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Mass Spectrometric Identification of Molecular Species of Phosphatidylcholine and Lysophosphatidylcholine Extracted from Shark Liver

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The profile and structural characterization of molecular species of phosphatidylcholine (PC) and lysophosphatidylcholine (LysoPC) from shark liver using liquid chromatographic/electrospray ionization mass spectrometry (LC-ESI/MS) and tandem mass spectrometry (MS/MS) are described for the first time in this paper. The presence of (i) a relatively high content of ether PC species, such as 1-*O*-alkyl- and 1-alk-1′-enyl-2-polyunsaturated PC species (about 20%), and (ii) a high percentage of docosahexaenoic acid (DHA)-containing LysoPC (about 27%) is the characteristic of this marine material. 1-Hexadecanoyl-2-docosahexaenoyl-PC (16:0/22:6; about 24%) and 1-docosahexaenoyl-2-hydroxyl-LysoPC (22:6; about 27%) are the two most abundant species in shark liver. The other polyunsaturated PC species including ether PC are tentatively identified as 1-heptadecanoyl-2-docosahexaenoyl-PC (17:0/22:6), 1-octadecyl-2-docosahexaenoyl-PC (alkyl-18:0/22:6), 1-hexadecyl-2-docosahexaenoyl-PC (alkyl-16:0/22:6), 1-octadecenyl-2-eicosapentaenoyl-PC (alkyl-18:1/20:5), 1-octadecenyl-2-eicosatetraenoyl-PC (alkyl-18:1/20:4), 1-hexadecyl-2-docosapentaenoyl-PC (alkyl-16:0/22:5), 1-(1*Z*-hexadecenyl)-2-docosahexaenoyl-PC (alkenyl-16:0/22:6), and 1-octadecenoyl-2-docosahexaenoyl-PC (18:1/22:6). These results establish that high contents of ether DHA-PC and DHA-LysoPC species can be obtained from shark liver.

KEYWORDS: Phosphatidylcholine; ether phosphatidylcholine; lysophosphatidylcholine; docosahexaenoic acid; mass spectrometry; tandem mass spectrometry

INTRODUCTION

Phosphatidylcholine (PC) and lysophosphatidylcholine (LysoPC) are the two natural phospholipid classes existing in the mixture form of molecular species. Biochemical and biophysical functions of phospholipids (*1*) are well documented and appear to be determined by the fatty acid composition of lipids. PC and LysoPC are also good substrates of phospholipase D for the preparation of other classes of phospholipids and lysophospholipids by transphosphatidylation.

Omega-3 long-chain polyunsaturated fatty acids play vital roles in many aspects of human nutrition. Inadequate dietary intake has been increasingly associated with a number of human health disorders, including increased risk of cardiovascular disease and cancer in adults, and delayed or impaired neurological and cognitive development in infants. Marine fish and algae oils, which are enriched in docosahexaenoic acid (DHA; 22:6ω-3)-containing neutral lipids, and ethyl DHA are commercially available products that have shown a benefit in alleviation of coronary atherosclerosis (*2*). The brain is more highly enriched than most other tissues in long-chain polyunsaturated fatty acids. The deficiency of essential fatty acids, in particular DHA, in the brain markedly affects neurotransmission, membrane-bound enzymes, the permeability of cell membranes, and ion channel activities (*3*). Due to the nature of the chemical structures of DHA neutral lipids (*4*), however, it is an intriguing question whether the lipids may act as transports for further delivery of DHA to target tissues such as the brain, although it has been reported that feeding neutral lipids increases the level of plasma free fatty acids that can be incorporated into plasma phospholipids, the main sources to provide DHA to the brain (*5*). Studies have indicated that DHA-enriched phospholipid diets modulate age-related alterations in the brain (*6*), but the route by which DHA-PC is transported to the brain is involved in a deacylation and reacylation cycle whereby the fatty acid is liberated by phospholipases and is reincorporated by acyltransferases. It is probably the main reason why the amount of plasma DHA phospholipids reaches a maximum about 9–12 h after ingestion of DHA-enriched PC or DHA-neutral lipids in humans (*7, 8*).

Ether phospholipids and lysophospholipids are usually present in various mammalian tissues and cells as minor components,

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existing together with diacyl or monoacyl molecular species carrying the same polar headgroup. There are two predominant types of ether bonds in the phospholipids. One form is represented by the plasmalogens (with 1-alk-1′-enyl fatty chain(s) linked to the *sn* – 1 position of the glycerol backbone), which is the most abundant subclass of phospholipids in most tissues. The other form is alkyl ether phospholipid species that contain 1-*O*-alkyl fatty chain(s) linked to the *sn* – 1 position of the glycerol backbone. Due to the nature of their chemical structures, however, ether phospholipids cannot be hydrolyzed by phospholipase A_1-like enzymes (*9, 10*) and may be catalyzed by phospholipase A_2-like enzymes at very slow rates (*10*). Furthermore, the hydrolysis of lysophospholipid species, which contain only one fatty acid chain that is usually linked to the *sn* – 1 position of the glycerol backbone, is also limited in the metabolism (*11*). Therefore, DHA-ether PC and DHA-LysoPC could be new types of transports of DHA to the brain. But the preparation of such special DHA lipid species by chemical synthesis is expensive (*12*), especially on a large scale. In the present paper, the profiles and chemical structures of the molecular species of shark liver PC and LysoPC are characterized for the first time by liquid chromatography/electrospray ionization mass spectrometric (LC/ESI-MS) analysis and tandem mass spectrometry (MS/MS). Based on published studies, the potential use of shark PC and LysoPC as transports of DHA to the brain is tentatively proposed.

MATERIALS AND METHODS

Chemicals. Chemical grade acetone, ethyl acetate, and ethanol, as well as analytical grade chloroform, methanol, ammonia hydroxide, and L-serine, were obtained from Sigma Company (St. Louis, MO). Phospholipase D (*Streptomyces* sp.) was obtained from BIOMOL Company (Plymouth Meeting, PA).

Extraction of Crude Phospholipids and Purification of PC and LysoPC. A fresh shark (*Carcharhinus melanopterus*) liver was obtained in a fish market near the city of Medan in Indonesia. The weight of the wet shark liver was about 1170 g.

The fresh liver was transported on ice (withdrawn 60–70 min after death). Tiny liver fragments were made with an electronic blender and then mixed with 20 volumes of cold acetone (4 °C) while stirring for 2 h. After removing the acetone, the liver fragments, now pellets, were roughly dried under nitrogen, and then the dried pellets were homogenized with 40 volumes (v/v) of ethyl acetate/ethanol (2/1 v/v) (*13*), while stirring for 3 h at room temperature. After filtration and evaporation of the solvent under vacuum, about 152 g of the lipid extract was obtained.

