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ARTICLES

Comparative Determination of Fatty Acid Composition of Low-Molecular Components of Blood Plasma by Three Mass Spectrometry Techniques: the 'Old–New' Exercise in Lipidomics[1]

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Abstract—Fatty acid composition of lipid low-molecular components of human blood plasma was determined by the combination of three analytical techniques: gas chromatography mass spectrometry and electrospray high-resolution mass spectrometry/tandem-mass spectrometry with direct flow injection. More than two tens of volatile fatty acid methyl esters were identified in lipid extracts subjected to methanolysis. When direct injection electrospray mass spectrometry was employed, 21 lysophosphatidylcholines and 25 phosphatidylcholine small isomer groups were recognized. Fatty acid distributions over the analytical signal intensity in groups of methyl esters and lysophosphatidylcholines were demonstrated to be similar to each other and highly correlated. The fatty acid distribution for phosphatidylcholine was correlated to those to a lesser extent. At the same time, the analytical signal intensity of lysophosphatidylcholines and phosphatidylcholines was observed to be highly correlated to concentrations of these lipid compounds in blood plasma determined by the similar technique of direct injection mass spectrometry and available in the literature.

Keywords: mass spectrometry, metabolomics, lipidomics, blood plasma, fat acids, phospholipids, phosphatidylcholines

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INTRODUCTION

Progress in metabolomics [1] and its constituent part, lipidomics [2–5], leads to several scientific and practical aims. The basic scientific aim is to understand how life works, how living organisms function, and what the nature of vital functions is related to metabolism processes and metaboloms. For example, the research of a lipidom have been caused by the fundamental role of lipids as units in cell membranes and energy suppliers, their participation in signal transduction, cell-cell interaction, regulation of physiological functions, and so on [2–5].

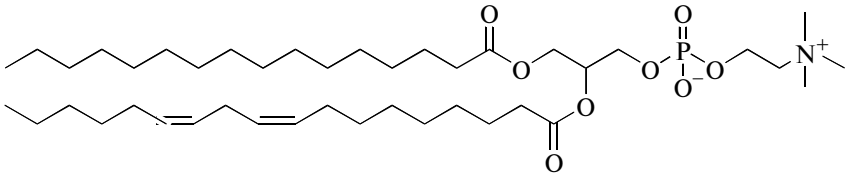
The practical goal of the research in metabolomics/lipidomics, which is the contribution to advances of medicine including clinical diagnostics, is equally important. A lipid content in blood and different biological matrices have been related to such diseases as atherosclerosis, cardiovascular ones, obesity, and metabolic syndrome [2–5]. In diagnostics, pathogenic bacteria have been widely identified according to characteristic fatty acids or chromatographic/mass chromatographic profiles of these compounds or their derivatives, particularly esters (see [6]).

The diagnostic value of fatty acids is not only expressed by those identification procedures. Residues of these compounds are contained in numerous classes and groups of lipids (Table 1). Therefore, the general or detailed fatty acid composition of biosamples is the important feature of lipidom and the marker of many diseases and states of living organisms. Potentialities in determining the composition as well as general advances of lipidomics depend on the progress in analytics, especially in such highly sensitive and selective techniques as mass spectrometry and its combination with chromatography. For many years, the fatty acid composition of biosamples has been fruitfully studied by means of gas chromatography mass spectrometry. In doing this, free and bound fatty acid have been derivatized to volatile compounds the content of which characterizes the sum of lipids.

Given the fact that intact lipids are usually nonvolatile compounds, the appearance of mass spectrometry techniques based on electrospray and laser ionization/desorption resulted in the current progress of lipidomics. That was also caused by a widely use of high resolution mass spectrometers and tandem mass spectrometers equipped with those ion sources. With these instruments, many lipid structures with certain

[1] The article was translated by the authors.

Table 1. The variety and some examples of lipids

Classification unit	Compounds	The number in the database [9]*
Class, subclass	Lipids	28663 (1861)
	—glycerophospholipids	6287 (789)
	—glycerophosphocholines	1282 (489)
	—phosphatidylcholines	860 (299)
Sum composition	Phosphatidylcholine(34:2) ≡ PC(34:2)	16 (16)
Isomer group	$C_{42}H_{80}NO_8P$	13 (10)
Detailed composition	Phosphatidylcholine (16:0/18:2) ≡ PC(16:0/18:2)	2 (2)
	Phosphatidylcholine (16:0/18:2(9Z,12Z)) ≡ PC(16:0/18:2(9Z,12Z))	2 (2)
Individual compound	 1-Palmitoyl-2-linoleoyl-*sn*-glycero-phosphatidylcholine	1 (1)

* The number of answers when searching, in parentheses: the number of detected and quantified compounds.

fatty acid residues before considered as only probable were actually detected.

Nevertheless, not all the challenges of the lipidomics progress were met. The detailed study of the biological role of lipids and related compounds evidently depends on how specifically their compositions in biosamples were determined. This analytical problem is far from being completely solved. Now only the part of known lipid compounds was both detected and quantified in biological matrices (Table 1). The reason is that such efficient techniques of biochemical analysis as high resolution mass spectrometry and tandem mass spectrometry just recently appeared. In addition, different analytical techniques including mass spectrometry ones were found out not to result in fully the same identification of lipids [7]. Therefore, it is very essential to use different analytical techniques in combination with each other. However, such studies have rarely performed [7, 8].

Commonly, results of in-depth study of biosample compositions may depend on not only general mass spectrometry techniques but also particular types and brands of instruments. From this it follows the goal of this research as the further study of intertechnique, interlaboratory, and interinstrument reproducibility of molecular composition data of lipids in biosamples by the example of the low-molecular fraction of human blood plasma. The research objectives were as follows.

—A comprehensive exploration of the fatty acid composition of lipids of blood plasma from which proteins were removed. For this purpose, the same sample preparation and the combination of three analytical techniques: (a) gas chromatography mass spectrometry, (b) high resolution electrospray mass spectrometry with the flow/syringe injection of solutions, and (c) corresponding tandem mass spectrometry technique, were used.

—Identification of major compounds expressing fatty acid composition of the lipid mixture and estimation of their quantitative relationships.

—Comparison of composition data acquired by (a) different techniques, (b) for different lipid classes, and (c) in different laboratories, i.e. between our different results and between those and the literature data.

It was intentionally decided to achieve these aims and objectives in a complicated manner, i.e. without the use of analytical standards.

Abbreviations and Symbols

ESI—electrospray ionization, FA—fatty acid(s), FAME—fatty acid methyl ester(s), FI—flow injection of liquids into mass spectrometer, FID—flame ionization detector, GC—gas chromatography, HRMS—high resolution mass spectrometry, LPC—lysophosphatidylcholine(s), MS—mass spectrometry, MS^2—tandem mass spectrometry, RI—retention index(ices), PC—phosphatidylcholine(s), TFA—trifluoroacetic acid, TLC—thin-layer chromatography, h—oxygen atom in oxidized phospholipid; A, %—relative area of chromatographic peak recorded in the

total ion current mode; A_{corr}, %—the same quantity with the correction for the response factor; I, %—relative intensity of mass peak; Δm, ppm—the difference between experimental and theoretical/formula mass; i.u.—RI unit.

