LIQUID CHROMATOGRAPHIC DETERMINATION OF THE SPECIES COMPOSITION OF MEMBRANE LIPIDS AND THEIR DERIVATIVES

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This review gives quantitative results of the separation of individual classes of polar glycerolipids of natural and synthetic origin into their individual species. Universal quantitative criteria calculated from the fatty-acid composition of the species obtained or of their mixtures are proposed for determining the degree of reliability of these results. The fractionation of the initial acyl-containing glycerolipids, their N- and O-derivatives with high hydrophobicity, the products of the enzymatic hydrolysis of the native lipids (diacylglycerols, phosphatidic acids) and also the lipophilic O-derivatives of these products is considered. For all these compounds, the results of their separation by the methods of TLC and HPLC both in the form of the adsorption chromatography of the coordination complexes with silver and also in the form of reversed-phase chromatography are discussed.

As a rule, polar glycerolipids* consist of several classes, each of which combines species of lipids containing one and the same hydrophilic head group but diacylglycerol (DAG) radicals differing from one another. Each individual species within a particular class of lipids is characterised by a definite number of carbon atoms (m_i) and olefinic bonds (e_i) in their fatty-acid (FA) radicals. Thus, the species composition ("fine structure") of the lipids of any class is the qualitative composition and amounts of the individual species in mole-% of the sum of the concentrations for the species of lipids of the class [1].

The properties of cell membranes and their main components – lipids – depend not only on the total FA composition of the latter but also on their species composition [2], and therefore the investigation of the fine structure of lipids is an important task of modern membranology. The main method of such an investigation is usually chromatography. Because of the complex species composition of natural lipids the use for the analysis of a particular class of lipids of only one given variety of chromatography does not provide the possibility of obtaining fractions of lipids of an individual species. For the analytical or preparative separation of a distinct class of lipids into individual species several varieties of chromatography are usually employed successively, and these include liquid chromatography (LC) and gas-liquid chromatography (GLC).

GLC is not infrequently used for fractionating various volatile lipid derivatives [3, 4]. However, the preparation of these derivatives is usually associated with a greater or smaller disturbance of the native nature of the initial lipids and with partial losses of them. In addition, the GLC of lipids can be carried out only at a high temperature, which leads to their further breakdown and the absorption of part of the sample in the GLC column. At the present time attempts are being made at the GLC separation of lipids according to e_i on polyester liquid phases [5, 6]. On intense heating, these phases are unstable, and therefore it is possible to use only nonpolar phases which, however, are capable of

*Below, natural polar glycerolipids are designated by the term "lipids." The compositions of all mobile phases mentioned in the text of our review are shown in parts by volume, and the ratios between the components of the stationary phases in proportions of the total mass of these phases.

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effecting fractionation only according to (m_i). All this greatly complicates the analysis and decreases the volume of information obtained on GLC even in association with mass spectrometry. Finally, the GLC of lipids is a specialized method requiring expensive equipment. Therefore, in the present review, the GLC method is mentioned only in application to the analysis of the FA composition of lipids. Narrowly specific methods of LC analysis of the species composition of sphingolipids and the plasmalogen forms of lipids are not considered, either.

In this review we consider the methods and the immediate results of the LC determination of the species composition of diacylglycerolipids. The analysis of these lipids has already been described in a number of reviews [3, 4, 6, 7] but in these the main attention was devoted to the GLC method. In contrast to this, LC analysis is carried out at room temperature and, frequently, without preliminary chemical modification of the lipids. Today the native lipids and their derivatives, the products of the hydrolysis of lipids – (DAGs) – and also various lipophilic derivatives of DAGs are used for such analysis. Not only individual methods of LC – adsorption TLC, high-performance LC of the π-complexes of olefinic compounds with silver ions (Ag^+-TLC, AG^+-HPLC) and reversed-phase chromatography (RP TLC, RP HPLC) – but also various combinations of the methods are used for the separation of these compounds [8-10].

Anlaysis of the species composition of lipids or their derivatives not infrequently begins with Ag^+-TLC. This method is based on the fact that π-complexes arise between the olefinic bonds in the aliphatic chains of the lipids and Ag^+ ions in the adsorbent [11]. The mobility of these complexes in Ag^+-TLC is inversely proportional to the number of olefinic bonds in the aliphatic chain, and therefore under Ag^+-TLC conditions the mixture of lipids being analyzed is, as a rule, separated into individual fractions which will subsequently be called e-fractions. The process of obtaining e-fractions is called e-fractionation. In each of these fractions are usually grouped lipid molecules containing the same number of olefinic bonds, which is characteristic for this e-fraction.

At the same time, in performing the e-fractionation of lipids and their derivatives with e = 2 and 3 a separation may take place between compounds with one and the same value of e, but with different FA compositions. Thus, the polarity of unsaturated species of lipids depends not only on e, but also, in part, on their FA composition.

The e-fractions isolated (or the initial mixture of lipids) can, in their turn, where necessary, be separated with the aid of RPC according to their equivalent lipophilicity L (= m – 2e) with the formation of the corresponding L-fractions. This type of separation is called L-fractionation [12]. Earlier, for its performance, among other varieties of RPC it was RP TLC on silica gel coated with a thin film of higher normal hydrocarbons that was most frequently used. For this purpose, RP HPLC on 3-10 μm silica gel with a hydrophilic coating formed by the chemical grafting of hydrocarbon chains by treating the SIO_2 with alkylcholorosilanes is usually employed [13].

At the present time, tens of publications on the species composition of lipids have already appeared and the chromatographic results that had to be used in our review are extremely numerous. For a more complete coverage of the information on the question under consideration the initial results on the e- and L-fractionation of lipids given in the literature has been treated mathematically. From the chromatograms given by the authors have been calculated the parameters of chromatic separation of the lipids [9] – the selectivity α, the separation factor R, the height equivalent to a theoretical plate (HETP), and the number of such plates N, and also the results of the GLC analysis. The LC composition of individual fractions and individual species of lipids have also been subjected to recalculation. These results are expressed briefly in the form of the calculated number of olefinic bonds $e_c = \sum_i (0.02\ a_i e_i)$ and the calculated equivalent lipophilicity of the DAG residue in a glycerolipid $L_c = \sum_i (0.02\ a_i L_i)$ where $L_i = m_i - 2e_i$ is the theoretical lipophilicity of the i-th FA in the mixture of FAs. In the equations given, a_i (the amount of the i-th FA in the mixture of FAs) is expressed in mole-%. However, in many cases it is necessary to calculate e_C and L_C by using the FA composition found in mass-%, since only these results are given in the original publications.

In the following stage of mathematical treatment, by comparing the calculated values of e_C and L_C with the nominal values of e and L for the corresponding fractions given by the

authors for each fraction j of the mixture of lipids analyzed we determined the relative deviation of e_c from e and L_c from L (%): $(s_e)_j = 100|e - e_c|/e$ and $(s_L)_j = 100|L - L_c|/L$.

Finally, using the calculated deviations sj, we found the most general integral index of the reliability of the results of the determination of the species composition of the given mixture of lipids – the mean relative deviation $\bar{s}_e = \sum_j (s_e)_j / n_e$ and $\bar{s}_L = \sum_j (s_L)_j / n_L$, where ne and n_L are the numbers of fractions isolated in its e- or L-fractionation respectively.

Thus, for comparing the results of qualitative species composition obtained by identical or different methods in different laboratories, we have been forced to use not these results themselves but only the values of their reliability which, consequently, have served as a universal criterion of the "suitability" of the particular results of analysis. We may mention, incidentally, that the reliability of a found quantitative species composition of lipids can also be evaluated by the direct comparison of the FA composition of these lipids calculated from it with the FA composition actually found. Nevertheless, hitherto such an estimate has been made in only rare cases [14].

It must be emphasized that in many papers on the e- or L-separation of lipids there is no information whatever on the FA compositions of their individual fractions [15, 16]. In such cases the identification of individual species was most frequently based on mass spectrometric results [17-19]. In other investigations of this type, the determination of the qualitative species composition was based on the results of a comparison of the fractions being analyzed according to chromatographic mobility with the corresponding standards representing individual species of lipids [20-22]. In all these cases identification bore an only preliminary nature, and all investigations of this type were, even in the best case, only qualitative.

It must be mentioned that it is also impossible to judge the degree of reliability of qualitative results obtained in the course of any fractionation without taking into account results on the recovery of the mass of lipids after their separation, i.e., the amounts of lipids in the sum of the fractions isolated as a percentage of the initial amount. However, hitherto these results have likewise been calculated only very rarely. In the present review we give all the recovery figures stated by the authors.

ANALYSIS OF NATIVE LIPIDS

To investigate the species composition of lipids, unchanged – native – lipids are frequently used. The first attempt to fractionate the latter with the aid of the Ag^+-TLC method was made by Kaufmann et al. [23]. The adsorbent used in their work was a mixture of silica gel (SiO_2) and $AgNO_3$ (7:3), and the mobile phase used was a mixture of $CHCl_3$ + Et_2O + AcOH (97:2.3:0.5). Under our conditions, soybean and egg lecithins (phosphatidylcholines – PCs) were separated into ten and seven e-fractions, respectively, and in this way it was shown for the first time that the classes of lipids are multicomponent mixtures. At the same time, there is no information in these papers on the identification of the lipids of the fractions obtained and their quantitative composition, and it was impossible to reproduce the results obtained in another laboratory [11].

