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Isolation and Purification of DHA from Skipjack Orbital Tissue Oil

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Several methods were examined for purification of docosahexaenoic acid(DHA, 22:6n-3) from skipjack *Euthynnus pelamis* orbital tissue oil, a marine by-product, and a modified method for isolation of a high purity DHA was proposed.

Skipjack orbital tissue contained 55.4% of total lipid(TL), and DHA accounted for 23.7% of the TL.

Application of low-temperature crystallization and urea inclusion compound methods to the orbital fatty acid mixture resulted in increases of DHA concentrations to approximately 46% and 61%, respectively. These methods were suitable for large production of DHA with relative low purity because of the simple purification procedure. DHA of approximately 74% in purity was obtained by silver nitrate aqueous solution method, but the method gave a very low recovery(<10%). Silver nitrate-impregnated silica column chromatography was suitable for purification of a high purity DHA(purity, >98% and recovery, >90%). A modified method, silver nitrate-impregnated silica column chromatography combined with low-temperature crystallization(two step purication method) was proposed as the most effective method to obtain DHA with high purity(99.9 %) from the skipjack orbital oil.

Key Words: Skipjack Orbital Oil, Purification method, DHA

Introduction

In 1978, Dyerberg *et al.* demonstrated that the low incidence of cardiovascular diseases such as myocardial infraction and atheroscleosis in Greenland Eskimos may be associated with their food habit; consuming large amounts of fish and fish products containing high level of polyunsaturated fatty acid(PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid(DHA). Epidemiological studies on a fishing village in Japan have been carried out by Hirai *et al.*(1980). They also suggested that a high intake of EPA from fish contributes to the low incidence of cardiovascular diseases of the fishermen. Thereafter, many studies on the physiological role of fish oils have been carried out by a number of workers. As a result, it is known that PUFA have an inhibitory effects on the incidence of hypertension(Singer *et al.*, 1983), coronary heart disease(Bang *et al.*, 1980), rheumatic diseases(Kremer *et al.*, 1985), and cancers(de Bravo *et al.*, 1986). Most of the physiological studies on PUFA have been focused to EPA along with the development of a purification technique for EPA from fish oils.

Recently, it was suggested that DHA influences on the improvement of learning ability(Enslen *et al.*, 1991) and retina reflection ability(Akpalaba *et*

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al., 1986) and the control of senile dementia(Suzuki and Wada, 1988), differing from EPA in these physiological roles. Nowadays, EPA is on the market at about 95% in purity, whereas DHA on the market is only about 25% purity(Yazawa and Kageyama, 1991), because the purification technique of DHA has not been well established. Most fish oils are rich in EPA, while skipjack and tuna oils are rich in DHA, particularly in their orbital tissue oils. The head containing the orbital tissue in skipjack is generally abandoned or used a little for fertilizer when skipjack are manufactured as canned foods. Therefore, the orbital tissue oil could be an available resource for DHA. The production of the high purity DHA from marine by-products may be offered to many physiologists at a low price as well as increasing utilization of marine by-products.

In this study, several methods for purification of PUFA described previously(Gunstone *et al.*, 1976; Christie, 1982; Yazawa and Kageyama, 1991) are applied for DHA purification from the skipjack orbital tissue oil. And author describes that the modified purification procedure(two step purification method) resulted in a highly purified DHA.

Materials and Methods

Samples

Skipjack *Euthynnus pelamis* head was obtained from a canned factory in Chungmu, Korea. The skipjack head was separated into orbital tissue and total lipid(TL) extracted with a mixture of chloroform/methanol according to the Bligh and Dyer procedure(Bligh and Dyer, 1959). TL content was determined gravimetrically.

Hydrolysis of TL and esterification of fatty acids

An aliquot of TL was saponified at 85℃ for 1 hr with 1N KOH-ethanol and the free fatty acids liberated from the saponifiables with 6N HCl were extracted with diethyl ether. An aliquot of the fatty acid mixture was converted to their corresponding methyl and ethyl ester derivatives with BF_3-methanol and 5% HCl-ethanol, respectively.

