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Separation of major polar lipids in *Pecten maximus* by high-performance liquid chromatography and subsequent determination of their fatty acids using gas chromatography

P. Soudant[a], Y. Marty[b,*], J. Moal[a], J.F. Samain[a]

[a]*IFREMER Centre de Brest, BP 70, 29280 Plouzané, France*
[b]*URA CNRS 322, Faculté des Sciences et Techniques, BP 809, 29285 Brest, France*

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Abstract

An easy method for the separation of major polar lipid classes by HPLC is described. Maximum resolution was achieved by an automated combination of a silica gel column and a diol column. Polar lipid analysis of the larvae and gonads of *Pecten maximus* showed the presence of a particular glycolipid especially rich in 22:6($n-3$) and the predominance of 20:4($n-6$) in the phosphatidylinositol. The phosphatidylcholine and phosphatidylethanolamine (diacyl form + alkenylacyl) were the major fractions. The plasmalogen form (25% in larvae, 34% in gonads) was essentially composed of polyunsatured fatty acids of 20 and 22 carbons in the *sn*-2 position.

1. Introduction

Phospholipids are essential constituents of all biological membranes. Both the nature of polar head groups and the esterified fatty acids influence the physico-chemical properties and the associated cellular functions of these membranes [1,2]. The main phospholipid classes of the animal kingdom, phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC), are found in molluscs but with some notable differences. Thus the presence of plasmalogens (1-O-alk-1′-enyl-2-acylglycerophospholipid) in substantial amounts has been shown in a number of marine molluscs, including pectinids [3–6]. These plasmalogens are found mainly bound to PE, but are also present in the PS and PC. In addition, the bivalves often lack sphingomyelin (SM) [7,8], a class generally present in all mammals and all marine vertebrates. Other groups of polar lipids such as glycolipids (GLY) [9,10] and phosphonolipids [8,11] can also be relatively abundant in molluscs.

The phospholipids of a majority of marine species including fishes, crustaceans and molluscs are rich in polyunsaturated fatty acids (PUFA), mainly of the ($n-3$) series concentrated on the $sn-2$ position of the glycerol backbone of the molecule [12]. These PUFA with 20 and 22 carbon atoms and more than three double bonds are essential for survival, growth and reproduction of molluscs [13,14]. The amounts of the PUFA in the phospholipids, especially of 22:6($n-3$), have been shown to remain relatively constant during larval development in

* Corresponding author.

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Pecten maximus [15]. However, the fatty acid composition of total phospholipids can become modified by the mono-species algal diet that is low in either 22:6($n-3$) or 20:5($n-3$) which, in turn, leads to perturbations in metamorphosis and growth during larval development in this species [16]. These results emphasize the importance to be placed on the composition of fatty acids in studies on phospholipids in molluscs.

Until now, thin-layer chromatography (TLC) was the analytical technique used in most studies on phospholipids of molluscs. The results obtained with this are limited to an estimation of the total amount and to a percentage determination of each class of the phospholipids. The qualitative and quantitative composition of fatty acids in phospholipids thus remains little elucidated. For this purpose, the high-performance liquid chromatography (HPLC) is very useful.

Among the existing HPLC methods [17–19], some permit the separation of a wide range of phospholipids but are not suitable for measuring the amounts of plasmalogens in molluscan phospholipids from a single injection. Use of an acidic mobile phase [20–22], which transforms plasmalogens into lyso-1-phosphatidyl, renders this possible. However, these methods, developed for use with mammalian tissues, can hardly be adopted for marine molluscan tissues. The molluscan phospholipids are characterized by a great variety of fatty acids [23], particularly in the major lipid classes, with the result that a large number of molecular species exist for each class. Their separation by HPLC results in wider peaks and lower resolution.

The objective of this study was to isolate by HPLC, with good resolution, the molluscan plasmalogens and PI, PS, PE and PC phospholipid classes and to determine the fatty acid composition of each of these. The scallop *Pecten maximus* was used for this study.

2. Experimental

2.1. Reagents and standards

Phospholipid [= cardiolipin (CL), PI, PS, PE, PC, lyso-PS (LPS), lyso-PC (LPC), lyso-PE (LPE) and sphingomyelin] and glycolipid standards [mono- and digalactosyl diglycerides (MGDG and DGDG)] were obtained from Sigma (St. Quentin Fallavier, France). PC with deuterated stearic acid was obtained from Larodan (Malmö, Sweden). Reagents and solvents were obtained from Supelco (Bellefonte, PA, USA) [14% boron trifluoride (BF_3) in methanol], Merck (Darmstadt, Germany) (carbon disulfide) and Fisons (Longhborough, UK) (acetonitrile, methanol, hexane and chloroform).

2.2. Instrumentation

Separation of phospholipids was carried out with a Merck–Hitachi HPLC system consisting of an L6200 gradient pump and a UV detector, and equipped with a Gilson (Villiers-le-Bel, France) Model 203 fraction collector. Two columns, a LiChrospher Si100 (5 μm; 25 × 0.4 cm I.D.) and a LiChrosorb diol (5 μm; 25 × 0.4 cm I.D.) (Merck), were combined in this system. Two mechanized Rheodyne Model 7010 valves were used to connect/disconnect the two columns. The fractions were evaporated in a vacuum centrifuge (Speed-Vac) connected to a nitrogen inlet.

The fatty acid methyl esters (FAME) were analysed in a Model 9001 gas chromatograph (Chrompak, Middleburg, Netherlands) equipped with an on-column injector, a flame ionization detector and an HP3388A integrator.

