Novel route to mono- and diglycerides synthesis in miniemulsion catalyzed by lipases
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1. INTRODUCTION

Lipids are key elements in the chemistry of life. Most organisms use the supramolecular chemistry inherent to phospholipids to form their exterior and compartmental membranes. Many plants and animals store chemical energy in the form of triglycerides, which are sparingly soluble in water. For the metabolic turnover of these and other biochemicals, they produce esterases, enzymes which can hydrolyze bonds of water-soluble esters. Esterases which can hydrolyze triglycerides at the water/oil boundary are termed lipases or more systematically triacylglycerol hydrolases, and those which attack phospholipids are termed phospholipases.\(^1\) Both types of enzymes have recently received considerable attention.\(^2\) - \(^13\) Whereas phospholipases are involved in key metabolic events such as membrane turnover and signal transduction, lipases have diverse functions in the degradation of food and fat. They have qualified as valuable drugs against digestive disorders and diseases of the pancreas. They also find applications in biotechnology (in particular as detergent additives) and as catalysts for the manufacture of specialty (oleo) chemicals and for organic synthesis. Their broad synthetic potential is large due to the fact that lipases, in contrast to most other enzymes, accept a wide range of substrates. They are quite stable in organic solvents, and thus, depending on the solvent system used, can be applied to hydrolysis reactions or ester synthesis. One of those applications is the synthesis of monoglycerides (MG) and diglycerides (DG), which are used in food industry and are used as emulsifiers in cosmetics and pharmaceuticals for the controlled-release preparations.\(^14\) The most frequently used method to produce MG and DG is the glycerolysis. In principle, there are two possibilities of glycerolysis, one where free fatty acid (FFA) and glycerol are mixed, and another one where triglyceride (TG) and glycerol are mixed together in a 1:2 ratio.

In 1958 Sarda and Desnuelle defined lipases in kinetic terms based on the phenomenon of interfacial activation.\(^15\) This lipase activation takes place at water/oil boundary. This aspect of the lipolysis will be discussed later, but it can already be forecast that the larger this interface is, the better the yield, and the faster the conversion is. The miniemulsion presents a reaction field offering a sizeable surface area which would meet the lipase activation requirements. An innovative route to mono- and diglycerides synthesis is delivered in this thesis to obtain these materials in miniemulsion. The purpose is thus to show the appropriateness of the miniemulsion technique to the lipolysis and the glycerolysis of triglycerides under different reaction conditions compared with others dispersion processes. In this dissertation a method to prepare a tricaprylin miniemulsion with a subtly chosen lipase friendly surfactant is presented. Then an analytical method is also investigated, developed and
optimized for the products of the tricaprylin lipolysis based on a specific up work, and on the combination of well-known analytical methods.

The range of reaction conditions includes the influence of different types of lipases, the influence of the homogeneity for the miniemulsion, and the influence of the procedure to shift the yield towards mono- and diacylglycerols, described by the glycerolysis. First, different types of lipases are compared. Their behavior in emulsion is known from the literature, and this is interesting to consider their regioselectivity and activity against tricaprylin in miniemulsion. Secondly, after choosing a lipase showing the best characterizations towards the tricaprylin lipolysis among the screened lipases for the following parts of the dissertation, the influence of the homogeneity of the miniemulsion on the variation of the tricaprylin lipolysis velocity, but also the nature and yield of the products formed under those conditions is studied. Thirdly, the influence of the specific interface induced by the miniemulsion on the velocity of the lipolysis of tricaprylin is investigated as a key factor of the catalytic activity. As a fourth part of this dissertation, the velocity, the nature and the yields of theirs products between the glycerolysis and the lipolysis of the tricaprylin in miniemulsion are compared. Finally, after having considered the lipase catalyzed products of the lipolysis against the tricaprylin, it is interesting to consider the yield of the hydrolysis at the thermodynamic equilibrium as a function of different bases.
2. THEORETICAL SECTION

2.1. Emulsions

An emulsion is a mixture of two immiscible liquids. One substance, the dispersed phase, is dispersed in the other, the continuous phase. Examples of emulsions include milk, butter and margarine, espresso, and mayonnaise, etc. In butter and margarine, a continuous liquid oil-phase surrounds droplets of water (water-in-oil emulsion) also known as inverse emulsion. In the following only the direct emulsion (oil-in-water emulsion) will be considered.

Emulsions tend to have a turbid appearance, because the many interfaces (the boundary between the phases is called the interface) scatter light that passes through the emulsion. Emulsification is the process by which emulsions are prepared. It is now interesting to understand the concept of interfaces and of surface tension. The surface is the apparent discontinuity between two phases in contact, typically the interface between two phases. Intuitively the interface is seen as a geometrical (immaterial) surface delimiting two phases (see Figure 1).

In reality the interface is a continuous transition between the two phases, a “limit layer” (see Figure 2) consisting of few atomic layers with a thickness of about 10 Å. Actually this interface is considered as a limit layer described in the thermodynamic model of Gibbs (see Figure 3). This layer is a “surficial phase” between two voluminal phases having its own thermodynamic characteristics (state variables $U, T, S, \mu_i,...$) and is described by the Gibbs equation.$^{[16]}$
To understand the phenomena of the interface, the interaction energy has to be considered. Intermolecular interactions, described by the Lennard-Jones like potential, are present between neighbor molecules with an attraction force of $1/r^6$. A molecule completely surrounded by other molecules will have a favorable energetic state. At the interface between two immiscible phases, the resulting force attracts a molecule into the related phase (see Figure 4).

A work needs to be provided to bring a molecule to the interface; it is the potential energy of the molecules at the interface. The work $\delta W$ to increase the area $A$ of the interface by $dA$ is proportional to the same increase of interface $dA$:

$$\delta W = \gamma_{la} \cdot dA \quad (1)$$

The surface tension $\gamma_{la}$ can be defined as the energy to be provided to increase the surface of the phase of 1 m². It is possible to reduce the surface tension between the two phases by using a surfactant.
Surfactants, also known as tensides, are wetting agents that lower the surface tension of a liquid, allowing easier spreading, and lower the interfacial tension between two liquids. The term surfactant is a blend of "surface acting agent". Surfactants are usually organic compounds that are amphipathic, meaning they contain both hydrophobic groups (their "tails") and hydrophilic groups (their "heads"). Therefore, they are soluble in both organic solvents and water. The term “surfactant” was coined by Antara Products in 1950. The direct action of the surfactant is then to reduce the provided and required work to form an emulsion. The term emulsion is subdivided into macroemulsion, miniemulsion, and microemulsion with regard to some parameters, such as droplet size, amount of surfactant, stability, etc. The droplet sizes for macroemulsions, miniemulsions, and microemulsions are usually in the range of several micrometers, hundreds of nanometers, and some tens of nanometers, respectively. The amount of surfactant used for emulsification and the stability of the emulsion are also used to differentiate these three kinds of emulsion. While in microemulsions the amount of surfactant - frequently in combination with a cosurfactant - often exceeds the amount of the dispersed phase, in miniemulsions and macroemulsions the surfactant content can be lower than 1% compared to the dispersed phase. Only microemulsions are thermodynamically stable, although, macro- and miniemulsions can be prepared in such way that their structure remains unchanged over longer periods of time, even up to years, through a kinetic stabilization. Furthermore, in the microemulsions, the aqueous phase may contain salt(s) and/or other ingredients, and the "oil" may actually be a complex mixture of different hydrocarbons and olefins. Moreover, while for the preparation of macro- and miniemulsions, one has to use shear forces (mechanical stirring, ultrasound, etc), microemulsions are spontaneously formed. Finally miniemulsions are stable against molecular diffusion (Ostwald ripening), whereas macroemulsions and microemulsions are not. Miniemulsions will be described in details in the following part.

2.2. Miniemulsion

Destabilization and breaking of emulsions can take place either by coalescence or by molecular diffusion degradation (Ostwald ripening). Stabilization of emulsions against coalescence can be obtained electrostatically or sterically by the use of surfactants. In order to create a stable emulsion of very small droplets, which is, for historical reasons, called a miniemulsion (as proposed by Chou et al.\textsuperscript{[17]}) the droplets must be also stabilized against
molecular diffusion degradation also called Ostwald ripening (a monomolecular process or $\tau_1$ mechanism) and against coalescence by collisions (a bimolecular process or $\tau_2$ mechanism).

The preparation of an emulsion results in a distribution of droplet sizes. Even when the surfactant provides sufficient colloidal stability of droplets, the outcome of this size distribution is determined by their different droplet sizes and therefore by the induced different Laplace pressures within the different droplets.

It is now interesting to understand the concept of the Laplace pressure.[18-21] If the state of a liquid system is defined by the extensive values surface $A$, the number of molecules $N$, the volume $V$, conjugated to the following intensive values, respectively $\gamma_{la}$ (surface tension),

$$\mu = \frac{dF}{dN} \quad (F \text{ is the Helmholtz free energy, defined by } F = U - TV \text{ and } \mu \text{ is the chemical potential}),$$

$$p = -\frac{dF}{dV} \quad (\text{pressure})$$

and if the chemical potential is constant (unique physical phase), then the Gibbs energy is:

$$G(V, A, T) = F(V, N, A, T) - \mu N = pV - \gamma_{la}A \quad (2)$$

When the interface is deformed and its volume varies, the induced work is the sum of pressure work and of surface tension work:

$$dW = -pdV + \gamma_{la}dA \quad (3)$$

With $p$ and $\gamma$ constant during this work, it can be noticed that this relation is also the opposite of the infinitesimal variation of the Gibbs energy:

$$dW = -dG \quad (4)$$

At the equilibrium, the work of capillary forces to pressure forces; the energy variation used to deform the surface compensates the energy variation given to modify the volume; if the balance is zero and $dG = 0$, it corresponds to a minimum of the free enthalpy, i.e.:

$$pdV = \gamma_{la}dA \quad (5)$$
As seen before, the phases tend to minimize their free surface $A$ in order to reduce the cohesion energy deficit. As soon as a liquid phase is only subjected to external and internal pressure forces, respectively $p_e$ and $p_i$, and anymore to external forces, like gravity, its minimal free surface is a sphere. The variation of its surface is:

$$dA = 8\pi R dR$$  \hspace{1cm} (6)$$

and its volume one is:

$$dV = 4\pi R^2 dR$$  \hspace{1cm} (7)$$

The relation $dW$ becomes also:

$$dW = -\Delta p dV + \gamma_\mu dA$$  \hspace{1cm} (8)$$

with $\Delta p = p_i - p_e$

The condition giving $dG = 0$ leads to the following relation:

$$\Delta p = \frac{\gamma_\mu}{2R}$$  \hspace{1cm} (9)$$

The Laplace pressure is thus higher in small droplets than in big ones. This leads to the diffusion of the dispersed phase from the small droplets through the continuous phase into the big ones, this is the Ostwald ripening. If the droplets are not stabilized against molecular diffusion, small ones will eventually disappear, increasing the average droplet size. The Ostwald ripening is influenced by the size and the polydispersity of the droplets. Moreover the rate of this diffusion process is linked to the solubility of the monomer in the continuous (water) phase of the emulsion. This property will be used for the stability of the miniemulsion. The addition of a sufficient number of molecules (a third component), whose solubility in the continuous phase is less than that of the dispersed phase one and which are therefore entrapped within the droplets, can provide stabilization against Ostwald ripening.\[^{22}\] An “osmotic stabilization” takes place, since the trapped molecules induce an osmotic pressure, which counteracts the Laplace pressure.
In the case of direct miniemulsions an (ultra-)hydrophobe like hexadecane or perfluorodimorphinopropane is often used as osmotic reagent.

2.3. Preparation and homogenization of miniemulsions

The formation of emulsions starts from a premix of the phases, which contain surface-active agents. The emulsification process requires the deformation and disruption of droplets, in order to increase the specific surface area of the emulsion. These newly formed interfaces have to be stabilized by surfactants.

Different methods can be used to promote the homogenization of emulsions to miniemulsions. Simple stirring was frequently used at the beginning of the miniemulsions research. However, the energy transferred by those techniques is not enough to get a narrow and homogeneous droplets distribution. Therefore a much higher energy to reduce large droplets into smaller ones is required, significantly higher than \( \gamma \Delta A \), the work needed to increase of \( \Delta A \) the surface of the dispersed phase, since the viscous resistance during agitation absorbs most of the energy. The excess energy is dissipated as heat.

The use of ultrasonication is very common as a source of high energy, particularly for the homogenization of small quantities, whereas rotor-stator dispersers with special rotor geometries, microfluidizers or high-pressure homogenizers are favorable for the emulsification of larger quantities. The first report about power ultrasound emulsification appeared in 1927. Several possible mechanisms of droplet formation and disruption under the influence of longitudinal density waves have been reported. One is the formation of droplets as a consequence of unstable oscillations of the liquid-liquid interface. These capillary waves may occur and have a contribution only if the size of droplets to be disrupted is larger than the wavelength of the capillary waves. For ordinary systems of oil and water this wavelength is known to be in the range of 10 \( \mu \text{m} \), which is the usual size of droplets in a premix for continuous emulsification. Therefore, in such a system the droplet formation or disruption by capillary waves is hardly conceivable.

The oscillation and subsequent disruption of droplets due to the action of sound is regarded as a mechanism related to that of capillary waves. The corresponding resonance radius at a frequency of 20 kHz is once more in the region of some 10 \( \mu \text{m} \). This process has to be taken into account only for a small fraction of droplets with diameters exactly matching the resonance frequencies. For the case of a usual broad droplet size distribution in an emulsion premix, a wide range of sound frequencies would be required in order to have this mechanism became the leading one.
The mechanism of cavitation is generally considered as crucial under practical conditions.\textsuperscript{[28]} Parameters positively influencing cavitation in liquids improve the emulsification in terms of smaller dispersed phase droplet size just after being disrupted. Cavitation bubbles can lead to implosion which induces intensive shock waves inside the surrounding liquid and the formation of high velocity liquid jets with huge elongation fields.\textsuperscript{[29]} This may cause droplet disruption around the collapsing bubble.

However, the exact process of droplet disruption, due to ultrasound induced cavitation, is not yet fully understood. At constant energy density, the droplet size decreases when adding stabilizers, whereas the viscosity of the oil in water-in-oil emulsions has no effect.\textsuperscript{[30]} The factors influencing the droplet size within miniemulsions are the following: the ratio of the dispersed phase to the continuous one, the density of the dispersed phase, the solubility and the amount of surfactant. Initially the droplet size in miniemulsions is a function of the mechanical agitation time and of its intensity.\textsuperscript{[31]} The droplet size is also evolving rapidly during the sonication towards a pseudo-stable state. Once this state is reached, it has been found that the size of the monomer droplet size is a function of the applied mechanical energy, assuming that a required minimum one is used. At the beginning of the homogenization, the droplet size distribution is still quite broad, however by continuous fusion and fission processes, the polydispersity is decreasing and the miniemulsion reaches then a stable state\textsuperscript{[32]} (see Figure 5).

With increasing time of ultrasound, the droplet size decreases and therefore the entire interface oil/water increases. But the size of the droplets will reach a limit conditioned by the equilibrium set at the above-mentioned different phenomena ruling the formation of the
miniemulsion. Since a constant amount of surfactant has now to be distributed onto a larger interface, the interfacial tension, as well as the surface tension at the air/miniemulsion interface, increases while the droplets are not fully covered by surfactant molecules.

2.4. Description of lipases and of its properties – Attractive applications

2.4.1. Structure and mechanism

What exactly is a lipase? This is not enough to say that it is a carboxyl esterase which specifically hydrolyzes triacylglycerols. In 1958 Sarda and Desnuelle defined lipases in kinetic terms, based on the phenomenon of interfacial activation.\[^{[15]}\] It includes the activity of lipases is low on monomeric substrates but strongly enhanced once an aggregated “supersubstrate” (such as an emulsion droplet or a micelle of micellar solution for instance) is formed above its saturation limit. This property is quite different from that of the usual esterases acting on water-soluble carboxylic ester molecules. For a long time lipases were considered as a special category of esterases which are highly efficient at hydrolyzing molecules aggregated in water.

Esterases which can hydrolyze triglycerides at the water/oil interface are termed lipases or, more systematically, triacylglycerol hydrolases [EC 3.1.1.3], and those which attack phospholipids are termed phospholipases (various entries under [EC 3.1.x]).\[^{[1]}\] EC stands for Enzyme Class, EC 3 defines the class of hydrolases, EC 3.1, the class of hydrolases acting on ester bonds, EC 3.1.1 defines the carboxylic ester hydrolases and EC3.1.1.3 the triacylglycerol lipases which are described in this dissertation.

The protein structure underlying these observations remained a mystery until a few years ago. Only in 1990 were the first two lipase structures solved by X-ray crystallography. They revealed lipase structures revealing a unique mechanism, unlike that of any other enzyme. Their three-dimensional structures suggested that interfacial activation might be due to the presence of an amphiphilic peptidic loop covering the active site of the enzyme in solution, just like a lid or flap.\[^{[33, 34]}\] From the X-ray structure of cocrystals between lipases and substrate analogues, there is a strong indirect evidence that, when contact occurs with a lipid/water interface, this lid undergoes a conformational rearrangement which renders the active site accessible to the substrate (see Figure 6).\[^{[35, 36]}\]
Figure 6: Structure of Mucor miehei lipase in closed (A, C) and open form (B, D). A and B (side view): the catalytic triad (yellow) and secondary structure elements showing the α/β-hydrolase fold common to all lipases. C and D (top view): space-filling model, colored by decreasing polarity (dark blue - light blue - white - light red - dark red). Upon opening of the lid, the catalytic triad (yellow) becomes accessible (D), and the region binding to the interphase becomes significantly more apolar.\cite{36}

However, within the framework of the European Lipase Project, carried out from 1990 to 1994 by 24 laboratories in 8 nations, structural and numerous biochemical data on highly purified lipases provided evidence that not all lipases subscribe to the phenomenon of interfacial activation.\cite{37} Thus, the lipases from Pseudomonas glumae\cite{38} and Candida antarctica (type B),\cite{39} whose tertiary structure is known, both have an amphiphilic lid
covering the active site but do not show interfacial activation. Among the pancreatic lipases whose tertiary structures have been solved, human pancreatic lipase contains a lid with 23 amino acid residues and shows interfacial activation,[40] whereas coypu lipase does not, although it has a lid of homologous size.[41] The lipase of the guinea pig, an enzyme which shows no interfacial activation, features a “mini-lid” composed of only five amino acid residues.[42] Thus, neither the phenomenon of interfacial activation nor the presence of a lid domain is appropriate criteria to classify an esterase into the lipase subfamily. For classifying an esterase as a lipase, the safest experimental evidence remains to be, as in the early days of research on this enzyme, whether or not it can hydrolyze long-chain acyl glycerols.[43]

All 12 lipases, whose structure has hitherto been elucidated, are members of the “α/β-hydrolase fold” family with a common architecture composed of a specific sequence of α-helices and β-strands (see Figure 7).[33, 34, 38, 39, 44-49]

Figure 7: Canonical fold of α-/β-hydrolases.[50] α-helices are indicated by cylinders and β-strands are indicated by shaded arrows. The topological position of the active-site residues is shown by a solid circle; the nucleophile is the residue after β-strand 5, the Asp/Glu residue is after β-strand 7, and the histidine residue is in the loop between β-8 and α-F.
They hydrolyze ester bonds by means of a “catalytic triad”, composed of a nucleophilic serine residue activated by a hydrogen bond in relay with histidine and aspartate or glutamate (see Figure 8). Serine proteases (“detergent proteases”) follow essentially the same mechanism.

Figure 8: Catalytic mechanism of lipases based on a “catalytic triad” of serine (nucleophile), histidine, and aspartate, or glutamate (connected through a hydrogen bond). The tetrahedral intermediate is stabilized by an “oxyanion hole”. The numbering of amino acids in this example refers to lipase from Rhizopus oryzae.\[^{36}\]

As pointed out already, a unique structural feature common to most lipases is a lid or flap composed of an amphiphilic \(\alpha\)-helix peptide sequence, which in its closed conformation (i.e., in the absence of an interphase or organic solvent) prevents access of the substrate to the catalytic triad. After the lid has opened, a large hydrophobic surface is created to which the hydrophobic supersubstrate (usually the oil drop) binds. This presumed mechanism is supported by the X-ray structures of lipases covalently complexed with hydrophobic inhibitors such as alkyl phosphonates, cycloalkyl phosphonates, or alkyl sulfonates. They
reveal an open lid, suggesting that the phosphonates mimic the transition state for acylation and the sulfonates for deacylation of natural triacyl glycerol ester substrates.\textsuperscript{[51, 52]} To summarize these findings, all lipases investigated so far exhibit a surprising degree of structural and functional similarity, regardless of the organism from which they were isolated and even if the observed amino acid sequence homologies are low. The coherent features of lipases are summarized in Table 1.

In spite of these similarities, subtle variations in the architecture of the substrate binding site may have a strong effect on the catalytic properties, the temperature, and stability of a lipase in a solvent. As these differences are of paramount importance for the selection of an individual lipase for a desired application, recent research has emphasized this aspect.

Table 1: Important features of lipases.\textsuperscript{[51, 52]}

<table>
<thead>
<tr>
<th>Feature</th>
<th>Details</th>
<th>Remarks</th>
</tr>
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<tbody>
<tr>
<td>Mechanism based on a “catalytic triad” made up of nucleophilic serine, histidine, and aspartate/glutamate</td>
<td>Found in over 30 lipases, including those from psychrophilic or thermophilic microorganisms; the only exception is replacement of aspartate for glutamate. Substitution of serine for cysteine by site-directed mutagenesis led to strongly reduced activity.</td>
<td>Related hydrolases show much greater variability in catalytic mechanism. In the case of proteases, the amide bond can be hydrolyzed through nucleophilic attack by hydroxyl (serine, threonine) or thiol groups (cysteine), or through electrophilic attack by a carboxyl group (aspartate/glutamate) or a metal ion (Zn\textsuperscript{2+}).</td>
</tr>
<tr>
<td>Consensus sequence at the active serine residue</td>
<td>The consensus sequence for over 30 cloned lipases is a “nucleophilic elbow” at the end of a (\sigma)-sheet and is composed of -Gly/Ala-X-Ser-X-Gly-.</td>
<td>Other hydrolases show greater variability.</td>
</tr>
<tr>
<td>Most lipases feature a lid structure</td>
<td>A lid composed of an amphiphilic peptide loop covers the active site of the enzyme in the inactive state.</td>
<td>No lid was observed in esterases or proteases, but some lipases have no lid or just a small lid.</td>
</tr>
<tr>
<td>All lipases are members of the “(\alpha/\beta)-hydrolase fold” family</td>
<td>The structure is composed of a core of predominantly parallel (\beta)-strands surrounded by helices. The active nucleophilic serine residue rests at a hairpin turn between a (\beta)-strand and an (\alpha)-helix.</td>
<td>Many other hydrolases (esterases, acetylcholine esterases, serine proteases, carboxypeptidases, dehydrogenases) and even a haloperoxidase show a similar structural motive, which suggests evolutionary relationships.</td>
</tr>
</tbody>
</table>

After considering the structure of the lipase, it is interesting to underline the characteristics of the substrate, the glycerides, and to describe the stereochemistry of its substrate to first understand the sn-1,3 lipase denomination, and then its behaviour in water.
Glycerides are lipids possessing a glycerol (another name for which is propan-1, -2, -3-triol) core structure with one or more fatty acyl groups, which are fatty acid-derived chains attached to the glycerol backbone by ester linkages. Glycerides with three acyl groups (triglycerides or neutral fats) are the main form of fatty energy storage in animals and plants. Since the plane of symmetry of the glycerol molecule is a prochiral plane, the two primary hydroxyl groups are stereochemically distinct (i.e., they are enantiotopic groups). A Fischer projection of a glycerol molecule is shown in Figure 9 with the secondary alcohol chain branching off to the left of the main hydrocarbon chain; the carbon atoms are numbered 1, 2, and 3 working downwards. With this system of numbering, glycerol becomes sn-glycerol (i.e. stereospecifically numbered glycerol). This makes it possible to obtain unambiguous, expressions for the stereoisomeric forms of (phospho)glycerides. Natural phospholipids, for example, all belong to the sn-glycero-3-phosphate series. In the case of natural triacylglycerols, the fatty acids which esterify positions sn-1, sn-2 and sn-3 are often different, which results in various chiral substrates.

Figure 9: Fischer representation of a triacylglycerol molecule. Identification of potentially hydrolysable ester bonds.

The four main classes of biological substances are carbohydrates, proteins, nucleic acids, and lipids. The first three of these substances have been clearly defined on the basis of their structural features, whereas the property which is common to all lipids is a physicochemical one. Lipids are in fact a group of structurally heterogeneous molecules which are all insoluble
in water but soluble in apolar and slightly polar solvents such as ether, chloroform and benzene.

Lipids have been classified by Small\cite{53} depending on how they behave in the presence of water (see Figure 10). This makes it possible to distinguish between polar and apolar lipids (e.g. hydrocarbon, carotene). The polar lipids can be further subdivided into three classes. Class I consists of those lipids which do not swell in contact with water and form stable monomolecular films (these include triacylglycerols, diacylglycerols, phytols, retinols, vitamin A, K and E, waxes and numerous sterols). The class II lipids (which include phospholipids, monoacylglycerols and fatty acids) spread evenly on the surface of water, but since they become hydrated, they swell up to form well-defined lyotropic (liquid crystalline) phases such as liposomes. The class III lipids (such as lysophospholipids and bile salts) are partly soluble in water and form unstable monomolecular films, and beyond the critical micellar concentration level, micellar solutions.

