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Structural elucidation of molecular species of pacific oyster ether amino phospholipids by normal-phase liquid chromatography/negative-ion electrospray ionization and quadrupole/multiple-stage linear ion-trap mass spectrometry

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Abstract

Although marine oysters contain abundant amounts of ether-linked aminophospholipids, the structural identification of the various molecular species has not been reported. We developed a normal-phase silica liquid chromatography/negative-ion electrospray ionization/quadrupole multiple-stage linear ion-trap mass spectrometric (NPLC-NI-ESI/Q-TRAP-MS^3) method for the structural elucidation of ether molecular species of serine and ethanolamine phospholipids from marine oysters. The major advantages of the approach are (i) to avoid incorrect selection of isobaric precursor ions derived from different phospholipid classes in a lipid mixture, and to generate informative and clear MS^n product ion mass spectra of the species for the identification of the *sn*-1 plasmanyl or plasmenyl linkages, and (ii) to increase precursor ion intensities by "concentrating" lipid molecules of each phospholipid class for further structural determination of minor molecular species. Employing a combination of NPLC-NI-ESI/MS^3 and NPLC-NI-ESI/MS^2, we elucidated, for the first time, the chemical structures of docosahexaenoyl and eicosapentaenoyl plasmenyl phosphatidylserine (PS) species and differentiated up to six isobaric species of diacyl/alkylacyl/alkenylacyl phosphatidylethanolamine (PE) in the US pacific oysters. The presence of a high content of both omega-3 plasmenyl PS/plasmenyl PE species and multiple isobaric molecular species isomers is the noteworthy characteristic of the marine oyster. The simple and robust NPLC-NI-ESI/MS^n-based methodology should be particularly valuable in the detailed characterization of marine lipid dietary supplements with respect to omega-3 aminophospholipids.

Keywords

Ether phospholipids; Ether phosphatidylserine; Ether phosphatidylethanolamine; LC/Q-TRAP-MS^3; US pacific oyster; Omega 3 phospholipids; Omega 3 lipid dietary supplement

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1. Introduction

Lipids from marine animals contain abundant amounts of polyunsaturated fatty acids (PUFAs), in particular the omega-3 PUFAs including docosahexaenoic acid (DHA; 22:6 ω-3) and eicosapentaenoic acid (EPA; 20:5 ω-3). Although the omega-3 fatty acids are essential for life, their bioavailability is low when delivered by oral route using traditional triglyceride forms of administration, especially for promoting brain functions [1–4]. Liposomes prepared from natural lipid mixtures extracted from marine animals appeared to be superior candidates for DHA/EPA supplementation. The greater bioavailability of omega-3 PUFAs measured after phospholipid liposome ingestion, compared with triglycerides ingestion, is supported by a number of animal and clinical studies [5–7]. It has been also reported that decreases in brain plasmalogen phospholipids, one of the earliest biochemical changes detected in Alzheimer disease (AD), are specific to brain regions with AD pathology [8], and that the plasmalogen ether phospholipid precursors can be supplied by dietary supplements. Therefore, the exploration of such marine lipid sources that could supply both plasmalogen and omega-3 fatty acid precursors is of importance in the fields of marine biology, food chemistry and nutritional science.

Marine oysters contain abundant ether molecular species of aminophospholipids [9–13] including plasmanyl/plasmenyl PS, plasmanyl/plasmenyl PE and related lysophospholipids (see Fig. 1 for structures). The detailed structural identification of the various molecular species, however, has not been reported. Although a combination of liquid and gas chromatography or liquid chromatography/mass spectrometry (LC/MS) has been employed in the analysis of phospholipids, it is difficult or even impossible to distinguish isobaric ether species [14]. An LC/MS based methodology for the structural elucidation of various ether molecular species of aminophospholipids has not been established. For example, an approach for the structural differentiation of plasmenyl PS (1-O-alk-1′-enyl-2-acyl PS) from plasmanyl PS (1-O-alkyl-2-acyl PS) is needed.

Tandem mass spectrometry of intact lipids is a highly specialized technique that has been widely used in structural elucidation of molecular species of various ether phospholipids [15–17]. The major advantage of this technique is to be able to generate informative molecular and fragment ions for characterization of an intact lipid molecule, its polar-head group, and composition and location of radyl linkages at the *sn*-1 and *sn*-2 positions of the glycerol backbone [18,19]. Zemski and Murphy [20] reported an approach to identify plasmenyl PE in a lipid mixture by normal-phase LC separation followed by the MS^2 analysis of their protonated molecules. Furthermore, Hsu and Turk described a series of the positive-ion ESI tandem mass spectrometric approaches for the structural elucidation of ether phosphatidylcholine (PC) [21,22], ether lysophosphatidylcholine (LysoPC) [21,22] and ether PE [23] by collisionally activated dissociation of their lithiated adduct precursor ions coupled to multiple-stage ion-trap mass spectrometry. Although identification of marine plasmanyl and plasmenyl molecular species can be performed directly by the above methods, it is time-consuming to purify the lipids from biological samples. It is also difficult to elucidate the chemical structures of ether aminophospholipids species such as PS in a lipid mixture using the positive-ion ESI/MS^n.

The negative-ion ESI/MS is a sensitive method to structurally analyze PE [24], PS [25,26], phosphatidylinositol (PI) [27], phosphatidic acid [28], phosphatidylglycerol [29], cardiolipins [30] and the corresponding lysophospholipids [31–33]. Furthermore, Hsu and Turk [34] have shown that the negative-ion ESI coupled to multiple-stage ion-trap mass spectrometry can be used to differentiate molecular species of plasmanyl PE and plasmenyl PE. Both alkenoxide anions ($[O–CH{=}CH–R_1]^-$) and a glycerol backbone based phosphoethanolamine ion in the MS^3 product ion spectra, which are generated from $[M–H]^-$

→ $[M–H–R'_2–CH{=}C{=}O]^-$ precursor ions, are diagnostic fragments for characterizing the *sn*-1 plasmenyl linkage, whereas the above fragment ions are absent in the MS^3 product ion spectra of plasmanyl PE [34]. Although the MS^3 product ion profile of plasmanyl PS has been reported [34], a full process to differentiate plasmenyl PS from plasmanyl PS has not yet been described.

Shotgun lipidomics combined with MS^2 precursor/neutral loss scans [35,36] or MS^n analyses have been shown to be powerful in profiling of phospholipid species in biological samples without LC separation of various lipid classes [37]. The major disadvantages of this method, however, are that: (i) it is possible to generate mixed product ion spectra of isobaric precursor ions, which are derived from different phospholipid classes present in a crude lipid extract, and (ii) it is difficult to determine the structures of minor molecular species in each phospholipid class. For example, abundant $[M-15]^-$ peaks of PC species can be generated by the negative-ion ESI/MS, but the isobaric precursors of PE species also appear in the mass range of 700–850 Da when crude lipids are directly infused into the mass spectrometer without LC separation of lipid classes. For this reason, the normal-phase LC separation of individual phospholipid classes from a crude marine lipid extract prior to MS^n analyses is necessary.

Herein, we first investigated the MS^3 product ion pattern of oyster plasmenyl PS species by the normal-phase liquid chromatography negative ion ESI/quadrupole-multiple stage linear ion-trap mass spectrometry (NPLC-NI-ESI/Q-TRAP-MS^3), and then established a full product ion fragmentation pathway for the structural differentiation of plasmenyl PS from plasmanyl PS. A combination of NPLC-NI-ESI/MS^3 and NPLC-NI-ESI/MS^2 is shown to be particularly useful in the structural elucidation of various molecular species of ether aminophospholipids extracted from the US pacific oysters, including (i) characterization of the *sn*-1 radyl linkage, (ii) composition of the *sn*-2 fatty acid chains, (iii) differentiating multiple isobaric isomers of a diacyl/alkylacyl/alkenylacyl molecular species mixture, and (iv) elucidating chemical structures of minor molecular species in a lipid mixture. Because PS and PE are the major lipid classes and contain abundant ether molecular species in some marine animals, the robust NPLC-NI-ESI/MS^n-based methodology described here is particularly valuable in aminophospholipidomics of marine animals.

2. Materials and methods

2.1. Materials

Analytical grade methyl tert-butyl ether, chloroform, methanol, ammonium chloride and HPLC grade water were obtained from VWR Company (Radnor, PA, USA).

2.2. Lipid extraction

The US pacific oysters (*Crassostrea sikamea* and *Crassostrea gigas*) were obtained from an oyster farm in the State of Oregon in the US (Oregon Oyster Farm Inc., Newport, OR, USA). The animals were cultivated in seawater (between 4 and 10 °C) with a natural diet of mixed microalgae everyday. The oysters were transported alive to our laboratory on ice overnight, and the whole body was collected the following day after removal of the shell. After homogenization with an electric blender, the total lipids were extracted three times with methyl tert-butyl ether (MTBE) according to the published method [38]. The upper phase was collected and dried under a nitrogen stream. The samples were re-dissolved in MTBE and stored at −20 °C after gently flushing with argon and were analyzed within 2 weeks of preparation. The major advantages of using MTBE over classical chloroform/methanol procedures include (i) better recoveries for most major lipid classes; and (ii) cleaner lipid extracts for mass spectrometric analyses of various phospholipids.

2.3. Acid treatment of extracted crude lipids

For the general identification of plasmenyl PS and plasmenyl PE in crude lipids, acid treatment was performed by exposure of the total lipids extract of the oyster (*C. sikamea*) to acetic acid fumes. Approximately 100 μg of the lipids in MTBE were dispersed on a small silica gel TLC plate (Silica Gel 60; 2.5 cm × 7.5 cm; EMD Chemicals Inc., Gibbstown, NJ, USA), and then the dried plate was placed at room temperature for 4 h in a small glass container with saturated acetic acid fumes. The lipids were then extracted twice from the silica gel using the published method [38], and were analyzed by the NPLC/NI-ESI/MS^n as described below.

