

Characterization of ω -3-Docosahexaenoic Acid-Containing Molecular Species of Phospholipids in Rainbow Trout Liver

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Four major phospholipids from rainbow trout liver were purified by chromatography, and the phospholipid classes and their molecular species were quantitatively and qualitatively determined by ^{31}P nuclear magnetic resonance, gas chromatography, liquid secondary ion mass spectrometry, and liquid secondary ion mass spectrometry combined with tandem mass spectrometry. Trout liver phospholipids consist mainly of glycerophosphatidylcholine (GPC; 67%), glycerophosphatidylethanolamine (GPE; 22%), glycerophosphatidylserine (GPS; 10%), and glycerophosphatidylinositol (GPI; 1.6%); the contents of 22:6 ω -3 fatty acid in the first three phospholipid classes were 34% (GPC), 31% (GPE), and 27% (GPS), respectively, and abundant molecular species were 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (GPC 16:0-22:6) (see Table 2 for phospholipid molecular species designations), 1-eicosaenoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine (GPE 20:1-22:6), 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine (GPE 18:0-22:6), 1-oleoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine (GPE 18:1-22:6), 1-linoleoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine (GPE 18:2-22:6), 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine (GPE 16:0-22:6), 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoserine (GPS 16:0-22:6), and 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoserine (GPS 18:0-22:6). GPI in trout liver lacks molecular species containing 22:6 ω -3 fatty acid. The study clearly indicates that a very high content of 22:6 ω -3 fatty acid-containing molecular species within phospholipids, mainly within GPC and GPE, is characteristic of rainbow trout liver.

Keywords: *Docosahexaenoic acid; phospholipid molecular species; phosphatidylcholine; phosphatidylethanolamine; phosphatidylinositol; phosphatidylserine; polyunsaturated fatty acid; trout liver*

INTRODUCTION

The functions of phospholipids in a number of biological processes have been widely described. Various phospholipases participate in the metabolism of phospholipids; phospholipases A₁ and A₂ attack the fatty acyl ester bonds at the *sn*-1 and *sn*-2 positions in the glycerol backbone, respectively, resulting in the release of free fatty acids and their subsequent metabolism via enzymatic pathways or rapid reacylation into the different molecular species of glycerophospholipids and other lipid classes.

Clinical investigations have demonstrated that a docosahexaenoic acid (22:6 ω -3)-containing fish oil diet is associated with a reduced incidence of coronary arterial disease (Leaf and Weber, 1985; Rapp *et al.*, 1991; Shekelle *et al.*, 1985), and possible mechanisms have been proposed (Bruckner *et al.*, 1987; Childs *et al.*, 1990; Kinsella *et al.*, 1990; Ruyle *et al.*, 1990). Although humans are able to synthesize 22:6 ω -3 fatty acid from 18:3 ω -3 fatty acid (α -linolenic acid), a diet of marine lipids containing this fatty acid is a major exogenous intake method. It has been reported that 22:6 ω -3 fatty acid is differentially incorporated into cellular glycerophospholipids and that the molecular species containing this polyunsaturated fatty acid esterified at the *sn*-2 position are better substrates for phospholipase A₂ (Na *et al.*, 1990).

