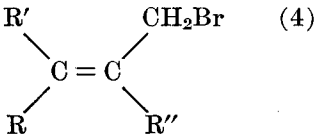

can be attributed to the presence of isomers III and IV. The presence of the characteristic band for the corresponding chloro-olefin, 7.94 μ to 8.01 μ, could not be confirmed because of an interfering triglyceride band.

The ratio of isomers I and II to isomers III and IV formed by hydrohalogenation is not known. That it is a constant for the hydrochlorination reaction, however, was shown by the reproducibility and the consistent mathematical relationship between absorptivity measurements at 11.05 μ obtained when synthetic mixtures of *Sterculia foetida* oil and corn oil were subjected to the hydrochloric acid treatment. Similar absorptivity measurements on HBr-titrated samples, on the other hand, indicated that this ratio varied from experiment to experiment.

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[Received September 28, 1962—Accepted November 13, 1962]

A Method for the Structural Analysis[1] of Triglycerides and Lecithins

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Abstract

The structural analysis of lecithins and triglycerides is described. The procedure is carried out on 2–5 mg of sample by a combination of reductive ozonolysis and thin-layer chromatography (TLC). The ozonides as well as the aldehyde "cores" derived from reduction of the ozonides are separated by TLC and analyzed quantitatively by densitometry. The constituent saturated fatty acids of the separated aldehyde "cores" are methylated and analyzed by gas-liquid chromatography (GLC). The scope of the method is demonstrated by the analysis of several synthetic and natural triglycerides and several lecithins isolated from natural sources.

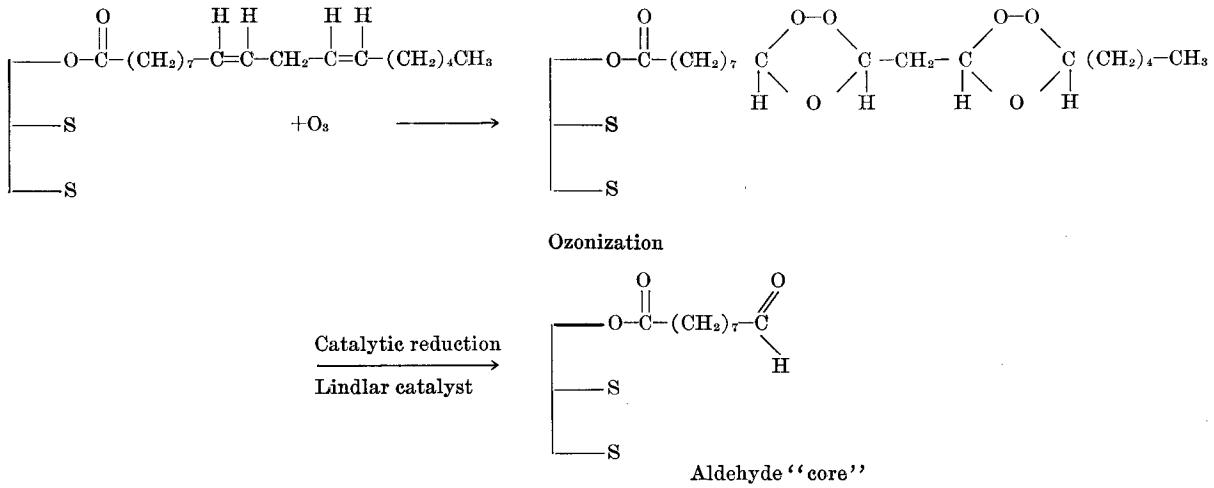
Introduction

GENERALLY, the component triglycerides of fats and oils are determined on the basis of the fatty acid composition of simple fractions. In some methods, the fractionation is carried out directly on the fats or oils (9,37,41,44,45,46), in others after oxidation of the unsaturated fatty acid constituents (20, 22,25,48). Pancreatic lipase hydrolysis, together with fatty acid analysis, has also been used to obtain information on triglyceride structure (1,6,31,32,42,43).

