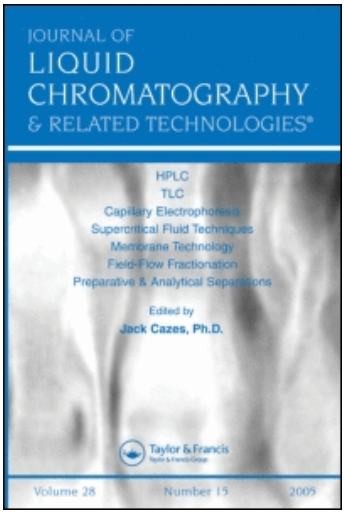

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DETERMINATION OF MOLECULAR SPECIES OF TRIACYLGLYCEROLS FROM HIGHLY UNSATURATED PLANT OILS BY SUCCESSIVE APPLICATION OF SILVER ION AND REVERSED PHASE TLC

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ABSTRACT

A method for the quantitative determination of molecular species of triacylglycerols in highly unsaturated plant oils by consecutive use of different TLC techniques is described. Silver ion TLC in both analytical and preparative mode has been followed by reversed phase TLC to give results compatible with those obtained with RP-HPLC and capillary GLC. The method has been applied to corn and cotton seed oils. The number of the separated and quantified triacylglycerol species in these oils prevails those reported in the literature.

INTRODUCTION

The detailed determination of the triacylglycerol (TAG) composition of natural fats and oils has always been one of the most important but difficult tasks in the lipid analysis. The analyte is a complex mixture of molecular species with very similar chemical properties and chromatographic separation is absolutely necessary in

order to obtain reasonable results. It is accepted now that there is no single chromatographic method capable to provide complete resolution of all components of a natural TAG mixture. A properly chosen sequence of chromatographic separations provides much more detailed and unambiguous information on sample composition [1] than does any single method irrespectively of the principles and instrumentation used. Among the chromatographic techniques available, silver ion chromatography has a key position in that it separates triglycerides on the basis of a single molecule property - degree of unsaturation [2]. Subsequent fractionation by high temperature gas liquid chromatography (GLC) reversed-phase thin-layer chromatography (TLC) or reversed-phase high performance liquid chromatography (RP-HPLC) is based on the different chain-length or overall polarity of the TAG molecules.

The great value of the complementary separations of TAG by silver ion chromatography and reversed phase chromatography was recognised long ago and the achievements has been recently reviewed [3]. Naturally, in the early seventies most attention was paid to the combination of silver ion TLC (Ag-TLC) and reversed-phase TLC (RP-TLC). Unfortunately, in those days both Ag-TLC and RP-TLC were messy techniques that were not easy to control. The results were mainly qualitative [2]. *In situ* quantitation was examined [4] but found no wide application and messy procedures which included scrapping, elution and transmethylation of the zones, and GC analysis of the component fatty acids were usually applied. Most of the drawbacks of both Ag-TLC and RP-TLC were, however, successfully overcomed and at present a well established procedures which include *in situ* quantitation by densitometry are available [4-9]. On the other hand, for a long period, RP-HPLC was considered as the only technique capable to solve all problems of the TAG analysis. However identification of dienoic and trienoic plant TAG was and definitely is neither easy nor complete and detection problems hampered the quantitative analysis in great extent. Thus, the sequential application of Ag-TLC and RP-TLC, provided the most detailed quantitative information on the TAG structure of sunflower oil, olive oil and peanut oil [7,9] before the successful utilization of a stable silver ion column by Christie [10] converted the powerful combination of Ag-HPLC and RP-HPLC into a handy and convenient analytical procedure [11,12,13]. However, a limited number of natural TAG mixtures has been analysed by the complementary application of these two methods at present and most of the published results relayed on RP-HPLC only.

Obviously, TLC techniques can not compete in speed with the HPLC, but are efficient alternatives to the more expensive and sophisticated HPLC procedures. In the preparative mode presented bellow Ag-TLC can be a successful aid to subsequent RP-HPLC. Moreover, it is demonstrated in this work that the combination of silver ion TLC and reverse phase TLC is capable to resolve complex mixtures of unsaturated seed oils TAG such as corn and cotton. The number of the separated and quantified TAGs species in these oils prevails those reported in the literature.

