Synthesis of Polyunsaturated Fatty Acid-Enriched Triglycerides by Lipase-Catalyzed Esterification

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ABSTRACT: This paper reports on the synthesis of triglycerides by enzymatic esterification of polyunsaturated fatty acids (PUFA) with glycerol. A PUFA concentrate obtained from cod liver oil was used to optimize the reaction to favor triglyceride synthesis with lipases. The type and amount of lipase and organic solvent, glycerol content, temperature, water content, and amount and time of addition of molecular sieves were studied. The optimal reaction mixture and conditions were: 9 mL hexane, 60ºC, 0.5% (vol/vol) water, 1 g molecular sieves added after 24 h of reaction, glycerol/fatty acid molar ratio 1:3 and 100 mg of Novozym 435 (Novo Nordisk A/S) lipase. Under these conditions, an enriched triglyceride yield of 84.7% containing 27.4% eicosapentaenoic acid and 45.1% docosahexaenoic acid was obtained from a cod liver oil PUFA concentrate.
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KEY WORDS: Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), lipase-catalyzed esterification, polyunsaturated fatty acids (PUFA), triglyceride synthesis.

The n-3 polyunsaturated fatty acids (n-3 PUFA) have been recognized for their important role in health. Eicosapentaenoic acid (EPA) can affect the circulatory system and can help to prevent arteriosclerosis and thrombosis (1), and docosahexaenoic acid (DHA) is important in the development of the central nervous system of infants (2,3). For medical or dietetic purposes, PUFA may be administered in different forms: as free fatty acids (FFA), as ethyl esters, or as triglycerides (TG). FFA seem to be the best absorbable pharmaceutical form for preventing cardiovascular diseases (4) and the ethyl ester is being used for treating cancer cachexia (associated chronic weight loss) (5). However, in other applications, the pharmaceutical industry demand is for the more natural TG form. For example, highly pure trieicosapentanoyl glycerol (tri-EPA) was used as an infusion for the study of thrombotic disorders in humans (6).

Perhaps the best possibility for obtaining PUFA-enriched TG is by lipase-catalyzed reactions. Lipases are known to catalyze mild esterification reactions with the formation of specific compounds. Since the early 1980s, the use of enzymes in nonaqueous media has been extensively studied as a tool in organic synthesis. To carry out bioconversion of lipophilic compounds effectively, the use of organic solvents in the reaction systems is important, as they can improve the solubility of hydrophobic substrates in water. Li and Ward (7) carried out an in-depth study of the best conditions for esterification of glycerol and n-3 PUFA concentrate using eight commercially available lipases in organic solvent. Maximal yield (up to 92.4%) was obtained with lipase PS-30 from *Pseudomonas* and lipase IM-60 from *Mucor miehei*. However, because lipases PS-30 and IM-60 are 1,3-specific, much less TG than monoglycerides (MG) or diglycerides (DG) was formed. With lipase PS-30, the concentrations of MG, DG, and TG obtained were 23.8, 40.6, and 18.1%, respectively. Better TG synthesis was obtained from DHA ethyl ester using lipase from *Candida antarctica* immobilized on macroporous acrylic resin (sp 435). More than 95% of DHA was converted to TG by esterification at 50ºC for 23 h. The reaction was carried out under vacuum to eliminate the ethanol synthesized (8).

The authors had previously developed a three-step method to obtain highly pure PUFA from cod liver oil (9) and from the marine microalgae *Isochrysis galbana* (9,10) and *Phaeodactylum tricornutum* (11,12). In the work reported in this paper, our goal has been to optimize reaction conditions for the production of commercially viable highly EPA- and DHA-rich TG by esterification with glycerol of a PUFA concentrate obtained from cod liver oil. In an upcoming paper these optimized conditions are applied to obtain highly EPA-enriched TG from microalgal lipids.

MATERIALS AND METHODS

Chemicals and materials. The lipases, Lipozyme IM and Novozym 435, were donated by Novo Nordisk A/S (Bagsvaerd, Denmark). Lipozyme IM, which is derived from *M. miehei,* is immobilized on macroporous anion exchange resin and has a 1,3-positional specificity. Novozym 435, derived from *C. antarctica*, is immobilized on macroporous acrylic resin, contains 2–3% water, and has 1,3-positional specificity in some reactions and none in others. Lipase PS, donated by Amano Enzyme Europe Ltd. (Milton Keynes, United Kingdom), was derived from *Pseudomonas*. Analytical-grade glycerol and organic solvents (hexane and isooctane) were obtained from Sigma Chemical (St. Louis, MO)

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and Panreac (Barcelona, Spain); 4 Å molecular sieves were obtained from Sigma Chemical Co. Water was purified in a Milli-Q system (Millipore Co., Bedford, MA).

Preparation of n-3 PUFA concentrates. Commercial cod liver oil (Acofarma, Barcelona, Spain) was saponified to obtain FFA as described elsewhere (9). The fatty acid composition of the extract is given in Table 1. The PUFA concentrate was obtained from FA extract of cod liver oil by the urea method as described by Robles Medina *et al.* (9). The FA composition of the PUFA concentrate is also given in Table 1.

