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Automated Identification and Quantification of Glycerophospholipid Molecular Species by Multiple Precursor Ion Scanning

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We report a method for the identification and quantification of glycerophospholipid molecular species that is based on the simultaneous automated acquisition and processing of 41 precursor ion spectra, specific for acyl anions of common fatty acids moieties and several lipid class-specific fragment ions. Absolute quantification of identified species was linear within a concentration range of 10 nM−100 μM and was achieved by spiking into total lipid extracts a set of synthetic lipid standards with diheptadecanoyl (17:0/17:0) fatty acid moieties, representing six common classes of glycerophospholipids. The automated analysis of total lipid extracts was powered by a robotic nanoflow ion source and produced currently the most detailed description of the glycerophospholipidome.

The molecular composition of lipid species is a key determinant of the physical state of cellular membranes.[1] Glycerophospholipids are the major components of biological membranes,[2] consisting of a glycerol phosphate backbone with a headgroup attached at the *sn*-3 position and two fatty acid (FA) or fatty alcohol moieties attached to the remaining two positions via, respectively, ester or ether bonds. Cells produce an assortment of structurally and functionally distinct lipid species by combining different headgroups with FA or fatty alcohol moieties with a varying number of carbon atoms and double bonds. To understand how the full lipid complement (also termed the lipidome[3,4]) impinges upon diverse cellular processes, it is important to characterize and quantify lipids as individual molecular species. This means that, for each glycerophospholipid species, the headgroup and both hydrocarbon moieties impinges upon should be determined.

Collision-induced dissociation of molecular anions of glycerophospholipids produces abundant acyl anions of their fatty acid moieties.[5−12] By selecting their m/z for multiple precursor ion scattering (MPIS) on a hybrid quadrupole time-of-flight mass spectrometer,[13−15] the FA composition of a large number of molecular species could be simultaneously determined in total lipid extracts.[16,17] Thus, MPIS advanced the characterization of lipidomes compared to the conventional analysis by precursor or neutral loss scanning that only annotates lipid species by their lipid class and sum formula (the total number of carbon atoms and double bonds) of their FA moieties.[7,18−21] The specificity arising from the accurate selection of m/z of fragment ions by the high mass resolution time-of-flight analyzer extended the dynamic range of precursor ion scans[17,22] and enabled the identification of low abundant molecular species from various classes of glycerophospholipids comprising unique FA moieties.[23]

However, MPIS spectra acquired from total lipid extracts are exceedingly complex and thereforeunder high-throughput settings.[16,17] The identification of molecular species of glycerophospholipids typically required manual reviewing, matching, and

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annotation of more than 40 simultaneously acquired precursor ion spectra, which, considering the large number of detected precursors and a more than 10-fold difference in their abundance, was extremely laborious. Furthermore, only relative quantification of individual species was possible since no methods of absolute quantification (including the selection of internal standards and isotope intensity correction algorithms[3]) were available. This severely limited the scope and impact of MPIS-driven lipidomics and prompted the development of algorithms and their software implementation for rapid, quantitative, and automated interpretation of large amounts of MPIS data.

Here we describe a methodology for the identification and quantification of molecular species of glycerophospholipids by automated interpretation of MPIS spectra that has been implemented in a dedicated software termed Lipid Profiler. Endogenous species of common lipid classes could be simultaneously quantified using a set of synthetic lipid class-specific diheptadecanoyl (17:0/17:0) internal standards using a novel algorithm for the isotopic correction of peak intensities adjusted to specific features of MPIS spectra. Combined with an automated nanoelectrospray chip-based ion source,[24] the entire approach lends itself to automated high-throughput quantitative analysis of complex lipidomes.

MATERIALS AND METHODS

Chemicals and Lipid Standards. All common chemicals were purchased from Sigma Chemicals (St. Louis, MO) and were analytical grade. Solvents, water, methanol (both LiChrosolv grade), and chloroform (LC grade), were from Merck (Darmstadt, Germany). Synthetic lipid standards (except PI 17:0/17:0) and lipid extracts were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

Synthesis of PI 17:0/17:0. 1,2-Diheptadecanoyl-*sn*-glycero-3-phosphoinositol was synthesized according to the procedure of Filthuth and Eibl.[25] The phosphoramidite of 2,3,4,5,6-penta-*O*-acetyl-DL-myo-inositol was coupled to 1,2-diheptadecanoyl-*sn*-glycerol (obtained by acylation of *sn*-3-*O*-benzylglycerol with heptadecanoyl chloride followed by hydrogenolysis), followed by oxidation and deprotection.[25] The final product was purified using a silica column and eluted with a gradient of $CHCl_3$/MeOH/aqueous ammonia 90:9:1−60:36:4 (v/v/v), yielding the ammonium salt of PI 17:0/17:0. The purity and identity of the compound was assessed by thin-layer chromatography, quantitative determination of phosphorus content,[26] and tandem mass spectrometry.

Sample Preparation for Mass Spectrometric Analysis. The concentration of lipid species in stock solutions was determined by the phosphate assay.[26] Standards and lipid extracts were prepared in the specified concentrations in $CHCl_3$/MeOH 1:2 (v/v) containing 5 mM ammonium acetate. Lipid extraction of MDCK II cells was performed as previously described.[16]

Quadrupole Time-of-Flight Mass Spectrometry. Lipids were analyzed in negative and positive ion modes on a modified QSTAR Pulsar *i* quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada) equipped with a robotic nanoflow ion source NanoMate HD System (Advion Biosciences, Inc., Ithaca, NJ). Ionization voltage was set to 1.05 kV, gas pressure to 0.1 psi, and the ion source was controlled by Chipsoft 6.3.2 software (Advion Biosciences, Inc.). Samples were loaded into microtiter plates (Eppendorf AG, Hamburg, Germany) and 10-μL aliquots were aspirated and infused into the mass spectrometer at a flow rate of ~250 nL/min. The instrument was calibrated in MS/MS mode using a synthetic lipid standard 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine as previously described.[27] MPIS was performed as previously described.[16,17] The analytical quadrupole Q1 was operated under unit mass resolution settings with 30-ms dwell time and step size of 0.2 Da. The collision energy was linearly ramped from 45 eV at m/z 620 to 60 eV at m/z 920. The m/z of fragment ions selected for MPIS are listed in Supporting Information Table 1S. The m/z of fragment ions were selected within a range of 0.2 Da. Peak enhancement[15] (trapping of fragment ions in the collision cell) was applied according to the instructions of the manufacturer and controlled by Analyst QS 1.1 software (Applied Biosystems/MDS Sciex).

Lipid Profiler Prototype Software. The software (MDS Sciex) operates together with Analyst software to identify and quantify lipid species detected by MPIS. Optionally, it could also process spectra acquired on triple quadrupole and linear ion trap mass spectrometers (data not shown). It was written in Visual Basic and uses a stand-alone lipid database (Microsoft Access), which stores information on the m/z and fragment specificity of lipid classes and the applied precursor ion scans (see Supporting Information Table 1S). Lipid species were identified by matching, within a user-defined tolerance, the m/z of precursor ions detected in MPIS spectra to the candidate m/z calculated using the database. Lipid Profiler software employed two isotopic correction algorithms to calculate the intensity of lipid precursors within overlapping isotopic clusters,[3,20] which are explained in detail in the Appendix. Inquires about Lipid Profiler software should be made directly to MDS Sciex.

Absolute quantification of identified species relied upon a spiked mixture of six synthetic internal standards having two diheptadecanoyl FA moieties: PA 17:0/17:0, PE 17:0/17:0, PG 17:0/17:0, PS 17:0/17:0, PC 17:0/17:0, and PI 17:0/17:0. The concentration of an endogenous lipid species of the PX class with the FA moieties FA_i and FA_j was calculated as

$$[\mathrm{PX\ FA}_i{\sim}\mathrm{FA}_j] = \frac{I(\mathrm{PX\ FA}_i{\sim}\mathrm{FA}_j)}{I(\mathrm{PX\ 17{:}0/17{:}0})} \times [\mathrm{PX\ 17{:}0/17{:}0}]\,\frac{\rho_{\mathrm{PX\ 17{:}0/17{:}0}}}{\rho_{\mathrm{PX\ FA}i{\sim}\mathrm{FA}j}} \quad (1)$$

where [PX 17:0/17:0] stands for the concentration of the internal standard of the same PX class; $I(\mathrm{PX\ FA}_i{\sim}\mathrm{FA}_j)$ is the sum of intensities (or areas) of the monoisotopic peaks of the corresponding precursor detected in the precursor ion scans specific for the FA moieties FA_i and FA_j; I(PX 17:0/17:0) is the intensity (area) of the monoisotopic peak of the precursor ion of the internal standard detected in the precursor ion scan specific for FA 17:0.

