Analysis of EPA and DHA in the viscera of marine fish using gas chromatography

De-Yong Zhang[1]*, Xiao-Lu Xu[1], Xiu-Ying Shen[2], Yu Mei[1] and Hui-Ying Xu[1]
[1]College of Biology and Environmental Engineering, Zhejiang Shuren University, Hangzhou, China
[2]College of Biology and Chemical Engineering, Zhejiang University of Science and Technology, Hangzhou, China

Abstract: The viscera of 10 kinds of marine fishes were collected for fish oil extraction and detection of DHA and EPA, two most important polyunsaturated fatty acids. The fish oil extraction ratio for the evaluated fishes varied from 0.95% to 10.18% (wt%). *Pseudosciaena crocea* presented the highest fish oil yield, followed by *Mustelus manazo*, *Hippoglossus* and *Sciaenopsocellatus*. A gas chromatography method was then established for analysis of EPA/DHA. The EPA concentration (in methyl ester form) in the fish oil varied from 1.39 to 10.65(mg/g). *Epinephelus awoara* presented the highest EPA concentration ($p<0.05$), followed by *Epinephelussp*, *Sciaenopsocellatus* and *Hippoglossus*. The DHA concentration (in methyl ester form) in the fish oil varied from 0.58 to 37.02 (mg/g). *Epinephelus awoara* presented the highest DHA concentration ($p<0.05$), followed by *Sciaenopsocellatus*, *Pseudosciaena crocea* and *Hippoglossus*. No strict positive correlation between the EPA/DHA concentration and the sea depth where the fish live was observed. The fishes living in middle depth presented highest EPA/DHA concentration.

Keywords: Marine fish; viscera; EPA; DHA; gas chromatography.

INTRODUCTION

EPA (5, 8, 11, 14, 17-eicosapentaenoic acid) and DHA (4, 7, 10, 13, 16, 19- docosahexaenoic acid) are two important omega-3 polyunsaturated fatty acids (PUFA). Both of them belong to essential fatty acids that the human body needs but cannot produce. Therefore, they must be consumed through food or supplements (Russell and Bürgin-Maunderemail, 2012). EPA and DHA were confirmed to benefit the functions of various systems in human body, including cardiovascular health, brain health, eyesight health, etc. For cardiovascular health, their functions include inhibiting platelet condensation, performing antithrombosis, helping vasorelaxation, raising HDL level, decreasing LDL and cholesterol level, and so on (Harris *et al*., 2013; Minihane, 2013; Nicholson, 2013). For brain health, their functions include improving brain cell development, improving brain function, improving memory and learning ability, preventing senile dementia, and so on (Janssen and Kiliaan, 2013; Luchtman and Song, 2013). For eyesight, they could strengthen retinal reflection ability (Kawakita *et al*., 2013; Ramkumar *et al*., 2013). In addition, they also benefit the therapy of diabetes inflammation, kidney disease and various cancers (Azizi-Soleiman *et al*., 2013; Halade *et al*., 2013; Hutchins-Wiese *et al*., 2014; Liu *et al*., 2013; Sorensen *et al*., 2014).

Polyunsaturated fatty acids are recommended for patients with wide-ranging chronic diseases, including coronary heart disease, rheumatoid arthritis, dementia, and depression (Russell and Bürgin-Maunderemail, 2012). Health authorities in many countries recommend increased intake of EPA/DHA. For example, European health authorities recommend at least 0.45-0.50g/day EPA+DHA to maintain good health (French Agency for Food, Environmental and Occupational Health & Safety, 2008; Health Council of the Netherlands, 2006; Scientific Advisory Committee on Nutrition, 2004). The mean daily intake of EPA+DHA and ALA (α-linolenic acid) in Australian adults is 0.175g and 1.07g, respectively. And a daily dietary intake of 0.5g EPA+DHA plus 2.0g ALA was recommended to lower the risk of coronary heart disease, 1.0g EPA+DHA plus 2.0g ALA for patients with documented coronary heart disease, and 1.2-4.0g EPA+DHA for patients with elevated serum triglyceride levels (Heart Foundation of Australia, 2008). Therefore, a sharp increase of consumption of EPA/DHA is occurring worldwide recently. Marine fish is the best direct source of EPA and DHA. There have been many investigations on the PUFA content in the marine fish from USA, Europe, Malaysia, and so on. Generally, several to several dozens of percentage of EPA/DHA could be found in the lipids of these marine fish. High content was frequently found in such species as Menhaden, Cod, Herring, Mackerel, silver catfish and so on (Gebauer *et al*., 2006; Wan Rosli *et al*., 2012). Totally, the marine fish presented much higher EPA/DHA level than other foods. While, the distribution of EPA/DHA in the viscera of marine fish has rarely been discussed till now. The lipids of tuna filet, head, viscera, liver, and gonads fished in Tunisian waters were examined, showing a lipid content varied from 1.5% to 14.2%. The major fatty acids classes were PUFA, among which the highest proportion is EPA/DHA. The results showed that fish viscera could provide satisfying level of EPA/DHA. But the content of EPA/DHA changed dramatic between winter and summer, resulting in a

