CHROM. 23 963

Reversed-phase thin-layer chromatography of diacylglycerols in the presence of silver ions

V. P. Pchelkin and A. G. Vereshchagin

Laboratory of Lipid Metabolism, Institute of Plant Physiology, Academy of Sciences, Botanicheskaya 35, Moscow 127276 (Russia)

ABSTRACT

Each of the SS, SL and SLe groups of 1,2-*rac*-diacylglycerols (DAGs) in which S is a residue of stearic (St) or palmitic (P) acid and L and Le are residues of linoleic and linolenic acid, respectively, were separated into individual species using reversed-phase thin-layer chromatography in the presence of silver ions (Ag^+-RP-TLC). This technique, combined with adsorption Ag^+-TLC (Ag^+-TLC + Ag^+-RP-TLC, in the form of two-dimensional TLC), afforded the complete separation of all the components of the DAG model mixture obtained by glycerolysis of St, P, L, Le and oleic acid (O) esters. All fifteen individual DAG species were identified: StSt-, StP-, StO-, PP-, PO-, OO-, StL-, PL-, OL-, LL-, StLe-, PLe-, OLe-, LLe- and LeLe-glycerols. Their Ag^+-RP-TLC mobilities relative to that of *rac*-1,2-LeLe ($R_{1,2\text{-LeLe}}$) were inversely proportional to the equivalent lipophilicity of their Ag^+ complexes L_3 ($R_{1,2\text{-LeLe}} = 1.47 - 0.04 L_3$). In terms of separation selectivity of the DAG model mixture, the Ag^+-TLC + Ag^+-RP-TLC technique exceeds reversed-phase high-performance liquid chromatography and is roughly equal to gas chromatography.

INTRODUCTION

In previous studies on the molecular species composition of the diacylglycerol (DAG) model mixture using adsorption thin-layer chromatography of their coordination complexes with silver ions (Ag^+-TLC), we identified eight out of the fifteen theoretically possible DAG species [1,2]. At the same time, in addition to these eight individual species, we discovered three DAG groups of mixed molecular species composition, *viz.*, SLe-, SL- and SS-glycerols, were S are residues of stearic (St) or palmitic (P) acid and Le and L are linolenate and linoleate, respectively [3]. The separation of each of these groups into individual species by Ag^+-TLC has not been achieved. Therefore, we attempted to attain the same goal by using another mode of TLC, *viz.*, reversed-phase TLC in the presence of silver ions (Ag^+-RP-TLC), which has previously been report-

ed to exceed the Ag^+-TLC in terms of separation selectivity of individual DAG species [2]. Several DAG samples of a relatively simple molecular species composition (see below) served as standards to identify individual DAG species in the model mixture [3].

EXPERIMENTAL

Preparation and fractionation of rac-1,2-DAGs

The model mixture [1] and the standards [3] of DAGs were obtained as described previously [3]. All DAG preparations were separated into their positional isomers and subsequently only *rac*-1,2-DAGs were used [3]. For one-dimensional Ag^+-RP-TLC fractionation of the latter [2], the entire surface of a Silufol plate (15 × 15 cm) (Kavalier, Sklárny, Czechoslovakia) was sprayed with a 1% (w/v) methanolic solution of silver nitrate [3] and then impregnated with a 10% (v/v) benzene solution of *n*-tetradecane [1]. From 20 to 50 μg of *rac*-1,2-DAGs were applied at the starting line of this plate. They were separated into individual spe-

Correspondence to: Dr. V. P. Pchelkin, Laboratory of Lipid Metabolism, Institute of Plant Physiology, Academy of Sciences, Botanicheskaya 35, Moscow 127276, Russia.

cies and groups of DAGs in a completely vapour-saturated chamber for continuous-flow TLC (see Fig. 1 in ref. 4) using a 5% (w/v) boric acid solution in methanol saturated with silver nitrate and *n*-tetradecane [2] as the mobile phase.

Prior to two-dimensional TLC, the entire plate was sprayed with the silver nitrate solution (see above), and 50 μg of *rac*-1,2-DAG mixture were applied at point A (Fig. 1). DAGs were separated by continuous-flow Ag⁺-TLC in direction 1 on region I of the plate for 6 h using a silver nitrate-saturated mixture of chloroform and isopropanol (99:1, v/v) as the mobile phase [3]. The plate was kept in a hood for 0.5 h, region II was impregnated with *n*-tetradecane and then SL and Sle zones formed by Ag⁺-TLC were further separated as above in direction 2 by continuous-flow RP-TLC.

