DOI 10.5012/bkcs.2011.32.4.1170

High Accuracy Mass Measurement Approach in the Identification of Phospholipids in Lipid Extracts: 7 T Fourier-transform Mass Spectrometry and MS/MS Validation

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Received September 29, 2010, Accepted February 1, 2011

In the present study, the approach of high accuracy mass measurements for phospholipid identifications was evaluated using a 7 T ESI-FTMS/linear ion trap MS/MS. Experiments were carried out for porcine brain, bovine liver, and soybean total lipid extracts in both positive and negative ion modes. In total, 59, 55, and 18 phospholipid species were characterized in the positive ion mode for porcine brain, bovine liver, and soybean lipid extracts, respectively. Assigned lipid classes were PC, PE, PEt, PS, and SM. In the negative ion mode, PG, PS, PA, PE, and PI classes were observed. In the negative ion mode, for porcine brain, bovine liver, and soybean lipid extracts, 28, 34, and 29 species were characterized, respectively. Comparison of our results with those obtained by other groups using derivatization-LC-APCI MS and nano-RP-LC-MS/MS showed that our approach can characterize PC species as effectively as those methods could. In conclusion, we demonstrated that high accuracy mass measurements of total lipid extracts using a high resolution FTMS, particularly, 7T FTMS, plus ion-trap MS/MS are very useful in profiling lipid compositions in biological samples.

Abbreviation: PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEt, phosphatidylethanol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphtidylserine; SM, sphingomyelin

Key Words : Lipids, Fourier transform ion cyclotron resonance mass spectrometer, Linear ion trap, Accurate mass measurements, Lipid database

Introduction

Fourier transform-ion cyclotron resonance mass spectrometry (FT-ICR MS or FTMS) offers an ultrahigh mass resolution and extraordinary mass accuracy, which has thus led to its wide applications in protein/peptide analysis.[1-3] FTMS can provide a mass resolution on the order of 90,000 below *m/z* 1,000 and allow a mass accuracy of 10 ppm or better in a broad band analysis.[4] These advantages become even more powerful in the analysis of small molecular species such as lipids that generally have relatively low mass values and exist within a narrow mass range, particularly, *m/z* 400-1,000.[5-8] Indeed, a number of groups have shown that FTMS is a very effective tool in lipid analysis.[9-14] For example, Ivanova *et al.* successfully utilized direct infusion electrospray ionization (ESI) FTMS to monitor the changes in the glycerophospholipid compositions of total lipid extracts obtained from intact and permeabilized RBL-2H3 (mucosal mast cell line) cells.[9] More recently, Taguchi and coworkers reported that ESI-FTMS coupled with liquid chromatography (LC) was useful in identifying the phospholipid molecular species of *Caenorhabditis elegans*.[10] They also demonstrated that the high resolving power of FTMS could distinguish the two phospholipid species of the same class but with the slightest mass difference: i.e., $\Delta m = 0.036$ Da between 35:2 diacyl (*m/z* 730.539) and 36:2 alkyl-acyl (*m/z* 730.575) phosphatidylethanolamine (PE).

MALDI FTMS has also been found to be very powerful in lipid structural analysis and in the rapid profiling of the lipid species in whole cells or tissues.[15-17] Wilkins and coworkers developed a data analysis strategy that utilizes a mass defect plot (fractional mass versus whole number mass) for the identification of lipid species detected in the high-resolution MALDI FTMS spectra.[4,16,17] Using only accurate mass measurements and a simple set of rules related to mass defects, a majority of ion-peaks below *m/z* 1,000 could be distinguished into twelve lipid classes. The classified lipids were further identified using their in-house lipid database with more than 50,000 entries. In addition, through the examination of the lipid database, they revealed that the minimum accuracy requirement for the correct assignment of the ion peaks is 21.38 ppm, under the assumption that the cation species attached to the compound of interest is already known. However, when the cation species is not known, the requirement becomes by far more strict; i.e., 0.38 ppm. This finding strongly suggests that the accurate mass measurement approach coupled with a direct infusion ESI-FTMS can be successfully used in lipid analysis as long as the cation species is well defined.

The direct infusion ESI-FTMS approach for the analysis of total lipid extracts has clear advantages and disadvantages when compared with LC-MS/MS. With LC-MS/MS, more

comprehensive analysis results can be expected in comparison with the direct infusion ESI-FTMS approach. However, LC-based analysis requires 2-dimensional LC separations. The first dimension is the normal-phase chromatography which can separate complex lipid species into several lipid classes and the other is reversed-phase separation which refines the lipid species within the same lipid class into individual lipid species with different fatty acid structures. Obviously, the shorter analysis time and the ease with which the comparison between two lipid extracts obtained under different conditions can be made are the advantages of the direct infusion ESI-FTMS approach. Even with such obvious merits, application of direct infusion ESI-FTMS in lipid profiling has not been widely made. A main reason for this, we believe, has to do with potential false-positive identification, particularly when complex biological lipid samples are examined.

In the present study, we analyzed standard lipid extracts using direct infusion ESI-FTMS and MS/MS tandem mass spectrometry. This study demonstrates that ESI-FTMS lipid identifications made based on the accurately measured mass values provide a rapid and reliable lipid analysis tool. Further, the analysis results for standard lipid extracts will serve as a good reference for future research.

Experimental

Materials and Sample Preparation. Total lipid extracts from porcine brain, bovine liver, and soybean tissues dissolved in a solution of chloroform and methanol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). For each extract, the vendor provided a rough estimate of their lipid constituents; porcine brain: phosphatidylethanolamine (PE, 16.7 wt %), phosphatidylserine (PS, 10.6 wt %), phosphatidylcholine (PC, 9.6 wt %), phosphatidic acid (PA, 2.8 wt %), phosphatidylinositol (PI, 1.6 wt %), and others (58.7 wt %), bovine liver: PC (42 wt %), PE (22 wt %), PI (8 wt %), cholesterol (7 wt %), lyso-phosphatidylinositol (1 wt %), and others (21 wt %), soybean: PC (24 wt %), PE (18 wt %), PI (11.5 wt %), lyso-phosphatidylinositol (4.6 wt %), PA acid (4.3 wt %), and others (37 wt %) (the analytical method was not specified by the vendor). A volume of 100 μL of each lipid extract solution was mixed with a 100 μL solution of chloroform and methanol 2:1 (v/v) and then vortexed for 10 min at room temperature. The resulting solution was subjected to brief centrifugation at 10,000 rpm for 10 min. For the positive ion mode mass analysis, 10 μL of acetic acid was added to the supernatant solution, vortexed, and centrifuged for another 5 min. For the negative ion mode analysis, no additional treatment was performed. HPLC grade organic solvent (chloroform and methanol) and acetic acid were purchased from Sigma (Seoul, Korea) and used without further purification.

