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Nanoflow liquid chromatography–tandem mass spectrometry for the characterization of intact phosphatidylcholines from soybean, bovine brain, and liver

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Abstract

Nanoflow liquid chromatography–electrospray ionization tandem–mass spectrometry (nanoLC–ESI–MS–MS) was applied for the characterization of intact phosphatidylcholine (PC) lipid molecules using a homemade reversed phase capillary column with a pulled tip for direct ESI at positive ion mode. Prior to the analytical column, a short capillary trapping column was utilized for on-line pre-concentration via microcross connection. Separation of intact phosphatidylcholines in the nanoflow LC column was carried out using a binary gradient elution method at 300 nL/min. The structures of the eluted PC components were determined by analysis of the typical fragment ions of PC molecules obtained from collision-induced dissociation (CID) after each precursor scan in mass spectrometry. In the current study, nanoflow LC–ESI–MS–MS analysis of PC molecules demonstrated the ability to obtain clear structural information, such as alkyl chain lengths and the degree of unsaturation with a protonated molecule ($[M + H]^+$) and its characteristic fragment ions ($[M + H{-}RCH_2COOH]^+$, $[M + H{-}RCH{=}C{=}O]^+$, and $[M + H\text{-}184]^+$). Results from the nanoflow LC–ESI–MS experiment showed the limit of detection at 3.5 fmol for the 14:0/14:0-PC standard. This technique then was applied to intact PC extracts from soybean, bovine brain, and liver without derivatization and resulted in the identification of 28, 25, and 39 phosphatidylcholines, respectively. The LC–MS–MS method has been shown to be useful for the analysis of low concentration PC molecules in biological samples.

Keywords: Nanoflow liquid chromatography; Phosphatidylcholine; Separation of intact phosphatidylcholines; Electrospray ionization; Mass spectrometry; Tandem mass spectrometry; Soybean PC; Bovine brain PC

1. Introduction

Phospholipids are major constituents of cell membranes and are found among tissues and subcellular compartments as mixtures of various molecular species depending on the type of polar head groups and the degree of unsaturation of the acyl chains [1–4]. Due to the diversity in molecular structure, the compositional variation of phospholipids in cell membranes affects their fusion with other cell membrane compartments, as well as cell growth and death [5,6]. In particular, it has been reported that phospholipid composition is closely related to various diseases such as diabetes and inflammation [7,8]. Recently, lipidomics, the study of the changes in lipid metabolism and their signaling processes, is growing in relation to the understanding of the function of proteins in proteomics research. In lipidomic studies, it is important to understand the changes in phospholipid composition and their relative abundance in various biological samples.

Analysis of phospholipids has been performed using chromatographic separation methods coupled with mass spectrometry: high performance liquid chromatography (HPLC) [9–11], gas chromatography–mass spectrometry (GC–MS) [12–14], and LC–MS [15–17]. While GC–MS has been used widely for the analysis of phospholipids due to its capability of providing excellent resolution and quantitation, it requires the derivatization of lipid molecules into volatile components before separation [12,18]. However, the derivatization of lipid molecules may result in the loss of analyte molecules during each processing step. This is an important consideration when analyzing

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phospholipids in small amounts of cells and tissues. For these reasons, analysis of intact phospholipids using LC methods has attracted more interest since the advent of electrospray ionization (ESI) in MS [17,19,20]. Since ESI offers improved sensitivity, low spectral background signals, and good compatibility with LC, the LC–ESI–MS technique is being utilized widely [4,21–24]. In addition, tandem mass spectrometric analysis using collision-induced dissociation (CID) of lipid molecules has provided a useful means to characterize molecular structure by examining fragment ions with [25] or without HPLC separation [20,26–30]. While each method (LC–MS or GC–MS or direct ESI–MS–MS) is useful for analysis of a specific phospholipid when the target is limited, it is difficult to obtain information on compositional changes of phospholipids in biological samples simultaneously. Since phospholipids are so diverse in structure, high resolution and high sensitivity chromatographic separation of intact phospholipids with high through-put must be assured prior to the MS analysis. This is particularly important for the simultaneous profiling of complex mixtures of phospholipids in small amount of cells and tissues. However, performance of HPLC separation of intact phospholipids is still behind the level that is sufficient to carry out the simultaneous profiling of lipid mixtures.

In this study, a nanoflow LC–ESI–MS–MS technique was applied for the separation of intact phosphatidylcholine (PC) species at a very low concentration level with tandem mass spectrometric analysis (or CID) to achieve the simultaneous identification of the molecular structure of PCs. Separation was carried out using a homemade capillary column (C_{18} reversed phase) with a pulled tip for direct ESI at positive ion mode, and a short capillary trapping column was utilized prior to an analytical column for on-line sample clean-up and for full automation. This technique allowed us to enlarge the identified number of intact PCs from soybean, bovine brain, and liver.

