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Identification of individual diacylglycerols by adsorption thin-layer chromatography of their coordination complexes

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ABSTRACT

Diacylglycerols (DAGs) obtained by glycerolysis of a mixture of cocoa butter, poppy seed and linseed oils were separated by preparative thin-layer chromatography (TLC) into their positional isomers. An isomerically pure model mixture of *rac*-1,2-DAGs was further separated into eleven fractions using adsorption TLC of coordination complexes of unsaturated DAG species with silver ions on an analytical scale. The mobility of the separate TLC zones of this mixture, which included residues of stearic (St), palmitic (P), oleic (O), linoleic (L) and linolenic (Le) acids, was compared with that of standard *rac*-1,2-DAG zones. The comparison demonstrated that the model mixture was composed of the following individual DAG species: StO-, PO-, OO-, OL-, LL-, OLe-, LLe- and LeLe-glycerols, in addition to SS-, SL- and SLe-glycerols in which S is St or P. The different mobilities of these DAG species can be accounted for by the differences in the relative polarities of the Ag^+ complexes of O, L and Le, which were found to be 1.03, 2.46 and 5.45, respectively. There was a strong negative correlation between the mobility and the polarity of the DAG species.

INTRODUCTION[a]

Fractionation of a model mixture of diacylglycerols (DAGs) by reversed-phase thin-layer chromatography (RP-TLC) has been reported previously [1]. The distribution of individual fatty acid residues between DAG molecular species was found to be close to random (see below), and therefore this mixture could include as many as fifteen DAG species. Because of this complexity, the complete separation of the mixture into individual species of DAGs was not achieved, and so a new attempt was made to identify them. In order to increase the separation selectivity of DAGs, we

[a] Major abbreviations: DAGs and TAGs = di- and triacylglycerols, respectively; FAMEs = fatty acid methyl esters; St, P, O, L, Le, S, U = residues of stearic, palmitic, oleic, linoleic, linolenic, saturated and unsaturated fatty acids, respectively; e = number of olefinic bonds in a DAG molecule; Δe = difference in e values between two adjacent DAG zones; n and N = number of major fatty acid and DAG molecular species in their mixtures, respectively; Ag^+-TLC = adsorption TLC on silver nitrate-impregnated silica gel; RP-TLC = reversed-phase TLC; $hR_F = 100R_F$; $hR_{1,3\text{-LL}} = 100R_{1,3\text{-LL}}$ = mobility of a DAG zone in relation to that of *rac*-1,3-LL.

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tested other modes of TLC which differ from RP-TLC in the mechanism of lipid fractionation, in particular adsorption TLC of the coordination complexes of unsaturated lipids with silver ions (Ag^+-TLC). To identify DAGs in the model mixture, we compared their Ag^+-TLC mobilities with those of standard DAGs of a relatively simple species composition. In this paper we report the results of the identification and discuss the separation selectivity of individual DAGs differing in their unsaturation.

EXPERIMENTAL

Materials

Purification of solvents and the preparation of the DAG model mixture and a mixture of trimethylborate and methanol were performed as described previously [1,2]. Mobile phases for TLC contained 10 ppm butylated hydroxytoluene and 0.9 ppm DL-α-tocopherol (Serva, Heidelberg, Germany) as antioxidants. Bromine, silver nitrate, boric acid, urea, glycerol (all analytical-reagent grade), *p*-toluenesulphonic acid (pure) and LSL_{254} silica gel (5–40 μm) with 13% gypsum (Chemapol, Prague, Czechoslovakia) were used as received. Glass plates with a permanent adsorbent layer were prepared as described previously [3] except that water, not toluene, was used to suspend the mixture of glass powder and silica gel.