EXPERIMENTAL

Materials, Chemicals and Samples

All reagents and solvents were analytical grade. Solvents were distilled before use. Petroleum ether was a b.p. 40-60°C fraction. Diethyl ether was peroxide-free, and chloroform, when used as a mobile phase component, was treated to remove the stabilizing alcohol. Dimethyldichlorosilane (DMDS) was purchased from Fluka (Switzerland) and was used as a silanizing reagent. Kieselguhr G and silica gel G were obtained from Merck (Germany). Sulfuryl chloride (Merck, Germany) and 50% solution of sulfuric acid in ethanol were used as charring reagents.

Corn and cotton oils were purchased from local suppliers.

TAG fraction was isolated by preparative silica gel TLC (1 mm thick layer) with a mobile phase of petroleum ether - acetone, 100:10 (v/v). The purified TAG were dissolved in hexane to give a 0.5% solution.

A standard mixture was prepared by mixing equal quantities of purified TAGs from lard and sunflower oils purified as described above; added to this mixture was certain amount of tristearine in order to increase the proportion of the trisaturated TAG (SSS, S, for saturated fatty acid moiety) to a reasonable value. This mixture was used to identify the TAGs from SSS to DDD (D-dienoic fatty acid moieties). A pure TAG fraction from tangerine oil with known composition [14,15] was used to identify TAGs which contained linolenic acid (trienoic fatty acid or T).

Ag-TLC

Quantitative mode

The procedure is described in details elsewhere [5,14,15]. Briefly, TAG classes differing in unsaturation were separated on 19 x 4 cm glass plates, coated

with ca 0.2 mm silica gel G layer and impregnated by dipping with a 0.5% methanolic solution of silver nitrate. An aliquot of 5-10 μl of the sample (about 20-40 μg) was applied to a plate. Plate was developed with a defined volume of the mobile phase in open cylindrical tanks (24 cm x 5 cm i.d) and the whole volume was allowed to pass through the plate. It was then dried (1 hour at 110°C), and treated consecutively with bromine and sulphuryl chloride vapours (30 min each, in closed tanks and in fume-cupboard). The separated TAG classes were finally charred by heating at 180-200°C on temperature-controlled metal plate.

Preparative mode

Preparative Ag-TLC was carried out as described in [6]. Namely, TAG classes were separated on 20x20 cm home-made glass plates covered with ca. 1 mm thick silica gel G layer which contained 5% silver nitrate. Plates were sprayed with 2',7'-dichlorofluorescein and TAG zones were vizualised under UV light. They were scrapped, transferred to small chromatographic columns and eluted with diethyl ether. The purity and identity of each zone was checked by analytical Ag-TLC after cochromatographing with the reference TAG mixture and the source oil, applied alongside. The solvent was removed by evaporation under nitrogen and samples were redissolved in hexane to give a 0.1% solution.

Quantitative RP-TLC

The procedure described in reference [9] was applied. In brief, 19 x 4 cm glass plates covered with ca. 0.2 mm thick Kieselguhr G layer were first treated for 6 hours with vapours of DMDS and then washed by a single elution with methanol. A 5-10 μl aliquot of the 0.1% TAGs chloroform solution was applied on the plate and developed twice in closed cylindrical tank (dimensions as shown above), each time with fresh 3 ml of the mobile phase to a solvent front of 17 cm. A mixture of acetone/acetonitrile/water was used as a mobile phase. The ratio acetone/acetonitrile was kept constant, 7/3 (v/v), while the proportion of water was varied depending on the TAG composition.

Plates were dried (at 110°C for 1 hour) and separated TAG species were vizualized by spraying with 50% ethanolic sulphuric acid and heating at 200-220°C for about 5 min over a temperature-controlled metal plate.

Densitometry.

The densities of the charred spots were measured by a Shimadzu CS-930 densitometer, equipped with DR-2 Shimadzu integrator, in the zigzag reflection mode at 450 nm. Beam-slit was varied from 0.4x0.4 mm to 1.2x1.2 mm depending on the separation achieved. The quantity of each spot was presented as relative area percent, as derived from the integrator.

