Kinetics of the enzymatic hydrolysis of triglycerides in o/w emulsions
Study of the initial rates and the reaction time course

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ABSTRACT

The hydrolysis of emulsified tributyrin by Thermomyces lanuginosus lipase (TLL) has been studied by titrimetric and colorimetric measurements as well as by thin-layer chromatography (TLC). A kinetic model have been developed and applied to the initial reaction rates as well as to simulate the time course of the hydrolysis reaction at several temperatures, lipase concentrations and volume fractions of tributyrin. The model successfully predicted the initial reaction rates, even at saturating enzyme concentrations, with a relative deviation of less than 5%. The reaction-progress curves predicted by the model also agree with the experimental results significantly. The combined analysis both of the experimental results and of the simulations suggests that the hydrolysis of tributyrin by TLL proceeds through two separate stages which partially overlap. In the first one, the main reaction is the hydrolysis of one of the outer ester groups (sn-1 or 3) of the tributyrin molecule. The second stage starts when the interface becomes saturated of diglyceride, and involves mainly the hydrolysis of the remaining outer ester bond. The results found offer valuable information on the time course of the enzymatic hydrolysis of emulsified triglycerides, which is useful for some of the applications of these enzymes, such as free fatty acid production, wastewater treatment or washing processes.

1. Introduction

Hydrolysis of emulsified triglycerides by lipases (E.C. 3.1.1.3) has great practical importance, as it represents a more attractive alternative for fatty acid production than the traditional chemical process [1]. Additionally, triglyceride emulsions (O/W) also constitute the environment in which lipases exert their catalytic action in nature as well as in other important and promising applications of these enzymes, such as the treatment of wastewater with a high fat content [2] or in washing processes [3–5], where lipases enable a more efficient removal of oily stains, especially at low temperatures [3,5].

Despite these facts, the most comprehensive studies on lipase kinetics have been conducted with monolayers [6], micelles [7,8] and phospholipid vesicles [9]. These systems, though thermodynamically stable and easier to characterize than emulsions, only partially reproduce the performance conditions of lipases in the aforementioned applications. Thus, in order to optimize lipase performance in hydrolytic and cleaning applications, the kinetics of lipase action in O/W emulsions must be studied in detail.

It is also remarkable that virtually all the kinetic studies on the hydrolysis of emulsified triglycerides by lipases published so far [10–14] consider only the initial reaction rates—that is, the kinetic models developed in these studies are never applied to predict the reaction time course, which is of key importance in simulating the operation of enzymatic bioreactors as well as in gaining a more complete insight into the mechanism of lipase action in washing and wastewater treatment processes. Very recently, Hermansyah et al. [15] have proposed a kinetic model for the lipase-catalyzed hydrolysis of triglycerides which assumes a Ping Pong Bi mechanism with competitive inhibition by the fatty acid at the three stages of the reaction. The model satisfactorily predicts the progress of the reaction over time, but results in a large set of kinetic and adsorption constants which, in addition, are difficult to validate by comparing them to previously publish results. Moreover, and despite the greater complexity of this model, it only allows for a slight improvement of the fittings when compared to those obtained with a Michaelis–Menten-type model. Nevertheless, a better comprehension of the changes taking place in the reaction medium during hydrolysis would allow optimization of working conditions for lipases in each application.

However, analysis of the progress of lipolysis over time is a difficult task, as reaction products, once released, can be distributed between the aqueous or the organic phase, depending on their pre-
dominantly hydrophilic or lipophilic character. In addition, some of the hydrolysis products, such as free fatty acids, mono- and diglycerides, are surface-active compounds and, consequently, tend to accumulate at the interface, thereby decreasing the interfacial tension and interfering with the action of lipases. This results in a rapid reduction of the reaction rate, found by several authors [5,13–16] on performing the hydrolysis of vegetable oils in O/W emulsions.

Another difficulty frequently found in most of the already published studies on enzymatic hydrolysis of emulsified triglycerides [10–14] is about the determination of the specific interfacial area of the substrate emulsions, which is a key parameter for the kinetic modelling. Estimation is normally accomplished by the use of empiric equations, such as the Calderbank’s [17], which implies a loss of accuracy compared to its direct measurement.

In a previous study [18], we also developed a simplified kinetic model for tributyrin hydrolysis, based on the calculation of the concentration of surface ester bonds. This model proved valid for low and saturating enzyme concentrations, although it was necessary to use a different equation for each one of these limiting situations.

In the present work, a kinetic model for the enzymatic hydrolysis of triglycerides in O/W emulsions has been developed, using the same assumptions as in Tsai and Chang’s study [12]. This model has been used to perform a systematic kinetic study of the enzymatic hydrolysis of triglycerides in O/W emulsions, varying enzyme concentration, oil phase volume fraction and temperature. Tributyrin was chosen as a model substrate, because butyric acid mainly distributes in the aqueous phase, thus making it easier to describe the surface behaviour of the system as the reaction progresses. To ensure a more accurate determination of the model parameters, a greater number of hydrolysis experiments, as compared to previously published studies, have been carried out. The model, applied to the initial reaction rates, proves valid for low and high (saturating) enzyme concentrations, the relative deviation between the experimental and predicted values being less than 5%.

The kinetic model has also been applied to predict the time course of the hydrolysis of emulsified tributyrin, with useful conclusions being drawn about the changes in the droplet composition during the reaction. Several assumptions, made on the spatial arrangement of tri- and diglyceride molecules at the surface of the organic-phase droplets, were finally confirmed by the analysis of the experimental results.

2. Materials and methods

2.1. Products and reagents

The enzyme used was the commercial lipase Lipolase® 100, from Thermomyces lanuginosus (TL), supplied by Novozymes A/S, Bagsvaerd, Denmark, with a reported molecular weight of 31,700 Da. The protein content in the commercial enzymatic preparation was determined gravimetrically, precipitating the protein with acetone, separating it by centrifugation (8000 rpm, 30 min), and drying the resulting precipitate (60 °C, 24 h). The value was 3.01% by weight. The specific activity of the enzyme is 100,000 LU/g of the commercial product (one LU represents the amount of enzyme that releases 1 μmol/min of butyric acid at 30 °C and pH 7.0). TLL possesses sn-1,3 specificity, i.e., it preferentially cleaves the ester bonds on the positions sn-1 and sn-3 of the triglyceride molecule.

Tributyrin (99% richness) was supplied by Merck (Darmstadt, Germany) and used without further purification. To stabilize the tributyrin emulsions an emulsification reagent, for which the active ingredient was gum arabic (from Merck) was used.