Detection and identification of individual DAG species

Soluble silver salts [3] and *n*-tetradecane [1] were removed from the plate and individual TLC zones of UU- and SS-DAGs, where U is Le, L or oleate (O), were revealed with phosphomolybdic acid (PMA). SS-DAGs which did not react with PMA were revealed with a 10% copper sulphate solution in an 8% solution of phosphoric acid (CS–PA) [2]. Treatment with CS–PA also revealed SU- and UU-DAGs. The mobility of different DAG zones was expressed as $x \pm s$, where $x = 100R_{1,2\text{-LeLe}} = hR_{1,2\text{-LeLe}}$ is the arithmetic mean of individual measurements of the mobility of a given zone in relation to that of the *rac*-1,2-LeLe zone ($R_{1,2\text{-LeLe}}$) multiplied by 100 (*h*) and *s* is the absolute standard deviation of these measurements; *rac*-1,2-LeLe was selected as a mobility standard because it exceeded other molecular species of the model mixture in its Ag⁺-RP-TLC mobility. Other methods were the same as those used previously [1–3].

RESULTS AND DISCUSSION

DAG standards

Previously, we have fractionated the DAG model mixture by one-dimensional Ag⁺-RP-TLC directly, without its preliminary separation into non-symmetrical and symmetrical *rac*-1,2- and *rac*-1,3-positional isomers, as the mobilities of both during reversed-phase chromatography were shown to be very similar [2]. However, the present study revealed (see below) that complete separation of the mixture could be achieved only by combining Ag⁺-RP-TLC with adsorption Ag⁺-TLC, *i.e.*, by two-dimensional TLC (see Experimental). In addition, Ag⁺-TLC mobilities of various positional isomers within each species present in the initial model mixture were shown to be different [3]. It is clear that direct two-dimensional TLC of this mixture would result in a very complicated separation pattern which would be useless for the identification of individual DAG species, and therefore nothing but isomerically pure components of the model mixture and DAG standards (see Experimental) could be employed for both Ag⁺-TLC and Ag⁺-RP-TLC in the present study [1–3]. Only non-symmetrical *rac*-1,2-DAG isomers were used in the experiments described below because, on the one hand, the ultimate goal of
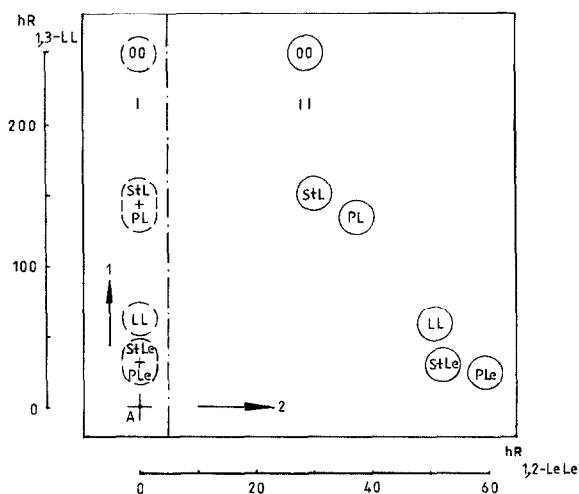
Fig. 1. Two-dimensional thin-layer chromatogram of a mixture of six *rac*-1,2-diacylglycerol species. A = starting point; I and II = regions of the plate non-impregnated and impregnated with *n*-tetradecane, respectively; 1 = direction of mobile phase [silver nitrate-saturated chloroform–isopropanol (99:1, v/v)] migration during Ag⁺-TLC; 2 = direction of mobile phase [5% (w/v) boric acid solution in methanol, saturated with silver nitrate and *n*-tetradecane] migration during Ag⁺-RP-TLC. Zone limits Ag⁺-TLC of DAGs in region I and after Ag⁺-RP-TLC in region II are indicated by broken and solid lines, respectively. $hR_{1,3\text{-LL}} = 100R_{1,3\text{-LL}}$ = mobility of DAG zones in relation to that of *rac*-1,3-dilinoleoylglycerol [3]. For other abbreviations, see Experimental and Table III.

TABLE I

FATTY ACID COMPOSITION OF DAG STANDARDS

Standard No.	Fatty acid composition (mol%)[a]				
	St	P	O	L	Le
1	3.1	67.0	29.9	–	–
2	69.3	3.2	25.3	2.2	–
3	49.6	27.8	2.1	20.5	–
4	44.0	30.1	1.2	2.2	22.5

[a] Data from ref. 3. For abbreviated designations of fatty acid species, see text.

our studies is the chromatographic determination of the molecular species composition of non-symmetrical sn-1,2-DAGs obtained from native polar glycerolipids [1] and, on the other, on liquid chromatography of DAGs of the same composition on achiral stationary phases rac-1,2-DAGs always coincide with sn-1,2-DAGs in their mobility [5].