Esterification reaction. A typical reaction mixture for glyceride synthesis consisted of glycerol, 2 g (about 20 mmol); PUFA concentrate, 0.4 g (about 1.2 mmol); hexane, 3 mL; water, 50 µL (1% on total volume); and lipase, 25 mg. This reaction mixture was placed in 50-mL Erlenmeyer flasks with silicone-capped stoppers under argon atmosphere, to avoid degradation of PUFA. The suspension was incubated at 50ºC and agitated in a water-bath shaker at 175 rpm; 1 g of molecular sieves was added after 1 h incubation to remove the water formed during the reaction. The reaction was stopped by addition of an acetone/ethanol mixture (1:1, vol/vol). Then the lipase and molecular sieves were separated by filtration and the volume of filtrate was adjusted to 25 mL by addition of acetone/ethanol. This mixture, product of the reaction, was stored under argon atmosphere at –20ºC until analysis.

Identification of reaction products and estimation of the degree of esterification (ED) and percentage and yield of TG. Products of reaction (FFA, MG, DG, and TG) were identified by thin-layer chromatography (TLC) followed by quantitative gas chromatography (GC). TLC plates were precoated with silica gel G-25 (Aldrich Chemical Co., Milwaukee, WI) that had been activated by heating at 100ºC for 20 min. The samples (0.2 mL) were spotted directly on the plate alongside authentic standards. The plate was developed in chloroform/acetone/methanol (95:4.5:0.5, vol/vol/vol). Spots of each lipid were visualized by developing the plate with iodine vapor in a nitrogen stream. Fractions corresponding to each lipid type were scraped from the plates and methylated according to the method of Lepage and Roy (13). Methylation and methyl ester analysis by GC have been described elsewhere (10). The ED represents the percentage of initial FA consumed in the reaction mixture as determined by TLC–GC and by acid-base titration (A-B) of FFA with 0.025 N NaOH. The acidity of the reaction mixture was first determined without FA to correct the volume of NaOH solution consumed in the titration of the reaction mixture. ED used in the work was the average of the values obtained by the two methods. The percentages of the individual types of glycerides over total glycerides (MG/GLY, DG/GLY, and TG/GLY) and TG yield were calculated from the results of the TLC–GC analysis. Thus, for example, the TG/GLY ratio is the FA present as TG and the total FA esterified by glycerol, and the TG yield is the ratio of FA present as TG to the initial FA in the reaction mixture; the yield was obtained by multiplying ED and TG/GLY.

TABLE 1
Fatty Acid Composition (% of total fatty acids) of the Extract and the PUFA Concentrate from Cod Liver Oil and the Triglycerides

Fatty acids	Extracts[a]	PUFA concentrate[b]	Triglycerides[c]
14:0	3.5	0.7	0.9
16:0	10.8		
16:1n-7	6.3	0.7	1.0
16:2n-4	0.9	1.7	1.3
16:3n-4	0.5	0.4	
16:4n-1		0.4	0.3
18:0	2.6		
18:1n-9	17.4		
18:1n-7	4.1		
18:2n-6	1.7	0.9	1.0
18:3n-3	1.4	1.3	1.5
18:4n-3	3.0	10.3	10.9
20:1n-9	10.4		
20:4n-6	0.4	1.0	0.9
20:4n-3	0.8	2.4	2.2
20:5n-3 (EPA)	9.5	26.2	26.2
22:5n-3	1.1	1.5	1.5
22:6n-3 (DHA)	13.9	47.8	44.7
Others	11.9	4.7	7.7
TG yield (%)			84.4

[a]Extraction of fatty acids (FA) by saponification of cod liver oil.
[b]Urea/fatty acid ratio 4:1, crystallization temperature 4°C, solvent methanol.
[c]The esterification was carried out at 60°C for 48 h. Reaction mixtures contained 41.6 mg glycerol, 0.4 g polyunsaturated fatty acids (PUFA) concentrate (ratio glycerol/FA 1:3 molar), 9 mL hexane, 0.5% water, 100 mg N-435 (Novo Nordisk A/S, Bagsvaerd, Denmark) and 1 g molecular sieves at 24 h.

RESULTS AND DISCUSSION

Esterification activity of the various lipases. The esterification of glycerol and n-3 PUFA concentrate in an organic solvent by the three lipases is compared in Table 2. Lipozyme IM showed a high ED (61%), but a low percentage of TG, and lipase PS produced a lower ED but a higher percentage of TG in total glycerides. N-435 had the highest ED and TG yield and was therefore selected for study of its optimal reaction

TABLE 2
Esterification Activity of Lipases[a]

	Commercial name of enzyme		
	Lipozyme IM	Novozym 435	Lipase PS
Specificity	1,3-specific[b]	Depends on the reactant[c]	1,3 specific[b]
ED[d] (%)	61.2	82.4	12.6
MG/GLY[e] (%)	17.4	14.9	45.8
DG(1,2)GLY[f] (%)	4.4	21.9	Not detected
DG(1,3)/GLY[g] (%)	63.4	44.0	21.4
TG/GLY[h] (%)	14.8	19.1	32.8
TG yield[i] (%)	9.1	15.7	4.1

[a]Reaction conditions: 3 mL hexane, 30°C, 2.0 g glycerol, 0.4 g PUFA concentrate (ratio glycerol/FA 16:1 molar), 25 mg enzyme, 24 h.
[b]Li and Ward (7).
[c]Novo Nordisk A/S (see Table 1 for address).
[d]Degree of esterification: FA in glycerides/initial FA.
[e,f,g,h]FA in MG, DG(1,2), DG(1,3), and TG, respectively/FA in total glycerides.
[i]FA in TG/initial FA. Abbreviations: ED, degree of esterification; MG, monoglycerides; DG, diglycerides; GLY, glycerides; TG, triglycerides. See Table 1 for other abbreviations.

conditions and was used to esterify the cod liver oil. Lipase N-435 produced MG, DG(1,2), DG(1,3), and TG (Table 2) in proportions that depended on reaction conditions. No positional specificity was observed with this enzyme.

