# Preparation of Highly Purified Concentrates of Eicosapentaenoic Acid and Docosahexaenoic Acid

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**ABSTRACT:** Because of the complexity of marine lipids, polyunsaturated fatty acid (PUFA) derivatives in highly purified form are not easily prepared by any single fractionation technique. The products are usually prepared as the ethyl esters by esterification of the body oil of fat fish species and subsequent physicochemical purification processes, including short-path distillation, urea fractionation, and preparative chromatography. Lipase-catalyzed transesterification has been shown to be an excellent alternative to traditional esterification and short-path distillation for concentrating the combined PUFA-content in fish oils. At room temperature in the presence of *Pseudomonas* sp. lipase and a stoichiometric amount of ethanol without any solvent, efficient transesterification of fish oil was obtained. At 52% conversion, a concentrate of 46% eicosapentaenoic acid (EPA) plus docosahexaenoic acid (DHA) was obtained in excellent recovery as a mixture of mono-, di-, and triacylglycerols. The latter can be easily separated from the saturated and monounsaturated ethyl esters and converted into ethyl esters either by conventional chemical means or enzymatically by immobilized *Candida antarctica* lipase. Urea-fractionation of such an intermediary product can give an EPA + DHA content of approximately 85%.
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**KEY WORDS:** *Candida antarctica*, DHA, enrichment, EPA, esterification, fish oil, lipase, n-3 fatty acids, *Pseudomonas* sp.

Utilization and production of marine oils have long traditions in the Nordic countries, as evidenced by 800-year-old formal regulations (1). During this time, it was observed that persons eating flesh from marine animals displayed special physiological characteristics (2), and in the medieval ages, cod-liver oil in some districts was regarded as obligatory for pregnant women (3). One hundred years ago, Heyerdahl (4) proposed that a polyunsaturated fatty acid was responsible for the therapeutic action of cod liver oil, and he proposed to give it the name "therapic acid." After World War II, Notevarp (5,6) pioneered the connection between polyunsaturated fatty acids (PUFA) and the incidence of cardiovascular diseases. Modern interest was initiated by epidemiological studies carried out by Bang and Dyerberg (7), who found a decreased incidence of coronary heart diseases in the Greenland Eskimo population compared to Danes. This beneficial effect has been attributed to the intake of n-3 PUFA, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

The growth of new markets for n-3 PUFA has resulted in the demand for commercial production of high-purity concentrates of EPA and DHA. Because of the complexity of marine raw materials, PUFA in highly purified form are not easily prepared by a single fractionation technique. Usually, a combination of techniques is used, the combination of which depends upon the fatty acid composition of the starting oil and the desired concentration and purity of PUFA in the final product.

Cod-liver oil and other fish oils are winterized (cooled and filtered) before sale to avoid precipitation when refrigerated. This practice, to a degree, increases the content of PUFA in these oils. Fish body oils produced in this way typically are declared to contain "30%" EPA plus DHA. However, these values often reflect area percentages as analyzed by gas chromatography (GC), and normally the weight percentages are significantly lower (8). To further increase the PUFA concentration, it is necessary to resort to chemistry.

The lability of PUFA esters to heat precludes the distillation through traditional columns. However, PUFA ethyl esters may be separated by short-path distillation without any apparent decomposition. Because the degree of fractionation that can be achieved with this method is limited, significant concentration effects can only be obtained at low yields.

Urea forms solid complexes with straight-chain organic compounds. When fatty acids or their esters are added to a solution of urea, a crystalline complex is formed with the more saturated fraction of the acids or esters. The crystals can be removed, leaving a raffinate that is highly concentrated in PUFA or PUFA esters. The principle of urea fractionation was discovered by Bengen in 1940 (9,10). An early and extensive review of urea complexation as a tool in separation processes was given by Swern and Marklin (11). Urea complexation as a fractionation tool, however, involves large amounts of solvents, chemicals, and by-products. The volumes involved are illustrated by the following scale-up experiment: 29.800 kg

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of ethyl esters of fish oil, containing approximately 28% EPA plus DHA, was distilled by a two-step molecular distillation to give 9.575 kg product that contained approximately 50% EPA plus DHA; precipitation with 16.150 kg urea gave 18.890 kg of urea complex and 3.140 kg PUFA concentrate (84% EPA plus DHA) that was further purified (12). The combined recovery of EPA and DHA was approximately 57% after molecular distillation, and approximately 31% after urea fractionation.

On a commercial scale there is a need to reduce the bulkiness of the PUFA concentration process. In this paper, we show that a facile concentration of PUFA can be obtained by introducing an enzymatic step to the overall process.

