Modification of Menhaden Oil by Enzymatic Acidolysis to Produce Structured Lipids: Optimization by Response Surface Design in a Packed Bed Reactor

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ABSTRACT: Structured lipids from menhaden oil were produced by enzymatic acidolysis in a packed bed reactor. Response surface methodology was applied to optimize the reaction. Lipozyme IM from *Rhizomucor miehei* lipase was the biocatalyst, and caprylic acid was the acyl donor. Parameters such as residence time, substrate molar ratio, and reaction temperature were included for the optimization. High incorporation of acyl donor and retention of high levels of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids in the original menhaden oil were obtained. Good quadratic models were obtained for the incorporation of caprylic acid and for the content of EPA plus DHA retained, by multiple regression with backward elimination. The coefficients of determination (R^2) for the two models were 0.91 and 0.87, respectively. The regression probabilities (P) were below 0.003 for both models. Also, the predicted values from the two models had linear relationships with the observed responses. All parameters studied had positive effects on the incorporation of caprylic acid, but only residence time and substrate molar ratio had negative effects on the content of EPA plus DHA retained. The optimal conditions generated from models were temperature = 65°C, substrate molar ratio = 4–5, and residence time = 180–220 min. Incorporated caprylic acid did not replace DHA, but the content of EPA decreased somewhat with an increase in caprylic acid incorporation.

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KEY WORDS: Fish oil, lipase-catalyzed acidolysis, Lipozyme IM, menhaden oil, packed bed reactor, response surface methodology, *Rhizomucor miehei,* structured lipids.

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) of fish oil have great potential for the prevention of coronary artery disease and treatment of patients with this problem (1–3). Some of their most important effects are the prevention of arrhythmia and the inhibition of ventricular fibrillation and consequent cardiac arrest. EPA has several antithrombotic actions, particularly by inhibiting the synthesis of thromboxane A2, and the prostaglandin that causes platelet aggregation and vasoconstriction. Fish oils retard the growth of atherosclerotic plaque by inhibiting both cellular growth factors and the migration of monocytes. EPA and DHA promote the synthesis of beneficial nitric oxide in the endothelium. Fish oils have a profound hypolipidemic effect, especially the lowering of plasma triacylglycerol. Also, fish oils have a mild blood pressure-lowering effect in both normal and mildly hypertensive individuals. These composite effects suggest a prominent therapeutic role for fish oils in the prevention and treatment of coronary artery disease.

Structured lipids (SL), which contain long-chain fatty acids (C_{14}–C_{22}) and medium-chain fatty acids (C_8–C_{12}), respectively, where each group is located specifically at the *sn*-2 position or *sn*-1,3 positions, have also attracted great attention for their special nutrition (4,5). SL have beneficial effects on immune function, nitrogen balance, and improved lipid clearance from the bloodstream (6). Free fatty acids liberated from dietary food during absorption are metabolized more easily if they are medium- or short-chain. Long-chain fatty acid monoacylglycerols can be absorbed directly. Therefore, essential or desired fatty acids are most efficiently utilized from the *sn*-2 position in acylglycerols. In accordance with this, SL are rapidly hydrolyzed by pancreatic lipase and absorbed efficiently into mucosal cells. They are useful for the treatment of lipid malabsorption (7–9).

As a consequence, the combination of fish oil fatty acids and medium-chain fatty acids to form structured lipids will give optimal nutrition. It was reported that SL with EPA and DHA predominantly in the *sn*-2 position of the triacylglycerol were a more readily absorbed source of EPA and DHA than when located at the *sn*-1,3 positions or randomized. They are absorbed as 2-monoacylglycerols. Studies also indicated that SL containing DHA had effects on visual and auditory performance as well as brain, liver, and adipose tissue lipid and fatty acid profiles (10–12). Menhaden oil has a high content of EPA and DHA, mainly located at the *sn*-2 position (13). *Rhizomucor miehei* lipase has an *sn*-1,3 regiospecificity, and a weak activity on those long-chain n-3 fatty acids in fish oils (14), especially DHA (15). These properties suggest the possibility of producing SL that incorporate maximal medium-chain fatty acids, and at the same time retain as much

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as possible of the EPA and DHA in the original menhaden oil. In this paper, SL from menhaden oil was synthesized in a packed bed bioreactor. Response surface methodology (RSM) was applied to reduce the experimental number and help optimize the reaction (16). The parameters studied for the optimization included flow rate, reaction temperature, and substrate molar ratio. The SL was produced under the optimal conditions to verify the optimization.