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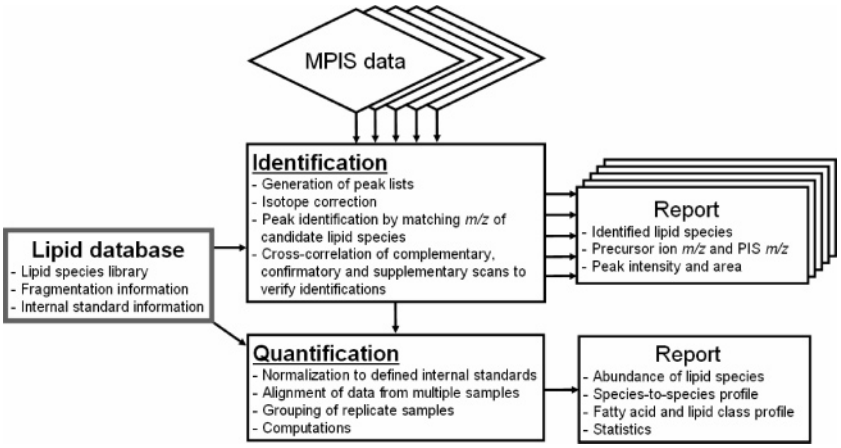

Figure 1. Workflow of automated processing of MPIS data by Lipid Profiler.

The intensities (areas) of monisotopic peaks were adjusted to represent the total intensity of the isotopic cluster. To this end, they were multiplied by a factor equal to $1/\rho$, where ρ is the intensity of the monoisotopic peak relative to the total intensity of all peaks in the isotopic cluster. It was calculated from the theoretical isotopic distribution of the corresponding lipid species and was previously termed as type I isotope correction factor.[3]

The mole percent of the quantified lipid species relative to all identified lipid species in the analyzed sample was calculated as

$$\eta(\mathrm{PX\ FA}_i{\sim}\mathrm{FA}_j) = \frac{[\mathrm{PX\ FA}_i{\sim}\mathrm{FA}_j]}{\sum_{\mathrm{X},i,j}[\mathrm{PX\ FA}_i{\sim}\mathrm{FA}_j]} \qquad (2)$$

A workflow diagram of MPIS data processing by Lipid Profiler software is presented in Figure 1.

RESULTS AND DISCUSSION

Identification of Lipid Species by MPIS. Figure 2 shows how MPIS for acyl anions of three FAs and a headgroup-specific fragment ion identified a glycerophospholipid molecular species. An equimolar mixture of synthetic PE 18:0/18:2, PE 18:1/18:1, PE 18:0/18:1, and PE 18:0/18:0 was directly infused into a quadrupole time-of-flight mass spectrometer. MPIS analysis was performed in negative ion mode by simultaneously acquiring precursor ion spectra for the PE headgroup fragment ion (PIS m/z 196.0, Figure 2A), and acyl anions corresponding to FA 18:2 (PIS m/z 279.2, Figure 2B), FA 18:1 (PIS m/z 281.3, Figure 2C), and FA 18:0 (PIS m/z 283.3, Figure 2D), respectively. The PE headgroup scan detected three precursor ions at m/z 742.6, 744.6, and 746.6, corresponding to PE species with the sum formulas PE 36:2, PE 36:1, and PE 36:0, respectively (Figure 2A). Matching peak profiles in the PE headgroup scan, and the three FA scans, revealed that PE 36:2 was composed of two individual molecular species: (a) an asymmetric PE 18:0~18:2 species, as determined by the simultaneous detection of the precursor ion with m/z 742.6 by FA 18:2 scan (PIS m/z 279.2, Figure 2B) and by FA 18:0 scan (PIS m/z 283.3, Figure 2D), and (b) the symmetric PE 18:1/18:1 species detected by FA 18:1 scan (PIS m/z 281.3, Figure 2C). Similarly, MPIS identified PE 36:1 (m/z 744.6) and PE 36:0 (m/z 746.6) species as PE 18:0~18:1 and PE 18:0/18:0, respectively (Figure 2C,D).

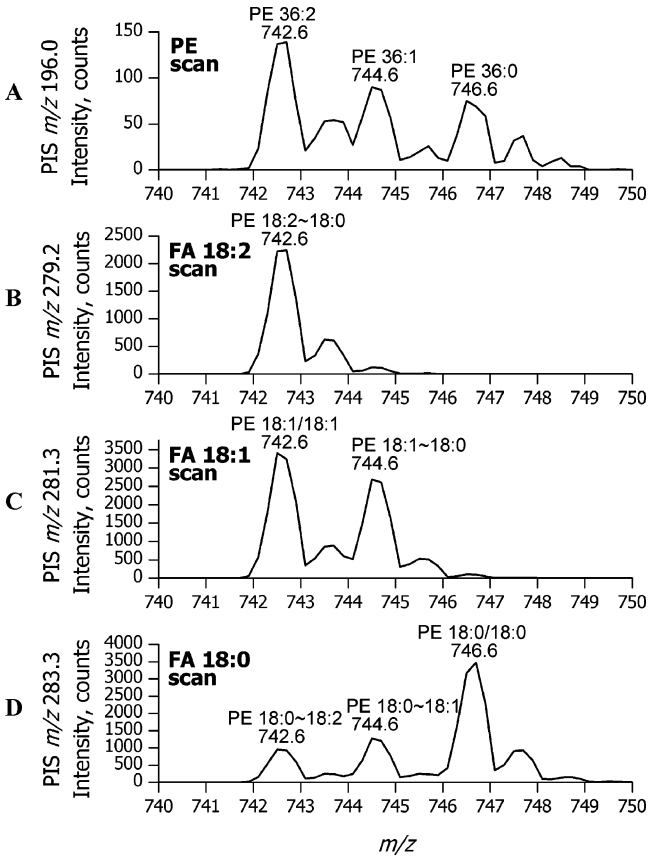

Figure 2. Identification of individual molecular species of PEs by MPIS. The equimolar mixture of PE 18:0/18:2 (m/z 742.6), PE 18:1/18:1 (m/z 742.6), PE 18:0/18:1 (m/z 744.6), and PE 18:0/18:0 (m/z 746.6) was analyzed by MPIS. (A) PIS m/z 196.0 spectrum (PE headgroup scan). (B) PIS m/z 279.2 spectrum (FA 18:2 scan). (C) PIS m/z 281.3 spectrum (FA 18:1 scan). (D) PIS m/z 283.3 spectrum (FA 18:0 scan). Detected lipid precursors are designated by their sum formulas in (A) and by their molecular composition in other panels.

Asymmetric glycerophospholipid species often occur as positional isomers, i.e., species with the inverted position of FA moieties on the glycerol phosphate backbone (e.g., PE 18:0/18:2 vs PE 18:2/18:0). Previous studies demonstrated that, in the MS/MS and MPIS spectra of asymmetric PE and PC species, the acyl anion of the *sn*-2 FA moiety was 2−3-fold more abundant than

that of the *sn*-1 FA moiety.[10,16,28,29] The ratio of precursor ion intensities at *m/z* 742.6 (annotated as PE 18:0~18:2) differed by 2-fold in the PIS *m/z* 279.2 spectrum (FA 18:2 scan) and in the PIS *m/z* 283.3 spectrum (FA 18:0 scan), respectively (Figure 2B,D). This indicated that an asymmetric PE species with *m/z* 742.6 comprised FA 18:0 and FA 18:2 moieties at the *sn*-1 and *sn*-2 position, respectively (i.e., PE 18:0/18:2). Similarly, the relative abundance of the precursor peaks in the PIS *m/z* 281.3 (FA 18:1 scan) and PIS *m/z* 283.3 spectra (FA 18:0 scan) identified the PE species at *m/z* 744.6 as PE 18:0/18:1. We note, however, that in MS/MS and MPIS spectra of anionic glycerophospholipids (i.e., PA, PS, and PG), the relative intensity of acyl anions is reversed—the acyl anion of the *sn*-1 FA moiety is more abundant than the acyl anion of the *sn*-2 FA moiety.[11,12] If required, the relative amount of positional isomers could be accurately determined by MS^3 analysis on an ion trap mass spectrometer.[16]

Identification of Lipid Species by Lipid Profiler Software. Lipid Profiler deciphered MPIS spectra and identified molecular species of glycerophospholipids, essentially as outlined above. The identification of asymmetric glycerophospholipid species required that the same precursor ion was detected by two complementary FA scans (e.g., the detection of precursor ions with *m/z* 742.6 by scans for FA 18:0 and FA 18:2, Figure 2B,D) and its *m/z* matches the expected sum composition (e.g., PE 36:2). The identification of symmetric glycerophospholipid species relied on the detection of a precursor ion by a single FA scan; however, both matching criteria equally applied. Hence, the precursor ion with *m/z* 742.6 should match the sum formula of PE 36:2, since it was detected by PIS *m/z* 281.3 for the acyl anion of FA 18:1 (Figure 2C).

