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Kinetics of lipase-catalysed interesterification of triolein and caprylic acid to produce structured lipids

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Abstract: The influence of the molar ratio caprylic acid/triolein, enzyme concentration and water content on the kinetics of the interesterification reaction of triolein (TO) and caprylic acid (CA) were studied. The enzyme used was the 1,3-specific *Rhizomucor miehei* lipase. Data modelling was based on a simple scheme in which the acid was only incorporated in positions 1 and 3 of the glyceride backbone. In addition, it was assumed that positions 1 and 3 of the triglycerides were equivalent and that the events at position 1 did not depend on the nature of the fatty acid in position 3 and vice versa. Monoglycerides and diglycerides were not detected during the experiments. This was attributed to the low water content of the immobilised enzyme particles. The value of the equilibrium constant, *K*, for the exchange of caprylic and oleic acids was 2.7, which indicated that the incorporation of caprylic acid into triglycerides was favoured compared with the incorporation of oleic acid. Simple first order kinetics could describe the interesterification reaction. Using this model and the calculated equilibrium constant, the apparent kinetic constants were calculated. The model fitted all the experimental data except for the CA/TO molar ratios larger than 6. Moreover, the interesterification reaction rate had a maximum value at CA/TO molar ratios of 4–6 mol mol^{-1}.

Keywords: equilibrium interesterification; kinetics of lipase-catalysed reaction; triolein; caprylic acid; structured lipids; interesterification

NOTATION

$area_X$	Oleic or caprylic acid chromatographic areas in GC analysis
$area_{IS}$	Internal standard chromatographic area in GC analysis
A	Free fatty acid liberated from a native triglyceride (oleic acid)
CA	Caprylic acid
CA/TO	Caprylic acid/triolein ratio (mol mol^{-1})
e_T	Enzyme concentration (g g^{-1} of catalyst particle)
E	Enzyme or lipase
EA, EM	Acyl–enzyme complexes of the native fatty acid (A) and the odd fatty acid (M)
F_{ATe}	Molar fraction of the native fatty acid (oleic acid) in triglycerides at equilibrium
F_{MT}	Molar fraction of the medium chain fatty acid (caprylic acid) incorporated in triglycerides
F_{MTe}	Molar fraction of the medium chain fatty acid (caprylic acid) incorporated in triglycerides at equilibrium
F_X	Response factor of oleic or caprylic acids in GC analysis
GA_2	Native triglyceride (triolein)
GAM	Triglyceride with one molecule of M incorporated
GC	Gas chromatography
GM_2	Structured triglyceride (two molecules of M incorporated)
k	Kinetic constant for reactions (2) and (3), defined by eqns (19), (20) and (21) (m^6/mol g h)
k_{dl}, k_{il}	Kinetic constants for the direct and the inverse reaction (15), defined by eqn (16)
k_{d2}, k_{i2}	Kinetic constants for the direct and the inverse reaction (17), defined by eqn (18)
K	Equilibrium constant for reactions (2) and (3), defined by eqn (4), $K = K_1 = K_2$
K_E	Average equilibrium constant for acyl–enzyme formation, defined by eqn (13)
K'_Y	Equilibrium constant for reactions (10) and (11), defined by eqn (12)

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K_1	Equilibrium constant for reaction (2), defined by eqn (4)
K_2	Equilibrium constant for reaction (3), defined by eqn (4)
L	Long chain fatty acid
LCT	Long chain triglycerides
m_E	Amount of enzyme (mg)
m_0	Initial caprylic acid/triolein molar ratio
M	Medium chain free fatty acid
$[M]_0$	Initial caprylic acid concentration
MCT	Medium chain triglycerides
MLM	Structured triglycerides with medium chain fatty acids in the 1 and 3 positions and a long chain fatty acid in the 2 position of the glycerol backbone
r_0	Initial reaction rate, defined by eqn (31) (mol $g^{-1} h^{-1}$)
r_1	Reaction rate for reaction (2), defined by eqn (16)
r_2	Reaction rate for reaction (3), defined by eqn (18)
t	Time (h)
ST	Structured triglycerides
TG	Triglyceride
$[TG]_0$	Initial concentration of triglycerides
TLC	Thin-layer chromatography
TO	Triolein
V	Organic phase volume
w_0	Water/triolein molar ratio
W	Water
[–]	Concentration (M)
ε_1	Extent of reaction (2) with respect to $[TG]_0$, defined by eqn (24)
ε_2	Extent of reaction (3) with respect to $[TG]_0$, defined by eqn (25)

1 INTRODUCTION

Medium-chain triglycerides (MCT) are used to provide a dense and readily absorbed energy source in patients with pancreatic insufficiency and other malabsorption problems.[1,2] MCTs may constitute 20–50% of the total fat in some liquid nutritional formulas intended for hospital patients requiring enteral feeding.[3] However, MCTs alone do not contain essential fatty acids and need to be supplemented with long-chain triglycerides (LCT) containing essential fatty acids (linoleic, linolenic or arachidonic acid) to provide balanced nutrition in enteral and parenteral nutrition products.[4] However, problems persist when lipid malabsorption problems exist. Structured lipids (SL) are triglycerides containing suitable fatty acids within each molecule. SLs are absorbed more readily than MCT and LCT mixtures,[5] therefore SLs are potentially useful for patients with lipid malabsorption problems. SL of the MLM type are triglycerides which contain medium-chain fatty acids (M) in positions 1 and 3 and an essential long-chain fatty acid (L) in position 2 of the glycerol backbone. This fatty acid distribution facilitates the absorption of SL, because pancreatic lipase is 1,3 specific and mainly hydrolyses the 1 and 3 positions of triglycerides.[6] In addition, this hydrolysis is accelerated when the fatty acids in positions 1 and 3 are medium-chain fatty acids, because of the higher affinity of the pancreatic lipase for these fatty acids.[7]

The simplest and most direct route for the synthesis of SL of the MLM type is by the interesterification between LCT and medium-chain free fatty acids, catalysed with a 1,3-specific lipase.[8–12] Most of the research about this reaction is directed towards studying the effect of variables such as the type of lipase and the lipase/substrate ratio, the reaction medium (solvent type or absence of solvent), substrate concentrations, amount of water, temperature and operational mode. Another important aspect to consider is the acyl-migration, which gives rise to undesired changes in the position of fatty acids within the glyceride molecules. Acyl-migration occurs in partial glycerides (monoglycerides and diglycerides), that are the necessary and unavoidable intermediates in lipase-catalysed interesterification. Many factors can influence acyl-migration, including the presence of an acyl donor (such as strong acid), water activity, non-polar solvents, enzyme supports with surface charges, high temperature and long reaction times.[13]

A critical step in the design of reactors using immobilised lipase is the formulation of rate equations that describe the performance of the lipase and utilise these data to optimise the control of the reactors. A kinetic study based on the reaction mechanism, all the relevant components (fatty acids, glycerides, glycerol, water and intermediates complexes), acyl-migration, specificity of lipases, temperature, etc, would lead to rate equations with too many parameters for an easy numerical solution. For these reasons, much of the published work on the interesterification process merely points out the effects of the operational variables on the process, or seeks to optimise the variables by statistical design of experiments.[13,14] However, for a rational design of the reactor and the optimisation of its performance, a kinetic model of the process needs to be investigated. Use of a model system containing only a few different chemical species or approaches that lump the various chemical components into a few representative groups can be used to simplify the modelling problem.