2. Materials and methods

2.1. Phosphatidylcholine standards:

1,2-Dilauroyl-phosphatidylcholine (12:0-12:0 PC), 1,2-dimyristoyl-PC (14:0-14:0, PC), 1-oleoyl-2-palmitoyl-PC (18:1-16:0 PC), 1-stearoyl-2-oleoyl-PC (18:0-18-1 PC), 1,2-distearoyl-PC (18:0-18:0 PC), 1,2-diarachidoyl-PC (20:0-20:0 PC) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Phosphatidylcholine mixtures from soybean, bovine brain and liver also were obtained from Sigma–Aldrich. All samples were prepared initially in 30/60/10 $CH_3OH/CHCl_3/CH_3CN$ containing 0.1% formic acid (FA); and for further dilution 90/10 CH_3OH/CH_3CN containing 0.1% FA was used. All solvents used were HPLC grade.

2.2. Nanoflow LC/electrospray ionization tandem mass spectrometry (nanoflow LC–ESI–MS–MS)

The schematics of an automatic nanoflow LC system with on-line sample trapping column is illustrated in Fig. 1. The analytical column (150 mm × 75 μm) and trapping column (5 mm × 100 μm) were prepared in-house. The tip at the end of capillary tubing (75 μm I.D., 360 μm O.D.) from Polymicro Technology (Phoenix, AZ, USA) was pulled by flame resulting in a tip diameter of approximately 10 μm and about 1 mm in length of the tip was filled with a sol–gel frit as described previously [31]. The pulled tip capillary with an end frit was packed with methanol slurry of 5 μm 100 Å Magic C_{18} from Michrom BioResources (Auburn, CA, USA) at a constant pressure (1000 psi) of He. The trapping column was made with silica tubing (100 μm I.D., 360 μm O.D.) in which the end frit (2 mm in length) was prepared in the same way and was packed initially with 5 μm 200 Å Magic C_{18} for 5 mm. The trapping column and the analytical column were connected via a PEEK microcross as shown in Fig. 1 and a platinum wire was used as an electrode

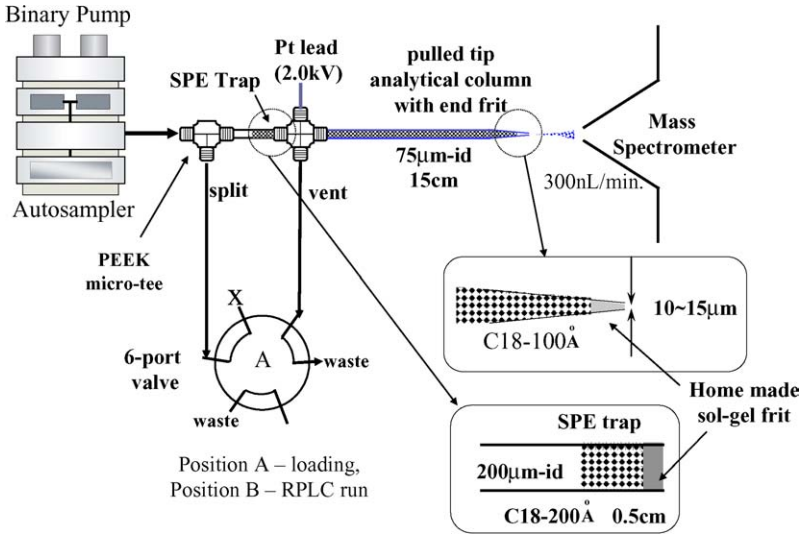

Fig. 1. System configuration of nanoflow LC–ESI–MS–MS using the homemade capillary column and the trapping column. The enlarged pictures show the tip of columns filled with frit implemented by sol–gel process.

to supply an electrospray ionization voltage as described in the literature [31–33].

Nanoflow LC separation was carried out using the model 1100 HPLC system from Agilent Technologies (Palo Alto, CA, USA). A binary gradient elution for RPLC was utilized for the separation of phosphatidylcholine samples with the following mobile phases: A: 50/30/20 CH_3CN/H_2O/isopropanol (IPA), B: 90/10 IPA/CH_3CN. Both mobile phase solutions contained 0.1% formic acid. Sample was loaded from an autosampler at a flow rate of 4 μL/min with a six-way valve configuration of A shown in Fig. 1 using mobile phase A. Injection volume was set as less than 1 μL for all runs. During the sample loading, the end of the vent tubing was open so that sample could be delivered to the trapping column at a relatively high speed. After loading, the valve configuration was changed to 60° so that the split tubing end was open with the vent tubing closed, and then RPLC separation began with a gradient elution. During separation mode, a flow rate of 7 μL/min was delivered from the binary pump, but the final flow rate at the analytical column was adjusted to be 300 nL/min by controlling the length of the split tubing (capillary I.D. 25 μm). After finishing each gradient elution, the column was re-equilibrated for at least 20 min with solvent A.

