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Hydrophilic interaction liquid chromatography–mass spectrometry of (lyso)phosphatidic acids, (lyso)phosphatidylserines and other lipid classes

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ABSTRACT

The goal of this work is a systematic optimization of hydrophilic interaction liquid chromatography (HILIC) separation of acidic lipid classes (namely phosphatidic acids—PA, lysophosphatidic acids—LPA, phosphatidylserines—PS and lysophosphatidylserines—LPS) and other lipid classes under mass spectrometry (MS) compatible conditions. The main parameters included in this optimization are the type of stationary phases used in HILIC, pH of the mobile phase, the type and concentration of mobile phase additives. Nine HILIC columns with different chemistries (unmodified silica, modified silica using diol, 2-picolylamine, diethylamine and 1-aminoanthracene and hydride silica) are compared with the emphasis on peak shapes of acidic lipid classes. The optimization of pH is correlated with the theoretical calculation of acidobasic equilibria of studied lipid classes. The final method using the hydride column, pH 4 adjusted by formic acid and the gradient of acetonitrile and 40 mmol/L of aqueous ammonium formate provides good peak shapes for all analyzed lipid classes including acidic lipids. This method is applied for the identification of lipids in real samples of porcine brain and kidney extracts.

1. Introduction

Phosphatidic acids (PA) are found in small amounts in biological membranes, but they are crucial biosynthetic precursors of all phospholipids (PL) and signaling molecules in biochemical and physiological processes in cells [1–3]. PA are a source of diacylglycerols (DG), which are precursors for triacylglycerols (TG), phosphatidylcholines (PC) and phosphatidylethanolamines (PE) via the Kennedy pathway [4], and precursors of phosphatidylglycerols (PG), phosphatidylserines (PS), phosphatidylinositols (PI) and cardiolipins via cytidine diphosphate DG (CDP-DG) pathway [1]. Lysophosphatidic acids (LPA) are biosynthetic precursors with regulatory functions in the mammalian reproduction system. PS are important components of cellular membranes, where they comprise 5–10% of total PL with important roles in the regulation of apoptosis, lipid synthesis and transport [1,3]. Lysophosphatidylserines (LPS) belong to signaling PL with the important role during inflammatory processes [5]. PA and PS are acidic lipids with the potential to carry two negative charges—both charges on the phosphate group for PA, while one negative charge is on the phosphate and the second one on the carboxylate group in case of PS (Fig. 1).

The comprehensive lipidomic analysis of various biological tissues is a challenging task due to the extreme complexity of individual lipid classes varying in the structure, attached functional groups, polarity, dissociation and ionization behavior, *etc.* The important part of lipidomic analysis is the quantitation, which can be performed using the MS with the direct infusion (shotgun lipidomics) [6–8] or the liquid chromatography–mass spectrometry (LC/MS) approaches [9,10]. Shotgun lipidomics enables fast and robust analysis using precursor ion and neutral loss scans characteristic for the fragmentation behavior of functional group of individual lipid classes [6–8]. LC/MS approaches can be divided into the lipid class separation according to the polarity using HILIC mode for polar lipid classes [9–11] or normal phase (NP) LC for nonpolar lipid classes [12,13]. Individual lipid species can be separated according to the fatty acyl chain length and the number of double bonds using reversed phase (RP) LC [11,14–16] or non-aqueous reversed-phase (NARP) LC [17–19]. Recently, the coupling of ultrahigh-performance supercritical fluid chromatography and mass spectrometry (UHPSFC/MS) approach [20] has

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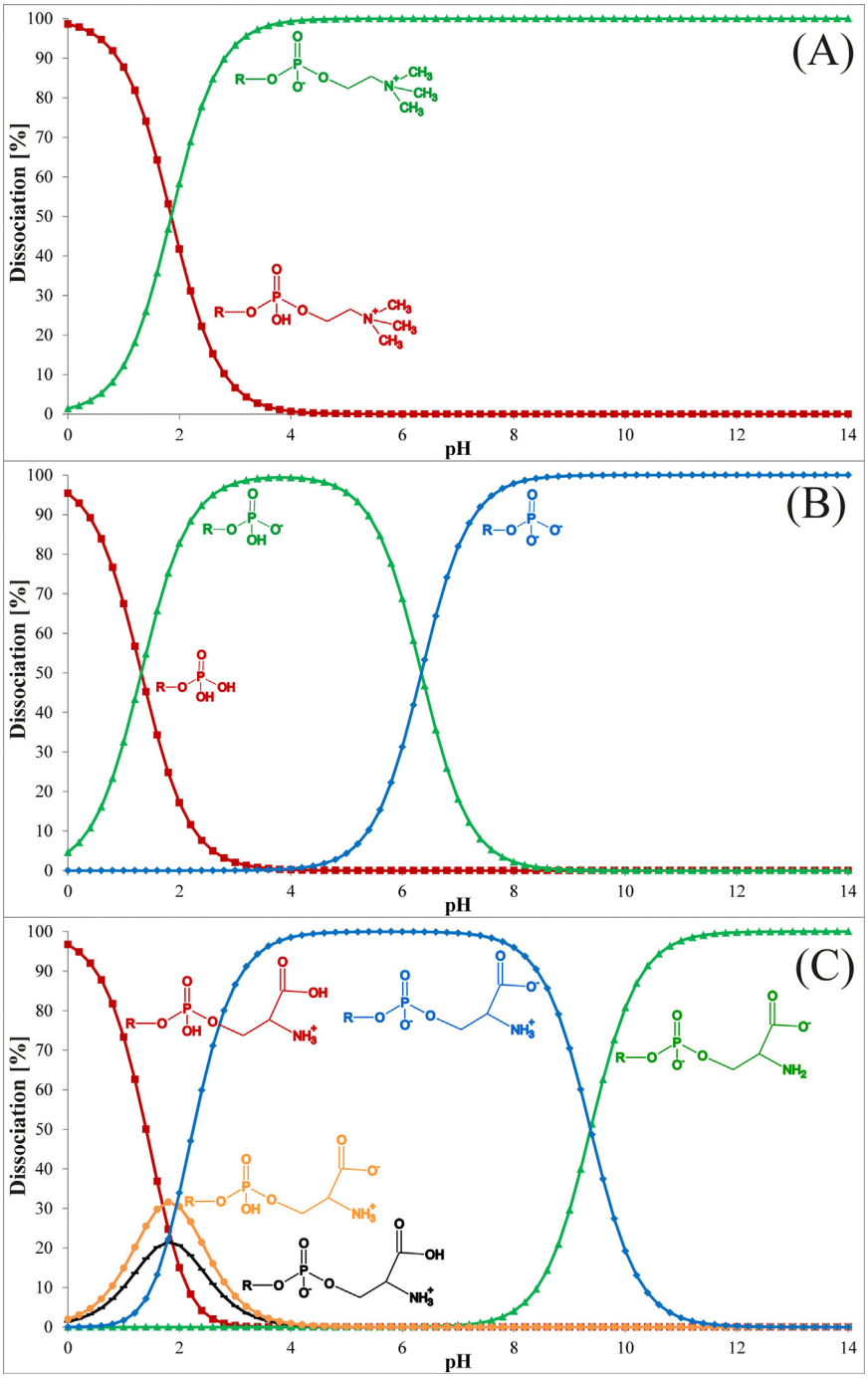

