Separation of EPA and DHA in Fish Oil by Lipase-Catalyzed Esterification with Glycerol

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ABSTRACT: The objective of this study was to investigate the use of lipases as catalysts for separating EPA and DHA in fish oil by kinetic resolution based on their FA selectivity. Esterification of FFA from various types of fish oils with glycerol by immobilized *Rhizomucor miehei* lipase under water-deficient, solvent-free conditions resulted in a highly efficient separation of EPA and DHA. Reactions were conducted at 40°C with a 10% dosage of the lipase preparation under vacuum to remove the coproduced water, thus rapidly shifting the reaction toward the products. The bulk of the FA, together with EPA, were converted into acylglycerols, whereas DHA remained in the residual FFA. As an example, when FFA from tuna oil comprising 5% EPA and 25% DHA were esterified with glycerol, 90% conversion into acylglycerols was obtained after 48 h. The residual FFA contained 78% DHA and only 3% EPA, in 79% DHA recovery. EPA recovery in the acylglycerol fraction was 91%. The type of fish oil and extent of conversion were highly important parameters in controlling the degree of concentration.

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KEY WORDS: DHA, EPA, esterification, n-3 fatty acids, fish oil, glycerol, kinetic resolution, lipase, Lipozyme RM IM, *Rhizomucor miehei* lipase.

Various beneficial biochemical and pharmacological effects of marine oils on human health have been studied comprehensively (1). They are attributed to the long-chain n-3-type PUFA characteristic of marine oils, notably *cis*-5,8,11,14,17-EPA and *cis*-4,7,10,13,16,19-DHA. In the beginning, the emphasis was almost exclusively on the beneficial effects of EPA on cardiovascular disease and various inflammatory diseases. This resulted in high demands by the pharmaceutical industry for concentrates with high EPA content as well as by the health food industry for EPA as food supplements (2). Recently, there has been a dramatic shift in interest toward DHA and its beneficial effects on pregnancy, infant nutrition, brain and nervous system development, and, most recently, depression and various mental disorders (3,4).

Various physical methods and combinations of methods are available for enriching EPA and DHA and their concentrations in fish oil (5,6). Lipases have found applicability in such enrichment based on their FA selectivity, which can be used in concentrating EPA and/or DHA by kinetic resolution. Lipases offer many advantages over the physical methods currently in use. They are ideally suited to esterification processes involving the highly labile PUFA because of the mild conditions under which they act (7). Their catalytic efficiency is high, so a relatively low amount of enzyme is required, especially when immobilized, which enables their reuse and enhances their productivity. There are numerous reports in the literature involving biotransformations of n-3 PUFA from marine oil by commercially available lipases (6,8,9). As a general rule, saturated and monounsaturated FA are good substrates for lipases, whereas the polyunsaturated ones are inadequate. The preference of lipase for EPA over DHA is a common feature of the commercially available lipases that display activity toward the long-chain n-3 PUFA. The reason is believed to relate to the carbon–carbon double bond in closest proximity to the carboxyl group being located one bond closer in DHA. This presumably adds some strain to the active site of these enzymes to accommodate DHA properly (10). *Pseudomonas* lipases are an exception to this rule in that they have been observed to display a slight preference for DHA over EPA (11).

Lipases can be used to make concentrates of EPA and DHA in a whole range of compositions highly efficiently and in very high to excellent yields. They can be used to concentrate EPA together with DHA (11), or they can offer a strong discrimination between EPA and DHA, which can be used to concentrate EPA or DHA individually (12). The ability of the *Rhizomucor miehei* lipase to discriminate against DHA has been used for the enrichment of DHA from fish oil FA by selective esterification (12,13) or transesterification (14) reactions. Other lipases that have been used to concentrate DHA are *Candida rugosa* (15,16), *Rhizopus delemar* (17), *Geotrichum candidum* (18), and *R. niveus* (19) lipases. We previously described such a separation of EPA and DHA, where esterification of various fish oil FFA with ethanol was conducted at room temperature under atmospheric pressure using immobilized *R. miehei* lipase (Lipozyme RM IM) (12). This report describes a related approach to separate EPA and DHA in fish oil based on esterification of fish oil FFA with glycerol by Lipozyme RM IM at 40°C under vacuum without a solvent. The bulk of the FA including EPA were converted into a mixture of acylglycerols, i.e., MAG, DAG, and TAG, whereas DHA remained in the residual FFA. This is illustrated in Scheme 1.

