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**RELATIVE QUANTITATION
OF PHOSPHATIDYLCHOLINES WITH INTERFERED
MASSES OF PROTONATED AND SODIATED
MOLECULES BY MATRIX-ASSISTED LASER
DESORPTION/IONIZATION MASS SPECTROSCOPY**

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This work presents a computational method for deconvolution of interfered peaks of protonated and sodiated phosphatidylcholines (PCs) to solve the problem of relative quantitation of these PCs. The method is based on the fact that adducts of PCs with proton and sodium ion give unique characteristic peaks in tandem mass spectra. The intensities of these peaks were used to find out contribution of protonated and sodiated PCs to the formation of interfered peak in full-scan mass spectrum and, further, to correlate peak intensities that correspond to PCs in full-scan mass spectrum with PC's concentrations. The model was calibrated on synthesized PCs and verified for applicability in the range of Na⁺ ion concentrations from trace to physiological one.

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Introduction

Phosphatidylcholines are important and the most numerous lipids in cells. They participate in cell signaling, organ physiology and can be associated with different diseases [1-4]. These facts determine the necessity to develop methods for qualitative and quantitative analysis of lipid composition of biological samples. Soft ionization methods of mass spectrometry (MS) and their combination with liquid chromatography separation technique are recognized as the most powerful methods in the lipidomic analysis. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) can be used as a fast method for screening of lipids composition and for estimation of their relative concentrations. Its distinctive features are fast sample preparation, short measurement time and robustness. Another important application of MALDI-MS is direct profiling of tissue lipids.

PCs are registered in MALDI-MS as protonated molecules and adducts with alkali metal ions such as sodium and potassium. Even trace amounts of alkali metal ions could cause that intensities of these peaks are comparable to intensities of protonated molecules peaks. An interference between peaks of sodiated and protonated PCs is observed for PCs with two aliphatic chains with a general formulae PC $m:n$ and PC $(m+2):(n+3)$, where m is the total carbon number of fatty acyls and n is the double bond number. Such overlapping of peaks hampers quantitative and qualitative estimation of lipids composition.

Several variants of solving the problem of peaks interference were suggested previously [5-9]. Adding lithium [5], potassium [6], or cesium [7,8] in the MALDI matrix solution was attempted to circumvent the obstacle of peaks interference. However, protonated PCs and SMs were still easily detected with their alkalinated counterparts in most of the MALDI spectra acquired by this approach. Another method is desalting samples to remove sodium cations prior to measurements using ion exchange resins or washing out step in the case of tissues imaging [9]. But this methods could cause sample composition change.

The fastest and the most reliable method of interfered peaks deconvolution is to resolve the peaks on the instrumentation level. The difference between sodiated and protonated molecules, e.g., for $[\text{PC } 18:1/18:1 + \text{Na}]^+$ and $[\text{PC } 18:1/20:4 + \text{H}]^+$, is $808.5851 - 808.5827 = 0.0024$, which requires the resolving power over 3.4×10^5 to separate them. But this is achievable on mass spectrometers with Fourier transform ion cyclotron resonance analyzer only.

The present work deals with the idea of overlapping peaks deconvolution. It is suggested to use the information from characteristic fragmentation and the following data processing for the deconvolution. The method allows to avoid additional sample pretreatment and can be used on any tandem mass spectrometer.

Experimental

Chemicals and Standards

Dichloromethane, ethanol, methanol (all HPLC/MS grade), 4-dimethylaminopyridine (DMAP), dicyclohexylcarbodiimide (DCC), 2,5-dihydroxybenzoic acid (DHB) and NaCl were purchased from Sigma-Aldrich (St. Louis, MO, USA). Oleic (18:1) and arachidonic (20:4) fatty acids (FA) were obtained from NuChekPrep (Elysian, MN, USA), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC 18:1) was from Avanti Polar Lipids (Alabaster, AL, USA). Deionized water was prepared with a Demiwa 5-roj purification system (Watek, Ledec nad Sázavou, the Czech Republic).

Samples Preparation

Synthesis of PCs from LPC was performed with a procedure similar to one used for triacylglycerols synthesis described earlier [10]. 5.1 mg 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine, 5.6 mg DMAP and 10.9 mg DCC were dissolved in 1 ml dichloromethane. 3 mg each fatty acid were dissolved in 0.3 ml dichloromethane. Then 0.2 ml solution with LPC and 0.2 ml of that with each fatty acid were stirred in a vial for 2 hours at ambient temperature.

