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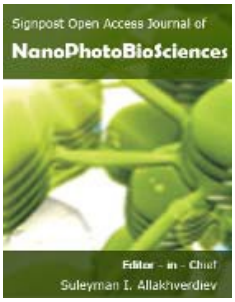

Quality Control Criteria for Liquid Chromatographic Separation of Membrane Lipids with Nanoscale Silver Clusters

Vasily P. Pchelkin* **and Dmitry A. Los**

Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya street 35, Moscow 127276, Russian Federation
*Corresponding author: E-mail: pchel_vp@ippras.ru

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«The right quality and uniformity are foundations of commerce, prosperity and peace...»
Deming W.E. (1986) «Out of the Crisis»

Abstract

The hydrophobicity (equivalent lipophilicity) of the individual lipid molecules and the degree of their unsaturation are the most important physicochemical characteristics of lipids. Complex mixtures of native lipids may be separated into fractions (each of which is normally characterized by its own hydrophobicity and/or its own unsaturation) by various methods of liquid chromatography (LC), including reversed-phase high-performance liquid chromatography (RP HPLC) and reversed-phase thin-layer chromatography (RP-TLC) as well as absorption and reversed-phase TLC (silver ion TLC and silver ion RP-TLC, respectively). Analysis of separated

mixtures of free rac-1,2-diacylglycerols from plant seeds revealed new virtual parameters of interaction between fatty acid (FA) double bonds and silver cations as nanoscale Ag_3 clusters. To compare the results of the quantitative analyses of molecular species composition obtained either by different methods or by identical methods in various laboratories, we used not the analytical results themselves but the values of their accuracy. These values served as universal criteria of the "fitness" of the results. The accuracy can be determined by the direct comparison of the calculated FA composition of separated lipid fractions with the experimentally determined composition. Our results together with other data on LC fractionation of phenethyl and phenacyl FA esters demonstrated that parameters of their separation are closely related to the variations of chemical potentials of each unsaturated lipid molecules (free energy values of their mass transfer between two liquid phases during RP-TLC or RP-HPLC fractionation in the presence of silver salts). The properties of coordination complexes of these natural lipid molecules with silver ions can be explained by the newly introduced virtual molecular parameters: relative polarity (p) and equivalent lipophilicity (L_3). Unsaturated FAs demonstrate strong correlation between lipophilicity and coordination numbers of silver atoms interacting with FAs in nanoscale clusters. Usually two olefin bonds interact with one silver atom, however, Ag_3 clusters are also possible. In general, the ionic and electronic conductivity of thin films consisting of lipid-silver clusters depends on the degree of FA unsaturation. We provide some examples of possible structures of new organic semiconductors with the controlled molecular architecture and different types of electronic conductivities.

Keywords

Quality control criteria; Chromatography; Fatty acids; Lipids; Silver nanoclusters

Introduction

Natural polar glycerolipids, or *sn*-1,2-diacylglycerols (*sn*-1,2-acyl$_2$Gros), form the structural basis of the biological membranes. Each individual molecular species of the acyl lipid class carries two fatty acid residues, which are characterized by an additive carbon number *(m)*, total olefinic bond number (*e*) (Equation 1):

$$e = (e_i)_1 + (e_i)_2, \quad (1)$$

as well as by nominal equivalent lipophilicity (Equation 2):

$$L = (L_i)_1 + (L_i)_2 = (m_i - 2e_i)_1 + (m_i - 2e_i)_2 \quad (2)$$

where L_i is the theoretical equivalent lipophilicity of *i*-th fatty acid (FA) residue; the latter is characterized by a definite number of carbon atoms (m_i) and of olefinic linkages (e_i) in each FA residue esterified to *sn*-1 or *sn*-2 position ($m_{sn\text{-}1}$, $e_{sn\text{-}1}$ or $m_{sn\text{-}2}$,

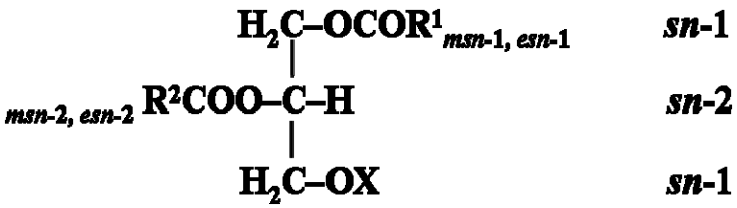


Figure 1: The total structural formula of free *sn*-1,2-diacylglycerol and their natural derivatives. R^1 – a fatty acyl chain at position *sn*-1 with $m_{sn\text{-}1}$ carbon atoms and e_{sn}-1olefinic linkages; R^2 – a fatty acyl chain at position *sn*-2 with $m_{sn\text{-}2}$ carbon atoms and e_{sn}-2 olefinic linkages; X = H, Gal, Gal, Gal_2, Gal_3, Gal_4, PO(O)OR; R = H, Cho, Etn, Ser, Ins, Gro, Ptd (Pchelkin, Vereshchagin, 1981; Figure 1):

$e_{sn\text{-}2}$ respectively) that correspond to virtual *s*tereo chemical numbering of the C-atoms of the glycerol (Gro) moiety.

Tremendous number of articles is devoted to studies of FA composition of lipid molecular species. Presentation of the original results in the concise form requires mathematical data processing (Pchelkin, Vereshchagin, 1992a). The experimental gas chromatographic (GC) data can be expressed as the calculated number of olefinic bonds (Das et al., 1982) in two FA residues of a molecule (Equation 3):

$$e_c = 2 \times \Sigma(a_i \times 10^{-2} \times e_i) \quad (3)$$

or as the calculated equivalent lipophilicity (Equation 4):

$$L_c = 2 \times \Sigma(a_i \times 10^{-2} \times L_i), \quad (4)$$

where a_i is the content of *i*-th FA in the mixture [Σa_i = 100 mole %].

Next stage of data processing involves the comparison of the calculated e_c and L_c values (Equations 5 and 6) with the respective nominal *e* and *L* values followed by subsequent evaluation of the relative deviations of e_c from *e* and L_c from *L* (s_e and s_L respectively, %%) for each j fraction of individual lipid class subjected to analysis with Equations 3 and 4.

$$s_e = 100\ |e_c - e|/e \quad (5)$$

&

$$s_L = 100\ |L_c - L|/L \quad (6)$$

Finally, the calculated deviation values are used to introduce the integral accuracy criteria for the molecular species composition analysis of each lipid class, *i.e*., the mean relative deviation.

This value can be calculated with Equations 7 and 8:

$$S_e = \Sigma(s_e)_j/n_e\ \% \quad (7)$$

&

$$S_L = \Sigma(s_L)_j/n_L\ \% \quad (8)$$

where n_e and n_L are the number of fractions obtained during *e*- and *L*-fractionation, respectively, of a particular lipid class (Pchelkin, Vereshchagin, 1992a).

Hydrophobic (methylene) selectivity (α) represents *size selectivity* during reversed-phase (RP) HPLC of a mixture of homologous lipophilic compounds (*m, m+1, ...*) in aqueous methanol mobile phase (*i.e.,* their *L*-fractionation). This α value is defined as a change of separation factor when the size of solute changes by 15.68 ml/mole without changing the energy of interaction with water (Golushko et al., 2007a). The unit of the *size selectivity* is defined as a change in the logarithmic separation factor (lnα) when the surface of solute in water changes by 1.0 cm^2/mole. The unit of the *polar selectivity* is defined as a change in the same factor when the value of the interaction energy of the solute with water changes by 1.0 kJ/mole (Golushko et al., 2007b).

Standard difference of Gibbs free energy of two homologous lipophilic mixtures during their phase transfer [$\Delta G_{CH2}^o = -R_gT \times \ln\alpha_c$] was calculated by Zhao & Carr (1999). It was demonstrated that, with the temperature rise from 298 to 323K, the α_c values decrease from 0.557 to 0.378, whereas the ΔG_{CH2}^o level varied from –330 to –318 cal/mole, *i.e*., from –1.38 to –1.33 kJ/mole (Yan et al., 2000). This ΔG_{CH2}^o = –275 cal/mole (1.15 kJ/mole) value was calculated for RP HPLC particles with chemically bonded octyl groups, whereas the ΔG_{CH2}^o = –314 cal/mole (1.31 kJ/mole) value was obtained for these particles with octadecyl groups (Zhao, Carr, 1999). Recently, a value of 0.4 kJ/mole has been determined for an aqueous methanol / *n*-hexadecane system (Rafferty *et al.*, 2007). We used these parameters for the estimation of $\Delta\mu_x/R_gT$ variations for major native esterified FAs in the presence and in the absence of silver ion clusters (Sections 3.1 and 3.4).

2. Nanoscale Silver Clusters

The interaction of three simplest unsaturated hydrocarbon molecules with a single silver atom may be considered as a suitable model for *e*-fractionation of complex unsaturated lipid mixtures (Figure 2). Basic principles of molecular dynamics simulation of a task-specific ionic liquid based on silver-olefin complex has been recently described (Jiang, Dai, 2008). Binding of the third C_2H_4 to Ag^+ is favorable, although the energy required for this process is lower than that for the first two C_2H_4 groups. This mechanism involves a concerted chain of reaction, in which one Ag^+ ion accepts one C_2H_4 and donates another ethylene to a nearby Ag^+ ion via a state of shared C_2H_4 groups between silver atoms.

An equal distance of 2.54 Å was determined between the silver ion and all three olefin molecules in the $Ag(C_2H_4)_3^+$ complex. Atomistic insights into this separation process have been described by Jiang and Dai (2008). At the same time, a crystal structure of zeolite A was determined, which contained Ag_4Cl_4 nanoclusters with reduced 1,3,5-tripyrylium dimmers with remarkable interplanar spacing (Kim et al., 2008).

$$Ag^+ + C_2H_4 \rightarrow Ag(C_2H_4)^+ \qquad E_1 = -1.42 \text{ eV}$$
$$Ag(C_2H_4)^+ + C_2H_4 \rightarrow Ag(C_2H_4)_2^+ \qquad E_2 = -1.20 \text{ eV}$$
$$Ag(C_2H_4)_2^+ + C_2H_4 \rightarrow Ag(C_2H_4)_3^+ \qquad E_3 = -0.47 \text{ eV}$$

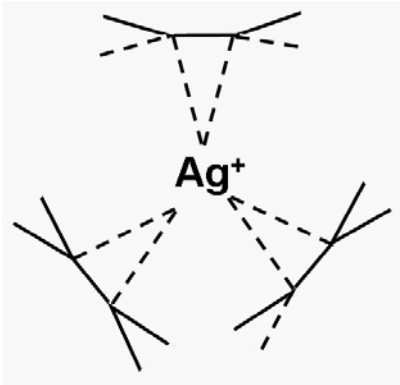


Figure 2: Interaction of silver ion with three ethylene molecules.

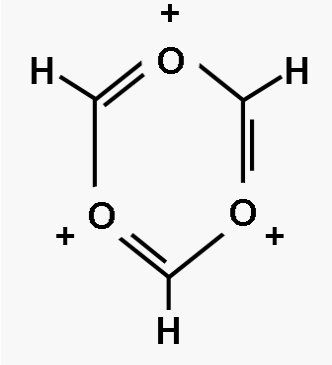


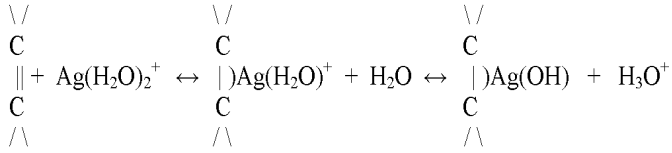
Figure 3: Structure of $C_3H_3O_3^{3+}$ cation.

