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Mechanistic model for the lipase-catalyzed alcoholysis of triacylglycerols

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Abstract

The enzymatic alcoholysis of triolein and an oil highly rich in polyunsaturated fatty acid with ethanol to obtain 2-monoacylglycerols (2-MG) was studied. Two *sn*-1,3 specific lipases were used to catalyze this reaction: Lipozyme® IM from *Mucor miehei* and lipase D from *Rhizopus oryzae*. The experimental results were acceptably fitted to a mechanistic kinetic model that considers the formation of an acyl–enzyme complex and the isomerization of 2-monoacylglycerols (2-MG) by acyl migration to 1(3)-monoacylglycerols (1(3)-MG). The results of the alcoholysis reaction were both qualitatively and quantitatively dependent on the lipase used. When using Lipozyme IM the process was controlled by the acyl migration of the 2-MG to 1(3)-MG, which finally gave rise to glycerol. In contrast, when using lipase D, no acyl migration occurred and the process was controlled by the formation of 1(3),2-DG and 2-MG. The yields of 2-MG obtained with lipase D (almost 80%) were therefore greater than those obtained using Lipozyme IM in the same experimental conditions. The proposed kinetic model predicted the experimental results of the alcoholysis as a function of the processing intensity (lipase amount × reaction time/reaction volume, $m_E t/V$) irrespective of whether acyl migration took place. It also allowed the kinetic parameters of all the processes involved to be calculated.
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1. Introduction

A lipase-catalyzed alcoholysis is a reaction between an ester and an alcohol that results in the substitution of the ester-alkyl group by the alcoholic-alkyl group:

$$R_1COOR_2 + R_3{-}OH \overset{\text{lipase}}{\longleftrightarrow} R_1COOR_3 + R_2{-}OH$$

If the ester were a triacylglycerol (TG), and the lipase were specific for the extreme positions of TG (*sn*-1 and *sn*-3), the alcoholysis could be written as:

$$LLL + 2ROH \overset{\text{lipase}}{\longleftrightarrow} OH{-}L{-}OH + 2L{-}R$$

where LLL is a TG in which the three acyl groups are long-chain polyunsaturated fatty acids, OH–L–OH is the 2-MG and L–R the corresponding esters, which are the major reaction products.

Monoacylglycerols (MG), as well as diacylglycerols (DG), are used extensively as emulsifiers in the food and pharmaceutical industries because they are biodegradable, biocompatible and non-toxic compounds [1,2]. Monoacylglycerols are included in the Inactive Ingredient Guide for pharmaceutical uses by the UK-FDA and accepted in the majority of food legislations. Their European Union food additive code is E471.

Alcoholysis followed by esterification of the 2-MG produced, is also being used for the synthesis of valuable structured triacylglycerols (STs) for medical purposes with an MLM structure (i.e. medium-chain fatty acids (M) located at positions *sn*-1 and *sn*-3 of the glycerol backbone and a functional long-chain fatty acid (L) located at position *sn*-2) [3–5]. The advantages of this two-step process for producing STs with respect to the lipase-catalyzed acidolysis of LLL (a single step process) are the feasibility of obtaining purer STs, a

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Nomenclature

A	fatty acid; acyl group; dimensionless concentration of fatty acids
$\overline{AAA}$	glycerol backbone with three acyl groups (TG)
[AAA]	concentration of triacylglycerol (mol/l)
B	monoalcohol (ethanol); dimensionless concentration of monoalcohol
BA	monoalcohol ester of the fatty acids present in the precursor oils; dimensionless concentration of the ester
CA	caprylic acid
$\bar{D}$	dimensionless parameter defined in Eq. (24)
DG	diacylglycerols
1(3),2-DG	1(3),2-diacylglycerols
1,3-DG	1,3-diacylglycerols
DGa	dimensionless concentration of 1(3),2-DG
DGb	dimensionless concentration of 1,3-DG
$[DG]_a$	concentration of 1(3),2-DG
$[DG]_b$	concentration of 1,3-DG
DHA	docosohexaenoic acid
E	enzyme
[E]	concentration of enzyme
$[E]_T$	total concentration of active enzyme (free and the acyl–enzyme complex)
EA	acyl–enzyme complex
[EA]	concentration of acyl–enzyme complex
EPA	eicosapentaenoic acid
EPAX4510	commercial oil rich in EPA
G	glycerol; dimensionless concentration of glycerol
[G]	glycerol concentration
GC	gas chromatography
k_A	exchange kinetic constant of an acyl group (A) between an acylglycerol and the enzyme; A enters the acylglycerol (Eqs. (1)–(4) and (7)–(10))
k_{-A}	exchange kinetic constant of an acyl group (A) between an acylglycerol and the enzyme; A leaves the acylglycerol (Eqs. (1)–(4) and (7)–(10))
k_{BA}	exchange kinetic constant of A between the alcohol and the enzyme; A enters the ester (Eq. (11))
k_{-BA}	exchange kinetic constant of A between the alcohol and the enzyme; A leaves the ester (Eq. (11))
k_{CA}	exchange kinetic constant of an acyl group (A) from an acylglycerol to free enzyme; defined by Eq. (29)
k_{CB}	exchange kinetic constant of an acyl group (A) from the ester to the free enzyme; defined by Eq. (29)
k_{CI}	kinetic constant of the isomerization of MG; A moves from position 2 to positions 1 or 3; defined by Eq. (30)
k_i	kinetic constant of the isomerization of MG; A moves from position 2 to positions 1 or 3 (Eqs. (5) and (6))
k_{-i}	kinetic constant of the isomerization of MG; A moves from positions 1 or 3 to position 2 (Eqs. (5) and (6))
K_A	equilibrium constant for formation of the acyl–enzyme complex between a free fatty acid and the enzyme (Eq. (12))
K_{Be}	equilibrium constant for the esterification between the monoalcohol and the acyl–enzyme complex (Eqs. (31) and (11))
K_{Ge}	equilibrium constants for the reaction of exchange of an acyl group between an acylglycerol and the enzyme (Eq. (31))
K_{Ie}	equilibrium constant for the isomerization of the MG (Eq. (31))
L	long-chain fatty acid
LLL	TG of long-chain fatty acids
L–R	long-chain fatty acid ester
m_E/V	lipase amount/reaction volume ratio (including the mass of the enzyme support where the lipase is immobilised)
m_0	initial alcohol/TG molar ratio
M	medium-chain fatty acid
MG	monoacylglycerols
1(3)-MG	1(3)-monoacylglycerols
2-MG	2-monoacylglycerols
$[MG]_a$	concentration of 2-MG
$[MG]_b$	concentration of 1(3)-MG
MGa	dimensionless concentration of 2-MG
MGb	dimensionless concentration of 1(3)-MG
MLM	structured TG with medium-chain fatty acids (M) located at positions *sn*-1 and *sn*-3 of the glycerol backbone and a functional long-chain fatty acid (L) located at position *sn*-2
MTBE	methyl-*tert*-butyl ether
$\bar{N}$	dimensionless parameter defined in Eq. (23)
PBR	packed-bed reactor
r_G	glycerol formation rate (Eqs. (44), (45) and (52))
STs	structured triacylglycerols
$t(m_E/V)$	processing intensity; product of time and the enzyme amount/reaction volume ratio
TG	triacylglycerols, dimensionless concentration of triacylglycerols
[TG]	concentration of triacylglycerols
TLC	thin-layer chromatography
TO	triolein
W	water
W_0	initial water content
$\otimes$	position not occupied in the glycerol back bone

Greek symbol

λ	dimensionless reaction time (Eq. (28))

reduction of the reaction time and the possibility of reducing acyl migration [6].

