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Effects of fatty acyl chain length, double-bond number and matrix on phosphatidylcholine responses in matrix-assisted laser desorption/ionization on an Orbitrap mass spectrometer

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RATIONALE: Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is used for the fast qualitative and quantitative analysis of phosphatidylcholines (PC). Fatty acyl chain lengths and the number of double bonds (DB) affect relative responses of PC; hence the determination of correction factors of individual PC is important for the accurate quantitation. The signal intensity in MALDI-MS strongly depends on the matrix; therefore, the following matrices typically used in lipidomics are studied in the present work: 2,5-dihydroxybenzoic acid (DHB), 1,5-diaminonaphthalene (DAN) and 9-aminoacridine (9AA).
METHODS: Series of PC with various fatty acyl chain lengths are synthesized for this study. PC concentrations over two orders of magnitude are studied with MALDI-MS. These experiments provide sets of calibration curves for each of the synthesized PC and the further analysis of parameters of calibration curves is performed.
RESULTS: Correction factors for PC decrease with increasing fatty acyl chain length for all matrices. These dependences are steeper for unsaturated PC than for saturated ones. MALDI matrices also have a significant effect on this dependence. The weakest dependence on fatty acyl chain length is found for saturated PC in 9AA. In the case of the other matrices, the effect of fatty acyl chain length on the response is essential for both saturated and unsaturated PC. Calibration curves and parameters of calibration curves for both saturated and monounsaturated PC are fitted by a linear function with regression coefficients decreasing in the order 9AA > DAN > DHB.
CONCLUSIONS: Differences in relative responses for PC in MALDI-MS measurements must be taken into account for accurate quantitation. Parameters of calibration curves can be used for the determination of PC concentrations using a single internal standard (IS). This method gives good results for the 9AA matrix, but the reproducibility of measurements for the DHB and DAN matrices is lower and the method can be used for a rough estimation only. These matrices are less convenient for the quantitation of PC. Copyright © 2015 John Wiley & Sons, Ltd.

Phosphatidylcholines (PC) are important and highly abundant lipids in biological samples. They play essential roles as constituents of biomembranes,[1] regulators for various biological processes such as homeostasis, metabolism, cell signaling and organ physiology.[2] These functions of PC are associated with several diseases connected with deviations from the normal lipid expression.[3–6] Quantitative lipidomic analysis can be used for disease biomarker discoveries. Electrospray ionization mass spectrometry (ESI-MS) is commonly used in lipidomic studies.[7–11] Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is also widely embedded in such investigations due to its high sensitivity, high speed of analysis, robustness and easy sample preparation.[12,13] MALDI-MS is mainly used in qualitative analysis, but MALDI-MS applications in quantitative analysis have been reported as well.[14–16]

A key problem for the accurate quantitation of lipids is different MS responses of various lipid classes and species. This means that the molar ratio of some lipid species is not equal to corresponding MS peak intensities ratio, i.e., different lipids may have different detection efficiency.[17–19] Several factors influence the overall detection efficiency, such as the ionization efficiency, the fragmentation efficiency, the efficiency of transfer to the gas phase, etc. Some of these factors are dependent on the molecular structure, while others are dependent on the composition and properties of a sample in general. PC consist of polar head groups containing choline and phosphoric acid residues, the glycerol backbone and fatty acyl, alkyl or alkenyl hydrophobic chains attached to *sn*-1 and *sn*-2 positions on the glycerol backbone. MS studies of lipids showed that PC are preferentially ionized and detected as protonated molecules or adducts with alkali metal ions.[13,20–23] Mainly the type of polar head group determines differences in the detection efficiency between classes of phospholipids. These differences could even result in the signal suppression of one lipid class by another

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one.[19] Fatty acyl chains determine differences in detection efficiency between lipid species within one class. The effect of fatty acyls is not as strong as for the polar head group, but it is not negligible. Previous ESI-MS studies have shown that both fatty acyl chain length and its degree of unsaturation have a significant influence.[17,18] To the best of our knowledge, no systematic study has been done so far for MALDI-MS quantitation of PC and therefore only an assumption is used that differences among MALDI responses are negligible inside the same lipid class.[24] The effect of fatty acyls could differ for MALDI and ESI techniques, because ionization mechanisms are not identical as well. One of the assumptions of the acyl chain influence in ESI-MS was that the difference in chains causes differences in the surface activity and, hence, different ionization efficiency in electrospray droplets and also can influence evaporation of molecules to the gas phase from the surface of a droplet.[18] In MALDI, the ionization depends on the matrix and its cocrystallization with sample molecules.[12] MALDI strongly depends on the sample structure, which is influenced by the analyte concentration, the ratio of analyte and matrix, solvent used for analyte and matrix preparation.[12,13,22,25,26] The detection efficiency can be dramatically different for the same species with various matrices or solvents used for the sample preparation.[13,25–27] Therefore, these factors must be taken into account during the development of MALDI-MS methods applicable for the quantitation.

Reliable quantitation in MS requires the use of an internal standard (IS) with a composition similar to quantified compounds, preferably isotopically labeled analogues. This approach is not feasible in the lipidomic quantitation, because tens to hundreds of lipid species may be present inside some lipid classes; therefore, certain simplifications must be introduced. Typically, the quantitation of lipid species inside the class is related to one or more IS from this class, but the IS cannot occur in studied samples, e.g., lipids with odd numbers of carbon atoms in fatty acyl chains.

The goal of the present work is the determination of effects of fatty acyl chain lengths and the DB number on the detection efficiency of PC in MALDI-MS experiments using 2,5-dihydroxybenzoic acid (DHB), 1,5-diaminonaphthalene (DAN) and 9-aminoacridine (9AA) as matrices, which are typically used for the lipidomic analysis.[27–29] The applicability of the final method with correction factors is used in the quantitation of PC in a human plasma sample.

