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# High-Throughput and Comprehensive Lipidomic Analysis Using Ultrahigh-Performance Supercritical Fluid Chromatography–Mass Spectrometry

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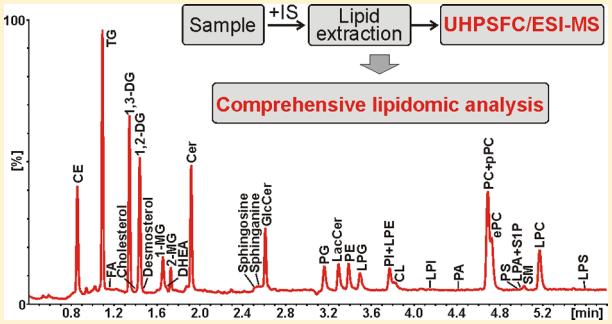
Supporting Information

**ABSTRACT:** New analytical approach for high-throughput and comprehensive lipidomic analysis of biological samples using ultrahigh-performance supercritical fluid chromatography (UHPSFC) with electrospray ionization-mass spectrometry (ESI-MS) is presented in this work as an alternative approach to established shotgun MS or high-performance liquid chromatography-MS. The lipid class separation is performed by UHPSFC method based on 1.7 $\mu$m particle-bridged ethylene hybrid silica column with a gradient of methanol–water–ammonium acetate mixture as a modifier. All parameters of UHPSFC conditions are carefully optimized and their influence on the chromatographic behavior of lipids is discussed. The final UHPSFC/ESI-MS method enables a fast separation of 30 nonpolar and polar lipid classes within 6 min analysis covering 6 main lipid categories including fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterols, and prenols. Individual lipid species within lipid classes are identified based on positive and negative-ion full-scan and tandem mass spectra measured with high mass accuracy and high resolving power. Developed UHPSFC/ESI-MS method is applied for the analysis of porcine brain extract as a complex lipidomic sample, where 24 lipid classes containing 436 lipid species are identified. The method is validated for the quantitative analysis of lipid species in biological tissues using internal standards for each lipid class. This high-throughput, comprehensive and accurate UHPSFC/ESI-MS method is suitable for the lipidomic analysis of large sample sets in the clinical research.






Lipidomics has had growing interest in recent decades because lipids are recognized as biologically active compounds having many functions in the human organism. They are a major form of energy storage in mammals, a source of fat soluble vitamins and essential fatty acids, main structural components of biological membranes, or act as cell signaling molecules.[1−3] According to the definition suggested by the LIPID MAPS consortium, lipids are hydrophobic or amphipathic small molecules that may originate entirely or in part by carbanion-based condensations of ketoacyl thioesters and/or by carbocation based condensations of isoprene units.[4] This definition covers a wide range of compounds, which can be divided into 8 categories and many subcategories according to their structures.[5] The extreme structural diversity of lipids in real biological samples is challenging for analytical techniques used in the lipidomic analysis due to large differences in physicochemical properties of individual species. At present time, two main analytical strategies are used in lipidomics,[6,7] such as the direct infusion mass spectrometry (MS) approach (known as shotgun MS) and the high-performance liquid chromatography (HPLC)/MS approach.

The shotgun MS methods use the characteristic fragmentation behavior of individual lipid classes detected by MS/MS scans (i.e., precursor ion, neutral loss and selected reaction monitoring scans) on triple quadrupole or quadrupole-linear ion trap mass spectrometers[2,8−10] or based on the measurement of MS and MS/MS spectra using mass analyzers with high resolving power and high mass accuracy.[11−14] In general, shotgun MS methods are considered to be high-throughput (analysis time is usually 10−30 min according to the number of scans) and easier to automate with robotic systems compared to HPLC techniques. On the other hand, these methods are less convenient for the direct resolution of some isobaric species or isomers and the identification of low abundant species because of possible ion suppression. Shotgun lipidomics with differential ion mobility have been used for the resolution of some isobaric and isomeric species.[15]

HPLC/MS methods usually provide more comprehensive information about the lipid composition, but they are typically more time-consuming with the analysis time in tens of minutes up to several hours in case of special methods for the separation of isomers. Hydrophilic-interaction liquid chromatography (HILIC)[16−21] enables the separation of lipids into lipid classes according to their polarity, but usually nonpolar lipids (e.g., TG,

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CE, Chol, etc.) elute nonresolved close to the system void volume. Therefore, normal-phase (NP) HPLC is used for the resolution of nonpolar lipid classes.[16,21−24] Reversed-phase (RP)-HPLC is widely used for the separation of individual lipid species according to the carbon number (CN) and the double bond (DB) number.[17,19,25−28] Nonaqueous reversed-phase (NARP)-HPLC[29−31] systems are preferred for the separation of nonpolar lipid species, such as TG. Nowadays, ultrahigh-performance liquid chromatography (UHPLC) with sub-two-micrometer particles is often used in the lipidomic analysis,[27,32,33] because it offers a significant reduction of the analysis time, the peak width (sensitivity increase) and the solvent consumption.

Supercritical fluid chromatography (SFC), especially using subtwo μm particles as ultrahigh-performance SFC (UHPSFC),[34] shows a great potential as the comprehensive and high-throughput screening method for the large number of samples in different omic fields including lipidomics. UHPSFC is not yet widely established in the lipidomic analysis and so far only SFC using conventional HPLC columns with 5 μm particles has been used. In general, C18 columns are most frequently used in SFC lipidomic analysis. SFC using C18 column has been used for the profiling of lipids in samples as intact molecules[35,36] or after their derivatization (methylation),[37] for the separation of TG,[38] oxidized PC,[39] carotenoids,[40] or TG regioisomers.[41] SFC using silver-ion HPLC columns has been used for the separation of TG from vegetable oils.[42]

The goal of our work is the development of new analytical strategy for the high-throughput and comprehensive lipidomic analysis of biological samples applicable for large clinical studies. We present new high-throughput UHPSFC/ESI-MS method using subtwo μm particle bridged ethylene hybrid silica column for the separation of wide range of nonpolar and polar lipid classes in one analysis including the identification and quantitation of individual lipid species using ESI-MS. The potential of this lipidomic method is demonstrated on the analysis of porcine brain extract as a complex lipidomic sample.