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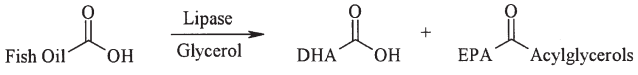

SCHEME 1

EXPERIMENTAL PROCEDURES

The fungal lipase from *R. miehei* (Lipozyme RM IM) and yeast lipase from *C. antarctica* (Novozym 435) were supplied immobilized as a gift from Novozymes A/S (Bagsvaerd, Denmark). Other microbial lipases were supplied by Amano Enzyme Europe Ltd. (Milton Keynes, England), and the porcine pancreas lipase was supplied by Sigma Chemical Company (St. Louis, MO). All were used directly as powders without any preadjustment or optimization of pH. Refined herring oil (5.5% EPA; 8.0% DHA), sardine oil, and sardine oil ethyl esters (16.8% EPA; 12.3% DHA), Chilean fish oil (20.0% EPA; 7.2% DHA), and tuna oil (5.2% EPA; 24.5% DHA) were all provided by Pronova Biocare A/S (Sandefjord, Norway). They were used without any further refinement. Reagent grade sodium hydroxide, anhydrous magnesium sulfate, and hydrochloric acid (37% w/w) were obtained from Merck (Darmstadt, Germany), and glycerol (99%) was obtained from Sigma. All were used without further purification. Solvents were obtained from Acros Organics (Geel, Belgium); all were of analytical grade and used without further purification.

Analysis. Analytical TLC plates (DC Alufolien Kieselgel 60 F_{254}) were obtained from Merck. FA analyses were performed on methyl esters using a PerkinElmer 8140 gas chromatograph with a 30 m capillary column, DB-225 30 N 0.25 mm (J&W Scientific, Folsom, CA) with hydrogen as a carrier gas, according to a previously described procedure (20). Preparative TLC was conducted on silica gel plates from Sigma (Art. 5721) after washing with a 50:50 (vol/vol) mixture of chloroform/methanol and heating at 110°C for 30 min. Elution was performed with an 80:20:1 (by vol) mixture of petroleum ether/diethyl ether/acetic acid to separate TAG, FFA, DAG, and MAG. Rhodamine 6G (Merck) was used to visualize the bands, which were subsequently scraped off and the fat methylated as described previously (20), then analyzed for FA. When quantitative analysis of individual lipid classes was required, it was necessary to add an accurately weighed internal standard (19:0 methyl ester from Sigma) to each fraction before methylation. The amount of standard (Wt_{Std}) used was approximately 10% of the estimated percentage weight of each lipid class in the sample. When calculating the weight of each lipid class, the type of alcoholic moiety was accounted for by using correction factors (CF) according to the equation shown below. The CF used were 1.211, 1.049, 0.995, and 0.951 for MAG, DAG, TAG, and FFA, respectively (21).

$$Wt_{Class} = CF \times \left(\frac{Wt_{Std} \times 100\%}{\%\,Area_{Std}} \right) \quad [1]$$

