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METHODS

Separation and Detection of Plasmalogen in Marine Invertebrates by High-Performance Liquid Chromatography with Evaporative Light-Scattering Detection

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Abstract We have developed a new method for determining ethanolamine plasmalogen contents in marine invertebrates. This quantification method involves derivatization of ethanolamine glycerophospholipid (EtnGpl) subclasses, alkenylacyl (plasmalogen), diacyl, and alkylacyl subclasses, by enzyme treatment and acetylation, followed by separation and detection by high-performance liquid chromatography (HPLC) with evaporative light-scattering detection (ELSD). This method enabled complete separation of the subclasses, and the limit of detection for plasmalogen was 200 ng (260 pmol). The peak area of plasmalogen by ELSD was unaffected by the degree of unsaturated fatty acids in EtnGpl, in contrast to ultraviolet (UV) detection. Thus, this method enables accurate determination of plasmalogen contents in various species containing marine products possessing abundant polyunsaturated fatty acids (PUFA). The method developed here was applied to marine invertebrates available in Japan. The examined marine invertebrates showed a wide range of plasmalogen contents ranging from 19 to 504 μmol/100 g wet wt. The plasmalogen levels in samples except those of class Cephalopoda and Crustacea were more than 60 mol% of EtnGpl.

Keywords HPLC–ELSD · EtnGpl subclass · Plasmalogen · Diradylglycerol · Marine invertebrate · TLC · LC–MS

Abbreviations

CerPCho	Sphingomyelin
ChoGpl	Choline glycerophospholipid
ELSD	Evaporative light-scattering detection
ESI-TOF MS	Electrospray ionization-time of flight mass spectrometry
EtnGpl	Ethanolamine glycerophospholipid
GC	Gas chromatography
HPLC	High-performance liquid chromatography
LC–MS	Liquid chromatography–mass spectrometry
MS	Mass spectrometry
NPH	2,4-Dinitrophenylhydrazine method
PakCho	1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine
160/22:6-PakCho	1-O-hexadecyl-2-octadecenoyl-sn-glycero-3-phosphoethanolamine
PakEtn	1-O-alkyl-2-acyl-sn-glycero-3-phosphoethanolamine
PlsEtn	1-O-alkenyl-2-acyl-sn-glycero-3-phosphoethanolamine
160/22:6-PlsEtn	1-O-hexadecenyl-2-docosahexaenoyl-sn-glycero-3-phosphoethanolamine
160/18:1-PlsEtn	1-O-hexadecenyl-2-octadecenoyl-sn-glycero-3-phosphoethanolamine
PtdEtn	1,2-diacyl-sn-glycero-3-phosphoethanolamine

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181/18:1-PtdEtn	1,2-di-octadecenoyl-sn-glycero-3-phosphoethanolamine
SerGpl	Serine glycerophospholipid
TIC	Total ion current chromatogram
TLC	Thin layer chromatography
UV	Ultraviolet

Introduction

Ethanolamine glycerophospholipid (EtnGpl) is a major class of glycerophospholipids found in biological membranes. Moreover, these subclasses exist in three forms with alkyl, alkenyl, or acyl linkages at the sn-1 position of the glycerol moiety (1-O-alkyl-2-acyl-sn-glycero-3-phosphoethanolamine, PakEtn; 1-O-alkenyl-2-acyl-sn-glycero-3-phosphoethanolamine, PlsEtn; and 1,2-diacyl-sn-glycero-3-phosphoethanolamine, PtdEtn, respectively). The alkenylacyl form is called "plasmalogen" [1]. While plasmalogen is restricted in most tissues and cells of animals, the nervous system shows high plasmalogen levels [2]. Moreover, plasmalogen levels were reported to be specifically decreased in postmortem brains from Alzheimer's disease patients [3–5]. Plasmalogen can also prevent cell death by scavenging singlet oxygen ($^1O_2$) and superoxide (O_2^-) at its alkenyl (vinyl ether) linkages, as demonstrated by mutant cells that are deficient in an enzyme for plasmalogen biosynthesis [6]. While these findings suggest that plasmalogen has important physiological roles, its absorption and metabolism are not fully understood, mainly due to a lack of plasmalogen resources [7, 8].

Following bovine spongiform encephalopathy outbreaks, use of bovine brain as a plasmalogen resource became difficult. On the other hand, most EtnGpl exists as plasmalogen in some marine invertebrates [9, 10], such as *Crassostrea gigas*, *Mytilus edulis* [11], *Strongylocentrotus intermedius*, and *Halocynthia roretzi* [12]. These marine invertebrates are readily available around island nations, such as Japan, and can, therefore, be used as new plasmalogen resources.

There are several methods for quantifying plasmalogen that measure either plasmalogen aldehydes with 2,4-dinitrophenylhydrazines [13, 14], iodine caught in a vinyl ether bond [15], lyso-EtnGpl with thin layer chromatography (TLC) [1, 2, 16], or dimethyl acetal with gas chromatography (GC) [17, 18]. However, these methods have some drawbacks in terms of sensitivity and selectively. Other conventional methods, e.g., derivatizing diradylglycerols after phospholipase C treatment, enable complete separation of EtnGpl subclasses [19, 20]. Generally, they are identified by ultraviolet (UV) detection. However, UV detection is affected by double bonds and is, therefore, not suitable for analyzing marine products that are rich in polyunsaturated fatty acids (PUFA). On the other hand, high-performance liquid chromatography (HPLC) with evaporative light-scattering detection (ELSD), which is insensitive to the mobile phase solvents and thus allows direct quantification, has been applied to lipid analyses [21–23]. Unlike UV detection, ELSD is relatively unaffected by double bonds and length of fatty acids [24].

In this study, a method was developed for determining EtnGpl subclasses consisting of derivatization by phospholipase C treatment and acetylation, and separation and detection by HPLC–ELSD. This method was applied to 24 marine invertebrates available in Japan.

Materials and Methods

Materials

Crassostrea gigas, *Patinopecten yessoensis*, *Todarodes pacificus*, *Paroctopus dofleini*, *Marsupenaeus japonicas*, and *Halocynthia roretzi* were purchased from local supermarkets in Sendai, Japan. Other marine invertebrates were collected at Onagawa Bay in northwest Japan. 1-O-hexadecenyl-2-octadecenoyl-sn-glycero-3-phosphoethanolamine (16:0/18:1-PlsEtn), 1-O-hexadecenyl-2-docosahexaenoyl-sn-glycero-3-phosphoethanolamine (16:0/22:6-PlsEtn), 1,2-di-octadecenoyl-sn-glycero-3-phosphoethanolamine (18:1/18:1-PtdEtn), and 1-O-hexadecyl-2-octadecenoyl-sn-glycero-3-phosphoethanolamine (16:0/22:6-PakCho) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Phospholipids (Phospholipid Kit) and 60 wt% plasmalogen of EtnGpl from bovine brain were purchased from Doosan Serdary Research Laboratories (Toronto, ON, Canada). Phospholipase C from *Bacillus cereus* type III was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methanol, *n*-hexane, isopropanol, and chloroform were of HPLC grade, and other reagents were of extra-pure grade.