Effect of variables on the ED and TG yield. First, the influence of each variable was studied independently. ED and TG yield were measured after 24 h of reaction, although, as thermodynamic equilibrium was not reached after this time, the results obtained are the average rates measured in the first 24 h and not the maximal ED or TG yield that could possibly be obtained.

Effect of organic solvent on glyceride synthesis. Organic solvents produce various physicochemical effects on enzyme molecules and the effects depend upon the kinds of organic solvents and enzymes used. Conformational changes in enzymes, when suspended in organic solvents, have been reported to result in alteration of substrate specificity and affinity of substrates for enzymes (14). Although large conversion yields have also been reported without organic solvents (8,15,16), those authors used monounsaturated oleic acid. In a study performed in our laboratory on the conditions affecting PUFA stability, PUFA peroxidation was shown to be greatly retarded when carried out in an organic solvent such as hexane (unpublished). Li and Ward (7) tested eight organic solvents with lipase PS-30 and lipase IM-60 (from *Pseudomonas* sp. and *M. miehei*, respectively) with the greatest activity observed in hexane and isooctane. Therefore we tested both these solvents with lipase N-435 (Table 3). Under the experimental condition used, the ED obtained with and without the two solvents was similar; however, the TG yield obtained with the two solvents was larger than with no solvent. The conversion to TG was similar with both solvents, although slightly better with hexane (Table 3). Solvent volume had little influence on ED or TG yield within the range studied, although the TG yield seemed to increase with volume of hexane up to 9 mL.

TABLE 3
Effects of the Type and the Amount of Organic Solvent on the ED and TG Synthesis

Solvent	Volume (mL)	ED[a] (%)	TG/GLY[b] (%)	TG yield[c] (%)
None		92.4	28.6	26.4
Hexane	3[d]	95.0	42.6	40.5
	6[d]	92.0	39.7	36.5
	9[d]	92.7	51.0	47.3
Hexane	9[e]	90.8	65.4	59.4
	12[e]	90.5	58.0	52.5
Isooctane	3[d]	93.9	42.5	39.9
	6[d]	93.0	37.8	35.1
	9[d]	91.7	29.5	26.8

[a,b,c]As in Table 2.
[d]50°C, 50 μL water, 2.0 g glycerol, 0.4 g PUFA (ratio glycerol/FA 16:1 molar), 1 g molecular sieves added at 4 h, 33 mg of lipase N-435, and reaction time 24 h.
[e]50°C, 100 μL water, 2.0 g glycerol, 0.4 g PUFA (ratio glycerol/FA 16:1 molar), 1 g molecular sieves at 24 h, 100 mg of lipase N-435, and reaction time 48 h.
See Tables 1 and 2 for abbreviations. See Table 1 for company source.

Effect of temperature on esterification. Kosugy and Azuma (8) suggested that low temperatures might be preferable for synthesizing TG from EPA, because EPA is polymerized during the reaction and lower temperature and shorter time more effectively prevent thermal decomposition of the PUFA. However, as shown in Figure 1, this work did not find a significant effect of temperature on ED or TG yield between 30 and 60ºC.

Effect of initial water content on glyceride synthesis. Lipase-catalyzed esterification reactions are reversible and, as water is one of the products of reaction, the equilibrium is affected by the amount of water in the reaction mixture. A small amount of water is essential for maintaining enzyme activity; hence the water content needs to be optimal. Table 4A shows how, in an initial series of experiments, the water content does not affect ED between zero and 2% water but does seem to enhance the synthesis of TG. As expected, once the water content exceeds 2%, the ED decreases with increasing water content. Additional experiments were carried out under other conditions to further optimize water content between 0.5, 1, and 2% vol/vol (Table 4A), and a 1% water content was found to be optimal.

Ergan *et al.* (17) demonstrated the importance of removing the water produced in the reaction to increase yield. This was attempted by addition of molecular sieve as a dehydrating agent. The results obtained in the first series of experiments shown in Table 4B indicate that a lower ED is obtained without molecular sieves than with added molecular sieves. Also, ED decreases when the amount of sieves is increased. A sieve amount of 1 g seems to be optimal for obtaining a high ED; however, an abnormally large yield of TG was obtained without the molecular sieves. While the time of addition of molecular sieves does not affect ED (Table 4B), TG seems to increase when the molecular sieves are added at later time. Similar results were obtained by Ergan *et al.* (17). In conclusion, a large amount of molecular sieves added earlier in the reaction seems to eliminate too much water and the enzyme activity decreases (13.5% of TG with 4 g of sieves added after 1 h). A small amount of molecular sieves added later in the reaction is therefore preferable.