Hydrolysis of fish oil. Fish oil TAG (100 g, approximately 0.11 mol) were added to a solution of sodium hydroxide (38 g, 0.95 mol) in distilled water (100 g, 5.55 mol) and ethanol (96%, 350 mL). The mixture was refluxed under nitrogen until the reaction was completed (less than 1 h), as determined by analytical TLC [eluted with 1:9 (vol/vol) ethyl acetate/petroleum ether]. The mixture was allowed to cool to room temperature and then acidified by carefully adding an aqueous solution of 4.0 M hydrochloric acid until slightly acidic. The temperature was not allowed to rise significantly, in which case ice was added to cool the mixture. The resulting mixture was extracted twice with a 50:50 (vol/vol) mixture of petroleum ether and diethyl ether. The combined organic fractions were washed several times with distilled water until almost neutral in pH, and then dried over anhydrous magnesium sulfate. The organic solvent was removed *in vacuo* on a rotary evaporator, finishing on high vacuum for 2 h at 50°C to afford the fish oil FFA in approximately 95% yield.

Lipase-catalyzed esterification. In a typical procedure, the immobilized *R. miehei* lipase preparation (1.0 g) was added to a mixture of fish oil FFA (10.0 g, approximately 34.5 mmol) and glycerol (1.59 g, 17.3 mmol). The resulting enzyme suspension was gently stirred at 40°C on a magnetic stirrer hot plate under continuous vacuum (0.01–0.1 Torr). The volatile water produced during the progress of the reaction was continuously condensed into a liquid nitrogen-cooled trap. Small samples (200–300 mg) were withdrawn from the reaction mixture by the aid of a Pasteur pipet at appropriate time levels. During sampling, the vacuum was released and replaced by nitrogen atmosphere. Care was taken that no unreacted glycerol escaped from the reaction. The enzyme particles were separated off by filtering the sample through a cotton wool plug located inside a second Pasteur pipet. Approximately 50 mg of the residual oil was accurately weighed and dissolved in isopropanol (25 mL). The resulting mixture was titrated with an aqueous 0.02 M sodium hydroxide solution, using phenolphthalein as an indicator. Blank titration of the isopropanol was performed on a regular basis. Preparative TLC was applied to separate the acylglycerols (not further fractionated except for the individual class weight percentage analysis) and the residual FFA, each of which was subsequently methylated and subjected to GLC to reveal their FA composition. The productivity studies were performed in an identical manner using an overhead stirrer (100 g FFA, 16 g glycerol, 10 g lipase) and terminated after 16 h.

Screening experiments. The esterification screening experiments were performed under conditions identical to those described above using sardine oil FFA (1.0 g), glycerol (160 mg), and lipase (100 mg). The reactions were terminated after 24 h and the products were analyzed as before. In the glycerolysis reactions involving sardine oil ethyl esters (1.0 g), glycerol (145 mg), and lipase (100 mg), the same conditions were used as for the esterification reactions. The extent of conversion was determined by the internal standard method described above as the weight percentage of acylglycerols formed. The CF for ethyl esters was exactly the same as that for DAG (1.049).

RESULTS AND DISCUSSION

The chief objective of this study was to develop a successful lipase-catalyzed process for separating and concentrating EPA and DHA in fish oil by kinetic resolution. EPA concentrates

with low DHA content are in high demand for treatment of various specific EPA-related health effects. Likewise, DHA concentrates with low EPA content are also in high demand, for infant formulas, for example. Kinetic resolution (12) occurs if the rate constant for EPA (k_{EPA}) is not equal to the rate constant for DHA (k_{DHA}). Fast processes are favorable for kinetic resolution, and any sort of equilibrium must be avoided to get maximal discrimination between EPA and DHA. Lipozyme RM IM is an immobilized 1,3-regioselective *R. miehei* fungal lipase commercially available from Novozymes A/S in Denmark. We previously described the application of Lipozyme RM IM to separate EPA and DHA highly successfully in the esterification of fish oil FFA with a twofold stoichiometric amount of ethanol at room temperature without a solvent (12). From that work, it was evident that EPA is a much better substrate for this lipase than DHA, resulting in substantial enrichment of DHA in the residual FFA. However, in terms of successfully separating the ethyl esters and the FFA fraction highly enriched with DHA by short-path distillation, the approach based on esterification of ethanol was not considered feasible. This relates to an inadequate difference in the b.p. between DHA as a free acid and the longest-chain FA, including EPA, as ethyl esters.