The objective of this study was to investigate the equilibrium and kinetics of structured triglyceride synthesis by interesterification of a model reaction system consisting of caprylic acid and triolein. A simple mathematical model is proposed which accounts for the effects of variables such as the concentrations of substrates and/or products, the substrate molar ratio, the amount of lipase, the water content and the reaction time. The proposed model may be of value in the design of reactors and to simulate the influence of the above-mentioned variables on reactor performance.

2 MATERIALS AND METHODS

2.1 Chemicals and materials

The 1,3-regiospecific *Mucor miehei* lipase sold as Lipozyme® IM was donated by Novo Nordisk A/S (Bagsvaerd, Denmark). The lipase was supplied immobilised on a macroporous anion exchange resin and had a 1,3 positional specificity. Lipozyme IM contained 2–3% water, determined by Karl Fischer titration (compact titrator microKF 2026, Crimson, Alella, Spain). Analytical-grade triolein, caprylic acid and hexane were obtained from Sigma Aldrich (St Louis, MO). Molecular sieves (4 Å) were also obtained from Sigma Aldrich.

2.2 Interesterification reaction

A typical reaction mixture for structured triglycerides synthesis consisted of triolein (100 mg, 0.113 mmol), caprylic acid (65.2 mg, 0.453 mmol), hexane (3 cm^3), water (1 mm^3) and lipase (25.2 mg). In all the experiments the amounts of triolein and hexane were kept constant. This reaction mixture was placed in 50-cm^3 Erlenmeyer flasks with silicone-capped stoppers. The mixture was incubated at 50 °C and agitated in an orbital shaker at 200 rpm. In a previous paper it was shown that an increase in the agitation rate did not affect the experimental results.[15] The reaction was stopped at different times by removal of immobilised lipase by filtration. The filtrate containing the reaction products was stored at −20 °C until analysis. All the reactions were carried out in triplicate and the standard deviations were always less than 8%.

2.3 Identification of reaction products and estimation of the molar fraction of caprylic acid incorporated into triglycerides

Hexane from the reaction mixture was removed by vacuum evaporation. Then 4.4 cm^3 of 0.5 mol dm^{-3} KOH (in 20% v/v ethanol solution) was added to 165.2 mg of the reaction mixture and the glycerides were extracted three times with hexane (3 cm^3). These glycerides (monoglycerides, diglycerides and triglycerides) were identified by thin-layer chromatography (TLC) followed by quantitative gas chromatography (GC). TLC analysis has been described elsewhere.[15] Fractions corresponding to each glyceride type were scraped from the plates and methylated by direct transesterification with acetyl chloride/methanol (1:20 v/v) by following the method of Lepage and Roy.[16] These methyl esters were analysed with a Hewlett Packard 4890 gas chromatograph (Avondale, PA) fitted with a capillary column of fused silica (Omegawax, 0.25 mm × 30 m, 0.20 μm standard film, Supelco, Bellefonte, PA) and a flame-ionisation detector. Nitrogen was the carrier gas and the total column flow was 36 cm^3 min^{-1}. The oven temperature program was as follows: 150 °C for 3 min, from 150 °C to 185 °C at 10 °C min^{-1}, 185 °C for 10 min, from 185 °C to 240 °C at 10 °C min^{-1}, and finally 240 °C for 12 min. The signal was analysed and integrated by an on-line computer. The amount of oleic and/or caprylic acid was calculated by eqn (1):

$$\text{Fatty acid (mg)} = 0.125\frac{F_X area_X}{area_{IS}} \qquad (1)$$

where 0.125 mg was the amount of the internal standard (19:0), F_X was the response factor of oleic or caprylic acid (1.13 and 1.49, respectively), $area_X$ was the oleic or caprylic acid chromatographic area and $area_{IS}$ was the internal standard chromatographic area.

2.4 Identification of structured triglycerides

The glycerides extracted from the product mixture were analysed with a Shimadzu (Kyoto, Japan) high performance liquid chromatograph (HPLC) equipped with an evaporative light scattering detector (ELSD) (Eurosep Instrument, Cergy-Pontoise, France). Triglyceride molecular species were separated by reverse-phase HPLC on a Beckman (San Ramon, CA) Ultrasphere C_{18} column (5 μm particle size, 80 Å pore size (4.6 mm × 25 cm)). Separations were obtained using acetone–acetonitrile as the eluent and following the method of Akoh and Huang.[11]

3 MODELLING: EQUILIBRIUM AND KINETIC EQUATIONS

3.1 Equilibrium

For interpreting the data obtained at equilibrium, we used the following simplified reaction scheme:

$$GA_2 + M \Leftrightarrow GAM + A \qquad (2)$$

$$GAM + M \Leftrightarrow GM_2 + A \qquad (3)$$

where GA_2 is the native triglyceride (triolein) and only the fatty acids in positions 1 and 3 are indicated. In eqns (2) and (3) M is the odd fatty acid (caprylic acid), A is the fatty acid liberated from the original triglyceride (oleic acid), GAM is a triglyceride with one molecule of M incorporated, and GM_2 is the structured triglyceride (two molecules of M incorporated). This simple model assumes that no reaction occurs at position 2 of triglycerides. From this scheme we can write the following equilibrium expressions:

$$K_1 = \frac{[GAM][A]}{[GA_2][M]} \qquad K_2 = \frac{[GM_2][A]}{[GAM][M]} \qquad (4)$$