EXPERIMENTAL

Chemicals and Samples

The following solvents, chemicals, and reagents were used: deionized water (conductivity ≤ 5 μ*S*/cm), acetonitrile (sort 0, Cryochrom, Russia), methanol (LC grade, Merck, Germany), hexane (sort 1, Cryochrom, Russia), methylene chloride (chemically pure grade, Component-Reaktiv, Russia), TFA (LC MS Ultra grade, Fluka, USA), *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Supelko, USA), magnesium (chip, sort MG, Lenreaktiv, USSR), iodine (crystalline, chemically pure grade), sodium hydroxide (chemically pure grade), sodium sulfate (anhydrous, Lenreaktiv, USSR), sodium chloride (>99.5%, Sigma, USA), sulfuric acid (concentrated, especially pure grade, Sigma-Tek, USA), phenolphthalein indicator (Moscow Alkaloid Factory, USSR).

Absolute methanol was prepared by the method [10] and saturated with hydrogen chloride up to 1 M concentration. Hydrogen chloride was prepared by the interaction of sulfuric acid and sodium chloride. Reaction was completed at the required saturation of methanol that was controlled by the titration with sodium hydroxide at the presence of phenolphthalein indicator.

Blood samples were provided by three volunteers.

Sample Preparation

Lipid extraction. The 5-mL blood samples were centrifuged at the rate of 3000 rpm for 10 min. Then 1 mL of plasma was separated, 2 mL of acetonitrile was added to remove proteins. The mixture was shaked for 15 min and centrifuged at the rate of 5000 rpm for 10 min. Liquid phase was decanted, mixed with 6 mL of methylene chloride, shaked for 10 min, and centrifuged at the rate of 3000 rpm for 10 min. The methylene chloride layer was separated and evaporated to dryness in a stream of nitrogen.

FAME preparation for GC–MS. The dry residue was dissolved in 1 mL of 1 M HCl solution in methanol and heated in a closed glass vial. Then 1 mL of water and 1 mL of hexane were added, the mixture was shaked for 3 min using the vortex mixer and was settled. The hexane layer was removed, and the extraction was repeated. The combined hexane layers were dried with sodium sulfate and were separated from the drying agent. Then the latter was washed with hexane, which was added to the extract. Hexane was evaporated in a stream of nitrogen.

To this dry residue, 50 μL of BSTFA silylating reagent was added, and the mixture was heated at 60°C for 5 min. The obtained mixture containing FAME and, in a lesser degree, different compounds (see below), was analyzed.

Extract solution for FI–ESI–MS. The dry residue following the extraction procedure with methylene chloride, was dissolved in 1 mL of the 4 : 1 mixture of acetonitrile and water, diluted by 10 times with 0.05% TFA, and analyzed.

INSTRUMENTS AND ANALYTICAL CONDITIONS

GC–MS

The experiments were performed on a QP2010 Plus gas chromatograph mass spectrometer (Shimadzu, Japan) equipped with HP Ultra 2 GC column (25 m × 0.2 mm × 0.25 μm, Agilent, USA). Injections (1 μL) were performed at 280°C in split mode (split ratio 1 : 15). The oven temperature was programmed from 50°C (3 min) to 290°C (13 min) at 10°C/min. The carrier gas was helium at the flow of 1 mL/min. The interface temperature was maintained at 280°C and the ion source was heated to 250°C. The m/z range was 35–550.

For everyone from three plasma samples, total ion current chromatograms, selected ion mass chromatograms, and mass spectra related to intensive chromatographic peaks, were acquired. Relative areas of chromatographic peaks (A, 100% for the principal peak) were used for quantitative estimations.

FI–ESI–MS

The analyses were performed on maXis 4G high resolution tandem mass spectrometer (Bruker, Germany). FI was at the flow of 3 μL/min. ESI was in the positive ion mode, the m/z range was 50–1500. There were standard conditions (as default settings) for ESI and acquisition of MS^1 spectra, the external mass calibration was used. The MS^2 conditions were as follows: the spectra were composed of 10–30 scans, the isolation width for precursor ion selection was ≥8 Da, collision energy was 35 and 40 eV.

IDENTIFICATION OF LIPIDS

GC–MS

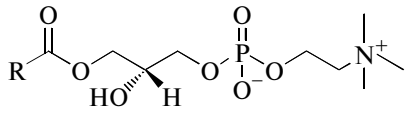
Volatile analytes were identified by their EI mass spectra using the NIST'08 mass spectra library. Identification versions providing the best similarity between experimental and reference spectra, were accepted. In doing so, we took into account, first, spectral match factors, estimated by the GCMS Solutions program and, second, the visual spectral similarity. In the case of positive identification, the latter was expressed in the same mass numbers of principal

peaks, including those of molecular ions, and the same order of their intensities.

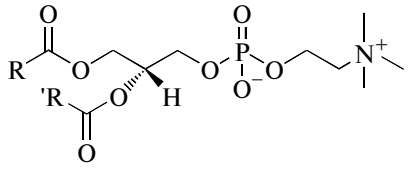
The reference RI [11] were also taken into consideration. They were obtained in different experimental conditions and therefore recalculated in this work by the correlation between those data and our retention times for compounds (FAME of saturated FA) unambiguously identified by their spectra. The difference between calculated and reference FAME RI in absolute value was as follows: monounsaturated FA, 5–12 i.u; FA with two, three, and four and more double bonds, 11–13, 1 (the only value for FAME of C20:3), 20–29 i.u., respectively. In cases of larger differences mainly observed for very low peaks, MS identification was considered as conditional and usually not taken into account.

FI–ESI–MS

Components of lipid extracts were identified by their ESI mass spectra in three stages. In the first stage (MS^1) molecular formulas were determined by corresponding accurate mass of $[M + H]^+$ ions. The candidate molecules were selected from lipidomics databases [9, 12] on the basis of the small difference of experimental and reference mass ($|\Delta m|$).



I, R = alkyl or alkenyl



II, R, R' = alkyl or alkenyl

The next stage of identification was performed by the MS^2 technique, resulting in the determination of lipid classes. Various classes of these bio compounds usually have different sets of characteristic fragment ions [12, 13] that was used for such group identification. It was proved that LPC (**I**) and PC (**II**) were predominantly detected (see below).

The third stage is the recognition of individual lipids or small groups of such compounds. It was taken into consideration that the same elemental composition of many lipids (see Table 1) usually corresponds to a group of individual compounds generated by varying FA residues. Those analytes were selected from databases [9, 12] and also proposed according to major FA determined by the GC-MS technique. For each candidate compound, MS^2 spectra were simulated on the base of principal fragmentation pathways of LPC (**I**) and PC (**II**) characterizing their FA [12, 13], i.e. the loss of acyl groups as corresponding FA (RCO_2H and $R'CO_2H$) and ketenes ([(R–H)=C=O] and [(R'–H)=C=O]).

Then the availability of such fragment ion peaks in the experimental MS^2 spectra were found out. The criterion used for identification of individual compound (or more properly, the sum of its position isomers) was not less than the double presence of one or several peaks of characteristic ions related to the loss of FA residues in the same or different MS^2 spectra. The latter are different in, first, the collision energy and, second, the precursor ion mass/formula. The second case should be explained.

