PARTIAL CHARACTERIZATION OF A BACTERIAL ACYLTRANSFERASE ENZYME FOR POTENTIAL APPLICATION IN DAIRY PROCESSING

by

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Declaration

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SUMMARY

This study describes:

- the evaluation of the current, and potential assay methods for the quantification of cholesterol, cholesteryl esters and free fatty acids in milk and the application thereof;
- an account of the difficulties associated with the usage of FoodPro® Cleanline, an enzyme preparation used as processing aid, during ultra-high temperature processing of milk;
- the development of activity assays which can be used for the kinetic characterization of glycerophospholipid cholesterol acyltransferase, the active enzyme in FoodPro® Cleanline;
- the development of an accurate and facile activity assay, and the validation thereof, which can be used for the validation of enzyme activity prior to dosage of milk with FoodPro® Cleanline.

OPSOMMING

Hierdie studie beskryf:

- die evaluering van die huidige, en potensiële, metodes vir die kwantifisering van cholesterol, cholesteriel esters en vryvetsure in melk, sowel as die toepassing van hieridie metodes;

- ‘n verduideliking van die moeilikhede wat ondervind word gedurende die gebruik van FoodPro® Cleanline, ‘n ensiempreparaat vir gebruik as ‘n verwerkingshulpmiddel, tydens ultrahoë-temperatuurprosessering van melk;

- die ontwikkeling van aktiwiteitsbepalings metodes vir gebruik in kinetiese karakterisering van gliserofosfolipied cholesterol asieltransferase, die aktiewe ensiem in FoodPro® Cleanline;

- die ontwikkeling van ‘n akkurate, eenvoudige aktiwiteitsbepalings metode, en bevestiging van hierdie metode, wat gebruik kan word vir kwalitieitskontrole alvorens die dosering van melk met FoodPro® Cleanline.

In memory of Jacobus Malan Hayward

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ABBREVIATIONS

1,2-dioleoyl-sn-glycerop-3-phosphocholine	DOPC
Acid degree value	ADV
Additional layer	A layer
Apolipoprotein A1	Apo-A1
Cholesteryl ester breakdown products	CEBP
Clean in position	CIP
Dalton	Da
Distilled water	dH_2O
Extra cellular products	ECP
Flame ionization detection	FID
FoodPro® Cleanline	FPCL
Free fatty acids	FFA
Gas chromatography	GC
Generally regarded as safe	GRAS
Glycerophospholipid cholesterol acyltransferase	GCAT
High performance liquid chromatography	HPLC
High temperature short time	HTST
Horseradish peroxidase	HRP
Isopropyl β-D-1-thiogalactopyranoside	IPTG
Lecithin cholesterol acyltransferase	LCAT
Lieberman-Burchard	LB
Lipopolysaccharides	LPS
Lysophospholipids	LPL
Mass spectrometry	MS
Maximal velocity	V_{max}
Methyl tert-butyl ether	MTBE
Michaelis constant	K_m
Molar extinction coefficient	ε
Molar	M
Multilamellar vesicles	MLV
Nanogram	ng
Nanometer	nm
Nitrophenol	pNP
N-Methyl-N-(trimethylsilyl) trifluoroacetamide	MSTFA
Non-esterified fatty acids	NEFA
para-Nitrophenol butyrate	pNPB
Parts per million	ppm
Research and development	R&D
Single ion monitoring	SIM
Small unilamellar vesicles	SUV
Sodium dodecyl sulfate polyacrylamide gel electrophoresis	SDS-PAGE
Standard operating procedure	S.O.P
Sub-Saharan Africa	SSA
Surface layer	S layer
Thin layer chromatography	TLC
Trimethylchlorosilane	TMCS
Ultra high temperature	UHT
Ultraviolet	UV

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CHAPTER 1

INTRODUCTION

At present, milk is considered to be one of the safest foods commercially available. It is therefore difficult to comprehend that barely 200 years ago milk was considered “as deadly as Socrates‟ hemlock”[1]. At that time, milk was commonly consumed at ambient temperature without prior thermal treatment. Because milk is a rich source of nutrients, designed to sustain the mammalian neonate, it provides the optimal conditions for the proliferation of, often fatal, microorganisms at ambient temperatures. As a result the usage of milk often resulted in high mortality rates, especially in infants. During the early 19th century Louis Pasteur discovered that mild heat treatment of foods can increase shelf life by killing the microorganisms responsible for spoilage[2]. This treatment was subsequently applied to milk intended for use by infants. Although thermal treatment of milk was rigorously opposed, the introduction of thermally processed milk coincided with a sharp decrease in infant mortality.

Since the advent of thermal treatment of milk, fouling has been a major problem leading to increased costs due to plant down-time and the need for cleaning chemicals. Thermal processing results in the denaturation of heat labile milk proteins, mainly β-lactoglobulins, and a decrease in the solubility of minerals such as calcium phosphate[3–5]. The denatured proteins subsequently form aggregates which adhere to the heat transfer surfaces of indirect thermal processing plants in a process known as fouling[3,4]. At temperatures in excess of 105°C this layer of proteinaceous material acts as a scaffold for the subsequent deposition of minerals resulting in mineral fouling[3]. The foulant layer reduces the heat transfer capacity of the heat exchangers by insulating the heat source from the product. To maintain a constant product temperature, the temperature of the heating medium needs to be increased. As a result, the energy cost of processing increases with the degree of fouling. Fouling may also result in product deterioration by ineffective heating and contamination with dislodged deposits[5]. In order to avoid excessive fouling, the heat exchangers should be cleaned on a regular basis (at least once a day). A typical clean in position (CIP) cycle can take as much as 2 hours of processing time and requires high volumes of harmful cleaning chemicals. These factors greatly increase the overall processing cost during the production of UHT milk. As a result, fouling, and the prevention thereof, has been exhaustively investigated[4–6].

Ultra high temperature (UHT) treatment of milk involves the treatment of milk at 142°C for a minimum of 2 seconds. This form of thermal treatment is sufficient to destroy all vegetative microbial cells and their spores. As a result, UHT treated milk has an increased shelf life when stored at ambient temperatures. In combination with a high nutritional value, UHT milk has great potential as nutritional aid in areas without sufficient cold chain storage infrastructure and areas struck by natural disasters. However, mineral fouling, during UHT processing, greatly increases the overall cost of production, reducing its availability in rural areas.

Since it is known that protein fouling precedes mineral fouling, and is a prerequisite thereof, it is conceivable that elimination of protein fouling could also eliminate mineral fouling. It has been shown that protein fouling can be reduced by stabilization of proteins denatured during thermal processing. This was achieved by the addition of emulsifiers, such as lecithin, to raw milk prior to thermal processing[7–9]. However, the use of lecithin for this purpose, in drinking milk, not only introduces an allergen, but is also prohibited by law. More recent studies have indicated that the addition of lipases can increase cheese yield during production. It was subsequently shown that this increase is due to the production of amphoteric surface active lysophospholipids by phospholipid hydrolysis. It has furthermore been shown that emulsions stabilized by lysophospholipids have increased heat stability and a lower sensitivity to flocculation by Ca^{2+} and Mg^{2+}[10–12]. This increase in heat stability has been attributed to the formation of lysophospholipid-protein complexes at droplet interfaces[12–15]. As such, the addition of lipases to raw milk, prior to thermal processing, may reduce fouling by increasing the heat stability of milk proteins. However, although the reaction of lipases with phospholipids yields lysophospholipids, this interaction also results in the formation of free fatty acids. Free fatty acids are known to increase the acid degree of milk resulting in an increase in rancidity. Furthermore, milk contains various native lipases which need to be inactivated as quickly as possible to prevent spoilage of the raw milk. Addition of foreign lipases is therefore not recommended.

Members of the genus Aeromonadaceae have been shown to produce a lipase-like enzyme glycerophospholipid cholesterol acyltransferase (GCAT, EC 2.3.1.43) which is able to transfer a fatty acyl chain from the two-position of phospholipids to a suitable acceptor such as cholesterol. In this reaction lysophospholipids and cholesteryl esters are produced. Free fatty acids are therefore sequestered in cholesteryl esters and would, as a result, not increase the acid degree value of raw milk. This enzyme has since been isolated, cloned, expressed and purified by DuPont® for the potential application in the dairy industry, to reduce fouling during UHT treatment, under the commercial name FoodPro® Cleanline (FPCL). Since enzyme

catalysis is highly specific, the use of enzymes could enable production of products with predetermined qualities. This information could furthermore be applied to development of a dosage model which could be used to predict the final product qualities. However, to the best of our knowledge, the kinetic characteristics of GCAT catalysis, which would allow for the accurate dosing of this enzyme, have not yet been published.

During pilot- and full scale FoodPro® Cleanline (FPCL) application trials, at different venues, conflicting results were obtained. The results were furthermore not consistent when the trials were repeated. The reason for these inconsistencies could not be explained. For successful industrial marketing and application of FPCL, these difficulties had to be overcome and eliminated. For this reason, and for quality control purposes, accurate and reproducible methods for the quantification of FPCL reaction products must be in place. Availability of kinetic data for the interaction between GCAT and its substrates would enable dosage optimization to produce a product with predetermined characteristics.

For the results presented in this thesis FPCL application trials were repeated in an attempt to determine the potential problems which may be associated with the commercial application of FPCL. Methods for the quantification of GCAT reaction products, cholesteryl esters and FFA in milk was evaluated. These methods were subsequently used during the kinetic characterization of the GCAT enzyme. The thesis concludes with a description of the development of a novel activity assay, and kinetic verification thereof, for the accurate determination of FPCL activity. This work is presented in Chapter 5. Finally, the newly developed assay will be used for FPCL applicability studies. The current work therefore had the following aims:

1. To perform pilot- and factory scale FPCL application trials in order to define the difficulties associated with the usage of FPCL.
2. Evaluation of the current, and potential, assay methods for the quantification of GCAT reaction products in milk and the application thereof.
3. The adaptation of existing activity assays to enable kinetic characterization of GCAT.
4. The development of an accurate GCAT activity assay for use in FPCL activity validation.
5. The verification of the newly developed assay by comparison to assays performed using the natural substrates for GCAT.

The studies conducted will be presented as follows:

Chapter 2 presents an introduction and overview of fouling in the dairy industry followed by a review of the GCAT enzyme. The current understanding of fouling and the existing methods for its prevention are addressed in detail. The history, purification, molecular characteristics and mechanism of action of the GCAT enzyme will subsequently be discussed with particular attention to the mechanism of action using different substrates, both natural and synthetic.

Chapter 3 reviews the current, and potential, methods for quantification of FPCL activity. The current difficulties associated with FPCL activity monitoring will be discussed with specific reference to the nature of the natural substrates, phospholipids and cholesterol. Potential substrates for use in kinetic characterization will be described in detail. The possibility for application of synthetic substrates for the development of a facile continuous activity assay will be reviewed, concluding with the outstanding work in enzyme characterization.

In Chapter 4 the current method for quantification of FPCL reaction products and free fatty acids are described and evaluated. The results from pilot- and factory scale FPCL trials performed throughout 2012 will be presented and discussed with particular mention to the previously found inconsistencies. This chapter will conclude in the identification of the possible reasons, and remedies, for the conflicting results obtained in previous studies conducted with FPCL elsewhere.

Chapter 5 describes the evaluation of different methods for the quantification of FPCL reaction products in milk. These methods will subsequently be used in the development of enzyme activity assay which would enable kinetic characterization of GCAT and the accurate monitoring of FPCL activity. The current work will attempt to validate the newly developed assay method by comparison of kinetic parameters obtained from kinetic studies. This chapter will conclude in the use of synthetic substrates for the evaluation of FPCL applicability in different milk processing methods.

In conclusion, Chapter 6 presents an overview of the results obtained during the course of this study. The current difficulties with FPCL activity monitoring and evidence for the current theory for the catalytic mechanism of GCAT will be discussed. This chapter will conclude in the discussion of potential substrates for use in GCAT activity studies.

CHAPTER 2

THERMAL PROCESSING IN THE DAIRY INDUSTRY

2.1 INTRODUCTION

Milk is a complex biological mixture, consisting of proteins, carbohydrates, minerals, lipids, vitamins and trace elements, essential to normal physical and mental development. Large scale production of milk is a challenging exercise. The nutrient rich nature of milk provides optimal conditions for microbial growth leading to the rapid spoilage of the raw product. As a result, various processing techniques have been applied to enhance the safety and quality of the final product, including extending the shelf life. Ultra-high temperature (UHT) processing can extend product shelf life for up to 12 months[16]. As thermal processing is an energy intensive process, wherein products are heated at least once, the efficiency of the heating process is of paramount importance in order to ensure economic viability. Temperature induced fouling, however, reduces processing efficiency significantly, leading to product deterioration and an increased overall processing cost [5,17].

Fouling of heat exchangers by dairy products has been a major problem since the advent of thermal processing of dairy products in the 1930s[4]. The fouling phenomenon, in the dairy industry, can be described as the temperature induced formation of deposits on the heat exchanging surfaces. Deposit formation reduces the heat transfer efficiency of the heat exchanger by insulating the product from the heat source. Consequently, overall processing cost is significantly increased by the need for additional energy input due to reduction of heat transfer efficiency, manpower, loss in productivity, cleaning chemicals and the environmental impact[3,18]. Fouling may furthermore result in product deterioration, by contamination with dislodged deposits, and inefficient heating during thermal processing. For these reasons, daily intermediate plant shutdown and cleaning is essential to maintain optimal product quality and processing efficiency[3].

Although a significant volume of research has been done into the reduction of temperature-induced fouling, the underlying factors affecting fouling are still poorly understood. Recent advances in lipase enzyme technology have shown promise by harnessing the natural emulsifying potential of milk lipids. Lipases hydrolyze phospholipids to yield lysophospholipids (LPL) which can stabilize denatured milk proteins. The use of lipases in various industrial

applications is well established. Enzyme catalysis is highly specific, active under mild reaction conditions and reduces non-specific by-products[19,20]. Enzymes are furthermore active at low concentrations and are often fully inactivated by UHT processing temperatures. For these reasons enzymes are well suited for use in the food, and especially the milk industry. In the ensuing section the current understanding of the mechanism of dairy fouling and the practical procedures for its limitation will be discussed in more detail.

2.2 FOULING

2.2.1 Types of fouling

The composition of the deposit layer on heat exchangers varies in relation to processing temperature. Two types of fouling have been described. Type A fouling describes the deposition of proteinaceous material at temperatures between 80 and 105°C[4,21]. The deposit layer formed in type A fouling, generally known as protein fouling, occurs as a relatively soft bulky layer consisting of 50-60% protein, 30-50% minerals and 4-8% fat[3,4,18]. Although milk proteins constitutes only a small amount of the total milk solids (< 5%), they account for more than 50% of the fouling deposits in type A fouling.

The two major whey proteins are β-lactoglobulin and α-lactalbumin, however, β-lactoglobulin is the major protein involved in fouling due to its heat sensitive nature[3,5,18]. Upon heating of milk, the β-lactoglobulins denature and expose reactive sulfhydryl groups[3,5]. This denatured form of β-lactoglobulin is known as the activated form of the protein since it is able to associate with itself or other milk proteins via disulfide bond formation to form protein aggregates[3,21]. The exact mechanism of fouling is not yet fully understood, however, a relationship between denaturation of β-lactoglobulin and UHT fouling has been previously established[22]. Figure 2.1 depicts the proposed mechanism of fouling as described by Britz and Robertson[21]. As shown in Figure 2.1, β-lactoglobulins are activated by heat, resulting in increased aggregation and, ultimately, deposition and fouling of the heat exchangers.

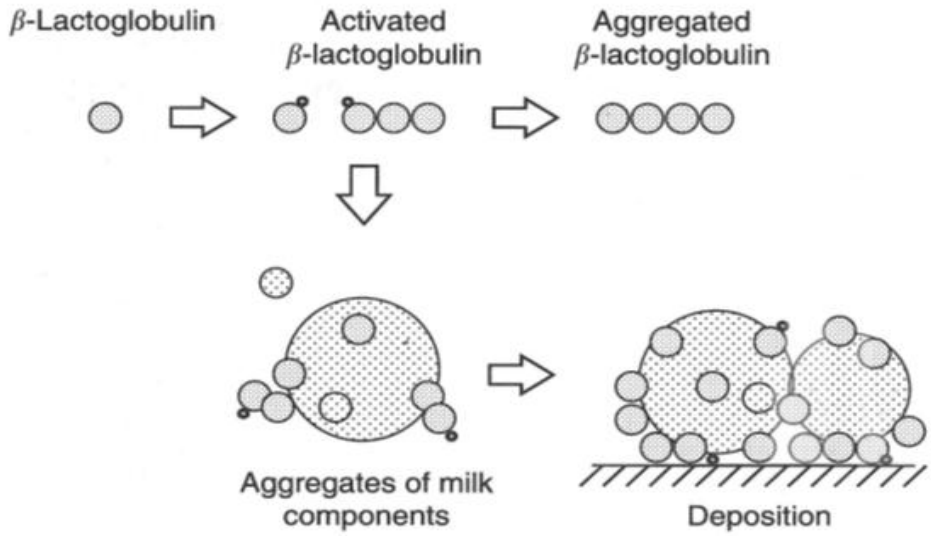


Figure 2.1 Mechanism of fouling wherein β-lactoglobulin is activated by thermal processing, resulting in the formation ,and adhesion, aggregates on the heat exchange surfaces[21].

Type B fouling, otherwise known as mineral fouling, occurs when processing is performed at temperatures in excess of 105°C. The deposit layer in mineral fouling is hard, granular in structure and consists of 70-80% minerals (mainly calcium phosphate), 15-20% protein and 4-8% fat[4]. The mineral content of milk is only about 1% in weight, however, minerals such as calcium phosphate constitutes up to 70% of the deposits of Type B fouling[23]. The solubility of calcium phosphate decreases with increasing temperature. Consequently, mineral fouling occurs due to a local supersaturation of calcium phosphates next to the heat exchanging surfaces upon precipitation of these minerals[17,23]. The mechanism of mineral fouling is better understood than that of protein fouling, however, a complete discussion of this process is beyond the scope of this thesis.

2.2.2 Mineral deposition is preceded by protein deposition

Fouling has generally been regarded as at least a two-step process, starting with an induction period followed by adsorption of proteins to the heat exchanging surfaces[4,5,24]. It was concluded by Foster[25], that mineral deposition occurs only after the exchanger surface has been covered by a thin layer of proteinaceous material. Mineral depositions subsequently diffuse through the deposit layer to the deposit-metal interface[26,27]. Protein fouling therefore acts as a scaffold for mineral fouling to build on. This theory is supported by the observation that an increase in the calcium content of milk, prior to thermal processing, increases the degree of fouling by decreasing the denaturation temperature of β-lactoglobulin[5,26,28]. Addition of potassium iodate on the other hand reduces fouling by oxidizing the sulfhydryl groups exposed by heat induced

protein denaturation[26,27]. Oxidation of these groups prevents protein aggregation resulting in a reduced degree of fouling. These results indicate that protein denaturation and aggregation is the key step in fouling. Supportive of this, various authors have shown that fouling is increased with an increase in milk protein concentration[26,29]. A reduction in protein fouling would therefore lead to an overall reduction in fouling.

2.2.3 Preventative measures for fouling

Although UHT fouling may be practically unavoidable, it may be minimized by various factors. The most important factors affecting milk fouling are the operating conditions in the heat exchanger, the composition of milk and the type and characteristics of heat exchangers. Milk composition varies with the age, breed, feeding conditions, season, udder health status and the stage of lactation of the cow[30,31]. Seasonal changes in the total milk protein concentration have been shown to affect fouling directly. However, these factors cannot be readily controlled in an attempt to reduce fouling. Considerable attention has therefore been concentrated on the operating conditions as well as the characteristics of the heat exchanger. Such attempts have, however, been met with limited success.

2.2.3.1 Operational measures for minimization of fouling

During thermal processing various operational parameters, such as the air content, velocity/turbulence, and temperature, contribute to fouling of the heat exchanger[5]. The solubility of air in milk decreases with an increase in temperature. Dissolved air is consequently released during thermal processing resulting in the formation of air bubbles. Deposit formation is mitigated when the air bubbles form next to heating surfaces. These bubbles subsequently act as nuclei for deposit formation due to local overheating of the heating surfaces[4–6]. Bubble formation may be suppressed by including a degassing step prior to thermal processing, or by increasing the operational back pressure. An increase in back pressure will act to keep the dissolved air in solution. An increase in flow velocity and turbulence has also been shown to reduce fouling. Higher flow velocities act to increase fluid shear stresses promoting deposit re-entrainment[5]. Inclusion of a preheating step may also reduce fouling by promoting the denaturation of β-lactoglobulin, and its association with casein (κ-casein) micelles[3,7]. The casein micelles not only associate with denatured protein, but also with calcium phosphate reducing the availability of the main constituent of type B fouling[3].

The surface characteristics of indirect heat exchangers also affect the rate of deposit formation. Adsorption of proteins to the heat exchanging surfaces is the rate limiting step in fouling. Surface modifications such as electro-polishing and surface coatings can therefore reduce surface roughness and wettability. Such modifications may reduce the adhesion strength of deposits to the heating surfaces. However, surface treatment is only beneficial until the heating surface has been covered with a layer of deposits. Whey protein adsorption leads to a demetallizing effect of the heat exchanger surface[4]. The benefit of surface treatment may therefore not lie with the reduction of fouling during thermal processing, but with simplifying the removal of deposits after processing.

2.2.3.2 Alternatives to conventional steam heating

Although commercial direct heating plants generally use superheated steam as the heating medium, other heating methods such as microwave-, ohmic- and induction- heating are available that do not require any heating medium. These methods have been gaining popularity due to a reduction in final product dilution as well as a reduction in fouling[3,5]. However, even with these methods, fouling cannot be eliminated completely.

Microwave heating methods have been employed in various industrial applications due mainly to its heating efficiency, energy saving and compactness. Unfortunately microwave systems have a limited lifespan. This method is therefore not economically viable for all applications[5]. In ohmic heating an electric current is passed through the product causing an uniform increase in temperature due to resistance against the current by the fluid[32]. This method increases efficiency by eliminating inefficient mechanisms such as heat transmission from a heating medium to the product. Ohmic heating have various advantages among which a lack of moving parts, uniform heating and instantaneous start/stop cycles are the most important. Nevertheless, the electrode surfaces are susceptible to deposition and erosion[5,32]. The use of high voltages also necessitates additional safety requirements. In induction heating product temperature is increased by oscillation of magnetic fields inside electric coils. This oscillation causes an electric current which heats the product[5]. Induction heating systems are energy efficient since no heat is wasted during heat transmission. The main disadvantage of induction heating systems is the dependence on magnetic boilers. Stainless steel, predominantly used in the dairy industry for hygienic purposes, is not magnetic and can therefore not be used for induction heating.

Fouling in the abovementioned heating systems are furthermore more problematic than with conventional heating. A deposition layer causes a difference in product heating properties,

resulting in non-uniform heating. Localized heating in the deposit layer promotes further fouling by creating a temperature difference on the fluid/deposit interface[5]. The major disadvantage of the above mentioned heating procedures is that existing UHT plants cannot be readily adapted to employ these heating methods. Altering heating systems would require re-fitting of the entire plant, resulting in extended periods of downtime. The use of these heating systems is therefore limited in established dairy industries.

2.2.4 The use of additives

A significant amount of research has been focused on the minimization of fouling by the optimization of operational parameters, such as inclusion of a preheating step, and the type and characteristics of the heat exchangers. Unfortunately these approaches have been met with limited success, mainly due to the complex nature of milk. As discussed previously, the composition of milk cannot be readily controlled. However, milk composition can be modified by the addition of various additives such as emulsifiers and enzymes. The use of such additives, however, necessitates adherence to national, and international, regulations with regards to labeling.

2.2.4.1 Emulsifiers

Emulsifiers are widely used in the dairy industry in order to control the stability and molecular structure of various products. Such additives are surface active amphiphiles, which act as biosurfactants. In colloidal solutions, such as milk, surfactants are able to associate with both the aqueous and oil phase at the interface where it acts to lower the surface or interfacial tension[7]. It has been shown that the natural emulsifier lecithin can be used to increase the heat stability of homogenized and concentrated milks[7]. The exact mechanism whereby lecithin increases heat stability is not yet fully understood. However, it has been proposed that, as with inclusion of a preheating step prior to UHT processing, lecithin promotes complex formation between κ-casein and β-lactoglobulin[7,33,34]. Various authors also believe that lecithin-protein interactions may play a role in the heat stability of some dairy products[7–9].

The use of lecithin may therefore reduce fouling by promoting protein stability during thermal processing of milk. However, the Foodstuffs, Cosmetics and Disinfectants Act (No. 54 of 1972), prohibits the use of any additive, including lecithin, in milk without proper labeling[35]. Milk products containing additives should be labeled accordingly, and is considered a blended-milk product as opposed to drinking milk. Furthermore, food grade lecithin is mainly a soy product

which is considered an allergen. The use of lecithin to reduce fouling is therefore not allowed during the production of products classified as drinking milk.

2.2.4.2 Enzymes

Since the ancient times, enzymes have played an indispensible role in food production. In the dairy industry, enzymes are used for many applications such as the production of cheese (rennet), to increase customer satisfaction (lactase enzymes) and to improve overall product texture and flavor (lipases). Since enzymes catalyze specific reactions, they can be applied to yield specified products. The most common enzymes currently used in the dairy industry include rennet, proteases, lactases, catalases and lipases[36].

Recently, a class of phospholipases, phospholipase A_1 (EC 3.1.1.32), have been applied in the dairy industry to increase cheese yield[37]. Phospholipase A_1 catalyzes the 1-position specific hydrolysis of phospholipids yielding LPL and free fatty acids (FFAs) as products[11,38–40]. LPL are a class of amphoteric surface-active surfactants which are less hydrophobic than their phospholipid counterparts[11]. An increase in water-solubility results in increased dynamic surface activity due to a higher concentration in the aqueous phase[10,11,15]. Emulsions stabilized by LPLs have been shown to have improved heat stability and a lower sensitivity to flocculation by Ca^{2+} and Mg^{2+} [10–12] . Furthermore, emulsions stabilized by LPL are less susceptible to fluctuations in pH[41,42]. Such emulsions therefore have improved stability over a wider pH and temperature range.

The increase in heat stability of emulsions, stabilized by LPL, has been attributed to the formation of surface-active LPL-protein complexes at droplet interfaces[12–15]. These complexes subsequently prevent the formation of protein-protein interactions in the interfacial layers. As discussed earlier, it has generally been accepted that milk fouling is at least a two-step process starting with protein deposition on the heat exchanger. Inhibition of protein-protein interactions by LPL may therefore lead to a reduction in fouling. Addition of phospholipases to raw milk prior to thermal processing may lead to a reduction in fouling by production of LPLs which can act as protein stabilizers.

2.3 GLYCEROPHOSPHOLIPID CHOLESTEROL ACYLTRANSFERASE

2.3.1 The use of glycerophospholipid cholesterol acyltransferase in the dairy industry

Since protein fouling is the major step during heat induced fouling, it is conceivable that a reduction in protein fouling would result in an overall reduction of the fouling rate. Members of

the genus Aeromonadaceae (formerly Vibrionaceae) produce a surface active enzyme, glycerophospholipid cholesterol acyltransferase (GCAT), which shares similarity with members of the lipase family of enzymes. As described earlier, heat denaturation and protein-protein complex formation of β-lactoglobulin have been considered to be the rate limiting step in fouling during thermal processing of dairy products [3,21]. Treatment of raw milk with GCAT, prior to UHT treatment, results in the conversion of native milk phospholipids to yield surface active LPLs and, either free fatty acids or cholesteryl esters depending on the absence or presence of cholesterol. The formed LPLs subsequently associate with β-lactoglobulin and caseins, increasing their heat stability. This increase in stability results in a reduction of free activated β-lactoglobulin. The stabilization of activated β-lactoglobulin effectively removes the main fouling substrate, reducing the rate of fouling.

2.3.2 Origin of GCAT

Throughout history the genus *Aeromonas* have gained much notoriety as pathogen of both cold- and warm- blooded organisms. Motile *Aeromonas* species are responsible for large losses in fish raised in ponds and reticulating systems. Members of this genus have furthermore been indicated as a common contaminant of a variety of raw foods[43]. The genus *Aeromonas* was first proposed in 1936 Kluyver and Van Niel[44] to accommodate a family of enteric bacterium-like microorganisms that are ubiquitous in aquatic environments and have polar flagella in the motile form[45,46]. The current description includes the characteristics of being gram negative, non-spore-forming, facultative anaerobic rods which are catalase and oxidase positive and metabolize carbohydrates fermentatively [45,47–50]. *Aeromonas* spp. can be subdivided into mesophilic motile and psychrophilic non-motile species. Organisms of the former group include *A. hydrophila, A. caviae, A. sorbia, A. veronii and A. schubertii* which have a single polar flagellum and are commonly found in warm water environments (between 5-41 °C). The latter group is clustered around *A. salmonicida* which is more common in colder environments with temperatures ranging from 5-25 °C[43,45,47,51].

Aeromonads are among the most common waterborne bacteria in the world, frequently causing disease among aquatic organisms including fish, frogs, turtles and alligators and have recently been considered as emerging human pathogens[47,52]. Mesophilic species of *Aeromonas* have been associated with a wide range of human infections of which gastroenteritis is the most common[53]. Members of the psychrophilic group are not human pathogens since these organisms are not able to survive at temperatures in excess of 25°C. They, however, are responsible for considerable losses in feral and cultured fish[47]. It is important to note that

although *Aeromonads* are major fish pathogens, they form part of the normal intestinal microflora of healthy fish[47]. The mere presence of *Aeromonads* is therefore not an indication of disease. The pathogenicity of these organisms are related to physiological and environmental stresses which are most commonly associated with fish under intensive culture[47]. For this reason, high mortality rates are often observed in cultured fish.

Historically *Aeromonas* species have been designated to the eubacterial family *Vibrionaceae* based primarily on phenotypic expression[45,46,50,52,54]. As a result, since creation of the genus *Aeromonas*, classification of organisms to this family has been in a state of flux, often leading to confusion and controversy. Definition of the exact taxonomic position of *Aeromonas* species has previously been difficult. These organisms share several phenotypic properties defined to be characteristic of each of the two eubacterial families, the Vibrionaceae and Enterobacteriaceae[46]. However, Colwell *et al.*[46] provided substantial molecular genetic evidence indicating that *Aeromonas* have a sufficiently distinct phylogenetic history from the aforementioned eubacterial families to warrant exclusion from the family Vibrionaceae. These authors subsequently proposed a distinct new family of eubacteria, the Aeromonadaceae, to accommodate *Aeromonads*.

2.3.3 Extracellular products of Aeromonads

Aeromonas species produce various extracellular products (ECP) that contribute to the pathogenicity and virulence of these organisms[45,51,55]. The ECPs have been shown to consists of a wide array of enzymes that actively degrade a variety of complex protein, polysaccharide, mucopolysaccharide, and lipid-containing molecules[49,55]. Characterization of the ECPs from *Aeromonas* indicated that the primary toxins produced are proteases, haemolysins, leukocytolysins, cytotoxins, lipases and the weak haemolysin, GCAT[51,56–58]. Virulent strains of *A. salmonicida* produce a further virulence factor, the surface layer (S layer), in addition to the ECPs[59–63]. The S layer, originally known as the additional layer (A layer), is a supplementary layer external to the outer membrane composed of a tetragonally arrayed protein of 50 kDa (A-protein). The S layer is tethered to the cell surface by lipopolysaccharide[55,61,63–65]. It was argued that this layer is a principle virulence factor of *A. salmonicida*, since mutants lacking the S-layer are avirulent[64]. The exact role of the S-layer is not yet fully understood, however, it has been hypothesized that this layer may be involved in protection, molecular sieving, cell adhesion, surface recognition, morphogenesis and autoaggregation[60]. Autoaggregation is considered essential for virulence[59,60,66]. Loss of the S layer results in the loss of the ability to autoaggregate along with a concurrent attenuation in virulence, although at least moderate virulence may be

retained in non-autoaggregating strains[59,63]. The S layer is furthermore thought to physically protect cells against bacteriophages since cells exhibit phage sensitivity in the absence of the S layer[61,66].