The molar fraction of the fatty acid M incorporated into the triglycerides at the equilibrium (F_{MTe}) and the molar fraction of the native fatty acid which remains in the original triglyceride at the equilibrium (F_{ATe}) are given as follows:

$$F_{MTe} = \frac{[GAM] + 2[GM_2]}{3[TG]_0}$$

$$F_{ATe} = \frac{3[GA_2] + 2[GAM] + [GM_2]}{3[TG]_0} \qquad (5)$$

where $[TG]_0$ is the initial concentration of triglycerides. $[TG]_0$ is equal to the total concentration of triglycerides, as it was experimentally observed that the concentrations of mono- and diglycerides were zero in almost all the experiments carried out in this work. Also,

$$F_{ATe} = 1 - F_{MTe} \tag{6}$$

and, therefore, the concentrations of the free fatty acids of both M and A, at equilibrium, are given by:

$$[M] = [M]_0 - 3[TG]_0 F_{MTe} = m_0[TG]_0 - 3[TG]_0 F_{MTe} \tag{7}$$

$$[A] = 3[TG]_0(1 - F_{ATe}) \tag{8}$$

where $[M]_0$ is the initial concentration of caprylic acid and m_0 is the initial caprylic acid/triolein molar ratio (equal to $[M]_0/[TG]_0$). Combining eqns (4)–(8) results in the following equation for calculating the average equilibrium constant from the F_{MTe} experimental results:

$$(3F_{MTe} - 2)\left(\frac{m_0}{3F_{MTe}} - 1\right)^2 K^2 + (3F_{MTe} - 1) \times \left(\frac{m_0}{3F_{MTe}} - 1\right)K + 3F_{MTe} = 0 \tag{9}$$

in which the two equilibrium constants K_1 and K_2 were considered equal by assuming that the positions 1 and 3 are equivalent and that the events at position 1 do not affect the nature of the fatty acid at position 3 (and vice versa). This assumption has been done to simplify the development and application of the proposed model, however there is experimental evidence that the 1 and 3 positions are significantly different at low conversions for the *Rhizomucor miehei* lipase.[17–19] However, this circumstance has no influence on the obtained results because the 1(3), 2-diglycerides are considered as an unique component.

3.2 Kinetics

We initially investigated whether the internal and external mass-transfer processes had a significant influence on the overall conversion rate. This influence can be disregarded because the observable Thiele modulus for the external mass-transfer processes ranged between 0.004 and 0.013 for the large and small catalyst particles, respectively.[20] This Thiele modulus represents the relative reactant concentration drop between the organic solution and the catalyst particle surface, which is provoked by the external mass-transfer.[21] Similarly, the influence of the internal mass-transfer can be disregarded because the Thiele modulus for the internal mass-transfer through the porous particle ranged between 0.03 and 0.2.[20] This modulus quantifies the influence of the diffusion into the catalyst pores on the overall process rate and this influence can be considered negligible for values lower than 0.3.[21] Because of the negligible mass-transfer effects, the two independent reactions, (2) and (3), are expected to occur on both the external and internal surfaces of the biocatalyst particles at concentrations of both reactants and products equal to the concentrations in the organic phase, which are the concentrations determined by analysis. Assuming that only the intermediates of appreciable life in which the enzyme participates are the acyl–enzyme, EA and EM, and that these complexes are in equilibrium with the free fatty acids, we can write

$$E + A \Leftrightarrow EA + W \tag{10}$$

$$E + M \Leftrightarrow EM + W \tag{11}$$

$$K'_Y = \frac{[EA][W]}{[E][A]} = \frac{[EM][W]}{[E][M]} \tag{12}$$

Equation (12) further assumes that for both oleic acid (A) and caprylic acid (M) the rate of acyl–enzyme formation does not depend on the type of the fatty acid. The water concentration [W] can be considered constant, because the water is produced in the hydrolysis step and consumed in the esterification step (the two interesterification steps) and that at any time the triglyceride concentration and the free fatty acid concentration remain practically constant. Considering this, eqn (12) can be written as:

$$K_E = \frac{[EM]}{[E][M]} \tag{13}$$

An enzyme mass balance leads to

$$[E] = \frac{e_T}{1 + K_E([A] + [M])}$$

$$[EA] = \frac{K_E e_T[A]}{1 + K_E([A] + [M])} \tag{14}$$

$$[EM] = \frac{K_E e_T[M]}{1 + K_E([A] + [M])}$$

where e_T is the total concentration of the enzyme per gram of the biocatalyst particles. The exchange of the fatty acid must occur through the formation of the corresponding diglyceride; however, since the diglyceride concentration is negligible, the first interchange step can be expressed as follows:

$$EM + GA_2 \underset{k_{i1}}{\overset{k_{d1}}{\Leftrightarrow}} EA + GAM \tag{15}$$

The rate expression for the above scheme is:

$$r_1 = k_{d1}[GA_2][EM] - k_{i1}[GAM][EA] = \frac{k_{d1}K_E e_T\left([GA_2][M] - \frac{k_{i1}}{k_{d1}}[GAM][A]\right)}{1 + K_E([A] + [M])} \tag{16}$$

Similarly, the second step of the process can be

expressed as

$$EM + GAM \underset{k_{i1}}{\overset{k_{d1}}{\Longleftrightarrow}} EA + GM_2 \tag{17}$$

and the corresponding rate expression is:

$$r_2 = k_{d2}[GAM][EM] - k_{i2}[GM_2][EA] = \frac{k_{d2}K_E e_T\left([GAM][M] - \frac{k_{i2}}{k_{d2}}[GM_2][A]\right)}{1 + K_E([A] + [M])} \tag{18}$$

r_1 and r_2 are the rate of disappearance of the native triglyceride (GA_2) and the rate of production of the structured triglyceride (GM_2), respectively, expressed in mol/(h g of biocatalyst). Because in the interesterification reaction the free fatty acid concentration (ie $[A] + [M]$) remains constant and equal to the initial concentration of free fatty acid (ie $[M]_0 = m_0[TG]_0$), eqns (16) and (18) can be written as:

$$r_1 = k\left([GA_2][M] - \frac{1}{K}[GAM][A]\right) \tag{19}$$

$$r_2 = k\left([GAM][M] - \frac{1}{K}[GM_2][A]\right) \tag{20}$$

where

$$k = \frac{k_d K_E e_T}{1 + K_E m_0 [TG]_0} \tag{21}$$

and

$$K = \frac{k_{d1}}{k_{i1}} = \frac{k_{d2}}{k_{i2}} \tag{22}$$

The above relationships assume that the exchange in position 1 does not depend on the nature of fatty acid in position 3 (or vice versa) (ie $k_{d1} = k_{d2} = k_d$ and $k_{i1} = k_{i2} = k_i$). In eqns (19) and (20) the terms within parentheses represent the distance from the equilibrium (ie the driving force) and the kinetic constants k contain the total concentration of active enzyme per gram of catalysts particles and will also depend on $[TG]_0$ and m_0. In these equations, if the concentrations are expressed in mol m^{-3} and the reaction rates in mol g^{-1} h^{-1}, the kinetic constant k will have the dimensions of m^6/(mol g h). This model has some features in common with the model proposed by Lortie *et al*[22] for the synthesis of triolein from glycerol and oleic acid. Thus, both models propose kinetic equations of first order with respect to each reactant. The models disregard the enzymatic reaction at position 2 and consider the positions 1 and 3 as identical. The relationships between r_1 and $d[GA_2]/dt$ and r_2 and $d[GM_2]/dt$ are:

