Production of Structured Lipids by Acidolysis of an EPA-Enriched Fish Oil and Caprylic Acid in a Packed Bed Reactor: Analysis of Three Different Operation Modes

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Structured triacylglycerols (ST) enriched in eicosapentaenoic acid (EPA) in position 2 of the triacylglycerol (TAG) backbone were synthesized by acidolysis of a commercially available EPA-rich oil (EPAX4510, 40% EPA) and caprylic acid (CA), catalyzed by the 1,3-specific immobilized lipase Lipozyme IM. The reaction was carried out in a packed bed reactor (PBR) operating in two ways: (1) by recirculating the reaction mixture from the exit of the bed to the substrate reservoir (discontinuous mode) and (2) in continuous mode, directing the product mixture leaving the PBR to a product reservoir. By operating in these two ways and using a simple kinetic model, representative values for the apparent kinetic constants (k_X) for each fatty acid (native, L_i or odd, M) were obtained. The kinetic model assumes that the rate of incorporation of a fatty acid into TAG per amount of enzyme, r_X (mole/(h g lipase)) is proportional to the extent of the deviation from the equilibrium for each fatty acid (i.e., the difference of concentration between the fatty acid in the triacylglycerol and the concentration of the same fatty acid in the triacylglycerol once the equilibrium of the acidolysis reaction is reached). The model allows comparing the two operating modes through the processing intensity, defined as $m_L t/(V[TG]_0)$ and $m_L/(q[TG]_0)$, for the discontinuous and continuous operation modes, respectively. In discontinuous mode, ST with 59.5% CA and 9.6% EPA were obtained. In contrast, a ST with 51% CA and 19.6% EPA were obtained when using the continuous operation mode. To enhance the CA incorporation when operating in continuous mode, a two-step acidolysis reaction was performed (third operation mode). This continuous two-step process yields a ST with a 64% CA and a 15% EPA. Finally, after purifying the above ST in a preparative silica gel column, impregnated with boric acid, a ST with 66.9% CA and 19.6% EPA was obtained. The analysis by reverse phase and Ag^+ liquid chromatography of the EPA-enriched ST demonstrated that the CA was placed in positions 1 and 3 and the EPA was occupying position 2 of the final ST.

Introduction

There is an increasing interest for clinical nutrition purposes in the production of structured triacylglycerols (ST) with medium-chain fatty acids (M) located at positions 1 and 3 of the glycerol backbone and functional long-chain PUFAs (L) at the position 2 (MLM). ST have been used in absorption studies (*1*, *2*) and for clinical nutrition (*3*). The structure MLM of the ST facilitates its absorption because pancreatic lipase is 1,3-specific and hydrolyzes ester bonds at positions 1 and 3 in triacylglycerols (TAG) (*4*). Moreover, it shows higher activity toward medium-chain than long-chain fatty acids, especially PUFAs (*5*). The liberated medium-chain free fatty acids are directly absorbed into the portal vein, and the 2-monoacylglycerols (with the essential long-chain fatty acid) are well absorbed via the lymphatic route (*2*).

The simplest and direct route for the synthesis of ST of the MLM type is the acidolysis between long-chain TAG and medium-chain free fatty acids catalyzed with a 1,3-specific lipase (*6*–*12*). The lipase offers high catalytic efficiency, specificity, and selectivity by incorporation of the required acyl group into a specific position of native TAG. Acyl migration is a major problem in the synthesis of ST in batch reactors because this causes undesired changes in the position of fatty acids within the acylglycerol molecules (*13*). Acyl migration occurs in partial acylglycerols (monoacylglycerols and diacylglycerols), which are the necessary and unavoidable intermediates in lipase-catalyzed interesterification. Many factors can influence acyl migration, including the presence of an acyl donor, as acids, water activity, nonpolar solvents, enzyme supports with surface charges, high temperature, and long reaction times (*10*). In batch reactors the high substrate/enzyme ratio requires long reaction times to reach equilibrium and consequently results in acyl migration. In contrast, the continuous enzymatic reactor with the lipase immobilized into a

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column is advantageous over batch reactor in reducing acyl migration (*13*).

Most of the research about the synthesis of ST is directed toward studying the effect of variables such as the type of lipase and the lipase/substrate ratio (*9*), the reaction medium (*9*), substrate concentrations, content of water (*11*), temperature (*9*), and operational mode (*6, 8, 10*). However, little has been reported on the kinetics of lipase-catalyzed interesterification between free fatty acids and heterogeneous TAG and the formulation of rate equations that describe the performance of the lipase, optimize the control of reactors, and permit a more rational design of the reactor. Among the scarce works related to acidolysis kinetics, Reyes and Hill (*14*) carried out a detailed study of the acidolysis between free fatty acid and a heterogeneous TAG such as olive oil and milk fat and proposed a kinetic model that accounts for the effect of the concentration of all chemical species participating in the interesterification reaction. Recently, we have proposed a simple kinetic model based on rate equations of first-order with respect to each reactant to describe the acidolysis of triolein and caprylic acid (*15*). Also, little information has been reported on the magnitude of rate constants in the esterification reaction. However this information is essential for understanding how operational variables affect reaction rates and for a rational design and scaling up of interesterification reactors. In a previous paper (*12*) the authors calculated average reaction rates and kinetic constants of exchange of caprylic acid (CA) and native fatty acids of cod liver oil by admitting that the rate of incorporation of a fatty acid into TAG by a unit amount of enzyme is proportional to the separation from the equilibrium. Thus, a simple equation was proposed for the prediction of the fatty acid composition of the ST at the exit of a packed bed reactor (PBR) where the lipase was immobilized. For the acidolysis reaction in the continuous PBR the lipase amount/(flow rate $\times$ substrate concentration) ratio could be considered as the intensive variable of the process for use in scale-up of the PBR (*12*).

