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Focused lipidomics by tandem mass spectrometry

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Abstract

In this paper we performed focused analyses of phospholipids by using the data of precursor ion scanning and neutral loss scanning of their polar head groups and fatty acyl moieties for the specific search of categorical phospholipids. By using precursor ion scanning or neutral loss scanning of polar head groups in the positive ion mode, more sensitive identification were obtained than that in the negative ion mode. Precursor ion scanning of carbonic anions in the negative ion mode was also effective to identify molecular species of phospholipids having specified fatty acyl moieties. By using these analytical methods, the detection limits of individual metabolites are going up to 5–20-fold of former conventional methods. The important factor is that by focusing in some limited categories of molecules, detection limit is greatly enhanced, thus minor but important molecules can be detected. Moreover, combination of LC–MS/MS and focused scanning for head group was revealed to be useful to identify very minor molecular species in the focused class of phospholipids.

Keywords: Lipidomics; Metabolomics; Phospholipids; Tandem mass

1. Introduction

Lipidomics is one of the prominent areas of metabolomics. Lipids are classes of molecules thought to be very important, not only as energy source or constituents of biological membrane, but also as functional molecules concerning the many regulation steps in biological process.

In the analysis of lipids by mass spectrometry, several different approaches have been reported [1,2]. Most popular methods have been used in metabolic analysis were single ion monitoring (SIM) and multiple reaction monitoring (MRM). These methods were normally used in combination with HPLC as LC–MS. The individual molecules were identified from their retention time and *m*/*z* value. In the case of MRM, essentially the combination with the detection of precursor ions and major fragment ions were used. Even in this analysis, the electrospray ionization (ESI) makes it possible to detect more than 10 molecules by a single LC analysis. MRM is commonly used in the quantitative analysis by mass spectrometry. But both in SIM and MRM analyses, the target molecules to be analyzed are needed to be defined in advance, and the data of their molecular masses and their fragments should be preliminarily required to set the analytical conditions. On the other hand, the comprehensive analysis of lipids by soft ionization is essentially used for the crude lipid mixture containing many different lipid metabolites [2–4], and whole molecules existing in the samples were expected to be identified as much as possible. In this case, without preliminary structural information for the metabolites before mass analysis, the significant profiling data can be obtained. Even for lipid mixture, some focuses in the molecules are effective to detect important factors in some occasions. For this

Abbreviations: MS, mass spectrometry; ESI, electro spray ionization; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SM, sphingomyelin; CID, collision induced dissociation; LC, liquid chromatography; HPLC, high performance liquid chromatography

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purpose, a precursor ion scanning method and a neutral loss scanning method are both very important for lipid analyses by MS [5–11]. These methods are used for comprehensive analysis of the categorical metabolites with structural similarities. The important factor is that by focusing in some limited categories of molecules, detection limit is greatly enhanced, thus minor but important molecules can be possible to detect. We tried to make up optimal collisional conditions for individual molecules to use these methods for the detection of specified class of phospholipids.

Adding to the comprehensive analysis, focused or targeted analyses for categorical components are very important. It is very difficult to obtain exact identification of all metabolites even in the limited classes of molecules such as lipid metabolites. This is caused by different extraction efficiency of individual metabolites, different solubility in analytical solvents, different ionic efficiency and broad dynamic ranges of their existence in biological samples. Even in the case of proteomics, it is very difficult to detect small amounts of peptides or proteins in mammalian plasma because of very wide dynamic ranges of protein contents in plasma. This is exactly the same in metabolites in most of biological samples.

In this paper we emphasized the importance of focusing on the specified category of lipid metabolites for the effective and sensitive identification of lipids [10,11]. For this purpose I selected precursor ion scanning and neutral loss scanning by triple quadrupole mass spectrometry for focused lipidomics. These methods are some variations of tandem mass spectrometry with ESI. The feature of these methods is comprehensive detection of focused categorical metabolites within the samples. Also, a combination of LC–MS/MS and focused scanning for head group was tested.

In this paper we performed focused analysis on individual classes of phospholipids, or on phospholipids which contained specific fatty acyl moieties. Also effectiveness of the specific search tool for their identifications is introduced.

2. Experimentals

2.1. Materials

All phospholipid standards were purchased from Avanti Polar Lipids (Alabaster, AL). All the solvents were HPLC grade, and were purchased from Wako Pure Chemicals (Osaka, Japan). Deionized water was obtained from a Milli-Q water system (Millipore, Milford, MA, USA).

2.2. Extraction of phospholipids from cells

Cultured cells (10^6), such as human monocyte THP-1 were washed with phosphate buffered saline (5 mM Na–phosphate buffer, pH 7.6 containing 150 mM NaCl), by three times centrifugation at 1200 × *g* for 5 min. Washed cells were extracted by the Bligh & Dyer's method [12]. Concentrated lipid extract was dissolved in with 100 μl of chloroform/methanol (2:1) and stored at −20 °C.

2.3. Extraction of phospholipids from rat spleen and liver

A spleen (1 g) or liver (5 g) of adult female Wister rat was homogenized with chloroform/methanol (1:2) and phospholipids were extracted by the Bligh and Dyer's methods. The total lipid extract was dried under a gentle stream of nitrogen and was dissolved in 4000 μl of chloroform-methanol (1:1).

2.4. Electrospray ionization mass spectrometrical (ESIMS) analysis of phospholipids

The ESIMS analysis was performed on a quadrupole-linear iontrap hybrid MS, 4000 QTRAP™ (Applied Biosystems/MDS Sciex, Concord, ON, Canada), with an AD10 VPμ HPLC system combined with an AD10 VPμ auto sampler (Shimadzu, Kyoto, Japan).

The extracted phospholipids were directly subjected to ESIMS analysis. The mobile phase compositions were acetonitril:methanol:water = 6:7:2 (0.1% ammonium formate). The flow-rate was 4 μl min^{-1}. The mass range of the instrument was set at *m*/*z* 400–950. One-thousand Da/s of the scan speeds was used. The trap fill-time was set at 3 ms in the positive ion mode, and 5 ms in the negative ion mode. The ion spray voltage was set at 5500 V in the positive ion mode and −4500 V in the negative ion mode. Nitrogen was used as curtain (value of 10) and collision gas (set to high). The declustering potential was set at 20 V to minimize in-source fragmentation. Both Q1 and Q3 resolution were set to "unit". Collision gas was at a value of 4 and collision energy on fragment ion dependent. Typically, 2 μl of sample was applied.

2.5. Precursor ion scanning and neutral loss scanning of polar head or fatty acid

Precursor ion scanning and neutral loss scanning were operated on a 4000Qtrap with flow injection at 4–10 μl/min. Optimum conditions for collision induced decay (CID) were selected by individual fragments or neutral loss. The level of collision energy is very important to the sensitive identification of focused molecules [13–16]. Optimal conditions to detect the proper precursor ions and neutral losses were obtained by MS/MS analyses of each phospholipid classes preliminary.

Automatic and programmed scanning for each class of phospholipids were succeedingly operated in 5 s/scan. Total cycle times for 8 min were used for total head group scanning, and 20 min were used for total carbonic anion scanning.