Mass Spectrometry. Experiments were performed on a linear ion trap-7 Tesla Fourier transform mass spectrometer (ThermoElectron Corp., San Jose, CA, USA). Data acquisition and analysis were made using *X*calibur version 1.4. (Note that our version of *X*calibur did not allow for internal calibration.) Before lipid mass analysis, external mass calibration/tuning was performed in the positive ion mode using a standard solution specified by the manufacturer: Caffeine (*m/z* 195), MRFA (*m/z* 524), and Ultramark mix (*m/z* 1222, 1522, and 1822) in a methanol/water solution containing 1% acetic acid. Since the instrumental function of automatic gain control (AGC) can minimize mass shifts that may arise from the space charge effect, external calibration is expected to provide a reasonable calibration effect. The sample solutions prepared as above were infused directly through a home-pulled fused silica capillary emitter (i.d. = 75 mm) at a flow rate of 0.2-0.5 μL/min using a syringe pump (Harvard Apparatus 22, Holliston, MA, USA). A potential difference of +1.8 ~ +2.2 kV and –1.8 ~ –2.3 kV were applied between an electrospray emitter and an inlet of the mass spectrometer in the positive and negative mode, respectively. The temperature of the ion-transfer capillary was set to 220 ºC. Ion transmission into the linear trap was optimized with the calibrant solution. The applied capillary potential was +7 V (–7 V in the negative ion mode), and the tube lens potential was set to +120 V (–120 V in the negative mode). The AGC targets for the full-scan linear-trap and FTICR cell were 3×10^4 and 5×10^5, respectively. The resolving power of the FTICR mass analyzer was set to 100,000 ($m/\Delta m_{50\%}$ at *m/z* 400). For the ESI FT mass spectrum, the mass range of *m/z* 150- 2,000 was scanned at a rate of 1 s/scan. A total of 100 transients were averaged to obtain both FTICR mass spectra and linear-ion trap MS/MS spectra. For each sample, five ESI-FTMS spectra were obtained. For collisionally-activated dissociation (CAD), only the linear ion trap was utilized for fragmentation and detection. In CAD, almost every ion peak was isolated with a 1.0 *m/z* isolation window, and then was subjected to collision activation with 25-30% normalized collision energy. As a collision gas, ultrahigh purity helium (99.999%) was used. The obtained mass spectra were processed using Xtract (ThermoElectron Corp., San Jose, CA, USA) to produce a list of monoisotopic masses.

Databases. The "lipid MAPS" (http://www.lipidmaps.org/tools/ms/) database is used for lipid identifications. For the positive ion mode mass analysis, H^+, Li^+, Na^+, and K^+ adduct species were all considered since these cation species can possibly co-exist. But no Li^+ and Na^+ adduct species was found and only a few K^+ adduct species were observed in our mass spectra. For the negative mode analysis, $(M–H)^-$ anions and CH_3COO^- adducts were searched.

Results and Discussions

Lipid profiling was performed using ESI-FTMS for three different total lipid extracts, porcine brain, bovine liver, and soybean, in both the positive and negative ion modes. The results obtained with the direct infusion ESI-FTMS approach combined with a linear ion trap MS/MS will be presented below.

Porcine Brain.

Positive ESI-FTMS Spectrum: A positive ion ESI-FTMS spectrum averaged for 100 transients is shown in Figure 1.

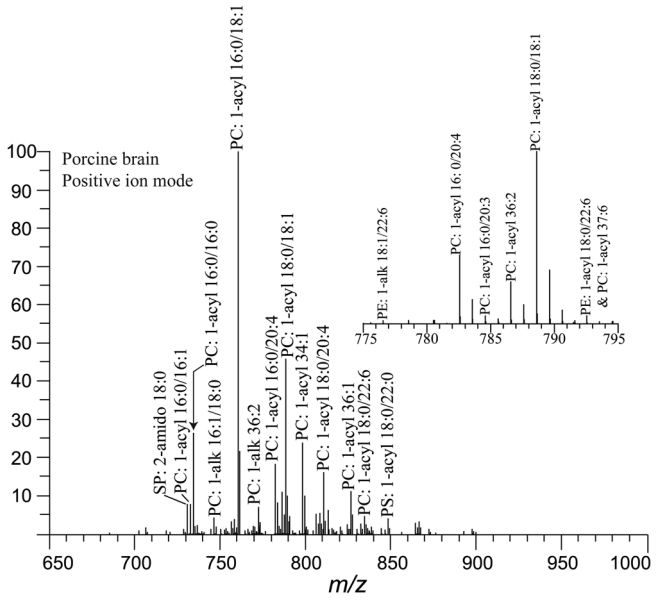

Figure 1. A positive ion ESI-FTMS spectrum for porcine brain total lipid extracts. Inset: an enlarged spectrum in the range of *m/z* 775-795.

Most of ion peaks appear in the region of *m/z* 700-900, indicating that the detected ions primarily belong to phospholipid species rather than neutral lipids that show relatively lower *m/z* mass values. Furthermore, the presence of some phospholipid classes was also confirmed by a low abundant peak observed at *m/z* 184 (not shown in Figure 1), which is a signature MS/MS (in-source fragmentation) fragment for PCs and SMs. In addition, the low abundance of these ion peaks also assures that in-source conditions were relatively mild, which is a pre-requisite for this type of study. A relatively low mass resolution was set: i.e., $m/\Delta m_{50\%}$ = 100,000 (at *m/z* 400), in order to increase the detection sensitivity. However, the ion observed in the mass spectrum generally showed a lower resolution, $m/\Delta m_{50\%}$ = ~55,000. This is presumably due to the broadening effect of averaging (100 scans). Nevertheless, the mass resolution was high enough for us to discern the adjacent isotopic peaks clearly, as shown in the inset of Figure 1.

