Lipase activity in Swedish raw milk

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Abstract

Bovine milk contains several enzymes which can affect the quality of milk. One of these enzymes are lipases which hydrolyse the triacylglycerides in milk. In this process off-flavours, odour and product defects are generated. By controlling the enzymes activity, milk quality can be increased and therefore milk and dairy products can be stored for a longer time. This is desirable since the world demand for long shelf-life milk and milk products is increasing.

To monitor the quality of raw milk and control the enzyme activity, a well-functioning method to detect lipase activity is desired. Thus, the aim of this thesis was to investigate and improve an existing method to determine lipolytic activity which is based on a fluorescent approach. With this method, a sensitive fluorescent measurement of the lipase activity directly in the natural milk environment is possible.

Once the method was evolved, raw milk samples from different regions in Sweden and from different origins, i.e. farm and dairy, were investigated and evaluated for possible differences in lipase activity. No significant difference was found between samples from farm and dairy origins. Between the different regions a significant difference was discovered. The lowest lipase activity in milk was found in milk from the south (Skånemejerier) followed by milk from the mid region of Sweden (Arla) and the highest lipase activity was seen in milk from the north (Norrmejerier). The lipase activity was also correlated to other previously measured properties of the milk. A significant negative correlation to some long chain fatty acids could be seen. This indicates that long chain fatty acids inhibit lipase activity in the milk which has previously been observed in the adipose tissue of rats and in goats milk. The long chain fatty acid content in milk can be influenced by the feed. Thus, the results of this thesis indicate that the raw milk quality could be improved by increasing the amount of long chain fatty acids in the milk through the feed and thereby reduce lipase activity.

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Popular science summary

Lipases are enzymes that split the fat in milk. This reaction is called lipolysis and results in the release of free fatty acids. These free fatty acids give off-flavours, odour, and product defects to the milk. By controlling the enzyme activity, milk quality can therefore be increased and the shelf life of milk and dairy products can be extended. This is desirable since the world's demand for long shelf-life milk and milk products is increasing.

Hence, it is useful to have a well-functioning method which can detect lipase activity to monitor the quality of raw milk. There are some existing methods to measure the lipase activity. However, the optimal assay for routinely measurements of dairy products for predictive purposes is still not devised. One method was published that measures the lipase activity in the natural milk environment, which has a limited number of experimental steps and has a high sensitivity. However, previous attempts to repeat this method failed. Therefore, the scope of this project was to further investigate that method and improve it.

The theory behind this method is to add a substrate to the milk and let it incubate for a specified time. This substrate consists of a fatty acid that is attached to a molecule called 4-methylumbelliferyl. This compound is non-fluorescent unless a lipase splits off the fatty acid to yield one molecule of highly fluorescent 4-methylumbellifery and one molecule fatty acid. Thus, if there is more lipase activity present, more fatty acids are split off and the fluorescence is higher. A fluorescence measurement is not possible in a turbid media like milk and therefore the turbidity needs to be decreased before a fluorescence measurement can be carried out. In milk, the fat droplets and proteins that are present in form of casein micelles cause the turbidity. The basic procedure to remove turbidity is a defatting step, followed by the addition of two solutions to remove the turbidity of the milk by unfolding the protein and make it go into solution. Furthermore, these added solutions stop the enzyme conversion by lowering the pH and thereby denature the lipase. Fluorescence intensity is highly dependent on pH and thus the last solution also rises the pH to a near neutral pH where acceptable fluorescence intensities are seen.

By adjusting the pH as it was specified in the previously published method, the method to detect the lipolytic activity showed to be working. However, the variation between data was high and thus further investigation on optimal pH and experimental procedures finally resulted in a lower variation. Also, the centrifugation step and incubation temperature were optimized to attain a more reliable method. It was possible to reduce the limit of detection and quantification of lipase activity and thereby not just attain a more robust but also more sensitive method in comparison to the previously published method. The newly developed method was validated by incubating the substrate in the milk for different times and checked for a linear relation. Also, a comparison of a fresh milk and the same fresh milk which was frozen for different times showed that there is no difference in lipase activity between a fresh and frozen sample. This is important since the measurements carried out later were done on frozen samples.

With the developed method, raw milk samples from different regions of Sweden were examined and evaluated for possible differences. The samples were taken both on dairy and farm level. No significant difference between these origins could be seen. However, all three regions were significantly different from each other - the milk from Skånemejerier (south of Sweden) exhibited the lowest lipase activity followed by milk from Arla (mid Sweden) and the highest

lipase activity was detected in milk from Norrmejerier (north of Sweden). The lipase activity results were also correlated to other properties of the milk measured previously. These properties include the fat content, amount of fatty acids, and bacterial count of the milk. However, none of the properties had a significant correlation except for some long chain fatty acids which showed a negative correlation to the enzyme activity. This indicates that a high amount of certain long chain fatty acids reduces the lipolytic activity. This effect has been seen in the adipose tissue of rats and in goat's milk before. Since the amount of long chain fatty acids in the milk can be influenced by the feed, a hypothesis is that the lipase activity could be reduced by changing the feed of the cows.

Content

List of Abbreviations and Symbols

[E]	Enzyme concentration
[S]	Substrate concentration
4-MU	4-methylumbelliferyl
4-MUB	4-methylumbelliferyl butyrate
4-MUL	4-methylumbelliferyl laurate
CV	Coefficient of Variance
FA	Fatty acids
FFA	Free fatty acids
ICH	International Conference on Harmonisation
LCFA	Long chain fatty acids
LOD	Limit of Detection
LOQ	Limit of Quantification
LPL	Lipoprotein lipase
MEDELI	Method for Determination of lipolytic activity from Krewinkel et al. (2016)
p-NP	*p*-nitrophenyl
RFU	Relative Fluorescence Unit
s	Slope of calibration curve
S.D.	Standard deviation
SCFA	Short chain fatty acids
TAG	Triacylglycerides
UHT	Ultra-high temperature
UMB	Umbelliferone
σ	Standard deviation

1 Introduction

Lipases can impair the sensory quality as well as the storage stability of milk by hydrolysing triacylglycerides (TAG) into free fatty acids. Even low lipase activities can cause off-flavours in dairy products (Krewinkel et al, 2016). At the same time, changes in dairy distribution patterns, i.e. the centralization of the dairy industry, result in increased storage periods of raw milk. Furthermore, there is a shift in the export market, consumer expectations as well as an increased demand for milk products with extended shelf life (Krewinkel et al., 2016; Murphy et al., 2016). All this results in a greater demand for dairy products with high quality standards. To produce those high-quality dairy products, dairies require higher raw milk quality (Murphy et al., 2016). A reliable test system for the determination of the lipase activity is therefore desirable to monitor and guarantee the quality of milk products (Krewinkel et al., 2016).

Krewinkel et al. (2016) published a method to determine lipolytic activity in milk called MEDELI. This method is based on a fluorescent approach. It is possible to measure lipase activity directly in the natural milk utilizing tailored fluorometric substrates with this method. A defatting step (centrifugation) is carried out before the milk is incubated with a substrate solution and treated with a stop and a neutralising solution to clarify the milk. The paper suggests that this process enables a sensitive fluorometric measurement. However, prior trials to replicate this method have failed. Therefore, the method is further investigated and improved. With the developed method, raw milk samples are measured.

The report is divided into two parts: the development of the method and the measurement of 45 raw milk samples from different origins and regions in Sweden.

1.1 Aim

It is the aim of this degree project to further investigate the method to determine lipolytic activity in milk proposed by Krewinkel et al. (2016) and improve it. Once the method is evolved, raw milk samples from three different regions of Sweden are investigated and evaluated for possible differences. Some samples are collected on farm level and others on dairy level. Thus, a comparison between these two levels will be carried out too. Furthermore, the measured lipase activity is compared to other properties of the milk previously measured.

1.2 Hypothesis

A hypothesis is that by further investigating the method after Krewinkel et al. (2016) it can be improved in regard of sensitivity and robustness. Another hypothesis is that by controlling lipase activity in raw milk the shelf life can be extended.

1.3 Environmental aspects

By controlling the lipase activity in raw milk, the shelf life can be extended which will result in less product waste and therefore in a more sustainable food production.

2 Theoretical Background

2.1 Composition and structure of milk

Milk consists of water, fat, proteins, lactose, and minerals as principle ingredients. However, there are also trace amounts of other substances like pigments, enzymes, vitamins, phospholipids and gases (Walstra et al., 2006). The average composition of milk can be seen in Table 1. Nevertheless, fresh raw milk often varies in composition, structure, and properties. The basic cause for this are e.g. genetic factors, the stage of lactation, illness of the cow, or the feed. At the dairy there are only small variations in milk composition since milk of many cows from several farms is mixed (Walstra et al., 2006). Milk has an average pH of 6.7 at room temperature (McSweeney & Fox, 2013).

Table 1: Average composition of the main components in milk (Walstra et al., 2006).

Component	***Average composition (%)***	***Range in variation (%)***
Moisture	87.1	85.3-88.7
Fat	4.0	2.5-5.5
Proteins	3.3	2.3-4.4
Lactose	4.6	3.8-5.3
Minerals (Ash)	0.7	0.6-0.8

Milk is a complex product since it has a heterogeneous composition and three physical states are present. Whey proteins are present in solution, the larger micellar caseins make it a colloidal suspension and the fat creates an oil-in-water emulsion (Humbert et al., 2006). The sizes of the different components can be seen in Table 2. The existence as dispersion gives milk the white colour (Walstra et al., 2006).

Table 2: Average size of components in milk (Bylund, 2015).

Size (mm)	***Type of particles***
10^{-2} *to* 10^{-3}	Fat globules
10^{-4} *to* 10^{-5}	Casein-calcium phosphates
10^{-5} *to* 10^{-6}	Whey proteins
10^{-6} *to* 10^{-7}	Lactose, salts and other substances in true solution

For the young calf, milk offers a nutritionally complete food. Also for humans it provides good nutrition by being rich in nutrients. However, since it contains many nutrients in significant quantities, microorganisms, especially bacteria, find good growth conditions in this medium too. Especially if the milk is at high ambient temperature, bacteria can grow (Walstra et al., 2006). Milk from healthy cows is generally sterile. However, as soon as the milk leaves the udder, the temperature decreases, and it can come in contact with for example oxygen and bacteria. These factors can lead to changes in the milk of physical, chemical, biochemical, and microbial nature. Biochemical changes are due to enzyme activity, e.g. lipase. To inhibit or reduce the changes, milk is cooled to about 4 °C. Particularly the growth of microorganisms and the enzyme action will be reduced in this manner. Therefore, it is important to constantly cool the milk, also during transport to the dairy and subsequent storage (Walstra et al., 2006).

2.1.1 Milk Fat

As can be seen in Figure 1, the milk fat is present as globules and is dispersed in the milk serum, making it an oil-in-water emulsion (McSweeney & Fox, 2006). The fat globules are 0.1-20 µm in diameter size with an average diameter of 3-4 µm. The membrane which surrounds the globules is 10-20 nm thick and has a complex composition consisting of phospholipids,

lipoproteins, cerebrosides, proteins, nucleic acids, enzymes, trace elements (metals) and bound water (Bylund, 2015). The membrane displays a barrier between the plasma and the core lipids and protects the globules against coalescence (Walstra et al., 2006). The fat consists primarily of TAG, other components are di- and monoglycerides, fatty acids (FA), sterols, carotenoids, and vitamins (McSweeney & Fox, 2006). Only a small part of the lipids is present outside the fat globules. Accounting for around 98 % of the total fat, TAG influence considerably the properties of the milk fat, for instance the hydrophobicity, density, and melting characteristics. This is due to the composition of FA in the TAG. They do not only vary in chain length (between 2 and 20 carbon atoms) but also in saturation (0 to 4 double bonds) (McSweeney & Fox, 2006; Walstra et al., 2006). The most abundant FA are myristic, palmitic, stearic, and oleic acids (Bylund, 2015). Depending on the fatty acid, part of the fat in the globules can crystallize at temperatures below 35°C (Walstra et al., 2006). McSweeney & Fox, (2006) mention a wide melting range of the milk fat from about -35°C to 38°C. This is also due to fat being the component in milk with the widest variability in concentration and composition (McSweeney & Fox, 2006).

Since the fat globules are the largest and lightest particles in the milk, they tend to cream. The creaming rate follows Stokes' Law (Bylund, 2015). Due to their low density, the fat globules can be concentrated and separated from the rest of the milk components. The creaming occurs either due to gravity or is induced through centrifugation. The latter represents a more efficient solution (Walstra et al., 2006).

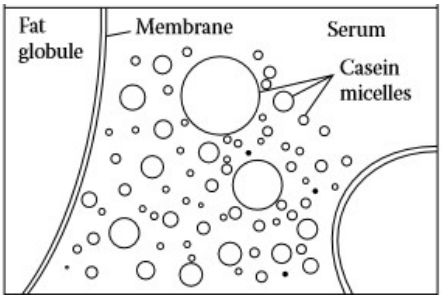

Figure 1: Milk at a magnification of 50000: Casein micelles and fat globules can be seen surrounded by serum. The fat globules have a thin outer layer (membrane) (from Walstra et al., 2006).

2.1.2 Milk Proteins

The main protein in milk is casein accounting for 80% of the total protein. There are four different forms of casein: α_{S1}-, α_{S2}-, β-, and κ-casein (Walstra et al., 2006). The caseins are unique for milk. They are phosphorylated during synthesis and aggregate into micelles by calcium being bound to phosphorus. All four forms of casein are present in the micelles, however, especially κ-casein is important for stabilizing the micelle as it protrudes from the surface (McSweeney & Fox, 2013). Since micelles have an open structure, they also contain water (Walstra et al., 2006). The micelles contain around 5000 molecules and range from 50 to 500 nm in diameter with an average of ~150 nm. One reason for the milks white appearance is the scattering of light by the casein micelles (McSweeney & Fox, 2013).

The remaining 20% of the total protein are whey (or serum) proteins. The milk serum or whey is defined as milk without the fat globules and casein micelles (Walstra et al., 2006; McSweeney & Fox, 2013). The whey proteins are present as monomers or as small quaternary structures (McSweeney & Fox, 2013). By contributing 12 % of total protein, β-lactoglobulin is the main whey protein (Walstra et at., 2006; McSweeney & Fox, 2013). Furthermore, there are also

various minor proteins, like α-lactalbumin, serum albumin, whey acidic protein, immunoglobulins, and enzymes (McSweeney & Fox, 2013).

2.2 Enzymes in milk

Enzymes are active proteins that can trigger chemical reactions and influence the course and speed of those reactions. Enzyme activity is specific and one enzyme only catalyses one type of reaction (Bylund, 2015). The activity of an enzyme depends on several factors. Principally, the reaction rate is proportional to the enzyme concentration [E]. However, the rate also depends on the substrate concentration [S]. In most enzyme activities in milk, [S] stays significantly in excess and therefore the initial reaction rate v_i is close to V_{max} (Figure 2 (a)). An exception is for instance the action of chymosin on κ-casein during milk clotting, where the substrate can eventually be used up (Walstra et al., 2006). Other factors that strongly influence enzyme activity are temperature and pH. Typically, the optimum temperature range for enzymes is between 25 and 50 °C (Bylund, 2015). As can be seen in Figure 2 (b), curve 2 shows that enzyme reactions follow Arrhenius kinetics. Though, the enzyme will start to denature and thereby lose activity at high temperatures. Curve 1 shows this event with an optimum and maximum temperature. The resulting v(T) depends on the enzymes access time to the substrate since the denaturation is not extremely fast (Walstra et al., 2006). This is displayed in curve 3. Therefore, T_{opt} and T_{max} depend on time and on heating rate (Walstra et al., 2006). The denaturation temperature varies from one type of enzyme to another (Bylund, 2015).

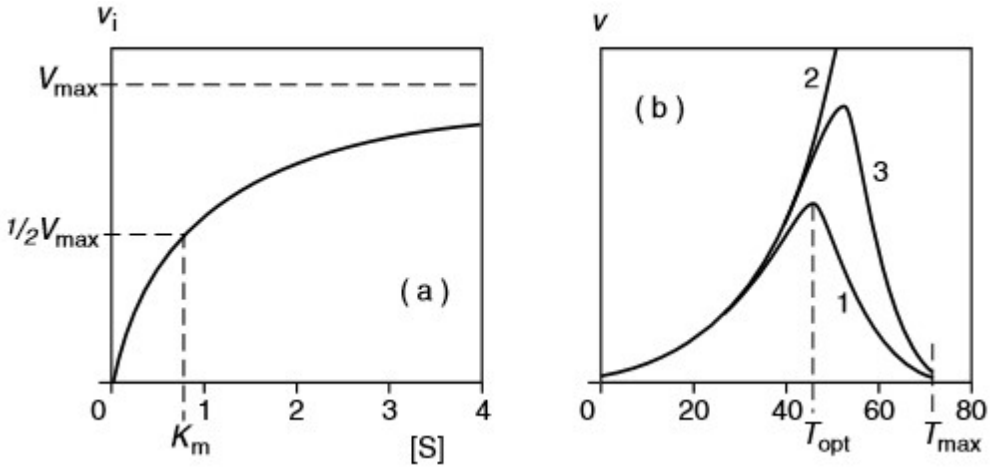

Figure 2: a) Effect of substrate concentration [S] on the initial reaction rate v_i (a) and b) example for the effect of temperature on the rate v of proteolysis caused by plasmin) (from Walstra et al., 2006).

Other factors that are affecting the enzyme action are for example the solvent properties as these can influence the conformation of the enzyme molecule. By changing the enzymes conformation, the enzyme activity and affinity for the substrate (K_m) is also affected (Walstra et al., 2006). For instance, the optimum pH range varies from enzyme to enzyme. Some work best in acid solutions and others prefer an alkaline environment (Bylund, 2015). Inhibitors can hinder enzyme action by e.g. binding to the active site of the enzyme (competitive inhibition) or by affecting the enzyme conformation. Some enzymes also need cofactors to be entirely active (Walstra et al., 2006).

The enzymes in milk are either originated in the cow's udder (native or indigenous enzymes) or they come from bacteria (Antonelli, 2002; Walstra et al., 2006). Milk contains about 70 different indigenous enzymes (Fox & Kelly, 2006). These native enzymes are associated with different parts of the milk. Many enzymes are linked to the fat globule membrane. Others are dispersed in the serum or located in the casein micelles (Walstra et al., 2006). However, milk

enzymes do not often alter the milk significantly even though the enzyme is present in high concentrations since the substrate is not present in milk (Fox & Kelly, 2006; Walstra et al., 2006). Some enzymes exhibit beneficial characteristics, e.g. antimicrobial functions (Walstra et al., 2006). Other enzymes are inactive since the environmental conditions are unsuited, e.g. pH or redox potential (Fox & Kelly, 2006). Lipoprotein lipase (LPL) for example is not extremely active in fresh milk even though this enzyme is available in high concentrations. Yet, during storage those enzymes can still cause spoilage (Walstra et al., 2006).

Since milk is rich in nutrients, has a neutral pH and high water activity, it offers ideal growth conditions for many bacteria (Vithanage et al., 2016). The microbiota in raw milk consists of microorganisms that contaminate the milk after the milk has left the udder, e.g. from the equipment or the cowshed. The cold temperature during storage selects for psychrotolerant microorganisms (von Neubeck et al., 2015). The most frequently isolated bacteria from refrigerated raw milk are Pseudomonas and Bacillus (Vithanage et al., 2016). Many of them produce enzymes, e.g. lipases and peptidases. Some of these microbial produced enzymes are greatly heat resistant and can survive pasteurisation or ultra-high temperature (UHT) treatment (von Neubeck et al., 2015). In products like cheese those enzymes can offer beneficial effects by providing aroma (Fox, 2003). However, in most other products, non-inactivated enzymes can lead to off-flavours or other quality defects before the expiry date is reached. Especially in dairy products with a long shelf life like milk powder or UHT milk this might be an issue (von Neubeck et al., 2015). Nevertheless, some enzymes are used as marker for quality tests and to monitor the efficiency of heat treatments (Walstra et al., 2006). Indigenous enzymes do not exhibit any beneficial effects to the nutritional or organoleptic properties of the milk and therefore their destruction by heat is aimed for in dairy processes (Fox & Kelly, 2006).