Mass Spectrometry

10 μ l of each sample were mixed with 10 μ l 0.5 mol l⁻¹ DHB in methanol. 0.7 μ l of mixture was deposited on the stainless steel sample plate and dried using a gentle stream of nitrogen. Each sample was deposited in ten wells and measured using LTQ Orbitrap XL with MALDI (Thermo Scientific, Waltham, MA, USA). MALDI mass spectra were acquired in the positive-ion mode in the mass range m/z 100-2000 with the laser energy 15 μ J per laser shot. Mass spectrum from one point was obtained as a result of summation of 7 laser shots with 3 sweep shots in advance. Spectra for each well were measured from 30 randomly distributed points. The final spectrum for each sample was obtained by averaging of 300 spectra from ten wells to obtain the representative averaged spectrum. Mass spectra were converted with msConvert tool [11] and preliminarily processed with home-made program based on the MALDIquant package [12].

Results and Discussion

The products of synthesis were investigated with MALDI-MS to confirm the presence of the compounds of interest. Figure 1 represents informative region of the positive-ion mass spectra of samples mixed with DHB matrix solution. The peaks present in mass spectra according to accurate mass correspond to protonated molecules of PC 18:1/18:1 (2.8 ppm mass accuracy) (Fig. 1a) and PC 18:1/20:4 (1.9 ppm mass accuracy) (Fig. 1b). PC 18:1/18:1 and PC 18:1/20:4 lipids will be designated further as PC1 and PC2. Peaks of adducts with sodium $[\text{PC}+\text{Na}]^+$ and potassium $[\text{PC}+\text{K}]^+$ cations are also present (Fig. 1). Intensities of peaks of sodiated molecules are of 80-90 % from protonated ones.

