



# Lipidomic profiling of biological tissues using off-line two-dimensional high-performance liquid chromatography–mass spectrometry

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## ABSTRACT

Lipids are important components in all biological tissues having many essential roles associated with the proper function of the organism. Their analysis in the biological tissues and body fluids is a challenging task due to the extreme sample complexity of polar lipids and to their amphiphilic character. In this work, we describe a new method for the characterization of the lipid composition in various tissues, using off-line two-dimensional coupling of hydrophilic interaction liquid chromatography (HILIC) and reversed-phase (RP) high-performance liquid chromatography coupled to electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) mass spectrometry. In the first dimension the total lipid extracts are fractionated using HILIC into individual lipid classes. In total, 19 lipid classes (+3 regioisomeric pairs) that cover a wide range of polarities are separated in one analytical run, which is the highest number of analyzed lipid classes reported so far. The lysophospholipid regioisomers are also separated in HILIC mode followed by the identification based on the characteristic ESI mass spectra. The collected fractions of the various lipid classes are further separated in the RP mode, which offers an excellent resolution of the individual lipid species. Their ESI or APCI mass spectra give correct information on the fatty acid composition and on the individual regioisomeric positions on the glycerol skeleton. Off-line coupling of both modes enables the comprehensive analysis of plant and animal samples as illustrated on the analysis of egg yolk, soya and porcine brain tissues.

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## 1. Introduction

Lipidomics is a branch of metabolomics that involves the identification and quantification of cellular lipid molecular species and their interactions with other lipids, proteins and metabolites [1]. Lipidomics contributes towards the understanding how lipids function in a biological system and for the elucidation of the mechanism of lipid-based diseases including atherosclerosis, diabetes, cancer, cardiovascular diseases, obesity, Alzheimer's disease, etc. [1,2]. Lipids may be broadly defined as hydrophobic or amphiphilic small molecules that originate entirely or in part by carbanion-based condensations of thioesters and/or by carbocation-based condensations of isoprene units [3]. Lipids are mostly water-insoluble molecules that represent a wide range of various polar and non-polar compounds containing fatty acids and their derivatives [4]. Lipids have a variety of functions within cells including the source of energy, fat-soluble vitamins, essential fatty acids, structural components of cell membranes, transmission of information in cells, biosynthetic precursor, etc. Lipids can be divided into eight basic categories according to Fahy et al. [3,5]: fatty acyls, glycerolipids,

glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides. The first and very important step in the lipid analysis involves the extraction of lipids from the biological material. The standard method of the lipid extraction was introduced by Folch et al. [6] and later modified by Bligh and Dyer [7]. This method is based on the use of chloroform/methanol/water as a ternary solvent mixture, which separates into two layers: upper (aqueous) layer containing non-lipid compounds and bottom (chloroform) layer containing lipid compounds.

High-performance liquid chromatography (HPLC), thin-layer chromatography (TLC) and gas chromatography (GC) can be applied for specific tasks in the lipidomic analysis. GC is a routine method for the fatty acid profiling after the transesterification to fatty acid methyl esters. In the hydrophilic interaction liquid chromatography (HILIC), individual lipid classes are separated according to their polarity using various silica [8–12] or diol columns [13–17]. Common types of mobile phase systems are the following: chloroform/methanol/water [13,14] and hexane/2-propanol/water [12,15–17]. Formic acid, ammonium acetate, ammonium hydroxide, ammonia, triethylamine and methyl tert-butyl ether can be used as additives. Various types of C<sub>18</sub> [18–25] and C<sub>8</sub> [26,27] columns in reversed-phase (RP) HPLC systems with mobile phases containing methanol/acetonitrile/water [20,22,28], methanol/water [21,23,25], acetonitrile/water [24,26]

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and 2-propanol/acetonitrile/water [18,19] have been reported. Non-aqueous RP (NARP) HPLC with various types of C<sub>18</sub> columns and the mixtures of organic solvents as the mobile phase (acetonitrile, 2-propanol, hexane, dichloromethane, etc.) is used for the separation of non-polar triacylglycerols (TGs) [29–33].

The lipidomic analysis deals with an enormous sample complexity, therefore one-dimensional chromatographic separation may not be sufficient for comprehensive lipidomic characterization. More abundant species can mask trace components, which leads to the loss of information with the potential biological relevance. Two-dimensional (2D) HPLC offers the opportunity to separate complex lipidomic mixtures according two molecular properties, e.g., hydrophobic character of the molecule (RP-HPLC) and electrostatic forces related to the compound polarity (HILIC). 2D-HPLC of lipids can be performed either in on-line [30,34–36] or off-line [37–39] mode. The advantage of off-line 2D is the full optimization of separation conditions in both dimensions [37], but this technique is more laborious and time consuming. On-line 2D-HPLC can be automated, but the chromatographic resolution in the second dimension is sacrificed at cost of sampling time at the first dimension, typically 1 min [30,34,35]. Numerous papers can be found on the detailed 2D-HPLC characterization of one lipid class (typically TGs) [30,34,35,37], but only few works are devoted to the comprehensive lipidomic analysis [36,38,39]. The best separation found in the literature has presented the identification of 721 lipid species from 12 lipid classes [36].

Electrospray ionization (ESI) [10,12,13,18,19,21–24,26,27,40–44] and matrix-assisted laser desorption–ionization (MALDI) [39,45–49] are the most widely used ionization techniques for the identification of polar lipids, while atmospheric pressure chemical ionization (APCI) [31–33,50] is preferred for non-polar lipids. ESI mass spectra in the positive-ion mode provide accurate information on the molecular weight (MW) and on the presence of individual polar head groups. In the ESI negative-ion mode, the presence of [RCOO]<sup>−</sup> fragment ions correspond to the individual fatty acids esterified on the glycerol skeleton. MALDI is often used in connection with TLC separation of lipid classes [45].

The main goal of our work is the development of non-target HPLC/MS method applicable for the comprehensive characterization of wide range of lipid classes in biological tissues, because the HPLC/MS methods used so far in lipidomics are based on the selected reaction monitoring of predefined mass transitions, but they cannot be applied for the determination of all unknown lipids. We use HILIC in the first dimension for the fractionation of total lipid extracts into individual lipid classes, which are then analyzed

using RP separation with ESI or APCI-MS identification of individual species. The developed method is applied for the analysis of lipid composition in selected animal and plant tissues.

## 2. Experimental

### 2.1. Materials

Acetonitrile, 2-propanol, methanol (all HPLC gradient grade), chloroform (HPLC grade, stabilized by 0.5–1% ethanol) and ammonium acetate were purchased from Sigma–Aldrich (St. Louis, MO, USA). De-ionized water was prepared with Demiwa 5-roi purification system (Watek, Ledeč nad Sázavou, Czech Republic) and by Ultra CLEAR UV apparatus (SG, Hamburg, Germany). Standards of lipids containing oleic acid ( $\Delta^9$ cis-C18:1) (i.e., TG, CE, FA, PG, LPG, PI, CA, LPI, pPE, PE, LPE, pPC, PC, SM and LPC – see Table 1 for the definition of lipid abbreviations) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Individual lipid species are annotated according to their fatty acid composition, e.g., 1-octadecenoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphatidylcholine is annotated as 18:1/22:6-PC. Spherisorb Si (250 × 4.6 mm and 150 × 4.6 mm, 5 μm, Waters, Milford, MA, USA), Atlantis Si (150 × 2.1 mm, 3 μm, Waters), Nova-Pak Si (150 × 3.9 mm, 4 μm, Waters), SunFire Si (250 × 4.6 mm, 5 μm, Waters), Purospher Star NH<sub>2</sub> (250 × 4 mm, 5 μm, Merck, Darmstadt, Germany), porous shell particles column Ascentis Si (150 × 2.1 mm, 2.7 μm, Sigma–Aldrich), porous shell particles column Kinetex HILIC (150 × 2.1 mm, 2.6 μm, Phenomenex, Torrance, CA, USA), Ascentis HILIC (150 × 2.1 mm, 3 μm, Sigma–Aldrich) and SeQuant ZIC-HILIC (150 × 2.1 mm, 3.5 μm, SeQuant, Umea, Sweden) columns were used for the optimization of HILIC separation. Hypersil Gold C<sub>18</sub> (150 × 3 mm, 5 μm, Thermo Scientific, Waltham, MA, USA), Luna C<sub>18</sub> (250 × 4.6 mm, 150 × 4.6 mm and 250 × 3.0 mm, 5 μm, Phenomenex), Ultracarb ODS 30 (150 × 3.2 mm, 5 μm, Phenomenex), Nova-Pak C<sub>18</sub> (300 × 3.9 mm and 150 × 3.9 mm, 4 μm, Waters), Purospher Star C<sub>18</sub> (250 × 4 mm, 5 μm, Merck), Zorbax SB-C<sub>18</sub> (150 × 4.6 mm, 3.5 μm, Agilent Technologies, Waldbronn, Germany), Eclipse XDB-C<sub>18</sub> (150 × 4.6 mm, 5 μm, Agilent Technologies), porous shell particles column Ascentis Express C<sub>18</sub> (150 × 2.1 mm, 2.7 μm, Sigma–Aldrich) and porous shell particles column Kinetex C<sub>18</sub> (150 × 2.1 mm, 2.6 μm, Phenomenex) columns were used for the optimization of RP-HPLC separation. Samples of egg, soya and porcine brain were purchased at local stores.

