

## Separation and quantification of *sn*-1 and *sn*-2 fatty acid positional isomers in phosphatidylcholine by RPLC-ESIMS/MS

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**Endogenous phosphatidylcholine in biological membranes exists as isomers with acyl moieties at the *sn*-1 or *sn*-2 positions of the glycerol backbone. However, detailed biochemical information on these positional isomers is not generally available. This study is the first report on the separation and identification of positional isomers of endogenous phosphatidylcholine using reversed-phase LC-ESIMS/MS. The separation of positional isomers in PC was achieved by using ultra performance LC, which uses a high-resolution HPLC system. To identify positional isomers in individual PC species, their lyso-PC-related fragments and fatty acids, which were obtained by MS/MS analysis in the negative ion mode, were used. From the application results of biological samples, the lipid extracts of mouse brain were found to be abundant in PC containing 22:6 at the *sn*-1 position of the glycerol backbone. However, the lipid extracts from mouse heart and liver were not abundant in positional isomers. This achievement demonstrates that the relative amounts of positional isomers in various tissues or molecular species differ. These results will be useful for the clarification of the biological mechanisms of remodelling enzymes such as phospholipase and acyltransferase. Thus, our report provides a novel and critical milestone in understanding how molecular composition of phospholipids is established and their biological roles.**

**Keywords:** Phosphatidylcholine/positional isomer/liquid chromatography/mass spectrometry.

**Abbreviations:** PC, phosphatidylcholine; SAFA, saturated fatty acid; MUFA, monounsaturated FA; PUFA, polyunsaturated FA; RPLC, reversed phase liquid chromatography; ESI, electrospray ionization; MS, mass spectrometry.

Phosphatidylcholines (PCs) are important not only as structural and functional components of cellular

membranes, but also as precursors of various lipid mediators (1, 2). In mammalian cells, PCs are synthesized through the Cytidine di-phosphate (CDP)-choline pathway or through methylation of phosphatidylethanolamine (PE) (3). Saturated (SA) and monounsaturated fatty acids (MUFAs) are generally linked at the *sn*-1 position and polyunsaturated fatty acids (PUFAs) tend to be located at the *sn*-2 position of the glycerol backbone. However, the fatty acid (FA) combination at the *sn*-1/2 positions can be different in tissues, cells, organelles or phospholipid (PL) classes. For example, pulmonary surfactant (4) has PC of 16:0/16:0 (SAFA/SAFA) at the *sn*-1/2 positions and the *sn*-1/2 acyl moieties of retina tissue are abundant in 22:6/22:6 (PUFA/PUFA) PC, PE and phosphatidylserine (PS). Furthermore, endogenous PLs in biological membranes exist also as *sn*-1 or *sn*-2 positional isomers (5–7). The FA residues of PL are rapidly turned over, a process regulated by several lipid-metabolizing enzymes such as phospholipase A<sub>s</sub> (PLA<sub>s</sub>) (8–10) and acyltransferases (4, 11–14). However, biochemical information at this level of detail is rarely reported. Thus, it is critical to learn the pattern and abundance of FA linkages at the *sn*-1 or *sn*-2 positions of PLs in order to understand their biological roles.

The FA content of PLs has been determined by following the conventional method. First, the *sn*-1 or *sn*-2 ester bond of the PLs was hydrolysed using lipase or PLA<sub>2</sub> and the FAs were purified and separated by thin layer chromatography (TLC). These FAs were analysed by gas chromatography (GC) or high performance LC (HPLC) using UV detection. However, these methods are not suitable for determining the FA combination at the *sn*-1/2 positions. Furthermore, it is difficult to calculate the accurate abundances of FAs, because they are hydrolysed by non-specific enzymes *in vitro*.

Recently, it has become possible to analyse molecular species in PL classes using liquid chromatography electrospray ionization mass spectrometry (LC-ESIMS). In the study of lipidomics, LC-ESIMS is an effective technique (15–19). We previously described normal phase (NP) and reversed phase (RP) LC-ESIMS analytical methods for PLs (20–22). Precursor ion scanning or neutral loss scanning methods are also very useful, as reported earlier (23, 24). Use of data based on detection of specific fragment ions or of neutral losses, such as fatty acyl moieties and the phosphoryl base obtained from glycerophospholipids, can be very effective in identifying individual molecular species (25–29). For example, a specific fragment at *m/z* 184 (phosphoryl choline) is

well-known and has been used as a specific target in searching for choline-containing PLs such as PC, lyso-PC (LPC) and sphingomyelin (SM) species using product ion and precursor ion scanning in the positive ion mode (17, 24, 26–28). Furthermore, by using ammonium formate as an elution buffer, both PCs and SMs were detected as  $[M + \text{HCOO}]^-$  ions in the negative ion mode. These formate adduct ions were easily dissociated and detectable as  $[M - \text{CH}_3]^-$  ions by MS/MS (30, 31). Indeed, PCs and SMs were detected by scanning for a neutral loss of 60 Da ( $\text{HCO}_2 + \text{CH}_3$ ). In addition, identification of the FA combination in the *sn*-1/2 positions of individual molecular PC species was speculated by their LPC- (formed from di-acyl PCs by loss of one acyl chain as a ketene or as a free FA) related fragments, which were obtained by MS/MS analysis in negative ion mode. In brief, the loss of ketene from the *sn*-2 substituent in  $[M - \text{CH}_3]^-$  ions was preferentially observed, and relative amounts of such positional isomers of PCs could be identified by MS/MS fragmentation (5, 6). However, published methods could not determine the abundance of positional isomers in endogenous PCs because they eluted at the same retention time. Thus, improvement of the separation of positional isomers by LC is necessary for accurate identification and quantification of the FA composition of individual PL molecular species.

In this study, we present a method for quantification and identification of FA *sn*-1 and *sn*-2 positional isomers in individual molecular species of PC using RPLC-ESIMS/MS. The analyses of molecular species of PC are performed by neutral loss scanning on a quadrupole linear ion trap mass spectrometer and the separation system was an ultra performance LC (UPLC) with a C18 (1.7  $\mu\text{m}$ , 150 mm  $\times$  1.0 mm) column. Highly selective and sensitive analysis can be achieved by using this separation system for positional isomers of PLs. Our study is the first report on the separation and identification of positional isomers of endogenous PC using RPLC-ESIMS/MS.