The detailed composition of the emulsification reagent has been described elsewhere [18]. The final emulsion consistently contained 0.1 g of gum arabic/mL of tributyrin.

A phosphate buffer solution (0.5 mM) was used to prepare the stock solution of enzyme, as well as the subsequent dilutions used in the hydrolysis experiments.

The rest of the reagents used in the experiments (supplied by Panreac, Barcelona, Spain) were analytical grade or better.
2.2. Preparation of the substrate emulsion

The tributyrin emulsions were prepared at a fixed volume fraction of 0.05, and afterwards diluted with a phosphate buffer solution to the final desired concentration. The emulsification was carried out by mechanical dispersion using an UltraTurrax® stirrer (Ika-Werke, Staufen, Germany) at 13,000 rpm for 1 min in glass beakers.

2.3. Enzymatic hydrolysis of tributyrin

The enzymatic hydrolysis of tributyrin was performed at pH 7.0 and different temperatures (30, 40, 50 and 60 °C). The pH-stat method [19] was used to monitor the reaction time course. Solutions of NaOH at concentrations of 0.05–1.0N, depending on the experiment performed, were used as the titrating agent. The titrations required by this methodology were conducted with the help of an automatic device (718 STAT Titrino from Metrohm, Herisau, Switzerland).

The hydrolysis reaction was started by placing 15 mL of the substrate emulsion in the reactor and, once the test temperature was reached, 1 mL of enzyme solution was added, while keeping the system continually stirred and thermodynamically controlled. The volume of the titrating agent added to the reaction vessel was recorded for 20 min. Each experiment was performed in triplicate. The volume fraction of tributyrin in the aqueous emulsion was changed between 0.0023 and 0.0469 and the enzyme concentration in the reaction medium was varied between 5.93 × 10⁻⁴ and 1.48 μmol/L.

To complement the results found with the pH-stat method, the composition of the reaction medium was analyzed using two other techniques:

(i) The colorimetric method for determining esterified fatty acids proposed by Stern and Shapiro [20] was adapted and employed to measure the concentration of glycerol monobutyrate (monobutyrin) in the aqueous phase. This technique is based on the reaction of carboxylic acid esters with hydroxylamine to form hydroxamic acids, which further cause a red color reaction with ferric chloride in acid medium. The colour development was measured in a Helios α spectrophotometer (Spectronic Analytical Inst., Leeds, UK) at 540 nm. Aqueous solutions of glycerol triacetate were used to make the corresponding calibration curve.

(ii) A qualitative TLC analysis of the reaction medium was performed to detect the presence of tributyrin at different times throughout the reaction. TLC plates were developed with hexane–diethyl ether (1:1, v/v) at ambient temperature. The plates were then air-dried, dipped in a 5% phosphomolybdic acid solution and heated to reveal the spots.

For the above-mentioned analyses, the reaction was stopped by adding 15 mL of hydrochloric acid (2 M) to the reaction medium and putting the mixture into boiling water for 10 min. Once cooled, the whole mixture was extracted with 3 × 10 mL of n-hexane. The resulting organic phase was then used for the qualitative determination of tributyrin by TLC, whereas the aqueous phase was analyzed for monobutyrin by the Stern and Shapiro method. As a control experiment an emulsion sample, before reaction, was subjected to the same procedure used for the reaction samples.

2.4. Estimation of the initial reaction rates

For the calculation of the initial reaction rates, the slope at time 0 of the hydrolysis curve (i.e., μmol of butyric acid released versus time) was estimated. For this, straight-line adjustments (by the least-squares method) were made of an increasing number of experimental points, until the value of the slope of these straight lines began to decrease [21]. This method proved to be the most appropriate one in a previous study [18]. The corresponding calculations were made by a program in MatLab®.

2.5. Conversion calculation

To quantify the reaction progress, we calculated the conversion as the fraction of hydrolyzed ester bonds at a certain time with respect to the total number of ester bonds initially present in the system. The number of hydrolyzed ester bonds can be calculated on knowing the volume of a standardized NaOH solution consumed over time to keep the pH constant at 7.0. The initial number of ester bonds will be three times the number of tributyrin molecules before the reaction, which can easily be calculated on knowing the volume fraction of tributyrin, its density and its molecular weight. All these considerations lead to Eq. (1), which was used to calculated conversion.

\[ x = \frac{n_{\text{but}} M_{\text{tb}}}{30v_{\text{tb}} \rho_{\text{tb}}} \]  

(1)

where \( n_{\text{but}} \) represents the moles of butyric acid released, \( M_{\text{tb}} \) the tributyrin molecular weight (302.37 g/mol), \( \alpha \) the volume fraction of tributyrin, \( v_{\text{tb}} \) the initial volume of the reaction mixture (15 + 1 mL) and \( \rho_{\text{tb}} \) is the density of tributyrin (1.035 g/mL). Obviously, conversion, as calculated from Eq. (1), should not be higher than 2/3, as TLL is an sn-1,3 specific lipase.

2.6. Droplet-size distribution of the tributyrin emulsions

The droplet-size distribution of the emulsions was determined by laser diffraction using a Coulter LS230 instrument (Beckman-Coulter, Miami, USA). This equipment is able to detect droplets ranging from 0.4 to 1000 μm in diameter. Measurements were performed before and after 20 min of reaction. The Sauter diameter (average volume/surface area), as supplied by the Coulter software, was used as the most representative mean diameter for the kinetic study. The average value of the Sauter diameter for all the emulsions prepared using the above-specified method was 5.88 μm. On ascertaining the Sauter diameter (\( d_{50} \)) the total specific interface area of the emulsion (\( \alpha_{r} \)) can be calculated from Eq. (2).

\[ \alpha_{r} = \frac{6\alpha}{d_{50}} \]  

(2)

3. Model development

3.1. Mechanism of the process

According to Martinelle et al. [22] and our previous results [18], T. lanuginosus lipase exhibits a pronounced interfacial activation, and consequently the hydrolysis of dissolved tributyrin can be considered negligible. Therefore, for the hydrolysis to take place, the enzyme must make contact with an ester bond located at the surface of the tributyrin droplets. The mechanism of this reaction is considered to occur in three stages:

(1) Adsorption or penetration of the enzyme in the aqueous–organic interface, involving the formation of the “activated” enzyme. This step was assumed to conform to the Langmuir isotherm, which implies that the enzyme–adsorption speed is proportional to the dissolved enzyme concentration and to the amount free interface.

\[ \frac{k_{r}}{k_{d}} \]  

(3)
(2) Joining of the “activated” enzyme with a hydrolysable surface ester bond and formation of the enzyme–substrate complex. This complex is an interfacial analogue of the Michaelis–Menten complex in homogeneous enzyme kinetics.