![Figure 10: A classification of biological lipids based upon their interaction in aqueous systems.\cite{53}](image)

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2.4.2. Occurrence and preparation of lipases

Lipases are ubiquitous enzymes and have been found in most organisms from the microbial,\(^{54-56}\) plant,\(^{57}\) and animal kingdom.\(^{58, 59}\) They are prepared either by extraction from animal or plant tissue, or by cultivation of microorganisms.\(^2\) Commercially available lipases are usually derived from microorganisms. Since the advent of genetic engineering techniques, an increasing number of lipases is being commercially manufactured from recombinant bacteria and yeasts. As an example, the “detergent lipase” from the fungus Humicula Lanuginosa is commercially produced in large scale (several 100 tons per year) through fermentation of Aspergillus Oryzae into which the gene coding for Humicula Lanuginosa lipase was cloned.\(^60\) Usually, lipases are just one member of a “hydrolytic enzyme cocktail” elaborated by an organism with the objective to sustain its growth. Often the lipase of interest must be separated from other esterases and proteases occurring in the crude enzyme preparation.\(^61, 62\)

Purification protocols are often laborious as the affinity of lipases is high not only to the oil/water boundary, but to any interphase of lower polarity than water (e.g. water-immiscible organic solvents, glass and polymeric surfaces, and air bubbles); lipases may irreversibly adsorb and denature at such interfaces.\(^63, 64\) The critical discussion of experiments on lipase catalysis reaches even beyond such difficulties, as many organisms produce mixtures of lipase isoforms which differ only marginally, for example by their glycosylation pattern.\(^65\) Limited proteolysis during maturation of a prolipase or during recovery in the presence of protease impurities may result in a heterogeneous mixture of enzymes with lipolytic activity, and ambiguities may exist in the taxonomic classification of the producing microorganism (see Table 2).
Table 2: Some of the ambiguities concerning commercially available lipases.\textsuperscript{[36]}

<table>
<thead>
<tr>
<th>Lipase source</th>
<th>Remarks</th>
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<tbody>
<tr>
<td>Candida rugosa (CRL)</td>
<td>An organism that was formerly classified as Candida cylindracea. Protein purification and cloning of the enzyme revealed that the organism produces at least five related lipase isoforms.</td>
</tr>
<tr>
<td>Geotrichum candidum (GCL)</td>
<td>Contains two isoforms differing in specificity towards $\Delta^9$-unsaturated fatty acids.</td>
</tr>
<tr>
<td>Rhizopus (RAL, ROL, RDL, RNL)</td>
<td>Although literature data suggest different specificities of the lipases from R. arrhizus, R. oryzae, R. delemar, and R. niveus, cloning and sequencing revealed nearly complete identity of all four enzymes.</td>
</tr>
<tr>
<td>Penicillium camembertii (PeCL)</td>
<td>Classified as lipase from P. cyclopium until 1990. Contains four lipase isoforms which differ in their glycosylation pattern.</td>
</tr>
<tr>
<td>Pseudomonas glumae (PGL)</td>
<td>Cloning and sequencing revealed identity with lipase from Chromobacterium viscosum (CVL).</td>
</tr>
</tbody>
</table>

Thus, quite a few results from the application of lipases to oleochemistry and organic synthesis may have originated from the use of a poorly defined enzyme cocktail which, among other activities, also included a lipase. Any result obtained with a commercial lipase should be scrutinized in view of potential masking effects from unrelated enzymes or other additives contained in the sample. Fortunately, many pure lipases, often obtained by recombinant technology, can now be purchased from enzyme suppliers. Table 3 summarizes commercially available lipases and their abbreviation codes used throughout this dissertation.
Table 3: Important commercially available lipases.\[36\]

<table>
<thead>
<tr>
<th>Origin</th>
<th>Code</th>
<th>M [kDa] (rounded)</th>
<th>Specificity</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>of mammalian origin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>human pancreatic lipase</td>
<td>HPL</td>
<td>50</td>
<td>sn-1,3</td>
<td></td>
</tr>
<tr>
<td>human gastric lipase</td>
<td>HGL</td>
<td>50</td>
<td>sn-3 (acid-stable)</td>
<td></td>
</tr>
<tr>
<td>porcine pancreatic lipase</td>
<td>PPL</td>
<td>50</td>
<td>sn-1,3</td>
<td>organic synthesis, digestive aid</td>
</tr>
<tr>
<td>guinea pig pancreatic lipase</td>
<td>GPL-RP2</td>
<td>48</td>
<td>sn-1,3</td>
<td></td>
</tr>
<tr>
<td>of fungal origin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida rugosa</td>
<td>CRL</td>
<td>60</td>
<td>non specific</td>
<td>organic synthesis</td>
</tr>
<tr>
<td>Candida antarctica B</td>
<td>CAL-B</td>
<td>60</td>
<td>sn-1,3</td>
<td>organic synthesis</td>
</tr>
<tr>
<td>Rhizomucor miehei</td>
<td>RML</td>
<td>30</td>
<td>sn-1,3</td>
<td>cheese manufacturing</td>
</tr>
<tr>
<td>Aspergillus oryzae</td>
<td>AOL</td>
<td></td>
<td></td>
<td>cheese manufacturing</td>
</tr>
<tr>
<td>Penicillium camemberti</td>
<td>PEL</td>
<td>30</td>
<td>sn-1,3</td>
<td>oleochemistry</td>
</tr>
<tr>
<td>Rhizopus delemar</td>
<td>RDL</td>
<td>41</td>
<td>sn-1,3</td>
<td>oleochemistry</td>
</tr>
<tr>
<td>Rhizopus oryzae</td>
<td>ROL</td>
<td>41</td>
<td>sn-1,3</td>
<td>oleochemistry</td>
</tr>
<tr>
<td>(phospholipase A1 activity)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhizopus arrhizus</td>
<td>RAL</td>
<td>41</td>
<td>sn-1,3</td>
<td>oleochemistry</td>
</tr>
<tr>
<td>of bacterial origin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas glumae</td>
<td>PGL</td>
<td>33</td>
<td>non specific</td>
<td>detergent enzyme, organic synthesis</td>
</tr>
<tr>
<td>Burkholderia cepacia</td>
<td>PCL/BCL</td>
<td>33</td>
<td>non specific</td>
<td>organic synthesis</td>
</tr>
<tr>
<td>Pseudomonas mendocina</td>
<td>PML</td>
<td>33</td>
<td>sn-1,3</td>
<td>detergents</td>
</tr>
<tr>
<td>Chromobacterium viscosum</td>
<td>CVL</td>
<td>33</td>
<td>sn-1,3</td>
<td>organic synthesis</td>
</tr>
<tr>
<td>Bacillus thermocatenulatus</td>
<td>BTL-2</td>
<td>43</td>
<td>sn-1,3</td>
<td></td>
</tr>
</tbody>
</table>

Lipases can be obtained commercially from many suppliers. Important original producers are Novo-Nordisk (Baegsvard, Denmark), Genencor International B. V. (Delft, The Netherlands), Boehringer-Mannheim (Mannheim, Germany), and Amano Co. (Nagoya, Japan).

2.4.3. Kinetics of lipase catalysis

As seen before, the “interfacial activation” phenomenon leads to the fact that the activity of lipases is enhanced on oil droplets in water (such as emulsions or miniemulsions) than on the same substrates in true monomeric solutions. It therefore emerged from the studies mentioned above that lipases might constitute a special category of esterases which are highly efficient at hydrolyzing molecules having a carboxylic ester group and are aggregated in water.

This property was used for a long time to distinguish between lipases and esterases. A conceptual shift has unfortunately occurred however, as the result of which “interfacial activation” has been taken to mean a hypothetical conformational change occurring as the result of interfacial adsorption.\[66\] Even worse “interfacial activation” has sometimes been
wrongly used to refer to the increase in the catalytic activity of an enzyme on a triacylglycerol substrate occurring in the presence of a tensioactive agent. The result of recent lipase research have nevertheless shown how careful one has to be about extrapolating any kinetic and/or structural characteristics observed to all lipases in general. The catalytic activity of many lipolytic enzymes has been measured using carboxylic esters which are partly soluble in water and many differences have been found to exist between the resulting profiles (see Figure 11).

In attempts to model enzyme kinetics sensu latu, the Michaelis-Menten mechanism has been recurrently used as a model. However, one of its underlying assumptions is that the enzymatic reaction must take place in an isotropic medium, i.e. both the enzyme and the substrate must be a part of the same phase. Hence, this mechanism cannot be used in its original form to model the action of lipolytic enzymes acting at the interface between a water phase and an (insoluble) lipid phase.\cite{54, 67} For this reason, a modified model has been proposed elsewhere\cite{68} which consists of two steps (see Figure 12): 1) the physical adsorption of lipase at the water lipid interface, which leads to activation of the lipase and hence as seen before to opening of the lid that would otherwise block the active site; and 2) the formation of the enzyme/substrate complex, which will eventually be hydrolyzed to give the product and regenerate the adsorbed enzyme (this second step may be described by a pseudo Michaelis-Menten mechanism occurring on an interphase, rather than in a bulk level).
Irrespective to the type of reaction catalyzed (i.e. hydrolysis, esterification, or transesterification), the most general accurate and accepted description of the catalytic action of lipases is a Ping Pong Bi Bi mechanism constituted of two major steps: 1) nucleophilic attack on the substrate ester bond by the oxygen atom of the hydroxyl group of Ser at the active site after opening of the lid (thus resulting in formation of an acylated enzyme complex and release of the alcohol moiety of the original substrate); and 2) hydrolysis of the acylated enzyme complex (thus resulting in formation of the product and regeneration of the enzyme). This tentative mechanism is depicted in Figure 13 using Cleland's schematic representation. It can be assumed in general that a reaction catalyzed by a lipase is described by the following chemical equation:
where $A_{ci}$ denotes an acid moiety of the $i$-th type, $A_{lj}$ an alcohol moiety of the $j$-th type, $E_{si,j}$, an ester moiety continuing an acid residue of the $i$-th type and an alcohol residue of the $j$-th type, and $W$ a water molecule.

Figure 13: Kinetic mechanism (Ping Pong Bi Bi) of lipase-catalyzed reactions involving multiple substrates/products, using Cleland's notation ($E$: enzyme; $E_s$: ester moiety; $F$: acyl enzyme, $A_l$: alcohol moiety; $A_c$: acid moiety; $W$: water; $i = 1, 2, \ldots, I$, $j = 1, 2, \ldots, J$).

Tan et Yin\textsuperscript{70} developed in 2005 a methodology to assess the kinetics constant of the intermediate reactions of glycerolysis and lipolysis. They considered the reaction system as a homogeneous phase because the used lipase is a free enzyme and not immobilized and a little amount of water was used in the experiment. For simplicity and with respect to the observations, in order to describe the selectivity of the lipase and the isomerization eventuality, the following assumptions were made:

1. All reactions are simple reactions.
2. The lipase is completely 1,3 position specific.
3. The lipase does not denature during these reactions.

Isomerization reactions of 2-monoglyceride into 1-monoglyceride and of 1,2(2,3)-diglyceride into 1,3-diglyceride are considered.

The considered ‘unit reaction’ is the hydrolysis according to the mechanism described in Figure 12. This reaction yields to three classes of products, diglycerides, monoglycerides, and
glycerol. The isomerization reactions of diglycerides and monoglycerides are also considered. The reactions then can be divided into four groups:

(1) Group I: Hydrolysis of tricaprylin:

\[
\begin{align*}
\text{tricaprylin} & \quad \text{1,2-dicaprylin} \\
\text{OCOR} + \text{OH} & \quad \text{OCOR} + \text{H}_2\text{O} + \text{RCOOH} \\
\text{OCOR} & \quad \text{OCOR} \\
\text{OCOR} & \quad \text{OH} \\
\end{align*}
\]

where \( k_1 \) is hydrolysis rate constant of the lipase for a single caprylic acid in tricaprylin; \( k_2 \) is the esterification rate constant for a single hydroxyl group in diacylglycerol.

(2) Group II: Hydrolysis of dicaprylin and esterification of monocaprylin:

\[
\begin{align*}
\text{1,2-dicaprylin} & \quad \text{2-monocaprylin} \\
\text{OCOR} + \text{H}_2\text{O} & \quad \text{OCOR} + \text{RCOOH} \\
\text{OH} & \quad \text{OH} \\
\end{align*}
\]

where \( k_3 \) is the hydrolysis rate constant of the lipase for a single caprylic acid in dicaprylin; \( k_4 \) is the esterification rate constant for a single hydroxyl group in monocaprylin.
(3) Group III: Hydrolysis of monocaprylin and esterification of glycerol and fatty acids:

\[
\begin{array}{c}
\text{OCOR} \\
\text{OH} \\
\text{OH} \\
\end{array} + H_2O \xrightleftharpoons[k_5]{k_6} \begin{array}{c}
\text{OH} \\
\text{OH} \\
\text{OH} \\
\end{array} + \text{RCOOH}
\]

1-monocaprylin \hspace{2cm} \text{glycerol}

where \(k_5\) is the hydrolysis rate constant of the lipase for a single fatty acid in monocaprylin; \(k_6\) is the esterification rate constant of lipase between caprylic acid and glycerol.

(4) Group IV: Acyl migration and isomerization of dicaprylin:

\[
\begin{array}{c}
\text{OCOR} \\
\text{OCOR} \\
\text{OH} \\
\end{array} \xrightleftharpoons[k_7]{k_8} \begin{array}{c}
\text{OCOR} \\
\text{OH} \\
\text{OCOR} \\
\end{array}
\]

1,2-dicaprylin \hspace{2cm} 1,3-dicaprylin

\[
\begin{array}{c}
\text{OH} \\
\text{OCOR} \\
\text{OH} \\
\end{array} \xrightleftharpoons[k_9]{k_{10}} \begin{array}{c}
\text{OCOR} \\
\text{OH} \\
\text{OH} \\
\end{array}
\]

2-monocaprylin \hspace{2cm} 1-monocaprylin

where \(k_7\) and \(k_8\) are the reaction rate constants for isomerization from 1,2-dicaprylin to 1,3-dicaprylin, and for the isomerization from 1,3-dicaprylin to 1,2-dicaprylin, respectively. \(k_9\) and \(k_{10}\) are the reaction rate constants for the isomerization from 2-monocaprylin to 1-monocaprylin, and for the isomerization from 1-monocaprylin to 2-monocaprylin, respectively.

The constants of the above-mentioned reactions are valid for a 5.0 wt.-% of catalyst with respect to the tricaprylin. According to the above-mentioned reactions, the following reaction rate equations can be obtained:
\[
- \frac{d[TG]}{dt} = k_1[TG][H_2O] - k_2[1,2-DG][FFA]
\]
\[
- \frac{d[1,2-DG]}{dt} = k_3[1,2-DG][H_2O] - k_4[2-MG][FFA] + k_5[1,2-DG] - k_6[1,3-DG]
\]
\[
- \frac{d[1,3-DG]}{dt} = k_7[1,3-DG][H_2O] - k_8[1-MG][FFA] - k_9[1,2-DG] + k_8[1,3-DG]
\]
\[
- \frac{d[1-MG]}{dt} = k_8[1-MG][H_2O] - k_9[G][FFA] - k_5[1,3-DG][H_2O] + k_4[1-MG][FFA]
\]
\[
- k_9[2-MG] + k_{10}[1-MG]
\]
\[
- \frac{d[2-MG]}{dt} = -k_4[1,2-DG][H_2O] + k_5[2-MG][FFA] + k_6[2-MG] - k_{10}[1-MG]
\]
\[
- \frac{d[FFA]}{dt} = -k_1[TG][H_2O] + k_2[1,2-DG][FFA] - k_5[1,2-DG][H_2O] + k_4[2-MG][FFA]
\]
\[
- k_8[1-MG][H_2O] + k_9[G][FFA]
\]
\[
- \frac{d[1-MG]}{dt} = -k_8[1-MG][H_2O] + k_9[G][FFA]
\]

This model was then developed and this differential equation system was solved by doing a simulation using a mathematical approximation method, the Runge-Kutta method and a calculation software, the Matlab program \(^{70}\) and then to compare the kinetics constants of the before-described kinetics done under different conditions.

The SI derived unit for measuring the catalytic activity of a catalyst is the katal, which is moles per second. The degree of activity of a catalyst can also be described by the turn over number (or TON) and the catalytic efficiency by the turn over frequency (TOF). The biochemical equivalent is the enzyme unit. The enzyme unit (U) is a unit for the amount of a particular enzyme. One U is defined as that amount of the enzyme that catalyzes the conversion of 1 micro mole of substrate per minute. The conditions also have to be specified: one usually takes a temperature of 30 °C and the pH value and substrate concentration that yield the maximal substrate conversion rate. However enzyme activity can be defined differently, that is the reason why it is always important to present the definition used to describe the activity. Table 4 presents the specific activity (U/mg) of different lipases.
Table 4: Specific activity of the lipase towards tributyrin (4:0)*, trioctanoin (8:0)* and triolein (18:0)* under non-optimized assay conditions.[71]

<table>
<thead>
<tr>
<th>Origin</th>
<th>Code</th>
<th>Concentration [mg/mL]</th>
<th>Specific activity [U/mg]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tributyrin</td>
</tr>
<tr>
<td>Candida antarctica A lipase</td>
<td>CAL-A</td>
<td>15.75</td>
<td>420^a</td>
</tr>
<tr>
<td>Candida antarctica B lipase</td>
<td>CAL-B</td>
<td>2.60</td>
<td>165^a</td>
</tr>
<tr>
<td>Candida rugosa lipase</td>
<td>CRL</td>
<td>2.45</td>
<td>1880^a</td>
</tr>
<tr>
<td>Chrombacterium viscosum lipase</td>
<td>CVL</td>
<td>0.38</td>
<td>500^a</td>
</tr>
<tr>
<td>Dog gastric lipase</td>
<td>DGL</td>
<td>4.00</td>
<td>570</td>
</tr>
<tr>
<td>Dog pancreatic lipase</td>
<td>DPL</td>
<td>1.40</td>
<td>5000^f,h</td>
</tr>
<tr>
<td>Fusarium solani cutinase</td>
<td>FSC</td>
<td>0.58</td>
<td>3180^g</td>
</tr>
<tr>
<td>Geotrichum candidum A lipase</td>
<td>GCA</td>
<td>0.20</td>
<td>46</td>
</tr>
<tr>
<td>Geotrichum candidum B lipase</td>
<td>GCB</td>
<td>0.03</td>
<td>14</td>
</tr>
<tr>
<td>Geotrichum candidum lipase (Münster)</td>
<td>GCM</td>
<td>2.00</td>
<td>140^b</td>
</tr>
<tr>
<td>Guinea pig pancreatic lipase</td>
<td>GPL</td>
<td>1.00</td>
<td>2000</td>
</tr>
<tr>
<td>Human gastric lipase</td>
<td>HGL</td>
<td>3.50</td>
<td>1000</td>
</tr>
<tr>
<td>Humicola lanuginosa lipase</td>
<td>HLL</td>
<td>2.00</td>
<td>3960^a</td>
</tr>
<tr>
<td>Human pancreatic lipase</td>
<td>HPL</td>
<td>2.00</td>
<td>8000^f,h</td>
</tr>
<tr>
<td>Lipoprotein lipase (bovine milk)</td>
<td>LPL</td>
<td>0.50</td>
<td>85^b</td>
</tr>
<tr>
<td>Rhizomucor miehei lipase</td>
<td>RML</td>
<td>2.80</td>
<td>8240^a</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa lipase</td>
<td>PAL</td>
<td>1.20</td>
<td>2167^a</td>
</tr>
<tr>
<td>Penicillium camemberti lipase</td>
<td>PEL</td>
<td>0.70</td>
<td>760^a</td>
</tr>
<tr>
<td>Pseudomonas fluorescens lipase</td>
<td>PFL</td>
<td>1.60</td>
<td>94^a</td>
</tr>
<tr>
<td>Pseudomonas glumae lipase</td>
<td>PGL</td>
<td>1.00</td>
<td>3500</td>
</tr>
<tr>
<td>Porcine pancreatic lipase</td>
<td>PPL</td>
<td>1.40</td>
<td>6000^f,h</td>
</tr>
<tr>
<td>Penicillium simplicissimum lipase</td>
<td>PS</td>
<td>0.65</td>
<td>330^a,i</td>
</tr>
<tr>
<td>Pseudomonas sp. lipase</td>
<td>PSp</td>
<td>1.80</td>
<td>416</td>
</tr>
<tr>
<td>Rhizopus arrhizus lipase</td>
<td>RAL</td>
<td>0.30</td>
<td>990</td>
</tr>
<tr>
<td>Rabbit gastric lipase</td>
<td>RGL</td>
<td>5.20</td>
<td>1000</td>
</tr>
</tbody>
</table>

Specific activities of all enzymes with trioctanoin and triolein as substrates are average values corresponding to 5% and 6% lipolysis yields, respectively. All lipolyses were carried out twice at 37°C and pH 8.0 (yields stated otherwise) under the following conditions: *NaCl 0.9%; ^NaCl 0.9%, CaCl₂, 2 mM; ^NaCl 0.9%, CaCl₂, 2 mM, BSA 1.5 µM; ^NaCl 0.9%, BSA 1.5 µM; ^NaCl 0.9%, NaTDC 2 mM; ^NaCl 0.9%, CaCl₂, 2 mM, NaTDC 2 mM; ^NaCl 0.9%, BSA 1.5 mM, NaTDC 2 mM; with colipase added in fivefold molar excess; pH 5.5; 25°C. pH 8.5; 6 °C. * (X:0): X stands for a triglyceride having a fatty chain length of X carbons and 0 stands for no unsaturation in the fatty chain.
2.4.4. Applications of lipases

2.4.4-i. Lipases as biocatalysts in organic synthesis

In view of their specific and limited function in metabolism, one should expect lipases to be of limited interest for the organic chemist. However, the opposite is true: chemists have discovered lipases to be one of the most versatile classes of biocatalysts in organic synthesis\[^{72-78}\] for a few simple reasons:

1. Owing to the large enzyme domains required for acyl group binding and the unpronounced structural features of acyl chains, lipases can accommodate a wide variety of synthetic substrates, while still showing regioselectivity or chiral recognition.
2. Lipases act at the water/lipid boundary, which exhibits high interfacial energy. To withstand the denaturing effect of the interface, lipases have evolved unusually stable structures that may survive even the effect of organic solvents.

2.4.4-ii. Lipases in oleochemistry

2.4.4-ii.a) Soaps and fatty acids
While the bulk of the 60 million tons of fats and oils produced every year are directly used in food, over five million tons are a renewable chemical feedstock for non-food-related applications (“oleochemistry”). One major chemical application of triglycerides is in the preparation of soap. Today’s preferred chemical processes (Monsavom, Sharples, or De Laval - Centripet processes) operate continuously and provide quantitative yields within minutes at 100 °C, independent from the origin of the fat or oil. Energy consumption is further minimized by energy recycling.\[^{79}\]

2.4.4-ii.b) Transesterified triglycerides
Many lipases exhibit \(sn\)-1,3 specificity and can therefore be used to regioselectively interesterify positions 1 and 3 of a natural triglyceride. However, the tendency towards acyl migration from the \(sn\)-2 to the \(sn\)-1 or \(sn\)-3 positions must be suppressed. So far, three types of modified triglycerides have gained in commercial importance. These are fats with improved spreadability, cocoa-butter equivalents, and highly digestive triglycerides.
2.4.4 -ii.c) Monoglycerides

In 1993 about 120,000 tons of monoglycerides and mixtures of mono- and diglycerides were manufactured through glycerolysis of triglycerides. After short-path distillation, monoglycerides of more than 90% purity are obtained. Monoglycerides are mild to strong emulsifiers depending on the length of the fatty chain (HLB value < 3.4, HLB: hydrophilic-lipophilic balance) permitted for use as food additives. Industrial applications include emulsification in food, cosmetics, and drug preparations. \[80-82\] Many reports deal with the preparation of monoglycerides through lipase catalysis, but the key problem remains that mixtures of mono- and diglycerides are formed.\[14, 83\] Several enzymatic preparation procedures enable the lipase-catalyzed synthesis of monoglycerides. Under commercial aspects, none of these procedures has been able to compete with the chemical manufacture of monoglycerides outlined above. Even when a lipase obtained from Penicillium roquefortii providing more than 90% of monoglycerides in a one-step glycerolysis reaction was used, the economic data were unsatisfactory. The use of lipases has proven to be advantageous, however, for the synthesis of partial glycerides containing labile substituents such as 8'-apo-β-carotinoic acid, which would not withstand chemical procedures.\[84\]

2.4.4-ii.d) Lipolysis and glycerolysis

Monoglycerides (MG) and diglycerides (DG) can be formed by controlled hydrolysis of triacylglycerols (lipolysis), esterification of glycerol and fatty acids, or acyl exchange between a slight excess of glycerol and triacylglycerols (alcoholysis or glycerolysis).

Currently glycerolysis is carried out at industrial level to form monoglycerides (MG) from glycerol and triglycerides (TG). Different aspects of this complicated reaction, which were studied extensively in the 1940s and 1950s, have been reviewed by Sonntag.\[82\] The reaction of TG with glycerol to yield partial glycerides is normally performed at high temperatures (250 °C) to increase the solubility of glycerol in the oil phase, which is only about 4% at room temperature. Alkali catalysts, such as NaOH, KOH, and Ca(OH)$_2$ are used to accelerate the process. At the end of the reaction time (usually about 4 h), the catalyst is neutralized and the reaction mixture is cooled rapidly. This step is crucial to minimize reversion of the reaction, because glycerol separates into a heavier, lower layer upon reduction of temperature. The resultant product is a mixture of MG, diglycerides (DG), and TG, as well as free fatty acids (FFA) and their metallic soaps. The MG yield is generally rather low at 30-40% and the
reaction product is distilled in a molecular still\(^1\) to achieve MG concentrations of about 90\%.

Glycerolysis is also conducted as the preliminary step for the production of alkyd resins and some detergents. The reaction is carried out on a considerable scale in various industries.\(^{[85]}\) Unfortunately, the high temperatures involved in the reaction often result in a dark-colored product, which changes the color of the product where the MG are used. In addition, the presence of FFA and their metallic soaps can lead to associate flavor and odor problems in the food products into which they are incorporated. Moreover, the utilization of solvents that can dissolve both oil and glycerol to decrease the high reaction temperatures also has been investigated.\(^{[82]}\) However, few solvents (phenols, cresols, 1,4-dioxane, and pyridine) promote the desired miscibility and they are not acceptable in trace amounts when MG/DG mixtures are compounded in food products.