Common enzymes in milk are among other phosphatases, catalases, proteinases, and lipolytic enzymes (Walstra et al., 2006; Bylund, 2015). Proteases are the most important enzymes from a technological perspective. The indigenous enzyme plasmin for instance is important for the ripening of hard cheese. Milk clotting enzymes like chymosin or pepsin coagulate the milk during cheese making. A third group present are the proteinases and peptidases that origin from microorganisms (and also from the starter culture) (McSweeney & Fox, 2013). However, residual proteolytic activity can induce an increase in viscosity, generate a bitter flavour and cause gelation in milk (Vithanage et al., 2016). Lipolytic enzymes break down fat into glycerol and free fatty acids (FFA) (McSweeney & Fox, 2013). This enzyme will be further described in the next section.

Usually, enzymes are inactivated by heat treatment. The inactivation occurs by unfolding (denaturation) of the enzyme molecule (Walstra et al., 2006). The temperature needed to inactivate the enzyme varies among the types of enzymes. However, some proteolytic and lipolytic enzymes produced by *Pseudomonas spp* are highly heat resistant. By pasteurisation or UHT treatment only a minor part of their activity is inhibited (Bylund, 2015).

2.3 Lipases

Lipases catalyse the hydrolysation of TAG to FFA and glycerol (Hasan et al., 2009). This breakdown is called lipolysis (Bylund, 2015). Lipases are ubiquitous enzymes that can be found in animals, plants, fungi, and bacteria (Gupta et al., 2004). Esterases are distinguished from lipases by their preference for soluble substrates (McSweeney & Fox, 2013). Shorter chain esters are more soluble and can therefore be used by esterases as well while longer-chain esters

are insoluble and can be used by lipases only (Deeth & Touch, 2000). However, early studies did not distinguish between lipases and esterases (McSweeney & Fox, 2013).

Most lipases are water-soluble enzymes which act on water-insoluble substrates (supersubstrates) (Beisson et al., 2000). Some enzymes hydrolyse the esters in solution, though LPL is active at the interface of oil and water (Walstra et al., 2006). This is because LPL is a highly surface active molecule. The enzyme binds reversibly to the lipid-water interface while the active site remains in solution and is thereby functional (Borgström & Brockman, 1984).

2.3.1 Lipoprotein Lipase

Lipolysis in raw milk is largely due to the indigenous enzyme LPL (Borgström & Brockman, 1984; Deeth & Fitz-Gerald, 2006). LPL comes from the mammary gland into the milk by spill-over. In the mammary gland it is involved in the synthesis of milk fat TAG. However, LPL has no biological function in milk (Borgström & Brockman, 1984; Deeth, 2006). This enzyme is a glycoprotein with two N-linked oligosaccharides which are essential for the activity of the enzyme. It is a homodimer with a molecular mass of ~100 kDa (Deeth, 2006). LPL is usually associated to the casein micelles by electrostatic bounds (Walstra et al., 2006; Deeth, 2006). It displays low substrate specificity, i.e. it catalyses the hydrolysation of long- and short-chain TAG, diacylglycerols, or monoacylglycerols (Borgström & Brockman, 1984). However, LPL features positional specificity: it favours the hydrolysis of primary ester bonds, meaning the *sn*-1 and *sn*-3 positions of the TAG molecule. Thus, *sn*-2 monoacylglycerols and FFA are generated (Borgström & Brockman, 1984; Deeth, 2006). Short chain fatty acids (SCFA) are concentrated in the sn-3 position of bovine milk TAG and thus are more often released by LPL (Deeth, 2006).

LPL is rather unstable to heat and can be inactivated by pasteurisation (72°C, 15 s). Therefore, it does not cause any or only little lipolysis in pasteurized milk and products made from pasteurized milk (Deeth, 2006). This enzyme is also not stable at acid pH and will therefore be inactivated in the stomach (Borgström & Brockman, 1984). LPL has a temperature optimum of ~33 °C and an optimum pH of ~8.5 (Walstra et al., 2006).

The LPL level in milk is low in early lactation (Borgström & Brockman, 1984). Milk contains about 10 to 20 nmol per litre of the enzyme (Walstra et al., 2006). However, the level increases towards the end of the lactation cycle, when the milk yield becomes low (Deeth & Fitz-Gerald, 2006; Walstra et al., 2006). Hence, seasonal variation in the degree of lipolysis can occur. Also, an increased number of milkings can lead to elevated lipolysis. This is because those conditions increase the leakage of lipoprotein from the blood into the milk (Walstra et al., 2006).

2.3.2 Spontaneous/ induced lipolysis

Theoretically, LPL can induce rapid hydrolysis of a large proportion of the milk fat. Nevertheless, this is not the case since the fat is protected by the milk fat globule membrane. In freshly secreted milk, this globule membrane is intact and establishes an effective barrier between LPL and the fat (Deeth, 2006). Other factors slowing down lipolysis are that the enzyme is bound to the casein micelles, the pH or ionic strength is suboptimal, presence of enzyme inhibitors or product inhibition occurs (Walstra et al., 2006).

Lipolysis can have two different origins: either it is spontaneous or induced. Spontaneous lipolysis can be brought about by cooling the raw milk to ~10 °C soon after secretion. Hence, it occurs at the farm only (Deeth, 2006). The milk of some cows is particularly susceptible to

this type of lipolysis. However, milk of other cows is resistant to spontaneous lipolysis. By mixing the different susceptible milk, lipolysis is slowed down since inhibitors are present. Therefore, mixed milk seldom becomes rancid (Walstra et al., 2006; Deeth, 2006). The main sources associated with spontaneous lipolysis are late lactation, poor-quality feed, and mastitis (Deeth, 2006).

Induced lipolysis occurs if the fat globule membrane is damaged by physical treatments like pumping or homogenization (Deeth & Fitz-Gerald, 2006; Borgström & Brockman, 1984). The more severe and the longer the physical forces are applied, the stronger is the disruption. Freezing and thawing can also disrupt the membrane (Deeth, 2006). Thereby, fat will be exposed and lipase can bind to the fat globules and hydrolyse the fat molecules (Bylund, 2015). Thus, agitation or homogenization of unpasteurised milk should be avoided since this would give rise to rapid lipolysis (Borgström & Brockman, 1984). If certain blood serum lipoproteins are present, the lipase can also absorb onto the fat and cause fast lipolysis (Walstra et al., 2006). The serum lipoprotein acts as activator protein for LPL (Borgström & Brockman, 1984).

Spontaneous and induced lipolysis both proceed during subsequent storage of the milk. However, most lipolysis will occur during the first 24 h when the milk is refrigerated (Deeth, 2006).

2.3.3 Microbial lipases

The principal lipases that are of microbial origin are produced by psychrotrophic bacteria (Deeth & Fitz-Gerald, 2006). They can be formed during milk storage or processing (Krewinkel et al., 2016). The lipases that are produced by these bacteria exhibit different characteristics than LPL (Deeth, 2006). Bacterial enzymes from psychrotrophic bacteria are heat stable - pasteurisation and UHT treatment cannot destroy them completely (Krewinkel et al., 2016). Therefore, they can have an exceptionally significant effect on the quality of stored products (Deeth & Fitz-Gerald, 2006). Most microbial lipases have their optimum pH for lipolytic activity in acid and neutral media (Hasan et al., 2009). The differences in the properties of LPL and microbial lipases are summarized in Table 3.

Table 3: Comparison of the characteristics of LPL and lipases from psychrotrophic bacteria (from Deeth, 2006).

Lipoprotein Lipase (LPL)	**Lipases from psychrotrophic bacteria**
Destroyed by pasteurisation	Stable to pasteurisation and UHT treatment
The milk fat globule membrane acts as a barrier to the lipid substrate	The membrane does not present a barrier
Activated by serum lipoproteins	Serum lipoproteins do not activate most microbial lipases
The effect is mostly associated with fresh milk and cream	The effect is mostly associated with stored products, e.g. UHT milk, cheese, butter, milk powder
Effect in cheese/ butter obvious at manufacture and does not change during storage	Effect in cheese/ butter obvious only after storage
High levels in (raw) milk	Only trace levels in (raw) milk

2.3.4 Consequences of lipolysis

The effects of lipolysis are twofold: firstly, flavours are produced and secondly the functionality is altered (Deeth, 2006). The generated flavours can be desirable but also off-flavours, i.e.

rancid, bitter, soapy, or astringent flavours are developed (Krewinkel et al., 2016). Especially short and medium chain FFA exhibit strong, undesirable flavours (Deeth, 2006). Already 1-2 % hydrolysed TAGs to FFAs give a rancid flavour to the milk (Borgström & Brockman, 1984). The term ‘‘rancid’’ is used to describe the off-flavour due to lipid oxidation as well, however, the two types of rancidity are clearly different in their origin and flavour (Deeth, 2006). FFA can also be the precursors for other flavour compounds, e.g. lactones, methyl ketones, or esters (Krewinkel et al., 2016). In some cheeses, flavours due to hydrolysed TAGs are also desirable (Deeth, 2006).

Functional defects can be the depression of its foaming ability when injected with steam, e.g. when making cappuccino. This is because partial glycerides, which are produced during lipolysis, are surface active and substitute the foam stabilizing proteins at the air-water interface of the air bubbles. Other functionality defects like impaired creaming ability during separation or an increased churning time in butter manufacture have been noted (Deeth, 2006).

In fresh milk there are only small amounts of FFA. With appropriate milking and storage conditions, milk can be kept for several days with minor further development of FFA. Only after prolonged storage or poor hygienic handling bacterial lipases can become important (Borgström & Brockman, 1984).

2.4 Method for detection of lipolytic activity (MEDELI) after Krewinkel et al. (2016)

This method was developed to offer a simple, sensitive, and reliable option to measure lipolytic activity directly in the natural milk environment. The basic procedure is a defatting step followed by an incubation with the substrates 4-MU butyrate (4-MUB) and 4-MU laurate (4-MUL). The non-fluorescent 4-MUB and 4-MUL are split by lipases into one molecule 4-methylumbelliferyl (4-MU) and the corresponding fatty acid. 4-MU has fluorometric properties which can be measured in the clarified sample. Thus, two solutions are added to remove the turbidity of the milk by dissolution of proteins and to adjust the pH (Krewinkel et al., 2016).

Three solutions are used for this method:

1. **Substrate solution**: This solution consists of 4-MUB and 4-MUL dissolved in 2-propanol.
2. **Stop solution**: This solution consists of guanidine hydrochloride (GuHCl) and HCl in water. It will shift the pH to below 2. Thus, lipases are denatured but 4-MU will not be destroyed for the fluorophore measurements.
3. **Neutralising solution**: This solution consists of Bis-tris, NaOH and EDTA disodium salt in water. It will help the clarification of the sample and shift the pH to 6.5. At this neutral pH, better fluorescence is observed and non-enzymatic hydrolysis of the substrate is not reported (Krewinkel et al., 2016).

The preparation steps include the following:

1. The milk sample is centrifuged (4 °C, 10 min, 20000g). This is carried out to remove milk fat since persisting milk fat would compete with the 4-MU derivate substrates that are added. Thus, a lower lipase activity would be measured.
2. The defatted milk is pipetted into a new reaction tube and is preincubated (800rpm, 40°C, 5 min in ThermoMixer).
3. The measurement starts by adding 25 µL of the substrate solution.
4. The reaction is stopped after 5 min by addition of 150 µL stop solution followed by short mixing.

5. 100 µL neutralising solution is added to neutralize and clarify the solution.
6. Milk samples that contain a higher fat content than 1.5% fat should have a second centrifugation step (4°C, 3 min, 20000g) for clarification.
7. The fluorescence released is determined by excitation at 355 nm and emission at 460 nm for the two substrates (4-MUB and 4-MUL) (Krewinkel et al., 2016).

The different steps are summarized in Figure 3.

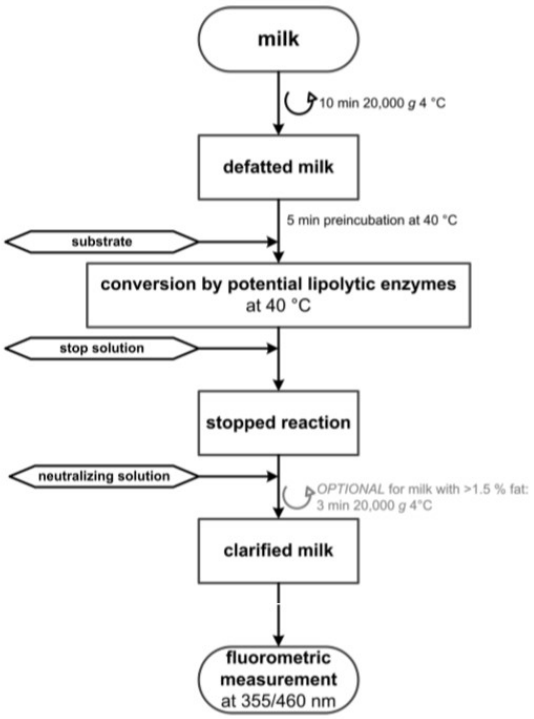

Figure 3: Procedure for the lipase activity measurement in milk after Krewinkel et al. (2016).

A blank value for the fluorescence measurement is determined by adding the stop solution to the milk before the substrate is added to the tube. A calibration curve with different 4-MU concentrations is prepared and should be checked after preparing new batches of the solutions due to high concentrations of compounds in the stop and neutralising solutions (Krewinkel et al., 2016). One katal of lipase activity corresponds to the release of 1 mol 4-MU within 1s.

This method showed to have several advantages in comparison with other methods to measure lipolytic activity. Firstly, the measurement is carried out in the unmodified milk sample without any enzyme extraction procedures. Therefore, the enzyme activity is measured in the natural matrix and the enzyme activity is not influenced as it happens in modified systems. This also implies, that there is no loss of formed analyte (4-MU) before the measurement, as may occur during extraction steps. Overall, this method offers a simple procedure without time consuming laborious pre-treatments. It was shown to be sensitive, reliable, robust and only requires a limited demand of apparatus (centrifuge and fluorimeter). Also, the limit of quantification (LOQ) is low compared to other methods (Krewinkel et al., 2016).

2.4.1 Clarification of the milk

Milk is turbid due to emulsified casein micelles and fat globules (Humbert et al., 2006). However, casein micelles scatter the light far less than the fat globules do. The reason for this is that they are smaller in size and inhomogeneous (Walstra et al., 2006). This turbidity prevents a direct photometric measurement (Krewinkel et al., 2016). Therefore, the sample needs to be pretreated by precipitation, centrifugation and/or filtration. A commercially available

'clarifying agent' (a mixture of organic solvents and detergents with an apparent pH >13) is available which renders casein micelles and fat globules soluble and thereby allows direct spectrophotometric measurements. However, it is important to assure that the clarifying agent does not induce hydrolysis of the remaining substrate, destroy any reaction product or hamper the spectrophotometric measurement (Humbert et al., 2006).

In the MEDELI the presence of scatterers and absorbers would influence the released fluorescence of the fluorophore (4-MU). The clarification is carried out by centrifugation and by addition of the stop and neutralising solution. In the centrifugation step the milk fat is removed. Therefore, the casein micelles cause the remaining turbidity. The casein micelles are solubilized by GuHCl in the stop solution with synergistic action of EDTA in the neutralising solution. EDTA will form complexes with calcium which lead to a destabilisation of the casein micelles (Krewinkel et al., 2016).

2.4.2 Fluorescence assay (4-MU)

Fluorescence assays are very useful analytical methods due to their high sensitivity, good selectivity, simplicity, speediness, and low cost (Zhi et al., 2013). In the method by Krewinkel et al. (2016), 4-MU is used as analyte for the detection of lipolytic activity. 4-MU is a synthetic coumarin compound that exhibits fluorescent properties. Table 4 shows, that the pH has an important influence on the fluorescence of 4-MU. This is because the molecular structure of 4-MU changes along with the change in pH (Zhi et al., 2013).

Table 4: Fluorescent properties of 4-MU at different pH (from Zhi et al., (2013)).

Condition	Near neutral	Weak alkaline pH 7.1-10.4	Acidic pH 2.0-6.7	Alkaline pH 10.8-13.4
	Strong fluorescence	Fluorescence intensity enhanced iso-fluorescence point formed at 330 nm	Fluorescence intensity declined	Fluorescence emission at 445 nm gradually quenched
Max excitation wavelength (λ_{ex})	320 nm	360 nm	320 nm	320 nm
Max emission wavelength ((λ_{em})	445 nm	445 nm	455 nm	445 nm

Figure 4 shows 4-MU at different conditions. 4-MU consists of a benzene and a lactonic ring. In strong acidic condition, it will be protonated and thereby be cationic (I). This will lead to a decrease in fluorescence intensity and a red shift in the emission wavelength. In near neutral conditions, 4-MU exists primarily as molecular form (II). This form has a strong fluorescence with λ_{ex} of 320 nm and λ_{em} of 445 nm. 7-hydroxyl proton dissociates in weak alkaline conditions and therefore 4-MU exists mainly as anion form (III). This form features a stronger fluorescence with λ_{ex} of 360 nm and λ_{em} of 445 nm. However, in strong alkaline conditions (pH>12), hydrolysis of the lactone bonds takes place (IV). This leads to a quenched fluorescence (Zhi et al., 2013). However, Deeth & Touch (2000) suggest that even though highest fluorescence is detected at weak alkaline conditions, the measurements are best performed at pH 6-7 because in the range of pH 7-9 the fluorescent characteristics of the 4-MU change markedly with ionisation so that the fluorescent response is excessively sensitive to slight changes in pH. Overall, 4-MU is considered as an excellent fluorophore (Zhi et al., 2013).

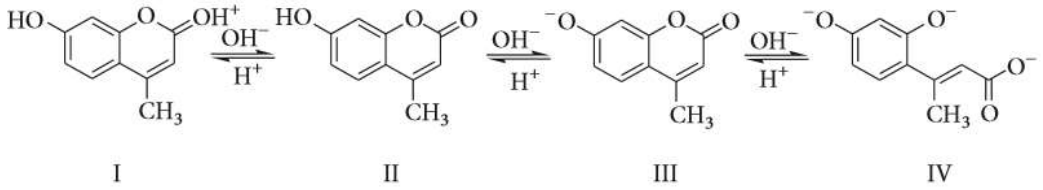

Figure 4: Conformation of 4-MU at different conditions: proton ionization and hydrolysis process (from Zhi et al., (2013)).

It was found that solvent polarity has a profound effect on the emission spectral properties of fluorophores and the sensitivity of the method. Therefore, water as solvent should be preferred over e.g. methanol (Zhi et al., 2013).

4-MU substrates are available with various FA of different chain lengths. Therefore, it allows the adaption to lipases with different substrate spectra. Alternative to the fluorimetric measurement, the utilization of chromogenic substrates, e.g. p-nitrophenol esters, which were described for lipase measurements before can work as well (Krewinkel et al., 2016).

2.5 Other lipase detection methods

There are numerous other methods to measure lipolytic activity. They can be categorized into the following types: titrimetric, colorimetric, spectroscopic (photometry, fluorimetry, infra-red), chromatographic (gas and liquid), radioactivity, interfacial tensiometry, turbidimetry, conductimetry, immunochemistry, and microscopy (Antonelli, 2002; Beisson et al., 2000; Deeth & Touch, 2000). The choice of method depends on the user's specific requirements. The sensitivity, availability of substrates, and ease of the procedure should be considered when assaying enzymes (Hasan et al., 2009).