MATERIALS AND METHODS

Materials. Refined, bleached, and deodorized (RBD) menhaden oil was provided by Zapata Protein, Inc. (Reedville, VA), already stabilized with 200 ppm tertiary butyl hydroxyquinone and 1000 ppm mixed tocopherols. The fatty acid composition and distribution (mol%) of the menhaden oil are given in Table 1. Water content was 0.03%. Caprylic acid (purity minimum 99%; water content 0.05%) was purchased from Sigma Chemical Co. (St. Louis, MO). Lipozyme IM, a commercially immobilized 1,3-specific lipase from *R. miehei*, was from Novo Nordisk A/S, Bagsvaerd, Denmark (water content, 2.3 wt%). All solvents and reagents for analyses were chromatographic or analytical grade.

Apparatus and bioreactor packing. The setup of the stainless steel bioreactor was similar to the glass bioreactor previously reported (17). The bed size of the reactor was 47 mm (i.d.) × 46 cm (length). Lipozyme IM (290 g) was packed following the method described before (17). The immobilized lipase was loaded directly into the column. The upper and lower ends of the bed were each layered with glass wool (3 and 2 cm in thickness, respectively). Nitrogen was passed through the packed bed to remove air. A FMI Lab pump (model QV) from Fluid Metering, Inc. (New York, NY) was used to feed the substrate. The bed was jacketed, and the temperature was maintained by a circulating water bath. Before pumping onto the enzyme bed, the substrates were preheated to a set temperature by a coiled heater. The packed bed was conditioned by feeding the substrate (menhaden oil and caprylic acid) overnight.

Experimental methods. Experiments on lipase-catalyzed acidolysis were conducted using menhaden oil and caprylic acid. When a new experiment was started, prepared substrates (0.15 wt% water, based on substrate, was added for all the experiments and dispersed by stirring for 20 min) were pumped into the enzyme bed at the set parameters. Samples were taken after 1,000 mL of products had been collected under the same set of parameters. Production (2 L) was carried out under optimal conditions.

Experimental design. A fractional factorial design with two star points according to the principle of RSM (16) was used in this work. The three factors chosen were reaction temperature (Te, °C), residence time (Rt, min) and substrate molar ratio (Sr, caprylic acid/menhaden oil). The settings for the factors was determined according to previous studies (17,18) as follows (low value/high value): substrate ratio, 3:5; residence time, 60:180; and temperature, 50:60. This gives a range of these factors of 2.318–5.682, 19–221, and 46.6–63.4, respectively, including the star points. The variables and their levels used are presented in Table 2.

Thin-layer chromatographic separation of triacylglycerols from the product mixtures. A general procedure similar to that previously described was used (19). A 20 × 20 cm coated with silica gel G (Fisher Scientific, Fairlawn, NJ) was used for the separation. Samples were dissolved in hexane before application to the plate. The developing solvent used was petroleum

TABLE 1
Fatty Acid Profiles and Distributions (mol%) of Menhaden Oil and Structured Lipids Produced at Optimal Conditions

Oils	Menhaden oil[a]			Structured lipids[b]		
sn	1,2,3	2	1,3	1,2,3	2	1,3
8:0				38.8	2.7	56.8
14:0	8.5	11.4	7.0	5.1	10.3	2.5
16:0	19.4	22.1	18.0	10.2	21.7	4.4
16:1	10.3	9.6	10.7	5.4	9.6	3.3
17:0	3.9	3.4	4.2	1.9	3.0	1.3
18:0	5.4	4.2	6.0	1.6	4.4	0.2
18:1	15.0	8.2	18.4	3.0	7.2	0.9
18:2	3.5	1.2	4.6	1.1	0.6	1.4
18:3	1.7	0.3	2.4	—[c]	0.3	—
18:4	3.6	0.5	5.2	0.3	0.4	0.2
20:1	1.4	0.7	1.8	0.6	0.8	0.5
20:5	11.9	17.1	9.2	11.8	16.7	9.4
22:5	2.5	2.8	2.4	3.0	3.4	2.8
22:6	12.9	18.4	10.1	17.2	18.9	16.3

[a]Refined, bleached, and deodorized menhaden oil (Zapata Protein, Inc., Reedville, VA).
[b]Production conditions in the text.
[c]Not detected.