2.4. Experimental conditions of NPLC/negative-ion ESI/MS^n

The NPLC/NI-ESI/MS was performed on an AB Sciex 5500 Q-TRAP hybrid mass spectrometer (Applied Biosystems, Foster City, CA, USA). The sample containing 1–5 (g of the lipids in 5–10 (l of MTBE was injected on to a silica normal-phase HPLC column (Ultra II 3-(m, Restek silica column (2.1 mm × 150 mm), Bellefonte, PA, USA) connected to an Agilent 1260 HPLC system. The phospholipids PE, PI/LysoPE, PS, PC and LysoPC are well separated on this column with the following program: 100% solvent A (chloroform/methanol/2 mM aqueous ammonium chloride, 80:19.5:0.5 by vol.) from 0 to 5 min; then a linear gradient of 100% solvent A to 100% solvent B (chloroform/methanol/water/2 mM aqueous ammonium chloride, 60:34.5:5:0.5; by vol.) from 5 to 30 min; and 100% solvent B from 30 to 35 min [39]. The flow rate was 0.35 ml min^{-1}. The ESI source temperature was set at 350 °C. The mass scan range used was from 200 to 1100 Da. Data acquisition and processing were controlled using the Analyst 1.5 software (Applied Biosystems, Foster City, CA, USA).

NPLC/NI-ESI/MS^n analyses of the oyster phospholipids were performed on the AB Sciex 5500 Q-TRAP hybrid mass spectrometer (Applied Biosystems, Foster City, CA, USA) as mentioned above. Lipid samples, corresponding to 10–50 μg of the lipids in 10–20 μl of MTBE, were introduced on to the silica HPLC column, and separated as above. NPLC/NI-ESI/MS^2 and/MS^3 analyses of PE, LysoPE and PS were performed with three stages (see below). Based on results obtained by the NPLC/NI-ESI/MS of oyster PE, LysoPE and PS, the precursor ions for the MS^2 and MS^3 analyses were selected. Collision energy for the MS^2 and MS^3 experiments were set at 35 eV and 40–45 eV, respectively. The mass scan range used was from 100 to 900 Da or from 100 to 500 Da.

2.5. Experiment for percentage profile of molecular species

The percentage profiles of molecular species of oyster PS, PE and LysoPE were performed by the LC/MS as described above. Approximately 2 μg of the lipids were injected into the column. Results were generated from the average intensities of $[M–H]^-$ ions of the molecular species on the basis of three replicate experiments. Various molecular species ionize to different extents, but the linearity of response of each molecular species can be found in a lower range of concentrations [40]. The reproducibility of the analyses was within 15% for the major species and better than 20% for the minor species. Based on the average intensities of $[M–H]^-$ ions, only percentage profiles [18,39,25] of molecular species of the oyster aminophospholipids were given in Tables 1, 3 and 5.

3. Results and discussion

The objective of the present study was to develop a sensitive and effective method for the structural elucidation of ether molecular species of aminophospholipids in marine animals, particularly the differentiation of the plasmanyl and plasmenyl linkages in the molecules. Because the marine lipid extracts are rich in phospholipids [2] and the aminophospholipids

and their ether molecular species are present as the major components in marine oysters [9–13], the normal-phase LC separation of individual phospholipid classes from each other prior to MS^n analyses is necessary, in order to avoid selecting isobaric precursor ions derived from different phospholipid classes present in the crude lipid mixture. This should also "concentrate" minor molecular species in a phospholipid class for the further MS^n analysis. Due to its high sensitivity and the additional information it provides, the negative-ion ESI/Q-TRAP-MS^n has been demonstrated to be a superior mass spectrometric approach to both qualitatively and quantitatively analyze various ether phospholipids [41–43], especially the characterization of plasmanyl/plasmenyl linkages at the *sn*-1 position of the glycerol backbone, and the analysis of minor species molecules in each phospholipid class.

3.1. Chromatographic separation of the lipids

Fig. 2a is the representative NPLC/NI-ESI/MS profile of the US pacific oyster *C. sikamea* lipids. Aminophospholipids were separated and eluted between 4 and 7 min (PE), 12 and 14.5 min (lysophosphatidylethanolamine (LysoPE)/PI), and 14.5 and 17 min (PS). A peak (marked with *), which was eluted between PE and LysoPE/PI, was tentatively identified as ceramide aminoethylphosphonate [9]. Fig. 2b shows the representative NPLC-NI-ESI/MS^3 (for PE and PS) and NPLC-NI-ESI/MS^2 profiles (for LysoPE) of the phospholipids. Although LysoPE and PI were co-eluted, the deprotonated molecules of LysoPE (in the mass range of 400–550 Da) and PI (in the mass range of 800–1000 Da) did not overlap each other.

3.2. Differentiation of molecular species by chemical treatment and mass determination

Molecular weights of pacific oyster *C. sikamea* PS species generated by the NPLC-NI-ESI/MS were inferred from their deprotonated molecules ($[M–H]^-$) and $[M–H-87]^-$ fragments [42], as shown in Fig. 3a. Twelve deprotonated molecules were tentatively designed as (i) diacyl PS species including 38:6 (*m*/*z* 806.6), 38:5 (*m*/*z* 808.6), 40:7 (*m*/*z* 832.6), 40:6 (*m*/*z* 834.6), 40:3 (*m*/*z* 840.6) and 40:2 (*m*/*z* 842.6); (ii) a second group due to plasmanyl PS/plasmenyl PS anions having 14 Da less than the diacyl PS species (see Table 1) including a38:6 or/and p38:5 (*m*/*z* 792.6 (806 – 14)), a38:5 or/and p38:4 (*m*/*z* 794.6 (808 – 14)), a38:2 or/and p38:1 (*m*/*z* 800.6), and a40:6 or/and p40:5 (*m*/*z* 820.6 (834 – 14)); and (iii) a third group relating to plasmenyl PS/plasmanyl PS anions having a 16 Da less than the diacyl PS species (see Table 1) including p40:6 or/and a40:7 (*m*/*z* 818.6 (834 – 16)), p40:5 or/and a40:6 (*m*/*z* 820.6 (836 – 16)), p40:2 or/and a40:3 (*m*/*z* 826.6 (842 – 16)). However, the plasmanyl and plasmenyl linkages in PS species cannot be differentiated in this procedure.

Differentiation of plasmenyl PS from others can be achieved by NPLC-NI-ESI/MS analysis of the lipids after acid treatment. Compared with ions in Fig. 3a, it is seen clearly in Fig. 3b that the intensities of $[M–H]^-$ anions at *m*/*z* 818.6, 826.6, 792.6, 794.6, and 800.6 were markedly lower or even absent after acid treatment, suggesting that the above deprotonated molecules correspond to the plasmenyl PS species.

3.3. NPLC/NI-ESI/MS^n to analyze plasmenyl PS/plasmanyl PS species

3.3.1. Fragmentation process of plasmenyl PS species by Q-TRAP-MS^3—In order to further differentiate the plasmenyl PS from plasmanyl PS species, the precursor ion at *m*/*z* 818.6 was analyzed by NI-ESI/MS^2 and NI-ESI/MS^3. Fig. 4a shows the MS^2 product ion spectrum of the precursor at *m*/*z* 818.6, corresponding to p40:6 PS or/and a40:7 PS species (also see Fig. 3 and Table 1). The ion at *m*/*z* 731 represents the loss of a serine group from its deprotonated molecule at *m*/*z* 818. Peaks at *m*/*z* 421 and 403 are fragments due to $[M–H-87-R'_2\text{-}CH{=}C{=}O]^-$ ($[731 – (327 – 17)]^-$) and $[M–H-87-R_2COOH]^-$ ($[731 – 328]^-$), which are formed, respectively, by the loss of a DHA-ketene and a DHA chain esterified at the *sn*-2 position [44] from the ion at *m*/*z* 731. The structural characterization

was supported by the presence of a weak fragment at *m*/*z* 327, corresponding to 22:6 fatty acid (DHA). It also suggests that the deprotonated molecule contains only one acyl chain at the *sn*-2 position since anions derived from the ether linkage at the *sn*-1 position cannot be formed by the MS^2 of the $[M–H]^–$ precursor ions. However, MS^3 product ion spectra of both $[M–H]^– \rightarrow [M–H-87]^–$ (*m*/*z* 818 → 731; Fig. 4b) and $[M–H]^– \rightarrow [M–H\text{-}87\text{-}R_2COOH]^–$(*m*/*z* 818 → 403; Fig. 4c) provide the characteristics of alkenoxide structure in the molecule by giving rise to an abundant anion at *m*/*z* 267 $[O–CH{=}{=}CH\ C_{16}H_{33}]^–$, suggesting the existence of a plasmenyl 18:0 linkage at the *sn*-1 position of the glycerol backbone, whereas this ion is absent in the MS^3 product ion spectrum of either $[M–H]^– \rightarrow [M–H-87]^–$ or $[M–H]^– \rightarrow [M–H\text{-}87\text{-}R_2COOH]^–$ of plasmanyl PS molecule (1-O-alkyl linkage [34]; and also see below). The ion at *m*/*z* 153 relates to a diagnostic ion of phospholipids. Taking together above information, this major species was identified as plasmenyl 18:0/22:6 PS (p18:0/22:6 PS) (see Table 1). The Scheme 1a shows the MS^3 fragmentation process of plasmenyl PS.