Gas-Liquid Chromatography(GLC)

The fatty acid methyl and ethyl esters were analyzed by GLC using Shimadzu GC 14A instrument (Shimadzu Seisakusho Co. Ltd., Kyoto, Japan) equipped with a CARBOWAX 20M fused silica wallcoated open-tubular column(25 $m \times 0.25$ *mm*, i.d.). The injector and detector were held at 250℃, and the column at 190℃ or 210℃. The split ratio was 1:50. Helium was used as the carrier gas at the constant inlet pressure of 1.5 kg/cm^2. The fatty acids were identified by comparing the retention time and equivalent chain length of the standard or literature(Ackman, 1986). The fatty acid methyl ester standards used were as follows; 14:0, 16:0, 18:0, 18:1, 18:2, 18:3, 20:0, 22:0, 22:1, 24:0(D-104, Serdary Research Lab., Inc.), 20:0, 20:1, 20:2, 20:3, 20:4(08A, Nu-Chek-Prep., Inc.), anteiso 15:0, 15:0, iso 16:0, 16:0, anteiso 17:0, iso 18:0, 18:0(MIX BR3, Larodan Fine Chemicals), and 22:6n-3(Sigma Chemical Co., Louis, MO, USA). The 20:5n-3 acid(Kurida Kougyo Ltd., Tokyo, Japan) was used as a standard after methylation.

Low-temperature crystallization method

An aliquot of the mixed fatty acids in ten volumes of hexane was placed in a flask, flushed with nitrogen and capped. The flasks were held at -60℃, -70℃, and -80℃ for 12 hr. The solution was filtered immediately through a Buchner funnel kept at the corresponding temperature. The precipitate was washed with cold hexane. The highly unsaturated fatty acids were recovered from precipitates and the mother liquor was concentrated in a rotary evaporator(Gunstone *et al.*, 1976). The content of recovered fatty acids was determined gravimetrically and the fatty acid compositions were analyzed with GLC after methylation.

Urea inclusion compound formation method

An aliquot of the mixed fatty acids was placed in about fifty volumes of 1M urea-methanol solution and then heated at 50℃ for 1 hr. The mixture were cooled and left at -60℃ for 2 hr. The precipitates formed were removed by filtration using a Buchner funnel and washed with the urea-methanol solution previously kept at -60℃. The filtrate

was evaporated to remove methanol. After addition of water, the highly unsaturated fatty acids were extracted with hexane. This extraction procedure was repeated. The content of recovered fatty acids was determined gravimetrically and the fatty acid compositions were analyzed by GLC after methylation.

Silver nitrate aqueous solution method

Fifty volumes of 1N $AgNO_3$ aqueous solution were added to an aliquot of the mixed fatty acids and the mixture was stirred. The solid substances containing saturated and lower unsaturated fatty acids were removed through a Buchner funnel. PUFAs were dissociated from the filtrates(Ag^+-PUFA complex) by adding water and shaking vigorously. The dissociated fatty acids were recovered with hexane(Yazawa and Kageyama, 1991). The content of recovered fatty acid was determined gravimetrically and the purity was checked by GLC after methylation.

Silver nitrate-impregnated silica column chromatography

About 50 *g* of silicic acid(70~230 mesh, E. Merck, Darmstadt, Germany) was added to 150 *ml* of 3% $AgNO_3$-ethanol solution and the mixture was stirred for 10~20 min. The ethanol was removed in a rotary evaporator and then left in an oven at 120℃ for 2 hr to activate the silicic acid(Christie, 1982). The silver nitrate impregnated silicic acid was packed into the glass open column(30 *cm*×2.5 *cm*, i.d.) with 10% acetone-hexane. An aliquot of fatty acid methyl esters(or ethyl esters) was placed on the column and sucessively eluted with 10% (400 *ml*), 20%(200 *ml*), 40%(200 *ml*) and 60% (200 *ml*) acetone-hexane at the flow rate of 1~2 *ml*/min. Ten *ml* portions of the effluent were collected with a fraction collector. Fatty acid composition in the collected effluent was determined by GLC after washing with 1% NaCl, followed by water to remove Ag^+ ion. The contents of DHA and EPA fractions were determined gravimetrically.

Low-temperature crystallization/silver nitrate-impregnated silica column chromatography(two step purification method)

In order to obtain a high purity DHA, the two step purification method that consists of a concentration step and a further purification step was applied. The fatty acid mixture was, first, concentrated to approximately 40% of DHA by the low-temperature crystallization method, and then the concentrates were further purified by silver nitrate-impregnated silica column chromatography described above section.

