Fractionation of Squid Visceral Oil Ethyl Esters by Short-Path Distillation

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ABSTRACT: Squid visceral oil contains high levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Its ethyl esters were fractionated by short-path distillation in this study. The elimination temperatures of squid visceral oil ethyl esters (SVOEE) ranged from 50 to 140°C, increasing with the carbon number of ethyl esters. The elimination temperature of cholesterol was higher than those of SVOEE. The SVOEE of *Illex argentinus* (SVOEE-A) was more advantageous as the raw material (feed) than that of *Ommastrephes bartrami* (SVOEE-B) for the isolation of EPA and DHA, because SVOEE-A contained less 20:1 and 22:1. When SVOEE-A originally containing 9.0% EPA, 14.7% DHA, and 1,121 mg/100 g of cholesterol was distilled from 50 to 150°C with 20°C interval, the 130°C distillate could give 15.5% EPA and 34.7% DHA with 99 mg/100 g of cholesterol, and the yield was 21.8%. The 150°C distillate could give 43.1% DHA with 496 mg/100 g of cholesterol. Furthermore, the distillates collected from 110 to 150°C contained 24.4 to 50.2% of EPA plus DHA, and their total yield was 58.3%. The final residue after 150°C distillation contained 77% of the total cholesterol in the initial SVOEE-A, and the yield was 6.0%.

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KEY WORDS: Docosahexaenoic acid, eicosapentaenoic acid, short-path distillation, squid visceral oil ethyl esters.

Fish oils high in n-3 polyunsaturated fatty acids (PUFA), especially *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA; 20:5) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA; 22:6), have many beneficial effects on human health and disease prevention and have attracted the research interests of medical and food scientists (1). DHA has received increasing attention recently owing to its specific functions in the brain and retina (2). As a consequence of popular awareness of these beneficial effects, increased consumption of n-3 PUFA has resulted in the demand for commercial production of high concentrates of EPA and DHA (3). However, since PUFA are readily oxidized, much care is required during their processing (4).

Squid is one of the major marine products in Taiwan, and its visceral oil is a rich source of EPA and DHA (5). Squid viscera are usually treated as offal; extracting the visceral oil will contribute to efficient use of squid. Furthermore, squid contains more DHA than EPA in its lipids and is therefore an ideal source for the isolation of DHA. However, the consumption of squid visceral oil is also restricted by its high cholesterol content.

There have been several attempts to isolate individual fatty acid esters by methods including high-performance liquid chromatography (6), silver resin chromatography (7), urea complexation (8), fractional vacuum distillation (9), and supercritical fluid extraction (10). The two chromatographic techniques and the urea method involve large amounts of solvents, chemicals, and by-products. Fractional vacuum distillation requires a high temperature which can result in decomposition or isomerization of PUFA. In addition to the supercritical fluid extraction method, short-path (molecular) distillation is an alternative method for obtaining concentrates of individual n-3 fatty acid esters (11).

In short-path distillation, the material is distilled under 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 (12). This technology is suitable for the separation, purification, and concentration of thermal labile substances with low vapor pressure. There are many examples of application related to food: the fractionation of milk fat (13), the recovery of carotenoids from palm oil (14), and the reduction of cholesterol in butter and lard (15).

The present study investigated the fractionation of squid visceral oil ethyl esters by short-path distillation. The objective was to produce fatty acid ethyl esters with high EPA and DHA but low cholesterol contents.

MATERIALS

Squid visceral oils were prepared from squids of two different species (*Illex argentinus* and *Ommastrephes bartrami*), respectively. The oils were obtained from Feng-I Co. (Kaohsiung, Taiwan) and stored at –20°C until use. Squid visceral oil ethyl esters (SVOEE) were prepared from refined and bleached squid visceral oil by ethylation reaction as described previously (5). The reaction was carried out in the dark under nitrogen at 60°C by mixing 500 g oil with 532 g 0.5 M sodium ethoxide in ethanol. After stirring for 10 min, the ethyl esters were separated from the reaction mixture by

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adding water. The ethyl esters were washed with distilled water until neutral and centrifuged to remove water. SVOEE-A was SVOEE prepared from the visceral oil of *I. argentinus*; SVOEE-B was from *O. bartrami*. Their principal fatty acid compositions and cholesterol contents are listed in Table 1 and Table 2, respectively.