The positive ESI-MS spectrum shows a large number of singly-charged ion, while multiply charged ion is not observed. The ion peak list was prepared by examining the ion cluster distributions of the observed ion-peaks. Here,

Table 1. A list of phospholipid species characterized in the positive ion mode for porcine brain total lipid extracts. The mass spectrum was obtained by averaging 100 transients. A '&' denotes K+ adduct. Superscript '#': lipid species also found in ref. 21

Class	Observed *m/z*	Theoretical *m/z*	ppm	Assignment
PC	720.5920	720.5902	2.50	1-alk 16:0/16:0
	728.5611	728.5589	3.02	1-alk 16:1/17:2
	#732.5558	732.5538	2.73	1-acyl 16:0/16:1
	#734.5713	734.5695	2.45	1-acyl 16:0/16:0
	744.5922	744.5902	2.69	1-alk 16:1/18:1
	746.6079	746.6058	2.81	1-alk 16:1/18:0
	754.5378	754.5382	0.53	1-acyl 16:1/18:3
	756.5540	756.5538	0.26	1-acyl 16:0/18:3
	#758.5718	758.5695	3.03	1-acyl 16:0/18:2
	#760.5860	760.5851	1.18	1-acyl 16:0/18:1
	770.6081	770.6058	2.98	1-alk 36:3
	772.6238	772.6215	2.98	1-alk 36:2
	780.5545	780.5539	0.77	1-acyl 16:0/20:5
	#782.5700	782.5695	0.64	1-acyl 16:0/20:4
	784.5884	784.5851	4.21	1-acyl 16:0/20:3
	#786.6031	786.6008	2.92	1-acyl 36:2
	#788.6183	788.6164	2.41	1-acyl 18:0/18:1
	792.5565	792.5539	3.28	1-acyl 37:6
	794.5713	794.5695	2.27	1-acyl 37:5
	794.6071	794.6059	1.51	1-alk 38:5
	796.5875	796.5851	3.01	1-acyl 17:1/20:3
	&798.5432	798.5415	2.13	1-acyl 34:1
	804.5542	804.5538	0.50	1-acyl 18:3/20:4
	806.5719	806.5695	2.98	1-acyl 18:1/20:5
	808.5876	808.5851	3.09	1-acyl 18:1/20:4
	810.6025	810.6008	2.10	1-acyl 18:0/20:4
	#814.6349	814.6321	3.44	1-acyl 18:0/20:2
	#816.6506	816.6477	3.55	1-acyl 38:1
	&826.5748	826.5728	2.42	1-acyl 36:1
	830.5708	830.5695	1.57	1-acyl 20:3/20:5
	832.5868	832.5852	1.92	1-acyl 20:2/20:5
	#834.6038	834.6008	3.59	1-acyl 18:0/22:6
	836.6205	836.6165	4.78	1-acyl 18:0/22:5
	838.6344	838.6321	2.74	1-acyl 18:0/22:4
	854.5722	854.5695	3.16	1-acyl 20:5/22:5
	856.5868	856.5851	1.98	1-acyl 20:5/22:4
	860.6182	860.6165	1.98	1-acyl 42:7
	870.6970	870.6947	2.64	1-acyl 42:2
	878.6565	878.6634	7.85	1-acyl 21:0/22:5
SM	731.6081	731.6061	2.73	SP 2-amido 18:0
	813.6871	813.6844	3.32	SP 2-amido 24:1
	841.7203	841.7157	5.47	SP 2-amido 26:1
PE	702.5451	702.5432	2.70	1-alk 16:1/18:1
	718.5768	718.5745	3.20	1-alk 16:1/19:0
	724.5279	724.5276	0.41	1-alk 16:3/20:2
	728.5229	728.5225	0.55	1-acyl 17:2/18:1
	750.5444	750.5433	1.47	1-alk 38:6
	752.5612	752.5589	3.06	1-alk 38:5
	768.5555	768.5538	2.21	1-acyl 18:1/20:3
	776.5611	776.5589	2.83	1-alk 18:1/22:6
	778.5769	778.5746	2.95	1-alk 18:0/22:6
	780.5926	780.5902	3.07	1-alk 40:5
	786.6031	786.6008	2.92	1-acyl 39:2
	792.5565	792.5539	3.28	1-acyl 18:0/22:6
	814.5384	814.5382	0.25	1-acyl 42:9
PS	848.6408	848.6375	3.89	1-acyl 18:0/22:0
	858.5269	858.5280	1.28	1-acyl 20:5/22:4
	862.6557	862.6532	2.90	1-acyl 20:0/21:0
	876.6737	876.6688	5.59	1-acyl 42:0

only the signals that show the complete distribution of both 'A' and 'A+1' were considered for database search. This procedure ensured that noise peaks were ruled out. The selected ion-peaks were then subjected to lipid database search. In the database search, a rather generous mass tolerance of 10 ppm was used from the observed monoisotopic *m/z* values of $(M+H)^+$. The 10 ppm mass tolerance is expected to increase, to some extent, the number of identified lipid species. However, considering that the mass tolerance of 21.38 ppm is known to be a required mass accuracy for the correct assignments of lipid species under the assumption of proton cationization, 10 ppm is not a loose criterion for accurate mass measurements.[16] On the other hand, when a multiple number of lipid candidates existed within a 10 ppm tolerance from the observed monoisotopic mass, only the candidate with the smallest delta-mass (Δm) was selected (Table 1-6). In the case where two closely-spaced peaks existed, both peaks were considered to find lipid candidates as long as these two peaks showed 'A' and 'A+1' isotopic distributions.

As a result, a total of 59 candidate lipids were found (Table 1). The number of identified lipid candidates could be improved by the introduction of a liquid chromatography (LC) separation prior to MS analysis. However, in the present study, no LC separation was performed. The lipid species in the table are denoted by the total number of carbon atoms in two fatty acid chains, i.e., *sn*-1 and *sn*-2, and also by the number of double bonds in the two chains. For example, PC (1-acyl 32:1) represents a phosphatidylcholine lipid species with a total of 32 carbon atoms in *sn*-1 and *sn*-2 positions and with one double bond in either *sn*-1 or *sn*-2, most likely in *sn*-2. The notations of 1-acyl and 1-alk indicate that the *sn*-1 fatty acid chain is linked to the glycerol backbone *via* the ester and the ether bond, respectively. When the fatty acid chains were explicitly identified in the MS/MS analyses, they are specifically denoted in the table; e.g., PC (1-acyl 16:0/18:3).

Lipid classes detected in the positive ion mode for porcine brain sample were PC, SM, PE, and PS. The lipid list includes 39 PCs, 3 SMs, 13 PEs, and 4 PSs. No PG, PA, or PI was found in the positive ion mode, which is consistent with previous reports from other groups.[18-20] Many of the candidate lipids in Table 1 are found to have a small delta-mass, Δm, i.e., 3 ppm or less, from the theoretical *m/z* values of the lipid species. The average difference was 2.62 ppm. This difference is small considering that no internal calibration was performed. The good mass accuracy was presumably due to the automatic gain control (AGC) function of the instrument used in this study.