Results and Discussion

Total lipid content and fatty acid compositions

Total lipid(TL) content of the skipjack orbital tissue was 55.4%, which was about 80-fold compared with that(0.7%) of skipjack muscle(Ohshima *et al.*, 1984). Table 1 shows the fatty acid compositions of the TL. The prominent fatty acids were 22:6n-3 (DHA, 23.7%), 16:0(21.2%), 18:1n-9(17.0%), 16:1 n-7(7.22%), 18:0(5.50%), and 20:5n-3(EPA, 5.24 %). The percentage of DHA in the TL was similar to that of skipjack muscle TL, whereas it was about 2-fold higher than those of mackerel(13.2%) and sardine(10.7%)(JAOA, 1989). Therefore, the skipjack orbital oil could be a good source of DHA. Furthermore, the utilization of marine by-products for DHA can provide a high value for skipjack food industry, because the skipjack head is mostly abandoned during processing of canned food.

Low-temperature crystallization method

Gas-liquid chromatograms of the concentrates obtained by low-temperature crystallization are shown in Fig. 1. The percentages of the concentrates obtained from the fatty acid mixture at -60℃, -70, *and* -80℃ were 28.3%, 27.9%, and 21.8%, respectively. The level of DHA and EPA in the concentrates at each temperature was 39.3% and 8.82 %, 42.4% and 9.54%, and 46.4% and 11.1%, respectively. Therefore, with this treatment at lower temperature, the level of DHA and EPA increased 2-fold higher than those in the original fatty acid mixture. From these results, it was suggested that the fatty acid mixture is necessary to be held at -70℃, to obtain DHA above 40% purity.

Table 1. Fatty acid compositions of the skipjack orbital oil*

Fatty acid	Area, %	Fatty acid	Area, %
14:0	3.57 ± 0.03	20:1n-7	0.12 ± 0.01
15:0 iso	0.26 ± 0.00	20:2n-6	0.31 ± 0.02
15:0	1.08 ± 0.01	20:3n-6	0.06 ± 0.02
16:0 iso	0.12 ± 0.00	20:4n-6	1.93 ± 0.03
16:0	21.2 ± 0.05	20:3n-3	0.15 ± 0.01
16:1n-7	7.22 ± 0.01	20:4n-3	0.36 ± 0.02
17:0	2.60 ± 0.02	22:0	0.23 ± 0.03
17:1n-8	0.99 ± 0.01	20:5n-3	5.24 ± 0.03
17:2n-8	0.23 ± 0.02	22:1n-11	0.14 ± 0.01
18:0	5.50 ± 0.01	22:1n-9	0.10 ± 0.01
18:1n-9+7	17.0 ± 0.03	21:5n-3	0.14 ± 0.01
18:2n-7	0.14 ± 0.00	22:3n-3	0.24 ± 0.01
18:2n-6	1.30 ± 0.02	22:5n-6	1.66 ± 0.04
18:2n-4	0.66 ± 0.01	22:5n-3	1.18 ± 0.07
19:0	0.15 ± 0.01	22:6n-3	23.7 ± 0.24
19:1+18:3n-4	0.28 ± 0.02		
18:3n-3	0.38 ± 0.01	Σ Saturated	35.1
19:3n-6	0.59 ± 0.01	Σ Monoenoic	26.4
20:0	0.35 ± 0.00	Σ Polyenoic	38.6
20:1n-9	0.87 ± 0.03	Total	100.1

* The data are presented as the mean ± standard deviation of three determinations.

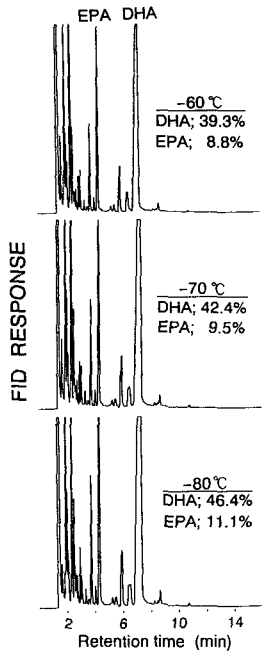

Fig. 1. Gas-liquid chromatograms of fatty acid methyl esters concentrated from the skipjack orbital oil by low-temperature crystallization method at -60℃, -70℃ and -80℃. Column; a CARBOWAX 20M fused silica wall-coated open-tubular, 25 *m*×0.25 *mm* i.d., Temp., 210℃; Injection temp., 250℃; Detector, FID; Carrier gas, He(1.5 *kg/cm*^{2}).