The methods measure either the reaction products (FFA), detect lipase-producing microorganisms or directly measure the lipase activity (Deeth & Touch, 2000). Factors like the pH, temperature, metal ions, organic solvents, detergents or surfactants can enhance or suppress lipolytic activity (Hasan et al., 2009). Therefore, it is important to perform the measurements in the original milk environment instead of in modified systems where the enzyme activity might be influenced (Krewinkel et al., 2016).

Since there are various different methods to measure lipolytic activity, not all of them can be further specified. However, three commonly used methods were chosen as examples and are described in the following sections.

2.5.1 Titrimetric (pH-stat method)

Titration methods are widely used to measure lipase activity in milk and milk products (Deeth & Touch, 2000). The reason for this is their simplicity and the use of inexpensive equipment. The basic principle of this type of method is as follows: FFA, which are formed during incubation of the lipase with an ester substrate, are titrated with an alkaline solution to a set pH and end point. The ester substrate is usually a triacylglycerol. The titration can be done either continuously during the lipase reaction, as in the pH-stat method, or subsequent to the reaction, as in solvent-extraction methods (Deeth & Touch, 2000). The pH-stat method is well-known and generally used as a reference lipase assay (Beisson et al., 2000). Figure 5 shows the basic principle of this method. The lipase activity is measured by injecting the enzyme into a thermostated reaction vessel which contains the mechanically stirred emulsion of natural or synthetic TAGs (substrate). The amount of added NaOH needed to neutralise the FFA released with time in order to maintain the pH at a constant end point value is measured to determine the lipase activity (Beisson et al., 2000; Hasan et al., 2009). The lipase activity is proportional

to the volume of base needed to maintain a constant pH during the time of measurement (Deeth & Touch, 2000). This quantitative method is sensitive within 1 µmol of released fatty acid per min. For levels lower than 0.1µmol per min this method is not reliable (Beisson et al., 2000; Hasan et al., 2009).

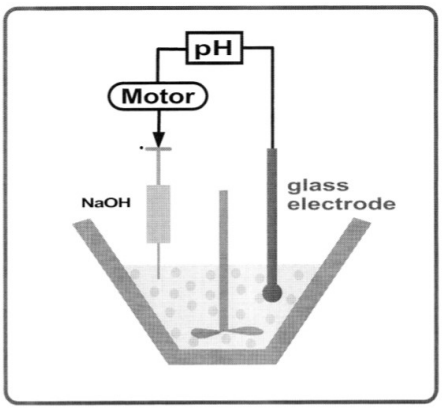

Figure 5: Depiction of the principle of the pH-stat method (from Beisson et al., (2000)).

Usually, olive oil, triolein, or tributyrin are used as substrates for this method. The latter one is mostly used as lipase substrate. However, tributyrin is not specific for lipases and can also be hydrolysed by esterases (Kademi et al.,2006).

The drawbacks of this method are its low sensitivity and a restricted pH range that can be investigated. This is because the end point value of the pH of the reaction medium needs to be equal or higher than the apparent pK_a value of the released FA in order to attain a partial ionisation so that the protons released will be detected (Beisson et al., 2000). A complete ionization of FFAs is attained at pH 9.0. Therefore, highest lipase activity can be found in a test medium adjusted to this pH value (Hoppe & Theimer, 1996). If the FFAs are not fully ionised at the selected pH value, the continuous titration is either highly inaccurate or impossible to perform, even if correction factors are applied (Beisson et al., 2000). The method is also time-consuming and therefore tedious for large-scale screening of lipolytic activity (Deeth & Touch, 2000).

2.5.2 Fluorescence assay

Several other fluorimetric assays to measure lipolytic activity based on 4-MU esters in defined buffer systems have been developed before (de Monpezat et al, 1990; Jacks & Kircher, 1967; Roberts, 1985). The method was originally developed by Jacks and Kircher (1967) who chose 4-MU since it is among the most intensely fluorescent substances known and lipolytic activity can be followed directly by their fluorescence. The hydrolysis of the acylated non-fluorescent 4-MU derivates is continuously monitored by measuring the increase of the fluorescent intensity of the enzyme reaction mixture due to the production of free 4-MU (Jacks & Kircher, 1967; Roberts 1985). The activity is expressed as the amount of 4-MU released per unit time by using a standard curve of the fluorescence of 4-MU (Deeth & Touch, 2000). De Monpezat et al. (1990) found that umbelliferone (UMB) has similar initial fluorescence properties as 4-MU. However, UMB is more stable than 4-MU and can be an alternative for the use of 4-MU, especially when detecting weak lipase activity.

The substrate emulsions are usually prepared by adding the ester in a water-soluble organic solvent like 2-methosyethanol or 2-propanol (Deeth & Touch, 2000; Krewinkel et al., 2016). A buffer, e.g. Tris-HCl, is added to the lipase sample and preincubated in a temperature-controlled

fluorimeter (Jacks & Kircher, 1967; Krewinkel et al., 2016). The reaction is started by adding the substrate solution and the fluorescence is recorded continuously at an excitation of 355 nm and emission at 460 nm for a specified timed. A calibration curve with increasing 4-MU concentrations is prepared to calculate the enzyme activity. One katal lipase activity corresponds to the release of 1 mol 4-MU within 1 s (Krewinkel et al., 2016).

Fluorescence intensity varies with temperature. Therefore, it is important to keep the same temperature throughout. Also, the buffer medium used may affect lipase activity. The chain length of the ester has an influence on the degree of lipolysis produced by a particular lipase. This is because shorter chain esters are more soluble and can act as esterase substrates while the longer-chain esters are insoluble and can be used by lipases only (Deeth & Touch, 2000).

Fluorimetric assays for lipolytic activity are simple, rapid and highly sensitive (Deeth & Touch, 2000). They are 4000-times more sensitive than colorimetric methods based on thioesters and 10000-times more sensitive than titrimetric methods based on use of a pH Stat (De Monpezat et al., 1990). However, the results can vary with ester chain length, temperature, pH and buffer used (Deeth & Touch, 2000). Also, milk samples cannot be measured directly with the previously described assays (Krewinkel et al., 2016).

2.5.3 Colorimetry assay

Various colorimetric methods based on β-naphtol esters have been developed to measure lipolytic activity. The basic principle is that the colourless hydrophobic β-naphthyl esters react with lipases and β-naphtol is generated. This reacts with a diazonium salt and this in turn leads to the formation of a coloured product (Paquette & McKellar, 1986). By spectrophotometrical measurements, the colour intensity is quantified which provides a measure of lipase activity (Deeth & Touch, 2000).

McKellar (1986) devised a method with β-naphthyl caprylate as substrate. The diazonium salt used is Fast Blue BB. The dye which is produced by the enzyme activity and subsequent reaction of β-naphtol with Fast Blue BB is extracted with ethyl acetate. The absorbance is measured at 540 nm (McKellar, 1986). Versaw et al. (1989) developed a modified method where an ethanol-ethyl acetate solvent system is used to clarify the sample instead of extracting the coloured product. This modification enables direct measurement of the lipase reaction products without a centrifugation step. Moreover, this modified method displays higher sensitivity with equal ease of use.

Both, the method of McKellar (1986) and of Versaw et al. (1989) found widespread application in milk and milk products. However, if no milk proteins are available, the extracellular lipase produced by *pseudomonas fluorescens* requires surface active agents like bile salts for activity (Deeth & Touch, 2000).

The methods based on β-naphthyl esters are simple, cheap, and relatively rapid. However, the methods showed to be unreliable and lower activity is measured when milkfat is present. It is discussed if this is due to a competition between β-naphthyl caprylate and milkfat, or reduced access of the enzyme to the ester. Furthermore, turbidity of the milk also influences the sensitivity of the measurement and thus a clarification needs to be carried out (Deeth & Touch, 2000).

Lipase activity can also be colorimetrically determined by the hydrolysis of *p*-nitrophenyl (*p*-NP) esters of FA with various chain lengths. This hydrolysis leads to the release of the alcohol

p-nitrophenol and the corresponding FFAs (Deeth & Touch, 2000; Hasan et al., 2009). For instance, Blake et al. (1996) developed a method with *p*-NP which was found suitable to detect lipase production by microorganisms during growth in dairy products. The enzyme activity is expressed as µmoles of p-nitrophenol released per minute (Hasan et al., 2009).

2.5.4 Conclusion

Lipase detection methods are diverse and difficult to compare (Chen et al., 2003). However, the optimal assay for routinely measurements of dairy products for predictive purposes is still not devised (Deeth & Touch, 2000). The main issue associated with all methods is the interference from milk lipids. Therefore, a centrifugation step should be applied to remove the fat. However, it was also reported that centrifugation leads to a 20 % loss of lipase activity in skim milk because of a redistribution of the lipase which is associated with the caseins. Table 5 shows that fluorimetric assays using 4-MU have the highest sensitivity. Nevertheless, this method still has the drawback of reduction of the fluorescence intensity by the fluorescence-suppressing effect of milk components like casein (Chen et al., 2003).

Table 5: Comparison of different lipase assays and their measurement conditions and sensitivity (from Chen et al., (2003)).

Method	Temperature, reaction time and pH	Estimated sensitivity
Titration	37°C, 30 min, pH 8.5	25 mol free fatty acids released $min^{-1} mL^{-1}$
Spectrophotometric	37°C, 30 min, pH 7.6	25 nmol *p*-nitrophenol released $min^{-1} mL^{-1}$
	40°C, 30 min, pH 7.2	40 nmol *β*-naphthol released $min^{-1} mL^{-1}$
Reflectance colorimetric	37°C, 10 h, pH 7.2	5 nmol oleic acid released $min^{-1} mL^{-1}$
Fluorimetric	37°C, 30 min, pH 6.5	35 pmol 4-methylumbelliferone released $min^{-1} mL^{-1}$

3 Materials and Methods

In the following section the different chemical solutions used are described as well as the measurement procedure for the samples.

3.1 Preparation of solutions

There are several solutions that are needed for this method to measure lipolytic activity. The chemicals used for those solutions are specified in Table 6 with their supplier and batch number.

Table 6: Specification of used chemicals with their supplier and batch number.

Chemical	Supplier	Batch
4-MUL	Santa Cruz Biotechnology (Dallas, TX, USA)	E0712
4-MUB	Sigma-Aldrich (St. Louis, MO, USA)	BCBQ8490V
4-MU	Sigma-Aldrich (St. Louis, MO, USA)	BCBP7855V
2-propanol	VWR Chemicals (Fortenay sous Bois, France)	17B134016
Bis-Tris	Sigma-Aldrich (St. Louis, MO, USA)	SLBQ9044V
EDTA-disodium salt	Sigma-Aldrich (St. Louis, MO, USA)	SLBP6452V
NaOH	Merck (Darmstadt, Germany)	B0643298 119
Hydrochloric acid 5 M	VWR Chemicals (Fortenay sous Bois, France)	15D270514
Guanidine hydrochloride solution 8 M	Sigma-Aldrich (St. Louis, MO, USA)	BCBV6566
Glycine	Merck (Darmstadt, Germany)	615K2148001
Lipoprotein Lipase from bovine milk	Sigma-Aldrich (St. Louis, MO, USA)	SLBT8246

3.1.1 Substrate solution

The substrate solution contains the two synthetic substrates 4-MUB (4-methyl-2-oxo-2H-chromen-7-yl butyrate) and 4-MUL (4-methyl-2-oxo-2H-chromen-7-yl dodecanoate). Both substrates are dissolved in 2-propanol with a concentration of 10 mM each.

3.1.2 Stop solution

To stop the enzyme conversion, an 8 M solution of GuHCl and 5 M solution of HCl is used. The Guanidine hydrochloride is used to denature the enzyme by unfolding the protein. It also exhibits strong chaotropic effects and therefore breaks down the structure of proteins (Biospectra, 2018). Denaturation can also be induced by acidic conditions (Haurowitz & Koshland, 2017). Therefore, hydrochloride is added to lower the pH < 2.

3.1.3 Neutralising solution

The neutralising solution is composed of 1 M Bis-tris (bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methan), 250 mM EDTA disodium salt (ethylenediaminetetraacetic acid disodium salt dihydrate), and 850 mM NaOH. This solution is used to obtain a buffered pH of 6.63. The EDTA disodium salt can act as a chelator of divalent ions like Ca^{2+} (Santa Cruz Biotechnology, 2017). Therefore, the EDTA forms complexes with the calcium and the casein micelles destabilize (Krewinkel et al., 2016). Thereby it will help the clarification of the sample. At the neutral pH, better fluorescence is observed compared to the low pH after the addition of HCl and non-enzymatic hydrolysis of the substrate is not reported (Krewinkel et al., 2016).

3.1.4 Standard solution

4-MU is dissolved in 2-propanol. This is used as a stock standard. On the day of analysis this stock standard is further diluted with clarified blank milk to concentrations of 20, 40, 60, 80, 100, and 120 µM of 4-MU.

3.1.5 Glycine buffer system

Glycine has a pK_a of 9.9 at 20 °C (Applichem, 2008). Therefore, it is suitable for a use at a pH around 10. Different glycine buffers were produced and tested:

- 1 M NaOH + 1 M Glycine in distilled water
- 1 M NaOH + 1 M Glycine + 0,25 M EDTA disodium salt in distilled water
- 2 M NaOH + 1 M Glycine + 0,25 M EDTA disodium salt in distilled water
- 3 M NaOH + 1 M Glycine + 0,25 M EDTA disodium salt in distilled water.

This buffer was used instead of the previously described neutralising solution.

3.2 Method Development – Procedures and Handling

The following parts describe steps used in the development of the method. They all follow the procedure specified in part 3.3 unless specified otherwise.

3.2.1 Milk

The milk used for the method development is pasteurized, unhomogenized milk (Skånemejerier, Åsens Lantmjölk, 2.9-3.1 % Fat). This milk was used for most experiments unless specified otherwise in the individual parts.

3.2.2 pH adjustment

The samples were measured with a pH meter (Metrohm 744 pH Meter) at room temperature. The samples used for pH measurements were scaled up in volume since the pH meter required a higher volume. Thus, it was scaled up ten times in comparison to the values specified in Table 7.

3.2.3 Investigation of the blank

The milk used for this test was UHT milk (1,5 % fat, Milbona, Lidl). The milk was only centrifuged once for 30 min at 5000 x g at 4 °C. The trials with pH adjusted tap water were handled exactly like the milk samples except that no centrifugation was applied. The water was pH adjusted with 1 M HCl and 1 M NaOH. The samples were incubated for 15 min at 40 °C. Also, two different volumes of milk were investigated (250 µL and 750 µL) in another experiment but with the same conditions.

3.2.4 Standard curves

Blank milk is used to prepare the standard curve. For a complete standard curve, 4620 µL blank milk is required. In this clarified milk, the standard solution is further diluted to concentrations of 20, 40, 60, 80, 100, and 120 µM of 4-MU. The calculation and dilutions for the standard curve can be seen in Appendix 1. When doing the comparison between a standard curve in water and milk 2.5 mL of each medium was incubated for 15 min at 40 °C before 1.5 mL GuHCl, 200 µL HCl, 250 µL substrate solution and 1 mL neutralising solution was added and the standard curve prepared as specified in Appendix 1 only without the standard at 120 µM. For milk, only three standard concentrations were prepared (20, 60, and 100 µM 4-MU).

3.2.5 Substrate concentration

The substrate concentration measurements were carried out on frozen raw milk attained from Skånemejerier. The milk was thawed at room temperature. The samples were only centrifuged once for 30 min at 5000 x g at 4 °C. It was incubated with different substrate solutions of different concentration: 2.5, 5, 7.5, 10, 14, and 16 mM. The samples were incubated for 15 min at 40 °C. For each concentration a blank was made with the corresponding substrate solution.

3.2.6 Incubation temperature

The milk used for this trial was only centrifuged once for 30 min at 5000 x g at 4 °C. For defining the incubation temperature, samples were prepared in triplicate and a complete standard curve was prepared as specified in Appendix 1 but without the standard at 120 µM. One set of samples and blank milk was incubated for 15 min at 37 °C and the other set of samples and blank milk at 40 °C.

3.2.7 Effect of standing time

The milk used for this trial was only centrifuged once for 30 min at 5000 x g at 4 °C. The samples were incubated for 5 min at 40 °C. The standing time was tested in two ways: after the neutralising solution was added and before the neutralising solution was added. The test after neutralising solution was added was tested with one tube which was measured after specified times (0, 30, 60, 90, 120, 150, and 180 min of standing time). For the test before the neutralising solution was added, 10 tubes were prepared and let stand for a specified time (0, 5, 10, 20, 30, 40, 50, 60, and 90 min). After this time, neutralising solution was added and measured.

3.2.8 Enzyme addition

The milk used for this trial was only centrifuged once for 30 min at 5000 x g at 4 °C. The enzyme (Lipoprotein Lipase from bovine milk, Sigma-Aldrich) was added in concentrations of 78.9, 39.4, 19.7, and 9.87 pkatal to the milk before it was incubated with substrate. Incubation took place at 40 °C for 5 min.

3.2.9 Fresh vs. frozen comparison

The milk used for this comparison is a pooled milk collected at four different farms in the region of Norrmejerier during indoor (winter) season. The milk was transported in cooled conditions and the fresh sample was measured one day after arrival. The samples were measured in triplicates with the procedure specified in part 3.3 except the thawing part. The fresh sample did not experience freezing and therefore did not need to be thawed. The other frozen samples were thawed on the day of measurement at room temperature. A standard curve for the measurement at each point of time was prepared. The samples were measured fresh and after 4, 11, 18, and 25 days of freezing time.

3.2.10 LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) are important performance characteristics when validating a method. The LOD is the lowest concentration of the analyte that can be detected but not necessarily quantified with the given test conditions. The LOQ is the lowest concentration of the analyte that can be quantified with acceptable accuracy under the given test conditions. There are various methods existing to estimate the LOD and LOQ (Shrivastava & Gupta, 2011). Thus, the LOD and LOQ are estimated in two different ways: like it is done in the MEDELI and as it is specified among others by the International Conference on Harmonisation (ICH). The ICH approach is based on the standard deviation (S.D.) of the response and the slope:

$$LOD = \frac{3.3 * \sigma}{s} \qquad LOQ = \frac{10 * \sigma}{s}$$

s – Slope of calibration curve
σ – S.D. of response (International Conference on Harmonization, 2005).

The σ is obtained by taking the S.D. of the blank response of twelve different blanks from different milk samples and different measurement days.
In the MEDELI, the LOD and LOQ is calculated by taking 6 and 9 times respectively the S.D. of the blank (Krewinkel et al., 2016). As in the ICH approach, the S.D. of the blank response is done from twelve different milk samples and different measurement days.

3.2.11 Glycine-Buffer System

The milk used for this trial was only centrifuged once for 30 min at 5000 x g at 4 °C. One blank and one sample was prepared for each glycine buffer (specified in part 3.1.5). The samples were incubated at 37 °C for 15 min. The glycine buffer was added instead of the neutralising solution. In some, but not all, trials GuHCl and HCl were added as well. After the measurement, the pH in each sample was measured.

3.3 Developed method – sample preparation and fluorimetric analysis

The investigated raw milk samples are received from three different regions in Sweden: Southern Sweden (Skånemejerier), mid Sweden (Arla) and northern Sweden (Norrmejerier). In each region 15 samples were taken. Of these 15 samples, five were taken from different silo tanks at the dairy and ten from silos of different farms. The samples were transported and stored in cooled conditions and frozen around 24 h after they arrived. The samples were taken between May and September and from then on stored in frozen conditions (-18 °C) until measurement.