EXPERIMENTAL

Materials

Dichloromethane, methanol, acetonitrile, 2-propanol, chloroform (all HPLC/MS grade), 4-dimethylaminopyridine (DMAP), dicyclohexylcarbodiimide (DCC), 2,5-dihydroxybenzoic acid (DHB), 1,5-diaminonaphthalene (DAN), 9-aminoacridine (9AA), and NaCl were purchased from Sigma-Aldrich (St. Louis, MO, USA). Myristic (FA 14:0), palmitic (FA 16:0), linolenic (FA 9Z,12Z,15Z-18:3), linoleic (FA 9Z,12Z-18:2), oleic (FA 9Z-18:1), stearic (FA 18:0), eicosapentaenoic (FA 5Z,8Z, 11Z,14Z,17Z-20:5), arachidic (FA 20:0), docosahexaenoic (FA 4Z,7Z,10Z,13Z,16Z,19Z-22:6), behenic (FA 22:0), and lignoceric (FA 24:0) fatty acids (FA) were purchased from NuChek Prep (Elysian, MN, USA); 1-heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (PC 17:0/0:0), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (PC 18:1/0:0), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (PC 16:0/16:0), 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine (PC 17:0/17:0), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (PC 18:1/18:1), 1,2-diarachidonoyl-*sn*-glycero-3-phosphocholine (PC 20:4/20:4), and 1,2-dibehenoyl-*sn*-glycero-3-phosphocholine (PC 22:0/22:0) were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

Human plasma was obtained from healthy volunteers in cooperation with the Faculty Hospital Olomouc based on the approval of the ethical committee at the Faculty Hospital Olomouc.

Synthesis and sample preparation

The synthesis of PC from lysophosphatidylcholine (PC *x:y*/0:0) was performed with a procedure similar to this one used for triacylglycerol synthesis described earlier.[30] PC 17:0/0:0 (4.5 mg) or PC 18:1/0:0 (5.2 mg) was mixed with 5.6 mg of DMAP and 10.9 mg of DCC and dissolved in 1 mL of dichloromethane. Volumes of 25 µmol of each FA were mixed and dissolved in 7 mL of dichloromethane. Then 0.2 mL of one PC *x:y*/0:0 solution and 0.2 mL of FA mixture were stirred in a vial for 2 h at ambient temperature. The described synthetic procedure was repeated three times both for PC 17:0/0:0 and PC 18:1/0:0, which yielded six series of PC to check the reproducibility of synthesized lipids ratio. Synthesized PC series were used without further purification.

Solutions of synthesized lipids were prepared by the dilution with methanol and mixed with the matrix for MALDI-MS measurements. The IS (PC 17:0/17:0) was added for the construction of calibration curves at the concentration comparable to the measured PC.

Solutions of synthesized lipids were prepared by dilution with methanol. The series contained dilutions in 3, 6, 10, 30, 60, 100 times. The IS (PC 17:0/17:0) solution in methanol was added for the construction of calibration curves at the concentrations 3 µmol/L and 30 µmol/L for PC 17:0/*x:y* and PC 18:1/*x:y*, respectively. The matrix solution is added for MALDI-MS measurements. The final sample contained a dissolved solution of synthesized lipids, IS and matrix solution in the ratio 1:1:1 (v/v/v).

The blood was collected into heparin-lithium tubes and centrifuged to obtain plasma. The lipid extract from plasma was prepared according to the modified Folch method.[31] A volume of 500 µL of sample was homogenized with 10 mL of chloroform/methanol (2:1, v/v). This mixture was filtered using a rough filter paper. Then 2 mL of 1 mol/L NaCl was added and centrifuged for 3 min at 2500 rpm. The chloroform layer containing lipids was evaporated by a gentle stream of nitrogen and dissolved in chloroform/2-propanol (1:1, v/v).

MALDI-MS

The following matrix solutions were used for MALDI experiments: 10 mg/mL of 9AA in 2-propanol/acetonitrile 60:40 (v/v), 77 mg/mL of DHB in methanol and 10 mg/mL of DAN in 2-propanol/acetonitrile 60:40 (v/v). 5 µL of each sample was mixed with 5 µL of matrix solution and 5 µL of IS. 0.7 µL of this mixture was deposited on the stainless steel sample plate

and dried. Each sample was deposited in four wells and measured using an LTQ Orbitrap XL with a MALDI source (Thermo Scientific, Waltham, MA, USA). MALDI mass spectra were acquired in the positive-ion mode in the mass range *m/z* 400–1000 with the laser energy of 15 μJ per laser shot for the DHB and 9AA matrices and 7 μJ for the DAN matrix. The optimal laser energy was determined by means of the LTQ Orbitrap XL software. Each mass spectrum was obtained as a result of summation of 3 to 5 laser shots. The number of laser shots was determined by the analysis of total ion current profile after shooting several times in one position. The decisive criterion was the decrease in total ion current by 10–20%. Spectra for each well were measured from 50 randomly distributed points. The final spectrum for each sample was obtained by averaging of 200 spectra from four wells to obtain the representative averaged spectrum. Mass spectra were converted with msConvert tool[32] and preliminarily processed with a home-made program based on the MALDIquant package.[33] Lipids nomenclature through the paper is in accordance with LIPID MAPS[34] terminology and the shorthand notation summarized in Liebisch *et al*.[35]

