Lipase-Driven, Room-Temperature Solubilization Processes of Deposited Fat-Based Stains

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Preface

The work presented in this thesis is based on research conducted at the Institute of Physical and Theoretical Chemistry (Faculty of Natural Sciences IV) at the University of Regensburg between January 2017 and January 2020.

This work would not have been possible without the help and support of many people to whom I would like to express my utmost gratitude.
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worked tirelessly to built a reliable machine that functioned through thousands of washing tests, requiring only minor maintenance. In case any issue arose, they repaired it on short notice and got the unit functioning again in very little time.

A special thanks I want to direct towards Hellmuth Schönsteiner who spent long hours getting the GC to work again so I could start my measurements. He also always helped me if I needed to find any chemicals that were tucked away somewhere in the cupboards and storage rooms within our group. I also want to thank Franiska Wolf who always made sure that anything we did not already have but was needed for my work was swiftly ordered.

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Franz Hippolyth Schermer

Abstract

The aim of this work was to investigate the processes that influence the washing efficiency of a lipase containing detergent on stained cotton fabric and, based on these findings, how to improve the washing performance at room temperature. To clarify the effectiveness of a lipase on a fat stain, the factors influencing the enzyme based washing performance had to be unravelled from the convoluted mixture of effects present during washing. Based on previous works performed by other members of this group, where the surfactant efficiency was of the most concern, strategies were developed and employed to increase the release of fat-based soil at room temperature. These strategies involved understanding the factors influencing the lipase's washing performance, defined by its activity, i.e. the turnover rate, and its efficiency, i.e. access to the substrate. By understanding these influences, the washing performance was increased without increasing the enzyme concentration.

Before employing the enzyme, the phase behaviour of fatty acids in surfactant solutions was studied. As the enzymatic hydrolysis of triglycerides yields fatty acids, it was assumed that they would dissolve better in the medium directly surrounding the stain compared to triglycerides. It was hypothesized that they would be incorporated into the micelles of the surfactant and act as co-surfactants. These tests were performed with the non-ionic surfactant Lutensol AO7, an ethoxylated linear alcohol with a chain length of C_{13}/C_{15}, the non-ionic polyalkylglycosides Glucopon 215UP and Glucopon 600CSUP mixed with the linear alkyl sulfate Texapon N70 and sodium dodecyl benzyl sulfonate. It was determined that only at extremely high surfactant concentrations palmitic acid can be dissolved sufficiently. The addition of co-solvents did not much improve the solubility. It was therefore determined that fatty acids could not just be dissolved at room temperature, even considering the higher surfactant concentration at the water/fat interface. Given the Krafft points of C_{16} and longer chained carboxylates, this is plausible.

An important cornerstone of this work were the detergency tests with stained cotton stripes. As the originally planned glass beaker tests proved to deliver unsatisfactory results, a testing unit was developed and built that would allow a higher throughput and increase the validity of the results. It was designed so that a constant temperature and a constant and consistent motion could be upheld. The machine itself is

outlined in the fourth part of this thesis. It proved invaluable for the experiments performed, giving access to reliable washing performance results. Initially, the results were used as directions which formulations showed synergistic behaviour and called for closer inspections in experiments that could isolate some of the plethora of effects taking place in the washing tests. Later, the testing unit allowed us to examine, if interactions determined with other methods did indeed influence the washing behaviour. With these tests it was found that certain co-solvents can not only increase the washing performance, but also increase the performance of the lipase, while others increased the dissolving of the fat but hindered the lipase. The testing of different stains also showed that under the conditions (very high soiling compared to amount of fabric) it is not possible to achieve conclusive results for every stain type. Also, the washing tests made it clear that experimentation to examine specific interactions was needed to determine, which processes influence the washing performance in which direction.

One of these experimental set-ups chosen to see, how additives could directly influence the lipase, were Langmuir-Blodgett (LB) trough tests. In a washing test environment, the co-solvent might swell the stain, which causes an increase in the fats surface area, which may lead to increased washing performance, due to the greater surface area the lipase can access. By creating a monolayer, however, this effect can effectively be excluded. In order to see, if LB trough experiments could show enzymatic activity, compression isotherms of several mixtures of tripalmitin and palmitic acid were recorded and a correlation of the isotherm with the molar content of palmitic acid was discovered. Lipase was then added to the subphase, a mixture of tripalmitin and palmitic acid spread on the surface and the system was allowed to incubate at 20 °C to see, if any changes in the palmitic acid content in the surface monolayer could be seen. It was indeed possible to track the increasing amount of palmitic acid. Co-solvents and other possible additives were added to the subphase along with the lipase. It was tested, if any difference in the hydrolysis of tripalmitin could be seen in the compression isotherms. In some cases that was possible, and increase in generated fatty acid (and therefore in enzymatic activity) was seen. However, this method was only applicable for additives with negligible surface activity. Any surface-active substances severely changed the isotherms, even if their subphase concentrations were as low as 10 ppm. They could not be tested with this method.

To gain further insight into the activity of the lipase in a detergent-like environment, an enzyme assay tracking the hydrolysed fat was suggested. The initial method relied on complete hydrolysis of the triglycerides, as the dye generated by the reaction chain corresponds to the amount of glycerol in the sample. This method proved to be unsuitable for the lipase used in the washing tests. Lipase concentrations greater 1'000 U/mL were needed to create enough dye for a colour change visible to the naked eye. Other experiments concluded that glycerol concentrations as low as 5 ppm should be enough to quantify the activity of the enzyme. It was therefore concluded that the lipase used does not generate a sufficient amount of glycerol in the 30 minutes of incubation used for this assay.

A method to experimentally access the degradation products of the enzymatic reaction with triolein and possibly quantify them directly, is high performance liquid chromatography (HPLC). A normal phase (NP) setup was chosen for the tests. The first step of the previous assay, the enzymatic reaction in a stirred emulsion was not changed. However, the further steps were quite different. After stopping the reaction, all samples were dried to remove all water before injecting the samples into the HPLC column. The residue of the samples was taken up in the same mixture that was used as the mobile phase and injected into the HPLC. Isocratic elution and UV-detection were used for the experiments. After measuring triolein, oleic acid, mono- and diolein standards, and creating calibration curves for oleic acid and triolein, it was discovered that 1,3-diolein is the main degradation product of the reaction of triolein and the lipase. No monoglycerides were detected. This very clearly showed why the originally planed assay was not suitable for the lipase used here. To verify the assay, several parameters, including the pH and water hardness of the emulsions and the temperature and enzyme concentration during incubation were modified. Since the results varied as expected, the experiments were continued with co-solvents and co-surfactants in the assay emulsions. Extreme increases of generated fatty acid were found for samples with Agnique AMD3L and 2-MTHF in the emulsions. For both of these co-solvents, 1,2-diolein was detected in the samples, which was not present in the samples without additives. The samples with 2-MTHF also showed small signals that indicated the presence of 1-monoolein, further cementing the hypothesis that the lipase's activity is increased in presence of 2-MTHF.

To see if the co-solvents had any influence on the additives stability, differential scanning calorimetry (DSC) measurements were performed. A sample containing the lipase was heated along with a blank containing the enzyme buffer but no enzyme. The degradation temperature of the lipase can be measured this way, as it will create a difference in the heat flow to the sample compared to the blank. By comparing the onset temperatures of those signals, destabilizing effects of additives can be seen. This method could only be used for a few systems, as the extremely high concentrations of enzyme and additive that were required here (1-5%) often caused the enzyme to degrade within seconds after mixing. For the systems that could be measured, it was found that 2-MTHF does indeed reduce the degradation temperature, suggesting it has an influence on the structure of the lipase, while adding Agnique AMD3L did not reduce the degradation temperature of the lipase.

Finally, to test the hypothesis that the co-solvents cause fat stains on fabric to swell, swelling tests with aqueous additive solutions and Biskin were performed. The hypothesis was that the swelling of the fat caused by an additive results in cracks and small fissures in the fat stains surface. The increased surface area allows the enzyme to access the stain easier. The increased washing performance results from the larger surface area that the enzyme can access. The results were mostly inconclusive and no quantitative statements can be drawn from them, due to large standard errors. However, it could be shown that an aqueous 2-MTHF solution does not increase the swelling of Biskin compared to a sample with pure water, while aqueous solutions of Agnique AMD3L and propylene glycol did increase the swelling of the fat stain.

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Part I

Introduction and Motivation

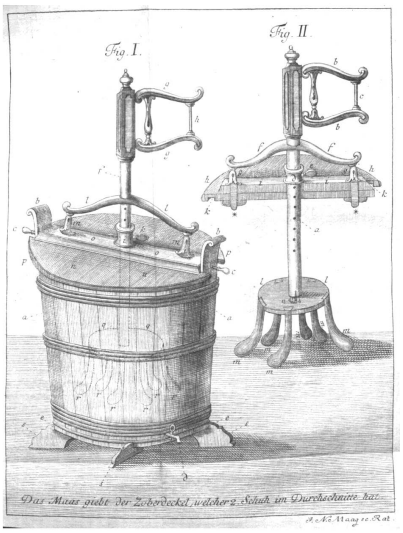


Figure 1: The design of a washing machine published by Jacob Christian Schäffer in 1766[1].

If one mentions the city of Regensburg, the city in which this thesis came into being, one might receive answers mostly praising the beauty of the city, or perhaps its rich history, dating back to the days of the Roman Empire or maybe complaints about the high rents and the difficulties of finding a small apartment, if one asks one of the over 30'000 students that study in Regensburg, either at the university or at the OTH. Laundry cleaning is not among the things that usually come to mind, but Regensburg has an important connection to that as well. Jacob Christian Schäffer's impact on how we clean our laundry is of no small importance, as he is widely considered as the inventor of the commercial washing machine, with his work "Die bequeme und höchstvortheilhafte Waschmaschine" published in 1766 in Regensburg[1]. Schäffer's design, which can be seen in Figure 1, differs quite a bit from the washing machines used in modern households, lacking the rotating drum design that is used today.

When Schäffer designed his washing machine, rotating drum machines were still 85 years away, as such a design was first patented in 1851 by James King. Half a century later, the first electrical washing machines were developed, and after the second world war, the first fully automatic washing machines were introduced. By the 1970s, these had replaced mechanical washing machines and washing basins almost completely[2, 3].

Since the days of Schäffer, not only washing machines have developed drastically. For centuries, the only available detergent was soap, initially produced from animal fat and wood ash. Later, plant oils replaced the animals fats and alkali the

wood ash. Use of such detergents was still common well into the 20th century. But soaps are pH and salt sensitive, they are skin irritant and may exhibit high Krafft temperatures, leading to poor solubility in water at low temperatures. For the last 60 years, technical surfactants, often sulfates and sulfonates, have been developed to increase the performance of laundry detergents and to avoid the detriments of natural soaps[4–7]. Today, laundry detergents are complex and highly specialized chemical formulations and a plethora of detergents is available on the market. Also, the temperatures used in modern laundry processes have significantly decreased. It is rarely required to use near-boiling temperatures today to remove stains, with the availability of specialized stain removers.

For several decades already, environmental concerns have been gaining importance in the public eye and now, in the face of climate change, ecological sustainability is in the centre of public discussion. This is also true for the chemical industry, were sustainable processes and compounds have become main goals in recent years[8, 9]. Anastas' and Warner's "twelve principles of green chemistry" have created a guideline for sustainability, that is widely known and taught today[10].
For detergents, these environmental concerns have initially been focused on ecotoxicity and biodegradability. The environmental impact of surfactants first gained public attention after the "foam mountains" had formed on rivers and in waste water treatment plants in the 1950s, which lead to legislation that set requirements for the biodegradation of surfactants[11–13]. By today, surfactants that are not readily biodegradable cannot be introduced to the market and detergents containing such surfactants would not find their way on the shelfs of supermarkets in the EU.
Still, development and improvement of detergents is an important field. The main aim today is to increase the performance and efficiency of the formulations, which allows a decrease of washing temperatures and water consumption. If less water is used that also needs less heating, electrical power consumption can be decreased substantially. A decrease of the temperature from 60 °C to 40 °C can decrease the energy consumption by 40 %[14]. For consumers to use lower washing temperatures, however, it is required that the detergent performance is enhanced, to achieve satisfying cleaning results.
A possible solution to increase the efficiency of laundry detergents and lower the washing temperatures while maintaining a good washing performance, are enzymes. The application of enzymes has shown a lot of promise in different industrial chemical

fields, due to advantages such as high selectivity, very specific reactions and that they can be applied in mild conditions and will still show high activity. Furthermore, enzymes are readily biodegradable and have not been known to accumulate in nature, which is a further advantage for sustainable applications[15–18]. But the advantages of enzyme application need not be limited to industrial processes, as the same advantages can be applied to a domestic process such as laundry cleaning.
Using enzymes to enhance the performance of a laundry detergent is not exactly a new idea. Already in 1914, Otto Röhm filed the first patent for a detergent with enzymes. He extracted the protease trypsin from animal pancreases. In the 1960s, the microbial production of proteases and amylases for detergents on a large scale began. In 1988, the first lipase for laundry detergents was introduced. Before that, enzymes that can dissolve fat were not available for detergents[19–22]. This was an important step, as removing fat stains from fabrics below the fats melting point is practically impossible, since solid fats can hardly be dissolved by the surrounding media present in a washing process, which was seen in early stages of this thesis. Breaking up the structure of a fat stain by an enzyme hydrolysing the triglycerides of the fat, however, might increase the stains solubility. It could then be removed without the necessity to heat the fat above its melting point.
However, knowledge concerning washing processes is still mostly empirical, and enzymes used in formulations are mostly tested for their storage stability and not their activity under washing conditions. To further increase the performance of laundry detergents, the underlying processes have to be understood. In the case of enzymes, the factors influencing their activity during the washing process must be investigated. Ideally, such an understanding can help to adjust the formulations, adding additives that help to achieve optimal activity and efficiency for the enzymes. This could then help towards developing formulations that show a high washing performance at low temperatures, reducing the energy consumption of washing processes while maintaining biodegradability and low ecotoxicity.

For this thesis, the low removal of fat stains in low temperature washing processes was investigated. Based on previous works within this group by Dengler[23], Wolfrum[24] and Zahnweh[25], experiments were designed to understand the underlying processes that influence the washing performance. A special focus was put on enzyme-driven processes. A lipase was employed to improve removal of triglyceride stains at room temperature (25 °C). Washing tests with an in-house designed and

built testing unit were performed to create a near-application environment to test if hypothesises held true in a more complex surrounding. To gain insight on the interaction of the lipase and the triglyceride, Langmuir-Blodgett trough tests were realized, where the lipase interacted with a triglyceride monolayer. To gain insight on the activity of the lipase and how to influence it, an assay with coupled HPLC analysis was developed. Additionally, DSC measurements were performed to see how formulation components that influence the lipase's activity act on its stability.

Much like the work of Dengler[23], which can be seen as a predecessor to this work and was aimed at improving the understanding of the role that surfactants play in washing processes and how to increase the surfactants efficiency and possibly improve the surfactants themselves, this work has a comparable goal, but instead of the surfactants, the main focus is on how lipase-driven washing can be improved in a similar way.

Part II

Fundamentals

1 Surfactants

1.1 General Information and Classification

The term surfactant is an abbreviation of Surface Active Agent" and is used to describe amphiphilic molecules[26]. The word amphiphilic is derived from the greek words "amphis" (both) and "philia" (love) and is used to describe a compound that exhibits both hydrophilic ("water-loving") and lipophilic ("fat-loving") properties. Surfactants are essential and indispensable in our daily live as they can be found in detergents and other washing products or in many care and cosmetics products. They are also used by the food industry as emulsifiers and have become vital to industrial processes in the paper and cellulose industry, the mining industry and metal processing, among others[4, 27].

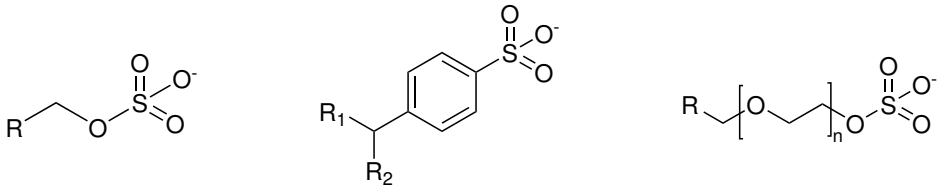
To achieve their amphiphilic properties, surfactants consist of a hydrophobic part, which is commonly a linear or branched alkyl chain with at least eight carbon atoms, often referred to as the "tail", and a hydrophilic headgroup. Due to this, surfactants exhibit surface activity and adsorb readily to surfaces, which is accompanied by the characteristic reduction of surface tension.

Historically, soap is the oldest surfactant known to mankind. It has been used since the antiquity[7] and is the prevalent surfactant in the world to this day[28]. Due to its disadvantages, mainly the alkaline reaction and sensitivity towards water hardness where insoluble "lime soaps" can be formed, the last century saw a beginning in development of synthetic surfactants, to substitute soap, as its downsides proved insurmountable. The first of these substitutes was an alkylated and sulfonated naphtalene compound, synthesized by German chemist Fritz Günther of BASF Ludwigshafen in 1917[29]. The importance of synthetic surfactants is increasing, as they can be tailormade for special applications and can avoid the disadvantages of soap. The global importance of surfactants manifests itself in a multi-billion dollar market. In 2016, the global turnover of surfactants was almost 31 billion USD and is expected to grow by an average of 3.1% per year up to 2024[30].

While for commercial applications surfactants may be classified according to their use, the most common and widely accepted classification of surfactants is by the charge of the hydrophilic headgroup into 4 classes: nonionic, anionic, cationic and

zwitterionic (often refered to as amphoteric)[4, 26, 29]. In the following, examples of each class and their most important properties will be given.

Anionic Surfactants

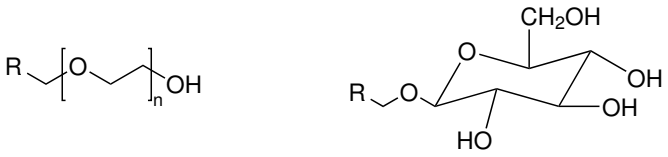


alkyl sulfate | alkyl benzenesulfonate | alkyl ether sulfate

Figure 1.1: The headgroups of important anionic surfactants.

The largest group of surfactants, making up about 50% of global production (excluding soap) are anionic surfactants[28, 31]. Common headgroups are sulfate, benzenesulfonate and carboxylate. They are found in most detergents and skin cleaning products and are also used in emulsifiers and foaming agents. A reason for their popularity is that their production is simple and cost efficient. The chain length of the alkyl chain is typically in the range of C12-C18. However, their applications are limited, due to their sensitivity to external conditions. Generally, they are sensitive to hard water, especially carboxylates, an effect that can be reduced by introducing a short polyoxyethylene chain between the headgroup and the alkyl chain. The presence of electrolytes can also affect their properties. Also, a combination with cationic surfactants is difficult, as it may cause precipitation[32].

Nonionic surfactants

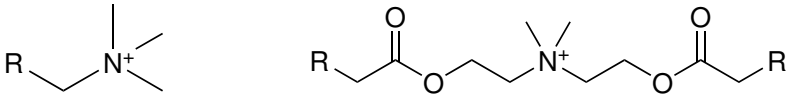


alcohol ethoxylate alkyl glycoside

Figure 1.2: The headgroups of important nonionic surfactants.

The second largest class in terms of production volume are nonionic surfactants[28, 31]. The polar head group of these surfactants bears no charge. Due to this, the presence of electrolytes or water hardness does not affect them and they are compatible with all other surfactant classes. This allows widespread use in complex formulations that are found in many modern commercial products. Commonly, nonionic surfactants are good detergents and exhibit good wetting and emulsification properties. In modern industrial synthesis, the degree of ethoxylation can be controlled precisely, allowing for tailor-made properties. However, they are not without limitations, as their physico-chemical properties are dependant on temperature. The water solubility of alcohol ethoxylates, which are the most important type of nonionic surfactants in terms of applications, decreases with increasing temperature. Alkyl glucosides, on the other hand, do not exhibit this behaviour, i.e. their water solubility increases with temperature[31, 32].

Cationic surfactants



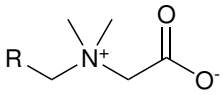
quat. alkyl ammonium alkyl ester quat

Figure 1.3: The headgroups of important cationic surfactants.

The third largest surfactant class, making up approximately 5-6% of the total surfactant production are cationic surfactants[31]. In most cases, the positive charge is carried by a nitrogen atom in the head group. Usually, they cannot be combined with anionic surfactants, although important exceptions were found where a combination led to synergistic properties[33]. They are generally neither good

detergents, nor good foamings agents, but can be very useful for specific purposes. Due to their positive charge, they adsorb strongly to surfaces, which are mostly negatively charged at neutral pH. Their high toxicity in comparison to other surfactant classes allows using cationic surfactants as bactericides. Like anionic surfactants, their physico-chemical properties are affected by the presence of electrolytes in solution[31, 32].

Zwitterionic Surfactants



alkyl betaine

Figure 1.4: The betaine head group, the most common head group of zwitterionic surfactants.

The smallest class in terms of production volume are zwitterionic surfactants. Their head group carries both a positive and a negative charge. They can be combined with all other surfactant classes and, since they possess no net charge, are not sensitive to water hardness or electrolytes. In general, they exhibit very low eye and skin irritation and are thus well suited for usage in personal care products[32].

1.2 Surfactant solubility

1.2.1 Solubility of ionic surfactants and "Krafft temperature"

Solubility in aqueous media is the requirement that surfactants need to meet to be used in any of the processes in this thesis. A key phenomenon observed in ionic surfactants is the very low solubility at low temperatures, with a drastic increase above a certain temperature threshold (see **Figure 1.5**). This is referred to as the "Krafft phenomenon". The Krafft temperature T_{Kr} or Krafft point is the temperature, at which the strong solubility increase starts. Below this temperature, no micelles are formed as the solubility is below the critical micellar concentration (cmc)[26, 32, 34]. Experimentally, T_{Kr} is usually accessed by determining the temperature at which a 1 wt-% aqueous surfactant solution clears[35].

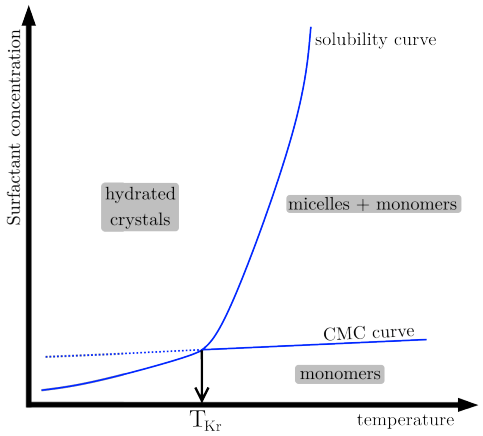


Figure 1.5: Temperature dependence of the cmc and surfactant solubility. The intersection of the two curves is referred to as the "Krafft temperature" T_{Kr}. The figure is based on [32] and [34].

Two counteracting thermodynamic forces can be identified that determine the Krafft temperature. The free energy of the solid crystalline surfactant and the free energy of the micellar solutions. While the former varies only slightly with different chain lengths or counterions, the latter can change drastically due to packing effects. Generally, a more regular packing decreases the free energy of the crystalline state, leading to a higher T_{Kr}[32]. The Krafft temperature also increases with the length of the linear chain, and simple sodium soaps already exhibit Krafft points above room temperature for chain lengths greater than 12[36]. As long chain surfactants are more efficient, a decrease of the Krafft temperature, achieved via an increase in the free energy of the crystalline state, is desirable.

One possibility to decrease the Krafft temperature is via branching of the alkyl chain or introducing double bonds. The reduced symmetry of the chain hinders regular crystal packing of the surfactant[32]. This is a less favourable method, as branched surfactants have shown to have poor biodegradeability[37, 38].

Another method to decrease T_{KR} is the introduction of a sterically demanding counterion instead of the alkali metal ions mostly found in anionic surfactants. It has been shown that introducing the sterically larger choline ion in place of the alkali ion can severely reduce the Krafft temperature[36, 39].

The packing conditions can also be influenced by introducing a polar group (usually an oxyethylene (EO) group) between the head group and the hydrophobic chain[32]. The EO groups are more flexible than the alkyl chain, which hinders crystal formation and precipitation of the surfactant and raises the free energy of the crystalline state[40, 41]. Commercially available surfactants make use of this effect.

Finally, the introduction of a third component can influence the Krafft temperature. Addition of polar co-solutes, e.g. alcohols can generally decrease T_{Kr}[32]. The decrease of T_{Kr} is directly proportional to the concentration of the alcohol and indipendent of surfactant concentration and is a result of a decreased cmc in the presence of alcohols[42, 43]. The addition of salt can also change the Krafft temperature in both directions and is heavily dependant on the head group and counterion of the initial surfactant. Also, it must be taken into account, whether a counterion is an organic molecule or a simple inorganic ion[24].

1.2.2 Solubility of nonionic surfactants

Nonionic surfactants exhibit a different temperature dependant solubility behaviour. Typically, they experience phase separation with increasing temperature with a characteristic cloud point. It is strongly dependant on the chain length, and in case of alcohol ethoxylates, on the number of EO groups[32, 44]. This behaviour can be explained by looking at the driving forces for the solubilisation of nonionic surfactants. The main driving force is the hydration of the head group and the formation of hydrogen bonds between the EO groups and the surrounding water. H-bond strength decreases with increasing temperature. When a certain threshold is reached, the solution becomes turbid, which is why the phenomenon is referred to as the cloud point[26]. The addition of anionic surfactants increases the cloud point. The increase is gradual until the cmc of the added surfactant is reached and a further addition of surfactant leads to a sudden jump in the cloud point. Adding electrolytes can either decrease (for salting-out types) or increase (for salting-in types) the cloud point. However, the effect is only visible, after a certain minimum concentration is reached[45].

1.3 Self-assembly of surfactants in aqueous solutions

As mentioned in the previous section, surfactants exhibit a critical micellar concentration (cmc) in aqueous solutions. Below the cmc, surfactants adsorb to the available interfaces (the air/water interface or the inner walls of a flask) to reduce the contact of their hydrophobic chain with the surrounding water. This adsorption

reduces the surface tension[46]. Adsorption phenomena and their implications will be explained in section 1.4. When the available interfaces are saturated, a further increase of surfactant concentration leads to the spontaneous formation of aggregates. These self-assembled structures are formed to prevent an increase of free energy in aqueous solution, as direct contacts of the alkyl chains and water are energetically unfavoured. At the cmc, micelles are formed, in which the hydrophobic parts are directed towards the interior of the aggregate and are thus isolated from the surrounding medium, while the hydrophilic headgroups are directed towards the surrounding water, as their hydration is energetically favoured. The molecules are assembled physically, not chemically, which means that the aggregates are dynamic and can change in size and shape[26, 46].

1.3.1 Critical micellar concentration

As already mentioned, below a certain concentration, surfactants are dissolved as monomers and adsorb to interfaces. As a certain concentration is reached, the surfactants start to spontaneously form aggregates, initially micelles in most cases. The concentration, at which this self-assembly begins is called the critical micellar concentration (cmc)[26, 32, 47].
Adsorption and aggregation effects of surfactants in solution can be explained via the "hydrophobic effect"[48]. This effect describes the interaction between water and a non-polar solute, which in this case is the hydrophobic chain of the surfactant. Two contributions constitute the hydrophobic effect. Firstly, the presence of the hydrophobic chain disturbes the strong hydrogen bond of the water molecules. To accommodate the hydrophobic group, a cavity is formed in the bulk water, which breaks up the strong hydrogen bonds of the water molecules. This constitutes a large increase in enthalpy (free energy). Secondly, water molecules rearrange themselves around the hydrophobic group. This structuring of the water molecules increases the entropy but decreases the enthalpy. Overall, this constitutes a small decrease in free energy which decreases with increasing temperature. The increase in free energy by the formation of the cavity outweighs the energy gain by the structuring of the water molecules, leading to aggregation of the surfactant[26, 47, 48].
The cmc can be determined precisely, as the formation of aggregates constitutes a sharp change in several physico-chemical properties of the solution[49]. Figure 1.6 shows the development of some physico-chemical properties in dependance of the surfactant concentration.

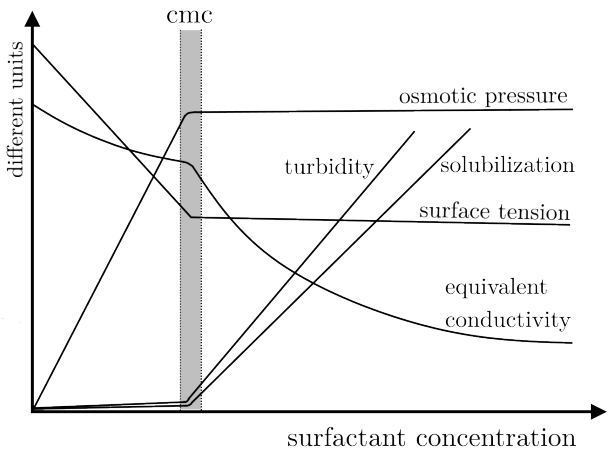


Figure 1.6: Schematic representation of the surfactant concentration dependant development of some physico-chemical properties of an aqueous surfactant solution. The figure is based on [49].

In applications, surfactants are used in concentrations above the cmc, as the required properties are only present then. It is therefore useful to be aware of the factors influencing micellization. Micellization is promoted by the aforementioned hydrophobic effect, but head group repulsion counteracts micellization, especially in ionic surfactants. These factors are heavily dependant on the molecular structure and also on system properties, e.g. temperature, cosolutes, etc.[47, 49]. When comparing surfactant structures and system properties to cmc values, several statements can be made.

For unbranched surfactants, the cmc increases with the length of the hydrophobic chain, due to the increasing influence of the hydrophobic effect. This logarithmic correlation can be described with Klevens equation[50]:

$$log(cmc) = A - BN \tag{1.1}$$

where A and B are constants specific for the surfactant and and N is the number of carbon atoms in the alkyl chain. The effect is more pronounced for nionionic surfactants compared to ionic ones[4, 26, 32, 49].

A branched surfactant or a surfactant with a double bond has a higher cmc compared to a linear, saturated surfactant with the same number of carbon atoms in the alkyl chain. This is due to a reduced hydrophobic effect[4, 5, 49].

The cmc of a nonionic surfactant is lower than that of an ionic one with the same alkyl chain, due to electrostatic repulsion of the headgroups[5, 49].

The present counter-ions have a strong effect on the cmc, dependant on their valency

and their interactions with the headgroup. The stronger the interaction, the lower the cmc, due to a reduced net charge of the head group[4, 5, 26, 49].
The cmc of a ionic surfactant in aqueous solution can be strongly reduced by adding salt[4, 5, 26].
Lastly, polar organic cosolutes can influence the cmc. Depending on their solubility behaviour in aqueous solution, an increase or decrease of the cmc is possible. Highly polar organic compounds, like ethanol or acetone, which are also highly water soluble, increase the cmc. They are hardly found in the interior of the micelles and instead increase the solubility of the monomeric surfactant, due to a decreased hydrophobic effect. Using less water soluble cosolutes or additives that prefer solubilization inside the micelle, like medium or long chain alcohols, can decrease the cmc, since these compounds can stabilize the micelles by decreasing the head group repulsion of the surfactant[4, 5, 32].

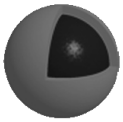
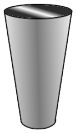
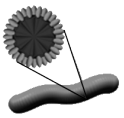
1.3.2 The packing parameter

Spherical micelles are the most well-known aggregate of surfactants in solution. However, surfactants can form aggregates of different sizes and shapes. This can be influenced by external parameters of the system, e.g. salt content or temperature[26]. Prediction of the shape of aggregates is possible via the "packing parameter" p defined by Israelachvili et. al.[51]. It connects the geometry of the surfactant molecule with the resulting shape of aggregates. It is defined as the following:

$$p = \frac{V}{a_0 l} \tag{1.2}$$

where V is the volume of the hydrophobic tail, a_0 the effective surface area per molecule at the micelle-water interface and l the length of the tail.
The packing parameter concept is a powerful tool to predict micelle shape and changes induced by changes in experimental conditions[46]. Table 1.1 shows an overview of the expected aggregate in relation to the packing parameter and gives examples for general surfactant structures that exhibit this behaviour. The area a_0 of the headgroup is the main factor in changing the packing parameter, as the ratio of V/l is nearly constant for commonly used surfactants. A larger headgroup results in more cone shaped packing shape, leading to spherical micelles. A decrease in a_0 increases the packing parameter, resulting in bilayers or inverted structures[52].

Table 1.1: The packing shapes of surfactants and structures they form in dependence of the packing parameter. Based on [52, 53].

p	Surfactant type	Packing shape	Formed structure
$< \frac{1}{3}$	Single-chained surfactants with large headgroup area	Cone 	spherical micelle 
$\frac{1}{3} - \frac{1}{2}$	Single-chained surfactants with small headgroup area	truncated cone 	cylindrical micelle 
$\frac{1}{2} - 1$	Double-chained surfactants with large head group areas and flexible chain	truncated cone 	vesicle, flexible bilayer 
~ 1	Double chained surfactants with small head group area or rigid chain	cylinder 	planar bilayer 
> 1	double chained surfactants with small head group area and large/bulky chains, nonionic lipids	inverted truncated cone, wedge 	Inverted micelles 

1.4 Surfactant adsorption to surfaces

As mentioned earlier, the word surfactant is an abbreviation of surface active agent. Due to their amphiphilic nature, surfactants adsorb to interfaces, leading to a decrease in surface tension. The reason behind the adsorption of surfactants to interfaces is a reduction of free energy in the system. As mentioned in section 1.3.1, the hydrophobic effect explains this energetically unfavourable restructuring of the bulk water when interacting with the hydrophobic chain of the surfactant. By reducing the contact with the alkyl chain, the free energy of the system is lowered. Initially, the surfactant molecules adsorb to interfaces. The kinetics of this process are influenced by parameters like temperature, concentration and viscosity. The structure and size of the surfactant itself play a significant part in the process[4, 5, 54].

In theory, the adsorption of surfactants can be approached by defining an excess concentration Γ_s of the surfactant at the interface (see equation 1.3). It is the excess concentration of surfactant s at the interface i compared to the bulk solution. The excess amount of surfactant at the interface is defined as n_s^i and A_i is the area of the interface i[5, 26, 55].

$$\Gamma_s = \frac{n_s^i}{A_i} \tag{1.3}$$

Changes of the Gibbs free energy G of a system are given by the following fundamental equation[55]:

$$dG = -SdT + Vdp - Ad\gamma + \sum_s \mu_s dn_s \tag{1.4}$$

where

- S is the entropy
- T is the temperature
- V is the volume
- p is the pressure
- A is the area of the interface
- γ is the interfacial tension
- μ is the chemical potential
- n_s is the amount of substance s

The change ins Gibbs free energy can also be written as[55]:

$$dG = \sum_s \mu_s dn_s + \sum_s n_s d\mu_s \tag{1.5}$$

Comparing this to equation 1.4 leads to the Gibbs-Duhem equation for interfaces[55]:

$$SdT - Vdp + Ad\gamma + \sum_s n_s d\mu_s = 0 \tag{1.6}$$

In order to access the excess concentration Γ_s, this equation has to be divided by the area of the interface A. If one then assumes that temperature and pressure are

constant, the Gibbs equation (equation 1.7) remains, which relates the interfacial tension to the shift in chemical potential μ_s and the excess concentration Γ_s[55].

$$d\gamma + \sum_s \Gamma_s d\mu_s = 0 \tag{1.7}$$

For a binary mixture, e.g. water and a surfactant with the assumption of constant temperature and pressure and a reasonable dividing surface to have an excess concentration of water Γ_1=0 and a sufficient dilution for $d\mu_2 = RTdlnc_2$, i.e. the activity coefficient of compound 2 ≈1, to be valid, the Gibbs adsorption isotherm is given as[5, 26]:

$$\Gamma_2 = -\frac{d\gamma}{RTdlnc_2} \tag{1.8}$$

This is valid for uncharged species. For ionic compounds, the dissociation with regards to other system components, like salts, has to be considered. With equation 1.8, the surface excess concentration of a surfactant in aqueous solution is experimentally accessible by surface tension measurements. Aqueous solutions of surfactants yield positive excess concentrations. For solutions of electrolytes however, an increase in surface tension upon increasing electrolyte concentration is possible, which yields a negative excess concentration[5, 26, 55].

1.5 Environmental and Toxicological Aspects

The widespread use of surfactants in industrial processes and many household products, such as laundry detergents, cleaners, cosmetics and care products leads to an inevitable release of surfactants into the environment as well as to exposure of humans to surfactants[29]. This causes health and environmental risks due to toxicity towards humans, ecotoxicity or accumulation in ecosystems of surfactants. Therefore, their impact on ecosystems, both in terms of biodegradability and ecotoxicity as well as potential safety or health hazards must be known. The awareness of ecological issues caused by surfactants has drastically increased in recent decades, which lead to a shift from traditional surfactants to new renewable ones with bio-based raw materials and low toxicity[56]. The aspect of toxicity illuminates potential hazards of surfactants during their assigned usage, e.g. during application on the skin or the harm arising from the possibility of accidental oral uptake. The aspect of biodegradability seeks to find potential dangers after the use of surfactants, when

they reach ecosystems through waste-water, where they should degrade instead of accumulating, which causes potential harm to those ecosystems.

1.5.1 Biodegradeability and Ecotoxicity

As mentioned in the beginning of this chapter, surfactants are used extensively world-wide, leading to a considerable discharge into the environment. Surfactants are mainly released to the environment via waste-water, either through direct discharge of effluents into aquatic systems, through sewage treatment plants or via the use of sewage sludge containing partially degraded surfactants[11, 47].

Historical aspects

Before the second World War, soaps were the predominant surfactant class. A shift towards synthetic, high performance surfactants began in the late 1940s with the introduction of tetrapropylenebenzene sulfonate. Its low production cost and excellent performance quickly made it the most important synthetic surfactant of the western world. However, in the late 1950s, a severe disadvantage of this surfactant class came to light, when large "foam mountains" formed on rivers and in waste water treatment plants. These events made the environmental impact of surfactants evident, which no one had considered before.
It was found that theses branched surfactants accumulated in the environment significantly, as they resisted degradation by bacteria[12]. It was also discovered, that these surfactants were quite toxic towards aquatic organisms. In the early 1960s, legislation banning the use of these surfactants and implementing minimum standards for biodegradation of surfactants was passed and test methods to determine biodegradability were developed[29, 38, 47].

Legislation in Europe and test methods

Soon after the initial environmental problems occured, governments started passing legislation to counter these issues. In 1961, Germany passed a law that required detergents to be biodegradable[13]. Other countries soon followed with similar legislation. In 1973, the European Economic Community (EEC) issued its first directives on surfactants (73/404/EEC and 73/405/EEC), seeking to harmonize legislation in its member states. The initial directive addressed legislation on surfactants,

demanding an average biodegradability of 90% for all surfactants of the four main categories (anionic, cationic, nonionic and zwitterionic) placed on the market, while the second directive published on the same day established a test methodology for primary degradation[57, 58]. Standardized tests were developed by the Organization for Economic Co-operation and Development (OECD) and the International Organization for Standardization (ISO), which were included in follow up directives on detergents. Testing methodology to differentiate between primary and ultimate biodegradation was established and EEC directives to assess environmental risk of surfactants were passed[47, 59, 60].
As the previous directives had been significantly amended due to them being outdated, all previous directives were replaced by the Detergents Regulation (648/2004/EC) published in 2004[61]. It was put into effect in member states in October 2005. The new legislation is far more inclusive and prescriptive than previous legislation, which never included testing on and control of cationic or zwitterionic surfactants. The Detergents Regulation aims to clarify its scope, containing an exact definition of surfactants as well as a list of what is legally considered a detergent application. It imposes stricter requirements on biodegradability that surfactants have to meet in order to be allowed to be sold in the European market, demanding for surfactants to be "ready biodegradable" with no derogation to be used in common "non-exceptional" applications. This is a main difference to previous legislation, which only accounted for "primary biodegradability". The new regulation also updated testing methods, accounting for improved methods based on technical progress[60].
As of today, the directive has been amended in 2012 with the directive 259/2012/EC, limiting the use of phosphorous compounds in consumer laundry detergents and automatic dishwasher detergents to limit "eutrophication" risks[62].

Biodegradability

Biodegradation is a term describing the breakdown of chemical compounds by living organisms, mostly referring to microorganisms. It is vital to remove organic compounds from the environment. Generally, when regarding the end products of biodegradation of surfactants, two types of degradation can be distinguished. "Primary degradation" refers to the functional breakdown of the substance, determined by a substance-specific analytical method. For a surfactant, this means, that

the surfactant properties are lost. This is already enough to decrease foaming and aquatic toxicity of surfactants. "Ultimate biodegradation", on the other hand, is defined as the complete breakdown of the compound. For a surfactant, this means that the initial molecule has been converted to CO_2, CH_4, water, mineral salts and biomass. The complete removal of surfactants from the environment is only possible through "ultimate degradation"[11, 29, 59]. "Ready biodegradable" surfactants are those, that reach a sufficient amount of ultimate degradation within a specified time-frame, defined by the OECD[59].

For waste-water treatment in sewage plants, aerobic and anaerobic biodegradation are important. Commonly, in europe, waste-water is not discharged into the environment untreated, but into sewage plants, where the waste water is treated before discharge into the environment. In the mid 1990s, it had been found that the influents of rivers in Germany from waste-water treatment plants still contain traceable amounts of linear alkylbenzene sulfonates (LAS). This has lead to the increased biodegradability demands in the Detergents Regulation, to avoid accumulation of surfactants in aquatic systems[47].

In the sewage treatment, some surfactants absorb to the sludge fraction, which is treated in settling plants, commonly under anaerobic conditions. This sewage sludge is usually disposed on agricultural land after treatment, which leads to possible exposure of surfactants that degrade poorly under anaerobic conditions to the environment. This lead to an increase in legislative demands for anaerobic biodegradability of surfactants in the Detergents Regulation (648/2004/EC)[59, 60].

In plants as well as in the environment, the aerobic digestion path is the main degradation pathway of surfactants. In general, two pathways can be differentiated. In the first, the degradation begins with the separation of the hydrophilic head group from the alkyl chain, which is then attacked oxidatively. In the second mechanism, the oxidation of the alkyl chain commences without a prior cleaving of the chain and the headgroup. In both cases the surfactant looses its amphiphilic properties[59, 63]. A detailed report on the biodegradability of surfactant classes can be found in [59].

Available data suggests that alkyl sulfates and alkyl ether sulfates, as well as lineal alkyl ethoxylates, alkyl(poly)glycosides and soaps are both primarily and ready biodegradable under anaerobic and aerobic conditions. Linear alkylbenzene sulfonates are primarily and ready biodegradable under aerobic conditions, but degrade poorly under anaerobic conditions. Generally, biodegradability decreases if

the aromatic group moves from a terminal position on the alkyl chain to a more central one. As already mentioned, branched alkylbenzene sulfonates resist biodegradation, which is why they are banned in the EU and North America. Alkylphenol ethoxylates are a surfactant class that is banned in Europe even though these surfactants are primarily and ready biodegradable. However, their degradation products, namely nonylphenol and ethoxylated nonylphenol compounds, are far more toxic than the surfactant they originate from, which resulted in the surfactants being banned[11, 60, 63, 64].
The biodegradability of cationic surfactants is generally poor under aerobic conditions and no anaerobic degradation is known. This may be either due a lack of an appropriate metabolic pathways, or because of a toxic effect of cationic surfactants on anaerobic microorganisms[11]. Only quaternary ammonium salts are primarily and readily biodegradable under aerobic conditions[59].
Commercially used zwitterionic surfactants are known to be primarily and ready biodegradable under aerobic and anaerobic conditions[47, 59].

Ecotoxicity

As mentioned above, it was found that surfactants can still reach aquatic systems, even after waste-water treatment. The sewage sludge commonly applied to agriculturally used land also often carries surfactants that were not properly degraded. Additionally, a direct discharge into the environment is also possible[47]. Therefore, the toxicity towards living organisms in the environment, i.e. the ecotoxicity of surfactants is an important factor in determining the environmental impact of a surfactant. The toxicity towards water organisms is of the greatest concern within the ecotoxicity of surfactants, as the greatest part of surfactants discharged into the environment is discharged into aquatic systems.
Standardized and widely accepted ecotoxicological test to examine toxicity towards aquatic organisms exist and are legally required to be performed and passed to a pre-defined level by the EU if a detergent product containing surfactants is to be sold on the European market. Tests can discern between acute and chronic or subchronic toxicity.
Aquatic toxicity tests are performed according to ISO and OECD standards on[47]:

- Bacteria, representing microorganisms that have to degrade organic matter like surfactants (e.g.ISO 11348-1)

- Algae as plant representatives (e.g. ISO 10712)
- Daphnia, a planktonic crustacean, as a representative for plant feeding animals (e.g. ISO 6341)
- Fish, representing an organism of a higher trophic level (e.g. ISO 10229)

Acute toxicity describes biological effects that occur within a short period of time and/or after short-term exposure. The results of these tests, among others, are the LC_{50}/EC_{50} (median lethal/effect concentration) values. These are statistically derived concentrations of a substance over a defined period of exposure (24, 48 or 96 h). Chronic and subchronic tests, on the other hand, seek to determine the harmfull properties of a substance after a long-term exposure in relation to the tested organisms live span. The results of these tests are the LOEC (lowest observed effect concentration) and NOEC (no observed effect concentration, i.e. the highest concentration that yielded no long-term effect). Knowledge of chronic risks is important to assess environmental risk in natural conditions[47, 60].

