Rapid Densitometric Determination of Triglyceride Groups by Argentation Thin Layer Chromatography

D. CHOBANOV, R. TARANDJISKA, and R. CHOBANOVA, Institute of Organic Chemistry, Bulgarian Academy of Sciences, Sofia 13, Bulgaria

ABSTRACT

A method has been developed for the quantitative thin layer chromatographic determination of the triglyceride groups, differing in unsaturation, based on densitometry of the charred bands. Complete separation was achieved by continuous development in an open glass jar. The need for correction coefficients was avoided through addition of bromine to the double bonds prior to charring in the presence of sulphuryl chloride. The accuracy and precision of the method were evaluated on a standard mixture and on sunflower oil, olive oil, lard, cocoa butter, and beef tallow. The method is applicable for triglyceride group analysis of the most common fats and oils which contain saturated, monoene, and diene acids.

INTRODUCTION

Barrett et al. (1,2) introduced argentation thin layer chromatography (TLC) for separating natural triglycerides according to their degrees of unsaturation. Direct densitometric measurement of the spots made their method simple and rapid. However, the need for correction coefficients for quantitation of the peaks constrained Kaufmann et al. (3) to use the two-dimensional technique for this purpose. For the same reason, many workers prefer to elute each zone from a preparative plate and measure the recovered fractions by such methods as gravimetry (4,5), gas liquid chromatography of the methyl esters with added internal standard (6,7), colorimetry after chromotropic (8-10) or hydroxamic acid (11,12) reaction, and titration after periodate-oxidation (13). These methods are very useful as part of more detailed analyses by consecutive chromatographic and enzymatic procedures (14) but are laborious and time-consuming for a routine practice.

Several modifications in the method of Barrett et al. (2) with respect to preparation of the chromatoplates, development, and especially charring of zones has led to the rapid and simple procedure of quantitative analysis described in this paper. Presented here are results of its application to both a standard mixture and some natural oils and fats, used to obtain accuracy and precision data.

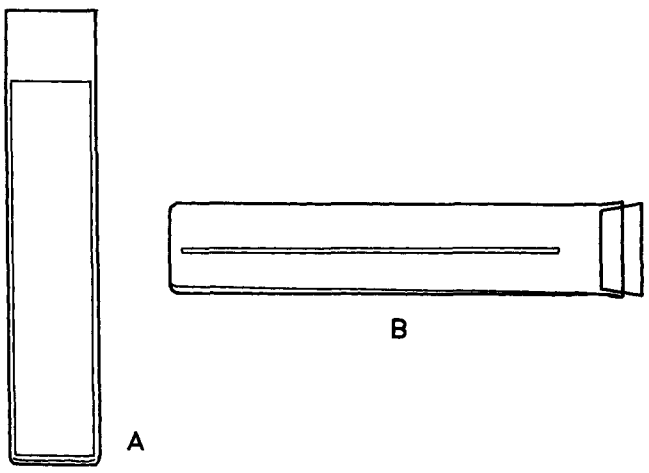

FIG. 1. Cylindrical jars for continuous ascending development (A), impregnation with 0.5% methanolic silver nitrate (A), and exposure to bromine and sulphuryl chloride vapors (B). Dimensions correspond to those of the plate (4 x 19 cm).

EXPERIMENTAL PROCEDURES

Acetone, heptane, and petroleum ether (br 40-70 C) were redistilled reagent grade solvents. Sulphuryl chloride and Silica Gel G for TLC were purchased from Merck (Darmstadt, W. Germany). The triglycerides (TG) of the oils and fats (commercial samples) were isolated by preparative TLC on silica gel, using petroleum ether:acetone (100:8, v/v). Trilinolein, triolein, and tristearin were isolated from sunflower oil, olive oil, fully hydrogenated lard, respectively, by a preparative argentation TLC method that will be published elsewhere.

