Molecular Species Analysis of Phosphoglycerides from the Ripe Roes of Cod (*Gadus morhua*)

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Molecular species of the 3,5-dinitrobenzoyl derivatives of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) were quantitated by UV detection at 254 nm after reversed-phase HPLC using solvent systems modified from Takamura *et al.* (*Lipids 21,* 356–361, 1986). Three isocratic solvent systems were used and a total of 39 different molecular species detected. Four species, 16:0/20:5, 18:1/20:5, 16:0/22:6 and 18:1/22:6 contributed 67.2% and 61.8% of PC and PE respectively but only 23.0% of PI. In PI the most important species was 18:0/20:4 at 36.7% but this species only constituted 0.7% in each of PC and PE. Small amounts of dipolyunsaturated species were also found in PC and PE.
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The determination of the fatty acid composition of tissue lipids by gas liquid chromatography of fatty acid methyl esters has been a routine analytical tool of the lipid biochemist for many years. Recently the development of HPLC methods for analyzing the molecular species composition of phosphoglycerides has permitted an investigation of biomembrane phospholipids at a new level of complexity and has given fresh insight into the organization of the biomembrane. A variety of methods has been developed using intact or derivatized phospholipids and several types of detection systems. One of the most successful methods is that of Patton *et al.* (1) which employed an isocratic elution system of intact phosphoglycerides on a reversed-phase (C18) column with UV detection at 205 nm. This system has been widely used for studies involving polyunsaturated fatty acids (PUFA) but cannot give quantitative information on all the molecular species present since detection at 205 nm depends on the presence of double bonds in the unsaturated fatty acids. Post-column fluorescence detection or flame ionization detection does allow quantitative analysis of intact phosphoglycerides (2,3).

However, the most common quantitative methods rely on preparing derivatives of the phosphoglycerides by hydrolysis with phospholipase C followed by acylation of the diacylglycerol to give acetyl, benzoyl or dinitrobenzoyl derivatives (4–7). These derivatives are separated into molecular species on reversed-phase columns and detected with refractive index or UV detectors (3,6). Most molecular species analyses have been of PC but an additional advantage of derivatization is that all the glycerides are converted to similar compounds which can therefore all be separated using the same chromatography conditions. In this paper molecular species analyses of PC, PE and PI from the ripe roes of cod *(Gadus morhua)* were determined as 3,5-dinitrobenzoyl derivatives. A third solvent system was developed to complement the system of Takamura *et al.* (8) and resolve the coeluting species found by these workers.

Abbreviations: PUFA, polyunsaturated fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol. Molecular species are abbreviated as follows: e.g., 16:0/20:5 PC is 1-palmitoyl-2-eicosapentaenoyl-*sn*-glycero-3-

MATERIALS

Ripe roes were excised from cod caught off Gourdon, south of Aberdeen, Scotland, and stored at −20°C. *Bacillus thuringiensis* type strain IAM 12077 was obtained from the National Culture of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, Scotland.

Phospholipase C from *Bacillus cereus* (Sigma Type XIII), butylated hydroxytoluene (BHT), 3,5-dinitrobenzoyl chloride, dipalmitolein, diolein and the following phosphatidylcholines were obtained from Sigma Chemical Co., Poole, Dorset, UK: egg yolk PC, dilauryl PC, dimyristoyl PC, dipalmitoyl PC, distearoyl PC, 1-stearoyl 2-arachidonyl PC.

Merck TLC and HPTLC plates coated with silica gel 60, Analar grade glacial acetic acid, methyl acetate, propan-1-ol, propan-2-ol and pyridine were purchased from BDH Ltd., Poole, Dorset, UK. All other solvents of HPLC grade were from Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland.

Ultrasphere ODS and Ultrasphere C8 HPLC columns (25 × 0.46 cm, 5 micron particle size) were obtained from Altex/Beckman (Beckman Instruments UK Ltd., Progress Road, Sands Industrial Estate, High Wycombe, Bucks, UK).

METHODS

Purification of phosphoglycerides. Total lipid was extracted from freeze-dried homogenates of eggs taken from four fish by the method of Folch *et al.* (9). Approximately 3 g of lipid were obtained from 100 g wet weight of cod roe (10). Solvents routinely contained 0.01% (w/v) BHT and samples were stored at −20°C under N_2 between preparative procedures.