A. salmonicida is the causative agent of furunculosis, a septicemic salmonid disease characterized by necrotic lesions and general liquefaction of internal tissues[67]. The ECPs produced by *A. salmonicida* contains a cocktail of potential toxins. Munro and co-workers[68] showed that when a total ECP preparation from *A. salmonicida* was intraperitoneally injected, all the symptoms characteristic of furunculosis could be reproduced. This observation prompted a search for the major ECP toxin(s). Since the necrotic lesions characteristic of furunculosis are proteolytic in nature, it was argued that the major toxin would most likely be a protease. Ellis *et al.*[69] subsequently isolated a single 70 kDa serine protease which was injected intramuscularly into juvenile Atlantic salmon. The result was compared with a total unfractionated ECP preparation with similar proteolytic activity. Interestingly, the injection of purified protease yielded significantly reduced necrosis when compared to the unfractionated ECP preparation. These results indicate that other toxins are important for virulence, or that other ECP enzymes act synergistically with the protease to produce the characteristic symptoms of furunculosis. Various authors subsequently showed that a mixture of purified protease and haemolysin (GCAT), termed T lysin by Titbal and Munn[70], was as efficient in producing muscle lesions in rainbow trout and Atlantic salmon as whole ECP[59,71,72]. Supportive of such a synergistic mechanism, it was observed that purified haemolysin incompletely lyses erythrocytes, only in the absence of protease[59]. Since intramuscular injection of a cocktail of protease and GCAT yielded the characteristic symptoms of furunculosis, it was argued that these enzymes are the major virulence factors produced in the ECPs of *A. salmonicida*.

Bernheimer *et al.*[73] was the first to described the hemolytic effect of ECPs from *Aeromonads* when they showed the presence of phospholipase- A and C activities in the ECPs from *A. hydrophila*. MacIntyre and Buckley[74] subsequently indicated that filtered supernatants of *A. hydrophila* had the ability to produce free fatty acids, cholesterol esters, and deacylated water-soluble products when incubated with erythrocyte membrane glycerophospholipids. This activity was initially attributed to a high molecular weight enzyme complex between GCAT and the phospholipases described by Bernheimer *et al.*[74,75]. However, it was subsequently revealed that all the reactions are catalyzed by the single GCAT enzyme which exhibit phospholipase, acyltransferase and lysophospholipase activity[76]. In the native state GCAT forms a complex with lipopolysaccharide (LPS) and acts as a heamolysin, leukocytolysin and a cytotoxin[56].

The role of the 70 kDa serine protease and GCAT-LPS as major virulence factors was, however, discounted when Vipond and co-workers[67] showed, with the use of defined deletion mutants, that neither the protease nor GCAT is essential for the development of diseases. It was subsequently proposed that the activity of the serine protease may play a role in activation of protoxins, in particular GCAT (will be discussed later), in addition to tissue destruction at the site of infection[67]. GCAT, on the other hand, is able to transfer an acyl chain to various straight chain alcohols[77]. Since it was consistently observed that acyl transfer is maximal when cholesterol is the acceptor, it implies that the *in vivo* function of GCAT is not in cellular metabolism, as *Aeromonas* do not contain cholesterol[77,78]. It is therefore more likely that GCAT serves an accessory role during infection rather than cellular metabolism by enhancing nutrient acquisition and cell proliferation by providing essential amino acids needed for growth [67,79]. Consequently, it was concluded that although these enzymes are not solely responsible, in combination with other ECP enzymes, they aid in the progression of furunculosis.

Although the role of GCAT as a major virulence factor during infection was discounted, the multiple reactions catalyzed by this enzyme attracted considerable attention for its biotechnological application[65]. The ensuing review will describe the molecular characteristics of GCAT.

2.3.4 Glycerophospholipid cholesterol acyltransferase

Early speculation that GCAT may contribute to the pathogenicity of *A. salmonicida* led Macintyre and co-workers to conduct a study to determine the extent of this enzyme's distribution in, the then family, Vibrionaceae[57]. Through these studies it was shown that all organisms of this family, with the exception of *P.shigelloides*, are able to produce cholesterol esters and LPL when egg yolk emulsions or human erythrocyte membranes are used as substrates. Interestingly, of all organisms examined, *Staphylococcus aureus* was the only organism of another family which showed GCAT activity on the same substrates. Prior to these studies the activity of GCAT was proposed to be that of an enzyme complex between an acyltransferase and a lipase. The report by Macintyre *et al.*[57] was the first instance where these activities were attributed to a single enzyme catalyzing multiple reactions.

The GCAT enzyme (EC 2.3.1.43), produced by all members of the Aeromonadaceae, is a ≈ 25 kDa enzyme that forms a complex with LPS, the complex having an estimated molecular mass of 2000 kDa [48,56]. Formation of this complex has a stabilizing effect on the GCAT enzyme, and protects the enzyme from inactivation by heat and proteolytic degradation[48,49,56].

Additionally, complex formation enhances the hemolytic activity and lethal toxicity eightfold[56]. The lipase activity of GCAT is, however, unaffected by complex formation with LPS[56]. Lee[56] proposed that complex formation with LPS may enhance the hemolytic activity of GCAT by aiding in membrane penetration, delivering the enzyme to the site where optimal substrates are present. Once inside the fish host, GCAT completely lyses its erythrocyte membranes[56,80,81]. Conversely, the enzyme is not able to lyse human erythrocytes. The phospholipid makeup of erythrocyte membranes therefore play a significant role during hemolysis [48,77]. The substrate specificity of GCAT will be discussed in more detail in the ensuing sections.

GCAT shares several features with the mammalian plasma enzyme lecithin:cholesterol acyltransferase (LCAT)[82,83]. As with LCAT, GCAT catalyzes the position 2 specific transfer of an acyl chain from a phospholipid donor to a suitable acceptor[77,84]. Like the mammalian enzyme, GCAT has no divalent cation requirement and its activity is stimulated by apolipoprotein A-1[76,77]. However, GCAT has far less stringent phospholipase substrate requirements than LCAT as all commonly occurring glycerophospholipids can function as substrates[77,78]. Based on the similarities with the mammalian plasma protein, and because it is far more stable, GCAT have received considerable attention, not only as a research tool, but also for its potential biotechnological applications[65].

2.3.4.1 Purification of GCAT

In order to study the function and mode of action of enzymes, large quantities of pure protein is needed. The purification of native GCAT from *A. salmonicida* is cumbersome, involving a complicated multistep purification procedure which ultimately yields small amounts of pure enzyme[65,76]. A recombinant method was therefore pursued. In 1990 Hilton and coworkers[65] described a method wherein large quantities of recombinant GCAT, from *A. hydrophila*, can be expressed in a mutant *A. salmonicida* host[65]. Selection of this mutant was based on the production of greatly reduced amounts of its own extracellular proteins in addition to reduced amounts of chromosomally encoded GCAT. Extracellular expression of recombinant GCAT can be induced with isopropyl β-D-thiogalactoside (IPTG), enabling enzyme purification from cell free culture supernatant by ammonium sulfate precipitation, centrifugation and successive gel filtration and ion exchange chromatography steps[65]. Ammonium sulfate precipitation recovers the enzyme bound to the outer membrane fragments from cell free culture supernatants. These fractions contain a small number of proteins resulting in a dramatic increase in specific enzyme activity[76]. GCAT can subsequently be selectively recovered by detergent solubilization and

centrifugation. This method typically yields 35-45 mg of pure GCAT from 2 L culture supernatant as compared to 1-2 mg pure protein from 3.6 L culture supernatant for native GCAT[65,76].

The use of GCAT in food products has recently received considerable attention due to the stabilizing effect that LPL can deliver to foodstuffs. Although *A. salmonicida* is not a human pathogen, this organism cannot be used as an expression host due to the risk of contaminating food products. In lieu of this, a modified form of GCAT from *A. salmonicida* has been successfully expressed in *Bacillus licheniformis*[85]. The gene encoding GCAT has been modified at a single amino acid, asparagine 80, for improved expression by this host. *B. licheniformis* is considered a class 1 contaminant agent under the NIH guidelines for research involving recombinant DNA technology and the use of such expression products are deemed safe for use in food products[86]. This host is furthermore generally regarded as safe (GRAS) and meets the criteria for a safe production organism[85]. Additionally, the host *B.licheniformis* (BRA7) has been modified, by GENENCOR®, to eliminate the production of several enzyme activities, protease and amylase, which could impede expression and/or purification[85]. Recombinant GCAT could thus be prepared in large quantities by submerged fed-batch culture fermentation followed by the same purification procedures as described above.

Interestingly, when GCAT is expressed in *E. coli,* no enzyme could be detected in the culture supernatant indicating that the enzyme is not secreted by this host. Similar observations were made with a variety of extracellular proteins, leading to the conclusion that *E. coli* does not poses the mechanistic ability for export of these proteins over the cell outer membrane[87].

2.3.5 GCAT genetics

2.3.5.1 Primary structure

Although *A. salmonicida* is a well-known pathogen of various aquatic organisms, most interest has been concentrated on the virulence factors of *A. hydrophila*. This is mainly because this organism is increasingly implicated in clinical human infections[45]. Most of the original enzymatic characterization was therefore focused on GCAT produced by *A. hydrophila*. There are, however, differences between the enzymes produced by these organisms although they catalyze similar reactions. The enzyme produced by *A. salmonicida* is smaller than that of *A. hydrophila*. However, polyclonal antibodies prepared against *A. salmonicida* GCAT do not cross-react with the enzyme produced by *A. hydrophila*, indicating significant difference between these two enzymes[87]. This observation, however, is difficult to explain since these

enzymes share 100% homology in antigenic regions of the polypeptide[88]. Apart from this, the enzymes share 93.7% homology in amino acid sequence[88]. From Figure 2.2 it can be seen that nine out of 21 non-identical residues are located within a region coding for 29 amino acids (residues 239-268 highlighted in yellow)[88]. Excluding this region, homology is 96.1%. The enzymes produced by these two organisms therefore compare well on a molecular level. The proposed active site residues remain conserved.

The primary structure of GCAT from *A. hydrophila* was first published in 1988 by Thornton *et al.*[87]. The open reading frame encodes a 281 amino acid protein, preceded by an 18 amino acid signal peptide, with a predicted molecular weight of 31 303 Da[87]. This , however, did not correlate with results obtained from SDS-PAGE analysis which indicated a molecular weight of approximately 35 kDa[65,87]. Subsequent studies revealed that an error was made in the first publication by Thornton *et al.*[65]. The error was found to be due to an error in reading frame translation. The corrected sequence, presented in Figure 2.2, encodes a protein of 35.1 kDa which correlates with the results from SDS-PAGE analysis of purified unprocessed GCAT[65].

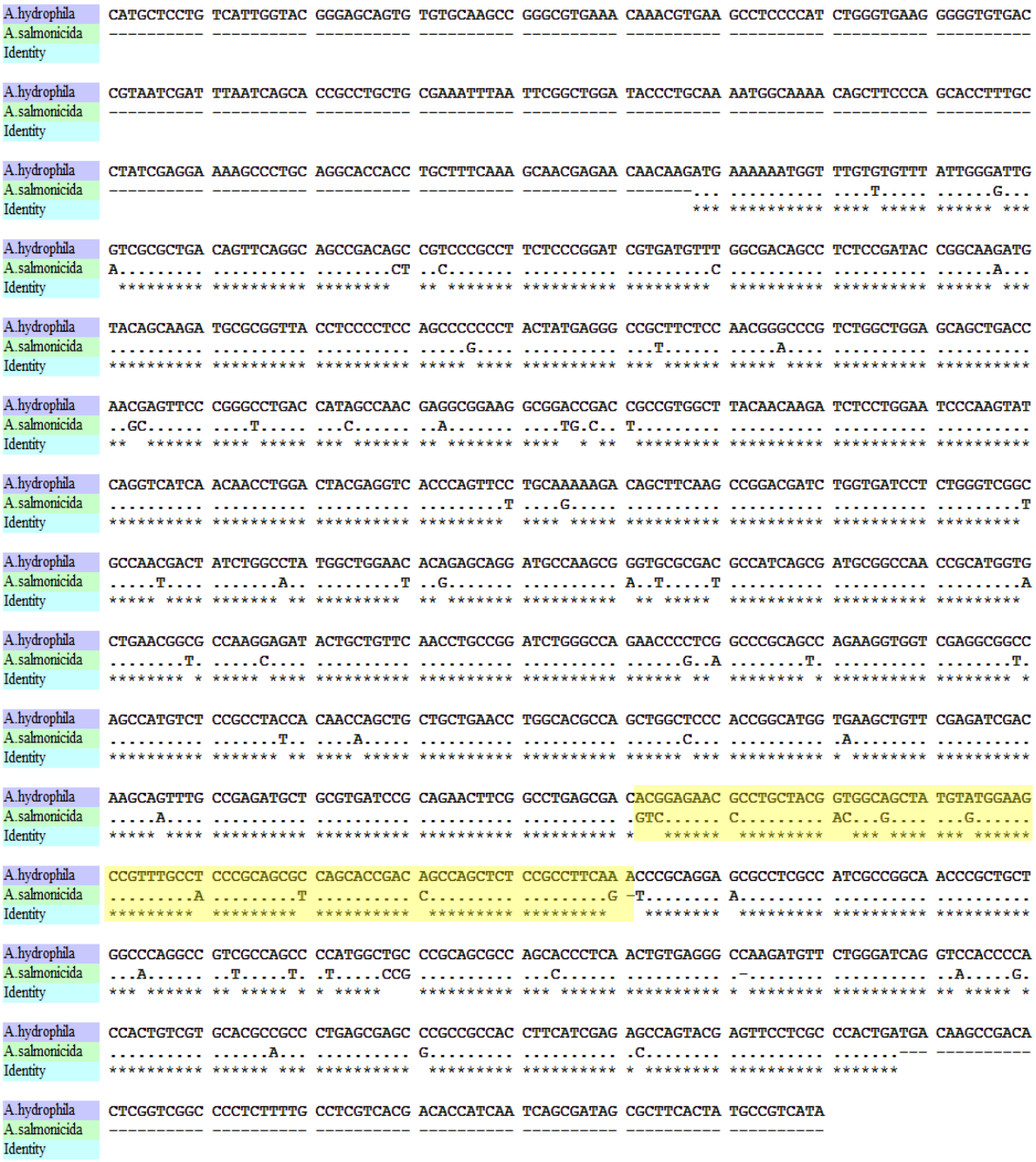


Figure 2.2 Comparison between the primary amino acid sequences of GCAT from *A. salmonicida* and *A. hydrophila*

During purification, the enzyme is reduced in size from 35 to 27 kDa. It was consequently suggested that, as with the hole forming protein aerolysin, the enzyme is secreted as a protoxin which may undergo post-translational modifications[89]. In support of this, trypsin treatment of the 35 kDa protein results in the formation of a protein with a molecular weight of 27 kDa as determined by SDS-PAGE. Once formed, the 27 kDa protein is resistant to further degradation, except when proteinase K is used. Two separate trypsin digestion sites were subsequently

identified (Figure 2.3). Trypsin treatment nicks the enzyme between two cysteine residues (cys^{225} and cys^{281}) resulting in the loss of a 3.7 kDa peptide located towards the C-terminal of the protein [83]. This yields two protein fragments, a 27 kDa fragment connected to a 4.7 kDa peptide via a disulfide bond.

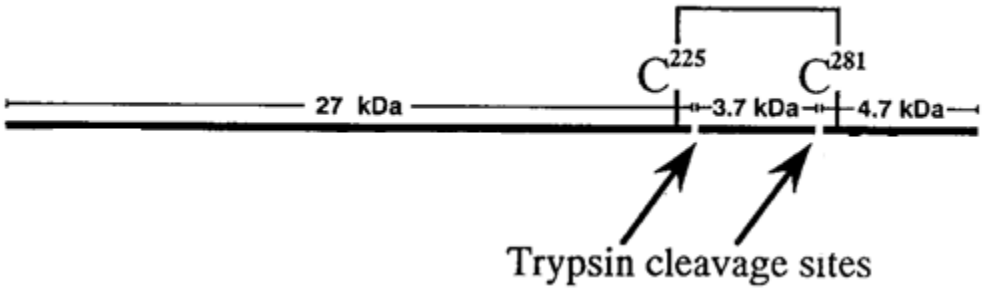


Figure 2.3. Trypsin cleavage sites in GCAT resulting in the loss of the the peptide encoded by amino acids 230 – 274. The positions of the cysteien residues that form a disulfide bond is indicated above the figure. Figure Adapted from source [83].

Hilton *et al.*[65] showed that, when trypsinized GCAT is treated with SDS-PAGE sample buffer containing mercaptoethanol, the protein migrates as a band of 27 kDa. However, after staining with Coomassie Blue, smaller peptides were observed at the front of the gel. Omitting mercaptoethanol from the sample buffer results in the migration of a single band corresponding to the combined sizes of the 27 kDa protein and the 4.7 kDa peptide, predicted to be produced from the second trypsin digestion site[65]. It was therefore concluded that since mercaptoethanol treatment yields two protein fragments, the two cysteines in GCAT are most likely joined by a disulfide bond, tethering the 4.7 kDa peptide to the rest of the protein[65].

Formation of the 27 kDa protein, tethered to the 4.7 kDa peptide via a disulfide bond, coincides with an increase in enzyme activity, suggesting that, under normal circumstances, GCAT is secreted from the host as an inactive pro-enzyme which is post transnationally activated by limited proteolysis[65]. Post-translational activation is therefore most likely a protection mechanism wherein the bacteria's own membranes are protected from self-inflicted damage during secretion[65,83]. Supportive of this, unprocessed GCAT is not able to penetrate lipid monolayers at surface pressures exceeding 20 mN/m whereas after trypsination it is able to degrade monolayers at pressures exceeding 40 mN/m[65]. Studies have shown that the surface pressures of natural membranes are approximately 30 mN/m [90]. GCAT is thus not able to penetrate membranes in the inactive, unprocessed form.

Trypsin treatment permits removal of the 3.7 kDa peptide enabling disulfide bridge formation between Cys^{225} and Cys^{281}. Earlier reports suggested that a disulfide bridge may be essential for enzyme activity and secretion of lipases[91,92]. It was furthermore reported that cysteine residues may play a role during enzyme catalysis in the human plasma protein LCAT[92]. Based on these reports, the role of cysteine residues during GCAT activity and secretion was investigated. GCAT contains a single pair of cysteine residues that form a disulfide bond. Site-directed mutagenesis of these residues indicated that the major role of the disulfide bond is to stabilize the three dimensional structure of the protein[83]. In the absence of the disulfide bond the protein is more susceptible to urea denaturation and degradation by proteases, along with a significant reduction in enzymatic activity. The reason for the reduction in enzymatic activity will become evident in the following section. The absence of the disulfide bond may permit the 4.7 kDa peptide to move away from the 27 kDa fragment, thereby exposing previously hidden sites to proteases[83].

2.3.5.1 Homology of GCAT with other enzymes

Initially the primary structure of GCAT was elucidated to determine the sequence similarities with the mammalian plasma enzyme, LCAT. Surprisingly, although these two enzymes catalyze similar reactions they differ considerably in primary structure. Sequence similarities are, however, reported among members of the lipase superfamily[87,93,94]. GCAT contains a sequence which is highly conserved in lipase enzymes from various host organisms. Based on data obtained from porcine pancreatic lipase, this region is believed to form the interfacial lipid-binding site of lipases[87]. Additionally, GCAT contains a second sequence which shares homology with the suggested active site[87]. Interestingly, Thornton *et al.*[87] showed that although the apparent sequence homology between the proposed active site of GCAT and that of pancreatic lipase is not convincing (Figure 2.4), this region is more homologous than that of the same sequence of LCAT. The same authors furthermore showed that both GCAT and pancreatic lipase are insensitive to levels of diisopropylphosphofluoridate that would render LCAT completely inactive. These results indicate alternative roles for the active site serine of these two enzymes.

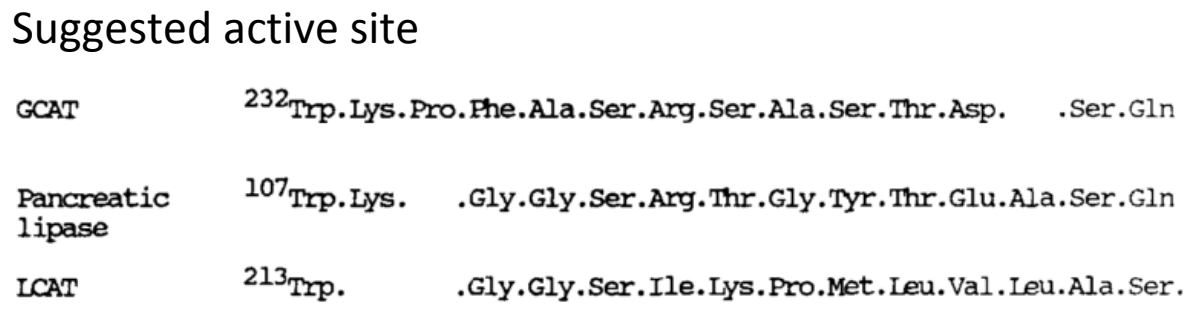


Figure 2.4. Amino acid homology between GCAT with the proposed active site of porcine lipase and a similar region in LCAT[87].

2.3.5.2 The active site residues

Evolution of the primary structure of proteins is constrained by its function. Based on the coevolution of protein primary structure and function, homologous proteins from diverse sources can often be grouped in families founded on the conservation of vital amino acid sequences[95]. The apparent similarity between the active site of GCAT and pancreatic lipase suggests that GCAT may be part of the lipase family of enzymes. In support of this, GCAT acts as a phospholipase when no acyl acceptor is available. The active site of lipases typically contains a conserved catalytic triad consisting of serine, aspartic acid and histidine[96]. In order to confirm if GCAT is indeed a member of the lipase family, the active site residues were determined.

The active site serine of lipases is contained in a small consensus region with the conserved sequence G-X-S-X-G[40]. This sequence is also conserved in members of other esterase families, including the serine proteases[40,97,98]. Data obtained from a range of chemical inactivation experiments proposed that the active site of GCAT, like serine proteases, consists of a Ser-His-Asp catalytic triad[40,99,100]. Hilton and Buckley[40] showed that Ser[16] is essential for the activity of GCAT. Chemical modification of this residue results in total loss of enzyme activity. In lipases with known three dimensional structures the G-X-S-X-G sequence, which contains the active site serine, forms a critically important loop between an α-helix and a β-sheet[96]. In GCAT however, Ser[16] is located in the sequence G-X-S-X-**S**[40,96]. The active site consensus is furthermore located in close proximity to the amino terminus which is in contrast to lipase enzymes wherein the active site is located closer to the middle of the protein[96,101]. Thus, although GCAT shares several features with lipases, this enzyme is different from lipases in the location of the active site serine.

All lipases with known primary structures have at least two hydrophobic amino acids preceding the G-X-S active site consensus sequence[102]. To determine the role of these residues in catalysis, they were mutated by site-directed mutagenesis[102]. During these studies Phe[13] was changed to a Ser in addition to mutation of the second serine following the active site serine to glycine. For comparative purposes this serine was also mutated to valine. These mutations not only decreased enzyme activity, but also resulted in a decreased secretion from the host organism. This is an indication that these residues may play a pivotal role in the correct folding and/or secretion of GCAT. Surprisingly, although mutation of the serine following the active site serine to a glycine brings the sequence into line with the lipase consensus, mutation resulted in an inactive enzyme. These results indicated the presence of at least two groups of lipolytic serine esterases. The latter group being one in which the active site serine is contained within the active site consensus G-X-S-X-S.

The observation that an inactive enzyme is obtained, when the second serine in the active site consensus of GCAT is mutated, led Upton and Buckley[101] to determine the extent of distribution of the G-X-S-X-S consensus in other enzymes[102]. Based on a BLAST search focused on short, highly conserved protein segments, it was found that seven other proteins have identical or very similar sequences. The consensus sequence for this region is FGDSLS preceded by three hydrophobic amino acids[101]. Evidence obtained from thioesterase I, produced by *E.coli,* and arylesterase, from *Vibrio mimicus,* suggests that the first serine in the consensus region is most likely the active site nucleophile as is the case in GCAT [101,103]. It was furthermore shown that all eight enzymes have at least four other “sequence blocks” in common which appear in the same order in each case. These observations provided evidence that the abovementioned enzymes represent a new family, or subfamily, of lipolytic enzymes. Discovery of the sequence blocks provided clues to the locations of the other active site residues which, at the time of publication, were not known for any of the enzymes.

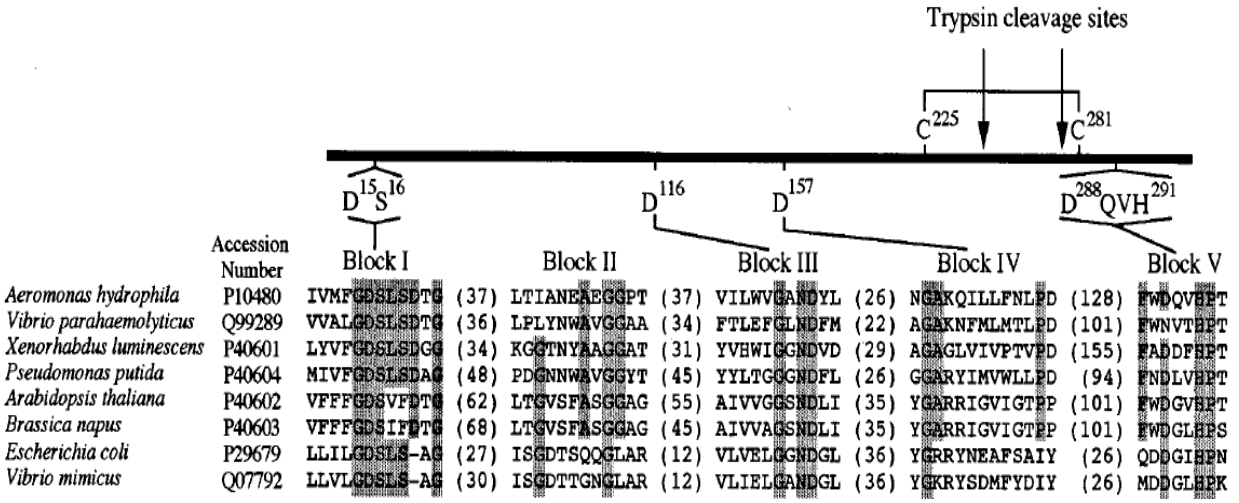


Figure 2.5. Protein sequence homology blocks as proposed by Upton and Buckley[101]. Each block compares potentially important amino acids in GCAT with other proteins possessing a G-X-S-X-S active site motif. The numbers in brackets are an indication of the number of amino acid residues between conserved blocks.

In 1996 Brumlik and Buckley[96] attempted to identify the active site histidine and aspartic acid residues by using the sequence homology blocks described above. The authors proposed that, for enzymes with similar catalytic activities, the active-site residues should be contained within the same sequence blocks. This assumption proved true as it was shown that the active-site serine, Ser[16], is located in block I in the conserved consensus, FGDSLS, described earlier.

GCAT contains 5 histidine residues of which only two are located in the 27 kDa protein fragment. Since it has previously been shown that the 27 kDa fragment retains activity after removal of the 4.7 kDa fragment, Hilton and Buckley[40] argued that only His[175] and His[180] can play a role in catalysis. Subsequent site-directed mutagenesis of His[175] and His[180] to asparagine yielded an active enzyme. In agreement with this, it was previously indicated that histidine is not required for the catalytic activity of pancreatic lipase, nor hepatic lipase [30,32]. These findings led to the assumption that histidine does not play a role during catalysis, distinguishing GCAT from other lipases[40]. However, this assumption was disproved upon further inspection of the data shown in Figure 2.5. In all the proteins sequences shown in Figure 2.5, block v is the only block that contains histidine, implicating this histidine, His[291] in GCAT, the most likely candidate for a role during catalysis as the remaining histidines are not found in conserved regions[96]. Brumlik and Buckley[96] showed that although the enzyme is secreted at normal levels, a completely inactive enzyme is obtained when His[291], located in the 4.7 kDa peptide, is replaced with asparagine. This finding is in contrast to a previous finding that enzyme activity is retained after removal of the 4.7 kDa fragment[65]. These authors subsequently suggested that refolding of the 27 kDa peptide most probably gave rise to a structure that had residual lipase activity [40].

Identification of the active site aspartic acid proved challenging since mutation of various aspartic acid residues not only resulted in reduced activity, but also reduced secretion to such an extent that sufficient protein could not be obtained for activity studies. Identification of the active site aspartic acid was therefore not possible without the sequence information gained from Figure 2.5. In Figure 2.5 the only blocks which contain aspartate residues in all the proteins are blocks I and III. The aspartate residues, Asp^{15} and Asp^{116} in GCAT, was thus the most likely to play a role in catalysis[96]. The role of each aspartate residue was subsequently determined by individual mutation of each residue. GCAT contains additional aspartate residues in blocks IV and V. Asp^{157} and Asp^{228} were thus also mutated to asparagines[96]. It was subsequently determined, by a process of elimination, that Asp^{116} is the most likely candidate for a role in catalysis since Asp^{116} is, not only the only aspartate residue conserved in the same block as all the other proteins, but this residue is also correctly positioned between the catalytic serine and histidine[96]. Mutation of this residue ultimately resulted in total enzyme inactivation.

Since the active site catalytic triad has been established, the role of other amino acid residues in catalysis was investigated. It was briefly discussed previously that the hydrophobic amino acid residues preceding the active site serine may be essential during the protein folding or secretion. Robertson *et al.*[102] attempted to determine if tyrosine plays a role in protein folding and/or secretion from the host cell. For this purpose, the authors nitrated the possible reactive tyrosine residues with tetranitromethane. Nitration of tyrosine resulted in an 80% loss in activity. The reactive tyrosyl residues were identified after treatment with cyanogen bromide and subsequent high performance liquid chromatographic (HPLC) analysis of the resulting peptides. Three fractions were found to contain reactive nitrated tyrosyl residues, although only two of the three nitrated residues could be detected by HPLC. The remaining residue was identified by sequencing the amino terminus containing the remaining possible reactive residue. In this fragment Tyr^{30} was shown to be nitrated. Based on the results obtained, the above residues were replaced with phenylalanine residues by site-directed mutagenesis. It was subsequently shown that Tyr^{230} is responsible for the loss in activity, since mutations of the other residues did not affect enzyme activity considerably[102].

The reduction in activity was specific for substrates consisting of phospholipids since the reaction rate was not as severely affected when p-nitrophenyl butyrate was used as substrate. From this data it can be deduced that GCAT interacts with these substrates in different manners. Furthermore, tetranitromethane treatment of the mutant, wherein Tyr^{230} was mutated, did not result in additional reduction in activity. The results thus indicated that, although mutation

of Tyr[230] affects the activity of GCAT when phospholipids are used as substrates, this residue is not essential to the activity of GCAT since the reaction rate is unimpaired when p-nitrophenyl butyrate is used as substrate. The authors[105] concluded that Tyr[230] most probably interacts with the carbonyl at the 2-position of the phospholipid and is thus essential during phospholipid binding in the substrate binding sites.

2.3.6 Catalytic activity and substrate specificity

As previously discussed, GCAT shares several features with lipases. The GCAT enzyme produced by *Aeromonads*, like lipases, is activated at lipid-water interfaces, have relatively non-specific esterase activity and does not require calcium as co-factor [65,102]. Although this enzyme will catalyze the hydrolysis of neutral- and phospholipids, it is distinguished from other lipases by the unusual ability to specifically carry out acyl transfer from the 2-position of phospholipids in the presence of a suitable acceptor such as cholesterol [83,96]. When no acceptor is available, the enzyme exhibits phospholipase A_2 (EC 3.1.1.4) activity[76]. GCAT will furthermore catalyze the hydrolysis of LPL and cholesteryl esters (EC 3.1.1.5) when no phospholipids are available. However, transacylation of 1-acyl-lysophosphatidylcholine is not catalyzed, leading to the conclusion that a specific interaction between the acyl donor and acceptor is needed for acyltransferase activity [78,106]. The main reactions catalyzed by GCAT are depicted in Figure 2.6.

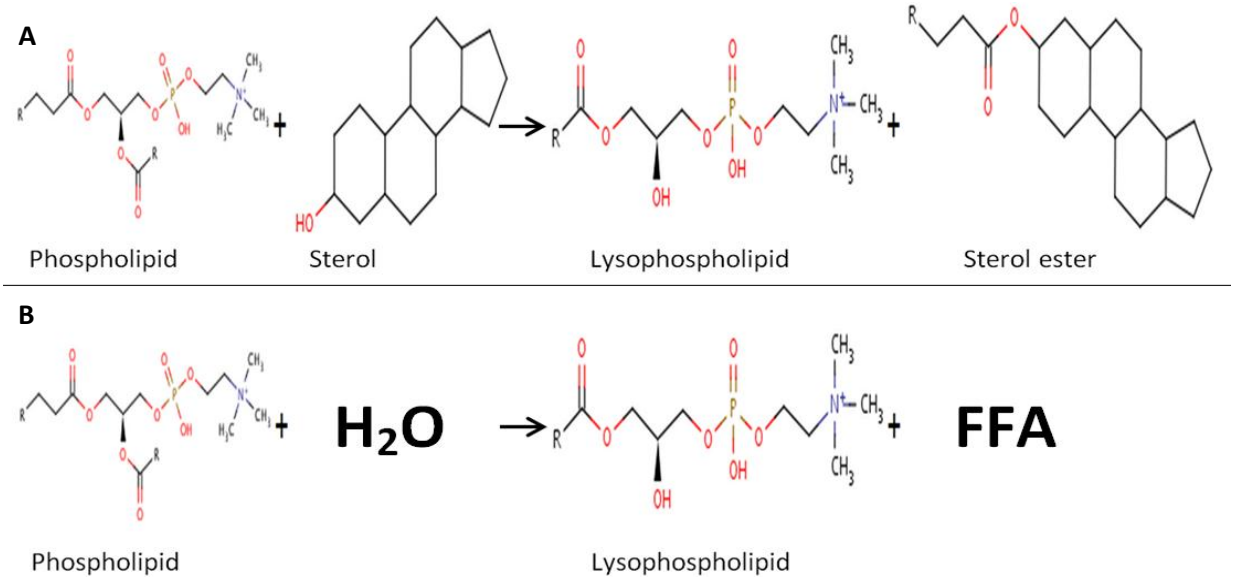


Figure 2.6. Reactions catalyzed by GCAT. A; Esterification of cholesterol with a fatty acyl chain from a phospholipid donor. B; Phospholipid hydrolysis by the lipase activity of GCAT in the absence of a suitable acceptor[107].