Bovine brain glycerophosphatidylserine (GPS) is a natural source of glycerophospholipids, which are enriched with 22:6 ω -3 fatty acid at the *sn*-2 position. Several pharmacological effects of GPS have been documented, including (i) enhanced release of acetylcholine from brain slices (Vannucchi and Pepeu, 1987), (ii) increased catecholamine turnover in the hypothalamus (Toffano *et al.*, 1978), and (iii) reduction of brain energy metabolism (Bigon *et al.*, 1979a). Lysophosphatidylserine molecular species, formed by a phospholipase-mediated reaction, have been proposed to be the active metabolites (Bigon *et al.*, 1979b). Clinical trials have shown the potential of bovine brain GPS liposomes in the alleviation of moderate mental deterioration, such as geriatric depressive disorders (Maggioni *et al.*, 1990), and Alzheimer's disease (Amaducci *et al.*, 1991) after a prolonged oral administration. The study performed on the effect of GPS on the release of acetylcholine from brain slices also demonstrated that bovine brain glycerophosphatidylethanolamine (GPE), which contains species with 22:6 ω -3 fatty acid at the *sn*-2 position, is only half as active compared to GPS on acetylcholine output and that bovine brain glycerophosphatidylcholine (GPC), which contains no 22:6 ω -3 acid, has no effect (Casamenti *et al.*, 1979). From these experiments, it appears that the 22:6 ω -3 fatty acid should be present to observe pharmacological effects. A better understanding of the role of the polar head group(s) of natural phospholipid liposomes containing 22:6 ω -3 fatty acid residues is warranted because it may contribute to a more rational selection of suitable lipids as therapeutic agents (Palatini *et al.*, 1991). To our knowledge, there have been no studies dealing with the specific role of the polar head group. The exploration of suitable

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sources of 22:6 ω -3 fatty acid-containing molecular species of phospholipids, especially GPC and GPE, is essential for further study of their pharmacological and therapeutic effects and obtaining insight into the structure-function relationship of the lipids.

MATERIALS AND METHODS

Chemicals. Chloroform, methanol, and phosphoric acid were obtained from Fisons Scientific Equipment (Loughborough, U.K.). *m*-Nitrobenzyl alcohol and diethanolamine were from Sigma Chemical Co. (Poole, Dorset, U.K.).

Separation of Trout Liver Phospholipids. Total lipids were extracted from a rainbow trout (*Oncorhynchus mykiss*, weighing approximately 300 g) liver (1.9 g; withdrawn in 40–50 min after death), obtained from a trout farm (Hockley Health, West Midlands, U.K.), according to the method of Folch *et al.* (1957). After the solvent was evaporated, the lipids were dissolved in chloroform/methanol (60:40 v/v) and then applied onto a short column (Chen *et al.*, 1990a) containing anion-exchange resin (Q-Sepharose Fast Flow, Uppsala, Sweden). Briefly, the resin was washed before use with (i) 2 \times 3 bed volumes of chloroform/methanol/1 M sodium acetate (60:40:8 v/v/v), (ii) 2 \times 5 bed volumes of chloroform/methanol/water (60:40:8 v/v/v), and (iii) 2 \times 10 bed volumes of chloroform/methanol (60:40 v/v). Nonacidic phospholipids were eluted with chloroform/methanol (55:45 v/v), and acidic phospholipids were obtained by elution with acetic acid/chloroform (6:1 v/v). GPS and GPI were further purified (Chen *et al.*, 1990b) from the acidic lipid fraction on an aminopropyl normal-phase HPLC column (4.6 \times 150 mm, 5 μ m, Sigma-Aldrich, Sigma Chemical Co., St. Louis, MO); GPE and GPC were prepared from the nonacidic lipid fraction by silica thin-layer chromatography (Vitiello and Zanetta, 1978).

Distribution of Phospholipids. The distribution of the major classes of phospholipids isolated from trout liver was determined directly on the total lipid extract by ^{31}P NMR (Bardamante *et al.*, 1990). The measurement was performed on a Bruker ACP-400 spectrometer (161.97 MHz). The same extract was also analyzed by thin-layer chromatography (Vitiello and Zanatta, 1978).

Gas Chromatographic Analysis of the Fatty Acids in the Phospholipids. Gas chromatographic analysis of 22:6 and other fatty acids in each phospholipid class was performed with a SP-2330 packing column (200 \times 0.3 cm) on a Pye Unicam gas chromatography system (Model Series 204, U.K.), using a temperature program from 180 to 230 $^{\circ}\text{C}$ at 2 $^{\circ}\text{C}/\text{min}$. The methylated fatty acids (Sattler *et al.*, 1991) were identified by comparing both GC retention times of fatty acid methyl ester standards (PUFA II, Sigma) and composition of fatty acids of total trout phospholipids (Turner *et al.*, 1989).