The 1,3,5-tripyrylium ring (*cyclo*-2,4,6-tridehydro-1,3,5-trioxanium, $C_3H_3O_3^{3+}$ cation, see Figure 3) isoelectronic with benzene, had not been heretofore reported (Kim et al., 2008).

This tripyrylium ring formation takes place in acidic methanol and water mixtures, and the silver ion may serve as a catalyst of this process:

$$6MeOH + 2H_2O \rightarrow [C_3H_3O_3]_2^{2+} + 10H_2 + 2OH^-$$

According to the author's note, one ring bonds to a 3-fold axis of an Ag^+ ion (Kim et al., 2008). The interplanar spacing of 2.43Å was calculated, which is formed due to four-electron σ-bonding of 12-center π* orbitals and polar forces:



Stabilization of silver nanocluster in olefinic polymers is also accompanied by the processes of the π-complex formation with Ag atoms (Figure 4; Pomogailo et al., 1997).

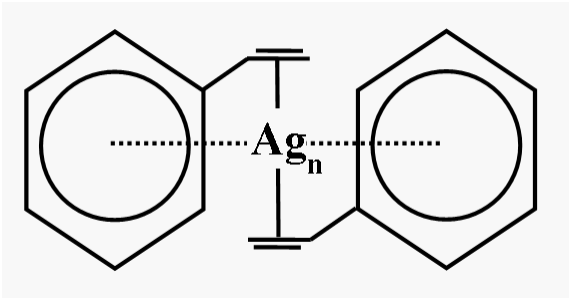


Figure 4: Structure of π-complex of the silver nanocluster and styrol molecules.

These new data were also included in characterization of coordination complexes of major native esterified FAs with silver ions (Section 4).

3. Separation Methods of Membrane Lipids

During the years, considerable efforts have been made to develop the methods of chromatographic separation of natural acyl$_2$Gro mixtures formed after the hydrolysis of polar glycerolipids into individual acyl$_2$Gro species. However, such mixtures are still not suitable for chromatographic separation due to insurmountable limitations caused by their FA composition. To overcome this problem, we prepared an artificial acyl$_2$Gro mixture with predetermined FA composition. This model mixture, obtained by transesterification of plant oils with glycerol (Gro), served as an adequate substitute for natural samples (Figure 5).

Along with separation of these mixtures into individual acyl$_2$Gro species, we also examined their FA composition. Such quantifications are obligatory for the use of these reference species as the standards in further investigation of the natural acyl$_2$Gros to uncover the patterns that govern the clustering of FA residues among the glycerol molecules.

The individual FAs of plant polar lipids are quite diverse, however, five major FAs are always present: palmitic (Pam or 16:0), stearic (Ste or 18:0), oleic (Ole or 18:1), linoleic (Lin or 18:2) and linolenic (αLnn or 18:3) acid. For this reason we used only these FA residues for preparation of acyl$_2$Gro mixtures.

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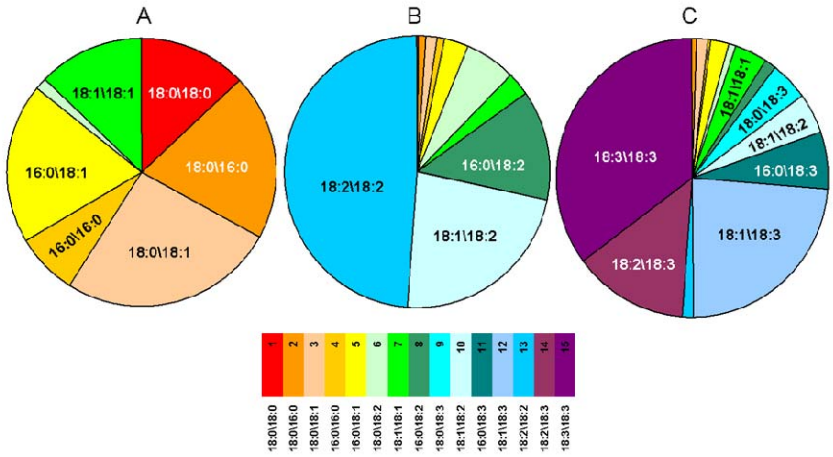


Figure 5: Random molecular species composition of acyl$_2$Gro samples prepared from cocoa butter (A), poppy seed oil (B) and linseed oil (C) by transesterification with glycerol (Gro).

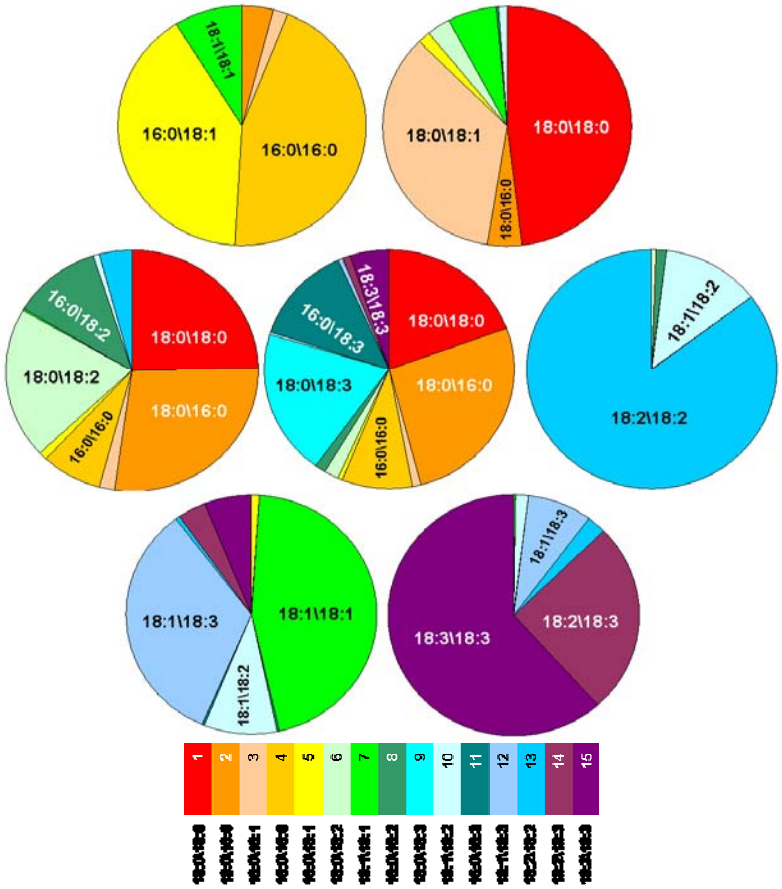


Figure 6: Random molecular species composition of seven synthetic *rac*-1,2-acyl$_2$Gro preparations from cocoa butter, poppy seed oil, and linseed oil.

Optimal starting material for transesterification was an equimolar fat mixture that contained approximately equal amounts (20% each) of these five FAs (n = 5). This was achieved by mixing cocoa butter, poppy seed oil and linseed oil according to the Equation 9:

$$k_1(a_i)_A + k_2(a_i)_B + k_3(a_i)_C = 20\ \%, \quad (9)$$

where k_1, k_2, and k_3 are the molar proportions of oils in the mixture ($k_1 + k_2 + k_3 = 1$) and a_i is the amount of FAs (a_i, mol.%) in acyl$_2$Gros from each of these oils (A, B, and C in Figure 5). This mixture was subjected to transesterification and subsequent silica gel column chromatography of the products yielding an acyl$_2$Gro mixture composed of 20 ± 3 mole % of each of the above mentioned FA residues. According to the random distribution theory, the distribution of n FA residues between acyl$_2$Gro species may occur in such way that the total number of acyl$_2$Gro molecular species (N) is composed of monoacid (n) and diacid [$n(n$-1)/2] acyl$_2$Gro molecular species (Equation 10):

$$N = n(n + 1)/2; \quad (10)$$

where n = 5, N = 15.

Analyses performed to establish molecular species composition of this mixture were based on the GC determination of contents of individual FA residues in separated acyl$_2$Gro fractions obtained by HPLC or TLC (Pchelkin, Vereshchagin, 1981).

3.1. TLC of Free Diacylglycerols

The initial model mixture of diacylglycerols consisted of *rac*-1,2- and 1,3-acyl$_2$Gros, as estimated by TLC (Pchelkin, Vereshchagin, 1981). Only purified *rac*-1,2-acyl$_2$Gro positional isomers had been further used in our study, since they behaved similarly to natural *sn*-1,2-acyl$_2$Gros during TLC separation (Pchelkin, Vereshchagin, 1992b).

Eight samples of the synthetic model *rac*-1,2-acyl$_2$Gros (Fig. 6) have been separated by adsorption silver nitrate TLC (Ag-TLC) into following individual species: SteOleGro (*18:0\18:1*), PamOleGro (*16:0\18:1*), Ole$_2$Gro (*18:1\18:1*), OleLinGro (*18:1\18:2*), Lin$_2$Gro (*18:2\18:2*), OleLnnGro (*18:1\18:3*), LinLnnGro (*18:2\18:3*), and Lnn$_2$Gro (*18:3\18:3*). In addition, three molecular groups [*SS*Gro (*S\S*), *S*LinGro (*S\18:2*), and *S*LnnGro (*S\18:2*), in which *S* is saturated Ste or Pam, have been identified (Figure 7; Pchelkin, Vereshchagin, 1991).

The formation of separated fractions during Ag-TLC depends on the relative polarity parameter (p) of unsaturated acyl$_2$Gro molecular species in coordination complexes with silver ions (Table 1, column 4).

It was suggested that the p values are additive:

$$p = (p_i)_I + (p_i)_{II}, \quad (11)$$

where $(p_i)_I$ and $(p_i)_{II}$ are the polarity parameters of the 1st and 2nd FA residues (Pchelkin, Vereshchagin, 1991).

Selectivity of resolution values (α) of unsaturated *rac*-1,2-acyl$_2$Gro molecules (in relation to *rac*-1,2-*S/S*) correlated with the relative polarity parameter (p) of acyl$_2$Gros during their fractionation in a chloroform-isopropanol mobile phase (99:1, v/v) in the presence of silver ions (Figure 7). The p_i values of Ole, Lin, and Lnn may be calculated from the mobilities (R_x, x = 1,3-Lin$_2$Gro) of saturated (*S/S*) acyl$_2$Gros [$(R_x)_{SS}$] and of Ag^+ complexes of the monoacid unsaturated acyl$_2$Gros (*UU*): Ole$_2$Gro, Lin$_2$Gro, or Lnn$_2$Gro [$(R_x)_{UU}$].

Setting these data into the empirical Equation 12

$$p_i = \ln[(R_x)_{SS}/(R_x)_{UU}] \quad (12)$$

yields that the p_i values of Ole, Lin, and Lnn residues are approximately equal to 1.0, 2.5, and 5.5, respectively (Pchelkin, Vereshchagin, 1991).

