TABLE IV

Fatty Acid Composition of the Partially Hydrogenated Samples of Rapeseed Oil Studied in Three Experiments (% by weight)

Fatty acid	Exp. I		Exp. II		Exp. III			
					A[c]		B[d]	
	P.E.[a]	Si[b]	P.E.	Si	P.E.	Si	P.E.	Si
$C_{12:0}$	trace	trace	trace	trace	trace	trace	trace	trace
$C_{14:0}$	trace	trace	trace	trace	trace	trace	trace	trace
$C_{16:0}$	4.0		4.1		3.7		3.5	
$C_{16:1}$	0.3		0.4		0.6		0.5	
Total C_{16}	4.3	4.1	4.5	4.0	4.3	3.9	4.0	3.6
$C_{18:0}$	8.2		6.4		6.2		7.2	
$C_{18:1}$	34.2		34.8		37.6		37.2	
$C_{18:2}$ I	1.6		4.6		3.7		3.4	
$C_{18:2}$ II[e]	4.7		3.1		1.8		1.9	
$C_{18:2}$ III	1.4		2.5		1.2		1.0	
$C_{18:3}$ I[e]	....		....		0.3		0.2	
$C_{18:3}$ II	0.8		....		....		....	
Total C_{18}	50.9	50.4	51.4	52.7	50.8	50.7	50.9	51.2
$C_{20:0}$	2.5		1.7		2.2		2.1	
$C_{20:1}$	11.3		10.9		10.3		10.5	
Total C_{20}	13.8	14.5	12.6	12.7	12.5	12.5	12.6	12.8
$C_{22:0}$	3.8		3.6		2.8		4.1	
$C_{22:1}$	27.2		27.9		29.6		28.4	
Total C_{22}	31.0	31.0	31.5	30.6	32.4	32.9	32.5	32.4

[a] Butanediol-succinate polyester column.
[b] Silicone column.
[c] Hydrogenated by conditions used in shortening manufacture and randomly rearranged.
[d] Hydrogenated by conditions used in margarine manufacture.
[e] Natural isomer.

other factor of apparent significance in affecting body-weight gain of rats fed hydrogenated rapeseed oil was the occurrence of isomers of linoleic acid, possibly 9,15-octadecadienoic and 12,15-octadecadienoic acids derived from linolenic acid. Geometric isomers of linoleic acid were found to have no effect on essential fatty acid activity (15,16), but positional isomers were not similarly investigated. From these studies it is shown that hydrogenation of rapeseed oil increases, does not change, or decreases the growth-promoting qualities of rapeseed oil, depending at least in part on the final fatty acid composition. Low gains in weight were associated with a decrease of natural linoleic acid and the presence of other octadecadienoic acids.

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Determination of Mono-, Di-, and Triglycerides by Molecular Distillation and Thin-Layer Chromatography[1,2]

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Analysis of mixtures of mono-, di-, and triglycerides by molecular distillation and thin-layer chromatography is described.

Mono- and diglycerides undergo appreciable acyl migration through the effect of heat during molecular distillation. Nevertheless this technique may be used for the quantitative analysis of mixtures of mono-, di-, and triglycerides, provided there are no substances present which catalyze disproportionation.

Thin-layer chromatographic (TLC) analysis of mono-, di-, and triglycerides is fast and simple and can be carried out on a micro-scale with a high degree of accuracy and precision. It also is extremely sensitive, permitting the quantitative estimation of as little as 0.1% of a single component in a mixture.

The routine analyses of α- and β-monoglycerides and of 1,2- and 1,3-diglycerides also may be performed by this method.

THE ANALYSIS of mixtures of mono-, di-, and triglycerides is usually carried out by column chromatographic procedures, employing selective adsorption (1,6) or partition techniques (12). In general, these methods are time-consuming and involved, since many fractions must be collected and analyzed for a single determination. Furthermore the precision and accuracy of analyses by these methods, in general, is less than desirable for many analytical purposes.

Described are two procedures for the analysis of

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mixtures of mono-, di, and triglycerides. One employs analytical molecular distillation, the other, thin-layer chromatography (TLC). The latter appears to be especially well-suited to use as a general method for the quantitative as well as qualitative analysis of these compounds.