Buckley[77] observed that, although GCAT has no absolute requirement for specific phospholipids as acyl donors, the enzyme has a strong preference for phospholipids substituted with short-

chain or unsaturated fatty acids. In experiments with fish and human erythrocytes as substrates, it was shown that the enzyme effectively lyses fish erythrocytes. However, incubation with human erythrocytes resulted in incomplete lysis, although hydrolysis and transacylation of the membrane phospholipids still occurred[56]. This is due to difference in membrane phospholipid makeup of fish and human erythrocytes. It has been established that fish tissues are much richer in polyunsaturated fatty acids and, additionally, the phosphatidylcholine content of erythrocyte membranes are about 30% higher than in human erythrocytes[56]. GCAT is thus well suited to digestion of fish tissues during *A. salmonicida* infection.

In contrast to the nonspecific requirements of acyl chain donors, GCAT displays considerable specificity toward acyl acceptors. Although a variety of alcohols can act as acyl acceptors, preference is shown toward steroids with 3βOH-groups and trans A:B rings, with cholesterol being the most preferred acceptor[40,77,78,106]. Steroids with 3αOH-groups and planar A:B rings do not act as acceptors, but as inhibitors of acyl transfer[77]. It was therefore argued that a hydrogen bond between a donor and acyl acceptor is a minimum requirement for enzyme-catalyzed acyl transfer[77,106]. When the donor is hydrogen-bonded to water, simple hydrolysis of the ester occurs[77,106]. Buckley[77] subsequently suggested that cholesterol is a good acceptor because it is able to interact maximally with the donor through van der Waals interactions, enabling the formation of the hydrogen bond. Molecules containing 3αOH-groups or puckered ring systems inhibit hydrolysis, and acyl transfer, by interacting with the acyl donor in the enzyme pocket. However, because the molecule is not oriented correctly, hydrogen bond formation is not possible[77]. This mechanism of inhibition effectively removes the enzyme substrates, and occupies the enzyme catalytic site.

While attempting to assess whether the glycerophospholipid backbone is an absolute requirement for enzymatic hydrolysis, Buckley[78] found that GCAT catalyzes the hydrolysis of synthetic p-nitrophenyl ester substrates. These substrates were also effective acyl donors for the enzyme-catalyzed esterification of cholesterol[78]. In experiments using p-nitrophenyl butyrate it was found that the apparent V_{max} values are similar to those obtained using phospholipid substrates, although this data was not published[78] . Nitrophenyl esters are valuable tools for kinetic studies of the enzyme, although a comparison between the kinetic parameters, K_m and V_{max}, is essential in order to rationalize the results obtained.

Results from studies using nitrophenyl substrates indicated that GCAT is a nonspecific esterase which preferentially catalyzes acyl transfer in the presence of a suitable acceptor[78]. In contrast

to the lack in specificity displayed by the use of nitrophenyl substrates, acyl transfer to cholesterol is specific to the 2-position when a phospholipid is the acyl donor[76–78]. It was subsequently proposed that the positional specificity is based on the saturated fatty acid preferences of GCAT[78]. Buckley[78] examined this possibility by comparing the enzyme's action on 1-plamitoyl-2-oleoyl-phosphatidylcholine and on 1-oleoyl-2-palmitoyl-phosphatidylcholine. The results of these studies confirmed that acyl transfer is 2-position specific as more than 80% of the fatty acid transferred to cholesterol was oleate, when 1-plamitoyl-2-oleoyl-phosphatidylcholine was used, and more than 80% was palmitate when the latter phospholipid was used [78]. The acyltransferase activity of GCAT is thus not dependent on the fatty acid preference since acyl chain saturation had no effect on the transferase activity. These results confirmed that a specific interaction between acyl donor and acceptor is essential for acyl transfer.

The mammalian plasma protein LCAT catalyzes similar reactions as GCAT. However, these enzymes differ based on the acyl donor preference. Additionally, LCAT has an absolute requirement for the activator, apolipoprotein A-1 (Apo-A1)[76]. LCAT activity can furthermore be activated by the presence of albumin. Although GCAT has no co-factor or activator requirements, it has been reported that enzyme activity is augmented in the presence of both albumin and apolipoprotein A1[76,108]. Albumin most likely activates the enzyme by complex formation with lysolecithin and fatty acids whereas Apo-A-I interacts selectively with the surface of liposome substrates[76]. Although both enzymes catalyze the acylation of cholesterol, when a suitable acyl donor is present, these enzymes differ based on the optimal cholesterol/phospholipid ratios. Cholesterol ester production by LCAT is maximal at cholesterol/phospholipid ratios of 1:3, 1:4, 1:6 or 1:7 with Apo-A1 present, whereas GCAT activity have been shown to be maximal with liposomes consisting of cholesterol/phospholipid in 1:1 ratio[76]. The mammalian enzyme can catalyze the acylation of lysolecithin by a mechanism requiring low density lipoprotein, while the bacterial enzyme catalyzes only the hydrolysis of LPL[76,109]. The lysophospholipase activity of GCAT is only active when no other substrates are available.

2.3.7 3D protein structure

The function of proteins *in vivo* is, to a large degree, determined by its interaction with other molecules in its immediate environment. The degree of interaction is dictated by the 3D structure of the protein. Elucidation of the 3D structure of a specific protein may therefore yield

important information regarding the mechanism of catalysis. The 3D structure of GCAT, however, remains to be determined despite recent advances in both experimental techniques and computational prediction methods.

Computational protein structure prediction is based on the conservation of key sequences among proteins with similar functions. Homologous proteins can be recognized by primary structure comparison with a database of proteins with known structures. However, some proteins require chaperones, some are inherently disordered, and some undergo post translational modification. Computational prediction cannot accurately predict the effect of post-translational modification and the effect of chaperones directly from the primary protein structure. Since GCAT undergoes post-transnational activation, the loss of a 3.7 kDa peptide, and does not share direct homology with other enzyme families, the 3D structure varies greatly when different prediction software is used.

2.3.8 Application of GCAT in the dairy industry

Since the GCAT enzyme has no specific phospholipid substrate requirements, and does not require any co-factors. The versatility and wide range of non-specific substrate requirements makes it an ideal candidate for application in the food industry. For these reasons, GCAT has been employed as a processing aid during ultra-high temperature processing under the commercial name FoodPro® Cleanline (FPCL), developed by DuPont®. Treatment of milk, and other dairy products, with FPCL can increase thermal processing efficiency by reducing UHT plant fouling. Moreover, the GCAT enzyme is completely inactivated by UHT treatment and it has no subsequent technological function in the final product. The use of FPCL in UHT milk therefore meets the requirements for classification as a processing aid since: i. it cannot be consumed as a food by itself ii. it is intentionally added during processing of raw material to fulfill a technological purpose and iii. remains present in the final product without affecting the overall milk content[110]. Milk containing FPCL may therefore be classified as drinking milk without additional labeling requirements.

Other phospholipases may be used to hydrolyze milk phospholipids to LPL, which would act to reduce fouling. However, during this reaction, the free fatty acids liberated from phospholipids accumulate. Phospholipases do not poses transferase activity which sequester free fatty acids as fatty acyl esters such as cholesteryl esters. Free fatty acids are responsible for the rancid, unpleasant taste which is often associated with spoilt milk[111]. Milk with an acid degree value (ADV), an indication of potential milk rancidity, of more than 1 (0.25% $^{m}/_{m}$) is considered rancid

and should be discarded[112,113]. Furthermore, milk contains various inherent and microbial lipases which should be inactivated as soon as possible since these enzymes accelerate milk spoilage. Consequently, addition of foreign lipases would be counterintuitive. For this reason, GCAT is particularly well-suited for use in the dairy industry.

2.4 CONCLUSION

The GCAT enzyme produced by *Aeromonads* is unique in its catalytic activity with plant- and animal lipids. Although the mechanism of catalysis has been elucidated, to the best of our knowledge, no kinetic parameters for any of the reactions catalyzed by GCAT have been published. Surprisingly, various authors have described activity assays whereby the K_m and V_{max} values for this enzyme could be calculated. It therefore appears that these assays are not as straight forward as the authors would suggest. Knowledge of the kinetic parameters would provide a better understanding of the reaction between GCAT and its substrates.

Ideally, familiarity with the kinetic parameters, K_m and $V_{max,}$ would allow for the prediction of product formation at a particular GCAT dosage and incubation time. By manipulating the dosage and incubation time of GCAT in milk processing, specific characteristics (e.g. final FFA and cholesterol concentration) of the product can be altered to meet the demands of the client. These advantages, together with the fact that no current kinetic data is available stimulated our interest to characterize the GCAT enzyme in terms of its kinetic parameters. It was therefore necessary to first evaluate quantification techniques for each of the reaction products in order to develop an accurate activity assay. Since the FoodPro® Cleanline preparation will be used in the dairy industry, this preparation was used in all subsequent experiments described in this thesis.

CHAPTER 3

GCAT ACTIVITY MONITORING AND KINETICS – A BRIEF OVERVIEW

3.1 INTRODUCTION

Phospholipases play a major role in various cellular processes including lipid metabolism, signal transduction and host defenses. As a result, this group of enzymes is amongst the world's most extensively studied enzymes. In recent years, phospholipases, including GCAT, have attracted considerable attention as biotechnological tools in various industries including the food, dairy detergent, textile, pharmaceutical, cosmetic and fuel industries[40,114]. For successful industrial application a rapid and reliable activity assay is needed in order to evaluate the degree of enzymatic lipid hydrolysis and/or ester synthesis. A large number of techniques have been described whereby lipolytic activity of enzymes can be determined[96,115]. However, apart from catalyzing the hydrolysis of phospholipids, GCAT preferentially catalyzes acyl transfer in the presence of suitable acyl acceptors. In contrast to lipase activity monitoring, determination of transferase activity typically involves the use of radiolabeled substrates[40,78,116]. The use of radiolabeled substrates allows for accurate activity monitoring, however, for large scale commercial application radioactive waste production is of major concern. Substrate preparation for such assays are also time consuming and difficult to reproduce.

To overcome the difficulties associated with GCAT reaction monitoring with phospholipid substrates, various synthetic substrates have been described. However, since enzymes do not necessarily react equally with different substrates, kinetic analysis is vital in order to determine the validity of newly developed assays by comparison of the kinetic parameters. This chapter will describe the current and potential methods for GCAT activity monitoring and kinetic characterization for commercial application.

3.2 AVAILABLE ASSAYS FOR GCAT ACTIVITY MONITORING

As discussed in Chapter 2, GCAT shares several features with the lipase family of enzymes. As such, this enzyme catalyzes the hydrolysis of phospholipids in the absence of an acyl acceptor. For the determination of the hydrolytic activity of lipases, numerous assays have been described. However, since phospholipids do not possess chromogenic properties, which enable continuous reaction monitoring, these assays often rely on the use of subsequent enzymatic

reactions which yield a detectable product[115,117]. When using these methods kinetic analysis can only be performed in a discontinuous manner since the quantification of reaction products is dependent on subsequent reactions. Although discontinuous assays have several advantages[118], quantification of hydrophobic reaction products (FFA and cholesteryl esters) often require solvent extraction followed by separation and quantification of reaction products and substrates. Separation of cholesterol and cholesterol esters can be achieved chromatographically, however, detection and quantification is difficult, especially in complex media. Various attempts have been made to overcome the difficulties associated with the use of lipids as substrates in GCAT assays[78]. In these methods activity is monitored by either the utilization of substrates or product formation when incubated with experimental or synthetic substrates. The major lipase assays currently used are listed below.

3.2.1 Assaying activity by utilization of substrate

a. *Nephelometry* – Lipase activity is monitored as a reduction in the absorbance of a triacylglycerol emulsion as a function of time. This method is sensitive, but artifacts can interfere with activity determination[115].
b. *Turbidimetry* – Lipolytic activity is measured as the increase in absorbance of a Tween 20 solution in the presence of $CaCl_2$. The increase in absorbance is due to the release of free fatty acids from Tween 20. The liberated FFAs associate with $CaCl_2$ and precipitate causing an increase in absorbance at 500nm[119].
c. *Interfacial tensiometry* – Enzyme activity is monitored as a reduction in surface tension of a lipid suspension as a function of time at an air-water interface. The reduction in surface tension is due to the solubilization of lipolytic reaction products. This method is highly sensitive, however, it is still unknown if this method is representative of enzyme reactions occurring at oil-water interfaces[115].
d. *Atomic force microscopy* – This method was developed by Nielsen *et al.*[120] and relies on real time imaging of the formation of increasingly large defects (holes) in the surface of lipid bilayers. The increase in hole area can be recorded as a function of time and analyzed by purpose built software. Specific enzyme activity can be calculated as the increase in interface hole size as a function of time, assuming that a single enzyme molecule is acting at each hole.

3.2.2 Assaying activity by formation of reaction products

e. *Titrimetry* – Titrimetric and indicator dye methods, such as the pH-stat method, are generally used as a reference lipase assay[121]. This method can be used for the

quantitative analysis of lipase activity, and is sensitive to within 1µmol of product released[122]. Titrimetry is still used to validate newly developed lipase assays.

f. *Quantification of FFA release* – Various methods have been developed for the quantification of liberated FFA. These methods often rely on FFA binding to a chromogenic dye followed by spectrophotometric detection[115,121,123]. Enzyme coupled FFA assays have also been described. These methods rely on the conversion of FFA to their coenzyme A derivatives which can subsequently be oxidized by a chromogenic substrate resulting in a colour or fluorescence, depending on the substrate probe used[124,125]. Chromatographic detection has also been described[126,127]. However, such methods separate and detect individual FFAs. For quantification of total FFAs, a standard for each individual FFA is needed. Radiolabeled substrates may be used to overcome this drawback.

g. *Flourometric assays* – These assays often rely on the lipase induced displacement of fluorescent fatty acid probes from fatty acid binding proteins[115,128]. Wolf *et al.*[129] described a sensitive fluorescent assay using the naturally occurring fluorescent probe 2-parinaroyllecithin. In this assay, the liberated parinaric acid binds to albumin resulting in increased fluorescence intensity which can be quantified as a function of time.

h. *Use of synthetic substrates* – In order to overcome the difficulties associated with the use of hydrophobic lipid substrates, the use of water soluble nitrophenyl based substrates have been described for the assay of lipase enzyme activity[130,131]. During the reaction of lipases with nitrophenol containing fatty acyl substrates, the ester bond is hydrolyzed to yield chromogenic nitrophenol and a fatty acid. This reaction will be discussed in more detail in section 3.4.3.

Upon comparison of the different assay methods Vorderwulbecke *et al.*[132] indicated that no universal lipase assay exist[115]. This is mainly because lipases, although often very similar, regularly differ dramatically in terms of enzymatic activity and substrate preference. No single assay can accurately quantify the hydrolytic activity of all lipases. Furthermore, although the abovementioned assay systems may be used to determine the lipolytic activity of lipase enzymes, they cannot monitor the transferase reaction catalyzed by GCAT since no acyl acceptor is available. For this reason lipase assays have been adapted to incorporate suitable acyl acceptors for GCAT catalysis. The use of such assays have also been described and relies on the use of experimental lipid membranes impregnated with cholesterol as acyl aceptor[39,40,76,78]. During these studies, the use of synthetic nitrophenol containing fatty acyl

substrates was also investigated. It has been hinted that reaction rates obtained from assays using egg yolk lecithin and para-nitrophenol butyrate (pNPB) are similar, although this data was never published[78]. Since the use of pNPB would enable continuous GCAT reaction monitoring, it would be of interest to investigate this possibility further.

3.3 GCAT ACTIVITY MONITORING

3.3.1 Shortcomings of the current method of GCAT activity screening

As mentioned briefly in the preceding sections, high throughput activity screening is essential for the successful industrial application of enzymes. Although some of the assays described in Sections 3.2.1 and 3.2.2 can be applied to accurately quantify GCAT activity in commercial enzyme preparations, these methods are labour intensive, time consuming and require skilled personnel. These methods are furthermore not automatable and often require expensive specialized equipment. For these reasons the enzyme activity of the commercial GCAT preparation, namely FoodPro® Cleanline, is only determined at central distribution posts in Europe, Asia and the U.S.A prior to shipment to the client. The current method of activity monitoring relies on the comparison of FPCL activity to an activity standard range prepared from a GCAT stock with known activity. This assay is performed in an endpoint styled comparative assay wherein the samples with unknown activity is incubated with the activity standards for exactly 10 min. Following incubation, the FFA content of each sample is quantified using the NEFA HR FFA quantification kit. Although this method is fully automated and eliminates some of the difficulties experienced with other assays, such as a need for enzyme inactivation, this is not the most appropriate assay available. Because enzyme activity is quantified by comparison to a “standard enzyme”, errors in the quantification of the standard enzyme activity would result in under- or over estimation of all subsequent sample activities. Such an error may result in large scale product losses. Moreover, enzyme activity is not static and can be unfavorably affected by many factors ranging from improper storage to agitation during transport. Because enzyme activity is only quantified at designated distribution posts, enzyme activity cannot be guaranteed upon delivery to the client. Furthermore, prolonged storage, as would be common with small dairies, may also result in loss of enzyme activity over time. Since dosage is based on a volume per volume basis (ppm) rather than on enzyme activity, accurate activity data is essential to ensure appropriate dosage, preventing large scale product losses.

A sufficiently facile assay, which can be performed on-site, would be advantageous to the marketability of the commercial enzyme preparation since dosage may be adjusted more

accurately based on enzyme activity. To date no such activity assay is available. However, as described in Section 3.2.2, synthetic substrates have been used to determine lipase activity. Among these, nitrophenyl based substrates are the most attractive candidates, based on the ease of use and versatility. Although pNPB have been shown to act as a substrate for the GCAT enzyme, it remains unclear if this substrate can be used for the accurate and sensitive quantification of enzyme activity.

In order to evaluate if pNPB may be used for activity quantification, the kinetics of GCAT catalysis, using pNPB as substrate, must be compared to kinetics obtained using the natural substrate, lecithin. Since the rate of enzyme catalysis often varies significantly when using different substrates, the definition of a single unit of enzyme activity would not necessarily be the same for both substrates. The definition of a unit of enzyme activity should therefore be defined by comparison to activity data obtained from assays using lecithin as substrate. However, although seemingly straight-forward, a kinetic study of GCAT has never been described. This is mainly due to the complex nature of phospholipid substrates in buffered systems.

3.3.2 The current model of the GCAT reaction

In earlier reports of phospholipid hydrolysis by GCAT, Hilton and Buckley[82] described a two-step interaction of the enzyme with phospholipid substrates as depicted in Figure 3.1. In this mechanism GCAT first binds the phospholipid polar head groups, followed by membrane penetration and formation of the enzyme substrate complex. This mechanism is similar to interfacial lipid hydrolysis by lipases[133].

During its reaction with water-insoluble substrates, such as phospholipids, GCAT, like lipases, act at water-lipid interfaces[40,82,96]. Studies have shown that the overall catalytic rate of lipases are regulated by the physicochemical character and organizational dynamics of the interface[133,134]. Reactions catalyzed by GCAT, and other lipases, are therefore two-dimensional in character since phospholipid/interfacial binding precedes formation of the enzyme substrate complex as presented in Figure 3.1. In many cases, interfacial binding have been shown to be rate limiting[133]. In contrast, Hilton and Buckley[82] indicated that binding of the polar head groups may not be essential to GCAT catalysis.

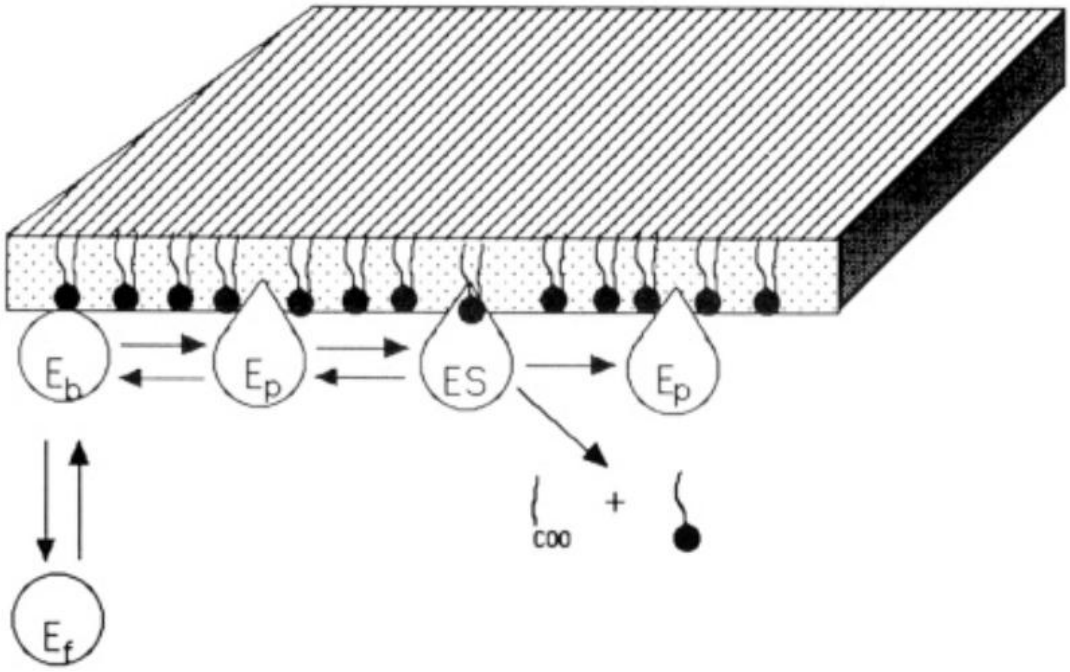


Figure 3.1 Illustration of the two-step interfacial reaction mechanism of GCAT as proposed by Hilton and Buckley[82]. In this representation, formation of the enzyme-substrate complex is preceded by enzyme binding to the polar head groups of phospholipids.

More recently, Hilton *et al.*[39] suggested that GCAT does not require the interfacial activation which characterizes lipases. The mechanism whereby GCAT catalyzes interfacial phospholipid hydrolysis is therefore still not fully understood. However, Buckley[76] suggested that hydrogen bonding between the hydroxyl group of the acyl acceptor and the ester linkage of the donor may play an important role in the active site of the enzyme. Since cholesterol is incorporated into membranes so that the hydroxyl group of cholesterol is in alignment with the ester bond of phospholipid, this scenario appears feasible.

Since, to the best of our knowledge, no kinetic data exists for either of the reactions catalyzed by GCAT, there is no kinetic data to support either of the possibilities described. It therefore remains possible that rapid equilibrium is reached, and GCAT catalysis occurs via a Michaelis-Menten kinetic model. In order to explore this possibility, kinetic assays are required.

3.4 SUBSTRATES FOR GCAT KINETIC ANALYSIS

3.4.1 Small unilamellar vesicles as enzyme substrates

Due to the hydrophobic nature of the substrates, GCAT activity assays have often relied on the preparation of the experimental lipid vesicle substrates; liposomes, as depicted in Figure 3.2[135].

Upon hydration of dried lipid cakes, phospholipid membranes spontaneously form due to the unfavorable interaction between hydrophobic phospholipids and water[136]. The hydrated lipid cakes subsequently become fluid and swell. Agitation of the swollen lipid cakes results in the formation of energetically favorable multilammelar vesicles (MLVs). Once formed, the size of MLVs can be reduced by applying energy in the form of mechanical mixing (extrusion) or sonication[135–137]. Sonication of MLV suspensions results in the formation of small unilamellar vesicles with an average size of 250Å[136].

MLVs are often too large or heterogeneous for use in enzyme activity studies. SUVs are therefore among the most widely used experimental phospholipid membranes[39,77,78,136]. The use of SUV's as experimental substrates for GCAT activity assays have been described in combination with radiolabeled lipids and/or sterols[56,76,78,82,84,138]. However, although the use of radiolabeled substrates increases sensitivity and eliminates the need for extraction controls and internal standards, for large scale industrial application radioactive waste production is a major concern. The preparation of SUVs is furthermore laborious and time consuming.

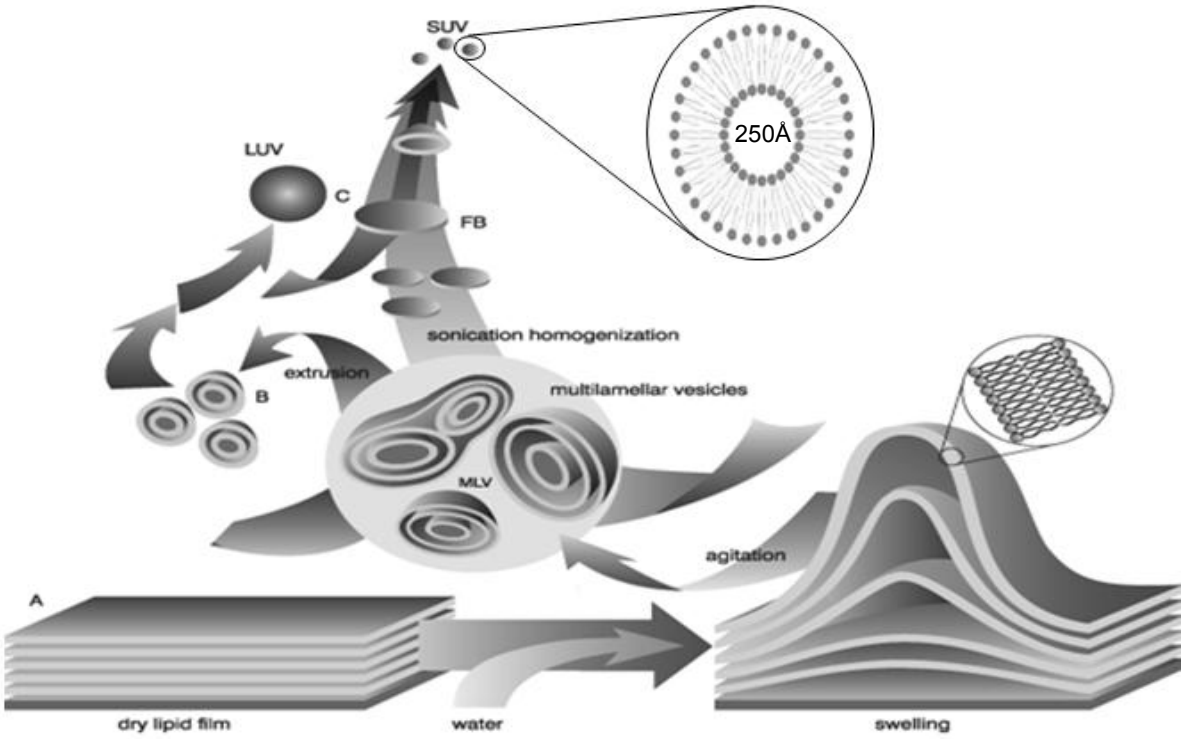


Figure 3.2. General depiction of vesicle formation starting with dried lipid films. Multilammelar vesicle formation occurs spontaneously as a result of hydration. Preparation of smaller vesicles require additional energy input[139].

SUVs can be prepared by using either a probe or a bath sonicator[136]. Selection of a specific sonication method is dependent on the lipid suspension required. Probe sonication is mostly used when high energy in small volumes is required. As the sample volume is increased, energy distribution is significantly affected. Uneven energy distribution may reduce the ability to form uniformly sized lipid vesicles. Probe sonication is therefore mostly used for high lipid concentrations or viscous suspensions with small volumes[136]. However, due to the small sample size and high energy input, there is a significant risk of lipid degradation due to excessive heat production and increased gas exchange[136]. It is therefore essential that sonication temperature is regulated at all times. Lipid suspensions may also be contaminated with titanium particles released from the probe. These particles must be removed by centrifugation prior to use of the vesicles. For these reasons bath sonication is more widely used for the preparation of SUVs.

Bath sonication enables the preparation of larger volumes of SUVs. Because of the larger volumes, ultrasonic irradiation is more homogeneous leading to increased reproducibility[136]. Furthermore, temperature regulation is not as crucial as with probe sonication because the heat generated by sonication is easily absorbed by the bath. However, it is important to maintain sonication temperature above the transition temperature (T_m) of the lipid with the highest T_m in the suspension[136,139]. The T_m is an indication of when a specific lipid will undergo transition from the solid gel state to the fluid state[136,140]. At temperatures below T_m the packaging of phospholipid head groups is weakened and the gel phase fluidity is increased[136]. Above T_m the membranes condense causing a reduction in area, a closer packaging and a decrease in fluidity[136]. Therefore, when temperature regulation is required, as is the case with saturated lipid suspensions, the bath temperature may easily be regulated with a thermostated water bath. Although bath sonication has several advantages over probe sonication, when larger volumes are required, bath sonication would not yield a homogeneous suspension of SUVs[136]. This is due to a larger area of ultrasonic radiation[136]. If vesicles of uniform size are required, chromatography or centrifugation may be used to remove larger liposomes.

3.4.2 Incorporation of cholesterol into SUVs

Cholesterol is an important component of biological membranes. Apart from decreasing the permeability of the membrane to fluids such as water, cholesterol also plays a role in stabilization of the phospholipid bilayers[136,141–144]. As can be seen from Figure 3.3.A, cholesterol contains a planar steroid nucleus composed of four fused rings (A,B,C and D), a β-hydroxyl group attached to the 3-carbon of the A ring and an aliphatic side chain[136,142,143]. Being

amphipathic in nature, cholesterol is inserted into lipid bilayers with the hydroxyl group oriented toward the aqueous phase and the aliphatic side chain aligned with the fatty acyl chains of phospholipids as presented in Figure 3.3.B [136,142]. The hydroxyl is in alignment with the ester linkages of the phospholipids, while the rigid steroid rings associates with the first few carbons of the phospholipid acyl chains[145]. This association reduces the freedom of movement of the phospholipid chains, which improves membrane rigidity and reduces membrane permeability to water[136,146].

Cholesterol cannot form bilayer structures in the absence of phospholipids. However, in the presence of phospholipids cholesterol is able to incorporate into membranes in high concentrations (typically 2:1 molar ratios)[136]. The distribution of cholesterol in membranes is furthermore not random. It has been shown, with space filling models, that membrane components are packed in a two-dimensional lattice of linear arrays, with cholesterol rows altering rows of phospholipid at equimolar concentrations[136,145]. Cholesterol concentrations in excess of 50 mol % would disrupt the linear arrays of alternating cholesterol and phospholipid, decreasing the ability to form intact SUVs[136]. At concentrations higher than 50 mol %, it was shown that phase transition is also eliminated and the enthalpy of phase change is reduced to zero, decreasing the fluidity of the membranes, and inhibiting hydrocarbon crystalization[136,145].