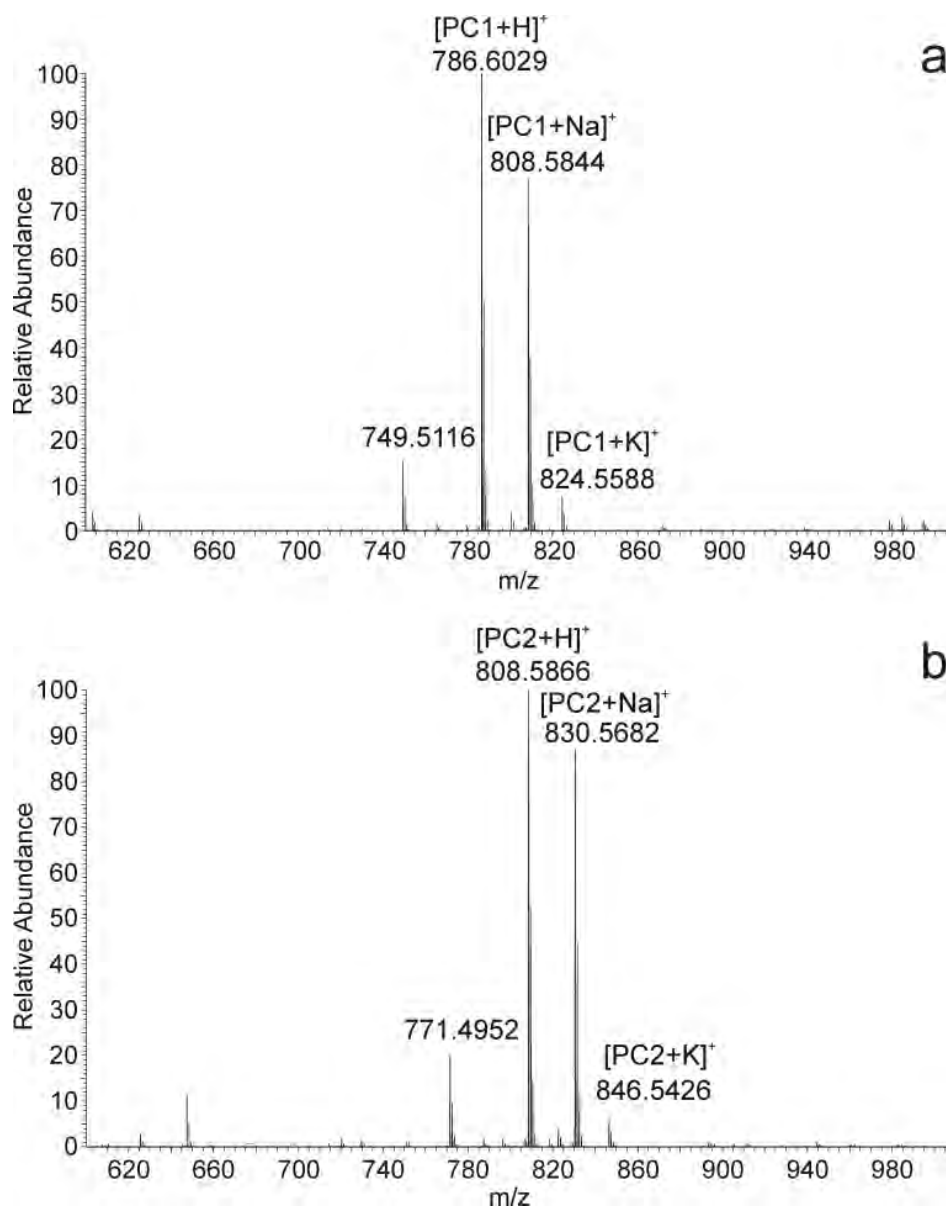


Fig. 1 Positive-ion MALDI mass spectra of synthesized PC: a – PC 18:1/18:1; b – PC 18:1/20:4