Neutral lipids were separated by preparative TLC using hexane/diethyl ether/acetic acid (70:30:1, v/v/v). The origin material was eluted from the silica with chloroform/methanol/water (5:5:1, v/v/v), and the eluate was dried by rotary evaporation under vacuum at 35°C. The polar lipid fraction contained 46% PC, 20% PE, 3.0% PI, 1.4% PS and smaller amounts of sphingomyelin, lyso-PC and phosphatidic acid determined as phospholipid-bound phosphorus (10).

Polar lipid classes were separated by TLC using methyl acetate/propan-1-ol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, v/v/v/v/v) (11), and visualized under UV light after spraying with 0.1% (w/v) 2,7-dichlorofluorescein in methanol. The PC and PI fractions were further purified by TLC in chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v/v/v/v) and detected as before. From the lipids 2,7-dichlorofluorescein was removed by extracting with a 2% (w/v) solution of

was determined by GLC of fatty acid methyl esters (FAME) in a Canberra Packard 436 gas chromatograph fitted with a CP Wax 52 CB fused capillary column (50 m × 0.32 mm i.d.) (Chrompack UK Ltd., London) using hydrogen as carrier gas (12).

Preparation of 3,5-dinitrobenzoyl derivatives. One mg portions of PC and PE were hydrolyzed with phospholipase C from *B. cereus* using a two phase system of 1 ml diethyl ether and 1 ml 0.01 M Tris-SO_4 pH 7.4 at room temperature (13). Phosphatidylinositol was hydrolyzed with a PI-specific phospholipase C prepared from *B. thuringiensis* by the method of Ikezawa and Taguchi (14). At the end of the incubation diacylglycerol (DAG) and residual phospholipid were extracted by the addition of 1 ml 0.88% (w/v) KCl and 8 ml chloroform/methanol (2:1, v/v). Lipids were dried under N_2 and stored overnight *in vacuo*. By TLC 1,2-diacylglycerol was purified using hexane/diethyl ether/acetic acid (50:50:1, v/v/v), detected with 2,7-dichlorofluorescein, eluted with hexane/diethyl ether (1:1, v/v) and dried under N_2 and finally *in vacuo* for 2 hr before derivatization. Only trace amounts (estimated to be 1 or 2%) of unhydrolyzed phospholipid remained after phospholipase C digestion. The 1,2-diacylglycerols were acylated in dry pyridine with 3,5-dinitrobenzoyl chloride at 60°C under N_2 for 45 min (8). After extraction and washing (8) the purity of the 1,2-diacyl, 3-dinitrobenzoyl glycerol derivatives was checked by HPTLC in hexane/diethyl ether/acetic acid (70:30:1, v/v/v). All the diacylglycerol was converted to the dinitrobenzoyl derivative.

HPLC of 1,2-diacyl-3-dinitrobenzoyl glycerol derivatives. Molecular species were separated on reversed-phase columns at room temperature (19–21°C) using three isocratic solvent systems with a Pye Unicam 4010 pump, detected at 254 nm with a Pye Unicam 4020 detector and quantitated using a Shimadzu C-R3A integrator. Solvent 1 (methanol/propan-2-ol, 95:5, v/v, 1.0 ml/min) and solvent 2 (acetonitrile/propan-2-ol, 80:20, v/v, 1.0 ml/min) were used with a C18 column as described by Takamura *et al.* (8). A third solvent (methanol/water/acetonitrile, 93:5:2, v/v/v, 1.2 ml/min) was developed for use with a C8 column.