2.3. Culture of adults and larvae

Adults of *P. maximus* were collected from the Bay of Brest in February 1993 and transferred to the IFREMER's hatchery at Argenton near Brest, France. After emptying their gonads by a thermal shock, the scallops were held for 11 weeks in rearing tanks with a coarse sandy bottom. The temperature of the circulating ambient seawater was maintained at 15°C and the scallops were fed a standard mixture of microalgae (*Pavlova lutheri*, *T-Isochrysis*, *Skeletonema costatum*, *Chaetoceros calcitrans*) at a rate of $8 \cdot 10^9$ cells per scallop per day. The larvae of *P. maximus* were reared in a 400-l cylindro-conical

vessel and were fed a mixture of microalgae (*P. lutheri*, *T-Isochrysis*, *S. costatum*).

2.4. Sample preparation and extraction of lipids

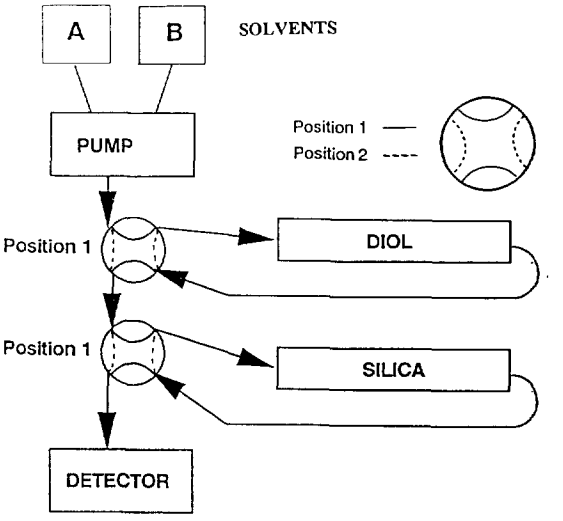
After acclimatization for 11 weeks, gonads of three specimens were taken at random and ground at −180°C with a Dangoumeau homogenizer. About 50 000 larvae (19 days old) were recovered on a pre-ignited (overnight at 450°C) GF/D filter (3-μm porosity) by filtration. The gonad samples and the larvae were placed separately in tubes containing $CHCl_3$–MeOH (2:1, v/v) containing 1% butylated hydroxy-toluene (BHT), closed under nitrogen and frozen at −20°C. A known amount of PC with two strands of deuterated stearic acid was added to the samples as a primary internal standard.

2.5. Determination of total polar lipids with a silica gel microcolumn

An aliquot of the sample was evaporated to dryness and lipids were recovered with three washings of 500 μl each of $CHCl_3$–MeOH (98:2). This was placed on top of a silica gel microcolumn [30 × 5 mm I.D. Kieselgel, 70–230 mesh (Merck), previously heated to 450°C and deactivated with 5% water] [15]. The neutral lipids were eliminated with 10 ml of $CHCl_3$–MeOH (98:2). The polar lipids were recovered with 10 ml of MeOH, a known amount of 23:0 fatty acid (as a secondary internal standard) was added and the mixture was taken for methylation and analysis by GC.

2.6. Separation of phospholipid classes by HPLC

The isocratic method [20] was modified by using two columns with different characteristics [an OH-bound silica gel column (diol) and a classical silica gel column] and a binary mobile phase composed of solvent A (acetonitrile) and solvent B [acetonitrile–methanol–phosphoric acid (93:5:1.5, v/v/v)] at a flow-rate of 1 ml min^{-1}. The connections between the Si and diol columns and the solvent system are shown schematically in Fig. 1. The changeover between



	Solvents		Valve (position)	
TIME	A	B	DIOL	SILICA
0 min	100	0	1	1
10 min	0	100	1	1
20 min	0	100	2	1
50 min	0	100	1	2
70 min	100	0	1	1

Fig. 1. Schematic diagram showing the elution procedure using the silica gel and diol columns.

the columns was done by using two electrically operated six-way valves. After drying under nitrogen, the samples were recovered twice in 50 μl of $CHCl_3$–MeOH (98:2) and manually injected in the 200-μl loop of the HPLC system. The amount of the total lipid thus injected varied between 50 and 150 μg. The phospholipid classes were separated at room temperature and detected by UV spectrophotometry at 205 nm. The separation of the different classes, GLY, CL, PI, PS, PE, PC, LPC, LPE and SM, and their identification were confirmed by two-dimensional TLC following the methods described earlier [24]. The phospholipid fractions were collected automatically in Wheaton vials containing a known amount of 23:0 fatty acid (secondary internal standard) and 20 μg of BHT (anti-oxidant) and were taken for methylation and fatty acid analysis by GC.

2.7. Determination of FAME by gas chromatography

The total polar lipid fractions obtained by microcolumn separation and the phospholipid fractions isolated by HPLC were concentrated at 40°C in a vacuum centrifuge, but were not allowed to dry completely so as to prevent the degradation of unsaturated fatty acids in the acidic phase, in particular those associated with lyso-1-phosphatidyl ethanolamine (LPE) derived from hydrolysis of plasmalogens. The phospholipid-bound fatty acids were transesterified by heating with 14% BF_3 in methanol for 10 min at 95°C [25]. After cooling, the FAME were extracted with CS_2 [26]. The organic phase was evaporated, taken up in hexane and the FAME were separated in a DBWAX capillary column (25×0.32 mm I.D.; 0.2-μm film thickness) programmed from 150 to 250°C at 3°C min^{-1}, with hydrogen as the carrier gas. The fatty acids were identified by their retention times with reference to standards.