Lipid Extraction

EtnGpl subclass analysis was performed as described in Fig. 1. Samples were freeze-dried, crushed, and kept at a temperature below −30 °C for further processing. The freeze-dried samples (about 20 mg as total lipid) were added to 20 volumes of chloroform/methanol (2:1, v/v) and vortexed for 1 min. The total lipids were prepared according to the method of Folch et al. [25]. The total lipids were redissolved in 500 μL of chloroform/methanol (2:1, v/v).

Phospholipid Analysis

Phospholipid contents in the total lipids were determined according to the method described by Rouser et al. [26].

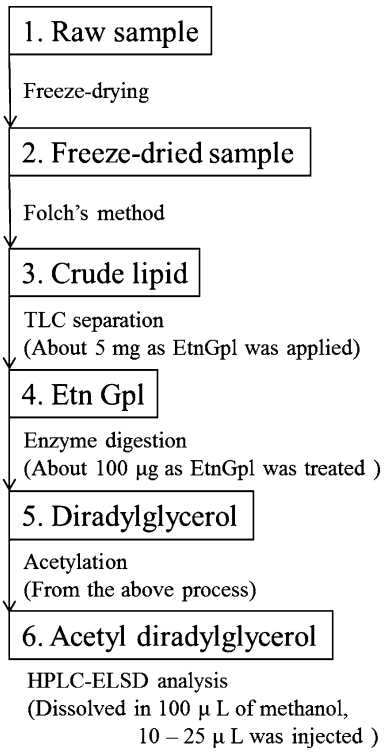

Fig. 1 Determination for EtnGpl subclasses. The measurements of each process were as follows: *1* wet weight by weighing, *2* dry weight by weighing, *3* total lipid weight by weighing, phospholipid content by Rouser method and content of phospholipid subclasses by HPLC–ELSD, *4* EtnGpl content by HPLC–ELSD, and *6* content of acetyl diradylglycerol subclasses by this developed method (the values were corrected by the recovery ratio of derivatized 18:1/18:1-PtdEtn)

Phospholipid classes were analyzed by HPLC–ELSD [27]. The silica column was LiChrosorb SI100 (4.6 × 250 mm, φ 10 μm; Waters Corporation, Milford, MA, USA) with a binary gradient consisting of solvent A [chloroform/methanol/30 % ammonium hydroxide (80:19.5:0.5, by vol)] and solvent B [chloroform/methanol/water/30 % ammonium hydroxide (60:34:5.5:0.5, by vol)]. The gradient profile was as follows: 0–14 min, 100 % B linear gradient; 14–24 min, 100 % B. The flow rate was 1.0 mL/min, and the column was maintained at a temperature of 35 °C. The post-column ELSD was a SEDEX model 55 (Sedere, Vitry sur Seine, France), kept at an evaporation temperature of 60 °C and pressure of 2.0 bar (2.7 L/min) for nebulization gas (nitrogen). The photomultiplier sensitivity was adjusted to a gain of 8.

Phospholipid Class Separation

On TLC (Silica 60, Merck, Darmstadt, Germany), phospholipid classes were separated by chloroform/methanol/acetone/water (80:25:10:4, by vol) and EtnGpl was detected by primulin. The EtnGpl fraction was extracted by the method of Bligh and Dyer [28].

Derivatization of EtnGpl Subclasses

To subfractionate EtnGpl into the diacyl, alkenylacyl, and alkylacyl subclasses, EtnGpl was derivatized by a modification of the method of Guan et al. [29]. About 100 μg of EtnGpl was redissolved in 1.5 mL of diethyl ether saturated with water. Phospholipase C was dissolved in 100 mM Tris–HCl (pH 7.4), and 500 μL of this solution (containing 1.0 unit) was added to the dissolved EtnGpl. Incubation was performed at 37 °C for 30 min. After incubation, 500 μL of distilled water and 1.0 mL of diethyl ether were added and centrifuged at 2,500 rpm for 5 min. After separation of the layers, 2.5 mL of diethyl ether was added to the lower layer and centrifuged. The separated upper layers were mixed and dried under nitrogen. The residue was dissolved in 15 μL of benzene, 15 μL of acetate anhydride, and 30 μL of 0.68 M 4-dimethylaminopyridine in pyridine. The reaction was mixed at 37 °C for 3 h. After the reaction, 1.8 mL of methanol, 100 μL of 100 mM Tris–HCl (pH 8.5) and 1.2 mL of petroleum ether were added and centrifuged. Then, 2.4 mL of petroleum ether was added to the lower layer. The upper layers were collected, dried under nitrogen, and redissolved in 100 μL of methanol.

Liquid Chromatography–Mass Spectrometry (LC–MS) Analysis for Derivatized EtnGpl

Diradylglycerols and acetyl diradylglycerols were confirmed by HPLC with electrospray ionization-time of flight mass spectrometry (ESI-TOF MS). The C18 column was TSK-GEL ODS-80Ts (4.6 × 250 mm, φ 5 μm; TOSOH Co., Tokyo, Japan), and the mobile phase consisted of methanol/0.5 M ammonium acetate (99:1, v/v). The flow rate was 1.0 mL/min, and the column was maintained at a temperature of 28 °C. The ESI-TOF MS was a Mariner (Applied Biosystems, Carlsbad, CA, USA). Electrospray ionization was performed in positive ion mode. Spray voltage, nozzle potential, nozzle temperature, and nebulizer gas flow rate were set to 2,000 V, 150 V, 150 °C, and 3.0 mL/min, respectively.

HPLC–ELSD for Acetyl Diradylglycerols

Acetyl diradylglycerols were analyzed by HPLC–ELSD. The silica column was a Zorbax Rx-SIL (4.6 × 250 mm, φ 5 μm; DuPont, Wilmington, DE, USA), and the mobile phase was *n*-hexane–isopropanol (100:0.25, v/v). The flow rate was 1.5 mL/min, and the column was maintained at a temperature of 36 °C. The ELSD conditions were as described above. Standards were made from 16:0/18:1-PlsEtn and 18:1/18:1-PtdEtn and the purities were determined by GC [30]. The value of each sample was corrected

by the ratio of derivatized 18:1/18:1-PtdEtn under the same conditions.