Materials

The following reference compounds were prepared in highly purified form: 1-monopalmitin (m.p. 76.5–77°C.), 1,3-dipalmitin (m.p. 72.2–72.5°C.), tripalmitin (m.p. 66.1–66.5°C., sap. val. 208.8), triolein (I.V. 85.7, sap. val. 189.9), monostearin (m.p. 81.0–81.5°C.), 1,3-distearin (m.p. 79.5–79.9°C.). A sample of pure 1,2-distearin (m.p. 69.5–70.0°C.) was obtained from A. de Freitas, Department of Biochemistry, University of Minnesota.

The 1-monopalmitin and 1-monostearin were synthesized by the method of Fischer (4), as described by Daubert *et al.* (3). The crude products were recrystallized several times from ethyl ether and purified further by silicic acid column chromatography (1). The final products were homogeneous by thin-layer chromatographic analysis; periodate analysis (13) indicated that they were pure 1-monoglycerides.

The 1,3-dipalmitin and distearin were prepared *via* trityl derivatives (2,7) and purified in a similar manner.

Tripalmitin and triolein were prepared by transesterification of the corresponding pure methyl esters with triacetin, using sodium methoxide as a catalyst (8). Most of the excess methyl ester and monoglyceride impurities were removed by several extractions of a Skellysolve F solution of the crude triglycerides with 90% ethanol. The bulk of the diglyceride impurity was removed by low-temperature fractional crystallization. Traces of impurities remaining were separated by silicic acid column chromatography (1).

Procedures

Molecular Distillation. The analysis of mixtures of mono-, di-, and triglycerides by this technique was performed with a still essentially the same as that described by Paschke, Kerns, and Wheeler (10). Distillations were conducted on 0.5 to 2 g. of material at a pressure of $<10^{-3}$ mm. of mercury. Free fatty acids, glycerol, and simple esters were analyzed at 75–80°C., monoglycerides at 145–150°C., and diglycerides at 200–210°C. The amount of triglyceride was usually estimated by difference although this fraction may also be determined by distillation at 250–260°C.

A typical distillation curve which shows the separation of methyl oleate, monopalmitin, dipalmitin, and refined corn oil representing triglyceride, is presented in Figure 1. In this distillation the time interval between temperature increments was lengthened more than usual to demonstrate that there is little overlapping of components. A comparison of the analytical values with the known composition indicated that mono-, di-, and triglycerides are separated quantitatively by this technique.

Thin-Layer Chromatography (TLC). About 10 μg. samples are spotted with a microsyringe on the base of glass plates 2 in. x 8 in. with a thin layer of silica gel G, prepared according to Stahl (15,16) and Mangold and Malins (9). The plates are placed in a small glass jar, containing a small amount of various

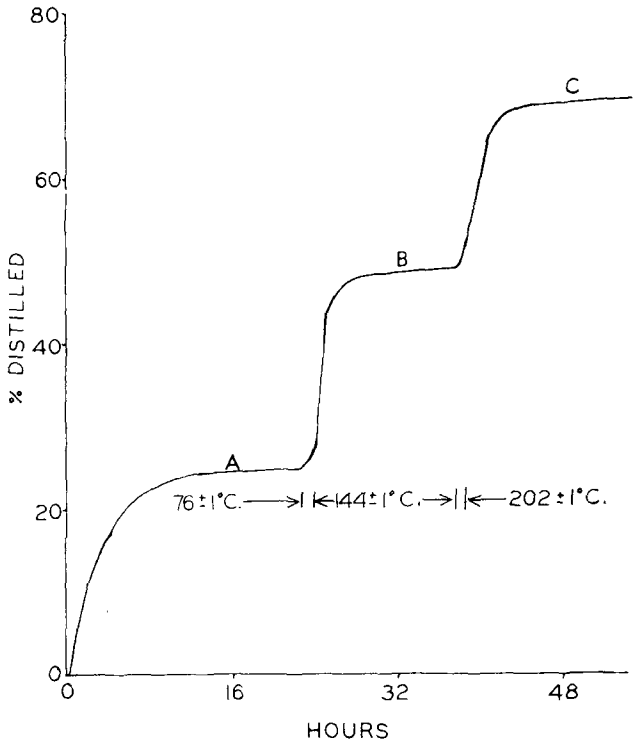

FIG. 1. Molecular distillation of a sample consisting of 23.6% methyl oleate, 23.9% monopalmitin, 23.2% dipalmitin, and 29.3% corn oil. Distillation analysis: A = 24.8% methyl ester; B = 24.0% monoglyceride; C = 21.4% diglyceride; triglyceride = 29.8% by difference.

mixtures of ethyl ether and Skellysolve F, and are developed in an ascending manner. Usually a developing time of 10 to 20 min. is sufficient to provide a separation of the components. After the plates are developed, they are held at room temperature for several minutes in a fume hood until the solvent evaporates. Then they are sprayed with 50% aqueous H_2SO_4 and heated on a hot plate to char the organic material, thereby locating the position of the spots.