Mass Spectrometry. Conventional liquid secondary ion mass spectrometry (LSIMS) and collisionally induced dissociation (CID) tandem mass spectra of the phospholipids were obtained with a VG70SEQ mass spectrometer (Fisons, Manchester, U.K.) of EBqQ design or a CONCEPT II HH four-sector mass spectrometer (Kratos Analytical Ltd., Manchester, U.K.) of EBEB configuration with scanning-array detection (Chen *et al.*, 1994). The samples were ionized using a cesium ion gun and analyzed in both the positive and negative ion modes. The phospholipids were dissolved in chloroform/methanol/water (58:4:0.2 v/v/v), and then 1–2 μL of the solution (containing approximately 5–10 μg of sample) was transferred to the surface of diethanolamine liquid matrix, without mixing (Chen *et al.*, 1989).

RESULTS

In the present paper, the molecular species of the four major phospholipids from rainbow trout liver have been characterized by a combination of complementary physicochemical methods, including ^{31}P nuclear magnetic resonance (^{31}P NMR), gas chromatography (GC), LSIMS, and LSIMS combined with tandem mass spectrometry (MS/MS). A very high content of 22:6 ω -3 fatty acid-

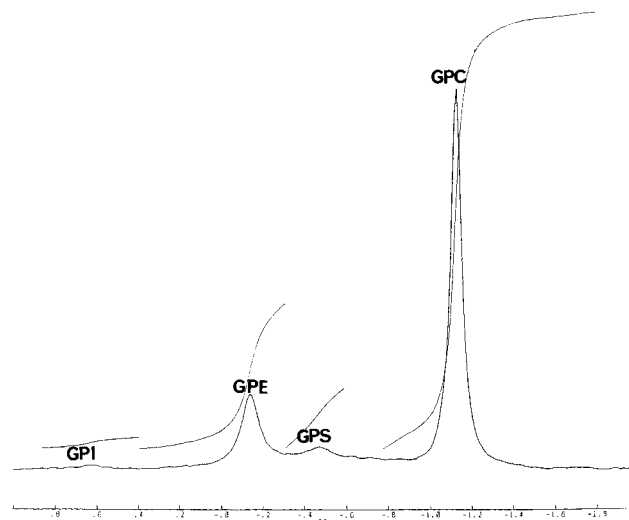


Figure 1. ^{31}P NMR spectrum obtained for glycerophospholipids isolated from rainbow trout.

Table 1. Distribution (Percent of Total Fatty Acid) of Fatty Acids in Rainbow Trout Liver Phospholipids

fatty acid	GPC ^a	GPE	GPS	GPI
16:0	31.0 \pm 0.24 ^b	17.3 \pm 0.3	22.7 \pm 0.2	36.5 \pm 0.4
16:1 n -7	5.0 \pm 0.3			
18:0	5.1 \pm 0.1	10.4 \pm 0.2	26.7 \pm 0.1	26.8 \pm 0.3
18:1 n -9	9.8 \pm 0.3	19.6 \pm 0.2	14.6 \pm 0.2	11.5 \pm 0.2
18:2	5.2 \pm 0.1	5.1 \pm 0.3		
20:1 n -11		7.8 \pm 0.1		
20:4 n -6				25.1 \pm 0.2
20:5 n -3	9.6 \pm 0.1	8.9 \pm 0.2	8.3 \pm 0.3	
22:6 n -3	34.2 \pm 0.4	31.1 \pm 0.3	27.4 \pm 0.3	
unidentified	5.1		1.1	

^a GPC, glycerophosphatidylcholine; GPE, glycerophosphatidylethanolamine; GPS, glycerophosphatidylserine; GPI, glycerophosphatidylinositol. ^b The results were obtained from GC analyses based on three replicate experiments of each phospholipid class.

containing molecular species within the GPC and GPE phospholipid classes could be demonstrated.

Distribution of Major Phospholipids and Fatty Acids within Phospholipid Classes. One hundred thirty milligrams of total lipids was obtained from a trout liver (1.9 g; wet material). In general, fish liver is 40–50% phospholipids (Gunstone *et al.*, 1986). Figure 1 illustrates the ^{31}P -NMR spectrum of phospholipids isolated from trout liver. The four major classes were qualitatively and quantitatively determined (Bardamante *et al.*, 1990) as GPC (67%), GPE (22%), GPS (10%), and GPI (1.6%). The distribution of the major fatty acids present in the four lipid classes is given in Table 1 (Turner *et al.*, 1989).