The position of the fatty acids in lecithin has been studied mainly by methods based on the specific action of lecithinase A (12,13,15,26,30,22,40). It was generally believed that lecithinase A was specific for the hydrolysis of the fatty acids in the α-position. However, recent evidence (16,47) indicates that the attack by this enzyme is directed to the fatty acids on the β-position. Although enzymatic methods provide information on the position of the fatty acids in both lecithin and triglycerides, they do not permit a direct determination of the positional arrangement of the fatty acids in the parent molecules.

Present methods for the structural analysis of triglycerides are laborious or time consuming, and require gram-wise amounts of material which limits their application with regard to both the number of analyses that may be performed and to samples which are available in relatively large amounts. These drawbacks have been largely eliminated in the analysis of triglycerides by a method described recently by the authors (34). Presented here are further developments in this method for triglyceride analysis and its application to the analysis of the positional arrangement of the fatty acids in lecithin.

The two basic reactions of the method are demonstrated on glyceryl-1-linoleate-2,3-distearate as follows:



[1] Supported in part by the U. S. Public Health Service, National Institutes of Health Research Grant H-5735 (C1).

The above triglyceride ozonide falls into the class having two ozonide groups; on reduction, the ozonide gives an aldehyde "core" with one aldehyde group. Evidence that the above reactions are essentially quantitative under the conditions described in the method is presented in another paper from our laboratory (35).

TLC is carried out on both the ozonides and the aldehyde "cores." The ozonides are separated on the basis of the number of ozonide group/molecule, the aldehyde "cores" are separated on the basis of the number of aldehyde groups/molecule.

Experimental

Materials

Lead-poisoned palladium catalyst. This catalyst is prepared according to the procedure of Lindlar (27) except that the final product is extracted with acetone and methylene dichloride to remove organic matter with which it is usually contaminated.

Silica Gel G (according to Stahl, Brinkmann Instruments, Inc., Great Neck, L. I., New York). The organic matter is extracted from this adsorbent with freshly distilled diethyl ether.

Solvents. All solvents are routinely dried and distilled before use.

Triglycerides. Triolein and tripalmitin were prepared by transesterification of pure methyl oleate and palmitate with triacetin using sodium methoxide as a catalyst (24). The crude product was purified by a partition between petroleum ether (B.P. 35–60C) and 90% ethanol, fractional crystallization from acetone and silicic acid column chromatography. The latter procedure was used for the purification of mono- and diglycerides as well as triglycerides and was carried out essentially as used by several investigators for the fractionation of neutral lipids (21,7,3, 11,19). In brief, about 2 g of the mixture of glycerides was separated in a 2.5 × 50 cm column of silicic acid pretreated according to Horning et al. (21). The triglyceride fraction was eluted with 5% diethyl ether in petroleum ether (B.P. 35–60C); the diglycerides were eluted next with 30% diethyl ether in petroleum ether and the monoglycerides were eluted last with diethyl ether. TLC was used to monitor the fractionation (36).

Glyceryl-2-oleate-1,3-distearate and glyceryl-1-stearate-2,3-dioleate were prepared by reaction of oleyl chloride with 1,3-distearin and 1-monostearin respectively, according to the procedure described by Hartman (17). The final products were purified by silicic acid column chromatography followed by crystallization from acetone.

Glyceryl-2-linoleate-1,3-distearate and glyceryl-2-stearate-1,3-dilinoleate were prepared by similar methods. The 1,3-diglycerides used in the above preparations were prepared via the trityl derivatives (8) and purified by silicic acid column chromatography followed by crystallization from ethanol. The monostearin was prepared by the Fischer method (10) and purified in a similar manner. The properties of the final triglyceride preparations are summarized in Table I.

The mp were determined by a capillary method corresponding essentially to the "regular complete method" described by Lutton et al. (28). All preparations were homogeneous by TLC analysis (36).

Natural triglycerides. Cocoa butter, olive oil, corn oil, and lard triglycerides were obtained free of other lipid constituents by silicic acid column chromatography. The I.V. and fatty acid composition of these triglycerides are reported in Table II.