Sodium ethoxide was purchased from Merck Co. (Darmstadt, Germany), and ethanol (99.5%, vol/vol) was obtained from Shimakyu's Pure Chemical Co. (Osaka, Japan). Ethyl tricosanoate and 5α-cholestane used as internal standards were purchased from Nu-Chek-Prep, Inc. (Elysian, MN).

EXPERIMENTAL PROCEDURES

Distillation. Short-path distillation of SVOEE was carried out in a Leybold KDL-4 still (Leybold AG, Hanau, Germany). The still has 4.3 dm^2 of evaporation surface and 2.2 dm^2 of condensing surface. The vacuum system was kept in the range of (0.5–3) × 10^{-3} mmHg during operation, and the internal condenser was cooled with flowing water (around 20°C). After the vacuum had been established, the SVOEE were passed over the evaporating surface at 50°C to remove traces of the high volatile compounds, dissolved air, and water. After passing all the samples over the evaporating surface at this temperature, the distillate was removed. The residue was then distilled at a higher temperature, and the distillate was removed again. This cycle of operation was repeated for as many temperature intervals as desired. SVOEE-A was distilled at Run A10 and A20, temperature intervals of which were 10 and 20°C, respectively. SVOEE-B was distilled at Run B10 and B20, temperature intervals of which were also 10 and 20°C, respectively. At each temperature, the distillate was collected and analyzed for fatty acid composition and cholesterol content.

Analytical methods. Analyses of the fatty acid composition were performed using a Varian 3400 gas chromatograph equipped with a 30 m × 0.25 mm i.d. SP-2330 column (Supelco, Inc., Bellefonte, PA) and a flame-ionization detector using hydrogen as the carrier gas with 1.5 mL/min flow rate. The injector and detector temperatures were both at 250°C. The column temperature was set at 160°C for 4 min and then programmed at a rate of 2°C/min to 200°C with a 4-min holding time at the final temperature. The weight of each fatty acid was calculated by comparing its peak area with that of ethyl tricosanoate (internal standard), and the fatty acid composition was expressed as weight percentage.

The cholesterol contents of the distillates were also measured by gas chromatography (16). Each 0.4 g of the sample was mixed with 1.0 mg 5α-cholestane as internal standard and then saponified with 15 mL of 2 N KOH ethanolic solu-

TABLE 1
The Fractionation Results of SVOEE-A During Short-Path Distillation Under A20 Conditions

	Initial feed	Distillate obtained at temperature (°C)						
Run A20	SVOEE-A[a]	50	70	90	110	130	150	Residue
Yield (%)		1.7	7.7	21.2	29.1	21.8	7.4	6.0
Cholesterol (mg/100 g)	1,121	921	33	16	57	99	496	14334
Fatty acid (wt%)								
14:0	3.4	5.9	23.4	7.2	0.3	0	0	
16:0	13.9	15.4	29.4	34.4	14.1	1.0	0.1	
18:1	13.1	13.0	9.4	17.8	23.7	7.4	0.6	
20:1	6.0	5.8	1.1	2.3	7.6	12.6	5.5	
20:5 (EPA)	9.0	9.0	2.4	5.1	13.8	15.5	4.5	
22:1	2.8	2.7	0.2	0.5	1.6	6.0	11.1	
22:6 (DHA)	14.7	14.7	1.4	2.7	10.6	34.7	43.1	

[a]Squid visceral oil ethyl esters of *Illex argentinus* (SVOEE-A); 250 g were used in this experiment. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

TABLE 2
The Fractionation Results of SVOEE-B During Short-Path Distillation Under B20 Conditions

	Initial feed	Distillate obtained at temperature (°C)						
Run B20	SVOEE-B[a]	50	70	90	110	130	150	Residue
Yield (%)		1.9	4.8	18.0	27.8	24.2	8.3	5.8
Cholesterol (mg/100 g)	2,278	2300	96	29	37	120	1020	29768
Fatty acid (wt%)								
14:0	2.5	2.8	22.0	5.4	0.2	0	0	
16:0	13.2	11.5	37.4	37.0	10.6	0.6	0.2	
18:1	17.3	14.8	16.1	26.1	26.9	7.2	0.5	
20:1	16.9	15.2	5.2	8.9	20.0	26.8	9.5	
20:5 (EPA)	4.5	4.0	1.8	3.2	6.3	5.7	1.3	
22:1	11.1	10.6	1.0	2.1	6.1	19.2	31.9	
22:6 (DHA)	12.7	11.9	1.8	3.0	9.0	24.1	24.8	

