Production of Triglycerides Enriched in Long-Chain n-3 Polyunsaturated Fatty Acids from Fish Oil

Stephen R. Moore* and Gerald P. McNeill

Unilever Research, Sharnbrook, Bedford MK44 1LQ, United Kingdom

ABSTRACT: Processes that combine enzymic and physical techniques have been studied for concentrating and separating eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish oil. *Candida rugosa* lipase was used in hydrolysis reactions to concentrate these acids in the glyceride fraction. By controlling the degree of hydrolysis, two products have been obtained, one enriched in total n-3 (~50%), the other enriched in DHA and depleted in EPA (DHA ~40%, EPA ~7%). The glyceride fraction from these reactions was recovered by evaporation and converted back to triglycerides by partial enzymic hydrolysis, followed by enzymic esterification. Both reactions were carried out with *Rhizomucor miehei* lipase. DHA-depleted free fatty acids from a *C. rugosa* hydrolysis were fractionated to increase the EPA level (~30%) and re-esterified to triglycerides by reaction with glycerol and *R. miehei.*

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KEY WORDS: *Candida rugosa,* DHA, enrichment, EPA, esterification, fish oil, hydrolysis, lipase, n-3 fatty acids, *Rhizomucor miehei.*

Fish oils are a readily available source of two long-chain n-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The recognition that these fatty acids play an important role in human health and nutrition (1) has led to much research into methods of extracting and concentrating these materials from marine oils. There are a number of physical fractionation methods. Crystallization in solvents (2) is one, which can be enhanced by combination with urea complexation (3,4). Distillation (5) and supercritical fluid extraction (6,7) are also techniques that have been used. These methods generally require fish oil fatty acids or fatty acid esters, although recently, low-temperature solvent fractionation of fish oil triglycerides has been described (8). In most cases the product is enriched in both DHA and EPA.

Another approach is the use of lipases. This has the advantage of relatively mild processing conditions at temperatures close to ambient. A number of lipases have been studied in a number of different reaction systems. Immobilized *Rhizomucor miehei* has been used to incorporate DHA and EPA into oils by means of acidolysis reactions (9,10). A *Pseudomonas* lipase (Amano lipase CES) was found to be effective in concentrating n-3 fatty acids of cod liver oil in alcoholysis reactions (11). *Geotrichum candidum* and *Candida rugosa (Candida cylindracea)* both show discrimination against longer-chain polyunsaturated fatty acids (PUFA) and have been used to prepare concentrates from fish and tuna oils in hydrolysis reactions (12–14). The *C. rugosa* lipase is of particular interest because it shows increasing discrimination against fatty acids in the range of C_{18} to C_{22}, as their chainlength increases (15).

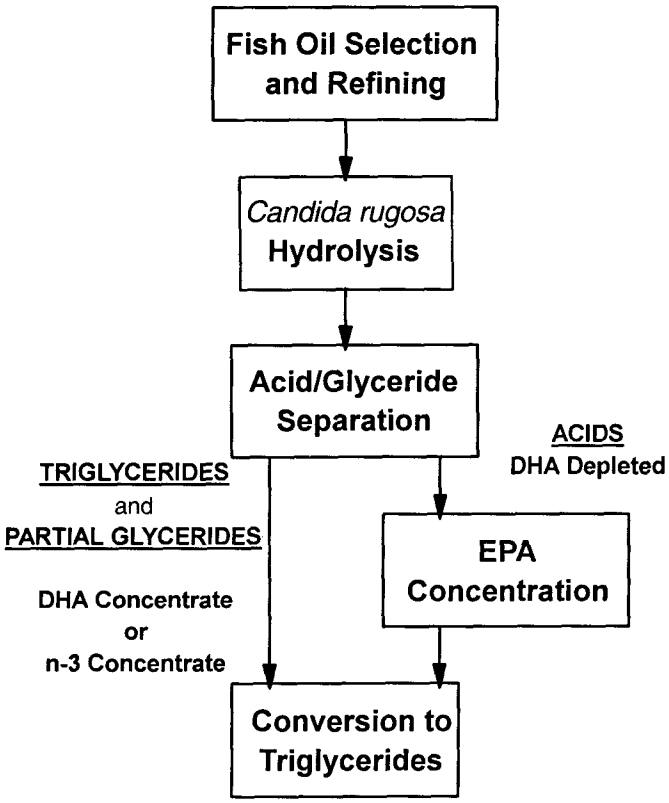
Most work to date has examined the overall concentration of n-3 PUFA in single processes. For the purposes of providing materials for formulating nutritionally functional foods, it would be useful to be able to provide both overall concentrates and isolates of individual acids. For example, DHA is recognized as being important for brain and eye development in infants. Some studies, however, have observed negative effects on growth when feeding DHA to infants in combination with high EPA levels (16). Also, these materials are susceptible to oxidation. Hence, there are stability benefits in being able to formulate products with only the required acid (DHA or EPA), while minimizing the total n-3 level.