**Table 1**

Abbreviations used for individual lipids classes in accordance with the literature [3,5] and typical neutral losses ( $\Delta m/z$ ) from protonated molecules [M+H]<sup>+</sup> and characteristic ions ( $m/z$ ) used for the identification in positive-ion ESI mass spectra.

Lipid class	Abbreviation	Neutral loss ( $\Delta m/z$ )	Characteristic ion ( $m/z$ )
Triacylglycerol	TG	–	[M+H–R <sub>i</sub> COOH] <sup>+</sup>
Cholesterol	Chol	–	369
Cholesteryl ester	CE	–	–
Fatty acid	FA	–	–
Cytidine diphosphate-diacylglycerol	CDP-DG	–	[M+H–R <sub>i</sub> COOH] <sup>+</sup>
Phosphatidylglycerol	PG	172	–
Lysophosphatidylglycerol	LPG	172	–
Glycosylceramide	GlcCER	162	–
Phosphatidylinositol	PI	260	–
Cardiolipin	CA	–	–
Lysophosphatidylinositol	LPI	260	–
Phosphatidylethanolamine plasmalogen	pPE	141	–
Phosphatidylethanolamine	PE	141	–
Lysophosphatidylethanolamine	LPE	141	–
Phosphatidylcholine plasmalogen	pPC	183	184
Phosphatidylcholine	PC	183	184
Sphingomyeline	SM	183	184
Lysophosphatidylcholine	LPC	183	184

## 2.2. Sample preparation

The total lipid extracts from lipid tissues were prepared according to Folch et al. [6] using chloroform/methanol/water system. Briefly, approximately 1 g of lipid tissue was homogenized with 20 mL mixture of chloroform/methanol (2:1, v/v) and the homogenate was filtered using a coarse filter paper. Then, 4 mL of 1 M NaCl was added and the mixture was centrifuged at 3000 rpm for 5 min at room temperature. The chloroform (bottom) layer containing lipids was evaporated by a gentle stream of nitrogen and redissolved in 2-propanol/water (1:1, v/v) for HILIC analysis.

## 2.3. HPLC/MS conditions

All HPLC experiments were performed on a liquid chromatograph Agilent 1200 series (Agilent Technologies). The UV detection at 205 nm and the Esquire 3000 ion trap analyzer with (Bruker Daltonics, Bremen, Germany) were coupled in series, where ESI is used for the detection of all polar phospholipid species, while APCI is preferred for non-polar lipids, such as TGs.

### 2.3.1. HILIC fractionation of lipid classes

In the first dimension, HILIC was used for the fractionation of total lipid extracts into lipid classes using Spherisorb Si column (250 × 4.6 mm, 5 μm, Waters), a flow rate of 1 mL/min, an injection volume of 10 μL, separation temperature of 40 °C and a mobile phase gradient: 0 min – 94% A + 6% B, 60 min – 77% A + 23% B, where A is acetonitrile and B is 5 mM aqueous ammonium acetate. The injector needle was washed with the mobile phase after each injection. Lipid classes were identified using ESI-MS in the mass range  $m/z$  50–1500 with the following setting of tuning parameters: pressure of the nebulizing gas of 60 psi, the drying gas flow rate of 10 L/min and temperature of the drying gas 365 °C. Fractions of lipid classes were collected manually, evaporated by a gentle stream of nitrogen and redissolved in the initial mobile phase composition for the 2D analysis. The volume for redissolution was selected according to the concentration of individual fractions in the range of 100–1000 μL.

### 2.3.2. RP-HPLC separation of polar lipids

In the second dimension, collected fractions of lipid classes were analyzed using two RP-HPLC methods according to their polarity. Fractions of polar lipids were analyzed using RP-HPLC with porous shell particles column Kinetex C<sub>18</sub> (150 × 2.1 mm, 2.6 μm, Phenomenex), the flow rate of 0.3 mL/min, the injection volume of 1 μL, separation temperature of 40 °C and the mobile phase gradient: 0 min – 75% A + 25% B, 100 min – 88% A + 12% B, where A is the mixture of acetonitrile/2-propanol (1:1, v/v) and B is 5 mM aqueous ammonium acetate. Polar lipids were identified using ESI-MS in the mass range  $m/z$  50–1500 with the following setting of tuning parameters: pressure of the nebulizing gas of 40 psi, the drying gas flow rate of 9 L/min and temperature of the drying gas 365 °C. The low energy collision induced dissociation tandem mass spectrometry (MS/MS) experiments were performed during HPLC/MS runs with the automatic precursor selection, the isolation width of  $m/z$  4, the collision amplitude of 1 V and helium as a collision gas.

### 2.3.3. NARP-HPLC separation of non-polar lipids

Fractions of non-polar lipids were analyzed by non-aqueous reversed phase (NARP) HPLC/APCI-MS with C<sub>18</sub> columns according to our previous work [31], *i.e.*, two Nova-Pak C<sub>18</sub> columns (150 × 3.9 and 300 × 3.9 mm, 4 μm, Waters) connected in series, the flow rate of 1 mL/min, the injection volume of 1 μL, separation temperature of 25 °C and the mobile phase gradient: 0 min – 100% acetonitrile, 106 min – 31% acetonitrile + 69% 2-propanol. Non-polar lipids were identified using APCI-MS in the mass range  $m/z$  50–1200,

pressure of the nebulizing gas of 70 psi, the drying gas flow rate of 3 L/min, temperatures of the drying gas and APCI heater 350 °C and 400 °C, respectively.