## EXPERIMENTAL SECTION

**Materials.** Acetonitrile, 2-propanol, methanol (all HPLC/MS grade), *n*-hexane, chloroform stabilized with 0.5−1% ethanol (both HPLC grade), ammonium acetate, ammonium formate and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was prepared with Demiwa 5-roi purification system (Watek, Ledeč nad Sázavou, Czech Republic) and by ultra CLEAR UV apparatus (SG, Hamburg, Germany). Carbon dioxide 4.5 grade (99.995%) was purchased from Messer Group Gmbh (Bad Soden, Germany). Standards of lipid class representatives were used for UHPSFC/ESI-MS method development, that is, CE, TG, FA, 1,3-DG, 1,2-DG, and 1-MG containing oleoyl acyls and cholesterol purchased from Sigma-Aldrich and Cer, PG, PE, LPG, PI, LPE, CL, LPI, PA, PC, pPC, ePC, PS, LPA, SM, LPC, and LPS containing oleoyl acyls, sphingosine, sphinganine, GlcCer d18:1/16:0, LacCer d18:1/16:0, S1P d17:1, desmosterol and DHEA purchased from Avanti Polar Lipids (Alabaster, AL, USA). Internal standards (IS) were used for the validation and quantitation, that is, CE 19:0, TG 19:0/19:0/19:0, FA 14:0, DG 19:0/0:0/19:0, and MG 19:0/0:0/0:0 purchased from Nu-ChekPrep (Elysian, MN, USA), D7-cholesterol, Cer d18:1/17:0, GlcCer d18:1/12:0, PG 14:0/14:0, LacCer d18:1/12:0, PE 14:0/14:0, LPG 14:0/0:0, LPE 14:0/0:0, PC 22:1/22:1, PC 14:0/14:0, SM d18:1/17:0, and LPC 17:0/0:0 purchased from Avanti Polar Lipids. Stock solutions of individual IS at the concentration of 2 mg/mL were prepared in a chloroform−2-propanol mixture (1:4). Stock solution of all IS was prepared by the mixing of 20 μL of each IS. Calibration solutions were prepared by the dilution in a hexane−chloroform mixture (7:3, v/v). Porcine brain was purchased at a local store. The total lipid extract of porcine brain for nontargeted identification of lipid species was prepared from 50 mg of the sample according to the modified Folch procedure,[18] then evaporated by a gentle stream of nitrogen and redissolved in 1 mL of a chloroform−2-propanol mixture (1:1, v/v). Ten microliters of the extract stock solution was diluted into 1 mL of the hexane−chloroform mixture (7:3, v/v) for nontargeted UHPSFC/ESI-MS analysis. The same procedure was used for the preparation of the total lipid extract for UHPSFC/ESI-MS quantitation with the addition of IS into the porcine brain sample before the extraction. Two microliters of LPE and DG, 4 μL of TG, Cer, PG, LPC, and MG, and 40 μL of FA, GlcCer, CE, D7-cholesterol, sulfatide, PE, PI, PC, and SM stock solutions of IS were added.

**UHPSFC/ESI-MS Conditions.** All UHPSFC experiments were performed on an Acquity UPC$^2$ instrument (Waters, Milford, MA, USA). The final method for lipidomic analyses used the following conditions: Acquity BEH UPC$^2$ column (100 mm × 3 mm, 1.7 μm, Waters), the flow rate 1.9 mL/min, the injection volume 1 μL, the column temperature 60 °C, the active back pressure regulator (ABPR) pressure 1800 psi and the gradient of methanol−water mixture (99:1, v/v) containing 30 mM of ammonium acetate as a modifier: 0 min, 1%; 5 min, 51%; 6 min, 51%. The injector needle was washed with the hexane−2-propanol−water mixture (2:2:1, v/v/v) after each injection.

UHPSFC instrument was connected with the mass spectrometer via the commercial interface kit (Waters) composed of two T-pieces enabling the backpressure control and mixing of column effluent with a makeup liquid. The mixture of methanol−water (99:1, v/v) at the flow rate 0.25 mL/min delivered by HPLC 515 pump (Waters) was used as a makeup liquid. The hybrid quadrupole−traveling wave ion mobility−time-of-flight mass spectrometer Synapt G2Si (Waters) in high-resolution mode with both positive-ion and negative-ion ESI modes was used in the mass range $m/z$ 50−1600 with the following setting of tuning parameters: capillary voltages 3.0 kV and 2.5 kV for positive-ion and negative-ion modes, respectively, the sampling cone 20 V, the source offset 90 V, the source temperature 150 °C, the drying temperature 500 °C, the cone gas flow 0.8 L/min, the drying gas flow 17 L/min, and the nebulizer gas flow 4 bar. Leucine enkephaline was used as the lock mass for all experiments. MS/MS experiments were performed on a transfer cell with the collision energy ramp from 20 to 35 eV.

**Method Validation.** The linearity, the limit of detection (LOD), and the limit of quantitation (LOQ) of developed UHPSFC/ESI-MS method were determined based on the analysis of spiked lipid brain extract as a matrix by IS with the final concentration 0.4, 2, 4, 40, 400, 1000, and 3000 ng/mL. Ten microliters per milliliter of the extract stock solution was used for the experiments. The linearity was determined from calibration curves of individual IS plotted as peak abundances vs concentrations of standard solutions and calculated by the linear regression. Each calibration point was measured in triplicate from one calibration solution. LOD and LOQ were


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determined from signal-to-noise ratios 3 and 10, respectively. Matrix effects were determined based on comparing the peak abundances of pure IS and IS spiked into the lipid brain extract as a matrix. The reproducibility of peak area was determined from 6 consecutive measurements of spiked porcine brain extract with IS for low (2 ng/mL) and high (1000 ng/mL) concentrations. The reproducibility of retention times was calculated as a standard deviation from 6 consecutive injections of the sample.

**Quantitative Analysis.** Final UHPSFC/ESI-MS conditions were used for quantitative analysis of lipid species in the porcine brain sample. Concentrations of lipid species were calculated from peak abundances of corresponding ions in lipid class ESI mass spectra after the isotopic correction related to the peak abundance of IS. Concentrations were calculated in pmol/mg of wet sample with average standard deviation of ±1.9%.

**Lipid Nomenclature.** LIPID MAPS nomenclature[4,5] and shorthand notation[43] were followed throughout this manuscript.