*Corresponding author: e-mail: deyonghz@163.com

seasonal change of PUFA (Selmi *et al*., 2008). Another report even showed a much higher EPA/DHA content in the viscera of squid. With an optimized extraction process, viscera of squid presented a 69.3% extraction ratio, with 50.81 of PUFA, 13.33% EPA and 29.91% DHA in the extracted oil (Liu *et al*., 2011).

China has a sea area of nearly 300 km^2 and a total coastline of 32000 km. More than 1700 species of marine fish have been found in the sea area of China and about 60 to 70 species of them belong to economic fish of high yield. Bohai Sea and Yellow Sea are rich in *Pseudosciaena polyactis Bleeker*, *Pacific Gadus macrocephalus*, *Clupea pallasi*, etc. East China Sea is rich in *Trichiurus lepturus*, *Pseudosciaena crocea*, *Pseudosciaena polyactis Bleeker*, *Architeuthis dux*, etc. South China Sea in rich in *Makaira*, *Eleotridae*, *Euthynnus yaito*, *Mustelus manazo*, *Chelonia mydas*, etc (Lin *et al*., 2007). In fish processing, viscera are generally considered waste products and often discarded. Therefore, it is of importance to explore the potential utilization of these viscera for extraction of bioactive molecules of high value. In the present study, some marine fish from the sea area of China were collected for extraction and analysis of the DHA/EPA in the viscera. The general level of DHA/EPA in the viscera and the difference between various species were the main concerns here.

MATERIALS AND METHODS

Chemicals and reagents

EPA methyl ester (C20:5) and DHA methyl ester(C22:6) standards (purity ≥ 99%) were bought from SIGMA Company (St. Louis, USA). KOH (analytically pure), ethanol (analytically pure), n-hexane (chromatographically pure), methanol (chromatographically pure) were bought from J&K Chemical Company (Beijing, China).

Treatment of fish oil sample under test

The viscera of 10 kinds of marine fish were provided by Zhejiang Ocean Family Co. LTD (Hangzhou, China). Backgrounds of the fish were summarized in table 1. The viscera were steamed at 90□ for 45 min for oil extraction. After a centrifugation at 6000 g for 15 min, the oil phase was collected as the fish oil sample for analysis. Fish oil reacted with methanol in alkaline conditions for formation of fatty acid methyl ester. Firstly, KOH-methanol solution was prepared. KOH of 2.8g was dissolved with some ultrapure water and was transferred into 100mL volumetric flask. After combing with 1.6g methanol, a dilution with water to 100mL final total volume was performed. For each kind of fish, 0.05g fish oil was dissolved with 5mL n-hexane in 10mL tube with lid, and was added with 2mL 0.5mol/L KOH-methanol solution prepared above. After 1min of violent shaking, ultrapure water was added up to the tube neck. The water layer was discarded, and the n-hexane was washed 3 times again using water. After a centrifugation for 5min at 4000g, the n-hexane layer was collected and diluted to 10mL final volume. After a dilution again by 5 times, 1.0μL of the final sample was loaded for detection.

Treatment of standards and establishment of standard curve

EPA methyl ester and DHA methyl ester were dissolved with n-hexane, which had been filtered using an organic membrane. A series of concentrations (0.05, 0.10, 0.50, 1.0, 2.0mg/mL) of mixed standard solutions containing both EPA methyl ester and DHA methyl ester were prepared. In addition, solution of single EPA methyl ester (2mg/mL) or single DHA methyl ester (2mg/mL) was also prepared for verifiable determination of sample peaks.

Gas chromatography conditions setting

Detector: Shimadzu GC-2014 gas chromatography. Chromatographic column: Agilent technologies, inc. 19091N-133 (30m x 0.250mm, 0.5μm); column oven temperature: initial temperature 180°C, rise to 220°C at 10°C/min speed, rise to 250°C at 8°C/min speed, maintain for 13min; Injection port temperature: 250°C; Detector temperature: 270°C; carrier gas and flow rate: N_2 (≥99.99%) 1.0mL/min; air 450mL/min; H_2 40mL/min; sample size: 1.0μL; split rate: 20:1. For each sample, 1.0μL of sample was loaded precisely for detection.