Identification of the Molecular Species. *Glycerophosphatidylcholine.* The molecular weights of the GPC species were inferred from their protonated molecules ($[\text{M} + \text{H}]^+$), produced by LSIMS (Fenwick *et al.*, 1983), and from their triple anions ($[\text{M} - 15]^-$, $[\text{M} - 60]^-$, and $[\text{M} - 86]^-$), which are generated by negative ion LSIMS (Munster *et al.*, 1986). The two acyl chains in a single molecular species were identified by the presence of carboxylated anions ($[\text{R}_1\text{COO}]^-$ and $[\text{R}_2\text{COO}]^-$), which are produced by CID of the $[\text{M} - 15]^-$ ions (Jensen *et al.*, 1986), or of $[\text{M} - 86 - \text{R}_2\text{COOH}]^-$ and $[\text{M} - 86 - \text{R}_2\text{CO} - \text{H}]^-$ product ions, which are formed by CID of the $[\text{M} - 86]^-$ ions (Huang *et al.*, 1991; Chen *et al.*, 1992). The positions of two acyl chains were determined on the basis of relative abundance differ-

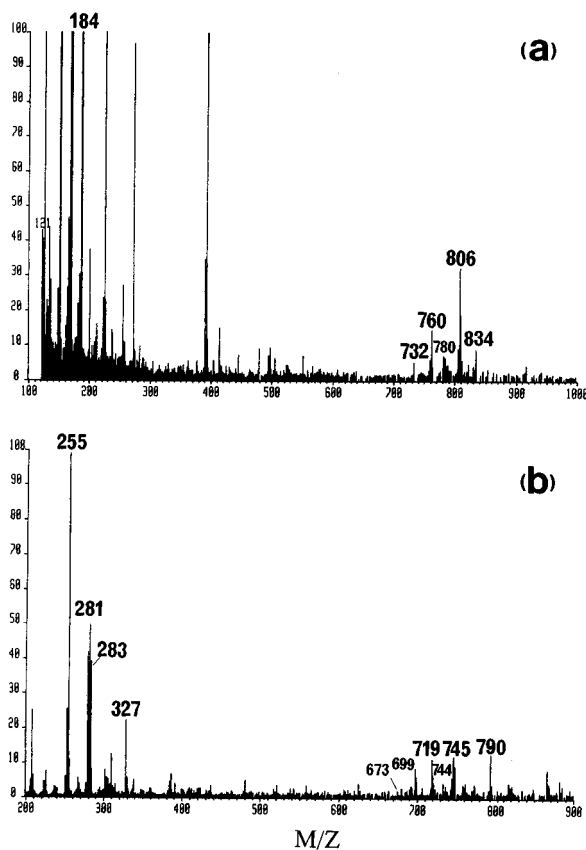


Figure 2. Conventional positive ion (a) and negative ion (b) liquid secondary ion mass spectra of rainbow trout liver. The spectra were recorded with a VG70SEG mass spectrometer.

ences between these anions. Figure 2 illustrates conventional positive ion (a) and negative ion (b) mass spectra of trout liver GPC. We identified nine molecular species (see Table 2), including three containing 22:6 fatty acid residue. Figure 3 shows the CID spectrum obtained for the $[M - 15]^-$ ion (m/z 795) of a GPC molecular species. Peaks at m/z 255 and 327 correspond to palmitic (16:0) and docosahexaenoic (22:6) carboxylate ions, respectively. Docosahexaenoic acid was found to be esterified at the *sn*-2 position because the signal intensity of the peak at m/z 327 is higher than that of the m/z 255 peak (Jensen *et al.*, 1986). This molecular species was thus identified as GPC 16:0-22:6 (see Table 2 for the designation of phospholipid molecular species). Table 2 lists the trout liver GPC species identified by

this method. The fatty acid residues in several minor species, 36:6, 34:2, and 32:1, could not be identified by tandem mass spectrometry because of lower intensities of their parent ions.