This work deals with the synthesis of ST of the MLM type with a high content of EPA in position 2. A high EPA content in ST was achieved by using an EPA-rich commercial oil (40% EPA) for the acidolysis reaction with CA. Immobilized 1,3-specific lipase (Lipozyme IM) was used as the catalyst. The immobilized lipase was disposed in a packed bed reactor (PBR) that was operated in two modes: by recirculating the reaction mixture through the PBR (discontinuous mode) and by directing the reaction mixture that comes out from the PBR to a product reservoir (continuous mode). The aims of this study were to investigate the kinetic of ST synthesis and simultaneously the effects of the flow rate of the reaction mixture through the PBR. A simple model is applied to compare both operation modes through the processing intensity, defined as the (lipase amount $\times$ reaction time)/(volume of reaction mixture $\times$ initial TAG concentration), $m_L t/V[TG]_0$, and the lipase amount/(flow rate of substrate mixture $\times$ initial TAG concentration) ratio, $m_L/q[TG]_0$, for the discontinuous and continuous operation modes, respectively.

Materials and Methods

Chemicals and Materials. Lipozyme IM was donated by Novo Nordisk A/S (Bagsvaerd, Denmark). This lipase contained 2−3% water as determined by Karl Fischer titration (compact titrator microKF 2026, Crimson, Alella, Spain) and was supplied immobilized on a macroporous anion-exchange resin. The enzyme showed a 1,3-posi-

Table 1. Fatty Acid Composition of the Original EPAX4510 Oil (F_{X_0}), Structured Triacylglycerols (ST) Produced in Discontinuous Mode Once Equilibrium of the Acidolysis Reaction Was Reached (F_{X_e}), and ST Produced in Continuous Mode (F_X Continuous)[b]

fatty acids	F_{X_0} (% mol)	F_{X_e} discontinuous (% mol)[a]	F_X continuous (% mol)[b]
8:0	0	59.5	51.0
16:0	6.9	4.4	3.0
16:1	3.0	1.7	1.3
18:0	6.8	4.0	3.1
18:1n-9	11.8	5.1	5.1
18:1n-7	6.9	5.0	3.9
18:4n-3	4.0	2.0	2.4
20:1n-9	5.7	3.4	2.5
20:4n-6	2.5	0.6	1.3
20:5n-3	40.4	9.6	19.6
22:1n-9	4.3	2.7	2.3
22:6n-3	7.7	2.1	4.5
total	100	100	100

[a] Operation conditions in discontinuous mode: 10−20 g of EPAX4510 oil, 10−20 g of CA (molar ratio CA//EPAX4510 = 6), 200−300 mL of *n*-hexane ($[TG]_0 = 0.036$−0.108 M), 30 °C, 2.2−2.5 g of Lipozyme IM immobilized into a 0.66 cm i.d. $\times$ 25 cm column, flow rate of 200 mL/h, time 27−114 h (data from ref 21). [b] Operation conditions in continuous mode: 20 g of EPAX4510 oil, 20 g of CA (molar ratio CA/EPAX4510 = 6), 200 mL of hexane ($[TG]_0 = 0.108$ M), 30 °C, 11 g of Lipozyme IM immobilized into a 1 cm i.d. $\times$ 40 cm column, flow rate of 15 mL/h.

tional specificity. Analytical-grade caprylic acid (CA) and hexane were obtained from Sigma Aldrich (St. Louis, MO). Table 1 shows the fatty acid composition of the commercial oil used (EPAX4510, Pronova Biocare, Norway). From this composition an average molecular weight for the EPAX4510 was 924.3 Da.

Acidolysis Reaction in the Packed Bed Reactor (PBR). Figure 1 shows a scheme of the reaction system. The immobilized lipase was packed into a glass column covered with aluminum foil to prevent photoinduced oxidation. The enzyme bed was held between two mobile perforated disks. The porosity in this confined bed was 0.46, and the bed density of catalyst was 0.36 g/cm³ (*12*). The substrate mixture was kept in a reservoir submerged in a termostated water bath. The mixture consisted of EPAX4510, 10 or 20 g; caprylic acid, 10 or 20 g; and hexane, 0−300 mL. The molar ratio of caprylic acid/EPAX4510 was $m_0 = 6$ in all the experiments. The initial EPAX4510 concentration was varied from 0.036 to 0.216 M. In addition some experiments were conducted without the solvent. The reaction mixture was pumped upward, through the column, by a peristaltic pump at flow rates between 15 and 406 mL/h. The column was jacketed for controlling the reaction temperature (30 °C).

A three-way valve was placed at the exit of the lipase bed, permitting two modes of operation (Figure 1): (1) by recirculation of the mixture that came out of the bed to the substrate reservoir (product recirculation) or (2) by directing the reaction mixture leaving the bed to an additional reservoir (continuous operation). When the system was operated with recirculation the reaction was monitored by sampling at different times (between 1.0 and 175 h) in the substrate reservoir. The content of the reservoir bottle was continuously agitated at 200 rpm during the reaction by a magnetic bar. These experiments were carried out with 2−3 g of lipase into a column of 6.6 i.d. $\times$ 250 mm length. When the packed bed reactor was operated in continuous mode sampling was done at the exit of the bed. These experiment were carried out with 11 g of lipase and a column of 1 cm i.d. $\times$ 400 mm length. The reaction mixture flow rate was varied be-
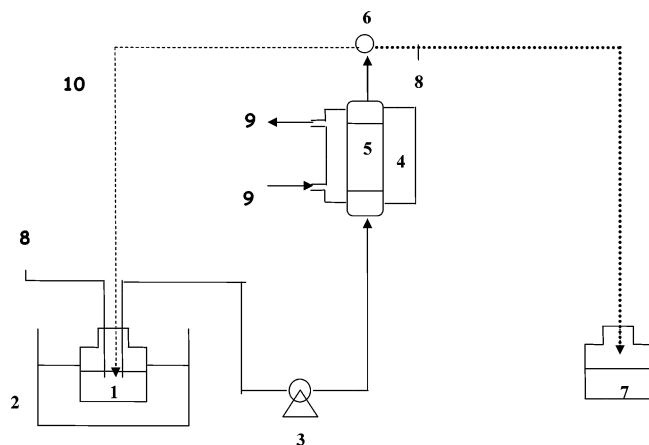
Figure 1. Immobilized lipase packed-bed reactor (PBR). (1) Substrate reservoir, (2) reactor temperature control, (3) peristaltic pump, (4) water jacket, (5) bed of immobilized enzyme, (6) three-way valve, (7) product reservoir, (8) sampling, (9) cooling/heating water, and (10) recirculation. The dashed line describes the flow of materials for recirculating operation; the dotted line describes the flow for the continuous mode.

tween 15 and 250 mL/h. The samples were stored at −20°C until analysis. All analyses were carried out in triplicate. The standard deviation was always below 6%.