\[ E^* + S^* \rightleftharpoons E^*S \tag{4} \]

(3) Separation of the intermediate formed in the products and release of the “activated” enzyme. This step was considered to be irreversible under the experimental conditions used, due to the instantaneous ionization of the butyric acid released.

\[ E^*S + H_2O \rightarrow E^* + S'OH + AH \tag{5} \]

In Eqs. (3)–(5), E represents the free enzyme, E* the activated (adsorbed) enzyme, a the free interfacial area of the emulsion, S’ a free hydrolysable surface ester bond, E’S the interfacial enzyme–substrate complex, S’OH the diglyceride formed at the interface and AH the butyric acid released in the hydrolysis. The corresponding elemental kinetic constants are \( k_a, k_{d}, k_1, k_{-1}, \) and \( k_2. \)

The butyric acid released is assumed to dissociate almost completely and to dissolve into the aqueous phase, due to its relatively high dissociation constant when compared to those of long chain fatty acids. Accordingly, the inhibition by fatty acid was not considered for the development of the model.

3.2. Arrangement of surface ester groups in the tributyrin droplets

The hydrolysis of emulsified tributyrin implies the direct contact between the enzyme and the ester bonds located on the surface of the tributyrin droplets, and thus accessible to the enzyme. The tetrahedral arrangement of the central carbon of glycerol in the tributyrin molecule and the three n-butyl hydrophobic groups bonded to its three positions suggest that the tributyrin molecule will appear more frequently with the edge formed by the hydrogen and an ester group in contact with the aqueous phase, Fig. 1(a). That is, only one of the ester groups is accessible from the aqueous phase.

The same reasoning indicates that when one of the ester group in the outer positions (sn-1 or 3) is hydrolyzed, the diglyceride formed will be retained in the organic phase, and, if it is found on the surface, will be preferentially arranged with the hydrogen and the free alcohol group towards the aqueous phase, orientating its remaining ester groups towards the core of the organic phase, as indicated in Fig. 1(b). Eventually, the remaining ester groups will become accessible to the enzyme due to the molecular movement, so the hydrolytic process will continue, but probably more slowly.

This implies that under normal conditions the hydrolysis reaction will proceed quickly up to one-third of the ester groups present in the emulsion, and after that it will go on more slowly. This conclusion is also suggested by the experimental results, as can be seen in Fig. 2, where the reaction-progress curve at different lipase concentrations is plotted up to 20 min. As the lipase concentration increases, the initial reaction rate follows the same trend, but the conversion at 20 min is consistently lower than approximately 0.33.

3.3. Variation of droplet-size during hydrolysis

If one admits that the diglyceride and a small fraction of the butyric acid released remain in the organic phase, it can be accepted, in principle, that the volume of the droplets will be only slightly affected by the hydrolytic process, in the same way as the volume fraction of the organic phase and the specific interfacial area of the emulsion. However, if one compares the droplet-size distribution (DSL) of the substrate emulsions before and after 20 min of reaction at two different enzyme concentrations (Fig. 3), a clear shift to the left of the DSL can be appreciated, indicating a decrease in the mean droplet diameter.

Given that in a control experiment without enzyme (data not shown), the DSL remained unaltered for 20 min under reaction conditions, the aforementioned decrease can be attributed only to the hydrolytic process. Interestingly, this variation was not reflected in the mean Sauter diameter of the emulsions, as supplied by the Coulter software, which remained practically constant or increased slightly after the reaction, as reflected in Table 1, where the Sauter diameters of the tributyrin emulsions before and after the reaction are listed for different lipase concentrations and tributyrin volume fractions. The increase in the Sauter diameter reflected in Table 1 may be caused by the relatively higher abundance of the droplets of greater size (above 20 µm approx.) after hydrolysis (see Fig. 3),

![Fig. 1](image1.png)

**Fig. 1.** Arrangement of the tributyrin molecule on the interface between the aqueous phase (W) and the organic phase (T) before (a) and after (b) the hydrolysis of an ester bond. \( R \) represents each one of the three n-butyl groups of the tributyrin molecule.

![Fig. 2](image2.png)

**Fig. 2.** Time course of the hydrolysis of tributyrin (0.0094, v/v) with different lipase concentrations (\( e_0 \)) at 35 °C. Conversion was calculated according to Eq. (1).
which can be explained by the generation of a significant amount of droplets smaller than 0.4 μm and thus undetectable for the equipment used. In any case, the observed behaviour indicates that the formation of monobutyrin may begin at relatively low conversions, even below one-third of the total ester bonds. As monobutyrin is fairly soluble in water it will distribute mainly in the aqueous phase, which would explain the shift of the DSD to the left.

The hydrolysis process can hence be considered to proceed in two different stages, which can partially overlap. The first one consists of the hydrolysis of an outer ester group of the surface molecules of tributyrin, which leads to the formation of glycerol dibutyrate (dibutyrin) remaining in the organic phase. As the concentration of surface molecules of tributyrin decreases, the hydrolysis of the remaining outer ester group of the dibutyrin molecules will begin, this stage being much slower than the first one, because it requires an appropriate arrangement of the surface molecules of dibutyrin.

3.4. Development of the reaction-rate equation

The reaction-rate equation can be developed from the three-step mechanism previously described by applying the stationary-state approximation to both intermediates in which the enzyme participates, $E$ and $E'S$, and considering both the enzyme and the interfacial-area balances (Eqs. (6) and (7), respectively), as the interfacial area of the emulsions limits the reaction rate when saturating enzyme concentrations are attained.

\[
e_0 = [E] + a_T([E'] + [E'S])
\]

(6)

\[
a_T = a + A_m a_T([E'] + [E'S])
\]

(7)

where $e_0$ represents the total concentration of enzyme, $a_T$ the total specific interfacial area of the emulsion and $A_m$ is the enzyme coverage, i.e., the interfacial area occupied by one enzyme molecule.

