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Analysis of 5,8,11,14,17-Eicosapentaenoic Acid and 4,7,10,13,16,19-Docosahexaenoic Acid in the Viscera of Marine Organisms Using Gas Chromatography

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A gas chromatography was established for analysis of 5,8,11,14,17-eicosapentaenoic acid and 4,7,10,13,16,19-docosahexaenoic acid in the viscera of marine organisms. The viscera of 6 kinds of non-fish marine organisms were then collected for oil extraction and detection of 4,7,10,13,16,19-docosahexaenoic acid and 5,8,11,14,17-eicosapentaenoic acid. The oil extraction ratio varied from 0.72 to 6.23 % (wt. %). *Scylla serrata* presented the highest oil yield, followed by *Architeuthis dux* and *Panopea abrupta*. The 5,8,11,14,17-eicosapentaenoic acid concentration (in methyl ester form) in the oil varied from 0.56 to 7.75 (mg/g). *Scylla serrata* presented the highest 5,8,11,14,17-eicosapentaenoic acid concentration ($p < 0.05$), followed by *Architeuthis dux* and *Panopea abrupta*. The 4,7,10,13,16,19-docosahexaenoic acid concentration(in methyl ester form) varied from 0.15 to 4.71 (mg/g). *Architeuthis dux* and *Scylla serrata* presented obviously higher 4,7,10,13,16,19-docosahexaenoic acid concentration than other species ($p < 0.05$).

Keywords: Marine organisms, Viscera, EPA, DHA, Gas chromatography.

INTRODUCTION

5,8,11,14,17-Eicosapentaenoic acid (EPA) and 4,7,10,13,16,19-docosahexaenoic acid (DHA) are two important omega-3 poly-unsaturated fatty acids. Both of them belong to essential fatty acids that human body needs but can hardly produce by itself. Therefore, they depend on consumption through food or supplements[1]. 5,8,11,14,17-Eicosapentaenoic acid and 4,7,10,13,16,19-docosahexaenoic acid 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 condensing, performing antithrombosis, helping vasorelaxation, raising HDL level, decreasing LDL and cholesterol level, *etc*.[2-4]. For brain health, their functions include improving brain cell development, improving brain function, improving memory and learning ability and preventing senile dementia and so on[5,6]. For eyesight, they could strengthen retinal reflection ability[7,8]. They also benefit the therapy of diabetes inflammation, kidney disease and various cancers[9-13]. Polyunsaturated fatty acids are recommended for patients with wide-ranging chronic diseases, including coronary heart disease, rheumatoid arthritis, dementia and depression[1]. Health authorities in many countries recommend increased intake of 5,8,11,14,17-eicosapentaenoic acid and 4,7,10,13,16,19-docosahexaenoic acid. For example, European health authorities recommend at least 0.45-0.50 g/day 5,8,11,14,17-eicosapentaenoic acid + 4,7,10,13,16,19-docosahexaenoic acid to maintain good health[14-16]. The mean daily intake of 5,8,11,14,17-eicosapentaenoic acid + 4,7,10,13,16,19-docosahexaenoic acid and ALA suggested in Australia is 0.175 g and 1.07 g, respectively[17]. A sharp increasing of consumption of 5,8,11,14,17-eicosapentaenoic acid/4,7,10,13,16,19-docosahexaenoic acid is occurring worldwide recently.

Most attentions on 5,8,11,14,17-eicosapentaenoic acid and 4,7,10,13,16,19-docosahexaenoic acid source were paid to marine fishes, which are rich in polyunsaturated fatty acids and a lot of other non-fish organisms have long been ignored. In addition to fish, there are a huge number of other marine organisms in the ocean. For example, over 1580 kinds of marine organisms have been confirmed in the beach in China. Mollusks (513 species) accounts for the largest proportionare, followed by seaweed (358 species) and crustaceans (308 species). This study aims to establish a gas chromatography for detection of the 5,8,11,14,17-eicosapen-taenoic acid and 4,7,10,13,16,19-docosahexaenoic acid in the viscera of some marine organisms and to analyze some typical samples.

