Phospholipids in Mediterranean Cephalopods

Vassilia J. Sinanoglou and Sofia Miniadis-Meimaroglou*

Food Chemistry Laboratory, Department of Chemistry, University of Athens, Panepistimiopolis Zographou, 15771 Athens, Greece.
Fax: (301) 7228815; (301) 7483415. E-mail: miniadou@chem.uoa.gr

* Author for correspondence and reprint requests

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Polar lipids of the cephalopods *Eledone moschata, Sepia officinalis* and *Todarodes sagittatus* mantle, represent 50.5%, 66.1% and 74.2% of wet tissue respectively. On the other hand the polar lipids of these three species of cephalopods constitute of 80.8%, 94.8% and 93.7% of phospholipids, respectively. The main phospholipids identified were phosphatidylcholine (52.2, 51.3 and 58.4% of total phospholipids respectively in the above mentioned species), phosphatidylethanolamine (18.1, 19.7 and 23.9%), sphingomyelin (10.7, 15.2 and 6.7%), lysophosphatidylcholine (3.1, 3.8 and 1.8%) and the unusual lipid ceramide aminoethylphosphonic acid (15.9, 10 and 9.2%).

The 56.8% of phosphatidylcholine in *Eledone moschata*, the 46% in *Sepia officinalis* and the 74.1% in *Todarodes sagittatus* refer to the structure of 1,2-diacyl-glycerocholine and the remaining percentage refer to the structure of 1-*o*-alkyl-2-acyl-glycerocholine or 1-*o*-alkyl-1-enyl-2-acyl-glycerocholine.

The 87.2% of phosphatidylethanolamine in *Eledone moschata*, the 81% in *Sepia officinalis* and the 90.7% in *Todarodes sagittatus* refer to the structure of 1,2-diacyl-glyceroethanolamine and the remaining percentage refer to the structure of 1-*o*-alkyl-2-acyl-glyceroethanolamine or 1-*o*-alkyl-1-enyl-2-acyl-glyceroethanolamine.

The major saturated fatty acids in phosphatidylcholine and phosphatidylethanolamine were C16:0 (30.3–67.5% and 23.2–54.5%) and C18:0 (3.6–17% and 15.4–28%), respectively, while the major unsaturated fatty acids in these lipids were C18:1n-9, n-7 (1.0–7.3% and 5.3–10.5%), C20:5n-3 (1.5–9.8% and 4,5–15.8%) and C22:6n-3 (12.5–42.0% and 7.0–11.3%), respectively.

Introduction

Cephalopod molluscs (octopuses, squids, etc) comprise one of the main fishery food resources commercially available in Greece, representing a high proportion of fishery products consumed. Phospholipids are the major structural lipids in fish classes and one of the major constituents in the cell membrane of plant animal and fungal organisms. Although the lipid composition of many marine molluscs is well documented, there is only a limited amount of information on the complete composition and structure of the molecular species of mollusc flesh phospholipids (De Koning, 1993). Membrane phospholipids are a complex mixture of molecular species containing a variety of fatty acyl acid head group compositions. In general, phospholipids contain higher proportions of the longer chain polyunsaturated fatty acids. It is widely accepted that chemical and physical properties of cell membranes depend on the phospholipid composition, which can affect membrane fluidity which in turn alter the activity of many membrane-bound proteins. Therefore, altering the phospholipid profile may bring about significant biological consequences (Stubbs and Smith, 1984; Muderhwa and Brockman, 1992; Zakim *et al.*, 1992).

Objective of the present work is the quantitative and qualitative determination of mantle phospholipid composition of three edible species of cephalopods (*Eledone moschata, Sepia officinalis* and

Abbreviations: EI electron ionization, ES electrospray, FA fatty acids, GC gas chromatography, HPLC high performance liquid chromatography, HPTLC high performance thin layer chromatography, l-PC lyso-phosphatidylcholine, MS mass spectrometry, NL neutral lipids, PC phosphatidylcholine, PE phosphatidylethanolamine, PhL phospholipids, PL polar lipids, PnL phosphonolipids, PUFA polyunsaturated fatty acids, Shm sphingomyelin, SPE solid phase extraction, TFA total fatty acids, TLC thin layer chromatography, TL total lipids, TPhL total phospholipids.

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Todarodes sagittatus) from Saronicos Bay (Greece), focusing on the isolation and identification of the structure of the molecular species of the main phospholipids [phosphatidylethanolamine (PE) and phosphatidylcholine (PC)].

Materials and Methods

Animals used

Thirty speciments (six composite samples, five cephalopods per composite) from three classes of cephalopod molluscs *Eledone moschata* (Mollusca, Cephalopoda, Octopodidae), *Sepia officinalis* (Mollusca, Cephalopoda, Sepiodea) and *Todarodes sagittatus* (Mollusca, Cephalopoda, Teuthoidae) were collected from Saronicos Bay (Greece) in November 1996. They were brought to the laboratory alive in sea water and immediately analysed as described below.

Extraction and separation of polar from neutral lipids

The mantle from each of the above invertebrates, after measuring the total weight of wet tissue, were homogenized in an Omni-Mixer (Sorvall Inc. Newtown, Connecticut, USA; 0 °C, 5 min at medium speed). Extraction of lipids was essentially effected according to Bligh and Dyer (1959). After phase equilibration, the lower chloroform layer (total lipids) was removed, concentrated in a rotary evaporator to dryness, redissolved in 200 ml of chloroform/methanol (9:1, by vol.) and stored at 0 °C.