The data on fatty acid composition in Table I demonstrate that standards 1 and 2 include large amounts of P and St, respectively, and standards 3 and 4 contain, in addition to S acid, mostly L and Le, respectively. From these data, a possible species composition of DAG standards 1–4 was calculated as shown in Table II [1–3]. From Table II it can be

TABLE II

CHARACTERISTICS OF MOBILITY AND LIPOPHILICITY OF INDIVIDUAL DAG SPECIES ON Ag⁺-RP TLC AND THEIR IDENTIFICATION IN STANDARDS 1-4

Mobility of DAG zones $(x \pm s)$[a]	DAG species content in standards 1–4: calculated (mol%)[b] and evaluated visually[c]				Staining behaviour with		Ag⁺-TLC data [3][d]	DAG identification	L_3 values of DAGs[e]
	1	2	3	4	PMA	CS–PA			
4 ± 1		49 +++	26 +++	20 +++	–	+	A	StSt	36
10 ± 2	4 ++	5 ++	29 +++	28 +++	–	+	A	StP	34
15 ± 1	2 +	35 +++	2 +		+	+	+	StO	33
19 ± 1	45 +++		8 ++	9 ++	–	+	A	PP	32
23 ± 1	40 +++	2 +			+	+	+	PO	31
30 ± 2	9 ++	9 ++	20 +++	3 +	+	+	B	OO + StL	30
38 ± 3			11 ++		+	+	B	PL	28
51 ± 2		4 ++	20 +++		+	+	C	LL + StLe	24
60 ± 2			15 +++		+	+	C	PLe	22

[a] Arithmethic means of individual measurements of $hR_{1,2\text{-LeLe}}$ and absolute standard deviations are shown (see Experimental).

[b] Calculated from the DAG fatty acid composition data in Table I according to the theory of random distribution of fatty acid residues between DAG molecules [1]. Standard 4 also contained the LeLe zone (5 mol%).

[c] Results of visual evaluation of the relative intensity of DAG zone staining are designated by –, +, ++, +++ (in ascending order).

[d] Unambiguously proved the presence of a given DAG species (+), SS-DAGs (A), SL-DAGs (B) or SLe-DAGs (C) in the model mixture [3].

[e] Equivalent lipophilicity $L_3 = m - 2p - u$, where m and p are total number of carbon atoms and relative polarity of DAG aliphatic chains, respectively, and u is the number of unsaturated fatty acid residues in the DAG molecule [1–3]. See text for other abbreviations.

seen that these standards include mainly SS and SU species in addition to a small amount of UU species. Hence it can be suggested that these standards would be suitable for the identification of respective DAG species in SS, SL and Sle groups of the DAG model mixture. Table II shows also that the separation of each of the standards 1–4 by Ag^+-RP-TLC yields 5–7 zones. Up to ten separate DAG zones were obtained.

Identification of individual DAG species in standards 1–4

The identification was based on the data in Table II which include both the parameters of Ag^+-RP-TLC mobility of DAGs and the results of their detection (see columns 6–8) and of the identification of different DAG species and DAG groups by Ag^+-TLC [3]. Below, when examining the properties of each TLC zone, these results are assumed to be known and, therefore, no detailed discussion of them is given.

Table II demonstrates that zones with $x = 60$ and 51 (referred to below as zones 60 and 51) display the highest TLC mobility (except the $x = 100$ zone corresponding to rac-1,2-LeLe). These zones occur almost exclusively in standard 4. From their staining behaviour, they belong to unsaturated DAGs, and from the comparative colour intensities of different zones of this standard they can be PLe and StLe, respectively. One must also take into account that PLe and StLe predominate among the SU-DAGs in the calculated standard 4 composition. Moreover, because the reversed-phase mobility of DAGs is known to be inversely proportional to their lipophilicity [1,2], PLe which is less lipophilic must exceed StLe in its mobility. Therefore, one must conclude that zone 60 represents an individual PLe species whereas in the standard 4 zone 51 contains StLe. At the same time, zone 51 occurs also in standard 3 as its most mobile component. This standard does not contain Le and includes only traces of O, but is rich in L (see Table I). Hence, in standard 3 zone 51 does not contain StLe, and includes only LL species. Therefore, the latter cannot be separated from StLe by Ag^+-RP-TLC.

The next in rank of mobility is zone 38, which was found only in standard 3. From the fatty acid composition of the latter (Table I) and the data on DAG species content and staining characteristics

(Table II), it can be suggested that this zone belongs to the SL group and can represent only one of the two alternative molecular species of this group (PL or StL), i.e., PL because it is the least lipophilic and most mobile of the SL species.

As regards zone 30, it occurs as a major component only in standard 3, in which L predominates among unsaturated fatty acids (see above). Therefore, in this instance zone 30 contains only the diacid StL species. At the same time, this zone is present in an appreciable amount in standard 1, which, apart from S, contains only O. In this standard, zone 30 is most mobile and, therefore, must contain the least lipophilic of all DAG species possible for this case. Because DAGs of this zone do not represent SS-DAGs (see columns 6 and 7), they can contain only OO- and/or PO-glycerols. If one takes into account that the calculated contents of these species in standard 1 are 40 and 9%, respectively, and that zone 3 is considerably inferior to the adjoining zones 23 and 19 in its colour intensity, one can conclude that in the case of standard 1 zone 30 includes only the one (monoacid) OO species [3]; hence, on Ag^+-RP-TLC OO has the same x as StL.