## Experimental procedures

### Materials

1,2-diacyl-*sn*-glycero-3-phosphocholine (PC) standards such as 16:0/18:2 PC (PLPC, 1-palmitoyl, 2-linoleoyl PC), 16:0/20:4 PC (PAPC, 1-palmitoyl, 2-arachidonoyl PC) and 16:0/22:6 PC (PDPC, 1-palmitoyl, 2-docosahexaenoyl PC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Soybean 15-lipoxygenase, sodium borohydride and deoxycholic acid were obtained from Sigma-Aldrich (St Louis, USA). All solvents were HPLC or LC-MS grade and the other chemical reagents were analytical grade. Solvents and other chemical agents were obtained from Wako Pure Chemicals (Osaka, Japan). Ultra pure water was obtained from a Milli-Q water system (Millipore, Milford, USA).

### Sample preparation for LC-MS

The brain, liver and heart of 8-week-old male C57BL/6J mice were homogenized with 7.5 ml chloroform/methanol (1/2 v/v) using a glass homogenizer, and the total lipids were extracted using the Bligh–Dyer method (32). The total lipid extract was dried under a gentle stream of nitrogen gas and was re-dissolved in methanol. The fractionated sample was stored at  $-30^\circ\text{C}$  when not in use.

### LC-ESIMS/MS system

The LC-ESIMS/MS analysis was performed using a 4000 Q-TRAP<sup>®</sup> quadrupole linear ion trap hybrid mass spectrometer (Applied Biosystem/MDS Sciex, Concord, ON, Canada) with an ACQUITY Ultra Performance LC<sup>®</sup> (Waters). The sample was subjected to LC-ESIMS/MS analysis by using the ACQUITY UPLC<sup>™</sup> BEH C18 (1.7  $\mu\text{m}$ , 150 mm  $\times$  1.0 mm) column. Sample (10  $\mu\text{l}$ ) was simply injected by the autosampler, and the total PL fractions were separated by a step gradient of mobile phase A (acetonitrile/water containing 15 mM ammonium formate = 90/10 v/v adjusted to pH 7.4 by the 28% aqueous ammonium) : mobile phase B (acetonitrile/methanol/isopropanol/water containing 15 mM ammonium formate = 47.5/45/2.5/5 v/v/v/v adjusted to pH 7.4 by the 28% aqueous ammonium) in ratios of 100:0 (0–10 min), 0:100 (10–20 min), 0:100 (20–60 min), 100:0 (60–61 min) and 100:0 (61–75 min) at a flow rate of 70  $\mu\text{l}/\text{min}$  and column temperature of  $30^\circ\text{C}$ .

### MS conditions

MS/MS analyses were performed in negative ion mode. The ion spray voltage was set at  $-4500\text{ V}$ . Nitrogen was used as both the curtain gas (set to 10 arbitrary units) and collision gas (set to 'high'). Neutral loss scanning to identify the PC species was performed as described earlier (28, 31). The characteristic fragmentation patterns of individual molecular PC species were determined by enhanced product ion scanning (EPI). These EPI experiments were performed as continuous, not data-dependent, measurements for obtaining mass chromatograms of characteristic product ions. The collision energy was set to 55 eV. The scan range of the instrument was set at *m/z* 200–900, at a scan speed of 4,000 Th/s. The Q0 trapping was set to 'on'. The linear ion trap fill-time was set to 10 ms. The declustering potential was set to  $-105\text{ V}$ . The resolution of Q1 was set to 'unit'.

### Preparation of oxidized PC by 15-lipoxygenase

PCs such as PLPC, PAPC and PDPC were oxidized by soybean 15-lipoxygenase. PCs were solubilized in 150  $\mu\text{l}$  of 0.2 M borate buffer (pH 9.0) containing 10 mM deoxycholate and reacted with 7.5  $\mu\text{g}$  (131,000 U/mg) soybean 15-lipoxygenase at room temperature for 30 min. The oxidation reaction was monitored by its absorbance at 234 nm. PC hydroxide was prepared by the reduction reaction of PC hydroperoxide using  $\text{NaBH}_4$  for 10 min on ice. The PC hydroxide was extracted using solid-phase extraction (SPE) (33, 34) (see below).

### Extraction of PC hydroxide

The PC hydroxide was extracted using SPE. Briefly, this procedure is described as follows. The oxidized PC fractions were diluted with 10 volumes of water adjusted to pH 3.0 with 0.1 N HCl. The samples were applied to preconditioned (20 ml of methanol and 20 ml of water) C18 Sep-Pak cartridges (500 mg; Waters, Millford, MA, USA), and washed with 20 ml of water, 10 ml of hexane and 10 ml of methyl formate to exclude non-volatile ions and detergents. The samples were eluted with 10 ml of methanol to obtain the PC hydroxides. The lipid extracts were dried under a gentle stream of nitrogen, dissolved in 1 ml of methanol and stored at  $-80^\circ\text{C}$  until use.

## Results

### Separation and identification of positional isomers of synthetic PC standards

In the first synthetic step, the substrate di-acyl PC is hydrolysed by PLA<sub>2</sub>. 1-Acyl LPL is produced by this reaction, and then a portion of 1-acyl-LPL is converted to 2-acyl-LPL as the FA at *sn*-1 gradually migrates to the *sn*-2 position. It is known that the ratio of 1-acyl LPL:2-acyl LPL is about 9:1. A condensation reaction of FA to 1-acyl LPL and 2-acyl LPL is performed in the final step, affording synthetic PC standards containing positional isomers (H. Nakanishi and R. Taguchi, unpublished data) (35, 36) (Fig. 1).