For the preparation of crude phospholipids, 25 g of the lipid extract was mixed with 30 volumes (v/v) of acetone, and then the mixture was stirred at 35 °C for 1 h. After filtration, the solution was kept at −20 °C overnight, leading to precipitation of phospholipids. After rapid filtration and subsequent drying of the solvent with nitrogen, crude phospholipids were obtained, consisting of about 80% PC, about 15% LysoPC, and about 5% other lipids, which were roughly monitored by thin-layer chromatography.

Both shark liver PC and LysoPC were further purified from about 2 g of the crude phospholipids by silica chromatography using an axial pressure in a 250 mL column equilibrated with chloroform/methanol (95/5). Gradient elution was performed stepwise with mixtures of chloroform/methanol 90/10 (v/v), 80/20 (v/v), 70/30 (v/v), and 60/40 (v/v). The shark liver PC was eluted with a mixture of chloroform/methanol/water 50/50/1 (v/v), while the LysoPC was eluted with a mixture of chloroform/methanol/water 50/50/5 (v/v). During the purification, phospholipid fractions were monitored by thin-layer chromatography.

Preparation and Purification of Ether LysoPC. About 150 mg of purified PC was dissolved in 18 mL of methanol in a 150 mL bottle, and then 2 mL of 5 M NaOH was added (*14*). This reaction mixture was left to react at room temperature for 18 h. Total lipids were extracted by adding 22 mL of methanol, 40 mL of chloroform, and 34 mL of water. After removing the upper-phase solution, the lower-phase was left at −20 °C for several hours. After concentration of the clear solution that contains ether LysoPC, the sample was loaded on a silica column (150 mL) for further purification. The ether LysoPC was eluted with a mixture of chloroform/methanol/water (50/50/2 v/v) and used for identifying the ether linkage of shark PC ether species.

Acidic Treatment of the Purified Ether LysoPC. Acidic hydrolysis was performed by incubation of purified ether LysoPC species (about 1 mg) with a mixture of 0.02 M hydrochloric acid in methanol (1 mL) and chloroform (0.5 mL) for 20 min at room temperature. A control sample was also treated as described above with a mixture of methanol (1 mL) and chloroform (0.5 mL). After the extraction of total lipids from control and acid-treated samples, the ether LysoPC species were analyzed by positive-ion ESI-MS.

Preparation of Transphosphatidylated PS and PA. A mixture of 2 mL of acetate buffer (0.2 M, pH 5.5), containing 40 mM of calcium chloride and 1 g of L-serine, was prepared at 45 °C and was then placed into a 20-mL disposable vial that contained about 100 mg of purified shark liver PC (*15*). The vial was flushed with argon. The enzymatic reaction was started by adding 5 units of phospholipse D (*Streptomyces* sp.) and was carried on for 18 h at 45 °C on a shaker. Once the reaction was over, the reacted solution was transferred to another container that contained 40 mL of a mixture of chloroform/methanol (2/1 v/v) and 6 mL of water. After extraction of total lipids, two separated phases were formed. Both PS and PA (a byproduct) were in the lower-phase. The remaining PS and PA in the upper-phase were re-extracted with 20 mL of chloroform. After the combined two organic phases were dried, the PS and PA were further purified by silica chromatography with a 150 mL column. The PA was eluted with a mixture of chloroform/methanol 85/15 (v/v), while the PS was eluted with a mixture of chloroform/methanol 55/45 (v/v).

LC/ESI-MS and MS/MS. For generating molecular species profiles of PC, LysoPC, and ether LysoPC derived from the PC, positive-ion LC/ESI-MS and ESI-MS were performed on a Micromass Platform LC/MS system (Micromass Ltd., Manchester, U.K.; Waters Co., Milford, MA), equipped with a standard ESI source and a quadrupole analyzer. Twenty microliters of each lipid sample in methanol/chloroform (1/1 v/v) was injected into the LC/MS system. The flow rate used was 0.35 mL/min. The PC, LysoPC, and ether LysoPC were separated on a silica HPLC column (3 μm, Thermo, silica column (2.0 mm × 150 mm), Foster City, CA), which was eluted with a linear gradient of 100% solvent A (chloroform/methanol/30% ammonium hydroxide 80/19.5/0.5 v/v) to 100% solvent B (chloroform/methanol/water/30% ammonium hydroxide, 60/34.5/5/0.5 v/v) for 15 min and then with 100% solvent B for other 10 min (*16*). The source temperature was at 350 °C. The mass range used was from 350 to 1000 Da. Data acquisition and processing were controlled using MassLynx software (Micromass Ltd., Manchester, U.K.). Both nontreated and acid-treated ether LysoPC samples were dissolved in mixtures of NaOH (20 mM)/chloroform/methanol (0.1/1/1 v/v) and analyzed by positive-ion ESI-MS.

For generating both positive-ion and negative-ion ESI-MS/MS product ion spectra, a Q-TOF mass spectrometer (Micromass, Manchester, U.K.) at Free University in The Netherlands was used. Nontreated ether LysoPC samples were dissolved in mixtures of LiOH (30 mM)/chloroform/methanol (0.1/1/1 v/v); the transphosphatidylated PA and PS samples were dissolved in chloroform/methanol (1/1 v/v). All lipid samples were introduced into the mass spectrometer via a Harvard Apparatus (Holliston, MA) model syringe pump at the flow rate 15 μL/min. Argon was used as collision gas. The quadrupole analyzer was used to select precursors of the lipids, and the time-of-flight analyzer was used for product ion analyses in mass scanning from 50 to 600 Da for the positive-ion ESI-MS/MS and from 200 to 1000 Da for the negative-ion ESI-MS/MS, respectively. The scan speed used was 1.0 s/scan.

RESULTS AND DISCUSSION

LC/ESI-MS Profiles of Shark Liver PC and LysoPC. The crude phospholipids extracted from shark liver were analyzed

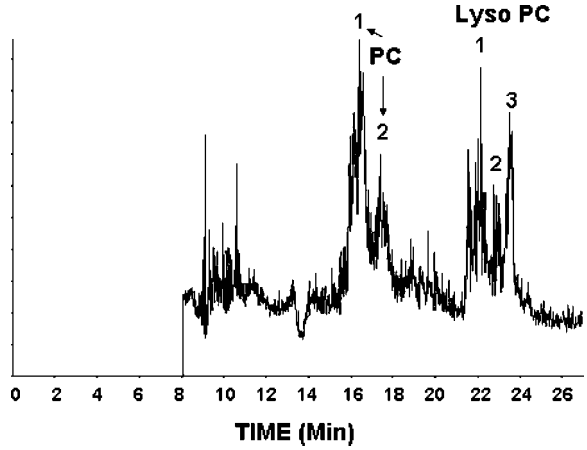


Figure 1. Total ion current profiles of positive-ion LC-ESI/MS of shark liver PC and LysoPC.