Operating in this way, it is possible to get to Eq. (8),

\[
r = \frac{k_2 e_0 [S^*]}{(K_{M}^{a} / K_{e} a a_T) + K_{M}^{a} + [S^*]}
\]

(8)

where the following constants have been introduced:

- $K_{m}^{a}$, Michaelis–Menten constant for the interfacial enzyme–substrate complex

\[
K_{M}^{a} = \frac{k_{-1} + k_{2}}{k_{1}}
\]

(9)

- $K_{e}$, equilibrium constant associated with the adsorption of the enzyme

\[
K_{e} = \frac{k_{4}}{k_{d}}
\]

(10)

During the initial stages of the reaction, the enzyme concentration is lower than that of surface ester bonds (see [18] for further details), and therefore we can assume that $[S^*] \gg [E'S]$ and thus $[S^*] = c_{s0}^*$ (which represents the total interfacial concentration of surface ester bonds). It should be pointed out that $c_{s0}^*$ is a constant value in our system, as it depends only on the chemical nature of the oily phase and on the physicochemical properties of the aqueous phase (pH, ionic strength and temperature), as previously stated by Verger et al. [6]. Accordingly, Eq. (8), when applied to initial reaction rates, can be rewritten as follows:

\[
r_0 = k_{ap} \frac{e_0}{1 + (1/K_{ap} a_T y_a)}
\]

(11)

where two new parameters, $k_{ap}$ and $K_{ap}$, have been introduced to designate the following sets of constants:

\[
k_{ap} = \frac{k_{2} c_{s0}^*}{K_{M}^{a} + c_{s0}^*}
\]

(12)

\[
K_{ap} = \frac{K_{e} (K_{M}^{a} + c_{s0}^*)}{K_{M}^{a}}
\]

(13)

As well as the fraction of free interfacial area:

\[
y_a = \frac{a}{a_T}
\]

(14)

Considering that $K_{M}^{a}$ and $c_{s0}^*$ will probably be of the same order of magnitude, as is the case in homogeneous enzyme kinetics, the kinetic parameters $k_{ap}$ and $K_{ap}$ virtually represent the kinetic constant $k_2$ and the equilibrium constant of the enzyme-adsorption, respectively, as can be deduced from Eqs. (12) and (13).
Operating with the interfacial area balance (Eq. (7)), we find that the fraction of free interfacial area is given by:

\[ y_a = \frac{1}{2} \left( 1 - \frac{1}{K_{ap} a_T^2} + 4 \frac{A_m e_0}{a_T} \right)^{1/2} \]  

(15)

Finally, the initial reaction rate, by substituting Eq. (15) in Eq. (11), is given by:

\[ r_0 = \frac{k_{ap} e_0}{1 + 2/K_{ap} a_T^2 \left[ 1 - (1/K_{ap} a_T^2) - (A_m e_0/a_T) \right]} \]

\[ + \sqrt{\left[ 1 - (1/K_{ap} a_T^2) - (A_m e_0/a_T)^2 \right] + (4/K_{ap} a_T^2)}} \]

(16)

The former equation describes the variation of the initial reaction rate with the enzyme concentration and the specific interfacial area of the emulsion, and can be used to estimate the kinetic parameters \( K_{ap}, K_{db} \) and \( A_m \).

Alternatively, Eq. (16) can be deduced from the set of equations proposed by Tsai and Chang [12], if the parameter \( f \), which is defined by the authors as the volume fraction of the oily phase, is set to one.

3.5 Applying the kinetic model to predict the reaction time course

The conversion referring to the initial concentration of ester bonds in the emulsion can be expressed as:

\[ x = \frac{3c_0 - c_e}{3c_0}, \quad c_e = 3c_0(1 - x) \]

(17)

where \( c_0 \) represents the initial molar concentration of tributyrin and \( c_e \) stands for the total concentration of non-hydrolyzed ester bonds in the system, from which the reaction rate can be expressed as:

\[ r = -\frac{dc_e}{dt} = 3c_0 \frac{dx}{dt} \]

(18)

The molar concentration of tributyrin in the system can be calculated on knowing its volume fraction, density and molecular weight, using Eq. (19).

\[ c_0 = \frac{\rho_{TB} V_{TB}}{M_{TB}} = 3.42 \alpha \text{ (mol/L)} \]

(19)

If we admit that the ester group of position 2 is not hydrolyzed, since the enzyme is sn-1,3-specific, at each time, it holds that:

\[ c_0 = c_{TB} + c_{DB} + c_{MB} \]

(20)

\[ c_e = 3c_{TB} + 2c_{DB} + c_{MB} \]

(21)

where \( c_{TB}, c_{DB} \) and \( c_{MB} \) are, respectively, the molar triglyceride, diglyceride and monoglyceride concentrations in the emulsion.

Assuming, at least in principle, that in the first stage of the hydrolysis reaction (up to a conversion of approximately 1/3), practically no monoglyceride was formed, Eqs. (20) and (21) can be simplified to:

\[ y_a = \frac{1}{2} \left( 1 - \frac{1}{K_{ap} a_T^2} - \frac{c_{MB}}{c_0} + \frac{c_{DB}}{c_0} \right) \]

\[ + \left[ 1 - \frac{1}{K_{ap} a_T^2} - \frac{c_{MB}}{c_0} + \frac{c_{DB}}{c_0} \right] \]

(22)

\[ y_a = \frac{1}{2} \left( 1 - \frac{1}{K_{ap} a_T^2} + 4 \frac{A_m e_0}{a_T} \right)^{1/2} \]

(23)

Operating with Eqs. (22) and (23) to eliminate the dibutyrin concentration, we get:

\[ c_{TB} = c_e - 2c_0 = c_0(1 - 3x) \]

(24)

and therefore,

\[ c_{DB} = 3c_0x \]

(25)

However, during the hydrolysis process, only the interfacial concentrations are relevant, so Eqs. (24) and (25) should be expressed in terms of interfacial concentrations. To do that, the balance of surface ester bonds during the first stage of the reaction, Eq. (26), should be taken into account.

\[ c_{20} = [S^*] + [E'S^*] + [S'OH] \]

(26)

where \([S^*]\) and \([S'OH]\) represent the interfacial concentration of surface ester bonds and surface alcohol groups, respectively.

The balance given by Eq. (26) can be further simplified considering that, under our experimental conditions, the molar concentration of surface ester bonds is at least 20 times higher than the molar concentration of enzyme [18]; and, consequently, it can be assumed that \([E'S^*] \approx [S^*] + [S'OH] \). Thus, admitting that the droplet composition is uniform during the hydrolysis, Eqs. (24) and (25) can be rewritten as:

\[ [S^*] = c_0(1 - 3x) \]

(27)

\[ [S'OH] = 3c_0x \]

(28)

Still, it is very possible that the diglyceride, once formed, tends to accumulate at the interface due to its higher hydrophilicity, despite the fact that according to the results of Hermansyah et al. [15], the adsorption constant for diolein is surprisingly lower than that of triolein, but the corresponding constant for monoolein is the highest one, as logically expected.