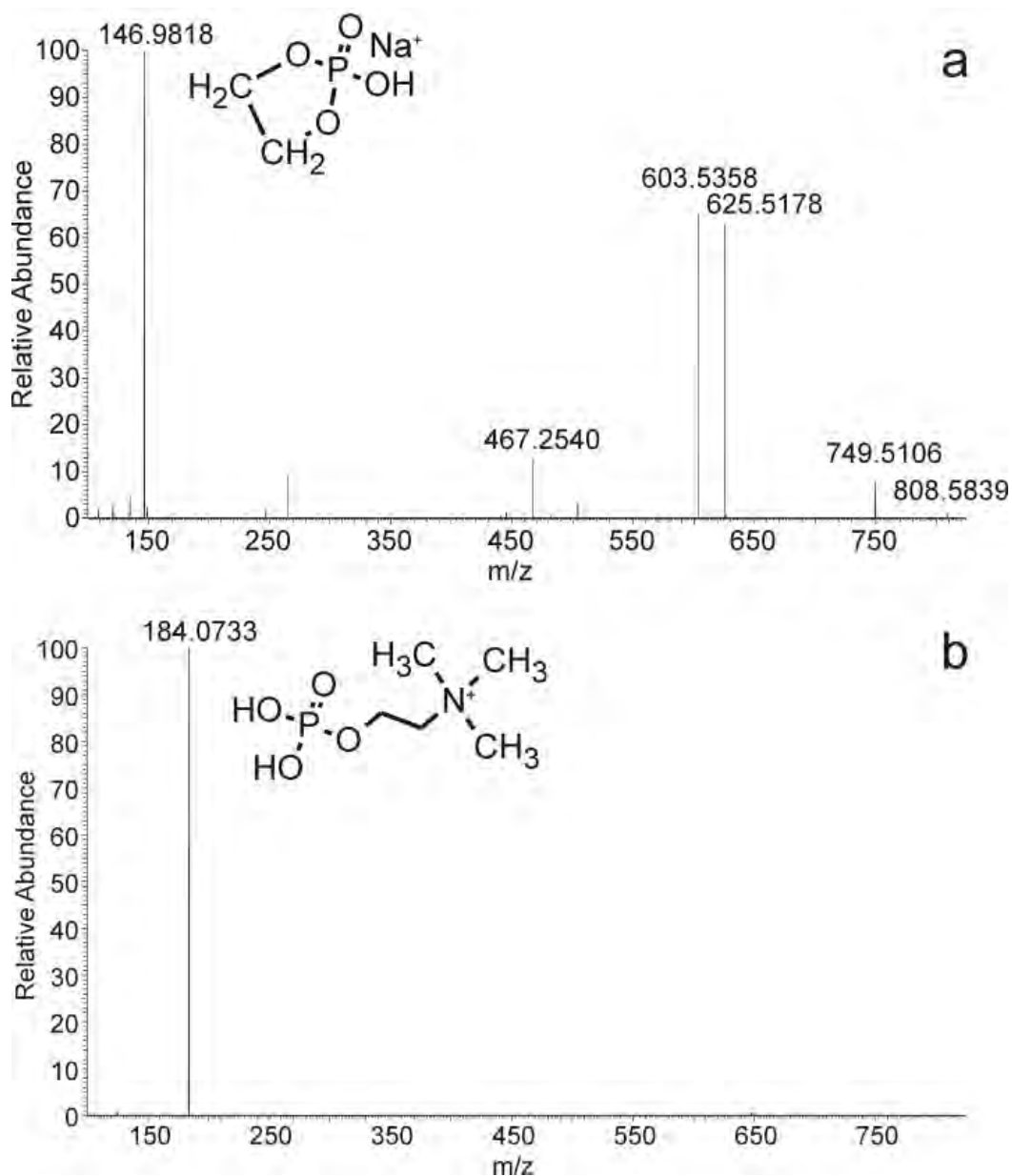


Fig. 2 Tandem spectra of sodiated and protonated phosphatidylcholines: a – $[PC1 + Na]^+$; b – $[PC2 + H]^+$. Structural formulae represent characteristic fragment ions used for further peaks deconvolution

Tandem mass spectra were obtained to confirm the chemical structure of the synthesized compounds. Tandem mass spectra of protonated and sodiated compounds are depicted in Fig. 2. Fragmentation of the precursor ion which is supposed to be sodiated PC resulted in the mass spectrum represented in Fig. 2a. Indeed, such kind of fragmentation is characteristic for the fragmentation of sodiated PCs [13]. The fragment peaks correspond to the loss of trimethyl ammonium group (m/z 749.5 in Fig. 2a), sodiated and protonated fragment ions with the neutral loss of the choline head group (m/z 625.5, 603.5, correspondingly), sodiated fragment ion with the loss of trimethyl ammonium and

one of fatty acyls (m/z 467.3) and the fragment ion corresponding to the rest of phosphocholine head group (m/z 147.0). Tandem mass spectrum represented in Fig. 2b corresponds to the fragmentation of protonated PC [13]. This fragmentation yields one fragment ion with m/z 184.1 which is PC head group ion. Fragment ions with m/z 147.0 and 184.1 are intensive and characteristic for sodiated and protonated precursor ions and thus could be used for the deconvolution of the overlapping peaks of corresponding precursors. Besides, these fragment ions are close to each other by mass which allows avoiding possible mass discrimination.

Calibration Procedure for Interfered Peaks Deconvolution

Figure 3 demonstrates scheme of the interfered peak formation. Both $[\text{PC1}+\text{Na}]^+$ and $[\text{PC2}+\text{H}]^+$ ions contribute to the I_2 intensity. The information about contribution of each ion to the I_2 intensity can be obtained from the fragmentation of precursor ions with $m/z(2)$. The resulting tandem mass spectrum represents a superposition of spectra from Figs 2a and 2b. It can be supposed that the intensity of specific peaks in the tandem spectrum should be proportional to the number of precursor ions of corresponding species and thus should be correlated with the contribution of these ions to the intensity of the peak in the full mass spectrum, resulting in the following relations: $I_{[\text{PC1}+\text{Na}]^+}^F \sim I_{[\text{PC1}+\text{Na}]^+}$ and $I_{[\text{PC2}+\text{H}]^+}^F \sim I_{[\text{PC2}+\text{H}]^+}$, where $I_{[\text{PC1}+\text{Na}]^+}^F$ and $I_{[\text{PC2}+\text{H}]^+}^F$ are abundances of characteristic fragment ions and they correspond to intensities of peaks with m/z 147.0 in Fig. 2a and with m/z 184.1 in Fig. 2b, respectively. The meaning of $I_{[\text{PC1}+\text{Na}]^+}$ and $I_{[\text{PC2}+\text{H}]^+}$ is shown in Fig. 3.