The samples are thawed at 10 °C in running cold water the day before measurement and kept in the fridge at 4 °C over night. The raw milk is then filled into tubes and centrifuged (BECKMAN COULTER, Allegra X-15R Centrifuge) for 30 min at 5000 x g and 4°C. Afterwards, the fat layer is removed with a small spoon and the bottom phase transferred into a new tube and shaken. This defatted milk is then centrifuged again at 4 °C and 12045 x g for 20 minutes with a mini centrifuge (Mini-Spin, Eppendorf). The top layer and bottom phase are discarded and the milk in the middle taken out and used for examination.

250 µL defatted milk is preincubated for 5 min at 37 °C. Afterwards 25 µL substrate solution is added and the sample incubated for 10 min at 37 °C in the incubator (Termaks Cooling Incubator). Subsequently, the enzyme activity is stopped by the addition of 150 µL GuHCl and 22 µL HCl. Finally, a near neutral pH is achieved by adding 100 µL neutralising solution. The blank is done by adding the substrate solution after the addition of GuHCl and HCl but before the neutralising solution. The milk and substrate solution used for the blank is heated for the same time as the samples. A standard curve is prepared with the blank milk with concentrations between 20 and 120 µm 4-MU. When running more than one sample, pooled milk from all samples is used for diluting the standard curve. The calculation of the standard curve can be seen in Appendix 1.

The samples are shortly vortexed (KEBO-Lab REAX 2000, serial No. 14460) after the addition of each solution to attain a homogeneous sample. Finally, the samples are measured with a fluorimeter (BMG Labtech, FLUOstar OPTIMA, serial number: 413-0151) at an excitation wavelength of 355 nm and emission wavelength of 460 nm. The samples are vortexed again directly before filling 100 µL into a 96-well plate (Greiner: Microplate, 96 well, PS F-Bottom(chimney well)). The gain is adjusted to the highest point of the standard curve (120 µm) with a required value of 80 %. However, this point is not included in the curve fitting of the remaining concentrations. The settings for the fluorometric measurements can be seen in Appendix 2.

Five different milk samples are measured at a time. The samples are measured in triplicates. Samples that have a CV higher than 10 % are measured again. Their activity is calculated by the standard curve which is diluted with a pooled blank made of all five milk samples. An example for how the enzyme activity is calculated can be found in Appendix 3. Furthermore, one blank for each individual milk is made.

Statistical analyses, calculations, and visualisations were carried out using IBM SPSS Statistics Version 24 and Microsoft Office Excel 2016. Significance was determined at $p \leq 0.05$. The general linear model is based on the following fixed effect model:

$$Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$$

Where: Y_{ij}=response variable (enzyme activity)
μ = overall mean
τ_i= *i*th treatment effect (origin/ region)
ε_{ij} = random error
i = treatment (origin/ region)
j= *j*th observation taken under treatment *i* (number of observations).

The enzyme activity is correlated with other properties of the milk measured at an earlier point. The fat content was measured with the MilkoScan^{TM} Mars Analyser. Microbiological measurements were carried out at Eurofins, i.e. the psychrotrophic bacteria was determined with a plate count method, total count with a Bactoscan FC and somatic cell count with COMBIFOSS 4000,6000,FT+. The FFA were determined using the method specified in the paper of de Jong and Badings (1990).

The correlation calculation is based on Pearson's correlation which requires four assumptions: the variables need to be continuous, have a linear relationship, no significant outliers present, and be approximately normally distributed (Laerds Statistics, 2013). Thus, outliers were identified with boxplots and excluded from the calculation of the correlation. The factors that were not normally distributed were checked with Spearman's rho as well to confirm the result.

4 Results

The result part is divided in the development of the method and the results of the milk samples that are measured with the developed method.

4.1 Development of the method

The method was developed by first establishing the pH in all sample preparation steps, i.e. after each addition of a solution to the sample. The pH was adjusted to the pH specified in the MEDELI by addition of HCl. The final amounts of all samples are specified in Table 7.

Table 7: Amounts of different solutions for the modified method to measure lipase activity in milk.

Amount (µL)	Solution
250	Defatted milk
25	Substrate solution
150	GuHCl
22	HCl
100	Neutralising solution

Different amounts of HCl were tested to investigate the ideal end-pH for the fluorescence measurement. All trials can be found in Appendix 4. The pH measurements were carried out in an experiment that was scaled up ten times in comparison to the volumes specified in Table 7. This is because a higher volume of sample was necessary for measuring with the pH meter. It was chosen to use 22 µL of HCl since this amount resulted in the lowest variation in data as can be seen in Table 8.

Table 8: The average variation (CV) at different amounts of added HCl.

Amount HCl (µL)	17	20	22
Sample (CV)	5.9	8.0	**1.7**
Blank (CV)	11.3	8.1	**5.5**
Both (CV)	9.0	8.0	**3.6**

Both, substrate and standard solution, are dissolved in 2-propanol and thus it was investigated how much 2-propanol contributes to the fluorescence of a sample. This was done by measuring milk samples and 2-propanol in comparison. The results showed that 2-propanol only contributed with around 0.7 % to the total fluorescence of the sample and can therefore be neglected. The data which this result is based on is displayed in Appendix 6.

The substrate solution was tested for its stability since it was suspected that 4-MUL and 4-MUB might hydrolyse over time into the fatty acid and the fluorescent molecule 4-MU. The stability was examined by carrying out a fluorimetric measurement of the substrate solution and 2-propanol at the same time. The gain for the same substrate solution was kept constant. By measuring the 2-propanol as well, the daily variation within the fluorimeter could be eliminated because it is assumed that 2-propanol should have constant fluorescence values over time. The difference between substrate solution and 2-propanol was used to follow the stability of the substrate solution over time. This resulted in a predicted stability of approximately two weeks of storage in cool conditions [Data not shown].

4.1.1 Treatment of the blank

The blank should be identical to the sample but should not contain the fluorophore. By comparing the result of the blank and that of the sample, the actual fluorescence of the fluorophore can be estimated by eliminating the background noise (Lakowicz, 2006). Therefore, it is important that the blank is treated as similar to the sample as possible. The blank

is made by adding the substrate solution after adding the stop solution, meaning that the enzyme is already denatured when substrate is added. Thereby, the fluorescence of the substrate is taken into account, but the enzyme cannot cleave the substrate anymore. The gain should also be identical when the sample and blank is measured. Table 9 shows that the volume that is incubated influences the fluorescence. A smaller volume results in significantly higher fluorescence values. Therefore, the blank should be prepared with the same volume as the sample.

Table 9: Influence of the volume on the relative fluorescence value (RFU) (raw data).

Volume	Fluorescence [RFU]
750 µL milk	35964 ± 215
250 µL milk	49291 ± 2433

As can be seen in Table 10, it is important to heat the substrate that is added after the incubation to the blank since this results in a higher blank value as if the substrate is not heated at incubation temperature. Furthermore, in the samples it is heated as well, and the treatment should be as similar to the samples as possible. Trials in pH adjusted water showed that ideally the substrate would be heated in the milk. However, heating in the milk is not possible since even in UHT-milk lipase activity can be seen. Therefore, the blank should be prepared with the substrate heated next to the milk as a compromise.

Table 10: Influence of the treatment of the substrate on the fluorescence of the blank in UHT milk (left) and pH adjusted water (right) (raw data).

Test in UHT milk	Fluorescence [RFU]
Milk & substrate not heated	25064 ± 642
Milk & Substrate heated	27932 ± 73
Substrate in UHT milk heated	57305 ± 157

pH adjusted water (pH ~6,7)	Fluorescence [RFU]
Water & substrate not heated	55179 ± 989
Water & substrate heated but separately	57158 ± 18
Substrate in water heated	58353 ± 221

4.1.2 Standard curve

The standard curve is necessary to estimate the enzyme activity out of the fluorescence data. An idea was to use a standard curve in water instead of milk to have a more constant curve without the natural variation from milk.

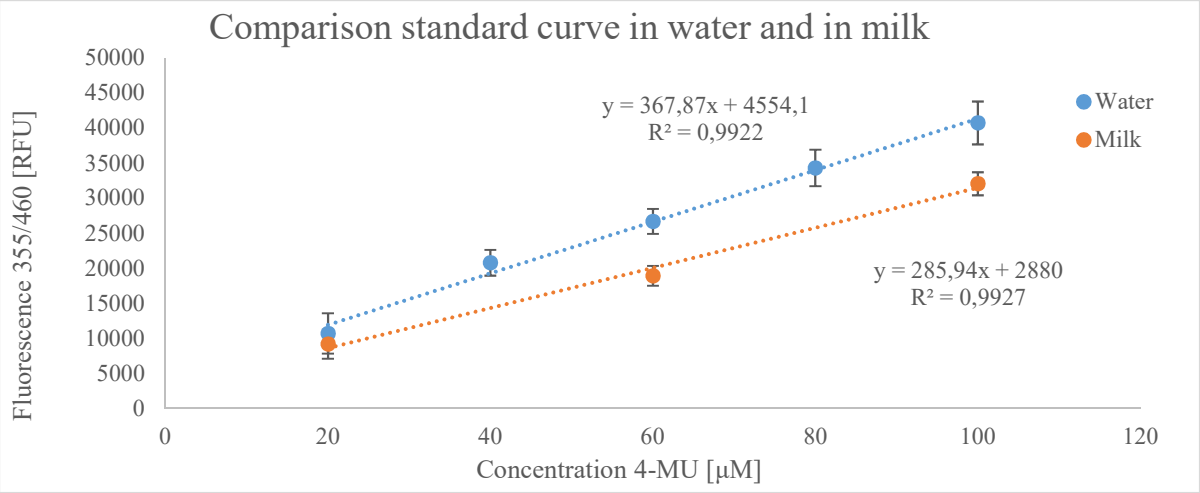

Figure 6: Comparison between a standard curve in milk and in water.

As can be seen in Figure 6, there is no significant difference between the standard in milk and in water at a concentration of 20 µM. However, the slope of the curves is different and therefore significant differences occur between the values at higher concentrations. Thus, it is not possible to prepare the standard curve in water instead of milk. However, to keep the natural variation as low as possible the standard curve is done in a pooled blank milk from all samples examined at the same time.

When preparing several standard curves, it was noticed that the highest point was repeatedly slightly lower than the linear expectation and therefore influences the overall correctness of the curve. This event can be seen in Figure 7 on the left. It can also be seen from this graph that R^2 increases with decreasing percentage of gain adjustment (● displays the highest gain adjustment with 95 % and results in the lowest R^2 whereas ◆ corresponds to a gain adjustment of 75 % and results in the highest R^2). However, when the gain is decreased, the curve slope also decreases. This means that the robustness of the method is reduced and thus the gain should not be decreased too much. Therefore, the gain was set at 80 % (symbol ■ in the graph). Figure 7 on the right shows an example for an improved curve fitting. Here, the highest standard (in this case 100 µM) was prepared and the gain adjusted to it but not included in the curve fitting. Hence, it was chosen to prepare an additional standard at 120 µM to adjust the gain to it but not to include this standard point in the curve fitting.

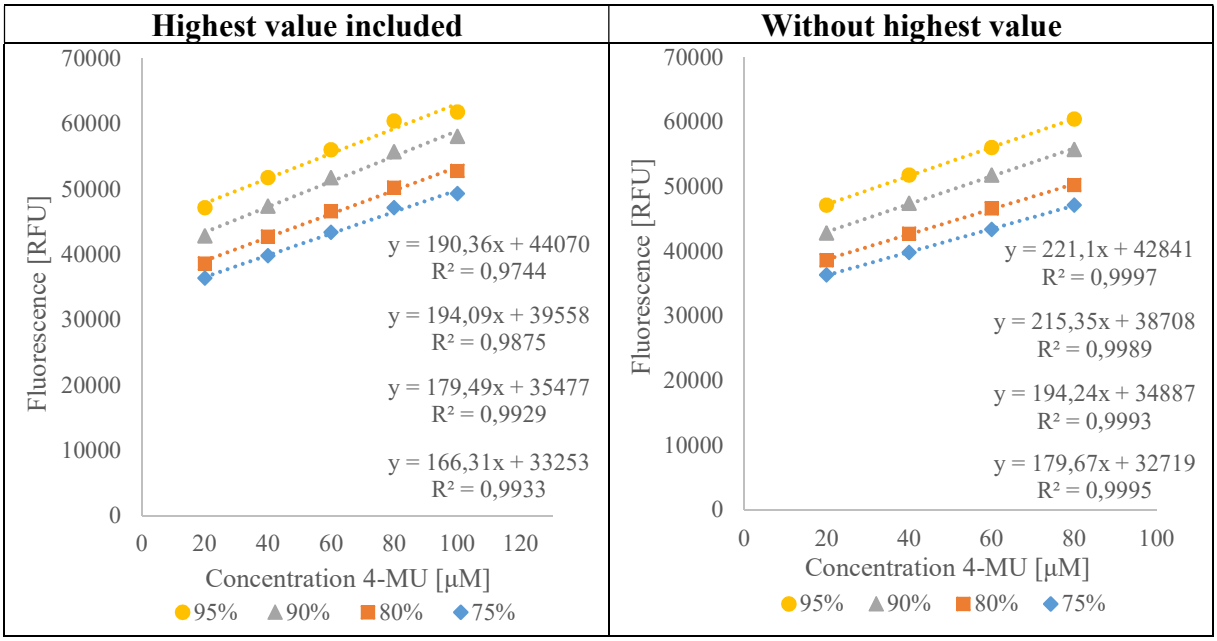

Figure 7: Comparison of the standard curve with and without the highest value at different gain adjustments.

4.1.3 Centrifugation steps

As described in the MEDELI, a second centrifugation step of the incubated and clarified milk is recommended for samples that contain more than 1,5 % fat. Trials showed that there is a visual difference in being less turbid after the second centrifugation step (see Figure 8). However, as can be seen in Table 11, there are no significant differences (p=0.07) in fluorescence values between a centrifuged and uncentrifuged sample. Hence, this step was eliminated.

Table 11: Comparison between a blank with and without a centrifugal step after clarification (raw data). (a): Mean values do not differ significantly ($p<0.05$)

Sample treatment	Fluorescence [RFU]
Centrifuged	6839 ± 814 (a)
Uncentrifuged	5718 ± 614 (a)

Nevertheless, an additional centrifugation step before addition of substrate showed to bring a greater difference between blank and sample. This can be seen in Table 12. Since a larger difference between blank and sample is desirable to attain a more sensitive measurement, this step was carried out before the addition of substrate.

Table 12: Average difference between blank and sample with and without a second centrifugation step. (a-b): Values differ significantly from each other ($p=0.027$)

	Without 2nd centrifugation	With 2nd centrifugation
Difference between sample and blank (%)	157 ± 18 (a)	197 ± 26 (b)

The visual difference between different centrifugation steps can be seen in Figure 8.

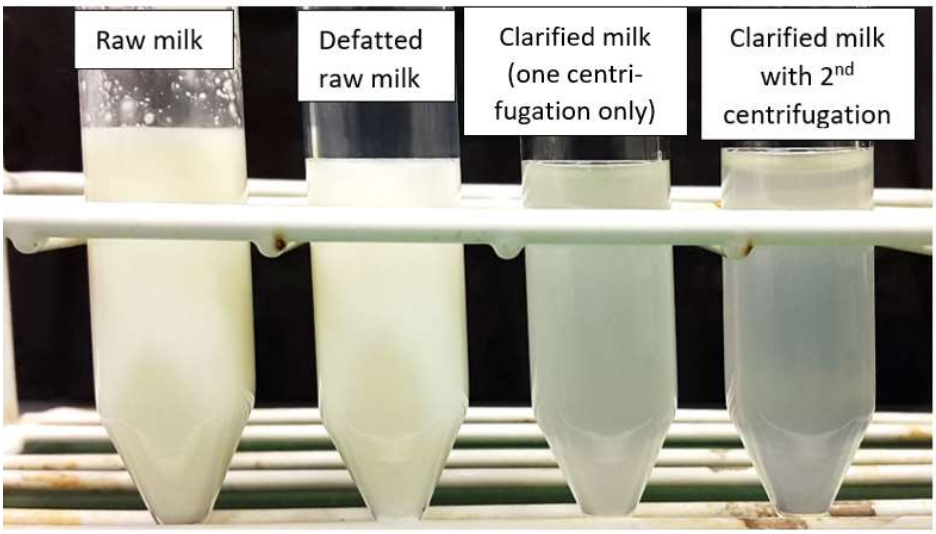

Figure 8: Comparison of turbidity of milk after different steps, i.e. from left: raw milk, defatted raw milk, clarified milk but with only one centrifugation and clarified milk after a second centrifugation step.

4.1.4 Substrate concentration

To investigate that the substrate is present in excess and not limiting the lipase activity, different substrate concentrations were examined. By doing so, it can be ensured that the enzyme conversion is close to V_{max}. The substrate concentration used in the original method (MEDELI) was 10 mM of each substrate (4-MUL and 4-MUB). Figure 9 shows the blank corrected values at different substrate concentrations. It can be seen that there is no significant difference between 10, 14, and 16 mM. However, to be certain that there is enough substrate, the incubation time in the final method was decreased from 15 minutes to 10 minutes and the temperature decreased from 40 °C to 37 °C.

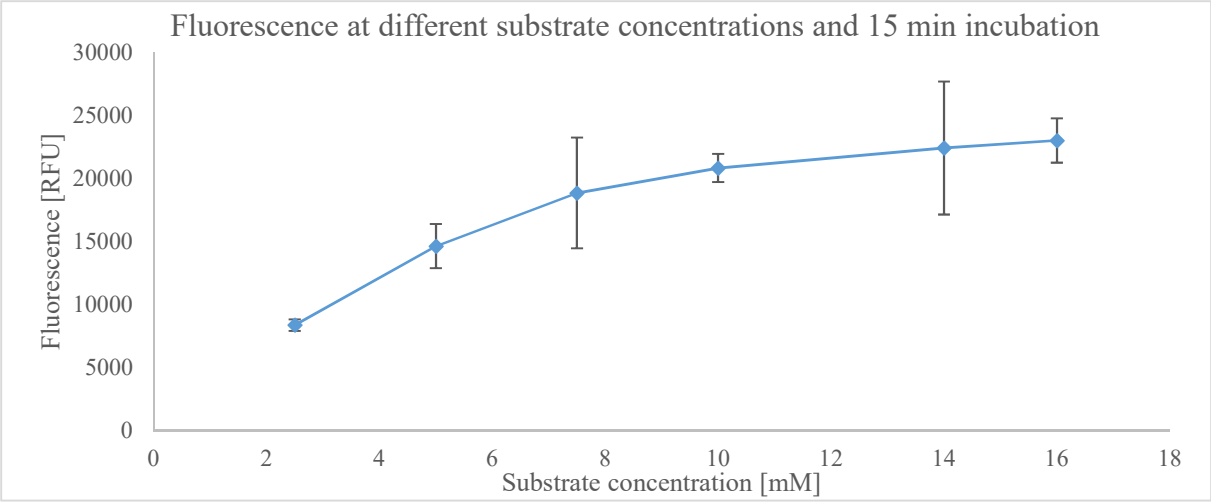

Figure 9: Enzyme conversion at different substrate concentrations. Each concentration point corresponds to equal molar amounts of 4-MUL and 4-MUB, i.e. 10 mM means 10 mM 4-MUL and 10 mM 4-MUB.

4.1.5 Incubation temperature

In the MEDELI an incubation temperature of 40 °C is used. However, in other articles measuring enzyme activity (Roberts, 1985; Wiederschain & Newburg, 2001; Chen et al., 2003), a temperature of 37 °C is applied. Thus, a comparison between the two incubation temperatures was made. Table 13 shows that the differences in fluorescence between the blanks at the two temperatures is fairly low with 2 %. The difference between the samples is slightly higher with 6 %. However, since the standard curves showed to have a different slope, as can be seen in Figure 10, the concentration of released 4-MU is higher at 40 °C than at 37 °C.