To compensate for this poor separation, a different approach was proposed. We decided to base this approach on esterification of FFA, obtained from fish oil hydrolysis, with glycerol instead of ethanol according to Scheme 1. A lipase such as Lipozyme RM IM was expected to convert the bulk of the FA present, including EPA, into acylglycerols, leaving the far more recalcitrant DHA untouched in the residual FFA. It was hoped that results comparable to those of ethanol, in terms of lipase activity and discrimination between EPA and DHA, would be offered by this process. It was also anticipated that by this modification the DHA-enriched FFA fraction could be successfully separated from the less-volatile acylglycerol fraction by short-path distillation. A number of lipase-catalyzed esterification processes have used glycerol (15,22). We previously reported on a highly efficient lipase-catalyzed esterification to prepare homogeneous TAG of EPA and DHA. This was accomplished by reacting glycerol with stoichiometric amounts of pure EPA or DHA as free acids in the presence of immobilized lipase from *C. antarctica* under essentially anhydrous, solvent-free, elevated temperature conditions with a continuous removal of the coproduced water under vacuum (23). There was little, but nonetheless noticeable, discrimination between EPA and DHA by that lipase in this process.

Fish oils are by far the most important source of EPA and DHA, with their total content typically within the range of 12–30%, depending on the fish species (5). Therefore, we were interested in investigating various types of fish oil as potential raw materials in the preparation of concentrates with high DHA content and perhaps concentrates high in EPA as well. We anticipated that fish oils rich in DHA, such as tuna oil, were suitable for preparing DHA concentrates by the process described in this paper, whereas fish oils rich in EPA, such as sardine oil and Chilean fish oil, were suitable raw materials for making EPA concentrates.

Esterification of herring oil FFA. The initial studies were based on herring oil FFA containing 5.5% EPA and 8.0% DHA. Glycerol was treated with two molar equivalents of FFA. This meant a 1.5-fold excess of hydroxyl groups, as based on the number of FFA equivalents participating in the reaction, with FFA thus being the limiting factor on which the percentage extent of conversion was based. The reaction was conducted at 40°C under vacuum (0.01 to 0.1 Torr). This aided the volatile water coproduced to be eliminated from the reaction directly on formation by condensation into liquid nitrogen-cooled traps as the reaction proceeded, thus rapidly shifting the reaction toward the products. This was essential to make the reaction nonreversible, thereby making it possible to obtain high recoveries and separation of the desired EPA and DHA products. To secure adequate agitation of the mixture of glycerol and the herring oil FFA, constituting a relatively low amount of PUFA, a reaction temperature of 40°C was necessary. Too high a temperature is undesirable for several reasons. High temperatures work against the lipase FA selectivity on which the kinetic resolution is based, EPA and DHA may be destroyed by prolonged exposure to high temperatures, and lipases are intolerant of high temperatures. A lipase dosage of 10% was used, as based on the weight of the FA substrate, which corresponds to a concentration of about 1% by weight (the commercially available immobilized Lipozyme RM IM preparation being about 10% lipase and 90% carrier). We preferred to use an immobilized form of the selected lipase, since not only does immobilization often enhance the activity of the enzyme, but it also improves its stability and aids its recovery and reuse—all factors that affect the economics and possible industrialization of the process. The results are demonstrated in Table 1.