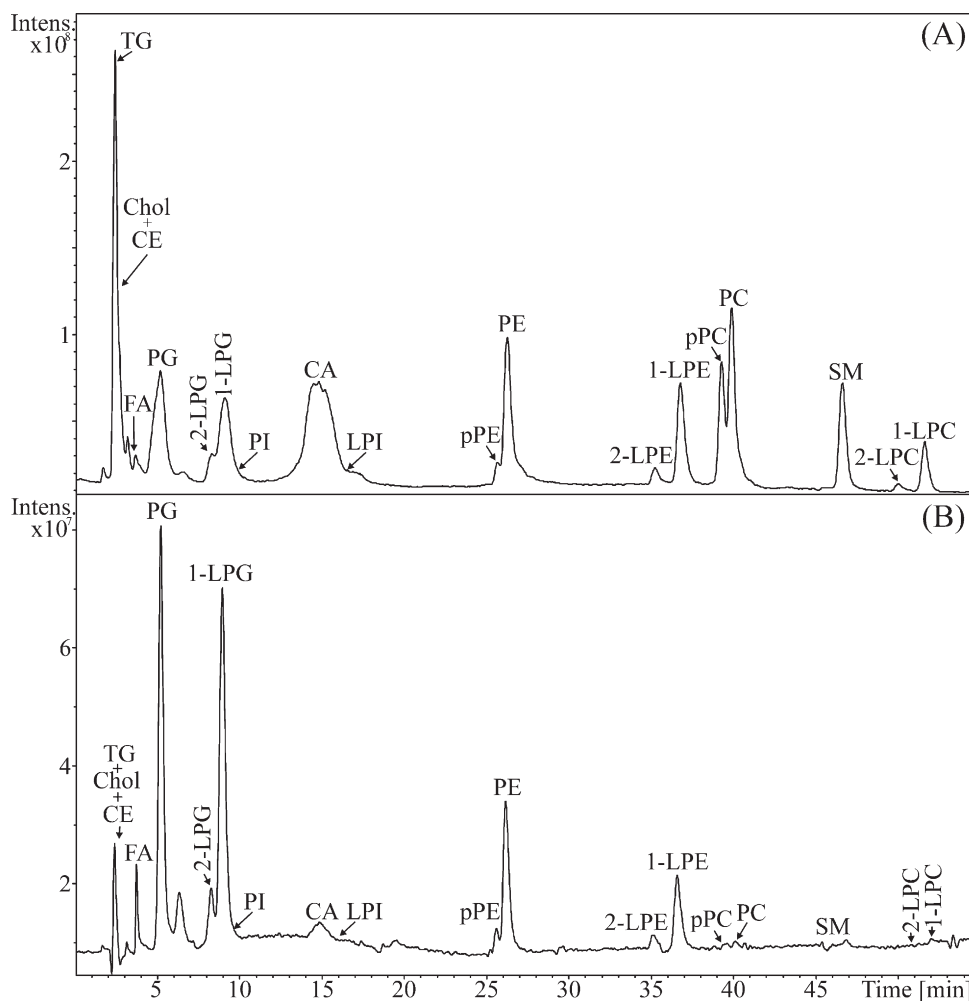
## 3. Results and discussion

### 3.1. Optimization of HILIC separation

The total lipid extracts prepared by the modified Folch extraction procedure, have been fractionated by HILIC into the individual lipid classes. First, chromatographic conditions have been carefully optimized (Figs. S1–S3) to achieve best separation of the maximum number of lipid classes. For this purpose, column packing, mobile phase composition (*i.e.*, type of organic solvent, concentration of water, concentration of additive and pH value), separation temperature and gradient steepness have been tested using a mixture of 16 standards of lipid classes represented by species containing oleic acid ( $\Delta^9$ *cis*-C18:1) and the total lipid extract from egg yolk containing lipid classes with a wide range of polarities. Nine commercially available silica columns with porous and porous shell particles designed especially for HILIC separation or conventional silica columns for NP-HPLC from various manufacturers have been tested (columns are listed in Section 2, representative chromatograms are shown in Fig. S1). Both types of HILIC and NP-HPLC columns provide the separation of lipid classes with similar results. Greater differences are observed among columns from different manufacturers due to diverse packing procedures and between porous vs. porous shell particles columns. Porous shell particles HILIC columns (Fig. S1A) permit a good resolution of the lipid classes, however, we have observed the limited sample capacity of these columns which makes them inconvenient for the fractionation. The best chromatographic resolution of critical classes with lower retention times and the ability to work with higher sample load are achieved with NP-HPLC silica column with porous particles (Spherisorb Si, 250 × 4.6 mm, 5 μm) (Fig. 1).

The mobile phase composition plays a critical role in the separation selectivity of HILIC. A number of common HPLC organic solvents in the mixture with aqueous ammonium acetate have been tested as the mobile phase components for the HILIC analysis of lipids, *e.g.*, acetonitrile, methanol, ethanol, 2-propanol, hexane and their mixtures. The type of organic solvent has a significant effect on the chromatographic resolution of lipids. Good resolution of lipid classes is obtained with the mixture of hexane/2-propanol/aqueous ammonium acetate, but poor reproducibility caused by a limited miscibility and increased background noise in HPLC/MS disfavors this mobile phase composition. The best selectivity, chromatographic resolution and low background noise are observed in acetonitrile/water mobile phase with the addition of ammonium acetate.

Water present in the mobile phase is adsorbed on the surface of the silica stationary phase and participates in the HILIC separation process [51]. Therefore, the water concentration is crucial for the separation selectivity and chromatographic resolution in HILIC separation of lipids, especially important is the initial percentage of water. The minimum amount of 4% of water in the mobile phase has been found as the basic prerequisite for the reasonable separation. The concentration of salts and pH value of mobile phase are also important parameters, especially for ionic lipids. The use of strong ion-pairing agents (*e.g.*, alkyl ammonium salts) can significantly improve the chromatographic resolution of charged compounds, but it causes a strong signal suppression and contamination of MS system. Volatile mobile phase modifiers (*e.g.*, ammonium acetate) yield almost comparable chromatographic separation. These agents are MS compatible, but their concentration should be kept as low as possible to reduce the ion suppression



**Fig. 1.** HILIC/ESI-MS analysis of lipid classes represented by standards containing oleic acid ( $\Delta 9_{cis}$ -C18:1) using Spherisorb Si column ( $250 \times 4.6$  mm,  $5 \mu\text{m}$ ) and ESI-MS detection in (A) positive-ion mode and (B) negative-ion mode. HPLC conditions: flow rate 1 mL/min, separation temperature  $40^\circ\text{C}$ , gradient 0 min – 94% A + 6% B, 60 min – 77% A + 23% B, where A is acetonitrile and B is 5 mM aqueous ammonium acetate. Peak annotation: TG: triacylglycerol, Chol: cholesterol, CE: cholesteryl ester, FA: fatty acid, PG: phosphatidylglycerol, 2-LPG: 2-lysophosphatidylglycerol, 1-LPG: 1-lysophosphatidylglycerol, PI: phosphatidylinositol, CA: cardiolipin, LPI: lysophosphatidylinositol, pPE: phosphatidylethanolamine plasmalogen, PE: phosphatidylethanolamine, 2-LPE: 2-lysophosphatidylethanolamine, 1-LPE: 1-lysophosphatidylethanolamine, pPC: phosphatidylcholine plasmalogen, PC: phosphatidylcholine, SM: sphingomyeline, 2-LPC: 2-lysophosphatidylcholine, 1-LPC: 1-lysophosphatidylcholine.

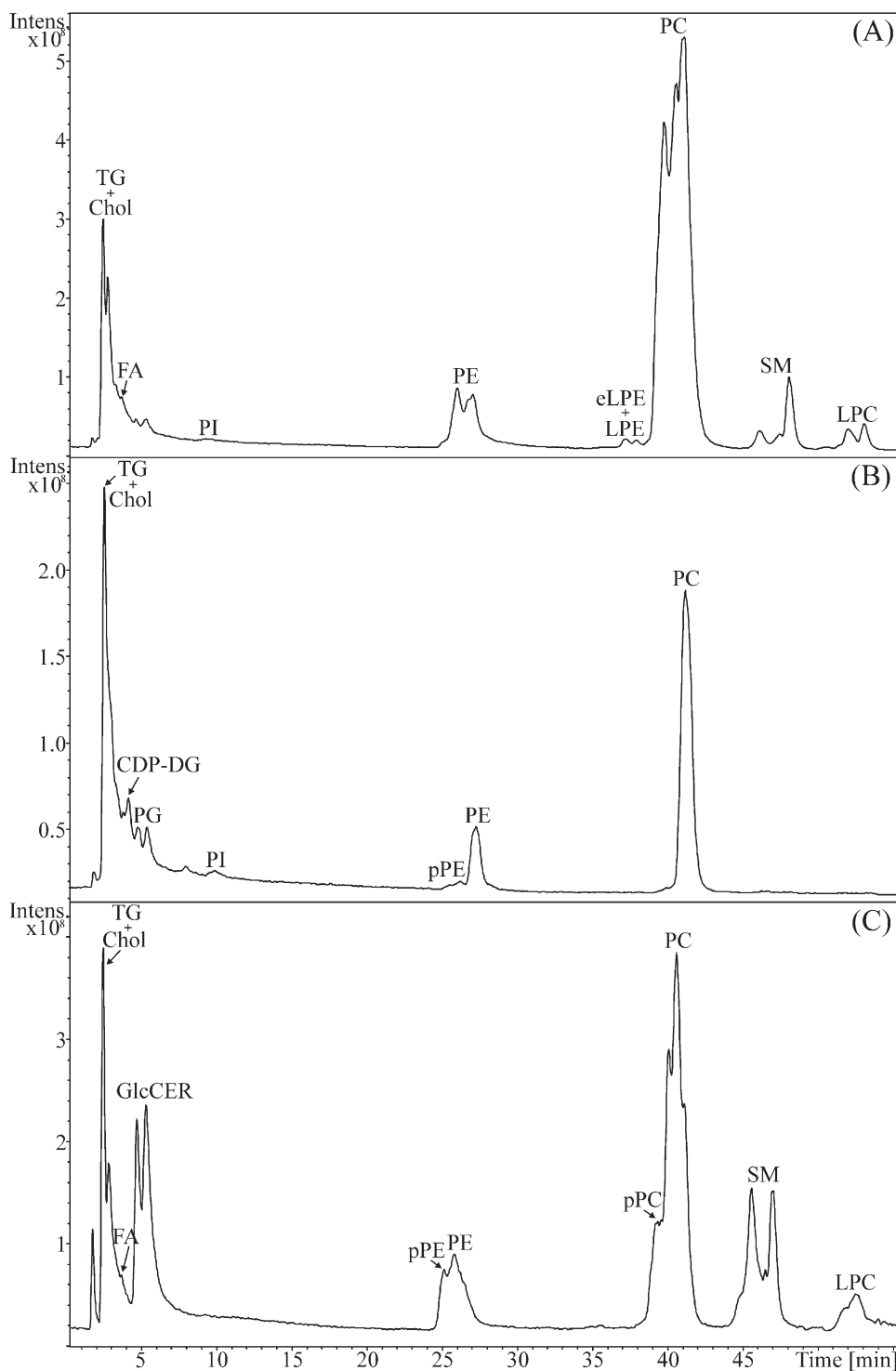
[52]. In our method, ammonium acetate concentration and pH value have only a small effect on retention times of lipid classes with higher retention times (LPC, SM, PC, LPE and PE), but rather significant effect for classes with low retention times, *i.e.*, PG, LPG, CA (Figs. S2 and S3). The salt concentration and pH value strongly influence the ionization efficiency of lipids, which is significantly lower in mobile phases without ammonium acetate (Fig. S2) and at lower pH values (Fig. S3). The best separation and signal intensity have been achieved in mobile phases with 5 mM aqueous ammonium acetate at the neutral pH. Changes of gradient steepness and separation temperature have only negligible effects on the chromatographic resolution.