TABLE 2
Actual Experimental Settings of the Factors and the Responses Therefrom for the Optimization of the Reaction by RSM[a]

ExpNo	OrderNo	Rt	Sr	Te	Inc	PUFA
1	4	59.4	3	50	27.8	28.7
2	14	169.2	3	50	26.0	26.8
3	16	59.4	5	50	22.9	30.2
4	8	169.8	5	50	36.4	22.0
5	13	60.0	3	60	26.3	26.2
6	9	170.4	3	60	29.5	29.4
7	15	59.4	5	60	26.6	28.3
8	3	196.8	5	60	37.1	25.2
9	2	21.0	4	55	20.6	28.6
10	6	201.6	4	55	30.7	26.3
11	1	117.6	2.318	55	27.5	26.1
12	12	117.6	5.682	55	33.6	25.0
13	10	117.6	4	46.6	28.4	27.5
14	17	117.0	4	63.4	39.8	25.9
15	5	117.0	4	55	32.3	25.6
16	11	117.6	4	55	32.7	27.8
17	7	117.6	4	55	31.5	27.9

[a]Abbreviations: RSM, response surface methodology; ExpNo, experiment numbers; OrderNo, the order of running; Rt, residence time (min); Sr, substrate molar ratio (caprylic acid/menhaden oil); Te, temperature (°C); Inc, the incorporation of caprylic acid (mol%); and PUFA, the total contents of eicosapentaenoic acid (EPA) plus docosahexaenoic acid (DHA) in the products (mol%).

ether/ethyl ether/acetic acid (80:20:0.5, vol/vol/vol). A 0.2% methanol solution of 2,7-dichlorofluorescein was used to visualize the bands. The triacylglycerol band was scraped off to a suitable stoppered test tube for methylation.

Triacylglycerol methylation. A method recommended by Christie (20) was used with some modification. Hexane (0.5 mL) was used to dissolve the triacylglycerols from the silica gel powder. After gentle shaking, 2 N sodium methoxide in methanol (0.15 mL) was added. The mixture was further shaken for 5 min at room temperature, then more *n*-hexane (0.3–0.5 mL) was added, followed by powdered anhydrous calcium chloride. The mixture was allowed to stand for 1 h before gas chromatographic (GC) analysis.

Fatty acid composition analysis. The fatty acid compositions of menhaden oil and SL were analyzed by GC as previously described (19). The contents of caprylic acid (Inc) and EPA plus DHA (PUFA) were obtained in molar percentages as listed in Table 2.

sn-*2 positional analysis.* The fatty acid distribution at the *sn*-2 position of the final product was determined by Grignard degradation with allyl magnesium bromide followed by isolation, methylation, and GC analysis of the *sn*-2 monoacylglycerol fraction (21).

Karl Fischer water content determination. The water content of menhaden oil and caprylic acid was determined by Karl Fischer method (720 KFS Titrino, Switzerland, using HYDRANAL titrant and solvents).

Statistical analysis. The responses were analyzed by means of Modde 4.0 (Umetri, Umeå, Sweden). Second-order coefficients were generated by regression analysis with backward elimination (16). The goodness of fit of the model was evaluated by the coefficients of determination (R^2 and Q^2) and the analysis of variances. The quadratic response surface model was fitted to the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X^2{}_i + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij} X_i X_j \qquad [1]$$

where Y is response variables, β_0 intercept, β_i first-order model coefficients, β_{ii} quadratic coefficients for the ith variable, β_{ij} interaction coefficients for the interaction of variables i and j, and X_i are independent variables.

RESULTS AND DISCUSSION

Model fitting. The targeted product of this study is to contain caprylic acid that is located at both the *sn*-1 and *sn*-3 positions, and EPA and DHA mainly at the *sn*-2 position. Owing to the weak activity of *R. miehei* lipase on EPA and DHA ester bonds, and its regiospecificity only on *sn*-1,3 positions, we expected that only fatty acids at *sn*-1,3 positions other than EPA and DHA would be replaced by caprylic acid. Therefore, the optimal product should retain all existing EPA and DHA and incorporate caprylic acid to a maximum possible degree. Certainly the reaction parameters will play an important role in producing such an optimal product. The responses and variable settings in Table 2 were fitted to each other with multiple regression. A good fit was obtained and no outliers were observed. Also, there was no decline of incorporation with the order of running as observed before (22), probably because the running time throughout the whole 17 experiments was only about 100 h, including preparation; and the enzyme activity showed little decrease in such a short time. Lipozyme IM is reported to be stable up to 3 mon at optimal conditions (23,24). The maintenance of some water in the substrates, and at the same content for all experiments, could also be one of the possible reasons for the stability of the enzyme.

