Enrichment of Eicosapentaenoic Acid and Docosahexaenoic Acid in Saponified Menhaden Oil

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ABSTRACT: Eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) in free fatty acids (FFA) derived from saponified menhaden oil were concentrated by the solubility differences of FFA-salts in organic solvent. FFA-salts were formed by adding NaOH to a solution containing FFA. A Buchner funnel was used to separate solid phases from liquids containing FFA-salts. FFA that are rich in EPA and DHA can be recovered from the liquid phase by the addition of 12 N HCl. The effects of reaction time, the amount of NaOH, and solvent used on the concentration of EPA and DHA were systematically investigated. With a total volume of 112 mL, made up of 1.85% 15 N NaOH, 88.1% acetone, and 10.0% FFA, a reaction temperature of 30°C, and a reaction time of 1 h, the resulting liquid phase contained 65.4 wt% EPA and DHA, with a corresponding yield of 41.5%. By replacing the acetone with a mixture of 45% acetone and 55% acetonitrile and then storing the liquid phase at –70°C overnight, the content and yield of EPA and DHA in the final liquid phase were 61.4 wt% and 66.2%, respectively.

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KEY WORDS: DHA, EPA, menhaden oil, solubility difference, solvent extraction.

Polyunsaturated fatty acids (PUFA) have been the subject of much attention in recent years because of their special physiological functions in human (1). Fish oil is an important natural source for PUFA, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The health-promoting effects of EPA and DHA in human subjects have been reviewed (2,3).

Various methods are available for obtaining concentrated EPA and DHA from fish oil, such as urea adducts (4), iodolactonization (5), high-performance liquid chromatography (6), supercritical fluid extraction (7), extraction by silver nitrate (8), and solvent extraction (9). Enzyme methods, including esterification (10), hydrolysis (11), and transesterification (12), have also been employed for the concentration of EPA and DHA. Although enzyme methods usually require more steps, thermal degradation of PUFA can be minimized because of their mild operating temperatures.

The solubility of a free fatty acid (FFA)-salt in solvent depends on factors such as the polarity difference between the FFA-salt and the solvent, the chain length, and the number of double bonds in the FFA. In this study, the solubility differences of FFA-salts in organic solvent were employed for the concentration of EPA and DHA in FFA derived from saponified menhaden oil. The effects of reaction time and the amounts of NaOH and solvent used on the concentration of EPA and DHA were studied systematically.

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MATERIALS AND METHODS

Materials. Refined menhaden oil and all fatty acid standards were purchased from Sigma Chemical Co. (St. Louis, MO). All solvents and reagents used were of high-performance liquid chromatographic or American Chemical Society grade.

Preparation of FFA. FFA were prepared according to the method described by Haagsma *et al.* (13) with some modifications. Typically, a 3.7 N NaOH solution was prepared by dissolving 48 g NaOH and 0.5 g Na_2EDTA in 160 mL water. To this solution, 160 mL ethanol was added. A mixture containing 100 g menhaden oil and 200 mL NaOH solution was heated at 60°C with magnetic stirring at 550 rpm for 1 h. Forty milliliters of water and 400 mL of hexane were then added, and the solution was stirred for 1 h. The upper layer was removed and discarded. To the lower layer 160 mL of water was added, and 12 N hydrochloric acid was then added to pH 1. The lower layer was removed by using a separating funnel and discarded. The FFA-containing upper layer was dried with anhydrous magnesium sulfate, and hexane was evaporated in a vacuum rotary evaporator at 35°C.

Determination of the composition of FFA. FFA were transformed into the corresponding methyl esters with trimethylsulfonium hydroxide (14). The composition of FFA was analyzed by a China Chromatography model 8700F (Taipei, Taiwan) gas–liquid chromatograph, equipped with a flame-ionization detector. The column used was SP-2330 (30 × 0.25 mm i.d.; Supelco, Bellefonte, PA). The temperature of the injector and the detector were set at 250 and 270°C, respectively. The column was held at 180°C for 9 min, then increased to 235°C at 15°C/min and held at 235°C for 8 min.