A photograph of a plate prepared in this manner, showing the separation of mono-, di-, and tripalmitin, is shown in Figure 2. The lower spot is monopalmitin, the center spot dipalmitin, and the upper spot tripalmitin. This plate was developed with a solvent, consisting of 30% ethyl ether in Skellysolve F. A 5-lb. chemical reagent jar is usually used as the developing jar for plates of this size.

For quantitative analysis the spots are measured with a densitometer (Photovolt Corporation, 52 and 501-A) with a stage attached to it for semiautomatic plotting of curves. The slit size of the densitometer was 1 x 7.5 mm., and readings were taken at each millimeter of travel over the length of the plate. No filter was used. Usually there was only a slight difference in background density from one end of the plate to the other, which varied linearly over the plate and had no effect on the accuracy of the measurements.

The curve given by the analysis of the plate shown in Figure 2 is presented in Figure 3. In actual practice three plates are used for the analysis of mono-, di-, and triglycerides because the R_f values for these compounds differ by so much that with a single solvent system, as shown in Figure 3, monoglycerides do not move off the base line whereas the triglycerides move almost with the front. Thus monoglycerides are

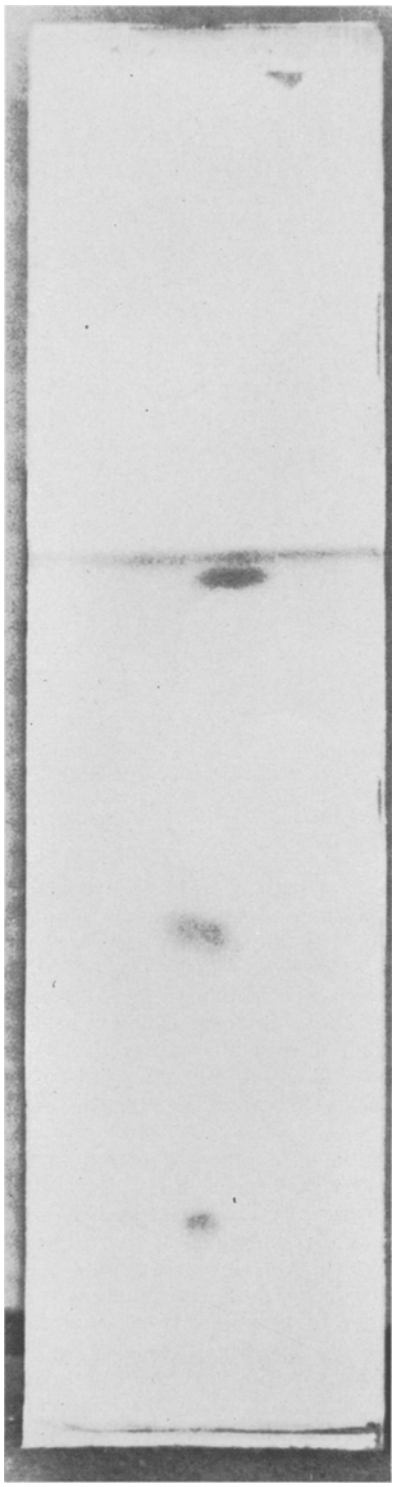

FIG. 2. Photograph of chromatographic plate, showing the separation of mono-, di-, and tripalmitin, lower, central, and upper spots, respectively.

analyzed on one plate with a solvent system, consisting of 90% ethyl ether and 10% Skellysolve F; the diglycerides are developed on another plate, using 30% ethyl ether in Skellysolve F; and the triglycerides are developed on a third plate with 10% ethyl ether in Skellysolve F. Both the triglycerides and the diglycerides run at or near the front in the first solvent system, which is used to analyze the monoglycerides. In the second solvent system the monoglycerides remain at the base of the plate, and the

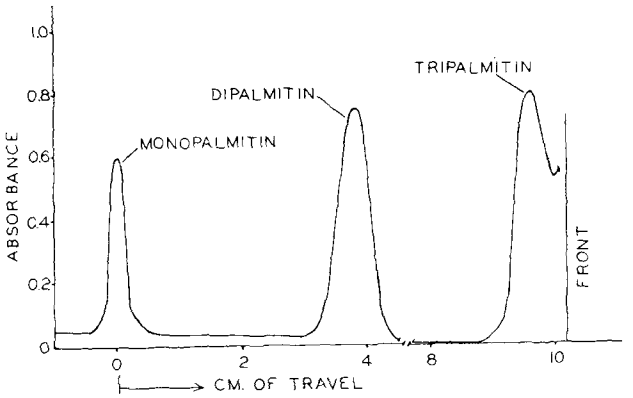

FIG. 3. Densitometric analysis of the chromatogram shown in Figure 2.

triglycerides move with the front, permitting optimum conditions for the analysis of diglycerides (Figure 3). Only the triglycerides migrate from the base line in the third solvent system.