Evaluation of precision and recovery ratio of the proposed method

One fish oil sample was detected repeatedly for 5 times. The peak areas were recorded and the relative standard deviation (RSD) was calculated for precision evaluation.

Nine fish oil samples containing three concentrations of EPA methyl ester and DHA methyl ester were prepared. The low dose group was prepared with 0.15g fish oil, 900μL of 10mg/mL EPA methyl ester and 1500μL of 10mg/mL DHA methyl ester. The middle dose group was prepared with 0.15g fish oil, 1125μL of 10mg/mL EPA methyl ester and 1875μL of 10mg/mL DHA methyl ester. The high dose group was prepared with 0.15g fish oil, 1350μL of 10mg/mL EPA methyl ester and 2250μL of 10mg/mL DHA methyl ester. The solutions above were treated as described for samples for detection and recovery ratio evaluation.

STATISTICAL ANALYSIS

The peaks of EPA methyl ester and DHA methyl ester were recorded, and the concentrations of them were calculated. The difference between different kinds of fish oil was analyzed using One-way ANOVA in SPSS (V11.5). Statistical significance was determined with α=0.05.

Table 1: Brief backgrounds of the fishes investigated in the study

Fish species	Living conditions	Distribution
Pseudosciaena crocea	Living mainly in the middle and lower water layer in the coastal and offshore waters within 80 metres; The spawning fish are photophobia, and fond of countercurrent and turbid waters.	Mainly in the offshore of China (from central area of Yellow Sea to the south area of QiongzhouStrait) and west coast of north Korea
Pampus argenteus	Living mainly in the sea area within 5-110 m; carnivorous; feeding on jellyfish and zooplankton.	Mainly distributed in India area of the western Pacific
Epinephelus awoara	Living mainly in the sea area of the island reef off the coast, gravel, coral reefs; generally not in groups.	Mainly distributed in the northwest Pacific Ocean
Trichiurus lepturus	Vertically moving as day-night cycle (inhabiting under water in day and rising to the surface water at night)	Widely distributed in the Indian Ocean and the Pacific coast
Panalichthysethostigma	Living in the sea water about 20-70 m in depth, with sand, gravel, or mixed substrate.	Mainly distributed in the east coast of the Atlantic ocean especially the northeast Atlantic
Paralichthys olivaceus	Living in the sea water of medium depth along the continental shelf (Some might enter fresh water area, even living there permanently); perched on a shallow sandy bottom; suitable for benthic life on the ocean floor.	Mainly in temperate waters
Mustelus manazo	Various marine environment	Widely distributed in tropical and subtropical oceans
Sciaenopsocellatus	Swimming in cluster quickly; showing obvious migratory behavior; wide temperature (optimum temperature of 10 to 30□) and wide salt (freshwater, brackish water and seawater) adaptive.	Mainly in the coast waters in the south Atlantic and Gulf of Mexico
Hippoglossus	Coldness adaptive benthic fish; sand latent; larvae living in nearshore shallow fatten (10 m to 30 m); migrate to deeper ocean (50m-90m) in cold seasons.	Widely distributed in tropical and temperate ocean; rich in the Yellow Sea and BohaiSea in China
Epinephelussp	Belong to tropical coral reefs and coastal fish; living in the shallow water with rock bottom. Feeding on invertebrate and fish.	Mainly in the Indian Ocean and the Pacific (from the southern Japan to the northern Australia)

RESULTS

Establishment of gas chromatographic method

As shown in fig. 1, the retention time for EPA peak and DHA peak was 10.221min and 13.913min respectively. With the concentration as the abscissa and the peak area as the ordinate, standard curves and regression equations for EPA and DHA were established, respectively. In a range of 0 to 2000μg/mL, a satisfying linear relationship between the concentration and the peak area was confirmed. The direct equations between the peak area (Y) and the concentration (X) in the loaded samples were established as equation (1) and equation(2). Then, the concentration of EPA or DHA (mg/g) in the fish oil could be calculated according to equation (3) or equation (4), respectively.

$$Y_{EPA}=5971.3X_{EPA}(\mu g/mL)+3.53;R^2=0.9991 \quad (1)$$

$$Y_{DHA}=5119.5X_{DHA}(\mu g/mL)+3.95;R^2=0.9993 \quad (2)$$

$$C_{EPA}(mg/g)=X_{EPA}(\mu g/mL)*50\ (mL)*10^{-3}/0.05g \quad (3)$$

$$C_{DHA}(mg/g)=X_{DHA}(\mu g/mL)*50\ (mL)*10^{-3}/0.05g \quad (4)$$

Precision and recovery ratio

For the precision experiment, five peak area values of 7.12, 6.99, 7.10, 6.90 and 7.12($\times10^5$) were observed for the repeated detection, respectively. The average value of the peak area was confirmed to be 7.05($\times10^5$), with a RSD value of 0.98%.