RESULTS AND DISCUSSION

Synthesis of PC standards

Two series of PC with different fatty acyl chain lengths and number of DB were synthesized for the investigation of these effects on the MALDI-MS detection efficiency. The synthesis is performed on the basis of commercially available standards of saturated (PC 17:0/0:0) and monounsaturated (PC 18:1/0:0) lysophosphatidylcholines. Two kinds of PC sets are synthesized by the reaction of lysophosphatidylcholines and the mixture of FA standards to prepare PC 17:0/*x:y* and PC 18:1/*x:y* series, where *x* is the carbon number (CN) and *y* is the DB number in the following fatty acyls in the *sn*-2 position: 14:0, 16:0, 18:3, 18:2, 18:1, 18:0, 20:5, 20:0, 22:6, 22:0 and 24:0 (details in the Experimental section). Saturated FA are selected to produce series of data points to reveal the general trends. The number of DB is varied by choosing lysophosphatidylcholines with different numbers of unsaturations in its fatty acyl. Only saturated and monounsaturated lysophosphatidylcholines are commercially available; therefore, reaction products with saturated FA allowed the detailed study of PC with zero and one DB only. FA with higher unsaturation are added for rough estimation of their responses with respect to the main series. The lengths of saturated FA cover the typical range in biological samples. The synthesized PC together with *m/z* values of their $[M+H]^+$ and $[M+Na]^+$ ions are summarized in Table 1. In addition, the PC 16:0/*x:y* series were synthesized and analyzed (data not presented). This demonstrates properties similar to that of PC 17:0/*x:y*. Presentation of the data for the PC 17:0/*x:y* series is related to the fact that it can be added to biological samples for data correction as it has odd CN which is relatively rare in nature.

MALDI-MS analysis of synthesized PC

Products of synthesis were investigated by MALDI-MS to confirm the presence of the compounds of interest. Zoomed *m/z* regions of $[M+H]^+$ ions in the positive-ion mass spectra of

Table 1. Theoretical and experimental *m/z* values of protonated and sodiated molecules in the PC 17:0/*x:y* and PC 18:1/*x*:0 series with measured mass accuracy

	$[M+H]^+$			$[M+Na]^+$		
Lipid	Theoretical	Experimental	Mass accuracy [ppm]	Theoretical	Experimental	Mass accuracy [ppm]
PC 17:0/14:0	720.5538	720.5548	1.4	742.5357	742.5367	1.3
PC 17:0/16:0	748.5851	748.5860	1.2	770.5670	770.5700[a]	3.9
PC 17:0/18:3	770.5694	770.5700[a]	0.8	792.5514	792.5527	1.6
PC 17:0/18:2	772.5851	772.5866	1.9	794.5670	794.5700[b]	3.8
PC 17:0/18:1	774.6007	774.6021	1.8	796.5827	796.5827	0.0
PC 17:0/18:0	776.6164	776.6176	1.5	798.5983	798.5997	1.8
PC 17:0/20:5	794.5694	794.5700[b]	0.8	816.5514	816.5523	1.1
PC 17:0/20:0	804.6477	804.6487	1.2	826.6296	826.6307	1.3
PC 17:0/22:6	820.5851	820.5861	1.2	842.5670	842.5680	1.2
PC 17:0/22:0	832.6790	832.6799	1.1	854.6609	854.6622	1.5
PC 17:0/24:0	860.7103	860.7111	0.9	882.6922	882.6936	1.6
PC 18:1/14:0	732.5538	732.5546	1.1	754.5357	754.5367	1.3
PC 18:1/16:0	760.5851	760.5859	1.1	782.5670	782.5692[c]	2.8
PC 18:1/18:3	782.5694	782.5692[c]	0.3	804.5514	804.5523	1.1
PC 18:1/18:2	784.5851	784.5858	0.9	806.5670	806.5687[d]	2.1
PC 18:1/18:1	786.6007	786.6015	1.0	808.5827	808.5826	0.1
PC 18:1/18:0	788.6164	788.6170	0.8	810.5983	810.5989	0.7
PC 18:1/20:5	806.5694	806.5687[d]	0.9	828.5514	828.5520	0.7
PC 18:1/20:0	816.6477	816.6481	0.5	838.6296	838.6303	0.8
PC 18:1/22:6	832.5851	832.5855	0.5	854.5670	854.5678	0.9
PC 18:1/22:0	844.6790	844.6796	0.7	866.6609	866.6616	0.8
PC 18:1/24:0	872.7103	872.7107	0.5	894.6922	894.6929	0.8

[a, b, c, d] *m/z* values are not resolved in our measurements.

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PC 17:0/*x:y* and PC 18:1/*x:y* series are presented for 9AA (Fig. 1), DHB (Supplementary Fig. S1) and DAN (Supplementary Fig. S2, see Supporting Information) matrices. Peaks present in mass spectra correspond to protonated or sodiated molecules of PC, which is confirmed by accurate *m/z* determination with the mass accuracy typically better than 3 ppm (Table 1). Peaks of sodiated PC are not observed at all or with a negligible intensity for samples measured with 9AA matrix, which is an advantage for the interpretation of spectra. Relative abundances of $[M+Na]^+$ ions for PC are about 10–60% related to $[M+H]^+$ ions for measurements with DHB and DAN matrices. Peaks at *m/z* 770, 794 (Supplementary Figs. S1(a), S2(a)) and *m/z* 782, 806 (Supplementary Figs. S1(b), S2(b)) correspond to overlapping signals of protonated and sodiated PC, which is known problem of MS analysis of phospholipids in the positive-ion mode,[36] because their resolution requires a resolving power of over 300,000. Tandem mass spectra are obtained to confirm the chemical structures of synthesized compounds. Supplementary Fig. S3 (see Supporting Information) shows a characteristic fragmentation of sodiated and protonated PC on the example of PC 18:1/18:1.[37,38] All calculations in the present investigation are performed for protonated species only to be able to compare results for the same ions for all three matrices. Besides, the ratio of intensities of sodiated and protonated PC can be different for different species because protonation is localized on the head group and the interaction with Na^+ can involve fatty acyl chains which wrap around the cation and such kinds of interaction with Na^+ may depend on chain lengths much stronger than the interaction of H^+.

