Fractionation of Urea-Pretreated Squid Visceral Oil Ethyl Esters

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ABSTRACT: Ethyl esters of squid *(Illex argentinus)* visceral oil contained 11.8% eicosapentaenoic acid (EPA) and 14.9% docosahexaenoic acid (DHA). The esters were treated with urea to increase the contents of EPA and DHA. The non-urea complexing ethyl esters of squid visceral oil contained 28.2% EPA and 35.6% DHA. This mixture was fractionated by molecular distillation to further increase the EPA or DHA content. The fraction collected in the 110°C distillate had an EPA content of 39.0% with 0.26 g/100 g of cholesterol, while the 130°C distillate contained 65.6% DHA and 0.42 g/100 g of cholesterol. Ethyl esters prepared from visceral oil of squid *Ommastrephes bartrami* had 4.5% EPA and 12.7% DHA. After urea pretreatment, the EPA and DHA contents were raised to 10.1 and 30.0%, respectively. When this mixture was further fractionated by molecular distillation, 16.9% EPA with 0.35 g/100 g cholesterol was found in the 110°C distillate and 52.6% DHA with 0.70 g/100 g cholesterol was found in the 130°C distillate. Cholesterol in the squid visceral oil ethyl esters was concentrated in the final residue of molecular distillation when the polyunsaturated ethyl esters were enriched by the urea complexation method prior to molecular distillation. For example, the cholesterol content in the ethyl esters from *O. bartrami* squid visceral oil was 2.28 g/100 g originally. It was enriched to 64.15 g/100 g in the final residue from the molecular distillation.

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Squid visceral oil contains 15 to 25% of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The DHA content is even higher than the EPA content (1). It is well documented that consumption of fish oils high in n-3 fatty acids such as EPA and DHA reduce the risk of atherosclerosis (2,3). In addition, DHA has been found to be important for the development of the brain, retina, and nervous system (4,5). Increasing the EPA and DHA content in fish oil has therefore attracted great attention (6,7).

Urea can be used to fractionate fatty acids or their esters into groups with different degrees of unsaturation (8,9). Urea generally crystallizes in a tetragonal form, but in the presence of certain aliphatic compounds it forms hexagonal prisms. Some aliphatic compounds will stay inside the hexagonal prism and form complexes with urea. This special property of urea can be used to separate saturated fatty acids or their esters, which are flexible straight-chain molecules, from unsaturated fatty acids or esters whose flexibility becomes less with the increase in the number of double bonds (10).

Molecular distillation can be applied to substances with a high boiling point. Material is distilled at high vacuum in an apparatus constructed in such a way that the distance traveled by the molecules between the evaporating and condensing surfaces is shorter than their mean free path (11). This technology has been applied to the fractionation of milk fat (12) and reduction of cholesterol in butter (13).

The purpose of this study was to increase the EPA and DHA contents in squid oil ethyl esters. Ethyl esters were treated with a urea solution first, and the non-urea complexing ethyl esters were further fractionated by molecular distillation. Ratios of EPA to the monoethylenic 20:1 and of DHA to 22:1 were employed to evaluate the fractionation efficiency. The cholesterol content of the product was also measured.

EXPERIMENTAL PROCEDURES

Materials. The crude visceral oils from two different varieties of squid were supplied by Feng-I Co. (Kaohsiung, Taiwan). The oils were separated by wet-rendering from the viscera, which were removed from the squid bodies as processing wastes. Squid visceral oil A was obtained from Argentine squid (*Illex argentinus*), and squid visceral oil B was obtained from North Pacific squid (*Ommastrephes bartrami*).

Ethyl esters of squid visceral oil were obtained from the crude visceral oils after chemical refining (removal of free fatty acids) and bleaching with acid clay (1). Ethyl esters were prepared by reacting 500 g oil with 532 g of 0.5 M sodium ethoxide/ethanol solution at room temperature under N_2 in the dark for 2 h. At the end of the reaction, distilled water was added to the reaction mixture to stop the reaction. The supernatant layer of ethyl esters from the reaction mixture was washed with distilled water until neutral. After removal of water by centrifugation (8000 × *g*, 10 min) the esters were stored at −20°C for later use.

