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LIPIDOMIC ANALYSIS OF PORCINE ORGANS USING HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY AND MASS SPECTROSCOPY

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The identification and quantitation of all lipids in complex biological tissues is the first step towards the understanding how lipids function in a biological system and the elucidation of the mechanism of lipid-related diseases including obesity, atherosclerosis, cancer, cardiovascular diseases, etc. Our optimized nontargeted method using hydrophilic interaction liquid chromatography (HILIC) coupled to electrospray ionization mass spectrometry (ESI-MS) was used for the characterization of the lipidome, mainly glycerophospholipids and sphingolipids in porcine brain, heart, kidney, liver, lung, spinal cord, spleen and stomach. Individual lipid classes are quantified based on the peak integration of individual lipid classes separated in the HILIC mode multiplied by their response factors and correlated by sphingosylethanolamine (d17:1/12:0) as a single internal standard. Subsequently, relative abundances of deprotonated molecules $[M-H]^-$ in negative-ion ESI mass spectra are used for determination of lipid species concentration inside individual classes. The approach using $[M-H]^-$ ions enables to obtain

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detailed information about phosphatidylethanolamines and their different forms of fatty acyl linkage on the glycerol skeleton (plasmalogens and ether analogs), phosphatidylinositols and hexosylceramides in studied organs. Our results provide important knowledge about lipid representations in vital porcine organs and can be applied for future metabolic studies on human to investigate serious lipid-related human disorders.

Introduction

Lipids are important components of cellular organisms and they are divided according to the structural and biosynthetic complexity into the following categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides [1,2]. Glycerophospholipids, together with sphingolipids and sterols, represent structural components of biological membranes. Fatty acids are building blocks for the synthesis of triacylglycerols (TG), which are mainly used for the energy storage and signaling. The balance among saturated, monounsaturated and polyunsaturated fatty acids is important for maintaining the optimum fluidity of membranes. In the human organism, polyunsaturated fatty acids (n-3 and n-6) are main components of brain and retina, as they are essential for the biosynthesis of prostaglandins, which decreases the production of inflammatory agents [3]. The main sources of polyunsaturated fatty acids are fish and other marine animals, but some animals can synthetize them from linolenic acid obtained from plant diets (herbivora). The biosynthesis of fatty acids in the organism occurs in the liver, adipose tissue and the lactating breast [4,5]. Glycerophospholipids are key constituents of lipid bilayers, which forms the separate environment of internal cells and participate in the cellular signaling and enzyme activation. Plasmenyl-lipids (plasmalogens) and plasmanyl-lipids (ethers) form approximately 15-20 % of glycerophospholipids in the human organism, and they have elevated levels in cancer tissues [5-7]. Sphingolipids are contained in most cell membranes, mainly in nervous membranes (brain and spinal cord). Their metabolism has been studied many times due to apparent physiological changes of sphingolipid structures and concentrations in various human diseases. Sterols are important in cellular functions, because they are precursors of fat-soluble vitamins and steroid hormones.

Lipidomic analysis of biological tissues is a daunting task due to the extreme complexity of the lipidome (amphipathic character of lipids with hydrophobic acyl tails and hydrophilic head groups). Most of described analytical methods enable to analyze only a certain part of lipids without the overall lipidomic picture. High-performance liquid chromatography (HPLC) - mass spectrometry (MS) coupling is a powerful technique in the characterization of glycerolipids, glycerophospholipids, sphingolipids, etc. Hydrophilic interaction

liquid chromatography (HILIC) and reversed-phase (RP) HPLC coupled to electrospray ionization (ESI) MS can be used for the separation of glycerophospholipids and sphingolipids [8-13] in animal tissues. The comprehensive lipidomic analysis is often performed using on-line or off-line two-dimensional chromatographic separation, which offers the opportunity to separate complex lipidomic mixtures according to two molecular properties [13-15].

The conventional quantitative techniques of lipids by MS are divided into the targeted or nontargeted analysis using various scans typical for triple quadrupole (QqQ) and hydride quadrupole — linear ion trap mass analyzers [8,9,12,16-21], and nontargeted analysis using MS/MS data obtained by instruments with ultrahigh resolving power (>100,000) and sub-ppm mass accuracy of Fourier transform (orbitrap and ion-cyclotron resonance) [12,22-24]. Our nontargeted HILIC-HPLC/ESI-MS method [25] applies the different approach for the quantitation of all separated lipid classes in the HILIC mode using the single internal standard (sphingosyl PE, d17:1/12:0) and lipid class specific response factors (RF). The disadvantage of previously described quantitative methods is the need of expensive internal standards for the quantitation. In contrast, ^{31}P nuclear magnetic resonance (NMR) [26,27] is accurate and nondestructive method, which does not need any previous optimization and calibration. On the other hand, ^{31}P NMR is not applicable to small amounts of samples or lipids without phosphorous atom and is not suitable for routine coupling with HPLC.