For mass spectrometric analysis, an LCQ Deca ion trap mass spectrometer from Thermo Finnigan (San Jose, CA, USA) was utilized. Electrospray ionization was carried out at a potential of 2.0 kV applied via Pt wire and at a capillary temperature of 180 °C. Phosphatidylcholine ions were detected in a data-dependent analysis mode. The acquisition method involved one MS precursor scan from 600 to 900 u followed by data-dependent MS–MS scans (40% normalized collision energy) throughout the study. The mass tolerance between the measured mono-isotopic mass and the calculated mass was 1.0 Da for the molar mass of a precursor and 1.0 Da for the mass of fragment ions. The acquired MS–MS spectra were analyzed manually.

3. Results and discussion

3.1. Nanoflow LC–MS–MS of phosphatidylcholine standards

Prior to the nanoflow LC–MS–MS analysis of phosphatidylcholine standards, the tandem mass spectrum of each standard molecule was obtained with direct infusion and the collected MS–MS spectra were utilized for the recognition of fragmentation pattern of PC molecules. Fig. 2 shows the base peak chromatogram (BPC) of the separation of six PC standards (12:0/12:0-PC, 14:0/14:0-PC, 18:1/16:0-PC, 18:0/18:1-PC, 18:0/18:0-PC, and 20:0/20:0-PC in an order of increasing

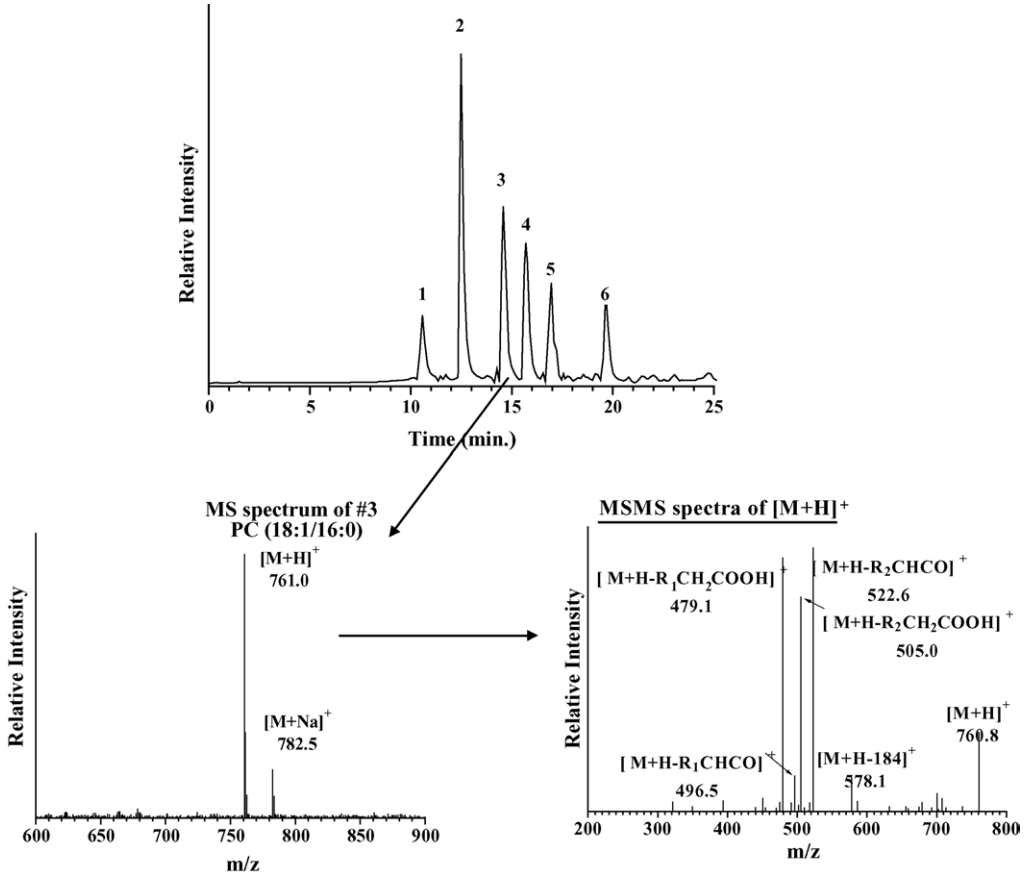

Fig. 2. Base peak chromatogram (BPC) of intact phosphatidylcholine standards (top) (No. 1: 12:0/12:0-PC, No. 2: 14:0/14:0-PC, No. 3: 18:1/16:0-PC, No. 4: 18:0/18:1-PC, No. 5: 18:0/18:0-PC, and No. 6: 20:0/20:0-PC) by nanoflow LC–ESI–MS spectra of the precursor ion of the peak No. 3 (1-oleoyl-2-palmitoyl-PC) (the lower left), and the resulting MS–MS spectra of the protonated 1-oleoyl-2-palmitoyl-PC. Mobile phase compositions for binary gradient elution were 50/30/20 CH_3CN/H_2O/IPA for A and 90/10 IPA/CH_3CN for B (Both solvents contained 0.1% formic acid). For gradient elution conditions, see Section 3.1.