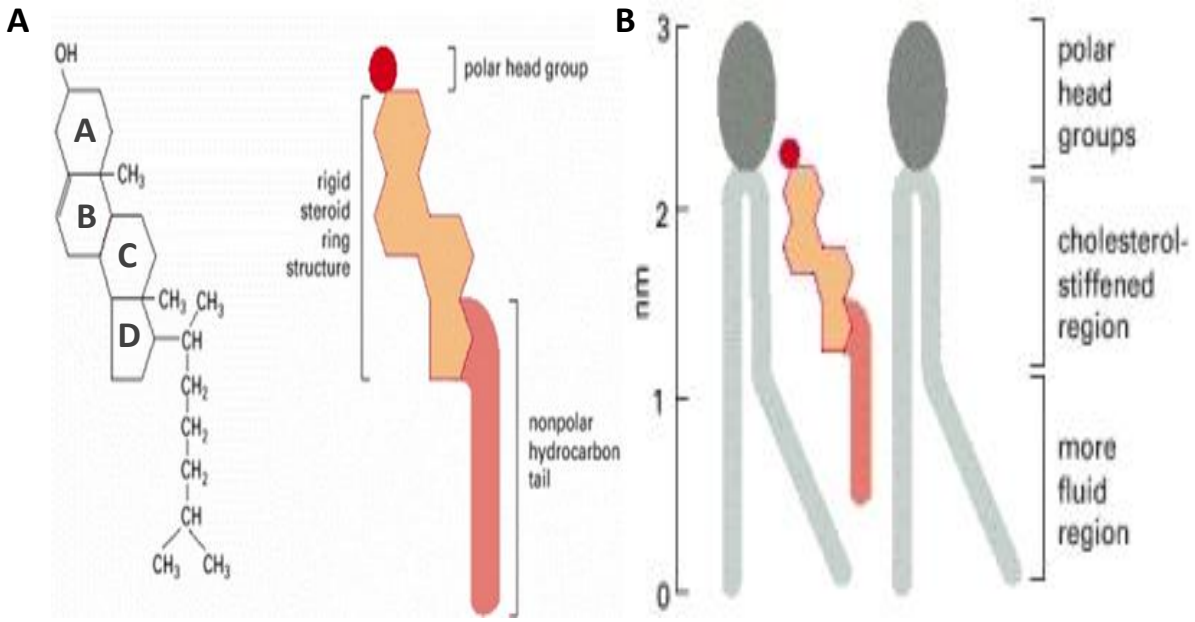


Figure 3.3. (A) Chemical structure of cholesterol indicating the different sections of the molecule, and (B) the position of cholesterol incorporation in lipid membranes (Figure recreated from sources [142,145])

SUVs may therefore be used to monitor both the lipase and transferase activities of GCAT catalysis since cholesterol is efficiently incorporated into the micellar membrane, even at high

concentrations. Vesicles containing cholesterol are well suited for general enzyme activity assays. However, quality emulsions are not easily obtained. In order to overcome the difficulties associated with preparation of quality emulsions, various novel approaches, such as the use of synthetic water soluble substrates and enzyme coupled assays have been described[115,132,147]. One such substrate is pNPB.

3.4.3 pNPB as substrate for GCAT activity monitoring

While studying the reaction mechanism of GCAT, Buckley *et al.*[77] showed that the hydrolysis of several synthetic nitrophenyl esters are also catalyzed. Among the substrates investigated, it was found that the reaction rate was the highest when pNPB was used. It has furthermore been shown that this substrate is also an effective donor for enzyme catalyzed acyl transfer to cholesterol. The use of nitrophenyl substrates enabled the continuous monitoring of GCAT hydrolysis. The reaction of pNPB with GCAT is depicted in Figure 3.4. In this reaction the ester bond is hydrolyzed by GCAT yielding butyric acid, or cholesteryl butyrate depending on the presence of cholesterol, and 4-nitrophenol (4NP). Under basic conditions, 4NP is deprotonated to yield 4-nitrophenolate which has an intense yellow color and a strong absorbance at 400 nm (ε_{400} =12000 M^{-1} cm^{-1})[78,148]. Substrate production can consequently be continuously monitored on a thermostated kinetic spectrophotometer. Since the reaction rate is monitored in real-time, activity quantification is more accurate and reproducible than conventional assays using lipid substrates. Assays using these substrates are furthermore readily scalable to enable high-throughput activity monitoring in microtiter plates.

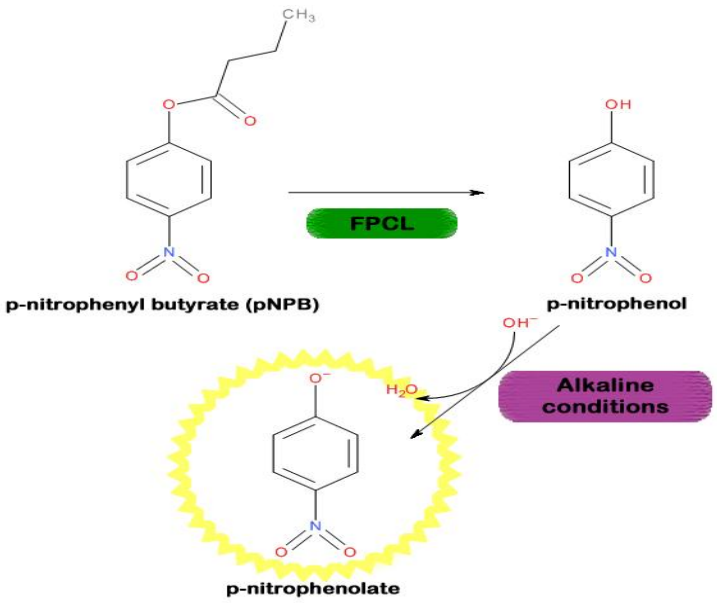


Figure 3.4. Enzymatic hydrolysis of pNPB by GCAT followed by subsequent deprotonation of pNP under basic conditions yielding an intense yellow colour (Figure adapted from ref [149]).

3.5 CONCLUSION

The use of SUVs as substrates for the study of hydrolytic enzymes is well established. However, its use for routine enzyme activity monitoring is limited by the difficulty to produce quality emulsions and the need for specialized equipment for the detection of reaction products[133,134]. As a result synthetic substrates, whose reaction products can be easily detected, are increasingly being used for routine analysis.

The use of pNPB enables continuous reaction monitoring of both reactions catalyzed by GCAT. However, it remains to be shown if pNPB can serve as sole substrate for activity monitoring. Comparison of the kinetic parameters obtained from studies using phospholipids and pNPB as substrate would indicate if this synthetic substrate can be used for routine activity determinations. Since no kinetic data is currently available, such studies may also provide evidence for the mechanism whereby GCAT interacts with substrates. This thesis will describe the development of assay methods, both continuous and discontinuous, which may be used for the kinetic analysis of GCAT catalysis. The data obtained from these studies will then be compared to define a single unit of enzyme activity when using pNPB as substrate under similar conditions.

CHAPTER 4

FOODPRO® CLEANLINE APPLICATION TRIALS

4.1 INTRODUCTION

From the discussion in Chapter 2 it is clear that fouling is a common phenomenon during ultra-high temperature (UHT) treatment of dairy products, resulting in an increase in plant downtime and input cost. As described in this chapter, DuPont® recently isolated a bacterial enzyme, glycerophospholipid cholesterol acyltransferase (GCAT, E.C: 2.3.1.43), which can reduce fouling by preventing the aggregation and precipitation of proteins denatured by UHT treatment. However, prior to commercial promotion it is essential to validate the efficiency of the commercial GCAT preparation, FoodPro® Cleanline (FPCL). For this purpose, accurate methods for the quantification of GCAT reaction products are crucial since incorrect dosage/usage of FPCL may lead to overproduction of free fatty acids (FFA). An overabundance of FFA may reduce the economic value of the final product by increasing product rancidity. In this chapter the current method for the quantification of GCAT reaction products was evaluated prior to application of FPCL in pilot and factory scale trials. This was important since this method was required for quality control purposes subsequent to each trial. This chapter will therefore describe the evaluation of this analysis method followed by the application of FPCL in various pilot and factory scale UHT trials.

4.2 MATERIALS AND METHODS

All FPCL preparations used in this section were from commercial preparations provided by DuPont® SSA Dairy innovations center Cape Town, South Africa. Unless specified otherwise Mat# A14076G190, Batch# 4861927699 FoodPro® Cleanline (FPCL) was used for all trials.

4.2.1 UHT product analysis

The free fatty acid (FFA) and cholesterol/cholesteryl ester content of milk samples, collected from pilot- and factory scale FPCL trials, were evaluated by gas chromatography using the method recommended by DuPont®, Bruges. In this section, this method will be evaluated.

Milk samples were prepared for gas chromatographic (GC) analysis by liquid-liquid ether extraction. For this purpose one mL milk was accurately measured into a 15 mL teflon-lined

screw cap tube containing 2 mL 96% ethanol and 1.5 mL deionized water. The contents were thoroughly mixed, followed by the addition of 0.3 mL NH_4OH. A phenolphthalein indicator (10 µl 20% ($^{w}/_{v}$)) was added to increase visualization of phase separation. This mixture was mechanically shaken for 5 min on an IKA Vibrax VXR shaker. After 5 min agitation, 5 mL methyl *tert*-butyl ether (MTBE) was added followed by shaking for 5 min. Petroleum ether (5 mL, 40 – 60 °C) was added and the mixture was again mechanically shaken for 5 min. The solvent top-layer was clarified by centrifugation at 3500 x *g* for 10min. The clear organic phase was subsequently transferred to a clean glass test tube and the extraction was repeated as follows:

Add 1 mL ethanol – Mechanically shake for 5 min.
Add 5 mL MTBE – Mechanically shake for 5 min.
Add 5 mL petroleum ether – Mechanically shake for 5 min.

The organic phase was again clarified by centrifugation as described above. The solvent phase was pooled with the first extract and the solvent was evaporated under a stream of nitrogen at 60 °C. The resulting lipid extract was prepared for GC analysis as described in the ensuing section.

Following lipid extraction, the extracts were dried under a stream of nitrogen at 60°C. The dried lipids were resuspended in a volume (1.5 mL) of heptane: pyridine (2:1) containing an internal standard of heptadecane. Aliquots of 500 µL were subsequently transferred to clean crimp cap vials and treated with 100 µl N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) containing 1% trimethylchlorosilane (TMCS). The vials were subsequently sealed and heated at 60°C for 15 min. Following derivatization, the samples were ready for GC-MS analysis.

GC-MS analyses of cholesterol and cholesteryl ester derivatives were performed using an Agilent 6890N GC (Agilent Technologies Wilmington, USA) coupled to an Agilent 5975 MS detector. A Restek (12723-127) 30m x 250 µm (inner diameter) x 0.25 µm (film thickness) Rtx®-5Sil MS Capillary Column was used for separation. Of each derivatized sample 1µl was injected using a CTC PAL autosampler in splitless-injection mode into the injection port, set at 300°C. High purity helium (99.999%) was used as the carrier gas at a constant flow of 1.0 mL/min. The GC oven temperature program applied was as follows: the initial temperature was set at 150°C, and held for 1 min. The oven temperature was then increased to 330 °C at a rate of 10°C/min and maintained at 330°C for 10 min. Full scan ($^{m}/_{Z}$ 35 – 700) and single ion monitoring (SIM) was used for detection. SIM was used to identify trimethylsilyl palmitate ($^{m}/_{Z}$ 313), trimethylsilyl stearate ($^{m}/_{Z}$ 341), cholesta-3,5–diene ($^{m}/_{Z}$ 368), cholesta-4,6–diene ($^{m}/_{Z}$ 368) and trimethylsilyl

cholesterol ($^{M}/_{Z}$ 458). The MS source temperature was set at 230°C and the MS quadrupole temperature at 150°C.

4.2.2 UHT trials

4.2.2.1 Pilot scale trials

Unless stated otherwise, all pilot scale trials were performed at DuPont® Nutrition and Health Cape Town (South Africa) using an OMVE HT220 HTST/UHT continuous flow processing system under the supervision of senior application specialist, Peter Lawson. Fresh pasteurized full cream milk was used for these trials. Prior to the addition of FPCL, the milk was subjected to various tests to ensure milk quality. These tests included analysis of the physical milk properties, i.e. % fat, % protein, % lactose and pH using a Milkoscan™ analyzer followed by an alcohol test with 75% ethanol. A volume of milk was subsequently spiked with 10 ppm FPCL and incubated at ≤ 5°C for at least 2 hours prior to UHT processing. Unspiked milk was processed in the same manner as a control.

Two successive full clean in position (CIP) and sterilization cycles were performed prior to UHT processing. The tubular heating and pre-heating sections were opened and the tubes and static mixers were visually inspected prior to processing. UHT processing was performed at 20 liter per hour, using tubular heat exchangers, at 142°C with 4 seconds holding time followed by cooling to <25°C for aseptic filling. The homogenizer pressure was set at 200 bar (first stage/second stage, 150/50) at 65°C and temperature (ΔT) and pressure differentials were noted every 30 min. Samples of 250 mL were collected for analysis 10 min after the arrival of product at the sampling valve. Samples were collected in heat-sealed UV-resistant plastic bottles and stored at 4°C until analysis. Following UHT processing, the heat exchangers were visually inspected for fouling prior to CIP.

4.2.2.2 Full scale factory trials

Factory trials were performed at Dewfresh in Leandra, South Africa under the supervision of the Director, Mr Stefan Swanepoel. For these trials fresh milk, previously standardized and clarified, was spiked with 10 ppm FPCL and incubated for 2 hours at 4°C with agitation. Thermal processing was performed at ≈6000 L per hour using a tubular UHT system (Finnah, Finnahmat 6500/065). Milk temperature was increased to 142°C and held for 6 seconds with the homogenizer pressure set at 200 bar (first stage/second stage, 150/50 at 65°C). The milk was

subsequently cooled to 20°C for aseptic packaging. ΔT values were noted every 30 min and 1 l samples were collected from the packaging line at random intervals for analysis.

4.3 RESULTS AND DISCUSSION

Unless specified otherwise all experiments were performed at least in duplicate, and the data presented as the mathematical mean.

4.3.1 GC-MS analysis

In order to ensure optimal product quality, a method for the quantification of GCAT reaction products (cholesterol, cholesteryl ester and FFA) is critical. The method currently used by DuPont® for evaluation of product quality is GC coupled to flame ionization detection (FID). Although this method is sufficiently sensitive for the low level detection of cholesterol, cholesteryl esters and FFA, it does not allow for peak identification without prior injection of a pure standard. Even when the retention time of a specific compound is known, peak overlapping may result in a decrease in detection sensitivity. In order to overcome this drawback, GC-mass spectrometry (GC-MS) may be used. This analysis technique allows for the identification of individual compounds based on their specific mass to charge ratio ($^{m}/_{Z}$) following ionization. Furthermore, the $^{M}/_{Z}$ values obtained can be compared to a library for peak identification without prior injection of a pure standard.

For the GC analysis of cholesterol and FFA, derivatization is required to improve gas chromatographic behavior and detectability by increasing volatility and/or stability of the compounds of interest. During derivatization reactive functional groups are silyated by MSTFA yielding trimethylsilyl derivatives of the compounds of interest. Reactive functional groups (such as an HO-R) are thus essential for derivatization. Figure 4.1 illustrates the general reaction mechanism for derivatization of an alcohol functional group by MSTFA and TCMS (99:1).

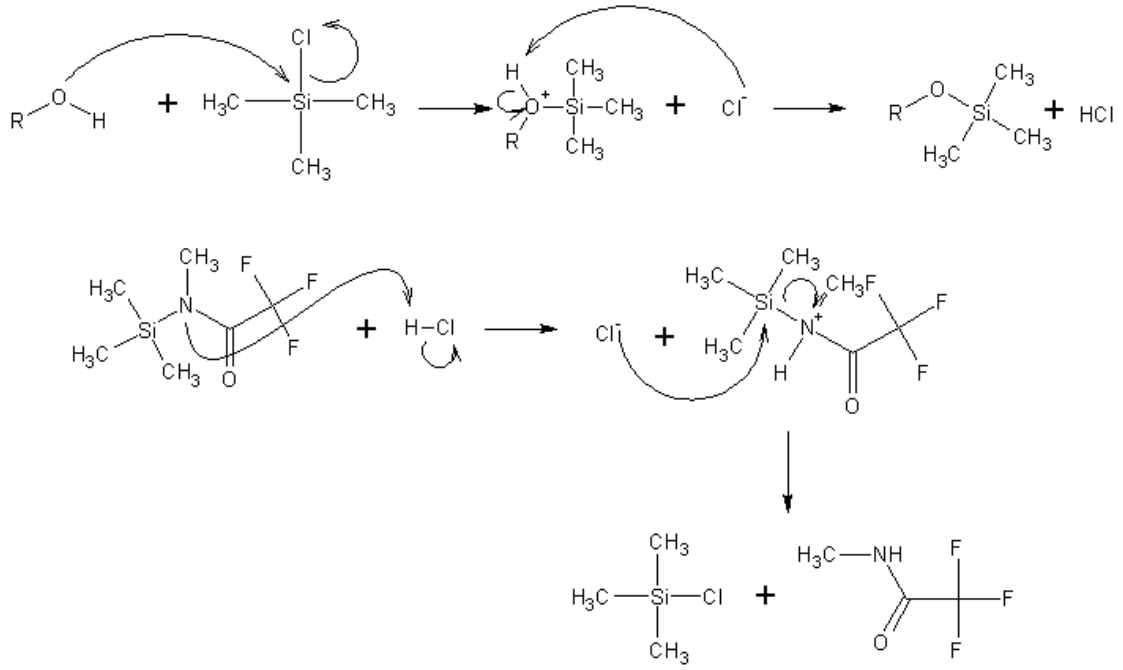


Figure 4.1 General reaction mechanism for silyation of an alcohol functional group using MSTFA and 1% TMCS as derivatizing reagents where R = cholesterol.

In this derivatization TMCS is the main silyl donor. However, TMCS only constitutes 1% of the derivatization reagent used. During derivatization TMCS acts as a catalyst that promotes TMCS formation via MSTFA cleavage. This method of TMCS cycling is used to prevent the excessive formation of HCl which would occur if TMCS is used as sole derivatizing reagent.

The chemical structures of cholesterol (a), cholesteryl stearate (b), cholesteryl palmitate (c) and heptadecane (d) are depicted in Figure 4.2. From these figures it can be seen that only cholesterol has a functional group that can be derivatized by silyation. As a result, derivatization should not affect any of the cholesteryl esters. Single peaks are therefore expected for each injected standard.

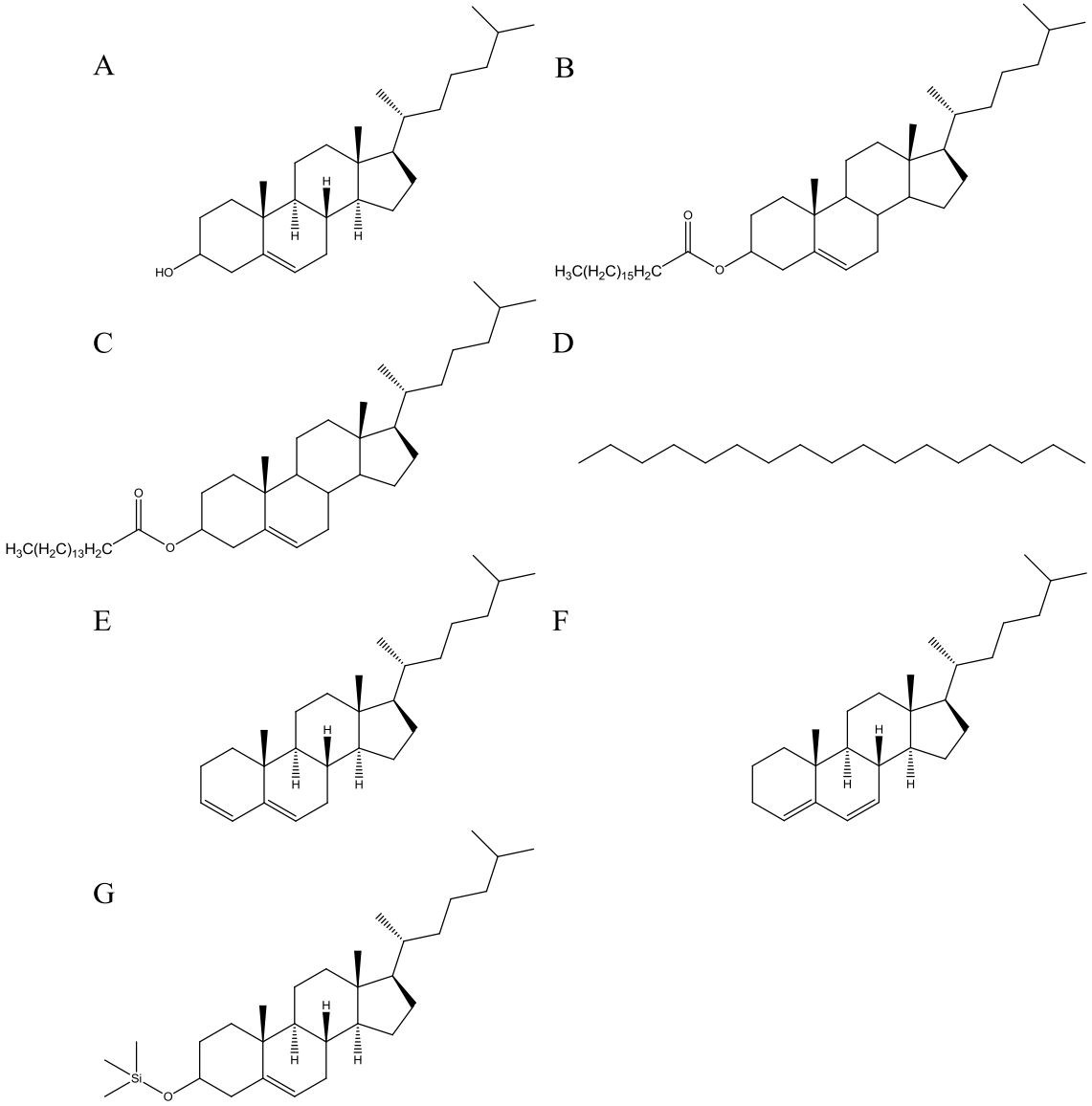


Figure 4.2 Chemical structures of (A) cholesterol, (B) cholesteryl stearate, (C) cholesteryl palmitate, (D) heptadecane, (E) cholesta 3,5 diene, (F) cholesta 4,6 diene and (G) cholesterol trimethylsilyl ether[150].

From Figure 4.3, however, it can be seen that multiple peaks are observed when pure cholesteryl ester standards were injected. Comparing the $^{m}/_{Z}$ values obtained for each peak to a library, it was found that the peaks at 9.18 and 10.92 min correspond to silyated FFAs corresponding to the acyl chain from each injected cholesteryl ester standard. The remaining peaks correspond to the cholesteryl ester breakdown products, cholesta-3,5–diene, cholesta -4,6–diene and cholesterol trimethylsilyl ether at 15.23, 16.04 and 17.62 min respectively as depicted in Figure 4.2 (E-F).

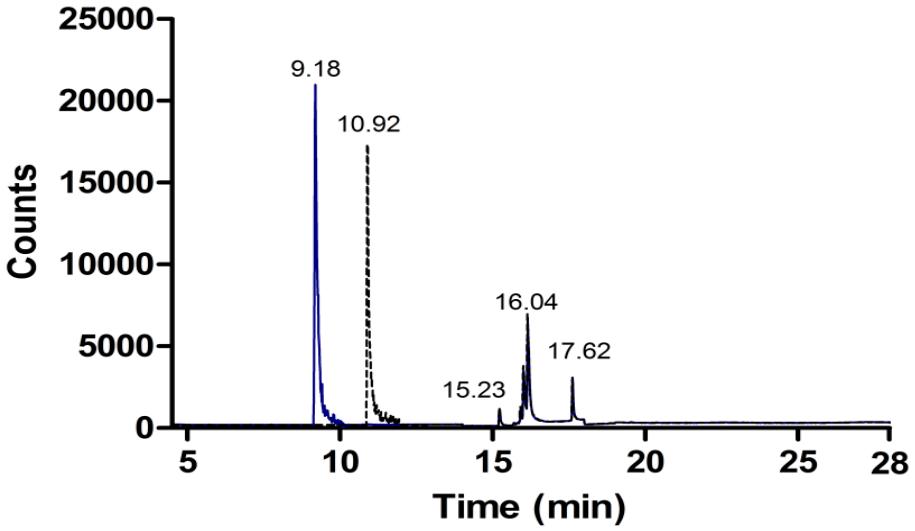


Figure 4.3 Chromatograms obtained after 1µl injection of 200 ng pure standards of cholesteryl palmitate (solid line) and cholesteryl stearate (dashed line) analyzed with GC-MS as described in the text.

To determine if cholesteryl esters were hydrolyzed during derivatization or ionization, MS and FID detection methods were compared. From Figure 4.4 it can be seen that multiple peaks are produced irrespective of detection method. These results suggest that cholesteryl ester breakdown occurs during derivatization rather than during ionization. Consistent with this, Onorato *et al.*[151] suggested that derivatization for GC-MS is complicated by isomerization and decomposition of lipid side chains during ester analysis[152].

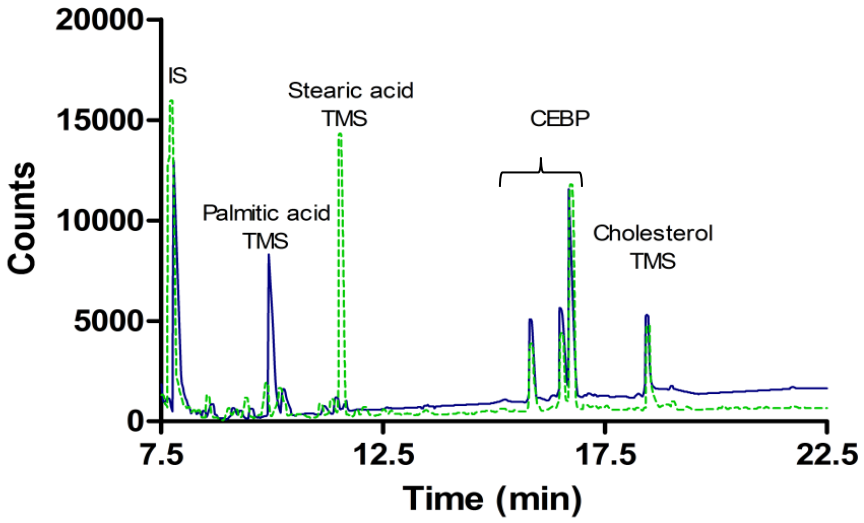


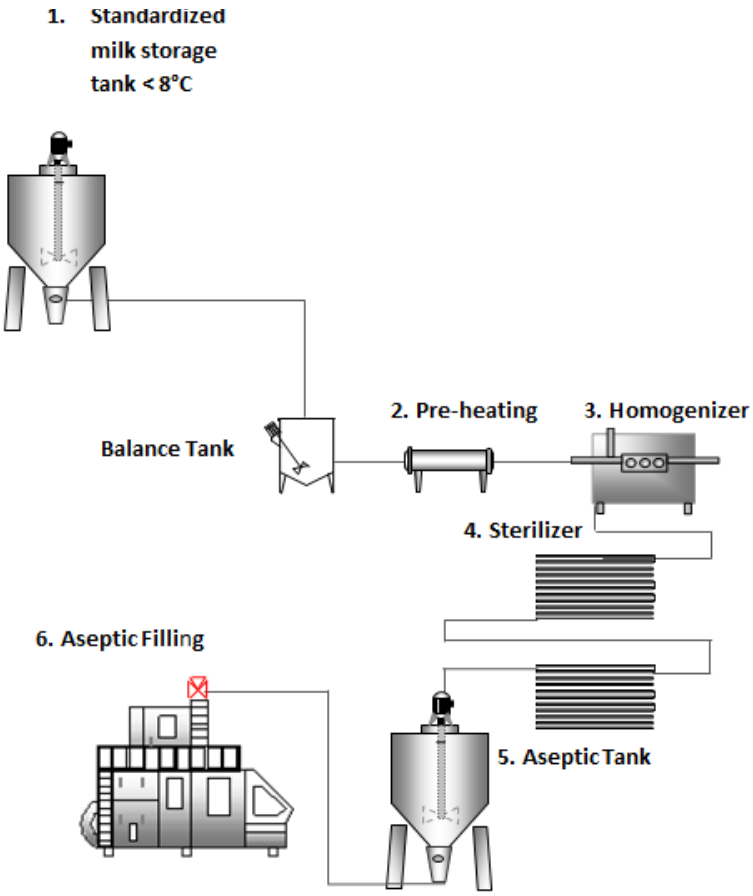
Figure 4.4 GC-FID chromatogram of 1µl injection of 200 ng pure standards of cholesteryl palmitate (solid line) and cholesteryl stearate (dashed line) with internal standard (IS) and cholesteryl ester breakdown products (CEBP).

GC analysis of a mixture consisting of cholesterol and cholesteryl esters would consequently result in an overestimation of the cholesterol content and an underestimation of the cholesteryl ester content. Furthermore, since FFAs are also a product of cholesteryl ester derivatization, this method is also not suitable for the quantification of FFAs in treated milk samples. Other methods for the quantification of cholesterol, cholesteryl esters and FFAs are therefore essential for accurate evaluation of GCAT reaction products.

4.3.2 UHT trials

The general process-flow of all trials performed is depicted in Figure 4.5. For pilot scale trials previously pasteurized commercial milk was used. In all trials performed milk was incubated for at least two hours at ≤ 5°C prior to UHT processing.

UHT Milk factory process-flow



Processing Parameters for commercial UHT (Indirect Plant) milk production:

1. Storage of standardised, pasteurized milk prior to UHT processing – Addition of FPCL
2. Pre-heating 65°C
3. Homogenize up-stream 200 Bar (150/50) at 65°C
4. UHT Treatment (Indirect) 142 - 144°C for minimum of 6 seconds
5. Cooling to less than 25°C, transfer to Aseptic Tank
6. Aseptic Filling - Filling temp 25°C

Figure 4.5 General UHT milk process-flow used during all trials.

Fouling cannot be observed directly during thermal processing. However, various operational parameters may be used in order to monitor fouling[153]. During these studies two detection

methods were used since these methods can be used universally. The first parameter used was ΔT. Deposit formation reduces the heat transfer capacity of the heat exchangers. The temperature of the heating medium must therefore be increased in order to maintain a constant product temperature of 142°C. The difference in temperature between the heating medium and product, referred to as ΔT, can therefore be used to monitor fouling. Fouling related increases in ΔT typically results in a fouling profile wherein ΔT remains fairly constant for the first few hours of processing followed by a rapid increase in the last hours of processing. This profile is due to a series of events that take place at the solid-liquid interface. As discussed in Chapter 2, mineral fouling is the predominant form of fouling during UHT processing. However, protein fouling precedes mineral fouling resulting in a "lag phase" wherein protein adsorption to the solid heat exchange surface takes place[4]. Once formed, the homogeneous proteinaceous layer acts as a scaffold for mineral deposition leading to mineral fouling. In order to ensure efficient operation of the UHT plant, it is advised to keep ΔT below 5°C. Once this value is reached, processing should be ceased and the heat exchangers cleaned by an intermediate rinse or full CIP.

In addition to temperature differentials, system back pressure may also be used to monitor fouling. Thermal processing is performed at temperatures above the normal boiling point of water at atmospheric pressure. To prevent flash boiling of the product in the holding tubes and/or heat exchangers, processing is performed at increased pressure. Product flashing may result in product burn-on and, consequently, off flavours. Deposit formation increases the system back pressure by obstructing the flow path of the product. In order to maintain a constant back pressure, pressure regulation valves are automatically adjusted in response to variations in back pressure. However, this regulation is limited. Once back pressure buildup exceeds the maximum capacity of the pressure regulation valve, system back pressure will increase rapidly due to a lack in regulation. In order to prevent damage to the holding tubes, UHT processing must be stopped and the processing plant cleaned.

4.3.2.1 Pilot scale trials

Full scale UHT plants operate at processing flow rates in excess of 5000 L product per hour. Due to these high volumes along with the high running costs, full scale trials are not financially viable for research and development (R&D) purposes. For R&D, pilot scale UHT plants are more suitable due to ease of operation, lower flow rates (up to 150 L per hour), and support for multiple heat exchangers. Using pilot scale plants, product development can be performed leaving full scale commercial UHT processing unhindered. For this reason, pilot plant trials were

conducted in order to evaluate the effect of FPCL on plant fouling and final product quality prior to factory application. During these trials ΔT and back pressure was used to monitor fouling.

Throughout 2012 various pilot scale trials were conducted to evaluate the effect of FPCL on UHT plant fouling and the final product quality. Initially, however, no significant difference could be demonstrated between enzymated and non-enzymated milk. Similar results were obtained for subsequent pilot scale trials performed (two additional trials). Upon investigation it was found that the FPCL used (Mat# A14076G190 Batch# 1781369165) had less than 25% of the expected activity. Failure to demonstrate a significant difference, between treated and untreated milk on UHT plant fouling, was subsequently attributed to a lack in enzyme activity.

The trial was subsequently repeated with fresh FPCL with a validated enzyme activity. When comparing the operational parameters (ΔT and back pressure) from these trials a number of differences were observed. As can be seen in Figure 4.6, there was a substantial difference in ΔT values. UHT processing performed without the addition of FPCL (control) resulted in a typical fouling profile wherein ΔT remained fairly constant for the first 2 hours of processing followed by a rapid increase in the last hour of processing. The maximum recommended ΔT was exceeded after 2.5 hours, and the trial was terminated to prevent damage to the holding tubes and static mixers. In contrast, the ΔT of milk spiked with FPCL (enzymated 1 and 2) did not increase substantially, even after 3.5 hours of processing.