In this work, we described application of nontargeted HILIC-HPLC/ESI-MS method for qualitative and quantitative description of the lipidome in porcine organs and the comparison of results with the previously published data [8,9,12,27-31]. Then we determined the distribution of individual lipid species inside lipid classes in porcine brain, heart, kidney, liver, lung, spinal cord, spleen and stomach using $[M-H]^-$ ions.

Experimental

Chemicals and Standards

Acetonitrile, propan-2-ol and methanol (all HPLC gradient grade), hexane and chloroform stabilized by 0.5-1 % ethanol (both HPLC grade), sodium methoxide and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-Dodecanoyl-heptadecasphing-4-enine-1-phosphoethanolamine (sphingosyl PE, d17:1/12:0) used as the IS for the nontargeted quantitation with RFs was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Brain, heart, kidney, liver, lung, spinal cord, spleen and stomach of pig (female at the age of 8 months) were obtained from the family farm of the first author.

Sample Preparation

Total lipid extracts for HILIC-HPLC/MS analyses were prepared according to the modified Folch procedure [32] using a chloroform - methanol - water system. Briefly, approximately 0.5 g of porcine organ and 50 μl 3.3 mg l^{-1} IS (sphingosyl PE, d17:1/12:0) were homogenized with 10 ml of chloroform - methanol (2:1, v/v) mixture for 5 min, and the homogenate was filtered using a coarse filter paper. Then, 2 ml 1 mol l^{-1} NaCl was added, and the mixture was centrifuged at 2500 rpm for 4 min at ambient temperature. The chloroform (bottom) layer containing lipids was taken and evaporated by a gentle stream of nitrogen and redissolved in chloroform - propan-2-ol (1:1,v/v) mixture for subsequent HILIC analyses.

HILIC-HPLC/ESI-MS of Individual Porcine Organs

Total lipid extracts of porcine organs were analyzed using a Spherisorb Si column (250×4.6 mm, 5 μm, Waters, Milford, MA, USA), a flow rate of 1 ml min^{-1}, an injection volume of 10 μl, column 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 [13]. All HPLC/MS experiments were performed on the liquid chromatograph Agilent 1200 series (Agilent Technologies, Waldbronn, Germany) coupled to the Esquire 3000 ion trap analyzer with ESI (Bruker Daltonics, Bremen, Germany). Individual lipid classes were identified and quantified using the total ion current in positive-ion and negative-ion modes in the mass range m/z 50 - 1000 with the following setting of tuning parameters: pressure of the nebulizing gas 60 psi, drying gas flow rate 10 l min^{-1} and temperature of the drying gas 365 °C. 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 $\Delta m/z$ 4, the collision amplitude of 1 V and helium as a collision gas. Data were acquired and evaluated using the Data Analysis software (Bruker Daltonics).

Results and Discussion

Lipid Classes Characterization of Porcine Organs Using HILIC-HPLC/ESI-MS

Figure 1 shows an overview of our quantitation approach used for the complex lipidomic characterization of eight porcine organs: brain, heart, kidney, liver, lung, spinal cord, spleen and stomach. This approach is based on our HILIC-HPLC/ESI-MS method [25] published recently for the characterization of three nonpolar lipid classes (triacylglycerols, cholesterol and cholesteryl esters) and fifteen polar lipid

classes, from which ten lipid classes belong to the group of phospholipids such as phosphatidylglycerols (PG), phosphatidylinositols (PI), plasmenylphosphatidylethanolamines (pPE), plasmanylphosphatidylethanolamines (ePE), phosphatidylethanolamines (PE), lysophosphatidylethanolamines (LPE), phosphatidylserines (PS), plasmenylphosphatidylcholines (pPC), plasmanylphosphatidylcholines (ePC) and lysophosphatidylcholines (LPC) and five to the group of sphingolipids such as ceramides (Cer), sulfatides, hexosylceramides (HexCer), sphingosylphosphatidylethanolamine (IS) and sphingomyelins (SM). The nontargeted quantitation of lipid classes is based on the peak integration of individual lipid classes separated in the HILIC mode multiplied by their response factors (*RF*) and correlated by a single IS (sphingosyl PE, d17:1/12:0). However, HILIC-HPLC method does not enable the separation of non-polar lipids (TG, Chol and CE), which coelute in one chromatographic peak close to the void volume of the system; therefore, their quantitation would not be reliable using this chromatographic system. Furthermore, the quantitation of Cer, HexCer and sulfatides cannot be performed due to wide and only partially resolved peaks occurring in HILIC-HPLC. In the