Two sets of densitometric results were obtained: Ag-TLC provided the quantitative data for the TAG classes differing in unsaturation and RP-TLC - for the TAG species differing in chain-length within a given class. Obviously, the Ag-TLC results were of vital importance as they were used as a base to recalculate the RP-TLC results and to produce the final data for the TAG composition of the sample.

RESULTS AND DISCUSSION

Ag-TLC

Cotton and corn oil are of certain industrial interest and have been intensively studied by Ag-TLC [16,17,18,19] and RP-HPLC [20,21]. Therefore, they were used in this study to demonstrate the ability of the combination of Ag-TLC and RP-TLC in TAG analysis, as it was possible to compare our results with those obtained by others.

Our experience in Ag-TLC revealed that three different developments on three different plates are necessary in case linolenic acid is present in the sample, even at contents lower than 1% [14,22]. The chromatographic conditions are presented in Table 1.

The first column presents the condition suitable for separation and determination of the SSS, SSM (M, for monoenoic fatty acid moiety) and SMM classes (Fig.1-A). SSS was not found in the examined oils even by heavy overloading. The SMM/SSM ratio was determined under this conditions.

On a separate plate, under the conditions presented in the second column we were able to separate all TAGs but SSM (Fig. 1B). These conditions did not provide satisfactory resolution of TAG classes with higher unsaturation than SDD. These TAG classes (denoted further in the text as "SPUTAG" (Sum of the Polyunsaturated TAG)) therefore were quantified as a sum. The critical point at

TABLE 1.

Chromatographic Conditions for the Separation of TAG Classes by Silver Ion TLC*

	Separation of S_3, SM_2 and S_2M			Separation of TAG from S_2M to T_3			Separation of the poly-unsaturated TAGs (Σp.u.)		
Oil	sample (μg)	mobile phase (v/v)	volume (ml)	sample (μg)	mobile phase (v/v/v)	volume (ml)	sample (μg)	mobile phase (v/v/v)	volume (ml)
cottonseed	35	PE:A 100:5 Fig.1A	7	30	PE:A:EA 100:4:2 Fig.1B	12	20	PE:A:EA 100:4:2 Fig.1C	12
corn	35-40	PE:A 100:5	7	30-35	PE:A 100:8 + 100:5	5 6	25-30	PE:A 100:8 + 100:5	5 6

* PE - petroleum ether (b.p. 40-60°C)
A - acetone
EA - ethylacetate
S - saturated, M - monoenoic, D - dienoic and T - trienoic fatty acid moieties

this stage was the sample size. Overload in densitometry leads to systematically lower results for the overloaded components. We used the ratio SPUTAG/MMD as a criterion to keep overload under control. The sample size which ensured maximal value of the ratio was considered suitable. Quantitation was considered correct in these cases only when SPUTAG/MMD remained constant.

A third plate was used to resolve the components of SPUTAG (Table 1, third column, Fig. 1C). The resolution was complete and enabled correct densitometric determination.

The mobile phase we usually use in Ag-TLC is binary with light petroleum and acetone being mixed in different proportions [5,14,15,22]. This mixture was suitable for corn but not for cotton TAGs. The specific TAG composition of cotton oil required a third component. Ethyl acetate was found suitable as it ensured the separation of TAGs which normally formed critical pair.

RP-TLC

Table 2 presents the chromatographic conditions used to resolve the TAG species within a TAG class by RP-TLC. Obviously, since the oils contained only one monoenoic - oleic, one dienoic - linoleic, and one trienoic fatty acid - linolenic, TAG chain length was determined by the chain-length of the saturated fatty acids.