In some glycerophospholipid species, the hydrocarbon moiety is attached to the *sn*-1 position of the glycerol phosphate backbone through an ether bond (plasmanyl species) or vinyl ether bond (plasmenyl species or plasmalogens).[30] Collision-induced dissociation of molecular anions of ether species produces abundant acyl anions of *sn-2* FA moieties, along with relatively low abundant headgroup fragments. Plasmenyl species also yield low abundant alkenoxide fragment ions produced from their *O*-alk-1′-enyl moieties (data not shown). Acyl anions are typically 20−100-fold more abundant compared to alkenoxide fragments. Alkenoxide fragments are isobaric with acyl anions of FA that differ by a single methylene group are isobaric ($\Delta m = 0.0364$ Da). Therefore, the assignment of precursor ions as ether or diacyl species relied upon the abundance difference between *sn*-2 and *sn*-1 related fragments: to recognize a precursor ion as an ether species, more than a 20-fold difference in abundance was typically required. Otherwise, this precursor was considered as a diacyl species. If the corresponding alkenoxide fragment was not detectable, the species was reported as an ether lipid without further assignment to a plasmanyl or plasmenyl class. Ambiguous assignments could be verified by manual inspection of precursor ion intensities, direct MS/MS analysis of relevant precursors, or both.[31,32]

The lipid species identification was further supported by the concomitant detection of the same precursor ions in confirmatory or supplementary precursor ion scans. Confirmatory scans use *m/z* of lipid class-specific fragment ions, such as PIS *m/z* 196.0—the PE headgroup scan. For example, the identification of PE 18:0~18:2 detected at *m/z* 742.6 by scans specific for FA 18:0 and FA 18:2 moieties (Figure 2B,D) was validated by detecting the same precursor by the PE headgroup-specific scan (Figure 2A). Supplementary scans utilize fragment ions that are common for lipids of all classes comprising specific FA moieties. For example, upon collision-induced dissociation, acyl anions of polyunsaturated FAs lose CO_2.[33,34] Corresponding *m/z* of neutral loss products could be included in the MPIS experiment and supported the identification of lipid species containing a polyunsaturated FA moiety, independently of the lipid class (see below).

A typical MPIS experiment utilized 41 simultaneously acquired precursor ion scans and recognized ~200 lipid precursors in a total lipid extract. The spectra were interpreted by Lipid Profiler software within 30 s on a conventional (Pentium 4) desktop computer. Within this time period, the software accessed the MPIS data file, produced a peak list with a user-defined threshold intensity, performed isotopic correction (see below), annotated precursor ions, and created the identification report (Figure 1, Supporting Information Figure 3S). Details on the quantification routines are presented below.

MPIS Enhances the Identification Specificity of Lipid Molecular Species. Next we evaluated the performance of MPIS in identifying molecular species of glycerophospholipids and compared it to conventional lipid class-specific precursor and neutral loss scans that are commonly applied in lipidomics. To this end, a commercially available PC extract from bovine heart was analyzed by PIS *m/z* 184.1 in positive ion mode (Figure 3A). This scan specifically detects PC and sphingomyelin species, yet it only annotates them with their sum formulas.[18,20] The same extract was analyzed by MPIS in negative ion mode by selecting *m/z* of 41 acyl anions of common FAs, as well as several lipid class-specific fragments (see complete list of fragment *m/z* in Supporting Information Table 1S). PIS *m/z* 184.1 and MPIS spectra were processed by Lipid Profiler software, which identified and annotated plausible PC precursors (Figure 3). Among other peaks, PIS *m/z* 184.1 scan detected abundant diacyl PC 34:2 at *m/z* 758.6 and putative ether species PC *O*-34:3 at *m/z* 742.7 (Figure 3A). The MPIS profile was in a full agreement with the PIS *m/z* 184.1 spectrum, but also provided a wealth of important details on the chemical structure of identified lipids. The diacyl PC 34:2 was detected as an acetate adduct at *m/z* 816.7 by precursor ion scans specific for FA 16:1, FA 16:0, FA 18:2, and FA 18:1 moieties (Figure 3B). The relative abundance of the precursor peaks in the corresponding FA scans determined the predominant location (*sn*-1 and *sn*-2) of FA moieties in both molecular species. Its major and minor isobaric components were identified as PC 16:0/18:2 (peak intensities ratio, 3) and PC 16:1/18:1 (peak intensities ratio, 5). The ether PC *O*-34:3 was detected by scans for FA 18:2 and FA 15:1/*O*-16:1 moieties at *m/z* 800.7 (Figure 3B and Supporting Information Figure 1S, PIS *m/z* 239.2, respectively). However, because of the peak intensity ratio of 100,

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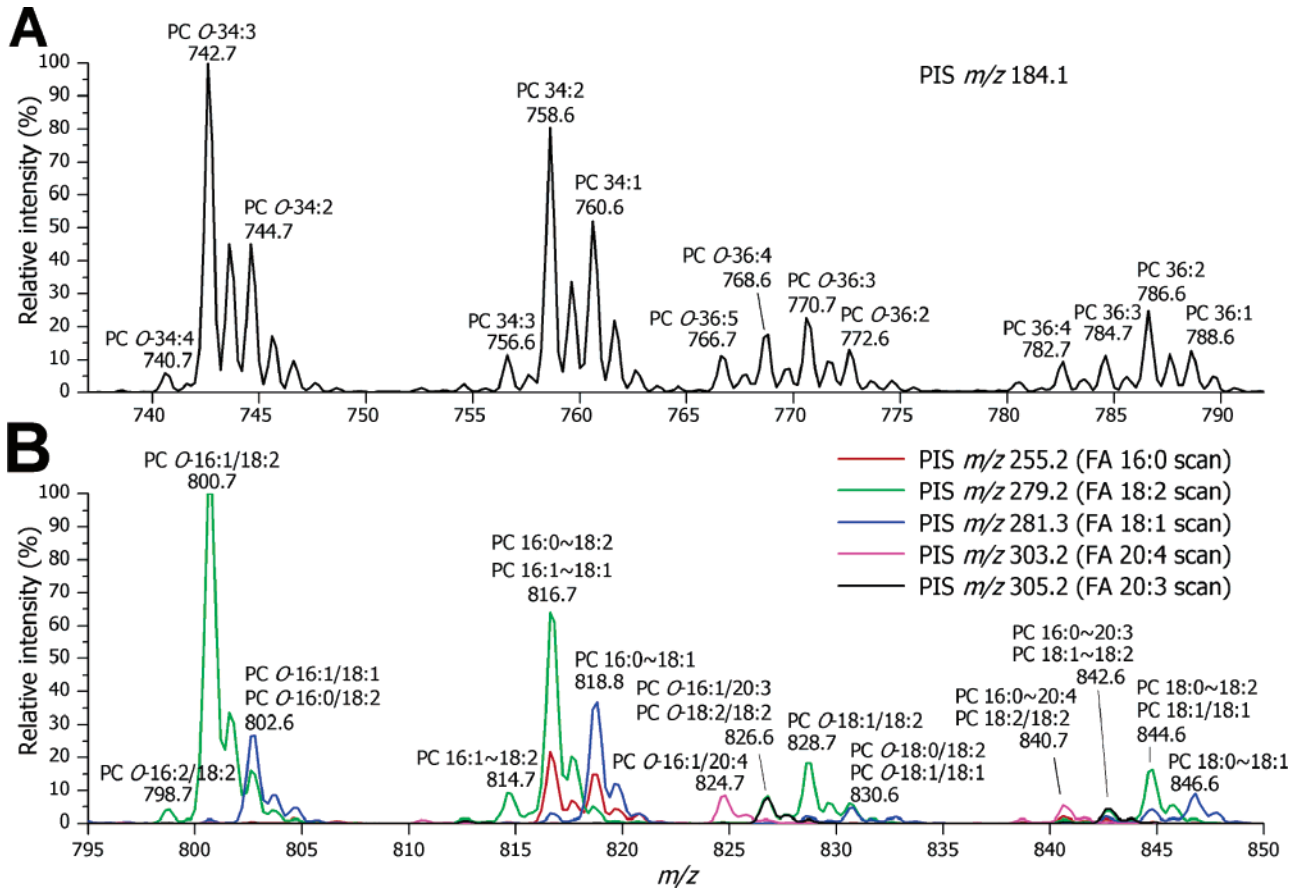

Figure 3. Spectral profiles obtained by lipid class-specific PIS and lipid species-specific MPIS. (A) PIS *m/z* 184.1 spectrum of bovine heart PC extract acquired in positive ion mode. Detected precursors are annotated as diacyl or ether species using a sum formula. Note that PIS *m/z* 184.1 is not capable of distinguishing isobaric diacyl species and ether species. Identified PC species are annotated by assuming that the major constituent of the detected precursor contains even numbered acyl, alkyl, or alkenyl chains. (B) FA profile of bovine heart PC extract obtained by MPIS analysis. In negative ion mode, PC precursors were detected as acetate adducts. For clarity, only 5 precursor ion spectra (out of 41 acquired) are presented (all acquired spectra are presented in Supporting Information Figure 1S). Identified precursor ions are annotated using a molecular formula that describes the FA moieties of the detected lipid species.

it was annotated as the plasmenyl species PC *O*-16:1/18:2, where the *O*-alk-1′-enyl moiety was 16:1 and the *sn*-2 moiety was FA 18:2. The fully automated interpretation recognized 30 isobaric PCs in the PIS *m/z* 184.1 spectrum, whereas 48 individual molecular species were revealed by MPIS (Table 1). The profile (including the assignment of ether species) was in full agreement with the independent analysis by the method of data-dependent acquisition.[31]

To further validate the automated interpretation of MPIS profiles, we analyzed, in the same way (however, in negative ion mode), commercially available PA, PE, PG, PS, and PI. The lipid class-specific scans (PIS *m/z* 153.0 for PAs, PGs, and PSs; PIS *m/z* 196.0 for PEs; and PIS *m/z* 241.0 for PIs) were acquired simultaneously with FA scans in the same MPIS experiments (see Supporting Information Table 1S) and interpreted by Lipid Profiler. We compared the number of species detected by the respective lipid class-specific scans (as annotated by sum formulas) and the number of species detected in FA scans (as annotated by molecular compositions). Altogether, the MPIS method increased the number of detected species in all classes, on average, by a factor of 1.8, compared to the conventional lipid class-specific precursor ion scans (Table 1).