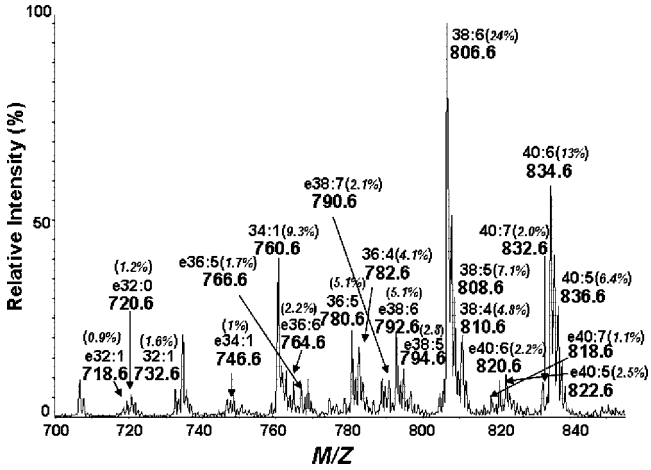


Figure 2. Positive-ion LC-ESI MS spectra of shark liver PC (e means ether; for example, e38:6 means ether-38:6).

by positive-ion LC/ESI-MS, as shown in **Figure 1**. The PC and LysoPC were eluted between 15.5 and 17.8 min and between 21.3 and 24.0 min, respectively. Two traces in the LC/MS profile of PC can be observed, corresponding to polyunsaturated fatty acid-containing PC species (PC peak 1 in **Figure 1**) and others (PC peak 2 in **Figure 1**), because polyunsaturated and non-polyunsaturated PC species can be roughly isolated with this silica HPLC. Three traces in the LC/MS profile of shark liver LysoPC can be also observed, due to mainly 22:6 LysoPC species (LysoPC peak 1 in **Figure 1**), mainly 20:4 and 20:5 species (LysoPC peak 2 in Figure 1), and others, including ether LysoPC molecules (LysoPC peak 3 in **Figure 1**).

The molecular weights of the PCs were inferred from their protonated molecules ($[M + H]^+$) (see **Figure 2**). The molecular species composition with numbers of total carbons and total double bonds of fatty acid chains within shark liver PC, as well as the tentative percentage abundance of the lipid species, are also shown in **Figure 2**.

LysoPC species were inferred from their protonated molecules ($[M + H]^+$), shown in **Figure 3**. Based on information generated by positive-ion LC/ESI-MS, molecular species compositions, with number of total carbons and number of total double bonds of fatty acid chains within shark liver LysoPC, as well as the tentative percentage abundance of the lipid species, are listed in **Table 1**. Because each LysoPC species contains only one fatty chain at the *sn* – 1 position (or at the *sn* – 2 position in the minor species), the molecular species are tentatively designed based on LC/ESI-MS analyses (see **Table 1**).

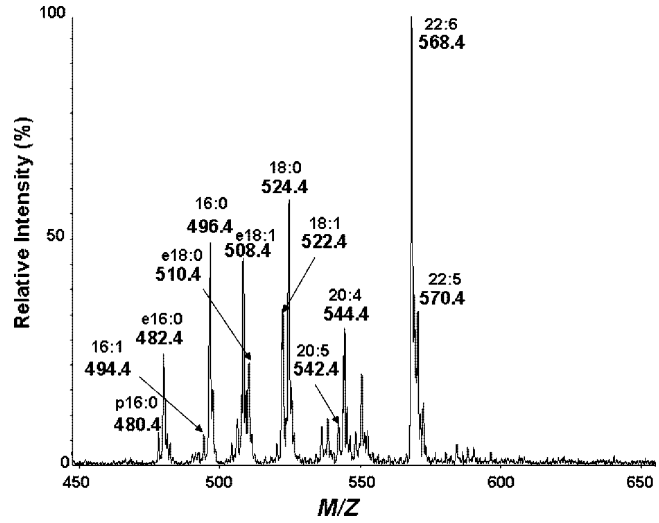


Figure 3. Positive-ion LC-ESI MS spectra of shark liver LysoPC (Number of total carbons (*X*) and number of total double bonds (*Y*) of fatty acid chains within shark liver PC are shown as *X*:*Y* (for example, 38:6); e means ether (for example, e38:6 means ether 38:6)).

Table 1. Protonated Molecules of Shark Liver Lysophosphatidylcholine Species by LC/MS

$[M + H]^+$	total carbons (*X*)	total double bonds (*Y*)[a]	percentage (%)[b]
480	16	1	1.7 ± 0.7 (plasmenyl 16:0)
482	16	0	6.5 ± 0.5 (plasmanyl 16:0)
494	16	1	1.1 ± 0.4 (16:1)
496	16	0	12 ± 3.2 (16:0)
508	18	1	9.3 ± 2.4 (plasmanyl 18:1)
510	18	0	5.0 ± 1.7 (plasmanyl 18:0)
522	18	1	6.4 ± 1.4 (18:1)
524	18	0	14 ± 2.7 (18:0)
542	20	5	2.4 ± 0.6 (20:5)
544	20	4	5.7 ± 0.5 (20:4)
568	22	6	27 ± 2.7 (22:6)
570	22	5	8.9 ± 1.2 (22:5)

[a] *X*:*Y* (For example 22:6), where *X* is the total carbon number of the fatty acids esterified at the *sn* – 1 position and *Y* is the total unsaturation degrees of fatty acid chains. [b] Semiquantitative results were generated from the average intensity of $[M + H]^+$ ions of the molecular species of shark liver LysoPC, based on three replicate experiments of liquid chromatography/mass spectrometry.

Identification of Alkyl Ether and Alkenyl Ether Lipid Species. The acidic hydrolysis of ether phospholipids, followed by chromatographic or mass spectrometric identification, is a common approach for distinguishing 1-*O*-alkyl (plasmanyl) and 1-*O*-alk-1′-enyl (plasmenyl) linkages because the double bond in the plasmenyl style of linkage is relatively unstable under acidic treatment in a short period of time. Recently, a MS/MS approach (*17*) has been reported for identifying structures of 1-*O*-alkyl- (plasmanyl 16:0) and 1-*O*-alk-1′-enyl- (plasmenyl 16:0) LysoPC, and the decision can be made based on the absence (plasmanyl) or presence (plasmenyl) of a prominent product ion at *m/z* 187 in the mass spectrum, arising from loss of a 1-hexadec-1-enyl group (a hexadec-1-enol), whish is generated by the MS/MS analysis of $[M + Li]^+$ of plasmenyl LysoPC species.