Nevertheless, experiments to determine the species compositions of lipids with the aid of Ag^+-TLC have continued. In the course of the preparative e-fractionation of the PCs from rat liver in the mobile phase $CHCl_3$ + MeOH + H_2O (65:35:4) the authors isolated only two e-fractions, which had complex FA compositions and were therefore not identified [24]. Another analysis of the species composition of the PCs and kephalins (phosphatidylethanolamines - PEs) of animal origin was made by Arvidson [25, 26]. Only for the fractions with e = 1 and e = 2 was there satisfactory agreement between e and e_c.

In the e-fractionation of both PCs and PEs the mobile phase $CHCl_3$ + MeOH + H_2O (65:25:4) remained the most popular for a long time, although the values of s_e on the use of this phase were frequently high, and $e_c < e$ was observed for all the fractions [26-29]. The values of $\bar{s}_e$ in one and the same series of experiments could differ from one another by an order of magnitude [29], which was probably explained by the inadequate reproducibility of the conditions of the e-fractionation of the FAs in parallel experiments. The recovery of the PCs usually did not exceed 75% [27]. Attempts to achieve a satisfactory separation of the polyenic species of PCs from one another were unsuccessful and it is apparently for this reason that there are no results on the chromatographic mobility of different e-fractions of

TABLE 1. Reliability of the Results of the Determination of the Species Composition of Individual Classes of Phospholipids from Animal Tissues by the Ag^+-TLC Method*

Nominal e values of the individual fractions	Individual classes of lipids								
	PCs [25]	PEs [26]	PCs [26]	PCs [27]	PCs [28]	PCs [29]	PEs [30]	PSs [32]	PCs [34]
1	0,93—1,03	0,96—1,07	0,97—0,98	0,88	0,76—0,94	—	—	1,14	**
2	1,88—2,06	1,72—1,78	1,98—2,00	1,92	1,86—2,00	1,79—2,01	1,83	**	1,94—2,11
3	2,81	—	—	2,52	2,30—2,88	—	—	—	—
4	3,36 3,66	3,70—3,72	3,84—3,91	3,42	**	3,60—3,92	4,38	**	4,01 4,05
5	4,40—4,49	—	—	—	—	—	—	**	—
6	4,67—5 53	4,84—5,14	4,72—5,08	—	—	—	5,70	5,77	**
$\bar{s}_e$, %	2,0—9,7	10,3—10,7	5,1—7,1	11,6	4,1—17,9	1,2—10,2	7,7	8,8	2,2—2,9

*Values of e_c are given. A dash in the column of the table means that the corresponding fraction was not detected in the mixture of lipids. The ratios of SiO_2 and $AgnO_3$ in the stationary phase were 7:2 [25], 91.67:8.33 [32], and 87.5:12.5 [30]; the ratios of $CHCl_3$, MeOH, and H_2O in the mobile phase were, for the PEs and PCs, 55:35:7 and 60:30:5, respectively [26]; for the PCs and PEs, 65:25:4 [25, 27, 29, 30, 34]; and for the PSs, 65:35:6.8 [32].
**The authors did not give the FA composition of this fraction.

PCs in the paper by Tinoco et al. [29].

The results obtained in the Ag^+-TLC of the PCs from beef heart must be regarded as no more successful [31]. The e-fractions isolated were distinguished by extremely complex FA compositions and they were difficult to identify not only with particular species of PCs but even from the values of e; the selectivity of the separation was apparently extremely low.

The preparative Ag^+-TLC of one more class of lipids from bovine brain — phosphatidylserines (PSs) — has also been performed [32]. The FA compositions of two of the five e-fractions of PSs so obtained were established: in a hexaenic fraction the value of s_e was low, while in a monoenic fraction it was extremely high (14%).

In addition to e-fractionation, other LC methods of analysis of the species composition of lipids exist. Thus, simultaneously with the Ag^+-TLC Arvidson performed the analytical L-fractionation with the aid of RP TLC of the initial PCs of rat liver or of the e-fractions obtained from them with e = 1, 4, and 6 [33]. He separated each of such fractions in the MeOH + H_2O (90:10)/n-$C_{11}H_{24}$ system with the formation of L-fractions having L = 26, 28, 30, 32, and 34. Although the selectivity of the separation of the latter from one another was, on the whole, high, not all these L-fractions were satisfactorily separated from the neighboring ones: thus, for the fractions with L = 30 and 32 from the e-fraction with e = 2 the value of α did not exceed 1.4.

Tinoco et al. used a combination of Ag^+-TLC and RP TLC for the preparative separation of the mixture of PCs from the same source. Judging from the FA compositions of the L-fractions obtained, agreement of L_c with L was observed only for the PCs with L = 30 from the e-fraction with e = 2. The values of $\bar{s}_L$ ranged from 0.3 to 2.6%, but the recovery of the lipids amounted to only 50% [29]. Thus, the results of this L-fractionation were not satisfactory.

In another investigation [34] also devoted to the analysis of the PCs of rat liver, Arvidson first used L-fractionation in the form of RPC on a column with hydrophobic Sephadex, and then the Ag^+-TLC of the L-fractions obtained. For the e-fractions $e \approx e_c$ was observed; however, satisfactory agreement between L and L_c was found only for the L-fractions consisting of monoenic PCs; the values of $\bar{s}_e$ and $\bar{s}_L$ were at the very same level as the corresponding parameters for the e- and L-fractions obtained by Tinoco et al. [29] or even superior to them. Later, in the L-fractionation of egg PCs on a column of Sephadex, Arvidson [34] succeeded in achieving an efficiency of separation comparatively high for this type of column (HETP = 0.3 mm, N = 1100). However, he succeeded in realizing the possibilities of

TABLE 2. Reliability of the Results of the Determination of the Species Composition of the PCs* by the Methods of RP TLC [29], Column RPC [34], and RP HPLC [35, 36, 38, 41]

Nominal values of L for the individual fractions	Source of the PCs		
	rat liver [29]	rat liver [34]	egg yolk [35]
24	—	—	—
26	—	—	26,24
28	28,48–28,91[1]	—	28,16[3] / 28,40[3]
30	29,41–29,64[1] / 30,00–30,22[2]	27,03–28,86	29,84[3] / 30,08[3]
32	31,64–31,81[2]	31,05[3] / 30,68–30,86[3]	32,00[3] / 32,00[3] / 32,00[3]
34	—	33,81–33,89	33,96
$\bar{s}_L$, %	0,58–2,16	2,58–3,69	0,43
Stationary phase	n-$C_{11}H_{24}$+SiO_2 (7:93)	Hydroxyalkoxypropyl–Sephadex (22% of HOPrO groups)	Nucleosil 5-C_{18}
Mobile phase	MeOH+H_2O (40:9)	MeOH+H_2O (4:1)	MeOH+1 mM aq. sol. KH_2PO_4 (95:5)

Nominal values of L for the individual fractions	Source of the PCs		
	egg yolk [36]	soybeans [38]	rat liver [41]
24	—	24,32	—
26	27,00	25,76	—
28	28,60[3] / 28,40[3]	27,99	28,00[3] 28,25[3] / 28,76[3]
30	29,80[3] / 29,92[3] 30,00[3] / 29,60[3]	29,87[3] / 29,91[3]	30,72[3] / 30,92[3]
32	31,20[3] / 32,00[3] / 31,42[3] 32,80[3] / 32,20[3]	31,70	32,12
34	33,84[3] 34,00[3] / 34,00[3]	—	—
s_L, %	1,17	0,66	1,62
Stationary phase	LiChrosorb RF-18	μBondapak C_{18}	Styrene–divinylbenzene
Mobile phase	MeOH+H_2O+MeCN+ +20 mM ChoCl/MeOH (8:3:90)	MeOH+H_2O (91:9→95:5)	MeCN+MeOH+H_2O (70:15:15)

*Values of L_C calculated from the FA compositions of the corresponding PC fractions are given.

[1]) From the fraction of PCs with e = 4

[2]) From the fraction of PCs with e = 2

[3]) On the L-fractionation of the PCs several fractions with one and the same value of L were obtained in each case; the authors do not state the cause of this phenomenon.

Note. See also the notes to Table 1.

this column completely only in the fractionation of monoenic PCs, and the whole analysis took several hours.

The use of RP HPLC (for example, on a column of μBondapak C_{18}) with the mobile phase $CHCl_3$ + MeOH + H_2O (10:100:10) and a refractometric detector [20] permitted a considerable acceleration of the L-fractionation of PCs. Its selectivity for synthetic saturated species

of PCs was high (α = 1.5-2.0), but it was considerably lower (α = 1.3) for the PCs of egg lecithin; in the latter case, the efficiency of L-fractionation was also low (HETP = 0.5 mm, N = 540-660). All this prevented a satisfactory separation of the L-fractions of egg PCs from one another.