Areas under the peaks are directly proportional to the amount of sample, but the proportionality constant varies with the structure of the compound. The reason for the quantitative difference in the development of the spots for compounds of different structures (per unit of carbon density) cannot be explained at present, but since it appears to be an inherent property, standard curves or conversion factors prepared thereby can be employed for quantitative analysis.

Standard curves for saturated mono-, di-, and tripalmitin are shown in Figure 4. The slopes of the standard curves vary slightly from one batch of plates to another, but the ratios of the slopes are constant. Thus conversion factors that are based on the relative slopes of a single set of standard curves may be employed, eliminating the necessity of running standard curves on each new batch of plates.

The size and density of the spots for triolein bear a linear relationship to the amount of sample (Figure 4), but the densitometrically-determined response is much greater for triolein than tripalmitin in spite of the fact that both compounds have essentially the same carbon densities (77.3 *vs.* 75.8%). Errors in the analysis of natural products, containing unsaturated and saturated fatty acids, will generally be less

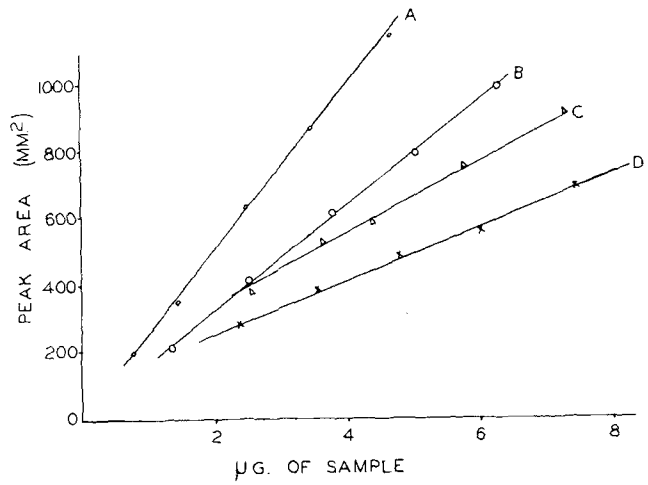

FIG. 4. Relation between peak area and sample size in the TLC analyses of A. triolein; B. tripalmitin; C. dipalmitin; D. monopalmitin.

than 5% because the unsaturation is usually more or less randomly distributed throughout the mono-, di-, and triglyceride components. However, since unsaturation exerts a profound effect on the development of the spots, it is frequently expedient to hydrogenate samples that contain an appreciable amount of unsaturation before they are quantitatively analyzed. This is carried out in a small Parr apparatus by the technique described by Schlenk *et al.* (14), in which about 20 samples can be hydrogenated simultaneously. The hydrogenation is carried out with a palladium-on-charcoal catalyst on a few mg. of sample dissolved in ethyl ether at room temperature, and at a pressure of about 50 lbs. Normally the samples are fully hydrogenated in a few minutes, but, as a precaution to insure complete reaction, a half-hour reaction time is usually employed. The catalyst is filtered and washed; the filtrate is analyzed directly after being made up to a known volume.

Results and Discussions

The molecular distillation and TLC analyses of two mixtures of mono-, di-, and tripalmitin are reported in Table I.

TABLE I

Analysis of Mono-, Di-, and Tripalmitin

Compound	Mixture 1 (%)			Mixture 2 (%)		
	TLC	Mol. dist.	Known comp.	TLC	Mol. dist.	Known comp.
Tripalmitin	43.4	44.3	43.6	10.6	12.4	11.3
Dipalmitin	30.8	30.1	31.1	10.0	10.6	10.5
Monopalmitin	25.8	25.6	25.3	79.4	77.0	78.2

Agreement between the known composition and the analytical values obtained by both methods was very good; the deviation was not more than 1.2 percentage units and usually much less. Each value represents a single analysis. However duplicate analysis may be performed within a deviation of less than $\pm 1\%$ by either method.