The best-fitting quadratic model by multiple regression and backward elimination was determined for both incorporation of caprylic acid (Inc) and the content of the polyunsaturated fatty acids, EPA plus DHA (PUFA). The statistics for the model coefficients and probability (P) values for two response variables were calculated. The two model equations for Inc (mol%) and PUFA (mol%) can therefore be written as follows:

$$\text{Inc} = 33.143 + 2.652\text{Rt} + 1.978\text{Sr} + 1.957\text{Te} - 3.278\text{Rt}^2 - 0.913\text{Sr}^2 + 3.031\text{Rt} \times \text{Sr} + 0.312\text{Rt} \times \text{Te} \qquad [2]$$

$$\text{PUFA} = 26.980 - 1.018\text{Rt} - 0.622\text{Sr} + 0.298\text{Rt}^2 - 0.412\text{Sr}^2 - 1.643\text{Rt} \times \text{Sr} + 1.429\text{Rt} \times \text{Te} \qquad [3]$$

All P values of the coefficients were below 0.1 after the models were refined (Table 3). The coefficients of determination (R^2) of the models for Inc and PUFA were 0.91 and 0.87, respectively. The P values for the multiple regression were 4.85×10^{-4} for Inc and 2.29×10^{-3} for PUFA. According to the analysis of variance, there was no lack of fit. Also, predicted results according to the models for Inc and PUFA were close to the observed responses from experiments. A general linearity was obtained for both Inc and PUFA (Fig. 1). This indicates that the models generated above represent the actual relationships between reaction parameters.

Main effects of parameters. Inc was affected by all three parameters studied. All parameters had a positive influence on Inc, but Rt was most significant. This observation was in

TABLE 3
Probability (P) of Coefficients for the Two Models[a]

Variables	Inc	PUFA
Intercept	3.378×10^{-11}	2.199×10^{-13}
Rt	1.619×10^{-3}	0.005
Sr	0.006	0.039
Te	0.006	NS
Rt × Rt	9.612×10^{-4}	0.094
Sr × Sr	0.047	0.057
Rt × Sr	0.003	1.078×10^{-3}
Rt × Te	0.087	2.660×10^{-3}

[a]NS, not significant. For other abbreviations, see Table 2.

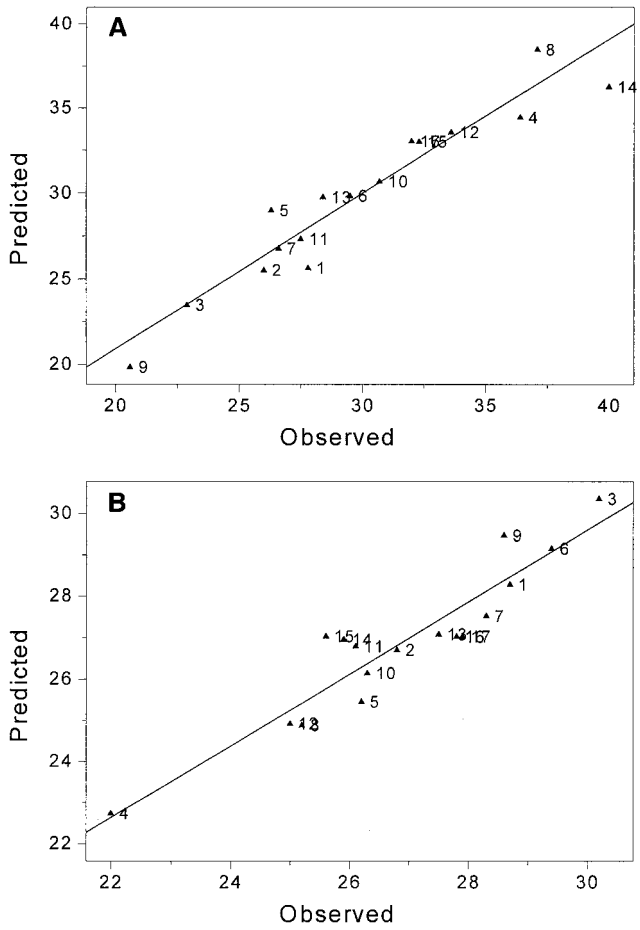

FIG. 1. The relationships between the observed results and the data predicted by the models. (A) Incorporation of caprylic acid (Inc), and (B) content of EPA plus DHA (PUFA). The numbers inside the graphs represent the experimental numbers. The solid line was obtained by regression. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid.

agreement with previous reports (18). At the same time, the second order parameters for Rt and Sr gave negative effects in which the former was more significant, and the interaction between Rt and Sr affected Inc positively. For the content of EPA plus DHA (PUFA) retained, only Rt and Sr had negative effects in which Rt was also more significant. Temperature had very little effect on PUFA. The second order parameter terms did not affect PUFA very much, however, the interaction between Rt and Sr had the most substantial effects on PUFA content, and the interaction between Rt and Te had a positive effect. This conclusion can also be seen in Equations 2 and 3.