The next zone, 23, be found only in standards 1 and 2 and, from the data in columns 6 and 7 (Table II), does not represent SS DAGs. Of the two major DAG zones of standard 1 which in its fatty acids contains 67% P and 30% O, this zone is the most mobile, i.e., the least lipophilic. It can be concluded that this zone represents one of the two possible SO species, namely PO.

Zone 19 (of the SS type) is the major component of standard 1 in which P predominates (see above). It can also be found in standards 3 and 4 where P makes up 28 and 30% of the total fatty acids, respectively. At the same time, zone 19 is absent in standard 2 where the P content does not exceed 3.2%. Therefore, zone 19 is composed of the disaturated PP species.

Zone 15 does not belong to the SS type (see columns 6 and 7) and is present almost exclusively in standard 2. Here O appears to be the only major unsaturated fatty acid, and among S fatty acids St predominated (Table I). Because PO and OO were identified previously in zones 23 and 30, respectively, it can be concluded that zone 15 is composed of only one individual SU species, i.e., StO [3].

The last but one zone, 10 (of the SS type), may

represent either StP or StSt. However, this zone was found to be intensely colored only in the standards rich in both St and P, *i.e.*, in standards 3 and 4. Therefore, this zone can consist of nothing but StP.

Finally, zone 4, which also belongs to the SS type, is present only in the standards (2–4) containing St as the major component and is absent from standard 1 where the St content does not exceed 3.1% (see Tables I and II). It is therefore evident that zone 4 is composed of the monoacid StSt species.

Identification of StL and StLe in the DAG model mixture by Ag⁺-TLC followed by Ag⁺-RP-TLC

As shown in the previous section, in the course of analysis of the standards of a simple species composition, the SS group of DAGs was separated into individual StSt, StP and PP molecular species, the SL group into StL and PL and the SLe group into StLe and PLe. At the same time, these data show that during Ag⁺-RP-TLC StL and StLe do not differ in their mobility from OO and LL, respecitively. Previously it has been shown that StL, StLe, OO and LL could be completely separated from each other by adsorption Ag⁺-TLC. However, the latter technique does not separate StL and StLe from PL and PLe, respectively [3]. Hence the six molecular species, *i.e.*, StL, StLe, PL, PLe, OO and LL, are close to each other in their TLC mobility and therefore the methods for their complete TLC separation must be considered in more detail.

Several variants of the qualitative composition of these DAGs in synthetic and natural mixtures are shown in Table III. It is seen that in order to deal with different variants, one must adopt different strategies. Thus, if the absence of OO and/or LL in a given DAG mixture (variants 3 and 7) has been established by Ag⁺-TLC, StL and/or StLe can be identified in it by the occurrence of zones 30 and/or 51, respectively, on Ag⁺-RP-TLC. Similarly, if PL and/or PLe were not found in a mixture by Ag⁺-RP-TLC (variants 1 and 5; 2 and 6), then the presence of StL and/or StLe will be unambiguously proved by Ag⁺-RP-TLC.

Finally, the most complicated variants which are also present in our model mixture (variants 4 and 8) include all six DAG molecular species listed above. In these instances the identification of StL and StLe species can be achieved only by two-dimensional chromatography of the mixture on the same plate

TABLE III

IDENTIFICATION OF StL AND StLe INDIVIDUAL SPECIES IN DAG MIXTURES OF DIFFERENT MOLECULAR SPECIES COMPOSITION USING THE SEPARATION OF THESE MIXTURES BY Ag⁺-TLC FOLLOWED BY Ag⁺-RP-TLC

DAG species[a]		Variants of DAG mixture species composition[b]			
A	B				
		1	2	3	4
StL	PL	−	−	+	+
	OO	−	+	−	+
		5	6	7	8
StLe	PLe	−	−	+	+
	LL	−	+	−	+

[a] A, StU species to be identified; B, DAG species accompanying StL and StLe in the model mixture and identified by Ag⁺-TLC (OO, LL) and Ag⁺-RP-TLC (PL, PLe).
[b] 1–8 are variant numbers; + and − indicate the presence and absence, respectively, of a given DAG species (column B) in a mixture.

(Ag⁺–TLC + Ag⁺-RP-TLC, see Fig. 1), *viz.*, by using Ag⁺-TLC in the first direction and Ag⁺-RP-TLC in the second. After separating DAGs by Ag⁺-TLC, OO and LL zones in addition to SL and SLe groups will be formed. In the second stage of two-dimensional TLC these groups are separated into StL, PL, StLe and PLe individual species as a result of their Ag⁺-RP-TLC.

Earlier, by Ag⁺-TLC we identified in the DAG model mixture LeLe, LLe, OLe, LL, OL, OO, PO and StO species [3], and now, by means of Ag⁺-TLC + Ag⁺-RP-TLC, PLe, StLe, PL, StL, PP, StP and StSt were also shown to be present, in addition to the species mentioned above. Hence our model mixture contains fifteen individual DAG species: LeLe, LLe, OLe, PLe, StLe, LL, OL, PL, StL, OO, PO, PP, StO, StP and StSt, *i.e.*, noting but the species which are formed as a result of the random distribution of the five fatty acid residues between molecules of glycerol [1].