### 3.2. HILIC fractionation of lipids

In HILIC mode (Figs. 1 and 2), lipids are separated into classes that are identified based on the neutral losses of their polar head groups and characteristic product ions in full-scan positive-ion ESI mass spectra (Table 1). In general, the retention mechanisms in HILIC are based on the adsorption of the solute on the column packing and/or the partitioning of the solute into a water layer that is formed on the surface of the column packing. These interactions belong to the group of electrostatic forces. The typical rule for the

retention of lipids in HILIC is that compounds with higher polarity or stronger dissociation are more retained, but other mechanisms are involved in case of Chol, FA, PG and LPG classes, where relatively low retention times in HILIC are observed that are comparable to neutral (non-polar) classes of TGs and CEs without any ionic or polar functional group (Fig. 1). Most lipid classes are baseline separated using our optimized HILIC method except for non-polar lipids (TG, Chol and CE) which are coeluting at retention times close to the void volume of the system. These classes are collected in one fraction, but TG and Chol can be easily separated using RP-HPLC mode in the second dimension. Lipids inside individual classes are partially separated according to the number of double bonds. Polyunsaturated species containing C22:6, C22:5 and C20:4 acids are less retained and they form partially separated peaks at the front of particular classes, for example, classes of PE, PC, SM (Fig. 2A and C) are partially separated into two or three peaks according to the unsaturation level of individual species.

The optimized HILIC method enables the partial separation of phospholipid plasmalogens from their phospholipid analogs, *i.e.*, pPE from PE (Figs. 1 and 2B and C) and pPC from PC (Figs. 1 and 2C). *Sn*-1 and *sn*-2 regioisomers of lysophospholipid are also separated in the HILIC mode (Fig. 1), *i.e.*, 2-LPG and 1-LPG, 2-LPE and 1-LPE, 2-LPC and 1-LPC. *Sn*-1 lysophospholipids have higher reten-

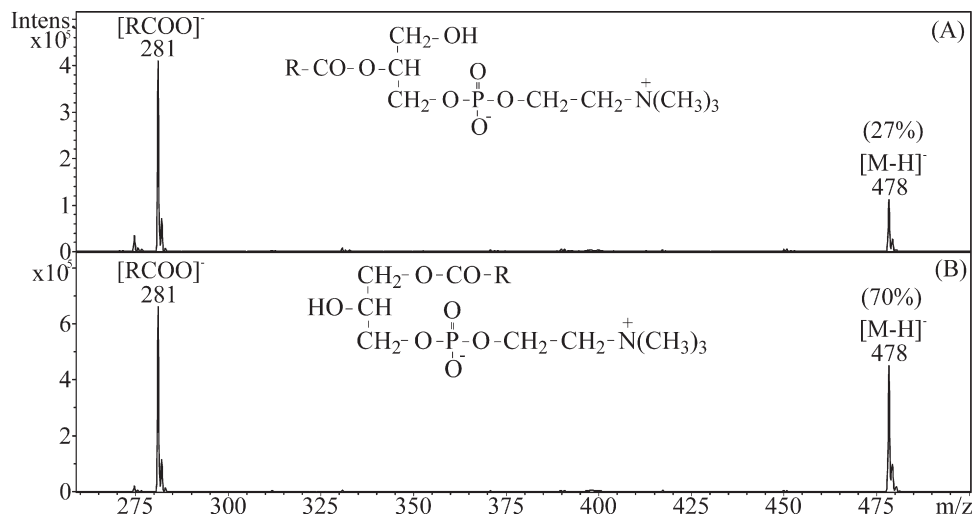


**Fig. 2.** HILIC with positive-ion ESI-MS detection of total lipid extracts from (A) egg yolk, (B) soya, and (C) porcine brain tissues. HPLC conditions and peak annotation are identical as for Fig. 1, CDP-DG: cytidine diphosphate-diacylglycerol, GlcCER: glycosylceramide.

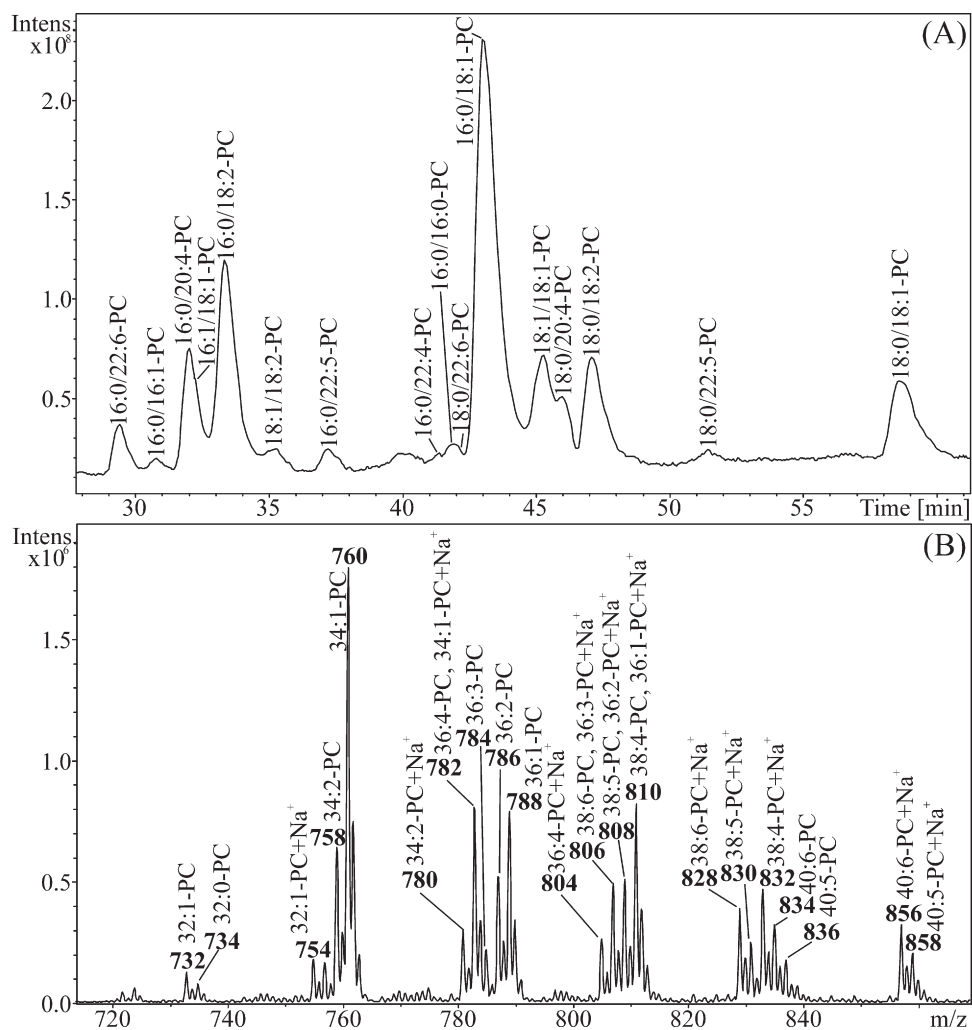
tion in comparison to *sn*-2 isomers. Individual regioisomers are identified by negative-ion ESI full-scan mass spectra based on the relative abundance of carboxylate ions  $[\text{RCOO}]^-$  (Fig. 3). The relative abundance of the  $[\text{RCOO}]^-$  ion formed by cleavage of the fatty acid from the *sn*-2 position of phospholipids is higher in comparison to the ion formed by cleavage from the *sn*-1 position [12]. This rule has been applied for the identification of lysophospholipid regioisomers, *i.e.*, the higher intensity of  $[\text{RCOO}]^-$  ion corresponds to *sn*-2 isomer. Ratios of relative abundances of  $[\text{RCOO}]^-/[\text{M}-\text{H}]^-$  ions for lysophospholipid regioisomers are

100/27 for 2-LPE (Fig. 3A) and 100/70 for 1-LPE (Fig. 3B), 100/75 for 2-LPC and 45/100 for 1-LPC, 100/30 for 2-LPG and 62/100 for 1-LPG. The identification of *sn*-1 and *sn*-2 isomers is confirmed by identical standards. The synthesis of lysophospholipids produces mainly *sn*-1 isomers due to the thermodynamic preference. The small concentration of *sn*-2 isomers is formed via the fatty acyl migration on the glycerol during the purification process, which is in accordance with our results that significantly higher concentration is observed for *sn*-1 lysophospholipids in comparison to *sn*-2 isomers (Fig. 1).