Optimization of the reaction. According to the equations generated by the models in the model fitting section, Inc and PUFA retained were affected not only by the first-order variables but also by the second-order factors and the parameter interactions. Therefore, Inc and PUFA will have a complex relationship with parameters that encompasses both first- and second-order polynomials and may have more than one maximum. Thus, the maximum Inc and PUFA cannot be directly obtained by solving the two equations, because they are so complicated that more than one solution might exist. The best way to evaluate the relationships between responses and parameters and interactions that existed herein is to analyze the contour plots for Inc and PUFA (Figs. 2 and 3, respectively). All three plots in Figure 2 gave similar relationships with respect to the effects of each parameter. The higher the Rt, Sr, and Te, the higher the Inc that was obtained. This agrees with the conclusions for the main effects in the above section. However, when Rt was beyond 180 min, Inc started to decrease. In Figure 3A, shorter Rt and higher Sr resulted in higher PUFA, or longer Rt and lower Sr gave a similar tendency. In Figure 3B, shorter Rt and lower Te resulted in higher PUFA content. In Figure 3C, lower Sr gave a higher PUFA and Te had little influence on PUFA, especially at higher substrate molar ratios. The optimal conditions were generated by the optimizer function of the software with interactive calculations in the range selected (Table 4). The two responses were selected at equal weights and both used for maximization. The targeted values for Inc and PUFA were 45 and 30 mol%, respectively. From Table 4, it can be seen that the general conditions for optimal production were: Te, 65°C; Sr, 4–5 mol/mol; and Rt, 180–200 min. By model prediction, a product containing 30 mol% of combined EPA and DHA, and 40 mol% of caprylic acid can be obtained at the above optimal conditions.

Relationship between Inc and PUFA. As expected, the incorporation of caprylic acid did not lead to replacement of EPA and DHA; instead the other fatty acids in the *sn*-1,3 positions were replaced. To check if this was really what happened, we set up relationships between Inc and PUFA retained, and the individual content of DHA (Fig. 4). As can be seen, PUFA content was slightly reduced with an increase in Inc. However, the content of DHA alone was nearly stable. This demonstrates that EPA at *sn*-1,3 positions can be partially removed by Lipozyme IM with subsequent incorporation of caprylic acid, especially if EPA is removed less efficiently than other fatty acids such as palmitic and oleic acids. DHA, on the other hand, was not replaced at all by caprylic acid. This agrees with the previous report (15) that it is possible to separate EPA from DHA by Lipozyme IM-catalyzed reactions

Verification of the model and optimization. Production was conducted under optimal conditions as suggested in the section above to produce 2 kg of structured lipids. The fatty acid composition and position distribution results of the final product are given in Table 1. The product contained 38.8 mol% caprylic acid and 29.0 mol% EPA plus DHA, which data were very close to the predicted values in such optimal conditions (Table 4). Fatty acids at the *sn*-2 position was analyzed by Grignard degradation, and the result showed that 35.6 mol% of combined EPA and DHA was retained at the *sn*-2 position and only 2.7 mol% caprylic acid migrated into the *sn*-2 position of the structured lipids.