Preparation of DAG standards

Fatty acid methyl esters (FAMEs) obtained by methanolysis of cocoa butter and poppy seed oil [1] were dissolved in 22% (w/v) methanolic urea solution and the resulting urea complexes of stearic (St), palmitic (P), oleic (O) and linoleic (L) acid methyl esters were precipitated by fractional crystallization at different temperatures [4]. Linolenic acid (Le) methyl ester was prepared from linseed oil as described previously [5]. The FAMEs thus obtained were used to prepare seven mixtures, in which the number of major FAME species (n) was two or three, as follows: (1) P + O; (2) St + O; (3) St + P + L; (4) St + P + Le; (5) O + L; (6) O + L + Le; (7) L + Le. The fatty acid composition of each FAME preparation was determined by gas chromatography (GC) [6].

Standard DAGs were synthesized by the modified method of Markley [7]. Each of the seven FAME mixtures (2 mmol) was mixed with glycerol (1 mmol), *p*-toluenesulphonic acid (2 mmol) and 10 ml of toluene, and incubated for 1 h at 118°C. The reaction mixture was used to isolate the sum of DAG isomers, *rac*-1,2- plus -1,3-DAGs [1], from which *rac*-1,2-DAGs used as standards were prepared [2].

Isolation of positional isomers of DAGs

For the preparative isolation of DAG positional isomers, 40 mg of the above standards were applied as a band on a starting line of a silica gel and boric acid layer (19:1; 200 × 200 × 0.5 mm) and separated for 1.5 h in a chamber for ascending TLC [1] using chloroform–acetone (98:2, v/v) as the mobile phase [8]. Layers were sprayed with a 0.001% aqueous solution of Rhodamine 6G, and the *rac*-1,2- and -1,3-DAG zones (R_F = 0.3 and 0.5, respectively) were revealed under UV radiation (254 nm). Each DAG isomer was eluted from the adsorbent for 1 h in an extractor [9] with

chloroform–methanol–trimethyl borate (2:1:0.1, v/v/v). The isomeric purity of the DAGs was determined by known methods [1]. Isomers of DAGs dissolved in benzene were stored in the cold in sealed ampoules [4].

Ag^+-TLC of DAGs

Prior to Ag^+-TLC, a plate with a permanent adsorbent layer (10 × 20 cm) was sprayed with a 1% (w/v) methanolic solution of silver nitrate [10,11]. As a result, the layer acquired about 5% (w/w) of silver nitrate. At the starting line of this plate, 20–50 μg of *rac*-1,2- or -1,3-DAGs were applied, and DAGs were separated for 3–23 h by continuous TLC [12] using a silver nitrate-saturated mixture of chloroform and isopropanol (99:1, v/v) as the mobile phase. Silver ions were removed from the adsorbent with water and the dry plate was held for 30 min in the presence of bromine vapour. DAG zones were revealed as described previously [3], and their mobility was expressed as $(hR_{1,3\text{-LL}})_i$ using $x \pm s$, where x is the arithmetic mean of individual measurements of mobility and s is the absolute standard deviation of these measurements. The relative intensity of DAG zones obtained during the analysis of a given standard mixture was assessed visually. Other methods were the same as those used previously [1,2].

RESULTS AND DISCUSSION

Composition of DAG positional isomers in the products of glycerolysis

A DAG model mixture was obtained by glycerolysis, and therefore it was a mixture of DAG positional isomers [1]. Previously we did not consider the role of these isomers in the analysis of the DAG composition by RP-TLC, because under these conditions they did not differ from each other in their TLC mobility [1]. However, in adsorption TLC such a separation within a given DAG molecular species does take place; this was first demonstrated by Barrett *et al.* [13] in the Ag^+-TLC of synthetic *rac*-1,3- and -1,2-DAGs. Therefore, before discussing the Ag^+-TLC fractionation of the DAG model mixture, it is necessary to consider the qualitative and quantitative composition of its positional isomers.