Spike and Recovery Experiment

To confirm the accuracy of this method, a known amount of plasmalogen was spiked into a biological sample and its response was recovered. We used 60 wt% plasmalogen from bovine brain as the additional plasmalogen, because the single species may have the inherent recovery by the polarity and structure. Briefly, 1, 2, and 4 mg (i.e., 0.6, 1.2, and 2.4 mg for plasmalogen) of 60 wt% plasmalogen were added to 180 mg of rat brain (1.2 mg for plasmalogen) before lipid extraction, and the recoveries were determined after this analytical process. This experiment was conducted in compliance with the policies and procedures detailed in the Animal Experiment Guidelines of Tohoku University.

Results

EtnGpl Derivatization

Reaction conditions of phospholipase C and acetylation were determined by pretesting EtnGpl standards (60 wt% plasmalogen of EtnGpl, 16:0/18:1-PlsEtn and 18:1/18:1-PtdEtn) to confirm diradylglycerols and acetyl diradylglycerols with TLC (data not shown) and total ion current chromatogram (TIC) of LC–MS (Fig. 2). Subsequently, under the same conditions, EtnGpl extracted from rat brains as biological samples was also confirmed to be completely reacted.

Separation and Detection of Acetyl Diradylglycerols by HPLC–ELSD

Three types of acetyl diradylglycerol, i.e., alkenylacyl, alkylacyl, and diacyl subclasses, could be completely separated from each other on a silica column with an isocratic system consisting of *n*-hexane–isopropanol (100:0.25, v/v) (Fig. 3). Acetyl diradylglycerol standards, alkenylacyl, alkylacyl, and diacyl subclasses, were derivatized from 16:0/18:1-PlsEtn, 16:0/22:6-PakCho and 18:1/18:1-PtdEtn, respectively.

The calibration curves were constructed with two acetyl diradylglycerol standards, alkenylacyl and diacyl derivatives, ranging in concentration from 1 to 10 μg. The calibration curves were expressed by the equation $y = ax^b$, which is a common feature of ELSD [21, 23, 31, 32], and the regression correlation coefficients were above 0.996 (Fig. 4), with detection limits of 200 ng/injection at a signal-to-noise ratio of 3. The calibration curves for

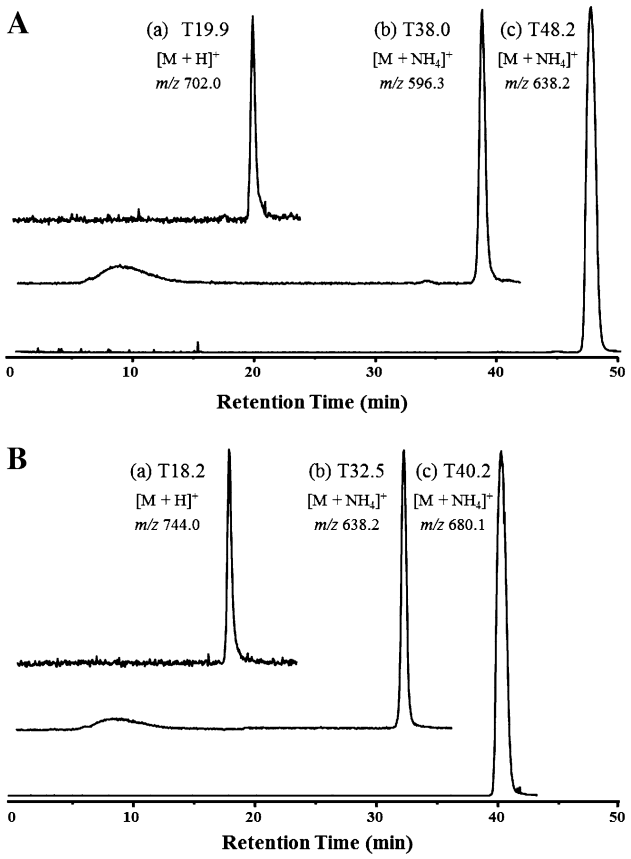

Fig. 2 Total ionization current chromatogram (TIC) of derivatized EtnGpl by LC–MS. **A** Derivatives from 16:0/18:1-PlsEtn, **B** derivatives from 18:1/18:1-PtdEtn; (*a*), (*b*), and (*c*) indicate original EtnGpl, diradylglycerol, and acetyl diradylglycerol, respectively

alkenylacyl and diacyl derivatives were nearly the same. The values of the alkenyl and diacyl derivatives were converted to those of plasmalogen and PtdEtn, respectively. The concentrations of PakEtn were determined by subtracting those of the plasmalogen and PtdEtn from the total EtnGpl because the spike and recovery test could not be performed without the PakEtn standard. The reaction recovery of the PakCho was different from those of plasmalogen and PtdEtn.

Subsequently, the accuracy of this method was confirmed by a spike and recovery experiment (Table 1).

Comparison of Methods for Determining Plasmalogen

The developed method using HPLC–ELSD was compared with conventional methods for plasmalogen (Table 2), i.e., the 2,4-dinitrophenylhydrazine method (NPH) [14], and separation and detection of acetyl diradylglycerols by TLC [33]. In rat brain, a higher level of plasmalogen was obtained by HPLC–ELSD than by the two other methods. Plasmalogen values obtained by TLC varied widely. In rat liver, plasmalogen was not detected by TLC.

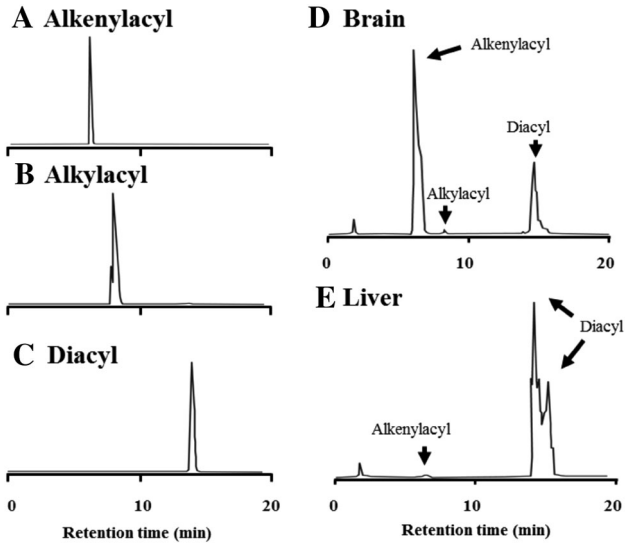

Fig. 3 HPLC–ELSD chromatograms of acetyl diradylglycerols. **a** Alkenylacyl subclass from 16:0/18:1-PlsEtn, **b** alkylacyl subclass from 16:0/18:1-PakCho, **c** diacyl subclass from 18:1/18:1-PtdEtn, **d** derivatives from rat brain EtnGpl, **e** derivatives from rat liver EtnGpl. Rat tissues were obtained from male Sprague–Dawley rats (7 weeks old) after fasting for 24 h