Purification of Triacylglycerols by Preparative Chromatography in a Silica Gel Column. Samples of ST were purified in a column (3 cm i.d. × 60 cm length) filled with silica gel (70−230 mesh ASTM, Scharlau) activated by heating at 120 °C for 2 h. To prevent that the silica gel could catalyze the acyl migration of the EPA from position 2 of the TAG to positions 1 and 3, the silica gel was previously impregnated with boric acid following a procedure described elsewhere (*16*). Silica gel (90 g) was mixed with 10 g of boric acid (Panreac, Barcelona) and 200 mL of methanol (Panreac). Then the methanol was evaporated in a vacuum evaporator. The separation of the different fractions (esters, triacylglycerols, and free fatty acids) was carried out by stepwise elution using different composition mixtures of hexane−ether as mobile phases and following the procedure described by Kates (*17*).

Identification of Fatty Acids and Estimation of Molar Fraction of Fatty Acids in Triacylglycerols. Hexane was removed from the product mixture in a vacuum evaporator. Acylglycerols (monoacylglycerols, diacylglycerols, and triacylglycerols) were extracted with (3 × 3 mL) hexane after adding 2 mL of 0.5 N KOH (20% ethanol solution) to 70 mg of the reaction mixture. These acylglycerols were identified by thin-layer chromatography (TLC) followed by quantitative gas chromatography (GC). TLC analysis has been described elsewhere (*18*). Fractions corresponding to each acylglycerol type were scraped from the plates and methylated by direct transesterification with acetyl chloride/methanol (1:20) using the method of Lepage and Roy (*19*). These methyl esters were analyzed by capillary gas chromatography (GC) following the procedure described in ref 12.

Identification of Structured Triacylglycerols. The acylglycerols extracted from the product mixture were analyzed with a Shimadzu (Kyoto, Japan) high performance liquid chromatograph (HPLC) equipped with an evaporative light scattering detector (ELSD) (Eurosep Instrument, Cergy-Pontoise, France). Triacylglycerol molecular species were separated by reverse-phase HPLC on a Beckman (San Ramon, CA) Ultrasphere C18 column (5 μm particle size, 80 Å pore size (25 cm × 4.6 mm)).

Separations were obtained using acetone−acetonitrile as the eluent and following the method of Akoh and Huang (*9*).

The regioisomeric composition of the final structured triacylglycerol was analyzed by HPLC with a ChromSpher 5 Lipids silver ion chromatography column (25 cm × 4.6 mm, from Chrompack, Middelburg, The Netherlands). A binary solvent gradient made of solvent A (*n*-hexane/2 propanol/acetonitrile 350:100:3.5 v/v/v) and solvent B (*n*-hexane/2 propanol/acetonitrile 350:100:10 v/v/v) was used. The column was eluted at a flow rate of 0.65 mL/min with a gradient of A for 3 min, A to B over 22 min, B for 8 min, and B to A over 4 min. The lipids species were also detected with the ELSD. The elution order and retention time of MLM and MML were confirmed according to the method reported by Han et al. (*20*).

Rate Equations. In a previous paper (*12*) an empirical kinetic model was proposed for obtaining representative values of the apparent kinetic constants, k_X, for each fatty acid, X (native, L_i, or odd, M), by admitting that the rate of incorporation of a fatty acid X into the triacylglycerols per amount of enzyme, r_X (mol/h g of lipase), is proportional to the extent of deviation from the equilibrium (i.e., the driving force) for each fatty acid in the form:

$$r_X = k_X(F_{X_e} - F_X) \tag{1}$$

F_X is the molar fraction of any fatty acid (odd or native) in the triacylglycerols and F_{X_e} is the molar fraction at equilibrium. r_X is positive for caprylic acid (M) and negative for the native oil fatty acids (L_i). To calculate reaction rates when the reaction occurs in an PBR, we determined the average reaction rate (r_{mX}, moles/(h g)) between the entrance and the exit of the lipase bed. r_{mX} can be obtained by means of a mass balance for any free fatty acid, X, applied to the bed; i.e.,

$$q[X]_{in} - r_{mX}m_L = q[X]_{out} \tag{2}$$

where m_L is the lipase amount in the bed (g), q is the substrate mixture flow rate through the lipase bed (mL/h), and $[X]_{in}$ and $[X]_{out}$ are the concentrations (moles/mL) of the free fatty acid (M or L_i) at the entrance and at the exit of the bed, respectively.

The relationships between the concentration of a free fatty acid (caprylic acid, $[X] = [M]$, or a native fatty acid, $[X] = [L_i]$) and its molar fractions in the triacylglycerols ($F_X = F_M$ or $F_X = F_{L_i}$) is given by the corresponding balances as follows:

$$m_0[TG]_0 = [X] + 3[TG]_0F_X = [M] + 3[TG]_0F_M \tag{3}$$

$$3[TG]_0F_{L_i0} = [X] + 3[TG]_0F_X = [L_i] + 3[TG]_0F_{L_i} \tag{4}$$

where $[TG]_0$ is the initial triacylglycerol concentration (i.e., the initial concentration of the starting oil), m_0 is the caprylic acid/triacylglycerol molar ratio, and F_{L_i0} is the initial molar fraction of the native fatty acids in the starting material. These balances ignore partial acylglycerols (mono- and diacylglycerols) because the concentrations of these acylglycerols were negligible in all of the experiments carried out. By substituting eq 3 (for caprylic acid) and eq 4 (for native fatty acid) into eq 2, we obtain

$$r_{mX} = \frac{3[TG]_0(F_{X_{out}} - F_{X_{in}})}{\dfrac{m_L}{q}} \tag{5}$$

where $F_{X_{out}}$ and $F_{X_{in}}$ are the molar fractions of the native fatty acids at the exit and at the entrance of the PBR, respectively. In this work we operated in two modes: in discontinuous, by recirculating to the substrate reservoir the reaction mixture leaving the bed, and in continuous.