Briefly, in the positive ion mode, precursor ion scanning at *m*/*z* 184 was used for choline-containing phospholipids. Neutral scanning of 141, 185, 189, and 277 Da were used for PE, PS, PG and PI respectively. In the negative ion mode, neutral

loss scanning of 60 Da (loss of HCOO + CH_3) and 87 Da (loss of serine $–H_2O$) were used for choline-containing phospholipids and serine-containing phospholipids, respectively. And precursor ion scanning at *m/z* 153 and *m/z* 241 in the negative ion mode were used for glycerol-containing phospholipids, and inositol-containing phospholipids, respectively. Precursor ion scanning of carbonic anions in the negative ion mode was used for the identification of the molecular species having specified fatty acyl moieties.

2.6. Reverse phase LC–MS/MS surveyed with precursor ion scanning and succeeding product ion scanning by data dependent manner

LC separations were achieved using a Deverosil C30 (Nomura Chemical, Japan) reverse-phase LC (RPLC) column (150 mm × 0.3 mm i.d.) at room temperature. The mobile phase compositions were acetonitrile:methanol: triethylamine = 25:75:1 (0.3% formic acid, pH 6.8). The mobile phase was pumped at a flow of 8 μl min^{-1} for an isocratic elution. Typically, 4 μl of sample was applied. This system was combined with PC-specific detection using neutral loss scanning of 60 Da and data-dependent scanning. Triggering neutral loss scanning to product ion scanning switching in a data-dependent manner was based on ion intensity. Precursor ions with the highest intensity in the spectra of neutral loss scanning were selected automatically for product ion scanning. The collision energy was set at −50 eV. One time of neutral loss scanning survey and succeeding five times of product ion scanning for 5 different precursor peaks were performed in order within 1 cycle event.

3. Results and discussion

Precursor ion scanning and neutral loss scanning are used for the comprehensive analysis of the categorical metabolites with the structural similarities.

3.1. Precursor ion scanning and neutral loss scanning of phospholipids

Fig. 1 shows shame for the instruction of precursor ion scanning of the carbonic anion originated from arachidonic acid, and neutral loss scanning of 87 Da (serine–H_2O) for serine containing phospholipids.

Table 1 shows *m/z* values of specific fragment ions and values of neutral loss for precursor ion scanning and neutral loss scanning of polar head groups and fatty acyl chains. In this experiments, adding to well known fragments for precursor ion scanning of acidic phospholipids such as *m/z* 153 for PG, or *m/z* 241 for PI in the negative ion mode, neutral loss from precursor ions detected as ammonium adducts of PI and PG in the positive ion mode were found to be specific and sensitive for detecting these phospholipids.

3.2. Identification of phospholipids classes by precursor ion scanning or neutral loss scanning of polar head groups

Fig. 2 shows a total ion spectrum (a) and a spectrum by neutral loss scanning of 87 Da (serine–H_2O) (b) of phospholipids extracted from rat spleen in the negative ion mode. Even minor peaks such as *m/z* 834.7, 836.7 and 838.7 in a total ion spectrum, were effectively identified as PS by this neutral loss scanning. Signal to noise values indicated in Fig. 2a and b shows about 10–20-fold difference in the sensitivities of detection limits in the data from total molecular related ions or data from neutral loss scanning. Table 2 shows identification results obtained by our lipid search tool, named "lipid search" (http://metabo.umin.jp). Major molecular species of PS were effectively identified. The description and renewed our search system will be acceptable in May at http://lipidsearch.jp and precise quantification methods will be reported elsewhere (in preparation). Fig. 3A shows a total ion spectrum (a) and a spectrum obtained by precursor ion scanning of *m/z* 196 (glycerol ethanolamine phosphate

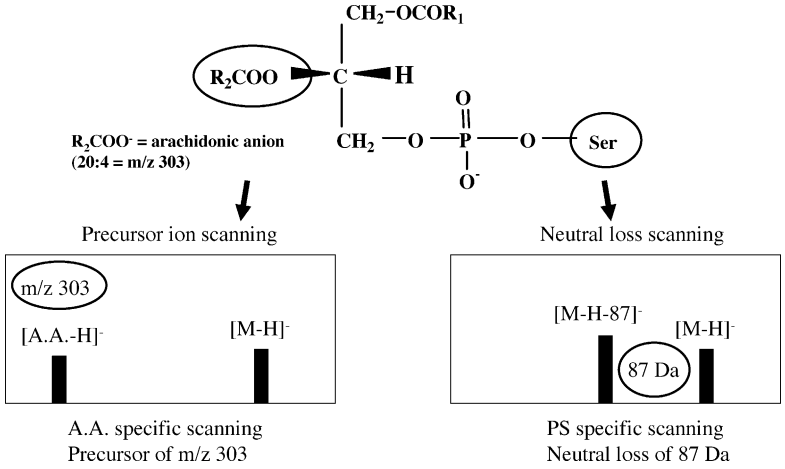


Fig. 1. Shame of precursor ion scanning and neutral loss scanning of the polar head group or carbonic anion of phospholipids. A.A., arachidonic acid.

Table 1
Precursor ion scanning and neutral loss scanning of individual classes of phospholipids in the positive and negative ion modes[a] and *m/z* values of carbonic anions for precursor ion scanning of phospholipids

	Positive	Negative
(a) Precursor ion scanning and neutral loss scanning of individual classes of phospholipids in the positive and negative ion modes		
PC	Pre *m/z* 184 (phosphoryl choline)	N-loss 60 Da ($HCOO + CH_3$)
PE	N-loss 141 Da (phosphoryl ethanolamine)	Pre *m/z* 196 (glycerol phosphoryl ethanolamine–H_2O)
PS	N-loss 185 Da (phosphoryl serine)	N-loss 87 Da (serine-H_2O)
PI	N-loss 277 Da (phosphoryl inositol + NH_4)	Pre *m/z* 241 (phosphoryl inositol–H_2O)
PG	N-loss 189 Da (phosphoryl glycerol + NH_4)	Pre *m/z* 153 (phosphoryl glycerol–H_2O)

Carbonic anions	Values of *m/z*	Carbonic anions	Values of *m/z*
(b) *m/z* Values of carbonic anions for precursor ion scanning of phospholipids			
14:0	227	20:5	301
16:1	253	20:4	303
16:0	255	20:3	305
18:3	277	20:2	307
18:2	279	20:1	309
18:1	281	22:6	327
18:0	283	22:5	329
		22:4	331

[a] Pre, precursor ion; N-loss, neutral loss.

