⁂A New Method for Glyceride Composition Determination by Colorimetry

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ABSTRACT

A new method was devised for determination of glyceride composition of fats by oxidation, fractionation of the derived azeleoglycerides by thin layer chromatography and quantitation of the fractions obtained by colorimetry. The method was first standardized with standard triglyceride mixtures and was then applied to vegetable oils and their interesterified mixtures. It appeared to work very well in all cases, including directed interesterified mixtures where the lipase hydrolysis method of glyceride estimation is unsuitable.

INTRODUCTION

Determination of glyceride composition of fats and oils is one of the most interesting fields of observation of fat chemists and demands attention by every possible mode of investigation. Although it may be said that, between 1956 and 1965, a revolution has occurred in this area by the invention of a series of new chromatographic and enzymatic techniques of very high degrees of sensitivity, none of them uses photometric methods for quantitation. We found it interesting, therefore, to carry out studies in an attempt to develop a colorimetric method of estimation of glycerides.

It was found suitable to make use of Palit and Ghosh's observation (1) that aqueous solutions of some dyes, upon extraction with benzene at an appropriate pH, yield extracts that are sensitive to acids, and therefore, estimation of carboxylic acids could be made based on this principle. The method involves a biphasic system and uses readily available, inexpensive chemicals. A method was already established by Chakrabarty et al. (2) for the quantitative estimation of fatty acids based on their reaction with Rhodamine 6G. Since azeleoglycerides contain free carboxyl groups, they were expected to behave similarly; this was found to happen in reality. It is established that Youngs' periodate-permanganate method of oxidation of fats (3) gives nearly quantitative yields of oxidized products with minimal side reactions (4). It seemed possible that, if the oxidized products (obtained by Youngs' method) of fats could be separated effectively by an appropriate method, and the separated fractions extracted quantitatively with benzene, then these aliquots would give a pink-colored solution with Rhodamine 6G of which optical density could be determined photometrically; this could be fruitfully used for the estimation of each individual CO_2H-containing species, and hence, for the estimation of glyceride composition of original fats.

It has been shown that azeleoglycerides could be separated effectively by thin layer chromatography (TLC) on silicic acid (5,6). Our method thus involved the following steps: (a) oxidation, (b) separation of azeleoglycerides by TLC and extraction, and (c) estimation of the fractions by colorimetry.

The samples subjected to this new method of determining glyceride composition included standard triglyceride mixtures, seven vegetable oils (to compare the values obtained by other standardized methods) and a series of interesterified fats prepared from the mixtures of the oils already mentioned, in view of their possible use in various segments of edible and nonedible industries. The glyceride composition of these fats has been the subject of prolonged research in our laboratory (7-9).

EXPERIMENTAL PROCEDURE

Apparatus and Reagents

The spectrophotometer used was a Beckman Du visible spectrophotometer. The glassware consisted of glass-stoppered test tubes (20-ml capacity), glass-stoppered conical flasks and other standard equipment. All glassware used was scrupulously cleaned with warm chromic acid, then throughly washed with distilled water, and finally dried in a hot air-oven. The reagents used were: (a) benzene purified with cold, concentrated sulfuric acid (AR), which was then washed free of acid, dried over anhydrous sodium sulfate and distilled; (b) standard solutions in benzene (concentration = 1 μmol/ml) of fatty acids which are common constituents of lipids, e.g., palmitic, stearic and oleic acids; (c) standard solutions in benzene of single azeleoglycerides obtained by oxidation of standard triglyceride samples. Standard triglycerides were obtained by a series of silver nitrate TLC followed by partition chromatography of the crude fractions obtained by low-temperature solvent crystallization of suitable oils; (d) phosphate buffer solution (0.2 M KH_2PO_4 + 0.2 M NaOH, pH 10-12); (e) Rhodamine 6G (0.005% solution in benzene).

Preparation of Rhodamine 6G Reagent

Rhodamine 6G (10 mg) was dissolved in 10 ml of phosphate buffer, and immediately extracted with 200 ml of benzene. The aqueous layer was discarded, and the clear orange-yellow-colored benzene layer transferred into a thoroughly cleaned and dried amber-colored glass bottle, and preserved over solid sodium hydroxide beads in the dark.