Total lipids were fractionated by solid phase extraction (SPE) using the method of Berger *et al.* (1992). Phase separation of neutral and polar lipids was achieved by the same method as modified by Mastronicolis *et al.* (1996).

Iatroscan Analysis of polar lipids

Lipid mixtures were separated on silicic acid-coated quartz rods, Chromarods (Type SII), and then quantitated by passing the rods through a hydrogen flame ionization detector (F. I. D.) (Kramer, 1980; Tanaka *et al.*, 1980). Mixtures of polar lipids were evaporated to dryness and redissolved in chloroform/methanol (9:1, by vol.) at a concentration of 0.03 g ml^{-1}. From each solution of polar lipids, 0.5–1.0 μl was spotted on each of 10 silica gel Chromarods (Type SII). The polar lipids were developed in chloroform/methanol/water (70:35:3.5, by vol.) to a height of 17 cm, and the rods were scanned in an Iatroscan TH-10 Analyzer, Mark II (Iatron Laboratories, Inc., Tokyo, Japan.), equipped with a hydrogen flame ionization detector and connected to an integrator. The flame ionization detector was operated with a hydrogen flow-rate of 160 ml min^{-1} and an air flow-rate of 2 l min^{-1} The scanning speed was 240 mm min^{-1}. A two-pen linear recorder (Fischer Recorball, Model 5000) was used at 10 mV full-scale deflection and a chart speed of 0.47 cm s^{-1}. The standards used were ceramides, cardiolipine, phosphatidylcholine, phosphatidylethanolamine, lyso-phosphatidylcholine, lyso-phosphatidylethanolamine, phosphatidylserine, sphingomyelin standards of the Sigma Chemical Co (Sigma-Aldrich Company, Dorset, U. K. and St. Louis, MO).

Isolation and purification of individual phospholipids

Individual phospholipid components were recovered from chromatoplates after polar lipids separation by preparative TLC using a solvent system consisting of chloroform/methanol/glacial acetic acid/water (50:25:6:2, by vol.). The individual spots, visualized by exposure to iodine vapors, were scraped into a vial and extracted from the silica gel using the solvent system of the Bligh-Dyer procedure, i.e. chloroform/ methanol/ water (2:2:1, by vol.). After phase separation, chloroform extracts were evaporated and the residual lipids were used for further chromatographic analysis. The extracts of individual lipids were rechromatographed on HPLC for confirmation of purity.

HPLC of phospholipids (Andrikopoulos et al., 1986)

Individual phospholipid components containing 10 mg of lipids were dried and the residue was redissolved in chloroform to a solution 10%. Quantitative and qualitative analysis, was performed with a Jasco 880-PU HPLC, (Intelligent HPLC Pump Jasco 880–31 Solvent Mixing Module) chromatographer, equipped with a Jasco 875 UV spectrometric detector at 214 nm. Phospholipid components were extracted on a Partisil SCX 10 μm column (250×4.6, 25 mm i.d.) (Jones Chromatography Ltd,

Mid Glamorgan, UK). The isocratic system acetonitrile/methanol/water (350:150:35 v/v/v) was used as the mobile phase at a flow-rate of 1.5 ml min^{-1} in a press of 34–40 kg cm^{-2}. A Hewlett Packard HP3396A integrator (Hewlett Packard, Palo, Alto, CA.) was used.

The solvents used were Lichrosolv solvents gradient grade (E. Merck, Darmstadt, Germany). The standards used were phosphatidylcholine, phosphatidylethanolamine, lyso-phosphatidylcholine, lyso-phosphatidylethanolamine, phosphatidylserine, sphingomyelin standards (Sigma-Aldrich Company).

Separation of phospholipids by two-dimensional HPTLC

Individual phospholipids were further separated by two-dimensional HPTLC analysis carried out on precoated 10x10 silica gel 60 G plates (E. Merck, Darmstadt, Germany). 10–20 μg of sample were applyed as spots at the lower right-hand corner of the plate, 1.0 cm from each edge. The solvent system for the 1_{st} dimension consisted of chloroform/methanol/acetic acid/water (50:25:6:2, by vol.) and for the 2 nd dimension consisted of chloroform/methanol/water (65:25:4, by vol.). Visualization of spots was effected by exposure to iodine vapors, by spraying with ninhydrin reagent (solution 0.2% (w/v) of ninhydrin in ethanol) for detection of amino-groups and by spraying with phosphomolybdenum blue reagent (Dittmer and Lester reagent) for detection of phospholipids.

Quantitative analysis of phospholipids

Total phosphorus and phosphonate phosphorus were determined by the method of Long and Staples (1961) and the method of Kapoulas *et al.* (1984), respectively. Esters were determined by the method of Snyder and Stephens (1959), glyceryl ethers by the method of Hanahan and Watts (1961), plasmalogens by the method of Gottfried and Rapport (1963) and long chain bases by the method of Lauter and Trams (1962). Mild alkaline hydrolysis was performed with 0.1 ml NaOH (1.2 N) in methanol (50%, by vol.), 45 °C, 20 min (Wells and Dittmer, 1966). Chloroform-soluble products of phospholipid components were analysed by single-dimensional TLC analysis carried out on precoated 20x20 silica gel 60 G plates (E. Merck, Darmstadt, Germany). Solvent systems consisted of chloroform/methanol/acetic acid/water (50:25:6:2, by vol.) and of chloroform/methanol/water (65:25:4, by vol.). Visualization of spots was effected by exposure to iodine vapors, by spraying with ninhydrin reagent (solution 0.2% (w/v) of ninhydrin in ethanol) for detection of amino-groups and by spraying with phosphomolybdenum blue reagent (Dittmer and Lester reagent) for detection of phospholipids. The standards used were phosphatidylcholine, phosphatidylethanolamine, lyso-phosphatidylcholine, lyso-phosphatidylethanolamine, phosphatidylserine, sphingomyelin standards of the Sigma Chemical Co.