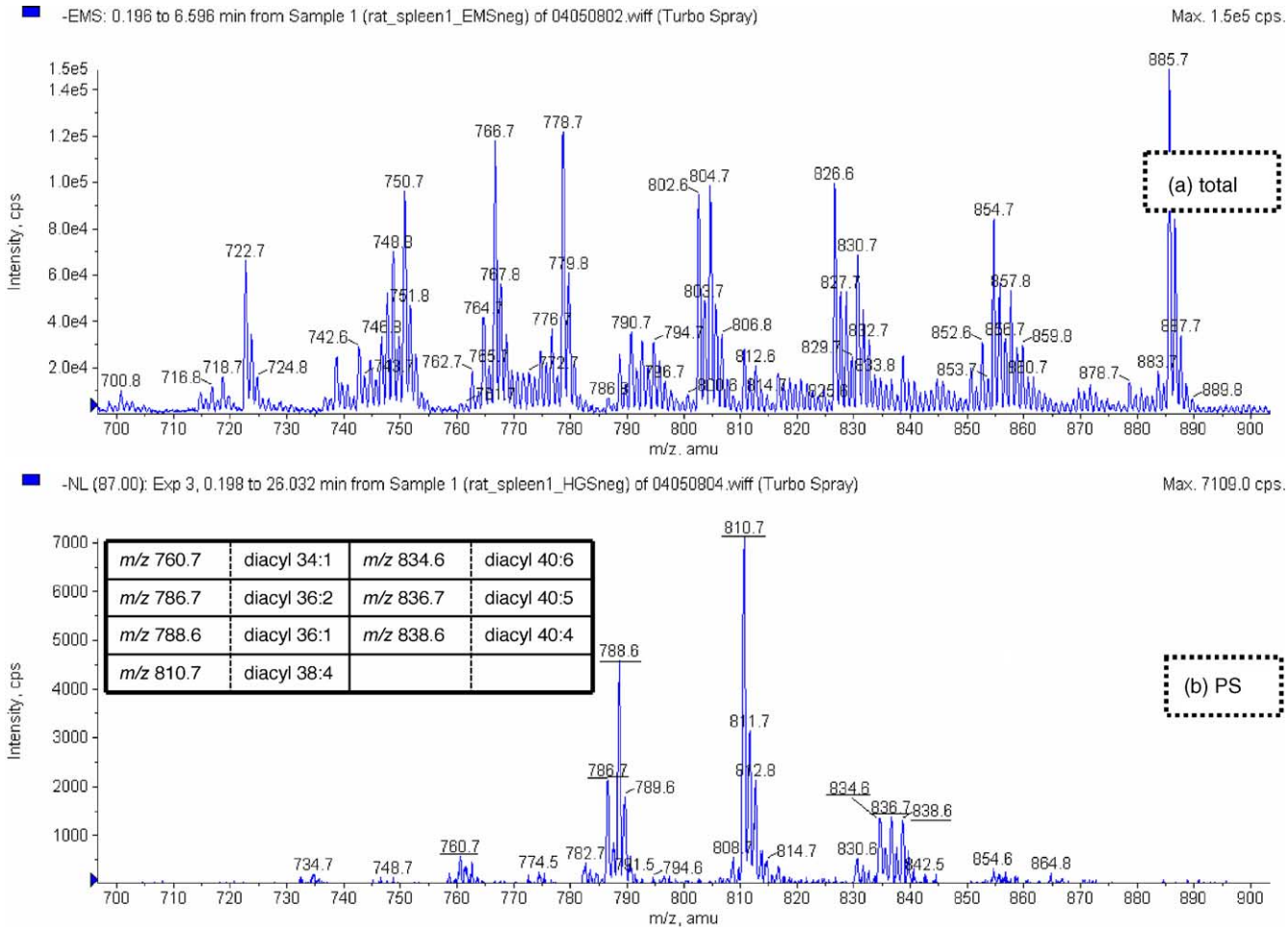


Fig. 2. Detection of phosphatidylserine in the lipid mixture extracted from rat spleen by neutral loss scanning of 87 Da in the negative ion mode. (a) A total ion spectrum of phospholipids extracted from rat spleen in the negative ion mode. (b) A mass spectrum of neutral loss scanning of 87 Da (serine–H_2O).

Table 2
Identification of molecular species of phosphatidylserine by the data from neutral loss scanning in the negative ion mode

m/*z*	Intensity	Molecular species	
524.4	5020	lysoPS,1-acyl,18:0	
760.6	2547	PS,1-acyl,34:1	
782.6	2137	PS,1-acyl,36:4	
786.6	11239	PS,1-acyl,36:2	
788.6	21904	PS,1-acyl,36:1	PS,1-alk,38:8
808.7	1989	PS,1-acyl,38:5	
810.6	38813	PS,1-acyl,38:4	
812.7	2658	PS,1-acyl,38:3	
830.6	2708	PS,1-acyl,40:8	PS,1-alk,40:1
834.7	8529	PS,1-acyl,40:6	
836.7	4796	PS,1-acyl,40:5	
838.7	4542	PS,1-acyl,40:4	

Phospholipids were designated as follows: PS 34:1, where 34: means the summed number of carbon atoms at both the *sn*-1 and *sn*-2 positions, and: 1 means the summed number of double bonds at both the *sn*-1 and *sn*-2 positions. Then, 1-acyl means 1-acyl 2-acy, while 1-alk means 1-alkyl, 2acyl or 1-alkenyl, 2-acyl. PS, phosphatidylserine.

Table 3
Identification of molecular species of phosphatidylethanolamine by the data from neutral loss scanning in the positive ion mode

m/*z*	Intensity	Molecular Species
716.5	109920	PE,1-acyl,34:2
718.5	75862	PE,1-acyl,34:1
740.5	177432	PE,1-acyl,36:4
742.5	86785	PE,1-acyl,36:3
744.6	225015	PE,1-acyl,36:2
746.6	106054	PE,1-acyl,36:1
752.6	62093	PE,1-alk,38:5
754.6	52149	PE,1-alk,38:4
764.5	112740	PE,1-acyl,38:6
766.5	216364	PE,1-acyl,38:5
768.6	892332	PE,1-acyl,38:4
770.6	70242	PE,1-acyl,38:3
782.6	30041	PE,1-alk,40:4
790.5	57267	PE,1-acyl,40:7
792.6	160293	PE,1-acyl,40:6
794.6	132254	PE,1-acyl,40:5
796.6	108036	PE,1-acyl,40:4

PE, phosphatidylethanolamine; alk, alkyl or alkenyl.