Procedures for Micro Analysis of the Fatty Acids

A suitable volume of the test sample in benzene solution was added from a pipette into a glass-stoppered test tube. The volume was brought up to 4 ml with purified benzene, 2 ml of Rhodamine 6G reagent was added, and the tube was stoppered and shaken for a few seconds to ensure uniform mixing of the contents. A pink color developed almost immediately. After 30 min (although a shorter interval is permissible), the optical density of the solution was measured at 535 nm. A reagent blank without the sample was run under identical conditions and used to adjust the instruments to a transmittance of 100%.

Calibration curves were constructed with known concentrations of palmitic acid, stearic acid and oleic acid, all of which were identical. This also confirms the previous report (2).

Preparation of Standard Triglyceride Samples

Standard triglyceride samples (PPP, PPO, POO, StStO, StOO, OOO) were prepared by: (a) crude fractionation of suitable oils by low-temperature solvent crystallization (10);

TABLE I

Fatty Acid Compositions of the Standard Triglycerides

Sample	Molecular percentages						
	14:0	16:0	18:0	18:1	18:2	20:0	Others
Tripalmitin	--	100	--	--	--	--	--
Dipalmitolein	--	74.6	--	25.4	--	--	--
Dioleopalmitin	--	25.0	--	75.0	--	--	--
Distearolein	--	--	73.7	26.3	--	--	--
Dioleostearin	--	--	24.4	75.6	--	--	--
Triolein	--	--	--	99.6	0.4	--	--
Trilinolein	--	--	--	0.3	99.7	--	--

(b) argentation TLC of the fractions obtained to separate the triglycerides according to unsaturation (11). TLC plates (20 x 40 cm) were coated with Silica Gel G slurry containing 15% $AgNo_3$. Each sample (50-100 mg) was applied in band form in one plate. The plates were developed in usual ascending manner with 99:1 chloroform/methanol solution. The bands were visualized under UV light after spraying 2',7'-dichlorofluorescein solution. The fractions were extracted by the process of Hill et al. (12) using 1% sodium chloride solution in 90% methanol and 90:10 diethyl/methanol containing 0.1 mg BGT/ℓ; (c) liquid liquid partition chromatography (13) of triglycerides containing the same number of double bonds (e.g., PPO and StStO). Kieselguhr plates (0.5 mm) were impregnated with liquid paraffin. Sample size in each plate was 10-20 mg. The plates were developed with 80:20 acetone/methanol saturated with liquid paraffin. The bands were scraped into a small chromatography column (silicic acid) and eluted with diethyl ether (14).

The standard triglycerides thus obtained by a series of TLC were finally tested by (a) argentation TLC and liquid-liquid partition TLC to find whether they gave single spots, and (b) gas liquid chromatography (GLC), which showed that the fatty acid compositions nearly conformed to the ideal values as shown in Table I (In the case of methyl ester of tripalmitin, an internal standard, viz., methyl laurate was added for proper identification. Other fatty acid compositions were calculated from their respective peak areas measured by height of each peak multiplied by width at half-height).

Preparation of Standard Azeleoglyceride Samples and Calibration Curves

About 50 mg of each triglyceride sample was oxidized by Youngs' periodate-permanganate oxidation method (3) because the method gives nearly quantitative yields. The oxidized azeleoglycerides were extracted quantitatively with chloroform, desolventized and standard solutions were prepared by dissolving 1 μmol/ml of benzene. Suitable aliquots were mixed with 2.0 ml Rhodamine 6G, optical densities were measured and calibration curves were constructed (Figs. 1 and 2).

Azeleoglycerides from dipalmitolein and distearolein gave the same straight lines; dioleostearin and dioleopalmitin also behaved similarly. To estimate the amount of trisaturated glycerides, suitable quantitites were hydrolyzed according to the micromethod given by Kates (15). The liberated fatty acid was estimated colorimetrically as before, and the quantity of GS_3 measured accordingly.

