Phospholipids of marine origin: the orange roughy (*Hoplostethus atlanticus*)

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Fillets of deep-skinned orange roughy (*Hoplostethus atlanticus*) were found to contain 5.46% total lipids consisting of as much as 93% non-digestible wax esters. The fillets therefore act as a mild laxative, which probably contributes to their popularity on the American and Japanese markets. In addition, the fillets had remarkably low levels of cholesterol and phospholipids (seemingly irreplaceable constituents of cell membranes) of 0.033% and 0.17%, respectively. These concentrations are much lower than those of pelagic fish and it is not clear what replaces them in the membranes of this long-lived, deep-water fish. The phospholipid composition was, however, similar to that of other fish species, consisting of 51% phosphatidylcholine, 22% phosphatidylethanolamine, 10% sphingomyelin, 7% cardiolipins, 5% phosphatidylinositol, 3% phosphatidylserine and 2% lyso phosphatidylcholine. The non-phosphorylated lipids consisted of 96.3% wax esters, 1% cholesterol esters, 0.6% free fatty acids, 2% triacylglycerols and 0.1% free cholesterol. The fatty acid compositions of the phospholipids and the non-phosphorylated lipids were different, the phospholipids having a high level of docosahexaenoic acid (C22:6 *n*-3) of 40.4%, whereas the non-phosphorylated lipids had a high level of oleic acid (C18:1 *n*-9) of 58.3%. Almost two thirds of the alcohols of the wax esters from the non-phosphorylated lipids consisted of mono-unsaturated fatty alcohols (C14:1 to C24:1), whereas the remaining one third were saturated alcohols (C16:0 to C24:0).

Introduction

The orange roughy (*Hoplostethus atlanticus*) is a long-lived, deep-water fatty fish that inhabits the oceans at depths of 800–2000 m and that can reach an estimated age of 150 years.[1] For spawning purposes, dense aggregations gather at the tops of sea mounts ('hot spots'). It was extensively exploited in New Zealand and Tasmania in the 1980s and is now caught also off the coasts of Namibia and Madagascar.[2,3] The annual catch in Tasmania was more than 34 000 tonnes in 1989 but has since declined to approximately 7000 tonnes.[2] Only about one third of the fish is edible and is sold as frozen fillets on the American, European and Japanese markets, where it is popular because of its bright, attractive appearance and firm texture.[4] The remaining two thirds, consisting of frames, heads, swim bladders and skins, is treated as waste material, sometimes being used simply as fertilizer.[2] The orange-coloured oil comprising about 18% of the whole fish consists largely of liquid wax esters. It has found limited use as a degreasing solvent but most of it is exported to Japan for use in cosmetics and lubricating oils in the steel industry.[2,5]

Orange roughy press oil has been thoroughly examined in Tasmania,[2] New Zealand[5,6] and Japan.[7] That work was concerned mainly with the composition of the neutral lipids; the only attempt at analysing more complex lipids was that of Body,[8] who determined the composition of the lipids extracted from the roe of the orange roughy. The complete composition of orange roughy phospholipids is not available in the literature, so we decided to determine this in our institute. It seemed especially interesting to establish whether the phospholipid composition of the fish differed from that of non-deep-water species such as pilchard (*Sardina ocellata* Jenyns) and hake (*Merluccius capensis* Castelnau), whose phospholipid composition was determined in our laboratory.[9–11]

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Experimental

General. Deep-skinned orange roughy fillets packed in polythene bags were transported from a Namibian fishing factory by air in frozen condition to our laboratory and stored at –40°C until subjected to lipid extraction and analysis, which took place within one week.

Chemical analyses. Phosphorus was determined according to Bartlett's method[12] and the water-soluble compounds choline, ethanolamine, serine and *myo*-inositol according to procedures developed in our institute.[13–15] The determination of the proportions of choline in phosphatidylcholine (lecithin), sphingomyelin and lyso-phosphatidyl choline (lysolecithin) was accomplished by chromatography on silicic acid-impregnated paper followed by staining with Edicol Supra Ponceau and spectrophotometry at 510 nm.[10]

The free fatty acid (FFA) content was determined by titration with 0.02 M aqueous sodium hydroxide in hot neutralized ethanol using phenolphthalein as indicator and the result expressed as percentage oleic acid. FFA content was corrected for the presence of acidic phospholipids and converted into genuine FFA content after phosphorus determination.[16]

Free and esterified cholesterol were determined by gas chromatography as described elsewhere.[17,18] Glycerol was determined as its tri-*o*-trimethylsilyl ether using *meso*-erythritol as internal standard according to a recently published procedure.[19]