In the previous nano-ESI FTMS studies of *C. elegans* by the Taguchi group, they identified the same class of phospholipids with the slightest mass differences.[10] For example, PE (diacyl 35:2, *m/z* 730.53868) and PE (alk-acyl 36:2, *m/z* 730.57507) with a mass difference of 0.03639 Da could be distinctly identified. In our spectra, a couple of similar cases were observed between the isomers of PCs and/or PEs. For example, PC 1-acyl 16:0/20:5 (*obs. m/z* 780.5545) and PE 1-alk 40:5 (*obs. m/z* 780.5926) with a mass difference of 0.0381 Da (*cal.* 0.0363 Da) were observed (see Figure 2). The other examples are PC 1-acyl 37:5 (*obs. m/z* 794.5713)/ PC 1-alk38:5 (*obs. m/z* 794.6071) and PC 1-alk 16:1/17:2 (*obs. m/z* 728.5611)/PE 1-acyl 17:2/18:1 (*obs. m/z* 728.5229).

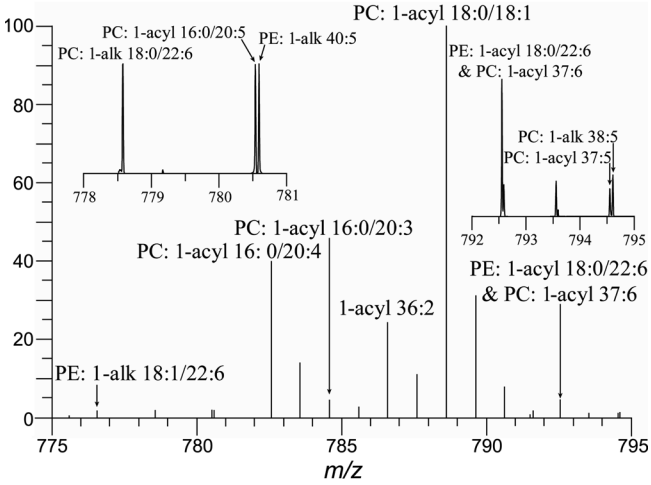

Figure 2. A zoom-out partial mass spectrum of Figure 1 in the region *m/z* 775-795.

It is also notable that, in some cases, lipid species with a K^+ adduct were also found even with an excess acetic acid treatment; PC 1- acyl 34:1 and PC 1-acyl 36:1. However, no sodium or lithium adduct peak was observed.

Identification of lipid species based on the accurately measured mass values has previously been shown to be effective.[4,10-17] Even with the disadvantage of relatively low sensitivity, the direct-infusion ESI-FTMS approach is very easy to implement and also provides a good method to compare the global lipid distributions of two total lipid extracts under inspection.

MS/MS Studies: The lipid species preliminarily assigned as described above were subjected to collisionally activated dissociation (CAD) using a linear ion trap mass spectrometer. MS/MS data can confirm the lipid class and also provide information of the fatty acid chains. That is, tandem mass spectrometry data enables us to evaluate whether the candidate lipid species obtained on the basis of accurately measured mass values (Table 1) are correctly assigned or not. Furthermore, detection of head group fragments makes it possible for us to distinguish PC and PE which cannot be otherwise discerned.

Upon collisional activation, a head group and/or one or more fatty acid chains were lost from the lipid molecules, which allowed us to identify the lipid class and fatty acid chains. The head group losses used in the assignment were as follow: PC, –59 and –183 Da; SM, –59 and –183 Da; PE, –43 and –140 Da; PS, –87 and –184 Da. Figure 3 illustrates an example of the product ion spectrum for the molecular ion peak at *m/z* 760.5860 (tentatively assigned as PC 1-acyl 34:1). The signals at *m/z* 701.6 and 577.6 represent $(M+H–N(CH_3)_3)^+$ and $(M+H–Choline)^+$, respectively, indicating a PC or SM species. The even-numbered mass value of $(M+H)^+$

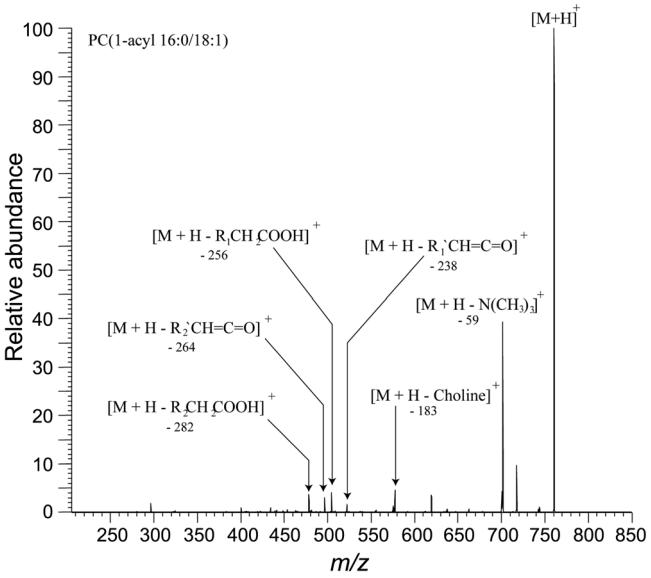

Figure 3. MS/MS spectrum for the peak at *m/z* 760.5860 (porcine brain) obtained using the positive-mode linear ion trap mass spectrometer.

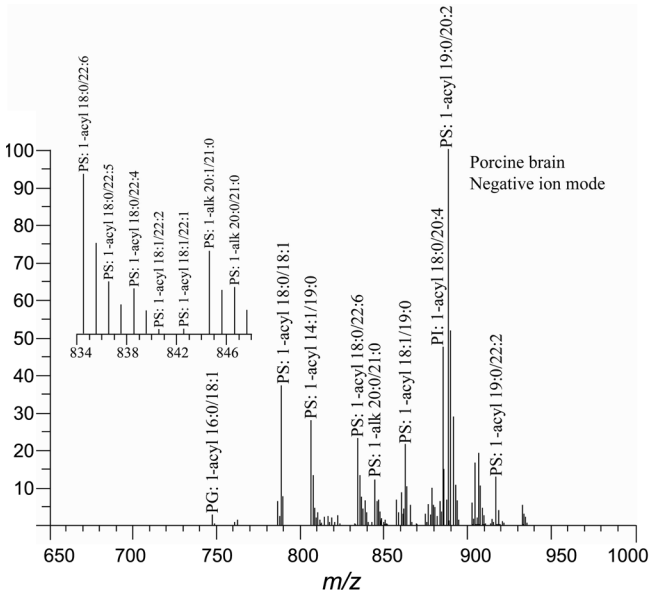

Figure 4. A negative ion ESI-FTMS spectrum for porcine brain total lipid extracts. Inset: an enlarged spectrum in the range of *m/z* 834-848.

precursor ions ensures that it is a PC species. The fragments at *m/z* 478.5 and 504.4 are $(M+H–R_2CH_2COOH)^+$ and $(M+H–R_1CH_2COOH)^+$, respectively, indicating that the two fatty acids are 1-acyl 16:0/18:1 or 1-acyl 18:1/16:0. With the fragment ions only, it was not possible to clearly assign a proper order to the *sn*-1 and *sn*-2 fatty acids, but it is very likely that the lipid is 1-acyl 16:0/18:1, since a double bond is usually present in the *sn*-2 position.