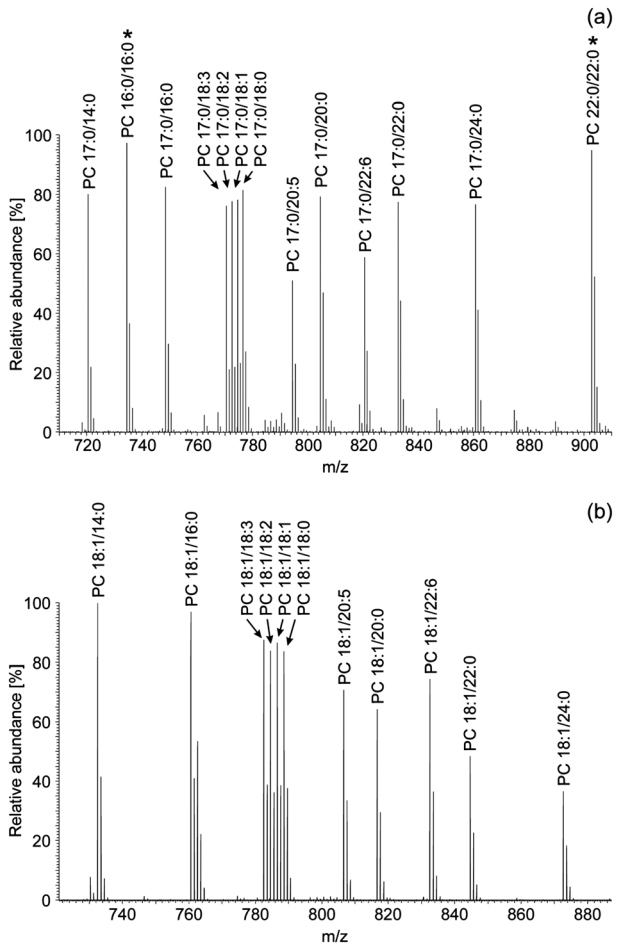

Figure 1. Positive-ion MALDI mass spectra of synthesized PC measured using 9AA as the matrix: (a) PC 17:0/*x:y* series with added PC 16:0/16:0 and PC 22:0/22:0 at identical concentrations (labeled by asterisks) and (b) PC 18:1/*x:y* series. The result after averaging of 200 mass spectra from four wells with the same sample is shown.

Concentrations of analyzed PC are estimated for each synthesized mixture using the standard addition analysis[16] to enable the selection of the appropriate IS concentration. Determined concentrations are around 0.06 mmol/L for the PC 17:0/*x:y* series and 0.8 mmol/L for the PC 18:1/*x:y* series with the relative standard deviation (RSD) approximately 5%. Based on this information, concentrations of IS added to each dilution of synthesized series are 3 µmol/L and 30 µmol/L for PC 17:0/*x:y* and PC 18:1/*x:y* correspondingly. Synthesized PC compounds were not purified and some amounts of initial substances (lysophosphatidylcholine and fatty acids) are present in the mixtures modeling the presence of other lipids. The crucial question of our work concept is the confirmation of the initial assumption that the reaction really provides equimolar mixtures of synthetized PC.[39–41] For this purpose, an equimolar mixture of two PC standards is added to synthesized samples (Fig. 1(a)). Peaks of added standards of PC 16:0/16:0 and PC 22:0/22:0 are labelled with asterisks. Slopes of trend lines built for intensities of equimolar standards and synthesized species have close values (–0.029 and –0.032, respectively). Peak intensities of synthesized PC in mass spectra obtained with 9AA matrix also demonstrate a similar dependence as for the equimolar mixture of standards known from the literature.[26] The reproducibility of normalized intensities of synthesized species produced in three different replicative reactions is demonstrated by standard deviations in Supplementary Fig. S4 (see Supporting Information).

MALDI mass spectra depend on the structure of measured compounds, matrix, solvent and the concentration ratio of matrix to analyte. Dependences of absolute intensities of PC in positive-ion MALDI mass spectra on the concentration are shown for the three studied matrices (Fig. 2). Dependences show the same trend for all studied matrices. Absolute intensities increase up to $5*10^{-4}$ mol/L, then the signal starts to decrease. This behavior could be explained by the spot structure after the cocrystallization with the matrix and the variation in matrix-to-analyte ratio. For example, in the case of DHB, the structure of the spot is uniform without visible matrix crystals for samples with high concentrations of lipids. The matrix-to-analyte ratio for these samples is about 50:1 to 500:1, while the optimal ratio is typically at 1000:1 and higher.[12] Such excess of lipids may result not in an increase in their MALDI-MS signal, but in hampering of the function of DHB as a matrix. With the increase in the matrix relative amount, polycrystals could be formed, which improves incorporation of lipids into the matrix structure and thus improving lipid-matrix interaction resulting in the increase in lipid signal intensities. The observed effect depends on the overall concentration of lipids, which may be attributed to the influence on the sample structure. Their measurement allows the

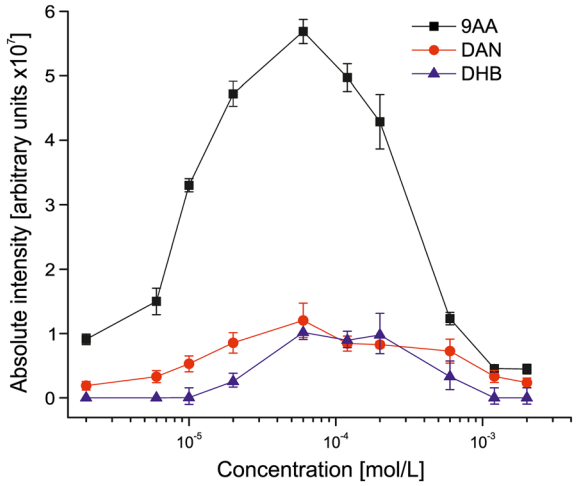

Figure 2. Dependences of absolute intensities of $[M+H]^+$ ions of PC 32:1 on the concentration measured using 9AA, DAN or DHB matrix.

concentration region with the monotonic dependence of peak intensities to be determined, which is used for further experiments.