Evidently, the random DAG model mixture (see below) obtained by glycerolysis of esters of fatty acids A and B (where A = B and also A ≠ B) contains non-symmetrical positional isomers, *i.e.*, *sn*-1,2-(AB) and *sn*-2,3-(AB) stereoisomers; their sum, according to the IUPAC nomenclature [14], is referred to as *rac*-1,2-AB. In turn, the natural *sn*-1,2-(AB) stereoisomers include the positional isomers of fatty acid residues, *i.e.*, *sn*-1-A,2-B- and *sn*-1-B,2-A-glycerols. Similarly, *sn*-2,3-(AB) stereoisomers, which do not occur in nature, include *sn*-2-A,3-B- and *sn*-2-B,3-A-glycerols. Together with *rac*-1,2-AB, the DAG model mixture also contains symmetrical isomers, *i.e.*, *sn*-1-A,3-B- and *sn*-1-B,3-A-glycerols; the sum of these stereoisomers is designated as *rac*-1,3-AB.

During adsorption TLC, *rac*-1,2- and -1,3-AB completely separate from each other (see above), regardless of the presence of silver ions in the mobile phase, whereas resolution within a given positional isomer does not occur [11]. Thus, synthetic *rac*-1,2-DAGs do not differ from natural *sn*-1,2-DAGs in their TLC mobility. Therefore, only a *rac*-1,2-DAG mixture was used as a model mixture in this study. This mixture was isolated from the glycerolysis products by preparative adsorption TLC on

boric acid-containing silica gel, because the presence of this acid enhances the difference in TLC mobility between the positional isomers [8]. Non-symmetrical and symmetrical DAG isomers thus obtained proved to be chromatographically pure.

Determination of the number of zones formed during Ag^+-TLC of the rac-1,2-DAG model mixture

Fatty acid residues present in the DAG model mixture vary in their unsaturation

TABLE I

IDENTIFICATION OF INDIVIDUAL DAG SPECIES IN SEPARATE Ag^+-TLC ZONES OF DAG STANDARDS AND RELATIVE POLARITIES OF COORDINATION COMPLEXES OF THESE DAG SPECIES (p_{DAG})

$hR_{1,3\text{-LL}}$ of DAG zones ($x \pm s$)	Possible DAG species composition (mol%)[a] and visual evaluation of their relative contents[b] in standards 1–7							DAG identification (e values)	p_{DAG}[c]
	1	2	3	4	5	6	7		
700 ± 9	49 +++	53 +++	63 +++	57 +++	–	–	–	SS (0)	0 0 0
488 ± 7	[2] +	35 +++	[2] +	–	–	–	–	StO (1)	1.03 1 1
344 ± 8	40 +++	[2] +	–	–	–	–	–	PO (1)	1.03 1 1
250 ± 6	9 ++	7 ++	–	–	–	46 +++	–	OO (2)	2.06 2 2
147 ± 9	–	[3] +	31 +++	[3] +	2 +	–	–	SL (2)	2.46 2.1 1.9
94 ± 6	–	–	–	–	12 ++	9 ++	[2] +	OL (3)	3.49 3.1 2.9
60 ± 4	–	–	4 ++	–	86 +++	–	3 +	LL (4)	4.92 4.2 3.8
33 ± 3	–	–	–	35 +++	–	–	–	SLe (3)	5.45 4.4 5.1
20 ± 3	–	–	–	–	–	35 +++	[8] ++	OLe (4)	6.48 5.4 6.1
10 ± 2	–	–	–	–	–	3 +	25 +++	LLe (5)	7.91 6.5 7.0
3 ± 1	–	–	–	5 ++	–	7 ++	62 +++	LeLe (6)	10.90 8.8 10.2

[a] Calculated from the data of DAG fatty acid composition in Table II. Figures in square brackets are the content of minor DAGs.

[b] –, +, ++, and +++ represent visual evaluation of the relative intensities of DAG zones.

[c] Top, middle and bottom figures are p_{DAG} values calculated by using p_i, p'_i and p''_i, respectively.