Determination of Glyceride Compositions of Standard Triglyceride Mixtures

The standard triglycerides were mixed in suitable ratios. Each mixture (100 mg) was oxidized by Youngs' method (3) and the oxidized mixture was extracted quantitatively with chloroform. The total oxidized mixture, comprising azeleoglycerides and other oxidized components, was applied in horizontal band form on a Silica Gel G (BDH) plate (0.5 mm thick). Standard azeleoglycerides were plotted to the side for identification.

The plates were developed in usual ascending manner with 60:40 hexane/diethyl ether and the bands were visualized with iodine. Four distinct bands were visible and identified as GS_3, GS_2A, GSA_2 and GA_3 by comparison of R_f values of standard azeleoglycerides. Iodine was removed from the plates by blowing nitrogen; the azeleoglyceride bands were scraped carefully and extracted quantitatively with chloroform. The fractions were then

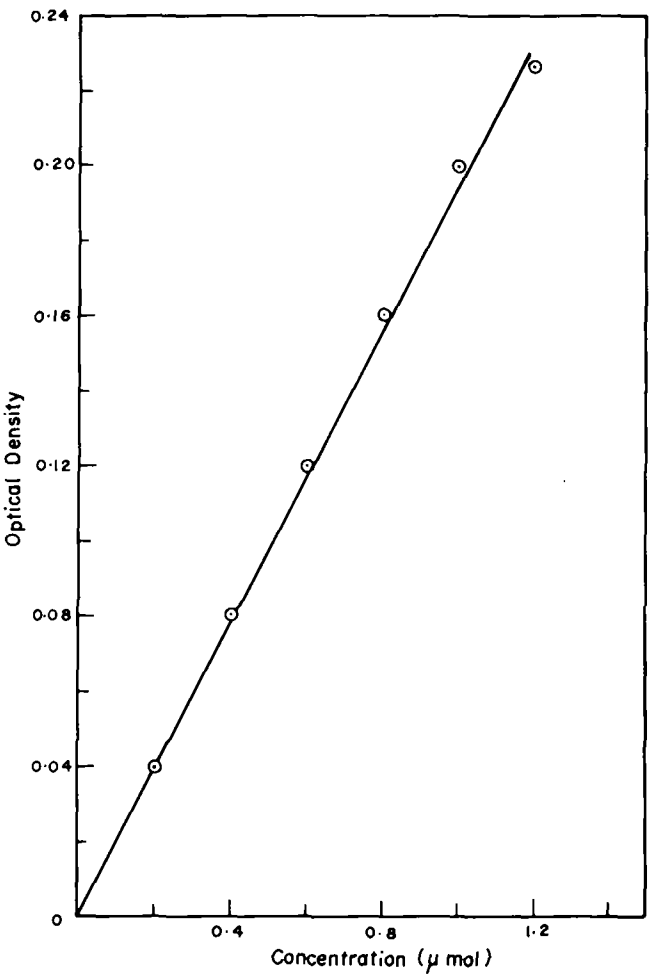

FIG. 1. Calibration graph for GS_2A. S-saturated acid group; A—azelaic acid group.

desolventized, redissolved in 4 ml of purified benzene and mixed with 2 ml of Rhodamine 6G solution. Optical densities were measured as before, at 535 nm, and the fractions were estimated from calibration curves (Fig. 3).

Determination of Glyceride Compositon of Individual Vegetable Oils and Interesterified Fats Obtained from Their Mixtures

The triglyceride compositions of seven vegetable oils were determined by the method described earlier, using 100-mg samples in each case.

The oils examined included (a) rice bran (*Oryza sativa*), cottonseed (*Gossypium hirsutum*), peanut (*Arachis hypogoae*), sunflower (*Helianthus annus*), mowrah (*Madhuca latifolia*), sal (*Shorea robusta*) and palm (*Elaeis guineensis*). Figure 4 shows the chromatogram of standard azeleoglyceride samples with the azeleoglyceride mixture obtained from palm oil.