Fig. 1. Dissociation equilibria of (A) PC, (B) PA and (C) PS calculated by the Marvin demo software in the pH range 0–14, where R means $C_{17}H_{33}COOCH_2(C_{17}H_{33}COO)CHCH_2$-.

been introduced with the applicability for both polar and nonpolar lipid classes. The quantitative analysis in LC/MS approaches was described using internal standards (IS) per each lipid class [9] or the combination of single IS and response factors of individual lipid classes related to this IS [21]. The lipid class separation using HILIC or NP mode is more convenient for LC/MS quantitation, because lipid class IS are coeluting with lipid species inside particular lipid classes, therefore they are ionized at identical matrix conditions similarly as for shotgun lipidomics. This way of lipidomic quantitation should provide the most reliable data. Successful separations of PA and PS in HILIC mode have been previously reported [22–28], but they are based on the use of additives not well compatible with MS detection with possible strong ion suppression and memory effects, such as the use of alkyl ammonium salts as ion-pairing agents [29].

The main goal of this work is the optimization of HILIC/MS method for the analysis of the highest number of individual lipid classes with the emphasis on acidic lipid classes, such as PA, PS and their lysoderivatives using MS compatible conditions. We describe the separation in HILIC mode using a silica hydride column, which enables the characterization of 18 lipid classes including PA, PS and regioisomeric pairs of lysoderivatives in one analytical run. The developed method is applied for the analysis of lipid class composition of porcine brain and kidney.

2. Experimental

2.1. Chemicals and standards

Acetonitrile, 2-propanol, methanol (all HPLC/MS grade), chloroform stabilized by 0.5–1% ethanol (HPLC grade), ammonium formate, ammonium acetate, formic acid, acetic acid and sodium chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was prepared with a Milli-Q Reference Water Purification System (Molsheim, France). Standards of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (PC 18:1/18:1), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (2-LPC 18:1), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (PE 18:1/18:1), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (2-LPE 18:1), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (PG 18:1/18:1), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoglycerol (2-LPG 18:1), 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (PS 18:1/18:1), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoserine (2-LPS 18:1), 1,2-dioleoyl-*sn*-glycero-3-phosphatidic acid (PA 18:1/18:1), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphatidic acid (2-LPA 18:1), *N*-oleoyl-sphing-4-enine (Cer d18:1/18:1), *N*-palmitoyl-1-glucosyl-sphing-4-enine (HexCer d18:1/16:0), *N*-palmitoyl-1-lactosyl-sphing-4-enine (Hex2Cer d18:1/16:0) and *N*-oleoyl-sphing-4-enine-1-phosphocholine (SM d18:1/18:1) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Standards of 2-lysophospholipids contain small amounts of regioisomeric 1-lysophospholipids. The lipid nomenclature follows the LIPID MAPS system and the shorthand notation for lipid structures [30]. Biological samples (porcine brain and kidney) were obtained from the local farm.

2.2. Sample preparation

Briefly, 1 mg of lipid class standard was dissolved in a mixture of 200 μL of chloroform and 800 μL of 2-propanol. The lipid standard mixture was prepared by mixing of 10 μL of PC, 2-LPC, PE, 2-LPE, PG, 2-LPG, Cer, HexCer, Hex2Cer, SM and 50 μL of PA, 2-LPA, PS and 2-LPS. Biological samples (porcine brain and kidney) were prepared according to a modified Folch extraction [31] using a chloroform–methanol–water system. Briefly, 250 mg of tissue was homogenized for 3 min with 5 mL of chloroform–methanol (2:1, v/v) mixture and the homogenate was filtered using 0.2 μm Teknokroma syringe filter with regenerated cellulose (Teknokroma, Barcelona, Spain). Subsequently, 1 mL of 1 mol/L NaCl was added, and the mixture was centrifuged at 3000 rpm for 5 min at the ambient temperature. The chloroform bottom layer containing lipids was evaporated by a gentle stream of nitrogen and redissolved in chloroform-2-propanol (1:1, v/v) mixture for HILIC/MS analyses.