peak number) using the homemade nanoflow LC column described in this study. The binary gradient RPLC separation was carried out by varying the mobile phase composition of (A) 50/30/20 CH_3CN/H_2O/IPA and (B) 90/10 IPA/CH_3CN. Both mobile phases contained 0.1% (v/v) formic acid. After finishing sample loading in solvent A, the gradient was changed to 80% B for 30 s and was maintained for 10 min. The gradient then was increased to 90% B for 5 min and maintained until the end of the run. After finishing a run, the gradient was decreased to 0% B for 1 min and maintained for at least 20 min for column re-conditioning. A similar separation could be achieved using isocratic elution with 80% mobile phase B but in this case, the last PC molecule (20:0/20:0-PC) was not resolved well (data not shown). The flow rate during the gradient separation was kept at 300 nL/min and the eluted PCs were analyzed by ESI–MS–MS with the precursor scan and data-dependent MS–MS scan in sequence. Each precursor scan showed a clear protonated molecular peak along with a sodium adduct molecular ion peak, simultaneously. In case of the peak number 3 (1-oleoyl-2-palmitoyl-PC (18:1-16:0 PC)), $[M + H]^+$ along with $[M + Na]^+$ peaks were shown exclusively at *m/z* 761.0 and 782.5, respectively. The MS–MS spectrum of the selectedmolecule (*m/z*: 761.0) (lower right side of Fig. 1) contained fragment ions at *m/z* 496.5 and 522.5, corresponding to the neutral loss of the fatty acid group as a ketene at *sn*-1 ($[M + H–R_1CH=C=O]^+$) and *sn*-2 ($[M + H–R_2CH=C=O]^+$), respectively. The latter ion was more abundant than the former, which supports the premise that during the fragmentation process, the loss of ketene at *sn*-2 is sterically more favorable than that at *sn*-1. This observation is similar to the proposed mechanism for fragmentation of phosphatidylethanolamine [27] and of PCs [28]. The ions at *m/z* 479.1 and 505.0 are the result of the loss of the fatty acid moiety at *sn*-1 ($[M + H–R_1CH_2COOH]^+$) and *sn*-2 ($[M + H–R_2CH_2COOH]^+$), respectively. The ion *m/z* 578.1 ($[M + H\text{-}184]^+$) is thought to be derived from the loss of the polar head group $[C_5H_{13}NPO_4]^+$ (*m/z* 184) under positive ionization. This result supports the premise that the molecule contains a choline head group [21]. Another diagnostic for the presence of a choline head group is known as an “ion of neutral loss of trimethylamine”. When the same molecule was examined by direct infusion to ESI–MS–MS without LC separation, neutral loss of trimethylamine from the sodium adduct of the molecular ion yielding an ion ($[M + Na–N(CH_3)_3]^+$) could be found easily as an intense peak. However, neutral loss of trimethylamine from molecules, $[M + H\text{-}59]^+$ ions, in the LC–MS–MS run were negligible in intensity or were not observed. In Fig. 2, a peak at *m/z* 702.6 may represent $[M + H\text{-}59]^+$, but these types of ions were not clearly found in other LC–MS–MS spectra of components 1–6 (except 3). This could be due to the dilution of alkaline metal ions in the sample solution by mobile phase solvents during LC separation.

3.2. Limit of detection (LOD) in nanoflow LC–MS

In order to estimate LOD for the separation and characterization of intact phosphatidylcholine lipids using the current nanoflow LC–ESI–MS technique, the 14:0/14:0-PC standard was tested by decreasing its concentration and running it at the same flow rate (300 nL/min.) with an isocratic elution of 80% B mobile phase. The MS detection was made in the selected ion monitoring (SIM) mode at *m/z* 679 by lowering injection amount from 1.5 pmol to 3.5 fmol and the ion chromatograms were shown in Fig. 3. Injections were made by sequentially and repeated for three times. Based on calibration of the peak area vs. concentration ($r^2 = 0.999$), LOD was calculated as 3.5 fmol (2.4 pg) at a signal-to-noise ratio of 3. The result showed a good linear relationship between the peak area and the range of injected amounts (3.5 fmol ~ 1.5 pmol) and the RSD was less than 0.6% ($n = 3$). The LOD value in this study was lower than that (8 fmol) obtained with capillary LC–ESI–MS method [24].