Water-soluble products of phospholipid components were analysed by paper chromatographic analysis, carried out on Whatman paper No.1 with the ascending (6–7h) and descending (7–8h) technique using solvent systems consisting of phenol/water/ethanol/acetic acid (80:20:12:10, by vol.) and of phenol/ethanol/acetic acid (100:12:10, by vol.), respectively. The individual spots, for detection of amino groups visualized by spraying with ninhydrin reagent (solution 0.2% (w/v) of ninhydrin in ethanol), for detection of phospholipids by spraying with Hanes-Isherwood reagent and for detection of choline by spraying with Dragendorff reagent. The standards used were L-α-glycerylphosphorylcholine, L-α-glycerylphosphorylethanolamine and 2-aminoethylphosphonic acid standards of the Sigma Chemical Co.

Electrospray ionization mass spectrometry

Phosphatidylcholine and phosphatidylethanolamine were identified by electrospray ionization mass spectrometry. ESI mass spectra were acquired on a Fisons VG Quatro SQ, SN 5147 triple quadrupole mass spectrometer (Fisons Instruments, Altrincham, WA) (Conditions: Q, SN 5147), equipped with an electrospray interface and data-handling system. Aliquots (10 μl) of the sample solution (10^{-3}–10^{-4} mol l^{-1}) were injected directly into the electrospray source via a loop injector Rheodyne 7125 into a stream of solvent (methanol/water 80:20, by vol. containing 1% ammonium acetate) at a flow-rate of 2 μl min^{-1}.

Parameters for anions (ES-): Capillary 3.00 Kvolt, HV lens 0.46 Kvolt, focus 40 Kvolt, skim-

mer 46 Kvolt, source temp 80 °C, ion energy 1.9 Volts, mass range 100–1000.

Parameters for cations (ES+): Capillary 3.30 Kvolt, HV lens 0.38 Kvolt, focus 40 Kvolt, skimmer 45 Kvolt, source temp 70 °C, ion energy 2.4 Volt, mass range 100–1000.

The mass spectrometer was scanned over the m/z range of interest and the mass scale was calibrated by injected solutions of standards in methanol/water (80:20, by vol.) containing 1% acetic ammonia. The standards used were phosphatidylcholine, phosphatidylethanolamine, lyso-phosphatidylcholine, lyso-phosphatidylethanolamine, phosphatidylserine, sphingomyelin standards of the Sigma Chemical. The solvents used were Lichrosolv solvents of gradient grade (E. Merck, Darmstadt, Germany).

Methanolysis of lipids gas chromatography and gas chromatography/mass spectrometry analysis of fatty acid methyl esters. (Sinanoglou and Miniadis-Meimaroglou, 1999)

Methyl esters of the fatty acids contained in phospholipid fractions were prepared as follows: A sample containing 20–50 mg of lipids was dried before trans-esterification and the residue was redissolved in 0.75 ml n-hexane; then 0.1 ml of 2 N potassium hydroxide in methanol was added and the solution was mixed for 2 min in a vortex mixer (Velp scientifica, Italy), dried over anhydrous sodium sulfate and left for 25 min. After phase separation the upper layer of n-hexane containing the fatty acid methyl esters was removed and immediately injected into the gaschromatograph.

Quantitative and qualitative analysis, was performed on a HRGC Mega 2 Series 8560 MFC 800 (Fisons Instruments) gas chromatograph, equipped with a EL 980 CE Instruments FID (flame ionization detector) (Hellenic Labware). A fused silica capillary column of high polarity was used (Supelco SP 2340, 60 m X 0.32 mm i.d. and 0.2μm film thickness; Supelco, Inc., Bellefonte, PA, USA). This polymeric stationary phase was a non-bonded poly(biscyanopropylsiloxane). The carrier gas was hydrogen at a pressure of 15 psi. The make-up gas was atmospheric air at a pressure of 15 psi and hydrogen was supplied to the FID at a pressure of 9 psi. The temperature of injector was 230 °C and of detector 250 °C. The temperature was programmed at 150 °C for 15 min, raised from 150 to 170 °C at a rate 2 °C min^{-1}, held constant at 170 °C for 10 min, raised from 170 to 215 °C at 4 °C min^{-1} and held at 215 °C for 15 min. The duration of the analysis was 62 min. In the GC/MS analysis system a mass spectrometer VG Trio-2000 mass spectrometer (Fisons Instruments) replaced the FID. Electron ionization (E. I.) was produced by accelerating electrons from a hot filament through a potential difference, usually of 70 eV. In both GC and GC/MS methods, the fatty acid methyl esters were identified by comparison with a standard mixture of fatty acid methyl esters purchased from Sigma. This was made in our laboratory using a mixture of fatty acid methyl ester standards (5 mg of each) from Sigma Chemical Co: lauric acid M-E, myristic acid M-E, palmitic acid M-E, stearic acid M-E, arachidic acid M-E, *cis*-10-heptadecenoic acid M-E, oleic acid (*cis*-9) M-E, vaccenic acid (*cis*-11) M-E, linoleic acid (*cis*-9,12) M-E, γ-linolenic acid (*cis*-6,9,12) M-E, α-linolenic acid (*cis*-9,12,15) M-E, *cis*-11-eicosenoic acid M-E, *cis*-11,14,17-eicosatrienoic acid M-E, arachidonic acid (*cis*-5,8,11,14) M-E, *cis*-5,8,11,14,17-eicosapentaenoic acid M-E, erucic acid (*cis*-13) M-E, *cis*-7,10,13,16-docosatetraenoic acid M-E, *cis*-4,7,10,13,16,19-docosahexaenoic acid M-E.