In another laboratory, after the L-fractionation of egg PCs by the RP HPLC method the FA compositions of all the thirteen fractions obtained were analyzed by GLC and mass spectrometry [35]. In this work, the value of s_L for the majority of the fractions did not exceed 1% and the value of $\bar{s}_L$ was only 0.4%. The fractions isolated consisted mainly of pure individual species of PCs; at the same time, some L-fractions each contained several species of PCs with different values of L. In this case, apparently, fractionation had taken place by several mechanisms simultaneously.

A still large number of fractions (15) was obtained as a result of the preparative RP HPLC of 15 mg of PCs from the same material in a gradient mobile phase using a UV detector [36]. This fractionation was carried out with high efficiency (HETP = 0.6 mm, N about 15,000). After the GLC analysis of the FA compositions of the fractions, 23 individual species of PC were identified in them; the recovery of the PCs was 93 ± 2.5%, and the value of $\bar{s}_L$ was 1.17% [36].

For the L-fractionation of the PCs, PEs, PSs and phosphatidylinositols (PIs) from rat liver with the aid of RP HPLC, a mixture of 30 mM solution of choline chloride in MeOH, a 25 mM aqueous solution of KH_2PO_4, MeCN, and AcOH (90.5:7:2.5:0.8) was tested as the mobile phase; the stationary phase, as previously [20, 35], was octadecylsilylsilica gel [37]. In this case, the recovery of lipids was high (96.3 ± 5.3%). However, in this case complete separation between the individual L-fractions was not achieved.

If a mixture of MeOH + H_2O is used as the mobile phase in RP HPLC then for the majority of L-fractions of highly unsaturated lipids of plant origin it is possible to achieve their satisfactory separation from one another [38]. Thus, by using a linear gradient of the composition of this mobile phase Crawford et al. isolated from soybean PCs six L-fractions. Although the selectivity of the separation was low (α = 1.17-1.27), thanks to the satisfactory efficiency of the column (HETP = 0.1 mm), the value of the separation factor proved to be extremely considerable: for example, in the separations of the fractions with L = 30 and 32 from one another it reached 8.7. The authors identified the fractions on the basis of results on the FA compositions; values of L_C and s_L found proved to be within acceptable limits.

For the RP HPLC of a mixture of synthetic single-acid PCs in the mobile phase MeCN + H_2O + H_3PO_4 (90:10:0.1), Compton et al. used a column with 5-μm particles of silica gel containing grafted-on octadecyl groups [39]. A feature of the L-fractionation of PCs under these conditions was that in a number of cases individual PCs with one and the same nominal value of L were separated from one another on the basis of the values of the capacity factor k'. Such differences were the greater the higher degree of unsaturation of the lipids subjected to fractionation. In our view, this was due to the comparatively low lipophilicity of the stationary phase used by these authors, which was distinguished by an inadequately high density of the grafted hydrophobic groups on the surface of the particles.

The results of another investigation [40] may serve as an independent confirmation of this hypothesis. Thus, on the RP HPLC of lipids with a phase of constant composition – a mixture of MeOH + H_2O + MeCN (8:1:1) – it was shown that, depending on the brand of stationary phase used, the value of R changed by a factor of more than two (from 3.0 to 7.4). It must be pointed out that this study was one of the first in which a mass detector was used.

Simultaneously, a detector of the same type was tested by Christie and Hunter [41]. On the RP HPLC of 0.5-2.0 mg of rat liver PCs they isolated six L-fractions and determined their FA compositions. However, for some of them the value of s_L was very high. Investigations into the field of use of a mass detector for the HPLC of lipids are continuing [42-44].

A cycle of investigations devoted to use of a plasma-ionization detector in HPLC of plant lipids [45-49] is of interest. Because of noise during the detection of the lipid fractions, the workers concerned renounced the use of buffer solutions as mobile phases [47]. They proposed for the L-fractionation of PCs of vegetable origin the mixture MeCN + MeOH + AcOH + H_2O + EtPrNH (89.8:6.8:1.5:1.0:0.9) and obtained six fractions [48]. On the RP HPLC of phosphatidylglycerols (PGs) from Dunaliella salina in the mobile phase EtPrNH + AcOH +

MeOH + MeCN (0.15:0.25:34.85:64.75) three fractions were isolated [46]. Nevertheless, under the conditions of the experiment no separation was achieved of mixtures of the individual species 18:2/18:3 + 18:3/18:3 and 18:0/18:3 + 18:1/18:2.

In this cycle of investigations examples are also given of the L-fractionation of mono- and digalactosyl-DAGs (MGDAGs and DGDAGs, respectively) isolated from D. salina and Arabidopsis thaliana [47, 49]. On RP HPLC in the mobile phase MeOH + H_2O (96:4), eight fractions were obtained, but it is impossible to evaluate the quality of L-fractionation from the available information. Earlier, Lynch et al., using a column with Biophase 5 ODS and the mobile phase MeOH + 1 mM aqueous KH_2PO_4 solution (95:5), also separated MGDAGs and DGDAGs from the chloroplasts of the alga D. salina into eight and six fractions, respectively [50].

An example of a successful separation of plant galactolipids with the aid of RP HPLC on a column of Spherisorb 10 (ODS) in the mobile phase $CHCl_3$ + MeOH (88:12) [51] deserves attention. By this method the MGDAGs and DGDAGs of wheat leaves each yielded two L-fractions for which the L_C values practically coincided with the theoretical figure (L_C = 28.02 and 30.03), while the value of s_L did not exceed 0.05%.

Thus, RP HPLC on particles of increased lipophilicity with grafted-on stationary phases may be considered the most promising method for the L-fractionation of native lipids [52-57].

The best-known example of the highly selective e-fractionation of native glyco- and sulfolipids is provided by Ag^+-TLC investigations of leaf lipids [58-61]. As in the case of phospholipids [25-33], for the e-separation of MGDAGs and DGDAGs a mixture of $CHCl_3$ + MeOH + H_2O was used as the mobile phase; in the analysis of tri- and tetragalactosyl-DAGs 2-4% of AcOH was added to it [60]. Later, for the e-fractionation of the MGDAGs isolated from chloroplasts, the water in this mobile phase was replaced by acetone [61]. It is possible that the analytical Ag^+-TLC of native glycolipids can be carried out successfully only at a relatively low temperature (+4°C). In MGDAGs and DGDAGs under the given conditions it was possible satisfactorily to separate from one another and to identify with the aid of mass spectrometry eleven and seven individual species in them, respectively [60].

In concluding this section, it must be mentioned that with the aid of existing chemical or enzymatic methods it has so far rarely been possible to convert, in satisfactory yield, glycolipids into less polar derivatives and thereby to raise the selectivity of the separation of their e- or L-fractions from one another. Today, therefore, analysis of glycolipids in the native form remains practically the only method of determining their species composition.

ANALYSIS OF LIPIDS IN THE FORM OF THEIR DERIVATIVES

As is known, the polar functional groups present in native lipids are capable of forming hydrogen bonds and dipole-dipole interactions. This greatly lowers the selectivity of the chromatographic fractionation of such lipids [1]. By raising the lipophilicity of the components being analyzed it is possible to achieve a considerable rise in this magnitude. Various methods are used to obtain more hydrophobic lipid derivatives. For example, for PEs (I) the O-methyl-N-dinitrophenyl derivatives (II) are synthesized [62].

It is obvious that (II) can be obtained only from lipids containing a free amino group – PSs, PEs, etc. As an example of the e-fractionation of the derivatives (II) we can give the Ag^+-TLC of (II) from egg PEs. This led to the isolation of six e-fractions; for the majority of them the values of e_C and e were close, but for the hexaenic fraction s_e amounted to 7% [3]. Today, derivatives (II) are used very rarely [63, 64].