TABLE I

Analyses of Triglycerides

Triglyceride	I.V. (Wijs)	m.p. Form I	60C n_D
Glyceryl-2-oleate-1,3-distearate	27.5	43.9–44.3C at .25C/min	1.4490
Glyceryl-2-linoleate-1,3-distearate	54.8	36.8–36.9C at .025C/min.	1.4524
Glyceryl-1-stearate-2,3-dioleate	58.2	23.7–24.0C at .06C/min	1.4515
Glyceryl-2-stearate-1,3-dilinoleate	119.0	9.0–9.6C[c] at 1C/min	1.4595
Triolein	85.7	5.6–6.0C at .5C/min	1.4675[a]
Tripalmitin	.....	66.9–67.4C at .6C/min	1.4380[b]

[a] At 25C.
[b] At 80C.
[c] Form II or Form III.

Fatty acid analyses were performed on methyl esters by gas-liquid chromatography (GLC) using an F & M Model 609 Hydrogen Flame instrument with an 8′ × ⅛″ column of ethylene glycol succinate polyester phase on 100–120 mesh Gaschrom P at 180C. The methyl esters for the GLC analyses were prepared by transmethylation of a few mg (or less) of sample by heating it in refluxing 0.5 N anhydrous methanolic HCl for about one hr. After the transesterification was completed as indicated by TLC analysis, the methyl esters were extracted into diethyl ether, washed with distilled water, and dried in the usual manner.

Lecithins. Egg lecithin was isolated from the lipid of fresh egg powder by a combination of dialysis (4) and silicic acid chromatography using the general technique of Rhodes and Lea (38,39).

Lecithins were also isolated from crude soybean lecithin (Glidden Co., Chicago, Ill.), wheat germ oil (Viobin Corp., Monticello, Ill.) and fresh beef spinal cord lipid (Geo. A. Hormel & Co., Austin, Minn.). Crude lecithins were obtained from these lipids by acetone precipitation and then purified by silicic and chromatography by the same procedure used for the isolation of egg lecithin as follows: About 2 g of crude lecithin was placed on the top of the column (2.5 × 50 cm) of silicic acid and eluted with various ratios of chloroform-methanol starting with chloroform. Traces of neutral lipid, in particular, were removed by elution with chloroform. Then the bulk of the cephalin was eluted with an 8:2 (v/v) ratio of chloroform-methanol. When, toward the end of the elution of the cephalin, a small amount of lecithin started to emerge from the column, the ratio of chloroform to methanol was changed to 7:3 (v/v). The remainder of the cephalin (containing some lecithin) was eluted almost immediately, and then the lecithin fraction was collected. The entire procedure was monitored by TLC. The final products were homogeneous by TLC and, as far as could be determined, contained no impurities. Table III shows the fatty acid composition and N/P ratios of the final products. The lecithin isolated from beef spinal cord was not analyzed.

TABLE II

Fatty Acid Composition of Natural Triglycerides

Source	I.V. (Wijs)	C_{16} saturated	C_{16} monoene	C_{18} saturated	C_{18} monoene	C_{18} diene
Lard	58.3	26.0	2.5	15.4	44.7	11.4
Cocoa butter	39.7	24.6	.....	32.6	40.8	2.0
Olive oil	88.3	9.4	0.95	2.2	77.5	9.9
Corn oil	132.7	12.9	.....	.....	27.3	59.8

TABLE III

Analyses of Lecithins

Source	Fatty Acid Composition								
	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Linolenic	% N	% P	N/P
Egg[a]	37.0	0.6	12.4	31.4	12.0	1.0	1.78	3.90	1.01
Wheat germ	18.7	.....	.....	13.1	63.6	4.6	1.63	3.64	1.00
Soybean	12.5	.....	5.4	12.3	64.0	5.8	1.71	3.66	1.03

[a] Arachidonic 2.7%; Eicosapentaenoic 0.8%; Docosahexaenoate 2.1%

Structural Analysis of Triglycerides

A sample of 2–5 mg is ozonized in N-pentane according to the procedure described by Privett and Nickell for the determination of double bond structure (35).

The ozonides are separated into classes based on the number of ozonide groups in the molecule by thin-layer chromatography (TLC) on Silica Gel G with various ratios of diethyl ether and petroleum ether (B.P. 35–60C). The ratio of these solvents depends on the range of the ozonide classes to be separated. For example, classes of ozonides containing zero to 4 ozonide groups are separated with 15% of diethyl ether in petroleum ether as shown in Figure 1.