Table 13: Comparison of the fluorescence and 4-MU concentration at incubation temperatures of 37 °C and 40 °C (raw data).

	37 °C	**40 °C**	**Difference (%)**
Blank (RFU)	24306 ± 111	24840 ± 175	2
Sample (RFU)	44144 ± 324	47086 ± 231	6
Concentration 4-MU (µM)	57.5 ± 1.1	69.7 ± 0.7	17

A small variation in fluorescence has a smaller influence on the concentration of 4-MU at 37 °C than at 40 °C due to the different slopes of the standard curves. Since the enzyme activity is lower at 37 °C the substrate is also more likely to be in excess as mentioned in the previous section.

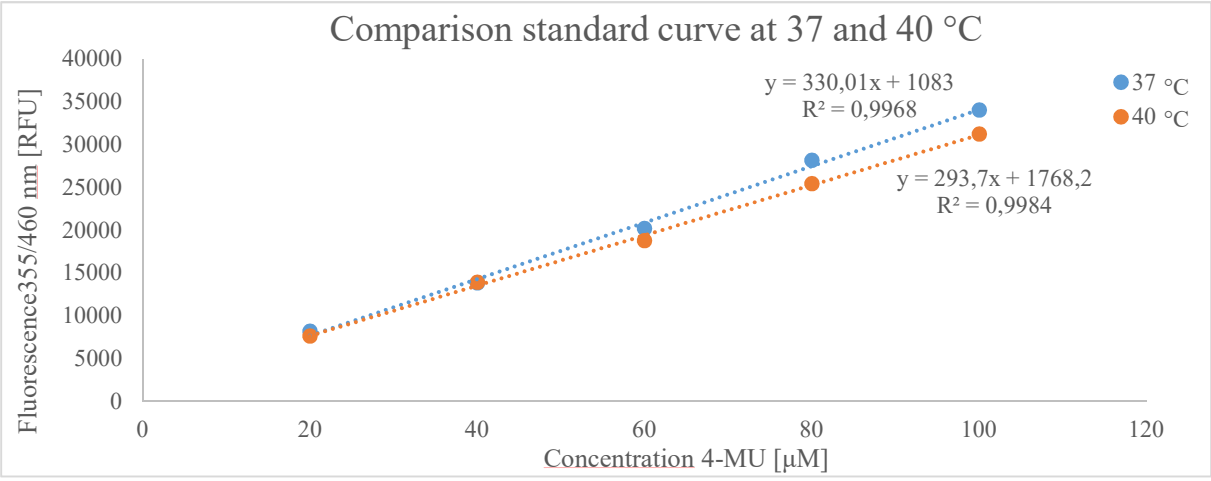

Figure 10: Comparison of standard curves at 37 °C and 40 °C with an incubation time of 15 min.

4.1.6 Effect of standing time

Krewinkel et al. (2016) specify in their article that the clarified milk should have stable fluorescence values for at least 360 minutes with a variation of ± 4 %. However, Figure 11 showed that the fluorescence is increasing linearly right from the beginning. Therefore, it is important to keep the same times for all samples and blanks.

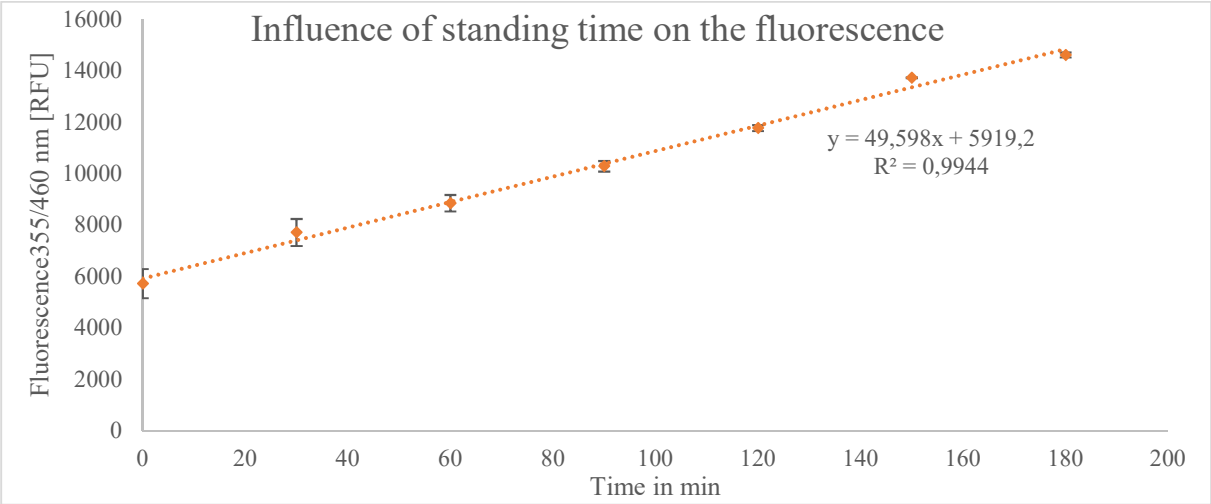

Figure 11: Development of fluorescence over time after the neutralising solution is added.

It is also stated in the article that the fluorescence should be stable for at least 50 minutes with added stop solution but prior to adding the neutralising solution. This could be seen as well in measurements displayed in Figure 12. The fluorescence values were stable for at least 90 minutes. The variation in data is due to small dirt particles in the well detected later. However, there is no trend in fluorescence values visible over at least 90 minutes.

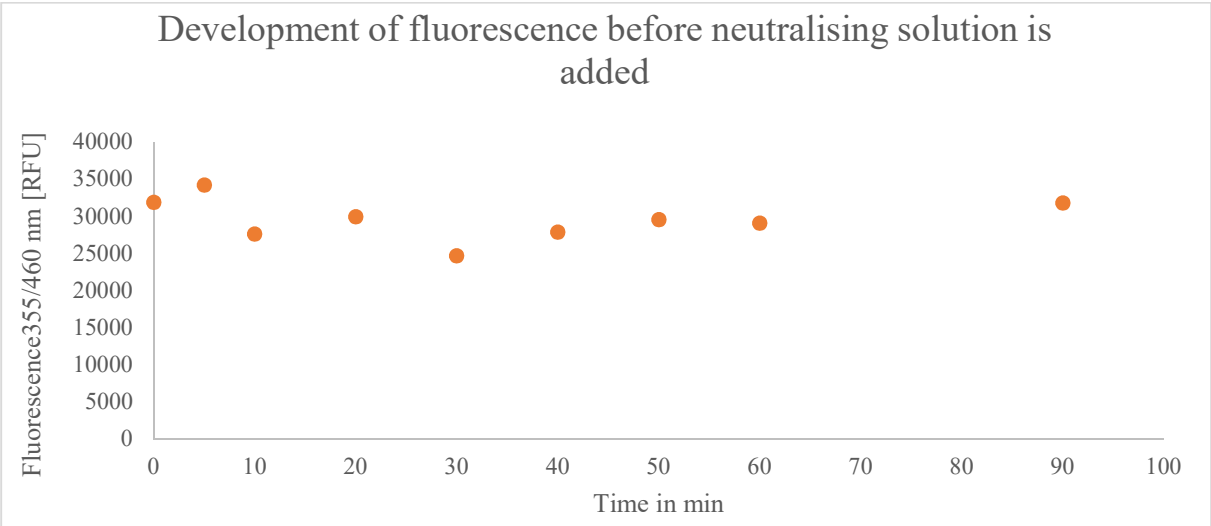

Figure 12: Development of fluorescence over time after addition of stop solution but prior to adding neutralising solution.

4.1.7 Linearity

The method was further validated by inspecting if there is a linear correlation between the incubation time and concentration of released 4-MU. First, a trial with incubation times of 5, 30, 60, and 90 minutes was performed. However, since already after 30 minutes the concentration of generated 4-MU was higher than the highest point of the standard curve, the test was repeated with shorter incubation times of 5, 10, 15, and 20 minutes. Figure 13 shows

that a linear relation is existing and that the method is performed in the linear range of the instrument.

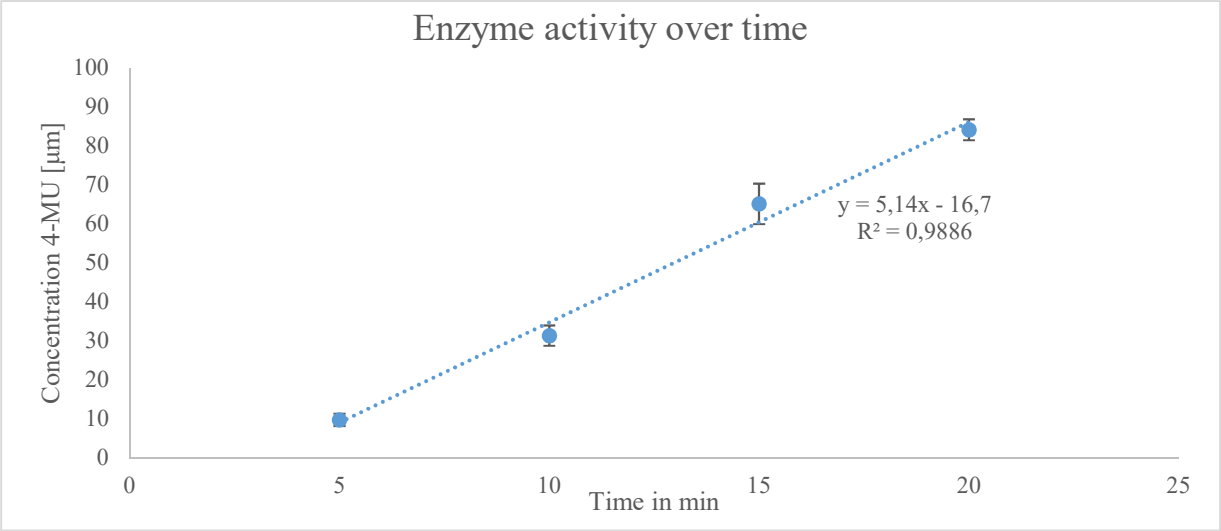

Figure 13: The concentration of released 4-MU after different incubation times.

The ability to differentiate between different enzyme concentrations was also verified by adding a known amount of enzyme (bovine LPL) to the milk before it was incubated with substrate. The result can be seen in Figure 14. There is a linear increase in lower concentrations between 10 and 40 pkatal. Between 40 and 80 pkatal the fluorescence remained on the same level.

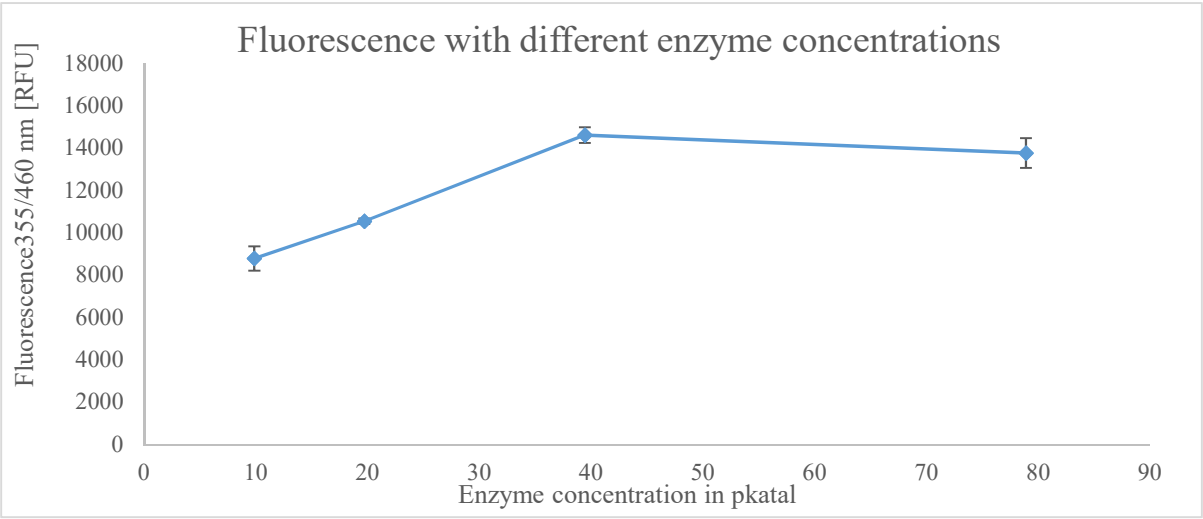

Figure 14: Fluorescence values after the addition of different enzyme concentrations.

4.1.8 Variation in Data

It is desirable that the variation within replicates is as low as possible. As an acceptable variation a CV of the raw fluorescence data of 10 % was defined. Samples that had a higher CV than 10 % were measured again.

To examine the variation of the equipment (fluorimeter), blanks from the same tube were measured in eight different wells. The result can be seen in Table 14 and the CV is reasonably low with 0.6 %. To compare this, the inter assay variation of technical replicates from eight different tubes were measured which resulted in a variation of about 4.9 %. The same was carried out with samples from eight different tubes and resulted in a CV of 2.8 %.

Table 14: Variation of the equipment and technical replicates (n=8).

	CV (%)
Blank from the same tube	0.6
Blank from different tubes	4.9
Sample from different tubes	2.8

However, other sample runs showed that some outliers exist like the two red marked values in Table 15. Since these values are all replicates from the same milk this should not occur and further trials were carried out to reduce the variation and outliers.

Table 15: Example for unexplained outliers in sample measurements (raw data).

	Fluorescence [RFU]	Average (RFU)	S.D.	CV (%)
Sample 1	44750	48576	7086	14.6
Sample 2	45520			
Sample 3	43933			
Sample 4	57447			
Sample 5	43987			
Sample 6	63423			
Sample 7	42899			
Sample 8	46648			

The variation became less when the samples were vortexed directly after the addition of a solution and also by vortexing directly before adding to the measurement plate. Furthermore, it was seen that the pipette tip should be properly fixed and exchanged after using it for four tubes.

One important factor that can also influence the fluorescence is the pH. The pH variation can be seen in Table 16. The pH after the addition of different solutions is to a high degree consistent within the technical replicates but also between different biological replicates.

Table 16: Stability of pH after the addition of different solutions.

	Within technical replicates n= 4		**Between biological replicates** n=2	
	Mean	**CV**	**Mean**	**CV**
After GuHCl	6.24 ± 0.004	0.07	6.5 ± 0.007	0.12
After HCl	0.57 ± 0.009	1.5	0.58 ± 0.02	3.26
After Neutralising solution	6.7 ± 0.02	0.3	6.7 ± 0.02	0.33

The samples were stored in a frozen state in tubes containing 15 mL each. To see how much a sample from the same milk but from different frozen tubes varies, a comparison between the variation within the same tube and between different tubes was done. The detailed results can be seen in Appendix 5. The summarized result is presented as average of all the CVs in Table 17. The variation within the same frozen milk tube is distinctly lower than from different frozen tubes. However, the CV of the raw data was still below the acceptable variation of 10 %.

Table 17: Summary of the variation in data within the same tube as well as in between different tubes.

	CV within same tube (%)	**CV in between tubes (%)**
Average raw data	1.7	3.7
Average enzyme activity	4.6	10.2

4.1.9 Fresh vs. frozen milk

To see if there is a difference in lipase activity when the samples are frozen a comparison between fresh and frozen milk samples was carried out. This is important since the measurements are done on frozen milk samples. The test was carried out by measuring the same milk sample when it was fresh and after it was frozen for different times. During the measured time of up to 25 days no trend in the data could be seen. There was some variation in data but this is within the usual variation of the method (CV of 5.8 %). The individual results are presented in Table 18.

Table 18: Comparison of enzyme activity of a fresh sample compared to frozen samples with different freezing times.

Sample	Lipase activity (pkat/mL)
Fresh	132.2
Frozen for 4 days	127.8
Frozen for 11 days	122.6
Frozen for 18 days	134.4
Frozen for 25 days	143.2

4.1.10 LOD and LOQ

The LOD and LOQ is calculated with two different approaches as specified in part 3.2.10. The results of the different approaches can be seen in Table 19. The individual data on which these results are based on is displayed in Appendix 7.

Table 19: Calculated LOD and LOQ with two different approaches.

	Approach after ICH (pkat/mL)	**Approach after Krewinkel et al., 2016** (pkat/mL)
LOD	29.1	23.4
LOQ	88.2	49.9

4.1.11 Glycine-Buffer-System

Zhi et al. (2013) investigated the fluorescence spectra of 4-MU at different pHs. Figure 15 shows that highest fluorescence values can be detected at a pH around 10. At this pH a slight difference in pH will not lead to a drastic difference in fluorescence since there is a plateau.

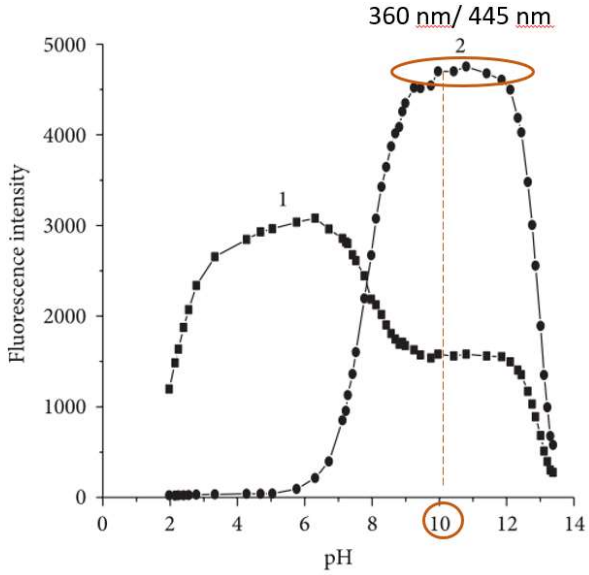

Figure 15: The dependence on pH of the fluorescence of 4-MU (from Zhi et al., (2013)).

It can also be seen from Figure 15, that at pH~6.6, where fluorescence of the MEDELI is measured, there is a great change in fluorescence if the pH changes slightly. Therefore, it was thought to change the buffer pH to around 10. To do this, a change to a buffer which is more suitable at this pH range (glycine-buffer) was made.

The results for the trials with this buffer system can be seen in Table 20. Further pre-trials are presented in Appendix 8. It can be seen that the samples that were measured at a pH ~10 had more or less no difference between the blank and the sample. Therefore, this buffer system and a measurement at pH range of ~10 cannot be used to measure the lipolytic activity with this substrate.

Table 20: Fluorescence results of the trials with the Glycine-Buffer-System (raw data) and their corresponding pH.

	Glycine-1 M NaOH-EDTA-GuHCl	**Glycine-2 M NaOH-EDTA-GuHCl**	**Glycine-3 M NaOH-EDTA-GuHCl**	**Glycine-3 M NaOH-EDTA-GuHCl-HCl**
Blank (RFU)	50290	57650	58533	749
Sample (RFU)	59484	57846	57786	3501
% Difference	18.3	**0.34**	**-1.28**	467.4
pH	7.82	**9.79**	**10.92**	2.62

4.2 Measurement of the samples

The samples were measured according to the specified procedure described in part 3.3. The enzyme activity of all 45 measured samples is displayed in Appendix 9. Some samples were measured twice to see the reproducibility of the method and confirm the results. The comparison of the second measurement can be seen in Appendix 10. The average of the two measured results is taken for further calculation.