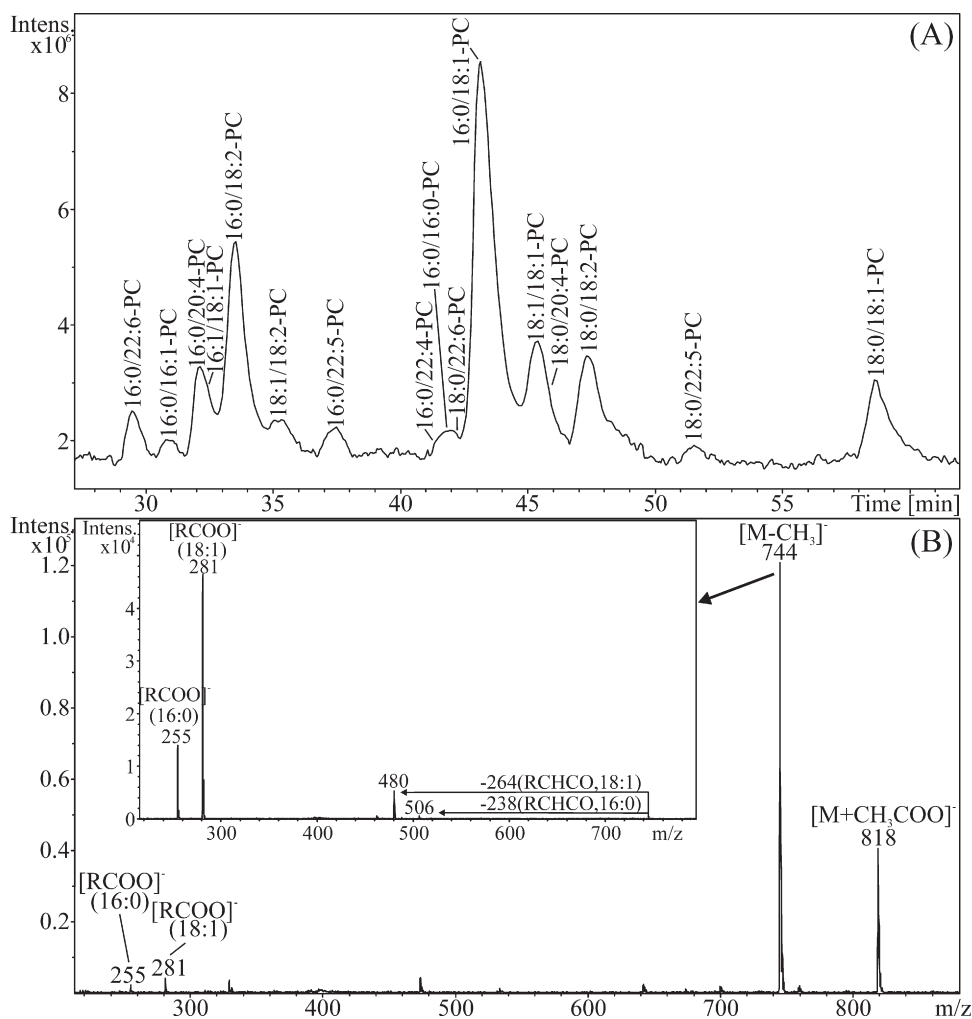




**Fig. 3.** Negative-ion ESI mass spectra of (A) 1-hydroxy-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine (2-LPE), and (B) 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphatidylethanolamine (1-LPE) standards.



**Fig. 4.** Comparison of (A) RP-HPLC/ESI-MS analysis of PC fraction from egg yolk in the positive-ion mode and (B) positive-ion ESI mass spectrum of PC fraction from HILIC. HPLC conditions: porous shell particles column Kinetex C<sub>18</sub> (150 × 2.1 mm, 2.6 μm), flow rate 0.3 mL/min, separation temperature 40 °C, gradient 0 min – 75% A + 25% B, 100 min – 88% A + 12% B, where A is the mixture of acetonitrile/2-propanol (1:1, v/v) and B is 5 mM aqueous ammonium acetate.



**Fig. 5.** RP-HPLC/ESI-MS analysis of (A) PC fraction from egg yolk in the negative-ion mode and (B) negative-ion ESI mass spectrum of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (16:0/18:1-PC) with inset of MS/MS spectrum of  $m/z$  744 used for determination of regioisomeric position of acyl chains. HPLC conditions are identical as for Fig. 4.

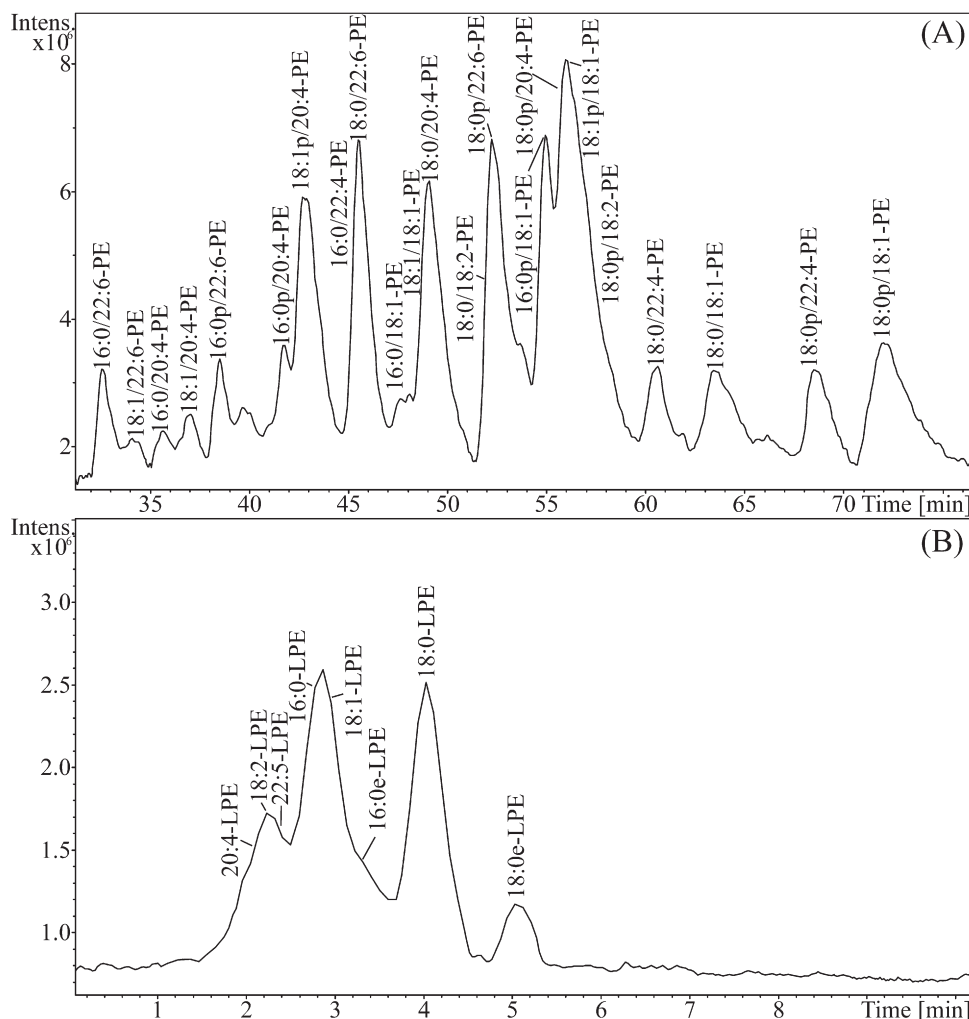
### 3.3. Reversed-phase HPLC/MS analysis of lipids

RP-HPLC method has been developed for the analysis of lipid fractions in the second dimension using  $C_{18}$  column and acetonitrile/2-propanol/aqueous ammonium acetate gradient. The special attention has been paid to the optimization of chromatographic conditions to achieve the highest number of separated species. Nine  $C_{18}$  columns with various column packing from different manufacturers have been tested (columns are listed in Section 2, representative chromatograms are shown in Fig. S4). Porous shell  $C_{18}$  column provides the best chromatographic resolution for most lipid species within a reasonable retention window (Figs. 4–7 and Fig. S4A). Similarly to HILIC, water concentration in the mobile phase is especially important at the beginning of gradient, which is one of the most critical parameter affecting the chromatographic resolution of lipids in RP-HPLC. Higher concentration of water significantly improves the chromatographic resolution and also the signal to noise ratio (Fig. S5). Water has also well pronounced impact on retention times of polyunsaturated species, which are retained more strongly at higher concentrations of water than other species, which can result in changed retention order (Fig. S5). The concentration of ammonium acetate does not show a visible effect on the chromatographic resolution or retention times of lipids, but it improves the ionization efficiency of lipids (Fig. S6). Other parameters, such as pH value of mobile phase (Fig. S7), gradient steepness

(Fig. S8) and separation temperature, have negligible effects on the RP-HPLC separation.