Glass plates (4 x 19 cm) were cut from spectral thin plates and roughened on the face to be coated as recommended by Barrett et al. (2) A series of five uniform sized plates was coated with a slurry of Silica Gel G (4 g) in distilled water (10 ml) to give a layer 150-180 μ thick by a spreader similar to that of Goodall (15). The chromatoplates were allowed to dry overnight at room temperature. They were then impregnated by vertically dipping (3) in a 0.5% methanolic silver nitrate solution, using a glass cylinder (Fig. 1-A). The air-dried chromatoplates were activated 5 min in an oven at 110 C prior to use.

A self-made glass sample applicator, constructed on the principle described by Achaval et al. (16), was used to apply the sample solution in heptane as an even, narrow, 5 mm long band. Usually 15-35 μl of 0.1% sample solution was used, depending on triglyceride (TG) composition. Larger volumes were carefully applied, when necessary, in 10 μl portions several times on the initial band.

Continuous ascending development in an open glass cylindrical jar (Fig. 1-A) was applied instead of that used in sandwich type equipment. Attempts to achieve resolutions like those in the chromatograms of Figure 2 for 19-cm long plates failed when single or multiple developments (8,10) in closed tanks were used. A filter paper disk with nearly the same diameter was placed on the even bottom of the jar, providing uniform development. Usually 5 ml of a petroleum ether:acetone (100:4, v/v) mixture was used per plate at room temperature to obtain good separation for the samples studied. All the developing solvent was allowed to move through the layer and evaporate at the upper end of the plate.

The plates were heated in an oven at 100 C for at least 1 hr, then placed for 30 min in a closed horizontal cylindrical jar over a uniform layer of liquid bromine and transferred for 30 min in a second jar over liquid sulphuryl chloride, as shown in Figure 1-B. In this way, the chromatogram is evenly saturated with the vapors of the charring agent. The plates were then heated 10-15 min at 150-180 C over a temperature-controlled metal plate. The zones appeared as dark brown to near black bands on an almost white background.

Densities of the charred substances were measured for a band along the chromatogram, on which the zones were situated, the two spot-free sides being covered with a glass screen to avoid scattering of light. A Carl Zeiss Densitometer (Model ERI 65 m, Jena) with no filter, adjusted for highest sensibility of transmitted light, was used. The peaks

of the differential densitometric curve were measured by triangulation. For that reason, only those densitograms showing smooth and almost straight base lines were used for quantitative evaluation. This was especially valid for the partially overlapping peaks, whose areas were measured by means of the common rule of interpolation. The absolute area under each peak was taken as relative percent, which corresponded to the wt content of the substance.

The same procedure was applied for TLC quantitative determination of the methyl esters of the oils and fats. The pure TGs were converted into methyl esters by the method of Hartman et al. (17).

A mixture of equal parts of the chromatographically purified TGs of sunflower oil and lard and a small amount of tristearin was separated and the bands identified, using the data of Kaufmann et al. (5). Identification of the bands of the oils and fats under study was made with the aid of this reference mixture by comparing the bands on one and the same chromatogram. Literature data were also used for the purpose.

RESULTS AND DISCUSSION

Comment on the Procedure

The solvent mixture was adjusted to the smallest possible amount of silver nitrate because of its negative effect during charring. The acetone content should be tested and, depending on temperature and TG composition, adjusted before use if necessary.

It is important to develop the chromatograms in an open jar to obtain the following advantages: (a) better resolution of bands, (b) maintenance of each zone under the same conditions, (c) simple equipment, and (d) night development.

Double bond cleavage was supposed to take place during charring at elevated temperatures in the presence of silver nitrate. The shorter chain fragments formed evaporate probably in part, thus lowering the values for the unsaturated TGs. The results of Barrett et al. (2) could be explained by this phenomenon. Because preliminary hydrogenation of the sample (18,19) is not applicable in this case, addition of bromine to the double bonds prior to charring with sulphuryl chloride (20) was found to be very important in this study. The need for correction coefficients was thus avoided (Table I).