Urea fractionation. Ethyl esters (250 g) were mixed with urea, cyclohexane, and methanol at a 1:3:3:0.12 weight ratio. The urea complexation reaction was allowed to proceed at 20°C for 3 h while stirring. The filtrate of the reaction mixture was washed with 500 mL 0.5% hydrochloric acid and then

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washed three times with 500 mL distilled water followed by drying with anhydrous sodium sulfate. The dried filtrate was purified by passage through silicic acid (100 g), and the solvent (cyclohexane) was removed by a rotary evaporator to obtain the non-urea complexing fraction (NUCF) of ethyl esters. The fatty acid composition and cholesterol content of the ethyl esters were determined by gas chromatographic analysis.

Molecular distillation. The NUCF of squid visceral oil ethyl esters was further fractionated by molecular distillation (model KDL-4; Leybold-Heraeus, Hanau, Germany). Details of the equipment and fractionation procedure have been described elsewhere (14). The initial distillation temperature was set at 50°C. It was increased by 20°C intervals to a final temperature of 130 or 150°C. The distillate collected at each distillation temperature was weighed, and the fatty acid composition and cholesterol content were determined.

Analysis of fatty acid composition. Around 10 mg of the squid visceral oil ethyl esters sample was accurately weighed, and a 1-mL *n*-hexane solution of 1 mg/mL ethyl tricosanoate was added as an internal standard. Analyses of the fatty acid composition were carried out by gas chromatography (14). The weight of each fatty acid was calculated by comparing its peak area with that of ethyl tricosanoate, and the fatty acid composition was expressed as a weight percentage.

Analysis of cholesterol. The cholesterol content of the ethyl esters was measured by gas chromatography (14). Cholesterol content was expressed as grams of cholesterol per hundred grams of sample.

RESULTS AND DISCUSSION

Urea fractionation. Ethyl esters A prepared from *I. argentinus* visceral oil had 11.8% EPA by weight and 3.8% 20:1, and the respective contents of DHA and 22:1 were 14.9 and 1.7%. The ratios of EPA to 20:1 and DHA to 22:1 were quite high. Ethyl esters A was therefore an ideal material for the concentration of EPA and DHA by urea fractionation and molecular distillation.

The results of urea fractionation are shown in Table 1. Most of the saturated and monounsaturated ethyl esters complexed with urea, and EPA and DHA were concentrated in the NUCF. The EPA content increased from 11.8 to 28.2% whereas 20:1 decreased from 3.8 to 0.0%. Similarly, the DHA content increased from 14.9 to 35.6%, and 22:1 decreased from 1.7 to 0.9%. Although the EPA and DHA content increased more than twofold after urea fractionation, the cholesterol content also increased. Since cholesterol does not complex with urea, the cholesterol content increased from 0.74 to 1.78 g/100 g in the NUCF.

Ethyl esters B prepared from *O. bartrami* visceral oil had only 4.5% EPA but 20:1 was quite high (16.9%). The DHA content was 12.7%, and 22:1 was 11.1%. The ratios of EPA to 20:1 and DHA to 22:1 were much lower than in ethyl esters A. Concentrating EPA and DHA in ethyl esters B directly by molecular distillation was not easy, since the elimination curves of EPA and DHA overlapped, respectively, with those of 20:1 and 22:1 to a great extent (14). As Table 1 shows, urea fractionation increased the EPA content from 4.5 to 10.1%, while 20:1 decreased from 16.9 to 4.8%. The DHA content increased from 12.7 to 30.0% while 22:1 decreased from 11.1 to 3.5%. Urea fractionation significantly improved the ratios of EPA to 20:1 and DHA to 22:1, and therefore the NUCF might benefit from further concentration by molecular distillation. Unfortunately, cholesterol increased at the same time from 2.28 g/100g to 5.79 g/100g.