Fragments of the same protonated molecules could be present in various MS^2 spectra differed in the nominal m/z value of the precursor ion because the isolation range setting was as wide as several Da (see above). Two or three initial $[M + H]^+$ precursor ions (principal isotope forms are discussed), with the mass difference of ±2 Da or ±4 Da between them, fall into the window at a similar selection probability. In this case, characteristic fragment ions of different compounds might be or not be of the same mass. The latter implied relatively reliable identification, the former meant that identification was just possible/ambiguous.

In the identification procedure, only fragment ions whose peak intensities were larger than the 0.1–0.2% threshold and corresponding $|\Delta m|$ differences did not exceed the certain value, were taken into consideration. The mass tolerance criterion was chosen from experimental MS^2 spectra of lipids identified as LPC. As these compounds contain the only FA residues, their mass spectra are rather simple and fragment ions are unambiguously recognized. The average $|\Delta m|$ value for fragment ion peaks was found to be (7 ± 6) Da, with 96% values not exceeding the 25 Da limit. It was the maximum mass deviation, which was chosen for identification of peaks as those of fragment ions of PC and LPC (see below) rather than noise or signals of different components of the samples.

COMPARISON OF MASS AND CHROMATOGRAPHIC PEAK INTENSITIES

In determination of the quantitative FA composition of plasma extracts, relative intensities of chromatographic and mass signals related to derivatives of the same FA (or isobaric FA groups), were compared. Intensity values were averaged over three samples. Interdependencies of these quantities obtained for different techniques and compound groups and correlations with data extracted from the literature sources were approximated by functions providing the best correlation coefficients (expressed as R^2). In doing so, additional calculations were made.

Combinatorial Probability

The correlation in the FA composition between FAME, LPC, and PC was explored by comparing chromatographic peaks (as relative areas of FAME ones in GC–MS) and/or mass signals (as relative intensities of $[M + H]^+$ ion peaks of LPC and PC in ESI–MS^1). The correlation between signals of FAME and LPC containing the only FA residues was directly determined.

The comparison of the PC peaks appeared to be more complicated because these compounds contain two FA residues, different or the same ones. For those, the comparison was made with the PC formation probability expressing the conditional concentration of different FA in samples under the analysis. The intensity of FAME (GC–MS) or LPC (ESI–MS^1) peaks was logically considered to be the measure of those concentrations. This method of calculation is illustrated here for the instance of the principal peak belonging to PC(34:2) in ESI–MS^1 spectra and the GC–MS data.

The PC(34:2) total formula covers individual compounds (more properly, isomer groups) of predominantly PC(16:0/18:2), PC(16:1/18:1), and PC(14:0/20:2). Different FA combinations cannot be considered because of a low concentration of corresponding FA. The formation probability of each constituent of the PC(34:2) mixture is the product of the presence probability of two corresponding FA, i.e. the product of relative intensities of their signals. In the case of PC(16:0/18:2), the principal constituent of the mixture, the probability is proportional to the product of $2 \times A_{16:0} \times A_{18:2}$, where $A_{16:0}$ and $A_{18:2}$ are the peak relative area of FAME of 16:0 and 18:2, respectively. The 2 coefficient is due to the existence of two position isomers: PC(16:0/18:2) and PC(18:2/16:0). For $A_{16:0}$ and $A_{18:2}$ being 100 and 49.4%, respectively, the probability under consideration is $2 \times 100\% \times 49.4\%$ or, in another expression, $2 \times 1.00 \times 0.494 \approx 0.99$. Probabilities for the isobaric/isomer groups of PC(16:1/18:1) and PC(14:0/20:2) are 0.055 and 0.034, respectively, and the total probability for the PC(34:2) sum is $0.99 + 0.055 + 0.034 \approx 1.08$. The similar calculations were made for other PC; obtained probabilities were normalized to the major total composition, i.e. PC(34:2).

For the correlation of mass peak intensities of PC and LPC, the similar calculation were made where LPC peak intensities in ESI–MS^1 spectra were used instead of chromatographic peak areas of corresponding FAME. For example, the formation probability of the PC(16:0/18:2) constituent is proportional to the product of $2 \times I_{LPC(16:0)} \times I_{LPC(18:2)}$, where $I_{LPC(16:0)}$ and $I_{LPC(18:2)}$ are peak relative intensities of LPC(16:0) and LPC(18:2), respectively.

Analogous combinatorial calculations were performed in the research [7].

Response Factors

Correlation of various types of mass spectra was established by comparing not only intensities of chromatographic and mass spectrometric signals but related quantities also expressing relative analyte amounts. There was the estimation of molar concentrations of FAME and PC performed here by introducing correction coefficients (as response factors), extracted from the literature or obtained by the treatment of the literature data.

In GC–MS, the response factor (equals to the ratio of the chromatographic peak area to the analyte mass concentration) is known for many FAME, with their values differing by at most a factor of 2 [14]. To estimate the relative molar concentration of one or another FAME in analyzed samples, the experimental chromatographic peak area was divided by the ratio of the response factor to corresponding molar mass. Response factors were not available for some compounds, and those were estimated according to the regularity of variations of reported values [14].

In the case of ESI, there are known PC responses which vary in a wide range, namely decrease by 2.5–5 times in the series of saturated FA when passing from PC(24:0) to PC(48:0) and increase by up to 40% in passing from saturated to unsaturated FA. The dependence on the molar concentration is also observed [15]. Response factor values calculated according to the data of that report and averaged over the concentration were used to recalculate relative intensities of PC mass peaks and to compare them to other quantities.

RESULTS AND DISCUSSION

Main compound groups detected in the analyzed samples and connected to the major analytical signals are FAME (derivatized lipids, GC–MS), LPC, and PC (ESI–MS). Corresponding mass spectra and chromatograms, identification results, and other obtained data are shown in Figs. 1–4 and given in Tables 2–4. The obtained results will be discussed next in the compound groups noted above.

Volatile Derivatives of Lipids

The identified FAME and corresponding relative intensities of chromatographic signals are given in Table 2; a typical chromatogram is shown in Fig. 1a. Identification of major peaks, with the only exception (C20:3, with the abnormal RI value), presents few problems. Reference mass spectra and RI of identified FAME of unsaturated FA were referred to, predominantly, geometric *Z*-isomers (*cis*-isomers) which prevail in nature and among the records in used databases [9, 12]. Chromatograms also revealed tens of very low peaks (A ≤ approximately 0.1%) superposed to the major peaks and therefore hardly identified.