A fixed effect model was produced to statistically evaluate the results. For this model, different prerequisites must be met: it was tested for orthogonality, normality, and homogeneity of variance. The test results are presented in Appendix 11. The test for orthogonality exhibited that there is no correlation between parameter (Region and Origin). It also showed that the data is normally distributed and the variance is homogeneous. Hence, all three prerequisites were met. The boxplots in Figure 16 visualize the enzyme activity results and point out differences between groups, variations and outliers. It can be seen that two outliers exist but since there was no error in calculations these were included in the model.

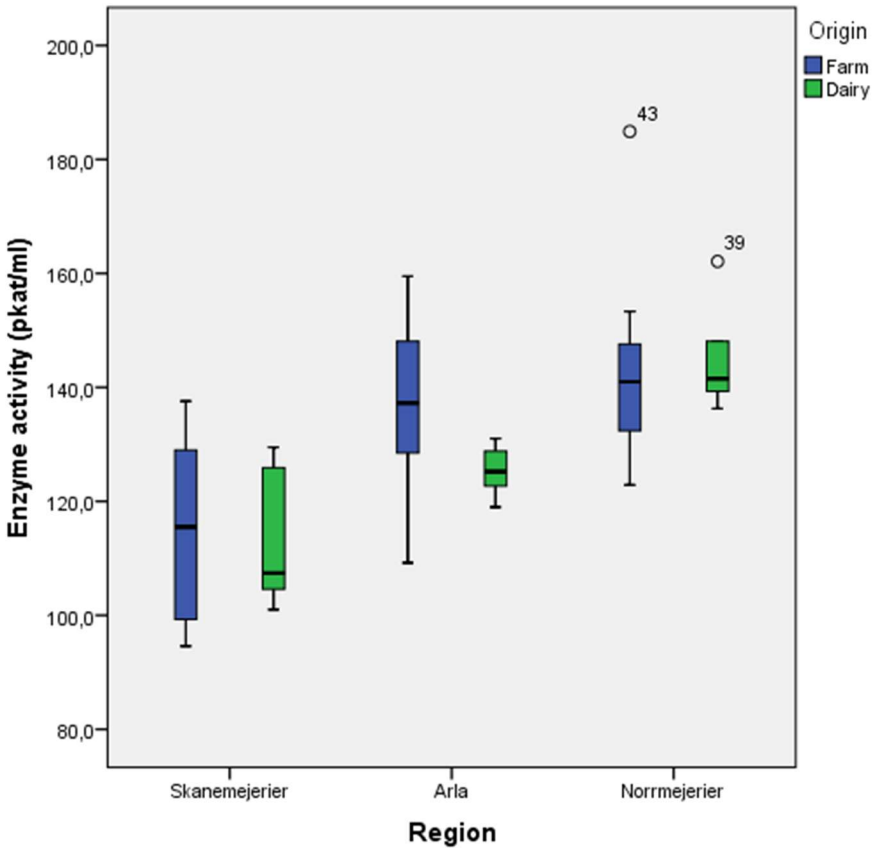

Figure 16: Boxplots of the enzyme activity results with their distribution among different groups and outliers.

Main and interaction effects are calculated in the model which is presented in Appendix 12. There is a main effect present when looking at the region. No significant difference exists between origins (farm/ dairy). Also, an interaction effect between the region and origin is not existent. Thus, a new model was calculated without the interaction effect which can be found in Appendix 13. The model has an R^2 of 0.449. A pairwise comparison pointed out that all three regions are significantly different from each other ($p<0.05$). Figure 17 displays that milk from Skånemejerier has a significantly lower lipase activity with 114.9 ± 14 pkat/mL than milk from Arla and Norrmejerier which show lipase activities of 133.5 ± 14 pkat/mL and 144.4 ± 15 pkat/mL, respectively. The milk from Arla and Norrmejerier are also significantly different from each other regarding the lipase activity. Figure 17 also shows that the activity at the dairy level is slightly lower than at the farm level; however, this difference is not significant (p-value 0.364).

The model is confirmed by checking for normality, homogeneity of variances, and residuals. The results in Appendix 14 indicate that all three assumptions are fulfilled. By plotting the factors against the standardized residual of the enzyme activity the variation within the different factors can be seen. The variation among the different regions and in between farm and dairy level is displayed in Appendix 15. Samples from Norrmejerier have a slightly higher variation than samples from Arla and Skånemejerier. The variation in between farm samples is higher than in between dairy samples.

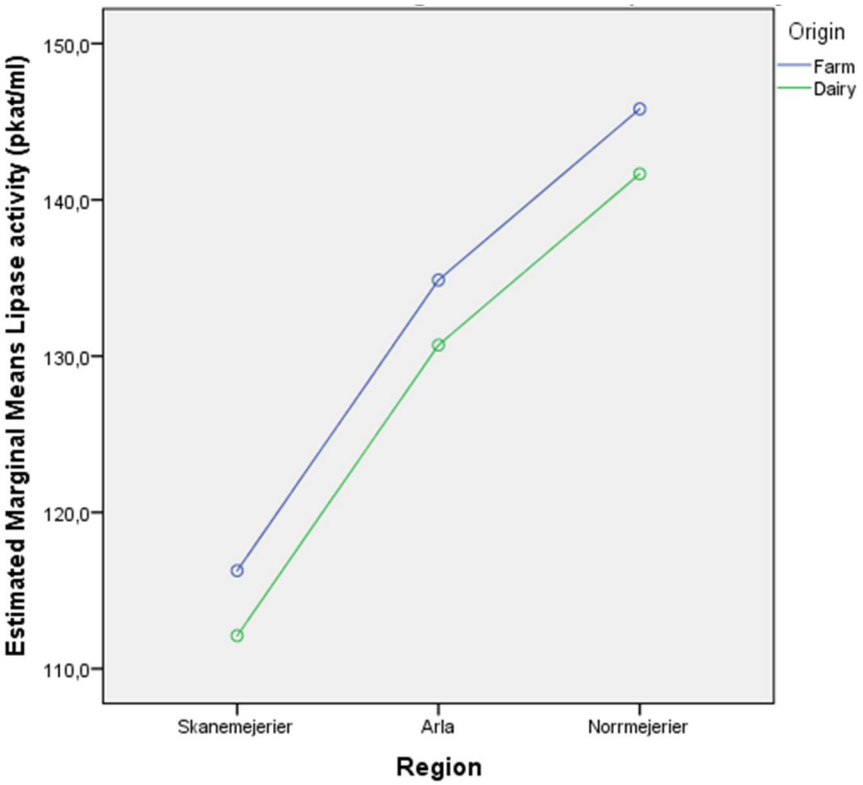

Figure 17: Enzyme activity in different regions and the comparison between farm and dairy level.

Prior to the lipase activity measurements, the same milk samples were investigated on other properties, among others their fat content, amount of FFA, psychrotrophic bacteria, their somatic cell count, and total cell count. This data was used for an analysis of a possible correlation with lipase activity. At 95 % significance level no significant correlation could be seen between the lipase activity and the other factors except for the total FFA. The statistical analysis results for all factors can be seen in Appendix 16. The statistical significant correlation to the total amount of FFA is displayed by a linear regression line in Figure 18. This indicates, that the higher the amount of FFA the lower the enzyme activity and vice versa.

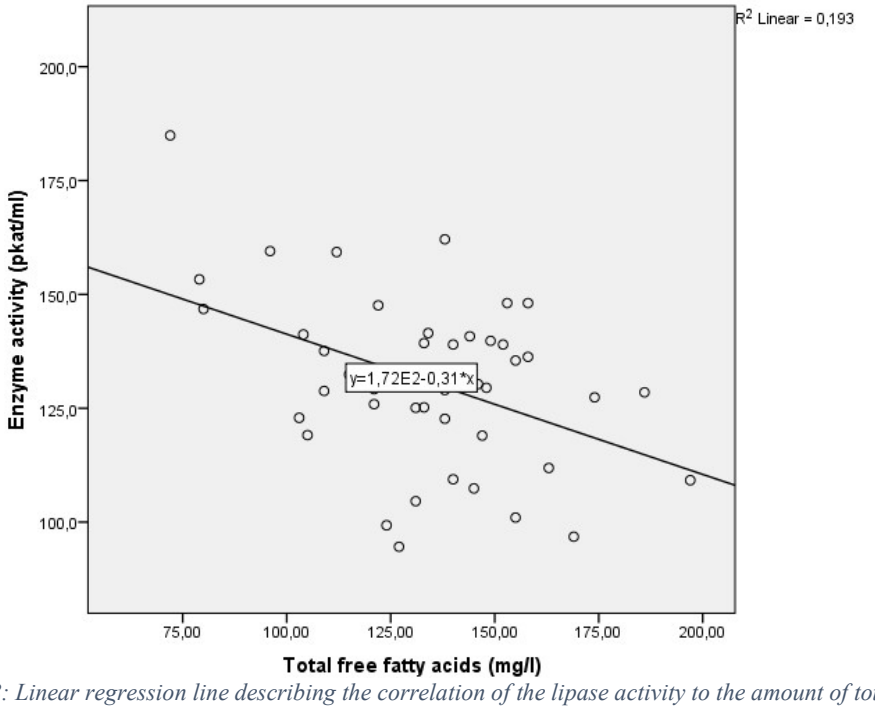

Figure 18: Linear regression line describing the correlation of the lipase activity to the amount of total FFA.

Based on this result the individual FFA were tested for correlation as well. No significant correlation was present between the lipase activity and all the short and mid chain fatty acids as well as oleic acid (C18:1) and linoleic acid (C18:2). However, a significant negative correlation could be seen with palmitic acid (C16:0), stearic acid (C18:0), and α-linolenic acid (C18:3). All these results are also displayed in Appendix 17. Figure 19 illustrates the statistical significant correlation of palmitic acid (16:0), stearic acid (C18:0) and α-linoleic acid (C18:3) to the lipase activity.

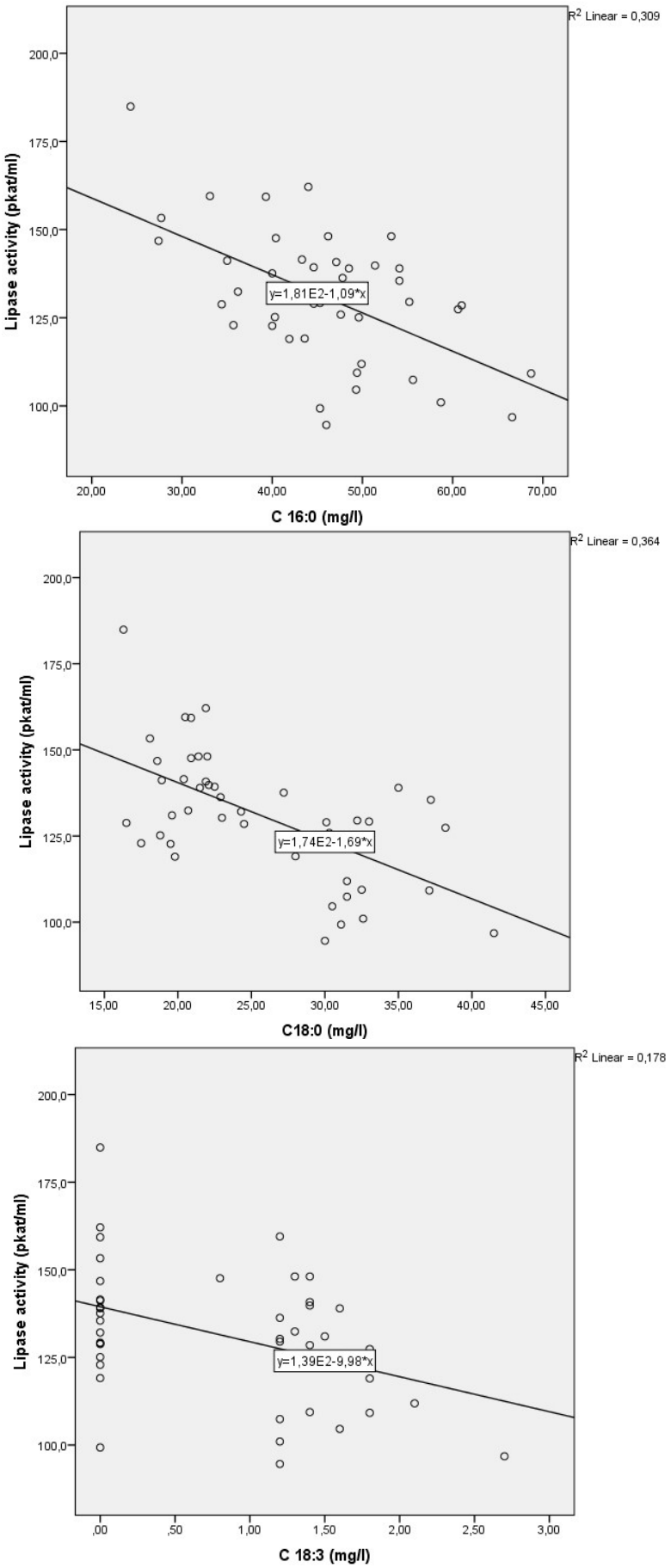

Figure 19: Linear regression line describing the correlation of palmitic acid (C16:0; top); stearic acid (C18:0; middle) and α-linoleic acid (C18:3; bottom) to lipase activity.

5 Discussion

5.1 Development of the method

By adjusting the pH to the values specified in the MEDELI, the method showed to be working and therefore the main aim of the thesis was fulfilled. However, more tests were carried out to further investigate the method. Especially the variation in data was large in the beginning and it was tried to attain a more robust and at the same time sensitive method. The CV appeared to be the lowest when adding 22 µL of HCl. This is reasonable since the final pH will be closest to the pK_a of the buffer system and therefore variations in pH are less likely. This is important since a slight change in pH has a big influence on the fluorescence.

The variation in data was tried to be reduced in several other ways as well. First, the procedure was carried out by adding the different solutions to all tubes and then they were closed and vortexed. However, it was found that vortexing the samples directly after the addition of a solution to one tube could reduce the variation. Presumably, this direct mixing to attain a homogeneous sample is especially important when adding the neutralising solution. The neutralising solution might not be evenly spread when it is pipetted into the tube and thereby the pH can be higher at certain points leading to a hydrolysis of substrate as could be seen when using the glycine-buffer-system. Thus, direct mixing could help decreasing this risk.

By changing the pipette tip after some samples it was possible to also reduce the variation in data. It was seen that after a certain usage of one tip there was some liquid remaining in the tip which was not supposed to be there. Thus, the results are more exact when taking a new tip after a specified number of samples.

Samples from different frozen tubes exhibited a higher CV than milk from the same frozen tube. This can have two reasons: First, the milk that was frozen was not homogeneous. Secondly, there could be a difference in the handling during the centrifugation and separation steps. These are the only steps that took place until the milk was split up in more smaller tubes and thus the higher difference should have occurred also within the same frozen milk tube. It cannot be determined which reason applies or whether even both reasons combined cause the higher CV. Nevertheless, the variation is still within the acceptable range and thus its origin was not further investigated.

The sensitivity of the developed method in comparison to the MEDELI is slightly reduced since in the MEDELI the dilution of the milk sample by addition of the different solutions is lower with a factor of 2.1. In the developed method the dilution factor is 2.19. The difference is caused by the fact that in the MEDELI the stop solution consists of 8 M GuHCl and 1 M HCl in water as one solution. However, the preparation of this solution is hazardous and thus it was chosen to add a ready-made 8 M GuHCl solution and another 5 M HCl solution. Therefore, the dangerous step of producing the stop solution was eliminated but two solutions need to be added in order to stop the enzyme conversion. By adding two different solutions instead of one, the total volume of added solutions to the milk sample became slightly higher.

The sensitivity of the method could be improved by implementing the second centrifugation step before the sample is incubated. This step brings a larger difference between blank and sample. This is reasonable since more fat is removed and thereby less fat in the milk can interfere with the added substrate which results in apparently lower lipase activity. Also, this does not result in more work steps as specified in the MEDELI since the second centrifugation step after the incubation could be eliminated.

The second centrifugation step of the clarified milk proposed by Krewinkel et al. (2016) brought about a visually less turbid sample but there was no significant difference in fluorescence between a centrifuged and uncentrifuged sample. Thus, the background fluorescence causing particles could not be removed by this step. Further, Krewinkel et al. (2016) mention in their article that turbidity destroys the linear correlation between concentrations of fluorophores and their fluorescence measurements. However, the prepared standard curves showed that, also without the second centrifugation of the clarified milk and therefore a more turbid sample, a linear correlation of different concentrations of the fluorophore could be seen in the measured values.

The incubation temperature is chosen to be 37 °C since the slope of the standard curve is steeper at this temperature. Thereby, slight changes in fluorescence do not have such a big impact on the concentration of 4-MU as they have at 40 °C. Thus, the results should be more robust. At 37 °C elevated enzyme activity is still noticeable and thus a sensitive measurement possible.

A new standard curve is prepared for the measurement of every five samples. This is important since environmental effects should be considered. As could be seen with the result of the standing time of the clarified milk, the fluorescence increases linearly over time. Therefore, a blank for each measurement is important to subtract any effect of time from the samples.

The experiment with the standing time after the addition of GuHCl and HCl but prior to adding the neutralising solution showed that GuHCl and HCl effectively deactivate the enzyme since no increase in fluorescence could be found there over at least 90 minutes. Thus, the increase in fluorescence after the milk is neutralized is most likely due to hydrolysing of the substrate. This will happen in the blank as well as in the sample and in the blank milk the standard curve is prepared in. Therefore, this hydrolysing is taken into account by subtracting the blank from the samples.

When looking at the blank, it was noticed that a higher volume had a lower fluorescence than a lower volume. A possible reason for this is that the time until the larger volume reaches the incubation temperature is longer compared to the time need in a smaller volume. It is desirable to produce a higher volume of blank since this is needed to produce the standard curve. However, due to the finding that the volume significantly influences the fluorescence, more tubes are prepared with the same volume as the sample and pooled after the addition of all solutions. In this way also the variation between blanks and standard curves can be reduced since it is a mixture of several individual tubes.

The method is validated by incubating the sample for different times. A linear correlation is a prerequisite for a valid method. This was the case in the developed method which shows again the applicability of it. It was also tested by adding different amounts of enzyme to the milk. A linear correlation is also visible at concentrations between 10 and 40 pkatal. However, no increase in fluorescence could be detected between 40 and 80 pkatal. First it was thought that this is the case because the substrate is used up. However, in the measured raw milk samples significantly higher concentrations of around 130 pkatal were measured. In raw milk it was previously found that the substrate concentration is in excess and not limiting the process. Thus, the experiment with the added enzyme should be repeated to confirm that the result at 80 pkatal was not just a measurement error.

The comparison between the fresh and frozen milk do not show a trend in the data. The variation between the different freezing times are within the measurement error of this method. Thus,

there is no influence between a fresh and a frozen sample for at least 25 days of frozen storage. This means, that the enzyme is not destroyed by freezing or thawing and that the thawing procedure is suitable. This is important since the samples vary in the amount of time that they have been frozen.

The LOD and LOQ show quite different values when comparing the different calculation approaches by ICH and Krewinkel et al. (2016). However, in both approaches the LOQ is below the lowest measured sample and therefore all attained data is valid. The values were also compared to the LOD and LOQ attained by Krewinkel et al. (2016). Table 21 shows that both the LOD and LOQ obtained within this thesis are below the values in the MEDELI and thus an improvement of their method could be achieved. It can also be seen that both methods are able to detect significantly lower lipase activity in comparison to a pH-stat approach.

Table 21: Comparison of the LOD and LOQ of the developed method to other methods.