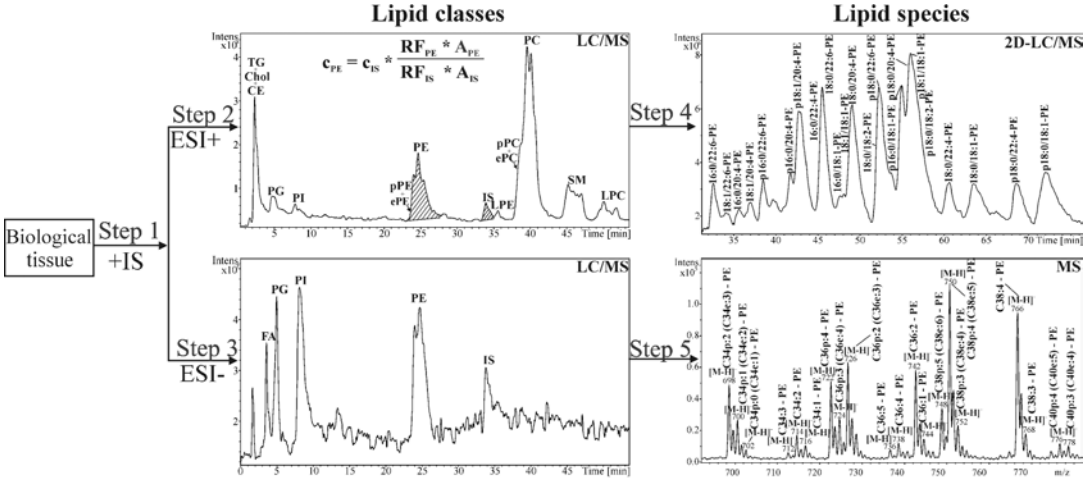

Fig. 1 Schematic overview of our nontargeted lipidomic approach: step 1/ extraction with addition of internal standard, step 2/ HILIC-HPLC/MS quantitation in positive-ion ESI, step 3/ HILIC-HPLC/MS in negative-ion ESI, step 4/ negative-ion ESI mass spectrum of separated lipid class in HILIC-HPLC (for details, see Experimental)

case of PS, the obtained data are semi-quantitative (labeled with asterisks) due to the peak tailing, which causes problems with the peak integration. Reliable quantitative data are obtained for PE, LPE, PC, SM and LPC. Table I shows the comparison of our quantitative data of porcine brain and previously reported data [27,29,30] for human or rat brain. Concentrations (in mmol g^{-1}) or relative abundances (in %) are similar in spite of different analytical methods and different type of brain samples. Relative abundances of individual lipid classes in all porcine organs are reported in Fig. 2, where the relative abundance of PC is in the

range 32-40 %, PE 26-35 %, SM 5-19 %, PI up to 20 %, PS up to 12 %, LPE up to 8 %, LPC up to 7 % and PG up to 5 %. Only spleen contains all quantified lipid classes (i.e., PC, PE, PI, SM, PS, LPE, LPC and PG). In contrast to spleen, the lung and spinal cord are composed from the smallest number of lipid classes (i.e., PC, PE, PI, SM and PG or PS). The comparison of lipid class concentrations in individual porcine organs confirms that PC and PE are the most abundant polar lipids in porcine organs. The highest concentrations of PC and PE are observed in the brain and the lowest ones in the lung and stomach. HILIC-HPLC/ESI-MS method enables to determine total concentrations of lipid classes, but the next step is the determination of concentrations of individual lipid species inside these classes. For this reason, the detailed characterization of selected lipid classes is done using two different approaches described in next two chapters.

Table I Comparison of concentrations or relative abundances of individual lipid classes in brain (porcine, human or rat) obtained by our HILIC-HPLC/ESI-MS method and previously published TLC [30], ^{31}P NMR [27] and HPLC/ELSD [29] data

Lipid class	HILIC-HPLC/ESI-MS		TLC [30]	^{31}P NMR [27]	HPLC/ELSD [29]	
	Porcine		Human	Human	Rat	Rat
	Concentration μmol g^{-1}	Relative abundance, %	Concentration μmol g^{-1}	Relative abundance, %		
PI	4.5	5	3.7	5.3	3.3	5.3
PE	30.7	34.3	29	37.6	37.6	37.6
LPE	2	2.3			1.4	
PS	10.6 [a)]	13.1 [a)]	9	12.5	12.7	15.6
PC	33.2	37.1	22.1	37.6	36.9	37.6
SM	6.5	7.3		1.9	4.8	1.9
LPC	0.8	0.9			1.5	
Others				0.7	0.95	2

[a)] Only semi-quantitative data are determined for PS due to the peak tailing

Analysis of Individual Lipid Species Using [M-H]$^-$ Ions in Negative-ion ESI Mass Spectra

This approach enables the characterization of individual lipid species inside lipid classes based on relative abundances of deprotonated molecules [M-H]$^-$ in the negative-ion HILIC-HPLC/ESI-MS (i.e., PE, PI, PG, LPE, HexCer and sulfatides).