[a]Squid visceral oil ethyl esters of *Ommastrephes bartrami* (SVOEE-B); 250 g were used in this experiment. See Table 1 for other abbreviations.

tion at 40°C overnight. Water (20 mL) was added after the reaction was completed. The solution was extracted three times with 15 mL *n*-hexane. The combined extract was washed twice with 10 mL of 0.5 N KOH aqueous solution and washed another three times using 10 mL of water. The washed extract was dehydrated with anhydrous sodium sulfate and then concentrated to a volume of 1–2 mL. Of the concentrate 1 μLwas injected into the Varian 3400 gas chromatograph equipped with a 30 m × 0.25 mm i.d. DB-1 column (J & W Scientific, Folsom, CA) and flame-ionization detector. The injector, column, and detector temperatures were all set at 250°C. Hydrogen was used as the carrier gas, and the flow rate was 1.5 mL/min. The cholesterol content was expressed as milligrams of cholesterol per 100 grams of sample.

RESULTS AND DISCUSSION

Elimination curves. Cyclic short-path distillation was employed to separate SVOEE according to the distillation temperature. Figure 1 shows the changes in fatty acid compositions of the distillates vs. distillation temperature for Run A10. These curves are called the elimination curves (12). The temperature at the maximum of the peak was known as the elimination temperature. As shown in Figure 1, the elimination temperatures of SVOEE-A, ranging from 50 to 140°C, were found to increase with the carbon number of the ethyl esters. The principal fatty acid ethyl esters in SVOEE-A were 16:0, 18:1, EPA, and DHA, which displayed elimination temperatures at 80, 100, 110, and 130°C, respectively. Hence, the rate of evaporation is in the order of 16:0 > 18:1 > EPA > DHA. However, it must be noted that the elimination temperature is not an absolute quantity for the substance, but is also dependent on both the apparatus and the operating conditions (12). In other words, the elimination curve gives a characteristic but relative temperature for each kind of fatty acid ethyl ester. Gray and Cawley (17) investigated the influence of structures on the elimination maximum of fatty acids and found that one methylene group could raise the elimination maximum by 5°C and nonconjugated double bond would lower the maximum by 2°C. In the present study, however, we could not distinguish the elimination temperature between 18:1, 18:2, 18:3, and 18:4, because the distillation temperature was raised at an interval of 10°C.

SVOEE-B contained a high percentage of 20:1 and 22:1, whose carbon number is the same as EPA and DHA, respectively. SVOEE-B was distilled at Run B10, and their elimination curves are shown in Figure 2. The elimination temperature of 20:1 (120°C) is 10°C higher than that of EPA (110°C), and 22:1 is also 10°C higher than that of DHA (130°C). However, the elimination curve of EPA overlapped with that of 20:1 to a great extent. The overlapping of the elimination curves indicates the poor separation that could be expected with short-path distillation of a mixture containing EPA and 20:1. Similarly, the separation between DHA and 22:1 was also inefficient during cyclic distillation of SVOEE-B.

The elimination temperature of cholesterol was above 150°C, as shown in Figures 1 and 2, respectively. Hickman (18) found that the elimination maximum of cholesterol was near 170°C, and Fletcher *et al.* (19) reported that the elimination maxima of certain saturated cholesterol esters were higher than that of free cholesterol. Hence, the above results implied that cholesterol could remain in the final residue after distillation at 150°C.

Distillation conditions. Since the polyunsaturated EPA and DHA are thermally labile changes caused by the distillation temperature should be taken into consideration. In a previous investigation (20), the thermal change of EPA ethyl ester began at 159°C and reached a maximum at 206°C; DHA ethyl ester began at 166°C and had a maximum at 217°C. Wijesundera *et al.* (21) showed that the geometrical isomers of EPA were formed during prolonged heating at 220°C. Although the residence time on the evaporating surface is as short as 10 to 15 s, it is still suggested that the distillation temperature should not exceed the thermal change temperature of EPA ethyl ester.