Thin-layer chromatography. Thin-layer chromatography (TLC), used for identification of the lipids, was carried out on small glass plates (4 cm × 8 cm) coated with silica gel 60 (Merck, Kieselguhr F 254). Neutral lipids were separated into classes in the solvent system hexane:diethyl ether:acetic acid (85:15:1). They were visualized by dipping the plates into an aqueous solution of 30 g cupric acetate and 80 g phosphoric acid per litre, followed by heating in an oven for 30 min at 120°C. Wax esters and cholesterol esters had a R_f value of approximately 0.80, triacylglycerols 0.55, FFA 0.20, free cholesterol 0.10, whereas phospholipids remained at the origin.[20] Phospholipids were separated with the solvent system choroform:methanol:water (25:10:1), cardiolipins had an R_f value of about 0.90, phosphatidylethanolamine, phosphatidylserine plus phosphatidylinositol 0.70, phosphatidylcholine 0.30, sphingomyelin 0.20 and lyso-phosphatidylcholine 0.10. Suitable standards for characterization of the lipids were from Sigma (St Louis).

Extraction and purification of the lipids. A composite sample of 498 g orange roughy fillets was extracted three times with one litre of chloroform:methanol (2:1 volume ratio) in a Waring blender. The combined extracts were washed three times in a large volume of water and the chloroform solution dried over anhydrous sodium sulphate.[21] After filtration by gravity and evaporation of the chloroform on a rotary evaporator, the lipids were dried on a freeze drier, resulting in 27.2 g (5.46%) of straw-coloured oil with a phosphorus content of 0.11%. This yellow oil solidified to an off-white waxy substance in the refrigerator.

Separation of the phospholipids from the non-phosphorylated lipids. Orange roughy lipids (21.6 g; %P 0.11) were separated into non-phosphorylated lipids 96.9% (20.9 g, %P 0.005) and phospholipids 3.1% (0.68 g, %P 3.6) by chromatography on a column (180 mm × 32 mm) of 45 g of silicic acid (Merck), activated overnight at 120°C according to the procedure of Nevenzel *et al.*[22] The non-phosphorylated lipids were eluted with 500 ml of hexane:diethyl ether (9:1 v/v) and the phospholipids with 250 ml methanol. The phospholipids moved as a yellow band down the column and, after evaporation of the methanol on a rotary evaporator, were isolated as a honey-coloured flaky material, while the non-phosphorylated lipids appeared as a colourless oily liquid.

Determination of the lipid composition

Phospholipids

The phospholipid composition was determined by qualititative and quantitative analysis of choline, ethanolamine and serine obtained after

hydrolysis in 2 M hydrochloric acid at 120°C for 48 h as described previously.[10,13] *Myo*-inositol was determined by gas chromatography using scyllitol as internal standard as described elsewhere.[15] The complete composition of the phospholipids could be calculated using the average molecular weight of the fatty acids (see below). Cardiolipins (those phospholipids not containing a nitrogenous base or *myo*-inositol) were qualitatively identified by TLC as described by Christie,[20] whereas their quantitative estimation was accomplished by subtracting the sum of all other phospholipid classes from 100%.

Non-phosphorylated lipids

These lipids were separated into a major fraction (97.6%) consisting of wax and cholesterol esters and a minor fraction consisting of triacylglycerols, FFA and free cholesterol by chromatography on a column (180 mm × 32 mm) of 45 g of activated silicic acid. The major fraction was eluted with hexane:diethyl ether (99:1 v/v) and the minor fraction with hexane:diethyl ether (95:1 v/v).

Fatty acids and fatty alcohols

The fatty acid and fatty alcohol composition of the various lipids was determined by gas chromatography of their respective methyl esters and acetates on a Hewlett Packard 6890 gas chromatograph. Methyl esters were prepared by refluxing a portion (0.1–0.3 g) of the lipids with 5 ml of 0.5 M sodium hydroxide in methanol for 30 min in the presence of some boiling chips. Subsequently, 5 ml of a 15% solution of boron trifluoride in methanol was added and refluxing continued for another 5 min, whereupon 5 ml of hexane was added and the mixture refluxed for another 5 min.

The flask was removed and sufficient saturated aqueous sodium chloride was added to raise the hexane solution into the neck of the flask. The methyl esters in the hexane were removed with a Pasteur pipette and injected into the gas chromatograph. The methyl esters were separated on a 50 m × 0.32 mm Machery Nagel Permabond capillary column using hydrogen as carrier gas. The temperature was programmed from 150°C to 280°C rising at 4°C/min, while the injection port remained at 250°C and the detector at 300°C.[20] The average molecular weight of the fatty acids was calculated from their composition.