Over the last decade, lipase-catalyzed glycerolysis under milder reaction conditions has received increasing attention in an effort to minimize the above-mentioned flavor, odor, and color problems associated with conventional glycerolysis. And more precisely, the glycerolysis carried out by a lipase has got attention due to its mild reaction conditions and position specific products such as 2-monoglyceride by 1,3-specific lipase.\(^{[86]}\)

In principle, there are two ways of glycerolysis, one where free fatty acid (FFA) and glycerol are mixed and another one where triglyceride (TG) and glycerol are mixed together in a 1:2 ratio. The glycerolysis can be performed in a solvent-free system, in a presence of water or organic solvent such as tert-butanol and tert-pentanol,\(^{[87]}\) a mix of acetone and isooctane,\(^{[88]}\) etc. It can be noticed that increasing water in glycerol phase increases the FFA content.\(^{[88]}\) Furthermore, other authors found that 10–12\% water in glycerol was suitable for glycerolysis.\(^{[89]}\)

It is interesting here to report the results published about the compared study of hydrolysis and glycerolysis of 1,3-palmitin-2-olein (POP) by the 1,3-position specific lipase from Rhizopus Arrhizus.\(^{[70]}\) The experiments were performed in a free solvent system at 37 °C. Quantitative analyses of products were conducted by using an HPLC system with an

\(^{1}\)Molecular Still Separation has been around since the 1930's. High vacuum distillation, which has been termed "molecular" or "short path" distillation, is a process tool to separate mixtures of organic or silicon compounds. The degree of separation is a function of the difference in molecular weights of any distilled mixture. The greater the variance in the molecular weights, the purer the distillate. The closer the molecular weights of the mixture, the less efficient is the desired fractionation, Molecular Still Separation is normally used in the oil industry to separate components from the distillate (waste stream of a deodorizer). For example Vitamin E (tocopherol) is removed from soybean oil distillate. This process is not normally used in oil processing because it has been shown to be very costly and ineffective in removing some components.
evaporative light-scattering detector (ELSD). The analytical column was a C18 column (5 μm, 250 mm x 4.6 mm). The time courses of the hydrolysis are depicted in Figure 14.

Figure 14: Time course of the lipolysis of POP taken from the literature.[70] (A) TG, 1,2-olein-3-palmitin (+), 1,3-palmitin-2-olein (○); (B) FFA, oleic acid (◇), palmitic acid (□); (C) MG, 1-monoolein (×), 2-monoolein (○), 1-monopalmitin (△); (D) DG, 1-palmitin-3-olein (—), 1-palmitin-2-olein (■).

The time course of POP first decreases rapidly. Then the time course tends to reach an asymptotical value comprised between 35 and 40% (see Figure 14, A). The almost opposite profile is observed for the palmitic acid reaching an asymptotical value comprised between 14 and 16% after a fast increase. The time course of oleic acid increases, but remains under 2% after 24 h (see Figure 14, B). The time course of 2-monoolein reaches a maximum of about 6% after 6 h and decreases to 5.5% after 24 h, the 1-monoolein (1-MG) and 1-monopalmitin (1-MG) show a comparable increasing profile in the first 12 h remaining under 1%, then the 1-monoolein keeps on increasing and reaches 2% after 24 h, while the 1-monopalmitin remains constant under 1% (see Figure 14, C). Considering the diglycerides, the time course of 1-palmitin-2-olein (1,2-DG) increases and reaches a maximum of about 35% after 6 h and...
decreases to about 30% after 24 h. The time course of 1-palmitin-3-olein (1,3-DG) increases and reaches about 2% after 12 h and remains then almost constant (see Figure 14, D).

The observation of Figure 14 shows that all acylglycerols are products of the lipolysis. However a critical examination shows also that only small yields for 1,3-DG and 1-MG are observed. RAL seems to be sn-1,3 selective enzyme, 1-MG and 1,3-DG are almost not formed, just 1,2-DG are formed yielding 2-MG.

The glycerolysis is compared to the above-described lipolysis. The time courses of the glycerolysis of POP are depicted in Figure 15.

As for tricaprylin, the time course, depicted in Figure 15, first decreases rapidly and then reaches an asymptotical value comprised between 5 and 10%. (see Figure 15, A). For the free fatty acid, the same profile can be observed, but it reaches only 3% and remains constant during the whole kinetics (see Figure 15, B). As for the MG, where both profiles show a
transient accumulation and where the lipolysis reaches a maximum of 6% after 6 h before decreasing till about 5% after 24 h, the glycerolysis reaches about 25% after 8 h and seems to remain constant afterwards (see Figure 15, C). Considering the DG, the time course of 1-palmitin-2-olein shows a transient accumulation culminating at 25% after 6 h and as it starts to decrease, the time course of 1-palmitin-3-olein starts to increase and reaches almost 45% after 24 h. The time courses of 1,3-dipalmitin increases regularly and reaches 5% after 24 h. The 1,3-diolein content remains under 2% during the whole kinetics (see Figure 15, D). The glycerolysis leads to significant higher content in monoglycerides and diglycerides than the lipolysis.

Others glycerolysis pathways were investigated; for examples, MG have been prepared from corn oil\textsuperscript{[90]} and tallow\textsuperscript{[91]} with immobilized lipases. By keeping the temperature below a specific temperature (30-46 °C), saturated fatty acids could be concentrated in the MG fraction during the lipase-catalyzed glycerolysis of palm oil,\textsuperscript{[92]} beef tallow, and lard.\textsuperscript{[93]} MG yields as high as 90% could be achieved with incubation times up to four days\textsuperscript{[94]} A membrane bioreactor also has been used for the glycerolysis of olive oil, but MG yields were low.\textsuperscript{[95]} However, the use of an in-line adsorption column connected to a membrane bioreactor to remove MG as they form improved overall yield.\textsuperscript{[96]} A microemulsion system also has been used to hydrolyze TG to obtain 2-MG.\textsuperscript{[97]} Yields of up to 80% were obtained however; contamination of the product with the surfactant was cited as a disadvantage.
3. RELEVANT METHODS FOR CHARACTERIZATION

3.1. Dynamic light scattering (DLS)

One of the most popular methods in colloid analysis is the so-called dynamic light scattering (DLS) or photon correlation spectroscopy (PCS). This technique enables the determination of particles sizes and their size distribution in dispersion in a range of 1-2000 nm. The method of DLS is based on the observation of the Brownian motion of submicrometer particles in diluted solutions.

When light hits small particles the light scatters in all directions (Rayleigh scattering) so long as the particles are small compared to the wavelength (< 250 nm). If the light source is a laser and thus is monochromatic and coherent, then one observes a time-dependent fluctuation in the scattering intensity. These fluctuations are due to the fact that the small molecules in solutions are undergoing Brownian motion and so the distance between the scatterers in the solution is constantly changing with time. This scattered light then undergoes either constructive or destructive interference by the surrounding particles and within this intensity fluctuation information is contained about the time scale of movement of the scatterers.

The principle of DLS is based on the Doppler Effect. The frequency of a moving source will be displaced, whereas the absolute value of the displacement depends on the speed of the particles. The phases of each scattered wave fluctuate in time. Therefore, the intensity of the scattered wave fluctuates also randomly. As the colloidal movement of the particle, which takes place in solution, is very slow, the frequency displacement is also extremely small and cannot be observed directly in this frequency range. The spectral broadening of the scattered light cannot be solved experimentally. The scattered light contains all information about the diffusion movement of the particles enabling the determination of the particles size.

The photomultiplier registers the number of photons that hit the detector during different intervals of time, which are called “channel width” (see Figure 16). The value of the diffusion coefficient of small particles is bigger than that of big particles and thus the signal changes more. It is hence possible to find a correlation between the coefficient of diffusion and the fluctuation in time of the scattered intensity. The autocorrelation function of the intensity is calculated and can be plotted versus the channel width. The average diffusion coefficient of the particle and its standard deviation are extracted from the fitting of this curve.
Using the Stokes-Einstein relation, the hydrodynamic averaged intensity radius can be assessed from the diffusion coefficient $D$:

$$R_H = \frac{k_B T}{6\pi \eta_0 D}$$  \hspace{1cm} (10)

with $\eta_0$ the viscosity of the medium, $k_B$ the Boltzmann constant, and $T$ the absolute temperature.

![Schematic presentation of a typical dynamic light scattering machine.](image)

Figure 16: Schematic presentation of a typical dynamic light scattering machine.\textsuperscript{[98]} PMT stands for photomultiplier tube.

### 3.2. Nuclear magnetic resonance

In its simplest form, nuclear magnetic resonance (NMR) is the study of the properties of molecules containing magnetic nuclei by applying a magnetic field and observing the frequency at which they come into resonance with an oscillating electromagnetic field. Larmor frequencies (which will be further described and explained in the next paragraphs) of nuclei at the fields normally employed typically lie in the radiofrequency region of the electromagnetic spectrum, so NMR is a radiofrequency technique. When applied to proton spins, the technique is called proton magnetic resonance ($^1\text{H-NMR}$).

This method is based on a physical phenomenon, the resonance. When two pendulums share a support and one is set in motion, the other is forced into oscillation by the motion of the common axle. As a result, energy flows between the two pendulums. The energy transfer occurs most efficiently when the frequencies of the two pendulums are identical. The
condition of strong effective coupling, when the frequencies of two oscillators are identical, is called resonance and the excitation energy is said to resonate between the coupled oscillators. Resonance is the basis of a number of everyday phenomena, including the response of radios to the weak oscillations of the electromagnetic field generated by a distant transmitter, but also in spectroscopic applications.

Many nuclei possess a spin angular momentum. A nucleus with spin quantum number \( I \) (which may be an integer or a half-integer and is never negative) has the following properties:

1. An angular momentum of magnitude \( \hbar \sqrt{I(I+1)} \);
2. A component of angular momentum \( m_i \hbar \) around an arbitrary axis, where \( m_i = I, I-1, \ldots, -I \);
3. If \( I > 0 \), a magnetic moment with a constant magnitude and an orientation that is determined by the value of \( m_i \).

To say that a nucleus has a magnetic moment means that, to some extent, it behaves like a small bar magnet. According to the second property, the spin, and hence the magnetic moment, of the nucleus may lie in \( 2I+1 \) different orientations relative to the axis. A proton has \( I = \frac{1}{2} \) and its spin may adopt either of two orientations; a \( ^{14}\text{N} \) nucleus has \( I = 1 \) and its spin may adopt any of three orientations. For interest of this dissertation, \( spin - \frac{1}{2} \) nuclei shall be considered, which are nuclei with \( I = \frac{1}{2} \). As well as protons, \( spin - \frac{1}{2} \) nuclei include \( ^{13}\text{C}, ^{19}\text{F}, \) and \( ^{31}\text{P} \) nuclei. The state with \( m_i = +\frac{1}{2} \) is denoted \( \alpha \) and the state with \( m_i = -\frac{1}{2} \) is denoted \( \beta \). It is worth bearing in mind that two very common nuclei, \( ^{12}\text{C} \) and \( ^{16}\text{O} \), have zero spin, and hence zero magnetic moment and so are invisible in magnetic resonance.

The nuclear magnetic moment of a nucleus is denoted \( \mu \). The component of the nuclear magnetic moment on the axis, \( \mu_Z \), is proportional to the component of spin angular momentum on that axis \( m_i \hbar \):

\[
\mu_Z = \gamma \hbar m_i
\]

The coefficient of proportionality \( \gamma \) is called the magnetogyric ratio of the nucleus. Theories of nuclear structure are not yet sufficiently advanced for \( \gamma \) to be calculated reliably and it is treated as an empirical quantity.
The magnetic moment is often expressed in terms of the nuclear $g$-factor $g_N$ and the nuclear magneton $\mu_N$:

$$\gamma \hbar = g_N \mu_N \quad \mu_N = \frac{e\hbar}{2m_p} = 5.051 \times 10^{-27} \text{ JT}^{-1} \quad (12)$$

where $m_p$ is the mass of the proton. The nuclear magneton is about 2000 times smaller than the Bohr magneton, so nuclear magnetic moments are about 2000 times weaker than the electron spin magnetic moment.

In quantum mechanical terms, the nuclear magnetic moment of a nucleus can align with an externally applied magnetic field of strength $B_0$ in only $2I + 1$ ways, either parallel or opposing $B_0$. The energetically preferred orientation has the magnetic moment aligned parallel with the applied field ($\text{spin} + \frac{1}{2}$) and is often given the notation $\alpha$, whereas the higher energy anti-parallel orientation ($\text{spin} - \frac{1}{2}$) is referred to as $\beta$ (see Figure 17). The rotational axis of the spinning nucleus cannot be oriented exactly parallel (or anti-parallel) with the direction of the applied field $B_0$ (defined in our coordinate system about the $z$-axis) but must precess about this field at an angle with an angular velocity of $2\pi \nu_L$ (see Figure 18).

![Figure 17: The nuclear spin energy levels of a spin $-\frac{1}{2}$ nucleus (e.g. $^1\text{H}$ or $^{13}\text{C}$) in a magnetic field. Resonance occurs when the energy separation of the levels matches the energy of the photons in the electromagnetic field.](image-url)

Figure 17: The nuclear spin energy levels of a spin $-\frac{1}{2}$ nucleus (e.g. $^1\text{H}$ or $^{13}\text{C}$) in a magnetic field. Resonance occurs when the energy separation of the levels matches the energy of the photons in the electromagnetic field.
In a magnetic field $B_0$, the $2I + 1$ orientations of the nucleus have different energies:

$$E_{m_i} = -\mu_z B = -\gamma B m_i$$  \hfill (13)

These energies are often expressed in terms of the Larmor frequency $\nu_L$:

$$E_{m_i} = -m_i \hbar \nu_L$$  \hfill (14)

where $\nu_L = \frac{\gamma B}{2\pi}$.

The stronger the magnetic field is, the higher the Larmor frequency is. A field of 12 T corresponds to a Larmor frequency of about 500 MHz for protons.

The energy separation of the two states of spin $-\frac{1}{2}$ nuclei (see Figure 17) is the needed energy to observe a typical NMR transition:

$$\Delta E = E_\beta - E_\alpha$$

$$\Delta E = \frac{1}{2} \gamma \hbar B - \left(\frac{1}{2} \gamma \hbar B\right)$$

$$\Delta E = \gamma \hbar B = \hbar \nu_L$$  \hfill (15)

For a proton is $\gamma_p = 26.75 \times 10^7$ rad.T$^{-1}$.s$^{-1}$ and $B_0 \sim 2$ T, $\Delta E = 6 \times 10^{-26}$ J. The relative populations of the higher ($N_\beta$) and lower ($N_\alpha$) energy levels at room temperature are given by the Boltzmann law:

$$\frac{N_\beta}{N_\alpha} = e^{\frac{\Delta E}{kT}} \approx 0.999.$$  \hfill (16)
For NMR, this means that the probability of observing a transition from \( N_\alpha \) to \( N_\beta \) is only slightly greater than that for a downward transition, \( i.e. \) the overall probability of observing absorption of energy is quite small. This relationship also explains why a larger \( B_0 \) favors sensitivity in NMR measurements, increasing as it does the difference between the two Boltzmann levels and why NMR becomes more sensitive at lower temperatures.

If the sample is bathed in radiation of frequency \( \nu \), the energy separations come into resonance with the radiation when the frequency satisfies the resonance condition:

\[
h \nu = \gamma h B = h \nu_L
\]  

(17)

That means, there is resonance when \( \nu = \nu_L \) and the radiation has the Larmor frequency. At resonance there is strong coupling between the nuclear spins and the radiation; strong absorption occurs as the spins make the transition \( \alpha \rightarrow \beta \).

The potential energy of the precessing nucleus is also a function of \( \theta \) is the angle between the direction of the applied field and the axis of nuclear rotation:

\[
E = -\mu B_0 \cos \theta
\]  

(18)

If energy is absorbed by the nucleus, then the angle of precession, \( \theta \), will change. For a nucleus of spin \( +\frac{1}{2} \), absorption of radiation "flips" the magnetic moment so that it opposes the applied field (the higher energy state)(see Figure 19).

![Figure 19](image-url)  

Figure 19: The nuclei absorb energy \( \Delta E \) (provided by a RF pulse), resulting in a change of the spin states, this phenomenon is the resonance.
There are two types of NMR spectrometers, continuous-wave (CW) and pulsed or Fourier-Transform (FT-NMR). Today CW-NMR spectrometers have largely been replaced with pulsed FT-NMR instruments. In low-resolution CW instruments electromagnets were cooled with water and magnets in FT-NMR spectrometers are cooled with liquid helium.

A continuous-wave NMR instrument consists of the following units: a magnet to separate the nuclear spin energy states; at least two radiofrequency channels, one for field/frequency stabilization and one to furnish RF irradiation energy; a sample probe containing coils for coupling the sample with the RF field; a detector to process the NMR signals; a sweep generator for sweeping either the magnetic or RF field through the resonance frequencies of the sample; and a recorder to display the spectrum (see Figure 20).

The spectrum is scanned by the field-sweep method or the frequency-sweep method. In the frequency-sweep method, the magnetic field is held constant, which keeps the nuclear spin energy levels constant, then the RF signal is swept to determine the frequencies at which energy is absorbed. In the field sweep method, the RF signal is held constant, then the magnetic field is swept, which varies the energy levels, to determine the magnetic field strengths that produce resonance at fixed resonance frequency.

Fourier-transform NMR spectrometers use a pulse of radiofrequency radiation to cause nuclei in a magnetic field to flip into the higher-energy alignment (see Figure 21). The length of the
RF pulse is 1-10 µs and is wide enough to simultaneously excite nuclei in all local environments. The interval between pulses $T$ is typically one to several seconds. During $T$, a time-domain RF amplified induced signal measured with the detection coil is called free induction decay (FID) of free precession signal. The FID signal represents different amortized sinusoids, function of the time and is emitted as nuclei return to their original state (see Figure 24).

An FID can be detected with a radio-receiver coil that is perpendicular to the static magnetic field. The FID signal is digitized and stored in a computer for data processing. Ordinary the time-domain decay signals from numerous successive pulses can be summed and averaged to improve the signal-to-noise ratio. The result is then converted to a frequency-domain signal by a Fourier transformation. The resulting frequency-domain output is similar to the spectrum produced by a scanning continuous-wave experiment.

![Fourier transformation nuclear magnetic resonance (FT-NMR) spectrometer](image)

Figure 21: Fourier transformation nuclear magnetic resonance (FT-NMR) spectrometer.\[99\]

It will now be considered how an observable measure can be produced. As mentioned above, to observe a resonance, an energy enabling the transition $\alpha \rightarrow \beta$ is needed. In the case of a Fourier transformation spectrometer, energy is provided by a second magnetic field $B_1$ whose
intensity is $10^6$ lower than the intensity of $B_0$. $B_1$ is applied with short pulses (of the order of \(\mu\)s) to get the resonance. $B_1$ rotates perpendicularly to and around $B_0$ at the angular velocity $2\pi\nu$ (see Figure 22). The angle of rotation $\theta$ depends on the gyromagnetic ratio $\gamma$ of the nucleus, the amplitude $B_1$ of the RF pulse and the length of time $t_w$, the RF pulse is applied:

$$\theta = \gamma B_1$$  \hspace{1cm} (19)

Figure 22 illustrates the rotation of $M_0$ by application of the RF pulse for sufficient time to rotate $M_0$ by $90^\circ$ ($\theta = \frac{\pi}{2}$ radians). That pulse is called a $90^\circ$ or $\frac{\pi}{2}$ pulse. The application of the $B_1$ field for twice as long ($\theta = \pi$ radians) will result in inversion of $M_0$.

![Figure 22: Rotation of the magnetization $\mathbf{M}_0$.](image)

The quantum mechanical analogs of the $90^\circ$ and $180^\circ$ pulses are illustrated in Figure 23. The $90^\circ$ pulse produces an equalization of populations in the two energy states and the $180^\circ$ pulse produces an inversion of populations so that the high energy state has a larger number of nuclear spins.
The Fourier transformation allows getting the signal understandable. A signal function of the frequency representing the final NMR spectrum is depicted in Figure 24.

The spectrum is described by a mathematical expression of the Fourier transformation:

\[ f(\omega) = \int_0^\infty f(t)e^{-i\omega t} dt \quad (20) \]

It is important to realize that only a small proportion of "target" nuclei are in the lower energy state (and can absorb radiation). There is the possibility that by exciting these nuclei, the populations of the higher and lower energy levels will become equal. If this occurs, then there

\[ \text{equilibrium} \]

\[ \text{90° pulse} \]

\[ \text{180° pulse} \]
will be no further absorption of radiation. The spin system is saturated. The possibility of saturation means that the relaxation processes has to be taken into account which would allow nuclei to return to the lower energy state.

How do nuclei in the higher energy state return to the lower state? Emission of radiation is insignificant because the probability of re-emission of photons varies with the cube of the frequency. At radio frequencies, re-emission is negligible. The focus has to be set on non-radiative relaxation processes. Ideally, the NMR spectroscopist would like relaxation rates to be fast - but not too fast. If the relaxation rate is fast, then saturation is reduced. If the relaxation rate is too fast, line-broadening in the resultant NMR spectrum is observed.

There are two major relaxation processes:
- spin-lattice (longitudinal) relaxation $T_1$,
- spin-spin (transverse) relaxation $T_2$.

It will be first considered the spin-lattice relaxation. The sample in which the nuclei are held is called the lattice. Nuclei in the lattice are in vibrational and rotational motion, which creates a complex magnetic field. The magnetic field caused by motion of nuclei within the lattice is called the lattice field. This lattice field has many components. Some of these components will be equal in frequency and phase to the Larmor frequency of the nuclei of interest. These components of the lattice field can interact with nuclei in the higher energy state and cause them to lose energy (returning to the lower state). The energy that a nucleus looses increases the amount of vibration and rotation within the lattice (resulting in a tiny rise in the temperature of the sample).

The longitudinal relaxation time, $T_1$, characterizes the rate at which the longitudinal $M_z$ component of the magnetization vector recovers. It is thus the time it takes for the signal to recover 63% [1-(1/e)] of its initial value before being flipped into the magnetic transverse plane. The name spin-lattice relaxation refers to the time it takes for the spins to give the energy they obtained from the RF pulse back to the surrounding lattice in order to restore their equilibrium state.

The sample in which the nuclei are held is called the lattice. Nuclei in the lattice are in vibrational and rotational motion, which creates a complex magnetic field. The magnetic field caused by motion of nuclei within the lattice is called the lattice field. This lattice field has many components. These components of the lattice field can interact with nuclei in the higher energy state and cause them to lose energy (returning to the lower state). The energy, that a
nucleus looses, increases the amount of vibration and rotation within the lattice (resulting in a tiny rise in the temperature of the sample).

The relaxation time, $T_1$ (the average lifetime of nuclei in the higher energy state), is dependant on the magnetogyric ratio of the nucleus and the mobility of the lattice. As mobility increases, the vibrational and rotational frequencies increase, making it more likely for a component of the lattice field to be able to interact with excited nuclei. However, at extremely high mobilities, the probability of a component of the lattice field being able to interact with excited nuclei decreases.

$M_Z$ is increasing exponentially towards $M_{Z_0}$:

$$\frac{dM}{dt} = -\frac{1}{T_1} (M_Z - M_{Z_0}) \quad (21)$$

The integration of (21) gives as represented Figure 25:

$$M_Z = M_{Z_0} (1 - e^{-\frac{t}{T_1}}) \quad (22)$$

![Figure 25: Spin-lattice relaxation after saturation of the spin system.](image)

The spin-spin relaxation, $T_2$, describes the interaction between neighboring nuclei with identical precessional frequencies but differing magnetic quantum states. In this situation, the nuclei can exchange quantum states; a nucleus in the lower energy level will be excited, while the excited nucleus relaxes to the lower energy state. There is no net change in the populations of the energy states, but the average lifetime of a nucleus in the excited state will decrease. This can result in line-broadening.
\( T_2 \) characterizes the rate at which the \( M_{xy} \) component of the magnetization vector decays in the transverse magnetic plane. It is the time it takes for the transverse signal to reach 37\% (1/e) of its initial value after flipping into the magnetic transverse plane.

After RF pulse, \( M_x \) and \( M_y \) shift out of the phase and decrease exponentially:

\[
\frac{dM_x}{dt} = -\frac{M_x}{T_2} \quad \text{and} \quad \frac{dM_y}{dt} = -\frac{M_y}{T_2}
\]

The integration of (23) gives:

\[
M_x = M_y = M_0 e^{-\frac{t}{T_2}}
\]

The types of information accessible via high resolution NMR include:
- Functional group analysis (chemical shifts),
- bonding connectivity and orientation (J coupling),
- through space connectivity (Overhauser effect),
- molecular conformations,
- chemical dynamics via analysis of the lineshapes.

It could be thought that all \(^1\text{H}\)s absorb at the same frequency. Yet, in practice different nuclei absorb at different frequencies.

As seen above, an atomic nucleus can have a magnetic moment (nuclear spin), which gives rise to different energy levels and resonance frequencies in a magnetic field. The total magnetic field experienced by a nucleus includes local magnetic fields induced by currents of electrons in the molecular orbitals (note that electrons have a magnetic moment themselves). The electron distribution of the same type of nucleus (e.g. \(^1\text{H}, ^{13}\text{C}, ^{15}\text{N}\)) usually varies according to the local geometry (binding partners, bond lengths, angles between bonds, ...) and with it the local magnetic field at each nucleus. This is reflected in the spin energy levels (and resonance frequencies). Thus the resonance frequency depends on the “effective” magnetic field that a proton “feels”. This can differ for different types of \(^1\text{H}\) due to local electron currents that counteract the applied field. This phenomenon is called shielding.
The field seen by the nuclei will be different:

\[ B_{\text{eff}} = B_{\text{app}} (1 - \sigma) \]  

(25)

\( \sigma \) is the screening constant typically \( 10^{-6} \).

It will absorb the related energy as represented Figure 26. The magnetic field induced spin energy is then because of the shield effect:

\[ E_{\text{int}} = -\gamma \hbar m_s B_{\text{app}} (1 - \sigma) \]  

(26)

The size of the chemical shift is given with respect to a reference frequency or reference sample, usually a molecule with a barely distorted electron distribution.

Figure 26: Shift of frequency due to screening is at the origin of the NMR spectrum, this is the chemical shift.