In our MS/MS studies, a head group loss was mostly observed, while the detachment of a fatty acid chain was not always observed; for example, PC 1-alk 36:3 at *m/z* 770.6081, PC 1-alk 36:2 at *m/z* 772.6238, PC 1-acyl 36:2 at *m/z* 786.6031, PC 1-acyl 37:6 at *m/z* 792.5565, PC 1-acyl 37:5 at *m/z* 794.5713, PC 1-alk 38:5 at *m/z* 794.6071, PC 1-acyl 34:1 at *m/z* 798.5432, PC 1-acyl 38:1 at *m/z* 816.6506, PC 1-acyl 36:1 at *m/z* 826.5748, PC 1-acyl 42:7 at *m/z* 860.6182, and PC 1-acyl 42:2 at *m/z* 870.6970. Even when only the head group loss was observed, it was possible to confirm the presence of a preliminarily assigned lipid. Within an allowed mass tolerance range (e.g., 10 ppm), only a few lipid species of different classes are usually present. Thus, the identification of the lipid class using the fragment ion peak arising from a head group loss was possible.

The prevailing loss of a head group and fatty acid chain as was observed above is somewhat different from what was previously reported by Hsu *et al.*[21] They found that collisional activation of the protonated phophatidylcholine molecular ions generally give rise to a product ion at *m/z* 184 (choline$^+$), instead of resulting in loss of a head group or fatty acid chain, and in contrast, the lithiated molecular ions tend to produce the loss of a head group or fatty acid chain. This discrepancy is likely to be due to the use of two slightly different activation methods; in our case, MS/MS was performed in a linear ion trap mass spectrometer, while Hsu used a triple quadrupole mass spectrometer. In addition, our instrument software did not allow us to monitor the mass region around *m/z* 184 when relatively high mass molecular ions are subjected to CAD. Thus, we had to rely only upon head group/fatty acid chain loss peaks in identifying the lipid species.

Negative ESI-FTMS Spectrum and MS/MS Confirmations: An ESI FTMS spectrum for the porcine brain lipid extracts was also obtained in the negative ion mode (Fig. 4). The overall peak pattern of this spectrum is quite different from that of Figure 1. In the negative ion mode, lipid classes of PG, PS, PA, PE, and PI are known to be detected. In the cases of PS and PE, they can be detected in both positive and negative ion mode. Table 2 was prepared based on the list of monoisotopic masses observed in the mass spectrum. In the negative ion mode, a total of 28 lipid species were found; 1 PG, 22 PSs, and 5 PIs. The delta-masses of the observed lipid species were as good as the ones detected in the positive ion mode. A few ion peaks with a CH_3COO^- adduct were also found; for example, PS 1-acyl 14:1/19:0 at *m/z* 806.5462, PS 1-acyl 18:1/19:0 at *m/z* 862.6094, PS 1-acyl 19:0/20:2 at *m/z* 888.6253, and PS 1-acyl 19:0/22:2 at *m/z* 916.6569. When compared with the lipid species identified in positive mode, no lipid species was identified in both positive and negative modes.

MS/MS confirmation was performed also for all the ion peaks observed in the negative mode. For the identification of lipid classes, the following head group losses were used: PG, –74 and –171 Da; PS, –87 Da; PI, –240, –258, and –298 Da. Figure 5 demonstrates MS/MS mass spectrum for PS 1-acyl 16:0/18:1 at *m/z* 760.5138. The –87 Da loss peak clearly shows that this lipid species is PS, and other fragments were used for identifying fatty acid chains. Based on the MS/MS results, the correct assignments for all ion peaks

Table 2. A list of phospholipid species characterized in the negative ion mode for porcine brain total lipid extracts. A '*' denotes (M+CH3COO-)- peak

Class	Observed *m/z*	Theoretical *m/z*	ppm	Assignment	Class	Observed *m/z*	Theoretical *m/z*	ppm	Assignment
PG	747.5189	747.5181	1.07	1-acyl 16:0/18:1	PS	840.5787	840.5760	3.21	1-acyl 18:1/22:2
PS	760.5138	760.5134	0.53	1-acyl 16:0/18:1		842.5934	842.5916	2.14	1-acyl 18:1/22:1
	762.5662	762.5654	1.05	1-alk 18:0/17:0		844.6449	844.6437	1.42	1-alk 20:1/21:0
	786.5305	786.5290	1.91	1-acyl 18:1/18:1		846.6615	846.6593	2.60	1-alk 20:0/21:0
	788.5458	788.5447	1.39	1-acyl 18:0/18:1		860.6397	860.6386	1.28	1-acyl 19:0/22:0
	*806.5462	806.5496&	4.22	1-acyl 14:1/19:0		*862.6094	862.6119	2.9	1-acyl 18:1/19:0
	810.5299	810.5290	1.11	1-acyl 18:0/20:4		886.5547	886.5603	6.32	1-acyl 22:4/22:4
	812.5466	812.5447	2.34	1-acyl 18:0/20:3		*888.6253	888.6279&	2.93	1-acyl 19:0/20:2
	814.5609	814.5603	0.74	1-acyl 18:0/20:2		*916.6569	916.6592	2.51	1-acyl 19:0/22:2
	816.5764	816.5760	0.49	1-acyl 18:0/20:1	PI	857.5192	857.5185	0.82	1-acyl 16:0/20:4
	818.6295	818.6280	1.83	1-alk 20:0/19:0		869.5573	869.5550	2.65	1-alk 18:1/20:4
	834.5302	834.5291	1.32	1-acyl 18:0/22:6		883.5388	883.5342	5.21	1-acyl 18:1/20:4
	836.5472	836.5447	2.99	1-acyl 18:0/22:5		885.5512	885.5498	1.58	1-acyl 18:0/20:4
	838.5623	838.5603	2.39	1-acyl 18:0/22:4		909.5517	909.5499	1.98	1-acyl 18:0/22:6

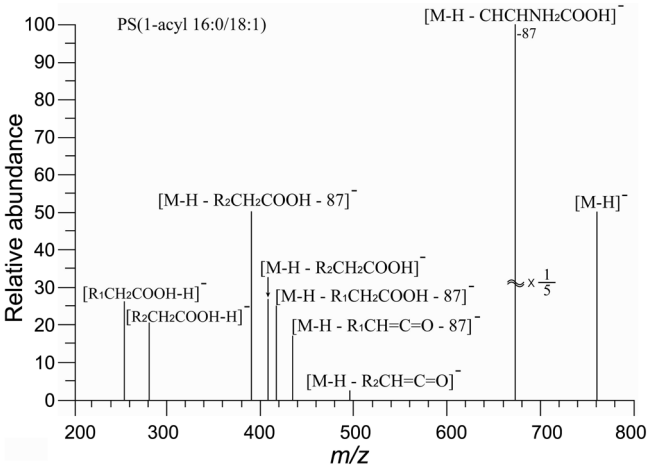

Figure 5. MS/MS spectrum for the peak at *m/z* 760.5138 (porcine brain) obtained using the negative-mode linear ion trap mass spectrometer.

were ensured. These results clearly indicate that the direct infusion ESI-FTMS accurate mass measurements offer an easy and reliable lipid identification strategy also in the negative ion mode.