In the RP-HPLC method (Figs. 4–7), lipid species are separated according to the acyl chain lengths and the number of double bonds. The chromatographic pattern partially fits to the well known model used for TGs [31–33] (Fig. 8), where the equivalent carbon number (ECN) is defined as the total carbon number (CN) of fatty acyls minus two times the double bond (DB) number ( $ECN = CN - 2DB$ ). The retention of lipids in RP-HPLC increases proportionally to their ECN, except for polyunsaturated species containing fatty acyls with four and more double bonds, which are retained more strongly and they elute in higher ECN groups. For example, 16:0/22:6-PC with  $ECN = 26$  and 16:0/20:4-PC with  $ECN = 28$  elute in the group with  $ECN = 30$  (Fig. 4A). The same chromatographic behavior is observed for all lipid classes in RP-HPLC (Figs. 4–7). Plasmalogen (pPE) and ether (eLPE) lipids (Fig. 6) have higher retention times in RP-HPLC in comparison to their phospholipid (PE and LPE) analogs.

ESI-MS and MS/MS spectra in both the positive-ion and negative-ion modes are used in the RP-HPLC/MS analysis of complex lipids. Full-scan positive-ion ESI mass spectra (Fig. 4B) allows the determination of molecular weights based on protonated molecules  $[M+H]^+$ , sodium  $[M+Na]^+$  and potassium  $[M+K]^+$  adducts. In the positive-ion ESI-MS mode, the neutral losses of polar head groups afforded characteristic fragment ions are observed



**Fig. 6.** RP-HPLC/ESI-MS analysis in the negative-ion mode of (A) PE fraction containing plasmalogens (pPE) from porcine brain and (B) LPE fraction containing alkyl ethers (eLPE) from egg yolk. p refers to plasmalogens and e refers to alkyl ethers. HPLC conditions are identical as for Fig. 4.

(Table 1). Whereas, in the negative-ion ESI-MS mode (Fig. 5B), we observed the deprotonated molecules  $[M-H]^-$ , the adducts with chloride  $[M+Cl]^-$ , acetate  $[M+CH_3COO]^-$  and/or the loss of methyl  $[M-CH_3]^-$  with low relative abundances of fatty acyl fragment ions  $[RCOO]^-$ . The latter are used for the determination of the fatty acid composition of the individual species. The relative abundances of aforementioned product ions in the low-energy MS/MS analyses (inset of Fig. 5B) provide clear information for the determination of their *sn*-1 or *sn*-2 positions based on the higher intensity of  $[RCOO]^-$  product ions formed by losses from the *sn*-2 position. Fig. 4 shows the comparison of RP-HPLC/ESI-MS analysis of PC fraction and ESI mass spectrum of the peak of whole PC group from HILIC. First, the fraction of PC is collected during the HILIC analysis and then this fraction can be either analyzed by RP-HPLC/ESI-MS (Fig. 4A) or measured directly by ESI-MS without a chromatographic separation (Fig. 4B). RP-HPLC/ESI-MS enables the determination of fatty acid combinations in individual lipids including the determination of their *sn*-1 or *sn*-2 positions even for trace concentrations (Fig. 4A), while the direct ESI-MS is sufficient only for the determination of carbon number and double bond number (Fig. 4B). In addition, the presence of sodiated molecules  $[M+Na]^+$  complicates the identification in the spectra measured by direct infusion ESI-MS, because they have identical nominal masses as protonated molecules of lipids differing by two methylene units and three double bonds,

e.g., protonated 36:4-PC has the same nominal mass  $m/z = 782$  as sodiated 34:1-PC. Their resolution requires a high-resolution mass analyzer or chromatographic separation (Fig. 4A).

The fraction of non-polar TGs containing cholesterol has been analyzed using our previously developed NARP-HPLC method with APCI-MS detection using  $C_{18}$  column in the total length of 75 cm, separation temperature 25 °C and the gradient of acetonitrile/2-propanol [31] (Fig. 8). Cholesterol is clearly separated from TGs in NARP-HPLC. Individual TGs are separated according to their ECN as described in more details earlier [31–33]. Most TGs inside one ECN group are separated according to acyl chain lengths and the double bond number. TGs with higher number of double bonds have lower retention times than saturated TGs with the same ECN, e.g., 18:1/18:1/18:1-TG has slightly lower retention in comparison to 18:1/18:1/16:0-TG or even 16:0/18:1/16:0-TG, all with ECN=48. APCI-MS is suitable for the identification of TGs due to their non-polar character. Individual TG species and their fatty acid composition are identified based on their full-scan positive-ion APCI mass spectra containing both abundant fragment ions and  $[M+H]^+$  and  $[M+NH_4]^+$  ions. Ratios of fragment ions  $[M+H-RCOOH]^+$  are used for the determination of fatty acids stereochemical positions, because the neutral loss of fatty acid from *sn*-2 position provides the fragment ion with lower relative abundance in comparison to *sn*-1 and *sn*-3 positions.



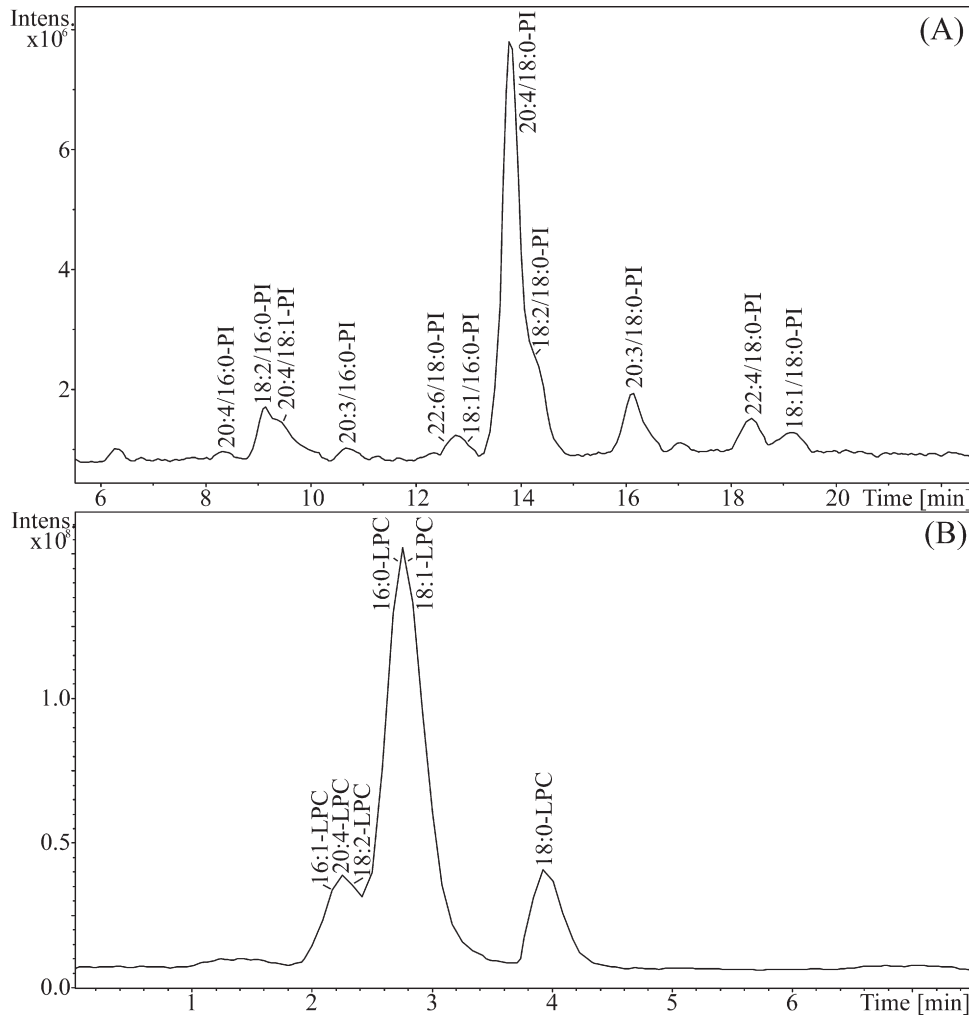


Fig. 7. RP-HPLC/ESI-MS analysis in the positive-ion mode of (A) PI, and (B) LPC fractions from egg yolk. HPLC conditions are identical as for Fig. 4.