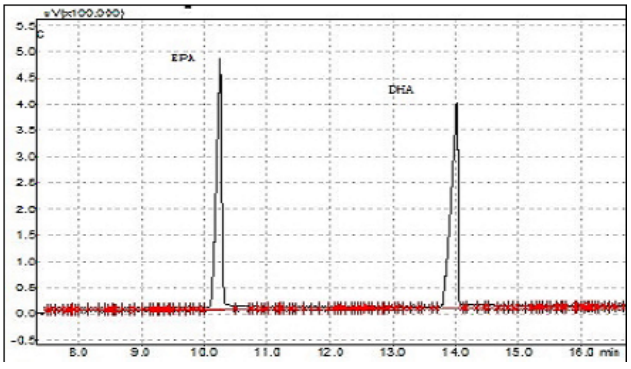

Fig. 1: Absorption peak of EPA methyl ester and DHA methyl ester

The results in the recovery ratio experiment were shown in table 2 and table 3. The recovery ratio for EPA methyl ester and DHA methyl ester falls into a range between 98.84% and 100.80%. The average recovery ratio for EPA methyl ester and DHA methyl ester was 100.80% and 98.84% respectively. And the RSD value for EPA methyl ester and DHA methyl ester was 1.86% and 0.95% respectively.

Table 2: Preparation of standard chemicals

Tube No.	Concentration (mg/mL)	
	EPAmethyl ester	DHA methyl ester
1	0.05	0.05
2	0.1	0.1
3	0.5	0.5
4	1	1
5	2	2
6	2	0
7	0	2

Fish oil yields and the concentration of EPA and DHA

As shown in table 4, the fish oil extraction ratio varied from 0.95% to 10.18% (wt%). *Pseudosciaena crocea* presented the highest fish oil yield ($p<0.05$), followed by *Hippoglossus, Mustelus manazo, Sciaenopsocellatus, Paralichthys olivaceus, Epinephelus awoara, Panalichthysethostigma, Epinephelussp, Pampus argent*eus and *Trichiurus lepturus*. The EPA concentration (in methyl ester form) in the fish oil varied from 1.39 to 10.65 (mg/g). *Epinephelus awoara* presented the highest EPA concentration ($p<0.05$), followed by *Epinephelussp*, *Sciaenopsocellatus*, *Pseudosciaena crocea*. *Panalichthysethostigma, Mustelus manazo, Pampus argenteus, Trichiurus lepturus and Paralichthys olivaceus.* The DHA concentration (in methyl ester form) in the fish oil varied from 0.58 to 37.02(mg/g). *Epinephelus awoara* presented the highest DHA concentration, followed by *Sciaenopsocellatus*, *Pseudosciaena crocea*, *Hippoglossus, Mustelus manazo, Epinephelussp, Pampus argenteus, Trichiurus lepturus, Panalichthysethostigma and Paralichthys olivaceus*

DISCUSSION

Gas chromatography is one of the widely used methods for fatty acid analysis currently. To facilitate the analysis, the fatty acids in the sample are usually transformed into methyl ester form, which is relatively easy for gasification. The process includes hydrolyzation of fat and formation of fatty acid methyl esters through reaction with methanol (Metcalfe *et al*., 1966). As to esterification, three methods, acidic methyl esterification, alkaline methyl esterification and trimethylsilyl-diazomethane methyl esterification are usually taken (Liu, 1994; Metcalfe *et al*., 1966; Wilhelm *et al*., 1959). Incomplete esterification is often observed in acidic methyl esterification. Trimethylsilyl-diazomethane methyl esterification works better for free fatty acids. Therefore, alkaline methyl esterification using KOH- methanol was performed in the present study. The gas chromatography method established in the present study showed satisfying precision and recovery ratio for detecting EPA/DHA using standard mixture.