For PE, PS and PC, two lyso forms (the lyso-1-phosphatidyl resulting from hydrolysis of plasmalogens by the mobile acid phase and the biogenic lyso-2-phosphatidyl characterized by the absence of fatty acid chain in position 2) can occur. In HPLC separation, the two forms are partially resolved. The lyso forms of PC were absent from our samples. As lyso-serine was eluted along with lyso-ethanolamine, the term PLSM is used to denote all the lyso forms derived from PS and PE.

The fatty acids were designated following the formula $C{:}X(n-y)$, where C is the number of carbon atoms, X is the number of double bonds and y is the position of the first double bond counted from the CH_3 terminus.

2.8. Calculation of the amounts of phospholipid classes

The method of Seewald and Eichinger [21] was used to calculate the respective amounts of each phospholipid from the quantitative spectrum of the fatty acids obtained by GC. The mean molecular mass of a phospholipid class was calculated from the percentages of each fatty acid and their respective molecular masses.

In the case of plasmalogens, the PE being dominant with respect to PS, the calculation of phospholipid amount was carried out with the molecular mass of the PE core. Besides, as the fraction collected has lost its aldehyde chain in position 1, the molar mass takes into account the molecular mass of the PE core plus the mean molecular mass of the fatty acid in position 2 plus the mean molecular mass of the aldehyde in position 1. The last value was estimated from the composition of dimethyl acetal identified and determined from the FAME of the total PL fraction obtained by silica gel microcolumn separation.

3. Results and discussion

3.1. Optimization of the HPLC separation of phospholipids by combining the Si and diol columns

The combination of the two columns was intended to improve the separation of the phospholipid classes, maintaining at the same time a simple solvent system (isocratic beyond 10 min). The elution order of the different classes is not the same with the two columns: with the diol column it is GLY, PI, PC, PS, PE, LPC, SM and LPE whereas with the silica gel column it is GLY, PI, PS, PE, LPE, PC, LPC and SM.

Because of the diversity of molecular species, the two columns, when used separately, do not provide the resolution necessary for an analysis of the molluscan phospholipids. The diol column does not provide a separation of the PI and PC as well as the PS and PE classes of a molluscan lipid extract. This, however, provides a good resolution with the samples where the phospholipid classes are less rich in molecular species. The silica gel column, on the other hand, does not have enough resolution for the two major fractions, PC and LPE, present abundantly in the molluscs. In addition, the retention times of the last two classes, LPC and SM, in the elution are

too high to be compatible with good resolution of those eluted first using the same system of solvents. A combination of the two columns in the following manner, therefore, provides for an improvement in the resolution.

At the time of injection of the lipidic extract, the two columns are aligned in a series (Fig. 1). When the GLY, PI, PS, PE and PC classes have moved out, i.e., after 19 min of elution, the diol column, with the most polar phospholipid classes (LPE, LPC and SM) still within, is disconnected. This procedure permits the subsequent separation of GLY, PI, PS, PE and PC classes in the silica gel column with very good resolution, which until now was least resolved in the diol column. Once the elution of these five classes in the silica gel column is complete, the diol column is reconnected to the system and the silica gel column is disconnected. This permits the elution of the most polar classes (LPE, LPC and SM) in a relatively short time, using a single mixture of acetonitrile–methanol–85% phosphoric acid (93:5:1.5, v/v/v).

Figs. 2 and 3 show a separation of phospholipid standards and a lipid extract of the gonads of female *P. maximus* obtained by using the two columns in combination.

Utilization of a linear polarity gradient, between pure acetonitrile and the mixture, during the first 10 min of elution when both columns are serially connected, results in a better resolution of glycolipids of *P. maximus* and also of the vegetable galactolipids such as MGDG and DGDG. When the lipid extracts do not contain glycolipids, this phase of separation can be omitted. The presence of phosphoric acid in the mobile phase improves the resolution of the peaks and also hydrolyses the vinyl ether bond in position 1 of the glycerol of the plasmalogens, as proposed by Kawasaki et al. [27] and demonstrated by Seewald and Eichinger [21]. This offers the possibility of determining separately the fatty acids lyso-derived from plasmalogens.

The phospholipids are not directly quantified by UV absorption measurements since the absorption coefficients depend on their fatty acid composition [28]. This quantification is done by a subsequent analysis in GC of the composition of the fatty acids of the phospholipid classes isolated. The smallest amount that can be detected

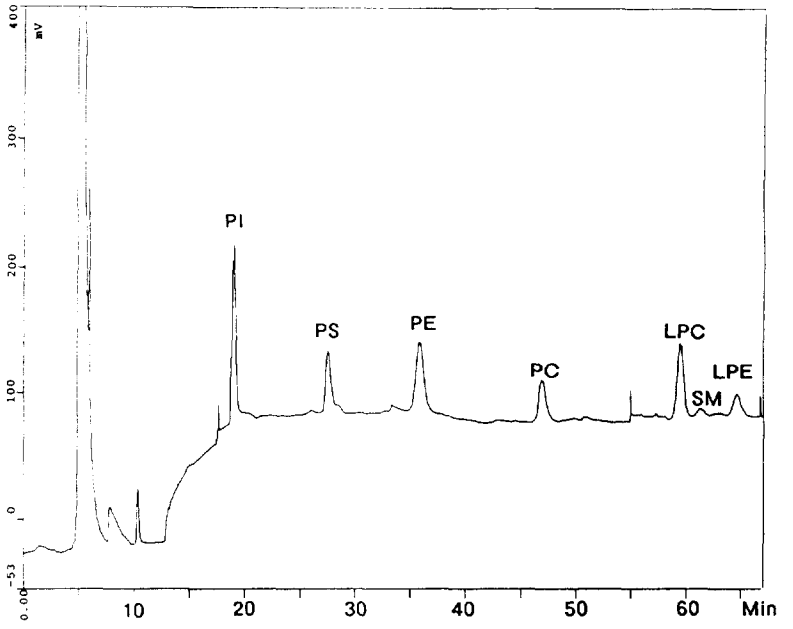


Fig. 2. HPLC separation of phospholipid standards (12.5 μg each of PI, PS, PE and PC and 16 μg each of LPC, SM and LPE).