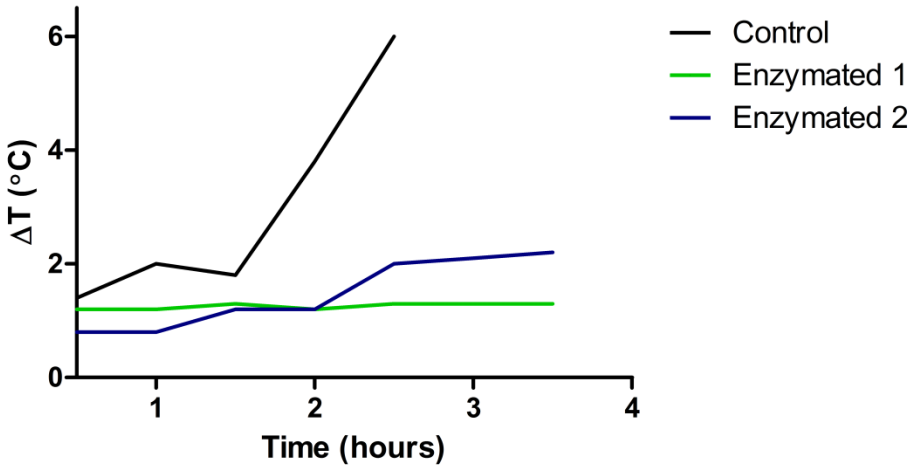


Figure 4.6 ΔT fouling profiles of UHT processing performed on milk samples with and without FPCL. In this figure Enzymated 1 and 2 represents duplicate trials performed using FPCL.

From Figure 4.7 is can be seen that enzymation also resulted in a reduced rate of back pressure buildup. The system back pressure for the control trial was below that of trial 2 for the

first 2 hours of processing. However, a rapid pressure increase of 4 bar (ca. 85%) was observed 30 min prior to termination of the trial. In contrast, system back pressure remained constant for at least 60 min prior to the end of trials performed with enzymated milk. Compared to the control, the system pressure for enzymated trials 1 and 2 increased by 33% and 66% respectively. Although a substantial increase in operational back pressure was observed for the second enzymated trial, this increase was substantially less (44%) than that of the control trial. The back pressure of the trials with FPCL was furthermore stable prior to termination of the experiment while the pressure of the control trial increased at a constant rate.

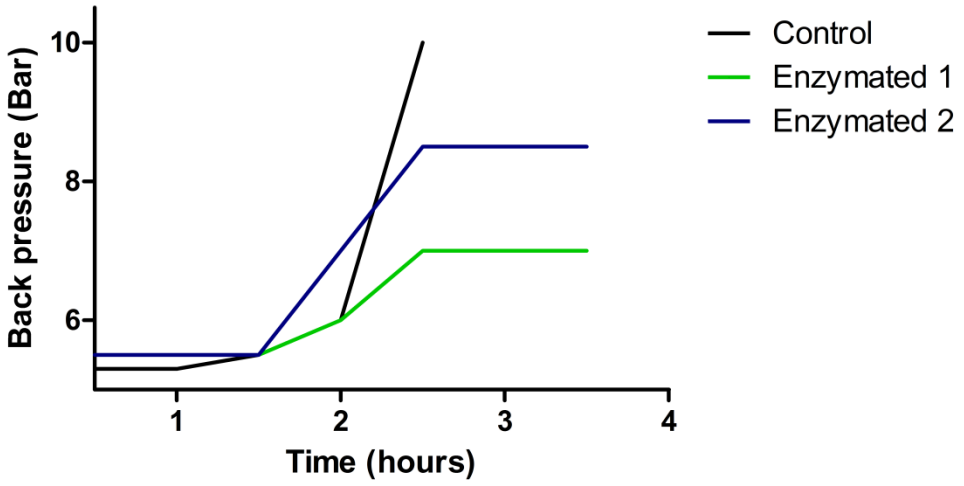


Figure 4.7 Graph indicating an increase in system backpressure as a result of deposit formation during UHT plant fouling.

Following thermal processing the static mixers were opened and visually examined. Figure 4.8 shows the visual inspection of the static mixers of trials without (A) and with FPCL (B). Although a layer of fouling deposit was observed in both trials, this was substantially less on the product side mixer (encircled in red) for the trial with FPCL, even after an additional hour of processing. Furthermore, the overall fouling layer of the control trial was more brown in colour indicating caramelization. Caramelization, and burn-on, imparts a burnt taste on the products, reducing the product appeal[154]. Excessive burn-on may also lead to a increased cleaning time and damage to the heat exchangers. It is therefore recommended to avoid burn-on at all costs.

The results obtained from the pilot plant trials indicated that the use of FPCL could increase thermal processing time by up to 30%. Although the data for a single trial was discussed, the results were highly reproducible after obtaining fresh FPCL. The results also suggest that

caramelization and burn-on may be reduced with the use of FPCL, resulting in an increased product appeal.

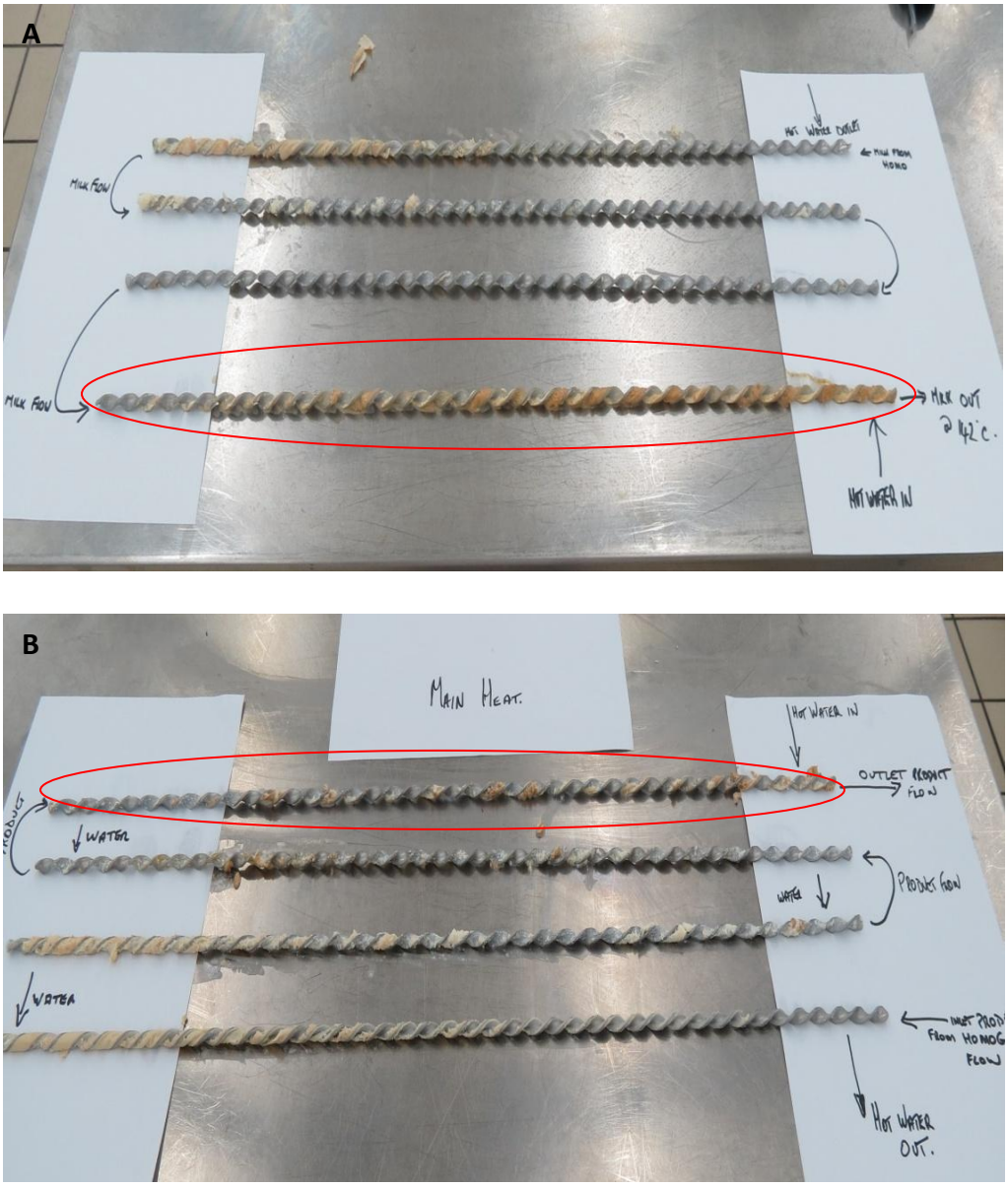


Figure 4.8 Static mixers of the OMVE HT220 HTST/UHT pilot scale thermal processing plant subsequent to thermal treatment of fresh pasteurized full cream milk (A) with FPCL and (B) without FPCL.

4.3.2.2 Full scale factory trials

Based on the success of the pilot scale trials, full scale factory trials were performed as described in Section 4.2.2.2. In these trials fouling was monitored by system back pressure. Unfortunately no previous ΔT data was available as control because, under normal operation, fouling is monitored solely based on back pressure. Future trials would therefore include ΔT

data for control processing runs as well as runs with enzymated milk. The UHT processing plant used has a maximal pressure limit of 21 bar. Once this pressure was reached, processing is terminated in order to prevent damage to the heat exchangers during processing.

During normal production, low fat milk is processed prior to full cream milk in attempt to reduce fouling. In order to enable the use of previous processing data as a reference, the FPCL trial was performed similarly. An equal volume (25000 L) of low fat milk, also containing FPCL, was therefore processed prior to full cream milk. From each batch, two samples were collected at the beginning and the end of production. Samples were collected *ca.* 10 min apart.

From the results presented in Figure 4.9 it can be seen that, although no control data is available, ΔT remained constant throughout the trial. The maximal ΔT value obtained during this trial was 0.8°C. This was significantly lower than the 5°C ΔT limit, indicated as the red line, even after prolonged processing.

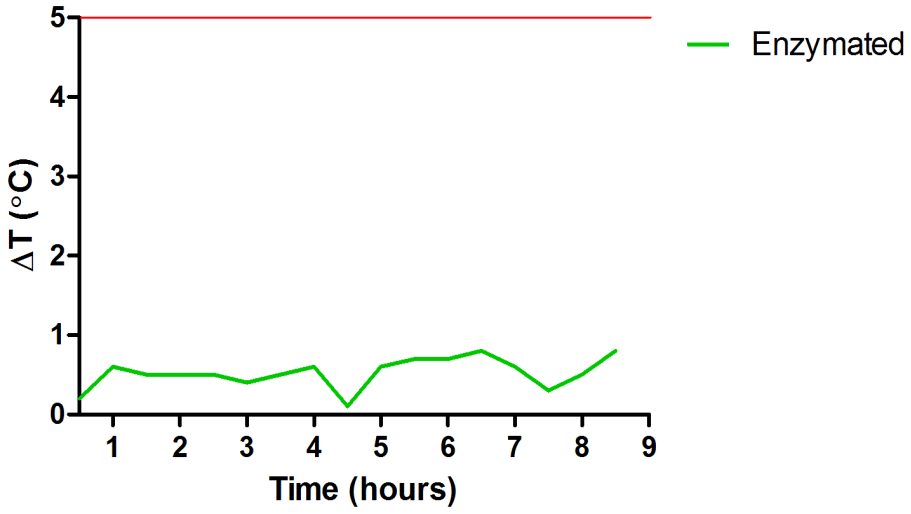


Figure 4.9 ΔT values obtained from full scale UHT production with the addition of FPCL In this figure the red line indicates the maximum back pressure limit.

The pressure data obtained from the full scale factory trials, conducted at Dewfresh, is depicted in Figure 4.10 . Upon comparison with the data obtained from pilot scale studies (Figure 4.7) a similar back pressure profile was observed for the control trial without FPCL. During these trials, as with pilot scale trials, system back pressure remained constant for the first hour of processing. This was followed by a gradual increase in back pressure and a rapid spike 30 min prior to termination of UHT processing. In comparison, addition of 10 ppm FPCL resulted in a more gradual increase in back pressure and an elimination of the pressure spike.

Under normal processing conditions, without the addition of FPCL, an intermediate wash was required after 4.5h of processing (≈26500 L milk). With the addition of 10 ppm FPCL the processing time was increased to 8.5 h. However, the venue for the trial was a medium scale production facility and has a maximal production capacity of 50000 L milk per day. At a processing rate of 6000 L per hour this limit was reached after 8.5 h. The results presented suggest that the 8.5 h could be improved on even further if larger storage tanks were available. Repeating the trials described at a larger venue will therefore be an interesting avenue to persue.

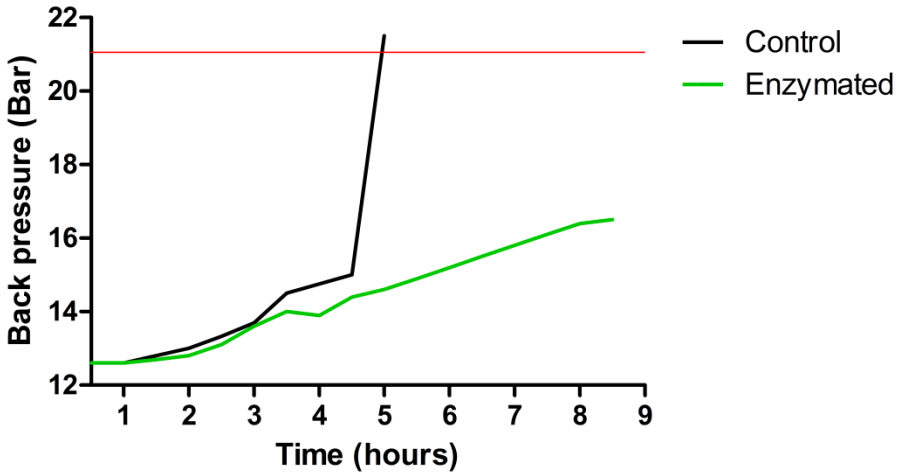


Figure 4.10 Back pressure profile of full scale UHT trials.

The studies conducted in this section illustrate the benefit of FPCL on UHT processing efficiency. However, the increase in efficiency was only achieved when the enzyme activity was within the specified range. Because FPCL is dosed on a volume per volume basis (ppm) rather than on enzyme activity the results obtained in the first pilot scale trials illustrate the need for enzyme activity confirmation prior to dosage. Currently the enzyme activity of FPCL is exclusively determined at designated distribution posts of which there are none in Sub-Saharan Africa. The commercial FPCL preparation is therefore distributed as having between 900 and 1100 units of enzyme activity (Addendum A). Since enzyme activity is not static, and may be affected by various factors including incorrect handling and storage, the results obtained illustrate the need for a facile and accurate method for enzyme activity verification prior to use. To date, no such assay has been described.

Availability of an FPCL activity assay, which is sufficiently simplistic and accurate, would increase the commercial value of FPCL by instilling confidence in the product quality. Such an

assay could also be used in determining the applicability of FPCL in different processing methods such as the Scania method used for the production of 2% milk and 40% cream.

4.4 CONCLUSION

The data presented in this chapter clearly indicates the benefit of FPCL during UHT processing of milk. These benefits include an increase of up to 100% in the total uninterrupted thermal processing duration. The results presented suggest that the use of FPCL will consequently result in an increase in the commercial profit margin of UHT milk. However, for successful application accurate methods for the monitoring of GCAT reaction products are needed. In this study product analysis by GC analysis, using the proposed method, resulted in hydrolysis of cholesteryl esters during sample preparation. A different method of product analysis is therefore required. Various enzyme linked kits are commercially available for this purpose, however, its use in milk analysis first needs to be evaluated.

The results obtained from pilot scale studies furthermore indicated a need for a facile method for GCAT activity verification in FPCL. Accurate activity data is essential to the successful application of GCAT in a commercial setting. This was especially evident from the initial pilot scale trials wherein no substantial effect could be shown after addition of FPCL. Availability of a rapid and accurate assay would not only increase the marketability of FPCL, but may also be used in the evaluation of applicability to different processing methods.

Based on the findings shown in this chapter, the following chapters will describe investigations of various methods which may be used for the quantification of GCAT reaction products in milk. These methods will subsequently be used for the development in an accurate reproducible activity assay for GCAT activity monitoring. Finally, the methods described will be used for the analysis of milk samples obtained from the pilot- and factory scale trials described in this chapter.

CHAPTER 5

EVALUATION OF METHODS FOR THE QUANTIFICATION OF GCAT REACTION PRODUCTS AND THE PARTIAL KINETIC CHARACTERIZATION OF GCAT

5.1 INTRODUCTION

The mechanism of action and molecular characteristics of GCAT have been the topic of extensive research. The lack of co-factor requirement and stability under diverse reaction conditions makes GCAT an attractive candidate for application in industries such as dairy. However, for successful industrial application, various quality control procedures must exist whereby enzyme reaction products can be quantified. In Chapter 3 it was shown that the current method for quantification of cholesteryl esters and free fatty acids (FFA) results in the hydrolysis of cholesteryl esters yielding cholesterol and FFAs as products. The need for an accurate, facile, activity assay was furthermore illustrated in this chapter. The difficulties discussed could result in large financial losses due to product quality deficiencies. In the case of GCAT, incorrect dosage and/or incubation times may lead to excessive FFA production which could render an entire batch of milk unusable. Accurate methods for the quantification of FFA, cholesterol, cholesterol esters and enzyme activity are therefore vital. This chapter describes the evaluation of chromatographic and enzymatic methods for the quantification of cholesterol, cholesteryl esters and FFA. These methods were subsequently used for FPCL quality control analysis and the development of an activity assay for the partial kinetic characterization of GCAT.

5.2 MATERIALS AND METHODS

Unless specified otherwise, all reagents and solvents used were of analytical grade and purchased from reputable scientific suppliers. Cholesterol/cholesteryl ester quantitation kit was obtained from Abcam® (Cambridge, United Kingdom). The NEFA free fatty acid (FFA) quantification kit was acquired from WAKO Chemicals (Neuss, Germany). Pierce BCA protein quantification kit was acquired from Pierce chemical company (Rockford Ill, USA). 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), Fluka para-Nitrophenyl butyrate (pNPB), 4-nitrophenol (NP) and cholesterol was obtained from Sigma-Aldrich (St Louis MO, USA). 26(27) - ^{14}C cholesterol (53 mCi/mmol) was purchased from Amersham International (United

Kingdom). Kieselgel 60-F_{254} thin layer plates were from Merck (Darmstadt, Germany). All solvents used for normal phase high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) were dried using 4Å molecular sieve (Merck Darmstadt, Germany) and filtered through 0.45 µm HVHP filter paper (Millipore) prior to use. Greiner bio-one 96 well flat bottom microtiter plates were purchased from Greiner (Frickenhausen, Germany)

Unless stated otherwise, all enzyme solutions used were obtained from DuPont® SSA Dairy Innovation Center Cape Town, South Africa. The enzyme solutions used were from commercial stocks and were stored as per manufacturer instructions. Unless specified otherwise Mat# A14076G190, Batch# 4861927699 FoodPro® Cleanline (FPCL) was used for all assays. Protein content was determined using the Pierce bicinchoninic acid (BCA) protein determination kit with bovine serum albumin as standard. Food grade deoiled soy lecithin (Solec™ F), used for activity studies, was obtained from Solae™.

5.2.1 Lipid extraction

Prior to sample extraction and quantification of GCAT reaction products, lipid extraction methods were evaluated in order to ensure maximal extraction efficiency. Percentage recovery was calculated by spiking full cream milk with ^{14}C labeled cholesterol (80 000 counts per minute). Each extract was resuspended in 100 µl chloroform and the radioactivity was counted in a liquid scintillation counter. The following extraction methods were evaluated.

Method 1 – Hexane extraction with saponification

One mL milk was accurately measured and added to a 15 mL teflon-lined screw cap tube containing 1 mL 10% ($^{w}/_{v}$) ethanolic KOH. The mixture was subsequently incubated for 30 min at 70 °C. The unsaponifiable supernatant was then extracted with 5 mL hexane and 2 mL deionized water. The extraction mixture was mechanically shaken for 5 min using an IKA Vibrax VXR shaker. After 5 min agitation, the organic upper phase was quantitatively transferred to a clean glass test tube. Hexane extraction was repeated three times.

The resulting extract was evaporated to dryness at 60 °C under a stream of nitrogen using a Techne sample concentrator. The extracted lipids were suspended in 100% hexane and directly subjected to HPLC analysis.

Method 2 – Hexane extraction Method 1 but without saponification.

Method 3 – Ether extraction as described in Chapter 4 Section 4.2.1 but without derivatization.

5.2.2 Chromatographic analysis

All HPLC solvents and sterol standards were purchased from Sigma-Aldrich (Darmstadt, Germany). All solvents were HPLC grade, and were filtered prior to use through a Millipore 0.45 µM pore size type HVHP filter. A Finnigan Surveyor (Thermo Scientific) running ChromQuest (version 4.2.34) software was used for all HPLC analysis. Fresh skim milk, used as matrix, was obtained from a local supermarket.

Preparation of sample and standard solutions

Milk fat was extracted using Method 3 described in Section 4.2.1. The dried extracts were dissolved in 100% ethanol by sonication followed by centrifugal filtration, using a Millipore type HVHP 0.45 µm pore size centrifugal filter, at 3500 x *g* for 10 min in a Hermle Z400 centrifuge. Lipid extracts were quantitatively transferred to clean 1.5 mL crimp cap HPLC vials (National Scientific) and subjected to HPLC analysis.

A standard solution, containing 1 mg/mL cholesterol, cholesteryl palmitate and cholesteryl stearate was prepared by dilution of 20 mg/mL stock solutions with 100% ethanol. A series of standards with concentrations ranging from 0 to 0.250 mg/mL was prepared by serial dilution in fat free milk prior to lipid extraction. All standards were prepared in duplicate. Of each sample/standard 20 µl was injected onto the HPLC column using a Finnigan Surveyor Autosampler plus fitted with a 25 µl sample loop. In order to minimize cross contamination resulting from multiple injections, the standards were run subsequent to the extracted milk samples. Prior to injection of the standards a solvent blank was injected in duplicate.

5.2.2.1 Reverse phase HPLC methods

Various solvents were tested as mobile phase for isocratic separation of cholesterol and cholesteryl esters. For sterol analysis the following C18 columns were compared: Waters Symmetry (4.6 x 150 mm), Waters Atlantis (3.9 x 150 mm) and a Phenominex Luna (4.6 x 150 mm). After changing the mobile phase, the column was equilibrated with at least 10 column volumes, or until a stable baseline was obtained.

Prior to injection of spiked milk extracts, standard solutions, containing 200 µg of each sterol standard, were injected to determine the retention times for each standard. The retention times obtained were subsequently stored in a method library to enable the integration of each peak after completion of the run. The column temperature was maintained at 30°C in order to

compensate for variations in ambient temperature. The general HPLC method used was similar with each solvent system and is described in Table 5.1.

Table 5.1 General method used for isocratic HPLC analysis of milk extracts.

Flow rate:	1.5 mL/min
Injection volume:	20 µl
Detection wavelength:	210 nm
Spectral scan range:	400 – 190 nm
Column oven temperature	30 °C

After a satisfactory solvent system was identified, the separation profiles of different C18 columns were compared. Each column was thoroughly equilibrated prior to standard injection as described previously. Columns were compared based on the retention times, peak areas and peak resolution of injected standards.

5.2.2.2 Normal phase thin layer chromatography method

Solvent systems consisting of hexane and isopropanol or ethyl acetate were compared with TLC using silica backed Kieselgel 60-F_{254} plates. The retention of individual and mixed standards was compared with varying ratios of solvent. TLC plates were developed by spraying with 50% sulfuric acid followed by heating at 100 °C for 10 min. For kinetic studies of GCAT a mobile phase consisting of 95:5 hexane: ethyl acetate was used.

5.2.3 Cholesterol quantification using modified Lieberman-Burchard reagent

Based on an assay described by Kim and Goldberg[155], the cholesterol content of aqueous samples were quantified following solvent extraction using a modified Lieberman-Burchard (LB) reagent. Solvent extracted lipids were resuspended in 100 µL hexane prior to analysis. To prepare LB reagent, 40 mL glacial acetic acid was added to 44 mL ice-cold acetic anhydride. To this mixture 6 mL ice-cold concentrated sulfuric acid was added. The final concentrations of reagents were therefore 40:44:6 glacial acetic acid, acetic anhydride and sulfuric acid.

The LB assay described in the text was adapted to a microtiter plate based assay with a final volume of 200 µL per assay. All incubations were performed at 37°C. The optimal absorbance wavelength of LB reagent was subsequently determined. This was achieved performing an UV spectral scan (800-480 nm) of a 1 mg/mL cholesterol standard in hexane on a Biotek Powerwave 340 microtiter plate reader in Greiner bio-one flat bottom microtiter plates. The optimal incubation time was determined by reading the absorbance of 1 mg/mL standard in LB reagent in five minute increments at the wavelength determined above for 40 min. The effect of

assay volume on incubation time was evaluated by increasing the assay volume to 1 mL. Sample absorbance was measured every 30 seconds in a Beckman DU-65 recording spectrophotometer at 37°C for a total of 60 min. Variations in the ratio of sample to LB reagent were investigated by increasing the volume of standard in relation to LB reagent. The results were evaluated by comparison of the absorbance values (minimum and maximum) of a standard range of cholesterol standards ranging from 0 – 1 mg/mL. The ratios assayed were 10:190, 20:180 and 40:160 ($^{\mu l}/_{\mu l}$) standard: LB reagent.

5.2.4 Enzyme coupled GCAT product quantification assays

5.2.4.1 Free fatty acid quantification

Free fatty acids were quantified using the NEFA HR(2) FFA quantification kit with oleic acid as standard. The kit was adapted for use in a microtiter plate assay. The efficiency of this adaptation was evaluated as the regression fit of an oleic acid standard dilution series. All incubations were performed at 37°C.

Assay procedure:

To 20 µL standard/sample, 110 µL reagent R1 was added followed by 5 min incubation at 37°C. The absorbance of the reagent blank was subsequently determined at 540 nm in a Multiscan EX (Labsystems) microtiter plate reader. After reading the absorbance of the reagent blank, 55 µl reagent R2 was added to each well and incubated at 37°C for an additional 5 min. Following the final incubation the absorbance of each well was determined at 540 nm. The final absorbance was calculated by subtracting the first reading from the second. The final absorbance and concentrations of the standards were subsequently plotted to produce a linear calibration curve. The concentration of FFA in each sample was calculated using this calibration curve.

5.2.4.2 Cholesterol/Cholesteryl ester quantitation

Total cholesterol and cholesteryl esters were quantified using an enzyme based quantitation kit provided by Abcam Biochem. For quantification of cholesterol/cholesteryl esters in milk, milk samples were homogenized by Ultra Turrax and diluted 10-fold with distilled water (dH_2O) prior to each assay. Assays were performed with a 35 µl diluted milk sample as per manufacturer instructions. All incubations were performed at 37°C. The fluorescence values were determined on a Thermo Varioskan multiplate reader with using an excitation wavelength of 535 and an emission wavelength of 590.

Enzyme-based cholesterol and cholesteryl esters quantification relies on the use of three enzymes namely cholesterol esterase, cholesterol oxidase and horseradish peroxidase (HRP). Figure 5.0 depicts the assay principle. In the first reaction, cholesteryl esters are hydrolyzed to cholesterol and FFA by the action of cholesterol esterase. Cholesterol is subsequently oxidized, by cholesterol oxidase, to cholest-4-ene-3-one and H_2O_2. The non-fluorescent probe, ADHP, is subsequently oxidized by HRP, with H_2O_2 acting as the electron source, to yield highly fluorescent resorufin (Ex/Em = 535/590)[156,157]. Free cholesterol content can be quantified by omission of cholesterol esterase in the first reaction. Cholesteryl ester content is subsequently calculated as the difference between total cholesterol (with cholesterol esterase added) and free cholesterol.

$$\text{Cholesterol esters} \xrightarrow{\text{Cholesterol Esterase}} \text{Cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholest-4-ene-3-one} + H_2O_2$$

$$H_2O_2 + \text{ADHP (non-fluorescent)} \xrightarrow{\text{HRP}} \text{resorufin (fluorescent)}$$

Figure 5.0 General reaction principle for the enzyme-based quantification of total and free cholesterol[156]

5.3 ENZYME ACTIVITY ASSAYS

Prior to all kinetic analysis of FPCL, the protein content of the stock solution was quantified using the Pierce BCA protein determination kit with bovine serum albumin as standard. Each protein determination was performed in triplicate as per the manufacturer instructions. The commercial FPCL preparation contains multiple components. Enzyme spike concentrations were subsequently quantified as amount protein, in µg, instead of the mass of the FPCL used.

5.3.1 Phospholipase activity assay

5.3.1.1 Substrates for phospholipase activity quantification

Food grade lecithin was used as substrate for assay development purposes. Prior to all assays, the lecithin powder was “washed” with acetone in order to remove residual FFAs and other contaminants. This was achieved by suspending 50 g dry lecithin powder in 500 mL acetone with stirring for 20 min. The acetone was subsequently removed by vacuum filtration through Whatman no. 1 filter paper on a Buchner funnel. The FFA content of a dried powder sample

was determined, and washing was repeated until no residual FFA was present. Typically, two wash steps were sufficient.

For kinetic analysis of the hydrolytic activity of GCAT, SUVs, consisting of egg yolk lecithin, were prepared as described by Rautenbach[158]. A dilution series of lecithin, between 0.05 – 2 mM, was prepared from a 20 mg/mL stock solution in chloroform, in chemically clean thin-walled glass test tubes. The chloroform was evaporated under a stream of nitrogen to leave a thin lipid film on the glass. The lipid was subsequently resuspended in a 20 mM Tris-HCl, pH 7.0, buffer containing 160 mM KCl flushed with N_2. This suspension was sonicated in a sonic bath filled with a 1% Triton X-100 solution until the suspension was translucent. Optimal sonication time was initially determined by sonicating a lipid suspension for various time periods and visually comparing the translucency and FFA content of the resulting suspensions with each other.

5.3.1.2 Enzyme inactivation studies

In order to prevent phospholipid hydrolysis following aliquot collection, enzyme activity had to be quenched. For this purpose, three enzyme inactivation methods, i.e. heat-, perchloric acid- and liquid nitrogen- inactivation, were evaluated. Evaluation of each method included assessment of the effect of treatment on the phospholipid substrate and efficient enzyme inactivation. Inactivation assays were performed by incubating a volume of FPCL in 2.5% ($^{m}/_{v}$) lecithin at 37°C. Prior to addition of enzyme to the phospholipid substrate, a 100 µL sample was collected as baseline control. The substrate was subsequently spiked with 441 ng enzyme per mL substrate. 100 µL aliquots were collected at 2 min intervals. Inactivation treatment was applied to each sample as follows:

i. Heat inactivation:

The collected samples were transferred to sample tubes pre-equilibrated to 90°C in an OMEG Scientific heating block. Each sample was heat treated for 15 min.

ii. Method of perchloric acid inactivation:

The collected sample was transferred to a sample tube containing 10 µL perchloric acid. Following inactivation, the acid was neutralized with 10 M KOH. The amount of KOH needed for neutralization was evaluated using pH indicator strips (Merck Darmstadt, Germany). Once the

optimal volume of KOH was established, the experiment was repeated and the FFA quantified as described.

iii. Liquid nitrogen inactivation:

The collected aliquots were rapidly transferred to plastic thin walled 250 µL polymerase chain reaction tubes and immersed in liquid nitrogen. After all aliquots were collected, the samples were heat treated for 15 min at 90°C.

Following heat treatment, all samples were centrifuged at 3000 x *g* for 5 min and the FFA content of the supernatant was quantified using the NEFA HR FFA quantification kit as described previously. The results from each assay were compared in terms of linearity of the collected samples when plotted, as well as the FFA content of the baseline blank samples following treatment.

5.4.1.3 Enzyme activity assay

The hydrolytic activity of GCAT, using acetone washed lecithin, was quantified by adding 840 ng protein to a 2.5% ($^{m}/_{v}$) lecithin solution at 37°C in poly top glass vials. A 100 µL aliquot was collected at 0 min followed by aliquots at 2 min intervals for a total of 10 min. Enzyme activity in all the aliquots collected was completely inhibited as described in Section 5.3.1.2.iii. Following enzyme inactivation, the FFA content was quantified as previously described in Section 5.2.4.1. The FFA concentration was plotted as a function of time to yield individual rate equations.

