

Selective Enrichment of EPA and DHA by Adduct Formation of Fatty Acids of Fish Oil and Urea

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Abstract

A modified method of urea adduct formation, in which water and organic solvent were used as the wetting agent and the reaction medium, respectively, is suggested to obviate methanolysis and to ease recovery in the separation of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish oil. With the new method the fraction in which the total content of EPA, DHA and their precursors is more than 80% could be obtained. Although the total content of precursors in the concentrate was hardly affected by the kind of the wetting agent or the organic solvent, the content of EPA and DHA varied significantly depending on the organic solvent. This finding made it possible to selectively enrich the desired components. After DHA-enriched fraction (DHA is 50%) was obtained by using pentane, EPA-enriched fraction (EPA is 53%) could be obtained from the residue of DHA-enriched fraction by using heptane.

Key words: EPA and DHA, urea adduct, wetting agent, organic solvent, enrichment

Introduction

Epidemiological studies have revealed that human populations who are consuming 200-400g fish/day (*e.g.*, Greenland Eskimos) are less prone to coronary heart disease as compared with those who are consuming limited amount of fish⁽¹⁻³⁾. It is believed that the important components yielding this epidemiological result are polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), especially with reference to EPA. It is well known that fish is a plentiful source of those fatty acids. Thus more consumption of fish may be beneficial to the reduction of atherosclerosis and coronary heart disease, the leading cause of debility and death in many countries.

Considering the dietary habit of many people, it seems very difficult to consume daily such a large amount of fish. If provided in a concentrated form of EPA or DHA, the intake volume can be reduced markedly. Among the available separation methods of PUFA from fats and oils⁽⁴⁻⁶⁾, urea inclusion formation⁽⁷⁾ is of value for industrial-scale production. Although numerous methods of preparing urea inclusion compounds have been described, most of the preparative techniques commonly used methanol as both a reaction solvent and a wetting agent of

urea. The procedures, however, suffer from the recovery of PUFA after filtration and methanolysis of fatty acids during the urea adduct formation. With an organic solvent and small amount of methanol as the reaction medium and the wetting agent, respectively, the conventional methods could be improved in the aspect of recovery of PUFA, but methanolysis is still a problem.

This paper describes a new way of adduct formation of urea and fatty acids and the factors affecting the efficiency of the procedure with respect to the yield and the content of EPA, DHA and their precursors in the concentrate.

Materials and Methods

Materials

Partially refined fish oil was a gift of Seoul Food Industrial Company, Ltd. (Seoul, Korea). Urea was purchased from Duksan Pharmaceutical Co., Ltd. (Kyunggi-Do, Korea) and the standard methyl esters of fatty acids were obtained from Sigma (St. Louis, USA). Organic solvents were of analytical grade.

Preparation of fatty acids

Preparation of fatty acids was carried out according to the procedure of Haagsma *et al.*⁽⁸⁾. Fish oil was hydrolyzed in NaOH solution containing Na₂EDTA. After saponification, the unsaponifiable

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matter was removed by adding hexane to the solution, stirring the mixture for 1 hr, and discarding the hexane layer. Upon lowering the pH of the remaining solution to 1.0, two layers appeared again and the upper hexane layer containing the free fatty acids was evaporated in a rotary evaporator at 30°C. This preparation was used as the starting material for concentration.

Urea adduct formation

In order to investigate the potential of water, formamide, acetonitrile, or alcohols as a wetting agent of urea, 1.2 ml of each wetting agent and 30 g of urea were added to pentane solution containing 10g of fatty acids. The mixture was intermittently shaken overnight at room temperature to form the adduct of urea and fatty acids. The mixture was then filtered through a glass filter and thoroughly washed with the same organic solvent. The combined filtrate was washed with water to remove unreacted urea. The fatty acid composition of this fraction was analyzed after evaporating the organic solvent.

In an experiment to study the effect of organic solvents on the fatty acid compositions in the concentrate, 1.2 ml of water was used as wetting agent, and the weight ratio of urea to fatty acids was 3. The amounts of agent and urea are recommended amount by Swern⁽¹⁰⁾.

Preparation of DHA- or EPA-enriched fraction

To 10g of fatty acids dissolved in pentane, 30g of urea and 0.6 ml of water were added. The mixture was allowed to form urea adduct as described above. After the reaction, it was filtered through a glass filter and the filtrate was evaporated to be the DHA-enriched fraction.