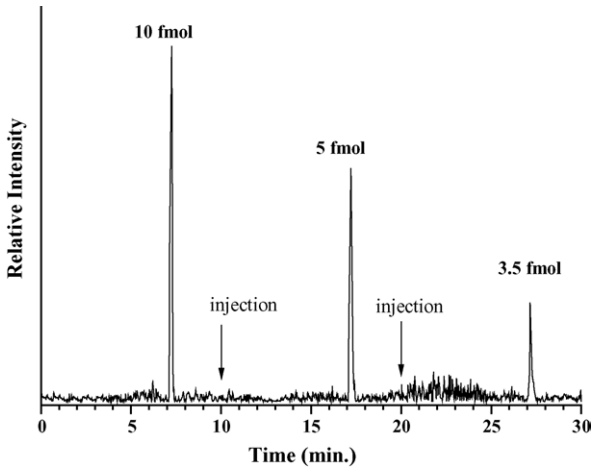

Fig. 3. Ion chromatograms of 14:0/14:0-PC standard by selected ion monitoring mode at *m/z* 679. Runs were made sequentially by decreasing injection amount at an isocratic elution of 80% B mobile phase used in Fig. 2 at a flow rate of 300 nL/min. This figure shows only three chromatograms (10, 5, and 3.5 fmol). From peak area calibration, LOD was calculated as 3.5 fmol at S/N = 3 with RSD of 0.6% ($n = 3$).

3.3. Nanoflow LC–MS–MS of natural phosphatidylcholine extracts

Fig. 4 shows the base peak chromatogram of phosphatidylcholines from the soybean sample obtained by nanoflow LC–ESI–MS at the same flow rate used in Fig. 2. The mobile phase compositions were the same as used in Fig. 2 but the binary gradient condition was modified as follows: it began with 40% B (90/10 IPA/CH_3CN with 0.1% FA) with an initial increase to 50% B for 20 min, ramped to 90% for 10 min, and then maintained until the end of the run. Experiment was carried out with precursor scan followed by each data dependent MS–MS scan. Characterization of soybean PCs was carried out in the same manner used in Fig. 2. As shown in Fig. 4, 28 PC molecules were identified from each CID spectrum with an injection of 5 ng of soybean PC mixture. The CID mass spectra of species 3, 8, 63, and 64 (peak numbers in Fig. 3) in Fig. 5 revealed that characteristic fragment ions to distinguish acyl chain structures and choline head groups were clearly shown in CID spectra even though the peak intensity of the species number 3 was very low in the chromatogram.

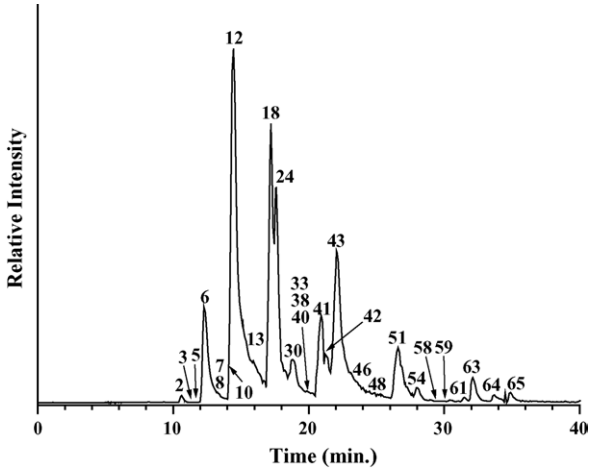

Fig. 4. Base peak chromatogram of the intact phosphatidylcholine mixtures from soybean obtained by nanoflow LC–ESI–MS. Chromatogram was extracted from precursor scan runs. Mobile phases were the same as used in Fig. 2 except for the gradient elution conditions (see Section 3.3). The numbering system for each component follows that given in Table 1.

In Fig. 5a, the ion at *m/z* 545.7 represented the fragment ion without the choline head group, $[M + H\text{-}184]^+$, from 14:0-18:3-PC molecule, and the loss of myristic acid and linolenic acid appeared with the ions at *m/z* 500.3 ($[M + H{-}R_1CH_2COOH]^+$) and 450.7 ($[M + H{-}R_2CH_2COOH]^+$), respectively. The data shown in Fig. 5a also supported the loss of fatty acid as a form of ketene at *m/z* 518.9 and 468.6 for *sn*-1 and *sn*-2, respectively.

In the CID experiments in this study, the protonated molecular peaks ($[M + H]^+$, *m/z* = 729.5) were observed at a significant intensity compared to those of the other fragment ions of all four species shown in Fig. 5. It was evident that the three characteristic fragment ions (removal of fatty acid, ketene form, and choline head) appeared clearly for the other three PC species (14:0/18:2-PC for No. 8, 22:0/18:2-PC for No. 63, and 23:0/18:2-PC for No. 64) except the CID of peak number 8 (14:0/18:2-PC in Fig. 5b) did not show the fragment ion of $[M + H\text{-}184]^+$. The other three spectra (Fig. 5b–d) showed a similar fragmentation pattern as observed in Fig. 5a. The four PC species in Fig. 5 were among the newly found 12 species in this study and the list of the total 28 soybean PCs were listed in Table 1 . Newly found species are marked with an asterisk in Table 1. In the literature [3], the same species were studied by LC–MS using atmospheric pressure chemical ionization (APCI); however, experiments were performed using the conversion of PCs to diacylglycerol nicotinate derivatives. While the derivatization reaction in that study [3] provided an improved HPLC separation, only 16 molecular species were identified. The current work demonstrated the capability of separating intact phosphatidylcholines without derivatization and the number of identified PCs using nanoLC–MS–MS technique was greater. This could be due to the ability to separate low concentration molecular species from high abundant phosphocholine components with the nanoflow LC system used in this study, a higher efficiency of electrospray ionization at nanoflow injection using a capillary pulled tip column interface, and the simultaneous identification