The same assay was used for kinetic analysis with SUVs consisting of DOPC or purified egg yolk lecithin (Sigma-Aldrich) as substrate. In these assays 1.2 mL SUV suspension of each substrate concentration was prepared, and 1 mL was used for each assay. Incubations were performed in 8x40 mm shell vials in an OMEG Scientific heating block maintained at 37°C. To the substrate solution, 500 ng protein was added and a total of six aliquots were collected at timed intervals starting with T_0 (no enzyme). Rate equations were prepared as described above and kinetic analysis was performed using GraphPad Prism® 5 software.

5.3.2 Acyl transferase assay

For monitoring the transferase reaction catalyzed by GCAT, SUVs consisting of DOPC and cholesterol were prepared as described in section 5.3.1.1 to a final volume of 1.2 mL. In these assays, 26(27) - ^{14}C cholesterol (53 mCi/mmol) was added as reaction tracer. Incubations were performed in 8x40 mm shell vials in an OMEG Scientific heating block maintained at 37°C.

After preparing the SUV substrate suspension, 1 mL was transferred to a chemically cleaned shell vial and equilibrated to 37°C for 10 min. Following equilibration, 210 ng protein was added and 6x100µl aliquots were collected starting at T_0. The enzyme activity of each aliquot was terminated by the addition of 5 mL dichloromethane in 15 mL teflon-lined screw cap culture tubes. Prior to lipid extraction 300 µl dH_2O containing 0.05% ($^w/_v$) phenolphthalein and 1% ($^w/_v$) NH_3OH was added to each sample to increase the visibility of phase separation. Lipids were extracted by mechanical shaking for 10 min using an IKA VXR Vibrax basic at 1500 RPM. Each sample was subsequently centrifuged at 3000 x *g* for 10 min. The aqueous phase of each sample was removed by aspiration and the residual solvent was evaporated under a stream of nitrogen at 50°C. The dried lipids were subsequently dissolved in 50 µl chloroform and separated using TLC.

For analysis, 20 µL of each sample was applied to a 10 x 20 cm TLC plate and cholesterol and cholesteryl esters were separated with hexane containing 5% ($^v/_v$) ethyl acetate as mobile phase. The plates were developed by spraying with diluted sulphuric acid (50% ($^v/_v$)) as described in Section 5.2.2.2. Radiolabeled cholesterol and cholesteryl oleate spots (corresponding to standards) were excised, added to 4 mL scintillation fluid and counted in a liquid scintillation counter (Perkin Elmer Tri-Carb 2810 TR). Rate equations, for the formation of cholesteryl oleate, were prepared and the reaction rate, in mM/min, was determined. Units of activity was subsequently converted to µmol/min using Equation 5.1. Each assay concentration, used for Michaelis-Menten kinetic analysis, was performed in triplicate.

$$\mu mol/\min = mM/min \times \ assay\ volume\ (mL)$$

Equation 5.1 Equation for the conversion enzyme activity, in mM/min, to µmol/min.

5.3.3 Continuous assays using pNPB as substrate

The activity of GCAT was continuously monitored spectrophotometrically at 400 nm and 37°C on a Biotek Powerwave 340 microtiter plate reader by the release of pNP from pNPB. The liberated pNP was quantified by comparison to a calibration curve prepared from a 10 mM pNP stock. Prior to each assay a known amount of FPCL (mg protein) was diluted with assay buffer (10 mM HEPES-buffer, 0.5 M NaCl and 0.1% Triton X-100, pH 7.0) to yield a maximal absorbance change of ≈1.0 AU/min. In our hands a 16 000-fold dilution of the FPCL stock (≈88 ng protein) was sufficient for all samples assayed. The diluted enzyme solution was kept on ice when not in use, and was prepared fresh every two hours. The final assay volume for this

method was 200 µL and it was sufficiently sensitive to detect GCAT activity in milk samples dosed with 10 ppm FPCL. Milk samples were diluted 10 fold prior to the assay.

Assay procedure:

A calibration curve of pNP was prepared in the range between 0.001 and 0.2 mM (assay concentration), from a 10 mM pNP stock. Of each standard 10 µL was added to 190 µL assay buffer in a microtiter plate and the contents were mixed by swirling the plate in a figure of eight movement.

Activity assay:

The following volumes of reagents were added to the wells of a 96-well microtiter plate. At least four assay blanks were run in concord with the samples in order to determine the assay background absorbance.

	Blank	**Sample**
Assay buffer	90 µL	90 µL
Substrate solution	10 µL	10 µL

After addition of the substrate solution the plate was swirled in a figure of eight movement to mix the contents of each well. Of the diluted enzyme solution/milk sample 150 µL was added to the well adjacent to the well containing the assay substrate to ensure similar temperature equilibration. The plate containing diluted enzyme and substrate solution was subsequently equilibrated to 37°C inside a thermostated plate reader. After 5 min equilibration the following was transferred to the wells containing assay buffer and substrate solution.

	Blank	**Sample**
Assay buffer	100 µL	0 µL
Enzyme solution	0 µL	100 µL

Immediately after the equilibrated enzyme solution was added, the absorbance of each sample/blank was measured at 400 nm in 5 second intervals at 37°C for 1 minute using the Biotek Gen5 software. The pNP concentration for each data point was subsequently determined by comparison to a calibration curve. pNP formation (in mM) was plotted as a function of time, and rate equations were constructed.

For kinetic analysis of GCAT, the same assay procedure was employed. However, a dilution series of pNPB substrate from 0.01 – 3.5 mM (assay concentration) was used instead of a

constant substrate concentration of 10 mM. Each substrate concentration was repeated in quadruplicate. Product formation was quantified by comparison to a calibration curve of pNP. The rate of product formation (mM/min) was subsequently converted to standard enzyme activity units (U) in µmol product formed per minute by using Equation 5.2. Specific activity was calculated using Equation 5.3. The calculated enzyme activity units were subsequently plotted against substrate concentration. Michaelis-Menten analysis was performed on this data using GraphPad Prism® 5 software.

$$U = concentration\ pNP(mM)\ X\ assay\ volume\ (mL)$$

Equation 5.2. Equation for the conversion of reaction rate, in $mM.min^{-1}$, to units of enzyme activity, in $\mu mol.min^{-1}$.

$$Specific\ U = \frac{concentration\ pNP(mM) X\ assay\ volume\ (mL)}{Protein\ (mg)}$$

Equation 5.3. Equation for the conversion of units enzyme activity, in µmol/min, to specific activity in µmol/min/mg protein.

5.3.4 Evaluation of FPCL for use in the Scania processing method

Following the development of a facile and accurate GCAT activity assay the acceptability for the use of FPCL in the Scania processing method was evaluated in fat free buffered systems using the synthetic substrate pNPB. In these assays 1 mL Novo buffer* was spiked with 3 ppm FPCL and incubated at 65°C. Aliquots of 100 µL were subsequently collected at 0, 10, 20, 30, 60 and 90 min. Collected samples were stored on ice until activity quantification. Residual enzyme activity was subsequently comparatively determined with pNPB as substrate using the microtitre plate assay described in Section 5.3.3. Unspiked buffer was used as experimental control.

5.4 DATA ANALYSIS

All experiments were performed at least in triplicate, unless stated otherwise, and the data presented as the mathematical mean. Data presentation, analyses and statistics were performed using GraphPad Prism® 5 software.

* Novo buffer: 2.7mM sodium citrate, 10 mM phosphate, 7.91 mM citric acid, 19.43 mM potassium hydroxide, 4.08 mM magnesium chloride, 5.1 mM calcium chloride and 3.33mM sodium carbonate pH 6.5

5.5 RESULTS AND DISCUSSION

5.5.1 Liquid chromatographic analysis

For chromatographic analysis of milk lipids, lipid extraction is required. Before attempting chromatographic analysis of milk samples, three lipid extraction methods were evaluated and compared in terms of cholesterol recovery. With the use of radiolabeled cholesterol it was shown that extraction Method 3 recovered 94% of the labeled cholesterol compared to 79% and <10% for Methods 1 and 2 respectively.

Lipid hydrolysis results in the formation of glycerol and FFA moieties. These moieties will not be extracted by hexane due to their more polar nature. As can be seen in Figure 5.1, extraction Method 1 yielded an extract containing fewer lipids which results in reduced peak overlapping. However, lipid hydrolysis also results in the hydrolysis of cholesteryl esters. This method of extraction is therefore only viable for use in total cholesterol quantification. Omission of saponification resulted in less of the total lipid being extracted. For these reasons extraction Method 3 was used for all subsequent extractions in preparation for GC and HPLC analysis.

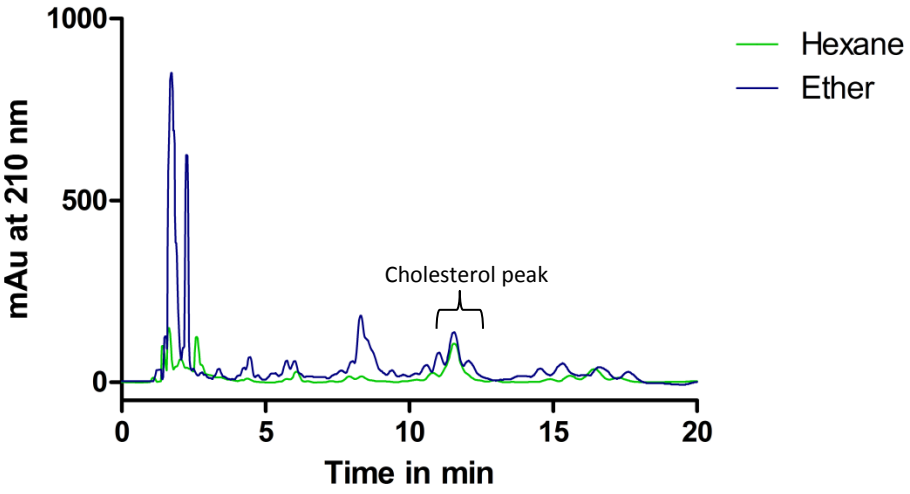


Figure 5.1 Comparison of extraction methods with HPLC-UV using a Waters Symmetry C_{18} column with methanol as mobile phase at 1 mL/min.

The use of HPLC for quantitative purposes has been well established due to its ease of operation, reproducibility and accuracy. HPLC analysis has the advantage that cholesterol can be readily detected using a UV detector. However, the low maximum UV absorbance of cholesterol at 210 nm, severely limits the usable polar solvent range. With the exception of

acetonitrile, most polar solvents have UV cut-off values above 210 nm, resulting in high background absorbance and decreased resolution.

The low polarity of long-chain cholesteryl esters furthermore results in limited solubility in polar organic solvents. This reduces the useable solvent range for HPLC analysis even further. A cholesteryl ester solubility assay was subsequently performed to evaluate possible polar solvents for separation of cholesterol and cholesteryl esters. From these studies, it was shown that both cholesteryl stearate and cholesteryl palmitate remains in solution when diluted from a 10 mg/mL stock in ethanol. Ethanol was subsequently used as the main solvent for RP-HPLC analysis. Figure 5.2 shows the retention of a cholesterol standard by C_{18} columns from different manufacturers. Ethanol, isopropanol and water (93:3:5) was used as mobile phase. As can be seen, the retention of cholesterol varies significantly between different columns under similar conditions. Increased retention times would result in an increased run duration and solvent usage. In order to reduce overall run duration, the Waters Atlantis column was used for all subsequent analyses.

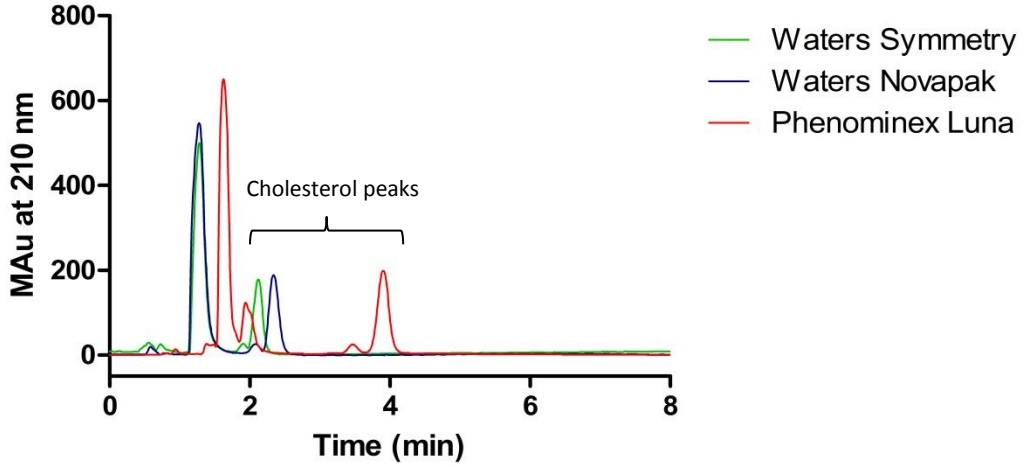


Figure 5.2 Retention profiles for cholesterol on C_{18} columns from different manufacturers. The columns tested were Waters Symmetry, Phenominex Luna(2) and a Waters Atlantis.

A standard mixture consisting of cholesterol, cholesteryl stearate and cholesteryl palmitate was subsequently injected (4 µg) using the same solvent as described above. The result for this separation is depicted in Figure 5.3. As can be seen, both cholesteryl esters are strongly retained. As a result, the peak shape and resolution is significantly reduced. In order to improve peak shape and decrease retention, mobile phase water was substituted with methanol. This resulted in the reduction of total analysis time to 8min.

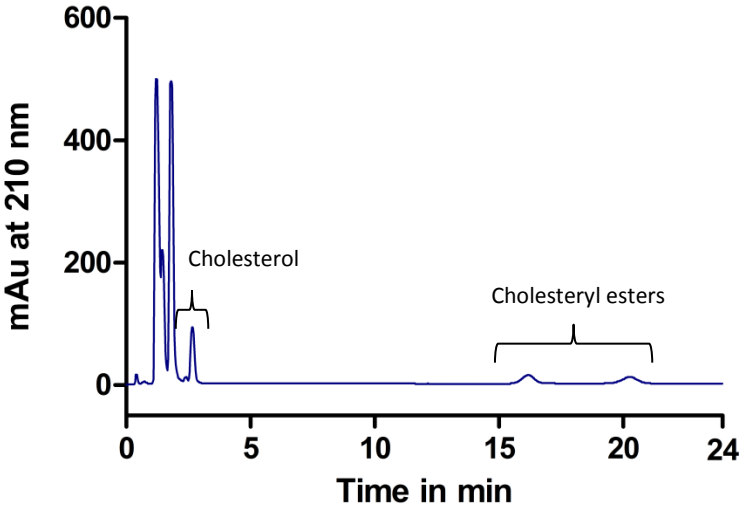


Figure 5.3 HPLC separation of a standard mix containing 200 µg/mL cholesterol and cholesteryl ester standards with ethanol isopropanol and water (93:3:5) as mobile phase. A Waters Atlantis C_{18} column was used for separation.

Since the HPLC method is intended for the separation and quantification of cholesterol and cholesteryl esters in milk samples, fat free milk, spiked with standards, were used as sample matrix in subsequent studies. Figure 5.4 depicts the HPLC separation of fat free milk spiked with 0.2, 0.1 and 0.01 mg/mL ($^{m}/_{v}$) of the standard mixture as described previously. From this figure it can be seen that the overall separation profile is similar for each sample with the exception of the cholesterol and cholesteryl standard peaks

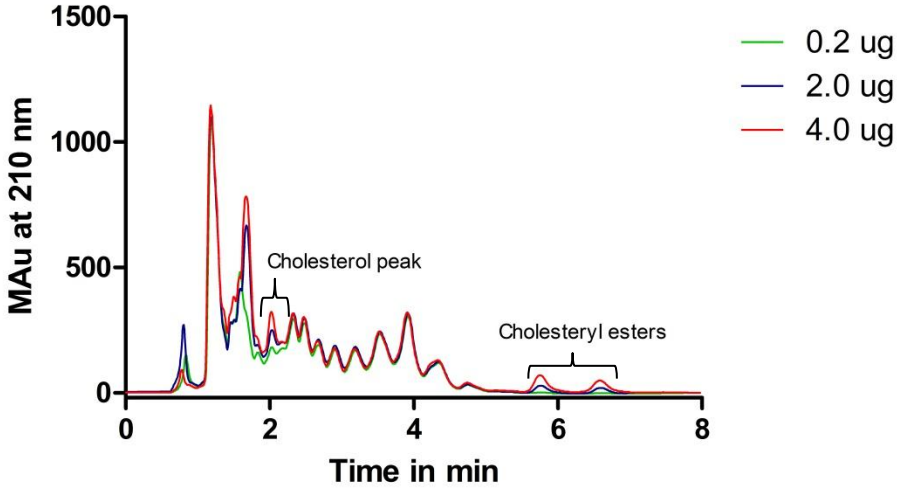


Figure 5.4 HPLC analysis of fat free milk spiked with cholesterol and cholesteryl ester standards. HPLC conditions: Waters Atlantis C18 column with ethanol: isopropanol and methanol (93:3:5) as mobile phase.

The HPLC method was validated against a microtiter plate assay using Liebrman-Burchard reagent as will be described in the following section. This assay was selected due to its sensitivity, and because samples are extracted and prepared for analysis in the same manner. These results suggest that although analysis of milk lipids result in high background, this can be compensated for using fat free milk as matrix.

5.5.2 Cholesterol quantification using a modified Lieberman-Burchard reagent

Cholesterol quantification using the assay described by Kim and Goldberg[155], with a modified LB reagent, was successfully scaled to a micro-assay with a final volume of 200 µL. When adding LB reagent directly to aqueous samples, the reaction mixture reacts violently resulting in a reduction of assay sensitivity. Consistent with this, Kim and Goldberg[155] observed a 50% reduction in sensitivity when 8% ($^{v}/_{v}$) water was added to each assay. This is mainly due to the acidic nature of the LB reagent. For this reason, lipid extraction and suspension in an organic solvent, such as hexane, is required in order to accurately quantify cholesterol in aqueous samples such as milk.

As can be seen in Figure 5.5, performing a spectral scan of LB reagent containing a cholesterol standard, following 20 min incubation at 37°C, indicated that LB reagent has an optimal absorbance at 615-625 nm when incubated with cholesterol. This result was consistent with previous descriptions of assays using LB reagent[155,159]. The absorbance of all subsequent assays was consequently measured at 620 nm.

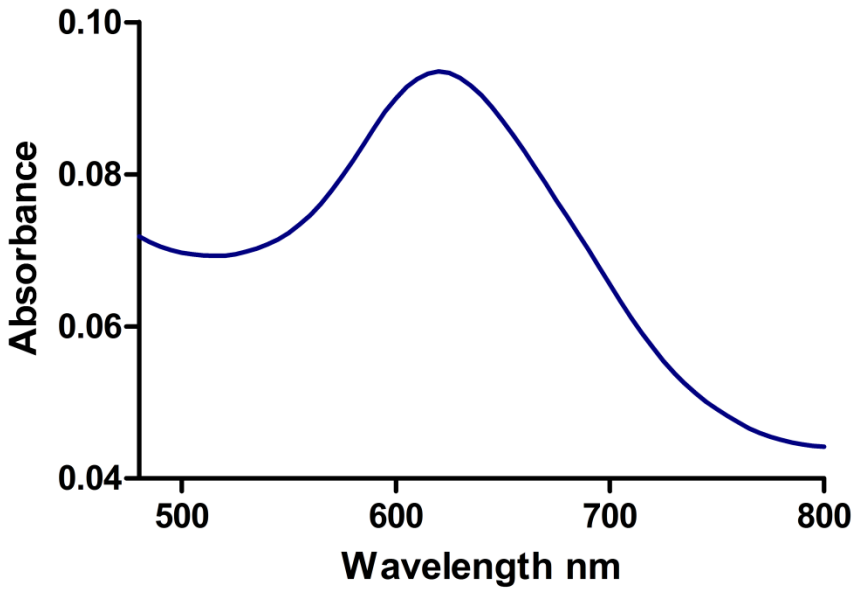


Figure 5.5 Spectral scan (480 – 800 nm) of LB reagent containing a cholesterol standard in a final assay volume of 200µl following 20 min incubation at 37°C**.**

The effect of incubation time was subsequently evaluated in order to determine if the final assay volume has an effect on assay sensitivity. As can be seen from Figure 5.6.A, maximal absorbance for the microtiter plate assay was reached after 25 min incubation at 37°C. After this point the absorbance steadily decreased at a rate of 0.0007 absorbance units per min. This was, however, not the case for a final assay volume of 1 mL containing the same ratio (1:4) cholesterol standard: LB reagent. As can be seen in Figure 5.6.B, maximal absorbance was achieved after 10 min incubation at 37°C. After 10 min, the absorbance decreased at a rate of 0.0092 absorbance units per min. Since the optimal incubation time was not constant when different assay volumes were compared, this result suggests that this assay does not scale in a linear fashion and optimization for each assay volume is essential.

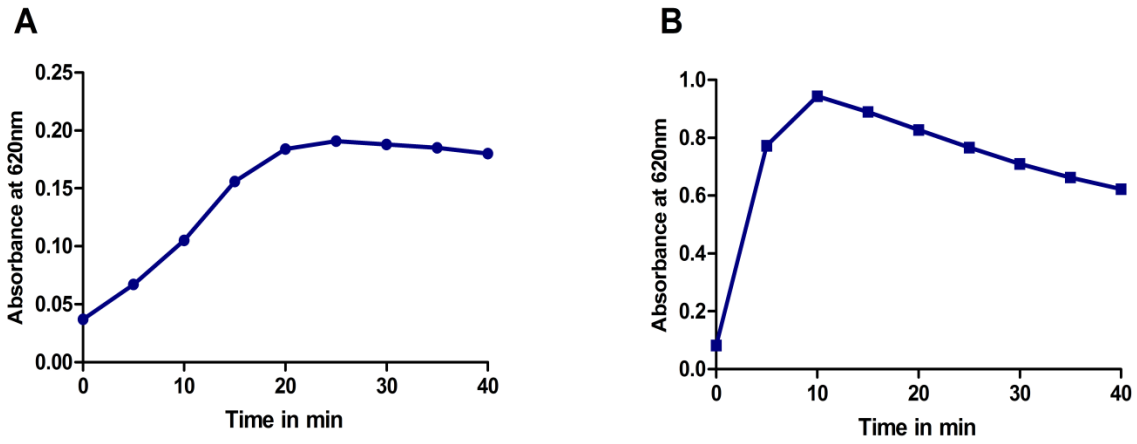


Figure 5.6 Comparison of optimal incubation time for the micro-assay (A) and assay with a final volume of 1 mL (B). Each assay was performed with 4µg cholesterol standard and incubated at 37°C for the times indicated and determined at 620nm.

The ratio of sample to LB reagent did not have an effect on the linearity of the assay. As can be seen from Figure 5.7, the regression line formula and R^2 was not significantly affected by variations in the ratio of standard to LB reagent. A linear increase in absorbance was observed for an increase in the ratio of cholesterol to LB reagent.

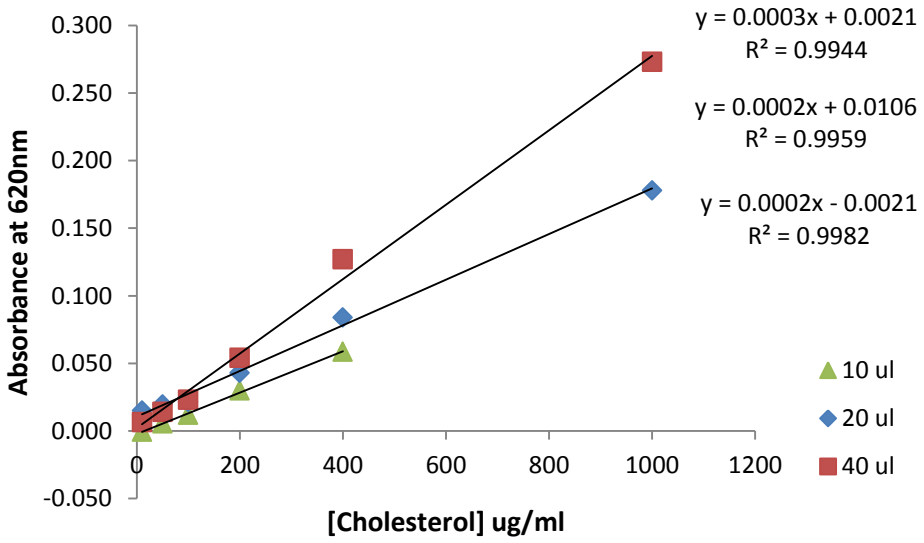


Figure 5.7 The effect of sample to LB reagent volume on assay linearity and final absorbance.

In order to validate the results obtained, the cholesterol content of an egg yolk extract (prepared by Method 3 in Section 5.2.1) was determined using each assay (20 µl and 40 µl sample). Both assays yielded a final cholesterol concentration of ≈498 µg/mL egg yolk extract. Based on the

results described above, all subsequent microtiter plate assays were performed using the conditions described in Table 5.2 .

Table 5.2 Volumes and incubation conditions for cholesterol quantification in lipid extracts.

Volume sample (µL)	20
Volume LB reagent (µL)	160
Incubation time (min)	25
Incubation temperature	37°C

The assay described was linear in a cholesterol concentration range from 26 µM to 26 mM and can be used for the simultaneous quantification of multiple lipid extracts. This method was furthermore not affected by cholesteryl esters present in the extracted sample.

5.5.3 Enzyme coupled GCAT product quantification assays

5.5.3.1 Free fatty acid quantification

As discussed in Chapter 3, the FFA content of samples can be quantified using titemetry or chromatographic analysis. However, these methods require relatively large sample volumes and/or individual standards for each FFA present. The use of enzyme-based assay kits enables the simultaneous quantification of multiple samples without the need for sample extraction and individual FFA standards.

The NEFA HR kit is intended for use with automated analysis systems such as the Konelab 20XT Clinical Chemistry Analyzer. For this study the kit protocol was adapted for use in a microtiter plate based assay by reducing the individual reagent volumes. The efficiency of this adaptation was evaluated as the regression fit of an oleic acid standard dilution series. As can be seen from Figure 5.8, the NEFA HR(2) kit was successfully adapted to a microtiter plate based assay for a concentration range between 0.05 and 1 mM oleic acid. Using this assay, the FFA content of low sample volumes (10 µl) could be accurately quantified in a two-step 10 min spectrophotometric assay.

Due to the specificity of the enzymes present, only FFA is quantified without interference from lipids present in the sample. The use of the NEFA HR(2) assay enabled quantification of enzyme activity in small reaction volumes, thereby reducing the quantity of substrate required during activity assays. This kit could furthermore be used for the quantification of total FFA in milk samples obtained from pilot- or full-scale FPCL trials. These results will, however, be discussed in more detail in the following sections.

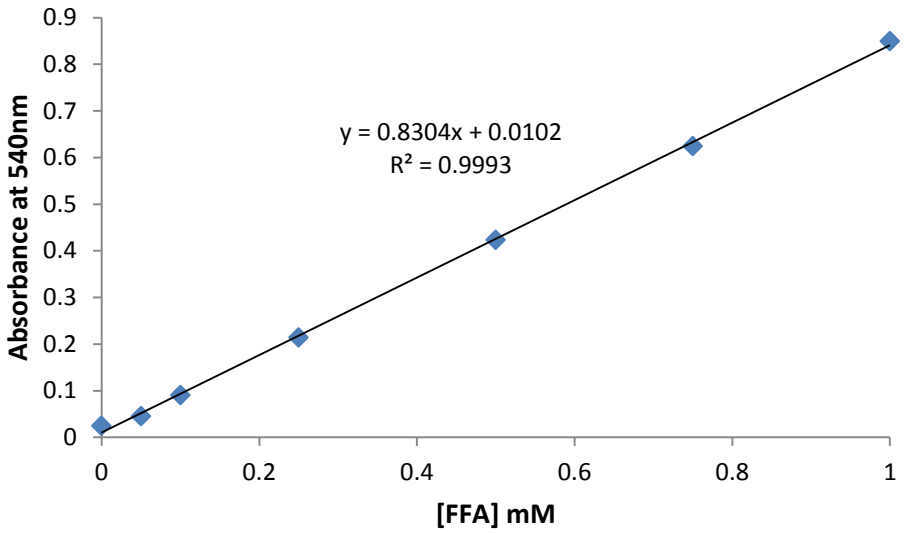


Figure 5.8 Standard curve produced by serial dilution of a 1 mM oleic acid standard. The regression line does not pass through zero due to low-level background absorbance.

5.5.3.2 Cholesterol/Cholesteryl ester quantification

Although the cholesterol content of milk samples could be accurately quantified using the LB assay described previously, this assay requires lipid extraction prior to quantification. Additionally, in order to quantify total cholesterol (cholesterol and cholesteryl esters), ester hydrolysis is required prior to analysis. Total cholesterol quantification, using LB reagent, is therefore time consuming and labor intensive. The use of enzyme based kits allows for the rapid quantification of both cholesterol and cholesteryl esters in a microtiter plate fluorescent assay.

This assay proved sufficiently sensitive to quantify cholesterol and cholesteryl esters in milk samples. However, although activity assays could be performed successfully with the use of the cholesterol/cholesteryl ester quantification kit, these kits are expensive (up to $5 per well). The use of quantification kits were therefore limited to analysis of milk samples collected from pilot- and full-scale FPCL trials.

5.5.3.3 Quantification of cholesterol and cholesteryl esters in milk samples obtained from FPCL trials

Subsequent to all pilot scale trials described in Section 3.2.2.1, the cholesterol and cholesteryl ester content of the collected samples were determined as described in this section. The average cholesterol conversion data for these trials are depicted in Figure 5.9. The % cholesterol conversion was calculated as the percentage difference between total cholesterol and free cholesterol subsequent to UHT treatment. As can be seen, the conversion of

cholesterol to cholesteryl esters was similar for all trials. An average cholesterol conversion of 68% was achieved for all the trials performed. From this figure an increase in the cholesteryl ester of the control samples can be observed. However, milk cholesterol content is known to vary with the season. This result is therefore most likely due to lipid variations with a change in season.

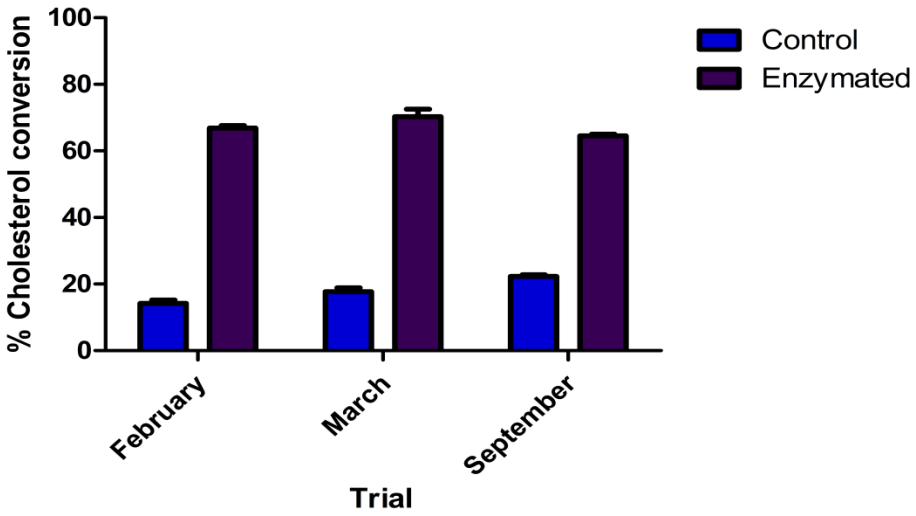


Figure 5.9 Cholesterol conversion data from pilot trials performed in 2012. The cholesterol content of each sample was determined in duplicate.

Figure 5.10 depicts the cholesteryl ester content of each sample collected from the factory trial described in Chapter 4 Section 4.2.2.2. The cholesteryl ester content is calculated as the amount of cholesteryl ester in relation to total cholesterol. As can be seen, the cholesteryl ester content again did not differ substantially, irrespective of milk fat content and incubation time. Although this would suggest that maximal cholesterol conversion had been achieved, this was not reflected by a substantial increase in the FFA content as depicted in Figure 5.11. Furthermore, no significant trend could be observed in the FFA content of the collected samples.