Acyl migration within the 2-MG (i.e. the change of the acyl group at the *sn*-2 position to any of the two extreme positions, *sn*-1 or *sn*-3, of the glycerol backbone) gives rise to undesired changes in the position of the acyl groups, yielding a 1(3)-MG. This isomerization process has been extensively studied [7–11].

The two-step process developed by Soumanou et al. [4] has been developed in batch reactors, but acyl migration is still a major problem in the synthesis of STs in these reactors and this decreases the yield of the target ST. The high substrate/enzyme ratios used in a typical reaction mean that a long time is required to attain equilibrium, which inevitably results in acyl migration. In this sense, Mu et al. [12] and González Moreno et al. [13] have recently reported that an enzymatic packed-bed reactor may have advantages over the batch reactor with respect to reducing acyl migration.

On the other hand, unlike the wealth of information pertaining to the kinetics of lipase-catalyzed hydrolysis and esterification reactions in non-aqueous media, there is a shortage of reliable data on the kinetics of lipase-catalyzed alcoholysis between alcohols and homo/heterogeneous triacylglycerols (TG). The only attempt to describe the ethanolysis reaction has recently been reported by Torres et al. [14,15]. These authors have studied the kinetics of the lipase-catalyzed ethanolysis of fish and borage oils using a generalized Michaelis–Menten mechanism to describe the ethyl ester formation of the primary fatty acid present in the precursor oils. However, the relative lack of information about kinetics is an additional problem for a rational design and scale-up of alcoholysis reactors. This work aims to study the alcoholysis of long-chain polyunsaturated fatty acids TG (LLL) as a first step for producing MLM-type STs, based on Soumanou's two-step process, using either homogeneous or heterogeneous TG in both batch and packed-bed reactors [16]. The effects of the amount of enzyme, its support and the reaction time were investigated with two different lipases. In addition, a mechanistic model for alcoholysis is proposed. This model is based on the elemental reaction events that may occur in this process.

2. Experimental

2.1. Chemicals and materials

The lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) used were from *Rhizopus oryzae* (lipase D, from Amano Pharmaceutical Co. Ltd., Nagoya, Japan) and from *Mucor miehei* (Lipozyme®IM, donated by Novo Nordisk A/S, Bagsvaerd, Denmark). Lipozyme®IM was supplied immobilised on a macro porous anion exchange resin containing 3–5% water. However lipase D was immobilised in our laboratory. Water content of the immobilised lipases was determined by Karl–Fischer titration (Compact titrator microKF 2026, Crimson, Alella, Spain). The enzymes showed *sn*-1,3 positional specificity.

Pre-washed Celite® 545 (Fluka, Buchs, Switzerland) was used as support for adsorptive immobilization of lipase D. The immobilization was carried out by precipitation using chilled acetone as described elsewhere [16]. The lipase D immobilised on Celite® contained 0.2–0.4% water, as determined by Karl–Fischer titration.

Analytical-grade caprylic acid (CA), triolein (TO) and molecular sieves (4 Å) were obtained from Sigma–Aldrich (St. Louis, MO). The commercial oil EPAX4510 was supplied by Pronova Biocare (Norway). The main fatty acids contained in this oil are eicosapentaenoic acid, EPA (40.4%), docosohexaenoic acid, DHA (7.7%) and oleic acid (11.8%). The average molecular weight calculated for the EPAX4510 was 924.3 Da. The chemicals and solvents used were of reagent grade and purchased from common commercial suppliers.

2.2. Alcoholysis reaction in the batch reactor

Typically, pure triacylglycerol (triolein, 500 mg) or the commercial polyunsaturated fatty acid oil (EPAX4510, 525 mg), with initial concentrations between 0.14 and 0.28 M dissolved in methyl-*tert*-butyl ether (MTBE), was placed in 50 ml Erlenmeyer flasks with silicone-capped stoppers. MTBE was chosen because it efficiently suppresses acyl migration [10]. Dry ethanol (dried over 4 Å molecular sieves, activated by heating overnight to 200 °C) was added to final concentrations ranging from 0.095 to 2.28 M. The ethanol/TG molar ratio, m_0, ranged between 2 and 10. The reaction mixture was incubated and agitated in an orbital shaking air-bath with temperature control (37 °C) at 200 rpm during the reaction time. The reaction was started by adding amounts (between 14 and 600 mg) of immobilised lipase and was stopped by separation of lipase by filtration. The reaction products were stored at −20 °C until analysis. All analyses were carried out in triplicate.

2.3. Alcoholysis reaction in the packed-bed reactor (PBR)

Details regarding the PBR used have been previously reported [17]. The immobilised lipase (2 g) was packed into a glass column (6.6 i.d. × 250 mm length) covered with aluminium foil to prevent photo-induced oxidation. The enzyme bed was held between two mobile perforated disks. The substrate mixture was kept in a reservoir submerged within a thermostated water bath. The mixture consisted of EPAX4510, 33.4 g; dry ethanol, 10.0 g; and MTBE, 200 ml. The ethanol/EPAX4510 molar ratio was $m_0 = 6$. The initial EPAX4510 concentration was 0.036 M. The reaction mixture was pumped upward through the column by a peristaltic pump at a flow rate of 45 ml/h. The column was jacketed to control the reaction temperature (37 °C). The reactor was operated by recirculating the mixture leaving the packed bed to the substrate feed reservoir which was continuously agitated at 200 rpm. The reaction was followed by sampling the reaction mixture inside the substrate reservoir at different times (between 1.0 and 144 h). The samples were stored at −20 °C until analysis. All analyses were carried out in triplicate.

2.4. Identification of the reaction products and estimation of the molar fraction of fatty acids in the acylglycerols

Excess MTBE and ethanol were removed from the product mixture in a vacuum evaporator. Acylglycerols (MG, DG and TG) were identified by thin-layer chromatography (TLC) followed by quantitative gas chromatography (GC). TLC analysis has been described elsewhere [18]. The separation of positional isomers of MG was carried out by boric acid-impregnated TLC [19]. 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 [20]. These methyl esters were analysed with a Hewlett Packard 4890 gas chromatograph (Avondale, PA) connected to a capillary column of fused silica Omegawax (0.25 mm × 30 m, 0.20 μm standard film; Supelco, Bellefonte, PA), and a flame-ionization detector. GC details are described elsewhere [17]. The standard deviation was always below 10%.