## RESULTS AND DISCUSSION

**UHPSFC/ESI-MS Method Development.** Precise, comprehensive, and high-throughput methods are required for the analysis of large sample sets in current lipidomics. Our lipidomic approach is based on UHPSFC lipid class separation followed by ESI-MS identification and quantitation of individual species. UHPSFC with 1.7 $\mu$m particle size column is used in this work as the method providing high chromatographic efficiency together with high speed of analyses. First, the comprehensive nontargeted UHPSFC/ESI-MS analysis of representative or pooled sample is performed to describe the lipidomic composition of studied samples. The total lipid extract is separated into lipid classes using UHPSFC enabling their direct identification according to retention times. Individual lipid species within lipid classes are identified using positive-ion and negative-ion MS and MS/MS spectra with high mass accuracy and high resolving power. Then, the targeted quantitative analysis of identified lipid species in individual samples is performed using the UHPSFC/ESI-MS. Concentrations of individual lipid species are obtained from their relative abundances related to the IS.

In our work, we follow a generally accepted strategy of quantitative lipidomic analysis using IS per each lipid class added before the extraction, which covers different extraction efficiencies and MS responses among lipid classes, while differences among individual species within the class given by different acyl composition are not considered. IS are selected based on the detailed knowledge of lipidomic composition from the nontargeted UHPSFC/ESI-MS analysis of representative sample without IS. Lipid species which are not detected in the representative sample or having concentrations below LOQ are used as IS (see Experimental Section), that is, species containing deuterium atoms (D7-cholesterol), odd number fatty acyls (e.g., CE 19:0, DG 19:0/0:0/19:0, etc.), short or long fatty acyls (e.g., PE 14:0/14:0, PC 22:1/22:1, etc.) similarly to previous works.[10,13] Prerequisite for the precise quantitation is identical ionization conditions for IS and determined lipids, mainly a mobile phase composition and a matrix, which is especially important for the gradient elution. This requirement cannot be fulfilled with widely used C18 columns in the SFC analysis, where species within the lipid class are separated according to the composition of fatty acyls and elute in different retention times under the different mobile phase composition within the gradient. For this reason, we have decided to use HILIC-like separation of lipids into lipid classes, where lipid species and lipid class IS coelute in one peak under the identical mobile phase and matrix composition. Moreover, ion suppression effects among lipid classes are avoided as they are separated into individual peaks. For this purpose, UHPSFC dedicated column with bridged ethylene hybrid silica stationary phase is selected for the lipid class separation and individual parameters of the UHPSFC separation are carefully optimized with respect to the separation of the highest number of lipid classes within the shortest analysis time.

A polar modifier has to be used to increase the polarity of mobile phase for the analysis of lipids due to their wide range of polarities from rather nonpolar up to ionic species. The composition of the modifier is one of the most critical parameters influencing the UHPSFC separation of lipids. Different organic solvents have been tested, such as 2-propanol, ethanol, methanol, acetonitrile and their mixtures. Methanol is the best compromise between the chromatographic and ionization efficiency and also provides the best solubility of ammonium acetate necessary for the separation of lipids, which is poorly soluble in 2-propanol and almost insoluble in acetonitrile. Mixtures of acetonitrile or 2-propanol with methanol have also been tested, but they do not bring significant improvement compared to pure methanol. The partial improvement of the peak shape mainly for polar lipids (eluting after 2.7 min) is achieved with the addition of small amount of water into the modifier (Supporting Information Figure S-1). The selectivity is not influenced by the addition of water into the modifier except for LacCer and PE species, which are baseline resolved at 1% of water in the modifier (3.45 and 3.59 min), but coelute at 3% (3.46 min). The significant effect mainly on the peak shape of polar species is observed for the concentration of ammonium acetate in the modifier (Figure 1). Without ammonium acetate, only nonpolar lipid classes eluting before 2.0 min are separated, while polar lipids provide very broad peaks. The peak shape of polar lipids is significantly improved by increasing the concentration of ammonium acetate, while the separation of nonpolar lipids is not influenced. The ionization efficiency of lipids is also influenced by the addition of ammonium acetate into the modifier. The peak areas of TG, MG, GlcCer, and LacCer are reduced to half with addition of 5 mM of ammonium acetate, but the peak area of CE is doubled (Supporting Information Table S-1). The ionization efficiency of lipids is almost constant within the range from 5 to 30 mM of ammonium acetate and starts slightly deteriorate at 50 mM, therefore the concentration of 30 mM is selected. The addition of acetic acid or ammonium hydroxide has also been tested, but the effect on the chromatographic separation is negligible, so they are not used in the final method.

The analysis time is shorter with increased initial concentration of the modifier, but the low concentration (1%) is selected for the gradient, because it significantly improves the separation of nonpolar lipid classes (Supporting Information Figure S-2). Peak widths are slightly improved with the increase of the modifier gradient steepness, while the separation of lipids is not significantly influenced, which perfectly fits with the requirement for the high-throughput method (Supporting Information Figure S-3). As expected, the increased flow rate does not influence too much the chromatographic resolution using UHPSFC (Supporting




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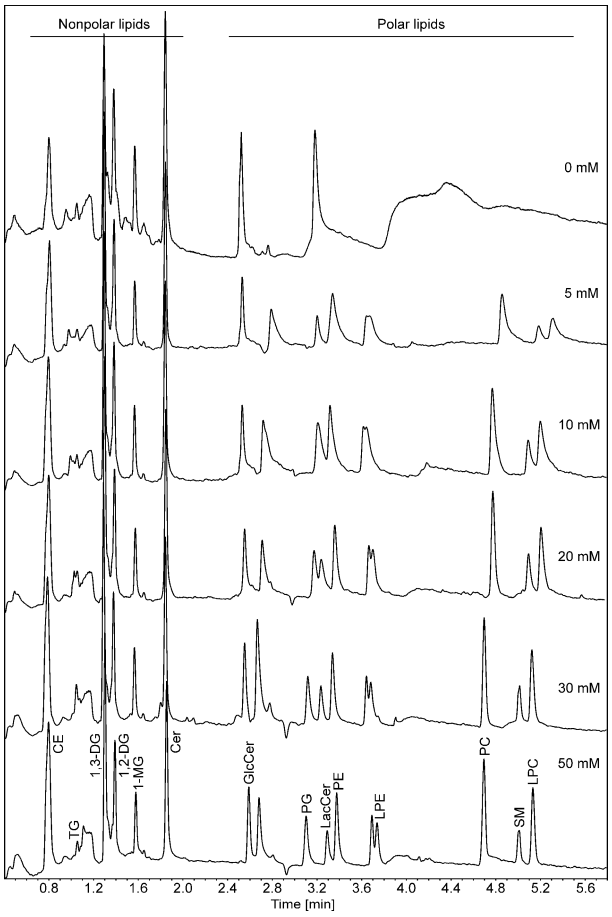


