Paper Chromatography of Unsaturated Glycerides*

By

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The separation of glycerides from the other lipids and the separation of mono-, di-, and triglycerides from each other have been successfully achieved by chromatography. However, it seems considerably difficult to separate a mixture of triglycerides into individual triglyceride members which differ in the component fatty acids and in the position of these fatty acids in the glyceride molecule, although a few chromatographic separations have hitherto been attempted for this purpose[1-4].

In the present studies a reversed-phase paper chromatography of synthetic and natural glycerides has been developed by employing the mercuration technique, by which good separations of unsaturated fatty acid esters and lecithins by paper chromatography have previously been attained[5-7].

Experimental

Materials. Unsaturated mono-, di-, and triglycerides composed of oleic or linoleic acid as unsaturated acids were synthesized by usual methods. The products were purified by solvent extraction, fractional crystallization, and chromatography. The analytical data for these synthetic glycerides proved their purity as shown in Table 2. The fact that the glycerides thus prepared gave only a single spot, respectively, on the paper chromatogram by the mercuration method under-described also suggested their high purity.

Several different samlpes were tested for each kind of natural fats in order to ascertain that the chromatogram was characteristic for the fat from definite species. For examples, the characteristics of fats used for densitometric measurement were as follows (acid value, saponification value, and iodine value by Wijs method, in order, being indicated in parentheses) : coconut oil (4.1, 258.5, 8.3), cacao butter (1.3, 194.5, 38.1), olive oil (3.7, 195.2, 81.3), castor oil (0.4, 179.2, 84.9), peanut oil (0.6, 190.3, 98.4), rapeseed oil (1.7, 173.9, 100.2), cottonseed oil (0.4, 194.6, 106.8), sesame oil (2.0, 191.4, 111.0), soybean oil (0.1, 192.8, 124.2), linseed oil (1.1, 193.9, 178.6), tung oil (0.5, 193.0, 165.9). These fats were used for paper chromatography after removal of the free fatty acids.

Mercuration. To about 30 mg. of glycerides (or glycerides in a small amount of ether), more than 20% excess of mercuric acetate** in 1 ml. of absolute methanol (or in a mixture of 0.7 ml. of absolute methanol and 0.3 ml. of methyl acetate) was added. After refluxing at 80°C. for 30 min., the mixture was cooled, and 2~3 ml. of ether or benzene and about 20 ml. of distilled water were successively added with shaking. The upper organic layer separated was directly used as the sample solution for paper chromatography.

For isolation of the mercurated glycerides the above organic layer was separated, washed with distilled water repeatedly, and dried with anhydrous sodium sulfate. After evaporating the solvent *in vacuo*, the residue was dried in a vacuum desiccator placed in a dark chamber. The mercurated glycerides were obtained as a colorless or light yellow viscous oil or semisolid which on heating with dilute hydrochloric acid liberated the original glycerides.

Paper Chromatography. From 0.5 to 3 μl. of the sample solutions, containing 10~300 μg. of each mercurated glyceride, were applied on a line 5 cm. from the lower edge of a sheet of Toyo No.

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** In methanol solution 1 molecule of mercuric acetate adds to 1 double bond.

2 filter paper. After spraying the paper evenly and repeatedly with petroleum hydrocarbons or tetralin, it was immediately developed by the ascending technique in a glass cylinder at 30°C. for 5~6 hr.; the composition and amounts of the stationary and developing solvents used are listed in Table 1. The paper was then removed, air-dried overnight, and sprayed with a 0.2% solution

Table 1 Solvent Systems and Development Distance

Solvent system abbreviation	Stationary solvent		Developing solvent (ratio by vol.)	Distance travelled (cm.)
	Composition	Amount impregnated (ml./100 cm^2 of paper)		
MAP	Petroleum hydrocarbon (b.p. 140—170°C.)	1.2	Methanol—acetic acid—petroleum hydrocarbon (b.p. 140—170°C.) (10 : 2 : 2)	20—23
MAP′	Petroleum hydrocarbon (b.p. 185—215°C.)	0.6	Methanol—acetic acid—petroleum hydrocarbon (b.p. 185—215°C.) (10 : 2 : 1)	25—27
MAT	Tetralin	1.0	90% (v/v) Methanol—acetic acid—tetralin (10 : 2 : 1)	20—23

of diphenylcarbazone in alcohol. The mercurated unsaturated glycerides appeared as purple or bluish purple spots.