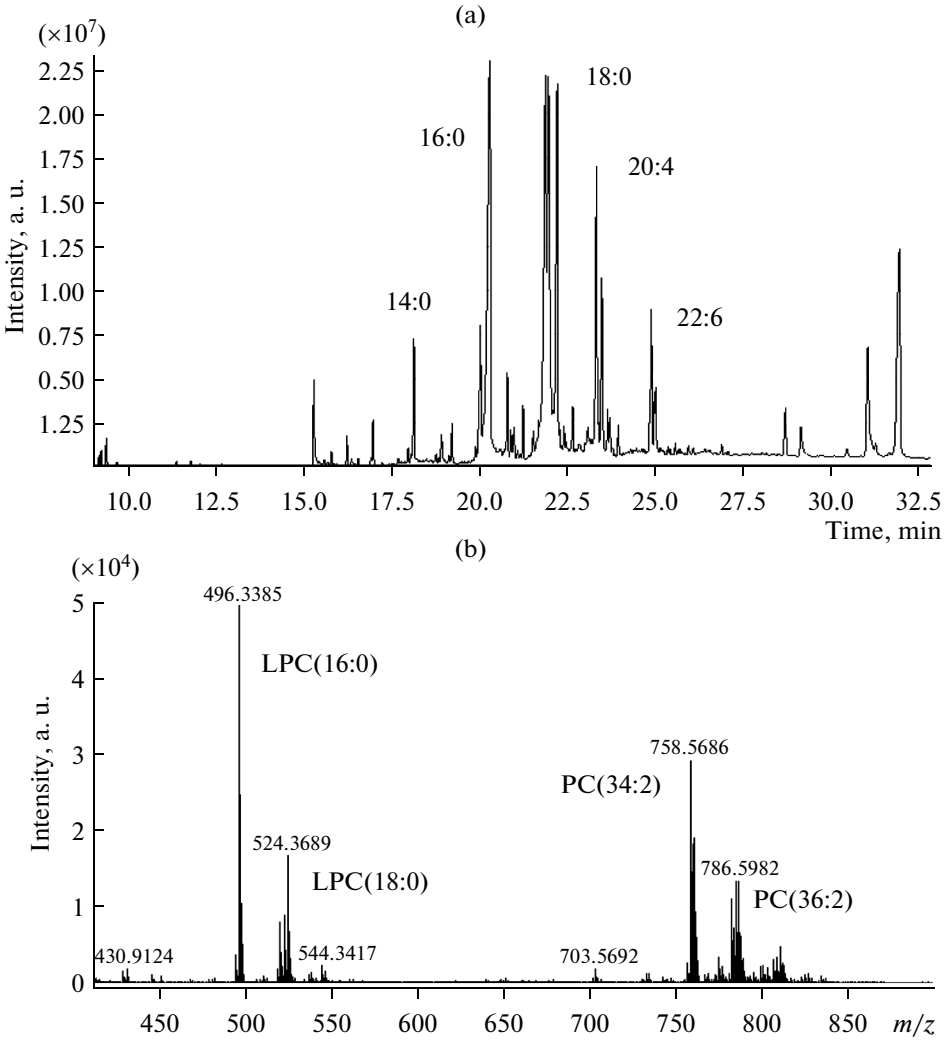


Fig. 1. (a) Chromatogram (total ion current, GC–MS) of the derivatized lipid extract, (b) ESI–MS^1 spectrum of the lipid extract. Specified are some major FAME, LPC, and PC.

It is common to consider that the area of chromatographic signals (Table 2) or that corrected for response factors is proportional to the FAME concentration in analyzed mixtures. In any case data of Table 2 semi quantitatively correspond to the total content of FA in blood plasma reported in the literature [7,16] (Fig. 2a). We note that here the introduction of corrections to relative area values slightly increased correlation coefficients (R^2 are larger by 0.04–0.06). In comparison with the literature data for individual compounds obtained by the GC–FID technique, our results were higher for FAME of C18:0, C20:4, C22:6 and lower for those of mainly C18:1 and C18:2.

We also note that lipids are very often extracted from blood plasma or serum using the mixture of methanol and chloroform (the Folsh solution), e.g., see [3, 7]. Recovery of some lipid groups by this solution may be not the same as that by methylene chloride in our study. To some extent, it may be resulted in, e.g., imperfect data correlation shown in Fig. 2a. Nevertheless, even when the extraction is not full, methylene chloride and chloroform as analogous solvents provide a similar recovery of nonpolar compounds from the same group, LPC or PC. Therefore when signal relative intensities of compounds with different FA from the same lipid group, are compared with corresponding literature data (see below), non-identity of sample preparation procedures can be disregarded.

Lysophosphatidylcholines

Recognition results of these analytes are given in Table 3 and Fig. 1b. From 21 compounds, 12 LPC were identified by the criterion: $|\Delta m| < 5$ ppm. All peaks having the larger mass deviation were of relatively low intensity. Herewith they superposed to other peaks of various origins that contributed to the mass

measurement error. Identification of those LPC is certain because the vast majority of them were detected in the same matrix (blood [9]).

Major LPC were also characterized by MS^2 spectra (Table 3, Fig. 3a). The spectra contained fragment ion peaks which characterized this lipid class as a whole and also certain individual compounds (the fragmentation pathways, see [12, 13]). The former were fragments with *m/z* 104 ($HO(CH_2)_2N^+(CH_3)_3$), 184 ($H_2PO_4(CH_2)_2N^+(CH_3)_3$), 240 ([M(**I**) + H – $RCO_2H]^+$), and 258 ([M(**I**) + H – (R–H)=CO]$^+$). Individual LPC were characterized by the ions of $[M + H]^+$, [M + H – H_2O]+ and [M + H – HPO_4^- $(CH_2)_2N^+(CH_3)_3]^+$ and the lost neutral species of RCO_2H and (R–H)=CO. With that every MS^2 spectrum usually contained fragment peaks of two or three precursor ions having mass which differed by 2 or 4 Da.

We also note that obtained spectra were not capable of distinguishing individual position LPC isomers (the position of acyl group in the glycerol moiety and the localization of double bonds in residues of unsaturated acids); determination of geometric isomers is proved even more complicated. In this case, one should be guided by the common idea of preferred isomers: 1-acylglicerides and most abundant FA identified here, see Table 2.

The relative intensity of LPC peaks correlates very well with the literature data on the content of these compounds in blood (Fig. 2b). The correlation with the relative area of FAME peaks (data of GC–MS, see above) is also strong (Fig. 2c). Corrections to chromatographic peak areas, which are response factors, do not improve the correlation.

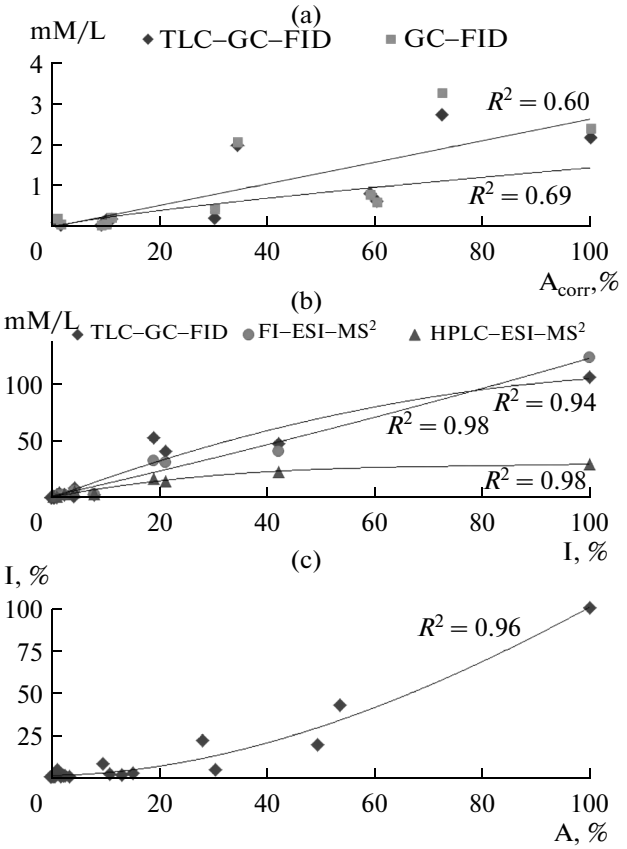


Fig. 2. (a) Correlation of the relative area (A_{corr}, corrected for different molar response factors) of chromatographic signals of various FAME and their concentration in blood plasma according to the literature data obtained by GC–FID techniques [7, 16] (in the research [7] lipids were prefractionated into groups by the TLC technique). (b) Correlation of the relative intensity of LPC mass peaks in ESI–MS^1 spectra and their concentration in blood plasma according to the literature data obtained by techniques of TLC–GC–FID and FI–ESI–MS^2 [7] and HPLC–ESI–MS^2 [3]. (c) Correlation of the relative intensity of LPC mass peaks in ESI–MS^1 spectra and the relative area of chromatographic signals of corresponding FAME (GC–MS). The approximation by the linear, power, or polynomial (quadratic expression, cubic quaternion) function.