2.3. LC/MS conditions

Lipidomic analyses were performed on a liquid chromatograph Agilent 1290 Infinity series (Agilent Technologies, Waldbronn, Germany) coupled with the Esquire 3000 ion trap analyzer (Bruker Daltonics, Bremen, Germany). The optimization of HILIC method was performed using Acquity UPLC BEH HILIC column (150 × 2.1 mm, 1.7 μm, Waters, Milford, MA, USA), Ascentis Si (150 × 2.1 mm, 3 μm, Sigma–Aldrich), Kinetex HILIC (150 × 2.1 mm, 2.6 μm, Phenomenex, Torrance, CA, USA), Spherisorb Silica (150 × 4.6 mm, 10 μm, Waters), Acquity UPC^2 Torus DEA (100 × 3 mm, 1.7 μm, Waters), Acquity UPC^2 Torus 2-PIC (100 × 3 mm, 1.7 μm, Waters), Acquity UPC^2 Torus Diol (100 × 3 mm, 1.7 μm, Waters), Acquity UPC^2 Torus 1-AA (100 × 3 mm, 1.7 μm, Waters), and Cogent Diamond Hydride (250 × 4.6 mm, 4 μm, Microsolv, Eatontown, NJ, USA).

The final LC/MS method for the analysis of individual lipid classes in biological samples is the following: Cogent Diamond Hydride column (250 × 4.6 mm, 4 μm, Microsolv), a flow rate of 1 mL/min, an injection volume of 3 μL, column temperature of 40 °C and a mobile phase gradient: 0 min—99.7% A + 0.3% B, 60 min—75% A + 25% B, where A was acetonitrile with formic acid and B was 40 mmol/L aqueous ammonium formate with pH 4 adjusted by formic acid. The pH was measured by portable pH meter Checker (Hanna Instruments, Woonsocket, RI, USA). For all mobile phases used in this work, acetonitrile (phase A) contained the identical amount of formic acid as used for pH adjustment of aqueous solution (phase B) to keep the constant concentration of formic acid during the gradient elution. Individual lipid classes were detected in positive- and negative-ion ESI modes in the mass range *m*/*z* 50–1000 with the following setting of tuning parameters: scan speed 13,000 *m*/*z* /s, pressure of the nebulizing gas 60 psi, drying gas flow rate 10 L/min and temperature of the drying gas 365 °C, high-voltage capilary 4000 V and the target mass 800.

3. Results and discussion

3.1. Dissociation equilibria of lipids in aqueous solutions

Our previously published LC/MS method [11] using silica column Spherisorb Si (250 × 4.6 mm; 5 μm, Waters) and the mobile phase consisting of acetonitrile and 5 mmol/L of aqueous ammonium acetate enables the separation of most commonly occurring polar lipid classes except for acidic lipid classes (PA, LPA, PS and LPS) due to very broad tailing peaks under these conditions. For this purpose, we have reoptimized the selection of column packing material and the mobile phase composition (mainly pH, the type and concentration of additives). The dissociation equilibria for selected lipid classes in aqueous solutions are theoretically calculated using the Marvin demo software (ChemAxon, Budapest, Hungary) in the whole pH range from 0 to 14 (Fig. 1). The acidobasic dissociation equilibrium of PC (Fig. 1A) is a representative example of neutral PL. The pH 4 and higher should be selected to ensure that only one dissociation form is present in the solution (as zwitterions, see Fig. 1A). The similar behavior is observed for all neutral lipid classes. PA may occur in three forms depending on the pH (Fig. 1B): neutral molecules for pH < 2, deprotonated molecules in between pH 2 and 6 and doubly deprotonated molecules for pH > 6. Only one ionization form should be present in the solution to avoid the deterioration of chromatographic peaks, such as almost exclusive presence of deprotonated form at a narrow pH range of 3.6–4.0 (Fig. 1B). The most complicated situation is observed for PS class, where five different forms exist (Fig. 1C), which results in serious difficulties observed in HILIC separations of PS. The singly negatively charged molecule is present for the pH region from 4 to 7. The overlap of three above described pH regions is extremely narrow, because only at pH 4 all lipid classes are present in one dissociation form without the equilibrium with another form. This calculation is performed only for the aqueous solution, not considering the methanolic part of the mobile phase, but experimentally determined optimal separation conditions at pH 4 perfectly match with these considerations, as discussed in more detail later. The addition of formic acid is used for pH adjustment of aqueous part of the mobile phase. The identical amount of formic acid is also added to acetonitrile to keep the constant concentration of formic acid during the gradient run.

The dissociated ionic species formed from acidic lipids (see Fig. 1) in aqueous solutions compete for protons from silanol groups on the surface of silica stationary phase, so the quality and purity of the stationary phase is a basic prerequisite for the successful separation in addition to the pH selection. Properties of the sta-

Table 1
Tailing factors and normalized peak capacities per 1 cm of column length for individual lipid standards using nine different columns.