(number of olefinic bonds, *e*) from 0 to 3 [1]. Therefore, DAG species with $e = 0$–6 can be assumed to be present in this mixture. On the basis of this assumption, it could be expected that during Ag^+-TLC of the model mixture seven *e* fractions of DAGs would be formed, because during the Ag^+-TLC of FAMEs only separation according to *e* has been observed [11], each zone differing from the adjacent zone by one *e* unit (*i.e.*, $\Delta e = 1$). In reality, however, the total number of zones formed during this separation turned out to be eleven instead of seven (*cf.*, Table I). Thus, the DAG separation was not limited by the $\Delta e = 1$ rule, and hence many adjacent Ag^+-TLC zones did not differ from each other in their *e* values (*i.e.*, $\Delta e = 0$). At the same time this number (eleven) was less than the possible total number of DAG molecular species in the model mixture (fifteen [1]); therefore, some zones seem to be heterogeneous, *i.e.*, to contain more than one DAG molecular species.

The same number of zones (eleven) was observed after Ag^+-TLC of the *rac*-1,3-DAG mixture. These isomers always exceeded the corresponding *rac*-1,2-DAG molecular species in their relative mobility. For example, the latter was equal to 100 for *rac*-1,3-LL compared with 60 for *rac*-1,2-LL. In the studies described below, the only *rac*-1,3-DAG species used was *rac*-1,3-LL, which served as a standard in the calculation of the relative mobility of individual *rac*-1,2-DAG molecular species ($hR_{1,3\text{-LL}}$; see Experimental).

Standards for the identification of individual rac-1,2-DAG molecular species in separate Ag^+-TLC zones

In order to determine the *rac*-1,2-DAG molecular species composition in each of the eleven Ag^+-TLC zones obtained, all of them were compared with respect to their mobility with various *rac*-1,2-DAG standards (see Experimental). These standards were synthesized by catalysed transesterification of FAMEs and glycerol in toluene [7], because this reaction could be carried out at a lower temperature than the glycerolysis without the solvents used earlier for preparing the model mixture [1]. Thus, both the mixture and the standards were the products of transesterification, differing from each other only in the number of fatty acid molecular species used for the synthesis. Hence, the possible DAG molecular species composition can be described in both instances in terms of the same distribution theory, *viz.*, the theory of random distribution [1].

According to this theory, the distribution of fatty acid residues between the products of glycerolysis, including DAGs, occurs in such a way that the probability of formation of a given DAG molecular species is determined on the one hand by the molar content of its component fatty acids in the original FAME mixture, and on the other by the number of a given fatty acid residue (one or two) in this species. Finally, the calculation of the random distribution is based on the assumption that all DAGs are formed by a completely random esterification of each hydroxyl group, and the rate of this reaction does not depend on the nature of fatty acid residues.

The qualitative composition, *i.e.*, the possible total number of major DAG molecular species (N) in each *rac*-1,2-AB standard, disregarding AB and BA positional isomers (see above), is made up of monoacid (n) and diacid [$n(n-1)/2$] DAG molecular species, and so is equal to $N = n(n+1)/2$ [1]. The fatty acid composition of both the standards and the original FAME mixtures is shown in Table II. Both lipid classes appear to be generally similar in this respect, each standard including 3–5 fatty acid species. However, only the major species (n), *i.e.*, those exceeding 6% of the total,

TABLE II

FATTY ACID COMPOSITION OF ORIGINAL FAME MIXTURES AND RESULTING DAG STANDARDS

Standards		Fatty acid composition (mol%)				
No.	Class	St	P	O	L	Le
1	FAMEs	4.0	69.8	26.2	–	–
	DAGs	3.1	67.0	29.9	–	–
2	FAMEs	68.8	2.9	25.8	2.5	–
	DAGs	69.3	3.2	25.3	2.2	–
3	FAMEs	48.4	23.8	0.6	27.2	–
	DAGs	49.6	27.8	2.1	20.5	–
4	FAMEs	44.0	26.4	1.2	1.8	26.6
	DAGs	44.0	30.1	1.2	2.2	22.5
5	FAMEs	–	1.1	6.8	92.1	–
	DAGs	–	0.7	6.9	92.4	–
6	FAMEs	–	0.5	65.3	6.2	28.0
	DAGs	–	0.8	67.4	7.0	24.8
7	FAMEs	–	–	3.0	16.3	80.7
	DAGs	–	–	5.2	16.2	78.6