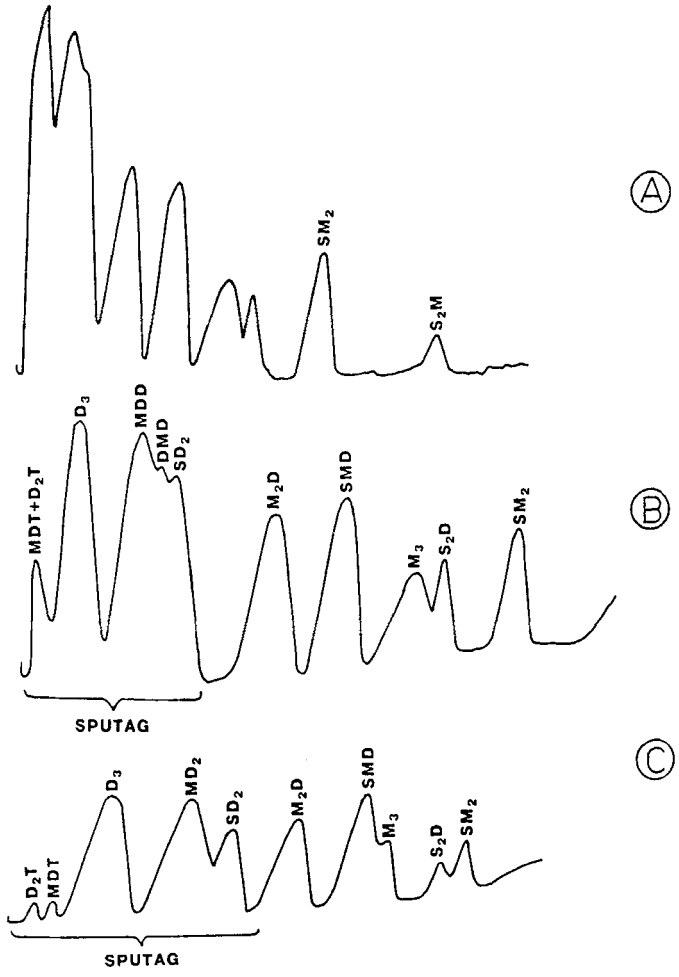

FIGURE 1

Typical resolution of the TG groups of cottonseed oil. For the experimental conditions see Table 1.

TABLE 2.

Water Proportion in the Mobile Phase acetone/acetonitrile/water, 70:30:X for the Separation of Triacylglycerol Classes into Molecular Species by RP-TLC

TG class[a]	TG species[b]	PN[c]	water proportion, by volume
S_2M	PPO, PStO, StStO	48, 50, 52	12
SM_2	POO, StOO	48, 50	14
S_2D	PPL, PStL, StStL	46, 48, 50	12
SMD	POL, StOL	46, 48	18
SD_2	PLL, StLL	44, 46	20

[a] For the abreviations see the footnote to Table 1
[b] The order of designation does not indicate positional isomers, P - palmitic; St - stearic; O - oleic; L - linoleic fatty acid moieties
[c] Partition number PN=C-2n; C - number of carbon atoms
n - number of double bonds

A three component mobile phase was used. Acetone/acetonitrile ratio was kept constant, 7:3 (v/v). Water was suitable modifier [6] and its proportion in the mobile phase was gradually increased with increasing overall unsaturation of the TAG class. In a previous study a simple approach to predict the water proportion necessary for a good separation was established [6].

Quantitation.

It is a well known that while the fatty acid composition of a given seed oil can vary depending on the climate, genetic or variety factors [23,3], the TAG composition varies little, with the proportion between the different unsaturation classes remaining roughly constant and unique [24].

The TAG compositions of cotton and corn oils, as being determined in this study, are presented in Table 3. As already noticed, (see Experimental) resolution and quantitative results obtained by Ag-TLC were the core of the whole analysis. The quantitative result for a given TAG class was taken to recalculate the data for the component molecular species obtained by RP-TLC. Measures were taken to ensure the necessary accuracy and precision of the analysis (see [8] where this problem was studied in details).

TABLE 3.

TG Composition of Cotton and Corn Oils as Determined by Successive Application of Ag-TLC and RP-TLC*