Glycerophosphatidylethanolamine. The deprotonated ($[M - H]^-$) and protonated ($[M + H]^+$) molecules of GPE, formed by negative ion and positive ion LSIMS, respectively, provided the molecular weights of the different molecular species. The two acyl chains in a GPE species were identified by the $[R_1COO]^-$ and $[R_2COO]^-$ anions, which are obtained by CID of the $[M - H]^-$ ions (Jensen *et al.*, 1987; Kayganich and Murphy, 1992), or of $[R_1COOCH_2CH]^+$ and $[R_2COOCHCH_2]^+$ as well as $[R_1COOCH_2CHOCH_2 + H]^+$ and $[R_2COOCHOCH_2CH_2 + H]^+$ peaks, which are formed by CID of the $[M + H]^+$ ions (Chen and Li, 1994). The relative abundances of $[R_2COO]^-$ and $[R_2COOCHCH_2]^+$ product ions, which are higher than those of $[R_1COO]^-$ and $[R_1COOCH_2CH]^+$ ions, allow for the determination of the positions of the two acyl chains (Kayganich and Murphy, 1992). Figure 4 shows the conventional negative ion LSIMS spectrum obtained for trout liver GPE. The molecular weights of the various species were inferred from the deprotonated molecules (Table 3). Figure 5 illustrates the CID spectrum obtained for the $[M - H]^-$ ions (m/z 762) of a molecular species (Figure 4). The two fatty acyl chains were characterized on the basis of the ions at m/z 255 (palmitic acid; 16:0) and 327 docosahexaenoic acid; 22:6). The higher signal intensity of the peak at m/z 327 ($[R_2COO]^-$) compared to that of the ion at m/z 255 ($[R_1COO]^-$) indicates that the 22:6 fatty acid is esterified at the *sn*-2 position (Kayganich and Murphy, 1992). This lipid species was thus identified as GPE 16:0-22:6. The other molecular species of trout liver GPE, analyzed using this mass spectrometric method, are listed in Table 3.

Glycerophosphatidylserine and Glycerophosphatidylinositol. The conventional negative ion and positive ion LSIMS analyses of GPS resulted in the formation of deprotonated and protonated molecules of molecular species (Jensen *et al.*, 1986; Chen *et al.*, 1990b). The composition and position of the fatty acyl groups in a GPS species were readily inferred from the $[R_1COO]^-$ and $[R_2COO]^-$ product ions, which are formed by CID of the $[M - H]^-$ ions, and from the relative abundance differences between the two anions, respectively. The relative abundance of the carboxylate product ion derived from cleavage at the *sn*-2 position ($[R_2COO]^-$)

Table 2. Molecular Species of Rainbow Trout Liver GPC As Determined by LSIMS and MS/MS

$[M + H]^+$	$[M - 15]^-$	$[M - 60]^-$	$[M - 86]^-$	CID of $[M - 15]^-$		molecular species ^a
				$[R_2COO]^-$	$[R_1COO]^-$	
834						40:6 (18:0-22:6)*
820						39:6*
806	790	745	719	327 (100) ^b	255 (35)	16:0-22:6**
804						38:7 (16:1-22:6)*
780						36:5 (16:0-20:5)*
760	744	699	673	281 (100)	255 (32)	16:0-18:1**
758						34:2 (16:0-18:2)*
732						32:1 (16:0-16:1)*

^a Abbreviations used to designate phospholipid species: *X:Y* (for example 40:6), where *X* is the total carbon number of the fatty acids esterified at *sn*-1 and *sn*-2 positions and *Y* is the total number of unsaturation degrees of fatty acid groups. The code *X:Y* is usually used for expressing general information on fatty acids in the molecular species, which is obtained by conventional LSIMS or other ionization methods. *n:m-N:M* (for example, 16:0-22:6), where *n* is the total number of carbons in the *sn*-1 fatty chain, *m* is the total number of double bonds in the fatty acid esterified at the *sn*-1 position, *N*, is the total number of carbons in the *sn*-2 fatty chain, and *M* is the total number of double bonds in the fatty acid esterified at the *sn*-2 position. The *n:m-N:M* code is often used for indicating the positions of the two fatty chains in the glycerol backbone, which can be determined by tandem mass spectrometry. *, obtained by conventional mass spectrometry. **, obtained by tandem mass spectrometry. ^b Relative abundance.