The approximate molar percentage of FA accommodated in the acylglycerols, as based on the initial number of moles of FFA present in the reaction, was used to monitor the progress of the reaction in terms of conversion. This was determined by titration of the residual FFA in samples withdrawn periodically from the reaction mixture as the reaction was allowed to proceed. Although this measure offers only an estimated conversion, since no attempts were made to account and correct for the glycerol moiety of the acylglycerols, it provides useful and easily obtained information on the progress of the reaction. The area percentage provided by GLC analysis of the residual FFA and the acylglycerol product mixture was used to designate the extent of enrichment of EPA and DHA into these fractions directly as the esterification reaction proceeded. Preparative TLC was used to separate the FFA and the acylglycerols, which were not further fractionated into MAG, DAG, and TAG, unless otherwise stated. The recovery or yield of EPA or DHA in the acylglycerols or the residual FFA was defined as the mole percentage representing the proportion of the total number of moles in the initial fish oil FFA. It was calculated by the following equation, with the main factor contributing to the inaccuracy being the %Fraction (%Conversion for the acylglycerols or 100% – %Conversion for the FFA fraction), as shown for DHA:

TABLE 1
Esterification of Herring Oil FA[a]

Time		Acylglycerols				Residual FFA			
		Area%		Rec.%		Area%		Rec.%	
(h)	Conv.%	EPA	DHA	EPA	DHA	EPA	DHA	EPA	DHA
1	30.4	3.4	0.5	14.5	1.4	8.1	15.7	84.5	98.6
2	59.0	3.8	0.6	30.1	3.8	8.5	21.9	60.9	96.2
3	60.2	4.7	1.0	45.3	5.5	8.6	26.2	54.7	94.5
7	79.5	5.5	1.7	81.9	17.4	4.7	31.2	18.2	82.6
12	87.4	6.0	2.3	92.2	25.5	3.5	46.7	7.8	74.5
24	89.3	6.0	2.9	96.3	32.4	1.9	50.6	3.1	67.6

[a]Conv.%, conversion—molar percentage of FA accommodated in the acylglycerols based on the total number of moles of FFA in the reaction as determined by titration. Area%, area percentage of EPA or DHA as provided by GLC FA analysis; Rec.%, recovery (or yields) of EPA or DHA in the acylglycerol or residual FFA fraction, defined as the proportion of the total molar content in the initial fish oil. Reaction conditions: FFA/glycerol (2:1, mol/mol), 10% *Rhizomucor miehei* lipase (based on the weight of FFA), 40°C, vacuum (0.01–0.1 Torr).

$$\%\mathrm{DHA}_{\mathrm{Rec.}} = \%\mathrm{Fraction} \times \left(\frac{\mathrm{Area}\%\mathrm{DHA}_{\mathrm{Fraction}}}{\mathrm{Area}\%\mathrm{DHA}_{\mathrm{Initial}}} \right) \qquad [2]$$

Excellent results were obtained for the herring oil FFA, and high extents of conversion into acylglycerols were obtained in a relatively short reaction time, as shown in Table 1. It is evident from these results that glycerol can act as an excellent alcoholic acyl acceptor for a Lipozyme RM IM-catalyzed esterification of marine oil FFA, provided that certain critical reaction conditions are followed. At these low-pressure conditions, and despite the resulting low water content, the enzyme must retain its essential water content, which is required for maintaining catalytic function (24), remarkably well. Very high recovery of both DHA in the residual FFA (82.6%) and EPA in the acylglycerol product (81.9%) was observed, as well as a favorable DHA-to-EPA ratio of nearly 7:1, as based on area percentage, after only a 7-h reaction time. The results were further improved after 12 h, when the ratio of DHA to EPA had increased to 13:1 with the residual FFA comprising 46.7% DHA and 3.5% EPA, with 74.5% DHA recovery. After 24 h, at 89.3% conversion, that ratio was 27:1, with the recovery of DHA still nearly 70%. With this process, the relatively inexpensive herring oil may serve as a suitable raw material for producing concentrates of the desired tuna oil composition, i.e., approximately 5% EPA and 25% DHA. Such a composition is under strong demand for use in infant formulas (5). It was demonstrated that the same lipase preparation was reusable in 20 subsequent 16-h runs on a 100-g scale, in which 85–90% conversion was obtained consistently without any apparent deterioration of lipase performance.