	Tailing factor [*1]									Normalized peak capacity/cm [*2]								
	Acquity	Ascentis	Kinetex	Spherisorb	Diol	2-PIC	DEA	1-AA	Hydride	Acquity	Ascentis	Kinetex	Spherisorb	Diol	2-PIC	DEA	1-AA	Hydride
Cer	1.4	1.1	0.9	1.3	1.0	1.3	1.5	1.3	1.1	4	3	6	4	10	15	17	12	6
HexCer	1.2	0.9	1.1	1.8	2.9	3.4	1.1	2.6	1.3	1	1	1	2	3	4	4	2	2
Hex2Cer	1.8	1.1	1.1	2.6	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	1.3	1	1	1	2	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	2
PG	1.6	4.1	1.5	2.2	1.5	1.3	1.6	1.3	1.0	2	2	2	2	5	15	17	12	3
PA	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	1.6	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	2
LPG	1.1	1.0	0.9	1.3	2.2	1.6	1.3	2.4	1.0	3	1	2	1	2	5	9	12	2
PE	2.1	1.0	1.4	2.0	2.2	1.7	1.7	1.4	1.1	4	4	4	4	2	15	9	3	4
PS	n.d.[*3]	2.6	1.4	0.9	n.d.[*3]	1.7	2.2	n.d.[*3]	1.3	n.d.[*3]	1	1	1	n.d.[*3]	8	2	n.d.[*3]	4
LPA	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	1.6	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	n.d.[*3]	1
LPE	1.5	1.1	1.5	1.1	1.8	1.7	1.4	1.4	1.0	5	6	6	5	5	15	17	3	6
LPS	n.d.[*3]	1.0	n.d.[*3]	n.d.[*3]	n.d.[*3]	1.4	1.1	n.d.[*3]	1.4	n.d.[*3]	2	n.d.[*3]	n.d.[*3]	n.d.[*3]	8	6	n.d.[*3]	3
PC	1.6	1.4	1.1	1.5	1.8	1.8	1.7	1.6	1.4	5	4	6	4	10	15	6	6	2
LPC	1.3	1.3	1.2	1.3	1.6	1.6	1.3	1.6	1.0	5	4	6	4	5	15	9	3	4

[*1]The tailing factor is calculated as the ratio of the full width in 5% of peak height and two times of the front half width, $T_f = w_{0.05}/2 \times f$ [32].
[*2]The peak capacity is calculated as the ratio of the gradient time and full width in half of peak maximum, $P_C = 1 + (t_g/1.7 \times w_{1/2})$ [33], divided by the column length in cm.
[*3]n.d. – not determined due to wide tailing peaks.

tionary phase could seriously affect peak shapes of acidic lipids, which may result in the peak tailing. Silica columns contain free silanol groups, which exhibit the dissociation equilibrium of acidic protons depending on the mobile phase composition. Dissociated molecules of acidic analytes can also participate in the competition for protons, which could result in the stronger retention of deprotonated acids on the stationary phase and also peak tailing.

3.2. Unmodified silica columns

First, 4 commercially available silica columns (Fig. 2) with porous, porous shell and sub- 2 μm particles are tested using the lipid standard mixture, as described in the Experimental part. Representative chromatograms from different silica columns are measured under the same LC/MS conditions (except for the optimal flow rates used for particular columns) with the mobile phase consisting of acetonitrile, 20 mmol/L of aqueous ammonium formate and pH adjustment by formic acid. Silica columns varying in the particle size and manufacturers provide different results in terms of peak shapes and chromatographic resolution of separated lipid classes, but the retention order of lipid classes is almost identical (see Fig. 2). Table 1 shows the chromatographic efficiency of tested columns for individual lipid classes reported as their tailing factors and peak capacities per cm of the column length. The tailing factor is calculated as the ratio of the full width in 5% of peak height and two times of the front half width of peak, $T_f = w_{0.05}/2 \times f$ [32]. The peak capacity is calculated as the ratio of the gradient time and the full width in the half of peak height, $P_C = 1 + (t_g/1.7 \times w_{0.5})$, divided by the column length in cm [33]. The retention order of regioisomers of lysoderivates is assigned by negative-ion ESI mass spectra using relative abundances of carboxylate ions $[RCOO]^-$, which has been described in our previous work [11]. Acquity UPLC BEH HILIC column provides good peak shapes of all lipid classes except for wide and tailing peaks of PA, LPA, PS and LPS classes (Figs. 2 A and S1). The similar situation is observed for Kinetex HILIC (Fig. 2C) and Spherisorb Si (Fig. 2D) columns. The best peak shapes for acidic lipid classes are observed on Ascentis Si column (Fig. 2B).

The next step is the optimization of mobile phase composition, such as the concentration of ionic additives and pH. Ascentis Si column is used for this optimization due to the best results obtained in the previous step. Effects of pH of the mobile phase on the separation are illustrated in Figs. S2 and S3. The effect of pH on the retention behavior of PA and LPA is better visualized in the graph of normalized retention times *vs.* pH (Fig. 3) showing notable increases of PA and LPA retention at increased pH values. Differences in the retention behavior of most lipid classes except for PA and LPA are negligible, but significant changes of both retention times and peak shapes are observed for PA and LPA even for fine tuning of pH shown in Fig. S3. Peak shapes of acidic lipid classes are also seriously affected by the concentration of ammonium formate (20 and 60 mmol/L) in the mobile phase with the constant pH (Fig. S4). Increased concentration of ammonium formate improves peak shapes of PS and LPS, but on the other hand reduces the overall signal intensity of acidic lipids probably due to suppression effects. Unfortunately, peak shapes of PA and LPA are not improved at all (Fig. S4). The best separation on the silica column is achieved with fully porous particle column Ascentis, the mobile phase consisting of acetonitrile and 20 mmol/L of aqueous ammonium formate and pH adjusted to 3.4 by formic acid, but except for acidic lipid classes with strongly tailing peaks (Fig. 2B).