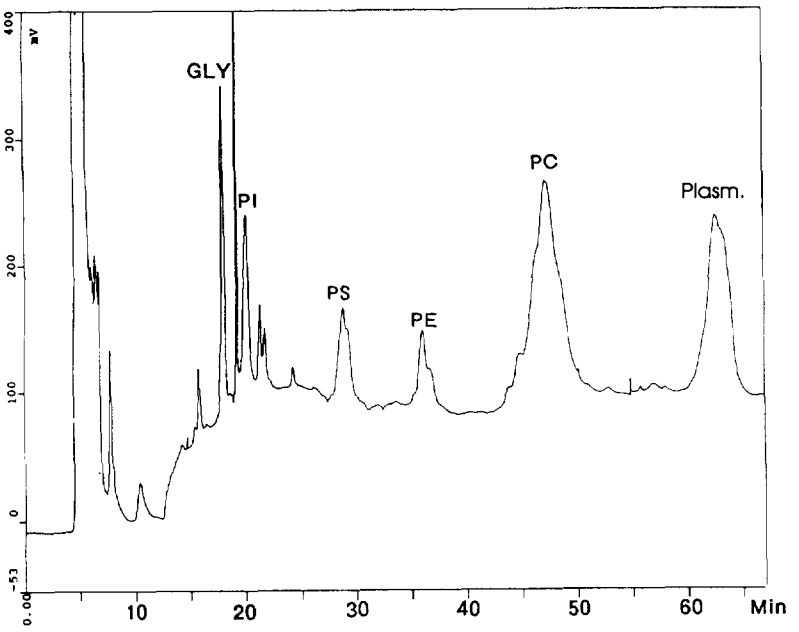


Fig. 3. HPLC separation of polar lipids of female *P. maximus* gonads. The plasmalogen (LPE + LPS) peak corresponds to acid hydrolysis products (lyso-1-phosphatidyl) of the plasmalogens of PE and PS.

is 1 μg of total fatty acids for the least abundant phospholipid classes. In practice, injection of 20 μg of total phospholipids in the lipid extract is sufficient for the GC determination of the fatty acids of the six polar lipid fractions (glycolipid, PI, PS, PE, PC. lyso-PE + PS) in *P. maximus*. The determination of fatty acids from five replicate injections of a standard solution containing PE with 60% plasmalogens, PC and LPC gave coefficients of variation of 7% for LPC, 8% for PC, 10% for non-plasmalogen PE and 7% for LPE. The cumulated amounts of each fatty acid through all classes, obtained after HPLC separation, corresponded to those found in the total phospholipid fraction obtained after passage of the lipid extract in the silica gel microcolumn. As far as retention times are concerned, the diol column is remarkable for its stability for all phospholipid classes including the most polar ones such as LPE, LPC and SM. The silica gel column shows an appreciable and steady reduction in the retention times during the course of the day, noticeable particularly with the PE and PC phospholipid classes (the maximum variation was of the order of 8% for PC). Storage overnight under acetonitrile was sufficient to regenerate the Si column and re-obtain the initial retention times.

3.2. Determination of different phospholipid classes and their bound fatty acid composition in female gonads and larvae of P. maximus

Distribution of the classes

The phospholipid amounts (Table 1) and their fatty acid composition (Tables 2 and 3) were

Table 1
Percentage composition of phospholipid classes in the gonads and larvae of *P. maximus*

Class	Gonads		Larvae	
	Mean	S.D.	Mean	S.D.
GLY	5.9	0.5	9.0	1.5
PI	8.2	0.6	9.3	1.7
PS	7.6	0.5	7.9	1.5
PE	5.3	1.0	9.1	1.5
PC	39.1	3.6	39.7	2.8
LPE[a]	34	3.6	25	2.1

[a] LPE = plasmalogens.

Table 2
Fatty acid composition expressed as a percentage of total moles of complex lipid classes of *P. maximus* gonads (mean and S.D.; $n = 3$)