The glyceride compositions were also determined by Coleman's lipase hydrolysis method (16). The hydrolysis was done with triacylglycerol lipase (steapsin from hog pancreatin, Sigma) at 37.5 C in ammonium chloride/ammonium hydroxide buffer, using a bile salt suspension of the oil in the presence of calcium chloride and stopped by the addition of 4 N HCl. The values obtained were compared to determine the efficiency of our method.

The prospects of interesterification of suitable blends of the oils in various ratios (by standard process, 17) were subjects of intensive research in the authors' laboratory (7-9). Their glyceride compositions were determined by a number of methods, viz., traditional permanganate oxidation method (7), silver nitrate TLC and subsequent GLC with an internal standard (9) lipase hydrolysis (9) and others. Therefore, it seemed valuable to estimate their glyceride compositions by this colorimetric method by the procedure shown. Table IV shows that the glyceride composition of interesterified fats could be determined very efficiently by this method.

DISCUSSION

Table II shows that the quantity estimated by our method for different types of standard triglycerides agrees quite well with the amounts taken.

Moreover, when the results obtained for individual triglyceride classes present in natural vegetable oils are compared with those obtained by the standard method of lipase hydrolysis (16), it is found that the difference in values between the two methods is never greater than 3 units, although when the percentage of error is calculated, it is sometimes quite significant (Table III). These fats may, however, be regarded as sufficiently strong basis for the establishment of the accuracy and sensitivity of the method for a wide range of triglyceride mixtures.

No other method of determination of glyceride composition involves quantitation of azeleoglycerides by the

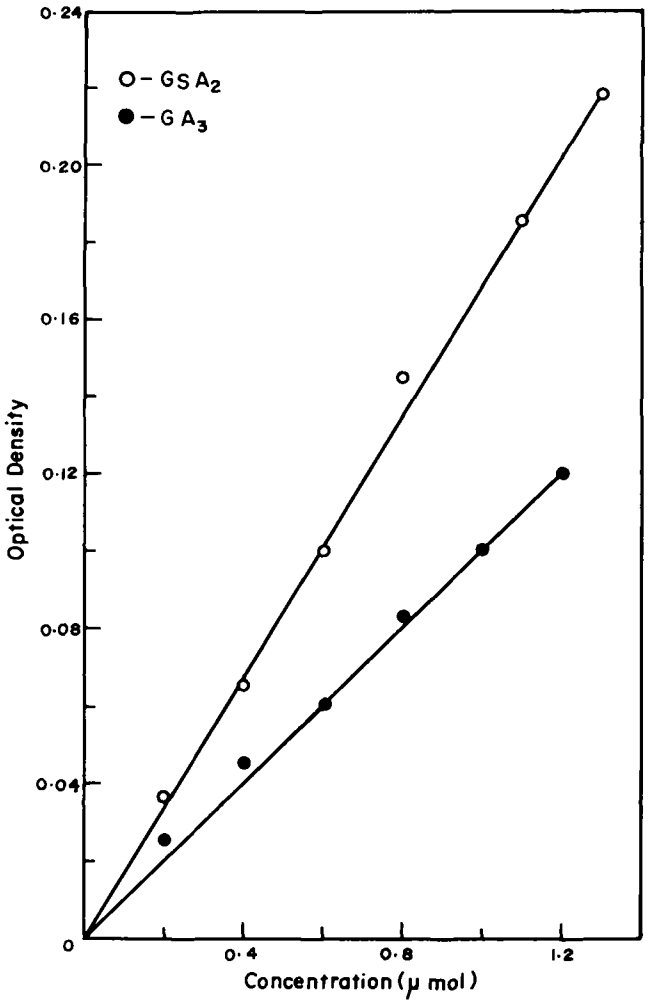

FIG. 2. Calibration graph for GSA_2 (S—saturated acid group; A—azelaic acid group) and GA_3.