Substrate ratio. Table 2 displays the results when glycerol was treated with 3 instead of 2 equiv of herring oil FFA under the esterification conditions. This change resulted in far less satisfactory results, where 80% conversion was reached in almost 80 h, compared to only 7 h in the previous case. Moreover, a dramatic decrease in lipase discrimination between EPA and DHA, as well as poor recovery of DHA in the residual FFA, was observed. When using this 3:1 molar ratio between FFA and glycerol, i.e., an exact stoichiometric amount, the molar equivalents of FFA and glycerol hydroxyl groups are equal. This means that 100% conversion is dependent on 100% TAG formation. To form TAG, the hydroxyl group located at the mid-position of the glycerol moiety must be involved. Many lipases, including *R. miehei* lipase, are 1,3-regioselective and act preferably or exclusively at the primary end-positions of glycerol, and transformations involving the mid-position may depend on acyl migration processes (23), which are usually much slower than the lipase-catalyzed transformations. The effect of this is to slow the overall reaction rate down considerably, thereby working against the kinetic resolution. This explains our inferior results when using the less-beneficial molar ratio of 3:1. When using the 2:1 ratio, as in the previous example, high conversion did not depend on formation of high levels of TAG, only MAG and DAG formation.

TABLE 2
Esterification of Herring Oil FA[a]

Time		Acylglycerols				Residual FFA			
		Area%		Rec.%		Area%		Rec.%	
(h)	Conv.%	EPA	DHA	EPA	DHA	EPA	DHA	EPA	DHA
3	21.5	5.7	3.5	27.0	7.2	7.7	12.4	73.0	92.8
6	30.8	6.9	3.1	27.6	11.1	5.9	11.1	72.4	88.9
10	34.7	6.6	3.0	33.7	11.8	6.3	11.9	66.3	88.2
24	45.1	6.6	3.7	43.6	18.7	6.2	13.2	56.4	81.3
58	72.1	6.1	5.5	72.7	45.1	6.3	17.3	27.3	54.9
80	82.5	5.7	5.6	83.3	58.5	5.4	18.7	16.7	41.5

[a]See Table 1 for abbreviations and reaction conditions. In this experiment, a substrate ratio of 3:1 FFA/glycerol was used. All other conditions remained the same.

TABLE 3
Esterification of Tuna Oil FA[a]

Time		Acylglycerols				Residual FFA			
		Area%		Rec.%		Area%		Rec.%	
(h)	Conv.%	EPA	DHA	EPA	DHA	EPA	DHA	EPA	DHA
1	16.8	3.4	1.6	12.0	1.3	5.1	24.5	88.1	98.7
4	45.5	3.7	1.6	31.6	3.4	6.6	36.9	68.4	96.6
7	57.6	3.6	1.7	39.5	4.8	7.4	45.7	60.5	95.2
11	64.2	4.1	1.9	52.2	6.7	6.8	48.1	47.8	93.4
24	74.2	5.2	2.8	71.4	10.3	6.0	71.1	28.6	89.8
48	90.0	6.7	5.0	90.9	21.4	2.9	77.5	9.1	78.7

[a]See Table 1 for abbreviations and reaction conditions.

Further experiments on various FFA/glycerol ratios (1:1, 1.8:1, 2:1, 2.5:1, and 3:1) implied that the 2:1 ratio was indeed optimal. All further experiments in this report were therefore conducted at this 2:1 ratio. With the 2:1 ratio, no TAG formation would occur in theory. However, individual acylglycerol class analysis by preparative TLC implied that at 80% conversion, the acylglycerol mixture comprised 9% MAG, 76% DAG, and 15% TAG. At a comparable conversion using the ratio of 2.5:1, the TAG content increased to 22%, with the MAG and DAG contents remaining at 11 and 67%, respectively. Similar trends were observed for the other fish oil types tested, with noticeable variations in the MAG, DAG, and TAG ratios.