Fatty acid[a]	GLY		PI		PS		PE		PC		LPE[b]		Total	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
14:0	–	–	–	–	–	–	1.4	0.8	2.6	1.1	–	–	1.3	0.6
15:0	–	–	1.1	1.8	–	–	–	–	0.7	0.2	–	–	0.4	0.2
16:0	3.2	0.5	2.7	0.9	4.1	2.0	12.8	2.0	23.3	3.2	0.9	0.3	12.4	1.1
17:0	–	–	–	–	–	–	1.7	0.2	0.8	0.2	–	–	0.5	0.1
18:0	2.3	0.2	33.8	2.7	44.6	3.4	28.1	5.8	4.9	0.8	0.9	0.3	11.2	0.5
16:1($n-9$)	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16:1$n-7$)	–	–	–	–	–	–	–	–	3.7	1.2	–	–	1.7	0.7
18:1($n-9$)	1.6	1.1	2.4	1.9	1.1	0.4	2.7	1.2	2.7	0.3	0.7	0.4	2.0	0.6
18:1($n-7$)	–	–	1.1	1.0	1.1	0.5	2.9	0.6	4.7	0.5	0.5	0.2	2.7	0.4
20:1($n-11$)	–	–	–	–	–	–	–	–	0.2	0.1	0.5	0.2	0.3	0.0
20:1($n-9$)	–	–	5.1	0.8	1.7	0.6	1.3	0.1	0.7	0.3	2.5	0.3	1.6	0.2
20:1($n-7$)	–	–	1.4	0.3	–	–	–	–	0.3	0.1	1.2	0.4	0.6	0.1
18:2($n-6$)	–	–	1.5	1.5	0.2	0.0	1.2	0.9	2.1	0.4	–	–	1.3	0.4
18:2($n-4$)	–	–	–	–	–	–	–	–	0.7	0.4	–	–	0.3	0.2
18:3($n-6$)	–	–	–	–	–	–	–	–	0.4	0.1	–	–	0.2	0.0
18:3($n-3$)		–	–	–	–	–	–	–	1.3	0.1	–	–	0.6	0.1
18:4($n-3$)		–	–	–	–	–	1.0	0.6	2.3	0.2	–	–	1.1	0.2
18:5($n-3$)	–	–	–	–	–	–	–	–	–	–	–	–	–	–
20:2($n-6$)	–	–	–	–	–	–	–	–	–	–	–	–	–	–
20:3($n-6$)	–	–	0.2	0.3	0.6	0.0	–	–	0.5	0.1	–	–	0.3	0.1
20:4($n-6$)	0.8	0.3	36.7	4.7	4.0	1.1	4.9	1.1	2.1	0.3	7.5	1.2	6.6	0.2
20:4($n-3$)	–	–	0.2	0.2	0.5	0.4	–	–	0.4	0.2	–	–	0.4	0.2
20:5($n-3$)	2.2	0.2	6.2	2.0	11.5	3.4	30.4	1.7	18.4	0.5	32.7	4.0	19.6	0.8
21:4($n-6$)	–	–	–	–	0.7	0.0	–	–	0.8	0.2	0.7	0.3	0.5	0.1
21:5($n-3$)	0.9	0.2	–	–	0.5	–	–	–	1.0	0.1	0.3	0.1	0.6	0.1
22:4($n-6$)	–	–	–	–	–	–	–	–	1.4	0.5	1.6	1.0	1.0	0.5
22:?	–	–	–	–	14.0	1.2	1.7	0.3	1.3	0.3	4.2	2.1	2.9	0.7
22:5($n-6$)	1.7	0.5	1.7	0.7	3.6	1.6	1.7	0.3	2.5	0.5	7.4	1.8	3.5	0.9
22:5($n-3$)	5.4	1.8	–	–	0.7	0.0	–	–	2.4	1.4	0.7	0.2	1.7	0.8
22:6($n-3$)	81.8	2.1	6.1	1.0	12.7	3.6	8.2	1.0	17.8	2.1	37.5	2.5	24.7	2.7
To.Sat.	5.5	0.5	37.5	1.6	48.7	1.8	44.0	4.4	32.4	3.0	1.8	0.6	25.8	1.0
To.Mon.	1.6	1.1	9.9	1.4	4.0	1.4	6.8	1.5	12.4	1.5	5.5	0.9	8.8	1.4
To.($n-9$)	1.6	1.1	7.5	2.2	2.9	1.0	3.9	1.3	3.4	0.1	3.2	0.7	3.6	0.6
To.($n-7$)	–	–	2.5	0.8	1.1	0.5	2.9	0.6	8.7	1.4	1.8	0.4	5.0	0.9
To.Poly.	92.9	0.8	52.5	1.4	49.1	1.9	49.2	3.2	55.3	2.8	92.6	1.3	65.3	2.3
To.($n-4$)	–	–	–	–	–	–	–	–	0.7	0.4	–	–	0.3	0.2
To.($n-6$)	2.5	0.8	40.1	3.3	8.4	1.4	7.7	2.0	8.9	1.0	16.6	1.8	12.8	0.9
To.($n-3$)	89.4	2.1	12.4	2.0	25.5	1.0	39.7	1.8	42.7	3.6	70.9	4.8	48.2	3.5
($n-3$)/($n-6$)	36.2	14.0	0.3	0.1	3.0	0.4	5.1	1.2	4.9	0.9	4.3	0.7	3.8	0.5
22:6/20:5	37.6	4.0	1.0	0.5	1.1	0.6	0.3	0.0	1.0	0.1	1.1	0.1	1.3	0.1
22:5/20:4	2.1	0.1	0.0	0.0	0.9	0.6	0.4	0.1	1.2	0.2	1.0	0.3	0.5	0.1

[a] To.Sat. = total saturated fatty acids; To.Mon. = total monounsaturated fatty acids; To.($n-9$) = total monounsaturated fatty acids ($n-9$); To.($n-7$) = total monounsaturated fatty acids ($n-7$); To.Poly. = total polyunsaturated fatty acids; To.($n-4$) = total polyunsaturated fatty acids ($n-4$); To.($n-6$) = total polyunsaturated fatty acids ($n-6$); To.($n-3$) = total polyunsaturated fatty acids ($n-3$).
[b] LPE = plasmalogens.

measured on gonads of three adult females and on four lots of 19-day-old larvae. In *P. maximus*, PC and PE (non-plasmalogen PE + plasmalogen PE) were the major classes (>70% of the total polar lipids) and the PI and PS were the minor ones (<20%). We also found the existence of a

Table 3
Fatty acid composition expressed as a percentage of total moles of complex lipid classes of 19-day-old *P. maximus* larvae (mean and S.D.; $n = 3$)