Statistical analysis

We examined 30 speciments (six composite samples, five cephalopods per composite) from three classes of cephalopods molluscs. Each analysis was conducted nine times.

Statistical analysis: Data are reported as mean and standard deviation.

Results and Discussion

General observations

The amounts of total lipid extracted from the mantle of *Eledone moschata, Sepia officinalis* and *Todarodes sagittatus*, according to the procedure of Bligh-Dyer (1959) were 20.0 g kg^{-1}, 14.0 g kg^{-1} and 17.0 g kg^{-1}, respectively (average for 30 specimens of each of the studied invertebrates), as was already reported by Sinanoglou and Miniadis-Meimaroglou (1999). Total phospholipids, of the mantle of the above invertebrates, after separation from neutral lipids by solid phase extraction were

quantified by weighing and amounted to 40.8, 62.7 and 69.5% of total lipids of these species. The latter is similar to the value of 76.6% quoted by Mason *et al.* (1973) for the lipids extracted from squid rhabdome segments and the value of 75% obtained for *Loligo vulgaris* (De Koning, 1993). The lipid phosphorus content of total phospholipids was 46.0, 36.2 and 41.6 g kg^{-1} respectively, which is similar to the value of 35.0 g kg^{-1} obtained for *Octopus vulgaris* (De Koning, 1972) and 36.7 g kg^{-1} obtained for *Loligo vulgaris* (De Koning, 1993). The phospholipid fraction contained 24.9 ± 0.3, 31.5 ± 0.4 and 17.3 ± 0.2% (mol/mol) glyceryl ethers, 128.0 ± 0.6, 132.9 ± 0.7 and 150.0 ± 0.6% (mol/mol) esters, 25.3 ± 0.4, 24.0 ± 0.3 and 15.6 ± 0.3% (mol/mol) sphingosine and 17.4 ± 0.2, 14.4 ± 0.3 and 11.8 ± 0.2% (mol/mol) plasmalogens, respectively.

Phosphatidylethanolamine and phosphatidylcholine were the major phospholipid components of *E. moschata*, *S. officinalis* and *T. sagittatus* mantles, and their proportion in total phospholipids ranged from 18.1% to 23.9% for phosphatidylethanolamine and from 52.2 to 58.4% for phosphatidylcholine (Table I). There was also determined the unusual lipid ceramide aminoethylphosphonic acid which ranged between 15.9–9.2% of total phospholipids. As minor phospholipid components sphingomyelin ranged from 6.7 to 15.2%, of total phospholipids and lyso-phosphatidylcholine ranged from 1.8 to 3.8% of total phospholipids (Table I).

For different cephalopod species (S.Africa) it was reported that *Octopus vulgaris* (De Koning, 1972), *Loligo vulgaris* (De Koning, 1993) and the abalone *Haliotis midae* (De Koning, 1966b) phospholipids consist of phosphatidylethanolamine (30, 29 and 32% of total phospholipids, respectively), phosphatidylcholine (42, 56 and 41%) phosphatidylserine (5, 2 and 5%), phosphatidylinositol (4, 2 and 5%), sphingomyelin (3, 5 and 1%), lyso-phosphatidylcholine (0, 3 and 0%) and the unusual lipid ceramide aminoethylphosphonic acid (13, 3 and 6%).

Quantitative analysis of individual phospholipids

Individual phospholipid components were separated by preparative TLC with the solvent system consisting of chloroform/methanol/glacial acetic acid/water (50:25:6:2, by vol.) and the extracted lipids were purified by HPLC. Phosphatidylethanolamine was given a peak at 6–6.5 min and phosphatidylcholine was given a peak at 11.9–12.4 min in all of the invertebrates studied.

Mantle lipid phosphorus content of *E. moschata*, *S. officinalis* and *T. sagittatus* phosphatidylethanolamine was 47.1, 35.5 and 41.4 g kg^{-1} and of phosphatidylcholine 47.5, 39.2 and 36.1 g kg^{-1}, respectively. The concentration of glyceryl ethers in PE was found to be 12.8 ± 0.2, 19.0 ± 0.3 and 9.3 ± 0.1% of total PE, respectively, and in PC 43.2 ± 0.5, 54.0 ± 0.6 and 25.9 ± 0.4% of total PC, respectively. The concentration of plasmalogens in *E. moschata, S. officinalis* and *T. sagittatus*, mantle PE was found to be 10.13 ± 0.1, 9 ± 0.1 and 5.2 ± 0.1% of total PE and in PC 30.1 ± 0.4, 24 ± 0.3 and 17.9 ± 0.2% of total PC, respectively. The molar ratios for the total phosphorous, diacyl, alkenylacyl (plasmalogens) and alkylacyl PE were 1.00 : 0.87 : 0.11 : 0.02, 1.00 : 0.81 : 0.09 : 0.10 and 1.00 : 0.91 : 0.05 : 0.04, respectively. The molar ratios for the total phosphorous, diacyl, alkenylacyl (plasmalogen) and alkylacyl PC were 1.00 : 0.57 :

Table I. Phosphoipids profile of the molluscs studied as determined by iatroscan.