3.3. Modified silica columns

The comprehensive optimization of HILIC separation described in the previous chapter did not result in acceptable peak shapes of acidic lipid classes, therefore the investigation has continued with modified silica columns with different chemistries (Fig. 4). The knowledge of the role of pH and modifier concentration from the previous optimization is also taken into account. The diol column is the second most widespread column in the HILIC mode recommended mainly for acidic compounds. The retention behavior of lipids on Acquity UPC^2 Torus Diol (100 × 3 mm, 1.7 μm) is different in comparison with unmodified silica columns (Figs. 4 A and S5A). All lysoderivates are strongly retained on the diol column and they elute at higher retention times. PA, LPA, PS and LPS are not detected due to the strong peak tailing. Acquity UPC^2 Torus 2-PIC (100 × 3 mm, 1.7 μm) column containing bonded 2-picolylamine on the silica surface is recommended for a wide range of acidic, basic or neutral compounds. The application to the lipid standard mixture provides a significant improvement of chromatographic resolution and peak shapes of PS and LPS classes (Figs. 4 B and S5B), but unfortunately not for PA and LPA. The retention behavior of some ceramides (Cer and HexCer) is similar with unmodified silica surface, but the peak tailing of Hex2Cer containing large glycosylated part is worse. Acquity UPC^2 Torus DEA (100 × 3 mm, 1.7 μm) column containing bonded diethanolamine should be orthogonal to Torus 2-PIC phase, however in reality both types of stationary phases provide similar retention behavior and peak shapes of all lipid classes but with the lower signal intensity (Figs. 4 C and S5C). The last type of modified silica column is Acquity UPC^2 Torus 1-AA (100 × 3 mm, 1.7 μm) containing bonded 1-aminoanthracene, which is recommended for neutral compounds, such as polar and

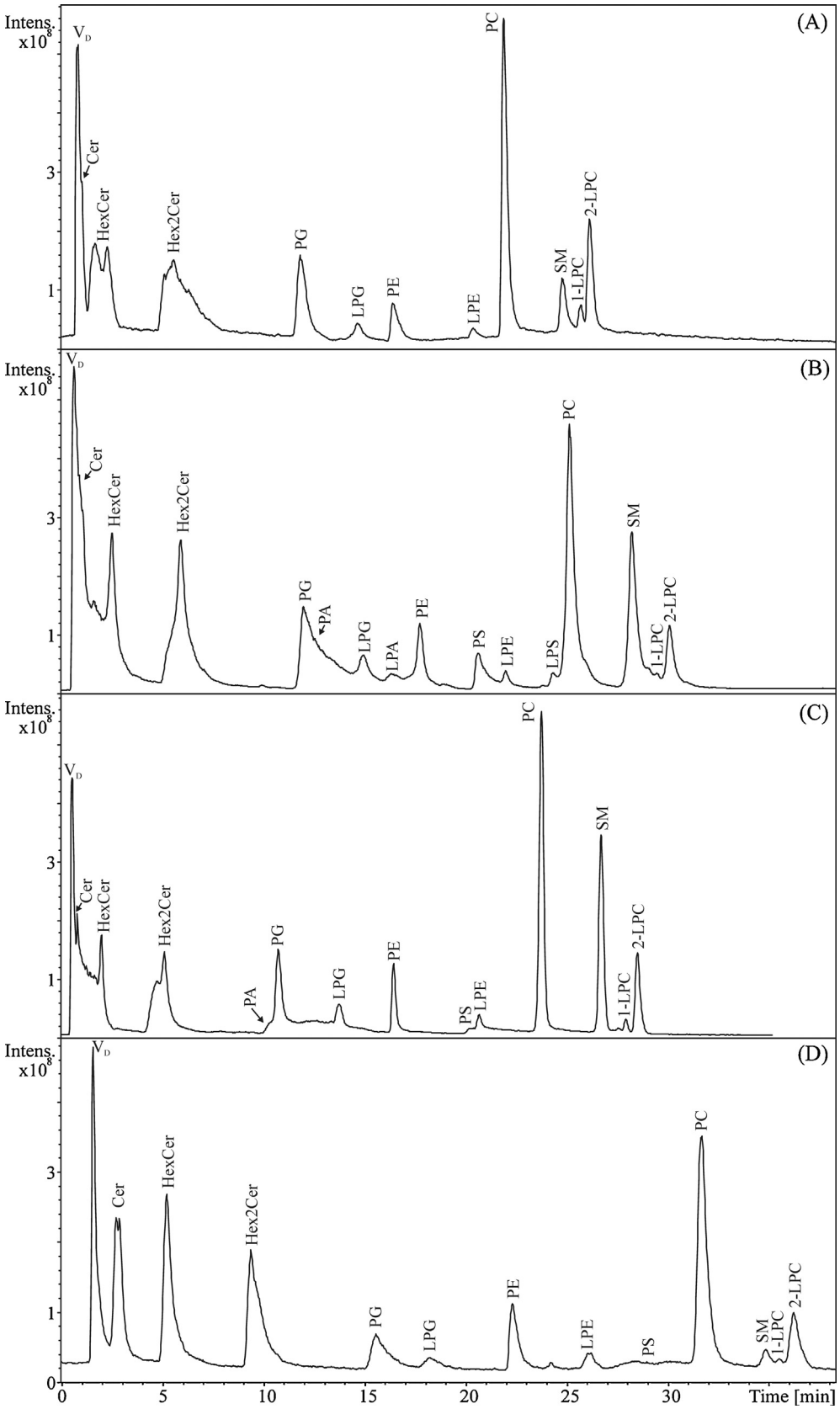

Fig. 2. Positive-ion HILIC/ESI–MS of lipid standard mixture measured using unmodified silica columns: (A) Acquity UPLC BEH HILIC (150 × 2.1 mm, 1.7 μm) column, flow rate 0.5 mL/min, (B) Ascentis Si (150 × 2.1 mm, 3 μm) column, flow rate 0.6 mL/min, (C) Kinetex HILIC (150 × 2.1 mm, 2.6 μm) column, flow rate 0.6 mL/min, and (D) Spherisorb Si (150 × 4.6 mm, 10 μm) column, flow rate 1 mL/min. Conditions: gradient 0 min—99.7% A + 0.3% B, 40 min—79.9% A + 20.1% B, where A is acetonitrile and B is 20 mmol/L of aqueous ammonium formate, pH 3.5 adjusted by formic acid. Peak annotation: Cer—ceramide, HexCer—hexosylceramide, Hex2Cer—dihexosylceramide, PG—phosphatidylglycerol, LPG—lysophosphatidylglycerol, PA—phosphatidic acid, LPA—lysophosphatidic acid, PE—phosphatidylethanolamine, LPE—lysophosphatidylethanolamine, PS—phosphatidylserine, LPS—lysophosphatidylserine, PC—phosphatidylcholine, SM—sphingomyelin, LPC—lysophosphatidylcholine.