The accumulation of the diglyceride at the interface can be included in the model equations by introducing a surface/bulk partition coefficient for dibutyrin, \( K_r \), which will obviously be greater than one. With the introduction of this partition coefficient, Eq. (28) should be rewritten as:

\[ [S'OH] = 3c_0x K_r \]

(29)

Considering Eq. (29) together with the balance of surface ester bonds, Eq. (26), we get:

\[ [S^*] = c_0(1 - 3x) \]

(30)

Finally, replacing Eq. (30) in Eq. (8), we obtain the following expression for the reaction rate:

\[ r = \frac{k_{ap} e_0(1 - 3x)}{1/K_{ap} a_T^2 y_a + (K_{M} + c_{DB}(1 - 3x))/(K_{M} + c_{DB})} \]

(31)

The fraction of free interfacial area, \( y_a \) (i.e., \( a/a_T \)), can be calculated from Eq. (15), but replacing \([S^*]\) according to Eq. (30), which leads to:

\[ \frac{c_{MB}}{c_0} = \frac{K_{M} + c_{DB}}{K_{M} + c_{DB}(1 - 3x)} \]

\[ + \left[ 1 - \frac{1}{K_{ap} a_T^2} - \frac{c_{MB}}{c_0} + \frac{c_{DB}}{c_0} \right] \]

\[ + \left[ 1 - \frac{1}{K_{ap} a_T^2} - \frac{c_{MB}}{c_0} + \frac{c_{DB}}{c_0} \right] \]

(32)

Operating with Eqs. (18), (19), (31) and (32), and introducing the dimensionless parameter \( \beta \), defined by Eq. (33), we finally get to Eq. (34), which expresses the reaction rate as a function of the conversion, the model parameters being \( k_{ap}, K_{ap}, A_m \) and \( \beta \).

\[ \beta = \frac{c_{MB}}{K_{M} + c_{DB}} \]

(33)

\[ \frac{dx}{dr} = \frac{k_{ap} e_0/10.26(1 - 3x)}{G + (2/K_{ap} a_T^2 y_a^2) - (A_m e_0/a_T) \left[ 1 - (G/K_{ap} a_T^2) - (A_m e_0/a_T) \right] + (1 - (G/K_{ap} a_T^2) - (A_m e_0/a_T))^2 + 4(G/K_{ap} a_T^2) \]^{1/2}} \]

(34)
The symbol $G$ in Eq. (34) stands for the following expression:

$$G = 1 - \beta + \beta (1 - 3K_x x)$$  \hspace{1cm} (35)

Eq. (34) can be used to simulate the progress of the reaction over time using the values of the model parameters calculated from the fit of the initial reaction rates, and testing several values of $K_x$. The value of the dimensionless parameter $\beta$ was considered to be 0.5, since $c_{\alpha 0}$ was assumed to have a constant value in our system [6] and, by analogy with homogeneous systems in which the Michaelis–Menten constant represents the substrate concentration required to reach one-half maximum velocity, $K_x$ should be of the same order of magnitude as $c_{\alpha 0}$. The theoretical simulations were carried out with the help of a computer program developed with the MatLab® software package.

4. Results and discussion

4.1. Influence of gum arabic on lipase interfacial action

As gum arabic was used to stabilize all the emulsions prepared, it makes sense to analyze its possible effect on lipase adsorption at the oil–water interface. In a previous work [23], the lipase from T. lanuginosus was found to be more active (4.3-fold) on tributyrin when it was pre-emulsified with gum arabic. Similar results were obtained by Tiss et al. [24]. Therefore, gum arabic not only shows no significant interference with lipase contact, but exerts a positive effect on lipase activity, which can be attributed to the increase in the interfacial area of the tributyrin emulsion, which is a key factor for lipase action.

4.2. Initial reaction rates

The variation of the initial reaction rate with enzyme concentration as well as with the volume fraction of tributyrin at 30 °C is shown in Fig. 4. As can be seen, initial reaction rate reached a maximum asymptotic value for an enzyme concentration higher than 0.3 μmol/L when the volume fraction of tributyrin is equal to 0.0094. However, at a higher volume fraction of tributyrin (0.0188), the initial reaction rate increased linearly with the enzyme concentration up to 0.6 μmol/L. Therefore, the maximum rate attained depends on the interfacial area of the emulsion, indicating that the saturation of the interface due to enzyme-adsorption may occur under our experimental conditions. Similar results were found at all the temperatures assayed, as reflected in Fig. 5.

Applying the kinetic model previously developed to these results, by using a program in MatLab®, we found the optimum values of the three model parameters ($k_{ap}$, $K_{ap}$ and $A_m$), i.e., those values which minimize the sum of the absolute value of the residuals. The excellent agreement between the observed and predicted initial rates can be seen in Fig. 4 (results for 30 °C) and Fig. 5 (all other temperatures). The model satisfactorily predicts the saturation phenomenon observed when the enzyme concentration is increased while keeping constant the volume fraction of tributyrin (Fig. 4a). An analogous behaviour, which is also predicted by the model, was found when the volume fraction of tributyrin, and hence the specific interfacial area of the emulsion, was varied while keeping constant the enzyme concentration (Fig. 4b).

The resulting values of the model parameters are shown in Table 2 together with the relative deviation between the predicted and observed reaction rates, as calculated according to Eq. (36). It is remarkable that the relative deviation never exceeded 5%.

$$\text{rel. dev.} (\%) = \frac{\sum |(r_{\text{obs}} - r_{\text{cal}})|}{\sum r_{\text{obs}} \times 100} \hspace{1cm} (36)$$

![Fig. 4. Initial rates of tributyrin hydrolysis at 30 °C and pH 7.0. The experimental (points) and predicted (solid lines) results are compared. The initial reaction rate was studied as a function of (a) enzyme concentration, $e_0$, and (b) tributyrin volume-fraction, $\alpha$.](Image 443x644 to 680x944)

![Fig. 5. Influence of lipase concentration on the initial rate of tributyrin hydrolysis at temperatures between 40 and 60 °C, pH 7.0 and $\alpha=0.0094$. The experimental (points) and predicted (lines) results are shown (40 °C, full line; 50 °C, long dashed line; 60 °C, short dashed line).](Image 445x428 to 678x583)

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>$k_{ap}$ (s$^{-1}$)</th>
<th>$K_{ap}$ (L$^2$/cm$^4$)</th>
<th>$A_m$ (cm$^2$/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>4.8</td>
<td>1559</td>
<td>5.059 × 10$^{-9}$</td>
</tr>
<tr>
<td>40</td>
<td>3.6</td>
<td>1821</td>
<td>3.542 × 10$^{-9}$</td>
</tr>
<tr>
<td>50</td>
<td>3.4</td>
<td>1988</td>
<td>2.038 × 10$^{-9}$</td>
</tr>
<tr>
<td>60</td>
<td>4.8</td>
<td>2005</td>
<td>1.093 × 10$^{-9}$</td>
</tr>
</tbody>
</table>
To estimate the enzyme coverage \( (A_m) \), we calculated the average value of those found at different temperatures (see Table 2), resulting in a value of \( 2.98 \times 10^{11} \text{ cm}^2/\text{mol} \) or \( 4950 \text{ Å}^2/\text{molecule} \). This result is of the same order of magnitude as, although slightly higher than, that reported by Martinelle et al. [22] for the same enzyme, of \( 3400 \text{ Å}^2/\text{molecule} \). The higher value of \( A_m \) found in this study can be explained considering that, in our system, the active site of the enzyme is occupied by a substrate molecule, which may slightly increase the cross-sectional area of the enzyme. On the contrary, Martinelle et al. [22] studied the adsorption of TLL on a polystyrene surface, where the enzyme active site remained unoccupied. Similar values of enzyme coverage can be found in literature [25].