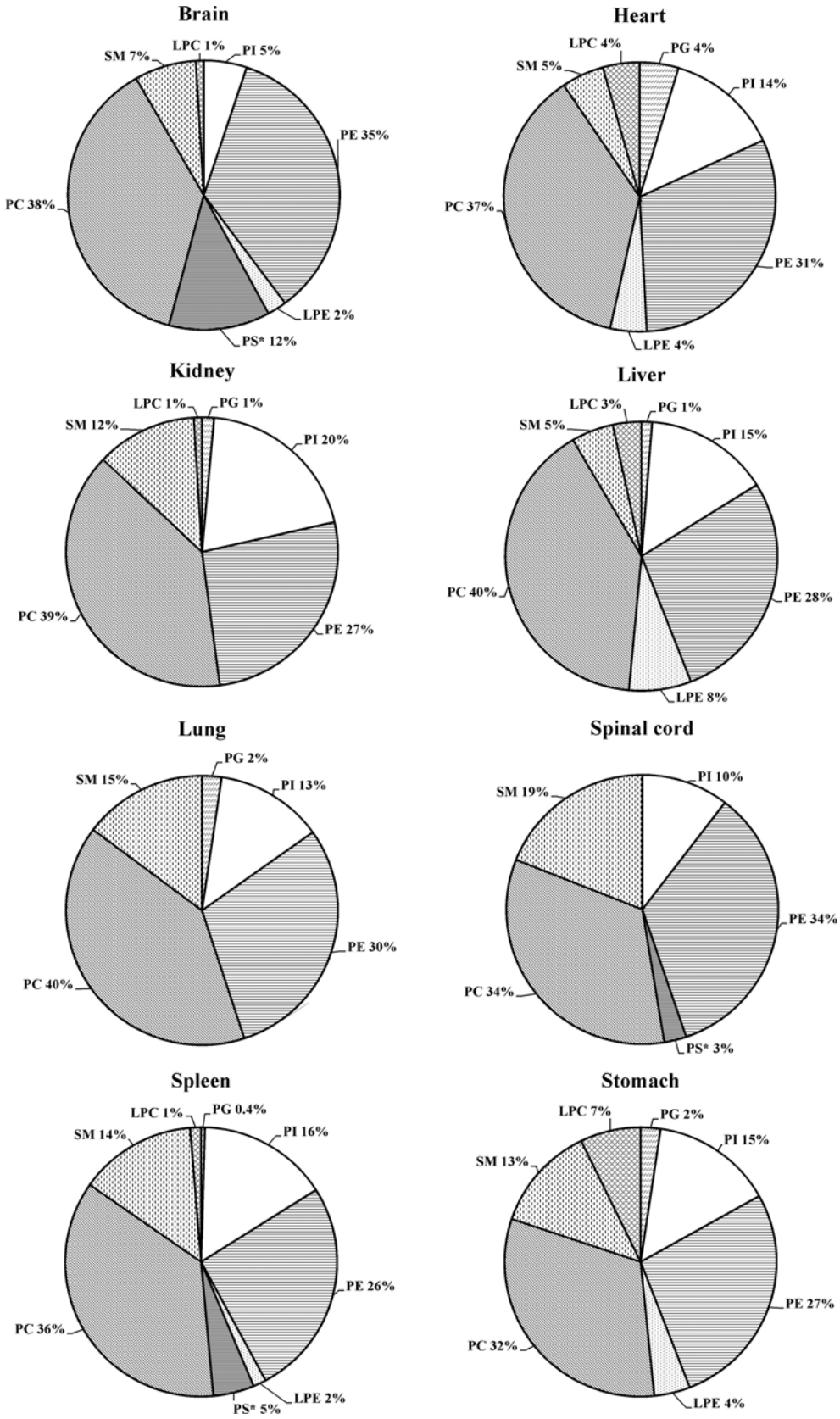

Fig. 2 Relative abundances [%] of monitored lipid classes in porcine organs using nontargeted quantitation in positive ESI-mode with single internal standard and response factor approach