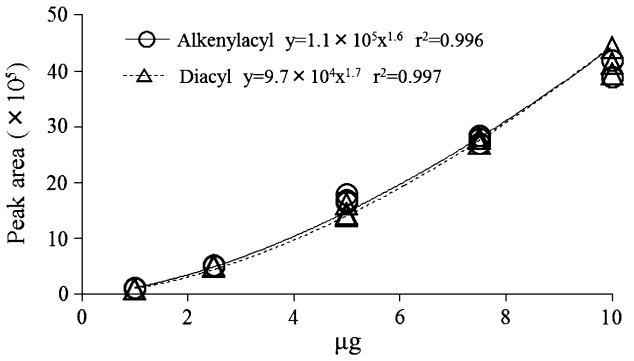

Fig. 4 Calibration curves of acetyl diradylglycerol standards in HPLC–ELSD. The calibration curves were constructed with acetyl diradylglycerol standards of different concentrations (1–10 μg/injection). Three determinations for each standard were given at different concentrations

Table 1 Spike and recovery test for HPLC–ELSD method

Spiked amount of plasmalogen (mg)			Recovery (%)
Rat brain 180 mg (1.2 mg for plasmalogen)	+	0.6	102.0 ± 2.0
		1.2	91.6 ± 3.8
		2.4	90.6 ± 2.6

Values are means ± standard deviation ($n = 5$)

Lipid Contents in Marine Invertebrates

The moisture and lipid contents of the marine invertebrates shown in Table 3 were investigated (Table 4). Most of the samples examined had moisture and total lipid contents of 70–90 % and 5–20 % (dry wt), respectively. The liver of *Halocynthia roretzi* had the highest moisture content, while the viscera of *Todarodes pacificus* had the lowest moisture content and the highest total lipid content.

Table 2 Comparison of methods for determining plasmalogen contents in rat tissues

	Plasmalogen contents of EtnGpl (mol%)		
	HPLC–ELSD	NPH	TLC
Rat brain	61.4 ± 1.5	45.3 ± 0.6	46.1 ± 9.5
Rat liver	3.5 ± 0.4	2.3 ± 1.1	Trace

Values are means ± standard deviation ($n = 5$)

The phospholipid contents were very high in *Asterina pectinifera* and muscles of *Todarodes pacificus*. In *Asterias amurensis* and the viscera of the invertebrates examined, total lipids consisted mostly of non-phospholipid.

As shown in Table 5, EtnGpl contents of phospholipid were as follows: >35 mol%, *Acanthopleura japonica*, *Cellana grata*, *Chlorostoma argyrostoma*, *Septifer virgatus*, *Stichopus japonicus* (red sea cucumber), and the muscle of *Paroctopus dofleini*; 34–35 mol%, *Thais bronni*, *Nucella heyseana*, *Mytilus galloprovincialis*, *Asterias amurensis*, and the liver of *Halocynthia roretzi*. In *Stichopus japonicus* (green sea cucumber), the gonad of *Strongylocentrotus nudus*, and the muscle of *Todarodes pacificus*, ChoGpl constituted about 70 mol% of the phospholipid. The contents of others were high in *Halichondria panacea* and *Halichondria japonica*.

EtnGpl subclass contents in marine invertebrates

Table 5 shows the EtnGpl subclasses present in marine invertebrates. Plasmalogen levels of EtnGpl were as follows: >90 mol%, *Anthopleura midori* (Fig. 5a), *Anthopleura japonica*, *Thais bronni*, *Mytilus galloprovincialis*, *Crassostrea gigas*, *Asterias amurensis* (Fig. 5b), *Asterina pectinifera*, and the gonad of *Strongylocentrotus nudus*; 80–90 mol%, *Acanthopleura japonica*, *Nucella heyseana*, *Chlorostoma argyrostoma*, *Septifer virgatus, Stichopus japonicus* (red and green sea cucumbers), and the muscle and liver of *Halocynthia roretzi* (Fig. 5c, d, respectively). In *Todarodes pacificus*, PtdEtn constituted about 60 mol% of EtnGpl. PakEtn content was high in the muscle of *Paroctopus dofleini*. In *Halichondria panacea* and *Halichondria japonica*, EtnGpl subclasses were not analyzed because of a lack of separation of EtnGpl by TLC.

Plasmalogen contents of fresh and dried samples are shown in Table 6. Plasmalogen contents in 100 g of fresh samples were as follows: >400 μmol, *Septifer virgatus*, *Asterias amurensis*, and *Asterina pectinifera*; 300–400 μmol, *Thais bronni* and *Nucella heyseana*,; 200–300 μmol,

Table 3 Taxonomic classification of the marine invertebrates examined

Phylum	Class	Scientific name	Common name
Porifera	Demospongiae	*Halichondria panicea*	Marine sponge
		Halichondria japonica	
Coelenterata	Anthozoa	*Anthopleura midori*	Sea anemone
		Anthopleura japonica	
Mollusca	Polyplacophora	*Acanthopleura japonica*	Chiton
	Gastropoda	*Thais bronni*	Univalve
		Nucella heyseana	
		Cellana grata	
		Chlorostoma argyrostoma	
		Tugali gigas	
	Bivalvia	*Mytilus galloprovincialis*	Blue mussel
		Septifer virgatus	Purplish bifurcate mussel
		Crassostrea gaigas	Oyster
		Patinopecten yessoensis	Scallop
	Cephalopoda	*Todarodes pacificus*	Cuttlefish
		Paroctopus dofleini	Octopus
Arthropoda	Crustacea	*Marsupenaeus japonicus*	Prawn
Echinodermata	Echinoidea	*Strongylocentrotus nudus*	Sea urchin
		Hemicentrotus pulcherrimus	
	Asteroidea	*Asterias amurensis*	Starfish
		Asterina pectinifera	
	Holothuroidea	*Stichopus japonicus*	Red sea cucumber
			Green sea cucumber
Protochordata	Ascidiacea	*Halocynthia roretzi*	Sea squirt

Anthopleura midori, *Anthopleura japonica*, *Cellana grata*, *Tugali gigas*, the gonad of *Strongylocentrotus nudus*, and the gonad of *Hemicentrotus pulcherrimus*. Plasmalogen contents of dried samples tended to be the same, except for samples with high moisture and low phospholipid contents, such as *Chlorostoma argyrostoma* and the liver of *Halocynthia roretzi*.