## EXPERIMENTAL PROCEDURES

The experimental procedures with *Pseudomonas* sp. lipase (PSL) and *Pseudomonas fluorescens* lipase (Amano Enzymes Europe Ltd., Milton Keynes, United Kingdom), including analytical procedures, are as given in Haraldsson *et al.* (13). Immobilized *Candida antarctica* lipase (CAL) (SP435, 1–2% water content) was obtained by Novo-Nordisk (Bagsvaerd, Denmark). The fish oil triglycerides, originating from sardine oil, were provided by Pronova Biocare a.s. (Bergen, Norway), and contained 14.9% EPA and 9.8% DHA. They were used without further refinement. The short-path distillation was carried out in a Leybold KDL 4 still (Leybold AG, Hanau, Germany). Analyses of environmental pollutants (preconcentration and analysis by GC with electron capture detection) were performed at Handels- und Umweltschutzlaboratorium Dr. Wiertz - Dipl. Chem. Eggert – Dr. Jörissen GmbH, Hamburg, Germany.

*CAL ethanolysis.* Immobilized CAL was added to an acylglycerol mixture (2.5 g; approximately 8.5 mmol of ester equivalents; initial composition 25.0% EPA and 15.1% DHA; produced by PSL-catalyzed transesterification of fish oil) and absolute ethanol (0.80 g, 14.4 mmol). The resulting enzyme suspension was gently stirred at room temperature under nitrogen. Samples were collected at appropriate times and analyzed as described before (13). The reaction was discontinued by separating the enzyme by filtration after 22 h.

*Analyses of lipid classes.* Analysis of lipid classes after short-path distillation was performed by high-performance size-exclusion chromatography. Ten mg of the substance to be examined was weighed into a 10-mL volumetric flask, dissolved in tetrahydrofuran (THF) and diluted to 10.0 mL with THF. The chromatographic separation was carried out with Waters Ultrastyragel columns (0.3 m by 7.8 mm i.d.), packed with styrene–divinylbenzene copolymer, one column with pore size 100 Å and two columns with pore size 500 Å (Waters, Milford, MA; Cat. no. 10570 and 10571) connected in series. THF was used as a mobile phase at a flow rate of 0.8 mL/min. A Waters Model 410 Differential Refractometer (Waters) was used as a detector, and a VG Multichrom 2 Chromatography Data System (VG Data Systems, Altrincham, United Kingdom) for quantitation.

## RESULTS AND DISCUSSION

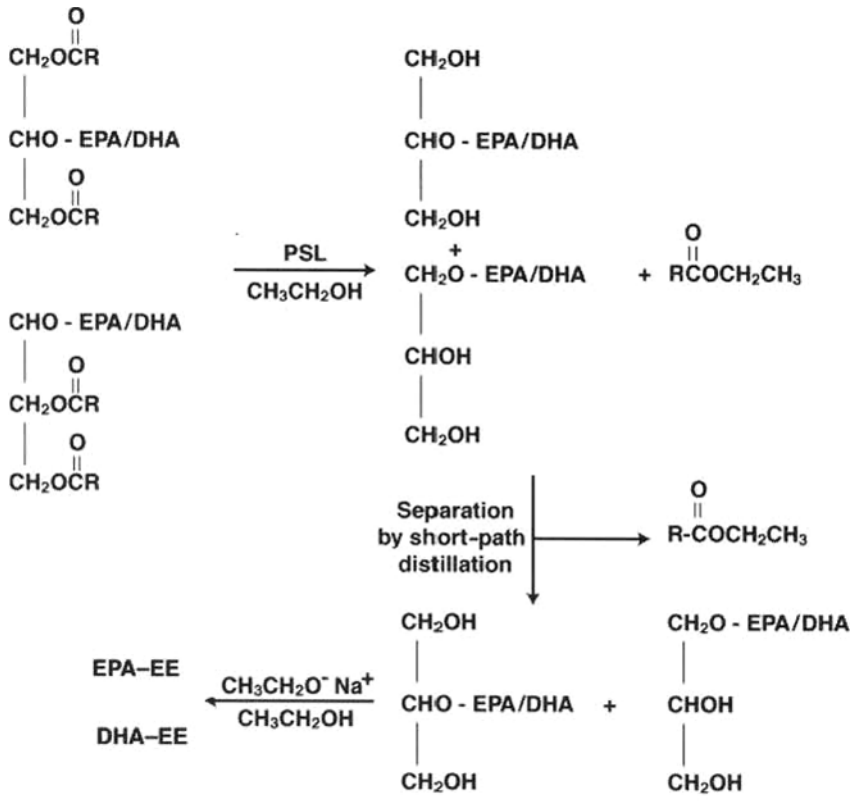
*Biocatalyzed transesterification.* To reduce the large reaction volumes in the concentration of EPA and DHA, the possibility of finding an enzymatic procedure for selectively transesterifying fatty acids other than EPA and DHA was investigated. In Scheme 1 is displayed the outline of principle for concentration of EPA ethyl ester (EPA–EE) and DHA ethyl ester (DHA–EE) *via* lipase-catalyzed transesterification [*Pseudomonas* sp. lipase (PSL); R, any fatty acid]. Detailed information regarding the enzymatic work is given by Haraldsson *et al.* (13). The work that followed showed that lipase-catalyzed transesterification was an excellent alternative to traditional esterification and short-path distillation in concentrating the combined EPA and DHA content in fish oils, and that it can take place as part of an integrated process for the production of highly purified concentrates of these fatty acids (14). At room temperature, in the presence of PSL and a stoichiometric amount of ethanol, without any solvent, a highly efficient transesterification reaction of the fish oil (14.9% EPA and 9.8% DHA) was obtained. At 52% conversion (Table 1), a concentrate of 46% EPA and DHA (Table 2) was obtained in excellent recovery as a mixture of mono-, di-, and triacylglycerols.

