Lipid Analysis of PUFA in the New Millennium and Future Trends

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Abstract : Polyunsaturated fatty acids (PUFA), especially highly unsaturated fatty acids such as DHA and EPA have a significant potential of being functional fatty acids for preventing myocardial infarction, psoriasis, bronchial asthma, and other diseases. The PUFA analysis of lipids has provided fundamental information for evaluating these functional lipids for understanding the human metabolism.

Structure lipids with different positional distributions of PUFA in acylglycerol are also needed in order to identify the health benefits of the lipid. However, no single and simple method has been completely established to analyze the triacylglycerol molecular species of the marine lipid. The authors have developed a new standard method for DHA analysis by NMR. The DHA content and relative quantification of the n-3 fatty acids in the marine lipid were accurately and rapidly obtained using 300-500 MHz proton NMR.

Recent interests have been summarized as the health benefits since it has been recognized that there are many difficulties in the analysis of geometric and positional isomers of conjugated linoleic acid. Furthermore, in the field of fish oil utilization, the undesirable flavor of the product should be restricted to prevent odors as reversed grassy smell from PUFA. Analysis of fish flavor was examined by the method of solid phase micro extraction (SPME) for identifying flavor compounds from oxidized fish oil by heating with micro oven and other conditions.

For the utilization of fish oil after getting the precise analysis of PUFA, how to prevent the oxidation is important. Inositol hexaphosphate as one of the natural antioxidants has the potential for the preventing of fish oil oxidation.

Lipid analysis should be undertaken with the methods of non-destructive and observational analyses in the new millennium. The future trends of lipid analysis might include the key words of rapid, accurate, less solvent, and non-destructive instrumentation.

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Key words : lipid analysis, PUFA, fatty acid, DHA, NMR, SPME, flavor, fish oil, lipid oxidation, antioxidant

1 Introduction

Lipid analysis is widely used in many fields such as physical and nutritional biotechnology, oil refining and producing factory, structural identification in lipid world at institutes, organizations, universities, and companies. This article concentrates polyunsaturated fatty acid (PUFA), especially related to the field of food science, mainly connected to fish oil. PUFA, particularly highly unsaturated fatty acid (HUFA) such as DHA has a significant potential of being

functional lipid for preventing myocardial infarction, psoriasis, bronchial asthma and other diseases (1).

DHA analysis in food materials gives us a lot of fundamental information for evaluating the physiological and functional effects for understanding the human nutrients. Therefore, the following subjects are concentrated in the article.

* DHA analysis by NMR : which has organized as the collaborative work together with several countries. TG molecular species by LC-MS : which has selected as expected research to under-

stand of lipid properties.

* Flavor analysis of fish oil: this is the fundamental information to utilize the PUFA in food science and technology.

* Other topics like conjugated linoleic acid (CLA) and natural antioxidants for preventing PUFA oxidation.

2 Instrumental Analysis of PUFA

It is important for us to obtain the basic information about PUFA mainly contained in fish oil accumulated through the food chain in the sea. In Japan DHA is produced naturally from the discarded parts of conventional and popular fish such as tuna, bonito, and sardine.

Two groups of fish species around Japanese island are 1) teleost with about 3600 species and 2) cartilaginous fish with 900-1000 species, including 380-400 sharks as shown in Fig. 1.

2.1 DHA Analysis by Proton-NMR

The exact DHA content is important to be a production, which will be considered as an indispensable criterion for judging the quality of nutrients in marine products. Therefore, DHA content in fish oil should be strictly analyzed by accurate instrumental method. For this purpose, gas chromatography (GC)

is the most popular equipment so far. However, GC method has time-consuming involving considerable manipulation steps and it may cause oxidation of lipids, and also sometimes may give us wide deviation depending on the using columns. GC also gives us to pose difficulty in correct identification of each fatty acid in the peaks obtained (2).

From these unstable facts on GC, proton NMR method was evaluated to calculate DHA content and obtain the ratio of n-6/n-3 fatty acids in fish oil. DHA position connected to the acylglycerol structure is also analyzed by carbon NMR.

The experiments were undertaken as the international cooperation project by the support of Marinform 21 in Japan, Fats and oils division of IUPAC. The collaborative works were performed with Norway, Italy, France, Denmark, and Japan.

Figure 2 is the chart of the assignment of signals of DHA, n-3 fatty acids, and the internal standard, by the method of proton-NMR with the magnetic field of 500 MHz (3).

For quantification of DHA, signal indicated at around 2.38 ppm derived from C2 and C3 methylenes of DHA structure and other signal of non-DHA is indicated at around 2.28 ppm is derived from C2 methylene of the substance.

The proportion of total n-3 fatty acids to n-6 fatty

Fig. 1 Fish Species Around Japanese Island.