Bovine Liver. The same experimental and interpretation approach was used for the identification of lipid species in the bovine liver total lipid extracts: i.e., both in the positive and negative ion modes. The detected ions were all phospholipids species. This was probably due to high ionization efficiency of phospholipids. It may also be related to the sample preparation procedure for total lipid extracts, but the information regarding the sample treatment could not be obtained from the vendor. As shown in Table 3, in positive ion mode, 55 lipid species were identified. Among those identified are 42 PCs, 12 PEs, and 1 PEt. For bovine liver extracts, no PS or SM was found. In addition, there was no alkali metal adduct.

In Table 3, it is noteworthy that there were found a large number of PC and PE isomers. For example, a peak found at *m/z* 760.5873 was assigned as PC 1-acyl 16:0/18:1 and PE 1-acyl 18:1/19:0, indicating that these two lipid species coexist. The coexistence of these two species was revealed by MS/MS of the peak at *m/z* 760.5873. As shown in Figure 6, MS/MS of this peak gave rise to $(M–59)^+/(M–183)^+$ and $(M–43)^+/(M–140)^+$ peaks, which indicate the presence of PC and PE species, respectively. There were also found a number of fragments which reveal identities of fatty acid chains. Other examples of this case were also found for [PC 1-alk 16:1/17:1, PE 1-alk 18:1/18:1, *m/z* 730.5760], [PC 1-acyl 33:1, PE 1-acyl 36:1, *m/z* 746.5719], [PC 1-acyl 17:0/18:2, PE 1-acyl 17:2/21:0, *m/z* 772.5873], [PC 1-acyl 17:0/18:1, PE 1-acyl 17:1/21:0, *m/z* 774.6035], [PC 1-acyl 16:0/20:4, PE 1-acyl 39:4, *m/z* 782.5705], [PC 1-acyl 18:0/18:2, PE 1-acyl 18:2/21:0, *m/z* 786.6031], and [PC 1-acyl 38:6, PE 1-acyl 19:0/22:6, *m/z* 806.5706].

In negative ion mode, 34 lipid species were identified; 17 PGs, 1 PE, 7 PSs, and 9 PIs (see Table 4). Fatty acid chains were revealed for all the lipid species, except for PS 1-acyl 40:6, PS 1-acyl 40:5, and PS 1-acyl 40:4.

Soybean. For soybean lipid extracts, 18 and 29 lipid species were identified in the positive and negative ion modes, respectively (see Table 5 and 6). In the MS/MS studies, fatty acid chains were all identified without exception. The number of lipid species identified in the positive ion mode, i.e., 18 lipid species, is much less than those found in the porcine brain (59 species) and bovine liver (55 species). The found lipid species included 11 PCs and 7 PEs, but no PS, SM, or PEt was found. No alkali metal adduct was observed. As found for porcine brain and bovine liver, there were also found three PC and PE isomers for soybean total extracts in the positive ion mode: PC 1-acyl 14:0/18:2, PE 1-acyl 17:0/18:2, *m/z* 730.5378; PC 1-acyl 16:0/16:0, PE 1-acyl 16:0/19:0, *m/z* 734.5709; PC 1-acyl 16:0/18:3, PE 1-acyl 18:3/19:0, *m/z* 756.5560. These isomers were clearly identified in the MS/MS experiments for the isobaric precursors, i.e., the

Table 3. A list of phospholipid species characterized in the positive mode for bovine liver total lipid extracts. A '&' denotes K+ adduct. Superscript '†' and '#': lipid species also found in ref. 20 and 21, respectively

Class	Observed *m/z*	Theoretical *m/z*	ppm	Assignment
PC	728.5603	728.5589	1.92	1-alk 16:1/17:2
	730.5760	730.5745	2.05	1-alk 16:1/17:1
	†#732.5558	732.5538	2.73	1-acyl 32:1
	†#734.5718	734.5695	3.13	1-acyl 32:0
	742.5771	742.5745	3.50	1-alk 34:3
	746.5719	746.5695	3.21	1-acyl 33:1
	†#756.5560	756.5538	2.91	1-acyl 16:0/18:3
	†#758.5720	758.5695	3.30	1-acyl 16:0/18:2
	†#760.5873	760.5851	2.89	1-acyl 16:0/18:1
	#764.5609	764.5589	2.62	1-alk 16:1/20:5
	766.5769	766.5745	3.13	1-alk 16:1/20:4
	768.5555	768.5538	2.21	1-acyl 15:0/20:4
	768.5934	768.5902	4.16	1-alk 16:0/20:4
	†#772.5873	772.5851	2.85	1-acyl 17:0/18:2
	†#774.6035	774.6008	3.49	1-acyl 17:0/18:1
	778.5376	778.5382	0.77	1-acyl 16:1/20:5
	†780.5542	780.5539	0.38	1-acyl 16:0/20:5
	†782.5705	782.5695	1.28	1-acyl 16:0/20:4
	†#784.5877	784.5851	3.31	1-acyl 16:0/20:3
	†#786.6031	786.6008	2.92	1-acyl 18:0/18:2
	†788.6192	788.6164	3.55	1-acyl 18:0/18:1
	792.5568	792.5538	3.79	1-acyl 17:2/20:4
	792.5928	792.5902	3.28	1-alk 16:1/22:5
	794.5711	794.5695	2.01	1-acyl 17:1/20:4
	794.6098	794.6058	5.03	1-alk 16:1/22:4
	796.5871	796.5851	2.51	1-acyl 17:2/20:2
	804.5545	804.5538	0.87	1-acyl 18:2/20:5
	†#806.5706	806.5695	1.36	1-acyl 38:6
PC	†#808.5864	808.5852	1.48	1-acyl 38:5
	†#810.6026	810.6008	2.22	1-acyl 18:0/20:4
	820.5874	820.5852	2.68	1-acyl 17:1/22:5
	822.6038	822.6008	3.65	1-acyl 17:1/22:4
	830.5697	830.5695	0.24	1-acyl 40:8
	†832.5858	832.5852	0.72	1-acyl 18:2/22:5
	†#834.6024	834.6008	1.92	1-acyl 18:0/22:6
	#836.6193	836.6165	3.35	1-acyl 18:0/22:5
	†#838.6365	838.6321	5.25	1-acyl 40:4
	&846.5440	846.5416	2.84	1-acyl 38:5
	&848.5600	848.5572	3.30	1-acyl 18:2/20:2
	856.5863	856.5852	1.28	1-acyl 20:4/22:5
	858.6019	858.6008	1.28	1-acyl 20:3/22:5
	860.6192	860.6165	3.14	1-acyl 42:7
PE	730.5760	730.5745	2.05	1-alk 18:1/18:1
	738.5035	738.5069	4.60	1-acyl 36:5
	746.5719	746.5695	3.21	1-acyl 36:1
	752.5611	752.5589	2.92	1-alk 38:5
	760.5873	760.5851	2.89	1-acyl 18:1/19:0
	766.5392	766.5382	1.30	1-acyl 18:1/20:4
	772.5873	772.5851	2.85	1-acyl 17:2/21:0
	774.6035	774.6008	3.49	1-acyl 17:1/21:0
	778.5765	778.5746	2.44	1-alk 18:1/22:5
	782.5705	782.5695	1.28	1-acyl 39:4
	786.6031	786.6008	2.92	1-acyl 18:2/21:0
	806.5706	806.5695	1.36	1-acyl 19:0/22:6
PEt	869.7032	869.6994	4.37	1-acyl 46:2