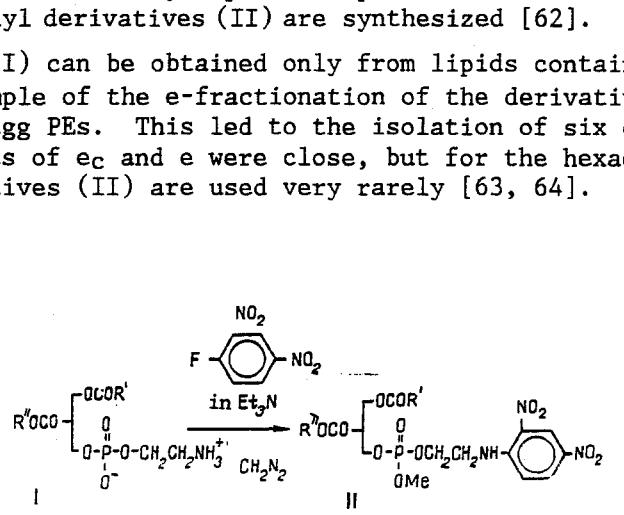


Fig. 1

TABLE 3. Reliability of the Results of a Determination of the Species Compositions of Various Lipophilic Derivatives of Individual Classes of Phospholipids by the Ag^+-TLC Method*

Nominal e values of the individual fractions	Derivatives of individual classes of lipids						
	II from PEs [62]	V from PSs [65]	VI from PSs [65]	VIII from PSs [14]	VIII from PEs [14]	VIII from PEs [67]	IX from PIs [68]
1	1,04	0,76	1,15	0,82	1,28	0,90	1,02
2	1,84[1] / 2,08[1]	2,04	1,68	1,82	2,03	2,12	2,22
3	3,00	1,22	2,01	1,14	2,74	1,97	2,57
4	4,12	3,41	3,57	3,53	2,40[1] / 4,23[1]	4,16	4,06
5	—	3,17	3,93	4,11	5,06	5,68	4,27
6	5,58	5,13	5,04	5,41	5,81	4,94	—
$\bar{s}_e$, %	4,3	25,3	18,8	21,4	12,5	10,3	22,7
Ratio of SiO_2 and $AgNO_3$ in the stationary phase	8:2			10:3		85:15	10:3
$CHCl_3$:MeOH:H_2O ratio in the mobile phase	98:2:0	65:25:4		80:20:3[2] / 97:3:0[3]		80:15:2[2] / 97:3:0[3]	$CHCl_3$+ +Me_2CO (75:25)

*For the designations of derivatives (II), (V), (VI), (VIII), and (IX), see text.

[1]) In the separation of (II) and (VIII) two subfractions were obtained in each case, with e = 2 and e = 4 because of the separation of individual species of (II) and (VIII) with the e-fractions from one another by chain-length.

[2]) For the separation of the fractions with e = 4-6 from one another.

[3]) For the separation of fractions with e = 0-3 from one another.

Note. See also the notes to Table 1.

For the e-fractionation of PEs and PCs (IV), in addition to the derivatives (II) their N-trifluoroacetyl derivatives – (III) and (V), respectively – are used. In the case of (IV) a by-product of the synthesis is the azlactone (VI) [65]. At first glance, (III) gives better results in Ag^+-TLC than the initial PEs. Thus, on the e-fractionation of a mixture of derivatives (III) obtained from rat liver PEs it was possible to separate these derivatives into seven fractions [65] while the initial PEs were separated into only four fractions [26]. However, the values of s_e for fractions (III) were considerable. Although on the Ag^+-TLC of mixtures of (V) and of (VI) from the identical material, these were each separated into six e-fractions, for the polyenic fractions of (V) and of (VI) the deviations of e_c from e were even higher than in the case of the fractionation of (III).

Having criticized the use of (V) and (VI), Bjerve used in place of (V) the N-trifluoroacetyl-O-methyl and N-acetyl-O-methyl derivatives of the aminolipids – (VII) and (VIII), respectively [14]. In the opinion of this author, the derivatives (VIII) give a better separation of e-fractions from one another in Ag^+-TLC than the derivatives (VII). For the Ag^+-TLC of a mixture of derivatives (VIII) obtained from PEs and PSs, Bjerve proposed to use two mobile phases with different polarities successively. He has given results on the FA composition of the e-fractions only for the derivatives (VIII). In the case of the (VIII) fractions with e = 2 and e = 5 from the PEs, satisfactory agreement was observed between e and e_c (s_e = 1.2 and 1.3%, respectively). In the fractionation of the derivatives (VIII) obtained from rat liver PSs the values of s_e were higher. As a result of the Ag^+-TLC of the derivatives (VIII) from the lipids of rat liver microsomes, nine fractions were isolated, with e = 0-6 [66]. For the majority of e-fractions of the derivatives (VIII) obtained from the PEs of the same source by other workers the values of s_e were likewise very high [67].

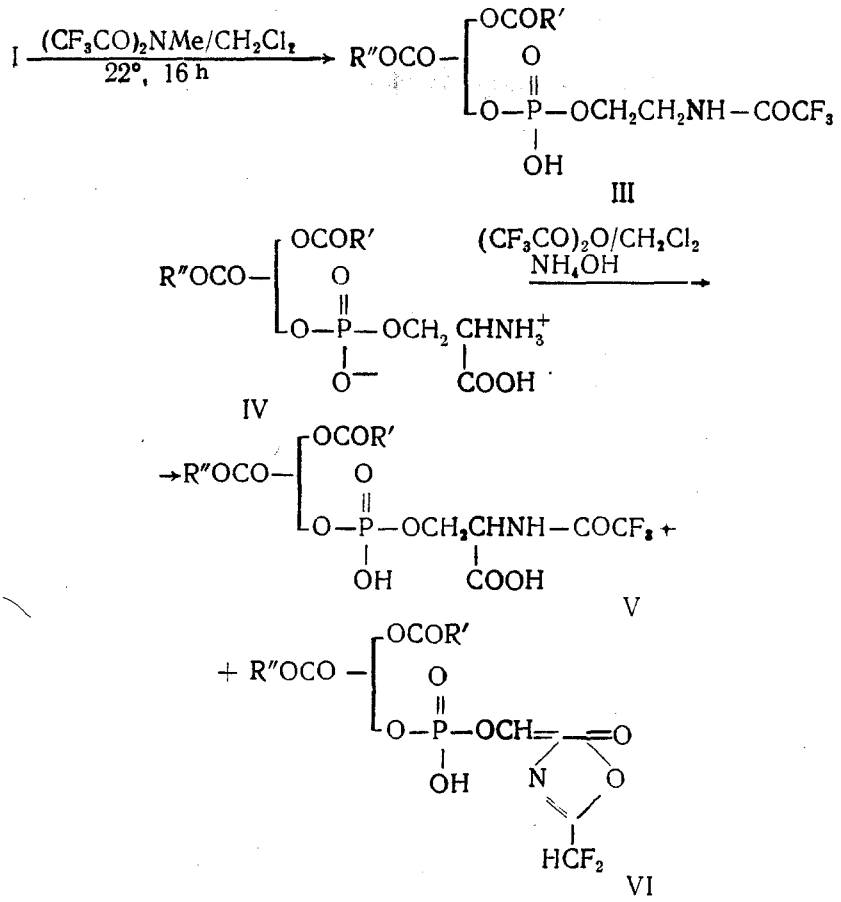


Fig. 2

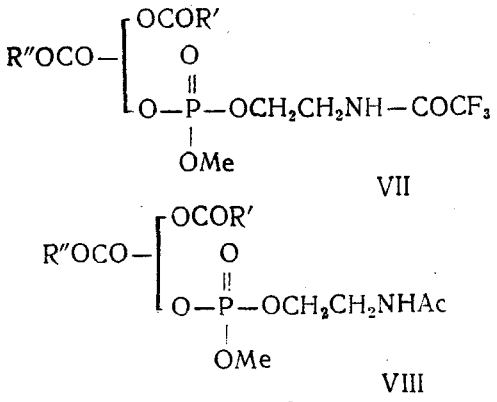


Fig. 3

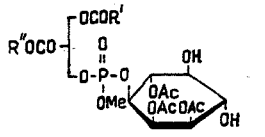


Fig. 4

In an investigation of another class of lipids – PIs – their O-triacetyl, and also their O-triacetyl-O-methyl derivatives (IX) were used [68]. The mixture of the derivatives (IX) was subjected to Ag^+-TLC in the mobile phase $CHCl_3$ + Me_2CO, and on their e-fractionation the value of s_e was extremely considerable. They have been used infrequently in recent years [69].

The dimethyl esters of phosphatidic acids – the derivatives (XII) – were used long ago to investigate the fine structure of lipids [70]. For this purpose, the PA derivatives (XII) were obtained after the hydrolysis of, for example, PCs (X) under the action of phospholipase D (EC 3.1.4.4).

The use of derivatives (XII) for the analysis of the species compositions of PCs, PIs and other lipids is possibly more promising. Thus, in the Ag^+-TLC of the derivatives (XII) synthesized from the same sample of PIs as derivatives (IX), the value of $\bar{s}_e$ was almost two times lower [68]. It must, however, be noted that the treatment of unsaturated PAs with

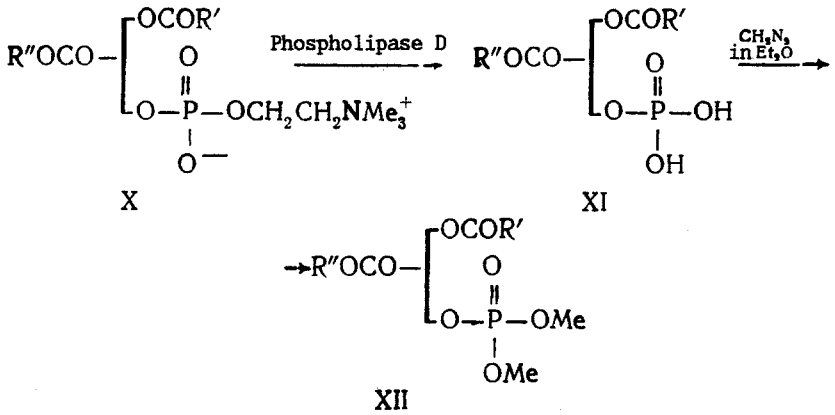


Fig. 5

TABLE 4. Reliability of the Results of the Determination of the Species Composition of the Dimethyl Derivatives of Individual Classes of Phospholipids by the Ag^+-TLC Method*