3. The alcoholysis model

The model is based on the following hypothesis: (1) due to the *sn*-1,3 positional specificity of lipases, only the fatty acids in the positions *sn*-1 and *sn*-3 of the triacylglycerol are exchanged; (2) the exchange in position 1 does not depend on the nature of the fatty acid in position 3 and vice versa and whether or not there is a residue present in position 3 and vice versa; (3) internal and external mass-transfer processes were negligible (i.e. the concentration of all reactants and products was equal to the concentration in the organic phase, which are the concentrations determined by analysis) [21]; (4) the only intermediate of appreciable lifespan in which the enzyme participates is the acyl–enzyme complex; (5) acyl migration is only produced in the 2-MG and it is not an enzymatic reaction although it may be catalyzed by the support in which the enzyme is immobilised. It is referenced that some supports, as the anionic exchange resin where Lipozyme IM is immobilised, catalyzes the acyl migration [10].

For interpreting the data obtained, we used the following sequence of reactions:

(a) Enzymatic deacylation of the TG through the acyl–enzyme complex to produce intermediate diacylglycerols (1,2-DG or 2,3-DG) and finally 2-monoacylglycerol (2-MG). The carbon 2 of diacylglycerols and monoacylglycerols is a quiral carbon and for this reason the 1,2-DG and 2,3-DG and the 1-MG and 3-MG have been considered:

$$\mathrm{E} + \overline{\mathrm{AAA}} \underset{k_\mathrm{A}}{\overset{k_{-\mathrm{A}}}{\Leftrightarrow}} \mathrm{EA} + \overline{\otimes\mathrm{AA}} \tag{1}$$

$$\mathrm{E} + \overline{\mathrm{AAA}} \underset{k_\mathrm{A}}{\overset{k_{-\mathrm{A}}}{\Leftrightarrow}} \mathrm{EA} + \overline{\mathrm{AA}\otimes} \tag{2}$$

$$\mathrm{E} + \overline{\otimes\mathrm{AA}} \underset{k_\mathrm{A}}{\overset{k_{-\mathrm{A}}}{\Leftrightarrow}} \mathrm{EA} + \overline{\otimes\mathrm{A}\otimes} \tag{3}$$

$$\mathrm{E} + \overline{\mathrm{AA}\otimes} \underset{k_\mathrm{A}}{\overset{k_{-\mathrm{A}}}{\Leftrightarrow}} \mathrm{EA} + \overline{\otimes\mathrm{A}\otimes} \tag{4}$$

(b) Isomerization of the 2-MG by acyl migration to 1(3)-monoacylglycerols (1(3)-MG):

$$\overline{\otimes\mathrm{A}\otimes} \underset{k_{-\mathrm{i}}}{\overset{k_\mathrm{i}}{\Leftrightarrow}} \overline{\mathrm{A}\otimes\otimes} \tag{5}$$

$$\overline{\otimes\mathrm{A}\otimes} \underset{k_{-\mathrm{i}}}{\overset{k_\mathrm{i}}{\Leftrightarrow}} \overline{\otimes\otimes\mathrm{A}} \tag{6}$$

(c) Formation of glycerol from the 1(3)-MG catalyzed by the *sn*-1,3 specific lipases:

$$\mathrm{E} + \overline{\mathrm{A}\otimes\otimes} \underset{k_\mathrm{A}}{\overset{k_{-\mathrm{A}}}{\Leftrightarrow}} \mathrm{EA} + \overline{\otimes\otimes\otimes} \tag{7}$$

$$\mathrm{E} + \overline{\otimes\otimes\mathrm{A}} \underset{k_\mathrm{A}}{\overset{k_{-\mathrm{A}}}{\Leftrightarrow}} \mathrm{EA} + \overline{\otimes\otimes\otimes} \tag{8}$$

(d) Formation of 1,3-DG from 1(3)-MG by esterification of the acyl group liberated from TG through the acyl–enzyme complex:

$$\mathrm{EA} + \overline{\otimes\otimes\mathrm{A}} \underset{k_{-\mathrm{A}}}{\overset{k_\mathrm{A}}{\Leftrightarrow}} \mathrm{E} + \overline{\mathrm{A}\otimes\mathrm{A}} \tag{9}$$

$$\mathrm{EA} + \overline{\mathrm{A}\otimes\otimes} \underset{k_{-\mathrm{A}}}{\overset{k_\mathrm{A}}{\Leftrightarrow}} \mathrm{E} + \overline{\mathrm{A}\otimes\mathrm{A}} \tag{10}$$

(e) Esterification of the acyl group liberated from TG through the acyl–enzyme complex with the monoalcohol (ethanol):

$$\mathrm{EA} + \mathrm{B} \underset{k_{-\mathrm{BA}}}{\overset{k_\mathrm{BA}}{\Leftrightarrow}} \mathrm{E} + \mathrm{BA} \tag{11}$$

where E is the enzyme and EA the acyl–enzyme complex. The species of acylglycerols are depicted by a horizontal line representing the glycerol backbone; under this line there are three positions: 1, 2 and 3, which can be occupied by acyl groups, A, or not occupied, ⊗. B represents the monoalcohol. The exchange kinetic constants of an acyl group (A) between an acylglycerol and the enzyme are represented by $k_{-\mathrm{A}}$ (when A leaves the acylglycerol) and k_A (when it enters the acylglycerol) while the exchange kinetic constants of A between the alcohol and the enzyme are $k_{-\mathrm{BA}}$ and k_BA, respectively. The kinetic constants of the isomerization reaction of MG (Eqs. (5) and (6)) are k_i (A moves from position 2 to positions 1 or 3) and $k_{-\mathrm{i}}$ (A moves from positions 1 or 3 to position 2).