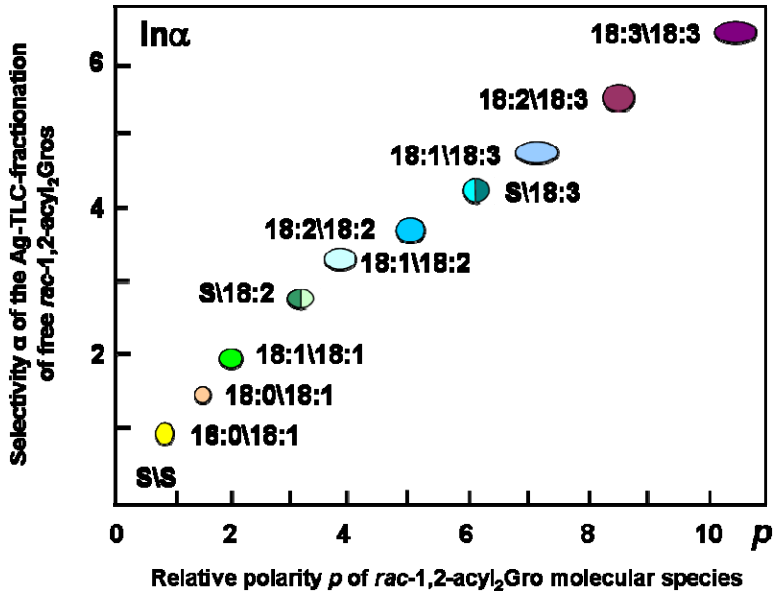


Figure 7: Selectivity values α of the Ag-TLC resolution of *rac*-1,2-acyl$_2$Gro molecular species (in relation to *rac*-1,2-S$_2$Gro) *vs* their relative polarity *p* parameter (Pchelkin, Vereshchagin, 1991). Color definitions are the same as in Figure 5 and 6.

It is interesting to note that these calculated p_i values are always close to the corresponding empirical $\sqrt{e_i^3}$ levels (± 0.3).

Using p_i and Equation 12, the *p* values for each acyl$_2$Gro molecular species have been calculated (Table 1, column 4). Indeed, it appears that the mobility of individual acyl$_2$Gros during Ag-TLC may be described by the *p* values. The acyl$_2$Gro species with the same *p* value cannot be separated from each other by Ag-TLC. This observation shows that there is a strong negative correlation between the *p* and R_x parameters of each acyl$_2$Gro molecule: for example, $R_{1,3\text{-Lin2Gro}} = 7 \times \exp(-0.5 \times p)$, $r = -0.979$ (Pchelkin, Vereshchagin, 1991). Mixed *rac*-1,2-*SS*Gro, *rac*-1,2-*S*LinGro, and *rac*-1,2-*S*LnnGro fractions, which could not be resolved by Ag-TLC, were separated into the individual molecular species by RP-TLC in the presence of silver ions (Ag-RP-TLC) (Pchelkin, Vereshchagin, 1992b). During Ag-RP-TLC in the methanol-boric acid-silver nitrate/*n*-tetradecane system (Figure 8), the mobility of unsaturated acyl$_2$Gro Ag-coordination complexes was determined by the nominal lipophilicity L_3 value(Table 1, column 8):

$$L_3 = [(L_3)_i]_I + [(L_3)_i]_{II} = (m_i - 2p_i - u_i)_I + (m_i - p_i - u_i)_{II}, \quad (13)$$

where

$$(u_i)_I + (u_i)_{II} = u, \quad (14)$$

The parameter *u* represents the total number of unsaturated FA residues located at 1st (I) and 2nd (II) *sn*-positions in the individual acyl$_2$Gro molecular species (Table 1, column 5).

Our data shows that the nominal $(L_3)_i$ values of single Ole, Lin, and Lnn residues in the acyl$_2$Gro molecule is equal to 15, 12, and 6, respectively (Table 1, underlined in column 8). We always observed the inverse correlation ($r = +0.999$,) between the mobility of acyl$_2$Gros and their lipophilicity: $R_{1.2\text{-Lnn2Gro}} = 1.47 - 0.04L_3$ (Figure 5; Pchelkin, Vereshchagin, 1992b).

Table 1. Qualitative characterization of molecular species composition of the random model diacylglycerol mixture.

acyl$_2$Gro molecular species	**Nominal acyl$_2$Gro molecular parameter**						
	m	*e*	*p*	*u*[a]	L_1[b]	L_2[c]	L_3[d]
1	2	3	4	5	6	7	8
Lnn$_2$Gro (*18:3\18:3)*	36	6	11	2	24	22	12
LinLnnGro (*18:2\18:3)*	36	5	8	2	26	24	18
OleLnnGro (*18:1\18:3)*	36	4	6.5	2	28	26	21
PamLnnGro (*16:0\18:3*)	36	3	5.5	1	28	27	22
SteLnnGro (*18:0\18:3*)	36	3	5.5	1	30	29	24
Lin$_2$Gro (*18:2\18:2*)	36	4	5	2	28	26	24
OleLinGro (*18:1\18:2*)	36	3	3.5	2	30	28	27
PamLinGro (*16:0\18:2*)	34	3	2.5	1	28	27	28
SteLinGro (*18:0\18:2*)	36	2	2.5	1	32	31	30
Ole$_2$Gro (*18:1\18:1*)	36	2	2	2	32	30	30
PamOleGro (*16:0\18:1*)	34	1	1	1	32	31	31
Pam$_2$Gro (*16:0\16:0*)	32	0	0	0	32	32	32
SteOleGro (*18:0\18:1*)	36	1	1	1	34	33	33
StePamGro (*18:0\16:0*)	34	0	0	0	34	34	34
Ste$_2$Gro (*18:0\18:0*)	36	0	0	0	36	36	36

a u – a total number of unsaturated (u) 1st (I) and 2nd (II) FA residues in each acyl$_2$Gro molecular species.
b equivalent lipophilicity $L_1 = m - 2e$ (Pchelkin, Vereshchagin, 1981).
c equivalent lipophilicity $L_2 = m - 2e - u$ (Pchelkin, Vereshchagin, 1991).
d equivalent lipophilicity of π-coordination complexes of unsaturated acyl$_2$Gro molecular species with Ag ions $L_3 = m - 2p - u$ (Pchelkin, Vereshchagin, 1992b).

It should be noted that RP-TLC of acyl$_2$Gros in the methanol-trimethylborate (93:7, v/v) / *n*-tetradecane system in the absence of silver ions revealed strong linear correlation (r = 1.0, Pchelkin, Vereshchagin, 1981) between R_f and L_1 values: $R_f = 1.64 - 0.04L_1$ [*i.e.*, $\ln\alpha = 0.2(36 - L)$; Figure 9].

The relationship between the polarity of the acyl$_2$Gro molecule and its chemical potential (μ) was suggested (Pchelkin, Vereshchagin, 1991), since theln($1/R_f$-1) is a part of the Equation 15:

$$\Delta\mu_x/R_gT = \ln\{[1/(R_f)_A - 1]/[1/(R_f)_B - 1]\}, \quad (15)$$

where $\Delta\mu_x$ is the difference between the μ values of A and B compounds differing in the presence of an X group in B; R_g is the universal gas constant; and T is the absolute temperature during RP-TLC fractionation. Transformation of the right part of the Equation 15 results in the following (Equation 16):

$$\ln\{[1/(R_f)_A - 1]/[1/(R_f)_B - 1]\} = \ln[(l_f - l_B)/(l_f - l_A)] + \ln(l_A/l_B) \quad (16)$$

where l_f, l_A, and l_B are TLC-distances of the solvent front of compounds A and B, respectively. These compounds may be represented by the standard *rac*-1,2-Ste$_2$Gro (*SS*) and *rac*-1,2-Lnn$_2$Gro (*UU*) samples.

It is well known that

$$(R_x)_{SS}/(R_x)_{UU} = (R_f)_{SS}/(R_f)_{UU} = l_{SS}/l_{UU} \quad (17)$$

Hence,

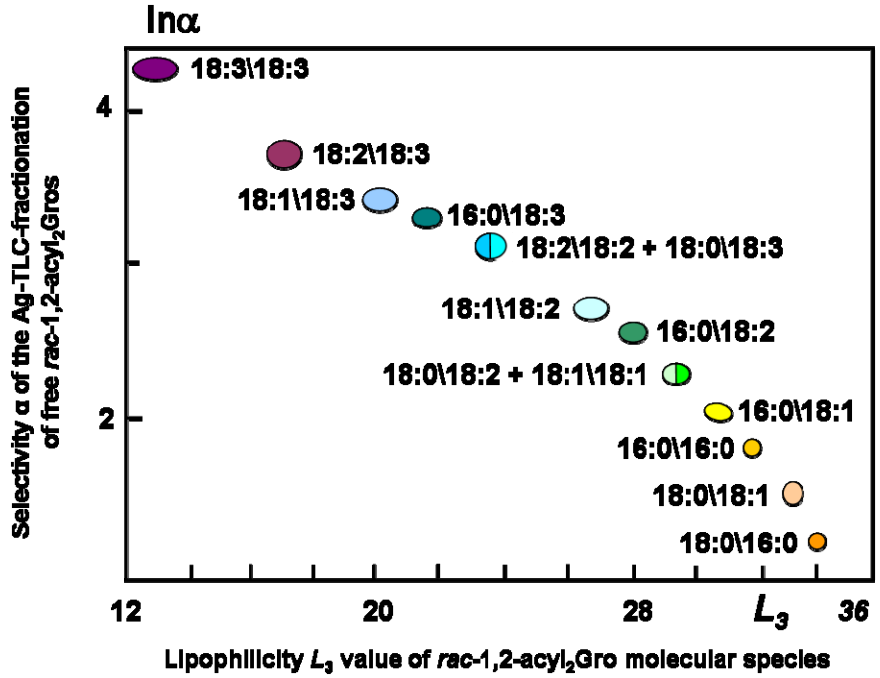


Figure 8: Selectivity values α of the Ag-RP-TLC resolution of acyl$_2$Gro molecular species (in relation to *rac*-1,2-Ste$_2$Gros) *vs* their lipophilicity L_3 values (Pchelkin, Vereshchagin, 1992b). Color definitions are the same as in Figures 5 and 6.