The TLC of the ozonides of corn oil (Fig. 2) demonstrates the separation of triglycerides containing up to six ozonide groups. This separation was effected with 20% diethyl ether in petroleum ether (B.P. 35–60C.). The results in Figures 1 and 2 demonstrate that triglyceride ozonides differing by only one ozonide group can be separated by TLC.

Quantitative TLC. Approx 50 μg of mixed ozonides is chromatographed on a 5 × 20 cm plate. The spots are made visible by charring them by heating the plate in an oven at 180C for 25 min after spraying it with chromic-sulfuric acid (29). This reagent is prepared by saturating 80% aqueous H_2SO_4 with $K_2CR_2O_7$. The spots are measured by densitometry using a densitometer (Photovolt Corp, New York, N.Y.) equipped with a specially designed stage for holding thin-layer plates. No filter is used in the densitometer, and measurements are made with a 1 mm × 1 mm slit. Figure 3 shows a typical densitometer curve of the analysis of a standard mixture of triglyceride ozonides.

The amount of each component is represented by the product of the max density (peak height) and the diameter of the spot (base of the peak). These values are corrected for the relative amount of carbon lost through degradation of the ozonide group during the charring process. Since ozonides are immediately decomposed by strong acids and heat, only the residual saturated carbon skeleton of the triglycerides is charred; the low mol wt fragments cleaved from each unsaturated fatty acid moiety evaporate from the plate. The correction for the amount of carbon lost during charring by fission of the ozonides is made on the basis of the analysis of the aldehyde "cores" described below which determines the number of unsaturated fatty acids in each triglyceride ozonide class. For example, the correction for an ozonide class containing two monomeric ozonide acids such as that given by a triglyceride containing two oleic acids and one saturated acid would be on the basis of a loss of 18 carbon atoms. The number of carbon atoms contributed by the saturated fatty acids is determined by a GLC analysis of the aldehyde "cores" as described below. The results of the analysis of a standard mixture of triglycerides (Table IV) shows that the values calculated in this manner agree closely with the known composition.

After the analytical separation of the ozonide classes is carried out, another sample is chromatographed for the isolation of the individual classes of ozonides for further analysis. About 10 spots of about 100 μg each are placed on a 20 × 20 cm plate and developed under the same conditions used for quantitative analysis. The positions of the spots are located by spraying the plate with 0.1% alcoholic 2,7 dichlorofluorescein and viewing it under ultraviolet light (29). Each row of spots is then scraped off the plate in a band of adsorbent and recovered by extraction with methylene chloride, the solvent in which the subsequent reductive fission is performed.

Each of the recovered ozonide classes is fissioned by catalytic reduction as previously described (34) using the Lindlar catalyst.

About 50 μg of the mixture of aldehyde "cores" from each ozonide class is chromatographed on a 5 × 20 cm. chromatoplate for quantitative analysis. The spots are charred and analyzed by densitometry as described above. From this analysis, the types of triglycerides in each ozonide class are determined in terms of the number of their constituent saturated and unsaturated (aldehyde) fatty acids. For example, the ozonide class containing 2 ozonide groups is analyzed for its content of S_1O_2 and S_2L_1 where O is oleate and L is linoleate.

Samples of each aldehyde "core" are also separated by preparative TLC for a GLC analysis. About ten 50 μg spots of the sample are chromatographed on a 20 × 20 cm plate under the same conditions used for quantitative analysis. The plate is sprayed with 2,7 dichlorofluorescein and viewed under ultra-

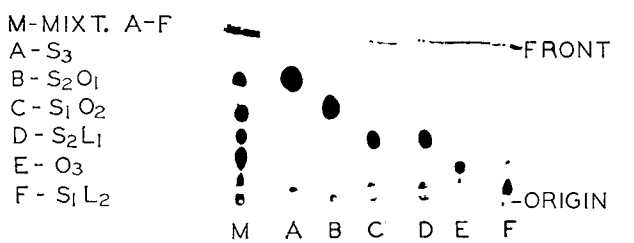


FIG. 1. TLC separation of standard mixtures of triglyceride ozonides (15% diethyl ether in petroleum ether B.P. 35–60C). S = stearate, O = oleate,, L = linoleate.