TABLE I

Accuracy and Precision Data for a Standard Mixture from 20 Analyses

	Relative % by wt		
	Trilinolein	Triolein	Tristearin
Known value	34.5	30.6	34.9
Mean value	35.3	29.6	35.0
Standard deviation	±0.8	±0.8	±0.9
Relative SD	±2.2	±2.5	±2.6
Mean error	±0.8	±1.0	±0.1
Relative error	±2.3	±3.3	±0.3

The polarity of the eluent can be conveniently correlated with its volume, used for continuous development. For example, TGs with up to four double bonds can be better separated using 10 ml of a petroleum ether:acetone (100:2, v/v) mixture. The distances between the bands of the more unsaturated TGs increase with polarity, a suitable eluent being 4 ml of petroleum ether:acetone (100:12, v/v) for plates, impregnated by dipping in 1% methanolic silver nitrate.

Accuracy and Precision Data

A standard mixture of tristearin, triolein, and trilinolein was used to judge the accuracy and precision of the method. Under the conditions of the procedure, chromatograms were obtained in which the three TGs were situated ca. on the same positions as those in Figure 2. Statistical calculations were conducted on a series of 20 chromatograms for the content of each TG. The results are presented in Table I.

Both the accuracy and precision data are almost equal for the three TGs, irrespective of their unsaturation, R_f value, and shape of spot. Inasmuch as the amounts are almost equal, it follows from the SD values that no systematic error could be expected. It is thus obvious that the bromination of the separated TGs is a necessary step in the procedure.

Similarly, the method was examined for analysis of natural TG mixtures. Five oils and fats, differing in unsaturation, were selected so as to estimate the precision when TG is present in negligible or considerable amounts. The results of three chromatograms for a sample were used to calculate the SD for each TG present. Both the TG group

TABLE II

Triglyceride Group Composition of Oils and Fats

Triglyceride group	Relative % by wt				
	Sunflower oil	Olive oil	Lard	Cocoa butter	Tallow
D_3[a]	24.6[b] ± 0.7[c]	-	-	-	-
MDT	-	0.9 ± 0.2	-	-	-
M_2T	-	1.0 ± 0.4	-	-	-
DMD	30.0 ± 0.6	1.6 ± 0.4	1.8 ± 0.3	-	-
MDD			1.3 ± 0.2	-	-
DSD	18.8 ± 0.3	1.1 ± 0.1	1.4 ± 0.2	0.7 ± 0.1	-
SDD			1.6 ± 0.2		-
M_2D	8.9 ± 0.2	12.2 ± 1.5	7.6 ± 1.1	0.4 ± 0.1	-
SMD	10.6 ± 1.0	6.4 ± 1.4	18.1 ± 2.4	2.8 ± 0.2	1.3 ± 0.4
M_3	1.3 ± 0.3	39.4 ± 1.8	8.0 ± 0.8	0.7 ± 0.1	5.9 ± 1.2
S_2D	2.0 ± 0.3		7.0 ± 0.7	9.7 ± 0.8	1.5 ± 0.2
SM_2	2.7 ± 0.8	29.5 ± 2.4	27.9 ± 1.3	13.5 ± 0.6	35.5 ± 1.0
SMMt	-	-	-	-	1.7 ± 0.9
SSM	1.1 ± 0.1	7.9 ± 0.2	20.5 ± 2.6	70.3 ± 0.2	13.9 ± 0.7
SMS					22.5 ± 1.2
S_2Mt	-	-	-	-	0.8 ± 0.3
S_3	-	-	4.9 ± 0.5	1.9 ± 0.8	16.8 ± 0.6

[a]Acyl groups in the triglycerides: S = saturated, M = *cis*-monoene, Mt = *trans*-monoene, D = all-*cis*-diene, and T = all-*cis*-triene. The order of the letters is not important, except when positional isomers are being separated.

[b]Mean value of triplicate analysis.

[c]Standard deviation.