Fatty acid[a]	GLY		PI		PS		PE		PC		LPE[b]		Total	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
14:0	–	–	–	–	–	–	3.8	0.6	4.1	0.7	0.4	0.2	2.3	0.5
15:0	–	–	–	–	–	–	–	–	0.8	0.3	–	–	0.4	0.2
16:0	5.8	2.0	8.4	0.2	5.8	1.2	15.0	1.6	19.8	1.0	2.8	2.5	12.6	0.8
17:0	–	–	–	–	–	–	1.1	0.1	0.6	0.1	0.1	0.2	0.4	0.1
18:0	3.2	1.4	20.6	1.4	41.7	1.5	14.0	0.5	3.7	0.6	1.5	0.5	9.4	0.5
16:1($n-9$)	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16:1($n-7$)	–	–	1.5	0.3	–	–	3.6	0.2	3.2	0.3	–	–	1.9	0.1
18:1($n-9$)	2.4	1.2	3.1	0.3	1.8	0.8	7.1	0.5	6.8	0.4	2.9	1.5	4.9	0.3
18:1($n-7$)	5.0	1.6	8.4	1.6	3.0	1.4	7.7	0.3	7.3	0.4	2.5	0.2	6.0	0.2
20:1($n-11$)	–	–	–	–	1.5	1.0	–	–	0.1	0.1	1.8	0.2	0.5	0.1
20:1($n-9$)	–	–	7.8	1.2	2.7	0.3	1.3	0.0	0.7	0.0	7.1	0.4	2.7	0.4
20:1($n-7$)	–	–	1.4	0.2	–	–	0.3	0.2	0.1	0.1	2.4	0.1	0.6	0.1
18:2($n-6$)	–	–	–	–	–	–	2.2	1.1	3.5	0.4	–	–	1.7	0.4
18:2($n-4$)	–	–	–	–	–	–	–	–	0.4	0.2	–	–	0.2	0.1
18:3($n-6$)	–	–	–	–	–	–	–	–	0.6	0.2	–	–	0.3	0.1
18:3($n-3$)	–	–	0.8	0.3	0.6	0.4	1.8	0.1	2.5	0.2	–	–	1.4	0.1
18:4($n-3$)	–	–	1.3	0.4	0.5	0.3	5.4	0.5	4.1	0.1	–	–	2.5	0.0
18:5($n-3$)	–	–	–	–	–	–	–	–	–	–	–	–	–	–
20:2($n-6$)	–	–	–	–	–	–	–	–	–	–	–	–	–	–
20:3($n-6$)	–	–	–	–	–	–	–	–	0.4	0.1	0.5	0.1	0.3	0.0
20:4($n-6$)	0.6	0.1	13.9	0.5	1.9	0.2	1.9	0.2	0.8	0.1	2.0	0.1	2.6	0.2
20:4($n-3$)	–	–	–	–	–	–	–	–	0.2	0.1	0.4	0.1	0.2	0.1
20:5($n-3$)	3.0	0.7	16.3	2.0	8.4	0.9	24.4	1.8	10.7	0.7	11.8	0.3	12.0	0.6
21:4($n-6$)	–	–	0.4	0.1	0.8	0.1	–	–	0.6	0.1	0.9	0.1	0.5	0.0
21:5($n-3$)	0.4	0.3	0.4	0.1	0.6	0.3	0.4	0.1	0.8	0.1	1.3	0.0	0.7	0.0
22:4($n-6$)	–	–	0.1	0.1	–	–	–	–	0.3	0.0	0.6	0.0	0.3	0.0
22:?	–	–	0.1	0.2	11.2	1.3	0.5	0.2	0.4	0.2	2.8	1.2	1.7	0.4
22:5($n-6$)	3.3	0.3	3.0	0.6	5.2	0.6	2.0	0.3	4.8	0.3	13.9	1.1	5.8	0.2
22:5($n-3$)	1.3	0.1	0.4	0.2	1.0	0.5	–	–	0.9	0.1	1.9	2.3	0.9	0.4
22:6($n-3$)	74.8	5.6	12.4	1.3	13.4	1.7	7.4	0.7	21.8	0.9	42.3	2.0	27.2	1.0
To.Sat.	9.0	3.1	29.0	1.4	47.5	2.4	33.9	2.1	29.1	0.9	4.8	3.1	25.0	1.3
To.Mon.	7.4	2.4	22.0	2.4	8.9	2.5	20.1	0.6	18.2	0.3	16.7	1.7	16.7	0.4
To.($n-9$)	2.4	1.2	10.8	1.3	4.5	1.1	8.4	0.5	7.5	0.4	10.0	1.5	7.6	0.3
To.($n-7$)	5.0	1.6	11.2	1.9	3.0	1.4	11.7	0.4	10.6	0.5	4.9	0.3	8.6	0.2
To.Poly.	83.6	5.4	48.9	1.4	43.6	4.8	46.0	1.6	52.8	0.9	78.5	4.6	58.2	1.1
To.($n-4$)	–	–	–	–	–	–	–	–	0.4	0.2	–	–	0.2	0.1
To.($n-6$)	3.9	0.4	16.9	0.8	7.1	0.6	6.1	1.3	10.5	0.5	17.1	1.0	10.9	0.6
To.($n-3$)	79.2	5.3	31.2	1.1	23.9	3.3	39.0	2.7	40.1	0.8	56.4	3.6	44.2	0.9
($n-3$)/($n-6$)	20.3	2.5	1.9	0.1	3.3	0.3	6.6	1.6	3.8	0.2	3.3	0.1	4.1	0.3
22:6/20:5	25.8	6.8	0.8	0.2	1.6	0.1	0.3	0.0	2.0	0.2	3.6	0.1	2.3	0.1
22:5/20:4	5.5	0.8	0.2	0.0	2.8	0.5	1.1	0.2	6.0	1.2	6.8	0.8	2.3	0.2

[a,b] See Table 2.

particular glycolipid (GLY). This GLY fraction represents 6% and 9% of the total polar lipid content of the gonads and larvae of *P. maximus*, respectively. Initial attempts at identification showed that, in contrast to that described by De Moreno et al. [10] in the gonads of *Mytilus platensis*, the GLY fraction in *P. maximus* did not contain phosphate.