Assuming that the acyl–enzyme complex is at equilibrium with the free fatty acids, we can write:

$$\mathrm{EA} + \mathrm{W} \Leftrightarrow \mathrm{E} + \mathrm{A}, \qquad K_\mathrm{A} = \frac{[\mathrm{EA}][\mathrm{W}]}{[\mathrm{E}][\mathrm{A}]} \tag{12}$$

This reaction is responsible for the generation of free fatty acids, generally in very small amounts due to the small amount of water (W) in the reaction mixture. If this reaction is at equilibrium, the free enzyme/acyl enzyme complex ratio will

only depend on: (i) the initial water content of the reaction mixture; (ii) the K_A value; (iii) the water partitioning between the organic phase and the immobilised enzyme. This means that for a given enzymatic reaction system and a fixed temperature, the enzyme/acyl–enzyme ratio would be a function of the initial water content, W_0, and therefore it will be verified that:

$$\frac{[E]}{[EA]} = F(W_0) \tag{13}$$

Taking into account that the *sn*-1 and *sn*-3 extreme positions of the glycerol backbone are equivalents, we can write:

$$[\overline{AA\otimes}] = [\overline{\otimes AA}], \qquad [\overline{\otimes\otimes A}] = [\overline{A\otimes\otimes}] \tag{14}$$

and, therefore, the following independent concentrations can be defined:

$$[TG] = [\overline{AAA}], \qquad [DG]_a = [\overline{AA\otimes}] + [\overline{\otimes AA}] = 2[\overline{AA\otimes}],$$
$$[DG]_b = [\overline{A\otimes A}] \tag{15}$$

$$[MG]_a = [\overline{\otimes A\otimes}], \qquad [MG]_b = [\overline{A\otimes\otimes}] + [\overline{\otimes\otimes A}] = 2[\overline{A\otimes\otimes}],$$
$$[G] = [\overline{\otimes\otimes\otimes}] \tag{16}$$

where, admitting that the enzyme is fully *sn*-1,3 specific, $[DG]_a$ and $[MG]_a$ would be the only DG and MG produced (Eqs. (1)–(4)) and no glycerol would be produced. However, if acyl migration takes place (Eqs. (5) and (6)) $[MG]_b$ will be formed and, therefore, free glycerol (Eqs. (7) and (8)) and DG $[DG]_b$ (Eqs. (9) and (10)) could also be formed.

From this mechanism, by carrying out an enzyme balance and applying the hypothesis of steady state to the acyl–enzyme complex (EA), the following dimensionless differential equations, which allow the resolution of the model, have been obtained:

$$\frac{d\,TG}{d\lambda} = -2\bar{D}\,TG + \frac{1}{K_{Ge}}\bar{N}\,DGa \tag{17}$$

from reactions (1) and (2)

$$\frac{d\,DGa}{d\lambda} = \left(2\bar{D}\,TG - \frac{1}{K_{Ge}}\bar{N}\,DGa\right) - \left(\bar{D}\,DGa - \frac{2}{K_{Ge}}\bar{N}\,MGa\right) \tag{18}$$

from reactions (1)–(4)

$$\frac{d\,MGa}{d\lambda} = \left(\bar{D}\,DGa - \frac{2}{K_{Ge}}\bar{N}\,MGa\right) - 2\frac{k_{CI}}{k_{CA}}\left(MGa - \frac{1}{2K_{Ie}}MGb\right) \tag{19}$$

from reactions (3)–(6)

$$\frac{d\,MGb}{d\lambda} = 2\frac{k_{CI}}{k_{CA}}\left(MGa - \frac{1}{2K_{Ie}}MGb\right) + 2\left(\bar{D}\,DGb - \frac{1}{2K_{Ge}}\bar{N}\,MGb\right) - \left(\bar{D}\,MGb - \frac{2}{K_{Ge}}\bar{N}G\right) \tag{20}$$

from reactions (5)–(10)

$$\frac{d\,DGb}{d\lambda} = -2\bar{D}\,DGb + \frac{1}{K_{Ge}}\bar{N}\,MGb \tag{21}$$

from reactions (9) and (10)

$$\frac{d\,BA}{d\lambda} = \frac{k_{CB}}{k_{CA}}(\bar{N}(m_0 - BA) - K_{Be}\bar{D}\,BA) \tag{22}$$

from reaction (11). Where

$$\bar{N} = \frac{(2TG + DGa + 2DGb + MGb) + \frac{k_{CB}}{k_{CA}}BA}{\frac{1}{K_{Ge}}(DGa + MGb + 2MGa + 2G) + \frac{k_{CB}}{k_{CA}K_{Be}}(m_0 - BA) + (2TG + DGa + 2DGb + MGb) + \frac{k_{CB}}{k_{CA}}BA} \tag{23}$$

$$\bar{D} = \frac{\frac{1}{K_{Ge}}(DGa + MGb + 2MGa + 2G) + \frac{k_{CB}}{k_{CA}K_{Be}}(m_0 - BA)}{\frac{1}{K_{Ge}}(DGa + MGb + 2MGa + 2G) + \frac{k_{CB}}{k_{CA}K_{Be}}(m_0 - BA) + (2TG + DGa + 2DGb + MGb) + \frac{k_{CB}}{k_{CA}}BA} \tag{24}$$

with the initial condition:

$$\lambda = 0, \quad TG = 1, \quad DGa = 0, \quad DGb = 0, \quad MGa = 0, \quad MGb = 0, \quad BA = 0 \tag{25}$$

In Eqs. (17)–(25) all the concentrations have been written in dimensionless variables by dividing the concentrations of any product within the reaction mixture by the initial concentration of triacylglycerols, $[TG]_0$, i.e.:

$$TG = \frac{[TG]}{[TG]_0}, \qquad DGa = \frac{[DG]_a}{[TG]_0}, \qquad DGb = \frac{[DG]_b}{[TG]_0},$$
$$MGa = \frac{[MG]_a}{[TG]_0}, \qquad MGb = \frac{[MG]_b}{[TG]_0} \tag{26}$$

$$G = \frac{[G]}{[TG]_0}, \qquad B = \frac{[B]}{[TG]_0}, \qquad BA = \frac{[BA]}{[TG]_0} \tag{27}$$

λ is the dimensionless reaction time, which has been introduced as:

$$\lambda = tk_{CA}\frac{m_E}{V} \tag{28}$$

where m_E/V is the lipase amount/reaction volume ratio (including the mass of the enzyme support where the lipase is immobilised).

k_{CA} and k_{CB} are kinetic constants that appear because k_{-A}, k_{-BA}, respectively, must be transformed in order that the concentration of enzyme used appears (m_E/V, that include the weight of the support where the enzyme is immobilised) rather than the total active enzyme concentration $[E]_T$ (which includes the free enzyme and the enzyme as acyl–enzyme complex) which figures in the kinetic equations derived initially from the reaction mechanism. Then, we can write:

$$k_{-A}[E]_T = k_{CA}\frac{m_E}{V}, \qquad k_{-BA}[E]_T = k_{CB}\frac{m_E}{V} \tag{29}$$