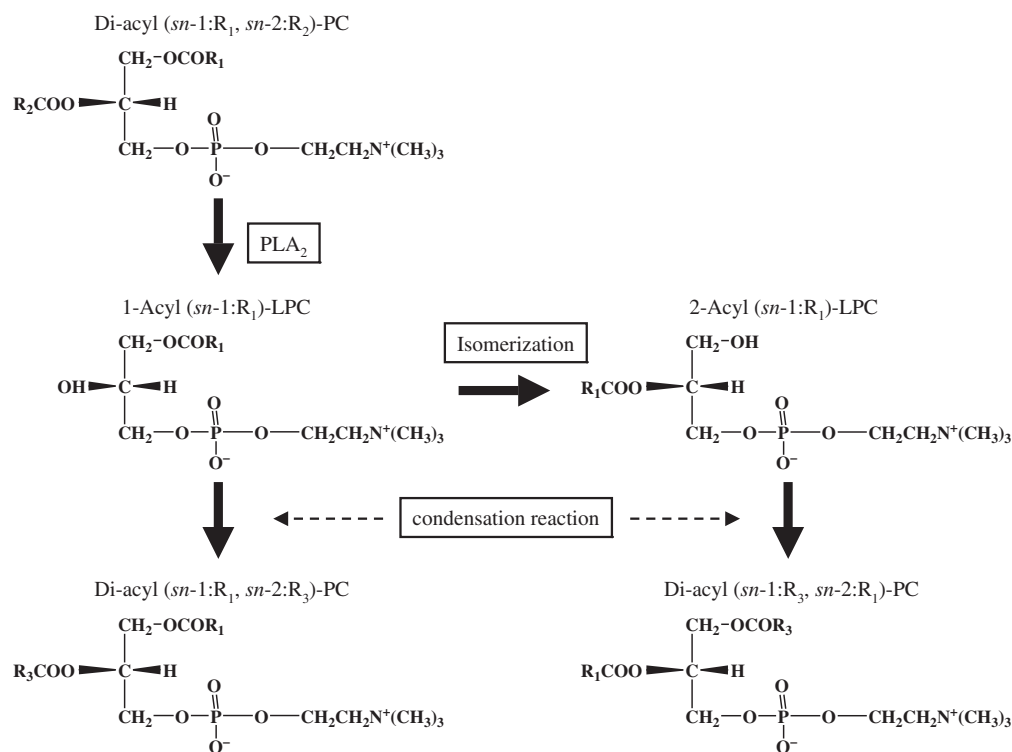


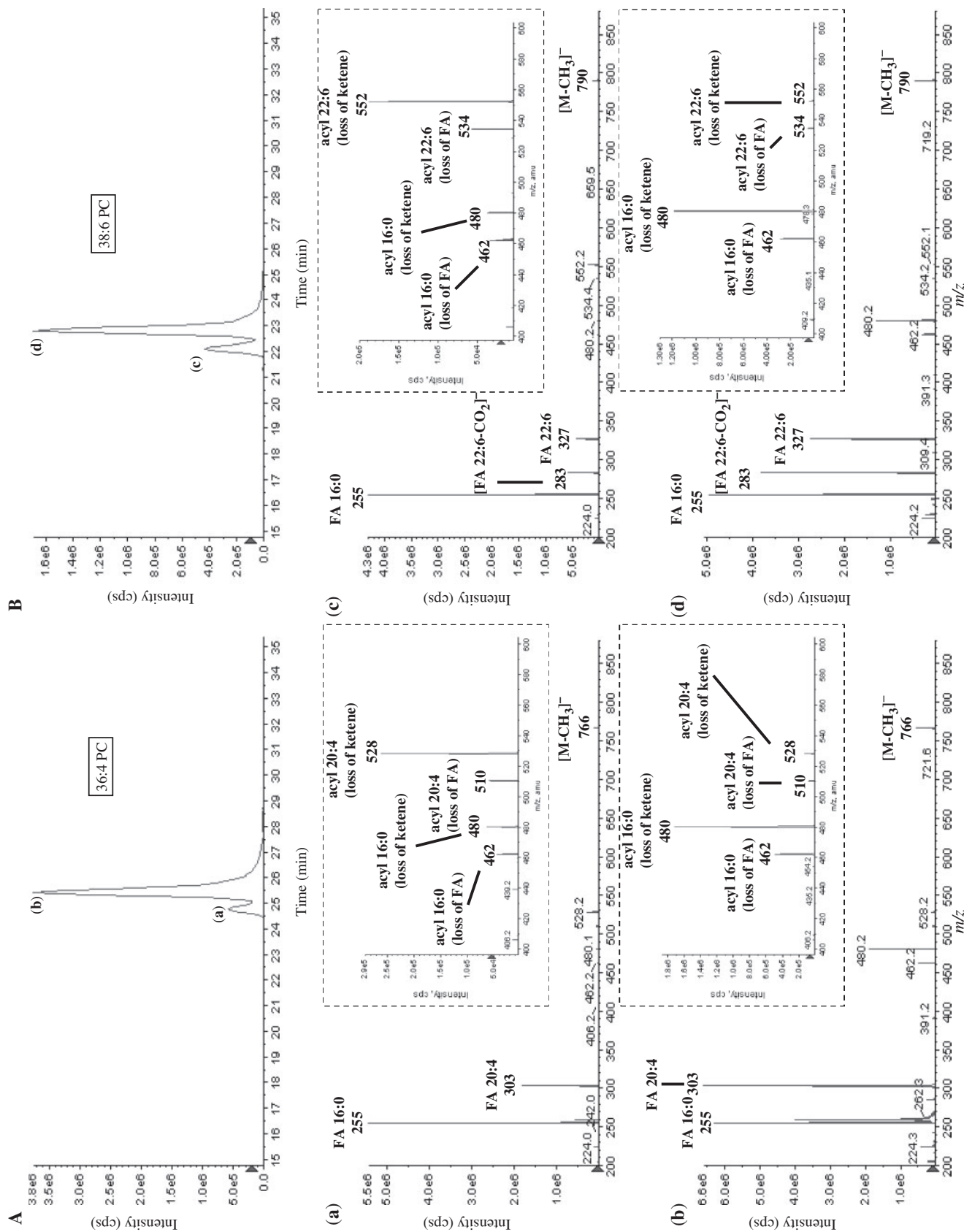
Fig. 1 The production mechanism of positional isomers in synthetic phosphatidylcholine.

To identify positional isomers of endogenous PCs, we first established a separation system using UPLC, which is simply a high-resolution HPLC system using a high-pressure pump and very small particle silica gel. Generally, the molecular species of PC are separated by RPLC using gradient analysis. However, under these conditions, positional isomers are detected as a single peak. Therefore, we used isocratic conditions for separation of the positional isomers of synthetic PC standards, such as 16:0/20:4- and 16:0/22:6-PC (Fig. 2).

Identification of PC molecules was performed using triple-stage quadrupole MS. The choline-containing PLs such as PCs and SMs were detected specifically in negative ion mode by neutral loss scanning for 60 Da, which corresponds to a loss of ( $\text{HCO}_2 + \text{CH}_3$ ) from the  $[\text{M} + \text{HCOO}]^-$  precursor ions. Under these conditions, the PC species all have even nominal (integral)  $m/z$  values, and the SM species all have odd  $m/z$  values.