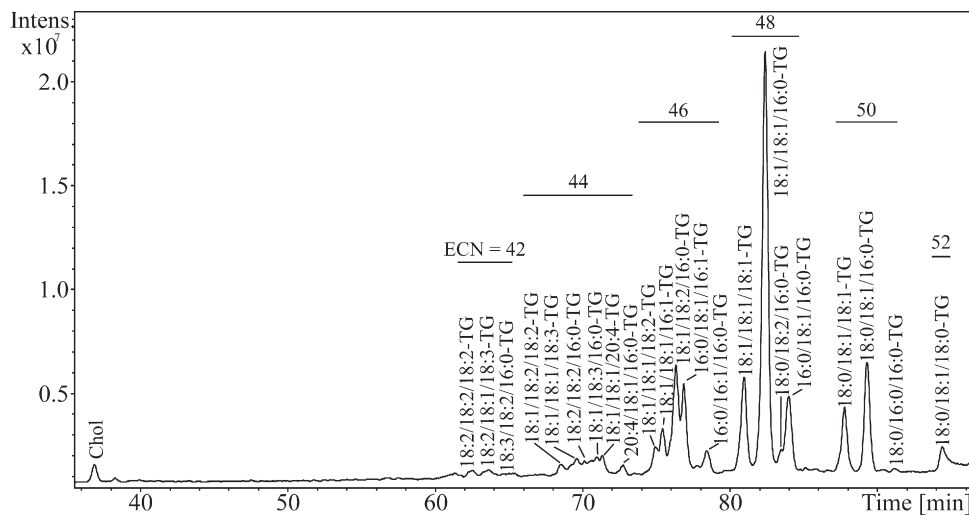
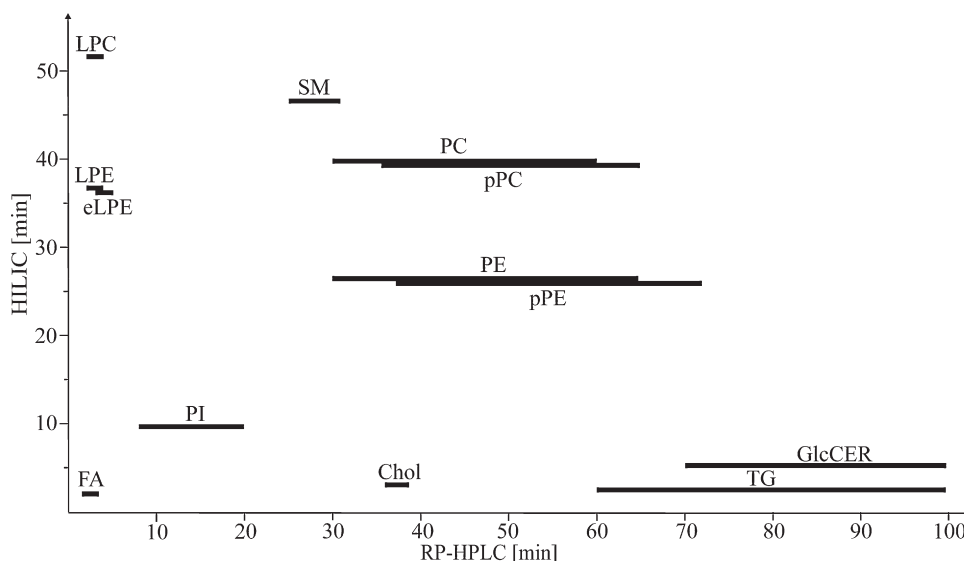


Fig. 8. NARP-HPLC/APCI-MS analysis in the positive-ion mode of TG fraction containing cholesterol from egg yolk. HPLC conditions: two Nova-Pak C<sub>18</sub> columns (150 × 3.9 and 300 × 3.9 mm, 4 μm, Waters) connected in series, flow rate 1 mL/min, separation temperature 25 °C, gradient 0 min – 100% acetonitrile, 106 min – 31% acetonitrile + 69% 2-propanol.



**Fig. 9.** 2D plot compiled from HILIC and RP-HPLC retention times of lipids detected in studied samples. Retention times of Chol and TG species are from NARP-HPLC analysis. HPLC conditions are listed in Section 2.

### 3.4. Off-line 2D-HPLC/MS analysis of samples

Optimized off-line 2D-HPLC/MS method is applied for the characterization of lipid composition in egg yolk, soya and porcine brain tissues (Figs. 2 and 4–8). Fig. 9 depicts 2D plot compiled from retention times of lipid classes using HILIC and individual lipid species using RP-HPLC. The plot shows an excellent orthogonality of both modes, which enables detailed lipidomic characterization and also the potential of this system for on-line 2D-HPLC/MS lipidomic analysis. In total, 15 lipid classes have been identified in analyzed samples using optimized HILIC method. The tested biological samples do contain 10 (egg yolk, porcine brain) or 8 (soya) different lipid classes. TG, Chol, PE and PC classes are present in all samples. The most abundant classes are TG and PC. eLPE in egg yolk (Figs. 2A and 6B) and pPC and pPE in soya (Fig. 2B) and porcine brain (Fig. 2C) samples are identified. In egg yolk and porcine brain samples, both *sn*-1 and *sn*-2 regioisomers of LPE and LPC are detected with a strong preference of *sn*-1 isomer over *sn*-2 isomer. More than 150 lipid species are identified using RP-HPLC/MS of fractions in analyzed samples. The number of lipid species varies among classes, which is given by fatty acid composition of the sample and their combinations on the glycerol skeleton. For example, the sample of egg yolk contains 22 species of TG and 1 Chol (Fig. 8), 6 FA (not shown), 11 PI (Fig. 7A), 14 PE (not shown), 6 LPE and 2 eLPE (Fig. 6B), 16 species of PC (Figs. 4A and 5A), 6 SM (not shown) and 6 LPC (Fig. 7B). TGs are composed from fatty acids containing 0–3 double bonds and 16 or 18 carbons except for 18:1/18:1/20:4-TG and 20:4/16:0:18:1-TG. Complex lipids are composed from the wide range of fatty acids containing 0–6 double bonds and 16–22 carbon atoms. They often have the combination of one saturated or monounsaturated acid and one polyunsaturated acid, e.g., 16:0/22:6-PC. The strong preference of unsaturated fatty acids in the *sn*-2 position is observed for all identified glycerolipids except for PI, where saturated fatty acids are favored in *sn*-2 position (Fig. 7A). In total, 9 fatty acids in egg yolk, 6 in soya and 9 in porcine brain samples are observed.

## 4. Conclusions

This work presents off-line 2D combination of HILIC fractionation in the first dimension followed by the RP-HPLC analysis of collected fractions in the second dimension. Both systems show

an excellent orthogonality enabling the comprehensive characterization of lipid composition in complex biological samples as demonstrated on egg yolk, soya and porcine brain tissues. Lipids are separated into classes according to their polarity and electrostatic interactions in HILIC mode, while the retention of lipids in RP-HPLC is governed by acyl chain lengths and the number of double bonds. The separation of *sn*-1 and *sn*-2 lysophospholipid regioisomers in HILIC mode is achieved by carefully optimized chromatographic conditions. Higher relative abundance of fatty acyl ions formed by the cleavage of fatty acid from *sn*-2 position in negative-ion ESI mass spectra enables the differentiation of lysophospholipid regioisomers. RP-HPLC mode also provides the resolution of plasmalogen and ether phospholipids in analyzed samples. Fatty acid composition of individual lipids and positions of fatty acyls on the glycerol skeleton are determined based on their mass spectra. Unsaturated fatty acids are preferentially esterified in *sn*-2 position in all lipid classes except for PIs, where saturated fatty acids are favored in this position for unknown reason. Off-line 2D-HPLC/MS analysis of lipids is time consuming method, but it provides valuable information for the identification of species at trace concentrations in comparison to one-dimensional HPLC or even the direct infusion analysis.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.05.081.