The following equations can be used to find contributions of different ion species into formation of interfered peak

$$I_{[\text{PC1}+\text{Na}]^+} = \frac{I_2}{I_{[\text{PC1}+\text{Na}]^+}^F + I_{[\text{PC2}+\text{H}]^+}^F} I_{[\text{PC1}+\text{Na}]^+}^F \quad (1)$$

$$I_{[\text{PC2}+\text{H}]^+} = \frac{I_2}{I_{[\text{PC1}+\text{Na}]^+}^F + I_{[\text{PC2}+\text{H}]^+}^F} I_{[\text{PC2}+\text{H}]^+}^F \quad (2)$$

It is expected that the intensities of peaks in the full-scan mass spectrum are proportional to concentrations of corresponding compounds in a sample. PCs are represented in a mass spectrum mainly by protonated and sodiated adducts. Taking these two facts into account, it can be deduced that

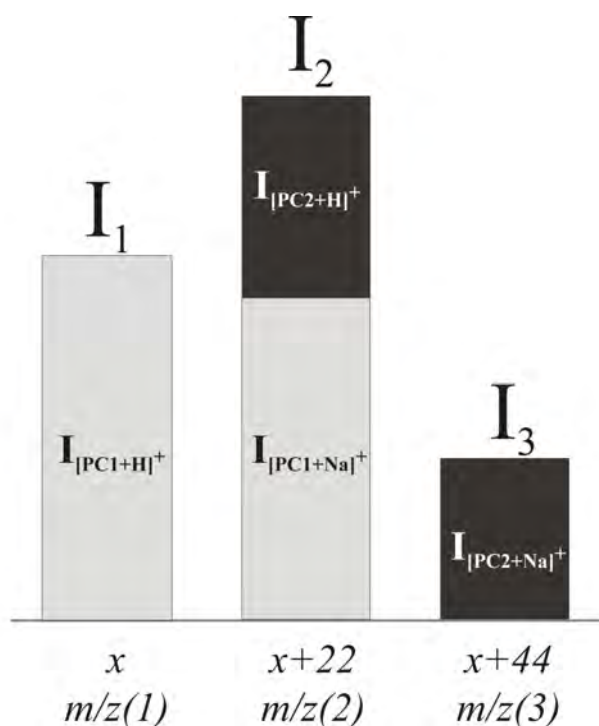


Fig. 3 Scheme of interfered peak formation in full-scan mass spectrum

$$\frac{I_{\text{PC1}}}{I_{\text{PC2}}} = \frac{I_{[\text{PC1+H}]^+} + I_{[\text{PC1+Na}]^+}}{I_{[\text{PC2+H}]^+} + I_{[\text{PC2+Na}]^+}} \sim \frac{c_{\text{PC1}}}{c_{\text{PC2}}} \quad (3)$$

where I_{PC1} and I_{PC2} are summarized abundances of protonated and sodiated lipid molecules, $I_{[\text{PC1+Na}]^+}$ and $I_{[\text{PC2+H}]^+}$ are determined by Eqs (1) and (2), $I_{[\text{PC1+H}]^+}$ and $I_{[\text{PC2+Na}]^+}$ are peak abundances from the full mass spectrum and c_{PC1} and c_{PC2} are concentrations of corresponding lipids. $I_{[\text{PC1+H}]^+}$ and $I_{[\text{PC2+Na}]^+}$ correspond to $m/z(1)$ and $m/z(3)$ in Fig. 3 and for the present work, these are abundances of peaks with m/z 786.6 and 830.6 (Fig. 1). Solutions with PC 18:1/18:1 and PC 18:1/20:4 were mixed at various ratios and investigated with MALDI-MS to find the coefficient of the proportionality in Eq. (3). The full-scan mass spectra and tandem spectra of m/z 808 were measured for all the prepared samples. Using Eqs (1) and (2) and the data from the acquired mass spectra, the dependence for the values in Eq. (3) were built (Fig. 4). The analysis of the data showed that the best linear regression is applicable for the data points corresponding to the samples for which $\frac{c_{\text{PC1}}}{c_{\text{PC2}}} < 2$. The function describing the linear dependence is

$$\frac{I_{\text{PC1}}}{I_{\text{PC2}}} = 1.193 \frac{c_{\text{CP1}}}{c_{\text{CP2}}} + 0.0624 \text{ with the coefficient of determination } R^2 = 0.996. \text{ The}$$

expression reverse to Eq. (3) was used to cover the full range of the PC1 and PC2 concentration ratios. For this case the linear function best fitted the data points is

$$\frac{I_{PC2}}{I_{PC1}} = 0.7783 \frac{c_{CP2}}{c_{CP1}} + 0.023 \text{ with the coefficient of determination } R^2 = 0.9989.$$