**Figure 1.** Effect of ammonium acetate concentration in the modifier on the UHPSFC/ESI-MS analysis of lipid class standards. UHPSFC conditions: Acquity BEH UPC$^2$ column (100 × 3 mm, 1.7 $\mu$m, Waters), the flow rate 1.9 mL/min, column temperature 60 °C, the ABPR pressure 1500 psi and the gradient of methanol with ammonium acetate as a modifier: 0 min, 2%; 5 min, 52%; 6 min, 52%. Peak annotation: CE, cholesteryl esters; TG, triacylglycerols; DG, diacylglycerols; MG, monoacylglycerols; Cer, ceramides; GlcCer, glucosylceramides; PG, phosphatidylglycerols; LacCer, lactosylceramides; PE, phosphatidylethanolamines; LPE, lysophosphatidylethanolamines; PC, phosphatidylcholines; SM, sphingomyelins; LPC, lysophosphatidylcholines.

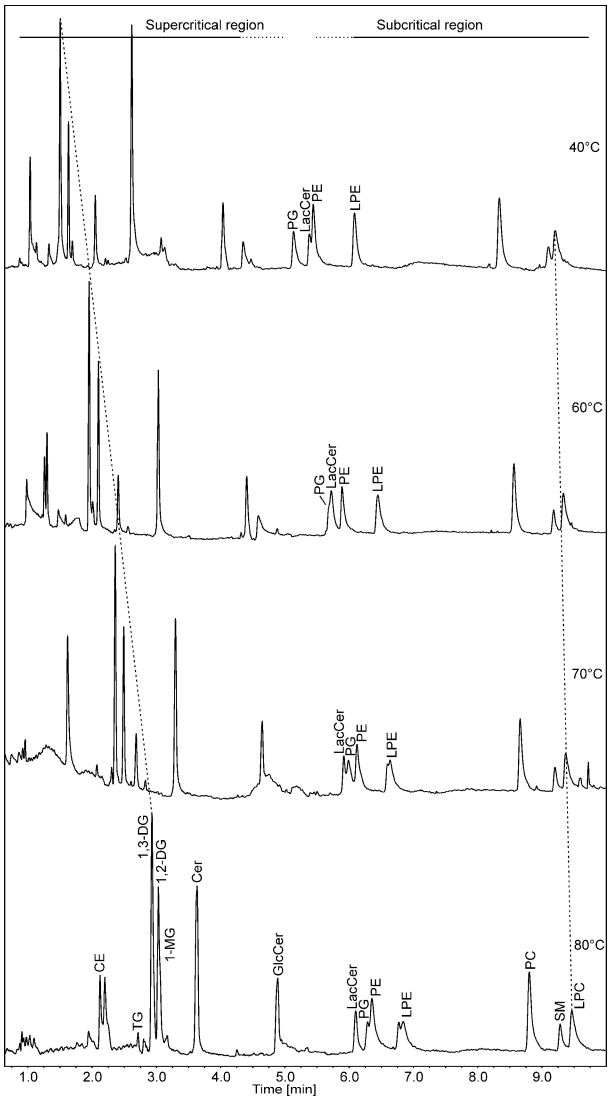


**Figure 2.** Effect of column temperature on the UHPSFC/ESI-MS analysis of lipid class standards. Dashed lines highlight changes of the retention behavior of TG (left) and LPC (right) species. UHPSFC conditions: Acquity BEH UPC$^2$ column (100 × 3 mm, 1.7 $\mu$m, Waters), the flow rate 1.9 mL/min, the ABPR pressure 1500 psi and the gradient of methanol with 10 mM of ammonium acetate as the modifier: 0 min, 0%; 10 min, 50%; 11 min, 50%. Peak annotation: CE, cholesteryl esters; TG, triacylglycerols; DG, diacylglycerols; MG, monoacylglycerols; Cer, ceramides; GlcCer, glucosylceramides; PG, phosphatidylglycerols; LacCer, lactosylceramides; PE, phosphatidylethanolamines; LPE, lysophosphatidylethanolamines; PC, phosphatidylcholines; SM, sphingomyelins; LPC, lysophosphatidylcholines.

Information Figure S-4), which is in accordance with well-known fact that the optimum theoretical plate height lies in a wide range of linear velocities.[34] It has to be noted that multiple parameters are changed by the change of flow rate, such as the column pressure drop and the gradient volume to column volume ratio. The flow rate of 1.9 mL/min is used, which enables the high-throughput analysis with short retention times and acceptable system backpressure.

In this work, no significant effect on the chromatographic efficiency is observed with the change of active back pressure regulator pressure (Supporting Information Figure S-5), therefore the pressure of 1800 psi is used as the lowest recommended value for a good reproducibility of retention times. The column temperature (Figure 2) does not significantly influence the chromatographic efficiency, but it causes some minor changes in the chromatographic selectivity. This can be demonstrated on the retention order of PG and LacCer standards, that is, PG elutes before LacCer at 40 and 60 °C, but after at 70 and 80 °C. The temperature 60 °C is used, which is a common value for UHPSFC analyses and provides a lower resistance and system backpressure.

UHPSFC effluent is mixed with the makeup fluid to ensure the ionization of lipids using ESI. The flow rate and the composition of makeup fluid have been optimized with respect to the ionization efficiency of lipids. The separated species are diluted by the makeup fluid lowering their concentration and sensitivity; therefore, the lowest possible flow rate of 0.25 mL/min is used, which is sufficient for stable electrospray and good ionization efficiency of lipids. The composition of the makeup fluid has also been optimized with the best results obtained for the mixture of methanol−water (99:1, v/v), which corresponds to the composition of the modifier in the final method.