were used to calculate N. In the standards 1, 2, 5 and 7 (where $n = 2$) $N = 3$, and in those with $n = 3$ (3, 4 and 6) $N = 6$. Thus, the N value in each standard was much less than $N = 15$ ($n = 5$) in the DAG model mixture [1]. Therefore, the molecular species composition in a given standard could be relatively easily determined and in turn used for identifying individual DAG species in the model mixture.

The quantitative composition, *i.e.*, the random concentration of each molecular species in the DAG standards (A_1, A_2, ..., A_N%), can be calculated by using the concentrations of individual fatty acid residues in these standards (a, b, ..., %). For each monoacid DAG species $A_1 = a^2/100$, and for the diacid species $A_2 = 2ab/100$, the factor of 2 being used in this equation to take into account both positional isomers AB and BA of diacid DAGs. DAG contents below 2%, *i.e.*, below the usual sensitivity limit when using the Ag^+-TLC technique, were disregarded and the remaining ones approximated to the nearest unit percent.

The results of the "random" calculation of both qualitative and quantitative DAG molecular species composition in the standards 1–7 and the data on the Ag^+-TLC mobility of the latter are shown in Table I. It can be seen that the standards as a whole formed eleven TLC zones, as was also the case with the original DAG model mixture (see above), whereas the number of major zones in each standard varied from three to five.

Identification of individual DAG species

Using the data in Tables I and II, let us consider the identification procedure for DAGs present in each of these zones, beginning with the TLC zone characterized by the lowest mobility ($hR_{1,3\text{-LL}} = 3 \pm 1$) and referred to below as zone 3. It can be seen that this zone can be found in the standards containing Le (Nos. 4, 6 and 7), although it is only in standard 7 that this zone is the predominant one because of its high Le

content. Thus, zone 3 contains only the LeLe species characterized by the maximum *e* number ($e = 6$) possible for our model mixture.

Zone 10 adjoining the LeLe can only be found in standards 6 and 7, which, unlike all the other standards, include both L and Le. On the transition from standard 6 to standard 7 there is a rise in the L level from 7.0 to 16.2%, and an increase in the zone 10 content (from + to + + +). At the same time, this zone is absent from standards 3 and 4, which contain only one or other of the two polyenic fatty acids. Therefore, this zone cannot be composed of monoacid DAGs (LL or LeLe); it can contain only a single DAG species, *viz.*, LLe, the only possible combination of L and Le.

The next zone, 20, is present mainly in standard 6, characterized by an elevated content of O and Le. Therefore, it may represent either OLe or OO. The first possibility is more likely because there is a positive correlation between the relative intensity of zone 20 in standards 6 and 7 and their O content. The latter hypothesis is improbable as zone 20 is absent from standards 1 and 2, which must certainly contain diolein. Therefore, we conclude that zone 20 includes only OLe.

Zone 33 occurs only in standard 4, in which saturated (S) fatty acids account for 85% of its total fatty acids whereas the unsaturated (U) fatty acids are composed predominantly of Le. In addition to zone 33 this standard contains only trace amounts of LeLe and also a highly non-polar zone 700 present also in standards 1 and 2 (SS, see below). Therefore, zone 33 can include only SLe, where S is either P or St.

Zone 60 is the major component in standard 5, where L accounts for 92% of total fatty acids. Therefore, this zone can include only a single individual DAG species, *i.e.*, LL. As regards zone 94, it can be detected only in the standards devoid of S (Nos. 5–7). Therefore, DAGs in this zone can only be of the UU type, *i.e.*, either OL or OO. However, the latter hypothesis should be discarded owing to the absence of zone 94, together with zone 20 (see above), in standard 1. It is inferred that zone 94 is composed of the diacid OL species.