1.5.2 Toxicity

The toxicity of surfactants towards humans can be accessed with a multitude of available tests. Toxicity tests are differentiated between local effects, meaning effects directly at the point of contact with the surface of the body (e.g. skin irrition) and systemic effects, meaning effects that can be detected after the substance has entered the organism (e.g. carcinogenity). Generally, the toxicity of a surfactant originates from an interaction with biological structures, such as membranes or enzymes. These interactions can damage cells and inhibit enzymes. Even at low concentrations, surfactants can bind to membranes, altering the permeability. At higher concentration, membrane lysis is possible, a concept that is explicitly employed when investigating cell membranes[29, 65].

Local Toxicity

Determination of local toxicity is important when investigating surfactants, as the main applications of surfactants are in household products such as shampoos, cleaning agents or laundry detergents, where skin or eye contact cannot be avoided or may even be intended. Numerous *in vivo* and *in vitro* tests, precisely adapted to

their respective problems have been developed. For skin tests, two effects are important: the effect of the surfactant on the skin and the ability to penetrate the skin. Generally, skin penetration of surfactants is very low for ionic surfactants and somewhat higher for nonionic surfactants, but still low enough that they are not considered potential hazards. On the other hand, ionic surfactants have shown to be more skin irritant than nonionic surfactants, with sodium dodecyl sulfate (SDS) being a prominent example of a skin irritant surfactant that still sees use. However, for formulations where skin contact is desired, milder alklyl ether sulfates are preferred anionic surfactants[7, 29, 32]. The skin toxicity of surfactants originates from their ability to emulsify lipids, defatting the skin. The removal of this protective layer results in an increased permeability of the skin, a loss of moisture and symptoms like roughness, scaling or inflammatory symptoms. The irritancy potential of surfactants depends strongly on their concentration. Also, a dependance on the length of the alkyl chain was found for anionic surfactants, showing that surfactants with linear, saturated C10 to C12 chains have the highest damage potential[7, 29, 65].
Generally, cationic surfactants show a higher skin toxicity, followed by anionic surfactants and nonionic surfactants. For products marketed as "skin sensitive" zwitterionic surfactants, such as betaines, are often used, as they are known to be very mild[32].

Systemic toxicity

Aside from toxicity of a surfactant upon contact with skin or mucous membranes, it is also important to know toxic effects caused by a compound after it enters the organism, either through oral ingestion or through resorption. In this context, acute toxicity is important in case of poisoning, but as the acute toxicity for commercial surfactants has been shown to be low (in the same order of magnitude as table salt or sodium bicarbonate in fact), chronic toxicity is of larger significance here. Surfactants play a large role in any cleaning process, so the consequences of a life-long exposure of small quantities is of special interest. These consequences may include potential carcinogenity or mutagenity, which is why thorough studies of systemic long-term effects of surfactants are necessary[7, 32].
All important classes of surfactants have in the past been tested for chronic effects. Experiments with animals as well as with humans have been performed where

prolonged oral uptake beneath the lethal dose was examined. Surfactant concentrations of 0.1 to 1.0% in feed did not show any toxic effects[7, 66].
While ready resorbtion of surfactants in the gastrointestinal tracts is known, especially for anionic and nonionic surfactants, the lack of toxic effects, combined with the lack of accumulation of surfactants in the body can be attributed by their rapid metabolism in animals and humans, where the alkyl chains are oxidized. It was also found that metabolites of these processes are eliminated through excretion[29].
All four major surfactant classes were examined thoroughly on their carcinogenity, mutagenity and teratogenity. None of the commercially available surfactants possesses carcinogenic activity, acts teratogenic and exhibits next to no mutagenic activity with only some negligible exceptions being found for certain cell tests[7, 67].

2 Triglycerides

2.1 General Information

The main focus of this thesis is the removal of fat and oil from textile surfaces. Fats and oils are triesters of glycerol (propane-1,2,3-triol), which are referred to as triglycerides. Generally, a fat is a triglyceride that is solid at room temperature, while an oil is a triglyceride that is liquid at room temperature. Figure 2.1 shows the general structure of triglycerides. The three fatty acids, R_{1-3}, can all be the same, but they can also be up to three different ones in naturally occuring fats[68, 69].

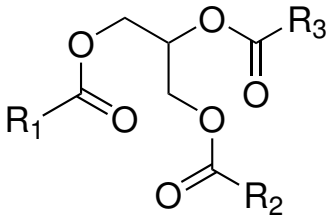


Figure 2.1: Schematic structure of a triglyceride composed of three fatty acids R1, R2 and R3 and glycerol.

Triglycerides are the main components of vegetable oils and fat originating from animals[70, 71]. It has also long been known, that soil originating from humans has a high triglyceride content[72, 73], which is why its removal from textiles (e.g. clothing) is of importance.

Triglycerides are recognized as an essential component in human nutrition, providing and storing energy. They also serve as carrier for oil soluble vitamins[74]. The chain length of the fatty acids varies, in natural fats commonly between C10 and C24, with the majority of fatty acids having alkyl chain lengths of 16, 18 or 20. Natural fatty acids are unbranched and have an even number of carbon atoms. Saturated fatty acids contain no double bonds, while mono- or polyunsaturated fatty acids contain one or several double bonds respectively[75–77]. The physico-chemical properties of a triglyceride are heavily dependant on the composition of its fatty acids. The packing of natural unsaturated fatty acids is unfavoured, since they are usually found in cis configuration. This leads to a decrease of the melting point[68].

A common nomenclature system used as a shortcut for fatty acid nomenclature is Cx:y, with x representing the number of carbon atoms and y the number of double bonds. A common fatty acid used in soaps, lauric acid is written as C12:0 in this nomenclature. Oleic acid, a common fatty acid found in olive oil, as C18:1[78]. Other important natural fatty acids are, among others, palmitic acid (C16:0) and linolenic acid (C18:3). The structures of these fatty acids can be seen in Figure 2.2.

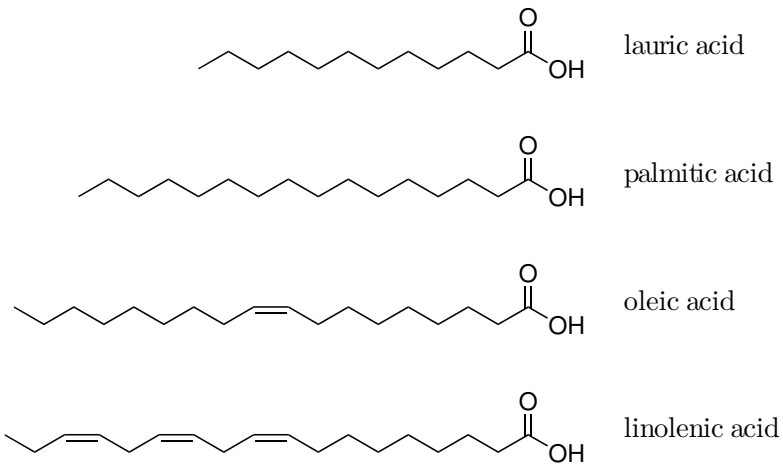


Figure 2.2: Structural formulas of some important natural fatty acids.

Mono- and diglycerides may also occur in natural fats. They are products of incomplete esterification of glycerol. Monoglycerides, which occur at only very low concentrations naturally, can be used as emulsifiers[79]. The emulsification properties of monoglycerides may be of importance in laundry washing, as generating monoglycerides from triglycerides via enzymatic cleaving may help emulsifying fatty soil.

2.2 Crystal structure of triglycerides

In solid state, fats are present in a form of crystal networks, maintaining specific polymorphic crystalline structures[80, 81]. Malkin introduced a terminology for the most common morphologies named α, β' and β in order of increasing melting points[82]. However, Malkins nomenclature gave no information on the crystal structure. A structural identification of the morphologies later characterised the packing of the carbon chains within the subcell as the dominating factor of crystallization. The three corresponding subcell structures (see fig. 2.3 are hexagonal (H) for α, orthorhombic-perpendicular ($O_\perp$) for β' and triclinic-parallel ($T_{//}$) for β[83, 84].

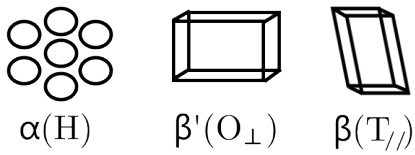


Figure 2.3: Common polymorphic crystal structures of triglyceride subcells. The figure is based on [80]

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The relative stability of the three morphologies is dependant on the three fatty acids of the triglyceride. Kinetically, the α form is favoured, but it is thermodynamically the least stable form. The β form is usually the most stable form for triglycerides, with highly crystalline ordered hydrocarbon chains. If the three alkyl chains are identical or at least very similar, e.g. all chains are saturated and their chain length is within 4 carbon atoms of each other, the stability increases from α, β' to β. However, when the chemical structures of the three fatty acids vary greatly, e.g. through differences in chain length or unsaturation, the β' structure may be favoured over the β form. An example where this behaviour has been observed is 1,3-distearoyl-2-ricinoleyl-glycerol[80, 85, 86].
The chain length structure of triglycerides is also important for the crystal structure. Double-chain length packing occurs for triglycerides with similar fatty acid structures, while triple chain length packing appears for triglycerides with largely different fatty acids. The morphology and the packing severely influences the physicochemical properties of the fat[80]. Figure 2.4 shows the two packing structures.

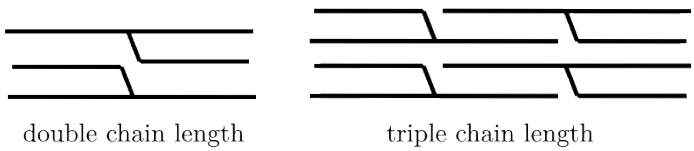

Figure 2.4: Double and triple chain length packing structures of triglycerides. The figure is based on [80]

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3 Laundry detergency processes

3.1 General information

While laundry detergency is an age-old and well-researched process, its complexity allowed for only empirical studies for a long time[87]. Surfactants have always played a major role in detergency processes. For well over a thousand years, soaps were exclusively used for cleaning and have been replaced by synthetic surfactants only after the second world war[7]. Their content in modern detergency formulations ranges from 15 to 40%. The increasing environmental awareness of our time provides a challenge to the 60 billion dollar global detergent market and necessitates a deeper understanding of the underlying processes to further improve washing performance[88]. Developing sustainable, environmentally tolerable detergency products that are highly efficient even at lower concentrations (compared to older detergency products) is highly demanded. Another goal is the reduction of water consumption and electricity. The latter is reached through more efficient washing machines on the one hand, but predominantly by reducing the washing temperature on the other hand, as the washing temperature is the main energy contributor to machine based washing processes[89]. Decreasing the washing temperature form 60 °C to 40 °C decreases the energy consumption by about 40% and decreasing the washing temperature further to 30 °C can save another 30% of electricity[14]. However, decreasing the temperature typically also decreases the washing power, so highly efficient detergents are neeeded.

In modern household laundry, common textile materials are hydrophilic cotton, hydrophobic polyester or blended fabrics of the two. Due to their different properties, effective soil release may not be possible with the same agents. As commercial detergent formulations have to account for all kinds of soil on very different fabrics, they are quite complex[90].

Aside from the already mentioned properties, mechanical properties during the washing process also play an important role as well as the type and age of the soil [87, 90, 91]. Due to the overall complexity of detergency processes, the following section only covers factors relevant for the work performed during this thesis.

3.2 Cotton Fibres

As the nature and properties of materials relevant for the removal of fatty substances is vast, it was decided to choose cotton as a relevant material for laundry processes and focus the experiments on the soil removal from cotton fibres.To gain a better understanding of the relevant processes, it is of avail to closer regard the structure of cotton fabric.
A single hair of cotton consists of several layers. From outside to inside, these are: the cuticle, the primary wall, the winding layer, the secondary wall and the lumen. Figure 3.1 illustrates the structure of a single fibre of cotton. A countless amount of those single fibres form a twine. The layered structure of the fibre results in a number of porous spaces. Therefore, cotton fibres can be regarded as a complex, microscopic sponge-like structure [92]. Due to this structural characteristic, cotton fibres are able to adsorb 32 wt-% of water [93]. This leads to the characteristic swelling of the fibres, which is important for its cleaning properties [94, 95].

This, however, cannot be applied to processed fabrics, where swelling and slippering is commonly reduced, which severly changes washing properties of the fibres [96, 97].

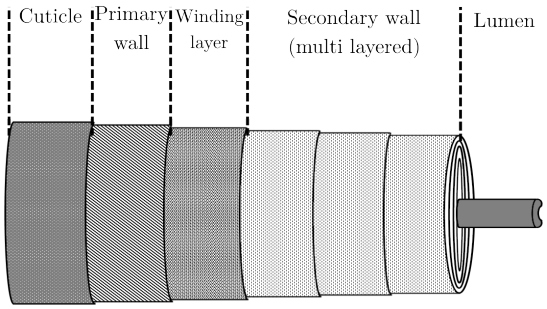

Figure 3.1: Schematic representation of a cotton fibre with its various layers[98].

Untreated cotton fibres consist of the following components [99, 100].

- 80-90% cellulose
- 6-8% water
- 1.0-1.8% ash
- 0.5-1.0% waxes and fats
- 0-0.1% proteins

However, before the fibres are spun into twines, they are treated with sodium hydroxide solution. During this process, the waxes and fats in the fibres are saponified and the pectins and other impurities are removed. In the next step, the fibres are bleached and mercerized. This adds to the luster of the fibres and enhances sorption properties. Finally, the fibres are dried. Processed fibres consist of 99% cellulose [100, 101].

3.3 Soil

3.3.1 Composition of Soil

Soil found on common laundry either originates from the human body or the environment. Soil that originates from humans has a high content of fatty substances. Table 3.1 shows the typical composition of fatty soil secreted from the human body[72, 73, 87, 102].

Table 3.1: Average composition of fatty substances secreted by the human body. Based on [87].

Component	Content (%)
Free fatty acids	22-27
Wax and sterol ester	20-22
Triglyceride	25-35
Diglyceride	6-10
Squalene	10-15
Sterol	2-5
Paraffin	0.5-1.5

Soil from the environment has a lower content of fatty substances compared to soil originating from humans. Lubricating oil or greases from machinery and oils and fat from food or cosmetics provide sources of fat based soil from the environment. Particulate soil from the environment is mainly made up of clay minerals. Table 3.2 shows an average composition of soil originating from the environment[87, 102, 103].

Table 3.2: Average composition of soil from the environment. Based on [87].

Component	Content (%)
Water soluble components	10-15
Ether soluble components	8-12
Moisture	2-5
Organic substances (e.g. fats, fibres, soot, etc.)	20-25
Ash, containing:	50-55
Fe_2O_3	10-12
MgO	1-2
CaO	7-9
SiO_2	23-26

3.3.2 Soiling Mechanism

Soiling in laundry detergency refers to a process that takes place when soil comes into contact with a fabric and is retained on the surface of that fabric. The soil adheres to the surface as a consequence of interactions (e.g. van-der-Waals forces, mechanical forces, electrostatic forces, oil bonding) between the substrate (in this case fabric) and the soil. Soiling either takes place through direct contact with another soiled surface or by contact with a liquid containing soil or by contact with airborne soil. The mechanisms are different for particulate soil and liquid soil and have to be treated separately. Both types can also occur together and while solid particles are more likely to adhere to a surface that is already soiled with a liquid (i.e. oil bonding), the same cannot be said for the opposite situation[87, 90, 104].
A liquid soil can change its shape while interacting with the surface while a solid particulate soil's shape is not prone to variation. Figure 3.2 illustrates the different adsorption to a surface in different systems. A change in the environment has an influence on the geometry of the system with a liquid soil, which indicates changes

in the soil-substrate interaction. In contrast to this, the geometry of the particulate soil-substrate system does barely, if at all, react to changes in the environment[90]. Furthermore, the position of the soil on the fabric has to be considered. Soil can penetrate the inter-fibre and inter-yarn spaces, the irregularities of the fibre surface (especially important for fine soil particles in highly surface textured fibres) and within the crevices and pores of the fabric by mechanical entrapment or occlusion[87, 104, 105].

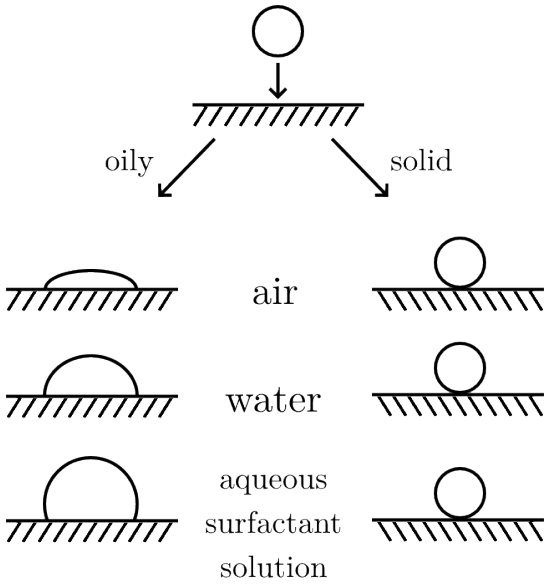

Figure 3.2: Interaction and contact area of a liquid and a particulate soil with a substrate depending on the environment. The figure is based on [90]

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For liquid (oily) soils the phenomenon of wettability or relative surface energy has to be taken into account. If the surface free energy of the soil is lower than that of the fabric, the soil should wet the surface. Additionally, the driving force of the distribution of an oil in a fabric is the net effect of the oil trying to maintain a minimum liquid-air interface and the capillary forces which promote the advance of the liquid into the fabric (see equation 3.1)[87, 106, 107].

$$p = \frac{2\gamma_{SO} cos\theta}{r} \tag{3.1}$$

The capillary pressure p is defined by the surface tension of the oil and the substrate γ_{SO}, the contact angle θ of the fluid on the fabric and r, which is a parameter characterizing the air filled core separating the fibres that depends on the fabrics structure[107, 108]. If θ is smaller than 90°, p will be positive and the liquid soil

will soak into the fabric and wet it[87].
In this case, the substrate is simply treated as a plane as this simplifies the consideration for the oil droplet shape. The contact angle θ between the liquid drop and the fabric is dependant on the different interfacial tensions occurring in the system (see Figure 3.3). The different interfacial tensions are γ_{SO}, between the substrate and the oil, γ_{SE}, between the substrate and the environment and γ_{OE}, between the liquid and the environment[109].

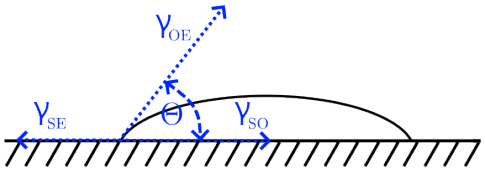

Figure 3.3: Contact angle of a liquid droplet on a plane solid surface showing the forces at the three phase boundary[110, 111].

Young's equation (equation 3.2) relates the surface tensions and gives the contact angle θ[90, 108, 109].

$$cos\theta = \frac{\gamma_{SE} - \gamma_{SO}}{\gamma_{OE}} \tag{3.2}$$

When the environment changes, the interfacial tensions change, which results in a change of the shape of the oily soil droplet on the fabric. This is an important factor in detergency when considering how the soil is released, which will be the consideration of the following section.
In contrast to liquid soil, the shape of particulate soil is mostly independent of the environment. Even if one assumes a partial flattening of the particulate soil where it comes into contact with the surface, this distortion is negligible on a macroscopic scale and the geometry of solid soiling is mostly constant. In a system driven purely by dispersion-type interaction forces, the adhesion of solid soil to a fabric is always weaker than that of a liquid. However, if electrical interactions are present and must be taken into account, the situation changes. The situation of adhesion and release of solid soil on a flat solid substrate resembles that of the coagulation of a colloidal dispersion in that case. Therefore, it can be described via the DLVO theory[87, 90].

3.3.3 Soil release mechanism

A unified mechanism for soil release does not exist, as the possible combinations of substrate and soil with different properties is too vast and the mechanism itself depends on the nature of the substrate and the soil. The focus of this thesis is the release of solid and liquid fat-based soil from cotton fibres. As cotton is porous in its structure, soil will not only be found on the surface. Therefore, water and cleaning agent have to be able to diffuse into the fabric. Works of Miller[109] and Dillan[112] could show, that the release of soil from cotton can be mechanically treated like the release of soil from hard surfaces, which was investigated and described by Bäckström[113]. It was shown that different mechanisms exist for release of liquid (oily) soil and particulate soil[91]. Both of these mechanisms shall be described here. It must be noted however, that in realistic household soil, a mixture of both types of soil is usually present.

Removal mechanism of liquid soil

Erik Kissa found that the washing process for liquid soil can be separated into three steps [91, 114, 115].

1. **Induction period**: Water and cleaning agent diffuse to the soiled surface.
2. **Soil removal period**: Interaction between the agent and the soil. Removable aggregates are formed.
3. **Final period**: Aggregates are transported away from the surface.

In the first period, very little soil is removed. It is dominated by the slow diffusion of the cleaning agent into the fibres' pores. The main removal of contaminations happens in the second phase[109].
The removal of liquid soil can be described via two different models: the "roll-up" mechanism and the "emulsification" mechanism[109]. Figure 3.4 illustrates both mechanisms.
The "roll-up" mechanism is the main mechanism for oily soil removal on hydrophilic fibres such as cotton. It can be explained by looking at Young's equations once again:

$$cos\theta = \frac{\gamma_{SE} - \gamma_{SO}}{\gamma_{OE}} \tag{3.3}$$

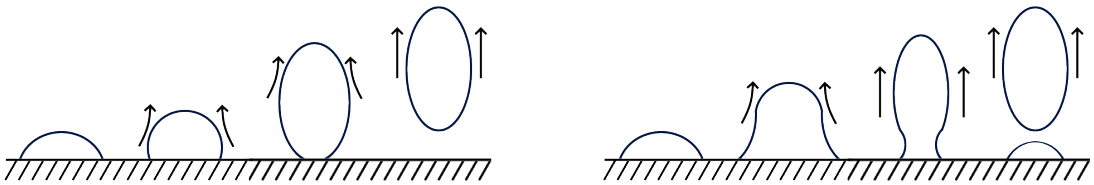

Figure 3.4: Schematic illustration of the "roll-up" and "emulsification" mechanism of liquid soil release. The figure is based on[109].

When the fabric is wetted by the detergent, surfactants adsorb to substrate-environment (SE) and oil-environment (OE) interface. This causes a reduction in the interfacial tensions γ_{SE} and γ_{OE}. This results in an decrease in $cos\theta$ which means that θ increases. If γ_{SE} is reduced enough, $\gamma_{SE} - \gamma_{SO}$ will be negative and θ values will be increased to >90°. A "roll-up" of the liquid soil can then be observed. With increasing surfactant adsorption, the contact angle increases further and if it reaches 180°, the liquid soil drop will be completely discharged from the fabric. At contact angles between 90° and 180°, this spontaneous discharge will not be observed, but agitation and some mechanical input during the process will increase removal. For cotton fibres, the roll-up behaviour is enhanced when compared to flat surfaces with similar hydrophilicity, as cotton fibres will take up water and swell in aqueous solution (see section 3.2), which increases the hydrophilicity of the fabric surface[5, 109, 116].
For hydrophobic textiles, e.g. polyesters, very small initial θ values can be observed, with the fabric surface completely covered in oil. In this case, the surfactant cannot undercut the oil, θ stays below 90 ° even after addition of the detergent and no roll-up takes place. Instead, the emulsification mechanism becomes the most important factor[112, 116, 117]. The low interfacial tension at the oil-detergent interface promotes this process, as it allows for easy deformation of the oil film from which small emulsion droplets are formed[109]. For a complete removal of the oil from the substrate, additional mechanisms such as mechanical work and hydraulic currents in the detergent solution are required[5].
For liquid soils with a high amount of polar groups present, another mechanism has been reported. At the soil-detergent interface, intermediate phases (e.g. liquid crystals) form due to the interaction of the polar components of the soil and the adsorbing surfactants. These intermediate phases are then broken off by agitation during the process and emulsified in the aqueous phase, which allows a new

intermediate layer to form[109, 118].
Finally, it is possible that liquid drops are directly solubilized into the micelles if the surfactant is present above the cmc and if a significant excess of surfactant is present at the oil-detergent interface. This may be the case in systems that form intermediate layers at the soil-detergent interphase[109, 119].

Regardless of the mechanism, soil removal can be improved by agitation and increased temperature. Agitation of the system enhances the transport of surfactant to the surface and therefore increases adsorption, which accelerates roll-up and other removal processes. After the soil is released, agitation can increase the transport rate of emulsified soil away from the substrate. That increasing temperature increases detergency performance is widely accepted. Specific effects of increased temperature are for example melting of solid soil, which is then accessible for the "roll-up" mechanism and a decrease in viscosity for liquid soils, which increases deformability of the interface and thus enhances removal processes. Furthermore, diffusion is increased at higher temperatures, which means the diffusion of surfactants towards the soil and the diffusion of emulsified soil away from the fabric is increased[91, 113].

Removal mechanism of solid soil

The removal of solid soil (e.g. crystallized organic compounds or inorganic materials) is mainly driven by the reduction of the adhesion forces between the soil and the substrate. The attractive forces, mainly van der Waals forces, have to be overcome in order to facilitate removal. Additionally, solid soil removal is often greatly dependant on mechanical action during a detergency process. Other than mechanical input, solid soil removal depends on the following mechanisms.
Wetting of the substrate and soil. Initially, the wetting of both the fabric and the soil reduces attractive van der Waals forces. The reduced net adhesion results from a formation of electrical double layers at the fabric/water and soil/water interfaces. These double layers usually have the same sign on both the fabric and the soil which leads to a repulsion[5].
The adsorption of surfactant molecules at the fabric/water and soil/water interfaces. For anionic surfactants, the adsorption leads to an increased surface charge on both the fabric and the soil, resulting in increased repulsion. Furthermore, the surfactant layers on both soil and fabric advance to the point where the soil and the

fabric are in contact (see figure 3.5). This leads to the development of a disjoining pressure which reduces the adhesion between the soil and the surface. As nonionic surfactants generally do not influence the surface charge and therefore do not generate electrostatic repulsive forces, this disjoining pressure is the decisive factor for them[5, 7].

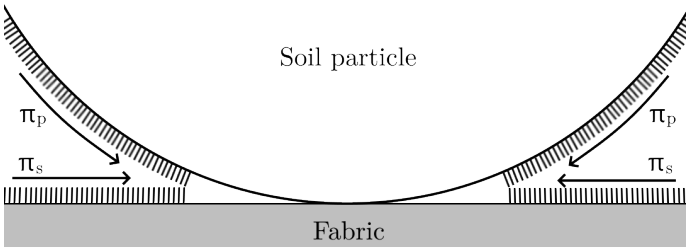

Figure 3.5: Schematic illustration of reduced adhesion forces between soil and fabric induced by adsorption of the surfactant. π_p is the splitting pressure of the surfactant layer on the soil particle, π_s is the splitting pressure of the surfactant layer on the fabric surface. The figure is based on[7].

A theoretical background for the repulsion forces can be approached via the DLVO theory [120]. This theory was developed to explain coagulation of colloidal particles, but it can be applied to solid soil adhesion to a fabric by making some assumptions. The fabric is considered a particle of infinite size and since solid soil is not homogenous, the process is a heterocoagulation[7, 90, 121].
In the DLVO theory, the total potential $V(tot)$ of the interaction is calculated from the (usually) attractive van der Waals forces $V(vdW)$ and the (usually) repulsive forces of the electrical double layer $V(el)$. It is assumed that both contributions are additive (equation 3.4).

$$V(tot) = V(el) + V(vdW) \tag{3.4}$$

This approach does not include other types of interaction, e.g. steric interactions of surfactants adsorbed at the interfaces. Furthermore, for soil adsorption, $V(el)$ does not need to be repulsive, as double layers of unequal charge density may be present. Also, the van der Waals forces, which are normally attractive, can be repulsive for dissimilar particles in a third medium[90, 122].

3.4 Commercial laundry detergents

3.4.1 Composition of laundry detergents

Laundry detergency, in the broadest sense, is the removal of undesirable components from textiles by an aqueous surfactant solution. It is a complex process that involves multiple interactions between the detergent solution, the soil and the fabric, which were examined in the previous section. Surfactants play a key part in any form of detergency, due to their amphiphilicity. Their ability to change interfacial properties by adsorbing to an interface is a key requirement of any cleaning process[7].
A plethora of laundry detergent products is available on the market. About half of the global surfactant consumption is made up by domestic cleaning products. Laundry detergents contribute the largest portion of this half. Most modern laundry detergents are highly complex formulations, consisting of more than 20 components, each serving a specific function[7, 47]. The main components of household laundry detergents are[6]:

- Surfactants
- Builders, such as zeolithes, to suppress calcium deposition on textiles
- Enzymes, which facilitate the removal of large and insoluble organic compounds
- Dye transfer inhibitors, which suppress deposition of dissolved pigments
- Optical brighteners, which make fabrics appear brighter
- Bleaching agents and activators, which remove organic stains by oxidation
- Fragrances, which serve only cosmetical purposes
- Filling materials, to simplify dosing and enhance pourability

In commercial laundry detergents, mixtures of nonionic and anionic surfactants are used to achieve a high washing efficiency. Commonly, in modern products, linear alcohol ethoxylates, linear alkyl (ether) sulfates and linear alkyl benzene sulfonates are combined. Nonlinear surfactants, due to their poor biodegradability do not see significant use. Cationic surfactants are used mainly in fabric softeners, as they do not exhibit significant cleaning performance, but instead their impact on the fibre surface makes the fabric appear softer to the touch[5, 7, 47].

3.4.2 Enzymes in laundry detergents

General Information

The use of enzymes in laundry detergents dates back over 100 years. In 1914, german scientist Otto Röhm filed the first patent for a detergent containing enzymes[19]. He used the protease trypsin, extracted from animal pancreases. Large-scale microbial production of enzymes began in the 1960s. However, for decades, enzymes in detergents were limited to proteases and amylases. Only with the introduction of "Lipolase" in 1988 by Novo Industri, fat dissolving enzymes became available for laundry detergents. Previous lipases were inactivated by the high pH conditions of laundry washing processes[20–22]. Lipolase is still used to this day and was also used in this thesis.
Today, industrially produced enzymes are a multi-billion dollar market. The second largest share of this market, with about one quarter, is made up by enzymes for detergents, with the only larger sector being enzymes for medicinal purposes. Enzymes are also used in the food industry and in textile production. The addition of enzymes to laundry detergents led to a significant increase in the efficiency of washing processes. They make washing at lower temperatures feasible, and remove the need for washing at temperatures of more than 80 °C. This does not only reduce wear of clothes that were continuously hot washed, it also severely reduces energy consumption of washing processes. Furthermore,since enzymes are proteins, they are completely biodegradable and do not leave toxic degradation products that accumulate in the environment.[22, 123].

Enzymes are a very complex topic that will be only presented briefly here. Knowledge of enzymes and their function dates back to studies from the late 19th century. Generally, enzymes are proteins that catalyse specific reactions by reducing the activation energy of that reaction[124, 125]. In 1894, Fischer proposed the "lock and key" model, explaining the specific reactions of enzymes with a single substrate[126]. This principle is still taught as a fundamental basis of enzymes. This model, however, suggests, that the active site of an enzyme is rigid, fitting the substrate exactly. This is not quite true, as enzymes are flexible structures. The substrate binding to the enzyme causes a change in shape. To explain this, Koshland proposed the "induced fit" model, which explains the stabilization of the transition state of an enzyme during the reaction it catalyses[127].

The name of an enzyme is often derived from the substrate it reacts with or the reaction it catalyses, with the suffix -ase added. A unified nomenclature for enzymes was developed by the International Union of Biochemistry and Molecular Biology. Each enzyme is described by an "Enzyme Commission number" or EC-number. The first level of this system categorizes enzymes based on their mechanism. These categories are[124, 125, 128]:

1. Oxidoreductases, which catalyze oxidation/reduction reactions
2. Transferases, which transfer functional groups
3. Hydrolases, which catalyze hydrolization of various bonds (e.g. lipases)
4. Lyases, which cleave bonds by other reaction types than oxidation or hydrolysis
5. Isomerases, which catalyze isomerization changes in the substrate
6. Ligases, which covalently join two molecules

A set of four numbers separated by periods is required to fully specify an enzymatic reactions. Within the EC number system, different enzymes catalysing the same reaction are given the same set of numbers. The sections two through four of the EC system are necessary to specify an enzymatic reaction precisely (e.g. mechansim, their substrate and the products)[128].

Lipolase

The lipase used for the experiments within this thesis is the previously mentioned Lipolase. It is produced biochemically by a modified aspergillus mold, to which the gene expressing the lipase has been transferred[21, 129]. It was the first time a lipase suitable for the high pH environment of laundry detergency was successfully produced at a commercial level. Before the introduction of Lipolase, no enzymes that hydrolyse fats were available for laundry detergents and generally fats had to be removed by washing at sufficiently high temperatures causing them to melt, as the surfactants in the detergent do not solubilize solid fats[91, 113]. Lipases do not require a cofactor and generally have a high catalytic activity, so industrial and commercial application for them is of interest[130–132].
The lipase commonly referred to as Lipolase is a lipase produced from Asperigillus

oryzae. Lipases are triacylglycerol acylhydrolases and are classified in the EC-system under the EC-number 3.1.1.3[133]. Enzymes with this EC-number catalyse the hydrolisis of ester bonds in triacylglycerols, preferably the ester bonds on the outer positions of the triacylglycerol. Generally, the reaction takes place at the water/oil interphase, due to the poor solubility of long chain triglycerides in water on the one hand, and structural properties of the lipase on the other[131, 134]. An important structural aspect that lipases share is the "hydrophilic lid" in front of the active site within the binding pocket. The lid is built of an α-helix structure that has hinge-like segments on its edges. The outer surface of the lid is hydrophilic, while the inner surface that points towards the active site is hydrophobic. Due to this structural peculiarity, the lipase is only active at an interface with an hydrophobic substrate. In the aqueous phase, the lid remains closed, hiding the active site, effectively deactivating the lipase[132, 135–137]. At an interface with a hydrophobic compound (e.g. a triacylglyceride), the enzyme undergoes a structural change. The lid opens and the lipase becomes active. Only when the lid is open, a substrate can enter the binding pocket and the hydrolysis reaction takes place. This lid is crucial for the lipase's selectivity and activity[131, 132, 138–140].
As a part of this thesis was to find a way to increase the activity of the lipase, finding a way to facilitate the opening of the lid might lead to a beneficial effect.

4 Characterization methods

4.1 Colorimetric analysis

To evaluate the washing performance of detergent solutions, a colorimetric approach was chosen. The method itself, which will be explained closer in the experimentals section, is based on the method established by Dengler[23].
When light interacts with an object, it is either transmitted, absorbed or reflected. For the colour of an object that a spectator will observe, only the latter is relevant. The human eye perceives colour via three cone-shaped receptors, which are sensitive to blue, red and green light, respectively[141]. Human perception is highly subjective though, and a precise mathematical description for colour and instrumentation to quantify colour was needed[142]. Colour control was standardized by the Comission Internationale de l'Eclairage (CIE), and their CIELab system is still widely used today. Initially, the XYZ colour space was introduced, which aimed to have a method of colour analysis that was analogous to human colour perception. Each of the three coordinates represents one of the cone-shaped receptors. Tristimulus photometers, which utilize this principle by using a red, green and blue filter, through which the light reflected off a sample passes, have been built. They gave X, Y and Z values of the reflected light of a sample as results. They have since been replaced, as the XYZ colour space proved to be not uniform and thus incapable of determining colour differences. The L*a*b* colour space was introduced by Hunter, which was later refined by the CIE to form the CIELab colour space, which is the standard used today for colour evaluation in textiles[143, 144]. The L-axis is called lightness and gives a value from 0 for black to 100 for white. The a-axis is called the red-green axis. It shows positive values for red and negative values for green. The b-axis is the blue-yellow axis and results in positive values for yellow and negative ones for blue[143]. Figure 4.1 illustrates the CIELab color space viewed from the blue side of the b-axis.

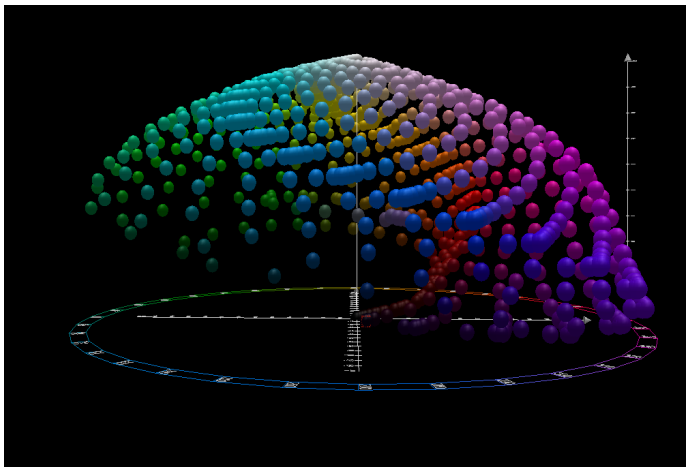

Figure 4.1: The CIELAB colour space viewed across the blue-yellow axis. The Y-axis represents the L* values increasing from bottom to top, the chromatic circle on the bottom shows the highest chromatic values of the a*/b* plane. This picture is published by H. Everding under the CC BY-SA 4.0 license[145].

Within the CIELab system, all colours can be plotted in a three dimensional space. The L*, a* and b* values are calculated via a nonlinear transformation of the measured XYZ values in reference to a white point n with the values $X_nY_nZ_n$ (equations 4.1-4.6)[144].

$$X_1 = \frac{X}{X_n} \tag{4.1}$$

$$Y_1 = \frac{Y}{Y_n} \tag{4.2}$$

$$Z_1 = \frac{Z}{Z_n} \tag{4.3}$$

$$L^* = 116Y_1 - 16 \tag{4.4}$$

$$a^* = 500(X_1 - Y_1) \tag{4.5}$$

$$b^* = 200(Y_1 - Z_1) \tag{4.6}$$

For the tests performed in this thesis, the exact colour of the textiles was not the factor of interest. Instead, the change in colour of the textile in the course of the performed washing tests was investigated. The value used here was the total change

in colour ΔE^*. It was calculated by using the changes of the L*, a* and b* values (equation 4.7)[143, 144, 146]. The ΔE value does have its limits, as equal values can be obtained from different colours. As only a single dye was used for all tests here, the initial colour of all tested textiles was within a rather small margin. Therefore, this drawback can be ignored here.

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \tag{4.7}$$

4.2 Chromatographic methods

4.2.1 General information

On its own, chromatography is not a "characterization method" like this section's name would suggest. Rather, it is a method to separate compounds of a mixture to enable subsequent qualification and quantification. It must therefore be coupled with a suitable detection method.
The term chromatography can literally be translated to "colour writing", as it is derived from the greek words "chroma", meaning "colour" and "graphein", which means "to write". It is mostly accepted that chromatopgraphy was discovered by Tswett in 1906, when he separated chlorophyll and xanthophyll with a glass column filled with calcium carbonate. He observed different coloured sections within the column, which caused the coining of the term chromatography[147, 148].
The basic principle of chromatography is the separation of the components of a mixture through their distribution in two immiscible phases. Generally, one of these phases is immobile (the stationary phase, e.g. a porous solid or a liquid film) while the other one, the mobile phase, which can be liquid or gaseous is moving through the stationary phase, carrying the component mixture. Separation of the mixture occurs from repeated sorption/desorption processes of the components during the movement along the stationary phase in the direction of the mobile phase flow. A dissimilarity of those sorption/desorption processes between the mixture components leads to a separation. Chromatography can be divided into three main categories, depending on the aggregate state of the mobile phase: gas chromatography (GC), liquid chromatography (LC), and supercritical fluid chromatography (SFC)[148].

The separation of the compounds in chromatography is based on different mechanisms.

In GC, if the stationary phase is a liquid, which is the more common arrangement in GC, separation occurs because of interfacial adsorption and differences in the gas-liquid partitioning of the components. If the stationary phase is solid, interfacial adsorption or size exclusion govern the separation, if a solid with controlled pore size is used. If a supercritical fluid is used as the mobile phase, the stationary phase ca be a liquid or a solid. Separation is governed by interfacial adsorption and absorption processes. In case of a liquid mobile phase, the stationary phase is usually a solid. The mechanisms governing separation can be based on interfacial adsoprtion, size exclusion for solids with defined pore sizes or on electrostatic interactions due to the charge of an analyte. For adsorption, absorption or partition effects, the velocity with which a compound is transported through a column is mainly dependant on the affinity of a substance to the stationary phase and the ability of the mobile phase to resorb the analyte from the stationary phase[148, 149].

As mentioned, analysis of a chromatographic separation requires the coupling of a sufficient detector to the column. The plot of the results this detector records is referred to as a chromatogram. Signal intensity of the detector (e.g. UV/VIS, refractive index) is plotted against the time from the injection of the analyte into the column until the signal appeared in the detector. The time and analyte needs to pass through the column and into the detector is called the retention time t_r. The minimum time a compound needs to pass through the column is called permeation time t_m. A compound eluting at t_m is not retained at all by the stationary phase and its flow rate is equal to the flow of the mobile phase. The retention time of a compound is the main factor that allows analysis of compounds qualitatively. It is achieved by injection and elution of reference standards under the same conditions. Compounds can also be analysed quantitatively, since the area under the signal is proportional to the amount of substance that was detected. To achieve this, a suitable calibration with reference standards of known concentrations is necessary[148, 149].

4.2.2 The Van Deemter equation

The separation of mixtures in chromatography is based on a transition of the compounds between the mobile phase and the stationary phase. The transitions establish distribution equilibria of the compounds between the two phases. However, due to a constant movement of the mobile phase, this equilibrium is continuously disturbed.

Thus, the separation of mixtures in a chromatography column can be divided in a series of theoretical separation stages, similar to the concept of theoretical plates originating from distillation columns. This concept is commonly used to explain chromatographic separation processes[148, 150, 151].
As the separation is described by a series of adsorption and distribution processes at each theoretical plate, the efficiency of the separation is greater, the more of these theoretical plates are present in the columns. Factors like the length of the column and the surface properties, particle size and packing density of the stationary phase influence the separation efficiency. Van Deemter, Zuiderweg and Klinkenberg explained how the height of the theoretical plates (and therefore its number in a column with given length) is dependant on the linear velocity of the mobile phase. They made out three terms which influence the height of a theoretical plate H. These are combined in the Van Deemter equation (figure 4.2), which can be used to calculate the minimum height H which therefore leads to the best possible separation in a given system[148, 152].

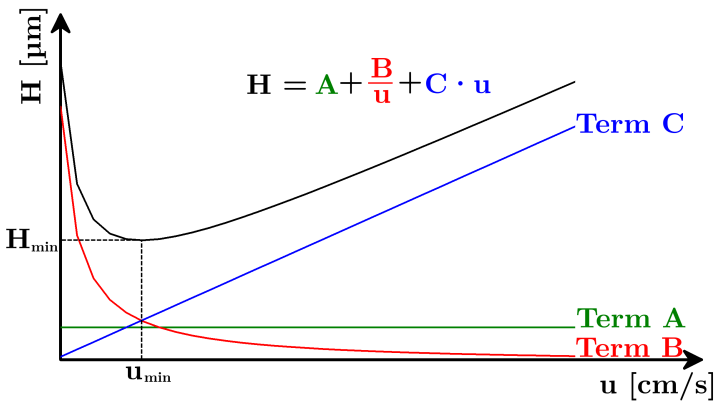

Figure 4.2: Plot of the van Deemter equation calculating the theoretical plate height as a function of the mobile-phase velocity. The green, red and blue curve show the plots of the three terms making up the van Deemter equation[148, 152].

The A term represents eddy diffusion or turbulent diffusion. It is independent of the mobile phase flow rate and therefore a constant value for a given column. It is caused by no-ideal packing in the column, leading to flow irregularities and a statistical distribution of the distance the mobile phase (and the compounds it carries) has to travel through the column. The B term represents longitudinal molecular diffusion. Analytes, while being generally moved in the direction of the mobile phase flow, can experience motion in all directions by means of diffusion. The diffusion

coefficient of the mobile phase greatly influences the B-term. The term is reciprocally proportional to the linear velocity of the mobile phase, meaning its influence gets smaller the larger the flow rate is. The C term represents resistance to mass transfer. It increases linearly with the flow rate. The mass transfer of the analyte is dependant on the adsorption and desorption processes occurring in the column[148, 152].
In a chromatographic separation, it is desired to reach H_{min}, as this gives the best possible separation in a given system. Ideally, the analysis time of the process should also be as short as possible, which is why the flow rate at which H_{min} is reached should be as high as possible. This can be achieved, for example by a small particle size (in LC) or low film thickness (in GC) of the stationary phase, which influences the terms A and C. A homogenous distribution of the particle size and packing density of the stationary phase leads to a preferable B term. Furthermore, longitudinal diffusion can be hindered with a smaller column diameter, which influences the terms A and B. Optimizing these parameters leads to a more efficient and faster separation of the compounds. It must also be noted that the sorption processes occuring in the column are temperature dependant. Increasing the column temperature decreases adsorption to the stationary phase[148].