After evaporating the filter cake to dryness under a stream of nitrogen, 100 ml of heptane were added. The slurry was refluxed for 1 hr at the boiling point of the solvent to dissociate the urea complexes, cooled to room temperature, and then stood overnight to reform urea adduct. Again, non-adduct forming fraction was collected. This is the EPA-enriched fraction.

Analysis of fatty acid composition

According to the procedure of AOCS⁽⁹⁾, fatty acids were esterified and the methyl esters were

analyzed with a Varian gas chromatograph, model Vista 6000. A stainless-steel column (3m × 2mm id) packed with Silar 7 CP on Chromosorb W-HP (Supelco Inc., Bellefonte, USA) was operated by temperature programming from 185 to 235°C. The response of a flame ionization detector was analyzed with a Data System model DS601 of Varian and each component was identified by comparing it to the retention times of standard methyl esters of fatty acids.

Results and Discussion

Since the use of methanol is the cause of unwanted methanolysis in preparing a concentrate of omega-3 fatty acids from fish oil by the conventional urea adduct formation, one of the plausible ways to improve these methods may be the replacement of methanol with other solvents. When urea adduct formation was tried by using pentane as a reaction medium and water, formamide, acetonitrile or alcohols as the wetting agent of urea, the compositions of EPA, DHA and their precursors (Omega-3 series of C18:3, C20:4 and C22:5 are known to be the precursors of EPA and DHA) in the non-adduct forming fractions are shown in Table 1. The total contents of them were higher than those in the starting material, meaning that all the reagents tested could be used as the wetting agent of urea. The common property of the reagents is that they can dissolve or wet urea and are immiscible with the reaction solvent. Water seems to be the most preferable wetting agent because it is quite a bargain and absolutely free from toxicity.

It was interesting that although the total content of EPA, DHA and their precursors was hardly affected by the kind of wetting agent, the content of EPA and DHA varied. EPA ranged from 15.5% to 25.9% and DHA from 40.2% to 49.1%. The weights of each concentrate obtained from 10g of the starting material varied from 1.6g to 2.2g as shown in Table 1.

One of the advantages in using water as the wetting agent is that more diverse organic solvents can be used as the reaction medium for urea adduct formation because it is the reagent with the highest polarity and thus immiscible with most of the organic solvents. Ten different organic solvents were

Table 1. Effect of the wetting agent on the enrichment of EPA, DHA and their precursors by urea adduct formation method

Wetting agent	Fatty acid composition (area %)				Weight obtained (g)
	EPA	DHA	Σ Precursors of EPA and DHA	Others	
Starting material	15.5	9.8	7.4	67.3	10.0
Water	15.5	49.1	14.9	20.5	1.7
Acetonitrile	19.4	46.1	15.3	19.2	1.8
Formamide	15.5	48.8	15.4	20.3	1.6
Ethanol	25.9	40.2	15.7	18.2	2.2
Methanol	21.7	44.5	15.0	18.8	1.9

Table 2. Effect of the organic solvent on the enrichment of EPA, DHA and their precursors by urea adduct formation method

Organic solvent	Fatty acid composition (area %)				Weight obtained (g)
	EPA	DHA	Σ Precursors of EPA and DHA	Others	
Starting material	15.5	9.8	7.4	67.3	10.0
Methylene chloride	35.8	24.2	16.3	23.7	3.1
Heptane	35.8	30.6	13.8	19.8	2.9
Benzene	35.3	26.4	16.0	22.3	3.1
Xylene	34.7	25.5	15.3	24.5	3.3
Ethyl ether	33.9	23.6	15.4	27.1	3.8
Cyclohexane	26.2	31.8	14.8	27.2	2.5
N-hexane	22.5	42.8	15.0	19.7	2.0
Isooctane	19.3	48.0	14.5	18.2	1.8
Pentane	15.5	49.1	14.9	20.5	1.7
1,4-dioxane	14.8	9.2	7.2	68.8	10.2
N-hexane ^{a)}	17.2	10.5	8.1	64.2	8.0

^{a)}In this case, urea adduct formation was carried out without the wetting agent.

tested for the urea adduct formation. Table 2 lists the fatty acid compositions of the concentrates. The total content of EPA, DHA and their precursors increased from 32.7% in the starting material to more than 72.8% in the concentrate when 9 kinds of water-immiscible organic solvents except 1,4-dioxane were used as reaction media for urea adduct formation, indicating that the immiscibility of organic solvent with the wetting agent is an essential property to be used as the reaction solvent.