Compared with the MS^3 product ion pathway of plasmenyl PE [34], beside the presence of alkenoxide fragments an abundant ion at *m*/*z* 196 (Scheme 1b), which is derived from $[M(PE)\text{-}H\text{-}R'_2\text{-}CH{=}{=}C{=}{=}O]^–$ precursor ion, can be also observed in the spectra as the second diagnostic fragment of plasmenyl PE structure (Scheme 1b), whereas this type of fragment, corresponding to the ion at *m*/*z* 239 (Scheme 1a), is absent in MS^3 product ion spectra of plasmenyl PS. It could be explained by the fact that the precursor ions due to $[M(PS)\text{-}H\text{-}R'_2\text{-}CH{=}{=}C{=}{=}O]^–$ cannot be formed from deprotonated molecules of PS species (Scheme 1a). However, the formation of abundant fragment $[M–H\text{-}87]^–$, corresponding to a loss of serine group from deprotonated molecules of PS species, are favorable (Scheme 1a), and the MS^3 of $[M–H]^– \rightarrow [M–H-87]^–$ can generate diagnostic fragments due to alkenoxide structures for differentiating plasmenyl PS from plasmanyl PS.

3.3.2. NPLC/NI-ESI-MS^n to analyze mixture of plasmanyl/plasmenyl PS species —Fig. 5a shows the NPLC/EI-ESI/MS^2 product ion spectrum of the PS precursor at *m*/*z* 820.6 (a40:6 or/and p40:5; also see Fig. 3). The ion at *m*/*z* 733 was formed by the loss of a serine group from its deprotonated molecule. The presence of anions at *m*/*z* 327 (FA 22:6) and 329 (FA 22:5) suggests that this is composed of the two PS species containing two different fatty acid chains esterified at the *sn*-2 position. However, the MS^3 product ion spectrum of $[M–H]^– \rightarrow [M–Serine]^–$ (*m*/*z* 820 → 733; Fig. 5b) gives a pair of fragments at *m*/*z* 403 and 267 $[O\ CH\ CH\ C_{16}H_{33}]^–$. One of the species was identified as plasmenyl 18:0/22:5 PS (p18:0/22:5 PS). Fig. 4c shows the MS^3 product ion spectrum of $[M–H]^– \rightarrow [M–Serine\text{-}R_2COOH]^–$ (*m*/*z* 820 → 405). An ion at *m*/*z* 405 is due to a plasmanyl 18:0 linkage [45]. A weak ion also appeared at *m*/*z* 269, which probably corresponds to a 17:0 fatty acid [18]. The two isobaric species isomers in the PS molecule were thus identified as plasmanyl 18:0/22:6 (a18:0/22:6 PS), and/or 17:0/22:6 diacyl PS.

Another major deprotonated molecule at *m*/*z* 826 (see Fig. 3 and Table 1) was identified as plasmenyl 18:0/22:2 PS (see Table 2 for supporting fragments for structural elucidation of the species), based on the fragment ions at *m*/*z* 335 (FA 22:2), 403 $[M–H\text{-}87\text{-}R_2COOH]^–$ and 267 $[O–CH{=}{=}CH–C_{16}H_{33}]^–$, which were obtained by the MS^3 of *m*/*z* 826 → 739 (spectra not shown). A previous study has also reported the presence of 22:2 (Δ7,15) as a major fatty acid in marine oyster ether phospholipids [10]..

3.3.3. Analysis of minor ether PS species by NPLC/NI-ESI-MS^n—Because the NPLC separation of individual phospholipid classes was performed before MS^n analyses, precursor ions due to HPLC-concentrated oyster PS minor species can be correctly selected and further structurally elucidated easily. Fig. 6a shows the MS^3 product ion spectrum of precursors at *m*/*z* 792 → 705 $[M–H\text{-}87]^–$. This anion is due to the two ether species,

identified as plasmenyl 18:0/20:5 PS (p18:0/20:5 PS), and plasmanyl 16:0/22:6 PS (a16:0/22:6 PS), respectively. The identification is supported by the appearance of fragments at *m*/*z* 421 [M–H-87-R_{2-1}-CH═C═O]$^-$, ([705 – (301 – 17)]$^-$), *m*/*z* 403 [M–H-87-R_{2-1}COOH]$^-$, (R_{2-n} is due to the different *sn*-2 fatty acid chains (*n* = 1–2)), ([705 – 302 (FA 20:5)]$^-$), and *m*/*z* 267 [O–CH═CH–$C_{16}H_{33}$]$^-$ for p18:0/20:5 PS; as well as *m*/*z* 395 [M–H-87-R_{2-2}-CH═C═O]$^-$ ([705 – (327 – 17)]$^-$), *m*/*z* 377 [M–H-87-R_{2-2}COOH]$^-$ ([705 – 328 (FA 22:6)]$^-$) or [plasmanyl 16:0]$^-$ ([405 (plasmanyl 18:0) – (CH_2CH_2)]$^-$) for a16:0/22:6 PS (Fig. 5a and Table 2). Fig. 6b and c are the MS^3 product ion spectra of the precursor ions at *m*/*z* 794 → 707 [794 – 87]$^-$ and *m*/*z* 800 → 713 [800 – 87]$^-$, respectively. The deprotonated molecule at *m*/*z* 794 (a38:5 PS or/and 37:5 diacyl PS) relates to a mixture of an alkylacyl and a diacyl species, identified as plasmanyl 18:0/20:5 PS (a18:0/20:5 PS), and 17:0/20:5 diacyl PS, based on fragments at *m*/*z* 423 [M–H-87-R'_2-CH═C═O]$^-$ ([705 – (301 – 17)]$^-$), *m*/*z* 405 [707 – 302 (FA 20:5)]$^-$ ([plasmanyl 18:0]$^-$) [45]; and *m*/*z* 269 (FA 17:0) [18]; the latter deprotonated molecule at *m*/*z* 800 corresponds to an alkenylacyl species, identified as plasmenyl 18:0/20:1 PS (p18:0/20:1 PS), supported by the presence of fragments at *m*/*z* 421 [M–H-87-R'_2-CH═C═O]$^-$ ([713 – (309 – 17)]$^-$), *m*/*z* 403 [M–H-87-R'_2COOH]$^-$ ([713 – 310 (FA 20:1)]$^-$) and *m*/*z* 267 [O–CH=CH–$C_{16}H_{33}$]$^-$ (Table 2). A previous study also reported the presence of 20:1 (n-11) as a major fatty acid in marine oyster ether phospholipids [10].

3.3.4. Analysis of PS species of oyster C. gigas by NPLC/NI-ESI-MS^n—The molecular species of PS from a second pacific oyster *C. gigas* were also characterized using the above approaches. The major ether PS species are similar to those found in the oyster *C. sikamea* PS, identified as plasmenyl 18:0/22:6 PS (p18:0/22:6 PS), plasmanyl 18:0/22:6 PS (a18:0/22:6 PS), and plasmenyl 18:0/22:2 PS (p18:0/22:2 PS), which correspond to their deprotonated molecules at *m*/*z* 818.6, 820.6 and 826.6, respectively. However, the minor plasmanyl PS species of oyster *C. gigas* are different from those of *C. sikamea* PS. In order to further characterize the minor PS species, the MS^2 precursor ion scan (PIS) was performed. Supplementary 1a and 1b shows the NPLC/MS^2 PIS of *m*/*z* 269 (due to the FA 17:0) to form deprotonated molecules at *m*/*z* 794 (17:0/20:5 diacy PS) and 820 (17:0/22:6 diacyl PS), as well as the PIS of *m*/*z* 405 (due to the plasmanyl 18:0 [45]) to generate deprotonated molecules at *m*/*z* 794 (plasmanyl 18:0/20:5 PS (a18:0/20:5 PS)), *m*/*z* 802 (plasmanyl 18:0/20:1 PS (a18:0/20:1 PS)), *m*/*z* 820 (plasmanyl 18:0/22:6 PS (a18:0/22:6 PS)), *m*/*z* 822 (plasmanyl 18:0/22:5 PS (a18:0/22:5 PS)) and *m*/*z* 828 (plasmanyl 18:0/22:2 PS (a18:0/22:2 PS)).

Table 1 lists the molecular species of both *C. gigas* and *C. sikamea* PS, in order to clearly show differences in both their chemical structures and percentages. Table 2 lists the MS^2 and MS^3 product ions of [M–H]$^-$ and [M–H]$^-$ → [M–H-87]$^-$ precursors of plasmenyl PS and plasmanyl PS molecular species of the two US pacific oysters identified by NPLC/NI-ESI/Q-TRAP-MS^n.

3.4. Analysis of diacyl and ether PE species by NPLC/NI-ESIMS^n

3.4.1. Profiling of oyster PE species—Fig. 7 shows NI-ESI mass spectra of the oyster *C. sikamea* PE before (a) and after (b) treatment by acetic acid fumes. Fifteen protonated molecules were detected and tentatively identified (Table 3) as (i) diacyl PE species including 36:6 (*m*/*z* 734.6), 36:5 (*m*/*z* 736.6), 38:7 (*m*/*z* 760.6), 38:6 (*m*/*z* 762.6), 38:5 (*m*/*z* 764.6), 40:7 (*m*/*z* 788.6), and 40:6 (*m*/*z* 790.6); (ii) a second group of anions having 14 Da less than the diacyl PE species including a38:6 or/and p38:5 (see Table 2 for the further explanation) (*m*/*z* 748.6; (760 – 14)), a38:5 or/and p38:4 (*m*/*z* 750.6; (764 – 14)), p40:6 (*m*/*z* 774.6; (788 – 14)), and a40:6 or/and p40:5 (*m*/*z* 776; (790 – 14)); and (iii) a third group of

anions at *m*/*z* 802 (p42:6; (774 + 28)) and *m*/*z* 800 (p42:7). However, the plasmanyl and plasmenyl linkages in the species cannot be further identified in this procedure.

3.4.2. Analysis of plasmanyl/plasmenyl molecular species—Hsu and Turk [34] have reported fragmentation processes for structurally differentiating plasmenyl PE from plasmanyl PE by the MS^3 of $[M–H]^- \rightarrow [M–H\text{-}R'_2\text{-}CH{=}C{=}O]^-$ precursor ions. On the basis of the fragment pathway (Scheme 1b) [34], we developed the NPLC/NI-ESI/Q-TRAP-MS^3 approach to structural elucidation of molecular species of the US pacific oyster PE. We also found that both plasmenyl and plasmanyl PE species are present in the oysters.