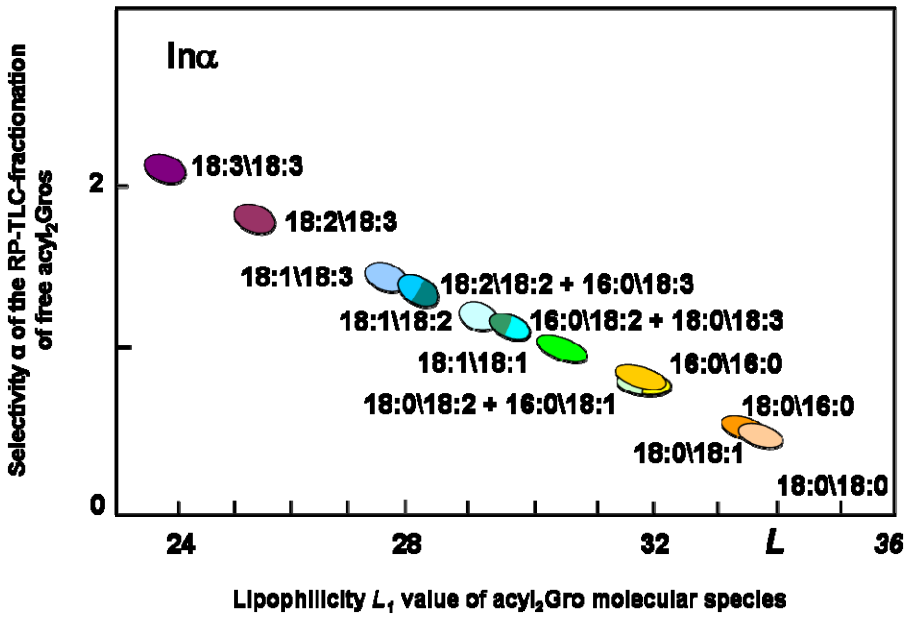


Figure 9: Selectivity values α of the RP-TLC resolution of acyl$_2$Gro molecular species (in relation to Ste$_2$Gro) *vs* their lipophilicity L_1 values (Pchelkin, Vereshchagin, 1992b). Color definitions are the same as in Figures 5-8.

$$\ln\{[1/(R_f)_{SS} - 1]/[1/(R_f)_{UU} - 1]\} = \ln[(l_f - l_{UU})/(l_f - l_{SS})] + \ln(l_{SS}/l_{UU}) \quad (18)$$

and

$$\Delta\mu_x/R_gT = \ln\{[(R_x)_f - (R_x)_{UU}]/[(R_x)_f - (R_x)_{SS}]\} + \ln[(R_x)_{SS}/(R_x)_{UU}] = C + D \quad (19)$$

Differences between μ values of these saturated (*SS*) and unsaturated (*UU*) samples may be calculated by the Equation 19 with known R_f or $R_{1.2\text{-Lnn2Gro}}$ parameters obtained during RP-TLC or Ag-RP-TLC separations (Pchelkin, Vereshchagin, 1981, 1992b).

The component C in the Equation 19 is almost identical for individual acyl$_2$Gro molecules separated by RP-TLC and Ag-RP-TLC, whereas the component D is always variable (Pchelkin, 2000). The value of D increased 2-fold for *rac*-1,2-Lnn$_2$Gros during Ag-RP-TLC if compared to RP-TLC. Accordingly, the equivalent lipophilicity of two Lnn residues of a single Lnn$_2$Gro molecule dropped to 12 methylene units, *i.e.,* 2.19 **:** 0.18 = 12 = 1 + 11 = C + D (Pchelkin, 2000).

Thus, the equivalent lipophilicity L_3 value of the *rac*-1,2-Ste$_2$Gro molecule ($L_3 = L_2 = L_1 = m = 36$) is 3-times higher than the equivalent lipophilicity L_3 value of each *rac*-1,2-Lnn$_2$Gro molecule ($L_3 = 12$) with the same carbon number $m = 36$). This comparison can be also applied to other eight unsaturated acyl$_2$Gro molecules with $m = 36$. Real equivalent lipophilicity levels of the coordination complexes with silver ions are always well reflected by the above mentioned L_3 values (Table 1).

After LC fractionation of unsaturated acyl$_2$Gros in the presence of silver ions, the data on FA composition can be recalculated. Instead of *e* and *L* values, the experimental results may be expressed for each LC fraction as the calculated value of the relative polarity of π-complexes of these acyl$_2$Gros with silver ions (p_c value, Equation 20):

$$p_c = 0.02\ \Sigma(a_i p_i) \quad (20)$$

or by the calculated equivalent lipophilicity of these π-complexes [$(L_3)_c$, Equation 21]:

$$(L_3)_c = 0.02\ \Sigma[a_i\ (L_3)_i]. \quad (21)$$

The content of each esterified individual FA residue in the mixture of this LC fraction (mole %) is expressed as

$$a_i = 100\ A_i/[M_i\ (\Sigma A_i)\ \Sigma(A_i/M_i\Sigma A_i)] \quad (22)$$

where A_i is a peak area of i^{th} fatty ester according to the recorder response of an argon β-ionization GC detector and M_i is a molecular weight of this ester (Equation 22; Pchelkin, 1993).

This content can be also calculated as

$$a_i = 100\ B_i/[M_i\ \Sigma(B_i/M_i)] \quad (23)$$

where B_i is a peak area of the *i*-th FA ester obtained by GC with a flame ionization detection (Equation 23; Pchelkin, 1993).

The next step of data processing involves the comparison of the calculated p_c or $(L_3)_c$ values with the respective nominal p or L_3 parameters (Equations 24 and 25; Pchelkin, 1997a,b):

$$s_p = 100\ |p_c - p|/p\ \% \quad (24)$$

& $$s_{L3} = 100\ |(L_3)_c - L_3|/L_3\ \%. \quad (25)$$

Finally, each total value of the mean relative deviation for all acyl$_z$Gro fractions in this test can be calculated using Equations 26 and 27:

$$S_p = \Sigma(s_p)_j/n_e\ \% \quad (26)$$

& $$S_{L3} = \Sigma(s_{L3})_j/n_{L3}\ \%, \quad (27)$$

where n_{L3} is the number of these fractions obtained during RP-LC separation of unsaturated acyl$_z$Gros in the presence of silver ions (Pchelkin, 1997a,b).

3.2. Reversed-Phase HPLC of Native Triacylglycerol Mixtures

We compared the real value of the *u* parameter of unsaturated lipids during RP-LC in the absence and presence of silver ions. Previously, this value was reported to be equal for each unsaturated FA residue in the individual unsaturated $acyl_3Gro$ molecules during Ag-RP-LC fractionation (Vereshchagin, 1965). In this case, the *u* value represents the additive parameter for each a cyl_zGro molecule (Equation 28):

$$u = (u_i)_1 + ... + (u_i)_z, \quad (28)$$

where *u* is a total number of unsaturated FA residues in this molecule (u = 1, ..., z), and $(u_i)_z$ is a presence (*U*) or an absence (*S*) of the unsaturation into the z^{th} FA residue. In other words, $(u_i)_z = 1$, or $(u_i)_z = 0$.

These called "*equivalent average chain length*" was described by Podlaha and Toregard (1989) during RP-HPLC of native $acyl_3Gro$ mixtures. These length parameters were equal to 15.05, 12.73, and 10.81 for Ole, Lin, and Lnn residues, respectively; and close to the nominal $(L_2)_i$ values (Equation 29):

$$(L_2)_i = L_i - u. \quad (29)$$

This corresponds to the experimental u_i values of 0.95, 1.27, and 1.19 for Ole, Lin, and Lnn, respectively (Pchelkin, 1997a). Another group reported similar chain length values of 15.42, 12.83, and 10.41 (Rezanka, Mares, (1991), and the corresponding u_i values of 0.58, 1.17, and 1.59, respectively. Previously, other u_i levels have been reported for Ole (0.6) and Lin (0.7) (Barron, Santa-Maria, 1989).

Bornaz et al. (1991) introduced another parameter (f_i) for calculation of the theoretical carbon number (TCN) of native $acyl_3Gro$ molecules.The absolute values of this parameter for Ole_3Gro, Lin_3Gro, and Lnn_3Gro were 2.47, 2.25, and 2.12, respectively. These values are close to 3, *i.e.*, they are equal to the additive *u* level. The u_i values are almost equal to 1 or 0.96 ± 0.17 (Pchelkin, 1997a). One may conclude that, during RP-LC of unsaturated $acyl_zGros$, the absolute *u* value does not depend on the presence or absence of silver ions in the mobile phase. This is also true for RP-HPLC fractionation of other natural oils (Laakso, Christie, 1991). Thus, the FA composition data of RP-HPLC or Ag-HPLC separated fractions (z = 3) may be recalculated as (Equation 30):

$$(L_2)_c = 0.01\ z\ \Sigma[a_i\ (L_2)_i]. \quad (30)$$

Examination of 130 $acyl_3Gro$ fractions originated from herring oil sample (Laakso, Christie, 1991) revealed strong negative correlation (r = -0.982) between the calculated $(L_2)_c$ and nominal L_2 values. The determined lipophilicity level of individual molecules of unsaturated lipids resolved by RP-LC reflects their nominal L_2 values most accurately (Pchelkin, 1997a).

The following stages of calculations of the mean relative deviation values employ the Equations 31 and 32:

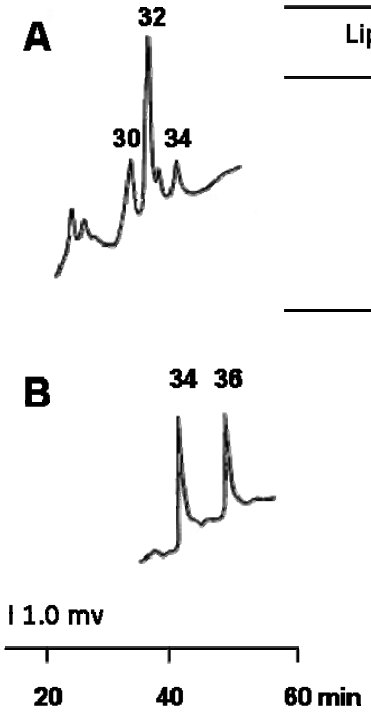
$$s_{L2} = 100\ |(L_2)_c - L + u|/(L - u)\ \% \quad (31)$$

$$\&\qquad S_{L2} = \Sigma(s_{L2})_j/n_L\ \%. \quad (32)$$

These equations may be also used for the estimation of the accuracy of the molecular species composition analysis of individual complex mixtures of neutral lipids.

3.3. Reversed-Phase HPLC of Diacylglycerols and Their Lipophilic Derivatives

Apart of triacylglycerols, we have made an attempt to estimate the relationship between the visually observed RP-HPLC retention of major $acyl_2Gro$ molecular species and their lipophilicity. Previously, we observed that the values of lipophilicity of individual $acyl_2Gro$ components of model mixtures significantly varied under different



Lipid Fraction Lipophilicity L_1	30	32	34
Capacity Factor k'	5.33	6.25	7.92
Plate Number N	4020	2860	4560
Separation Selectivity α		1.17	1.27
Resolution R_s		1.69	3.16

Total Carbon Number m	34	36
Capacity Factor k'	7.03	8.76
Plate Number N	3540	5240
Separation Selectivity α	1.22	
Resolution R_s	2.82	

Figure 10: RP-HPLC separation of free *sn*-1,2-acyl$_2$Gros from egg yolk lecithin(A), and of standard synthetic *sn*-1,3-StePamGro (34) and *sn*-1,3-Ste$_2$Gro (36) mixture (B).

test conditions (Pchelkin, Vereshchagin, 1992b). We defined the lipophilicity as L or L_1 during RP-HPLC of dimethylborate ethers of acyl$_2$Gros in the methanol-trimethylborate mobile phase and the octadecyl silica gel column (Pchelkin, 1998). Later, lipophilicity was defined as L_2 regardless of the type of RP-LC separation of pure *rac*-1,2-acyl$_2$Gro isomer (Pchelkin, Vereshchagin, 1992b).

To define the most satisfactory characteristic of individual acyl$_2$Gro molecules resolved by RP-HPLC (L_1 or L_2 values), we introduced the correlation coefficient (r) and the relative retention parameter (lg$k$') (Pchelkin, 1997a). The retention of acyl$_2$Gro molecules is always proportional to their total lipophilicity value, whereas the selectivity is mostly dependent on the L_2 parameter. The relationship between lg$k$' and L_2 levels is always linear (lg$k$' = 0.033L_2 - 0.33, r = 0.999) (Pchelkin, 1997a).