Urea inclusion compound method

Fig. 2 shows the gas-liquid chromatogram of the concentrate obtained from the skipjack orbital fatty acids by urea inclusion compound method. Approximately 25.6% of concentrates was obtained from the original fatty acid mixture. The percentages of DHA and EPA of the concentrate were 61.1% and 10.7%, respectively. These percentage are 2.5-fold higher than those of the original fatty acid mixture.

This method has been widely used for purification of linoleic or linolenic acids from vegetable oils (Swern and Parker, 1953; Johnson and Ali, 1961; Gunstone *et al.*, 1976), whereas rarely used for DHA or EPA from marine oils. Haagsma *et al.* (1982) reported that percentages of DHA and EPA were 44.6% and 27.6% in the concentrates(26.5%) obtained from cod liver oil(included 11.7% of DHA

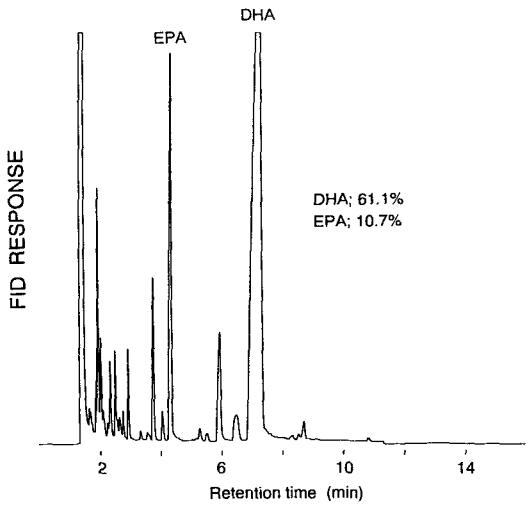

Fig. 2. Gas-liquid chromatogram of fatty acid methyl esters concentrated from the skipjack orbital oil by urea inclusion compound method. Conditions are the same as in Fig. 1.

and 12.1% of EPA) by urea inclusion compound method. These results were lower in the percentage of DHA but higher in EPA than those of the present study. These differences are attributable to the differences in the fatty acid compositions between both original oil samples used.

Silver nitrate aqueous solution

Fig. 3 shows gas-liquid chromatogram of DHA and EPA purified by silver nitrate aqueous solution method. Approximately 8.3% of PUFA-rich fraction was obtained from the original fatty acid mixture by this method. The DHA and EPA percentage of the fraction were 74.0% and 16.5%, respectively. The percentage of DHA in the fraction obtained by this method was higher than that in the fraction obtained with the low-temperature and urea inclusion compound method described above, whereas the former method was poor in recovery compared with the latter. DHA fraction obtained in this study were lower in purity than that of Yazawa and Kageyama(1991), but similar in recovery.

The methods described above, for example, low-temperature crystallization, urea inclusion compound and silver nitrate aqueous solution methods, are unsuitable for isolation of DHA and EPA with purities above 90%. However, low-temperature crystallization and urea inclusion compound methods may be suitable for large scale separation of partially purified DHA and EPA fractions from the original fatty acid mixture. This partially purified DHA fraction was further treated with the procedure as will be mentioned later, to obtain DHA with higher purity.

Silver nitrate-impregnated silica column chromatography

Gas-liquid chromatograms of DHA methyl and ethyl esters and EPA methyl ester purified by silver nitrate-impregnated silica column chromatography are shown in Fig. 4, 5, and 6, respectively. The DHA methyl ester was purified to 98.4%(recovery, >90%) and the ethyl ester to 90.8%(recovery, >94%). On the other hand, EPA methyl ester was purified to 84.0%(recovery, >84%). This method was evaluated as the most effective method among the others described above to obtain the high purity DHA from the skipjack orbital oil. Teshima *et al.*(1978) studied on the purification of EPA and

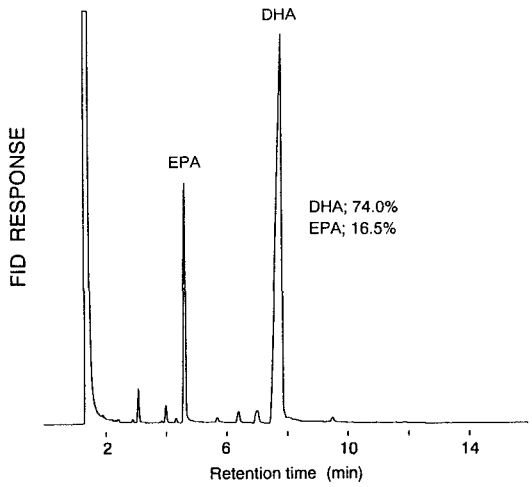

Fig. 3. Gas-liquid chromatogram of fatty acid methyl ester concentrated from the skipjack orbital oil by silver nitrate aqueous solution method. Conditions are the same as in Fig. 1.