Thus, k_{CA} and k_{CB} include the corresponding kinetic constants (k_{-A} and k_{-BA}, respectively) and the ratio of active enzyme/total enzyme (including the support where the lipase is immobilised). In addition, if acyl migration were catalyzed by the enzyme support in which the enzyme is immobilised, it can be admitted that its kinetic constant (k_i) is proportional to the concentration of enzyme support and therefore to the concentration of enzyme (m_E/V). Then, we can write:

$$k_i = k_{CI}\frac{m_E}{V} \tag{30}$$

K_{Ge} and K_{Be} are the equilibrium constants for the reaction of exchange of an acyl group between an acylglycerol and the enzyme (reactions (1)–(4) and (7)–(10)) and between the ethanol and the enzyme (reaction (11)), respectively. K_{Ie} is the equilibrium constant for the isomerization reaction of the MG (reactions (5) and (6)), i.e.:

$$\frac{k_{-A}}{k_A} = K_{Ge}, \qquad \frac{k_{-BA}}{k_{BA}} = K_{Be}, \qquad \frac{k_i}{k_{-i}} = K_{Ie} \tag{31}$$

In Eqs. (20), (23), (24) and (27) G is the dimensionless glycerol concentration, which can be calculated from the acylglycerol balance:

$$G = 1 - (TG + DGa + DGb + MGa + MGb) \tag{32}$$

Similarly, in Eq. (27) B is the dimensionless monoalcohol concentration, which can be calculated from a monoalcohol balance taking into account the initial ethanol/triacylglycerol molar ratio, $m_0 = [B]_0/[TG]_0$, i.e.:

$$B = m_0 - BA \tag{33}$$

Rate Eqs. (17)–(22) show that at equilibrium (i.e. when reaction rates are equal to zero) the following conditions are met:

(a) When reactions (1)–(4) are at equilibrium the driving forces of Eqs. (17) and (18) are null and therefore the following equations are fulfilled:

$$\frac{\bar{N}\,DGa}{\bar{D}\,TG} = 2K_{Ge} = \frac{2k_{-A}}{k_A} \tag{34}$$

$$\frac{\bar{N}\,MGa}{\bar{D}\,DGa} = \frac{K_{Ge}}{2} = \frac{k_{-A}}{2k_A} \tag{35}$$

and combining both equations we can obtain

$$\frac{DGa^2}{TG\,MGa} = 4 \tag{36}$$

(b) When reactions (5) and (6) are at equilibrium the following is fulfilled (see kinetic equations (19) and (20)):

$$\frac{MGb}{MGa} = 2K_{Ie} = \frac{2k_i}{k_{-i}} \tag{37}$$

(c) When reactions (7) and (8) are at equilibrium we can write (see kinetic equation (20))

$$\frac{\bar{N}G}{\bar{D}\,MGb} = \frac{K_{Ge}}{2} = \frac{k_{-A}}{2k_A} \tag{38}$$

(d) When reactions (9) and (10) are at equilibrium we can write (see the driving forces in kinetic equations (20) and (21))

$$\frac{\bar{N}\,MGb}{\bar{D}\,DGb} = 2K_{Ge} = \frac{2k_{-A}}{k_A} \tag{39}$$

(e) When reaction (11) is at equilibrium it follows that (see the driving force in kinetic equation (22))

$$\frac{\bar{N}(m_0 - BA)}{\bar{D}\,BA} = K_{Be} = \frac{k_{-BA}}{k_{BA}} \tag{40}$$

and combining Eqs. (39) and (40) we obtain

$$\frac{BA\,MGa}{(m_0 - BA)DGa} = \frac{K_{Ge}}{2K_{Be}} = \frac{k_{-A}k_{BA}}{2k_Ak_{-BA}} \tag{41}$$

4. Model simplifications

4.1. Acyl migration as limiting step

If the acyl migration steps (reactions (5) and (6)) control the global process rate, all the preceding reactions, (1)–(4), and the subsequent ones, (7)–(11), will be at equilibrium. The equilibrium of reactions (1)–(4) imply that the driving forces of Eqs. (17) and (18) are null and therefore Eqs. (34) and (36) are fulfilled. Also, when the reactions (7)–(11) are at equilibrium Eqs. (38)–(41) are fulfilled. In addition, the equilibrium of reactions (7)–(11) when ethanol is in excess, means that 1(3)-MG (MGb) will be present in a very small amount and consequently there will be no 1,3-DG (DGb), because they will rapidly be transformed into free glycerol (Eqs. (7) and (8)). In these conditions Eq. (32) is transformed into

$$G = 1 - (TG + DGa + MGa) \tag{42}$$

and

$$\frac{dG}{d\lambda} = -\left(\frac{d\,TG}{d\lambda} + \frac{d\,DGa}{d\lambda} + \frac{d\,MGa}{d\lambda}\right) \tag{43}$$

and taking into account Eqs. (17)–(19) we can write:

$$\frac{dG}{d\lambda} = 2\frac{k_{CI}}{k_{CA}}\left(MGa - \frac{1}{2K_{Ie}}MGb\right) \tag{44}$$

Finally, taking into account (28) and considering that $MGb \approx 0$, Eq. (44) is reduced to:

$$\frac{dG}{d\left(t\frac{m_E}{V}\right)} = 2k_{CI}\,MGa \tag{45}$$

Eq. (45) means that the glycerol formation rate should be fitted to an irreversible first order reaction with respect to the concentration of 2-MG (MGa).

4.2. Absence of acyl migration

If no acyl migration takes place, 1(3)-MG will not be formed (i.e. MGb = 0) and consequently neither will 1,3-DG (i.e. DGb = 0). Free glycerol will, therefore, not be released (i.e. G = 0) and thus Eqs. (17)–(22) are reduced to:

$$\frac{d\,TG}{d\lambda} = -2\bar{D}\,TG + \frac{1}{K_{Ge}}\bar{N}\,DGa \tag{46}$$

$$\frac{d\,DGa}{d\lambda} = 2\left(\bar{D}\,TG - \frac{1}{2K_{Ge}}\bar{N}\,DGa\right) - \left(\bar{D}\,DGa - \frac{2}{K_{Ge}}\bar{N}\,MGa\right) \tag{47}$$

$$\frac{d\,MGa}{d\lambda} = \bar{D}\,DGa - \frac{2}{K_{Ge}}\bar{N}\,MGa \tag{48}$$

$$\frac{d\,BA}{d\lambda} = \frac{k_{CB}}{k_{CA}}(\bar{N}(m_0 - BA) - K_{Be}\bar{D}\,BA) \tag{49}$$

where the dimensionless parameters $\bar{N}$ and $\bar{D}$ are given by Eqs. (23) and (24), but in this case DGb = MGb = G = 0. The initial condition is:

$$\lambda = 0, \quad TG = 1, \quad DGa = 0, \quad MGa = 0, \quad BA = 0 \tag{50}$$

At equilibrium, the driving forces of Eqs. (46)–(49) will be null and Eqs. (37) and (42) are fulfilled.