Six ether LysoPC species (spectrum not shown) are tentatively identified as plasmanyl 18:1 (*m/z* 508, $[M + H]^+$; the most abundant), plasmanyl 16:0 (*m/z* 482, $[M + H]^+$; the second most abundant), plasmanyl 18:0 (*m/z* 510, $[M + H]^+$; the third most abundant), plasmenyl 16:0 (*m/z* 480, [M +

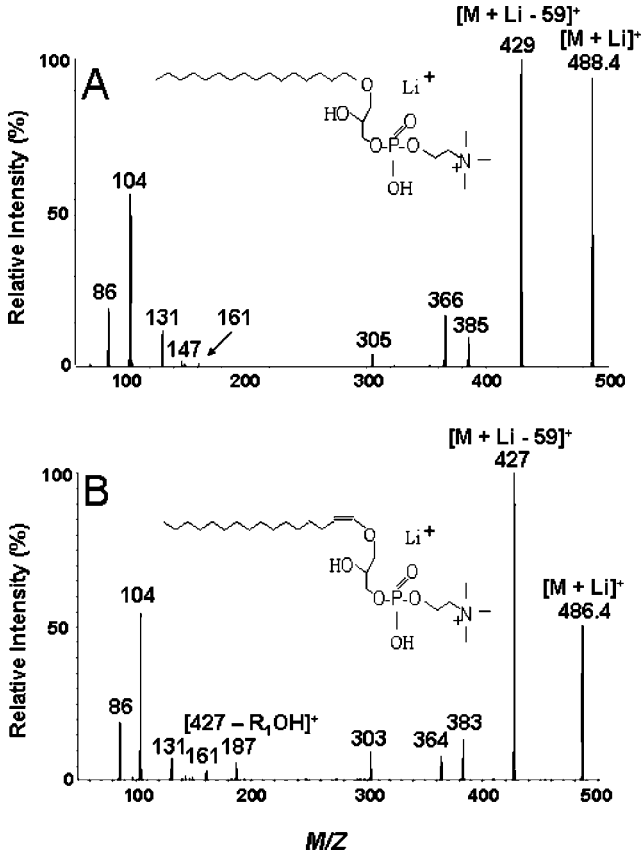


Figure 4. Product ion MS/MS mass spectra of $[M + Li]^+$ precursors of (**A**) *m/z* 488 and (**B**) *m/z* 486.

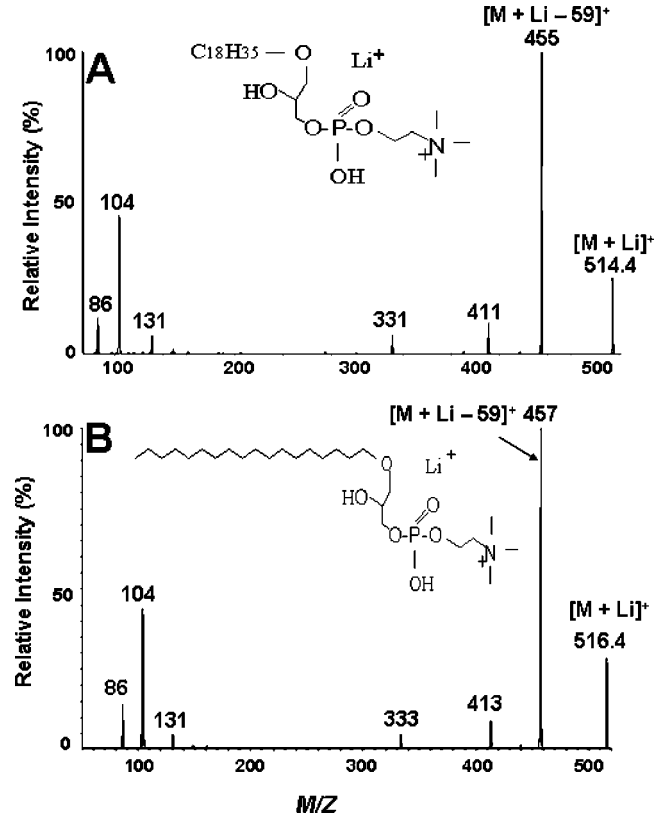


Figure 5. Product ion MS/MS mass spectra of the $[M + Li]^+$ precursors of (**A**) *m/z* 514 and (**B**) *m/z* 516.

$H]^+$; the fourth most abundant), plasmanyl 14:0 (*m/z* 476, $[M + H]^+$; a minor species), and plasmenyl 14:0 (*m/z* 474, $[M + H]^+$; another minor species), corresponding to the ether bond linkage at the *sn* – 1 position of shark liver ether PC species. The designation is supported by the following experiments. After a 20 min acidic treatment of the ether LysoPC species, followed by positive-ion ESI-MS analyses, it was observed that the intensity ratio of the ion at *m/z* 504 ($[M + Na]^+$; alkyl 16:0) to the ion at *m/z* 502 ($[M + Na]^+$; alkenyl 16:0) in the mass spectrum was increased, compared with that of the ions generated from the two nontreated ether species (spectrum not shown). This suggests that the peak at *m/z* 480 ($[M + H]^+$) or *m/z* 502 ($[(M + Na)^+$] should be due to a plasmenyl 16:0 species because the fatty chain with the 1-alk-1′-enyl type of ether linkage was partly hydrolyzed with the acidic treatment. This designation was confirmed by the results obtained by MS/MS analyses of $[M + Li]^+$ precursors of the species (**Figure 4**). A peak at *m/z* 187 appeared only in the product ion spectrum (**Figure 4B**) of the $[M + Li]^+$ precursor at *m/z* 486, which was formed by the loss of a 1-hexadec-1-enol group from a plasmenyl 16:0 species (*17*), compared with the product ion spectrum (**Figure 4A**) of the $[M + Li]^+$ precursor at *m/z* 488 (plasmanyl 16:0).