Densitometer Curves. The chromatograms employed for the measurement of spots by densitometer were prepared by running the sample lined along the original line 2.5 cm. wide until the solvent front of MAP′ (*cf.* Table 1) reached to a height of 25.6 cm. above the line for 5.5 hr. The absorbance of spots was measured in a densitometer (Toyo Roshi Co.) by using a yellow filter. The slit size of the densitometer was 1×27 mm., and readings were taken at 1-mm. intervals. The densitometer curves were made being based on the R_F values and the absorbances corrected for background absorption.

Methanolysis. These paper-chromatographic techniques were applied to follow the progress of methanolysis. After removal of free fatty acids, each 100 mg. of sesame oil glycerides (Sap. V., 190.8; I. V., 114.0) in 2 ml. of a 2% solution of sulfuric acid in absolute methanol were refluxed at 80°C. for 2, 5, 10, 20, 40, 80, 160, and 320 min., respectively. The mixture was cooled rapidly, and 10 ml. of ether and 6 ml. of water were added. The ether extract was neutralized with a sodium carbonate solution, washed with water, and dried over anhydrous sodium sulfate. After removal of the ether, the residue and the original glycerides were converted into their mercuric acetate addition compounds as described above. The sample solutions thus obtained were chromatographed on paper in parallel.

Hydrolysis by Pancreatic Lipase. In order to outline the process of enzymic hydrolysis of triglycerides, a paper-chromatographic analysis was attempted with cacao butter (Sap. V., 195.8; I. V., 37.3) which was purified by removing the free fatty acids in advance. The hydrolysis conditions were the same as those described by Mattson and Beck[8], except that 0.5 g. of the cacao butter was taken as the sample triglycerides and the digestion was carried out with 350 mg. (or 100 mg.) of pancreatic lipase (steapsin) for 7.5 min. (or 15 min.). At the end of the reaction period the pH was adjusted to 2 with 1 *N* hydrochloric acid, and 25 ml. of ethanol was added. The lipids were then extracted with ether, and the ether solution was washed with water repeatedly, neutralized with potassium hydroxide, and again washed with water. After drying the solution with anhydrous sodium sulfate, the ether was evaporated. The mixture of glycerides thus obtained was mercurated in the usual manner and it was subjected to paper chro-

matography.

Results and Discussion

Addition of Mercuric Acetate. The most suitable solvent for mercuration was methanol or methanol—methyl acetate. Almost complete addition of mercuric acetate was attained by heating at 80°C. for 30 min.

R_F Values of Mercurated Synthetic Glycerides. The R_F values of the mercury addition compounds of twelve synthetic glycerides containing oleic or linoleic acid as unsaturated acids are shown in Table 2. It has been found that, in the case of

Table 2 Synthetic Unsaturated Glycerides and R_F Values of Their Mercury Addition Compounds

Glyceride	Abbr.	M. p., °C.	Sap. V.	I. V.	OH V.	R_F of mercurated glyceride	
						MAP[a]	MAT[a]
1-Monoolein	O	34	156.5	70.9	315.6	0.88	0.75
1, 3-Diolein	OO	24	180.3	81.7	91.0	0.60	0.20
1-Palmito-3-olein	PO	46	187.8	42.2	94.4	0.46	0.11
1-Stearo-3-olein	SO	55	180.0	40.1	90.9	0.39	0.07
Triolein	OOO	.....	189.7	85.8	.....	0.45	0.06
1-Palmito-2, 3-diolein	POO	19—20	195.5	58.7	.....	0.22	0.06
1-Stearo-2, 3-diolein	SOO	22	188.9	57.1	.....	0.16	0.03
1, 2-Dipalmito-3-olein	PPO	35	201.0	30.3	.....	0.08	0.02
1, 2-Distearo-3-olein	SSO	39	188.4	28.0	.....	0.05	0.01
1-Palmito-2-stearo-3-olein	PSO	33—34	194.6	28.8	.....	0.06	0.01
1-Stearo-2-palmito-3-olein	SPO	36	194.9	29.7	.....	0.07	0.02
Trilinolein	LLL	.....	191.2	173.1	.....	0.81	0.52

[a] As for the solvent systems MAP and MAT, see Table 1.

oleoglycerides, even a neutralized glyceride containing no free oleic acid often gives two very weak spots of definite R_F values ; one (R_F 0.76 (with MAP), 0.54 (with MAT)) corresponds to that of mercurated oleic acid, and the other (R_F 0.66 (with MAP), tailed) remains unknown. Although these facts show a possibility of the slight hydrolysis of glycerides during mercuration, it seems practically negligible in amount. No methanolysis during mercuration in methanol has also been proved. Contamination with unreacting mercuric acetate in sample solution has completely prevented by using the ether or benzene layer as the sample solution as described above. The clear-cut separations of synthetic mono-, di-, and triglycerides, especially of some individual triglyceride members of different unsaturation from each other, have been achieved by this paper chromatography.