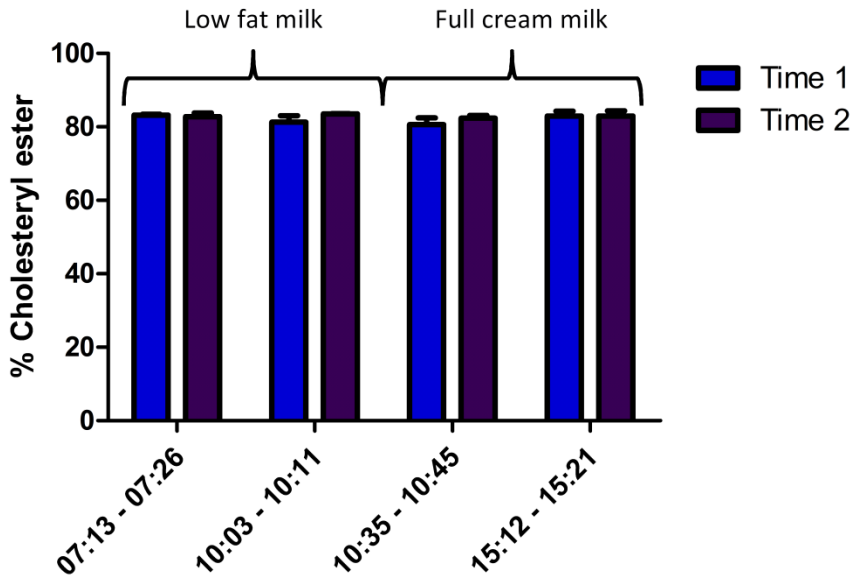


Figure 5.10 % Cholesteryl ester of each sample calculated as the amount cholesteryl ester in relation to total cholesterol content. The X axis depicts the sample collection times for each duplicate.

The FFA quantification method described in Section 5.2.4.1 measures FFA in molar concentrations. Percentage FFA was subsequently calculated using the molecular weight of oleic acid as reference. The values depicted in Figure 5.11 are therefore estimates. In this figure the red line indicates the maximal allowed amount of FFA. As can be seen addition of FPCL did not substantially increase the milk FFA content, even after prolonged incubation (07:26 – 10:11).

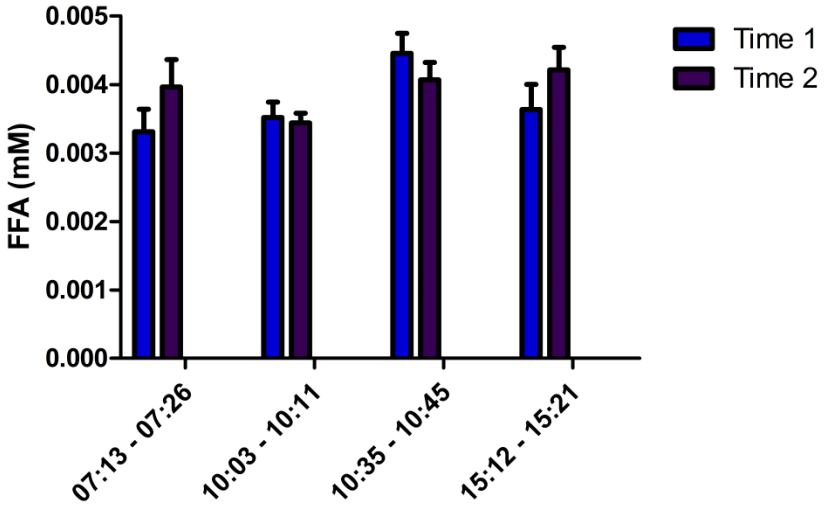


Figure 5.11 Comparison of the % FFA of the collected samples. In this figure samples were duplicate samples were collected 10 min and 2.5h from each other. The maximal suggested % FFA before the product loses commercial appeal is estimated at ≈0.25 % FFA.

5.5.4 Enzyme activity assays

FPCL, containing GCAT as the active enzyme, is intended for industrial application. A rapid and accurate assay for activity quantification is therefore essential to ensure optimal dosage. In order to increase economic viability, this assay should also allow for high throughput screening of multiple samples simultaneously. As discussed in Chapter 3, various methods are available whereby the activity of GCAT may be quantified. However, these methods are labour intensive, time-consuming and often rely on the use of radiolabeled substrates.

The use of synthetic substrates enables continuous reaction monitoring, yielding more accurate activity quantification. However, it has not yet been shown if the units of enzyme activity are similar when compared to assays using natural substrates. For this reason, it is important to compare these assays and determine the kinetic parameters of GCAT with the use of different substrates. Comparison of the kinetic parameters would give an indication if pNPB may be used as substrate for accurate activity quantification.

5.5.4.1 Phospholipase activity assay using pNPB as substrate

Due to the labour intensive nature of enzyme activity determination using lecithin as substrate, synthetic substrates for lipase enzyme activity monitoring were evaluated. Nitrophenol substrates are well-known for their use in the enzyme activity quantification of various enzymes such as lipases, neutral phosphatases, β-galactosidase and α-amylases[160–162]. Furthermore, pNPB has been shown previously to be an effective substrate for the GCAT enzyme[78]. During its reaction with pNPB, GCAT hydrolyzes the ester bond yielding nitrophenol and a free fatty acid. Under basic conditions, the resulting nitrophenol is deprotonated to form para-nitrophenolate which has a strong absorbance at 400nm.

The use of pNPB therefore enables the continuous monitoring of product formation. Due to the stoichiometric ratio of nitrophenol to free fatty acid (1:1), enzyme activity is defined as the amount of enzyme that liberates 1 nmol of nitrophenol per minute under assay conditions[148,163]. However, this definition of enzyme activity is generic and does not necessarily apply to all enzymes assayed. The definition of a single unit of enzyme activity, using pNPB as substrate, should therefore be defined by comparison to assays using natural substrates.

Continuous assays are more accurate, reproducible and less time-consuming than end-point style assays. A microtiter plate assay was consequently developed whereby the hydrolytic activity of GCAT could be quantified using pNPB. In order to determine the pNPB concentration

at which substrate saturation is reached, full kinetic analysis was performed with pNPB concentrations ranging from 0.01 – 3.5 mM spiked with a diluted FPCL preparation containing 88.44 ng protein. In Figure 5.12 it can be seen that saturation was reached at ≈1mM pNPB. Michaelis-Menten kinetic analysis of the data obtained yielded a maximum specific reaction velocity of 292.4 μmol.min^{-1}.mg protein^{-1} and a Michaelis constant (K_m) of 0.09 mM. Subsequent activity assays were therefore performed with 10 mM pNPB to ensure saturation.

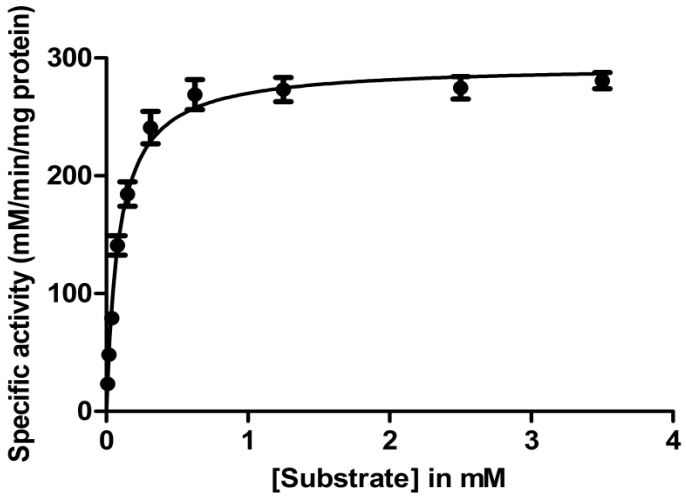


Figure 5.12 Michaelis-Menten kinetic analysis of the lipase activity of GCAT using the assay using pNPB as substrate as described in the text (N = 3, R^2 = 0.98).

A unit of enzyme activity, for the continuous microtiter plate assay, was subsequently defined by comparison to assays performed using lecithin as substrate. The results of activity assays using both lecithin and pNPB are presented in Table 5.3. As can be seen, the values for pNPB did not correlate well with the results from the assay performed with lecithin when it was assumed that the definition for a unit of activity was the same for both assays (pNPB (1)). Based on these results, the definition for a single unit of enzyme activity was adapted as follows:

Definition: *1 Unit of enzyme activity, when using pNPB as substrate, is the amount of enzyme (in mg) which will produce 0.250 μmol pNP per minute under assay conditions.*

Table 5.3 Comparison of enzyme activity determined with the use of lecithin and pNPB. Total enzyme activity per mL FPCL is calculated by multiplication of U with the total amount of protein present in FPCL (14.74 mg/mL).

Substrate	Reaction rate mM/min	Reaction rate μmol/min	μmol/min/mg (U)	U/mL FPCL
Lecithin	0.0188	0.056	66.96	987.05
***pNPB (1)**	0.1200	0.024	272.73	4020.00
***pNPB (2)**	0.1200	0.024	68.18	1005

* pNPB 1 was calculated using the standard definition for a unit of enzyme activity while pNPB (2) was calculated using the adapted definition for a single activity unit described above.

Applying this definition (pNPB 2) yielded similar activity results when compared to the assay performed using lecithin as substrate. Although the data presented here indicate the results from a single assay, with three replicates, these results were highly reproducible. This data suggests that these assays may be used interchangeably for enzyme activity screening. The results were furthermore consistent with the activity data stipulated in the FPCL specification sheet presented in Addendum A.

5.5.4.2 GCAT kinetic analysis

As mentioned previously, to the best of our knowledge, no kinetic data for the GCAT enzyme have been published. Familiarity with the kinetic parameters, K_m and V_{max} should provide more evidence if pNPB may be used as a substrate for GCAT activity screening. If this is shown to be the case, the newly developed assay may also be used to determine optimal dosage concentration and incubation time during industrial application of FPCL.

The natural substrates for GCAT have no measurable chromogenic properties. A discontinuous assay was therefore developed in order to study the kinetic characteristics of GCAT. In this assay GCAT was incubated with a SUV substrate suspension consisting of DOPC or purified egg yolk lecithin. To enable measurement of the transferase activity, cholesterol was incorporated into the SUV as described in the methods section. Aliquots were subsequently collected at timed intervals and the enzyme activity was quenched. Enzyme activity was monitored by the production of FFA or cholesteryl ester. After development of the assay, SUV preparation was evaluated and optimized.

As mentioned earlier, various methods have been described to inactivate the enzyme activity. These methods often include the use of acids, such as trichloroacetic- or perchloric acid. However, as can be seen in Figure 5.13 the use of perchloric acid resulted in substrate phospholipid hydrolysis and, subsequently, high levels of FFA. As a result, FFA liberation was not linear, and the results of assays inactivated with acid could not be used. Since GCAT is heat sensitive and loses enzyme activity when exposed to temperatures in excess of 80°C, heat inactivation was subsequently evaluated. From these studies it was shown that enzyme activity was destroyed by heat treatment. However, FFA production was once again not linear indicating that enzyme activity was not destroyed in a consistent manner. Each aliquot was therefore first flash-frozen, using liquid nitrogen prior to heat inactivation. From Figure 5.13 it can be seen that using this method FFA liberation was more linear. Enzyme inactivation by flash freezing and heat treatment (Section 5.3.1.2 Method iii) was therefore used for all subsequent assays.

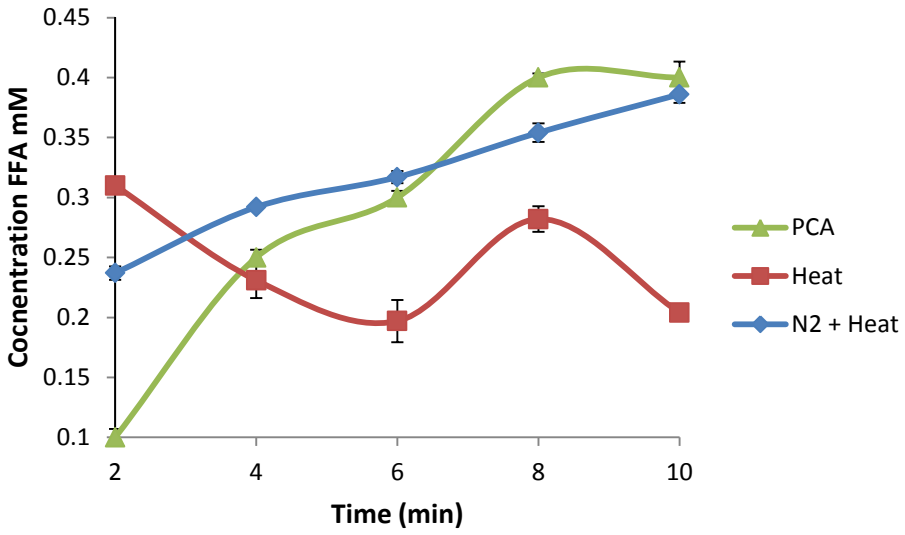


Figure 5.13 Comparison of enzyme inactivation methods as evaluated by the linearity of FFA liberation.

The transferase activity of GCAT was evaluated using SUVs consisting of DOPC, cholesterol and radiolabeled cholesterol as substrate. The newly developed assay was sufficiently sensitive to monitor transferase activity at substrate levels as low as 10 µM cholesterol and phospholipid. During earlier studies, performed by Buckley *et al.*[39], it was shown that the acyltransferase activity of GCAT is maximal when equimolar concentrations of cholesterol and phospholipid is used. The transferase activity of GCAT was therefore initially monitored with SUV suspensions consisting of equimolar concentrations of cholesterol and DOPC. During these assays the vesicle suspension was sonicated for a total of 40 min.

Kinetic analysis of the transferase activity of GCAT, using SUVs consisting of equimolar concentrations of cholesterol and DOPC, is depicted in Figure 5.14. From this figure it can be seen that the transferase activity of GCAT is inhibited at substrate concentrations in excess of 0.250 mM. The decrease in transferase activity coincided with a decrease in reproducibility of the data. Excluding the data of substrate concentrations above 0.250 mM and extrapolating the curve yielded an apparent K_m of 0.071 mM and a V_{max} = 52.67 µmol/min/mg protein. Using this data, the total activity of FPCL was calculated to be 776 units/g FPCL. However, since saturation was not reached, this value was not accurate.

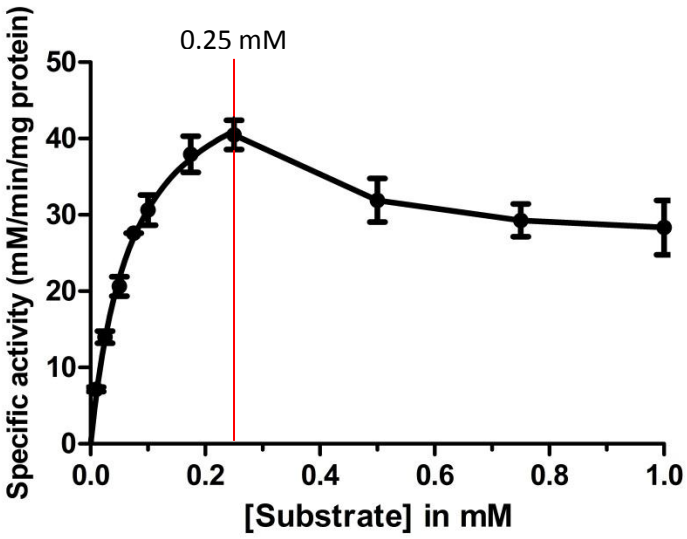


Figure 5.14 Michaelis-Menten kinetic analysis of GCAT using a substrate consisting of equimolar concentrations of DOPC and cholesterol (N = 3). The red line indicates the point, at 0.250 mM, where inhibition starts.

The effect of SUV composition on enzyme catalysis was subsequently investigated. As discussed in Chapter 3, cholesterol cannot form vesicles independently of phospholipid. Therefore, in order to study the effect of cholesterol on enzyme catalysis independently from phospholipid, SUVs consisting of 0.6 mM DOPC and varying concentrations of cholesterol were prepared. A preliminary study was conducted wherein single assays were performed. The result from this study is presented in Figure 5.15 where the x-axis represents the ratio of cholesterol to phospholipid. An inhibition profile was again observed. Moreover, enzyme catalysis is inhibited at cholesterol/phospholipid ratios above 1:1. Previous studies by New[136] suggests that cholesterol to lipid ratios of 1:1 should yield optimal vesicles since cholesterol is incorporated intermediate to each lipid molecule, forming ordered structures at these ratios. The inhibition profile could therefore not be explained based on substrate composition.

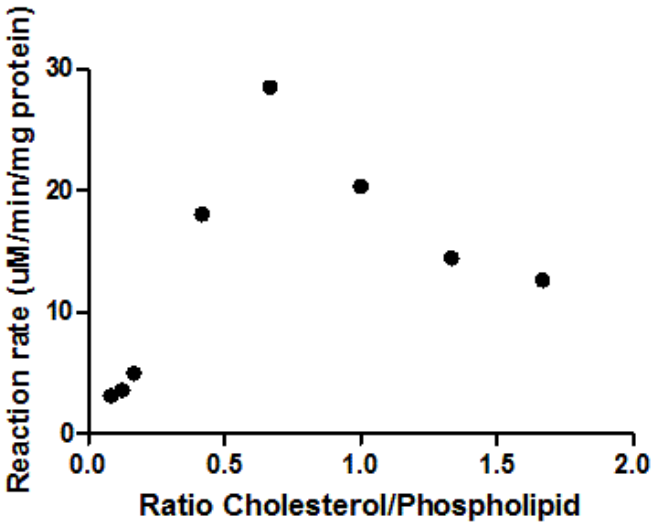


Figure 5.15 Results of a preliminary study of wherein the effect of substrate composition on GCAT activity was investigated (N = 1).

Although the data presented were obtained using SUV suspensions consisting of DOPC and cholesterol, similar results were obtained using purified egg yolk lecithin. The result may be due to various factors, however, the most likely reasons is either the inability of DOPC to form correctly structured SUVs at concentrations in excess of 0.25 mM or allosteric product inhibition. Attention was therefore shifted to evaluation of vesicle formation.

In order to evaluate the factors governing vesicle formation, SUVs were prepared as described previously without addition of cholesterol. Since milk contains various phospholipid species, purified lecithin from egg yolk was used as substrate for structural and kinetic analysis. SUVs consisting of egg yolk lecithin were prepared as described in the previous section by 40 min sonication. Figure 5.16 depicts the results from a lipase activity assay using SUVs prepared with 0.2 mM lecithin spiked with 1 µg protein. As can be seen from this figure, the initial FFA concentration is 57% of the total phospholipid concentration prior to addition of the enzyme. Furthermore, although the initial velocity of the first replicate appears linear, this result was not reproducible in replicate 2.

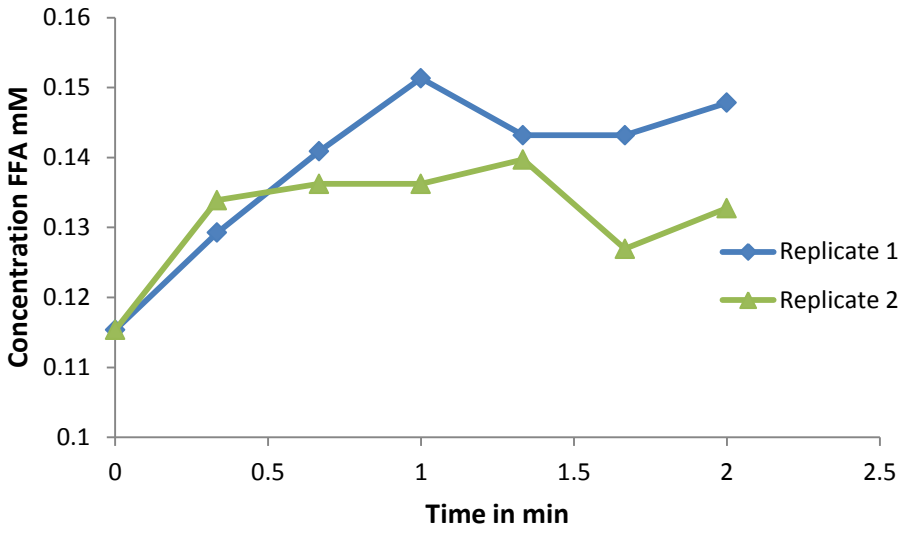


Figure 5.16 GCAT activity assays with SUVs consisting of purified egg yolk lecithin as substrate.

The high levels of initial FFA may be due to two factors namely, enzyme over-dosage or hydrolysis of the phospholipid substrate during sonication. Eklund[164] suggested that sonication up to 60 min does not result in significant phospholipid hydrolysis. The effect of enzyme concentration was therefore evaluated using SUVs prepared in the same manner as described previously with 0.5 mM lecithin. The substrate suspension was subsequently spiked with 420 and 210 ng protein respectively. As can be seen from Figure 5.17, the reduction of spike concentration did not have a significant effect on assay linearity and initial FFA concentration. This is especially evident since the 210 ng spike had a higher initial FFA concentration than the assay spiked with 420 ng protein. Furthermore, there was no significant formation of FFA even after a prolonged incubation period. These results suggest that the vesicular structure is compromised during its preparation.

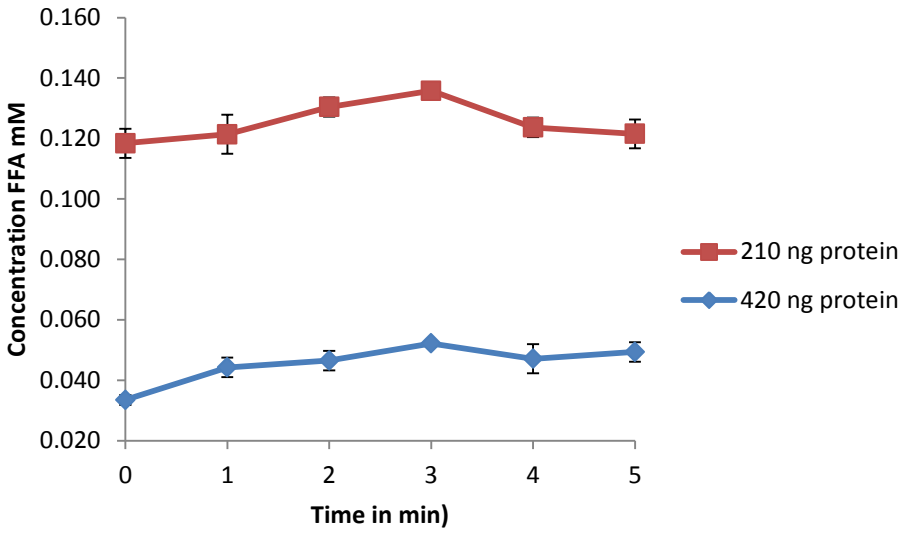


Figure 5.17 Evaluation of the effect of enzyme spike concentration on assay linearity.

The effect of sonication time on vesicular formation was consequently investigated by sonication of 0.25 mM lecithin suspensions for different time periods. The SUV suspension was subsequently spiked with 1 µg protein and aliquots were collected at 2 min intervals. As can be seen from Figure 5.18, sonication time had a substantial effect on assay linearity. However, from the initial FFA values presented in Table 5.4 it can be seen that sonication times in excess of 20 min results in substantial hydrolysis of the phospholipid substrate. Sonication times of 10 and 20 min did not differ substantially with respect to initial FFA concentration and final assay reaction rate. However, there was still a substantial initial increase in FFA concentration after addition of enzyme. These results suggest over-dosage of the substrate suspension with enzyme. Based on these results, total sonication time was reduced from 40- to 20 min and enzyme spike concentration was reduced to 515 ng per mL substrate. Lecithin concentrations ranging from 0.01 to 1.5 mM were used for kinetic analysis.

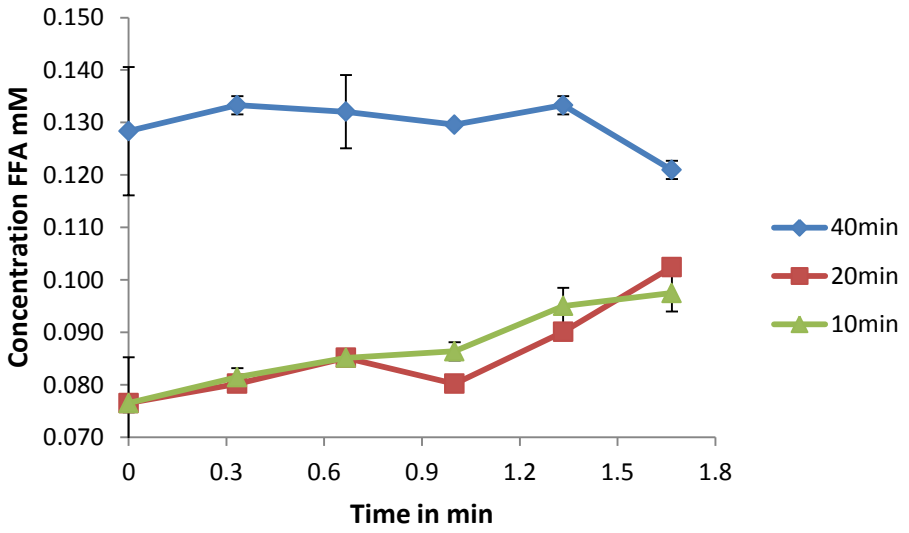


Figure 5.18 Effect of sonication time on the linearity of an activity assay using 0.250 mM substrate.

Table 5.4 Comparison of assay linearity in response to an increase in total sonication time.

Sonication time (min)	Initial FFA (mM)	Reaction rate mM/min
10	0.042	0.0126
20	0.042	0.0132
40	0.078	-0.0034

Figure 5.19 depicts the results from kinetic analysis using SUVs consisting of both lecithin and DOPC respectively. From this figure it can be seen that the phospholipase activity of GCAT followed an inhibitory profile at substrate concentrations in excess of 0.17 mM, irrespective of the phospholipid species used. Previous results suggest that the initial FFA content of substrate suspensions plays a major role in assay linearity and quality. The FFA content of substrate suspensions, prior to the addition of enzyme, was subsequently quantified and shown to be below 2 µM. It therefore appears that 20 min sonication still results in the structural disintegration of SUVs. The total sonication time was consequently reduced to from 20- to 10 min and the assay was repeated.

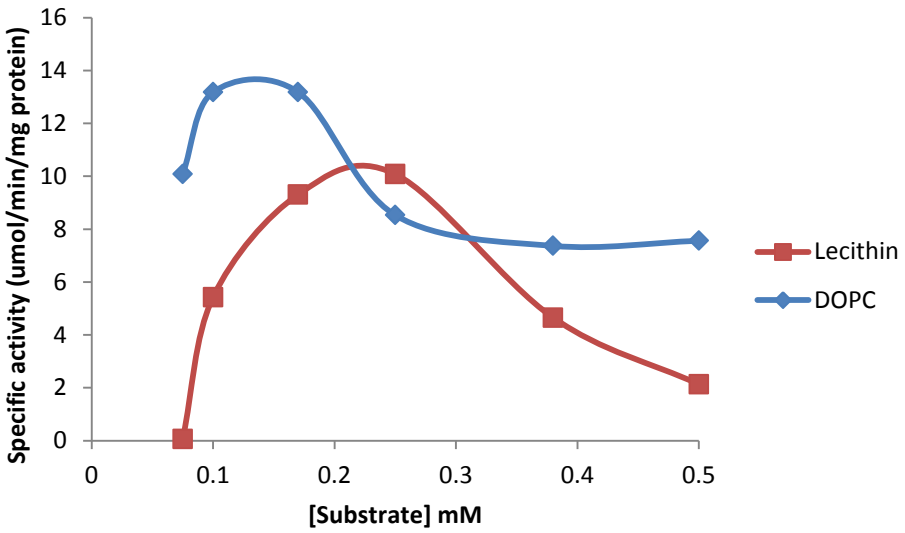


Figure 5.19 Comparison of SUV composition by means of kinetic analysis. Each point on the graph indicates a single assay.

A reduction in the total sonication time had a major effect on assay linearity and reproducibility. As can be seen from Figure 5.20, a decrease in sonication time eliminated the inhibition profile previously observed. However, this resulted in a concurrent reduction in overall reaction velocity. Furthermore, a general decrease in reaction velocity was observed for substrate concentrations up to 0.25 mM.

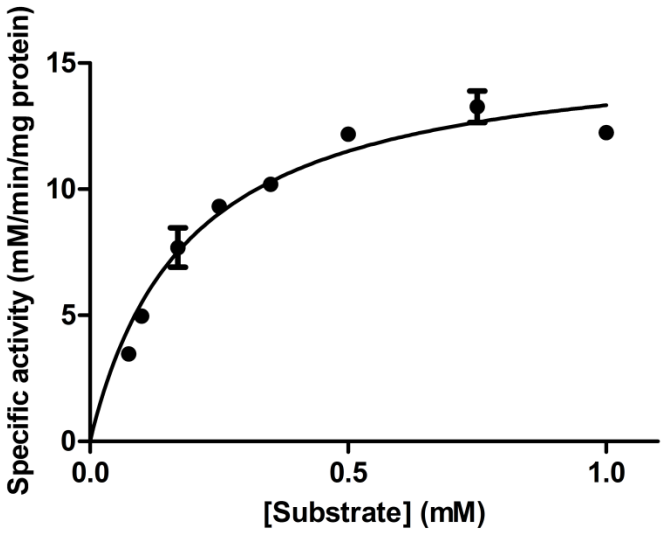


Figure 5.20 Kinetic analysis of GCAT lipase activity with SUVs consisting of purified egg yolk lecithin sonicated for a total of 10 min (N = 3, R^2 = 0.93).

Although a reduction in overall initial velocity was observed, kinetic analysis of GCAT was performed using SUVs consisting of lecithin concentrations ranging between 0.075 and 1.0 mM

prepared by sonication for 10 min. When plotted, the data yielded a kinetic profile consistent with Michaelis-Menten catalysis. Kinetic analysis of the data yielded a V_{max} of 15.84 and a K_m of 0.190 mM. Therefore, although a reduced initial reaction rate was observed, the V_{max} value obtained was higher than the highest reaction velocity from Figure 5.19, 12.17 µmol/min/mg protein.

The kinetic parameters obtained from the assays described, using both pNPB and lecithin, are presented in Table 5.5. It is expected that the K_m for each substrate would differ, and this is certainly the case. In order to prove that pNPB may be interchangeably used as substrate for activity monitoring of GCAT, the V_{max} values should correspond. From the data presented in Table 5.5, it can be seen that the V_{max} values differ substantially. As a result, the final activity per gram FPCL differs by 76%. However, the vesicular structure was shown to be compromised during preparation of SUVs, resulting in a reduced reaction rate. The values obtained during kinetic characterization using SUVs consisting of lecithin may therefore be an underestimation of actual hydrolytic activity.

Table 5.5 Comparison of kinetic parameters and final enzyme activity per mL FPCL obtained using pNPB and lecithin as substrate.

Assay substrate	K_m	V_{max}	Total units per gram FPCL
pNPB	0.09	292.40	1005.0
Lecithin	0.19	15.84	233.50

Since it was shown that excessive sonication results in the inhibition of enzyme catalysis at substrate concentrations above 0.2 mM, even in the absence of cholesterol, the transferase reaction catalyzed by GCAT was reevaluated. For these assays the same assay procedure described earlier was used. Sonication time was reduced from 40- to 20 min since sonication times below 20 min did not yield translucent SUV suspensions.

From the results presented in Figure 5.21 it can be seen that, as was the case with phospholipase assays, a reduction in the total sonication time resulted in elimination of the inhibitory profile of enzyme catalysis for the transferase reaction. However, enzyme saturation could not be achieved with substrate ratios between 0.05:1 and 2.5:1 (cholesterol: phospholipid). A further increase in the cholesterol content of SUVs resulted in a loss of liposomes as observed by a rapid decline in enzyme activity. The data obtained was extrapolated and the apparent K_m and V_{max} was determined to be 2.83 mM and 102 µmol/min/mg protein respectively.

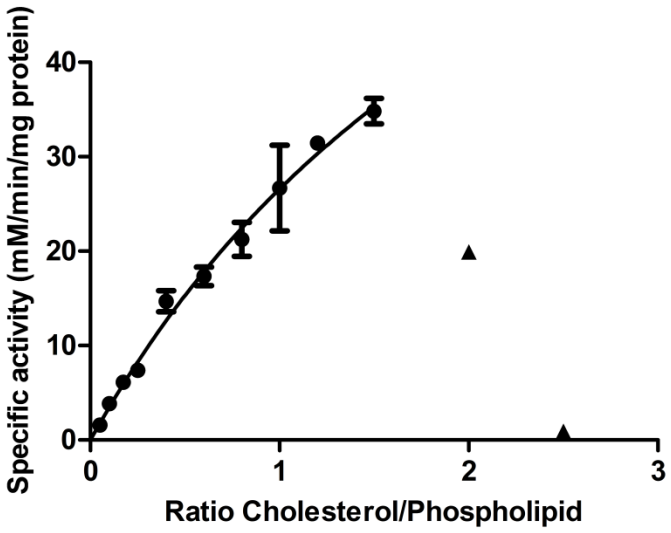


Figure 5.21 Kinetic analysis of the transferase activity of GCAT using optimized substrates (N= 3).

5.5.5 FPCL application analysis

This section describes application of the newly developed pNPB activity assay in the evaluation of the applicability of FPCL in dairy UHT processing.