*Separation by short-path distillation.* The EPA- and DHA-containing acylglycerols can be easily separated from the saturated and monounsaturated ethyl esters by short-path distillation. Short-path distillation is not usually regarded as being suitable for difficult separations. However, in this process it proved to be surprisingly advantageous. The effect was demonstrated in an experiment in which the total EPA and DHA in the acylglycerol fractions analyzed was 40.1% by GC area percentages (Tables 3). The product was formed by adding PSL (100 g, 25,200 activity units/g) to fish oil (1000 g, approximately 1.13 mol) and absolute ethanol (170 g, 3.70 mol). The resulting enzyme suspension was stirred gently with a magnetic stirrer at room temperature under nitrogen for 50 h. A centrifuge was used for separating the reaction mixture from the lipase (5,000 rpm for 10 min). The reaction mixture (902 g) was degassed by passing through the short-path distillation still under vacuum at 80°C to remove volatile components to give an acylglycerol/ethyl ester mixture (844 g). The mixture (756 g) was distilled in the short-path distillation still at 125°C, 0.005 mbar. This gave a distillate (359 g,

**TABLE 1**
**Composition of Acylglycerol/Ethyl Ester Mixture After Partial Ethanolysis of Fish Oil with *Pseudomonas* sp. Lipase (PSL) at 20°C[a]**

| | 1 h | 2 h | 4 h | 8 h | 13 h | 24 h |
|---|---|---|---|---|---|---|
| MG | 5.0 | 9.4 | 13.0 | 17.9 | 16.8 | 15.9 |
| DG | 29.1 | 35.6 | 31.6 | 23.3 | 24.9 | 25.9 |
| FFA | 2.6 | 2.9 | 2.5 | 2.7 | 2.7 | 2.7 |
| TG | 42.9 | 17.9 | 14.2 | 9.1 | 7.5 | 5.7 |
| EE | 20.4 | 34.2 | 38.7 | 47.0 | 48.1 | 51.8 |

[a]Results in weight percentage after different reaction times. MG, monoacylglycerols; DG, diacylglycerols; FFA, free fatty acids; TG, triacylglycerols; EE, ethyl esters.




SCHEME 1

47.4%) and a residue (388 g, 51.3%). The fractions had the compositions shown in Table 4. The residue after short-path distillation contained as much as 47.3% EPA plus DHA, compared to 40.1% in the acylglycerol fractions. The distillation step results in a 10.6% monoacylglycerol fraction in the distillate, which is predominantly monoacylglycerols of fatty acids with chainlength below $C_{20}$. In this way, little or no EPA or DHA is lost into the distillate. Similarly, a small part of the ester fraction will remain with the residual acylglycerol mixture. This will be predominantly $C_{20}$ and $C_{22}$ fatty acids, such as EPA and DHA. Accordingly, part of the EPA and DHA that has been transesterified will still remain in the residue fraction after distillation. The recovery from the starting fish oil was approximately 88%, a significant increase from the two-step short-path distillation from ethyl esters.