Effect of glycerol/PUFA ratio on glyceride synthesis. The initial glycerol/FA ratio affects the equilibrium of the reaction. Ergan *et al.* (16) found that the stoichiometric ratio (i.e., 1:3) was optimal for the highest production of TG with Lipozyme IM-20 (from *M. miehei*) in the absence of organic solvents. An excess of glycerol decreased the TG yield. With a similar lipase (Lipozyme IM-60 from *M. miehei*), in the presence of an organic solvent, Li and Ward (7) observed that glyceride synthesis increased with increasing glycerol content, although, as previously noted, low TG was obtained. The effects of the glycerol/PUFA ratio on esterification by lipase N-435 are shown in Figure 2. Initially, ED increased with the amount of glycerol, then remained constant between glycerol/FA ratios of 1:1 and 16:1 (where an excess of glycerol is present). Similar results were obtained by Li and Ward (7). However, the TG synthesis is maximal at the stoichiometric

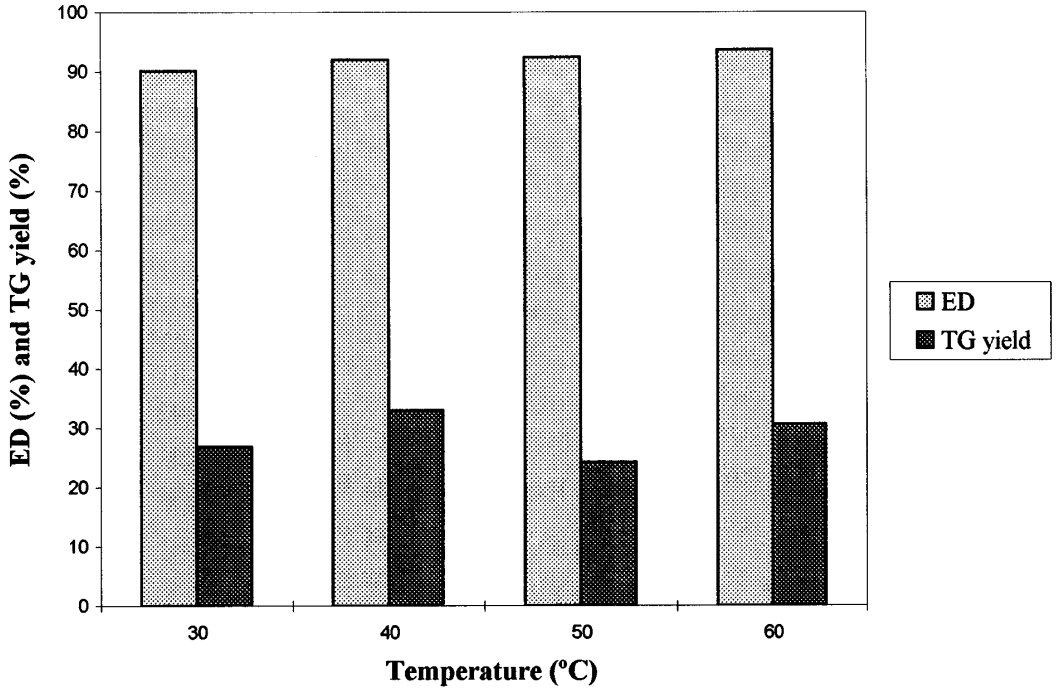

FIG. 1. Effect of temperature on degree of esterification (ED) and triglyceride (TG) yield. Esterification conditions: 3 mL hexane, glycerol/fatty acid (FA) molar ratio 16:1, 1% (vol/vol) water, 1 g molecular sieves added at 1 h, 25 mg of lipase N-435 (Novo Nordisk A/S, Bagsvaerd, Denmark), and 24 h of reaction.

glycerol/FA ratio of 1:3. This result agrees with Ergan *et al.* (16) and seems logical because with an excess of glycerol the equilibrium is displaced toward esterification, increasing ED, but formation of TG decreases because the excessive glycerol favors the formation of MG and DG. The formation of TG over MG and DG is maximal at the stoichiometric ratio of 1:3. With a glycerol/FA ratio of 24:1, ED decreases probably because such a great excess of glycerol increases polar interaction between the glycerol and the enzyme; glycerol coats the powdered immobilized lipase, inhibiting the reaction by limiting substrate and product diffusion (15).

Effect of lipase N-435 content on glyceride synthesis. Figure 3 shows that with very little lipase N-435, ED is over 90%, indicating a high activity for this enzyme. This ED also remains constant, which indicates that esterification equilibrium has been reached. On the other hand, TG yield increased

TABLE 4
Effect of Water Content (A) and the Amount and Addition Time of Molecular Sieves (B) on the ED and TG Synthesis

(A) Water (% vol/vol)	ED[a] (%)	TG yield[b] (%)	(B) Addition time (h)	Molecular sieves (g)	ED[a] (%)	TG yield[b] (%)
0[c]	92.7	23.8	—[c]	—	83.5	30.5
1.0[c]	92.4	24.2				
2.0[c]	92.8	30.3	1[c]	1.0	92.4	24.2
5.0[c]	77.7	—		2.0	89.7	16.4
10.0[c]	52.7	—		4.0	83.3	13.5
0.5[d]	93.9	40.1	11[d]	0.5	90.9	53.2
1.0[d]	89.0	45.7		1.0	91.4	60.2
2.0[d]	83.1	28.8	8[e]	1.0	94.6	58.7
			11[e]	1.0	91.4	60.2
			24[e]	1.0	93.6	64.4

[a,b]As in Table 2.
[c]9 mL hexane, 50°C, 1% water (Table 4B), 2.0 g glycerol, 0.4 g PUFA (glycerol/FA 16:1 molar), 1 g molecular sieves at 1 h (Table 1A), 25 mg lipase N-435, and 24 h reaction time.
[d]9 mL hexane, 40°C, 2.0 glycerol, 0.4 g PUFA (glycerol/FA 16:1 molar), 1 g molecular sieves at 11 h, 100 mg lipase N-435, 24 h reaction time.
[e]9 mL hexane, 40°C, 1% water, 2.0 glycerol, 0.4 g PUFA (glycerol/FA 16:1 molar), 100 mg lipase N-35, and 48 h reaction time. See Tables 1 and 2 for abbreviations. See Table 1 for company source.