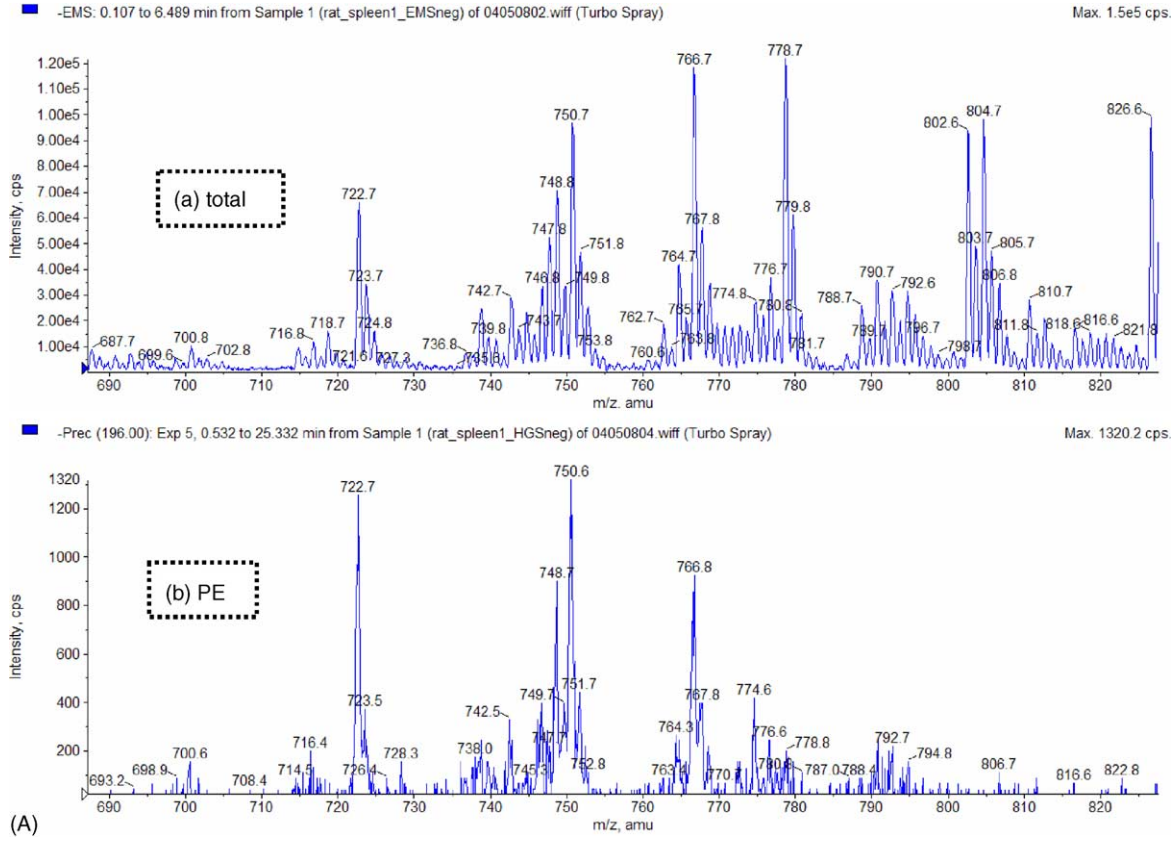


Fig. 3. (A) Detection of phosphatidylethanolamine in the lipid mixture extracted from rat spleen by precursor ion scanning of *m*/*z* 196 in the negative ion mode. (a) A total ion spectrum of phospholipids extracted from rat spleen in the negative ion mode. (b) A mass spectrum of precursor ion scanning of *m*/*z* 196 (glycero phosphoryl ethanolamine–H_2O). (B) Detection of phosphatidylethanolamine in the lipid mixture extracted from rat spleen by neutral loss scanning of 141 Da in the positive ion mode. (a) A total ion spectrum of phospholipids extracted from rat spleen in the positive ion mode. (b) A mass spectrum of neutral loss scanning of 141 Da (phosphoryl ethanolamine).

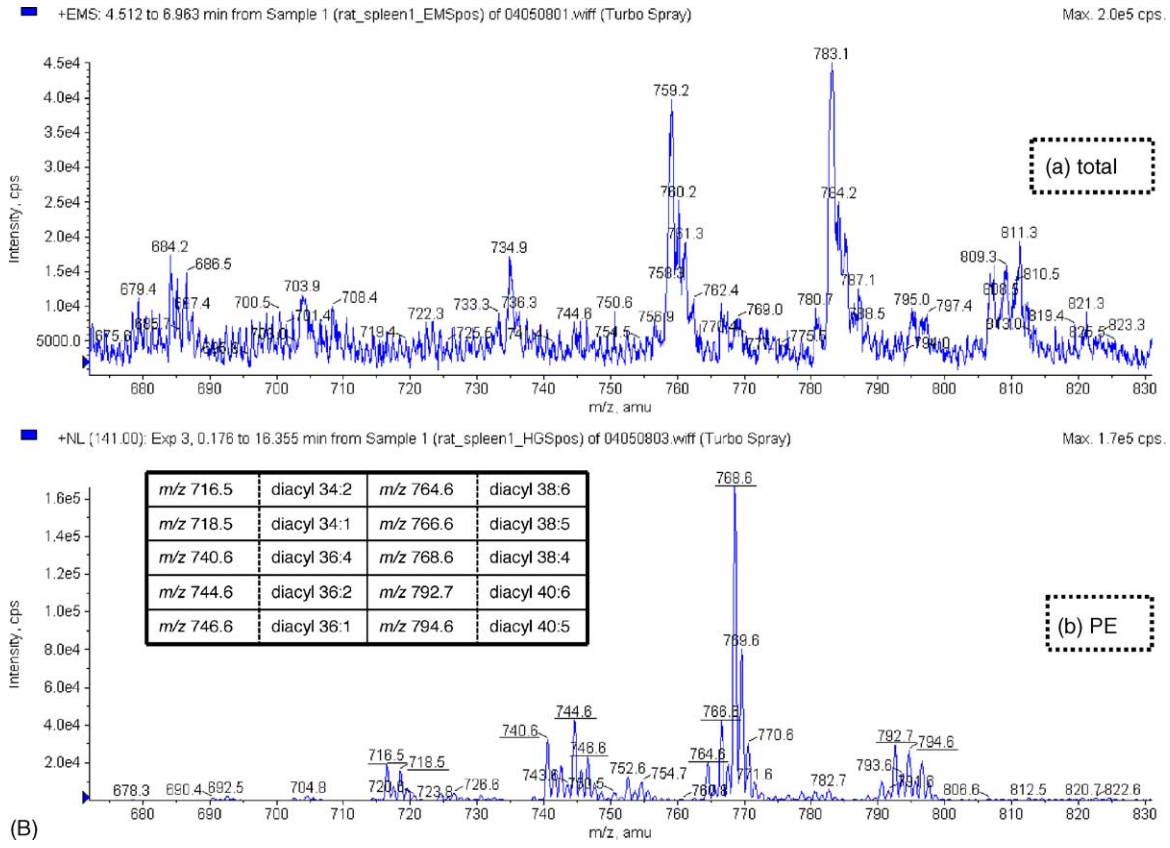


Fig. 3. (*Continued*).

–H_2O) (b) of phospholipids extracted from rat spleen in the negative ion mode.

Fig. 3B shows total ion spectrum a) and a spectrum obtained by neutral loss scanning of 141 Da (phosphoryl ethanolamine) of phospholipids extracted from rat spleen in the positive ion mode. More sensitive detection results were obtained in the positive ion mode. Table 3 shows identification results obtained by Lipid Search. Major molecular species of PE were effectively identified.

Fig. 4 shows a total ion spectrum and spectra of precursor ion scanning and neutral loss scanning of each polar head groups of total phospholipids extracted from THP-1 cells in the positive ion mode using automatic programmed scanning. In the positive ion mode, precursor ion scanning at *m*/*z* 184 was used for choline-containing phospholipids. Neutral loss scanning at 141, 185, 189, and 277 Da were used for PE, PS, PG and PI respectively. In the positive ion mode, more sensitive detection of each class pf phospholipids was obtained than that in the negative ion mode.

The data obtained by neutral loss scanning or precursor ion scanning of polar head fragments can be effectively applied to our search window "lipid Search" by checking squares of the specified classes of phospholipids in the identification window for the analysis of obtained mass spectrum. Most of the major molecular species of each class of phospholipids were detected comprehensively as same in Tables 2 and 3 (data not shown). In the detection mode of polar head scanning, partially quantitative data can be obtained from the intensities of detected precursor of each scanning. This is because the same class of phospholipids with identical polar head group displayed comparable ion intensities proportional to their chain length and number of double bonds. Thus we are using these data for detecting the changes in profiling of different samples. We think this method is very useful for partial quantitation in biochemical experiments.