Compared with MS^2 product ion spectra of PS species, carboxylic anions due to the fatty acid chains are significantly abundant in PE spectra, and composition and location of the acyl chains can be identified readily, even in the minor molecular species. Fig. 8a shows the MS^2 product ion spectrum of a precursor anion at *m*/*z* 750 (a38:5 or/and p38:4 or/and 37:5). Abundant carboxylic anions at *m*/*z* 301 and 257 (301 – 44) [36], *m*/*z* 329, *m*/*z* 315, *m*/*z* 269 and *m*/*z* 255 correspond, respectively, to 20:5, 22:5, 21:5,17:0 and 16:0 fatty acid chains in the molecule. Anions at *m*/*z* 466 $[750\text{-}R'_{2-1}\text{-}CH{=}C{=}O]^-$, ($R'_{2-n}$ is due to the different *sn*-2 fattyacid chains (*n* = 1–3)), $[750 – (301 – 17)]^-$, 448 $[750\text{-}R_{2-1}COOH]^-$ ($[705 – 302$ (FA 20:5)$]^-$) and a fragment at *m*/*z* 405 (plasmanyl 18:0), formed by the MS^3 of *m*/*z* 750 → 466, all suggests the presence of plasmanyl 18:0/20:5 PE (a18:0/20:5 PE). Fragments at *m*/*z* 438 $[750\text{-}R'_{2-2}\text{-}CH{=}C{=}O]^-$ ($[750 – (329 – 17)]^-$) or [466 (plasmanyl 18:0) – (CH_2–CH_2)]$^-$ (Fig. 8a) indicates the presence of plasmanyl 16:0/22:5 PE (a16:0/22:5 PE) species. The combination of the MS^2 carboxylic fragments at *m*/*z* 301 (abundant; the *sn*-2-20:5) and 269 (weak; the *sn*-1-17:0) and the MS^3 product ions (Fig. 8b) at *m*/*z* 466 and 269 (the *sn*-1-17:0), as well as the MS^2 fragment ions (Fig. 8a) at *m*/*z* 315 (abundant; the *sn*-2-21:5), *m*/*z* 255 (weak; the *sn*-1-16:0) and *m*/*z* 452 $[750\text{-}R'_{2-3}\text{-}CH{=}C{=}O]^-$ ($[750 – (315 – 17)]^-$) further suggest the existence of the two diacyl species 17:0/20:5 PE and 16:0/21:5 PE. The presence of a small amount of heneicosapentaenoic acid (21:5 (ω-3) or Δ 6,9,12,15,18) has been reported in marine animals [9–13,46].

Fig. 9a shows the MS^2 product ion mass spectrum of a PE precursor ion at *m*/*z* 776.6. Carboxylic anions at *m*/*z* 329 and 285 (329 – 44), *m*/*z* 327 and 283 (327 – 44) [36], and *m*/*z* 301 relate, respectively, to 22:5, 22:6 and 20:5 fatty acids esters in the PE species. This identification was further supported by the appearance of ions at *m*/*z* 464 $[M–H\text{-}R'_{2-1}\text{-}CH{=}C{=}O]^-$, R'_{2-n} is due to the different *sn*-2 carbon–hydrogen chains (*n* = 1–3), ($[776 – (329 – 17)]^-$), *m*/*z* 466 $[M–H\text{-}R'_{2-2}\text{-}CH{=}C{=}O]^-$ ($[776 – (327 – 17)]^-$) and *m*/*z* 492 $[M–H\text{-}R'_{2-3}\text{-}CH{=}C{=}O]^-$ ($[776 – (301 – 17)]^-$), corresponding to losses of the 22:5-ketene, 22:6-ketene and 20:5-ketene groups from their shared precursor anions. However, the MS^3 product ion spectra of both $[M–H]^- \rightarrow [M–H\text{-}R'_{2-2}\text{-}CH{=}C{=}O]^-$ (*m*/*z* 776 → 466) and $[M–H]^- \rightarrow [M–H\text{-}R'_{2-1}\text{-}CH{=}C{=}O]^-$ (*m*/*z* 776 → 464; spectrum not shown) provide diagnostic fragments of plasmanyl and plasmenyl structures in the two species by giving rise to ions at *m*/*z* 405 (plasmanyl 18:0) (Fig. 9b), as well as *m*/*z* 267 $[O–CH{=}CH–C_{16}H_{33}]^-$ and 196 (Scheme 1b) (not shown), suggesting the co-existence of plasmanyl 18:0/22:6 PE (a18:0/22:6 PE) and plasmenyl 18:0/22:5 PE (p18:0/22:5 PE) species. Fig. 9c shows the MS^3 product ion spectrum of *m*/*z* 790 → 492. The minor species was structurally elucidated as plasmenyl 20:0/20:5 PE (p20:0/20:5 PE), and the designation of the molecular species was based on the presence of a pair of fragments at *m*/*z* 431 [405 (plasmanyl 18:0) + (CH_2CH_2)]$^-$ and 295 $[O–CH{=}CH–C_{18}H_{37}]^-$. Finally, the diacyl species 17:0/22:6 was also identified, on the basis of an ion at *m*/*z* 269 in the MS^3 product ion of *m*/*z* 776 → 466 (Fig. 9c).

3.4.3. Differentiation of multiple isobaric isomers and minor species of oyster PE by NPLC/NI-ESI-MS^n—Fig. 10a shows the LC/MS^2 product ion spectrum of a PE

precursor at *m*/*z* 790.6 (40:6 or/and p41:5 PE). Carboxylic anions at *m*/*z* 327 (FA 22:6) and 283 (327 – 44), *m*/*z* 301 (FA 20:5) and 257 (301 – 44), *m*/*z* 329 (FA 22:5), *m*/*z* 309 (FA 20:1), *m*/*z* 315 (FA 21:5) [9–13,44–47], *m*/*z* 343 (FA 23:5) [44–47], and *m*/*z* 355 (FA 24:6) are due to the fatty acids esterified at the *sn*-1 and *sn*-2 positions of the glycerol backbone. The structural elucidation of the species was further supported by the appearance of several groups of fragment ions at (i) *m*/*z* 480 $[M–H\text{-}R_{2–1}\text{-}CH{=}C{=}O]^-$, $[790 – (327 – 17)]^-$ and 462 $[M–H\text{-}R_{2–1}\ COOH]^-$, ($R_{2–n}$ is due to the different *sn*-2 fatty acid chains (n = 1–6)), ($[790 – 328$ (FA 22:6)$]^-$) originating from 18:0/22:6 PE (40:6 PE); (ii) at *m*/*z* 506 $[M–H\text{-}R'_{2–2}\text{-}CH{=}C{=}O]^-$ ($[790 – (301 – 17)]^-$) and 488 $[M–H\text{-}R_{2–2}\ COOH]^-$($[790 – 302$ (FA 20:5)$]^-$) originating from 20:1/20:5 PE (40:6 PE); (iii) at *m*/*z* 478 $[M–H\text{-}R'_{2–3}\text{-}CH{=}C{=}O]^-$ ($[790 – (329 –17)]^-$) for the characterization of 18:1/22:5 PE (40:6 PE); (iv) *m*/*z* 492 $[M–H\text{-}R'_{2–4}\text{-}CH{=}C{=}O]^-$ ($[790 – (315 – 17)]^-$) and *m*/*z* 295 $[O–CH{=}CH–C_{18}H_{37}]^-$ (see Fig. 9c) for determination of plasmenyl 20:0/21:5 PE (p41:5 PE); (v) *m*/*z* 464 $[M–H\text{-}R'_{2–5}\text{-}CH{=}C{=}O]^-$ ($[790 – (343 – 17)]^-$) and *m*/*z* 267 $[O–CH{=}CH–C_{16}H_{33}]^-$ for the identification of plasmenyl 18:0/23:5 PE (p41:5 PE); (vi) *m*/*z* 452 $[M–H\text{-}R'_{2–6}\text{-}CH{=}C{=}O]^-$ ($[790 – (355 – 17)]^-$) originating from 16:0/24:6 PE (40:6 PE). To our knowledge, this is the first report to show the differentiation of six isobaric diacyl/etheracyl species isomers mixture in a PE molecule from marine lipids.

Isobaric diacyl/etheracyl species isomers mixtures were also found in other PE species, such as shown in the MS^2 product ion spectrum of the precursor ion at *m*/*z* 762 (Fig. 10b) (38:6 or/and p39:5), along with the groups of supporting fragments for structural designation of (i) at *m*/*z* 327 (FA 22:6; abundant); 283 (327 – 44), *m*/*z* 255 (FA 16:0; weak), *m*/*z* 452 $[M–H\text{-}R'_{2–1}\text{-}CH{=}C{=}O]^-$, $[762 – (327 – 17)]^-$ and *m*/*z* 434 $[M–H\text{-}R_{2–1}\ COOH]^-$($[762 – 328$ (FA 22:6)$]^-$) for 16:0/22:6 PE (38:6 diacyl PE); (ii) *m*/*z* 301 (FA 20:5; abundant)/257 (301 – 44), *m*/*z* 281 (FA 18:1; weak), *m*/*z* 478 $[M–H\text{-}R'_{2–2}\text{-}CH{=}C{=}O]^-$ ($[762 – (301 – 17)]^-$) and *m*/*z* 460 $[M–H\text{-}R_{2–2}\ COOH]^-$ ($[762 – 302$ (FA 20:5)$]^-$) for 18:1/20:5 (38:6 diacyl PE); (iii) *m*/*z* 329 (FA 22:5) and *m*/*z* 450 $[M–H\text{-}R'_{2–3}\text{-}CH{=}C{=}O]^-$ ($[762 – (329 – 17)]^-$) for 16:1/22:5 PE (38:6 diacyl PE); and (iv) *m*/*z* 315 (FA 21:5), *m*/*z* 464 $[M–H\text{-}R'_{2–4}\text{-}CH{=}C{=}O]^-$ ($[762 – (315 – 17)]^-$) and *m*/*z* 267 $[O–CH{=}CH–C_{16}H_{33}]^-$ for plasmenyl 18:0/21:5 PE (p18:0/21:5 PE) (spectra not shown; see Table 4).