The identification specificity of species with long and unsaturated FA moieties was notably improved. Hence, MPIS revealed that PE *O*-38:6 detected at *m/z* 748.7 by PE-specific headgroup scan PIS *m/z* 196.0 comprised at least three individual species: PE *O*-18:2/20:4, PE *O*-18:1/20:5, and PE *O*-16:1/22:5. At the same time, PE 34:1 detected at *m/z* 716.6 was single-species PE 16:0/18:1 (data not shown). Similar results were obtained by the analysis of extracts of other lipid classes and corroborated by independent data-dependent lipid profiling.[31]

Table 1. Number of Lipid Species Identified by Lipid Class-Specific Scans and by MPIS Analysis[a]

lipid source	lipid class-specific scan (PIS *m/z*)	no. of species identified by lipid class-specific scan	no. of species identified by FA-specific scans	factor diff[b]
chicken egg PA extract	153.0[c]	15	24	1.6
bovine heart PE extract	196.0[c]	27	60	2.2
porcine brain PS extract	153.0[c]	15	24	1.6
chicken egg PG extract	153.0[c]	14	26	1.9
bovine heart PC extract	184.1[d]	30	48	1.6
bovine liver PI extract	241.0[c]	20	37	1.9

[a] Each lipid extract was analyzed in triplicate. Detected precursor ions were identified by Lipid Profiler software. Only species identified in each of the three independent analyses were counted. [b] The number of species identified by MPIS divided by the number of species identified by a corresponding class-specific scan. [c] Performed in negative ion mode. Detected species were annotated by sum formula. [d] Performed in positive ion mode. Detected species were annotated by sum formula.

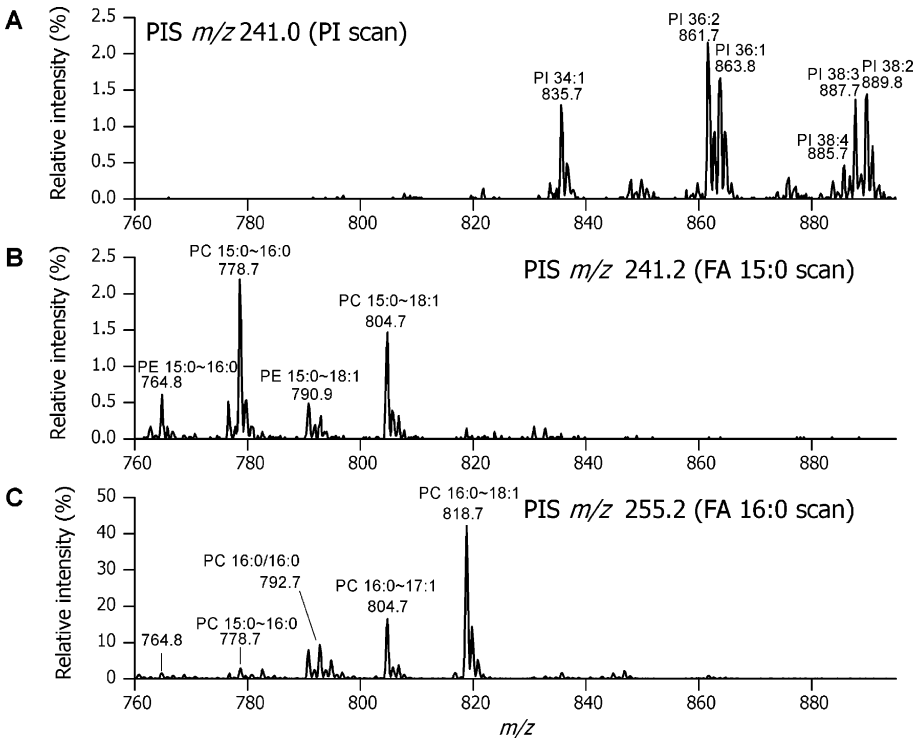

Figure 4. Specific precursor ion scans distinguishing PI species from glycerophospholipids comprising FA 15:0 moieties, although their characteristic fragments, they are isobaric. A lipid extract of MDCK II cells was analyzed by MPIS in negative ion mode. (A) PI species were detected by PIS *m*/*z* 241.0 and annotated by sum formula. (B) FA 15:0 containing lipids of all classes were detected by PIS *m*/*z* 241.2 and annotated by molecular formulas. (C) FA 16:0 containing lipids of all classes were detected by PIS *m*/*z* 255.2 Note that PC 15:0−16:0 (*m*/*z* 778.7) was detected both in FA 15:0 and FA 16:0 scans. Peak intensities are normalized to the most abundant precursor ion at *m*/*z* 742.7 (PE 18:1/18:1) detected by PIS *m*/*z* 281.3 (FA 18:1 scan).

High Mass Resolution of the Time-of-Flight Analyzer Improves the Specificity of MPIS. The identification of lipid species by Lipid Profiler relies on the specificity of precursor ion scans. Here we demonstrate that, because of the high mass resolution of the time-of-flight mass analyzer, MPIS distinguishes lipid species whose specific fragments, potentially suitable for precursor ion scan profiling, are isobaric.

Collision-induced dissociation of PIs yields the class-specific headgroup fragment ion with *m*/*z* 241.01 ($C_6H_{10}O_8P$).[9] However, it is isobaric with the acyl anion of FA 15:0 having *m*/*z* 241.22 ($C_{15}H_{29}O_2$), which, despite having the odd number of carbon atoms, is common in mammalian glycerophospholipids.[16,35]

A total lipid extract of MDCK II cells was analyzed by MPIS as described above. As anticipated, PIS *m*/*z* 241.0 revealed a profile of PI species (Figure 4A), which, however, did not overlap with the PIS *m*/*z* 241.2 profile (Figure 4B) that is specific for glycerophospholipids with FA 15:0 moieties. The detected species were identified by Lipid Profiler by considering other FA scans that were acquired in parallel. One of these scans, specific for glycerophospholipids containing FA 16:0 moieties, is presented in Figure 4C as a reference. The ratio of intensities of the precursor peaks detected by FA 16:0 and FA 15:0 scans was equal to 1, thus indicating that the precursor at *m*/*z* 778.7 was an equimolar mixture of PC 15:0/16:0 and PC 16:0/15:0. In other lipid species, the FA 15:0 moiety was mostly located at the *sn*-1 position: PC 15:0/18:1 (*m*/*z* 804.7, peak intensities ratio, 5), PE 15:0/16:0 (*m*/*z* 764.8; peak intensities ratio, 3), and PE 15:0/18:1 (*m*/*z* 790.9, peak intensities ratio, 6). Interestingly, the FA 16:0 scan identified another lipid with the odd-numbered FA moiety PC 16:0/17:1 (Figure 4C), which was confirmed by the FA 17:1 scan (data not shown).

MPIS Identification of Lipid Species Having a Polyunsaturated FA Moiety. Acyl anions of polyunsaturated FAs, produced by the collision-induced dissociation of molecular anions of diacyl and ether glycerophospholipids, yield additional satellite fragments by neutral loss of CO_2.[33,34] Their *m*/*z* were included in the list of fragments for MPIS (Supporting Information Table 1S) as a supplementary mean to validate the identification of corresponding molecular species. For example, MPIS profiling of a bovine heart PE extract identified four low abundant PE species containing the FA 20:5 moiety, which were simultaneously detected in scans specific for FA 20:5 and FA 20:5-CO_2 (Figure 5).

Neutral loss of CO_2 from polyunsaturated acyl anions has two important implications for lipid profiling. First, loss of CO_2 from the acyl anion of docosahexaenoic acid FA 22:6 yields a fragment ion with *m*/*z* 283.2431 ([FA 22:6−CO_2]$^-$) that is isobaric with the acyl anion *m*/*z* 283.2642 of abundant stearic acid FA 18:0, and therefore, additional caution should be taken when using *m*/*z* of this fragment in supplementary PIS. However, most importantly, loss of CO_2 directly affects the quantification accuracy of FA 22:6-containing species, which, as we demonstrate below, can be improved by using a correction factor together with MPIS profiles.

(35) Connor, W. E.; Lin, D. S.; Thomas, G.; Ey, F.; DeLoughery, T.; Zhu, N. *J. Lipid Res.* **1997**, *38*, 2516−2528.

