Determination of Triglyceride Structure via Silver Nitrate-TLC[1]

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Abstract

A procedure for the quantitative analysis of component triglycerides by thin-layer chromatography (TLC) with silver nitrate-silicic acid coated plates in combination with lipase hydrolysis is described. The method is demonstrated on synthetic triglyceride mixtures and applied to several natural fats.

The results indicate that the fatty acids of natural fats are not distributed among the triglycerides in accordance with any simple mathematical formula and methods based on mathematical distribution patterns cannot be generally applied.

Introduction

SILICIC ACID IMPREGNATED with silver nitrate is being used widely in various ramifications for the analysis of triglycerides after the method of de Vries (1). In accordance with the known property of the argentation of olefinic linkages (2,3), de Vries (1,4) demonstrated that triglycerides may be fractionated by column chromatography on silicic acid impregnated with silver nitrate on the basis of the degree and geometric configuration of the unsaturation. The use of silver nitrate as an adsorbent for TLC fractionation was first demonstrated by Morris (5). Barret et al. (6) expanded the TLC technique to the separation of triglycerides and developed a method for the determination of triglycerides based on the densitometry of charred spots similar to that employed by Privett and Blank (7) except that phosphoric acid was used instead of sulfuric acid-chromic acid in the charring procedure. Recently the analysis of triglycerides by a combination of analytical techniques in conjunction with fractionation by silicic acid impregnated with silver nitrate has been described by a number of investigators (8–10). Various aspects of the analysis of triglycerides by multiple procedures, which include a fractionation with silicic acid impregnated with silver nitrate, have been elaborated on by Kaufmann et al. (11), Litchfield (12) and McCarthy (13). Described here is a simple method for the application of the technique in conjunction with lipase hydrolysis.

Experimental

Materials and Methods. Tripalmitin and triolein, as well as the methyl esters used in this study, were obtained from The Hormel Institute and were of >99% purity. The preparation of the diacid triglycerides 1-oleo-2,3-dipalmitin and 1-palmito-2,3-diolein used in the standard triglyceride mixtures has been previously described (7). Corn oil and fresh prime steam lard were obtained from commercial sources. A sample of rat liver triglycerides was obtained from a group of animals receiving 10% lard as their sole source of dietary fat. The fat was extracted from the freshly excised livers of the animals with chloroform:methanol (2:1, v/v). The triglycerides were then separated from the other lipid components by silicic acid column chromatography. The homogeneity of the triglyceride fraction was determined by silicic acid TLC.

Triglyceride mixtures. Two mixtures were prepared by a combination of interesterification and randomization to provide a triglyceride composition with a wide variation in unsaturation. One mixture was prepared from an approx equal molar mixture of methyl palmitate, oleate and linoleate. Another mixture was prepared from an approx equal molar mixture of methyl palmitate, oleate and linolenate. These mixtures of esters were simultaneously interesterified and randomized by stirring them with triacetin containing ca. 0.2% sodium methoxide under a high vacuum at room temp for one hr (during this time the methyl acetate was removed and collected in a cold trap) and then at 60C for two hr to complete the randomization. The triglycerides formed in the reaction were freed of impurities by silicic acid column chromatography. The triglyceride composition was determined from the fatty acid composition by calculation on the basis of a random distribution of the fatty acids as shown in the results (Table III).

Fatty acid composition of triglycerides was determined by GLC of the methyl esters. Methylation was carried out by heating 1–10 mg sealed in a glass ampule with 2 ml of 5% HCl in methanol under an atmosphere of nitrogen in a boiling water bath for about one hr.

GLC was carried out with an F & M Scientific Co. Model 609 Flame Ionization instrument equipped with a 6 ft x ¼ in. column packed with 8% ethylene glycol succinate polymer (EGSS-X phase, Applied Science Laboratory) on Chromosorb P at 175C and a carrier gas flow of 55 ml/min.

TLC of triglycerides was carried out on 20 x 20 cm chromatoplates coated with silicic acid impregnated with silver nitrate as described by Barret et al. (6). Two solvents were employed. The low unsaturated triglycerides (3 and less double bonds) were chromatographed with 0.8% methanol in chloroform. Triglycerides containing more than three double bonds were chromatographed with 2–3% methanol in chloroform, depending on the degree of unsaturation.