4.3. Influence of temperature on the rate and equilibrium constants

The rate constant \( k_{ap} \) increased with temperature in the range from 30 to 50 °C, but it appeared to remain almost constant between 50 and 60 °C. This tendency can be attributed to the greater importance of the deactivation of TLL at 60 °C [26]. The temperature dependence of the rate constant \( k_{ap} \) between 30 and 50 °C, when fitted to the Arrhenius equation gives an apparent activation energy \( (\Delta E) \) of 9.93 kJ/mol. The value found for the activation energy of the reaction is of the same order of magnitude as that reported by Al-Zuhair et al. [27] for the hydrolysis of palm oil by lipase of 5.02 kJ/mol. In addition, it proves comparable with the values found by other authors for the enzymatic hydrolysis of different triglycerides, both in emulsion, 8.15 kJ/mol [28] and in reverse micelles, 29.3 kJ/mol [29] or 19.7 kJ/mol [30]. These results suggest that the interfacial curvature may affect the decomposition of the activated enzyme–substrate complex, leading to an increase in the activation energy (by two- or three-fold) in micellar systems when compared to emulsions. In reverse micellar systems, such as those used in studies [29] and [30], lipases are confined in water pools of a few-nanometer size (2–20 nm according to Bohidar and Behboudnia [31] for the system water–AOT–isooctane). This diameter is considerably lower than the mean droplet-diameter commonly found in O/W emulsions (one to several hundreds microns).

Moreover, the variation of the equilibrium constant \( K_{ap} \), with temperature was fitted to the van’t Hoff equation, which allowed us to determine the enthalpy change for enzyme-adsorption \( (\Delta H_\text{a}) \). The fit was quite satisfactory, with an adsorption enthalpy of −43.1 kJ/mol. This value, higher than those typical of a physical adsorption process, is consistent with the fact that lipase interfacial adsorption implies a conformational change in the enzyme, commonly referred to as “activation”.

4.4. Application of the model to previously published data

For a better validation of the proposed model, it has been fitted to the initial reaction rates reported by several authors who have studied the enzymatic hydrolysis of triglycerides in aqueous emulsions. Results reported by Ekiz et al. [11], who conducted the hydrolysis of tributyrin using a lipase from \( \text{Candida cylindracea} \), Albasi et al. [13], for sunflower oil hydrolysis by \( \text{C. cylindracea} \) lipase, and Al-Zuhair et al. [14], for palm oil hydrolysis by \( \text{Candida rugosa} \) lipase, have been plotted in Fig. 6 together with the initial reaction rates predicted by the model proposed in this study, showing good agreement between them. All these studies were performed using a stirred tank reactor, and the specific interfacial area of the emulsion was determined by means of empirical correlations, such as those of Calderbank [17] and Tablarides and Bapat [32].

A program in Matlab was used to calculate the optimum value of the model parameters \( (k_{ap}, K_{ap} \text{ and } A_m) \) in each case. The estimated values are listed in Table 3 together with the relative deviation (Eq. (36)) and the main features of each work. Studies [13] and [14] do not declare the protein content of the commercial enzyme-preparation they used, therefore, in order to make the estimates more comparable, enzyme concentration has been expressed in grams of commercial product by unit volume of the reaction mixture. Doing so, the values found for the rate constant \( (k_{ap}) \) and for the enzyme coverage \( (A_m) \) in fact represent the product of these constants by the fraction of active enzyme in the commercial product. Therefore, the ratio \( k_{ap}/A_m \), which is also included in

![Fig. 6. Model (Eq. (16)) fittings of the results reported by Ekiz et al. [11] (a), Albasi et al. [13] (b) and Al-Zuhair et al. [14] (c). The specific interfacial areas are indicated. Solid lines represent model predictions. For more details on these studies see Table 3.](image-url)
Table 3
Model (Eq. (16)) parameters calculated with the results reported by Ekiz et al. [11], Albasi et al. [13] and Al-Zuhair et al. [14]

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Substrate</th>
<th>Lipase</th>
<th>T (°C)</th>
<th>(k_{ap}) (mol/(g s))</th>
<th>(K_{ap}) (L²/cm⁴)</th>
<th>(A_{m}) (cm²/g enz. prep.)</th>
<th>(k_{ap}/A_{m}) (g/(s cm²))</th>
<th>Rel. dev. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[11]</td>
<td>Tributyrin</td>
<td>Candida cylindracea</td>
<td>35</td>
<td>(7.11 \times 10^{-4})</td>
<td>(7.88 \times 10^{-9})</td>
<td>(5.05 \times 10^{4})</td>
<td>(1.41 \times 10^{-8})</td>
<td>3.4</td>
</tr>
<tr>
<td>[13]</td>
<td>Sunflower Oil</td>
<td>Candida cylindracea</td>
<td>37</td>
<td>(1.73 \times 10^{-2})</td>
<td>(1.33 \times 10^{-9})</td>
<td>(1.30 \times 10^{5})</td>
<td>(1.33 \times 10^{-7})</td>
<td>9.9</td>
</tr>
<tr>
<td>[14]</td>
<td>Palm Oil</td>
<td>Candida rugosa</td>
<td>45</td>
<td>(6.29 \times 10^{-3})</td>
<td>(1.09 \times 10^{-9})</td>
<td>(7.52 \times 10^{5})</td>
<td>(8.37 \times 10^{-11})</td>
<td>8.8</td>
</tr>
<tr>
<td>This study</td>
<td>Tributyrin</td>
<td>Thermomyces lanuginosus</td>
<td>40</td>
<td>(1.64 \times 10^{-1})</td>
<td>(6.43 \times 10^{-9})</td>
<td>(2.76 \times 10^{5})</td>
<td>(5.95 \times 10^{-9})</td>
<td>2.3</td>
</tr>
</tbody>
</table>

For an easier comparison, the kinetic parameters found in this study are also included.