4.2.3 Gas chromatography (GC)

In GC, a gas, or a mixture of gases is used as the mobile phase, thus the name gas chromatography. Due to this, any sample analysed with this method needs to be vaporized, which limits this method to analytes that are not only thermally stable to temperatures up to 300 °C or more, but also sufficiently volatile. However, derivatization methods, like alkylation or silylation, exist that can convert unsuitable compounds into derivatives which can be analysed via GC. The stationary phase in GC is usually an immobilized liquid film or solid particles[148].
Figure 4.3 shows a simplified scheme of a typical GC system. The carrier gas (commonly helium and nitrogen) is connected to the column via a flow controller, which provides a constant stream of carrier gas (the mobile phase). The sample is introduced into the system via an injector, commonly an autosampler. The injection can either be performed via a split/splitless injector or on-column. If a split/splitless injector is used, the sample is injected into an evaporation chamber. In split mode, a part of the vaporized sample is guided to the column, which is useful for a high sample concentration. In splitless mode, the whole sample is introduced into the

column, which is useful for trace analysis. If on-column injection is used, the whole sample directly injected into the column in liquid state. It evaporates in the heated column. This method is useful for thermally labile compounds[148, 149].
At the end of the column, the outlet is connected to a detector, which generates an electric signal that is evaluated at a connected computer to create the final chromatogram. The injector, column oven and detector are temperature controlled and temperatures can be adjusted during the separation to increase selectivity. Usually, only the oven temperature is adjusted during a measurement while the injector and detector temperatures remain constant. As mentioned before, increasing the temperature reduces adsorption, leading to shorter elution times[148, 149].

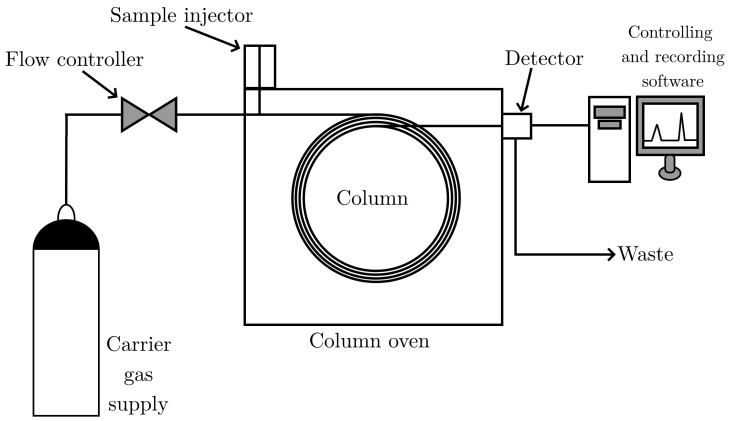

Figure 4.3: A simplified scheme of a GC system.

Columns

Two types of columns can be differentiated in GC chromatography. The first one are packed columns, which are usually made of glass or metal and filled with tightly packed solid particles (e.g. silica gel, activated carbon, aluminium oxide, polymers). The particles may also be coated with a liquid film. The inner diameter of theses columns typically ranges from 2 to 4 mm and their length is in practice limited to 5 m, due to the packing's resistance to gas flow. Longer packed columns require extreme gas pressure, which has proven impractical in real life. Packed columns can deliver up to 15'000 theoretical plates. Due to their limited separation efficiency, their usage is declining and today, capillary columns make up the vast majority of GC columns[148, 149].
Capillary columns are open-tubular columns. They are made of fused silica and the stationary phase is an immobilized liquid film on the inside of the tube. Their inner

diameter is typically around 0.25 mm and their length ranges from 10 to 60 m. The number of theoretical plates possible with this type of column ranges from 150'000 to 500'000. As cross-linked, surface-bonded polysiloxane based stationary phases enabled thicker and more thermally stable coatings, these columns started to replace packed columns due to their far higher separation efficiency. By today, they are far more common than packed columns. To adjust the polarity of the stationary phase, various sidegroups can be employed with polysiloxane. Dimethylsiloxane coatings are used if very nonpolar stationary phases are desired. The addition of phenyl groups can slightly increase polarity. A common non-polar column is the HP-5 column, which contains 5% phenyl- and 95% methyl-siloxane. Polyethylene glycol offers high polarity, but is very sensitive to oxygen and the thermal stability is low. The thickness of the stationary phase film influences the properties as well. A thicker film increases the capacity of the column, but decreases the separation efficiency. Film thicknesses range from below 1 μm up to several μm[148, 149].

Detectors

The detector which was used for GC experiments in this thesis is the flame ionization detector (FID). It is the most common detector in GC. It offers high sensitivity and a large linear range and a signal that is directly proportional to the amount of substance. It is however, a destructive method and cannot detect compounds like water, nitrogen or carbon dioxide. The detection is based on ions formed during combustion of organic compounds in a hydrogen flame. The anode is placed at the nozzle of the detector and the collector cathode is positioned above the flame. Radicals generated during combustion of compounds eluting to the detector break down to ions and generate a current between the electrodes. This current is the detected signal[149, 153].
Other detectors found in GC chromatography are thermal conductivity detectors (TCD), mass spectrometry detectors (MSD), flame photometric detectors (FPD), nitrogen-phosphorous detectors (NPD), electron capture detectors (ECD) and atomic emission detectors (AED)[148, 149, 153]. As they were not employed during this thesis, they are not further explained here.

4.2.4 High performance liquid chromatography (HPLC)

High-pressure or high-performance liquid chromatography (HPLC) is a chromatographic separation method that uses organic solvents as mobile phase and tightly packed solid particles as stationary phase. The high packing density of the stationary phase within the column leads to high pressures during separation (up to 400 bar). This high pressure combined with the tightly packed stationary phase with well defined particle and pore size as well as surface properties leads to a very efficient separation when compared with other LC methods. HPLC can be used to separate compounds which are thermally unstable or not volatile. The only requirement for a sample is sufficient solubility in an adequate solvent or solvent mixture that can be used as mobile phase.[148].
Figure 4.4 shows a simplified scheme of an HPLC system. The organic solvent(s) used as mobile phase are transported through a degasser to remove residual gases that could cause issues if they were to be pumped into the column. The solvents are then mixed unpressurised and further transported to the solvent pump. It is possible to use several pumps (e.g. one per solvent). The mixture is then pumped to the sampling loop commonly equipped with an autosampler to ensure safe and reproducible sample injection. The mixture now dissolved in the mobile phase is then transported to the column. The column oven ensures that the temperature remains constant throughout the separation. After the column, the separated compounds reach the detector, that generates a signal which is sent to a computer where a suitable software creates the chromatogram[148, 149, 154].

Columns

The columns used for HPLC chromatography are distinguished by the properties of the stationary phase used for a particular column. Two general types of stationary phases can be differentiated. In normal phase HPLC (NP-HPLC), uncoated polar particles like silica gel or aluminium oxide are used as stationary phase. In this case, the mobile phase must be a non-polar organic liquid. Normal phase columns are best suited to separate non-polar and non-ionic compounds, which would exhibit very high retention times in columns with non-polar stationary phases. Higher polarity of a substance increases the retention time in NP-HPLC. Today, this type of HPLC is rarely employed and sees use only in special cases where a non-polar stationary phase is not feasible[148, 154].

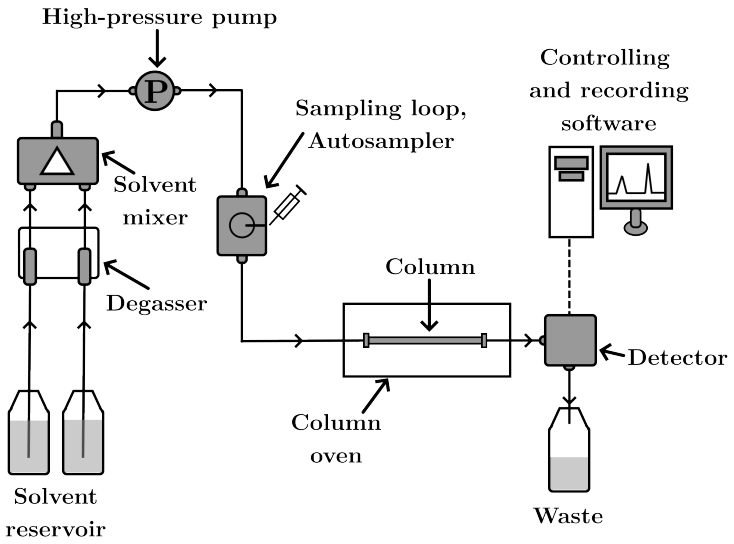

Figure 4.4: A simplified scheme of a HPLC system.

The more commonly found columns are those for reverse phase HPLC (RP-HPLC). The name is derived from the fact the the polarity of the stationary phase and mobile phase is inverted to "regular" LC methods. This method also uses silica gel particles as the stationary phase. To achieve a non-polar surface, the silanol groups are chemically modified, usually with linear alkyl chains. Common modifications include a linear octyl cahin (RP-8) or linear octadecyl chains (RP-18). Mixtures of short and long chain alkyl endgroups can also be used to achieve specific properties and to ensure that no residue unmodified silanol groups remain. The technique of modifying the silanol groups is called "end-capping". RP-HPLC sees far more use than NP-HPLC and is used for the separation of compounds in chemical, biochemical, pharmaceutical and medical fields. Its popularity stems in part from the fact that aqueous samples can be used without pretreatment, making this method especially suitable for biogenic compounds. The expected retention times are inverted to NP-HPLC, with a decreasing polarity increasing the retention time of a compound[149, 154].

The sizes of the spherical particles used in HPLC range from 2 to 15 μm. Commonly, a small distribution of the particle size is desired. The smaller the particles, the higher the separation efficiency but also the required pressure. Solid particles with mean diameters smaller than 2 μm exist, but they require pressures of over 1'000 bar. However, instrumentation that can achieve such pressures leads to highly efficient separation with lower analysis times[148, 154, 155].

Mobile phases

In HPLC, the mobile phase needs to be chosen carefully. It can be a pure solvent or a mixture of solvents. Also, the composition of the mobile phase can be changed during the separation process. This method is called gradient elution. Due to the change in eluting power of the mobile phase during separation, gradient elution can be applied to reduce the analysis time of strongly retarding substances. Isocratic elution, on the other hand is a method where the composition of the mobile phase remains constant throughout the analysis. The selection of suitable solvents depends on the stationary phase (NP or RP) but also very much on the mixture that is to be eluted and analysed[148, 154].
A way to evaluate solvents for chromatography is by "eluting power". It is a measure of a solvents ability to elute the analyte in a given column. Naturally, the eluting power is dependant on the stationary phase. In NP-HPLC the eluent strength increases with increasing polarity of the solvent, in RP-HPLC, where the stationary phase is non-polar, the series is inverted[148, 156].

Detectors

The detector most commonly used with HPLC analysis is the photometric UV/VIS detector. Lambert-Beer's law (equation 4.8) is used to correlate the absorbance of the sample within the flow cell with its concentration. Due to the previous separation, compounds of the mixture can be measured individually within the detector.

$$A = \varepsilon l c \tag{4.8}$$

In this equation, A is the absorbance of the sample, l is the path length through the sample, ε is the molar absorptivity at the measured wavelength and c is the sample concentration. Modern diode array detectors can record wavelengths from 190 to 950 nm[148, 149, 157].
Other detectors used in HPLC are fluorescence detectors, mass spectrometers (HPLC-MS), refractive index detectors, conductivity and electrochemical detectors[157].

4.3 Langmuir-Blodgett Trough

The Langmuir-Blodgett (LB) trough is a laboratory apparatus used to prepare monolayers of amphiphilic molecules on a liquid surface. These monolayers can then be compressed and expanded and their properties examined. Coupled with a Wilhelmy plate, the monolayers effect on the surface pressure as well as the monolayers compression behaviour can be examined. Furhtermore, it is possible to coat a solid substrate with one or multiple monolayers by immersing the substrate, compressing the Langmuir film on the trough to the desired density, an then carefully removing the substrate[26, 158, 159].
A Langmuir-Blodgett trough (figure 4.5) like the one used for this thesis consists of a thermostated trough made from polytetrafluoroethylene (PTFE). The trough is commonly wide and long but less than a centimeter deep to ensure a large surface compared to the required volume of subphase. Usually, water is used as subphase. On one end of the trough, a barrier rests on the surface. This barrier is connected to a mechanism that can slide it at a constant speed parallel to the trough towards the Wilhelmy plate, compressing a prepared monolayer. The Wilhelmy plate is a partially immersed thin plate (made of cellulose or platinum) that is used to measure the interfacial tension on the troughs surface. It is connected to an electrobalance. This way the surface pressure can be accessed experimentally. A setup like this is referred to as a Langmuir balance. By constantly measuring the surface pressure while the monolayer is compressed via the barrier, the surface pressure - molecular area isotherm can be recorded. This way, the properties of the monolayer can be evaluated[26, 159, 160].

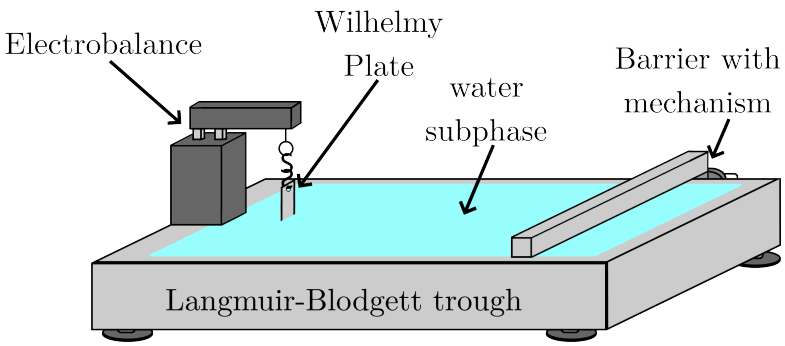

Figure 4.5: A schematic representation of a LB trough with a Wilhelmy plate attached to an electrobalance (the Langmuir balance setup).

Film formation

Langmuir films are assemblies of amphiphilic molecules on the surface of subphase, which is usually water. The LB trough is designed to create and influence such films. As the barrier touches the surface, moving it compresses the molecules on the water surface. The resulting increase in surface pressure can be measured with a Wilhelmy plate attached to a electrobalance. To create such a film the amphiphile has to be dissolved in a volatile solvent that does not dissolve in water (e.g. chloroform). Concentrations of about 1 mg/mL are sufficient. About 20-100 µL of the solution are then carefully spread on the surface with a micro syringe. This way, the solution will spread on the surface and the solvent will evaporate. This leads to a low density film of the amphiphilic compound on the water surface. Due to the low density of molecules on the surface, they can freely move on the water an behave similar to a two dimensional gas. This situation is therefore termed a 2D-gaseous phase. The surface pressure is low, each molecule occupies a large molecular area and the surface pressure rises only slowly upon compression. In the surface pressure - molecular area isotherm in figure 4.6 the gaseous like phase is the part of the curve under A. Upon compression, the molecular surface area decreases. A change in compressibility signifies the 2D-phase transition into the two dimensional liquid-like phase. The surface pressure begins to rise steeper as the area per molecule decreases. In the example in figure 4.6, this is the part of the isotherm under B. As the barrier is closed further and all the fatty acid chains begin to align, the transition to the nearly incompressible solid-like phase is seen. In figure 4.6, this is the part of the isotherm under C. Here, a dense monolayer is formed and the molecules begin to form 2-D lattice structures. This highly organized film with two dimensional crystalline structures can be visualized with a Brewster angle microscope. If coating of a substrate is desired, the monolayer should not be further compressed, as further compression will lead to a collapse of the monolayer, noticeable by a sudden decrease in surface pressure (the part of the curve under D in figure 4.6)[160–162].

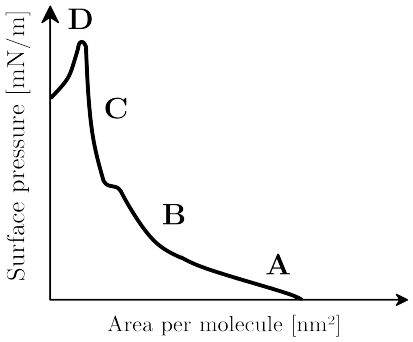

Figure 4.6: A schematic representation of a surface pressure - molecular area isotherm. A: gaseous phase. B: liquid phase. C: solid phase. D: film collapse.

Wilhelmy method

The Wilhelmy method, named after its inventor Ludwig Wilhelmy, is a method to determine the surface tension between a liquid-air interface or a liquid-liquid interface[163]. It is a very common method to measure interfacial tensions. For Langmuir balance measurements, a plate made from cellulose is commonly used. It is suspended from an electrobalance and partially submerged in the liquid. The plate is wetted by the liquid which exerts a downwards force on the plate. That downward force is measured by the balance. By using a cellulose plate, complete wetting of the plate can be ensured[159, 162, 164]. Figure 4.7 shows the set-up of the method.

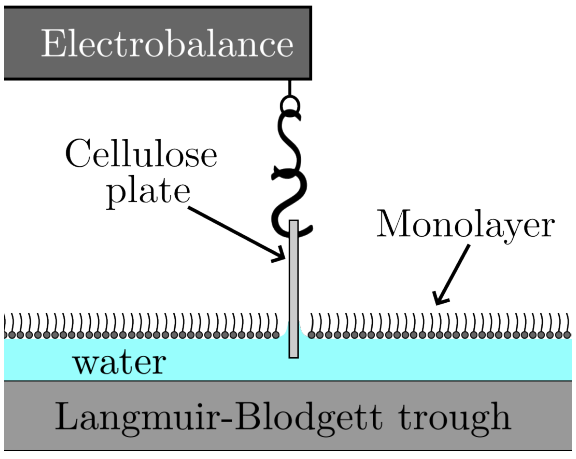

Figure 4.7: The Wilhelmy plate method used to record surface pressure - molecular area isotherms.

The downward force exerted on the partly immersed plate, which is measured by the connected balance, is equal to the weight of the plate (first bracket in equ. 4.9) and the force resulting from the surface tension of the liquid (second bracket in equ. 4.9), minus the buoyancy of the plate in the liquid (third bracket in equ. 4.9)[164, 165].

$$F = [m_p g] + [2(t_p + w_p)\gamma_l cos(\Theta)] - [\rho_l V_p g] \tag{4.9}$$

with

- m_p: mass of the plate
- g: gravitational acceleration
- t_p: thickness of the plate
- w_p: width of the plate
- γ_l: surface tension of the liquid
- Θ: contact angle of the liquid on the plate
- $\rho_l V$: density of the liquid
- V_p: Volume of the submersed part of the plate

This method can be used to obtain the surface tension of various liquids. It is also possible to measure advancing and receding contact angles with the Wilhelmy plate method by moving the plate upwards and downwards in a controlled manner[163, 165].
By using a cellulose plate, which soaks with water, perfect wetting can be ensured, leading to a contact angle of 0°and therefore a cos(Θ) of 1. During the compression, the plate is not moved, leading to a constant buoyancy. As the weight of the plate is also constant, both of these factors can be eliminated by zeroing the balance before the compression. The measured value is then directly proportional to surface pressure Π, which is the difference between the surface tension of the liquid γ_l and that of the film γ_f[164, 165].

$$\Pi = \gamma_l - \gamma_f \tag{4.10}$$

Modern filmbalances, connected to and controlled by a software, show the surface pressure Π directly in mN/m.

4.4 Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) is a thermoanalytical method to determine the thermodynamic behaviour of a sample. It compares the amount of heat required to increase the temperature of a sample with a reference. This amount of heat is then plotted as a function of temperature. Both sample and reference are kept at equal temperatures throughout the measurement. This method allows the detection of phase transitions and of heat capacities[166].
As both sample and reference are heated simultaneously, phase transitions in the sample lead to differences in the heat flow required to hold the sample as well as the reference at the same temperature. When plotted against temperature, these differences show as positive or negative peaks. An endothermic process shows as a positive peak, since more heat is required to maintain the sample's temperature, e.g. the melting of a solid sample absorbing heat while undergoing the phase transition. An exothermic process (e.g. crystallization) is shown as a negative peak in the plot, as the sample emits heat, and requires less heat flow to maintain its temperature[166, 167].
To obtain such curves, sample and reference are placed in thermally isolated measuring cells. Independent ovens are used to heat both sample and reference. A thermal element measures the temperatures of both cells and the ovens are regulated to compensate any differences that occur between the sample and the reference. Figure 15.1 shows a simplified scheme of a DSC set-up[166, 167].

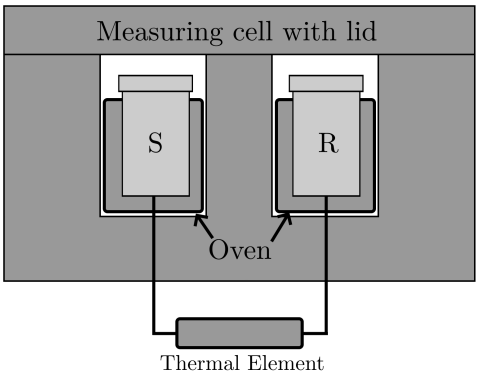

Figure 4.8: A simplified schematic view of a DSC setup. R: Reference. S: Sample

DSC offers high certainty and reproducibility while requiring very little amounts of sample. It gives direct experimental access to the enthalpies of phase transitions. By integrating the peaks in the plot, the peak area can be calculated. This area is

proportional to the transition enthalpy. The sample's molar mass, exact mass in the sample cell and a device specific calibration factor have to be considered[166, 167]. Within this thesis however, the temperature of the phase transition was of more concern as its enthalpy, as the experiments wanted to determine which compounds stabilize or destabilize a lipase. Figure 4.9 shows an example of a typical endothermic peak that a DSC measurement could yield.

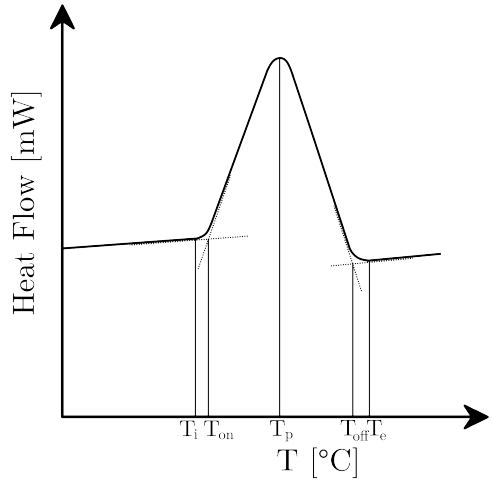

Figure 4.9: An example of an endothermic melding peak of a DSC measurement. A baseline drift can appear due to changed heat capacity after the melting process[168].

The onset point T_{on} of the transition peak is determined by linear interpolation of the baseline and the onset flank of the peak. The beginning temperature is T_i. T_p marks the maximum of the peak. The offset point T_{off} is calculated in a similar way as the onset point and T_e marks the peak's end. The heating rate of the measurement can influence the width of the peak, leading to changes in the maximum and the offset. The onset point is far less dependant on the heating rate, which is why it was used to compare changes in the phase transition temperature of the lipase[166, 168].

Part III

Experimental Methods

5 Materials

Surfactants

The following surfactants used in this thesis were all provided by BASF SE (Ludwigshafen, Germany):
Lutensol AO7 (nonionic, C13/C15-Oxo alcohol + 7EO, purity >99%), Glucopon 215UP (nonionic, C8-C10 alkyl polyglycoside, purity approx. 64%), Glucopon 600CSUP (nonionic, C10-C16 alkyl polyglycoside, purity approx. 53%), Texapon N70 (anionic, C12-C14 ethoxylated sulfate, purity 70%), Maranil DBS/LC (anionic, linear C10-C13-alkyl benzene sulfonic acid, purity >99%) and the prototype surfactants RA0089-21 (cationic, C12EO4-choline, ∼25%) and RA0089-24(cationic, C12EO1-choline, 26%).
Sodium dodecylbenzenesulfonate (≥81%, SDBS) was purchased from Sigma-Aldrich (Steinheim, Germany).

Glycerides and fatty acids

Tripalmitin (>80%), monopalmitin (>95%) and triolein (>80%) were purchased from TCI Deutschland GmbH (Eschborn, Germany). Palmitic acid (>98%) was purchased from Sigma-Aldrich.
For HPLC measurements, additional glycerides and fatty acids in higher purity were purchased from Sigma-Aldrich. These were oleic acid (>99%), 1-monoolein (>99%),2-monoolein (neat oil), diolein (≥99%, mixture of 1,3- and 1,2-isomers), and triolein (>99%).
Biskin® (Peter Kölln GmbH & Co. KGaA, Elmshorn, Germany) was obtained from a local supermarket.

Additives

In the context of this thesis, an additive is a compound that was used in a low concentration in a detergent mixture. It does not mean a certain class of chemicals.
2-methyl tetrahydrofuran (2-MTHF, ≥99%) was purchased from Alfa Aesar (Kandel, Germany). 1,2-Propandiol (≥99%) was purchased from Merck (Darmstadt, Germany). Triacetin (99%), Tributyrin (97%), DL-1,2-Isopropylideneglycerol (Solketal, ≥97%), L-Pyroglutamic acid (≥99%) and Dihydrolevoglucosenon (Cyrene, ≥98.5%)

were purchased from Sigma-Aldrich.
Plurafac® LF7319 (alkoxylated, predominantly unbranched fatty alcohols, ≥90%), Agnique® AMD3L (lactic acid dimethyl amide, ≥99%), Agnique® AMD10 (decanoic acid dimethyl amide, ≥99%), Agnique® AMD10 (C8-C10 fatty acid dimethyl amide, ≥99%) and Cetiol OE (Di-n-octylether, ≥99%) were kindly supplied by BASF SE.

Enzymes

All enzymes used in the course of this thesis were purchased from Sigma-Aldrich (Steinheim, Germany).
Lipases from Thermomyces lanuginosus (solution, >100,000U/g) and Aspergillus oryzae (solution, >100,000 U/g and lyophilized powder, ∼50 U/mg), both sold as Lipolase 100L were used throughout all tests where cleaving of triglyceride was investigated. Glycerokinase from Cellulomonas sp. (lyophilized powder, 25-75 units/mg), Glycerol 3-phosphate Oxidase from Aerococcus viridans (lyophilized powder, ≥70 units/mg) and Peroxidase from horseradish (lyophilized powder, ∼150 units/mg) were used for the lipase enzyme assay.

Solvents

Unless stated otherwise, Millipore water ($\geq 18 M\Omega$) was used for all experiments.
Chloroform (≥99.8%) was purchased from Fisher Scientific (Schwerte, Germany), n-dodecane (≥99%) was purchased from Alfa Aesar (Kandel, Germany). Methyl tert-butyl ester (MTBE, ≥99.8%, for HPLC), 2-propanol (≥99.9%, for HPLC), n-hexane (≥97%, for HPLC) and ethanol (p.a.) were purchased from Sigma-Aldrich.

Acids and Bases

Formic acid (reagent grade, ≥96%) was purchased from Sigma Aldrich (Steinheim, Germany). Hydrochloric acid solution (37%, p.a.) was purchased from Fisher Scientific (Schwerte Germany). Sodium hydroxide pellets (≥99%, p.a.) and potassium hydroxide pellets (≥85%, p.a.) were purchased from Merck (Darmstadt, Germany). Trichloroacetic acid (≥99%, p.a.) was purchased from Carl Roth (Karlsruhe, Germany).

Salts

Potassium dihydrogen phosphate (p.a.), di-potassium hydrogen phosphate (p.a.), magnesium chloride hexahydrate (p.a.), magnesium sulfate heptahydrate (p.a.) and

calciumsulfate dihydrate (p.a.) were purchased from Merck (Darmststadt, Germany). Choline chloride was purchased from Sigma-Aldrich, Choline-EO3-chloride was kindly provided by BASF SE.

Chemicals required for the lipase enzyme assay

Adenosine 5'-triphosphate (ATP) disodium salt hydrate (≥99%), N,N-Diethyl-m-toluidine (99%), 4-Aminoantipyrine (≥99%, p.a.), Ethylenediaminetetraacetic acid trisodium salt hydrate (EDTA-Na_3,≥95%) and 2-(N-Morpholino)ethanesulfonic acid (MES) buffer solution (0.5 M in H_2O) were purchased from Sigma-Aldrich.

Other

Trimethylsulfonium Hydroxide solution (TMSH, 0.2mol/L in Methanol) was purchased from TCI. Sudan Black B was purchased from Sigma-Aldrich. For the detergency tests, non-soiled, white cotton fabric without optical brighteners (article number 211) was purchased from Swissatest Testmaterialien AG (St.Gallen, Switzerland).

Sodium citrate dihydrate (≥99%), Choline dihydrogencitrate (≥98%) and Ethylenediaminetetraacetic acid (EDTA,≥98%) were purchased from Sigma-Aldrich. Trilon® M solution (aqueous solution of the trisodium salt of methylglycinediacetic acid, ∼40%) was provided by BASF SE.

Persil Universal detergent powder, which was used as a detergent reference, was obtained from a supermarket.

6 Methods

6.1 Determination of Fatty Acid Solubility

6.1.1 Optical Evaluation

Mixtures of water and surfactant from 0 to 100% (m/m) surfactant were prepared. To each mixture, PA was added stepwise in steps of 1% (m/m). After each addition, the mixture was stirred for several minutes. The samples were then evaluated optically. PA was added to each sample until it no longer cleared when stirred.

6.1.2 Gas Chromatography (GC)

Gas Chromatography (GC) measurements were performed with a Hewlett Packard HP 6890 Series GC system equipped with a flame ionization detector (FID). A non-polar HP-5 (5% phenyl-and 95% methyl-siloxane) capillary column (30m x 0.32mm i.d., 0.25 µm film thickness) was used for separation. Analyses were performed with a constant helium flow of 1.0 mL/min. The GC was equipped with a split/splitless injector at a temperature of 300 °C. A HP 6890 Autosampler was used to inject 1 µL of the sample in split mode using a split ratio of 20:1. The FID was maintained at a temperature of 300 °C. The temperature of the oven was initially set at 70 °C. This temperature was maintained for 1 minute. Then the temperature was increased at a rate of 40 °C per minute to 210 °C. This temperature was then maintained for 7.5 minutes. Analysis of each sample was carried out three times.
To quantify PA in the samples an internal standard (IS) method using n-dodecane was employed. To this purpose, samples with a known concentration of PA and n-dodecane were prepared for each batch measurement to obtain the calibration factor K_f. The same amount of dodecane was added to all samples during preparation.
Samples were prepared by over-saturating surfactant solutions with PA and subsequent filtration with 0.45 µm CA syringe filters. Samples were then completely dried and the residue was dissolved in 3 mL MTBE and filtrated once more with 0.45 µm PTFA syringe filters. 80µL of methylating reagent (TMSH in methanol) was added to 100µL of filtrated sample to obtain the methyl esters of the fatty acids

in the sample, which is necessary for GC detection of long chain fatty acids[169]. 20µL of internal standard solution (20 mg/mL n-dodecane in MTBE) was added to all samples which were then measured by GC/FID.

6.2 Langmuir-Blodgett Trough

A NIMA601 film balance by KSV NIMA (Gothenburg, Sweden) was used for the experiments. The film balance was connected to a computer where the NIMA516 software was used to record measurements. A thermostat was connected to the filmbalance and set to 20 °C. The solutions for the tests were also cooled to this temperature. 300 mL of cooled millipore water or testing solution (millipore water, lipase, additive) was filled into the trough of the filmbalance. 50 µL of a 0.1 wt-% solution of a mixture of tripalmitin and palmitic acid (ratio dependant on the measurement) in chloroform were then carefully spread on the surface. After allowing the solvent to evaporate for a few minutes and after an additional incubation time in case of measurements with lipase in the solution, the monomolecular film was compressed at 20 cm^2/s. The surface pressure was recorded continuously throughout the measurement with a Wilhelmy plate connected to a sensor. The compression was stopped only after the collapse of the monolayer.

6.3 Detergency Tests

The detergency tests were performed on the in-house built detergency testing unit (DTU) which is described in detail in part IV. Figure 6.1 shows the DTU.

The procedure of the detergency tests can be divided into several parts.

Preparation of dyed soil

Two types of soil were used for the detergency tests. Both were prepared manually. 0.5 wt-% sudan black B was added to the fat (either Biskin or a 60/40 wt-% mixture of Triolein and Tripalmitin). The mixture was then homogenized by melting the fat and stirring for 30 minutes.

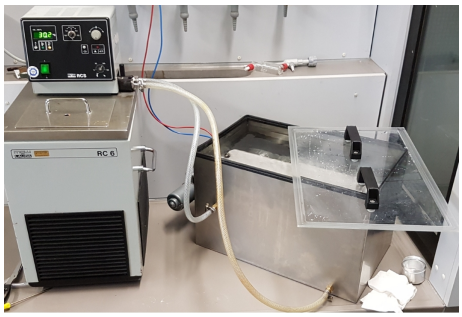

Figure 6.1: The detergency testing unit used for the detergency tests connected to a cryostat.

Preparation of textile stripes

The cotton cloth described in the materials section was cut into 5x2 cm stripes. As four stripes were used per beaker, the stripes were cut into 4 different shapes, to ensure their identifiability after testing. After cutting the stripes into their shapes, each group of four was weighed.
To dye the stripes, a 10% (v/v) solution of the dyed soil in chloroform was prepared. The stripes were dipped into the solution for 5 seconds and then hung up to dry inside a fume hood.
After drying, a colorimetrical measurement with a Elrepho SE071 spectrophotometer by Lorentzen & Wettre (Munich, Germany) was performed. 10 points were measured for each stripe. Also, the stripes were weighed again in the same groups of four to determine the degree of soiling.

Preparation of detergent

A stock solution of 24.4 wt-% $MgSO_4$ and and 16.9 wt-% $CaSO_4$ was prepared. The concentrations were chosen in such a way, that a 1:100 dilution of the stock solution with millipore water led to a total water hardness of 14 °dH. From this stock solution, water with the desired water hardness was prepared for all washing tests that required water hardness.
The surfactants were dissolved in the prepared water. All further components (additives, co-surfactants, builders, buffers) were added when the surfactant concentration was as desired. A CG843 pH meter by Schott (Mainz, Germany) was used to measure the pH of the detergent solution, which was adjusted with 1M NaOH solution

to a range from 8 to 9, in order to match the desired pH of a laundry detergent formulation. The exact value for each system was agreed upon with BASF, according to their internal results and experience. If required, Lipase was added directly before the tests.

Performing the detergency test

Beakers were prepared by adding five 8 mm balls made from stainless steel into each beaker and then adding 30 mL detergent solution. Four textile stripes were then added to each beaker. All beakers were closed and put into the DTU. The testing unit was set to 25 °C. Tests were performed at 60 rpm motor speed for 30 minutes. Then, the beakers were taken out of the DTU and the stripes immediately removed from the beakers and rinsed. Rinsing was performed by dipping the stripes into a glass beaker filled with 200 mL millipore water that was stirred at 300 rpm for 10 seconds. The stripes were then hung to dry overnight inside a fume hood.

Analysis

After drying, stripes were weighed in the preassigned groups of four. The brightening of the stripes was determined via colorimetric measurements with the Elrepho SE071. 10 points were measured for each stripe. The principal of this method is explained in chapter 4.1.

6.4 Enzyme assay

The assay developed to determine the activity of the enzyme is based on a method published by Sigma-Aldrich[170]. This assay is based on a 4 step reaction that produces a dye that can be measured at 545 nm. The 4 reaction steps are:

$$\text{Triglyceride} + H_2O \xrightarrow{\text{lipoprotein lipase}} \text{Glycerol} + 3\,\text{Fatty acid} \tag{6.1}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow[Mg^{2+}]{\text{glycerol kinase}} \text{Glycerol}-3\text{-Phosphate} + \text{ADP} \tag{6.2}$$

$$\text{Glycerol}-3\text{-Phosphate}+O_2 \xrightarrow{\text{L}-\alpha\text{-glycerophosphate oxidase}} \text{Dihydroxyacetone}-\text{Phosphate}+H_2O_2 \tag{6.3}$$

$$2\,H_2O_2+\text{4-Aminoantipyrene}+\text{N, N}-\text{Diethyl}-\text{m-toluidine} \xrightarrow{\text{peroxidase}} \text{Quinoneiminie dye}+4\,H_2O \tag{6.4}$$

6.4.1 Preparation of reagents

Reagents were prepared and stored according to the assay procedure by Sigma Aldrich[170].The following solutions were prepared:

- K-phosphate buffer, pH 7.0, 0.1 M
- Lutensol AO7 solution 5 wt-%
- Trichloroacetic acid (TCA) solution, 0.2 M
- MES-buffer, pH 6.5, 0.05 M
- Colour developing reagent (see below)
- Enzyme diluent (K-phosphate buffer, pH 7.5, 0.02 M, containing 2 mM $MgCl_2$ and 0.5 mM Na_3 – EDTA)

The color developing reagent was prepared by dissolving the following components in 200 mL MES buffer (pH 6.5, 0.05 M):

- 4.0 mL Lutensol AO7 solution
- 0.04 mL N,N-Diethyl-m-toluidine
- 4.0 mg 4-Aminoantipyrine
- 24.2 mg ATP disodium hydrate
- 40.7 mg $MgCl_2 \cdot {}_6H_2o$
- 200 units Glycerol kinase
- 500 units L-α-Glycerophosphate oxidase
- 300 units Peroxidase

6.4.2 Preparation of emulsions

To prepare the assay, suitable Triglyceride in water with surfactant emulsions were prepared. Initial emulsions were prepared with 10% fat concentration in mind. The emulsions consisted of either Tripalmitin, Triolein or Biskin and Lutensol AO7 dissolved in the K-phosphate buffer at pH 7.0. Since the fat concentration was very high, total surfactant concentrations of up to 1.5% were required to achieve sufficient emulsion stability to conduct the assay. Since such a high amount of surfactant can be detrimental for the activity of the lipase, emulsions with only 0.1% total surfactant were also prepared. For these emulsions, the concentration of the fat was only 1.0% and only triolein was used, as emulsions with Biskin and Tripalmitin at this surfactant concentration were not sufficiently stable. Two methods for sample preparation were tested. For both methods, emulsions were stirred with a laboratory stirrer at 800 rpm for 10 to 15 minutes. If the fat was solid at room temperature, the sample was heated up to the fats melting point during stirring. After that one half of the samples was homogenized with a Polytron PT3100 homogenizer by Kinematica (Luzern, Switzerland) at 20'000 rpm for 3 minutes. The other half was sonicated with a UP200Ht sonicator by Hielscher Ultrasonics (Teltow, Germany). Sonication was performed for 30 seconds twice with a 30 second pause in between. The sonicator was set to 110 W and 0.5 s pulses.
The droplet size of the emulsions was determined by random sampling with a Zetasizer Nano ZS by Malvern Panalytical GmbH (Kassel, Germany).

6.4.3 Assay Procedure

1. Hydrolysis reaction

For the first step in the assay procedure, 2 mL fat emulsion were mixed with 0.2 mL Lipase solution. Lipase solution was prepared by mixing the lipase used for the assay with the enzyme diluent solution. This was necessary to obtain the desired enzyme volume activity. The mixture was stirred for 30 minutes at room temperature. After 30 minutes, the reaction was stopped by adding 1 mL of TCA solution. To obtain blanks, the enzyme was added after the TCA addition. Samples and Blanks were filtered through Whatman No. 42 filter paper (pore size approx. 2.5 µm) after the hydrolysis reaction.

2. Colour change reaction

3 mL of the colour developing reagent were mixed with 0.05 mL of the obtained filtrate. The mixture was stirred at 40 °C for at least 25 minutes to ensure completion of the colour change reaction. Longer reaction times (up to 4 hours) did not seem to have any influence on the colour intensity of the sample. It was noted that samples had to be stored protected from light if they were not immediately measured after the colour change reaction.

3. UV/VIS spectroscopy

Samples were measured within 24 hours after the colour change reaction. Optical densities against MES buffer solution (used for the colour developing reagent) of the samples at 545 nm were determined with a Lambda 18 photometer by Perkin Elmer (Waltham, MA, USA). Polystyrene cuvettes with 10 mm pathlength were used for the measurements.

6.5 Enzyme assay with HPLC analysis

To determine the exact products of enzymatic degradation by the employed lipase, a normal-phase high pressure liquid chromatography (NP-HPLC) method was developed.

6.5.1 HPLC method

Experiments were performed with a Waters HPLC system consisting of two waters 515 HPLC pumps, a Waters 717plus Autosampler and a Waters 2487 UV/VIS detector. To seperate the samples a Chromegasphere SI60 column (10µm particle size, 60 Å pore size, 250x4.6 mm) by ESI Industries (West Berlin, NJ, USA), which was heated to 40 °C, was used. The injected volume was 10 μL. Samples were eluted at 1.8 mL/min. For measurements with isocratic elution, a mixture of 90% hexane and 10% 2-propanol was used. In case a gradient solution was used, solution A was pure hexane and solution B was hexane/2-propanol/formic acid (60:39.9:0.1). The gradient was adjusted so that the overall ratio of hexane to 2-propanole was 99:1 at the beginning of each measurement, then B was increased over 6 minutes to reach a ratio of 80:20 where it remained for 2 minutes and then returned to its original ratio over 4 minutes. The detection wavelength was 190 nm. Analysis of each sample was carried out three times. Samples were prepared in triplicates. The software "Empower 3" was used to record and evaluate the chromatograms. The apex track algorithm available in the software was used to calculate the area of the peaks, which also allows to account for overlapping peaks.
The oleic acid concentration was determined quantitatively via an external standard calibration. A calibration curve with 6 oleic acid concentrations from 5 to 30 mg/mL was generated. For each concentration, three solutions were prepared from high purity oleic acid and hexane/2-propanol/formic acid (90:9.9:0.1) independently. For each solution, three measurements were carried out.

6.5.2 Preparation of emulsions

Fat and surfactant concentrations were not changed compared to the UV/VIS assay. However, only Triolein was used for the HPLC analysis and all samples were homogenized with the Polytron PT3100 homogenizer. For this method a 0.02 M K-phosphate buffer at pH 7.5 (containing no $MgCl_2$ or Na_3-EDTA) was used.

6.5.3 Assay procedure

1. Hydrolysis reaction

For the first step in the assay procedure, 10 mL fat emulsion were mixed with 0.02 mL Lipase solution. Lipase solution was prepared by mixing the lipase used for the assay with the enzyme diluent solution. This was necessary to obtain the desired enzyme volume activity, which was 2 U/mL. The mixture was stirred for 30 minutes at 37 °C. After 30 minutes, the reaction was stopped by adding 2 mL of TCA solution to each sample and subsequently cooling each sample in an ice bath. To obtain blanks, the enzyme was added after the TCA addition. Samples and Blanks were then dried completely by evaporating the water.

2. HPLC measurements

Dried samples were dissolved in hexane/2-propanole/formic acid (90:9.9:0.1) and subsequently filtrated through 0.45 nm PTFE syringe filters and then measured by HPLC/UV.

6.6 DSC

DSC measurements were performed on a Setaram Micro DSC III by Setaram Instrumentation (Caluire, France) with the software Setsoft 2000 controlling the DSC. Samples containing 1% Lipolase and 0.5 to 5.0% of either additive or 1:1:1 surfactant mixture were prepared along with blanks containing everything except the lipase (including 1,2-propanediol and $CaCl_2$, which were part of the lipase buffer in the solution delivered by Sigma Aldrich). The sample size for a measurement was 800 µL. Samples were measured from 25 °C to 95 °C. The heating rate was 1 °C per minute. The onset points of occurring endothermic and exothermic peaks were used to determine changes in the thermal denaturation of the lipase in the presence of additives.

6.7 Swelling tests

The ability of a mixture of natural fats to swell, i.e. to adsorb water was investigated. 5 mL of liquid were added to approximately 500 mg of Biskin®. After 30 minutes, the solution was removed and the change in mass of the fat was noted. Samples were weighed again after 3 hours and after 24 hours to see if residue liquid remained. Samples were stored in open containers between weighing. The solutions tested here aside from water were 3% solutions of promising additives in water. Further tests included solutions with 800 ppm of the 1:1:1 surfactant mixture and/or 1 ppm of Lipolase. The same additives were screened for the solutions with surfactant and lipase. This was to see if swelling of fat increased in the presence of additives, which would be an advantageous interaction for detergency processes.

Part IV

In-House-Built Detergency Testing Unit (DTU)

7 General Information

A reliable method to determine the performance of washing solutions on fat based soils has been of interest for the work of this group for a while. Initial tests performed by Dengler[23] and Wolfrum[24] were based purely on the ability of the washing solutions to dissolve fatty soils and did not take into account any physical processes that influence washing performance. Dyed soil was applied to a textile stripe, which was subsequently immersed into a detergent solution in a glass beaker. The solution was stirred with a magnetic stirrer. All experiments were carried out at ambient temperature without temperature control. After a washing time of 20-30 minutes, the detergent solution was replaced with millipore water to and stirred for an additional 20-30 minutes to remove residual detergent. The preparation and drying of the textile stripes was equal to the procedure described in 6.3.
At the start of this project, the same method was intended to be employed, but since initial results with the formulations used here proved inconclusive, a new method for reliable detergency or washing performance tests was developed. In the course of this development, a testing unit was designed and built which was used for all further tests. The structure shall be presented in this chapter. A user or instruction manual for the DTU, which includes the function and a general working procedure can be found in the Appendix part of this thesis.
The design of the DTU is based of the commercially available Launder-Ometer® by SDL Atlas, which was developed for colour fastness tests. The principle is the same. The heart of the unit is a rotating axis within a thermostated water tank to which several stainless steel beakers are fixed. The detergent and textile samples are within these beakers. A major difference is the size. Compared to the Launder-Ometer® the DTU was shrinked significantly. The volume of the DTU's beakers is 100 mL each, while the commercially available unit operates with 550 or 1200 mL beakers. Furthermore, the Launder-Ometer® operates with a constant rotor speed and dynamic temperature profiles, while the DTU was designed to work with a constant temperature during a measurement while the rotor speed can be adjusted anytime.