Here, we describe experiments that were carried out to produce, as triglycerides, an overall n-3 concentrate, a DHA concentrate (with depleted EPA levels) and an EPA concentrate (with depleted DHA levels) from fish oils. The processes exploit the chainlength specificity of *C. rugosa* lipase in hydrolysis reactions and combine this with other physical separation processes and lipase reactions to prepare the final materials.

EXPERIMENTAL PROCEDURES

Overall process scheme. Production of the three concentrates described above is given in Scheme 1. By controlling the level of hydrolysis, a reaction with *C. rugosa* lipase is used to either concentrate the total n-3 acids or DHA in the glyceride fraction. The free fatty acids and glycerides are then separated. These glycerides consist of triglycerides and partial glycerides. To convert this system back to substantially all triglycerides, a partial hydrolysis, followed by an enzymic esterification, is carried out. The purpose of the partial hydrolysis is to generate glycerol, which can be removed by washing and drying of the oil, leaving the free fatty acids and partial glycerides in the correct stoichiometry for re-esterification to triglyceride.

*To whom correspondence should be addressed.



SCHEME 1

The free fatty acids, removed after the *C. rugosa* hydrolysis and which are depleted in DHA, are subjected to a concentration step to increase the EPA level, and are then converted to triglyceride by lipase-catalyzed esterification with glycerol.

Materials. Chilean fish oils with suitable starting compositions in terms of their DHA and EPA levels were selected and refined according to the methods disclosed in Unilever Patent EP 304 115 (17). These methods include a soda/silicate "boiling" process after neutralization, bleaching with high levels (~4%) of acid-activated bleaching earth, and deodorization below 190°C. The initial free fatty acid composition of the oils used for each product is given in Table 1.

Candida rugosa AY lipase, in powdered form, was supplied by the Amano Pharmaceutical Co. Ltd. (Nagoya, Japan). SP392 catalyst, *R. miehei* immobilized on an ion exchange resin, was supplied by Novo Nordisk Bioindustries U.K. Ltd.

Candida rugosa *hydrolysis. Candida rugosa* AY lipase was dissolved in demineralized water and added to the fish oil. A ratio of 0.5:1 water to oil (w/w) was used in the preparation of the n-3 and EPA concentrates, and 1:1 was used for the DHA material. The reaction was carried out at 25°C under nitrogen with stirring. When the required level of free fatty acids had been produced, the reaction was stopped by heating to 90°C. The inactivated enzyme solution was separated, and the oil was then washed and dried by heating under vacuum.

Acid/glyceride separation. After the *C. rugosa* hydrolysis, the free fatty acids were separated from the mixed triglycerides and partial glycerides in a Fischer shortpath evaporator (model KD 500/S). The process was run at a temperature of 190°C, with a flow rate that gave a residence time, in the unit, of about 1 min.

Conversion of triglyceride and partial glyceride mixture to triglycerides: Step 1—Partial hydrolysis. Partial hydrolysis of the glyceride fraction was used in the triglyceride resynthesis process (described above), for the production of the n-3 and DHA concentrates. This was achieved as follows. Demineralized water and the oil were mixed together in a weight ratio of 1:1. Immobilized *R. miehei* lipase (SP392 ex Novo Nordisk) was added. The reaction was allowed to proceed, with stirring under nitrogen, to the required free fatty acid level. The enzyme and water were separated by settling. The oil was then washed several times to remove glycerol generated during the reaction, and then dried under vacuum.