The variations of nuclear magnetic resonance frequencies of the same kind of nucleus (27), due to the screening induced by the variations in the electron distribution, are characterized by the chemical shift. Typically the chemical shift due to screening relative to a standard, the trimethylsilane (TMS), is measured:

\[ \nu_i - \nu_{\text{ref}} = \frac{\gamma B_{\text{app}}}{2\pi} (\sigma_{\text{ref}} - \sigma_i) \]  

(27)
The different positions of the frequency are measured as $\delta$ in ppm:

$$10^{-6} \delta_i = \frac{V_i - V_{ref}}{V_{ref}} \approx (\sigma_{ref} - \sigma_i) \quad (28)$$

The magnitude of the shielding depends on how the motion of electrons modifies the local field (interaction between field and electron angular momentum).

Qualitatively, proton NMR spectra can be interpreted by considering electronegativity of bond functional groups. Higher electronegativity pulls electrons away from the nuclei, lowering the resonance field and giving a larger $\delta$.

Other nuclei have larger chemical shifts because of the larger electron density (see Figure 27).

---

**Figure 27**: Proton chemical shift ranges for samples in CDCl$_3$ solution. The scale is $\delta$ relative to TMS at $\delta = 0$ ppm.$^{100}$

It has been discussed how the chemical shift of an NMR absorption is affected by the magnetic field $B_z$ produced by the presence of neighboring electrons. Now it is interesting to examine how the magnetic field produced by neighboring nuclei $B_n$ affects the appearance of the $^1$H NMR absorption. The effect occurs through the interaction between nuclear spins and bonding electron spins rather than through space. First the NMR absorption of a hydrogen nucleus labeled A with only one neighboring hydrogen nucleus in a vicinal position labeled X
(see Figure 28) is considered. It will be also assumed that $H_\alpha$ and $H_\gamma$ have significantly different chemical shifts.

![Figure 28: Representation of geminal and vicinal hydrogens.](image)

$H_\gamma$ will have approximately equal probability of existing in either the low energy $\alpha$-state or high energy $\beta$-state. Again because of the small energy difference between the low and high energy states, the high energy state is easily populated by excitation from thermal energy. For those molecules in which $H_\gamma$ exists in the low energy state, about half the molecules in the sample, its magnetic field $B_n$ will be subtracted from the magnetic field $B_\sigma-B_\pi$ and for those molecules in which $H_\gamma$ exists in the higher energy state, again about half the molecules, its magnetic field $B_n$ is added to $B_\sigma-B_\pi$.

It has to be considered that whether $B_n$ for a particular spin state adds to or subtracts from $B_\sigma$ is a function of the number of intervening bonds; this phenomenon does not usually affect the appearance of the signal and will not be explained here but results from the mechanism of coupling involving interaction of nuclear spins with electron spins. For the example of vicinal coupling (3 intervening bonds), the $B_n$ field is negative for $H_\gamma$ in the $\alpha$-spin state; for geminal coupling, $B_n$ is positive for $H_\gamma$ in the $\alpha$-spin state. Geminal coupling occurs between protons of different chemical shift bonded to the same carbon (2 intervening bonds); this situation will be discussed later.

As a consequence of the $B_n$ field in a vicinal system, at fixed external magnetic field $B_\sigma$, a lower frequency $\nu$ will be required to achieve resonance for those molecules which have $H_\gamma$ in the $\alpha$-state than for those molecules which have $H_\gamma$ in the $\beta$-state. The NMR signal for $H_\alpha$ will appear as a two line pattern (doublet) as shown in Figure 29. The $H_\gamma$ is said to split the absorption $H_\alpha$ into a doublet and the two protons are coupled to each other. The intensity of the two lines will be equal since the probability of $H_\gamma$ existing in the $\alpha$- or $\beta$-states is approximately equal. The chemical shift, which is defined as the position of resonance in the absence of coupling, is the centre of the doublet. Just as $H_\gamma$ splits the signal of $H_\alpha$ into a doublet, $H_\alpha$ splits the signal of $H_\gamma$ into a doublet. The overall splitting pattern consisting of two doublets is called an AX pattern.
When the molecule bears two equivalent vicinal protons, four possibilities exist for their combined magnetic fields: both are in \( \alpha \)-spin states, one is in the \( \alpha \)-spin state and one in the \( \beta \)-spin state and vice versa, or both in the \( \beta \)-spin state. These four possibilities have about equal probability and the appearance of the NMR signal is a 3-line pattern, a triplet (see Figure 30), with intensities 1:2:1 because the effect of \( \alpha\beta \) and \( \beta\alpha \) are equal. With one adjacent proton in the \( \alpha \)-spin state and the other in the \( \beta \)-spin state, the effect of the \( B_s \) field becomes zero and the center line of the triplet is the position of the chemical shift. The two \( H_X \) protons, seeing only one proton, split the \( H_A \) signal into a triplet and the \( H_A \) proton, seeing two equivalent protons, splits the two \( H_X \) protons into a doublet. The overall splitting pattern consisting of a triplet and a doublet is called an \( AX_2 \) pattern.
Three chemical shift equivalent vicinal protons $H_X$ split the absorption of $H_A$ into a quartet with intensity pattern 1:3:3:1 as shown in Figure 31. The chemical shift is the center of the quartet. The three $H_X$ protons split the $H_A$ signal into a quartet and the $H_A$ proton splits the signal for the three $H_X$ protons into a doublet. The overall splitting pattern consisting of a quartet and a doublet is called an AX$_3$ pattern.

Figure 31: Splitting of an NMR signal into a quartet as a result of the $B_n$ field produced by three vicinal protons.

The spacing between the lines of a doublet, triplet or quartet is called the coupling constant. It is given the symbol $J$ and is measured in units of Hertz (cycles per second). The magnitude of the coupling constant can be calculated by multiplying the separation of the lines in $\delta$ units (ppm) by the resonance frequency of the spectrometer in megahertz. Some spectroscopists place a number before the symbol $J$ to designate the number of bonds linking the coupled nuclei (colored orange below). Using this terminology, a vicinal coupling constant is $3J$ and a geminal constant is $2J$.

The following general rules summarize important requirements and characteristics for spin 1/2 nuclei:

1) Nuclei having the same chemical shift (called isochronous) do not exhibit spin-splitting. They may actually be spin-coupled, but the splitting cannot be observed directly.

2) Nuclei separated by three or fewer bonds (e.g. vicinal and geminal nuclei) will usually be spin-coupled and will show mutual spin-splitting of the resonance signals (same $J$'s), provided they have different chemical shifts. Longer-range coupling may be observed in molecules having rigid configurations of atoms.
3) The magnitude of the observed spin-splitting depends on many factors and is given by the coupling constant $J$ (units of Hz). $J$ is the same for both partners in a spin-splitting interaction and is independent of the external magnetic field strength.

The splitting pattern of a given nucleus (or set of equivalent nuclei) can be predicted by the $n+1$ rule, where $n$ is the number of neighboring spin-coupled nuclei with the same (or very similar) $J$’s. If there are 2 neighboring, spin-coupled, nuclei the observed signal is a triplet ($2+1=3$); if there are three spin-coupled neighbors the signal is a quartet ($3+1=4$). In all cases the central line(s) of the splitting pattern are stronger than those on the periphery. The intensity ratio of these lines is given by the numbers in Pascal’s triangle related to the Fibonacci series as shown in Figure 32. Thus a doublet has 1:1 or equal intensities, a triplet has an intensity ratio of 1:2:1, a quartet 1:3:3:1,… etc.

![Pascal's Triangle and its related Fibonacci series](image)

Figure 32: Pascal’s Triangle and its related Fibonacci series.

If a given nucleus is spin-coupled to two or more sets of neighboring nuclei by different $J$ values, the $N+1$ rule does not predict the entire splitting pattern. Instead, the splitting due to one $J$ set is added to that expected from the other $J$ sets (see Figure 33). It has to be noticed that there may be fortuitous coincidence of some lines if a smaller $J$ is a factor of a larger $J$. 

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Coupling constants are independent of the external magnetic field and reflect the unique spin interaction characteristics of coupled sets of nuclei in a specific structure. As noted earlier, coupling constants may vary from a fraction of 1 Hz to nearly 20 Hz, important factors being the nature and spatial orientation of the bonds joining the coupled nuclei. In simple, freely rotating alkane units such as CH₃CH₂X or YCH₂CH₂X the coupling constant reflects an average of all significant conformers and usually lies in a range of 6 to 8 Hz. This conformational mobility may be restricted by incorporating the carbon atoms in a rigid ring and in this way the influence of the dihedral orientation of the coupled hydrogens may be studied (see Figure 34).
The structures of cis and trans-4-tert-butyl-1-chlorocyclohexane (see Figure 34) illustrate how the coupling constant changes with the dihedral angle ($\phi$) between coupled hydrogens. The inductive effect of chlorine shifts the resonance frequency of the red colored hydrogen to a lower field ($\delta$ ca. 4.0 Hz), allowing it to be studied apart from the other hydrogens in the molecule. The preferred equatorial orientation of the large tert-butyl group holds the six-membered ring in the chair conformation depicted in the drawing. In the trans isomer this fixes the red hydrogen in an axial orientation; whereas for the cis isomer it is equatorial. The listed values for the dihedral angles and the corresponding coupling constants suggest a relationship, which has been confirmed and clarified by numerous experiments. This relationship is expressed by the Karplus equation (see Figure 35).

Figure 34: Fischer projections of the conformations of cis and trans-4-tert-butyl-1-chlorocyclohexane.[100]

Figure 35: The Karplus curve allows predicting the coupling constant between two vicinal protons along their stereochemical configuration.[100]
4. RESULTS AND DISCUSSION

This study of the lipase catalyzed lipolysis in miniemulsion is concentrated on tricaprylin. Tricaprylin is of interest since it is often used in the field of lipidology, because of its wide field of applications in the field of food as surfactant, in the medicinal field, or in the bioenergy field as bio-diesel. Moreover, this particular triglyceride was chosen because of its defined short fatty chains (C8) with a melting point at 9 °C. It means that at room temperature tricaprylin is liquid, which allows a formulation of a stable miniemulsion at room temperature. Additionally, the defined fatty chain length simplifies the analytics.

4.1. Analysis of different products

The work-up and analysis of the different products, which are presented in the following chapter, will enable to determine the quantitative and qualitative composition of the obtained products mixture, through the analysis of self-obtained standards.

4.1.1. 1H-NMR spectroscopy of acylglycerols of tricaprylin

Quantitative analyses of lipolysis products are conducted by using 1H NMR in deuterated chloroform. Besides serving as solvent, CDCl₃ is also the reference material for chemical shift values with residual chloroform giving a peak at 7.259 ppm. For sake of consistency, the integration value of the terminal methyl protons in the fatty acid chains is chosen as reference in all spectra and assigned the value 3.00. In accordance with the IUPAC nomenclature, the carbon atoms are counted from the carbon carrying the carboxylic acid or ester group in the fatty acid chain, with this carbon being C1. The assignments of the most common proton signals relevant to fatty compounds have been compiled in the literature[102] and are given in Table 3.
Table 3: Assignments of proton signals in the $^1$H-NMR spectra of fatty compounds in CDCl$_3$;\textsuperscript{[102]} see also references therein; all values relative to tetramethylsilane (TMS) $\delta = 0$ ppm).

<table>
<thead>
<tr>
<th>Structure</th>
<th>Description</th>
<th>Shift Values (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-CH$_3$</td>
<td>terminal methyl in alkyl chain</td>
<td>0.85-0.90 (triplet)</td>
</tr>
<tr>
<td>($\omega$1)CH$_2$</td>
<td>saturated alkyl chain</td>
<td>1.2-1.3</td>
</tr>
<tr>
<td>-CH$_2$-</td>
<td>acyl C-3, saturated chains</td>
<td>1.58</td>
</tr>
<tr>
<td>-CH$_2$-</td>
<td>acyl C-4 to C-(\omega3). Saturated chains; ($\omega$2)CH$_2$, saturated chain</td>
<td>1.2-1.3</td>
</tr>
<tr>
<td>R$_3$C-H</td>
<td>saturated</td>
<td>1.4-1.7</td>
</tr>
<tr>
<td>-CH$_2$-COOR</td>
<td>acyl C2</td>
<td>2.1-2.3 (triplet)</td>
</tr>
<tr>
<td>COOR-CH$_3$</td>
<td>methyl in acetoxy</td>
<td>1.9-2.6 (singlet)</td>
</tr>
<tr>
<td>-O-CH$_3$</td>
<td>methoxy ether, aliphatic</td>
<td>3.3-3.8 (singlet)</td>
</tr>
<tr>
<td>-O-CH$_3$</td>
<td>methyl ester, aliphatic</td>
<td>3.6-3.8 (singlet)</td>
</tr>
<tr>
<td>-CH-OH</td>
<td>$sn$-2 in glycerol</td>
<td>3.75 (multiplet)</td>
</tr>
<tr>
<td>-CH$_2$-OH</td>
<td>$sn$-1 or $sn$-3 in glycerol</td>
<td>3.6 (doublet)</td>
</tr>
<tr>
<td>-O-CH$_2$-</td>
<td>aliphatic saturated alcohol or ether</td>
<td>3.4-3.7</td>
</tr>
<tr>
<td>-CH$_2$-O-CO-R</td>
<td>$sn$-1 or $sn$-3 esterified glycerol</td>
<td>4.2-4.4</td>
</tr>
<tr>
<td>-CH$_2$-O-CO-R</td>
<td>$sn$-2 esterified glycerol</td>
<td>5.1-5.2 (quintet)</td>
</tr>
<tr>
<td>-CH$_2$-O-R</td>
<td>$sn$-1- or $sn$-3-O-alkylglycerol</td>
<td>3.5-3.6</td>
</tr>
<tr>
<td>-CH-O-R</td>
<td>$sn$-2-O-alkylglycerol</td>
<td>3.6-3.7</td>
</tr>
<tr>
<td>R-OH</td>
<td>hydroxyl proton</td>
<td>3.0-5.3</td>
</tr>
<tr>
<td>R-OH</td>
<td>carboxyl</td>
<td>10.5-12.0</td>
</tr>
</tbody>
</table>

It is possible to quantify the obtained acylglycerols with $^1$H-NMR. The components were first analyzed separately.

The acylglycerols were obtained by a lipase PS-catalysed lipolysis carried out at 35 °C in miniemulsion using tricaprylin as substrate. The different products of this reaction are separated qualitatively using the preparative thin layer chromatography (TLC) (see Figure 36).
The possible products of the tricaprylin lipolysis in miniemulsion are 1-, and 2-monoglycerides; 1,2- and 1,3-diglycerides; tricaprylin, glycerol, and caprylic acid. The $^1$H-NMR spectra of the different fractions in CDCl$_3$ are shown in Figure 37. The $^1$H-NMR spectra of caprylic acid and glycerol are not depicted, because both of them are not soluble in CDCl$_3$, but in DMSO-$d_6$. Therefore they are presented later in this paragraph.

It should be noted that 1,2-diglyceride and 1,3-diglyceride are collected together, since their respective $R_f$ values are very close to each other and 1-monoglyceride is also collected with 2-monoglyceride. That is the reason why traces of 1,2-dicaprylin in the spectrum of 1,3-dicaprylin can be observed and a significant amount of 1,3-dicaprylin in the 1,2-dicaprylin fraction $^1$H-NMR spectrum.
Figure 37: $^1$H-NMR spectra in CDCl$_3$ of the different TLC fractions: tricaprylin; 1,3-diglyceride; 1,2- and 1,3-diglycerides; 1- and 2-monoglycerides.

It can be noticed, that the peak with a chemical shift between about 4.90 and 5.20 ppm is specific to the different $^1$H-NMR spectra of tricaprylin, 1,2-diglyceride, and of 1-, 2-monoglyceride, will be useful later in order to quantify a part of the different lipolysis products.

The separated and analyzed products will be used as standards for $^1$H-NMR in the following to quantify their respective amount in the reaction mixture.

The determined $^1$H NMR chemical shift values of the different tricaprylin acylglycerol protons are presented in Table 4.
Table 4: $^1$H NMR chemical shift values ($\delta$) of tricaprylin and its acylglycerols protons in CDCl$_3$.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$H_A$</th>
<th>$H_A'$</th>
<th>$H_B$</th>
<th>2-H</th>
<th>3-H</th>
<th>$CH_2$</th>
<th>$CH_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tricaprylin</td>
<td>4.295(dd)$^a$</td>
<td>4.147(dd)$^b$</td>
<td>5.265(tt)$^c$</td>
<td>2.316/2.310(t)$^d$</td>
<td>1.602(m)</td>
<td>1.283(m)</td>
<td>0.881(t)$^e$</td>
</tr>
<tr>
<td>1,3-DG</td>
<td>4.189(dd)$^f$</td>
<td>4.134(dd)$^g$</td>
<td>4.083(m)</td>
<td>2.348(t)$^h$</td>
<td>1.632(m)</td>
<td>1.285(m)</td>
<td>0.881(t)$^i$</td>
</tr>
<tr>
<td>1,2-DG</td>
<td>4.235(dd)$^j$</td>
<td>4.188(dd)$^k$</td>
<td>5.084(m)</td>
<td>2.334(dt)</td>
<td>1.633(td)$^l$</td>
<td>1.291(m)</td>
<td>0.882(t)$^m$</td>
</tr>
<tr>
<td>1,2-DG</td>
<td>$H_A/H_A'$: 3.731(d)$^n$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-MG</td>
<td>4.206(dd)$^o$</td>
<td>4.150(dd)$^p$</td>
<td>5.084(m)</td>
<td>2.351(t)$^q$</td>
<td>1.633(m)</td>
<td>1.294(m)</td>
<td>0.882(t)$^r$</td>
</tr>
<tr>
<td>1-MG</td>
<td>$H_A$: 3.698(dd)$^s$</td>
<td>$H_A'$: 3.600(dd)$^t$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-MG</td>
<td>3.832(d)$^u$</td>
<td>3.832(d)$^v$</td>
<td>4.929(p)$^w$</td>
<td>2.351(t)$^x$</td>
<td>1.633(m)</td>
<td>1.294(m)</td>
<td>0.882(t)$^y$</td>
</tr>
</tbody>
</table>

J values (Hz): $a$ 4.32, 11.89; $b$ 5.96, 11.89; $c$ 4.35, 5.92; $d$ 7.55/7.52, 7.67; $e$ 6.87; $f$ 4.40, 11.28; $g$ 5.51, 11.56; $h$ 7.56; $i$ 6.86; $j$ 5.69, 11.94, 9.35; $k$ 4.45, 11.30, 9.35; $l$ 6.61, 12.85; $m$ 6.84; $n$ 4.75; $o$ 4.67, 11.65; $p$ 6.07, 11.65; $q$ 7.55; $r$ 6.84; $s$ 3.96, 11.44; $t$ 5.80, 11.44; $u$ 4.72; $v$ 4.76, 4.72; $w$ 7.55.

$H_A$/H$_{A'}$ are the glyceryl protons in the cases of 1-MG, and 1,2-DG in $\alpha$-position of the alcohol group. The corresponding $^1$H-NMR spectra are given in the appendix.

Concerning glycerol (see Figure 38), the $^1$H-NMR spectrum in DMSO-d$_6$ is depicted in Figure 39.

![Figure 38: Structural representation of the most stable conformation of the glycerol molecule.](image)

The shifts of the protons of the glycerol are assigned as follows: the distorted double doublet at $\delta = 3.358$ ppm ($J_{AA'} = 5.08$ Hz, $J_{AB} = 10.90$ Hz) is due to the shift of $H_{A'}$. The distorted double doublet at $\delta = 3.291$ ppm ($J_{AB} = 5.43$ Hz, $J_{AA'} = 10.83$ Hz) is due to the shift of $H_A$. It can be noticed that the $H_A$ proton, respectively the $H_A'$ proton, and the $H_B$ proton are stronger coupled in glycerol than in any other above-mentioned acylglycerols, but that the $H_A$ proton,
and the $H_A$ proton are not so strongly coupled than in any other acylglycerols. The free rotation of the sigma bonds of the glycerol “backbone” is probably responsible of those changes.

Figure 39: $^1$H-NMR spectrum of glycerol in DMSO-$d_6$

In this spectrum, it is not possible to assign the peak corresponding to the $H_B$ proton. A quintet is expected to be found at $\delta = 3.415$ ppm, but just a multiplet can be observed, whose coupling constant cannot be determined and whose integration is more than three times too big to correspond to this proton.

The doublet at $\delta = 4.495$ ppm ($J = 4.78$ Hz) and the triplet at $\delta = 4.420$ ppm ($J = 5.63$ Hz) correspond to ether protons. The glycerol, used for this $^1$H-NMR spectrum seems to be a mix of glycerol and dimers of glycerol (see Figure 40). The sum of all different peaks integrals from Figure 39 equals 10, thus confirming this suggestion.
However the quantitative determination of glycerol is not possible. Indeed Lutensol AT50 is used as non-ionic surfactant to prepare the miniemulsion. The surfactant Lutensol AT50 is a poly(ethylene oxide)-hexadecyl ether with an EO block length of about 50 units. The signal of Lutensol is exactly emerging with similar chemical shifts than glycerol in $^1$H-NMR of DMSO-$d_6$, i.e., between $\delta = 3.25$ and 3.50 ppm. Lutensol AT50 cannot be removed, because during the purification step, the mono- and diglycerides, which are soluble not only in organic solvents but also in water, could not be removed as well. An other method to quantify the glycerol amount is based on the compilation of the results obtained from the FFA acid-base titration and from $^1$H-NMR as described later in this thesis.

As for the caprylic acid, the $^1$H-NMR spectrum of which is depicted in Figure 41, the different protons characteristic of the caprylic fatty acid chain can be recognized, as already seen in the above-described acylglycerols $^1$H-NMR spectra. However, it can be noticed that the triplet at $\delta = 2.186$ ppm corresponding to the protons carried by the carbon C$_2$ in the caprylic acid (see Figure 41 and Figure 42) is slightly shifted more upfield than the ones related to the different acylglycerols in CDCl$_3$, i.e. at about $\delta = 2.3$ ppm (see Table 4). This observation could be used to assess the relative amounts of fatty acid in the raw product.
4.1.2. Determination of caprylic acid content by acid-base titration

Caprylic acid is released in the reaction system during the lipolysis. As seen before it is possible to see and to quantify the caprylic acid from $^1$H-NMR spectrum in DMSO-d$_6$. However because of the significant $^1$H-NMR-emerging relative error (about 5%) an other analytical method is preferred for the quantification of the released fatty acid amount in the raw product. Indeed, this is the analysis of free fatty acid by acid-base titration with NaOH of samples dissolved in ethanol, whose relative error is almost negligible (<1%). The comparison of the released fatty acid amount with the ester function groups present on tricaprylin leads to the expression of the saponification yield:

$$\%FFA_{\text{released}} = \frac{n_{FFA}}{3n_{TGini}}$$

(29)
4.2. Kinetics of lipolysis of tricaprylin by lipase in miniemulsion

In the following part, the lipolysis of tricaprylin in miniemulsion is described. A direct miniemulsion was prepared with 20.17 wt.-% of tricaprylin as disperse phase with respect to the continuous phase. Lutensol AT50 was used as non-ionic surfactant. It was chosen because of its inertness towards lipases. Lipases were used in catalytic amounts (5.0 wt.-% with respect to tricaprylin).

A series of different kinetics were performed as a function of the type of lipases and of the droplet size by varying the surfactant amount (see Table 5).

Two types of lipases were used for the kinetics. First of all, lipases with a specific regioselectivity, sn-1,3 lipases were chosen. The Rhizopus Arrhizus lipase (RAL) is of high interest because it is a sn-1,3 lipase showing a particular high activity towards tricaprylin as presented in Table 5 (see samples RAL1XX3, RAL1XX5, RAL2XX6, RAL3XX2, and RAL4XX2). The Rhizomucor Michei lipase (RML) is also interesting because it is a sn-1,3 lipase presenting lots of application in the field of monoglycerides synthesis (see sample RML1XX1). Finally, the Pseudomonas Cepacia lipase (lipase PS) (PS1XX1) was chosen because of its absence of regioselectivity. The influence of the miniemulsion homogeneity was investigated with RAL as lipase (see samples RAL1XX3 and RAL1XX5). To study the influence of the miniemulsion droplets, different surfactant concentrations were used with the lipase RAL. In order to shift the yield towards the monoglycerides by adding glycerol, the glycerolysis of tricaprylin by RAL in miniemulsion was also studied (see sample RAL3XX2).

The used temperatures are known to be the optimal temperatures for the lipase activity.\[70, 103-105\]

Table 5: Description of the series of kinetics.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Surfactant [wt.-%]</th>
<th>Droplet size [nm]</th>
<th>Temperature [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAL1XX3a</td>
<td>RAL</td>
<td>355 ± ca. 10 %</td>
<td>37</td>
</tr>
<tr>
<td>RAL1XX5</td>
<td>RAL</td>
<td>289 ± 9.35 %</td>
<td>37</td>
</tr>
<tr>
<td>RAL2XX6</td>
<td>RAL</td>
<td>645 ± 6.90 %</td>
<td>37</td>
</tr>
<tr>
<td>RAL4XX2</td>
<td>RAL</td>
<td>219 ± 5.14 %</td>
<td>37</td>
</tr>
<tr>
<td>RAL3XX2b</td>
<td>RAL</td>
<td>314 ± 13.44 %</td>
<td>37</td>
</tr>
<tr>
<td>RML1XX1</td>
<td>RML</td>
<td>270 ± 4.25 %</td>
<td>40</td>
</tr>
<tr>
<td>PS1XX1</td>
<td>PS</td>
<td>301 ± 9.3 %</td>
<td>35</td>
</tr>
</tbody>
</table>

\(^{a}\) miniemulsion with a low homogeneity, \(^{b}\) glycerolysis ratio (tricaprylin/glycerol) (mol/mol) (1/2). XX stands for the different samples composing a whole kinetics.
For the analysis of the reactions, titration and NMR experiments are used. In order to be able to evaluate the results, standards enable to assign the different signals of the $^1$H-NMR spectra to its relative raw products of the lipolysis of the tricaprylin in miniemulsion and to study the kinetics of the enzymatic lipolysis of the tricaprylin in solution.