Positive-ion ESI-MS analyses of acid-treated ether LysoPC species also showed that the intensity ratio of the ion at *m/z* 532 ($[M + Na]^+$) to the ion at *m/z* 530 ($[M + Na]^+$) is almost unchanged (spectra not shown), indicating that the two species are not sensitive to acidic attack. **Figure 5** shows product ion spectra of $[M + Li]^+$ of the two species (at *m/z* 516 (**Figure 5A**) and at *m/z* 514 (**Figure 5B**)). The absence of a fragment at *m/z* 215 in both spectra, which could be formed by the loss of the corresponding 1-alk-1-enol group (compared with the appearance of a peak at *m/z* 187 in the product ion spectrum (**Figure 4B**) of the $[M + Li]^+$ precursor of the plasmenyl 16:0 species, confirms that the both ether LysoPC species are plasmanyl LysoPC species. However, the double bond position within the plasmanyl 18:1 species, corresponding to the ion at *m/z* 514 $[M + Li]^+$, remains unknown. The ether linkage of a minor ether LysoPC species cannot be further identified properly, but the peak at *m/z* 474 probably corresponds to plasmenyl 14:0.

Location of Fatty Acid Chains in PC Species. It has been reported (*18*) that the location of diacyl chains in PS and PA species can be determined by MS/MS analyses of their deprotonated molecules based on a general rule that the intensity ratio of the two carboxylate anions ($[R_1COO]^-/[R_2COO]^-$) is greater than 1. We also reported (*19*) that the structure of ether phospholipid species, including 1-alkyl-2-acyl and 1-alk-1′-enyl-2-acyl, can be characterized by (i) the presence of a pair of informative product ions corresponding to [phosphatidic acid – H – $R_2COOH]^-$ (abundant) and [phosphatidic acid – H – $R'_2CHC{=}O]^-$ (weak) and (ii) the absence of the carboxylate anion due to $[R_1COO]^-$ in their mass spectra, which are generated by MS/MS analyses of the $[M - H]^-$ precursors of PA and PS species. Although informative fragmentation on the location of fatty chains in PC species can also be generated by MS/MS analyses of $[M - 15]^-$ precursors of PCs (*20, 21*), it is difficult to characterize structures of minor and ether species because of the relatively lower intensities of the precursors, compared with those of the $[M - H]^-$ ions of PA and PS species. Although a new approach to identify the location of fatty chains in PC species by MS/MS analyses of $[M + COOCH_3]^-$ precursors has been reported (*22*), a special C_{30}-HPLC column is needed in the analysis.

Figure 6 shows negative-ion ESI mass spectra of transphosphatidylated PA (**A**) and PS (**B**), corresponding to the molecular species listed in **Table 2**. **Figure 7** shows product ion spectra of 38:6 PA (*m/z* 719) and 38:6 PS (*m/z* 806), generated by

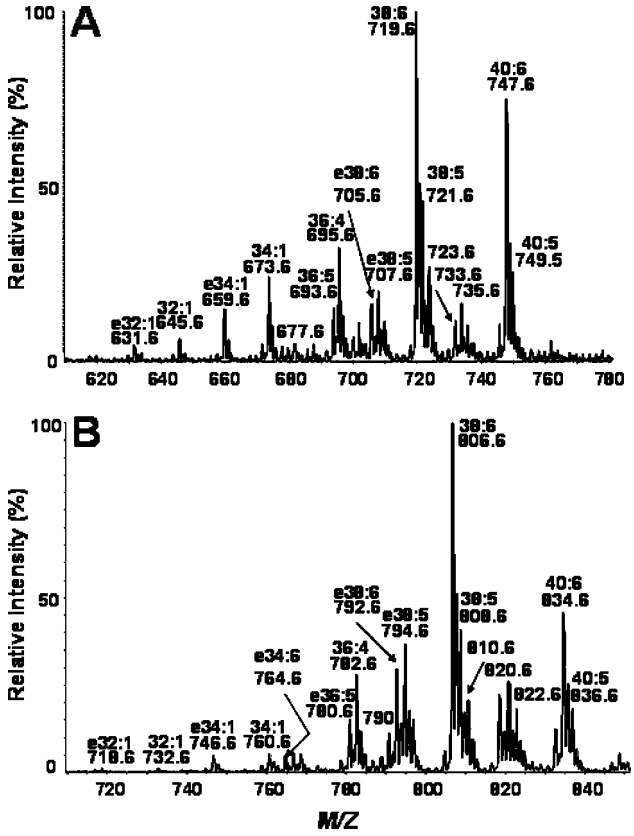


Figure 6. Negative-ion ESI mass spectra of (**A**) transphosphatidylated PA species and (**B**) transphosphatidylated PS species (see Table 2 for the identification of all molecular species of PA and PS corresponding to their deprotonated molecules $[M - H]^-$; e means ether; for example, e38:6 means ether-38:6 and e18:1 means ether-18:1).

negative-ion MS/MS of the two deprotonated molecules. Carboxylate fragments at *m/z* 255 and *m/z* 327 correspond to a 16-carbon fatty acid and a 22-carbon fatty acid with six double bonds, and the abundance of the ion at *m/z* 255, which is derived from the *sn* – 1 position, is greater than that of the peak at *m/z* 327, which is derived from the *sn* – 2 position (*18*). Fragment ions at *m/z* 391 and *m/z* 409 relate to ion structures of $[M - H - R_2COOH]^-$ or $[M - H - 87 - R_2COOH]^-$ and $[M - H - R'_2CHC{=}O]^-$ or $[M - H - 87 - R'_2CHC{=}O]^-$, along with peaks at *m/z* 463 ($[M - H - R_1COOH]^-$ or $[M - H - 87 - R_1COOH]^-$) and *m/z* 481 ($[M - H - R'_1CHC{=}O]^-$). Based on deprotonated molecules of PA and PS and MS/MS-generated product ions of the $[M - H]^-$ precursors, this species is identified as 1-hexadecanoyl-2-docosahexaenoyl-PC (16:0/22:6 PC).

Figure 8 shows product ion mass spectra of ether 38:6 PA (*m/z* 705) and ether 38:6 PS (*m/z* 792) obtained by MS/MS analyses of the two $[M - H]^-$ precursors. Based on the fragments at *m/z* 377 ($[M - H - R_2COOH]^-$ or $[M - H - 87 - R_2COOH]^-$; abundant) and *m/z* 395 ($[M - H - R'_2CHC{=}O\ ]^-$ or $[M - H - 87 - R'_2CHC{=}O\ ]^-$; weak), as well as peaks at *m/z* 403 ($[M - H - R_2COOH]^-$ or $[M - H - 87 - R_2COOH]^-$; abundant) and *m/z* 421 ($[M - H - R'_2CHC{=}O]^-$ or $[M - H - 87 - R'_2CHC{=}O]^-$; weak) in the spectra (also see **Scheme 1**), it suggests that the $[M - H]^-$ precursor corresponds to the two different molecular species, indicating the existence of a 22-carbon fatty acid with six double bonds, a 20-carbon fatty acid with five double bonds at the *sn* – 2 position, and two different ether fatty chains linked to the *sn* – 1 position. Taken together with structural information on deprotonated molecules of PA and PS, MS/MS-generated fragment ions, and the bond linkage of ether PC species, the two species are tentatively identified as 1-hexadecyl-2-docosahexaenoyl-PC (alkyl-16:0/22:6 PC; major) and 1-octadecenyl-2-eicosapentaenoyl-PC (alkyl-18:1/20:5 PC; minor).

