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Lipid composition of the liver oil of the ray, *Himantura bleekeri*

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Abstract:

If rays are traditionally fished for their caudal fins, the rest of the body is wasted, except part of the skin that can be transformed into leather. Liver oil of the ray, Himantura bleekeri, was characterized in terms of lipid class composition and fatty acid profile. Liver oil content was high and represented 54% of the liver weight (w/w). Neutral lipids were predominant (92%), major components being triacylglycerols (63%). Other neutral lipids identified were hydrocarbons, sterol esters, mono- and di-acylglycerols, free fatty acids, sterols and glyceryl ethers. Polyunsaturated fatty acids of the n−3 series, namely eicosapentaenoic and docosahexaenoic acids, were high (4% and 16%, respectively). Cholesterol was the major component in the sterol fractions (free or esterified). Hydrocarbons were up to 30 carbons and squalene was present at the level of 22%. Thus, this liver oil proved to be an adequate source of n−3 fatty acids and other valuable lipidic compounds.

Keywords: Ray liver oil; Fatty acids; Lipids; PUFA; EPA; DHA; Squalene; Sterol

1. Introduction

The traditional Inuit diet was known to be rich in long-chain n-3 polyunsaturated fatty acids (PUFAs) and believed to account for the low incidence of cardiovascular disease in those populations (Mnari et al., 2007). This observation is the starting point of the increasing interest of these fatty acids (Bergé & Barnathan, 2005). Indeed, PUFAs and particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are nowadays recognized to prevent inflammatory, arrhythmias, hypertension and triacylglycerolemia, atherosclerosis, autoimmune disorders (Mnari et al., 2007) and to display a variety of beneficial effects in area ranging from foetal development to cancer prevention (Connor, 2000).

Fish oils are the main commercial sources of EPA and DHA. However, oils from elasmobranches (particularly the sharks) also contain large quantities of squalene, a 30-carbon isoprenoid. As this compound occurs naturally in the surface of the human skin (Navarro-Garcia, Pacheco-Aguilar, Vallejo-Cordova, Ramirez-Suarez, & Bolanos, 2000) it is currently used in cosmetics, pharmaceuticals and sunscreens (Wetherbee & Nichols, 2000).

Among elasmobranches, ray fishes are traditionally caught around the world. However only a few parts are eaten, the caudal fins and most of the rest is considered as waste (body, viscera, skin,…). Few authors have studied the lipid composition of those by-products notably liver and gonads and they have revealed the high proportion of lipids into (Pal, Banerjee, Patra, Patra, & Ghosh, 1998; Ould El Kebir, Barnathan, Siau, Miralles, & Gaydou, 2003; Navarro-Garcia, Bringas-Alvarado, Pacheco-Aguilar, & Ortega-Garcia, 2004a; Navarro-garcia, Pacheco-Aguilar, Bringas-Alvarado, & Ortega-Garcia, 2004b).

As indicated by Navarro-Garcia et al. (2004b), little information has been reported for ray liver oil. In the present investigation, the lipids and fatty acids of the liver of the commercial ray *Himantura bleekeri* were studied for the first time. This ray, belongs to the family *Dasyatidae* of the class *Elasmobranchii*. This cartilaginous fish lives in Indo-pacific ocean and its traditionally fished for flesh and skin (to produce leather) (Blyth, 1860).

Results are intent to be used to explore the commercial potential of such biomass as a source of marine oils and particularly PUFAs.

2. Materials and methods

2.1. Sample collection

Himantura bleekeri was collected on the east coast of India (Chennai), during February 2004. Liver was isolated and kept frozen (-18°C) until analysis.