The molecular species containing PUFA in cod roe PC were identified by collecting the peaks after HPLC of the intact PC using the solvent system of Patton *et al.* (1), and preparing FAME to identify the component fatty acids by GLC. A number of synthetic standards were also used to construct plots of log relative retention time (RRT) against effective carbon number at the C1 position of the glyceride (1,8). A convenient reference peak was 16:0/22:6 and RRTs were calculated relative to this species. Sometimes added to samples as additional

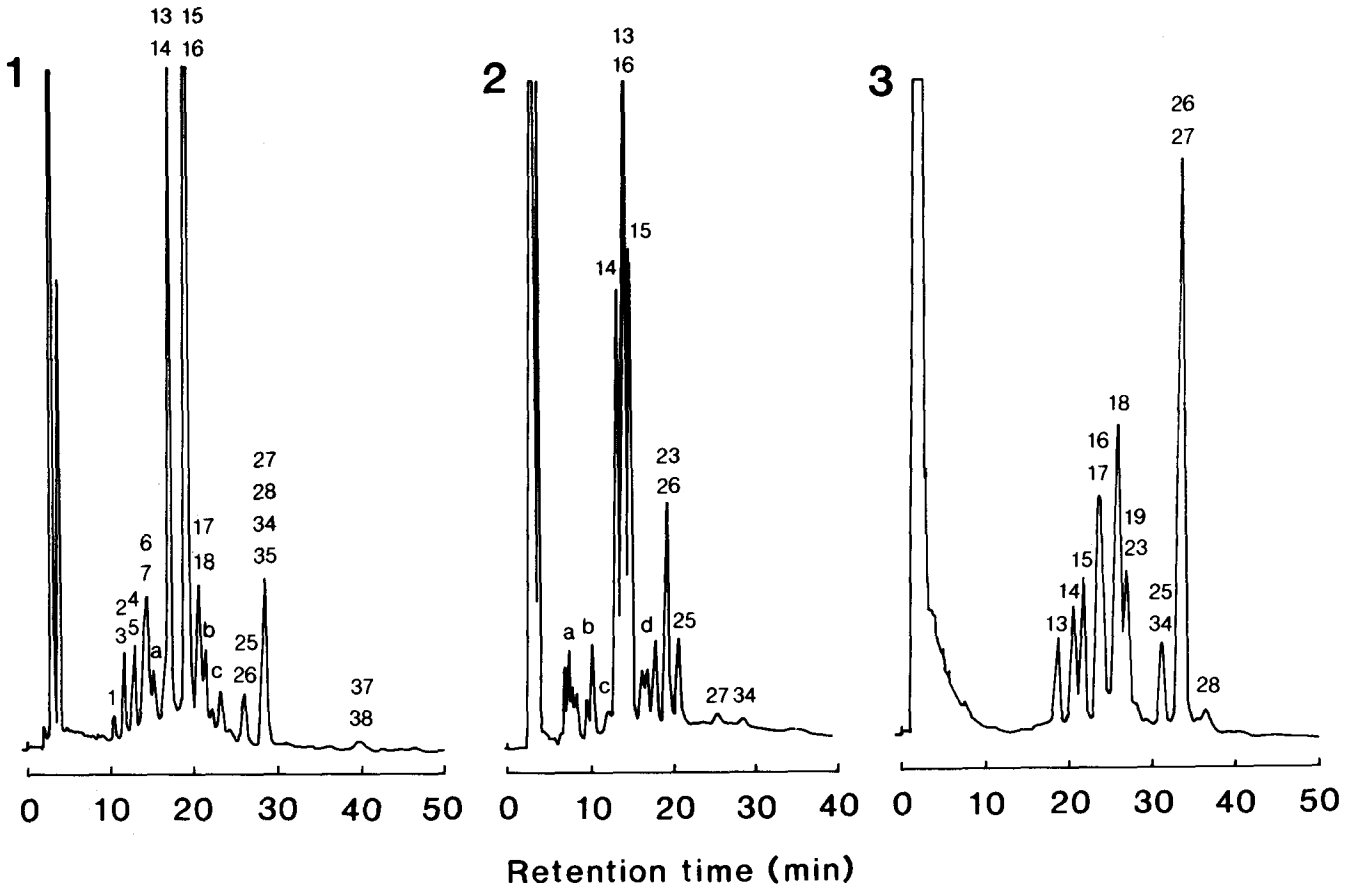

FIG. 1. Molecular species separations of the 3,5-dinitrobenzoyl derivatives of cod roe PC (1) in methanol/propan-2-ol 95:5 (v/v) 1.0 ml/min, cod roe PE (2) in acetonitrile/propan-2-ol 80:20 (v/v) 1.0 ml/min and cod roe P1 (3) in methanol/water/acetonitrile 93:5:2 (v/v/v) 1.2 ml/min. The absorbance range at 254 nm was 0.04 units in 1 and 2, and 0.02 units in 3, with c. 40 μg, c. 40 μg and c. 10 μg of material injected respectively. Peak numbers refer to the molecular species shown in Table 2, but space did not permit the individual numbering of all peaks. Lettered peaks or groups of peaks contain the following species: in profile 1, a = 8, 9, 10, 11; b = 19, 20; c = 21, 22 and 23, 24;