3.3. Identification of phospholipids by precursor ion scanning of fatty acyl derivatives

Also focused analyses of phospholipids by the specified fatty acyl derivatives were performed by precursor ion scanning in the negative ion mode. Fig. 5 shows a total ion spectrum and spectra of precursor ion scanning of carbonic anions of total phospholipids extracted from THP-1 cells in the negative ion mode using automatic programmed scanning. Most of the molecular species of phospholipids with indicated fatty acyl chains were selectively identified (data not shown). Precursor ion scanning used were *m*/*z* 253, 255, 279, 281, 283, 301, 303, 305, 307, 309, 327, 329, and 331 for 16:1, 16:0, 18:2, 18:1, 18:0, 20:5, 20:4, 20:3, 20:2, 20:1, 22:6, 22:5, and 22:4 respectively.

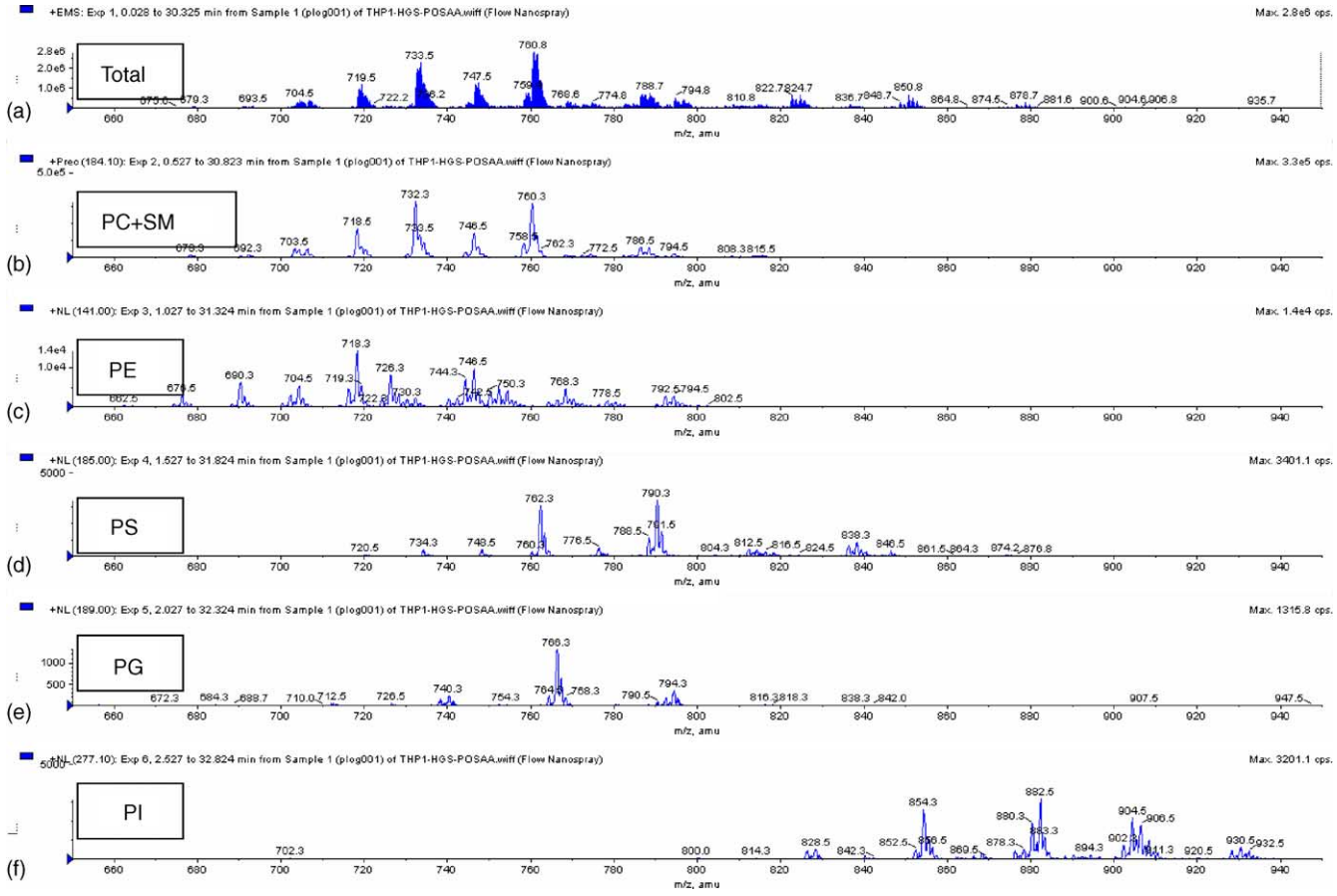


Fig. 4. Identification of individual molecular species of focused phospholipid classes by precursor ion scanning and neutral loss scanning of their head groups in the positive ion modes. Extracted total lipid mixture from THP-1 cells was subjected to precursor ion scanning of *m*/*z* 184 and neutral loss scanning of 141, 185, 189, and 277 Da. (a) EMS mode analysis of total lipids in the positive ion modes. (b) Precursor ion scanning at *m*/*z* 184 for PC and SM. (c) Neutral loss scanning of 141 Da for PE. (d) Neutral loss scanning of 185 Da for PS. (e) Neutral loss scanning of 189 Da for PG. (f) Neutral loss scanning of 277 Da for PI.

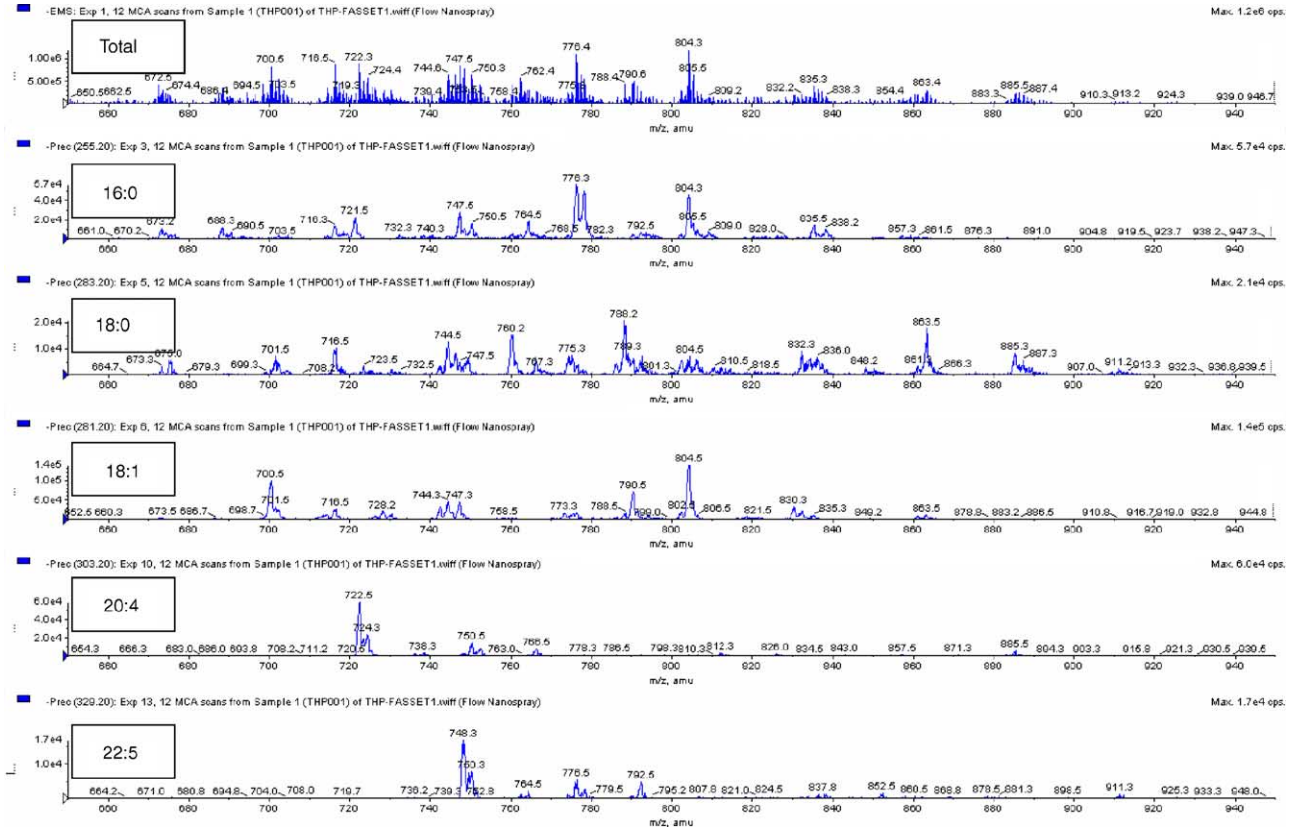
Because one fatty acyl moiety was selected as a fragment ion, another fatty acyl moiety and the polar head are most often identified from the molecular related mass value with a help of other information. As a result of the detection in the search window of Lipid Search, most probable individual lipid molecular species are indicated as a pair of fatty acyl chains simultaneously. In this search window, different classes of phospholipids containing specified fatty acyl chains such as an arachidonic acid can also be effectively identified. We also found that neutral loss scanning of fatty acid or carbonyl ketene are also very effective to identify glycerolipids with specified fatty acyl chains.