It is evident that the Ag⁺-TLC + Ag⁺-RP-TLC technique which ensures complete separation of the mixture of fifteen DAG species, *i.e.*, a mixture of a very complex molecular species composition will be all the more suitable for the analysis of individual *sn*-1,2-DAGs obtained from plant polar glycero-

lipids because in the latter instance the number of species formed is usually lower than in our mixture. For example, the identification of StL and StLe will be required only rarely because the level of St in membrane lipids does not usually exceed 4%, and therefore StL and StLe are usually present there in only trace amounts, if at all.

Relationship between the Ag⁺-RP-TLC mobility of individual DAG species and the equivalent lipophilicity of their coordination complexes

Having at our disposal the results of DAG identification and the x values*a* of different TLC zones (Table II), we attempted to determine the relationship between the mobility of individual DAGs on Ag⁺-RP-TLC and characteristics of their molecules. One of the most important is known to be lipophilicity [6]. Previously this characteristic has been shown to vary under different TLC conditions. For example, during RP-TLC of free DAGs in a methanol–trimethyl borate–n-tetradecane system, *i.e.*, in the absence of silver ions, lipophilicity has been defined by us as

$$L_1 = m - 2e \qquad (1)$$

Where m and e are the total numbers of carbon atoms and olefinic bonds in the fatty acid chains, respectively [1]. Later, in preliminary experiments on the separation of a mixture of DAG positional isomers by Ag⁺-RP-TLC, the lipophilicity of DAG coordination complexes was expressed as

$$L_2 = m - 2e - u \qquad (2)$$

where u is the number of unsaturated fatty acid residues in the DAG molecule [2]. However, further studies have demonstrated that the Ag⁺-TLC mobility of these complexes was mainly determined by their polarity, p. Like e, polarity is inversely proportional to the equivalent lipophilicity of Ag⁺–DAG complexes. At the same time, polarity reflects their properties more accurately than the number of olefinic bonds, e [3]. Therefore, to calculate the lipo-

philicity parameter of rac-1,2-DAGs on Ag⁺-RP-TLC, e values in eqn.2 were substituted by p values taken from Table II in ref. 3. Calculated parameters

$$L_3 = m - 2p - u \qquad (3)$$

approximated to the nearest whole number are shown in Table II.

To establish which of the three lipophilicity parameters, L_1 [1], L_2 [2] or L_3 (Table II), is the most satisfactory characteristic of DAG properties on Ag⁺-RP-TLC, we calculated correlation coefficients, r, between each of these parameters and x values of the respective DAG species (Table III). The values were -0.976, -0.972 and -0.999, respectively, and confirm, first of all, the above conclusion that the mobility of individual DAGs on RP fractionation is always inversely proportional to their lipophilicity. Moreover, these results show that during Ag⁺-RP-TLC the selectivity of DAG separation is dependent mostly on the L_3 parameter. This relationship is linear ($x = 147 - 4 L_3$, $r = +0.999$), the number of DAG chromatographic zones is equal to the total number of different L_3 values and separation between individual DAG species takes place only when their L_3 values differ by $\geqslant 1$, *i.e.*, at $\Delta L_3 \geqslant 1$.

It should be emphasized that on RP-TLC of DAGs in the absence of silver ions there was also a linear relationship between their hR_F and L_1 values: $hR_F = 164 - 4 L_1$ [2]. However, under these conditions, the DAG model mixture was separated only into seven zones with $\Delta L_1 \geqslant 2$, whereas the presence of silver ions resulted in the formation of twelve zones, indicating a significant increase in the selectivity of DAG separation in the RP system [2].

Comparison of Ag⁺-TLC + Ag⁺-RP-TLC with other chromatographic techniques in the selectivity of DAG separation

Above it was stated that the proposed technique ensured the complete separation of a mixture containing fifteen individual DAG species and embraces a wide range of their lipophilicity. Nevertheless, for the definitive evaluation of the practical significance of this technique, it was necessary to compare it with other modern procedures for DAG fractionation [7] in the separation selectivity of natural DAGs and their derivatives. As a criterion of

---

a These values for pure rac-1,2-DAGs presented in Table II were not always equal to the respective mobility characteristics obtained earlier by the same method when using a mixture of DAG positional isomers [2].

selectivity we applied the relative retention of two neighbouring peaks or chromatographic zones as suggested by Schoenmakers [8]:

$$\alpha_{j,i} = t_{R,j}/t_{R,i} \qquad (4)$$

where $t_{R,i}$ and $t_{R,j}$ are retention times of the first (i) and next (j) eluting peaks. Peaks i and j were considered to be separated only at $\alpha_{j,i} > 1.1$. The values of

$hR_{1,3\text{-}LL}$ [3] and $hR_{1,2\text{-}LeLe}$ (Table II) show that the relative retention of one or another DAG species during separation, if any, by Ag$^+$-TLC and Ag$^+$-TLC + Ag$^+$-RP-TLC was much in excess of $\alpha_{j,i} = 1.1$. Therefore, when comparing the two TLC techniques with other fractionation procedures it was assumed that the former allowed the separation of eight and the latter of fifteen DAG species (Table IV).