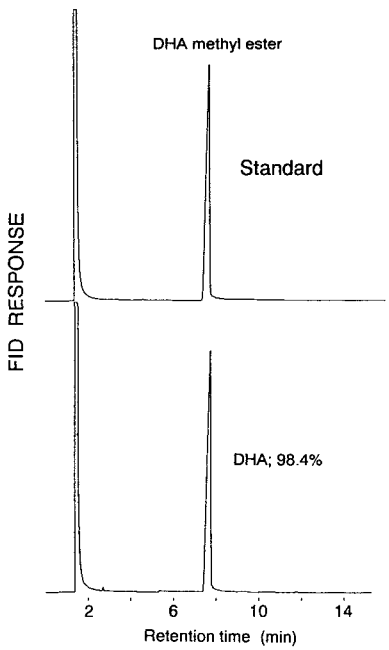

Fig. 4. Gas-liquid chromatograms of DHA methyl ester purified from the skipjack orbital oil by silver nitrate-impregnated silica column chromatography. Conditions are the same as in Fig. 1.

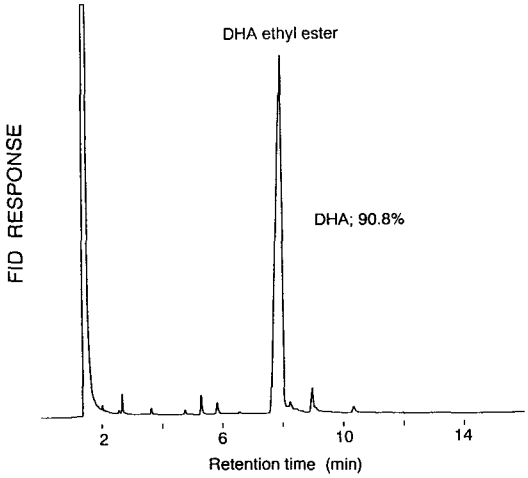

Fig. 5. Gas-liquid chromatogram of DHA ethyl ester purified from the skipjack orbital oil by silver nitrate-impregnated silica column chromatography. Conditions are the same as in Fig. 1.

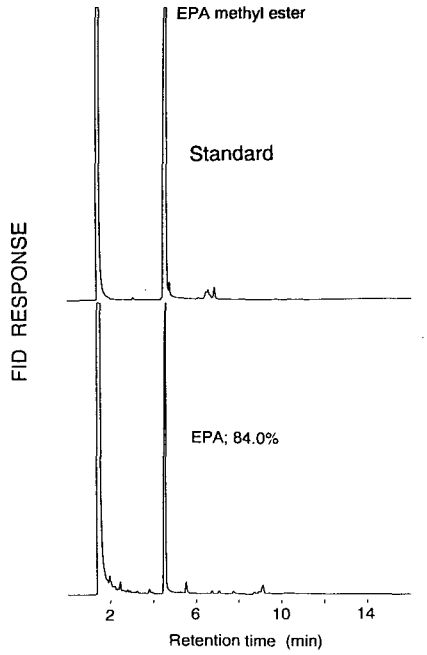

Fig. 6. Gas-liquid chromatograms of EPA methyl ester purified from the skipjack orbital oil by silver nitrate-impregnated silica column chromatography. Conditions are the same as in Fig. 1.

DHA from squid-liver oil by the same method, using petroleum ether:diethyl ether(98:2, v/v) as an eluent. From those results, they reported that EPA was purified to 97~100%(recovery, 49.4%), and DHA to 99~100%(recovery, 19.0%). These results were superior to those of the present study in the aspect of purity, but not in the aspect of recovery.