2.2. Lipid extraction

Lipids were extracted following the Folch method (Folch, Lees, & Sloan Stanley, 1957). To 5 g of raw material, 33 ml of methanol were added and mixed for at least 30 min at room temperature. Then 66 ml of chloroform was added. Solution was stirred for 30 min. After eliminating the non-soluble part by filtration, 0.2 volumes of calcium chloride (0.9% in distilled water) were added. After decantation, in a separatory funnel (12 hours at 4°C), the organic phase was recovered, and the solvent evaporated. Lipid fractions were stored in chloroform at –20°C for future use (Figure 1)

2.3. Lipid class separation by Column chromatography

85mg of lipid was subjected to column chromatography using Chromabond SiOH column (Macherey-Nagel, Düren, Germany) as described by Dreyfus et al. (Dreyfus, Guerols, Freysz, & Hicks, 1997). The column was conditioned with 20 ml of chloroform. The neutral lipids, glyco-, and phospholipids were eluted by chloroform (20 ml), acetone/methanol (9:1, 30 ml) and methanol (20 ml), respectively. Solvents were evaporated and each class of lipids was estimated by weighing with a microbalance. All fractions were kept in chloroform at –20°C before use.

2.4. Saponification of lipid extract

The total lipid sample (10 mg) was refluxed with a 1M solution of potassium hydroxide in 95% ethanol (2 ml) for 1 hour (Christie, 1989c). The solution was cooled to room temperature then 5 ml of

water were added and the mixture was extracted thoroughly with hexane-diethyl ether (1:1, v/v; 3 x 5 ml). The organic phase was washed with water, dried over anhydrous sodium sulphate and the non-saponifiable materials were recovered by removing solvent in a rotary evaporator. The water washings were added to the aqueous layer, acidified by 6M hydrochloric acid and further extracted with diethyl ether- hexane (1:1, v/v; 3 x 5 ml). Free fatty acids (FFA) were recovered by washing extract with water, drying it over anhydrous sodium sulphate and removing solvents by evaporation. FFA were kept in chloroform à -20°C before use.

2.5. Neutral lipids components identification by thin layer chromatography

Analytical TLC was performed for neutral lipids fraction on 20 x 20 cm chromatoplates coated with silica gel G (0.25 mm thickness; Macherey-Nagel, Düren, Germany). Elutions were obtained by using hexane/ether/acetic acid (70:30:1, v/v/v) according to Mangold. (Mangold, 1961; Mangold, 1964).

Lipids compounds were visualized by spraying successively with two specific reagents: - firstly, with a primuline solution (5 mg in 100 ml of acetone/water, 80:20, v/v), which allows lipid spots identification under a UV lamp (Bergé, Gouygou, Dubacq, & Durand, 1995) - secondly, with a solution of sulphuric vanillin leading to colorized spots after heating slowly until 180°C during 20 min.

All the lipids were identified by comparing their retention factor (Rf) with those of authentic standards (SIGMA, St Quentin Fallavier, France).

2.6. Quantitative analysis of neutral lipid classes by thin layer chromatography

Preparative TLC were performed on 20 x 20 cm chromatoplates (0.5 mm thickness; Macherey-Nagel, Düren, Germany) with 50 mg of neutral lipid. Compounds were separated by using the same eluting system as analytical TLC. Spots were scraped in glass tubes and eluted from the silica by two washes (2 x 10 ml) with two solvent mixtures: hexane/diethyl ether (50:50 ; v/v), and hexane/chloroform/methanol (10:10:10 ; v/v/v). Proportions of compounds were directly estimated in weight after solvent removal.

2.7. Derivatisation of neutral lipid classes

2.7.1. Free fatty acids and those linked to glycerol

500 mg of lipid were evaporated under nitrogen and transmethylated by contact with methanol/sulphuric acid (98:2) in excess for one night at 50°C. After cooling, 1 ml of distilled water and 2 ml of hexane were added and vortexed. The upper organic phases containing fatty acid methyl esters (FAMEs) were collected and assayed by GC-FID and GC-MS. (Christie, 1993)

Picolinyl esters were prepared by treatment of FAME's with potassium tert-butoxyde and 3-hydroxymethylpyridine during 45 min at 45°C. Derivatives were extracted with 2ml of hexane and 1 ml of water. The upper organic phase was dried with sodium sulphate column. Derivatives were dried under a flush of nitrogen and dissolved into 1 ml of hexane for injection (Dubois, Barthomeuf, & Bergé, 2006).