Molecular distillation. The NUCF of ethyl esters were further fractionated by molecular distillation to increase the EPA

TABLE 1
Urea Fractionation of Squid Visceral Oil Ethyl Esters Prepared from *Illex argentinus* (ethyl esters A) and *Ommastrephes bartrami* (ethyl esters B)

	Ethyl esters A		Ethyl esters B	
	Before fractionation	After fractionation	Before fractionation	After fractionation
Yield (wt%)[a]		26.7		22.0
Cholesterol (g/100 g)	0.74	1.78	2.28	5.79
Fatty acid (wt%)				
14:0	4.2	0.6	2.5	0.3
16:0	16.3	0.0	13.2	0.2
16:1	3.4	0.1	1.8	1.6
18:0	1.5	0.0	3.7	0.0
18:1	14.0	0.3	17.3	8.5
18:2	1.8	0.7	0.8	1.2
18:3	1.4	0.6	0.4	0.7
20:1	3.8	0.0	16.9	4.8
20:5 (EPA)	11.8	28.2	4.5	10.1
22:1	1.7	0.9	11.1	3.5
22:6 (DHA)	14.9	35.6	12.7	30.0
Recovery of EPA (wt%)[b]		63.9		49.6
Recovery of DHA (wt%)[b]		64.0		52.1

[a]Weight percentage based on the original ethyl esters.
[b]Recovery of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) based on the initial contents in ethyl esters.

TABLE 2
Fractionation Results of Urea-Pretreated Squid Visceral Oil Ethyl Esters of *I. argentinus* During Molecular Distillation

		Distillate obtained at temperature (°C)				
	Feed[a]	70	90	110	130	Residue
Yield (%)[b]		9.4	21.2	29.4	18.8	4.7
Cholesterol (g/100 g)	1.78	1.33	0.63	0.26	0.42	21.08
EPA (wt%)	28.2	26.8	32.4	39.0	13.4	
DHA (wt%)	35.6	25.7	22.4	36.5	65.6	

[a]The non-urea complexing fraction of squid visceral oil ethyl esters of *I. argentinus*.
[b]Weight percentage based on the feed. For abbreviations see Table 1.

and DHA contents. Ethyl esters were fractionated by an initial molecular distillation at 50°C. The distillation temperature was raised in increments of 20°C. The distillate collected at each distillation temperature was recovered and the residue was redistilled at the next higher temperature. Table 2 presents the results of molecular distillation of ethyl esters A after urea fractionation. The EPA content was maximized in the distillate from 110°C. The EPA content increased from 28.2% in the starting NUCF esters to 39.0% in the distillate. The cholesterol content in this distillate was lowered to 0.26 g/100 g, even lower than the raw material before urea fractionation. The yield of this fraction of distillate was 29.4%. The distillate from 130°C contained the maximum amount of DHA, which was 65.6%, but the cholesterol content was only 0.42 g/100 g. The yield of this high-DHA distillate was 18.8%. The combined 110 and 130°C distillates, which had a total EPA and DHA content higher than 75%, showed a 48.2% yield. It can thus be concluded that nearly half of the NUCF esters can be concentrated to a distillate with an EPA and DHA content of 75% after molecular distillation. The cholesterol content was quite concentrated in the residue after 130°C distillation. This residue had 21.08 g/100 g cholesterol. It could be a good source of cholesterol. The yield was 4.7%.

The results of molecular distillation of ethyl esters B after urea fractionation are presented in Table 3. As in Table 2, the EPA content also reached a maximum in the 110°C distillate. It increased from 10.1% in the starting NUCF esters to 16.9%

TABLE 3
Fractionation Results of Urea-Pretreated Squid Visceral Oil Ethyl Esters of *O. bartrami* During Molecular Distillation

		Distillate obtained at temperature (°C)					
	Feed[a]	70	90	110	130	150	Residue
Yield (%)		2.7	10.1	27.4	28.3	5.3	9.7
Cholesterol (g/100 g)	5.79	0.74	0.29	0.35	0.70	1.80	64.15
EPA (wt%)	10.1	8.3	10.8	16.9	11.1	3.5	
DHA (wt%)	30.0	9.8	10.9	23.4	52.6	53.6	

[a]The non-urea complexing fraction of squid visceral oil ethyl esters of *O. bartrami*. For abbreviations see Table 1.