There have been many investigations about the PUFA, especially EPA/DHA in various marine fish worldwide. Some observations showed that the concentration of EPA/DHA was closely related to many factors, such as specie, season, sexuality, body part, developmental stage, food intake and so on. As to the species, for example, a database from US department of agriculture showed that such fish as Menhaden, Salmon, Cod, Herring, Mackerel, Scad, Muroaji generally present high quantity of EPA/DHA (Gebauer *et al*., 2006). An investigation from Europe also observed high EPA/DHA level in such fish as Mackerel, Herring and Trout(Claudia *et al*., 2012). Another report from malaysia coast confirmed the highest level of DHA in Sixbar grouper and EPA in Barramundi (Wan Rosli *et al*., 2012). As to season and body distribution, for example, Little tuna(Euthynnus alletteratus) showed an obvious higher total fat and n3-PUFA(mainly EPA/DHA) level in winter than in summer. The head, viscera, fillet of little tuna were also compared in that report, the head and viscera showed relatively high concentration of EPA/DHA (Selmi *et al*., 2008). By-products during processing of squid were also evaluated for oil production, the viscera were confirmed to contain high quantity of PUFA too, especially EPA and DHA. With the optimized procedure by the authors, the oil extraction ratio reached about 70%, with 50.81% PUFA (consisted of 13.33% EPA and 29.91% DHA) in the oil(Liu *et al*., 2011). As mentioned above, the distribution of EPA/DHA in the discarded fish viscera hasn't been discussed so much till now. In the present study, all of the investigated fish presented relatively high level of EPA/DHA, showing optimistic potential for fish oil production. But different species showed significant difference in oil yield and EPA/DHA concentration. Such fish as *Epinephelus awoara*, *Pseudosciaena crocea*, *Sciaenopsocellatus and Hippoglossus* seem to provide higher concentrations of EPA/DHA.

The relation between EPA/DHA and the water depth was another concern in the present study. To analyze the relationship between the EPA/DHA concentration and the depth where the fish live, the living background of the fishes were investigated. Based on the data, the fish live in a rough increasing order of depth as follow: *Pampus argenteus* (assigned as 30m), *Panalichthysethostigma* (assigned as 45m), *Epinephelus awoara* (assigned as 50m), *Hippoglossus* (assigned as 75m), *Pseudosciaena crocea* (assigned as 80m), *Epinephelussp* (assigned as 100m), *Sciaenopsocellatus* (assigned as 110m), *Mustelus*

Table 3: EPA recovery ratio

Tube No.	Sample quantity (mg)	Control quantity (mg)	Detected value (mg)	Recovery rate (%)
1	18.03	10.00	28.12	100.32
2	17.55	10.00	27.60	100.18
3	17.98	10.00	28.09	100.39
4	17.12	11.00	28.63	101.81
5	16.95	11.00	28.90	103.40
6	16.80	11.00	28.99	104.28
7	17.82	14.00	31.60	99.3
8	17.76	14.00	31.42	98.93
9	17.84	14.00	31.40	98.62

Table 4: DHA recovery ratio

Tube No.	Sample quantity (mg)	Control quantity (mg)	Detected value (mg)	Recovery rate (%)
1	24.34	15.00	38.55	97.99
2	24.66	15.00	38.46	96.76
3	24.46	15.00	38.69	98.05
4	22.78	19.00	41.60	99.57
5	22.69	19.00	41.50	99.54
6	22.66	19.00	41.34	99.23
7	23.54	22.00	45.20	99.25
8	23.67	22.00	45.50	99.63
9	23.86	22.00	45.64	99.52

Table 5: Fish oil yields and the concentration of EPA and DHA in the fish oil

Fish	Fish oil yield (wt%)	EPA concentration (mg/g)	DHA concentration (mg/g)
Pseudosciaena crocea	10.18±0.81	5.73±0.03	12.10±0.23
Mustelus manazo	6.63±0.52	3.14±0.02	9.32±0.30
Epinephelus awoara	5.40±0.22	10.65±0.06	37.02±1.01
Panalichthysethostigma	4.05±0.15	3.99±0.06	1.93±0.05
Sciaenopsocellatus	5.82±0.41	6.12±0.12	13.64±0.13
Hippoglossus	6.63±0.29	5.97±0.08	9.53±0.08
Epinephelussp	3.86±0.26	8.43±0.04	7.84±0.18
Pampus argenteus	3.38±0.34	2.26±0.09	3.58±0.11
Trichiurus lepturus	0.95±0.07	1.56±0.09	2.04±0.10
Paralichthys olivaceus	5.51±0.02	1.39±0.11	0.58±0.45

manazo (assigned as 200m), *Paralichthys olivaceus* (assigned as 250m), *Trichiurus lepturus* (assigned as 300m). No positive correlation between the concentration and the depth was observed. The general principle seems to be that the fish living in middle depth water present the highest EPA/DHA concentration. Neither the shallow layer fish nor the deepest layer fish showed highest concentration of EPA/DHA.

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