Discussion

This study demonstrated a new HPLC–ELSD procedure using enzyme treatment and acetylation for the separation and quantitative analyses of plasmalogen in marine invertebrates. The procedure has several advantages. Cyclopentane, which consists mainly of conventional mobile phase, is expensive [20, 29]. This new mobile phase consists of *n*-hexane and isopropanol. Therefore, this method can be performed to separate acetyl diradylglycerols at relatively low cost. In addition, ELSD is little affected by double bonds and length of fatty acids, in contrast to UV. The calibration curves for alkenylacyl forms from 16:0/18:1-PlsEtn and 16:0/22:6-PlsEtn were also nearly the same ($y = 1.1 \times 10^5 x^{1.6}$ and $y = 1.2 \times 10^5 x^{1.6}$, respectively).

In comparison with other methods, the HPLC–ELSD method yielded higher values of plasmalogen. In previous reports for the brain of adult rats with various methods, plasmalogen accounted for 55–57 mol% of EtnGpl [34–38]. In contrast, a lower value was reported using the aldehyde derivatives of alkenylacyl chains (41.7 mol%) [39], and use of HPLC for diradylglycerol derivatives yielded a higher value (65.9 mol%) [29]. It is known that diradylglycerol derivatives are stable, whereas aldehyde and the derivatives of alkenylacyl chains are unstable [40]. The background is very much higher by TLC than by HPLC–ELSD. In contrast, the plasmalogen level (61.4 mol% of EtnGpl) of the rat brain analyzed with HPLC–ELSD was about the same as that reported previously by a number of methods including a reliable method using radioactive isotopes [29]. In addition, this method was confirmed by the spike and recovery procedure.

Recently, new methods with mass spectrometry (MS) were reported for determination of plasmalogen, such as GC–MS [41] and LC–MS/MS [42, 43], and were shown to be applicable to measurement of plasmalogen with high sensitivity. Although these methods enable analysis of molecular species and fatty acids, it is difficult to obtain total plasmalogen because of the requirement for standards

Table 4 Moisture and lipid contents in the marine invertebrates examined

	Part	Moisture (wet wt%)	Lipid contents (wet wt%)		
			Total lipids	Phospholipids	Non-phospholipids
Halichondria panicea	Whole	84.7	1.1 (7.0)[a]	0.3 (1.7)	0.8 (5.4)
Halichondria japonica	Whole	85.2	1.1 (7.1)	0.4 (2.6)	0.7 (4.5)
Anthopleura midori	Whole	75.9	2.3 (9.7)	1.0 (4.3)	1.3 (5.4)
Anthopleura japonica	Whole	79.3	2.5 (11.9)	0.9 (4.4)	1.6 (7.5)
Acanthopleura japonica	Whole	66.5	0.8 (2.4)	0.4 (1.2)	0.4 (1.2)
Thais bronni	Whole	76.8	1.8 (7.7)	0.9 (3.7)	0.9 (4.0)
Nucella heyseana	Whole	77.4	1.9 (8.5)	0.8 (3.4)	1.2 (5.1)
Cellana grata	Whole	82.6	1.0 (5.9)	0.6 (3.3)	0.5 (2.6)
Chlorostoma argyrostoma	Whole	92.4	0.4 (5.3)	0.2 (2.6)	0.2 (2.7)
Tugali gigas	Whole	78.9	3.6 (17.3)	0.8 (3.7)	2.9 (13.7)
Mytilus galloprovincialis	Whole	81.8	1.0 (5.3)	0.4 (2.1)	0.6 (3.2)
Septifer virgatus	Whole	80.4	3.3 (16.9)	1.0 (5.1)	2.3 (11.8)
Crassostrea gaigas	Whole	83.3	1.6 (9.6)	0.5 (3.1)	1.1 (6.4)
Patinopecten yessoensis	Whole	73.0	2.5 (9.4)	0.7 (2.6)	1.8 (6.8)
	Muscle + mantle	79.8	0.8 (4.1)	0.5 (2.5)	0.3 (1.6)
	Viscera	81.6	4.7 (25.4)	0.7 (3.7)	4.0 (21.7)
Todarodes pacificus	Whole	73.1	4.3 (16.0)	1.2 (4.5)	3.1 (11.5)
	Muscle	76.2	2.1 (8.9)	1.4 (6.0)	0.7 (2.9)
	Viscera	57.4	16.9 (39.6)	0.9 (2.1)	16.0 (37.5)
Paroctopus dofleini	Muscle	83.8	0.7 (4.5)	0.4 (2.8)	0.3 (1.7)
Marsupenaeus japonicus	Whole	72.6	2.7 (9.8)	0.8 (3.0)	1.9 (6.8)
	Muscle	73.4	1.1 (4.3)	0.8 (3.0)	0.3 (1.3)
Strongylocentrotus nudus	Gonad	82.1	2.8 (15.5)	0.8 (4.6)	2.0 (11.0)
Hemicentrotus pulcherrimus	Gonad	84.2	2.7 (17.2)	0.7 (4.5)	2.0 (12.7)
Asterias amurensis	Whole	78.5	7.5 (35.0)	1.1 (5.2)	6.4 (29.7)
Asterina pectinifera	Whole	81.6	3.2 (17.4)	1.3 (6.8)	1.9 (10.5)
Stichopus japonicus-red	Whole	88.6	0.5 (4.6)	0.2 (1.6)	0.3 (3.0)
Stichopus japonicus-green	Whole	88.7	0.6 (5.5)	0.2 (1.8)	0.4 (3.7)
	Muscle	90.4	0.4 (4.6)	0.1 (1.5)	0.3 (3.1)
	Viscera	84.0	0.8 (5.0)	0.3 (1.9)	0.5 (3.1)
Halocynthia roretzi	Muscle	83.3	1.7 (10.4)	0.6 (3.8)	1.1 (6.6)
	Liver	98.2	0.4 (20.7)	0.1 (3.1)	0.3 (17.7)

Values are means ($n = 5$) except moisture and non-phospholipids. Moisture and non-phospholipids were determined as follows: [(fresh weight − freeze-dried weight)/fresh weight] and (total lipids − phospholipids), respectively

[a] Figures in parentheses show the contents in mg/100 g dry wt

of each molecular species. Moreover, the equipment required for MS is very expensive. On the other hand, ELSD is relatively inexpensive and enables analysis of total plasmalogen regardless of the kind of fatty acid. Thus, this method is suitable for screening a wide range of samples.

Using the HPLC–ELSD method, we searched for plasmalogen resources in marine invertebrates. Although limited numbers of samples were collected from local markets and Onagawa Bay, the EtnGpl subclass compositions of different species are shown in Table 7. With the exception of the class Cephalopoda and Crustacea, the marine invertebrates examined had high plasmalogen contents (>60 mol% of EtnGpl). Species with especially high levels of EtnGpl were *Asterias amurensis*, *Asterina pectinifera*, *Strongylocentrotus nudus*, *Anthopleura midori*, and *Anthopleura japonica*. The levels of plasmalogen in fresh samples of *Asterias amurensis*, *Asterina pectinifera*, and *Septifer virgatus* were very high.