Figure 9 is the product ion mass spectra of *m/z* 707 (ether-38:5 PA) and *m/z* 794 (ether-38:5 PS). On the basis of two MS/MS-generated pairs of fragments of PA and PS at *m/z* 403 ($[M - H - R_2COOH]^-$ or $[M - H - 87 - R_2COOH]^-$; abundant) and *m/z* 421 ($[M - H - R'_2CHC{=}O]^-$ or $[M - H - R'_2CHC{=}O]^-$; weak), as well as *m/z* 377 ($[M - H - R_2COOH]^-$ or $[M - H - 87 - R_2COOH]^-$; abundant) and *m/z* 395 ($[M - H - R'_2CHC{=}O]^-$ or $[M - H - 87 - R'_2CHC{=}O]^-$; weak), the two species are tentatively identified as 1-octadecenyl-2-eicosatetraenoyl-PC (a18:1/20:4 PC) and 1-hexadecyl-2-docosapentaenoyl-PC (alkyl-16:0/22:5 PC).

Furthermore, other representative polyunsaturated PC species are tentatively identified as 1-heptadecanoyl-2-docosahexaenoyl-PC (17:0/22:6; **Figure 10A**) and 1-octadecyl-2-docosahexaenoyl-PC (alkyl-18:0/22:6; **Figure 10A**), corresponding to the deprotonated molecule of PS at *m/z* 820 ($[M - H]^-$) and MS/MS-generated fragments at *m/z* 269 ($[R_1COO]^-$; 17:0; a weak peak derived from 17:0/22:6 only), *m/z* 405 ($[M - H - 87 - R_2COOH]^-$; an abundant fragment), and*m/z* 423 ($[M - H - 87 - R'_2CHC{=}O]^-$; a weak peak), as well as 1-octadecenoyl-2-docosahexaenoyl-PC (18:1/22:6; **Figure 9B**), which is characterized by deprotonated molecule of PS at *m/z* 832 ($[M - H]^-$) and MS/MS-generated product ions at *m/z* 281 ($[R_1COO]^-$; 18:1; most abundant), *m/z* 417 ($[M - H - 87 - R_2COOH]^-$; abundant), and *m/z* 435 ($[M - H - 87 - R'_2CHC{=}O]^-$; weak). Table 2 lists the molecular species thus deduced for the shark liver PC obtained by MS/MS analyses of deprotonated molecules of transphosphatidylated PA and PS species.

Potential Use of Shark Liver PC and LysoPC. The relatively high percentage of long chain polyunsaturated fatty acid-containing ether PC species, including plasmanyl and plasmenyl DHA-PC, and DHA-LysoPC species in shark liver is reported for the first time in this paper. An alternative approach, based on published methods (*17–19*), for determining the fatty acid location of various molecular species of shark liver PC by tandem mass spectrometric analyses of deprotonated molecules of the transphosphatidylated PA and PS species is described as well. The advantage of the approach is that MS/MS-generated fragments are informative for structurally identifying various ether PA and PS species. The formation mechanism of such lipid profiles present in shark liver is not a major point of discussion here. However, our findings have provided information that DHA ether PC and DHA LysoPC species are enriched in shark liver. Compared with DHA-enriched PC species extracted from microalgae (*7*), squid skin (*23, 24*), and the liver of fresh water fish (*25*), the percentages of 16:0/22:6 and 14:0/22:6 species in the above materials are higher than those of the PCs from shark liver. Furthermore, microalgae PC species contain no arachidonic and eicosapentaenoic acids (*7*).

The long-term goal of our studies is to study and develop shark liver PC and LysoPC as carriers for the delivery of DHA to the brain. Based on metabolic roles of ether phospholipids and lysophospholipids, DHA carried by ether DHA-PC and DHA-LysoPC could be transported to the brain faster than DHA carried by DHA-PC (*26–30*). After DHA is ingested as shark liver PC, most of the DHA-ether PC species may cross through the intestinal barrier easily (*31*), because the hydrolysis rate of DHA-ether PC by phospholipases can be significantly slowed down (*10*), and most of the species may not be involved in a deacylation and reacylation cycle. Shark liver LysoPC enriched

Table 2. Product Ions of Collision-Induced Dissociation of $[M - H]^-$ Ions of Transphosphatidylated Phosphatidylserine and Phosphatidic Acid, and the Assignment of PC Molecular Species from Shark Liver

$[M - H]^-$	$[M - H]^-$	$[PA - R'_1CHC{=}O]^-$	$[PA - R_1COOH]^-$	$[PA - R'_2CHC{=}O]^-$	$[PA - R_2COOH]^-$	$[R_1COO]^-$	$[R_2COO]^-$	PC species assignment	$[M + H]^+$
718 (PS)	631 (PA)			365 (w)[a]	347 (a)[b]			p14:0/18:1[c,d]	718
732 (PS)	645 (PA)			381 (w)	363 (a)	239		14:0/18:1	732
746 (PS)	659 (PA)			395 (w)	377 (a)		281	a16:0/18:1[e]	746
760 (PS)	673 (PA)	435	417	409 (w)	391 (a)	255 (a)	281 (w)	16:0/18:1	760
764 (PS)	677 (PA)			367 (w)	349 (a)			a14:0/22:6	764
766 (PS)	679 (PA)			367 (w)	349 (a)			a14:0/22:5	766
780 (PS)	693 (PA)	455	437	409 (w)	391 (a)	255 (a)	301 (w)	16:0/20:5	780
782 (PS)	695 (PA)	457	439	409 (w)	391 (a)	255 (a)	303 (w)	16:0/20:4	782
790 (PS)	703 (PA)			393 (w)	375 (a)			p16:0/22:6	790
792 (PS)	705 (PA)			395 (w)	377 (a)		327	a16:0/22:6	792
				421 (w)	403 (a)		327	a18:1/205	
794 (PS)	707 (PA)			421 (w)	403 (a)			a18:1/20:4	794
				395 (w)	377 (a)			a16:0/22:5	
806 (PS)	719 (PA)	481	463	409 (w)	391 (a)	255 (a)	327 (w)	16:0/22:6	806
808 (PS)	721 (PA)			409 (w)	391 (a)	255		16:0/22:5	808
810 (PS)	723 (PA)			437 (w)	419 (a)	283 (a)	303 (w)	18:0/20:4	810
818 (PS)	731 (PA)			421 (w)	403 (a)			a18:1/22:6	818
820 (PS)	733 (PA)			423 (w)	405 (a)			a18:0/22:6	820
				423 (w)	405 (a)	269		17:0/22:6	
822 (PS)	735 (PA)			423 (w)	405 (a)			a18:0/22:5	822
832 (PS)	745 (PA)			435 (w)	417 (a)	281		18:1/22:6	832
834 (PS)	747 (PA)	481	463	437 (w)	419 (a)	283 (a)	327 (w)	18:0/22:6	834
836 (PS)	749 (PA)			437 (w)	419 (a)	283		18:0/22:5	836