When the synthetic PCs are analysed by product ion scanning of their  $[\text{M} + \text{HCOO}]^-$  ions in negative ion mode, the PCs  $[\text{M} + \text{HCOO}]^-$  yield demethylated PC  $[\text{M} - \text{CH}_3]^-$  ions that lose ( $\text{HCO}_2 + \text{CH}_3$ ) from the precursor ions. The demethylated LPC-related ions are detected as fragment ions in two different forms, as  $[\text{M} - \text{CH}_3 - \text{RCH}_2\text{COOH}]^-$  resulting from neutral losses of free FAs, and as  $[\text{M} - \text{CH}_3 - \text{RCH}=\text{C}=\text{O}]^-$  resulting from losses of ketenes. We previously reported that 1-acyl-demethylated-LPC fragment ions produced by loss of the *sn*-2 moiety are detected as a peak higher than 2-acyl-demethylated-LPC fragment ions produced by loss of the *sn*-1 moiety (31). Furthermore, in the case of the loss of the

acyl group at *sn*-2, the abundances of  $[\text{M} - \text{CH}_3 - \text{RCH}=\text{C}=\text{O}]^-$  ions were higher than those of the corresponding  $[\text{M} - \text{CH}_3 - \text{RCH}_2\text{COOH}]^-$  ions, but in the case of *sn*-1 substituents the reverse was true (Fig. 2). For example, collision-induced dissociation (CID) of the backward peak in the mass chromatogram of the  $m/z$  826 (36:4-PC)  $[\text{M} + \text{HCOO}]^-$  yielded  $m/z$  480 (acyl 16:0 demethylated LPC, loss of ketene,  $[\text{M} - \text{CH}_3 - \text{C}_{18}\text{H}_{37}\text{CH}=\text{C}=\text{O}]^-$ ) and  $m/z$  510 (acyl 20:4 demethylated LPC, loss of FA,  $[\text{M} - \text{CH}_3 - \text{C}_{14}\text{H}_{29}\text{CH}_2\text{COOH}]^-$ ) at higher abundances than their corresponding ions at  $m/z$  462 (acyl 16:0 demethylated LPC, loss of FA,  $[\text{M} - \text{CH}_3 - \text{C}_{18}\text{H}_{37}\text{CH}_2\text{COOH}]^-$ ) and  $m/z$  528 (acyl 20:4 demethylated LPC, loss of ketene,  $[\text{M} - \text{CH}_3 - \text{C}_{14}\text{H}_{29}\text{CH}=\text{C}=\text{O}]^-$ ). Thus, the precursor ion of the backward peak was assigned to the PC containing 16:0 at *sn*-1 and 20:4 at *sn*-2 (Fig. 2B). Conversely, the precursor ion of the forward peak was assigned to the PC containing 20:4 at *sn*-1 and 16:0 at *sn*-2, because these related-fragment ions by CID of the forward peak were reversed (Fig. 2A). Similarly, the backward peak in the mass chromatogram of the  $m/z$  850 (38:6-PC) yielded  $m/z$  480 ( $[\text{M} - \text{CH}_3 - \text{C}_{20}\text{H}_{41}\text{CH}=\text{C}=\text{O}]^-$ ) and  $m/z$  534 (acyl 22:6 demethylated LPC, loss of FA,  $[\text{M} - \text{CH}_3 - \text{C}_{14}\text{H}_{29}\text{CH}_2\text{COOH}]^-$ ) at higher abundances than their competing fragment ions at  $m/z$  462 ( $[\text{M} - \text{CH}_3 - \text{C}_{20}\text{H}_{41}\text{CH}_2\text{COOH}]^-$ ) and 552 (acyl 22:6 demethylated LPC, loss of ketene,  $[\text{M} - \text{CH}_3 - \text{C}_{14}\text{H}_{29}\text{CH}=\text{C}=\text{O}]^-$ ), respectively. The precursor ion of the backward peak was assigned to the PC containing 16:0 at *sn*-1 and 22:6 at *sn*-2 (Fig. 2D). Conversely, the precursor ion of the forward peak was assigned to the PC containing



**Fig. 2 Separation and identification of synthetic phosphatidylcholines.** PCs were detected as  $[M + HCOO]^-$  ions in the negative ion mode by using ammonium formate as the elution buffer. The mass chromatogram of  $m/z$  826 and  $m/z$  850 for synthetic 16:0/20:4-PC (A) and 16:0/22:6-PC (B) was separated into two peaks. MS/MS fragments of each forward peak and backward peak were (a), (c) and (b), (d), respectively. The formate adduct ions detected were easily dissociated and detectable as  $[M - CH_3]^-$  ions by MS/MS. The demethylated LPC-related ions were then detected as fragment ions in two different forms, as  $[M - CH_3 - RCH_2COOH]^-$  resulting from neutral losses of FAs, and as  $[M - CH_3 - RCH = C = O]^-$  resulting from losses of ketenes. 1-Acyl-demethylated-LPC fragment ions produced by loss of the *sn*-2 moiety are detected as peaks higher than 2-acyl-demethylated-LPC fragment ions produced by loss of the *sn*-1 moiety. A similar result was reproducibly obtained. cps, count per second. FA, fatty acid.

22:6 at *sn*-1 and 16:0 at *sn*-2 (Fig. 2C). Thus, the positional isomers of the *sn*-1/2 acyl moieties of PC could be identified from these LPC-related fragment ions and FA fragment ions.

#### Quantification of positional isomers

Quantification of the positional isomers of FAs was performed using peak areas if the peaks were separated. For example, the peak at *m/z* 826 (36:4-PC)  $[M+HCOO]^-$  was separated into two peaks at retention times of 24.5–25.0 and 25.0–7.0 min (Fig. 2). From the fragmentation pattern of the parent ion  $[M+HCOO]^-$ , the forward and backward peaks were identified as 20:4/16:0- and 16:0/20:4-PC, respectively. Thus, the approximate relative abundance of 20:4/16:0- and 16:0/20:4-PC was 10:90 from each of the peak areas.

$$\text{Relative abundance} = 100 \times \frac{\text{Peak area of } 16:0/20:4\text{- or } 20:4/16:0\text{-PC}}{\text{Sum of each peak area of } 20:4/16:0\text{- and } 16:0/20:4\text{-PC}}$$

Similarly, the forward and backward peaks of the *m/z* 850 (38:6-PC)  $[M+HCOO]^-$  were identified as 22:6/16:0- and 16:0/22:6-PC, respectively, and the relative abundance of 22:6/16:0- and 16:0/22:6-PC was 18.1:89.9 (Fig. 2 and Table 1). Quantitative calculation was possible in the linear range of 5–1,000 pmol/10  $\mu$ l. Typical regression lines were: 16:0/20:4 PC,  $y = 5.0E7x - 3.0E7$  ( $r^2 = 0.9971$ ); 20:4/16:0 PC,  $y = 4.0E6x - 2.0E6$  ( $r^2 = 0.9977$ ); 16:0/22:6 PC,  $y = 2.0E7x - 1.0E7$  ( $r^2 = 0.9987$ ); and 22:6/16:0 PC,  $y = 4.0E6x - 2.0E6$  ( $r^2 = 0.9988$ ).