This approach cannot be applied to the positive-ion mode due to the presence of both protonated molecules $[M+H]^+$ and sodium adducts $[M+Na]^+$ ions, because $[M+Na]^+$ ions have identical nominal masses as $[M+H]^+$ ions of lipids having two methylene units and three double bonds (DB). The resolving power (*RP*) required for the differentiation of these species is over 300 thousand (calculated from *m*/*z* 800: $RP = 800/(21.98435\text{-}21.98195) = 333\times10^3$). Such *RP* is achievable only on the ion cyclotron resonance mass spectrometer; therefore, decided for the quantitation of PE, PI and HexCer species in the negative-ion mode. For PE species, the type of fatty acyl linkage to the glycerol skeleton was also taken into account, i.e., the most commonly known ester-linked fatty acyls at both *sn*-1 and *sn*-2 positions (diacyl) are referred to as PE, ether-linked fatty acyls at the *sn*-1 position (1-alkyl-2-acyl) as ethers (ePE) and vinyl ether-linked fatty acyls at the *sn*-1 (1-alkenyl-2-acyl) as plasmalogens (pPE). Plasmalogens and ethers having identical retention times in the HILIC mode and identical $[M-H]^-$ ions (e.g., plasmalogen C38:4 has the same nominal mass *m*/*z* = 751 as ether C38:5) cannot be distinguished using this approach. Nevertheless, ether lipids are present in animal tissues as the minority; therefore, they are reported in parentheses (Fig. 3 and the discussion). The comparison of relative abundances of individual lipid species of PE and pPE (ePE) in all the analyzed porcine organs is shown in Figs 3A and 3B, respectively. Fatty acyls are annotated by their CN:DB (carbon number : double bond number). PE (Fig. 3A) containing saturated and monounsaturated fatty acyls with 16 and 18 carbon atoms are prevailing. The highest relative abundance of PE corresponds to species containing 38 carbon atoms and four DBs (typically C18:0 and C20:4 acyls). Polyunsaturated PE are present mainly in brain (typical composition C40:6, C40:5 and C40:4) and their lowest relative abundances are observed in heart. Lung, spinal cord and spleen contain the long-chain (more than 20 carbon atoms) monounsaturated or saturated fatty acyls (C40:1 and C40:2 species), which are typical of sphingolipids, but they occur in small amounts in phospholipids as well. There are two cases, where it is not possible to exclude the presence of pPE species in PE (mixtures of C36:0 with C38p:6 and C38:0 with C40p:6) due to identical *m*/*z* values observed in ESI spectra. Average parameters of aCN and aDB described in the legend are calculated from relative abundaces as weighted arithmetic means. Values of aCN are around 37 carbon atoms, but aDB varies from 2 for spinal cord to 3.3 for heart. Average parameters are useful for the simple overall characterization of particular sample type, as demonstrated earlier for TGs [33].

The representation of pPE (ePE) in various organs shows some interesting features (Fig. 3B). The highest relative abundance of pPE (ePE) is observed in kidney for C36:4 (typically C16:0 and C20:4). The most abundant pPE (ePE) species contains fatty acyls with four and more DBs, which shows that plasmalogens may serve as a source of polyunsaturated fatty acids [3]. Values of aCN in pPE (ePE) species are from 36.7 (heart and kidney) to 38.0 (liver). aDB of