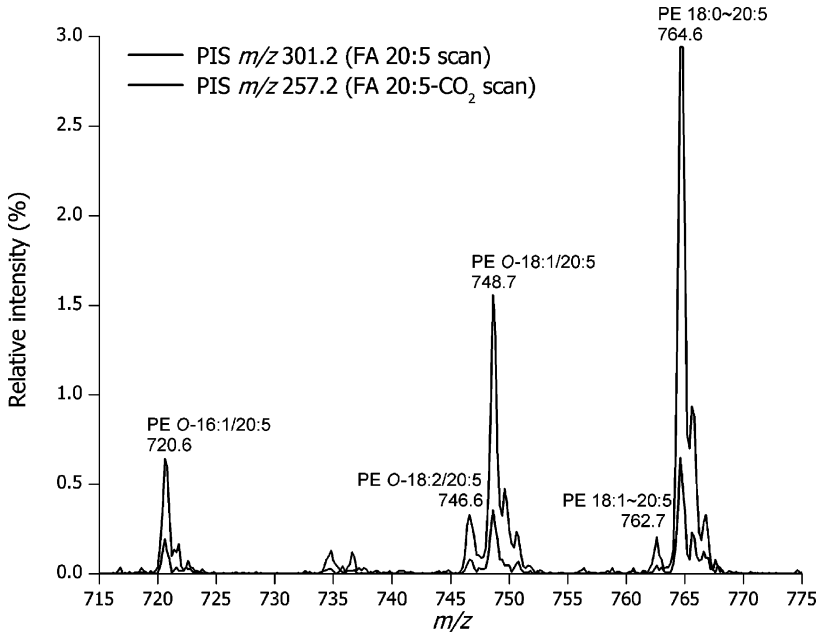

Figure 5. Validating the identification of lipid species containing a FA 20:5 moiety by supplementary scan for FA 20:5-CO_2 fragment. Bovine heart PE extract was subjected to MPIS analysis. Scans acquired for FA 20:5 (PIS *m/z* 301.2) and FA 20:5−CO_2 (PIS *m/z* 257.2) allowed the specific identification of FA 20:5-containing PE species. Peak intensities were normalized to the most abundant peak with *m/z* 766.6 detected by FA 20:4 scan (PIS *m/z* 303.2) that corresponded to PE 18:0~20:4.

We define the correction factor α_{PX} as a ratio of the peak intensities of the precursor from the lipid class PX (i.e., PA, PE, PG, PS, PC, PI) detected by PIS *m/z* 283.3 (FA 22:6-CO_2 scan) and PIS *m/z* 327.2 (FA 22:6 scan). They were determined in a separate experiment using available synthetic standard(s), such as PC 16:0/22:6, PE *O*-16:1/22:6, PA 16:0/22:6, and PG 16:0/22:6, under the fixed instrument settings (most importantly, the collision energy offset). The lipid class-specific correction factors were then used to adjust the intensities of the corresponding endogenous lipid precursors detected by PIS *m/z* 283.3 (FA 18:0 and FA 22:6-CO_2 scan):

$$I_{\text{FA 18:0}} = I_{\text{PIS } m/z\ 283} - \alpha_{PX} I_{\text{PIS } m/z\ 327.2}$$

The same correction factors also adjusted the intensity of the precursor peak at PIS *m/z* 327.2 (FA 22:6 scan):

$$I_{\text{FA 22:6}} = (1 + \alpha_{PX}) I_{\text{PIS } m/z\ 327.2}$$

To test the correction accuracy, we analyzed an equimolar mixture of synthetic isobaric plasmenyl PE *O*-16:1/22:6 (*m/z* 746.5130) and diacyl PE 18:0/18:0 (*m/z* 746.5705) species by MPIS. The correction factor α_{PE} was estimated as 0.56 (±0.05) by separately analyzing PE *O*-16:1/22:6 under the same instrument settings (Table 2). Applying the correction factor to the reference peak intensity of PE *O*-16:1/22:6 at PIS *m/z* 327.2 (FA 22:6 scan) allowed us to distinguish the contributions of the two species to the intensity at PIS *m/z* 283.3 (FA 18:0 and FA 22:6-CO_2 scans) and, thereby, correctly determine their molar ratio (Table 2). The precursor of PE 18:0/18:0 was not detectable by PIS *m/z* 327.2 (FA 22:6 scan).

Isotope Correction of Peak Intensities for MPIS Quantification. Correction of peak intensities within overlapping isotopic clusters improves the confidence of identification of low abundant lipid species and the quantification accuracy.[3,20] Here we demonstrate that a dedicated isotope peak intensity correction algorithm enabled reconstruction of the bona fide isotopic distributions of lipid species in MPIS experiments.

TOF MS and MPIS spectra of synthetic PE 18:1/18:1 are presented here as an example. The isotope profile observed in TOF MS spectrum was in a good agreement with the profile computed from its elemental composition $C_{41}H_{77}NO_8P$ (Figure 6). However, in PIS spectra, the isotope profiles were perturbed because only a subset of the isotopic population of the intact precursor was detected. For example, the PIS *m/z* 281.3 (FA 18:1 scan) spectrum matched the isotope distribution calculated for the neutral fragment of the PE 18:1/18:1 that lost the acyl anion of FA 18:1 ($C_{23}H_{44}NO_6P$) (Table 4). The isotope profiles of the precursor in the PIS *m/z* 282.3 spectrum and PIS *m/z* 283.3 spectrum (FA 18:0 scan) also differed from the profile of the intact species, but agreed with the calculated isotopic abundances (Table 4). Details on the isotope correction algorithms employed in Lipid Profiler are provided in the Appendix. Importantly, summing up the isotopic peak intensities in the PIS *m/z* 281.3, PIS *m/z* 282.3, and PIS *m/z* 283.3 spectra (Figure 6B−D) recreated the isotopic profile of the intact molecule (Figure 6A). For further quantitative analysis and reports, Lipid Profiler operated with the total intensities of isotopic clusters computed using the ρ correction factor (type I isotope correction factor[3]) as described in Materials and Methods.

Quantification of Glycerophospholipid Species by MPIS. Han et al.[3,36] demonstrated that quantification of lipid species in total lipid extracts could rely upon a single internal standard per each analyzed lipid class, if applied together with the isotope correction of intensities of their monoisotopic peaks. The internal

(36) Han, X. L.; Yang, J. Y.; Cheng, H.; Ye, H. P.; Gross, R. W. *Anal. Biochem.* **2004**, *330*, 317−331.

Table 2. Quantification of the Isobaric Species PE *O*-16:1/22:6 and PE 18:0/18:0 Using a Predetermined Correction Factor

analyte	PIS *m/z* (scan)	relative intensity[a]	α_{PE} estimate[b]
PE *O*-16:1/22:6[c]	327.2 (FA 22:6)	1	0.56 (±0.05)
	283.3 (FA 18:0/FA 22:6-CO_2)	0.56	
PE *O*-16:1/22:6 + PE 18:0/18:0 (1:1)[d]	327.2 (FA 22:6)	1	
	283.3 (FA 18:0/FA 22:6-CO_2)	2.22	

Quantification of Isobaric Species Using the Correction Factor

	scan (PIS *m/z*)	relative intensity[a]	molar ratio
PE *O*-16:1/22:6	FA 22:6 (327.2)	1	(1 + 0.56)/1.66 =
	FA 22:6-CO_2 (283.3)	0.56	0.938 (±0.002)
PE 18:0/18:0	FA 18:0 (283.3)	1.66	

[a] Normalized to the intensity of the precursor with *m/z* 746.5 detected by PIS *m/z* 327.2 (FA 22:6 scan). [b] αPE was calculated as the intensity ratio of precursor ions with *m/z* 746.5 detected by PIS *m/z* 283.3 and PIS *m/z* 327.2. [c] Synthetic plasmenyl species PE *O*-16:1/22:6 (*m/z* 746.51) was analyzed three times by MPIS to estimate αPE correction factor. [d] Equimolar mixture of synthetic isobaric PE *O*-16:1/22:6 and PE 18:0/18:0 (*m/z* 746.57) was analyzed three times by MPIS.

standards were selected such that their *m/z* was out of the range typical for endogenous species, and the analysis was performed using an "intrasource separation" method that enhanced preferential ionization of certain lipid classes.[37]

Here we demonstrate that molecular species of glycerophospholipids of various classes could be simultaneously quantified by MPIS using a one-class/one-standard approach, combined with collision energy ramping and dedicated isotope correction algorithm. Synthetic diheptadecanoyl species of major glycerophospholipid classes, PA 17:0/17:0, PE 17:0/17:0, PG 17:0/17:0, PS 17:0/17:0, PC 17:0/17:0, and PI 17:0/17:0, were employed as internal standards. All of them were detectable by PIS *m/z* 269.3 (FA 17:0 scan) (Figure 7A), and their mixture was spiked at the low-micromolar concentration into total lipid extracts. Ramping the collision energy compensated for *m/z*-dependent differences in the yield of acyl anions (Figure 7B). The PIS profile of these six diheptadecanoyl species (Figure 7A) was reproducible and served as an internal quality control for the efficiency of lipid extraction and ionization. None of them were detectable in total lipid extracts from *Escherichia coli*, *Saccharomyces cerevisiae,* and mammals, although we found PE 17:0/17:0 in *Caenorhabditis elegans* (data not shown). Importantly, these standards (except PI 17:0/17:0) are commercially available, and since they are not isotopically labeled, their precursor and fragment peaks have natural isotopic profiles.

To evaluate the quantification dynamic range, we analyzed the dilution series of synthetic lipid standards that are common in biological membranes (e.g., PE 18:1/18:1) within the concentration range of 1 nM−100 μM, whereas the concentration of each of the six diheptadecanoyl standards was fixed at 0.25 μM. Within this range, the instrument response was linear for all analyzed species with the slope value of approximately one, for all studied lipid classes (data not shown).