Sample	PE		PnL		PC		Shm		l-PC	
	% of TL	% of PhL	% of TL	% of PhL	% of TL	% of PhL	% of TL	% of PhL	% of TL	% of PhL
Eledone moschata (octopus)	7.40 ± 0.36	18.13	6.50 ± 0.27	15.93	21.30 ± 1.25	52.20	4.36 ± 0.19	10.74	1.26± 0.19	3.10
Sepia officinalis (squid)	12.34 ± 0.45	19.67	6.28 ± 0.25	10.00	32.22 ± 1.98	51.33	9.54 ± 0.21	15.20	2.38± 0.21	3.80
Todarodes sagittatus (squid)	16.64 ± 0.57	23.95	6.42± 0.22	9.25	40.58 ± 2.31	58.40	4.61 ± 0.13	6.70	1.24± 0.13	1.80

FID area percents were corrected to weight% according to [total weight (area lipid/ area total lipid) x 100]. Data are expressed as wt% of total lipids and wt% of total phospholipids and represent means ± standard deviation of six composite samples, five cephalopods per composite.

0.30 : 0.13, 1.00 : 0.46 : 0.24 : 0.30 and 1.00 : 0.74 : 0.18 : 0.08, respectively. The above lipids were analysed by two-dimensional HPTLC. Phosphatidylethanolamine revealed three components (1, 2, 3) in *E. moschata*, two components (1, 2) in *S. officinalis* and two components (1,2) in *T. sagittatus*. The component (1) co-chromatographed with the phosphatidylethanolamine standard in all of the invertebrates studied and the other ones had a lower Rf in the 2nd dimension than that of the component (1). This was considered to be due to differences in the nature of their constituent fatty acids or to the different point of substitution of the glycerolgroup of ethanolamine. All of the components were positive to ninhydrin and to phosphomolybdenum blue reagents. Phosphatidylcholine was migrated as a single spot (positive to phosphomolybdenum blue). Samples of the phosphatidylethanolamine and phosphatidylcholine were subjected to mild alkaline hydrolysis. Lipid phosphorus content of *E. moschata*, *S. officinalis* and *T. sagittatus* of phosphatidylethanolamine alkaline stable product was 6.12, 6.74 and 3.72 g kg^{-1} and of phosphatidylcholine were 20.42, 21.17 and 9.38 g kg^{-1}, respectively. As determined by TLC (solvent system consisted of chloroform/methanol/acetic acid/water 50:25:6:2, by vol) alkaline stable product of phosphatidylethanolamine was migrated as one spot (positive to ninhydrin and to phosphomolybdenum blue), co-chromatographed with standard of lyso-phosphatidylethanolamine and the alkaline stable product of phosphatidylcholine was migrated as one spot (positive to phosphomolybdenum blue) co-chromatographed with the standard of lyso-phosphatidylcholine. As determined by ascending and descending paper chromatographic analysis, the water-soluble product of phosphatidylethanolamine was migrated as one spot (positive to ninhydrin and to Hanes-Isherwood reagent), co-chromatographed with L-α-glycerylphosphorylethanolamine standard and the water-soluble product of phosphatidylcholine was migrated as one spot (positive to Hanes-Isherwood reagent and to Dragendorff reagent), co-chromatographed with L-α-glycerylphosphorylcholine standard.

Fatty acid composition of phosphatidylethanolamine and phosphatidylcholine

Fatty acid composition of phosphatidylethanolamine and phosphatidylcholine, of cephalopod mollusc mantles studied was determined using GC and GC/MS (Kishiro and Yasuda, 1988). The data on fatty acids for phosphatidylethanolamine and phosphatidylcholine of the studied cephalopod molluscs, as well as the proportion of the saturated, monounsaturated and polyunsaturated fatty acids of the total fatty acids contained in each of the phospholipid fractions are given in Table II. Fatty acid profiles of phosphatidylethanolamine and phosphatidylcholine fractions of the studied cephalopods presented a differentiation much more in the proportions than in the variety of fatty acids (Table II). Phosphatidylethanolamine and phosphatidylcholine of cephalopod molluscs contained high proportions of palmitic acid C16:0 (23.2–54.5% of PE total fatty acids and 30.3–67.5% of PC total fatty acids) and stearic acid C18:0 (15.4–28.0% of PE total fatty acids and 3.6–17% of PC total fatty acids). Although there is only a limited amount of information in literature on the fatty acid composition of phosphatidylethanolamine and phosphatidylcholine of cephalopod molluscs, it seems that the main saturated fatty acids are the same. (Jangaard and Ackman, 1965 for squid *Illex illecebrosus*; De Koning, 1972; 1993 for *Octopus vulgaris* and for *Loligo vulgaris*).

Oleic acid (C18:1 n-9) and eicosaenoic acid (C20:1 n-9) were found in proportions that ranged from 5.3% to 10.5% and from 2.0% to 4.5%, respectively of PE total fatty acids and from 1.0% to 7.3% and from 0.5% to 4.4%, respectively of PC total fatty acids. So *S. officinalis* and *T. sagittatus* (squid) have been found to be richer in oleic acid (C18:1 n-9) and eicosaenoic acid (C20:1 n-9) than *E. moschata* (octopus).