8 Requirements

The general function of the DTU is to perform detergency or washing performance tests. During development, several key requirements were defined which were crucial in the design of the unit.

- Temperature controlled detergency testing with a pre-defined temperature that can be adjusted between measurements
- A constant motion similar to that of a washing machine, to account for the mechanical input of machine based washing on soil removal
- Sample containers that are small enough for laboratory scale tests
- Significantly higher throughput compared to glass beaker detergency tests

With the chosen design for the unit, all the pre-defined requirements could be met.

9 DTU structure

Figure 9.1 shows the general design of the DTU and the central axis which holds the 15 beakers. In order to save costs and reduce required maintenance, the unit was not built with an internal thermostat. Instead, a standard laboratory thermostat is connected to the unit. This means that a thermostat failure does not necessarily result in a longer downtime of the DTU, as another thermostat can simply be connected.

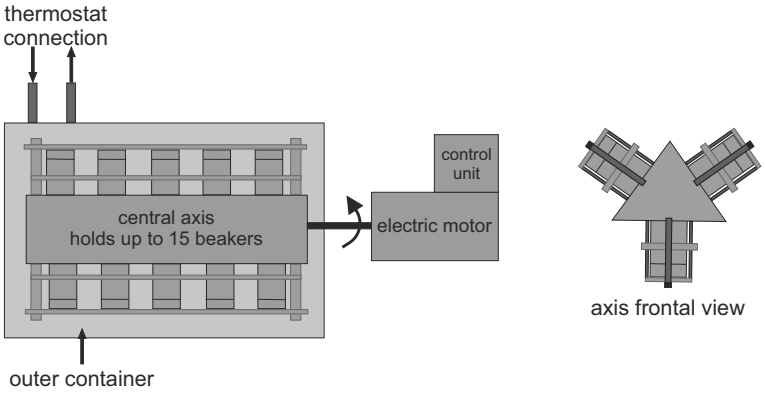

Figure 9.1: Left: Design draft of the DTU; Right: Frontal view of central axis

Figure 9.2 shows the DTU ready for testing with all beakers mounted.

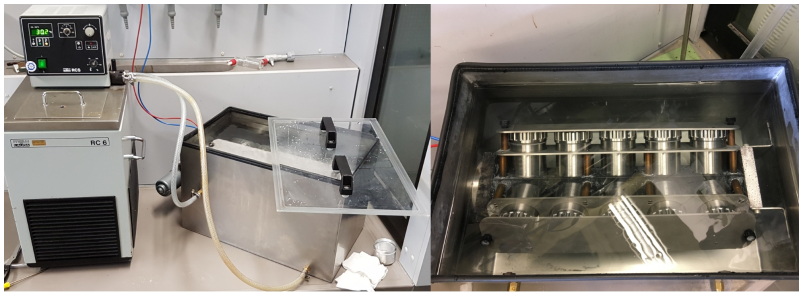

Figure 9.2: Left: complete DTU with a standard laboratory cryostat connected. Right: View inside the container with the axis beeing fully mounted with beakers.

Part V

Results and Discussion

10 Investigation of Fatty Acid Solubility in Surfactant Solutions

10.1 Introduction

The initial goal set at the beginning of this work was to find ways to increase fat-based soil release from fabric at low washing temperatures. This proves a challenge since it involves removing solid fats below their melting point. Liquefied soil is commonly easier removed due to the roll-up mechanism[5, 90]. As such, solubilization of soil had been identified in previous works as a driving force for soil release. However, it had also been found that the solubilization of solid triglycerides even in presence of organic solvents was low[23]. As mentioned, the central concept of this thesis was the incorporation of a lipase that cuts the triglycerides into smaller components, which are easier to remove from the surface. Those smaller components may be solubilized more easily than triglycerides. Furthermore, salts of long chain fatty acids ($RCO_2^-M^{n+}$, where M is a metal cation with charge n) make up the surfactant class of soaps. They may therefore be incorporated into the micelles of the detergent solution or adsorb to the remaining soil increasing soil release (see soil release mechanism in section 3.3.3), effectively causing a local increase in surfactant concentration and therefore increasing detergency performance.

To see an indication if such a solubilization of fatty acids was present in a detergent formulation, the solubility of palmitic acid in a variety of detergent solutions was tested. Ternary phase diagrams were prepared to compare the solubility of palmitic acid at different surfactant concentrations with different surfactants/surfactant mixtures. The surfactant comparison was done to determine which surfactant or surfactant mixture is best suited to increase fatty acid solubility in water-rich systems. The purpose of the ternary phase diagrams was to determine how much the fatty acid solubility can be increased if the surfactant concentration is higher than in the bulk of a washing mixture, as surfactant concentrations are expected to be higher at the soil/water and fabric/water interface, since surfactants adsorb to the soil and the fabric[4, 5].

Additionally, solubility tests with various additives were performed. These additives

were commonly organic and applied as co-solvents. The goal was to further increase fatty acid solubility, in accordance to work performed by Dengler[23], whose thesis can be seen as a predecessor to this one.

10.2 Ternary Phase Diagrams

10.2.1 Water/Tripalmitin(TP)/Lutensol AO7

A mixture with only one nonionic surfactant and no cosolutes was used as a basic reference. As the surfactant was available at near 100% purity, very high surfactant concentrations were also investigated. Figure 10.1 shows the obtained phase diagram.

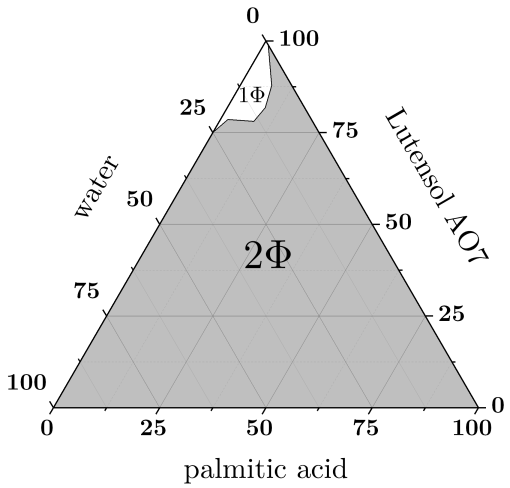

Figure 10.1: Ternary phase diagram of water/TP/Lutensol AO7 at 298 K and pH=10 (KOH). The area marked with 1Φ gives the monophasic region. The area marked with 2Φ gives the region where a turbid emulsion was formed.

This experiment, where the solubility of palmitic acid was evaluated optically, showed macroscopic solubility of palmitic acid only at very high surfactant concentrations, presumably in the area of reverse micelles.
This concentration range is, however, far outside the reach of concentrations in a washing process. The bulk surfactant concentration in laundry detergency is usually below 1000 ppm, although it is higher at the surface of the soiled fabric, since surfactants adsorb to surfaces. However, it is rather unlikely that local surfactant concentrations >80% will occur.
As this method was not precise enough to determine very small solubilities of

palmitic acid in water-rich (water content ≥99%) surfactant solutions, the aforementioned GC method was developed to determine the fatty acid concentration in saturated solutions of palmitic acid with surfactant concentrations between 0.1 and 1.0%. For the phase diagrams, this translates to a magnification of the water-rich corner of the diagram (see figure 10.2).

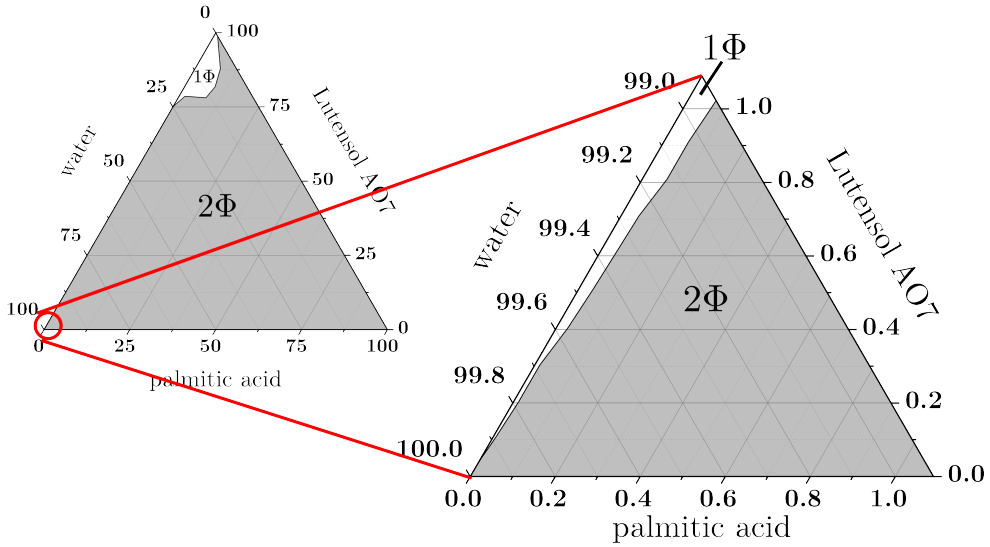

Figure 10.2: Magnification of the water-rich area of the ternary phase diagram of water/TP/Lutensol AO7 at 298 K and pH=10 (KOH). The area marked with 1Φ gives the monophasic region. The area marked with 2Φ gives the region where a turbid emulsion was formed. For all further solubility investigations, only the concentration range of the magnified diagram was examined.

10.2.2 Comparison of Surfactant Systems

Further experiments aimed to find a surfactant system that shows the highest possible solubility for palmitic acid (PA) at room temperature. The following surfactants and surfactant mixtures were used for the experiments. The phase diagrams of these systems are shown in Figure 10.3.

- Lutensol AO7
- Lutensol AO7/Texapon N70 (1:1)
- Lutensol AO7/Texapon N70/SDBS (1:1:1)
- Glucopon 215UP/Texapon N70 (1:1)
- Glucopon 215UP/Texapon N70/SDBS (1:1:1)
- Glucopon 600CSUP/Texapon N70/SDBS (1:1:1)

All of the curves show low concentrations for palmitic acid in the examined range. To improve clarity of the actually determined PA concentrations, Table 10.1 compares the PA concentrations of the 6 systems at 1% total surfactant. The error of the method is +/- 10 ppm

Table 10.1: Palmitic acid dissolved in various surfactant solutions at 1% total surfactant. The pH was set to 10 with KOH.

Surfactant	**Dissolved PA (ppm)**
Lutensol AO7	670
Lutensol AO7/Texapon N70 (1:1)	330
Lutensol AO7/Texapon N70/SDBS (1:1:1)	200
Glucopon 215UP/Texapon N70 (1:1)	110
Glucopon 215UP/Texapon N70/SDBS (1:1:1)	130
Glucopon 600CSUP/Texapon N70/SDBS (1:1:1)	100

None of the tested mixed detergent systems could match the solubility power of the pure Lutensol system. However, the overall solubility of PA in the water-rich area was poor for all examined systems. It appears that cleaving the triglycerides and dissolving the resulting fatty acids will not be sufficient to increase the washing power in a laundry detergent.

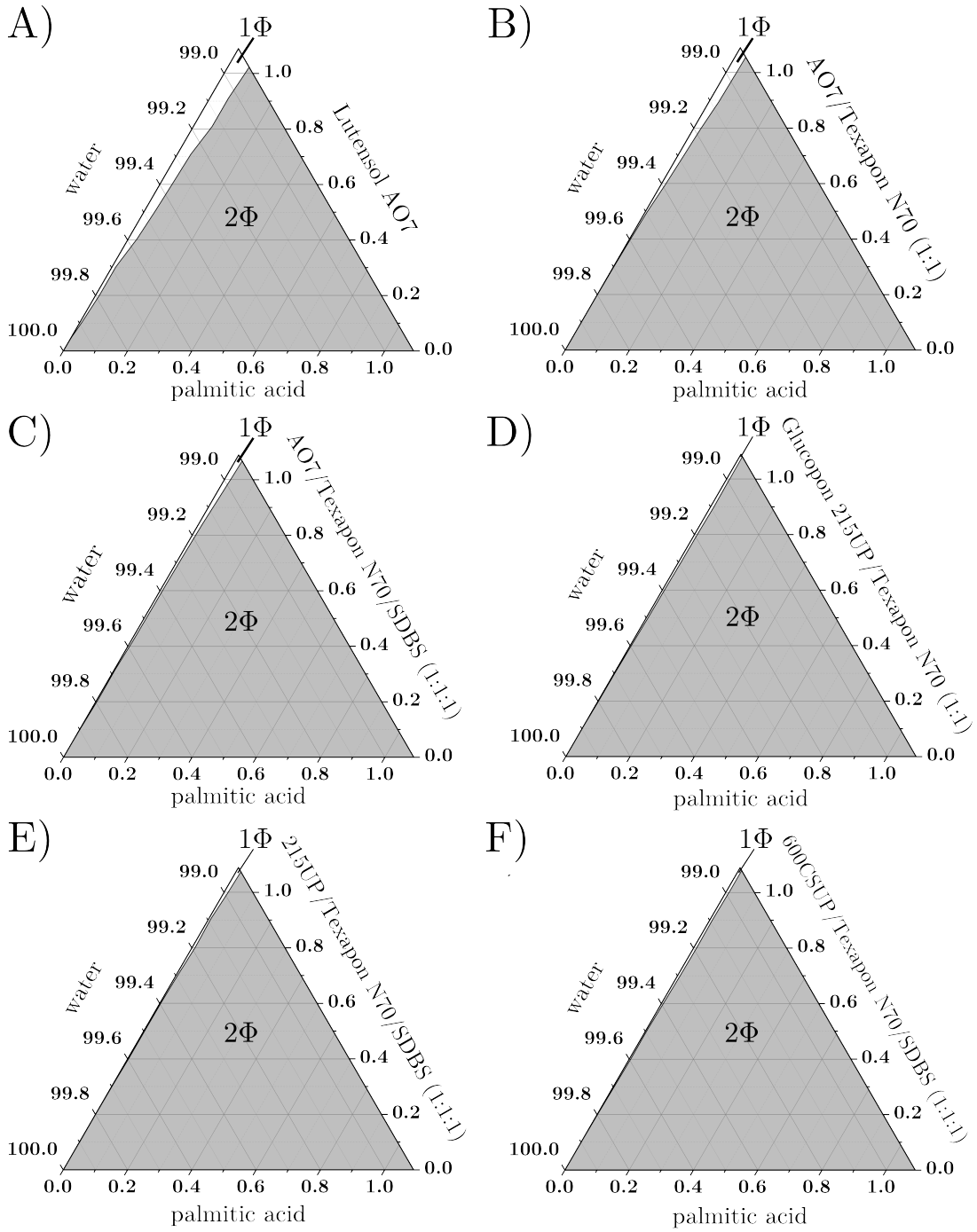

Figure 10.3: Ternary phase diagrams of water/TP/surfactant systems at 298 K and pH=10 (KOH). The area marked with 1Φ gives the monophasic region. The area marked with 2Φ gives the region where a turbid emulsion was formed.

10.2.3 Comparison of Hydroxides

As previously mentioned, the pH for the tested systems was 10, as washing processes are usually performed at a basic pH. The cation of the hydroxide might influence the solubility. Studies by Wolfrum[24] found that the use of choline can have a positive influence on detergency processes, decreasing the Krafft temperature of anionic surfactants due to their bulkiness.

For these tests, the pH value was left at 10, but instead of KOH, choline hydroxide (ChOH) and choline EO_3 hydroxide ($ChEO_3OH$) were used. The hydroxides were prepared from their chlorides via ion-exchange chromatography. Silver nitrate was used to determine if the removal of chloride had been successful.
The obtained phase diagrams of these systems are shown in Figure 10.4. Table 10.2 shows the amount of dissolved PA at 1% total surfactant. The corresponding results from the previous section, were KOH was used, are included for comparison.

Table 10.2: Comparison of palmitic acid dissolved in various surfactant solutions at 1% total surfactant with different hydrocxides. The pH was 10. The mixture ratio of surfactants was always 1:1:1.

Surfactant	**Dissolved PA (ppm)**
Lutensol AO7/Texapon N70/SDBS (KOH)	200
Lutensol AO7/Texapon N70/SDBS (ChOH)	210
Lutensol AO7/Texapon N70/SDBS ($ChEO_3OH$)	270
Glucopon 215UP/Texapon N70/SDBS (KOH)	130
Glucopon 215UP/Texapon N70/SDBS ($ChEO_3OH$)	90

While a slight increase in dissolved palmitic acid was determined for the Lutensol system when $ChEO_3OH$ was used instead of KOH, the amount of dissolved PA decreased for the Glucopon system. Overall, the inclusion of choline via the hydroxide does not seem to have a drastic effect on the solubilization properties.

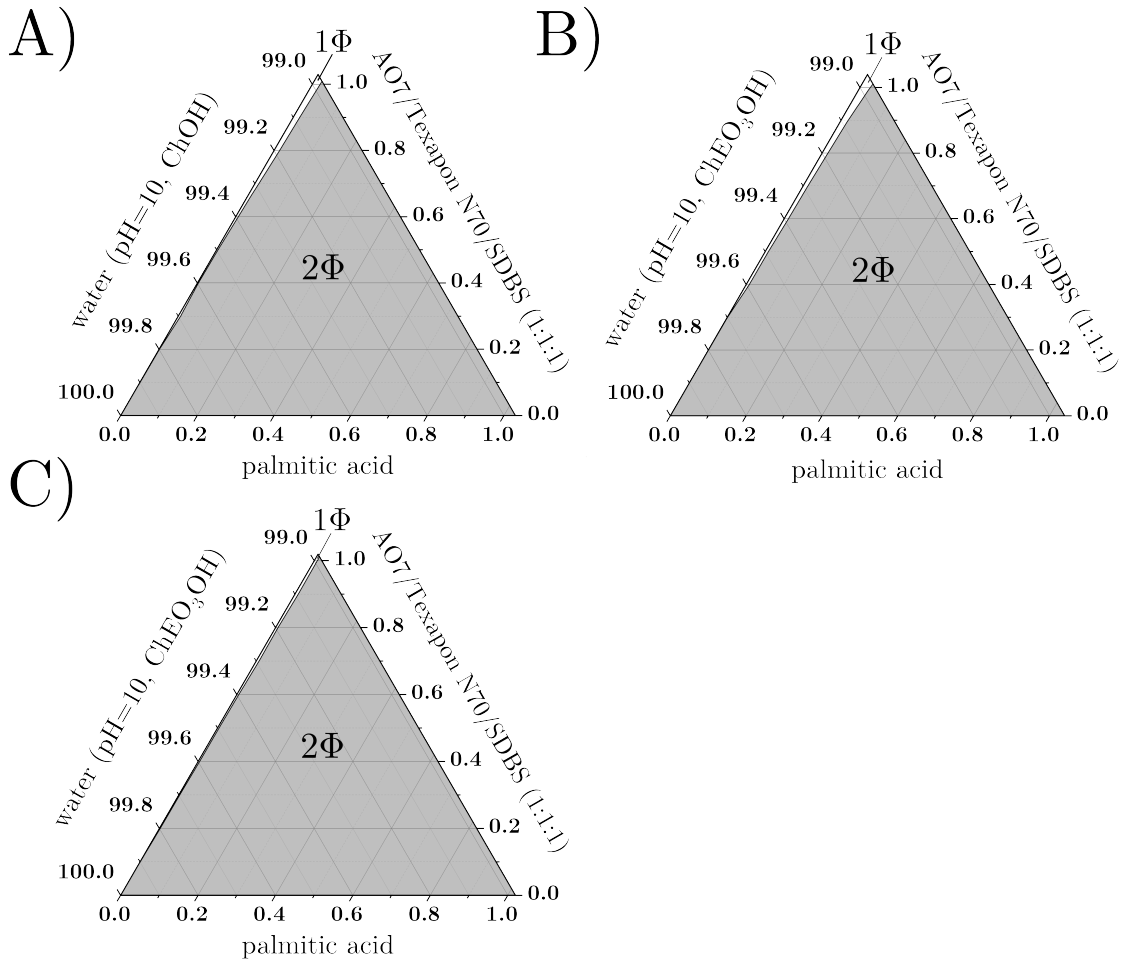

Figure 10.4: Ternary phase diagrams of water/TP/surfactant systems at 298 K and pH=10. The area marked with 1Φ gives the monophasic region. The area marked with 2Φ gives the region where a turbid emulsion was formed.

10.3 Comparison of Additives

Initially, the solubility tests had been ceased, as the results showed only very low palmitic acid solubility in detergent solutions, meaning that a higher solubility of a fatty acid compared to the triglyceride could not be the reason for an increased washing performance in the presence of a lipase.

However, to determine if the increased washing performance of formulations with additives were due to an increase in fatty acid solubility, solubility experiments were resumed with additives from detergency tests. For these experiments, several changes were made compared to the previous washing tests. Instead of creating a phase diagram from a multitude of samples with varying concentrations, the surfactant

concentration was kept constant across all experiments. The maximum PA solubility (i.e. the highest PA concentration where a monophasic solution could be obtained) at this surfactant concentration was determined. Additionally, propane-1,2-diol and ethanol were added in constant concentrations, as this background-matrix was also used in the detergency tests.
The chosen concentrations were:

- 0.8% total surfactant (1:1:1 mixture of AO7, Texapon N70 and SDBS)
- 0.25% propane-1,2-diol
- 0.09% ethanol
- 0.1% additive

For each component, this is exactly the tenfold concentration used in the detergency tests. This choice was made for two main reasons:

1. Two safely quantify palmitic acid concentrations via GC, a sufficiently high concentration is needed.
2. It is assumed that the concentration of surface active surfactants and of (organic) solvents is higher at the stain/water interface compared to the bulk phase.

Figure 10.5 shows the comparison of the obtained PA solubility for the tested additives. Table 10.3 shows the exact values that were obtained. The last two additives used are cationic surfactants supplied by BASF SE, which were actually part of the work of Zahnweh[25] in a similar project at the Kunz group at the University of Regensburg. In their regular concentration range, the cationic surfactants proved problematic for the lipase activity. They were therefore incorporated here as additives with lower concentrations.
Relatively, a significant increase in the range of +50% could be achieved for some additives. With +72%, the most significant increase could be seen for the cationic surfactant C12EO4Ch, which means that a higher surfactant concentration seems more beneficial than adding additives that are not amphiphilic. The overall concentrations, however, are still in the ppm range and on their own probably not sufficient to explain an increase in washing performance.

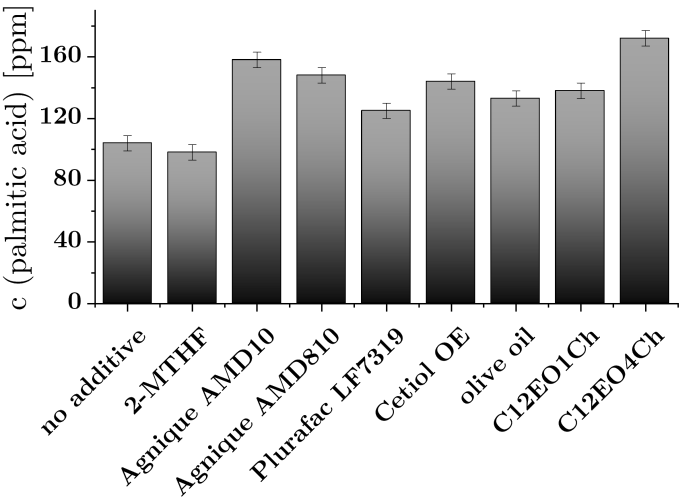

Figure 10.5: Highest achieved palmitic acid concentration for monophasic mixtures. All systems contain a 1:1:1 Lutensol AO7, Texapon N70 and SDBS surfactant mixture, propane-1,2-diol and ethanol. All systems except for the first one contain 1000 ppm of the additive denoted underneath the respective column.

Table 10.3: Comparison of palmitic acid dissolved in a 1:1:1 surfactant solution of Lutensol AO7, Texapon N70 and SDBS with a propane-1,2-diol and ethanol background and various additives with different properties. The pH was set to 10 with KOH.

Surfactant	**Dissolved PA (ppm)**
no additive	104
2-Methyl-THF	100
Agnique AMD10	158
Agnique AMD 810	148
Plurafac LF7319	125
Cetiol OE	144
olive oil	133
C12EO1Ch	138
C12EO4Ch	172

10.4 Conclusion

The overall conclusion that can be drawn from the solubility tests is that except for very high surfactant concentrations, the solubility of palmitic acid is very low, and will probably not exceed 100 ppm in the environment expected in a washing process. This means that the higher performance of a detergent that is expected when an enzyme is added, does not originate from a higher solubility of the fatty acid that the enzyme generated when hydrolysing the fat. The solubility of the fatty acid is extremely low and overall differs only little from the triglyceride solubility it originates from.

One of the ideas formed at the beginning of this thesis was that the fatty acids removed from the triglycerides would dissolve and function as co-surfactants, increasing the washing performance *in-situ* and thus helping to remove the mono-, di- and triglycerides that remain on the fabric. The low solubility found for palmitic acid in the surfactant solution at room temperature appears to contradict this notion. Indeed, when looking at the Krafft temperature of sodium palmitate, which is approximately 60 °C[171], or that of potassium palmitate, which is approximately 15 °C lower[36], this is not too much of a surprise.

It has been found that the addition of sterically demanding counter-ions, such as tetramethylammonium and choline can severely reduce the Krafft temperature of surfactants. For the here employed palmitate, Krafft temperatures below room temperature can easily be reached [24, 36, 39]. For this to work with the *in-situ* generated fatty acid on the stain, enough choline would have to be available near the interface.

The use of choline derivatives was already tested extensively by Wolfrum[24]. Since the solutions for the solubility tests were set to pH 10 by adding hydroxides, it was tested if choline hydroxide and choline-EO_3 hydroxide would increase the solubility significantly. While an increase could be detected in most cases, the overall solubility of the palmitate was still rather low.

Dengler's[23] work with organic solvents to increase the solubility of triglycerides was the origin of the idea to use such solvents as additives in a surfactant solution to increase the solubility of the fatty acid. A lot of organic solvents (e.g. chloroform, methanol or benzene) were not tested, since they are either toxic or cancerogenic and can therefore not be used in a detergent. Another type of additive was to use amphiphilic molecules to act as co-surfactants. Interestingly, only 2-MTHF did not

show any increased fatty acid solubility in these tests. This was the same co-solvent that had seemed promising in washing tests[23]. The other additives did increase the solubility of palmitic acid, but not to a degree that it would allow the palmitic acid to act as an *in-situ* generated co-surfactant. The conclusion that can be drawn from this was that the increased washing performance that was achieved with a lipase acting on the stain (which is presented in the next section) cannot be due to severely increased solubility of the generated fatty acids compared to their triglycerides as the solubility remains similarly low.

Previous investigations by Dengler have shown that liquid triglycerides can easily be dissolved[23]. Here, however, only a solid fatty acid was tested. Natural fats always contain a mixture of solid and liquid fats and fatty acids with different chain lengths and degrees of saturation. In the case where fatty acids with shorter chains are released from the triglycerides by the lipase, they might actually dissolve better, if their Krafft temperature is exceeded during the process. Also, the removal of solid fats in a natural mixture might be enhanced if the surrounding liquid fats are removed and the structure of the stain is broken up. This will be discussed further in the next section, where experiments with Biskin, which is a fat mixture derived from plants, are performed.

11 Laundry Detergency Tests

11.1 Introduction

A goal of this thesis was to have an application-oriented washing test to be able to examine the washing performance of the formulations in regard to a laundry detergency application. As already mentioned, this led to the development of the DTU, since previous tests proved to be poorly reproducible and not particularly close to washing in a machine, while commercial testing units were usually severely over-sized for the needs of these experiments.
For this thesis, the washing performance at room temperature was of interest. Due to increasing environmental concerns calling for sustainable solutions in our daily lives, a reduction of washing temperature is of interest, as it severely reduces the energy consumed by the washing machine[14]. However, this reduction of temperature must not lead to a reduction of the washing efficiency if it is to be accepted among consumers. As Venkatesh showed that the soil of typical household laundry consists mostly of fat based substances[87]. For these substances, washing above the melting point would be the more efficient method, as the roll-up mechanism can be utilized. Yet, at low temperatures, many fatty substances will still be solid. Previous studies by Dengler have shown that surfactant can only marginally decrease the melting point of tripalmitin (TP) and that solubilization works only for liquid triglycerides[23]. A concept introduced in Dengler's work was to create microemulsions by addition of solvents and co-surfactants to the detergent mixture to remove solid triglycerides and avoid redeposition.
However, as it was shown, breaking up the crystalline structure of a deposited triglyceride without melting it, is barely possible[23, 24]. Therefore, the influence of lipases was investigated in this work. A concept developed was, that if enough of the deposited solid fat was broken into fatty acids and mono-/diglycerides, the crystal structure of the fat on the fabric would become less stable and could thus be removed more efficiently.
The influence of the lipase "Lipolase 100L" on the washing performance at room temperature was tested here in a variety of systems. Several surfactant systems were tested, based on their performance in the intitial solubility tests. Solvent-based

additives were screened with the DTU. The additives were chosen based on work done by Dengler[23] and based on desired solvent properties (e.g. lipophilicity). Initially, two mechanisms were assumed for the function of the additives. Firstly, that the additives would increase washing performance by solubilizing fatty acids cleaved by the enzyme (which stops redeposition) and secondly, that their lipophilicity may cause a swelling of solid triglyceride stains, increasing the surface area of the stain which also increases accessibility of deposited triglycerides for the lipase. While the possibility of enzyme inhibition by additives was considered, an increase of enzyme activity through the presence of additives was initially not taken into account.
The influence of various builders in hard water on the enzyme driven washing performance was also investigated. A further factor of washing performance, namely the temperature, was not investigated here. Washing tests at higher temperatures, very often 40 °C and higher, have been done before[172–174] and were not the focus of this thesis, which aimed to achieve a high washing performance at temperatures <30 °C. To this end, two types of dyed soil were used: Biskin and a 40:60 TP/TO mixture.
The washing performance of the various systems was determined colorimetrically. A colorimeter using the L*a*b* system was used to compare the ΔE values of stained cotton stripes before and after washing. A higher value translates to a higher washing performance, as more soil has been removed.

This section features measurements that deal with the influence of water hardness. During the course of the experiments, the german standard to indicate water hardness (°dH) was always used. This standard is defined as a measure for the total hardness of water, consisting of both the influence of Ca ions and Mg ions in water. If the concentration of both calcium and magnesium in the water is known (in mg/L), the total hardness can be calculated with the following formula:

$$^{\circ}dH \approx 0.14 \cdot [\text{Ca-content in mg/L}] + 0.23 \cdot [\text{Mg-content in mg/L}] \tag{11.1}$$

Officially, this form of denoting water hardness is no longer valid, according to European law. To unify all the different national methods of indicating water hardness, especially in relation to application of laundry detergents, the Detergents Regulation[61] called for an European wide standard. This regulation was made national law in Germany with the detergents law of 2007[175]. By these new laws and regulations, water hardness is to be stated in mmol $CaCO_3$ per liter and magnesium

ions are not considered directly. It is, however, still common in Germany to denote the hardness of tap water as the total hardness (Ca and Mg ions) in °dH. Therefore a conversion of mmol/L $CaCO_3$ to °dH has been defined. 1 °dH equates to 0.1783 mmol/L of $CaCO_3$. Due to common practices of both water suppliers to use the total hardness in °dH for local tap water and also of manufacturers of laundry detergents to indicate dosage recommendations for their detergents (in relation to water hardness) in °dH, it was decided to also use °dH for the experiments in this thesis where the water hardness was relevant[176].

11.2 Washing Performance of Surfactant Systems

The initial tests with the DTU were to find out if the solubility behaviour of the surfactant systems translates to their washing performance. No additives were used in these tests. The water hardness was artificially adjusted to 5 °dH with no builder used in the formulation. This was done to reduce possible influences from other formulation components as far as possible. Hardness was added to the water by recommendation of the surfactants' supplier, BASF SE, to achieve optimal surfactant performance. A washing test with pure water was performed as a blank. Detergency power of pure water is expected to be very low, since fats do not dissolve in water. Figure 11.1 shows a comparison of the obtained ΔE values of the stripes used for the washing tests of the various surfactant systems. Table 11.1 shows the obtained ΔE values. The total surfactant concentration during these tests was 1000 ppm (1 g/L). The used soil was Biskin with a soiling degree of 25±2%. The pH value was set to 8.5 using 1M KOH solution.

Table 11.1: Averaged ΔE values and standard errors represented in figure 11.1.

System	**ΔE without lipase**	**ΔE with lipase**
1) Lutensol AO7	15.5±2.3	14.8±2.3
2) Glucopon 215UP	9.3±2.5	14.6±1.6
3) Glucopon 600CSUP	25.5±1.1	19.7±1.7
4) Lutensol AO7/Texapon N70/SDBS	20.9±1.1	24.4±1.6
5) Glucopon 215UP/Texapon N70/SDBS	25.0±1.0	26.6±1.9
6) Glucopon 600CSUP/Texapon N70/SDBS	25.0±1.0	26.1±2.0
7) Lutensol AO7/Texapon N70/Maranil DBS	20.3±1.2	23.1±1.0
8) no surfactant	8.4±1.4	7.8±1.9

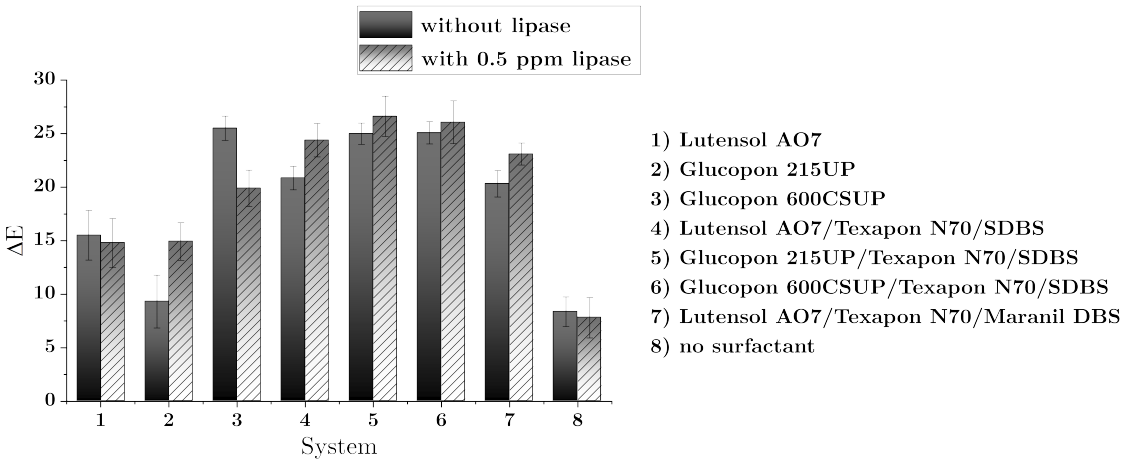

Figure 11.1: Averaged ΔE values of cotton stripes determined with a colorimeter by measuring them before and after washing with various surfactant solutions. Biskin dyed with sudan black B was used for all stripes in this batch. The sample labeled "no surfactant" was only washed with water.

The results of this series of experiments suggest that solubilization behaviour and washing performance do not necessarily correlate. The highest solubility of fatty-acid was seen for pure Lutensol AO7, which shows a low washing performance compared to the 1:1:1 mixtures, which all exhibited low solubility power. Furthermore, the washing performance upon enzyme addition, which should result in a higher fatty acid content of the soil due to enzymatic cleaving, was also low and in two of three cases even decreased in previous experiments. As expected, all surfactant solutions performed a lot better than just washing with pure water, which does not dissolve fat or sudan black B, with the exception of the Glucopon 215 UP solution, which only gave a slight increase in washing performance compared to pure water. The best results without enzyme were achieved for the 1:1:1 mixtures with Glucopons. However, they showed only slight performance increases upon lipase addition, ranging from about 4% (for the system with 600CSUP) to about 6% (for the system with 215UP). On the other hand, the two tested 1:1:1 systems with Lutensol AO7, while showing a poorer performance without lipase, showed an increase of removed soil of approximately 15%. Replacing SDBS with Maranil DBS/LC did little to change the overall performance. However, unlike SDBS, Maranil is liquid at room temperature and therefore better suited for use in liquid laundry detergents. Theoretically, the solution using only Glucopon 215UP showed the highest increase in removed material upon lipase addition (57%). However, even with such a stark increase, the overall performance of that system was still low.

These tests led to the decision that further detergency tests would be done with the Lutensol AO7/Texapon N70/SDBS mixture, as it showed good washing performance and a good increase of removed soil upon enzyme addition. In a later stage, SDBS was replaced by Maranil DBS/LC. Since this did not show any influence in the base washing performance, the two alkyl benzene sulfonates were treated as interchangeable. For later tests, a propylene glycol and ethanol background was also added, as these components can be found in washing formulations to increase storage stability of enzymes.

11.3 Employing Natural Soaps as Co-Surfactants

As explained in the theory section of this work, a soap is a salt of a long chain carboxylic acid. Soaps are still used in laundry detergents and can easily be derived from natural sources, like coconut fat, by completely hydrolysing the fats and removing the glycerol. A theory why the addition of lipase helps to increase the washing performance made at the beginning of this thesis was that the generated fatty acids would act as surfactants and further facilitate the removal of the remaining soil. Increasing the activity of the enzyme would help to further enhance the soil removal, as more fatty acids would be generated.
From this theory it was derived that directly adding fatty acids to the detergent would further improve the soil removal. Instead of only having *in-situ* generated fatty acids, a small portion of fatty acids would be present on the surface from the beginning on and help break up the soil and remove it. This could speed up the removal process as it would eliminate the time the lipase needs to generate a sufficient amount of fatty acids to help solubilize the soil.
In order to come closer to a complete detergent formulation and test this hypothesis, Edenor coconut fat was employed. At 800 ppm total surfactant concentration, the coconut fat concentration to be employed was 95 ppm. As soaps are detergents, the concentration of the other three detergents of the 1:1:1 system was lowered accordingly so that the total surfactant concentration remained unchanged.
The hydrolysed coconut fat was tested at a temperature of 25 °C and a pH of 8.5. The water hardness was adjusted to 5 °dH and no builders were used. Biskin soil was used for the tests. Figure 11.2 and Table 11.2 show the results.
No difference in the washing performance was obtained for these detergency tests. It seems that replacing a part of the 1:1:1 mixture with an equal amount of natural

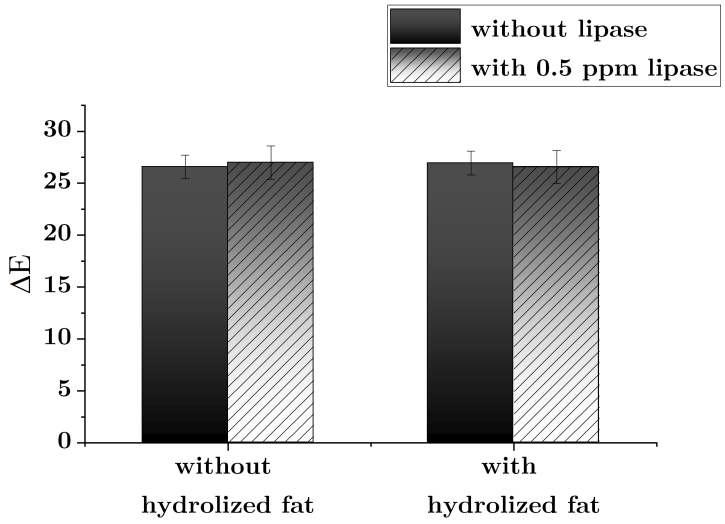

Figure 11.2: Averaged ΔE values of cotton stripes soiled with Biskin mixture dyed with sudan black B determined with a colorimeter by measuring them before and after washing with the 1:1:1 surfactant system with either no hydrolized coconut fat or with 95 ppm hydrolized coconut fat (inclued in the 800 ppm total surfactant concentration).

Table 11.2: Averaged ΔE values and standard errors represented in Figure 11.2.

System	**ΔE without lipase**	**ΔE with lipase**
Without hydrolized fat	26.6±1.1	27.0±1.6
With hydrolized fat	26.9±1.1	26.6±1.6

soaps does not influence the washing performance at all. Also, for these tests, no increase in the washing performance, when lipase was added, could be seen. There was no explanation for this behaviour that occurred in this batch. However, the use of hydrolysed coconut fat was not completely discarded. As mentioned, soaps are still used in laundry detergents. Therefore, in washing tests that called for a formulation closer to a market-ready detergent, the Edenor coconut fat was still employed.

11.4 Influence of Water Hardness and Builders

To evaluate the influence of water hardness and builders on the enzyme performance in the washing tests, four builders were chosen for comparison. These were sodium citrate, choline citrate, EDTA and Trilon M (BASF). The water hardness was set to 14 °dH, and builders were added to achieve a residue water hardness of 10 °dH, 7.5 °dH, 5 °dH and 3°dH. These tests were performed with a constant propylene glycol (250 ppm) and ethanol (90 ppm) background. The pH was set to 8.5. The total surfactant concentration was set to 800 ppm and the Edenor hydrolysed coconut fat was used. The stain used for the builder comparison was the TP/TO 40/60 mixture. Due to the extremely large number of experiments, comparison of builders was limited to 5 °dH for the Biskin stain and tests with all water hardnesses were only performed with choline citrate. The soiling degree of both stains on the cotton fabric was 25±2%. Table 11.3 and Figure 11.3 show the results of the colorimetrical analysis of the stripes.

Table 11.3: Averaged ΔE values and standard errors represented in Figure 11.3.

	Choline citrate		**Sodium citrate**	
Water hardness	**ΔE without lipase**	**ΔE with lipase**	**ΔE without lipase**	**ΔE with lipase**
14 °dH	10.3±1.5	3.0±0.9	10.3±1.5	3.0±0.9
10 °dH	8.8±1.1	3.7±0.4	10.7±1.3	5.3±0.9
7.5 °dH	13.4±0.6	9.6±0.0	9.0±1.1	8.6±1.1
5 °dH	14.7±1.0	16.8±0.8	10.8±1.2	8.1±0.9
3 °dH	12.5±0.7	10.9±0.9	12.0±0.9	7.6±0.8
	Trilon M		**EDTA**	
Water hardness	**ΔE without lipase**	**ΔE with lipase**	**ΔE without lipase**	**ΔE with lipase**
14 °dH	10.3±1.5	3.0±0.9	10.3±1.5	3.0±0.9
10 °dH	12.1±1.1	11.8±0.8	14.5±1.0	10.7±1.0
7.5 °dH	18.5±1.0	12.5±0.9	14.4±0.9	14.7±0.8
5 °dH	15.3±0.7	11.6±0.8	16.1±0.8	13.2±0.9
3 °dH	16.7±1.3	13.9±1.6	13.8±1.5	15.0±0.9

It is noticeable that for many of these tests with the TP/TO stain, the washing efficiency actually decreased upon enzyme addition, especially if the residue water

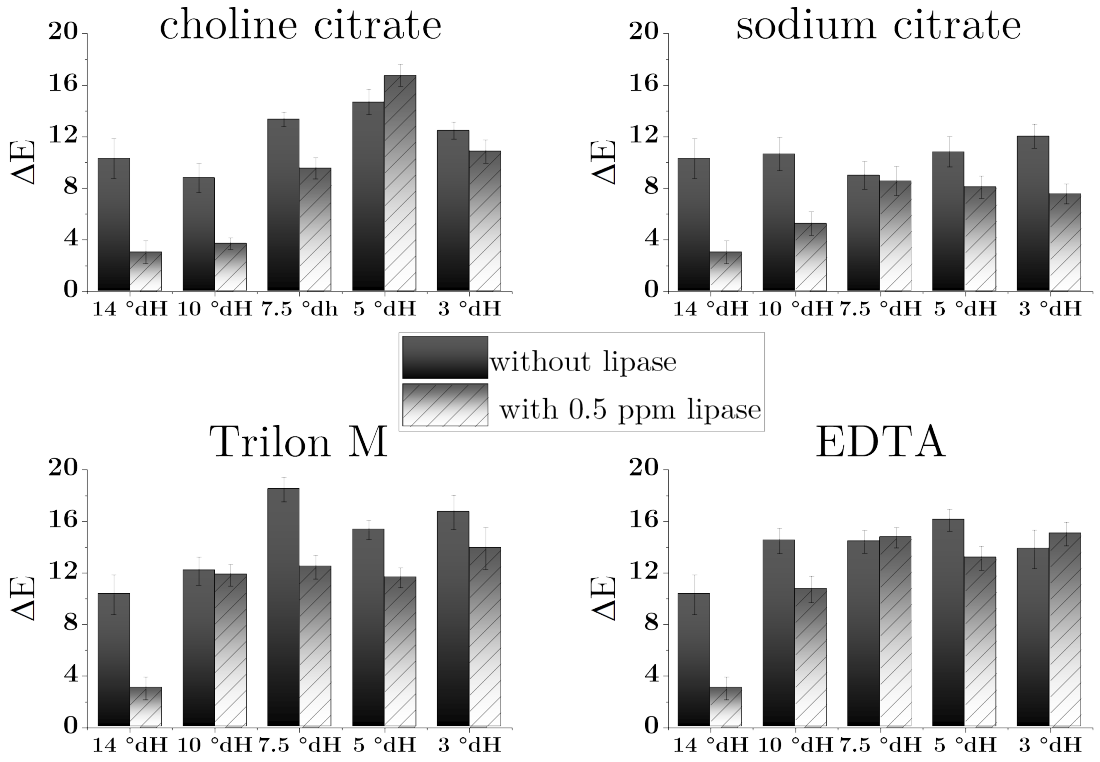

Figure 11.3: Averaged ΔE values of cotton stripes soiled with a TP/TO 40/60 mixture dyed with sudan black B determined with a colorimeter by measuring them before and after washing with various surfactant solutions. The 14 °dH sample was without any builders and is the same for all 4 batches.

hardness was high. Ideally, a low residue water hardness should remain in a washing process, as a high water hardness can inactivate ionic surfactants[177]. A maximum of the washing performance with the lipase deployed was achieved with choline citrate at 5 °dH. The formulations with sodium citrate showed relatively low washing performance results, while the washing performance with Trilon M was very high without enzyme, but the ΔE results of the tests with enzyme stayed around 12 at every tested water hardness. For EDTA, the results were overall rather similar without enzyme across all tested hardnesses, except for 3 °dH, where a slight decrease was noted. At lower water hardnesses the washing performance with enzyme remained in the range of the results without enzyme.