To test whether the quantification method was applicable for analyzing complex mixtures of endogenous lipids, we analyzed a

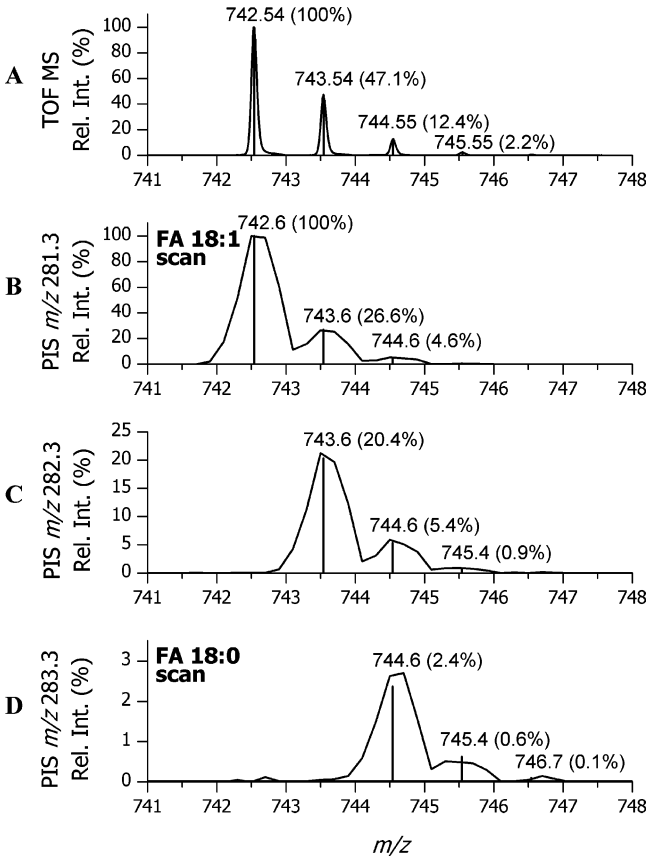

Figure 6. Comparison of isotopic profiles of the synthetic standard PE 18:1/18:1 in TOF MS and PIS spectra. Peak intensities in all precursor ion scans (panels B−D) were normalized to the intensity of the monoisotopic peak at *m/z* 742.6 in the PIS *m/z* 281.3 spectrum. The calculated isotopic distributions are presented as vertical bars, and respective values are in parentheses (see Table 4 for details). (A) TOF MS spectrum; (B) PIS *m/z* 281.3 spectrum (FA 18:1 scan); (C) PIS *m/z* 282.3 spectrum; (D) PIS *m/z* 283.3 spectrum (stands for FA 18:0 scan). Note that summing up the intensities of isotopic peaks in precursor ion spectra (panels B−D) recreates the isotopic profile of the intact PE 18:1/18:1 detected by TOF MS (panel A).

(37) Han, X.; Yang, K.; Yang, J.; Fikes, K. N.; Cheng, H.; Gross, R. W. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 264−274.

Table 3. Possible Distribution of Isotopes between Fragment Ions and Complementary Neutrals

	subpopulations detectable by PIS for the fragment F_n[b]			
precursor[a]	F_0	F_1	F_2	F_3
M_0	F_0N_0			
M_1	F_0N_1	F_1N_0		
M_2	F_0N_2	F_1N_1	F_2N_0	
M_3	F_0N_3	F_1N_2	F_2N_1	F_3N_0
...				

[a] The precursor column indicates the various isotopic forms of the precursor ion M. [b] The column presents possible isotopic combinations of charged fragment F and neutral fragment N. The sum of isotopes in the complementary fragments equals the number of isotopes in the precursor. The columns F_0, F_1, ..., indicate which subset of the isotopic population of the precursor will be detectable at the corresponding F_0, ..., F_n specific precursor ion scan.

dilution series of an *E. coli* polar lipid extract spiked with the fixed concentration of the same diheptadecanoyl standards and quantified the absolute amounts of PE 16:0/17:1 and PG 16:0/19:1—the two most abundant species among all detectable PEs and PGs. The concentrations of PE 16:0/17:1 and PG 16:0/19:1, calculated using eq 1, were plotted against the total lipid concentration in the extract (in mg/L) and total concentration of phosphate (in μM), as determined by phosphate analysis (Figure 8). Similar to the results obtained with synthetic standards, the signal intensity of both quantified species changed linearly within, approximately, 10 nM−100 μM the total sample phosphate with a limit of quantification[31] better than 1 and 30 nM for PE 16:0/17:1 and PG 16:0/19:1, respectively. The total molar concentration of all identified PE and PG species equaled 89% of the total sample phosphate content, with the remaining 11% corresponding to cardiolipins, which are poorly ionizable under the applied infusion conditions (data not shown). The PE and PG class species equaled 78 and 11% of the total sample phosphate content, respectively, which was in good agreement with previous reports.[38]

Using MPIS, together with a set of diheptadecanoyl internal standards, we profiled commercially available polar lipid extracts from porcine brain and bovine heart. Automated identification, annotation, isotopic correction, and quantification of lipid species were performed using Lipid Profiler software. The absolute concentration of each species (e.g., PE 18:0/20:4) was determined and converted to mole percent by normalizing to the sum of the concentrations of all identified glycerophospholipid species (Figure 9A). The comparative analysis of brain and heart extracts suggested multifaceted differences in their molecular lipid composition. The most abundant species in the brain tissue were PC 16:0/18:1, PS 18:0/18:1, PE *O*-18:2/18:1, and PC 18:1/18:1, compared to PC *O*-16:1/18:2, PE 18:0/20:4, PC 16:0/18:2, PC 16:0/18:1, and PE *O*-16:1/18:2 in the heart tissue (Figure 9A).

MPIS methodology provided a comprehensive and quantitative description of the glycerophospholipidome, which can be processed, displayed, and compared in several ways, such as the direct quantitative species-to-species comparison (Figure 9A), or emulated total FA profile (Figure 9B) (typically obtained by gas chromatography/mass spectrometry) or lipid class profile (Figure 9C) (typically obtained by thin-layer chromatography or normal-phase liquid chromatography). For example, the emulated FA profile showed that brain glycerophospholipids were enriched in FA 18:1, and contained similar amounts of FA 18:0 and FA 16:0. They also comprised a diverse pool of polyunsaturated FA 22:6, FA 22:5, FA 22:4, and FA 20:4 that, taken together, equaled 15 mol % of all FA moieties (Figure 9B). In comparison, heart glycerophospholipids were enriched in FA 18:2 (undetectable in brain glycerophospholipids) and FA 20:4 and contained similar amounts of FA 18:1, FA 18:0, and FA 16:0 (Figure 9B). At the same time, the lipid class-specific profiling showed that PEs were equally abundant in brain and heart, whereas the two tissues showed noticeable differences in the amounts of PCs, PSs, and PIs (Figure 9C).

Despite being a sensitive, versatile, and robust analytical tool, MPIS approach requires the optimization of the sample preparation protocol and several instrument control settings. In particular, it is important to minimize in-source fragmentation of lipid precursors, adjust the collision energy ramping and collision gas pressure for best signal response, optimize the enhancement (q_2 ion trapping) settings,[15] and control the intensities of detected peaks to avoid saturation of the TOF detector. Settings of the ion source (NanoMate HD) must allow stable and reproducible spray with the flow injection rate of 200−300 nL/min. Sample preparation should minimize the content of chloride anions in the sprayed analyte; otherwise, the enhanced abundance of chloride adducts with PCs (in addition to the desired acetate adducts) complicates the identification of PC species. If, for any reason, MPIS fails to achieve unequivocal identification of certain molecular species, it is worth reducing the sample complexity by micropreparative thin-layer chromatography or liquid chromatography.[39,40]

Table 4. Calculated Isotope Abundance of the Precursor Ion PE 18:1/18:1 and Its Fragments Detectable on Precursor Ion Scans

precursor[a]		isotope distribution detectable by precursor ion scans [b,c]		
m/z	isotope distribution	$^{(0)}$FA 18:1 m/z 281.25	$^{(1)}$FA 18:1 m/z 282.25	$^{(2)}$FA 18:1 m/z 283.26
742.54	1	1 (=1 × 1)	0	0
743.54	0.471	0.266 (=1 × 0.266)	0.204 (=0.204 × 1)	0
744.55	0.124	0.046 (=1 × 0.046)	0.054 (=0.204 × 0.266)	0.024 (=0.024 × 1)
745.55	0.022	0.006 (=1 × 0.006)	0.009 (=0.204 × 0.046)	0.006 (=0.024 × 0.266)

[a] Relative abundance of isotopic peaks of PE 18:1/18:1 ($C_{41}H_{77}O_8NP$: 1, 0.471; 0.124; 0.22) acyl anion of FA 18:1 ($C_{18}H_{33}O_2$:1; 0.204; 0.024), and complementary neutral fragment ($C_{23}H_{44}O_6NP$: 1; 0.266; 0.046; 0.006) were calculated using Analyst. [b] Isotope distribution of the combinations of fragments were calculated as described in the Appendix. [c] $^{(0)}$FA 18:1, $^{(1)}$FA 18:1, and $^{(2)}$FA 18:1 stand for precursor ion scans for the fragments whose m/z correspond to monoisotopic, first and second isotopic peaks of acyl anion of FA 18:1, respectively.