### References

- [1] A.D. Watson, J. Lipid Res. 47 (2006) 2101.
- [2] M.R. Wenk, Nat. Rev. Drug Discov. 4 (2005) 594.
- [3] E. Fahy, S. Subramaniam, R.C. Murphy, M. Nishijima, C.R.H. Raetz, T. Shimizu, F. Spener, G. van Meer, M.J.O. Wakelam, E.A. Dennis, J. Lipid Res. 50 (2009) S9.
- [4] W.W. Christie, <http://lipidlibrary.aocs.org/index.html>, American Oil Chemists' Society, 2011.

- [5] E. Fahy, S. Subramaniam, H.A. Brown, C.K. Glass, A.H. Merrill, R.C. Murphy, C.R.H. Raetz, D.W. Russell, Y. Seyama, W. Shaw, T. Shimizu, F. Spener, G. van Meer, M.S. VanNieuwenhze, S.H. White, J.L. Witztum, E.A. Dennis, *J. Lipid Res.* 46 (2005) 839.
- [6] J. Folch, M. Lees, G.H.S. Stanley, *J. Biol. Chem.* 226 (1957) 497.
- [7] E.G. Bligh, W.J. Dyer, *Can. J. Biochem. Physiol.* 37 (1959) 911.
- [8] D.Y. Wang, W.M. Xu, X.L. Xu, G.H. Zhou, Y.Z. Zhu, C.B. Li, M.M. Yang, *Food Chem.* 112 (2009) 150.
- [9] A. Cascone, S. Eerola, A. Ritieni, A. Rizzo, *J. Chromatogr. A* 1120 (2006) 211.
- [10] P.M. Hutchins, R.M. Barkley, R.C. Murphy, *J. Lipid Res.* 49 (2008) 804.
- [11] U. Sommer, H. Herscovitz, F.K. Welty, C.E. Costello, *J. Lipid Res.* 47 (2006) 804.
- [12] M. Pulfer, R.C. Murphy, *Mass Spectrom. Rev.* 22 (2003) 332.
- [13] E. Hvattum, C. Rosjo, T. Gjoen, G. Rosenlund, B. Ruyter, *J. Chromatogr. B* 748 (2000) 137.
- [14] S. Uran, A. Larsen, P.B. Jacobsen, T. Skotland, *J. Chromatogr. B* 758 (2001) 265.
- [15] L.Q. Pang, Q.L. Liang, Y.M. Wang, L. Ping, G.A. Luo, *J. Chromatogr. B* 869 (2008) 118.
- [16] C. Wang, S.G. Xie, J. Yang, Q. Yang, G.W. Xu, *Anal. Chim. Acta* 525 (2004) 1.
- [17] S. Harrabi, W. Herchi, H. Kallel, P.M. Mayer, S. Boukhchina, *Food Chem.* 114 (2009) 712.
- [18] E.J. Ahn, H. Kim, B.C. Chung, G. Kong, M.H. Moon, *J. Chromatogr. A* 1194 (2008) 96.
- [19] D.Y. Bang, E.J. Ahn, M.H. Moon, *J. Chromatogr. B* 852 (2007) 268.
- [20] V. Ioffe, T. Kalendarev, I. Rubinstein, G. Zupkovitz, *J. Pharm. Biomed. Anal.* 30 (2002) 391.
- [21] C.A. Lytle, Y.D. Gan, D.C. White, *J. Microbiol. Methods* 41 (2000) 227.
- [22] K. Retra, O.B. Bleijerveld, R.A. van Gestel, A.G.M. Tielens, J.J. van Hellemond, J.F. Brouwers, *Rapid Commun. Mass Spectrom.* 22 (2008) 1853.
- [23] K. Sandra, A.D. Pereira, G. Vanhoenacker, F. David, P. Sandra, *J. Chromatogr. A* 1217 (2010) 4087.
- [24] Y.Q. Xia, M. Jemal, *Rapid Commun. Mass Spectrom.* 23 (2009) 2125.
- [25] F.G. Xu, L. Zou, Q.S. Lin, C.N. Ong, *Rapid Commun. Mass Spectrom.* 23 (2009) 3243.
- [26] B. Barroso, R. Bischoff, *J. Chromatogr. B* 814 (2005) 21.
- [27] N. Mazzella, J. Molinet, A.D. Syakti, A. Dodi, J.C. Bertrand, P. Doumenq, *Rapid Commun. Mass Spectrom.* 19 (2005) 3579.
- [28] E.M. Hein, L.M. Blank, J. Heyland, J.I. Baumbach, A. Schmid, H. Hayen, *Rapid Commun. Mass Spectrom.* 23 (2009) 1636.
- [29] J. Cvačka, O. Hovorka, P. Jiroš, J. Kindl, K. Stránský, I. Valterová, *J. Chromatogr. A* 1101 (2006) 226.
- [30] P. Dugo, T. Kumm, M.L. Crupi, A. Cotroneo, L. Mondello, *J. Chromatogr. A* 1112 (2006) 269.
- [31] M. Holčápek, M. Lísa, P. Jandera, N. Kabátová, *J. Sep. Sci.* 28 (2005) 1315.
- [32] M. Lísa, M. Holčápek, *J. Chromatogr. A* 1198 (2008) 115.
- [33] M. Lísa, M. Holčápek, H. Sovová, *J. Chromatogr. A* 1216 (2009) 8371.
- [34] E.J.C. van der Klift, G. Vivó-Truyols, F.W. Claassen, F.L. van Holthoorn, T.A. van Beek, *J. Chromatogr. A* 1178 (2008) 43.
- [35] P. Dugo, T. Kumm, B. Chiofalo, A. Cotroneo, L. Mondello, *J. Sep. Sci.* 29 (2006) 1146.
- [36] H.G. Nie, R.R. Liu, Y.Y. Yang, Y. Bai, Y.F. Guan, D.Q. Qian, T. Wang, H.W. Liu, *J. Lipid Res.* 51 (2010) 2833.
- [37] M. Holčápek, H. Velínská, M. Lísa, P. Česla, *J. Sep. Sci.* 23 (2009) 3672.
- [38] T. Houjou, K. Yamatani, M. Imagawa, T. Shimizu, R. Taguchi, *Rapid Commun. Mass Spectrom.* 19 (2005) 654.
- [39] Y. Sato, T. Nakamura, K. Aoshima, Y. Oda, *Anal. Chem.* 82 (2010) 9858.
- [40] P.H. Axelsen, R.C. Murphy, *J. Lipid Res.* 51 (2010) 1244.
- [41] K. Ekroos, C.S. Ejsing, U. Bahr, M. Karas, K. Simons, A. Shevchenko, *J. Lipid Res.* 44 (2003) 2181.
- [42] C.S. Ejsing, T. Moehring, U. Bahr, E. Duchoslav, M. Karas, K. Simons, A. Shevchenko, *J. Mass Spectrom.* 41 (2006) 372.
- [43] A. Takatera, A. Takeuchi, K. Saiki, T. Morisawa, N. Yokoyama, M. Matsuo, *J. Chromatogr. B* 838 (2006) 31.
- [44] A.A. Karlsson, P. Michelsen, A. Larsen, G. Odham, *Rapid Commun. Mass Spectrom.* 10 (1996) 775.
- [45] B. Fuchs, R. Suss, J. Schiller, *Prog. Lipid Res.* 49 (2010) 450.
- [46] N. Goto-Inoue, T. Hayasaka, T. Taki, T.V. Gonzalez, M. Setou, *J. Chromatogr. A* 1216 (2009) 7096.
- [47] A. Rohlfing, J. Muthing, G. Pohlentz, U. Distler, J. Peter-Katalinic, S. Berkenkamp, K. Dreisewerd, *Anal. Chem.* 79 (2007) 5793.
- [48] K.A. Al-Saad, W.F. Siems, H.H. Hill, V. Zabrouskov, N.R. Knowles, *J. Am. Soc. Mass Spectrom.* 14 (2003) 373.
- [49] J. Cvačka, A. Svatoš, *Rapid Commun. Mass Spectrom.* 17 (2003) 2203.
- [50] W.C. Byrdwell, *Lipids* 36 (2001) 327.
- [51] P. Jandera, Extreme chromatography: faster, hotter, smaller, in: W.C. Byrdwell, M. Holčápek (Eds.), *Hydrophilic Interaction Chromatography – An Excellent Method for Separation of Polar Samples*, American Oil Chemists' Society, 2011, p. 47.
- [52] M. Holčápek, K. Volná, P. Jandera, L. Kolářová, K. Lemr, M. Exner, A. Církva, *J. Mass Spectrom.* 39 (2004) 43.