It was remarkable that the composition of EPA and DHA varied significantly according to the organic solvents with the total content of them nearly unchanged. Pentane and isooctane were found to be effective for the concentration of DHA, while for that of EPA, heptane, methylene chloride, benzene, xylene and ethyl ether were more effective. For example, when the organic solvent was changed from

pentane to methylene chloride the composition of DHA was reduced to half level, while that of EPA was doubled when the organic solvent was changed from pentane to methylene chloride. An attempt to correlate the selectively enriched phenomena of EPA or DHA with the chemical or physical properties of the organic solvent failed. In this regard, it is necessary to continue investigations on why the fatty acid composition of the non-adduct forming fraction varies depending on the wetting agent or the organic solvent. If the wetting agent, water, was not added, urea adduct was not formed and thus EPA, DHA and their precursors were hardly enriched. The very slight increase of their contents in the concentrate seemed to be due to the water inherent in the urea. Depending on the reaction medium, the yields of DHA ranged from 77% to 91%, while those of EPA varied tremendously in the ran-

Table 3. Effect of the amount of water or the weight ratio of urea to fatty acids on the enrichment of EPA, DHA and their precursors by urea adduct formation method

Organic solvent	Weight ratio of urea to fatty acid	Water (μ l)	Fatty acid composition (area %)			
			EPA	DHA	Σ Precursors of EPA and DHA	Others
Pentane	1	200	21.4	15.0	10.5	53.1
	2	150	32.0	27.9	15.6	24.5
	2	400	31.8	27.7	15.6	24.9
	2	600	31.8	26.5	15.3	26.6
	3	200	23.3	43.2	15.4	18.1
	3	600	15.5	49.1	14.9	20.5
	3	900	15.2	49.6	14.6	20.6
	5	1,000	15.2	49.3	14.7	20.8
Heptane	1	300	31.4	22.8	13.1	32.7
	2	250	33.9	24.3	13.9	27.9
	2	500	33.1	24.4	13.7	28.8
	3	300	36.9	30.3	13.9	18.9
	3	600	35.8	30.6	13.8	19.8
	3	900	35.8	29.1	13.6	21.5
	3	1,300	32.7	26.8	14.0	26.5
	4	500	34.4	34.8	14.0	16.8
	4	1,000	33.5	34.9	13.8	17.8

ge of 17-83%. Heptane seemed to be the most effective organic solvent for the concurrent concentration of both EPA and DHA.

It can be inferred from the result of Table 2 that the successive fractionation of fatty acids with pentane followed by heptane may selectively enrich DHA and EPA, respectively. To determine the proper conditions for the selective enrichment, the fatty acid compositions of the concentrates obtained at the various water contents and weight ratios of urea to fatty acids were analyzed (Table 3). When pentane was used to fractionate DHA more efficiently, the contents of DHA increased by 3 times as compared with the content of the starting material at the 2:1 weight ratio of urea to fatty acids. When the ratio is above 3, DHA comprised a half of the concentrate, which corresponds to 5 times as much as that of the starting material. The total contents of EPA, DHA and their precursors reached nearly 80% of the concentrate. On the other hand, when heptane was used as the reaction solvent to enrich EPA, the weight ratio of urea to fatty acids did not have a significant effect on the enrichment of EPA if it was above 1. DHA content, however, ranged broadly from 22.8% to 34.9%. Other conditions being the same, the amount of water did not seem to affect significantly the enrichment of EPA and

DHA if it is within a range from 2% to 6.7% as weight percent against urea. The amount of water below or above this level was not desirable for concentration of the desired fatty acids.

It is fortunate that the optimal conditions of separating DHA- and EPA-enriched fractions with pentane and heptane, respectively, was found at almost the same amount of water and weight ratio of urea to fatty acids. Without adding or subtracting water and urea, further procedure to obtain EPA-enriched fraction can be carried out with the filter cake remained after separating the DHA-enriched fraction from the starting material. To selectively enrich EPA, the filter cake was evaporated to dryness under a stream of nitrogen and 2 volumes of heptane were added. After refluxing the slurry for 1 hr at the boiling point of the solvent to dissociate the urea complexes, it was cooled to room temperature and then stood overnight to reform the urea adduct. Again the non-adduct forming fraction was collected and the fatty acid composition was analyzed. As shown in Table 4, EPA composed more than a half of the concentrate and the total content of EPA, DHA and their precursors was 75.2%. DHA was the secondly abundant fatty acid, but its level was reduced as compared with the starting material. This makes it more plausible to use this EPA-