The total number of the major and minor acyl$_2$Gro chromatographic peaks is equal to the total number of different L_2 values, and satisfactory separation of individual acyl$_2$Gro species takes place only when the nominal L_2 value is over 1 ($\Delta L_2 > 1$).

The m values, which are close to the nominal L_2 values of the identical molecular species, have been earlier obtained by RP-HPLC with UV detection for three classes of acyl$_2$Gro lipophilic derivatives. The relationship between lg$k$' and m was linear: lg$k$'= $a \times m - b$, where values ranged from 0.055-0.160 for a to 1.54-2.56 for b (Batley et al., 1980, 1982; Bishop, 1987; Cantafora, Masella, 1992).

RP-HPLC of free acyl$_2$Gros also revealed the linear relationship between the lg$k$' and L_1 values: lg$k$' = $0.035 \times L_1 - 0.41$. Only seven peaks with $\Delta L_1 = 2$ have been detected during RP-HPLC fractionation of the *rac*-1,2- plus *rac*-1,3-acyl$_2$Gro model

mixture. RP-HPLC elution profiles and parameters of three L_1 (= 30, 32, and 34) fractions of free *sn*-1,2-acyl$_2$Gros from egg yolk lecithin and from the simplest standard saturated synthetic *sn*-1,3-StePamGro plus *sn*-1,3-Ste$_2$Gro mixture are represented in Figure 10.

Separation of the *sn*-1,2-acyl$_2$Gros obtained *via* phospholipase C hydrolysis of commercial egg lecithin gave three major and two minor fractions. The R_s values varied from 1.7 to 3.2, whereas the selectivity value α was equal to 1.22 ± 0.05 (Pchelkin, 1997a).

When a height equivalent theoretical plate (HETP) of the chromatographic column was near 10 μm, and its total theoretical plate number was more than 10,000, twelve individual peaks were observed with a nonselective detection during separation of the *rac*-1,2-acyl$_2$Gro model mixture. This indicated a significant increase in separation selectivity in such RP-HPLC system (Pchelkin, 1997a).

The ΔL_2 values (Δu, *i.e.*, a minimal real distance between the nominal L_2 and observed L_o levels compared to that of saturated acyl$_2$Gro standard with known carbon number) for each of the PamLnnGro/OleLnnGro, PamLinGro/OleLinGro, Ole$_2$Gro/PamOle/Pam$_2$Gro,andSteOleGro/StePamGro groups, characterized by similar L values, are equal to 0.59 ± 0.14 (Pchelkin, 1997a).

Similarly to the s_L criteria (Equation 6) for each j fraction of a lipid mixture subjected to the RP-HPLC, new quantitative criteria q_L was introduced for the comparison of the observed $(L_o)_j$ values to the respective nominal $(L_1)_j$ values or $(L_2)_j$ relative deviations, and for a subsequent evaluation of the relative deviations of $(L_o)_j$ from $(L_1)_j$ or $(L_2)_j$ (Equations 33 and 34):

$$(q_{L1})_j = 100\ |(L_o)_j - (L_1)_j|/(L_1)_j\ \% \qquad (33)$$

$$\&(q_{L2})_j = 100\ |(L_o)_j - (L_2)_j|/(L_2)_j = 100\ |\Delta u|/(L_2)_j\%. \qquad (34)$$

Finally, according to the S_L criteria (Equation 8), the deviation values $(q_L)_j$ were used to calculate the integral criteria of an accuracy of RP-HPLC separation of a certain lipid mixture, *i.e.*, its mean relative deviation (Equation 35):

$$Q_L = \Sigma(q_L)_j/n_L\ \%, \qquad (35)$$

where n_L is the total number of a satisfactory detected lipid fractions (Pchelkin, 1997a).

Thus, RP-HPLC retention data of different acyl$_2$Gro molecular species shows that the Q_{L2} values are usually equal to 0.1-3.2 % only, whereas the respective Q_{L1} are higher and vary from 0.4 to 4 %. The Q_{L2} value of all RP-HPLC fractions of free *rac*-1,2-acyl$_2$Gros is near 1.1 % (Pchelkin, 1997a).

RP-LC separation of our model acyl$_2$Gro mixture was efficient and selective due to the formation of the dimethylborate esters in the mobile phase (Pchelkin, Vereshchagin, 1981). Later, fine RP-HPLC separation was also achieved by Ha and Thompson (1991) on an octadecyl silica gel column with a theoretical plate number more than 25,000 in a non-borate mobile phase. The Q_{L2} levels were near 1.2 % for five PamXGro/OleXGro pairs (X = Pam, Ole, Lin, or Lnn) of the natural mixture of free *sn*-1,2-acyl$_2$Gros. The order of elution was well reflected by the nominal L_2 values (Pchelkin, 1997a).

In this respect, special attention should be paid to a recent set of retention data obtained by RP-HPLC for acyl$_2$Gro acetate (acyl$_2$GroAc) molecular species of various burning bush (*Euonymus* family) seed oils: *E. maackii, E. macropterus, E. hamiltonianus, E. sieboldianus, E. sacrosanctus, E. maximowiczianus*, *E. bungeanus, E. europaeus,* and *Celastrus* (*C. orbiculatus, C. scandens, C. rugosus* (Turtygin et al., 2012). The SB-C18 silica gel columns with three acetone and acetonitrile mobile phases have been applied for these RP-HPLC tests. Calculations define the selectivity coefficient α in a range of 1.09-1.32. Experimental equivalent carbon

numbers (ECNs) of separated acyl$_2$GroAc fractions (Lnn$_2$GroAc, LinLnnGroAc, Lin$_2$GroAc, OleLnnGroAc, PamLnnGroAc, OleLinGroAc, PamLinGroAc, Ole$_2$GroAc, and PamOleGroAc) were equal to the observed L_o levels: 25.40-26.09, 27.57-28.16, 29.67-30.15, 30.05-30.46, 30.77-31.10, 32.02-32.37, 32.76-32.97, 34.34-34.54, and 35.05-35.15, instead of nominal ECN values (26, 28, 30, 30, 30, 30, 32, and 34, respectively). It was reported that the $(q_L)_j$ values for three mobile phases are close to 0.2-3.8%, whereas the Q_{L1} values vary insignificantly from 1.6 to 1.8 % (Turtygin et al., 2012).

Three variants for each fatty acid substitution in different acyl$_3$Gro molecules have been pointed and calculations provided:

a) Δ(αLnn→Lin) = lgk'(Lnn$_2$LinGro) – lgk'(Lnn$_3$Gro) = 0.107,
b) Δ(Lin→Ole) = lgk'(Lin$_2$OleGro) – lgk'(Lin$_3$Gro) = 0.121,
c) Δ(Ole→Pam) = lgk'(PamLin$_2$Gro) – lgk'(OleLin$_2$ Gro) = 0.036.

The lg$k'_{(PamLin2Gro)}$ and lg$k'_{(OleLin2Gro)}$ difference is equal to 0.036 when its $|(L_o)_j - (L_2)_j|$ value or its u_j level is near 1.09 (Deineka et al., 2008; Turtygin et al., 2012), which is close to our data.

3.4. RP-HPLC and Silver Ion RP HPLC of Fatty Acid Phenethyl and Phenacyl Esters

Nikolova-Damyanova et al. (1993) estimated lipophilicity values of the unsaturated FAs during RP-HPLC and Ag-RP-HPLC fractionation of more lipophilic esters. We used that data (lnk') (m = 18) for calculation of $\Delta\mu_x/R_gT$values for three fatty acid phenethyl (BnCH$_2$acyl) and phenacyl (BzCH$_2$acyl) esters in comparison to phenethylstearate or phenacylstearate (differences in lnk'_x to lnk'_{Ste}, or $\Delta\mu_{BnCH2Ste}/R_gT$ to $\Delta\mu_{BzCH2Ste}/R_gT$ values, *i.e.*, the $\Delta\mu_{Ste}/R_gT$ levels; Equations 36-39):

$$\Delta\mu_{BnCH2Ste}/R_gT = 0.23e_i = 0.18(1.3e_i) \quad (36)$$

$$\Delta\mu_{BzCH2Ste}/R_gT = 0.22e_i = 0.18(1.2e_i) \quad (37)$$

$$\Delta\mu_{BnCH2Ste}/R_gT = 0.88e_i = 0.18(4.9e_i) \quad (38)$$

$$\Delta\mu_{BzCH2Ste}/R_gT = 0.61e_i = 0.18(3.4e_i) \quad (39)$$

These values were also proportional to the e_i numbers in all three unsaturated FA residues, but $\Delta\mu_x$ values were variable for different lipid molecules with the same e_i number, although with different lipophilic groups.

Fractional chain length (FCL) values obtained by RP-HPLC in the absence (FCL_O) and in the presence of silver ions (FCL_{Ag}) were also estimated (Nikolova-Damyanova et al. 1993). Similarly to acyl$_2$Gro, the FCL_{Ag} value of trienoic molecular species was almost equal to the $2p_i$ number. Nevertheless, FCL_O and FCLAg values of both phenethyl and phenacyl FA derivatives with the same m number are proportional to their nominal e_i numbers.

4. New Virtual U-Acyl Parameters during Ag-RPLC and Quality Control Criteria

4.1. Variations in Chemical Potentials of Unsaturated FA Residues in Ag-RP-LC System and Their Equivalent Lipophilicity Values

First step of the estimation of variations in chemical potentials includes calculation of the Fractional Chain Lengths (FCL) during RP-LC fractionation of unsaturated lipids (U = Ole, Lin, Lnn) in the absence (FCL_O) or in the presence (FCL_{Ag}) of silver ions. These variations may be calculated for the π-complexes of each monoacid class of unsaturated lipids with silver ions (Equation 40):

$$2(p_i - e_i + 0.5) = \pi_U = (L_1)_i - (L_3)_i = FCL_O - FCL_{Ag} - 1. \quad (40)$$

This can be also calculated with Equations 2 and 13.

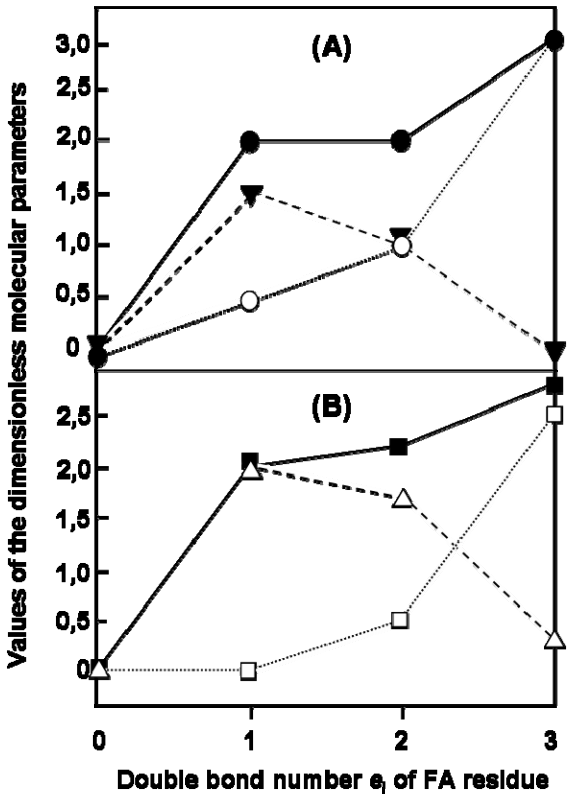


Figure 11: Correlation between the degree of unsaturation e_i of esterified C_{18}-FA residues of $acyl_2Gro$ molecules and their values of dimensionless molecular parameters in the presence of silver ions: φ_U (●), π_U (▼), β_U(○) in (A), and λ_U (■), ψ_U (Δ), ε_U (□) in (B).