A high concentration of zone 147 was found only in standard 3, which contains mainly S and L. Obviously, DAGs of this zone have nothing to do either with the SS type (because of the absence of this zone in standard 1, see above) or with the UU type, as LL is located in zone 60, and the presence of OO can be excluded because of the low O content in standard 3. It is therefore evident that zone 147 is composed only of SL.

Zone 250 was found in standard 6 and thus should belong to the UU type (see above). We believe that this zone includes only the monoacid OO species because all the other UU DAGs have already been identified. Moreover, O makes up to 67% of the total fatty acids in standard 6. Diolein occurs also in standards 1 and 2, which have a higher O content than any other standard except 6.

Zones 344 and 488 are present almost exclusively in standards 1 and 2, respectively. It is evident that these DAGs do not belong to either the UU or the SS type (see above), and should therefore represent only the SU DAGs. We consider that zones 344 and 488 are composed of PO and StO, respectively. This conclusion is based mainly on the fatty acid composition of standards 1 and 2 (Table II). Moreover, in standard 3, in which the St content is 1.3 times higher than that of P and the calculated StO and PO concentrations are 2.5 and 0.8 mol-%, respectively, there is the clear zone 488 (StO) whereas zone 344 (PO) cannot be observed.

Finally, standards 1–4 have one more zone, 700, which exceeds any other zone in its mobility. All UU and SU DAGs having already been identified (see above), this

zone should contain only SS species, which are known not to form coordination complexes and therefore cannot be separated by Ag^+-TLC [11].

Hence Ag^+-TLC makes it possible to identify in standards 1–7 eight individual DAGs, *viz.*, StO, PO, OO, OL, LL, OLe, LLe, and LeLe, *i.e.*, all UU and SO species possible. It follows also from our data that these standards contain SS, SL and SLe components. Their species composition cannot be determined by Ag^+-TLC (see above); the maximum number of individual DAG species in these components is 3, 2 and 2, respectively. The eleven zones found in standards 1–7 coincided in their TLC mobilities with the respective zones of the model mixture [2]. The data for DAG identification in this mixture can be considered to be accurate because the fatty acid composition of DAGs used as standards was established unambiguously (see above). Therefore, the GC and mass spectrometric identification of DAGs in separate zones were not carried out.

It can be expected that Ag^+-TLC will be suitable for determining the species composition of DAG radicals of highly unsaturated polar glycerolipids of plant origin because they are usually characterized by low levels of S and SS ($\leqslant 20$ and $\leqslant 4\%$, respectively), and of SL and SLe. At the same time, complete identification of DAGs in mixtures with similar S contents to our model mixture would require the combination of Ag^+-TLC with some other chromatographic techniques.

Selectivity of DAG separation by Ag^+-TLC

The comparison of the fatty acid composition of DAG species identified above with their mobility on Ag^+-TLC fractionation makes it possible to assess the selectivity of this separation. From Table I it can be seen that at $\Delta e \geqslant 1$ these species always separate from each other (A-type separation). This rule has been demonstrated repeatedly for both DAGs and other neutral lipids [15].

At the same time, when separating DAGs with $e = 1$–4, it has been established that their mobility depends not only on e, but also on other factors, because in this instance there was a separation of DAG species with $\Delta e = 0$ (B-type separation, see above). In this instance there were three modes of separation: mode B_1 involves the resolution of UU DAGs with the same e value (*e.g.*, LL and OLe), mode B_2 the separation of UU DAGs from respective SL and SLe DAGs with strongly asymmetric structures (*e.g.*, OL from SLe, and OO from SL) and mode B_3 the separation of SO DAGs differing in the length of their unsaturated acyl residues (StO and PO).

We are unaware of any B_1- and B_3-mode fractionation experiments performed so far, but the B_2-mode separation has been reported [16,17]. For example, when DAGs obtained by hydrolysis of animal lecithins were fractionated using a mixture of chloroform and ethanol (96:4) as the mobile phase, the hR_F values of OL and SLe were 58 and 44 and those of OO and SL were 75 and 64, respectively [17]. These data are close to our results (Table I).