5. Results and discussion

5.1. Alcoholysis of triolein catalyzed by Lipozyme® IM in a batch reactor

On the whole, enzymatic reaction rates are directly proportional to the concentration of active enzyme. Therefore, if no denaturalization of the enzyme occurs, the significant variable is the product of the catalyst concentration and time, i.e. $(m_E/V)t$. This value represents the processing intensity of the enzymatic reaction. Fig. 1 shows the dimensionless concentration of the reaction products (MG, DG, TG and G) versus the processing intensity for the experiments carried out with an initial ethanol/triolein molar ratio, m_0, equal to 10. Similar figures were obtained with the results obtained at ethanol/triolein molar ratios of 6 and 2. Nonetheless, for a given processing intensity, the amount of glycerol produced at $m_0 = 2$ is lower than the one obtained at $m_0 = 10$ and $m_0 = 6$, because in the former case the initial ethanol content is the lowest one used and therefore the equilibrium is less displaced to the formation of products (data not shown). Fig. 1 shows that from a very low processing intensity (approximately 250 h(g/l)) the concentration of TG is nearly null, the DG rapidly decreased and, finally, the MG tend to disappear from the reaction mixture. In contrast the dimensionless concentration of glycerol (G) asymptotically tends to 1. Since Lipozyme® IM, is an *sn*-1,3 specific lipase, the generation of G must be a consequence of the acyl migration within the MGa for processing intensities above 250 h(g/l). These data are explained below.

5.2. Processing intensities above 250 h(g/l)

If the acyl migration step is controlling the alcoholysis reaction at these processing intensities, Eq. (45) must be met. This equation indicates that acyl migration leads to the glycerol formation, due to the rapid disappearance of the 1(3)-MG. To

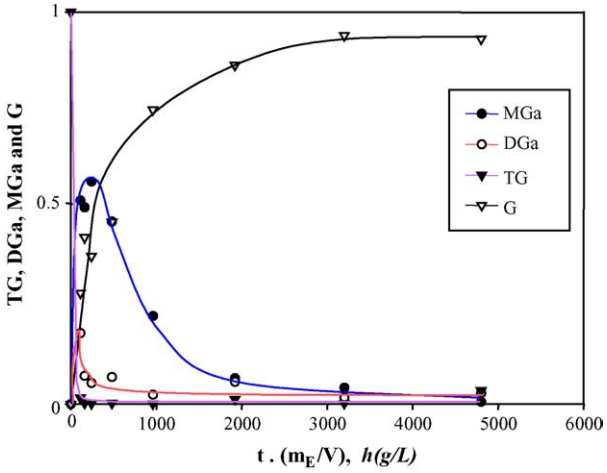

Fig. 1. Alcoholysis of triolein catalyzed with Lipozyme® IM: influence of the processing intensity, $t(m_E/V)$, on the dimensionless concentrations of the alcoholysis products. Ethanol/triolein molar ratio, m_0 = 10, 37 °C.

validate Eq. (46) the following empirical equation was fitted to the glycerol data depicted in Fig. 1:

$$G = \frac{t\frac{m_E}{V}}{351 + t\frac{m_E}{V}} \quad (51)$$

whose derivative, with respect to $t(m_E/V)$, allows us to predict the glycerol formation rate:

$$r_G = \frac{dG}{d\left(t\frac{m_E}{V}\right)} = \frac{351}{\left(351 + t\frac{m_E}{V}\right)^2} \quad (52)$$

According to Eq. (45) the glycerol formation rate should be proportional to the dimensionless concentration of 2-monoleín, MGa. Fig. 2 shows that Eq. (45) reproduces the experimental results very well. The lineal regression of data shown in Fig. 2 leads to a relative rate constant for the acyl migration process, k_{CI}, equal to 0.00058 l/g h.

On the other hand, from Eqs. (34), (36), (37) and (39) one can write:

$$\left(\frac{DGb}{DGa}\right)_{equilibrium} = \frac{K_{Ie}}{2} \quad (53)$$

This means that the equilibrium constant for the isomerization reaction could be calculated from the DGb/DGa ratio. Previous experiments of partial hydrolysis of triolein carried out in our laboratory at 50 °C allowed us to calculate the 1,3-diolein (DGb)/1,2(2,3)-diolein (DGa) ratio at equilibrium [22]; Table 1 shows the distribution of acylglycerols at equilibrium and consequently, the equilibrium constant for the isomerization process at 50 °C would be:

$$\left(\frac{DGb}{DGa}\right)_{equilibrium} = 2.32 \pm 0.30 \quad (54)$$

and, therefore, taking into account Eq. (53), the equilibrium constant for the isomerization reaction calculated is

Table 1
Partial hydrolysis of triolein (TG) at 50 °C catalyzed by Lipozyme® IM (Influence of the initial water content, W_0 (initial moles of water per mole of triolein), on the distribution of acylglycerols at equilibrium [22])

W_0	MGa + MGb	DGa	DGb	TG
0.356	0.005	0.040	0.115	0.840
0.460	0.015	0.075	0.150	0.760
0.848	0.030	0.090	0.190	0.690
1.340	0.060	0.105	0.245	0.590

$K_{Ie} = 4.6 \pm 0.6$. This K_{Ie} value is of the same order of magnitude as that which can be calculated from data reported by Lortie et al. [11], who studied the isomerization of 1,3-diolein to 1,2 diolein and determined the relative rate constants for both the direct and the reverse reaction at 60 °C and consequently the equilibrium constant of this reaction. From the data of these authors and using Eq. (33), the equilibrium constant calculated is $K_{Ie} = 2$, at 60 °C. The small difference between our equilibrium constant and the one calculated from Lortie's data could be attributed mainly to the difference in the temperature used. Since the enthalpy of this isomerization reaction must be very small, a slight variation of K_{Ie} with temperature may also be expected.

5.3. Processing intensities below 250 h(g/l)

Fig. 1 shows that for processing intensities below 250 h(g/l) the concentration of MGa increases with the processing intensity up to a steady value. If we assume that no acyl migration occurs at these low processing intensities, then the MGa are not transformed into MGb and consequently neither DGb nor glycerol are produced. Fig. 3 shows the evolution of the dimensionless concentration of alcoholysis products (MGa, DGa, TG and ethyl ester of oleic acid, BA). These results clearly show that acyl migration was almost negligible (i.e. no glycerol was formed) as the balance MG + DG + TG ≈ 1. According to the proposed model, the variation of MGa, DGa,

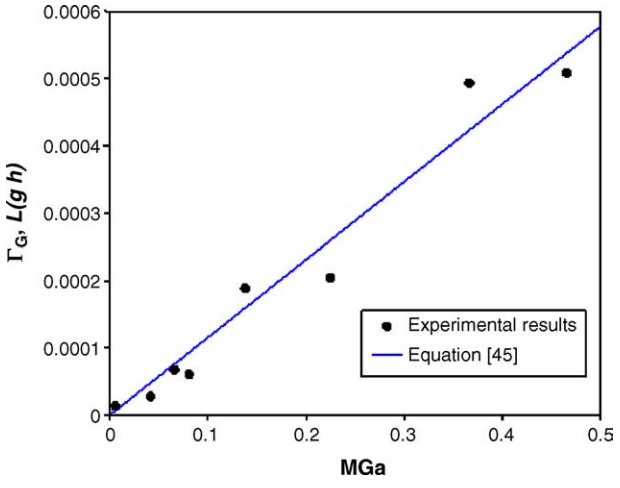

Fig. 2. Alcoholysis of triolein catalyzed with Lipozyme® IM: relationship between the glycerol production rate, r_G, and the dimensionless concentration of MGa. $m_0 = 10$; processing intensities, $t(m_E/V) > 250$ h(g/l).