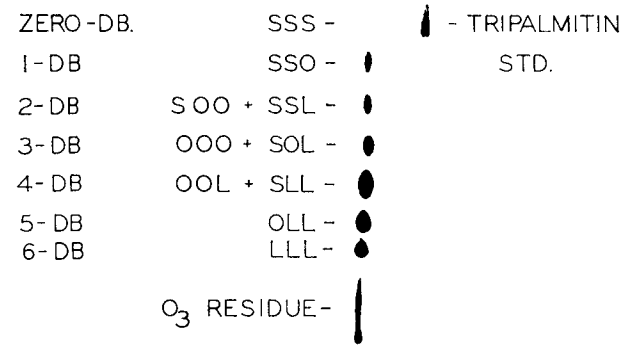


FIG. 2. TLC separation of corn oil ozonides (20% diethyl ether in petroleum ether B.P. 35–60C). S = Saturated fatty acids (palmitic acid), O = oleic acid, L = linoleic acid.

TABLE IV
Analyses of Standard Mixtures Triglyceride Ozonides

Triglyceride	Ozonide class	Known	No. 1	No. 2	No. 3	Avg
P_3	0	9.5	7.4	6.8	7.3	7.2
S_2O_1, S_1O_2	1	15.2	16.3	15.0	16.0	15.7
S_2L_1, O_3	2	41.5	41.6	43.0	42.8	42.5
	3	33.8	34.7	35.2	33.9	34.6

violet light to locate the position of the spots (bands). Each row of spots is then scraped from the plate in a band of adsorbent and recovered by extraction with freshly distilled diethyl ether. The recovered samples are transmethylated as previously described and the saturated fatty acid composition determined by GLC.

The final triglyceride composition is calculated on the basis of the analysis of the ozonide classes, the triglyceride types of each ozonide class (from the analysis of the aldehyde "cores"), and the saturated fatty acid composition of the "cores" of each triglyceride type. The analysis of several natural triglycerides are summarized in Table V.

Structural Analysis of Lecithins. Lecithins are analyzed by essentially the same method as triglycerides, that is, by reductive ozonolysis followed by analysis of the aldehyde "cores" by TLC. At present, the fractionation of the lecithin ozonides prior to catalytic reduction has not been investigated. This step is not as important in the analysis of lecithins as in the analysis of triglycerides because the "cores" of all four lecithin types can be separated by TLC.

The TLC of the lecithin aldehyde "cores" is carried out by the reversed phase system of Malins and Mangold (33) using Silicone (Dow Corning 200 fluid viscosity 10 cs) as the stationary phase and 85:15 (v/v) acetic acid:water as the mobile phase. The separated "cores" are analyzed by densitometry after being charred as described above. The silicone used as the stationary phase does not interfere with the charring process. The densitometry of the four natural lecithins described above is shown in Figure 4.

Identification of the peaks (spots on the chromatoplate) in Figure 4 was made on the following basis. Peak I (with the lowest R_f value) was due to the fully saturated type. This was confirmed by the analysis of a sample of fully hydrogenated lecithin. Infrared analysis, as well as TLC, showed that the hydrogenated lecithin was not altered by the reductive ozonolysis process. Peak II was attributed to the "core" of the α-saturated-β-unsaturated type on the basis of the analysis of egg lecithin which has been shown to consist primarily of this type (16,17). Peak III was attributed to the "core" from the α-unsaturated-β-saturated type. This was deduced from the following considerations. Analysis of the "cores" representing Peaks II and III by GLC (after transmethylation of the "cores") showed that they contained saturated as well as unsaturated fatty acids. Since wheat germ lecithin gave two well-defined peaks representing these types, it was evident from its fatty acid composition that they were not due to a difference in unsaturated fatty acid composition, and they were obviously not due to a difference in saturated fatty acids because these consisted entirely of palmitic acid. Thus, it was concluded that peak II represented the α-saturated-β-unsaturated type and peak III the α-unsaturated-β-saturated type.