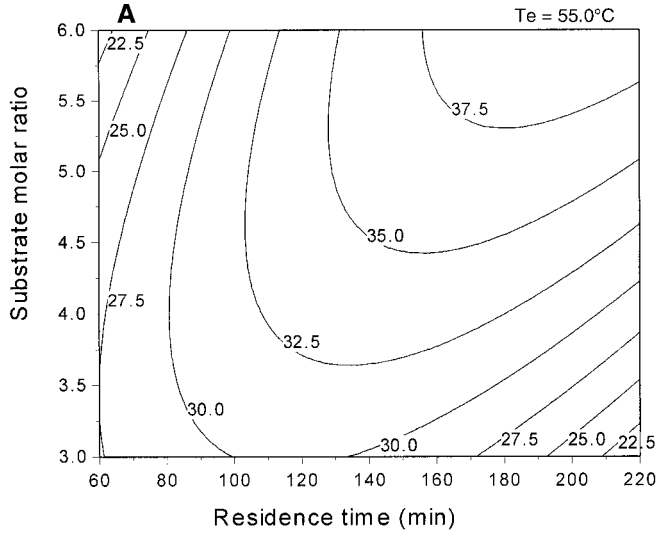

FIG. 2. Contour plots for Inc. (A) Residence time (Rt, min) vs. substrate molar ratio (Sr, mol/mol), (B) Rt (min) vs. temperature (Te, °C), and (C) substrate molar ratio (Sr, mol/mol) vs. Te (°C). For other abbreviation see Figure 1.

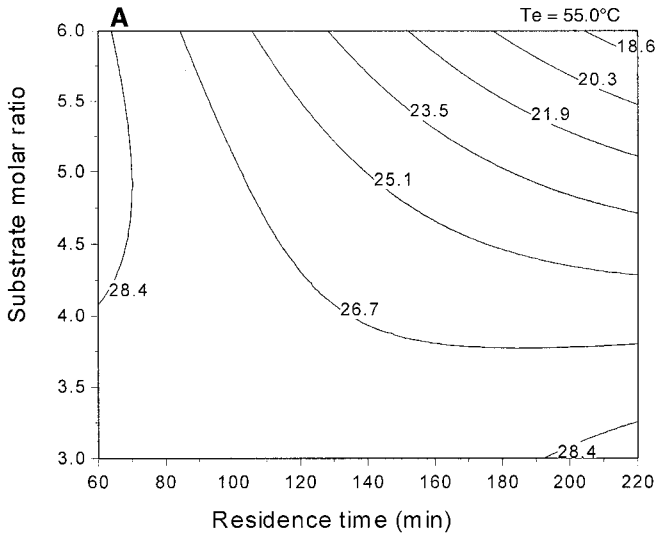

FIG. 3. Contour plots for the content of EPA plus DHA (PUFA). (A) Rt (min) vs. Sr (mol/mol), (B) Rt (min) vs. Te (°C), and (C) Sr (mol/mol) vs. Te (°C).

TABLE 4
Optimal Conditions and Responses Generated by the Models Assisted by the Software of Modde 4.0[a,b]

No.	Rt	Sr	Te	PUFA	Inc
1	193.4022	4.2446	64.9998	29.0543	37.4957
2	202.8599	4.2819	64.9999	29.2866	36.9955
3	63.8903	4.3907	50.0000	29.7993	25.6569
4	186.8713	4.6261	64.9999	27.7495	39.6328
5	188.6047	4.4658	64.9999	28.2651	38.8558
6	199.1882	3.9665	64.9998	30.0658	35.4703
7	186.4005	4.6522	64.9999	27.6605	39.7580
8	186.7029	4.5844	64.9999	27.8682	39.4620

[a]Modde 4.0 (Umetri, Umeå, Sweden).
[b]The ranges of parameters selected were: Rt, 60–220 min; Sr, 3–6 mol/mol; and Te, 50–65°C. The set targets were 45% for Inc and 30% for PUFA (EPA + DHA). For abbreviations, see Table 2.

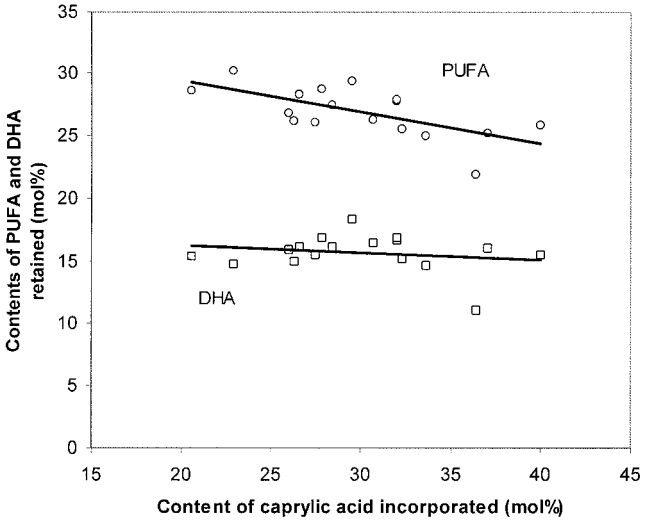

FIG. 4. Relationships between the incorporation of caprylic acid and the content of EPA plus DHA (PUFA), and individual content of DHA. For abbreviations see Figure 1.

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