Step 2—Re-esterification of mixed glycerides and free fatty acids. Immobilized *R. miehei* lipase (SP392 catalyst) was also used for the re-esterification process. This was carried out at 55°C under vacuum to remove water generated during the reaction. The reaction was continued until the free fatty acid level had fallen to below 5%. The catalyst was then removed by filtration, and the oil was refined according to the method given above.

Concentration of EPA. The acids from a *C. rugosa* hydrolysis were subjected to an acetone fractionation at −60°C. A solvent-to-oil ratio of 4:1 was used. The olein fraction, containing the EPA concentrate, was collected by filtering the crystallized slurry. The solvent was removed from this fraction by evaporation under vacuum.

Esterification of EPA-containing free fatty acids with glycerol. A slight molar excess of the concentrated EPA acids, from the above fractionation, was added to glycerol in a batch vessel. SP392 catalyst was added. The esterification was car-

TABLE 1
Fatty Acid Composition of Fish Oils (wt%)[a]

	14:0	14 Un/15	16:0	16:1	16 Un/17	18:0	18:1	Other 18	20:0	20:5	Other 20	22:0	22:6	Other 22
Feed oil for n-3 concentrate	6.5	0.9	15.6	8.0	5.9	3.4	15.1	5.3	0.2	16.2	4.4	0.1	13.2	5.3
Feed oil for DHA concentrate	5.5	1.6	16.8	6.6	4.8	4.8	19.2	4.8	0.2	10.1	5.1	—	14.2	6.5
Feed oil for EPA concentrate	7.0	1.0	18.3	7.9	5.9	3.8	13.6	5.1	0.2	15.9	3.9	—	12.3	5.1

[a]Refer to text for details of products and materials. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Un, unsaturated.

ried out at 55°C with vacuum applied. At the end of the reaction, the catalyst was separated by filtration, and the oil was refined according to the method previously referenced.

Analysis. Fatty acid compositions were determined by fatty acid methyl ester gas chromatography by the method given in AOCS Ce 1b-89 (18). Free fatty acids were determined by titration against standard sodium hydroxide solution. Partial glyceride contents were determined by silica gel high-performance liquid chromatography.

RESULTS

Production of a total n-3 concentrate. The production process for the total n-3 concentrate is described above and outlined in Scheme 1. The composition of the glyceride fraction at each step in the process is shown in Table 2.

The hydrolysis with *C. rugosa* lipase was stopped when the free fatty acid level had reached 60%. At this stage, the total n-3 content in the glyceride fraction had increased to 50%. This represented an overall enrichment of 1.6. The increase in EPA level, however, was relatively small, compared to a more than doubling of the DHA level. Removal of the free fatty acid from this mixture by short-path evaporation at 190°C did not significantly alter the fatty acid composition in the remaining triglyceride and partial glyceride fraction.

The partial hydrolysis with SP392 catalyst, part of the process for converting the glyceride mixture back to triglyceride (see above), was taken to 20% free fatty acid. This level of hydrolysis generated sufficient glycerol such that, after its removal by washing and drying, the system could be re-esterified to >95% triglycerides, again by using SP392 catalyst. The above processes and final refining, once again, did not affect the total n-3 levels in the product.

DHA concentrate. The DHA concentrate was prepared from a starting oil with an initial DHA/EPA ratio greater than 1 (Table 1). This facilitated achieving a high final DHA/EPA ratio. The composition at each stage in the production process, described in the Experimental Procedures section and in Scheme 1, is given in Table 3. The hydrolysis reaction, with *C. rugosa* lipase, was taken to 80% free fatty acid, resulting in almost a tripling of the initial DHA level to about 40%. At this level of hydrolysis, the EPA level was also reduced over its starting value, leading to a final DHA/EPA ratio of 5.3. Partial glyceride levels in the oil, after *C. rugosa* hydrolysis and acid removal, were similar to those found during the production of the n-3 concentrate, described above.