The presence of 23:5 fatty acid has also been reported in some marine lipids. For example, very long chain (ω-3) fatty acids were found in the blubber of seals (*Phocahispida*) from northern fresh or seawater. Thus, 23:5, 24:3, 24:4, 24:5, 24:6, 26:5, 26:6 and 28:7 fatty acids were found at a concentration not exceeding 0.2% each [47–49]. In Ophiuroidea (*Brittle star*), a 24:6 (ω-3) fatty acid has been observed at concentrations of 3–15% of total fatty acids, especially in some phospholipids [48,49]. Our results show for the first time that 21:5 (ω-3) and 23:5 (ω-3) fatty acid – containing PE molecular species also occur in the US pacific oysters as the minor species.

The major molecular species of PE in the pacific oyster *C. sikamea* were similar to those found in the oyster *C. gigas* although their percentage composition differed from each other (see Tables 3 and 4).

3.5. Analysis of oyster LysoPE species by NPLC/MS^2

As shown in Table 5, eight molecular species of LysoPE were identified from the pacific oysters by NPLC/NI-ESI/MS^2 of the $[M–H]^-$ precursor ions of the LysoPE, and based on the MS^3 product ion fragmentation pathways of plasmanyl and plasmenyl PE species [34]. The most abundant species is the plasmenyl 18:0 isomer.

4. Summary and conclusions

We have investigated the product ion pattern of plasmenyl PS species by the silica normal-phase liquid chromatography negative ion ESI/quadrupole-multiple stage linear ion-trap mass spectrometry (NPLC-NI-ESI/Q-TRAP-MS^3), and described a full MS^3 fragment pathway for the structural differentiation of plasmenyl PS from plasmanyl PS. We further developed an approach for the structural elucidation of ether molecular species of serine and ethanolamine phospholipids from marine oysters. The present study shows that a combination of NPLC-negative-ion ESI/MS^3 and NPLC-negative-ion ESI/MS^2 is particularly valuable in the detailed characterization of marine lipid dietary supplements with respect to omega-3 aminophospholipids, including (i) characterizing the *sn*-1 radyl linkages, and composition of the *sn*-2 fatty acid chains, (ii) differentiating multiple isobaric isomers of a diacyl/etheracyl molecular species in a mixture, and (iii) elucidating chemical structures of minor molecular species in a lipid mixture. Because marine oysters are very popular as sea food, and they contain relatively large percentage of omega-3 fatty acids in the form of phospholipids, the structural elucidation of the major phospholipid classes as well as their “minor” molecular species is of importance in evaluating nutritional value of these delicacies, especially with regard to supplying the requirements of omega-3 fatty acids and plasmalogens. Although fish oils (mainly in the form of triglycerides) are widely marketed for DHA/EPA supplementation, new omega-3 sources other than triglycerides, i.e. free fatty acids, ethyl EPA/DHA esters and more recently phospholipids are emerging [2]. In fact, marine phospholipids are considered to be a more efficient dietary source of omega-3 polyunsaturated fatty acids than triglycerides or free fatty acids [4–7]. Further studies in animals, on the effect of oyster feeding on the omega-3 fatty acid and plasmalogen content of the tissues, especially the brain, are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

NPLC-NI-ESI/Q-TRAP-MS	normal-phase silica liquid chromatography/negative-ion electrospray ionization/quadrupole multiple-stage linear ion-trap mass spectrometry
PS	phosphatidylserine
PE	phosphatidylethanolamine
LysoPE	lysophosphatidylethanolamine
MTBE	methyl tert-butyl ether

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2012.05.035.

HIGHLIGHTS

- Development of a simple and robust NPLC-NI-ESI/Q-TRAP-MS^n method for ω-3 aminophospholipidomics of marine animals.
- Establishment of a full fragmentation pathway for the structural differentiation of plasmenyl PS from plasmanyl PS.
- Structurally differentiate up to six isobaric species from a phosphatidylethanolamine molecule in the US pacific oysters.

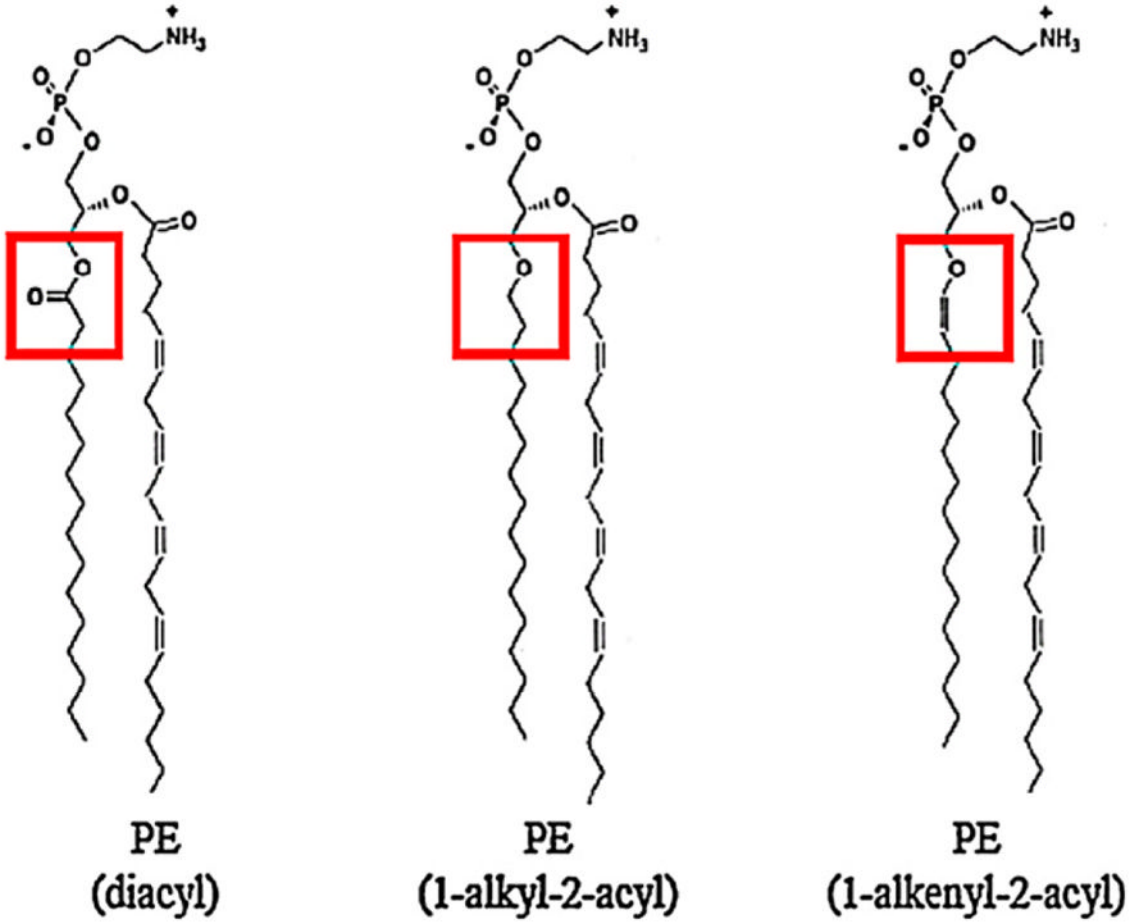


Fig. 1.
Molecular species structures of diacyl PE (left), plasmanyl/acyl PE (1-O-alkyl-2-acyl PE) (central), and plasmenyl/acyl PE (plasmalogen PE) (right).

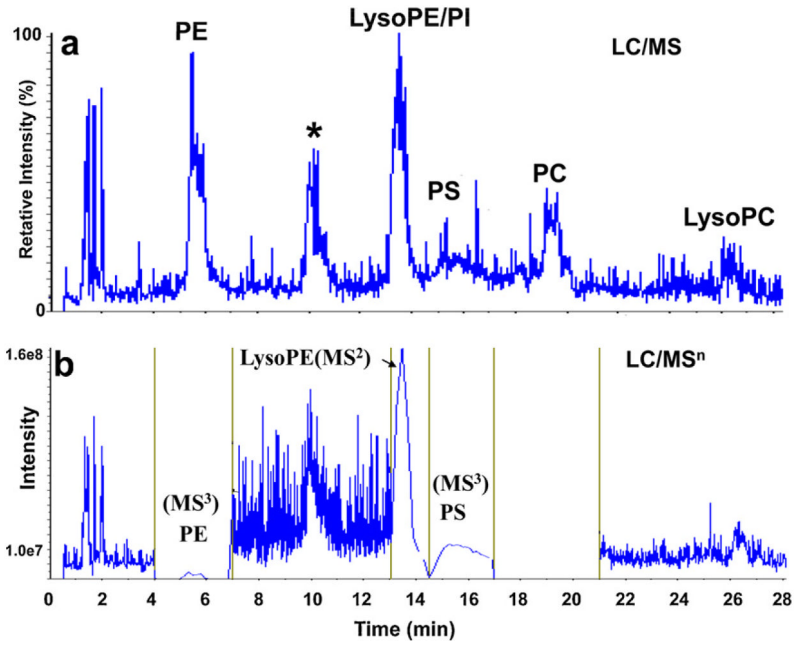


Fig. 2.

(a) Normal-phase silica LC-NI-ESI/MS total ion chromatography of the US pacific oyster *Crassostrea sikamea* lipid extract (a peak marked with * was tentatively identified as ceramide aminoethylphosphonate [9]); and (b) normal-phase silica LC-NI-ESI/MS^3 ion chromatography of the oyster phosphatidylethanolamine (PE) and phosphatidylserine (PS), and Normal-phase silica LC-NI-ESI/MS^2 of the oyster lysophosphatidylethanolamine (LysoPE).