5.5.5.1 Evaluation of the applicability for the use of FPCL in the Scania processing method

During the production of 2% milk for commercial purposes using the Scania method, the fat content of raw milk is reduced from ≈3.8% to 2% by an in-line standardization process yielding 2% milk and ≈40% fat/cream. In this process the whole milk temperature is increased to between 62 and 64°C in order to guarantee high quality cream. This holding temperature is sufficient to increase milk-fat fluidity and inactivate native milk lipase enzymes, reducing hydrolysis of free fat. The optimum holding time for this process is between 15 and 30 min prior to pasteurization. In order to prevent excessive FFA formation, the holding time, including filling and emptying, should not exceed four hours[165]. Excessive production of FFA by lipase enzymes reduces the beating ability of cream, and should thus be avoided.

As described in Chapter 2, GCAT is active at water-lipid interfaces. The enzyme would therefore be present in the cream fraction after separation from whole milk during processing. For successful application, using the Scania method, FPCL should be fully inactivated during holding at 62-64°C. Retention of FPCL activity after holding will result in excessive FFA production, thereby reducing the commercial value of the cream. Inactivation studies were

therefore conducted at 65°C in Novo buffer. In these studies a buffered system was used since this allowed for low-level detection of GCAT activity without interference from milk lipids.

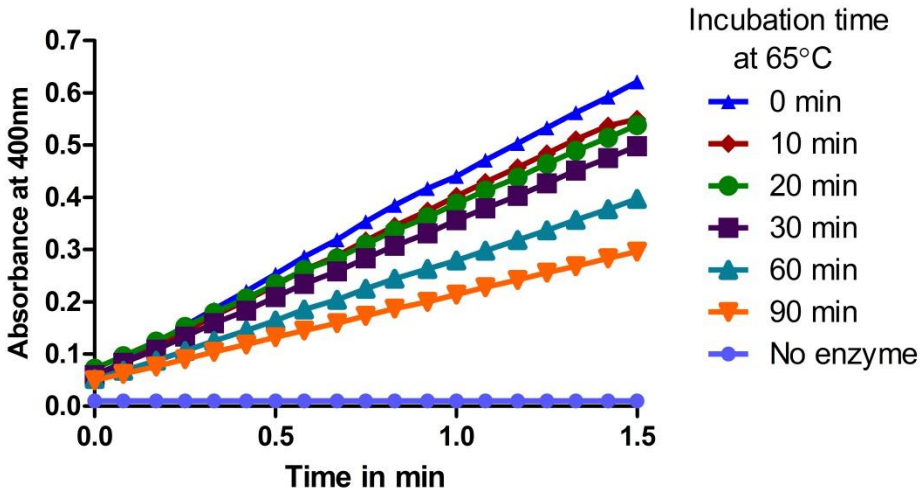


Figure 5.22 The effect of prolonged incubation of FPCL at 65°C in Novo buffer on GCAT activity.

As can be seen in Figure 5.22, incubation at 65°C for 90 min resulted in a significant (ca. 52%) reduction in enzyme activity. However, when compared to the control sample, it can be seen that enzyme activity was retained even after prolonged incubation. These results suggest that FPCL cannot be used for processing using the Scania method since residual GCAT activity is retained.

5.5.5.2 Evaluation of FPCL activity subsequent to UHT processing

During UHT processing the activity of GCAT is irreversibly inhibited. If enzyme activity is retained in the final product, excessive FFA production would lead to rapid product deterioration. In order to ensure that UHT processing effectively inhibited the enzyme, activity assays were performed on milk samples collected from the factory trial described in Chapter 4 Section 4.3.2.2. Enzyme activity was monitored using the assay described in Chapter 5 Section 5.3.3. In these assays fresh pasteurized milk was spiked with 10 ppm FPCL as a positive control while a spiked sample boiled at 90°C for 10min was used as negative control.

The results from these studies are depicted in Figure 5.23 below. In Figure A the activity of the GCAT enzyme was determined in collected samples using the newly developed pNPB assay. Figure B shows that enzyme activity can be detected at dosage levels of 10 ppm when compared to a no-enzyme control. From the figures it is clear that the GCAT enzyme was completely inhibited in all samples following thermal processing. Due to the low levels of dosage

and the complete inactivation following UHT treatment, residual GCAT will not play any significant role in the final product.

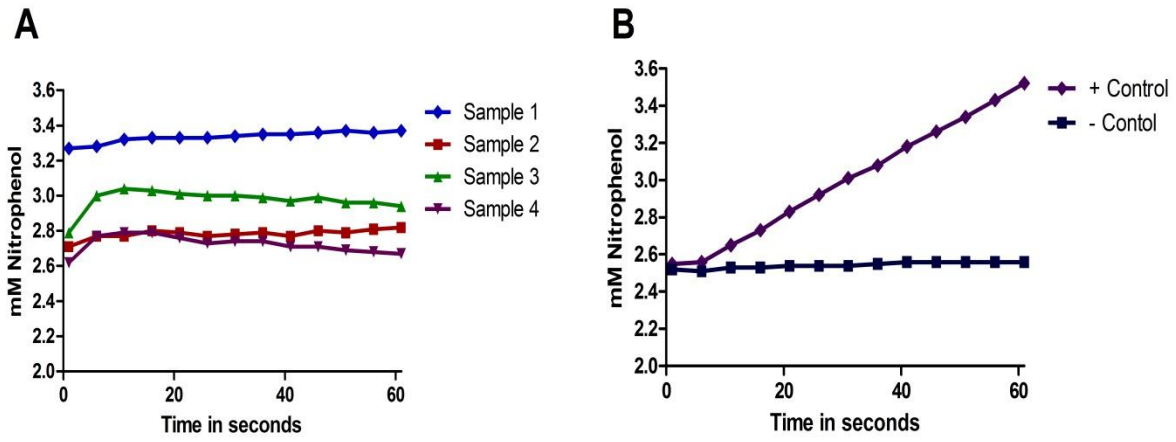


Figure 5.23 Residual enzyme activity remaining following thermal processing at 142°C for 6 seconds for (A) UHT processed milk samples collected from the factory trial performed at Dewfresh. (B) Spiked and unspiked fresh pasteurized milk used as assay controls.

5.6 CONCLUSION

This chapter described the evaluation of techniques for the quantification of GCAT reaction products and its use in the development of an enzyme activity assay. Activity screening of GCAT have traditionally been determined using SUVs consisting of phosphatidylcholine and cholesterol as substrate coupled with scintillation detection. Although accurate, these methods are labour intensive and require specialized instrumentation. Furthermore, it is clear from the data presented that the organizational parameters of SUVs greatly affect the enzyme reaction rate.

Although the data presented did not yield accurate kinetic data, it was shown that various parameters during the preparation of SUVs influence enzyme catalysis. It was furthermore shown that the initial reaction rate of GCAT, when using SUVs as substrates, is greatly affected by the sonication time during SUV preparation. This fact has not been reported previously. Although 10 min sonication yielded translucent SUV suspensions, the initial reaction rate was significantly lower than that of similar suspensions prepared with 20 or 40 min sonication. On the other hand, extensive sonication (40 min) yielded a reduced reaction rate at substrate concentrations in excess of 0.250 mM. It was found that sonication for 20 min yielded the best results during kinetic studies although maximal velocity is was not achieved.

The use of both SUVs and pNPB as substrates for reaction monitoring of GCAT have been described previously[40,78]. However, to the best of our knowledge, a kinetic study of the GCAT reaction using either substrate has not been described in literature. The previously described activity assays are furthermore not appropriate for kinetic studies due to the end-point styled approach. These studies also did not include a thorough investigation into substrate preparation and vesicle formation.

The current study is the first account wherein the available assays for GCAT activity, using SUVs as substrates, have been adapted, and optimized, to discontinuous assays for the kinetic characterization of both the reactions catalyzed by GCAT. Furthermore, although further investigation is needed, the data obtained from these studies provide evidence for the two step reaction mechanism proposed by Hilton and Buckley[82] (Chapter 3, Section 3.3.2). Disruptions in the three dimensional structure of the SUVs, such as that caused by phospholipid hydrolysis during sonication, may result in shielding of phospholipid head groups. This would reduce enzyme-head group interactions ultimately resulting in a reduced enzyme reaction rate. This scenario is supported by the finding that a reduction in sonication time and cholesterol: phospholipid ratios in excess of 2.5:1, resulting in a reduction in SUV formation, also resulted in a decrease in enzyme reaction rate.

This study furthermore provides the first description of a microtiter plate assay, using pNPB as substrate, which may be used for the facile and accurate monitoring of FPCL enzyme activity for commercial application. Although the kinetic data obtained could not substantiate the use of pNPB as sole substrate for activity monitoring, it was shown, using food grade lecithin as substrate, that assays using pNPB and lecithin may be used interchangeably during reaction monitoring of GCAT in FPCL. Furthermore, upon request from DuPont® Industrial Biosciences, France, the newly developed microtiter plate assay has been submitted for validation to replace the current method of activity assay. Once validated by DuPont® scientists, the described assay will be implemented as the new global standard operating procedure (S.O.P) for commercial FPCL activity assay, replacing the current assay.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

The past 200 years have seen great advances in food safety. Thermal processing of dairy products prior to consumption is undoubtedly one of the most significant of all. Although the evidence is circumstantial, the infant mortality rate fell dramatically following the implementation of thermal processing of milk[2]. Today ultra-high temperature (UHT) processing of milk yields a product which is not only high in nutritional value but also has the added benefit of an extended shelf life when stored at ambient temperatures. However, as should be clear from the preceding sections, production of UHT milk is a difficult exercise. Temperature induced fouling increases the overall cost of thermal processing by increasing energy consumption and plant down-time. The need for hazardous cleaning chemicals, such as caustic soda and acetic acid, is furthermore harmful to the environment. Because of these difficulties, the overall production cost of UHT milk is increased, limiting its availability in rural areas where it is most needed.

Consequently, fouling, and the prevention thereof, has been exhaustively investigated[4–6]. Although great advances have been made in the reduction of fouling during UHT processing, mainly by optimization of the processing parameters, fouling is still a major problem. The use of enzymes in milk and dairy products has enjoyed limited success in the past. However, addition of the bacterial enzyme, glycerophospholipid cholesterol acyltransferase (GCAT) to raw milk prior to thermal processing has shown promise. The results presented in the current study suggests that the correct use of the commercial enzyme preparation, FoodPro® Cleanline (FPCL), can increase thermal processing duration by up to 50%. However, preliminary trials indicated that the current methods used for quantification of GCAT reaction products, cholesteryl ester and free fatty acids, are not satisfactory for quality control purposes. Optimal enzyme activity can furthermore not be guaranteed upon delivery at the client since activity testing facilities are limited to Europe, Asia and the Americas. In order to ensure successful commercial application, the methods for quality control needed to be re-evaluated and alternatives offered.

Enzyme catalysis is highly specific. As a result, the quality of the final product can be manipulated by adjusting enzyme dosage strength. If the mechanistic characteristics of the enzyme used are known, this information can be used to predict the extent of enzyme catalysis

following incubation. Conversely, if kinetic data for the GCAT enzyme could be produced, this information could be used for the development of a dosage model whereby final product cholesteryl ester and FFA content can be predicted. Availability of such a model would greatly increase the marketability of FPCL since this will allow product tailoring according to the needs of the client. This data may also be used for the validation of newly developed assays. However, assays for the study of the catalytic mechanism for GCAT have not been described previously. For this purpose, accurate methods for the quantification products of GCAT catalysis are crucial. Furthermore, the initial studies conducted illustrated the need for a facile activity assay whereby GCAT activity in FPCL can be validated.

Therefore, the aim of this thesis was to evaluate methods available for quantification of GCAT reaction products. Apart from its uses for quality control purposes, these methods were also employed during the development of an activity assay whereby the kinetic mechanism of GCAT catalysis could be studied. The use of water soluble nitrophenol substrates for GCAT activity monitoring have been described previously[78]. Para-nitrophenol butyrate was subsequently used as substrate for the development of a rapid, reliable and practical assay for FPCL activity validation. During validation of this newly developed assay, the kinetic parameters obtained from kinetic studies were compared to that obtained from kinetic studies using pNPB as substrate. Additionally, the activities obtained from this assay and assays performed using food grade deoiled soy lecithin as substrate was compared.

In order to gain further insights into the potential difficulties associated with FPCL application, various pilot- and factory scale UHT trials were performed. The samples collected from these trials were analyzed to evaluate the extent of FPCL usage on total cholesterol and FFA content following thermal processing. For the purpose of this study pilot scale FPCL application trials were performed at DuPont® SSA Dairy innovations center Cape Town during the first half of 2012. The initial results indicated no significant difference between trials with- and without FPCL. Similar results were obtained during subsequent trials. The enzyme activity of FPCL was consequently determined using an assay based on the formation of free fatty acids following incubation with lecithin as substrate. It was found that the FPCL solution used had less than 25% of the specified activity. When the trials were repeated with fresh FPCL significant differences were observed. With the addition of 10 ppm FPCL the ΔT spike observed in control trials was eliminated, even after prolonged processing. Operational back pressure buildup was also greatly reduced. Visual inspection of the static mixers furthermore revealed less browning of the deposits during the enzymated trial. A decreased degree of browning would not only

affect the taste of the final product, but will also result in easier cleaning following processing. Similar results were obtained during full scale factory application trials.

Although the initial trials were perceived as failures, the results obtained were invaluable since they illustrated a major deficiency in the current marketing model. The recommended dosage for FPCL is 10 ppm. Because enzyme activity can be undesirably affected by various factors, including improper handling and storage, dosage on a volume per volume basis may result in unreliable and erroneous results, such as those observed during the initial pilot scale trials. The studies conducted therefore demonstrated the need for an accurate easy to use activity assay which could be performed on-site prior to FPCL dosage. The availability of enzyme activity data would enable dosage optimization resulting in an increase in product reproducibility. Additionally, if enzyme activity is known, this data may be combined with catalytic data of the GCAT enzyme to enable dosage adjustment to obtain a product with customer-specified characteristics.

Various authors have described assays whereby the mechanism of GCAT catalysis can be investigated, however, complete kinetic characterization of this enzyme has not yet been successfully achieved[40,48,78,166]. This can be ascribed to a combination of difficulties in obtaining quality emulsions during the preparation of experimental phospholipid substrates and the wide range of possible substrates[133]. Apart from the problems accompanying the preparation substrates for kinetic analysis, neither the substrates nor the products have measurable chromatographic characteristics which would allow facile quantification. The assays described were therefore performed in end-point style using radio-labeled substrates which could be monitored by scintillation counting[40,78]. End-point style assays allows for general activity monitoring. However, such assays have the disadvantage that deviations from the classical Michaelis-Menten model of catalysis are not considered.

In contrast to kinetic analysis using water soluble substrates, lipases, when incubated with phospholipases, yield a sigmoidal kinetic profile[167]. Although the mechanism of GCAT catalysis is still not fully understood, it is known that, GCAT, like lipases, catalyze phospholipid hydrolysis at lipid-water interfaces. As such the interaction of this enzyme with its substrates is at least a two-step reaction[82,133]. During the first step of this reaction GCAT binds the phospholipid head groups in the interface. This is followed by interfacial penetration and formation of the enzyme-substrate complex[82]. It is has been proposed that this binding is, at least in part, determined by the packaging of the acyl chains[82]. Although the mechanism of catalysis remains vague, end

point assays are not sufficient for kinetic analysis of GCAT catalysis. Availability of kinetic data can furthermore be used for the validation of novel activity assays by comparison of the kinetic parameters obtained.

For kinetic analysis of GCAT, accurate methods for the quantification of substrates and/or reaction products are needed. Gas chromatography (GC) coupled to flame ionization detection (FID) is currently used for the quantification of cholesterol, cholesteryl esters and free fatty acids in samples obtained from FPCL application trials. However, sample preparation resulted in the destruction of cholesteryl esters. Consistent with this, previous reports by Onorato *et al.*[151], indicated that derivatization results in the breakdown of cholesteryl esters. The liberation of cholesterol from cholesteryl esters, prior to analysis, will subsequently result in an overestimation of the total free cholesterol present in samples containing both cholesterol and cholesteryl esters. As a result, this method is not suitable for quality control purposes. Alternative methods for the quantification of GCAT reaction products were therefore evaluated.

The use of high performance liquid chromatography (HPLC) for the quantification of cholesterol and cholesteryl esters have been described previously[159,168,169]. However, although the hydrophobic nature of cholesteryl esters result in increased retention on C_{18} HPLC columns, the low maximum UV absorbance of cholesteryl esters results in reduced detection sensitivity. Accurate HPLC analysis of mixtures containing both cholesterol and cholesteryl esters are therefore limited to samples containing relatively high levels (ppm) of these compounds. Milk contains on average 140 ppm cholesterol[105]. However, while evaluating the existing HPLC methods for the quantification of cholesterol and cholesteryl esters, it was shown that these methods are not sufficiently sensitive. This is because milk is a complex biological fluid containing various compounds which could result in peak overlapping. To compensate for this phenomenon, the standards in this study were prepared in fat free skim milk. The method described in this study was shown to enable low level detection of cholesterol and cholesteryl esters in extracted milk samples. This method requires liquid-liquid sample extraction prior to analysis. HPLC analysis of milk samples is therefore time consuming and labour intensive. This method is furthermore not sufficiently sensitive for the low levels of detection required for kinetic studies.

Commercially available enzyme based assay kits were subsequently evaluated. It was shown that although these methods allows for accurate low level detection of cholesterol, cholesteryl esters and FFAs, its use for kinetic studies is limited due to the costs involved. These costs

could be reduced by purification of the individual enzymes needed from its bacterial hosts; however, this was beyond the scope of the current study. For low level FFA determination in milk, the NEFA HR FFA quantification kit (WAKO, Germany) was adapted to a microtiter plate assay. The adaptation resulted in a 50% increase in the total number of assays with one kit. The cholesterol/cholesteryl ester quantification kit, however, could not be optimized further. Its use was therefore limited to quality control in samples obtained from FPCL trials.

An alternative method involving the use of a stable Lieberman-Burchard (LB) reagent was subsequently evaluated for cholesterol quantification during kinetic studies. This method yielded the sensitivity needed for kinetic studies; however, solvent extraction was required prior to cholesterol determination. Furthermore, with this assay only free cholesterol content can be detected. The use of this method is therefore time consuming, labour intensive and requires extraction controls for accurate determination of cholesterol in aqueous media. The WAKO NEFA HR FFA quantification and Abcam cholesterol/ cholesteryl ester quantification kits were therefore used for quantification of GCAT reaction products in milk obtained from application trials. For kinetic studies, the NEFA HR FFA proved sufficiently inexpensive and sensitive for use in determination of FFAs produced during the study of the hydrolytic activity of GCAT when incubated with phospholipids. The transferase activity was monitored with the use of radiolabeled tracers since its use enables low level detection of substrates and products and eliminates the need for extraction controls.

While investigating the mechanism of action for GCAT, Buckley[78] showed that this enzyme could also hydrolyze p-nitrophenyl esters. This author subsequently suggested that p-nitrophenyl butyrate (pNPB) is hydrolyzed at a rate comparable to phosphatidylcholine hydrolysis. The supportive data for this statement was, however, never published. The use of pNPB could overcome the difficulties associated with the use of phospholipids as substrates since these are non-interfacial monomeric water-soluble substrates[170]. In this study pNPB was used as substrate for the development of a continuous microtiter plate assay for GCAT activity monitoring. The benefits of this assay include increased sensitivity, low-cost, ease of use, accuracy, reproducibility, versatility and automatability. The assay developed proved sufficiently sensitive for activity monitoring in milk samples spiked with 10 ppm FPCL. However, validation was required prior to commercial application.

For validation, the kinetic parameters obtained using the newly developed pNPB assay was compared with that obtained using phospholipids as substrates. The assay described by

Buckley[76] was subsequently adapted to a discontinuous assay in an attempt to determine the kinetic parameters, K_m and V_{max}, of GCAT catalysis. These adaptations included evaluation of methods for complete enzyme inactivation following time-point collection and optimized monitoring of product formation. Although not optimal, discontinuous assays are suitable for the kinetic study of enzyme catalysis. However, complete enzyme inactivation, subsequent to time-point collection, is essential. Enzyme inactivation using acids have been used successful previously. In this study acid treatment resulted in substrate destruction. Enzyme activity was subsequently irreversibly inhibited using liquid nitrogen followed by heat treatment at 90°C for 15 min. The hydrolytic reaction was monitored by FFA formation. During the initial studies conducted, using pNPB and de-oiled food grade soy lecithin as substrates, it was found that, in contrast to the report by Buckley[78], the reaction rates were not similar when it was assumed that the definition of a unit of enzyme activity is similar. However, this may be due to the use of different substrates during the studies conducted. The definition was subsequently redefined as follows: *1 Unit of enzyme activity, when using pNPB as substrate, is the amount of enzyme (in mg) which will produce 0.25 µmol p-nitrophenol per minute under the defined assay conditions.* The results from the described studies indicated that the newly developed assay may be used for accurate determination of enzyme activity in FPCL. This assay is furthermore sufficiently simplistic for use by moderately skilled personnel and does not require highly specialized equipment.

While attempting to determine the kinetic parameters of GCAT catalysis it was found that the reaction velocity of catalysis is significantly affected by the total sonication time during the preparation of SUVs. This result suggested that the organizational integrity of SUVs may be compromised by extended sonication. In contrast to this, Eklund[164] reported that sonication up to 60 min does not result in significant phospholipid hydrolysis. In the present study an increase in total sonication time resulted in increased levels of FFAs in the SUV suspension. However, these conflicting findings may be ascribed to different sonication intensities for the sonicators used. Nevertheless, extended sonication resulted in a reduced reaction velocity at substrate concentrations in excess of 0.25 mM to yield an inhibitory profile (Figure 5.14). Decreasing the total sonication time to 10 min resulted in abolishment of the observed inhibition pattern. However, reduction of the sonication time resulted in a reduced overall reaction velocity.

Since the organizational parameters of the SUVs play a major role during catalysis, these results propose that the formation of the enzyme-substrate complex is preceded by binding to the head groups of phospholipids in the interface as proposed by Hilton and Buckley[82]. For this

model the phospholipid head groups should be un-obscured and accessible to the GCAT enzyme. Phospholipid hydrolysis results in the formation of lysophospholipids. The overall characteristics of lysophospholipids differ significantly from that of phospholipids[42,171,172]. As such, the packaging order of lysophospholipids in SUVs would be different from that of phospholipids resulting in "kinks" in the walls of the resultant SUV. These abnormalities in the vesicular structure would reduce the accessibility of the phospholipid head groups to GCAT. Although it remains possible that the observed reduction in enzyme activity may be due to inhibition by FFAs, the observation that phospholipid:cholesterol ratios in excess of 2.5:1 results in a dramatic decrease in reaction velocity suggest that this is not the case. It has been shown that cholesterol is organized alternately with phospholipids in SUV membranes at equimolar concentrations[136]. At phospholipid:cholesterol ratios in excess of 2:1 insertion of cholesterol result in disruptions in the organizational structure of SUVs during its preparation. Such disruptions would not only affect the vesicular structure but also accessibility to the phospholipids by GCAT. These disruptions could subsequently be visualized as the reduction in enzyme reaction velocity observed.

For lipases, interfacial binding is considered to be the rate-limiting step during the reaction with lipids. As a result, the kinetic profile for the interaction of lipases with lipids is characterized by a lag-phase[167]. After formation of the enzyme-substrate complex, the catalytic steps take place thereby regenerating the enzyme at the interface. As a result, interface binding and penetration is not required and lipase catalysis can hereafter be described by Michealis-Menten kinetics[133,167,173]. However, studies by Hilton and Buckley[82] reported that GCAT interfacial binding is not rate limiting as is the case with lipases. Since no lag phase was observed, these results propose that, as suggested by these authors, rapid equilibrium is reached and GCAT catalysis can be described by Michaelis-Menten kinetics.

Although the kinetic studies conducted yielded important information regarding the reaction mechanism of GCAT catalysis, we were not able to obtain accurate kinetic data. This is due to the complex nature of SUVs as substrates for kinetic studies. As described above, the overall reaction velocity varied with sonication time, illustrating the absolute requirement of intact interfaces. The apparent kinetic parameters were determined to be as follows (Table 6.1):

Table 6.1 Kinetic parameter obtained from kinetic studies conducted in the current study

Parameter	Hydrolytic activity	Acyltransferase activity	pNPB assay
K_m	0.190 mM	2.83 mM	0.09 mM
V_{max}	15.84 µmol/min/mg protein	102 µmol/min/mg protein	292.4 µmol/min/mg protein

As described previously, GCAT preferentially catalyzes acyl transfer in the presence of a suitable acyl acceptor[40,87]. Although not accurate, the kinetic results presented suggest that this is due to a low affinity for cholesterol. As such, cholesterol is less strongly bound in the active site of the protein enabling an increased rate of product release. Since lysophospholipids do not partake in subsequent reactions, an increased affinity would result in a lower V_{max}. This was evident when the apparent V_{max} values were compared.

The difficulties experienced during the studies conducted have also been described during biophysical studies of the human lecithin cholesterol acyltransferase[170,174]. In order to overcome these difficulties, the use of catalytically inert ether phosphatidylcholine analogues was subsequently suggested.[170,174,175]. The chemical strength of the ether bond would enable prolonged sonication without hydrolysis[174]. Experimental substrates could subsequently be incorporated into previously prepared SUVs without phospholipid hydrolysis. However, it has been shown that these substrates may affect catalysis by acting as activators or inhibitors for catalysis[174]. The effect of these catalytically inert substrates should therefore be evaluated prior to its use in kinetic studies. This was, however, beyond the scope of this study.

To ensure successful application, at least 60% of the free cholesterol should be converted during incubation without excessive formation of FFAs. With the use of the described methods, it was shown that two hours incubation at 4°C was sufficient to convert up to 80% of the free cholesterol to cholesteryl ester. Evaluation of the total FFA in milk indicated that dosage with FPCL did not result in the overproduction of FFAs. Along with the 50% increase in total processing duration, these results indicate that FPCL may be used to reduce the overall cost of UHT processing, without a decrease in product quality.

Since the newly developed activity assay, using pNPB as substrate, was sufficiently sensitive to determine enzyme activity in milk samples spiked with FPCL, this assay was used for application studies. The Scania processing method is used for the production of 2% milk and 40% cream in a single process. However, the cream is held at a temperature below the inactivation temperature of GCAT for at least 30 min prior to pasteurization[165]. Since GCAT is

active at lipid-water interfaces, it will also be present in the cream. In this study it was shown that the cream holding temperatures is not sufficient to destroy GCAT activity. These results therefore indicate that FPCL may not be used for processing with the Scania method. To enable the use of FPCL in this processing method, a GCAT mutant with increased heat sensitivity may be produced by site-directed mutagenesis. However, mutation may also affect the overall catalytic activity of the GCAT enzyme. Therefore, currently the use of FPCL is limited to use in the production of UHT milk.

In conclusion, the data presented in this study, as discussed above, clearly demonstrate the benefits of FPCL during thermal processing. The studies furthermore revealed the current difficulties, and remedies, associated with the application of FPCL in the dairy industry. To the best of our knowledge, the current work describes the first partial kinetic characterization of the GCAT enzyme. The data obtained from these studies provided evidence for the reaction mechanism of GCAT catalysis yielding a better insight into the substrate specificity exhibited by this enzyme. Finally, a facile and accurate microtiter plate activity assay was described whereby FPCL activity could be validated prior to dosage.

Taken together the commercial application of the methods described in this study would increase FPCL marketability by enabling product tailoring and quality validation. Furthermore, the newly developed assay has, upon request from DuPont®, been submitted for validation to potentially replace the current global standard operating procedure for activity quantification. Although complete kinetic characterization was unsuccessful, this study not only described the first kinetic study of GCAT but also provided the first evidence for the proposed mechanism of catalysis suggested 23 years ago[82]. The studies furthermore provided alternative methods which could overcome the difficulties experienced. Finally, the current study brings us one step closer to a global reduction in the total cost for UHT milk, thereby increasing its availability in areas where it is most needed.

In future studies the use of catalytically inert ether phosphatidylcholine species will be evaluated for use as scaffold for full kinetic characterization of GCAT. During these studies, the enzymes needed for enzyme-linked quantification of GCAT reaction products could be cloned, expressed and purified from experimental hosts, thereby reducing the overall cost per assay. Alternative methods for the preparation of SUVs, such as extrusion, will also be investigated in order to evaluate the effect on vesicle formation. The degree of substrate degradation will be evaluated by microscopy and FFA liberation. Alternative assay methods, such as interfacial tensiometry,

will also be attempted since this method would allow continuous activity monitoring using the natural substrates, lecithin and cholesterol. Finally, in order to enable in-factory activity verification, the plausibility dip-strip type activity assays will be investigated. These assays will rely on the use of either natural or synthetic substrates immobilized on a suitable surface. Enzyme activity could subsequently be estimated by comparison to a colored activity standard sheet similar to the modern-day pH strip method.

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Valid from: October 6, 2009

ADDENDUM

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PRODUCT DESCRIPTION - PD 233496-1.1EN

Foodpro® Cleanline

Description

Foodpro® Cleanline is a liquid acyltransferase enzyme produced by a selected strain of Bacillus licheniformis.

Application areas

Dairy products based on fresh or re-constituted milk

Usage levels

Should be used in a dosage range : 5-50 LATU/Kg milk.

Directions for use

Foodpro® Cleanline can be added directly to the milk. Activity will depend on process variables such as pH and temperature, contact time, concentration and nature of substrates.
As an indication: Incubation time at 10 ppm dosage level is 20 minutes at 40°C up to 6 hours at 10°C.

Composition

Protein (enzymes)
Water
Glycerol
Potassium Sorbate
Trisodium citrate dihydrate

Physical/chemical specifications

Physical form	liquid
Colour*	brown
Activity	900 - 1100 units/g

*Colour may vary from batch to batch.

Microbiological specifications

Total viable count	less than 50000 /ml
Coliforms	less than 30 /ml
E.Coli	absent in 25 ml
Salmonella species	absent in 25 ml
Antibiotic activity	negative by test

Heavy metal specifications

Arsenic	Less than 3 mg/kg
Cadmium	Less than 0.5 mg/kg
Lead	Less than 5 mg/kg
Mercury	Less than 0.5 mg/kg

Storage

Foodpro® Cleanline should be stored dry and cool (max. 10°C/50F).

The shelf life of Foodpro® Cleanline is 6 months when stored as recommended in unbroken packaging.

Packaging

28 kg blue plastic jerry can

Purity and legal status

Foodpro® Cleanline meets the specifications for enzyme preparation laid down by the Joint FAO/WHO Expert Committee on Food Additives and the Food Chemicals Codex.

As legislation regarding its use in food may vary from country to country, local food regulations should always be consulted concerning the status of this product. Advice regarding the legal status of this product may be obtained on request. Foodpro® Cleanline is Generally Recognized As Safe (GRAS) in the United States for use in dairy applications.

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Valid from: October 6, 2009

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PRODUCT DESCRIPTION - PD 233496-1.1EN

Foodpro® Cleanline

Safety and handling

Inhalation of enzyme dust and mists should be avoided. In case of contact with the skin or eyes, promptly rinse with water for at least 15 minutes. For detaled handling information, please refer to the appropriate Material Safety Data Sheet, the Enzyme Technical Association (ETA) handbook Working Safely With Enzymes, and the Association of Manufacturers and Formulator of Enzyme Products (Amfep) handbook Guide to the Safe Handling of Microbial Enzyme Preparations. All are available from Danisco.

GMO status

The microorganism used for production of Foodpro® Cleanline was developed by recombinant DNA techniques.

Allergens

The table below indicates the presence (as added component) of the following allergens and products thereof (according to US Food Allergen and Consumer Protection Act (FALCPA), 2004, Standard 1.2.3 of the Joint Australia New Zealand Foods Standards Code, and Directive 2000/13/EU as amended).

Yes	No	Allergens	Description of components
	X	Cereals containing gluten	
	X	Crustaceans	
	X	Eggs	
	X	Fish	
	X	Peanuts	
(X)		Soybeans	Used as fermentation substrate and considered consumed during fermentation.
	X	Milk (incl. lactose)	
	X	Nuts	
	X	Celery	
	X	Mustard	
	X	Sulphur dioxide and sulphites (>10 mg/kg)	
	X	Lupin	
	X	Molluscs	

*Danisco has determined that fermentation nutrients are outside the scope of US and EU food allergen labeling requirements (1,2) . However, Australia/New Zealand does not consider fermentation nutrients to be outside the scope of local allergen labeling requirements. Allergens and products thereof which are used in the recovery process or in the formulation of an enzyme product, are declared in the table above. 1) Position paper sent by the Enzyme Technical Association to the FDA on September 12, 2005 (see, http://enzymetechnicalassoc.org/Allergen%20psn%20paper-2.pdf) 2) Summarized in the position paper of the Association of Manufacturers and Formulators of Enzyme Products: http://www.amfep.org/documents/AmfepstatementScopeAllergyLabellingDirf000.pdf

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