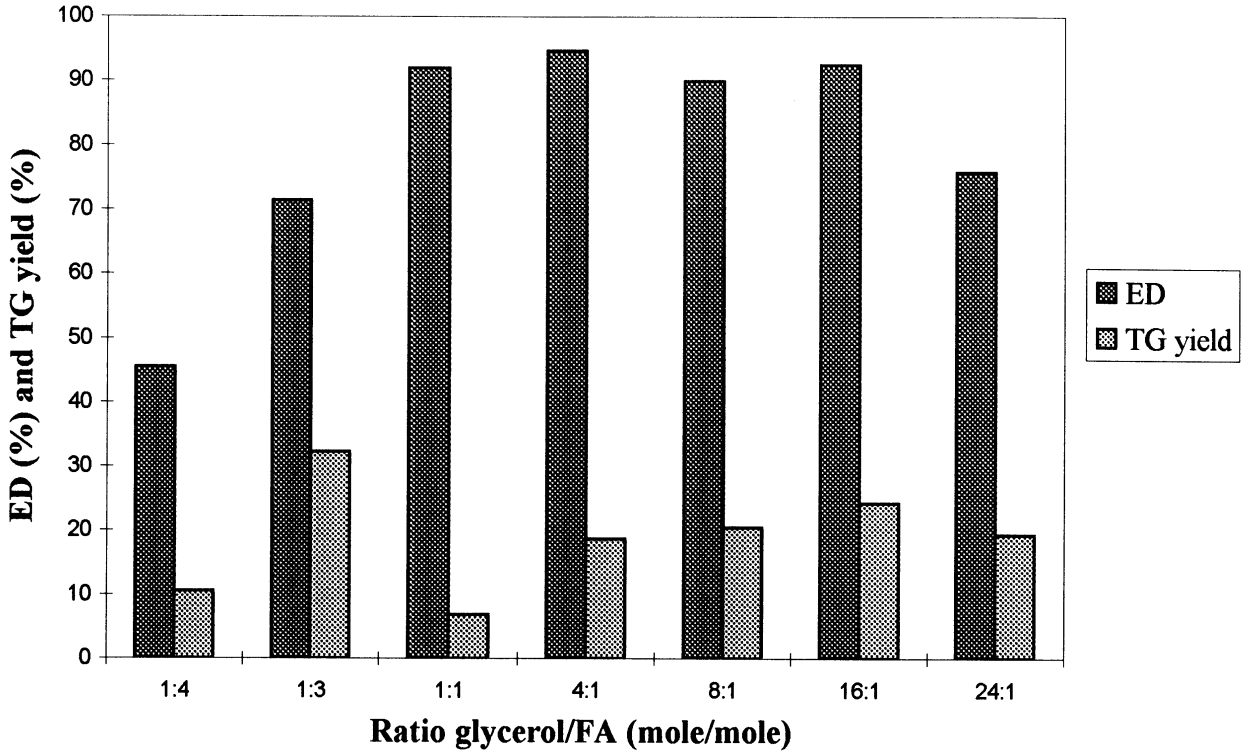

FIG. 2. Effect of glycerol/fatty acid ratio on ED and the TG yield. Esterification conditions: 3 mL hexane, 1% (vol/vol) water, 1 g molecular sieves added at 1 h, 50°C, 25 mg of lipase N-435, and 24 h of reaction. See Figure 1 for abbreviations and company source.