Environmental pollutants, such as pesticides, tend to concentrate in the ethyl ester fraction after lipase ethanolysis. A fish oil mixture, having undergone partial lipase-catalyzed ethanolysis, was analyzed before and after short-path distillation. The starting material contained 0.03 mg/kg of combined dichlorodiphenyltrichlorethane (DDT) and DDT metabolites. α-Hexachlorocyclohexane (α-HCH), hexachlorobenzene (HCB), and toxaphene were not detected. After distillation,

**TABLE 2**
**Concentration of EPA and DHA in Different Lipid Classes After Partial Ethanolysis of Fish Oil with PSL at 20°C[a]**

| | Area % (EPA + DHA) | | | | | |
|---|---|---|---|---|---|---|
| | 1 h | 2 h | 4 h | 8 h | 13 h | 24 h |
| MG | 27.5 | 29.0 | 32.8 | 36.5 | 40.9 | 43.8 |
| DG | 32.0 | 34.8 | 39.7 | 42.2 | 47.4 | 47.2 |
| FFA | 5.5 | 5.4 | 9.9 | 12.6 | 12.4 | 18.9 |
| TG | 29.0 | 33.5 | 41.9 | 44.0 | 45.6 | 44.3 |
| FE | 2.6 | 2.6 | 3.5 | 4.4 | 5.0 | 6.4 |
| MG/DG/TG | 30.0 | 33.6 | 38.7 | 40.5 | 44.9 | 45.8 |

[a]Area percentage after different reaction times is based on the integration of the corresponding chromatograms obtained by gas chromatography (GC). EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. See Table 1 for other abbreviations.

**TABLE 3**
**Composition of Acylglycerol/Ethyl Ester Mixture After Partial Ethanolysis of Fish Oil with PSL at 20°C for 50 h[a]**

| | | EPA | | DHA | | EPA + DHA |
|---|---|---|---|---|---|---|
| Lipid class | wt%[b] | area%[c] | rec%[d] | area%[c] | rec%[d] | area%[c] |
| MG | 17.1 | 17.0 | 18.0 | 17.8 | 28.9 | 34.8 |
| DG | 26.0 | 28.9 | 53.1 | 14.5 | 42.1 | 43.4 |
| FFA | 2.8 | 5.7 | 1.2 | 6.0 | 1.9 | 11.7 |
| TG | 8.9 | 28.8 | 19.3 | 11.8 | 12.2 | 40.6 |
| FE | 46.2 | 2.6 | 8.4 | 2.9 | 14.8 | 5.5 |
| MG/DG/TG | 52.0 | 25.0 | 90.4 | 15.1 | 83.2 | 40.1 |

[a]See Tables 1 and 2 for abbreviations.
[b]Results in weight percentage as determined by preparative thin-layer chromatography.
[c]Results in area percentage as determined by GC.
[d]Recovery percentage as determined by GC, based on the total amount of EPA or DHA in the initial oil (Ref. 13).

**TABLE 4**
**Composition of Fractions After Separation by Short-Path Distillation of the Reaction Mixture from the PSL-Catalyzed Ethanolysis of Fish Oil[a]**

| | TG (%)[b] | DG (%)[b] | MG (%)[b] | EE (%)[b] | EPA (%)[c] | DHA (%)[c] | EPA + DHA (%)[c] |
|---|---|---|---|---|---|---|---|
| After degassing | 11.2 | 35.5 | 19.7 | 33.6 | 16.4 | 11.0 | 27.4 |
| Distillate | — | 0.9 | 10.6 | 88.5 | 3.6 | 3.4 | 7.0 |
| Residue | 18.5 | 54.0 | 25.2 | 2.3 | 29.1 | 18.2 | 47.3 |

[a]See text for experimental details. See Tables 1 and 2 for abbreviations.
[b]Results in area percentage as determined by high-performance size-exclusion chromatography.
[c]Results in area percentage as determined by GC.

the ethyl ester fraction contained up to 0.03 mg/g combined DDT and DDT metabolites, and up to 0.005 mg/g α-HCH, 0.03 mg/g HCB, and 0.4 mg/g toxaphene. The target acylglycerol residue did not contain detectable amounts of pesticides. The results show that, for some pesticides, the levels in the starting material are just below the detection limit. For DDT, some of the pollutant can be removed by degassing of the partially transesterified product mixture. The results show that enzymatic ethanolysis of fish oil can be an efficient way of removing environmental pollutants from n-3 concentrates.

*Integrated production process.* The enzymatic alcoholysis reaction can serve as the initial step in an integrated production process for EPA and DHA concentrates. The acylglycerol mixture from this step can be converted into ethyl esters, either by conventional chemical means or enzymatically. Urea fractionation of such an intermediary product, based upon traditional procedures (15,16), gives an EPA and DHA content of approximately 85%.