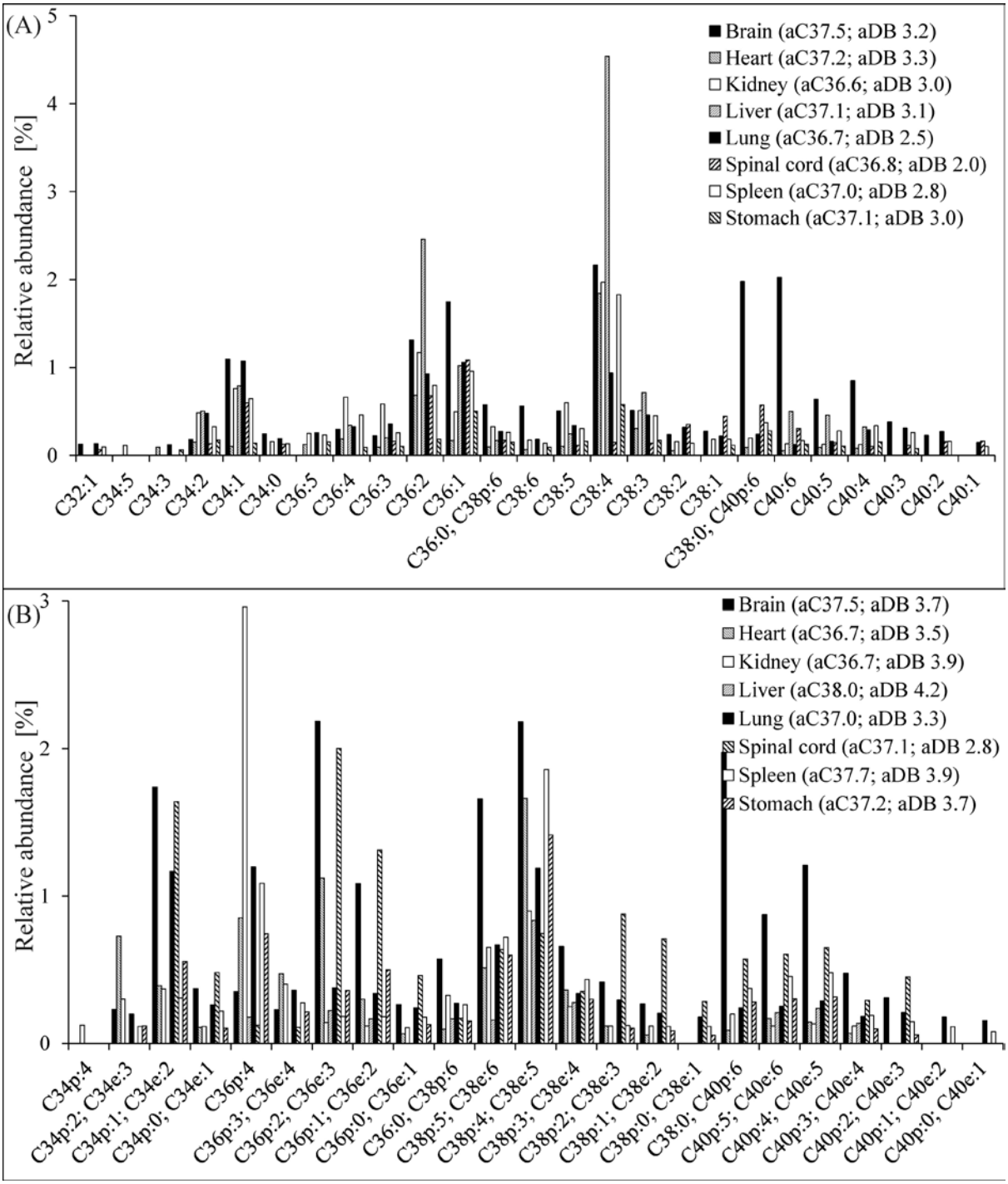

Fig. 3 Relative abundances [%] of individual species of (A) PE (1,2–diacyl) and (B) pPE (1-alkenyl-2-acyl) and ePE (1-alkyl-2-acyl) determined using relative abundances of $[M-H]^-$ ions in negative-ion HILIC-HPLC/ESI-MS and calculated average carbon numbers (aCN) and average double bonds (aDB) numbers in porcine organs

individual porcine organs varies from 3.3 (lung) to 4.2 (liver), except for spinal cord (2.8) given by higher abundances of pPE (ePE) species containing fatty acyls with 0 to 3 DBs. In contrast to PE, long-chain saturated and monounsaturated fatty acyls in individual pPE (ePE) occur in spleen and stomach.

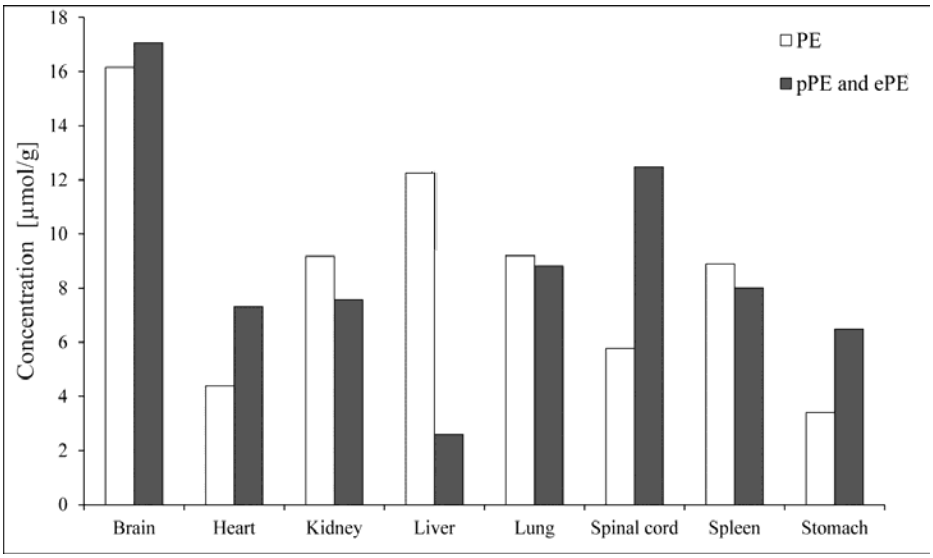

Fig. 4 Comparison of total concentrations of PE (individual species shown in Fig. 3A) and sum of pPE and ePE species (individual species shown in Fig. 3B) in porcine organs