The enrichment of EPA and DHA in FFA. Typically, 10 g FFA was mixed with 100 mL solvent (acetone unless otherwise specified) and preheated at 30°C for 30 min. Two and

one-tenth milliliters 15 N NaOH solution (1.2 times the equivalent of FAA) was added and the solution was then stirred with a magnetic stirrer at 550 rpm for 1 h. Solid and liquid phases were separated by means of a Buchner funnel. Acetone in the liquid phase was removed by evaporation; 80 mL distilled water was then added, and FFA were recovered from the corresponding sodium salts by adding 12 N hydrochloric acid to pH 1. FFA were then extracted by using hexane. Hexane was removed by using a vacuum rotary evaporator at 35°C until a constant weight was achieved. The yield of EPA and DHA is defined as the ratio of the weight of recovered EPA and DHA in the liquid phase to the weight of EPA and DHA in saponified menhaden oil. In studying the effect of acetonitrile, mixtures of acetone and acetonitrile were used as solvent. The liquid phase resulting from the reaction between NaOH and FFA was collected and stored at –70°C overnight. The precipitate was collected by filtration and discarded.

Statistics. Mean absolute deviations, defined as

$$\sum_{i=1}^{3} |S_i - \bar{S}|/3 \qquad [1]$$

were used in all figures and tables.

RESULTS AND DISCUSSION

Effect of reaction time. As shown in Figure 1, the yield of EPA and DHA in the liquid phase decreased rapidly while the content of EPA and DHA in FFA increased only slightly as the reaction proceeded. At a reaction time of 1 h, the content of EPA and DHA reached 65.4% with a corresponding yield of 41.5%. From saponified squid visceral oil, which had an initial EPA plus DHA content of 28.9%, Jan obtained enriched FFA which contained 70–78% EPA plus DHA with a corresponding yield of 46–48% by a similar method (15). Our result shows that at a comparable yield, we can obtain a total EPA plus DHA content of only *ca.* 65.4%. In this work, a reaction time of 1 h was chosen for determination of the concentration of EPA and DHA in FFA derived from saponified menhaden oil.

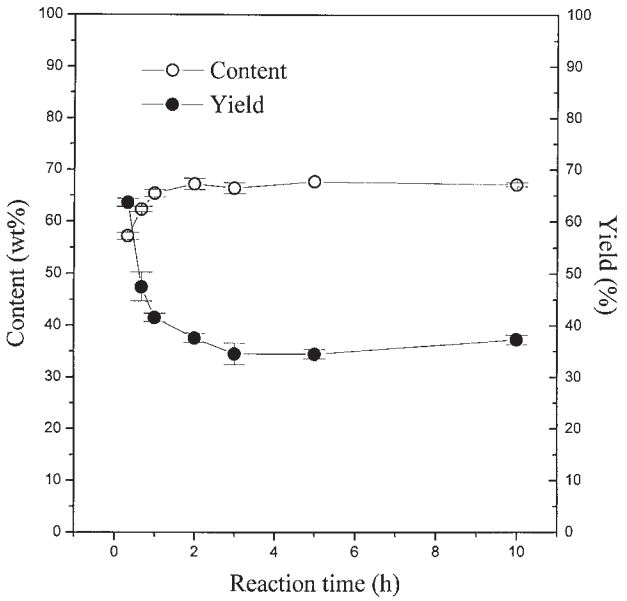

FIG. 1. Effect of reaction time on the content of eicosapentaenoic acid plus docosahexaenoic acid (EPA + DHA) and yield in the liquid phase. Reaction conditions: 100 mL acetone, 10 g free fatty acids (FFA), 2.1 mL 15 N NaOH, temperature 30°C, magnetic stirrer speed 550 rpm. Error bars represent mean absolute deviations.

TABLE 1
Effect of NaOH Concentration on the Content and Yield of EPA + DHA in the Liquid Phase[a]

Concentration of NaOH (N)	Content (wt%)	Yield (%)
2	38.3 ± 1.2	74.5 ± 2.5
5	54.8 ± 1.3	63.9 ± 2.9
10	64.2 ± 1.1	47.0 ± 3.5
15	65.4 ± 0.7	41.5 ± 0.8

[a]Reaction conditions: 100 mL acetone, 10 g free fatty acids (FFA), 2.1 mL NaOH, temperature 30°C, magnetic stirrer speel 550 rpm. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Error terms represent mean absolute deviations.