4.2.1. Typical exploitation of the results obtained by the Pseudomonas Cepacia lipase-catalyzed lipolysis of tricaprylin in miniemulsion at 35 °C

The aim of this paragraph is to present a typical exploitation of the results obtained by the Pseudomonas Cepacia lipase (PS)-catalyzed lipolysis of tricaprylin in miniemulsion at 35 °C after 5 h.

A typical $^1$H-NMR spectrum of this kinetics presenting a mixture of saturated C$_8$-acylglycerols is depicted in Figure 43.

Figure 43: Typical $^1$H-NMR (400 MHz) spectrum in CDCl$_3$ of saturated C$_8$-acylglycerols, which are the products of the lipolysis of tricaprylin in miniemulsion by the Pseudomonas Cepacia lipase (PS) at 35 °C after 5 h.
As seen in the analytical part, the first spectral range of the $^1$H-NMR spectrum from $\delta = 0.0$ ppm to 3.2 ppm corresponds to the protons of fatty chains of the acylglycerols of tricaprylin. Since the different acylglycerols have the same chemical shift for those protons, it is not possible to estimate any result from those peak areas. Therefore, the spectral range from $\delta = 3.78$ ppm to 5.35 ppm is considered in the evaluations.

The evolution of the lipolysis with the time of the $^1$H-NMR spectrum (depicted in Figure 46) range is observed in Figure 44.

Figure 44: Kinetics of the lipolysis of tricaprylin by the Pseudomonas Cepacia lipase (PS) at 35 °C illustrated with the evolution of $^1$H-NMR spectrum of the raw product.
Two parts are considered in Figure 44, the left [5.40 ppm - 4.80 ppm] and the right side [4.40 ppm – 3.80 ppm]. The left side depicts the evolution of three peaks characteristic for tricaprylin, 1,2-dicaprylin and 2-monocaprylin. The right side depicts the evolution of three signals, corresponding to 1,3-dicaprylin, 1-monocaprylin and once again to 2-monocaprylin. It can be noticed that 1,2-dicaprylin and traces of 2-monocaprylin are already accumulated after 15 min.

A critical examination of the signal of 1,3-dicaprylin at $\delta = 4.051/4.4$ ppm shows that there is a superposition of different signals corresponding to different products of the lipolysis. They have to be taken into account for the exploitation of the $^1$H-NMR spectra (see Figure 45). One must bear in mind that the full signal corresponding to the protons of 1,3-diglyceride appears in this spectral range. The only possibility to get the area relative to the quantity of 1,3-diglyceride, is to subtract the area of the signal at $\delta = 4.051/4.4$ ppm from the areas related to the protons corresponding to tricaprylin (4H); 1,2-diglyceride (2H); 1-monoglyceride (2H).

Figure 45: Comparison between the superposition of (A): the following $^1$H-NMR spectra of the references (tricaprylin - 1,3-diglyceride - 1,2; 1,3-diglycerides – 1-; 2-monoglycerides) depicted in the analytical part (see Figure 37) and (B): the $^1$H-NMR spectrum of the raw products of the lipolysis of tricaprylin in miniemulsion by the Pseudomonas Cepacia lipase (PS) after 5 h at 35 °C.
The area related to 1,3-diglyceride can be assessed with the following equation:

\[
s_{1,3-DG} = s_{[\delta=4.05-4.4]} - (2 \cdot s_{1-MG} + 4 \cdot s_{TG} + 2 \cdot s_{1,2-DG}) \tag{29}
\]

with \(s_X\) standing for the area related to the peak of product X and \(s_{[\delta=4.05-4.25]}\) for the area related to the peak in the spectral range \([\delta=4.05-4.4\text{ ppm}]\).

To develop a method for the determination of the produced glycerol amount from the \(^1\)H-NMR spectra in CDCl\(_3\), the mass balance must be considered and leads to the following system:

\[
\begin{align*}
n_{TGini} &= n_{TG} + n_{DG} + n_{MG} + n_G \\
n_{FFA} &= n_{DG} + 2n_{MG} + 3n_G
\end{align*}
\tag{30}
\]

where

- \(n_{TGini}\) is the tricaprylin molar number introduced,
- \(n_{TG}\), the tricaprylin molar number during the lipolysis,
- \(n_{DG}\), the dicaprylin molar number during the lipolysis,
- \(n_{MG}\), the monocaprylin molar number during the lipolysis,
- \(n_G\), the glycerol molar number during the lipolysis,
- \(n_{FFA}\), the caprylic acid released by the acylglycerols during the lipolysis.

The following equation (31) can easily be concluded from the above-mentioned system (30):

\[
n_G = \frac{1}{3} n_{DG} + \frac{2}{3} n_{MG} - \frac{1}{3} \sum_{X=M}^T n_{XG} - \frac{n_{FFA}}{3n_{TGini}} - 1
\tag{31}
\]

where \(\sum_{X=M}^T n_{XG} = n_{TG} + n_{DG} + n_{MG}\) and \(\frac{n_{FFA}}{3n_{TGini}}\) are directly obtained from the titration of the free fatty acid (see equation 29).
It can be assumed that \( n_{XG} = s_{XG} \), where \( s_{XG} \) is the NMR peak area of each acylglycerol.

Using the above-described equations, it is now possible to exploit the \(^1\)H-NMR spectra for an evaluation of the kinetics. The \(^1\)H-NMR spectrum of the lipase PS-catalyzed lipolysis of tricaprylin in miniemulsion at 40 °C after 5 h (see Figure 43) is developed as an example. The titration of the caprylic acid gives for this sample 44.9% conversion.

![Figure 46: Expanded region at \( \delta = 3.78-5.35 \) ppm of the \(^1\)H-NMR (400 MHz) spectrum in CDCl\(_3\) of saturated C\(_8\)-acylglycerols, which are the products of the lipolysis of tricaprylin in miniemulsion by the Pseudomonas Cepacia lipase (PS) at 35 °C after 5 h (see Figure 43).](image)

Due to the value of the integrals and the value of the titration yield, one can determine the relative amount of acylglycerols in this sample (see Table 6).
Table 6: Exploitation of $^1$H-NMR spectrum depicted Figure 46 and relative amount in acylglycerols.

<table>
<thead>
<tr>
<th>Lipolysis Products</th>
<th>Relative amount [mol%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-monocaprylin</td>
<td>8.5</td>
</tr>
<tr>
<td>2-monocaprylin</td>
<td>21.8</td>
</tr>
<tr>
<td>1,2-dicaprylin</td>
<td>38.6</td>
</tr>
<tr>
<td>1,3-dicaprylin</td>
<td>6.5</td>
</tr>
<tr>
<td>tricaprylin</td>
<td>15.0</td>
</tr>
<tr>
<td>glycerol</td>
<td>9.6</td>
</tr>
<tr>
<td>caprylic acid</td>
<td>44.9</td>
</tr>
</tbody>
</table>

*Presentation of possible kinetics models*

The model described in the experimental part (see Paragraph 2.4.3.) is considered. A critical examination of the equations system shows that following approximations can be done, while considering the first equation of the system:

$$\frac{-d[\text{TG}]}{dt} = k_1[\text{TG}][H_2O] - k_2[1,2-DG][\text{FFA}]$$

It can be assumed that at the beginning of the tricaprylin hydrolysis the concentration of 1,2-dicaprylin and caprylic acid are negligible. One obtains:

$$\frac{-d[\text{TG}]}{dt} = k_1[\text{TG}][H_2O]$$

In the situation described by Equation 32-b, the reaction fits a second-order reaction kinetic model with respect to water and tricaprylin. However since the water represents the continuous phase, the concentration can be considered to be constant. In this case, the reaction would fit a first-order kinetic model.

The integral method is then applied to correlate the experimental data. For this purpose, a differential rate equation based on the disappearance of functional groups is constructed by assuming that the reaction is first- or second-order under the applied conditions during the first 2.5 h of the reaction. After this time, this kinetic model is no more valid because of the consumption of the formed products.
By assuming first as above-mentioned that the lipolysis reaction is first-order with respect to tricaprylin at the beginning of the reaction, the following rate equation is obtained:

\[
k' \cdot t = \ln \left( \frac{[TG]_0}{[TG]} \right)
\]

(33)

where \([TG]_0\) and \([TG]\) are the concentrations of tricaprylin at the initial time and at time \(t\), respectively. \(k'\) can be described as:

\[
k' = k \cdot [H_2O]^n
\]

(34)

where \(k'\) is the observed overall rate constant, \(k\) is the overall rate constant or velocity, \([H_2O]\) is the average concentration of water, considered as constant and \(n\) is the reaction order with respect to water and as above-mentioned (see Equation 32-b) is considered to have the value one.

Based on the above considerations, the rate data from the lipolysis reaction would fit with Equation 33. To test this rate equation, \(\ln \left( \frac{[TG]_0}{[TG]} \right)\) is plotted against time (see e.g. Inlet Figure 48).

In the case of a non-applicability of the first-order reaction kinetic model, i.e. of a significant decrease in the correlation coefficient and in the coefficient of determination, the second-order reaction kinetic model has to be considered:

\[
k' \cdot t = \frac{1}{[TG]} - \frac{1}{[TG]_0}
\]

(35)

To test this rate equation, \(\frac{1}{[TG]} - \frac{1}{[TG]_0}\) is plotted against time (see e.g. Inlet in Figure 49).

In both cases, the least-square approximation is applied fitting a straight line to the experimental data and in each case the correlation coefficient, \(r\), and the coefficient of determination, \(r^2\), are determined.
Kinetics of the isomerization of monoglycerides and diglycerides

It is important to study the kinetics of the isomerization of monoglycerides and diglycerides in water and in CDCl$_3$ in order to know its influence towards the yield of the products and to know the reliability of the $^1$H-NMR measurement in this thesis. Indeed these isomerization reactions are poorly described in the literature, since too many different parameters responsible for the isomerization have to be taken into account. Empirically it is known that 1,2-DG isomerizes to the thermodynamically more stable 1,3-form on storage, especially if there is any polar solvent present. If they do not isomerize on storage,$^{[97, 105]}$ they almost can certainly do so during analysis. The result is an equilibrium mixture of two forms with approximately 3:1 ratio of 1,3- to 1,2- form. MG isomerize resulting in approximately 90% 1-MG at the equilibrium state. That is also the reason why one cannot purchase pure 2-MG standards as the reaction occurs even in the solid state.

The lipolysis of tricaprylin in miniemulsion by the lipase PS was carried out and inhibited after 5 h by addition of SDS. Kinetics of the isomerization of monoglycerides and diglycerides were then carried out in water at 35 °C over 20 h and in CDCl$_3$ at room temperature over 24 h. The results are depicted in Figure 47.

The observation of Figure 47 shows that the relative amounts of monoglycerides and diglycerides are almost constant over the time of 24 h. Only a slight rise of 1-monocaprylin and 1,3-dicaprylin (relative amounts) respectively and a slight decay of 2-monocaprylin and of 1,2-dicaprylin are noticed. However the variation over the kinetics can be neglected since it remains under 5 mol%.
Figure 47: Time course of isomerization of mono- and diglycerides in H$_2$O at 35 °C: (A) 1-monocaprylin, (B) 2-monocaprylin, (C) 1,2-dicaprylin, (D) 1,3-dicaprylin and in CDCl$_3$ at room temperature: (A) 1-monocaprylin, (B) 2-monocaprylin, (C) 1,2-dicaprylin with, (D) 1,3-dicaprylin.

The results of the kinetics are discussed in the following part. The standard deviation for the exploitation of $^1$H-NMR is determined and lies between 1 and 5 mol%, and therefore about 5 mol% standard deviation on the $^1$H-NMR measurement are assumed in this thesis.

4.2.2. Description of kinetics

4.2.2-i. Description of kinetics of tricaprylin lipolysis in miniemulsion carried out with the lipases RML, RAL, and lipase PS at respective different optimal temperatures

Figures 49, 50 and 51 depict the time course of tricaprylin lipolysis in miniemulsion catalyzed by RAL at 37 °C, by lipase PS at 35 °C and by RML at 40 °C, respectively. The different acylglycerols and the caprylic acid are plotted as molar fractions of the initial tricaprylin concentrations.
Figure 48: Time courses of the products of tricaprylin (TG) lipolysis in miniemulsion by RAL at 37 °C: (A) caprylic acid ■, (B) 1-monocaprylin ●, (C) 2-monocaprylin ▲, (D) 1,2-dicaprylin with ▼, (E) 1,3-dicaprylin ●, (F) tricaprylin ▲, (G) glycerol ▼. Inset: Plot of $\ln \left( \frac{[TG]}{[TG_0]} \right)$ versus reaction time to test first-order reaction.

It can be seen in Figure 48, that the tricaprylin time course decreases exponentially (see inset in Figure 48) and reaches the value of 18.0 mol% after 2.5 h. Afterwards only a slight decrease is observed (17 mol% after 24 h). For the 1-monoglyceride a bell-shaped profile is observed, it increases rapidly within the first 3 h till 4 mol% and then decreases and reaches the value of 2 mol% after 5 h. After that it stays almost constant at 2 mol%. There is a transient accumulation of 2-monoglyceride, it increases till 35 mol% after 3 h and then decreases regularly till 18 mol% after 24 h. The same profile is observed for the 1,2-diglyceride, a transient accumulation until 45 mol% is detected after 6 h and then a very slow decrease to reach the value of 40 mol% after 24 h. 1,3-diglyceride first presents also a bell-shaped profile, showing a maximum of 3 mol% after 1.5 h. Before it decreases till 2 mol%
after 5 h and then remains almost constant. Glycerol shows two linear increase domains. A first one reaches the value of 4 mol% after 2 h and a second one the value of 21 mol% after 24 h. The caprylic acid time course increases till 42 mol% after 5 h and then seems to tend asymptotically towards 55-60 mol%, reaching the value of 48 mol% after 24 h.

The plot of $\ln\left(\frac{[TG]_0}{[TG]}\right)$ versus reaction time shows that this reaction is a first-order one (see inset in Figure 48).

In the case of the lipase PS (see Figure 49), tricaprylin decreases along a hyperbolic decay (see inset in Figure 49) to first reach the value of 17 mol% after 2 h and further slightly decreases to the value of 13 mol% after 24 h. As for the 1-monoglyceride, a bell-shaped profile is first observed. It increases rapidly till 11 mol% after 3 h and decreases till the value of 9 mol% after 5 h and then slowly decreases till 7 mol% after 24 h. A transient accumulation was observed for 2-monoglyceride. It increases till 24 mol% after 3.7 h and then decreases regularly to reach the value of 11 mol% after 24 h. The same kind of profile is observed for the 1,2-diglyceride, a transient accumulation until 42 mol% after 0.75 h and then a continuous decrease till the value of 11 mol% after 24 h. 1,3-diglyceride first presents also a bell-shaped profile, showing a maximum of 9 mol% after 2.5 h, before it decreases till 6 mol% after 9 h and is then constant. Glycerol shows a linear increase and reaches the value of 38.0 mol% after 24 h. The caprylic acid increases till 45 mol% after 5 h and then seems to tend asymptotically towards 60-65 mol%, reaching the value of 61 mol% after 24 h.
Figure 49: Time courses of the products of tricaprylin lipolysis in miniemulsion by the lipase PS at 35 °C: (A) caprylic acid ■, (B) 1-monocaprylin ●, (C) 2-monocaprylin ▲, (D) 1,2-dicaprylin with ▼, (E) 1,3-dicaprylin ●, (F) tricaprylin ▲, (G) glycerol ▼. Inset: Plot of $\frac{1}{[TG]} - \frac{1}{[TG]_0}$ versus reaction time to test second-order reaction. TG stands for tricaprylin.

The asymptotical behavior of the tricaprylin time course (see Figure 49, A) can be understood as obtaining the equilibrium in the lipolysis reaction. Lipolysis can be divided into three events. These include i) substrate partitioning to the lipid/water interface, ii) enzyme partitioning and iii) catalysis at the interface.\cite{106} Patton and Carey\cite{107} showed that diacylglycerol formed during fat digestion by human pancreatic lipase in the presence of bile salts and colipase, remains in the oil phase while monoacylglycerol enters the aqueous phase. Lagocki et al.\cite{108} studied the hydrolysis of trioctanoylglycerol and the partition of 1,2-dioctanoylglycerol between the lipid and the aqueous phase. They concluded that the diester remains on the surface, while both 2-monooctanoylglycerol and octanoic acid enter the aqueous phase. Finally, Scow et al.\cite{109} indicated that diacylglycerol formed by the action of lipoprotein lipase remains and spreads at the interface. Consequently, it can be assumed that a
layer of diacylglycerol is formed at the interface between the lipid and the aqueous phase leading to the asymptotical behavior of the tricaprylin time course.

The plot of \( \frac{1}{[TG]} - \frac{1}{[TG]_o} \) versus reaction time demonstrates that this reaction follows a second-order kinetics model compared to the first-order kinetics of the reaction with RAL as enzyme (see inlet of Figure 49).

![Graph](image)

Figure 50: Time courses of the products of tricaprylin lipolysis in miniemulsion by RML at 40 °C: (A) caprylic acid ■, (B) 1-monocaprylin ●, (C) 2-monocaprylin ▲, (D) 1,2-dicaprylin with ▼, (E) 1,3-dicaprylin ◆, (F) tricaprylin ▲, (G) glycerol ▲. Inset: Plot of \( \frac{1}{[TG]} - \frac{1}{[TG]_o} \) versus reaction time to test second-order reaction.

In the case of RML (see Figure 50), the time course of tricaprylin first decreases along a hyperbolic decay (see inset in Figure 50) till a value of 21 mol% after 3.0 h and then till a value of 17 mol% after 24 h. As for the 1-monoglyceride, a bell-shaped profile is first observed. It increases rapidly to reach 6 mol% after 2.5 h and decreases to reach the value of
3 mol% after 5 h; it then slowly decreases till 2 mol% after 24 h. There is a transient accumulation of 2-monoglyceride, it increases till 30 mol% after 2.5 h and then decreases regularly to reach the value of 20 mol% after 24 h. The same kind of profile is observed for the 1,2-diglyceride, a transient accumulation until 46 mol% after 2.5 h and then a continuous decrease to reach the value of 11 mol% after 24 h. 1,3-Diglyceride first presents also a bell-shaped profile, showing a maximum of 3 mol% after 0.25 h, before it decreases till 2 mol% after 3 h and is then almost constant. Glycerol shows here a linear increase and reaches the value of 26 mol% after 24 h. The caprylic acid time course increases till 42 mol% after 5 h and then seems to tend asymptotically towards 60 mol%, reaching the value 52 mol% after 24 h. The plot of \( \frac{1}{[TG]} - \frac{1}{[TG]_0} \) versus reaction time shows that this reaction obeys as in the case of using PS as enzyme a second-order kinetics (see inlet Figure 50).

In the following paragraphs, comparisons of product time courses are depicted. The dotted lines, which are depicted in the following diagrams, are a support for the reader, giving a tendency for the respective observed time courses.

4.2.2-ii. Influence of the different type of lipases

Three lipases were used to catalyze the lipolysis of tricaprylin in miniemulsion in order to study the influence of the lipase on the yield, the velocity and their respective activity in miniemulsion. The different product amounts of the reactions are compared (see Figure 51 and Table 7).
Figure 51: Comparison of the time courses of the lipolysis of tricaprylin in miniemulsion by different lipases (RML: RML1XX1 ■, RAL: RAL1XX5 ●, lipase PS: PS1XX1 ▲): (A) tricaprylin, (B) caprylic acid, (C) 1-monocaprylin, (D) 2-monocaprylin, (E) 1,2-dicaprylin, (F) 1,3-dicaprylin, (G) glycerol.
A comparable profile is observed for tricaprylin for the three lipases (see Figure 51), as seen before, the time course of RAL first decreases exponentially (see Figure 48, first order) and the time courses of lipase PS and RML first decrease along a hyperbolic decay (see Figure 49 and 50, second order). Then all time courses tend to reach an asymptotical value comprised between 15 and 20 mol% (A). The tricaprylin hydrolysis is slightly faster with lipase PS (see Table 7). A similar behavior is also observed for the caprylic acid. It must be noticed however that the caprylic acid time course from lipase PS is slightly above the both other time courses after 24 h (B). Large differences are detected for thermodynamically stable 1-monoglyceride. The yield is twice to four times bigger for the lipase PS than for the both other lipases, RAL and RML (C). For the 2-monoglyceride, comparable time courses are observed reaching a maximum of about 30 mol% after 3 h and 20 mol% after 24 h. The 2-monoglyceride time course obtained in lipase PS catalyzed system is slightly below the two other time courses (D). Considering the 1,2-dicaprylin, the time courses of the three lipases are quite similar till reaching their maximum, but a difference can be observed between RML and RAL whose yield reaches almost 40 mol% and lipase PS whose yield falls down to about 25 mol% (E). Drastic differences are also observed between the 1,3-diglyceride time course obtained in the system catalyzed by lipase PS and the RAL and RML systems (F). The 1,3-diglyceride time course from lipase PS increases till 9 mol% after 2.5 h, whereas RAL and RML reach only 3 mol% and then remain at 2 mol%. Finally, the time courses of glycerol are all linear. Comparable profiles are observed over 24 h for RML and RAL, but here also, for lipase PS a yield almost twice bigger than glycerol is depicted in Table 7 (G). The different maxima, which are characteristic for the above-depicted time courses, are shown in Table 7.

The observation of Figure 48 to 50 attests that all acylglycerols are products of the lipolysis. However a critical examination shows also that only small yields of 1,3-diglyceride and 1-monoglyceride in the cases of RML or RAL lipolysis are observed. In the case of the lipase PS, despite it is proved not to have a specific selectivity, it is noticed that the yield of (thermodynamically instable) 2-monoglyceride (70 mol% 2-monocaprylin out of both monoglycerides) and of 1,2-diglyceride (82 mol% 1,2-dicaprylin out of both diglycerides) are relatively bigger than the others respective stereoisomers (1-monoglyceride, and 1,3-diglyceride), which are actually the expected stereoisomers of a sn-1,3 regioselective lipase lipolysis.

In order to obtain high 1-MG and 1,3-DG ratios, lipase PS is favored, while for high 2-MG and 1,2-DG amounts, RAL is favorable. This can be explained due to the selectivity. Indeed, RAL seems to be the most sn-1,3 selective enzyme. 1-MG and 1,3-DG are almost not formed,
just 1,2-DG are formed yielding to 2-MG. RML shows a lower sn-1,3 selectivity than RAL towards the lipolysis of tricaprylin in miniemulsion. Indeed, a lower 1,3-DG ratio is observed, but a higher 1-MG ratio than for RAL is noticed.

Table 7: Comparison of the representative maxima of time courses of the different products of tricaprylin lipolysis in miniemulsion by respectively RML@40 °C, RAL@37 °C, and lipase PS@35 °C.

<table>
<thead>
<tr>
<th></th>
<th>RML1XX1 (mol%, time)</th>
<th>RAL1XX5 (mol%, time)</th>
<th>PS1XX1 (mol%, time)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG</td>
<td>21.0 @ 3 h</td>
<td>16.9 @ 3 h</td>
<td>16.5 @ 2 h</td>
</tr>
<tr>
<td>FFA</td>
<td>41.8 @ 5 h</td>
<td>42.2 @ 5 h</td>
<td>44.9 @ 5 h</td>
</tr>
<tr>
<td>1-MG</td>
<td>5.8 @ 2.5 h</td>
<td>3.2 @ 3 h</td>
<td>10.8 @ 5 h</td>
</tr>
<tr>
<td>2-MG</td>
<td>29 @ 2.5 h</td>
<td>34.5 @ 3 h</td>
<td>23.8 @ 3.7 h</td>
</tr>
<tr>
<td>1,2-DG</td>
<td>45.5 @ 2.5 h</td>
<td>46.3 @ 6 h</td>
<td>42.1 @ 0.75 h</td>
</tr>
<tr>
<td>1,3-DG</td>
<td>2.9 @ 2.5 h</td>
<td>3.3 @ 1.5 h</td>
<td>9.0 @ 2.5 h</td>
</tr>
<tr>
<td>G</td>
<td>26.3 @ 24 h</td>
<td>21.2 @ 24 h</td>
<td>38.0 @ 24 h</td>
</tr>
</tbody>
</table>

The order of the tricaprylin decay is determined for the tricaprylin yield of the above-described different kinetics. Following the best coefficient of determination, the overall rate constants for the tricaprylin hydrolysis time courses are determined for the different kinetics. The results are summarized in Table 8.

Table 8: The rate constants for the tricaprylin hydrolysis time courses with RML, RAL and lipase PS.

<table>
<thead>
<tr>
<th></th>
<th>RML1XX1</th>
<th>RAL1XX5</th>
<th>PS1XX1</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k'$ [h$^{-1}$]</td>
<td>1.3721</td>
<td>0.7128</td>
<td>2.5383</td>
</tr>
<tr>
<td>first-order</td>
<td>$r$ 0.9668</td>
<td>0.9890</td>
<td>0.6408</td>
</tr>
<tr>
<td></td>
<td>$r^2$ 0.9347</td>
<td>0.9781</td>
<td>0.4107</td>
</tr>
<tr>
<td>second-order</td>
<td>$r$ 0.9926</td>
<td>0.9816</td>
<td>0.9106</td>
</tr>
<tr>
<td></td>
<td>$r^2$ 0.9853</td>
<td>0.9635</td>
<td>0.8293</td>
</tr>
</tbody>
</table>

As it can be seen in Table 8, the reaction with RAL followed first-order kinetics during the first 2.5 h. On the other hand, for the reactions conducted with RML and PS, there is a decrease in the correlation coefficient and in the coefficient of determination. Moreover, the
coefficient of determination for RML is bigger for the second-order kinetics modeling than for the first-order one. This means that the reaction with RML can be fitted with the second-order kinetics model. The coefficient for PS is also bigger for the second-order kinetics modeling than for the first-order one. However its value hardly reaches 0.83. The percentage of original uncertainty remains significant (about 17 mol%). The second-order kinetics model describes better the lipase PS lipolysis in miniemulsion.

As for the kinetics constants, it is observed that the hydrolysis of tricaprylin by lipase PS in miniemulsion is almost two times faster than by RML and more than three times faster than by RAL (see Table 8).

The characterizations of the miniemulsion droplets, which are performed by DLS, allow establishing a relation with the used lipases. The number of lipase units per droplet, the relative surface area per lipase, and the number of ester bonds per lipase are calculated and depicted in Figure 52.