TABLE IV

ESTIMATE OF SEPARATION SELECTIVITY OF INDIVIDUAL DAGs AND THEIR DERIVATIVES BY RP-HPLC

sn-3-O-Derivatives	Number of DAG species		Non-separable DAG species (fractions)[a]	Estimate of separation selectivity	Ref.
	Total	Separated			
Free DAGs	15	8	StSt + StP + PP, StL + PL, StLe + PLe	0.53	3[b]
	15	15	–	1	This work[c]
	5	5	–	1	9
Acetates	7	3	II	0.43	10
	12	8	II	0.67	11
	8	6	III	0.75	12
	7	3	II, III	0.43	13
tert.-Butyldimethylsilyl ethers	9	3	II, III	0.33	14
Benzoates	7	5	II	0.71	15
	3	3	–	1	16
	8	2	II, III	0.25	17
	10	4	I–III	0.40	18
	7	4	II	0.58	19
	7	5	II	0.71	20
Metoxybenzoates	5	5	–	1	21
	12	6	I, III, LL + Ole	0.50	22
Nitrobenzoates	4	4	–	1	23
	8	3	II, III	0.38	24
	8	3	II, III	0.38	25
	8	2	I–III	0.25	26
	8	4	II, III	0.50	27
	8	2	I–III	0.25	28
	9	3	I–III	0.33	29
	3	3	–	1	30
	7	5	III	0.71	31
Naphthylurethanes	7	3	II, III	0.43	32
	9	3	I–III	0.33	33
	7	5	LL + PLe	0.71	34
	4	4	–	1	35
Anthroates	7	3	StSt + StP, II	0.43	36

[a] Non-separable species: I = StP + StO; II = PP + PO + OO + StL; III = PL + OL.
[b] Ag$^+$-TLC.
[c] Ag$^+$-TLC + Ag$^+$-RP-TLC.

The value of the "estimate of separation selectivity" (*ESS*) was expressed as the ratio between the total number of DAG species being analysed and the number of species which were separated from each other. Of course, when considering all individual DAG species referred to by other workers we took into account only those species which happened also to be present in our model mixture [1]. It is evident that a maximally accessible value of *ESS* (the case of complete separation of all the components of a DAG mixture) is 1. The shortcoming of calculating *ESS* in the way devised here is caused by the fact that analyses of DAG mixtures performed by various workers include different total numbers of individual species. Therefore, the estimates obtained by this method should be considered as preliminary values.

Let us first consider reversed-phase high-performance liquid chromatography (RP-HPLC), which is now widely used for DAG species analysis. *ESS* values of *sn*-3-O-derivatives of DAGs calculat-

ed by us using the mobility data obtained by this technique are presented in Table IV. It can be seen that in most studies *ESS* was significantly lower than 1 because many DAG species were not separated by RP-HPCL. Identical mobilities of these species were obtained because they did not differ from each other in their L_1 values (see above). Previously, the same relationship was observed when separating a DAG model mixture by RP-TLC [1]. Most frequently there was no separation between the components of fractions I, II and III with $L_1 =$ 34, 32 and 30, respectively (their molecular species composition is shown in Table IV). Moreover, the LL + OLe and LL + PLe pairs with the same $L_1 =$ 28 and StSt + StP also failed to be separated in some instances. Values of *ESS* = 1 were observed by only a few workers and, moreover, the number of individual DAG species which are taken into account in the present study and were included in their analyses did not exceed five (see above), *i.e.*, it was much less than in our model mixture. Hence Ag$^+$-TLC +

TABLE V

ESTIMATE OF SEPARATION SELECTIVITY OF INDIVIDUAL DAG DERIVATIVES BY GC

sn-3-O-Derivatives	Number of DAG species		Non-separable DAG species (fractions)[a]	Estimate of separation selectivity	Ref.
	Total	Separated			
Acetate	3	3	—	1	37
	7	5	VI	0.71	38
	14	10	VII, LLe + LeLe	0.71	39
	3	3	—	1	40
	10	4	IV–VI	0.40	41
Trimethylsilyl ethers	4	4	—	1	42
	6	2	V, OO + LL	0.33	43
	7	3	VI, PL + OL	0.43	44
	7	5	VI	0.71	38
	3	3	—	1	45
	15	13	VII	0.87	39
	7	7	—	1	46
	6	2	VI	0.33	47
	13	9	V, OL + StLe	0.69	48
	9	6	VI	0.67	49
	14	14	—	1	50
	8	8	—	1	51
	10	10	—	1	52
Tert.-Butyldimethylsilyl ethers	15	13	VII	0.87	39
	6	6	—	1	53
	5	3	VI	0.60	54
	10	4	IV–VI	0.40	55

[a] Non-separable species: IV = StP + PO; V = PLe + StSt + StO; VI = StO + OO + StL + OL; VII = LL + Ole.