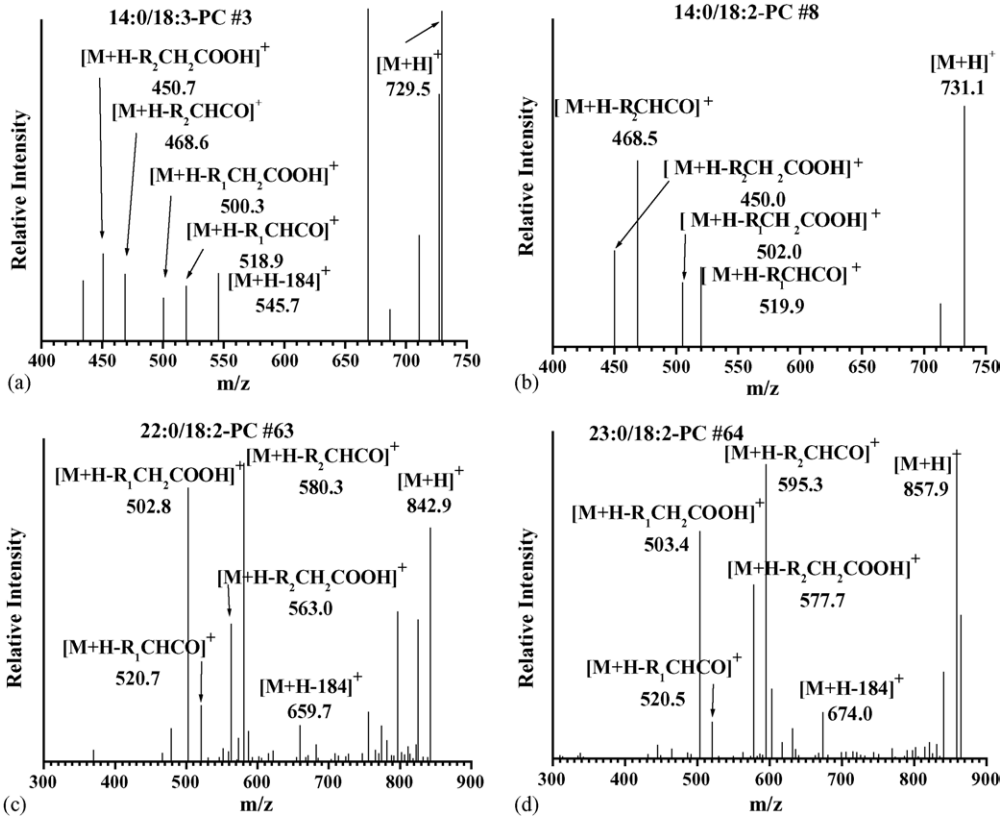

Fig. 5. Data dependent MS–MS spectra of the protonated PC species numbered with (a) 3, (b) 8, (c) 63, and (d) 64 shown in Fig. 4.

Table 1
List of identified molecular species and relative amount of phosphatidylcholines from soybean, bovine brain, and bovine liver