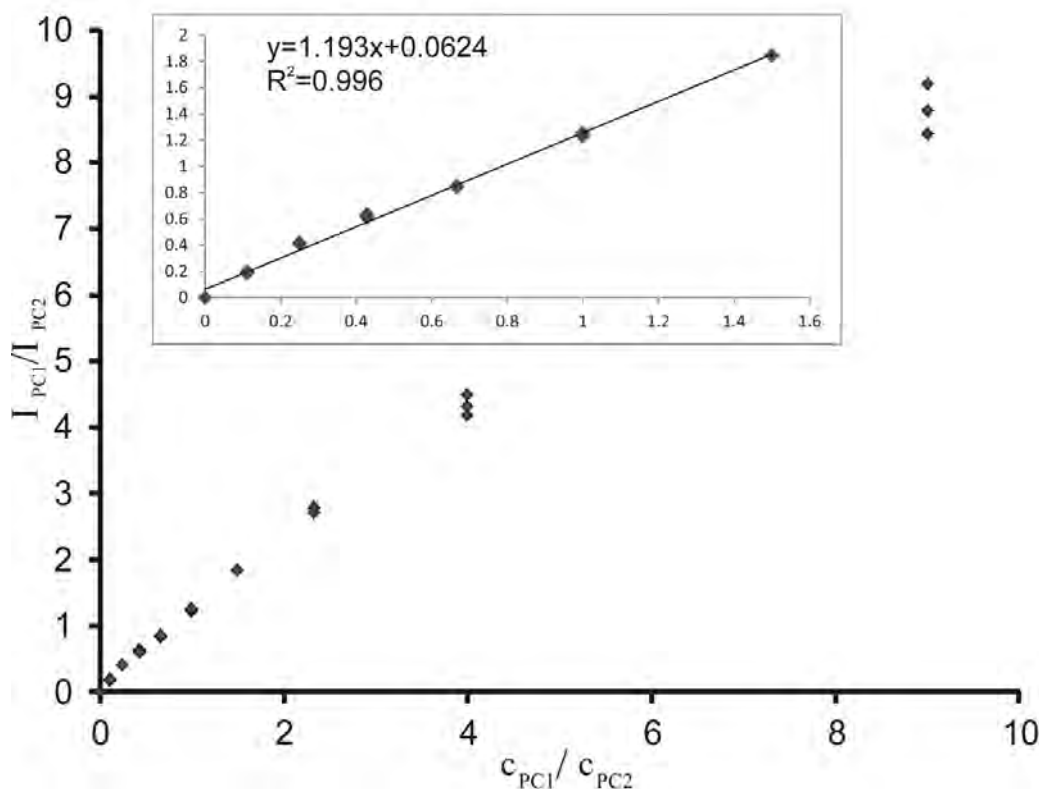


Fig. 4 Calibration curve of summarized peak intensity ratio vs concentration ratio according to Eq. (3)

Checking Stability of Model on Various NaCl Concentrations

A set of samples containing PC 18:1/18:1 and PC 18:1/20:4 in ratio 1:1 (v/v) and concentrations of NaCl in the range from trace amounts to physiological concentration (9 mg ml^{-1}) were prepared and measured to check, if suggested ratios work for different concentrations of Na^+ ions. Abundances of characteristic fragment ions TE $I_{[PC1+H]^+}$ and $I_{[PC2+Na]^+}$ and thus the ratio between them changed

significantly (Figs 5a and 5b). But the value $\frac{I_{[PC1+H]^+} + I_{[PC1+Na]^+}}{I_{[PC2+H]^+} + I_{[PC2+Na]^+}}$ is nearly constant

in the used range of NaCl concentrations (Fig. 5c) and its relative standard deviation is about 8 %. This experiment confirmed the applicability of the developed model for the samples with NaCl concentration up to the physiological one.