EXPERIMENTAL

5,8,11,14,17-Eicosapentaenoic acid methyl ester (C20:5) and 4,7,10,13,16,19-docosahexaenoic acid methyl ester (C22:6) standards (purity = 99 %) were bought from SIGMA Company (St. Louis, USA). Potassium hydroxide (analytically pure), ethanol (analytically pure), *n*-hexane (chromatographically pure), methanol (chromatographically pure) were bought from J&K Chemical Company (Beijing, China). Shimadzu GC-2014 gas chromatography (Japan). The viscera of 6 kinds of non-fish marine organisms (Table-1) were provided by Zhejiang Ocean Family Co. LTD (Hangzhou, China).

Gas chromatography setting: Chromatographic column: Agilent lechnologies, inc. 19091N-133 (30 m × 0.250 mm, 0.5 μm); 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 13 min; Injection port temperature: 250 °C; Detector temperature:270 °C; carrier gas and flow rate: N_2 (≥ 99.99 %) 1 mL/min; air 450 mL/min; H_2 40 mL/min; sample size: 1 μL; split rate:20:1. For each sample, 1 μL of sample was loaded precisely for detection.

Preparation of the standards and establishment of the standard curve: 5,8,11,14,17-Eicosapentaenoic acid methyl ester and 4,7,10,13,16,19-docosahexaenoic acid methyl ester were dissolved with *n*-hexane which had been filtered using organic membrane. A serial of concentrations of mixed standard solutions containing both 5,8,11,14,17-eicosapentaenoic acid methyl ester and 4,7,10,13,16,19-docosahexaenoic acid methyl ester were prepared. In addition, solution of single 5,8,11,14,17-eicosapentaenoic acid methyl ester or single 4,7,10,13,16,19-docosahexaenoic acid methyl ester was also prepared for verifiable determination of sample peaks (Table-2).

Treatment of viscus oil samples: The viscera were steamed at 90 °C for 45 min for oil extraction. After a centrifugation at 6000 g for 15 min, the oil phase was collected as viscus oil sample for analysis. Viscus oil then reacted with methanol in alkaline conditions for formation of fatty acid methyl ester. For each kind of fish, 0.05 g viscus oil was dissolved with 5 mL *n*-hexane in 10 mL tube with lid and was added with 2 mL 0.5 mol/L KOH-methanol solution (2.8 g KOH, 1.6 g methanol, diluted using water to 100 mL final total volume). After 1 min of violent shaking, some ultrapure water was added for washing. Discarding the water layer and washing the *n*-hexane 3 more times using water. After a centrifugation for 5 min at 4000 g, the *n*-hexane layer was collected and was diluted to 10 mL final volume. After a 5 × dilution, 1 μL of the final sample was loaded for detection.

TABLE-2
PREPARATION OF STANDARD CHEMICALS

Tube no.	3. 5,8,11,14,17-Eicosapentaenoic acid (EPA) methyl ester (mg/mL)	4. 4,7,10,13,16,19-Docosahexaenoic acid (DHA) methyl ester (mg/mL)
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

Evaluation of the precision and the recovery ratio of the gas chromatography: Fish oil samples extracted from *Pseudosciaena crocea* were chosen for precision and the recovery ratio evaluation. For precision, a sample was detected repeatedly for 5 times. The peak areas were recorded and the relative standard deviation (RSD) was calculated for precision evaluation. For recovery ratio, 9 samples containing three concentrations of 5,8,11,14,17-eicosapentaenoic acid methyl ester and 4,7,10,13,16,19-docosahexaenoic acid methyl ester were prepared. The low dose group was prepared with 0.15 g fish oil, 900 μL of 10 mg/mL 5,8,11,14,17-eicosapentaenoic acid methyl ester and 1500 μL of 10 mg/mL 4,7,10,13,16,19-docosahexaenoic acid methyl ester. The middle dose group was prepared with 0.15 g fish oil, 1125 μL of 10 mg/mL 5,8,11,14,17-eicosapentaenoic acid methyl ester and 1875 μL of 10 mg/mL 4,7,10,13,16,19-docosahexaenoic acid methyl ester. The high dose group was prepared with 0.15 g fish oil, 1350 μL of 10 mg/mL 5,8,11,14,17-eicosapentaenoic acid methyl ester and 2250 μL of 10 mg/mL 4,7,10,13,16,19-docosahexaenoic acid methyl ester. The solutions above were treated as described for samples for detection and recovery ratio evaluation.