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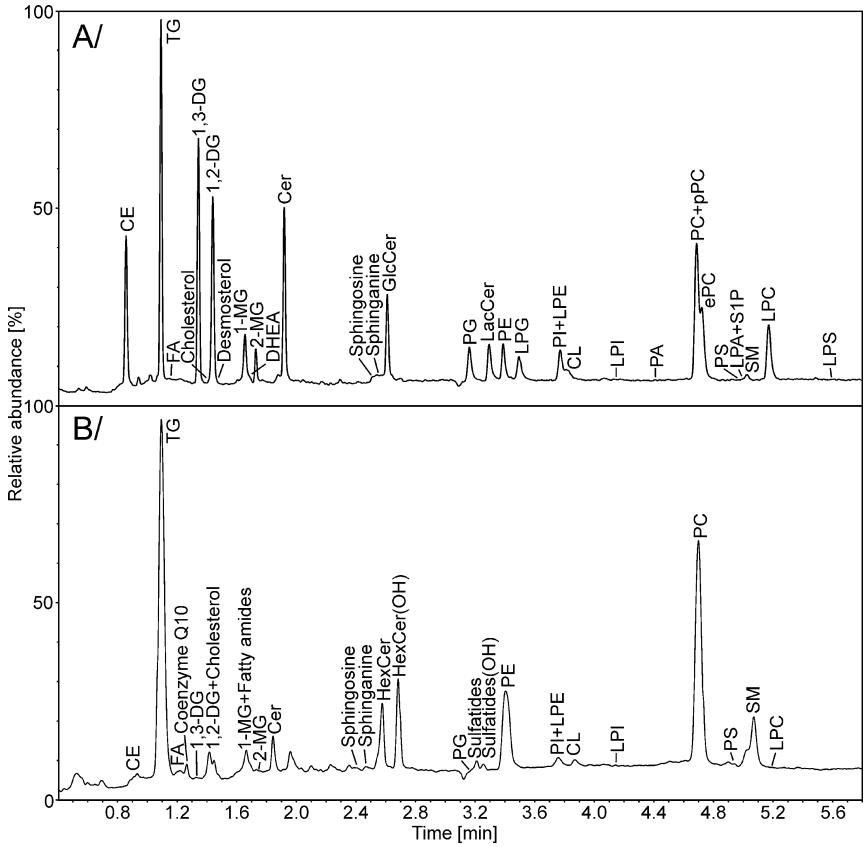


**Figure 3.** Positive-ion UHPSFC/ESI-MS chromatograms of the mixture of lipid class standards (A) and the total lipid extract of porcine brain (B). UHPSFC conditions: Acquity BEH UPC$^2$ column (100 × 3 mm, 1.7 $\mu$m, Waters), the flow rate 1.9 mL/min, the column temperature 60 °C, the ABPR pressure 1800 psi and the gradient of methanol−water mixture (99:1, v/v) containing 30 mM of ammonium acetate as the modifier: 0 min, 1%; 5 min, 51%; 6 min, 51%. Peak annotation: CE, cholesteryl esters; TG, triacylglycerols; FA, fatty acids; DG, diacylglycerols; MG, monoacylglycerols; DHEA, dehydroepiandrosterone; Cer, ceramides; GlcCer, glucosylceramides; HexCer, hexosylceramides; PG, phosphatidylglycerols; LacCer, lactosylceramides; pPE, 1-alkenyl-2-acyl phosphatidylethanolamines (plasmalogens); ePE, 1-alkyl-2-acyl phosphatidylethanolamines (ethers); PE, phosphatidylethanolamines; LPG, lysophosphatidylglycerols; PI, phosphatidylinositols; LPE, lysophosphatidylethanolamines; CL, cardiolipins; LPI, lysophosphatidylinositols; PA, phosphatidic acids; PC, phosphatidylcholines; pPC, 1-alkenyl-2-acyl phosphatidylcholines; ePC, 1-alkyl-2-acyl phosphatidylcholines; PS, phosphatidylserines; LPA, lysophosphatidic acids; S1P, sphingosine-1-phosphate; SM, sphingomyelins; LPC, lysophosphatidylcholines; LPS, lysophosphatidylserines.

**UHPSFC Separation of Lipids.** Figure 3 shows the separation of lipid class representatives (Figure 3A) and the total lipid extract of porcine brain (Figure 3B) using the final UHPSFC method (see Experimental Section). In our UHPSFC method, lipids are mainly separated according to their polarity into lipid classes. Retention times increase with the increased lipid polarity similarly to HILIC or NP-HPLC separations. In total, up to 30 lipid classes from 6 different lipid categories covering the wide range of nonpolar and polar lipids are separated within 6 min analysis. Under optimized UHPSFC conditions, most of lipid classes are baseline or at least partially separated. Similarly to HPLC separations, the strong peak tailing is observed for UHPSFC of acidic lipids, such as PA, PS, LPA, S1P and LPS, which complicates their precise quantitation. This behavior can be overcome by the use of stronger additives, such as alkylamines,[44] but these additives are not compatible with MS detection, because they cause severe memory effects.[45]

In addition to the class separation, lipid species within individual lipid classes are partially separated according to the fatty acyl composition (Figure 4). Retention times of all lipids increase with increased DB number, as demonstrated on reconstructed ion chromatograms of TG with 54 carbon atoms and the different number of DB (Figure 4A). The different situation is observed for the species differing in fatty acyl lengths, where the retention behavior of lipids differs for individual lipid classes. Retention times of TG increase with the fatty acyl length (Figure 4B), which is observed also for other nonpolar lipid classes, such as CE, FA, DG, MG, fatty amides and Cer (Supporting Information Table S-2). On the other hand, retention times of polar lipids decrease for longer fatty acyls, which can be demonstrated on the separation of PC species (Figure 4C). The retention behavior of nonpolar and polar lipids using UHPSFC can be correlated with HPLC using HILIC and NP modes.[17,22] Retention times of nonpolar lipids increase with the higher number of DB both in NP-HPLC and UHPSFC systems, but UHPSFC also provides the partial separation of lipid species differing in fatty acyl lengths. No separation is observed in the HILIC mode for polar lipids containing 0 to 3 DB, but species with 4 and more DB are less retained, which is reversed to UHPSFC. On the other hand, lower retention times for longer fatty acyl species are observed for HILIC mode, which is the same as for UHPSFC of polar lipids. It seems that there is a mixed-mode retention mechanism using our UHPSFC method changing from NP mechanism for nonpolar lipids to HILIC mechanism for polar lipids. This