No.[b]	Molecular species	Relative amount (%)		
		Soybean	Bovine brain	Bovine liver
1	14:0/14:0		0.06	
2	18:3/18:3	0.27		
3	14:0/18:3	0.06[a]		
4	14:0/16:1		0.05	
5	16:1/18:3	0.05[a]		
6	18:2/18:3	6.44		
7	15:1/18:2	0.06[a]		
8	14:0/18:2	0.11[a]		
9	16:0/20:5			0.67
10	16:1/18:2	0.30		
11	16:0/18:3			0.93
12	18:2/18:2	29.01	0.52	
13	18:1/18:3	0.68		
14	16:0/22:6		0.73	0.61
15	14:0/16:0		3.82	
16	14:0/18:1		3.30	
17	16:0/20:4		1.16	0.39
18	16:0/18:2	13.55	1.06	
19	17:0/20:5			0.06[a]
20	16:0/22:5			2.71
21	16:0/16:1		0.24	0.33
22	16:1/18:1			0.83
23	16:0/18:2			4.47
24	18:1/18:2	12.78	0.41	0.19
25	18:0/20:5			2.82
26	16:0/20:3			6.17
27	15:0/18:1			0.34
28	18:1/20:3			0.91
29	17:0/22:5			0.33
30	18:0/18:3	4.63		0.36
31	16:0/17:1		1.44	
32	17:1/18:1		0.20	
33	19:1/18:2	0.12[a]		
34	16:0/22:4		0.44	2.44
35	18:0/22:6		0.49	1.43
36	18:1/22:5			0.11
37	18:0/20:4			4.80
38	17:0/18:2	0.17		0.32
39	18:0/22:5			5.43
40	16:0/16:0	0.13	3.05	0.28
41	16:0/18:1	4.18	42.05	8.03
42	18:1/18:1	3.08	2.79	4.29
43	18:0/18:2	14.61		9.59
44	17:0/20:3			0.12
45	18:0/20:3			9.06
46	17:0/18:1	0.05[a]	1.52	1.08
47	18:0/22:4			5.49
48	19:0/18:2	0.06[a]		0.23
49	18:0/21:3			21.59
50	16:0/18:0		2.00	0.20
51	18:0/18:1	5.03	29.42	
52	17:0/18:0			0.22
53	18:0/20:2		0.72	0.43
54	20:0/18:2	1.19		
55	18:0/19:1			1.24[a]
56	19:0/18:1		0.67	
57	18:0/22:3			0.23
58	22:0/18:3	0.14[a]		
59	18:0/18:0	0.09[a]	0.43	0.29[a]
60	18:0/20:1		3.09	1.00[a]
61	20:0/18:1	0.37[a]		
62	18:1/22:1		0.33	
63	22:0/18:2	1.74[a]		
64	23:0/18:2	0.49[a]		
65	24:0/18:2	0.61[a]		

Relative amount was based on the area of the extracted ion chromatogram of each molecular species from precursor scans of the nanoLC–ESI–MS run.
[a] Newly found in this work compared to reference [3].
[b] Numbers are applied through Figs. 4 and 6.

by tandem mass spectra. Improvement in LC separation can be due partly to the incorporation of a gradient elution method. However, the LC peaks observed in Fig. 4 appeared to be broader than what was observed with the separation of derivatized PCs. Peak broadening can originate from the elution of positional isomers having the same molecular weight but with different locations of double bonds at the alkyl chains of PC in the biological samples. The chromatogram of soybean PCs in Fig. 4 showed tailing phenomena for most peaks. These were attributed from the use of pre-concentration trap. When sample was loaded directly to the analytical column without the pre-concentration trap attached in Fig. 1, the tailing disappeared (chromatogram not shown). In this case, it took a long time for sample loading. Sample was delivered at 1.5 μL/min with the valve position A (to bypass analytical column) for 10 min, flow rate was decreased to 300 nL/min with the change of valve position to B, and then maintained for 20 min. Compared to the method using on-line pre-concentration trap, the number of identified PCs from on-column injection was not greatly improved. From this reason, on-line pre-concentration method was utilized for the analysis of brain PC samples.

From a nanoflow LC–MS run, each identified precursor ion ($[M + H]^+$) was extracted into a selected ion chromatogram and the peak area of each species was calculated for comparison with other species. Recently, it was reported that the difference in ionization efficiency of PC molecular species at positive ion mode was nearly the same (about 10% in relative error) while at negative ion mode the difference was significant depending on the length of the acyl chains [4]. Based on this result, the relative peak area of each species was calculated as an indication of relative abundance (Table 1). It was noteworthy that 7 out of 12 newly found species for soybean PCs were mostly the low concentration (around or less than 0.1%) PC species (14:0/18:3, 14:0/18:2, 15:1/18:2, 19:1/18:2, 17:0/18:1, and 18:0/18:0). The largest difference in the relative abundance among soybean PC species was about 600-fold based on the peak area of precursor ions.

Fig. 6 shows the two BPCs obtained with phosphatidylcholine mixtures from (a) bovine brain and (b) bovine liver samples. Gradient elution conditions for the two runs were adjusted separately to enhance the separation of the complex PC molecules contained in each sample. The compositions of mobile phases A (50/30/20 CH_3CN/H_2O/IPA with 0.1% FA) and B (90/10 IPA/CH_3CN with 0.1% FA) used in Fig. 6a were