Statistical analysis: The peaks of 5,8,11,14,17-eicosapentaenoic acid methyl ester and 4,7,10,13,16,19-docosahexaenoic

TABLE-1
BRIEF BACKGROUNDS OF THE FISHES INVESTIGATED IN THE STUDY

Species	Living conditions	Distribution
Sepioidea	Generally moving to the coastal shallow sea in the spring, and moving to the offshore deep water in winter	Distributed extensively in most oceans, especially in the tropical and temperate district
Portunus trituberculatus	Lurking underwater in the day, and foraging in the night with obvious phototaxis. Adaptive water temperature of 8-31 °C; Adaptive salinity of 13-38 ‰	Mainly distributed in Japan, Korea, Malaysia, the Red Sea and Guangxi province of China
Panopea abrupta	Embedded type shellfish; Perched substrate of sand and mud, with 3-18 m of water depth; Buried depth of about 50-80 cm; Adapted to water temperature of 3-23 °C	Living originally in the north pacific coast of USA and Canada. Introduced to many areas later, including the southeast coast of China
Abalone	Attached to the rocks; Crawling in reef tents and holes	Distributed worldwide, along the coastal waters of every continent (except the Atlantic coast, the Caribbean, and the USA East Coast)
Scylla serrata	Beach-habitating swimming type crabs; living in intertidal mudflats or silt on the tidal flats; Staying in the puddles on the beach or rock seams	Distributed in the west Pacific tropical/subtropical waters in India, the Southeast Africa and the Red Sea
Architeuthis dux	Generally living in the upper/middle layer of shallow sea; Moving vertically in a range over 100 m	Mainly distributed in the shallow sea in tropical and temperate zones

acid methyl ester were recorded and the concentrations of them were calculated. The difference between different kinds of marine organisms was analyzed using One-way ANOVA in SPSS (V11.5). Statistical significance was determined with $\alpha = 0.05$.

RESULTS AND DISCUSSION

Establishment of gas chromatography: As shown in Fig. 1, the residence time for 5,8,11,14,17-eicosapentaenoic acid peak and 4,7,10,13,16,19-docosahexaenoic acid peak was 10.221 and 13.913 min, respectively. With the concentration as the abscissa and the peak area as the ordinate, standard curves and regression equations for 5,8,11,14,17-eicosapentaenoic acid and 4,7,10,13,16,19-docosahexaenoic acid are established, respectively. In a range of 0 to 2000 μg/mL, a satisfying linear relationship between the concentration and the peak area was confirmed.

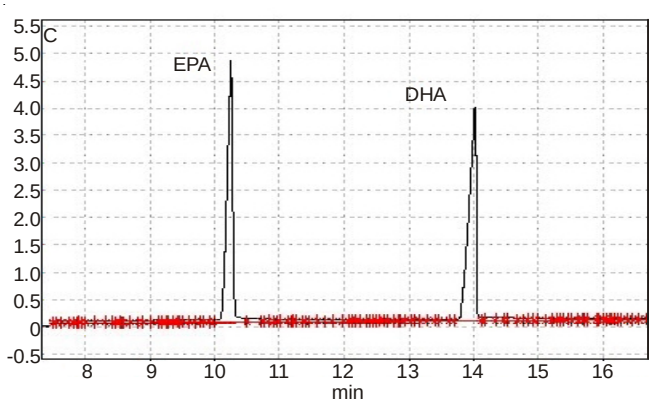

Fig. 1. Absorption peak of 5,8,11,14,17-eicosapentaenoic acid methyl ester and 4,7,10,13,16,19-docosahexaenoic acid methyl ester

The direct equations between the peak area (Y) and the concentration (X) in the loaded samples were established as eqn. 1 and 2. Then, the concentration of 5,8,11,14,17-eicosapentaenoic acid or 4,7,10,13,16,19-docosahexaenoic acid (mg/g) in the viscus oil could be calculated according to eqn. 3 or 4, respectively.

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

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

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

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

Precision and recovery ratio: For the precision experiment, five peak area values of 7.12, 6.99, 7.10, 6.90 and 7.12 ($\times 10^5$) was observed for the repeated detection, respectively. The average value of the peak area value was confirmed to be 7.05 ($\times 10^5$), with a RSD value of 0.98 %.