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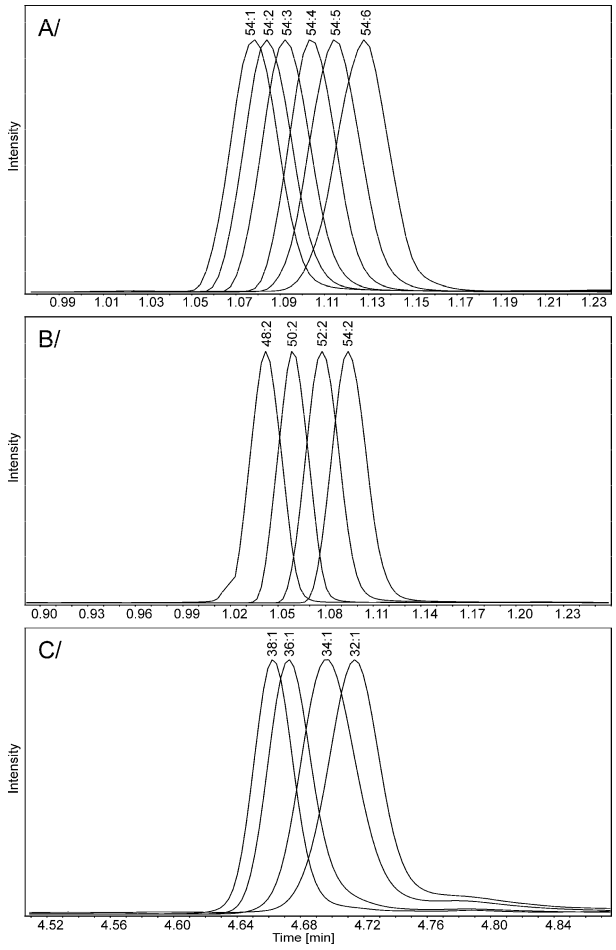


**Figure 4.** Effects of DB number and fatty acyl chain length on the retention behavior of lipids using UHPSFC/ESI-MS. Reconstructed ion chromatograms from the analysis of porcine brain extract: A/TG with 54 carbon atoms and the different number of DB, B/TG with 2 DB and the different number of carbon atoms and C/PC with 1 DB and the different number of carbon atoms. UHPSFC conditions are identical as for Figure 2.

phenomenon is probably caused by the mobile phase composition during the gradient. Almost pure $CO_2$ is used at the beginning of the analysis close to nonpolar mobile phases used in NP-HPLC. Then, the concentration of polar modifier is increased during the analysis changing from NP to HILIC conditions. This behavior enables the separation of both nonpolar and polar species in one analysis, which is not usually achieved by NP or HILIC modes or with poor reproducibility of retention times.

The addition of modifier changes the critical point of the mobile phase and usually 20−30% of modifier at maximum can be used to keep supercritical conditions. In this work, up to 51% of the modifier had to be used for the elution of polar lipid classes (e.g., PC, SM, LPC, etc.). It is evident that the mobile phase changes from the supercritical to subcritical fluid during the analysis due to high increase of modifier concentration. This can be clearly demonstrated by the influence of column temperature on retention times of lipids (Figure 2). In general, higher temperature induces a lower density of mobile phase leading to the increase of retention times.[34] Retention times of lipids increase with higher temperature using UHPSFC (as shown in Figure 2), but this effect is more pronounced in the supercritical region corresponding to approximately 1% to 26% of the modifier (up to 5 min). For example, the change of retention time of TG is 1.43 min between 40 and 80 °C, while the change for LPC is only 0.26 min. The similar behavior is observed for the ABPR pressure (Supporting Information Figure S-5). Retention times of lipids decrease with higher pressure under supercritical conditions due to the increase of mobile phase density, but changes in the subcritical region are slightly lower. Although properties of used mobile phase change significantly during the analysis, the reproducibility of retention times is very good.

**UHPSFC/ESI-MS Identification of Lipids.** Retention times from the UHPSFC analysis and the ESI mass spectra measured with high mass accuracy and high resolving power in both positive- and negative-ion modes are used for the unambiguous identification of individual lipid species (Supporting Information Table S-2). The total lipid extract is separated into lipid classes using the UHPSFC enabling the direct identification of lipid class based on the comparison of retention times with standards. The class separation of lipids also avoids the ion suppression effects among different lipid classes and improves the identification of isobaric (e.g., PC 36:1 vs PS 36:2) and trace species. Then, the averaged mass spectrum of lipid class chromatographic peak is used for the determination of lipid species level (Supporting Information Figure S-6), that is, the number of carbon atoms and DB of attached fatty acyl/alkyls. The partial separation of individual species within lipid classes according to fatty acyl lengths and the number of DB provides another supporting information for their identification. The fatty acyl composition of individual species can be determined using $[RCOO]^-$ ions in negative-ion MS/MS spectra.[17]

The ionization and fragmentation behavior of individual lipid classes using the UHPSFC/ESI-MS is similar to HPLC/ESI-MS. The most abundant ions in positive-ion UHPSFC/ESI-MS full scan mass spectra are protonated molecules $[M + H]^+$ (base peaks for fatty amides, sphinganine, LacCer, PE, LPE, CL, PC, SM, and LPC), adducts with ammonium ion $[M + NH_4]^+$ (TG and coenzyme Q10) and neutral losses of water $[M + H - H_2O]^+$ (DG, cholesterol, MG, Cer, sphingosine and LacCer), attached fatty acyls $[M + H - acyl]^+$ (CE) or phosphoglycerol $[M + H - H_2PO_4CH_2CHOHCH_2OH]^+$ (PG and LPG) and sulfo $[M + H - SO_3]^+$ (sulfatides) groups. UHPSFC/ESI-MS mass spectra provide relatively low abundance of sodium adduct ions, which reduces the risk of incorrect identification between $[M + H]^+$ and $[M + Na]^+$ ions, because the difference $\Delta m/z = 22$ also corresponds to additional 2 methylene units minus 3 DB. The relative abundance of $[M + Na]^+$ ions is below 1.5% for CE, TG, PC, SM, and LPC, from 3 to 10% for DG, Cer, PG, PE, LPG, and LPE and more than 20% only for MG, GlcCer and LacCer. The data are also correlated with negative-ion mass spectra and retention times of lipid species within lipid classes to unambiguously confirm the identification of all reported lipids. Positive-ion MS/MS spectra of identified classes provide well-known characteristic fragment ions and neutral losses observed in HPLC/MS, such as the phosphocholine fragment ion $m/z = 184$ ($[H_2PO_4CH_2CH_2N(CH_3)_3]^+$) for moieties containing choline (PC, LPC, and SM), $m/z = 369$ ($[M + H - H_2O]^+$ or $[M + H - acyl]^+$) for cholesterol containing lipids (cholesterol and CE) or fragment ions corresponding to ceramide bases (Cer, GlcCer, and LacCer). The neutral loss of phosphoethanolamine $\Delta m/z = 141$ ($H_2PO_4CH_2CH_2NH_2$) is observed for PE and neutral losses of fatty acyls for TG, DG, and MG. In the negative-ion mode,