3.4. Combination analysis by RPLC–MS/MS with focused scanning of polar head groups

Combination of RPLC–MS/MS and neutral loss scanning is effective to detect individual molecular species with

Fig. 5. Identification of individual molecular species of THP-1 cell phospholipids having focused specified carbonic anions by precursor ion scanning in the negative ion mode. (a) EMS mode analysis of total lipids in the negative ion modes. (b) Precursor ion scanning at *m*/*z* 255 for 16:0 fatty acid. (c) Precursor ion scanning at *m*/*z* 283 for 18:0 fatty acid. (d) Precursor ion scanning at *m*/*z* 281 for 18:1 fatty acid. (e) Precursor ion scanning at *m*/*z* 303 for 20:4 fatty acid. (f) Precursor ion scanning at *m*/*z* 329 for 22:5 fatty acid.

Fig. 6. Mass chromatograms of RPLC/ESI-MS/MS of phospholipids mixture from rat liver by neutral loss scanning of 60 Da in the negative ion mode. The molecular species of phospholipids from rat liver were separated by RPLC–MS using the C30 reverse-phase column. When using a reverse-phase column, phospholipids were eluted in order from the hydrophilic molecules to the hydrophobic molecules. In phospholipids, the length of fatty acyl chains mainly influenced to the elution order in each molecular species. PC species were detected specifically by neutral loss scanning of 60 Da in the negative ion mode. The isotopic peaks of other molecular species are indicated by asterisks (*). (a) Total ion chromatogram. (b) Mass chromatogram of *m*/*z* 800 (34:3 diacyl PC) by neutral loss scanning of 60 Da. (c) Mass chromatogram of *m*/*z* 802 (34:2 diacyl PC) by neutral loss scanning of 60 Da. (d) Mass chromatogram of *m*/*z* 804 (34:1 diacyl PC) by neutral loss scanning of 60 Da. (e) Mass chromatogram of *m*/*z* 806 (34:0 diacyl PC) by neutral loss scanning of 60 Da.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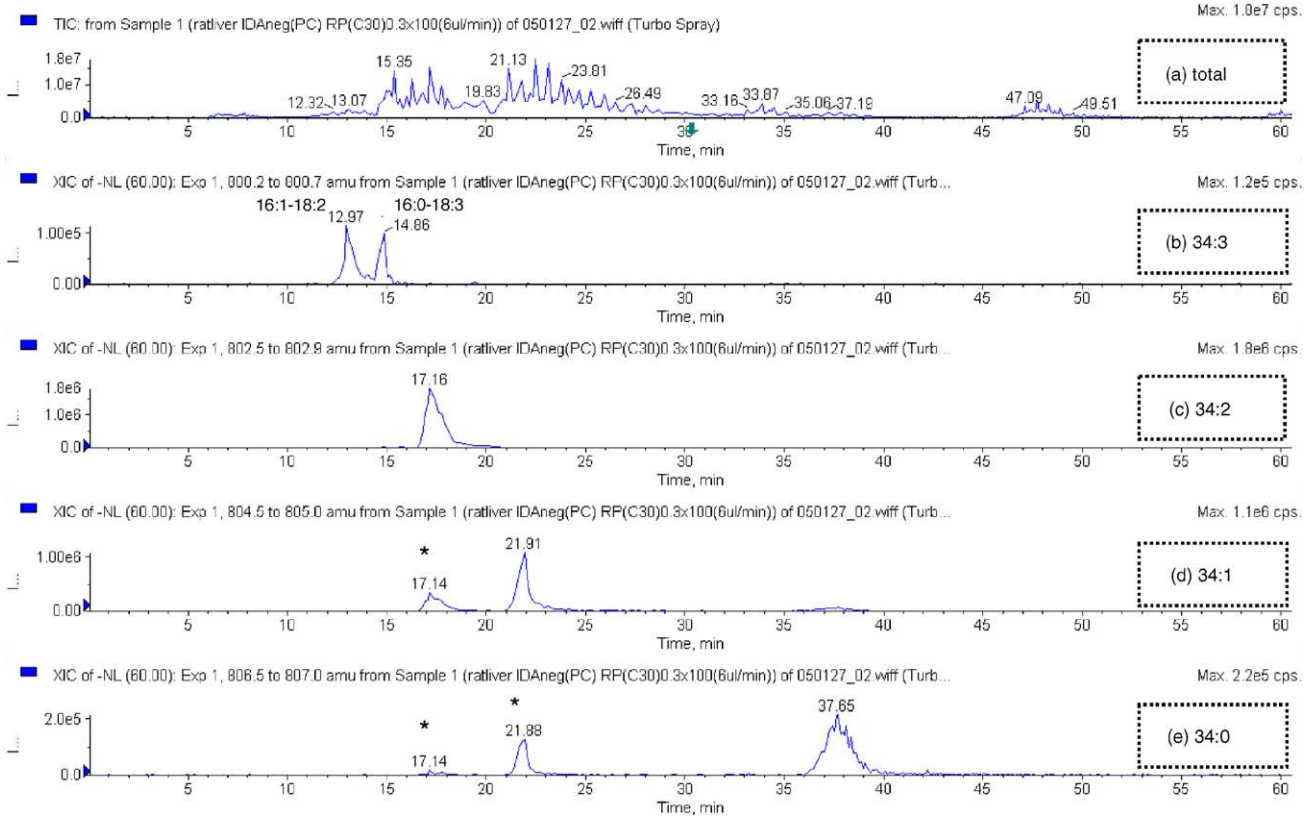
Max. 1.2e6 cps.
Total
-Prec (255.20): Exp 3, 12 MCA scans from Sample 1 (THP001) of THP-FASSET1.wiff (Flow Nanospray)
Max. 5.7e4 cps.
16:0
-Prec (283.20): Exp 5, 12 MCA scans from Sample 1 (THP001) of THP-FASSET1.wiff (Flow Nanospray)
Max. 2.1e4 cps.
18:0
-Prec (281.20): Exp 6, 12 MCA scans from Sample 1 (THP001) of THP-FASSET1.wiff (Flow Nanospray)
Max. 1.4e5 cps.
18:1
-Prec (303.20): Exp 10, 12 MCA scans from Sample 1 (THP001) of THP-FASSET1.wiff (Flow Nanospray)
Max. 6.0e4 cps.
20:4
-Prec (329.20): Exp 13, 12 MCA scans from Sample 1 (THP001) of THP-FASSET1.wiff (Flow Nanospray)
Max. 1.7e4 cps.
22:5
m/z, amu