Relations between Structure and R_F Values. From the data gained in these experiments, the following relations between structure and R_F values have been observed : (a) Increasing the number of double bonds or hydroxyl groups increases the R_F values, particularly more markedly in the latter. (b) The presence of saturated alkyl chains in the glyceride molecule considerably lowers the R_F values. (c) With glycerides of equal unsaturation and of the same number of hydroxyl groups, the greater the molecular weight of a glyceride, the lower was the R_F value. (d) The positional isomerism resulting from differences in the point of attachment of fatty acid radicals and hydroxyl groups of glycerol (e. g., PSO and SPO)

seems to have some effect on the R_F values though slightly. With the synthetic glycerides containing oleic acid, the following relative order of the R_F values may be given with the solvent system MAP (*cf.* Table 2) : O>OO>PO>OOO>SO>POO>SOO>PPO>SPO, PSO>SSO.

Paper Chromatography of Natural Triglycerides. The paper chromatograms of several natural fats by the mercuration method are shown in Figs.

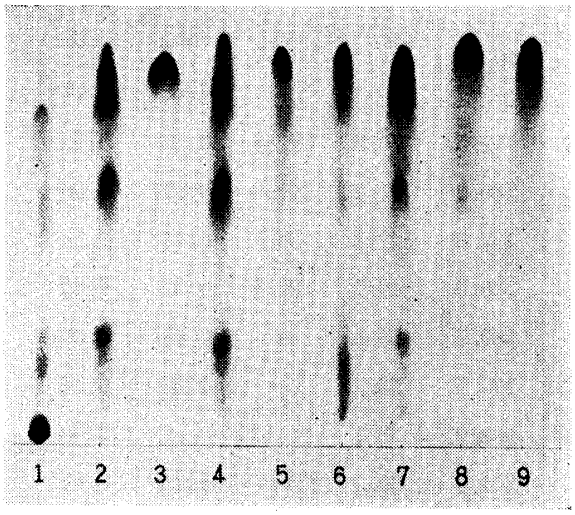

Fig. 1 Paper chromatogram of vegetable fat glycerides as their mercury addition compounds: 1, cacao butter ; 2, olive oil ; 3, castor oil; 4, peanut oil; 5, rapeseed oil; 6, cottonseed oil ; 7, sesame oil ; 8, soybean oil ; 9, tung oil. Solvent system : methanol—acetic acid—petroleum hydrocarbon (MAP).

1 and 2 as examples. As the amounts of unsaponifiable matter are generally slight in these vegetable fats, it may be considered that practically all the spots separated and detected on paper are those of the component triglycerides. The chromatogram of individual natural fat is very characteristic and reproducible showing some tailing, except that of castor oil which contains triricinolein for the most part and gives a single clear-cut spot. The tendency to tailing, however, may be due to the partial overlapping of the spots of individual triglyceride members and neither to the gradual decomposition of mercurated glycerides during development nor to the unsuitableness of solvent systems. Generally, many spots or bands of different intensities have been observed on the chromatogram of a natural fat, with some partial overlapping in the region of higher R_F values.

In this paper chromatography the natural triglyceride mixtures seem to be separated mainly depending upon their unsaturation as in synthetic glycerides. From the results of the simultaneous parallel development of mercurated synthetic and natural glycerides and the paper-chromatographic analysis of the component fatty acids in the spots separated on paper, it has been roughly concluded that, among intense-colored spots, the spots of R_F 0.00-0.10, 0.14-0.25, 0.45-0.55, and above 0.55 correspond to those of the glycerides having 1, 2, 3, and 4 or more double bonds, respectively, with the exception of castor oil. Therefore the natural fats of higher unsaturation give the intense-colored spots of higher R_F values, the increase of unsaturation also somewhat increasing the color intensity of spots. Fully saturated glycerides remained on the original points without any coloration.

Methanolysis of Sesame Oil. The progress of methanolysis could be finely followed by this paper-chromatographic technique. As an example the paper chromatogram showing the progress of methanolysis of sesame oil is presented in Fig. 3.