Table 3, should not be affected by the way of expressing the enzyme concentration, as well as the equilibrium constant \(K_{ap}\). As expected, the values found for \(A_{m}\) (cm²/g enz. prep.) and, particularly, for \(K_{ap}\) are similar to each other and to those calculated with our own data. However, the rate constant \(k_{ap}\) and consequently the ratio \(k_{ap}/A_{m}\) are relatively higher for the results of Albasi et al. [13], especially when compared to the estimate found for the results reported by Al-Zuhair et al. [14], although both groups used a commercial vegetable oil as the substrate and the specific interfacial area of the emulsion is very similar (Fig. 6). It should be pointed out that the initial reaction rates reported by Albasi et al. are considerably higher (see Fig. 6 for a better comparison) than those reported by the rest of the authors, particularly if one considers that commercial vegetable oils always contain a small amount of free fatty acids which can act as surface-active agents, thus decreasing lipase activity [25,33]. This fact is consistent with the values reported by Al-Zuhair et al. [14]. Moreover, the smaller values of \(K_{ap}\) for sunflower oil and palm oil, compared to those found for tributyrin, may also reflect a less efficient adsorption of the lipase on the oil/water interface due to the presence of free fatty acids already absorbed onto it. It is also remarkable the similarity of the model parameters calculated with our own results and with those of Ekiz et al. [11], which also used tributyrin as the substrate but no stabilizer to prepare the emulsions. This fact may indicate that gum arabic, which we used as a stabilizer, does not interfere significantly with lipase action at O/W interfaces.

4.5. Reaction time-course
Initially, Eq. (34) was used to predict the progress of the hydrolysis reaction with \(\beta\) equal to 0.5 and letting \(K_r\) equal to 1, i.e., assuming no accumulation of diglyceride at the interface. On doing so, the agreement between experimental and predicted results was highly satisfactory at low enzyme concentrations (data not shown). However, at high (saturating) enzyme concentrations the predicted results significantly deviate from the observed ones, particularly as the conversion approaches one-third. When other values of the parameter \(\beta\) were assayed, always within the range from 0 to 1, the same tendency prevailed, and no significant improvement was achieved.

If the effect of enzyme deactivation is neglected, since it was found not to be significant at 30 °C in a previous work [26], the accumulation of dibutyrin at the surface must be assumed in order to explain the observed behaviour. To do that the partition coefficient \(K_r\) must be set to a value greater than one. The corresponding simulations (with \(\beta = 0.5\)) are shown in Fig. 7, where the corresponding experimental results have also been plotted. A good agreement between predicted and observed values can be seen, regardless of

![Fig. 7](image-url) Simulations according to Eq. (34) of the time course of tributyrin hydrolysis (\(\alpha = 0.0094\)) at 30 °C and different lipase concentrations: (a) \(5.93 \times 10^{-4}\) μmol/L; (b) \(2.97 \times 10^{-2}\) μmol/L; (c) \(2.97 \times 10^{-1}\) μmol/L; (d) \(5.93 \times 10^{-1}\) μmol/L. Solid points correspond to the experimental results. Error bars represent ± the standard deviation of three replicates.
the enzyme concentration used, until conversion reaches approximately 0.25. Once the interface is saturated with dibutyrin the hydrolysis of the remaining outer ester-bond becomes significant, which may explain the deviations occurring at conversions higher than 0.25. This second stage of the reaction, considerably slower than the first one, was not considered when developing Eq. (34), causing the predicted conversions to diverge from the experimental ones when approaching 1/3.

However, it was not possible to find a unique value of the partition coefficient $K_r$ valid for the entire range of enzyme concentrations assayed. On the contrary, $K_r$ followed an increasing trend with enzyme concentration, as can be seen in Fig. 8, and attained a maximum for saturating enzyme concentrations ($e_0 > 2.97 \times 10^{-1} \mu\text{mol/L}$). Thus, the rise in the partition coefficient paralleled that of the reaction rate, reaching a maximum also when the reaction rate reached its highest possible value at a given volume fraction of tributyrin. This result suggests that the difference in composition between the bulk and the surface of the droplet is more accentuated when reaction rate increases up to its maximum value. Under such conditions the diffusion rate of the diglyceride from the surface of the droplets to the bulk and that of the triglyceride in the opposite direction may not be sufficient to ensure the homogeneity of the droplet composition. Thus, the higher the generation rate of dibutyrin at the surface the greater will be its accumulation rate at the interface.

An appropriate model for such a situation would have to include the equations describing diffusion in spherical coordinates. However, an adequate approximation to the aforementioned effect is to express the partition coefficient as an empirical function of enzyme concentration, as in Eq. (37).

$$K_r = 1.30 - 0.30 \exp(-9.0 \times 10^6 e_0)$$

(37)

In summary, the above-discussed results suggest that both the greater tendency of the diglyceride to remain attached to the surface as well as the diffusional limitations occurring at high reaction rates determine the accumulation of the diglyceride at the interface and thus a change in the reaction mechanism before conversion reaches 1/3. Once the interface is saturated with diglyceride, which happens in a very short time at high enzyme concentrations, the generation of monoglyceride, a slower reaction stage, will predominate, causing the DSD to move to the left, i.e., towards smaller droplet-sizes.

To confirm such hypotheses, two different hydrolysis experiments were planned, so that similar conversions were attained using different enzyme concentrations:

(i) Experiment (a): Short-duration experiment at high (saturating) enzyme concentrations ($0.2967 \mu\text{mol/L}$). After 4 min, the reaction was stopped and monobutyrin concentration was measured by colorimetry.

(ii) Experiment (b): Long-duration experiment at low (non-saturating) enzyme concentrations ($0.0059 \mu\text{mol/L}$). Monobutyrin concentration was measured by colorimetry at 45 min (experiment b.1) and 185 min (experiment b.2). In both cases, the reaction medium was also analyzed for tributyrin by TLC.