When operating in discontinuous mode, with **recirculation** of the reaction mixture through the lipase bed, the operation was under non-steady state and eqs 2 and 5 involved that the accumulation term was neglected. However, when the enzyme amount was low and the flow rate large (and therefore the conversion due to a single passage of the substrate mixture through the bed was low) and the reaction mixture volume held in the feed reservoir was large compared with the volume in the bed, the change of [X] with time due to the accumulation was indeed negligible compared with the change of [X] due the enzymatic reaction in the bed.

To facilitate the interpretation of the experiments carried out by this procedure, it was necessary to work with a high circulation flow rate to eliminate the possible influence of the external mass transport effects in the bed. Also, the feed reservoir needed to be perfectly mixed. To ensure that the latter condition was attained, the reaction mixture reservoir was agitated continuously.

When the system operated with recirculation, if the feed reservoir is perfectly mixed, a differential mass balance of any free fatty acid X in the feed reservoir provides

$$q[X]_{out}\,dt = q[X]_{in}\,dt + V\,d[X] \qquad (6)$$

because the mixture leaving the bed is identical to that entering the reservoir and vice versa. In eq 6 V is the volume of reaction mixture in the reservoir. This equation can be rewritten in the form

$$V\left(-\frac{d[X]}{dt}\right) = q([X]_{in} - [X]_{out}) \qquad (7)$$

The comparison of eqs 2 and 7 shows that if the previously indicated hypotheses are fulfilled and the conversion in a passage of the reaction mixture through the bed is sufficiently small, we can conclude that

$$\frac{V}{m_L}\left(-\frac{d[X]}{dt}\right) = r_{mX} \qquad (8)$$

The first term of eq 8 corresponds to the reaction rate (r_X) in a perfectly mixed batch reactor. According to the proposed model, r_X is given by eq 1. Therefore, considering that $r_X = r_{mX}$ and considering eqs 3 and 4, we have

$$r_X = \frac{3[TG]_0\,V}{m_L}\frac{dF_X}{dt} \qquad (9)$$

Equaling expressions 1 and 9 and integrating between time zero and a time t, we obtain

$$F_X = F_{X_e} + (F_{X_0} - F_{X_e})\exp\left(-\frac{k_X m_L t}{3[TG]_0 V}\right) \qquad (10)$$

Equation 10 represents the variation of the fatty acid composition of the structured triacylglycerols with time in a perfectly mixed discontinuous reactor.

When we operated in **continuous** mode, the reaction mixture that comes out from the bed is directed to a product reservoir (Figure 1). In this case the composition of the reaction mixture at the entrance of the bed is

constant and the system will operate in steady state. At these conditions the average reaction rate is given by eq 5, being $F_{X_{in}} = F_{X_0}$.

To link the average rate (eq 5) with the kinetic eq 1, it was necessary to hypothesize about the degree of mixing in the PBR. To establish the equivalence between a continuous and a discontinuous reactor we applied the hypothesis of plug flow within the bed. This hypothesis is logical because in the continuous operation greater masses of enzyme and beds of high length/diameter ratio were used and, therefore, appreciable conversions due to a passage of the reaction mixture through the bed were obtained. The hypothesis of plug flow can be introduced by means of a mass balance for any fatty acid into the triacylglycerols, X, to the differential lipase amount (dm_L) contained in a differential thickness of bed:

$$q3[TG]_0 F_X + r_X\,dm_L = q3[TG]_0(F_X + dF_X) \qquad (11)$$

where F_X and $F_X + dF_X$ are the molar fractions of the native fatty acid (L_i) or caprylic acid (M) at the entrance and at the exit of the differential thickness of bed considered. Substituting the kinetic eq 1 and integrating between the entrance ($F_X = F_{X_0}$, $m_L = 0$) and the exit of the PBR ($F_X = F_X$, $m_L = m_L$) we obtain

$$F_X = F_{X_e} + (F_{X_0} - F_{X_e})\exp\left(-\frac{k_X m_L}{3[TG]_0 q}\right) \qquad (12)$$

This equation allows predicting the molar fractions of any native (L_i) and caprylic acid (M) in the structured triacylglycerols at the exit of the reactor when operating in continuous mode. Comparison of eqs 10 and 12 shows that continuous and batch reactor should give similar results whenever the following condition occurs:

$$\left(\frac{m_L t}{V[TG]_0}\right)_{discontinuos} = \left(\frac{m_L}{q[TG]_0}\right)_{continuous} \qquad (13)$$

This equation is useful to compare both operation modes through a new parameter: the processing intensity, which corresponds to the left-hand side of eq 13, for discontinuous mode, or to the right-hand side, for continuous mode. Equation 13 is based on the hypothesis that the PBR behaves as a differential reactor when it operates in discontinuous mode and on the hypothesis that a plug flow is fulfilled within the PBR when it operates in continuous mode. Equation 13 implies that the reaction rate (and the corresponding expression for the equation rate) is the same in both reactors and the external mass transfer resistance is not significant in the PBR.

Results and Discussion

Acidolysis of EPAX4510 and CA in the PBR: Product Recirculation (Discontinuous Mode). The composition of the structured triacylglycerols (ST) obtained at the equilibrium (F_{X_e}) is depicted in Table 1. This table shows that the CA incorporation was significant (59.5%), whereas the EPA content was only 9.6%. This percentage is low if we take into account that the original oil contained 40.4% EPA (Table 1). Since the 1,3 positional specificity of the lipase used is contrasted, this result may indicate that the major amount of EPA in the original oil was bonded in the exchangeable 1−3 extreme position of the glycerol backbone. Therefore, operating in discontinuous mode, this commercial fish oil, despite its high EPA content, might not be a suitable raw
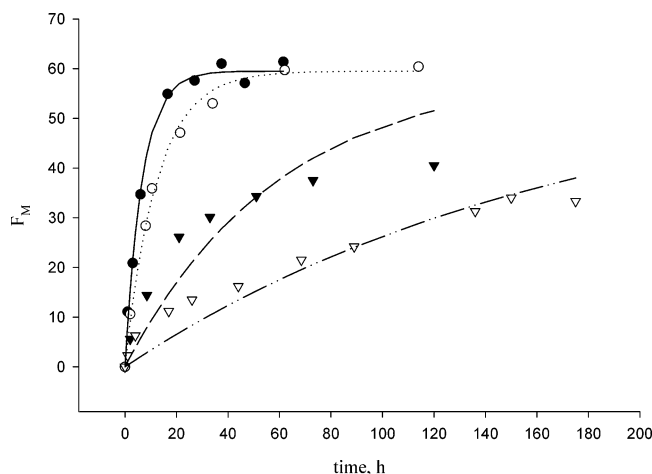
Figure 2. Influence of time and TAG concentration on the mass fraction of CA (F_M) in the ST produced by acidolysis of EPAX4510 and CA in the PBR with recirculation: (●) $[TG]_0 = 0.036$ M; (○) $[TG]_0 = 0.108$ M; (▼) $[TG]_0 = 0.216$ M; (▽) without solvent. The lines were obtained by using eq 10, the values of F_{X_0} and F_{X_e} of Table 1, and the initial concentration of TAG ($[TG]_0$) previously indicated. Acidolysis conditions: 10–20 g of EPAX4510, 10–20 g of C8:0, 100–300 mL of hexane, 2.5 g Lipozyme IM, 200 mL/h. Experiment without solvent: 20 g of EPAX4510, 20 g of C8:0, 0 mL of hexane, 3.0 g of Lipozyme IM, 30 mL/h.