Phosphatidylcholines

Identified PC are presented in Table 4. As in the case of LPC, most (two thirds) molecular formulas were identified by the criterion: $|\Delta m| < 5$ ppm. Peaks having the larger mass deviation were mainly of relatively low intensity. Errors in mass measurement were probably due to a superposition of low peaks of various origins. Identification of PC sum formulas is certain, as in the case of LPC, because, first, most of them were detected earlier in this matrix (blood, see Table 4) and, second, interpretation results of MS^1 spectra were confirmed by MS^2 spectra.

The latter determine the variety of detected individual compounds (Table 4, Fig. 3b). As in the case of LPC, one could observe fragment ion peaks typical for this lipid class and characteristic ion signals of individual compounds (their mass spectra, see [12, 13]). The first were fragments with *m/z* 184, $H_2PO_4(CH_2)_2N^+(CH_3)_3$. Individual PC were characterized by the fragment ions formed by the loss of the same neutral species ([M + H – $N(CH_3)_3]^+$ and [M + H – HPO_4^- $(CH_2)_2N^+(CH_3)_3]^+$) and also by two pairs of fragments following the loss of FA and corresponding ketenes: [M(**I**) + H – $RCO_2H]^+$ and [M(**I**) + H – (R–H)=CO]$^+$, [M(**II**) + H–R'$CO_2H]^+$ and [M(**II**) + H – (R'–H)=CO]$^+$. As was noted above, the presence of fragments of two or three precursor ions having mass differed by 2 or 4 Da in every MS^2 spectrum, complicated the spectral interpretation. However, some fragments were characteristic that provided reliable identification of approximately 20% individual PC variants. Literature data on the PC composition in biosamples have usually been less detailed: sum compositions were mainly available in publications. One may point out the reports [23, 24] as exemptions.

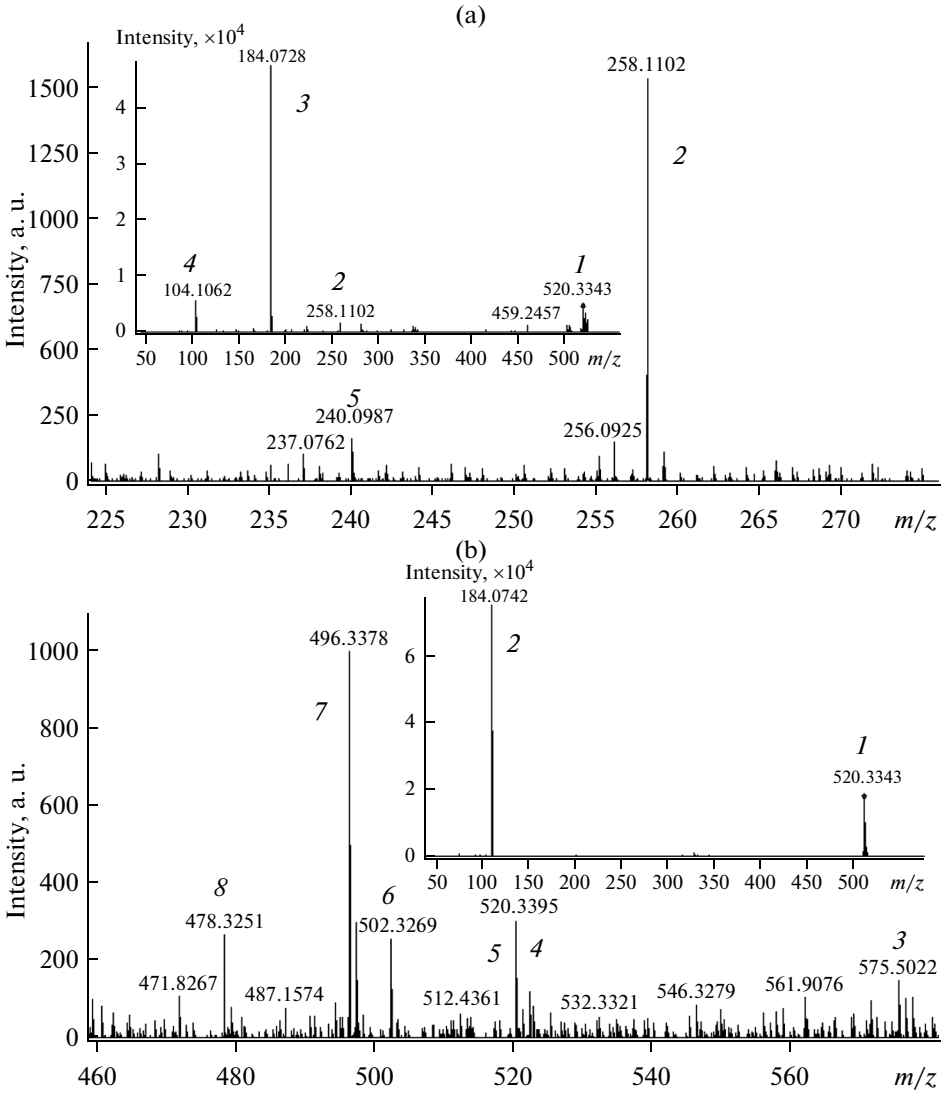


Fig. 3. Partial and total (inserted) MS^2 spectra of two phospholipids. (a) LPC(18:2), precursor ion with *m/z* 520 (*1*). Fragment ions: *2*—$[M + H–C_{16}H_{29}CH{=}CO]^+$, *3*—$H_2PO_4(CH_2)_2N^+(CH_3)_3$, *4*—$HO(CH_2)_2N^+(CH_3)_3$, *5*—$[M + H–C_{17}H_{31}CO_2H]^+$. (b) PC(16:0/18:2), precursor ion with *m/z* 758 (*1*). Fragment ions: *2*—*m/z* 184 ($H_2PO_4(CH_2)_2N^+(CH_3)_3$), *3*—575 ([M+H—$HPO_4^-$ $(CH_2)_2N^+(CH_3)_3$]), *5*—520 $[M+H—C_{14}H_{29}CH{=}CO]^+$, *6*—502 $[M+H—C_{15}H_{31}CO_2H]^+$, *7*—496 $[M + H–C_{16}H_{29}CH{=}CO]^+$, *8*—478 $[M+H—C_{17}H_{31}CO_2H]^+$. There is also the small peak with *m/z* 522 (*4*, near the peak *5*), which may be the fragment of the isomer PC(16:1/18:1) or the heavier PC(16:0/18:1).