TABLE III

Calculated and Found Fatty Acid Group Composition of the Oils and Fats

	Relative % by wt									
	Sunflower oil		Olive oil		Lard		Cocoa butter		Tallow	
Acid group	Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found
D[a]	64.3[b]	63.2[c]	8.3	11.6	14.9	12.8	4.9	4.0	0.9	1.1
M	22.9	23.2	73.7	70.3	45.5	47.3	32.0	33.7	42.8	43.3
Mt	-	-	-	-	-	-	-	-	0.8	1.6
S	12.8	13.7	18.0	18.1	39.5	39.9	63.0	62.2	55.4	54.0

[a]Acyl groups in the triglycerides: S = saturated, M = *cis*-monoene, Mt = *trans*-monoene, D = all-*cis*-diene. The order of the letters is not important, except when positional isomers are being separated.

[b]Calculated from triglyceride group composition in Table II.

[c]Found by argentation thin layer chromatography methyl ester analysis.

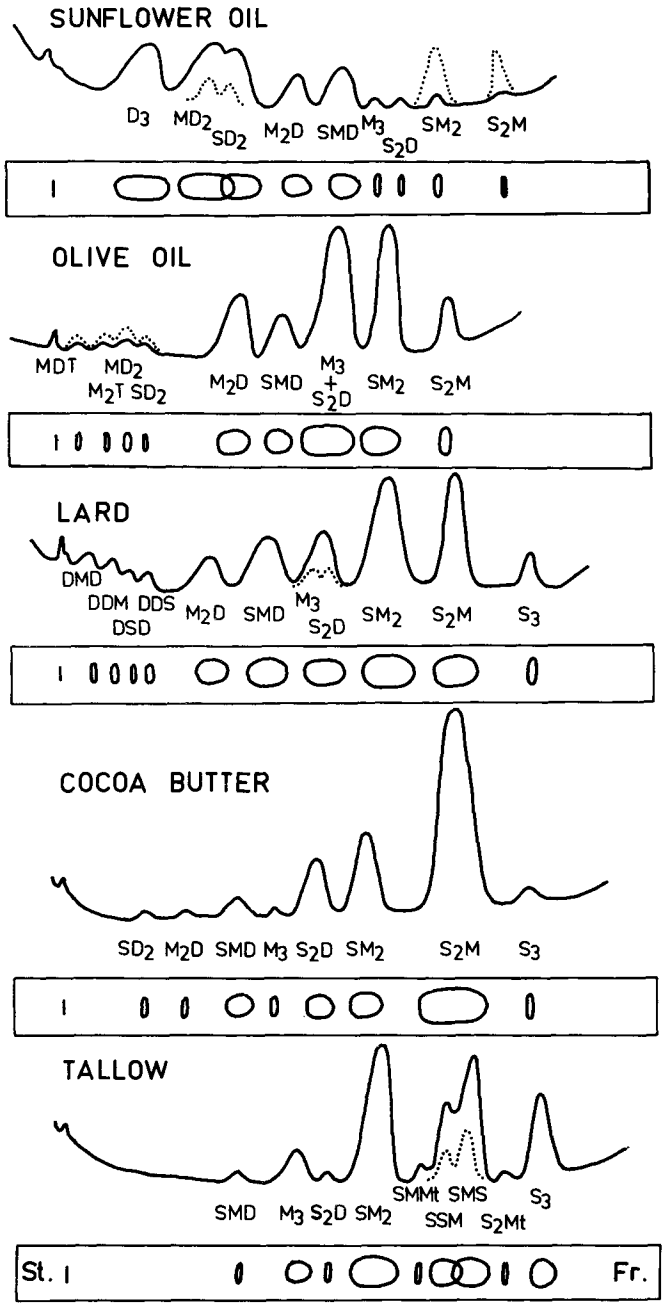

FIG. 2. Separation of oils and fats by argentation thin layer chromatography. For the sake of clarity, chromatograms are diagrammatically depicted. Chromatographic conditions are described in Experimental Procedures. Acyl groups in the triglycerides: S = saturated, M = *cis*-monoene, Mt = *trans*-monoene, D = all-*cis*-diene, and T = all-*cis*-triene. The order of the letters is not important, except when positional isomers are being separated. St = start, Fr = front.

composition and the respective SD values are given in Table II, which shows that the SD values are reliable enough for such a small number of analyses. A good coincidence between the SD values for a TG group in the different oils and fats was observed. The precision is not influenced by a systematic error again.