Another parameter influencing MALDI mass spectra is the laser energy. The optimal laser energy is determined using standard procedures of the LTQ Orbitrap XL software and varied within ±30% around that value. No significant variations in responses to synthesized PC are observed.

One of the reasons that could cause discrimination in signal intensity is that during the sample drying, the moving edge of the evaporating droplet can support processes similar to those in the chromatography resulting in uneven analyte distribution. These processes may result in response differences over local regions of the sample spot. The measurement in multiple random points over a sample spot improves the representative data sampling. Relative intensities for synthesized PC from spectra corresponding to randomly distributed spots over one well with a sample are shown in Supplementary Figs. S5–S7 (see Supporting Information). Similar picture is observed for other spots. Figures represent data for matrices used in the study. Relative standard deviations of absolute intensities are relatively high. They are around 20, 16 and 38% for samples measured with 9AA, DHB and DAN, respectively. At the same time, relative intensities vary to a smaller extent. Relative standard deviations of intensities normalized to the internal standard are around 7.6, 8.9 and 13.6% for samples measured with 9AA, DHB and DAN. In addition, deviations of normalized intensities are correlated demonstrating relatively even distribution of compounds over the spot. Analysis of Supplementary Fig. S8 (see Supporting Information) shows that differences between observed values of normalized intensities for different PC are significant compared with ranges of signal deviation.

Response dependences on fatty acyl length and DB number

Calibration curves for the synthesized PC are constructed after the addition of PC 17:0/17:0 as the IS to prepared series with different concentrations. Logarithmic plots of the normalized signal of $[M+H]^+$ ions *vs.* the molar ratio of PC to IS are shown in Fig. 3 for 9AA matrix with the RSD approximately 3% (Supplementary Fig. S9, see Supporting Information, represents similar data for two other replicative syntheses). The average of the regression coefficients is 0.997 ± 0.003 (Supplementary Table S1, see Supporting Information). Calibration curves can be described by a linear equation:

$$y = s * x + i \quad (1)$$

where s and i are parameters corresponding to the slope and intercept of the calibration curves and

$$y = \log\left(\frac{I_x}{I_{IS}}\right), x = \log\left(\frac{c_x}{c_{IS}}\right) \quad (2)$$

where I_x, c_x are the intensity and concentration of synthesized PC, I_{IS} and c_{IS} are the intensity and concentration of the IS (PC 17:0/17:0). Parameters of these dependences are summarized in Supplementary Table S1 (see Supporting Information). Dependences of s and i on the CN of fatty acyls are constructed based on this data (Fig. 4). Dots corresponding to parameters of calibration curves of lipids that differ in chain length only can be well approximated with linear functions (Table 2):

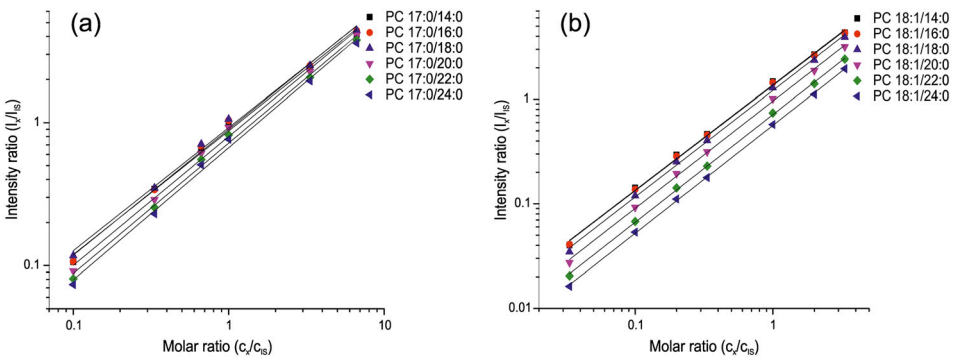

Figure 3. Logarithmic correlation of normalized intensities of $[M+H]^+$ ions and normalized molar ratios of synthesized PC to IS (PC 17:0/17:0) measured using 9AA as the matrix: (a) PC 17:0/*x*:*y* and (b) PC 18:1/*x*:*y* series. The result is for one synthetic procedure.