Fig. 2 An Example of the Manual Peak Separation for Ethylene Glycol Dimethyl Ether (EGDM) Signals, Docosahexanoic Acid (DHA) C2,3 and Other Fatty Acids, C2 Methylenes Signals, and n-3 and Other Fatty Acids Methyl Signals. Each framed Region (a, b, c) is enlarged above (3).

acids could be also determined simultaneously by comparing the methyl signal at 0.81-0.89 ppm of non n-3 fatty acids, and methyl signal at 0.90-0.98 ppm derived from n-3 fatty acids.

Methanol, 1,4-dioxane, and ethylene glycol dimethyl ether (EGDM) were compared to select the best internal standard in the NMR determination. Among those internal standards, EGDM was the smallest relative errors of less than 7%. Therefore, EGDM was sited to be internal standard and the methylenes and methyls of EGDM are indicated at 3.45-3.55 ppm and 3.30-3.40 ppm, respectively, as shown in **Fig. 2**.

Using this condition, the DHA content was analyzed and also the data obtained by NMR was compared with that of GC.

Table 1 is the result of DHA content and n-3 fatty acids proportion by proton-NMR and GC analyses. Samples 1, 2, 3, 4, and 5 were bonito oil unrefined, tuna from Yaizu city, tunas from Chousi city in Japan, and salmon from Norway, respectively. The highest coefficient of variation (CV) was less than 2% except the highest ones around 3%. By checking the NMR analytical data, it is recognized that DHA content appeared higher, and n-3 proportion is lower than

those obtained by GC analysis.

In this study, the pulse repetition time was 30 second, which was judged to be practical and to give reproducible results. Sample preparation requires only two steps: sample weighing and preparation of internal standard solution. This simplicity of NMR offers a great advantage over GC method since fish lipid deteriorates easily due to their high content of PUFA.

Additionally, NMR is a nondestructive method and therefore allows us to re-use small samples, if further experiments are needed.

The method is proposing to JOCS and IUPAC as official standard method.

2.2 DHA Analysis by Carbon NMR

Furthermore, **Fig. 3** shows the assignment of DHA position in the TG by carbon-NMR. The connection of DHA to α or β position of TG could easily identified because the chemical shifts of α - and β -positions of DHA are 172.4 ppm and 172.0 ppm, respectively. Signals of other fatty acids are shifted. Using the chemical shift, the DHA position of TG in fish oils can be analyzed.

The middle in the figure is percentage in the β -positions of DHA and EPA in TG of fish oil. The

Table 1 Comparison of DHA Content from the Interlaboratory ^1H NMR Analysis between Japan and Norway Together with GC Data.

Sample oil Data		DHA content (mg/g)			DHA proportion (mol%)			n-3 fatty acids proportion (mol%)		
		Norway [#]	Japan [#]	GC	Norway [#]	Japan [#]	GC	Norway [#]	Japan [#]	GC
No.1	Average	248.17	276.07	267.58	27.03	27.45	24.50	32.05	32.08	33.60
Bonito	CV	0.64	1.33	1.67	0.78	0.46	2.49	0.08	0.04	2.03
No.2	Average	123.79	122.57	123.40	11.93	12.01	11.00	22.56	22.24	21.62
Tuna	CV	4.01	1.38	0.73	2.98	0.43	1.71	0.85	0.40	1.35
No.3	Average	214.51	208.27	215.70	20.65	20.71	20.16	29.08	28.79	29.93
Tuna	CV	2.72	0.91	0.20	0.66	2.02	1.43	0.05	0.73	0.89
No.4	Average	217.97	206.73	215.42	21.18	20.75	19.69	32.22	31.79	32.11
Tuna	CV	3.84	0.07	0.72	1.59	0.47	1.35	0.23	0.34	1.07
No.5	Average	111.70	111.07	105.99	10.65	10.76	10.18	28.03	28.13	28.60
Salmon	CV	3.27	0.95	1.47	0.88	0.32	1.15	0.32	0.33	0.74

Ethylene glycol dimethyl ether was used as internal standard.

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^1H NMR analysis with 30s pulse repetition time. **Fig. 3** Chemical Shift of DHA by NMR and the Ratio of DHA at β -Position.

circle indicates DHA and the triangle shows EPA. It is easily recognized that DHA is connected in the β -position rather than that of EPA.

The figure below is the groups of tuna and bonito, sardine, and other fish. Tuna and bonito have higher DHA portion at β -position than sardine. It could be recognized that NMR analysis of DHA is more precise than that of GC method.

2.3 Analysis of TG Molecular Species by LC-MS

For utilization of fish oil, the analysis of TG molecular species is another requirement in recent years from the points of structured lipids related to the physical and metabolic functions.

To identify fish oil molecular species of TG is needed to understand and utilize those oils for metabolic cycle. LC-MS instrumentation is one of recent topics to analyze the molecular species. Recently, catfish has examined as the one of popular fish in fresh water in the world to have relatively simple fatty acid composition rather than marine fish species.