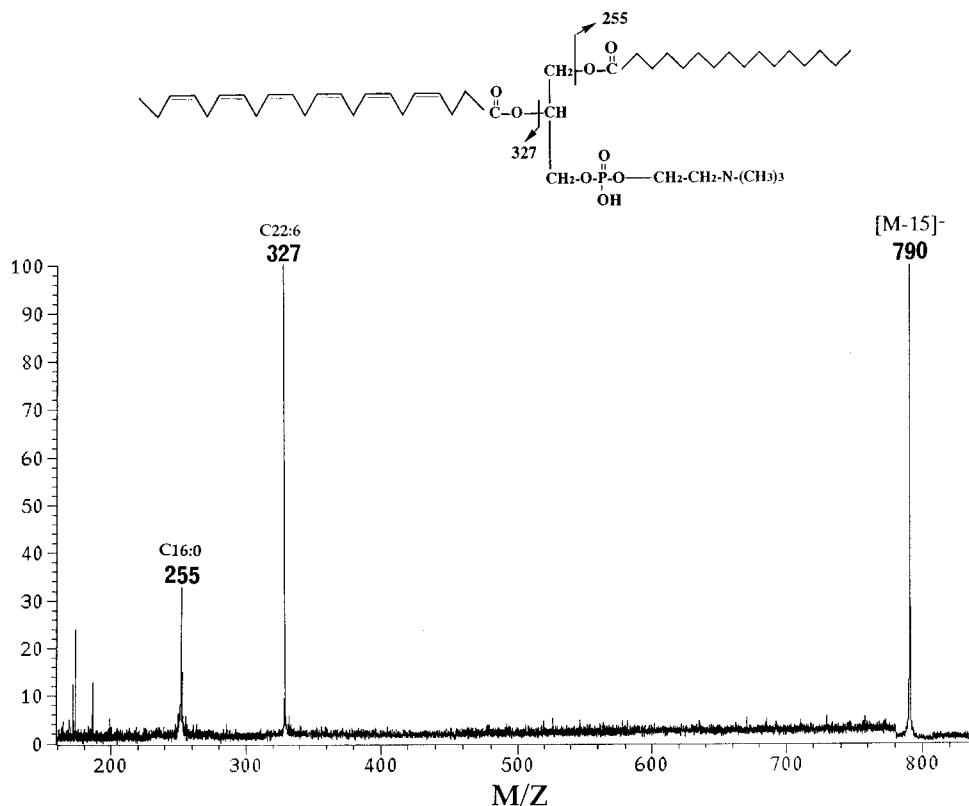


Figure 3. CID spectrum obtained for the $[M - 15]^-$ ion of GPC 16:0-22:6, generated by LSIMS. The MS/MS analysis was performed with a Kratos CONCEPT II HH mass spectrometer.

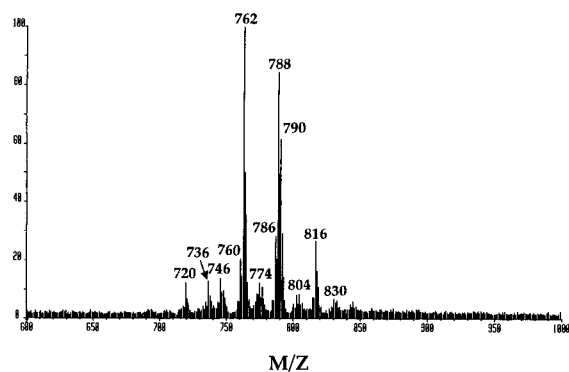


Figure 4. Conventional negative ion liquid secondary ion mass spectrum obtained for GPE isolated from rainbow trout liver. The spectrum was recorded with a VG70SEQ mass spectrometer.