The $\Delta\mu_{Ste}/R_gT$ levels and $\Delta\mu_{SS}/R_gT$ values derived from retention data of monoacid unsaturated $acyl_2Gro$ molecules appear to be almost directly proportional to the parameter (FCL_O - FCL_{Ag} - 1) or to π_U numbers (Pchelkin, 2000). The $\Delta\mu_\pi/R_gT$ value is sometimes equal to 0.18, which corresponds to the level of the methylene unit component ($\Delta\mu_{CH2}/R_gT$).

Second step of this estimation involves the division of $\Delta\mu_{Ste}/R_gT$ and $\Delta\mu_{SS}/R_gT$ values by this 0.18 level (Pchelkin, 2000). This operation allows estimation of the influence of number of methylene groups and double bonds in different FA moieties on the formation of π-complexes with silver ions π_i (Equation 41). The π_U levels of each unsaturated FA residue of natural lipids are obtained by this operation. In the case of application to the calculated $(L_1)_i$ and $(L_3)_i$ values, possible discrepancies between the π_U and φ_U levels are always well compensated by the additional β_U parameter:

$$0.5(L_1)_i - 0.5(L_3)_i = \varphi_U = \pi_U + \beta_U = p_i - e_i + 0.5 + \beta_U \quad (41)$$

The results of these calculations are presented in Fig. 11A.

Another mathematical operation provides the opportunity to estimate the total effect of double bonds FA on Ag-RP-LC fractionation of unsaturated lipids in respect to $(L_2)_i$ and $(L_3)_i$ values:

$$\lambda_U = \psi_U + \varepsilon_U = p_i - e_i + \varepsilon_U = 0.5(L_2)_i - 0.5(L_3)_i = (FCL_O - FCL_{Ag})_i/2, \quad (42)$$

where ψ_U and ε_U are olefinic bond and carbonyl bond FA components, respectively (Pchelkin, 2000).

Final calculations are shown in Figure 11B. The ψ_U parameter of each unsaturated FA residue has always the same value for all lipids with the same e_i number, whereas the level of the ε_U parameter remains constant only for some fatty acid residue. When e_i number increases from 1 to 3, the ψ_U value also varies from zero to 3, whereas ε_U level always decreases. The above mentioned values and levels are nearly constant for all classed of lipid derivatives. Thus, if the e_i number is minimal (as in case of Ole_2Gro), then possible involvement of the FA carbonyl group into formation of π-complexes with silver ions will be maximal. The real experimentally observed level for this monoenoic FA residue is reflected by a coordination number of these ions (Evstigneeva, Pchelkin, 2006).

Similar parameters applied above for description of molecular lipid properties may be also used for characterization of coordination complexes of major native esterified polyenoic FAs (Figure 12) with silver ions (Nikolova-Damyanova et al., 1993). The calculated ψ_U values are equal to 3, 4, and 5 during Ag-RP-HPLC fractionation of arachidonoyl, eicosapentoyl, and docosahexaenoyl lipophylic derivatives; their p_i levels are near 7, 9, and 11, respectively (Evstigneeva, Pchelkin, 2001). Thus, these p_i values are always higher than the e_i numbers, and they are close to the $1.8e_i$ values. The p_i values may be approximately described by the $(2e_i - 1)$ numbers. These numbers are quite close to the variable coordination number of silver atoms that are associated in triangular Ag_3 clusters (Pchelkin, 2003; Evstigneeva, Pchelkin, 2006).

Finally, the equivalent chain lengths (ECLs) of both phenethyl and phenacyl FA derivatives with the same m_i number and their novel equivalent lipophilicity values (*i.e.,* L_4, …, L_7 levels) have been calculated based on data of RP-HPLC (Equations 43-44) and Ag-RP-HPLC (Equations 45-46) (Pchelkin, 2000):

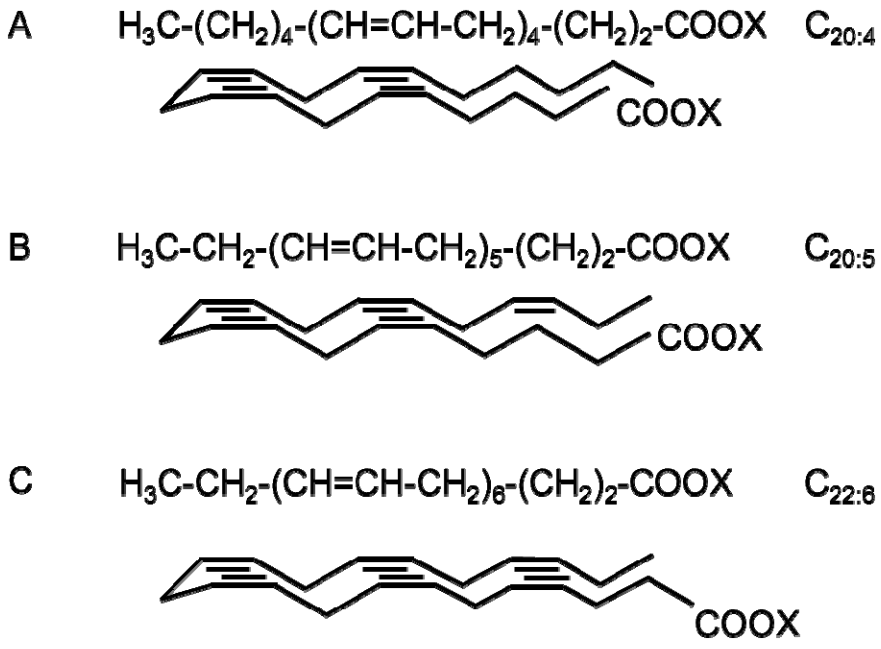


Figure 12: Structure of the native polyenoic fatty acid esters. (A) $C_{20:4}$, (B) $C_{20:5}$, (C) $C_{22:6}$. X = $CH_2CH_2C_6H_5$, phenethyl; or $CH_2COC_6H_5$, phenacyl.

$$L_4 = L_1 + \psi_U \quad (43)$$

$$L_6 = m_i - \lambda_U \quad (44)$$

$$L_5 = L_3 + \psi_U - 2u_i \quad (45)$$

$$L_7 = m_i - 2\lambda_U - u_i \quad (46)$$

Also deviations $(q_L)_j$ of these ECLs (%%) from the L_4, …, L_7 values have been estimated (Pchelkin, 2000):

$$(q_{L4})_j = 100\ |(ECL)_j - (L_4)_j|/(L_4)_j \quad (47)$$

$$(q_{L5})_j = 100\ |(ECL)_j - (L_5)_j|/(L_5)_j \quad (48)$$

$$(q_{L6})_j = 100\ |(ECL)_j - (L_6)_j|/(L_6)_j \quad (49)$$

$$(q_{L7})_j = 100\ |(ECL)_j - (L_7)_j|/(L_7)_j \quad (50)$$

The respective mean relative deviations Q_L (Equation 35) have been found: the Q_{L1} and Q_{L3} deviation levels are higher than 10%, whereas the Q_{L4}-Q_{L7} levels are close to or lower than 5.0% (Pchelkin, 2000).

Therefore, RP-HPLC and Ag-RP-HPLC retentions of these mono-, di-, and trienoic esters are satisfactory described by the L_4, …, L_7 values similarly to the situation, when the RP-TLC or RP-HPLC fractionation of unsaturated acyl$_2$Gro molecules are reflected and described by the L_1 and L_2 levels (Pchelkin, 2000).

4.2. Quantitative Estimation of Basic Lipid Molecular Parameters of Pure LC Standards

Earlier we introduced the e_c, p_c, L_c, $(L_2)_c$, and $(L_3)_c$ parameters and the corresponding s_e, s_p, s_L, s_{L2}, and s_{L3} values calculated from the known A_i and B_i numbers (Equations 22 and 23). New algorithm allows the computer simulation of data obtained for individual lipid mixtures with $n = 5$ and more.

According to this algorithm, the GC analysis of five samples of major native fatty acids may be processed (Figure 13; Pchelkin, 1993).

In these experiments, the major lipid components always displayed 96% of the desired purity value, and the relative standard deviations (RSD) of independent measurements were always below 0.3. The s_e, s_p and s_L levels were equal to 3, 3.2 and 1 %, respectively, *i.e.*, they did not exceed 5 % (Pchelkin, 1993). Thus, the s_L value can be considered as the upper border of the satisfactory (qualitative) resolution of two compounds, when R_s is equal to or exceeds 1 (Peeters et al., 1988).

When the amount of the major component reaches 99 %, a mathematical model gives the s_{L2} and s_p values of 0.2 ± 0.1 and 1.0 ± 0.3 %, respectively (Pchelkin, 1997b). The homogeneity of 99.7 % gives the s_{L2} and s_p levels of 0.04 ± 0.01 and 0.2 ± 0.1% for every lipid fraction, where the R_s parameter is above 1.5 (Peeters et al., 1988).

4.3. Quality Control of Molecular Parameter Values for LC Fractions of Native Glycerolipids

Final calculated data was obtained after Ag-TLC of molecular species of some phospholipid classes from animal tissues (Pchelkin, 1997b). Phosphatidylcholine (PtdCho), as a major component of rat liver tissues; was subjected to LC fractionation. The PtdCho fractions with $p = 1$ and 2 fit to p_c parameters, while fractions with $p > 2.5$ usually have $p_c < p$. The s_p values of p fractions rise progressively as the degree of lipid unsaturation increases.

Similarly to PtdCho, nominally the mono-, di-, tetra-, and hexaenoic phosphatidylethanolamine (PtdEtn) fractions invariably showed $p_c < p$ (Pchelkin, 1997b). The S_p value was approximately 2 times higher the S_e value (Das et al., 1982). The S_p values in many fractions were very high, and the p parameter often exceeded the p_c level (Pchelkin, 1997b).

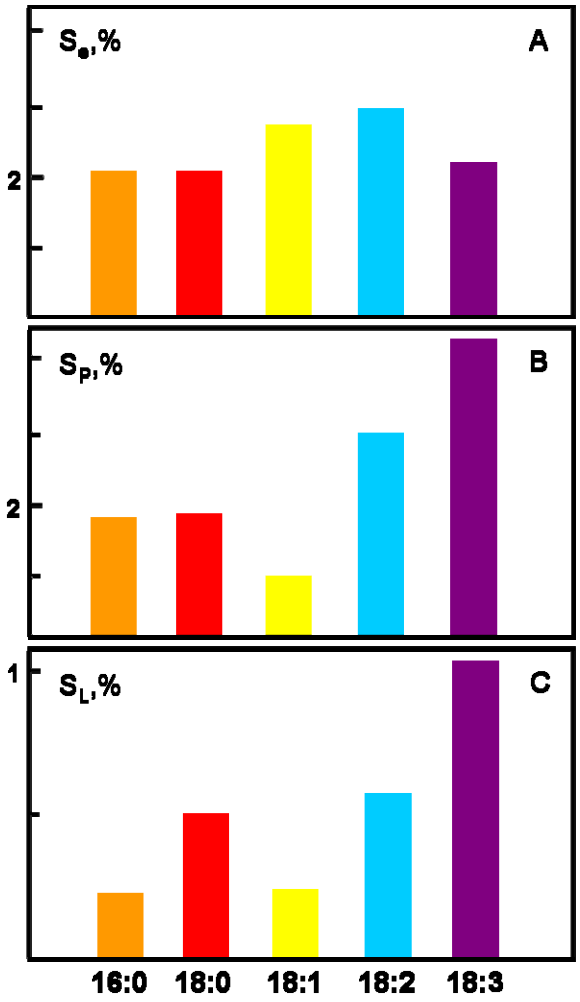


Figure 13: Real levels of S_e (A), S_p (B), and S_L (C) values of monoacid lipid standards.