Although plasmalogen levels of EtnGpl in some marine invertebrates were higher in the present study than those reported previously [10, 12, 44], the differences were thought to be due to the instability of derivatives and sensitivity as described above. Moreover, Joseph previously reported that there are seasonal changes in lipid content of Mollusca because of the reproductive cycle [45].

Table 5 Phospholipid contents in the marine invertebrates examined

	Part	Phospholipid contents (phospholipid mol%)				
		EtnGpl	SerGpn	ChoGpl	CerPCho	Others
Halichondria panicea	Whole	21.2 ± 2.0 (69 ± 7)[a]	4.8 ± 0.8 (16 ± 2)	19.8 ± 1.7 (65 ± 6)	3.8 ± 0.5 (12 ± 2)	50.5 (164)
Halichondriajaponica	Whole	11.1 ± 1.0 (56 ± 5)	6.1 ± 0.5 (30 ± 2)	19.4 ± 2.5 (99 ± 13)	Trace	63.4 (319)
Anthopleura midori	Whole	19.8 ± 0.8 (262 ± 11)	24.6 ± 1.6 (325 ± 21)	30.9 ± 1.3 (409 ± 17)	Trace	24.8 (311)
Anthopleura japonica	Whole	23.9 ± 0.8 (280 ± 9)	23.7 ± 1.3 (278 ± 15)	32.1 ± 1.3 (376 ± 15)	Trace	20.4 (225)
Acanthopleurajaponica	Whole	35.8 ± 2.2 (187 ± 12)	5.4 ± 0.6 (28 ± 3)	45.5 ± 4.3 (239 ± 23)	Trace	13.3 (71)
Thais bronni	Whole	34.6 ± 1.4 (381 ± 16)	11.1 ± 0.7 (122 ± 7)	49.1 ± 1.8 (541 ± 20)	Trace	5.3 (57)
Nucella heyseana	Whole	34.7 ± 2.4 (343 ± 24)	13.6 ± 1.4 (135 ± 14)	50.7 ± 4.2 (501 ± 42)	Trace	1.0 (8)
Cellana grata	Whole	38.3 ± 2.4 (284 ± 18)	Trace	41.6 ± 2.8 (307 ± 21)	Trace	20.1 (152)
Chlorostoma argyrostoma	Whole	38.6 ± 1.7 (98 ± 4)	Trace	44.0 ± 3.3 (112 ± 8)	Trace	17.3 (45)
Tugali gigas	Whole	33.8 ± 1.6 (334 ± 16)	Trace	45.8 ± 5.1 (454 ± 51)	7.5 ± 0.4 (75 ± 4)	12.8 (134)
Mytilus galloprovincialis	Whole	34.9 ± 2.2 (176 ± 11)	18.1 ± 1.4 (91 ± 7)	29.7 ± 2.1 (149 ± 11)	Trace	17.3 (84)
Septifer virgatus	Whole	36.8 ± 2.4 (474 ± 31)	12.0 ± 1.7 (155 ± 22)	33.3 ± 3.0 (428 ± 39)	Trace	17.9 (226)
Crassostrea gaigas	Whole	22.7 ± 1.4 (154 ± 10)	15.2 ± 1.7 (103 ± 11)	28.2 ± 1.5 (192 ± 10)	Trace	33.8 (226)
Patinopecten yessoensis	Whole	29.7 ± 2.9 (269 ± 26)	12.9 ± 1.5 (116 ± 14)	36.4 ± 3.9 (329 ± 35)	Trace	21.0 (187)
	Muscle + mantle	31.3 ± 3.7 (207 ± 25)	12.2 ± 1.9 (81 ± 12)	35.4 ± 3.8 (235 ± 25)	Trace	21.1 (138)
	Viscera	19.5 ± 2.3 (173 ± 21)	8.9 ± 1.4 (79 ± 12)	35.6 ± 4.2 (315 ± 37)	Trace	36.0 (317)
Todarodes pacificus	Whole	29.7 ± 2.1 (466 ± 33)	8.9 ± 0.8 (139 ± 13)	54.9 ± 1.6 (859 ± 25)	4.2 ± 0.3 (66 ± 5)	2.3 (37)
	Muscle	29.3 ± 1.7 (543 ± 32)	Trace	68.4 ± 5.1 (1263 ± 95)	Trace	2.3 (53)
	Viscera	17.2 ± 2.1 (199 ± 24)	12.0 ± 2.0 (139 ± 23)	46.7 ± 4.7 (540 ± 55)	9.2 ± 1.8 (07 ± 21)	14.8 (169)
Paroctopus dofleini	Muscle	37.1 ± 2.8 (215 ± 16)	13.1 ± 1.2 (75 ± 7)	42.2 ± 2.8 (244 ± 16)	Trace	7.6 (43)
Marsupmaeusjaponicus	Whole	28.4 ± 2.4 (303 ± 26)	Trace	60.7 ± 4.5 (646 ± 48)	9.2 ± 1.5 (97 ± 16)	1.7 (26)
	Muscle	31.6 ± 4.2 (327 ± 44)	Trace	52.8 ± 6.2 (546 ± 64)	10.0 ± 1.8 (04 ± 19)	5.7 (67)
Strongylocentrotus nudus	Gonad	23.6 ± 1.3 (246 ± 14)	Trace	73.5 ± 2.7 (767 ± 28)	Trace	2.9 (36)
Hemicentrotus pulcherrimus	Gonad	32.5 ± 0.8 (295 ± 7)	Trace	57.1 ± 2.1 (520 ± 19)	Trace	10.4 (101)
Asterias amurmsis	Whole	34.4 ± 2.5 (504 ± 37)	4.5 ± 0.5 (65 ± 7)	54.3 ± 3.0 (794 ± 44)	Trace	6.8 (105)
Asterina pectinifera	Whole	24.9 ± 1.8 (405 ± 29)	10.2 ± 0.9 (166 ± 15)	42.4 ± 3.3 (690 ± 54)	Trace	22.4 (361)
Stichopus japonicus-red	Whole	35.5 ± 3.1 (83 ± 7)	7.0 ± 1.1 (16 ± 3)	50.4 ± 2.6 (118 ± 6)	2.3 ± 0.4 (5 ± 1)	4.8 (12)
Stichopus japonicus-green	Whole	23.4 ± 1.6 (62 ± 4)	Trace	73.1 ± 3.1 (196 ± 8)	Trace	3.5 (10)
	Muscle	21.4 ± 1.5 (39 ± 3)	Trace	65.6 ± 5.1 (121 ± 9)	Trace	13.0 (25)
	Viscera	23.0 ± 2.2 (90 ± 9)	8.6 ± 1.3 (34 ± 5)	36.3 ± 3.3 (141 ± 13)	2.3 ± 0.4 (9 ± 2)	29.7 (115)