Relationship between the selectivity of Ag^+-TLC separation of individual DAG species and the relative polarity of their coordination complexes[a]

The data in Table I suggest that in the course of separation according to modes

[a] The term relative polarity of coordination complexes of unsaturated DAG species with silver ions means the affinity of these complexes to the highly polar stationary phase of the Ag^+-TLC system [11]. This term is referred to subsequently simply as "polarity (of lipids)".

A and B the elution order of individual DAG species is inversely proportional to their relative polarity (p_{DAG}). To test this suggestion we had to calculate the p_{DAG} value. To this end we assumed that p_{DAG} values are additive, being similar in this respect to TAG polarity values reported earlier [18]. From this viewpoint, the value of p_{DAG} is equal to the sum of polarities of fatty acid residues (p_i) forming a given DAG species:

$$p_{DAG} = \sum_i (n_i p_i) \quad (1)$$

where n_i is the number of ith fatty acid residues in this species. In turn, p_i values were calculated from the mobilities of disaturated DAGs (SS) and Ag^+ complexes of monoacid diunsaturated DAGs (UU), OO, LL and LeLe (Table I). By substituting these data in the equation

$$p_i = \ln [(hR_{1,3\text{-LL}})_{SS}/(hR_{1,3\text{-LL}})_{UU}] \quad (2)$$

obtained empirically, it was shown that the p_i values of O, L and Le residues are 1.03, 2.46 and 5.45, respectively.

It was of interest to compare these values with those obtained earlier when separating Ag^+ complexes of other neutral lipid classes, *viz.*, FAMEs and TAGs [18,19]. FAMEs were separated by countercurrent distribution between *n*-hexane and 0.2 *M* silver nitrate in aqueous methanol (9:1) [20]. In this experiment, on transition from O ($e = 1$) to L ($e = 2$) and from O to Le ($e = 3$), the affinity of the ith FAME to the polar phase of the system, *i.e.*, its p_i value, did not increase 2- and 3-fold, respectively, but increased to a much greater extent. In the opinion of Scholfield *et al.* [20], this relationship occurs because on the formation of coordination complexes every olefinic bond of L and Le polarizes the adjoining one, the degree of such polarization being directly proportional to the number of methylene groups located between the interacting double bonds. Thus, the qualitative relationships between O, L and Le residues as regards their polarity found by these authors [20] were similar to those found in our work; however, they failed to obtain quantitative data to characterize this parameter.

The hypothesis of Scholfield *et al.* [20] was confirmed by Gunstone and Padley [18], who attempted for the first time to assess semiquantitatively the "complexing power" (p'_i) between the O, L and Le residues on the one hand and the silver ions on the other on Ag^+-TLC of natural TAGs including these residues (see above). In further discussion we assume that the p'_i values are directly proportional to the polarity of the fatty acid residues. According to Gunstone and Padley [18], $p'_S = 0, p'_O = 1, p'_L = 2 + a$ and $p'_{Le} = 4 + 4a$, where $a < 1$ is some fraction. Let $a = 0.1$, then $p'_L = 2.1$ and $p'_{Le} = 4.4$.

Finally, when using Ag^+-TLC to separate FAMEs, Grynberg and Ceglowska [19] introduced the concept of "relative force of complex formation" of an ith fatty acid residue (f.c.$_i$). This concept was similar to that of Gunstone and Padley's "complexing power", but its calculation was based on the results of TLC rather than on an arbitrary assumption:

$$\text{f.c.}_i = p''_i = [(1 - R_F)_i - (1 - R_F)_S]/[(1 - R_F)_O - (1 - R_F)_S] \quad (3)$$

It follows from eqn. 3 that p''_O is always equal to 1. As regards p''_L and p''_{Le}, it has been shown that in a mobile phase similar to that used in our work (chloroform–methanol [19]) they were equal to 1.9 and 5.1, respectively.