TABLE 2
Composition of Fractions During Preparation of n-3 Concentrate[a]

	EPA (wt%)	DHA (wt%)	DPA (wt%)	Total (wt%)	DG (wt%)	MG (wt%)	FFA (wt%)
Refined Chilean fish oil	16.2	13.2	2.3	31.7	1.9	<0.1	<0.1
Glycerides after *Candida rugosa* hydrolysis	18.5	28.8	4.4	51.7	—	—	58.1
Glycerides after evaporation	18.5	28.8	4.4	51.7	28.5	0.9	2.2
Glycerides after partial hydrolysis	18.5	28.8	4.4	51.7	24.2	5.1	2.2
Triglycerides after re-esterification	18.5	28.8	4.4	51.7	4.6	<0.1	1.9
Refined product	18.6	28.5	4.7	51.8	4.6	<0.1	<0.1

[a]Refer to the Experimental Procedures section for details of overall production route and individual process steps. DPA, docosapentaenoic acid; DG, diglyceride; MG, monoglyceride; FFA, free fatty acids. See Table 1 for other abbreviations.

TABLE 3
Composition of Fractions During Preparation of DHA Concentrate[a]

	EPA (wt%)	DHA (wt%)	DHA/EPA	DG (wt%)	MG (wt%)	FFA (wt%)
Refined Chilean fish oil	10.1	14.2	1.4	1.2	<0.1	<0.1
Glycerides after *Candida rugosa* hydrolysis	7.5	39.5	5.3	—	—	80.2
Glycerides after evaporation	7.5	39.5	5.3	24.3	0.8	3.3
Glycerides after partial hydrolysis	7.5	39.5	5.3	26.1	4.7	22.8
Triglycerides after re-esterification	7.5	39.5	5.3	4.2	<0.1	6.5
Refined product	7.5	40.6	5.4	4.0	<0.1	<0.1

[a]Refer to the Experimental Procedures section for details of overall production route and individual process steps. See Tables 1 and 2 for abbreviations.

TABLE 4
Composition of Fractions During Preparation of EPA Concentrate[a]

	EPA (wt%)	DHA (wt%)	EPA/DHA
Refined Chilean fish oil	15.9	12.3	1.3
Acids from hydrolysis	12.3	2.4	5.1
Fractionated acid olein	33.2	5.9	5.6
Fractionated acid stearin	2.3	0.8	—
Triglycerides after esterification and refining	33.3	6.0	5.6

[a]Refer to the Experimental Procedures section for details of overall production route and individual process steps. See Table 1 for abbreviations.

And once again, a partial hydrolysis to about 20% free fatty acid allowed final re-esterification to >95% triglycerides (see production of total n-3 concentrate above).

EPA concentrate. The acids used in the preparation of the EPA concentrate (see Experimental Procedures section and Scheme 1 for process description) were obtained by evaporating the free fatty acids from a 60% hydrolysis reaction with *C. rugosa* lipase. The acid composition is shown in Table 4. The starting oil, used in this hydrolysis reaction, was of a similar composition to that used for the production of the n-3 concentrate (Table 1). These acids, therefore, would be representative of those produced by that process. Although the acids were depleted in both EPA and DHA, the decrease in EPA level was relatively small compared to the fivefold reduction in DHA level. The resultant EPA/DHA ratio was 5:1 with an EPA level of 12.3%.

A 35% yield of olein was achieved from solvent fractionation, with an EPA level of 33.2%. Recovery of EPA into the olein was, therefore, better than 85%. These acids were esterified, by the procedure described above, to yield a material that contained >95% triglyceride after refining.

DISCUSSION

The final composition of the materials produced here depends on the relationship between the initial fish oil composition and the selective hydrolysis. This has been investigated separately in laboratory experiments (19). Figures 1 and 2 show data from that work, which illustrates the principles involved. The DHA and EPA levels in the glycerides, as a function of degree of hydrolysis, are shown for an oil of similar starting composition to that used for the n-3 concentrate. The total DHA + EPA, within the glycerides, rises almost linearly with amount of hydrolysis. The graph also shows, however, the chainlength discrimination of *C. rugosa* lipase between EPA and DHA. There is an initial increase in the EPA level, as lower-chainlength fatty acids are preferentially hydrolyzed away. Ultimately, however, EPA itself is hydrolyzed preferentially to DHA, and its concentration falls compared to its initial level. The overall increase in n-3, therefore, is due to the increasing DHA concentration, which is reflected in the DHA/EPA ratio, which also increases (Fig. 3).

