedure to a variety of oils containing unusual acids, a series of analyses was made of three oils and one standard unsaturated methyl ester, in different I.V. ranges, to ascertain precision and accuracy. A statistically replicated experimental design provided results (Table I) indicating an *SE* of approximately the same magnitude *SD* ± 4) regardless of degree of oil unsaturation. Sample size, variations in paired combinations, and degree of unsaturation did not influence the results, but there was some effect on the values caused by the order of injections, the trend being toward lower values for the first samples injected.

TABLE I

Comparative Iodine Values (I.V.) and Hydrogen Iodine Values (H.I.V.) on Various Oils Having No Unusual Acids (4 I.V. determinations per oil and 36 H.I.V. determinations at different sample sizes and sequence of injection)

Oil	H.I.V. (Standard Error)		I.V., AOCS
Crambe	87	(± 0.7)	90
Soybean	131	(± 0.7)	132
Linseed	182	(± 0.7)	180
Methyl 10-Undecenoate[a]	131	(± 0.7)	124

[a]Calcd. I.V. 128. Compound 99.8% pure

The Brown hydrogenation procedure was next applied to a variety of seed oils which contain fatty acids of less common structure. The hydroxyl-containing castor and *Lesquerella* oils (Table II) showed normal hydrogen uptake and good agreement between I.V. and H.I.V. However, values for *L. lescurii* oil show only fair agreement with that calculated on the basis of GLC composition. This discrepancy may be due either to inadequate determination of the hydroxydiene in the oil (36) by GLC or to the presence of other components yet unrecognized. For *Dimorphotheca* the I.V. value as expected was lower than H.I.V. due to incomplete halogenation of the conjugated dienoid system in the dimorphecolic acid moiety. The absence of substantial dehydration, of hydrogenolysis of dimorphecolic acid, or of any appreciable effect on the epoxide rings in the *Vernonia* and *Euphorbia* (20) oils, which are both high in vernolic acid, attests to the usefulness of the particular catalyst and procedure in assessing the degree of unsaturation of an oil. Other workers have found hydrogenolysis of functional groups (13,18,37) in long-chain fatty chains bearing α-hydroxy conjugated diene or epoxide groups over Pt and Pd on carbon catalysts in ethanol soultion at room temperature using more extended reaction times. As with *Dimorphotheca* oil, unsaturation in other systems (tung, isano, *Osyris* oils) (Table II) containing conjugated olefinic or acetylenic linkages is accurately determined by H.I.V. and most inadequately estimated by conventional I.V. procedures. In oils with a very low (*Cuphea*) or a very high (*Lithospermum*; tetraene-containing) I.V. and that contain no unusual structures, I.V. and H.I.V. and calculated values are in close agreement. In other oils—*Petroselinum, Limnanthes, Thalictrum*—agreement is not as close as may be desired. Reasons for the disparity in value have not yet been explored; but the first is known to contain large quantities of unsaponifiables, the second a predominance of C_{20} and C_{22} acids, some of which have less common position and type of unsaturation (5,34) and the third an unusual *trans* acid in substantial amt (4); and any one of these components may influence the methods employed for analysis. The seed oil from *Leonotis*, a mint, is now under investigation at the Northern Laboratory and is suspected to contain unsaturation resistant to rapid hydrogenation. The good agreement between I.V. and

TABLE II

Hydrogen Iodine Values (H.I.V.) of Seed Oils Having Unusual Fatty Acid Composition, with Iodine Values (I.V.) by Usual Procedures or as Calculated from GLC Analysis for Comparison

Species (% unusual components)		I.V. (GLC)	H.I.V.	I.V. (AOCS)
Castor	(85)	80	84	84
Lesquerella lasiocarpa	(54)	81	84	84
Lesquerella lescurii	(35)	123	137	137
Dimorphotheca species	(65)	147	152	126
Vernonia anthelmintica	(80)	88	90	99
Euphorbia lagascae	(58)	88	88	88
Isano	(82)	369	363	146
Osyris alba	(60)	195	203	113
Tung	(84)	235	248	159
Lithospermum tenuiflorum	(20)	227	225	224
Cuphea llavea	(85)	10	10	13
Petroselinum crispum	(86)	104	94	106
Limnanthes douglasii	(82)	84	78	85
Thalictrum dipterocarpum	(50)	187	180	162
Leonotis nepetaefolia	(14)	103	98	91
Sterculia foetida	(>50)		85	84

H.I.V. of the *Sterculia* oil shows that its cyclopropenoid structure is readily saturated without substantial ring-opening. As noted for epoxides and α-hydroxy conjugated dienes, other investigators (40) have also found hydrogenolysis of functional groups when cyclopropenoid acids are reduced under usual conditions involving longer times at atmospheric pressure in the presence of Adams catalyst.

Most samples reached a definite pressure end point in 1-5 min in our application of the Brown procedure, dependent in part on experimental conditions such as rate of stirring. Occasionally, however, the end point was not so well defined and the reaction tapered off toward the end, with 15-20 min required until there is no further dropwise introduction from the buret. Although further experience with this analytical method is needed for complete evaluation of its potential, we are impressed with the breadth of its applicability, the speed with which an answer may be obtained on a sample of oil, and the precision and accuracy that can be achieved if suitable care is taken in the determination.

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Determination of Polar Lipids: Quantitative Column and Thin-Layer Chromatography

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Abstract

The structures of the polar lipid classes of plants and animals are presented, their nomenclature discussed, and suggestions are presented for clarification of nomenclature. The three general types of quantitative chromatographic procedures (column chromatography, thin-layer chromatography, and combinations of column and thin-layer chromatography) available for polar lipids are reviewed and a new quantitative two-dimensional thin-layer chromatographic procedure is presented. Useful quantitative procedures employing columns of cellulose, silicic acid, silicic acid mixed with silicate, magnesium silicate, and ion exchange celluloses are presented. New findings with diethylaminoethyl cellulose columns are described. New quantitative procedures employing silicic acid, magnesium silicate, or diethylaminoethyl cellulose column chromatography with quantitative thin-layer chromatography are described.

THE PURPOSE OF THE present communication is to review the general classes of polar lipids and their nomenclature, and to present some of the chromatographic techniques available for their determination. Since the nomenclature of polar lipids is unsatisfactory in several respects, this subject is considered in detail. A new quantitative two-dimensional thin-layer chromatographic procedure is presented and two new procedures employing column chromatography (silicic acid or magnesium silicate) with quantitative thin-layer chromatography (TLC) are presented.

Lipid Classes and Nomenclature

Glycerol Lipids

There are two general groups of polar lipids: the glycerol lipids and the sphingolipids. Each of these main groups can be subdivided into subgroups: phospholipids and lipids without phosphorus. Figures 1–17 show schematically some of the classes of polar lipids. The glycerol lipids are shown in Figures 1–10. Phosphatidic acid, phosphatidyl glycerol, and diphos-