$$r_1 = \frac{V}{m_E}\left(\frac{-d[GA_2]}{dt}\right) \qquad r_2 = \frac{V}{m_E}\left(\frac{d[GM_2]}{dt}\right) \tag{23}$$

where m_E is the amount of enzyme and V is the volume of the organic phase. The latter is practically constant and equal to the initial volume of solvent. If ε_1 and ε_2 are defined as the extent of the reactions (2) and (3) with respect to the initial concentration of triglycerides, respectively, ie:

$$\varepsilon_1 = \frac{[TG]_0 - [GA_2]}{[TG]_0} \qquad [GA_2] = [TG]_0(1 - \varepsilon_1) \tag{24}$$

$$\varepsilon_2 = \frac{[GM_2]}{[TG]_0} \qquad [GM_2] = [TG]_0\varepsilon_2 \tag{25}$$

and taking into account that

$$\begin{aligned} [TG]_0 &= [GA_2] + [GAM] + [GM_2] \\ [M] &= [M]_0 - [GAM] - 2[GM_2] \\ [A] &= [M]_0 - [M] \end{aligned} \tag{26}$$

we can deduce that:

$$\frac{d\varepsilon_1}{dt} = k\left(\frac{m_E}{V}\right)[TG]_0 \times \left((1 - \varepsilon_1)(m_0 - \varepsilon_1 - \varepsilon_2) - \frac{1}{K}(\varepsilon_1 - \varepsilon_2)(\varepsilon_1 + \varepsilon_2)\right) \tag{27}$$

$$\frac{d\varepsilon_2}{dt} = k\left(\frac{m_E}{V}\right)[TG]_0 \times \left((\varepsilon_1 - \varepsilon_2)(m_0 - \varepsilon_1 - \varepsilon_2) - \frac{1}{K}(\varepsilon_2)(\varepsilon_1 + \varepsilon_2)\right) \tag{28}$$

These equations, with the initial conditions:

$$t = 0; \quad \varepsilon_1 = 0; \quad \varepsilon_2 = 0 \tag{29}$$

are the mathematical model that represents the progress of the reaction. The model assumes perfect mixing and this assumption is satisfactory for the reaction system used. This work experimentally determined that the molar fraction of the caprylic acid incorporated into the triglycerides (F_{MT}) was related to ε_1 and ε_2 by the following equation:

$$F_{MT} = \frac{[GAM] + 2[GM_2]}{3[TG]_0} = \frac{\varepsilon_1 + \varepsilon_2}{3} \tag{30}$$

The numerical integration of the model equations was carried out with the values of the kinetic parameters that best fitted the experimental results. MatLab 5.1 was used for the numerical integration.

4 RESULTS AND DISCUSSION

4.1 Identification of the molecular species

Figure 1 shows the HPLC chromatogram of a final reaction mixture of a typical interesterification reaction after removing nearly all the free fatty acids. In this figure the HPLC retention times, according to the total carbon number and polarity for oleic acid, dicaprylolein (MLM), monocaprylolein and triolein,

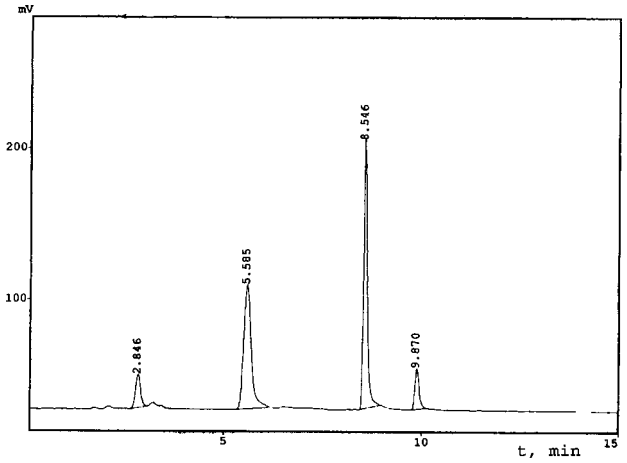

Figure 1. HPLC chromatogram of structured triglycerides. The retention times correspond to oleic acid (2.85), dicaprylolein (5.59), monocaprylolein (8.55), triolein (9.87). Reaction conditions: 100 mg triolein; 65.2 mg caprylic acid (m_0=4.0); 3 cm^3 hexane; m_E=16.8 mg; t=6 h; w_0=0.371.

were 2.8, 5.6, 8.5 and 9.9 min, respectively. These retention times have been determined by using standards, and are very similar to those reported by Akoh and Huang.[11] This chromatogram confirms that only the molecular species shown in eqns (2) and (3) take part in the interesterification reaction. Further analysis by TLC confirmed that the formation of monoglycerides and diglycerides was negligible, which is logical because nearly all the experiments were carried out without adding water, except for the water contained in the immobilised lipase. However, our results differ from those reported by Goderis *et al*[23] In their study of the interesterification of triolein and palmitic acid, under controlled water activity, these authors found appreciable concentrations of diglycerides at equilibrium. This difference is due to the fact that these authors used triglyceride and carboxylic acid concentrations appreciably lower (≈7-fold lower) than those used in this work and, therefore, the percentage of produced diglycerides, with respect to total glycerides, was higher than the percentage of diglycerides obtained in this work.

4.2 Effect of initial caprylic acid/triolein molar ratio, m_0

Figure 2 shows that the rate of interesterification attains a peak with increasing m_0 values and then declines when m_0 increases further. Therefore, it seems that an excess of caprylic acid above a molar ratio of approximately 6, diminishes the reaction rate. This observation concurs with the data of Kuo and Parkin,[24] and is explained by the acidification of the enzyme layer caused by an excess of the free fatty acids.[24] However, since the hydrolysis of triglycerides to diglycerides should precede the exchange of fatty acid, this decrease may also be provoked by the decrease of intermediate diglycerides that give rise to an increase in the free fatty acid concentration. This result is consistent with the results of Akoh and Huang,[11] who, working with the same system, found that the maximum proportion of dicapryl, oleilglycerol (C37 in their nomenclature) was obtained with a molar ratio range of 4–6. However, Akoh and co-workers found that when using capric acid ($C_{10:0}$) instead of caprylic acid ($C_{8:0}$), the capric acid that incorporated into triglycerides was similar for CA/TO molar ratios of both 6 and 8.[25] This could be due to the roughly 16% difference in the pK_a of those fatty acids (4.85 and 4.93 for the caprylic acid and capric acid, respectively).[26] Similarly, Mu *et al*[27] reported that there was an increase in the incorporation of caprylic acid into sunflower oil when the molar ratio increased to 8 (Fig 7 of their work); however, these authors operated with the lipase packed into a fixed bed reactor using very high lipase/caprylic acid ratios which may have decreased substrate inhibition.