	LOD	LOQ
Developed method	23 pkat/ mL	50 pkat/ mL
MEDELI	41 pkat/ mL*	63 pkat/ mL*
pH-stat	14.3 nkat/ mL*	21.5 nkat/ mL*
* Data from Krewinkel et al., 2016		

Overall, the issue with this method is that the lipase activity is underestimated by including the centrifugation steps. As mentioned earlier, it was reported that centrifugation leads to a 20 % loss of lipase activity in skim milk because of a redistribution of the lipase which is associated with the caseins. However, this issue arises for all methods applying a centrifugation step and thus not only for the here developed approach. Furthermore, it was mainly wished to do a relative comparison between samples from different regions and origins; therefore, all samples experience the same loss in activity and so the differences are still visible.

The glycine-buffer system did not work because at a pH between 8-10 spontaneous alkaline hydrolysis of the substrate 4-MUB to 4-MU is reported (Roberts, 1985). Therefore, the substrate will get hydrolysed in both sample and in blank and as a result no difference in the actual enzyme activity is visible. This background fluorescence showed to be 5-10 times larger at higher pH compared to pH 7 or lower (Roberts, 1985). However, the actual approach to measure at a pH around 10 can result in a more sensitive and robust method if another substrate is used that is not susceptible to hydrolysis in this pH range. For example, UMB was found to have similar initial fluorescence properties as 4-MU. However, UMB is more stable than 4-MU and can be an alternative for the use of 4-MU (De Monpezat et al., 1990). For example, Fink & Koehler (1970) measured sulfatase activity with this substrate at a pH of 10 and were able to increase the sensitivity of their measurement by doing so. Thus, further investigations could be carried out to improve the method by using other substrates.

5.2 Measured Samples

The average lipase activity of all measured raw milk samples is 130 pkat/mL milk (from Appendix 9). The raw milk measured in Krewinkel et al. (2016) had a lipase activity of 19.2 $pkat_{4\text{-}MUL}$/mL milk. It is hard to compare these results since they only used 4-MUL as substrate on which only particular lipases can act. In this project the total lipase activity was determined, which means that 4-MUB is included as well. This substrate can be hydrolysed by esterases too since SCFA are more soluble. However, the LOD of the MEDELI was 41 pkat/mL and thus their measured data is distinctly below the LOD. Therefore, it is unclear how precise and reliable this data is. Furthermore, the raw milk in the MEDELI was taken from the university

owned agricultural experiment station and thus might have experienced less storage time than the samples used for this project. However, it can be said that this data is in the same order or magnitude.

The measured activity is presumably related to LPL which is usually present in high levels in raw milk. This assumption is reinforced by the fact that there is no significant correlation between the lipase activity measurements and the psychrotrophic bacteria cell count, as well as somatic cell count and total bacterial cell count. It should be considered that LPL will be inactivated during a heat treatment and thus the enzymes causing spoilage in long shelf life products are of microbial origin. Nevertheless, if LPL activity is lower before the heat treatment also the threshold until which rancidity can be noticed is at a later point.

The variation in the measured data was reasonably small and thus none of the samples experienced a CV of the raw data higher than 10 % and needed to be measured again. The average CV was 3.1 % and is thus smaller than the CV defined in the MEDELI of 8.8 %.

The samples that were measured repeatedly on different days had some acceptable variation and others had higher variations. These variations can partly be explained by using different tubes of frozen milk which showed before that they had a CV of around 10 %. On top of that, the samples measured again were the ones which showed a bit higher or lower results in the first place and thus might have been a measurement error. Therefore, the higher variation can be due to different measurement days, different frozen tubes, but also measurement errors.

The variation within the farm samples is higher than in the dairy samples. This is not a surprising result since the milk at the dairy is a mixture of milk from many different farms. Thus, variations from an individual farm can be compensated by mixing in the silo tanks with milk from other farms. The milk of some cows is particularly susceptible to spontaneous lipolysis whereas milk of other cows is resistant to it. By mixing the different susceptible milk, lipolysis is slowed down since inhibitors are present. The difference in variation between regions is not as distinct as with the origins. The highest variation was seen between samples from Norrmejerier. In this region two outlier were identified which are responsible for this higher variation.

Overall, the measured results are satisfactory even though some variation within replicates existed. However, significant results could be seen, i.e. that the lipolytic activity is lowest in the area of Skånemejerier and highest in the north of Sweden. The difference might come from the feed and thus an analysis of the feed in the different regions could be carried out. Another factor could be different predominant breeds in these three areas.

The significant negative correlation between the lipase activity and long chain fatty acids (LCFA) was unreckoned as it was expected to result in a positive correlation. It was thought that a high lipase activity results in a greater amount of FFA. However, the opposite phenomenon occurred. Different articles suggest that lipolysis is suppressed by LCFA (Kirkland et al., 1994; Kalderon et al., 2012). These authors discovered this phenomenon in the adipose tissue of rats. The impact of suppression of the LPL activity by oleic acid is visualized in Figure 20.

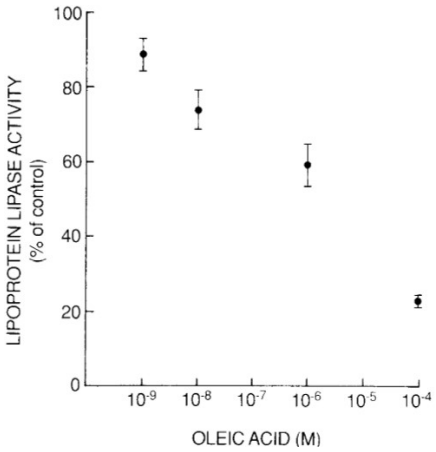

Figure 20: Suppression of LPL activity by oleic acid (from Kirkland et al., 1994).

The impact of this inhibition of lipase activity depends on the acyl chain length of the fatty acid. With increasing chain length, the effect became larger (Kirkland et al., 1994). This effect is featured in Table 22. According to Ferreira & Patton (1990) hydrophobic solutes, i.e. LCFA, are dissolved in the fat droplets partition between the interior oil phase and the surface monolayer where the lipolysis occurs. Thus, they absorb to the substrate interface and thereby inhibit lipolysis. Furthermore, the FA can also compete with the triglycerides for the active site region and inhibit lipolysis in this way as well. Nevertheless, Kalderon et al. (2012) mentions that the mode of action how LCFA suppress adipose lipolysis is not clear. However, this finding does correspond to the correlation determined within this thesis between the lipase activity and LCFA. Thus, the results attained within this thesis indicate that a suppression of lipase activity could occur in the presence of LCFA also in milk.

Table 22: Effects of different fatty acids and one alcohol on the LPL activity (from Kirkland et al., 1994).

Agent (10^{-4} mol/L)	LPL Activity (% of control)
Butyric acid	98.4 ± 5.5
Lauric acid	64.3 ± 7.0
Palmitic acid	50.1 ± 3.1
Palmitoleic acid	75.7 ± 7.8
Oleic acid	22.7 ± 1.9

Anderson (1982) stated that lipolysis is influenced by many factors but that the FFA concentration is poorly correlated with milk lipase activity. However, it has been found that LPL is subject to strong product inhibition also in milk. This means that the enzyme binds FA and thereby the LPL action is controlled by ensuring that the products are not formed more rapidly as they can be taken up by the tissue. This inhibition is not caused by SCFA because they are relatively water-soluble and therefore do not accumulate around sites of lipolysis on the fat droplets (Olivecrona et al., 2003). Consequently, it can be assumed that the LCFA are responsible for this product inhibition. However, no other connection to bovine milk could be found on how LCFA can be used to reduce LPL activity in bovine milk. Nevertheless, in goat milk the LPL activity and spontaneous lipolysis was reduced when fat was added to the goats' diet (Chilliard et al., 2003). Chilliard et al. (2003) explain this by the theory that supplemental lipids need to be taken up and thus more mammary LPL is needed at the basal membrane of secretory cells and thus decreasing the secretion of LPL into the milk. Kirkland et al. (1994) also found that the lipase activity can be influenced by the diet - animals or humans who had a high-fat diet exhibited lower lipase activities than subjects that were given a diet high in carbohydrates. In bovine milk, the fat composition and content is easily influenced by the feed

of the cow (Kadegowda et al., 2008). The milk fat consists of preformed FA which are taken up directly from feed, but also from body mobilization, and FA newly synthesized in the mammary gland (Poulsen et al., 2012). The LCFA are derived from the diet with the exception of oleic acid (C18:1), which is produced from stearic acid (C18:0) by the Δ9-desaturase system in the mammary gland (Kadegowda et al., 2008). This contrasts with C4:0 to C14:0 which are solely synthesized in the mammary gland (Poulsen et al., 2012). It is well established that more dietary fat results in a greater proportion of 18-carbon fatty FA and thus a smaller proportion of the shorter chain FA (Staples, 2006). For example, butterfat added to the feed showed to increase C > 16:0 FA. Cottonseed oil or tallow in the diet of lactating cows is reported to increase stearic and oleic acids in milk fat but does not alter the yield of palmitic acid. The yield of 16:0 and 18:1 can be increased by adding a mixture of palmitic acid and total C18 FA to the feed (Kadegowda et al., 2008).

Thus, by adjusting the feed the lipolytic activity of LPL could be influenced. However, further experiments should be carried out to confirm this result and examine which FA in particular suppress the lipase activity. The feed could be adapted to the specific FA that presumably inhibit lipase activity to see if this is reflected in a lower lipase activity in the milk as well.

6 Conclusion

In conclusion it can be said that a sensitive, easy, and robust method was developed which was the aim of this thesis. In comparison to the MEDELI, a lower LOD and LOQ could be achieved and thus the sensitivity increased. Even in UHT milk activity could be detected in contrast to the MEDELI. This gives reason to believe that this method is also suitable to measure heat treated milk. Nevertheless, the activity in UHT milk was not further examined and would give opportunity for further investigation which was not within the scope of this project. The number of experimental steps is about equal with the MEDELI since both methods apply two centrifugation steps but at different times. There is one more solution that is added within the here developed method. However, the hazardous preparation of the stop solution used in the MEDELI is eliminated. Overall, the robustness of the method could be increased by further investigating the method and lowering the CV within samples. The final measurement procedure is summarized in a flow chart in Figure 21.
Not included in the investigation of the method was the effect of temperature since the fluorescence also varies with it. However, all samples were measured at room temperature and it was assumed that this is not varying much due to a ventilation system in the laboratory.

The samples that were measured with the developed method showed significant difference regarding the region, i.e. the lipase activity is lower in the south of Sweden than in the north. Presumably those differences occur due to different feed or breeds. No significant difference was seen between farm and dairy level. However, a negative correlation between the lipase activity and certain LCFA could be seen. This indicates that LCFA suppress the lipase activity. By changing the feed of the cow, the amount of LCFA in the milk can be influenced and thereby lipase activity in raw milk reduced. However, further trials are needed to confirm this result.

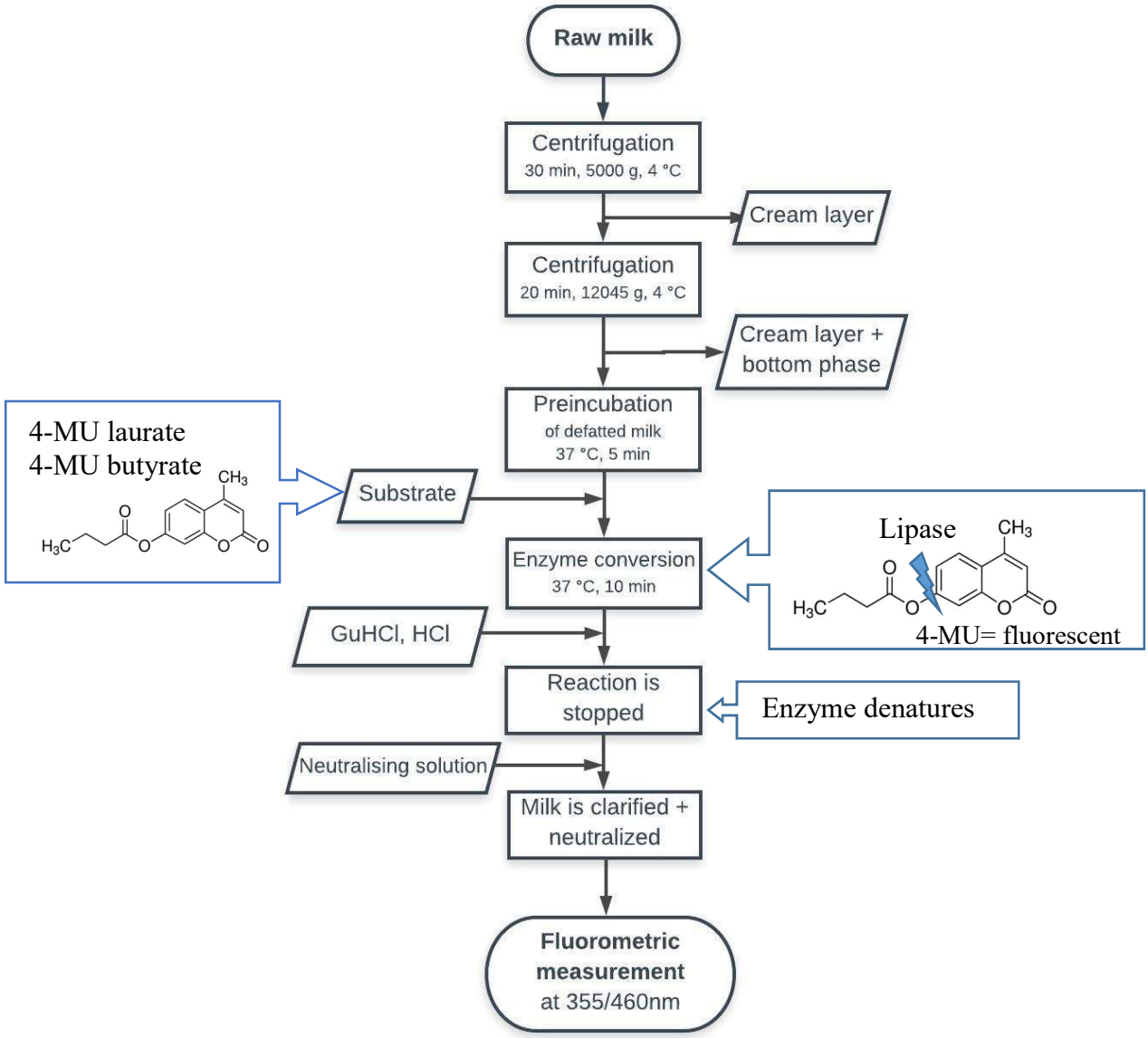

Figure 21: Flowchart of the final developed method to measure lipolytic activity in the natural milk environment. By centrifugation the raw milk is defatted before it is incubated with a substrate on which the lipases can act during a conversion time of 10 min. Afterwards the reaction is stopped, neutralized and the milk clarified before a fluorometric measurement is carried out.

7 Future Perspective

Further investigations could be carried out to improve the method by using another substrate and measure in different pH regions. This could lead to a more sensitive and robust method. Another opportunity for improvement could also be to test the fluorescence at other wavelengths. The available fluorimeter only had four different filters and a measurement at all desired wavelengths is not possible. This can be especially interesting when measuring at the pH of around 10 since other optimum wavelengths exist there (Zhi et al., 2013).

Additional tests should be performed to confirm the finding that LCFA suppress lipolytic activity. Also, it should be examined which FA in particular suppress the lipase activity. The feed could be adapted to the specific FA that presumably inhibit lipase activity to see if this is reflected in a lower lipase activity in the milk as well.

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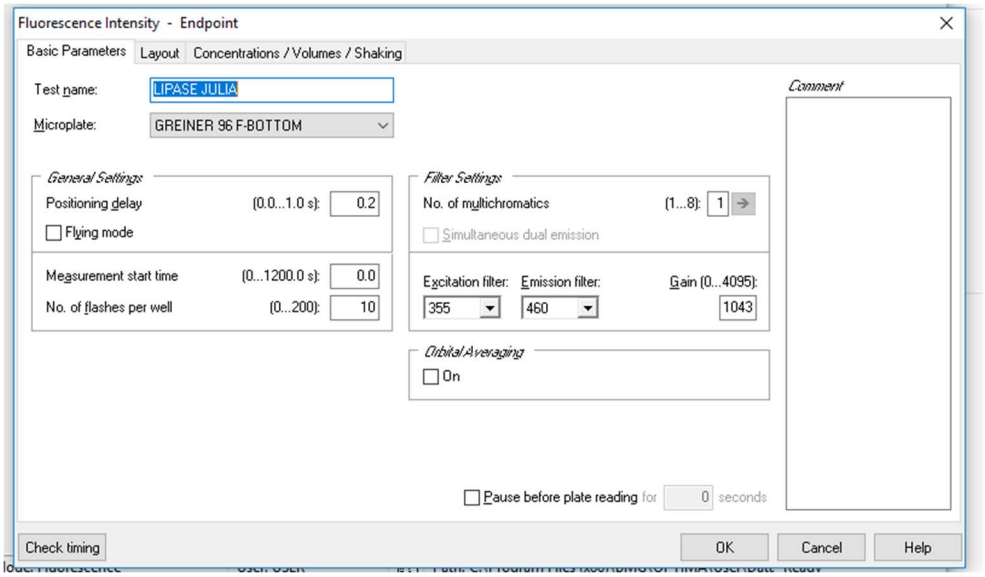
Appendices

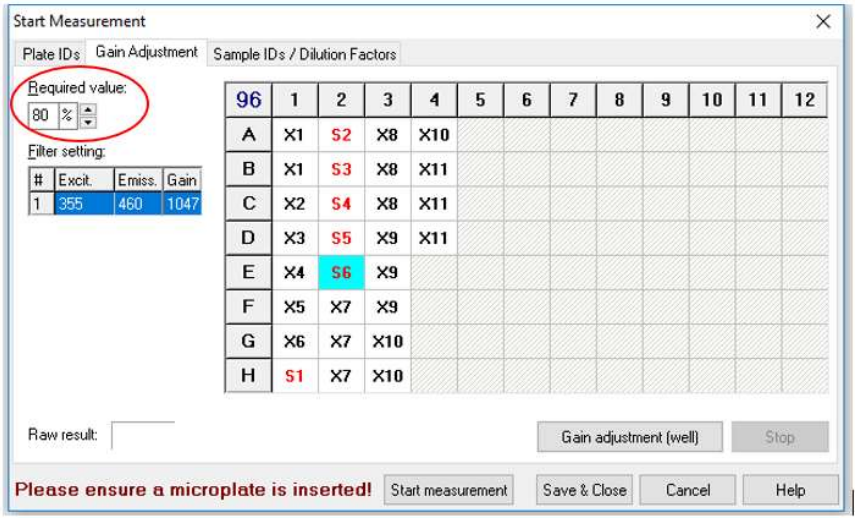
1 Calculation of the standard curve

Standard solution concentration: 0.055015 M = 55.015 mM = 55015 µM

Conc. µM	Dilution factor	Volume stock (µL)	Volume milk (µL)	Volume total (µL)	Actual dilution factor	Actual conc. (µM)
120	458.46	200	720	920	460	119.6
100	550.5	100	450	550	550	100.03
80	687.7	100	590	690	690	79.73
60	916.9	100	820	920	920	59.80
40	1375.4	50	590	690	1380	39.87
20	2750.8	50	1350	1380	2760	19.93

2 Settings of the fluorimeter





3 Calculation of the enzyme activity

V_{milk}: 0.25 mL
V_{total}: 0.547 mL → Dilution factor: 2.19

$$1\ \text{katal} = 1\ \frac{mol\ 4\text{-}MU}{s}$$

Example:

$$40.45\ \mu M\ \text{4-MU} = 40.45\ \frac{\mu mol}{l} = 0.04045\ \frac{\mu mol}{ml} = 40.45\ \frac{nmol}{ml}$$

$$40.45\ \frac{nmol}{ml} * 2.19 = 88.59\ \frac{nmol}{ml} * \frac{1}{670\ s} = 0.1322\ \frac{nmol}{ml*s} = 132.2\ \frac{pkatal}{ml}$$