TABLE 4
Fractionation Results of Squid Visceral Oil Ethyl Esters Prepared from *O. bartrami*[a]

	EPA fraction		DHA fraction	
Sample	[EPA] (wt%)	Cholesterol (g/100 g)	[DHA] (wt%)	Cholesterol (g/100 g)
Original mixture	4.5	2.28	12.7	2.28
After urea pretreatment	10.1	5.79	30.0	5.79
After molecular distillation	16.9	0.35	52.6	0.70

[a]For abbreviations see Table 1.

in the distillate. The cholesterol content of this distillate was lowered from 5.79 to 0.35 g/100 g. The yield of this distillate was 27.4%. The DHA content in the 130°C distillate had 52.6% DHA with a yield of 28.3%. The cholesterol content in this distillate was 0.70 g/100 g. Cholesterol was very concentrated in the 150°C residue, which had 64.15 g/100 g cholesterol with a yield of 9.7%, even higher than that obtained from the NUCF of ethyl esters A.

Results of fractionation of squid oil ethyl esters B by urea pretreatment and molecular distillation are summarized in Table 4. Urea pretreatment raised the EPA content by 2.2-fold, and molecular distillation of the NUCF esters improved its concentration by 3.8-fold with a reduction in cholesterol by 6.5-fold. Urea pretreatment improved DHA content by 2.4-fold, and molecular distillation of the NUCF esters increased its content by 4.1-fold. The reduction of cholesterol in the DHA-rich fraction after molecular distillation was not so effective as in the EPA-rich fraction, possibly because the elimination temperature for DHA was higher than that for EPA.

REFERENCES

1. Liang, J.H., and L.S. Hwang, Ethylation Reaction of Squid Visceral Oil, *Food Sci., Taiwan 24*:408–418 (1997).
2. Kromhout, D., E.B. Bosschieter, and C.D.L. Coulander, The Inverse Relation Between Fish Consumption and 20-Year Mortality from Coronary Heart Disease, *New Engl. J. Med. 312*: 1205–1207 (1985).
3. Kinsella, J.E., Food Components with Potential Therapeutic Benefits: The n-3 Polyunsaturated Fatty Acids of Fish Oil, *Food Technol. 28*:89–97 (1986).
4. Connor, W.E., and S. Reisbick, Essential Fatty Acids: The Importance of n-3 Fatty Acid in the Retina and Brain, *Nutr. Rev. 50*:21–29 (1992).
5. Yokota, A., Functions and Clinical Applications of Docosahexaenoic Acid, *Jpn. J. Clin. Nutr. 83*:619–624 (1993).
6. Nilsson, W.B., E.J. Gauglitz, Jr., and J.K. Hudson, Supercritical Fluid Fractionation of Fish Oil Esters Using Incremental Pressure Programming and a Temperature Gradient, *J. Am. Oil Chem. Soc. 66*:1596–1600 (1989).
7. Breivik, H., G.G. Haraldsson, and B. Kristinsson, Preparation of Highly Purified Concentrates of Eicosapentaenoic Acid and Docosahexaenoic Acid, *Ibid. 74*:1425–1429 (1997).
8. Ackman, R.G., W.M.N. Ratnayake, and B. Olsson, The "Basic" Fatty Acid Composition of Atlantic Fish Oils: Potential Similarities Useful for Enrichment of Polyunsaturated Fatty Acids by Urea Complexation, *Ibid. 65*:136–138 (1988).
9. Domart, C., D.T. Miyauchi, and W.N. Sumerwell, The Fraction-

ation of Marine-Oil Fatty Acids with Urea, *Ibid. 32*:481–483 (1955).
10. Gunstone, F.D., and F.A. Norris, *Lipids in Foods Chemistry, Biochemistry and Technology*, Pergamon Press Inc., New York, 1983, p. 16.
11. Krell, E., Separation Processes, in *Handbook of Laboratory Distillation*, Elsevier Scientific Publishing Company, New York, 1982, pp. 292–306.
12. Arul, J., A. Boudreau, J. Makhlouf, R. Tardif, and T. Bellavia, Fractionation of Anhydrous Milk Fat by Short-Path Distillation, *J. Am. Oil Chem. Soc. 65*:1642–1646 (1988).
13. Armando, L., B. Paolo, M. Carlo, F. Liliana, V. Stefania, F. Enzo, and B. Pierre, A New Short-Path Distillation System Applied to the Reduction of Cholesterol in Butter and Lard, *Ibid. 71*:609–614 (1994).
14. Liang, J.H., and L.S. Hwang, Fractionation of Squid Visceral Oil Ethyl Esters by Short-Path Distillation, *Ibid. 77*:773–777 (2000).

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