The relative abundance of positional isomers can be roughly calculated by the peak intensity of demethylated-LPC fragments rather than FA fragments, even in the case that the parent ions such as 34:0-PC (16:0/18:0- and 18:0/16:0-PC), 34:1-PC (16:0/18:1- and 18:1/16:0-PC) and 34:2-PC (16:0/18:2- and 18:2/16:0-PC) were not detected as the separate peak (5–7, 31). However, the peaks due to only FA fragments are not suitable for quantification of positional isomers, because PUFA fragments yield  $[FA-CO_2]^-$  ions from the facile elimination of

decarboxylation from  $[FA]^-$ . For example, *m/z* 327 yields a fragment at *m/z* 283 by facile elimination of 44 Da. Thus, the signal observed at *m/z* 283 may be due to a mixture of the stearate anion plus this secondary fragmentation product of the primary carboxylate ion at *m/z* 327. Such observations serve as a warning about methods using relative abundances of carboxylate fragment ions to define positional isomerism in PCs.

#### Quantification and separation of positional isomers from biological samples

We confirmed the relative amounts of the positional isomers of PCs from the lipid extracts of mouse tissues such as brain, liver and heart. PC profiles of the brain, liver and heart were shown in Fig. 3. Brain mainly had the 16:0/16:0, 16:0/18:1, 18:0/18:1, 16:0/22:6 and 18:0/22:6 species and liver mainly had the 16:0/18:2, 18:0/18:2, 16:0/22:6 and 18:0/22:6 species. Furthermore, heart had more abundantly the 16:0/22:6 and 18:0/22:6 species (Fig. 3).

From the analysis of mouse brain extracts, positional isomers of 36:4-PC (*m/z* 826) or 38:6-PC (*m/z* 850) could be separated into two peaks, and the retention times matched the standard PCs (Figs 4 and 5). In addition, the peaks of 36:4-PC (*m/z* 826) and 38:6-PC (*m/z* 850) were identified as 20:4/16:0-, 16:0/20:4-PC and 22:6/16:0-, 16:0/22:6-PC, respectively, from MS/MS fragments (Fig. 4). The approximate relative abundances of 20:4/16:0-:16:0/20:4-PC and 22:6/16:0-:16:0/22:6-PC were 9.2:90.8 and 30.6:69.4, respectively (Table 1). Interestingly, the lipid extracts from mouse heart and liver mainly consisted from 16:0/20:4- and 16:0/22:6-PC, and 20:4/16:0- and 22:6/16:0-PC were not detected (Fig. 5). This is a new and intriguing result that suggests the relative abundance of positional isomers is different in different tissues or molecular species. Thus, these results indicate that FA remodelling enzymes each have specific characteristics in tissues, cells, organelles or PL classes.

#### Separation of positional isomers by oxidation

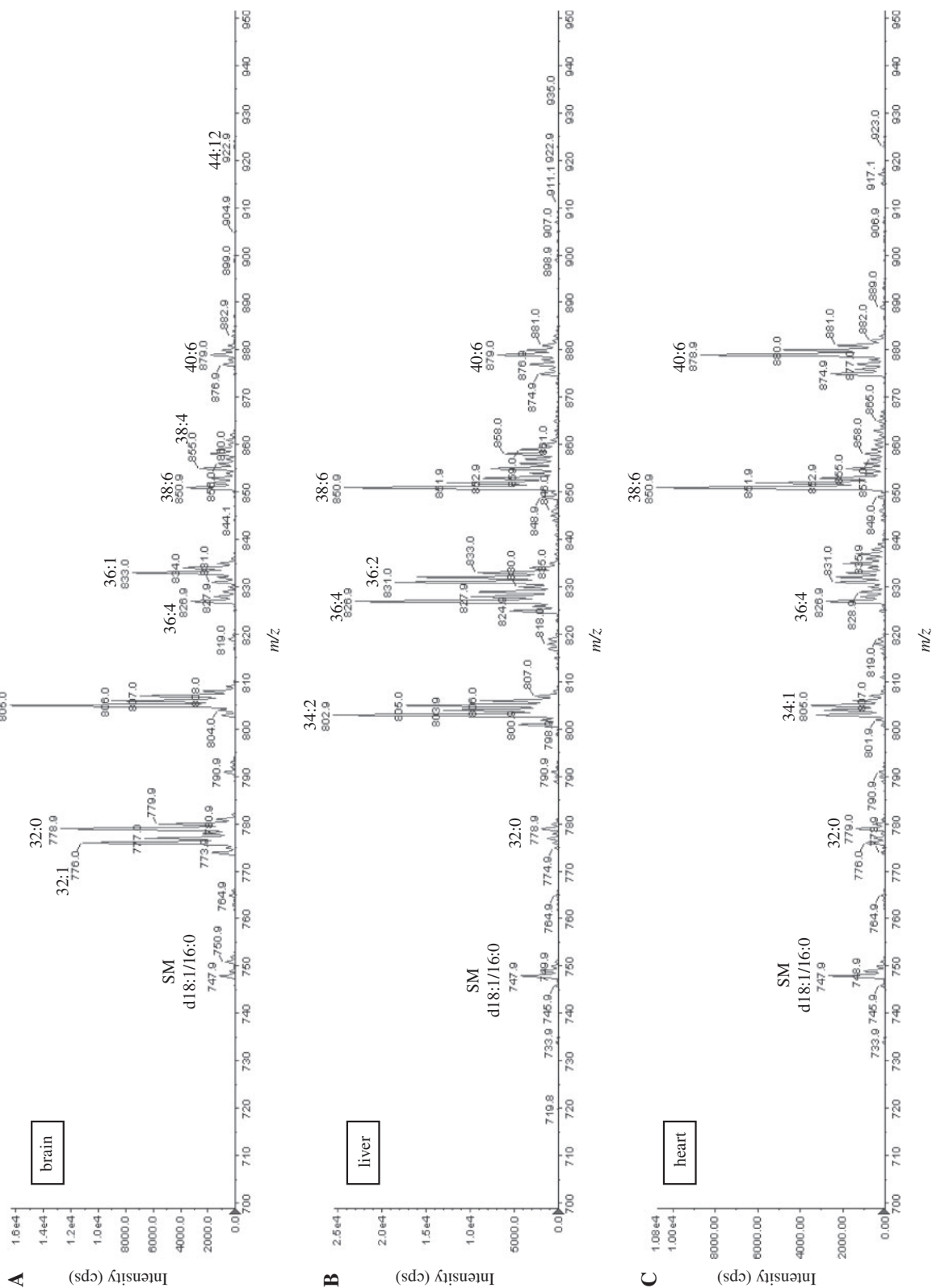
The aforementioned experiments revealed that 16:0/22:6-PC could be separated by the UPLC system. On the other hand, 16:0/18:2-PC (*m/z* 802  $[M+HCOO]^-$ ) was not detected as a separate peak (Fig. 6). These results suggest that more polar PCs are more easily separated by RP columns. To test the hypothesis that the separation is highly influenced by the polarity of the fatty acid, oxidized PCs were measured by RPLC-ESIMS/MS. Oxidized PCs were prepared by 15-lipoxygenase from authentic PCs such as PLPC (34:2-PC), PAPC (36:4-PC) and PDPC (38:6-PC). As a result, the oxidized PLPC (34:2+O-PC) separated into 18:2+O/16:0- and 16:0/18:2+O-PC. Furthermore, oxidized PAPC (36:4+O-PC) and PDPC (38:6+O-PC) were clearly separated into 20:4+O/16:0-, 16:0/20:4+O-PC and 22:6+O/16:0-, 16:0/22:6+O-PC, respectively (Fig. 6).