*CAL-catalyzed ethanolysis.* Immobilized CAL has been documented to have good activity toward both EPA and DHA (17). This is in agreement with the results shown in Table 5. The data show that CAL is suitable for the ethanolysis of the acylglycerol mixture from the PSL reaction. Complete conversion was obtained after 22 h at room temperature. Virtually all EPA and DHA in the acylglycerol mixture from the PSL reaction had been converted into the ethyl esters. This required less than a twofold stoichiometric amount of ethanol, as based on the number of ester equivalents left in the residual acylglycerol mixture. As with the PSL reaction, this means a significant reduction of reaction volume compared to chemical ethanolysis, where ethanol also acts as a solvent. Despite its efficiency, CAL discriminates between EPA and DHA in favor of the former fatty acid, in agreement with published observations (17). After the PSL treatment, there are small amounts of EPA and DHA present as free acids as a result of hydrolysis side reactions. In conventional chemical ethanolysis reactions under basic conditions, the free fatty acids are lost as soaps. On the other hand, the CAL reaction esterifies these free fatty acids and acylglycerols to ethyl esters, thus increasing the recovery of both EPA and DHA.

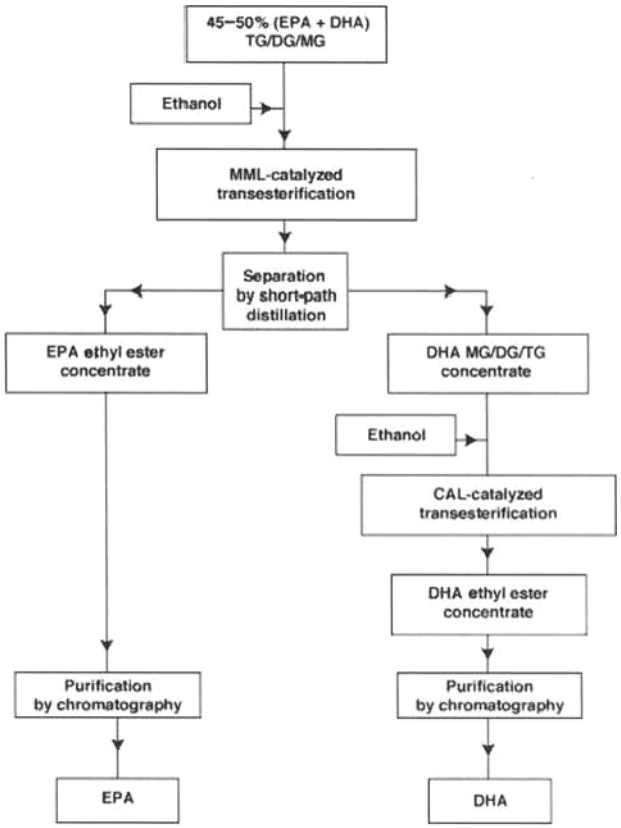
**TABLE 5**
**Results from Ethanolysis of Acylglycerol Mixture with *Candida antarctica* Lipase at 20°C[a]**

| Time (h) | Conversion (%) | Area % EPA | Area % DHA | Recovery EPA (%) | Recovery DHA (%) |
|---|---|---|---|---|---|
| 1 | 40.2 | 28.1 | 7.1 | 47.1 | 20.8 |
| 3 | 68.1 | 25.5 | 8.2 | 72.6 | 40.4 |
| 6 | 76.6 | 27.0 | 10.3 | 83.4 | 55.4 |
| 22 | 100.0 | 25.3 | 14.8 | 98.0 | 100.0 |

[a]See Table 2 for abbreviations.

*Sequential use of various lipase.* If it is desirable to produce a substantially pure EPA and DHA concentrate, a process as schematically illustrated in Scheme 2 may be proposed. Here, PSL is used to achieve an initial concentration of EPA and DHA. In subsequent steps, an enzyme like *Mucor miehei* lipase (MML), which selectively favors the esterification of EPA rather than DHA, might be used, thereby permitting a good separation of these two acids. Finally, an enzyme such as CAL can be used to effect the ethanolysis of the DHA-enriched acylglycerol mixture.

*Chromatography.* For very pure products, the final purification step will often include chromatography. As the recirculation of eluent represents a major part of the total cost of the chromatographic separation process, the economy of the process may be significantly improved if the organic eluent in preparative chromatography is substituted with a supercritical fluid (18,19). Recently, preparative simulated moving-bed chromatography has shown good results for purification of EPA and DHA (20,21). This technique gives much higher




SCHEME 2

productivity and uses less solvent than traditional production-scale high-performance liquid chromatography. The productivity of the process is further increased by using a pressure gradient with supercritical carbon dioxide as the eluent.

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