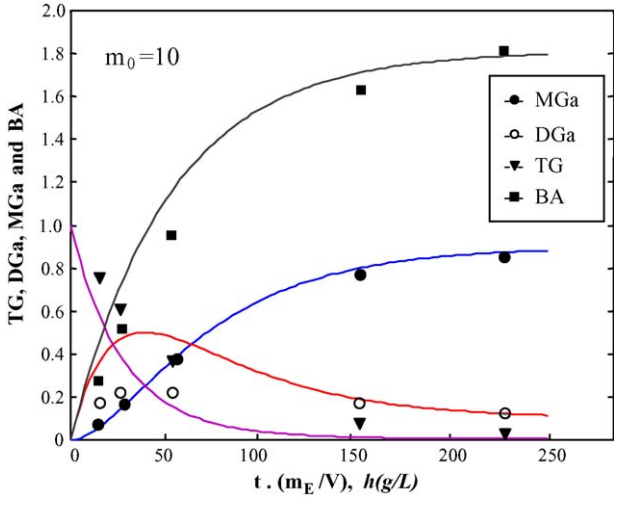

Fig. 3. Alcoholysis of triolein catalyzed with Lipozyme® IM at low processing intensities, $t(m_E/V) < 250$ h(g/l), $m_0 = 10, 37$ °C. Continuous lines represent the predicted values using Eqs. (46)–(50).

TG and BA with processing intensity must be governed by Eqs. (46)–(50). These equations can be solved by using four parameters: two kinetic constants (k_{CA} and k_{CB}, Eq. (29)), and two equilibrium constants (K_{Ge} and K_{Be}, Eq. (31)). The solution of 46–50 using MatLab® provided the values for the apparent kinetic and equilibrium constants shown in Table 2. The continuous lines in Fig. 3 correspond to the simulated values and show that the kinetic constants for the exchange of an acyl group from an extreme position in the glycerol backbone are independent of the fact that the other positions may or may not be occupied by a fatty acid.

5.4. Alcoholysis of triolein catalyzed by lipase D

This set of experiments was carried out with lipase D immobilised in our laboratory on Celite®. Fig. 4 shows the variation of the dimensionless concentration of 2-MG (MGa), BA, DGa and TG with the processing intensity. It can be seen that the concentrations vary sharply with the processing intensity up to an equilibrium value, which is reached at relatively low processing intensities, such as 700 h(g/l), and remain constant for all the processing intensities tested. Since the equilibrium concentration of MGa remains constant, no acyl migration took place with this enzyme and therefore neither DGb nor glycerol were produced. The experimental conditions were absolutely identical for the two enzymes used. The only difference between data shown in Figs. 1 and 4 comes from the enzyme and the enzyme support used. Obviously, the higher the processing intensity the larger the amount of enzyme support in the reaction medium was. Therefore, it is quite likely that the acyl migration occurring when Lipozyme® IM was used (Fig. 1) may be catalyzed by the enzyme support, constituted by an anionic exchange resin. This hypothesis concurs with data reported by Millqvist Fureby et al. [10], who showed that the support material for Lipozyme® IM catalyzed the acyl migration process. However, the support of lipase D was acid-washed Celite®, which ruled out the presence of any ionic group on its surface, and this support could not, therefore, have catalyzed the acyl migration. Again this hypothesis agrees with Millqvist Fureby et al. [10] who showed that Celite® washed with acid, did not catalyze any acyl migration process.

The solution of (46)–(50) by using MatLab®, provided the values for the kinetic and equilibrium constants shown in Table 2. These parameters (k_{CA}, k_{CB}, K_{Be}, K_{Ge}) were of the same order of magnitude, but the kinetic constants obtained when using lipase D are higher than the ones obtained when using Lipozyme® IM, which indicates that lipase D is more appropriate for the alcoholysis process than Lipozyme® IM.

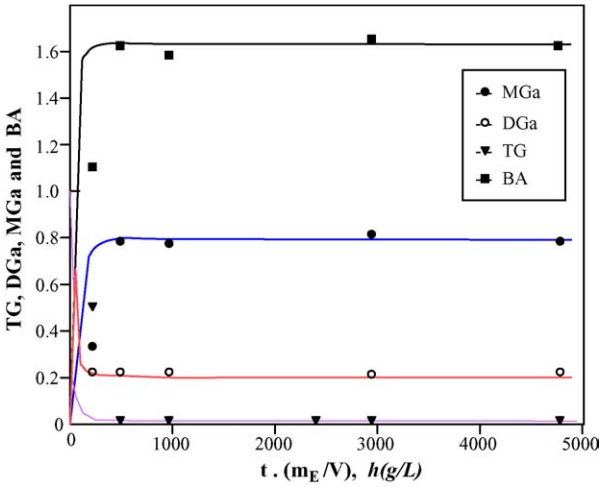

Fig. 4. Alcoholysis of triolein catalyzed with lipase D immobilised on Celite®. Influence of the processing intensity, $t(m_E/V)$, on the dimensionless concentration of alcoholysis products. $m_0 = 10$, 37 °C. Continuous lines represent the predicted values using Eqs. (46)–(50).

5.5. Alcoholysis of EPAX4510 catalyzed by lipase D in a packed-bed reactor (PBR)

Having verified that lipase D provided larger amounts of MGa during alcoholysis of triolein and taking into account the optimal experimental conditions obtained when using this homogeneous TG, we carried out the alcoholysis of heterogeneous commercial oil rich in EPA (EPAX4510), in a packed-bed reactor. The variation of the dimensionless concentrations of the different reaction products with the processing intensity is shown in Fig. 5. For MGa and all the other reaction products, concentrations of equilibrium were reached. The reactor was operated by recirculating the reaction mixture through the PBR. The change in composition with only one passage through the PBR was very low at the flow rate used (45 ml/h). In these conditions the PBR may be considered a discontinuous stirred tank reactor and we could apply the proposed model when no acyl migration occurs (Eqs. (46)–(50)). Solving these equations using MatLab®, the kinetic parameters shown in Table 2 (k_{CA}, k_{CB}, K_{Be}, K_{Ge}) were obtained. These kinetic and equilibrium parameters minimised the sum of the squared differences (SSD) between the experimental and the calculate results,

$$\mathrm{SSD} = \sum(\mathrm{TG_{exp}} - \mathrm{TG_{calc}})^2 + \sum(\mathrm{DGa_{exp}} - \mathrm{DGa_{calc}})^2 + \sum(\mathrm{MGa_{exp}} - \mathrm{MGa_{calc}})^2 + \sum(\mathrm{BA_{exp}} - \mathrm{BA_{calc}})^2 \quad (55)$$