Another possible alternative explanation of a decrease in the reaction rate at high caprylic acid content could be related to the water activity. The solubility of water in the fatty acids increases as the chain length of the fatty acid decreases. Therefore, as the caprylic acid content increases, the overall water activity would be expected to decrease, this in turn may reduce the reaction rate. In effect, the catalytic activity of Lipozyme IM seems to be maximal for a water activity of 0.50,[28] and, therefore, an increase of the concentration of components that decreases the water activity can also decrease the reaction rate.

Considering that the results obtained for the longest reaction times for m_0 of 2 and 4 (F_{MT} (32 h) = 0.427 and F_{MT} (18 h) = 0.562, respectively) are close to the equilibrium values, the equilibrium constants can be calculated by eqn (9). These equilibrium constants are K = 2.75 and 2.66 for the CA/TO values 2 and 4, respectively. These values are acceptably close and indicate a value of K of the order of 3 for the exchange of oleic acid and caprylic acid at 50 °C, the K values

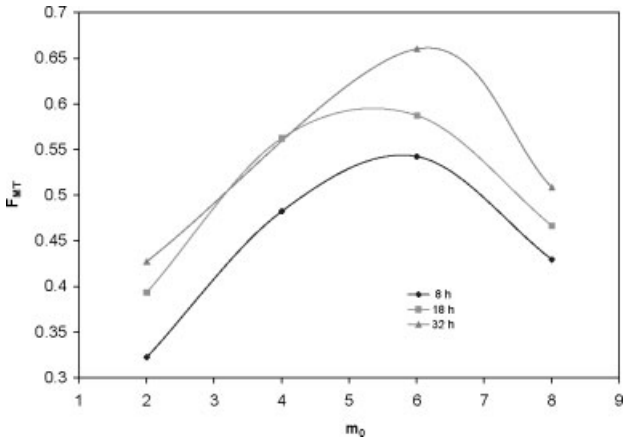

Figure 2. Effect of caprylic acid/triolein molar ratio, m_0, and time on the molar fraction of caprylic acid incorporated into the triglycerides, F_{MT}. m_E=25.2 mg; w_0=0.371.

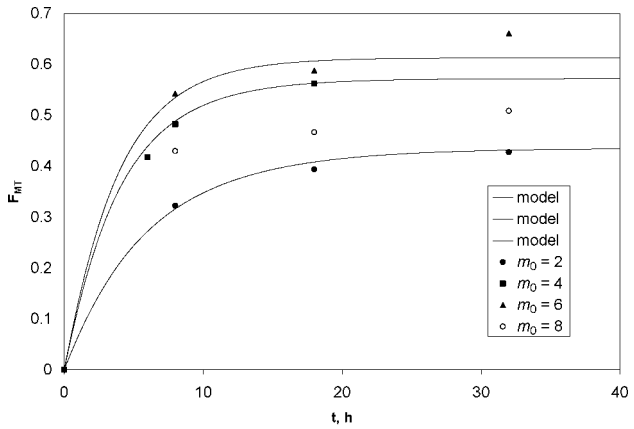

Figure 3. Effect of time and caprylic acid/triolein molar ratio, m_0, on the molar fraction of caprylic acid incorporated in triglycerides, F_{MT}. Fit of the experimental results to the kinetic model (eqns (27–29). m_E=25.2 mg, w_0=0.371.

suggest that the incorporation of caprylic acid is favoured relative to oleic acid. The same can be stated for a molar ratio of 6, but the value obtained of F_{MT} = 0.66 indicates a total displacement of oleic acid and, therefore, the experimental errors in this zone have a strong influence in the value of K obtained (eg if the F_{MT} value were 0.61, which is within the maximum standard deviation of 8%, the K value obtained by eqn (9) would be also 2.7). In contrast, for the molar ratio of 8 the F_{MT} values are still far from the equilibrium composition and eqn (9) cannot be applied to these experiments.

Using eqns (27–29) with the previously calculated equilibrium constant (K = 2.7), the apparent kinetic constants that minimised the deviation between the experimental and calculated results were determined. Figure 3 shows that this model fits the experimental data corresponding to the molar ratios 2, 4 and 6 quite well. However, these equations did not fit the results obtained with a molar ratio of 8.0, which again indicates that the presence of excess of free fatty acid alters the enzymatic activity.

Figure 3 also shows that the F_{MT} values never exceed 0.67, which supports the hypothesis that only positions 1 and 3 participate in the exchange (ie eqns (2) and (3) are valid) and also suggests a negligible influence of the acyl-migration. This last hypothesis agrees with other results obtained in our laboratory from the hydrolysis of triolein (data not shown), where it was observed that the acyl-migration took place through the monoglycerides. The experiments depicted in Fig 3 were carried out in the absence of added water and the formation of monoglycerides and diglycerides was negligible, as shown in Fig 1.

Table 1(A) shows the apparent second order kinetic constants, k, that provide a better fit to the experimental results shown in Fig 3 and the initial reaction rate, r_0, calculated by using eqn (31), as a function of m_0:

$$r_0 = k[\mathrm{TG}]_0[\mathrm{M}]_0 = k\ m_0[\mathrm{TG}]_0^2 \qquad (31)$$

The maximum initial reaction rate is seen to correspond to the molar ratio of 6, as was also concluded from Fig 2. In this figure it can also be observed that the experimental results obtained at a caprylic acid/triolein molar ratio of 8 clearly are not adjusted by the model, because these results are between the interval of the results corresponding to the molar ratios of 2 and 4. The k values corresponding to the molar ratios of 2, 4 and 6 were an acceptable fit for eqn (21):

$$1/k = 2.25\ 10^6 + 8130\ m_0[\mathrm{TG}]_0 \qquad r^2 = 0.959$$

$$k = (4.4\ 10^{-7})/(1 + 0.0036\ m_0[\mathrm{TG}]_0) \qquad (32)$$

However, eqn (32) did not fit the k value for the molar ratio of 8, which indicated that, apart from the greater acyl–enzyme formation, there was some other effect that decreased the reaction rate, as has been commented on previously.