[a] w: weak ion. [b] a: abundant ion (see refs 18, 19, and 21 in the text for information about product ions generated by CID MS/MS of PA and PS). [c] *n:m/N:M* (for example 16:0/18:2), where *n* is the total number of carbons in the *sn* – 1 position, *m* is the total number of double bonds in the fatty acid chain at the *sn* – 1 position, *N* is the total number of carbons in the *sn* – 2 position, and *M* is the total number of double bonds in the fatty acid chain at the *sn* – 2 position. [d] In the notation p16:0/16:0, p means plasmalogen (1-alk-1′-enyl or plasmenyl) linkage. [e] In the notation a16:0/18:1, a means alkyl linkage (1-*O*-alkyl or plasmanyl).

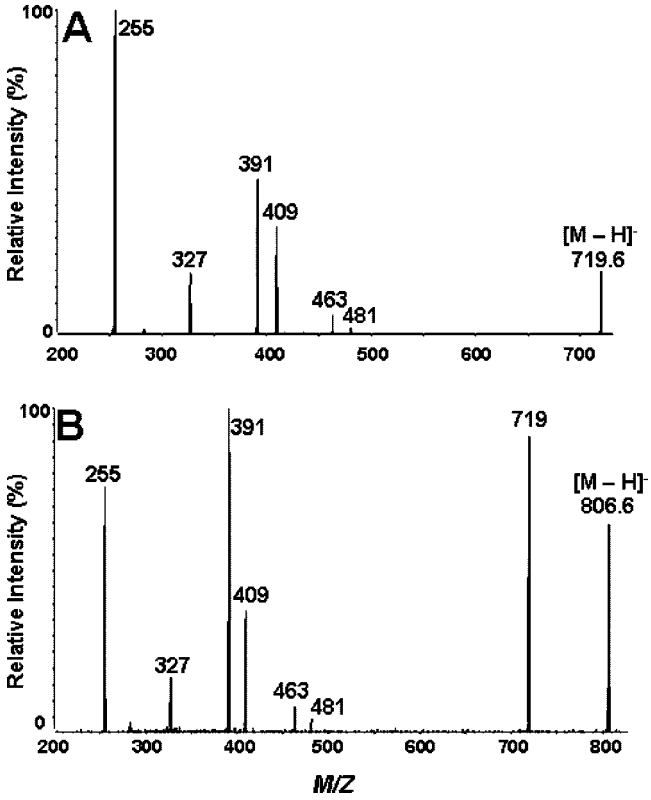


Figure 7. Product ion MS/MS spectra of $[M - H]^-$ precursors of (**A**) *m/z* 747 in **Figure 5A** due to transphosphatidylated 38:6 PA and of (**B**) *m/z* 806 in **Figure 5B** corresponding to transphosphatidylated 38:6 PS.

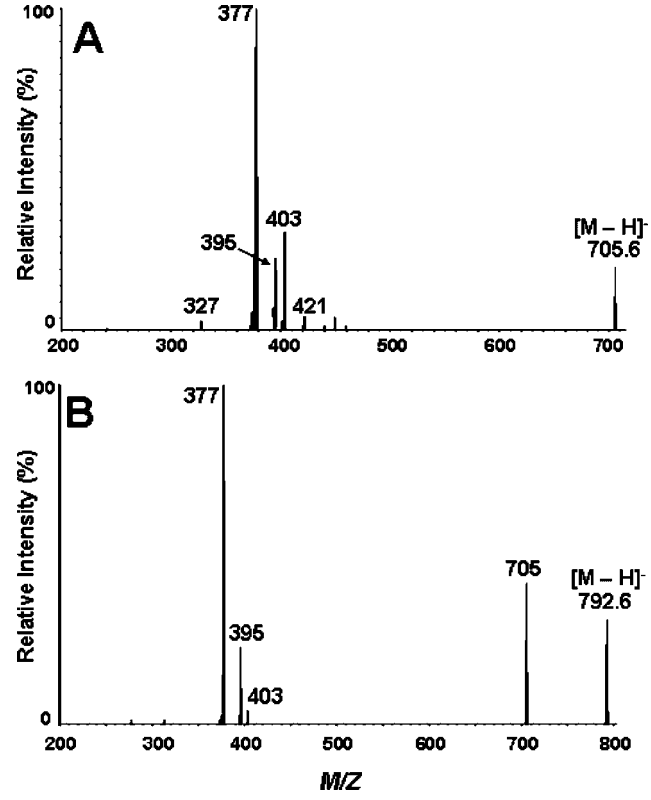
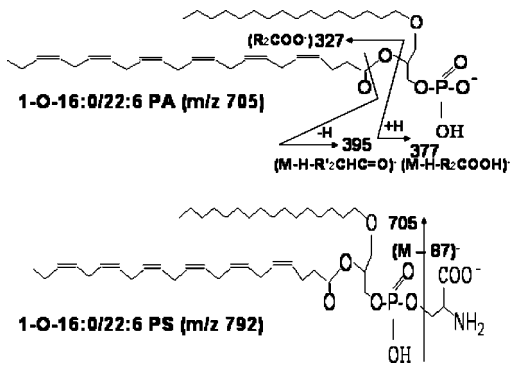


Figure 8. Product ion MS/MS spectra of the $[M - H]^-$ precursors of (**A**) *m/z* 705 in **Figure 5A** due to transphosphatidylated ether-38:6 PA and of (**B**) *m/z* 792 in **Figure 5B** corresponding to transphosphatidylated ether-38:6 PS.