The relative peak area of oxidized PCs is approximately the same as the peak area of PCs (Table 1).

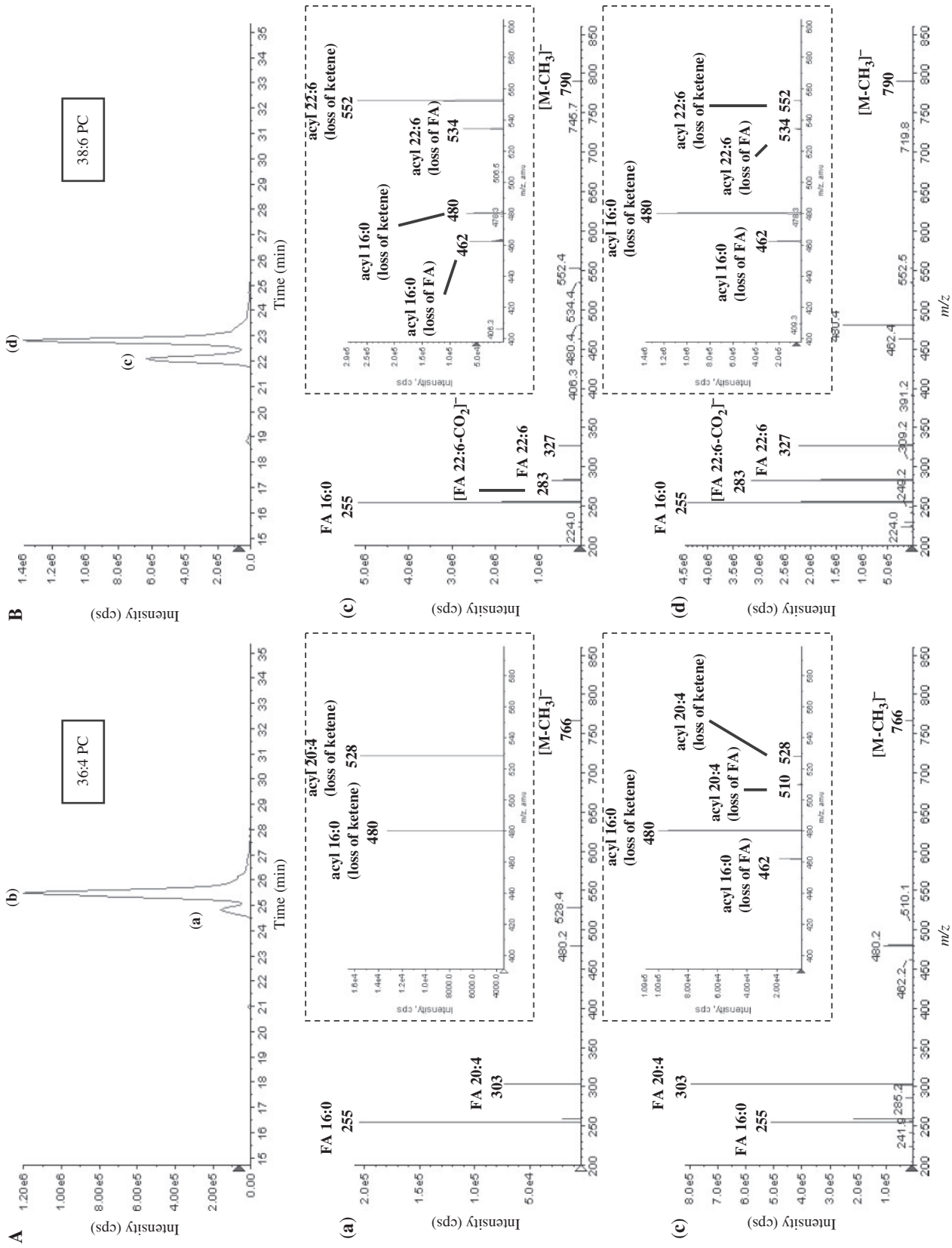
**Table 1. The relative abundance of positional isomers in phosphatidylcholines.**

Precursor ion ( <i>m/z</i> )	Molecular species	Standard (%)	Mouse brain (%SD) ( <i>n</i> = 5)
826	16:0/20:4	90	90.8 ± 0.62
	20:4/16:0	10	9.2 ± 0.62
850	16:0/22:6	81.9	69.4 ± 1.41
	22:6/16:0	18.1	30.6 ± 1.41
818	16:0/18:2+O	89.3	—
	18:2+O/16:0	10.7	—
842	16:0/20:4+O	91.5	—
	20:4+O/16:0	8.5	—
866	16:0/22:6+O	84.1	—
	22:6+O/16:0	15.9	—