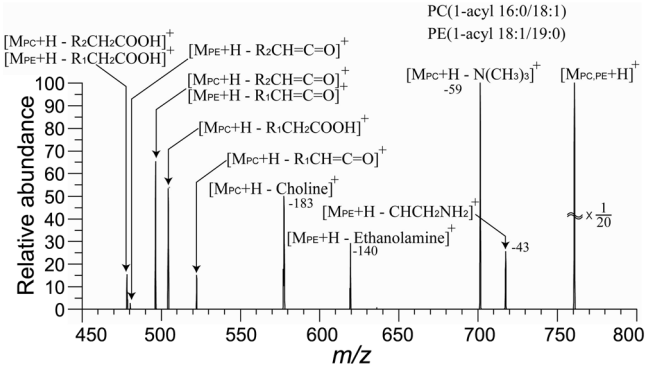

Figure 6. MS/MS spectrum for the peak at *m/z* 760.5873 (bovine liver) obtained using the positive-mode linear ion trap mass spectrometer.

peaks at *m/z* 730.5378, 734.5709, and 756.5560.

In the negative ion mode, 8 PGs, 7 PAs, 4PEs, 1PS, and 9 PIs were identified. No CH_3COO^- adduct was found. Mass accuracies were also very satisfactory, giving 2.21 ppm delta-mass on average. It is also noteworthy that PE 1-acyl 18:2/18:3 and PE 1-acyl 18:2/18:2 were found both in the positive and negative ion mode (see Table 5 and 6). They appeared at *m/z* 738.5083 and 740.5249 in Table 5 and at *m/z* 736.4937 and 738.5095 in Table 6. However, it is not clear whether or not these species found both in the positive and negative ion modes are the identical lipid species since the double bond positions could be different in the fatty acid chains.

To summarize, for porcine brain, bovine liver, and soybean lipid extracts, accurately measured monoisotopic mass values combined with MS/MS results could identify a large number of phospholipid species in a simple experimental setup.

Comparision to Other LC-MS(/MS) Results. The effectiveness of the direct infusion ESI-FTMS plus MS/MS approach can be evaluated by comparing its analysis results with those obtained from other methodologies, such as LC-atmospheric pressure chemical ionization (APCI) mass spectrometry conducted after conversion to diacyglycerol nicotinate derivatives and the nanoflow-reversed phase (RP) LC-MS/MS approach.[22,23] In those methods, experiments were conducted only in the positive ion mode, and thus the comparison is made only for the results obtained in positive mode.

Analysis of our results revealed that comparable numbers of lipid species, particularly PCs, were identified compared with those identified using the derivatization-LC-APCI MS and nano-RP-LC-MS/MS methods (see Table 1, 3, & 5). For porcine brain lipid extracts, nano-RP-LC-MS/MS identified 25 PC species, while 39 PC species were observed in our method. Comparison of both results reveals that 10 PC

Table 4. A list of phospholipid species characterized in the negative ion mode for bovine liver total lipid extracts

Class	Observed *m/z*	Theoretical *m/z*	ppm	Assignment	Class	Observed *m/z*	Theoretical *m/z*	ppm	Assignment
PG	745.5032	745.5025	0.94	1-acyl 16:0/18:2	PE	766.5393	766.5392	0.13	1-acyl 18:0/20:4
	747.5188	747.5181	0.94	1-acyl 16:0/18:1	PS	786.5297	786.5290	0.89	1-acyl 18:0/18:2
	759.5182	759.5181	0.13	1-acyl 17:0/18:2		788.5456	788.5447	1.14	1-acyl 18:0/18:1
	761.5348	761.5338	1.31	1-acyl 17:0/18:1		810.5290	810.5290	0.00	1-acyl 18:0/20:4
	767.4870	767.4869	0.13	1-acyl 18:2/18:3		812.5454	812.5447	0.86	1-acyl 18:0/20:3
	769.5028	769.5025	0.39	1-acyl 18:2/18:2		821.5342	821.5338	0.49	1-acyl 40:6
	771.5188	771.5181	0.91	1-acyl 18:1/18:2		823.5497	823.5495	0.24	1-acyl 40:5
	773.5342	773.5338	0.52	1-acyl 18:0/18:2		825.5661	825.5651	1.21	1-acyl 40:4
	775.5504	775.5494	1.29	1-acyl 18:0/18:1	PI	857.5190	857.5185	0.58	1-acyl 16:0/20:4
	793.5029	793.5025	0.50	1-acyl 18:2/20:4		859.5348	859.5342	0.70	1-acyl 18:0/18:3
	795.5188	795.5182	0.75	1-acyl 18:1/20:4		861.5502	861.5498	0.46	1-acyl 18:0/18:2
	797.5345	797.5338	0.88	1-acyl 18:0/20:4		863.5662	863.5655	0.81	1-acyl 18:0/18:1
	799.5505	799.5494	1.38	1-acyl 18:0/20:3		883.5341	883.5342	0.11	1-acyl 18:0/20:5
	801.5661	801.5651	1.25	1-acyl 18:2/20:0		885.5502	885.5498	0.45	1-acyl 18:0/20:4
	803.5812	803.5807	0.62	1-acyl 18:1/20:0		887.5672	887.5655	1.92	1-acyl 18:0/20:3
	827.5820	827.5807	1.57	1-acyl 20:0/20:3		911.5661	911.5655	0.66	1-acyl 18:0/22:5
	833.6283	833.6277	0.72	1-acyl 20:0/20:0		913.5823	913.5811	1.31	1-acyl 18:0/22:4