Effect of NaOH concentration. Table 1 shows that when the number of moles of NaOH added is constant, which is 1.2 times the molar equivalent of FFA, the content of EPA and DHA increases with NaOH concentration while the corresponding yield decreases. Acetone is the medium responsible for the enrichment of PUFA. The solubility of a sodium salt of fatty acid in acetone depends on the chain length and number of double bonds of the fatty acid. When a low concentration NaOH was used, the water it contains extracted a significant amount of salts of PUFA as well as salts of saturated and monounsaturated fatty acids into the liquid phase. The net result is low content and high yield of PUFA in the liquid phase. The converse is true with high concentrations of NaOH. A NaOH concentration of 15 N was chosen in this work.

Effect of amount of NaOH added. As the number of moles of 15 N NaOH added increased, the content of EPA and DHA increased while the yield decreased as can be seen from Table 2. On the basis of the results shown in Table 2, a compromise between yield and concentration of EPA and DHA was made by choosing 0.21 mL 15 N NaOH/g FFA as the amount of NaOH used in this work.

Effect of the amount of acetone. The amount of acetone used has little effect on the content of EPA and DHA. However, use of a large amount of solvent favors high yield as can be seen

TABLE 2
Effect of the Amount of 15 N NaOH Added (number of equivalents of FFA) on the Content and the Yield of EPA + DHA in the Liquid Phase[a]

Number of equivalents of FFA	Content (wt%)	Yield (%)
0.8	51.8 ± 1.2	83.9 ± 0.6
1.0	56.4 ± 4.3	67.4 ± 3.3
1.2	65.4 ± 0.7	41.5 ± 0.8
1.4	67.2 ± 1.2	27.4 ± 0.9
1.6	73.1 ± 0.2	9.6 ± 0.6

[a]Reaction conditions are the same as those in Table 1, except the amount of NaOH. For abbreviations see Table 1.

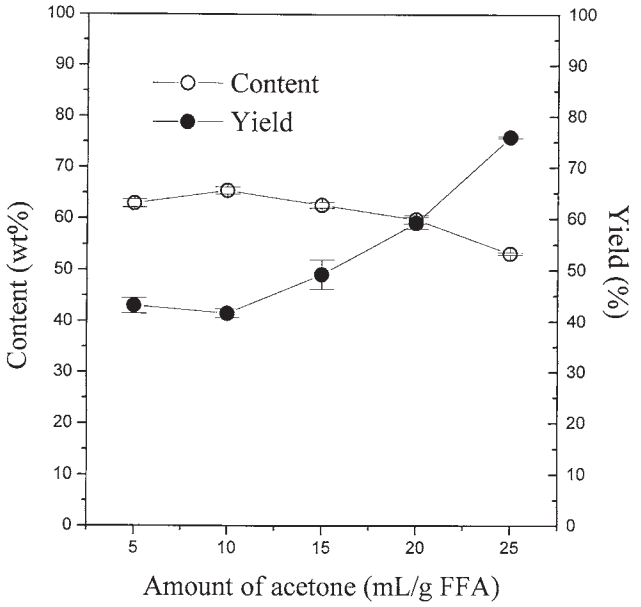

FIG. 2. Effect of the amount of acetone on the content and the yield of EPA + DHA in the liquid phase. Reaction conditions are the same as those in Figure 1 except for the amount of acetone. For abbreviations see Figure 1.

from Figure 2. The amount of solvent used in this work was set at 10 mL acetone/g FFA.

Effect of cold treatment on the liquid phase. After a reaction time of 1 h, the solid phase was separated and discarded. The liquid phase that had been collected was stored at −70°C overnight, and solid formed was removed. As shown in Figure 3 (with amount of acetonitrile = 0 mL/g FFA), EPA and DHA content and yield in the remaining liquid are 70 wt% and 33%, respectively. The corresponding content and yield of EPA and DHA without cold treatment are 65.4 wt% and 41.5%, respectively, as can be seen from Figure 1 (with a reaction time of 1 h).