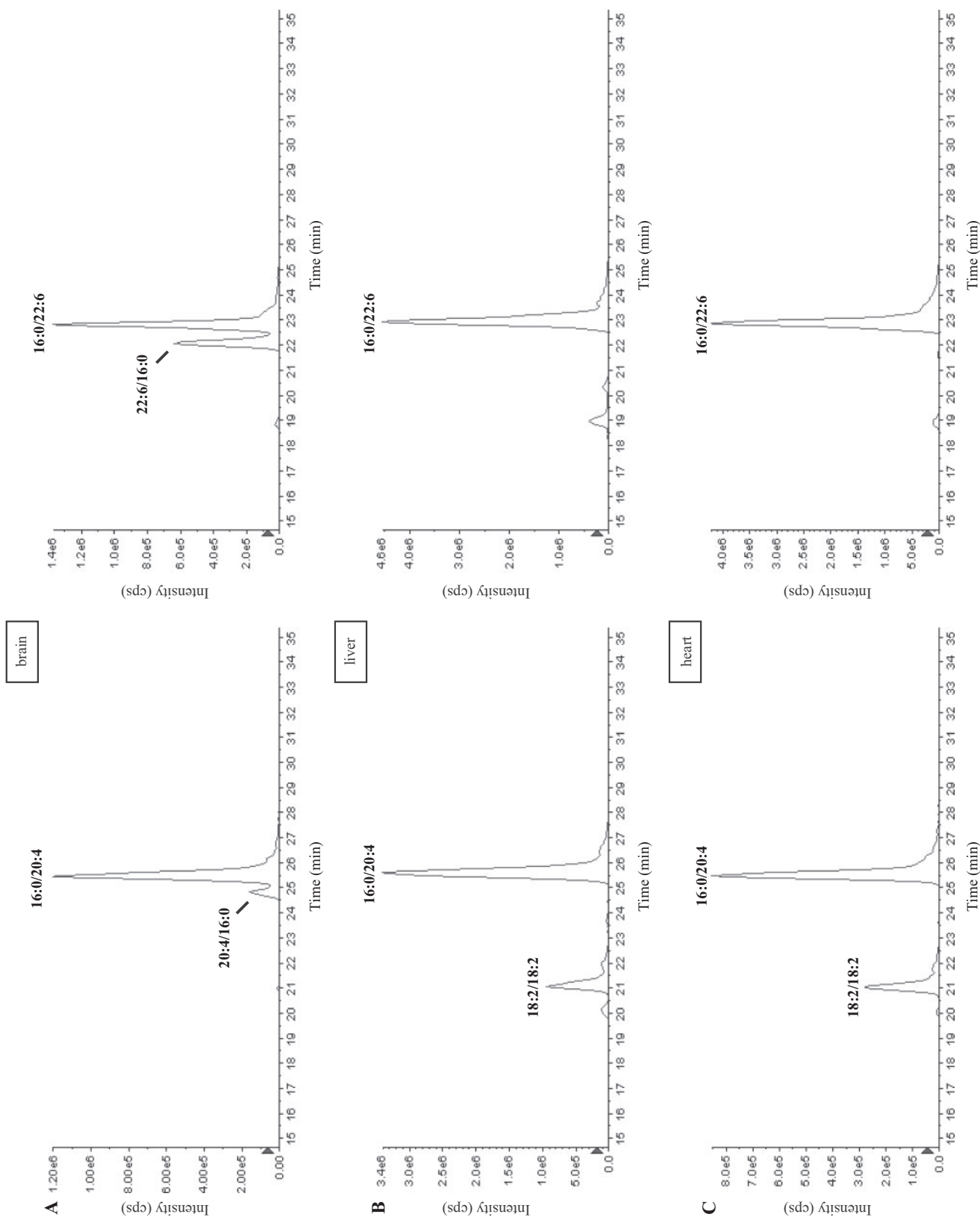
SD, standard deviation.



**Fig. 3 Profiles of phosphatidylcholine molecular species from mouse brain, heart and liver.** (A) The brain PC mainly consisted from 32:0 (16:0/16:0), 32:1 (16:0/18:1), 34:1 (16:0/18:1), 38:6 (16:0/22:6) and 40:6 (18:0/22:6) species. (B) The liver PC mainly consisted from 34:2 (16:0/18:2), 36:2 (18:0/18:2), 36:4 (16:0/20:4), 38:6 (16:0/22:6) and 40:8 (18:0/22:6) species. (C) The heart PC mainly contained 38:6 (16:0/22:6) and 40:8 (18:0/22:6) species. Dashed lines indicate major molecular species. cps, count per second.