It can be seen that p'_i and p''_i are close to the respective p_i values (see above). At the same time, there is a considerable difference between p'_L and p''_L and between p'_{Le} and p''_{Le} values, f.c.$_i$ being even lower than the number of olefinic bonds in the L residue.

Using p_i and eqn. 1, we calculated p_{DAG} values for all DAG molecular species (see Table I). These values were used to verify our initial suggestion (see above) by the correlation coefficients r between p_{DAG} and $hR_{1,3\text{-LL}}$. The results of this calculation are given in Table III. It can be seen that there is a strong negative correlation between these parameters ($r = -0.804$; $p > 99\%$). Thus, as regards A, B_1 and B_2 separation modes, our suggestion was fully justified. At the same time, the B_3-mode separation between StO and PO could not be explained from this viewpoint, because these species did not differ from each other in their p_{DAG} values.

A similar correlation ($p > 99\%$) was found after replacing p_i in eqn. 1 with p'_i and p''_i (Table I). Nevertheless, it was of interest to compare the values of p_i, p'_i and p''_i as regards their accuracy. To this end they were used to calculate not only the polarity of free DAGs but also that of individual species of other lipid classes, *viz.*, DAG acetates and TAGs; the Ag^+-TLC mobility data of the latter (hR_F) were obtained earlier [15–17,21,22]. The polarity of these species calculated by substituting p_i, p'_i and p''_i into eqn. 1 was used to calculate correlation coefficients between p and hR_F (Table III). It

TABLE III

CORRELATION BETWEEN RELATIVE POLARITIES OF COORDINATION COMPLEXES OF INDIVIDUAL SPECIES BELONGING TO DIFFERENT NEUTRAL LIPID CLASSES AND Ag^+-TLC MOBILITY OF THESE COMPLEXES

$-r \cdot 1000$ values calculated using $hR_{1,3\text{-LL}}$ (Table I) and hR_F values found by other workers [15–17,21,22].

Lipid class	Polarity values of unsaturated fatty acid residues used for calculating lipid polarity according to eqn. 1			Ref.
	p_i	p'_i	p''_i	
DAGs	804	821	779	This work
	983[a]	968[a]	989[a]	16
	997	988	976	17
	946[a]	921[a]	893[a]	15
	998	993	984	15
DAG acetates	956	969	920	21
	991	995	980	15
	987	993	970	15
	977	962	944[a]	15
TAGs	989	979	966	21
	944	835[a]	830[a]	21
	980	975	966	22
	962	951	932	15
	809[a]	802[a]	818[a]	15

[a] Accurate at the $95\% < p < 99\%$ level; other r values are accurate at the $p > 99\%$ level.

can be seen that all correlations are accurate at $p > 95\%$. At the same time, when using p_i, p'_i and p''_i values, the correlation at $p > 99\%$ was observed in eleven, ten and nine cases, respectively. Thus, the relative polarity of unsaturated fatty acid residues on Ag^+-TLC is most accurately reflected by the p_i values.

The correlation between p and R_M values, which were calculated from R_F values [23], was similar to that described above (data not shown).

In conclusion, it should be stressed that the relationship between the polarity of an individual DAG species and its Ag^+-TLC mobility is logarithmic (see eqns. 1 and 2). Therefore, it can be suggested that there is some connection between the polarity of the DAG molecule and its chemical potential (μ), as $\ln(1/R_F - 1)$ is a member of the well known equation

$$\Delta\mu_X = RT\ln[(1/R_{F_A} - 1)/(1/R_{F_B} - 1)] \quad (4)$$

where $\Delta\mu_X$ is the difference between the μ values of A and B homologues, differing in the presence of an X group in B, R is the universal gas constant and T is the absolute temperature [23]. It must be recognized that eqn. 4 can be applied only to liquid–liquid systems. However, when separating FAME coordination complexes in such systems, *i.e.*, by reversed-phase partition chromatography [24] and countercurrent distribution [20], there was the same polarity relationship between O, L and Le residues as that described above.

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