Fig. 5.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Max. 1.8e7 cps.
(a) total
XIC of -NL (60.00): Exp 1, 800.2 to 800.7 amu from Sample 1 (ratliver IDAneg(PC) RP(C30)0.3x100(6ul/min)) of 050127_02.wiff (Turb...
Max. 1.2e5 cps.
16:1-18:2
16:0-18:3
(b) 34:3
XIC of -NL (60.00): Exp 1, 802.5 to 802.9 amu from Sample 1 (ratliver IDAneg(PC) RP(C30)0.3x100(6ul/min)) of 050127_02.wiff (Turb...
Max. 1.8e6 cps.
(c) 34:2
XIC of -NL (60.00): Exp 1, 804.5 to 805.0 amu from Sample 1 (ratliver IDAneg(PC) RP(C30)0.3x100(6ul/min)) of 050127_02.wiff (Turb...
Max. 1.1e6 cps.
(d) 34:1
XIC of -NL (60.00): Exp 1, 806.5 to 807.0 amu from Sample 1 (ratliver IDAneg(PC) RP(C30)0.3x100(6ul/min)) of 050127_02.wiff (Turb...
Max. 2.2e5 cps.
(e) 34:0
Time, min

Fig. 6.

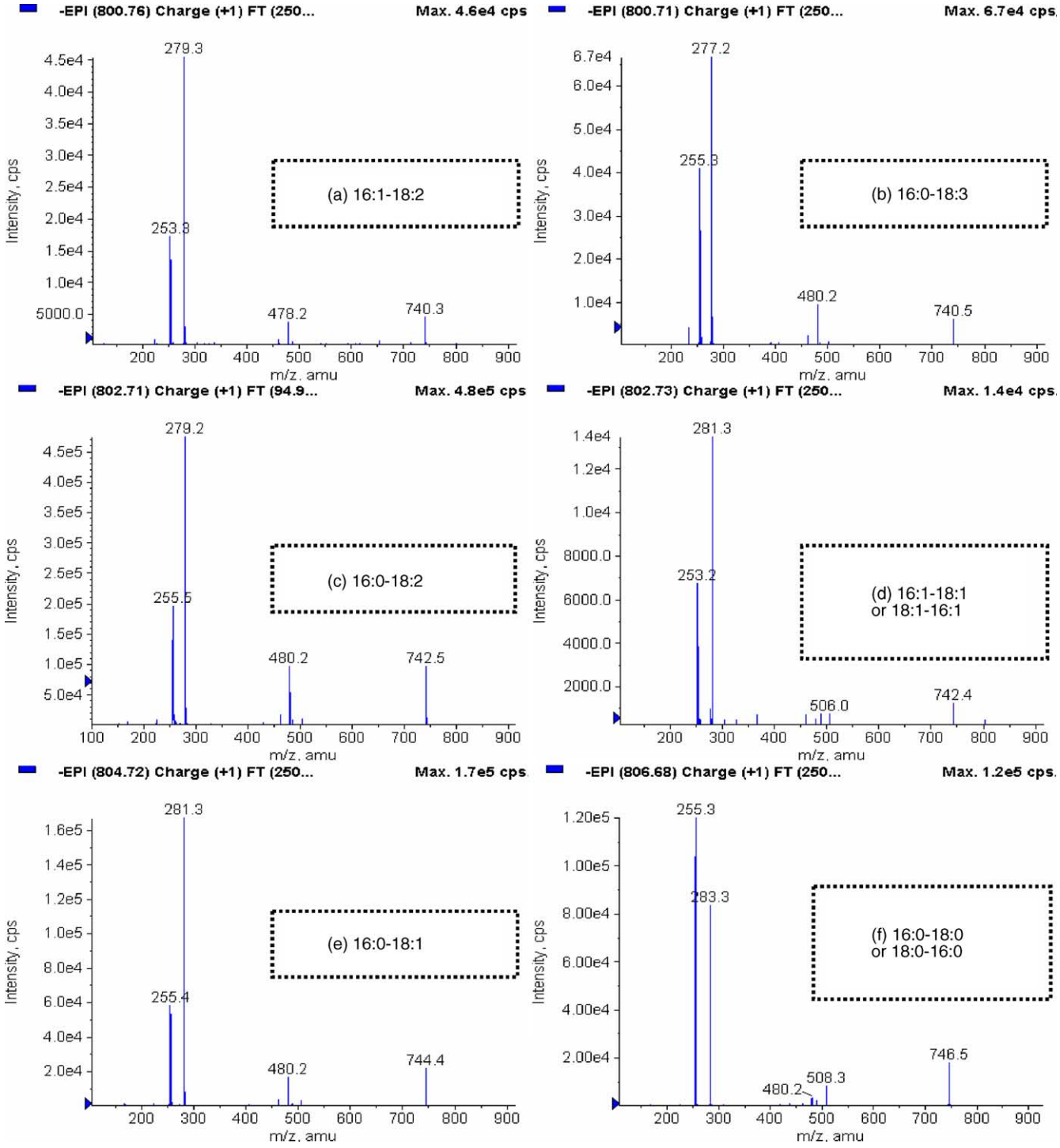

Fig. 7. RPLC–MS/MS spectra of $[M + HCO_2]$ ions obtained by data dependent scanning succeeding to neutral loss scanning of 60 Da in the negative ion mode for a phospholipids mixture from rat liver. The molecular species of phospholipids from rat liver were separated and identified by RPLC/ESI-MS/MS using the C30 reverse-phase column. This system was combined with PC-specific detection using neutral loss scanning of 60 Da and data-dependent scanning. Precursor ions with the highest intensity in the spectra of neutral loss scanning were selected automatically for product ion scanning. (a) MS/MS spectrum of *m/z* 800 (16:1–18:2 diacyl PC). (b) MS/MS spectrum of *m/z* 800 (16:0–18:3 diacyl PC). (c) MS/MS spectrum of *m/z* 802 (16:0–18:2 diacyl PC). (d) MS/MS spectrum of *m/z* 802 (16:1–18:1 diacyl PC or 18:1–16:1 diacyl PC). (e) MS/MS spectrum of *m/z* 804 (16:0–18:1 diacyl PC). (f) MS/MS spectrum of *m/z* 806 (16:0–18:0 diacyl PC or 18:0–16:0 diacyl PC).