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Table 1. Calibration Parameters of Internal Standards (IS) in the Porcine Brain Matrix Using UHPSFC/ESI-MS Method

| IS | calibration range [pmol/mL] | slope | correlation coefficient | LOD[a] [pmol/mL] | LOQ[b] [pmol/mL] | matrix effect |
|---|---|---|---|---|---|---|
| | | | positive-ion mode | | | |
| CE 19:0 | 0.5−4200 | 0.624 | 0.994 | 0.1 | 0.4 | 100 |
| TG 19:0/19:0/19:0 | 0.4−1200 | 69.590 | 0.993 | 0.07 | 0.2 | 78 |
| FA 14:0 | n.d.[c] | n.d.[c] | n.d.[c] | n.d.[c] | n.d.[c] | n.d.[c] |
| DG 19:0/0:0/19:0 | 0.6−1600 | 35.354 | 0.996 | 0.2 | 0.7 | 103 |
| D7-cholesterol | 100−7100 | 0.043 | 0.992 | 74 | 247 | 76 |
| MG 19:0/0:0/0:0 | 1−2800 | 7.561 | 0.993 | 1.4 | 4.6 | 91 |
| Cer d18:1/17:0 | 0.8−1900 | 22.926 | 0.996 | 0.1 | 0.4 | 89 |
| GlcCer d18:1/12:0 | 65−4300 | 0.678 | 0.991 | 66 | 220 | 86 |
| PG 14:0/14:0 | 60−4100 | 0.403 | 1.000 | 99 | 329 | 78 |
| LacCer d18:1/12:0 | 55−3600 | 0.403 | 0.997 | 33 | 111 | 108 |
| PE 14:0/14:0 | 65−4400 | 0.444 | 0.998 | 90 | 298 | 147 |
| LPG 14:0/0:0 | 95−6400 | 0.169 | 1.000 | 114 | 379 | 157 |
| LPE 14:0/0:0 | 100−6500 | 0.150 | 0.998 | 375 | 1252 | 83 |
| PC 22:1/22:1 | 0.5−3100 | 1.402 | 0.999 | 0.4 | 1.5 | 87 |
| PC 14:0/14:0 | 6−4100 | 1.688 | 0.998 | 12 | 41 | 78 |
| SM d18:1/17:0 | 0.6−3900 | 2.235 | 0.997 | 0.4 | 1.3 | 83 |
| LPC 17:0/0:0 | 9−5700 | 1.833 | 0.999 | 18 | 61 | 85 |
| | | | negative-ion mode | | | |
| CE 19:0 | n.d.[c] | n.d.[c] | n.d.[c] | n.d.[c] | n.d.[c] | n.d.[c] |
| TG 19:0/19:0/19:0 | n.d.[c] | n.d.[c] | n.d.[c] | n.d.[c] | n.d.[c] | n.d.[c] |
| FA 14:0 | 2−4600 | 1.338 | 0.996 | 0.3 | 1.0 | 92 |
| DG 19:0/0:0/19:0 | 0.6−1600 | 4.611 | 1.000 | 1.1 | 3.5 | 83 |
| D7-Cholesterol | n.d.[c] | n.d.[c] | n.d.[c] | n.d.[c] | n.d.[c] | n.d.[c] |
| MG 19:0/0:0/0:0 | 6−2800 | 0.870 | 0.999 | 7.3 | 24 | 86 |
| Cer d18:1/17:0 | 0.8−1900 | 19.708 | 0.998 | 0.01 | 0.03 | 96 |
| GlcCer d18:1/12:0 | 3−4300 | 1.261 | 0.993 | 4.2 | 14 | 82 |
| PG 14:0/14:0 | 3−4100 | 1.235 | 1.000 | 4.8 | 16 | 94 |
| LacCer d18:1/12:0 | 3−3600 | 0.971 | 0.991 | 5.1 | 17 | 85 |
| PE 14:0/14:0 | 3−4400 | 0.855 | 0.994 | 5.8 | 19 | 98 |
| LPG 14:0/0:0 | 5−6400 | 0.930 | 0.997 | 5.0 | 17 | 87 |
| LPE 14:0/0:0 | 5−6500 | 0.561 | 1.000 | 5.5 | 18 | 88 |
| PC 22:1/22:1 | 0.5−3100 | 0.326 | 0.999 | 0.8 | 2.5 | 89 |
| PC 14:0/14:0 | 0.6−4100 | 0.337 | 0.999 | 0.5 | 1.7 | 84 |
| SM d18:1/17:0 | 0.6−3900 | 0.523 | 0.992 | 0.02 | 0.06 | 97 |
| LPC 17:0/0:0 | 0.9−5700 | 0.266 | 0.999 | 0.6 | 2.1 | 96 |

[a]LOD: limit of detection ($S/N$ = 3). [b]LOQ: limit of quantitation ($S/N$ = 10). [c]n.d.: not detected.



base peaks of spectra are mostly deprotonated molecules $[M - H]^-$, except for DG, PC, SM, and LPC providing mainly adduct ions with acetate $[M + CH_3COO]^-$. Relatively high abundances of $[M + CH_3COO]^-$ ions are also observed for Cer, GlcCer, and LacCer (70−95%). CL species exhibit $[M - 2H]^{2-}$ ions, which can be used for their identification even in the lower mass range, such as $m/z$ = 50−1000 usually used in lipidomic analyses. Negative-ion MS/MS spectra show mainly $[RCOO]^-$ ions corresponding to the fatty acyl/alkyl composition.