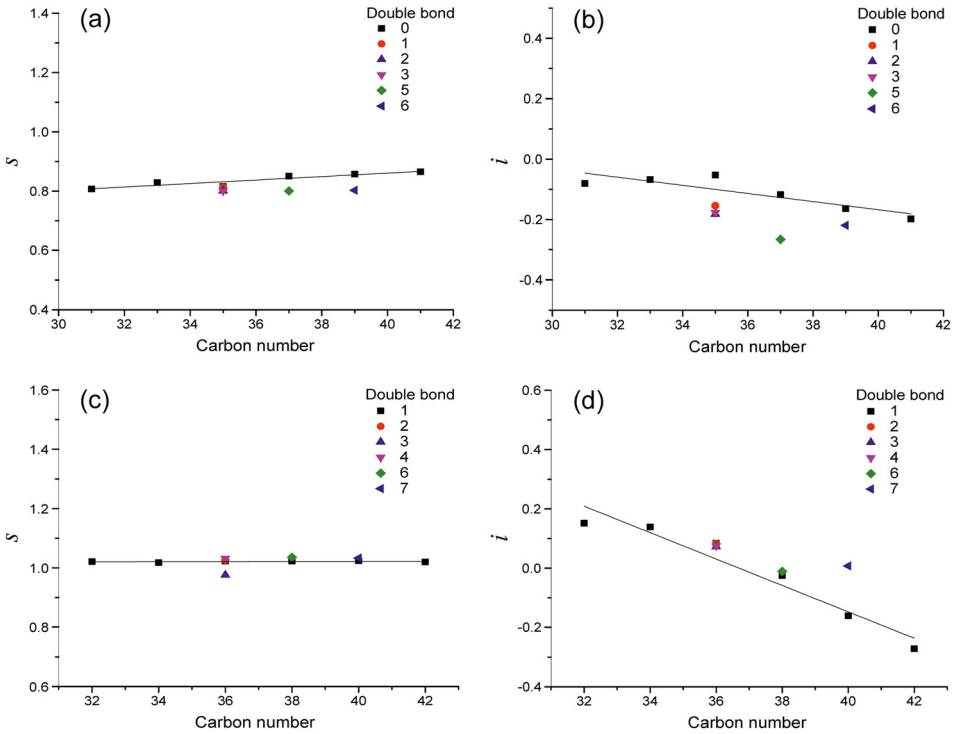

Figure 4. Linear dependences of: (a) slopes (*s*) for PC 17:0/*x vs.* the carbon number, (b) intercepts (*i*) for PC 17:0/*x*:*y vs.* the carbon number, (c) slopes (*s*) for PC 18:1/*x*:*y vs.* the carbon number, and (d) intercepts (*i*) for PC 18:1/*x*:*y vs.* the carbon number. Samples measured using 9AA as the matrix. The result is for one synthetic procedure.

Table 2. Coefficients of linear fitting of corresponding parameters (*s* and *i*) of calibration curves in their dependence on CN (Fig. 5 and Supplementary Figs. S6 and S7, see Supporting Information)

Matrix	Lipid series	Parameter of calibration curve	Slope[a]	Intercept[b]	Regression coefficient (r^2)	Power of analysis	p-value
9AA	PC 17:0/x:0	s^c	0.006	0.673	0.826	0.97	0.01
		i^d	–0.013	0.391	0.703	0.80	0.02
	PC 18:1/x:0	s^c	0.003	0.917	−0.153	-	0.59
		i^d	–0.041	1.470	0.924	1.00	0.00
DHB	PC 17:0/x:0	s^c	–0.038	2.108	0.956	1.00	0.00
		i^d	0.001	0.007	−0.248	-	0.94
	PC 18:1/x:0	s^c	–0.083	4.739	0.946	1.00	0.00
		i^d	–0.099	5.511	0.789	0.94	0.01
DAN	PC 17:0/x:0	s^c	0.031	–0.158	0.738	0.86	0.02
		i^d	0.021	–1.367	0.257	0.20	0.17
	PC 18:1/x:0	s^c	0.002	0.900	0.382	0.32	0.11
		i^d	–0.029	0.998	0.947	1.00	0.00

[a]Slope of the linear dependence of calibration curve parameter on the carbon number.
[b]Intercept of the linear dependence of calibration curve parameter on the carbon number.
[c]Slope of the calibration curve.
[d]Intercept of the calibration curve.

$$s = 0.006 * \mathrm{CN} + 0.673 \quad (3)$$

$$i = -0.013 * \mathrm{CN} + 0.391 \quad (4)$$

for the series of saturated PC and

$$s = 0.003 * \mathrm{CN} + 0.917 \quad (5)$$

$$i = -0.041 * \mathrm{CN} + 1.470 \quad (6)$$

for monounsaturated PC. The dependence is not very steep, which is represented by the values of multipliers of CN close to zero. Values of the slope for PC 18:1/*x*:*y* are less dependent on CN than for PC 17:0/*x*:*y* and the trend is reversed for intercepts. Figure 4 illustrates the effects of CN and DB on the relative responses of PC. Intensities of saturated PC are very similar (Fig. 1(a), PC 17:0/*x*:0 series), but intensities of monounsaturated species decrease for

higher CN (Fig. 1(b), PC 18:1/*x*:0 series). The influence of DB number can be estimated from the series of PC 17:0/18:0, PC 17:0/18:1 and PC 17:0/18:2 showing a small incremental decrease in intensity for unsaturated PC. This effect is much stronger for polyunsaturated PC, e.g. the comparison of the pairs PC 17:0/20:0 and PC 17:0/20:5, PC 17:0/22:0 and PC 17:0/22:6 (Fig. 1(a)) with the relative signal decrease of *ca*. 25–30% for polyunsaturated fatty acyls related to saturated ones.

For the explanation of *s* and *i* values, the following expression can be deduced from Eqns. (1) and (2):

$$\frac{I_x}{I_{IS}} = 10^i \left(\frac{c_x}{c_{IS}}\right)^s \quad (7)$$

which demonstrates the relationship between MS peak intensity of a compound and its concentration and also influence of parameters of calibration curves on this relationship. It is obvious from Eqn. (7) that *s* determines the shape of the dependence and *i* plays a role in the determination of the scaling factor. Thus, taking into account Eqns. (3)–(6) and values in Supplementary Table S1 (see Supporting Information) and Fig. 4, one can conclude that the dependence of the intensity on the concentration is nearly linear as values of *s* are close to 1 and that the scaling factor in Eqn. (7) is more strongly influenced by the fatty acyl chain lengths in the case of monounsaturated PC than for saturated PC.

Several points corresponding to polyunsaturated PC are also depicted in Fig. 4. It is impossible to build dependences similar to PC 17:0/*x*:*y* and PC 18:1/*x*:*y* due to the lack of series of data points. Nevertheless, it is evident that these points are relatively close to the monounsaturated PC series (Figs. 4(c) and 4(d)) and, thus, Eqns. (5) and (6) can be used for a rough estimation of parameters of calibration curves for polyunsaturated PC as well.

Accounting the dependence of parameters of the calibration curve on CN for saturated PC may result in corrections to the determined concentration of about 10%, which is determined by the largest difference between the peak intensities of PC 17:0/14:0 and PC 17:0/24:0. This value is comparable to the experimental error in MALDI analysis of saturated PC with 9AA. The assumption about equal responses can be accepted. In the case of unsaturated PC, the picture is different and the value of correction can reach 60% for PC with CN difference equal to 10. Thus taking into account the influence of the acyl chain length can improve the estimation of concentration of unsaturated PC.