The LPC (lyso 1 or lyso 2) and the biogenic lyso-2-phosphatidyl PE and PS were not detected

in our samples. Hence the PLSM corresponds to lysol-phosphatidyl PE and PS. Complementary separation by HPLC without an acidic mobile phase [29] confirmed the absence of biogenic LPE and LPS and showed that the alkenyl-acylphosphatidylserine is on an average 1.5% of the total PLSM. This presence of plasmalogens in PS (about 6% total serine content) is specific to molluscs [5]. In view of the preponderance of PE in the aminophospholipids, the plasmalogen fraction can be considered as essentially deriving from the alkenylacylphosphatidylethanolamine sub-class (75% of total PE content). The plasmalogen content of aminophospholipids represents 34% and 25%, respectively, of the total of polar lipids of gonads and larvae of *P. maximus*. This agrees with the data reported for molluscs by Dembitsky [3].

TLC analyses confirmed the existence of these phospholipid classes. These also showed that SM was absent in the lipidic extracts of *P. maximus* but that phosphonolipids were present. The phosphonolipids, particularly the ceramide aminoethylphosphonate (CAEP) [30,31], have been shown in many invertebrate species. It has been suggested that this phosphonolipid replaces the sphingomyelin of the vertebrates, even if some molluscs possess both of these [7,8]. For this type of compound (ceramides), the fatty acid, bound by an amine bond, is not transesterified by BF_3 after 10 min of heating at 100°C, in contrast to glycerophospholipids [32,33]. Its fatty acid content, therefore, does not influence the fatty acid composition of total polar lipids. A systematic comparison of total fatty acids in phospholipids before and after HPLC separation confirmed that the recovery of the fatty acids was complete.

Individual class composition

The fatty acid composition of the GLY fraction did not differ between the gonads and larvae of *Pecten maximus*. This fraction is characterized by high amounts of PUFA: 93% and 84% of the total fatty acids in the gonad and larvae, respectively, with 22:6($n-3$) accounting for 82 and 75% of them. This predominance of 22:6($n-3$) is of particular interest since this glycolipid can thus also contain up to 30% of the total of 22:6($n-3$) of the complex lipids whereas it represents only 6 and 9% of polar lipids, respectively, in the gonads and the larvae.

The PI fraction is characterized by the presence of large amounts of a polyunsaturated fatty acid of the ($n-6$) series, the 20:4($n-6$). This accounts for 37% of PI fatty acids in the gonad and is equivalent to 60% of the 20:4($n-6$) of complex lipids. The other important fatty acid was 18:0, and hence the couple 18:0–20:4($n-6$) is the major molecular species of PI. Bell and Sargent [12] have demonstrated this characteristic composition in another mollusc *Chlamys islandica*. This fatty acid composition of PI appears to have a wide distribution across the animal kingdom and therefore suggests a common cellular role. In man, it is known to have generally two main functions: the production of second messengers, inositol triphosphate (IP_3) and diacylglycerol (DG), [34] and supply of arachidonic acid, precursor of prostaglandins, thromboxanes and leukotrienes.

In spite of having a nearly same percentage of total PUFA (about 50% of the total of the fraction), the female gonads of adult *P. maximus* and the larvae show significant differences in the amounts of individual polyunsaturated fatty acids. The PUFA of the PI of the gonad are largely dominated by 20:4($n-6$) (37% of the total of the fraction) whereas the larvae have a much larger spectrum distributed between 20:4($n-6$) (13.9%), 20:5($n-3$) (16.3%) and 22:6($n-3$) (12.4%). These differences suggest a specific role for PI, depending on the developmental stage.

In the PS, the PUFA represent 49.1 and 43.6% of total fatty acids in the gonads and larvae, respectively. A fatty acid with 22 carbon atoms, probably with three double bonds uninterrupted by a methyl (NIM), is one of the principal fatty acids (14% for the gonads and 11.2% for the larvae), as already shown in the phospholipid fraction of the *P. maximus* larvae [15]. This 22-carbon fatty acid characterizes the PS of the gonads and the larvae of *P. maximus*. It is perhaps NMIT, shown by Dunstan et al. [35] to occur in small amounts in the oyster and tenta-

tively identified from a sponge extract [36]. Being absent from food, it can only be of endogenous origin and biosynthesized from mono-unsaturated fatty acids, as suggested by Dunstan et al. [35], even though the biosynthesis of polyunsaturated long-chain fatty acids is absent or very weak in molluscs. In other marine animals, the fatty acids of PS are generally dominated by 22:6($n-3$).

The other characteristic fatty acids in the PS are 22:6($n-3$), 20:5($n-3$) and 22:5(n − 6). The 22:6($n-3$) was present at 12.7 and 13.4%, respectively, in the gonads and larvae. Corresponding percentages for 20:5($n-3$) are 11.5 and 8.4%, and for the 22:5($n-6$) 3.6 and 5.2%. These three PUFA, in association with 18:0 (42–45%), constitute the three major molecular species of PS. The fatty acid compositions of this fraction in gonads and larvae are very similar, which suggests a similar metabolic role for PS, independent of the developmental stage. In human tissues, this class is located rather on the internal layer of the membranes, which implies an intracellular role [37].