Low-temperature crystallization/silver nitrate-impregnated silica column chromatography(Two step purification method)

Fatty acid methyl esters were subjected to low-temperature crystallization method(first step) and subsequently to silver nitrate-impregnated silica column chromatography(second step). The fatty acid mixture(23.7% of DHA) were purified to 46.4 % of DHA by the first step. As shown in Fig. 7, from the DHA-rich samples concentrated by the fi-

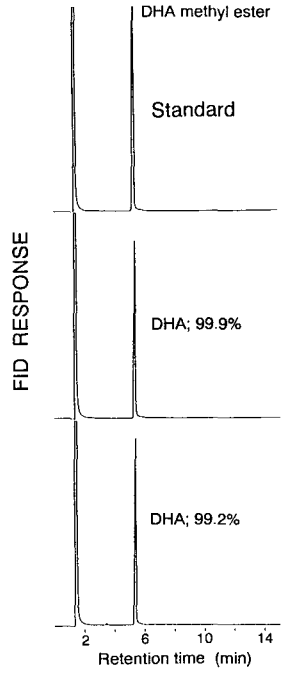

Fig. 7. Gas-liquid chromatograms of DHA methyl ester purified from the skipjack orbital oil by silver nitrate-impregnated silica column chromatography combined with low-temperature crystallization(two step purification method). Conditions are the same as in Fig. 1, except that column temperature is 230℃.

rst step, the fractions of DHA with 99.2% purity (recovery, 18.6%) and DHA with 99.9% (recovery, 52.4%) were obtained by the second step.

Tokiwa *et al.*(1981) reported that DHA methyl ester was purified to 85.5% (recovery, 53.3%) from the previously concentrated fatty acid methyl ester (included 30% of DHA) by a reversed phase high performance liquid chromatography. The DHA preparation was insufficient in both purity and recovery compared with that of the present study. On the other hand, Hayashi and Kishimura(1993) studied on the preparation of n-3 PUFA ethyl ester concentrates from skipjack orbital tissue oil by silica column chromatography. As the results, they reported that DHA and EPA were concentrated to 82% and 11%, respectively. This method, however, resulted in a mixture of DHA and EPA.

Consequently, silver nitrate-impregnated silica column chromatography combined with low-temperature crystallization was evaluated as the most effective method in both aspects of the purity and recovery of DHA from the skipjack orbital oil among the methods described above. However, this study was carried out on a laboratory scale. Therefore, further study needs to examine on isolation of DHA with higher purity from skipjack orbital tissue oil in larger scale.

In 1990, the production of canned tuna commodity was about 37,000 M/T, accounting for 64% of total production of canned fishery commodities in Korea(Ministry of Agriculture, Forestry and Fisheries, 1991). In tuna cannery, about 40% of fish body(edible part) is used as raw materials and the remain is abandoned or used slightly as fertilizer. Therefore, non-edible part of skipjack, a marine by-product, can be used as an available resource for DHA.

In the present study, it is expected that the production of the high purity(>99.0%) DHA from the skipjack orbital tissue oil may raise the added value in skipjack(or tuna) food industry as well as be supplied to physiologists at a low price. Furthermore, its evailability could boost utilization for medical supplies and use as supplementary food for human health.

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가다랭이 안와조직으로부터 DHA의 추출 및 정제

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가다랭이 안와油로부터 docosahexaenoic acid(DHA)를 정제하기 위해 기존의 방법들을 적용하여 비교 검토하고, DHA의 효과적인 정제를 위해 조작방법을 개량하였다.

가다랭이 안와조직의 총지질은 55.4%였으며, 이 중 DHA는 23.7%였다. 저온분별결정법과 요소결정법을 적용한 결과 순도에서 각각 약 46% 및 61%의 DHA가 얻어졌다. 이들 방법들은 순도면에서는 다소 떨어지나, 정제조작이 단순하여 다량의 DHA 분리에 적합하였다. 질산은 수용액법은 상기 2가지 방법에 비하여 순도면에서는 약간 개선되었으나, 회수율이 대단히 낮았다(10% 이하). 질산은 함침 실리카 칼럼 크로마토그래피법은 고순도 DHA의 정제방법으로써 적합하였다(순도 98% 이상, 회수율 90% 이상). 결과적으로 저온분별결정법과 질산은 함침 실리카 칼럼 크로마토그래피법을 조합한 개량법(2단계 정제법)이 가다랭이 안와油로부터 고순도 DHA(99.9%)의 정제를 위한 가장 효과적인 방법으로 평가되었다.