The influence of builders was also investigated with the Biskin stain. For these tests, all other parameters, including the soiling degree, were left unchanged compared to the tests with the TP/TO stain. Looking at the results with choline citrate

as builder (Figure 11.4 and Table 11.4), water hardness does not seem to have a large influence on removing the Biskin stain. The overall washing performance was in the same range across all tested hardnesses with only a slight increase at 7.5 °dH. Also the performance with and without lipase always were always very close to one another, which is different from the previous results where no builders were used and water hardness was artificially set to 5 °dH. In those tests, a boost from present lipase was clearly visible.

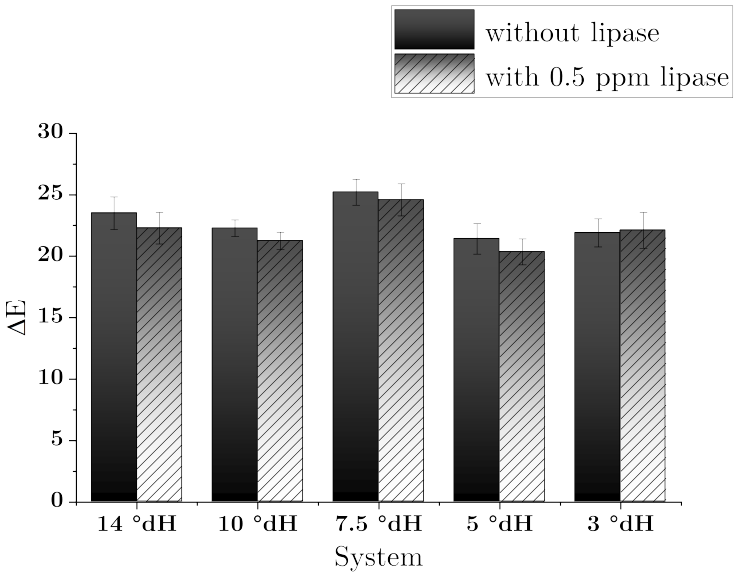

Figure 11.4: Averaged ΔE values of cotton stripes soiled with Biskin mixture dyed with sudan black B determined with a colorimeter by measuring them before and after washing with the 1:1:1 surfactant system. Water was prepared with a hardness of 14 °dH. Choline citrate was added as a builder to the detergent formulations in the right concentration to achieve the desired water hardness. No builder was added to the 14 °dH sample.

Table 11.4: Averaged ΔE values and standard errors represented in Figure 11.4.

System	**ΔE without lipase**	**ΔE with lipase**
14 °dH	23.5±1.3	22.3±1.3
10 °dH	22.3±0.7	21.2±0.7
7.5 °dH	25.2±1.0	24.5±1.3
5 °dH	21.4±1.2	20.4±1.1
3 °dH	21.9±1.1	22.1±1.5

As previous tests without builders were mostly conducted at 5 °dH, the four builders tested with the TP/TO stain were compared at a residue water hardness of 5 °dH. Results (see Figure 11.5 and Table 11.5) were inconclusive here, as washing performances were similar for all tested builders. All systems, except the one with Trilon M, where similar results for tests with lipase added compared to those without lipase. For the system with Trilon M, there seems to be a slight decrease, even though it is still within the measurement's margin of error. It seems that, when using the Biskin stain, residue water hardness and the nature of the used builder plays a lesser role compared to the TP/TO soil.

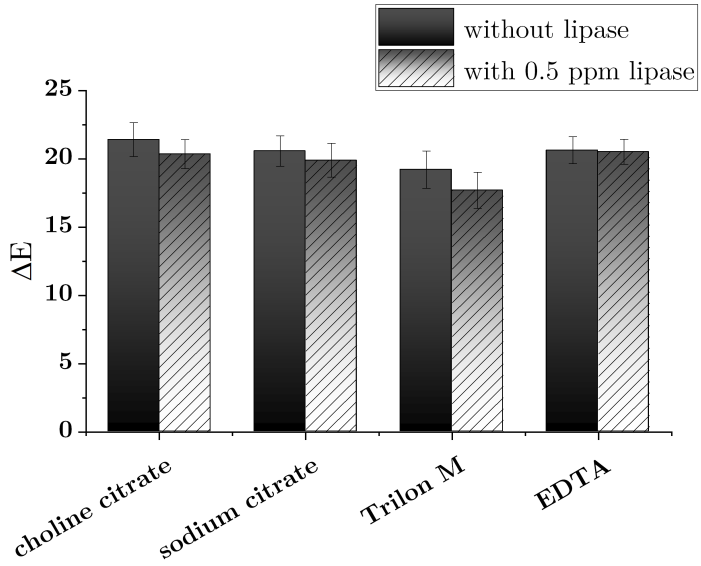

Figure 11.5: Averaged ΔE values of cotton stripes soiled with Biskin mixture dyed with sudan black B determined with a colorimeter by measuring them before and after washing with the 1:1:1 surfactant system. Water was prepared with a hardness of 14 °dH. Various builders were used and added in concentrations to achieve a residual water hardness of 5 °dH.

Table 11.5: Averaged ΔE values and standard errors represented in Figure 11.5.

System	**ΔE without lipase**	**ΔE with lipase**
choline citrate	21.4±1.2	20.4±1.1
sodium citrate	20.6±1.1	19.9±1.2
Trilon M	19.2±1.4	17.7±1.3
EDTA	20.6±1.0	20.5±0.9

11.5 Detergency Tests with Additives

To increase the washing power in an enzyme based washing process, another approach was tested. As mentioned, the concept of solvent-based additives stems back from the thesis of Dengler[23]. Usually, these solvents would dissolve fat quite well in their pure state (e.g. pure 2-MTHF dissolved about 42% Biskin). In theory, the solvents, co-solvents, or co-surfactants chosen as additives show some affinity to the fat-based soil and are therefore found in a higher concentration on the soil surface compared to the bulk solution. Due to this, the dissolving of the soil is facilitated and the washing performance increases. The concentration of the additive on the soil surface is unlikely to be high enough to dissolve the soil on its own, but if enough solvent is present, the stain might swell, increasing its surface area which not only facilitates soil release processes as more surfactant can adsorb to the surface but which also increases the accessibility of the soil for the lipase, resulting in an increased lipase efficiency. In theory, this should result in an increased washing performance boost upon enzyme addition when compared to performance boost gained from adding lipase to a detergent formulation without additive. A lack of performance increase would, on the other hand, indicate that the presence of the additive decreases the enzyme's activity (i.e. its turnover rate).
Initially, these tests were performed with the Biskin stain only. In a later stage, a "finalized list" of additives was tested with both stains.

Determining an Ideal Additive Concentration

Initially, the amount of additive that is required to be effective was estimated. Washing tests with varying additive concentrations were performed. The idea was that the performance increase seen by the addition of solvents would only increase up to a certain point. After that, a further increase of additive concentration would only benefit the washing performance sub proportionally, if at all. For these tests, the 1:1:1 system consisting of Lutensol AO7, Texapon N70 and SDBS was used. No background or natural fat based co-surfactant was used during these tests and water hardness was set to 5 °dH without adding builders. The total surfactant concentration was 1000 ppm, the pH was 8.5. 1 ppm lipase was used in order for positive effects to be more pronounced. Figure 11.6 and Table 11.6 show the results of the initially tested three additives.

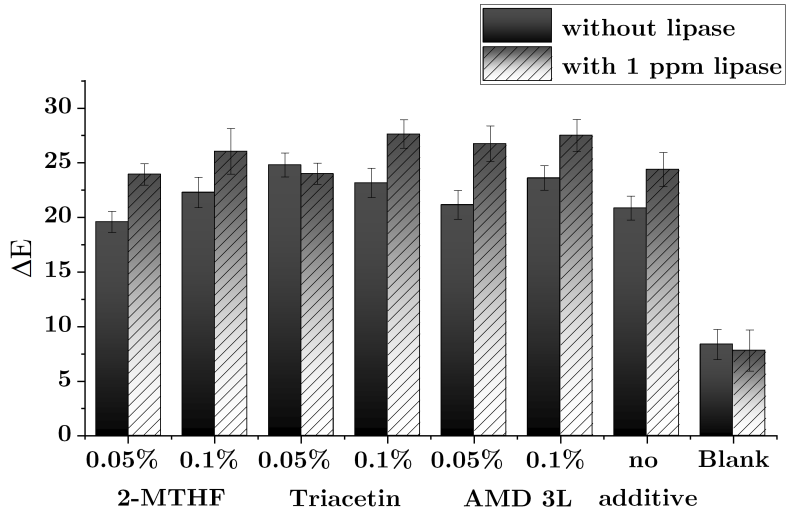

Figure 11.6: Averaged ΔE values of cotton stripes soiled with Biskin mixture dyed with sudan black B determined with a colorimeter by measuring them after soiling and after washing. The sample labeled no additive is merely the 1:1:1 system without any further components. For the blank sample, only water was used to wash the stripes.

Table 11.6: Averaged ΔE values and standard errors represented in Figure 11.6.

System		ΔE without lipase	ΔE with lipase
2-MTHF	**0.05%**	19.6±1.0	23.9±1.0
	0.1%	22.3±1.4	26.0±2.1
Triacetin	**0.05%**	24.8±1.1	24.0±1.0
	0.1%	23.2±1.3	27.6±1.3
Agnique AMD 3L	**0.05%**	21.1±1.3	26.7±1.6
	0.1%	23.6±1.1	27.5±1.5
no additive		20.9±1.1	24.4±1.6
Blank		8.4±1.4	7.8±1.9

With the exception of the sample with the lower triacetin concentration, the addition of a solvent-based additive always caused a significant increase in washing performance. It is, however, notable here that the performance increase of the samples where 0.1% additive was used in the washing solution did not perform significantly better than those with 0.05%. Therefore, these concentrations are both already higher than the optimum that was attempted to be found. Further additive washing tests with varying concentrations of 2-MTHF were thus performed to see how far

the concentration of additive can be decreased without loosing its beneficial effect. For the test batch with 2-MTHF concentrations between 10 ppm and 1% ($\hat{=}$ 10'000 ppm) were used (see Figure 11.7 and Table 11.7). Most conditions were kept equal to the previous batch except for the total surfactant concentration, which was lowered to 800 ppm and the lipase concentration, which was reverted back to 0.5 ppm. Both changes were done to be closer to concentrations expected in actual laundry washing. Furthermore, in this case, there is a sample where the additive concentration is slightly above the surfactant concentration (the 1000 ppm additive sample). This is an advantageous setup to see, if any additive concentration above the total surfactant concentration is actually detrimental for the washing performance.

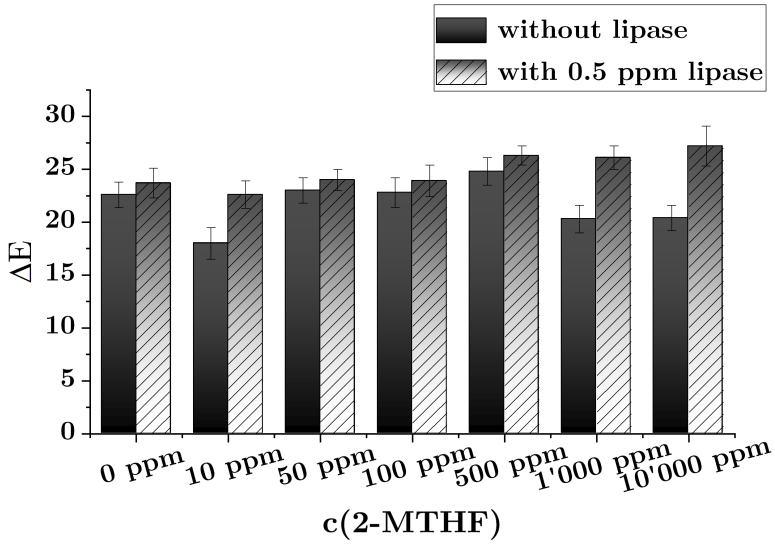

Figure 11.7: Averaged ΔE values of cotton stripes soiled with Biskin mixture dyed with sudan black B determined with a colorimeter by measuring them before and after washing with the 1:1:1 surfactant system with 2-MTHF added in various concentrations.

The results show that while the washing performance decreases when the additive concentration is greater than the total surfactant concentration, the performance boost gained from adding lipase increases further if more 2-MTHF is added. This seems to indicate that while 2-MTHF might hinder the surfactants from releasing the soil if c(additive)>c(surfactant), the lipase actually benefits from a higher concentration of 2-MTHF. The highest additive concentration that remained below the total surfactant concentration was 500 ppm. The results achieved here overall were good (highest washing performance before lipase addition and good boost upon enzyme addition) but it was decided that further additive tests will be performed with 100 ppm additive, as a laundry washing formulation that results in

Table 11.7: Averaged ΔE values and standard errors represented in Figure 11.7.

c(2-MTHF)	ΔE without lipase	ΔE with lipase
0 ppm	22.6±1.2	23.7±1.4
10 ppm	18.0±1.5	22.6±1.3
50 ppm	23.0±1.2	24.0±1.0
100 ppm	22.8±1.4	23.9±1.5
500 ppm	24.8±1.3	26.3±0.9
1'000 ppm	20.3±1.3	26.1±1.1
10'000 ppm	20.4±1.2	27.2±1.9

500 ppm 2-MTHF in the washing process would contain too much 2-MTHF in the concentrate. Lower additive concentrations however, seem to be too low too see a significant influence of the additive on the overall performance.

Additive Effectiveness in Presence of Stabilizing Formulation Components

Commonly, propylene glycol is used in liquid laundry detergents to stabilize washing enzymes, giving the product a longer shelf life. Even the solution the lipase was delivered in by Sigma Aldrich contained 25% propylene glycol. It was therefore important to see if any interdependencies with propylene glycol occur if an additive that on its own enhances the washing performance with lipase (like 2-MTHF) is present. For these tests a constant propylene glycol concentration of 267 ppm, which is a third of the total surfactant concentration, was chosen. Other than that, these tests were performed analogous to the concentration series of 2-MTHF in the previous section. This was done to see if the varying ratio of propylene glycol and 2-MTHF causes significant differences. Figure 11.8 and Table 11.8 show the results of these tests.

The results here, on first glance, seem to indicate that using 2-MTHF with a propylene glycol background is not beneficial for the washing performance. The rather high washing performance in the sample that contained no 2-MTHF was unexpected, as generally, while prolonging shelf life, propylene glycol was not considered to be beneficial for the washing performance. When adding 2-MTHF, a similar trend to the previous batch without propylene glycol becomes visible. A higher 2-MTHF content generally leads to a higher washing performance increase when lipase is also

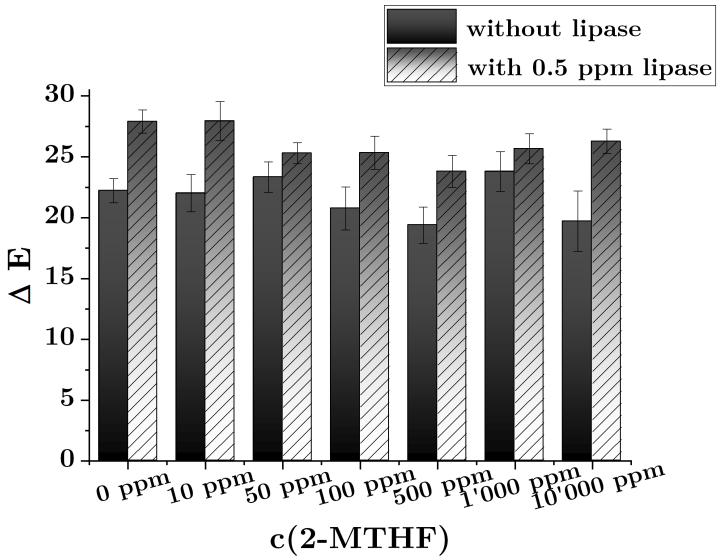

Figure 11.8: Averaged ΔE values of cotton stripes soiled with Biskin mixture dyed with sudan black B determined with a colorimeter by measuring them before and after washing with the 1:1:1 surfactant system with 2-MTHF added in various concentrations. The washing solutions of every sample contained 267 ppm propylene glycol in this batch.

Table 11.8: Averaged ΔE values and standard errors represented in Figure 11.8.

c(2-MTHF)	ΔE without lipase	ΔE with lipase
0 ppm	22.2±1.0	27.9±1.0
10 ppm	22.0±1.5	27.9±1.6
50 ppm	23.3±1.3	25.3±0.9
100 ppm	20.8±1.8	25.3±1.4
500 ppm	19.4±1.5	23.7±1.3
1'000 ppm	23.7±1.6	25.7±1.2
10'000 ppm	19.7±2.5	26.3±1.0

added, but if the additive concentration exceeds the total surfactant concentration, the base washing performance (without enzyme) decreases, removing any benefit gained from the relative washing performance increase with lipase. For this batch, 100 ppm 2-MTHF seem again an ideal middle ground, as the base washing performance is not decreased, while the increase of the performance with lipase does not significantly increase at higher 2-MTHF concentrations. The 1'000 ppm sample is somewhat of an outlier here, showing high base performance but a poor increase when lipase is added.

Comparing Possible Additives for a Laundry Detergent

With the formulation growing more complex, a set of possible additives was chosen, which were to be tested in a detergent mixture. The total surfactant concentration was kept at 800 ppm. A 1:1:1 mixture of Lutensol AO7, Texapon N70 and Maranil DBS/LC was used, with 95 ppm hydrolized natural coconut fat as co-surfactant (included in the 800 ppm). A constant background of 250 ppm propylene glycol and 90 ppm ethanol was added, which are both used to ensure a long shelf life of the enzymes in the detergent. Concentration of additives was 100 ppm. The pH was set to 8.5. Water with a hardness of 14 °dH was used and choline citrate was added to reach a residue hardness of approx. 5 °dH. For these tests, all additives were tested with both the Biskin and the TP/TO stain and the temperature was left at 25 °C. Figure 11.9 and Table 11.9 show the results for the tests with Biskin stain.

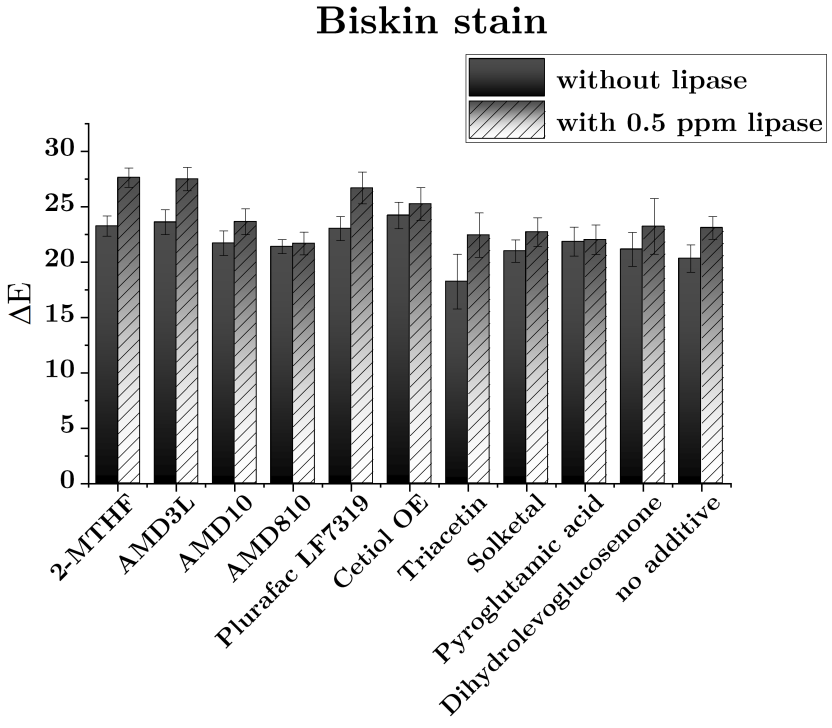

Figure 11.9: Averaged ΔE values of cotton stripes soiled with Biskin mixture dyed with sudan black B determined with a colorimeter by measuring them before and after washing with the 1:1:1 surfactant system with the listed additives at a concentration of 100 ppm each.

Table 11.9: Averaged ΔE values and standard errors represented in Figure 11.9.

Additive	**ΔE without lipase**	**ΔE with lipase**
2-MTHF	23.2±0.9	27.6±0.9
Agnique AMD3L	23.6±1.1	27.5±1.1
Agnique AMD10	21.7±1.1	23.6±1.2
Agnique AMD810	21.4±0.6	21.7±1.0
Plurafac LF7319	23.0±1.1	26.7±1.4
Cetiol OE	24.2±1.2	25.2±1.5
Triacetin	18.2±2.5	22.4±2.0
Solketal	21.0±1.0	22.7±1.3
Pyroglutamic acid	21.9±1.3	22.0±1.3
Dihydrolevoglucosenone	21.1±1.5	23.2±2.5
no additive	20.3±1.2	23.1±1.0

These results show that some additives (e.g. 2-MTHF and Agnique AMD3L) appear to not only increase the base washing performance of the detergent formulation but they also lead to an enhanced "boost" when lipase is added, i.e. the relative increase in washing performance from adding lipase is greater with additive than it is without. The additives were chosen either to act as a co-solvent or to act as a co-surfactant. If the relative increase upon enzyme addition with additive is similar to the sample without, the additive and the lipase do not interact and any benefit gained from the additive is solely based on interaction with the soil, e.g. an increased solubility of the soil in the surrounding medium. If the performance increase from adding lipase is diminished with additive (as is the case for e.g. Agnique AMD810), that suggests that the additive has a negative influence on the lipase, e.g. by destabilizing its active conformation. The meaning of these results, when combined with insight gained from other experiments, will be more closely examined in the next part of this thesis.

The results with the TP/TO stain (Figure 11.10 and Table 11.10) allowed for less of an overall insight. For a lot of the tested additives, washing performance decreased upon enzyme addition. This was somewhat unexpected, as even at a complete deactivation of the lipase by an additive, the washing performance should have remained the same. At a higher water hardness, a decrease in performance may be possible, as the fatty acids released by the enzymatic cleaving of the triglycerides could immediately precipitate as lime soap, redepositing on the fabric and effectively sealing

it off for further cleaning. However, a builder was used for this test batch to prevent this.

Overall a slight increase in washing performance was observed for the system without any additives and a strong increase for the system with 2-MTHF. Other than that, the washing performance either decreased upon enzyme addition or remained within margin of error. For some additives, even the washing performance without enzyme decreased compared to the system without any additive. For some systems (Agnique AMD10, Cetiol OE and Solketal), however, it increased strongly when no enzyme was used and decreased a lot when lipase was added. An explanation for that might be that the temperature used is actually too low for the TP/TO stain. At 25 °C, this stain might not be reproducibly removable in the DTU, meaning that results may spread a lot and appear rather random. Washing at a higher temperature or for a longer time might be beneficial when the TP/TO stain is used, but was refrained from in this thesis.

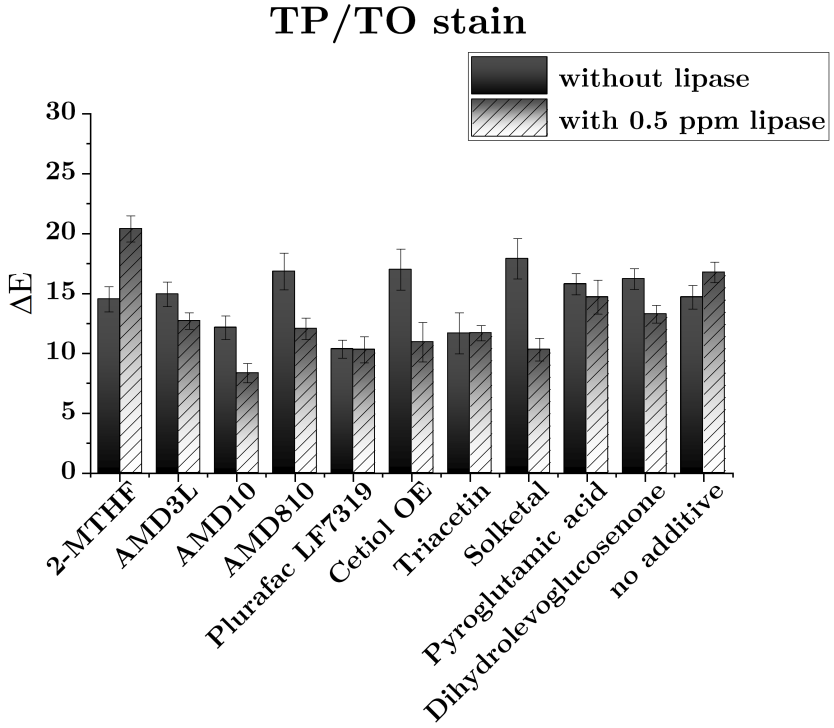

Figure 11.10: Averaged ΔE values of cotton stripes soiled with Biskin mixture dyed with sudan black B determined with a colorimeter by measuring them before and after washing with the 1:1:1 surfactant system with the listed additives at a concentration of 100 ppm each.

The overall lower ΔE values compared to the washing tests with the Biskin stain

are due to the staining process itself. When TP/TO is used, the stained stripes are somewhat lighter in colour even when the degree of soiling is the same. This leads to smaller differences between stained and washed stripes, resulting in smaller ΔE values.

Table 11.10: Averaged ΔE values and standard errors represented in Figure 11.10.

Additive	**ΔE without lipase**	**ΔE with lipase**
2-MTHF	14.5±1.1	20.4±1.1
Agnique AMD3L	14.9±1.0	12.7±0.7
Agnique AMD10	12.1±1.0	8.3±0.8
Agnique AMD810	16.8±1.5	12.1±0.9
Plurafac LF7319	10.3±0.7	10.3±1.1
Cetiol OE	17.0±1.7	10.9±1.6
Triacetin	11.7±1.7	11.4±0.6
Solketal	17.9±1.7	10.3±0.9
Pyroglutamic acid	15.8±0.9	14.7±1.4
Dihydrolevoglucosenone	16.2±0.9	13.3±0.7
no additive	14.7±1.0	16.8±0.8

11.6 Conclusion

The first conclusion drawn from the washing tests is that designing and building the testing unit was a success, as the washing tests could not only be increased in quantity, but also in terms of quality. The initially designed tests that were performed in previous works (e.g. [23, 24]) lacked the mechanical input that laundry cleaning in a washing machine has. Internal discussions came to the conclusion that the mechanics of a washing process are very important and tests ignoring that input completely and only regarding solubility may not properly represent the performance that a formulation would show in a real-life scenario. As mentioned, the DTU was designed to represent a washing application as realistically as possible while staying within the scale of laboratory experiments. As actual washing machines require several kilograms of laundry, the ability to downscale to a few grams of fabric was very important.

In the beginning, room temperature washing tests with the old glass beaker method were attempted, but as even well-known brands of laundry detergents performed poorly and without recognizable reproducibility, they were completely discarded. With the testing unit, reproducibility was increased, as the experimental conditions could be controlled precisely. Also, with the inclusion of mechanical effects, the performance of formulations improved to a plausible level. Overall, the testing unit was used extensively during this work, with over 2'000 washed cotton stripes.

Initial tests were performed with various surfactant systems. As they had shown different amounts of dissolved fatty acid, it was tested if their ability to dissolve fats transferred directly to the washing performance. Biskin was chosen as model soil, as it represented a good mixture of natural fats. These tests showed that washing performance and solubility behaviour do not correlate, which had been expected. The highest solubility of palmitic acid had been seen for pure Lutensol AO7, a nonionic surfactant. In the washing tests, a formulation containing only Lutensol AO7 and no further surfactant, showed very poor results. The 1:1:1 mixture of a nonionic surfactant, a sulfate and a benzene sulphonate, a mixture commonly used in commercial laundry detergents, exhibited the best washing performance. This was the first sign that, in order to increase the washing performance, the solubility behaviour is not the right property to look at.
Bueno et al. discovered that the stain removal kinetics for solid soils are mostly dependant on the mechanical input. They described that for solid soils, increased mechanical action leads to higher rates of removal[178]. Removing the stain does not necessarily dissolve it. However, it seems that dissolving it is not necessary. They also explained that the mechanical input is less important for liquid soils. This would explain the disparity between the washing tests and the solubility tests, as only solid fatty acid was considered for the solubility tests. These tests were also designed to specifically ignore physical input. This shows that the washing tests, where chemical and mechanical influences are considered, are much better suited to test if a formulation holds up in an application-near environment.

Since it was assumed that fatty acids acting as co-surfactants can facilitate further removal of a fatty stain, hydrolysed Edenor coconut fat was employed. The concentration used was well below that of the regular surfactants. No performance boost in washing performance could be seen, with or without enzyme. This may

be due to the fact that the surfactant concentration was lowered to incorporate the coconut fat, which essentially just replaced one surfactant with another. It is generally accepted that higher concentrations of detergent lead to higher soil removal [179]. However, the efficiency of a surfactant at equal concentration must also be taken into account[24, 180, 181]. It appears that the hydrolysed coconut fat is not more efficient than the surfactant system.

Water hardness tests were performed to see how the washing performance changes when different builders are employed, especially in regard to the performance of the enzyme. Builders are needed in any laundry detergent, since very hard water will impede the washing performance, as removed fats may redeposit on the fabric as lime-soaps[29, 182]. For these tests, generally a lower water hardness was better. When sodium citrate was used with stripes soiled with TP/TO, washing results were clearly worse and the enzyme's activity seemed to decrease, when Trilon M was used, while choline citrate worked best. For the Biskin stain, the influence of the builder was hardly visible, with Trilon M performing only slightly below average.

For the additive concentration the most important result is that if the additive concentration surpasses the surfactant concentration, the overall washing performance decreases to a level, where even an increased performance boost of the lipase, which was seen for very high 2-MTHF concentrations, cannot counterbalance the loss of washing performance. There seems to be an interaction that hinders the surfactants if the additive concentration is too high, e.g. the soil surface and the media directly surrounding it change in properties too much for an efficient roll-up to occur.

The additives chosen for comparisons were picked to test different properties. Some, like 2-MTHF, were mostly chosen to function as co-solvents, and were expected to be present on or within the fat stain, possibly swelling it, with partition coefficients more towards the fat side. Others were more amphiphilic with a surfactant-like structure, like Cetiol OE, acting as co-surfactants in the mixture. For the tests with Biskin stain, all additives except for Triacetin did increase the washing performance, mostly by small margins. But the performance increase by the additive itself was not the concerning factor here. The increase gained in washing performance after adding the enzyme was the most important factor. The comparisons with the systems without enzymes were made to see if the relative increase of

washing performance after adding the enzyme is bigger in a system with additive than it is in the base system without additive. Such an increase would show a synergistic interaction between the enzyme and the additive, signaling that they could be combined for a more effective laundry detergent. These systems would also have priority in further evaluations that should discern how this synergy is achieved. Table 11.11 shows the relative washing performance increase after lipase addition of the systems in the Biskin stain batch.

Table 11.11: Relative washing performance increase, calculated from the ΔE values of the additive sweep with Biskin stain.

Additive	**Relative performance increase**
2-MTHF	19.0%
Agnique AMD3L	16.5%
Agnique AMD10	8.8%
Agnique AMD810	1.4%
Plurafac LF7319	16.1%
Cetiol OE	4.1%
Triacetin	23.1%
Solketal	8.1%
Pyroglutamic acid	0.5%
Dihydrolevoglucosenone	10.0%
no additive	13.8%

By comparing the ΔE values of the samples with enzyme to those without enzyme, it can be calculated that 2-MTHF, Agnique AMD3L, Plurafac LF719 and Triacetin lead to a relative washing perfomance increase after enzyme addition that was greater than the increase of the system without additive. For the Triacetin samples, the overall performance was still rather bad, even if this system showed the greatest relative performance increase after enzyme addition. But the addition of the short-chain triglyceride caused a severe decrease in performance without enzyme. Of the other two additives that have proven beneficial for the lipase's performance in a washing test scenario, 2-MTHF and Agnique AMD3L fall under the co-solvent approach. Two possibilities arise here: either their solvent properties cause them to "soak" into the fat stain, effectively swelling it. This could cause gaps to appear in the stain, increasing its surface, giving the lipase a larger area to attack. The increase in washing performance then stems from an increased accessibility of the fatty stain

for the enzyme, increasing its efficiency, while the activity, i.e. the turnover rate, remains unchanged. The other possibility is that the additive directly increases the turnover rate of the enzyme, increasing its activity, e.g. by stabilizing the transition state during the enzymatic hydrolysis of the fat or by facilitating the opening of the lipase's lid covering the active site. It has been shown that the activity of lipases can indeed be increased in aqueous solutions with low amounts of other compounds, e.g. ionic liquids[183, 184]. The used enzyme lipolase has, as many lipases, an α-helix lid with a hydrophilic outer surface that is closed in an aqueous environment and covers the active site. The lid only opens at a hydrophobic interface and the lipase becomes active[131, 132, 138, 139]. If the lid opening could be facilitated by a co-solvent, the activity of the lipase can be increased. Solvent-induced lid opening has been studied, and it was shown that lid opening is favoured in a less polar environment and can be driven by the solvent, independent of a substrate molecule[185–188]. These findings favour the possibility of increased activity. A combination of the two effects is also within the realm of possibilities. Further experiments were designed to illuminate what causes the synergistic effect in the washing tests.

Plurafac LF7319, on the other hand, is a nonionic surfactant, consisting of alkoxylated, mainly unbranched fatty alcohols. The higher surfactant content facilitates the removal of the fat by the mechanisms explained in section 2.3.3, as is expected at increased surfactant concentration[179]. The synergistic effect from enzyme addition is likely from the higher surface area the enzyme can access through the increased removal by the surfactant. Plurafac LF7319 was the only amphiphilic additive that did not destabilize the lipase in the washing tests, which explains why this system outperforms the other systems with similar additives.

Similar comparisons cannot be securely made for the tests with the TP/TO stain, as results varied strongly and the results with enzyme added were, for a significant part of the experiments, worse than those without. It is possible that the high soiling degree of the stripes compared with the low temperature that was used makes reproducible washing with these conditions and the TP/TO stain impossible, as this stain was more persistent than Biskin.

12 Langmuir-Blodgett Trough Tests

12.1 Introduction

While the washing tests did show how the washing performance of a detergent formulation can be influenced with a lipase and additives, the systems tested are overall too complex to determine the influence of specific interactions that occur during washing. Even if simplified formulations are used, too many factors play into the overall washing process. In order to be able to properly explain phenomena observed in the washing tests, the system has to be simplified further to isolate components in a way that specific interactions will be exposed.
One way that this goal could be achieved is to test the lipase on a well defined triglyceride monolayer instead of on a stained cloth with unknown surface geometry. As the lipase would cleave the triglycerides, the monolayer's properties would change, since the fatty acid content in the monolayer would increase. In the terms of a Langmuir-Blodgett balance, where the lipase is present in the subphase, this would lead to a change in the surface pressure - area isotherm, which can be detected with a Wilhelmy plate. By recording the isotherm after a certain incubation time and modifying the subphase further by adding additives, influences on the lipase's activity can be detected. However, this method is limited to additives that experience no or only very little surface activity and are found in the bulk phase of the Langmuir trough and not in the monolayer.
For the first tests, no enzyme was used. Instead, isotherms of mixtures of tripalmitin and palmitic acid were recorded to see how an increasing fatty acid content influences the isotherm. Later, lipase was added in various concentrations and isotherms were recorded after different periods of time to assess which combination of lipase concentration and incubation time leads to a satisfying base line that is visibly different from a system without lipase. Only then additives were added, at a concentration that does not change the monolayer when no lipase is present, but is high enough to have an impact on the lipase. Ideally, these isotherms could be compared to the mixtures of tripalmitin and palmitic acid recorded previously in order to estimate how much fatty acid has been generated by the lipase.

12.2 Determining the Influence of Free Fatty Acid in a Mixture with its Triglyceride

Initial tests were designed to determine the suitability of this method for the considered purpose. To this end, mixtures of tripalmitin and palmitic acid in chloroform were prepared. The mixtures were then used to prepare monolayers on the LB trough and the surface pressure - area isotherms of these mixed monolayers were measured. As the curves for palmitic acid and tripalmitin differ significantly (tripalmitin does hardly show any visible phase transitions), it was expected to see a continuous development from pure tripalmitin to pure palmitic acid. The isotherms of the mixtures are shown in Figure 12.1. It should be noted that the area is given as the total area of the monolayer in cm^2 instead of the more conventional area per molecule in either nm^2 or $Å^2$. This is because mixtures of molecules with different molar masses were used. With an averaged molar mass of the mixture, an average molecular area might be determined, but as that average molar mass would be different for each mixture, comparisons would be difficult. So, it was decided to use the total monolayer area instead.

The measurements show that the influence of PA can hardly be seen below a molar ratio of 1:1. The isotherm of the mixture with equal molar ratio is the first one that shows a visible phase transition that originates from the palmitic acid. At lower palmitic acid fractions, any effect on the acid is too small to be visible. For the 1:1 and the 40:60 curve, a change in the isotherm that is similar to a 2D-phase transition of a pure fatty acid appears at a surface pressure of approximately 50 mN/m. For the 20:80 isotherm, this phase transition shifts to the lower end of the curve. It is assumed, that this is the liquid-like state of the fatty acid, as the 20:80 isotherm begins to resemble the isotherm of pure palmitic acid.

These results led to the decision that more isotherms for mixtures with a molar fraction of palmitic acid of at least 0.5 have to be measured. The goal was to find a characteristic that is proportional to the molar fraction of palmitic acid.

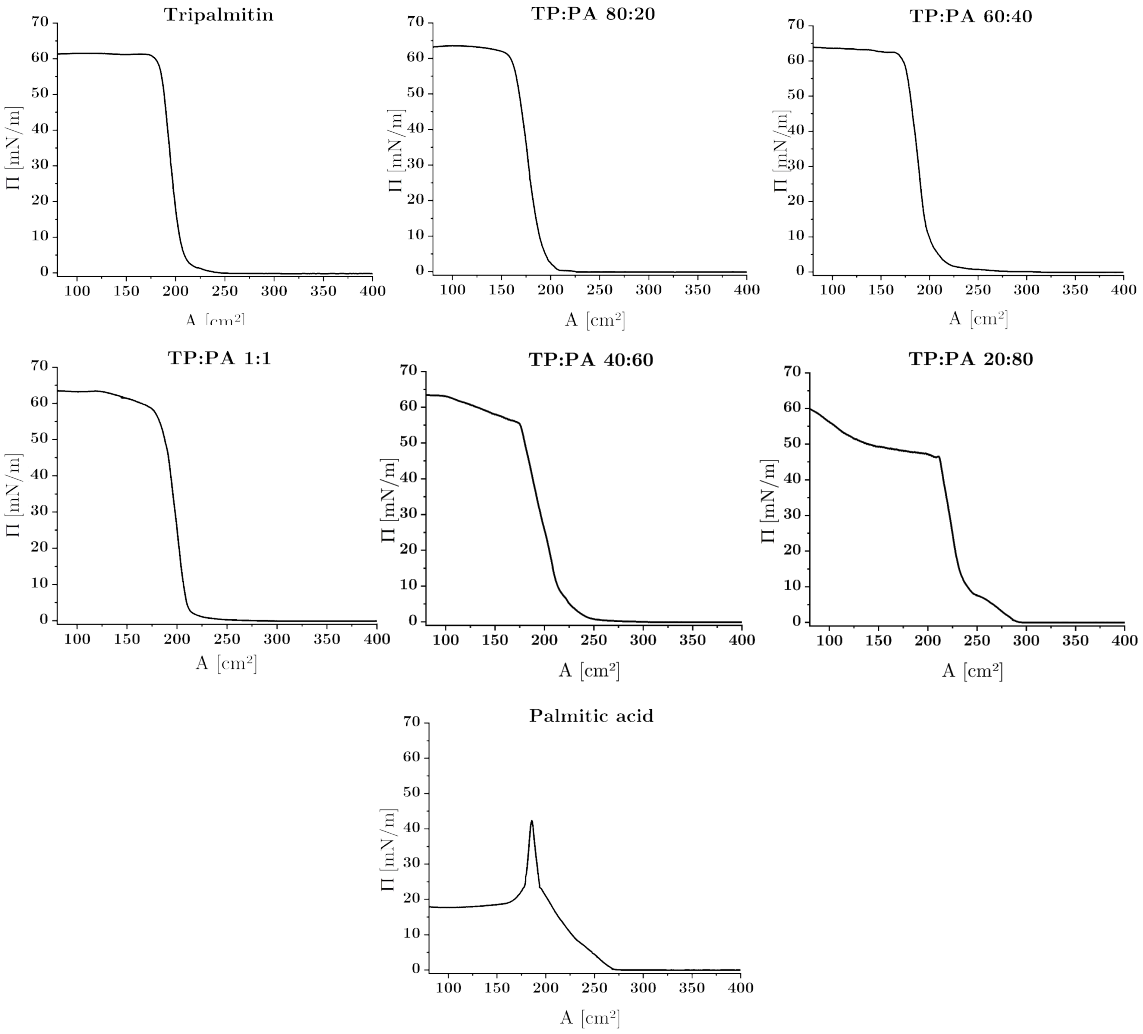

Figure 12.1: Surface pressure - area isotherms of mixtures of palmitic acid (PA) and tripalmitin (TP). Ratios are molar and given as TP:PA.

12.3 Finding a Characteristic for the Palmitic Acid Content in the Isotherms that is Suitable for a Calibration

With increasing palmitic acid content in the monolayer mixture, a region began to show in the isotherms that appeared to be a 2D-liquid phase. It was assumed, that this area is partially proportional to the palmitic acid fraction in the sample. This area will in the following be referred to as compressible area. An isotherm with equal molar amounts of palmitic acid and tripalmitin, was inspected more closely.

Three possible ways to analyse the isotherms were considered (Figure 12.2).

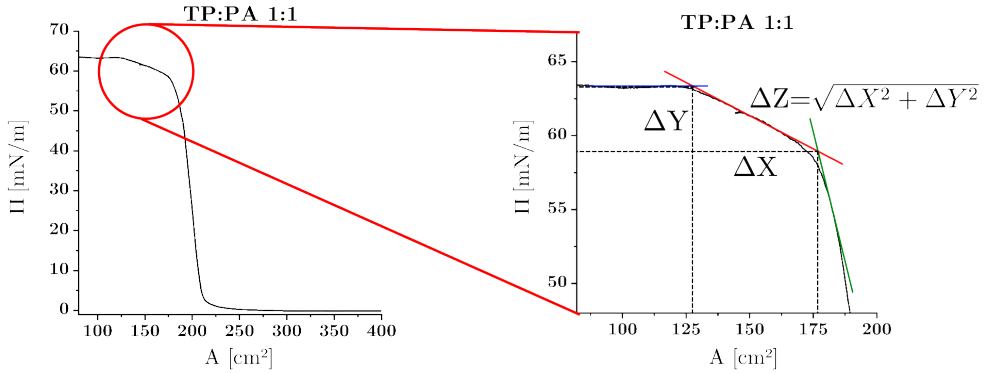

Figure 12.2: A closer inspection of the compressible area of the LB-isotherm of the 1:1 TP:PA mixture. By extrapolating the areas with different slopes, a length (ΔZ), height (ΔY) and width (ΔX) of the compressible area can be determined.

This analysis led to three values associated with the compressible area. The ΔX defines the change in surface area of the compressible part of the isotherm. ΔY represents the change in surface pressure during this compressible phase. ΔZ is the distance between the two intersections of the linear slope representing the compressible area with the two adjacent linear slopes. ΔZ was considered because the slope of the compressible area changed with increasing palmitic acid concentration and might therefore be relevant in finding a characteristic that is directly proportional to the palmitic acid fraction in the LB-film.
Subsequently, the compressible areas of all isotherms with a PA molar fraction between 0.5 and 1 were analysed in this fashion. In Figure 12.3, ΔX, ΔY and ΔZ were plotted against the molar fraction of palmitic acid.

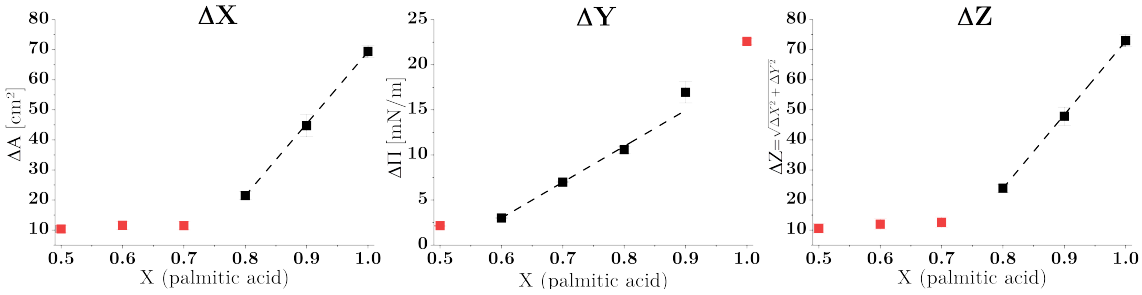

Figure 12.3: Plots of ΔX, ΔY and ΔZ of the compressible areas of isotherms of TP:PA mixtures with a molar fraction x of PA of at least 0.5. Red coloured data points were not considered in the linear functions shown between the black data points.