Table 4
PC species from rat liver identified by RPLC/ESI-MS/MS with neutral loss scanning of 60 Da in the negative ion mode

m/z	Retention time (min)	Molecular species
540.5	7.2	LPC,1-acyl,16:0
564.4	7.0	LPC,1-acyl,18:2
566.5	6.6	LPC,1-acyl,18:1
568.5	7.8	LPC,1-acyl,18:0
588.5	6.2	LPC,1-acyl,20:4
612.4	6.1	LPC,1-acyl,22:6
762.6	28.4	PC,1-alk,16:1–16:0
764.6	32.2	PC,1-alk,16:0–16:0
774.7	13.5	PC,1-acyl,14:0–18:2
778.7	23.9	PC,1-acyl,16:0–16:0
788.8	14.8	PC,1-alk,16:0–18:2
790.7	18.8	PC,1-alk,16:0–18:1
792.8	52.0	PC,1-alk,16:0–18:0
798.6	12.2	PC,1-acyl,14:0–20:4
800.7	14.9	PC,1-acyl,16:0–18:3
800.7	13.5	PC,1-acyl,16:1–18:2
802.7	17.2	PC,1-acyl,16:0–18:2
802.7	15.5	PC,1-acyl,16:1–18:1
804.7	21.7	PC,1-acyl,16:0–18:1
806.7	37.9	PC,1-acyl,16:0–18:0
812.7	14.0	PC,1-alk,16:0–20:4
816.8	20.2	PC,1-alk,16:0–18:2
818.6	46.3	PC,1-alk,18:0–18:1
818.8	27.5	PC,1-alk,18:1–18:0
822.8	11.6	PC,1-acyl,14:0–22:6
824.7	12.7	PC,1-acyl,16:1–20:4
824.7	13.7	PC,1-acyl,16:0–20:5
826.7	13.3	PC,1-acyl,18:2–18:2
826.7	16.3	PC,1-acyl,16:0–20:4
828.6	19.8	PC,1-acyl,18:1–18:2
828.7	18.9	PC,1-acyl,18:0–18:3
828.8	18.3	PC,1-acyl,16:0–20:3
830.7	24.7	PC,1-acyl,18:0–18:2
830.7	20.7	PC,1-acyl,18:1–18:1
830.7	22.7	PC,1-acyl,16:0–20:2
832.7	32.0	PC,1-acyl,16:0–20:1
832.7	33.9	PC,1-acyl,18:0–18:1
834.7	34.2	PC,1-acyl,18:0–18:0
836.7	13.2	PC,1-alk,16:0–22:6
836.7	19.1	PC,1-alk,16:1–22:5
840.7	19.7	PC,1-alk,18:0–20:4
844.6	31.2	PC,1-alk,20:0–18:2
846.6	13.0	PC,1-alk,20:1–18:0
	11.1	PC,1-acyl,18:2–20:5, PC,1-acyl,18:3–20:4
848.6	13.6	PC,1-acyl,16:0–22:7
848.6	11.7	PC,1-acyl,16:1–22:6
850.7	15.4	PC,1-acyl,16:0–22:6
850.7	13.2	PC,1-acyl,18:2–20:4
852.7	16.7	PC,1-acyl,16:0–22:5
852.7	14.6	PC,1-acyl,18:2–20:3
852.7	16.1	PC,1-acyl,18:1–20:4
852.8	19.5	PC,1-acyl,18:0–20:5
854.6	17.2	PC,1-acyl,18:2–20:2
854.7	19.9	PC,1-acyl,16:0–22:4
854.7	22.5	PC,1-acyl,18:0–20:4
856.7	27.4	PC,1-acyl,18:0–20:3
858.6	35.9	PC,1-acyl,18:0–20:2
858.7	52.4	PC,1-acyl,18:2–20:0
860.7	49.0	PC,1-acyl,18:0–20:1
860.9	48.2	PC,1-acyl,14:0–22:1
862.6	17.9	PC,1-alk,18:1–22:6
864.8	18.4	PC,1-alk,18:0–22:6
868.7	28.5	PC,1-alk,20:0–20:4
870.4	13.3	PC,1-alk,22:3–18:0
874.6	12.4	PC,1-acyl,18:2–22:6
876.7	18.6	PC,1-acyl,18:0–22:7
876.7	14.7	PC,1-acyl,18:1–22:6
878.7	16.2	PC,1-acyl,20:2–20:6
878.7	21.2	PC,1-acyl,18:0–22:6
878.8	18.8	PC,1-acyl,16:0–24:6
880.7	26.4	PC,1-acyl,18:0–22:5
882.8	30.0	PC,1-acyl,18:0–22:4
892.6	26.9	PC,1-alk,20:0–22:6
898.6	11.9	PC,1-acyl,20:4–22:6
902.8	15.4	PC,1-acyl,20:2–22:6
904.7	19.7	PC,1-acyl,20:1–22:6
906.7	33.6	PC,1-acyl,20:0–22:6
922.6	10.9	PC,1-acyl,22:6–22:6
924.6	12.0	PC,1-acyl,22:5–22:6

low amounts of focused class of phospholipids at the level of exact fatty acyl pairs. This method is applicable without preliminary separation of different class of phospholipids. Major molecular species can be analyzed without LC separation, but in this case precursor ions sometimes containing other class of phospholipids with close molecular related mass values (*m/z*), thus obtained fragments were contaminated and difficult to identify exact molecular species. Thus, we combined RPLC–MS/MS and neutral loss scanning for exact identification of minor molecular species. By the separation with RPLC, phospholipids can be separated very effectively at the level of molecular species even in the same *m/z* values (Fig. 6). In this system first separation was obtained in retention time of LC between 10 and 40 min separation ten or more molecular species might be eluted in the same retention time. Then by second separation, selected mass corresponding *m/z* peaks detected by neutral loss scanning of 60 Da ($HCOO + CH_3$) as choline-containing phospholipids are obtained. Then third separation is individual *m/z* values obtained as mass chromatogram of neutral loss of 60 Da. And final identification was obtained as fragment ions in MS/MS spectra from selected individual *m/z* values obtained by data dependent scanning (Fig. 7).

As indicated in Table 4, di-polyunsatulated molecular species such as 22:6–22:6 PC can be also identified. This molecular species were very minor species in liver, but is reported to exist densely in mammalian retina.

This method is useful for further confirmation of unexpected minor molecules, such as oxidized or other kinds of derivatives suspected to be included in focused classes.

Quantitation of the amounts of different pairs of fatty acyl chains with same *m/z* value and the same polar head group can be obtained from the peak area of mass chromatogram of neutral loss scanning (Fig. 6).

In this paper precursor ion scanning or neutral loss scanning are revealed to be useful techniques which can be even

applicable as the combination with single LC–MS system. We think above mentioned method will be useful as an other option for multidimensional LC–MS/MS system.

4. Conclusion

Method for focused lipidomics by mass spectrometry was examined. Precursor ion scanning and neutral loss scanning of polar head group of phospholipids were very useful for detecting most of all molecular species within the same class of phospholipids. And these data were useful for profiling of these lipids in biological samples. Precursor ion scanning of carbonic anions was also very useful to identify molecular species of phospholipids having specified fatty acyl chains.

With automatic programmed analysis system for these scanning modes and with automatic identification tool of Lipid Search, more than 300 molecular species of phospholipids were identified within 2 h after extraction of total phospholipids.

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