is found to be lower than that of the anions resulting from fragmentation at the *sn-1* position ($[R_1COO]^-$). This conclusion is based on the results obtained by CID MS/MS of $[M - H]^-$ ions of GPS 18:0-18:1 and GPS 18:1-18:0 isomers (Chen and Li, 1996) and bovine brain GPS 18:0-18:1 (Jensen *et al.*, 1986). Although the product ions enabling characterization of the fatty acid residues in a GPS species can be also generated by CID MS/MS of its $[M + H]^+$ ions, the negative ion method appears to be at least 10 times more sensitive than the positive ion method for the analysis of natural GPS (Chen and Li, 1994).

GPS and GPI appear to be minor phospholipid classes in trout liver; the molecular species are listed in Table 3. Figure 6 shows the CID spectrum obtained for the $[M - H]^-$ ion (at m/z 834) of GPS 40:6. The presence of a serine group in this molecule is supported by a signal at m/z 747 $[M - 88]^-$. The two fatty acid residues were characterized by product ions at m/z 283

Table 3. Molecular Species of Rainbow Trout Liver GPE As Determined by LSIMS and MS/MS

$[M - H]^-$	CID of $[M - H]^-$		molecular species ^a
	$[R_2COO]^-$	$[R_1COO]^-$	
830			43:7*
816	327 (100) ^b	309 (35)	20:1-22:6**
804			41:6*
802			41:7*
790	327 (100)	283 (40)	18:0-22:6**
788	327 (100)	281 (50)	18:1-22:6**
786	327 (100)	279 (45)	18:2-22:6**
776			39:7*
774			39:8*
762	327 (100)	255 (39)	16:0-22:6**
760			38:7*
746			p-38:6 (p-16:0-22:6) ^{c,*}
736			36:5 (16:0-20:5)*
720			p-36:5 (p-16:0-20:5)*

^a See footnote a in Table 2. *, obtained by conventional mass spectrometry. **, obtained by tandem mass spectrometry. ^b Relative abundance. ^c Plasmalogen molecular species.

(stearic acid; 18:0) and 327 (docosahexaenoic acid; 22:6), of which the 22:6 residue is present at the *sn-2* position. This species was thus identified as GPS 18:0-22:6. Other molecular species of trout liver GPS were 16:0-22:6 and 18:1-22:6 (see Table 4).

GPI was found to lack 22:6 fatty acid-containing molecular species (spectrum not shown). Two abundant GPI species in trout liver were 16:0-22:4 (m/z 858) and 18:0-20:4 (m/z 868), which were characterized on the basis of $[M - H]^-$ ions, formed by negative ion LSIMS (see Table 4).

DISCUSSION

Marine fish oil has been widely used in nutritional and medical studies because it is the only concentrated source of 22:6 ω -3 fatty acid (Turner *et al.*, 1989; Bell

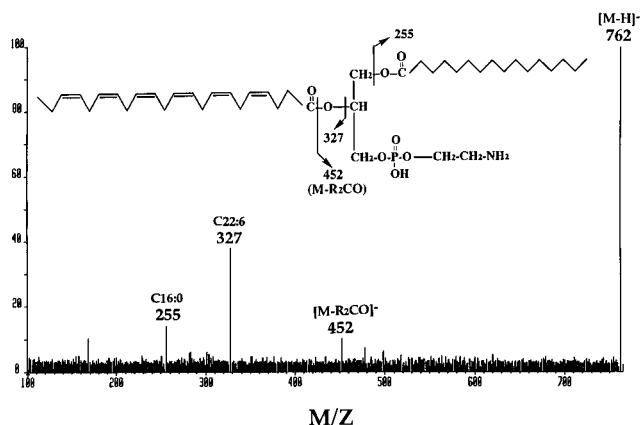


Figure 5. CID spectrum obtained for the $[M - H]^-$ ion of GPE 16:0-22:6, generated by LSIMS. The spectrum was recorded in a VG70SEQ mass spectrometer.