Triglyceride Structural Analysis. A sample of 10–20 mg of triglyceride, free of other lipids, was chromatographed in bands on a 20 x 20 cm chromatoplate in one or both of the solvent systems described above. The bands of separated triglycerides were located by spraying the chromatoplate with 2,7-dichlorofluorescein (0.1% in ethanol) and viewing it under UV light. Each band was marked and scraped off the plate into a 100-ml beaker containing 20 ml of 5% methanol in ethyl ether. A precisely known wt (usually ca. 0.5 mg) of methyl pentadecanoate was added to the solution which was then fitlered through a small sintered glass funnel and washed several times with fresh 5% methanol in ether. An amt of low boiling

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TABLE I

Fatty Acid Composition of Selected Fraction in the Analysis of Rat Liver Triglycerides

	Total fraction	β-Monoglycerides
S	4.2	5.3
M	52.9	90.0
D	2.0	4.7
15:0*	40.9	

S = saturated acids.
M = monoenoic acids.
D = dienoic acids.
* Represents added internal standard.

petroleum ether (bp 35–60C), equal to about one-third of the volume, was added to the filtrate which was then washed three times with ca. 30 ml of distilled water. The solution was dried over anhydrous sodium sulfate, filtered, reduced in volume to 1–2 ml by evaporation and transferred to a small ampule. The last traces of solvent were then evaporated under reduced pressure. The sample was sealed in an atmosphere of nitrogen with ca. 2 ml of dry 5% methanolic HCl and heated in a boiling water bath for one hr. The methyl esters were recovered, diluted to a known volume with carbon disulfide and analyzed by GLC.

Another sample of each fraction was obtained from additional chromatoplates for lipase hydrolysis. The hydrolysis technique was similar to the general procedure described by Mattson and Volpenhein (14) scaled down to a semi-micro method. The monoglycerides produced by the lipase hydrolysis were isolated by preparative TLC by the general technique used for the analysis of these compounds (15,16) and methylated with dry methanolic HCl as described above. The fatty acid composition of the β-monoglycerides was then determined by GLC of the recovered methyl esters.

The amt of each triglyceride fraction was determined on the basis of the GLC analysis of the known wt of the internal standard. The distribution of the fatty acids among the triglycerides in each fraction was determined from the total fatty acid composition and that of the β-monoglycerides obtained by lipase hydrolysis as illustrated by the following example:

Triglyceride mixture

S	S	M	S = saturated fatty acids
S	D	M	D = dienoic fatty acids
D	S	M	M = monoenoic fatty acids
I	II	III	

This mixture of triglycerides (I, II and III) was obtained as a single band on the chromatography of the sample of rat liver triglycerides, the complete analysis of which is tabulated in the results (Table V). It should be pointed out that only when the amounts of I and II are relatively low in relation to III are all of these triglycerides found in the same hand. The triglyceride composition of any band can be readily determined from a consideration of its R_f value and from its fatty acid composition. Correction for overlapping of bands also can be made from a considera-

TABLE II

Analysis of a Standard Mixture of Triglycerides

	1	2	3	AV	Known	Abs. error
PPP	25.6	27.0	26.1	26.2	25.6	+0.6
PPO	20.4	22.1	23.4	22.0	22.4	−0.4
POO	25.6	19.6	22.6	22.6	23.2	−0.6
OOO	28.4	31.4	27.9	29.2	28.8	+0.4

P = palmitic acid.
O = oleic acid.

TABLE III

Analysis of a Randomized Mixture A, Figure 1, of Triglycerides

TG type	Found	Calc. Rand.
PPP	5.2	4.9
PPO	10.4	9.5
POP	4.3	4.8
POO	8.4	9.3
OPO	5.0	4.7
OOO	4.7	4.6
PPL	8.7	7.4
PLP	3.3	3.7
POL	7.3	7.2
OPL	7.4	7.2
OLP	6.8	7.2
OOL	6.4	7.1
OLO	3.8	3.6
PLL	5.3	5.6
LPL	2.7	2.8
OLL	5.3	5.5
LOL	3.0	2.7
LLL	2.0	2.1

P = palmitic acid.
O = oleic acid.
L = linoleic acid.

tion of the fatty acid composition in relation to those of adjoining bands unless the resolution is very poor. The fatty acid composition of the total fraction and that of the β-monoglycerides produced by lipase hydrolysis is summarized in Table I.