Ag⁺-RP-TLC significantly exceeds RP-HPLC in the selectivity of separation.

There are also a large number of studies in which DAG mixtures of different types of derivatives were simultaneously fractionated, quantified and identified by gas chromatography (GC). ESS values of *sn*-3-O-acetates and silyl ethers of DAGs separated by this method are presented in Table V. These results indicate that in general the separation selectivity achieved by GC was significantly higher than that in RP-HPLC experiments. In many instances it was close to the selectivity of separation achieved by Ag⁺-TLC + Ag⁺-RP-TLC [39,48,50,52]. At the same time, the number of DAG species separated from each other never reached fifteen because no workers succeeded in separating LL from OLe (fraction VII in Table V). In addition, the components of fractions IV–VI in Table V frequently did not differ from each other in their mobilities. Finally, it must be stressed that the selectivity of GC fractionation usually slightly decreased with increasing DAG unsaturation, whereas the reverse was true when separation was carried out using Ag⁺-TLC + Ag⁺-RP-TLC.

Hence, in the selectivity of separation of our DAG mixture, the technique of Ag⁺-TLC + Ag⁺-RP-TLC is at least equal to GC and even exceeds it in certain respects. Both methods are also about equal as regards the amount of laboratory work and manipulations required. At the same time, the advantage of Ag⁺-TLC + Ag⁺-RP-TLC as compared with HPLC and GC is that its application does not require elaborate equipment. In addition, when using Ag⁺-TLC and Ag⁺-RP-TLC there is no dependence on the synthesis of various DAG derivatives. This is essential for performing DAG separations by RP-HPLC and GC, although it is frequently accompanied by the migration of acyl residues, *i.e.*, a breakdown of the native structure of DAGs [7].

In conclusion, the possiblity of simultaneous DAG determination is usually regarded as an advantage of GC as compared with planar chromatography. However, inspection of a considerable body of the results of such experiments shows that they lack sufficient accuracy [7]. Thus, the recovery of trimethylsilyl ethers of DAGs after GC is far below 100% [56]. This seems to be caused by the high-temperature oxidation of polyunsaturated DAG

molecular species and partial retention of DAG samples in the GC column [56]. Therefore, it could be assumed that is is more advisable to carry out the quantitative determination of lipids by liquid chromatography.

REFERENCES

1 V. P. Pchelkin and A. G. Vereshchagin, *J. Chromatogr.*, 209 (1981) 49.
2 V. P. Pchelkin and A. G. Vereshchagin, *Appl. Biochem. Microbiol. USSR*, 24 (1989) 667.
3 V. P. Pchelkin and A. G. Vereshchagin, *J. Chromatogr.*, 538 (1991) 373.
4 D. H. van den Eijden, *Anal. Biochem.*, 57 (1974) 321.
5 P. Michelsen, E. Aronsson, G. Odham and B. Åkesson, *J. Chromatogr.*, 350 (1985) 417.
6 A. G. Vereshchagin, *Izv. Akad. Nauk SSSR, Ser. Biol.*, No. 1 (1981) 54.
7 V. P. Pchelkin and A. G. Vereshchagin, *Adv. Chromatogr.*, 32 (1991) 87.
8 P. J. Schoenmakers, *Optimization of Chromatographic Selectivity —a Guide to Method Development*, Elsevier, Amsterdam, 1986, p. 5.
9 Y. Kondoh and S. Takano, *J. Chromatogr.*, 393 (1987) 427.
10 Y. Nakagawa and L. A. Horrocks, *J. Lipid Res.*, 204 (1983) 1268.
11 Y. Nakagawa, T. Sugiura and K. Waku, *Biochim. Biophys. Acta*, 833 (1985) 323.
12 K. Itoh, A. Suzuki, Y. Kuroki and T. Akino, *Lipids*, 20 (1985) 611.
13 Y. Nakagawa, K. Waku, A. Hirose, Y. Kawashima and H. Kozuka, *Lipids*, 21 (1986) 634.
14 S. Pind, A. Kuksis, J. J. Myher and L. Marai, *Can. J. Biochem. Cell Biol.*, 62 (1984) 301.
15 M. L. Blank, M. Robinson, V. Fitzgerald and F. Snyder, *J. Chromatogr.*, 298 (1984) 473.
16 S. J. Robins and G. M. Patton, *J. Lipid Res.*, 27 (1986) 131.
17 P. V. Subbaiah and H. Monshizadegan, *Biochim. Biophys. Acta*, 963 (1988) 445.
18 M. Careaga-Houck and H. Sprecher, *J. Lipid Res.*, 30 (1989) 77.
19 B. Banerji, P. V. Subbaiah, R. E. Gregg and J. D. Bagdade, *J. Lipid Res.*, 30 (1989) 1907.
20 A. P. Simoes, G. N. Moll, B. Beaumelle, H. J. Vial, B. Roelofsen and J. A. F. Op den Kamp, *Biochim. Biophys. Acta*, 1002 (1990) 135.
21 M. Batley, N. H. Packer and J. W. Redmond, *Biochim. Biophys. Acta*, 710 (1982) 400.
22 D. G. Bishop, *J. Liq. Chromatogr.*, 10 (1987) 1497.
23 M. Batley, N. H. Packer and J. W. Redmond, *J. Chromatogr.*, 198 (1980) 520.
24 M. Kito, H. Takamura, H. Narita and R. Urade, *J. Biochem.*, 98 (1985) 327.
25 H.Takamura, H. Narita, R. Urade and M. Kito, *Lipids*, 21 (1986) 356.
26 M. Ishinaga and K. K. Carroll, *Biochem. Cell Biol.*, 66 (1988) 1163.