Figure 52: Characterization of the miniemulsion droplets and its relation to the used lipase. (A) droplet size [nm], (B) lipase/droplets ($\times 10^3$), (C) nm²/lipase, (D) ester bond/lipase ($\times 10^3$).

MW: RAL $\sim$ 43000 g/mol, RML $\sim$ 29500 g/mol, PS $\sim$ 33000 g/mol.
Figure 52 presents that the average droplet diameter of the miniemulsions is between 270 and 300 nm (A). The same amount of lipase (150 µg) is used for the different reactions. With respect to the respective molecular weight, it has to be noticed that the RAL concentration in the miniemulsion is smaller than the others resulting in a lower ratio lipase/droplet than for the other lipases (B). It explains also why the ratio of the surface per lipase (C) and the ratio of the ester bond per lipase (D) is bigger for RAL than for the other lipases. One lipase covers about 22 nm² (RML and PS) to 31 nm² (RAL) of the droplet surface. However, this variation of lipase amount does not seem to be a limiting parameter for RAL since it shows the relative fastest velocity (see Table 8) justifying the catalytic role of the lipase. A synopsis of the characteristics of the different samples is summarized in Table 9.

Table 9: Synopsis of the characteristics of the miniemulsion droplets and the relation to the used lipase.

<table>
<thead>
<tr>
<th></th>
<th>RML a1XX1</th>
<th>RAL b1XX5</th>
<th>PS c1XX1</th>
</tr>
</thead>
<tbody>
<tr>
<td>d droplets [nm]</td>
<td>270±4.25%</td>
<td>289±9.35%</td>
<td>301±9.3%</td>
</tr>
<tr>
<td>Lipase/droplet (x10³)</td>
<td>10.082±13.30%</td>
<td>8.44±30.75%</td>
<td>12.544±30.57%</td>
</tr>
<tr>
<td>nm²/lipase</td>
<td>22.786±4.44%</td>
<td>31.078±10.31%</td>
<td>22.786±10.25%</td>
</tr>
<tr>
<td>Ester bond/lipase (x10³)</td>
<td>3.760</td>
<td>5.481</td>
<td>4.207</td>
</tr>
</tbody>
</table>

a MW ~ 29,500 g/mol; b MW ~ 43,000 g/mol; c MW ~ 33,000 g/mol.

The results are consistent with the experiments which are described in the literature. Lipolyses of tricaprylin by different lipases in (macro)emulsion were performed earlier by Rogalska et al. [71] In their case, the emulsions were stabilized by the enzyme and no additional surfactant is used resulting in micrometer droplets and a broad droplet size distribution. They observed the activity and the regioselectivity of RML and RAL (see Table 4) but also for the lipase Pseudomonas sp. (PSp), which is similar to lipase PS and is obtained through cloning and sequencing the identity with the lipase PS [36]. The authors mentioned a relatively higher activity towards trioctanoin for RAL than for RML and PSp. There are different possibilities (under different conditions and for different substrates) to define the activity of a lipase. It will be simply considered that in an emulsion the activity of the following lipases at 37 °C towards tricaprylin is classified as follows:

\[
a(\text{RAL}) \gg a(\text{PSp}) \sim a(\text{RML})
\]
From the kinetics, the actual activity of the enzymes in miniemulsion can be determined. It means that in miniemulsion and under the applied conditions, the lipase activity can be expressed as units/milligram (U/mg), where 1 U is defined as the microequivalents (µmol) of caprylic acid liberated per hour (related to 1 mg trioctanoin). The lipase activities in miniemulsion towards trioctanoin are presented Table 10. The lipase PS shows a significant higher activity (15.4 U/mg) in miniemulsion than RML and RAL (10.9 U/mg).

Table 10: Specific activity of the lipase towards trioctanoin (8:0) under described conditions.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Code</th>
<th>Concentration [mg/mL]</th>
<th>Specific activity [U/mg] Trioctanoin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhizopus Arrhizus lipase</td>
<td>RAL</td>
<td>8.90</td>
<td>10.90a</td>
</tr>
<tr>
<td>Rhizomucor miehei lipase</td>
<td>RML</td>
<td>8.90</td>
<td>10.90b</td>
</tr>
<tr>
<td>Pseudomonas Cepacia lipase</td>
<td>PCL/PS</td>
<td>8.90</td>
<td>15.40c</td>
</tr>
</tbody>
</table>

a RAL1XX5; b RML1XX1; c PS1XX1.

In miniemulsion the behavior of the lipases are different, according to the following classification is observed:

\[ a(PS) > a(RAL) \sim a(RML) \]

Moreover concerning the lipase regioselectivity in a self-emulsified emulsion, as mentioned before, the lipases RAL and RML present a sn-1,3 selectivity and the lipase PS does not show any specific selectivity for the hydrolysis. The results depicted in Figure 51 confirm the regioselectivity of RAL and RML with about 90% of 2-MG vs. 10% of 1-MG and with also about 90% of 1,2-DG vs. 10% of 1,3-DG. As for the lipase PS, it can not be spoken about the absence of selectivity. Indeed the results of Figure 51 depict for the lipase PS about 30% of 1-MG vs. 70% of 2-MG and about 20% of 1,3-DG vs. 80% of 1,2-DG. So the lipase PS seems to show rather a sn-1,3 selectivity in miniemulsion, however the selectivity is less pronounced than in the case of RAL and RML. It can be concluded, that in miniemulsion, the lower reactivity is coupled with a higher 1,3-sn selectivity.

Finally, it is interesting to compare the results of the present work with the results obtained by Tan and Yin.\(^{[70]}\) The authors carried out the lipolysis of 1,3-palmitin-2-olein with RAL in emulsion and published diagrams presenting a comparable profile for the hydrolysis of 1,3-palmitin-2-olein (see Paragraph 2.4.4-ii.d). But the triglyceride time course does not decrease as fast as in miniemulsion, it only reaches 30% after 6 h (versus 17 mol% after 3 h for RAL.
against tricaprylin in miniemulsion) and tends to reach an asymptotical value comprised between 25 and 30% (versus about 15 mol% in miniemulsion). A similar behavior is also observed for the free fatty acid released during the lipolysis, the same profile is observed, however it is noticed that it only reaches about 14% after 12 h (versus 42 mol% after 3 h). The regioselectivity of RAL appears also comparable with what is observed in miniemulsion, 1-monoglyceride is almost not found. As for the 2-monoglyceride, a comparable time course is also observed, but reaching a maximum of about 6% after 6 h (versus 35 mol% after 3 h) and a further decrease to 5% after 24 h (versus about 20 mol% after 24 h). Considering the 1,2-diglyceride, its yield reaches almost 35% after 5 h (versus 46 mol% after 6 h) and falls down to about 30% after 24 h (versus 40 mol% after 24 h). Almost no 1,3-diglyceride is formed. Even though the substrate is significantly different than tricaprylin (C16/C18 versus C8 for the tricaprylin), RAL seems to have a higher activity in miniemulsion.

4.2.2-iii. Influence of the miniemulsion homogeneity

Two different lipolysis reactions were carried out at 37 °C with lipase RAL to study the influence of the miniemulsion homogeneity in the enzymatic lipolysis. In order to understand better the mechanism of the lipolysis of tricaprylin in miniemulsion, the homogeneity of the droplets was varied. The kinetic stability of the miniemulsion was assured by the presence of a hydrophobic agent, the hexadecane. It has to be uniformly distributed within the droplets. If hexadecane is not uniformly distributed in the dispersed phase before being mixed with the continuous phase and before the sonication, the system then will slowly lead to a process of phase separation of both phases. Independently of the miniemulsion stability, during the lipolysis a slow phase separation can be noticed, due to the formation and the release of fatty acid which is hydrophilic and partially soluble in the continuous phase. Thus the released acid will diffuse into the continuous phase inducing the formation of a diffusion gradient destabilizing the whole miniemulsion leading then to phase separation. The phase separation was maximized when the lipolysis in miniemulsion was carried out over several days. This phase separation, which is induced by an increase of the droplet size, appeared directly at the beginning of the lipolysis in the case of the less stable miniemulsion, whereas it appeared after about 3 h in the case of the stable miniemulsion. The reaction yields of the different products as a function of miniemulsion homogeneity (homogeneous: RAL1XX5 and less homogeneous: RAL1XX3) are compared in Figure 53.
Figure 53: Comparison of the time courses of the lipolysis of tricaprylin in miniemulsion (less homogeneous miniemulsion: RAL1XX3 ▲, homogeneous miniemulsion: RAL1XX5 ●): (A) tricaprylin, (B) caprylic, (C) 1-monocaprylin, (D) 2-monocaprylin, (E) 1,2-diglyceride, (F) 1,3-diglyceride, (G) glycerol.
A comparable profile is observed for tricaprylin (see Figure 53); the time courses first decrease exponentially and then reach an asymptotical value comprised between 14 and 17 mol% (A). A similar curve is detected for the caprylic acid. The same profile is observed and a comparable linear profile for the time courses of glycerol is seen (B). For the other time courses, considerable differences are detected. For 1-monoglyceride, the yield is almost ten times bigger for the less homogeneous miniemulsion than for the homogeneous one (C). Also for the 2-monoglyceride, drastic differences are seen between both times courses. Whereas both profiles show a transient accumulation of 2-monocaprylin, the time course of the homogeneous miniemulsion reaches a maximum of 35 mol% after 3 h and then decreases to about 20 mol%. The 2-monocaprylin time course from the less homogeneous miniemulsion system reaches only 9 mol% after 3 h and decreases to about 7 mol% after 24 h (D). Considering the 1,2-dicaprylin, the time courses are also dramatically different. The yield of the homogeneous miniemulsion reaches 46 mol% after 5 h, and then decreases slowly till 40 mol% after 24 h. In the case of the less homogeneous miniemulsion, a transient accumulation reaches a maximum of 27 mol% after 2.5 h, and then a minimum of 14 mol% after 5 h is reached. Surprisingly, it increases afterwards again and reaches the value of 25 mol% after 24 h, whereas it has been expected to decrease or at least to remain constant (E). Concerning the 1,3-diglyceride, the time course obtained by the less homogeneous miniemulsion system depicts a fast increase reaching 31 mol% after 3 h and then a slow decrease reaching about 26 mol% after 24 h. In the homogeneous miniemulsion, the time course of 1,3-diglyceride hardly reaches 3 mol% after 1.5 h and slowly decreases to 2 mol% after 24 h (F). Finally, the time courses of glycerol are both linear and Figure 53 depicts similar glycerol yields after 24 h for both homogeneous and less homogeneous systems (G). The different maxima, which are characteristic for the above-depicted time courses, are shown in Table 11.

The homogeneity obviously influences the selectivity of the lipase. Indeed, the lipase in the less homogeneous system behaves almost as a non-specific lipase. After 3 h, not only an unexpected accumulation of 1,3-dicaprylin reaching more than 30 mol% (even more than with lipase PS, see Table 7) is observed, but also a continuous accumulation of 1,2-dicaprylin reaches a maximum of about 30 mol% after 2.5 h. Additionally a consequent and then expected accumulation of 1-monocaprylin reaching 37 mol% takes place after 4 h. On the same manner, correspondences between the 1,2-dicaprylin kinetics profile and the 2-monocaprylin should be noticed, since the product of the 1,2-dicaprylin hydrolysis by a sn-1,3
lipase is the 2-monoglyceride. But a small yield of about 9.0 mol% is observed for the 2-monocaprylin (see Table 11).

The homogeneous miniemulsion favors the formation of 1,2-DG and 2-MG. Almost no 1,3-DG and consequently no 1-MG are found. RAL in the homogeneous miniemulsion shows an high sn-1,3 selectivity, whereas the less homogeneous miniemulsion yields to 1,3-DG and to 1-MG, but almost to no 2-MG and to only a small amount of 1,2-DG. The acylglycerols obtained in the less homogeneous miniemulsion seem to show a loss of selectivity of RAL, and thus are different from the acylglycerols obtained by Tan and Yin. Indeed the authors observed reaction products issued from a sn-1,3 regioselective mechanism lipolysis.

Table 11: Comparison of the representative maxima of time course of the different products of tricaprylin lipolysis in miniemulsion by a respectively less and better homogeneity of the miniemulsion.

<table>
<thead>
<tr>
<th></th>
<th>less homogeneous miniemulsion</th>
<th>homogeneous miniemulsion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RAL1XX3 (mol%, time)</td>
<td>RAL1XX5 (mol%, time)</td>
</tr>
<tr>
<td>TG</td>
<td>14.8 @ 3 h</td>
<td>16.9 @ 3 h</td>
</tr>
<tr>
<td>FFA</td>
<td>41.3 @ 3 h</td>
<td>42.2 @ 5 h</td>
</tr>
<tr>
<td>1-MG</td>
<td>36.6 @ 3.8 h</td>
<td>3.2 @ 3 h</td>
</tr>
<tr>
<td>2-MG</td>
<td>9.0 @ 2.5 h</td>
<td>34.5 @ 3 h</td>
</tr>
<tr>
<td>1,2-DG</td>
<td>27.3 @ 2.5 h</td>
<td>46.3 @ 6 h</td>
</tr>
<tr>
<td>1,3-DG</td>
<td>31.3 @ 3 h</td>
<td>03.3 @ 1.5 h</td>
</tr>
<tr>
<td>G</td>
<td>15.8 @ 24 h</td>
<td>21.2 @ 24 h</td>
</tr>
</tbody>
</table>

It is observed that the homogeneous miniemulsion leads to a higher selectivity to form 1,2 DG and 2-MG. This can be understood as a consequence of a better defined reaction environment. Indeed less rearrangements of the enzymes on the droplet surface may happen in the homogeneous miniemulsion during the lipolysis, since less desorption/adsorption steps of the lipase may occur during the reaction, but also no related diffusion gradient takes place in the reaction field.

The order of the exponential decay is determined for the tricaprylin yield of the above-described different kinetics. The related overall rate constants for the tricaprylin hydrolysis time courses are determined and depicted in Table 12.
Table 12: The overall rate constants for the tricaprylin hydrolysis time courses with RAL in the cases of a homogeneous and a less homogeneous miniemulsion.

<table>
<thead>
<tr>
<th></th>
<th>less homogeneous RAL1XX3</th>
<th>homogeneous RAL1XX5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k$ [h$^{-1}$]</td>
<td>0.6873</td>
<td>0.7128</td>
</tr>
<tr>
<td>first-order $r$</td>
<td>0.9802</td>
<td>0.9889</td>
</tr>
<tr>
<td>$r^2$</td>
<td>0.9608</td>
<td>0.9781</td>
</tr>
<tr>
<td>second-order $r$</td>
<td>0.9735</td>
<td>0.9816</td>
</tr>
<tr>
<td>$r^2$</td>
<td>0.9478</td>
<td>0.9635</td>
</tr>
</tbody>
</table>

As it can be seen in Table 12 and as already observed before (see inset in Figure 49), the reaction with RAL followed first-order kinetics during the first 2.5 h. However, for the reaction RAL1XX3, whose miniemulsion homogeneity is lower, there is a slight decrease in the correlation coefficient and the coefficient of determination.

The coefficients of determination depicted in Table 12 show that the first-order reaction kinetics modeling describes better the profile of the tricaprylin hydrolysis in both homogeneous and less homogeneous miniemulsions. The homogeneous miniemulsion leads to the high value of $k'$, since the reaction interface remains constantly large for the entire reaction course. This might be due to a decrease of the interfacial area throughout the reaction.

The characterizations of the miniemulsion droplets, which are performed by DLS, allow establishing a relation between the yields and the homogeneity of the miniemulsion. The number of lipase units per droplet, the relative surface per lipase and the number of ester bonds per lipase are determined and depicted in Figure 54.

Before starting the reaction, the average droplet size in the less homogeneous miniemulsion is about 355 nm, and about 290 nm in the homogeneous case.
Figure 54: Characteristics of the miniemulsion droplets and the relation to the used lipase. 
(A) droplet size [nm], (B) lipase/droplets ($\times 10^3$), (C) nm²/droplet, (D) lipase/ester bond ($\times 10^3$).

The droplet size of the initial miniemulsion is slightly bigger in the case of the less homogeneous miniemulsion (about 355 nm versus about 289 nm, see Figure 54 and Table 13). This difference may be due to the relative homogeneity difference existing already before the reaction. The lipase per droplet ratio and the interfacial area per lipase are dramatically influenced by this difference. Indeed the lipase per droplet ratio is almost two times bigger and the interfacial area is about 20% smaller for the less homogeneous system RAL1XX3 than for the homogeneous one.
Table 13: Synopsis of the characteristics of the miniemulsion droplets as function of the homogeneity and the relation to the used lipase RAL.

<table>
<thead>
<tr>
<th></th>
<th>less homogeneous</th>
<th>homogeneous</th>
</tr>
</thead>
<tbody>
<tr>
<td>$d$ droplets [nm]</td>
<td>355 ± ca 10 %</td>
<td>289 ± 9.35 %</td>
</tr>
<tr>
<td>Lipase/droplet (x10^3)</td>
<td>15.649 ± ca. 33.10%</td>
<td>8.444 ± 30.75%</td>
</tr>
<tr>
<td>nm²/lipase</td>
<td>25.300 ± ca. 9.10%</td>
<td>31.078 ± 10.31%</td>
</tr>
<tr>
<td>Ester bond/lipase (x10^3)</td>
<td>5.481</td>
<td>5.481</td>
</tr>
</tbody>
</table>

* MW ~ 43,000 g/mol.

But even though the above-mentioned differences between the interfacial area at the beginning of the reaction and the fact that the interface area decreases, no significant consequences on the kinetics rate constants of the tricaprylin hydrolysis are depicted (see Figure 54). However the results from Table 12 show that the velocity for the kinetics conducted in the homogeneous miniemulsion is slightly higher than for the less homogeneous one.

Again from the kinetics, a specific activity of the lipases in both situations can be calculated. The activity of RAL in the case of the less homogeneous miniemulsion is lower (10.50 U/mg versus 10.90 U/mg), but it remains comparable (see Table 14).

Table 14: Specific activity of the lipases towards trioctanoin (8:0) under described conditions.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Code</th>
<th>Concentration [mg/mL]</th>
<th>Specific activity [U/mg]</th>
<th>Trioctanoin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhizopus Arrhizus lipase</td>
<td>RAL</td>
<td>8.90</td>
<td>10.90^a</td>
<td></td>
</tr>
<tr>
<td>Rhizopus Arrhizus lipase</td>
<td>RAL</td>
<td>8.90</td>
<td>10.50^b</td>
<td></td>
</tr>
</tbody>
</table>

* RAL1XX5 (homogeneous miniemulsion), ^b RAL1XX3 (less homogeneous miniemulsion).

4.2.2-iv. Influence of the droplet size

Different reactions at 37 °C with the lipase RAL were performed to study the influence of the droplet size of the miniemulsion in the enzymatic lipolysis. As already mentioned before, lipases are known for their interfacial activation mechanism. A variation of the miniemulsion droplet size leads consequently to a variation of the whole interface area, (see Figure 55) and then influences the lipase interfacial activation mechanism positively or negatively.
Droplets with an average size in the range of 220 nm to 465 nm were obtained by using different concentrations of non-ionic surfactant (Lutensol AT50): i) 0.5 wt.-% (RAL2XX6), ii) 1.0 wt.-% (RAL1XX5) and iii) 2.0 wt.-% (RAL4XX2).

Figure 55: Influence of the surfactant concentration on the droplet size of tricaprylin miniemulsion and on its whole surface.

The different product amounts of the above-described reactions are then compared (see Figure 56).
Figure 56: Comparison of the time courses of the lipolysis of tricaprylin by RAL at 37 °C in miniemulsion (RAL2XX6, 0.5 wt.-% surfactant, 465 nm ■; RAL1XX5, 1.0 wt.-%, 289 nm ▲; RAL4XX2, 2.0 wt.-%, 219 nm ▲): (A) tricaprylin, (B) caprylic acid, (C) 1-monocaprylin, (D) 2-monocaprylin, (E) 1,2-dicaprylin, (F) 1,3-dicaprylin, (G) glycerol.
A comparable profile is observed for tricaprylin (see Figure 56), the time courses first decrease exponentially and then reach an asymptotical value comprised between 15 and 20 mol%. It is also seen that the slope is less steep for the reaction of the miniemulsion with the largest droplet size (RAL2XX6, 0.5 wt.-% surfactant, 465 nm) than for smaller droplets (RAL1XX5, 1.0 wt.-% surfactant, 289 nm; and RAL4XX2, 2.0 wt.-% surfactant, 219 nm). However the general profile of these time courses remains similar (A). Comparable profiles for caprylic acid are observed (B). One observes almost similar linear profiles for the glycerol reaching almost 20 mol% after 24 h for the miniemulsions with the largest and the smallest droplet size (RAL2XX6, 0.5 wt.-%, 465 nm; RAL4XX2, 2.0 wt.-%, 219 nm). Only for the miniemulsion with the intermediate droplet size (RAL1XX5, 1.0 wt.-%, 289 nm) the glycerol time course reaches about 7 mol% after 2.5 h and then it steeply decreases (G). The time courses for 2-monoglyceride depict the same behavior, the curves describe a fast increase reaching about 30 mol% after 3 h and then a slow decrease reaching about 18 mol% for the reaction of RAL2XX6 and RAL1XX5 and about 26 mol% for the reaction of RAL4XX2 (D). The profiles for 1,2-diglyceride are also comparable, the curves describe a fast increase reaching about 43 mol% after 3 h and then a slow decrease reaching about 40 mol% (E). Only for 1-monoglyceride (C) and 1,3-diglyceride, slight differences are observed, the time courses stay below 7 mol% and are thus in the uncertainty range. The time courses of 1-monocaprylin depict the same behavior, a first fast increase followed by a transient accumulation reaching respectively 2.4 mol% after 2.5 h, 3.5 mol% after 3 h and 5 mol% after 5.5 h are observed for following respective reaction of RAL2XX6, RAL1XX5, and RAL4XX2 and then decrease respectively towards the asymptotical values of 3 mol%, 2 mol%, and 1 mol% respectively. Considering the time courses of 1,3-diglyceride, they depict a fast increase followed by a transient accumulation whose respective maxima are 3 mol% after 2.5 h, 3 mol% after 1.5 h and 7 mol% after 1 h for the following respective kinetics of RAL2XX6, RAL1XX5 and RAL4XX2 then decrease respectively towards the asymptotical values of 2 mol%, 2 mol%, and 1 mol% (F). Table 15 depicts the representative maxima of the different time courses.

In brief it can be noticed that the smaller the droplet size is, the higher the 1-MG yield is; and the smaller the droplet size is, the less steep the 2-MG time course is. The droplet size directly affects the reaction, only the selectivity is not significantly influenced by those variations.

Table 15: Comparison of the representative maxima of time course of the different products of tricaprylin lipolysis in miniemulsion by different surfactant (Lutensol AT50) concentration respectively 0.5 wt.-% (RAL2XX6), 1.0 wt.-% (RAL1XX5), 2.0 wt.-% (RAL4XX2).
The order of the exponential decay is determined for the tricaprylin yield of the above-described different kinetics. The overall rate constants for the tricaprylin hydrolysis time courses are determined and summarized in Table 16.

Table 16: The overall rate constants for the tricaprylin hydrolysis time courses with RAL in the cases of different miniemulsion droplet size.

<table>
<thead>
<tr>
<th>Droplet size</th>
<th>465 nm</th>
<th>289 nm</th>
<th>219 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAL2XX6 (mol%, time)</td>
<td>RAL1XX5 (mol%, time)</td>
<td>RAL4XX2 (mol%, time)</td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>21.2 @ 3 h</td>
<td>16.9 @ 3 h</td>
<td>15.5 @ 3 h</td>
</tr>
<tr>
<td>FFA</td>
<td>40.8 @ 5 h</td>
<td>42.2 @ 5 h</td>
<td>44.7 @ 5 h</td>
</tr>
<tr>
<td>1-MG</td>
<td>2.4 @ 2.5 h</td>
<td>3.5 @ 3 h</td>
<td>04.9 @ 5.5 h</td>
</tr>
<tr>
<td>2-MG</td>
<td>29.2 @ 3 h</td>
<td>34.5 @ 3 h</td>
<td>33.3 @ 3 h</td>
</tr>
<tr>
<td>1,2-DG</td>
<td>43.1 @ 3 h</td>
<td>46.3 @ 6 h</td>
<td>42.8 @ 3 h</td>
</tr>
<tr>
<td>1,3-DG</td>
<td>03.3 @ 2.5 h</td>
<td>3.3 @ 1.5 h</td>
<td>06.5 @ 1 h</td>
</tr>
<tr>
<td>G</td>
<td>18.5 @ 24 h</td>
<td>21.2 @ 24 h</td>
<td>16.4 @ 24 h</td>
</tr>
</tbody>
</table>

As can be seen in Table 16 and as already observed before (see inset in Figure 49), the reaction with RAL followed a first-order kinetics during the first 2.5 h. The smaller the droplet size is, the faster the kinetics also is. This can be explained by the much higher interfacial area which can be used for the reaction.

It is then possible to characterize the size of the miniemulsion droplets by DLS and to establish a relation with the used lipases (see Figure 57).
Figure 57: Characteristics of the miniemulsion droplets and the relation to the used lipase.

(A) lipase/droplet (×10³), (B) nm²/droplet, (C) surfactant/droplet (×10³), (D) nm²/surfactant.

Figure 57 depicts that with increasing surfactant concentration, the amount of lipase per droplet decreases rapidly (A); the surface area per lipase increases linearly (B); the surfactant molecules per droplet decreases to an asymptotical value (about 100×10³) and the surface area per surfactant molecule decreases regularly (D). However it must be noticed, that for a constant amount of ester bonds per lipase, the smaller the droplet size is, the smaller the interfacial area per surfactant molecule is. This means that an increase of the surfactant density occurs for the small miniemulsion droplet size. Moreover the smaller the droplet size, the larger the interfacial area per lipase. This means that the increase of the surfactant density occurs despite the increase of the whole interfacial area per lipase. A synopsis of the characteristics is depicted in Table 17.
Table 17: Synopsis of the characteristics of the miniemulsion droplets with different droplet sizes and the relation to the used lipase RAL.