The 15:0 represents 0.50 mg of added standard. Therefore, the total amt of the fraction = [(S+D+M)/15:0] × 0.50 mg = [(4.2 + 52.9 + 2.0)/40.9] × 0.50 = 0.72 mg. In this particular analysis the total sample = 5.91 mg. Therefore, the percentage of this fraction = (0.72/5.91) × 100 = 12.2%. Since the amt of monoenoic acid (M) in this fraction arises entirely from III, the amt of III = (%M/%15:0) × 0.50 mg = (52.9/40.9) × 0.50 = 0.65 mg. The amt of I + II = 0.72 mg − 0.65 mg = 0.07 mg. The individual amt of I and II are calculated from the relative amt of S and D acids in the monoglycerides. It can be seen that together they make up 10% of the total fatty acids. S makes up 53% and D 47% of this fraction (the 10%, Table I). Therefore, the amt of I = (53/100) × 0.07 and the amt of II = (47/100) × 0.07 which is 0.04 and 0.03 mg, respectively. The percentage of each of types I, II and III in the total = 0.7, 0.5 and 11.0% as reported in the results (Table V).

It may be observed that percentage composition of each triglyceride type in the above example may also be calculated directly from the fatty acid composition of the β-monoglyceride and that of the total fraction. For an example of the application of the method of calculation to a more complex mixture of triglycerides, the reader is referred to another report (17) from this laboratory on the application of the method to milk fat.

Results and Discussion

The type of separations of triglycerides that may be effected by silver nitrate-silicic acid adsorbents with mixtures of chloroform-methanol is illustrated in Figure 1. Identification of the components of each band in Figure 1 was made on the basis of fatty acid composition, comparison of R_f values of simple triglycerides of different degrees of unsaturation and from a consideration of the fatty acid composition of the original preparation. Nine separate bands can be distinguished in the chromatography of triglyceride mixture A, and 8 bands in triglyceride mixture B using a solvent system of 0.8% methanol in chloroform. The results in Figure 1 show that the fractionation of triglycerides does not occur strictly on the basis of the total unsaturation. That some bands contain mixtures of triglyceride classes, a class being defined as those with the same total unsaturation, shows that

TABLE IV

Triglyceride Structural Analysis of Natural Fats

	Lard		Corn oil		
	Found	Random	Found	Random	Reduction ozonolysis (7)
SSS	2.4	4.9	tr	0.3	....
SSM	24.1	13.8	1.6	1.7	1.0
SMS	tr	6.9			
SMM	3.5	19.7	4.6	3.4	3.4
MSM	29.8	9.8			
MMM	8.5	14.0	3.9	2.4	2.2
SSD	4.5	3.1	2.7	3.3	2.7
SDS	0.1	1.5			
SMD	0.1	4.4	5.6	4.6	
MSD	14.5	4.4	0.6	4.6	14.9
SDM	0.3	4.4	7.7	4.6	
MMD	4.3	6.3	8.0	9.5	13.5
MDM	2.3	3.1	5.2	4.7	
SDD	2.4	1.5	13.9	9.1	15.5
DSD			0.5	4.6	
MDD	3.2	2.1	19.2	18.9	26.6
DMD			5.0	9.5	
DDD	tr	0.2	21.5	18.9	20.3

tr = trace.
S = saturated acids.
M = monoenoic acids.
D = dienoic acids.

the type of fatty acids, as well as the total unsaturation, influence the separation. A common example of this type of overlapping of classes is that of triolein and monolinoleo-dipalmitin. The same type of overlapping of triglyceride classes have also been observed by Kaufmann et al. (10) using benzene-ether solvent mixtures. Since de Vries (4) and Subbaram and Youngs (9) reported a fractionation of triglycerides by total unsaturation, presumably their system was not as efficient or the finite separations within classes went unrecognized. Another factor which influences the fractionation by silver nitrate-silicic acid TLC is the concn of the components. Generally a component will have a higher relative R_f value when it is present as a major component than when it is a minor component.