The TG molecular species between Thai and Japanese species are shown in Figs. 4 and 5.

The right side of the above in Fig. 4 is the chart of TG obtained from the skin oil of Thai catfish, while the left one is the sample obtained in Japan.

As one of example, the peak 7 of Thai species is shown in the below in Fig. 4, where the fragment pattern at positive ions are indicated right side and negative ions are shown in the left side. The fragment of m/e 859.5 shows the molecular ions of the TG, and the pattern of diacylglycerol (DG) has two fragments. The m/e 603.5 is the fragment without palmitic acid

Fig. 4 Comparison of HPLC Patterns of Japanese and Thai Catfish and the Fragments of Peak 7 on the HPLC Chromatogram of Thai Catfish.

Fig. 5 Comparison of Molecular Species between Japanese and Thai Catfish.

and the m/e 577.5 means DG disconnected of oleic acid from the TG.

The negative fragments indicate oleic and palmitic acids separated from the glycerol structure. From these results, each of TG molecular species is easily identified.

As shown in Fig. 5 Japanese species complicated on TG molecular species combined with higher amount of DHA. The difference of the molecular species

between Thai and Japanese is based on the different of feed in both countries. The chicken droppings are used as the feed for catfish in Thailand, and the fish meals from sardine are mainly used in Japan.

Figure 6(4) shows the HPLC patterns of TG molecular species of tuna, bonito, and sharks. Sharks contained high amount of DHA and especially hammer head shark contains higher amount of tri-DHA than other species.

* indicated the poster number in JAWC 2000

Fig. 6 Comparison of LC Chromatograms of Triglyceride Molecular Species of Fishes (4).

Fig. 7 Major Volatile Compounds Identified in Crude Bonito and Tuna Oils.

3 Lipid Flavor of Fish

In the field of fish oil utilization, the undesirable flavor of the product should be restricted to prevent odors from PUFA as reversed grassy smell.

Big-eye and southern blue-fin tuna were used as the high content of DHA oil in the fish oils, while yellow-fin has slightly low levels.

3.1 Flavor Compounds from Fish Species

Figure 7 shows the pattern of the flavor compounds from the head oils of big-eye tuna and bonito (5). The analysis was performed using 500 mg oil heated at 30°C, and the volatile compounds were purged to polymer tenax TA at purge flow rate 50 mL/min for 20 min. After 10 min desorption at 170°C, volatiles were analyzed by GC-MS.

The identified 45 compounds were composed 86.5% of all flavor compounds of the sample.

Heptane, 2-ethylfuran, ethyl vinyl ketone, hexanal, *trans*-2-pentenal, 1-penten-3-ol were significant compounds from the tuna head oil.

On the other hand, bonito oil contains almost the same compounds as well as additional compounds such as propanal, 2,3-pentanedione, and 2,4-heptadienal.

The organoleptic evaluation indicates the difference of the fishy flavor between tuna and bonito, showing that bonito has a much heavier and complicated odor than tuna oil flavor. It might be considered that bonito flavor is more complicated than that of tuna flavor derived through the oxidation.

In both oils, 1-penten-3-ol was the major component at 18% and 15% in bonito and tuna, respectively. The threshold of the substance is known to be 9.2 ppb, which contributes to the grassy odor of the marine oil. For instance, from the report of the volatile compounds in sardine oil, 1-penten-3-ol contained 22% and propanal contained 17%. Both are influencing compounds for their sardine like fishy odor. Marine flavors from bonito and tuna oils are also influenced by the high amount of 1-penten-3-ol, along with other aldehydes, especially with propanal to produce the

Fig. 8 Peak Patterns and Flavor Compounds Produced from Bonito Triglyceride Through the Different Oxidative Conditions.

heavy fishy flavor.

2-pentylfuran has been identified only in bonito oil, which was presumably formed by the autoxidation of linoleate, and was identified as the reversion compounds and imparts a bean and grassy flavor when added to the oil.

Aldehydes such as hexanal, heptanal and *trans*-2-hexenal were also found in high concentrations. These compounds contribute to the formation of the heavy green, cut grassy aroma in the oils. Particularly, tuna oil contained a high amount of *trans*-2-hexenal. This compound contributes most of the grassy flavor to the tuna oil.

3.2 Analysis of Fish Oxidized Flavor by SPME

The recent analytical method was examined by solid phase micro extraction (SPME) for identifying flavor compounds from oxidized fish oil by heating with micro oven at 170°C and also the autoxidized oil at 30°C prepared from bonito with high content of n-3 fatty acids. For the analysis of flavor compounds, both oils were maintained with the evaluation by the anisidine value at 40 (6).