<table>
<thead>
<tr>
<th></th>
<th>RAL2XX6</th>
<th>RAL1XX5</th>
<th>RAL4XX2</th>
</tr>
</thead>
<tbody>
<tr>
<td>droplets [nm]</td>
<td>465±6.90%</td>
<td>289±9.35%</td>
<td>219±5.14%</td>
</tr>
<tr>
<td>Lipase/droplet (x10³)</td>
<td>35.169±22.16%</td>
<td>8.444±30.75%</td>
<td>3.674±16.23%</td>
</tr>
<tr>
<td>nm²/lipase</td>
<td>19.315± 7.41%</td>
<td>31.078± 10.31%</td>
<td>41.012± 5.42%</td>
</tr>
<tr>
<td>Ester bond/lipase (x10³)</td>
<td>5.481</td>
<td>5.481</td>
<td>5.481</td>
</tr>
<tr>
<td>Surfactant/droplet (x10³)</td>
<td>225.547±22.16%</td>
<td>108.294±30.75%</td>
<td>94.248±16.23%</td>
</tr>
<tr>
<td>nm²/surfactant</td>
<td>3.012± 7.41%</td>
<td>2.423±10.31%</td>
<td>1.599± 5.42%</td>
</tr>
</tbody>
</table>

MW (a) ~ 43,000 g/mol.

As mentioned before, the lipase catalysis depends on the interfacial activation. So the bigger the surface area of the disperse phase is, the better the yield of the reaction products is. The surface area per lipase increases regularly, whereas the velocity of the tricaprylin hydrolysis tends to reach an asymptotical value (see Figure 58).

![Figure 58: Relation between the overall rate constants of tricaprylin hydrolysis by RAL and the normalized surface of droplets (per gram of miniemulsion) with the same tricaprylin content (20.17%).](image)

From the data, a specific activity of the lipases as function of the miniemulsion droplet size can be calculated. The activity of RAL depicts a maximum for the intermediate droplet size (10.90 U/mg, RAL1XX5, 289 nm). The activity variation is comparable with the variation observed for the kinetics rate constants (see Table 18).
Table 18: Specific activity of the lipase RAL towards trioctanoin (8:0) for different droplet sizes.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Code</th>
<th>Concentration</th>
<th>Specific activity [U/mg] Trioctanoin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhizopus Arrhizus lipase</td>
<td>RAL</td>
<td>8.90</td>
<td>7.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rhizopus Arrhizus lipase</td>
<td>RAL</td>
<td>8.90</td>
<td>10.90&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rhizopus Arrhizus lipase</td>
<td>RAL</td>
<td>8.90</td>
<td>10.50&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> RAL2XX6 (465 nm), <sup>b</sup> RAL1XX5 (289 nm), <sup>c</sup> RAL4XX2 (219 nm).

Correlating the above-mentioned observation, it is noticed, that even though the interfacial area per lipase increases with a factor of 2 (between 465 nm and 219 nm, see Table 17), the velocity of the tricaprylin hydrolysis does not increase with a comparable factor, but reaches an asymptotical value of about 0.72 h<sup>-1</sup> for the smallest miniemulsion droplet size (see Figure 58). This means that a shielding effect affects the reactivity due to the presence of surfactant and decreases the velocity of small droplets where percentage wise a higher coverage of the droplet surface by surfactant is observed. This shielding effect is also noticed in the observation of the activity (see Table 18).

4.2.2-v. Glycerolysis versus lipolysis

The aim of the glycerolysis is to shift the yield towards the formation of monoglycerides by adding glycerol. A molar ratio of tricaprylin to glycerol 1:2 is used for the glycerolysis. The differences between a standard lipolysis (RAL1XX5, where no glycerol is added, droplet size: 289 nm) and the glycerolysis in miniemulsion (RAL3XX2, droplet size: 314 nm) are also compared by performing the reaction of the tricaprylin glycerolysis with lipase RAL at 37 °C. The results are depicted Figure 59. Since glycerol is added at the beginning of the glycerolysis, the time course of glycerol is determined by subtracting the initial amount of glycerol from the measured amount of glycerol.
Figure 59: Comparison of the time courses of the lipolysis of tricaprylin by RAL at 37 °C in miniemulsion (RAL1XX5 ■, RAL3XX2 + glycerol ●): (A) tricaprylin, (B) caprylic acid, (C) 1-monocaprylin, (D) 2-monocaprylin, (E) 1,2-dicaprylin, (F) 1,3-dicaprylin, (G) glycerol.
A comparable profile is observed Figure 59 for tricaprylin. The time courses first decrease exponentially and then reach an asymptotical value comprised between 15 and 20 mol%. It can be noticed that the hydrolysis is faster in the case of the lipolysis than in the case of the glycerolysis, which may be due to the relative tricaprylin concentration towards the constant lipase amount. The presence of the glycerol in the case of the glycerolysis leads to a lipase catalyzed esterification, reducing the available lipase amount for the tricaprylin hydrolysis (A). For the caprylic acid, the same profile can be observed with the lipolysis faster than the glycerolysis (B). Linear profiles for the time courses of glycerol are observed (G). As for the time courses of the other products, remarkable differences are detected. For 1-monoglyceride, the yield is two times higher for the glycerolysis reaching 8 mol% after 2.5 h than for the lipolysis reaching only 4 mol% after 3 h (C). For the 2-monoglyceride, where both profiles show a transient accumulation, the lipolysis reaches a maximum of 35 mol% after 3 h before decreasing till about 22 mol% after 24 h and the glycerolysis reaches 24 mol% after 3 h before a slow decrease leading to 12 mol% after 24 h (D). Considering the 1,2-dicaprylin, the time courses are not significantly different. The time course obtained from the lipolysis depicts a fast increase with a maximum at 46 mol% after 5 h and the glycerolysis time course a maximum at 42 mol% also after 5 h (E). For the 1,3-diglyceride time courses, the glycerolysis time course depicts a transient accumulation reaching 22 mol% after 3 h stabilizing after 24 h at about 10 mol%, whereas the lipolysis time course remained at about 3 mol% after 1.5 h (F). In Table 19, the representative maxima of the different above-described time courses are summarized.

The glycerolysis leads to the formation of 1-monoglyceride and 1,3-diglyceride, and also to 2-MG (23.7 mol%). RAL is known to be sn-1,3 selective (see Table 3 and 4) and since the educt mix is also composed of glycerol, the (trans)esterification of the glycerol at the position sn-1 and sn-3 is observed, leading to the formation of 1-monocaprylin and 1,3-dicaprylin. On the other hand, the lipolysis of tricaprylin takes also place in the glycerolysis reactive field, leading to the expected 2-monoglyceride and 1,2-diglyceride. It is noticed that the yields in 1,2-DG and 2-MG, (while they are comparable), are with 5 mol% to 10 mol% lower in the case of the glycerolysis than in the case of the lipolysis (see Table 19). This fact may be due to a better selectivity in the case of the lipolysis. This result can be understood with the fact that lipases are regioselective only for the ester splitting, but less (or not) for the ester formation.

The hydrolysis of tricaprylin is slightly faster in the case of the lipolysis ($\Delta = \text{ca } 20 \text{ mol\%}$) than in the case of the glycerolysis (see Figure 59). A part of the lipase might be consumed for
the (trans)esterification of the glycerol and the other part for the hydrolysis of the tricaprylin. Considering the slopes of the other time course curves, it is detected that the formation of the different products is relatively faster for the lipolysis than for the glycerolysis (see Figure 59).

Table 19: Comparison of the representative maxima between the time course of the different products of tricaprylin lipolysis in miniemulsion and the one of tricaprylin glycerolysis in miniemulsion.

<table>
<thead>
<tr>
<th></th>
<th>without glycerol</th>
<th>with glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RAL1XX5 (mol%, time)</td>
<td>RAL3XX2 (mol%, time)</td>
</tr>
<tr>
<td>TG</td>
<td>16.9 @ 3 h</td>
<td>23.1 @ 3 h</td>
</tr>
<tr>
<td>FFA</td>
<td>42.2 @ 5 h</td>
<td>41.8 @ 5 h</td>
</tr>
<tr>
<td>1-MG</td>
<td>3.2 @ 3 h</td>
<td>8.2 @ 2.5 h</td>
</tr>
<tr>
<td>2-MG</td>
<td>34.5 @ 3 h</td>
<td>23.7 @ 3 h</td>
</tr>
<tr>
<td>1,2-DG</td>
<td>46.3 @ 6 h</td>
<td>42.2 @ 5 h</td>
</tr>
<tr>
<td>1,3-DG</td>
<td>3.3 @ 1.5 h</td>
<td>21.6 @ 3 h</td>
</tr>
<tr>
<td>G</td>
<td>21.2 @ 24 h</td>
<td>23.3 @ 24 h</td>
</tr>
</tbody>
</table>

The order of the exponential decay is determined for the tricaprylin yields of the above-described different kinetics. The overall rate constants for the tricaprylin hydrolysis time courses are determined and depicted in Table 20.

Table 20: The overall rate constants for the tricaprylin hydrolysis time courses with RAL in the cases of a homogeneous and a less homogeneous miniemulsion.

<table>
<thead>
<tr>
<th></th>
<th>without glycerol</th>
<th>with glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RAL1XX5</td>
<td>RAL3XX2</td>
</tr>
<tr>
<td>$k \text{ [h}^{-1}\text{]}$</td>
<td>0.6873</td>
<td>0.5037</td>
</tr>
<tr>
<td>first-order</td>
<td>$r$</td>
<td>0.9889</td>
</tr>
<tr>
<td></td>
<td>$r^2$</td>
<td>0.9781</td>
</tr>
<tr>
<td>second-order</td>
<td>$r$</td>
<td>0.9816</td>
</tr>
<tr>
<td></td>
<td>$r^2$</td>
<td>0.9635</td>
</tr>
</tbody>
</table>

As can be seen in Table 20 and as already observed before, the reaction with RAL followed a first-order kinetics during the first 2.5 h. However, for the reaction of RAL3XX2, i.e., the glycerolysis, there is a slight decrease in the correlation coefficient and in the coefficient of
determination. The coefficients of determination of the second-order kinetics modeling are smaller than those of the first-order one. Therefore first-order reaction kinetics modeling describes better the profile of the tricaprylin hydrolysis also for the glycerolysis. It can be noticed that the addition of glycerol slows down the tricaprylin hydrolysis.

It is then possible to characterize the size of the miniemulsion droplets by DLS measurements and to establish a relation with the used lipases. The number of lipase units per droplet, the relative surface area per lipase, and the number of ester bonds per lipase are determined and depicted in Figure 60.

![Figure 60: Characteristics of the miniemulsion droplets and its relation to the used lipase.](image)

(A) droplet size [nm], (B) lipase/droplet \(\times 10^3\), (C) nm\(^2\)/droplet, (D) lipase/ester bond \(\times 10^3\).

A synopsis of these characterizations is depicted in Table 21. Due to the slightly larger size in the case of RAL3XX2, the number of lipase per droplet increases and the area per lipase slightly decreases.
Table 21: Characteristics of the miniemulsion droplets and its relation to the used lipase.

<table>
<thead>
<tr>
<th></th>
<th>without glycerol</th>
<th>with glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RAL1XX5</td>
<td>RAL3XX2</td>
</tr>
<tr>
<td>(d) droplets [nm]</td>
<td>289±9.35%</td>
<td>314±13.44%</td>
</tr>
<tr>
<td>Lipase/droplet ((\times 10^3))</td>
<td>8.444±30.75%</td>
<td>10.828±45.98%</td>
</tr>
<tr>
<td>nm²/lipase</td>
<td>31.078±10.31%</td>
<td>28.604±10.31%</td>
</tr>
<tr>
<td>Ester bond/lipase ((\times 10^3))</td>
<td>5.481</td>
<td>5.481</td>
</tr>
</tbody>
</table>

\[^a\] MW \(\sim 43,000\) g/mol.

The RAL activity in the case of the lipolysis and the glycerolysis are compared in Table 22. It can be noticed that the activity of RAL is significantly higher in the case of the lipolysis than in the case of the glycerolysis as it is observed for the velocity of tricaprylin hydrolysis.

Table 22: Specific activity of the lipases towards trioctanoin (8:0) under described conditions.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Code</th>
<th>Concentration [mg/mL]</th>
<th>Specific activity [U/mg] Trioctanoin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhizopus Arrhizus lipase</td>
<td>RAL</td>
<td>8.90</td>
<td>10.90[^a]</td>
</tr>
<tr>
<td>Rhizopus Arrhizus lipase</td>
<td>RAL</td>
<td>8.90</td>
<td>7.30[^b]</td>
</tr>
</tbody>
</table>

\[^a\] RAL1XX5, \[^b\] RAL3XX2.

As outlined previously, the glycerolysis of fats and oils employing alkaline catalyst represents the process currently used in industry for the large-scale synthesis of monoacylglycerols (MAGs). The enzymatic glycerolysis offers the advantage of a high space-time yield because 3 mol monoacylglycerols per mol triacylglycerol could be formed, whereas in the hydrolysis of triacylglycerols with sn-1,3 specific lipases 2 mol fatty acid are synthesized per mole monoacylglycerol. Initiated by the work of Yamane et al.\[^{110}\] lipase catalyzed glycerolysis received considerable attention and several papers dealt with the glycerolysis reaction of model fats and a variety of natural fats and oils. The reactions were performed in reverse micelles,\[^{90, 111, 112}\] in the presence\[^{113, 114}\] or absence\[^{113}\] of organic solvents and in a solvent-free-solid-state system.\[^{89, 94, 115, 116}\] Problems employing the hydrophilic substrate glycerol in organic solvents and analytical problems for the quantitative determination of partial glycerides were described by Ferreira-Dias and Fonseca.\[^{117}\] Yamane et al.\[^{96}\] studied the glycerolysis reaction of corn oil by Pseudomonas fluorescens lipase in a batch system without the addition of surfactants or emulsifiers. At 40 °C and 3.7% water content in the glycerol
phase, 20.4% MG content is determined. In this thesis, the glycerolysis reaction of tricaprylin by using Rhizopus Arrhizus lipase in a miniemulsion is studied. At 37 °C, a yield of more than 30 mol% MG is determined after 3 h. Here also, whereas RAL is known as a sn-1,3 selective lipase, more than 20 mol% 1,3-DG content is determined after 3 h and even more than 40 mol% 1,2-DG content after 5 h.

It is interesting to compare the results described in the literature by Tan and Yin.\cite{70} They carried out the glycerolysis of 1,3-palmitin-2-olein in emulsion by RAL and published diagrams presenting a comparable profile for the hydrolysis of 1,3-palmitin-2-olein (see Paragraph 2.4.4-ii.d). The time course does not decrease as fast as in miniemulsion, it reaches 10% after 10 h (versus 23.1 mol% after 3 h for RAL against tricaprylin in miniemulsion) and tends to reach an asymptotical value comprised between 5 and 10% (versus about 15 mol% in miniemulsion). A different and remarkable behavior is observed for the free fatty acid released during the lipolysis, the same profile is observed, however it is noticed that it only reaches about 2.5% after 4 h (versus 41.8 mol% after 3 h in miniemulsion) and remains almost constant. The loss of selectivity of RAL is more significant in emulsion than in miniemulsion. Indeed 1-monoglyceride reaches about 25% after 10 h (versus 8.2 mol% after 2.5 h in miniemulsion), whereas 2-monoglyceride yield remains under 5% (versus 23.7 mol% after 3 h in miniemulsion). Analyzing the 1,2-diglyceride, its time course shows a transient accumulation reaching about 25% after 5 h (versus 46.3 mol% after 6 h) and falls down to about 5% after 24 h (versus 40 mol% after 24 h). A drastic difference is observed for the time course of 1,3-diglyceride, its time course reaches more than 40% after 24 h (versus 22 mol% after 3 h). Here also whereas the substrate is significantly different than tricaprylin (C16/C18 versus C8 for the tricaprylin), RAL seems to have a better activity in miniemulsion, only the loss of sn-1,3 selectivity is more significant in emulsion.

4.2.2-vi. Hydrolysis in miniemulsion

The aim of this paragraph is to compare the lipase-catalyzed lipolysis with the hydrolysis of tricaprylin in miniemulsion by different bases under different conditions (base/tricaprylin ratio, temperature) and to determine whether a regioselective hydrolysis is possible. As bases sodium hydroxide, ammonia, potassium carbonate, triethylamine, hydroxylamine and caustic soda were chosen. The reaction conditions and the results are summarized in Table 23 and 24. Sodium hydroxide leads to full hydrolysis. The weaker base ammonia leads to quantitative reactions with tricaprylin in miniemulsion when it is used in large excess (1:30) at room temperature. In a ratio of 1:18 with respect to the ammonia mole number and at 80 °C, a
reaction takes place resulting in the formation of 4.2 mol% of 1,3-diglyceride, 0.7 mol% of 1,2-diglyceride and 0.9 mol% of 1-monoglyceride. The other conditions do not lead to any reaction (see Table 23).

Potassium carbonate, which is a weaker base than ammonia, also leads to a quantitative reaction with tricaprylin in miniemulsion when it is used in large excess (1:30) at room temperature.

In a ratio of 1 to 18 with respect to the base mole number and at room temperature, a reaction is observed with 1.7 mol% of 1,3-diglyceride, 0.4 mol% of 1-monoglyceride, and 0.1 mol% of 2-monoglyceride. In a ratio of 1 to 18 with respect to the base mole number but at higher temperature (80 °C), a reaction is observed with 6.9 mol% of 1,3-diglyceride, 2.4 mol% of 1,2-diglyceride, 1.0 mol% of 1-monoglyceride and 0.1 mol% of 2-monoglyceride.

In a ratio of 1 to 6 with respect to the base mole number and at room temperature, a reaction is observed with 2.2 mol% of 1,3-diglyceride, 0.7 mol% of 1,2-diglyceride, 1.3 mol% of 1-monoglyceride and 0.3 mol% of 2-monoglyceride. In a ratio of 1:6 in respect to the base mole number but at 80 °C, a reaction is observed with 1.8 mol% of 1,3-diglyceride, and 0.5 mol% of 1-monoglyceride.

The triethylamine does not lead to any reaction in the presence of tricaprylin.

The hydroxylamine leads to a reaction in miniemulsion at room temperature with tricaprylin in a ratio of 1:30 with respect to the base and. 3.6 mol% of 1,3-diglyceride, 0.9 mol% of 1,2-diglyceride, 0.5 mol% of 1-monoglyceride and 0.1 mol% of 2-monoglyceride are formed during the reaction.
Table 23: Different bases used for the hydrolysis in miniemulsion under mentioned conditions and results.

<table>
<thead>
<tr>
<th>Base</th>
<th>Ratio tricaprylin: base (mol)</th>
<th>Temperature</th>
<th>Result*</th>
</tr>
</thead>
<tbody>
<tr>
<td>sodium hydroxide</td>
<td>1:6</td>
<td>room temp.</td>
<td>full reaction</td>
</tr>
<tr>
<td>ammonia, 25% solution</td>
<td>1:6</td>
<td>room temp.</td>
<td>no reaction.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80 °C</td>
<td>no reaction</td>
</tr>
<tr>
<td></td>
<td>1:18</td>
<td>room temp.</td>
<td>no reaction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80 °C</td>
<td>reaction</td>
</tr>
<tr>
<td></td>
<td>1:30</td>
<td>room temp.</td>
<td>full reaction</td>
</tr>
<tr>
<td>potassium carbonate</td>
<td>1:6</td>
<td>room temp.</td>
<td>reaction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80 °C</td>
<td>reaction</td>
</tr>
<tr>
<td></td>
<td>1:18</td>
<td>room temp.</td>
<td>reaction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80 °C</td>
<td>reaction</td>
</tr>
<tr>
<td></td>
<td>1:30</td>
<td>room temp.</td>
<td>full reaction</td>
</tr>
<tr>
<td>triethylamine</td>
<td>1:6</td>
<td>room temp.</td>
<td>no reaction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80 °C</td>
<td>no reaction</td>
</tr>
<tr>
<td>hydroxylamine</td>
<td>1:30</td>
<td>room temp.</td>
<td>reaction</td>
</tr>
</tbody>
</table>

*“reaction” stands for formation of acylglycerols and “full reaction” for quantitative hydrolysis of tricaprylin.

Table 24: Relative concentration (mol%) of acylglycerols at equilibrium of the tricaprylin hydrolysis in miniemulsion with different bases and under different conditions.

<table>
<thead>
<tr>
<th>Base</th>
<th>1-MG</th>
<th>2-MG</th>
<th>1,2-DG</th>
<th>1,3-DG</th>
<th>triglyceride</th>
<th>glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH 1:6 (RT)</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>NH₃ 1:18 (80 °C)</td>
<td>0.9</td>
<td>0.0</td>
<td>0.7</td>
<td>4.2</td>
<td>93.4</td>
<td>0.8</td>
</tr>
<tr>
<td>NH₃ 1:30 (RT)</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>K₂CO₃ 1:6 (RT)</td>
<td>1.3</td>
<td>0.3</td>
<td>0.7</td>
<td>2.2</td>
<td>95.5</td>
<td>0.0</td>
</tr>
<tr>
<td>K₂CO₃ 1:18 (RT)</td>
<td>0.4</td>
<td>0.1</td>
<td>0.0</td>
<td>1.7</td>
<td>97.8</td>
<td>0.0</td>
</tr>
<tr>
<td>K₂CO₃ 1:30 (RT)</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>K₂CO₃ 1:6 (80 °C)</td>
<td>0.5</td>
<td>0.0</td>
<td>0.0</td>
<td>1.8</td>
<td>97.6</td>
<td>0.1</td>
</tr>
<tr>
<td>K₂CO₃ 1:18 (80 °C)</td>
<td>1.0</td>
<td>0.1</td>
<td>2.4</td>
<td>6.9</td>
<td>89.6</td>
<td>0.0</td>
</tr>
<tr>
<td>NH₂OH 1:30 (RT)</td>
<td>0.5</td>
<td>0.1</td>
<td>0.9</td>
<td>3.6</td>
<td>94.3</td>
<td>0.6</td>
</tr>
</tbody>
</table>
The hydrolysis of tricaprylin by the above-mentioned different weak bases leads, compared to sodium hydroxide as strong base, to either low yields or to quantitative reaction.

An excess of base with respect to tricaprylin in a ratio 1:30 is needed to observe a full hydrolysis in the case of ammonia and potassium carbonate and a ratio 1:18 leads only to poor yields of different hydrolysis products.

However it can be noticed that when a reaction takes place, 1,3-diglyceride, the most thermodynamically stable isomer is favorably formed compared to 1,2-diglyceride, which is less thermodynamically stable. The same conclusion can be done as for the monoglycerides, the most thermodynamically stable isomer; the 1-monoglyceride is preferentially formed. However the relative contents of those products remain very low.

So it could be spoken about a thermodynamic selectivity, the activity depends on the temperature, a threshold energy barrier needs to be overstepped, to lead to the quantitative reaction.
5. SUMMARY AND CONCLUSIONS

In summary, it could be shown that the lipolysis and glycerolysis in miniemulsion catalyzed by lipases lead to a significant increase of selectivity and presents an important route to obtain mono- and diglycerides. The miniemulsion offers an interfacial area, which allows increasing the yields and the kinetics of reactions. The reaction mechanism of most lipases is ruled by the interfacial activation between the oil phase and the aqueous phase. In this dissertation, a screening of lipases has been done and a sn-1,3 specific lipase was chosen not only to study the lipolysis and the glycerolysis in miniemulsion but also the influence of the homogeneity of the miniemulsion and the influence of the miniemulsion interfacial area through the variation of the miniemulsion droplet size.

Mono- and diglycerides have wide applications in food, cosmetics and pharmaceuticals. Glycerolysis is carried out at industrial level to form monoglycerides from glycerol and triglycerides. Unfortunately, the high temperatures involved in the reaction often result in a dark-colored product. In addition, the presence of free fatty acid and their metallic soaps can lead to associated flavor and odor problems as for examples in the food products into which they are incorporated. The lipolysis route has been abandoned because of its poor yields. The glycerolysis catalyzed by a lipase has got attention due to its mild reaction conditions and regiospecific products such as 2-monoglyceride by sn-1,3 specific lipase.

First of all, the lipolyses of tricaprylin catalyzed by the Rhizopus Arrhizus lipase, by the Pseudomonas Cepacia lipase, and by the Rhizomucor Miehei lipase were carried out and compared using the free fatty acid titration and $^1$H-NMR. Their respective products time courses show an exponential decay of tricaprylin hydrolysis in the case of RAL and a hyperbolic decay of tricaprylin hydrolysis in the cases of RML and lipase PS. The kinetics revealed also a significant transient accumulation of 1,2-dicaprylin and 2-monocaprylin as well as no production of 1,3-dicaprylin or 1-monocaprylin except for the lipase PS, for the RAL catalyzed glycerolysis and for the RAL catalyzed lipolysis in an unhomogeneous miniemulsion. The results showed the sn-1,3 selectivity of RAL and RML with about 10% of 1-MG vs. 90% of 2-MG and with also about 10% of 1,3-DG vs. 90% of 1,2-DG in homogeneous miniemulsion. For the lipase PS, where the selectivity does not depict the same specificity, it can not be spoken about the absence of selectivity as observed in the literature.

In fact the observations for the tricaprylin lipolysis in miniemulsion by lipase PS showing about 30% of 1-MG vs. 70% of 2-MG and about 20% of 1,3-DG vs. 80% of 1,2-DG prove
that lipase PS seems to show what could be described as a lower sn-1,3 selectivity in miniemulsion.

Not only the regioselectivity, but also the activities of those lipases present a different classification in miniemulsion than in emulsion. Lipase PS shows indeed the best activity:

\[ a(PS) > a(RAL) \sim a(RML) \]

The following experiments were performed with the Rhizopus Arrhizus lipase.

Secondly, the homogeneity of the miniemulsion was observed to be a parameter influencing not only the activity but also the regioselectivity. The Rhizopus Arrhizus lipase which is known to show sn-1,3 selectivity, it was used in homogeneous and a less homogeneous tricaprylin miniemulsion. In fact, the lipolysis is slightly faster in the homogeneous miniemulsion, but the selectivity is drastically influenced by the loss of homogeneity. The rearrangements of the lipase at the interface oil/water mainly take place during, among others, the coalescence process, which results in the loss of selectivity.