Table 5. A list of phospholipid species characterized in the positive ion mode for soybean total lipid extracts. Superscript '†' and '#': lipid species also found in ref. 20 and 21, respectively

Class	Observed *m/z*	Theoretical *m/z*	ppm	Assignment	Class	Observed *m/z*	Theoretical *m/z*	ppm	Assignment
PC	716.5246	716.5225	2.93	1-acyl 13:0/18:2	PC	†814.6355	814.6321	4.17	1-acyl 18:2/20:0
	#730.5378	730.5382	0.55	1-acyl 14:0/18:2		#842.6666	842.6634	3.80	1-acyl 18:2/22:0
	†#734.5709	734.5695	1.91	1-acyl 16:0/16:0	PE	730.5378	730.5382	0.55	1-acyl 17:0/18:2
	†756.5560	756.5538	2.91	1-acyl 16:0/18:3		734.5709	734.5695	1.91	1-acyl 16:0/19:0
	†#780.5557	780.5539	2.31	1-acyl 18:2/18:3		738.5083	738.5069	1.90	1-acyl 18:2/18:3
	†#782.5700	782.5695	0.64	1-acyl 18:2/18:2		740.5249	740.5225	3.24	1-acyl 18:2/18:2
	†#786.6034	786.6008	3.31	1-acyl 18:0/18:2		744.5562	744.5538	3.22	1-acyl 18:0/18:2
	804.5538	804.5538	0.00	1-acyl 18:2/20:5		756.5560	756.5538	2.91	1-acyl 18:3/19:0
	810.6005	810.6008	0.37	1-acyl 18:2/20:2		758.5710	758.5695	1.98	1-acyl 18:2/19:0

Table 6. A list of phospholipid species characterized in the negative ion mode for soybean total lipid extracts

Class	Observed *m/z*	Theoretical *m/z*	ppm	Assignment	Class	Observed *m/z*	Theoretical *m/z*	ppm	Assignment
PG	721.5040	721.5025	2.08	1-acyl 16:0/16:0	PE	714.5094	714.5079	2.10	1-acyl 16:0/18:2
	743.4885	743.4868	2.29	1-acyl 16:0/18:3		736.4937	736.4923	1.90	1-acyl 18:2/18:3
	745.5039	745.5025	1.88	1-acyl 16:0/18:2		738.5095	738.5079	2.17	1-acyl 18:2/18:2
	747.5204	747.5181	3.08	1-acyl 16:0/18:1		740.5254	740.523	2.43	1-acyl 18:1/18:2
	767.4882	767.4869	1.69	1-acyl 18:2/18:3	PS	836.5419	836.5447	3.35	1-acyl 18:1/22:4
	769.5038	769.5025	1.69	1-acyl 18:2/18:2	PI	819.5048	819.5029	2.32	1-acyl 16:0/17:2
	771.5204	771.5181	2.98	1-acyl 18:1/18:2		831.5050	831.5029	2.53	1-acyl 16:0/18:3
	773.5355	773.5338	2.20	1-acyl 18:0/18:2		833.5198	833.5185	1.56	1-acyl 16:0/18:2
PA	669.4511	669.4501	1.49	1-acyl 16:0/18:3		845.5202	845.5185	2.01	1-acyl 17:1/18:2
	671.4669	671.4657	1.79	1-acyl 16:0/18:2		847.5364	847.5342	2.60	1-acyl 17:0/18:2
	673.4810	673.4814	0.59	1-acyl 16:0/18:1		855.5048	855.5029	2.22	1-acyl 18:2/18:3
	693.4517	693.4501	2.31	1-acyl 18:2/18:3		857.5203	857.5185	2.10	1-acyl 18:2/18:2
	695.4673	695.4657	2.30	1-acyl 18:2/18:2		859.5368	859.5342	3.02	1-acyl 18:1/18:2
	697.4832	697.4814	2.58	1-acyl 18:1/18:2		861.5519	861.5498	2.44	1-acyl 18:1/18:2
	699.4986	699.4970	2.29	1-acyl 18:0/18:2					

species were identified in both studies, and they are denoted with a '#' mark in the 'observed *m/z*' column in Table 1. In the case of bovine liver extracts, 40 and 39 PC species were found using the above-mentioned two other methods, respectively, in comparison to 42 species identified using our method. The derivatization-LC-APCI MS (†, Table 3) and nano-RP-LC-MS/MS (#, Table 3) resulted in an identification of 18 and 16 phospholipid species, respectively, in common with our method, even though individual common components were a little different. For soybean lipid extracts, 16 and 28 PC species were characterized in those two methods; 11 PCs were monitored in our analysis. Six species were observed by each of the two methods, respectively, common to our results.

Conclusions

In this study, a direct infusion positive and negative ion ESI-FTMS method combined with ion-trap MS/MS were applied for the identification of phospholipid molecular species in porcine brain, bovine liver, and soybean lipid extracts. Our main focus was to evaluate whether this approach is valid for the analysis of phospholipid species. Accurate mass measurements of lipid extracts provided a large number of lipid candidates when searched for by comparison with lipid databases ('Lipid MAPS') with a maximum mass tolerance of 10 ppm. With mass values only, 59, 55, and 18 species were found in the positive mode for porcine brain, bovine liver, and soybean lipid extracts, respectively. The assigned lipid classes were PC, SM, PE, PS, and PEt. In the negative ion mode, mainly, PG, PS, PA, PE, and PI classes were observed. For porcine brain, bovine liver, and soybean lipid extracts, 28, 34, and 29 species were characterized. When compared with the results obtained from the derivatization-LC-APCI MS and nano-RP-LC-MS/MS, our approach revealed comparable numbers of PC species, which clearly demonstrates that our method is also useful in the profiling of lipid extracts.

Acknowledgments. This work was supported by Korea Research Foundation grant funded by the Korean government (MOEHRD, Basic Research Promotion Fund, KRF-2008-314-C00166) and Seoul R&BD program (PA090889). HBO is very thankful to Prof. M. H. Moon (Yonsei University) for encouragements and discussions. Y. H. Kim was supported by grant (G30123) from the Korea Basic Science Institute.

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