material to produce MLM type ST with the functional fatty acid (i.e., the EPA) in the position 2.

Figures 2 and 3 show the time variation of CA and EPA contents, respectively, at different initial concentrations of EPAX4510, $[TG]_0$, and different CA concentrations. All the experiments, except for the one carried out without solvent, were performed at a mixture flow rate through the bed of 200 mL/h. This flow rate was selected because mixture flow rates over 75 mL/h had been previously proved not to influence the reaction rate when an initial TAG concentration equal to 0.036 M was used (*21*). In the experiment carried out without solvent, the mixture flow rate was of 30 mL/h, and in this case the external and internal mass transfer resistance could have also been important.

Figures 2 and 3 show that the initial concentration of the starting fish oil, $[TG]_0$, strongly influenced the acidolysis rate. This influence was very significant for the concentration 0.216 M and when no solvent was used. These results were fitted to the model represented by eq 1. If, as commented, the acidolysis reaction is independent of the mixture reaction flow rate, the PBR operating in this way will behave as a discontinuous perfectly mixed batch reactor and therefore data will have to fit to eq 10. The lines in Figures 2 and 3 show that the percentage of CA incorporated into the ST and the percentage of EPA in the ST, respectively, fitted to eq 10 (similar figures were obtained for the rest of native fatty acids in the original oil, data not shown). These figures show that the fitting is satisfactory for CA and EPA, particularly for the two lowest triacylglycerols (TAG) concentrations tested. In these cases the deviation found was always below 10%, which is quite acceptable considering the many manipulations necessary for the analysis of the samples. For experiments carried out at the highest concentration (0.216 M) and without solvent the deviation was somewhat greater. Table 2 shows the values of the kinetic constant k_X that best fitted to eq 10 and the initial reaction rates calculated by eqs 9 and 10.

Figures 2 and 3 and Table 2 show that the higher the initial TAG concentration, $[TG]_0$, the lower the reaction
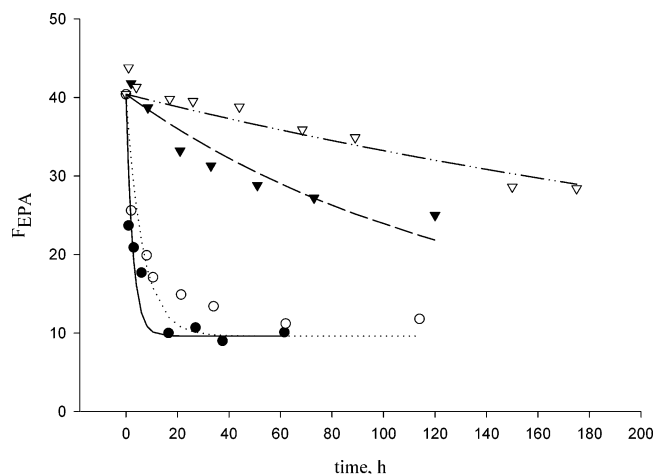

Figure 3. Influence of time and TAG concentration on the mass fraction of EPA (F_{EPA}) in the ST produced by acidolysis of EPAX4510 and CA in the PBR with recirculation: (●) $[TG]_0 = 0.036$ M; (○) $[TG]_0 = 0.108$ M; (▼) $[TG]_0 = 0.216$ M; (▽) without solvent. Lines were obtained using the model (eq 10) and the same parameters indicated in Figure 2. Acidolysis conditions as in Figure 2.

Table 2. (Top) Acidolysis of EPAX4510 and CA in the PBR in Discontinuous Mode at Several TAG Concentrations: Apparent Kinetic Constants, k_X, and Initial Reaction Rates (r_{X_0}) for Exchange of CA (8:0) and EPA (20:5n-3) (Borrom) Acidolysis of EPAX4510 and CA in the PBR in Continuous Mode: Apparent Kinetic Constants, k_X, and Initial Reaction Rates (r_{X_0}) for Exchange of CA, EPA, DHA (22:6n-3) and Oleic Acid (18:1n-9)

	Discontinuous (or Recirculation) Mode[a]			
	$[TG]_0 =$ 0.036 M	$[TG]_0 =$ 0.108 M	$[TG]_0 =$ 0.216 M	without solvent
	k_X (mol/g lipase·h)			
8:0	0.0022	0.0021	0.00044	0.00013
20:5n-3	0.0057	0.0040	0.00020	0.00017
	r_{X_0} (mol/g lipase·h)[a]			
8:0	0.132	0.124	0.0259	0.0074
20:5n-3	−0.176	−0.123	−0.0061	−0.0051

	Continuous Mode	
	k_X (mol/g lipase·h)	r_{X_0} (mol/g lipase·h)[a]
8:0	0.00186	0.111
20:5n-3	0.000851	−0.0262
22:6n-3	0.000583	−0.00326
18:1n-9	0.00335	−0.0224

[a] Initial reaction rates calculated from eq 1 taking into account the kinetic constants, k_X, and the values of F_{X_e} (Table 1).