A similar study was performed for the same PC samples measured with DHB or DAN matrices. The results of this study are summarized in Supplementary Figs. S10–S13 and Supplementary Tables S2 and S3 (see Supporting Information). In general, lipids measured with DHB and DAN also show dependences of their responses on fatty acyls, but this dependence is much stronger than for the 9AA matrix. Parameters of linear dependences are fitted with regression coefficients on average equal to 0.91 ± 0.06 for DHB and 0.98 ± 0.00 for DAN, which is somewhat worse compared to 9AA. Relative standard deviations of peak intensities are also higher, on average18% for DHB and 40% for DAN. These facts make cautious the use of data and equations obtained with DHB and DAN for predictive measurements without using several IS.

Table 2 shows parameters of calibration curves for all three matrices. Similar data are calculated for all three replicate syntheses and obtained parameters are close to each other within errors of measurements and calculations.

Application of the developed method for PC standard mixtures and human plasma

The developed method was first applied for the analysis of mixtures of PC standards with known concentrations. Concentrations of these standards are calculated using information about mass spectrometric intensities with and without accounting for differences in relative responses of PC. In the first case, concentrations are obtained by multiplying the ratio of the PC intensity to IS with IS concentration. In the second case, they are calculated with Eqn. (7) using the data from Table 2. Comparisons of PC concentrations known from the mixture preparation with concentrations calculated from MS data are presented in Tables 3–5. Errors in Tables 3–5 are found as difference between concentrations known from preparation and from MS data divided by the concentration known from the preparation and multiplied by 100. Results show that

Table 3. Concentrations of lipid standards in mixtures 1 and 2 prepared by weighing and determined from MALDI measurements using 9AA matrix with and without the use of response correction

	Concentration [μmol/L]			Relative error [%]	
Lipid	Weighed	Without response correction	With response correction	Without response correction	With response correction
			Mixture 1		
PC 16:0/16:0	3.0	3.5 ± 0.3	2.8 ± 0.1	18	5
PC 18:1/18:1	3.0	2.7 ± 0.2	2.6 ± 0.1	10	13
PC 20:4/20:4	3.0	2.2 ± 0.2	3.2 ± 0.1	26	8
PC 22:0/22:0	3.0	3.2 ± 0.2	3.0 ± 0.1	6	1
			Mixture 2		
PC 16:0/16:0	1.2	1.8 ± 0.1	1.2 ± 0.0	47	2
PC 18:1/18:1	6.0	6.3 ± 0.4	5.9 ± 0.3	5	1
PC 20:4/20:4	0.4	0.3 ± 0.0	0.4 ± 0.0	30	10
PC 22:0/22:0	15.0	13.7 ± 1.0	15.2 ± 3.7	9	1

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Table 4. Concentrations of lipid standards in mixtures 1 and 2 prepared by weighing and determined from MALDI measurements using DHB matrix with and without the use of response correction

	Concentration [μmol/L]			Relative error [%]	
Lipid	Weighed	Without response correction	With response correction	Without response correction	With response correction
			Mixture 1		
PC 16:0/16:0	3.0	4.0 ± 0.8	3.0 ± 1.7	32	2
PC 18:1/18:1	3.0	2.8 ± 0.8	2.7 ± 0.8	6	10
PC 20:4/20:4	3.0	2.5 ± 0.5	3.3 ± 0.7	17	10
PC 22:0/22:0	3.0	1.3 ± 0.8	3.3 ± 0.3	57	9
			Mixture 2		
PC 16:0/16:0	1.2	1.9 ± 0.4	1.4 ± 0.2	59	18
PC 18:1/18:1	6.0	6.5 ± 2.4	6.5 ± 2.2	8	9
PC 20:4/20:4	0.4	0.3 ± 0.1	0.4 ± 0.0	19	4
PC 22:0/22:0	15.0	3.3 ± 2.1	9.3 ± 5.8	78	38

Table 5. Concentrations of lipid standards in mixtures 1 and 2 prepared by weighing and determined from MALDI measurements using DAN matrix with and without the use of response correction

	Concentration [μmol/L]			Relative error [%]	
Lipid	Weighed	Without response correction	With response correction	Without response correction	With response correction
			Mixture 1		
PC 16:0/16:0	3.0	3.1 ± 1.0	2.8 ± 0.2	20	6
PC 18:1/18:1	3.0	3.1 ± 0.8	3.2 ± 0.2	3	8
PC 20:4/20:4	3.0	3.6 ± 0.7	3.5 ± 0.1	20	17
PC 22:0/22:0	3.0	1.9 ± 0.5	1.9 ± 0.1	36	36
			Mixture 2		
PC 16:0/16:0	1.2	1.3 ± 0.3	1.1 ± 0.1	12	5
PC 18:1/18:1	6.0	6.5 ± 1.6	6.3 ± 2.0	8	5
PC 20:4/20:4	0.4	0.5 ± 0.1	0.4 ± 0.0	19	12
PC 22:0/22:0	15.0	24.8 ± 7.0	7.4 ± 4.1	65	50

taking into account the effect of fatty acyl parameters on PC detection efficiency significantly improves the determination of concentration of PC by MALDI-MS. Parameters of monounsaturated PC are used in calculations for polyunsaturated PC due to the lack of polyunsaturated PC standards, so the precision of concentrations for highly unsaturated PC may be slightly reduced.

The method was also applied for a sample of human plasma from a healthy volunteer. The positive-ion MALDI mass spectrum of this sample is presented in Fig. 5. PC 34:1 and PC 36:2 species are quantified by the method of IS addition. The results of this quantitation are summarized in Table 6 together with concentrations calculated using the ratio of PC peak intensity to the IS without and with the response factor. The errors listed in Table 6 were calculated in a way similar to the errors in Tables 3–5, but the concentration found with the IS addition method is used as the reference. This data also demonstrates a satisfactory agreement between concentrations found with different methods and a decrease in error using response factors is observed in most cases. Table 7 demonstrates results on PC concentrations in the human plasma normalized to PC 34:0 with and without correction, compared with the normalized data from the literature.[42] The data for some PC correlate well, but in some cases higher differences are observed, which may be attributed to the fact that human plasma samples are not identical.