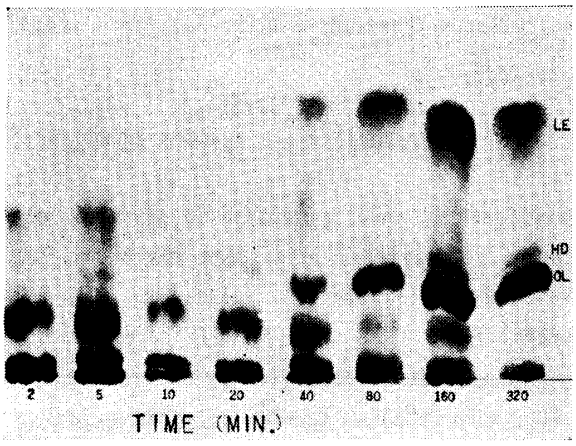

Fig. 3 Paper chromatogram showing the progress of methanolysis of sesame oil. Solvent system: 90% methanol—acetic acid—tetralin (MAT). The spots of methyl esters formed are indicated by : HD, hexadecenoate ; OL, oleate ; LE, linoleate.

Diminishing the unsaturated glycerides and consequent formation of unsaturated methyl esters during methanolysis can be observed on this paper chromatogram in due course. Most glycerides were converted into methyl esters by heating for 320

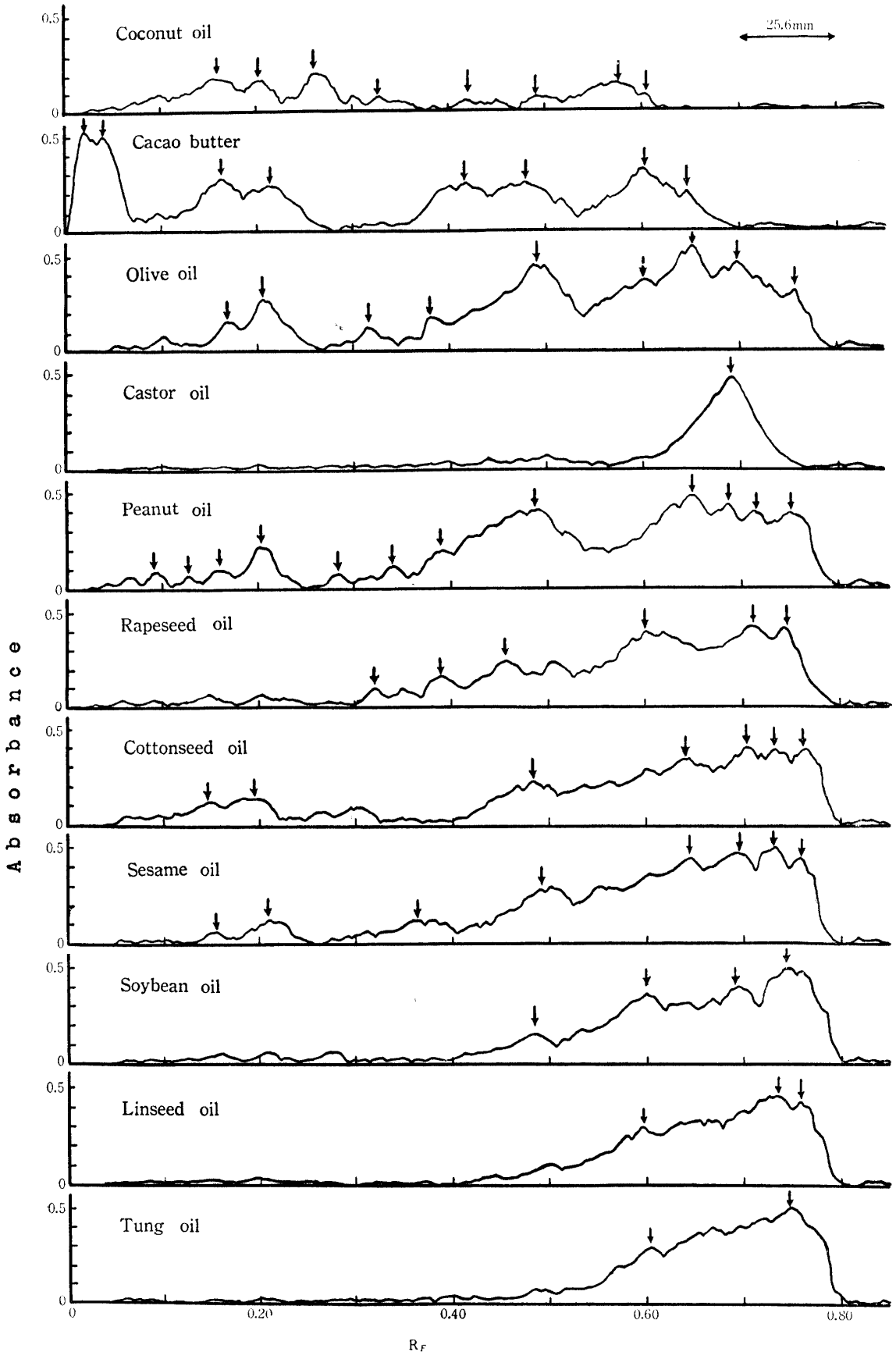

Fig. 2 Densitometer curves obtained from the paper chromatograms of mercurated vegetable fat glycerides. Solvent system: methanol—acetic acid—petroleum hydrocarbon (MAP'). Arrows indicate the positions visually distinguishable as distinct spots on the chromatograms.

min., though a small proportion of unchanged unsaturated glycerides still remained as the bands near origin. The method has proved useful for investigation of ester interchange.