The precision and accuracy of the method described here is indicated from the analysis of the standard mixture of triglycerides and the analysis of mixture A of randomized triglycerides (Fig. 1) show in Table II and III, respectively. 0.8% Methanol in chloroform was used as the developing solvent in both of these analyses.

The silver nitrate-lipase procedure, like the ozonolysis method (7), provides an analysis of triglycerides on the basis of the number and type of unsaturated fatty acids. However, the silver nitrate-lipase method gives, in addition, information on positional arrangement and, because of the characteristics of the argentation, a more detailed analysis of the more highly unsaturated glycerides. That the separation does not occur as a simple function of the number of double bonds, but is influenced by the constituent fatty acids, is well demonstrated from the results on the randomized sample (Table III).

TABLE V

Structural Analysis of Rat Liver Triglycerides

Triglyceride type	$AgNO_3$—Lipase technique %		%	VanderWal method (20) %
SSS	1.8	SSS	1.8	2.3
SSM	6.9	SSU	7.6	5.6
SSD	0.7			
SMS	10.3	SUS	10.8	18.5
SDS	0.5			
SMM	51.3			
SMD	3.9	SUU	61.5	44.0
SDM	6.3			
MSM	1.4	USU	1.8	3.3
DSM	0.4			
MMM	11.0			
MMD	1.4	UUU	13.6	26.3
MDM	1.2			
Others*	2.9		2.9	.....

S = saturated acids.
M = monoenoic acids.
D = dienoic acids.
U = unsaturated fatty acids.
* Contains a mixture of SD_2, MD_2 and D_3.

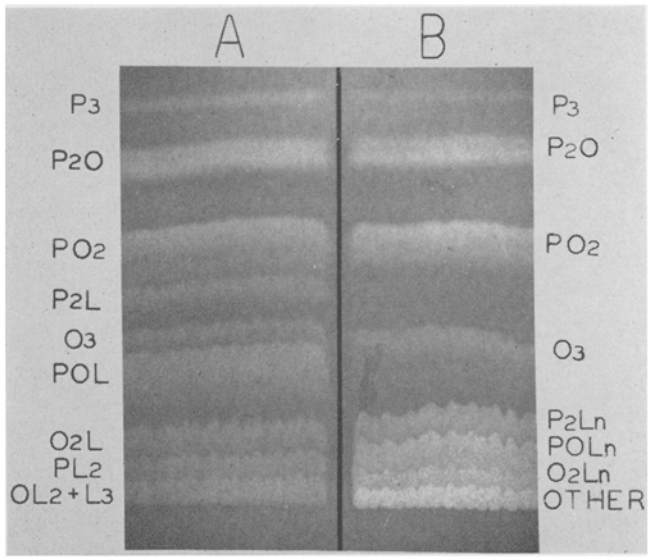

FIG. 1. A—Randomized mixture of triglycerides prepared from palmitate, oleate and linoleate. B—Randomized mixture of triglycerides prepared from palmitate, oleate and linolenate. P = palmitic acid, O = oleic acid, L = linoleic acid, Ln = linolenic acid.

The amt of each fraction separated by silver nitrate-TLC may be determined by a glycerol analysis as described by Litchfield et al. (8) or by the oxidation technique described by Jurriens et al. (18), for example. However, since a fatty acid analysis must be made in any event, the use of a methyl ester as an internal standard is very convenient for the determination of the amt of material in each fraction.

Application of the method to natural fats is illustrated by the results in Table IV on corn oil and lard. In these analyses the less unsaturated triglycerides were developed in a solvent system of 0.8% methanol in chloroform and a second plate developed in 2% methanol in chloroform was used to separate the higher unsaturated triglycerides. The random distribution for these fats is calculated merely for comparison with the experimental values. Comparison of the present results on corn oil and those previously obtained by ozonolysis reduction (7) were in good agreement as shown in Table IV. Unfortunately, the same sample of lard which was analyzed previously (7) was not available for re-analysis and because lard varies in composition (19) results on different samples are not strictly comparable.