Various fish oils as potential raw materials. Table 3 shows the results when DHA-rich FFA from tuna oil, comprising 5.2% EPA and 24.5% DHA, were treated under the esterification conditions with Lipozyme RM IM. Considerably lower reaction rates can be noticed for the tuna oil as compared to herring oil FFA, presumably due to the much higher DHA content of tuna oil. The results are by no means bad, however. After 24 h, at 74.2% conversion, the residual FFA contained 6.0% EPA and 71.1% DHA in 89.8% DHA recovery, which is highly advantageous. At 90.0% conversion after a 48-h reaction time, the FFA residue contained only 2.9% EPA and 77.5% DHA, the latter in 78.7% recovery. It is evident from these results that tuna oil FFA are definitely a potential raw material for highly enriched DHA concentrates. According to Table 1, a tuna oil-like composition was obtained for the much cheaper herring oil FFA after less than 7 h, which presumably may be treated by a subsequent enzymatic step to afford a composition similar to that described for the tuna oil FFA.

Table 4 displays the results when sardine oil FFA, comprising 16.8% EPA and 12.3% DHA, were treated under the glycerol esterification conditions with Lipozyme RM IM. The results show that the lipase displayed very good discrimination between EPA and DHA. Owing to a relatively high initial EPA content in the sardine oil, the DHA-to-EPA ratio was never high, making the oil a less suitable raw material than tuna oil for preparing high-DHA concentrates by this process. However, good separation between EPA and DHA was obtained, which is best noticed from the EPA and DHA composition in the acylglycerols. For instance, 80.0% conversion into acylglycerols was obtained after 28 h. The residual FFA comprised 13.2% EPA and 50.0% DHA, whereas the acylglycerols comprised 20.1% EPA and 3.5% DHA. The recovery of both DHA into the residual FFA fraction and EPA into the acylglycerol products remained very high, at 78.4 and 85.9%, respectively.

When EPA-rich FFA from Chilean fish oil, comprising 20.0% EPA and 7.2% DHA, were treated with Lipozyme RM IM under the same esterification conditions, the results displayed in Table 5 were obtained. Acylglycerols of a highly favorable EPA-to-DHA ratio were clearly obtained, with a very high conversion rate. The method illustrated could therefore form the basis of a process for preparing not only a concentrate of DHA but also EPA, depending on the type of fish oil used. This fastest-reacting oil reached 73.0% conversion in only 3 h, at which the acylglycerols contained 23.0% EPA and just 1.9% DHA, an EPA-to-DHA ratio of 12:1. The recovery was 75.3% for EPA and 24.5% for DHA in the acylglycerols. At the same conversion, the residual FFA comprised 20.4% EPA and 15.8% DHA, with reversed recovery values. After 6 h,

TABLE 4
Esterification of Sardine Oil FA[a]

Time		Acylglycerols				Residual FFA			
		Area%		Rec.%		Area%		Rec.%	
(h)	Conv.%	EPA	DHA	EPA	DHA	EPA	DHA	EPA	DHA
1	18.6	12.1	1.0	12.0	1.5	20.4	14.3	88.0	98.5
2	39.4	14.3	1.2	31.4	4.0	20.4	18.7	68.7	96.0
3	48.3	13.4	1.2	38.2	4.9	21.7	22.4	61.8	95.1
5	53.6	16.3	1.4	45.4	5.5	22.1	28.1	54.6	94.4
7	68.0	16.7	1.9	60.9	11.0	22.7	32.0	39.1	89.0
28	80.0	20.1	3.5	85.9	21.6	13.2	50.0	14.1	78.4

[a]See Table 1 for abbreviations and reaction conditions.