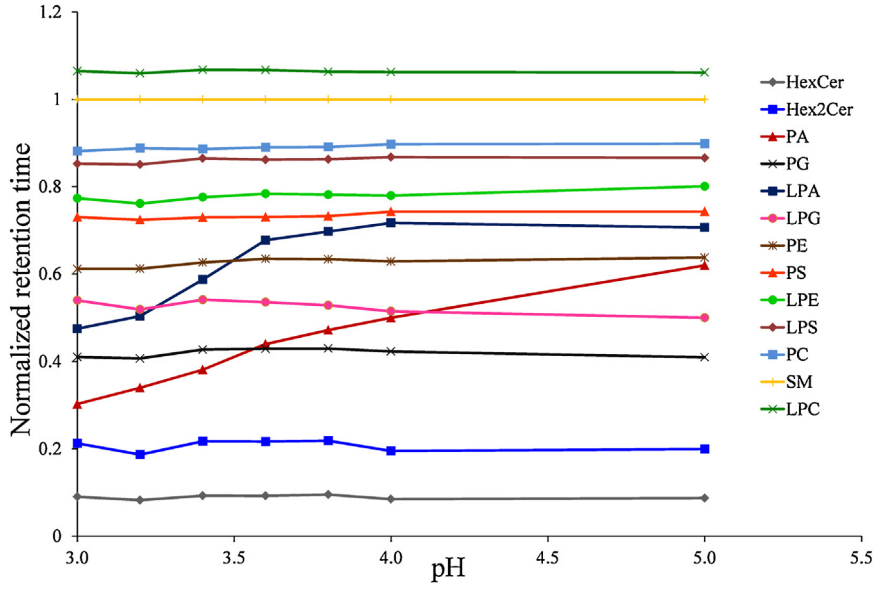

Fig. 3. Dependencies of normalized retention times (related to SM peak) of lipid standard mixture on pH using Ascentis Si column. Chromatograms are shown in Figs. S2 and S3.

nonpolar steroids, lipids and hydrophobic compounds. This column exhibits a higher retention of Cer, which results in their separation from the peak of system void volume. On the other hand, peak shapes of other lipid classes (Figs. 4 D and S5D) are the worst among all modified silica columns tested. The best separation is achieved on Acquity UPC^2 Torus 2-PIC column, but except for bad peak shapes of PA, LPA and Hex2Cer classes. Modified silica columns provide the best results in terms of the peak capacity (Table 1), but the major goal of this work to detect acidic lipid classes is not achieved similarly as for unmodified silica columns.

3.4. Hydride silica columns

Free silanol groups (Si—OH) on the surface of common silica columns are replaced by silicon-hydride (Si—H) bonds in case of so called hydride columns, which eliminates the acidic protons from silanols, but this reaction probably does not have 100% coverage. The silanol surface has a strong association with water, but silica hydride particles are slightly hydrophobic with only weak attraction of water. This type of columns can be operated in three chromatographic modes: RP, NP and aqueous NP [34]. The hydride column could enable the separation of both polar and nonpolar lipids using the dual retention mechanism RP and aqueous NP modes, which depends on the solvent composition. The separation of lipid standards using the hydride column provides excellent results for acidic classes PA, LPA, PS and LPS, and also the separation of Cer from the peak of system void volume (Fig. 5). The relative standard deviations for six consecutive injections are in the range of 0.5–2.2% (Table S2). The pH (Fig. S6), type and concentration of additives (Figs. S7 and S8) and the slope of mobile phase gradient are carefully optimized as well. Higher pH (Fig. S6) has a positive effect on peak shapes of most PL but the negative effect of sphingolipids (Cer, HexCer, Hex2Cer and SM). Significant changes in signal intensities are observed for Cer, which is approximately ten times lower at pH 6 compared to pH 3.5 (Fig. S6). Ammonium formate and ammonium acetate buffers as typical LC/MS volatile additives are compared (Fig. S7). The signal intensities are approximately two times higher for ammonium acetate buffer (Fig. S7A), but peak shapes and resolution of acidic lipid classes are significantly better for ammonium formate buffer (Fig. S7B). The optimization of ammonium formate concentration (Fig. S8) shows that most lipid classes have almost identical retention times except for PA and LPA, which have higher retention and better peak shapes with higher concentration of ammonium formate. However, increased concentrations also cause certain loss of sensitivity due to suppression effects, so the best compromise is 40 mmol/L of aqueous ammonium formate. The application of final method using the mobile phase consisting of acetonitrile and 40 mmol/L of aqueous ammonium formate and pH 4 adjusted by formic acid for lipid standard mixture is shown both for positive-ion (Fig. 5A) and negative-ion (Fig. 5B) ESI–MS. The optimal pH correlates well with dissociation equilibria shown in Fig. 1. The hydride column provides higher peak capacities for most lipid classes than unmodified silica columns, but lower than some modified silica columns (Table 1). The tailing factors for the most lipid classes are close to 1 except for PA and LPA (1.6 in both cases), but it is by far the best result among all tested conditions in this study, where tailing factors cannot be calculated at all due to completely deteriorated peak shapes of PA and LPA.