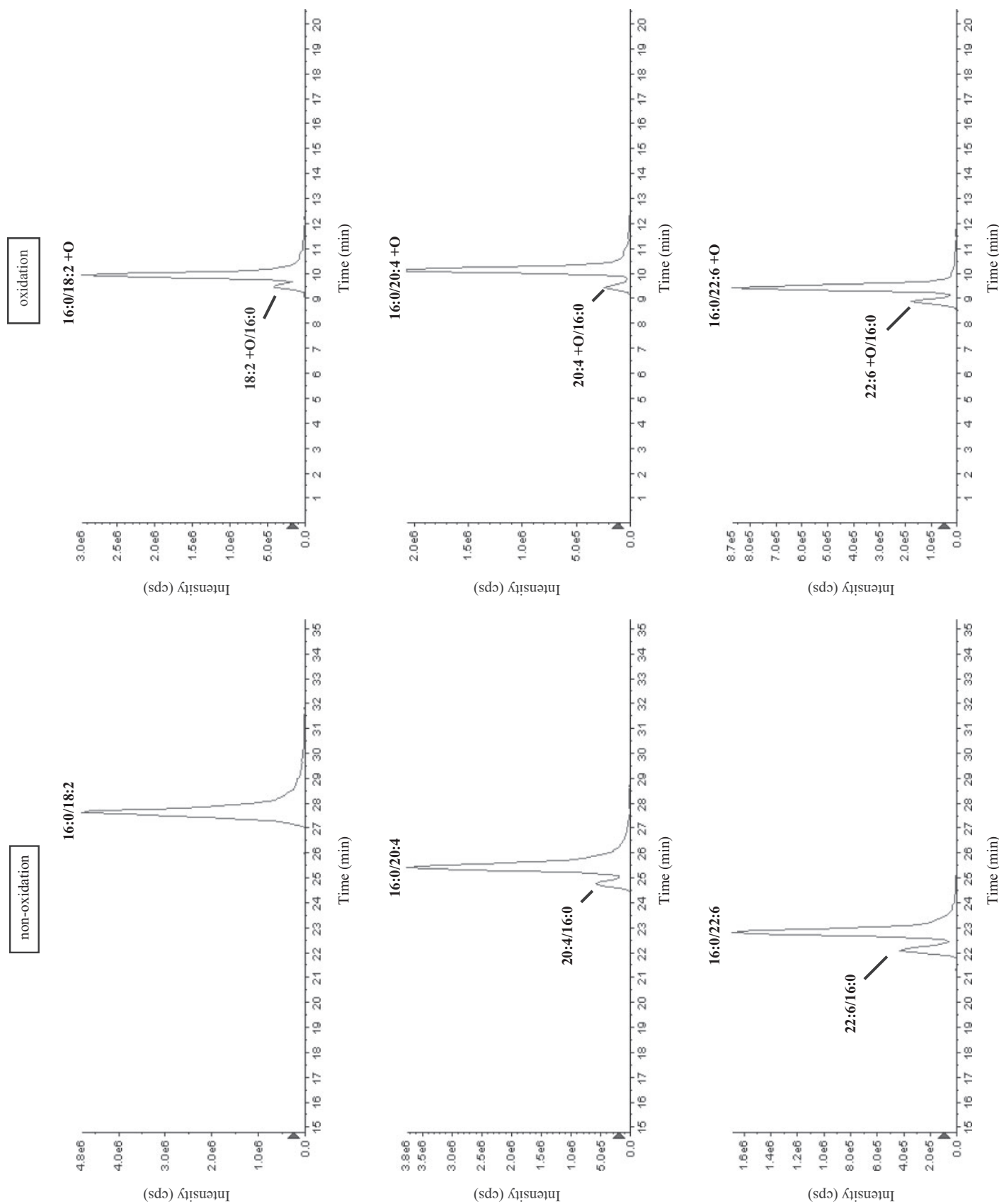


**Fig. 4 Separation and identification of endogenous phosphatidylcholines.** The mass chromatograms of 16:0/20:4-PC (*m/z* 826) and 16:0/22:6-PC (*m/z* 850) from mouse brain lipid extracts are shown in A and B, respectively. MS/MS fragments of each forward peak and backward peak were (a), (c) and (b), (d), respectively. These fragmentations were matched with synthetic PCs (see Fig. 2). The results are representative of five independent experiments. cps, count per second. FA, fatty acid.



**Fig. 5** Analyses of the positional isomers of PCs from the lipid extracts of mouse tissues such as brain, liver and heart. PC species of the brain lipid extracts were separated as positional isomers (A), whereas the lipid extracts from mouse heart (C) and liver (B) were not separated. The results are representative of five independent experiments. cps, count per second.





**Fig. 6 Separation of the positional isomers by oxidation.** Oxidized PCs were prepared by 15-lipoxygenase from authentic PCs such as PLPC (34:2-PC), PAPC (36:4-PC) and PDPC (38:6-PC). The positional isomers of PCs were more clearly separated as their oxidized forms. In particular, the oxidized PLPC (34:2 +O-PC) separated into 18:2 +O/16:0- and 16:0/18:2 +O-PC. MS/MS fragments of each oxidized PCs is shown in Supplementary Fig. S2. A similar result was reproducibly obtained. cps, count per second.

By oxidation of PUFA on *sn*-1 position, the peak is shifted in the earlier elution time much larger than those on *sn*-2 position (21, 37, 38). Thus, these results show that the ratio of positional isomers is not influenced by oxidation. Rather, we concluded that oxidation is useful to calculate the relative abundance of positional isomers of other PLs in addition to PCs.

## Discussion

Cellular membranes contain several classes of glycerophospholipids, which have numerous structural and functional roles in cells. And the FA combination at these *sn*-1/2 positions is different in tissues, cells, organelles or PL classes. For example, the PC of mouse brain had a lot of palmitic acid (16:0)- or oleic acid-containing molecular species and those of liver had a lot of linoleic acid (18:2)-containing molecular species. In addition, DHA (docosahexaenoic acid; 22:6)-containing molecular species existed abundantly in the mouse heart (Fig. 3). On the other hand, the PS and PI in these tissues mainly had the 18:0/22:6 and 18:0/20:4 species, respectively (Supplementary Fig. S1). It is assumed that the differences in main molecular species are closely related to physiological functions of each tissue.

FA remodelling systems for PLs including acyl-CoA: lysophospholipid acyltransferases, CoA-dependent and CoA-independent transacylation systems, lysophospholipase/transacylase, phospholipases A<sub>1</sub> and A<sub>2</sub> are involved in the biosynthesis of these molecular species (4, 8–14). In addition, the distribution and substrate specificity of these enzymes are different. Thus, these differences cause different distribution of PL molecular species such as *sn*-1/2 di-PUFAs, di-SAFAs and di-MUFAs. For example, PUFA/PUFA-, especially 22:6/22:6-PLs, are known to be abundant in the specific area of brain tissue and retina (H. Nakanishi *et al.*, unpublished data) (39, 40). Additionally, pulmonary surfactant has PC of 16:0/16:0 (SAFA/SAFA) at the *sn*-1/2 positions and the *sn*-1/2 acyl moieties of lipid droplet are abundant in 18:1/18:1 (MUFA/MUFA) PC (4, 41). Therefore, the analysis at molecular species level that uses the MS in understanding these functions is indispensable.

This study is the first report on the separation and quantification of positional isomers of endogenous PC using RPLC-ESIMS/MS. High separation and sensitivity can be achieved by using this separation system for the positional isomers of PCs. In addition, this achievement shows that the relative amounts of positional isomers between various tissues or molecular species differs.

The PC molecular species containing 22:6 at the *sn*-1 position of the glycerol backbone existed abundantly in the mouse brain (Fig. 5). However, heart and liver were not abundant in positional isomers of PC species (Fig. 5). It is notable that the ratio of the positional isomer of each molecular species was different in the organs. These different existence ratios of isoforms in the different organs seem to be caused by the

significantly high-speed remodelling process, which is shorter than that needed for getting around to equilibrium level of lyso PL isoforms, ~9:1 for *sn*-1/*sn*-2 (H. Nakanishi and R. Taguchi, unpublished data). Furthermore, the remodelling enzymes such as acyltransferase, transacylase and phospholipase are involved in not only the remodeling of FAs but also the synthesis and degradation of some bioactive lipids and their precursors. Therefore, we assume that it is necessary for mouse brain that there is a lot of PC molecular species containing 22:6 at the *sn*-1 position as precursors of 22:6/22:6 species. Thus, these results suggest the existence of specific enzymes for the remodeling of DHA in the mouse brain. DHA plays a critical role in the development and function of the nervous system (42–44). In addition, PUFAs including DHA are also considered as major molecules of radical trap controlling oxidative stress within the cell (45, 46). DHA-containing molecular species were abundant in heart than brain (Fig. 3). We consider that the position of DHA is not affected as anti-oxidative mechanism. However, DHA is included in the *sn*-2 position of the glycerol backbone in general. Taken together, we speculate from our observation the existence of novel physiological function of DHA/DHA PC in the brain.

Our reports provide a novel and critical milestone in understanding how the molecular composition of PLs is established and the biological implications of this process. The present paper deals only with separation and quantification of individual molecular PC species, and other PLs class studies are now underway. In the future, it may be possible to determine not only the biological mechanism of the remodeling enzymes but also the biological implications of diversity of glycerophospholipids in cellular membranes.

## Supplementary Data

Supplementary Data are available at *JB* online.

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## Conflict of interest

None declared.

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