The slightly low value for the molecular distillation analysis of the monoglyceride in the sample that contains the high level of monopalmitin appears to be due in part to a small amount of disproportionation because the analytical values on pure monoglycerides (Table II) by this technique are almost always slightly low. No disproportionation appears to take place on the distillation of pure diglycerides however.

Although the analysis by molecular distillation (Table I) agreed well with the known composition, an appreciable amount of acyl migration occurred on the distillation of both mono- and dipalmitin. This was demonstrated by conducting a periodate analysis (α-monoglyceride content) before and after molecular distillation and on the distillate after redistillation of pure 1-monopalmitin and 1-monostearin (Table II).

About 40% of 1,3-dipalmitin was converted to the 1,2-form during the distillation of this compound. This was determined by TLC analysis of the distilled product, the results of which are shown in Figure 5. This analysis was carried out with a solvent system that consisted of 30% ethyl ether in Skellysolve F. The 1,2- and 1,3-diglycerides were identified by comparison with pure 1,2- and 1,3-diglyceride standards.

TLC is a very sensitive method of analysis, as demonstrated in Figure 6, which shows the analysis of a sample of monopalmitin with 0.1% added tripalmitin. By overloading the plate, the small amount of added tripalmitin was detected. It may be quantitatively determined by reference to the appropriate standard curve. This analysis was carried out with 10% ethyl ether in Skellysolve F. Percentages of the same order of diglyceride impurities in monoglycerides may be detected almost equally as well by selecting the proper ratio of ethyl ether and Skellysolve F.

TABLE II

Periodate Analysis of Molecularly Distilled Monoglycerides

Sample	% Monoglycerides	
	Periodate analysis (13)	Molecular distillation
Original monopalmitin	99.9	98.9
First distillate	96.7	98.2
Second distillate	88.8	.....
Original monostearin	99.8	98.1
First distillate	94.8	98.7
Second distillate	90.2	.....

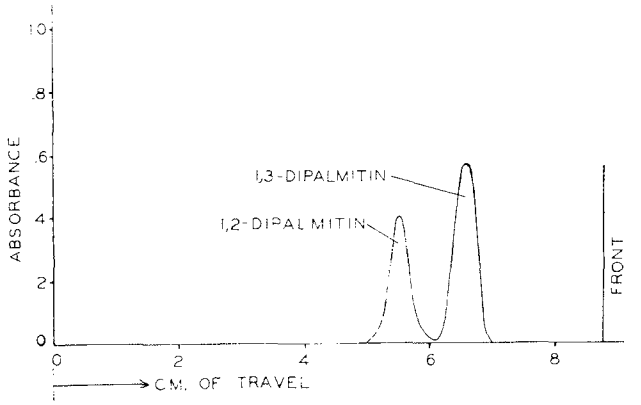

FIG. 5. TLC analysis of the distillate of highly purified 1,3-dipalmitin molecularly distilled at 205°C.

The analysis of three laboratory preparations of monoglycerides, representing animal, vegetable, and marine oil products, by TLC and molecular distillation are reported in Table III. These products were prepared according to the conditions described by Gruger *et al.* (5). Considerable disproportionation occurred on the distillation analysis of the crude preparations, as evidenced by the lack of agreement between the distillation and periodate analyses. The disproportionation apparently was caused by the presence of small amounts of foreign substances, possibly soaps or catalysts not removed by the normal

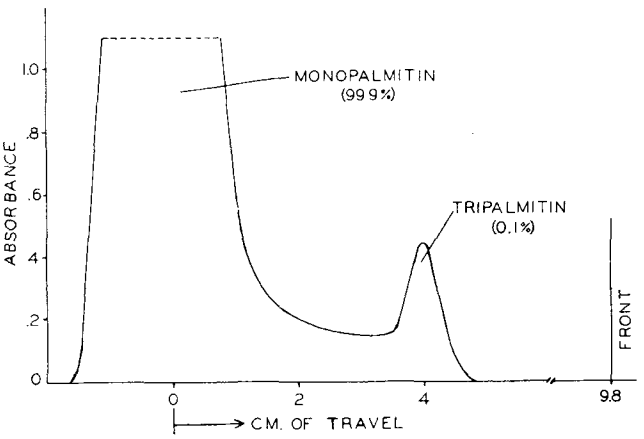

FIG. 6. TLC analysis of monopalmitin, containing 0.1% added tripalmitin.

TABLE III

Analysis of Natural Monoglyceride Preparations

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

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

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

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

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

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

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

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

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

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