Table 5 continued

	Part	Phospholipid contents (phospholipid mol%)				
		EtnGpl	SerGpn	ChoGpl	CerPCho	Others
Halocynthia roretzi	Muscle	15.5 ± 0.6 (128 ± 5)	Trace	38.2 ± 1.5 (314 ± 12)	8.4 ± 1.3 (68 ± 11)	37.9 (316)
	Liver	34.7 ± 1.3 (23 ± 1)	16.0 ± 0.9 (11 ± 1)	43.4 ± 4.1 (30 ± 3)	Trace	5.9 (4)

Values are means ± standard deviation ($n = 5$) except where otherwise noted. Others were determined as follows: [phospholipids − (EtnGpl + SerGpl + ChoGpl + CerPCho)]. Average molecular weights used were 769, 825, 771, 760, and 775 for EtnGpl, SerGpl, ChoGpl, CerPCho, and others, respectively. Trace indicates less than 0.05

[a] Figures in parentheses show the contents in μmol/100 g wet wt

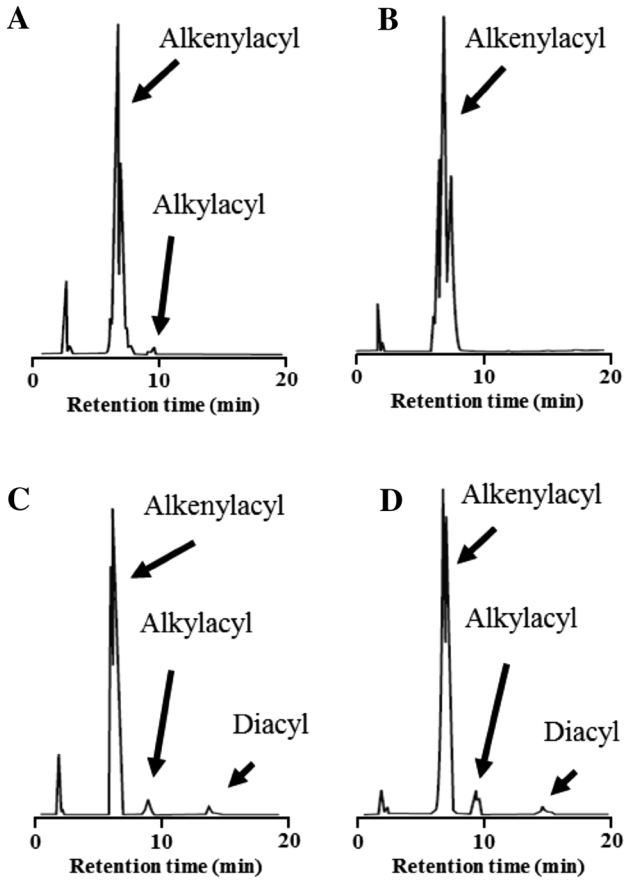

Fig. 5 HPLC–ELSD chromatograms of acetyl diradylglycerols from EtnGpl of marine invertebrates. **a** *Anthopleura midori*, **b** *Asterias amurensis*, **c** *Halocynthia roretzi* muscle, **d** *Halocynthia roretzi* liver. **a**, **b**, **c**, and **d** were injected with 15, 10, 20, and 20 μL as step 6 in Fig. 1, respectively

In the muscle of *Halocynthia roretzi*, the amounts of the total lipid, phospholipid, and plasmalogen were lower in the present study than those reported previously, whereas the plasmalogen level of EtnGpl was higher [12]. Thus, there may also be seasonal changes in plasmalogen levels.

In *H. panacea* and *Halichondria japonica*, plasmalogen was not analyzed because EtnGpl could not be separated by TLC. Ceramide galactoside had an Rf value near EtnGpl and was detected by primulin. In *Halichondria japonica*, it was reported that the main glycerosphingolipid was ceramide digalactoside [46]. Because of the increased hydrophilicity, it was inferred that ceramide digalactoside was consistent with the position of EtnGpl. Although this separation for screening was performed by TLC, it is expected that separation would be improved by HPLC.

The class Asteroidea was reported previously to have high plasmalogen levels of EtnGpl [47]. In addition, two starfish examined were also rich in plasmalogen. However, starfish are too small and require a great deal of effort for collection and dissection; therefore, it is difficult

Table 6 Comparison of amounts of EtnGpl subclasses in the marine invertebrates examined