Nominal e values of the individual fractions	Source of dimethyl derivatives of the PAs (XII)					
	PIs [68]	PCs [70]	PCs [71]	PIs [74]	PIs [75]	PAs [75]
0	0,00	0,00	—	—	**	—
1	1,07	0,91	1,02	0,84	**	0,96
2	2,36	1,42[1] / 1,93[1]	1,94[1] / 2,00[1]	1,88	1,90	1,93
3	2,30	2,27	2,88	2,29	2,63	3,20
4	3,34	3,75	3,62	3,97	3,98	4,08
5	**	2,75	4,38	4,47	**	**
6	**	4,01	5,30	5,85	**	**
$\bar{s}_e$, %	12,9	20,4	6,1	9,8	6,0	4,2
Ratio of SiO_2 and $AgNO_3$ in the stationary phase	10:3	90:10	83:17		8:2	
$CHCl_3$:MeOH:H_2O ratio in the mobile phase	49:1:0	180:20:1	90:10:1[2] / 98:2:0[3]		44:1,2:0	

[1]) On the e-fractionation of the derivatives (XII), in each case two subfractions with e = 2 were obtained because of the separation of the individual (XII) species within the e-fractions from one another according to chain length.
[2]) For the separation of the fractions with e = 4-6 from one another.
[3]) For the separation of the fractions with e = 0-3 from one another.
*, **See notes to Table 1.

diazomethane frequently leads to a disturbance of the native structure of the lipids. It is apparently for this reason that after the separation of the mixture of derivatives (XII) by the Ag^+-TLC method the values of e_C for the penta- and hexaenic fractions of derivatives (XII) did not exceed 2.75 and 4.01, respectively, while s_e reached 20.4% [71].

The results of the simultaneous e-fractionation on one and the same plate of the highly unsaturated derivatives (XII) obtained from the PCs of herring and eggs are of interest [72]. As usual, a mixture of $CHCl_3$ + MeOH + H_2O was used as the mobile phase for the Ag^+-TLC of the derivatives (XII); here their separation took place in two stages: in the mobile phase $CHCl_3$ + MeOH (98:2) it was possible to separate only fractions with $e \leqslant 3$. The zones of the derivatives (XII) obtained were then subjected to a second e-fractionation on the same plate but using a more polar mobile phase [70]. A preparation of herring PCs was separated into four fractions with nominal e values of 4, 5, 6 and $\geqslant 11$. In Renkonen's opinion, the last fraction was a mixture of 20:5/22:6 and 22:6 species [72]. On the separation under the same conditions of the derivatives (XII) synthesized from egg PCs, fractions with e = 3, 4, and 6 were

obtained [72]; satisfactory correspondence between the e and e_C values was obtained only for the mono-, di-, and trienic fractions of the derivatives (XII).

For Ag^+-TLC the latter are used even today [73]. For example, the derivatives (II) including those obtained from the PIs of lymphocytes were the object of an investigation undertaken by Waku et al. [74, 75]. The values for the polyenic fractions of the derivatives (XII) isolated as the result of e-fractionation were appreciably lower than those found previously.

Experiments are known on the e-separation of derivatives (XII) by the Ag^+-HPLC method. Thus, comparatively recently, a mixture of derivatives (XII) obtained from egg PCs was subjected to such fractionation using not only silica gel impregnated with $AgNO_3$ but also an organic polymer continuing Ag^+, ether being used as the mobile phase in both cases [76]. The second of these variants of the fractionation of the derivatives (XII) gave somewhat better results, but here again it was possible to achieve complete separation from one another only for the di- and trienic species of derivatives (XII).

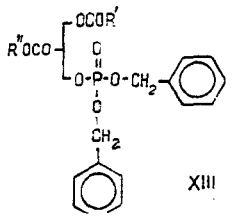
As an example of the successful L-fractionation of derivatives (XII) we can give the results of the RP TLC of these derivatives from rat liver PCs in the Me_2CO + MeCN + H_2O (65:35:5)/n-$C_{11}H_{24}$ system [77]. The fractions of derivatives (XII) isolated consisted of almost pure individual species (16:0/18:2 with L_C = 30.14, and 18:0/18:2 with L_C = 32.08; $\bar{s}_L$ = 0.36%). A satisfactory result ($\bar{s}_L$ = 0.86%) was obtained in the RP TLC of di-, tetra-, and hexaenic fractions of derivatives (VIII) from PEs of the same material in the MeOH + H_2O (85:15)/ n-$C_{11}H_{24}$ system [67]. On the repeated RP TLC of the L-fractions of (VIII), the value of $\bar{s}_L$ did not exceed 0.46%; the isolated monomolecular fractions consisted of individual species of derivatives (VIII) with purities of 90.1-97.6%.

The results of the first experiments on the fractionation of derivatives (XII) using RP HPLC [78] are of interest. The mobile phases in the analysis of the derivatives (XII) obtained from egg PCs were the mixtures MeOH + H_2O (96:4) and MeCN + H_2O (94:6), the k' values being almost one and a half times greater in the second case. In each of these experiments three fractions were isolated with L = 30, 32, and 34, but the authors give the results of an analysis of their FA compositions only for the first variant. For these (XII) fractions the L_C values were 29.91, 31.89, and 33.61 ($\bar{s}_L$ = 0.60%) and the main individual species were 16:0/18:2, 16:0/18:1, and 18:0/18:1, respectively.

The use of Me_2CO, MeCN + MeOH, and mixtures of EtOH, n-PrOH, or THF with H_2O as mobile phases decreased the selectivity of separation from one another of the L-fractions containing disaturated species of derivatives (XII) and also other L-fractions [78-80]. However, by using pure MeCN it is possible to effect the satisfactory L-fractionation of derivatives (XII) on columns with 10-μm particles of Partisil ODS; with the aid of such columns it was possible to isolate eight L-fractions in each case from mixtures of derivatives (XII) of egg and soybean PCs [79].

At the present time, HPLC is the standard method of fractionating complex mixtures of many organic compounds, HPLC with a UV detector being used most frequently [9]. It is obvious that in this way it is possible to fractionate only those lipid derivatives that possess fairly strong absorption in the UV region. As such derivatives it has been proposed to use the dibenzyl esters of PAs (XIII), the sensitivity of the detection of which with a UV detector is twenty times higher than for the derivatives (XII) [81].

For the L-fractionation of the derivatives (XIII) in the mobile phase MeCN + H_2O (95:5) a HPLC column with particles of μBondapak has been proposed [81]. In this way it has been possible to separate a mixture of derivatives (XIII) obtained from egg PCs into nine fractions. It is interesting that (XIII) species with one and the same value of L (for example, 16:0/18:1



XIII

Fig. 6

and 18:0/18:2) were separated from one another in the course of such analysis, i.e., e-fractionation took place; in the case of the RP HPLC of derivatives (XII) no such phenomenon was observed. It may be assumed that in the RP HPLC of mixtures of derivatives (XIII) of complex species composition this phenomenon will interfere with the reliable identification of the fractions from their L values. Other disadvantages of the RP HPLC of derivatives (XIII) may probably be the extremely high value of k' for disaturated (XIII) species [81]. The derivatives (XIII) have not come into wide use, and subsequently even the workers who proposed them returned to the use of derivatives (XII); apparently the L-fractionation of the latter gave a more satisfactory result [78, 79].

It is impossible not to mention lipophilic derivatives of native lipids containing no phosphorus – glycolipid acetates [82, 83]. On e-fractionation with the aid of Ag^+-TLC of preparations synthesized from wheat lipids, mobile phases of the same composition as for the separation as DAGs (see below) were used. The authors do not give detailed results of the experiments performed. We know no other examples of the separation of galactolipid acetates.

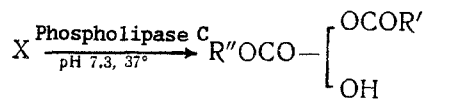
Thus, in the course of the last twenty years, various derivatives of native phospholipids have been used for LC. Nevertheless, the range of use of these derivatives in the LC of lipids is still limited. This is possibly connected with the fact that in their synthesis it is frequently impossible to retain the structures of all the species of initial lipids, and fractionation rarely leads to satisfactory results. The appearance of new lipophilic derivatives of the PAs will probably make it possible to improve the parameters of the separation of the individual lipid fractions from one another [84].

ANALYSIS OF LIPIDS IN THE FORM OF THE PRODUCTS OF THEIR HYDROLYSIS (FREE DAGs)

As is well known, the polar groups of glycerolipids being analyzed can be eliminated and give DAGs considerably exceeding the initial polar lipids with respect to lipophilicity and, consequently, also with respect to the selectivity of chromatographic separation and therefore more suitable for LC fractionation. Another important advantage of DAGs is the fact that with their use it is possible under identical conditions of LC to determine the species composition of all those classes of glycerolipids that undergo conversion into DAGs with no disturbance of their composition and structure. At the same time, it must be emphasized that the use of DAGs for the LC of lipids can lead to a certain loss of the information contained in the native lipid molecules. For example, after the conversion into DAGs of lipids labeled in the polar radicals with ^{32}P and ^{15}N it now becomes impossible to determine the distribution of radioactivity in the individual species of these lipids.