All three plots show a linear dependence across a part of the measured mole fraction range. The ΔX and ΔZ plots show a linear correlation for between X(PA)=0.8 and

X(PA)=1.0. Both reach a minimum in the range $0.7 < X(PA) < 0.8$. Since all three values in that range are practically the same, this range cannot be used for a calibration of the molar fraction of palmitic acid.
The ΔY plot shows a linear dependence between a molar fraction of palmitic acid of 0.6 and 0.9. In the range of $0.5 < X(PA) < 0.6$, there seems to be a minimum for $\Delta\Pi$. The value for pure palmitic acid, on the other hand, seems to high compared to the other values of $\Delta\Pi$. It is outside the linear range and cannot be considered for a calibration of X(PA).
In the course of the experiments, the change in surface pressure ($\Delta\Pi$) of the compressible area was used as the preferred calibration for the molar fraction of palmitic acid. Since a mixture with a molar ratio of TP:PA of 1:1 was used for experiments with lipase in the subphase, a calibration that can be used from a molar fraction of 0.6 on seems more suitable. Also, using the other two options would result in working relatively close to a system with only palmitic acid and differentiating between additives might prove difficult. The linear functions of the three plots are:

- For ΔX:
$$\Delta A = 239.55 \cdot X(PA) - 170.21 \tag{12.1}$$
- For ΔY:
$$\Delta\Pi = 39.82 \cdot X(PA) - 20.89 \tag{12.2}$$
- For ΔZ:
$$\Delta Z = 245.09 \cdot X(PA) - 172.18 \tag{12.3}$$

12.4 Finding a Suitable Enzyme Concentration and Incubation Time for Analysis

The next step was to test various enzyme concentrations (in the subphase) and incubation times and to determine, how much palmitic acid had been released by the enzymatic reaction. In theory, since the enzyme is active at a water/fat interphase, cleaving of triglycerides in the monolayer should be possible. This would lead to a change in the expected surface pressure - area isotherm after incubation, as the mole fraction of palmitic acid would be higher than the initial 0.5.
The first measurements were performed with an enzyme concentration in the subphase of 1 ppm. This proved way too high, as the presence of enzyme on the

monolayer was showing in the isotherm. It was calculated that approximately 0.17 ppm is the amount of substance (in this case the 1:1 TP:PA mixture) used to form the triglyceride/fatty acid monolayer. It was deemed necessary that the enzyme concentration in the bulk needed to be significantly lower than the amount of fat on the monolayer. The next tests were performed with a lipase concentration of 25 ppb. At this concentration, recording an isotherm without spreading a substance on the subphase first, led to a flat line, which means that there was no significant amount of enzyme present at the surface. As the trough was cooled to 20 °C during the measurement, an initial incubation time of 30 minutes was chosen to account for both the lower activity of enzymes at lower temperatures and the fact that the lipase is only present in the subphase and must diffuse to the monolayer. Lower incubation times were also tested. Figure 12.4 shows the initial measurements with enzyme compared to an isotherm of a 1:1 mixture without enzyme.

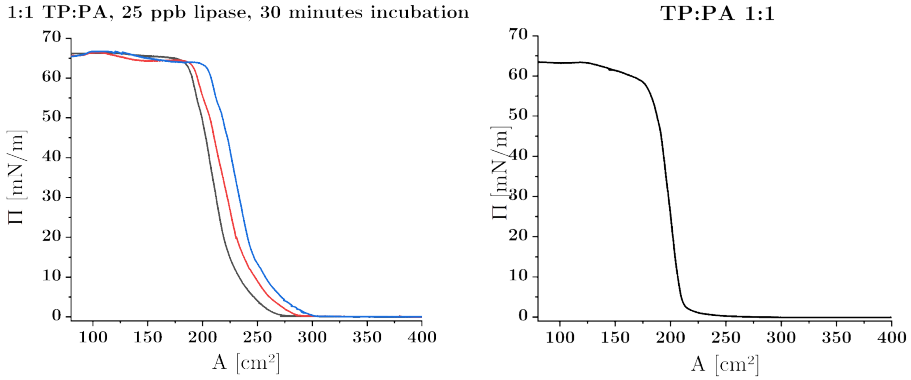

Figure 12.4: Isotherms of 1:1 TP:PA mixtures at 20 °C. Left: 3 equal measurements with 25 ppb lipase in the subphase and 30 minutes incubation time. Right: without lipase.

What can be seen here, is that a change in the progression of the surface pressure between approximately 300 and 220 cm^2 indicates a phase transition. But unlike for the mixtures without lipase, the end of this transition is not clearly visible. Instead, the increase of the slope appears more continuous, making evaluation difficult, as it is unclear how two of the three linear functions should be fitted to the isotherm. A possible answer to this is that the recording of the isotherm itself is too slow, and the amount of free palmitic acid increases too fast during the compression. This would indictate, that the lipase concentration is still too high.
Nevertheless, a test with additive under these conditions was performed. 10 ppm 2-MTHF was added to the subphase. The result can be seen in Figure 12.5 on the left. On the right, an isotherm of palmitic acid is shown for comparison. This

result indicates that the enzyme concentration and/or the incubation time is still too high, as the graph appears very similar to palmitic acid, which suggests that all of the tripalmitin was completely consumed by the enzyme. The less clean look of the isotherm in conjunction with the film collapsing at a surface pressure of 18 mN/m suggests that too much substance was loaded on the trough to form the monolayer. In this context, this makes sense, since in the case of complete cleaving, the molar amount of palmitic acid is three times the original amount of tripalmitin. While this result clearly shows an increased activity of the enzyme, since complete cleaving was not seen before without 2-MTHF, it also meant that the method still had to be adjusted to gain results that can be evaluated.

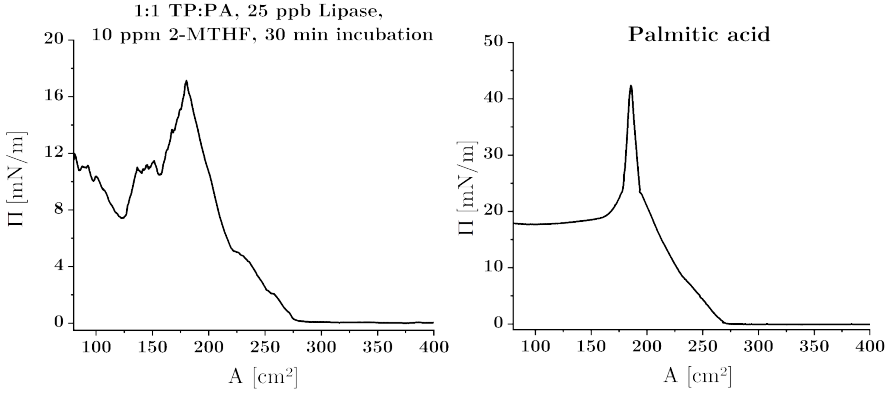

Figure 12.5: Langmuir isotherms at 20 °C. Left: 1:1 TP:PA mixture monolayer with 25 ppb lipase and 10 ppm 2-MTHF in the subphase and 30 minutes incubation time. Right: palmitic acid monolayer without lipase.

Following these experiments, the enzyme concentration was lowered further as the lipase concentration was still too high for proper evaluation. 5 and 10 ppb lipase concentration were tested. This meant that 0.5 μL of the 2% enzyme stock solution were added to either 1 L or 2 L of water that served as subphase to achieve such a low concentration.

Figure 12.6 shows a comparison of isotherms with 5 ppb Lipase, 10 ppb Lipase and 25 ppb Lipase in the subphase. The incubation time was 30 minutes for each enzyme concentration.

The experiments show that 5 ppb lipase is too little for the chosen incubation time. Longer incubation times than 30 minutes were deemed unfavourable, due to time requirements of the measurements. While 25 ppb seemed an appropriate concentration, tests with 2-MTHF showed that the enzyme concentration was still too high.

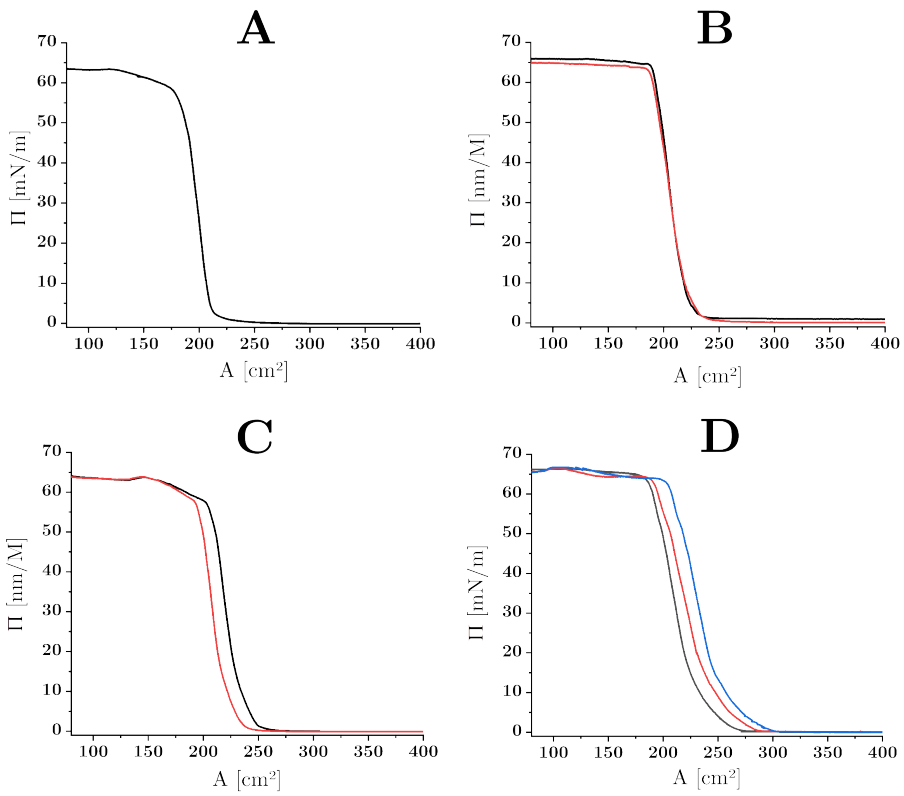

Figure 12.6: Langmuir isotherms at 20 °C with a 1:1 TP:PA mixture monolayer and 30 minutes incubation time before the isotherm was recorded. A: no lipase in the subphase. B: 5 ppb lipase in the subphase. C: 10 ppb lipase in the subphase. D: 25 ppb lipase in the subphase.

This is further supported by the comparison in Figure 12.7, which shows different incubation times with 25 ppb total enzyme. As they are all quite similar regardless of the incubation time, two things are possible: The enzymatic reaction without additive reaches a limit well before the isotherm can be measured (measurements take about 10 minutes per isotherm) or the influence of the lipase on the surface pressure isotherm is still too great and makes evaluation difficult. Considering these results, it was decided to continue measurements with 10 ppb lipase concentration.

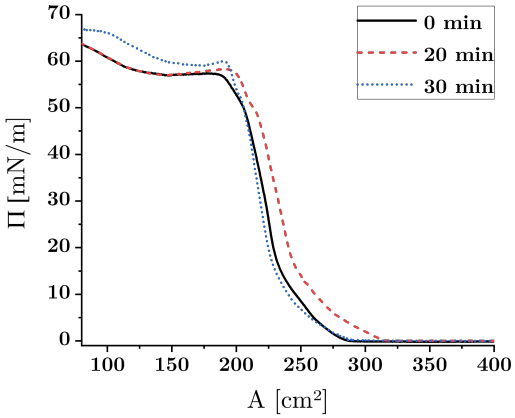

Figure 12.7: Langmuir isotherms at 20 °C with a 1:1 TP:PA mixture monolayer and 25 ppb lipase in the subphase with varying incubation times.

12.5 Comparison of Isotherms with Additives added to the Subphase

With the proper values for the lipase concentration and incubation time finally determined, comparison of additives was started. To ensure that enough additive is present below the monolayer to influence the enzymatic cleaving of the triglycerides, a relatively high additive concentration of 50 ppm was chosen, since it was expected that the additives were mostly equally distributed in the subphase with little to no surface excess beneath the TP:PA monolayer. Additives with an amphiphilic, surfactant-like structure could not be tested with this method, as they would definitely be present in excess at the surface and severly influence the surface pressure - area isotherm, making any evaluation of their influence on the enzyme difficult if not outright impossible.

All additives were tested without enzyme in the subphase initially, to determine if further evaluation is possible, which was not the case for Agnique AMD10 and AMD810. These molecules are dimethylamides of octanoic acid and decanoic acid. Due to the alkyl chains, these molecules would accumulate on the surface and influence the monolayer strongly.

Figure 12.8 shows isotherms of monolayers that were originally a 1:1 TP:PA mix. All of them were incubated for 30 minutes. The concentration of the lipase was 10 ppb and that of the additive 50 ppm, if not stated otherwise.

The top two isotherms are examples of measurements where evaluation was possible. The bottom two show isotherms where the additives are too surface active and

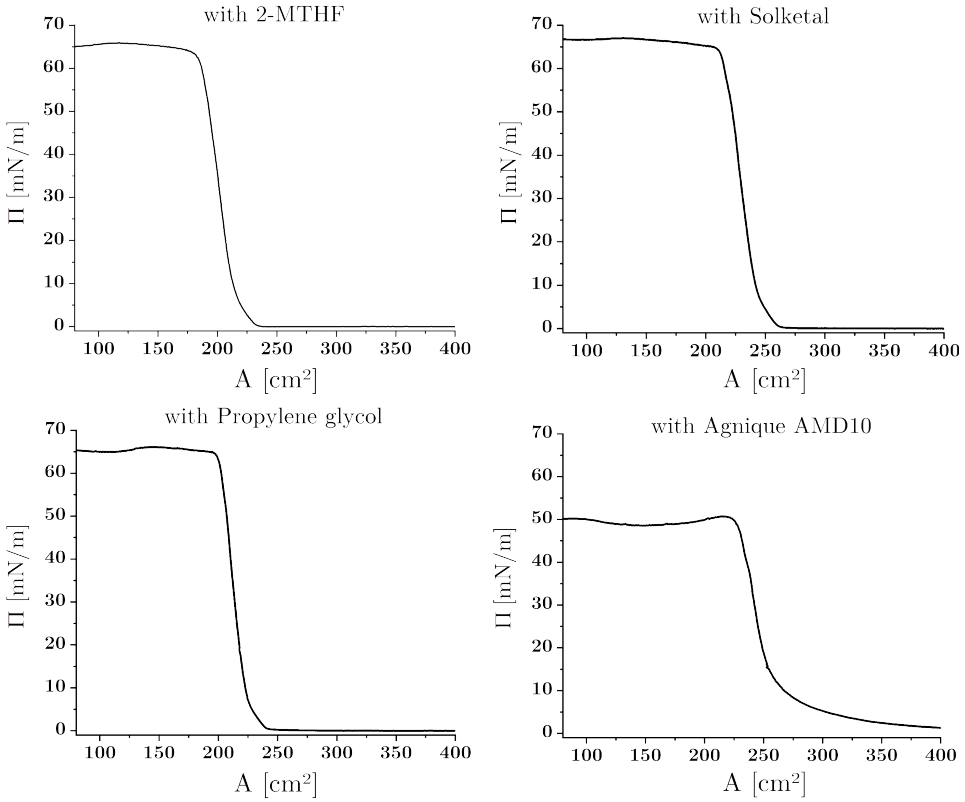

Figure 12.8: Surface pressure - area isotherms of samples where additive was added to the subphase. Agnique AMD10 was an example where proper evaluation was not possible, as the additive itself was very surface active and influences the isotherm significantly.

aggregate at the monolayer, making any evaluation impossible. Due to the high surface activity of the additives, the isotherms look the same regardless of whether lipase is used or not.

For the additives, where the increased enzymatic activity on the monolayer can be evaluated, it was attempted to calculate the mole fraction of palmitic acid that was present in the monolayer after incubation with the lipase. For 2-MTHF it was clear that there was a significant increase. Solketal also showed that the amount of free fatty acid on the monolayer had definitely increased. Figure 12.9 shows the results of the calculation, with the calibration function of $\Delta\Pi$.

It is important to note that this method is in the best case semi-quantitative and the exact percentages should be taken with a grain of salt. It can show tendencies of additives that increase the enzymatic activity, but it cannot calculate a precise turnover rate. The results suggest, however, that some additives do indeed directly increase the activity, i.e. the turnover rate of the enzyme. At the beginning of

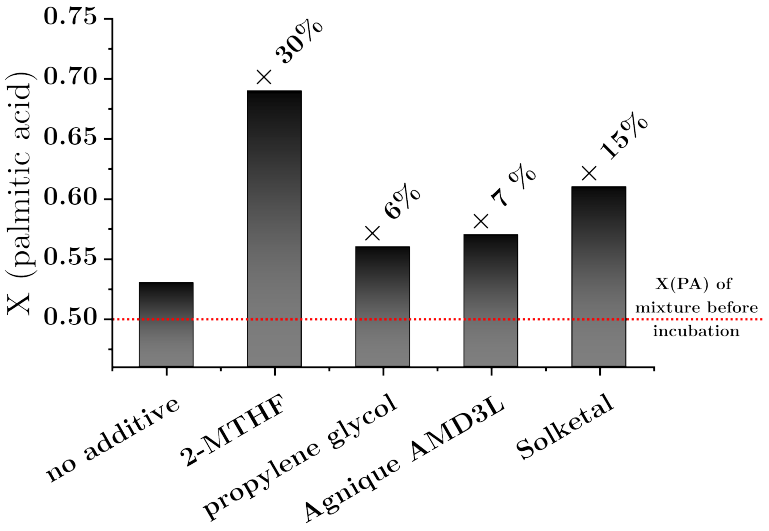

Figure 12.9: The mole fractions calculated from surface pressure - area isotherms of monolayers with 10 ppb lipase and 50 ppm enzyme in the subphase after 30 minutes incubation. The dotted line indicates the mole fraction of palmitic acid before incubation. The percentages given are the relative increase of the mole fraction compared to the sample without additive in the subphase.

the thesis, the hypothesis for the additives was that they would swell the fat stain and therefore make it more accessible for the enzyme, increasing the enzyme's efficiency, while the turnover rate remains the same. An increased presence of fatty acid in the Langmuir monolayers indicated by a change of the recorded isotherms, however, suggests that the increased accessibility of the stain is not the only possible function of additives. As the fat layer in these experiments is monomolecular, meso- and macroscopic effects, like swelling, cannot be responsible for the increased enzymatic activity. An increased turnover rate could be a possible explanation for the observations of the LB trough experiments. To verify this hypothesis, a more precise method had to be employed, which is why the lipase assay that the following sections deal with was developed.

12.6 Conclusion

The first insight that was gained from the experiments conducted here is that in a mixture of a triglyceride and its fatty acid, a correlation between the isotherm and the fatty acid content can indeed be drawn, albeit in a limited range. A linear correlation of $\Delta\Pi$ with the mole fraction of palmitic acid in the monolayer could be shown in a certain range. The area this linear correlation appeared in was not

present at all if pure tripalmitin was used. This correlation was of the most interest here and was used for further evaluation when using an enzyme.
Finding a balance between enzyme concentration in the subphase and incubation time for results that can be used to draw conclusions concerning the lipase's activity proved to be a rather delicate matter. The main issue is that the amount of fatty acid and/or triglyceride needed to form a monolayer is only about 200 ppb. Enzyme concentrations like they were used in the washing tests would be at least twice that amount. But since the enzyme is dissolved in the subphase before adding the triglyceride on the surface, lowering the enzyme concentration too far leads to extremely long incubation times until a significant change in the surface pressure - area isotherm can be seen. On the other hand, leaving the enzyme concentration too high leads to a complete hydrolysis in a few seconds. An enzyme concentration that worked well here was 10 ppb.
With the experiment conditions defined, it was tested how additives in the subphase change the amount of fatty acid on the monolayer after incubation with the lipase. The main issue here was, that it was impossible to use amphiphilic additives, as they would be found in excess on the surface and influence the monolayer strongly. In that case, no effect of the enzyme acting on the monolayer was visible, due to the additive changing the isotherm too strongly. Additives with very little surface activity that are likely to be evenly distributed in the subphase could not be used in extremely low concentrations as their influence on the enzyme working beneath the monolayer would be completely negligible. In the end, conclusive results could be gained for a few additives. For 2-MTHF, the result was a 30% increase in the molar fraction of palmitic acid compared to the isotherm without additives. The result for AMD3L was a little unexpected, as the increase of generated palmitic acid was rather low. This, however, suggests that the reason AMD3L shows a synergistic washing performance increase is not an interaction with the enzyme directly, as the activity of the enzyme does not seem to be increased too greatly. Instead, as previously mentioned, it is possible that the soil is swelled by the AMD3L and thus its surface is increased, enlarging the surface area the enzyme can access. The result for Solketal was a little surprising, as this additive performed poorly in the washing tests, where it reduced the performance of the lipase compared to a sample without additive. The washing test results, however, might be caused by another detrimental interaction that cannot be seen in the LB trough tests.

13 Lipase Assay with Photometric Analysis

13.1 Introduction

As mentioned in the introduction to the previous section, it became clear in the course of this thesis that there was a need to track the activity of the lipase in presence of additives to determine, which of the suggested additives increase the lipase's activity. Standardized assays to determine enzymatic activity exist for all enzyme classes. Commonly those assays consume ATP during one of their reaction steps and create a dye during the reaction which can be quantitatively determined with a regular photometer. The more dye is created in a certain amount of time at a certain temperature, the higher the enzyme's activity is.
Such a standard lipase assay was modified to fit the necessary requirements of this work. The assay procedure is explained in section 6.4. The aim was to find a method that can measure the activity of the lipase quantitatively. As the amount of dye created by the four step reaction directly corresponds to the amount of glycerol created by the lipase in the first step, this method was deemed fitting. Its procedure is relatively simple and for analysis a common UV/VIS photometer is sufficient. Since the initial hydrolysis reaction happens isolated from the later reaction steps, the reaction medium can easily be modified. The aim of the conducted experiments was to determine the influence possible detergent additives have on the activity of the lipase used in the washing tests. From these results, conclusions were drawn on how an enzyme-based washing procedure can be directly influenced and its performance increased by the addition of compounds that are beneficial for the lipase.
The initial work performed was to find a way to have uniform fat emulsions which could serve as the basis for the hydrolysis reaction. The preparation method for these emulsions had to ensure two things. Firstly, the stability of the emulsions over a period of time was long enough that the emulsion's properties do not change as much as to influence the result of the assay. Secondly, that the particles' size of the fat in the emulsion was consistent through all batches, as the particles' size determines the surface area of available substrate for the lipase. Also, tests without fat or enzyme, but instead specific concentrations of glycerol had to be performed to determine the concentration range of glycerol, in which the amount of generated

dye is linearly dependant on the lipase's activity.
After these initial experiments, the next step was to find the concentration of lipase necessary to create reproducible results, high enough to be safely over the quantifiable limit with some space for detrimental additives to still remain quantifiable, but low enough to see the effects of beneficial additives without leaving the linear range of generated dye during the assay procedure.
Here, a problem became evident. Extremely high lipase concentrations were necessary to create enough dye to quantify the lipase activity. Initially, a high surfactant concentration in the fat emulsions was blamed for this, but changes to the emulsions did not solve this issue. Therefore, the intent of the following section is to show how this initially developed method was unsuitable for the purpose it was to fulfil and thus why the HPLC based assay was developed.

13.2 Stability of Emulsions for the Assay

Since the assay published by Sigma-Aldrich[170] had to be adjusted, the first step was to determine the stability of the fat emulsions and suspensions with sufficiently high fat concentration. Lutensol AO7 was used as surfactant. Two methods of creating the emulsions were tested, homogenization with a high performance homogenizer and sonication with a sonicator probe (as explained in section 6.4). Table 13.1 shows an overview of the stabilities of the prepared emulsions.

Table 13.1: Stability of fat solutions. Biskin is only usable in emulsion using 1.5% total surfactant concentration, and sonication creates more stable solutions than homogenization.
ˆ : Aggregates formed after a while, usable if processed properly
* : Constant agitation required (e.g. magnetic stirring, ca. 800 rpm)

Solution	Homogenization	Sonication
Triolein + 0.5% AO7	Stable	Stable
Triolein + 1.0% AO7	Stable	Stable
Triolein + 1.5% AO7	Stable	Stable
Biskin + 0.5% AO7	Unstable	Unstable
Biskin + 1.0% AO7	Unstable	Unstable
Biskin + 1.5% AO7	Mostly unstableˆ	Semi-stable*
Tripalmitin + 0.5% AO7	Semi-stable*	Unstable
Tripalmitin + 1.0% AO7	Semi-stable*	Unstable
Tripalmitin + 1.5% AO7	Semi-stable*	Semi-stable*

While sonication resulted in better solution stability, it created an issue, where fat particles were small enough to penetrate or clog the filter. For this reason, homogenized solutions were used for the measurements.
A further batch was tested where the total surfactant concentration was limited to 0.1%. To ensure stable emulsions, only triolein was used and the fat concentration was lowered to 1%, since it was assumed that 1% fat should be enough substrate for the lipase. Homogenized mixtures were used here as well, because the sonicated emulsions were again difficult to filtrate without clogging the filter.

13.3 Lipase Assay Results

13.3.1 Pre-tests with Glycerol

To determine the amount of glycerol necessary to generate enough dye to quantify the enzymatic activity, preliminary tests with glycerol solutions of various concentrations were performed. The overall process of the assay was changed as little as possible. The glycerol dilutions were prepared so that the glycerol concentrations were 1, 5, 10, 50, 100, 500 and 1000 ppm after adding the 2 mL colour-developing reagent. The solutions were incubated for 15 minutes and then measured without further dilution. Figure 13.1 shows the determined absorbances for each sample.

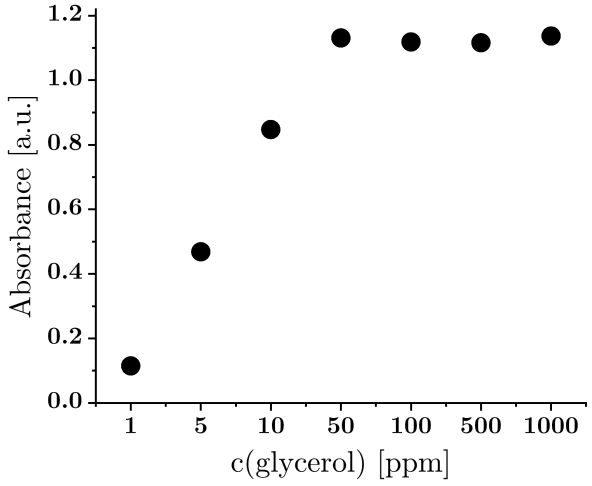

Figure 13.1: Optical density measurements at 545 nm of the glycerol dilution series.

The measurements show a linear increase of the absorbance until a saturation is reached at 50 ppm. The sample volume was 2.05 mL. This means that the assay

can detect as little as 2 µg of glycerol and a saturation is already reached if 100 µg of glycerol are present. Ideally, for quantification, the amount of generated glycerol after the hydrolization reaction of the lipase to be tested should be between 5 and 50 µg.

13.3.2 Initial Measurements

Initial assay measurements were performed with emulsions with 1.5% total surfactant and 10% fat. For TP and TO, homogenized emulsions were used. Since the Biskin emulsions were only stable enough if sonicated, sonicated Biskin emulsions were employed here.

The first step was to determine the amount of lipase necessary to generate enough dye to quantify the activity of the lipase. The lipase solution supplied by Sigma-Aldrich was listed with an activity of 100'000 U/mL and was diluted accordingly. Figure 13.2 shows the results of the absorbance measurements.

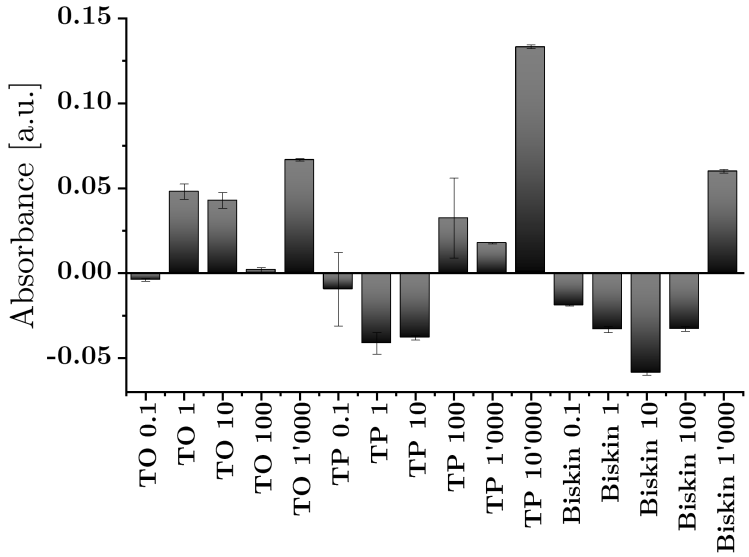

Figure 13.2: Optical density measurements at 545 nm of the order of magnitude sweep of the lipase concentration for all fats. Lipase concentrations are in U/mL. The concentrations given are those in the mixture after mixing the enzyme diluent with fat emulsion. Only the TP 10'000 sample showed a change in colour that was visible to the naked eye.

A significant colour change (as measured by change in optical density or absorbance at 545 nm in a 1 cm cuvette) could only be obtained, if the enzyme concentration was at 10'000 U/mL. For several samples the absorbance was smaller than the Blank (which results in negative values). This was unexpected, as the enzyme

concentration in the washing tests was only 2-2.5 U/mL and showed a visible impact on the washing performance. Also, since a few micrograms of glycerol were shown to be sufficient to generate a visible change in colour, this meant that hardly any glycerol was produced, except at extremely high lipase concentrations.
To see if the assay was sufficient to determine the impact of an additive on the activity of the lipase, an additive sweep with Biskin emulsions was performed. Due to previous results, the enzyme concentration used for this assay batch was 1'000 U/mL and the homogenized Biskin emulsion with 1.5% Lutensol AO7 was used again. Since the fat and the surfactant concentrations were very high, compared to the washing tests, the additive concentration was increased in a similar magnitude, compared to the washing tests, which is why 0.2% and 2.0% additive were added to the emulsions.

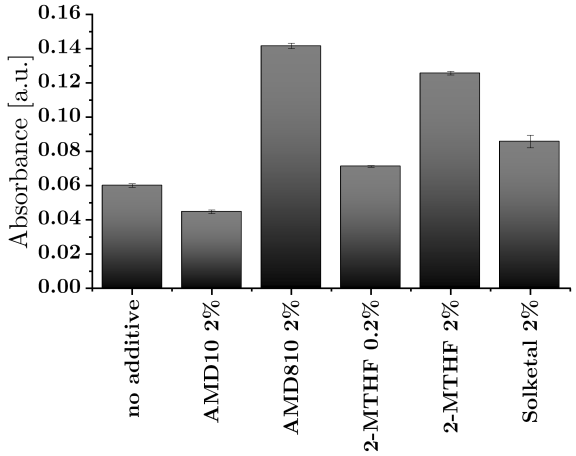

Figure 13.3: Optical density measurements at 545 nm to show the impact of additives on enzyme activity. Concentration in % indicates wt% additive in the lipase solution. Biskin emulsions were used and the enzyme concentration was 1'000 U/mL.

While the results indicate that the assay can theoretically be used to show the impact of additives on the activity of the lipase, the overall determined absorbance values are much too low, considering the high enzyme concentration. One hypothesis was that the surfactant concentration in the emulsions was too high and denatured the enzyme. Subsequently, it was decided to lower the total surfactant concentration to 0.1%, which is just marginally higher than the concentration used in the washing tests.

13.3.3 Measurements with lower Surfactant Concentration

The influence of a severely lowered surfactant concentration in the emulsions on the lipase performance in the assay was examined. In order to produce emulsions stable enough to perform the hydrolysis reaction of the assay, the fat concentration had to be lowered as well. As TO emulsions had proven the most stable before, these measurements were only performed with TO emulsions with a surfactant concentration of 0.1% and a fat content of 1%. Once again, a concentration sweep of the enzyme was performed to determine the concentration required to quantify the enzymatic activity. Figure 13.4 shows the results of these measurements.

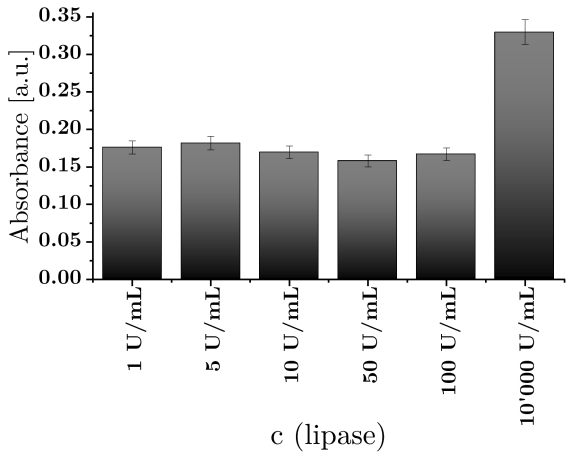

Figure 13.4: Optical density measurements at 545 nm of the concentration sweep of the lipase with the new emulsions with lower surfactant and fat concentration.

The results were very similar to the ones obtained with the emulsions with higher surfactant and fat concentrations. While the absorbance values were generally somewhat higher and never below the blank, only the sample with 10'000 U/mL lipase concentration showed any significant colour change visible to the naked eye. The other solutions were visually indistinguishable from the blank. This indicates, that the issue was not the high surfactant concentration, but another one altogether.

13.4 Conclusion

The initial assumption that the assay with the original emulsion with high surfactant content did not work because the surfactant denatured the lipase is not correct. Even with lower surfactant concentration (in a range similar to the detergency test),

no significant enzyme activity could be measured, except at very high enzyme concentrations. Considering that the assay created detectable amounts of dye with as little as 2 µg of glycerol present and a saturation was already reached if 100 µg glycerol were present in the solution, the conclusion has to be that the lipase used here does not generate glycerol, unless used at very high concentrations that are unrealistic for any application. However, that does not mean that the lipase does not function, it just means that the assay method that was employed here cannot detect its activity. It is possible that the lipase only cleaves one or two fatty acids from a triglyceride at normal concentrations, which does not generate any glycerol and is therefore not detected by the assay.
The next step was to find a method that can not only determine the actual products of the enzymatic hydrolysis but ideally also quantify those products, which would in turn allow to make conclusions about the turnover rate of the lipase in the presence of additives.

14 Lipase Assay with HPLC analysis

14.1 Introduction

Since the main result of the original assay was that the lipase used for the detergent formulation does not generate a significant amount of glycerol at regular enzyme concentrations, a new method had to be found in order to determine the main product(s) of the enzymatic reaction with the used lipase and ideally quantify them at the same time.
The method chosen here was a normal phase HPLC. This type of HPLC is rather uncommon nowadays. Typically, most HPLC measurements use a reverse phase setup, where the stationary phase is hydrophobic. In HPLC, this is commonly achieved by coating the silica particles in the column with linear alkyl chains between C8 and C18. In that case, polar solvents are used as mobile phase.
Due to the nature of the analyte of this work, reversed phase HPLC was not feasible, as long chain fatty acids, mono- and diglycerides were to be analysed. Eluting these molecules in a C18 coated column would likely take very long and use a large amount of solvent, or they could possibly also clog the column altogether. Therefore, a NP-HPLC setup with a polar column with uncoated silica particles and a non-polar mobile phase was chosen.
It was decided to use only triolein emulsions for the HPLC assay. Since triolein is liquid at room temperature, the triolein emulsions were more stable than those with tripalmitin. Also, it was simpler to only use one specific triglyceride, as several triglycerides at once (or a broader mixture like Biskin) would have produced convoluted chromatograms.
After preparing calibration curves for triolein and oleic acid as well as determining where the signal of the mono- and diglycerides appear, assay samples were examined. The first step (the hydrolyzation reaction) was not changed from the original assay, as it was intended to discover, why that assay had failed. Additives from the washing tests were included in the emulsions in the same way as with the photometric assay.

14.2 Initial tests and calibration

Based on publications where tri-, di-, and monoglycerides and fatty acids had been successfully separated using NP-HPLC[189–191], a method was developed that would not only allow to separate and identify the glyceride derivates, but to quantify at least one significant component of the mixture that would then allow to make conclusive statements on the lipase's activity in this sample. In order to keep the analysis simple, a single, pure triglyceride was to be used for this method. Triolein was chosen, for reason mentioned above. Also, Triolein, being an unsaturated fat, enabled the UV/VIS detection of C-C double bonds at 190 nm[192].

The first step was to develop a suitable method to determine the composition of the glyceride after hydrolysis by the enzyme. In accordance with [189], a gradient elution was chosen. The composition of the mixture can be found in section 6.4. Measurements with this method could not be evaluated, due to the extreme baselinedrift drowning any signals in the second half of the measurement. Figure 14.1 shows an example of such a measurement. This was not unexpected, as Ritchie and Jee[189] warned of the formic acid disturbing analysis by UV-absorption detection, which is why they switched to RI-detection. As RI-detection was not available here, the method had to be adjusted.

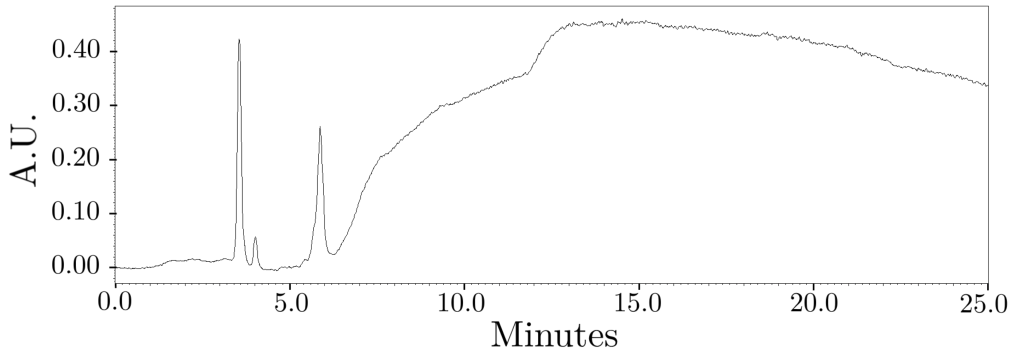

Figure 14.1: A chromatogram of a triolein/oleic acid 1:2 mixture with gradient elution and 0.1% formic acid in the mobile phase. The baseline drift makes evaluation of signals after 6 minutes impossible.

A switch to isocratic elution solved the problem of the baseline drift. It led, however, to the problem that the baseline itself was relatively high and made evaluation of samples with low concentrations difficult as the signals were very small. Once again, the formic acid was to blame for this. Not using forming acid, however, was not

possible, as in that case, the oleic acid signal became to wide and started to overlap with other signals. Figure 14.2 shows a comparison between a sample without any formic acid used (left) and one where formic acid was added to the mobile phase and isocratic elution was used (right).

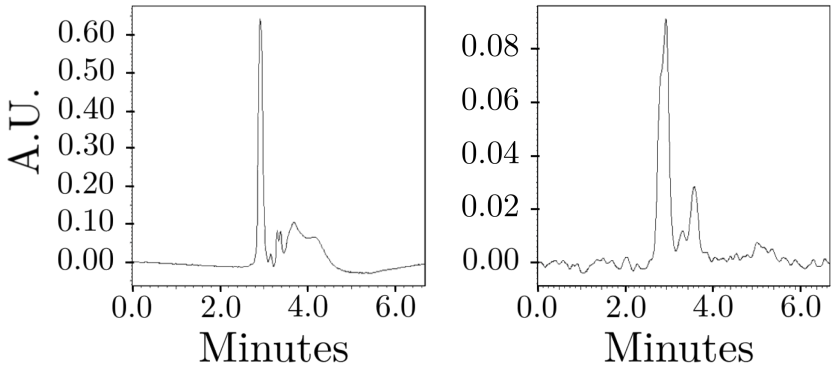

Figure 14.2: Comparison of two chromatograms of a 1:2 triolein/oleic acid mixture. Left: Without any formic acid, the oleic acid is present both protonated and de-protonated, leading to a broad signal that makes evaluation difficult. Right: With 0.1% formic acid added to the mobile phase, causing the oleic acid peak to be much narrower but also the signals to be very small (note the different scaling of the Y-axis).

To tackle the issue with the small signals, a new approach was tested. Instead of adding the formic acid to the mobile phase, 0.1% formic acid was added to the sample and another test-run was performed. This time, the results were far better. It appears that the formic acid in the sample is sufficient to obtain clean oleic acid peaks and overall too little to have an impact on the chromatogram. Figure 14.3 shows an example of such a successful test-run.

Even though the signal peaks were still relatively small, they were easily high enough compared to the baseline to continue with these conditions and further evaluate the mixtures. Due to this, tests with all possible degradation products were performed to find the elution times of the single compounds with the set conditions. Figure 14.4 is a chromatogram showing all possible components of the enzymatic degradation of triolein. The figure, however, does not show a single run, but several runs with different components (triolein and oleic acid in the first, both dioleins in the second and the monooleins in the third) superimposed onto one another to create a singular overview in one figure.

With these initial results in mind, the method now had to be calibrated to be able

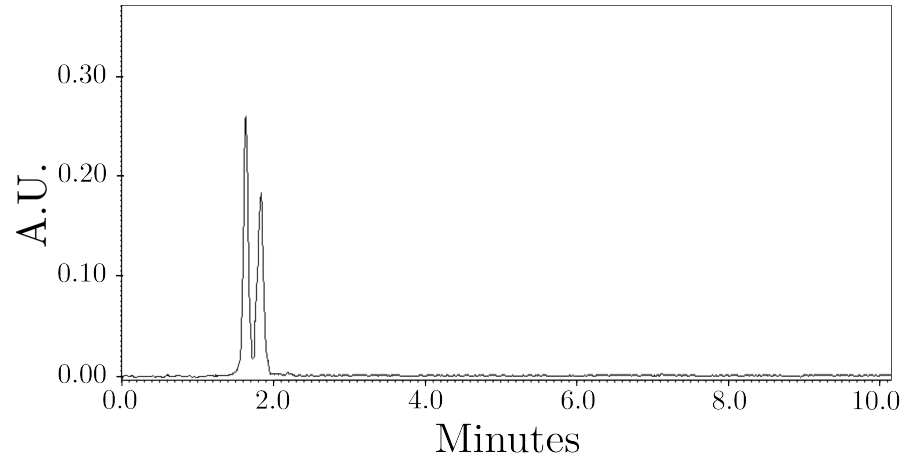

Figure 14.3: Chromatogram of a 1:2 mixture of triolein and oleic acid with 0.1% formic acid in the sample and isocratic elution with the hexane/2-propanol (90:10) mixture as mobile phase.

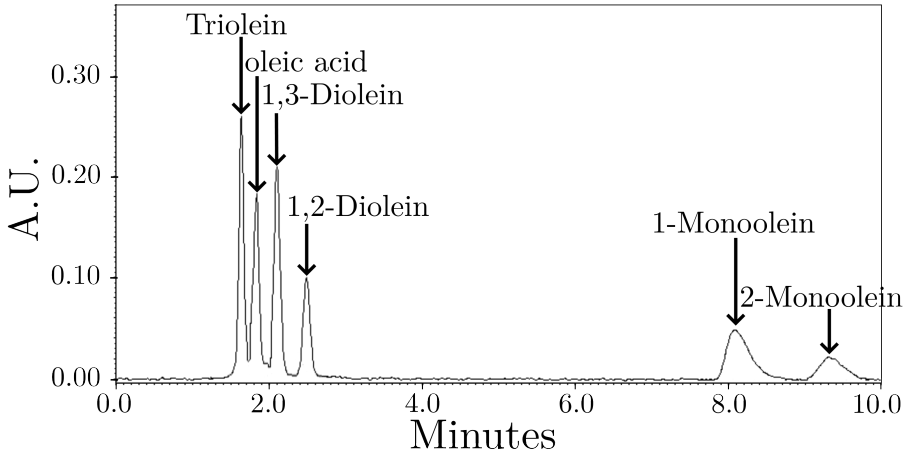

Figure 14.4: Chromatogram showing triolein, oleic acid and both mono- and dioleins that be present after hydrolysis of the triolein by the lipase. This overview was created from three separate chromatograms.

to quantify the samples from the enzyme assay. It was decided to use an external calibration method and prepare calibrations for two of the possible components. Firstly, triolein, and secondly oleic acid. For triolein, the idea was to determine the decrease in concentration compared to a blank from the same batch. The presence of oleic acid is the simplest indicator of enzymatic activity in the sample and should be detectable even at low turnover rates, where determination of a diolein might be difficult. Also, for these two components, the availability of calibration standards was a lot better.

The calibration was done by creating concentration series for triolein as well as oleic

acid. The peak areas of the substances were analysed and plotted against the concentration. Each calibration sample was measured three times and three series were created for each compound, creating overall 9 measurements for each concentration of each component. Figure 14.5 shows the calibration functions created for triolein.