Sometimes S_p values differ by almost an order in the same series of experiments. Fractionation of native PtdCho mixtures according to their lipophilicity parameter by different RP-LC methods have been reported for rat liver, egg yolk, and soybean seeds (Crawford et al., 1980). The S_{L2} values varied from 0.6 to 3.3 % in these experiments (Pchelkin, 1997a). High values have been obtained during RP-HPLC of the PtdCho preparation: S_{L2} of 0.63% for egg yolk (Smith, Jungalawala, 1981) and 0.52% for soybean seed (Crawford et al., 1980) were reported. Thus, $(L_2)_c$ and S_{L2} determined for major PtdCho fractions remained within permissible limits.

Another notable example is a complex «tandem» Ag-HPLC and RP-HPLC separation of the oil acyl$_3$Gro sample from North Atlantic herring fish. Ag-HPLC gave 11 fractions that have been further separated by RP-HPLC into 6-19 fractions (Laakso, Christie, 1991). The reported error values in these experiments were rather low: $0<S_L<5.2\%$; $0.4<S_{L2}<0.7\%$; $S_e = 4.0\%$; $S_p = 2.2\%$ (Pchelkin, 1993).

Silver ion RP paper chromatographic separation of linseed oil provided the calculated s_{L3} values for individual acyl$_3$Gro fractions that did not exceed 6%, whereas the total S_{L3} level is equal to 1.5% (Pchelkin, 1993).

4.4. Quality Control of Nominal Molecular Parameter for TLC Fractions of Glycerophospholipid Derivatives

We also recalculated the experimental data for the Ag-TLC fractionation of some native phospholipid mixtures (Pchelkin, 1997b). A good agreement between p_c and p values (S_p = 5.5%) was found in major fractions of the *O*-methyl-*N*-dinitrophenyl derivatives from egg PtdEtn. The p_c and p values were similar for all fractions except the hexaenoic fraction. By contrast, trifluoroacetamide PtdEtn-derived *p*-fractions were characterized by high S_p values. The discrepancy between p_c and p values of the polyenoic trifluoroacetamide derivatives of phosphatidylserine (PtdSer) and azlactone of PtdSer (respectively, S_p = 27.5% & S_p = 21.5%) was even higher than in the case of a trifluoroacetamide PtdEtn-derived fractions. Ag-TLC fractionation of *O*-methyl-*N*-acetyl derivatives of PtdEtn (S_p = 11.6%) gave a fair agreement between p_c and p values in the p = 2.5 fraction. However, separation of these derivatives of PtdSer from rat liver (S_p = 19.0%) resulted in higher s_p values (Bjerve, 1982). The s_p values (S_p = 11.8%) of *p*-fractions of *O*-methyl-*N*-acetyl amino lipids obtained from rat liver in another experiment were, in general, also very high, similarly to s_p values obtained during fractionation of *O*-acetyl and *O*-methyl-*O*-triacetyl derivatives of phosphatidylinositol (PtdIns) (S_p = 39.4%).

Quality control data of Ag-TLC determination of molecular species composition of dimethyl phosphatidates derived from several phospholipid classes is also available (Pchelkin, 1997b). Ag-TLC of the acetate derivatives synthesized from PtdIns demonstrated two-times lower total S_p values if compared to tests for this sample. A good agreement between p and p_c values was recorded only for mono- and dienoic fractions of dimethyl esters of phoshatidic acid. The corresponding s_p values (S_p = 10.6-19.0%) were often very high (Waku et al., 1982; Sugiura, Waku, 1984).

4.5. Quality Control of Nominal Molecular Parameter Values for TLC Fractions of Free acyl$_2$Gros and Their Acetate Derivatives

Free acyl$_2$Gros from animal PtdCho and PtdEtn are less polar compounds than native phospholipids Therefore higher selectivity values could be obtained during their Ag-TLC-separation. The total S_p values in these experiments ranged from 2.4 to 23.1%. In two cases the total S_p values of acyl$_2$Gro fractions were comparatively low (Pchelkin, 1997b). No satisfactory resolution of polyenoic acyl$_2$Gro fractions was achieved in these experiments. The S_p values in many cases were extremely high and exceeded 5%. Even lower S_p values have been obtained by van Golde and van Deenen; we also contributed to fractionation of acyl$_2$GroAcs by Ag-TLC and reached the lowest S_p values of 0.8-3.6% for the derivatives of PtdCho and PtdEtn (Pchelkin, 1997b).

Ag-TLC of the acyl$_2$Gros acetate derivatives displayed p_c values close to the nominal p; the error was usually not very high ($|p_c - p| < 0.1$), and S_p values did not exceed 3% in each independent experiment (Das et al., 1982; Masuzawa et al., 1984). For some phospholipid classes, the S_p values were extremely high (Aveldaño, Bazan, 1983).

4.6. Estimation of Molecular Species Composition of Model Diacylglycerol Mixture

The routine LC fractionation of lipid classes allows the estimation of the molecular species composition and the parameters of resolution for separated fractions. An attempt to quantify the molecular species composition of model free acyl$_2$Gro mixture has been made (Pchelkin, 1998). LC parameters of fractions obtained by adsorption Ag-TLC, column RP-LC, RP-TLC, or RP-HPLC (Table 2) and the quality control data (Table 3) have been measured and calculated.

The major parameters of Ag-TLC-separation (Table 2) were within permissible limits during second (B) and third (C) variants. Therefore these data may be

Table 2. Preparative Ag-TLC of the model diacylglycerol mixture according to degree of unsaturation of its molecules and quality homogenous control of its separated fractions.

Parameter	Ag-TLC Variant *		
	A	**B**	**C**
Resolution R_S	2.5 ± 0.8	4.9 ± 3.0	2.7 ± 1.2
Selectivity α	1.80 ± 0.38	1.81 ± 0.52	1.80 ± 0.36
S_e	4.2 ± 1.6	6.2 ± 5.1	5.8 ± 1.1
S_p	4.0 ± 3.2	2.8 ± 2.6	2.8 ± 0.9
Mobile phase	$CHCl_3$+ MeOH (98:2)	$CHCl_3$+ MeOH (99:1)	$CHCl_3$+ MeOH(98:2)
Stationary phase	1% $AgNO_3$ / SiO_2	1% $AgBO_2$ / SiO_2	2% $AgNO_3$ / SiO_2
Silica Trade Mark	Silufol®	Silcoplate®	Silica gel LSL®

* Pchelkin, 1998

Table 3. Preparative RP-LC variants of the model diacylglycerol mixture according to the equivalent lipophilicity values and permanent quality control of separated fractions.

Parameter	RP-LC Variant *		
	column RP-LC	**RP-HPLC**	**RP-TLC**
Resolution R_S	0.7 ± 0.4	0.8 ± 0.2	2.5 ± 0.9
Selectivity α	1.59 ± 0.56	1.19 ± 0.04	1.43 ± 0.06
S_{L1}	3.3 ± 3.2	1.5 ± 0.4	1.3 ± 0.3
S_{L2}	1.2 ± 0.6	0.8 ± 0.4	0.6 ± 0.3
Mobile phase	MeOH+$(MeO)_3B$	MeOH+$(MeO)_3B$	MeOH+$(MeO)_3B$ (93:7)
Stationary phase / Silica Trade Mark	*n*-tetradecane (10%) / Chromaton®NDMCS	LiChrosorb®RP-18 + Spherisorb® 5ODS	*n*-tetradecane (10%) / Silcoplate®

* (Pchelkin, 1998)

used to quantify the corresponding molecular species composition.

The respective parameter values of RP-TLC-fractionations of this mixture (Table 3) had lower levels than during the column separations with RP-LC and RP-HPLC. For this reason these data may be also used to quantify the molecular species composition.

The combination of two LC methods gives unique possibility to estimate the quantitative molecular species composition of the model $acyl_2$Gro mixture (Table 4; Pchelkin, 1998).

Table 4 shows that the distribution of FA residues between $acyl_2$Gro molecules is random. It implies that a satisfactory result during this LC fractionation refers to 1% level of the absolute standard deviation (s_{abs}) from the arithmetical mean of relative concentration of the individual molecular species in the model mixture. This level is suitable for the quantitative LC determination of the molecular species composition of free *sn*-1,2-$acyl_2$Gros of various phospholipid classes. The mean accuracy of RP-TLC and RP-HPLC separations of the major fractions can be considered as satisfactory.

4.7. Molecular Species Composition of Free *sn*-1,2-Diacylglycerols from Native Phosphatidylcholines

Various original methods of LC fractionation provide the necessary warranty for satisfactory data

processing during quantitative determination of native phospholipids, which contain *sn*-1,2-acyl$_2$Gros. Originally, such analysis had been performed for animal PtdCho samples obtained by phospholipase C hydrolysis (Table 5). The S_L values determined by GC analysis of the RP-HPLC fractions (*sn*-1,2-acyl$_2$Gro sample from egg yolk PtdCho) did not exceed 1.3 (S_{L1}) and 0.9% (S_{L2}) (Pchelkin, 1997a, 1998). Similar values (~1.2%) have been reported earlier (Smith, Jungalawala, 1981; Cantafora et al., 1983).

The results of RP-TLC fractionation of free *sn*-1,2-acyl$_2$lGros from sunflower seed lecithin are shown in Figure 14. The detailed scheme of these preparations is presented in Supplementary Figure S1. The S_{L1} and S_{L2} values of the *sn*-1,2-acyl$_2$Gros from from the PtdCho of sunflower seeds are 1.3 and 0.6%, respectively (Pchelkin, 1998). The experimentally determined composition of molecular species of these native PtdChos (Figure 14A) is very close to the calculated random composition (Figure 14B).

At the same time, the s_p values for Ag-TLC-separated fractions of free *sn*-1,2-acyl$_2$Gros from crude lecithin of *Panax ginseng* root cell culture did not exceed 6,2 %, and the corresponding total S_p value was 2,1 % (Pchelkin et al., 2004; see Supplementary Figure S2 for a detailed scheme of preparation of these samples).