TG classes (number of double bonds)	TG MOLECULAR SPECIES				COTTON				CORN			
					Ag-TLC + RP-TLC	CGC	RP-HPLC	RP-HPLC	Ag-TLC + RP-TLC	CGC	RP-HPLC	RP-HPLC
						ref.20	ref.20	ref.21		ref.20	ref.20	ref.21
S_3	PPP	16:0	16:0	16:0	-	0.5	-	0.4	-	-	-	-
(0)	PPS	16:0	16:0	18:0	-	0.1	-	-	-	1.9	0.4	-
	MiPO	14:0	16:0	18:1	0.4	0.6	-	-	-	-	-	-
S_2M	PPO	16:0	16:0	18:1	2.3	6.2	1.5	4.4	0.6	3.9	1.5	-
(1)	PStO	16:0	18:0	18:1	0.9	1.2	0.5	0.3	0.3	0.5	0.1	-
	StStO	18:0	18:0	18:1	tr.	0.2	-	-	0.2	0.3	-	-
	StAO	18:0	20:0	18:1	-	-	-	-	0.1	-	-	-
SM_2	POO	16:0	18:1	18:1	2	4.4	2	2.4	3.5	4	2.5	2.2
(2)	StOO	18:0	18:1	18:1	1.4	0.6	0.7	0.2	0.6	0.7	0.3	-
	AOO	20:0	18:1	18:1	1.4	-	-	-	0.2	-	-	-
	MiPL	14:0	16:0	18:2	0.9	1.7	0.6	-	-	-	-	-
S_2D	PPL	16:0	16:0	18:2	11.8	8.1	7.7	15.8	1.3	-	-	2
(2)	PStL	16:0	18:0	18:2	1.2	2.3	1.1	-	0.8	1.9	2.8	1.7
	PAL	16:0	20:0	18:2	-	-	-	-	0.5	-	-	-
M_3 (3)	OOO	18:1	18:1	18:1	0.7	2.6	1.6	1.4	5.5	4.1	3.1	3.7
SMO	POL	16:0	18:1	18:2	13.4	10.4	10.9	12.8	10.7	13.8	10.4	9.9
(3)	StOL	18:0	18:1	18:2	2	1.3	0.9	1	1.9	1.7	1.2	1.5
	AOL	20:0	18:1	18:2	-	-	-	-	0.7	-	-	-
M_2D (4)	OOL	18:1	18:1	18:2	5.5	6.1	5.1	4.7	13.7	11.2	10.9	10.4
	MiLL	14:0	18:2	18:2	-	1.1	-	-	-	-	-	-
SD_2	PLL	16:0	18:2	18:2	20.5	20.8	24.5	25.4	8.4	17.1	16.4	15.2
(4)	StLL	18:0	18:2	18:2	4.5	1.7	1.1	1.8	1.7	2.1	1.9	1.8
	ALL	20:0	18:2	18:2	-	-	-	-	0.2	-	-	-
S_2T (3)	PPLn	16:0	16:0	18:3	-	-	1.4	-	-	-	0.1	-
MD_2 (5)	PoLL	16:1	18:2	18:2	}14.4	0.1	0.3	-	}27.1	-	-	-
	OLL	18:1	18:2	18:2		14.1	15.4	11.8		18.8	22.5	26.1
M_2T (5)	OOLn	18:1	18:1	18:3	tr.	-	-	-	-	-	0.3	-
D_3 (6)	LLL	18:2	18:2	18:2	17.4	15.9	23.2	15.6	19.2	17.7	25.6	25.2
MDT (6)	OLLn	18:1	18:2	18:3	0.3	-	-	-	1.7	-	0.6	-
D_2T (7)	LLLn	18:2	18:2	18:3	0.2	-	1.5	-	1.2	0.3	1.2	2.1

* For the abreviations see the footnotes to Table 1 and 2; Mi and 14:0 - myristic acid; 16:0 - palmitic acid; 18:0 - stearic acid; A and 20:0 - arachidic acid; B and 22:0 - behenic acid; Po and 16:1 - palmitoleic acid; 18:1 - oleic acid; 18:2 - linoleic acid; 18:3 - linolenic acid moieties.

Results obtained in this work were compared with those reported by others where chromatographic methods, like gradient RP-HPLC and capillary GLC [20,21] were employed. Between the numerous papers only these were chosen where the TAG composition has been determined experimentally and not by calculations [25].

Cotton oil. This oil is widely used for nutrition purposes either alone or in mixtures with soybean oil [26] and was, therefore, intensively studied and analyzed [16-21]. It has a relatively high content of saturated fatty acids (25-30%) which is not typical for the most abundant seed oils. In total, 19 molecular species have been determined in this study. Minor components such as MiPO, PStO, MiPL, OOO, OLLn, LLLn, and MiPO have been determined. Of these, MiPO has been determined with capillary GLC only [20] and OLLn has not been found at all. Our results are in a good general agreement with those works where Ag-TLC followed by GLC have been applied [16-19].