Also as in the LPC group, MS^2 spectra did not provide direct evidences on the position isomerism. That was determined by different data, e.g. on the basis of preferable FA isomers (GC–MS, Table 2). As for the position of different FA residues bonded to the glycerol moiety, there may be both isomers, and for the pair of saturated and unsaturated FA, the 1st (R in (**II**)) and 2nd (R' in (**II**)) position, respectively, are commonly specified [7].

The $[M + H]^+$ peak intensity of different PC (as sum compositions, MS^1) and corresponding signals of FAME (GC–MS) or LPC (ESI–MC^1) cannot be directly compared, because the compound classes contain not the same number of acyl groups (two and one, respectively). Therefore, it is more meaningful to compare PC peak intensities to corresponding combinatorial probabilities derived from the distribution of monoacyl compounds with different FA residues over their signal intensities (see above).

The correlation with the GC–MS data was noticeable and not strong (Fig. 4a). Replacing the signal intensity with the relative molar amount of FAME and PC affected slightly on the correlation (R^2 decreased from 0.58 to 0.55). The mismatch of the two data series

is mainly due to the low content (I < 3%) of PC(32:0), probably, the palmitic acid derivative of PC(16:0/16:0). With that, FAME and LPC of this FA provided the principal signal (I = 100%). Removing this point significantly improves the correlation ($R^2 = 0.89$, Fig. 4a).

The similar trends is observed for PC when comparing with LPC peak intensities but correlation was less strong ($R^2 = 0.79$ without PC(32:0), Fig. 4b). These data lead to the general conclusion that the FA distribution in PC and other lipid groups are not the same in many respects.

It should be noted that in the case of two from three studied samples the relative intensity of the principal PC peak (*m/z* 758) was lower than that from the LPC group (*m/z* 496), see Fig. 1b. The total ion current of PC and LPC are generally comparable. Meanwhile, the LPC content in blood is much lower than that of PC [3]. Apparently, this imbalance can be explained by very high sensitivity of ESI MS in relation to LPC.

The correspondence of the PC peak intensities in MS^1 spectra to the literature data on the content in blood, is ambiguous (Fig. 4c). On the one hand, the correlation is not high for the blood analysis results obtained by the HPLC–ESI–MS^2 technique [3]. On the other hand, as in the case of LPC, the strong correlation ($R^2 = 0.98$, Fig. 4c, and 0.97, recalculation for molarities) was observed for the analogous technique (FI–ESI–MS^2). Apparently, such correlation is due to both the similar composition of analyzed samples and the evident similarity of used techniques (there was no chromatographic separation, the syringe introduction of samples was applied), although that in the research [7] was implemented on a triple quadrupole platform with the use of multiple reaction monitoring for precursor ion fragmentation.

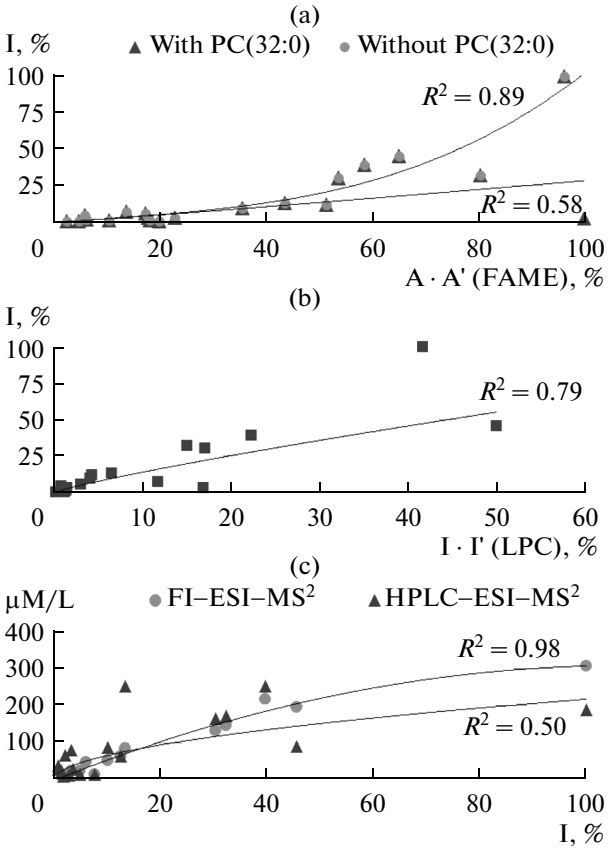


Fig. 4. Correlation of the relative intensity of PC mass peaks in ESI–MS^1 spectra and (a) the product of relative chromatographic peak areas of corresponding FAME (GC–MS), (b) the product of relative mass peak intensities of corresponding LPC (ESI–MS^1), (c) the PC concentration in blood plasma according to the literature data obtained by techniques of FI–ESI–MS^2 [7] and HPLC–ESI–MS^2 [3].

Oxidized Glycerophospholipids

Three groups of signals in the MS^1 spectra of LPC (Table 3) and PC (Table 4) belong to compound ions whose molecular formulas contain one oxygen atom more than other analyte formulas of these groups. Corresponding MS^2 spectra are similar to those of LPC (**I**) and PC (**II**), at that the neural species RCO_2H and [(R–H)=CO] or $R'CO_2H$ and [(R'–H)=CO] lost from $[M + H]^+$ precursor ions contain the extra oxygen atom in hydrocarbon chains of unsaturated FA. By analogy with the results of reports [18–22], we assigned peaks in MS^1 spectra under discussion and corresponding MS^2 spectra to oxy derivatives of the phospholipids. In those, hydroxyl group is bonded to one from carbon atoms of hydrocarbon groups of R in LPC (**I**) (two compounds, Table 3) and R/R' in PC (**II**) (five items, Table 4).

The additional evidences for identification of just these compounds may be that there were signals which, basing on the selected recognition criteria, could be assigned to fragment ions typical for the breakdown of oxidized FA chains (with the molecule loss of C_6H_{12}, $C_6H_{12}O$, C_9H_{16}, $C_9H_{16}O$, $C_{12}H_{20}$, $C_{15}H_{24}$, $C_{15}H_{24}O$) [18–20]. However, corresponding peaks intensities were low (as a rule, $\ll 1\%$), and not all from them could be differed from noises (spectral spikes).

It should be noted that there is a few information in respect to oxidized LPC in the literature. Possibly, their identification in other studies was prevented by the presence of PC having the same molecular formula (Table 3, the footnote). We also note that FAME of oxy FA were not detected here that implies the absence of oxy derivatives in the samples under analysis [25]. Derivatives of unsaturated FA are known to be susceptible to easy oxidation [18–22]. Thus, it is possible that the formation of oxidized PC and LPC occurred when handling samples before acquiring mass spectra or in the mass spectrometer itself.