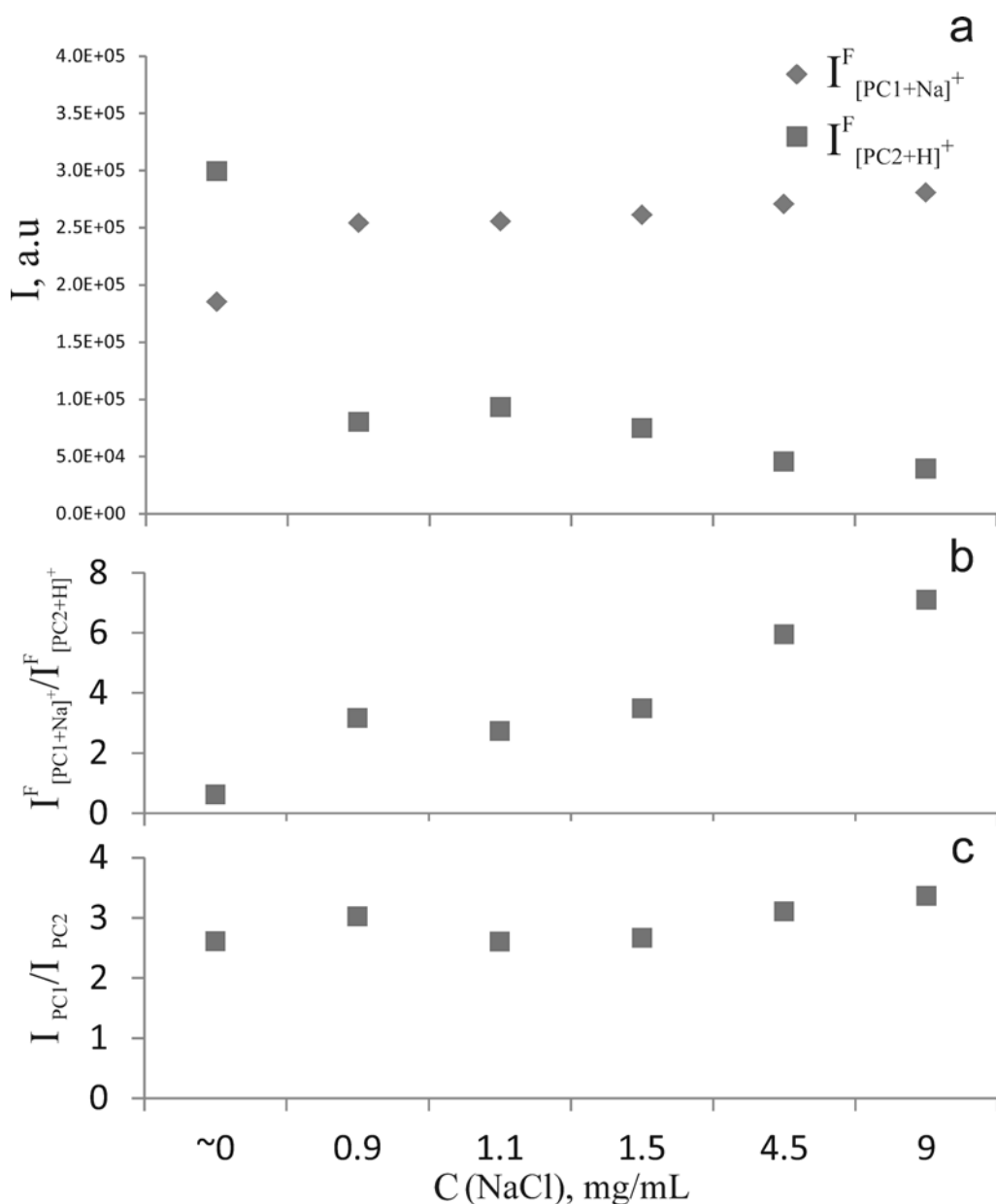


Fig. 5 Dependences of peaks intensities and their ratios on NaCl concentration. a – abundances of characteristic fragment ions; b – ratio of abundances of characteristic fragment ions; c – ratio of PC intensities according to Eq. (3)

Conclusion

The model was suggested for the deconvolution of the overlapping peaks of protonated and sodiated adducts of PCs. The model demonstrated a linear dependence of the summarized abundances ratio on the concentration ratio of PCs under consideration. This fact supposes possibility not only to deconvolute the overlapping peaks but also to estimate relative concentrations of PCs. The model turned out to be stable to changes in sodium ion concentration.

Abbreviations

DCC	dicyclohexylcarbodiimide
DHB	2,5-dihydroxybenzoic acid
DMAP	4-dimethylaminopyridine
FA	fatty acid
LPC 18:1	1-oleoyl-2-hydroxy- <i>sn</i> -glycero-3-phosphocholine
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
MS	mass spectrometry
PCs	phosphatidylcholines

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