In the free fatty acid fraction (Fig. 2), the EPA level rises back to its initial concentration in the fish oil as hydrolysis progresses. Due to hydrolysis of some DHA, the EPA/DHA ratio falls (Fig. 3) and is highest during the early stages of hydrolysis.

With respect to the materials produced here, this has a number of impacts. First, for the production of a concentrate, a balance needs to be found between initial n-3 levels, the level of enrichment required and the final yield of product. The fish oil used in these experiments is toward the top end of n-3 levels for common, commercially available oils. And at a hydrolysis level of 60%, it represents a good compromise between yield and final n-3 content. Beyond this level, the increasing yield loss is probably not compensated for by the small gain in concentration. Second, for the production of a DHA concentrate, the oil used should preferentially have a favorable DHA/EPA ratio and as high a DHA level as possible. Tuna oil, for example, has been used (14,20). Its cost, however, is high and its availability is limited. The oil used here is representative of the more common commercial oils with a suitable ratio and DHA content. A final level that approaches those found with tuna oil is reached. This is, however, at the cost of final product yield, because 80% hydrolysis is required. Third, for the production of an EPA concentrate, a subsequent enrichment step is needed, as the acid fraction always contains less EPA than the starting oil. This is facilitated by choosing an oil with a high EPA level. Due to the high degree of discrimination which *C. rugosa* shows against DHA, however, it is not necessary to have a low-DHA oil. This offers the opportunity to use the same fish oil for the production of a n-3 concentrate and an EPA product. There are a number of techniques that can be used for acid/glyceride separation. Liquid/liquid or supercritical fluid extraction, selective adsorption/desorption, and neutralization are all possible. The key requirement is that, whichever method is chosen, it should not affect the final composition, for example, by causing polymerization or oxidation of these highly unsaturated acids.

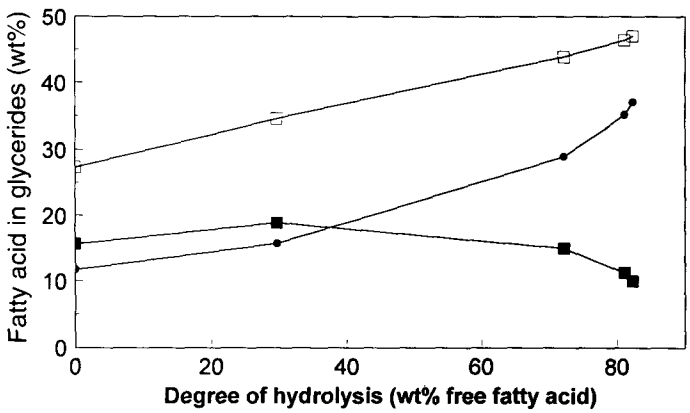


FIG. 1. Concentration of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in glycerides (wt%) during hydrolysis of fish oil by *Candida rugosa* lipase. EPA (■), DHA(●), EPA + DHA (□).

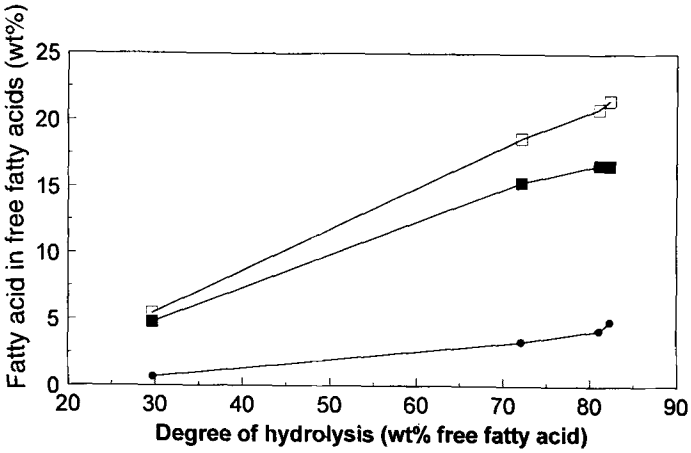


FIG. 2. Concentration of EPA and DHA in free fatty acids (wt%) during hydrolysis of fish oil by *Candida rugosa* lipase. EPA (■), DHA(●), EPA + DHA (□). See Figure 1 for abbreviations.