Table 2. FAME identification results and FAME chromatographic signal areas

FA	A, %
C12:0	0.5–1.7
C13:0	2.7–3.6
C14:0*	8.5–13
C15:0	2.1–3.2
C16:1 (9)**	4.3–19
C16:0*	100
C17:0*	2.9–5.5
C18:4***	0.1–0.2
C18:3 (6,9,12)****	1.0–2.9
C18:2 (9,12)	24–77
C18:1 (9)	21–43
C18:0	51–56
C19:0	0.6–1.3
C20:5 (5,8,11,14,17)	0.2–0.6
C20:4 (5,8,11,14)	26–35
C20:3 (8,11,14)	12–19
C20:2 (11,14)	1.6–3.0
C20:1 (11)	1.0–2.8
C20:0	1.3–2.6
C22:6 (4,7,10,13,16,19)	12–14
C22:5 (7,10,13,16,19)	2.8–4.9
C22:4 (7,10,13,16)	1.9–3.9
C22:1 (13)	0.3–0.6
C22:0	0.6–0.9
C23:0	0.04–0.4
C24:1 (15)	0.6–0.8
C24:0	0.3–0.4

* Including the minor contribution of the isomer containing a branched alkyl group.

** Including the minor isomer C16:1(11).

*** The mixture of two isomers available in comparable amounts.

**** Conditional identification.

Other Compound Groups

We briefly note that major representatives of other lipid groups were detected in the samples. They are the volatile derivatives of cholesterol and, to lesser degree, some other sterols (the GC–MS technique). According to ESI–MS^1, the lipid mixture contains phosphatidylethanolamines [26] and possibly other phospholipid groups (relative peak intensities do not exceed a few percent).

CONCLUSIONS

The principal research results, which were obtained by means of the combination of three mass spectrometry techniques, can be divided into two parts:

—identification of FA contained in the total lipid composition and of the representatives of two classes of phospholipids: PC and LPC;

—the intensity correlation of mass and chromatographic signals of representatives of different lipid classes containing the same FA residues (or the same isomer/isobaric FA groups).

Identification of more than two tens of major FAME presented a few special problems due to the availability of rather characteristic reference EI mass spectra and also reference RI. Identification of minor analytes from this group predictably proved to be more challenging that was caused by the superposition of their peaks with intense signals of other analytes in chromatogram, by similarity to other mass spectra, and by the absence of reliable RI values. In this regard, a diagnostic value of FA (see [6]) at their low content may be not high.

Identification of LPC and PC was complicated here by the fact that used express MS techniques related to the fast sample injection were not selective enough due to the absence of chromatography and relatively wide mass window for the selection of precursor ions in MS^2. Nevertheless, the sum composition of LPC and PC, small groups of LPC position isomers, and 1/5 such groups for PC, were eventually determined. The results of GC–MS analysis and the reference data further clarified identification of individual analytes.

The distributions of FAME formed from various lipid classes and LPC over the amount of different FAs are similar to each other that provided their strong correlation. That means that principal FA and relationships of their concentrations are the same in these analyte groups. It also provides a potentiality of cross-interpretation of different data sets, e.g. information acquisition on the FA structure from EI–MS spectra, and of mutual estimation of quantitative content of certain lipids. The distribution of FA in triglycerides, PC, shows lower correlation with results obtained for FAME and LPC at the indirect data comparison that is mainly due to the low concentration of PC(32:0),

Table 3. Identification results of glycerophospholipids with molecular mass of 450–600 Da*. Mass measurement accuracy, relative peak intensity

m/z	MS[1]			Individual compounds
	formula	$\|\Delta m\|$, ppm	I, %	
468	$C_{22}H_{46}NO_7P$	1.9	0.7–1.8	LPC(14:0)
494	$C_{24}H_{48}NO_7P$	1.2	6.1–8.4	LPC(16:1)
496	$C_{24}H_{50}NO_7P$	2.1	73–100	LPC(16:0)
518	$C_{26}H_{48}NO_7P$	5.5	2.4–5.5	LPC(18:3)
520	$C_{26}H_{50}NO_7P$	0.1	17–18	LPC(18:2)
522	$C_{26}H_{52}NO_7P$	2.6	15–25	LPC(18:1)
524	$C_{26}H_{54}NO_7P$	3.4	26–56	LPC(18:0)
536	$C_{26}H_{50}NO_8P$ **,***	9.5	2.4–3.2	LPC(h18:2)
538	$C_{26}H_{52}NO_8P$ ***,****	7.8	3.2–3.5	LPC(h18:1)
542	$C_{28}H_{48}NO_7P$	13	0.6–1.9	LPC(20:5)
544	$C_{28}H_{50}NO_7P$	6.4	3.5–4.8	LPC(20:4)
546	$C_{28}H_{52}NO_7P$	6.6	2.2–3.5	LPC(20:3)
548	$C_{28}H_{54}NO_7P$	2.3	≤1.2	LPC(20:2)
550	$C_{28}H_{56}NO_7P$	4.7	0.7–1.9	LPC(20:1)
568	$C_{30}H_{50}NO_7P$	15	0.9–2.0	LPC(22:6)
570	$C_{30}H_{52}NO_7P$	6.1	0.5–0.9	LPC(22:5)
572	$C_{30}H_{54}NO_7P$	4.7	0.3–1.5	LPC(22:4)
574	$C_{30}H_{56}NO_7P$ ***,*****	7.1	traces	LPC(22:3)
576	$C_{30}H_{58}NO_7P$	2.2	0.3–1.3	LPC(22:2)
578	$C_{30}H_{60}NO_7P$	3.3	traces	LPC(22:1)
580	$C_{30}H_{62}NO_7P$	2.6	traces	LPC(22:0)

* Here, the *m/z* values are rounded. The underlined compounds were characterized by MS^2 spectra. All lipids, with the exemption of formulas marked with footnote***, are contained in databases [9, 12] and were detected in blood [9].

** The contribution of PC(2:0/16:1) was observed.

*** Not available in databases [9, 12].

**** The contribution of PC(2:0/16:0) was observed.

***** Detected, e.g. in the work [17].

Table 4. Identification results of glycerophospholipids with molecular mass of more 700 Da*. Mass measurement accuracy, peak intensity