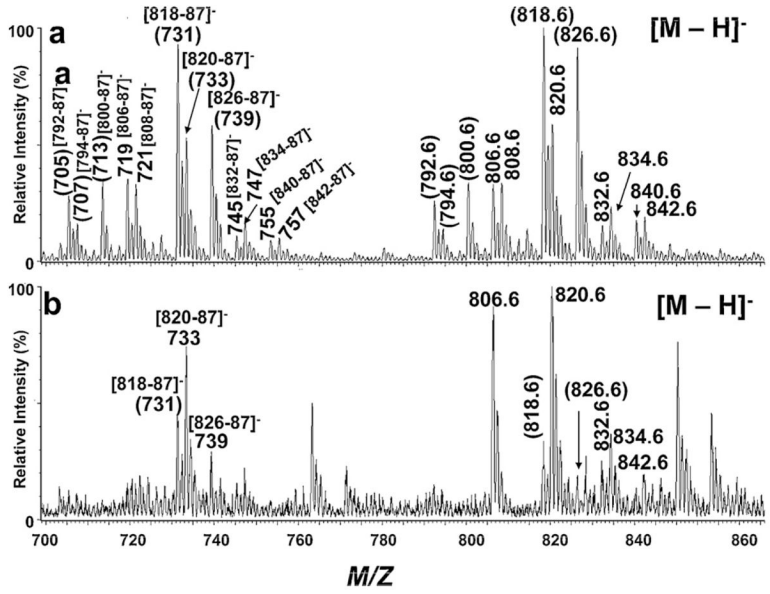


Fig. 3.

NPLC-NI-ESI/MS spectra of the US pacific oyster *Crassostrea sikamea* PS before (a) and after (b) acid treatment.

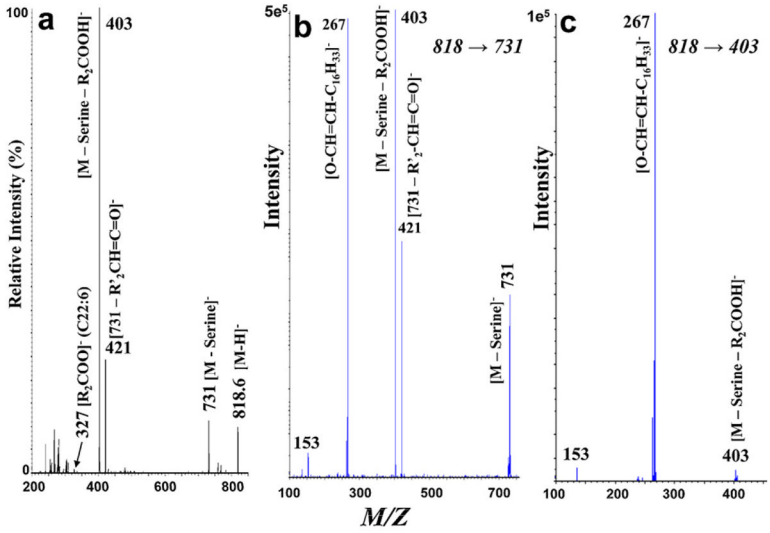


Fig. 4.

NPLC-NI-ESI/MS^2 product ion spectrum of (a) a precursor anion at *m*/*z* 818.6 (p18:0/22:6 PS) from the oyster PS; and NPLC-NI-ESI/MS^3 product ion spectra of (b) precursor ions at *m*/*z* 818 → 731 and (c) *m*/*z* 818 → 403.

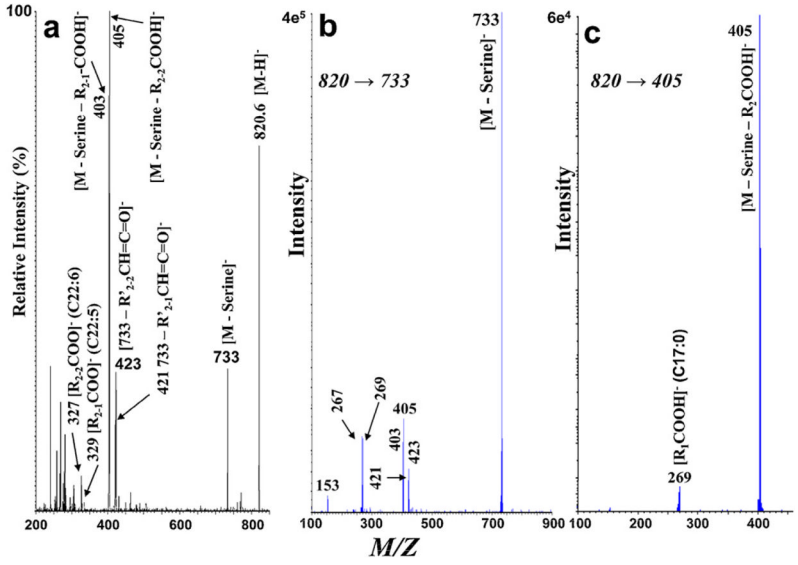


Fig. 5.

NPLC-NI-ESI/MS^2 product ion spectrum of (a) a precursor anion at *m*/*z* 820.6 (a mixture of a18:0/22:6 PS, p18:0/22:5 PS and 17:0/22:6 PS) from the oyster PS; and NPLC-NI-ESI/MS^3 product ion spectra of (b) precursor ions at *m*/*z* 820 → 733 and (c) *m*/*z* 820 → 405.

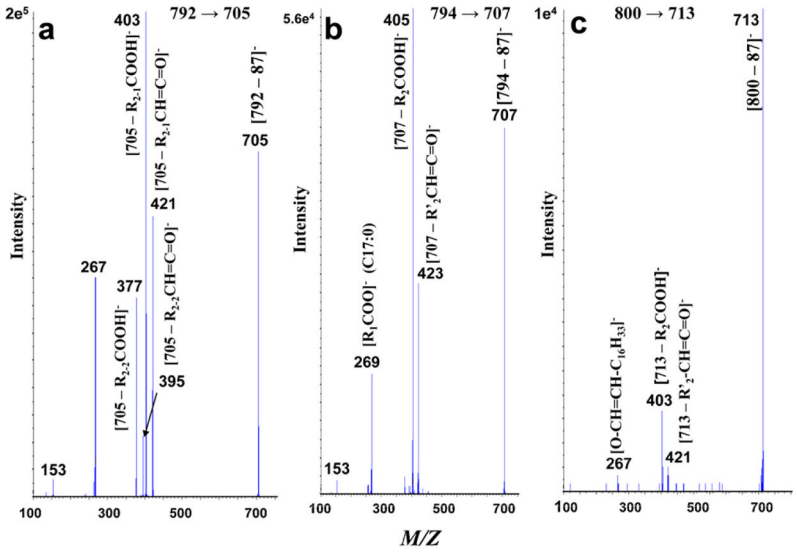


Fig. 6.

NPLC-NI-ESI/MS^3 product ion spectra of (a) precursor ions at *m*/*z* 792 → 705 (a mixture of p18:0/20:5 PS and a16:0/22:6 PS), (b) precursor ions at *m*/*z* 794 → 707 (a mixture of a18:0/20:5 PS and 17:0/22:6 PS) and (c) precursor ions at *m*/*z* 800 → 713 (p18:0/20:1 PS).

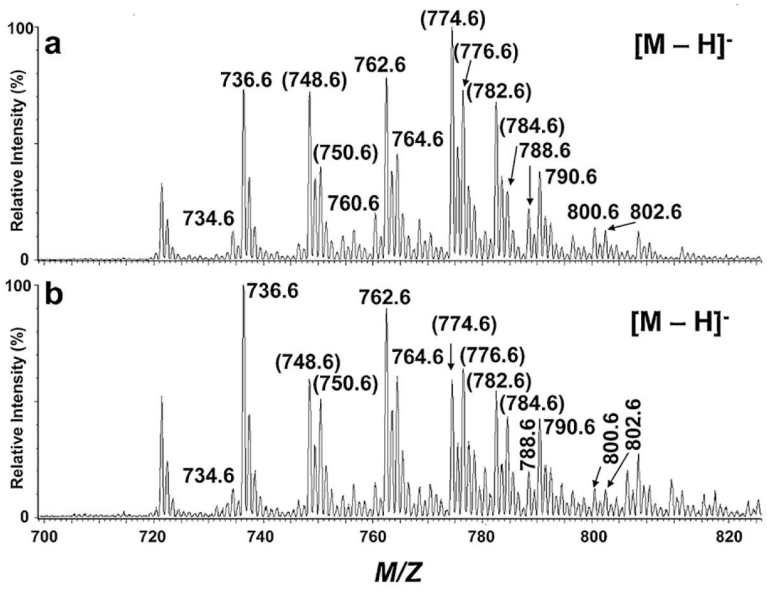


Fig. 7.

NPLC-NI-ESI/MS spectra of the US pacific oyster *Crassostrea sikamea* PE before (a) and after (b) acid treatment.

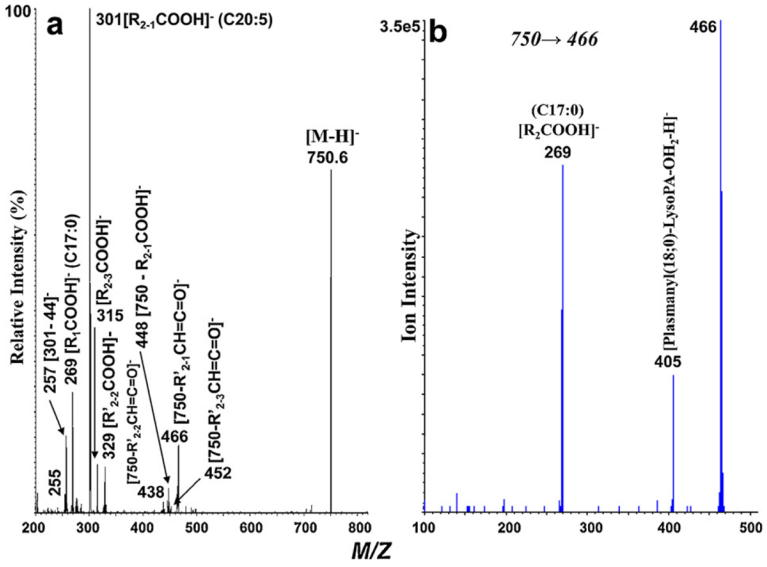


Fig. 8.