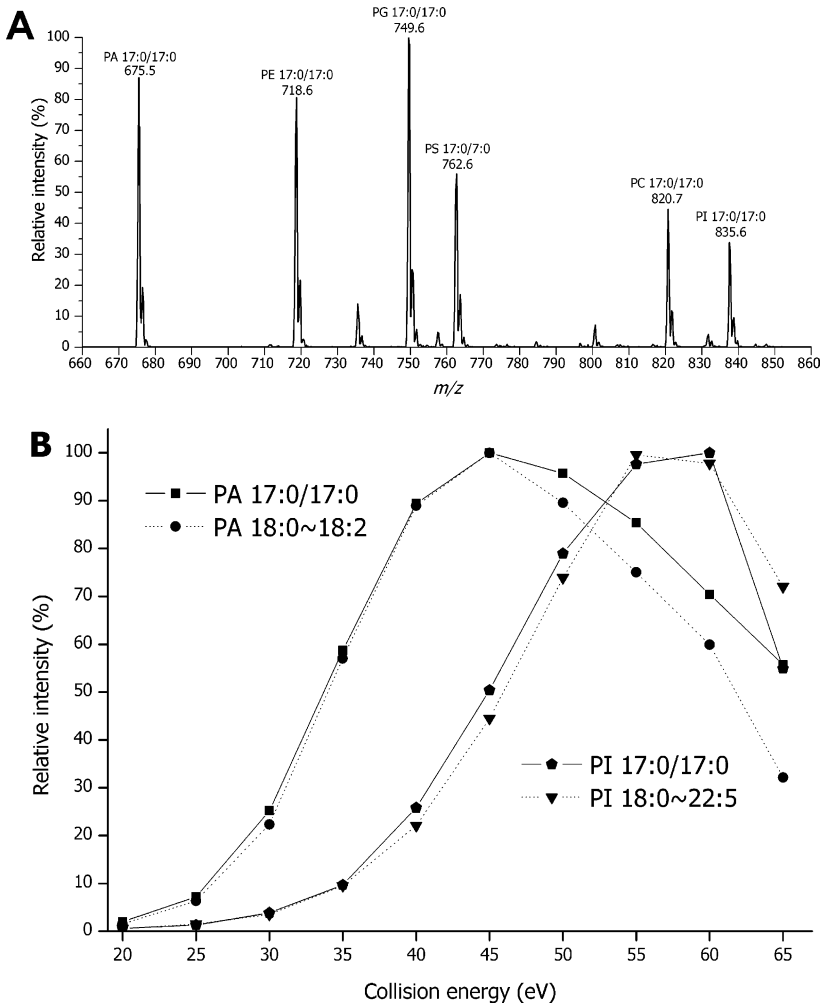

Figure 7. (A) PIS *m*/*z* 269.3 (FA 17:0 scan) spectrum of an equimolar mixture of PA 17:0/17:0, PE 17:0/17:0, PG 17:0/17:0, PS 17:0/17:0, PC 17:0/17:0, and PI 17:0/17:0. Each lipid species was spiked to a final concentration of 250 nM. Collision energy was ramped from 45 eV at *m*/*z* 620 to 60 eV at *m*/*z* 920 for optimal signal response. (B) Relative intensity of peaks of acyl anions produced at different collision energy offsets. Precursor ions of PA 17:0/17:0 (*m*/*z* 675.5, squares), PA 18:0∼18:2 (*m*/*z* 699.5, circles), PI 17:0/17:0 (*m*/*z* 837.6, pentagons), and PI 18:0∼20:5 (*m*/*z* 911.6, triangles). Total intensity of acyl anions at the indicated collision energy was normalized to the total intensity at the optimal collision energy. The average intensity determined in two independent experiments, performed under the same instrument settings, are presented.

CONCLUSION AND PERSPECTIVES

We developed an analytical routine for the automated deciphering of MPIS spectra, which includes the identification, annotation, isotopic correction of peak intensities, and quantification of molecular species of glycerophospholipids. The quantification relied upon a set of six diheptadecanoyl (17:0/17:0) synthetic internal lipid standards, covering common glycerophospholipid classes, and was linear within a concentration range of 10 nM−100 μM. The MPIS methodology produced a comprehensive and quantitative description of the complex ensemble of glycerophospholipid species by the direct analysis of total lipid extracts of cells or tissues. The analysis time was within 30 min/sample and the approach lent itself to high-throughput lipidomics.

Quantification of glycerophospholipids in total lipid extracts typically relies on spiked internal standards, representing lipids of the quantified lipid classes, and the acquisition of lipid class-specific precursor or neutral loss scans.[18,20] This methodology is straightforward and powerful, yet it fails to distinguish isobaric species often present in lipid extracts (e.g., PC 18:0/18:2 and PC 18:1/18:1). By applying a combination of multiple FA-specific, lipid class-specific, and supplementary precursor ion scans bundled in a single MPIS experiment, it has become possible to distinguish and quantify isobaric diacyl and ether species. The specificity and quantification capabilities of the MPIS approach were supported by the linearity of calibration curves of individual lipid species, acquired from a total lipid extract (Figure 8), by matching MPIS and conventional PIS profiles acquired from the same sample in independent experiments[31] and in Figure 3 and, finally, by a good agreement between the composition and relative abundance of endogenous lipid species detected by MPIS and a mass spectrometry independent approach.[16,35] Thus, MPIS methodology has improved the scope and precision of the characterization of glycerophopholipidomes without affecting the analysis throughput, since all required structure-specific scans were acquired in parallel and rapidly deciphered by the dedicated Lipid Profiler software. The approach has now been extended to other lipid classes (e.g.,

(38) Vance, D. E., Vance, J. E., Eds. *Biochemistry of Lipids, Lipoproteins and Membranes*, 3 ed.; Elsevier: Amsterdam, 1996.
(39) DeLong, C. J.; Baker, P. R. S.; Samuel, M.; Cui, Z.; Thomas, M. J. *J. Lipid Res.* **2001**, *42*, 1959−1968.
(40) Sommer, U.; Herscovitz, H.; Welty, F. K.; Costello, C. E. *J. Lipid Res.* **2006**, *47*, 804−814.

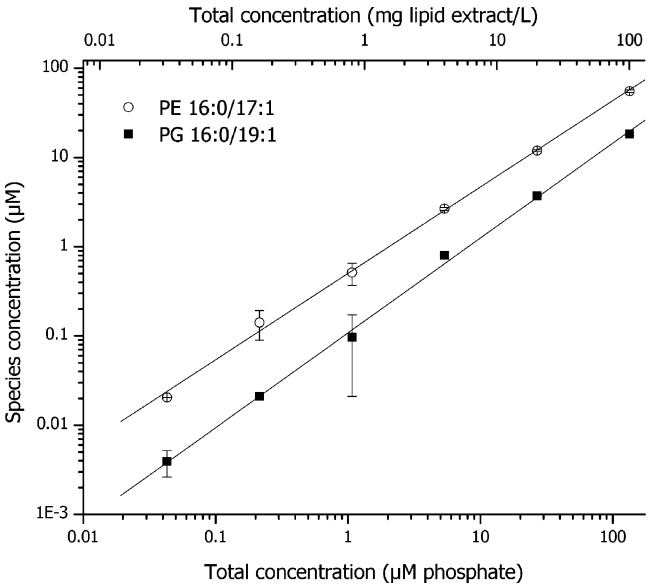

Figure 8. Dynamic range of MPIS quantification in the *E. coli* polar lipid extract. The set of synthetic internal standards (each at a final concentration of 0.25 nM) was spiked into an *E. coli* polar lipid extract. MPIS spectra were acquired as described in Materials and Methods and individual species identified and quantified using Lipid Profiler software. The estimated concentrations of the abundant PE 16:0/17:1 (*m*/*z* 702.5) and PG 16:0/19:1 (*m*/*z* 761.5) were plotted as a function of the total lipid concentration (in mg/L; upper *x*-axis) and total sample phosphate content (lower *x*-axis).

sphingomyelins, inositol-containing sphingolipids, ceramides, hexosylceramides) (data not shown), which can be profiled either simultaneously with glycerophospholipids or within the same experimental setup under different instrument settings.[37,41] With some modifications, the method can also cover the identification and quantification of sterols[42,43] and glycerolipids.[44] However, the most comprehensive characterization and quantification of individual glycerophospholipid species, including *sn*-1/*sn*-2 positional isomers, requires the combination of quadrupole time-of-flight and ion trap mass spectrometers,[16,41] which can be further complemented by orifice ozonolysis to determine the localization of double bond in FA moieties.[45]

MPIS methodology is a versatile tool for quantifying absolute differences between lipidomes.[46] Once a comprehensive data set has been acquired, it can be dissected and reported in multiple ways. Once integrated with a multivariate data analysis, the MPIS method can serve as an efficient screening tool for charting the perturbations in the molecular lipid composition under a variety of physiological and pathophysiological conditions. Importantly, the changes in lipid composition uncovered by MPIS can be immediately validated by targeted MS/MS experiments performed on perturbed lipid precursors via automated data-dependent acquisition.[31]

Abbreviations: FA, fatty acid; FA N:M, fatty acid comprising *N* carbon atoms and *M* double bonds in its hydrocarbon backbone; PA, phosphatidic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; PX N:M, lipid molecule(s) of PX class comprising, in total, *N* carbon atoms and *M* double bonds in the fatty acid moieties; PX FA_i/FA_j, a lipid molecule of PX class with FA_i moiety at the *sn*-1 position and FA_j moiety on the *sn*-2 position of the glycerol phosphate backbone; PX $FA_i{\sim}FA_j$, a lipid molecule of PX class (or a mixture of isomeric molecules) comprising FA_i and FA_j moieties at unidentified positions of the glycerol phosphate backbone; Prefix *O*, (PX *O*-...) indicates ether species of the PX class. Plasmanyl and plasmenyl species (where known) are annotated separately; PIS, precursor ion scanning; PIS *m*/*z* 281.3 stands for scanning for precursor ions that produce a fragment ion with *m*/*z* 281.3 upon collision-induced dissociation; MPIS, multiple precursor ion scanning.