An explanation for the difference in the polarity of the "cores" of these two types was given by infrared analysis (Fig. 5) which showed that the P=O band in the "core" giving peak II was shifted from 7.9 μ to a lower wavelength overlapping the P–O–C band at about 8.5 μ. Such a shift is due to a decrease in the electronegativity of the substituent groups attached to the P=O group (2) and explains the lower polarity of the "core" represented by this peak (II). The structures of the "cores" of these types may be represented as follows:

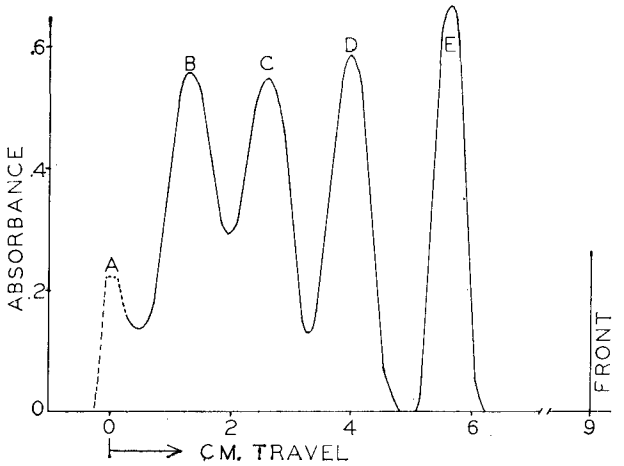
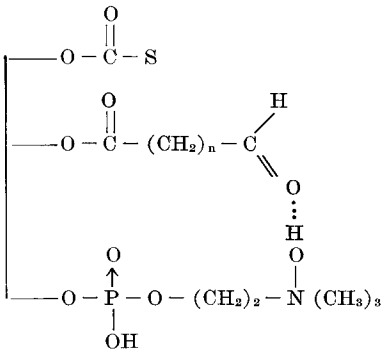
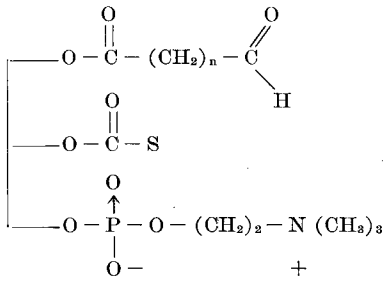


FIG. 3. Densitometer curve of a mixture of triglyceride ozonides. A = ozonide residues, B = ozonide of glyceryl-1-stearate-1,3-dilinoleate, C = ozonide of triolein, D = ozonide of glyceryl-1-stearate-2,3-dioleate, E = ozonide of 2-oleate-1,3-distearate.

Peak II Core



Peak III Core



S represents saturated fatty acids.

Further evidence for the above structure for the "core" representing peak II is the similarity of the region of the spectrum from 8.0 to 9.0 μ (Fig. 5) to that of phosphatidylserine (18) which also has a free P–OH group. A broad band at about 4.0 μ in the spectrum of the "core" represented by peak II (Fig. 4) also indicated the presence of a P–OH group as well as an associated aldehyde group in this compound.

TABLE V

Analyses of Triglycerides
(Wt %)

	S_3	S_2O_1	S_1O_2	S_2L_1	O_3	SOL	SL_2	OL_2	O_2L	L_3
Corn oil	0	1.0	3.4	2.7	2.2	14.9	15.5	26.6	13.5	20.3
Lard	7.0	23.6	19.4	11.9	14.6	23.5	.....	.....	.....	.....
Cocoa butter	2.0	74.7	17.0	6.3	.....	.....	.....	.....	.....	.....
Olive oil	.....	Trace	12.1	4.6	44.9	21.4	Trace	.....	17.2	.....

S = saturated fatty acids, O = oleiç, L = linoleic.

Since peak IV (Fig. 4) represented the most polar "core," it was derived from the fully unsaturated lecithin. This was confirmed by analysis of the constituent acids (as esters) by GLC which showed that it contained only fragments from unsaturated fatty acids.

The quantitative analysis of the lecithin types was made from the densitometer curves (Fig. 4) and is reported in Table VI.

These analyses were calculated on the basis of the average chain length for the constituent saturated fatty acids and that a 9 carbon atom half-ester-half-aldehyde residue remained in the "core" for each unsaturated fatty acid on reductive ozonolysis.