In the non-plasmalogen PE fraction, the PUFA varied between 46 and 49.2% of the total of fatty acids. The PUFA were dominated by 20:5($n-3$) (30.4% for the gonads and 24.4% for the larvae), though the 22:6($n-3$) are generally the dominant fatty acids of phospholipids. This suggests a specific role for 20:5($n-3$) associated with the non-plasmalogen PE in bivalves. Koizumi et al. [38] described a similar composition for the diacyl form of the PE of *Crassostrea gigas*, with a predominance of 20:5($n-3$) (22.3%) among the PUFA.

In the PC, the major phospholipid class, the saturated fatty acids were dominated by 16:0 (up to 23.3% in the gonads). This dominance of 16:0 in the PC distinguishes this class from others; in fact, the saturated chains of PS and PI are dominated by 18:0 and the non-plasmalogen PE presents an intermediate composition. This characteristic has already been found in *Crassostrea gigas* [35] and in the gonads of *Chlamys islandica* [12]. This composition of saturated fatty acids is equally the same in fishes and higher vertebrates. The PUFA represent 55.3% and 52.8% of the total of fatty acids of this fraction, respectively, in the gonads and the larvae. In the latter, the 22:6($n-3$) (21%) dominates over 20:5($n-3$) (10%), whereas in the gonads both are present in equivalent proportions (18%). Similarly in the ($n-6$) series, 22:5($n-6$) is dominant over 20:4($n-6$) in the larvae (5 and 1%, respectively) whereas in the gonads both were in equal amounts (2%). The PC is considered to be the biochemical crossroads in the remodelling of cellular phospholipids by a deacylation–reacylation cycle and shows a rapid turnover for incorporation of PUFA in comparison with other phospholipids [37]. In animals, this appears to favour a selection of 22:6($n-3$) whereas the food supplies mainly 20:5($n-3$). Nevertheless, differences in regulation between the larvae and gonads of *P. maximus* certainly exist and can influence the fatty acid composition of PC.

In the fraction denoted LPE, which corresponds mainly to the plasmalogens of the PE, and to a lesser extent to those of PS, the fatty acids are highly unsaturated, accounting for 92.6% in the gonads and 78.5% of total fatty acids in the larvae. These are nearly exclusively the 20- or 22-carbon fatty acids belonging mainly to the ($n-3$) series. This characteristic has also been observed in the plasmalogens of human origin [39]. Earlier studies have shown that the 20- and 22-carbon PUFA are preferentially incorporated in the plasmalogens. Several roles have been attributed to the plasmalogens: antioxidants, terminal storage of arachidonic acid, receptor mediators, substrate for biosynthesis of the platelet activation factor (PAF) and a determinant role in the physico-chemical properties of biomembranes. However, none of these functions have been totally proved [40]. It has also been suggested [5] that the plasmalogens are strongly involved in the membrane permeability phenomena in response to environmental stress in marine animals.

In the LPE fraction, as in the PC but in a pronounced manner, 22:6($n-3$) is found in higher amounts (43%) than 20:5($n-3$) (12%) in

the larvae, but in the gonads both occur in equivalent amounts (37 and 30%, respectively). This difference in the C_{22}/C_{20} ratio between the larvae and the gonad is more striking in the $(n-6)$ series, with a ratio of 7.2 for 22:5$(n-6)$/20:4$(n-6)$ for the larvae and 0.9 for the gonads. This may reflect a structural necessity, more particularly for the larvae, to maintain higher proportions of 22-carbon PUFA in the plasmalogens. The 22:6$(n-3)$, for which a primordial structural role has been suggested in *P. maximus* [15,16], could be associated with the permeability mechanisms in which the plasmalogens have been implicated. On the other hand, the maintenance of larger amounts of 20:5$(n-3)$ and 20:4$(n-6)$ in the plasmalogens of the female gonads can, as in human plasmalogens, reflect a storage function for prostaglandin precursors involved in reproduction.

This distribution of PUFA between the alkenyl acyl PE form [plasmalogen PE rich in 22:6$(n-3)$] and the diacyl PE form [non-plasmalogen PE rich in 20:5$(n-3)$] seen in the phospholipids of *P. maximus* has also been observed in *Crassostrea gigas* [38]. This suggests the existence of a metabolic independence, as in humans, of the biosynthesis pathways and the regulation of phospholipids having an ether bond as against the diacyl forms. These imply a complex coordination of peroxisomes, cytosol and endoplasmic reticulum [40]. This plasmalogen fraction of *P. maximus* must therefore be considered to be different from non-plasmalogen fractions. The utilization of an acidic mobile phase during HPLC separation which allows a separate analysis of plasmalogen forms of phospholipids finds its usefulness here.

The coupling of the two columns (diol and Si) thus permits an optimum resolution of the principal polar lipid classes of *P. maximus*. This technique is a valuable tool for the determination of fatty acid composition and of the relative proportions of different phospholipid classes in the context of a study on physiological and cellular functions of this bivalve. In addition, the ability to separate the less polar lipids such as glycolipids has brought to light the existence of a glycolipid extremely rich in 22:6$(n-3)$ in *P. maximus*, associated with specific biological functions. A further advantage of this method is that it can be easily adapted to other biological material by using, for example, only diol column for extracts poor in molecular species.

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