4.3 Effect of lipase concentration

In general, for the enzymatic reactions, the reaction rate is directly proportional to the concentration of active enzyme. Therefore, if no denaturation of the enzyme occurs, the significant variable is the product of the enzyme concentration and time, ie $(m_E/V)t$. In effect, eqns (27–29) can be re-written in

Table 1. Effect of the caprylic acid/triolein molar ratio, m_0 (A), lipase amount, m_E (B) and water/triolein molar ratio, w_0 (C) on the apparent kinetic constant of second order (k) (eqns (27–29) and on the initial rate of interesterification (r_0) (eqn (31)) ($[TG]_0$=37.7 mol m^{-3})

(A) m_E=25.2 mg; w_0=0.371 mol mol^{-1}		
m_0 *(mol mol^{-1})*	k *(m^6/(mol g h))*	r_0 *(mol g^{-1} h^{-1})*
2.00	3.4 10^{-7}	9.7 10^{-4}
4.00	3.0 10^{-7}	17.1 10^{-4}
6.00	2.4 10^{-7}	20.5 10^{-4}
8.00	0.7 10^{-7}	8.0 10^{-4}
(B) m_0=4.00 mol mol^{-1}; w_0 range 0.062–0.496 mol mol^{-1}		
m_E *(mg)*	k *(m^6/(mol g h))*	r_0 *(mol g^{-1} h^{-1})*
4.2–33.6	2.7 10^{-7}	15.3 10^{-4}
(C) m_E=25.2 mg; m_0=4.00 mol mol^{-1}		
w_0 *(mol mol^{-1})*	k *(m^6/(mol g h))*	r_0 *(mol g^{-1} h^{-1})*
≈0	2.8 10^{-7}	15.9 10^{-4}
0.371	3.0 10^{-7}	17.1 10^{-4}
0.862	2.7 10^{-7}	15.3 10^{-4}
5.288	1.6 10^{-7}	9.1 10^{-4}
49.536	0.4 10^{-7}	2.3 10^{-4}

the form:

$$\frac{\mathrm{d}\,\varepsilon_1}{\mathrm{d}\left(\frac{m_E}{V}t\right)} = k[\mathrm{TG}]_0 \times \left((1-\varepsilon_1)(m_0-\varepsilon_1-\varepsilon_2) - \frac{1}{K}(\varepsilon_1-\varepsilon_2)(\varepsilon_1+\varepsilon_2)\right) \quad (33)$$

$$\frac{\mathrm{d}\,\varepsilon_2}{\mathrm{d}\left(\frac{m_E}{V}t\right)} = k[\mathrm{TG}]_0 \times \left((\varepsilon_1-\varepsilon_2)(m_0-\varepsilon_1-\varepsilon_2) - \frac{1}{K}(\varepsilon_2)(\varepsilon_1+\varepsilon_2)\right) \quad (34)$$

$$t = 0; \quad \varepsilon_1 = 0; \quad \varepsilon_2 = 0 \quad (35)$$

Thus, Fig 4 shows the incorporation of caprylic acid versus the product between the lipase concentration and the reaction time for the experiments carried out with an $m_0 = 4$, amounts of lipase between 4.2 and 33.6 mg, and reaction times between 1 and 24 h. The scatter of the data may be attributed, in part, to the different water content of the experiments represented. It should be noted that when the amount of lipase is increased the water content also increases, since the enzyme contains approximately 3% water (so when the amount of lipase was increased from 4.2 to 33.6 mg, the range of the water/TO molar ratio increased from 0.062 to 0.496). From Fig 4 it can be concluded that above a value of $(m_E/V)t = 75000$ (g h)/m^3, the interesterification rate diminishes appreciably and over 150000 (g h) /m^3 (ie 4.5 (g of lipase h/g of triolein)) the reaction seems to have reached the equilibrium. In Table 1(B) it can be observed that the value of the kinetic constant obtained by means of the adjustment ($k = 2.7\ 10^{-7}$ m^6/(mol g h)) practically coincides with the value previously obtained (Table 1(A)) for $m_0 = 4.0$ ($k = 3.0\ 10^{-7}$).

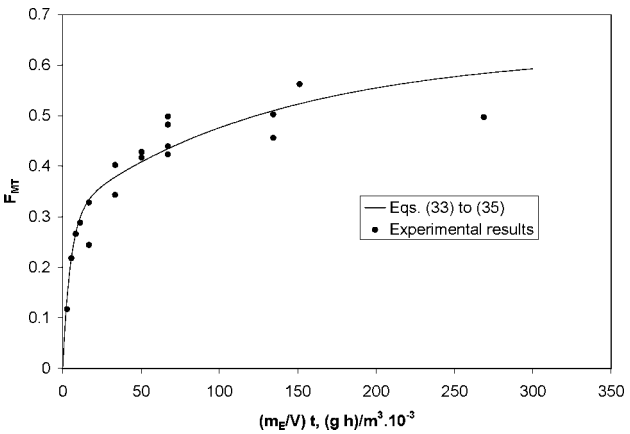

Figure 4. Effect of the product of the lipase concentration and time (m_E/V) (t) on the molar fraction of caprylic acid incorporated into the triglycerides, F_{MT}. Fitting of the experimental results to the kinetic model (eqns (33)–(35)). $m_0 = 4.00$ mol mol^{-1}; w_0 variation range 0.062–0.496 mol mol^{-1}.

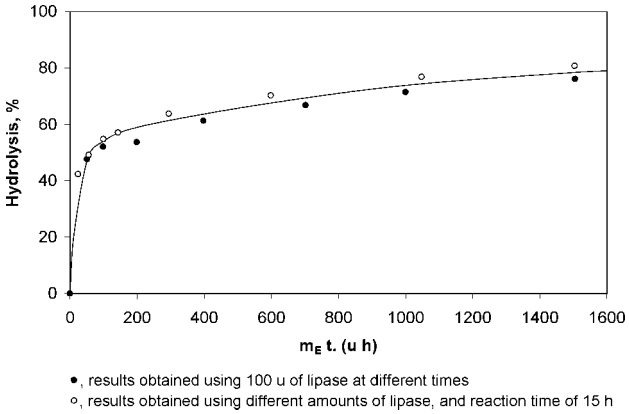

Figure 5. Effect of the product among the lipase amount (units of lipase activity, U) and reaction time, $m_E t$ (Uh), on the percentage of hydrolysis of borage oil. Data were recalculated from Figs 1 and 2 of Shimada *et al*.[29]

These results are consistent with the data obtained by Shimada *et al*,[29] who studied the hydrolysis of borage oil with the lipase of *Candida rugosa*. These authors found that the influence of both the amount of lipase and the reaction time were interchangeable. This is demonstrated in Fig 5, which was obtained from specific data derived from the influence of the reaction time and amount of lipase (Figs 1 and 2 of Shimada *et al*[29]). In Fig 5 all the experimental data fitted the same line, which effectively confirms that the $(m_E/V)t$ value is actually the significant variable defining the intensity of the enzymatic reaction. A similar conclusion was also obtained comparing the Figs 4 and 5 reported by Akoh and Huang,[11] although the values of the operational variables were not indicated with sufficient clarity to carry out quantitative calculations. Similarly, the same result can be observed more clearly in the work of Fomuso and Akoh[30] describing the interesterification of triolein and an equimolecular mixture of caproic and butyric acids.