Enzymic Hydrolysis of Cacao Butter. After hydrolyses of each 500 mg. of cacao butter with 350 mg. of pancreatic lipase (steapsin) for 7.5 min. and with 100 mg. of the lipase for 15 min., 401.1 mg. and 359.8 mg. of glycerides were recovered, respectively. The recovered glycerides and the original fat were mercurated and then chromatographed on paper in parallel, as shown in Fig. 4.

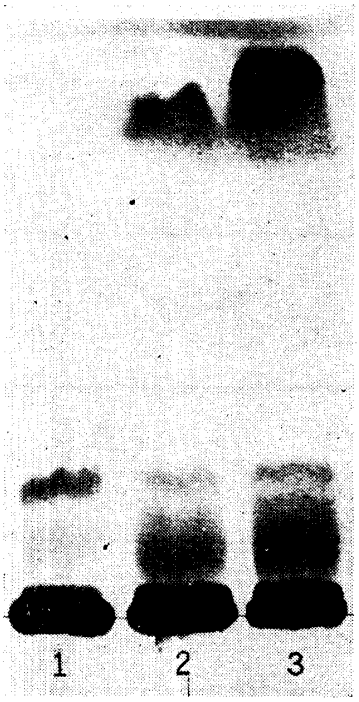

Fig. 4 Paper chromatogram showing the progress of enzymic hydrolysis of cacao butter by pancreatic lipase: 1, original cacao butter; 2, action of 350 mg. of lipase for 7.5 min.; 3, action of 100 mg. of lipase for 15 min. Solvent system: 90% methanol—acetic acid—tetralin (MAT).

The paper chromatogram presented in Fig. 4 elucidates the progress of enzymic hydrolysis, showing the appearance and increasing coloration of the spots of unsaturated mono-(R_F 0.80-0.90) ane diglycerides (R_F 0.08-0.19) as the hydrolysis products. The results obtained suggest the usefulness of this method in determining the fatty acid distribution in glycerides by enzymic hydrolysis.

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Summary

1. A reversed-phase paper-chromatographic method has been devised by which unsaturated glycerides can be separated and identified as their mercuric acetate addition compounds.

2. The method has been successfully used for the separations of synthetic unsaturated glycerides and of natural glyceride mixtures.

3. Some applications of this method to the investigations of ester interchange and enzymic hydrolysis of glycerides have been presented.

REFERENCES

1. Hamilton, J.G., and Holman, R.T. : J. Am. Chem. Soc., **76**, 4107 (1954).
2. Mangold, H.K., Lamp, B.G., and Schlenk, H. : J. Am. Chem. Soc., **77**, 6070 (1955).
3. Schlenk, H., Gellerman, J.L., Tillotson, J. A., and Mangold, H. K. : J. Am. Oil Chemists' Soc., **34**, 377 (1957).
4. Jáky, M. : Fette, Seifen, Anstrichmittel, **61**, 6 (1959).
5. Inouye, Y., Noda, M., and Hirayama, O. : J. Am. Oil Chemists' Soc., **32**, 132 (1955).
6. Noda, M., Hirayama, O., and Inouye, Y. : J. Agr. Chem. Soc. Japan, **30**, 106 (1956).
7. Inouye, Y., and Noda, M. : Arch. Biochem. Biophys., **76**, 271 (1958).
8. Mattson, F.H., and Beck, L.W. : J. Biol. Chem., **214**, 115 (1955).

不飽和グリセリドのペーパークロマトグラフィー

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要約

不飽和グリセリドを酢酸第二水銀付加化合物とし，逆相ペーパークロマトグラフィーで相互分離，確認する方法を考案した．この方法を合成不飽和グリセリドおよび天然油脂の構成グリセリドの相互分離，確認に利用した結果，良好な分離性を示すことを認め，またこの方法の特性についても検討した．本法はまた不飽和グリセリドの関与するエステル交換反応やリパーゼによる加水分解などの過程を追跡，精査できる簡易，有力な方法であることが証明された．