APPENDIX

Isotope Correction of MPIS Spectra. Expected isotope ratios are typically calculated by mass spectrometric software tools (e.g., Analyst) using binomial expansion.[47] These assume, however, that natural isotopes are randomly distributed in the molecule, and therefore, the isotope distributions in precursor ion spectra cannot be calculated this way since a specific fragment elemental composition is selected.

For the illustrative purpose, we define the intact singly charged molecular ion M having the isotope distribution represented by the population of ions $M_{k=\{0,1,2,3,...\}}$, where the subscript indicates the number of isotopes (e.g., ^{13}C, ^{2}H, ^{17}O, ^{15}N) in the molecule. The isotopic abundance of M is calculated using the binomial expansion mentioned above. Let us assume that, upon fragmentation, M produces the charged fragment ion F and the neutral fragment N. The isotope distributions of F and N are represented by $F_{i=\{0,1,2,3,...\}}$ and $N_{j=\{0,1,2,3,...\}}$, respectively, where the subscript indicates the number of isotopes. Note, that each additional isotope increases the masses of the precursor and fragments by 1 Da. Fragmentation of the precursor M_0 only produces a single combination of fragments F_0N_0; M_1 produces two populations F_0N_1 and F_1N_0; M_2 produces three populations F_0N_2, F_1N_1, and F_2N_0; etc. (Table 3). Table 3 shows that a precursor ion scan for the charged fragment F_0 will detect only the subset of precursor ions with isotopes localized in the neutral fragments (N_0, N_1, N_2, N_3, ...). Similarly, precursor ion spectra of fragments F_1, F_2, and F_3 will detect distinct subsets of precursor ions with additional isotopes localized in the complementary neutral fragment (Table 3).

The isotopic abundance of any combination of fragments F_i and N_j (Table 3) can be calculated as a product of the relative isotopic abundances of each of the two complementary fragments (f_i and n_j, respectively), which, in turn, are calculated from their elemental compositions. For example, the isotopic abundance of each subset of precursor ions detected in the precursor ion scan for F_0 (Table 3) is calculated as f_0n_0; f_0n_1; f_0n_2, ...

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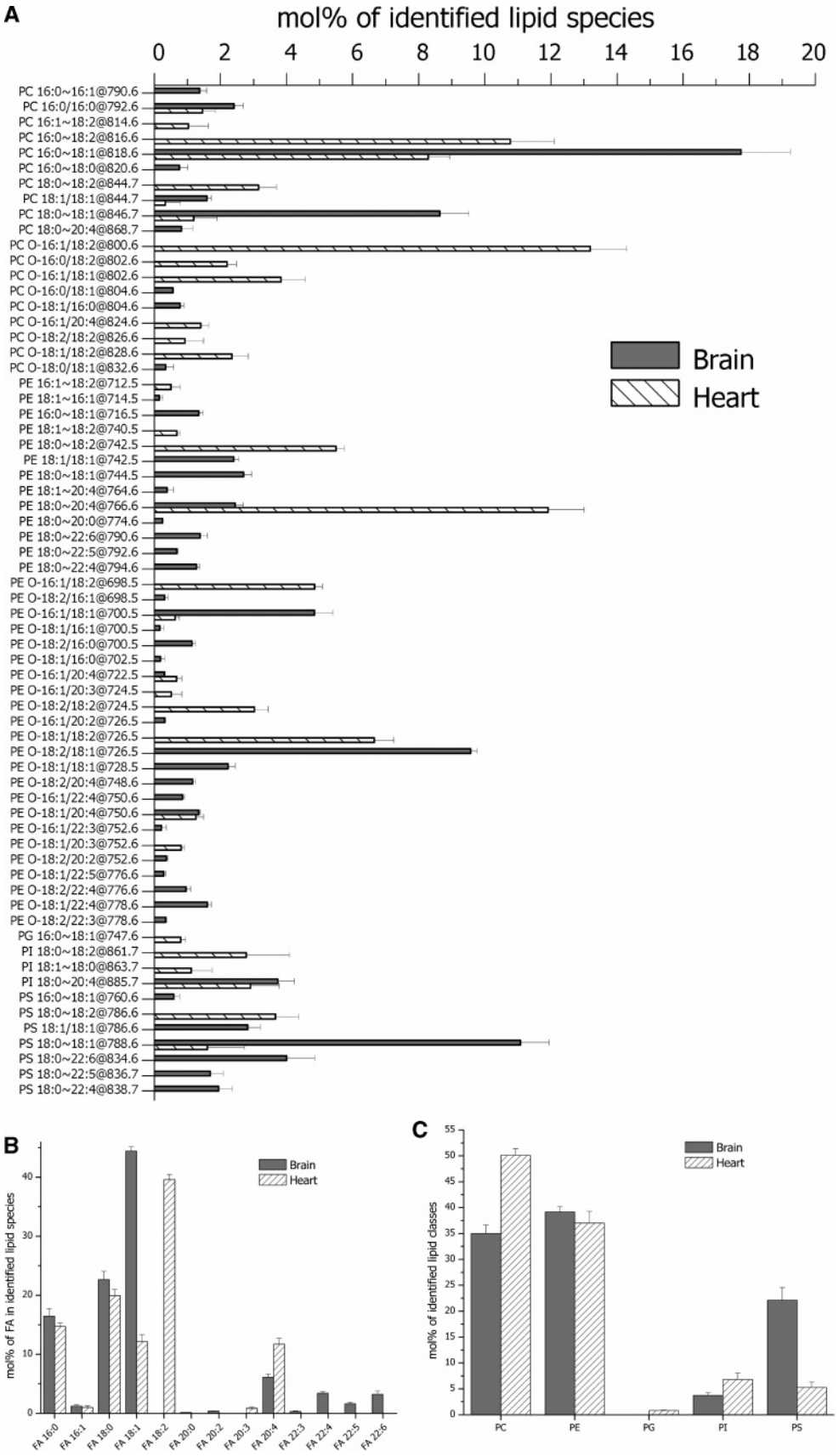

Figure 9. Comparative lipid analysis of total polar lipid extracts from porcine brain and bovine heart. (A) Species-to-species comparison. The mol % of identified lipid species were calculated as outlined in Materials and Methods. (B) Emulated total FA profile. The mol % of FA moieties were calculated as the sum of molar concentrations of lipid species in (A) containing the respective FA moiety, followed by normalization to the total molar concentration of all FA moieties. The FA concentration corresponding to symmetric lipid species was multiplied by a factor of 2 to account for two identical FA moieties. (C) Lipid class profile. The mol % of lipid classes was calculated as the sum of the mol % of lipid species (in panel A) of the respective lipid class. The MPIS analysis was repeated four times.

Let us consider the example in Figure 6, in which the precursor ion PE 18:1/18:1 ($C_{41}H_{77}O_8NP$) was monitored by TOF MS and PIS m/z 281.3, 282.3 and 283.3. The isotopic abundances of detected precursor ions in the precursor ion spectra were calculated as outlined above and presented in Table 4 and Figure 6.

Lipid Profiler software successively performs two types of isotope correction of MPIS data set. Intrascan isotope correction determines the individual abundance of precursors ions within overlapping isotopic clusters that are detected by the same precursor ion scan (e.g., PIS m/z 281.3, Figure 6B). It is required to resolve lipid species of the same class that differ by one double bond (e.g., PE 18:1/18:1 with m/z 742.5, and PE 18:0/18:1 with m/z 744.5, Figure 6B). However, it also helps to distinguish lipid species of different classes having similar m/z and the same FA moiety (e.g., the acetate adduct of PC 16:0/16:0 with m/z 792.6 and PG 16:0/22:6 with m/z 793.5). Within the spectrum, acquired by precursor ion scan for the fragment F, it proceeds from low to high m/z. For the intensity detected at the $m/z = M$, the software calculates the isotopic abundances of the neutral fragment N_1, N_2, N_3, ..., using the predicted elemental composition. Then it calculates the product of the intensity of the precursor M_0 and each of the isotopic abundance of N_1, N_2, N_3 and subtracts them from the intensities at the masses of M_1, M_2, M_3, ...

Interscan isotope correction is applied if an abundant lipid species, detected by precursor scan for the fragment with $m/z = X$, also produces interfering intensities in precursor scans for the fragments with $m/z = X + 1$ and $m/z = X + 2$ (e.g., PIS m/z 281.3 and PIS m/z 283.3, Figure 6B,D). For the intensity at $m/z = M$ detected by the precursor scan for the fragment ion F, the software checks if another precursor ion scan was acquired for the fragment with $m/z = F - 2$ and obtains the intensity of the peak with $m/z = M - 2$. Then the software calculates the expected intensity for the peak of $M - 2$ precursor acquired by the precursor scan for fragment F_2, multiplies it with the intensity of the same precursor recorded in the precursor ion scan for the fragment $F - 2$, and subtracts the value from the intensity at $m/z = M$ in the precursor ion scan for the fragment F. If the precursor scan for the fragment $F - 1$ was acquired, the $F - 1$ scan intensities are corrected using information from the precursor ion scan of $F - 2$, and the corrected $F - 1$ intensities are used to further correct the F scan data.

Since elemental compositions of lipid species and their fragment ions are known, the isotopic abundances were calculated and used as needed.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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