It is apparent from the results of the analysis of lard and the sample of rat liver triglycerides which is presented in Table V that no single mathematical relationship is applicable to these fats. The deviation from a random pattern is not large in corn oil, but is probably real. VanderWal's (20) method of triglyceride structural analysis is also not applicable to rat liver triglycerides. It is suspected that mathematical derivation of the distribution of fatty acids among triglycerides are incidental and such relationships can lead to only rough approximations of the actual composition of natural fats.

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Composition of Corn Oil

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Abstract

The composition of commercial corn oil from USA corn is remarkably constant. A total of 103 samples of refined corn oil produced over a period of 2.5 years were analyzed by the alkali isomerization procedure. Nearly 86% of the samples had an iodine value (I.V.) within one unit of the average value, 123.6. Linoleic content on a fatty acid basis, averaged 55.5%; 93% of the values were within two units of this value. All samples contained small amts of linolenic acid. This uniformity undoubtedly results from the system of corn marketing and buying which brings grain from the entire corn belt to the processing plants.

A number of corn oils were analyzed by GLC. The average linoleic acid content by this method was ca. 2.5 units higher than that found by the isomerization method. This difference may occur because GLC responds to all C-18 dienes equally while the alkali isomerization method responds only to conjugatable dienes. Possible sources of error in both methods of fatty acid analysis are discussed.

Corn oil samples taken over a 16-month period were analyzed by GLC. Average values were:

I.V.	Constituent fatty acids (%, fatty acid basis)					
(Wijs)	C-16:0	C-18:0	C-18:1	C-18:2	C-18:3	C-20:0
124.4	11.5	2.2	26.6	58.7	0.8	0.2

Although much of our experience has been with the alkali isomerization method, the GLC technique is preferred because it is simpler and yields more information on fatty acid composition. Another important advantage is that determination of the I.V. of the oil serves as a check on GLC results. The I.V. calculated from the GLC results, making allowance for 1.25% unsaponifiables in the case of corn oil, should be within a few units of the Wijs value.

Oils derived commercially from corns grown in other countries are generally more saturated than those from USA corn. The I.V. of the samples examined varied from 107–125, the linoleic acid contents from 42–56%. The relationship between I.V. and linoleic acid content established by others from hybrid corns holds fairly well for these samples.

Introduction

THE DEGREE OF UNSATURATION of oil from corn germ varies considerably. AOCS tabulation of "Physical and Chemical Characteristics of Oils, Fats and Waxes" shows a spread of 110–128 in I.V. (1). A much greater spread was found by Lofland et al. (2) on examination of strains of corn used in breeding programs. I.V. of the oils extracted from these corns varied from 88–147 and the linoleic acid content from ca. 20–70%. I.V. of the oils from the most common inbreds used by midwestern corn breeders ranged from 111–151 (3). This difference is reflected in the I.V. of the oils from 25 commercial midwestern hybrid corns which have been found to vary from 115–130 (4).

Despite this wide variation in the degree of unsaturation of corn oil from the individual hybrids, commercially available corn oil from midwestern corn (the pre-eminent USA source) is of relatively uniform composition. The purpose of this paper is to offer evidence to support this statement, to discuss methods of fatty acid analysis and possible sources of error, and to show that corn oils derived from corn grown in other parts of the world may differ greatly in composition from that grown in the Midwest.

Considerable information has been published on the composition of commercially available domestic corn oil. These data were reviewed and a number of analyses, including some extreme values, are cited in Table I. Some values are from work in which the sources of the corn oil are not clearly identified but were most likely domestic.

While these literature values will be discussed later, several observations are at once evident. The bulk of the results by all three of the analytical methods (methyl ester distillation, alkali isomerization and GLC) are quite similar for the principal fatty acids. Palmitoleic acid, almost invariably found by methyl ester distillation, was reported by only one of these investigators using GLC. Linolenic acid is almost always reported in results obtained in GLC and alkali isomerization. Results by the last two methods are in fair agreement.

Analytical Methods

Alkali Isomerization. Extreme care is required in carrying out the isomerization technique in order to obtain reproducible results. The method used in this laboratory is essentially that of Brice and Swain