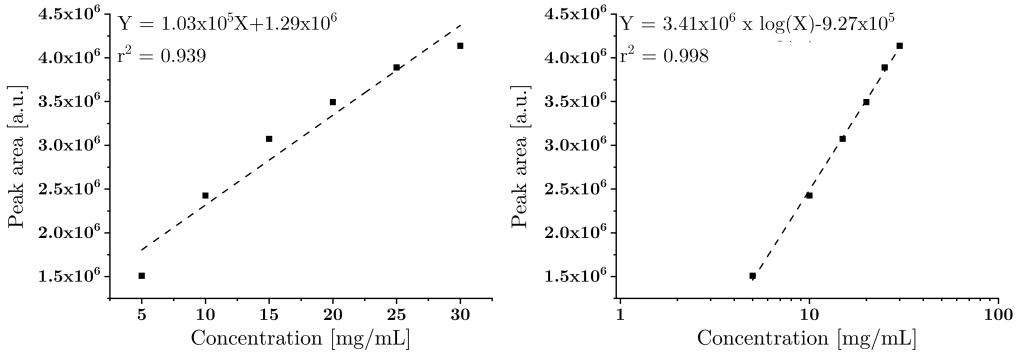

Figure 14.5: Linear (left) and logarithmic (right) calibration plots for triolein. The logarhitmic plot has a far better overlap with the obtained data, as shown by the r^2 values.

For oleic acid, the calibration was performed in the same way and calibration functions were calculated both linearly and logarithmically again. Figure 14.6 shows the calibrations plots of oleic acid.

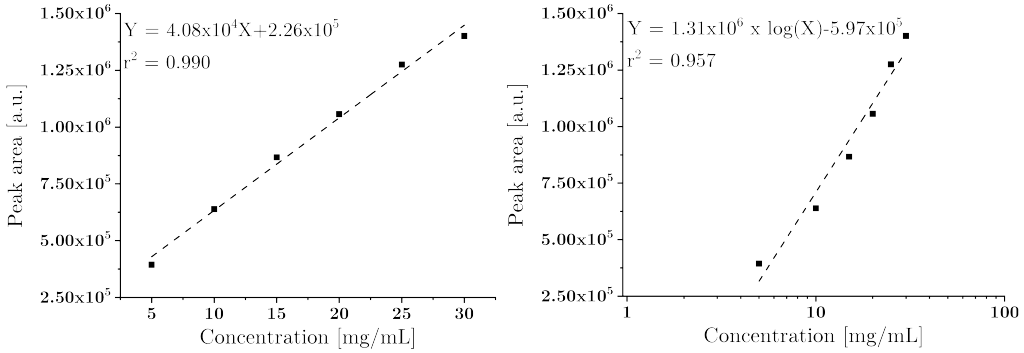

Figure 14.6: Linear (left) and logarithmic (right) calibration plots for oleic acid. Unlike for triolein, the better plot appears to be the linear one here, as shown by the r^2 values.

While for the triolein calibration, the logarithmic fit seemed to fit the data better, for the oleic acid, it is the other way around. The linear fit is here the one that fits the obtained data points better.
No further action was undertaken in regards to this matter. Since calibration functions were available, it was decided to move on to samples from the actual enzyme assay and see, how well the calibrations fare when used on those samples.

14.3 Analysis of Enzyme Containing Samples

14.3.1 Determining the Degradation Products of the Lipase

The next step was to take samples where the lipase was actually used on a triolein emulsion and analyse their contents. In order to understand, why the initial assay had failed, it was decided to keep the conditions as similar as possible to the original assay. The same pH 7.5 potassium phosphate buffer was used, even though it was suspected that the enzyme works better at higher pH. The enzyme concentration was 0.4 ppm (2 U/mL), incubation was done at 37 °C and for 30 minutes. The preparation and concentrations of the emulsions were left unchanged, and only emulsions with the lower total surfactant concentration of 1000 ppm were used.
The initial goal here was to determine, which degradation products can actually be expected from the enzymatic hydrolysis of triolein by the lipase. Figure 14.7 shows a chromatogram from this first batch of measurements. This chromatogram was created from a sample where only lipase was added to the emulsion at pH 7.5. The samples were then incubated as mentioned above. Stirring was done via a magnetic stirring plate at approximately 500 rpm.

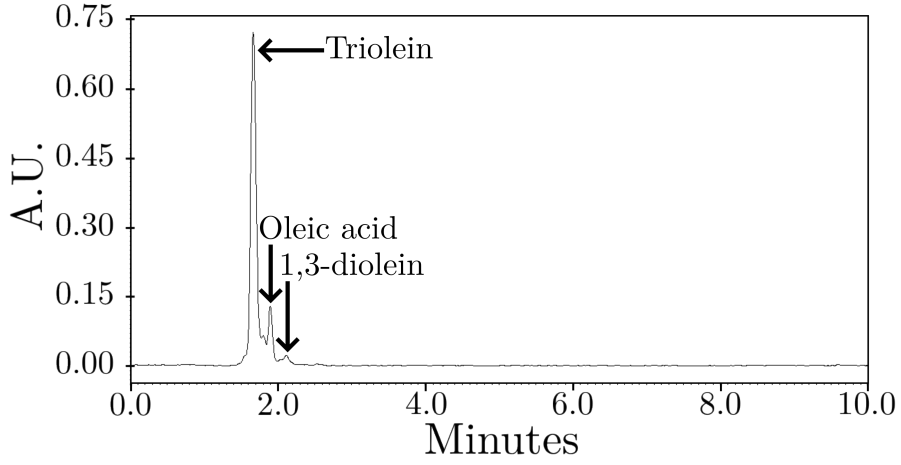

Figure 14.7: Chromatogram of an enzyme assay sample without any further additives added to the emulsion. The emulsion containing triolein was incubated for 30 minutes after adding 0.4 ppm lipase. After incubation, the sample was processed as described in section 3.2.5.

The results here show, why the originally planed assay was not suitable to determine the activity of the used lipase. The low amount of detected oleic acid, and the fact that only 1,3-diolein was detected of all possible degradation products

strongly suggests that this lipase does not generate any glycerol at "normal" concentrations. Instead, it only removes one fatty acid from the triglycerol, creating a 1,3-diglyceride in the process. The concentrations needed to create a sufficient amount of glycerol, i.e. to completely cleave the triglycerides present, was 10'000 U/mL, which translates to approximately 0.2% or 2000 ppm with the lipase used. Such concentrations are extremely unrealistic for any enzyme-driven process. As the chromatogram shows, at lower enzyme concentrations, the activity of this lipase cannot be quantified with the photometric assay as it only removes one fatty acid from the glyceride, not all three, as would be required.

14.3.2 Modifying the Assay's Parameters

As the next step, parameters of the assay were modified one by one to see the impact they had on the overall result. This was done to verify the HPLC method. The tested parameters were:

- Temperature
- pH value
- Enzyme concentration
- Water hardness

Only one parameter was changed at a time. The others were kept identical to the original batch, e.g., when the temperature was changed, the pH remained at 7.5, the enzyme concentration at 0.4 ppm and Millipore water, without residue water hardness, was used. Samples were prepared in sets of four and each sample was measured three times. Two blanks were prepared for each parameter modification. These were also measured three times each. The amount of oleic acid in the sample was then calculated from the peak area with the previously prepared calibration curves. The apex track algorithm of the "Empower 3" software was used to calculate the area of the peaks. This algorithm also allows to account for overlapping peaks. By substracting the mass of oleic acid found in the blank (the used triolein contained traces of oleic acid) from the mass of oleic acid found in the samples, the mass of oleic acid generated by the lipase could then be calculated. From this, the molar amount could be calculated, which was then divided by the incubation time to gain

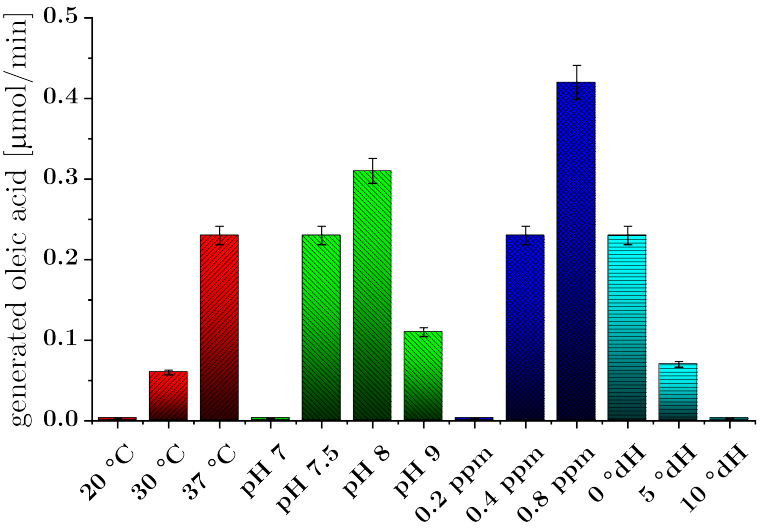

Figure 14.8: Lipase activity results for the parameter sweep. The 0 µmol of generated oleic acid per minute that were determined for some assay settings mean that the total mass of oleic acid in the sample was too close to the total mass in the blank to quantify, not necessarily that the enzyme was completely deactivated.

direct access to an activity parameter of the lipase (generated oleic acid per minute). Figure 14.8 compares the determined activities in the parameter sweep.
Most of those results are not surprising: A higher enzyme concentration leads to more oleic acid being generated, a lower temperature reduces the amount of generated oleic acid. That the results match the expectations for simple parameter changes means in this case that the method seems to function and does indeed allow to quantify the amount of oleic acid generated by the enzymatic hydrolysis of the triolein. The other parameter changes indicate that the lipase seems to function best at a pH of 8 and in purified water without residue water hardness present. At pH 7 and in hard water, too little fatty acid was generated to allow quantification.

14.3.3 The Influence of Additives on the Lipase

After clarifying if basic changes to the system are reflected in the results, the final step was to examine the influence that the various additives have on the lipase, as originally intended with the assay. It was decided to stick with the original parameters of the first HPLC assay test batch. The emulsions were prepared with the 20 mM potassium phosphate buffer with a pH of 7.5, the incubation temperature was kept at 37 °C, the incubation time at 30 minutes, the enzyme concentration at 0.4 ppm and purified water without residue water hardness was used. The additive

was added to the emulsion before the enzyme. The additive concentration was 100 ppm, equal to the concentration used in the detergency tests. For each additive, 4 samples and 2 blanks were prepared and were each measured three times. Figure 14.9 shows a comparison of the first three minutes of the chromatograms of assay samples without additive, with 2-MTHF and with Agnique AMD3L. This is enough to show the triolein, oleic acid and diolein signals. Due to the results of the first assay batch, monooleins were not expected.

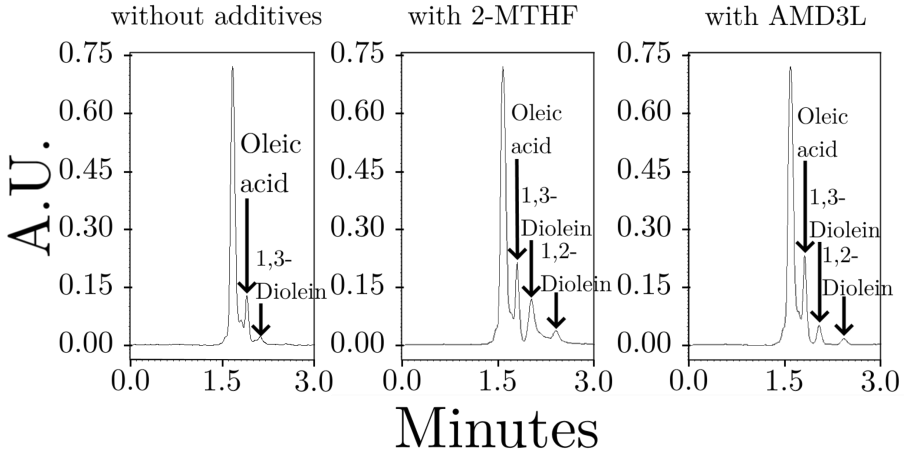

Figure 14.9: Section of the chromatograms of lipase assay samples without additives, with 100 ppm 2-MTHF and with 100 ppm Agnique AMD3L. The peaks of the visible degradation products of the hydrolysis reaction are marked.

Figure 14.9 compares the chromatogram shown in Figure 14.7 to the chromatograms of two additives which were deemed "good" additives in the detergency tests, i.e. additives that highly increase the washing performance with enzyme. One difference is immediately visible: the presence of a 1,2-diolein peak that is not visible in the chromatogram without additives. Also, the 1,3-diolein and the oleic acid peaks are significantly larger, if either of the additives is added. These results strongly indicate that the presence of these additives directly increases the activity of the enzyme and leads to a higher turnover rate. Also, the presence of 1,2-diolein suggests that the increased activity leads to a decreased selectivity, as not only the middle ester bond of the triglyceride is hydrolysed.

Upon closer inspection of the chromatograms, another finding was made supporting the increased activity hypothesis. As Figure 14.10 shows, the chromatogram of the sample that contained 2-MTHF showed a small and broad peak that becomes visible upon zooming into the chromatogram. This means that trace amounts of

1-monoolein were formed. Monoolein signals could not be found for any other sample that was tested in the HPLC during the course of this thesis. Two conclusions can be drawn from this: Firstly, these results support the increased activity hypothesis and secondly, they indicate a decrease in selectivity, as the presence of 2-MTHF seems to lead the lipase to also hydrolyse diglycerides, which it does not do normally at low concentrations, as indicated by the chromatogram of Figure 14.7.

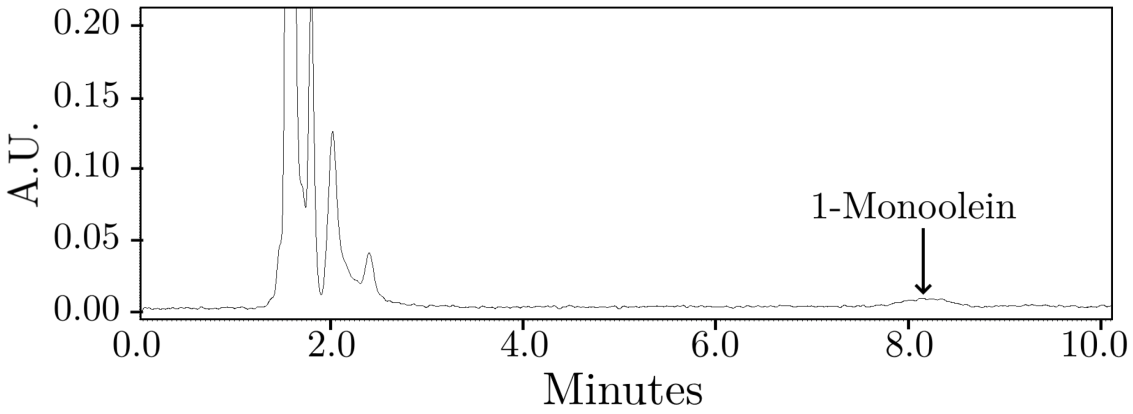

Figure 14.10: Chromatogram of an enzyme assay sample with 2-MTHF where the y-Axis scale was adjusted to magnify the 1-monoolein signal between 8 and 9 minutes.

With the method verified and qualitative information about the activity of the lipase gained from the chromatograms, a quantitative evaluation for the additives that were tested was the only thing left to do. The evaluation was done in the same way as the parameter sweep and Figure 14.11 shows the results. Once again, the generated molar amount of oleic acid per minute was used as an indicator of the enzymes activity.

As can be seen here, the only additive that was found to have a negative effect was Agnique AMD10, which was slightly unexpected, as this additive seemed to have a positive effect in the original assay with photometric analysis. That assay, however used 200 ppm of lipase for that particular batch and the determined absorbance was still very low (<0.2). This may further indicate that the originally planed assay was unfit to determine the activity of the lipase.

Looking at these results, the two additives mentioned in the comparison in Figure 14.9 stand out. The addition of Agnique AMD3L caused the generated amount of oleic acid to be tripled compared to the assay without additive and 2-MTHF even quadrupled the amount. These increases in oleic acid generation are backed by the aforementioned observation that for both additives the 1,3-diolein signal increased, and 1,2-diolein could be identified, which cannot be said of the chromatogram of the

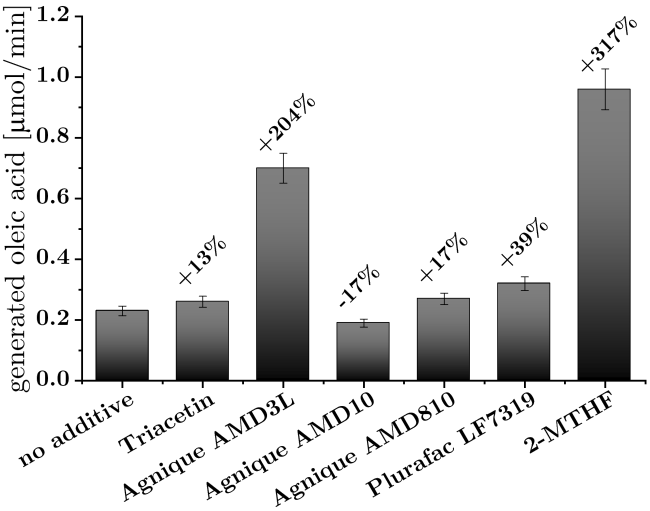

Figure 14.11: Lipase activity results for the additive sweep. The percentage values over the columns are the relative increase/decrease of the generated oleic acid, compared to the sample without additives.

assay without additive. Furthermore, for 2-MTHF, even 1-monoolein was identified, which was not visible in the chromatograms of any other assay sample. These results fortify the original assumption that some additives can indeed increase the turnover rate of the lipase.

The results also suggest a decreased selectivity of the lipase in the presence of these enzymes, which might be explained by the structure of the lipase mentioned in section 3.4.2. It is possible that the additives facilitate the opening of the hydrophilic lip, increasing the accessibility of the binding pocket. It was shown that solvent-induced lid opening is indeed possible[185, 186]. This however, might indicate a decrease in stability, due to the protective lid being opened more readily. To gain insight into possible stability changes of the lipase, DSC measurements were performed, which are presented in the following section.

14.4 Conclusion

Based on previous works where normal phase HPLC had been employed to separate lipids[189–191], an assay was developed to determine the turnover rate of a lipase. As expected, detecting the triolein and the possible degradation products of the enzymatic hydrolysis was possible, which tests with standards showed. Ritchie and Jee[189] warned of issues with a baseline drift when UV detection is used. These

issues occurred, but since RI detection was not available, a workaround had to be found. Others used evaporative light scattering detection (ELSD), separating and detecting mixtures of up to 16 lipid classes in one run[193]. As ELSD was not available, a method that works with UV detection was developed. As no fat mixtures were used, the sample solutions were much simpler in comparison to the other works, which proved helpful in finding a solution that works with UV detection. Triolein was chosen because oleic acid is an unsaturated fatty acid and the UV detection of unsaturated fats and fatty acids by detection of C-C double bonds had successfully been done before[192]. Tests showed that successful detection of the unsaturated lipids was indeed possible with the right experimental parameters.
The next step in developing the assay was to create calibration curves that can be used for quantification. Triolein and oleic acid were chosen for that. For triolein, the amount of triglyceride consumed by the assay was intended to be calculated. Oleic acid was chosen since it was easier to quantify than mono-, or diglycerides and was expected to be present at a high enough concentration, even at low enzymatic activity. An issue of the triolein calibration was that the dependence of the signal on the concentration was not linear, but logarithmic. An explanation was that the chosen concentrations were too high and a saturation was already reached. For oleic acid, the signal at equal concentrations was much smaller, since it contains only one double bond per molecule. For that reason, only the oleic acid calibration was used for further experiments.
To verify the assay, some parameters were modified that should result in obvious activity changes (e.g. temperature, enzyme concentration). The pH and the water hardness were also modified. The results showed that a higher temperature or a higher enzyme concentration results in more generated oleic acid, meaning a higher enzyme activity, as expected. The pH sweep showed an optimum of the lipase at pH 8 and the water hardness batch showed that the enzyme works best in Millipore water. These tests also revealed that the main reaction product of the lipase is the 1,3-diglyceride. No detectable amounts of monoglycerides were generated. These results show why the originally planed assay did not work. That assay needed glycerol, i.e. a complete cleaving of the triglyceride, to generate the detectable dye.
Finally, several additives were tested and compared to the base system, at pH 7.5, 0.4 ppm enzyme concentration and 37 °C incubation temperature. For 2-MTHF and Agnique AMD3L some changes were immediately visible. The oleic acid and 1,3-diolein signals were substantially larger. Also, for both additives, 1,2-diolein signals

became visible, suggesting that the increased activity leads to a decreased product specificity. Furthermore, small but noticeable monoolein signals were detected in the samples with 2-MTHF, suggesting that the increased activity leads to the lipase attacking diglycerides, which it normally does not in the relevant concentration range. This further strengthens the theory that 2-MTHF facilitates the opening of the hydrophilic lid, which dictates the enzymes activity and its selectivity[131, 132, 138]. Solvent-induced lid opening has been reported for solvents which lead to a less polar environment around the lipase[185, 186]. Such a shift towards the open-lid form of the lipase in the presence of 2-MTHF would explain the presence of monoolein, as the open binding pocket becomes more accessible for the surrounding substrate. In such a case, diglycerides might also reach the active site and be converted into a monoglyceride and a fatty acid.

Thus, it was no surprise when quantifying the generated oleic acid that 2-MTHF led to a huge increase in detected oleic acid. The amount detected in the base sample without additives was quadrupled when 2-MTHF was used. Agnique AMD3L also increased the amount of generated acid significantly. The other tested additives all led to small increases, except for Agnique AMD10, where a decrease of 17% compared to the base sample was detected, which suggests that the activity of the enzyme was decreased.

Overall, this assay proved to be a powerful tool to detect the activity of the lipase from standardized emulsions. Since the experimental approach was very much focused on exposing triolein to the lipase, the information gained from the results should be more easily interpretable than those of the washing tests, where a multitude of influences has to be considered. The assay could probably also be applied to other lipases.

It must be noted here that, if available, RI detection should definitely be considered for this assay. According to [189], it yields better results, as no workaround for the influence of formic acid has to be found. Such an acid needs to be added though, if any quantification is to be done. Without formic acid, the oleic acid signal was very broad and overlapped strongly with other peaks. It is also important to use a triglyceride of high purity (ideally as high as the calibration standard) for the emulsions, as impurities will definitely show in the chromatogram and disturb quantification attempts.

15 Differential Scanning Calorimetry (DSC)

15.1 Introduction

Another important factor to examine was the stability of the lipase in presence of additives. Any benefit gained from increasing the activity of the enzyme during washing might be nullified by a detrimental impact on the long-term stability.
A method to determine the stability of the lipase is via DSC. A sample and a blank are both heated up, while maintaining equal temperatures in both vessels. The heat required to increase the temperature of both sample and blank is measured. This way, the denaturing of the enzyme can be detected, since the required heat flow to the sample will differ from the heat flow to the blank, which does not contain lipase. By adding additives to the mixture, changes in the denaturation temperature can be detected. If this temperature is lowered, compared to a sample without additive, the additive in question destabilizes the enzyme. It is possible that additives that increase the lipase's activity lower its stability, meaning that the denaturation will occur at a lower temperature.
For the experiments here, relatively high concentrations of all components were needed to obtain visible peaks in the measurements. The difficulty was that for several systems, the required concentrations were high enough to denature the lipase at room temperature, before the sample could even be placed inside the DSC.

15.2 Results

As the concentrations required to obtain results that can be evaluated were rather high (1% enzyme, up to 5% surfactant/additive), a lot of systems could not be evaluated, since the lipase denatured immediately upon mixing. Also, the influence of surfactants and additives simultaneously could also not be evaluated, for that very reason. For the systems that could be measured, the onset temperature of the first peak was evaluated and the systems compared to the a blank system, which only contained lipase. Table 15.1 shows the comparison of the onset points of the successful measurements. For readability reasons, the heat flow - temperature plots are not featured here. They can be found in the appendix.

Table 15.1: Onset points of the denaturation peak of the lipase in the DSC measurements for all systems where the lipase did not denature before a measurement could be started.
*: Very small peak, due to beginning denaturation at room temperature over time. Complete room temperature denaturation after approximately 4 hours.

System	T_{on}
Buffer solution	65.4 °C
1:1:1 surfactant solution (5%)	57.3 °C
Agnique AMD3L (5%)	65.7 °C
2-MTHF (5%)	57.0 °C
Triacetin* (1%)	∼40 °C

The results show a decrease in thermal stability with the surfactant solution present. At such high concentrations, this is nothing unusual. That is also the reason why surfactants and additives could not be measured simultaneously, as adding 5% of each component led to degradation at room temperature.

15.3 Conclusion

Overall, the insight that was gained here is limited to a few additives, due to the aforementioned high substance concentrations. A few things could be learned here, though. As mentioned, the increased activity of the lipase in presence of 2-MTHF might be due to the hydrophilic lip of the lipase that usually only opens at a water/fat interphase being opened far more easily in the presence of 2-MTHF as a form of solvent-induced lid opening[185]. Since that exposes the binding pocket of the lipase, a reduced stability is not far-fetched.
Agnique AMD3L does not seem to have the same effect on the lipase, as it does not decrease the denaturation temperature. This suggests that the originally detected increase of the washing performance from samples with AMD3L does not stem from an increased lipase turnover rate, but from an interaction of the additive with the soil that increases its accessibility. This, however, is contradicted by the results from the HPLC assay. This will be more closely discussed in the next part, where the isolated results from the single experiments are considered in conjunction with one another.
For triacetin, measurements were only possible if started as quickly as possible after sample preparation and only if the triacetin concentration was severely reduced compared to 2-MTHF and AMD3L. Even at lower concentrations, triacetin denatured the lipase after a few hours at room temperature. The reason for this might be that acetic acid is generated. It is unclear if the enzyme cleaves the ester bonds of such a triglyceride. If the lipase does cleave the triacetin, however, this would explain the slow but reproducible denaturation. The hydrolysis of triacetate creates a steady release of acetic acid, lowering the pH of the solution, which, after a while, is enough to denature the lipase. The activity profiles of lipases concerning different chain lengths of the triglycerides' fatty acids have been studied[194–198] and it was found that shape and physico-chemical properties determine chain length specificity. But rarely organic acids with a chain shorter than C_6 are considered, as they do not really fall into the fatty acid category anymore. Studies with a similar lipase showed that the lipase can hydrolyse tributyrin[199], but that does not necessarily mean that triacetate is hydrolysed in a similar manner. While not specifically studied, a strong smell of butyric acid in washing tests where tributyrin was added, indicates that the lipase used here does indeed hydrolyse even short-chain triglycerides. For the DSC measurements, triacetin was used in a low enough concentration to dissolve

in the buffer, which would argue against the hydrolysis of the triacetate, due to the lack of a water-oil interface needed for the lipase to become active[131]. However, if triacetate facilitates solvent-induced lid opening[185], it could enable its own hydrolysis even without an interface. This would explain the slow denaturation at room temperature of all DSC samples containing triacetate, due to the increasing acetic acid concentration in the sample. In that case, triacetin cannot be used in detergent formulations with lipases.
Of these tests, AMD3L was the most interesting result, as it improved the washing performance in the detergency tests with enzyme, but does apparently not destabilize the lipase, like 2-MTHF.

16 Swelling Tests

16.1 Introduction

Most of the previous experiments had intended to determine the influence of formulation components on the lipase. During washing, however, several additives increased the washing performance even without the lipase present. That leads to the conclusion that there is an interaction between the additive and the soil that is beneficial for the soil removal.
The initial choice for the additives was organic co-solvents that were added to the detergent solution. It was theorized that their concentration at the stain is higher than in the bulk phase during the washing process. In past works, it was tested, if this would lead to an increased solubility of the fat, as these solvents can usually dissolve a significant amount of fat, if they are used without water[23]. It was, however, found during this thesis that adding small amounts of solvents to the detergent solution does not significantly increase fat solubility.
Even if the present additives do not significantly increase the solubility of the triglycerides in the soil, their presence might swell the soil, increasing its surface. Tests performed by BASF in Ludwigshafen support this hypothesis. A simple method to see, if any significant swelling can be observed is to use aqueous solutions of the additives and submerse a triglyceride pellet in such a solution. If an increased swelling can be detected, compared to submersing a similar pellet in water, this would indicate that the assumption made for the increased washing performance upon additive addition is correct.
The experiments performed here included different backgrounds to see, if they had an influence on the swelling behaviour. Initially experiments were performed with aqueous solutions of the additives. Then, either the lipase or the 1:1:1 surfactant mixture were added and finally the tests were performed with a background of both.

16.2 Results

For these experiments, the most important additives were picked for the first run. Four variations were tested for each sample: with/without lipase and with/without surfactant. For each variant, three samples were prepared. Figure 16.1 shows the relative change in mass of the fat samples without enzyme. Figure 16.2 shows the results with enzyme. The values presented here are those obtained three hours after removing the Biskin samples from the solutions.

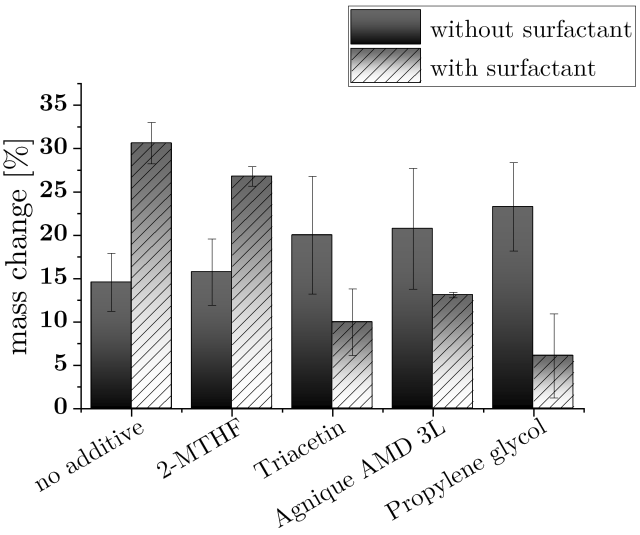

Figure 16.1: Relative mass increase of a Biskin sample after beeing exposed to water or an aqueous surfactant solution (1:1:1 mixture, 800 ppm total surfactant) or a 5% solution of the respective additive in either water or the surfactant solution. Mass increase was determined three hours after removing the Biskin from the solution.

The reason these tests were not continued with other additives is that very little information could be gained from these experiments. Generally, the presence of surfactant seemed to increase the swelling, with some exceptions. The aim was to see, if additives increase the swelling, which would have shown an interaction between the additive and the fat, and thus could have explained the increased performance of the enzyme in the detergency tests when additives are present. If the additives would increase the swelling of the fat stain, this could theoretically form "cracks" in the surface of the stain, which increases the surface area of the stain and therefore increases the efficiency of the lipase, by giving it more access to the stain. In this case, however, such an interaction could not be shown, as the error of the experiments with additive was far too large to make any definitive statements. Addition

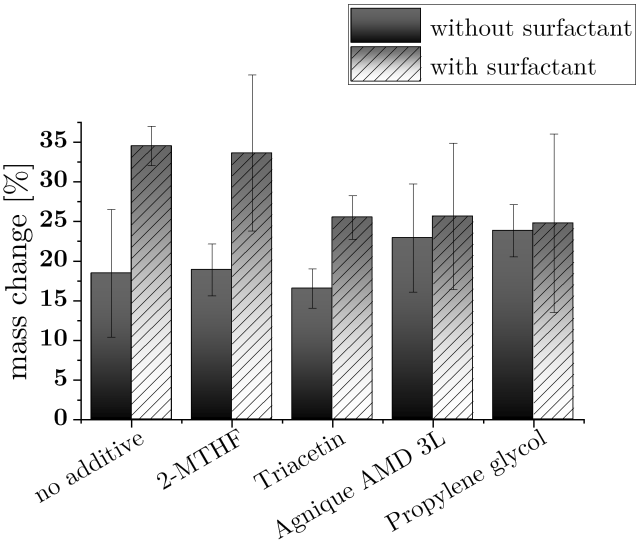

Figure 16.2: Relative mass increase of a Biskin sample after beeing exposed to water or an aqueous surfactant solution (1:1:1 mixture, 800 ppm total surfactant) or a 5% solution of the respective additive in either water or the surfactant solution. Mass increased was determined three hours after removing the Biskin from the solution. All solutions in this batch contained 1 ppm lipase.

of enzyme also does not seem to make any difference that was outside of the margin of error of the mass increase of the Biskin. Due to these results, the experiments were discontinued.

The swelling of the Biskin samples was also determined directly after removing them from the solutions and again after 24 hours, where most samples had returned to their original mass. As no conclusions could be drawn from these results that could not be gained from the mass after 3 hours, these results were moved to the appendix section.

16.3 Conclusion

As mentioned, the swelling tests were rather inconclusive, especially when surfactants were used. For the systems without surfactant, with or without enzyme, using 2-MTHF did not seem to increase the swelling on both the batch with lipase and the batch without it. While these results should not be over-interpreted, it is at least an indication that the performance increase, when combining 2-MTHF and the lipase in washing tests does indeed stem mostly from an increased lipase activity. For the purpose of this thesis, enzyme efficiency refers to a perceived increased activity because of a better accessibility of the substrate for the lipase, instead of an actual increase of the enzyme's turnover rate.

For Agnique AMD3L, a tendency to increase the swelling of the Biskin sample was detected. The errors are too large too make a quantitative statement, but qualitatively, a tendency is visible. That means that the synergistic effect from the washing tests might stem from the AMD3L's tendency to swell the fat stain, creating creases and openings that leaves the stain with a larger surface area to attack. The DSC and the LB trough results for AMD3L would support this theory, while the HPLC assay appears to support the theory of increased turnover rate.

Propylene glycol also seems to slightly increase the swelling of the Biskin. It is used in formulations to stabilize enzymes, prolonging its shelf-life. The buffer solution that the lipase was delivered in, contained 25% propylene glycol, for instance.

No clear tendency was seen for triacetin, as it seemingly increased the swelling in the first batch, but did not lead to any increase over the additive-free sample in the second batch.

Part VI

Finding Conclusive Explanations

17 Formulating Hypotheses from the Washing Tests

The results of the single experiments have already been discussed at the end of each section of the previous chapter. The aim of this chapter is to combine all these results into a conclusive overview, from which a deeper insight into the processes influencing the performance of the lipase in a complex washing formulation can be gained. Such an understanding of the processes and conditions in a washing application beyond the empirical level would greatly help to enhance specific properties. An important result from the washing tests was that any additive concentration should be well below the total surfactant concentration or the additive will reduce the washing performance. A possible explanation for this is that the additive, which for these tests were co-solvents, hinder removal processes, as outlined in the fundamentals part of this thesis. However, as an additive concentration that surpasses the surfactant concentration is not realistic for detergent formulations, this was not looked further into. For co-surfactants the result may possibly look different, but was not tested. Of the co-solvents that were used, 2-MTHF, Agnique AMD3L and triacetin stood out in the washing tests. Among the co-surfactants, Plurafac LF7319 increased the washing performance with lipase disproportionately high. For the co-solvents, two main theories were developed:

1. The co-solvents directly increase the turnover rate of the lipase. They are present in excess at the fat stain and either stabilize the transition state of the enzymatic hydrolysis, lowering the activation energy of the reaction, or they cause the hydrophilic lid of the lipase to open prematurely. Solvent-induced lid opening has been reported in literature[185, 186]. The exposed active site is more active and less selective. It is likely that such an effect would destabilize the lipase.

2. The co-solvents swell the fat stain, increasing its surface area. The lipase is only active on a water/oil interface, where the hydrophilic lid opens. In aqueous media, this lid blocks the active site[131, 132, 138]. Thus, the washing performance increases with the increased surface area of the stain as the

enzyme can access the stain better and work more efficiently. As the fat structure is broken up in this case, the remaining fat is more easily removed.

For the only co-surfactant that appeared to work synergistically with the enzyme, the reason of the improved washing performance probably stems from the increased surfactant concentration. The enhanced relative performance of the enzyme (compared to the system without additive) was relatively small. Surfactants can adhere to enzymes, denature them and disable them in a washing process. It seems that Plurafac LF7319 does not deactivate the lipase in the concentrations that were employed here. The surplus in relative performance could be similar to the second hypothesis for the co-solvents, specifically the increased surface area of the partially attacked stain. The increased surfactant content may cause an increase in the roll-up of the liquid soil[109], since it is more easily removed by surfactants than solid soil[87, 90]. The remaining solid soil is more exposed with a larger surface area. This allows the enzyme to work more efficiently.

Additives that reduce the effect of the enzyme in the washing process, on the other hand, might have an effect that is exactly opposing to the hypothesis for synergistic effects. They destabilize the enzyme, or their presence might impede the opening of the lid. From the washing tests alone, however, none of these theories could be confirmed. The system is far too complex, with the dyed fat being bound to a fabric of unknown and inhomogeneous surface geometry. Additionally, while the staining degree of the fat on the fabric could be controlled, its exact distribution was not always homogenous as some stains showed irregularities in their surface colour after the dyed soil had dried. Also, to achieve a more realistic simulation of a laundry washing process, mechanical effects were included in the tests. Overall, this led to a complexity in overlapping effects that called for the development of experiments that could examine specific interactions in a focused manner, in order to verify or falsify the formulated hypotheses.

18 Comparing the Hypotheses to the Experimental Results

18.1 Analysing the Results Towards Either Hypothesis

One of the hypotheses formulated is that the washing performance with a combination of the lipase and a good additive increases because the activity, i.e. the turnover rate of the enzyme is increased. Both the Langmuir-Blodgett trough tests and the assay were designed to specifically examine this possibility. The activity of lipases can be increased with the right additives, e.g. imidazolium derivates[183, 184]. Care must be taken concerning the stability and shelf-life of lipases in detergent formulations, as laundry washing products must still perform well months after production. It has, however, been found that lipases can be unstable in surfactant formulations[200]. Therefore, increasing the activity by sacrificing stability is detrimental in a mass market product.
The second formulated hypothesis was that the effect that increases the performance of the enzyme during the washing does not stem from any changes in the turnover rate of the lipase. Instead, the additive mixes with the stain, swelling it.

The experiments were mostly designed to examine if the first hypothesis could be validated. In order to show that the synergistic effect of a good additive combined with the lipase stems from an increase in the lipase's turnover rate, factors like swelling of the stain had to be excluded and the fatty acid generated by the lipase monitored. As long as a regular stained fabric was used, that was not possible, which is why the Langmuir-Blodgett tests were realized. The fat being only present at a monolayer should completely exclude swelling and increase of surface area.
The lipase assay was therefore designed to avoid the swelling of the static fat stain, immobilized on a fabric surface. Instead, emulsions were used and were kept stirred. It is, however, not impossible that co-solvents could stabilize these emulsions, leading to smaller fat particle sizes[201, 202]. Smaller particles would mean a larger

surface area, which could result in more generated fatty acid and an observed increase in activity. Therefore, on its own, the assay may not be sufficient to prove the solvent-induced lid opening.
DSC tests can show an interaction between the lipase and an additive. A change in the degradation temperature supports the hypothesis that additives directly interact with the enzyme.
Finally, partitioning coefficients might shed light on how the additive acts on the washing performance. COSMO-RS[203, 204] calculations on the partioning coefficient between water and triolein were performed at BASF SE for several possible co-solvents. These were kindly provided for use in this thesis. If a co-solvent is primarily found in the aqueous solution, it would support the lid-opening hypothesis.

When looking at the different results of the experiments, the washing tests showed that 2-MTHF, Agnique AMD3L and triacetin were co-solvents, where an increase of the enzyme's performance compared to a sample without additives was detected. In the Langmuir-Blodgett through tests, only 2-MTHF showed a significant increase in generated palmitic acid. The increase in palmitic acid content was only small for Agnique AMD3L, and triacetin could not be tested with the method. Interestingly, the compression isotherms of tests with solketal in the subphase seemed to indicate a somewhat larger increase in palmitic acid. Solketal performed poorly in the washing tests.
For further insight into the effects that influence the enzyme's performance in the washing tests, the HPLC assay was developed. Here, adding triacetin only led to a minor increase in oleic acid generation. For samples with Agnique AMD3L and 2-MTHF, the increase was an order of magnitude larger. Due to the extreme increases in generated oleic acid in the samples with these two additives, it is almost overlooked that Plurafac LF7319, the only co-surfactant that caused an increase in the enzyme's performance in the washing test, also led to a 39% increase in generated oleic acid, compared to a sample without additives. Either, the increased surfactant content led to a smaller droplet size in the emulsion, leading to a larger surface area of triolein the lipase could access, or the nonionic surfactant consisting of alkoxylated fatty alcohols creates an environment around the oil droplets in the emulsion that facilitates the opening of the lipase's lid.
Unfortunately, the experimental setup of the DSC tests did not allow to test many of the additives that were used in the washing tests, due to the high analyte

concentrations required for the samples. It did show, however, a decrease in degradation temperature for 2-MTHF. Since the stability of the lipase is dependant on the lid[131, 132, 138], a decreased stability might indicate that the lipase favours the open lid structure in the presence of 2-MTHF. For Agnique AMD3L, no decrease in degradation temperature was detected. The behaviour the lipase exhibited in the presence of larger amounts of triacetin is still not completely resolved. While the detected degradation temperature for samples that were measured fast enough decreased to about 40 °C, all samples did show denaturing of the lipase after some time at room temperature. If the lipase hydrolyses triacetin and thus generates acetic acid, this could lower the pH in the sample far enough to denature the enzyme, this would exclude triacetin from use in commercial products with this lipase. This was not detected in the washing tests, due to the low concentrations of additives and enzyme and due to the fact that all tests were started immediately after enzyme addition and were finished after 30 minutes. The DSC samples denatured at room temperature after a few hours.
The swelling tests were only performed for a few additives, since they were prone to large errors and therefore difficult to draw conclusions from. It seems that an aqueous solution of 2-MTHF does not swell a Biskin sample any more than an aqueous solution without it. The results indicate that aqueous solutions of Agnique AMD3L and propylene glycol swell a Biskin sample more than pure water. However, due to the large error of the averaged mass change, definitive statements prove difficult.

Finally, a look at the COSMO-RS calculations of the partitioning of co-solvents between triolein and water can be taken. Figure 18.1 shows the results of these calculations performed by BASF in Ludswigshafen.
From among the compounds tested as additives in this thesis that were included in the COSMO-RS calculations, only Agnique AMD10 and Agnique AMD810 strongly prefer the oil phase. Triacetin and 2-MTHF are both close to zero, favouring neither phase especially and Agnique AMD3L seems to strongly prefer the aqueous phase. This last result does contradict the swelling test results a bit, as they indicated an increase in swelling when using Agnique AMD3L. However, the large standard deviation of these tests could have led to false results.
The log(P) of 2-MTHF is interesting here. It favours the oil phase only very slightly. This can indicate that it will be found near the oil/stain interface due to an almost equal partition in both phases. There, it would be available for interaction with

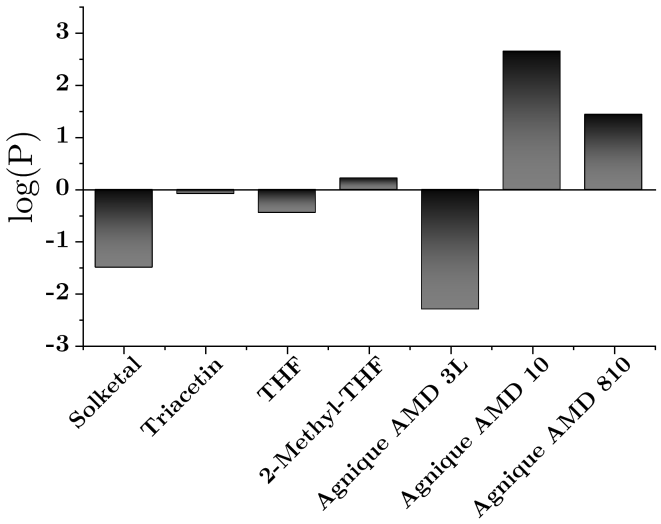

Figure 18.1: COSMO-RS calculations of partitioning coefficients between water and triolein for different additives. A positive log(P) value indicates a preference of the additive for the oil, a negative a preference for water. Kindly provided by Dr. Matthias Kellermeier, BASF SE.

the lipase that activates at a triglyceride interface by opening its lid[131]. Rehm et al. suggested that non-polar organic solvents, such as toluene, can stabilize an open-lid conformation to the point that it is favoured by the lipase[185]. Cajal et al. found that the activity of a lipase can be controlled by the composition and physical properties of the lipid interface[205].

The reduced denaturation temperature of the lipase in a solution containing 5% 2-MTHF suggests that 2-MTHF is non-polar enough to cause solvent-induced lid opening. As the lid is important for the stability of the lipase[131, 185, 188], a decreased thermal stability suggests a favoured open lid conformation. In washing tests, however, concentrations are a lot lower. But combining all insights gained suggests that 2-MTHF present on the interface can stabilize the lid opening and increase the activity of the lipase. Proving this will, however, require different experimentation techniques. One possibility may be the investigations of Belle, Ranaldi and their colleagues on the lid opening of the human pancreatic lipase via electron paramagnetic resonance spectroscopy by covalently binding a paramagnetic probe to the lid[206–208]. A similar method might be feasible for the lipase from *Asperigillus oryzae* used in this thesis.