3.5. LC/MS analysis of biological samples

The final LC/MS method using hydride column is applied for the characterization of lipid composition of total lipid extracts of porcine brain (Fig. 5C) and kidney (Fig. S9). 13 lipid classes are detected, namely sulfatides, Cer, HexCer, Hex2Cer, PG, PI, PA, PE, LPE, PS, PC, LPC and SM. More than 140 individual lipid species are identified based on characteristic ions (Table S1) observed in positive- and negative-ion ESI modes. Individual lipid species differ in attached fatty acyls, which are annotated by their total carbon number and double bond number (CN:DB). The annotation of lipids is done on the assumption that abundant lipids typically contain even number of carbon atoms and also supported by our knowledge of the composition of these samples from our previous works [11,35,36]. We identify 25 sulfatides, 1Cer, 19HexCer, 3Hex2Cer, 5 PI, 2 PA, 33 PE, 3 LPE, 12 PS, 21 PC, 7 SM and 3 LPC in the porcine brain, which is a comparable number of lipids with our previous work using off-line two-dimensional HILIC x RP-HPLC/ESI–MS method, which is extremely time-consuming and does not enable the analysis of acidic lipids classes [35]. In the porcine kidney, 21 sulfatides, 1Cer, 17HexCer, 3Hex2Cer, 3 PG, 5 PI, 33 PE, 11 PS, 15 PC, 6 SM and 3 LPC are detected.

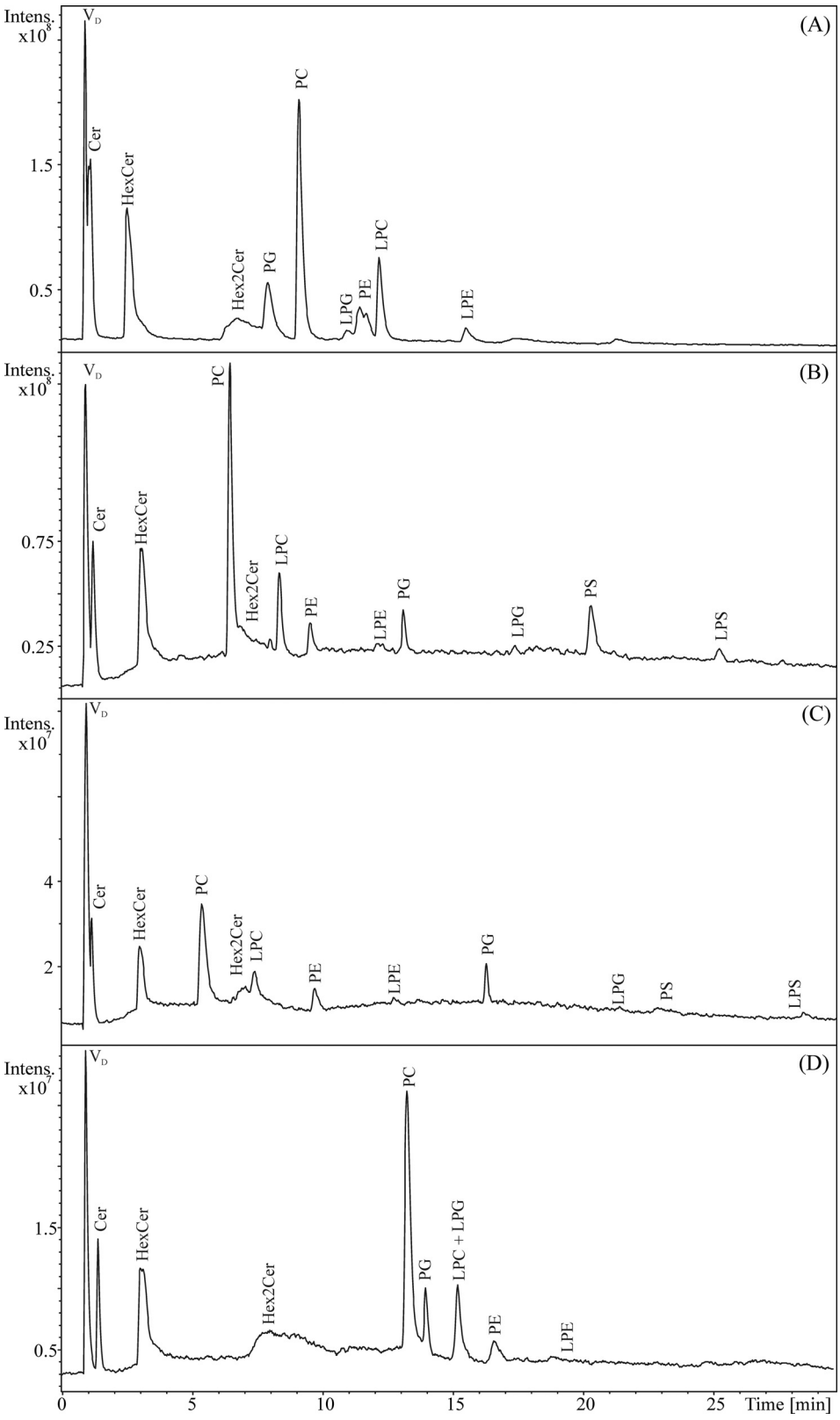

Fig. 4. Positive-ion HILIC/ESI-MS of lipid standard mixture measured using modified silica columns: (A) Acquity UPC^2 Torus Diol (100 × 3 mm, 1.7 μm), (B) Acquity UPC^2 Torus 2-PIC (100 × 3 mm, 1.7 μm), (C) Acquity UPC^2 Torus DEA (100 × 3 mm, 1.7 μm) and (D) Acquity UPC^2 Torus 1-AA (100 × 3 mm, 1.7 μm). Conditions: gradient 0 min—99.7% A + 0.3% B, 30 min—75% A + 25% B, where A is acetonitrile and B is 20 mmol/L of aqueous ammonium formate, pH 4 adjusted by formic acid.