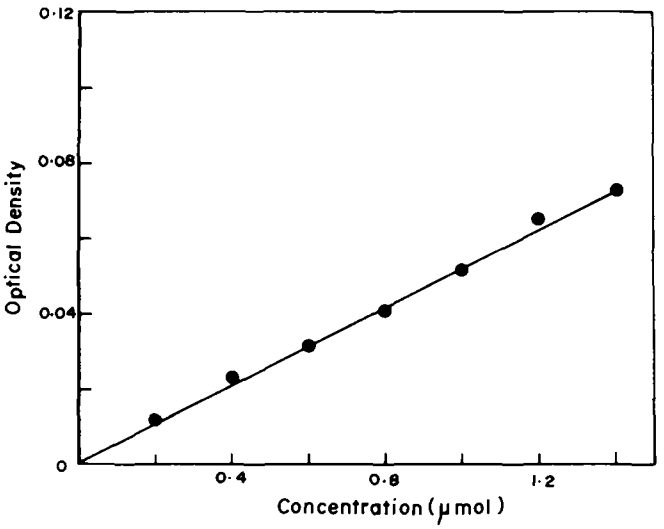

FIG. 3. Calibration curve for fatty acid.

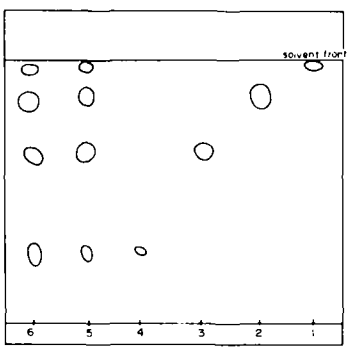

FIG. 4. 1-4—Standard GS_3, GS_2A, GSA_2, GA_3, respectively; 5, 6—oxidized palm oil mixture; solvent system—60:40, hexane/diethyl ether, visualized with iodine.

TABLE II

Estimation of Standard Triglycerides

Triglycerides	μmol taken	μmol found	Percentage of error
Tripalmitin	0.25	0.253	1.20
-do-	0.45	0.446	0.88
-do-	0.65	0.648	0.31
-do-	0.85	0.851	0.12
Dipalmitolein	0.20	0.200	--
Dipalmitolein	0.65	0.657	1.08
Palmitodiolein	0.30	0.31	1.33
Palmitodiolein	0.30	0.303	1.00
Dipalmitolein	0.90	0.91	1.11
-do-	0.45	0.44	2.21
	0.80	0.82	2.50
Triolein	0.20	0.20	--
Dipalmitolein	0.50	0.502	0.4
Triolein	0.60	0.609	1.5
Dipalmitolein	0.90	0.93	3.33
Triolein	0.80	0.81	1.25
Dioleopalmitin	0.30	0.31	3.33
Tripalmitin	0.80	0.79	1.25
Palmitodiolein	0.80	0.81	1.25
Triolein	0.80	0.81	1.25
Dipalmitolein	0.60	0.60	--
Palmitodiolein	0.50	0.49	2.0
Triolein	0.40	0.385	3.75
Tripalmitin	0.20	0.195	2.5
Dipalmitolein	0.30	0.31	3.33
Palmitodiolein	0.40	0.41	2.50
Triolein	0.20	0.20	--
-do-	0.90	0.89	1.11
	0.50	0.502	0.4
	0.60	0.60	--
	0.40	0.385	3.75
-do-	0.60	0.579	3.5
	0.70	0.72	2.86
	0.80	0.79	1.25
	0.60	0.60	--
-do-	0.80	0.795	0.63
	0.90	0.93	3.33
	1.00	1.01	1.00
	0.80	0.79	1.25

colorimetric method. The small size of sample required, efficient separation of azeleoglycerides by silica gel TLC, the stable pink color development with Rhodamine 6G, and remarkable speed (time required per analysis, 6-8 hours), accuracy, sensitivity and efficiency of the method all make the method quite suitable for use in routine analysis.

The method cannot, however, distinguish the different unsaturated acyl groups, as all of them would be converted to C_9 monobasic acids. When the percentages of only the four major classes of triglycerides are required, this method could be applied for routine analysis quite effectively.