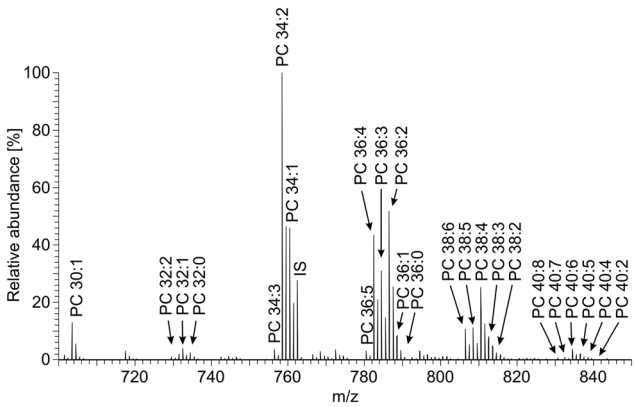

Figure 5. Positive-ion MALDI mass spectrum of human plasma lipid extract with added PC 17:0/17:0 as the internal standard (IS) and measured using 9AA as the matrix.

Table 6. Comparison of concentrations of selected PC in human plasma determined by the standard addition method and calculated from MALDI measurements with and without the use of response factors using different matrices

	Concentration [μmol/L]			Relative error [%]	
Lipids	Standard addition	Calculated without response factor	Calculated with response factor	Without response factor	With response factor
			9AA		
PC 34:1	82.2 ± 4.1	69.1 ± 6.7	74.0 ± 5.4	16	10
PC 36:2	96.8 ± 6.8	85.3 ± 7.7	102.9 ± 7.7	12	6
			DHB		
PC 34:1	82.2 ± 4.1	136.2 ± 29.5	111.8 ± 30.2	64	36
PC 36:2	96.8 ± 6.8	114.2 ± 24.4	115.1 ± 23.4	18	19
			DAN		
PC 34:1	82.2 ± 4.1	73.1 ± 10.6	71.78 ± 9.3	11	13
PC 36:2	96.8 ± 6.8	78.4 ± 12.2	88.8 ± 10.0	19	8

Table 7. Relative concentrations of PC in human plasma normalized on total PC content and multiplied by 1000 PC

	Relative concentration		
Lipid	Without response factor	With response factor	Literature data
PC 30:1	0.1 ± 0	0.1 ± 0	0.6 ± 0.1
PC 32:2	1.8 ± 0.1	1.2 ± 0.1	5.3 ± 0.2
PC 32:1	9.7 ± 0.2	6.3 ± 0.6	15.4 ± 1
PC 32:0	5.6 ± 0.4	3.6 ± 0.2	6.1 ± 0.3
PC 34:3	13.8 ± 0.7	10.8 ± 0.3	7.4 ± 0.5
PC 34:2	266 ± 25.1	196.9 ± 12	101.1 ± 7.5
PC 34:1	89 ± 1.2	92.5 ± 0.4	48 ± 4.4
PC 36:5	8.6 ± 0.5	8.3 ± 0.8	6.9 ± 0.6
PC 36:4	117.5 ± 11.2	108.3 ± 6.6	92.5 ± 5.9
PC 36:3	97.4 ± 2	90.1 ± 6.9	88.7 ± 7
PC 36:2	109.9 ± 0.5	128.7 ± 10.9	136.6 ± 9.7
PC 36:1	20 ± 1.6	19.1 ± 0.3	53.7 ± 7
PC 36:0	1.8 ± 0.1	1.8 ± 0	4.3 ± 0.8
PC 38:6	29 ± 0.4	33.7 ± 1.5	33.8 ± 2.6
PC 38:5	30.3 ± 0.9	35.2 ± 2.7	46.4 ± 4.8
PC 38:4	69.1 ± 1.9	78.9 ± 4	136.6 ± 11.3
PC 38:2	2.5 ± 0.1	3.1 ± 0.2	20.2 ± 3.1
PC 40:8	26.3 ± 2	37.6 ± 0.6	14.5 ± 1.3
PC 40:7	61.8 ± 4.7	86.7 ± 3	12.4 ± 2.4
PC 40:6	29.9 ± 2.9	42.6 ± 3.4	42.7 ± 5.5
PC 40:5	7.4 ± 0.3	10.9 ± 0.6	35.8 ± 5.4
PC 40:4	2.4 ± 0.1	3.6 ± 0.1	19.6 ± 3
PC 40:2	0 ± 0	0 ± 0	71.5 ± 8.6

Concentrations calculated from MALDI measurements using 9AA with and without the response factors and calculated from literature data.[42]

CONCLUSIONS

The present work demonstrates that the efficiency of PC detection in positive-ion MALDI-MS depends on fatty acyl chain length and DB number. Linear dependences are observed for all three matrices (9AA, DHB or DAN), while the steepness of these dependences differs for individual matrices and the saturation level of the PC. Relative responses of saturated PC with 9AA are the least dependent on the fatty acyl chain length and therefore the most convenient for quantitation applications. The effect of fatty acyl chain length on correction factors must be taken into account for DHB or DAN matrices with both saturated and unsaturated PC as well as with unsaturated PC with 9AA matrix. For a rough estimation, the results of PC quantitation obtained with 9AA matrix can be used without correction. Data obtained with DAN and especially with DHB are better to use mainly for the qualitative comparison or for the relative quantitation, taking into account the high signal deviation and strong dependence of PC responses on both CN and DB. Another way is to use one IS and the model for the calculation of PC concentrations, which accounts for differences in their responses. This model must be created in advance and based on preliminary investigation of PC with different compositions. This last approach provides good results for 9AA and slightly higher variations for DHB and DAN matrices.

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