Thirdly, the miniemulsion specific surface area was observed to play a determining role in the interfacial activation. The droplet size was varied between about 220 and 460 nm. This variation shows a relation between the lipase catalytic activity and the miniemulsion specific surface area. This relation is first linear for relative small specific surface area and then a saturation of the catalytic activity is observed for the higher specific surface area. This can be explained as a consequence of a shielding effect against the lipase induced by a droplet size related increasing surfactant density at the surface of the droplet. The lipase might then have difficulties to approach the surface anymore.

Fourthly, the glycerolysis reaction of tricaprylin by Rhizopus Arrhizus lipase in miniemulsion was studied. At 37 °C, over 30 mol% MG content 3 h and almost 60 mol% DG content after 3 h were determined. Whereas RAL is known as a sn-1,3 selective lipase, more than 20 mol% 1,3-DG content was determined vs. more than 40 mol% 1,2-DG content after 3 h.

Finally, the hydrolysis of tricaprylin by weak bases in miniemulsion was performed to study their selectivity and activity. Like the caustic soda, some weak bases show a strong uncontrolled activity until they are used at high concentrations and under defined temperature
conditions. Otherwise, the activity of the weak bases remains low. A thermodynamic selectivity is observed leading to the preferential formation of the thermodynamic stable isomers, 1,3-diglyceride and 1-monoglyceride.
6. ZUSAMMENFASSUNG


Zunächst wurden die Lipolysen von Tricaprylin katalysiert durch Rhizopus Arrhizus Lipase, Pseudomonas Cepacia Lipase und Rhizomucor Miehei Lipase durchgeführt und über Titration der freien Fettsäuren und \(^1\)H NMR Messungen verglichen. Die jeweiligen Produkt-Zeit-Verläufe für die Hydrolyse von Tricaprylin zeigen einen exponentiellen Zerfall bei der Verwendung von RAL und einen hyperbolischen Zerfall bei RML und der Lipase PS. Die Lipolyse-Kinetiken für RML und RAL legen offen, dass es zu einer vorübergehenden signifikanten Akkumulation von 1,2-Dicaprylin und 2-Monocaprylin kommt, während es keine Bildung von 1,3-Dicaprylin oder 1-Monocaprylin gibt. Die Lipase PS zeigt keine
Selektivität bei der Bildung der Mono- und Diglyceriden. Die RAL verliert ihre Selektivität in inhomogenen Miniemulsionen.

Die Ergebnisse zeigen eine sn-1,3-Selektivität der RAL und RML von etwa 10% für 1-MG gegenüber 90% für 2-MG und mit 10% für 1,3-DG gegenüber 90% für 1,2-DG in homogener Miniemulsion. Im Widerspruch zu Literaturangaben kann bei der Lipase PS nicht von einer Abwesenheit der Selektivität gesprochen werden, es wird lediglich eine geringeren Ausprägung der Selektivität erreicht in Vergleich zu RML und RAL erreicht. Vielmehr bestätigen die Beobachtungen für die Lipolyse von Tricaprylin in Miniemulsion mit Lipase PS eine Selektivität von 30% für 1-MG gegenüber 70% für 2-MG und von etwa 20% für 1,3-DG gegenüber 80% 1,2-DG, dass die Lipase PS ein Verhalten zeigt, das man als geringere sn-1,3-Selektivität in Miniemulsion beschreiben kann.

In Miniemulsion verändern sich nicht nur die Regioselektivitäten, sondern es ändern sich auch die Enzymaktivitäten dieser Lipasen. Die Lipasen lassen sich nach ihren Aktivitäten wie folgt ordnen:

\[ a(PS) > a(RAL) \approx a(RML) \]

Weitere Untersuchungen wurden aufgrund der bisherigen Ergebnisse am Modell der RAL durchgeführt.

Es wurde festgestellt, dass die Homogenität der Miniemulsion von entscheidendem Einfluss bezüglich der Regioselektivität und auch der Aktivität des Enzyms ist. Daher wurde der Einfluss der Homogenität bezüglich der sn-1,3-Selektivität in einer Tricaprylin-Miniemulsion genauer betrachtet. Tatsächlich kommt es zu einer schnelleren Reaktion in homogener Miniemulsion, dahingegen zeigt sich ein dramatischer Einfluss auf die Selektivität, wenn in einer inhomogenen Miniemulsion gearbeitet wird.

Die Glycerolyse von Tricaprylin durch Rhizopus Arrhizus Lipase in Miniemulsion zeigt einen MG-Gehalt von über 30 mol% und über 60 mol% des Diglycerides bei einer Temperatur von 37 °C nach 3 h. Obwohl RAL als sn-1,3-Lipase bekannt ist, ergibt sich ein Anteil von über 20% 1,3-DG und über 40% 1,2-DG nach 3 h.

Abschließend wurde die Hydrolyse von Tricaprylin durch schwache Basen in Miniemulsion durchgeführt; auch hier wurden die Selektivität und die Aktivität betrachtet. Wie die Natronlauge zeigen manche schwache Basen eine hohe unkontrollierte Aktivität, wenn sie in hohen Konzentrationen und bei hohen Temperaturen verwendet werden. Andernfalls bleiben die Aktivitäten gering. Hier zeigt sich keine kinetische Selektivität, sondern eine thermodynamisch motivierte Selektivität aufgrund der höheren thermodynamischen Stabilität des 1,3-Diglycerids und 1-Monoglycerids.
7. EXPERIMENTAL PART

7.1. Hydrolysis in miniemulsion

Preparation of the miniemulsion and syntheses

3.0 g of tricaprylin, 125 mg of hexadecane, and 11.875 g of a 1.0 wt.-% Lutensol AT50 solution in water (surfactant solution) are vigorously stirred for 1 h at room temperature. The miniemulsion is prepared by ultrasonicallyating the mixture with a Branson sonifier W450 digital (1/2” tip) at 90% amplitude continuously during 120 s under ice cooling.

Different bases (see Table 25) in various quantities are given to the miniemulsion and stirred at different temperatures (see Table 25) up to 24 h to reach the equilibrium.

Table 25: Different bases used for the hydrolysis in miniemulsion under mentioned conditions.

<table>
<thead>
<tr>
<th>bases</th>
<th>tricaprylin : base (mol)</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>ammonia, 25% solution</td>
<td>1:6</td>
<td>room temp.</td>
</tr>
<tr>
<td></td>
<td>1:18</td>
<td>80 °C</td>
</tr>
<tr>
<td></td>
<td>1:30</td>
<td>room temp.</td>
</tr>
<tr>
<td>potassium carbonate</td>
<td>1:6</td>
<td>room temp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80 °C</td>
</tr>
<tr>
<td></td>
<td>1:18</td>
<td>room temp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80 °C</td>
</tr>
<tr>
<td></td>
<td>1:30</td>
<td>80 °C</td>
</tr>
<tr>
<td>triethylamine</td>
<td>1:6</td>
<td>room temp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80 °C</td>
</tr>
<tr>
<td>hydroxylamine</td>
<td>1:30</td>
<td>room temp.</td>
</tr>
<tr>
<td>caustic soda</td>
<td>1:6</td>
<td>room temp.</td>
</tr>
</tbody>
</table>

7.2. Enzymatic lipolysis in miniemulsion

Preparation of the miniemulsion and synthesis

3.0 g of tricaprylin, 125 mg of hexadecane, and 11.875 g of a 1.0 wt.-% Lutensol AT50 solution in water (surfactant solution) are vigorously stirred for 1 h at room temperature. The miniemulsion is prepared by ultrasonicallyating the mixture with a Branson sonifier W450 digital (1/2” tip) at 90% amplitude continuously during 120 s under ice cooling.
A suspension of 150 mg Lipase Amano “PS” diluted in 5 mL of a 1.0 wt.-% Lutensol AT50 solution in water (surfactant solution) is given to the miniemulsion and stirred at 45 °C for 24 h to reach equilibrium. A solution of 150 mg SDS (inhibitant of lipase) in 5.0 g of distilled water is added to the miniemulsion in order to stop the reaction immediately and ensure determination of the conversion to distinct reaction times. The present water in the miniemulsion is then evaporated in oven at 45 °C under reduced pressure.

7.3. Enzymatic lipolysis in miniemulsion – Kinetics

Two methods for the preparation of the miniemulsion are considered.

7.3.1. Preparation of the miniemulsion – 1st Method

6.0 g of tricaprylin, 250 mg of hexadecane and 23.75 g of a 1.0 wt.-% Lutensol AT50 solution in water (surfactant solution) are vigorously stirred for 1 h at room temperature. The miniemulsion is prepared by ultrasonicating the mixture with a Branson sonifier W450 digital (1/2” tip) at 90% amplitude continuously during 120 s under ice cooling.

Two miniemulsions are then combined in order to obtain 60.0 g of miniemulsion. A suspension of 0.6 g lipase RAL in 20.0 g of surfactant solution and a solution of 0.6 g SDS (as inhibitor of lipase) in 20.0 g of distilled water are prepared. 11 vials are filled up with 5.0 g of the prepared miniemulsion, and then 1.65 mL of the prepared Lipase solution. The mixtures are stirred at 37 °C and then 1.667 mL of the SDS solution are added to the vials after 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, 12.0, and 24 h in order to measure the conversion by inhibition of the reaction. The vials are then dipped into liquid nitrogen to avoid the isomerization of the products and stored at -20 °C.

7.3.2. Preparation of the miniemulsion – 2nd Method

6.0 g of tricaprylin, 250 mg of hexadecane are mixed and added to 23.75 g of a 1.0 wt.-% Lutensol AT50 solution in water (surfactant solution). After vigorously stirring for 1 h at room temperature, the miniemulsion is prepared by ultrasonicating the mixture with a Branson sonifier W450 digital (1/2” tip) at 90% amplitude continuously during 120 s under ice cooling. Two separate prepared miniemulsions are combined and ultrasonicated once again for 60 s at 90% amplitude.

A suspension of 0.6 g lipase (see Table 2) in 20.0 g surfactant solution and solution of 0.6 g SDS (inhibitant of lipase) in 20.0 g of distilled water is prepared.
11 vials are filled up with 5.0 g of the prepared miniemulsion and then filled up with 1.65 mL of the prepared lipase solution.
The mixtures are stirred at the reaction temperature (see Table 26) and then 1.667 mL of the SDS solution are added to the vials after 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, 12.0, and 24 h to be able to measure conversion at these time after inhibition of the reaction.
The vials are then frozen in liquid nitrogen to avoid the isomerization of the products and stored at -20 °C until titration and preparation of the sample for NMR.

Table 26. Optimal activity temperatures of used lipases.

<table>
<thead>
<tr>
<th>Lipase</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL</td>
<td>35 °C</td>
</tr>
<tr>
<td>RAL</td>
<td>37 °C</td>
</tr>
<tr>
<td>RML</td>
<td>40 °C</td>
</tr>
</tbody>
</table>

7.4. Enzymatic glycerolysis in miniemulsion – Kinetics

Preparation of the miniemulsion
6.0 g of tricaprylin, 250 mg of hexadecane, and 2.377 g of glycerol are mixed and added to 23.75 g of a 1.0 wt.-% Lutensol AT50 solution in water (surfactant solution). After vigorously stirring for 1 h at room temperature, the miniemulsion is prepared by ultrasonicating the mixture with a Branson sonifier W450 digital (1/2” tip) at 90% amplitude continuously during 120 s under ice cooling.

Two separate prepared miniemulsions are combined and ultrasonicated once again for 60 s at 90% amplitude. A suspension of 0.6 g lipase RAL in 20.0 g of surfactant solution and a solution of 0.6 g SDS (inhibitant of lipase) in 20.0 g of distilled water is prepared. 11 vials are filled up with 5.3962 g of the prepared miniemulsion and then filled up with 1.65 mL of the prepared lipase solution. The vials are stirred at the reaction temperature (see Table 2) and then 1.667 mL of the SDS solution are added to the vials after 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, 12.0, and 24 h to be able to measure conversion at these time after inhibition of the reaction. The vials are then frozen in liquid nitrogen to avoid the isomerization of the products and stored at -20 °C until titration and preparation of the sample for NMR.
7.5. Methods

*Dynamic light scattering (DLS)*

The particle size analyses are conducted with a Malvern Zetasizer Nano-ZS ZEN 360 at a fixed angle of 173° equipped with a laser having a 633 nm wavelength. The data are processed using the Dispersion Technology Software 4.0. The sample are diluted in distilled water at a concentration of 1%.

*Nuclear magnetic resonance (NMR)*

Proton nuclear magnetic resonance (\(^1\)H-NMR) spectra are recorded on a Bruker Avance 400 spectrometer at 400 MHz in deuterated chloroform and dimethyl sulfoxide solvent with 64 scans. The data are processed using the Mestre-C Lite Version software. The reference for the calibration is relative to the singlet of TMS at 0.0 ppm.

*Preparative thin layer chromatography*

100 mg of the product is taken for the analysis with thin layer chromatography (TLC) method (with PLC plate 20 x 20 cm Silica gel F\(_{254}\) 2 mm Merck, developing chamber). The used eluent is composed of heptane/diethyl ether/acetic acid (55/45/1 - v/v/v).

100 mg of the product is analysed with thin layer chromatography (TLC) method (with PLC plate 20 x 20 cm Silica gel F\(_{254}\) 2 mm Merck, developing chamber). The used eluent is composed of heptane/diethyl ether/acetic acid (55/45/1 - v/v/v).

The reversible revelation is carried out through exposition of the TLC to solid iodine vapours. The different spots are scratched out and washed out successively with chloroform, ethylether and then with a little methanol addition for MG because of their stronger polarity.

*Acid-base titration*

The titrations of the free fatty acids released during the reaction are carried out with a titration device, a Schott Titronic Universal, using a 0.1 N sodium hydroxide solution, Merck c(NaOH) = 0.1 mol/L and a Inolab pH/Cond Level 1 WTW pH-meter. The data are processed with the Programme DosA, version 3.3.1, 2004, Nathalie Bonnin, software. To determine the equivalence, two methods are used assimilating concentration to activities. Those are the method of the derivate and the method of parallels (see Figure 61).
Figure 61: Titration of free fatty acid released by the lipase PS-mediated lipolysis of tricaprylin in miniemulsion after 3 hours.

**Freeze drying**

Freeze drying was performed with a Christ benchtop manifold freeze dryer ALPHA 2-4 D, whose ice condenser temperature is -83 °C and the pressure, inside the drying chamber, is 0.060 mbar and is assured by a Vacuubrand Chemistry-HYBRID-pump RC6.
8. REFERENCES

[100] [http://www.cem.msu.edu/~reusch/VirtualText/Spectrpy/nmr/nmr1.htm](http://www.cem.msu.edu/~reusch/VirtualText/Spectrpy/nmr/nmr1.htm).
9. APPENDIX

9.1. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>a(−)</td>
<td>lipase activity</td>
</tr>
<tr>
<td>CDCl₃</td>
<td>deuterated chloroform</td>
</tr>
<tr>
<td>CW</td>
<td>continuous-wave</td>
</tr>
<tr>
<td>1,2-DG</td>
<td>1,2-diglyceride</td>
</tr>
<tr>
<td>1,3-DG</td>
<td>1,3-diglyceride</td>
</tr>
<tr>
<td>DMSO-d₆</td>
<td>deuterated dimethyl sulfoxide</td>
</tr>
<tr>
<td>ELSD</td>
<td>evaporative light scattering detector</td>
</tr>
<tr>
<td>EO</td>
<td>ethylene oxide</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>FID</td>
<td>free induction decay</td>
</tr>
<tr>
<td>FT</td>
<td>Fourier transformation</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>k</td>
<td>overall rate constant or velocity</td>
</tr>
<tr>
<td>k’</td>
<td>observed overall rate constant</td>
</tr>
<tr>
<td>n</td>
<td>molar number</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>1-MG</td>
<td>1-monoglyceride</td>
</tr>
<tr>
<td>2-MG</td>
<td>2-monoglyceride</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>PCS</td>
<td>photon correlation spectroscopy</td>
</tr>
<tr>
<td>POP</td>
<td>1,3-palmitin-2-olein</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>PS</td>
<td>lipase PS or Pseudomonas Cepacia lipase</td>
</tr>
<tr>
<td>PSp</td>
<td>Pseudomonas sp. Lipase</td>
</tr>
<tr>
<td>r²</td>
<td>coefficient of determination</td>
</tr>
<tr>
<td>RF</td>
<td>Radio Frequency</td>
</tr>
<tr>
<td>RAL</td>
<td>Rhizopus Arrhizus lipase</td>
</tr>
<tr>
<td>RML</td>
<td>Rhizomucor Miehei lipase</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TMS</td>
<td>trimethylsilane</td>
</tr>
</tbody>
</table>
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>weight</td>
</tr>
</tbody>
</table>

### 9.2. Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>area</td>
<td>m²</td>
</tr>
<tr>
<td>B</td>
<td>magnetic field</td>
<td>T</td>
</tr>
<tr>
<td>D</td>
<td>diffusion coefficient</td>
<td>m²•s⁻¹</td>
</tr>
<tr>
<td>F</td>
<td>Helmholtz free energy</td>
<td>J</td>
</tr>
<tr>
<td>G</td>
<td>Gibbs energy</td>
<td>J</td>
</tr>
<tr>
<td>gN</td>
<td>nuclear g-factor</td>
<td>-</td>
</tr>
<tr>
<td>≤</td>
<td>reduced Plank constant</td>
<td>J•s</td>
</tr>
<tr>
<td>J</td>
<td>coupling constant</td>
<td>Hz</td>
</tr>
<tr>
<td>²J</td>
<td>geminal coupling constant</td>
<td>Hz</td>
</tr>
<tr>
<td>³J</td>
<td>vicinal coupling constant</td>
<td>Hz</td>
</tr>
<tr>
<td>kₘ</td>
<td>Boltzmann constant</td>
<td>J•K⁻¹</td>
</tr>
<tr>
<td>M</td>
<td>magnetization</td>
<td>A•m⁻¹</td>
</tr>
<tr>
<td>m₁</td>
<td>spin magnetic moment</td>
<td>J•T⁻¹</td>
</tr>
<tr>
<td>mₚ</td>
<td>mass of a proton</td>
<td>eV•c⁻²</td>
</tr>
<tr>
<td>N</td>
<td>number of particle in a thermodynamic system</td>
<td>-</td>
</tr>
<tr>
<td>p</td>
<td>pressure</td>
<td>Pa</td>
</tr>
<tr>
<td>Rₜ</td>
<td>hydrodynamic averaged intensity radius</td>
<td>m</td>
</tr>
<tr>
<td>S</td>
<td>entropy</td>
<td>J•K⁻¹</td>
</tr>
<tr>
<td>T</td>
<td>temperature</td>
<td>K</td>
</tr>
<tr>
<td>T₁</td>
<td>spin-lattice (longitudinal) relaxation</td>
<td>s</td>
</tr>
<tr>
<td>T₂</td>
<td>spin-spin (transverse) relaxation</td>
<td>s</td>
</tr>
<tr>
<td>U</td>
<td>internal energy</td>
<td>J</td>
</tr>
<tr>
<td>V</td>
<td>volume</td>
<td>m³</td>
</tr>
<tr>
<td>W</td>
<td>mechanical work done on a thermodynamic system</td>
<td>J</td>
</tr>
<tr>
<td>δ</td>
<td>chemical shift</td>
<td>ppm</td>
</tr>
<tr>
<td>γ</td>
<td>magnetogyracic ratio of a nucleus</td>
<td>rad•T⁻¹•s⁻¹</td>
</tr>
<tr>
<td>γₐ</td>
<td>liquid-air surface tension</td>
<td>N•m⁻¹</td>
</tr>
<tr>
<td>μN</td>
<td>nuclear magneton</td>
<td>J•T⁻¹</td>
</tr>
<tr>
<td>η₀</td>
<td>viscosity of the medium</td>
<td>Pa•s</td>
</tr>
</tbody>
</table>
\[
\begin{array}{lll}
\text{Symbol} & \text{Description} & \text{Unity} \\
\nu_{L} & \text{tangential velocity related to Larmor frequency} & \text{m}\cdot\text{s}^{-1} \\
\sigma & \text{screening constant} & \text{-} \\
\Phi & \text{dihedral angle} & \text{deg/rad} \\
\end{array}
\]

9.3. Chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Source, Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 N sodium</td>
<td>Merck, (c(\text{NaOH}) = 0.1 \text{ mol/l})</td>
</tr>
<tr>
<td>hydroxide solution</td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>Aldrich, glacial, 99.8%</td>
</tr>
<tr>
<td>Ammonia</td>
<td>Riedel-de-Häen, solution of aqueous ammonia, 25 wt.%</td>
</tr>
<tr>
<td>Deuterated chloroform</td>
<td>Deutero, 99.9%</td>
</tr>
<tr>
<td>Deuterated Dimethyl</td>
<td>Deutero, 99.9%</td>
</tr>
<tr>
<td>Sulfoxide</td>
<td></td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>Merck, technical product</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Aldrich, 99%</td>
</tr>
<tr>
<td>Lutensol AT 50</td>
<td>hexadecylmodificated polyethylene oxide, BASF, technical product, (C_{16}H_{33}(EO)_{50})</td>
</tr>
<tr>
<td>Heptane</td>
<td>Merck, technical product</td>
</tr>
<tr>
<td>Hexadecan</td>
<td>Aldrich, 99%</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>Merck, solution 50% in water</td>
</tr>
<tr>
<td>Potassium carbonate</td>
<td>Merck, &gt; 99%</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate, Aldrich, 99%</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>Merck, &gt; 99%</td>
</tr>
<tr>
<td>Tricaprylin</td>
<td>glycerol trioctanoate Sigma, &gt; 99%</td>
</tr>
<tr>
<td>Triethylamine</td>
<td>Merck, &gt; 99%</td>
</tr>
<tr>
<td>Trimethylsilane</td>
<td>Fluka, puriss. &gt;99.5%</td>
</tr>
</tbody>
</table>
9.4. Enzymes

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Source</th>
<th>Product description</th>
<th>Producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL</td>
<td>Pseudomonas Cepacia (Burkholderia cepacia)</td>
<td>Lipase PS</td>
<td>Amano</td>
</tr>
<tr>
<td>RAL</td>
<td>Rhizopus Arrhizus</td>
<td>Lipase from Rhizopus Arrhizus</td>
<td>Fluka</td>
</tr>
<tr>
<td>RML</td>
<td>Rhizomucor Michele</td>
<td>Novozym 388</td>
<td>Novozymes</td>
</tr>
</tbody>
</table>

9.5. $^1$H-NMR spectra of acylglycerols at 400 MHz in CDCl$_3$

The different products of lipolysis of tricaprylin in miniemulsion by the Rhizomucor Michele lipase at 40 °C were separated by TLC and analyzed by $^1$H-NMR so as to be used as standards. Their respective spectra are depicted in the following figures.

Figure 63: $^1$H-NMR spectrum of tricaprylin.
Figure 64: $^1$H-NMR spectrum of 1,3-diglyceride.

Figure 65: $^1$H-NMR spectrum of 1,2- and 1,3-diglyceride.
Figure 66: $^1$H-NMR spectrum of 1- and 2-monoglyceride.
10. DANKSAGUNG

Die vorliegende Arbeit, die zwischen November 2003 und Dezember 2007 in der Abteilung Organische Chemie III/Makromolekulare Chemie an der Universität Ulm entstanden ist, wäre ohne die Mithilfe einer Vielzahl an Personen nicht möglich gewesen und deshalb gebührt ihnen mein besonderer Dank:

Universität

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Privat

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11. LEBENSLAUF

Zu meiner Person

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geboren am 07.10.1976 in Marseille, Frankreich
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Seit 2003

Doktorarbeit an der Universität Ulm, Abteilung OCIII, mit Schwerpunkt Biokatalyse.

1997-2001

Chemiestudium in der „Ecole Nationale Supérieure de Chimie“, der Staatlichen Hochschule für Chemie, in Montpellier.
Abschluss: Chemiediplom und Master of Science mit Auszeichnung: Schwerpunkt Macromolekulare Chemie und Organische Chemie.

1994-1997

Vorbereitungsklasse (selektives Aufnahmeverfahren, dreijähriges Studium, das auf die Aufnahmeprüfung für die französischen Eliteschulen vorbereitet) im Gymnasium Lycée Stanislas, Paris, Frankreich.
Abschluss : Zulassung für die „Ecole Nationale Supérieure de Chimie“, Montpellier.

2002-2003

Technischer Vertriebsfachmann bei Chemspeed Ltd., Basel, Schweiz.
Tätigkeitsbereich: Verkauf und Werbung von Automaten zur parallelen Flüssig- und Festkörpersynthese an pharmazeutische und chemische Unternehmen.

2001-2002

Technischer Vertriebsfachmann bei Mettler-Toledo S.A. Autochem, Paris, Frankreich.
Tätigkeitsbereich: Verkauf und Werbung von Automaten zur parallelen Flüssig- und Festkörpersynthese (Bohdan und Myriad Inc.) an pharmazeutische und chemische Unternehmen.

Tätigkeitsbereich: Synthese und Herstellung von organischen und anorganischen Derivaten für Hydrophobe System.

Okt. 98 und Aug. 99  Berufspraktikum als Synthesetechniker im CNRS, Montpellier, Frankreich.
Tätigkeitsbereich: Synthese von Initiatoren für kontrollierte radikalische Polymerisation.


Sprachkenntnisse  Französisch (Muttersprache)
Englisch (fließend)
Deutsch (fließend)
Spanisch (Grundkenntnisse)

EDV-Kenntnisse  MS Office, Chemdraw, Isisdraw, Mestre-C, Win-NMR, Chromeleon (HPLC Software) – Netzwerkadministrator der Abteilung OC3, an der Universität Ulm.

Führerschein  PKW


~ Öffentlichkeitsarbeiter in der Studentenverbindung meiner Hochschule.


Cello spielen,
Schwimmen, Kampfsport, Snowboard und Ski fahren, Rugby.

Reisen – 7 Länder im Ausland, zahlreiche Aufenthalte in Großbritannien und in die USA.
12. ERKLÄRUNG


Ulm, den 19.12.2007

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Jean-Baptiste Doucet