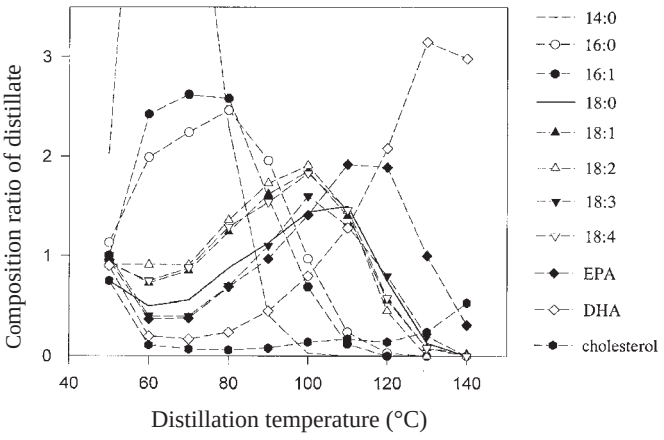

FIG. 1. Elimination curves of squid visceral oil ethyl esters of *Illex argentinus* (SVOEE-A) during short-path distillation under A10 conditions. Composition ratio of distillate is expressed as the ratio of the ethyl ester concentration in the distillate to that in the initial SVOEE. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

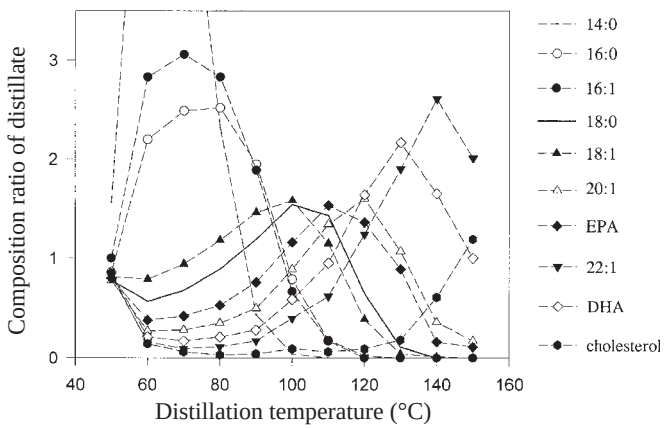

FIG. 2. Elimination curves of squid visceral oil ethyl esters of *Ommastrephes bartrami* during short-path distillation under B10 conditions. Composition ratio is the same as in Figure 1. See Figure 1 also for abbreviations.

In this study we also noticed that, when the temperature interval raised from 10 to 20°C, the elimination maximum of EPA shifted from 110 to 130°C, and that of DHA shifted from 130 to 150°C. In order to understand if the temperature interval has any influence on the separation efficiency, short-path distillation of SVOEE-A was performed at 10 and 20°C temperature intervals. The fatty acid ratio, *R*, is defined as the concentration of EPA plus DHA to that of 16:0 plus 18:1. And a separation efficiency, *S*, is used to eliminate the effect of feed composition on separation efficiency. Thus,

$$R = [\text{EPA} + \text{DHA}]/[16{:}0 + 18{:}1] \quad [1]$$

$$S = \log (R \text{ of the distillate}/R \text{ of the initial SVOEE}) \quad [2]$$

If the value of *S* is greater than zero, the distillate is higher in EPA plus DHA than the initial SVOEE.

Figure 3 shows the plots of S values against distillate percentage for Runs A10 and A20. At the end of distillation, the relative separation efficiency attained a value of 2.3 in Run A10, and a value of 1.9 in Run A20. Run A10 with a smaller temperature interval of 10°C exhibited a better separation efficiency and yielded a higher recovery of distillate with *S* value greater than zero. However, cyclic distillation with a smaller temperature interval needs more steps of distillation.