Table 4. Fatty acid composition (%) of starting material and EPA- or DHA-directed fraction

Fatty acid	Starting material	DHA-enriched fraction	EPA-enriched fraction
14:0 ^{a)}	6.9	0.7	0.9
15:0	0.5	0.3	—
16:0	16.5	0.1	0.7
17:0	0.9	0.4	1.2
18:0	2.9	2.3	0.7
14:1	0.2	—	0.4
16:1	7.9	1.1	4.0
17:1	1.0	0.3	1.7
18:1	11.6	0.6	2.8
20:1	5.8	0.2	0.6
22:1	1.6	2.6	1.3
18:2 ω 6	1.5	0.2	2.2
20:2 ω 6	0.3	0.2	0.4
18:3 ω 3	0.7	0.2	2.2
18:3 ω 6	0.4	0.7	0.3
20:3 ω 6	0.2	0.2	0.6
16:4 ω 3	2.0	6.0	2.3
18:4 ω 3	3.4	12.4	5.6
20:4 ω 3	1.0	1.6	3.2
20:4 ω 6	5.2	0.8	2.2
22:4 ω 6	—	0.4	—
20:5 ω 3	15.5	15.5	53.0
22:5 ω 3	2.1	0.7	6.1
22:5 ω 6	0.3	0.7	0.3
22:6 ω 3	9.8	49.1	7.0
Unknown	1.8	2.7	1.2
Weight obtained (g) 10		1.7	1.7

^{a)}Number of carbons: Number of double bonds

enriched concentrate as a cardioprotective agent and also for other medical applications⁽¹¹⁾ because recent reports claimed that DHA was found to be a strong inhibitor of prostaglandin biosynthesis⁽¹²⁾.

In the conventional methods of urea adduct formation, the fatty acids can be separated on the basis of chain length and degree of unsaturation—longer and more unsaturated fatty acids do not form urea adduct well⁽¹⁰⁾. However, the fatty acid compositions of all the prepared concentrates obtained under the various conditions in this study (Table 1-4 and the other data not shown) do not show such a trend. This seems to be natural, considering that the compositions of EPA and DHA varied according to the wetting agents or the organic solvents.

From 10g of fatty acids made from fish oil, 1.7g of DHA-enriched fraction and 1.7g of EPA-enriched fraction were obtained. Thus the procedure has the yields of 97.4 and 75.1% in terms of EPA

and DHA, respectively, when they were calculated based on the sum of the two fractions. As compared with the result observed by Haagsma *et al.*⁽⁸⁾, this method markedly improved the yield of EPA with that of DHA unchanged.

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어유 지방산과 요소의 부가화합물 형성을 이용한 EPA와 DHA의 선택적 농축방법

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포화지방산 또는 불포화지방산의 분리방법인 요소부가법이 지닌 가메탄을 분해(methanolysis)와 농축물 회수의 곤란함 등의 문제를 해결하기 위하여 지방산과 요소의 부가화합물 형성의 반응매질로 유기용매를 사용하고 요소의 습윤제로 물을 사용한 새로운 요소부가법을 제시하였다. 어유를 원료로 제조한 지방산을 헵탄, 헥산, 이소옥탄같은 비극성 유기용매에 용해시키고 요소와 요소의 습윤제인 물을 첨가하여 부가화합물 형성을 유도한 후 요소와 부가화합물을 잘 형성하지 않는 불포화지방산 분획을 회수하여 지방산 조성을 분석한 결과 EPA, DHA

및 이들의 전구물질을 포함한 고도불포화지방산이 80% 이상인 것으로 나타났다. 이 고도불포화지방산 농축물 내에 EPA와 DHA 함량은 사용하는 유기용매의 종류에 따라서 변화되었는데, 헵탄과 에틸 에테르 등을 사용할 경우에는 EPA의 농축에 효과가 높았으며 이소옥탄과 펜탄의 경우 DHA의 농축에 효과가 높았다. 이러한 특성을 이용하여 우선 펜탄을 사용하여 DHA를 농축하고 다시 헵탄을 사용하여 EPA의 농축을 시도한 바 어유 지방산으로부터 DHA가 50%인 농축물과 EPA가 53%인 분획을 각각 얻을 수 있었다.