	Part	EtnGpl subclasses (μmol/100 g wet wt)		
		Plasmalogen	PtdEtn	PakEtn
Halichondria panicea	Whole	–	–	–
Halichondria japonica	Whole	–	–	–
Anthopleura midori	Whole	255 ± 5 (1,060 ± 21)[a]	Trace	9 (39)
Anthopleura japonica	Whole	270 ± 10 (1,301 ± 48)	Trace	6 (26)
Acanthopleura japonica	Whole	168 ± 3 (502 ± 8)	11 ± 0 (33 ± 4)	9 (27)
Thais bronni	Whole	359 ± 6 (1,546 ± 27)	27 ± 0 (116 ± 5)	Trace
Nucella heyseana	Whole	307 ± 9 (1,358 ± 41)	40 ± 1 (181 ± 13)	Trace
Cellana grata	Whole	215 ± 2 (1,232 ± 11)	48 ± 0 (275 ± 31)	20 (117)
Chlorostoma argyrostoma	Whole	79 ± 4 (1,046 ± 56)	17 ± 1 (229 ± 10)	2 (31)
Tugali gigas	Whole	269 ± 15 (1,275 ± 73)	33 ± 2 (156 ± 5)	34 (163)
Mytilus galloprovincialis	Whole	161 ± 6 (887 ± 32)	8 ± 0 (44 ± 5)	6 (31)
Septifer virgatus	Whole	417 ± 23 (2,123 ± 119)	41 ± 2 (207 ± 19)	20 (103)
Crassostrea gaigas	Whole	141 ± 3 (844 ± 20)	11 ± 0 (65 ± 5)	Trace
Patinopecten yessoensis	Whole	179 ± 9 (662 ± 35)	40 ± 2 (150 ± 7)	49 (182)
	Muscle + mantle	133 ± 7 (660 ± 36)	26 ± 1 (131 ± 14)	43 (213)
	Viscera	137 ± 8 (744 ± 44)	25 ± 1 (135 ± 8)	11 (57)
Todarodes pacificus	Whole	113 ± 4 (420 ± 16)	270 ± 10 (1,003 ± 40)	84 (312)
	Muscle	106 ± 8 (448 ± 32)	340 ± 24 (1,429 ± 59)	93 (393)
	Viscera	60 ± 2 (141 ± 5)	113 ± 4 (264 ± 4)	30 (72)
Paroctopus dofleini	Muscle	86 ± 5 (531 ± 30)	47 ± 3 (293 ± 16)	85 (527)
Marsupenaeus japonicus	Whole	139 ± 3 (506 ± 30)	134 ± 3 (488 ± 8)	28 (101)
	Muscle	152 ± 1 (569 ± 5)	146 ± 1 (548 ± 16)	28 (104)
Strongylocentrotus nudus	Gonad	256 ± 12 (1,431 ± 67)	Trace	5 (29)
Hemicentrotus pulcherrimus	Gonad	230 ± 3 (1,453 ± 21)	47 ± 4 (297 ± 23)	21 (131)
Asterias amurensis	Whole	504 ± 22 (2,342 ± 100)	Trace	10 (48)
Asterina pectinifera	Whole	407 ± 37 (2,212 ± 199)	Trace	8 (46)
Stichopus japonicus-red	Whole	67 ± 2 (591 ± 16)	7 ± 0 (64 ± 6)	7 (60)
Stichopus japonicus-green	Whole	52 ± 5 (454 ± 40)	6 ± 1 (55 ± 4)	4 (38)
	Muscle	34 ± 1 (354 ± 8)	3 ± 0 (35 ± 1)	2 (25)
	Viscera	82 ± 2 (510 ± 10)	Trace	9 (57)
Halocynthia roretzi	Muscle	106 ± 3 (635 ± 18)	11 ± 0 (65 ± 5)	12 (69)
	Liver	19 ± 1 (1,106 ± 68)	2 ± 0 (98 ± 9)	3 (163)

Values are means ± standard deviation ($n = 5$) except PakEtn

[a] Figures in parentheses show the contents in μmol/100 g dry wt

to prepare large amounts of plasmalogen from starfish. On the other hand, the class Bivalvia was also reported to have high plasmalogen levels [11]. In the bivalves examined except *Patinopecten yessoensis*, plasmalogen accounted for about 90 mol% of EtnGpl. *Septifer virgatus* was also especially rich in plasmalogen, comparable to the starfish. *Septifer virgatus* and *Mytilus galloprovincialis* cause severe damage to the marine industry, and it is, therefore, possible to obtain these species in large amounts. Moreover, it is easy to separate the meat from the shell of bivalves; therefore, these species are expected to be good resources for plasmalogen.

With regard to the relation between plasmalogen levels of EtnGpl and taxonomic classification, Deuterostomia, including Echinodermata and Protochordata, contained high levels. Platyhelminthes [48] and Annelida [49], not examined in this study, were reported to contain high levels of plasmalogen. Thus, in Protostomia, lower organisms are thought to contain high levels of plasmalogen, whereas higher organisms, such as Arthropoda and the class Cephalopoda, contain low levels. In Deuterostomia, it was also reported that higher organisms, such as vertebrates, contain low plasmalogen levels except in the nervous system and testis [50–52].

Table 7 Contents of EtnGpl subclasses in the marine invertebrates examined

	Part	EtnGpl subclasses (EtnGpl mol%)		
		Plasmalogen	PtdEtn	PakEtn
Halichondria panicea	Whole	–	–	–
Halichondria japonica	Whole	–	–	–
Anthopleura midori	Whole	96.5 ± 1.9	Trace	3.5
Anthopleura japonica	Whole	98.0 ± 3.6	Trace	2.0
Acanthoplera japonica	Whole	89.4 ± 1.4	5.7 ± 0.7	4.9
Thais bronni	Whole	93.0 ± 1.6	7.0 ± 0.3	Trace
Nucella heyseana	Whole	88.5 ± 2.7	11.5 ± 0.8	Trace
Cellana grata	Whole	75.9 ± 0.7	16.9 ± 1.9	7.2
Chlorostoma argyrostoma	Whole	80.1 ± 4.3	17.5 ± 0.8	2.4
Tugali gigas	Whole	80.0 ± 4.6	9.8 ± 0.3	10.2
Mytilus galloprovincialis	Whole	92.1 ± 3.3	4.6 ± 0.5	3.3
Septifer virgatus	Whole	87.3 ± 4.9	8.5 ± 0.8	4.2
Crassostrea gaigas	Whole	92.8 ± 2.2	7.2 ± 0.6	Trace
Patinopecten yessoensis	Whole	66.7 ± 3.5	15.0 ± 0.7	18.3
	Muscle + mantle	65.7 ± 3.6	13.0 ± 1.4	21.3
	Viscera	79.5 ± 4.7	14.4 ± 0.8	6.1
Todarodes pacificus	Whole	24.2 ± 0.9	57.8 ± 2.3	18.0
	Muscle	19.7 ± 1.4	63.0 ± 2.6	17.3
	Viscera	29.5 ± 1.0	55.5 ± 0.8	15.0
Paroctopus dofleini	Muscle	39.3 ± 2.2	21.7 ± 1.2	39.0
Marsupenaeus japonicus	Whole	46.2 ± 1.1	44.5 ± 0.7	9.3
	Muscle	46.6 ± 0.4	44.9 ± 1.4	8.5
Strongylocentrotus nudus	Gonad	98.0 ± 4.6	Trace	2.0
Hemicentrotus pulcherrimus	Gonad	77.2 ± 1.1	15.8 ± 1.2	7.0
Asterias amurensis	Whole	98.0 ± 4.2	Trace	2.0
Asterina pectinifera	Whole	98.0 ± 8.8	Trace	2.0
Stichopus japonicus-red	Whole	82.8 ± 2.2	8.9 ± 0.8	8.3
Stichopus japonicus-green	Whole	83.2 ± 7.3	10.0 ± 0.7	6.8
	Muscle	85.5 ± 2.0	8.5 ± 0.3	6.0
	Viscera	90.0 ± 1.7	Trace	10.0
Halocynthia roretzi	Muscle	82.5 ± 2.3	8.5 ± 0.7	9.0
	Liver	80.9 ± 5.0	7.2 ± 0.7	11.9

Values are means ± standard deviation ($n = 5$) except PakEtn. PakEtn was determined as follows: [EtnGpl − (plasmalogen + PtdEtn)]. Trace indicates less than 0.05

In conclusion, a method for determining EtnGpl subclasses with ELSD was developed and was shown to be a powerful tool for searching for plasmalogen resources.

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