Fatty alcohols of the waxes were converted into their acetates by refluxing a small amount (0.1–0.3 g) with 10 ml of 1 M ethanolic potassium hydroxide for 30 min, whereupon 10 ml of water was added and the refluxing continued for another 10 min. After cooling to room temperature, 20 ml of diethyl ether was added, the mixture shaken and sufficient saturated aqueous sodium chloride added to let the ether rise into the neck of the flask. The ether solution was removed with a Pasteur pipette and filtered over anhydrous sodium sulphate into a dry 100 ml quickfit flask. The ether was evaporated on a rotary evaporator and 2 ml of dry pyridine and 1 ml of acetic anhydride were added. The flask was loosely stoppered and kept at 100°C for approximately 1 hour. The excess acetic anhydride and pyridine were removed in a stream of nitrogen while the flask was gently heated in a flame. The residue was taken up in about 3 ml of hexane and injected into the gas chromatograph. The gas chromatographic conditions were identical to those applied for the analysis of fatty acid methyl esters. Suitable standards for characterization of the fatty acids and alcohols were from Sigma.

Results and discussion

Lipid yield, Orange roughy fillets contained 5.46% lipids and approximately 93% of these lipids were non-digestible wax esters. The fillets therefore act as a laxative, similar to butterfish (*Lepidocybium flavobrunneum*), an oily fish that contains as much as 20% total lipids consisting of wax esters. Eating it causes severe diarrhoea, although the symptoms are relatively mild.[22] The mild laxative properties of the orange roughy are possibly one of the reasons the fish is held in such high esteem on American and Japanese markets.

The phospholipid content of the orange roughy fillets was only 0.17%, a value much less than that of hake flesh (1.5%) or of whole pilchard (0.9%).[9,10] Lovern, in reviewing the phospholipid content of sixteen edible fish species, concluded that compared with the large variations in triacylglycerol storage lipids, fish flesh phospholipids are reasonably constant. He quotes values for oily fish of between 0.7% and 0.8%, while non-fatty fish have somewhat lower values of between 0.5% and 0.6%.[23] Our work therefore shows a substantial difference between the phospholipid content of orange roughy, a deep-water fish virtually devoid of triacylglycerols, and other edible, non-deep-water fish species. The scarcity of glycerol in orange roughy as evidenced by low content of phospholipids and the virtual absence of triacylglycerols seems a peculiar characteristic of this deep-water fish. As phospholipids are an essential component in the structure of membranes, it is unclear what replaces them in the orange roughy muscle. This does not, however, apply to orange roughy roe as Body[8] found this contained as much as 1.5% phospholipids and 2.8% triacylglycerols.

Table 1. Main constituents of orange roughy (*Hoplostethus atlanticus*) phospholipids and their calculated composition.

Constituent	Percentage of total phospholipids
P	3.6
Total choline	9.14
Lecithin-choline	7.30
Sphingomyelin-choline	1.49
Lysolecithin choline	0.35
Ethanolamine	1.70
Serine	0.39
Myo-inositol	1.00
Calculated composition[a]	
Phosphatidylcholine (lecithin)	51
Phosphatidylethanolamine	22
Phosphatidylserine	3
Phosphatidylinositol	5
Lyso-phosphatidylcholine (lysolecithin)	2
Sphingomyelin	10
Cardiolipin[b]	7

[a]Quoted as whole numbers.
[b]Obtained by difference.

Phospholipid composition. Table 1 records the main constituents of the orange roughy phospholipids. The values of these together with the average molecular weight (303) of the fatty acids from the phospholipids allow the computation of its composition, which is also shown in the table. The phospholipid composition of the orange roughy was similar to that of other fish examined in our institute such as the pilchard, in that 51% consisted of phosphatidylcholine and 22% phosphatidylethanolamine compared with 53% and 25%, respectively, for the pilchard.[11] The minor amounts of 2%, 3%, 5% and 7% of lysophosphatidylcholine, phosphatidylserine, phosphatidylinositol and cardiolipins, respectively, were almost identical to those of pilchard phospholipids. The sphingomyelin content of the orange roughy phospholipids of 10% was somewhat higher than the 6% found in the pilchard.[9,11] This is probably also related to the scarcity of glycerol in the orange roughy, since sphingomyelin is not a glycerophospholipid.

Non-phosphorylated lipid composition. The composition of these lipids is recorded in Table 2. They consisted mainly (96.3%) of wax esters together with a small amount (1%) of cholesterol esters. The remainder consisted of triacylglycerols (2%), FFA (0.6%) and a small quantity of free cholesterol (0.1%). This composition differs considerably from that recorded for the non-phosphorylated lipids of orange roughy roe by Body,[8] who found they contained large amounts of triacylglycerols (69.5%), minor amounts of wax esters (4%) together with cholesterol esters (7%), FFA (1%) and a substantial amount of free cholesterol (18.5%).