reference species were 12:0/12:0 and 16:0/16:0. Solvent 1 was used to give the basic information on peak areas and the other two solvents were used to give the peak areas of individual species either directly or by subtraction. Each sample was chromatographed three times in each of the solvent systems and the standard deviations calculated. Where final peak areas were calculated by subtraction, the standard deviations of the contributing peaks were added to give the final error. All final analyses were corrected to 100%.

RESULTS AND DISCUSSION

The protocol described here employing three different isocratic solvent systems to separate 1,2-diacyl, 3-dinitrobenzoyl derivatives of phosphoglycerides offers significant advantages over earlier procedures. The method of Takamura *et al.* (8) which used the same derivatives and solvents 1 and 2 resulted in some coeluting species, including ones of major importance in tissues rich in (n-3)PUFA, e.g., 18:1/22:6 running with 16:0/22:6 in solvent 1 and with 16:0/20:5 in solvent 2. Therefore, a third solvent was developed from the system of Patton *et al.* (1), used for separating intact PC molecules, by removal of the choline-chloride and adjustment of the proportion of methanol, water and acetonitrile to give solvent 3 (methanol/water/acetonitrile, 93:5:2, v/v/v). However, with a C18 column this solvent gave very long run times with consequent peak broadening and diminished resolution, but it was found that a C8 column gave good separations in a much shorter time. The use of these three solvent systems allowed all coeluting species to be separated by at least one of the solvents. As little as 10 μg of material per run was used routinely. Typical separations are shown in Figure 1.

Each solvent system has slightly different characteristics. Solvent 1 separates according to the PUFA on C2 but does not resolve the saturated species from the monoene two carbons longer so that certain pairs such as 14:0/22:6 + 16:1/22:6 and 16:0/22:6 + 18:1/22:6 are unresolved. Solvent 2 separates these pairs with the monoene species running faster but unfortunately gives other coeluting pairs, e.g., 14:0/20:5 + 16:1/22:6 and 16:0/20:5 + 18:1/22:6. Solvent 3 separated all these pairs but there were other coeluting species later in the profile, e.g., 18:0/20:4 + 20:1/22:6.

Comparison of the fatty acid compositions of the phospholipids deduced from the HPLC molecular species analysis with those found by GLC of fatty acid methyl esters shows good agreement and validates the method (Table 1). The molecular species analysis often underestimates the minor fatty acids, e.g., 16:1, 18:2, 20:1 and 22:5, since small peaks containing these species can be unresolved from the large peaks of the major species.

Cod roe PC was found to comprise four main molecular species, with 18:1/20:5, 16:0/20:5, 18:1/22:6 and 16:0/22:6 making up two thirds of the sample, the last species contributing a third of the PC (Table 2). While a further 30 minor species were detected, 16:1/22:6 (4.7%) and 16:0/18:1 (4.9%) were the only other species present at greater than 2.6%. It was assumed in all the separations here that the more unsaturated fatty acid was on position 2 and it appeared that solvent systems 1 and 2 should, in some

TABLE 1

Comparison of the Fatty Acid Compositions Found by GLC and HPLC

	Mol % composition					
	PC		PE		PI	
	GLC	HPLC	GLC	HPLC	GLC	HPLC
14:0	1.8	2.1	1.2	0.6	0.3	tr
16:0	31.4	27.2	13.0	14.1	10.4	8.2
16:1(n-7 + n-9)	3.9	3.7	2.1	3.6	1.4	tr
18:0	1.7	2.4	5.4	3.5	21.2	24.5
18:1(n-7 + n-9)	14.7	16.1	21.8	22.1	22.4	16.1
18:2(n-6)	0.7	0.5	1.3	nd	nd	nd
20:1(n-7 + n-9)	1.0	0.8	5.4	5.3	2.5	1.4
20:4(n-6)	2.8	3.4	2.9	3.6	23.2	30.0
20:5(n-3)	14.1	12.9	16.0	16.3	7.7	7.9
22:5(n-3)	1.8	2.2	2.2	2.9	1.2	1.0
22:6(n-3)	26.2	29.1	28.7	27.9	9.6	10.8