2.7.2. Acetylation of sterols

2 ml of pyridine- acetic anhydride (2:1; v/v) were added to 500µg of isolated sterols at 45°C for 45 min. After cooling at ambient temperature, 2 ml of distilled water were added. Steryl acetates were extracted with 4 ml of hexane. The upper organic phase was evaporated under nitrogen and 1 ml of hexane was added. The derivatives were further analysed by Gas Chromatography-Mass Spectrometry (GC-MS).

2.8. Lipid identification by gas chromatography-mass spectrometry

Analysis of the lipid derivatives were performed by GC-MS on a Hewlett-Packard model 6890 series II gas chromatograph attached to an Agilent model 5973N selective quadripole mass detector. GC-MS was connected to a computer with Hewlett-Packard chemstation and the ionisation voltage used was 70 eV at 250°C. The temperature of injector and interface were maintained at 250°C and He was used as carrier gas under constant flow (1 $ml.min^{-1}$). Separation were realised on a CP-Sil 5 CB low bleed MS (60m x 0.25 mm i.d., 0.25 µm film thickness; Chrompack, Middleburg, The Netherlands).

FAMEs, picolinyl esters, acetates and hydrocarbons were analysed with various methods.

- For FAMEs oven temperature was programmed from 80 to 170°C at a rate of 30°C/min, then from 170 at 295°C with a rate of 3°C/min.
- Analysis of picolinyl esters were operated with an oven temperature linearly increasing from 220 to 270 °C at 0.4 °C/min.
- Sterols acetates were analysed with a linear programming. Oven temperature was programmed from 150 at 350°C at a rate of 3°C/min.
- For hydrocarbons (isolated by preparative TLC), oven temperature was programmed from 50 to 350°C at a rate of 2°C/min then kept constant at 350°C for 10 min.

2.9. Quantification of fatty acid from neutral lipids by gas chromatography-flame ionisation detector.

FAME's quantification was done by GC-FID using a gas chromatography (Auto system Gas Chromatography, Perkin Elmer) equipped with an auto-sampler and fitted with a split/splitless injector and flame ionisation detector. The separation was carried out with a BPX-70 capillary column (60m long, 0.25mm internal diameter, 0.25µm film thickness; SGE, Austinn, USA), containing a polar stationary phase (cyanopropyl-siloxane) (Berge, Debiton, Dumay, Durand, & Barthomeuf, 2002; Dumay, Barthomeuf, & Bergé, 2004).

3. Results and discussion

It is well known that rays like other elasmobranches store fat into liver (Kinsella, 1988). As expected, oil extraction from the liver of *Himantura bleekeri* confirmed that this organ is relatively rich in lipids (54% of the fresh matter). This is in accordance with previous studies on other elasmobranches' livers where lipid content ranged from 23 to 67% (Pal et al., 1998; Navarro-Garcia et al., 2000; Ould El Kebir et al., 2003; Navarro-Garcia et al., 2004a; Navarro-garcia et al., 2004b).

Neutral lipid was the major lipid class (92%), while glycolipids and phospholipids were present in relative low amounts (respectively 5% and 3%). Such distribution confirmed previous results obtained with liver oil of the ray *Dasyatis bleekeri* (Pal et al., 1998).

Composition of NL is presented in Table 1. Triacylglycerols (TAG) were found to represent about 2/3 of the NL. This predominance has been previously reported irrespective of the species of - rays 67% in *Dasyatis brevis* and 67% in *Gymnura marmorata* (Navarro-garcia et al., 2004b), 75% in *Rhinoptera steindechneri* (Navarro-Garcia et al., 2004a), 93% in *Dasyatis bleekeri* (Pal et al., 1998) or - sharks *G. curvier* (37.5%), *C. falciformis* (56.9%) (Navarro-Garcia et al., 2000). However high levels of NL particularly rich in TAG, are not specific of the group of the elasmobranches but can be extended to all liver oils of marine organisms (Pigott, 1996) as TAG are known to be the main energy source (Navarro-garcia et al., 2004b).