27 M. V. Bell, *Lipids*, 24 (1989) 585.
28 H. Takamura, K. Tanaka, T. Matsuura and M. Kito, *J. Biochem.*, 105 (1989) 168.
29 H. Takamura, H. Kasai, H. Arita and M. Kito, *J. Lipid Res.*, 31 (1990) 709.
30 M. Schlame, I. Horvath, Z. Forok, L. I. Horvath and L. Vigh, *Biochim. Biophys. Acta*, 1045 (1990) 1.
31 M. Schlame, L. I. Horvath and L. Vigh, *Biochem. J.*, 265 (1990) 79.
32 J. Kruger, H. Rabe, G. Reichmann and B. Rustow, *J. Chromatogr.*, 307 (1984) 387.
33 B. Rustow, D. Kunze, H. Rabe and G. Reichmann, *Biochim. Biophys. Acta*, 835 (1985) 465.
34 A. M. Justin, C. Demandre, A. Tremolieres and P. Mazliak, *Biochim. Biophys. Acta*, 836 (1985) 1.
35 P. Laakso and W. W. Christie, *Lipids*, 25 (1990) 349.
36 C. S. Ramesha, W. C. Pickett and D. V. K. Murthy, *J. Chromatogr.*, 491 (1989) 37.
37 Y. Ohno, I. Yano and M. Masue, *FEBS Lett.*, 50 (1975) 50.
38 J. Itabashi and T. Takagi, *Lipids*, 15 (1980) 205.
39 A. Kuksis, in H. Mangold (Editor), *CRC Handbook of Chromatography: Lipids*, CRC Press, Boca Raton, FL, 1984, p. 381.
40 D. A. Brengartner, *Anal. Chim. Acta*, 173 (1985) 177.
41 J. J. Myher, A. Kuksis, L. Marai and P. Sandra, *J. Chromatogr.*, 452 (1988) 93.
42 G. Casparrini, M. G. Horning and E. C. Horning, *Anal. Lett.*, 1 (1968) 481.
43 G. Casparrini, E. C. Horning and M. G. Horning, *Ann. Inst. Super Sanita*, 6 (1970) 189.
44 M. G. Horning, S. Murakami and E. C. Horning. *Am. J. Clin. Nutr.*, 24 (1971) 1086.
45 A. Kuksis, L. Marai and J. J. Myher, *J. Chromatogr.*, 273 (1983) 43.
46 A. Kuksis, J. J. Myher and L. Marai, *J. Am. Oil Chem. Soc.*, 62 (1985) 762.
47 M. S. Pessin and D. M. Raben, *J. Biol. Chem.*, 264 (1989) 29.
48 J. J. Myher and A. Kuksis, *J. Chromatogr.*, 471 (1989) 187.
49 J. J. Myher, A. Kuksis and S. Pind, *Lipids*, 24 (1989) 396.
50 T. Rezanka and M. Podojil, *J. Chromatogr.*, 463 (1989) 397.
51 L.-Y. Yang, A. Kuksis and J. J. Myher, *Biochem. Cell Biol.*, 68 (1990) 480.
52 J. J. Myher, A. Kuksis and L.-Y. Yang, *Biochem. Cell Biol.*, 68 (1990) 336.
53 P. Child, J. J. Myher, F. A. Knypers, J.A.F. Op den Kamp, A. Kuksis and L. L. M. van Deenen, *Biochim. Biophys. Acta*, 812 (1985) 321.
54 A. Kuksis, J. J. Myher and L. Marai, *J. Am. Oil Chem. Soc.*, 62 (1985) 767.
55 A. Kuksis, J. J. Myher and P. Sandra, *J. Chromatogr.*, 500 (1990) 427.
56 A. Dyer and E. Klopfenstein, *Lipids*, 12 (1977) 889.