m/z	MS[1]			MS[1] + MS[2]*	MS[2], possible variants**
	formula	$\|\Delta m\|$, ppm	*I*, %		
730	$C_{40}H_{76}NO_8P$	7.2	1.0–1.5	PC(32:2)	
732	$C_{40}H_{78}NO_8P$	7.1	2.1–3.3	PC(32:1)	
734	$C_{40}H_{80}NO_8P$ ***	5.2	1.5–2.5	PC(32:0)	
756	$C_{42}H_{78}NO_8P$	2.5	5.6–6.2	PC(34:3)	PC(16:0/18:3), PC(16:1/18:2)
758	$C_{42}H_{80}NO_8P$	0.7	60–100	PC(34:2)	PC(16:0/18:2), PC(16:1/18:1)
760	$C_{42}H_{82}NO_8P$	4.4	29–39	PC(34:1)	PC(16:0/18:1)
774	$C_{42}H_{80}NO_9P$	0.7	7.8–10	PC(h34:2)	PC(16:0/h18:2), PC(16:1/h18:1)
776	$C_{42}H_{82}NO_9P$	1.2	4.6–6.4	PC(h34:1)	PC(h16:1/18:0), PC(16:0/h18:1)
780	$C_{44}H_{78}NO_8P$	4.8	3.0–4.5	PC(36:5)	PC(16:0/20:5), PC(18:2/18:3), PC(16:1/20:4)
782	$C_{44}H_{80}NO_8P$	0.8	23–30	PC(36:4)	PC(16:0/20:4), PC(18:0/18:4), PC(18:1/18:3), PC(16:1/20:3), PC(18:2/18:2)
784	$C_{44}H_{82}NO_8P$	3.1	21–27	PC(36:3)	PC(16:0/20:3), PC(18:1/18:2)
786	$C_{44}H_{84}NO_8P$	2.5	29–33	PC(36:2)	PC(18:0/18:2), PC(16:0/20:2), PC(18:1/18:1)
798	$C_{44}H_{80}NO_9P$	3.6	5.0–6.0	PC(h36:4)	PC(16:0/h20:4), PC(h16:1/20:3), PC(h18:2/18:2), PC(h18:1/18:3), PC(18:1/h18:3), PC(16:1/h20:3)
800	$C_{44}H_{82}NO_9P$	1.1	4.7–5.5	PC(h36:3)	PC(16:0/ h20:3), PC(h18:1/18:2), PC(18:1/ h18:2), PC(16:1/ h20:2)
802	$C_{44}H_{84}NO_9P$	3.6	4.2–5.3	PC(h36:2)	PC(18:0/h18:2), PC(16:1/h20:1), PC(16:0/ h20:2), PC(18:1/h18:1)
804	$C_{46}H_{78}NO_8P$	3.1	≤1.5	PC(38:7)	
806	$C_{46}H_{80}NO_8P$	0.2	7.0–13	PC(38:6)	PC(16:0/22:6),PC(18:2/20:4), PC(18:1/20:5)
808	$C_{46}H_{82}NO_8P$	4.3	7.1–8.9	PC(38:5)	PC(18:0/20:5), PC(16:0/22:5), PC(18:1/20:4), PC(18:3/20:2), PC(18:4/20:1)
810	$C_{46}H_{84}NO_8P$	3.9	9.7–11	PC(38:4)	PC(18:0/20:4), PC(16:0/22:4), PC(18:3/20:1)
812	$C_{46}H_{86}NO_8P$	8.7	4.2–5.2	PC(38:3)	
814	$C_{46}H_{88}NO_8P$ ***	7.8	≤1.8	PC(38:2)	
830	$C_{48}H_{80}NO_8P$	3.3	0.5–0.8	PC(40:8)	
832	$C_{48}H_{82}NO_8P$	7.8	0.8–1.3	PC(40:7)	
834	$C_{48}H_{84}NO_8P$	5.6	2.1–3.3	PC(40:6)	
836	$C_{48}H_{86}NO_8P$ ***	12	1.1–1.9	PC(40:5)	

* Here, the *m/z* values are rounded. All sum compositions and listed variants of individual compounds, with the exemption of FA oxy derivatives, are contained in the databases [9]. Most sum compositions were detected in blood plasma [7].

** The underlined compositions were rather reliably identified by characteristic ions.

*** Substantial signal distortion due to its superposition with the isotope peak of PC ion having *m/z* lower by 2 Da.

the derivative of palmitic acid. This is the formal deviation from the similar statistical distribution in different lipid classes over FA residues.

On the other hand, the surprisingly strong correlation is observed between the $[M + H]^+$ peak intensity of analytes from LPC and PC groups and the literature data on their content in the blood plasma obtained by the FI–ESI–MS^2 technique with the use of the different instrumental platform. This may be due to the similar chemical composition of blood samples and the similarity in analytical methodology in the compared cases.

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REFERENCES

1. Metabolomics. http://en.wikipedia.org/wiki/Metabolomics.Accessed December 10, 2014.
2. Hu, C., Van der Heijden, R., Wang, M., et al., *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.,* 2009, vol. 877, no. 26, p. 2836.
3. Quehenberger, O., Armando, A.M., Brown, A.H., et al., *J. Lipid Res.,* 2010, vol. 51, no. 11, p. 3299.
4. *Lipidomics: Technologies and Applications*, Ekroos, K., Ed., Weinheim: Wiley, 2013.
5. Loizides-Mangold, U., *FEBS J.*, 2013, vol. 280, no. 12, p. 2817.
6. Mil'man, B.L. and Zhurkovich, I.K., *J. Anal. Chem.*, 2015, vol. 70, no. 10, p. 1179.
7. Psychogios, N., Hau, D.D., Peng, J., et al., *PloS One*, 2011, vol. 6, no. 2, e16957.
8. Simón-Manso, Y., Lowenthal, M.S., Kilpatrick, L.E., et al., *Anal. Chem.*, 2103, vol. 85, p. 2013.
9. *The Human Metabolome Database (HMDB)*. http://www.hmdb.ca. Accessed December 10, 2014.
10. *Organikum. Organisch-chemisches Grundpraktikum*, Berlin: VEB Deutsche Verlag der Wissenschaften, 1976.
11. Tret'yakov, K.V., *Retention Data*, NIST Mass Spectrometry Data Center, 2008. http://webbook.nist.gov/cgi/cbook.cgi?Source=2008TRE%2320876M&Units=SI&Mask=2000. Accessed December 10, 2014.
12. *LIPID MAPS Lipidomics Gateway.* http://www.lipidmaps.org. Accessed December 10, 2014.
13. LipidBlast. http://fiehnlab.ucdavis.edu/projects/LipidBlast. Accessed December 10, 2014.
14. Dodds, E.D., McCoy, M.R., Rea, L.D., et al., *Lipids*, 2005, vol. 40, no. 4, p. 419.
15. Koivusalo, M., Haimi, P., Heikinheimo, L., et al., *J. Lipid Res.*, 2001, vol. 42, no. 4, p. 663.
16. Kuriki, K., Nagaya, T., Tokudome, Y., et al., *J. Nutr.,* 2003, vol. 133, no. 11, p. 3643.
17. Li, Y., Song, X., Zhao, X., et al., *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.,* 2014, vol. 966, p. 147.
18. Reis, A., Domingues, M.R.M., Amado, F.M.L., et al., *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.,* 2007, vol. 855, no. 2, p. 186.
19. Domingues, M.R.M., Reis, A., and Domingues, P., *Chem. Phys. Lipids*, 2008, vol. 156, no. 1, p. 1.
20. Uchikata, T., Matsubara, A., Nishiumi, S., et al., *J. Chromatogr. A,* 2012, vol. 1250, p. 205.
21. Ding, J., Sorensen, C.M., Jaitly, N., et al., *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.,* 2008, vol. 871, no. 2, p. 243.
22. Reis, A., Domingues, P., Ferrer-Correia, A.J.V., and Domingues, M.R.M., *J. Mass Spectrom.*, 2004, vol. 39, no. 12, p. 1513.
23. Taguchi, R. and Ishikawa, M., *J. Chromatogr. A.*, 2010, vol. 1217, no. 25, p. 4229.
24. Bang, D.Y., Byeon, S.K., and Moon, M.H., *J. Chromatogr. A.*, 2014, vol. 1331, p. 19.
25. *Methyl Esters of Fatty Acids - Archive of Mass Spectra*. http://lipidlibrary.aocs.org/ms/arch_me/index.htm. Accessed December 10, 2014.
26. Phosphatidylethanolamine. http://en.wikipedia.org/wiki/Phosphatidylethanolamine. Accessed December 10, 2014.