4.4 Influence of the initial water/triolein molar ratio (w_0)

Lipases need a certain amount of water to maintain an optimal catalytic activity, but an excess of water contributes to the hydrolysis of triglycerides and facilitates the acyl-migration. The optimum amount of water in the reaction medium depends on the type of lipase and solvent used. There are several works describing the influence of the water and the solvent on lipase-catalysed reactions in organic media.[23,31,32] In general, the optimal content is found between zero and 10% (w/w water/lipase) and in many cases these values coincide with the amounts of water of that are retained in the support matrix during the immobilisation of the enzyme (usually 1–5%).

Figure 6 shows the variation of the molar fraction of caprylic acid incorporated into triglycerides with the initial water/triolein ratio and reaction time (in the experiment with added molecular sieves, the water

content was assumed to be practically zero, although it is obvious that the molecular sieves do not remove the water bound to the enzyme, but only the water in the organic phase). It is interesting to observe that for water/triolein molar ratios of ≤ 5.288 (ie up to 10 mg of added water) the equilibrium F_{MT} value ($F_{MTe} = 0.57$) was reached at approximately between 18 and 32 h and it was similar to the equilibrium value reached in the previous sections. However, for $w_0 = 49.536$ (100 mg of added water) F_{MT} was still 0.35 at 32 h of reaction. These results indicate that the equilibrium of the interesterification reaction is not influenced by the initial water content, as expected, and that for water contents between zero and 5.3 moles of water/mole of triolein, or between zero and 0.44 g of water/g of dry lipase, this equilibrium was attained between 18 and 32 h. However, as water contents were further increased, the reaction rate decreased appreciably.

The data presented in Fig 6 are also very similar to those obtained by Akoh and Huang.[11] These authors studied the influence of the water content on the interesterification between caprylic acid and triolein. The w_0 value varied between zero and 5.12, with reaction times up to 24 h. In these conditions, these authors attained equilibrium of the reaction at w_0 values and reaction times similar to those obtained in this work. Figure 6 also shows the application of the kinetic model represented by eqns. (27–29) to these set of results and Table 1(C) shows the values of the kinetic constants that provide better agreement with the experimental results. The initial reaction rates (Table 1(C)) were calculated by eqn (31). Both Fig 6 and Table 1(C) show that the reaction rate does not vary appreciably for w_0 values below 1 (or lower than 8% weight of water/weight of dry lipase); however, for higher values of the water content the reaction rate diminishes. This decrease is roughly linear with the logarithm of w_0, since from the k values that are shown in Table 1(C), it can be verified that k diminishes with the water content according to eqn (36):

$$k = 2.59\ 10^{-7} - 1.30\ 10^{-7} \log w_0; \quad r = 0.9994; \quad w_0 \leqq 0.862 \tag{36}$$

Table 1(C) shows that a maximum in the k and r_0 values occurs for $w_0 = 0.371$, equivalent to the water content of the lipase (3% by weight). Thus, it seems that the optimum water content of the lipase is around 3%, which indicates that water addition is not required as the residual water content is sufficient for the reaction. In this context, in a previous work of our group on the synthesis of triglycerides with the lipase Novozym 435, it was also verified that enzyme water contents of 2–3% (w/w) were sufficient for effective lipase-catalysed reaction.[33] The k values shown in Table 1 are consistent with data obtained by Lortie *et al*;[22] these authors proposed a similar model for the interesterification of oleic acid and triolein (their kinetic constants were in the range 0.26–1.8 $dm^3\ mol^{-1}\ h^{-1}$). By recalculating the k values of Table 1, taking into account the amount of enzyme and the reaction volume used, one can obtain k values in our experiment in the range 0.34–2.52 $dm^3\ mol^{-1}\ h^{-1}$.

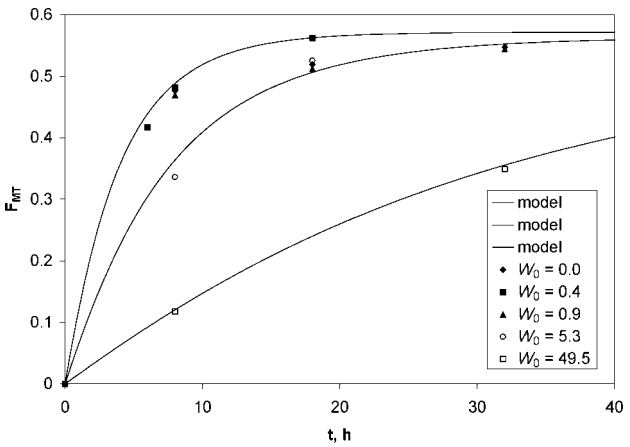

Figure 6. Effect of time and water/triolein molar ratio, w_0, on the molar fraction of caprylic acid incorporated into the triglycerides, F_{MT}. Fit of the experimental results to the kinetic model (eqns (27–29). $m_E = 25.2$ mg; $m_0 = 4.00$ mol mol^{-1}.

5 CONCLUSIONS

Figures 3, 4 and 6 prove that a simple kinetic model based on rate equations of first order respect to each reactant, can be used to describe quite complex lipase-catalysed reactions.[34] This suggests that there is a rate-limiting step that can be represented by relatively simple rate equations. The model developed here assumes that no reaction occurs at position 2 of triglycerides and that positions 1 and 3 are equivalent. However, strictly speaking, the positions 1 and 3 are not exactly equal, because of its pro-chiral nature. In this respect, the lipase Lipozyme IM has shown stereo-preference towards the 1 position over the 3 position.[19] However this stereo-specificity does not involve a major difference between the exchange reaction rates at the 1 and 3 positions. In this respect, Lortie *et al*[22] and Reyes and Hill[34] have reported an acceptable fit of their experimental results to models that do not consider stereo-specificity.

The simple rate equations proposed in this paper allow the derivation of useful information on the general behaviour of the system. The kinetic model proposed here takes into account: (1) the 1,3 specificity of lipase; (2) the absence of acyl-migration when partial glycerides are not formed, due to low water content; (3) the possibility of using the product of the enzyme concentration and the reaction time as the determining factor to define the intensity of treatment; and (4) the influence of water content only on the kinetics of the process and not on the equilibrium of interesterification.