The most characteristic polyunsaturated fatty acids of phosphatidylethanolamine and phosphatidylcholine were found to be docosahexaenoic acid (C22:6 n-3) and eicosapentaenoic acid (C20:5 n-3), which ranged between 7.0–11.3% and 4.5–15.8% of PE total fatty acids and between 12.5–42.0% and 1.5–9.8%, respectively of PC total fatty acids. It was also reported that docosahexaenoic acid and eicosapentaenoic acid are the most characteristic polyunsaturated fatty acids for marine organism phospholipids (De Koning, 1966a; De Koning and Evans, 1991; De Koning, 1993). The squid (*T. sagittatus*) phosphatidylcholine contained markedly more docosahexaenoic acid than the corresponding phosphatidylcholine of the octopus (*E.*

Table II. Fatty acid composition (w/w%) of phosphatidylethanolamine and phosphatidylcholine of the cephalopod molluscs studied.

Fatty acid	FA % (w/w) of total phospholipid fatty acids					
	E. moschata		*S. officinalis*		*T. sagittatus*	
	PE	PC	PE	PC	PE	PC
C12:0	–	–	–	–	–	–
C14:0	–	–	–	3.1 ± 0.07	1.3 ± 0.02	2.6 ± 0.04
C14:1n-5	–	–	–	–	10.1 ± 0.89	0.2[a]
C15:0	–	–	–	1.7 ± 0.04	2.3 ± 0.17	0.9[a]
C15:1	–	–	–	–	–	–
C16:0	54.5 ± 1 97	67.5 ± 2.25	23.2 ± 1.20	40.4 ± 1.11	24.6 ± 1.20	30.3 ± 1.16
C16:1n-9	–	–	–	0.3[a]	1.0[a]	–
C16:1n-7	–	–	–	1.5[a]	1.5[a]	0.4[a]
C17:0	–	–	–	0.4[a]	6.0 ± 0.54	0.2[a]
C17:1n-7	5.0 ± 0.13	–	3.0 ± 0.09	2.4 ± 0.18	2.5 ± 0.08	1.0[a]
C18:0	20.5 ± 1.61	17 ± 0.63	28.0 ± 1.50	9.3 ± 0.46	15.4 ± 0.63	3.6 ± 0.13
C18:1n-9, n-7	5.3 ± 0.22	1.0 ± 0.80	10.5 ± 0.45	7.3 ± 0.27	6.1 ± 0.49	3.7 ± 0.18
C18:2n-6	–	–	–	0.5[a]	–	0.2[a]
C20:1n-9	2.0 ± 0.08	0.5[a]	4.5 ± 0.24	4.4 ± 0.34	2.9 ± 0.02	1.9 ± 0.03
C20:3n-6	–	–	–	–	–	0.5[a]
C20:4n-6	1.2 ± 0.04	–	4.5 ± 0.38	2.8 ± 0.02	–	0.9[a]
C22:1n-9	–	–	–	0.3[a]	–	–
C20:5n-3	4.5 ± 0.11	1.5 ± 0.03	15.8 ± 1.61	8.8 ± 0.40	7.0 ± 0.22	9.8 ± 0.72
C22:4n-6	–	–	–	–	2.8 ± 0.03	0.3[a]
C22:5n-6	–	–	–	0.4[a]	5.2 ± 0.18	0.8[a]
C22:5n-3	–	–	–	1.3[a]	–	0.7[a]
C22:6n-3	7.0 ± 0.54	12.5 ± 0.89	10.5 ± 0.49	15.1 ± 1.11	11.3 ± 0.63	42.0 ± 1.81
Σsaturated	75.0	84.5	51.2	54.9	49.6	37.6
Σmonounsaturated	7.3	1.5	18	15.9	24.1	7.2
Σpolyunsaturated	17.7	14.0	30.8	29.2	26.3	55.2

FID area percents were corrected to weight% according to [total weight (area lipid/area total lipid) x 100]. Data are expressed as wt% of total fatty acids and represent means ± standard deviation of six composite samples, five cephalopods per composite. [a]SD values less than 0.01.

moschata) and squid (*S. officinalis*) while the phosphatidylethanolamine contents in docosahexaenoic acid were similar. Squids (*T. sagittatus* and *S. officinalis*) phosphatidylcholine and phosphatidylethanolamine contained markedly more eicosapentaenoic acid than the corresponding phospholipids of the octopus (*E. moschata*).

Fatty acids structure has been verified by GC-MS (E. I.) using standard fatty acid methyl esters of the Sigma Chemical Co. The analysis led to positive identification.

Mass spectrometry of phosphatidylcholine and phosphatidylethanolamine

The negative ion spectra of phosphatidylethanolamine (PE) yielded a characteristic peak at m/z 140, corresponding to the phosphoethanolamine head group-H^+: $(O\text{-}PO_3HCH_2CH_2NH_2)^-$ (Fig. 1) The positive ion spectra of phosphatidylcholine (PC) yielded a characteristic peak at m/z 184 corresponding to the phosphocholine head group + H^+ (Fig. 2). The positive ion spectra of phosphatidylethanolamine (PE) and the negative ion spectra of phosphatidylcholine (PC) yielded any peaks.