TABLE 5
Esterification of Chilean Fish Oil FA[a]

Time		Acylglycerols				Residual FFA			
		Area%		Rec.%		Area%		Rec.%	
(h)	Conv.%	EPA	DHA	EPA	DHA	EPA	DHA	EPA	DHA
1	29.4	15.2	0.6	22.0	3.1	22.4	7.8	78.0	96.9
2	56.4	19.7	1.1	50.2	10.3	25.2	12.8	49.8	89.7
3	73.0	23.0	1.9	75.3	24.5	20.4	15.8	24.7	75.5
4	78.4	23.5	2.4	83.0	32.5	17.4	18.4	17.0	67.5
6	82.1	25.6	3.1	87.5	37.5	16.7	23.9	12.5	62.5
12	82.3	22.7	5.4	86.9	48.5	15.8	25.7	13.1	51.5

[a]See Table 1 for abbreviations and reaction conditions.

82.1% conversion was reached, at which time the acylglycerols comprised 25.6% EPA and 3.1% DHA. The EPA recovery was very high, 87.5%. The residual FFA comprised 23.9% DHA in only 62.5% recovery, with 16.7% EPA content.

The results presented in Tables 1 and 3–5 nicely demonstrate that lipase can be used, even in a single step, to prepare concentrates highly enriched with EPA or DHA from fish oil without any additional concentration steps. These results unequivocally underline the great potential that Lipozyme RM IM may offer for concentrating both EPA and DHA, depending on the type of fish oil FFA being used as a substrate. They reveal different reaction rates, depending on the initial FA composition of the fish oil used. Thus, a slower reaction is expected for fish oil high in DHA. The rate and recovery of the reactions described in this paper were comparable to those obtained in our previous publication for the esterification of fish oil FFA with ethanol (12). The lipase displayed very high activity under extremely low water activity conditions, resulting in a high reaction rate that served the kinetic resolution very well.

From an industrial point of view, this approach may offer certain attractions. Numerous beneficial effects related to the use of lipase may apply in this respect, namely, mildness toward the highly labile n-3 PUFA; environmentally friendly solvent-free conditions that reduce the bulkiness of the reaction extensively; a commercially available lipase, already immobilized to ease industrialization; and high efficiency of the method.

Screening of various lipases. Both Lipozyme RM IM and the immobilized *C. antarctica* lipase (Novozym 435) offered high activity under the esterification reaction conditions of fish oil FFA and glycerol. In a preliminary study, a collection of 13 other commercially available lipases were screened for activity under these conditions. The following lipases were tested: the microbial lipases from *C. rugosa*, *Aspergillus niger*, *Penicillium roqueforti*, *Penicillium camembertii*, *R. delemar*, *R. niveus*, *Humicula lanuginosa*, *R. oryzae*, *C. lipolytica*, *R. javanicus*, *Pseudomonas cepacia*, and *P. fluorescens,* as well as the mammalian porcine pancreas lipase. All lipases were used directly as powders without any preadjustment or optimization of pH. The results revealed that sufficient activity under the esterification conditions was confined to only Lipozyme RM IM and Novozym 435, the latter lipase displaying inadequate discrimination between EPA and DHA.

It is conceivable that the poor activities demonstrated by these other enzymes occurred because they were tested as dry powders, whereas Lipozyme RM IM and Novozym 435 were immobilized preparations. Immobilization on an inert carrier is known in many cases to increase apparent enzyme activity, perhaps by causing a near single-layer deposition of enzyme and thereby increasing the efficiency of contact between enzyme and substrate. However, the *Pseudomonas* lipases immobilized on Amberlite XAD-7 did not show any improved activity, suggesting that the low activities are probably inherent features of the enzymes examined.

All of the lipases listed above were also tested for transesterification of sardine oil ethyl esters with glycerol (glycerolysis) under conditions similar to those for the esterification of FFA with glycerol. These results established that the glycerolysis reaction proceeded even slower in terms of conversion as compared to esterification. Only Lipozyme RM IM and Novozym 435 displayed any appreciable extent of conversion. In terms of selection between EPA and DHA by Lipozyme RM IM the results were not as beneficial as in the corresponding studies with fish oil FFA under the esterification reaction conditions. From these results, it appears that the attempt to use glycerolysis as an alternative to the esterification reaction failed.

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