The preparation of DAGs (XIV) from phospho- and glycolipids is now relatively simple [85, 86, 82, 87]. As a rule, Ag^+-TLC is used for the e-fractionation of the free DAGs, while $CHCl_3$ is most frequently the main component of the mobile phases [88]. For example, a mixture of $CHCl_3$ and EtOH (95:5) was used by Haverkate et al. for the analysis of the species composition of the DAGs obtained from spinach leaf PGs [89]. These authors isolated DAG fractions with e_c = 0.80, 0.83, 0.87, 1.84, and 3.24; the Rf values of these fractions are not given. The formation of three fractions with practically identical e_c values is of interest, although the authors do not explain it at all.

Table 5 gives the results of the e-fractionation of the DAGs obtained from rat liver PCs and PEs and also from the PCs of other materials of animal origin. In different investigations, the ratio of $CHCl_3$ and EtOH in the mobile phase varied from 98.5:1.5 to 90:10, and the proportion of $AgNO_3$ in the thin layer was between 5 and 33% [90-100]. The range of s_e values in these experiments was 0.8-14.9%; with an increase in the polarity of the mobile phases these magnitudes usually increased. It is impossible not to mention that here, simultaneous with e-fractionation, the separation of the DAGs according to chain length sometimes took place. For example, a mixture of DAGs of simple species composition obtained from the



XIV

Fig. 7

TABLE 5. Reliability of the Results of the Determination by the Ag^+-TLC Method* of the Species Compositions of the Free DAGs Obtained from Individual Classes of Phospholipids

Nominal e values of the individual fractions	Sources of the DAGs (XIV)		
	PCs [90]	PCs [92, 93]	PCs [94, 95]
0	0,09—0.40	0,04—0.42	—
1	1,00—1.16	0,95—1,04	0,94—1,05
2	2,00—2,14[1] / 1,55[1]	1,93—2,00[1] / 1,56—2,08[1]	1,99—2,10
3	2,57—3,17[1] / 2,70[1]	2,84—3,02[1] / 2,28—3,09[1]	2,88—3,16
4	4,04—4,14	3,79—4,10	3,52—3,61
5	—	—	4,72
6	—	—	5,65
$\bar{s}_e$, %	7,1—13,4	2,1—7,8	4.9—6,5

Nominal e values of the individual fractions	Sources of the DAGs (XIV)			
	PCs [96]	PCs [98]	PCs, PEs [99]	PCs [100]
0	—	—	0,04	—
1	0,92—0,99	1,00—1,38[1] / 1,16—1,26[1]	0,88—0,95	0,75—0,83[1] / 1,04—1,25[1]
2	1,76—2,03	1,94—1,96[1] / 2,00[1] / 2,18[1]	1,98—2,03	1,98—2,09
3	—	2,94[1] / 3,25[1]	3,17	2,74
4	3,62—3,85	3,90	3,46—3,75	3,30—4,41
5	—	4,92	—	—
6	—	5,86	5,18	—
$\bar{s}_e$, %	0,8—10,1	8,3—9,7	5,4—6,3	6,4—14,9

*The ratios of SiO_2 and $AgNO_3$ in the stationary phase were 8:1 [90, 100], 2:1 [93], 83:17 [94, 95], and 7:2 [100]; the ratios of $CHCl_3$ and EtOH in the mobile phase were 90:10 [89, 95, 100], 93:7 [90, 94, 99], 94:6 [93, 98], and 96:4 [92].

[1]) On the e-fractionation of the DAGs in each case several subfractions with identical e values were obtained because of the separation of individual DAG species within the e-fractions according to the chain length.

Note. See also the notes to Table 1.

PEs of Escherichia coli in Ag^+-TLC in the mobile phase $CHCl_3$ + EtOH (98:2) was separated into five fractions with e_C = 0.00, 1.03, 1.03, 2.00 and 2.00. Thus, here, in each case, two pairs of fractions with identical values of e but with different FA compositions were obtained [91]. The value of $\bar{s}_e$ in this case was relatively low (1.2%). Consequently, it may be assumed that the appearance of high values of $\bar{s}_e$ in the analysis of mixtures of DAGs of complex species composition is connected with the overlapping of different e-fractions with one another.

In spite of the disadvantages mentioned, mixtures of $CHCl_3$ and EtOH continued to be widely used for the Ag^+-TLC of free DAGs of natural origin [97-100]. Sometimes there has again been a separation of DAG fractions with identical e values according to chain length.

Satisfactory separation of polyenic fractions from one another has not always been observed, and the $\bar{s}_e$ value has frequently been extremely high [100]. At the same time, DAG fractions with e = 0, 1, 2, and 3 are usually separated from one another satisfactorily with the aid of Ag^+-TLC (α = 1.7-1.8). Recently, in analysis of DAG preparations obtained from lung-tissue PCs and PEs it was shown that the e_c values of these fractions differed from the nominal values by not more than 0.1 or coincided completely with them ($\bar{s}_e$ = 0.02-0.25%) [101]. Since Ag^+-TLC does not usually permit e-fractions each containing a single species of DAG to be obtained, this method must not infrequently be combined with others, especially RPC [102]. Thus, the satisfactory L-separation of free DAGs on a column with hydrophobic Sephadex was achieved by the use as mobile phase of a mixture of n-C_7H_{16}+ $CHCl_3$ (100:5); the R value for fractions of free DAGs with L = 30 and 32 obtained from rat liver PCs amounted to 1.5 [103].

In recent years, interest in the LC of free DAGs has fallen considerably. This situation can be explained primarily by the fact that derivatives of DAGs exceeding the initial DAGs in hydrophobicity have started to be more widely used for separation. Other factors limiting the use of free DAGs consist in the possibility of the isomerization during TLC of the sn-1,2-DAGs obtained from lipids, and, in the case of the L-fractionation of DAGs with the aid of RP HPLC, the low solubility of the saturated species of DAGs in polar mobile phases and the insensitivity of the UV detector for these DAG species. Further investigations are necessary to overcome these limitations and possibly, in time, the LC of free DAGs will attract the attention of research workers to the same degree as other methods of fractionating lipids used today in the analysis of their species composition [104].

ANALYSIS OF LIPIDS IN THE FORM OF DAG DERIVATIVES

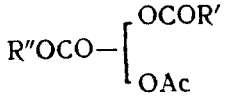
It was mentioned above that the free DAGs are more lipophilic than the initial lipids. Nevertheless, their LC separation frequently meets with failure. It is likely that even the DAGs are insufficiently lipophilic to give satisfactory results in the LC determination of the species composition of lipids. Many experiments have been performed on the fractionation of various DAG derivatives superior to the DAGs in hydrophobicity. Among them the most widely used have been the DAG acetates (XV) [105]. Thanks to the simplicity of their synthesis, the derivatives (XV) can be obtained from practically any class of glycerolipids.

Examples of the e-fractionation of the derivatives (XV) by Ag^+-TLC are known [106-114]. The most popular mobile phases in the analysis of (XV) have been the mixtures n-C_6H_{14} + Et_2O + AcOH and $CHCl_3$ + MeOH. With the aid of the latter of these mixtures, Privett et al. have separated a preparation of derivatives (XV) obtained from soybean PCs into six e-fractions [107]. The GLC analysis of the FA compositions of these fractions with an internal standard ($C_{15:0}$) showed that the value of $\bar{s}_e$ did not exceed 3%.

Kuksis et al., using the same mobile phase, separated a mixture of derivatives (XV) obtained from egg PCs into eight e-fractions [111]. However, even after repeated e-fractionation on the TLC plate no satisfactory separation of all the fractions of the derivatives (XV) from one another was apparently achieved in this case, since the results of the analysis of their FA compositions were given only for the monoenic fractions (e_c = 1.00).

Subsequently, Kuksis et al. performed the e-fractionation under the same conditions of a mixture of derivatives (XV) obtained from rat heart, kidneys, and blood plasma [115]. Here the values of e_c were almost always close to the nominal e values and $\bar{s}_e$ did not exceed 6.1%. For the fractions with e = 5 and 6 it proved impossible to calculate reliable values of e_c, since not all the FAs of these fractions were identified by the authors. The recovery of the lipids in this experiment was only 75 mole-% [115].