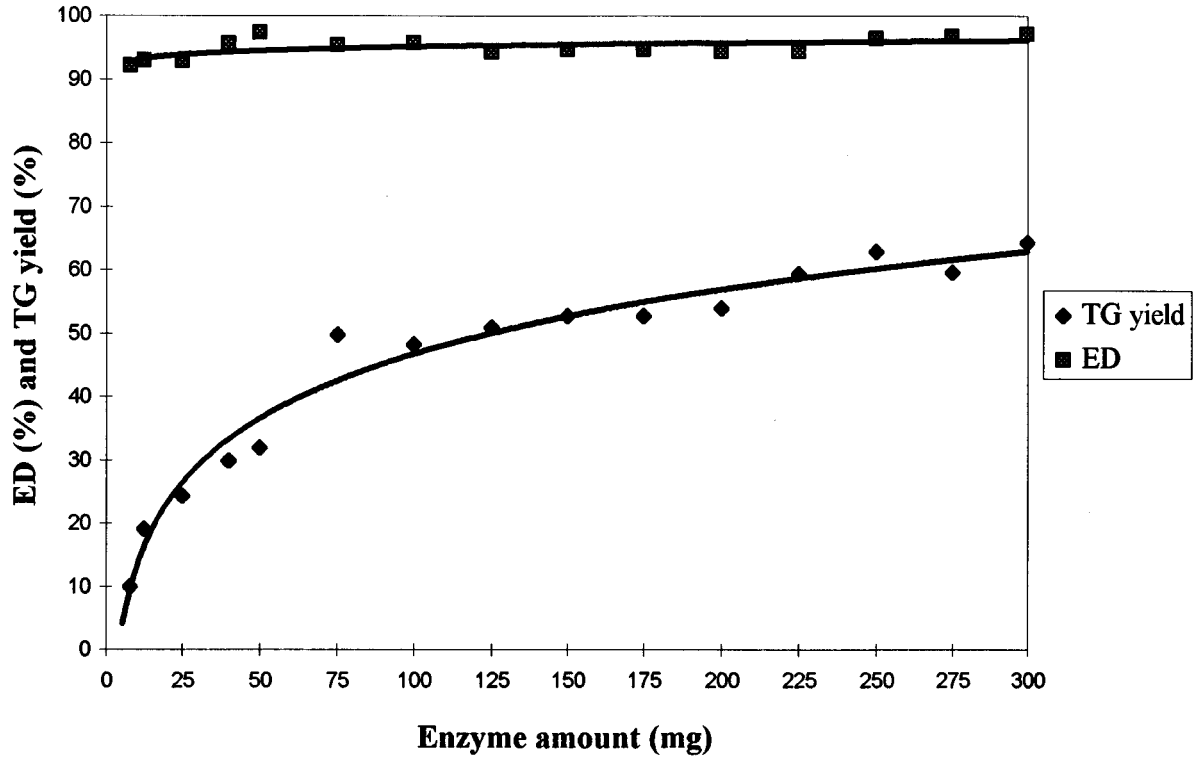

FIG. 3. Effect of enzyme (lipase N-435) amount on ED and TG yield. Esterification conditions: 3 mL hexane, glycerol/fatty acid molar ratio 16:1, 1% (vol/vol) water, 1 g molecular sieves added at 1 h, 50°C, and 24 h of reaction. See Figure 1 for abbreviations and company source.

quickly with the amount of lipase, confirming the nonspecificity of this enzyme: the formation of TG is controlled by enzymatic esterification in all three positions of the glycerol and not by isomerization of DG(1,3) to DG(1,2), which is the step that controls TG formation when the lipase is 1,3-specific (18). The rate of TG synthesis increased rapidly with increasing amount of lipase until the amount reached about 100 mg; thereafter the rate of increase was less significant.

Effect of the elapsed reaction time on glyceride synthesis. Esterification was performed to study the variation in ED and TG yields with elapsed reaction time under the best reaction conditions obtained in previous experiments (9 mL hexane, 1:3 mol/mol glycerol/FA ratio, 1% initial water content, 1 g of molecular sieves added after a long reaction time and 100 mg of lipase N-435) (Table 5A). The temperature was kept at 40ºC in order to preserve PUFA stability. A TG yield of 68.7% after 72 h of reaction was obtained at these experimental conditions. These results are lower than expected (for example, Table 4B shows that TG yield, of 60.2 and 64.4% were obtained after 48 h of reaction with a glycerol/FA ratio of 16:1). Moreover marked influence of some variables had not been observed in the previous study (e.g., volume of solvent, temperature, water content, etc.).

The foregoing results suggested strong interactions among variables, hence implying that the "one-at-a-time" variation of parameters was an unsatisfactory approach to optimization. Consequently, a statistical design of experiments was undertaken as detailed below.

Statistical design of experiments. Optimization of reaction conditions by the statistical Plackett-Burman design. (19) The experimental design described in Table 6 was used to elucidate the effects of the more important variables on TG yield. Based on the results, within the ranges studied, the most important variables (value of t_x) were the amount of enzyme, water content, and temperature; the amount of molecular sieves and PUFA content had a minor effect, and the influence of glycerol and solvent content and time of addition of molecular sieves negligibly affected the results. The sign of t_x shows that an increase in temperature and the amount of enzyme along with a decrease in water content increase the TG yield. An increase in the amount of molecular sieve and a decrease in the amount of PUFA also increased the TG yield, but to a lesser degree. Thus, for example, the high TG yield in trial 7 with only 25 mg of enzyme confirmed the importance of a high temperature and low water content. Table 7 shows the same results, but classified according to the amount of enzyme and the TG yield. This table also shows that the lowest results were obtained with a high water/molecular sieve ratio (experiments 10, 3, and 6) and with glycerol/FA ratios of 1:1 and 6:1 (experiments 10, 12, 5, 3, and 6); a large excess of glycerol (16:1) or a near-stoichiometric ratio (1:2.5) (experiments 9 and 8, respectively) being preferable. Therefore, the optimized conditions proposed based on statistically designed experiments are: 9 mL hexane, glycerol/FA ratio 1:3 (or 1/2.5), 0.5% of water, 1 g of molecular sieves added after 24 h of reaction, and 60ºC.

Esterification of glycerol and PUFA concentrates from cod liver oil under the optimized conditions. The statistically optimized conditions for synthesis of TG were employed to study the variation of the ED and the TG yield over time. A maximal TG yield of 84.7% was obtained after 48 h of reaction (Table 5B). Under these conditions both the ED and the TG yield reached a maximum, and no further increase could be obtained with longer reaction times, which meant that equilibrium had been reached.

Figure 4A shows the detailed change in composition of lipids during the course of the esterification reaction under the optimized conditions. Initially slow consumption of FFA was observed. After 24 h (when the molecular sieves were added), the rate of FFA consumption became faster. The TG formation rate was always faster than that of DG; the DG and MG percentages were negligible after 48 h of reaction. Because TG were derived from MG and DG, the percentages of these two molecular species depleted as there was no excess glycerol (glycerol/FA ratio 1:3). However, when the glyc-

TABLE 5
Esterification of the PUFA Concentrate from Cod Liver Oil with Glycerol in the Optimized Conditions

Elapsed time (h)	ED[a] (%)			TG/GLY[b] (%)	TG yield[c] (%)
	A-B[d]	TLC[e]	Average ED		
(A)[f]					
48	68.5	65.1	66.8	85.1	56.8
72	74.8	72.9	73.9	93.0	68.7
96	73.8	71.3	72.6	85.8	62.2
(B)[f]					
24	11.0	—	—	—	11.0
48	85.8	89.1	87.5	96.9	84.7
72	81.5	83.6	82.6	96.3	79.5
96	86.2	88.2	87.2	97.1	84.7

[a,b,c]As in Table 3.
[d]ED determined by titration of FFA with 0.035 N NaOH.
[e]ED determined by thin-layer chromatography (TLC).
[f](A) Glycerol/fatty acid 1:3 molar, 9 mL hexane, 1% water, 1 g of molecular sieves added at 24 h of reaction, temperature 40°C, and 100 mg of lipase N-435; and (B) glycerol/fatty acid 1:3 molar, 9 mL hexane, 0.5% (by vol) water, 1 g of molecular sieves added at 24 h of reaction, temperature 60°C, and 100 mg of lipase N-435. See Tables 1 and 2 for other abbreviations. See Table 1 for company source.