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REFERENCES

1 Bach AC and Babayan VK, Medium-chain triglycerides: an update. *Am Clin Nutr* **36**:950–962 (1982).
2 Gottschlich MM, Selection of optimal lipid sources in enteral and parenteral nutrition. *Nutr Clin Pract* 7:152–165 (1992).
3 Merolli A, Medium-chain lipids: new sources, uses. *Inform* **8**:597–603 (1997).
4 Haumann BF, Structured lipids allow fat tailoring. *Inform* **8**:1004–1011 (1997).
5 Christensen MS, Hoy CE, Becker CC and Redgrave TG, Intestinal absorption and lymphatic transport of eicosapentaenoic (EPA), docosahexaenoic (DHA), and decanoic acids: dependence on intramolecular triacylglycerol structure. *Am J Nutr* **61**:56–61 (1995).
6 Mattson FH and Volpenhein RA, The digestion and absorption of triglycerides. *J Biol Chem* **239**:2772–2777 (1964).
7 Bottino NR, Vandenburg GA and Reiser R, Resistance of certain long-chain polyunsaturated fatty acids of marine oils to pancreatic lipase hydrolysis. *Lipids* **2**:489–493 (1967).
8 Shimada Y, Sugihara A, Nakano H, Yokota T, Nagao T, Komemushi S and Tominaga Y, Production of structured lipids containing essential fatty acids by immobilized *Rhizopus delemar* lipase. *J Am Oil Chem Soc* **73**:1415–1420 (1996).
9 Shimada Y, Sugihara A, Maruyama K, Nagao T, Nakayama S, Nakano H and Tominaga Y, Production of structured lipid containing docosahexaenoic and caprylic acids using immobilized *Rhizopus delemar* lipase. *J Ferment Bioeng* **81**:299–303 (1996).
10 Shimada Y, Suenaga M, Sugihara A, Nakai S and Tominaga Y, Continuous production of structured lipid containing γ-linolenic and caprylic acids by immobilized *Rhizopus delemar* lipase. *J Am Oil Chem Soc* **76**:189–193 (1999).
11 Akoh CC and Huang KH, Enzymatic synthesis of structured lipids: transesterification of triolein and caprylic acid. *J Food Lipids* **2**:219–230 (1995).
12 Xu X, Balchen S, Hoy CE and Adler-Nissen J, Production of specific-structured lipids by enzymatic interesterification in a pilot continuous enzyme bed reactor. *J Am Oil Chem Soc* **75**:1573–1579 (1998).
13 Xu X, Skands ARH, Hoy CE, Mu H, Balchen S and Adler-Nissen J, Production of specific-structured lipids by enzymatic interesterification: elucidation of acyl migration by response surface design. *J Am Oil Chem Soc* **75**:1179–1186 (1998).
14 Huang K-H and Akoh C, Optimization and scale-up of enzymatic synthesis of structured lipids using RSM. *J Food Sci* **61**:137–141 (1996).
15 Robles Medina A, Esteban Cerdán L, Giménez Giménez A, Camacho Páez B, Ibáñez González MJ and Molina Grima E, Lipase-catalyzed esterification of glycerol and polyunsaturated fatty acids from fish and microalgae oils. *J Biotechnol* **70**:379–391 (1999).
16 Lepage G and Roy C, Improved recovery of fatty acid through direct transesterification without prior extraction or purification. *J Lipid Res* **25**:1391–1396 (1984).
17 Chandler IC, Quinlan PT and McNeill GP, Lipase-catalyzed synthesis of chiral triglycerides. *J Am Oil Chem Soc* **75**:1513–1518 (1998).
18 Chandler IC, Howarth OW, and Crout OHG, Measuring stereoselectivity in lipase-catalyzed acidolysis reactions by ultra-high resolution ^{13}C nuclear magnetic resonance. *J Am Oil Chem Soc* **78**:953–958 (2001).
19 Iwasaki Y, Yasui M, Ishikawa T, Irimescu R, Hata K and Yamane Y, Optical resolution of asymmetric triacylglycerols by chiral-phase high-performance liquid chromatography. *J Chromatography A* **905**:111–118 (2001).
20 Camacho Páez B, *Obtención de lípidos estructurados por acidolisis con lipasas inmovilizadas*, Doctoral Thesis, University of Almería (2001).
21 Doran PM, *Bioprocess Engineering Principles*, Academic Press Limited, London. pp 316–322 (1995).
22 Lortie R, Trani M and Ergan F, Kinetic study of the lipase-catalyzed synthesis of triolein. *Biotechnol Bioeng* **41**:1021–1026 (1993).
23 Goderis HL, Ampe G, Feyten MP, Fouwé BL, Guffens WM, Van Cauwenbergh SM and Tobback PP, Lipase-catalyzed ester exchange reactions in organic media with controlled humidity. *Biotechnol Bioeng* **30**:258–266 (1987).
24 Kuo S-J and Parkin KL, Substrate preference for lipase-mediated acyl-exchange reactions with butteroil are concentration-dependent. *J Am Oil Chem Soc* **70**:393–399 (1993).
25 Jennings BH and Akoh CC, Enzymatic modification of triacylglycerols of high eicosapentaenoic and docosahexaenoic acids content to produce structured lipids. *J Am Oil Chem Soc* **76**:1133–1137 (1999).
26 Sorber HA (Ed), *Handbook of Biochemistry. Selected Data for Molecular Biology*, 2nd edn, The Chemical Rubber Co, 18901 Cranwood Parkway, Cleveland, Ohio 44128 (1968).
27 Mu H, Xu X and Hoy C-E, Production of specific-structured triacylglycerols by lipase-catalyzed interesterification in a laboratory-scale continuous reactor. *J Am Oil Chem Soc* **75**:1187–1193 (1998).
28 Valivety RH, Halling PJ, Peilow AD and Macrae R, Relations between water activity and catalytic activity of lipases in organic media. Effects of supports, loading and enzyme preparation. *Eur J Biochem* **222**:461–466 (1994).
29 Shimada Y, Fukushima N, Fujita H, Honda Y, Sugihara A and Tominaga Y, Selective hydrolysis of borage oil with *Candida rugosa* lipase: two factors affecting the reaction. *J Am Oil Chem Soc* **75**:1581–1586 (1998).
30 Fomuso LB and Akoh CC, Enzymatic modification of triolein: incorporation of caproic and butyric acids to produce reduced-calorie structured lipids. *J Am Oil Chem Soc* **74**:269–272 (1997).
31 Zaks A and Klivanov AM, Enzymatic catalysis in nonaqueous solvents. *J Biol Chem* **263**:3194–3201 (1988).
32 Laane C, Boeren S, Vos K and Veeger C, Rules for optimization of biocatalysis in organic solvents. *Biotechnol Bioeng* **30**:81–87 (1987).
33 Esteban Cerdán LE, Robles Medina A, Gimenez Gimenez A, Ibáñez González MJ and Molina Grima E, Synthesis of polyunsaturated fatty acid-enriched triglycerides by lipase-catalyzed esterification. *J Am Oil Chem Soc* **75**:1329–1337 (1998).
34 Reyes HR and Hill CG, Kinetic modeling of interesterification reactions catalyzed by immobilized lipase. *Biotechnol Bioeng* **43**:171–182 (1994).