Investigations in the field of Ag^+-TLC of derivatives (XV) have been continued [110, 116, 117]; nevertheless, it has just been impossible to raise the yield of derivatives (XV) after e-fractionation. In a recent study carried out in the same laboratory, the recovery of lipids



XV

Fig. 8

TABLE 6. Reliability of the Results of the Determination by the Ag^+-TLC Method* of the Species Compositions of DAG Acetates Obtained from Individual Classes of Phospholipids or Mixtures of Them

Nominal e values for the individual fractions	Sources of the DAG acetates (XV)								
	[106]***	[109]***	PCs [110]	PCs [107]	PCs [113]	PIs [112]	PEs [115]	PCs [114]	PEs [114]
0	0,06	—	—	—	0,00	—	0,30	0,00	0,00
1	0,98	1,08	1,00	0,98	0,98–1,02	1,00	1,00	1,14	0,97
2	**	1,98	2,00	2,00	2,02–2,05	2,00	1,90	1,85	1,42
3	**	2,90	2,80[1] / 3,40[1]	2,95	2,15–2,98	3,00	—	2,99	2,62
4	3,74—3,75	3,38	3,40[1] / 4,00[1]	3,84	3,94–4,04	4,00	3,70	4,12	4,22
5	—	**	4,70[1] / 5,00[1]	4,72[1] / 5,04[1]	**	5,00	4,70	5,29	5,35
6	3,56—4,09	—	5,90[1] / 6,10[1]	—	**	—	5,90	6,29	6,91
$\bar{s}_e$, %	13,1—13,4	7,0	4,4	2,4	1,0—6,1	0,0	8,4	5,2	9,7
SiO_2:$AgNO_3$ ratio in the stationary phase	73:23	9:1	75:25	73:23			8:2	8:2	
Mobile phase	n.-C_6H_{14}+Et_2O+AcOH			$CHCl_3$+MeOH					
	70:30:1	2:1:0	75:35:1	99,2:0,8			44:1,2	99:1	

*, **See notes to Table 1.

***Mixtures of several classes of phospholipids.

[1]) In the e-fractionation of the derivatives (XV), two subfractions with one and the same nominal e value were obtained in each case.

did not exceed 73 ± 2% [117]. Earlier, the AG^+-TLC of the (XV) derivatives from bovine brain PIs performed in the same laboratory [116] gave a completely unusual result: for all the e-fractions of derivatives (XV) the FA compositions found experimentally agreed absolutely with those expected.

The results of experiments performed by other workers were considerably less impressive. As an example we can give the e-fractionation of derivatives (XV) from cytidine diphosphate DAGs likewise obtained from bovine brain performed by Thompson et al. [86, 118]. With the exception of the disaturated species of derivatives (XV), the separation of the e-fractions of this mixture from one another was clearly unsatisfactory; thus, the value of s_e for the tetraenic fraction of the (XV) amounted to 23.2% [86].

Investigations have been made with the aim of increasing the selectivity of the e-fractionation of derivatives (XV) by using other mobile phases in Ag^+-TLC, including mixtures of C_6H_6 + Et_2O [119], C_6H_6 + $CHCl_3$ + MeOH [120, 121], $CHCl_3$ + AcOH [122], and C_6H_6 + $CHCl_3$ [123]. Nevertheless, these mixtures, as well, do not usually ensure the successful fractionation of polyunsaturated species of derivatives (XV) [120]. In other experiments a mixture of petroleum ether and diethyl ether was used as the mobile phase for the e-separation of derivatives (XV), but its use did not lead to a rise in the selectivity of the separation of the unsaturated (XV) fractions from one another and did not decrease the value of $\bar{s}_e$; apparently, these fractions largely decomposed during analysis [109].

A mixture of petroleum ether, AcOH, and Et_2O is one of the most popular mobile phases for the TLC of neutral lipids [124]. Recently, using this phase for Ag^+-TLC, Das et al. separated a mixture of derivatives (XV) obtained from egg PCs into ten e-fractions [110]. These authors were the first to propose a formula for calculating the e_c values of all the fractions of derivatives (XV). According to our calculations, the value of $\bar{s}_e$ here was 4.4%.

Subsequently, Masuzawa et al. also calculated the e_C values of fractions of derivatives (XV) isolated in Ag^+-TLC on the basis of results on their FA compositions [115]. The value of $\bar{s}_e$ in this case was almost twice that in the preceding case. It must be wished that in the future the calculation of e_C values will become obligatory in the e-fractionation of lipids.

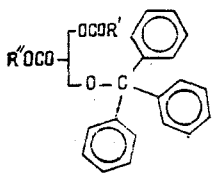
So far as concerns L-fractionation, here, for example, in one investigation the preparative RPC separation of disaturated species of derivatives (XV) obtained from the PCs of cows' milk whey [125] was carried out with the aid of "traditional" LC on a column of Celite coated with heptane; the mobile phase was a mixture of MeCN + MeOH (85:15). The FA compositions of the L-fractions of derivatives (XV) so obtained were determined. As also in RPC of the native PCs on Sephadex, the efficiency of the L-fractionation of the disaturated species of derivatives (XV) with L = 32, 34, and 36 rose with an increase in L. It is interesting that the value of R in this case did not differ from those achieved in the analysis of PCs by the RP HPLC method [20], but with respect to the selectivity of the separation of the L-fractions of derivatives (XV) from one another ($\alpha > 1.8$) they were superior to the corresponding species of native PCs ($\alpha < 1.5$). The results of an analysis of the FA composition were given only for the fractions of derivatives (XV) with L = 32; it was found that $L_C = 32.02$, and $s_L = 0.06\%$.

Subsequently, RPC was used to analyze the composition of the unsaturated species of derivatives (XV) obtained from rat liver PCs [77]. The fractions with L = 30 and 32 were separated from one another by the RP TLC method in the Me_2CO + MeCN + H_2O (1:8:2)/n-$C_{11}H_{24}$ system. Judging from the FA compositions, these fractions consisted to the extent of 98 and 97% of the individual species 16:0/18:2 and 18:0/18:2 ($L_C = 30.12$ and 32.04, respectively). The value of $\bar{s}_L$ in the experiment was only 0.26%, i.e., it was lower than on the L-fractionation in the same system of the derivatives (XII) obtained from the same PC preparation (see above)

Recently, 0.9 μmole of a mixture of derivatives (XV) from bovine brain PEs was separated by the RP HPLC method using a column with particles of Zorbax ODS; the mobile phase was MeCN + n-PrOH + tert-BuOMe + H_2O (72:18:8:2) [126]. The selectivity of the separation of the L-fractions of derivatives (XV) was apparently satisfactory in this case. It is possible that, as it is improved, RP HPLC will be used for the L-fractionation of derivatives (XV) considerably more frequently than has been the case hitherto [127-131].

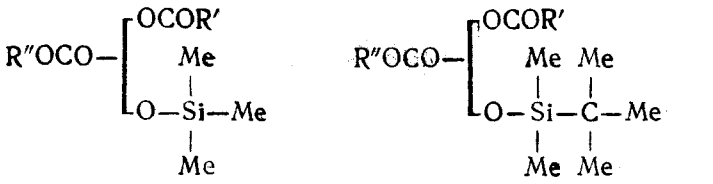
In LC, other lipophilic derivatives of DAGs – the trityl derivatives (XVI) [132] – have been less widely used than the derivatives (XV). On e-fractionation by the Ag^+-TLC method with C_6H_6 as the mobile phase, only saturated and mono- and diunsaturated fractions of derivatives (XVI) were isolated from a mixture of the derivatives (XVI) obtained from PCs of eggs and of Jensen's sarcoma; the values of $\bar{s}_e$ for the derivatives (XVI) from the PCs of these sources were 13.5 and 5.6%, respectively. Judging from the results of the FA composition of the mixture of polyenic species of derivatives (XVI), they had undergone pronounced decomposition [133]. However, the main factor preventing the wide use of derivatives (XVI) was apparently the instability of these compounds during chromatography on silica gel, leading to the formation of derivatives (XIV) [134]. It was subsequently [100] shown that the free DAGs were more suitable for determining the species compositions of lipids than their derivatives (XVI).

It must be emphasized that in the e-fractionation of the derivatives (XVI), as well, a separation from one another of fractions with identical values of e but with different chain lengths took place [132]; such separation has been observed previously in the e-fractionation of other derivatives and hydrolysis products of lipids (see above). So far as concerns the L-fractionation of the derivatives (XVI) by the RP TLC method, as recognized by the authors



XVI

Fig. 9



XVII XVIII

Fig. 10

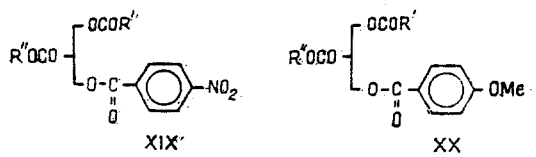
themselves, no satisfactory results were achieved here [132].

Together with the derivatives (XV) and (XVI), the trimethylsilyl derivatives of DAGs (XVII), which are currently in common use in GLC [4], have been used for the LC analysis of the species compositions of lipids [135]. These derivatives have not come into wider use for e-fractionation by the Ag^+-TLC method because of their low stability in polar mobile phases [136]. However, an example of the L-fractionation of derivatives (XVII) is known [103]. A mixture of these derivatives obtained from rat bile PCs was separated on a column of hydrophobic Sephadex with the use as mobile phase of a mixture Me_2CO + H_2O + n-C_7H_{16} + Py (87:13:10:1); as a result, four fractions were isolated, with L = 26, 28, 30, and 32. The value of R was, however, relatively low (∿1.1) and, in the opinion of the authors themselves, the L-fractionation of the free DAGs gave better results than the separation of their derivatives (XVII) [103].