The fatty acid composition of PC, PE and PI from cod roe found by GLC of FAME were converted from weight % to mol % using correction factors calculated from the molecular weights of the methyl esters relative to 16:0. The HPLC molecular species analysis gave area % which equates to mol % with a UV detection system. The fatty acid compositions were all corrected to 100%. tr = <0.1%; nd = not detected.

TABLE 2

Molecular Species Composition of PC, PE and PI from Cod Roe

	PC (mol %)	PE (mol %)	PI (mol %)
1. 20:5/20:5	0.5 ± 0.1	0.3 ± 0.1	—
2. 20:5/22:6 22:6/20:5	0.6 ± 0.2	0.8 ± 0.1	—
3. 22:6/22:6	1.3 ± 0.2	1.5 ± 0.3	—
4. 14:0/20:5	0.8 ± 0.2	tr	—
5. 16:1/20:5	1.1 ± 0.2	2.3 ± 0.1	tr
6. 14:0/22:6	2.1 ± 0.2	0.9 ± 0.1	tr
7. 16:1/22:6	4.7 ± 0.2	1.8 ± 0.1	tr
8. 14:0/20:4	0.6 ± 0.1	tr	—
9. 16:1/20:4	1.1 ± 0.2	0.3 ± 0.1	—
10. 14:0/22:5	0.8 ± 0.2	tr	—
11. 16:1/22:5	0.4 ± 0.1	2.0 ± 0.1	—
12. 16:1/18:2	tr	—	—
13. 16:0/20:5	15.2 ± 0.4	8.5 ± 0.6	2.6 ± 0.2
14. 18:1/20:5	6.1 ± 0.5	12.8 ± 0.6	5.1 ± 0.3
15. 16:0/22:6	31.0 ± 0.8	14.8 ± 0.4	5.9 ± 0.2
16. 18:1/22:6	14.9 ± 1.0	25.7 ± 0.7	9.4 ± 0.4
17. 16:0/20:4	2.6 ± 0.1	3.2 ± 0.1	6.2 ± 0.1
18. 18:1/20:4	1.9 ± 0.1	2.2 ± 0.2	16.7 ± 0.3
19. 16:0/22:5	0.5 ± 0.1	1.0 ± 0.2	1.3 ± 0.2
20. 18:1/22:5	1.3 ± 0.1	2.8 ± 0.2	0.4 ± 0.1
21. 16:0/18:2	tr	—	—
22. 18:1/18:2	tr	—	—
23. 18:0/20:5	0.9 ± 0.1	2.5 ± 0.2	7.5 ± 0.7
24. 20:1/20:5	0.3 ± 0.1	4.6 ± 0.2	0.6 ± 0.1
25. 18:0/22:6	1.7 ± 0.2	3.5 ± 0.4	4.8 ± 0.2
26. 20:1/22:6	0.6 ± 0.1	5.2 ± 0.5	1.6 ± 0.2
27. 18:0/20:4	0.7 ± 0.1	0.7 ± 0.3	36.7 ± 2.0
28. 20:1/20:4	—	0.7 ± 0.3	0.5 ± 0.3
29. 18:0/22:5	1.0 ± 0.2	—	tr
30. 20:1/22:5	0.3 ± 0.1	—	—
31. 18:0/18:2	tr	—	—
32. 20:1/18:2	tr	—	—
33. 16:0/16:0	—	—	tr
34. 16:0/18:1	4.9 ± 0.1	0.7 ± 0.1	0.2 ± 0.1
35. 18:1/18:1	1.1 ± 0.1	—	0.2 ± 0.1
36. 18:0/16:1	—	0.3 ± 0.1	—
37. 18:0/18:1	0.4 ± 0.1	—	—
38. 20:1/18:1 18:1/20:1	0.3 ± 0.1	—	—
39. 18:0/18:0	tr	—	—