EPA and DHA concentrates. Table 1 lists the results of fractionation SVOEE-A under A20 conditions with a temperature interval of 20°C. The SVOEE-A originally contained 9.0% EPA, 14.7% DHA, and 1121 mg/100 g of cholesterol. The distillate collected at 50°C (the initial distillation temperature) exhibited a composition similar to that of the initial feed and contained high volatile compounds with strong odors. This initial distillation stage exhibited an effect of deodorization. The 130°C distillate gave 15.5% EPA and 34.7% DHA with 99 mg/100 g of cholesterol, and the 150°C distillate had DHA content of 43.1% with 496 mg/100 g of cholesterol. The 110 to 150°C distillates contained 24.4 to 50.2% of EPA plus DHA, and the total yield of the distillates was 58.3%. The residue after 150°C distillation represented 6.0% of the initial feed mass and contained as much as 1.43×10^4 mg/100 g of cholesterol, which was 77% of the total cholesterol in the initial SVOEE-A.

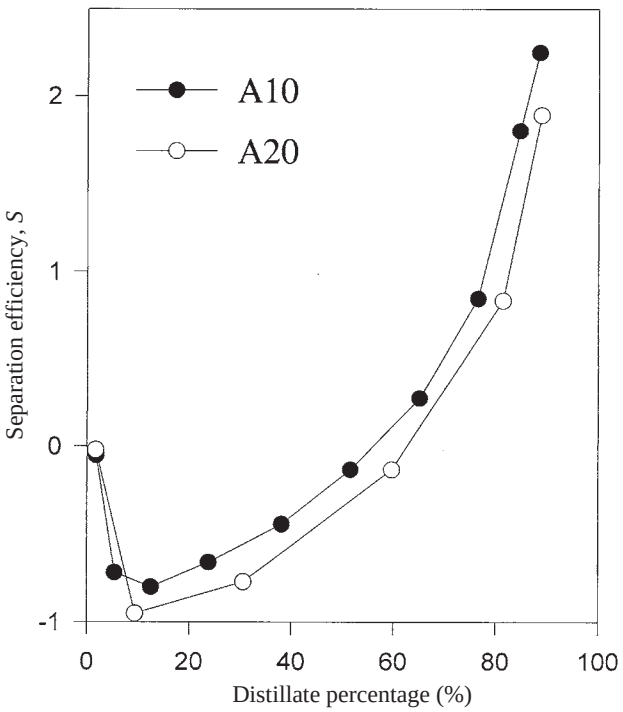

FIG. 3. The separation efficiency (*S*) during short-path distillation of SVOEE-A under different operating conditions. See Equation 2 for calculation of *s*, and Figure 1 for abbreviations.

TABLE 3
Results of Short-Path Distillation of SVOEE Under Different Operating Conditions

	Feed				Distillate	
Run no.	[EPA][a]	[DHA][a]	[EPA]/[20:1]	[DHA]/[22:1]	[EPA][a]	[DHA][a]
A10[b]	9.0	14.7	1.5	5.3	17.3	46.3
A20[b]	9.0	14.7	1.5	5.3	15.5	43.1
B10[c]	4.5	12.7	0.3	1.1	6.9	27.6
B20[c]	4.5	12.7	0.3	1.1	6.3	24.8

[a]Weight percentage. See Table 1 for abbreviations.
[b]From *Illex argentinus.*
[c]From *Ommastrephes bartrami.*

When SVOEE-B was distilled under B20 conditions with 20°C temperature interval, the following results were obtained (Table 2). The 130°C distillate contained 26.8% 20:1 and only 5.7% EPA. The cholesterol content in the 130°C distillate was 120 mg/100 g, compared to 2,278 mg/100 g in the feed. The 150°C distillate contained 31.9% 22:1, 24.8% DHA, and 1,020 mg/100 g of cholesterol. The final residue, representing 5.8% of the initial feed mass, contained 2.98×10^4 mg/100 g of cholesterol, which was 76% of the total cholesterol in the initial SVOEE-B.

Table 3 summarizes the results of this study. For the same feed material, separation with a 10°C interval gave higher concentrations of EPA and DHA than with a 20°C interval. Nevertheless, it is more preferable to carry out the distillation at 20°C interval from the economical point of view. When short- path distillation was performed under the same operating condition, it was apparent that feed containing higher percentage of EPA relative to 20:1 and DHA relative to 22:1 would result in distillates richer in EPA and DHA. Hence, SVOEE prepared from *I. argentinus* (SVOEE-A) are more suitable for the isolation of EPA and DHA using this technique. Cholesterol could be a by-product of this process.

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