All the ion spectras of phosphatidylethanolamine moieties yielded major peaks at m/z 255, 283, 301, 303, 327, 339 corresponding to ($RCOO^-$) for C16:0, C18:0, C20:5, C20:4, C22:6, C22:0 respectively (Fig. 1) and of phosphatidylcholine moieties at m/z 226, 256, 279, 284, 301, 308, 310, 312 and 337 corresponding to ($RCOO^-$ + H^+) for C14:1, C16:0, C17:1, C18:2, C18:0, C20:5, C20:3, C20:1, C20:0 and C22:1 respectively (Fig. 2). The molecular weights of phosphatidylethanolamine moieties ranged from 723 to 817 and of phosphatidylcholine moieties from 717 to 837.

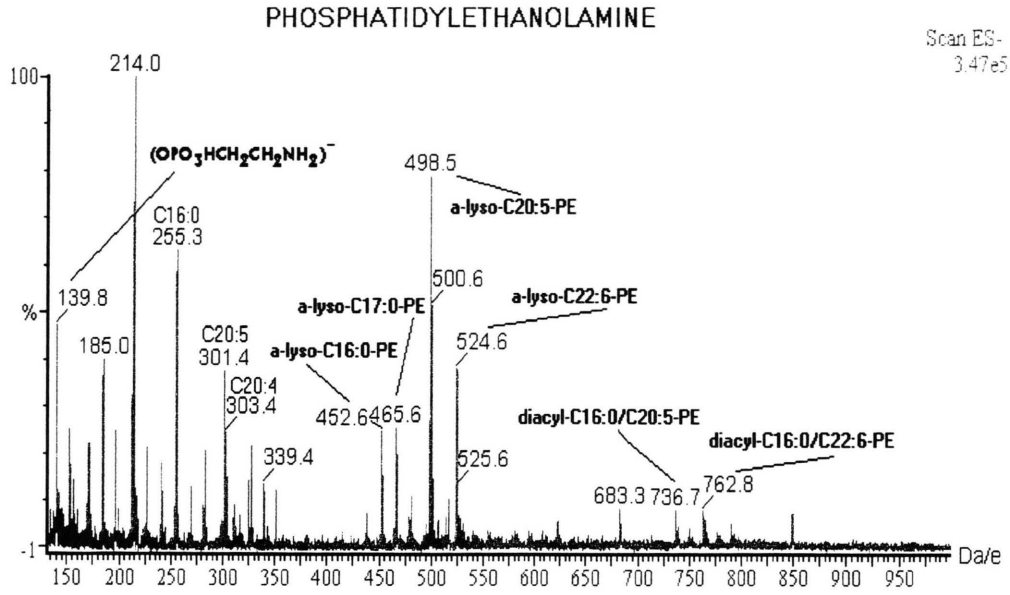

Fig. 1. Negative ion spectra of phosphatidylethanolamine.

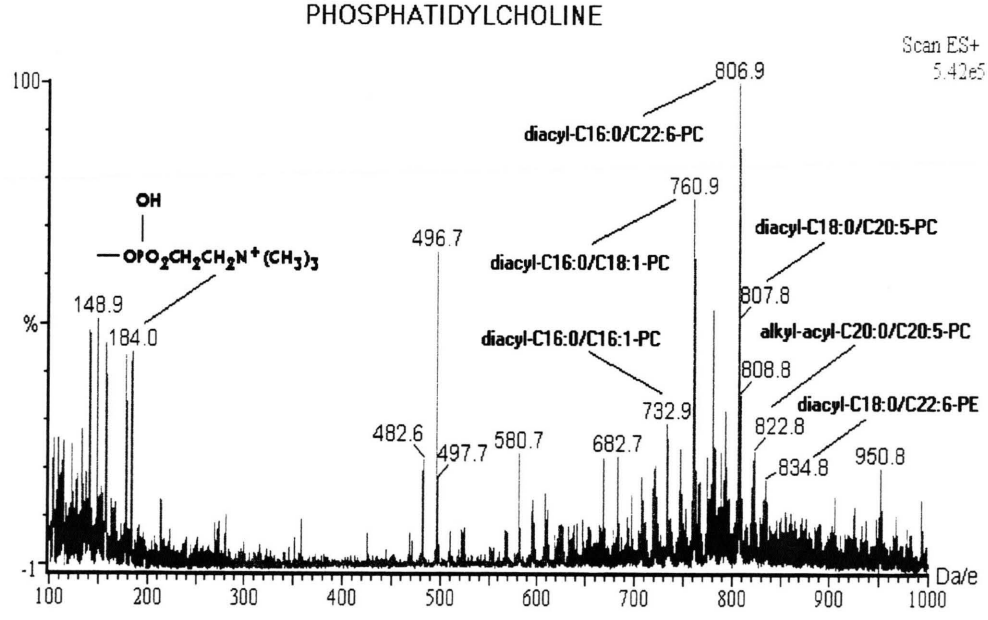

Fig. 2. Positive ion spectra of phosphatidylcholine.

By examining the molecular weights for combinations of fatty acid moieties, headgroups and acyl, alkyl or alkenyl linkages, it is possible to evaluate the possible combinations that will generate the parent ions of PE moieties (Kerwin *et al.*, 1994) (Table III) (Fig. 1) and PC moieties (Table IV) (Fig. 2). Over 55 and 50 molecular species of phosphatidylethanolamine (PE) and phosphatidylcholine (PC), respectivelly, were found in *E. moschata*, *S. officinalis* and *T. sagittatus*, using positive and negative ion electrospray mass spectra including diacyl, alkyl-acyl and alkenyl-acyl moieties.