The errors are given as ±1 standard deviation, rounded to the nearest decimal place (see text). For most of the minor species this considerably over estimates the error. It was assumed that the most saturated fatty acid was on the 1 position of the glyceride. tr =

TABLE 3

The Favored 1- and 2-Position Fatty Acids in Phosphatidylinositol from Cod Roe

	2-Position fatty acid (%)		
1-Position fatty acid	20:4(n-6)	20:5(n-3)	22:6(n-3)
16:0	38.7	16.2	36.9
18:0	74.9	15.3	9.8
18:1	52.2	15.9	29.4
	1-Position fatty acid (%)		
2-Position fatty acid	16:0	18:0	18:1
20:4(n-6)	10.3	61.1	27.8
20:5(n-3)	16.5	47.5	32.3
22:6(n-3)	27.2	22.1	43.3

e.g., 16:0/22:6 from 22:6/16:0, but not 16:0/18:1 from 18:1/16:0. In cod roe PC only 63.4% of species had a saturated fatty acid on C1, 34.1% had a monoene and 2.4% a PUFA. Of the 30 minor species detected, the most interesting were the diPUFA species 20:5/20:5, 20:5/22:6, 22:6/20:5 and 22:6/22:6 totalling 2.4% with the last species being the most important. Molecular species analyses are increasingly finding these highly unsaturated species in a variety of samples including fish muscle PC (4) and phosphoglycerides from bovine retina (15). The intriguing presence of diPUFA molecular species is contrary to earlier ideas on phospholipid structure, and their properties and role in biomembrane function remains to be elucidated.

In PE from cod roe the same four molecular species (16:0/20:5, 18:1/20:5, 16:0/22:6, 18:1/22:6) contributed over 60% of the total but their relative contributions were different, reflecting the greater abundance of 18:1 in PE. The major species contributing a quarter of the total sample was 18:1/22:6 (Table 2). In PE 18:0 and 20:1 species were much more important, totalling 17.2% compared with only 4.2% in PC. In PE only 36.5% of species contained a saturated fatty acid on C1, 60.9% held a monoene and again there was a small amount of diPUFA species totalling 2.6%. No alkenyl species were detected suggesting that PE plasmalogen was not present in this sample. Alkenyl species were readily separated in a sample of bovine brain PE containing about 50% plasmalogen.

It has long been known that mammalian PI is rich in 18:0 and 20:4(n-6) (16), and more recently this was also shown to be the case in fish tissues rich in (n-3)PUFA (10,17). It is therefore not surprising that 18:0/20:4 should be the major molecular species in PI as has been shown in several mammalian tissues (6,16). However, this appears to be the first molecular species analysis of PI from a tissue rich in (n-3)PUFA. The specificity of PI for 20:4(n-6), and especially the predominance of the 18:0/20:4 species is impressive. In PI 36.7% is this species while PC and PE each had only 0.7% (Table 2).

Table 3 shows the favored pairings for the six main fatty acids in PI. Phosphatidylinositol clearly shows a selectivity for an 18 carbon fatty acid on the C1 position and a 20 carbon PUFA on the C2 position. Thus 74.9% of 18:0 is paired with 20:4(n-6) while 61.1% of the 20:4(n-6) pair with about 15% of 20:5(n-3) on C2, but 22:6(n-3) is selectively paired with 16:0 or 18:1, the major species in PC and PE. Almost half the 20:5(n-3) is paired with 18:0 but only 22% of the 22:6(n-3) is with 18:0. Thus the pairing 18:0/22:6 is not favored and apparently selected against in PI, while in PC and PE there is no such specificity.

The unique molecular species composition of PI, most clearly shown in a tissue rich in (n-3)PUFA, reflects the pivotal metabolic role of PI in signal transduction through the biomembrane (18). In marine species rich in (n-3)PUFA, PI is clearly the main source of arachidonic acid for eicosanoid biosynthesis, while in terrestrial animals this role can also be filled by PC or PE plasmalogen (19,20). The extent to which the 18:0/20:5 species could be involved in such signalling activity is presently unclear both in fish and especially in humans, where dietary supplementation with fish oils to elevate the (n-3)PUFA content of tissue phospholipids is currently the basis for alleviating some circulatory and inflammatory disorders (21,22).

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