When the amount of monobutyrin produced and the number of hydrolyzed ester bonds are known, it is possible to calculate the distribution of glycerides in the system, which is presented in Table 4. To arrive at these results it has been assumed that, when extracting with n-hexane, monobutyrin completely dissolves in the aqueous phase whereas dibutyrin is fully retained in the organic phase. Therefore, figures in Table 4 should be considered only as estimates. Reaction progress during experiments (a) and (b) is also shown in Fig. 9, where the arrows indicate the moment when the reaction was stopped and the above-mentioned determinations were performed. As can be seen, reaction progresses fast at the beginning, particularly at high enzyme concentrations, experiment (a), until an abrupt change in slope occurs and a constant rate, but much slower than the initial one, is attained. As can be concluded from

![Fig. 8. Variation of the partition coefficient for the diglyceride, $K_r$, with enzyme concentration. The solid line represents the fitting of the experimental data to Eq. (37).](image)

![Fig. 9. Reaction progress curves for experiments (a) (□) and (b) (○). Arrows indicate the reaction point which corresponds to the results shown in Table 4 and Fig. 10.](image)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Temperature (°C)</th>
<th>$e_0$ (µmol/L)</th>
<th>$\alpha$ (v/v)</th>
<th>Reaction time (min)</th>
<th>Hydrolyzed bonds (%)</th>
<th>Ester bonds as tributyrin (%)</th>
<th>Ester bonds as dibutyrin (%)</th>
<th>Ester bonds as monobutyrin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>30</td>
<td>0.2967</td>
<td>0.0094</td>
<td>4</td>
<td>27.1</td>
<td>20.3</td>
<td>52.1</td>
<td>0.5</td>
</tr>
<tr>
<td>b.1</td>
<td>30</td>
<td>0.0059</td>
<td>0.0094</td>
<td>45</td>
<td>21.2</td>
<td>37.3</td>
<td>41.2</td>
<td>0.3</td>
</tr>
<tr>
<td>b.2</td>
<td>30</td>
<td>0.0059</td>
<td>0.0094</td>
<td>185</td>
<td>27.8</td>
<td>36.4</td>
<td>28.1</td>
<td>6.6</td>
</tr>
</tbody>
</table>

The three last columns indicate the percentage of ester bonds forming part of tri-, di- and monobutyrin molecules, respectively.
O/W emulsions has been developed and applied to the initial reac-
5. Conclusions
main process.
This fact confirms that, during the second reaction stage, the hydrol-
tributyrin are present in the reaction medium at 45 and 185 min.
of the TLC analysis indicate that significant and similar amounts of
ered appropriate to identify the presence of tributyrin. The results
varies with tributyrin concentration (data not shown) it was consid-
tributyrin does not react with phosphomolybdic acid, a white spot
non-hydrolyzed sample as a control, is shown in Fig. 10. Although
The corresponding TLC plate, which also includes the result of a
control experiment (emulsion) are also shown. Black arrows point to the white
spots which denote the presence of tributyrin.

Fig. 10. TLC plate indicating the presence of tributyrin after 45 and 185 min of hydrolysis with a lipase concentration of 0.0059 μmol/l. (experiment b). The results of a control experiment (emulsion) are also shown. Black arrows point to the white spots which denote the presence of tributyrin.

the results shown in Table 4 until the end of the first reaction stage, points “a” and “b.1” in Fig. 9, monobutyrin is scarcely produced. This stage probably finishes when the interface is saturated with dibu-
tryrin, which happens at higher conversions at saturating enzyme concentrations (0.27 in experiment [a] compared to 0.21 in experiment [b]). From that moment on, formation of monobutyrin from
dibutyrin predominates, so that the amount of tributyrin present in the system remains almost unaltered (see Table 4, experiment b.2).

This finding was also confirmed by TLC analysis of the reaction medium at the end of each reaction stage in experiment (b). The corresponding TLC plate, which also includes the result of a non-hydrolyzed sample as a control, is shown in Fig. 10. Although tributyrin does not react with phosphomolybdic acid, a white spot appears on the TLC plate after heating. Since the size of this spot varies with tributyrin concentration (data not shown) it was consid-
ered appropriate to identify the presence of tributyrin. The results of the TLC analysis indicate that significant and similar amounts of tributyrin are present in the reaction medium at 45 and 185 min. This fact confirms that, during the second reaction stage, the hydrol-
ysis of surface molecules of dibutyrin to yield monobutyrin is the main process.

5. Conclusions
A kinetic model for the enzymatic hydrolysis of triglycerides in O/W emulsions has been developed and applied to the initial reaction rates of the lipase-catalyzed hydrolysis of tributyrin as well as
to the reaction time-course, which is an issue barely addressed in the current literature.
As regards the initial reaction rates, the model gives a satis-
factory fit, with relative deviations equal or lower than 5%, not
only to our experimental results but to previously published ones.
Although tributyrin, a synthetic triglyceride was used as the sub-
strate, our model can also be applied to natural triglycerides of
greater practical importance, as can be concluded from the satis-
factory fit of results for sunflower oil [13] and palm oil [14]. It
should be pointed out that the three model parameters offer key
information on the system behaviour and properties, such as:
- the rate of the catalytic step (k_{ap});
- the affinity of the enzyme for the interface (K_{ap});
- the enzyme interfacial-coverage (A_{m}).

The proposed kinetic model also succeeded in predicting the
time course of the reaction when a partition coefficient for the
diglyceride was introduced. The results found, which were
confirmed by colorimetry and TLC measurements, indicate that tributyrin hydrolysis proceeds through two separate stages which partially overlap. In the first stage, the hydrolysis of one of the outer
ester groups (sn-1 or 3) of the triglyceride molecule to yield dibu-
tryrin is the predominant reaction. This stage, though expected to
be concluded when conversion attains 1/3 of the total ester bonds
in the system, was found to finish earlier, as the interface becomes
saturated with diglyceride, due to its surface-active character and
possible diffusional limitations. From this moment on, the second
stage of the reaction begins, consisting mainly in the hydrolysis of the remaining outer ester-bond of dibutyrin to yield monobu-
tryrin. Throughout the second reaction stage, which is considerably
slower than the first one, a significant amount of tributyrin remains
confined at the droplet core.

From a practical standpoint, the model we have presented in
this work represents a useful tool for the sizing of enzymatic reac-
tors for triglyceride hydrolysis in aqueous emulsions, even in the
presence of organic solvents as the diluent. The results reported in
this work are also of particular interest from the standpoint of the
application of lipases in detergency, as they suggest that, at satu-
rating enzyme concentrations, it takes only 4 min for the interface
to become covered with surface-active molecules generated by the
hydrolysis reaction (mainly diglycerides and free fatty acids). This
enhances the removal of oily stains from the surface to be cleaned
(clothes, glassware, inner surfaces of industrial equipment, etc.)
making it possible to achieve the desired cleaning effect at lower
temperatures and surfactant concentrations. Results found by our
group in washing assays with lipases [5,26] also point in the same
direction.

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PB1998-1293. M.G.R. also acknowledges a grant from the “Ministe-
rio de Educación y Ciencia” (Spain).

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