Discussion

Since the methods described here are relatively fast and can be carried out on a semimicro scale, they should have many applications in the analysis of triglycerides and lecithins, not heretofore practicable. The analysis of the natural triglycerides demonstrated the degree to which a triglyceride analysis can be performed at the present stage of the development of the method.

The pattern of the analysis of triglycerides is illustrated in Table VII. For example, a triglyceride containing three ozonide groups which yields one aldehyde would contain two saturated fatty acids and a trienoic acid. The saturated fatty acids can be determined by GLC analysis of the aldehyde "core." The identity of the unsaturated fatty acids can be deduced from the fatty acid composition of the original fat in most cases.

The method does not permit an analysis of the isomeric triglycerides or the multiple types which give 5 and 6 ozonide groups with the same classes of aldehyde "cores" (Table VII). However, some information may be obtained on the identity of the unsaturated fatty acids of these classes of ozonides by collection and analysis of the simple aldehydes cleaved on catalytic reduction of the ozonides by GLC as described by Privett and Nickell (35). It would appear that a long stride forward could be made in distinguishing isomers and members of other triglycerides separated only in groups by application of solid state infrared spectroscopy as applied by Chapman et al. (5) and/or by lipase hydrolysis techniques (1,31,32) in conjunction with the present method.

Another technique in the analysis of the triglyceride which is in the course of development, is the fission of the separated ozonides directly on the chromatoplate, followed by chromatography of the "core" in the second dimension. Such a technique would permit the analysis to be carried out on a micro scale as well as simplifying and shortening the procedure.

Since the method does not lend itself to the use of standard curves of pure reference compounds the charring conditions for quantitative analysis of spots by densitometry is very critical. If charring is carried out on a hot plate (over 200C) after spraying the plate with 50% aqueous H_2SO_4, as commonly practiced, an appreciable amount of evaporation occurs prior to complete oxidation which results in variable amounts of char (yield of carbon) from compound to compound. The yield of carbon on the charring of glycerides under these conditions is the resultant of two opposing processes—the ease of oxidation vs. the rate of evaporation of the compound. The difference in the yield of carbon (intensity of spots) on the charring of equal amounts of triolein

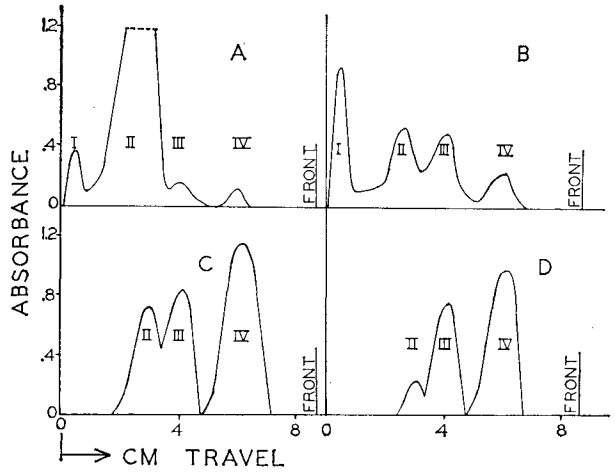


FIG. 4. Densitometer curves of four natural lecithin "cores" (reversed-phase system, stationary phase = silicone, mobile phase 85:15 v/v acetic acid-water). A = egg, B = beef spinal cord, C = soybean, D = wheat germ. I = α-β-saturated, II = α-saturated-β-unsaturated, III = α-unsaturated-β-saturated, IV = α-β-unsaturated.