**Validation of UHPSFC/ESI-MS Method.** Developed UHPSFC/ESI-MS method has been tested for the analysis of real samples represented by porcine brain extract as a complex lipidomic matrix. Table 1 shows calibration parameters of IS spiked into the porcine brain lipid extract using positive- and negative-ion ESI-MS. Obtained calibration curves (Supporting Information Figure S-7) of IS are linear within tested calibration ranges with correlation coefficients better than 0.991 in all cases. The positive-ion mode can be used for the quantitation of all tested lipid classes except for FA. In the negative-ion mode, CE, TG, and cholesterol are not detected. On the other hand, the negative-ion mode provides a better sensitivity for most classes indicated by lower LOD. Slopes of PC species slightly differ in the positive-ion mode (1.402 vs 1.688), while the difference is negligible in the negative-ion mode (0.326 vs 0.337). Slightly different ionization efficiencies can be expected for species differing by 16 carbon atoms and two double bonds in fatty acyls (i.e., 28:0 vs 44:2), but the same ionization behavior should be observed in both ionization modes. Another explanation of such behavior can be different influence of the matrix used for validation experiments to saturated and unsaturated species only in the positive-ion mode. The effect of the matrix on the response of IS (Table 1) has been evaluated from the analysis of pure IS and IS spiked into the porcine brain matrix. In negative-ion mode, the matrix effects for all IS are not significant ranging between 82% to 98%. The different behavior is observed in the positive-ion mode, where the influence of the matrix is much more pronounced. Especially ionization efficiencies of PE and LPG standards are significantly enhanced in the matrix (matrix effects 147% and 157%, respectively). The matrix has significant effect on the ionization efficiency of lipids, but these effects are identical for both lipid species and IS coeluting in one chromatographic peak. The reproducibility of peak areas has



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been calculated for IS added into the porcine brain lipid extract for 6 consecutive analyses. Average standard deviations of peak areas of IS are 2.9% and 8.2% for concentrations 1000 and 2 ng/mL, respectively.

The final UHPSFC/ESI-MS method provides an excellent intraday stability of retention times in the range ±0.01 min. A small continuous reduction of retention times of mainly polar lipids is observed during multiple consecutive days. This phenomenon of small retention shift on SFC columns is probably given by the formation of silyl ethers on the particle surface with alcohols from the mobile phase leading to the decrease of hydrophilicity,[46] but it does not affect the identification and quantitation of lipids. The process is reversible and silanols can be hydroxylated again using water. For this reason, the column is regenerated daily by 5 consecutive injections of 10 μL of water and acetonitrile into 100% of $CO_2$ at 0.5 mL/min flow rate (Supporting Information Figure S-8) or occasionally is flushed with water−acetonitrile mixture (1:1, v/v), which minimizes this effect.

**UHPSFC/ESI-MS Analysis of Porcine Brain.** Figure 3B shows the separation of the total lipid extract from the porcine brain using the final UHPSFC/ESI-MS method. The porcine brain is selected as a testing sample due to its high lipidomic complexity, which covers a high number of nonpolar and polar lipid classes with a wide range of polarities. 436 lipid species are identified and quantified in the porcine brain using the UHPSFC/ESI-MS method (Supporting Information Table S-2) from 24 different lipid classes and 6 main lipid categories including fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterols, and prenols. The number and profile of the identified species is comparable to recently published characterization of brain samples using conventional lipidomic methods. For example, 311 lipid species from 20 lipid classes have been identified in mouse cerebellum and hippocampus using the shotgun MS method, the instrument with 450 000 resolving power and different MS/MS methods.[47] 325 lipid species from 22 lipid classes have been quantified using HILIC and two RP-HPLC/MS methods in mouse and human brain tissues.[48]

The highest number of lipid species is identified for TG (72 species), PC (46), HexCer (40), sulfatides (35), PE (34), CL (32), FA (32), 1,2-DG (25), and SM (21). Glycosyl moieties in ceramide species, such as glucose and galactose, are not resolved using the UHPSFC/ESI-MS method and therefore they are annotated as hexosyl ceramides (HexCer). Lipid species with the various DB number are identified, from fully saturated up to highly polyunsaturated species containing 15 DB in case of CL 80:15. The lowest number of DB is observed for ceramides (Cer, HexCer, and sulfatides) and SM with 2 or at maximum 3 DB. Coenzyme Q10 as a representative of prenols is also identified in the porcine brain. The large number of PE and PC species with ether (ethers, ePE, ePC) and vinylether (plasmalogens, pPE, pPC) linkage are identified (Supporting Information Table S-2), but these species (ethers vs vinylethers) cannot be differentiated, so we report both possibilities.

## CONCLUSIONS

The application of lipid class separation using the UHPSFC/ESI-MS for the high-throughput and comprehensive lipidomic analysis is presented for the first time in this work. The main advantage of this method is short analysis time for the separation of both nonpolar and polar lipid classes comparable to established shotgun MS methods, but the separation dimension provides other benefits over direct infusion methods, such as easier identification of isobaric lipids based on retention times and the identification of trace species using reconstructed ion chromatograms. The quantitative analysis of lipids is also significantly improved, because the class separation completely avoids the ion suppression effects among lipid classes and individual species within the class are ionized together with IS under the same matrix effects. Results from the optimization of individual chromatographic parameters show a different behavior of lipid species under supercritical and subcritical conditions. The comprehensive analysis using the UHPSFC/ESI-MS method for a wide range of nonpolar and polar lipid classes is demonstrated on analyses of lipid class standards and the complex lipidomic sample. Obtained validation parameters show the applicability of the developed UHPSFC/ESI-MS method for the lipidomic analysis of real samples. The present work is a proof-of-concept of the use of UHPSFC/ESI-MS method for lipidomic analysis of large sample sets in clinical studies.

## ASSOCIATED CONTENT

### Supporting Information

Effects of water concentration, the modifier initial concentration, modifier gradient steepness, flow rate, and ABPR pressure on the UHPSFC analysis of lipid class standards, examples of ESI mass spectra of lipid classes, examples of calibration curves, effect of column regeneration on the UHPSFC analysis, effect of ammonium acetate concentration in the modifier on the peak areas of lipid class standards using UHPSFC/ESI-MS, and lipid species identified in porcine brain extract. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b01054.

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### Notes

The authors declare no competing financial interest.

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