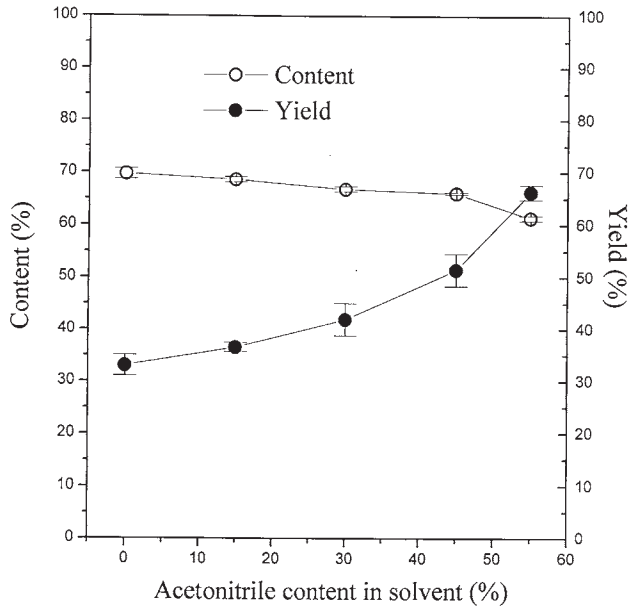

FIG. 3. Effect of the amount of acetonitrile on the content of EPA + DHA. Reaction conditions are the same as those in Figure 1 except that the total volume of acetonitrile and acetone is 100 mL. The mixture was stored at −70°C overnight before the solid that had formed was removed. For abbreviations see Figure 1.

TABLE 3
FFA Compositions of Saponified Menhaden Oil Before and After Enrichment

FFA	Before (wt%)	After (wt%) Solvent A[a]	Solvent B[b]
14:0	8.6 ± 0.3	0.8 ± 0.2	0.1 ± 0.0
16:0	16.2 ± 0.3	1.0 ± 0.3	ND
16:1	11.8 ± 0.8	2.7 ± 0.4	3.0 ± 0.5
18:0	2.9 ± 0.0	ND	ND
18:1	12.3 ± 0.5	1.7 ± 0.2	0.43 ± 0.2
18:2	1.2 ± 0.0	3.0 ± 0.3	3.0 ± 0.2
18:4n-3	2.6 ± 0.1	7.1 ± 0.4	5.8 ± 0.1
20:3n-3	1.1 ± 0.0	1.7 ± 0.2	1.7 ± 0.1
20:5n-3	16.3 ± 0.1	37.8 ± 0.2	36.4 ± 0.5
22.5n-3	3.2±0.0	3.9 ± 0.2	5.6 ± 0.5
22:6n-3	10.1 ± 0.1	27.6 ± 1.1	25.0 ± 0.7
Others	13.7 ± 0.7	12.6 ± 0.9	19.0 ± 0.3
Total n-3	33.3 ± 0.1	78.2 ± 0.4	74.5 ± 0.9
EPA + DHA	26.4 ± 0.1	65.4 ± 0.6	61.4 ± 0.3
Yield of EPA +DHA (%)	100 ± 0.0	41.5 ± 0.8	66.2 ± 1.4

[a]10 mL acetone/g FFA.
[b]5.5 mL acetonitrile and 4.5 mL acetone/g FFA. ND, not detected; for other abbreviations see Table 1.

Effect of acetonitrile. Fatty acids with different chain length and number of double bond have different solubilities in solvents with different polarity. Actually, acetonitrile had dramatic effects on the enrichment of EPA and DHA when it was used as a cosolvent. The liquid phase obtained from the reaction between NaOH and FFA was stored at −70°C overnight, solid was separated and discarded. As shown in Figure 3, the content of EPA and DHA decreased slightly while the corresponding yield increased rapidly with increasing amount of acetonitrile added. When a mixture of 45% acetone and 55% acetonitrile was used as the solvent with a solvent-to-FFA ratio of 10 mL/g, the content and the yield of EPA and DHA were 61.4 and 66.2%, respectively. Table 3 shows the FFA compositions of saponified menhaden oil before and after the enrichment. Most saturated and monounsaturated fatty acids were removed after the enrichment, especially when a mixture of acetone and acetonitrile was used as the solvent. The increases in weight percentage are more pronounced for the more unsaturated fatty acids.

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