NPLC-NI-ESI/MS^2 product ion spectrum of (a) a precursor anion at *m*/*z* 750.6 (a mixture of a18:0/20:5 PE, a16:0/22:5 and 16:0/21:5) from the oyster *Crassostrea sikamea* PE; and NPLC-NI-ESI/MS^3 product ion spectra of (b) precursor ions at *m*/*z* 750 → 466.

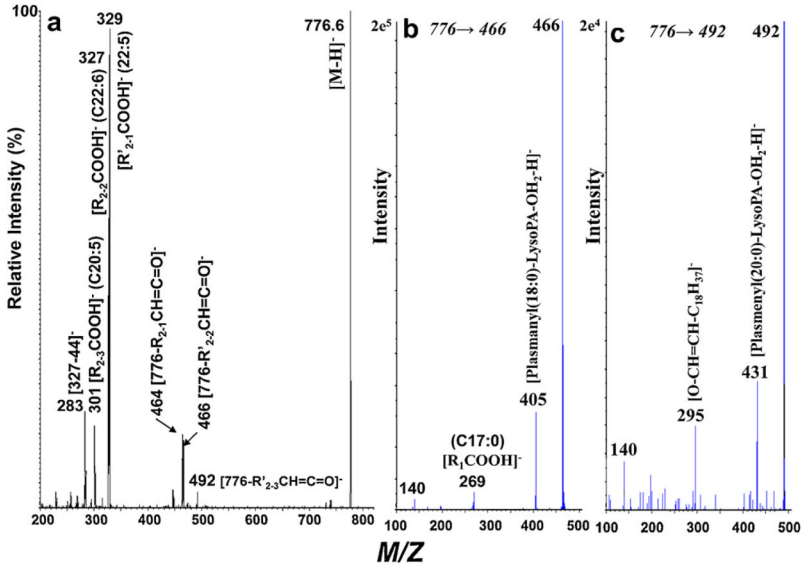


Fig. 9.

NPLC-NI-ESI/MS^2 product ion spectrum of (a) a precursor anion at *m*/*z* 776.6 (a mixture of p20:0/20:5, a18:0/22:6, p18:0/22:5 and 17:0/22:6 PE) from the oyster *Crassostrea sikamea* PE; and NPLC-NI-ESI/MS^3 product ion spectra of (b) precursor ions at *m*/*z* 776 → 466 and (c) *m*/*z* 776 → 492.

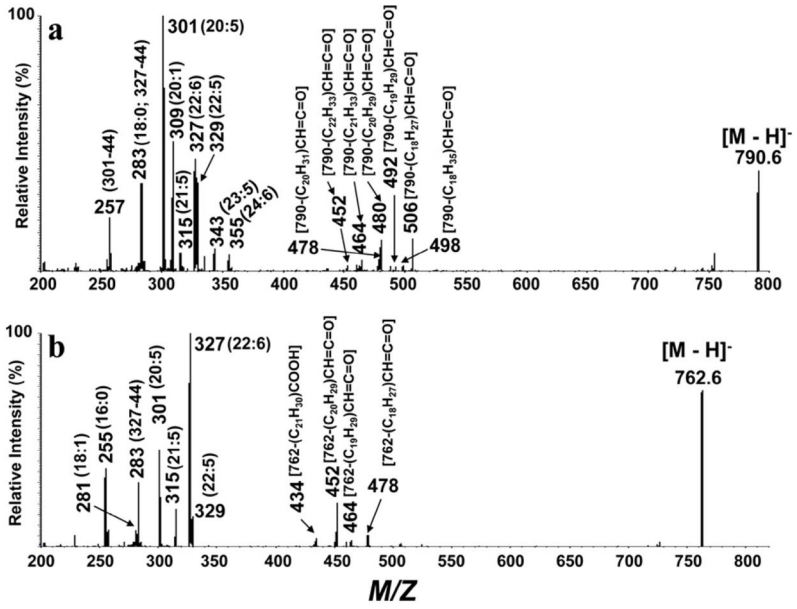
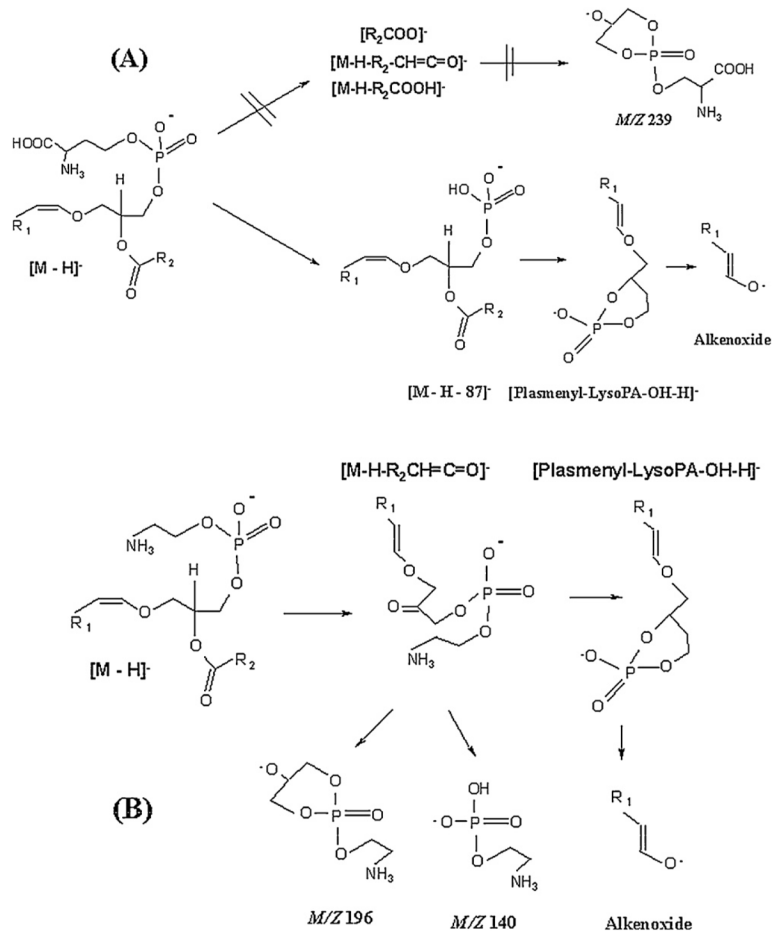


Fig. 10.

NPLC-NI-ESI/MS^2 product ion spectra of (a) a precursor ion at *m*/*z* 790.6; and (b) a precursor ion at *m*/*z* 762.6 From the US pacific oyster *Crassostrea sikamea* PE.



Scheme 1.

(a) the MS^3 product ion fragmentation process of plasmenyl PS molecular species and (b) the MS^3 product ion fragmentation pathway of plasmenyl PE molecular species.

Table 1

Deprotonated molecules of the US pacific oyster PS molecular species.

$[M–H]^-$	Total carbons (*X*):total double bonds (*Y*)[a]	Percentage (%)[b]	
		C. sikamea	*C. gigas*
842.6	40 (diacyl):2	4.2 ± 0.5	2.5 ± 0.3
840.6	40 (diacyl):3	4.0 ± 0.4	2.6 ± 0.4
834.6	40 (diacyl):6	5.0 ± 0.6	7.4 ± 0.8
832.6	40 (diacyl):7	3.2 ± 0.3	2.3 ± 0.4
828.6	40 (plasmanyl):2	5.3 ± 1.1	
826.6	40 (plasmenyl):2	19 ± 2.5	14 ± 2.4
822.6	40 (plasmanyl):5	1.0 ± 0.2	
820.6	40 (plasmanyl):6	13 ± 2.3	17 ± 2.2
	40 (plasmenyl):5		
	39 (diacyl):6		
818.6	40 (plasmenyl):6	22 ± 3.2	23 ± 2.0
808.6	38 (diacyl):5	7.0 ± 0.4	5.8 ± 0.5
806.6	38 (diacyl):6	7.0 ± 0.7	6.1 ± 0.6
800.6	38 (plasmenyl):1	7.3 ± 0.4	5.6 ± 1.1
794.6	38 (plasmanyl):5	2.7 ± 0.5	3.1 ± 0.5
	37 (diacyl):5		
792.6	38 (plasmenyl):5	5.6 ± 0.7	4.3 ± 0.7
	38 (plasmanyl):6		
Diacyl species		30.4	36.7
Ether species		69.6	63.3
Total DHA PS species		>40	>50

[a] *X:Y* (for example 40:2), where *X* is the total carbon number of the fatty acids esterified at *sn*-1 and *sn*-2 positions, and *Y* is the total unsaturation degrees of fatty acid groups.

[b] Results were generated from the average intensity of $[M–H]^-$ ions of pacific oyster PS species, based on three replicate experiments of NPLC-NI-ESI/MS.

Table 2

Q-TRAP MS^2 and MS^3 product ions of $[M–H]^-$ of US pacific oyster phosphatidylserine, and the assignment of molecular species.