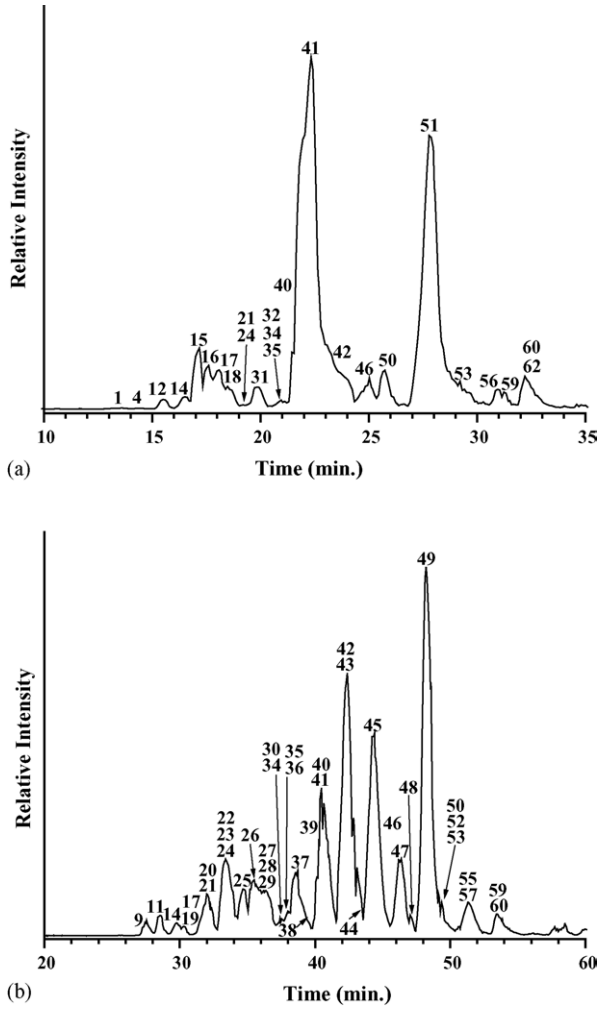

Fig. 6. Base peak chromatograms of the intact PC mixtures from a) bovine brain and b) bovine liver obtained by nanoflow LC–ESI–MS–MS. Mobile phases were 50/30/20 CH_3CN/H_2O/IPA with 0.1% FA for (a) and 90/10 IPA/CH_3CN with 0.1% FA for (b). For gradient elution conditions, see Section 3.3. The numbering follows the system given in Table 1.

the same as used in Fig. 4. The binary gradient condition of the run in Fig. 6a began with 40% of mobile phase B, which was increased to 50% B for 20 min, to 75% B for 10 min and then was maintained at 75% B until the end of the run. The run condition for Fig. 6b was modified from that of Fig. 6a in order to resolve the more complicated bovine liver PC mixtures. Mobile phase A in Fig. 6b was 70/30 CH_3CN/H_2O with 0.1% FA while mobile phase B was not altered from that of Fig. 6a. The gradient began with 0% mobile phase B, which was increased to 50% B for 10min. and then was maintained at 50% for 20 min. The mobile phase then was increased to 70% B for 15 min, 80% B for 10 min, and maintained at 80%. The injection amount varied from 2 to 30 ng of the brain sample and it was found that a large amount of sample injection resulted in peak broadening leading to overlapping of nearby eluting components. The latter was believed to cause suppression of the ionization of low abundant species that elute together with high abundant PC molecules, resulting in the failure of identification of structure. In the case of the bovine brain PC sample, a nanoLC–MS–MS experiment resulted with an identification of 25 PC species from 4 ng mixture as an optimum amount of injection. The identified molecular species of PCs are listed in Table 1. For the bovine liver sample in Fig. 6b, 39 PC species were identified. However, the injection amount of bovine liver sample was optimized at 30 ng. Since PC species contained in the bovine liver sample appeared to be more diverse in their chain lengths and the degree of unsaturation than those of the bovine brain sample, the gradient condition was adjusted to separate them over a longer time span (about 35 min. between the minimum and maximum retention times) whereas the other samples (soybean and brain samples) were made at 20–25 min. Comparison of the composition of the liver PC sample with those of the brain sample in Table 1 revealed that only 12 PCs out of 39 liver PC species appeared to overlap with those of the brain sample. In addition, the bovine liver PC mixtures contained more phosphatidylcholines with diverse alkyl chains as well as more double bonds than those of the brain sample.

4. Conclusion

In this study, a nanoflow LC–MS–MS method has been powerfully applied for the separation of intact phosphatidylcholines and the simultaneous characterization of molecular structure by confirming CID fragment ions. The current technique has demonstrated the LOD of 3.5 fmol for a standard PC molecule with the use of a homemade pulled tip capillary column at a nanoflow rate. Experiments using the PC extracts from biological samples (soybean, bovine brain, and bovine liver) were focused to optimize gradient elution conditions for the efficient separation of low concentration intact PC molecules for improved identification in ESI–MS–MS analysis. In the case of soybean PC mixtures, 28 PC molecules were identified, which was a much larger number than the reported number identified using derivatized PC molecules of the same species. Studies currently are on-going toward the characterization of other types of phospholipids and eventually for shotgun analysis of phospholipids from biological samples. The current study itself can be applicable to characterize the compositional differences between phosphatidylcholine species from biological fluids or tissues related to certain diseases such as breast cancer. However, a suitable database containing MS–MS information on phospholipids needs to be established in order to search PC molecules by computer processing.

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