in DHA could be "a fastest vehicle" for delivery of DHA to the brain. An early *in vivo* study (*32*) has demonstrated that, after intravenous injection of labeled LysoPC–albumin complex to adult squirrel monkeys, the exogenous labeled lipids can reach the brain in just 20 min. About 70% of the injected LysoPC species are taken up in the brain and then are acylated to form PC species. It has been reported that DHA-PC in neurons can be converted to DHA-PS by the PS synthase-1 (*33*). An *in vitro* experiment has demonstrated

Scheme 1. Fragmentation Pathway of 1-*O*-Alkyl-2-acyl-PA and -PS



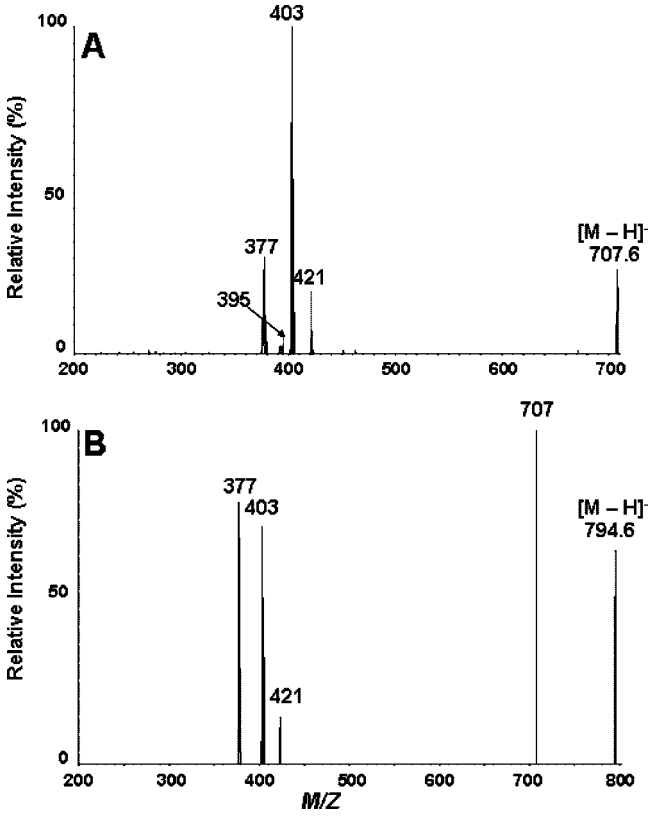


Figure 9. Product ion MS/MS spectra of the $[M - H]^-$ precursors of (**A**) *m/z* 707 in **Figure 5A** due to transphosphatidylated ether-38:5 PA and of (**B**) *m/z* 794 in **Figure 5B** corresponding to transphosphatidylated ether-38:5 PS.

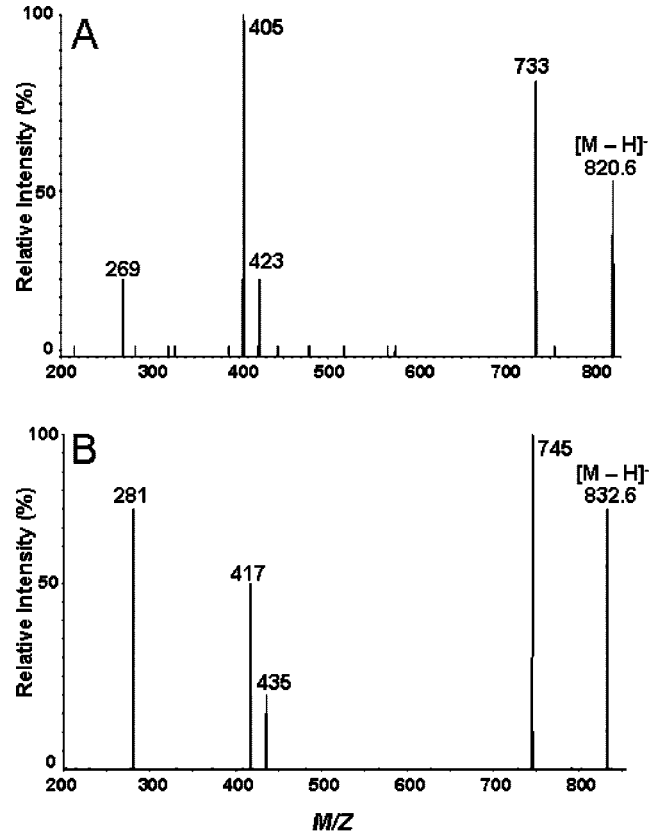


Figure 10. Product ion MS/MS spectra of $[M - H]^-$ precursors of (**A**) *m/z* 820 in **Figure 5B** due to transphosphatidylated ether-40:6 PS and 39:6 PS and of (**B**) *m/z* 832 in **Figure 5B** corresponding to transphosphatidylated 40:7 PS.

that the accumulation of DHA-PS in Neuro-2A cells may protect the neurons from apoptotic death (*34*).

In conclusion, both the profile and the fatty acid location of the PC and LysoPC species extracted from shark liver were tentatively determined by LC/ESI-MS and MS/MS. Our findings establish that shark liver is a natural resource enriched in not only DHA-PC but also ether DHA-PC and DHA-LysoPC. We propose that the latter two may be new types of carriers of DHA to the brain. Furthermore, studies on *in vitro* metabolism of shark liver PC and LysoPC with treatment of various phospholipases A_2, human LCAT, and human endothelial lipase are in progress in our laboratories.

ABBREVIATIONS USED

PC, phosphatidylcholine; LysoPC, lysophosphatidylcholine; PS, phosphatidylserine; PA, phosphatidic acid; DHA, docosahexaenoic acid; 17:0/22:6, 1-heptadecanoyl-2-docosahexaenoyl; a18:0/22:6, 1-octadecyl-2-docosahexaenoyl; a16:0/22:6, 1-hexadecyl-2-docosahexaenoyl; a18:0/20:5, 1-octadecyl-2-eicosapentaenoyl; a18:0/20:4, 1-octadecyl-2-eicosatetraenoyl; a16:0/22:5, 1-hexadecyl-2-docosapentaenoyl; p16:0/22:6, 1-(1*Z*-hexadecenyl)-2-docosahexaenoyl; 18:1/22:6, 1-octadecenoyl-2-docosahexaenoyl; LC/ESI-MS, liquid chromatography/electrospray ionization mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; ESI-MS/MS, electrospray ionization tandem mass spectrometry; MS/MS, tandem mass spectrometry; Q-TOF, quadrupole time of flight; HDL, high-density lipoprotein; LCAT, lecithin-cholesterol acyltransferase; BBB, blood–brain barrier.

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