TABLE 6
Effect of the Experimental Conditions on TG Yield After 48 h of Reaction (Plackett-Burman[a] Experimental Design) (19)

	Variables											
Experiment	T (°C)	Enzyme (mg)	Glycerol (mg)	Water (%)	Sieves (g)	Time[e] (h)	Hexane (mL)	PUFA (mg)	DVI[b]	DV2[b]	DV3[b]	TG yield (%)
1	60 (+)[c]	100 (+)	125 (–)	2.0 (+)	2.0 (+)	24 (+)	3 (–)	400 (–)	–	+	–	47.3
2	40 (–)[c]	100	2000 (+)	0.5 (–)	2.0	24	12 (+)	400	–	–	+	58.0
3	60	25 (–)	2000	2.0	0.5 (–)	24	12	1010 (+)	–	–	–	16.8
4	40	100	125	2.0	2.0	12 (–)	12	1010	+	–	–	35.5
5	40	25	2000	0.5	2.0	24	3	1010	+	+	–	25.0
6	40	25	125	2.0	0.5	24	12	400	+	+	+	7.8
7	60	25	125	0.5	2.0	12	12	1010	–	+	+	48.8
8	60	100	125	0.5	0.5	24	3	1010	+	–	+	60.5
9	60	100	2000	0.5	0.5	12	12	400	+	+	–	59.1
10	40	100	2000	2.0	0.5	12	3	1010	–	+	+	28.8
11	60	25	2000	2.0	2.0	12	3	400	+	–	+	34.2
12	40	25	125	0.5	0.5	12	3	400	–	–	–	26.9
t_x[d]	4.4	7.8	0.2	–5.7	2.3	–0.6	–0.3	–1.5			–	

[a]A fraction of the two-factorial design. Each line represents an experiment and each column represents an independent or [b]dummy variable. Each variable must have two different levels.
[c]The symbols (+) and (–) represent a high and low level, respectively, for each variable.
[d]Significance level of each variable by the *t* test; $t_x = Ex/\sqrt{V_{eff}}$; E_x is the difference between the average of the responses to high and low level and V_{eff} is the experimental error, $V_{eff} = \Sigma(E_d)^2/n$, E_d being the difference between the average of the responses to high and low levels of the dummy variables[c], and n is the number of dummy variables (20).
[e]Time of addition of molecular sieves. See Tables 1 and 2 for abbreviations.

TABLE 7
Influence of the Most Significant Variables on TG Yield[a]

	Independent variables							
Experiment	Enzyme (mg)	Water (%)	T (°C)	Sieves (g)	PUFA (mg)	Glycerol/PUFA (mole/mole)	Water/sieves (%/g)	TG yield (%)
8	100	0.5	60	0.5	1010	1:2.5	1.0	60.5
9	100	0.5	60	0.5	400	16:1	1.0	59.1
2	100	0.5	40	2.0	400	16:1	0.25	58.0
1	100	2.0	60	2.0	400	1:1	1.0	47.3
4	100	2.0	40	2.0	1010	1:2.5	1.0	35.5
10	100	2.0	40	0.5	1010	6:1	4.0	28.8
7	25	0.5	60	2.0	1010	1:2.5	0.25	40.8
11	25	2.0	60	2.0	400	16:1	1.0	34.2
12	25	0.5	40	0.5	400	1:1	1.0	26.9
5	25	0.5	40	2.0	1010	6:1	0.25	25.0
3	25	2.0	60	0.5	1010	6:1	4.0	16.8
6	25	2.0	40	0.5	400	1:1	4.0	7.8
t_x	7.8	–5.7	4.4	2.3	–1.5			

[a]From Table 6, classifying the experiments by the amount of enzyme and by the TG yield obtained. The time of addition of the molecular sieves and both the hexane and glycerine contents, which have very little impact on the process (i.e., t_x values close to zero, Table 6), have been excluded. This table includes the glycerol/PUFA and the water/molecular sieves ratios as variables affecting the esterification process. See Tables 1 and 2 for abbreviations.

erol/FA ratio used was 16:1, the changes in composition of lipids were quite different (Fig. 4B). Initially, rapid production of DG with fast consumption of PUFA was observed. Moreover, in the first 8 h no significant production of TG was detected, but afterward the DG content decreased at the same time the percentage of TG increased, whereas the free PUFA continuously decreased. This production profile was similar to the results published by Castillo *et al.* (15), but here the difference between the rates of TG and MG or DG formation was larger. Castillo *et al.* (15) used 1,3-specific lipases to esterify glycerol, making migration of the acyl group from position 1 or 3 to 2 necessary for TG formation; this isomerization reaction was the limiting step of TG synthesis. Because lipase N-435 is not 1,3-specific in this reaction, the TG are synthesized faster by esterification in position 1,2, and 3. On the other hand, the FA composition of the TG and PUFA concentrates used was similar (Table 1). This seems to indicate that lipase N-435 is not FA-specific, because all the FA are esterified by glycerol in identical proportions.