The results in the recovery ratio experiment were shown in Tables 3 and 4. The recovery ratio for 5,8,11,14,17-eicosapentaenoic acid methyl ester and 4,7,10,13,16,19-docosahexaenoic acid methyl ester falls into a range between 98.84 and 100.80 %. The average recovery ratio for 5,8,11,14,17-eicosapentaenoic acid methyl ester and 4,7,10,13,16,19-docosahexaenoic acid methyl ester was 100.80 and 98.84 %, respectively. The RSD value for 5,8,11,14,17-eicosapentaenoic acid methyl ester and 4,7,10,13,16,19-docosahexaenoic acid methyl ester was 1.86 and 0.95 %, respectively.

TABLE-3.
3. 5,8,11,14,17-EICOSAPENTAENOIC ACID (EPA) RECOVERY RATIO

Sample quantity (mg)	Control quantity (mg)	Detected value (mg)	Recovery rate (%)
18.03	10.00	28.12	100.32
17.55	10.00	27.60	100.18
17.98	10.00	28.09	100.39
17.12	11.00	28.63	101.81
16.95	11.00	28.90	103.40
16.80	11.00	28.99	104.28
17.82	14.00	31.60	99.3
17.76	14.00	31.42	98.93
17.84	14.00	31.40	98.62

TABLE-4
4. 4,7,10,13,16,19-DOCOSAHEXAENOIC ACID (DHA) RECOVERY RATIO

Sample quantity (mg)	Control quantity (mg)	Detected value (mg)	Recovery rate (%)
24.34	15.00	38.55	97.99
24.66	15.00	38.46	96.76
24.46	15.00	38.69	98.05
22.78	19.00	41.60	99.57
22.69	19.00	41.50	99.54
22.66	19.00	41.34	99.23
23.54	22.00	45.20	99.25
23.67	22.00	45.50	99.63
23.86	22.00	45.64	99.52

Viscus oil yields and the concentration of 5,8,11,14,17-eicosapentaenoic acid and 4,7,10,13,16,19-docosahexaenoic acid: As shown in Table-5, the viscus oil extraction ratio varied from 0.72 to 6.23 % (wt. %). *Scylla serrata* presented the highest oil yield, followed by *Architeuthis dux* and *Panopea abrupta*. The 5,8,11,14,17-eicosapentaenoic acid concentration (in methyl ester form) in the oil varied from 0.56 to 7.75 (mg/g). *Scylla serrata* presented the highest 5,8,11,14,17-eicosapentaenoic acid concentration($p < 0.05$), followed by *Architeuthis dux* and *Panopea abrupta*. The 4,7,10,13,16,19-

TABLE-5
VISCUS OIL YIELDS AND THE CONCENTRATION OF 3. 5,8,11,14,17-EICOSAPENTAENOIC ACID (EPA) AND 4. 4,7,10,13,16,19-DOCOSAHEXAENOIC ACID (DHA) IN THE OIL

Species	Oil yield (wt. %)	EPA (mg/g)	DHA (mg/g)
Sepioidea	2.23 ± 0.35	0.96 ± 0.28	1.23 ± 0.34
Portunus trituberculatus	0.80 ± 0.25	0.56 ± 0.29	0.51 ± 0.18
Panopea abrupta	3.78 ± 0.62	2.50 ± 0.85	0.15 ± 0.05
Abalone	0.72 ± 0.26	1.95 ± 0.46	3.34 ± 0.76
Scylla serrata	6.23 ± 1.02	7.75 ± 1.04	4.30 ± 0.59
Architeuthis dux	3.86 ± 0.93	5.97 ± 1.19	4.71 ± 1.01

docosahexaenoic acid concentration (in methyl ester form) varied from 0.15 to 4.71(mg/g). *Architeuthis dux* and *Scylla serrata* presented obviously higher 4,7,10,13,16,19-docosahexaenoic acid concentration than other species ($p < 0.05$).

Conclusion

The gas chromatography established in the present study is suitable for 5,8,11,14,17-eicosapentaenoic acid and 4,7,10,13,16,19-docosahexaenoic acid detection in the viscera of marine organisms.

The 6 kinds of marine organisms presented significant different oil yields and 5,8,11,14,17-eicosapentaenoic acid and 4,7,10,13,16,19-docosahexaenoic acid concentration. Such organisms as *Scylla serrata* and *Architeuthis dux* are promising candidates for 5,8,11,14,17-eicosapentaenoic acid and 4,7,10,13,16,19-docosahexaenoic acid extraction and utilization.

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