As expected, intermediate compounds of the biosynthesis of TAG are also found in minor proportion, they are free fatty acids (FFA), monoacylglycerol (MAG), 1,2 diacylglycerol (1,2-DAG) and 1,3 diacyglycerol (1,3-DAG). Sterols, hydrocarbons, steryl esters and glyceryl ethers were also found but under our conditions they were eluted with other components (see Table 1). A saponification step is thus needed to quantify and identify them (see below).

Total fatty acids and those linked to glycerol and sterols are presented in Table 2. Up to 13 different FA (3 saturated, 4 mono-unsaturated and 6 PUFAs) have been identified by GC-MS using FAME and picolinyl derivatives as previously described (Dubois et al., 2006). Their quantification was done by using GC/FID and FAME as former mentioned (Bergé et al., 2002). Palmitic acid (16:0) was found predominant (28%) while the level of saturated fatty acids was found to be 45%. Thus, unsaturated fatty acids represented more than the half of the total fatty acids (55%) as indicated before (Pal et al., 1998; Navarro-Garcia et al., 2000; Navarro-Garcia et al., 2004a; Navarro-garcia et al., 2004b). Among those unsaturated fatty acids, the n-3 PUFAs content was 27% with 16% of DHA and 4% of EPA which is in accordance with the marine liver oil compositions already found (Bergé et al., 2005). Indeed, in cod liver oil traditionally used as omega-3 PUFAs source, the amount of EPA and DHA was around 16% (Méndez, Gonzalez, Inocente, Giudice, & Grompone, 1996). Our study confirms previous results (Navarro-garcia et al., 2004b; Bergé et al., 2005) indicating that ray oil can be also an alternative source for omega-3 (18% of EPA and DHA in *G. marmorata*, and 16% for *Dasyatis brevis*). In addition to these omega-3, omega-6 fatty acids were also found: arachidonic acid (20:4 n-6) represented 4% and the level of linoleic acid (18:2 n-6) was 1%. The resulting ratio of n-3 to n-6 fatty

acids was 5.7 which is what it seems to be needed for the prevention of chronic diseases (Bergé et al., 2005).

After a saponification step, total sterols represented 46% of the unsaponifiable fraction (Table 3). Cholesterol was the only one found free while 12 were esterified with FA. Whatever the form (free or esterified), cholesterol was predominant and the others compounds were present in very low concentrations. Such results confirm a previous study on the liver oil of *Dasyatis bleekeri* where cholesterol was found at a level of 68% while 22-dehydrocholesterol, campesterol and sitosterol were detected in low amounts (Pal et al., 1998). These observations suggest that cholesterol best performs the structural function of sterols in membranes (Nes, 1974).

Hydrocarbons composition from the liver oil of *Himantura bleekeri* is presented in Table 4. *n*-alkanes were the major components (74%). Similar results have been previously published by Pal et al. (1998) for *Dasyatis bleekeri*. Up to 11 different hydrocarbons were identified. Squalene (22%) was at least twice more abundant than others, 9 alkanes were identified ranging from 17 to 28 carbons. Squalene is recognized as an important intermediate in biosynthesis of cholesterol. It is used as food additive and as precursor of squalane; the latter compound is utilized largely as an excipient in cosmetics and pharmaceuticals (Bordier, Sellier, Foucault, & Le Goffic, 1996). Its potential clinical uses are various as it may have several roles as antioxidant for human skin and eyes but also in detoxification of some xenobiotics (Kelly, 1999). This compound have been found in liver oil of many deep-sea shark species (Bordier et al., 1996; Wetherbee et al., 2000) but also in the ray *Dasyatis bleekeri* (Pal et al., 1998).

At least, as expected, glyceryl ethers were also detected. Such compounds have been previously found in ray liver and shark liver (Hayashi & Takagi, 1981; Bordier et al., 1996; Pal et al., 1998; Wetherbee et al., 2000; Navarro-Garcia et al., 2000). They are of interest since many benefits for human health like bacteriostatic and fungistatic properties, anti-inflammatory activities and hematopoietic effects, were attributed to those compounds (Bordier et al., 1996).