Figure 8 is the chromatogram after 8 days and two major unknown peaks are appeared in addition of the specific compounds of grassy odors such as 2,4-heptadienal, 2-octenal, and 4-heptenal. The unidentified peaks were observed in autoxidized fish oil that might be produced by the ring cleavage compound through the oxidation product based on n-3 fatty acid carbon structure.

The some major peaks derived in autoxidation were not observed on the same sample through the photoxidation.

4 Topics in the Lipid World and Antioxidants for Preventing PUFA Oxidation

For the utilization of fish oil after getting the precise analysis of PUFA, how to prevent the oxidation is important. It is not enough to prevent the fish oil with natural antioxidant though tocopherols and polyphenols in food are the most popular one.

The new compound founded at egg roe recently is the similar structure to α -tocopherol and more research will be needed to evaluate the antioxidant activity for fish oils.

4.1 CLA Analysis

Another topic of lipid analysis is conjugated linoleic acid (CLA). CLA discovered in 1966 has a great interest for the functional lipid in recent years for

Chelating effects of IP₂₋₆ on Fe²⁺ and Cu²⁺.

Synergistic effect of IP6 and α -Tocopherol.

Fig. 9 Antioxidant Activity of Inositol Phosphate Applied for Preventing Bonito Oil Oxidation.

* Numbers 1 and 2 show the different parts of the femur.

Fig. 10 Bone Density of Rats Measured by pQCT Method.

preventing the cancer. But after the discovery, little research has performed on the compounds.

After the paper of "A beef-derived mutagenesis modulator inhibits initiation of mouse epidermal

Fig. 11 Future Network of Lipid World and Related Subjects.

tumors by 7,12-dimethylbenz [a] anthracene", numerous of publications on CLA increased rapidly as more than 50 papers around in 1998 (7).

But among these papers, there is still little information on the analysis of the isomers of CLA. The analysis of the isomers is still difficult to be separated correctly.

The CLA analysis of commercial product by silver ion-high performance liquid chromatography using 3 series columns could be performed and obtained the good separation in 1998. The conjugated polyenoic fatty acids through using the derivatives of DMOX by LC-MS analysis are the target of many in the lipid world, but it remains the difficulties to identify all isomers.

4.2 Inositol Hexaphosphate for Antioxidant in Fish Oil

The inositol hexaphosphate (IP6) was examined as one of the natural antioxidants. IP6 is well known that it has the functional activity with the fiber and prevents the colon cancer. IP6 is the substance to prevent the absorption of minerals in the body, but it is well known that it is useful to diminish radical oxygen and lipid oxidation with iron and/or copper. The mechanism of the antioxidant effect for oil might be considered to be the potential of chelate activity with the phosphorus in the IP6 (8).

IP6 is hydrolyzed and decomposed *in vivo* to

produce IP1, 2, 3, 4, 5, and 6. Therefore, the activity of the antioxidant in each IP was analyzed. **Figure 9** is the result for bonito oil contained with high content of heme ion. The IP6 reacts with the highest prevention for the oxidation of bonito oil. This is now applying to use these compounds with tocopherols as the synergistic compound for the high content of PUFA in fish oil as shown in the below in **Fig. 9**.

4.3 Nondestructive Observation Analysis

The nondestructive observation analysis will be the major techniques in the new millenniums. **Figure 10** is the results of the study of "Influence of bonito DHA and the viscera extract and cuttle calcium on bone mineral density in rats" (9).

The picture obtained by p-QCT method is useful to identify the bone density of mammals using computer tomography.

In the picture of the **Fig. 10**, sample A4 is the result of non-calcium rat; G4 is the sample of taking together with DHA, calcium, and vitamin D. Observatory analysis is easy to admit the score for evaluation and assessment of the experiments.

This experiment revealed that the trabecular BMD level is maintained by the addition of DHA, the total and cortical BMD levels are maintained by the addition of vitamin D, and whole BMD is maintained by taking cuttle fish calcium together with vitamin D. As the future trend, it is needed to have more

combined research like nondestructive for evaluating the nutrient of lipid in mammals.

5 For Future Study in Lipid World

For the analysis, it is true that accurate, quick, reproducibility and deviation check among the organizations are important, but also it is needed to have less solvent for our safety and ecological clean circumstances for our earth.

The future trends of my image as shown in Fig. 11 on the lipid analysis, are supposed that Internet world will make us to exchange ideas with flush communication. All data analyzed and obtained should be transferred to some real and truth score library in the Internet.

In the library in the next new millennium, there will be established the lipid database and files in the World Wide Web site and it will be managed by nation organization to keep the accurate and real score by the analysis. The responsibility occurs to evaluate the analytical data and feedback to each people in anytime to need the real data and the evaluation through the Internet.

The idea how to establish the library of nation organization is that it might be supported by all countries and the effort of the center in lipid world such as mutual exchange of the organizations together with JOCS, AOCS, IUPAC, and AOAC.

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