rate for caprylic acid and EPA was. Table 2 shows that at the two lower concentrations (0.036 and 0.108 M) the rates of incorporation of CA into the TAG and the rates of displacement of EPA from the original TAG are similar. Nevertheless, at the concentration of 0.216 M the rate of displacement of EPA is significantly lower than the rate of CA incorporation (which is equal to the overall rate of displacement of all the native fatty acids). This result could represent an apparent advantage just for producing a ST enriched in EPA and CA. However, this advantage is counteracted by the fact that the overall rate of the exchange process was smaller than at the lower concentrations of native oil (0.036 and 0.108 M). The decrease of the acidolysis reaction rate when the initial concentration of TAG increases indicates that at high concentrations of native TAG (i.e., EPAX4510), the
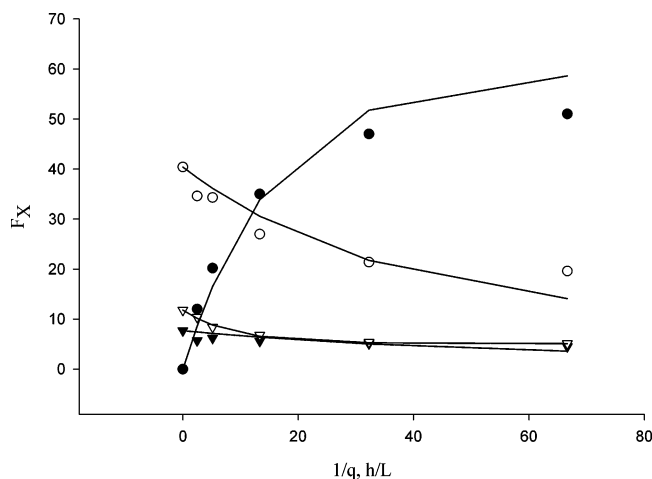
Figure 4. Influence of flow rate on the mass fraction of CA, EPA, DHA and oleic acid in the ST obtained by acidolysis of EPAX4510 and CA in the PBR operating in continuous mode: (●) C8:0; (○) EPA; (▼) DHA; (▽) oleic acid; the continuous lines were obtained from eq 12, the values of F_{X_0} and F_{X_e} of Table 1 and the initial concentration of TAG ([TG]$_0$) previously indicated. Acidolysis conditions: 20 g of EPAX4510, 20 g of C8:0, 200 mL of hexane ([TG]$_0$ = 0.108 M), 11 g of Lipozyme IM, column 1 cm i.d. × 40 cm length.

reaction rate could be influenced by diffusional limitations within the catalytic particles.

Acidolysis of EPAX 4510 and CA in the PBR: Continuous Operation. As commented above, the greatest CA incorporation into TAG (i.e., the largest k_X values) when operating with product recirculation was for [TG]$_0$ concentrations of 0.036 and 0.108 M (Table 2). At these concentrations the processing intensity, $m_L t/(V[TG]_0)$, requested to reach the equilibrium were 6500 and 6900 g lipase·h/mol TAG, respectively. These values were obtained by using the reaction times, calculated by eq 10, for an incorporation of 59%, close to the equilibrium (59.5%, Table 1). These processing intensities were used to carry out the acidolysis between EPAX4510 and CA in the PBR in continuous mode (i.e., directing the product reaction mixture coming out from the reactor to a product reservoir), taking into account the eq 13 (which roughly establishes the equivalence between the two operation ways). Thus, a processing intensity of 6700 g lipase·h/mol TAG (11 g lipase, 15 mL/h, and [TG]$_0$ of 0.108 M) was used in the first experiment carried out in continuous mode. Data of this experiment, corresponding to the maximum incorporation of CA, are shown in Table 1 (F_X continuous). It can be seen that the percentage of incorporation of CA into the TAG was 51%, below the 59% obtained in discontinuous mode, but most important is that the EPA content is almost 20%, much higher than the 9.6% obtained at the equilibrium when operating with the product recirculation mode (Table 1). These differences seem to indicate that when operating in continuous the reaction rate is lower than in the discontinuous mode and show that eq 13 is really only an approximation. Probably, at this low flow rate (15 mL/h), mass transfer phenomena affect to the reaction rate and also a deviation of the hypothesis of plug flow occurs.

In this operation mode the molar fraction of fatty acids in TAG is given by eq 12. This equation has been used to estimate the apparent kinetic constants of the main fatty acids (CA, EPA, DHA, and oleic acid). Figure 4 shows the experimental data and their fitting to eq 12 by using the F_{X_0} and F_{X_e} values for each fatty acid depicted in Table 1. The fitting is satisfactory for all of the fatty acids, with a maximum deviation inferior to 15%

and an average deviation lower than 6%. This result demonstrates that the hypothesis of plug flow is acceptably fulfilled, mainly at the highest values of liquid flow rate, q (Figure 4); at the lowest values of q the deviation between the experimental results and the model is greater (Figure 4) because at low flow rates a greater deviation of the plug flow hypothesis occurs. Table 2 shows the k_X values for CA, EPA, DHA and oleic acid that best fit the experimental results at [TG]$_0$ = 0.108 M. The k_X and r_{X_0} value for CA (0.00186 and 0.111 mol/(g h), respectively) are similar to the ones obtained in discontinuous mode for similar conditions (0.0021 and 0.124 mol/(g h)); however, the k_X and r_{X_0} values for EPA are appreciably lower, which represents an advantage with respect to the operation in discontinuous mode. This result also agrees with the high content of EPA and also DHA in the triacylglycerols coming out the PBR, 19.6% and 4.5%, respectively. Although in the continuous operation mode the CA incorporation did not reach the equilibrium value (51% against the 59.5% obtained in the discontinuous mode), this fact does not justify the important difference in the EPA content between the two operation modes (19.6% against 9.6% for the discontinuous mode). The different fatty acid composition of the ST obtained by both methods could be explained by admitting the existence of some degree of acyl migration of long-chain fatty acids from the 2-position to the extreme positions into the TAG, catalyzed by the anionic exchange resin support of the lipase (*22*). This acyl migration is greater when the system operates in the discontinuous mode because the residence time is much higher than in the continuous mode. The acyl migration must be through the mono and diacylglycerols (DAG) (*23*), but the concentration of these partial acylglycerols is negligible as a result of the almost total absence of water. Nevertheless, a small amount of DAG must have been formed because these products are essential mediators in the acidolysis reaction (*24*). In addition, the hexane also favors the formation of 1,3-DAG from 1,2-DAG (*22*). Therefore, to avoid acyl migration the time of contact between the TAG and Lipozyme IM should be kept at a minimum. Also, the enzyme support that catalyzes the acyl migration and the hexane should be changed (*22*). Furthermore, when a 1,2-DAG is transformed into a 1,3-DAG by acyl migration, this 1,3-DAG will be transformed into glycerol by successive hydrolysis because of the impossibility of the lipase to catalyze the incorporation of fatty acid into the position 2. For this reason the positional isomer MML does not appear in the final ST, as will be shown later (Figure 5b), and the concentration of DAG measured is always negligible.