The total cholesterol content of the orange roughy fillets was very low at 0.033 %, which is a quarter of the average value of 0.13% found for anchovy (*Engraulis capensis*), red eye (*Etrumeus whiteheadii*), maasbanker (*Trachurus trachurus*), pilchard (*Sardina*

Table 2. Analysis of orange roughy (*Hoplostethus atlanticus*) non-phosphorylated lipids and their calculated composition.

Constituent lipids	Percentage of non-phosphorylated lipids
Total cholesterol	0.66
Free cholesterol	0.10
Cholesterol as cholesterol ester	0.56
Free fatty acids	0.6
Glycerol	0.2
Class	
Wax esters	96.3
Cholesterol ester	1.0
Free cholesterol	0.1
Free fatty acids	0.6
Triacylglycerols	2.0

ocellata Jenyns), and lantern fish (*Lampanyctodes hectoris*), all non-deep-water fatty fish caught in South African waters.[18] As cholesterol, like phospholipids, is an essential component of membrane structures, it is also at present unknown what replaces it in orange roughy tissues. The cholesterol content of orange roughy roe calculated from the work of Body[8] is 0.91%, a value much higher than that of the orange roughy fillets.

Fatty acid and fatty alcohol composition. The fatty acid compositions of the total lipids, phospholipids and non-phosphorylated lipids of the orange roughy are recorded in Table 3. A striking feature of the fatty acid compositions of the total fillet lipids and the non-phosphorylated lipids is the abundance of mono-unsaturated fatty acids, ranging from palmitoleic acid (C16:1) to nervonic acid (C24:1). In particular, the high oleic acid (C18:1) content of 58.3% in the non-phosphorylated lipids is remarkable. In complete contrast, the phospholipid fraction had much less of these mono-unsaturated fatty acids, having only 14.1% of oleic acid (C18:1), but containing a high level of 40.4% of docosahexaenoic acid (DHA; C22:6 *n*-3). This is the highest level of DHA encountered in any of the phospholipids of marine origin analysed in our institute. Curiously, the level of eicosapentaenoic acid (EPA; C20:5 *n*-3) was only 4.7%, which is low compared to values of 13% and 9% of pilchard and hake flesh phospholipids respectively.[9,10]

The fatty alcohol distribution in orange roughy non-phosphorylated lipids is recorded in Table 4. This clearly shows that the major portion (65.3%) comprised the mono-unsaturated alcohols, from palmitoleyl alcohol (C16:1) to nervonyl alcohol (C24:1), the most abundant being eicosanol (C20:1) at 21.1%. The saturated alcohols were present in smaller amounts with palmityl alcohol (C16:0) as the predominant alcohol at 20.7%. Finally, the presence of only 0.5% of linoleyl alcohol (C18:2) should be noted. The composition of the fatty alcohols of the non-phosphorylated lipids is in complete agreement with that of the fatty alcohols of orange roughy press oil recorded by Tagaki, Itabashi and Aso.[7]

Table 3. Fatty acid distribution in orange roughy (*Hoplostethus atlanticus*) fillet total lipids, phospholipids and non-phosphorylated lipids (as percentage of the corresponding methyl esters).

Fatty acid	Total lipids	Phospholipids	Non-phosphorylated lipids
14:0	0.8	0.4	0.8
16:0	2.4	16.4	1.4
18:0	0.7	6.2	0.5
16:1	10.1	1.9	10.4
18:1 *n*-9	53.0	14.1	58.3
18:1 *n*-7	3.9	2.3	0.3
20:1	11.9		12.8
22:1	5.1		5.1
24:1	1.3	0.4	1.3
18:4		0.3	
18:2	0.8	0.8	0.8
20:4 *n*-6	0.8	5.5	0.8
20:5 *n*-3	2.2	4.7	2.1
20:4 *n*-3	0.5	3.9	0.5
22:6 *n*-3	6.0	40.4	4.4
22:5 *n*-3	0.5	2.7	0.5

Table 4. Fatty alcohol distribution in orange roughy (*Hoplostethus atlanticus*) non-phosphorylated lipids (as percentage of the corresponding acetates).

Fatty alcohol	Non-phosphorylated lipids
14:0	1.3
15:0	2.9
16:0	20.7
18:0	8.0
20:0	0.9
24:0	0.4
16:1	1.1
18:1	16.5
20:1	21.1
22:1	18.8
24:1	7.8
18:2	0.5

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