Other silyl derivatives of DAGs – their tert-butyldimethylsilyl ethers (XVIII) – are separated in Ag^+-TLC still less effectively than the derivatives (XVII) [137]. So far as concerns their L-fractionation, mixture of derivatives (XVIII) obtained from egg yolk and rat liver lipids have recently been separated into 18 fractions by the RP HPLC method on a column of Supelcosil RP 18 particles in a gradient of the mobile phase EtCN + MeCN (30:70 → 10:90) [138]. The fractions obtained were identified by mass spectrometry. The p-nitro- and p-methoxybenzoates of DAGs – (XIX) and (XX), respectively – are of interest for L-fractionation by the RP HPLC method. They are favorably distinguished from other DAG derivatives by strong absorption in the UV region of the spectrum that is independent of the FA composition of the lipids being analyzed. In HPLC, therefore, then they can be detected and determined quantitatively with the aid of an extremely sensitive UV detector.

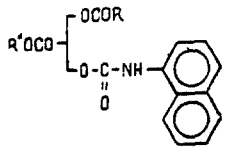
A mixture of eight saturated and monounsaturated (XIX) species was separated on a column containing 10-μm particles of Brownlee RP-18 using as mobile phase a mixture of iso-PrOH + MeCN (35:65) [21]. The authors gave the value of k' for some synthetic polyenic (XIX) species; the dependence of these values on the nominal total number of carbon atoms m in the two aliphatic chains of the (XIX) molecules were expressed by the equation log k' = 0.16 m – 2.31 [21]. The selectivity of the L-fractionation of the (XIX) species, apart from species with L > 32 was relatively low. According to a statement by the authors, the derivatives (XX) were more stable on storage than the derivatives (XIX); L-fractionation under identical conditions but on a column with LiChrosorb RP-18 permitted the separation of a mixture of derivatives obtained from total phospholipids of Escherichia coli into 14 fractions, and the dependence of the values of k' of the individual (XX) fraction on m corresponded to the linear equation log k' = 0.16 m – 2.31 [22]. It is obvious that the derivatives (XX) are more lipophilic than the derivatives (XIX).

Recently, 30 nmole of a mixture of DAG benzoates obtained from bovine brain PEs has been separated on a HPLC column with Ultrasphere ODS-5; the mobile phase was MeCN + iso-PrOH (70:30) [139]. In this paper it was emphasized that the use of such DAG derivatives permits the performance of quantitative determinations in HPLC with the aid of a UV detector. This has been confirmed by the results of other investigations devoted to the HPLC fractionation



XIX' XX

Fig. 11



XXI

Fig. 12

of derivatives (XIX) and (XX) [140-145].

Finally, we must mention the naphthylurethane derivatives (XXI) of DAGs, which have also been used for the HPLC of lipids [146]. For the RP HPLC of derivatives (XXI) a column of LiChrosorb RP-18 and a gradient of the mobile phase MeCN + H_2O (90:10 → 96:4) has been proposed; in contrast to the comparatively low temperatures of the analysis of the (XV), (XVIII), (XIX), and (XX) – 37, 30, 25, and 25°C, respectively – the column temperature for the RP HPLC of the derivatives (XXI) was high (60°C). On the L-fractionation under these conditions of a mixture of derivatives (XXI) obtained from rat liver microsome PCs, 19 (XXI) fractions were isolated. In some of them, several position isomers of the derivatives (XXI) with one and the same species composition were detected; consequently, isomerization of the derivatives (XXI) had taken place in the course of analysis. An additional feature of derivatives (XXI), thanks to which they will possibly assume a firm position in the HPLC of lipids, is pronounced absorption in the UV region of the spectrum [146-153].

Thus, various lipophilic derivatives of DAGs have been proposed for the LC analysis of the species composition of lipids. The most popular of them up to the present time have been the derivatives (XV) which can be used for e- and L-fractionation, and also for HPLC analysis. Such derivatives as (XIX) and (XXI) have begun to be used only in recent years and exclusively for L-separation by the RP HPLC method. Other hydrophobic derivatives of DAGs have not yet come into wide use in liquid chromatography [154-159].

* * *

The facts presented in the present review show that to obtain satisfactory results of the LC determination of the species compositions of lipids, the separation from one another of fractions characterized by different values of e or of L must be quantitative ($R \geqslant 1.5$). The oxidation of the samples during the performance of the whole experiment must also be brought to a minimum and the absence of foreign compounds interfering with the separation from them must be ensured. If all these conditions are satisfied, the error of the determination is usually small:

$$|e_c - e| < 0.1;\ |L_c - L| < 0.02;\ 0.8 \leqslant \bar{s}_e \leqslant 3\%;\ 0.05 \leqslant \bar{s}_L \leqslant 1\%.$$

By performing the corresponding calculation we have shown that in all cases when the values of s_e and s_L do not go beyond the upper limits of the ranges mentioned the proportion of main component in a fraction amounts to $\geqslant 96\%$.

In conclusion, it must be mentioned that at the present time Ag^+-TLC is being used most frequently for the e-fractionation of complex lipid mixtures. Another method for the e-separation of lipids – Ag^+-HPLC – has not yet found wide use, since for its use the initial concentration of silver ions in the HPLC column must be maintained throughout the whole analysis. As yet, the only means for achieving this aim conists in the use of a mobile phase containing Ag^+. If it is considered that for each fractionation a considerable volume of such a phase is required, it becomes obvious that the performance of the Ag^+-HPLC of polar glycerolipids is associated with great expense.

In recent years, the RP HPLC method has frequently been used for the separation of lipids. Furthermore, the L-fractionation of lipids on HPLC columns with 3-10 μm particles of silica gel containing grafted-on hydrocarbon chains has today almost completely displaced other LC methods for the L-separation of lipids. Nevertheless, with respect to the selectivity of L-separation of lipids, RP TLC on silica gel coated with a thin film of paraffinic hydrocarbons is superior to RP HPLC. However, RP TLC is difficult to automate, and therefore at the present time this method is little used for the analysis of the species compositions of lipids and their derivatives.

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AUTOCATALYTIC CONDENSATION OF 1,2-ORTHOESTERS OF SUGARS WITH 2,3-DIHYDROXY-1,4-NAPHTHOQUINONE (ISONAPHTHAZARIN)

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The autocatalytic glycosylation of isonaphthazarine and its derivatives with 1,2-orthoesters of D-glucose and maltose in chlorobenzene has been studied. It has been established that the ratio of mono- and bisglycoside forms depends on the acidity of the glycosylated hydroxy group and its steric accessibility. Free monoglycosides of 2,3-dihydroxy-1,4-naphthoquinone have been synthesized by deacetylation with sodium methoxide in methanol.

In a number of cases, the conversion of 1,4-naphthoquinones into acetylated glycoside derivatives leads to an enhancement of antitumoral [1], immunotropic [2], and antifungal [3] activity of the basic aglycons. There is information on the presence of O-glycosides of naphthoquinone in natural materials. Thus, among the products of the microbial transformation of a natural antitumoral naphthoquinone – lapachol– its 2-O-β-D-glycoside has been found [4]. The isolation from the roots of the African plant Sesamum angolese Welw. (Pedaliacae) of two new naphthoxirane derivatives and their glycosides, which possess antifungal and cytotoxic action, has been reported [5].

The glycosylation of hydroxynaphthoquinones is usually performed by the Koenigs–Knorr method [1, 2, 6]. We have proposed to use for this purpose the autocatalytic condensation of hydroxynaphthoquinone with sugar 1,2-orthoesters [7]. We later [8] reported the reaction of orthoesters of D-glucose and of maltose with isonaphthazarin.

The aim of the present work was a detailed study of the autocatalytic reaction of 1,2-orthoesters of D-glucose (I) and of maltose (II) with isonaphthazarin (III) and its derivatives (IV, V), and also of the possibility of the deacetylation of the acetylglycosides obtained. When the orthoesters (I) and (II) were condensed with the quinones (III-V) in boiling chlorobenzene, the acetylated glycosides (VI-XII) were obtained. The structures of these compounds were established by IR and ^1H and ^{13}C NMR spectroscopies. The presence in each case of an intense band of the valence vibrations of a carbonyl group in the 1650-1640 cm^{-1} region showed the p-quinoid structure of the aglycon. The ^{13}C spectra (Table 1) also agreed well with the suggested structures and with literature information [7]. For each of bisglycosides (VII) and (IX) five signals belonging to the carbon atoms of the aglycon were observed, which showed the existence of a plane of symmetry in their structures. For the same reason, coincidence of the signals of the carbon atoms of the carbohydrate radicals was observed. The β-configuration of the glycosidic bond in (VI-IX) was confirmed by the value of the chemical shift of the anomeric C-1' carbon atom (99-100 ppm) and the SSCC of

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