To identify the different types of TAG in the final reaction mixture produced (LLL or native, MLL or TAG with only one molecule of CA incorporated and MLM, or its positional isomer MLL), first the ST were fractionated by reverse-phase HPLC and then the positional distribution analysis in the target ST was carried out by silver ion liquid chromatography. Figure 5a shows the chromatogram obtained in RP-HPLC initial fractionation, and Table 3 shows the fatty acid composition of the different chromatographic fractions. Figure 5a shows the four more relevant peaks. The first one corresponds to the target product, MLM, because its composition in CA is 64%, which is almost the target composition (66.6%). However, as shown in Figure 5a and Table 3, ST with only a molecule of CA incorporated (MLL, peaks 2, 3, and 4) are also obtained. Then the ST corresponding to peak 1 was also analyzed by silver ion liquid chromatography with the aim of fractionate the two possible isomers MLM
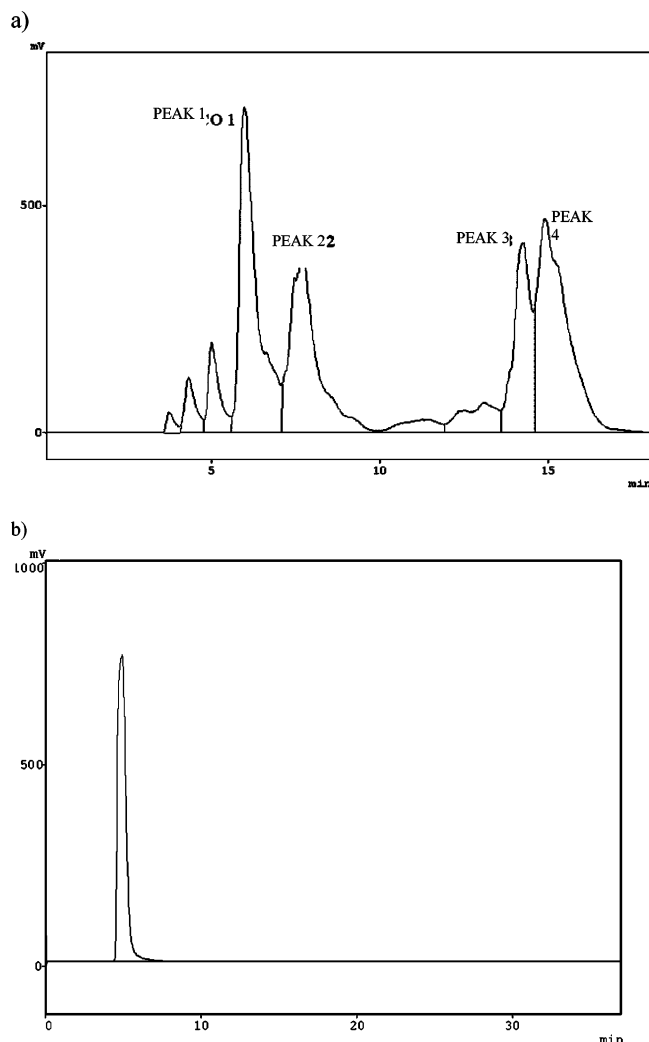
a)

b)

Figure 5. (a) Separation by reverse phase HPLC of the different triacylglycerols (TAG) produced by acidolysis of EPAX4510 and CA when using the ST obtained in the experiment carried out at 15 mL/h (see details of this experiment in Table 1). The fatty acid composition of the different peaks is shown in Table 3. (b) Separation by Ag$^+$ chromatography of the positional isomers (MLM and MML) of the TAG contained in the peak 1 which composition is shown in Table 3.

Table 3. Separation by Reverse Phase HPLC of Different TAG Contained in Produced ST[a]

fatty acids	ST	peak 1	peak 2	peak 3	peak 4
8:0	51.0	64.0	39.9	37.9	28.7
18:1n-9	5.1	2.1	0.9	8.3	8.2
18:1n-7	3.9	0.0	0.7	4.1	4.2
20:5n-3	19.6	22.8	35.1	18.5	18.4
22:6n-3	4.5	6.7	13.0	7.1	8.3

[a] The composition of the fractions 1−4 corresponds to the peaks 1−4 of the chromatogram shown in Figure 5a.

and MML. Figure 5b shows this chromatogram in which a single peak appears that, obviously, corresponds to the target structure MLM. This result demonstrates again the 1,3-specificity of Lipozyme IM. Also the high EPA content of the ST demonstrated that this operation mode is more suitable for the production of EPA-enriched ST than the discontinuous mode.