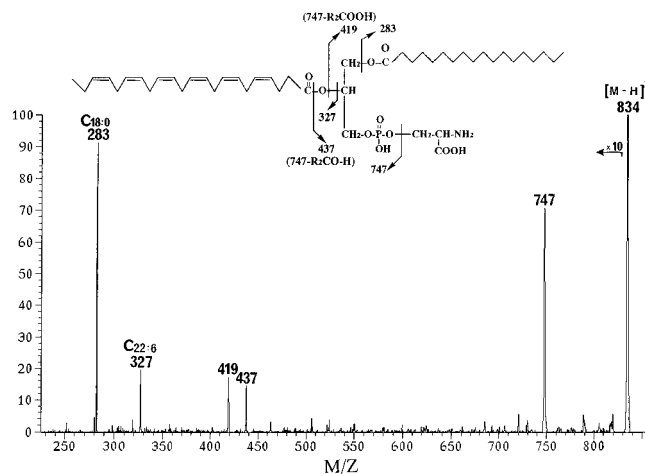


Figure 6. CID spectrum obtained for the $[M - H]^-$ ion of GPS 18:0-22:6, generated by LSIMS. The MS/MS analysis was performed with a Kratos CONCEPT II HH mass spectrometer.

Table 4. Major Molecular Species of Rainbow Trout Liver GPS and GPI As Determined by LSIMS and MS/MS

$[M - H]^-$	CID of $[M - H]^-$		molecular species ^b
	$[R_2COO]^-$	$[R_1COO]^-$	
834	327 (15) ^a	283 (100)	GPS 18:0-22:6**
832			GPS 40:7 (18:1-22:6)*
806	327 (17)	255 (100)	GPS 16:0-22:6**
804			GPS 38:7 (16:1-22:6)*
886			GPI 38:4 (18:0-20:4)*
858			GPI 36:4 (16:0-20:4)*

^a Relative abundance. ^b See footnote ^a in Table 2. *, obtained by conventional mass spectrometry. **, obtained by tandem mass spectrometry.

and Tocher, 1989). Because of the high content of 22:6 ω -3 fatty acid in fish oil, it is logical to assume that fish organs could also serve as a source of phospholipids species containing 22:6 ω -3 fatty acid. To our knowledge, the detailed molecular characterization of fish liver phospholipids has been performed only to a limited extent (Turner *et al.*, 1989).

Recently, Dey *et al.* (1993) have reported the molecular species characterization of liver phospholipids from marine and freshwater fish which were adapted to different temperatures. The content of 22:6 ω -3 fatty acid in the phospholipids of freshwater fish liver was found to be approximately 30%, and 22:6 ω -3 fatty acid-containing molecular species of the phospholipids were identified as GPC 16:0-22:6, GPE 16:0-22:6, GPE 18:0-

22:6, and GPE 18:1-22:6. This study also shows that GPE 18:0-20:4, GPE 16:0-18:1, and GPE 16:0-16:0 are major species in freshwater fish liver and that 18:0-20:4 and 16:0-18:1 species are absent from trout liver GPE.

The results obtained in the present study indicate that rainbow trout liver has a very high content of phospholipid species containing 22:6 ω -3 fatty acid at the *sn*-2 position, particularly GPC and GPE. Although bovine brain GPE also contains a high concentration of 22:6 ω -3 fatty acid-containing molecular species, the level of plasmalogen species with 22:6 fatty acid is relatively high.

Thin-layer chromatography coupled with phosphorus assay and high performance liquid chromatography and enzymatic treatment followed by GC are the classical approaches for the quantitative determination of phospholipid classes and for the structural identification of their molecular species from biological materials, respectively. In the present study, we have used a more direct, instrumental, mass spectrometric approach, which has the advantages of being less laborious and offering more specificity.

In conclusion, rainbow trout liver is a highly enriched natural resource of 22:6 ω -3 fatty acid-containing GPC and GPE molecular species. However, bovine brain is a more suitable biological material for obtaining GPS molecular species with 22:6 ω -3 fatty acid (Yabuuchi and O'Brien, 1968).

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