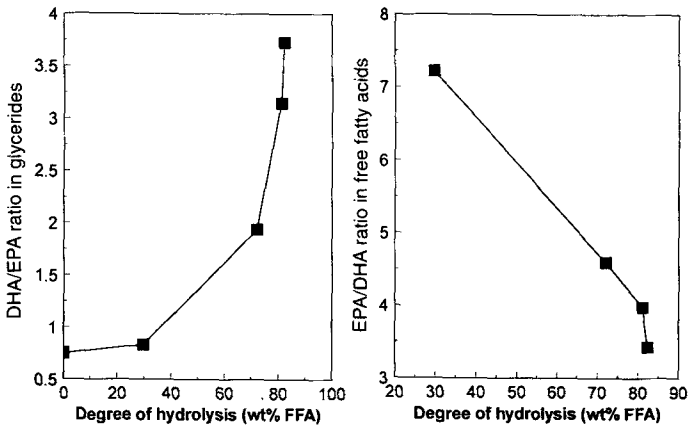


FIG. 3. DHA/EPA and EPA/DHA ratios in glyceride and free fatty acid fractions during hydrolysis of fish oil with *Candida rugosa* lipase. See Figure 1 for abbreviations.

Short-path evaporation, used in this study, proved suitable in this respect.

The purpose of the partial hydrolysis is to generate glycerol, which can be removed by washing, leaving a system of triglycerides, partial glycerides, and free fatty acids, which can be completely re-esterified to triglycerides. In this study, an *sn*-1-3 specific lipase was used. It is theoretically possible that such a lipase can be used to produce free fatty acid without generating glycerol, leaving 2-monoglyceride and 1,2-diglyceride. Partial glyceride isomerization, however, has been recognized for a long time (21), as has its consequences within enzymatic reactions (22). The isomerization itself favors 1(3)-monoglycerides and 1,3-diglycerides. Hence, in reality, hydrolysis of all fatty acid residues on some molecules does take place, even with a regiospecific lipase. Here, the hydrolysis was taken to a slight excess of free fatty acid over the level required for total re-esterification to triglyceride. And this proved to be sufficient to convert >95% of the glyceride. This is presumably due to the combined effects of the time allowed for reaction, the large excess of water present, and extraction of free glycerol into the aqueous phase, the latter effect preventing re-esterification of glycerol to partial glycerides.

As with the acid removal process, the concentration method used for the EPA-containing acids was one of a number that could have been employed. The results of low-temperature solvent fractionation, for example, should be comparable to urea complexation. Whichever method is chosen, it must minimize oxidation and polymerization. Solvent fractionation was suitable in this respect.

The use of *R. miehei* lipase in esterification reactions has been studied previously (23). In addition to the potential problems of regiospecificity, *R. miehei* also has been reported to show some selectivity against longer-chain fatty acids (24). This discrimination, however, is not absolute. By directing the reaction with water removal and allowing sufficient reaction time (~48 h), virtually complete conversion to triglyceride was achieved for both partial glycerides and glycerol with free fatty acids.

We conclude from these studies that combining fish oil selection with lipase and physical processing methods is a feasible means of producing a range of long-chain n-3 enriched products.