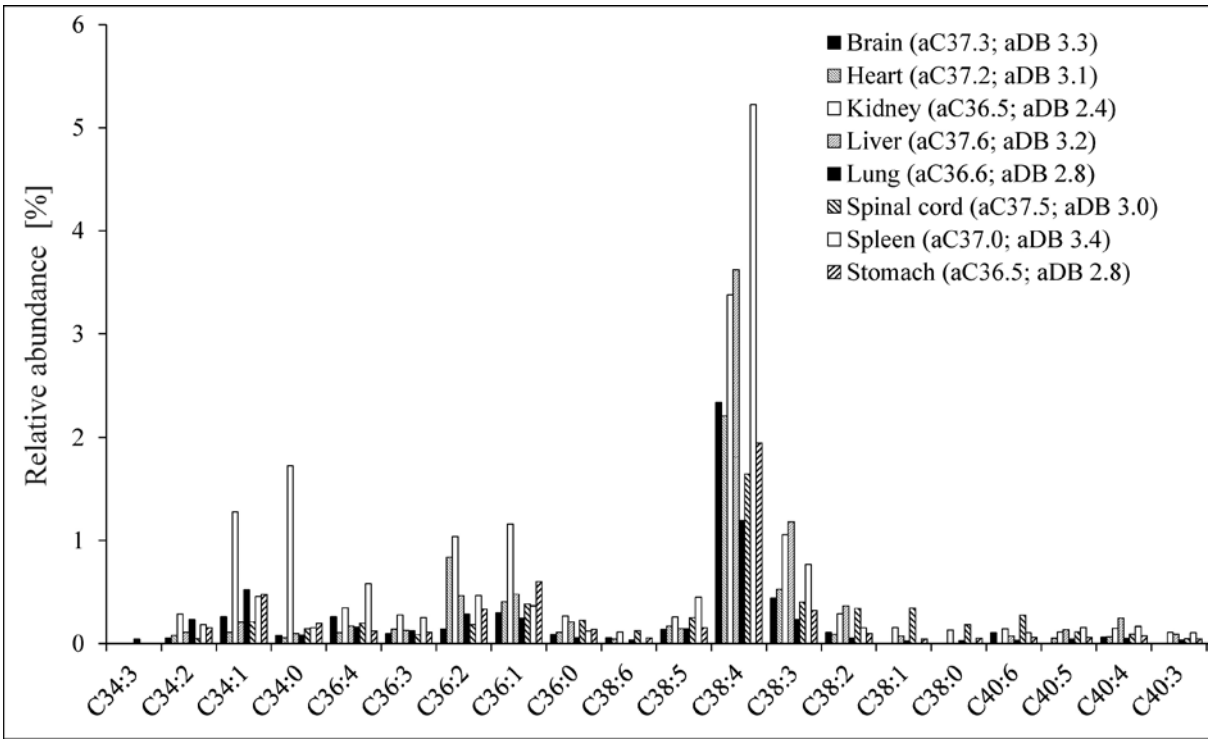

Fig. 5 Relative abundances [%] of individual PI species determined using relative abundances of $[M-H]^-$ ions in negative-ion HILIC-HPLC/ESI-MS and calculated average carbon numbers (aCN) and average double bond (aDB) numbers in porcine organs

In general, plasmalogens can form up to 70 % of PE in animal tissues, where they have many important functions [6,7]). Therefore, we compare the relative abundance of PE and pPE (ePE) in porcine organs (Fig. 4). The lowest amount of

pPE (ePE) is found in liver, which could be related with the biosynthesis of fatty acids in liver [6]. On the other hand, nervous tissues (brain and spinal cord) contain the highest amounts of pPE (ePE). The high relative proportion of pPE (ePE) is also observed in porcine heart and stomach. In these organs, plasmalogens have functions of antioxidants, signaling molecules and modulators of membrane dynamics [6,7]. Furthermore, individual lipid species of PI (Fig. 5) were determined, where the highest relative abundance in all the porcine organs corresponds to C18:0 and C20:4 fatty acyls (identical as for PE) with the highest abundance in spleen. High proportions of C34:0 and C34:1 PI species are determined in kidney unlike to other organs. Values of aCN are around 37 and aDB are from 2.4 (kidney) to 3.4 (spleen).