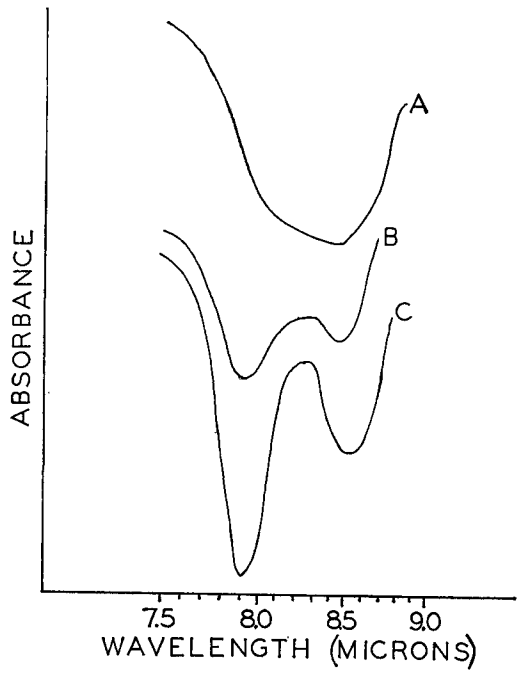


FIG. 5. Infrared spectra of: A = α-saturated-β-unsaturated lecithin "core," B = α-unsaturated-β-saturated lecithin "core," C = original egg lecithin.

TABLE VI

Structural Analysis of Lecithins
(Wt %)
(S = saturated f.a.; U = unsaturated f.a.)

Peak, Fig B type	I α–β–SS	II α–S–β–U	III α–U–β–S	IV α–β–UU
Source				
Egg	4.5	87.6	4.9	3.0
Soybean	none	20.3	24.7	55.0
Wheat germ	none	11.4	28.8	59.8
Beef spinal cord	22.1	33.1	28.9	15.9

and tripalmitin (36) is a good example of the influence of these factors. The difference between the yield of carbon on charring spots of mono-, di-, and triglycerides under these conditions (36) may also be explained on this basis. By carrying out the charring process below 200C and with chromic-sulfuric acid (29) which is a much stronger oxidizing agent than 50% aqueous H_2SO_4, differences in the amount of evaporation between glycerides is insignificant.

The use of the charring conditions described herein does not eliminate entirely the effect of R_f on the area of spots determined densitometrically with a slit just wider than the diameter of the largest spot as previously described (36). However, the error due to differences in R_f is eliminated through the use of the small slit as described here because the value obtained is a function of the product of the size of the spot and its max density and is independent of the R_f value. Such a technique, of course, could not be used if the spots assumed radically different shapes. The tacit assumption is that in addition to all spots having the same general shape, the change in diameter in one direction is proportional to that in the other direction with the increase in the size of the spot at the higher R_f values.

In the course of this study many samples of lecithins were analyzed. The structural analysis of lecithins, previously reported (29), showed that preparations from soybean and wheat germ contained small amounts of saturated types. In the present study the lecithins from these sources were not isolated in such a way as to preclude fractionation. The main objective in the present study was to obtain products free of impurities. Therefore, the analysis of the lecithin preparations reported here do not represent the total lecithins from these sources except perhaps in the case of egg lecithin where the initial isolation is carried out by dialysis.

Although egg lecithin consists mostly of one type, the α-saturated-β-unsaturated type, the detection of small amounts of diunsaturated and disaturated types is unequivocal and indicates the method described here for the analysis of lecithin types is more sensitive than enzyme methods.

TABLE VII

Separation Pattern of Ozonides and Aldehyde "Cores"

	Glyceride types (aldehyde cores)			
Ozonide class	S_3	A_1S_2	A_2S_1	A_3
0	3 S	.....	.....	.....
1	.....	M	.....	.....
2	.....	D	2 M	.....
3	.....	Tri	D + M	3 M
4	.....	Tet	M : D : + Tri: D :	M + M + D
5	.....	P	: Tet: D + : + M : Tri : :	: M : D + : + M : D + : + Tri: M :
6	.....	H	: : Tri: D : + + : Tet: Tri:	: : M + P :	: M : D + : + D : D + : + Tri: D :	: : M : + : M : + : Tet

M = Monoenoic acid(s); D = Dienoic acid(s); Tri = Trienoic acid(s); Tet = Tetraenoic acid(c); P = Pentaenoic acid(s); H = Hexaenoic acid(s); S_1 = one saturated acid; A_1 = one aldehyde residue in triglyceride.

Acknowledgment

The authors are indebted to Anthony DeFreitas of the Department of Agricultural Biochemistry of the University of Minnesota for preparation of glyceryl-2-linoleate-1,3-distearate and glyceryl-2-stearate-1,3-dilinoleate used in this study.

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[Received April 5, 1962—Accepted October 23, 1962]