REFERENCES

1. Newton, I.S., Food Enrichment with Long Chain n-2 PUFA, *INFORM 7*:169–177 (1996).
2. Stout, V.F., W.B. Nilson, J. Krzynowek, and H. Schlenke, in *Fish Oils in Nutrition*, edited by M.E. Stansby, van Nostrand Reinhold, New York, 1990, pp. 76–79.
3. Schlenk, H., Urea Inclusion Compounds of Fatty Acids, *Prog. Chem. Fats Other Lipids 2*:243–267 (1954).
4. Ratnayake, W.M.N., B. Olsson, D. Matthews, and R.G. Ackman, Preparation of Omega-3 PUFA Concentrate from Fish Oils via Urea Complexation, *Fat Sci. Technol. 90*:381–386 (1988).
5. Ackman, R.G., P.J. Ke, and P.M. Jangaard, Fractional Vacuum Distillation of Herring Oil Methyl Esters, *J. Am. Oil Chem. Soc. 50*:1–8 (1973).
6. Stout, V.F., and J. Spinelli, Polyunsaturated Fatty Acids from Fish Oils, U.S. Patent 4,675,132 (1987).
7. Rizvi, S.S.H., R.R. Chao, and Y.J. Liaw, Concentration of Omega-3 Fatty Acids from Fish Oil Using Supercritical Carbon Dioxide, in *Supercritical Fluid Extraction and Chromatography, ACS Sym. Ser.* 366:89–108 (1988).
8. Moffat, C.F., A.S. McGill, R. Hardy, and R.S. Anderson, The Production of Fish Oil Enriched in Polyunsaturated Fatty Acid Containing Triglycerides, *J. Am. Oil Chem. Soc. 70*:133–138 (1993).
9. Yamane, T., T. Suzuki, Y. Saheshi, L. Vikersveen, and T. Hoshino, Production of n-3 Polyunsaturated Fatty Acid Enriched Fish Oil by Lipase-Catalyzed Acidolysis Without Solvent. *Ibid.* 69:1104–1107 (1992).
10. Yamane, T., T. Suzuki, and T. Hoshino, Increasing n-3 Polyunsaturated Fatty Acid Content of Fish Oil by Temperature Control of Lipase-Catalyzed Acidolysis, *Ibid. 70*:1285–1287 (1993).
11. Zuyi, L., and O.P. Ward, Lipase Catalyzed Alcoholysis to Concentrate the n-3 Polyunsaturated Fatty Acid of Cod Liver Oil, *Enzyme Microb. Technol. 15*:601–606 (1993).
12. Tanaka, Y., J. Hirano, and T. Funada, Concentration of Docosahexaenoic Acid in Glyceride by Hydrolysis of Fish Oil with

Candida cylindracea Lipase, *J. Am. Oil Chem. Soc. 69*:1210–1214 (1992).
13. Tanaka, Y., T. Funada, J. Hirano, and R. Hashizume, Triglyceride Specificity of *Candida cylindracea* Lipase: Effect of Docosahexaenoic Acid on Resistance of Triglyceride to Lipase, *Ibid. 70*:1031–1034 (1993).
14. Shimada, Y., K. Marayama, S. Okazaki, M. Nakamura, A. Sugihara, and Y. Tominaga, Enrichment of Polyunsaturated Fatty Acids with *Geotrichum candidum* Lipase, *Ibid. 71*:951–954 (1994).
15. Lie, O., and G. Lambertsen, Fatty Acid Specificity of *Candida cylindracea* Lipase, *Fette Seifen Anstrichmittel 88*:365–367 (1986).
16. Carlson, S.E., The Role of PUFA in Infant Nutrition, *INFORM 6*:940–946 (1995)
17. Patent EP 304115.
18. *Official Methods and Recommended Practices of the American Oil Chemists' Society*, edited by D. Firestone, AOCS Press, 1996, Method Ce 1b–89.
19. McNeill, G.P., R.G. Ackman, and S.R. Moore, Lipase-Catalyzed Enrichment of Long-Chain Polyunsaturated Fatty Acids, Paper presented 87th AOCS Annual Meeting, Indianapolis, April 28–May 1, 1996.
20. Tanaka, Y., J. Hirano, and T. Funada, Synthesis of Docosahexaenoic Acid Rich Triglyceride with Immobilized *Chromobacterium viscosum* Lipase, *J. Am. Oil Chem. Soc. 71*:331–334 (1994).
21. Crossley, A., I.P. Freeman, B.J.F. Hudson, and J.H. Pierce, Acyl Migration in Diglycerides, *J. Chem. Soc.*:760–764 (1959).
22. Bloomer, S., P. Adlercreutz, and B. Mattiasson, Triglyceride Interesterifcation by Lipases. *Enzyme Microb. Technol. 14*:23–40 (1992).
23. Ergan, F., Glyceride Synthesis from Free Fatty Acids and Glycerol in Engineering of/with Lipases, edited by F.X. Malcata, Kluwer, (1996), pp. 421–434.
24. Hills, M.J., I. Kiewitt, and K.D. Mukherjee, Enzymatic Fractionation of Fatty Acids: Enrichment of γ-Linolenic Acid and Docosahexaenoic Acid by Selective Esterification Catalyzed by Lipases, *J. Am. Oil Chem. Soc. 9*:561–564.

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