Corn oil. A specific feature of this oil is the relatively high content of the symmetric positionally isomeric DMD TAG as found by enzyme hydrolysis [17]. It has been found that the high DMD content hampers the clear resolution of the SD_2/MD_2 classes by Ag-TLC [22,27]. To avoid the partial resolution of the positionally isomeric TAG, the plate was given two successive developments; the second mobile phase being of lower polarity (Table 1, columns two and three).

There is a good agreement between our quantitative Ag-TLC results and those reported previously, with one exception: the SD_2 and MD_2 classes. While the sum of these TAGs coincide very well with the published results, we found a higher quantity of MD_2 and lower for SD_2 then the reported. The reason might be that in [16] and [17] a combination of preparative Ag-TLC with GLC was used. Under the reported conditions of preparative Ag-TLC both TAGs migrate as two, not clearly resolved, zones and were isolated together. The high content of silver nitrate in the layer (13%) usually hampers strongly the detection under UV. Presumably, the zones haven't been precisely located and differentiated.

We determined 22 TAG species in corn oil and this number is higher than has been achieved by any chromatographic method so far. Recent communications employing RP-HPLC reported 19 [20] and 12 [21] species (Table 3). In some extent, the differences are due to the presence of low percents of stearic and

arichidic acids in corn oil. Exactly the minor TAG components containing these acids, were not resolved and determined by RP-HPLC. This is an obvious result since the total sample was injected onto the column, mixed peaks were inevitably formed and the minor component were presumably lost.

On the other hand, while the sum of PLL and OLL found in this work equals that in [20], we determined a much lower PLL content (Table 3). Under the conditions of [20], PLL and OLL differ by 0.51 ECN (0.64 TCN). These values seem to be not a sufficient difference for a base-line resolution of the two neighboring TAG. In the sequence of methods used in the present work PLL and OLL appeared in different unsaturation fractions and were quantified separately. We assume, therefore, that in the present work their proportion had been correctly determined.

Based on the TAG composition of the samples, their fatty acid compositions were calculated. The values obtained are compared with those determined directly by GLC in Table 4. There is a very good agreement especially if one takes into account that 19, respectively 22, TAG species were used to calculate the fatty acid composition. This is an evidence for the high accuracy of the analysis.

TABLE 4.

Comparison of the Calculated from TAG Data Fatty Acid Compositions of Cottonseed and Corn Oils with those Determined Directly by GLC *

Fatty acid composition	Cottonseed oil		Corn oil	
	GC	calcul. from TG	GC	calcul. from TG
14:0	0.9	0.4	<0.1	-
16:0	22.9	22.5	10.8	9.9
16:1	0.5	-	<0.1	-
18:0	1.9	3.3	1.5	1.9
18:1	16.8	17.9	29.0	31.3
18:2	56.4	55.5	57.5	55.4
18:3	0.3	0.2	0.6	0.9
20:0	0.2	-	0.2	0.5
20:1	-	-	0.1	-
22:0	0.1	-	<0.1	-

* Saturated fatty acids under 0.2% and monoenoic fatty acids under 1.0% could not be determined by the RP-TLC procedure used.

We assume that the results reported here are an illustration that the detailed TAG analysis requires preliminary fractionation by silver ion chromatography. Moreover, since it determines the accuracy of the final results, it is of vital importance for this resolution to be as complete and precize as possible. The second chromatographic stage in the analysis, irrespective of the technique applied (RP-TLC, GLC, RP-HPLC), uses then samples with simpler composition. Minor components could be unambiguously resolved, identified and determined as their proportions in the fraction are favourably changed.

We are convinced that the results presented here clearly show that despite its simplicity, TLC is able to provide results which are comparable and even superior to those obtained so far by HPLC.

We also believe that TLC has a certain advantage: the chromatogram on the plate, like a photograph, presents the real state of resolution under the chosen experimental conditions which helps a lot to change them toward the desirable direction. Moreover, identification of the components is much easier and unambiguous since a cochromatography with standards of known composition is always possible.

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