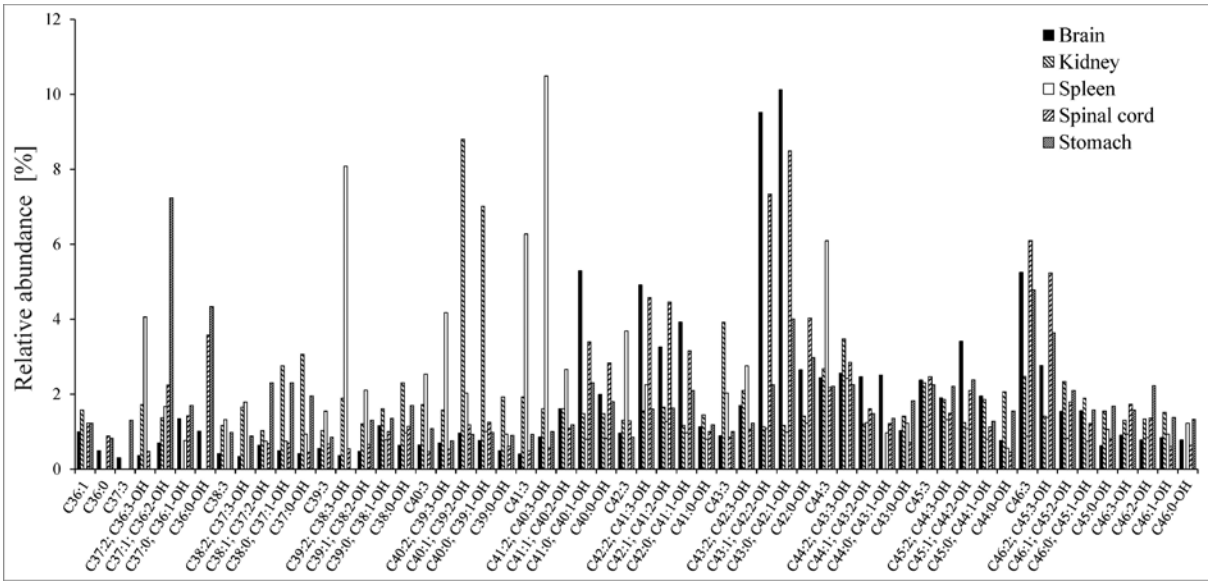

Fig. 6 Relative abundances [%] of individual HexCer containing nonhydroxy- or hydroxy-fatty acyls in brain, kidney, spleen, spinal cord and stomach determined using relative abundances of $[M-H]^-$ ions in negative-ion HILIC-HPLC/ESI-MS

This approach was also used for the characterization of relative abundances of individual sphingolipids (i.e., HexCer) containing nonhydroxy-fatty acyls or hydroxy-fatty acyls in porcine brain, kidney, spleen, spinal cord and stomach (Fig. 6). HexCer are the most abundant in nervous tissues (brain and spinal cord), where they form important components of neurons. Moreover, they are heavily represented in spleen due to various functions in the immune system. Each organ has different HexCer with the highest relative abundance, i.e., C42:1-OH (C43:0) and C42:2-OH (C43:2) in brain and spinal cord, while C36:2-OH (C37:1) and C36:0-OH in stomach. Kidney and spleen contain the most abundant nonhydroxy-fatty acyls such as C40:1 (C39:2-OH) and C40:0 (C39:1-OH) in kidney and C41:2 (C40:3-OH) and C39:2 (C38:3-OH) in spleen. As for plasmalogens and ethers, lipid species having identical values of $[M-H]^-$ ions for fatty acyls containing

nonhydroxy- and hydroxy-forms (e.g., HexCer containing C42:2-OH has the same nominal value of m/z = 825 as HexCer containing C43:2) cannot be distinguished using this approach. Fatty acyls in parentheses are less probable due to the odd carbon number or higher number of DBs, because saturated and monounsaturated fatty acyls are typical of glycosphingolipids.

Conclusion

Nontargeted HILIC-HPLC/ESI-MS method enables the characterization of individual lipid classes and their representation in vital porcine organs such as brain, heart, kidney, liver, lung, spinal cord, spleen and stomach. Concentrations of individual lipid classes in porcine organs were compared with previously published data. The determination of individual lipid species inside these classes was performed based on relative abundances of deprotonated molecules $[M\text{-}H]^-$ in the negative-ion ESI mode. The first approach using $[M\text{-}H]^-$ ions provides important information on PE and their plasmalogens or ether analogs, PI and HexCer in studied organs. The characterization of individual organs was performed using average parameters of CN and DB. The comprehensive lipidomic analysis provides the detailed knowledge of the lipidome, which will be applied for future metabolic studies on human to investigate serious lipid-related human disorders.

Abbreviations:

CE	cholesteryl ester
Cer	ceramide
Chol	cholesterol
CN	carbon number
DB	double bond
ESI	electrospray ionization
ePC	plasmanylphosphatidylcholine (ether)
ePE	plasmanylphosphatidylethanolamine (ether)
HexCer	hexosylceramide
HILIC	hydrophilic interaction liquid chromatography
HPLC	high-performance liquid chromatography
IS	internal standard
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
NMR	nuclear magnetic resonance
MS	mass spectrometry

PC phosphatidylcholine
PE phosphatidylethanolamine
PI phosphatidylinositol
PG phosphatidylglycerol
pPC plasmenylphosphatidylcholine (plasmalogen)
pPE plasmenylphosphatidylethanolamine (plasmalogen)
PS phosphatidylserine
QqQ triple quadrupole
RF response factor
RP reversed-phase
SM sphingomyelin
SRM selected reaction monitoring
TG triacylglycerol

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