MS^2 of $[M–H]^-$						**MS^3 of $[M- H] \rightarrow [M–H-87]^-$**			
$[M–H]^-$	**$[M–H-87]^-$**	**$[PA\text{-}R'_2\ CHC{=}O]^-$**	**$[PA\text{-}R_2\ COOH]^-$**	**$[R_2\ COOH]^-$**	**$[R_1\ COOH]^-$**	**$[PA\text{-}R'_2\ CHC{=}O]^-$**	**$[PA\text{-}R_2\ COOH]^-$**	**$[O–CH{=}CH–R_1]^-$**	**PS species assignment $[M–H]^-$**
842	755 (PA)	435	417	337(w)[a]	283(a)[b]	–	–	–	18:0/20:1[c]
840	753 (PA)	435	417	335(w)	283(a)	–	–	–	18:0/20:2
834	747 (PA)	437	419	327(w)	283(a)	–	–	–	18:0/22:6
832	745 (PA)	–	417	327(w)	281(a)	–	–	–	18:1/22:6
828	741 (PA)	–	405[d]	335	–	423(w)	405(a)	–	a18:0/22:2
826	739 (PA)	421	403[e]	335	–	421(w)	403(a)	267	p18:0/22:2[f]
822	735 (PA)	423	405	–	–	–	405	–	a18:0/22:5[g]
820	733 (PA)	423	405	327	–	423(w)	405(a)	–	a18:0/22:6
		421	403	329	–	421(w)	403(a)	267	p18:0/22:5
		423	405	–	–	–	405	–	17:0/22:6
							269 (R_1 COOH)		
818	731 (PA)	421	403	327	–	421(w)	403(a)	267	p18:0/22:6
808	721 (PA)	437	419	329(w)	283(a)	–	–	–	18:0/22:5
806	719 (PA)	437	419	327(w)	283(a)	–	–	–	18:0/22:6
800	713 (PA)	421	403	309	–	421(w)	403(a)	267	p18:0/20:1
794	707 (PA)	423	403	301	–	423(w)	405(a)	–	a18:0/20:5
		423	405	–	–	–	405	–	17:0/20:5
							269 (R_1 COOH)		
792	705 (PA)	421	403	301	–	421(w)	403(a)	267	p18:0/20:5
		395	377	327	–	395(w)	377(a)	–	a16:0/22:6

[a](w): weak ion.

[b](a): abundant ion (see Ref. [18] in the text for information of product ions generated by MS/MS of PA and PS).

[c]*n*:*m*/*N*:*M* (for example 18:0–20:1), where *n* is the total number of carbon in the *sn*-1 position and m is the total number of double bonds in fatty acid chain at the *sn*-1 position; where *N* is the total number of carbon in the *sn*-2 position and *M* is the total number of double bonds in fatty acid chain at the *sn*-2 position.

[d]The ion at *m*/*z* 405 can be used as a precursor scan for identification of all of plasmanyl 18:0 linkage PS species by NPLC-NI-ESI/MS^2.

[e] The ion at *m*/*z* 403 can be used as a precursor scan for identification of plasmenyl 18:0 linkage PS species or plasmanyl 18:1 linkage PS species by NPLC-NI-ESI/MS^2.

[f] p18:0–22:2: p means plasmenyl linkage (1-alk-1′-enyl or plasmalogen).

[g] a18:0–22:5: a means alkyl linkage (plasmanyl or 1-O-alkyl).

Table 3

Deprotonated molecules of the US pacific oyster PE molecular species.

$[M–H]^-$	Total carbons (*X*):total double bonds (*Y*)[a]	Percentage (%)[a]	
		C. sikamea	*C. gigas*
802.6	42 (plasmenyl):6	1.8 ± 0.4	1.8 ± 0.3
800.6	42 (plasmenyl):7	2.0 ± 0.7	2.6 ± 0.4
790.6	40 (diacyl):6	2.7 ± 0.6	3.5 ± 0.7
	41 (plasmanyl):6		
	41 (plasmenyl):5		
788.6	40 (diacyl):7	5.6 ± 0.5	3.5 ± 0.6
784.6	40 (plasmenyl):1	4.0 ± 0.8	1.7 ± 0.3
782.6	40 (plasmenyl):2	10 ± 0.8	6.5 ± 0.6
776.6	40 (plasmenyl):5	11 ± 1.3	9.7 ± 1.4
	40 (plasmanyl):6		
	39 (diacyl):6		
774.6	40 (plasmenyl):6	15 ± 1.2	23 ± 2.4
764.6	38 (diacyl):5	6.8 ± 0.4	7.4 ± 1.4
762.6	38 (diacyl):6	12 ± 1.7	8.3 ± 0.9
	39 (plasmenyl):5		
760.6	38 (plasmenyl):7	2.1 ± 0.4	2.5 ± 0.4
750.6	38 (plasmanyl):5	6.0 ± 0.8	7.5 ± 1.2
	37 (diacyl):5		
748.6	38 (plasmenyl):5	10 ± 1.7	12 ± 0.9
	38 (plasmanyl):6		
736.6	36 (diacyl):5	8.5 ± 0.9	8.9 ± 1.4
734.6	36 (diacyl):6	1.5 ± 0.3	1.1 ± 0.3
Diacyl species		37.1	32.7
Ether species		62.9	67.3
Total DHA PE species		>40	>40

[a]See Table 1 for explanation.

Table 4

Q-TRAP MS^2 and MS^3 Product Ions of $[M–H]^-$ of US Pacific oyster PE, and the assignment of the species.

MS^2 of $[M–H]^-$					**$]MS^3$ of $[M–H] \rightarrow [M–H\text{-}R'_2\ CHC{=}O]^-$**		
$[M–H]^-$	**$[M–H\text{-}R'_2\ CHC{=}O]^-$**	**$[M–H\text{-}R_2\ COOH]^-$**	**$[R_2\ COOH]^-$**	**$[R_1\ COOH]^-$**	**$[M–H\text{-}R'_2\ CHC{=}O]^-$**	**$[O–CH{=}CH–R_1\ ]^-$**	**PE species assignment**
802.6	492[d](a)[a]	474(w)[b]	327	–	492	295	p20:0/22:6[f]
800.6	490[e](a)	472	327	–	490	293	p20:1/22:6
790.6	506	–	301(a)	309(w)	–	–	20:1/20:5[c]
	492	–	315	–	492	295	p20:0/21:5
	480	–	327(a)	283(w)	480	–	18:0/22:6
	478	–	329(a)	281(w)	478	–	18:1/22:5
	464	–	343	–	464	267	p18:0/23:5
	452	–	355(a)	255(w)	452	–	16:0/24:6
788.6	478	460	327(a)	281(w)	478	–	18:1/22:6
784.6	464	–	337	–	464	267	p18:0/22:1
782.6	464	–	335	–	464	267	p18:0/22:2
	490	–	309	–	490	293	p20:1/20:1
776.6	492	–	301	–	492	295	p20:0/20:5
	466	–	327	–	466	–	a18:0/22:6[g]
	464	–	329	–	464	–	p18:0/22:5
	466	–	327(a)	269(w)	466	–	17:0/22:6
774.6	464(a)	446(w)	327	–	464	267	p18:0/22;6
	490(a)	472(w)	301	–	490	293	p20:1/20:5
764.6	480(a)	462(w)	301(a)	283(w)	480	–	18:0/20:5
	452	–	329(a)	255(w)	452	–	16:0/22:5
762.6	452(a)	434(w)	327(a)	255(w)	452	–	16:0/22:6
	478	–	301(a)	281(w)	478	–	18:1/20:5
	464	–	315	–	464	267	p18:0/21:5
760.6	450	–	327(a)	253(w)	450	–	16:0/22:6
750.6	466(a)	448(w)	301	–	466	–	a18:0/20:5
	452	–	315(a)	255(w)	452	–	16:0/21:5
	438	–	329	–	438	–	a16:0/22:5

MS^2 of [M–H]−					]MS^3 of [M–H] → [M–H-R′$_2$ CHC═O]$^-$		
[M–H]$^-$	[M–H-R′$_2$ CHC═O]$^-$	[M–H-R$_2$ COOH]$^-$	[R$_2$ COOH]$^-$	[R$_1$ COOH]$^-$	[M–H-R′$_2$ CHC═O]$^-$	[O–CH═CH–R$_1$]$^-$	PE species assignment
748.6	464(a)	446(w)	301	–	464	267	p18:0/20:5
	438(a)	420(w)	327	–	438	–	a16:0/22:6
	436	–	329	–	436	–	a16:1/22:5
736.6	452(a)	434(w)	301(a)	255(w)	452	–	16:0/20:5
734.6	450(a)	432(w)	301(a)	253(w)	450	–	16:1/20:5

See Table 2 for explanation of symbols.

Table 5

Deprotonated molecules of the US pacific oyster LysoPE molecular species.

$[M–H]^-$	MS^2 of $[M–H]^-$		Molecular species assig.	Percentage (%)[a]	
	$[R_1 COOH]^-$	$[O–CH═CH–R_1]^-$		*C. sikamea*	*C. gigas*
524.5	327 (283)	–	22:6 (acyl)	12.5 ± 0.2	7.7 ± 0.4
498.5	301 (257)	–	20:5 (acyl)	27.8 ± 1.7	16 ± 1.4
490.5	–	293	p20:1[b]	6.7 ± 0.6	8.8 ± 0.8
478.5	281	–	18:1 (acyl)	6.6 ± 0.5	6.2 ± 0.6
464.5	–	267	p18:0	31.7 ± 3.5	45 ± 3.4
452.5	255	–	16:0 (acyl)	5.7 ± 0.8	2.9 ± 0.7
450.5	253	–	16:1 (acyl)	5.6 ± 0.6	8.4 ± 0.9
436.5	–	239	p16:0	2.8 ± 0.7	5.6 ± 0.7
Monoacyl species				58.2	46.8
Ether species				42.8	53.2
ω-3 DHA species				12.5	7.7

[a]Results were generated from the average intensity of $[M–H]^-$ ions of US pacific oyster LysoPE species, based on three replicate experiments of NPLC-NI-ESI/MS.

[b]Plasmenyl LysoPE (plasmalogen species or p20:1).