Initially, it was assumed that the second hypothesis, the increased surface area increasing the lipase's efficiency as more substrate is available can be shown with Agnique AMD3L. The washing performance did increase while the LB trough tests

did not show much of an activity increase, however. The DSC measurements showed no decrease in denaturation temperature, while the HPLC assay tests did show a 200% increase in generated oleic acid. As it was already mentioned, this might have been caused by the co-solvent reducing the particle size of the oil droplets and therefore increasing the surface area of the enzyme's substrate[201, 202], which of course is beneficial for a surface-activating lipase. However, a small amount of 1,2-diolein was also found in the sample with Agnique AMD3L, which suggests a somewhat reduced selectivity. After the swelling tests did show a relative increase in mass compared to the sample without additive, it appeared that Agnique AMD3L is indeed an example of a co-solvent increasing the washing performance by increasing the accessibility of the substrate for the enzyme. However, as Figure 18.1 shows, Agnique AMD3L favours the aqueous phase. This indicates that also in a washing process, Agnique AMD3L will be predominantly found in the aqueous phase and not in the stain. Further experiments or possibly simulations will have to be performed to further understand this behaviour.

18.2 Results with 2-MTHF Support the Solvent-Induced Lid-Opening Hypothesis

The experiments designed to exclude the plethora of influences that are present in the washing tests gave insight into how the lipase reacts in presence of additives. Different additives, or co-solvents could be tested with different methods. 2-MTHF stood out in any method as a co-solvent that improves the performance of the lipase. Upon closer inspection, it seems that the reason for this is that this co-solvent facilitates the opening of the lipase's lid covering the active site. The results of experiments utilizing 2-MTHF can be summarized in the following way:

1. Washing tests: Caused a surplus in the washing performance increase of the lipase compared to the sample without additive.

2. Langmuir-Blodgett trough tests: Caused an increase of 30% in the palmitic acid content detected in the monolayer after incubation compared to the measurement without additives in the subphase.

3. HPLC assay: Quadrupled the amount of oleic acid detected in the chromatogram compared to the sample without additive.

4. DSC tests: Decreased the denaturation temperature of the lipase by 8.5 °C.
5. Swelling tests: No increase in the swelling could be detected.
6. COSMO-RS calculations: Does not prefer either oil or aqueous phase strongly.

Overall, these results favour the solvent-induced lid opening hypothesis, as explained in the previous section. While the swelling tests do not allow for any quantitative statement, the lack of mass increase of the Biskin sample, when 2-MTHF was used, was visible. In the compression isotherms of the LB trough test and in the HPLC assay, no other additive could match the increase in generated fatty acid of 2-MTHF. The assay results not only showed an increase in oleic acid, but it appears that 1-monoolein was generated during the 30 minutes incubation when 2-MTHF was used. This could not be shown for any other additive. While it is possible that swelling interactions may not be completely excluded in the emulsions of the HPLC assay, considering that additives were added to the emulsions before they were mixed, the monolayer of the Langmuir films cannot swell or increase their surface area. Finally, it was theorized that the stability of the lipase may be decreased when the lid is in the open conformation due to interactions with the co-solvent, as the lid is important for the enzyme's stability[131, 132, 138]. The DSC measurements showed a decrease in denaturation temperature of 8.5 °C when 2-MTHF was added to the enzyme buffer. It was found that solvents can induce lid opening in lipases and that the activity of the lipase can be influenced by the chemical composition at the interface[185, 205]. Together, all these results suggest that the increased washing perfomance of 2-MTHF stems from a solvent-induced or at least solvent-facilitated lid opening. To definitely prove such a behaviour, further experiments, like the suggested electron paramagnetic resonance spectroscopy[206–208] or even molecular dynamics simulations will be necessary.

19 Processes that Govern the Removal of Fat-based Stains from Laundry

While this thesis focused mostly on explaining the behaviour and performance of a lipase in a laundry cleaning process, the experiments also allowed for further conclusions on the underlying processes that need to be stirred in the right direction to achieve good washing performance at low temperatures.

The first finding was that predecessor works to this thesis had put too much emphasis on the ability of the washing formulation to dissolve the stain. The previous washing tests in glass beakers merely suspended a stained piece of fabric in an aqueous solution containing surfactants and further components of detergent formulations, dependant on the aim of the experiment. The fabric stripe was affixed above the beaker to ensure it does not move within the solution. Stirring was simply performed by a magnetic stirrer and the volume of washing liquor was very large compared to the amount of fabric (1 stripe no larger than 60 x 30 mm suspended in 100 mL solution). The results from this type of setup suggested dissolving the stain is the driving factor in cleaning the fabric. In the early stages of this thesis, it was found that results tend to correlate poorly with washing results from actual washing machines. Also, their reproducibility was too poor to determine influences of additives on a lipase. This resulted in the development and construction of the testing unit introduced in part IV of this thesis. This way, important mechanical influences of laundry washing could be considered.

Comparing the washing test results with results from solubility tests showed that a formulation's washing performance does not necessarily correlate with its ability to dissolve fats or fatty acids. It was concluded that fat removal from laundry is not mainly driven by solubilization of the fat, but instead by other removal processes. It was found that mechanical action, most importantly abrasive action, is a main driving force for the removal of natural fat and mineral oil-based soil[209, 210]. Such an action can be achieved by different pieces of fabric coming into contact during washing. The washing tests used four stripes per beaker, to include mechanical interaction from fabric friction. It is unlikely that soil removed this way will be completely dissolved, but it must still be transported away from the fabric.

Emulsification of insoluble fat-based soil might be an important driving force. Enhanced emulsification may also explain how some of the co-solvents or co-surfactants used as additives increased the washing performance before enzyme was added. The emulsifying abilities of surfactants are well known and used in other fields beside detergency, like drug delivery, food preperation, petroleum chemistry or as cement additives[27, 31, 177, 211–213]. How the surfactants act on the soil has been explained in the fundamentals section. The micellar solution surrounding the stain emulsifies the fat droplets that have been removed by the roll-up of emulsification mechanism. This ensures that the soil is transported away from the surface and does not redeposit[91, 114, 115]. It has, however, been found that maximum soil removal is not achieved by an ordinary micellar solution, but instead when the soil is incorporated into an intermediate phase, e.g. a microemulsion[109]. It is also known that co-solvents stabilize emulsions, reduce droplet sizes and help form microemulsions[201, 202, 214]. In that case, the co-solvents that improved the washing performance could increase the soil release from the surface by increasing the emulsification, possibly by creating an intermediate microemulsion near the surface. It could be shown in the past that microemulsions can significantly increase oily soil removal from fabrics and that soil removal depends on the emulsifying power of the formulation[215–220]. While the increase in soil removal that Tongcumpou and his colleagues [217] achieved was far greater than even the best co-solvent used in this thesis, they admitted that their experiments utilized formulations at concentrations which are not practical for commercial application. The formulations employed to test detergency here were always used at low concentrations in order to resemble conditions that are realistic for a real-life application.

Combining these findings with those in the previous section, a more complete picture of lipase-driven low temperature laundry detergency processes can be formed. A co-solvent approach can lead to a significant improval in soil release. However, it must consider how the emulsification of removed soil can be enhanced by the co-solvent and how the co-solvent will interact with the enzyme. A theoretical ideal case is a co-solvent or combination of co-solvents that facilitates the lid opening of the lipase and stabilizes the open lid form near the soil/water interface to increase the turnover rate and thus the performance of the lipase, while simultaneously increasing the emulsifying power of the formulation to effectively remove the fats and fatty acids from the surface.

Part VII

Summary and Outlook

The aim of this thesis was to improve the removal of fatty stains from fabric at room temperature. How surfactants and surfactant compositions influence this process had been the focus of previous works[23, 25], while here, the main focus were enzymes, or more precisely, lipases. Initially, it was considered that efficient hydrolysis of the triglycerides would increase the performance, due to the generated fatty acids dissolving considerably better in the washing liquor. Since results showed that this was not the case and the fatty acids do only marginally dissolve in a surfactant solution at room temperature, the processes that drive the washing performance had to be investigated more closely. The first step to clarify these processes was to build a washing test unit that could reliably perform the tests on a laboratory scale, as the previously used glass beaker tests did not consider mechanical influences like fabric friction and could not be properly temperature-controlled, leading to rather unreliable results. From the insight gained from these washing tests, specific tests were designed to reduce the plethora of influences and interactions present in washing tests to a few factors that could then be closely analysed.

The solubility tests showed that even mixtures of highly optimized synthetic surfactants will not dissolve sufficient amounts of long chain fatty acids at room temperature in a concentration range that is realistic for a laundry washing process. Only at extremely high surfactant concentrations (>80%), significant amounts (single digit percentages) of palmitic acid could be dissolved. At surfactant concentrations below 1%, only ppm amounts of palmitic acid could be dissolved. Also, a surfactant mixture of a non-ionic surfactant, a sulfate and a benzene sulphonate, which is optimized for laundry washing and that has proven itself in commercially available products, performed poorer than an equal amount of a pure non-ionic surfactant. While small amounts of co-solvents could lead to improvements of the fatty acid solubility of approximately 20-60%, the absolute solubility of palmitic acid in these formulations was still in the three digit ppm range. It was therefore concluded that the solubility of the fatty acids generated by the enzymatic hydrolysis of the fat cannot be the driving force of the improved washing performance.

As neither tripalmitin[23] nor palmitic acid can get dissolved or liquified at room temperature by a washing formulation with concentrations that can be expected in laundry cleaning processes, the washing process itself must be examined more closely. Initially, room temperature tests in glass beakers were performed, but they

proofed insufficient, the experimental setup only considering the chemical interaction of the washing liquor with the soiled fabric. A method had to be found that resembled laundry cleaning more closely, but was still practicable with on a small scale necessary for efficient laboratory testing. The testing unit that was designed and built met the required criteria and was an important cornerstone of this thesis. The first tests performed compared the washing performance of the surfactant mixtures to their ability to dissolve long chain fatty acids. The 1:1:1 mixture of a nonionic alcohol ethoxylate, an ethoxylated sulfate and a linear alkylbenzene sulphonate performed far better in the washing tests than the pure nonioninic surfactant, which performed a lot better in the solubility tests. This strengthened the assumption that the ability of a formulation to dissolve fat or a fatty acid is not the driving force of that formulations washing performance for a fat-based stain. The highest washing performance was seen for a system that replaced the alcohol ethoxylate with a polyalkylglycoside. But that system seemed to poorly function in combination with the lipase, so it was not used for further tests.

Natural soaps were employed as co-surfactants, as it was thought that the fatty acids generated by the lipase may function as co-surfactants and increase the washing performance. Adding a compound with similar composition might help to accelerate the removal process. No changes in washing performance were seen. This may be because the overall surfactant concentration was not increased, while *in-situ* generated co-surfactants increase the surfactant content at the water/soil interface.

The influence of water hardness and the used builder was then investigated. It was found that the influence on the washing performance is far greater for the TP/TO stain than it is for the Biskin stain. Generally, a low water hardness around 5 °dH seems to be ideal for the washing performance of a formulation, mainly because of the surfactants, not because of the lipase.

Co-solvents were also screened in the testing unit. It was found that the washing performance of the system is better if the co-solvent or additive is used well below the total surfactant concentration, as it will negatively influence the surfactants' performance if used at higher concentrations. The additve's influence on the enzyme's perfomance in the washing tests was also examined. It was found that the relative increase of the washing performance when lipase is added increases when also employing 2-MTHF, Agnique AMD3L, Plurafac LF7319 and Triacetin.

While the washing tests allow to find additives that can increase the lipase's performance, they do not allow to explain why the enzyme's performance is influenced,

as too many effects overlap to create the overall washing performance. Among the experiments designed to understand if the additives can directly influence the lipase's activity were Langmuir-Blodgett tests. Compression isotherms were recorded and correlated with the composition of the monolayer. Lipase was added to the subphase to examine changes to the monolayer composition after a pre-determined incubation time. Then, additives were added to the subphase to see if the change in the monolayer composition caused by the lipase is either enhanced or reduced. No surface active additives could be tested here. It could be shown that presence of 2-MTHF and Solketal in the subphase increased the amount of palmitic acid found on the monolayer after the incubation considerably, while adding Agnique AMD3L or propylene glycol to the subphase caused only a small increase compared to the measurements without additives.

To examine the enzyme's activity more closely, an assay was developed that generates a dye. The dye's concentration correlates directly with the activity of the lipase, detecting glycerol that is created by the cleaving of triglycerides by the lipase. It was shown that the dye generation shows a linear correlation to the glycerol concentration in the sample in a low ppm range (between 1 and approximately 30 ppm glycerol). No sufficient amount of dye was generated when the lipase was tested, except at very high enzyme concentrations (greater than 1'000 U/mL as compared to the 2-3 U/mL used in the washing tests). It was assumed that the lipase does not generate glycerol from triglycerides at a lower concentration in the 30 minutes incubation window that was chosen.

A different approach was then tested. To examine the products of the enzymatic reaction and possibly quantify those products in one step, a normal phase HPLC method was developed. It was shown that without other influencing factors, the lipase will only hydrolyse one fatty acid from the triglycerides. Beside the fatty acid, mostly 1,3-diglycerides are generated. The generation of fatty acid was examined, when parameters like pH, temperature and lipase concentration were modified. The results were as expected in this case. When additives that had performed well in previous tests were added, 1,2-diglycerides were found and in the case of 2-MTHF, even 1-monoolein. It could be shown that the amount of generated fatty acid in the emulsions can be severely increased with the right additives. Some additives can cause small increases. These were additives that, from their properties, should be treated

as co-surfactants. An increased surfactant concentration in an emulsion results in smaller droplet size, leading to a larger surface area that the surface-activating lipase can attack. For one additive, Agnique AMD10 a decrease in generated oleic acid was detected.

DSC measurments were attempted to check if the co-solvents and co-surfactants influence the thermal stability of the enzyme. Due to the high concentrations of both enzyme and additive required to analyze the DSC measurements, only a few tests could be performed, as the required 5% of additive in most cases led to a direct denaturation of the lipase at room temperature. It was found that Triacetin and 2-MTHF reduce the thermal stability of the lipase, while Agnique AMD3L does not have any effect on the thermal stability.

Finally, it was tested, if additive can influence the swelling of fat, when immersed in an aqueous solution. This was to show if in a laundry washing environment, the co-solvents interact with the stain. An increase in swelling could indicate that the additive will be found on or within the stain. The swelling might cause "cracks" in the stain, increasing its surface and reducing its structural stability. A larger surface area could increase the lipase's performance, since it is surface activating, and a reduced structural stability might facilitate the stain's removal. In that case, the increased enzymatic performance detected in the washing tests would not originate from an increase in the lipase's turnover rate. Instead, the lipase would work more efficiently, as it has a larger surface area it can access. The tests were, however, mostly inconclusive, as the standard deviation in samples of the same type was too large. The tests indicated that 2-MTHF does not increase the swelling of Biskin submersed in an aqueous solution. For Agnique AMD3L, however, the swelling did seem to increase.

The conclusions that can be drawn from this are that additives can influence the lipase's turnover rate. The activity of a lipase is dictated by a lid covering the active site[131, 138, 139]. Solvent-induced lid opening and activity control of a lipase via modifying the lipid interface has been described in literature[185, 205]. A co-solvent present at the water/soil interface that facilitates the opening of the lid may be the answer to increasing enzymatic washing performance at low temperatures.
Beyond the interactions with the lipase, which were the main focus of this work, it

could also be shown that co-solvents can increase soil release even without a lipase present. The theory that was applied based on studies on the influence of microemulsions on the soil release[215–220] is, that co-solvents can increase the emulsifying power of the detergent formulations, possibly by formation of microemulsions.

In future, further experiments will have to be performed to validate the hypothesis. Molecular dynamics studies might be necessary. Electron paramagnetic resonance spectroscopy has been successfully employed to investigate the lid opening of the human pancreatic lipase[206–208]. This might offer an experimental path that can be followed to examine if co-solvents can induce lid opening of the lipase from *Asperigillus oryzae* that was used here and that is used in laundry detergents, or any other lipase where solvent-induced lid opening may be relevant.
Another method to determine interaction of co-solvents with the lipase might be circular dichroism (CD), a method which uses circularly polarized light. The differential absorption of left- and right-handed polarized light is determined, which can be used to analyse chiral molecules, as they absorb the left-handed and right-handed polarized light differently[221]. It is commonly used to investigate the secondary structure of proteins[222]. CD has been been successfully employed to analyze structural properties of lipases and interactions on the structure in the absence of substrate[223–225]. The lid opening of a lipase is a structural change, albeit a small one, depending on the size of the lid. Therefore, CD may be useful to further study the lid opening of a lipase induced by a co-solvent, possibly both in presence and in absence of a substrate.

Part VIII

Appendix

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DSC Measurements

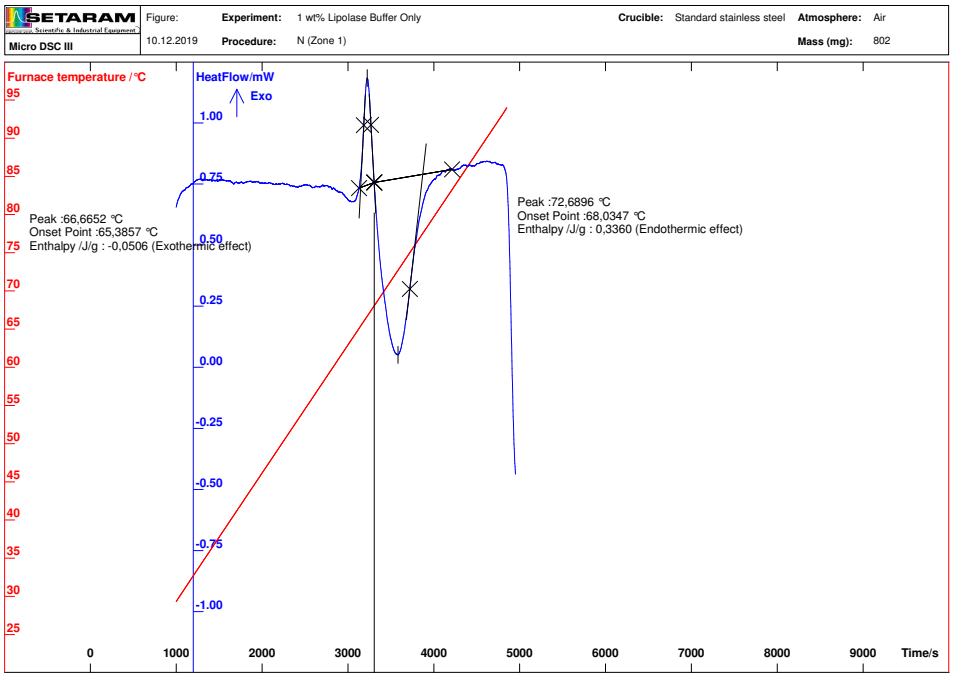

Figure 1: DSC measurement of Lipase in Buffer solution.

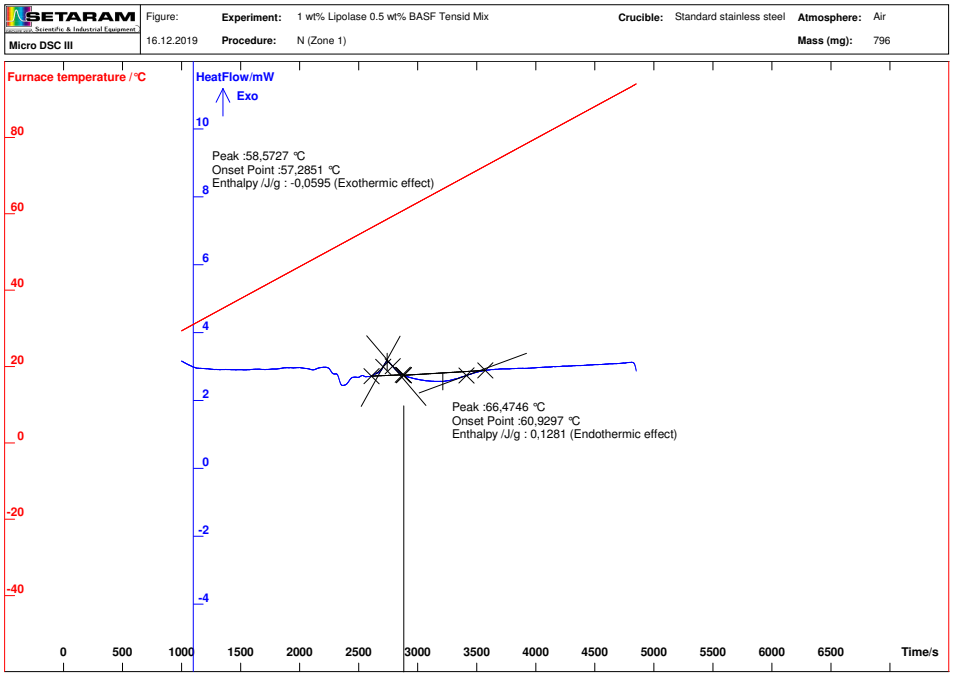

Figure 2: DSC measurement of Lipase in Buffer with 0.5% 1:1:1 surfactant mixture.

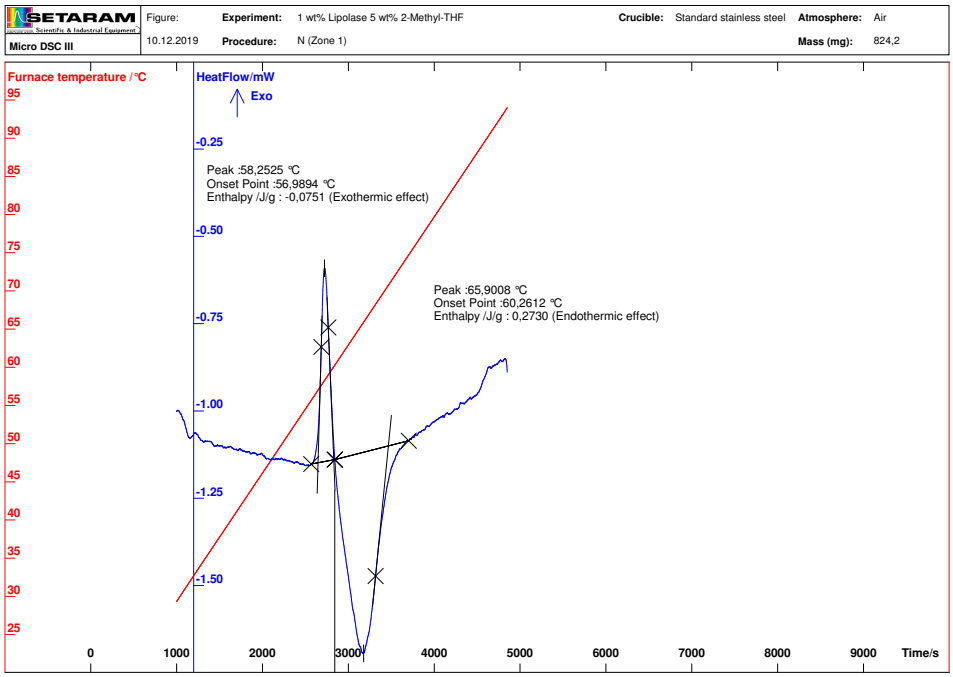

Figure 3: DSC measurement of Lipase in Buffer with 5% 2-Methyl-THF.

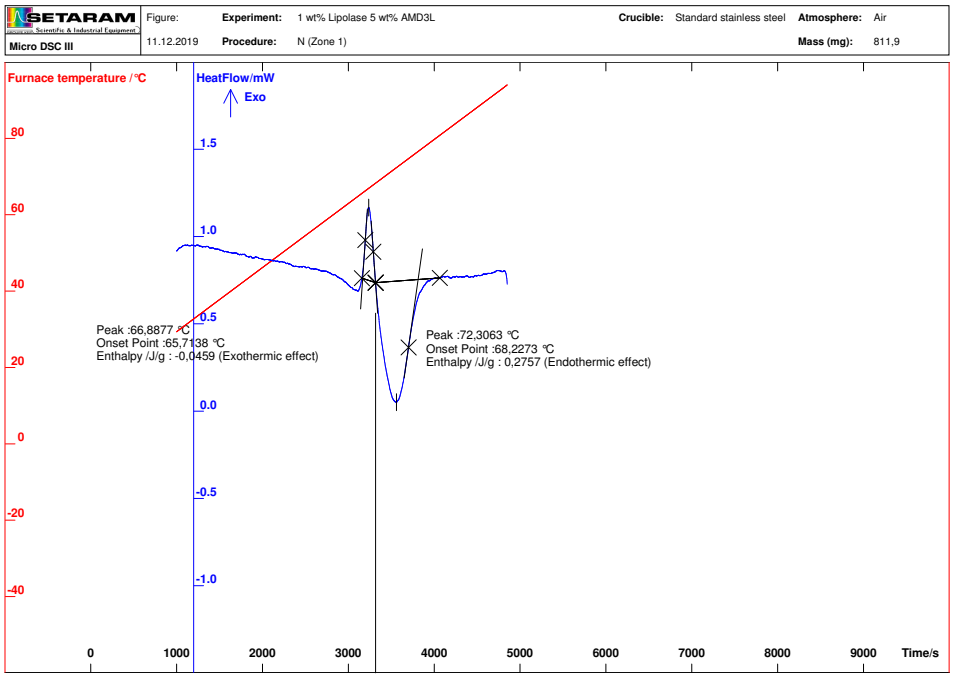

Figure 4: DSC measurement of Lipase in Buffer with 5% Agnique AMD3L.

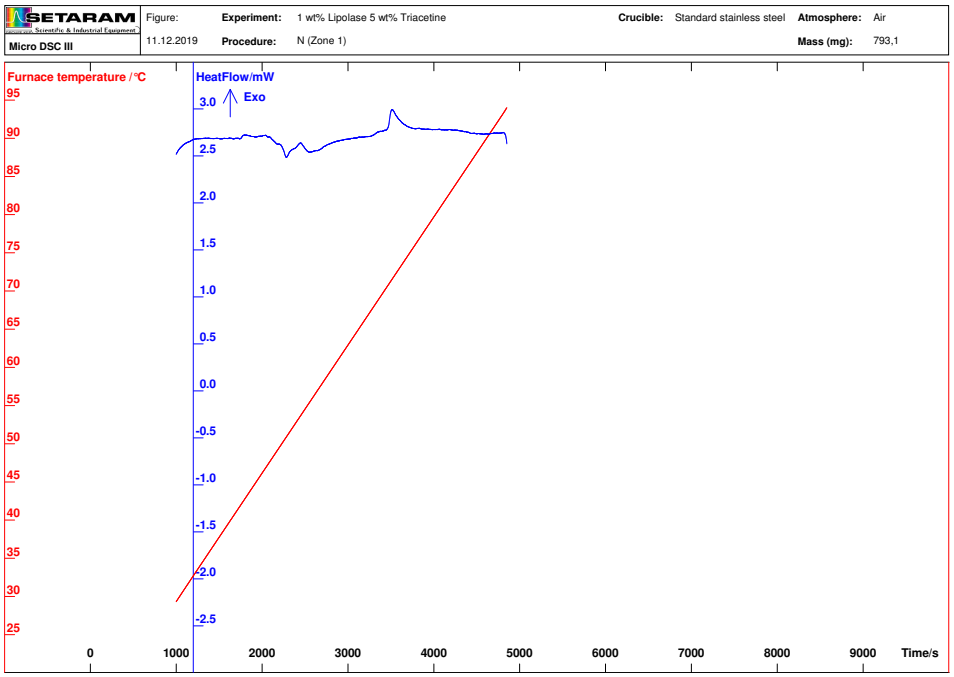

Figure 5: DSC measurement of Lipase in Buffer with 1% Triacetin.

Swelling tests

Mass increase directly after removal of solution

Without Lipase

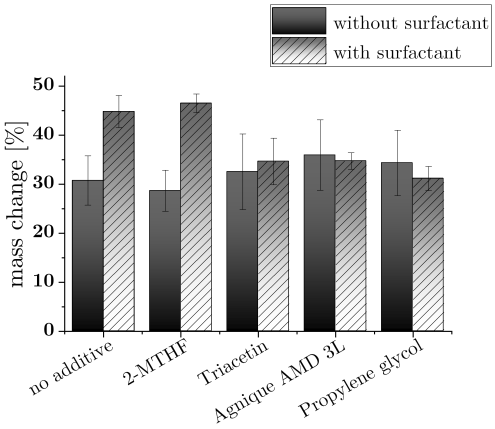

Figure 6: Relative mass increase of a Biskin sample after beeing exposed to water or an aqueous surfactant solution (1:1:1 mixture, 800 ppm total surfactant) or a 5% solution of the respective additive in either water or the surfactant solution. Mass increased was determined directly after removing the Biskin from the solution.

With Lipase

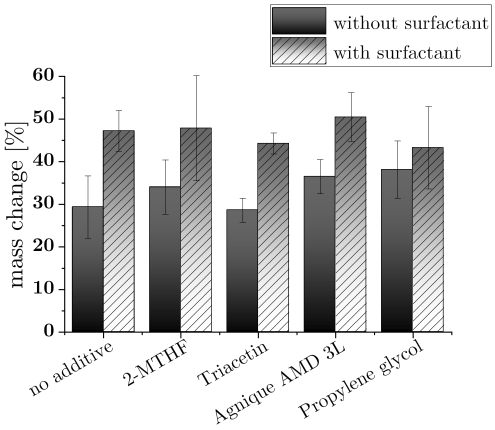

Figure 7: Relative mass increase of a Biskin sample after beeing exposed to water or an aqueous surfactant solution (1:1:1 mixture, 800 ppm total surfactant) or a 5% solution of the respective additive in either water or the surfactant solution. Mass increased was determined directly after removing the Biskin from the solution. All solutions in this batch contained 1 ppm lipase.

Mass increase after 24 hours

Without Lipase

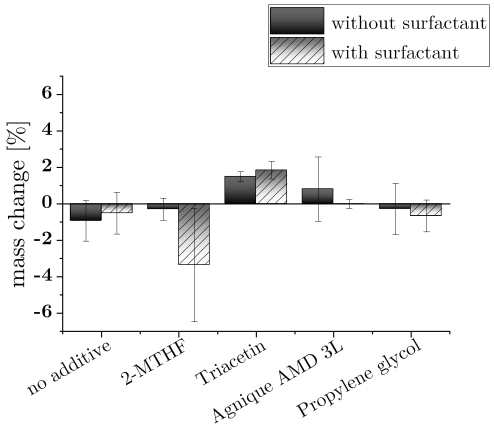

Figure 8: Relative mass increase of a Biskin sample after beeing exposed to water or an aqueous surfactant solution (1:1:1 mixture, 800 ppm total surfactant) or a 5% solution of the respective additive in either water or the surfactant solution. Mass increased was determined 24 hours after removing the Biskin from the solution.

With Lipase

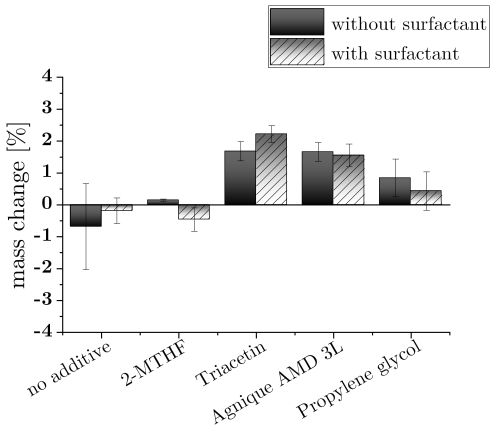

Figure 9: Relative mass increase of a Biskin sample after beeing exposed to water or an aqueous surfactant solution (1:1:1 mixture, 800 ppm total surfactant) or a 5% solution of the respective additive in either water or the surfactant solution. Mass increased was determined 24 hours after removing the Biskin from the solution. All solutions in this batch contained 1 ppm lipase.

User Manual: Detergency Testing Unit

DTU strucuture

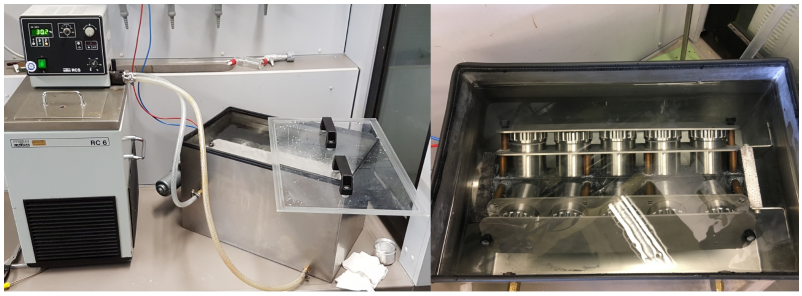

Figure 10: Left: complete DTU with a standard laboratory cryostat connected. Right: View inside the container with the axis beeing fully mounted with beakers.

In the following, the single components of the DTU along with their correct handling shall be explained.

Outer Container

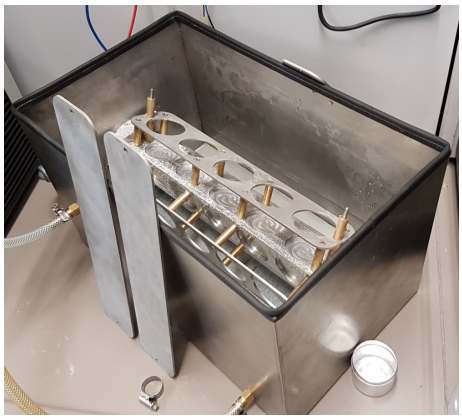

Figure 11: The empty outer container of the DTU. The beaker mounts on the central axis are well visible. The plates leaning on the side of the container are used to secure the beakers inside the mount.

The outer container of the unit is its casing and the water tank. It has two connectors for 8 mm (inner diameter) silicone hoses. The volume of water required to have all beakers be completely immersed at all times during measurements is approx. 30 l for the container. A higher overal voume will be needed, depending on the thermostat used. It is recommended to use only millipore water for two reasons. Firstly, to avoid limescale formation within the unit which could potentially damage it and secondly to avoid the growth of microorganisms in the thermostat or the unit.
The outer container has a lid which should always remain on the container during tests. This ensures that the set temperature will be maintained during measurements and reduces the risk of injuries from contact with the rotating axis.

Thermostat

A thermostat of sufficient size must be connected to the unit for proper functionality. The thermostat must be able to heat and maintain the temperature of 35-40 l of water (depending on the internal volume of the thermostat). If cooling is required (possible during summer and at low testing temperatures) a cryostat of sufficient size must be used.

Central Axis with beaker mount

The central axis (Fig. 11) on which up to 15 beakers can be mounted is the mechanical heart-piece of the unit. The closed beakers can be placed in each of the 15 drillings. To safely lock the beakers into position, a metal plate is screwed onto each side of the beaker mount. After the beakers have been placed into position, the plate is placed onto them, with the two threads at the end of each side of the beaker mount going through the holes at the end of each plate. The plate is then screwed tight with the appropriate locking nuts (Figure 12). No tool is needed to screw in the nuts, tightening them by hand is sufficient for a safe operation of the unit.

Figure 12: The locking nuts required to lock the beakers into place with metal plates.

Electric motor with control unit and power supply

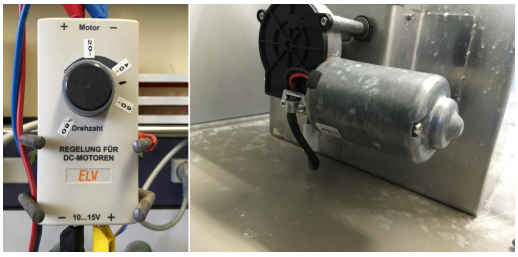

Figure 13: Left: The control unit of the electric motor. Right: The electric motor attached to the side of the unit.

The axis is powered by a 12 V DC electric motor which rotates the axis clockwise from the motors direction. The electric motor can be steplessly adjusted from 0 to 80 rpm via the control unit. It is strongly advised to not operate the unit above 60 rpm for more than a few minutes if more than half of the beakers are used, as it can lead to the motor overheating.

The 24 V, 5 A (type: MW OWA-120E-24) power supply can be directly plugged into a 230 V socket. The power supply is connected to the control unit via banana connectors. The same connector type is used for the connection of the control unit and the electric motor. Since the motor is a DC motor, the colour coding of the sockets at the motor, the control unit and the power supply must be taken into account. For the power supply connectors, make sure that the black and yellow connectors are matched. For the connectors of the electric motor, make sure that the blue and red connectors are matched. It is important not to interconnect sockets without matching colour, to avoid damaging the unit.

As soon as the unit is plugged in completely, it can be started via the rotary knob on the control unit. Note that the unit does not have an on/off switch. It is therefore recommended to unplug the DTU's power supply from the wall socket if the unit will not be used for a longer time.

Beakers

Figure 14: A 100 mL stainless steel beaker used to for the samples tested in the DTU.

The DTU was built with 15 stainless steel beakers with a volume of 100 mL each. Each beaker consists of three parts. The beaker itself, the screw cap and a rubber seal. The rubber seal is placed inside the cap before screwing it onto the beaker.
The beakers are the vessels used for the actual detergency tests. The solution and the textile stripes are placed inside the beaker before testing.
The cap must be screwed on carefully, as the continued opening and closing of the beakers causes significant stress to the thread and can damage it. As a rule of thumb, hand-tight screwing has proven to be sufficient to keep the beakers leak tight.
The beakers must be stored open and with the rubber seal removed from the cap, to ensure complete drying after cleaning the beakers.

Accessories

Stainless steel balls

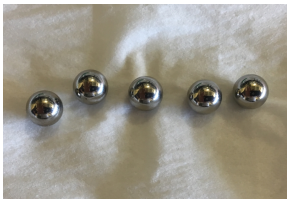

Figure 15: The 8 mm stainless steel balls used to simulate mechanical input in laundry washing processes.

To further simulate the mechanical input of a laundry washing process, five stainless steel balls with a diameter of 8 mm are placed inside of every beaker before a test. A sufficient amount (at least 75 balls) should always be stored close by the DTU.

Tools

Screw-cap spanner

A spanner that matches the caps of the beakers was also built. However, it must be used with care, as it is easily possible to screw the caps on to tightly with the spanner, causing damage to the thread of the cap or the beaker.

Beaker holder

To help closing the beakers more comfortably, a table-mounted beaker holder was built. Beakers placed on this holder will not turn while the cap is screwed on or off. It is strongly advised against combining the spanner and the holder for closing the beakers, as it is very easy to cause damage to the threads due to screwing the cap on to tightly. Hand-tight screwing of the cap while the beaker is placed on the holder is enough. On the other hand, placing a beaker on the holder and unscrewing the cap with the spanner might be necessary at times to unscrew a stuck cap.

Further tools

Further tools needed for operation of the DTU are hose clamps, to safely tighten the hoses connecting the unit with the thermostat to avoid leakages and a screwdriver to tighten/loosen the hose clamps.

It is also recommendable to have a pair of pliers at hand to remove hoses from the connectors, as they can be stuck when not removed for a longer time.

Working procedure

Preparation

Before any tests can be performed, several preliminary checks have to be performed.

Hoses
Make sure the hoses connecting the unit and the thermostat are correctly installed and tightened with hose clamps. Also make sure that the hoses are not damaged to avoid leakages.

Water
Check if enough water is in the unit and the thermostat. If the hose connection of the thermostat is positioned higher than that of the DTU, water may flow from the thermostat into the DTU's container. If the water level in the thermostat is too low, water from the DTU's container must first be filled into the thermostat. It is not recommended to use fresh water to fill up the thermostat as that may cause the DTU's container to overflow. Only refill the container with fresh water if the water level in the container is insufficient.
It is recommended to use only millipore water for the unit to avoid limescale deposition and to avoid infestation with microorganisms.

Electricity
Make sure that the electric motor is correctly connected with the control unit and that the control unit is correctly plugged into the power supply. Only then plug the power supply into a wall socket.
If everything is plugged in correctly, the motor should be able to be activated with the rotary knob.
CAUTION: To avoid injury, it is recommended to only turn on the electric motor with the container lid closed. It is important that the motor is not turned on if hands or other body parts are still inside the container.

Temperature setting
Turn on the thermostat and set the temperature that will be used for the test. Make sure that the circulation valve of the thermostat is opened, so that it can heat or cool the water in the DTU's container. When the thermostat shows that the set temperature is reached, wait for an additional 10 minutes before starting any tests to ensure the temperature in the unit is also correct.

Testing

In the following, general advice for testing procedures is given.
If tests are performed at temperatures deviating more than 5 °C from ambient temperature, it is recommended to heat/cool the detergent solution before the test to ensure the required temperature is reached quicker inside the beakers.
Stripes can be prepared from a variety of available textiles. The size of the beakers has been chosen to use smaller stripes (e.g. 5x2 cm). It is dependant on the type of test to be performed how many stripes are to be put inside of one beaker.
When preparing a test, it is advisable to stick to the following order:

- Place the rubber seals inside the caps
- Place the steel balls inside the beakers
- Fill the detergent solution in all beakers that will be used for the test
- Add the stripes to the beakers
- Close the beakers and immediately place them into the DTU
- Make sure to properly attach the metal plates holding the beakers in place
- Close the lid and start the test as soon as all beakers are mounted inside the testing unit

Note that the detergent solution will start to dissolve soil on the stripes as soon as they are placed in the beakers. A delay to the start of the test may falsify results.
If not all of the 15 beakers will be used, make sure to distribute the beakers evenly around the three sides of the beaker mount to ensure an even weight distribution around the axis.

Set the speed of the motor according to the needs of your testing method. As a rule of thumb, 50-60 rpm has proven sufficient for most tests.
When your test is complete, the following steps have to be performed:

- Set the electric motor to 0 rpm. Do not remove the lid before the axis has stopped
- Slowly turn the axis so that one side faces upward
- Unscrew the holding plate, remove it, the remove all beakers placed in this side of the mount
- Slowly turn the axis to the next side and repeat the previous step
- Repeat again for the third side
- If no further tests will be performed, turn of the thermostat and close the circulation valve
- Open the beakers and remove the stripes. If necessary, use the beaker holder and the screw-cap spanner

It is sufficient to clean the beakers with deionized water. If your testing method requires a rinsing step performed with the unit, it is recommended to flush the beakers once with millipore water, if the rinsing step is performed with millipore water. Rinsing with the DTU is performed in exactly the same way as a detergency test, only with rinsing solution instead of detergent.
When testing is complete, remove all rubber seals from the caps an clean the caps and seals separately with deionized water. The beakers, caps and seals can then be set to dry.

Maintenance

In general, the DTU works reliably and without failure. However, regular maintenance might still be required, especially when the unit is heavily used.

Cleaning the container

The water may need to be replaced regularly. Since the DTU is not sterile, the water might start to emit a poor smell and turn turbid after a while. In this case, all water must be removed from the unit. After removing all water, flush the unit with deionized water until the water is clear again. The same process should be performed with the connected thermostat. Then, wipe all surfaces inside the container with 2-propanol (technical grade). Let the unit dry over night before refilling it with millipore water.
In order to prevent the formation of microorganisms further, a chlorine based granulate for swimming pool disinfection can be used. A concentration similar to that recommended on the packaging has proven sufficient. Dissolve about 80-100 mg of the granulate in approx. 1.5 L of millipore water and then add the solution to the DTU. Turn on the electric motor at approx. 60 rpm for 1-2 minutes to ensure proper mixing.
It is advisable to add one spatula point of granulate weekly to the DTU's thermostat circuit for continued sanitation. The granulate must be dissolved in water before adding it to the DTU, as it only dissolves under strong agitation.

Replacing the fuse of the electric motor

The control unit of the electric motor is equipped with a 3,15 A fuse to protect the motor from damages. If the fuse has blown, unplug all cables from the control unit, then open the lid, by unscrewing the 4 screws marked on the left in Figure 16 and lifting off the lid. The fuse can be seen on the right of Figure 16. To remove it, lift it carefully with a small screw driver until it is out of the socket. The new fuse

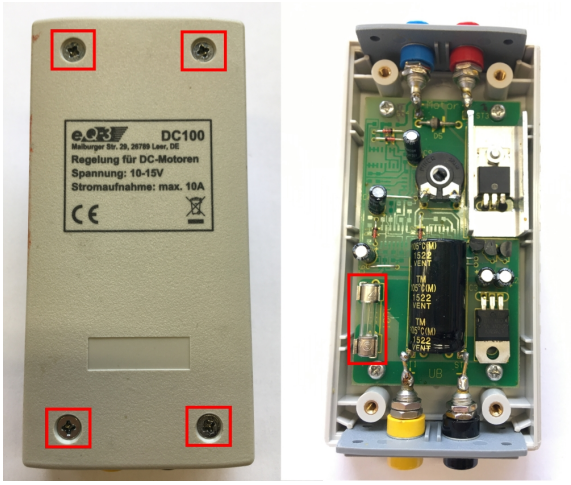

Figure 16: Left: The back lid of the control unit with the 4 screws that need to be unscrewed to open the lid marked with red squares. Right: The inside of the control unit. The fuse is marked with a red square.

can be pushed in by hand. A spare fuse should always be kept around the unit. If no spare fuse is available, ask for a replacement in the electronics workshop in the basement. Use the broken fuse as reference.

Leakages

Upon heavy usage, a leakage of the unit may occur. The most likely weak spot is the contact point of the motor and the axis. In such a case, unplug the unit immediately, empty the container and contact the mechanics workshop in the faculty's basement.

Beakers

Check before every test if the thread of any beaker and/or lid that is in use screws properly. If the thread screws only with difficulty, or there are noticeable blocking points, the thread must be repaired at the mechanics workshop. A damaged thread may result in a beaker not opening at all after a test.

Steel balls

Check if the steel balls have visible rust on their surface. Attempt to scrub rust of them, as it may falsify results. If the rust cannot be removed from a ball, it must be replaced.

Declaration

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

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Regensburg, den 27.04.2021

Franz Hippolyth Schermer