Table 2
Kinetic and equilibrium constants, k_{CA} and K_{Ge}, for the exchange of an acyl group (A) between an acylglycerol and the enzyme (reactions (1)–(4) and (7)–(10)) and for the exchange between the ethanol and the enzyme, k_{CB} and K_{Be} (reaction (11))

Alcoholysis system	k_{CA} (l/h g)	k_{CB} (l/h g)	K_{Ge}	K_{Be}
Triolein catalyzed by Lipozyme® IM, batch reactor	0.021 ± 0.001	0.029 ± 0.002	3.8 ± 0.05	0.95 ± 0.02
Triolein catalyzed by lipase D immobilised on Celite®, batch reactor	0.042 ± 0.001	0.107 ± 0.002	1.8 ± 0.05	0.90 ± 0.02
EPAX4510 catalyzed by lipase D immobilised on Celite®, packed-bed reactor	0.014 ± 0.001	0.056 ± 0.002	2.5 ± 0.05	0.84 ± 0.02

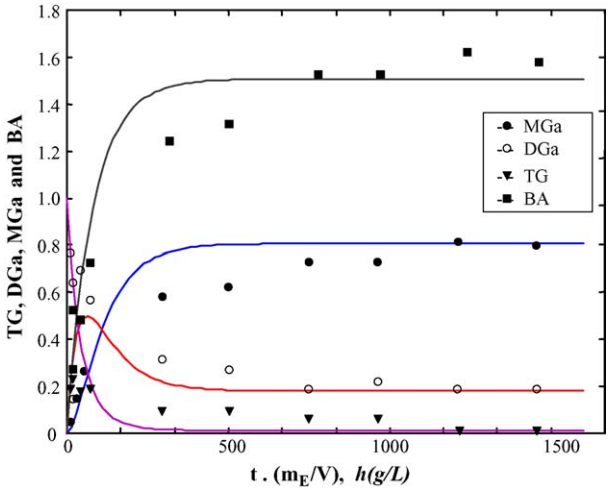

Fig. 5. Alcoholysis of EPAX4510 catalyzed with lipase D, immobilised on Celite®, in a packed-bed reactor. Influence of the processing intensity, $t(m_E/V)$, on the dimensionless concentration of alcoholysis products. Continuous lines represent the predicted values using Eqs. (46)–(50). Reaction conditions: 33.4 g EPAX4510, 10 g ethanol, $m_0 = 6$, 0.2 l of MTBE, 33.3 g of lipase D (2 g of lipase + 31.1 g of Celite®), 37 °C.

The deviations in the determination of the equilibrium and kinetic parameters (Table 2) correspond to the variation of each parameter that lead to an increase of 5% in the SSD value. The parameters found for EPAX4510 are of the same order of magnitude as those obtained with triolein (Table 2), which again demonstrates the validity of the model, since in this case a heterogeneous commercial oil was used and both kinetic and equilibrium constants for these reactions are very similar to those obtained for triolein. Nonetheless, the value of the kinetic constants k_{CA} and k_{CB} obtained with triolein and lipase D in a batch reactor are higher than those obtained for EPAX4510, which indicates that triolein is a more suitable substrate for alcoholysis than the commercial oil. As occurred in Fig. 3, Fig. 5 shows a reasonably good fit for both 2-MG and ethyl esters of the different fatty acids produced (the major reaction products), although poorer than the one obtained for triolein (homogeneous triacylglycerol) (Fig. 3), because we are now working with a heterogeneous TG, a completely different operational scale (33.4 g of TG as compared to 0.5 g) and a more complex operational procedure and reactor (PBR). Figs. 3 and 5 show that the curves corresponding to TG show a significant deviation with respect to the experimental data, the predicted concentrations of TG being lower than the experimental ones. The repetition of this result for two completely different starting oils, enzymes and reactors means that the rate constant (k_{CA} or k_{-A}) for the exchange of an acyl group in the TG (Eqs. (1) and (2)) is higher than it should be and it should be lower than the k_{-A} in the DGa and MGa (Eqs. (3) and (4)). In other words, once the first acyl group has been released, it seems easier for a second one to be liberated. Therefore, the assumption that the relative rate constant (k_{-A} or k_{CA}) for the exchange of an acyl group does not depend on whether other positions are occupied (which is one of the hypotheses of the model), led to predict values of the TG concentrations lower that the experimental, as can be seen in Figs. 3 and 5.

Table 2 also shows that K_{Be} is almost the same for the three alcoholysis systems used, which seems reasonable since this parameter only depends on the alcohol used (ethanol). Finally, the different values obtained for K_{Ge} could be attributed to the generality in the definition of the model, which is the trade-off for greater applicability, i.e. the experimental results corresponding to the hydrolysis and esterification of TG, 1,2-DG, 2,3-DG and 2-MG have been fitted by a relatively simple model with few kinetic parameters. The relation K_{Ge}/K_{Be} represents the equilibrium constant of alcoholysis, which should be independent of the lipase used; however, the heterogeneous nature of the system and the probable influence of the lipase support, which must be more important for the interaction TG–enzyme than for the interaction ethanol–enzyme, could justify the obtained results. In addition, the acyl migration produced with Lipozyme IM determined a greater error in the determination of the alcoholysis equilibrium of this system.

6. Conclusions

1. The kinetic model proposed for the lipase-catalyzed alcoholysis of TG describes the possible reactions related to the exchange of acyl groups, including acyl migration process. The model is based on elemental reaction events and assumes that no reaction occurs at position 2 of TG (lipase is *sn*-1,3 specific) and that positions 1 and 3 are equivalent. The kinetic model proposed demonstrated that the processing intensity (the product of the enzyme concentration and the reaction time) is the main parameter influencing the alcoholysis reaction. The rate equations of the proposed model allow the necessary equilibrium and kinetic constants to be estimated for a rational design of the alcoholysis reactor.
2. Figs. 1, 4 and 5 suggest that acyl migration is possibly catalyzed by the enzyme support of Lipozyme® IM. The anionic exchange resin employed in the immobilization of this lipase promoted the acyl migration (Fig. 1). In contrast, Celite® (a diatomaceous earth support) did not catalyze acyl migration (Figs. 4 and 5).
3. The proposed model fits acceptably the experimental results corresponding to all the fatty acids and the two oils, with only four parameters that correspond to the apparent rate and equilibrium constants. Moreover, the model fits acceptably the final products (MGa, BA and G) and approximately the intermediate products (DG and TG).

Acknowledgements

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