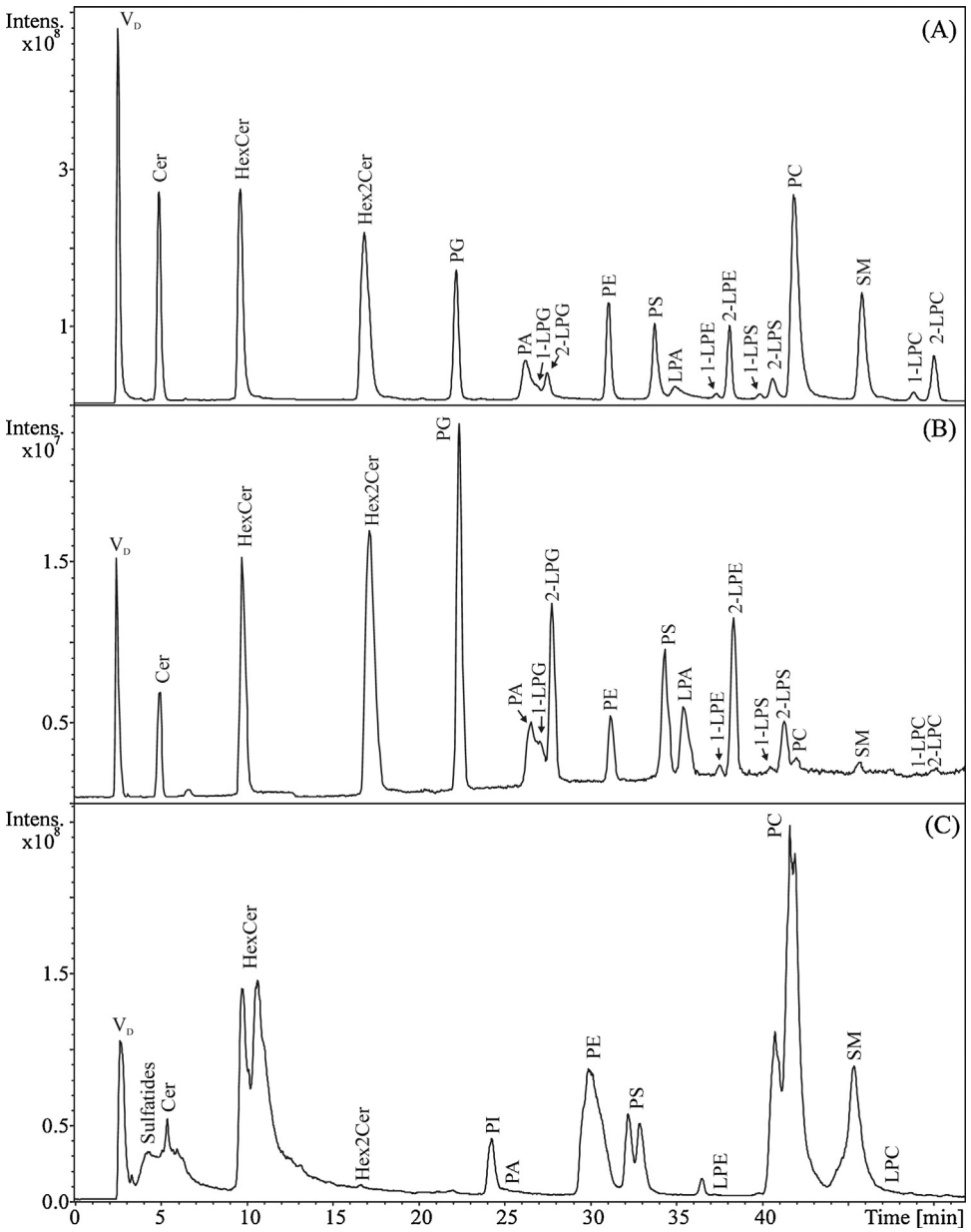

Fig. 5. Final HILIC/ESI-MS method using hydride column: (A) lipid standard mixture measured in the positive-ion mode, (B) lipid standard mixture measured in the negative-ion mode, (C) porcine brain extract measured in the positive-ion mode. Conditions: Cogent Diamond Hydride column (250 × 4.6 mm, 4 μm), flow rate 1 mL/min, gradient 0 min—99.7% A + 0.3% B, 60 min—75% A + 25% B, where A is acetonitrile and B is 40 mmol/L of aqueous ammonium formate, pH 4 adjusted by formic acid.

4. Conclusions

The optimized HILIC separation of acidic lipid classes PA, LPA, PS and LPS is convenient for the implementation in LC/MS lipidomic workflows, because the whole optimization is based only on conditions fully compatible with the MS detection, such as the use of volatile organic buffers and acids at lowest possible concentrations. The optimization of peak shapes of PA, LPA, PS and LPS has been a rather difficult task due to the necessity to optimize two critical parameters, such as the column type and pH value of mobile phase. Different dissociation forms for these acidic lipid classes can cause very broad and tailing chromatographic peaks and also seriously reducing the sensitivity, which explains why these lipid classes were not reported in previous works with HILIC/MS [9,10]. The best results are obtained on new hydride type column used under HILIC conditions and pH 4 selected in accordance with pH dependences of dissociation equilibria for PA and PS. The main advantage of hydride column is the replacement of acidic silanol groups Si—OH by hydride bonds Si—H, which reduces the risk of peak tailing of acidic lipids, as experimentally proved in our work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.01.064.

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