On the other hand, an indirect approach was applied to follow the accuracy of the method when oils and fats are analyzed. The fatty acid group composition, calculated from the TGs, was compared with that experimentally determined by TLC. The results, represented in Table III, show that the values are closely related and that a small random error is being admitted. Consequently, the average values of only three good chromatograms are reliable for the determination of the TG group composition of oils and fats containing the above acids.

Application to Natural Oils and Fats

Separations of the TG groups of the fats and oils studied are summarized in Figure 2.

A typical densitogram of Bulgarian sunflower oil (solid line) was used to calculate the areas of all the peaks. However, the peaks of MD_2 and SD_2 groups overlapped and that of S_2M was too small. The exact quantities of these groups were determined by means of two additional chromatograms. The ratio of MD_2 to SD_2 was established by separating a 5 μl of 0.1% sample solution, as shown by the dotted line. This ratio was used to divide their sum, known from the first chromatogram. Similarly, a third chromatogram was obtained with 80 μl sample solution to exactly determine the ratio of the peaks of S_2M and SM_2, the known quantity of the latter being used as "internal standard." In general the composition found is in good agreement with the values reported for Bulgarian sunflower oil (21,22) and other oils (5,23).

The four small peaks of the Spanish olive oil densitogram were identified by comparison with those reported by Kaufmann et al. (9). To increase accuracy, these groups were determined with the aid of a chromatogram, applying 40 μl of sample solution and using M_2D group as an internal standard in the manner already described. This separation is represented by the dotted line. Attempts to separate M_3 and S_2D groups failed due to the great quantity of the former. For that reason, the calculated amount of diene acid group was lower than the found one (Table III). The TG composition reported here is closely related to that found for olive oils with similar fatty acid contents (9,24-26).

The TG groups of lard were identified by the standard mixture. The groups M_3 and S_2D were separated as before by a second chromatogram starting from a smaller amount. The four peaks near the start line were identified as positional isomers of the groups MD_2 and SD_2. The two respective bands formed in a preparative chromatogram from larger sample were scraped, and their fatty acid com-

position corresponded to the above groups. Thus, it was concluded that their positional isomers are separated when a much smaller amount of sample is used for analysis. The position of each isomer was suggested from the theoretical considerations of the π-complexing of the silver ion. The composition of the lard TG groups is in general agreement with data published previously (7,27).

Cocoa butter was chosen as an example to prove the applicability of the method when a larger amount of a TG group is presented. The SD_2, M_2D, and M_3 groups showed too small peaks to be exactly evaluated. For that reason, a second separation was performed with 70 μl sample solution. The quantity of the SMD group, found in the first densitogram, was used as internal standard in the second run, the total composition of the fat then being recalculated from both densitograms. Taking into account these groups, a slightly lower value for S_2M resulted than that found by other workers (2,27-29). However, the calculated fatty acid composition was close to the estimated one (Table III), thus confirming the reported TG composition.

Nine TG groups were separated from beef tallow. Of special interest is the resolution of the two groups containing *trans*-monoene acids, i.e., SMMt and S_2Mt, identified by reference substances. Irrespective of the large quantity present, the positional isomers SSM and SMS were also determined, especially when a smaller amount was used (dotted line). Their respective positions are well known (1,2). The remaining groups were identified by the standard mixture and evaluated as described before. This composition of beef tallow (Table II) is comparable to that given by Luddy et al. (30), taking into account the discrepancy in the fatty acid contents.

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