It is interesting that experimentally determined composition of PtdCho molecular species is not too far from the calculated random composition (Figure 15); therefore, the accuracy of the LC determination of these molecular species is also satisfactory (Hadden et al., 1971). It is evident that nearly a half, or even more, of PtdCho may be represented

Table 4. Quantitative molecular species composition of the model diacylglycerol mixture.

acyl$_2$Gro molecular species	**Experimental values (weight%±s_{abs} %) found upon** *			
	GLC* «random distribution»	**RP-TLC****	**RP-HPLC****	**Ag-HPTLC****
Ste$_2$Gro	3.6 ± 0.2	3.5 ± 0.2	3.0 ± 0.8	
StePamGro	6.5 ± 0.0	5.8 ± 1.2	4.4 ± 1.8	} 14.0 ± 2.4
Pam$_2$Gro	2.9 ± 0.1	3.6 ± 0.6	2.8 ± 0.2	
SteOleGro	8.2 ± 0.2	10.1 ± 0.9	10.1 ± 1.5	8.3 ± 1.3
SteLinGro	8.8 ± 0.1	7.7 ± 0.3	7.7 ± 0.2	6.0 ± 0.9
PamOleGro	7.3 ± 0.1	8.4 ± 1.2	8.4 ± 0.5	9.2 ± 0.7
Ole$_2$Gro	4.6 ± 0.0	5.2 ± 2.1	5.5 ± 0.3	3.6 ± 0.7
PamLinGro	7.9 ± 0.2	9.4 ± 0.4	10.7 ± 3.8	8.1 ± 1.6
OleLinGro	9.9 ± 0.1	12.7 ± 1.0	11.9 ± 0.7	12.1 ± 1.5
Lin$_2$Gro	5.5 ± 0.1	7.1 ± 0.1	5.5 ± 0.7	7.0 ± 0.7
SteLnnGro	7.3 ± 0.2	5.7 ± 0.8	6.0 ± 1.1	4.7 ± 0.8
PamLnnGro	6.6 ± 0.1	5.3 ± 0.6	7.9 ± 1.4	5.6 ± 0.6
OleLnnGro	8.3 ± 0.0	6.0 ± 1.0	6.0 ± 1.0	7.4 ± 0.3
LinLnnGro	8.9 ± 0.1	5.6 ± 0.8	5.5 ± 0.4	7.9 ± 1.4
Lnn$_2$Gro	3.7 ± 0.0	3.9 ± 0.5	5.1 ± 1.3	6.1 ± 1.2
Sum±S_{abs}	100 ± 0.1	100 ± 0.8	100 ± 1.1	100 ± 1.1

The data according to Pchelkin, Vereshchagin, 1981*; Pchelkin, 1998**.

Table 5. Composition of molecular species of egg yolk phosp atidylcholines.

PtdCho molecular species	Lipid content, weight %, found by				
	1	**2**	**3**	**4**	**5**
StePamGro*P*Cho	0.0	0.5	0.0	0.3	0.1
SteOleGro*P*Cho	19.7	18.0	10.0	21.8	13.9
Pam_2Gro*P*Cho	0.0	0.6	0.5	0.4	0.0
SteLinGro*P*Cho	8.6	14.3	7.3	4,8	15.6
PamOleGro*P*Cho	}44.4	}47.5	37.0	39.3	38.9
Ole_2Gro*P*Cho			9.3	4.2	3.8
$SteAch_{\Delta 4}$Gro*P*Cho	2.0	6.3	3.7	2.7	2.7
PamLinGro*P*Cho	16.6	13.2	18.9	24.7	20.8
OleLinGro*P*Cho	1.8	1.3	3.2	0.4	1.8
$PamAch_{\Delta 4}$Gro*P*Cho	3.6	2.8	1.1	2.4	2.4

1. Hasegawa & Suzuki (1973); 2. Porter et al. (1979); 3. Smith & Jungalawala (1981); 4. Cantafora et al. (1983); 5. Pchelkin (1997a).

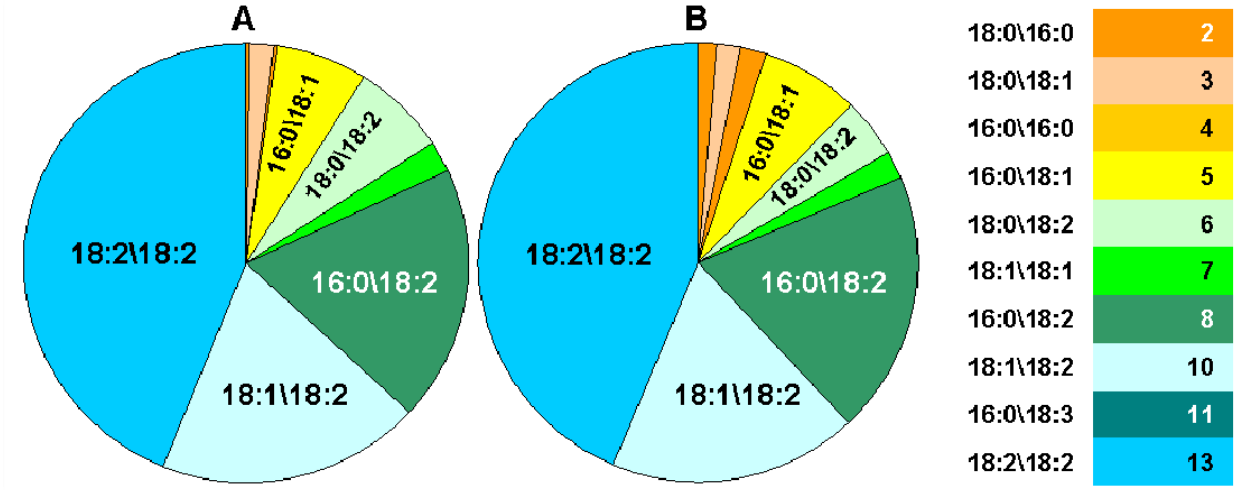


Figure 14: Experimentally determined (A) and calculated random (B) composition of molecular species of free *sn*-1,2-$acyl_2$Gros from sunflower seed lecithin (the same crude PtdCho sample) (Pchelkin, 1998).

by a single molecular species (16:0\18:2). This is often observed in polar (membrane), but not in neutral (storage) lipids.

5. Conclusions

The diversity of FA residues in different diacylglycerol molecules (n>4) complicates quantification of the LC fractionation results for complex mixtures of synthetic and/or native molecular species. However, it is now possible to perform a quality control of the molecular species composition and to estimate the upper limit, which exceeds the level of the integral accuracy criteria. According to the reported data, a ≤ 5 % value of the integral accuracy criteria corresponds to 1 % of

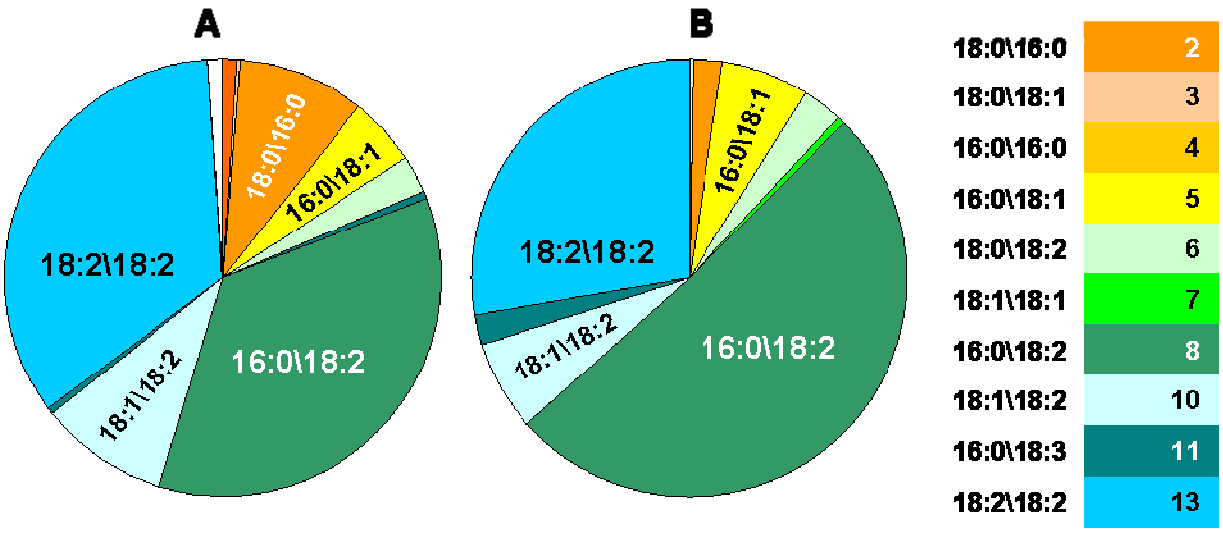


Figure 15: Calculated random (A) and experimentally determined (B) composition of free *sn*-1,2-acyl$_2$Gro molecular species from lecithin (the same crude PtdCho sample) of the root cell culture of *Panax ginseng* G-1(K)®(Pchelkin et al., 2004).

possible absolute standard deviation. This favors a satisfactory separation of the native lipid mixtures into individual molecular species.

If FA composition of natural lipids differs from that described here, a scheme may be employed to obtain suitable acyl$_3$Gro and acyl$_2$Gro mixtures with virtually unlimited range of FA residues. Such mixtures can be useful as standards and experimental samples for biochemical, chemical and technological studies. Since trienoic FA residues have a triplet of olefin bonds, silver atoms practically do not interact with the carbonyl groups of these residues (Pchelkin, 2000, 2003). The Ag atom predominantly interacts with this triplet, thus tightly stabilizing the entire structure. Combinations of three elements are wide spread in nature because three quarks in the nucleons of the stable isotopes of all chemical elements, including Ag, are strongly bound by nuclear forces. Silver atoms participate in the formation of polynuclear clusters during a complexation in a process of LC fractionation. This type of interaction causes a sharp decrease in the hydrophobicity of the cluster complexes. Despite of large molecular size of the FA chains, such complexes are capable of penetrating into various hydrophilic cell structures.

For example, bovine brain PtdSers are mainly represented by the molecular species that contain both monoenoic FA residues and docosahexaenoate (Salem et al., 1976; Salem, Abood, 1980). The existence of such structures provides additional information about operating mechanisms in the central nervous system. Coordination complexes of FAs with silver ions (or other transition metals) have weakly bound electrons at the external atom shells. This favors the formation of relatively stable domains with abnormally ionic conductivity in biomembranes. These domains alternate with highly hydrophobic insulating nanosized layers. Such structures resemble bulk memory cells with different types of electronic conductivity, which are widely used in modern computers.

Organic semiconductors have been synthesized several years ago (Zheng et al., 2007). Different electron conductivities of the *p-n*, *p-n-p*, and *n-p-n* types have been discovered in unsaturated copolymers with controlled molecular architecture

(Pomogailo, 1999; for an update see also Hanemann, Szabó, 2010). Recently, single molecules of chlorophyll *a* from spinach leaves have been used as the only source to engineer a four-step switching nanotransistor (Iancu, Hla, 2006).

It is likely that potential combinations of molecular species in polar glycerolipids of different biological membranes are limited by quite narrow range of environmental temperatures optimal for various metabolic pathways. When silver nitrate is introduced into the boron-containing chromatographic system, the equivalent lipophilicity for the π-complexes of unsaturated lipid molecules decreases, while the polarity range increases. This factor causes an increase in the total resolution selectivity of separate lipid fractions characterized by a certain generality of their monomolecular components.

The development of modern science is characterized by integration of knowledge gained by formerly specialized and isolated scientific disciplines. This integration process is accompanied by mutual bridging and enrichment. Here we demonstrated the interaction between π-complexes of silver with unsaturated lipids and with other organic compounds of natural or synthetic origin. This is just one example of how the interdisciplinary approach and combination of knowledge of lipid biology, chemistry, mathematics, and nanotechnology may open new ways for development of new methods for generation of new materials and/or their use for sustainability.

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