From the above results, it can be concluded that: (i) lipase N-435 is highly active in hexane when used to obtain both a

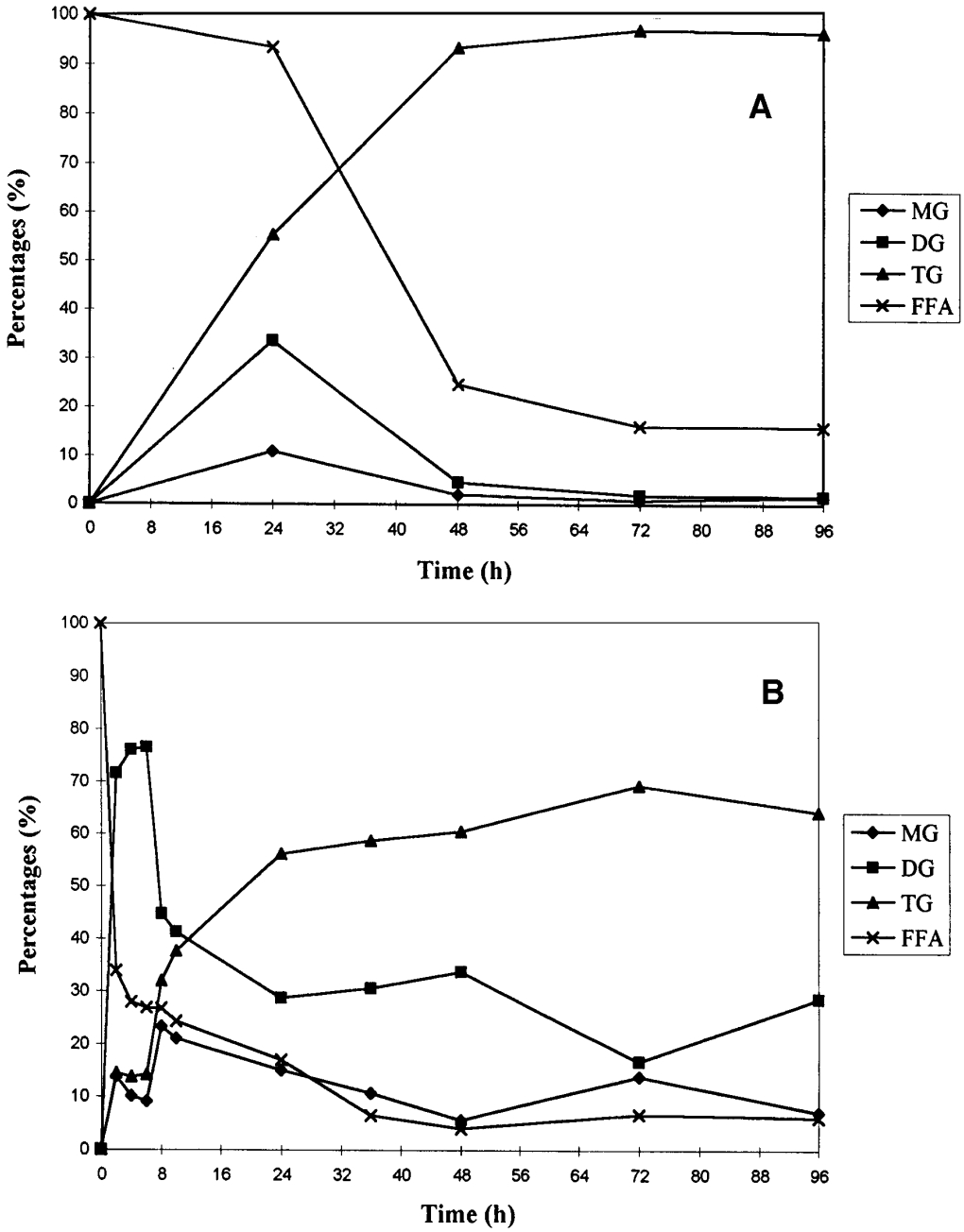

FIG. 4. Composition of glycerides synthesized. Esterification conditions: (A) 9 mL hexane, glycerol/fatty acid molar ratio 1:3 (41.7 mg glycerol and 0.4 g of PUFA concentrate), 0.75% (vol/vol) water, 1 g molecular sieves added at 24 h, 60ºC, and 100 mg of lipase N-435; (B) 9 mL hexane, glycerol/fatty acid molar ratio 16:1 (2.0 g glycerol and 0.4 g of PUFA concentrate), 1% (vol/vol) water, 1 g molecular sieves added at 24 h, 50ºC, and 100 mg of lipase N-435. See Figure 1 for other abbreviation and company source.

high ED and large TG yields; (ii) this lipase easily synthesizes highly concentrated EPA and DHA TG; (iii) the most important variables in obtaining high TG yield are the amount of enzyme, water content, and temperature; (iv) lipase N-435 is not 1,3-specific, and the TG yield increases with the amount of enzyme; (v) this immobilized lipase is highly thermostable and has been shown to be very active at 60ºC; (vi) a low initial water content (0.5% vol/vol) is necessary because water is a product of the reversible reaction of esterification, and some water is required for the enzyme activity; (vii) the water produced should be avoided near equilibrium—this can be done by using low water/molecular sieve ratios; (viii) the stoichiometric glycerol/FA molar ratio of 1:3 is optimal for the synthesis of TG from MG and DG.

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