Table IV shows that the method also permits estimation of the glycerides present in random and directed interesterified fats. This may be regarded as another interesting feature of the study, since the lipase hydrolysis method is not applicable to directed interesterified fats. Upon randomization, there is a reorganization of glyceride spectrum according to 1, 2, 3—random theory (18). The percentage of different glycerides agree quite well with those calculated theoretically. Upon directed interesterification, the proportions of GS_3 and GU_3 components always increase. The concentration of GSU_2 is decreased and that of GS_2U is increased or decreased according to fatty acid compositions.

The method would seem to be very useful for the routine analysis of the four major classes of glycerides present in natural and interesterified fats.

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TABLE III

Glyceride Compositions of Individual Oils by (a) Colorimetry and (b) Lipase Hydrolysis Method

Name of oil	Slip point (C)	Method	Molecular percentages			
			GS_3	GS_2U	GSU_2	GU_3
Rice bran	<0	a	--	20.5	49.7	29.8
		b	0.3	20.4	49.8	29.5
Cottonseed	<0	a	--	22.4	44.5	33.1
		b	0.6	21.3	47.2	30.9
Groundnut	<0	a	--	9.0	38.5	52.5
		b	0.2	8.1	39.5	52.2
Sunflower	<0	a	--	3.9	29.4	66.7
		b	–	2.6	28.5	68.9
Mowrah	28	a	--	49.2	39.2	11.6
		b	0.6	51.3	37.1	11.0
Palm	33	a	4.7	51.2	39.4	4.7
		b	5.4	48.9	39.2	6.5
Sal	28	a	1.9	63.6	31.3	3.2
		b	4.0	64.5	29.1	2.4

TABLE IV

Glyceride Compositions of Interesterified Fats by Colorimetry

	Name of oil mixture	Slip point (C)	Molecular percentages			
			GS_3	GS_2U	GSU_2	GU_3
1.	Ricebran and palm (1:1, w/w) (original mixture)	31	2.2	37.5	40.8	19.5
2.	-do- (Randomized)	33	7.2	29.4	43.6	19.8
3.	-do- (Directed interesterified)	35	11.0	30.5	28.1	30.4
4.	-do-	42	11.3	35.6	19.2	33.9
5.	Ricebran and cottonseed (1:1, w/w)	0	--	23.5	41.8	34.7
6.	-do- (Randomized)	17	1.0	20.6	45.1	33.3
7.	-do- (Directed interesterified)	35	9.9	35.6	11.9	42.6
8.	-do-	40	12.3	31.7	17.9	38.1
9.	Ricebran and mowrah (1:1, w/w) (original mixt.)	28	--	33.7	41.6	24.7
10.	-do- (Randomized)	33	7.8	28.6	42.5	21.1
11.	-do- (Directed interesterified)	35	12.6	28.1	30.1	29.2
12.	-do-	40	12.7	30.0	19.4	37.9
13.	Ricebran and sal (1:1, w/w) (original mixt.)	15	0.6	43.5	37.5	18.4
14.	-do- (Randomized)	36	9.8	27.5	43.1	19.6
15.	-do- (Directed interesterified)	38	10.2	29.6	25.5	34.7
16.	-do-	40	12.2	29.9	24.5	33.4
17.	Sal and cottonseed (1:1, w/w)	24	1.5	43.7	35.4	19.4
18.	-do- (Randomized)	41	9.9	35.8	35.7	18.6
19.	-do- (Directed interesterified)	43	10.6	37.5	33.3	18.6
20.	Sal and sunflower (1:1, w/w)	27	1.0	31.3	31.3	36.3
21.	-do- (Randomized)	38	9.3	21.5	41.1	28.0
22.	-do- (Directed interesterified)	43	9.7	22.3	38.7	29.2
23.	-do-	45	11.1	30.3	29.2	29.4
24.	Sal and groundnut (3:7, w/w)	26	--	20.2	47.1	32.7
25.	-do- (Randomized)	30	2.9	18.2	41.9	37.0
26.	-do-	36	10.4	13.6	32.4	43.6
27.	-do-	40	11.3	13.8	30.3	44.6

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