4 Adjusting and measurement of the pH after each step attained in a scaled-up experiment (10 times scaled up to usual measurement amount)

	Average pH									pH in MEDELI
Defatted milk	6.66 ±0.01									
After substrate solution	6.74 ±0.0									
After GuHCl	6.28 ±0.0	6.2 ±0.01	6.4 ± 0.01		6.25 ±0.007					<2
After HCl	--	0.17 ±0.01	0.38 ±0.02		0.58 ±0.02			0.64	1.08	
After Neutralising solution	10.67 ± 0.1	3,22 ±0.16	6,23 ±0.0	6,63 ±0.0	6.72 ±0.02	7.02	7.12	7.38	8.31	6.5
	Amount added GuHCl/ HCl									
GuHCL 8M (mL)	1.5	1.6	1.5	1.5	1.5	1.5	1.5	1.5	1.5	
HCl 5M (µL)	0	400	250	220	200	180	170	150	100	
The red fields were not measured again since they are assumed to be similar as the previously measured samples										

5 Variation of sample in between different frozen tubes compared to the variation within the same frozen tube

	n		Average conc. 4-MU [µM]	Average enzyme activity (pkatal)	CV
Variation in between frozen milk tubes	2	Sample 1	37.23 ± 3.98	128.4 ± 13.7	10.7
		Sample 2	39.58 ± 3.87	136.5 ± 13.4	9.8
Variation within the same frozen milk tube	2	Sample 1-tube 1	41.8 ± 4.5	144.1 ± 15.5	10.7
	2	Sample 1-tube 2	35.5 ± 1.3	122.3 ± 4.4	3.6
	2	Sample 1-tube 3	34.4 ± 0.5	118.8 ± 1.8	1.5
	2	Sample 2-tube 1	38.2 ± 2.1	131.6 ± 7.1	5.4
	2	Sample 2-tube 2	44.0 ± 0.5	151.6 ± 1.6	1.1
	2	Sample 2-tube 3	36.6 ± 1.9	126.3 ± 6.6	5.2

6 Contribution of 2-propanol to the total fluorescence

Sample	Fluorescence (RFU)
2-propanol	26
Milk sample 1	3423 ± 40
Milk sample 2	3924 ± 18

7 Data for the calculation of LOD and LOQ

Date examined	**Slope of standard curve**	**Blank value (RFU)**
06.02.2018	278.64	16540
07.02.2018	291.16	17193
13.02.2018	281.7	16871
14.02.2018	299.52	16482
15.02.2018	289.38	16027
16.02.2018	287.58	16366
21.02.2018	292.53	18467
22.02.2018	293.37	17695
23.02.2018	287.07	17903
26.02.2018	285.26	17062
27.02.2018	292.89	17927
28.02.2018	301.77	16872
Average	**290.1**	**17117**
S.D.	**6.7**	**741.5**
CV	2.3	4.3

8 First trials with the glycine-buffer system

	Blank (RFU)	**Sample (RFU)**	**Difference (RFU)**	**pH**
Glycine-NaOH-EDTA	42437	43259	822	8.86
Glycine-NaOH-EDTA-GuHCl	3655	17403	13748	7.59
Glycine-NaOH-EDTA-GUHCl-HCl	192	1437	1245	1.55
Glycine-NaOH	59216	59756	540	10.25
Glycine-NaOH-GuHCl	44154	43865	-289	9.69
Glycine-NaOH-GuHCl-HCl	169	1048	879	1.18

9 Enzyme activity results of all measured samples

Region	Origin	Date collected	Concentration 4-MU (mM)	Enzyme acitivty (pkat/ mL)
Skånemejerier	Farm	170531	32,2	111,9
	Farm	170531	39,9	137,6
	Farm	170531	37,4	129
	Farm	170531	38,3	132,1
	Farm	170531	34,5	119,1
	Farm	170614	28,6	99,3
	Farm	170614	36,3	125,1
	Farm	170614	27,8	96,8
	Farm	170614	27,5	94,6
	Farm	170614	31,8	109,4
	Dairy	170726	37,7	129,5
	Dairy	170726	31,1	107,4
	Dairy	170726	30,3	104,6
	Dairy	170726	29	101
	Dairy	170726	36,2	125,9
Arla	Farm	170620	36,9	127,4
	Farm	170620	37,2	129,2
	Farm	170620	31,4	109,2
	Farm	170620	40	139
	Farm	170620	39	135,5
	Farm	170907	40,8	139,8
	Farm	170907	43,3	148,1
	Farm	170907	37,1	128,5
	Farm	170907	45,8	159,3
	Farm	170907	45,9	159,5
	Dairy	170913	35,5	122,7
	Dairy	170913	38,3	131
	Dairy	170913	34,4	119
	Dairy	170913	37,2	128,8
	Dairy	170913	36,6	125,2
Norrmejerier	Farm	170810	37,8	130,3
	Farm	170810	42,8	147,6
	Farm	170810	38,4	132,4
	Farm	170810	40,7	140,8
	Dairy	170810	39,5	136,3
	Farm	170824	42,6	146,8
	Farm	170824	40,3	139
	Farm	170824	44,1	153,3
	Dairy	170824	47	162,1
	Dairy	170824	40,4	139,3
	Farm	170830	35,9	122,9
	Farm	170830	40,6	141,2
	Farm	170830	53,2	184,9
	Dairy	170830	42,6	148,1
	Dairy	170830	41,1	141,5

10 Comparison of samples that were measured on two occasions

Sample-ID	Date examined	Raw data (RFU)	Blank corrected (RFU)	CV of raw data (%)	CV of blank corrected (%)
S0614-4	28.02.	28533	13563	3,9	10,8
		28814	13844		
		27388	12418		
	15.02.	27036	10995		
		27145	11104		
S0726-1	13.02.	29185	12665	2,5	6,0
		30726	14206		
		30502	13982		
	27.02.	29185	12139		
		30726	13680		
		30502	13456		
S0726-4	07.02.	28234	11263	2,1	8,2
		27407	10436		
		28624	11653		
	27.02.	29033	13311		
		27848	12126		
		27860	12138		
A0906-3	21.02.	29485	12615	4,9	5,5
		29344	12474		
		29681	12811		
	27.02.	31280	13760		
		31602	14082		
N0830-5	21.02.	30943	12808	5,1	12,9
		29853	11718		
		30258	12123		
	28.02.	32832	14993		
		34111	16272		
		31700	13861		
S0531-1	23.02.2018	30310	13612	6,2	7,3
		30822	14124		
	07.02.2018	27694	12506		
		27249	12061		
S0614-5	16.02.2018	28278	12864	6,8	15,8
		26126	10712		
	28.02.2018	30309	15354		
		30084	15129		
		31072	16117		
A0913-1	16.02.2018	28332	11531	6,9	12,6
		29122	12321		
		27808	11007		
	27.02.2018	31764	14576		
		31434	14246		
A0913-5	20.02.2018	30621	13923	4,8	10,6

		28718	12020		
		28409	11711		
	28.02.2018	31378	14615		
		30658	13895		
		32111	15348		
N0830-1	20.02.2018	28863	12250	5,3	9,1
		28323	11710		
		28929	12316		
	28.02.2018	31750	14394		
		31323	13967		

11 Prerequisites for the preparation of the model

1. Test for orthogonality

Origin * Region Crosstabulation

Count

		Region			Total
		Skanemejerier	Arla	Norrmejerier	
Origin	Farm	10	10	10	30
	Dairy	5	5	5	15
Total		15	15	15	45

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	**,000[a]**	2	1,000
Likelihood Ratio	,000	2	1,000
Linear-by-Linear Association	,000	1	1,000
N of Valid Cases	45		

a. 0 cells (0,0%) have expected count less than 5. The minimum expected count is 5,00.

2. Test for normality

Tests of Normality

			Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Region	Origin	Statistic	df	Sig.	Statistic	df	Sig.
Enzyme_activity	Skanemejerier	Farm	,153	10	,200*	,936	10	**,510**
		Dairy	,285	5	,200*	,850	5	**,195**
	Arla	Farm	,155	10	,200*	,948	10	**,643**
		Dairy	,166	5	,200*	,979	5	**,928**
	Norrmejerier	Farm	,214	10	,200*	,866	10	**,090**
		Dairy	,250	5	,200*	,881	5	**,316**

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

3. Test for homogeneity

Test of Homogeneity of Variance					
		Levene Statistic	df1	df2	Sig.
Enzyme_activity	Based on Mean	1,038	5	39	**,409**
	Based on Median	,866	5	39	,513
	Based on Median and with adjusted df	,866	5	29,257	,516
	Based on trimmed mean	1,005	5	39	,427

12 Model for the statistical analysis of the measured enzyme activity

Tests of Between-Subjects Effects						
Dependent Variable: Enzyme_activity						
Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	7209,238[a]	5	1441,848	6,966	,000	,472
Intercept	678498,304	1	678498,304	3278,264	,000	,988
Region	6071,114	2	3035,557	14,667	**,000**	**,429**
Origin	173,056	1	173,056	,836	**,366**	,021
Region * Origin	342,717	2	171,358	,828	**,444**	,041
Error	8071,782	39	206,969			
Total	786740,220	45				
Corrected Total	15281,020	44				
a. R Squared = ,472 (Adjusted R Squared = **,404**)						

13 New model excluding the interaction effect

Tests of Between-Subjects Effects						
Dependent Variable: Enzyme_activity						
Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	6866,521[a]	3	2288,840	11,152	,000	,449
Intercept	678498,304	1	678498,304	3306,012	,000	,988
Region	6693,465	2	3346,733	16,307	**,000**	**,443**
Origin	173,056	1	173,056	,843	**,364**	**,020**
Error	8414,499	41	205,232			
Total	786740,220	45				
Corrected Total	15281,020	44				
a. R Squared = ,449 (Adjusted R Squared = **,409**)						

Pairwise Comparisons

Dependent Variable: Enzyme_activity

(I) Region	(J) Region	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
Skanemejerier	Arla	-18,593*	5,231	**,001**	-29,158	-8,029
	Norrmejerier	-29,547*	5,231	**,000**	-40,111	-18,982
Arla	Skanemejerier	18,593*	5,231	**,001**	8,029	29,158
	Norrmejerier	-10,953*	5,231	**,042**	-21,518	-,389
Norrmejerier	Skanemejerier	29,547*	5,231	**,000**	18,982	40,111
	Arla	10,953*	5,231	**,042**	,389	21,518

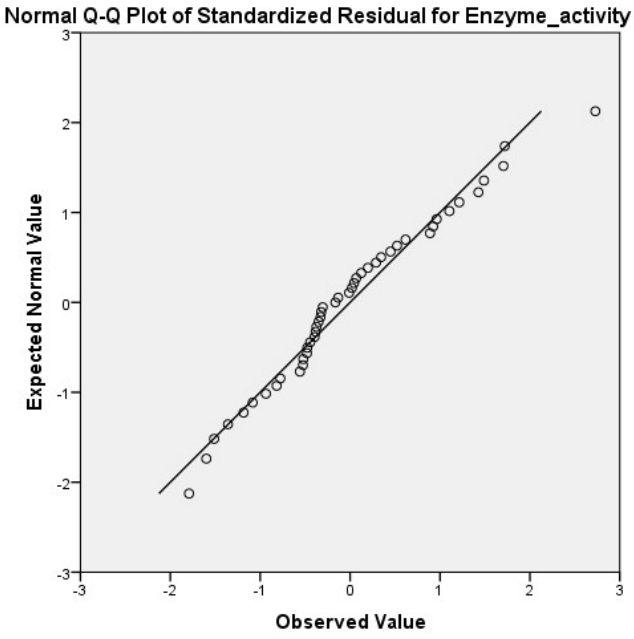
Based on estimated marginal means

*. The mean difference is significant at the ,05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

14 Confirmation of model

1. Check for normality



2. Check for homogeneity of variances

Levene's Test of Equality of Error Variances[a]

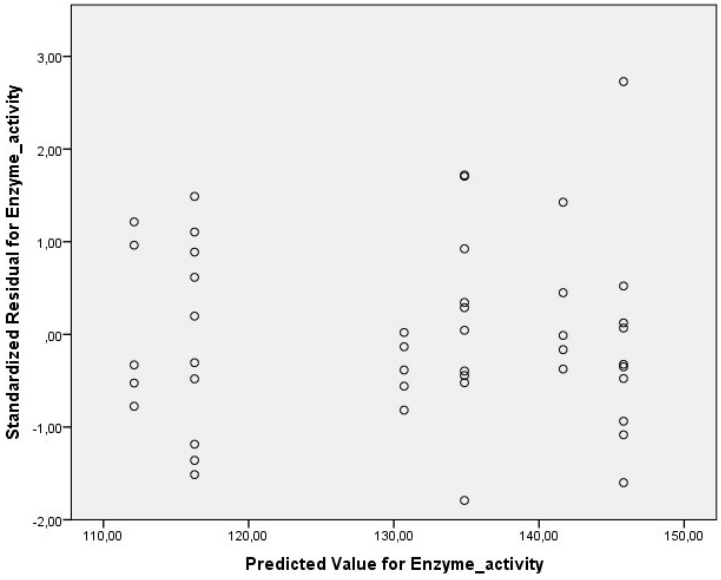
Dependent Variable: Enzyme_activity

F	df1	df2	Sig.
,752	5	39	**,590**

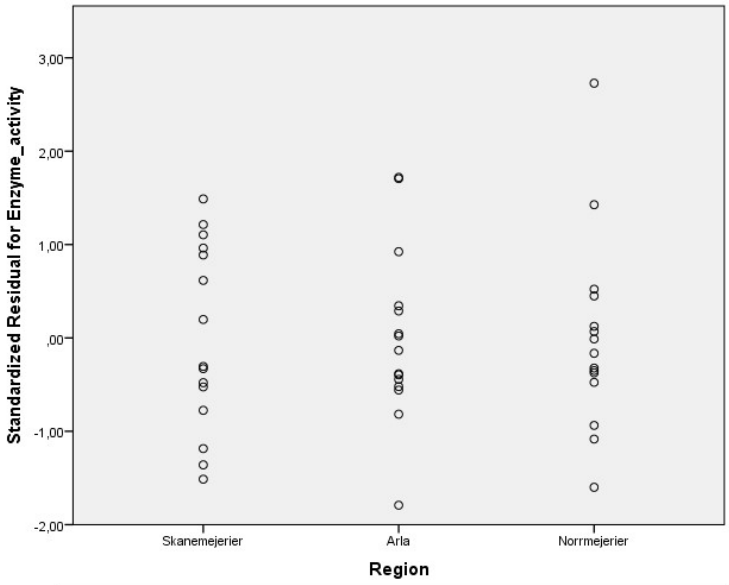
Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

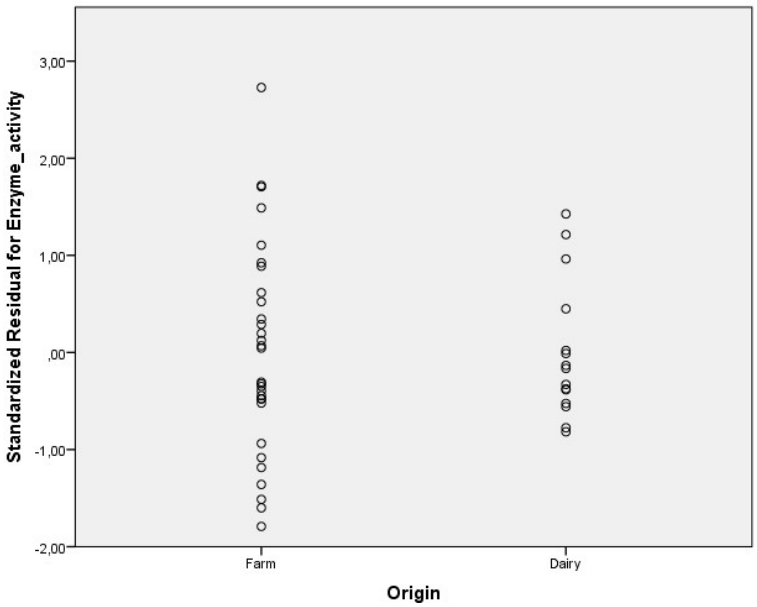
a. Design: Intercept + Region + Origin

3. Check for residuals



15 Variation of the different regions and origins





16 Normality test and correlation to other measured properties

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Enzyme_activity	,088	45	,200*	,975	45	**,440**
Fat_content	,118	45	,130	,973	45	**,371**
Total_free_FA	,083	45	,200*	,986	45	**,857**
Psychrotrophic_bacteria	,099	45	,200*	,952	45	**,061**
Somatic_cell_count	,074	45	,200*	,981	45	**,673**
Total_cell_count	,488	45	,000	,159	45	**,000**

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Correlations

		Enzyme_activity
Fat_content	Pearson Correlation	,167
	Sig. (2-tailed)	,310
	N	39
Total_free_FA	Pearson Correlation	**-,326***
	Sig. (2-tailed)	**,035**
	N	42
Psychrotrophic_bacteria	Pearson Correlation	-,291
	Sig. (2-tailed)	,058
	N	43
Somatic_cell_count	Pearson Correlation	-,107
	Sig. (2-tailed)	,501
	N	42
Total_cell_count	Pearson Correlation	-,018
	Sig. (2-tailed)	,910
	N	41

17 Normality and correlation to individual free fatty acids

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
C4_0	,094	45	,200*	,977	45	**,487**
C6_0	,063	45	,200*	,974	45	**,412**
C8_0	,073	45	,200*	,968	45	**,252**
C10_0	,086	45	,200*	,971	45	**,317**
C12_0	,105	45	,200*	,961	45	**,136**
C14_0	,066	45	,200*	,987	45	**,889**
C16_0	,089	45	,200*	,985	45	**,817**
C18_0	,189	45	,000	,920	45	**,004**

C18_1	,080	45	,200*	,989	45	**,937**
C18_2	,113	45	,186	,948	45	**,044**
C18_3	,283	45	,000	,822	45	**,000**

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Correlation

		Enzyme_activity
C4_0	Pearson Correlation	-,167
	Sig. (2-tailed)	,273
	N	45
C6_0	Pearson Correlation	-,230
	Sig. (2-tailed)	,129
	N	45
C8_0	Pearson Correlation	-,218
	Sig. (2-tailed)	,150
	N	45
C10_0	Pearson Correlation	-,208
	Sig. (2-tailed)	,171
	N	45
C12_0	Pearson Correlation	-,173
	Sig. (2-tailed)	,257
	N	45
C14_0	Pearson Correlation	-,280
	Sig. (2-tailed)	,062
	N	45
C16_0	Pearson Correlation	**-,556****
	Sig. (2-tailed)	**,000**
	N	45
C18_0	Pearson Correlation	**-,603****
	Sig. (2-tailed)	**,000**
	N	45
C18_1	Pearson Correlation	,007
	Sig. (2-tailed)	,964
	N	45
C18_2	Pearson Correlation	-,199
	Sig. (2-tailed)	,191
	N	45
C18_3	Pearson Correlation	**-,422****
	Sig. (2-tailed)	**,004**
	N	45

**.Correlation is significant at the 0.01 level (2-tailed)

18 Confirmation of correlation with Spearman's rho for non-normal data

Correlations with Spearman's rho			
			Enzyme_activity
Spearman's rho	Total_cell_count	Correlation Coefficient	-,069
		Sig. (2-tailed)	,668
		N	41
	C18_0	Correlation Coefficient	-,484**
		Sig. (2-tailed)	,001
		N	43
	C18_2	Correlation Coefficient	-,061
		Sig. (2-tailed)	,715
		N	38
	C18_3	Correlation Coefficient	-,384*
		Sig. (2-tailed)	,011
		N	43