Table III. Molecular species of phosphatidylethanolamine of the cephalopod molluscs studied.

M W	*m/z* (M-H$^+$)	Molecular species Diacyl PE	Alkenyl-Acyl PE	Alkyl-Acyl PE
723	722	C15:0/C20:5 C17:0/C22:5	–	–
735	734	C14:0/C22:6 C16:1/C20:5	–	–
737	736	C14:0/C22:5 C16:0/C20:5 C16:1/C20:4	–	–
739	738	C14:0/C22:4 C16:0/C20:4 C18:2/C18:2	–	–
749	748	C15:0/C22:6	–	–
751	750	C15:0/C22:5 C17:0/C20:5	–	–
753	752	C15:0/C22:4 C17:0/C20:4	–	–
763	762	C16:0/C22:6 C16:1/C22:5 C18:1/C20:5 C18:2/C20:4	–	–
765	764	C16:0/C22:5 C16:1/C22:4 C18:0/C20:5 C18:1/C20:4	–	–
767	766	C16:0/C22:4 C18:0/C20:4	–	–
769	768	C18:2/C20:1	–	–
777	776	–	C20:0/C20:5 C18:0/C22:5	C18:0/C22:6
779	778	–	C20:0/C20:4 C18:0/C22:4	C20:0/C20:5
781	780	–	–	C20:0/C20:4
787	786	C18:2/C22:6	–	–
789	788	C18:1/C22:6 C18:2/C22:5	–	–
791	790	C18:0/C22:6 C18:1/C22:5 C18:2/C22:4 C20:1/C20:5	–	–
793	792	C18:1/C22:4 C20:1/C20:4	–	–
795	794	C18:0/C22:4 C20:1/C20:3	–	–
797	896	C18:2/C22:1	–	–
805	804	–	C20:0/C22:5 C22:0/C20:5	C20:0/C22:6
807	806	–	C20:0/C22:4 C22:0/C20:4	C20:0/C22:5
809	808	–	–	C20:0/C22:4
813	812	C20:1/C22:6	–	–
815	814	C20:1/C22:5	–	–
817	816	C20:1/C22:4 C20:0/C22:5	–	–

By examining the negative ion spectra of PE species it is possible to characterize species according to a-lyso-phosphoethanolamine, which are contained in PE (Table V). These structures become as the products of the hydrolysis of the diacyl PE species and they ensure the presence of the acyl groups in the sn-2 position.

Table IV. Molecular species of phosphatidylcholine of the cephalopod molluscs studied.

M W	*m/z* (M-H$^+$)	Molecular species Diacyl PC	Alkenyl-Acyl PC	Alkyl-Acyl PC
717	718	–	C16:0/C16:0 C18:0/C14:0	C16:0/C16:1 C14:0/C18:1
719	720	–	–	C16:0/C16:0 C14:0/C18:0
731	732	C16:0/C16:1 C14:0/C18:1	–	–
733	734	C16:0/C16:0 C14:0/C18:0	–	–
745	746	–	C16:0/C18:0 C18:0/C16:0 C20:0/C14:0	C14:0/C20:1 C16:0/C18:1 C18:0/C16:1
747	748	–	–	C16:0/C18:0
757	758	C16:0/C18:2 C16:1/C18:1	–	–
759	760	C14:0/C20:1 C16:0/C18:1	–	–
761	762	C16:0/C18:0	–	–
765	766	–	C16:0/C20:4 C18:2/C18:2	C16:0/C20:5
773	774	–	C18:0/C18:0 C20:0/C16:0	C16:0/C20:1 C18:0/C18:1
779	780	C16:0/C20:5 C16:1/C20:4 C14:0/C22:5	–	–
781	782	C14:0/C22:4 C16:0/C20:4 C18:2/C18:2	–	–
787	788	C14:0/C22:1 C16:0/C20:1	–	–
791	792	–	C16:0/C22:5 C18:0/C20:5	C16:0/C22:6
795	796	–	C18:0/C20:3	C18:0/C20:4
803	804	–	–	C18:0/C20:0
805	806	C16:0/C22:6 C18:1/C20:5	–	–
807	808	C16:0/C22:5 C16:1/C22:4 C18:0/C20:5 C18:1/C20:4 C18:2/C20:3	–	–
819	820	–	C18:0/C22:5 C20:0/C20:5	C18:0/C22:6
821	822	–	C20:0/C20:4	C20:0/C20:5
833	834	C18:0/C22:6 C18:1/C22:5 C18:2/C22:4	–	–
837	838	C18:0/C22:4	–	–

These molecular weights cannot be generated for b-lyso alkenyl or alkyl PE species.

The identification of the alkyl and alkenyl fragment cannot be definitely ruled out both for the ion spectras of PE and PC species in electrospray mass spectrometry. The proposed combinations that will generate the parent ions of these species evaluated by examining the results of the determination of the molecule-groups of glycerinethers and plasmalogens and from the contents of fatty acids using GC-MS (Table II), in the studied invertebrates. These improve the presence both of alkenyl-acyl and alkyl-acyl PE and PC species.

Table V. Molecular species of α-lysophosphatidylethanolamine of the cephalopod molluscs studied.

M W	*m/z* ($M-H^+$)	α-lyso-acyl PE
439	438	C15:0
453	452	C16:0
467	466	C17:0
499	498	C20:5
525	524	C22:6
529	528	C22:4

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