4. Conclusion

In conclusion, this study demonstrates that the liver of the ray *Himantura bleekeri*, which is actually wasted, could be used as new raw material for oil production. Indeed, such oil, which could be obtained in relatively high amount, is an excellent source of omega-3 PUFAs, particularly in DHA. Also, the presence of squalene and glyceryl ethers can justify such exploitation for nutritional or cosmetic purposes.

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Table 1 : Distibution of neutral lipids from the liver of *Himantura bleekeri*.

	Rf	% (w/w)
Hydrocarbons/Steryl esters	0.94	6.3
Triacylglycerols	0.64	62.5
Free fatty acids/Fatty alcohols	0.37	8.8
1.3-diacylglycerols/sterols	0.21	8.3
1.2-diacylglycerols	0.17	6.9
Glyceryl ethers/Monoacylglycerols	0.02	7.3

Table 2 : Fatty acid composition of liver oil of *Himantura bleekeri*.

Fatty acid	Total FA (%)	MAG (%)	1,2-DAG (%)	1,3-DAG (%)	FFA (%)	TAG (%)	FFA linked to sterols (%)
C14:0	3.12	4.90	4.12	2.63	2.16	3.50	0.00
C16:0	28.33	36.86	30.56	27.50	24.58	32.33	29.29
C18:0	13.86	13.31	10.30	12.68	11.94	15.48	10.82
C16:1 w7	6.82	10.53	12.09	9.09	9.87	6.71	0.00
C18:1 w9	7.76	8.26	7.51	9.01	11.82	7.82	10.97
C18:1 w7	6.22	7.98	7.95	8.20	9.65	6.19	6.71
C20:1 w9	2.03	0.00	0.00	2.67	2.07	1.92	0.00
C18:2 w6	1.03	0.00	0.00	0.00	1.70	0.00	0.00
C20:4 w3	3.36	0.00	2.52	3.00	4.31	2.97	0.00
C20:4 w6	3.73	2.11	2.86	3.48	4.16	3.01	16.62
C20:5 w3	4.10	0.00	3.17	2.40	3.71	3.63	7.09
C22:5 w3	4.01	3.92	4.48	3.75	3.68	3.39	0.00
C22:6 w3	15.62	12.13	14.44	15.59	10.34	13.03	18.50
SFA	45.32	55.07	44.98	42.81	38.68	51.31	40.11
MUFA	22.84	26.77	27.56	28.97	33.41	22.64	17.68
PUFA	31.85	18.16	27.46	28.22	27.90	26.03	42.21
n-3 PUFA	27.09	16.05	24.60	24.74	22.04	23.02	25.59
n-6 PUFA	4.76	2.11	2.86	3.48	5.86	3.01	16.62

Table 3 : Composition of free and esterified sterols from the liver of *Himantura bleekeri*, analysed as acetates by gas chromatography.

	Percentage area (%)
Sterols (free)	
Cholesterol	100.00
Sterols (esterified)	
22-Dehydrocholesterol	0.89
Cholesterol	97.83
Unidentified	Traces
Desmosterol	0.09
Latosterol	Traces
24-Methylene-cholesterol	0.45
Campesterol (R)	0.52
22-Dehydro-brassicasterol (S)	0.07
24-Methyl-desmosterol	Traces
Unidentified	0.02
Sitosterol	0.02
Fucosterol	0.08

Table 4 : Hydrocarbon compositions in ray liver oil.

Hydrocarbon	Carbon number	Percentage (%)
Heptadecane	17	3.96
Octadecene	18	3.63
Heneicosane	21	4.42
Docosane	22	12.70
Tricosane	23	7.56
Tetracosane	24	13.81
Pentacosane	25	10.44
Hexacosane	26	9.92
Heptacosane	27	5.76
Octacosane	28	5.75
Squalene	30	22.05

Figure 1 : Flowsheet illustrating the different sample preparation steps