Acidolysis of EPAX4510 and CA in the PBR: Two-Step Continuous Operation (Third Operation Mode). ST containing 54.9% CA and 17.5% EPA from a continuous operation was subjected to a second acidolysis reaction to improve the percentage of CA incorporated (Table

Table 4. Production and Purification of ST Obtained in the PBR in Continuous Mode in Two Steps: Composition of ST Obtained in First and Second Acidolysis Steps[a], Compositions of Three Fractions Obtained in Purification of ST Obtained in the Second Acidolysis,[b] and Compositions of Fractions Corresponding to Peaks 1 and 2 [c]Obtained in the Separation of Fraction 3 (F3) by Reverse Phase HPLC[c]

fatty acids	ST 1st acidolysis	ST 2nd acidolysis[a]	fractions obtained in the purification of ST[b]			fractions of peak 1 and 2 (HPLC F3)[c]	
			F1	F2	F3	peak 1	peak 2
8:0	54.9	64.2	47.4	55.7	66.9	66.4	40.3
16:0	3.1	0.2	4.1	2.5	0.8	-	0.8
16:1	1.4	1.0	1.6	1.2	0.3	-	2.4
18:0	2.9	2.3	4.2	2.2	0.5	-	1.1
18:1n-9	4.5	4.1	6.0	3.5	1.1	0.6	2.1
18:1n-7	4.1	2.6	5.5	3.3	1.1	-	0.9
18:4n-3	2.0	2.2	1.5	2.1	3.2	2.2	0.8
20:1n-9	2.3	1.9	4.3	2.2	0.6	-	0.4
20:4n-6	1.2	1.0	1.7	1.3	0.6	0.3	1.9
20:5n-3	17.5	15.1	15.4	19.0	19.6	23.0	33.2
22:1n-9	2.2	1.8	3.0	2.2	1.5	1.1	2.5
22:6n-3	3.9	3.6	5.3	4.9	3.9	6.4	13.6
total	100	100	100	100	100	100	100

[a] Operation conditions: 3 g of TAG, 3 g of CA (molar ratio = 6), 30 mL of *n*-hexane, 10 g of Lipozyme IM immobilized in a 1 × 40 cm column, flow rate 10 mL/h. [b] Fractions obtained in the purification by silica gel (impregnated with boric acid) chromatography of the ST of the second acidolysis [c] Peaks 1 and 2 obtained in the separation of fraction 3 by reverse phase HPLC (Figure 5a).

4). Before this second reaction, the long-chain free fatty acids displaced from the original oil in the first reaction and present in the reaction mixture along with the target ST were removed. After that, fresh CA was again added. This second step was performed at a processing intensity of 9260 g lipase·h/mol TAG (i.e., 10 g of lipase, reaction mixture flow rate equal to 10 mL/h and $[TG]_0 = 0.108$ M). In these conditions a new ST with 64.2% CA (near the theoretical maximum value of 66.7%), 15.1% EPA, and 3.6% DHA was obtained (Table 4). Note that despite starting from a TAG with a 54.9% CA, the incorporation of CA in this second step did not exceed the theoretical value (66.7%). This confirm again the 1,3-specificity of Lipozyme IM.

The different TAG (and esters in general) present in the ST produced in this second step were fractionated in a stepwise silica gel chromatographic column, which had been previously impregnated with boric acid to prevent that the silica gel could catalyze any acyl migration inside the ST (*22*). Table 4 shows that the fraction 3, with a 66.9% CA and 19.6% EPA, correspond to the target product. Fractions 1 and 2 have CA contents between 33.3% and 66.6% and must contain significant percentages of ST with two molecules of CA.

Figure 6 shows the HPLC chromatogram obtained when separating the TAG present within the fraction 3, and Table 4 shows the composition of the peaks 1 and 2. This chromatogram shows fewer peaks than its equivalent for the major fraction obtained in a single step (Figure 5a). Peak 1 is the principal one with a fatty acid composition similar to that of fraction 3 (Table 4). The fraction corresponding to peak 1 has also been fractionated by Ag$^+$ HPLC, and the obtained chromatogram is identical to the one shown in Figure 5b, which corresponds again to the target MLM structure. This experiment demonstrated that TAG with the MLM desired structure can be produced with a high purity (64.2% CA, Table 4) by using a two-step process with a processing intensity of 9300 g lipase·h/mol TAG.
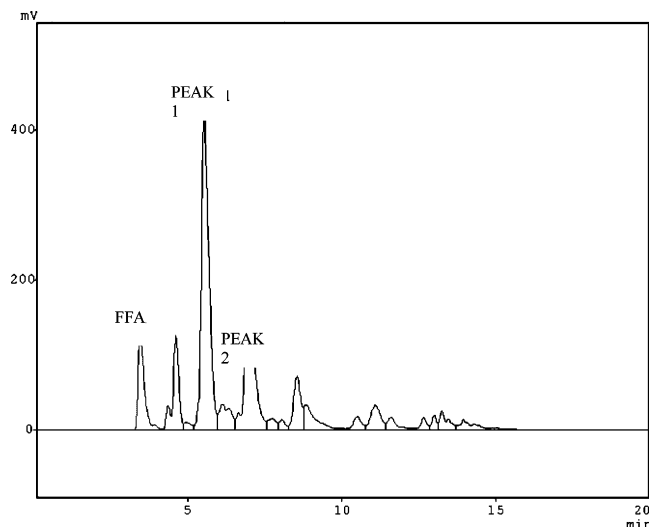
Figure 6. Separation by reverse phase HPLC of the different TAG produced by acidolysis of EPAX4510 and CA in a two steps process. The fatty acid composition of TAG is shown in Table 4.

Conclusions

The acidolysis reaction rate of heterogeneous commercial fish oil with CA, catalyzed by the lipase Lipozyme IM, has been adjusted to an empirical kinetic model that provided representative values of the apparent kinetic reaction constants for each fatty acid (native or odd). This model has fitted all experimental data and allows the simulation of the fatty acid composition of ST as a function of time (discontinuous operation with product recirculation) and at different mixture reaction flow rates through the bed (continuous operation way). Strictly speaking, this model is only applicable in the range of experimental conditions in which it has been obtained (see the captions of Figures 2−4). However, this model permits the scale-up of the ST production. Scaling-up should be done by keeping constant the processing intensity for the discontinuous operation and continuous operation (mass of enzyme × time/mol TAG, $m_L t/([TG]_0 V)$ and (mass of enzyme/(reaction mixture flow rate × TAG concentration)), $m_L/(q[TG]_0)$, respectively.

By operating in discontinuous mode, ST with 59.5% CA and 9.6% EPA was the final product; however the continuous operation mode yielded ST with 51% CA, mainly in the extreme 1 and 3 positions, and 20% EPA in the 2 position of the glycerol backbone. Therefore the continuous operation of the PBR is the selected option to produce ST with a high content of EPA in the position 2. In addition, to enhance the CA incorporation when operating in continuous mode, either an increase of the processing intensity or a two-step acidolysis can be considered.

Acknowledgment

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